Investigation of Measurement Methods of Antioxidant Activity and Involved Mechanisms

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Abstract: Bioactive food components are active ingredients in food or dietary supplements proven to have a role in health and they are safe for human consumption. These compounds exert their antioxidant effects by different mechanisms such as hydrogen atom transfer (HAT) or single electron transfer (SET) and their efficiencies can be evaluated by several methods such as ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), dipheny-picrylhydrazil (DPPH), Folin-Ciocaltue method (FCM), etc. In this review, these mechanisms and methods will be discussed in details.

Keywords: Bioactive compounds, Antioxidant, HAT, SET, Mechanisms of antioxidant action.

1. INTRODUCTION

1.1. Bioactive Compounds

Bioactive food components are active ingredients in food or dietary supplements derived from animal or vegetable sources, including materials necessary for human major nutritional needs, and materials proven to play a role in the health and safety of human beings. All active components in foods such as micro and macronutrients should be considered as bioactive food compounds. Bioactive compounds are classified into different groups according to their distinctive chemical structures and functions, for example phenolic compounds and their subclasses such as flavonoids. The biological activity of a chemical group is affected not only by the differences in chemical composition and structure, but also by factors such as bioavailability, distribution and metabolism. All positive and negative constructive effects of bioactive food components should be investigated in scientific models of risk - benefit. In these experiments, toxic components should not be considered as bioactive compounds.

Based on recommended daily intake (RDA), bioactive compounds by definition reflect their safety. Essential and none-essential nutrients according to their specific physiological functions should be considered as bioactive compounds. Bioactive compounds are present in common foods, food additives, dietary supplements and dietary foods. In recent decades, there have been apparent changes in the role of foods in promoting human health and the primary role of foods as sources of energy and body constituents has changed to biologically active components in human body. Thus the new term "functional food" was introduced in 1998, which is part of a daily human diet that is beneficial for health and helps reduce the risk of chronic diseases. In 80 AD, the Japanese defined these foods as: "food for specific health purposes".

Functional foods are:

Conventional foods containing bioactive natural materials such as fiber; Foods enriched with bioactive substances such as probiotics and antioxidants; Synthetic commercial nutrients such as prebiotics [1].

Reactive molecules of free radicals have been participated in many diseases such as cancers, atherosclerosis, diabetes, aging and neurological disorders. In order to have a healthy body the destruction caused by free radicals should neutralize by antioxidants. Nowadays there is a great interest in using natural antioxidants which include a wide range of bioactive compounds [2]. Factors which are affecting the type and amount of bioactive compounds in plant tissues include climate conditions, agronomical practices, harvest management and post-harvest storage conditions, genotypic differences, genus, plant age and species, processing and extraction conditions [1]. Analytical methods for identifying bioactive Compounds are as follows; UV-Vis spectrometry; Fourier transformer infrared spectrometry; Mass spectrometry; ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy; Determination of antioxidant activity of extract [2].

This study aimed to review the antioxidant properties of bioactive compounds, sample preparation to measuring antioxidant property, chemical reactions,

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involved mechanisms and effective factors in antioxidant effects and at last several practical methods for assessing antioxidant activity.

1.2. A Brief About Oxidation Reactions

Oxidizing characteristics of oxygen have a vital role in different biological actions, such as electron transport in adenosine-5'-triphosphate (ATP) production, while oxygen is essential for life, it can have destructive effects by means of cellular material oxidation [3]. Auto-oxidation and thermal oxidation of lipids, cellular oxidation pathways, and numerous physiological and biochemical processes in human body under normal circumstances produce two groups of radicals; reactive oxygen species (ROS) such as: superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), single oxygen ($^1O_2$) and hypochlorous acid (HOCl). The second group which includes reactive nitrogen species (RNS): nitric oxide ($NO^{\bullet}$) and nitrite proxy ($ONOO^-$) which form in $NO^{\bullet}$ reaction with superoxide during inflammatory processes [4]. Free radicals are highly active and toxic molecules with short half-life, originating from either inside (normal aerobic respiration, metabolism, and inflammation) or outside (pollution, sunlight, X-ray, extreme sports, smoking and alcohol) body stimuli having one or more none-paired electrons. Thus they attempt to obtain or lose electrons in body, and consequently causing damages to DNA, proteins, lipids and carbohydrates [2]. One of the most destructive effects of free radicals is lipid peroxidation that leads to cell membrane damage. For example, due to the effects of oxygen free radicals on the double bonds of unsaturated fatty acids and stimulation of membrane peroxidation chain reactions, unsaturated fatty acids and cells are ultimately destroyed. Lipid peroxidation impairs membrane of organism and changes the activity of its dependent enzymes and other proteins which can be potentially harmful to cells by releasing hydroperoxyl and alkoperoxyl radicals [5]. Lipid peroxidation thus changes the structure of unsaturated fatty acids, and reduces their fluidity and membrane potential negatively affecting cellular membrane permeability. With lipid peroxidation cellular wall and its performance will be affected. Also some oxidation products such as malone aldehyde react with biomolecules and show geotoxic and cytotoxic effects [6]. Cytotoxic metabolites derived from oxidation of low density lipids (LDL) can cause lipid peroxidation which plays an important role in the pathogenesis of atherosclerosis. Also free radicals participate in mechanism of cytochromes [7].

When fatty foods are exposed to air, light and heat, their taste, color and smell change due to oxidation and eventually they will spoil. The major products of auto-oxidation of lipids are tasteless and odorless hydroperoxides, which after decomposition form off-odor and off-flavor products such as aldehydes and ketones. In order to maintain food quality and increase shelf life, natural or synthetic antioxidants must be used [6, 7, 8]. Whenever the production of ROS and RNS in a system is more than the system capability to neutralize and remove them, oxidative stress does occur. The lack of anti-oxidative capacity causes imbalance and therefore, high concentrations of ROS cause damages to cellular lipids, proteins, and nucleic acids as well as their routine performances. Organisms protect themselves from ROS by multiple enzymatic systems and intracellular antioxidants, and foods can extend these natural defense mechanisms [9]. Antioxidants are compounds that prevent initiation or propagation steps of oxidation chain reactions and they are able to inhibit or repair oxygen damages to body's cells [10, 11]. Antioxidants by eliminating or preventing production of ROS and RNS through their oxidation can inhibit oxidative reactions and therefore, body always requires antioxidant sources continuously [12]. Generally speaking, the efficiency of an antioxidant is related to its surface, composition and distribution of hydrophobic and hydrophilic phases [13]. Vitamins C and E, carotenoids, xanthophylls, tannins and total phenolic are involved in the antioxidant index of a sample [14, 15]. Some amino acids such as cysteine, serine, histidine, tyrosine and arginine have antioxidant effects and can prevent oxidation of low density lipids (LDL). Anti-plaque effects of amino acids such as aspartate and glutamate are also attributed to their antioxidant properties. Aspartic, glutamic, serine, and valine amino acids probably through stabilizing mechanisms of free radicals are able to prevent production of malone de aldehyde and consequently inhibiting lipid peroxidation [6]. Antioxidants functions can be divided into two categories:

1. Break the chain: when a free radical absorbs or loses an electron, a second radical is formed and this process continues until chain breaking antioxidants, such as β-carotene, vitamins C and E terminate it and as a result, stabilize or convert the radical to a harmless product.

2. Way inhibitors: antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase prevent oxidation by reduction of chain formation. These kinds of

antioxidants by joining to initiator radicals can stop chain oxidation permanently. Effects of any antioxidant in the body depend on the type of involved free radical, how and where it is produced, and the place of the target damage.

Main Types of Antioxidants are as follow;

1. Plant chemicals (phytochemicals): Natural and safe antioxidants materials for the health of consumers like phenolic compounds, carotenoids, anthocyanins, tochopherols, ascorbic acid, flavonoids, minerals, enzymes and pectin.

2. Artificial antioxidants such as butilated hydroxyl anisol (BHA), butilated hydroxyl toloan (BHT), tert-butylhydroquinone (TBHQ), pyrogallol, etc.

Antioxidants sources in biological systems are categorized as follows:

Enzymes, such as superoxide dismutase, glutathione peroxidase and catalase; Large molecules, such as albumin, ceruloplasmin, ferritin and other proteins; Small molecules, such as ascorbic acid, glutathione, uric acid, tocopherols, carotenoids and polyphenols; Some hormones, like estrogen, angiotensin, and melatonin [16].

Antioxidants can exert various effects under different conditions and concentrations and as a result, act as antioxidant, per-oxidant or pro-oxidant. Vitamin E, for instance, has antioxidant properties in high doses, whereas at low concentrations it acts as per-oxidant. Vitamin E can also be a per-oxidant in the presence of peroxidase in LDL oxidation. Vitamin C at concentrations of 60-100 μM shows antioxidant properties, however at lower or higher amounts acts as per-oxidant [17]. Gallic acid shows both anti- and pro-oxidant effects, depending on its concentration in the lipid peroxidation system stimulated by Fe^{2+} or Cu^{+} [18]. Antioxidant capacity of plant foods is derived from the synergistic action of various antioxidants such as vitamins C and E, poly phenols, carotenoids, terpenoides, maillard reaction compounds and trace minerals. Daily intakes of main antioxidants like poly phenols, vitamins and carotenoids are 1000, 110 and 9.4 mg, respectively. Antioxidant capacity and concentration depend on species and ripeness grade. The antioxidant capacity of polyphenols gradually reduces after harvest. Also various post-harvest considerations such as storage conditions (time, temperature, and atmosphere composition) and processing factors (cutting, treatment time and temperature, addition of synthetic antioxidants) affect the antioxidant capacity of foods. Antioxidant capacity is an important factor for food science and technology and nutrition studies. Improvement in the standard assessment methods of total antioxidant capacity (TAC) is necessary and thus, most researches have focused on the profile-making methods such as free radical generation systems, reduction reactions, molecular target, the end point, solubility, hydrophobicity or hydrophilicity and so on. However, little attention has been paid to the crucial stages of sample preparation and extraction of antioxidants. Antioxidant capacity of food extracts is commonly measured in water-organic chemical solvents (methanol, ethanol, acetone, chloroform, etc.). An ideal solvent is the one that can extract the whole antioxidants present in a food sample, especially when complex compounds of carbohydrates and protein do not exist. As a result, significant amounts of antioxidants such as carotenoids and phenolics connected to fiber and proteins remain in the extraction residues, which in most chemical and biological studies are neglected. Antioxidants that are not extracted from a food matrix convert into bioactive compounds which are then released by digestive enzymes and intestinal microbial flora showing significant biological properties. In some foods, the amount of these none extractable antioxidants are more than the extractable polyphenols. For example, the antioxidant capacity of cocoa pulp, which is related to the presence of pro-anthocyanidins, or that of cereal residues, which are rich in hydrolysable tannins is greater than their extracts and therefore, the antioxidant capacity of the residues should be accounted [15].

2. SAMPLE PREPARATION AND ANTIOXIDANTS EXTRACTION

2.1. Plant Foods

Freeze dried foods are pulverized into particles having a diameter smaller than 0.5 mm in a centrifugal mill. Samples are stored under -20°C until analysis and the analysis must be done immediately after extraction. The purpose of this process is to obtain extractable antioxidants using water-organic solvents and non-extractable ones by acidic hydrolysis. 0.5 g sample is poured into a capped centrifuge tube; 20 mL acidic methanol/water (50:50 v/v, pH=2) is added and subsequently, the tube is shaken vigorously for an hour at room temperature. The tube is then centrifuged for 10 min at 2500 g and the supernatant is separated. 20 mL acetone/water (70:30 v/v) is added to the residues,

remixed and recentrifuged. Methanol and acetone extracts are mixed to determine the antioxidant capacity of the extractable fraction of the extraction process. The residues are subjected to 2 different acidic treatments in order to release the non-extractable antioxidants; residues are mixed with 20 mL methanol and 2 mL concentrated sulfuric acid and kept in a water bath at 85 °C with constant stirring for 20 h. Then the samples are centrifuged at 2500 g for 10 min and the supernatants are separated. After twice washing with distilled water, the final volume is kept at 50 mL. The antioxidant capacity of the residues refers to the hydrolysable tannins and other phenolic compounds linked to carbohydrates and proteins. The residues can be treated with HCl/butanol/$FeCl_3$ (5:95 v/v) at 100 °C for 3 h. The samples are centrifuged at 2500 g for 10 min and the supernatants are separated. After two times washing with HCl/butanol (5:95 v/v), the final volume is kept at 25 mL. The antioxidant capacity of the residues is related to the non-extracted, condensed tannins (proanthocyanidins) with water -organic solvents.

