

## Methods for Total Antioxidant Activity Determination: A Review

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### Abstract

An overview of the importance and mechanism of action of antioxidants, as well as of the methods of assessment of the antioxidant capacity is presented.

Antioxidants react through free radical or molecular oxygen quenching, being capable to either delay or inhibit the oxidation processes which occur under the influence of molecular oxygen or reactive oxygen species.

Antioxidants are responsible for the defense mechanism of the organism against the pathologies associated to the attack of free radicals, thus the intake of plant derived antioxidants is involved in the prevention of degenerative diseases caused by oxidative stress, such as cancer, Parkinson, Alzheimer or atherosclerosis.

The methods of antioxidant capacity evaluation, including spectrometry, chromatography and electrochemical techniques are detailed with respect to principles and analytical performances.

**Keywords:** Antioxidants; Oxidative stress; Reactive oxygen species; Antioxidant capacity; Antioxidant assessment

### Introduction

Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. They are used for the stabilization of polymeric products, of petrochemicals, foodstuffs, cosmetics and pharmaceuticals.

Antioxidants are involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals.

Endogenous antioxidants are enzymes, like superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds, such as uric acid, bilirubin, albumin, metallothioneins. When endogenous factors cannot ensure a rigorous control and a complete protection of the organism against the reactive oxygen species, the need for exogenous antioxidants arises, as nutritional supplements or pharmaceutical products, which contain as active principle an antioxidant compound. Amongst the most important exogenous antioxidants, vitamin E, vitamin C,  $\beta$ -carotene, vitamin E, flavonoids, mineral Se are well known, but also vitamin D and vitamin K<sub>3</sub>.

Exogenous antioxidants can derive from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc [1].

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, as well as the deterioration of fats and other constituents of foodstuffs [2].

### Health Benefits of Antioxidants

Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress, cancer prophylaxis and therapy, and longevity [3]. Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase

or other phenol oxidases, or even by plant tissues containing these enzymes [4-18].

The recommendations based on epidemiological studies are such, that fruits, vegetables and less processed staple foods ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract [19]. The explanation consists in the beneficial health effect, due to antioxidants present in fruit and vegetables [20]. There are numerous antioxidants in dietary plants: carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins [21]. Of the 50 analysed food products with high antioxidant content [20], 13 were spices, 8 were fruits and vegetables, 5 were berries, 5 were chocolate-based, 5 were breakfast cereals, and 4 were nuts or seeds. Considering the typical serving sizes, blackberries, walnuts, strawberries, artichokes, cranberries, brewed coffee, raspberries, pecans, blueberries, ground cloves, grape juice and unsweetened baking chocolate were at the top of the classification [20].

Fruit juices, beverages and hot drinks contain high amounts of antioxidants, like polyphenols, vitamin C, vitamin E, Maillard reaction products,  $\beta$ -carotene, and lycopene [22]. The consumption of fruit juices, beverages and hot drinks was found to reduce the morbidity and mortality caused by degenerative diseases [23-28]. Antioxidants are known to play a key role in the protective influence exerted by plant foods [28-32]. Epidemiologic studies that analyse the health

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implications of dietary components rely on the estimation of intakes of sample populations, which are found in databases that provide the compounds found in commonly consumed foods. Thus, the availability of appropriate and complete food composition data is vital. Due to the diversity of chemical compounds with antioxidant activity present in foodstuffs, complete databases of antioxidant contents are not yet available. In addition, levels of single antioxidants in foodstuffs do not necessarily reflect their total antioxidant potential (TAP) [22]; the total antioxidant potential depends on the synergic and redox interaction among the different molecules present in food [33,34]. Geographical differences in food composition should also be considered when regional surveys are performed.

The total antioxidant potential is a relevant tool for investigating the relationship between dietary antioxidants and pathologies induced by the oxidative stress. This was confirmed by the data obtained from a recent population-based control study, proving that diet TAP resulted in reduced risk of both cardiac and distal gastric cancer [35]. Several analytical methods were recently developed for measuring the total antioxidant capacity of food and beverages: these assays differ in the mechanism of generation of different radical species and/or target molecules and in the way end-products are measured [33,34,36-39].

The consumption of fruits and vegetables, as well as of grains and nuts, has been associated with reduced risk of chronic diseases [40-42]. Among food components fighting against chronic diseases, great attention has been paid to phytochemicals, plant-derived molecules endowed with steady antioxidant power. The cumulative and synergistic activities of the bioactive molecules present in plant food are responsible for their enhanced antioxidant properties. Hence, an appropriate investigation of the role of dietary antioxidants in disease prevention, should be based on a complete database of antioxidant-rich foodstuffs [40].

