A SHORT REVIEW ON THE PRINCIPLES OF DPPH ABTS, FRAP, CUPRAC AND FOLIN-CIOCALTEU ASSAY: ADVANTAGES AND DISADVANTAGES

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Abstract

At the present time, the world is suffering from the increasing jeopardy of incurable diseases like cancer, tumour, diabetes, arthritis, heart, brain or immune dysfunction etc., specially caused by the free radical induced oxidative damages. Therefore, the modern research community is very highly fascinated about the research related to the substances having potential antioxidant activity as these compounds are capable of preventing the free radical chain reactions and thereby can avert the ailments caused by oxidative damages. For this, simple, cost friendly, convenient, reliable and experimentally viable antioxidant activity assessment methods are very much significant. Due to the existence of numerous free radicals or oxidant sources and different kinds of antioxidant species with highly diverse chemical and physical characteristics, mechanism of action of antioxidants differ from each other, depending upon the nature of the substances involved in the process or other experimental parameters. Therefore, quite a number of antioxidant activity evaluation methods have been reported, each with their own advantages and disadvantages and selection of proper method with respect to the antioxidant and free radical type considering other important reaction parameters is very much imperative in order to have accurate estimation of the antioxidant capacity of the samples under study. In this short review, the author has attempted to analyse the principles behind the mechanism of action of certain electron transfer based antioxidant activity determination assays along with their advantages and disadvantages.

Keywords: Antioxidant activity, Electron transfer, free radicals, DPPH, ABTS, FRAP, CUPRAC, Folin-Ciocalteu assay

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Introduction

Oxygen, the most obligatory element for life, plays a fundamental role in diverse range of biological phenomena. However, it can aggravate the damage within the cell by oxidative events [1]. Even though the oxidation reactions are imperative for life, they can generate free radicals which originate chain reactions that instigate oxidative stress induced damage to cellular constituents [1, 2]. This damage is a primary contributor of aging and many of the serious degenerative ailments [3, 4]. More than about 100 disorders like cardiovascular disorders, hemorrhagic shock, neurodegenerative diseases, metabolic diseases, gastrointestinal ulcerogenesis, inflammatory bowel diseases are known to be ROS (reactive oxygen species) mediated, out of which, a few specific examples are - cancer, Alzheimers disease, Parkinson's disease, rheumatoid arthritis, cystic fibrosis, Down's syndrome, atherosclerosis, diabetes, cataracts, AIDS and ischemic reperfusion injury in various tissues including heart, brain, liver, kidney and gastro intestinal tract etc. Thus, in modern times, there is an intensification of research works related to the prevention of diseases by antioxidants, as antioxidants are the substances that are proficient in wiping up the free radicals and thereby they avert the free radicals from causing oxidative cell damages. Actually, an antioxidant is a molecule or agent, which, even if exist in a very lower amount in comparison to the amount of an substance that is prone to oxidation, can proficiently retard or restrain the adverse impacts of reactive oxygen and nitrogen species on usual human physiological functions. Antioxidants protect the cells through the termination of the chain reactions by eliminating free radical intermediates and restrain other oxidation reactions by being themselves oxidized. So, they are also termed as free radical scavengers. A healthy human body can biologically produce antioxidants, nevertheless the procedure is not 100% efficient in case of excessive and irresistible generation of free radicals and this ability also diminishes with age [2,3]. Thus, to maintain the body in healthy conditions as well as to prevent and cure various diseases, external supplementation of antioxidants is necessary, either through food sources, or via supplements or medicines. It is of enormous significance to the medical, nutritional experts as well as for bio-chemical, pharmaceutical, medicinal or food science researchers to estimate the antioxidant properties of various food constituents, natural extracts and various bio organic or bi-inorganic synthetic molecules [5-8].

For the measurement and analysis of antioxidant activities of various substances, a number of different methods have been proposed. However, there are many different aspects regarding this, which pose a lot of challenging issues with respect to the determination of antioxidant activities of molecules. One such aspect is that, in case of biological systems, there exist at least four different kinds of antioxidant sources: firstly, the enzymes like catalase, superoxide dismutase and glutathione peroxidise; secondly, the small molecules (ascorbic acid, uric acid, glutathione, carotenoids, tocopherol, polyphenols); thirdly, the big molecules (albumin, ferritin, ceruloplasmin, other proteins); and finally, various hormones like estrogen, insulin, melatonin, progesterone, etc. Alternatively, there exist manifold free radicals or oxidant species [e.g., $HO^{\bullet}$, $O_2^{\bullet-}$, $^1O_2$, $ROO^{\bullet}$, $ONOO^-$, $NO^{\bullet}$, $LOO^{\bullet}$]. The chemical and physical properties of different oxidants and antioxidants differ from each other. It has been observed that, in some cases, antioxidant molecules act via a single mechanistic pathway in a single system, and in some other cases, antioxidants have been observed to act via several mechanistic pathways even in the same system, depending on the reaction conditions [9, 10]. Thus, way of interaction of different antioxidant molecules might obviously be dissimilar in case of different free radical or oxidant species. The antioxidant properties of heterogeneous biological systems and food systems are known to be influenced by a tons of factors like, system pH, activation energy of antioxidant molecules, oxidation conditions of the systems, partitioning features of the antioxidant molecules between aqueous and lipid phases and the physical state of the oxidizable substrates, etc. In addition, the behaviour of the same antioxidant towards one oxidant might be different from its behaviour towards another oxidant. For instance, carotenoids do not usually work as good peroxyl radical scavenger in comparison to phenolic compounds and many other antioxidant species; but perform as outstanding scavenger of singlet oxygen, whereas, most other phenolic compounds and antioxidant molecules are observed to be almost ineffective towards singlet oxygen. Nevertheless, singlet oxygen, although it is a potentially harmful oxidant source, it cannot be considered as a free radical and therefore its response is not observed to occur via radical pathways. Rather, it interacts mainly by resulting additive products called

endoperoxides, a type of heterocyclic peroxides with -O-O- residue in the ring, subsequent reduction of which finally provides alkoxyl radicals and thus oxidative chain reactions are caused [9-11].

