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A Review on *In-vitro* Antioxidant Methods: Comparisions, Correlations and Considerations

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Abstract: This stimuli article provides an overview general aspect, their comparisons, correlations and considerations of various *In-Vitro* methods to measure the antioxidant defense system and to discuss a number of updated *In-Vitro* methods used for detection of antioxidant properties. This review article gives the information regarding the different methods that are used to perform the *In-Vitro* antioxidant activity. It emphasizes the method simplicity, time required, instrumentation which makes us to decide which method to be fallowed to perform antioxidant property based on the feasibilities afforded to determine it. It makes a glance regarding the advantage of different methods and which is most common method used in present days for effective analysis. On the other hand as there are advantage there may be some disadvantages which are also mentioned in this article. The research studies should also be carried mostly on the accurate methods for exact results which also act as a good reference for the further researches.

Key words: *In-Vitro* antioxidant methods, Cellular antioxidant activity, Cellular antioxidant activity, Semi quantitative analysis, Folin-Ciocalteu method.

Introduction

A antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by- products of cell metabolism.[1,2] Free radicals form when oxygen is metabolized or formed in the body and are chemical species that posses an unpaired electron in the outer (valance) shell of the molecule. This is the reason, why the free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA. These free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell[3]. Free radicals may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are $\mathbf{O^-_2}$ [superoxide], HO[hydroxyl] ,HO_2 [hydroperoxyl], ROO[peroxyl], RO[alkoxyl] as free radical and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly NO [nitric oxide], ONOO [peroxy nitrate], NO_2 [nitrogen dioxide] and N_2O_3[dinitrogen trioxide].[4,5] In a normal cell, there are appropriate oxidant: antioxidant balance. However, this balance can be shifted, when production species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Lipid peroxidation is oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading yield a wide range of cytotoxic products, most of which are aldehydes, like malondialdehyde (MDA), 4-hydroxynonrnal(HNE), Oxidative stress causes serious cell damage leading to a variety of human diseases[6] like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. Nutritional antioxidant deficiency also leads to oxidative stress, which signifies the identification of

natural anti-oxidative agents present in die consumed by human population.[7, 8]

1) Comparisons

***In-Vitro* determination of antioxidant capacity**

This approach has benefits over simply quantifying antioxidant components as it provides a measure of their effectiveness. The various conventional and latest methods comes under invitro are listed in table no.1. It is very difficult to select a suitable antioxidant assay method. Antioxidants act by several mechanisms and no one assay can capture the different modes of action of antioxidant. Conventional cuvette assay of radical scavenging activity is replaced by 96-well plate titer assay from past couple of years. Cuvette assay method uses UV-Visible spectrophotometer to see the absorbance, where as 96-well plate method uses ELISA plate reader for absorbance. The first method is very tedious, time consuming method, allows only 1 sample to read a time and requires high quantity of reagent where as the second method is time saving and it reads about 96 samples at a time, with small amount of reagent.

a) TLC Autography technique

The antiradical screening by thin layer chromatography (TLC) autography technique provides an easy, effective and rapid way to study plant extract profiles. No sample purification is needed as this technique provided a simultaneous separation and radical scavenging activity measurement of anti-oxidative compounds in plant extract.[9] qualitative as well as semi quantitative analysis of antioxidants can be done by this technique.

Qualitative analysis

In order to detect the antioxidant activity, a method based on the reduction of 2,2-diphenyl-1-picrylhydrazyl(DPPH) can be carried out. DPPH is a free radical stable at room temperature, which produces a violet solution in methanol. When the free radical reacts to an antioxidant, its free radical property is lost due to chain breakage and its color changes to light yellow. In the DPPH free radical scavenging capacity assay by TLC, the extracts that produced yellow are white spots in the purple background were considered as antioxidants.

Procedure in brief is-extracts resolved in the solvent is spotted on the silica-gel 60F 254 plates and develop the chromatogram in adequate solvent systems. Now all the plates can be sprayed with a methanolic solution of DPPH (2mg/ml). Thus, antioxidants appear as yellow bands on a light purple background. After spotting the extracts on the TLC plates, even uneluted plates also can be used to determine the qualitative antioxidant analysis. The uneluted plates also can immerse in 0.2% DPPH methanol solution and sample spots were evaluated for radical scavenging activity. The same method can be implemented to detect total phenolic and total flavonoid content just by changing the mobile phase solvent system and visualizing agent. Vanillin /H_2SO_4 reagent is sprayed on the plate and heating it at 110^0C, for 5 minutes and observed to detect different groups of compounds. Orange-yellow spots indicate poly phenolic compounds. The silicagel plate was sprayed with natural products-PEG reagent and observed at UV-365 nm, to detect the flavonoids as they appear as yellow-orange fluorescent spots.