Beverages

TAC in drinks is determined directly after dilution with water if necessary. To separately determine the antioxidant capacity of hydrophilic and hydrophobic compounds, ethyl acetate is mixed with drinks (ratio 1:1) and after shaking the samples for an hour, they are centrifuged for 10 min at 1800 g and finally the antioxidant capacity is determined in aqueous and organic phases.

Oils

TAC in vegetable oils is measured directly after dilution with ethyl acetate. Determination of the antioxidant capacity of compounds which is related to polar and non-polar fractions is separately done as follows; 5 mL oil is mixed with 5 mL methanol and strongly shaken for 20 min, then centrifuged for 10 min at 2500 g and the supernatant is recovered. Another 5 mL aliquot of methanol is added and the whole process is repeated. The antioxidant capacity in the methanol fraction (polar compounds) and the remaining oil (non-polar fraction) is measured directly after dilution with ethyl acetate.

2.2. Increasing the Efficiency of Antioxidants Extraction

Methods presented for extracting antioxidants from plant foods are often based on using water mixed with ethanol, methanol or acetone in different ratios because water increases the extraction efficiency to reach the optimal level. Another way to increase the efficiency of antioxidant extraction is using acidic solvents. Antioxidants are extracted from plant foods by a combination of at least 2 different solvents with different polarities, such as aqueous-organic solvents with different chemical structures like acidic methanol/water (50:50, v/v, pH=2) and then acetone/water (70:30, v/v). In commercial extraction of cocoa or red grapes, after the first extraction stage, a significant amount of antioxidant capacity remains in the samples which can be extracted in the second stage. Moreover, in the first stage of ferric reducing antioxidant power (FRAP) evaluation, by changing the solvent order about 78% more antioxidant capacity is extracted. The tests are done in the normal and nitrogen atmosphere in the capped centrifuge tubes. No significant difference is observed between the two treatments. The ratio of solid to solvent should also be considered because it increases the extraction efficiency of phenolic compounds to achieve an optimal level. An alternative for acidic hydrolysis to release hydrolysable phenolic in some foods such as cereals and nuts is alkali hydrolysis. However, this method should be associated with condensed tannins determination in dehydro-ascorbic acid -organic residues [15].

3. MAIN CHEMICAL REACTIONS OF ANTIOXIDANTS

3.1. Single Electron Transfer (SET) and Hydrogen Atom Transfer (HAT)

Antioxidants are capable of inactivating radicals with two major mechanisms, HAT and SET. Transfer of paired electrons with proton and HAT reactions may occur in parallel and therefore, the dominant mechanism in the system is determined according to structure, antioxidant properties, solubility, contribution coefficient and solvent.

Bond dissociation energy and ionization potential are two effective factors in determining the mechanism and efficiency of antioxidants.

Methods which are based on HAT mechanism measure the ability of antioxidants to disable free radicals by hydrogen donating.

$$(X^{\bullet} + AH \rightarrow XH + A^{\bullet})\ AH = \text{any H donor}$$

In HAT method, the relative reactivity is determined by bond dissociation energy of a H donor group with

usual amount of BDEΔ ~ 10 Kcal/ mol and the ionization potential <~ - 36 Kcal/mol. Reactivity assessments are based on kinetics competition. The HAT reaction is related to solvent and pH and it is usually completed in seconds to several minutes. The presence of reducing agents such as metals in HAT assessments is complex and apparently causes higher reaction ability.

SET based methods detect the ability of antioxidants to transfer single electron to reduce any compound, including metals, carbonyls and radicals.

$$X^{\bullet} + AH \rightarrow X^{-} + AH^{\bullet+} \quad (1)$$

$$AH^{\bullet+} \xrightarrow{H_2O} A^{\bullet} + H_3O^{+} \quad (2)$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O \quad (3)$$

$$M(III) + AH \rightarrow AH^{+} + M(III) \quad (4)$$

HAT and SET mechanisms in all samples often take place with a determined balance created by antioxidants structure and pH. Relative reactivity in SET methods is basically stabilized with deprotonation and ionization potential of an active functional group, and therefore SET reactions are pH dependent.

Generally, ionization potential values decrease with increasing pH and reflect more electron donation capacity by deprotonation. The SET mechanism is predominant in antioxidant compounds with Δ ionization potentials <-45 Kcal/mol. The correlation between the reduction potential and SET methods is proposed, but it is still unproven. SET reactions are usually slow and require long time to complete, so antioxidant capacity evaluations are mostly based on the percentage reduction in the product toward kinetic. When $AH^{\bullet+}$ half life is sufficient, secondary reactions cause major interferences in assessments and even lead to toxicity and mutations in living organisms. SET methods are very sensitive to ascorbic acid and uric acid which are very important ingredients in the maintenance of plasma and polyphenols reduction. Trace compounds and pollutants (particularly contaminant metals) specifically interfere with SET methods producing different results with poor reproducibility.

3.1.1. Methods Using HAT Mechanism

Among antioxidant evaluating methods, the oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), total oxidant scavenging capacity (TOSC), chemical luminescence (CL), β-carotene bleaching, low density lipids (LDL) oxidation.

3.1.2. Methods Using SET Mechanism

Ferric reducing antioxidant power (FRAP), total equivalent antioxidant capacity (TEAC), TRAP and copper reduction.

3.1.3. Methods Using HAT and SET Mechanisms Reactions

TEAC, 2,2 -Diphenyl-1-picrylhydrazyl (DPPH) and total phenolic method (FC) [16].

3.2. Principal Mechanisms Applied by Antioxidant Compounds

Reduction of metals such as iron and copper; Scavenging free radicals; Creating complexes with metal pro-oxidants (chelating); Quenching single oxygen; Stimulating anti-oxidative defense enzymatic activities [11].

4. FACTORS AFFECTING ANTIOXIDANT MECHANISMS AND CAPACITY

4.1. Type of Bioactive Compounds

4.1.1. Ascorbic Acid

Ascorbic acid, scurvy preventive agent, is the most abundant water-soluble antioxidant and a heat sensitive vitamin that can donate hydrogen atoms and form relatively stable ascorbyl free radicals with a half-life of about 10^{-5} sec [19]. The antioxidant effect of ascorbate is attributed to its capacity to remove reactive oxygen species (ROS) by reaction with superoxide radicals, hydrogen peroxide, hydroxyl radicals and single oxygen through the HAT mechanism. Also this compound removes RNS and prevents nitration reactions. Ascorbate is produced by reducing systems in organisms and as an antioxidant, it protects soluble cell components. Ascorbate contributes in reactions as a reducing agent that can be oxidized to dehydroascorbate. Biological studies of vitamin C have shown its protection role against DNA mutations [20]. However, ascorbic acid can act as a pro-oxidant in the presence of metals [10].

4.1.2. Phenolic Compounds

Polyphenols are the main plant components with antioxidant activity due to the reactivity of phenolic part (hydroxyl groups on aromatic rings) and it is believed that this property is basically related to their reducing properties through hydrogen or electron donating [12],

which play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen or peroxides destruction. Hydrogen donating properties of polyphenol compounds is responsible for preventing free radical production causing lipid peroxidation [21]. Phenolic compounds act as an antioxidant due to their ability in trapping superoxide anions. Generally, phenolic compounds in low concentrations show antioxidant behavior. In higher concentrations, they display pro-oxidant behavior and again by increasing the concentration they show antioxidant behavior. This situation always depends on the type (location and number of hydroxyl molecules) and the concentration of transition metal and phenolic compounds. In a study, the antioxidant capacity of limonene was determined in four ripen fruit tissues with β-carotene bleaching method. The capacity of the tissues and different genera differed from each other. The results of this study showed that citrus juices can effectively scavenge various reactive oxygen species or free radicals [22]. Polyphenolic flavonoid compounds with low molecular weight are found in all plant foods and based on the differences in the C ring, they are classified into different types, namely flavonols, flavones, isoflavones, flavonones, flavanols and anthocyanins. Quercetin, kaempferol and quercetagetin are flavonols while myricetin, naringin and naringenin belong to flavonones. For example, *in vitro* studies have demonstrated that proteins protect DNA against pro-oxidant activities of some flavonoids and polyphenolic compounds [23].

Flavonoids also chelate metal ions and therefore prevent them from participating in the production of free radicals. It is proposed that the antioxidant properties of phenolic compounds probably appear before they are absorbed in the alimentary tract.

Therefore, these compounds play an important role in protecting the gastrointestinal tract from oxidative damage and delay the progress of stomach, colon and rectum cancers. Protection ability is attributed to the scavenging of reactive nitrogen, chlorine and oxidative species produced by gastrointestinal tract or chemical compounds such as iron, ascorbate, heme proteins, lipid peroxides and nitrite in the stomach [24]. The most important group of polyphenols belongs to flavonoids, which show antioxidant ability because of their phenolic structure. Chalcones are a group of secondary plant metabolites which are natural precursors of flavones and dihydro flavones and several studies have shown that they have a very high antioxidant capability. Chalcones have many vital and medical activities such as are anti-inflammatory, anti-bacterial, anti-fungal and anti-cancer, that are attributed to their antioxidant properties as recipients of metal ions. These compounds reduce the intensity of oxidation reactions by different methods. The ability of blocking heavy metals plays an important role in the antioxidant ability of poly-phenolics, which can act as primary antioxidants. It means that they take part directly in the chain free radical reactions and prevent continuity. Poly-phenolic compounds are implicated in large number of biological effects including antibacterial, anti-inflammatory, antihistamine, protection against hepatitis, antiviral, anticancer, blood anti-coagulating and vessels protection. These performances are attributed to their antioxidant activity by various mechanisms, such as scavenging free radicals, reducing agents, pro-oxidant metal chelators, single oxygen quenchers and stimulating oxidative defense enzymatic activities. These mechanisms are conducted with two types of reactions; hydrogen atom transfer and single electron transfer [4]. Glycosides of phenolic compounds are usually weaker antioxidants than their aglycones. Additional hydroxyl groups with reducing capability can be released by hydrolysis [20]. Strong scavenging activities are attributed to free hydroxyl groups, but phenolic hydroxyl group in benzene ring participates in free radical scavenging activity to a great extent [21].

It was reported that protocatechuic acid stops double-stranded DNA separation by $OH^{\bullet}$ produced from Cu^{2+} reaction with H_2O_2. Chlorogenic acid can react with peroxyl radicals and inhibit the formation of $OH^{\bullet}$ by forming chelate with iron in the Fenton reaction. Researchers have shown that *p*-cumaric acid has an antioxidative effect against LDL due to chain breaking activities. Also a compound which has a pyrogallol nucleus is a significant chelator that can act as an antioxidant and reduce oxidative damages to biological molecules.

Researchers have found that polyphenols through their hydrogen donating capacity have antioxidant activity. In addition, metals chelating ability of polyphenols plays an important role in the protection against free radicals reaction produced by iron and copper and also inhibition of enzymes like lipoxidase [25]. The ability of phenolic compounds as antioxidant is related to the reducing properties of their phenolic hydroxyl groups which allow them to act as reducing agents, hydrogen donors and oxygen quencher antioxidants [11]. Anthocyanins are a group of plant phenolic compounds which show good antioxidant

properties. Anthocyanins at pH below 2 often exist in the form of flavyllium cations and so, absorb light at 510 nm, whereas decomposed anthocyanins in the polymeric form absorb light at pH below 2 and 4.5. Therefore in order to avoid interferences from absorbing materials, in the pH differential method, the difference in the absorbance at the 2 pH levels at the same wavelength is assessed [12].

4.1.3. Carotenoids

Carotenoids are fat-soluble antioxidants, which are widely distributed in nature and have absorbance at 470 nm. In the estimation of carotenoids, saponification with KOH-methanol is done in order to prevent fat interferences. Carotenoid estimation is affected by the presence of organic acids in tissues such as citric, oxalic and malic acids and thus addition of alkaline compounds to neutralize the acids is suggested.