The evaluation of the total antioxidant capacity (TAC) may be an appropriate tool to determine the additive antioxidant properties of plant foods [43]. The importance of TAC as a novel instrument to estimate the relationship between diet and oxidative stress-induced diseases, is presented in recent studies [44,45] showing a negative association between dietary TAC and the incidence of gastric cancer or the levels of C-reactive protein. In order to assess the overall intake of TAC in population studies, the TAC of 34 vegetables, 30 fruits, 34 beverages and 6 vegetable oils, of varieties most often consumed in Italy, has been analysed using three different assays [43]. Among fruits, the highest antioxidant activities were found in berries, among beverages, coffee had the greatest TAC, followed by citrus juices, which exhibited the highest value among soft beverages [43]. The TAC of spices, dried fruits, sweets, cereals, pulses, and nuts was determined with the aim to complete the Italian TAC database [40]. In fiber-rich foods where phenolics are present in free or bound forms, such as cereals, legumes, and nuts, the contribution of bound antioxidant compounds to the TAC value was evaluated [40].

Various berries and fruit types of less common fruit species are known to contain antioxidants [46]. The intake of high amounts of flavonoids, compounds endowed with antioxidant, antiproliferative and anti-inflammatory activity, may have a positive impact on human health, especially in the prevention of cancer and inflammatory diseases [46].

## The Mechanism of Action of Antioxidants

LMWAs (low molecular weight antioxidants) [47] are small molecules that frequently infiltrate cells, accumulate (at high concentrations) in specific compartments associated with oxidative damage, and then are regenerated by the the cell [48]. In human tissues, cellular LMWAs are obtained from various sources. Glutathione (GSH), nicotinamide adenine dinucleotide (reduced form), and carnosine [49] are synthesized by the cells; uric acid (UA) [50] and bilirubin [51] are waste products of cellular metabolism; ascorbic acid (AA) [52], tocopherols and polyphenols are antioxidants obtained from the diet.

Among these LMWAs, a considerable attention was focused on ascorbic acid (AA), known for its reductive properties and for its use on a wide scale as an antioxidant agent in foods and drinks [53]; it is also important for therapeutic purposes and biological metabolism.

Ascorbic acid is an antioxidant with therapeutic properties, which plays an important role in activating the immune response, in wound healing, in osteogenesis, in detoxifying the organism, in iron absorption, in collagen biosynthesis, in preventing the clotting of blood vessels, and in many other metabolic processes [54-56].

Vitamin C can be easily oxidized, its degradation being accelerated by heat, light and the presence of heavy metal cations [57-59]. Thus, due to its content variation, vitamin C represents an important quality indicator of foodstuffs [59] and contributes to the antioxidant properties of food [60-64].

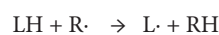
Special attention has been dedicated to the study of antioxidant action mechanism.

The excess free radicals circulating in the body oxidize the low density lipoproteins (LDL), making them potentially lethal; the excess free radicals can also accelerate aging processes and have been linked to other very serious pathologies, such as brain stroke, diabetes mellitus, rheumatoid arthritis, Parkinson's disease, Alzheimer's disease and cancer. Physiologically, the oxygenated free radicals are among the most important radical species. Reactive oxygen species (ROS) comprise species with a strong oxidizing tendency, both of a radical nature (the superoxide radical, the hydroxyl radical) and a non-radical nature (ozone, hydrogen peroxide) [65].

A number of chemical and physical phenomena can initiate oxidation, which proceeds continuously in the presence of (a) suitable substrate(s), until a blocking defence mechanism occurs [66]. Target substances include oxygen, polyunsaturated fatty acids, phospholipids, cholesterol and DNA [67].

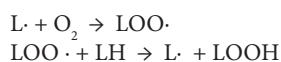
The essential features of oxidation via a free radical-mediated chain reaction are initiation, propagation, branching and termination steps [66]. The process may be initiated by the action of external agents such as heat, light or ionizing radiation or by chemical initiation involving metal ions or metalloproteins [68].

### Initiation



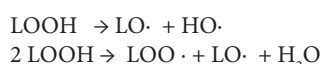
where LH represents the substrate molecule, for example, a lipid, with R· as the initiating oxidizing radical. The oxidation of the lipid generates a highly reactive allyl radical (L·) that can rapidly react with oxygen to form a lipid peroxy radical (LOO·).

## Propagation



The peroxy radicals are the chain carriers of the reaction; they can further oxidize the lipid, producing lipid hydroperoxides (LOOH), which in turn break down to a wide range of compounds [69], including alcohols, aldehydes, alkyl formates, ketones and hydrocarbons, and radicals, including the alkoxy radical (LO·).