As numerous reaction features and pathways are typically involved, a particular method to study the antioxidant activity of compounds will not be able to precisely work for the complete range of free radicals and antioxidant species that might get involved in a complex system [12]. Proper analysis is necessary in order to match the characteristics of existent radical sources and system properties with probable antioxidant action mechanism and this analysis is very much essential for the proper choice of suitable antioxidant activity study method. It is very important to observe that the antioxidant molecules are stable and do not react with other molecules in the system except the free radicals or oxidant species, antioxidants do not cause discolouration of the system or impart any off-colour or off-flavour in the system, especially in case of food systems. Due to these reasons, till date, there is no universally accepted single method for the precise quantitative antioxidant activity assessment procedure. Even, it was also observed that, the same antioxidant molecule demonstrate noticeably different efficiency against same type of free radicals in different systems [10, 11].

In this short review, we have attempted to analyse the principles of some highly recommended and most widely used electron transfer based antioxidant activity assays along with their advantages and disadvantages, specifically, DPPH, ABTS, FRAP, CUPRAC, Folin-Ciocalteu assay.

Types of Antioxidants

Commonly, antioxidants may be classified into two types:

(a) Primary or natural antioxidants
(b) Secondary or synthetic antioxidants.

A dietary antioxidant that can sacrificially quench reactive oxygen or reactive nitrogen species (ROS and RNS) in order to impede chain reactions induced by these free radical species, can be recognised as primary antioxidant. They are also known as free radical scavengers or chain-breaking antioxidants. When present in trace amounts, they are capable of either delaying or inhibiting the chain initiation by inactivating the free radicals; accordingly they can prevent chain initiation reactions and chain propagation reactions by interacting with alkoxyl and peroxyl radical species [13]. Secondary or synthetic antioxidants are phenolic compounds that can restrain the highly unstable oxidant molecules from getting produced at the very initial stage. They generally capture free radicals and in that way, they avert the oxidative damage by impeding the free radical chain reactions.

Alternatively, as reported by Ratnam and co-authors [14], antioxidants can be classified into two other groups-

a) enzymatic antioxidants
b) non-enzymatic antioxidants

Antioxidants that are endogenously generated, have low molecular weight and act as enzyme cofactors, are considered as enzymatic antioxidants; whereas non-enzymatic antioxidant molecules are usually sourced by dietary products. Again, dietary antioxidants can also be divided into a range of different classes. Among those classes, polyphenolic compounds correspond to the principal and biggest class. The term, polyphenols usually include different kinds of phenolic acids as well as flavonoids, within its meaning. The other different sections of dietary antioxidants consist of molecules of vitamins, carotenoids, organosulfurs and various minerals [15].

Reaction mechanisms

Analytical methods for determining antioxidant capacities have been categorized into two major groups depending upon the reaction mechanisms involved, namely, hydrogen atom transfer reaction-based methods and single electron transfer reaction-based methods. The final result obtained upon completion of end point is the same in case of both the mechanisms, but kinetic parameters and prospective side reaction or by-products varies. In most of the cases, electron transfer reactions and hydrogen atom transfer reactions might occur in an analogous manner, however, the predominant reaction mechanism, in case of a specific system under particular circumstances, is usually decided on the basis of factors like structural and physiochemical characteristics of antioxidants and free radicals or oxidant sources, medium of reaction, solubility parameters, partition coefficient, pH and temperature of the reaction, etc [16].

A. Hydrogen Atom Transfer (HAT) Mechanism

Hydrogen atom transfer based methods evaluate the typical free radicals scavenging aptitude of an antioxidant via donation of hydrogen atom. As an outcome, the free radicals are stabilized by formation of stable compounds and the antioxidant itself forms a stabilized radical [17], as shown in **Scheme 1**. Thus, the antioxidant, AH, reacts with the free radical, $R^{\bullet}$ by transferring a hydrogen atom to the radical and forms a stable radical $A^{\bullet}$ and stable compound RH.

$AH + R^{\bullet} \rightarrow RH + A^{\bullet}$ **(Scheme 1)**

HAT-based assays are more relevant in case of the radical chain-breaking antioxidant potential. As the HAT mechanism involves hydrogen atom donation, the bond dissociation enthalpy (BDE), regarding the H-atom donating group of the antioxidant molecule, is the main factor for the antioxidant activity assessment of a molecule. In general, this mechanism is dominant for those species which have BDE around 10 kcal/mol and ionization potential value lower than -36 kcal/mol. HAT reactions usually rely on pH of the system and solvent used in the measurements and are usually fairly swift, normally finished within seconds to minutes. Majority of the HAT-dependent assays scrutinize competition between kinetic parameters of the reactions and the evaluations are obtained using the curves so obtained. These kinds of methods usually utilize a synthetic free radical originator, like, 2,2′-azo bis(2-amidinopropane)dihydrochloride; 2,2′-azo bis(2,4-dimethylvaleronitrile), etc.; an antioxidant and an oxidizable molecular probe, like dichlorofluorescein, fluorescein, etc. Usually, peroxyl radicals are preferred as the reactive species in the HAT based assays due to their greater biological significance and longer half-life in comparison to other important radicals such as hydroxyl radicals and superoxide anion radicals. The HAT-based methods utilizing fluorescent probes have a similarity in mechanism with lipid peroxidation, but, in these measurements, most of the time, the antioxidant concentration is higher in comparison to the substrate concentration. This is opposite to real food systems as the substrate (e.g., lipid) concentration is very much higher than the antioxidant concentration in real food systems [18-20]. Thus it is dubious whether the antioxidant potential calculated via HAT-based methods by means of some chromogenic probe can be capable of demonstrating the conditions of actual foodstuff related systems. Some well recognised HAT based antioxidant activity assessment methods are-