Semi quantitative analysis

The diameter and intensity of the yellow spot depends on the amount of known amount and concentrations of the solutions. We can judge the potency of the test sample. Further more, R_f values for each hand can be calculated and photographs can be taken.

40 µg shows equal antioxidant property with that of 50 µg of rutin by this method[10]. A method was developed to measure the radical scavenging activity of compounds separated by reversed-phase TLC (RP-TLC) using phenolic acids as model analytes. TLC separation was followed by dipping the plate in a 0.04% (wt/vol) solution of 1, 1 – diphenyl-2-picrylhydrazyl (DPPH) in methanol. Reversed phase technique was applied for the measurement of free radical-scavenging activity of rapeseed meal fractions.[11] Bleaching of β-carotene on TLC is another method. After developing and drying, plates were sprayed with a 0.02% solution of β-carotene in CH_2Cl_2. plates were placed under natural light until discoloration of background. The yellow spots remaining indicated the presence of antioxidant substances. Absorbance at 517 nm was determined after 30 min and the percentage of activity was calculated.[12] Using TLC autography, a total of 58 extracts from various organs (aerial parts, leaves, flowers, fruits, roots) of 16 Turkish plants were tested for their antibacterial, antifungal, acetylcholinesterase inhibitory, antioxidant and radical scavenging activities. Antioxidant and radical scavenging activities were found to be predominant in highly polar extracts.[13] TLC – DPPH combined with Video scanning Documentation system including a HV-C20 3 x ½ " CCD video camera (Hitachi, Japan) can be connected with Reprostar 3 transilluminator cabinet, and for TLC plate imaging software supplied by Camag, Muttenz, Switzerland can be used.

The method was successfully applied to rapeseed meal extract. A RPTLC method combined with video scanning detection for quantitative evaluation of free radical scavenging activity of antioxidative fractions from rapeseed meal by using DPPH is reported. The activity was evaluated by measuring the area of bright

yellow bands against the purple background by a CCD video camera after dipping the plate in 0.04% (w/v) DPPH solution. DPPH scavenging activity of L-ascorbic acid and 17 well-known phenolic compounds including α-tocopherol, phenolic acids and flavonoids was determined by this TLC-DPPH method.

Advantages

This technique is easy, effective, and rapid way to study plant extract profiles. No sample separation is needed. Potency of sample can be known.

b) Cellular antioxidant activity (CAA) assay

Recently, scientists at cornell University proposed a new measure of antioxidant activity called the cellular antioxidant activity (CAA) assay, which they dubbed the "next step" in quantifying antioxidant activity. The new CAA method tests antioxidant compounds activity inside cells: An approach that probably provides a more accurate gauge of the antioxidant power of whole foods and individual antioxidant nutrients and compounds. Kellys Wolfe and Rui Hai Liu of cornell university's food science lab developed the new method in which antioxidant reaction will takes place inside the cell. This new approach is more biologically relevant as it accounts for uptake, metabolism, distribution and activity of antioxidant compounds in cells versus solely looking at antioxidant value. They applied the new technique to equal amount s (100 grams) of wild blueberries, cranberries, apples, red and green grapes. The results placed wild blueberries on top, followed by cranberries, apples, red grapes and green grapes.[14]

Advantages

More accurate guage of antioxidant power of whole foods and individual antioxidant nutrients and compounds. This approach is more biologically relevant as it accounts for uptake, metabolism, distribution.[14]

Disadvantages

Time consuming, costly.

c) Dye-substrate oxidation method

A novel microtiter plate assay was developed to determine the total peroxyl radical trapping activity of antioxidant extracted from marine organisms by measuring the inhibition rate of dye-substrate oxidation. They compared use of dihydrorhodamine-123, dihydrofluorescein and dichlorodihydrofluorescein as reduced substrates for oxidation by peroxyl radicals generated from 2,2-azobis(2-amidinopropane) dihydrochloride. The oxidation products of these highly reactive substrates are intensely colored dyes that absorb maximally in the wavelength region, 489 to 512 nm, and their concentrations were determined photometrically using a 96-well, microtiter plate reader. The microtiter plate method provides for concurrent multisample analysis with automated data storage, regression analyses, and calculation of oxidation inhibition rates. Dihydrorhodamine was selected as the preferred substrate for screening crude extracts, and typical assay results are presented. Novel lead antioxidants are selected from active extracts by chromatographic analysis with electrochemical detection. [15]

