Chapter

Recent Advances in Antioxidant Capacity Assays

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Abstract

This work presents a survey of the important antioxidant capacity/activity assays applied for a diversity of samples including plant extracts, foods, biological material, etc. The published materials are critically discussed, emphasizing the recent findings in the field. New and emergent antioxidant capacity assays, such as nanoparticles-based assay, are also presented. The discussion includes chemical-based methods as well as biochemical and cellular assays. Chemical methods detailed are radical/ROS-based scavenging assays (the trolox equivalent antioxidant capacity (TEAC/ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) assays, chemiluminescence methods, total radical-trapping antioxidant parameter (TRAP), total oxy radical scavenging capacity (TOSC), and β-carotene bleaching assays), non-radical redox potential-based assays (ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC), nanoparticle-based methods and electrochemical methods), metal chelation capacity and total phenolic content tests. The biochemical-based assays and *in vivo* assays discussed include the oxidation of low density lipoprotein (LDL), the thiobarbituric acid reactive substances (TBARS) and the cellular antioxidant activity (CAA) assays. While a direct link between the antioxidant capacity and health benefits is still a matter of debate, the antioxidant testing methodologies presented in this chapter remain valuable for the high efficiency and cost-effective evaluation of antioxidants, from compound discovery to quality control.

Keywords: antioxidant, total antioxidant capacity, reactive species, phenolic compounds, antioxidant assay, phytochemicals, food analytical method

1. Introduction

Antioxidants are classified in two categories: (1) primary or chain-breaking antioxidants, especially acting by scavenging reactive oxygen species/reactive nitrogen species (ROS/RNS) and (2) secondary or preventive antioxidants, that suppress the oxidation promoters such as metal ions, singlet oxygen, pro-oxidative enzymes and other antioxidants, commonly operating by transition metal ion chelation [1]. An antioxidant may operate directly or indirectly: directly by scavenging ROS/RNS species or by inhibiting their generation, indirectly, e.g., by up-regulating endogenous antioxidant defenses [2, 3]. Antioxidants can be also classified as enzymatic and non-enzymatic antioxidants. In the present review we shall discuss only the non-enzymatic antioxidants. The efficacy of an antioxidant depends on its antioxidant activity and/or its antioxidant capacity.

It should be stated from the very beginning that antioxidant activity and antioxidant capacity are two different terms. The antioxidant activity is linked to rate constant of an antioxidant against a specified free radical, whereas the antioxidant capacity represents the number of moles of a specified free radical, scavenged by an individual antioxidant present in the analyzed mixture [4]. Antioxidant activity is related especially to the reaction kinetics, whereas antioxidant capacity is related to the thermodinamics of the process regarding the oxidative conversion of an antioxidant and is connected with equilibrium constant of the process [5].

The antioxidant assays can target a specific compound (e.g., ascorbic acid, vitamin E, uric acid, etc.) or the total antioxidant capacity (TAC) given by the combined antioxidant capacities of all substances in a sample.

Antioxidant assays include direct and indirect methods. Direct assays are competitive, in which the produced reactive species simultaneously attack a „probe" and the antioxidant. Indirect assays are non-competitive, the redox reactions being simulated using an artificial probe, whose structural changes are measured by different techniques (spectroscopy, electrochemistry, or other methods).

The most common assays for TAC comprise: (i) the measurement of oxygen radical antioxidant capacity (ORAC) using different fluorescent probes [6], (ii) the Trolox equivalent antioxidant capacity based on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (TEAC/ABTS) [7], (iii) the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [8], (iv) the ferric reducing antioxidant power (FRAP) test [9], (v) the cupric reducing antioxidant capacity (CUPRAC) assay [10] and (vi) Folin–Ciocalteu's phenol reagent reducing capacity (for the content of total phenolics) [11, 12].

Extensive reviews regarding the methods for assaying antioxidant capacity/activity could be found in literature [1, 5, 6, 13–19] and a book has also been published recently [20] with a focus on the measurement of antioxidant activity and capacity. Several papers have discussed the advantages and disadvantages of different antioxidant assays, with a focus on method selection for specific requirements [14–16, 21, 22].

There are numerous research articles in literature pertaining to the evaluation of antioxidant methodology. However very few discuss the mechanistic steps involved in the respective reactions [23, 24]. In depth evaluation of ORAC, ABTS and DPPH methods were comprehensively presented [25]. Some important antioxidant assays in terms of mechanisms and kinetics of the involved reactions were evaluated [6, 14], while the mechanisms, advantages and disadvantages of different antioxidant assays were also described in [18, 26, 27].

A review of the main methods for monitoring the antioxidant capacity/activity of lipid-containing samples was presented in [28]. In addition, the determination of the antioxidant capacity of lipids via the flow injection analysis (FIA) coupled with chemiluminescence detection was specifically discussed in [29].

The role of antioxidants from a pharmaceutical perspective is presented in [30] and a review of the methodologies for the determination of biological antioxidant capacity *in vitro* is presented in [31].

Compiled information about antioxidants in terms of the chemistry, legislation and their application in foods as preservatives can be found in [32]. The extrapolation of laboratory data relative to the antioxidants' function and their implications on food production and human health, etc. is critically discussed in [33].

Some recent reviews [13, 34, 35] commented on the advance, applications, advantages and disadvantages of total antioxidant capacity assays. The contentions and limitations of some largely used antioxidant assays, hints for suitable assay selection, emerging techniques in antioxidant testing and future perspectives are provided in [5].

An interesting discussion is presented in [36] about the development of several TAC databases of foods, the development of methods for evaluating TAC in the diet, the application of TAC databases in epidemiological studies, the application of TAC

methods to biological fluids and the correlation between consumption of antioxidant rich-foods and the plasma TAC. The advantages and disadvantages of different TAC assays were also summarized.

Unfortunately many studies on TAC have reported disparate results regarding antioxidant capacity measured on the same material in different laboratories even by using the same analytical method, or in a particular laboratory by using different methods. Such discrepancies could be explained by the fact that the employed methods evaluate different things under various conditions, e.g., some measurements are done in homogenous solutions, other in suspensions, some methods evaluate hydrogen atom transfer capacity, other evaluate electron transfer capacity, etc.

Consequently, developing standardized antioxidant capacity methods might reduce the results spreading. A basic rationale to develop standardized antioxidant capacity methods for food, being provided in [37], which considered three candidates assays for standardization, i.e., ORAC, TEAC/ABTS and Folin Ciocalteu method.

Radicals are usually quenched by two mechanisms [6, 25], i.e., by transferring either an electron (ET) or a hydrogen atom (HAT) to transform the radical to a more stable species, albeit sometimes the mentioned mechanisms may not be well distinguished [37]. Consequently antioxidant capacity measurements may be in large, categorized as electron transfer (ET)- and hydrogen atom transfer, (HAT)-based assays.

In **ET–electron transfer assays,** one or more electrons are transferred to reduce the compounds of interest according to the following reaction schemes:

$$ROO^{\bullet} + AH / ArOH \rightarrow ROO^{-} + AH^{\bullet+} / ArOH^{\bullet+} + H_2O \rightarrow A^{\bullet} / ArO^{\bullet} + H_3O^{+} \quad (1)$$

$$ROO^{-} + H_3O^{+} \rightarrow ROOH + H_2O \quad (2)$$

$$M(III) + AH / ArOH \rightarrow AH^{\bullet+} / ArOH^{\bullet+} + M(II) \quad (3)$$

HAT–hydrogen atom transfer assays involve the transfer of a H atom to the target radical and eventual secondary quenching by radical recombination, as follows:

$$ROO^{\bullet} + AH / ArOH \rightarrow ROOH + A^{\bullet} / ArO^{\bullet} \quad (4)$$

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \quad (5)$$

where AH = any antioxidant with donatable H, ArOH = phenol or polyphenol, M = redox-active metal.

