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Technical Evaluation of Antioxidant Activity

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Abstract

There are several *in vitro* methods for evaluating antioxidant activity. This review article gives information regarding different methods that are used to measure the antioxidant defense system In addition; the advantages and shortcomings, as well as the specific cases of their application also are demonstrated. The chemical principles of methods based on biological oxidants comprise superoxide radicals scavenging ($O_2^{\cdot-}$); hydroxyl radical scavenging ($HO^{\cdot}$); hydrogen peroxide scavenging (H_2O_2); peroxyl radical scavenging ($ROO^{\cdot}$) and nitric oxide scavenging ($NO^{\cdot}$). Among the non-biological testing can highlight scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$ assay) and scavenging of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS assay). Furthermore, thiobarbituric acid reactive substances (TBARS) and protein carbonyl assays also have been described. This article will be a comprehensive ready reference for those who are interested on antioxidant study.

Keywords: Antioxidant capacity; Free radicals; Medicinal plants

Introduction

Free radicals are types of Reactive Oxygen Species (ROS), which include all highly reactive, oxygen-containing molecules. Types of ROS include the hydroxyl radical, the super oxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical and various lipid peroxides [1,2]. All these are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage [3]. In living organisms various ROS can be formed in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides [4].

Free radicals may be defined as chemical species associated with an odd or unpaired electron. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally achieve stable configureuration. They are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, liver diseases, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress among others [2,5].

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize free radicals. Thus, antioxidants are capable of stabilizing or deactivating free radicals before they attack cells [6]. An antioxidant is a substance that can efficiently reduce a pro-oxidant with concomitant formation of products having no or low toxicity. Indeed, a broader definition of antioxidant was suggested by Halliwell *et al.* in 1995 [7] as "any substance that when present at low concentrations, compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate". Therefore, according to this definition, not all reductants involved in a chemical reaction are antioxidants; only those compounds which are capable of protecting the biological target meet these criteria. This protection may be based on several mechanisms of action, namely: inhibition of generation and scavenging capacity against ROS/RNS (Reactive Nitrogen Species); reducing capacity; metal chelating capacity; activity as antioxidative enzyme; inhibition of oxidative enzymes [8,9].

Naturally there is a dynamic balance between the amount of free radicals produced in the body and antioxidants that protect the body against deleterious effects. Oftentimes, amount of antioxidant principles present under normal physiological conditions may be insufficient to neutralize free radicals generated. Therefore, it is obvious to enrich our diet with antioxidants to protect against harmful diseases. Hence there has been an increased interest in the food industry and in preventive medicine in the development of "Natural antioxidants" from plant materials [10]. That is why plants with antioxidant properties are becoming more and more popular all over the world. In this context, some of the most commonly used methods for *in vitro* determination of antioxidant capacity is reviewed in the following sections, where the chemical principles, recent applications as well as the advantages and shortcomings are outlined.

Methods

Scavenging capacity assays against stable, non-biological radicals

Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$ assay): This is the simplest and most widely reported method for screening antioxidant activity in foods and many plant drugs [11,12]. In this assay, the purple chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 518 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution (Figure 1). The activity is expressed as inhibitory concentration IC_{50}, that is the amount of antioxidant necessary to decrease by 50% the initial DPPH$^{\cdot}$ concentration [1,13]. The lower IC_{50}, the higher is the "antiradical efficiency". The main limitation of IC_{50} determination is