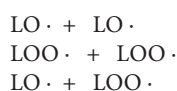
## Branching



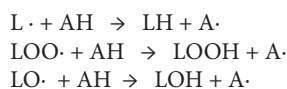
The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, in reactions similar to those involving hydrogen peroxide, yielding lipid peroxy and lipid alkoxy radicals.

## Termination

Termination reactions involve the combination of radicals to form non-radical products:



Primary antioxidants, AH, when present in trace amounts, may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxy or alkoxy radicals [70].



Secondary or preventative antioxidants are compounds that retard the rate of oxidation. This may be achieved in a number of ways, including removal of substrate or singlet oxygen quenching [66, 71].

## Methods of Total Antioxidant Capacity Assessment

The various analytical methods [72] of evaluation of the antioxidant capacity fall into distinct categories:

Antioxidant capacity assay	Principle of the method	End-product determination
<b>Spectrometry</b>		
DPPH	Antioxidant reaction with an organic radical	Colorimetry
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP	Antioxidant reaction with a Fe(III) complex	Colorimetry
PFRAP	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe <sup>3+</sup>	Colorimetry
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry
ORAC	Antioxidant reaction with peroxy radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein
HORAC	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein
TRAP	Antioxidant capacity to scavenge luminol-derived radicals, generated from AAPH decomposition	Chemiluminescence quenching
Fluorimetry	Emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength	Recording of fluorescence excitation/emission spectra

<b>Electrochemical Techniques</b>		
Cyclic voltammetry	The potential of a working electrode is linearly varied from an initial value to a final value and back, and the respective current intensity is recorded	Measurement of the intensity of the cathodic/ anodic peak
Amperometry	The potential of the working electrode is set at a fixed value with respect to a reference electrode	Measurement of the intensity of the current generated by the oxidation/reduction of an electroactive analyte
Biamperometry	The reaction of the analyte (antioxidant) with the oxidized form of a reversible indicating redox couple	Measurement of the current flowing between two identical working electrodes, at a small potential difference and immersed in a solution containing the analysed sample and a reversible redox couple
<b>Chromatography</b>		
Gas chromatography	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase	Flame ionisation or thermal conductivity detection
High performance liquid chromatography	Separation of the compounds in a mixture is based on the repartition between a solid stationary phase and a liquid mobile phase with different polarities, at high flow rate and pressure of the mobile phase	UV-VIS (e.g. diode array) detection, fluorescence, mass spectrometry or electrochemical detection

## Spectrometric Techniques

Spectrometric techniques [72-91] rely on the reaction of a radical, radical cation or complex with an antioxidant molecule capable to donate a hydrogen atom.

**The DPPH method:** [2,73-75] DPPH• (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH• does not dimerize, as happens with most free radicals. The delocalisation on the DPPH• molecule determines the occurrence of a purple colour, with an absorption band with a maximum around 520nm.

When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet colour. Therefore, the absorbance diminution depends linearly on the antioxidant concentration. Trolox is used as standard antioxidant [74, 75].

The spectrophotometric method with DPPH was applied to antioxidant capacity determination in fruit juices [75] and fruit (guava) extracts [74]. The standard curve was linear between 25 and 800mM Trolox [74]. Results are expressed in μM Trolox Equivalents/g fresh mass. Antioxidant activity of guava fruit methanol extracts, as determined by the DPPH method are comprised between 16.2 ± 1.0 and 32.0 ± 5.1μM TE/ fresh mass [74].

**The ABTS method:** The ABTS cation radical (ABTS<sup>•+</sup>) [76] which absorbs at 743 nm (giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization.

ABTS can be oxidized by potassium persulphate [43,74] or manganese dioxide [77], giving rise to the ABTS cation radical

(ABTS<sup>•+</sup>) whose absorbance diminution at 743 nm was monitored in the presence of Trolox [43,74,77], chosen as standard antioxidant.

The spectrophotometric method based on the absorbance diminution of ABTS cation radical was applied to antioxidant content determination in guava fruit extracts [74], fruit and vegetable extracts, soft beverages, alcoholic beverages, tea and coffee [43]. The standard curve was linear between 25 and 600 μM Trolox [74]. The values of the total antioxidant capacity of guava extracts ranged between 22.3 ± 0.9 and 37.9 ± 3.4 μM TE/ fresh mass [74].

The antioxidant activity of soft beverages, as determined by the ABTS method is comprised between 0.09 mM Trolox/liter for Cola and 3.30 mM Trolox/liter for grapefruit juice [43].

