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Antioxidant Capacity Assays. Chemical and Cellular-based Methods. Review

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Abstract: *The article is a continuation of the book chapter entitled Recent Advances in Antioxidant Capacity Assays published by IntechOpen in 2021. The various methods for determining antioxidant capacity in a variety of materials (plant extracts, biological material, foods, etc.) are discussed, with a special emphasis on articles published in recent years and especially on reviews. Both chemical methods for determining antioxidant capacity and cellular antioxidant capacity assays were presented. In addition to the review published in 2021, the following methods for the determination of antioxidant capacity were presented: crocin bleaching assay, Briggs-Rauscher reaction inhibition assay, ferri-cyanide-Prussian blue assay, cerric reducing antioxidant capacity assay and Anti Oxidant Power 1 (AOP1) assay. Several applications of the cellular antioxidant capacity assay were also presented in tabular form.*

Keywords: *antioxidant, reactive species, free radicals, antioxidant assay, phytochemicals, food analytical methods*

Introduction

Due to the increased occurrence of pathologies related to action of free radicals at various levels of body, increasing the capability of human organism to fight against free radicals' production and action became of outmost interest, antioxidants ensuring one of the main defense barriers. Consequently, studies on how to assess the efficacy of different formulations claiming antioxidant properties are focusing the attention of the scientific community.

The current paper is an updating of the information on the subject included in our work published in 2021 [1] aiming the actualization according to the state of the art on antioxidant capacity assay.

According to the reaction mechanisms antioxidants are generally acting either as

a. chain-breaking antioxidants; their effectiveness is based on their action as scavengers against reactive species -oxygen and/or nitrogen reactive species, (ROS/RNS); these compounds are recognized as primary antioxidants

b. preventive antioxidants; their effectiveness is based on their action of inhibition of oxidation promoters as pro-oxidative enzymes, or as chelating reagents for other oxidative status promoters as metallic ions, singlet oxygen, etc., these compounds are known as secondary antioxidants

According to the pathway of action, antioxidant action is a direct one (as are those acting as ROS/RNS scavengers) or indirect one (as are those able to inhibit ROS generation or those acting as regulators for endogenous antioxidants).

Based on specific structure, antioxidants are either enzymatic or nonenzymatic, the current paper is dealing with methods to assess antioxidant capacity of non-enzymatic antioxidants.

It is necessary to define the terms that are used when discussing about antioxidants properties, therefore further are clarified several terms.

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The antioxidant activity and antioxidant capacity of an antioxidant are two different terms. Thus, antioxidant activity is given by the reaction rate between antioxidant and a certain free radical, the antioxidant capacity is given by the number of moles of antioxidant reacting with a specific free radical, while the antioxidant efficacy of a substance is given by both the activity and antioxidant capacity of that compound.

Total antioxidant activity (TAC) refers to the summed antioxidant capacity of all substances in a sample to be analyzed.

The chemical variety of antioxidants make them tedious to separate and even identify. The term "antioxidant activity" evaluated by a specific assay gives only the chemical reactivity under the distinct conditions of the assay, such as temperature, reaction media, co-reactants, etc., therefore it is often erroneous to generalize the data as the "total antioxidant activity".

Methods for determining antioxidant activity can be direct or indirect methods. Direct assays are competitive because the antioxidant and a "probe" are attacked at the same time by the reactive species produced. In the case of indirect assays, a simulation of redox reactions is performed using artificial "probes" that react with the antioxidants and their antioxidant capacity is measured by evaluating some properties of the "probe" (electrochemical, spectrometric, chemiluminometric, etc.) [2]. Such a "probe" may be 2,2'-azino-bis(3-ethylbenzothiazole-6-sulphonate) radical cation, $ABTS^{\bullet+}$ or 2,2-diphenyl-1-picrylhydrazyl, $DPPH^{\bullet}$

In recent decades, numerous reviews have been published on the determination of antioxidant capacity reporting data from a diversity of sample, many of which were discussed in the previous work [1]. From these reviews, the most relevant are comprehensive works [2-11] several of them presenting information aiming to solve specific practical problems [6-8, 12, 13] However, only few of these are discussing the mechanisms and/or kinetics of the reactions involved in the determinations [3, 6, 10, 14-16] or the differences between the *in vivo* and *in vitro* methods for the evaluation of antioxidant activity/capacity [17]. Due to the complexity of the subject some controversies are born when approaching the determination of the antioxidant potentiality of a certain compound, methodologies to perform the screening and the analysis of natural and synthetic compounds antioxidants and pro-oxidants, including controversies, being discussed in [18].

Because of the large discrepancy of results for natural antioxidants assay in food systems, there is a stringent need for much better analytical in the field of antioxidant assessment.

When quenching the free radicals, antioxidants action by two basic mechanisms [3, 16] either by single electron transfer (SET) ore by hydrogen atom transfer (HAT) between antioxidant and free radical, thus converting the free radicals in more stable species, although a clear distinction between these two mechanisms is sometimes difficult [19]. Moreover, there are antioxidants quenching free radicals by the mean of both HAT and SET mechanisms. As results, when performing antioxidant capacity determination, the measurements and methods are either electron transfer (SET) and/or hydrogen atom transfer, (HAT) assays. Detailed discussion on this issue and on both types of mechanisms, SET and HAT, is provided in [1]. ROS and RNS methods generate the same end products regardless of the mechanism. A considerable influence can however have the characteristics of the system (e.g., *p*H, solvent, possibility of side reactions, etc.) and the kinetics of the process which is different for the SET or HAT mechanism [19]. Electron transfer is very fast (femto seconds).

In the attempt of providing comparable results the values of antioxidant capacity are generally expressed as equivalence of a compound considered as reference antioxidant such as Trolox or gallic acid, or as percent of antioxidant inhibition of a probe/substrate oxidation. Various analytical techniques are used to detect the changes generated by oxidation of the probe, the most frequently used being spectrophotometry, chemiluminescence, fluorescence, amperometry, etc.