In the absence of alkali, samples rich in polyphenols may interfere in the carotenoids estimation. It has been shown that KOH-methanol extraction may be a more suitable method for extraction and estimation of carotenoids in these samples [12]. Carotenoids especially β-carotene quenches single oxygen [11]. carotenoids are not chain breaking antioxidants and their ability to remove single oxygen is proposed to be chemically by different reaction mechanisms [23]. Carotenoids as ROS particularly as single oxygen and peroxyl radical quenchers are efficient. Gene regulation is one of the most apparent mechanisms in some pathogenic processes and it is influenced by carotenoids. Carotenoids are relatively resistant to thermal decomposition, while total phenolic and ascorbic acid contents are significantly sensitive to heat treatment [26]. Carotenoids are effective protecting agents against oxidative damage and single oxygen quenchers [18]. Carotenoids, phenolic compounds and other antioxidants are good peroxyl radical quenchers, but carotenoids have unique antioxidant ability in single oxygen quenching which other compounds are ineffective. Single oxygen is not a radical and it does not act through radical mechanisms, butit often forms endo-peroxides by addition to to double bonds which can be reduced to alkoxyl radicals and initiate chain radical reactions [16].

4.1.4. Minerals

In human nutrition, selenium, copper, zinc, iron and manganese are essential minerals for optimizing response of antioxidant enzymes. Groups that are present in heme proteins are similar to antioxidant enzymes of catalase, peroxidase and superoxide dismutase.

Superoxide dismutase decomposes superoxide radicals ($O_2^{\bullet -}$) to H_2O_2 and oxygen molecules, then catalase decomposes toxic H_2O_2 to water and prevents the formation of secondary hydroxyl radical. Negative effects of toxic H_2O_2 consist of affecting penetration of cell membranes, slow oxidation of some compounds, phagocytes destruction, and invasion of energy production systems by disabling glycolytic enzyme of glyceraldehyde-3- phosphate dehydrogenase and finally causing cell death.

H_2O_2 Degradation Reaction

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$$

Zinc participates in setting up the plant oxidative reactions through indirect reduction of iron absorption and translocation. Zinc enhances lipoxygenase as the most important peroxidation pathway enzyme and thereby stimulates lipid peroxidation. Generally, there is a balance between the amount of produced free radical and their scavenging rate from cell surface which maintains a plant in a relatively stable state. Oxidative stress appears when the amount of radicals in cell exceeds antioxidants compounds content. One of the most important plants defense systems for controlling and neutralizing free radicals is the induced synthesis of some plant antioxidants such as anthocyanins, carotenoids, antioxidant enzymes and phenolic composites like flavonoids. These compounds react with free radicals and convert them to their stable forms by donating electrons. Flavonoids and anthocyanins are important antioxidant compounds which destroy free radicals and prevent their expansion. It is probable that anthocyanins facilitate entrance of heavy metals into cell vacuoles and collect them from other sections. Research findings have shown that the amount of anthocyanins increases with increasing zinc levels and these compounds prevent membrane lipid peroxidation as a plant protective system against oxidative stress [27].

4.1.5. Vitamin E (α-Tocopherol)

Vitamin E is the best single oxygen quencher which acts as a chain breaking antioxidant [11].

4.1.6. Other Compounds

Antioxidant activities of glutathione and uric acid are exerted by scavenging free radicals and antioxidant abilities of isothiocyanates (including anti-nutritive compounds) inactivate single oxygen [28].

4.2. Processing Methods

4.2.1. Milling

Milling is another important factor affecting the antioxidant capacity. Samples heating and milling time in an oxidizing atmosphere during milling process should be kept at minimum. Milling should be done in a centrifugal mill, but hammer or shear mills should not be used because even under nitrogen atmosphere, a considerable loss is created in the antioxidant capacity. Particle size reduction increases the antioxidant capacity because it breaks the structure of food specific matrix and releases complexed antioxidants, reduces the traveled distance by the analyte to reach the surface and increases the particle's surface area resulting in the increased solvent penetration. However, it should be noted that particle size reduction also reduces the thermal stability and, and therefore the antioxidant capacity should be determined as closely as possible to the milling step to prevent decomposition of antioxidant compounds [23].

4.2.2. Blanching

Blanching is a thermal process of vegetables and fruits to a temperature enough to stop enzymatic activity and consequently stabilizing color, and reducing drying time and water losses without destroying the texture. Local people use acidic compounds to reduce or eliminate vegetable bitterness.

Several studies have shown that blanching improves the natural antioxidants compatibility and bioavailability of vegetables. In addition, blanching may cause some changes in physical properties and chemical composition of vegetables. In 2005, Oboh showed that blanching has significant effects on ascorbic acid content, total phenolic contents and antioxidant activity in green leafy vegetables. Recent studies have shown that blanching reduces antioxidant capacity in foods. Significant loss percentages (23-51%) in ascorbic acid content were observed in selected vegetables after blanching compared with crude ones. Ascorbic acid is a heat sensitive vitamin and numerous studies have shown that ascorbic acid is lost during heat treatment and blanching of vegetables. Also researchers have reported vitamin C decomposition by aerobic and anaerobic pathways. In addition, because ascorbic acid is soluble in water, it can be reduced considerably by blanching in boiling water. Hart and Scott (1995) indicated that blanching increases carotenoids and thus contribute to the reduction of β-carotene. Southon and Faulks (2001) acknowledged that carotenoids in plants are naturally linked to proteins in photosynthetic and physical structures.

Blanching breaks the natural link among vegetables components thus increasing the bioavailability of carotenoids. Due to the presence of conjugated double bonds in β-carotene, thermal processing can convert all natural *trans* β-carotene into *cis* form, increasing the amounts of β-carotene. It has been showed that the ethanolic extract of all blanched and raw vegetables has more antioxidant activity than the aqueous one. In this case blanching process shows different effects in different vegetables. Generally, phenolics in fruits and vegetables are connected to dietary fibers, proteins and sugars and form complex structures. Thermal processes break cell membranes and cause hydrolysis of broken links that have greater access to them. Conversely, a few researches have showed that phenolic compounds are heat sensitive so that thermal processes leach them into the boiling water in a few minutes. Processing due to improved antioxidant properties of natural compounds or formation of new compounds such as Maillard reaction products enhances antioxidant ability. In addition, thermal treatments can deform flavonoids and glycosides and form aglycones with higher antioxidant properties. Vegetable blanching does not necessarily reduce antioxidant properties. In some vegetables, blanching actually enhances the accessibility of antioxidant compounds and improves their natural compatibility. Therefore, suitable blanching conditions (time and temperature) are important for the maintenance of antioxidant properties [29]. Howard *et al*. (1999) reported that antioxidant activity loss in cooked tissues during blanching is due to a large vegetable surface in contact with water. Increasing the boiling time from 5 to 30 min significantly reduced the amount of antioxidants in the sample indicating antioxidants leaching into water. Interestingly, total radical scavenging activity (RSA) increased in green pepper tissues after boiling in water for 5 and 30 minutes. This phenomenon is not seen in other types of peppers. Yamaguchi *et al*. (2001) attributed the RSA enhancement in some vegetables after boiling to the prevention of oxidation by antioxidants due to thermal inactivation of oxidative enzymes. In addition, destruction of cell walls and substructures during boiling of vegetables causes the liberation of radical scavenger antioxidants. Gahler *et al.* (2003) attributed the improvement in the antioxidant activity of tomatoes upon heat treatment to the increased release of the phytochemicals like lycopene. Puupponen-pi-mia *et al.* (2003) reported that the $DPPH^{\bullet}$ index in water dropped to 23% and increased to

9% during blanching in cauliflower and cabbage, respectively [30].

4.2.3. Drying

Drying time and temperature as well as blanching significantly affect bioactive compounds losses. Drying conditions play an important role in determining the final product quality and maintenance of its antioxidant properties [31]. Reviews of antioxidant capacity of plant nutrients are done in dry powder. The antioxidant capacity of samples may be preserved or greatly destroyed depending on the drying method. Drying at high temperatures and long times reduces the antioxidant capacity. The situation is seen in orange byproducts or various tomato varieties exposed to different air drying conditions. However, the loss of antioxidant capacity by freeze drying is minimal. If this is not possible, the best option depending on the sample is vacuum drying at temperatures below 50-60 °C. Some researchers have observed an increase in the antioxidant capacity after certain drying processes due to the formation of new antioxidant compounds (Maillard compounds and polyphenols polymeric structures with more antioxidant capacity). This will be useful in optimization of these treatments to improve the antioxidant capacity of processed foods with high antioxidant. However, in raw food analysis, drying conditions should prevent the production of such new compounds because they don't exist in consumed foods [15].

4.2.4. Cooking

4.2.4.1. Effect of Cooking Methods on Radical Scavenging Activity (RSA)

RSA reduction in raw and cooked samples with different methods of microwave and frying did not show significant differences but after five minutes of boiling it decreased to less than 77% of its original value.

4.2.4.2. Effect of Cooking Methods on Total Phenolic Contents (TPC)

A positive correlation (R = 0.929) existed between TP and RSA in cooked peppers; the higher TP means the higher RSA. The results showed that after cooking, TP reduces in all samples but the reductions between the raw and, microwaved and fried samples are not significant. This can be probably attributed to polyphenoloxidase enzyme deactivation during heating which prevents polyphenols decomposition. Similar to RSA, the TP content in all samples of cooked tissues dropped significantly after 5 min and also showed a greater reduction after 30 min. Polyphenols decomposition depends largely on the processing time and vegetable size.

4.2.4.3. Effect of Cooking Methods on Ascorbic Acid Content (AsA)

Dehydro ascorbic acid does not have scavenging activity against $DPPH^{\bullet}$ or hydroxyl radicals, so only AsA is assessed. AsA losses during thermal treatment occurs by accelerating ascorbic acid oxidation to dehydro ascorbic acid then hydrolysis to 2,3-di-ketogluconic acid and eventually polymerization to other non-nutritive compounds [32]. Generally, microwave heating and frying do not affect RSA, TPC, AsA and total carotenoids content that are partially decompose during boiling in water. Antioxidant activity losses in cooked tissues with longer boiling time is greater.

These findings indicate that microwave cooking and frying is a better method to maintain bioactive compounds. When boiling in water is unavoidable, cooking time and the amount of water should be reduced to protect bioactive ingredients. Boiling water consumption is recommended because it contains bioactive compounds [30].

4.2.5. Freezing

4.2.5.1. Ascorbic Acid Content

Comparison the mean levels of ascorbic acid at different storage temperatures showed 64.5%, 10.7% and 8.9% reduction in ascorbic acid at -12, -18 and -24 °C, respectively. The main loss in ascorbic acid (31.4%) occurred at -12 °C after 15 days. These results confirm the findings of Ibenez *et al.* (1996), which stated that the severe reduction in ascorbic acid happens during the first 15 days of storage. The most probable reason for this phenomenon is the concentration of solutes in non-frozen phase during freezing process that causes an increase in the solute contents.

Ascorbic acid did not show significant differences at any temperature between two slow and quick freezing methods; although at -12 °C ascorbic acid reduction was significant. These results were in agreement with Ibanez *et al*. (1996), Klimezka and Irzyniec (1997) and Ulrich (1978), which reported ascorbic acid tendency to reduce fast at temperatures above -18 °C. Although the results were contradictory to Ibanez *et al.*, (1996), which reported that slow freezing method causes a greater reduction of ascorbic acid than quick freezing which probably is because of different varieties used in various experiments.

4.2.5.2. Anthocyanins Content

Comparison of the mean amounts of anthocyanin at -12 °C did not show any significant difference during the first 15 days of storage. The reduction rate was 40.2% for the whole period of storage which is less than the findings of Torreggiani (1999) that showed 65% reduction in tomato juice anthocyanins at -10 °C. It seems that differences may be due to different storage temperatures. A significant reduction in the mean amount of anthocyanin (34.3%) was observed at-18 °C between the first and 15th days of storage. Also at -24 °C, a significant reduction (up to 17.6%) was found only in the first 15 storage days which is in accordance with Polesello and Rampilli (1972). Anthocyanin contents showed significant differences at all temperatures and between two slow and quick freezing methods. This result was in agreement with Urbany and Horti (1992) that reported colorant foods changes in quick freezing. This change is probably due to anthocyanin pigment replacement in quick freezing. Effects of freezing methods on pH showed significant difference only at -18 °C. The separate effects of storage temperature, storage time and freezing method on the strawberry properties were significant.

4.2.5.3. Acidity and pH

Results for acidity demonstrated significant differences at all levels. Sample acidity showed a significant decrease (5%) at -12 °C at any time, but it was stable during the whole storage period at -24 °C. The average pH showed significant differences (5%) at day 15, second and third months. At -12 °C in the end of storage period pH was above 3.4 and this is one of the reasons of anthocyanin reduction at this temperature. At the end of the storage period, pH was above 3.4 and this could be one of the min reasons for anthocyanins reduction. Several factors including storage time, enzymatic and microbial changes affect the concentration of hydrogen ion, so pH is important in evaluating the efficacy and quality of a method [15].

