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Saeed Ahmad
The Islamia University of Bahawalpur Pakistan

Muhammad Adeel Arshad
The Islamia University of Bahawalpur Pakistan

Shakeel Ijaz
The Islamia University of Bahawalpur Pakistan

Umair Khurshid
The Islamia University of Bahawalpur Pakistan

Faisal Rashid
The Islamia University of Bahawalpur Pakistan

Rizwan Azam
The Islamia University of Bahawalpur Pakistan

Correspondence:
Muhammad Adeel Arshad
The Islamia University of Bahawalpur Pakistan

Review on methods used to determine Antioxidant activity

Saeed Ahmad, Muhammad Adeel Arshad, Shakeel Ijaz, Umair Khurshid, Faisal Rashid, Rizwan Azam

ABSTRACT
Antioxidants are helpful in the defense mechanism of body against different pathogens. The use of plant derived antioxidants is helpful against many degenerative diseases like Parkinson, Alzheimer and Cancer. This review gives an overlook to the different methods used to determine antioxidant capacity of different antioxidants. The explanation of analytical performances and principles of different methods used for the determination of antioxidant capacity involve chromatography, spectrometry and electrochemical techniques are discussed in detailed.

Keywords: antioxidants, chromatography, spectrometry, electrochemical

1. Introduction

The impact of atmospheric oxygen and other reactive oxygen species lead to oxidation processes and the compounds capable of inhibiting or delaying the effects of such processes are called antioxidants. Different products like foodstuffs, petrochemicals, pharmaceuticals and cosmetics are stabilized by antioxidants.

The defenses of an organism against the attacks of free radicals are made strong by antioxidants. Antioxidants occur in nature as both endogenous and exogenous species. Enzymatic compounds like catalase, glutathione peroxidase, superoxide and dismutase are included in endogenous antioxidants, also bilirubin, uric acid, albumina and metallothioneins are among the non-enzymatic endogenous antioxidants. The role of exogenous antioxidants comes into play when endogenous species are unable to provide full protection against the reactive oxygen.

Vitamin C, vitamin E, vitamin D, vitamin K_3, flavonoids, minerals and β-carotene are among the most effective and important exogenous antioxidants used as active ingredients in most pharmaceuticals and food supplements. Compounds like butylhydroxytoluene, butylhydroxyanisole, gallates etc are among the synthetic exogenous antioxidants, while they can be obtained from natural sources as well like flavonoids, vitamins, anthocyanins and some minerals [1].

In order to prevent harmful effects of free radicals in the body, also for the retrogression of food constituents and fats, interest in the use of antioxidants is fairly increasing [2].

2. Benefits of antioxidants

The relation of antioxidants to the cancer prophylaxis, therapy, longevity and oxidative stress has gained notable attention in recent days [3].

Recent studies suggest that the diseases related to oxidative stress like cancer, coronary heart disease, hypertension, obesity, diabetes and cataract are best protected by the use of vegetables, fruits and less processed foods [4]. This is made possible due to the positive health effects of vegetables and fruits containing beneficial antioxidants. Walnuts, cranberries, blackberries, strawberries, blueberries, raspberries, brewed coffee, cloves, grape juice, unsweetened chocolate are at the top of the food list containing antioxidants. On analyzing 50 food items containing high amount of antioxidants, 13 of them were spices, 5 were berries, 8 of them were vegetables and fruits, 5 were cereals and 4 of them were nuts [5].

Antioxidants like polyphenols, β-carotene, lycopene, vitamin C and vitamin E are found in large amounts in beverages, fruit juices and hot drinks [6]. The relationship between antioxidants obtained from the diet and oxidative stress can be checked and better understood by the use of the term total antioxidant potential (TAP). Recent studies have shown that the risk of both gastric cancers and cardiac problems are reduced by the total antioxidant potential in diet [7]. Different methods have been developed in order to estimate TAP capacity of dietary items including foods and drinks. There is a difference in the production of radical species and also in the end product measurement that is, how different analytical methods differ in their mechanisms. The interaction of redox and synergistic properties of different food molecules reflects the total antioxidant potential [8, 33, 34].