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Table 1. Classifications of antioxidant capacity/activity assays [1]

Chemical based assays	
Radical/ROS-based scavenging assays	The trolox equivalent antioxidant capacity (TEAC/ABTS) assay; 2,2'-azino-bis(3-ethylbenzothiazole-6-sulphonate ($ABTS^{\bullet+}$)
	DPPH assay; 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$)
	Oxygen radical absorbance capacity (ORAC) assay
	Chemiluminescence methods
	Crocin bleaching assay
	Briggs-Rauscher reaction inhibition assay
Non-radical, redox potential-based assays	Ferric reducing antioxidant power (FRAP) assay
	Cupric reducing antioxidant capacity (CUPRAC) assay
	Ferricyanide-Prussian blue assay
	Cerric reducing antioxidant capacity (CERAC assay)
	Nanoparticles based assays - colorimetric detection, AuNPs- and AgNPs- based assay; - electrochemical detection, AuNPs- magnetic NPs-based assays, etc. - silver nanoparticle antioxidant capacity (SNPAC) assays
	Electrochemical methods - cyclic voltammetry (CV) based assays - diferential pulse voltammetry (DPV) based assays - Square wave voltammetry (DWV) based assays - Amperometry, biamperometry-based assays
Live cell based assays	
	Cellular antioxidant activity assay (CAA)
	Anti Oxidant Power 1 (AOP1) assay

Underlining the aim of the current work, the main goal is to complete and update the respective topic based on recent data published in original papers or reviews starting from the presentation of methods for determining antioxidant capacity from a variety of samples as presented in the previous paper [1]

Chemical based assays

Radical/ROS scavenging assays

Scavenging ability toward stable free radicals $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ and oxygen radical absorbance capacity (ORAC) assay

The trolox equivalent antioxidant capacity (TEAC/ABTS) assay based on the use of $ABTS^{\bullet+}$ radical cation (2,2'-azino-bis(3-ethylbenzothiazole-6-sulphonate) together with DPPH assay based on the use of $DPPH^{\bullet}$ radical (2,2-diphenyl-1-picrylhydrazyl) [20] are some of the most widely used methods for determining the total antioxidant capacity (TAC) of plant extracts, food, clinical fluids, etc. The TEAC/ABTS assay is based on the ability of the test sample to decolorize a solution containing the $ABTS^{\bullet+}$ radical. The method can be applied to both hydrophilic and lipophilic samples.

TEAC/ABTS and DPPH methods have been discussed under multiple aspects in several reviews that can be considered as reference [1, 5, 10, 16].

In the very recent review [21] are presented general data on the TEAC/ABTS method but also the most important recent achievements on this method. Thus, the use of new methods for $ABTS^{\bullet+}$ radical generation, such as the use of peroxidase-like nanozyme is mentioned [20].

It was developed a gold doped copper hexacyanoferrate (Au@Cu-HCF) nanozyme-based colorimetric sensing device coupled with a smartphone camera for determination of antioxidant capacity as a “smart detector” [22]. The developed sensor (which is portable) was utilized with good results for TAC determination of citrus juice, lemon beverage and lotus root. The obtained results agree with that obtained with a commercial kit.

The TEAC/ABTS method has been adapted [23] to different analytical techniques such as HPLC, flow injection assay, stopped flow assay, automated analysis equipment etc.

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Noteworthy is the recently published review [24] in which the most important aspects of TAC determination in food and pharmaceuticals using spectrophotometric methods are presented. Thus it discusses: the importance of antioxidants and free radicals in the body, the process of oxidation and formation of free radicals, classification of natural antioxidants, antioxidant mechanism in radical scavenging and the most important spectrophotometric methods for determination of antioxidant capacity namely: TEAC/ABTS, DPPH, CUPRAC (cupric reducing antioxidant capacity), FRAP (ferric reducing antioxidant power), ORAC (oxygen radical absorbance capacity), TBARS (the thiobarbituric acid reactive substances) assays beta-carotene bleaching assay, total phenols with Folin Ciocalteu (FC) and targeted scavenging activitites: hydrogen peroxide scavenging, superoxide radical scavenging, nitric oxide scavenging, and peroxynitrite scavenging. The reactions involved in the determinations discussed are also indicated with a rich citation of the literature on the applications of the methods. A clear presentation of the general principles of the spectrophotometric methods discussed, as well as their advantages and disadvantages, is given. Unfortunately, the bibliographical indications on the applications of the methods discussed are not updated, with few papers published after 2020 being mentioned.

In another review [25] also gives a clear presentation of the importance of antioxidants, discusses the most important free radicals that can form in the systems studied and presents the spectrophotometric methods applied to the determination of antioxidant capacity (TEAC/ABTS, DPPH, CUPRAC, Folin Ciocalteu (FC), FRAP, ORAC, TRAP (Total radical-trapping antioxidant parameter). A review published in 2023 regarding ABTS methodology was published in [21].

The TEAC/ABTS method has many advantages: it can be applied to a wide variety of both natural and synthetic antioxidants, it is applicable over a wide *p*H range, it allows the determination of both lipophilic and hydrophilic antioxidants, it is easy to apply etc.

Important disadvantages of the method are: the results of the determinations depend on how the $ABTS^{\bullet+}$ radical was generated and the time interval set for the determinations. Also $ABTS^{\bullet+}$ radical (as well as the $DPPH^{\bullet}$ radical) is an artificial, metastable radical. Moreover $ABTS^{\bullet+}$ (and $DPPH^{\bullet}$ likewise) are N-centered and not O-centered radicals, raising questions on how the radical reactions take place with antioxidants in foods and natural systems. Reaction time can influence the results, as some antioxidants react rapidly and completely with the $ABTS^{\bullet+}$ ion, while others react slowly and incompletely; in the TEAC/ABTS assay the size of the antioxidant molecules and steric hindrances can greatly influence the results obtained.

