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Antioxidants: Activity determination and Identification

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Abstract
Antioxidants are gaining tremendous importance in our daily lives. These components intent to neutralize the free radicals formed in the body which are capable of damaging the body. Natural antioxidants added to foods may have a physiological impact on human health, particularly because they may reduce the risk of illnesses or they promote the delay of damaging free radicals effect, like cardiovascular diseases, intestinal cancer or infections symptoms. Synthetic antioxidants are often added to foods to retard oxidation and extend a helping hand in increasing shelf life. A variety of methods are used for determination of antioxidant activity. With the technological advances, identification of antioxidants is not a big deal. In the present article the various methods for antioxidant activity determination and for identification of antioxidants are discussed.

Keywords: antioxidants, free radicals, antioxidant activity, identification, oxidation

1. Introduction
Human body can be regarded as a complex system composed of natural enzymatic and non-enzymatic antioxidant defenses that strive to protect the body against damage caused due to free radicals or reactive oxygen species (ROS) and other oxidants. Free radicals are found to be major causative factors for the occurrences of many diseases like cancer and also for aging. Antioxidants provide protection against the damage caused by free radicals [1]. Antioxidants are compounds that are capable of delaying or inhibiting the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. Oxidation process normally occur in foods as well as in human body and the antioxidants serve as a defense mechanism against damage caused due to oxidation process They are used for the stabilization of polymeric products, of petrochemicals, foodstuffs, cosmetics and pharmaceuticals.
There is a growing interest of scientists in the concept of 'synergism' ie: A combination of antioxidants may be more effective rather than providing single entities. Antioxidants may contribute on a greater scale in improving the quality of life by preventing or postponing the occurrences of diseases. Due to this contribution incorporation and intake of antioxidants is considered to be of significance today and thus they are gaining a lot of importance in the field of healthcare and food industries [2].
Antioxidant activity can be termed as the ability to inhibit the process of oxidation. A good number of abstracts and research articles published, so far, for evaluating antioxidant activity of various samples of research interest were studied where 407 methods were come across, which were repeated from 29 different methods. These were classified as *In vitro* and *In vivo* methods and those are described and discussed below in this review article. Various methods are used to investigate the antioxidant property of samples (diets, plant extracts, commercial antioxidants etc.) [3]. This review article aims to accumulate commonly used methods that are used to evaluate the antioxidant property of various samples.
Antioxidant activity should not be concluded based on a single antioxidant test model. Several *In vitro* test procedures are carried out for evaluating antioxidant activities with the samples of interest. Another factor is that antioxidant test models vary in different respects. Therefore, it is difficult to compare fully one method to other one. Researcher has to critically verify methods of analysis before adopting them for his/her research purpose. Method for determining antioxidants should be adopted as per the suitability of researcher and availability of the materials [3].

2. Antioxidants
The basic concepts regarding antioxidants, free radicals and oxidative stress are discussed in our article, (Antioxidants: Extraction and application in food industry) [4]. Antioxidants are compounds of many different chemical forms, grouped together as they are capable of compensating the ill effects of free radicals and oxidation reactions in the food system and the human body. An antioxidant can be defined as: "any substance that, when present in low concentrations compared to that of an oxidizable substrate, delays or inhibits the oxidation of that substrate". Antioxidants were found attractive by the researchers as they were capable of inhibiting rancidity in oils and fats. Dietary sources like fruits, vegetables, tea, etc. are rich in antioxidants [4]. As the food industry, today is very much concerned with the incorporation of antioxidants in various food products newer techniques for the extraction, identification, purification, and recovery of antioxidants continue to emerge. With the development of these techniques, antioxidants have found many applications in the food industry today. The methods of antioxidant activity determination and identification are discussed further.

3. Methods for evaluating total antioxidant activity

Antioxidant activity is a parameter which cannot be estimated accurately on the basis of a single activity determination method. Many different methods have been developed for measuring the antioxidant activity of a sample. Figure 1 shows a flowchart representing the generalized methods to determine total antioxidant activity.

