CHAPTER

20

Assessment of Antioxidant Potential of Dietary Components

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OUTLINE

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Abstract

Antioxidants neutralize or mitigate the harmful effects of free radicals. Such antioxidants may be classed as either enzymatic or nonenzymatic. Some components in the diet act as important antioxidants as they may have direct antioxidant activity or are a component of antioxidant systems. Free radical-mediated stress arises when body fails to ameliorate the excess generation of free radicals. In such circumstances the need for supplementary or other dietary antioxidants arises. As a consequence, it is necessary to assay antioxidants status in subjects or antioxidant potential of novel dietary components. Several techniques have been developed to measure the antioxidant potential of dietary components. We describe antioxidants in general, then various platforms using spectroscopic, chromatographic, electrochemical, and photochemical methods. The following in assays and protocols are reviewed: hydrogen atom transfer, oxygen radical absorbance capacity, diphenyl-1-picrylhydrazyl radical scavenging, trolox equivalent antioxidant capacity, ferric-reducing antioxidant power, total radical-trapping antioxidant parameter, metal-chelating capacity, hydroxyl radical antioxidant capacity, diene conjugates, thiobarbituric acid reactive substances, hexanal, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, phycoerythrin, bleomycin–iron, copper-1,10-phenanthroline complex, peroxynitrite, lipid-soluble antioxidants, beta-carotene bleaching, hydroxyl radical scavenging, superoxide anion radical scavenging capacity, ferrous oxidation-xylenol orange, ferric thiocyanate, nonenzymatic in vivo and enzymatic in vivo assays.

Keywords: Antioxidants; Assays; Biosensors; Chromatography; Dietary compounds; Electrochemical; Free radical; Spectroscopy.

List of Abbreviations

CV Cyclic voltammetry
DMPO 5,5-dimethyl-1-pyrroline N-oxide
EPR Electron paramagnetic resonance
FRAP Ferric reducing antioxidant power
GC Gas chromatography
GGT Glutamyl transpeptidase activity
GPx Glutathione peroxidase
GR Glutathione reductase
GSH Reduced glutathione
GSSG Glutathione disulfide
GSt Glutathione-S-transferase
HAT Hydrogen atom transfer
HORAC Hydroxyl radical antioxidant capacity
HPLC High performance liquid chromatography
LPO Lipid peroxidation
NBT Nitro blue tetrazolium
ORAC Oxygen radical absorbance capacity
PCL Photochemiluminescence
RT Retention time
SET Single electron transfer
SOD Superoxide dismutase
TMM tetramethylmurexide
TPTZ 2,4,6-tripyridyl-s-triazine
USDA US Department of Agriculture
XO Xylenol orange

INTRODUCTION

Excess of free radicals causes oxidative stress and tissue damage in the body. Antioxidant compounds possess the ability to delay or inhibit these free radical-mediated processes. Thus, antioxidants decrease the risk of diseases by virtue of their reduction potential, metal-ion chelation, and radical scavenging activity (Fig. 20.1). Some, but not all, dietary components are known to act as antioxidants in biological systems. These enhance the endogenous enzyme systems, which have antioxidant activities such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT). Nonenzymatic compounds such as uric acid, albumin, and metallothioneins are also altered. Techniques based on colorimetry, spectrophotometry, chromatography, fluorimetry, and photometry are employed to assess the antioxidant potential of dietary components (Table 20.1).

Current research is focused on exploration of dietary antioxidant properties, their abundance, and role in oxidative stress-associated diseases such as cancer, cardiovascular disease, and neurodegeneration. Disease states such as HIV/AIDs are also associated with excessive generation of free radicals, either due to the disease process, therapeutic regimes or poor diets (Fig. 20.1). Additional antioxidant supply is necessitated when the endogenous antioxidative systems fails. In this regard, nutritional supplements/pharmaceutical products that are composed of antioxidant compounds play a vital role in maintaining optimal health and are recommended by some organizations.

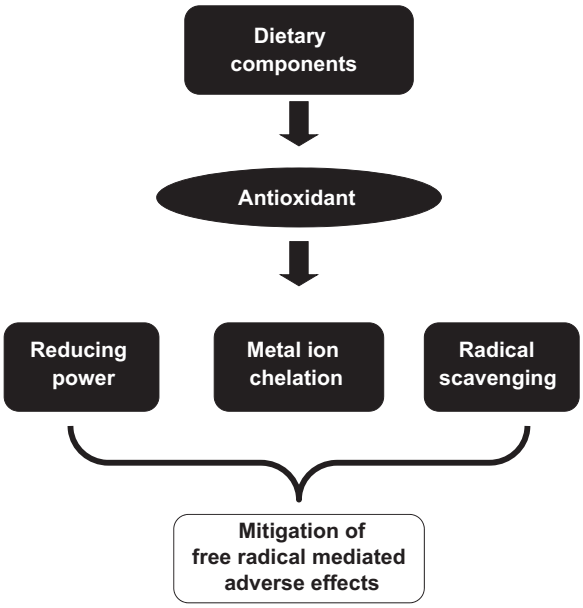

FIGURE 20.1 **Mechanism of antioxidant activity of dietary components.** Dietary components having antioxidant potential may exert their antioxidant activity by virtue of reducing power, metal ion chelation, and radical scavenging activity. By neutralizing the radical and ion-mediated damage dietary antioxidants may prevent the initiation and progression of various diseases.

TABLE 20.1 Techniques Used in the Assessment of Antioxidant Potential

Technique	Name of Assay or Platform	Principal Involved
Colorimetry	**DPPH** (2,2-diphenyl-1-picrylhydrazyl))	Antioxidant reacts with cationic, anionic radical, or metal complex producing colour in the solution that can be measured at particular wavelength.
	ABTS (2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)	
	FRAP (Ferric reducing antioxidant power)	
Fluorescence based	**ORAC** (Oxygen ׀ radical absorbance capacity)	Antioxidant reacts with different radicals produced by some radical-inducing agents. The reaction decreases the fluorescence intensity of a fluorescence compound used in the study
	HORAC (Hydroxyl radical antioxidant capacity)	
Chromatography	**GC** (Gas chromatography)	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase
	HPLC (High-performance liquid chromatography)	
Electrochemical techniques	**Cyclic voltammetry**	Intensity of current measured involving oxidation–reduction reactions
	Amperometry	
	Biamperometry	

Very often assays and physical platforms are described in terms of their acronyms such as DPPH, ABTS, etc. The full terms are described in the tables.