**The FRAP (ferric reducing antioxidant power) method:** The FRAP (ferric reducing antioxidant power) method relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine). The binding of Fe<sup>2+</sup> to the ligand creates a very intense navy blue color. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants [43,74,78]. Trolox [43] or ascorbic acid [78] were used as references.

The total antioxidant activity of white- and yellow-flesh nectarines [78] was evaluated by FRAP method, the results being expressed as Ascorbic Acid Equivalent Antioxidant Capacity, AEAC). The values ranged between 14.4 and 104.5 mg/100 of fruit.

**The ORAC (oxygen radical absorption capacity) assay:** [74,79, 80] the method measures the antioxidant scavenging activity against the peroxy radical, induced by 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), at 37°C. Fluorescein was used as the fluorescent probe. The loss of fluorescence was an indicator of the extent of the decomposition, from its reaction with the peroxy radical. Antioxidant activity of guava fruit methanol extracts were determined by the ORAC method. The standard curve was linear between 0 and 50 mM Trolox. The obtained results ranged between 18.2 ± 2.3 and 25.5 ± 1.6 μM TE/ fresh mass [74].

**The HORAC (hydroxyl radical averting capacity) assay:** [79,81]: this technique relies on the measurement of the metal-chelating activity of antioxidants, under the conditions of Fenton-like reactions. The method uses a Co(II) complex and hence evaluates the protecting ability against the formation of hydroxyl radical. Fluorescein is incubated with the sample to be analysed, then the Fenton mixture (generating hydroxyl radicals) was added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. Gallic acid solutions were used for building the standard curve.

**The TRAP (total peroxy radical trapping antioxidant parameter) assay:** the luminol-enhanced chemiluminescence (CL) [79,82] was exploited to monitor the reactions involving the peroxy radical. The CL signal is driven by the production of luminol derived radicals, resulted from the thermal decomposition of AAPH. The TRAP value was determined from the duration of the time period during which the sample quenched the chemiluminescence signal, due to the presence of antioxidants.

**The lipid peroxidation inhibition assay:** The lipid peroxidation inhibition assay method uses a Fenton-like system (Co(II) + H<sub>2</sub>O<sub>2</sub>), to induce lipid (e.g. fatty acid) peroxidation [79,83].

α-linolenic acid was chosen as a model substrate. It was mixed with the analysed sample, as well as with the Fenton-like mixture, to induce lipid peroxidation. After the end of the incubation, the concentration

of thiobarbituric acid-reactive substances (TBARS) was measured, as the index of lipid peroxidation. Lipid peroxidation was expressed in nmoles of TBARS per 1 ml of mixture α-linolenic acid/analysed sample.

**The PFRAP (potassium ferricyanide reducing power) method:** [84, 85] An absorbance increase can be correlated to the reducing ability of antioxidants/antioxidant extracts. The compounds with antioxidant capacity react with potassium ferricyanide, to form potassium ferrocyanide. The latter reacts with ferric trichloride, yielding ferric ferrocyanide, a blue coloured complex, with a maximum absorbance at 700 nm.

**The CUPRAC (cupric reducing antioxidant power) assay:** [84, 86] the standard antioxidants or extracts are mixed with CuSO<sub>4</sub> and neocuproine. After 30 min, the absorbance was measured at 450 nm. In the assay, Cu(II) is reduced to Cu(I) through the action of electron-donating antioxidants. Results are expressed in milligrams of Trolox per liter of extract.

**Fluorimetry:** fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength. In most cases, emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation. Fluorescence emission occurs when an orbital electron of a molecule relaxes to its ground state, by emitting a photon of light after being excited to a higher quantum state by some type of energy. Fluorescence assay has been used to antioxidant content determination [87-91].

The fluorescence spectroscopy has been applied for the determination of phenolic compounds in oils [87]. A method based on fluorescence is proposed to quantify the butylhydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) antioxidant concentration in biodiesel produced from sunflower and soybean oils. Fluorescence and excitation spectra of the solutions were recorded at room temperature using a spectrofluorimeter. The emission spectra were obtained under excitation at about 310 nm and fluorescence in the 320-800 nm range was evaluated. Biodiesel samples without BHA and TBHQ showed fluorescence band at about 420 nm, which can be attributed to tocopherols, inherent to the vegetable oils used in the biodiesel production. The addition of BHA and/or TBHQ is responsible for the occurrence of a fluorescence band around 330 nm. It was verified that the fluorescence intensity around 330 nm increases linearly as function of antioxidant concentration with correlation coefficient of about 1, regardless of the oil source and antioxidants.