As can be seen from the chemical reactions written above, regardless of the mechanism involved (ET or HAT), antioxidants scavenge ROS/RNS generating the same end products indifferent to mechanism involved, albeit kinetics and influence of system parameters, particularly solvent and pH, and potential for side reactions vary [37]. Moreover, HAT and proton coupled ET reactions may occur concurrently and the main mechanism in a particular system is determined by antioxidant properties and structure, partition coefficient, solvent, etc. [37].

The ET-based methods evaluate an antioxidant's reducing capacity (also of the probe for monitoring the reaction). Mainly HAT-based methods measure competitive reaction kinetics, and the determination is effected taking into account the kinetic curves. HAT-based assays mostly involve a synthetic free radical source,

an oxidizable probe, and an oxidant. An elaborate description of antioxidant mechanisms is well presented in several review papers [6, 13–16, 38].

Antioxidant capacity is expressed as equivalents of a reference antioxidant such as trolox, gallic acid, etc., or antioxidant inhibition against oxidation of the probe (generated by ROS). Oxidation of the probe is determined by different detection techniques, such as: spectrophotometric, fluorimetric, chemiluminescent, EPR, amperometric methods, cyclic voltammetry, etc.

A classification of the methods for the assessment of antioxidant capacity/activity discussed in this work is presented in **Table 1**.

Classifications		**Assays**	**References**
Chemical based assays			
Radical/ROS-based scavenging assays	HAT/ET assays (Mixed mode)	TEAC/ABTS assay	[1, 15, 18, 24, 34]
		DPPH assay	[1, 15, 34]
	HAT assay	ORAC assay	[1, 15, 25, 37]
		Chemiluminescence methods	[29, 37, 39, 40]
		TRAP assay	[13, 15, 41]
		TOSC assay	[15, 42]
		β-Carotene bleaching assay	[15, 34, 43]
Non-radical redox potential-based assays	ET assay	FRAP assay	[13, 15, 20, 34]
		CUPRAC assay	[21, 35, 44]
		Nanoparticles based assays	[15, 45–47]
		• colorimetric detection, AuNPs- and AgNPs-based assays	[15, 45–47]
		• electrochemical detection, AuNPs-based assays	[15, 45–47]
		• magnetic NPs-based assays	[48]
		Electrochemical methods	[49–51]
		• cyclic voltammetry (CV) based assays	[52, 53]
		• diferential pulse voltammetry (DPV) based assays	[54]
		• Square wave voltammetry (SWV) based assays	[55]
		• Amperometry, biamperometry-based assays	[56–58]
		Metal chelation capacity	[13, 59]
		Total phenolic content (TPC)	[60, 61]
Biochemical based assays and *in vivo* assays		Oxidation of low density lipoproteins (LDL) assay	[18, 62]
		The thiobarbituric acid reactive substances (TBARS) assay	[18, 63, 64]
		Cellular antioxidant activity assay	[18, 65, 66]

Table 1.
Classifications of antioxidant capacity/activity assays.

2. Chemical based assays

2.1 Radical/ROS scavenging assays

2.1.1 Scavenging ability toward stable free radicals ABTS$^{\bullet+}$ and DPPH$^{\bullet}$

2,2′-azino-bis(3-ethylbenzothiazole-6-sulphonate) radical cation, ABTS$^{\bullet+}$ and 2,2-diphenyl-1-picrylhydrazyl, DPPH$^{\bullet}$ are colored and stable free radicals that have been largely used to measure antioxidant capacity. DPPH$^{\bullet}$ is commercially available, but ABTS$^{\bullet+}$ must be produced from the oxidation of ABTS with chemical reagents such as $K_2S_2O_8$, MnO_2, etc. ABTS$^{\bullet+}$ is soluble in aqueous and in alcoholic media (λ_{max} 734 nm), while DPPH$^{\bullet}$ is soluble in different organic solvents (λ_{max} 517 nm, in ethanol). The chemical structures of ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ are presented in **Figure 1**.

The trolox equivalent antioxidant capacity (TEAC/ABTS) assay based on the use of ABTS$^{\bullet+}$ radical cation and DPPH$^{\bullet}$ radical-based (DPPH) assay are among the most used antioxidant capacity assays.

In TEAC/ABTS assays, the antioxidant capacity is evaluated as the capability of analyzed sample to diminish the color intensity after reacting with the ABTS$^{\bullet+}$ radical. This assay can be employed for lipophilic as well as hydrophilic compounds. The assay is technically simple, being widely applied for screening and habitual determinations. Most often, ABTS$^{\bullet+}$ is produced by oxidation of ABTS with $K_2S_2O_8$. The reaction of antioxidants with ABTS$^{\bullet+}$ is quite fast. Generally, the measurements are done after a fixed period of time. The TEAC/ABTS assays were recently investigated with regards to their basic chemistry, reaction stoichiometry and the reaction pathways behind the ABTS/potassium persulfate decolorization assay [24].

A recent review [67] of TEAC/ABTS assays gives a comprehensive insight into this approach for evaluating the antioxidant capacity, including different methods of ABTS$^{\bullet+}$ generation, experimental design, and quantification strategies, as well as TEAC value data collection obtained using a diversity of samples. Other recent reviews regarding both ABTS/TEAC and DPPH assays can be found in [1, 5, 18, 34].

A comprehensive critical evaluation of the TEAC/ABTS, DPPH, and oxygen radical absorbance capacity (ORAC) assays, presented in [25] discusses the different methods, the intrinsic mechanisms of reactions, the advantages and disadvantages, the limitations and recommendations for applications of the methods.

The TEAC method has several advantages:

- It allows the assessment of a plethora of synthetic as well as natural antioxidants (phenols, peptides, thiols, indols, flavonoids, aminoacids, carotenoids, tocopherols, vitamin C, etc.).

- It can be applied over a large pH range.

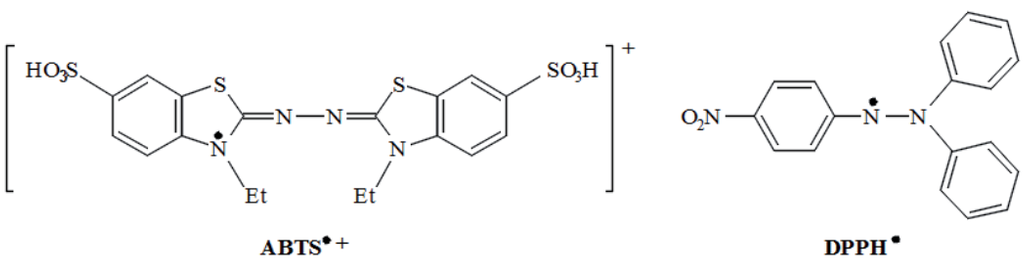

Figure 1.
Chemical structures of 2,2′-azino-bis(3-ethylbenzothiazole-6-sulphonate) radical cation, ABTS$^{\bullet+}$ and 2,2-diphenyl-1-picrylhydrazyl, DPPH$^{\bullet}$ radical.

- The solubility of $ABTS^{\bullet+}$ in buffered and organic media enables measurement of both hydrophilic and lipophilic antioxidant activities.

- ABTS is affordable and easy to use.

Disadvantages of TEAC include:

- For some antioxidants different TEAC values may be obtained, depending on the way in which $ABTS^{\bullet+}$ is generated and on the measurement time interval selected.

- $ABTS^{\bullet+}$ (the same is applicable for $DPPH^{\bullet}$) is a metastable radical that does not exist in nature, being a "non-physiological" radical.

- The results of the assays depend on the reaction time. Some antioxidants react very fast and completely while other react slowly or combine a mix of fast and slow reactions [68].

- In the TEAC the molecular size and steric hindrance is an important characteristic. The accessibility of polyphenolics with bulky substituents to the radical cation ABTS is sterically restricted.

The DPPH assay is low-cost and simple and consequently has been largely used in laboratory settings for many applications. The assay is based on measuring the decrease of the absorbance of $DPPH^{\bullet}$ radical (at a wavelength of 517 nm) as a result of its reaction with antioxidants from the sample. This method was criticized for lacking standardization in different stages of the analytical process [37].