Databases containing complete antioxidant contents are unavailable because of high diversity of antioxidant potential molecules present in the foodstuff. Risk of chronic diseases is reduced by the healthy consumption of vegetables, fruits, nuts and grains [9]. The focus has been given to the plant derived foodstuffs or phytochemicals with antioxidant capability. The increased antioxidant potential is due to the synergistic and cumulative activities of biomolecules present in the plant food. So, investigations on the role of antioxidants in chronic disease States should ideally be linked to the database of foodstuffs rich in antioxidants [9]. Antioxidants are also contained by different berries and less common fruits. Cancer and inflammation in humans can effectively be prevented by an impact of high intakes of flavonoids, furnished with antioxidants, largely due to their anti-inflammatory and antiproliferative properties [10].

In order to evaluate connection between oxidative stress induced diseases and diet, total antioxidant capacity (TAC) is regarded as an important tool in recent studies showing a negative relation between gastric cancer and dietary TAC [7]. TAC is also a novel tool to estimate if there is any additive antioxidant property present in the foodstuff. In Italy three different ways of assay analysis have been adopted in order to assess intake of TAC in overall population including TAC of 30 fruits, 34 vegetables, 34 beverages and 6 vegetable oils. With a goal to compile an Italian TAC database, the TAC of cereals, pulses, sweets, dried fruits, spices and nuts was ascertained. Amid beverages, the highest TAC was found in coffee, followed by soft beverages, among which citrus juices had the greatest value and the highest TAC values were found in berries among fruits [11].

3. Mechanism of Action

LMWAs (low molecular weight antioxidants) [12] are acquired from several different sources in human tissues. Uric acid [13] and bilirubin [14] are wastes produced as a result of cellular metabolism, nicotinamide adenine dinucleotide, carnosine [15] and glutathione are composed inside the cells, while tocopherols, polyphenols and ascorbic acid [16] are present in dietary items and obtained from them. Due to its reductive properties and large scale usage as an antioxidant agent, ascorbic acid (AA) among LMWAs is of most importance and receives a significant amount of focus [17]. It plays a pivotal role in triggering an immune response of the body, in osteogenesis. Also wound healing, collagen biosynthesis, iron absorption and prevention of vesicular clotting are among some of the significant roles of ascorbic acid [18, 19]. Degradation of ascorbic acid because of its easy oxidation by light , heat and heavy metal cations makes it an important quality indicator for different food items [20, 21].

Excess of free radicals are critical in this regard as they tend to oxidize low density lipoproteins, which in turn become potentially harmful. Acceleration of the aging process is also linked to the circulation of free radicals in the body. Also serious criticalities like diabetes mellitus, Parkinson's disease, rheumatoid arthritis, Alzheimer's disease, cancer and brain stroke are directly related to excess of free radicals. A very strong oxidizing tendency is exhibited by reactive oxygen species (ROS). The oxidizing tendency is for both radical nature and non-radical nature compounds. Superoxide radical and hydroxyl radical are the examples of radical natured molecules, while non-radicals include ozone and hydrogen peroxide [22].

Oxidation can be initiated by many different chemical and physical processes until a blocking defense mechanism takes place, otherwise in the presence of suitable environment, it may proceed continuously. Cholesterol, oxygen, phospholipids, fatty acids and DNA are the possible target substances. The chain reaction process of oxidation via free radicals involves a number of steps, including initiation, propagation, branching and termination. External agents like heat, light and ionizing radiations may initiate the process of oxidation, or else it may be chemically initiated by metal ions [23, 24].

3.1 Initiation

$LH + R\cdot \rightarrow L\cdot + RH$

Substrate molecule is represented by LH like lipid and R· as the initiating oxidizing radical. Oxidation of lipids causes the generation of a very reactive alkyl radical (L·) which can then speedily react with the oxygen to generate a lipid peroxyl radical (LOO·).

3.2 Propagation

$L\cdot + O_2 \rightarrow LOO\cdot$

$LOO\cdot + LH \rightarrow L\cdot + LOOH$

The chain carriers of the reaction are peroxyl radicals; they can further cause the oxidation of lipids, which result in the production of lipid hydroperoxides (LOOH), that then result into the breakdown into a variety of different compounds [69], and these compounds include aldehydes, ketones alcohols, alkyl formats and hydrocarbons and radicals which include alkoxyl radicals(LO) [25].