Sensory evaluation showed that the effect of freezing method on the desirability of frozen strawberry after storage (excluding tissue) was not important, while at -12 °C its desirability and quality reduced. Also samples stored at -18 and -24 °C had the highest quality in terms of color, texture, and flavor, but these characteristics in samples stored at -12 °C were not acceptable [33].

An extensive study was conducted about the effects of blanching/freezing and prolonged freezing on bioactive compounds in more than 20 different common vegetables. The results showed that the effects are strongly dependent on the plant species, while the dietary fiber content does not affect very much or it slightly increases. The minerals were stable in general, but losses of some soluble minerals through leaching into the water were observed. The results also showed that phenolic antioxidants and vitamins are more sensitive. Significant losses in the antioxidant activity and total phenols (20-30%) have been identified, for example it is shown that vitamin C content reduces in many vegetables up to one third and a little higher than its primary concentration during blanching and storage, respectively. Folic acid was very sensitive to blanching and more than half of it lost, but it was stable during freezing. Carotenoids and sterols did not affect by blanching or freezing processes [34]. In a study, ellagic acid, total phenol and vitamin C contents in a freshly frozen fruit, stored at -20 °C for a year of four raspberry varieties (Heritage, Autumn Bliss, Rubi and Zeva) in Spain were assessed using HPLC. The concentrations of ellagic acid 207-244 mg/Kg (fresh weight), total phenols, 137-1776 mg/Kg (fw) and vitamin C 221-312mg/Kg (fw) in two varieties of Rubi and Zeva were more than Autumn Bliss and Heritage. The amount of extracted ellagic acid, total phenol and vitamin C were slightly affected by the freezing process. At the end of the long-term freezing (12 months), no significant changes were observed in the total phenol content, but significant decreases were observed in ellagic acid (21-14%) and vitamin C (55-33%). The measured free radical scavenging capacity as antiradical performance depended on the season of harvest. The late varieties Rubi ($6.1*10^{-4}$) and Zeva ($10.17*10^{-4}$) showed more radical activity than the early types Heritage ($4.02*10^{-4}$) and Autumn Bliss ($4.63*10^{-4}$). Freezing process reduced antiradical amounts between 4 to 26% in four varieties, but antiradical activities remained unchanged after freezing process [35]. In this research, the correlation between free radical scavenging capacity and bioactive compounds (anthocyanins, ellagic acid, total phenol and vitamin C) in four Spanish raspberry varieties (Heritage, Autumn Bliss, Rubi and Zeva) and Spanish wild blackberry were evaluated on the samples during freezing and after freeze storage. Significant correlations were found between the free radical scavenging capacity and anthocyanin content and total phenols in the raspberry (r=0.85 and 0.83) and blackberry (r=0.84 and 0.68), but there was not a correlation between this factor and ellagic acid and vitamin C content. The key objective of this study was to select the most proper raspberry varieties for freezing regarding the stability of health-promoting

ingredients [36]. In a study, the effects of radiation, storage time and freeze drying on Rio Red grapefruit bioactive compounds were investigated. The grapefruit was exposed to 2 radiation dosages (control) and 300 Gy (^{137}Cs) and then stored up to 6 days. Finally, the pulp of irradiated and control grapefruit samples at the end of storage period was freeze dried and the bioactive and volatile compounds were extracted and analyzed by reverse-phase chromatography and gas chromatography, respectively. Frozen dried irradiated fruit pulp had greater flavonoid content (naringin and narirutin) in comparison with the control samples. Freeze drying reduced lycopene content, but β-carotene content decreased only in the controls. A decrease in d-limonene and myrecene contents in the irradiated fruits was observed six days after harvest [37].

4.2.5. Some other Methods

Tea activity properties increase significantly after roasting process. Nicoli *et al.* (1997) found a similar result about brown coffee with strong antioxidant properties. This phenomenon is often attributed to Maillard reaction products formed during roasting. Guillot *et al.* (1996) showed that there is a positive correlation between the degree of roasting in coffee and inhibition of lipid peroxidation in liver cell membranes of rats and the thermal process is responsible for the production of new antioxidants [18]. For example, it is shown that in the preparation of tomato puree, homogenization does not have an effect on tomato puree but changes were observed after pasteurization for 40 seconds at 98 °C. More folate in the homogenized and pasteurized samples was found due to more extraction from sub cell sections. Increasing in the pasteurization temperature up to 128 °C reduced the amount of ascorbic acid, total phenolic compounds and folate. Homogenization and pasteurization at 98 °C for 40 seconds increased the nutritional value and extractable folate of tomato puree and maintained its carotenoids. However, pasteurization temperatures above 98 °C were not recommended because of vitamin C and folate decomposition [26].

5. SOLVENTS, SEPARATED FRACTIONS AND INTERFERENCES EFFECTS

Extraction efficiency and activity are affected by solvent polarity. Water, methanol, ethanol, acetone, aqueous solvents and ethyl acetate are common solvents used for extraction. High enzymatic activity should be considered when the recovery of certain complex phytochemical compounds from plant byproducts is aimed. However, drying plant byproducts before extraction, immediate immersion in methanol and use of acidic conditions prevent materials from oxidation. Water pH determines the water solubility of compounds and affects hydrolysable fraction dissolution [38]. High temperatures improve the extraction efficiency through enhancing permeability of analytes and dissolution in solvents, but at high temperatures, bioactive compounds with antioxidant activity interact with other plant compounds preventing the extraction process. In addition, extraction time affects the stability of phenolic compounds through chemical and enzymatic decomposition, volatility losses or thermal decomposition. For example, despite the high antioxidant activity of banana peel extract, it generally acts as an antiradical factor rather than a lipid peroxidation inhibitor. Extracts prepared using different solvents and extraction conditions show different antioxidant activities suggesting that antioxidants or active compounds with different polarity are present in these extracts. Efficient extraction of antioxidants requires solvents in different polarities: polar antioxidants need polar solvents such as methanol, while ethyl acetate or chloroform are used for the extraction of hydrophobic antioxidants. Another way to increase the antioxidant extraction efficiency is using acidic solvents [15].

5.1. Interfering Substances

Ascorbic acid and sugars (mono and disaccharides) are major groups of interfering substances in plant extracts. Ascorbic acid spectacularly reacts with the FC reagent and measured amounts are almost equal to half of real gallic acid values. Measuring ascorbic acid because of its rapid reduction to dehydro ascorbic acid is complex. Dehydro ascorbic acid reacts with several compounds quickly. Researchers have found that the concentration of an ascorbic acid solution (similar to the concentration used for the extraction of phenolic compounds) at laboratory temperatures decreases about 70% per day and after several days at -20°C, very small amount of ascorbic acid maintains. Therefore the amount of ascorbic acid should be measured at the closest possible time to phenolic compounds assessment. Glucose, fructose and sucrose are main sugars in fruits and vegetables and they interfere with the methods for measuring phenolic compounds and antioxidant activities except for DPPH method, which requires hydrogen radical donation. Molar absorption coefficients of individual methods and sugars express their reactivity. However, in several

kinds of fruits with a relatively high concentration of sugars especially for the FCM method the greatest added value is 5%, whereas in very sweet fruits such as grapes, cherries, bananas, and peaches the amount of released phenolic compounds increase over 50%. In an alkaline environment, endiol reduction agents easily reduce compounds formed by polysaccharides. Ascorbate is a reducing factor too. Folin−Ciocalteu method (FCM) and acetoacetoxypropyl methacrylate (AAPM) reactions take place in an alkaline environment at pH= 10. Purines such as guanine (not guanosine), uric acid and xanthin react in the similar molar efficiency to mono-phenols. Adenine and pyrimidine react very little.

Proteins show a significant positive reactivity to tyrosine and tryptophan. Cysteine and glutathione produce almost half the molar absorption of mono-phenols. Carotenoids interfere in some methods, but their contents are usually low in fruits [20]. There are some interfering aspects in antioxidant capacity determination: first of all, the solvent is a key factor because the solvent polarity can affect the reaction mechanism. These aspects have been investigated in various foods or standards such as wine in ORAC, quercetin in DPPH, diet polyphenols in FRAP and wheat bran in ABTS. Another important factor is the presence of certain non-antioxidant compounds that take part in reactions and may show antioxidant capacity more than the actual values. For example, several amino acids are possible positive error sources in antioxidant capacity assessments. The same results have been observed in plasma evaluation by ORAC method, so that the values for complete plasma are much greater than those for deproteinized plasma. So, the protein content of samples should be taken into account when the results are analyzed. For total antioxidant capacity determination in vegetable oils, extraction is not necessary and analysis of samples can be done directly after diluting with normal hexane or ethyl acetate if required. Also, antioxidant capacity determination of polar and nonpolar compounds is separately possible and methanol extraction is essential. Oil antioxidants according to their distribution coefficients are present in polar and nonpolar sections. In oil tests, methanolic fraction may interfere with DPPH. After methanolic extraction, the compounds remaining in the sample are nonpolar since methanol extracts polar compounds as well as those with moderate polarity. Interestingly, the antioxidant capacity of the polar section (methanolic extracts) is larger than the whole oil (EC_{50} less), which could be related to interactions with lipid materials available in the whole oil that do not exist in methanolic extracts. Antagonistic effects, for example, are observed between quercetin and α-tocopherol in sunflower oil. It could also be attributed to polar paradox in which the hydrophobic antioxidants are more effective in polar conditions like methanol. Therefore, in order to compare the antioxidant capacity of oils, analysis should be performed in a similar extraction environment and evaluation method. In samples with high fat content such as nuts, fat may interfere with antioxidant capacity determination and thus, they should be defatted at room temperature before analysis. For example, a 0.5 g ground sample was placed in test tubes and 20 mL petroleum ether was added and after 20 min shaking and centrifugation for 10 min at 2500 g, the supernatant was collected. Then antioxidant capacity was separately measured in the oil and defatted materials containing extractable and non-extractable antioxidants. The antioxidant capacity of residues (related to hydrolysable tannins) was more than water-aqueous extracts (less EC_{50}). The results showed that oil contribution to total antioxidant capacity is much less than that of defatted section. In usual diet beverages, hydrophilic sections have greater contribution than hydrophobic ones in total antioxidant capacity. The bioavailability of ntioxidant capacity of beverages is more than solid foods because they do not require enzymatic action for antioxidants release [15].

6. PRACTICAL METHODS FOR ASSESSING ANTIOXIDANTS IN LABORATARY

6.1. Determination of Total Phenolic Contents

Total reducing capacity is measured by Folin-Ciocalteu method (FCM) which is well related to the reducing and antioxidant abilities of phenolic compounds. Proton dissociation of phenolic compounds creates phenolate anion, which reduces Folin-Ciocalteu reagent (FCR). FCM is not dedicated to phenolic compounds. Many none phenolic compounds in fruits, such as ascorbic acid and sugars can reduce reagent. Ascorbic acid reacts with the FCR considerably and the results are almost half of gallic acid values. Therefore, an increase in the determined phenolic compounds is seen in fruits with high ascorbic acid content. Ascorbic acid determination is complex, because of its rapid conversion to de hydro-ascorbic acid.

At first, FCR was used in proteins measurement because its active substance tends to react with tyrosine (containing phenolic groups). After many years

scientists applied this method to assess the total phenolic content of wine, and at present it has found many applications. This test measures the reducing capacity. Antioxidant capacity tests based on SET confirm a linear relationship between phenolic content and antioxidant activity. FCR is usually conducted by boiling a mixture of 100 g sodium tungstate (Na_2WO_4, $2H_2O$), 25 g sodium molybdate (Na_2MO_4, $2H_2O$), 100 mL concentrated HCl, 50 mL phosphoric acid 85% and 700 mL water. After boiling, 150 g lithium sulfate (Li_2SO_4, $4H_2O$) is added to the mixture and finally yellow FCR forms. Contamination with reducing substances changes its color from yellow to green, which by adding an oxidizer like bermine its color return to yellow. This unknown phenomenon is probably formed as a result of one or two electrons donated by the blue solution of $(Pmo\ W_{11}O_{40})^{4-}$. It is proposed that molybdenum reduces easily in complex and electron transmission reactions are done between reducing and molybdenum:

$$Mo\ (VI) + e \rightarrow Mo(v)$$

FCR substance is nonspecific for phenolic compounds and it may also react with non-phenolic compounds such as vitamin C and Cu (I). Phenolic compounds only in alkaline conditions (sodium carbonate solution at pH = 10) react with FCR [39].