a) Total radical trapping antioxidant parameter or TRAP assay
b) Lipid peroxidation inhibition capacity or LPIC assay
c) Oxygen radical absorbance capacity or ORAC assay
d) Inhibited oxygen uptake or IOC assay
e) Crocin bleaching nitric oxide radical inhibition activity
f) Hydroxyl radical scavenging activity
g) Scavenging of H_2O_2 radicals
h) Scavenging of super oxide radical, etc.

B. Single Electron Transfer (SET) Mechanism

Single electron transfer mechanism deals with the ability of a potential antioxidant to transfer one electron to reduce any compound (including metals, carbonyls, and radicals) and thereby becoming itself a radical cation [4, 12]. It involves a single step in which a radical cation, $AH^{\bullet+}$ is generated, as shown in **Scheme 2** and **3**. The adiabatic ionization potential is the determining parameter involved in this reaction and the low IP values indicate better antioxidant activity. SET reactions are typically slower and need longer times in order to reach completion, when compared to HAT reactions. The anion R^- thus formed is an energetically stable species containing an even number of electrons, while the cation radical $AH^{\bullet+}$ accordingly formed after transfer of an electron must be a stable species so that it does not interact with the substrate or other molecules [21].

$AH + R^{\bullet} \rightarrow AH^{\bullet+} + R^-$ **(Scheme 2)**

$M(III) + AH \rightarrow AH^{\bullet+} + M(II)$ **(Scheme 3)**

In single electron transfer mechanism, ionization potential (IP) of the reactive functional group plays the most noteworthy energetic factor for scavenging activity assessment. The lower the value of ionization potential of a specific functional group in a compound, smoother will be the electron transfer from it in a single electron transfer mechanism and greater will be the antioxidant potential. In general, the single electron transfer is followed by swift and reversible deprotonation in solution and its relative activity is also reliant on deprotonation, in addition to the ionization potential of the reactive functional group. The antioxidant activity pathway is primarily SET for materials with an IP> -45kcal/mol. In comparison to HAT pathway, the SET pathway is robustly dependent on reaction medium or solvent because of the probable solvent stabilization of the resultant cationic or charged species. The pH of the system

causes a significant influence on the reducing ability of antioxidant samples. Ordinarily ionization potential values declines with enhancing pH, signifying augmented electron-donating ability along with deprotonation. In acidic medium, reducing ability of the antioxidant molecules might get decreased because of probable protonation on the antioxidants, while basic medium might help in enhancing their reducing ability due to probable proton dissociation. SET reactions might possibly be reasonably slow and necessitate longer time period to achieve completion so these methods tend to traditionally assess the comparative percentage reduction in products instead of depending on the competitive kinetic parameters or instead of assessing total antioxidant potential.

SET-methods usually engage two different kinds of substances for their action; first one is an antioxidant molecule and second one is a chromogenic molecular probe, which interacts like an oxidant species and can take the electron provided by the antioxidant molecule. As a result of this reaction, the colour of the chromogenic probe alters which can be detected, usually by observing their absorbance using an UV-Vis Spectrophotometer. Amount of the change in colour can be considered to be proportional with respect to the concentration of the antioxidant samples under study, the higher the concentration, greater is the observed colour change. When the alteration in the colour seems to be stopped, it can be considered as the end point of the reaction. After careful monitoring of the absorbance, the change in the absorbance, i.e., ΔA values can be plotted against sample concentration in order to obtain a linear curve. From the slope, the reducing ability of the potential antioxidant can be measured, that is typically articulated with respect to some standard reference antioxidant, for example, as trolox equivalent, ascorbic acid equivalent or gallic acid equivalent, etc. In these cases, it is assumed that reducing ability corresponds to antioxidant ability, so that the proper co-relation can be made [18, 20-23].

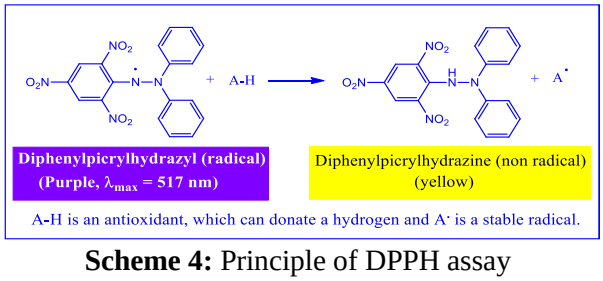
Some frequently used SET based methods are-

a) DPPH free radical scavenging assay
b) Trolox equivalent antioxidant capacity (TEAC) assay or ABTS free radical scavenging assay
c) Ferric reducing antioxidant power assay or FRAP assay
d) Folin-Ciocalteu assay (total phenolic assay by using the Folin-Ciocalteu reagent)
e) Cupric reducing antioxidant capacity assay or CUPRAC assay, etc.