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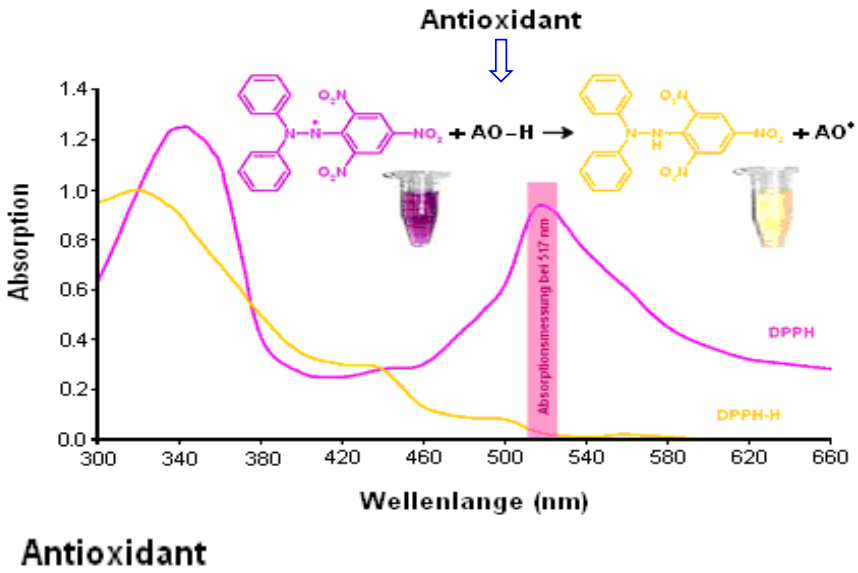

Figure 1: The structure of DPPH• radical and its reduction by an antioxidant (AO-H). (adapted of http://www.baltic-analytics.de/index.php?id=40).

that the percentage of radical scavenged is dependent of the initial concentration of DPPH• radical [14]. For this reason, it is more accurate to use the absorbance variation (or concentration of DPPH• consumed) rather than the percentage of the radical consumed. This absorbance value is further interpolated in a dose-response curve of a standard antioxidant such as ascorbic acid or Trolox and the results are expressed as equivalent concentration [7,15].

The steric accessibility of DPPH• radical is a major determinant of the reaction, since small molecules that have better access to the radical site have relatively higher antioxidant capacity. On the other hand, many large antioxidant compounds that react quickly with peroxyl radicals may react slowly or may even be inert in this assay. The inexistence of DPPH• or similar radicals in biological systems is also a shortcoming. In addition, the spectrophotometric measurements can be affected by compounds, such as carotenoids, that absorb at the wavelength of determination as well as by the turbidity of the sample [16]. The DPPH• assay is not suitable for measuring the antioxidant capacity of plasma, because proteins are precipitated in the alcoholic reaction medium. Finally, the DPPH• scavenging reaction is time-consuming and it may take 20 min up to 6 h. Recently, Magalhães *et al.* in 2006 [17] applied a mathematical model to the data collected within the first 3 min of DPPH• scavenging reaction to estimate the total DPPH• consumed [18]. This approach allowed a considerable reduction of the time taken for a single analysis for samples containing or originating slow reacting antioxidant compounds.

Despite the limitations abovementioned, the DPPH• radical is stable, commercially available, and does not have to be generated before assay like ABTS•+. Therefore, it is considered an easy and useful spectrophotometric method with regard to screening/measuring the antioxidant capacity of both pure compounds and complex samples.

Scavenging of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS•+) or Trolox equivalent antioxidant capacity (TEAC) assay: This method permits the measurement of antioxidant activity of mixtures of substances and hence helps to distinguish between additive and synergistic effects. The assay is based on interaction between antioxidant and ABTS radical cation (ABTS•+) which has a characteristic color showing maxima at 645, 734 and 815 nm [19-21].

ABTS assay measures the relative ability of antioxidant to scavenge the ABTS•+ generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard. The ABTS•+ is generated by reacting a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-green ABTS•+ by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum, during this reaction, the blue ABTS radical cation is converted back to its colorless neutral form (Figure 2). The results are usually expressed as Trolox equivalent antioxidant capacity (TEAC) [22].

The method is rapid and can be used over a wide range of pH values, which is useful to study the effect of pH on antioxidant mechanisms. Furthermore, the ABTS•+ radical is stable and soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/ samples. It also has good repeatability and is simple to perform; hence, it is widely reported. However, as the results obtained for samples are related to an antioxidant standard compound that shows different kinetic behavior, the results provided by this assay are dependent of time of analysis. ABTS assay is frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods [2].

Disadvantages of TEAC assay: ABTS•+ used in TEAC assay is not found in mammalian biology and thus represents a "non physiological" radical source. Thermodynamically, a compound can reduce ABTS•+ if it has a redox potential lower than that of ABTS (0.68 V) [15,16]. Many phenolic compounds have low redox potentials and thus react with ABTS•+. The TEAC reaction may not be the same for slow reactions, and it may take a long time to get the endpoint. Thus, by using an endpoint of short duration (4 or 6 min), one may be reading before the reaction is finished and result in lowered TEAC values.