Advantages

It provides fro concurrent multisample analysis with automated data storage, regression analyses, and calculation of oxidation inhibition rates. For screening crude extracts and typical assay results are presented.

d) Cupric Ion Reducing antioxidant capacity (CUPRAC)

CUPRAC method is Novel hydroxyl radical scavenging antioxidant activity assay for water-soluble antioxidants. Reactive oxygen species (ROS) may attack biological macromolecules giving rise to oxidative stress-originated diseases. Since OH is very short-lived, secondary products resulting from OH attack to various probes are measured. Although the measurement of aromatic hydroxylation with HPLC / electrochemical detection is more specific than the low-yield TBARS test, it requires sophisticated instrumentation. As a more convenient and less costly alternative, we can use p-aminobenzoate, 2,4- and 3,5-dimethoxybenzoate probes for detecting hydroxyl radicals generated from an equivalent mixture of Fe(II) + EDTA with hydrogen peroxide. The produced hydroxyl radicals attacked both the probe and the water soluble antioxidants in 37^0C incubated solutions for 2 h. The CUPRAC absorbance of the ethylacetate extract due to the reduction of Cu (II)-neocuproine reagent by the hydroxylated probe decreased in the presence of OH scavengers, the difference being proportional to the scavenging ability of the tested compound. A rate constant for the reaction of the scavenger with hydroxyl radical can be deduced from the inhibition of color formation. The second-order rate constants of the scavengers were determined with competition kinetics by means of a linear plot a A_0/A as a function of $C_{scavenger} / C_{probe}$ where A_0 and A are the CUPRAC absorbences of the system in the absence and presence of scavenger, respectively and C is the molar concentration of relevant species. The 2,4 – and 3, 5-dimethoxybenzoates were the best probes in terms of linearity and sensitivity. Iodide, metabisulfite, hexacyanoferrate (II), thiourea, formate, and dimethyl sulfoxide were shown by the modified CUPRAC assay to be more effective scavengers than mannitol, glucose, lysine, and simple alcohols as in the TBARS assay. The developed method is less lengthy, more specific, and of a higher yield than the classical TBARS assay. The hydroxyl radical scavenging rate

constants of ascorbic acid, formate and hexacyanoferrate(II) that caused interference in other assays could be easily found with the proposed procedure. [16] Apricots as five varieties of Malatya region are screened for antioxidant capacity by using CUPRAC. The novel reagent for the CUPRAC total antioxidant capacity assay, bis(neocuproine) copper(II) chloride, was easily accessible, stable, selective and responding to all antioxidants. Sulphite (normally contributing to the colour formed in the CUPRAC assay) was removed prior to assay on a strongly basic anion exchanger at pH 3 in the form of HSO. The CUPRAC findings correlated well with the results of ABTS /TEAC and Folin assays. This work reports for the first time the use of a novel spectrophotometric method (CUPRAC) for the assay of both total antioxidant capacity and sulphite levels of diverse apricot samples.[17]

Advantages

It requires sophisticated instrumentation. As a more convenient and less costly alternative. The developed method is less lengthy, more specific and of a higher yield than the classical TBARS assay.

Disadvantages

Sophisticated instruments are required which are more expensive.