In general, two of the most highly used antioxidant capacity assays, namely, the ABTS and DPPH assays are considered to be dependent on SET reaction pathway, although these two radicals can be scavenged not only by direct reduction through electron transfer pathways, but also by radical quenching through hydrogen atom transfer mechanism. In many cases, these two methods were reported to be HAT based, whereas many others reported as SET based. Factors, such as - type of antioxidant, medium or solvent, etc., can influence the dominating mechanism in a system [24, 25].

a) DPPH radical scavenging Assay

First reported by Brand-Williams *et al.* [26], DPPH assay is one of the simplest and most extensively used antioxidant activity assays. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a widely used stable organic nitrogen radical with a deep purple color, that usually demonstrates strong UV-Visible absorption band at 517 nm, attributable to its odd electron. Antioxidant molecules reduce the radical and thereby it transforms into the pale yellow coloured stable compound 2,2-diphenyl-1-picrylhydrazine and due to the pairing of electron, and the absorption diminishes **(Scheme 4).** The subsequent decolorization is recognised to be stoichiometric with respect to the quantity of electrons taken up. The DPPH radical scavenging potential is usually scrutinised by UV-Visible spectroscopy in which the reduction in absorbance of the reaction mixture is monitored at 515 to 528 nm, until the moment arrives when absorbance values become consistent, in hydrophobic or organic solvent [27].



Scheme 4: Principle of DPPH assay

Formerly, many researchers supposed that DPPH method followed the hydrogen atom transfer pathway; however modern research works revealed that it is basically an electron transfer based method and hydrogen-atom abstraction occurs in a marginalistic pathway [24-26]. The rate determining step of the reaction is between

DPPH radical and antioxidant that happens very swiftly via initial electron transfer and thereafter a very slow subsequent hydrogen atom transfer occurs, which especially relies on neutral hydrogen bond accepting solvents like methanol and ethanol. The rate and the mechanism of reaction between antioxidant and DPPH radical might be influenced by a range of factors, like temperature of the system, absolute as well as relative concentrations of DPPH radical solution and antioxidants, pH of the system, existence of oxygen, polarity and ionising character of solvents and existence of intermolecular or intramolecular hydrogen bonding interactions, etc., [28-32].

Advantages

DPPH radical solutions are extremely stable for a very long period if it is properly kept in a dark place. The radical stays as a free monomer, not only as solid, but also in solution and it is too stable as a monomer to dimerize. DPPH assay is the most comprehensively recommended antioxidant activity study method as it is extremely simple, uncomplicated, speedy and can be carried out in highly rudimentary chemical laboratories because it only requires a simple UV-Visible spectrophotometer. The extraordinary stabile nature of DPPH radical, economic and experimental viability makes the radical very much appropriate for analytical use in order to appraise the radical scavenging ability of different antioxidant samples for application in areas like medicine, food and cosmetics, etc. [28-35].

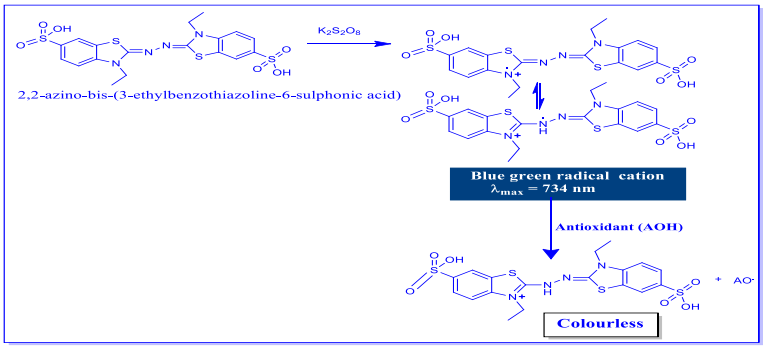
Disadvantages

Although DPPH radical has superior solubility in polar organic solvents (especially in alcohols), it is very disappointingly soluble in apolar solvents. At ambient temperature, it is nearly insoluble in water. So in this method, it is not possible to use water as a solvent, which is a vital drawback when we attempt to interpret the effectiveness of hydrophilic antioxidants. The assay does not follow a competitive kinetics based determination process as DPPH acts as the radical probe as well as the oxidant. Along with electron transfer and hydrogen transfer reactions, sometimes, its colour can be lost through some unrelated reactions as well. Steric accessibility plays a significant role in the reaction and small species can have superior access to the radical site resulting apparently higher antioxidant activity value in comparison to large species. Again, in cases when tested samples demonstrate spectra (for example, carotenoids) that overlap with that of DPPH near 517 nm, the interpretation becomes very much complicated. Although, it can be called a commonly used process for evaluating the antioxidant potentials in case of majority of food components and phenolic compounds, the changes in absorbance have to be cautiously monitored as the absorbance value can also be decreased by factors like light, oxygen and solvent properties, in addition to the antioxidant, which might give wrong assessment regarding the actual antioxidant activity of the samples. It was also reported that above a certain water content of the solvent, the antioxidant ability is reduced because of the coagulation of the DPPH radical making it less accessible for the antioxidants. DPPH radical only has slight resemblance with the extremely reactive, transient peroxyl radicals and therefore, certain antioxidants which might interact with peroxyl radicals in an extremely fast manner in vivo, may interact very little or may not interact with DPPH radical at all. The kinetics of the reaction has also observed to be non linear with the concentration of DPPH radical; so indication of antioxidant activity via EC_{50} value is quite problematic. It was also observed that DPPH radical react with eugenol in a reversible manner which may provide false and low activity readings in case of eugenol containing samples and other phenolic compounds with similar structures. Additionally, basic and acidic impurities that may possibly present in the solvent might also have an impact on the ionization equilibrium of phenolic compounds and consequently can originate an increase as well as diminution in the value of the calculated rate constants and thus can give false antioxidant activity results [28-35].