Dietary antioxidants include vitamins (C and E) and plant-based natural compounds (phenolics, carotenoids (which also have provitamin A activity), proanthocyanidins, benzoic acid derivatives, flavonoids, coumarins, stilbenes, lignans, lignins, and many others).[1] These components are absorbed with differential efficacy in the body and further subjected to various modes of xenobiotic and metabolism. Also, the dietary compounds may differ in their chemical interactions between with macromolecules within the diet. Digestive enzymes and bacterial microflora release potential bioavailable dietary components from the food matrix.[2] There is no comprehensive database available so far that lists out all the antioxidants present in food due to the enormous diversity of these compounds so Table 20.2 just lists some within the various categories. Further, the total antioxidant potential of food is not dependent on any one molecule's antioxidant potential, but it is the result of redox and synergic interaction between different moieties present in the diet.[3]

TABLE 20.2 List of Dietary Antioxidants

Category	Examples
Vitamins	Vitamin C
	Vitamin E
Pigments	Beta-carotene
	Lycopene
	Lutein
Phytochemicals	Phenolics
	Flavonoids
	Anthocyanins
Metals	Zinc
	Selenium

The list of dietary antioxidants is not exhaustive but illustrative only. Caution is required in interpreting the table: for example, selenium is a component of the enzyme glutathione peroxidase, which has the ability to detoxify lipid hydroperoxides. A deficiency of dietary selenium will reduce activities of blood glutathione peroxidase thus increasing oxidative stress. Vitamin E (comprising four tocotrienols and four tocopherols) on the other hand is a chain-breaking antioxidant that also requires vitamin C in the cycle of vitamin E regeneration. Many dietary components classified as antioxidants have complex biological roles, which also impact on molecular events in the cell.

Various phenomenon (heat, light, ionizing radiation, metal ions, and metalloproteins) are known to initiate oxidation process continuously in the presence of target substrate (oxygen, phospholipids, cholesterol, polyunsaturated fatty acids, and DNA) via a free radical-mediated chain reaction consisting of multiple steps i.e., initiation (Eq. 20.1), propagation (Eq. 20.2), branching (Eq. 20.3), and termination (Eqs. 20.4–20.6) steps.[4] The simple illustration of the reaction may be depicted as follows:

Initiation

$$LH + R^{\cdot} \rightarrow L^{\cdot} + RH \tag{20.1}$$

Propagation

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$
$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH \tag{20.2}$$

Branching

$$LOOH \rightarrow LO^{\cdot} + HO^{\cdot}$$
$$2\,LOOH \rightarrow LOO^{\cdot} + LO^{\cdot} + H_2O \tag{20.3}$$

Termination

$$LO^{\cdot} + LO^{\cdot} \rightarrow \text{Nonradical species} \tag{20.4}$$
$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{Nonradical species} \tag{20.5}$$
$$LO^{\cdot} + LOO^{\cdot} \rightarrow \text{Nonradical species} \tag{20.6}$$

(LH = substrate molecule; R· = initiating oxidizing radical; L· = allyl radical; LO· = alkoxyl radical; LOO· = lipid peroxyl radical; LOOH = lipid hydroperoxides; HO· = hydroxyl radical). The point suffix indicates the free radical or unpaired electron.

TECHNIQUES INVOLVED IN ANTIOXIDANT ASSESSMENT OF DIETARY COMPONENTS

Dietary components have various degree of solubility in different solvents. In terms of solubility these compounds may be polar, nonpolar, or midpolar. Prior to antioxidant assessment, a particular compound is extracted in an appropriate solvent or solvent system by using Soxhlet apparatus or other methods. The commonly used solvents are hexane, benzene, chloroform, ethyl acetate, acetone, methanol, or water. The extraction procedure concentrates the desired compound in a given sample. Extracts can further be concentrated by evaporation of the solvent. The extract is then subjected to various techniques to assess the antioxidant potential of dietary components (Fig. 20.2).

ANALYTICAL PLATFORMS

There are various analytical platforms and each have their own advantages (such as ease of use, levels of detection, ease of automation, etc.) and disadvantages (sophisticated machinery needs specialist training, may be expensive, prone to tube-blockage, etc.). Fig. 20.3 shows there is a rank order for the usage of different analytical platforms: chromatographic techniques are most popular. This is followed by colorimetric, fluorescence, electrochemical, biosensors, and photochemiluminescence (PCL), respectively (Fig. 20.3). However, the usages of different analytical platforms are also dependent on advances in physics, chemistry, and computing. In the ensuing text we describe some selective methods.

Electrochemical Techniques

Two dynamic electrochemical measurement techniques such as cyclic voltammetry (CV) and biamperometry are broadly applied to assess the antioxidant content in the given sample. CV experiments involve measuring the function of the electrode potential against time. Electrode potential is scanned linearly from the

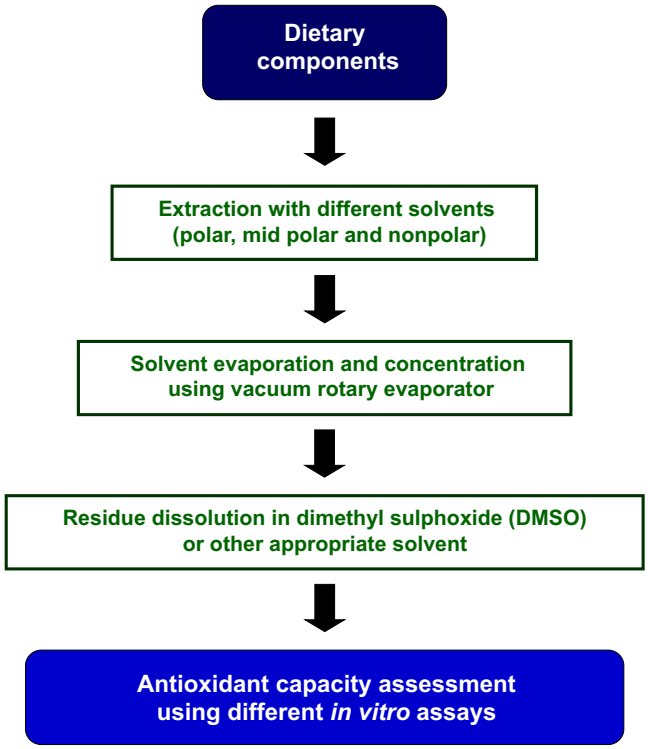

FIGURE 20.2 **Flow chart for the in vitro antioxidant activity assessment of dietary components.** Figure showing the steps of sample preparation and extraction using different polarity of solvents. The solvent is determined according to the chemical nature of the dietary component.