The criticism regarding DPPH assay is expressed even harder in [25]:"The DPPH reaction has been used as if it is a simplistic chemical "black box" – reagents are mixed and a number is generated, and the chemistry occurring between is ignored." In fact, antioxidant reactions with DPPH reagent are actually complex and reaction curves show multiple reactivity patterns [69]. DPPH reactions are very sensitive to the reaction medium, such as: water and solvent, pH, light exposure, dissolved oxygen, pH, etc. [69, 70].

The disadvantages of DPPH assay consist of the following:

- The evaluation of antioxidant capacity by the change in $DPPH^{\bullet}$ absorbance has to be carefully evaluated since the absorbance of $DPPH^{\bullet}$ after reaction with an analyzed sample may be diminished by some other factors (pH, O_2, light, type of solvent, etc.).

- Fixed-time assays may undervalue the radical scavenging capacities of slow-reacting antioxidants.

- Since the ionization of phenols – and consequently the reaction rates – are highly influenced by solvent composition and pH, the DPPH assay is not adequate to ranking antioxidant compounds and natural extracts.

In essence, the significant shortcomings of both TEAC/ABTS and DPPH assays are related to the intricacy of the mechanisms of reaction with antioxidants, the big influence of the experimental conditions on the obtained results, and the important difference between $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ chemical structures and those of free radicals existing in biological systems.

2.1.2 Oxygen radical absorbance capacity (ORAC) assay

The ORAC method determines the radical chain breaking capacity of antioxidants by measuring the blocking-up of peroxyl radical generated oxidation. The peroxyl radical reacts with a probe (usually fluorescent) to form a non-fluorescent product, and the process can be monitored with a good sensitivity by fluorescence. Antioxidant capacity is determined by measuring rate and amount of product generated over time. Competition between reaction of probe and antioxidants with the $ROO^{\bullet}$ radical (or other ROS/RNS) constitute the premise of the assay.

Peroxyl radicals ($ROO^{\bullet}$) are the main free radicals that act in lipid oxidation in biological environment under physiological circumstances and in foods. For this reason, ORAC assay could be considered to have a biological concern as a reference for antioxidant efficacy. Commonly, 2,2′-azo bis(2-methylpropionamidine) hydrochloride (AAPH) is employed as $ROO^{\bullet}$ source that generates peroxyl radical at a known rate at incubation in aqueous media. The reactions involved in ORAC assay are as follows:

$$AAPH + O_2 \rightarrow 2ROO^{\bullet} + N_2 \tag{6}$$

$$ROO^{\bullet} + Probe_{fluorescent} \rightarrow ROOH + Oxidised\ Probe_{non-fluorescent} \tag{7}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \tag{8}$$

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \tag{9}$$

The antioxidant capacity is measured by a diminished rate and through the quantity of product generated over time. A set of fluorescence decay curves can be obtained with or without antioxidants. The difference in the area under the curves (AUC) between the curves recorded in the presence and in the absence of the oxidant is considered to be a marker of the peroxyl radical scavenging capacity. Usually trolox (a standard antioxidant) is employed as reference and the obtained ORAC

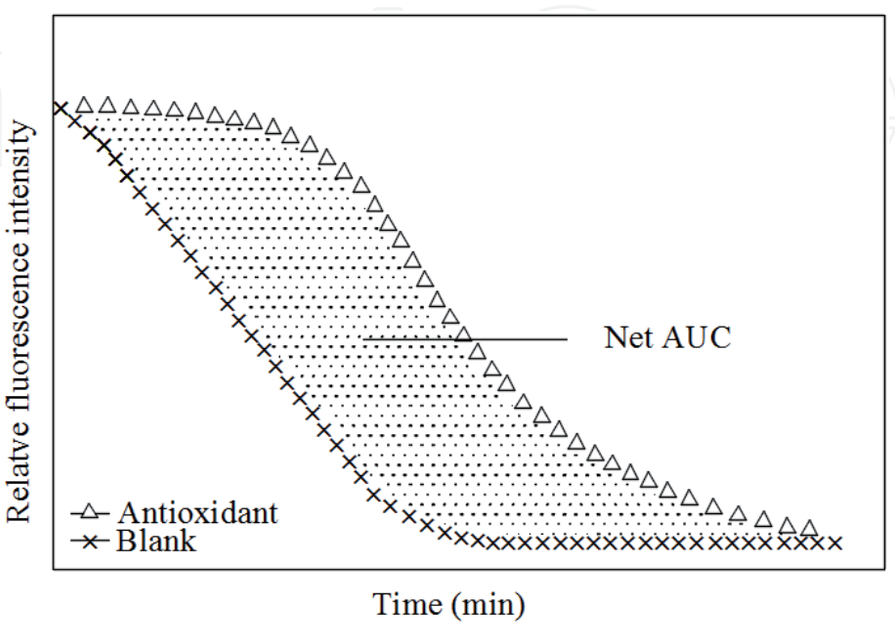

Figure 2.
ORAC antioxidant capacity of a sample expressed as the net AUC.

values are provided as trolox equivalents of the tested antioxidants. Data are shown as micromoles of trolox equivalents (TE) per liter or per gram of sample (μmol of TE/L or μmol of TE/g). The ORAC antioxidant capacity of a sample shown as the net area under the curve (AUC) is presented in **Figure 2**.

ORAC assay is a HAT-based method because it measures the capacity of hydrogen atom donating ability of antioxidants. β-phycoerythrin (β-PE), a protein obtained from *Porphyridium cruentum*, was employed as the fluorescent probe in the first studies. However, the use of β-PE in antioxidant assays has several shortcomings and can cause false ORAC values. The currently preferred fluorescent probes are fluorescein and dichlorofluorescein diacetate [37], as they are more stable and less reactive. Nevertheless, fluorescein may undergo undesired fluorescence quenching and side reactions [71] and other fluorescent probes have been suggested in consequence.

In order to measure both hydrophilic as well as 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 of the antioxidants [72, 73].

The ORAC method has the utility to be a simple and standardized assay, however, secondary reactions can occur, affecting the reported results. For example, it was reported that antioxidant-metal reactions could result in a smaller concentration of antioxidants and hence to a depreciation of the ORAC value [74].

The ORAC method can be readily automated and it is perhaps the most largely recognized of all the antioxidant methods.

2.1.3 Chemiluminescence methods

The fundamental chemistry of chemiluminescence measurements of antioxidants is based on the reaction of ROS/RNS species with special reagents to generate species in an excited state that light up (chemiluminescence). The chemical compounds that react with the initiating reactive species diminish the light generation. Hence, generally, chemiluminescence measurements for antioxidant capacity assay are based on competitive reactions. By changing the oxidant initiator (e.g., $O_2^{\bullet}$, $HO^{\bullet}$, $ROO^{\bullet}$, $ONOO^{-}$, HOCl, $^1O_2$, etc.) it is possible to measure the capacity of quenching of different ROS/RNS by an antioxidant [37]. Chemiluminescence is a highly sensitive analytical method. The detection limit is very low, below that of most chemical methods. The mainly used chemiluminescence reagents are luminol [37, 75–79], lucigenin [39], pholasin (a bioluminescent protein) [80] and peroxyoxalate [81]. Luminol is the main commonly employed aqueous chemiluminescent reagent. Luminol reacts with an oxidizing agent, hydrogen peroxide (in presence of a catalyst) to yield 3-aminophthalate in an excited electronic state, which emits light. Antioxidants can quench the produced ROS (by hydrogen peroxide) and diminish hydrogen peroxide-induced chemiluminescence.

Chemiluminescence method has been automated in flow-based assays, e.g., flow injection analysis (FIA) [29, 76, 79, 82], sequential injection analysis (SIA) [83, 84], multi-syringe FIA (MS-FIA) and multi commutation.

A review on antioxidant assays with chemiluminescence detection is presented in [40] and other more general reviews of antioxidant assays including methods with chemiluminescence detection are presented in [1, 16, 17].