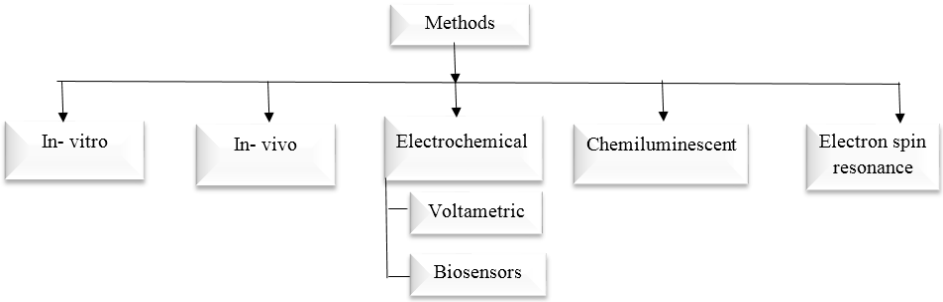

Fig 1: Methods of evaluating total antioxidant activity

3.1 *In vitro* and *In vivo* methods for determining antioxidant activity

a) *In vitro* methods: Generally *In vitro* antioxidant tests using free radical traps are relatively straightforward to perform. All the *In vitro* methods used in the quantification of total antioxidant activity involve the reactions of the reactive oxygen species with some reagents and the complex formed is VIS spectrometrically detected at a different wavelength in the case of color complex or is measured using a flour metrical detection [5].

b) *In vivo* methods: The *In vivo* action of antioxidants is due to the inhibition generation of the reactive oxygen species or by direct scavenging of free radicals [6]. In the case of *In vivo* methods, the antioxidants should be absorbed, transported, distributed and retained properly in the biological fluids, cells and tissues. The *In vivo* methods involve testing the antioxidant administration to animals at a specified dosage regimen. After a period of time, the animal blood or tissues are used for the assay. The *In vivo* antioxidant capacity may be measured using biological fluids and tissues (erythrocytes, urine, plasma, cerebrospinal fluids, saliva or tear) from humans or animals [7]. Some of the commonly used methods for determining antioxidant activity are listed in Table 1:

Table 1: *In vitro* and *In vivo* methods for determining antioxidant activity

***In vitro* Methods**	***In vivo* Methods**
DPPH scavenging activity	Ferric reducing ability of plasma
H_2O_2 scavenging activity	GSH estimation
Nitric oxide scavenging assay	GSHPx estimation
TEAC/ABTS	GSt
TRAP	SOD
FRAP	CAT
SOD	GR
ORAC	LPO
Metal chelating activity	LDL

A) *In vitro* Methods

i) DPPH scavenging activity

The molecule 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize. This delocalization of electron gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color. In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored. According to the sample extract (0.2 mL) is diluted with methanol and 2 mL of DPPH solution (0.5 mM) is added. After 30 min, the absorbance is measured at 517 nm. The percentage of the DPPH radical scavenging is calculated using the equation as given below % inhibition of DPPH.

DPPH radical = [(Abr- Aar/ Abr) x 100]

Where Abr is the absorbance before reaction and Aar is the absorbance after reaction has taken place [8].

ii) Hydrogen peroxide scavenging (H_2O_2) assay

Hydrogen peroxide is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH) that can initiate lipid peroxidation and cause DNA damage in the body. The ability of plant extracts to scavenge hydrogen peroxide can be estimated according to the method of A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (20–60 microgram/mL) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

% scavenged (H2O2) = (Ai-At/Ai) x100

Where Ai is the absorbance of control and at is the absorbance of test [9].

iii) Nitric oxide scavenging activity

The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent. Two (2) mL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer saline (pH 7.4) is mixed with 0.5 mL of sample at various concentrations (0.2–0.8 mg/mL). The mixture is then incubated at 25^0C. After 150 min of incubation, 0.5 mL of the

incubated solution is withdrawn and mixed with 0.5 mL of Griess reagent [(1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthyl ethylenediamine dichloride (0.1% w/v)]. The mixture is then incubated at room temperature for 30 min and its absorbance pouring into a cuvette is measured at 546 nm. The amount of nitric oxide radical inhibition is calculated following this equation:

% inhibition of NO radical = [(A0 - A1/ A1) x 100]

Where A0 is the absorbance before reaction and A1is the absorbance after reaction has taken place with Griess reagent [10].

iv) Trolox equivalent antioxidant capacity (TEAC) method/ABTS radical cation decolorization assay