The DPPH assay is a method with multiple applications in practice, due to its high sensitivity, simplicity, rapidity, reproducibility and also because it does not require special pre-treatment of the sample to be analyzed [26]. It is usually worked up in methyl alcohol medium. Determinations are made by monitoring the absorbance of the solution at 515-517 nm and the antioxidant capacity of the analyzed sample is correlated with the absorbance decrease. In the review

The DPPH method is widely used in practice, being simple, easy to apply and low cost. A disadvantage of the DPPH method is that the decrease in DPPH radical absorbance on which the determination is based may be due to other factors than just the antioxidants in the sample to be analyzed, such as: type of solvent used, ambient light, dissolved oxygen, *p*H, etc.) and thus errors may occur. The method has also been criticized for not being standardized in the different steps of the analytical process [19].

A major drawback of the TEAC/ABTS and DPPH methods is the very important difference between the chemical structure of the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals used in the determinations and the free radicals existing in biological systems.

In principle, the ORAC method measures the radical chain breaking capacity of antioxidants by evaluating their ability to neutralize peroxyl radicals used to generate oxidation. Determinations are usually made fluorimetrically. It uses a “probe” (which is fluorescent) with which the peroxyl radicals react, generating a non-fluorescent product. In the determinations, a competition takes place between the reaction of the $ROO^{\bullet}$ radical with the “probe” and the antioxidant.

ORAC (oxygen radical absorbance capacity) assay is biologically relevant because the peroxyl radicals ($ROO^{\bullet}$) used in the determination are the most important free radicals acting in lipid oxidation in biological systems and in food. Because of this, ORAC assay can be considered as a reference method for the determination of antioxidant efficacy. As a peroxyl radical generator, 2,2'-azo bis(2-methyl-propionamidine) hydrochloride (AAPH) is usually used. First, a set of curves showing the variation of fluorescence intensity in the absence and presence of a standard antioxidant of different concentrations is plotted. A calibration curve is then plotted by plotting the differences between the areas under the curves indicating the variations of fluorescence as a function of time in the presence and absence of the reference antioxidant at different concentrations. The greater the differences, the greater the antioxidant capacity of the sample. Trolox is usually used as the reference antioxidant. ORAC values measured for a sample to be analyzed are given in trolox equivalent units.

Since the ORAC method measures the ability of antioxidants to donate a hydrogen atom it is a HAT-based method. β -phycoerythrin (β -PE), a protein isolated from *Porphyridium cruentum*, was employed as the fluorescent probe in the first studies. Currently it is used as fluorescent "probe", fluorescein and dichlorofluorescein diacetate [19], as they are more stable. Other fluorescent probes have also been suggested. The use of pyrogallol red as a "probe" for ORAC method [27] resulted in good linearity, accuracy and precision of the assay. Another study, relying on p-aminobenzoic acid (PABA) as the fluorescent probe, showed ORAC values of liver and kidney [27]. The authors suggested the change of fluorescein with PABA in ORAC method for amino acids, plasma and thiol-type antioxidants (as inconsistent results were obtained for some thiols).

ORAC assay as originally proposed does not allow determination of hydrophobic chain breaking antioxidant capacity. In order to allow the determination of both hydrophilic as well as lipophilic antioxidants the ORAC method has been modified, using for this purpose a solution of 50% acetone/50% water (v/v) and 7% randomly methylated β-cyclodextrin which increases the solubility of the anti-oxidants [28].

The ORAC method can be easily automated and is probably the most appreciated method for the determination of antioxidant capacity.

Very recently the methods discussed in this subchapter have been applied to characterize different types of coffee [29] (by using ABTS, DPPH and ORAC assays); analyses of formulated soymilk dessert [30], ethanolic extracts from five ginger plants [31] of grape (Vitis vinifera) pomace [32] and analysis of Jerusalem artichoke (*Helianthus tuberosus L.)* leaves [33] (by using ABTS and DPPH assays).

A table of numerous applications of spectrometric and electrochemical nano-material-based assays for antioxidant assessment can be found in [1] published recently.

Chemiluminescence methods

ROS/RNS have the ability to react with certain reagents (chemiluminescence) and generate species in excited states that emit light (chemiluminescence). Based on this fact, new methods for the determination of antioxidants have been proposed, based on their ability to neutralize ROS/RNS and thus cause a decrease in chemiluminescence radiation intensity. The determination of antioxidants is based on the competition between antioxidants and chemiluminescence reagents for ROS/RNS species. The higher the antioxidant capacity of a substance, the lower the intensity of chemiluminescence radiation emitted. Luminol [19, 34-37] lucigenin [38] pholasin (a bioluminescent protein) [39] and peroxyoxalate [40] are used as chemiluminescent reagents. Luminol is the most widely used chemiluminescent reagent.

General reviews on the determination of antioxidant capacity, including chemiluminescence-based methods, are presented in [1, 8, 9]. The determination of the antioxidant capacity of lipids by using flow injection analysis with chemiluminescence detection are reviewed in [41].

Based on Co(II)-ethylenediaminetetraacetic acid (EDTA)-induced luminol-hydrogen peroxide chemiluminescence (luminol/Co(II)EDTA/H_2O_2) it were proposed methods for TAC determination of some *Rosmarinus officinalis L.* (rosemary) extracts [36] of fruit seeds extracts [37] and by employing

flow injection analysis method (FIA) for TAC determination of wines [35] and culinary and medicinal plants extracts [42]. The optimization of the experimental parameters of the above mentioned FIA method for TAC determination was further discussed in [43]. A good correlation has been found between the results for TAC determination of several plant extracts by using the chemiluminescence method reported in [36] and an amperometric method based on an electrochemical gold nanozyme-sensor [44]. In [45] a novel high throughput chemiluminescence method was described for screening antioxidants in herbal extracts and pure compounds.