The methods for the determination of lipid hydroperoxides and of the antioxidant capacity of lipids by using flow injection analysis with chemiluminescence reagents are reviewed in [29].

The TAC of some *Rosmarinus officinalis* L. (rosemary) extracts was measured by an in batch analytical method based on Co(II)-ethylendiaminetetraacetic acid

(EDTA)-induced luminol-hydrogen peroxide chemiluminescence (luminol/Co(II) EDTA/H_2O_2) [75]. The method allows for TAC determination in the range 10^{-5}–2.5 10^{-3} moles L^{-1} of gallic acid equivalent. The same in batch method was applied for the TAC determination of fruit juices and noncarbonated soft drinks [77] and fruit seeds extracts [85].

The luminol/Co(II)EDTA/H_2O_2 system with chemiluminescence detection was used also in a flow injection analysis (FIA) method for the total antioxidant capacity determination of wines [79] and culinary and medicinal plants extracts [76].

Amperometric TAC measurements of several plant extracts using an electrochemical gold nanozyme-sensor based on the enzyme-like catalytic activity of gold nanoparticles [58] were associated with those obtained from a chemiluminescence method reported in [75]. A good correlation has been found between the two methods (Pearson's correlation coefficient of 0.958).

A new microfluidic chemiluminescence method for fast determination of the TAC of apple and pomegranate juices and honey samples was reported in [86]. The method is based on the $NaHCO_3$-H_2O_2-Co^{2+} chemiluminescence reaction.

A chemiluminescence-sensing platform for the determination of natural antioxidants and imaging of their tissue distribution is reported in [87]. The chemiluminescence radiation is emitted upon the redox reaction of antioxidants (e.g., L-ascorbic acid) with quinones (e.g., menadione), in the presence of luminol.

Different chemiluminescent system that allow the evaluation of both hydrophilic and lipophilic antioxidants by using the same method were reported. Thus, lucigenin–hydrogen peroxide chemiluminescence in 2-propanol has been proposed to measure the activity of both hydrophilic and lipophilic antioxidants [88].

A peroxyoxalate–hydrogen peroxide–imidazol–fluorophore system was applied in the evaluation of antioxidants in olive oils and honey samples. The system relies on a furan dicarboxylate derivative as fluorophore [81].

2.1.4 Other radical/ROS scavenging assays

Total radical-trapping antioxidant parameter (TRAP) assay. This method generally measures the antioxidant's capability to interfere with the reaction between $ROO^{\bullet}$ (usually generated from AAPH) and a probe. It is relatively complex and laborious to perform [39 [37]. An early review of TRAP assay is presented in [89].

A TRAP assay for measuring total plasma antioxidant capacity used R-phycoerythrin (red protein pigments from the cells of red algae) as a fluorescent probe and AAPH, as $ROO^{\bullet}$ radical generator [41]. Fluorescence quenching was measured in absence and in presence of the analyzed antioxidant samples. The quantification of antioxidants is based on the duration of the lag phase.

Initiators for $ROO^{\bullet}$ radicals have been produced selectively by azides, enzymes (e.g., horseradish peroxidase) [90], or H_2O_2-hemin [91], etc. Some of the probes used in TRAP assays include fluorescein, dichlorofluorescein diacetate [92], R-phycoerythrin [93] and luminol [90].

It was reported that an important limitation of the TRAP assay is the use of the lag phase for determination of antioxidant capacity because not all antioxidants have a clear lag phase [94].

Total oxy radical scavenging capacity (TOSC) assay. The assay is based on the determination of antioxidants particularly toward three strong oxidants ($^{\bullet}OH$, $ROO^{\bullet}$, and $ONOO^{-}$) [15, 42]. In TOSC assay the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene by ROS and ethylene formation was determined by head space gas chromatography relative to a reference reaction. The antioxidants compete with KMBA for ROS and the formation of ethylene is inhibited.

The most important drawback of this assay is the long reaction time (hundreds of minutes) and the necessity of several chromatographic analyses for each experiment [21].

β-Carotene bleaching assay. This assay 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, owing to exposure to dissolved O_2, promoted by thermal induction. The measurements are done typically at 50 °C. Quantification is based on varying the rate at which β-carotene absorbance decays (at a wavelength of about 470–490 nm) in the presence of increasing concentrations of the antioxidant or prooxidant under evaluation. The decolorization is due to the breaking of π-conjugation by the addition reaction of radicals into a C=C bond of β-carotene [34]. The antioxidant capacity/activity is calculated in terms of % inhibition with regard to the reference.

An investigation of the experimental conditions that influence β-carotene bleaching assay is presented in [43] and in [95]. The β-carotene bleaching assay can screen both lipophilic and hydrophilic samples. It is sensitive to temperature, oxygen, pH and solvent effects and is time-consuming (an assay last hundreds of minutes).

2.2 Non-radical redox potential-based assays

2.2.1 Ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) assays

Ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) assays were reviewed in several recent papers [13, 14, 20, 21, 34, 35].

FRAP assay is based on antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe^{3+}-(TPTZ)_2]^{3+}$ to the blue colored ferrous complex, $[Fe^{2+}-(TPTZ)_2]^{2+}$ in acidic medium (pH 3.6). Measuring the increase in absorption at 593 nm monitors this reduction. The antioxidant capacity is expressed as μM Fe^{2+} equivalents or as a standard antioxidant equivalents. The FRAP assay is conducted at acidic pH 3.6 in order to prevent iron precipitation.

The reaction detects compounds with redox potentials lower than 0.7 V so FRAP is an adequate screen for the capacity to maintain redox status in cells or tissues. FRAP cannot measure compounds that act by radical quenching (H transfer), specifically bio-thiols (such as glutathione) and proteins [96]. For this reason the method is rather inadequate to measure the antioxidant capacity of intracellular fluids and human plasma/serum [97, 98].

Because the redox potential of $[Fe^{3+}-(TPTZ)_2]^{3+}$ is similar to $ABTS^{\bullet+}$ potential (0.68 V), similar compounds react in both the FRAP and TEAC assays. The FRAP mechanism is totally electron transfer and not mixed ET and HAT, and so in association with other antioxidant methods can be very useful in differentiating preponderant mechanisms with different antioxidants [37].

FRAP really determine only the reducing capacity based upon the ferric ion, which is not relevant to antioxidant capacity physiologically and mechanistically. However, in contrast to other assays of TAC, the FRAP method is simple, fast, inexpensive and robust and does not necessitate special equipment.

Cupric reducing antioxidant capacity (CUPRAC) assay. The method measures the reducing power of antioxidants to convert cupric (Cu^{2+}) to cuprous (Cu^{+}) ion. The copper reducing ability is measured by complexation of Cu^{+} with bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) or neocuproine (2,9-dimethyl-1,10-phenanthroline) the corresponding complexes having absorption maximum at 490 nm and 450 nm, respectively [99]. **Figure 3** presents the cupric reducing antioxidant capacity (CUPRAC) reaction mechanism.

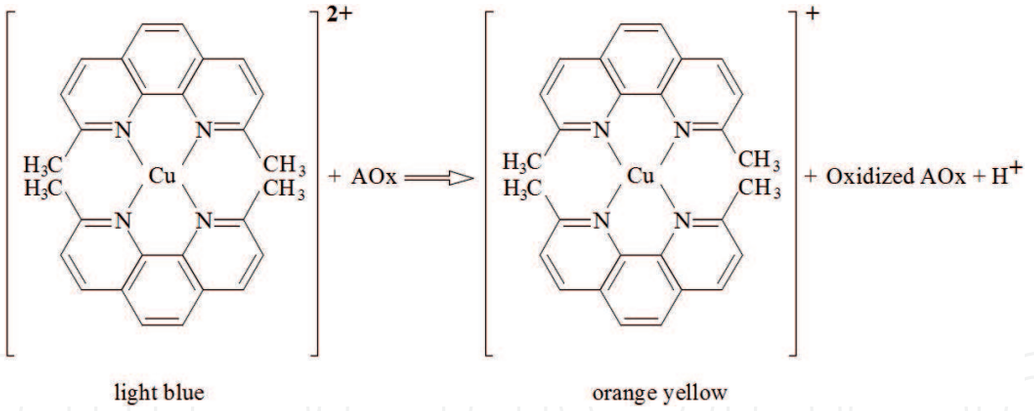

Figure 3.
Cupric reducing antioxidant capacity (CUPRAC) reaction mechanism.