This method, uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue–green chromophore ABTS + (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). ABTS radical cations are prepared by adding solid manganese dioxide (80 mg) to a 5 mM aqueous stock solution of ABTS (20 mL using a 75 mM Na/K buffer of pH 7). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, can be used as an antioxidant standard. A standard calibration curve is constructed for Trolox at 0, 50,100, 150, 200, 250, 300, and 35 micromolar concentrations. Samples are diluted appropriately according to antioxidant activity in Na/K buffer pH, 7. Diluted samples are mixed with 200 microliter of ABTS+ radical cation solution in 96-well plates, and absorbance is read (at 750 nm) after 5 min in a microplate reader. TEAC values can be calculated from the Trolox standard curve and expressed as Trolox equivalents (in mM) [11].

v) Total radical-trapping antioxidant parameter (TRAP) method

This method is based on the protection provided by antioxidants on the fluorescence decay of R-phycoerythrin (R-pE) during a controlled peroxidation reaction. The fluorescence of R-phycoerythrin is quenched by ABAP (2, 2'-azo–bis (2-amidino-propane) hydrochloride) as a radical generator. This quenching reaction is measured in the presence of antioxidants. The antioxidative potential is evaluated by measuring the decay in decoloration.120 microliter of diluted sample is added to 2.4 mL of phosphate buffer (pH 7.4), 375 microliter of distilled water, 30 microliter of diluted R-pE and 75 microliter of ABAP; the reaction kinetics at 38^0C is recorded for 45 min by a luminescence spectrometer. TRAP values are calculated from the length of the lag-phase due to the sample compared with standard [12].

vi) Ferric reducing-antioxidant power (FRAP) assay

This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2, 3, 5-triphenyl-1, 3, 4-triaza-2-azoniacyclopenta-1, 4-diene chloride (TPTZ) to the ferrous form at low pH. This reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer. Three milliliter of prepared FRAP reagent is mixed with 100 microliter of diluted sample; the absorbance at 593 nm is recorded after a 30 min incubation at 37^0C. FRAP values can be obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe3+ and expressed as mM of Fe2+ equivalents per kg (solid food) or per L (beverages) of sample [13].

vii) Superoxide radical scavenging activity (SOD)

The superoxide anion radicals are generated in 3.0 mL of Tris–HCl buffer (16 mM, pH 8.0), containing 0.5 mL of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract and 0.5 mL Tris–HCl buffer (16 mM, pH 8.0). The reaction is initiated by adding 0.5 mL phenazine methosulfate (PMS) solution (0.12 mM) to the mixture, incubated at 25^0C for 5 min and then the absorbance is measured at 560 nm against a blank sample [14].

viii) Oxygen radical absorbance capacity (ORAC) method

The test is performed using Trolox (a water-soluble analog of Vitamin E) as a standard to determine the Trolox Equivalent (TE). The ORAC value is then calculated from the Trolox Equivalent and expressed as ORAC units or value and is proportional to ''Antioxidant Power''.

This assay is based on generation of free radical using AAPH (2, 2-azobis 2-amidopropane dihydrochloride) and measurement of decrease in fluorescence in the presence of free radical scavengers. In this assay b-phycoerythrin (b-PE) was used as target free radical damage, AAPH as a peroxy radical generator and Trolox as a standard control. After addition of AAPH to the test solution, the fluorescence is recorded and the antioxidant activity is expressed as Trolox equivalent [15].The assay can be carried out in 96-well polypropylene fluorescence plates with a final volume of 200 microliter. Assays are conducted at pH 7.0 with Trolox (6.25, 12.5, 25, and 50 mol/L for lipophilic assays; 12.5, 25, 50 and 100 micromol/L hydrophilic assays) as the standard and 75 mM/L phosphate buffer as the blank. After the addition of AAPH, the plate is placed immediately in a multilabel counter preheated to 37^0C. The plate is shaken in an orbital manner for 10 s and the fluorescence is read at 1 min intervals for 35 min at the excitation wavelength of 485 nm and emission wavelength of 520 nm. Area-under-the-curve is calculated for each sample using Wallac Workout 1.5 software. Final computation of results is made by taking the difference of areas-under-the-decay curves between blank and sample and/or standard (Trolox) and expressing this in microliter of Trolox equivalents (TE) per g dry weight of sample (lM TE/g) [16].

ix) Metal chelating activity

Ferrozine can form a complex with a red color by forming chelates with Fe2+. This reaction is restricted in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe2+ complexes. Measurement of the color reduction determines the chelating activity to compete with ferrozine for the ferrous ions [17].0.1 mL of the extract is added to a solution of 0.5 mL ferrous chloride (0.2 mM). The reaction is started by the addition of 0.2 mL of ferrozine (5 mM) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA or citric acid can be used as a positive control [18].