3.3 Branching

$LOOH \rightarrow LO\cdot + HO\cdot$

$2LOOH \rightarrow LOO\cdot + LO\cdot + H_2O$

The breaking of lipid hydro peroxides generally involves the transition metal ion catalysis, and this reaction similar to those reaction which involve the hydrogen peroxide and this result into the production of lipid peroxyl and also lipid alkoxyl radicals.

3.4 Termination

Termination is the last step in this reaction and it involves the combination of the radicals and result into non-radical products:

$LO\cdot + LO\cdot$

$LOO\cdot + LOO\cdot$

$LO\cdot + LOO\cdot$

AH which is a primary antioxidant when it is present in trace amount it may either inhibit or delay the initiation step by reaction with lipid radical or it may stop the propagation step by reaction with alkoxyl or peroxyl radicals [26].

$L^{\cdot} + AH \rightarrow LH + A^{\cdot}$
$LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot}$
$LO^{\cdot} + AH \rightarrow LOH + A^{\cdot}$
Those compounds which retard the rate of oxidation process are called secondary or preventative antioxidants. This can be done by a number of ways, including the removal of substrate or may be by singlet oxygen quenching [23, 27].

4. Antioxidant capacity measurement methods
There are various analytical methods for the determination of antioxidant capacity and these methods belongs to different classes and these are given below:

1. **Spectroscopic Techniques**
2. **Electrochemical Techniques**
3. **Chromatographic Techniques**

4.1 Spectroscopic Techniques
Spectrometric techniques depend upon the reaction of radical, radical cation or complex, with an antioxidant molecule which is capable of donating a hydrogen atom [28-36].

4.1.1 DPPH Technique:
DPPH which is 2,2-diphenyl-1-picrylhydazyl. Due to the delocalization of the spare electron on the complete molecule, it is a stable free radical. As what happened with most free radicals DPPH does not dimerize. Purple colour appeared due to the delocalization of DPPH molecule with an absorption band around 520 nm.
The DPPH method was used in the determination of antioxidant capacity in many fruit extract and in many fruit juices. The result are expressed in uM Trolox Equivalents/g fresh mass and standard curve is linear between 25 and 800 mM Trolox. Guava fruit methanol extract has antioxidant activity between 16.2 ± 1.0 and 32.0 ± 5.1 μM TE/ fresh mass [30, 32].

4.1.2 ABTS Technique:
At 743 nm ABTS cation radical is absorbed by the loss of an electron by a nitrogen atom of ABTS. If Trolox or any other hydrogen donating antioxidant the atom of nitrogen quenches the hydrogen atom producing a decolorized solution [33].
Potassium phosphate or manganese dioxide can oxidized ABTS producing ABTS cation radical (ABTS+), its absorbance diminish at 743 nm in the presence of Trolox which is chosen as standard antioxidant. The ABTS cation radical method was applied to determine the antioxidant content in alcoholic beverages, tea, coffee and soft beverages. The standard curve was linear between 25 and 600 uM Trolox. The vales obtained by this method of guava extract was ranged in between22.3 ± 0.9 and 37.9 ± 3.4μM TE/ fresh mass.
Soft beverages antioxidant activity was also measured by ABTS method and it came between 0.09 mM Trolox/liter for the grape juice [11, 31, 32].

4.1.3 FRAP Technique:
The FRAP which is stand for ferric reducing antioxidant power. This method depends upon the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine). Navy blue colour is generated when Fe^{2+} bind with a ligand. The amount of antioxidants can be correlated with the amount of iron reduced and this amount of iron reduced is measured by absorbance. The reference used was ascorbic acid or Trolox.
By the FRAP method antioxidant activity of white and yellow-flesh nectarines was measured. The value ranged between 14.4 and 104.5mg/100 of fruit [11, 32, 34].

4.1.4 ORAC Technique:
ORAC stands for oxygen radical absorption capacity. The 2,2'-azobis-(2-amino-propane) dihydrochloride at 37°C induced the antioxidant scavenging activity against peroxyl radical and this activity is measured by this method. In this fluorescein was used as the fluorescent probe. The reaction with the peroxyl radical is because of the loss of fluorescence and loss of fluorescence is the indicator of amount of decomposition. The antioxidant activity of guava fruit extract with methanol was determined by this methanol and the standard curve obtained for this method was linear between 0 and the 50 mM Trolox. The guava extract result was lying between 18.2 ± 2.3 and 25.5 ± 1.6 μM TE/ fresh mass [32].