For the determination of natural antioxidants a chemiluminescence-sensing platform was proposed [46].

In a recent paper a flow injection chemiluminescence (FI-CL) method based on the Ag(III)-luminol system for the total polyphenol content analysis of tea and substitute tea was reported in [47].

Crocin Bleaching Assay

This method evaluates the inhibition capacity of antioxidants in protecting the bleaching of crocin (probe), a naturally occurring carotenoid derivative, with $ROO^{\bullet}$ radical generated through thermolysis of AAPH. The inhibition of crocin bleaching by an antioxidant compound is measured by monitoring the decrease in absorbance of the probe (crocin) at 443 nm ($\varepsilon = 1.3 \times 10^5$ L mol^{-1} cm^{-1}) for 10 min [7]. The crocin bleaching rate constants and antioxidant capacity of some phenolic antioxidants acquired by different assays were well correlated with each other. After some modifications the method was applied with good results for the analysis of plasma antioxidant capacity. In addition, a rapid HPLC-crocin bleaching assay system for on line measurement of antioxidants was reported in [48].

The advantages of the method are the usage of $ROO^{\bullet}$ radicals and polyunsaturated substrate and measurement of positive peaks in the chromatogram. The crocin bleaching assay has found few applications in food samples so far.

Methods for determining antioxidant capacity using Briggs-Rauscher (BR) oscillating reactions

In [49] the first report of the use of BR oscillating reaction to study the antioxidant properties of some compounds (from soybeans) is presented. In a second paper [50] a detailed study of a new method for the determination of antioxidant activity using BR oscillating reactions is described. Ten compounds were studied: pyrocatechol, ferulic acid, caffeic acid, resorcinol, homovanillic acid etc.).

When an antioxidant is introduced into the reaction medium, the oscillations stop immediately, and after a certain period of time (which depends on the type and amount of antioxidant used) the oscillations restart (with different characteristics from the initial ones). The inhibition time of the oscillations is linearly correlated with the concentration (amount) of antioxidant introduced into the reaction medium.

Briggs-Rauscher oscillating reactions take place in a reaction medium which usually contains malonic acid (MA) or a derivative of it (but can also be another organic compound), iodate anion in acidic medium, hydrogen peroxide and Mn^{2+} ions (catalyst). A series of iodination and oxidation reactions of organic compound take place, the main intermediates whose concentration oscillates during the reactions being: I_2, I^-, HOIO, HOI, $IO_2^{\bullet}$ and $HOO^{\bullet}$.

The inhibitory effect of antioxidants is explained by scavenging of $HOO^{\bullet}$ radicals. Oscillations were followed potentiometrically by using a iodide ion-selective electrode or a bright platinum electrode.

BR mixtures oscillate in a *p*H range 0.5 - 2.5 at 25°C, best at *p*H = 1.55, in the experimental conditions reported in [50].

Temperature has a great influence on oscillations and inhibition time, for this reason it must be controlled with an accuracy of fractions of a degree (or appropriate corrections must be made).

Representing the inhibition time as a function of antioxidant concentration gives calibration lines with different slopes, depending on the nature of the antioxidant. For an antioxidant one can calculate its relative activity in terms of concentration (RAC) by determining the ratio of the concentration of a standard antioxidant to its concentration, which gives the same inhibition time, or one can calculate its

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relative activity in terms of slope (RAS). The mechanism of the oscillating reaction (BR) is complex. [51, 52]

The method was initially applied to determine the antioxidant properties of some water-soluble compounds. Thus, the antioxidant capacity of coffee was determined according to its type (origin) as well as according to the method of obtaining the coffee brew [53].

Several studies have also been reported in the literature on the determination of the antioxidant capacity of some wines, the most relevant of which are the following [54, 55]. The BR oscillating reaction-based method for the determination of antioxidant activity has also been adapted for the analysis of some antioxidants or water-insoluble samples. Thus the antioxidant capacity of extra virgin olive oil was determined [56] by first dissolving the test sample in acetone (25% solution in water) which was then subjected to analysis. It was also applied to determine the antioxidant capacity of some beauty creams [57] by analysis of an emulsion of the test sample in isopropyl alcohol (12.5% in water). The presence of acetone or isopropyl alcohol in the reaction medium does not influence the oscillation time of the BR reaction or the inhibition time due to antioxidants in the sample

The vast majority of methods for determining antioxidant capacity using BR oscillating reactions are based on the use of Mn^{2+} as a catalyst. Another catalyst for the BR oscillating reaction has been reported in the literature, namely a nickel complex $NiL(ClO_4)^2$ where the ligand L in the complex is 5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4,11-diene [52, 58].

The results obtained when determining the antioxidant capacity of some natural phenolic compounds using BR oscillating reactions catalyzed by the above mentioned compound are similar to those obtained using Mn^{2+} ions as catalyst.

The analytical performance of methods for determining the antioxidant capacity of various compounds based on Briggs-Rauscher oscillating reactions is similar to that of other analytical methods used for this purpose.

The proposed new method is very cheap and easy to apply, since the reagents and apparatus required can be found in ordinary chemistry laboratories. Temperature has a great influence on the reaction oscillation time and the inhibition time determined by the antioxidants. To obtain correct results it is necessary that the temperature of the reaction mixture is carefully controlled (or corrections are made).

The *p*H of the solution at which the determinations are made is $pH \leq 2$ which is the *p*H of human gastric fluids and at which lipid peroxidation is most intense. The method allows useful *in vitro* information on the activity of some antioxidants to be obtained under *p*H conditions similar to those of human gastric juice, which is an important advantage.