b) ABTS radical scavenging assay or TEAC assay

ABTS or TEAC assay is based on the utilization of the colorimetric probe 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt radical cation (or $ABTS^{\bullet+}$) which can accept electrons, as well as hydrogen atoms provided by antioxidant samples. In ABTS assay, antioxidant activity is examined by relying on the capability of the studied compound to diminish the colour of ABTS radical cation by interrupting the initial oxidation with subsequent inhibition of the radical cation production, or by direct interaction with the previously formed radical cation **(Scheme 5)**. Miller and Rice-Evans reported the original TEAC for the first time and they utilized metmyoglobin-H_2O_2 to produce ferryl myoglobin at first, which was then allowed to react with ABTS to generate $ABTS^{\bullet+}$. However, it was revealed that, this assay results an overestimated

antioxidant capacity and therefore, certain modifications were reported with the use of oxidizing agents like potassium persulfate or solid manganese dioxide and also by using in situ electrochemical oxidation processes. Among these improvised versions, potassium persulfate based method is most highly recommended because of superior yields of the radical cation in addition to its radical/antioxidant inertness [26]. $ABTS^{\bullet+}$ exhibits maximum absorption bands near 645, 734 and 815 nm. As interaction of an antioxidant with ABTS radical cation results in its absorbance reduction to a non-zero value, it was recommended that the initial solution should be so prepared to exhibit an absorbance around 0.70 or 1.0 (normally corresponding to an original $ABTS^{\bullet+}$ concentration of 67 μM). Several polyphenolics or carotenoids might possibly represent overlapping absorbance around 400-450 nm, while 734 nm can be normally considered to be out of the range of such probable interference, which makes the wavelength of 734 nm the most preferable choice. So, in the study of the assay, radical cation solution is prepared in a suitable solvent and its initial absorbance at 734 nm is monitored, with the subsequent addition of the antioxidant, and then the decrease in the absorbance at 734 nm is monitored again after a period of 4 minutes which can be extended up to few hours [40-45].



Scheme 5: Principle of ABTS radical scavenging assay

Advantages

Concentrated stock solutions of ABTS radical cation can usually be stored in a refrigerator for long time as the radical cation in such conditions remains stable for several months. This assay is very simple to operate and reactions of $ABTS^{\bullet+}$ with antioxidants are very swift. It is stable over an extensive pH range, so this method is also suitable in cases, where the influence of pH over antioxidant activity mechanisms is needed to be analysed. $ABTS^{\bullet+}$ has the most attractive feature regarding its solubility as it can be solubilised in aqueous as well as in organic solvents and it is not influenced by ionic strength. So, in ABTS assay, activity of compounds can be efficiently studied in case of both hydrophilic and lipophilic antioxidant types [25]. TEAC method utilizing the ABTS oxidation via H_2O_2 and metmyoglobin only allows the evaluation of hydrophilic antioxidant molecules whereas the method using manganese dioxide as the oxidising agent allows the evaluation of lipophilic antioxidant molecules (e.g., carotenoids, tocopherols, etc.). When the reaction medium is altered from aqueous to ethanolic, this assay facilitates the analysis of both, hydrophilic and lypophilic type antioxidant compounds [42-45].

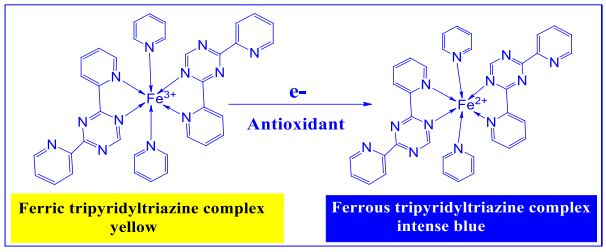
Disadvantages

Practically, TEAC value indicates the ability of the antioxidant molecules to interact with $ABTS^{\bullet+}$ rather than inhibiting the actual oxidative process. In case of many phenolic compounds and natural product extracts, this method has been observed to require a very long duration for proper completion of the reaction. Accordingly, by considering a fixed reaction end point with shorter duration, like 4 minutes, may provide false and less resultant antioxidant activity values or percentage inhibition values than the actual value, as the reaction might not be finished while monitoring the final absorbance. Differences in the resultant values were reported, when comparisons were done between the calculated antioxidant activity, with the assumption that the reaction is finished in a shorter time, along with the kinetic parameters, where the antioxidant and radical concentrations as well as the time required for depletion of the radical were also considered. For instance, BHA (butylated hydroxyanisole) exhibited superior antioxidant potential compared to ferulic acid when absorbance values were monitored at a certain end point, but it indicated slower kinetics in comparison to that of ferulic acid. Also, one important drawback of this assay is that ABTS

radical is nonexistent in biological systems and also it does not have any resemblance to radicals available in biological systems. Although ABTS radical cation is reported to be stable over an extensive pH range, most frequently maintained pH level is 7.4. However, the strength and stability of the radical cation at this pH has been observed to be tricky. In case of widely used standard antioxidants like trolox, ascorbic acid, etc., ABTS radical cation at this pH afforded consistent end-point values after 10 min, whereas, in case of standard phenolics, the results after 10 min period do not correspond to proper end-point values based on oxidation and only give estimated results only. Also, the antioxidant activities of standard phenolics were observed to be 5–20% greater at pH 7.4, in comparison to the results obtained at pH 4.5. From thermodynamical point of view, any substance having lower redox potential than ABTS (0.68 V), can cause the reduction of ABTS radical cation. Thus, it was mentioned that, quantitative estimation of antioxidant property by this method might be complicated or impossible at times, however, it is successful in order to obtain antioxidant activity orders with comparative assessments [42-45].