e) Cellular antioxidant activity

This method contains different principles to detect antioxidant property. It is based on solubilisation of the oils in aqueous buffer, labeling of the resulting emulsions with a suitable reporter fluorophore, which reflects lipid oxidation, and continuous monitoring of the decomposition process. Antioxidant capacity of number of non-refined seed oils is compared with that of refined oils by using this simple technique. And it was found that, most of the antioxidative components are removed from edible oils during refining process. [18] A novel fluorometric method has been developed to evaluate hydroxyl radical is generated by a Co(II) – mediated Fenton-like reaction, and the hydroxyl radical formation under the experimental condition is indirectly confirmed by the hydroxylation of p-hydroxybenzoic acid. The fluorescence decay curve of FL is monitored in the absence or presence of antioxidant, the area under the fluorescence decay curve (AUC) is integrated, and the net AUC, which is an index of the hydroxyl radical prevention capacity, is calculated by subtracting the AUC of the blank from that of the antioxidant. Gallic acid is chosen as a reference standard, and the activity of sample is expressed as gallic acid equivalents. The method is rigorously validated through linearity, precision, accuracy and ruggedness. A wide range of phenolic antioxidants is analyzed and the hydroxyl radical prevention capacity is mainly due to the metal-chelating capability of the compounds.[19] The hydroxyl radical scavenging capacity and efficacy of a novel organosiliceous anionic hydride compound, silica hydride, were quantified by a recently developed method. The method measures a direct relationship between the hydroxyl radical scavenging capability of the antioxidant compound and the linear decrease in signal from a fluorescent 2-hydroxyterephthalate product created by reacting a Fe^{2+}-EDTA complex in the presence of a potential radical scavenger. A fluorescence signal half-inhibition, IC_{50}, value of 1.4 ± 0.1 μm was obtained for silica hydride compounds. The validity of the analysis was verified by electron spin resonance spectroscopy, spectrophotometric analysis of NAD^+ / NADH ratios, mitochondrial membrane potential measurements and assays of reductions of both cytochrome C (Fe^{3+}) to cytochrome c (Fe^{2+}) and epinephrine to adenochrome reductions.[20]

Advantages

Antioxidant capacity of number of non-refined seed oils is compared with that of refined oils by using this simple technique.

f) Enhanced chemiluminescence (ECL)

ECL has been used to measure antioxidant capacity in biological fluids. The assay involves the chemiluminescent substrate luminal. Light emission occurs when the luminal is oxidized by hydrogen peroxide that is generated in a reaction catalyzed by horseradish peroxidase (fluid because the reaction HRP). This method can quantify the antioxidant capacity of a is sensitive to radical scavenging antioxidants that reduce the light output. A method of assay of the antioxidant activity of biological sample suspected of having such activity, is under patent and this method comprises the steps of (a) initiating a chemiluminescent reaction and allowing said reaction to progress, thereby to generate a level of luminescence, said level being selected from the group consisting of (i) A rising level between 90 to 100 % of maximum, (ii) The maximum (iii) A post-maximum substantially constant plateau level: (b) Adding said sample to said progressing chemiluminescent reaction, said sample causing said level of luminescence generated by said reaction to change when said sample has antioxidant activity: (c) Monitoring said change in the level of luminescence: and (d) Determining the antioxidant activity of said sample assayed by reference to that of samples of known antioxidant activity subjected to steps (a) to (c) above. The principle behind the enhanced chemiluminescent assay for TAC measurement is best described in the work by (Whitechead, et.al, 1992). To perform the enhanced chemiluminescence assay, a signal reagent (luminal plus para-iodophenol), which is a source of chemiluminescence, is mixed with

horseradish peroxidase (HRP)-linked immunoglobulin to produce ROS, which in turn is mixed with a substrate, hydrogen peroxide (H_2O_2). The power of the antioxidants in the seminal plasma to reduce the chemiluminescence of the signal reagent is compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), a water-soluble tocopherol analogue, and is measured as molar Trolox equivalents. Although accurate, this method is cumbersome and time consuming, because fresh signaling reagent solution must be prepared each time the assay is performed standardized with Trolox. Moreover, the signal reagent may reduce in intensity, adding another technical problem. Finally, expensive instrumentation (eg, luminometer) is needed to measure the chemiuluminescence, which means that this assay is often not readily available in a physician's office.[21]

Advantages

ECL has been used to measure antioxidant capacity of biological fluids. This method can quantify the antioxidant capacity of a is sensitive to radical scavenging antioxidants that reduce the light output.

Disadvantages

This method is cumbersome and time-consuming because fresh signaling reagent solution must be prepared. Finally, expensive instrumentation (eg. Luminometer) is needed to measure the chemiluminescence.

g) Ferric-raducing antioxidant power (FRAP) assay

In order to assess the modifying effect of tea flavonoids on plasma antioxidant status, a variety of methods has been employed. Commonly used is the FRAP assay. This is a colorimetric assay that measures the ability of plasma to reduce the intense blue ferric tripyridyltriazine complex to its ferrous form, thereby changing its absorbance.[22]

Advantages

It is simple, speedy, inexpensive, and robust does not required specialized equipment. It can be performed using automated, semiautomated, or manual methods.[22]