FRAP and CUPRAC have comparable values with TEAC values (with some exceptions) since similar redox potential probes are employed in the assay. The original CUPRAC assay has been modified in order to allow analysis of different samples, e.g., acetone/water medium containing methyl-β-cyclodextrin has been employed for simultaneous assay of hydrophilic and lipophilic antioxidants [100].

In another modified CUPRAC assay, an optical sensor containing immobilized CUPRAC reagent Cu^{2+}–neocuproine complex onto a perfluorosulfonate cation-exchange polymer (Nafion) membrane matrix was developed. The measurements of absorbance were done at 450 nm [101].

CUPRAC assay is more selective due to its lower redox potential than that of redox couples like Ce^{4+}/Ce^{3+} and Fe^{3+}/Fe^{2+} [102]. The CUPRAC assay have been discussed in a comprehensive review in [44].

2.2.2 Nanoparticles based assays

For the determination of antioxidants, nanoparticles (NPs) can be employed as electrochemical or colorimetric probes, components in chemical and biological detection systems, and for radical generation. Several reviews regarding TAC determination by using NPs can be found in literature [18, 45–47, 103].

Chemical reduction-based nanotechnological assays of colorimetric TAC measurements make use of the generation or growth of noble metal nanoparticles (AuNPs, AgNPs, etc.) upon reaction of Au^{3+} or Ag^{+} salts with antioxidant. The strong visible light absorption at a specific wavelength results from the surface plasmon resonance absorption of metal nanoparticles.

In a pioneering work, reported in [104] the antioxidant capacity of several phenolic acids was determined from the formation and growth of gold nanoparticles (AuNPs). The same experimental approach was employed in [105] to evaluate the antioxidant capacity of chrysanthemum extracts and tea beverages.

A comparison of a AgNPs-based method for TAC assays in different rapeseed varieties with those of several spectrophotometric methods (total phenolic with Folin–Ciocalteu reagent, FRAP and DPPH assays) was performed in [106]. A significant correlation (r: 0.59–0.91) was found between the spectrophotometric methods and the nanoparticle-based assay.

Another interesting alternative, an optoelectonic tongue based on an array of gold and silver nano-particles for analysis of a diversity of natural, synthetic and biological antioxidants is described in [107].

A portable nanoparticle based-assay for rapid and sensitive measurement of food antioxidants was proposed in [108] based on the use of immobilized ceria

(cerium oxide) nanoparticles. Due to the reversible oxidation state of cerium Ce^{3+}/Ce^{4+} on the NPs surface, nanoceria is capable of changing redox states and surface properties after interaction with antioxidants.

Furthermore, a novel chemical sensing array, based on metal oxide nanoparticles (i. e., cerium oxide, titanyl oxalate, TiO_2, Fe_2O_3, ZrO_2, ZnO and SiO_2) immobilized onto cellulose, was described as a portable and cheap paper-based colorimetric assay for polyphenol detection and field evaluation of antioxidant containing samples [109].

Last but not least, a novel method was proposed in [110] for evaluating the composition of mixtures of natural polyphenolic compounds by using an array of nano-oxides sensors and by chemometric analysis of the experimental data.

Some spectrometric and electrochemical nanomaterial-based assays for antioxidant assessment are presented in **Table 2**.

The nanoparticle-based assays to evaluate antioxidant capacity of natural products embody a novel and promising domain melding nanoscience with food and health research [18, 47].

	Antioxidant	**Nano-material**	**Detection principle**	**Real samples**	**Reference**
Spectro-metric	Total polyphenols in fat-rich samples	AuNPs	Detection of polyphenols in organic medium without extraction, by AuNPs formation at 540 nm	Chocolate, olive oil	[111]
	Polyphenols in food	AuNPs	Detection of polyphenol-mediated AuNPs formation from extracts via LSPR* by UV- visible spectroscopy at 540 nm	Tea, apple, pear, wine, honey	[112]
	Polyphenols	AuNPs	Au reduction, mild conditions, LSPR* detection	Fruit extracts	[113]
	Polyphenols	AgNPs	AgNPs seed-growth, LSPR* detection	Fruit juices, olive oils	[114]
	Polyphenols	AgNPs	AgNPs seed-growth, LSPR* detection	Ginger	[115]
	Total catechins evaluation	RhNPs	RhNPs LSPR* shifting	Teas	[116]
	Polyphenols	CdTe QDs**	CdTe QDs** fluorescence quenching inhibition	Teas	[117]
	Polyphenols	Graphene QDs	Graphene QDs fluorescence quenching	Olive oil extracts	[118]
	Phenolic acids	AuNPs on paper	Reduction of gold ions to AuNPs on paper sensors and measurement of the resultant color intensity	Tea, red wine	[119]

	Antioxidant	Nano-material	Detection principle	Real samples	Reference
	Flavonoids	Fluorescent gold nanocluster	Fluorescence quenching of gold nanoclusters imbedded into the cavity of bovine serum albumin tertiary structure	Serum, plasma, pharmaceutical analysis	[120]
Electrochemical	*tert*-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA)	Nanocarbon black	Measurements in the presence of the cationic surfactant CPB*** by square wave voltammetry using a carbon black paste electrode	Food samples and biodiesel	[121]
	Gallic acid	$NiAl_2O_4$ glassy carbon nanocomposite	Cyclic voltammetry and amperometry with a $NiAl_2O_4$ glassy carbon working electrode	Food samples	[122]
	Flavonoids (myricetin and rutin)	Single-walled carbon nanohorns	Based on host–guest supramolecular recognition concept	Human serum	[123]
	Antioxidant capacity	Multi-walled carbon nanotubes	Chronocoulometry at glassy carbon electrode modified with multi-walled carbon nanotubes	Red/white wine	[124]
	Antiioxidant capacity (o-diphenols)	Cerium (IV)oxide NPs	Polyphenols oxidation at quinones, quinones reduction at screen printed carbon electrode-CeO_2(IV) NPs	Red/white wine	[125]

*Laser surface plasmon resonance.
**Quantum dots.
***Cetylpyridinium bromide.

Table 2.
Spectrometric and electrochemical nanomaterial-based assays for antioxidant assessment.

2.2.3 Electrochemical methods

Electrochemical techniques emerged as an alternative strategy for a quick, precise, and cost-effective determination of the TAC of different samples, e.g., foods and beverages, plant extracts, etc. They circumvent some of the drawbacks of spectrophotometric methods such as long analyses and sample preparation time, the use of expensive reagent and undefined reaction time. These methods also enable the quantification of the antioxidant compounds, with very good sensitivity [126, 127] and sometimes, they permit determinations in the presence of compounds that interfere in other methods, such as the case of ascorbic acid in juice [128].

Electrochemical methods for antioxidant capacity/activity evaluation have been reviewed in [129] and more recently in [14, 49–51, 130]. The most commonly used electrochemical techniques for antioxidant assays in different samples are

cyclic voltammetry, differential pulse voltammetry, square wave voltammetry and amperometry.

Cyclic voltammetry (CV) [131]. The half-wave potential ($E_{1/2}$) of the registered cyclic voltammogram indicates a specific constituent in the analyzed sample (its ability to donate electrons) whereas the maximum current intensity indicates the concentration of a constituent. Antioxidants with similar structures have similar electron donating abilities and therefore similar half-wave potentials in cyclic voltammetry. Thus, when present in mixtures, they contribute globally to the observed features of the sample cyclic voltammogram.

Cyclic voltammetry has been widely used for evaluating the TAC of low-molecular weight antioxidants present in biological fluids, animal plasma, plants and fruits [52].