Total polyphenols were determined in supernatant by FCM through mixing 1 mL of supernatant with 1 mL of Folin-Ciocalteu reagent (FCR 1:3), 2 mL 20% sodium carbonate solution and 2 mL distilled water and finally, after an hour the absorbance at 700 nm was measured by spectrophotometer. The results are expressed as gallic acid equivalent per 100 g [19]. Total phenolic compounds extraction was assessed based on Naczk *et al.* (1992) method with some modifications. One gram samples of dry plant material with 20 mL acetone-methanol-water (7:7:6 v / v / v) were extracted three times at room temperature. The extracts were centrifuged at 6000 rpm for 10 min and then, the collected supernatants were centrifuged according to the slightly modified method of Hammer Schmidt and Pratt (1978) and were analyzed to determine total phenols. One mL test solution was combined with 10 mL deionized water and 2 mL Folin-Denis reagent. After 5 min, 2 mL saturated sodium carbonate solution was added and incubated for an hour at room temperature. Absorption was measured at 640 nm wavelength. Pyrochatecol solution was used as the standard for determining the total phenolic content [14].

6.2. Determination of Flavonoids Content

Flavonoids in food systems act as free radical scavengers and terminate chain radical reactions during triglyceride oxidation and they exert various biological effects such as antioxidant, anti-inflammatory and protect vascular actions. This complex of natural ingredients has been studied in fruit and vegetable carefully, but less attention has been paid to their presence in whole grains and nuts [25]. In a variety of citrus, flavonoids were identified as methoxylated flavones and flavone glycoside and were used as the natural indicator of commercial fruit juices. Recent studies have shown anticancer effects of citrus pectin, pulp, limonine and naringine against colon cancer in living systems. Citrus flavonoids like apigenin, naringenine, hespridine and nubiltineare were tested for their effects on retardation of colorectal cancer. The results showed that hespridine and nubiltine have greater anticancer effects [10]. Flavonoid content was measured using colorimetric method provided by Lenucci *et al*. (2006) with some modifications. Distilled water was added to 50 µL methanol extract/water to final volume 0.5 mL and then, 30 µL $NaNO_2$ 5% is added. After 5 min, 60 µL 10% $AlCl_3$ solution and subsequently after 6 min 200 µL NaOH, 1 M and 210 µL distilled water was added to the mixture. Absorbance was measured at 510 nm and the flavonoid content was expressed as equivalent mg catechine per gram of fresh material [22]. A good correlation was observed between flavonoid liocasine content and antioxidant activity [20]. Flavonoids are poly-phenolic compounds found in all plant food stuff and based on their C ring structures are categorized into different types of flavonol, flavone, isoflavones, flavonone and anthocyanins. Quercetin, kaempferol and quersetagenine are flavonol, whilst myresetin, naringine and naringenin are flavanone. Phenolic compounds are the main compounds responsible for the color and astringency of red wine. Flavan-3-ol, catechin, epicatechin, epicatechingallate, epigallocatechine-3-gallate and gallocatechine are the major groups of proanthocyanidin components. Diadzein and genistein are the primary known isoflavones and phytoestrogens [23].

Flavonoids can also chelate metal ions and thus prevent their participation in the production of free radical reactions. Health promotion properties of flavonoids based on their antioxidant activity has recently been challenged because flavonoid metabolites tend to reduce antioxidant activity. However it is proposed that the probable antioxidant

protection by phenolic compounds takes place before they can be absorbed into alimentary tract. Therefore, these compounds play an important role in protecting the gastrointestinal tract from oxidative damages and delay the progress of stomach, colon and rectum cancers. The protection ability is attributed to the scavenging of active nitrogen, chlorine and oxidative species produced by gastrointestinal tract or chemical reactions of compounds in the diet such as iron, ascorbate, heme proteins, lipid peroxides and nitrite in the stomach. Three different structural forms are responsible for flavonoids scavenging effects. First structure is orthodihydroxy or catechol group in B ring (3', 4'-OH) which stabilizes formed radicals. In the second structure, ring B is connected with 4-oxo group through C2-C3 double bond and in the third form, 3 - and 5-OH groups are linked with 4-oxo group. When the B ring has a group with 3 OH (3 ', 4', 5'-OH), the central OH link is the weakest bond because two other OH groups can form two hydrogen bonds with flavonoid radicals. Myricetin flavones based on the mentioned structural forms are efficient antioxidant flavonoids. Quersetagetin is a flavonoid with a structure similar to quercetin (with an additional 6-OH group). 6-methoxy luteolin only has 2 of the 3 mentioned structural forms (B ring orthocatechol and B ring conjugated with 40-oxo groups) and ano-methylated group on the A ring which does not affect antioxidant activity. Astragallin and narirotin have only one OH group linked to B ring and show less antioxidant activity than compounds with catechol group in the B ring. Quinovic acid and its glycosides also have antioxidant activity in plant extracts. Syringic acid is also a phenolic compound with mildantioxidant activity through linked OH group to the benzene ring [24]. Several *in vitro* methods have been applied for qualitative and quantitative determination of flavonoids such as HPLC with diode arrays detector and mass spectrometry [40]. Several researches have considered antioxidant activities of flavonoids compounds and the role of structural features participating in this activity. O-dihydroxy groups in B ring, the presence of double bond between carbon 2 and 3 in the conjugate mode with 4 - oxoin C ring and hydroxy groups 3, 5 and 4 - oxoin A and C rings are related with antioxidant activity. Phenolic acids such as cafeic, chologernic, ferolic, cinapic and *p*-qumaric acids are more active antioxidants than hydroxyl benzoic acid derivatives such as *p*-hydroxy benzoic acid, vanillic and syringic acids [41].

6.3. Determination of Flavonols Content

Flavonol content is determined as follows: 1 mL extract is mixed with 1 mL $AlCl_3$ (2 mg/mL) and 6 mL sodium acetate (50 mg/mL) and then, the absorbance is recorded at 440 nm wavelength after 2.5 h. Flavonol content is expressed as equivalents mg quercetin per gram of fresh material [22].

6.4. Determination of Ascorbic Acid Content

Humans have a variety of scavenger antioxidants including reduced glutathione (GSH), uric acid, α-tocopherol and ascorbic acid [23]. Biological studies have shown the protective role of ascorbic acid against DNA mutations [11].

Analysis of ascorbic acid in vegetables was carried out according to the Abushita *et al.* (1997) method. About 10 g chopped fruit was homogenized with 20 mL extract solution containing meta-phosphoric acid 0.3 M and acetic acid 1.4 M. The mixture was poured into a conical covered flask and mixed at room temperature for 15 min at 200 rpm with an orbital shaker. After 15 min, the mixture is filtered through Whatman No. 4 filter paper to obtain a clear extract. 2 mL of the collected supernatant was filtered through 0.45 µm membrane and immediately 20 µl of the filtrate was used for HPLC testing. Chromatographic conditions were as follows: C_{18} ultrasphere octadecylsilyl (ODS) hypersil column (250×4.6 mm, 5 µm particle size, Thermo scientific, Waltham, MA) equipped with a HPLC separation module (1100 HPLC series, Agilent Technologies, USA). Potassium acetate 0.1 M containing acetonitrile-water (50:50) at pH 4.9 with 1.5 mL/min flow rate as mobile phase, column temperature was maintained at 27°C and detection was performed at 254 nm wavelength [29]. In the calibration of the method ascorbic acid was used as a standard to assess extracts antioxidant activity [38]. Ascorbic acid content in plants was determined spectrophotometrically according to the Bajaj and Kaur method (1981). About 0.5 g plant material was extracted with 10 mL oxalic acid-EDTA solution for an overnight at room temperature. The extract was filtered through a filter paper and 2.5 mL of the extract was transferred to a 25 mL volumetric flask. Then reagents were added (2.5 mL oxalic acid–EDTA solution, 0.5 mL metaphosphoric acid-acetic acid, 0.1 mL sulfuric acid and 2 mL ammonium molybdate reagents) and finally, the volume was adjusted to 25 mL with distilled water. Molybdenum forms a blue complex with ammonium molybdate reduced by ascorbic acid and this reaction controls with measuring the absorption at 760 nm after a minute. Ascorbic acid content is determined using the calibration curve obtained from standard solutions [11].

6.5. Determination of Vitamin E Contents

0.5 g dried plant material is soaked in 20 mL ethanol for 30 min in a water bath at 85 °C. The chilled solution is filtered with a separator funnel, 10 mL heptane is added and the solution is shaken for 5 min. Then 20 mL sodium sulfate 1.25% is added and the solution is shaken for 2 min and allowed to form separated layers. Total tocopherols are determined based on the Contreras-Guzman and Strong method (1982) through reaction with copper ions and preformation of complex with 2, '2 – biquinoline (Cu II). Similarly, 0.5 mL α-tocopherol in ethanol is processed as sample and used as standard [11].

6.6. Determination of Carotenoids Content

Carotenoids are a group of natural pigments in fruits and vegetables responsible for colors that are abundant in peppers. Carotenoids present in peppers are often pro vitamin A (α-carotene, β-carotene and β-crypto xanthine) and xanthophils (oxygenated carotenoids) [30]. 0.5 g dried plant material is soaked in 30 mL hexane at room temperature, then it is extracted according to the Helrich method (1990) through warm saponification. Isolation of carotenes and xantophils from dried samples is performed using column chromatography. Hyflo Super-Cel as the stationary phase and hexane-acetone (9:1 v/v) and hexane-acetone-methanol (8:1:1 v/v/v) solutions as the mobile phase are used to separate carotenes and xanthophils. Carotene and xanthophil contents are determined spectrophotometrically at 436 and 474 nm wavelengths, respectively [11]. Carotenes in vegetables are extracted according to the Tee *et al.* (1997) method. About 10 g of chopped vegetables are mixed with 40 mL ethanol, 10 mL 100% potassium hydroxide and homogenized by mixing for 3 min. The mixture is saponified with the refluxing apparatus and heated for 30 min. Then the mixture is cooled to room temperature and shaken to prevent clumping. For extraction, the mixture is transferred into separating funnel and 50 mL normal hexane is added. The funnel is returned and it is discharged into the air and then shaken vigorously for a few seconds and layers are allowed to separate. The upper layer (hexane extract) is discarded and the aqueous layer is extracted again with 50 mL normal hexane for several times. The upper layers are collected and washed with distilled water in order to remove alkaline. 1% phenol phetalein solution is used as an indicator, which its color in the presence of alkali changes to pink. Then the water in the extract is removed by anhydrous sodium sulfate. The hexane residues are removed under vacuum at 45 °C using a rotary evaporator. The extract is diluted with hexane to volume of 10 mL and next, 2 mL of the concentrated extract is evaporated under nitrogen, re-dissolved in 2 mL acetone, passed through 0.45 µm membranes and finally 20 mL aliquots are injected to HPLC. A mixture of acetonitrile, methanol and ethyl acetate (88:10: 2 v/v/v) is used as mobile phase. Flow rate is 1 mL/min and detection wavelength is fixed at 450 nm [29].

6.7. Determination of Peroxide Value (PV)

Lipid peroxidation in fresh and processed foods is one of the main reasons of spoilage, reduced stability, off-flavor compounds, and undesirable quality and appearance. The radical species formed in the peroxidation process decompose fatty acids and other fatty compounds such as carotenoids, chlorophyll pigments and tocopherols [42, 43]. Generally, more unsaturated oils are more susceptible to oxidation [42]. In the presence of natural antioxidants, peroxidation is delayed and therefore these compounds are usually added to reduce the undesirable changes during processing and storage of fats and oils. Peroxide value is widely used as a criterion for unwanted reactions in food and the extent of peroxidation in biological samples [43]. When peroxide reaches a certain amount, various changes occur and volatile aldehydes and ketones are performed which create an unpleasant odor and taste in fatty substances. Peroxide value is expressed as mili-equivalent in 1000 g oil. Peroxide value cannot reflect the real antioxidant effects of phenolic compounds. Peroxide value does not increase continuously, but increases for a while to a certain level and then declines and creates by-products. Therefore to consider antioxidant effects, the oven test, peroxide value and thio barbitoric acid (TBA) should be examined [42, 71, 73, 75-77, 79-80, 82, 87].