Disadvantages

FRAP cannot detect species that act by radical quenching (H transfer), particularly SH group containing antioxidants like thiols, such as glutathione and proteins. [23, 24]

h) Total radical trapping antioxidant parameter (TRAP)

another assay which has been applied in human plasma is the total radical trapping antioxidant parameter (TRAP). In this assay, the rate of peroxidation induced byAAPH(2'-azobis(2-amidinopropane) hydrochloride) is monitored through the loss of fluorescence of the protein R-phycoerythrin (R-PE). In the TRAP assay the lag-phase induced by plasma is compared with that induced by Trolox in the same plasma sample.[24]

Advantages

Used for measurements of *in-vivo* antioxidant capacity in serum or plasma because it measures nonenzymatic antioxidants such as glutathione, ascorbic acid.[24]

Disadvantages

Many different end points have been used, so comparisons between laboratories are difficult. It is relatively complex and time consuming. It also requires a high degree of expertise and experience.

i) Oxygen radical absorbing capacity (ORAC) assay

Basically the same principle is applied as in the TRAP assay. The ORAC assay is another commonly applied antioxidant assay based on the ability of a test substance to inhibit the oxidation of B-phycoerythrin by reactive oxygen species, relative to Trolox. Proteins interfere with the analysis, partially protecting R-PE when all plasma antioxidants are exhausted. Determination of the lag-phase TRAP and ORAC assays can be performed with different radicals and thus different results will be obtained depending on the radical. For these reasons, results obtained with the TRAP or the ORAC assay in plasma have to be interpreted with care.[25]

Advantages

The advantage of the AUC approach is that it implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases. ORAC assay has been broadly applied in academy and in the food and dietary supplement industries as a method of choice to quantify AOC.[25]

Disadvantages

ORAC is limited to measurement of hydrophilic chain but ignores lipophilic antioxidants. It requires fluorometers, which may not be routinely available in analytical laboratories. Temperature control decreases reproducibility.

j) Trolox equivalent antioxidant capacity (TEAC)

This assay is based on the ability of molecules to scavenge the stable free radical of 2,2'- azinobis (3-ethylbenzothiozoline-6-sulfonic acid) in comparison with Trolox, a water soluble analogue of vitamin E. The activity of a compound is therefore expressed as TEAC. Of these assay, the ECL seems the least suitable to determine plasma antioxidant capacity because it relies on enzymatic activity. This technique has not been widely applied, which limits the possibility to compare results from different studies. All the other assays have been applied in plasma reproducibility.

k) ABTS {2,2' – azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)}

Miller et al (1993) described another technique for TAC measurement based on colorimetry. This assay is based on the principle that when 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) {ABTS} is incubated with a peroxidase (such as metmyoglobin and H_2O_2, a relatively stable radical cation, $ABTS^+$, is formed (see equation below). The formation of $ABTS^+$ on Interaction With Ferryl Myoglobin produces a relatively stable blue-green color, Measured at 600nm. Antioxidants in the fluid sample suppress this color production to a degree that is proportional to their concentrations. In this equation, $HX\text{-}Fe^{III}$ = metmyoglobin, X – $[Fe^{IV} = 0]$ = ferrylmyoglobin, ABTS = 2,2' – azino-di-[3-ethyl-bensthiazoline sulphonate].[38]

l) Folin-Ciocalteu method

The total flavonol content was expressed as rutin equivalent in mg/g or %W/W of the extracts. The $AICl_3$ method (Lamaison and Carnet, 1990) was used for determination of the total flavonoid content of the sample extracts. Aliquots of 1.5 ml of extracts were added to equal volumes of a solution of 2% $AlCl_3$ Y $6H_2O$ (2 g in 100 ml methanol). The mixture was vigorously shaken, and absorbance at 367 nm was read after 10 min of incubation. Flavonoid contents were expressed as mg catechin equivalent /g dry weight.[39] The Conjugated Dienes (CD) were quantified by measuring the absorbance at 234 nm, according to Esterbauer by as follows. A mixture of linoleic acid is emulsified with Tween 20 in phosphate buffer (pH 7), at a final concentration of 10mm was incubated alone (control) or with the plant extract (sample). The oxidation was initiated by the addition of freshly prepared copper sulphate. The oxidation can be stopped by cooling in an ice bath, in the presence of EDTA and BHT, Oxidation kinetics were determined at 37^0C , by measuring the absorbance at above mentioned nm for every 15 minutes over 270 minutes.[40] The qualification of TBARS was monitored, according to the ohkawa method. Briefly, the plant extract was added to linolenic acid emulsion in phosphate Buffer solution (PBS), and Tween20. The oxidation can be initiated by freshly prepared copper sulphate solution. After incubation at 37^0C for 3 hours, in obscurity, the reaction was stopped by cooling and adding EDTA. The BHT was used as a standard antioxidant. This preparation is combined with trichloroacetic acid thiobarbuturic acid, was heated in boiling water for 45 minutes and then cooled at room temperature. TBARS were extracted with n-butanol and the absorbance of the n-butanol layer was measured at 532 nm.[40]