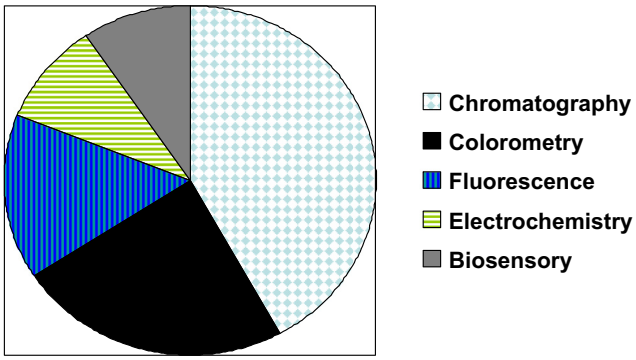

FIGURE 20.3 Pie chart showing popularity of different techniques used for antioxidant assessments. Relative proportion of various techniques *viz.*, chromatography (n = 2419); colorimetry (n = 1378); fluorescence (n = 871); electrochemistry (n = 547) and biosensory (n = 547)-based antioxidant assay. The number of chemiluminescence-based antioxidant assay (n = 6), used for assay of antioxidant assessment of compounds was too small to be shown in the pie chart. Data were generated by PubMed though it is expected that other data bases will produce the same relative proportions.

initial to final stage and once again to the initial value while respective current intensities are recorded. CV has been used to assess antioxidant potential of dietary components in vitro. This technique can also be applied to tissue homogenates, plasma, and plant extracts. The sensitivity of this method has been reported in terms of vitamin C.[5] The results obtained by the CV technique are comparable to that of spectrophotometric methods viz., ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays.

The biamperometric method is based on the basic principles of electrochemistry. In this technique, the current flowing between identical working electrodes with small potential difference, immersed in a reversible redox couple solution, is measured. The measurement is based on the redox couple indicated by the reaction of the analytes used. The sensitivity of the experiment is based on the specific reactions of particular redox pairs and analytes. Commonly used redox couples in biamperometric measurements are I_2/I^-, Fe^{3+}/Fe^{2+}, and Fe $(CN)_6^{3-}/Fe(CN)_6^{4-}$. Besides these, the DPPH·/DPPH redox pair is also used for the antioxidant measurement of samples. The reduced form of DPPH is produced when the antioxidant (analyte) reacts with the DPPH radical. The intensity of the current measured by the method is directly proportional to the residual concentration of the DPPH· in the test solution.[4] In this method, two identical working electrodes are used where the reduction and oxidation of DPPH· radical and DPPH occurs, respectively. This process maintains the reduced and oxidized form of the DPPH in equilibrium. Addition of antioxidant moieties into the solution decreases the concentration of DPPH radical, which generates the cathode current, which is measured.

Biosensor-Based Methods

The electron transfer property of oxidoreductases during catalytic reactions has often been exploited in biosensor applications. These enzymes are stable and do not require coenzymes or cofactors. Several studies reported the determination of antioxidant capacity using biosensor methods.[6–8] Superoxide radical, nitric oxide, glutathione, uric acid, ascorbic acid, and phenolic compounds can be monitored by using biosensors. DNA-based biosensors evaluate total antioxidant potential of the samples electrocatalytically.[9] In this method, a partially damaged DNA layer is adsorbed onto the electrode surface by OH radicals generated by the Fenton reaction. The subsequent electrochemical oxidation of the intact adenine bases generates an oxidation product that catalyzes the oxidation of nicotinamide adenine dinucleotide (NADH). Addition of antioxidant compounds scavenges hydroxyl radicals leaving adenine bases unoxidized and thus, increase the electrocatalytic current. Enzymes viz., tyrosinases, laccases, or peroxidases are used as biosensors for the detection of antioxidant moieties. Dietary polyphenols are detected by a specialized biosensor using an immobilized polyphenol oxidase.[4] Polyphenols exert their antioxidant activity through their hydroxyl groups. Tyrosinase-based biosensors detect the total amount of OH groups present in the polyphenols. This method provides an indirect evaluation of the antioxidant potential of sample that possesses hydroxyl groups. The results may be reported as trolox or gallic acid equivalents in mg/L.[4]

Chromatographic Methods

Multiple chromatographic techniques are being used in this field of antioxidant assessments. These methods include liquid, gas, affinity, and exchange chromatography. Antioxidant ingredients of dietary components have been analyzed by gas ion-chromatography with flame ionization detector and gas chromatography (GC) coupled with mass spectrometry. GC is used for volatile substances that do not decompose during the analytical process. Separation occurs between a stationary (liquid) and mobile (gas) phase. Microscopic layers of liquid and inert gas serve as mobile and stationary phases. The retention time (RT) of the analyte is quantitatively measured. Ionization and thermal conductivity detectors are the most commonly used GC detectors. High-performance liquid chromatography (HPLC) is another useful technique used for the estimation of antioxidant moieties in dietary components. This technique utilizes stationary phases, a pump, column, and a detector. The pump is used to move the mobile phase and analytes across the column with high pressure to provide a characteristic RT for the analyte. Usually, a diode array detector is used to obtain the additional information regarding the characterization of the analyte in the form of spectroscopic data. In one study, the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid–based radical scavenging activity of coffee was measured by using an HPLC system with postcolumn online antioxidant detection.[11] The sample was separated, and different elutes were subjected to a photodiode array detector and mixed with ABTS cation radical solution (which has a deep blue color). The absorbance of the solution was read at 720 nm by the detector. Quenching of radicals by antioxidant moieties resulted in the disappearance of the blue color, which was detected by a negative peak on the HPLC trace. If there were different antioxidant moieties in the given sample then each moiety would depict individual peak shifts. So the total HPLC-derived antioxidant potential of the given sample can be achieved by adding all the contributory peaks.[11] Fluorescence detection combined with HPLC was used to determine the antioxidant potential of propyl gallate, nordihydroguaiaretic acid, butylated hydroxyanisole, tert-butylhydroquinone, and octyl gallate in edible oils and foods.[4] Antioxidant potential of turmeric oil was also determined by various chromatographic methods.[10]

Fluorescence Spectroscopy

When a substance absorbs light or other electromagnetic radiation it emits the energy in the form of fluorescence. The emitted light has lower energy than the absorbed one. In other words, the light that has been emitted has a longer wavelength. When an excited electron relaxes to its ground state it emits photon energy in the form of fluorescence. This principle has been exploited to determine the antioxidant content of components.[4] Two different methods have been employed, based on the antioxidant component that needs to be analyzed. One includes recording of fluorescence and excitation spectra at different wavelengths, and the other method requires a strict pH control as the fluorescence intensity depends strongly on the pH value. Fluorescence methods are also used to explain the lateral organization of sterol in the biological membrane, which in turn affects the potentiality of antioxidant components.[12] This sheds light as to why some lipid-soluble antioxidants reflect adverse effects. As the cell membrane is an essential requirement for the proper functioning of cells, any disruption to it hampers its homeostatic metabolism and physiological processes. Few antioxidant compounds are soluble in both water and lipids. Through fluorometry, it is now known that these compounds disrupt the sterol organization by insertion into membrane bilayers leading to detrimental effect on the cells.