In [53] the results obtained for the TAC determination of 10 different fruit tea infusions using spectrophotometric methods (TEAC/ABTS, FRAP, DPPH and Folin–Ciocalteu's reagent total phenolic content) and by applying the CV method were reported comparatively.

In addition, CV has been used to measure the antioxidant capacity of a diversity of samples such as different winemaking by-products (pomace, skins, seeds, and stems) [132], propolis [133], edible oils [134] and berry fruits [135], among others.

Differential pulse voltammetry (DPV) has been applied for TAC assay of white and red wines [54] by using gallic acid as reference. The elaborated method is based on gallic acid electro-oxidation at carbon nanotubes-modified carbon paste electrode, at 350 mV (vs. Ag/AgCl) in 0.1 M phosphate buffer solution (pH = 2.50). The method enabled a reliable evaluation of the TAC for red and white wine samples, when glucose and ascorbic acid do not interfere.

Square wave voltammetry (SWV) has been used to analyze catechins in green and black teas [55] obtaining a detection limit of 40 nM for epigallocatechin gallate in green teas.

A databank of the content of antioxidants in food products was created based on amperometric measurements [56]. The antioxidants were quantified in 1140 food products, beverages, etc.

Amperometric, CV and DPV measurements using an electrochemical gold nanozyme-sensor [58] (based on the enzyme-like catalytic activity of gold nanoparticles), were used to evaluate the TAC of several plant extracts. The results of the amperometric measurements were compared to those from a chemiluminescence method for TAC assays [75] and a good correlation was found.

Biamperometric determinations are based on the reaction of the analyte with a redox pair such as I_2/I^-, Fe^{3+}/Fe^{2+}, $DPPH^{\bullet}$/DPPH, $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. $DPPH^{\bullet}$/DPPH biamperometry was used in the analysis of fruit juices for the determination of their TAC, using two identical Pt electrodes [57] and for tea, wine and coffee using glassy carbon electrodes [136].

Analytical characteristics of some electrochemical methods applied for the determination of antioxidants or total antioxidant capacity are presented in **Table 3**.

Electrochemical measurements of antioxidant capacity are redox-based methods with many advantages over conventional chemical assays since they are rapid and simple and do not require special chemical reagents or complicated sample preparation. Thus, they allow analysis of colored samples that do not permit direct evaluation by spectrophotometric techniques (e.g., wine and fruit juice) [150]. Electrochemical techniques allow also a large number of experimental parameters to be easily controlled and to register important information from a sample (e.g., the half-wave potential, the voltammetric charge, peak current intensity, etc.) that helps characterize different compounds from a sample [53]. These methods can be used to evaluate samples of whatever lipophilicity or hydrophilicity [151].

Electrochemical method	Electrode	Antioxidants	LOD/Linear range	Real samples	References
Cyclic voltammetry	Iridium-containing carbon (Ir-C)	Caffeic acid	0.0–25.0 mg L^{-1}	Wine	[137]
	Graphite Carbon microspheres Carbon nanotubes	Vanillic acid	2.85 μM 3.82 μM 4.13 μM/ 10–400 μM	Artificial wine solutions	[138]
	Glassy carbon	Curcumin	4.1 × 10^{-6} M	Spices	[139]
	Carbon ink chemically modified electrode containing [Cu(neocuproine$_2$)] (NO_3)$_2$	Trolox Gallic acid Ascorbic acid	2.51 × 10^{-5} M - -	Teas	[140]
	Glassy carbon electrode	TAC*	2–80 μmol L^{-1} trolox	Berry fruits	[135]
	Glassy carbon disc electrod	(+)-catechin as standard	0.0078 to 1 mM	Food grade oenological tannins	[141]
Differential pulse voltammetry	Carbon paste platinum	Ascorbic acid	0.02 mM/0.07–20 mM; 0.087 mM/ 0.31–20 mM	Fruit juices and wines	[142]
	Carbon nanotubes modified carbon paste	TAC* (vs gallic acid)	3.0 x 10^{-7} M/ 5.0 × 10^{-7}- 5.0 × 10^{-5} M	Red and white wines	[54]
	Dropping mercury	Gallic acid	0.3 μM/ 1.0–50 μM	Fruit juices	[143]
	Glassy carbon electrode surface activated by *in situ* chemical oxidation	Tertiary butyl hydroquinone	67 nM/ 1.0 μM – 1.1 mM	Jatropha biodiesel	[144]
Square wave voltammetry	4-[(4-decyloxyphenyl)- ethynyl]-1- methylpyridinium iodide modified glassy carbon	Total phenolic compounds (vs caffeic acid)	9.0 × 10^{-7} mol L^{-1}/ 9.9 × 10^{-7}- 3.8 × 10^{-5} mol L^{-1}	Total polyphenol content of Yerba mate extracts	[145]

Electrochemical method	Electrode	Antioxidants	LOD/Linear range	Real samples	References
	Screen printed electrode modified with CeNPs. (CeNPs/C/SPE)	Gallic acid Caffeic acid Quercetin *t*-resveratrol	7.0 μM 10.0 μM 9.0 μM 8.0 μM	White/red wines	[146]
Amperometry	Biosensor based on peroxidase-modified carbon paste	*t*-resveratrol Caffeic acid	0.023 mg L^{-1}/ 0.05–52 mg L^{-1} 0.020 mg L^{-1}/ 0.06–69 mg L^{-1}	Wine	[147]
Amperometry (flow injection)	Carbon nanotube modified-glassy carbon electrode	Gallic acid Catechin Quercetin Caffeic acid Trolox TAC* (vs trolox)	0.04 μM 0.02 μM 0.03 μM 0.08 μM 0.04 μM	Thai vegetables/herbs	[148]
Amperometry (flow injection)	Glassy carbon/carbon nanotubes/polyethyleneimine electrode	Caffeic acid, gallic acid Ferulic acid *p*-coumaric acid	< 0.1 μM/ 10^{-7}–10^{-4} M	Wines	[149]

**Total antioxidant capacity.*

Table 3.
Analytical characteristics of some electrochemical methods applied for the determination of antioxidants or total antioxidant capacity.

A disadvantage of the electrochemical methods of antioxidant capacity determination in complex media is the difficulty to analyze the macromolecules with antioxidant proprieties.

2.3 Metal chelating assay

Metal chelation capacity is evaluated by measuring the chelating effect of antioxidants for metal ions. Fe^{2+} ions are known to enhance lipid peroxidation trough Fenton reaction and also by decomposing lipid hydroperoxides into peroxyl and alkoxy radicals, which are more reactive. By Fenton reaction (written below), the ferrous ions produce $^{\bullet}OH$ radicals, which are highly reactive, and contribute appreciably to oxidative stress. The resulting hydroxy radicals cause damage to proteins, carbohydrates, cellular lipids and nucleic acids leading to cellular damage.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \tag{10}$$

Numerous metal ions such as Cu^{+}, Ti^{3+}, Cr^{2+}, and Co^{2+} and their complexes in their lower oxidation states react with H_2O_2 in a similar manner as Fe^{2+}, and the mixtures of these metal ions with H_2O_2 were named "Fenton-like" reagents [96].

Metal chelation capability could be used as an indicator of antioxidant capacity. Chelating agents stabilizing the oxidized form of the metal ions are effective as secondary antioxidants.

Commonly, metal chelation capacity is evaluated by determining the chelating effect of antioxidants for ferrous ion [59]. The evaluation of the metal-chelating activity of an antioxidant is based on the absorbance measurement of Fe^{2+}-ferrozine complex in presence and in absence of the analyzed sample. The decrease in absorbance of the solution after the introduction of test sample is related to the metal chelation capacity of the sample. The measurements are performed spectrophotometrically at 562 nm [13]. Ethylenediaminetetraacetic acid (EDTA) is generally used as a standard metal chelator. Metal chelation capability of different samples is expressed as EDTA equivalents.