2. Correlations

The developed method CUPRAC is less lengthy, more specific, and of a higher yield than the classical TBARS assay. Thus we can say that there is a correlation in between these two methods.[16] The CUPRAC finding correlated well with the results of ABTS/TEAC and Folic assays. This work reports for the first time the use of a novel spectrophotometric method (CUPRAC) for the assay of both total antioxidant capacity and sulphite levels of diverse apricor samples.[17] The hydroxyl radical scavenging capacity and efficacy of a novel organosiliceous anionic hydride compound, silica hydride, were quantified by a recently developed method. The method measures a direct relationship between the hydroxyl radical scavenging capability of the antioxidant compound and the linear decrease in signal from a fluorescent 3-hydroxyterephthalate product created by reacting an Fe^{2+}-EDTA complex in the presence of a potential radical scavenger. Hence we can say that there is a correlation.[20] Determination of the lag-phase TRAP and ORAC assays can be performed with different radicals and thus different results will be obtained depending on the radical. Hence we can say that there is no correlation. A comparison of the result of the assays showed that the ability to inhibit peroxidation of lipids in a liposomal system (LPIC) correlated well with the cytoprotective activities. If a single plant is assayed for its antioxidant activity by different technique, each technique gives the different results. Significant correlations were found between TPC, SASA and DPPH for the commercial coffee drubjs ub cguba.[29] A comparison of the antioxidant results of the three flavonoids (quercetin, rutin and catechin), shows a well correlation between LPO and ORAC methods. [30] Three assays were compared for the determination of total antioxidant capacity in human serum: the (ORAC) assay, the Randox Trolox-equivalent antioxidant capacity (Randox-TEAC) assay, and the ferric reducing ability (FRAP) assay. There was a weak but significant linear correlation between serum ORAC and serum FRAP. There was no correlation either between serum ORAC and serum TEAC or between serum FRAP and serum TEAC. Numerous publications applied the total phenols assay by FCR and an ET based antioxidant capacity assay and often found excellent lineal correlations between them.[31] The TEAC values for pure antioxidant compounds do not show clear correlation between TEAC values and the number of electrons an antioxidant can give away. The TEAC values of ascorbic acid (1.05), alfa – tocopherol (0.97), glutathione (1.28), and uric acid (1.01) are almost the same, although glutathione can normally donate one electron whereas the others are two electron reductants.[25] Radical trapping capacity

directly relates to the hydrogen atom donating ability of a compound and is not correlated to the redox potentials alone. [32]

3) Considerations of in-vitro antioxidant assay methods

Understanding of roles of various antioxidants and their activities is challenging. The use of one dimensional method to evaluate multifaceted antioxidants is not a complete analytical system. Due to the complexity of the composition of plant products, separating each antioxidant compound and studying it individually is costly and inefficient, not withstanding the possible synergistic interactions among the antioxidant compounds in plant products. The biggest problem is the lack of a validated assay that can reliably measure the antioxidant capacity of foods and biological samples. Several reviews have been published, and the opinions vary considerably. There seems to be no consensus of opinions, most probably due to the fact that the area of antioxidants is such a complex topic. There is considerable debate about which method is best and it is critical to understand that these tests are done in test tubes, not in people, since, there are different ways to measure antioxidant power, leaving research people seriously confused. The method used to measure and calculate the antioxidant activity has a major impact on the results because, both in foods and in vitro, oxidation reactions are complex. Liberation of antioxidants from foods in digestion may differ from liberation of same in extraction for antioxidant tests. Further, dietary antioxidants have to be absorbed and localized in active forms in the oxidation site to enable antioxidant effect. Antioxidants act by several mechanisms e.g. by donating hydrogen to radicals, reducing power, free radical scavenging activity, metal chelating ability, inhibition of β-carotene bleaching and quenching singlet oxygen. But unfortunately, most of the assay methods measures any one of the following.