Photochemiluminescence Methods

PCL assays involve the detection of free radicals by chemiluminescence detection generated by photochemical cleavage. Luminol is a photo inducer and auto oxidizer. It works as both the radical detection reagent and photosensitizer. In PCL assays, the auto oxidation is inhibited by a single or groups of antioxidant compounds at the nanomolar range. The antioxidant potential of the sample can be measured by studying the lag phase at different concentrations. The results are expressed as mmol equivalents of antioxidant activity of a reference compound (i.e., trolox) by using appropriate calibration curves. Some PCL methods have been developed with a combination of two different protocols. For example, measurement of antioxidant potential of water soluble (flavonoids, ascorbic acid, aminoacids) and lipid soluble (tocopherols, tocotrienols, carotenoids) components.[13] The PCL assay has few advantages over other methods:

- Easy and rapid to perform
- Do not require high temperatures for radical generation
- High sensitivity

SPECIFIC METHODS FOR ANTIOXIDANT ASSESSMENTS

Hydrogen Atom Transfer Assay

The transfer of a hydrogen atom is an important step to prevent the radical chain reactions. Hydrogen atom transfer (HAT)-based assays quantitate the ability of an antioxidant component to quench free radicals by virtue of hydrogen donation leading to the formation of a stable moiety. To understand the chemistry of the HAT assay, the following equation can be considered (Eq. 20.7):

$$AH + ROO\cdot \rightarrow ROOH + AO\cdot \tag{20.7}$$

Here, AH is an antioxidant component, ROO·, AO· are free radicals, and ROOH is a stable component. The equation reveals that AH donates a hydrogen atom to ROO· and transforms into a relatively stable free radical species i.e., AO·. Thus, there is a lesser possibility that the antioxidant free radical species (AO·) may be involved in the propagation of further radical reactions with initiation substrates.[14] Both phenolic and nonphenolic antioxidant compounds can undergo this mechanism when measuring their antioxidant potential. The aromatic ring in phenolic compounds shares the delocalized electron, which makes the radical stable.[15] If the hydrogen atom is weakly held to the antioxidant compound then the probability of being detached from its parent component increases, hence will react faster with the free radical. Therefore, in HAT-based assays, the bond dissociation enthalpy of the hydrogen-donating group of the antioxidant determines the relative antioxidant potential of the particular component.[16]

Single Electron Transfer Assays

Single electron transfer (SET) assays are used to detect the SET ability of a potential antioxidant to participate in the reduction of free radicals.[16] The assay is based on the color produced during the assay. The addition of an antioxidant compound to the experimental solution will decrease/increase the color intensity based on the type of assay. The transfer of single electrons from an antioxidant to active oxygen species results in a radical-cationic antioxidant complex. The complex is deprotonated through the interaction with aqueous medium. SET reactions involve the same reaction setup as discussed for HAT assays (described above). However, in terms of radical scavenging potential, SET reactions can be further be subjected to radical-propagation reactions. The half-life of the radical-cationic antioxidant complex is extended in SET type of assays.[14] The relative reactivity of the samples' antioxidant potential in SET assays is based on the deprotonation and ionization potential of the reactive functional group, which makes the reaction pH dependent. SET reactions occur at alkaline conditions. As pH increases then ionization potential decreases, which reflects the increased electron donating ability with deprotonation.[15]

Oxygen Radical Absorbance Capacity Assay

Oxygen radical absorbance capacity (ORAC) is one of the most common antioxidant activities used in research and has been applied to both clinical and food-based studies. For example, besides dietary supplements, this method has been used to measure the antioxidative capacity of fruits and vegetables, wines, juices, and nutraceuticals. In addition, it is used to determine total antioxidant ability of plasma or serum samples. A report published by US Department of Agriculture provided a list of ORAC values for about 60 different foods in the American diet (http://www.orac-info-portal.de/download/ORAC_R2.pdf). In principle, when a protein is subjected to oxidation by a radical it loses its conformation. Due to the loss of conformation, the fluorescence of the protein will decrease, which is quantitatively measured.[17] β-phycoerythrin was the originally used protein, which reacts with peroxyl radicals leading to the formation of a nonfluorescent product in the ORAC assay. Later, the method was modified with the use of synthetic nonprotein fluorescein (FL) (3′,6′-dihydroxyspiro[isobenzofuran-1[3H], 9′[9H]-xanthen]-3-one) as the fluorescence probe[18]. In a microplate well, an antioxidant and a free radical producing moiety are added along with a fluorescent molecule (fluorescein) followed by heating. Thermal degradation produces the free radical and reacts with antioxidant compounds and thereby decreases the hydrogen atom donating potential per antioxidant compound. This results in the loss of fluorescence as there is a decrease in radical concentration. Fluorescence intensity curves against time function are recorded at different excitation/emission wavelengths. A standard water-soluble antioxidant compound (e.g., trolox) is generated and compared with the area under the curve obtained by the addition of antioxidant. Thus, the results are expressed as standard antioxidant compound equivalents i.e., μM standard antioxidant compound equivalents (TE) per gram of sample.

Diphenyl-1-Picrylhydrazyl Radical Scavenging Assay

The DPPH assay is one of the oldest indirect methods used for the determination of antioxidant potential of various samples. This assay involves the HAT mechanism[19]. The assay relies on the principal that the purple-colored radical (DPPH·) chromogen in a solution of ethanol converts into a pale yellow hydrazine (DPPH-H) solution in the presence of a radical scavenging compound. This can be monitored at 515–528 nm[15].

Trolox Equivalent Antioxidant Capacity Assay

Miller et al.[20] demonstrated the use of the trolox equivalent antioxidant capacity (TEAC) assay for assessment of infant plasma antioxidant capacity. In principle, the ABTS anion is oxidized by peroxyl radicals leading to the formation of the ABTS cation radical with an intense color change, which is measured at an absorption maxima of 415, 645, 734, and 815 nm.[15] The presence of antioxidant compounds in the test solution will interfere with the interaction of ABTS anions and peroxyl radicals thereby decreasing the ABTS cation formation. This in turn reflects the antioxidant potential of the sample. Interference in the final result due to sample turbidity and/or other absorbing materials at given wavelengths is minimum at 734 nm. This assay is widely employed to assess the antioxidant potential of many food components.[15]

Ferric Reducing Antioxidant Power Assay

This assay is based on the oxidation and reduction of an iron complex. In this assay, the yellow-colored ferric 2,4,6- tripyridyl-s-triazine $[Fe^{3+}–(TPTZ)_2]^{3+}$ complex is reduced to the blue-colored ferrous complex $[Fe^{2+}–(TPTZ)_2]^{2+}$ in the presence of an antioxidant compound by electron donation. This is measured at 593 nm.[21] The assay is set up in acidic conditions and relates linearly with total reducing capacity of electron-donating moieties i.e., antioxidants. Originally, the method was used to assess the antioxidant capacity of human plasma, but later it was adopted to assess the antioxidant potential of dietary components with few modifications. The disadvantage of the FRAP assay is that the reaction is based on SET and therefore cannot detect antioxidants that involves HAT to reflect their antioxidant potential. A combination of the FRAP assay with any other method[15] addresses this issue.