B) *In vivo* methods

i) Ferric reducing ability of plasma: The antioxidative activity is estimated by measuring the increase in absorbance

caused by the formation of ferrous ions from FRAP reagent containing TPTZ (2, 4, 6-tripyridyl-s-triazine) and $FeCl_2.6H_2O$. The absorbance is measured spectrophotometrically at 593 nm. The method involves the use of blood samples that are collected from the rat retro-orbital venous plexus into heparinized glass tubes at 0, 7 and 14 days of treatment. Three mL of freshly prepared and warm (37^0C) FRAP reagent [1 mL (10 mM) of 2,4,6 tripyridyl-s-thiazine (TPTZ) solution in 40 mM HCl, 1 mL 20 mM $FeCl_2$.6-H2O, 10 mL of 0.3 M acetate buffer (pH 3.6)] is mixed with 0.375 mL distilled water and 0.025 mL of test samples. The absorbance of developed color in organic layer is measured at 593 nm. The temperature is maintained at 37^0C. The readings at 180 s are selected for the calculation of FRAP values [13].

ii) Reduced glutathione (GSH) estimation: GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. Deficiency of GSH in the lens leads to cataract formation. The tissue homogenate (in 0.1 M phosphate buffer pH 7.4) is taken and added with equal volume of 20% trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture is allowed to stand for 5 min prior to centrifugation for 10 min at 2000 rpm. The supernatant (200 microliter) is then transferred to a new set of test tubes and added with 1.8 mL of the Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid (0.1 mM)) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes are made up to the volume of 2 mL. After completion of the total reaction, solutions are measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from known GSH [19].

iii) Glutathione peroxidase (GSHPx) estimation: $GSHP_X$ is a seleno-enzyme two third of which (in liver) is present in the cytosol and one third in the mitochondria. GSHPx is found throughout the tissues. GSHPx measurement is considered in particular with patients who are under oxidative stress for any reason, low activity of this enzyme is one of the early consequences of a disturbance of the prooxidant/antioxidant balance [20].Cytosolic GPx is assayed via a 3-mL cuvette containing 2.0 mL of 75 mM/L phosphate buffer, pH 7.0. The following solutions are then added: 50 microliter of 60 mM/L glutathione reductase solution (30 U/mL), 50 L of 0.12 M/L NaN_3, 0.10 of 0.15 mM/L Na_2EDTA, 100 microliter of 3.0 mM/L NADPH, and 100 microliter of cytosolic fraction obtained after centrifugation at 20,000 g for 25 min. Water is added to make a total volume of 2.9 mL. The reaction is started by the addition of 100 microliter of 7.5 mM/L H_2O_2, and the conversion of NADPH to NADP is monitored by a continuous recording of the change of absorbance at 340 nm at 1 min interval for 5 min. Enzyme activity of GSHPx was expressed in terms of mg of proteins [21].

iv) Glutathione-S-transferase (GST): Glutathione-S-transferase is thought to play a physiological role in initiating the detoxication of potential alkylating agents, including pharmacologically active compounds. The reaction mixture (1 mL) consisted of 0.1 N potassium phosphate (pH 6.5), 1 mM/L GSt, 1 M/L l-chloro-2, 4-dinitrobenzene as substrate and a suitable amount of cytosol (6 mg protein/mL). The reaction mixture is incubated at 37^0C for 5 min and the reaction is initiated by the addition of the substrate. The increase in absorbance at 340 nm was measured spectrophotometrically [22].

v) Superoxide dismutase (SOD) method: It is estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 microliter of the lysate, 75 mM of Tris–HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol are added. An increase in absorbance is recorded at 420 nm for 3 min by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of auto oxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD is expressed as units/mg protein [23].

vi) Catalase (CAT): Fifty microliter of the lysate is added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. Catalase activity is measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H_2O_2, 43.6 M cm^{-1} was used to determine the catalase activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and is expressed as units per milligram of protein [24].