Non-radical redox potential-based assays

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

Ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) assays have been discussed in several reviews [5, 6, 12, 59-61]. The FRAP method is based on the reduction of the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe^{3+-}(TPTZ)_2]^{3+}$ by an antioxidant to the ferrous complex, $[Fe^{2+}(TPTZ)_2]^{2+}$ blue in color. Working *p*H is 3.6 to prevent precipitation of iron ions. The generation of $[Fe^{2+}(TPTZ)_2]^{2+}$ is measured spectrophotometrically at 593 nm. The determined antioxidant capacity is expressed in equivalents of a standard antioxidant or in μM Fe^{2+} equivalents. The method allows the determination of compounds with a redox potential lower than 0.7 V (which is the potential of the Fe^{3+}/Fe^{2+} couple). The method does not allow the determination of antioxidants that act by radical quenching (H transfer), e.g., biothiols or proteins [62].

The redox potential of $ABTS^{\bullet+}$ potential (0.68 V), is similar to the potential of $[Fe^{3+-}(TPTZ)_2]^{3+}$. For this reason similar compounds react in both the TEAC/ABTS and FRAP assays. FRAP reactions are carried out in acidic medium to limit Fe^{3+} hydrolysis (*p*H 3.6) where phenolic antioxidants are not dissociated. As a consequence, lower results than actual TAC are to be expected because phenols are oxidized much more slowly than the corresponding phenolates. FRAP and other Fe^{3+} reduction based

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SET methods were criticized for generating Fe^{2+} as the reduction product, which could give rise to the formation of ROS upon Fenton-like reactions with H_2O_2 (e.g., $^{\bullet}OH$), thus causing "redox cycling" of phenolics and yielding wrong TAC results.

Very recently the FRAP method has been applied to characterize different types of coffee [29] and for analysis of grape (Vitis vinifera) pomace [32]. Unfortunately the FRAP method, only determines the reducing capacity given by the Fe^{3+} ion which has very little relevance physiologically and mechanistically in terms of antioxidant capacity.

Unlike other TAC determination methods the FRAP method has however a number of advantages such as: simplicity, rapidity, cost effective and does not require special equipment.

Cupric reducing antioxidant capacity (CUPRAC) assay is based on the ability of antioxidants to reduce the Cu^{2+} ion to Cu^{+}. Cu^{+} ions are complexed with bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) or neocuproine (2,9-dimethyl-1,10-phenanthroline). The complexes formed have maximum absorption at 490 nm and 450 nm, respectively [63].

In the common CUPRAC method, the duration of an analysis is no more than 30 min. For fully exhibiting their antioxidant potency, flavonoid glycosides require acid hydrolysis to their corresponding aglycons. Some antioxidants may need high temperature incubation, so as to finish their reaction with the CUPRAC reagent [64].

An optical sensor [65] has been realized which has the CUPRAC reagent (Cu^{2+}- neocuproine complex) immobilized on a Nafion membrane. The colored Cu(I)-neocuproine cation was produced on the membrane without diffusing into solution. The sensor allows the determination of the reducing power of liquid samples without any pretreatment

Another variant of the CUPRAC assay based optical sensor [66] used a miniature reflectance spectrometer to measure reflectance changes at 530 nm Use of this type of sensor could significantly simplify the measurement and allow the *in situ* determination of antioxidant capacity of different samples.

The CUPRAC assay has advantages over FRAP assay for antioxidant determination in that all classes of antioxidants (including thiols) are measured and the copper reaction kinetics are faster than iron. The CUPRAC assay and its modifications together with its advantages over other similar SET-based TAC methods have been discussed in a comprehensive review in [67].

Ferricyanide (hexacyanoferrate(III))−Prussian blue assay, Cerric reducing antioxidant capacity (CERAC)

In the conventional method, hexacyanoferrate(III) (ferricyanide, $[Fe(CN)_6]^{3-}$ reagent is first incubated (at 50°C for 20 min) in a buffer at *p*H 6.6 with antioxidants generating the reduction product, hexacyanoferrate (II) (ferrocyanide, $[Fe(CN)_6]^{4-}$. Then is added Fe^{3+} to produce Prussian blue ($Fe_4[Fe(CN)_6]_3$) suspended in the medium. The absorbance measurements are done at 700 nm [6]. The initial ferricyanide/Prussian blue assay was optimized [68] The main modification consists of use at the same time of ferricyanide and Fe^{3+} in order to provide better redox conditions for different antioxidants. One drawback of this assay is the predisposition of the Prussian blue to precipitate. The precipitation can be prevented by the addition of a detergent (sodium dodecyl sulfate). The *p*H was settled to 1.7 for preventing the hydrolysis of Fe^{3+} ion. A shift in λ_{max} from 700 to 750 nm was observed in these conditions. This modified procedure was named the "ferric-ferricyanide assay" since it was not clear whether Fe^{3+} or ferricyanide was the actual oxidant. By further modification of this method [69] it was possible to evaluate hydrophilic and lipophilic antioxidants (e.g., β-carotene, α-tocopherol and BHT) in the same solution containing 1:9 (v/v) H_2O/acetone with or without 2% methyl-β-cyclodextrin.

Cerric reducing antioxidant capacity (CERAC)

A spectrophotometric CERAC method for determination of TAC of plant extracts by oxidation with Ce^{4+} sulfate in a medium of sulfuric acid at ambient temperature was described in [70]. The method

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relies on the evaluation of the remaining Ce^{4+} at 320 nm after oxidizing all antioxidants from the sample solution.