Total Radical-Trapping Antioxidant Parameter Assay

The total radical-trapping antioxidant parameter (TRAP) assay has been used to assess human plasma antioxidant capacity. In this assay, azo compounds such as 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and α,α′-azodiisobutyramidine dihydrochloride are added to the plasma/sample, which in turn produces peroxyl radicals. The oxidation is monitored by the oxygen consumed in the reaction. The antioxidative potential of the sample is measured by quantifying the adsorption of oxygen using oxygen electrodes. The red algal protein, R-phycoerythrin, is used as a fluorescence probe to detect the decrease in oxygen concentration in the experimental setup.[22] The assay comprises of lag time (induction period) compared with an internal standard i.e., trolox. The basic difference between ORAC and TRAP is that the former assay measures the area under the kinetic curve and the later measures lag time.[23]

Metal-Chelating Capacity Assay

Metal ions (e.g., iron and copper) are known to generate reactive oxygen species in biological systems. Iron is an essential mineral. The homeostatic mechanism in cellular metabolism maintains the required iron pool in the body. Slight imbalance in the free iron content in the biological fluids can be disastrous to the system. Iron is an active prooxidant known to promote peroxidation of lipids, which interrupts the integrity of cellular membranes and metabolism. The Fenton reaction is an iron-mediated free radical generation process, which occurs both in vitro and in vivo. It induces decomposition of hydroperoxides and the production of hydroxyl radicals.[15] In this assay, a transition metal ion is used to generate free radicals, which would complex with an antioxidant compound via coordination bonding. The formation of the complex between metal ions and antioxidant compounds is known as chelation. Therefore, the greater the chelating ability of antioxidant compounds, the higher is its antioxidant potential. When metal ion reacts with a substrate (tetramethylmurexide or ferrozine) it produces an intense color, which is measured at 485 and 562 nm. In the presence of an antioxidant compound, there is an inhibition of the metal ion–substrate chelation interaction thereby decreasing the color intensity.

Hydroxyl Radical Antioxidant Capacity Assay

This assay also measures metal ion chelating ability of antioxidant compounds. However, Co(II) complex is the metal ion source.[24] There is a good correlation between data generated from hydroxyl radical antioxidant capacity (HORAC), ORAC, and TRAP.[24]

Diene Conjugates

Lipid peroxidation (LPO) causes oxidative stress in the pathophysiology of various diseases. Quantification of conjugated dienes is a useful technique to study LPO, which evaluates the antioxidant potential of an inhibitory moiety. In this method, the oxidation of polyunsaturated fatty acids is initiated by a metal (copper, iron); chemical components in the assay medium (AAPH, DPPH) and heat application lead to the formation of diene conjugates.[25] The mechanism involves the abstraction of hydrogen from the CH_2 group, and stabilization of the product by molecular rearrangement leading to the formation of conjugated diene.

Thiobarbituric Acid Reactive Substances Assay

Thiobarbituric acid reactive substance (TBARS) assay is another method to detect lipid oxidation. This assay measures malondialdehyde (MDA), which is a split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of lipid substrates. The MDA reacts with thiobarbituric acid (TBA) forming a pink chromogen (TBARS), which is measured at 532–535 nm. In the course of time, few modifications have been incorporated into this method. However, one modification is in debate, i.e., the addition of ethanol in the test solution, as there is evidence that ethanol itself may act as an antioxidant.[26] In this assay, the substrate becomes oxidized with the addition of a metal ion (copper, iron), a free radical generating compound (AAPH) followed by addition of TBA. The extent of oxidation can be measured spectrophotometrically. The addition of any antioxidant moiety to the test solution inhibits the oxidation process, and the reduced chromogen formation indicates the antioxidant capacity. The result is quantified with a calibration curve using MDA or in term of percentage inhibition.[27]

Measurement of Hexanal

Hexanal (an unsaturated aldehyde) is one of the oxidative products formed by the lipid oxidation process. The decomposition of primary oxidation products gives rise to secondary products including hexanal. Antioxidant activity can be calculated as the percentage inhibition of one or more secondary oxidation products relative to controls. Both sensory and physicochemical methods have been used for the hexanal determination.[28] This method has an advantage of analyzing a single, well-defined, end product of the LPO process, which is lacking in other peroxidation detection methods.[15]

2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid Assay

ABTS is a highly water soluble and chemically stable compound. It acts as a peroxidase substrate and produces a metastable cation when subjected to oxidation by H_2O_2 or ferrylmyoglobin. The ABTS and its stable cation show absorption maxima at 342 and 419 nm, respectively.[29] The presence of an antioxidant compound inhibits the formation of $ABTS^{4+}$, which is measured spectrophotometrically. The lesser the absorption of the test solution at 734 nm, the greater is the potential of the antioxidant compound. The measurement of the hydrogen donating potential of the antioxidant sample at 734 nm minimizes the interference due to sample turbidity and other absorbing materials. Electron or hydrogen donating potential of antioxidants, to scavenge the ABTS radical cation in comparison to that of trolox is denoted as TEAC. It is equal to millimolar concentration of trolox solution with the antioxidant capacity equivalent to a 1 mM solution of the test substance.[29]

Phycoerythrin Assay

B-phycoerythrin and R-phycoerythrin are derived from natural sources (some bacteria and algae) and have been used as the target for free radical-mediated damage in in vitro assays. The fluorescence of the phycoerythrin protein is quenched by the peroxyl radical generated by thermal decomposition of AAPH. The introduction of

an antioxidant compound to the test solution will react and neutralize the peroxyl radical thereby increasing the fluorescence of the protein toward normal. The method has been significantly modified since studies on this reaction began. In one modification, phycoerythrin is used to assess the antioxidant potential of the sample against hydroxyl radical-mediated oxidative damage. OH radicals are generated from an ascorbate–Cu^{2+} system at copper-binding sites on macromolecules. This results in site-specific macromolecule damage. Differences in the areas under the phycoerythrin decay curves, between a sample and the blank, are expressed in trolox equivalents in the final results.[15]

Bleomycin–Iron Dependent Assay

Bleomycin binds to DNA by its bithiazole and terminal amine residues. It also forms a complex with metals ions using the amino-alanine pyrimidine-hydroxy histidine portion of the molecule. This assay was first used to measure nontransferrin bound iron in biological samples. Later, it was used to assess the prooxidant potential of food additives and/or nutritive components.[30] In the presence of O_2 and a reducing agent, the DNA can be degraded by the bleomycin–iron complex via ferric bleomycin peroxide. Hydroxyl radicals (produced sometimes due to decomposition) and bleomycin–iron (III) complex by themselves are not capable of inducing DNA damage. Therefore, this assay requires a reducing agent/hydrogen peroxide and oxygen for DNA damage to occur. The damage results in the release of free bases and base propenals. At low pH, these bases rapidly decompose on heating and react with TBA to form a TBA–MDA chromogen. The addition of dietary components to the reaction decreases the chromogen formation and so reflects its antioxidant potential.[31]