vii) Glutathione reductase (GR) assay: The ubiquitous tripeptide glutathione (GSH), which is the most abundant low molecular weight thiol in almost all cells, is involved in a wide range of enzymatic reactions. Livers (about 400 g) are obtained from killed rats (200–250 g).The livers are cut into small pieces and homogenized in 9 mL of 0.25 M ice-cold sucrose per g of rat liver in a blender. The homogenate is centrifuged for 45 min at 14,000 rpm. The pellets are suspended in a small volume of 0.25 M sucrose and centrifuged. The supernatants are combined with the previous centrifugate. The pooled material is adjusted to pH 5.5 with cold 0.2 M acetic acid and centrifuged again for 45 min at 14,000 rpm. The rate of oxidation of NADPH by GSSG at 30^0C is used as a standard measure of enzymatic activity. The reaction system of 1 mL contained: 1.0 mM GSSG,0.1 mM NADPH, 0.5 mM EDTA, 0.10 M sodium phosphate buffer (pH 7.6), and a suitable amount of the glutathione reductase sample to give a change in absorbance of 0.05–0.03/min. The oxidation of 1 micromolar of NADPH/min under these conditions is used as a unit of glutathione reductase activity. The specific activity is expressed as units per mg of protein [25].

viii) Lipid peroxidation (LPO) assay: LPO is an autocatalytic process, which mainly occurs due to cell death. Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals, which is an indicator of lipid peroxidation.
The tissues are homogenized in 0.1 M buffer pH 7.4 with a Teflon glass homogenizer. LPO in this homogenate is determined by measuring the amounts of Malondialdehyde (MDA) produced primarily. Tissue homogenate (0.2 mL), 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid and 1.5 mL of 8% TBA are added. The volume of the mixture is made up to 4 mL with distilled water and then heated at 95^0C on a water bath for 60 min using glass balls as condenser. After incubation the tubes are cooled to room temperature and final volume was made to 5 mL in each tube. Five mL of butanol: pyridine (15:1) mixture is added and the contents are vortexed thoroughly for 2 min. After

centrifugation at 3000 rpm for 10 min, the upper organic layer is taken and its OD is taken at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides can be expressed as n moles of thiobarbituric acid reactive substances (TBARS)/mg protein using an extinction coefficient of 1.56 x 10 ^{5} ML cm^{-1}[26].

ix) LDL assay: The isolated LDL is washed and dialyzed against 150 mmol/L NaCl and 1 mmol/L Na_2EDTA (pH 7.4) at 4^{0}C. The LDL is then sterilized by filtration (0.45lM), kept under nitrogen in the dark at 4^{0}C. LDL (100 microgram of protein/mL) is incubated for 10 min at room temperature with samples. Then, 5 micromole / L of CuSO4 is added, and the tubes are incubated for 2 h at 37^{0}C. Cu2+-induced oxidation is terminated by the addition of butylated hydroxytoluene (BHT, 10 micro M). At the end of the incubation, the extent of LDL oxidation is determined by measuring the generated amount of lipid peroxides and also by the thiobarbituric acid reactive substances (TBARS) assay at 532 nm, using Malondialdehyde (MDA) for the standard curve [27].

3.2 Electrochemical methods

Electrochemical methods are highly active for investigation of antioxidant compounds, assessment of antioxidant activity and measurement of electrochemical index. Different types of electrodes can be used for assay purposes. The devices may be stationary or flow through, and based on cyclic or differential pulse voltammetry as well as potentiostatic analysis. The methods are suitable for food control and monitoring the levels of antioxidant capacity in other biological samples and matrices.

A) Voltammetric methods

Voltammetric techniques are considered the most effective for studying antioxidant properties. Table 2 describes the Voltammetric methods for determining antioxidant activity.