Advantages of TEAC assay: TEAC assay is operationally simple; it has been used in many research laboratories for studying antioxidant capacity. TEAC values of many compounds and food samples have been reported. ABTS•+ reacts rapidly with antioxidants, typically within

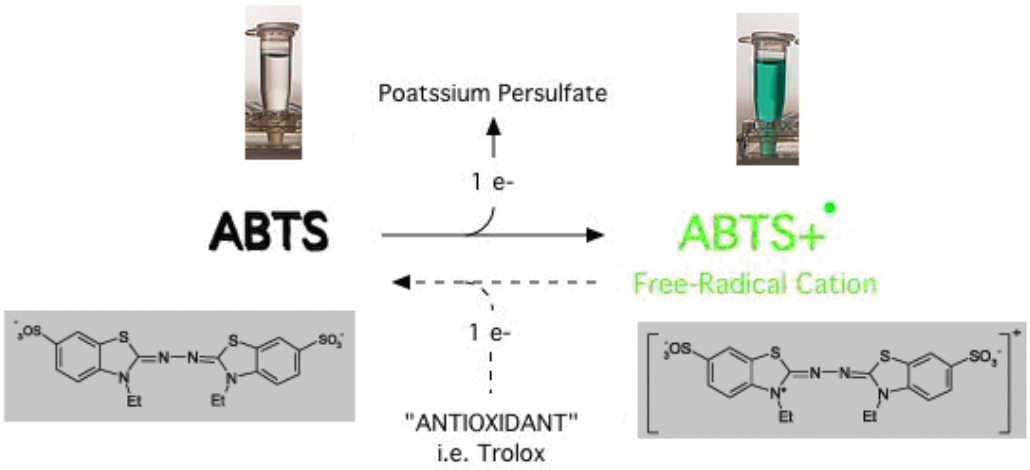

Figure 2: ABTS chemical reaction (adapted of Pannala *et al*. in 2011 [23]).

30 min. $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents and is not affected by ionic strength, so can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids.

Ferric reducing antioxidant power (FRAP assay): The ferric reducing ability of plasma (FRAP) assay measures the ability of plasma to reduce ferric ions, in the form of ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ). It is a simple, inexpensive and robust spectrophotometric technique [2,9]. However, the relevance of this assay is uncertain, as the assay reaction occurs by electron transfer, which does not mimic physiological situations. Furthermore, not all antioxidants are able to reduce Fe, antioxidants that act by H atom transfer are not detected, e.g. thiols. Another potential confounder is that the introduction of Fe may result in the generation of additional free radicals. FRAP values are calculated by measuring the absorbance increase at 593 nm and relating it to a ferrous ions standard solution or to an antioxidant standard solution (ascorbic acid, for instance). This method has also been adapted to 96 well microplate reader, giving better reproducibility and higher sample throughput [14], also was originally applied to plasma but has been extended to other biological fluids, foods, plant extracts and juices [14]. In summary, the FRAP assay is simple, inexpensive, and may offer a putative index of antioxidant capacity.

Radical-trapping antioxidant parameter (TRAP assay): The total radical trapping antioxidant potential (TRAP) assay measures the ability of antioxidants to buffer a reaction probe against peroxidation, using 2,2-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) or 2,2-azobis(2-amidinopropane) dihydrochloride (ABAP) as the free radical source. This assay also acts by H atom transfer. Usually, the oxidation of the probe is monitored using specialized equipment such as a fluorometer [9]. This reduces the applicability of the assay in a general laboratory setting. Antioxidant activity is determined by comparing the extension of the lag time for the appearance of the oxidized probe in the presence of the sample, to the corresponding times for Trolox. Thus the assay makes the assumption that all antioxidants in the sample exhibit a measurable lag time, proportional to their antioxidant capacity, which is not necessarily the case [15].