Copper-1,10-Phenanthroline Complex Mediated Assay

The copper-phenanthroline assay was designed to assess copper ions in biological samples. Later, it was applied to the assessment of the prooxidant action of food additives and/or nutrient components.[30] In this assay, H_2O_2 is produced by a copper-phenanthroline system, which damages the DNA. Hydroxyl radicals are involved in the damage of DNA caused by the copper-phenanthroline system. This damage is confined mainly to the DNA bases, unlike bleomycin–iron mediated DNA damage. To increase the DNA damage in this assay, reducing agents such as ascorbate and mercaptoethanol have been used. This assay is preferred where DNA solubility has been rendered by organic solvents.[31]

Peroxynitrite Involving Reaction-Based Assay

Peroxynitrite ($ONOO^-$) is an oxidant produced by the reaction between nitric oxide (NO) and superoxide radicals ($O_2{\cdot}^-$). It is known to produce oxidative stress by virtue of LPO, methionine, and sulfhydryl group oxidation in proteins, antioxidant depletion, and DNA damage. It is involved in nitration of tyrosine residues, which is considered as a marker for peroxynitrite-dependent damage in biological systems.[32,33] Enhanced levels of 3-nitrotyrosine are associated with various human diseases. Peroxynitrite radical scavenging by antioxidants based on tyrosine nitration serves as a useful tool to assess the antioxidant potential of dietary components.[34]

Lipid-Soluble Antioxidant Assay

This assay is similar to the FRAP method and is used to monitor the lipid-soluble antioxidants in dietary components. In this method, the organic residue is redissolved in propanol:acetone (2:1 v/v) containing 1% (v/v) Triton X-100. The rest of the procedure is as described for the FRAP assay.[35]

Beta-Carotene Bleaching Assay

This assay is used to assess the antioxidant potential of both volatile and nonvolatile compounds.[36] Linoleic acid and Tween-40 are dissolved in chloroform by boiling followed by the addition of beta-carotene. The mixture is evaporated till dryness followed by addition of oxygenated water, which forms an emulsion. Dietary components and standard antioxidant compounds are dissolved in ethanol to prepare another emulsified solution. Both the solutions are mixed, and the absorbance is recorded at 15 min interval, at 470 nm wavelength. The result is represented as percentage inhibition.[37]

Hydroxyl Radical Scavenging Assay

In a biological system, the hydroxyl radical is one of the most reactive free radicals. It can be generated by the Fenton reaction between ferrous iron and H_2O_2.[38] The reaction between dimethyl sulfoxide and H_2O_2 is also used to generate hydroxyl radicals.[39] Hydroxyl radical-mediated damage is assessed by different types of probes viz., deoxyribose, benzoate, and salicylate in colorimetric or fluorometric techniques. The antioxidant compounds, which possess the ability to scavenge the hydroxyl radical also, inhibit the radical-mediated damage in the assay. The results of the hydroxyl radical scavenging (HRSA) are generally expressed as a percentage of HRSA activity of the test sample. Electron paramagnetic resonance (EPR) techniques have also been exploited to assess HRSA activity with the help of spin-trapping agents, in addition to using probes. In this technique, a nitrone/nitroso compound reacts with free radicals to form a relatively stable adduct, which is measured with EPR spectroscopy, which produces a distinguishable adduct-specific spectrum. DMPO (5,5-dimethyl-1-pyrroline N-oxide) is commonly used to trap hydroxyl radicals leading to the formation of relatively stable DMPO-OH adducts, which are detected and quantified by EPR.[38] HRSA is a powerful tool for assessing the antioxidant potential of dietary components.

Superoxide Anion Radical Scavenging Capacity Assay

In this assay, the $O_2\cdot^-$ is generated through enzymatic/nonenzymatic superoxide anion reaction systems. Superoxide radical and uric acid are produced by a reaction catalyzed by xanthine oxidase acting on hypoxanthine or xanthine using O_2 as a cofactor.[40] In this reaction, an electron is transferred from NADH to O_2 present in the test solution. Occasionally, NADH is oxidized by phenazine methosulfate to produce $O_2\cdot^-$. Nitro blue tetrazolium (NBT) is used as a probe for the quantification of superoxide radical concentrations, by virtue of NBT reduction into a purple-colored formazan.[39] The antioxidant sample is incubated with phenazine methosulfate-NADH-NBT to assay its superoxide radical scavenging potential. The absorbance of the mixture is recorded at 562 nm against a blank. Similar to the HRSA, the superoxide radical anion assay also requires fluorometric probes. DMPO is used in both the assays to detect and trap the radicals because it cannot distinguish between superoxide and hydroxyl radicals. The use of EPR spectroscopy in combination with an appropriate spin trap agent may increase the reliability of the result.

Ferrous Oxidation-Xylenol Orange Assay

This assay is based on the oxidation of ferrous ions (e.g., hydroperoxides) into the ferric form followed by a reaction with a reagent containing xylenol orange (XO). This leads to the formation of a ferric-XO blue–purple color complex, which shows absorbance maxima at 550 nm. The method has been used to detect hydroperoxides in various samples. The presence of an antioxidant component in the given sample inhibits hydroperoxide formation by its electron donating ability to the ferric ion.[41]

Ferric Thiocyanate Assay

This assay is similar to ferrous oxidation-xylenol orange (FOX) except that the formed ferrous ion is monitored as a thiocyanate complex at 500 nm.[42] This assay also requires linoleic acid as a hydroperoxide source. The inhibition of the ferric thiocyanate (FTC) complex formation by compounds in samples is proportional to the antioxidant potential of the sample. The results of the assay are highly reproducible, but compounds having absorption maxima at 500 nm may produce false positive results in the assay. The assay in, combination with other assays viz., TBA, DPPH, FRAP, and ABTS, has been used to assess the antioxidant potential of various natural compounds.

Besides the above-discussed assays there are other assays that can be utilized to assess the antioxidant potential of dietary antioxidants viz., aldehyde/carboxylic acid assay; ascorbate, and ascorbate oxidase assays.[35,43] Recently, researchers have reported the assessment method for the dietary intake of antioxidant mineral such as selenium and zinc.[44] The antioxidant potential of these metals could be measured using various biological parameters (viz., oxidation of biological membranes, hemoglobin acetylation, DNA damage, formation of protein carbonyl content, etc.). These markers of oxidative damage are then correlated with the mineral intake.

METHODS FOR ANTIOXIDANT ASSESSMENTS: IN VIVO METHODS

In vivo antioxidant potential of the dietary components can be tested by administering them into laboratory animals viz., rat, mice, etc. at definite standardized dose for a specific time. The collected tissue and/or blood samples are assayed either enzymatically or nonenzymatically (Fig. 20.4) as discussed below:

Nonenzymatic In Vivo Assays

The ferric reducing ability of plasma is a simple, rapid, and useful assay to measure antioxidant potential in vivo. The principle is similar to the in vitro FRAP assay. In this method, the blood sample is collected (from the retro-orbital venous plexus or via other routes such as cardiac puncture under anesthesia) of experimental models into a heparinized tube. Samples containing antioxidants enhance the color intensity of the FRAP reagent by virtue of the formation of ferrous ion formation, which is measured at 593 nm.[45]

Reduced glutathione (GSH) is involved in cataract formation and renal amino acid reabsorption. Glutathione plays a role via oscillating between its two states i.e., reduced and oxidized forms. Its ratio is proposed to be a marker of oxidative balance. Dietary antioxidants maintain the balance between the two states. The quantification of GSH in tissue homogenates has been developed to assess antioxidant potential in vivo.[46] Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) dissolved in phosphate buffer is used in this assay.