Table 2: Voltammetric methods for determining antioxidant activity

Method	Description	Reference
Cyclic voltammetry	Cyclic voltammetry involves three electrodes: the working electrode (e.g., glassy carbon), the reference electrode (Ag–AgCl), and the auxiliary electrode (platinum wire). The working electrode is subjected to a constant potential rate of 100 mV/s and records the evolution curve called cyclic voltammogram. The two parameters of reducing power of a sample are: the peak potential related with individual compounds, and the anodic current related with the antioxidant concentration. Cyclic voltammetry has been applied with success in the extraction of antioxidants	28
Differential pulse voltammetry	It is a selective and sensitive technique, where the potential is changing linearly with the time (potential linear sweep) superimposed by the potential pulses of the amplitude between 10 and 100 mV for several milliseconds. Voltage pulse is applied at the end of the drop time; the total time of the drop is directed electronically by a drop knocker. The difference between the currents applied immediately before the pulse application and at the end is registered. Dependence of the difference between these two currents on the applied potential goes through maximum, so it has a peak shape. The position of the peak on the potential axis is given by the quality of analyte, and its height depends on the concentration of the analyte.	29,30
Square wave voltammetry	It uses a potential waveform. The advantage of SWV is that the entire scan can be performed on a single mercury drop in about 10 seconds. SWV saves time, reduces the amount of mercury used per scan by a factor of 100. SWV was found to be more sensitive in comparison with differential pulse voltammetry and has more extend dynamic range and lower limit of detection in comparison with the linear sweep voltammetry.	31
Amperometric measurement	The amperometric method is based on measurement of electric current resulting from oxidation of the substance (or the mixture) being studied on the surface of a working electrode at a certain voltage potential. The nature of the working electrode as well as the voltage potential applied determines the sensitivity of the amperometric method. The antioxidant activity may be measured by using the value of oxidation of such compounds on the working electrode of the amperometric detector. The signal is registered as differential dynamic curves. Amperometric method used for an evaluation of antioxidant capacity had the same advantages and disadvantages as the compared spectrophotometric methods.	32

B) Biosensors

(SOD) biosensor for the determination of the total and natural antioxidant capacity of red and white wines. The biosensor was obtained by coupling a transducer (an amperometric electrode for hydrogen peroxide, with a platinum anode maintained at a constant potential of +650 mV with respect to an Ag/AgCl/Cl− cathode) and the superoxide dismutase enzyme immobilized in a gel-like kappa-carrageenan membrane and concluded that antioxidant activity has a strong correlation with biosensor method [33]. The SOD biosensor method is a valid method for measuring the capacity of algae [34].A novel amperometric biosensor was developed based on immobilizing tyrosinase on the surface of an Mg–Al–CO3HTLc modified electrode for the detection of polyphenols [35]. A laccase-based biosensor to evaluate the antioxidant activity of wine [36].

3.3 Chemiluminescent methods

Chemiluminescence (CL) is a fast method for screening antioxidant activity. A suitable reagent for CL is luminol, due to its ability to scavenge the free radicals involved in the sequence leading to an excited electron (3-aminophtalatedianion), which emits light on return to its ground state. CL-detection is advantageous due to its high sensitivity, simplicity and based on stable and easy-to-handle reagents. CL-detection is advantageous due to its high sensitivity in comparison with the spectrophotometric assays (DPPH), moreover, the luminol-I2detection system is simple and based on stable and easy-to-handle reagents [37].

3.4 Electron spin resonance

Electron spin resonance (ESR) also called Electron Paramagnetic Resonance (EPR) spectroscopy. It is the most

promising technique for detecting and monitoring processes involving radicals. ESR spectroscopy provides qualitative and quantitative information about paramagnetic species having one or more unpaired electrons. Since hydroxyl radicals (OH) have a very short life-time, spin-trapping techniques with DMPO (5, 5-dimethyl-1-pyrroline-1-oxide) are used for its detection [38]. This method is the only one that detects free radicals involved in autoxidation. The ESR method was applied to measure antioxidant activity of coffee [39], aqueous and aroma extracts of squid miso prepared with Aspergillus oryzae-inoculated koji [40].

4. Isolation, purification, recovery and fractionation:

4.1 Isolation

It is important to isolate the antioxidants after the extraction process. Many methods were developed for the isolation of antioxidants. The conventional methods used for the isolation of antioxidants were laborious, time consuming, expensive and if not performed with accuracy led to decomposition of antioxidants [41]. High-speed counter-current chromatography (HSCCC) is considered to be a suitable method with high precision. A low risk of sample denaturation and low cost. HSCCC is successfully applied for many bioactive compounds (e.g., phenolics) from natural products [42]**.** High performance liquid chromatography (HPLC) proves to be a good method but it needs high precision, accuracy and technical skills. It is of low resolution and sensitivity. Other methods for antioxidant isolation and purification are butanol fraction extraction, Ultrafiltration, gel filtration chromatography and reversed phase HPLC.