An improvement of the above mentioned CERAC assay is presented in [71], consisting of a better selectivity over citric acid and simple sugars. A variant of the CERAC method based on the fluorimetric measurement of Ce^{3+} resulted in the reaction of Ce^{4+} with antioxidants was also reported [72]. Cerric reducing antioxidant capacity evaluation by using the reaction between analyzed antioxidant and amaranth [73] and Ce^{4+} was also employed for determination of antioxidant capacity. Calibration graphs for antioxidant capacity were obtained by measuring spectrophotometrically the amounts of unreacted dyes for known concentrations of a standard antioxidant. The methods were applied for the evaluation of antioxidants in medicinal plants and foodstuffs.

Nanoparticles based assays

Nanoparticle (NPs) can be used for the determination of antioxidants as an electrochemical or a colorimetric probe, for radical generation or as components in biological or chemical detection systems. In literature were reported several reviews regarding the employment of NPs for TAC determination [10, 74-77].

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 antioxidants. The formed nanoparticles intensely absorb radiation in the visible range at specific wavelengths due to surface plasmon resonance.

In [78], gold nanoparticle (AuNPs) method, silver nanoparticle (AgNPs) method and and iron oxide (IONPs nanoparticle method) were employed to evaluate the antioxidant capacities of Chinese wine and Zhuyeqing liquor. It was found a good correlation between developed method and FRAP method (correlation coefficients between 0.948 and 0.969).

An optoelectronic tongue (an array of gold and silver nanoparticles) for the determination of several types of natural, synthetic and biological antioxidants was reported in [79].

A novel methodology named SNPAC (silver nanoparticle antioxidant capacity), employing silver nanoparticles to assess antioxidant capacity and trolox as a standard antioxidant is reported in [80]. The method is based on the reduction of Ag^{+} ions in the presence of citrate-stabilized silver seeds to nanoparticles and measuring their absorbance due to surface plasmon resonance la 423 nm. The SNPAC method has been employed for the analysis of a diversity of pure antioxidants and complex mixtures.

A novel spectrophotometric ferricyanide/Prussian blue (PB) assay [81] for TAC determination was proposed, exploiting the formation of PB nano-particles in the presence of polyvinylpyrrolidone as stabilizer. Compared to similar common Fe^{3+}-based TAC assays, with this method much higher molar absorptivities were obtained for tested antioxidants and consequently the sensitivity of determinations was much better. Lower LOD and LOQ values were obtained for thiols.

A network of metal nano-oxides has also been proposed [82] (cerium oxide, titanyl oxalate, TiO_2, Fe_2O_3, ZrO_2, ZnO and SiO_2) for the realization of a portable paper-based colorimetric assay for polyphenols detection in various sample.

In another alternative, an electrochemical gold nanozyme sensor exploiting the ”enzyme like” catalytic activity of gold nanoparticles was applied for the TAC determination of plant extracts [44].

A network of metal nano-oxides (Al_2O_3, ZnO, MgO, CeO_2, TiO_2 and MoO_3) was made by impregnating them onto filter paper [83]. These can form coloured spots with some natural polyphenolic compounds: caffeic acid, gallic acid, ellagic acid, rosmarinic acid and quercitrin.

The image of the colored spots was scanned and analyzed with chemometric analysis by partial least squares regression. Actual and predicted quercetin concentrations are well correlated.

Three reviews regarding electrochemical nanosensors for the analysis of antioxidants [84, 85] and diagnosing oxidative stress [86] were published recently.

A table of numerous applications of spectrometric and electrochemical nano-material-based assays for antioxidant assessment can be found in [1] published recently.

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Electrochemical methods

Electrochemical methods have a number of advantages over spectrophotometric methods for determining antioxidant capacity. Thus, the preparation of samples for analysis is simpler, does not require the use of expensive reagents and the duration of determinations can be shorter. In addition, these methods allow determinations with high sensitivity or sometimes in the presence of compounds that may interfere with other analytical methods (e.g., determination of ascorbic acid in juices).

Relevant reviews on the determination of antioxidant capacity using electrochemical methods include [6, 87-89]. The most used electrochemical methods for the determination of antioxidant capacity are: cyclic voltammetry, differential pulse voltammetry, square wave voltammetry and amperometry.

Cyclic voltammetry (CV)

Most antioxidants are CV-active reducing agents and can thus be determined by CV on the basis of their redox potentials. CV was used for the evaluation of antioxidant capacity of different samples such as: animal plasma, fruits, plants and biological fluids.

A comparative study [90] on the determination of TAC of several tea infusions by using CV and spectrophotometric methods (FRAP, DPPH, TEAC/ABTS and total phenolic content) showed a good correlation of the data obtained, which can be easily explained by the fact that both types of methods are based on the mechanism of reaction antioxidant/oxidant SET (single electron transfer).

CV method has found applications for the determination of antioxidant capacity for a variety of samples such as berry fruits [91] propolis [92] winemaking by-products [93] etc.

Differential pulse voltammetry (DPV)

It was used for the evaluation of antioxidant capacity of white and red wines [94] without interference from glucose and ascorbic acid. Also, DPV and DPPH assays were applied for the determination of TAC and total phenolic content (TPC) in different infusions of coffee samples [95]. A good correlation was found between all three methods. The electrochemical method was shown to be practical, low cost and very useful to assay the antioxidant characteristics of coffee samples.

Square wave voltammetry (SWV)

It was applied with good results for the determination of catechins in black and green teas [96] The obtained detection limit of catechin in green teas was of 40 nM for epigallocatechin gallate.

Based on amperometric measurements [97] it was created a databank regarding the content of antioxidants in a diversity of samples (fish, dairy, cocoa, nuts etc.). It were determined not only water soluble antioxidants but also fat-soluble antioxidants.

Amperometric, CV and DPV determinations of antioxidant capacity of some plant extracts employing an electrochemical gold nanozyme-sensor [44] and chemilumescence determinations (by using a method reported in [36]) were compared and a good correlation was found.