LPO assays are also employed as in vivo makers of oxidative stress. MDA reacts with TBA to produce a pink color chromogen, which is measured spectrophotometrically at 532 nm wavelength.[47]

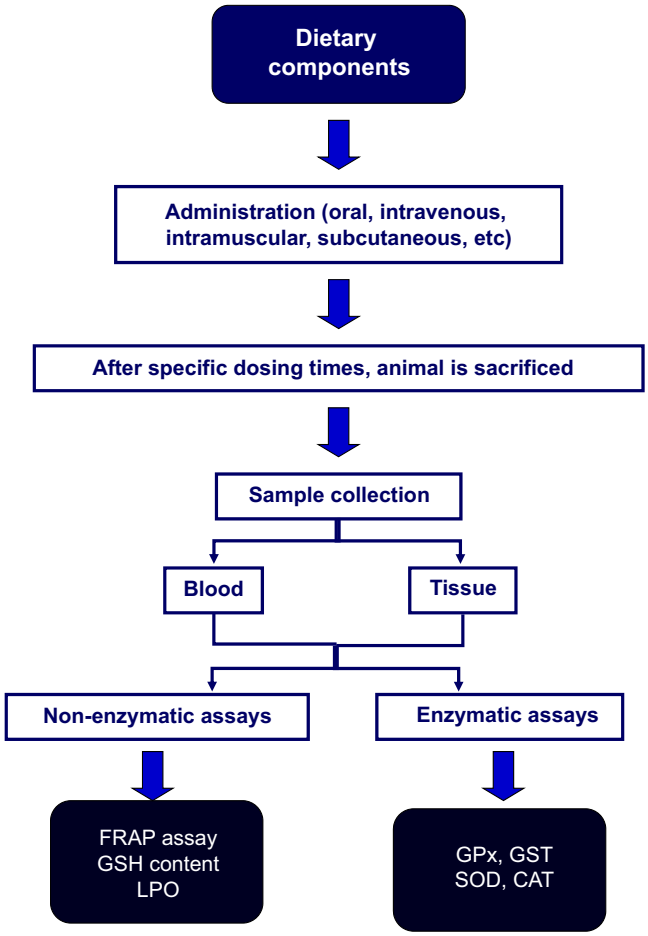

FIGURE 20.4 **Flow chart for the in vivo antioxidant assessment of dietary components.** This figure shows the steps involved in dietary component administration in experimental animals, sample collection, and different enzymatic and nonenzymatic assays for the in vivo antioxidant assessment of dietary components. *CAT*, catalase; *FRAP*, Ferric reducing antioxidant power; *GPx*, glutathione peroxidase; *GSH*, reduced glutathione; *LPO*, lipid peroxidation; *SOD*, superoxide dismutase.

Enzymatic In Vivo Assays

GPx is a seleno-enzyme that catalyzes the reaction of hydroperoxides with GSH to form glutathione disulfide and hydroperoxide. On the basis of its occurrence i.e., cellular, extracellular, biomembrane, and gastrointestine, there are four isoenzymes of GPx. It has been reported that the decrease in GPx activity is associated with imbalance between oxidative stress and antioxidants.[48] Reduced dietary selenium also reduces plasma GPx activity.

Glutathione-S-transferase is known to initiate detoxification of alkylating agents by catalyzing the reaction with the sulfhydryl group of glutathione, leading to the neutralization of their electrophilic sites and water-soluble byproducts.

SOD and catalase enzymes are involved in superoxide anion and hydrogen peroxide–neutralizing phenomenon. In general, red blood cell lysates are used as samples for the assay, details of which are described elsewhere.[49,50] Similarly, two other enzymes viz., glutamyl transpeptidase activity and glutathione reductase are also being used to assess the antioxidant potential of administered components in rodents.

In all these assays consideration must be given to the fact that storage conditions may affect the assay results so pilot assays need to be conducted.

SUMMARY POINTS

- A healthy diet-containing dietary antioxidants or components that contribute to an enhanced antioxidant status are necessary to eliminate the risk of various lifestyle and genetic diseases.
- Excess of free radicals produces an oxidative environment in the body resulting in initiation and progression of various diseases.
- Antioxidants mitigate free radical-mediated damage in the body.
- Various platforms are employed to assess antioxidant potential of dietary components including electrochemical, biosensory, chromatographic, fluorescence, and PCL methods.
- The following assays and protocols can be used to asses dietary components or the effects of treatments: HAT, ORAC, DPPH radical scavenging, TEAC, FRAP, total radical-trapping antioxidant parameter (TRAP), metal-chelating capacity, HORAC, diene conjugates, TBARS, hexanal, ABTS, phycoerythrin, bleomycin–iron, copper-1,10-phenanthroline complex, peroxynitrite, lipid-soluble antioxidants, beta-carotene bleaching, HRSA, superoxide anion radical scavenging capacity, FOX, FTC, nonenzymatic in vivo, and enzymatic in vivo assays.
- Assessment of antioxidant potential in dietary components may help lessen the effects of an imbalanced diet. Alternatively such information may be used to mitigate the effects of excessive free radicals due to disease, toxic agents, or drugs themselves, which cause free radical-mediated damage as unwanted side effects.

References

1. Lindsay DG, Astley SB. European research on the functional effects of dietary antioxidants-EUROFEDA. *Mol Aspects Med* 2002;**23**:1–38.
2. Parada J, Aguilera JM. Food microstructure affects the bioavailability of several nutrients. *J Food Sci* 2007;**72**:21–32.
3. Ramadan MF, Moersel JT. Impact of enzymatic treatment on chemical composition, physicochemical properties and radical scavenging activity of goldenberry (*Physalis peruviana* L.) juice. *J Sci Food Agric* 2007;**87**:452–60.
4. Pisoschi AM, Negulescu GP. Methods for total antioxidant activity determination: a Review. *Biochem Anal Biochem* 2011;**1**:1–10.
5. Chevion S, Roberts MA, Chevion M. The use of cyclic voltammetry for the evaluation of antioxidant capacity. *Free Radic Biol Med* 2000;**28**:860–70.
6. Giardi MT, Rea G, Berra B. *Bio farms for nutraceuticals: functional food and safety control by biosensors*. Landes Bioscience and Springer Science; 2010.
7. Barroso MF, De-los-Santos-Álvarez N, Delerue-Matos C, Oliveira MBPP. Towards a reliable technology for antioxidant capacity and oxidative damage evaluation: electrochemical (bio) sensors. *Biosens Bioelectron* 2011;**30**:1–12.
8. Cortina-Puig M, Noguer T, Marty JL, Calas-Blanchard CC. Electrochemical biosensors as a tool for the determination of phenolic compounds and antioxidant capacity in foods and beverages. In: *Biosensor in food processing, safety and quality control*. CRC Press; 2010.
9. Barroso MF, de-los-Santos-Álvareza N, Lobo-Castanón MJ, Miranda- Ordieres AJ, Delerue-Matos C, Oliveira MB, Tunon-Blanco P. DNA-based biosensor for the electrocatalytic determination of antioxidant capacity in beverages. *Biosens Bioelectron* 2011;**26**:2396–401.
10. Jayaprakasha GK, Jena BS, Negi PS, Sakariah KK. Evaluation of antioxidant activities and antimutagenicity of turmeric oil: a byproduct from curcumin production. *Z Naturforsch* 2002;**57**:828–35.
11. Stalmach A, Mullen W, Nagai C, Crozier A. On-line HPLC analysis of the antioxidant activity of phenolic compounds in brewed, paper-filtered coffee. *Braz J Plant Physiol* 2006;**18**:253–62.
12. Olsher M, Chong PLG. Sterol superlattice affects antioxidant potency and can be used to assess adverse effects of antioxidants. *Anal Biochem* 2008;**382**:1–8.