Fluorimetric methods of ascorbic acid determination are based on dehydroascorbic acid reaction with o-phenylene diamine [88,89]. This technique requires a strict control of pH, as the fluorescence intensity depends strongly of the pH value.

A fluorescence method was developed [90], to examine how membrane sterol lateral organization affects the potency of antioxidants. This information was used to evaluate possible adverse effects of lipid-soluble antioxidants, which was reported in recent clinical studies. In the presence of an antioxidant, the lag time produced during free radical-induced sterol oxidation in lipid vesicles reflects the potency of the antioxidant. The obtained data suggest that while ascorbyl palmitate is a more efficient antioxidant than its water-soluble counterpart as judged by the lag time, it can easily perturb sterol lateral organization by insertion into membrane bilayers, which could impose detrimental effects on cells.

Another fluorescence assay [91] measured the rate and extent of sterol oxidation in lipid bilayers. Dehydroergosterol (DHE), a

fluorescent cholesterol analog, is used as a probe and at the same time, as a membrane component. The assay can also be performed on bilayers containing a mixture of sterols including DHE and nonfluorescent sterols, such as cholesterol and ergosterol. The fluorescence intensity of DHE decreases on oxidation, so the rate and extent of free radical- or enzyme-induced sterol oxidation can be measured as a function of temperature and membrane composition. In agreement with the sterol regular distribution model, it is found that both free radical- and enzyme-induced sterol oxidation vary with membrane sterol content in a well defined alternating manner [91].

### Electrochemical techniques

Electrochemical techniques were also applied to antioxidant content and antioxidant capacity determination. Cyclic voltammetry and biamperometry are the most broadly used.

**Cyclic voltammetry:** Cyclic voltammetry is a type of potentiodynamic electrochemical measurement. In cyclic voltammetry experiments the working electrode potential is ramped linearly versus time. In cyclic voltammetry, the potential of a working electrode is linearly scanned from an initial value to a final value and back, while recording the respective current intensity.

When the value of a set potential is reached, the working electrode's potential ramp is inverted. This inversion can happen multiple times during a single experiment. The current at the working electrode is plotted versus the applied voltage to give the cyclic voltammogram.

The important parameters obtained from a cyclic voltammogram are the intensities of the cathodic and anodic peaks  $I_a$ ,  $I_c$ , the anodic oxidation potential ( $E_a$ ), and the cathodic oxidation potential ( $E_c$ ). All these values can be readily obtained from the voltammogram. In the case of a reversible system, the values of the intensities of the cathodic and anodic peaks are equal. For irreversible system, only the presence of one peak is noticeable on the voltammogram. Cyclic voltammetry (CV), shown to be a convenient methodology, has been validated for the quantitation of the Low Molecular Weight Antioxidant capacity of blood plasma, tissue homogenates, and plant extracts. Analysis of the CV tracing yields the values of (i) the biological oxidation potential,  $E$  and  $E_{1/2}$ , which relate to the nature of the specific molecule(s); (ii) the intensity ( $I_a$ ) of the anodic current; and (iii) the area of the anodic wave (S) [47]. The sensitivity of the method, as given by the slope of the calibration graph versus vitamin C was 15.175  $\mu\text{A}/\text{mM}$  ascorbic acid [47]. The antioxidant capacity of dry vegetal extracts [65] (expressed as mg of ascorbic acid equivalents) was determined by cyclic voltammetry performed at a glassy carbon working electrode. Green tea, black tea, rosemary and coffee, were selected and analysed to test the total antioxidant capacity of respective dry extracts. For the three extracts obtained from each matrix the antioxidant capacity was determined by the measurement of the anodic area of a cyclic voltammogram.

Of the substances tested, those in which dry methanol extracts display the highest total antioxidant capacity are: Green Tea > Black Tea > Rosemary > Arabica Coffee > Herb Tea > Acerola > Quality Tea > Acai. On the other hand, of the substances tested, those for which dry aqueous extracts display the highest total antioxidant capacity are: Green Tea > Black Tea > Arabica Coffee > Herb Tea > Rosemary > Acerola > Quality Tea > Acai. This implies that these two solvents do not always have the same extractive capacity for all the antioxidant substances contained in the various vegetal samples.

Cyclic voltammetry results of antioxidant capacity determination in

buckwheat products showed good correlation with the data obtained by spectrophotometry [92]. Cyclic voltammograms of analysed buckwheat extracts were useful for evaluation of the antioxidant capacity. The total charge below the anodic current waveform was correlated with the data obtained by the spectrophotometric method with ABTS<sup>+</sup> and DPPH. The changes in the antioxidant capacity of buckwheat and its products followed the changes in flavonoid composition.