These reactions have complex effects on physiological processes related to the beginning of tumors and other diseases, degradation, changes in cell membrane structure, protein and DNA. Spectrophotometric methods with UV-VIS detectors are usually used for determining peroxide values in food lipids such as oils, margarine and butter. These procedures usually use oxidation of Fe (II) to Fe (III) to react with different reagents and produce colored complexes including Fe (III)-thiocyanate in IDF (International Dairy Federation) method and Fe (III)-orange xylenolin FOX (ferrous oxidation-xylenol orange) method. These complexes have absorbance at 400-600 nm wavelengths that is usually measured near

its maximum point. Previous volumetric methods like iodometery are replaced with infrared detection technique in these new methods. Foods containing compounds with natural absorption in this range such as carotenoids interfere in spectrophotometric peroxide value determinations. Therefore, the following method to eliminate these drawbacks and determine the amount of peroxide values is proposed: 0.01-0.05 g sample is added to a 10 mL capped test tube and dissolved in 1 mL chloroform: acetic acid (2:3) solution by adding 100 µL Fe (II) and mixing for 15 s on a vortex mixer and then keeping for 10 min in the dark. 2 mL deionized water is added and 4 mL diethyl ether (containing 7 ppm BHT) is used to extract oil and pigment. Organic phase is discarded and the remaining ether in the aqueous phase is removed with N_2 flow for a few seconds. To determine Fe (III), 1 mL of the aqueous phase is transferred to a disposable plastic tube and mixed with 100 µL saturated ammonium thiocyanate solution. After 10 min, the absorbance at 470 nm is measured versus the aqueous solvent. Baseline spectrum correction was done at 670 nm. The reaction solvent containing all the reagents except the sample is used as control and its absorption is measured and subtracted from the absorption of samples. A stock solution of Fe (II) is prepared by gentle mixing of 0.4 g $BaCl_2$. $2H_2O$ solution in 50 mL deionized water and 0.5 g $FeSO_4$. $7H_2O$ in 50 mL deionized water. 2 mL concentrated hydrochloride acid is added to the filtered solution. This solution must be freshly prepared and should be examined by adding a few drops of thiocyanate to the test solution before usage. If pale pink color appears, the solution should be discarded.

Calibration of Fe (III)

A solution of Fe (III) 104 µg/mL is prepared from the standard stock solution [Fe (III) 1040 µg/mL with 1% HCl] by dilution with chloroform: acetic acid (2:3). For calibration, a series of Fe (III) solutions is prepared in the concentration of 0-10 µg/mL by consecutively diluting the stock solution. The calibration curve is plotted by drawing the absorbance at 470-670 nm versus Fe (III) solutions.

$$\text{PV (mequiv peroxide/Kg of sample)} = \frac{(A_{sm} - A_{bl})}{55.84 \times 2 \times m \times W_{sm}}$$

A_{sm} = sample absorbance at 470 nm, A_{bl} = solvent absorbance at 470 nm (both absorption are correct with subtracting the amounts of 670 nm absorbance), M = slope of Fe (III) calibration curve, 55.84 atomic mass of iron, Fe conversion to meq peroxide factor and Wsm g sample weight [43].

In a study, the antioxidant activity of olive leaf extract in linoleic acid system was assessed according to the official method of AOCS (1990) by measuring peroxide value. The peroxide values in the butanol and ethyl acetate extracted fraction had stronger antioxidant activity than hexane, chloroform, water and negative controls. The antioxidant efficiency of butanol and ethyl acetate fraction is proportional to total amount of flavonoids and predominant phenolics such as oleuropein, routine, and vanillin. The anti-oxidant activity of olive leaf components was affected by prevalent phenolic compounds such as oleuropein and their functional groups such as catechol. Therefore, high level of oleuropein and routines in olive leaf extract probably has beneficial effects on human chronic diseases, which are related to oxidative stresses [44].

6.8. Ferric Reducing Ability of Plasma Method (FRAP)

Ferric reducing antioxidant power assessment (FRAP) is a method which its application in the antioxidant evaluation of polyphenolic compounds is progressively increasing. The FRAP method is related to the reduction of [Fe (III)-TPTZ) $_2$] complex to [Fe (II)-TPTZ)$_2$] by an antioxidant and usually in none physiological conditions at low pH of about 3.6. TEAC and FRAP techniques measure the reducing ability [23, 58, 59, 65]. FRAP reagent that contains TPTZ (2, 4 and 6 - three (2-Pyrydyl)-s-Tryazyn), $FeCl_3$ and acetate buffer is mixed with distilled water and test samples (aqueous-organic) or blank (solvent). Maximum absorption value measurements are obtained at 595 nm wavelength after 30 min at 37°C. Torolox solutions with known concentrations are used for calibration. The results of FRAP method are expressed as Fe_2SO_4 equivalent, but later are expressed as Trolox according to the same results expression methods [15]. This method was initially introduced for assessing the reducing power in plasma, but later its application in plant assessments gained attention. This reaction evaluates the reduction amount of TPTZ to a colored product. Compounds with the reduction potential lower than 0.7 V (the reduction potential of TPTZ-Fe^{3+}) are identified with this method, thus FRAP is a reasonable screening of ability to maintain reducing conditions in cells and tissues. It seems that the reducing power is related to the degree of hydroxylation and the extent of conjugation in polyphenols. However, FRAP does not identify the compounds with radical disabling

mechanism (transition H) especially thiols and proteins. Since the reduction potential of Fe (III)-TPTZ values is comparable to ABTS $^{\bullet+}$ (0.68 V), the same compounds react in both FRAP and TEAC methods. The Reaction conditions are variable: TEAC and FRAP are performed at neutral and acidic pH, respectively (to maintain iron dissolution).

The reaction at low pH reduces the ionization potential; allows the electron transfer, increases the reduction potential and causes change in the dominant reaction mechanism. In a given antioxidants sequence FRAP values is usually less than the TEAC.

FRAP values often have a weak relationship with other antioxidants. Iron reducing ability has a little relation to radical disabling processes (transfer H) which are used by most antioxidants. Oxidation or reduction of radicals to ions stops radical chains and can reflect the reducing power and compounds ability to regulate the reducing degree in plasma and tissues. Generally, FRAP mechanism compared to the mixed SET and HAT is more electron transport, so its combination with other methods for the diagnosis of dominant mechanisms of the various antioxidants is very useful. Furthermore, since reduced metals are active radical chain propagators through reducing hydro peroxides to $RO^{\bullet}$, high levels of FRAP is probably related to the polyphenols tendency for peroxiding under some circumstances. Some flavones and flavanones with high FRAP values demonstrate this fact.

A freshly prepared FRAP reagent contains 2.5 mL TPTZ solution (2,4,6 - tripyridy-s-triazin) 10 mM in HCl 40 mM which are added to 2.5 mL of $FeCl_3$ 20 mM and 25 mL acetate buffer 3M (pH=3.6) that its temperature is brought to 36°C. For conducting this experiment, first aqueous solutions of sample 0.025, 0.05 and 0.1% (w/v) are extracted, and then ascorbic acid aqueous solution 0.005% (w/v) is prepared as the positive control and filtered through a 0.22 µm filter and sealed in sterilized containers. The containers are studied under four different ambient conditions: in the light (laboratory temperature), in the dark (laboratory temperature), refrigerator (4°C) and freezer (-20°C). The solutions are tested at specified times (0, 3, 7, 14 and 30 days). 1.5 mL of a fresh FRAP solution (10:1:1 ratio of acetate buffer, iron chloride and TPTZ reagent) is poured into the test tube and placed in a water bath for 5 min at 37 °C. Then 50 µL of sample is added to the corresponding tube and placed in the water bath for 10 min at 37 °C. The absorption is measured at 593 nm wavelength versus control (1.5 mL FRAP solution and 50 µL distilled water). Antioxidant concentrations of the samples are calculated using standard samples ($FeSO_4$ in 125, 250, 500 and 1000 µm concentrations) and the standard curve is plotted.

Advantages/Disadvantages

Reducing reactions in FRAP and TEAC methods take place in the 4 and 6 min, respectively, but it is not always correct. FRAP results are remarkably time-dependent. Phenols with high activity which link or break iron with different reactivity in the shortest time, for example four minutes give the best results. However, some polyphenols react slower and need longer reaction times of about 30 min. The FRAP method was tested for diet polyphenols in water and methanol. Absorption at 593 nm for polyphenols like caffeaic, tannic, ferolic, ascorbic acids and quercetin, even after several hours of reaction time slowly increase, therefore it probably does not show a complete and fixed absorb endpoint. The FRAP method does not measure thiol antioxidants such as glutathione and it only measures reducing ability based on ferric ion. In comparison with other total antioxidant power assessing methods, the FRAP method is a simple, fast, inexpensive and reliable procedure which does not need any complicated equipment [16].

6.9. Trolox Equivalent Antioxidant Capacity Method (TEAC)

This method gives relatively high values that are related to high reactivity of the ABTS reagent. The ABTS reagent is relatively unstable and under reaction conditions is spoilable. For example, there is a considerable decrease in absorption during incubation, which increases the measured values, and thus this decrease should be subtracted [20]. The lipophilic TEAC method is more reliable for determining hydrophobic antioxidants ability than the hydrophilic counterpart. According to the test results, maximum difference between hydrophilic TEAC values is less than three times, whereas this is nearly six times for hydrophobic TEAC values. The hydrophilic TEAC method probably shows the antioxidant capacity based on the reducing ability, but this method does not display a compound free radicals scavenging ability in a special environment such as biological membranes. However, it seems that hydrophobic TEAC better determine the antioxidant capacity of a hydrophobic compound [45, 61-64, 66, 69-70]. The TEAC method is based on the ability of compound to scavenge the long life stable colored radical cation of 2, '2 - azobis - (3 -

methyl - benzothiasoline) -6 - sulfonic acid ($ABTS^{+\bullet}$). TEAC values can be attributed to all compounds by comparing their scavenging capacity with trolox (artificial water soluble vitamin E) amounts. This method is widely used to measure the antioxidant capacity of basil, tropical fruits and wines [28]. The $ABTS^{\bullet+}$ radical has maximum absorption at the near-infrared area and 645, 734 and 815 nm wavelengths. TEAC reflects the ability of antioxidants for hydrogen or electron donation in order to scavenge the $ABTS^{\bullet+}$ radical in comparison to trolox. The absorbance of antioxidants decreases at 734 nm in a time-dependent scale. TEAC I measures hydrophilic antioxidant capacity, while carotenoids, tocopherol and other hydrophobic antioxidants are measured using TEAC II and TEAC III is used for both compounds [23]. The evaluation of antioxidant activity in aqueous and oily environments is performed with SET or HAT reactions. TEAC is done in three main modes: TEAC (I) is based on metmyoglobine activation by hydrogen peroxide in the presence of the ABTS reagent. TEAC (II) which the ABTS oxidation is done by MnO_2 and it is used in DNA oxidative damage consideration. TEAC (III) is based on the oxidation of ABTS by $K_2S_2O_8$ to $ABTS^{\bullet+}$ which is a stable nitrogen centered cationic radical containing a green-blue group with a maximum absorption at 734 nm wavelength. The antioxidant activity of natural products such as carotenoids, phenolic compounds and some plasma compounds is determined by monitoring colorless ABTS and absorption declines at 734 nm. Finally, the absorption values of samples are compared with standard trolox and the antioxidant capacity of extract is expressed as trolox equivalent concentration [16].

6.10. DPPH Method

DPPH is a stable free radical with a central nitrogen atom and absorption at 517 nm, and it is reduced by hydrogen or electron receiving processes resulting in the conversion of its color from purple to yellow. Compounds with this ability by SET and HAT reactions are regarded as an antioxidant. The lack of dependence on sample polarity is one of its advantages [46, 47].

Antioxidants interact with $DPPH^{\bullet}$ and convert it to 2, 2'- diphenyl-1-picrylhydrazilin at a very high speed through the hydrogen donating ability. The degree of color changes indicates antioxidant extract scavenging functionality due to its hydrogen donating ability [12]. DPPH is a fast and accurate method to estimate the antioxidant activity of fruit and vegetable extracts and its results are highly reproducible and comparable with other free radicals scavenging methods such as ABTS [10, 67-68].