13. Besco E, Braccioli E, Vertuani S, Ziosi P, Brazzo F, Bruni R, Sacchetti G, Manfredini S. The use of photochemiluminescence for the measurement of the integral antioxidant capacity of baobab products. *Food Chem* 2007;**102**:1352–6.
14. Wright JS, Johnson ER, Di Labio GA. Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J Am Chem Soc* 2001;**123**:1173–83.
15. Sveinsdottir H, Hamaguchi PY, Bakken HE, Kristinsson HG. Methods for assessing the antioxidative activity of aquatic food compounds. In: Kristinsson HG, editor. *Antioxidants and functional components in Aquatic foods*. John Wiley & Sons, Ltd; 2014. p. 151–74.
16. Gulcin I. Antioxidant activity of food constituents: an overview. *Arch Toxicol* 2012;**86**:345–91.
17. Zulueta A, Esteve MJ, Frígola A. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chem* 2009;**114**:310–6.
18. Ou B, Hampsch-Woodill M, Prior RL. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem* 2001;**49**:4619–26.
19. Braude EA, Brook AG, Linstead RP. Hydrogen transfer. Part V. Dehydrogenation reactions with diphenylpicrylhydrazyl. *J Chem Soc (Resumed)* 1954:3574–8.
20. Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 1993;**84**:407–12.
21. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996;**239**:70–6.
22. Wayner DDM, Burton GW, Ingold KU, Locke S. Quantitative measurement of the total peroxyl radical trapping antioxidant capability of human blood plasma by controlled peroxidation: the important contribution made by plasma proteins. *FEBS Lett* 1985;**187**:33–7.
23. Magalhaes LM, Segundo MA, Reis S, Lima JL. Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chim Acta* 2008;**613**:1–19.
24. Ciz M, Cizova H, Denev P, Kratchanova M, Slavov A, Lojek A. Different methods for control and comparison of the antioxidant properties of vegetables. *Food Control* 2010;**21**:518–23.
25. Heinonen IM, Lehtonen PJ, Hopia AI. Antioxidant activity of berry and fruit wines and liquors. *J Agric Food Chem* 1998;**46**:25–31.
26. Criqui MH. Ethanol or antioxidants? *BioFactors* 1997;**6**:421–2.
27. Du Z, Bramlage J. Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. *J Agric Food Chem* 1992;**40**:1566–70.
28. Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. *Analyst* 2002;**127**:183–98.
29. Rice-Evans CA, Miller NJ. Total antioxidant status in plasma and body fluids. *Methods Enzymol* 1994;**234**:279–93.
30. Aruoma OI. Nutrition and health aspects of free radicals and antioxidants. *Food Chem Toxicol* 1994;**32**:671–83.
31. Aruoma OI, Spencer JPE, Rossi P, Aeschbach R, Khan A, Mahmood N, Munoz A, Murcia A, Butler J, Halliwell B. An evaluation of the antioxidant and antiviral action of extracts of rosemary and Provençal herbs. *Food Chem Toxicol* 1996;**34**:449–56.
32. Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 1996;**9**:836–44.
33. Lancaster J. *The biological chemistry of nitric oxide*. New York: Academic Press; 1995.
34. Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat Res* 2003;**523**:9–20.
35. Hunter KJ, Fletcher JM. The antioxidant activity and composition of fresh, frozen, jarred and canned vegetables. *Innovative Food Sci Emerging Tech* 2002;**3**:399–406.
36. Pratt DE. Natural antioxidants of soybean and other oil-seeds. In: Simic MG, Karel M, editors. *Autoxidation in food and biological systems*. , New York: Plenum Press; 1980. p. 283–92.
37. Mallet JF, Cerati C, Ucciani E, Gamisana J, Gruber M. Antioxidant activity of fresh pepper (Capsicum annuum) cultivares. *Food Chem* 1994;**49**:61–5.
38. Lloyd RV, Hanna PM, Mason RP. The origin of the hydroxyl radical oxygen in the Fenton reaction. *Free Radic Biol Med* 1997;**22**:885–8.
39. Liang N, Kitts DD. Antioxidant property of coffee components: assessment of methods that define mechanisms of action. *Molecules* 2014;**19**:19180–208.
40. Chung HY, Baek BS, Song SH, Kim MS, Huh JI, Shim KH, Kim KW, Lee KH. Xanthine dehydrogenase/xanthine oxidase and oxidative stress. *Age* 1997;**20**:127–40.
41. Girao H, Mota C, Pereira P. Cholesterol may act as an antioxidant in lens membranes. *Curr Eye Res* 1999;**18**:448–54.
42. Kikuzaki H, Nakatani N. Antioxidant effects of some ginger constituents. *J Food Sci* 1993;**58**:1407–10.
43. Moon JK, Shibamoto T. Antioxidant assays for plant and food components. *J Agric Food Chem* 2009;**57**:1655–66.
44. Serra-Majem L, Pfrimer K, Doreste-Alonso J, Ribas-Barba L, Sa´nchez-Villegas A, Ortiz-Andrellucchi A, Henrı´quez-Sa´nchez P. Dietary assessment methods for intakes of iron, calcium, selenium, zinc and iodine. *Br J Nut* 2009;**102**:S38–55.
45. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Anal Biochem* 1996;**239**:70–6.
46. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;**82**:70–7.
47. Ohkawa H, Onishi N, Yagi K. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem* 1979;**95**:351–8.
48. Paglia DE, Valentin WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;**70**:158–69.
49. McCord J, Fridovich I. Superoxide dismutase, an enzymic function for erythrocuprin. *J Biol Chem* 1969;**244**:6049–55.
50. Aebi H. Catalase. *Methods Enzymol* 1984;**105**:121–6.