**The amperometric method:** The amperometric method involves the measurement of the intensity of the current that flows between a working electrode and a reference electrode, at a fixed (applied) value of potential. The current is generated by the oxidation/reduction of an electroactive analyte. The value of the potential is maintained at a set value with respect to a reference electrode [93-95].

The amperometric determination of the antioxidant activity [96] was based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH•) at the glassy carbon electrode. All the experiments were performed in a three-electrode electrochemical cell at 140 mV vs. Hg<sub>2</sub>Cl<sub>2</sub> | 3M KCl using ethanolic solution (40%) and 0.033M KCl in 0.033 M phosphate buffer, pH=7.4. The linear range obtained for Trolox in 100  $\mu\text{M}$  DPPH ethanol-water solution was up to 30  $\mu\text{M}$ , with a limit of detection of 0.05  $\mu\text{M}$ . The developed method was applied for the evaluation of antioxidant activity of some water or ethanol soluble pure antioxidant compounds and of several samples of tea, wine and some other beverages. The good correlation of results ( $R^2=0.9993$ ) expressed as Trolox equivalents, was obtained between the proposed amperometric method and the classic spectroscopic method [96].

**The biamperometric method:** The biamperometric method is based on the measurement of the current flowing between two identical working electrodes polarized at a small potential difference and immersed in a solution containing a reversible redox couple. Indirect biamperometric measurement relies on the reaction of the analyte with the indicating redox couple, its selectivity depending on the specificity of the reaction involving the oxidized or reduced form of the redox pair and the analyte. Fe<sup>3+</sup>/Fe<sup>2+</sup>, I<sub>2</sub>/I<sup>-</sup>, Fe(CN)<sub>6</sub><sup>3-</sup>/Fe(CN)<sub>6</sub><sup>4-</sup> are redox couples commonly used in biamperometric measurements [97].

A common redox pair chosen in biamperometric studies was DPPH•/DPPH. Antioxidants react with DPPH• (radical form) generating DPPH (reduced form), the intensity of the resulted current being proportional to the residual concentration of DPPH•, after its reaction with the analyte (antioxidant) [75, 98].

Two identical Pt [75] or glassy carbon [98] electrodes were used, where the reduction of the DPPH• radical and the oxidation of the reduced form (DPPH) take place as follows:



The reduction of DPPH• at electrode 1 gives rise to a cathodic current, while the oxidation of DPPH at electrode 2 generates an anodic current. In biamperometry, the controlled parameter is the potential difference between the two identical working electrodes. The potential values of the two electrodes are not controlled with respect to a reference electrode. The biamperometric detector response is linear with respect to that constituent of the redox couple which is present in lower concentration. Working conditions were chosen for a DPPH• (oxidized form) concentration smaller than DPPH concentration. Each antioxidant addition in a solution containing the redox couple DPPH•/DPPH decreases the concentration of the oxidized (radical) form and increases the concentration of the reduced form, thus generating a

current proportional to the concentration of antioxidant. In the case of the proposed method, the cathodic current is limited by the lower concentration of DPPH• (radical form) in the indicating mixture. The DPPH•/DPPH method was applied to the determination of the total antioxidant capacity in fruit juices [75], tea, wine and coffee [98]. The sensitivity of the method was 20.1 nA/ $\mu$ M of Trolox, while the limit of detection accomplished by the used measuring device was 0.05  $\mu$ M [98].

Another redox couple used in biamperometric antioxidant capacity assay is ABTS<sup>+</sup>/ABTS [99,100]. The ABTS cation radical was enzymatically produced by peroxidase in a tubular flow-through reactor. The performance of the bioreactor was tested at different concentrations of immobilized enzyme, ABTS and hydrogen peroxide. Interdigitated array microelectrodes were used as electrochemical sensors for the biamperometric determination. The results of antioxidant activity were determined using Trolox as a standard. The applied interdigitated electrode (IDE) detector accomplished a good sensitivity of 0.3 nA/ $\mu$ M Trolox and offered linear range between 20 to 500 $\mu$ M Trolox. Real samples like juices, tea and wine were analysed [99].

The ABTS cation radical was also produced bienzymatically, by using glucose oxidase and peroxidase. The linearity of IDE detector was tested in the range 20 $\mu$ M-2000 $\mu$ M and a good sensitivity of 0.165 nA/ $\mu$ M for Trolox solutions was obtained [100]. The interdigitated gold electrodes were used for biamperometric determination of antioxidant capacity of alcoholic beverages (wine and spirits) [100].