4.1.5 HORAC Technique:
HORAC is hydroxyl radical averting capacity. This is the method which measure the metal chelating activity of the antioxidant and the conditions provide are like the Fenton like reaction. Cobalt (II) complex is used in this method, which prevent the formation of hydroxyl radical. First of all fluorescein is incubated with the material which is required to be analyzed and then Fenton mixture was added into it. After the initial fluorescence was determined then after every minute reading was taken and every time shaking is done. A standard curve was obtained by using Gallic acid solution [29, 35].

4.1.6 TRAP Technique:
TRAP stands for total peroxyl radical trapping antioxidant parameter. In this method luminal enhanced chemiluminescence was used which involve the peroxyl radical. The thermal decomposition of AAPH causes the production of luminal derived radicals and from it CL signal is obtained. The value of TRAP was measured by that time period at which sample quenched the signal and this occurs due to the presence of antioxidants [29, 37].

4.1.7 PFRAP Technique:
This is potassium ferricyanide reducing power. The reducing ability of the antioxidant extract may be correlated to the increase in absorbance. The potassium ferrocyanide is formed when a compound with antioxidant ability react with the potassium ferricyanide. Then the product reacts with ferric trichloride forming ferric ferricyanide, which is blue coloured complex and it has a maximum absorbance at 700 nm [38, 39].

4.1.8 CUPRAC Technique:
This stands for cupric reducing antioxidant power. With neocuproine and $CuSO_4$ standard antioxidant or their extract are mixed. The absorbance was measured at 450 nm after time period of 30 min. The electron donating antioxidant act and cause the reduction of Cu(II) to Cu(I). the result of this method are written in mg of Trolox per liter of extract [38, 40].

4.1.9 Fluorimetry:
This method is also being used for the determination of antioxidant content. It is a phenomenon in which substances absorbs light and then emit it. Fluorescence occurs when an

electron moves from a lower energy state to a higher energy state and then come back to its lower energy state by emitting radiation. And the light which is emitted in this process has low energy and longer wavelength than the absorbed radiation. By this method we can determine the phenolic compound in oils.

A very strict control of pH is required if a fluorimetric method is used for the determination of ascorbic acid. O-phenylene with dehydroascorbic reaction occur in this.

By the help of fluorescence assay it can be determine how sterol lateral organization affects antioxidant potency and also the extent of sterol oxidation in lipid bilayers can also be determine by fluorescence assay [41-44].

5. Electrochemical Techniques:

The antioxidant capacity of various compounds is also frequently evaluated using electro chemical methods. Commonly used electrochemical methods are bioamperometric and cyclic voltammetric methods.

5.1 Cyclic Voltammetric Technique:

This method is based on the potentio-dynamic electrochemical evaluation. In this process the working electrode is ramped linearly to the time. The working electrode potential measured at different intervals in order to calculate the current intensity at respective electrode. On attaining the preset potential, the potential ramp of working electrode is reversed. In a single experiment this reversal can take place many times. Then the cyclic voltammogram is made by plating working electrode's current to the applied voltage. A cyclic voltammogram gives the following significant parameters such as cathodic oxidation potential, anodic oxidation potential, anodic peaks and cathodic peaks. Cyclic voltammetry has to be proved to be the most suitable method for the determination of the antioxidant capacity of low molecular weight substances as blood plasma, plant extracts [12]. The antioxidant capacity of some dried plant extracts was measured by cyclic voltammetry using carbon electrode. Then the results were expressed as ascorbic acid equivalents in milligrams. The samples were of coffee, black tea, Green tea and rosemary [22].

5.2 Amperometric technique:

It is based on the principle that the intensity of the current flowing between a reference and a working electrode is measured at a fixed potential. A current is produced by the oxidation/reduction reaction of an analyte whose value is fixed at a preset value as compared to the reference electrode [45].