c) Ferric reducing antioxidant power assay

The large majority of the commonly reported antioxidant activity evaluation methods are indirect methods that determine the capacity of antioxidant samples to avert the oxidative effects of reactive species that have been purposefully generated in the system under observation. In these kinds of methods, antioxidant activity brings on a lag phase and when the antioxidant ability of the tested sample get exhausted; an indication can be obtained by observing the change in the absorbance value. Alternatively the ferric reducing antioxidant power (FRAP) assay is a relatively simple, fast and direct method to calculate the total antioxidant activity or electron donating ability of antioxidant compounds [46-48]. So, it is a redox-linked colorimetric method, where an antioxidant sample is considered as a reducing agent and follows a non direct radical scavenging test in order to find out the total antioxidant activity of the sample. In this method, solution of ferric tripyridyltriazine salt is treated with the antioxidant present in the reaction mixture which donates an electron, and as a result, the Fe^{3+}-TPTZ solution is reduced to the ferrous form. The redox potential of such an antioxidant should be less than the redox potential of the half reaction: salt-Fe^{3+} + electron → salt-Fe^{2+}; under the same condition of reaction parameters [49, 50]. The ferric salt solution initially displays pale yellow colour and demonstrates an absorption band at 593 nm, but the colour changes to intense blue on the reduction of ferric ion to ferrous form (**Scheme 6**) and the absorbance value at 593 nm enhances.



Scheme 6: Reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ by antioxidant

The important reaction parameters like the temperature, pH of the system, volume of antioxidant and reagent and time period of reaction are fixed. From the changes in the absorbance values at 593 nm, the total antioxidant activity is determined by comparing the changes in the absorbance after addition of a known concentration of ferrous ion to the reagent, instead of the antioxidant sample under similar reaction parameters. The ferric ion is available in huge surplus in the reaction mixture. Accordingly, the reaction limiting factor is considered as the combined or total electron donating ability of reducing agents (i.e., the antioxidants) present in the reaction mixture [51].

Advantages

FRAP assay is very widely used as it is easier, uncomplicated, rapid, low-cost, low-maintenance and robust. The method does not necessitate any kind of specialized apparatus or particular expertise and can be carried out in automated, semiautomatic or manual manner. Also, it does not call for a critical reaction parameters or conditions or system environment. Pre-treatment of the samples is not required and the reagents used in this assay are stable and non-toxic and the stoichiometric features are consistent over a wide range. The results obtained in this method are highly accurate, found to be linear over a wide range and it shows a lag phase nature of estimation. In addition, this method has outstanding reproducibility and sensitivity [49-54].

Disadvantages

TEAC assay and FRAP assay both begins with the assumption that redox reactions progress so quickly that they reach to reaction completion

within 4 and 6 min, correspondingly, but this have not always observed to be the true in practice. Some of the polyphenolic compounds do not interact this fast and need longer reaction periods (like 30 minutes or more) for their recognition. In case of polyphenolic compounds like, ascorbic acid, caffeic acid, quercetin, ferulic acid and tannic acid, etc., absorption at 593 nm gradually keeps on augmenting even after a few hours of reaction period. Thus, similar to the case of TEAC method, a single absorption end-point of shorter duration might not signify a completed reaction. To be precise, FRAP only estimates the reducing ability of the molecules, relying on ferric ion, which cannot be actually considered to be appropriate as antioxidant activity, from mechanistic and physiological point of view. Fe^{2+} ion is also known for its pro-oxidant activity and it can generate the most reactive and detrimental free radical available in vivo, i.e., hydroxyl radical, from its interaction with hydrogen peroxide. As, according to this assay, antioxidant power is the potential of a compound to produce Fe^{2+} from Fe^{3+}, so it is highly possible that antioxidants like ascorbic acid and uric acid can cause reduction of Fe^{3+} in addition to the reduction of other reactive species and the capability to reduce ferric ion might or might not indicate the potential to reduce the other reactive species. However, all reducing agents that can reduce ferric ion, cannot be called as antioxidants. Compounds with electron-donating ability, having less redox potentials than the redox pair Fe^{3+}/ Fe^{2+}, might actually cause false addition to the calculated FRAP values and designate misleadingly higher values, even though they do not possess antioxidant property. On the other hand, antioxidant molecules with the capacity to cause efficient reduction of prooxidants, might not lead to effective reduction of ferric ion. For instance, glutathione is a very efficient antioxidant found in vivo, but the FRAP method have not been found to be applicable for glutathione or other thiols. Serious issues were observed if other Fe^{3+} species exist in the reaction mixture that can interact with metal chelating agents available in food components as such complexes are competent to react with the antioxidants. Many observations revealed that, there is no exact relationship of the quantity of electrons that can be donated by an antioxidant with its calculated FRAP value. Ascorbic acid, uric acid and α-tocopherol depict an equal FRAP value of 2.0, whereas bilirubin shows two fold higher value as compared to ascorbic acid which indicates that 1 mol ascorbic acid and 1 mol bilirubin can potentially reduce two fold (2 mol) and four fold (4 mol) Fe^{3+} ion. This result is completely contradictory as bilirubin and ascorbic acid both shows their ability as two electron donors [10, 49-54].