### Biosensors method

Oxidoreductases are the most oftenly used in biosensor applications because of their electron transferring properties during catalysis. These enzymes offer the advantages of being stable and in some situations do not require coenzymes or cofactors. There are several reviews and books referring to antioxidant and antioxidant capacity determination by biosensors [72, 101-104].

Potential applications of biosensors for evaluation of antioxidant status include monitoring of superoxide radical ( $O_2^{\bullet-}$ ), monitoring of nitric oxide (NO), monitoring of glutathione, monitoring of uric acid, ascorbic acid or phenolic compounds [104].

A carbon paste DNA-based biosensor for the electrocatalytic evaluation of total antioxidant capacity was constructed [105]. The method was based on the partial damage of a DNA layer adsorbed on the electrode surface by OH• radicals, generated by Fenton reaction and the subsequent electrochemical oxidation of the intact adenine bases, to generate an oxidation product that was able to catalyse the oxidation of NADH. The presence of antioxidant compounds scavenged hydroxyl radicals, leaving more adenine molecules unoxidized, and thus, increasing the electrocatalytic current of NADH measured by differential pulse voltammetry. Using ascorbic acid as a model antioxidant species, the detection of amounts as low as 50 nM ascorbic acid in aqueous solution was possible [105].

Frequently, polyphenols are the main contributors to the antioxidant capacity of several plants which contain them. Several amperometric biosensors for the detection of phenolic compounds have been developed, on the basis of enzymes, such as tyrosinase, laccase or peroxidase [106-109]. Biosensors for phenolic compounds were constructed by immobilizing polyphenol oxidase (PPO) into conducting copolymers prepared by electropolymerization of pyrrole with thiophene capped polytetrahydrofuran [108].

These enzyme-based biosensors allow the evaluation of the “total phenol content”. Since tyrosinase acts on the hydroxyl groups of phenolic compounds, the total amount of -OH groups in red wines was obtained through activity determination by enzyme electrodes. Results are reported in Gallic Acid Equivalent (GAE) as mg/l [108,110]. For polyphenol determination in vegetable extracts [111], an amperometric horseradish peroxidase-based biosensor was employed.

The biosensors were used for the determination of antioxidant capacity in wines, the results being consistent with those obtained by spectrophotometry [112,113] or in orange juices, by biosensors based on screen-printed electrodes [114]. For the analysis of commercial red wines, a multi-walled nanotube ionic liquid electrode with immobilized tyrosinase was used [113]. The sensing ranges were 0.01-0.08 mM in a phosphate buffer solution.

### Chromatographic methods

Chromatographic methods were often applied to antioxidant separation and detection, and used before spectrophotometrical or electrochemical assessment of the total antioxidant capacity.

**Gas chromatography:** Gas chromatography (GC) is a common type of chromatography used for separating and analysing compounds that can be vaporized without decomposition. The process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase. The mobile phase is usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support. The comparison of retention times is what gives GC its analytical usefulness. The most common detectors are the flame ionization detector and the thermal conductivity detector.

The antioxidant capacity of turmeric oil (responsible for its antimutagenic capacity) was also determined by chromatographic methods [115]. Turmeric oil and its fractions were analysed by gas chromatography with flame ionisation detector and gas chromatography coupled with mass spectrometry. Turmeric oil and its fractions were then tested for antioxidant activity using the carotene-linoleate model system and the phosphomolybdenum method. The quantitative antioxidant capacity of the turmeric oil and its fractions were measured spectrophotometrically through the phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex with a maximum absorption at 695 nm. The method using the carotene-linoleate [115, 116] model was based on the continuous measurement of the optical density, until the colour of  $\beta$ -carotene disappeared. Butylated hydroxyanisole (BHA) was used for the blank.

**HPLC (high performance liquid chromatography):** HPLC (high performance liquid chromatography) typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector to provide a characteristic retention time for the analyte. The detector (usually a diode array detector) may also provide additional information related to the analyte, (i.e. UV/Vis spectroscopic data for analyte if so equipped).

A pump provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows a better separation on columns of shorter length and ensures higher velocity. Normal-phase HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. Reversed phase HPLC has a

non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with  $RMe_2SiCl$ , where R is a straight chain alkyl group such as  $C_{18}H_{37}$  or  $C_8H_{17}$ . With these stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily.