The measurement of the antioxidant activity by an amperometric method is based on the reduction of the DPPH using carbon electrode [45]. These experiments are carried out in an electrochemical cell consisted of three electrodes at 140 mV vs. Hg_2Cl_2 3Mpotassium chloride using 40% ethyl alcohol solution and 0.033 M potassium chloride. The buffer used was phosphate buffer of pH 7.5.

5.3 Biamperometric Technique:

In this process, between two same working electrodes that is polarized at the minute difference in potential and immersed in a solution that contains a reversible redox couple. The current flows in this process are measured. This commonly used redox couple in biamperometric measurement is Fe^{3+}/Fe^{2+}, I_2/I^-, $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ [46]. Controlled parameters in biamperometry is potential difference between two same kind of working electrodes while the potential value of reference electrode is not controlled with respect to two electrodes.

5.4 Biosensors Technique:

Because of their electron giving nature, oxidoreductases are very commonly used biosensors. These are very stable in nature and do not require any other cofactor or chemical for their working. They have been broadly studied and discussed in the literature [47, 48]. Biosensors are employed for the determination of ascorbic acid, uric acid, phenolic compounds, super oxides and peroxides. In broader sense most of the antioxidant property of the plant extract is due to their polyphenolic constituents. These polyphenols are detected by the enzyme based biosensors. These enzymes include tyrosinase and lacease. The total phenolic content in the red wine were evaluated by the biosensors electrode using tyrosinase as it acts on the hydroxyl group of the phenols. The total phenolic content of the vegetable extracts were determined by using the peroxidase based biosensors. These results were calibrated [49, 51].

6. Chromatographic Techniques:

Chromatographic techniques are also employed for the assessment of the anti-oxidant capacity of the plant extracts. Of the chromatographic techniques, gas chromatography is used for these purposes.

6.1 Gas chromatography:

Gas chromatography is one of the primary type of chromatography that is extensively applied for the separation and analysis of stable but vaporizable compounds. The process is carried out by passing the mixture through the stationary phase which is liquid and the mobile phase in this type of chromatography is gas. Commonly used gases as mobile phase are helium and nitrogen that are considered to be inert. Whereas the stationary phase is a micro film of a liquid incorporated on to a non-reactive solid support. The retention time comparison gives its value. Usually thermal conductivity and flame ionization detectors are used in gas chromatography. Example of the sample analyzed for antioxidant capacity is turmeric oil. The procedure was done by using gas chromatography and mass spectrometer system. The detector used was flame ionization detector. The sample was then evaluated for anti-oxidant capacity by employing other two systems as carotene linoleate system and phosphor molybdenum method [52].

6.2 HPLC:

In HPLC, the analyte along with mobile phase is moved through a shorter length column compacted with fine particles of stationary phase with a higher pressure provided by the pump resulting in a cogent separation. The retention time is measured by a detector.

Normal phase HPLC involves the use of a non-aqueous and non-polar mobile phase along with a polar stationary phase to separate effectively the non-polar substances. While Reversed phase type of HPLC involves the use of aqueous and somewhat polar mobile phase along with a non-polar stationary phase. Well known stationary phase is silica which is used after treating it with trialkylchlorosilane RMe2SiCl to elute polar substances easily and to enhance retention time for substances with low polarity. On-line antioxidant activity is determined using a post column in the HPLC system. The eluate after separation of the components on HPLC column is subjected to a photodiode array detector. Then it is mixed with ABTS (2,2'-

azinobis-3-ethylbenzothiazoline-6-sulphonic acid) to determine absorbance at specific wavelength using a detector. On quenching any radical, bluish colour of the ABTS disappears showing a negative peak. Total antioxidant activity is obtained by adding all the individual peaks [53].

Another method involves the use of 5% acetic acid-acetonitrile-methanol mobile phase on C18 HPLC column using fluorescence detector. Comparing antioxidant standards with fluorescence spectra, sample peaks can be analyzed [54].

7. Conclusion

The antioxidants have gained a very good importance is mainly because of health benefits which are given by the antioxidants which have low molecular weight. The antioxidants work by preventing the attack of free radicals on the basic components of the body like nucleic acid or lipids.

Different methods can be used for the antioxidant determination and these methods include chromatography, spectrometry and electro analytical. By the help of these techniques total amount of antioxidant present in the food stuff can be determined.

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