In oils, DPPH is the best method for total and methanolic extracts. DPPH is the only method which is usually used in both aqueous-organic plant foods and vegetable oils. Expressing the results at the fixed endpoint which is usually used for the DPPH method evaluations assesses the inhibition percentage with comparison the changes created by blank and sample. However, the inhibition percentage depends on the radical concentration and samples, and therefore, doing a research comparing different initial values is not possible. In all fixed endpoint methods, reaction completion should be determined quite in the selected time [15]. In this technique, antioxidants are allowed to react with stable radicals in the methanol solution. A decrease in the concentration of $DPPH^{\bullet}$ is monitored with a decline in the absorption at a certain wavelength. $DPPH^{\bullet}$ in the radical form has an absorbance at about 515 nm, but it disappears after reducing by an antioxidant (AH) or a radical species ($R^{\bullet}$). The antioxidant activity is determined using DPPH as a free radical. An antioxidant solution in methanol is added to the methanolic DPPH solution. The absorbance at 515 nm is assayed until the reaction reaches to the fixed situation. The exact initial $DPPH^{\bullet}$ concentration in the reaction environment is calculated from the calibration curve determined by linear regression. The antiradical activity is defined as the needed antioxidant concentration to decrease the initial $DPPH^{\bullet}$ concentration by 50% (adequate concentration = efficient concentration = EC_{50} = mol 1^{-1}(AO) / mol 1^{-1}($DPPH^{\bullet}$)). The more antiradical power (ARP) means more efficient antioxidants [23]. Although the DPPH method represents values similar to the TEAC method, but its results are minimal. Trolox is the most widely used standard. Several times lower values for extracts are determined by the DPPH method compared to the TEAC method. This significant difference can be explained by the relatively more stability of $DPPH^{\bullet}$ and its less reactivity. This radical only reacts with more reactive phenolic compounds. It does not identify the less active phenolic compounds that still have antioxidant activity in human body. Glucose, fructose and sucrose are the most important carbohydrates present in fruits and vegetables. Exception for the DPPH method, these sugars interfere with methods for measuring antioxidant activities to various extents [20]. The $DPPH^{\bullet}$ lipophilic molecule reduction is almost equal to the number of available hydroxyl groups, which by donating hydrogen to $DPPH^{\bullet}$ convert its color

from dark purple to bright yellow. The absorption at 517 nm wavelength after 60 min indicates the amount of residual DPPH$^{\bullet}$. Therefore, the remaining amount of DPPH$^{\bullet}$ is inversely associated with the antioxidant radical scavenging ability. The antiradical activity increases with increasing the concentration of essential oils. EC_{50} is inversely associated with the antiradical activity. The less EC_{50} means the more antiradical activity [48].

0.3 mL almond extract at different concentrations were mixed with 2.7 mL methanolic solution containing DPPH$^{\bullet}$ at concentration of $6*10^{-5}$ mol/L. Mixture was vigorously mixed and remained 60 min in the dark. The DPPH radical reduction is determined by measuring the absorbance at 517 nm. The Radical scavenging activity (RSA) was calculated as a DPPH colorless percentage with the following equation:

%RSA= $\{(A_{DPPH}-A_S)\ /A_{DPPH}\}$ * 100: As = solution absorption containing known amount of a sample, A_{DPPH}=DPPH solution absorption.

An extract concentration with 50% radicals scavenging activity (EC_{50}) of %RSA graph versus extract concentration was calculated. BHA and α-tocopherol were used as standards [25].

6.11. Oxygen Radical Absorbance Capacity Assay (ORAC Method)

FRAP, ABTS, DPPH and ORAC are almost *in vitro* methods to determine the antioxidant activity. To obtain a reliable antioxidant capacity of food it is recommended to consider at least two methods (or even all) of these procedures [19]. ORAC measures the free radical scavenging capacity of a sample. The ORAC mechanism is a hydrogen atom transfer system and it is monitored by means of spectrophotometer. Because of the evaluation of kinetic variables and the analysis of samples with different concentrations, this method lasts 4-30 min. ORAC is applied to assess the antioxidant capacity of hydrophilic compounds and of course with some modifications, it is used to determine the antioxidant capacity of hydrophobic compounds. The ORAC results are usually expressed in a fixed end point and the results are interpreted in a trolox calibration curve and stated as trolox equivalent to that of µmol required trolox for the preparation of antioxidant capacity similar to the sample. More trolox equivalent means more sample antioxidant capacity [15, 59, 60]. The ORAC method measures the inhibition of peroxyl radical oxidation generated by antioxidant activity and thus reflects the antioxidants radical chain breaking activity through H atom transmission. In this method, the peroxyl radical reacts with a fluorescent probe to form a non-fluorescent product that can be determined easily by fluorescence assessment. The antioxidant capacity is determined by reducing the speed and amount of formed product over time:

$$\text{R-N=N-R} \xrightarrow{O_2} N_2 + 2ROO^{\bullet}$$

$$ROO^{\bullet} + \text{probe (fluorescent)} \rightarrow ROOH + \text{oxidized probe (loss of fluorescence)}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$ROO^{\bullet} + A^{\bullet} \xrightarrow{Fast} ROOA$$

B-phycoerythrin (B-PE), an isolated protein from the *porphyridium cruentum*is is used as fluorescent probe. The disadvantages of using B-PE in antioxidant evaluation are: 1 - B-PE has a large variety in reactions with peroxyl radicals which causes contradictions in the results; 2- B-PE upon exposure to light, becomes fluorescent; 3 - Polyphenols especially proanthocyanidins link to B-PE through nonspecific protein binds.

The latter two drawbacks lead to the recorded amounts of ORAC to be lower than actual get. Common fluorescent probes such as fluorescein (FL; 3', 6'-dihydroxydihydroxyspiro [isobenzofuran- 1 [3H], 9' [9H]- xanthen]- 3 -one) and dicholoro fluorescein (H2DCF- dA; 2', 7'- dichlorodihydrofluorescein diacetate) are more stable with less reactivity. Fluorescent products of oxidized fluorescein which are produced by peroxyl radicals are detected by LC-MS system and the reaction mechanism is expressed as the HAT reaction. The probe interaction with peroxyl radicals is monitored by fluorescence loss over time. The ORAC values are reported as trolox equivalent. Traditional antioxidant tests only follow lag phase expansion while antioxidant effects often extend well after the first stages of oxidation. To avoid underestimating the antioxidant activity and calculate the antioxidant effects of secondary products, the ORAC method monitors reaction for extended periods for example ≥ 30 min. The antioxidants protective effects can be calculated form net integrated areas under decreasing fluorescence curves.

The standard curve is plotted using the area under curve (AUC) for five trolox standard concentrations and sample trolox equivalent is calculated using a linear relationship Y = a + bX between trolox µm

concentration (Y) and net area under decreasing fluorescence curve (X = AUC_{sample}- AUC_{blank}). Results are expressed as µmol trolox equivalent (TE) per sample (liter or gram). The $ORAC_{FL}$ evaluation is limited to the hydrophilic chain breaking capacity only against peroxyl radicals. This method ignores important lipophilic antioxidants against lipid oxidation in all samples and also other physiologically active radicals. To expand the ORAC method usability for evaluating lipophilic antioxidants in addition to hydrophilic ones, a solution of acetone/water 50% (V/V) containing 7% randomly methylated β-cyclodextrin (RMCD) is used to solve antioxidants. Lipophilic and hydrophilic compounds are selectively extracted before analysis.

ORAC Advantages / Disadvantages

The ORAC test provides manageable sources of peroxyl radical and it models antioxidants reactions with lipids in foods and physiological systems. The identification of hydrophilic and hydrophobic antioxidants is possible by changing radical sources and solvents. This method is very sensitive to temperature, so temperature should be precisely controlled. Although fluorescent indicators are sensitive for detection, they need spectroflourimetry that normally is not available in laboratories. Long-term analysis (about 1 hour) is a problem which can be overcome with assessments using high output [16]. The comparison of these 4 methods is presented in Table **1**. The disadvantages of these 4 methods are summarized in Table **2**.

6.12. Total Radical Trapping Antioxidant Parameter Method (TRAP)

This method shows the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals derived from AAPH or ABAP (2,'2-

Table 1: Comparison of 4 Methods of Antioxidant Activity Evaluation

Method	Methods basis	mechanisms	instruments	Time (min)	Examined compounds
FRAP	Metal reduction	SET	UV-vis spectrophotometer	30	Hydrophilic
ABTS	Scavenging free radicals and reducing	SET	UV-vis spectrophotometer	7	Hydrophilic and after some modifications hydrophobic
DPPH	Scavenging free radicals	SET/HAT	UV-vis spectrophotometer	Longer time according to samples	Aqueous-organic extracts, vegetable oils
ORAC	Scavenging oxygen free radicals	HAT	Fluorimetery	30-40	Hydrophilic and after some modifications hydrophobic

Table 2: The Disadvantages of 4 Evaluating Antioxidant Activity Methods

Main drawbacks	Method
Other compounds may absorb at 595 nm Any compound with a redox potential lower than 0.77 V, although it dose not behave *in vivo* as an antioxidant, may reduce iron It is performed at a non-physiological pH	FRAP
Antioxidants, besides reacting with the radical to yield the original molecule, generate other compounds Reaction is not over at the usually taken 6 min The free radical used is not present *in vivo*	ABTS
Other compounds may absorb at 515 nm There may be an steric hindrance for molecules with a higher molecular weight The free radical used is not present *in vivo* and it is quiet stable, unlike radicals present in living organisms	DPPH
The kinetics of reaction may vary depending on the concentration of the antioxidant, what enables this method to be used for kinetics studies It measures the ability of antioxidants to scavenge peroxyl radicals, present *in vivo*; however, the procedure to generate these peroxyl radicals is not physiological Protein may have an interfering effect	ORAC

azobis (2-amidinopropane) di hydrochloride) and the target probe. Different methods use oxygen absorbance, fluorescence R-phycoerythrin or ABTS (2,'2 azinobis (3-ethylbenzothiazoline -6- sulfonic acid) as reaction probe. Basic reactions are similar to the ORAC technique. The method requires that the probe react with $ROO^{\bullet}$ in low concentrations, an indistinct spectroscopy change between initial and oxidized probes (to maximize sensitivity) and no radical chain reaction should occur after probe oxidation. The probe oxidation is measured by spectrophotometry or fluorescence. The antioxidant activity is determined when whole antioxidants are consumed. Antioxidants expand retardation time to advent oxidized probe and their ability is measured by percentage of reaction reduction. The TRAP values are usually expressed as retardation time or sample reaction time in comparison to the corresponding times for trolox.

TRAP Method Advantages / Disadvantages

This method is used for evaluating the antioxidant capacity in serum or plasma *in vivo*. It evaluates none enzyms like glutathione, ascorbic acid, α-tocopherol and β-carotene. Perhaps the biggest disadvantage or advantage of this method is the usage of too many end points which makes the comparison difficult. Using lag phase is based on the assumptions that all antioxidants show lag phase and that lag phase duration is relative to AOC (antioxidant capacity).

However, all antioxidants do not have a clear lag phase. In addition, the obtained amounts of the lag phase often underestimate AOC because the amount of involved antioxidants after lag phase is totally ignored. The TRAP method includes the initiation of lipid peroxidation with producing water soluble peroxyle radicals and it is sensitive to all known chain breaker antioxidants, but it is quite complicated, time consuming and needs much expertise. However, the TRAP method is used as a none-physiologic oxidative stress user (water-soluble peroxile radicals), but it is also adjustable with fat-soluble initiator [16]. Some antioxidant capacity evaluation methods express their results by referring to the lag phase, which defines as the time required by an antioxidant to operate its action before the oxidation process initiation. For example, this method is applied in LDL oxidation or total radical trapping oxidant parameter (TRAP). It should be noted that not all antioxidants show a clear lag phase and therefore it is not useful in determining the capacity of antioxidant activities of complex compounds such as plasma (applicable in plant foods and beverages) [15].

6.13. Copper Reduction Method

In some types of FRAP method, copper is applied instead of iron and as a result, new bio methods are presented such as AOP-490 and CUPRAC. These methods are based on the reduction of Cu (II) to Cu (I) by the combined antioxidant action of all samples (reducing agents). In the Bioxytech AOP-490 method, bathocuproine (2, 9 - dimethyl -4,7 – di phenyl -1, 10 - phenanthroline) forms a complex with Cu (I) at the ratio of 2:1, which results in a colored substance with maximum absorption at 490 nm. The reaction rate and concentration of products are followed by bathocuproine complex. The CUPRAC method produces a complex of neocuproine (2,9-di methyl-1,10- phenanthroline) with Cu (I) having maximum absorption at 450 nm. The dilution curve is produced by uric acid standards to convert the absorption of samples to uric acid equivalents. Phenanthroline complexes dissolve slightly in water and therefore, it should be diluted in organic solvents such as ethanol 95%. However, β-carotene does not react with the CUPRAC reagent in aqueous ethanol and it needs dichloroethane which limits its miscibility. The CUPRAC values are comparable with the TEAC values for poly phenols, while the FRAP values are usually significantly lower. Free copper and copper in complexes have less reducing potential than iron, but reacts more selectivity. Sugars, citric acid and usual interferences in FRAP are not oxidized in CUPRAC. SET is the main reaction in these methods.