The antioxidant activity using a HPLC system with post-column on-line antioxidant detection, based on 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid radical scavenging activity. The method was applied to the determination of antioxidant content of coffee [117]. Following separation of the coffee samples on the HPLC column, the eluate was directed to a PDA (photodiode array) detector and then mixed with a stabilised solution of the ABTS cation radical and the solution was directed to a detector monitoring absorbance at 720 nm. The ABTS cation solution has a deep blue colour, and any quenching of the radical results in a loss of colour indicated by a negative peak on the HPLC trace. The antioxidant contributions of individual HPLC peaks were added to give the total HPLC-derived antioxidant activity. The total antioxidant capacity of the green coffee determined with the on-line HPLC system was  $760 \pm 2.5 \mu\text{mol Trolox/l}$  and  $984 \pm 25.8 \mu\text{mol Trolox/l}$  for the roasted coffee [117].

A HPLC method with fluorescence detection [118] was developed for the determination of propyl gallate, nordihydroguaiaretic acid, butylated hydroxyanisole, tert-butylhydroquinone and octyl gallate in edible oils and foods. The HPLC separation was performed on a C18 column using a mixture of 5% acetic acid-acetonitrile-methanol as the mobile phase and monitored by using a fluorescence detector. Sample peaks were identified by comparison of the fluorescence spectra with those of antioxidant standards. Average recoveries of fortified antioxidants at 100 micrograms/g were 72.1-99.6%. Coefficients of variation were 0.7-7.2% [118].

The antioxidant activity of the extracts from root barks of adult plant and root of seedlings extracts [119] was evaluated by HPLC coupled to electrochemical detection (HPLC-ED).

The EICD electrochemical detector was composed of a glassy carbon working electrode, an Ag/AgCl reference electrode and a Pt electrode. The separation of the analytes was carried on a Gemini C18 column, using the isocratic mode and acetonitrile/water mixture containing acetic acid as mobile phase. The optimal potential of the standards was obtained on a hydrodynamic voltammogram, from the evaluation of peak areas vs applied potential vs Ag/AgCl [119].

The advantages and shortcomings of the antioxidant assessment methods were presented by Prior and colab [120]. Several articles highlight the advantages and disadvantages of in vivo and in vitro analysis [121-128].

The methods are chosen as a function of the nature of the sample and the comparison is valid only on the same sample types. The advantages of the analytical techniques can refer to the complexity of the necessary tools, to the simplicity of the applied procedure, to the duration of the analysis, to the biological relevance and the performances of the method (sensitivity, precision, accuracy, detection limit).

Determinations relying on photometric measurements (DPPH, ABTS and FRAP assays) are simple and rapid and need only a UV-Vis spectrophotometer to perform, which probably explains their widespread use in antioxidant screening.

Most methods can be rapidly automatized and some can be applied in vivo (e.g. ABTS assay). Nevertheless, the analytical signal is sometimes difficult to measure and does not account for all antioxidants.

TRAP assay has been criticized as being based on an unphysiological oxidative stress (water-soluble peroxy radicals), the FRAP assay does not measure thiol antioxidants, such as glutathione. The DPPH assay was considered as not based on a competitive reaction, because DPPH is both radical probe and oxidant. Interpretation is complicated when the tested compounds have spectra that overlap DPPH at 515nm. The FRAP assay is characterized by a fast kinetics (4-6 min) but in fact this is not always true. Some polyphenols react more slowly and require longer reaction times for detection, for example, 30min. Copper has advantages over iron for antioxidant assay, in that all classes of antioxidants, including thiols, are detected with little interference from reactive radicals, and the copper reaction kinetics are faster than in case of iron. CUPRAC assay is complete in minutes for ascorbic acid, uric acid, gallic acid, and quercetin, but requires 30-60 min for more complex molecules. ORAC method is based on a temperature-sensitive reaction. Hence, temperature control is essential.

Regarding the complexity of the analytical instruments, the photometric methods are the simplest, followed by voltammetric and chromatographic methods.

Voltammetry offers low detection limits, even when compared to more expensive techniques. It requires little sample preparation. This technique provides us with the advantage of a fast analysis as well as with the easiness and rapidity of the standard addition method application. Because of the low cost of the required equipment, as well as simplicity of the employed procedures, voltammetry appears to offer an attractive alternative to the titrimetric or instrumental methods, in particular in food quality control. It does not require complicated, expensive equipment and well-qualified personnel like chromatography, nor is it laborious or time consuming like the previously mentioned instrumental technique [59].

## Conclusions

The increasing interest gained by antioxidants is due to the health benefits provided mainly by natural sourced (exogenous) low molecular weight antioxidants. This consists in preventing the occurrence of oxidative-stress related diseases, caused by the attack of free radicals on key biocomponents like lipids or nucleic acids.

Various methods and analytical tools are employed for antioxidant content and total antioxidant capacity evaluation: spectrometry, electroanalytical methods, chromatography.

These techniques are able to offer a complete profile of the antioxidant content of foodstuffs.

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