This assay could be also applied *in vitro* to the evaluation of antioxidant activity of beverages and foods. Thus, it has been used to study green tea and red wine, expressing the results as Trolox equivalents, that is, Trolox micromoles that have the same scavenging peroxyl radical ability as one liter of beverage. In oil samples subjected to peroxyl radical attack by the lipophilic azo compound 2,20-azobis(2,4-dimethylvaleronitrile), the total antioxidant capacity of the oil is determined by oxygen consumption measured with a Clark-type electrode. In this method, TRAP is determined by measuring the length of time that oxygen uptake is inhibited [13].

Scavenging capacity assays against specific ROS/RNS

Superoxide radical anion ($O_2^{\bullet-}$) scavenging capacity assays: Superoxide radical anion ($O_2^{\bullet-}$) is produced as a result of the donation of one electron to oxygen. This radical arises either from several metabolic processes or following oxygen activation by irradiation. The analytical methods for determination of $O_2^{\bullet-}$ scavenging capacity make use of the system XOD/hypoxanthine or xanthine at pH 7.4 to generate superoxide anion radical [8,13]. To a minor extent, $O_2^{\bullet-}$ is also generated using a non-enzymatic reaction of phenazine methosulphate (PMS) in the presence of nicotinamide adenine dinucleotide (NADH). In both generation systems, $O_2^{\bullet-}$ may reduce nitroblue tetrazolium (NBT) into formazan, which is spectrophotometrically monitored at 560 nm [14]. However, this method is unsuitable for reactions with slow rate constants, and requires the presence of appropriate equipment. The capacity of extracts to inhibit the colour to 50% is measured in terms of EC_{50} [24,25].

Interest in the scavenging ability of the superoxide anion is largely due to its role in the production of the highly reactive hydroxyl radical in the presence of metal ions. However, this is not the only mechanism of oxidising lipids, and the ability to scavenge the superoxide anion is not necessarily effective in preventing lipid oxidation. In addition measurement of the scavenging ability of the superoxide anion must be interpreted with care as no equilibrium can be reached when superoxide radicals are generated constantly throughout the assay. Therefore, measurement of the superoxide radical is sufficiently problematic that the uses of these assays are not yet at a standard to recommend its reliability and utility [8].

Hydroxyl radical ($HO^{\bullet}$) scavenging capacity assays: The hydroxyl radical is formed by the combination of Fe(II) and hydrogen peroxide, which is a Fenton reaction [13]. Due to the high reactivity of hydroxyl radicals, almost anything in biological systems can be regarded as an $HO^{\bullet}$ scavenger. Hence, this task is not performed by any specific molecule or enzyme. Thus, the evaluation of direct scavenging of $HO^{\bullet}$ may be irrelevant for evaluation of antioxidant action of a compound or matrix, simply because very high concentrations of scavenger are

required to compete with adjacent molecules in vivo or in the food matrix for any $HO^{\bullet}$ generated. For these reason, it is more relevant and useful to quantify the capacity of putative antioxidants to scavenge or block the formation of its precursors ($O_2^{\bullet -}$, H_2O_2, HOCl) and/or to sequester free metal ions related to $HO^{\bullet}$ formation [14]. Scavenger compounds that act in this way would behave as preventive antioxidants.

Hydrogen peroxide (H_2O_2) scavenging capacity assays: Hydrogen peroxide (H_2O_2) is generated *in vivo*, under physiological conditions by peroxisomes, by several oxidative enzymes and by dismutation of superoxide radical, catalysed by superoxide dismutase. There is increasing evidence that H_2O_2, either directly or indirectly via its reduction product $OH^{\bullet}$, acts as a messenger molecule in the synthesis and activation of inflammatory mediators [13]. One of the most common methods for assessing the scavenging capacity against this molecule is based on the intrinsic absorption of H_2O_2 in the UV region. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity. This method involves *in-vitro* generation of hydroxyl radicals using Fe^{3+}/ascorbate/EDTA/H_2O_2 system using Fenton reaction. Scavenging of this hydroxyl radical in presence of antioxidant is measured. In one of the methods the hydroxyl radicals formed by the oxidation is made to react with DMSO (dimethyl sulphoxide) to yield formaldehyde. Formaldehyde formed produces intense yellow color with Nash reagent (2 M ammonium acetate with 0.05 M acetic acid and 0.02 M acetyl acetone in distilled water). The intensity of yellow color formed is measured at 412 nm spectrophotometrically against reagent blank. The activity is expressed as % hydroxyl radical scavenging [24].