a) Measuring its ability to donate an electron or hydrogen atom to a specific reactive oxygen species or to any electron acceptor. b) Testing its ability to remove any source of oxidative initiation. In addition food antioxidants may inhibit oxidation by several mechanisms. The dominant mechanism depends on conditions. For example in case of liberation of antioxidant from food into stomach, physical structure of food and temperature of the meal influence the dominant mechanism. But these factors do not take into the account in in-vitro antiradical activity methods. Further more, we are using oxidizing substrates, initiators, and other components in in-vitro assay, which may not be present in the digestive system. Hence, partitioning of oxidizing substrates, antioxidants and provident in the studied food is critical. Antioxidant activity may be distinctly different in bulk oils and multiphase foods, such as aemulsions.[33, 34, 35, 36, 37]

Table No:01, List of In-Vitro antioxidant methods

S.No	Name of the method
I	**Hydrogen Atom Transfer methods (HAT)**
1)	Oxygen radical absorbance capacity (ORAC) method
2)	Lipid peroxidation inhibition capacity (LPIC) assay
3)	Total radical trapping antioxidant parameter (TRAP)
4)	Inhibited oxygen uptake (IOC)
5)	Crocin bleaching Nitric oxide radical inhibition activity
6)	Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)
7)	Scavenging of H_2O_2 radicals
8)	ABTS radical scavenging method
9)	Scavenging of super oxide radical formation by alkaline (SASA)
II	**Electron Transfer methods (ET)**
1)	Trolox equivalent antioxidant capacity (TEAC) decolourization
2)	Ferric reducing antioxidant power (FRAP)
3)	DPPH free radical scavenging assay
4)	Copper (II) reduction capacity
5)	Total phenols by Folin-Ciocalteu
6)	N,N-dimethyl-p-Phenylenediamine (DMPD) assay

…Continued….Table No:- 01, List of In-Vitro antioxidant methods

S. No	Name of the Method
III	**Other Assays**
1)	Total oxidant scavenging capacity (TOSC)
2)	Inhibition of Briggs – Rauscher oscillation reaction
3)	Chemiluminescence
4)	Electrochemiluminescence
5)	Fluorometric Analysis
6)	Enhanced chemiluminescence (ECL)
7)	TLC bioautography
8)	Cellular antioxidant activity (CAA) assay
9)	Dye-substrate oxidation method

Table No:- 02, Summary of Antioxidant Assays

Antioxidant assay	Simplicity	Instrumentation Required	Biological relevance	Mechanism	Time required
ORAC	++	+	+++	HAT	++
TRAP	— —	— —	+++	HAT	+++
FRAP	+++	+++	— —	SET	— —
TEAC	+	+	—	SET	—
F-C	+++	—	—	SET	+
TLC Autography technique	+++	+	— — —	SET,HAT	— — —
CAA Asay	—	—	+++	HAT	+++
Dye-substrate Oxidation method	+	++	++	HAT	+
CUPRAC	+++	+++	— — —	HAT	+
Fluorometric analysis	++	++	+	HAT	+
ECL	— — —	+++	+++	HAT	+++
ABTS	+	+	+	HAT	+

+, ++, +++ = Desirable To Highly Desirable Characteristic.
—,——,——— = less desirable to highly undesirable characteristic.

Conclusion

Factors affecting oxidation reactions and antioxidant activities in foods and in vitro differ. The current approaches have still leaved many open questions. In vitro assays can only rank antioxidant activity for their particular reaction system and their relevance to in vivo health protective activities is uncertain. Therefore, it is prodent to use more than one type of antioxidant assay to measure antioxidant activities, and to include at least one assay that has biological relevance. Currently there is no convenient assay to evaluate antioxidant capacity in a food system, particularly in emulsions and foams, which are heterogeneous and the reaction cannot be easily monitored in real time. Conventional chemical analysis on these matrixes requires tedious sample treatments. Thus the efficiency is fairly low and the results are only qualitative. Our aim is to develop a high through out assay that can be used to monitor oxidation progress of an emulsion system in real time taking advantage of oxygen sensor coated micro-plate. The capacity of antioxidants in emulsion can thus be quantified and ranked. The assay will be a valuable tool for identifying better antioxidants for food preservations and cosmetic products.

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