Redox couples Fe^{3+}/Fe^{2+}, I_2/I^-, $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$, $DPPH^{\bullet}/DPPH$ were used with good results for the determination by biamperometry of the antioxidant capacity for a variety of samples. Thus $DPPH^{\bullet}/DPPH$ has been applied for the evaluation of TAC of fruit juices [98] and for tea, wine and coffee [99].

Compared to conventional methods, electrochemical methods for the evaluation of antioxidant capacity have a number of advantages, namely speed, simplicity and no special reagents are required. In addition, these methods allow the analysis of coloured samples that can be evaluated with difficulty using spectrophotometric methods, e.g., wine and fruit juice. These methods allow the analysis of both lipophilic and hydrophilic samples. A good correlation was obtained between the results gathered for TAC assays by using electrochemical assay and by alternative analytical approaches for different samples of foods and beverages. A disadvantage of these methods is the difficulty of analysing macromolecules with antioxidant properties.

A review of electrochemical sensors and biosensors used in assessing antioxidant activity [87], a review of electrochemical methods of antioxidant determination in plants and food extracts [100] and a review regarding antioxidant determination with the use of carbon-based electrodes [101] were published recently.

A table of numerous applications of electrochemical methods for the determination of antioxidants in a variety of samples can be found in [1].

Live cell assays

The methods for determining antioxidant capacity discussed so far are based on chemical hydrogen atom transfer (HA) and single-electron transfer (SET). These methods are applied in a cell-free environment and give us no information on the action of antioxidants in living systems., which is a serious limitation of the methods.

For this reason, new methods have been proposed for the determination of antioxidant capacity based on the use of live cells, the most important of which are the Cell Antioxidant Assay (CAA) and Anti Oxidant Power 1 (AOP1) assay.

Cell antioxidant assay (CAA)

This method has been discussed in several reviews [10, 12, 102, 103].

Cellular-based antioxidant activity assay (CAA) is clearly superior to conventional methods of determining antioxidant activity because the determinations are made within the cell medium [104]. Within cells the role of antioxidants is not limited to neutralization of reactive species (ROS/RNS), but in addition they play an important role in modulation of redox cell signaling, gene expression, and upregulation of antioxidant or detoxifying enzymes. In addition, the antioxidant properties of a substance are also determined by its bioavailability (given by its ability to cross the cell membrane, its distribution in the membrane or even its metabolism). For this reason, results obtained when determining antioxidant capacity using living cells are often much different from those obtained using chemical methods.

To perform a CAA determination a fluorescence probe namely cell-permeable non-polar 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is used. This molecule penetrates through cell walls, where it is deacetylated by cellular esterases and forms a polar molecule 2′,7′-dichlorofluorescin, nefluorescent (DCFH) that cannot migrate out of cells. By using 2, 2′-azo bis(2-amidinopropane) dihydrochloride (AAPH) (a chemical stressor) which can penetrate into the cell, peroxyl radicals are generated which oxidize DCFH to form fluorescent dichlorofluorescein (DCF). By monitoring its fluorescence, the degree of oxidation inside the cells can be determined. When antioxidants are introduced into the intracellular space, they partially neutralize the peroxyl radicals and cause a decrease in the intensity of the fluorescence signal, which is a function of the nature and concentration of the antioxidants. By using standard antioxidants, calibration curves can be plotted on the basis of which the antioxidant capacity of samples to be analyzed can be determined.

Human hepatocarcinoma HepG2, Caco-2 matured differentiated intestinal cells human gastric adenocarcinoma cell line AGS [105, 106] etc. were used as cells for the determinations. *Saccharomyces cerevisiae* cells were also used to measure antioxidant capacity by CAA of various types of samples [107]. The CAA methodology is much closer to a biological approach than the wellknown chemical methods for determination of antioxidant capacity.

The results of determining antioxidant capacity by CAA methods and chemical methods in general do not correlate well, which can be explained by the fact that the results obtained with the two types of assays can be influenced by very different factors. Some examples regarding the evaluation of natural compounds by using CAA are presented in Table 2.

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Table 2. Evaluation of some natural compounds by using CAA

Evaluated antioxidants	Cells used	Results	Ref..
Resveratrol-loaded chitosan-ɤ-poly(glutamic acid) NPs	HepG2	The solubility and stability of RSV-loaded NPs may be influenced by their particle size, which could be controlled by the chitosan and ɤ -PGA concentrations.	[108]
Sea buckthorn	HepG2	For different sea buckthorn subspecies the total phenolic acids and flavonoid aglycones were correlated with CAA and antiproliferation activities.	[109]
Buckwheat honey and manuka honey	HepG2	The CAA of manuka honey is lower than that of buckwheat honey.	[110]
Diosmetin	HepG2	Diosmetin had strong CAA, the antioxidant mechanism is associated to nonenzymatic and enzymatic defense systems.	[111]
Crataegus azarolus	Human Lymphoblast Cell K562	The extract of Crataegus azarolus showed a significant cellular antioxidant activity against ROS.	[112]
Different varieties of blueberry (*Vaccinium* spp.)	HepG2	CAA in different varieties varied about 3.9 times without phosphate buffer saline (PBS) wash, and 4.7 times with PBS wash. Anti-proliferation activity is positive related to CAA	[113]
Novel antioxidant pentapeptides	HepG2	Peptides with a lower α-helix content and a higher β-sheet content seem to be associated with antioxidant activity.	[114]
Kale and red cabbage	HepG2	According to the CAA assay, the stir-fried kale and red cabbage displayed the highest levels of antioxidant, comparatively with boiling and steaming.	[115]
Puha (*Sonchus oleraceus* L.)	HepG2	Foliar extracts of puha were proved to have useful antioxidant activities which is similar to those of extracts from blueberry fruits.	[116]
Kiwifruit (*Actinidia deliciosa*) cultivars	HepG2	It were evaluated four different subspecies of kiwifruit. It was found that the type of cultivar is associated markedly with the phytochemical profile and bioactivities.	[117]
Capsicum annuum L. cv Senise, Incorporation into liposomes,	HepG2	The extract showed no cytotoxic activity and reduced the intracellular ROS level in stressed cells. The antioxidant activity was further improved when the extract was loaded into liposomes.	[118]
Vegetable oil emulsions	HepG2	The medium-chain saturated triglycerides in vegetable oil emulsions resulted in a stronger CAA than that of long-chain saturated triglycerides.	[119]
Mixtures of quercetin and resveratrol		There is a synergistic increase in antioxidant effects at the cellular level by 19.13% when mixtures of resveratrol and quercetin are used.	[120]
Aronia extract and three enriched fractions	CaCo-2, HepG2, SH-SY5Y	Aronia extracts rich in polyphenols possesses broad cellular antioxidant effects and proanthocyaanidines are major contributors	[121]