However, the instability of the molecule remains problematic and there are technical difficulties to be overcome before this marker can be reliably used [9], in particular, standardisation of collection procedure, storage conditions and analytical techniques is necessary, particularly if data across studies is to be compared.

Peroxyl radical ($ROO^{\bullet}$) scavenging capacity assays: Peroxyl radicals ($ROO^{\bullet}$) are commonly found in food, natural products and biological samples and they are formed during lipid oxidation chain reactions (autoxidation). This is slightly less reactive than $HO^{\bullet}$ and thus possesses an extended half-life of seconds instead of nanoseconds [7]. Compared with other oxygencentered free radicals, peroxyl radicals are stable species capable of diffusing to remote cellular locations [13,26]. In general, methods for examination of $ROO^{\bullet}$ scavenging capacity measure the ability of an antioxidant to scavenge peroxyl radicals by hydrogen atom transfer reactions. In these assays a competitive scheme is applied, where antioxidants or target molecules react with $ROO^{\bullet}$. Hence, the assay system has three components: thermolabile azo-compound, which yields carbon-centered radicals ($R^{\bullet}$) that react fast with O_2 to give a steady flux of $ROO^{\bullet}$ radicals; oxidizable target; and antioxidant compounds [14].

Nitric oxide radical ($NO^{\bullet}$) scavenging capacity assays: Nitric oxide, because of its unpaired electron, is classified as a free radical and displays important reactivity's with certain types of proteins and other free radicals. *In vitro* inhibition of nitric oxide radical is also a measure of anti oxidant activity. This method is based on the inhibition of nitric oxide radical generated from sodium nitroprusside in buffer saline and measured by Griess reagent, the Griess reaction is frequently used for assessment of $NO^{\bullet}$ production by whole cells or enzymes [27]. Its application to *in vitro* determination of $NO^{\bullet}$ scavenging capacity is also frequent. In this case, the nitric oxide remaining after reaction with the test sample is measured as nitrite. It is important to emphasize that nitrate may also be formed, thus it should be reduced to nitrite prior to determination. In presence of scavengers, the absorbance of the chromophore is evaluated at 546 nm. The activity is expressed as % reduction of nitric oxide [24]. Standard curves were generated using sodium nitrite and results were expressed as percentage change from control response. Compared to other methods, this methodology is not straightforward, requiring the addition of several enzymatic reagents.

Thiobarbituric acid reactive substances (TBARS) Assay

Oxidative stress in the cellular environment results in the formation of highly reactive and unstable lipid hydroperoxides. Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid. Thiobarbituric Acid Reactive Substances (TBARS) assay was proposed over 40 years ago and is now the most commonly used of method to screening and monitoring lipid oxidation [15,28]. TBARS method has been used to evaluate a wide range of samples that include human and animal tissues and fluids, drugs, foods and natural products. The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. Even though there remains a controversy cited in literature regarding the specificity of TBARS toward compounds other than MDA, it still remains the most widely employed assay used to determine lipid peroxidation.

Figure 3 shown the principle of TBARS method, product formed acid-treated samples and standards, followed by the TBA reagent, are added to the included 96 well microplate (Step 1). The microplate is then incubated at 45-50°C for 2-3 hours, during which time the MDA in the sample reacts with the TBA reagent to produce a colored end product (Step 2). The microplate is read at 532 nm and the intensity of the color corresponds to the level of lipid peroxidation in the sample (Step 3). TBA reacts with MDA to form a pink chromagen, which can be detected spectrophotometricaly at 532 nm [14]. Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over time, depending upon the presence of anti-oxidants. In practice, TBARS are expressed in terms of malondialdehyde (MDA) equivalents [28]. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted.