In [38] the results obtained at the determination of metal chelation capacity for a number of antioxidants and extracts are presented. A study regarding the standardization of the experimental protocols to evaluate the capability to chelate Fe^{2+} (employing ferrozine as chromogenic reagent) and Cu^{2+} (employing pyrocatechol violet as the chromogen agent) is presented in [152]. This study used 96-well microplates and analyzed Brazilian coffees (n = 20).

2.4 Total phenolic content (TPC)

Total phenolic content (TPC) or Folin–Ciocalteu reducing (FCR) assay is an important parameter of total antioxidant capacity (TAC) and largely employed for evaluation of a diversity of samples. The TPC assay has been used for a long period as a measure of total phenolic content in natural products [37]. In this method, TPC values are evaluated as equivalents of gallic acid or another phenolic compound, e.g., caffeic acid, catechin, ferrulic acid, etc.

The Folin Ciocalteu reagent contains phosphomolybdic/phosphotungstic acid complexes, with added lithium sulfate and bromine, in strong basic medium (5–10% aqueous Na_2CO_3, pH 10–12) to generate the phenolate anion [153]. The TPC method is based on the measurement of the blue-colored chromophore (λ_{max} = 620–765 nm) generated as a result of reduction of Folin Ciocalteu reagent with phenols from the sample [154]. The reduction site is considered the molybdenum centre in the complex (Mo^{6+} ion is reduced to Mo^{5+} by phenols).

TPC assay is operationally simple, reproducible and convenient for evaluation of total phenolic for a variety of samples because the reagent is commercially available.

However many non-phenolic compounds e.g., ascorbic acid, aromatic amines, sulfur dioxide, some metal ions (Cu^{+} and Fe^{2+}), etc. can interfere by reducing Folin Ciocalteu reagent. Several methodologies have been studied to increase the selectivity of the TPC method for total phenolic determinations in plant extracts [60].

A critical review of the methods for the assays of TPC in food matrices is presented in [61]. The review focuses on the most used methods to measure by UV-Vis spectrometry the TPC, *o*-diphenols, flavonoids, flavonols, anthocyanins, and tannins. Examples of application of TPC assay for winemaking byproducts (seeds, skins, stems, and pomace) and in Venezuelean propolis are given in [155, 156], respectively.

The TPC assay is still widely employed. However solid phase extraction (SPE) was considered as clean-up step in only a few cases. When SPE was employed, the SPE-FCR assay presented excellent reproducibility [157].

TPC assays are low-cost, simple, do not require expensive equipment and they are used largely to evaluate a big diversity of samples.

3. Biochemical-based assays and *in vivo* assays

3.1 Oxidation of low density lipoprotein (LDL) assay

A review of this assay alongside other assays measuring lipid oxidation can be found in [62]. The oxidation of LDL generated by ROS/RNS was studied long ago. ROS play an very important role in the initiation, propagation and termination reactions of the LDL lipid peroxidation. The lipid peroxidation processes could be followed by different methods, e.g., UV spectrophotometry and/or chemiluminiscence techniques. As an initiator of LDL oxidation is commonly employed cupric sulfate. By using a spectrophotometric methods the formation of diene conjugates at 234 nm is measured. By using a chemiluminescence methods the emitted radiation is measured as a result of the formation of oxidative products. By mixing a cupric sulfate solution with LDL sample, the kinetic profiles correspond to the occurrence of a lag phase owing to the existence of endogenous antioxidants such as coenzyme Q and vitamin E in the LDL particle. Following the lag time, the peroxidation of lipids is measured as an growth of the analytical signal (absorbance or chemiluminescence intensity) that finally, after minutes or hours, hit a plateau. By adding an antioxidant to the reaction mixture the lag time is enhanced. The antioxidant capacity is evaluated by measuring of the lag time. The most important advantage of this method is the employment of a biological significant target.

3.2 The thiobarbituric acid reactive substances (TBARS) assay

Two review dedicated exclusively to TBARS assays are presented in [63, 64]. Important aspects of the TBARS assay such as state-of-the-art of the method, determination in physiological systems, assays in food systems and the employment of TBARS in antioxidant evaluation studies are presented in [63].

The thiobarbituric reactive substances (TBARS) assay is frequently used to evaluate lipid peroxidation. The method is based on the reaction of malondialdehyde (MDA) generated as an advanced product of unsaturated lipid degradation under the influence of ROS/RNS, with thiobarbituric acid (TBA) under acidic conditions and at high temperature (100 °C) [158]. It is obtained a characteristic colored product [MDA-$(TBA)_2$] which is measured spectrophotometrically at

532 nm. MDA is a marker of oxidative stress. It is formed from polyunsaturated fatty acids (PUFA) with at least three double bonds in their molecule. This method is not a selective assay for lipid peroxidation products because TBA reacts with a diversity of aldehydes, not only those generated in the lipid peroxidation process [14]. The lack of specificity of the method is emphasized by the designation: thiobarbituric acid reactive substances (TBARS). MDA formation is the most largely employed method for lipid peroxidation evaluation. The method was significantly enhanced by coupling with HPLC. Several food components such as sugar degradation products, proteins and Maillard browning products affect the measurements. The thiobarbituric acid reactive substances (TBARS) method is widely employed to evaluate antioxidant activity and lipid oxidation in a diversity of samples.

3.3 Cellular antioxidant activity (CAA) assay

Cellular-based antioxidant activity assays (CAA) are performed within the cell medium and are presumed to be biologically more appropriate than the respective chemical assays owing to their better representation of the physico-chemical characteristics of the medium [159]. At the cellular level the antioxidant outcome is not confined only to reactive species scavenging, but imply also gene expression, modulation of redox cell signaling and upregulation of detoxifying or antioxidant enzymes. Moreover, in order to assay antioxidant capacity/activity it is very important to take into consideration some features regarding the bioavailability of an antioxidant such as the uptake, the partitioning in membranes and the metabolism. CAA assay is very useful for the evaluation of a new antioxidant because the change of the redox state at the cellular level (caused by the antioxidant) is strongly influenced by the different cell components.

The principle of CAA is presented in **Figure 4**. The cell-permeable non-polar 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is used as a fluorescence probe. Within the cells this molecule is deacetylated by cellular esterases generating a polar molecule, 2′,7′-dichlorofluorescin (DCFH) which is captured in the cells. Afterwards, peroxyl radicals produced inside the cells from 2, 2′-azo bis(2-amidinopropane) dihydrochloride (AAPH) which cross easily the cellular membrane oxidize DCFH to form dichlorofluorescein (DCF) which is fluorescent. The fluorescence intensity generated within the cells is related with the extent of oxidation. The molecules with antioxidant properties scavenge peroxyl radicals and will decrease the generation of fluorescence. Consequently, the antioxidant activity of a sample can be evaluated by assessing the decrease in the cellular fluorescence.

Several reviews were published regarding this topic [18, 65, 66]. Based on the CAA concept introduced in [160], CAA was used to determine the antioxidant capacity of dietary supplements, foods and phytochemicals in cell cultures [159]. In this study, human hepatocarcinoma HepG2 cells were loaded with the redox sensor DCFH which is oxidized to fluorescent DCF by the $ROO^{\bullet}$ resulted from the thermal decomposition of AAPH. Antioxidants diminish the fluorescent radiation emitted by DCF. CAA is expressed as μmoles of quercetin equivalents per 100 μmol of tested pure compound or per 100 g product (vegetables, fruits, etc.). Several cell sorts have been employed for the CAA assay beyond HepG2, e.g., Caco-2 matured differentiated intestinal cells [161], human gastric adenocarcinoma cell line AGS [162], etc.

Cellular oxidative stress can also be elicited by exposing cell cultures to H_2O_2 (in the mM range) and then measuring fluorimetrically the oxidation of the probe (DCFH) [163].