4.2 Purification

Macroporous adsorption resins were used in the purification of bioactive constituents from natural extracts due to their high efficiency and have higher adsorption. Macroporous resins (MARs) are durable polar, non-polar or slightly hydrophilic polymers with high adsorption capacity. Advantages of MARs are mechanical strength, diverse structures, good performance, low costs and environmental friendliness. This method is time-consuming, laborious and requires large volumes of solvents [43].

4.3 Recovery

The food industry generates huge amounts of by-products from the processing of foods of plant origin. The disposal of these by-products is expensive and has a harmful effects on the environment. Many of these by-products could serve as a source of potentially valuable bio-active compounds [44].

1. A so called "5-Stages universal recovery processing" for the recovery of antioxidants from natural sources. These "5-stages" are: 1. Macroscopic pre-treatment, which implies: a. wet milling, thermal and/or vacuum concentration, b. mechanical pressing, freeze drying, centrifugation and microfiltration
2. Macro and micro-molecules separation–alcohol precipitation, ultrafiltration, isoelectric solubilization–precipitation and extrusion
3. Extraction—solvent, acid, alkali, microwave-assisted, steam diffusion, hydrodystillation, supercritical fluids
4. Isolation and purification—adsorption, chromatography, nanofiltration and electrodialysis
5. Product formation—spray- and freeze–drying emulsions [44].

4.4 Fractionation

The fractionation steps may enhance antioxidant properties of phenolic mixtures. One method used for the polyphenol fractionation involves column chromatography of Sephadex LH 20 or reversed C18 phase, where the phytochemicals are fractioned into several fractions according to the phenolic properties like weight, solubility and polarity. An ultrafiltration method for the recovery and fractionation of different phenolic classes from winery sludge. Ultrafiltration is among the conventional technologies that are utilized prior or after extraction [45].

5. Identification of antioxidants

Accurate identification of antioxidants in a particular fraction is essential for the particular application. Many instrumental techniques have been developed for the identification of antioxidants. Many researches regarding determination of antioxidant activity have been carried out till date and one or more identification techniques have been applied for the identification of antioxidants. The use of identification techniques provide accurate information about the presence of antioxidant compounds in a fraction. Table 2 summarizes some of the techniques and application for identification of antioxidants.

Table 3: Techniques and application for identification of antioxidants.

Technique	Application
High Performance Liquid Chromatography (HPLC)	▪ Analysis of carotenoids and phenolic compounds [46].
Thin-layer chromatography (TLC)	▪ TLC has been applied for the detection of lignin in Oriental medicinal plants [47].
Gas chromatography (GC)	▪ Presence of six lignans in Punicagranatum L fruit endocarp, pulp, seeds, wood knots and commercial juices is reported by the use of this technique [48].
Macroporous adsorption resins combined with ultrafiltration	▪ The absorption of flavonoids and phenolic acids on different resins has been studied [49]. ▪ Ginger polyphenol adsorption has been studied using an anion exchange resin, AmberliteIR-400 [50].
HSCCC	▪ HSCCC is used for the separation and isolation of tannins, anthocyanins and flavonoids [51].
Mass spectrometry	▪ Rapid identification of secondary metabolites from antioxidant extracts[52]
Liquid Chromatography–Mass Spectrometry (LC–MS)	▪ LC–MS allows the characterization of complex structures of grape polyphenols, such as procyanidins, proanthocyanidins, prodelphinidins, and tannins, and provides experimental evidence for structures that were previously only hypothesized [53].
Gas Chromatography–Mass Spectrometry (GC–MS)	▪ Used to separate, identify and quantify cis-and trans resveratrol in red wine [54].
Capillary electrophoresis	▪ Capillary electrophoresis is excellent for the simultaneous separation of 16 phenolic compounds (e.g.,rutin,myricetin,kaemppferol,quercetin,natitutin, morin,(−)-epicatechin,(+)-catechin,cinnamic acid, trans-resveratrol, ferulic acid, vanillic acid, gallic acid, caffeic acid

	and 3,4-dihydroxybenzoic acid) present in red, white and rosé wines [55].
Nuclear magnetic resonance (NMR)	▪ A prediction of total phenolic content, total anthocyanin content and total antioxidant capacity of sour cherries by applying multivariate data analysis to NMR data [56].
IR spectrometry	▪ Infrared spectroscopy has been used to evaluate phenolic changes occurring during the development of olive fruit [57].

6. References

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