d) Folin-Ciocalteu Assay

This assay is considered as one of the most widely used methods for the assessment of total phenolic content of various natural compounds, like fruits, vegetables, herbs, etc., and so this method is also termed as: "Total phenolic assay by using the Folin-Ciocalteu Reagent". Although the precise chemical character of the Folin-Ciocalteu reagent is properly not clear, it has been recognised to contain phosphomolybdic/phosphotungstic acid complexes. Folin-Ciocalteu assay measurements are dependent on electron transfer from phenolic compounds or other compounds having reducing abilities, in alkaline medium that finally results in blue complexes. The complexation can be spectrophotometrically monitored by observing the absorbance values at 750–765 nm. The intensity of the absorption bands have been reported to be linear with respect to the concentration of phenolic content in the reaction mixture. The interaction of phenolic compounds with Folin-Ciocalteu reagent only takes place at basic conditions, which is achieved to have pH-10, by using sodium carbonate solution [55-59]. This method was used by Singleton and Rossi [57] for the estimation of total phenols present in wine and thereafter this method was extended over a wide range of samples. They indicated certain important conditions so that the variation and inconsistency in results can be eliminated-

a) Appropriate volumetric ratio of the alkali and Folin-Ciocalteu reagent
b) Proper selection and utilization of reference standards like gallic acid,
c) Optimal temperature and reaction time period for the development of proper colour,
d) Scrutinizing the optical density at 765 nm that might help in minimizing the sample matrix led interference that is usually coloured,

Advantages

Although the exact chemical features of Folin-Ciocalteu reagent is uncertain, this method is very easy, simple and has very high convenience and amazing reproducibility. Numerous research works have followed the total phenol assay by Folin-Ciocalteu reagent along with various other antioxidant activity study methods like DPPH, TEAC, FRAP, etc., and most of the time, outstanding linear relationship has been found

between the antioxidant activity and total phenolic profiles of the compounds. Along with phenolic compounds, Folin-Ciocalteu reagent is also applicable for non phenolic substances as a number of non phenolic compounds were found to be successful in reducing it, as well [55-62].

Disadvantages

Along with the utilization of gallic acid for the reference standard, many other reference standard equivalents like tannic acid, catechin, caffeic acid, chlorogenic acid, ferulic acid equivalents have also been used. Without the standardization process, this method might provide difference in the magnitude of results upto many orders. Resultant absorbance values are reliant on the molecular structure of the compounds and are typically proportional with the quantity of interacting phenolic hydroxyl moieties. For instance, the absorbance provided by caffeic acid, that has two reactive hydroxyl groups and cathechin with three reactive hydroxyl groups are two and three fold higher, respectively, than the absorbance of phenol, having one such hydroxyl groups. Thus, if very highly reactive standard molecule is used as reference for calibration that may provide very elevated absorbance values producing very low results for the tested samples. This method is an aqueous phase method as it can be carried out in water, but inapplicable for lipophilic antioxidants. Folin-Ciocalteu reagent has not been considered to be particular for phenolics only, as it was observed to be efficiently reduced by a lot of non phenolic molecules. Thus, this method evaluates the reducing ability of a compound; however, the name of this method "total phenolic assay" does not reflect the same. Therefore, there is a continuous conflict about what is perceived in case of total antioxidant activity assays, whether they are only phenolic compounds or phenolic compounds along with reducing agents or with prospective metal chelating agents [10, 55-62].

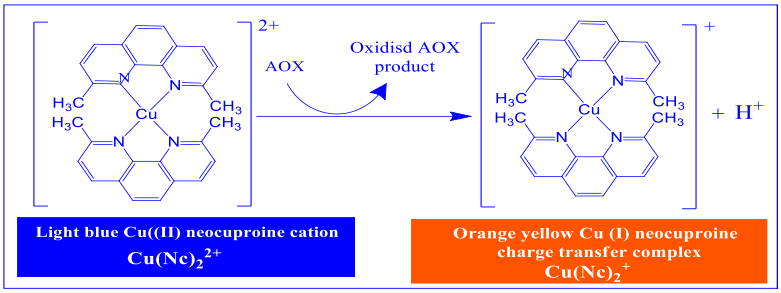
e) Cupric reducing antioxidant capacity assay