Saccharomyces cerevisiae cells were employed in a CAA assay to measure antioxidant capacity of different types of products in living systems [164]. Pretreatment of

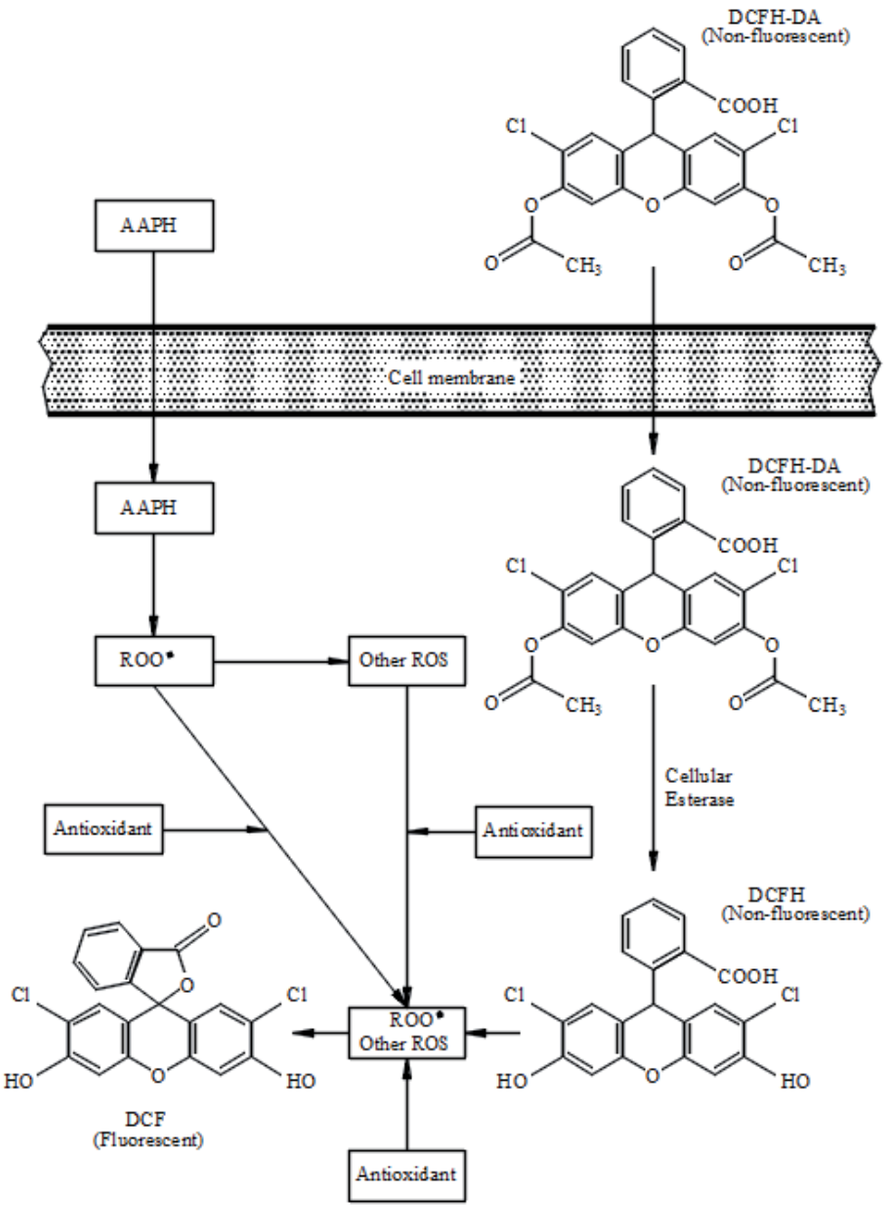

Figure 4.
Schematic presentation of cellular antioxidant activity assay. DCFH-DA, _ dichlorofluorescin diacetate; DCFH, 2′,7′-dichlorofluorescin; DCF, dichlorofluorescein; AAPH, 2, 2′-azo bis(2-amidinopropane) dihydrochloride.

the cells with different flavonoids [164] or mixtures of polyphenols [165] partially diminished the damage generated by H_2O_2.

S. cerevisiae as a model organism system for the antioxidant activity assessment of dietary natural products is reviewed in [65].

An investigation of antioxidant activities of 44 types of dark teas using the DPPH, ABTS, FRAP assays, and CAA assay (by using HepG2 cells) is reported in [166]. Correlation analysis indicated that there was a significant positive correlation between the levels of epigallocatechin gallate and the antioxidant activities evaluated using the ABTS and FRAP assays.

The CAA assay is an adequate and very good technique to measure the performance of antioxidants against oxidative stress. In this manner it is evaluated the capacity of a compound or a mixture of compounds to exercise an antioxidant response at the cellular level and to reduce intracellular oxidative stress, not just its capability as a reducing agent or its ROS/RNS scavenging ability. The CAA methodology is closer to a biological approach, and an antioxidant is regarded as a compound useful to modulate the redox state of the cell.

It is indicated to evaluate the antioxidant capacity of a sample by employing several chemical methods and CAA assays. The antioxidant capacities evaluated by CAA assays are not related well with their chemical values because the two types of methods are affected by very distinct factors.

4. Conclusions

Many studies were published concerning the antioxidant capacity of different products. However, with all these research efforts, a direct link between a food TAC value and health benefits was not found [12].

Taking into account the vast material published in the literature the following conclusion regarding the antioxidat assays can be drawn [14, 15]:

- The expression "total antioxidant capacity" (TAC) correspond to the cooperant effect of antioxidants existing in a sample (cumulative and maybe synergistic/antagonistic). It is a more adequate term to express the total antioxidant capability of a sample than the summation of individual antioxidant constituents.

- It is a stringent need to standardize the TAC assays and to formulate the results of measurements as equivalents of a standard material so that to enable relevant comparison between different methods and different samples [18].

- Most methods developed for TAC evaluation are not based on well detailed investigations of the chemical system involved in measurements (antioxidants interactions, pH, effect of solvents, kinetics, etc.) [16, 19].

- Many in vitro antioxidant methods are accomplished at pH values far from physiological pH and cannot have much sense for in *vivo* determinations of antioxidant effect.

- It is very useful to add a cellular-based assay to assess the analyzed sample capability to generate a cellular antioxidant response, in addition to its ability as a good scavenger of ROS/RNS [18].

- Potential mutual action of antioxidants (i.e., synergistic or antagonistic effects) or prooxidant actions of antioxidants (e.g., under the influence of the composition of the medium) should be taken into account [16, 167].

- For testing natural compounds it is necessary to employ several *in vitro* chemical-based assay that measures various facets of the reactivity of the antioxidants toward ROS/RNS [18]. Including a CAA assay is highly recommended [20].

Taking into account our evaluation regarding the state-of-the-art in the field of antioxidant capacity/activity assays we consider that the assessment regarding this subject expressed in [25] is correct, namely:" Twenty five years of antioxidant screening have NOT resolved issues of assay chemistry, standardization, and reporting; provided significant insight into chemical mechanisms and factors controlling antioxidant action; clearly connected *in vitro* assay chemistry to *in vivo* actions; established rate constants for reaction of antioxidants with radicals that are relevant in foods and biological tissues;...".

The *in vitro* antioxidant assays and the determination of total phenolic content employing colorimetric methods are not only used for the evaluation of potential beneficial effects of different products. There are also used for the quality control of natural products and foods [166, 168] where the antioxidant capacity of commercial samples, evaluated by *in vitro* assays, can be collated against reference materials. Hence, trends can be very valuable for comparing samples from the same materials. In food technology, *in vitro* antioxidant methods and TPC assay may be useful to assess, e.g., the antioxidant actions of herbal extracts on lipid-rich foods, the effects of processing steps on the stability of phenolic compounds from herbal extracts [168] employed to counteract lipid oxidation, or to obtain more antioxidant compounds from raw materials. In the area of active packaging, radical scavenging assays can contribute to assessing efficiency of antioxidant packaging formulations [169]. The *in vitro* methodologies for antioxidant and TPC assays are applied in routine quality control programs by food companies in many countries [170, 171]. The methodologies for antioxidant and TPC assays can be considered valuable high-throughput, low cost tools used to evaluate and find antioxidant sources and for quality control of foods and natural products.

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