Protein carbonyl assay

The indicator and marker most commonly used of protein oxidation is protein carbonyl content [28]. Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to cation binding locations on proteins and with the aid of further attack by H_2O_2 or O_2 can transform side-chain amine groups on several amino acids into carbonyls. Metal-catalyzed protein oxidation is not the only mechanism by which carbonyls are introduced into proteins, cigarette smoke and aldehydes have also been implicated in the oxidation of plasma proteins. The use of protein carbonyl groups as a biomarker of oxidative stress has some advantages in comparison with the measurement of other products of oxidation, because of the relative early formation and the stability of carbonylated proteins [8]. Most of the assays for detection of protein carbonyl involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenylhydrazone product, which can be analyzed spectrophotometrically at 360-385 nm. This can be quantified by different methods, such as spectrophotometric assay, HPLC, ELISA (enzyme-linked immunosorbent assay) and Slot blotting [30,31]. This assay can be used to measure oxidized protein

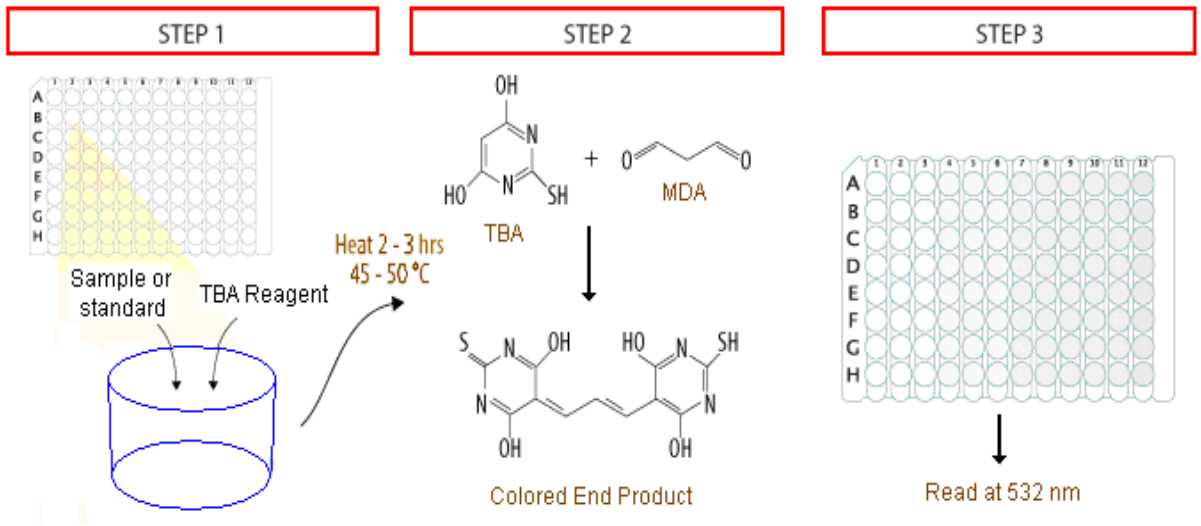

Figure 3: Assay Principle for the TBARS. (http://www.rndsystems.com/rnd_page_objectname_tbars_parameter_kit_measuring_oxidative_stress.aspx; adaptad).

or oxidative stress in plasma, serum, cell lysates, muscle, and tissue homogenates.

Discussion

Due to the variety of available methods, there is the need to use appropriate methodology for the antioxidant capacity investigation, because the assays differ from each other in terms of reaction mechanisms and conditions, as well the form that the results are expressed [5]. Alam *et al.* [2] describe that even when only one method is selected the results may varied accordingly the solvent's choice, time of reaction, pH and standard employed, amongst other not less important factors. These features make difficult to compare the data from different studies. Other important issue is the area of application, i.e., nutrition, pharmaceutical, botanical, chemical, pharmacological and agricultural, that may cause severe influence to the interpretation of the results. Therefore, outstanding to these multiple aspects, it is strongly suggested the use of various methods in order to acquire a more complete antioxidant profile. In fact, the combination of all approaches associated with the choice of methods that are commonly accepted, validated and standardized could be the key to better evaluate the antioxidant capacity [25,31].

In addition, considering all methods antioxidants *in vitro*, the DPPH is easiest and simple. Therefore, is commonly used for measuring the antioxidant capacity of both pure compounds and complex samples [18].

Conclusion

This review article is focused on *in vitro* commonly used methods of antioxidant evaluation. It was prepared based on plenty literature search. Presently, eleven *in vitro* methods are most used for antioxidant evaluation purpose. DPPH method is the most frequently used one for *in vitro* antioxidant activity. This article will be a comprehensive ready reference for those who are interested on antioxidant study.

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