CUPRAC assay was originally introduced by Apak *et al.*, [63] and is dependent on the reduction reaction of Cu^{2+} to Cu^{+} by the reducing action of antioxidant present in the reaction mixture. This method utilizes the Cu^{2+}-neocuproine reagent as chromogenic oxidant for its measurements. In CUPRAC assay, the solution of the antioxidant compound is mixed with neocuproine (i.e., 2,9-dimethyl-1,10-phenanthroline), copper chloride and ammonium acetate, by maintaining the system pH at 7, and after 30 minutes, the absorbance is monitored at 450 nm using an UV-Vis spectrophotometer. The initial light blue colour, attributable to the Cu(II)-neocuproine cation solution, transforms into orange-yellow colour, because of its reduced form, Cu(I)-neocuproine cation **(Scheme 7)**. Thus the products obtained from this process are buffered using the ammonium acetate medium. The reaction in CUPRAC assay needs 30 minutes for its completion in case of majority of the tested samples. In case of slow reacting antioxidants, the incubation temperature is required to be enhanced, so that their oxidation with CUPRAC reagent can be completed to obtain accurate measurement. The cupric ion concentration in the CUPRAC reagent is required to be in stoichiometric surplus to that of the neocuproine, so that the redox reaction proceeds to the right hand side. In practice the true oxidant is $Cu(Nc)_2^{2+}$ instead of Cu^{2+}, as the standard redox potential of $Cu(Nc)_2^{2+}/Cu(Nc)_2^{+}$ couple is 0.6 V, much greater to that of Cu^{2+}/Cu^{+} couple, that has a standard redox potential of 0.17 V. This can explain the reason why the polyphenols are more easily oxidized by Cu^{2+}-neocuproine than with Cu^{2+}. As this method does not utilize any kind of radical reagent, so it is found to be insensitive with respect to a variety of physical parameters including temperature, pH, sunlight, humidity, etc. Hydrolysis of flavonoid glycosides to their analogous aglycons is necessary in order to evaluate their proper antioxidant ability. Similarly, polyphenols have to be oxidized to their quinone forms and the product so obtained from reduction is the copper(I) chelate of bis(neocuproine), which displays maximum absorption bands at 450 nm. The reagent is allowed to get adsorbed on a cation-exchanger membrane and thereby an economical, linearly interacting antioxidant sensor is prepared [63-67].

Advantages

This method is distinguished to have advantage over other electron transfer methods due to the fact that operational pH range in this method is same as the physiological pH (i.e., pH 7), whereas FRAP assay mostly uses acidic pH (around 3.6) and Folin method generally uses alkaline pH (around 10) for their measurements. It has advantage over DPPH assay and Folin assay also on the fact that it can be used for hydrophilic as well as lipophilic antioxidants. The CUPRAC method is very advanced, yet very easy and straightforward antioxidant activity determination method which can be most extensively utilized to assess the antioxidant potentials of many of the

synthetic antioxidant samples as well as natural antioxidant species, counting phenolic acids or other polyphenolic compounds, flavonoids, thiols, hydroxycinnamic acids, vitamin E and ascorbic acid, trolox, etc.



Scheme 7: Redution of Cu((II) neocuproine reagent

In case of polyphenolic compounds, CUPRAC values have been found to be in similarity with TEAC values, while the frequently observed FRAP values were found to be noticeably lower. Compounds like citric acid and reducing sugars that are not actually antioxidants but can be oxidised in processes like FRAP assay, are generally un-oxidized by the CUPRAC reagent, because of the lesser redox potential displayed by this CUPRAC reagent. It is also useful for evaluating the ability of the antioxidants bearing –SH groups. The lower redox potential have the ability to augment redox cycling, which makes this process a superiorly sensitive evaluator of prospective pro-oxidant ability of antioxidant samples, as well. Other advantages of this method over other electron transfer based methods are: CUPRAC reagent is swift and adequately strong to cause successful oxidation of thiol-type antioxidants, while the FRAP assay is not successful for studying thiol type antioxidants; CUPRAC reagent have superior stability and accessibility than most of the chromogenic assays including ABTS or DPPH assay; the absorbance vs. concentration curves of CUPRAC assay have been observed to be linear above a broad range, in contrast to other assays that gives polynomial curves; and this method can be carried out very easily in regular laboratories and can be applied in a diverse manner. Thus, this method is applicable for all kinds of antioxidants, which is an advantage of utilization of copper over iron and it can be distinguished without having much intervention from fast reacting radicals and also the reaction kinetics involving copper reagent is faster to that of iron. Thus, with all these advantageous features, this assay is self-sufficient and it can be called a complete package of "antioxidant and antiradical assay altogether" [10, 25 63-66].

Disadvantages

It takes few minutes for completion in case of compounds like for vitamin E, uric acid, vitamin C, gallic acid, quercetin, whereas it needs 30-60 minutes in case of complex structures. Thus, it also has a problem with the selection of proper end point for completion of reaction in case of complex compounds; otherwise it will provide false results [10].

Conclusion

Dr. Richard Cutler, former Director of the National Institute of Aging, Washington once mentioned, "The amount of antioxidants in your body is directly proportional to how long you will live". Selection of proper foods with potent antioxidant activity or incorporation of antioxidant supplements in regular diet can prospectively diminish the jeopardy of serious life-style related degenerative ailments and can support us to augment the longevity of life. The use of proper antioxidant activity evaluation methods can assist us in understanding the antioxidant properties of various natural and synthetic compounds and thus can support us in choosing antioxidant rich foods, supplements etc. Most importantly, it can help various bio-chemical, medicinal, pharmaceutical researchers to find out various natural extracts and synthetic molecules that can be used for food preservation or for drug synthetic purposes, in order to combat free radical induced diseases. Through this short review article, a few of the electron transfer mechanism based antioxidant activity evaluation methods are analysed which follow similar mechanistic pathways but differ from each other in terms of their reaction conditions, oxidant and target probe, reaction expression and resultant values. In deciding the most suitable antioxidant activity evaluation method for a specific kind of species, characteristics of the antioxidant molecules, system features and conditions, solvent, pH, temperature and concentration of the

samples should be carefully observed. As already mentioned, till date, no single antioxidant capacity assessment method is successful for estimation of the activity of all kinds of antioxidants that may follow different single mechanisms in different systems, or multiple mechanisms in the same system. All these methods have advantages as well as various disadvantages that should be properly and thoroughly understood before selecting the reaction conditions, reaction durations, solvent, pH, temperature and other essential features of the reaction. So we hope that this article will provide additional insight towards the development of research related to antioxidant activity analysis of diverse kinds of natural and synthetic compounds.

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