Anti Oxidant Power 1 (AOP1) assay [122]

One of the major drawbacks of the CAA method is that a chemical stressor (AAPH) is used to determine the antioxidant activity, which generates a large amount of peroxyl radicals inside the cells, difficult to control, which negatively influences the determination of antioxidant capacity. In addition, these radicals are physiologically less relevant [12].

New methods and reagents for the determination of antioxidant capacity by CAA have been proposed to address these issues. For example, reagents have been used that can generate within cells a controlled population of ROS species, more physiologically relevant than peroxyl radicals. The phenomenon of photosensitization, which involves the presence of oxygen, light and a special reagent, photosensitizer (PS), has been used for this purpose. Thus, ROS-generating proteins (RGPs) have been proposed as PS [102, 123] which were originally used in photodynamic therapy (PDT) to selectively kill specific diseased cells with ROS generated by irradiation.

In the case of the AOP1 method, which is a live cell antioxidant assay, ROS are generated inside cells by photoinduction followed by measurement of fluorescence radiation in a time-dependent manner,

in the presence or absence of antioxidants. One of the photosensitizers used with good results is thiazole orange (TO) [124].

TO generates $^1O_2$ and $HO^•$ radicals inside cells under the action of light and also allows fluorimetric measurement of their "concentration [122]. To perform the determinations the sample to be analyzed is treated with TO, then light is applied to it, which generates intracellular ROS and implicitly fluoresces TO. The variation of the generated fluorescence as a function of time is then measured. For the determination of an antioxidant in a sample, the area under the fluorescence time variation curve recorded in the presence of a standard antioxidant is compared with that obtained in the presence of the analyzed antioxidant. Increasing the concentration or activity of an antioxidant within cells causes a decrease in the intensity of fluorescence radiation and hence the area under the curve recorded when measuring fluorescence as a function of time. The method has been applied for the characterization of standard antioxidants [122] and for the determination of antioxidants in plants [125].

The AOP1 method for determining antioxidant capacity has a number of characteristics that make it superior to the CAA method [102] namely:

- The AOP1 assay is based on the controlled generation of $^1O_2$ and other ROS that are more biologically relevant than the peroxyl radicals used in the CAA method;
- In the case of AOP1 we have a control of ROS generation by the light intensity used, a control that is lacking in the case of the CAA method;
- In the case of the AOP1 method, no washing is required. CAA requires rinses of the culture medium which disturbs the cell culture. No washes required in case of AOP1 method.
- AOP1 is easy to standardize, unlike CAA which is much harder to standardize.
- CAA does not differentiate between cytotoxic and antioxidant effects AOP1 can easily differentiate between cytotoxic and antioxidant effects.
- AOP1 measures the effects of antioxidants inside cells; CAA measures the effects of antioxidants in the plasma membrane of cells
- In CAA, DCFH-DA can auto-oxidize; in AOP1 the sensor is not directly involved in the oxidation process.

Conclusions

The interaction of antioxidants and free radicals both with each other and with other chemical species present in the different types of samples analyzed, implies the existence of highly complex chemical and/or biochemical equilibria that are only partially understood. For example: antioxidants may interact with each other (synergistic or antagonistic effects) or there may be pro-oxidant effects of antioxidants (under the influence of environmental composition), etc.

The chemical balances involving antioxidants and free radicals become even more complicated in the case of biological materials or living organisms.

For these reasons, although a wide variety of methods have been proposed to determine antioxidant capacity based on different principles, the results obtained with them are difficult to interpret. Most of them show smaller or larger deficiencies. Thus, in the case of the TEAC/ABTS and DPPH methods, the results are obtained using $ABTS^{•+}$ and $DPPH^•$ radicals, which are artificial radicals that do not exist in the natural environment; in the case of many methods, the determinations are made at *p*H values that are very different from the physiological *p*H and for this reason cannot make much sense for *in vivo* determinations; many methods for determining antioxidant capacity have not been studied in sufficient detail (antioxidant interactions, *p*H, effect of solvents, kinetics, etc.) to know the influence of different experimental conditions on the results obtained, etc.

Another shortcoming of the existing methods for determining antioxidant capacity is the lack of standardization, as very few of them are standardized methods, which makes it very difficult to compare the results obtained with the various methods.

For this reason, when we want to determine the antioxidant activity of a sample, it is advisable to use several chemical methods and in addition a cell-based assay, to evaluate the ability to generate a cellular response.

However, in vitro methods for the determination of antioxidant capacity can be considered useful, rapid and inexpensive methods for quality control of foods and natural products provided that the results obtained are collated against reference materials.

As far as active packaging is concerned, chemical methods for the determination of antioxidant capacity can be useful in evaluating the efficiency of different materials used for antioxidant packaging. At present chemical methods for determining antioxidant capacity are used on a large scale for routine quality control of foods.

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