Copper Reduction Method Advantages/ Disadvantages

The benefits of copper over iron in antioxidant assessments are such that all types of antioxidants including thiol are detected with little interference from active radicals and also, the reaction kinetics of copper are faster than those of iron. The AOP-490 method completes only in 3 min and CUPRAC for ascorbic acid, uric acid, gallic acid and quercetin in a few minutes, but for more complex molecules it lasts about 30 to 60 min. Therefore, copper reduction assessments for a mixture of antioxidants have similar problems in selecting appropriate reaction time [16].

6.14. Total Oxidant Scavenging Capacity Method (TOSC)

This method determines the antioxidants absorbance capacity especially against three major oxidants, including hydroxyl radicals, peroxile radicals and peroxy nitrite. This method gives an important result about different antioxidants evaluation with

different radical sources. A-keto-γ-methiol butyric acid (KMBA) is oxidized in this method and ethylene is formed. Time analysis of ethylene formation is followed by headspace GC analysis and the antioxidant capacity is determined through antioxidants ability to prevent ethylene formation towards control reaction. This method defines turning points during 300 min using under curve areas. Value- response linear curves are available from antioxidants reaction kinetics.

Advantages / Disadvantages of TOSC

In this method, the antioxidant capacity is determined against three major oxidants. However, this method is not easily adaptable for tests requiring high capacity for quality control and multiple GC injections of a single sample for measuring ethylene production. In the TOSC method kinetic, there is not a linear relationship between the TOSC inhibition percentage by oxidant source and oxidants concentration or diluent [49].

The comparison of some antioxidant activity determination methods is presented (Table **3**).

6.15. Conjugated Dienes TEST

Conjugated dienes are defined as sections within 2 double bonds separated by a single bond. This condition typically is present in poly unsaturated fatty acids by reaction with ROS and oxygen (forming mono hydro-peroxide). In this method, fresh LDL is isolated from blood samples and they are oxidized by Cu (II) or other substances such as AAPH, forming conjugated dienes that are monitored spectrophotometrically at 234 nm wavelength. The effects of antioxidant compounds with HAT mechanism are evaluated by reducing the absorption amounts at 234 nm. This method is appropriate for assessing a simple fatty acid and primary stage of lipid peroxidation. The main disadvantage of this method is the presence of biological and natural compounds with absorption at 234 nm, which hampers the access to sample actual absorption values [74, 86]. This method is similar to soy bean lipoxygenase activity inhibition assay.

6.16. Lipoxygenase Activity Inhibition Assay

Lipoxygenase is a biological target for many diseases such as asthma, heart disease, cancer and tumor. In mammalian cells, this enzyme has a key role in the biosynthesis of various biological regulator compounds such as hydroxy equsatetraenoic acid, leukotrienes, are lypocsin and hypocsilin. Thus, lipoxygenase is a potential target for drug design and discovery of inhibitors mechanism to treat a wide variety of disorders and autoimmune diseases. Antioxidants react non-specifically with lipoxygenase through scavenging radical intermediate and reduce their iron section [12]. The phenolic antioxidant activity is probably related to their reducing properties, which allow them to act as reducing agents or hydrogen atoms donors. In addition to their ability to chelate metals, they inhibit lipoxygenase and scavenge free radicals [25]. Enzymatic lipid peroxidation is measured

Table 3: Comparison Some Antioxidant Activity Determination Methods Major Indicators

Antioxidant assay	Simplicity	Instrumentation required	Biological relevance	Mechanism	Endpoint	Quantitation	Lipophilic and hydrophilic AOC
ORAC	++[a]	+	+++	HAT	Fixed time	AUC	+++
TRAP	---[b]	--specialized	+++	HAT	Lag phase	IC_{50} lag time	--
FRAP	+++	+++	--	SET	Time,varies	ΔOD fixed time	_ _ _
CUPRAC	+++	+++		SET	Time	ΔOD fixed time	_ _ _
TEAC	+	+	-	SET	Time	ΔOD fixed time	+++
DPPH	+	+	-	SET	Ic_{50}	ΔOD fixed time	-
TOSC	-	-	++	HAT	IC_{50}	AUC	_ _ _
LDL oxidation	-	+++	+++	HAT	Lag phase	Lag time	_ _ _
PHOTOCHEME	+	--specialized	++		Fixed time	Lag time or AUC	+++

[a]: +,++,+++ = desirable to highly desired characteristics. [b]: -, --, --- = less desirable to highly undesirable based upon this characteristic. [c]: The lipophilic is quantitated by AUC measured over a defined measuring time and the hydrophilic assay is quantitated based upon the lag phase.

by means of spectrophotometer with an increase in the lipid hydroperoxide absorption at 234 nm. Mango peel acetonic extracts show concentration-dependent lipoxygenase inhibitory activity. Unripe mango peel extracts have better inhibition than ripe ones and BHA. 1 mL mixture contains 250 µM linoleic acid substrate solution, 5 nM soy bean lipoxygenase and 50 mM Tris buffer (pH=9). In a study, different concentrations of mango peel extract were incubated with linoleic acid for 2 min before reaction initiation. The reduction in hydro-peroxide formation was calculated in the presence of peel extract or BHA. The control sample contained all reagents except the peel extract/BHA. [12].

6.17. Chemiluminescence (CL) Method

The identification of radical species is not easy because of their short shelf life and different chemical properties. Procedures for monitoring these radicals or effective compounds on radical's absorbance such as chemical luminescence methods are based on reactive substances such as luminole, and pyrogallol.

Luminol can increase luminescence in the presence of superoxide anion radicals. The antioxidant activity is monitored through HAT main reaction by a decrease in the chemical luminescence intensity caused by luminol and superoxide anion radical. This phenomenon is used to consider the effects of trapping or absorbing oxygen species compounds and enzymes such as sodium azide (NaN_3) for single oxygen, superoxide dismutase (SOD) enzyme for superoxide anion, catalase and peroxidase for H_2O_2. The chemistry of CL procedures is based on radical oxidizing reactions with excited species that are able to propagate the chemical luminescence (producing light by chemical methods). Any compound which can react with radical initiators prevents light production. Peroxyl radical oxidizing sources include horseradish peroxidase enzyme and H_2O_2-hemine. The reaction can disable different oxidizing species such as $O_2^{\bullet -}$, $OH^{\bullet}$, HOCl, $LOO^{\bullet}$, $OONO^{\bullet}$ and $^1O_2$ by changing initiators. Although lucigenin and bioluminescence proteins such as pholasin are commonly present. Luminol is the most important indicator component for trapping oxidants, intensifying long lasting and stable emissions. A continuous light output depends on the constant production of free radicals derived from *p*-idophenol, luminol and oxygen and also, the light emission is sensitive to radical scavenger antioxidants interferences. The antioxidant capacity is assessed as emission light declines.

6.18. β-Carotene Bleaching Assay

β-carotene reacts with peroxyl radical and creates β-carotene epoxide. β-carotene is accepted as radical scavenger or antioxidant. Lipids such as linoleic acid form peroxyl radical (LOO°) in the presence of ROS and O_2, which reacts with β-carotene to form a stable β-carotene radical, resulting in a decrease in the amount of β-carotene and its specific absorption at 473 nm. The HAT reactive antioxidants in solution can react with peroxyl radical competitively and the antioxidant effects are monitored by declines in the solution color. β-carotene by having 11 pairs of double bonds is very sensitive to free radical indirect oxidation and by linoleic acid oxidation becomes easily colorless. During oxidation, a hydrogen atom is separated from the active bis-alilic methylene group (on 11_{th} C linoleic acid between 2 double bonds). Consequently, penta-diene free radical forms and poly unsaturated β-carotene molecules absorb a hydrogen atom. Once the β-carotene molecule loses conjugated bond, its orange color reduced which is monitored spectrophotometrically [50].

β-Carotene Bleaching Inhibition

The almond extracts antioxidant activity was evaluated by linoleate β-carotene model system. β-carotene solution was prepared by dissolving 2 mg β-carotene in 10 mL chloroform. 2 mL of this solution was poured into a 100 mL round bottom flask. After chloroform remove at 40 °C under vacuum, 40 mg linoleic acid, 400 mg Tween 80 as an emulsifier and 100 mL distilled water were added and shaken vigorously. 4.8 mL of the aliquot phase of this emulsion was passed into test tubes containing 0.2 mL almond extract of different concentrations. The tubes were shaken and incubated in a water bath at 50 °C. Once the emulsion added to each tube, the absorption was measured at zero moment at 470 nm wavelength. The absorption readings at 20 min intervals were continued until control changes color. The lipid peroxidation inhibition is calculated by the following equation:

LPO inhibition= (amount of β-carotene after 2 h assessment/initial amount of β-carotene)$^{\times}$ 100

The extract concentration with half of antioxidant activity (EC_{50}) was calculated by designating the antioxidant activity percent versus the extract concentration. TBHQ was used as standard [25]. The β-carotene bleaching method is based on the essential oil ability to decrease β-carotene oxidative losses in a β-carotene/linoleic acid emulsion. β-carotene bleaching

rate decreases in the presence of antioxidants [46-51, 72, 81, 83-85, 88].

6.19. Deoxyribose Method

O_2^- and H_2O_2 in the reactions that require metal ions are converted to very active hydroxyl radical ($OH^•$) and several hydroxyl radical scavengers are used to study its role in biological systems. Some medical elements and compounds such as alopiuorinol, anti-inflammatory drugs and amygdaline have beneficial effects on hydroxyl radicals scavenging, which the rate constant of second order is required to prove this claim. This factor is determined by radiolysis which is a useful method in reactions containing $OH^•$ but it is expensive and unavailable. In a simple and cheap method, deoxyribose sugar decomposes with exposure to $OH^•$ generated by radiation or by Fenton systems. If the complex mixture of products is heated under acidic conditions, malone aldehyde is formed which is identified by its ability to react with thiobarbitoric acid (TBA) and the formation of pink material. In addition, deoxyribose is used to determine the formed $OH^•$ in biological systems. If Fe^{2+}-EDTA chelate is incubated with deoxyribose in phosphate buffer (pH = 7.4), $OH^•$ is formed. Any radical that may escape from scavenging by EDTA will react with deoxyribose:

$$Fe^{2+} - EDTA + O_2 \rightleftharpoons Fe^{3+} - EDTA + O_2^- \quad [1]$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad [2]$$

$$Fe^{3+} - EDTA + H_2 \rightarrow OH^- + {}^•OH + Fe^{3+} - EDTA \quad [3]$$

$${}^•OH + \text{deoxyribose} \longrightarrow \text{fragments} \xrightarrow[\text{TBA plus acid}]{\text{Heat with}} \text{MDA} \quad [4]$$

$$2TBA + MDA \rightarrow \text{choromogen} \quad [5]$$

It is likely that the decomposition rate of deoxyribose increases in the presence of reducing agent such as ascorbic acid in the reaction mixture:

$$Fe^{3+} - EDTA + \text{Ascorbate} \rightarrow Fe^{2+} - EDTA + \text{Oxidized Ascorbate}$$

Addition of H_2O_2 enhances the reaction speed strongly. Each added molecule (antioxidant) to the reaction mixture, which is able to react with $OH^•$ partly competes with deoxyribose for $OH^•$ depending on the reaction rate constant and its concentrations towards deoxyribose. Therefore, the decomposing rate of deoxyribose will decrease. The $OH^•$ derived from the reaction of Fe-EDTA complex with H_2O_2 in the presence of ascorbic acid can attack deoxyribose and produces a pink substance by heating with TBA at low pH. The added free radical scavengers compete with deoxyribose for the produced $OH^•$ and reduce chromogen formation. The rate constant for scavengers reaction with $OH^•$ is inferable by inhibiting the color formation. EDTA usage is essential in the reaction system. The inhibition of deoxyribose decomposition which is iron dependent in the absence of EDTA depends on scavenger ability to react with $OH^•$ and also its ability to form complex with iron ions. This method cannot be applied for some specific compounds such as strong metal chelators. Citrate gives contradictory results which may be related to its ability to bind metals. The reagent reaction with H_2O_2 probably decreases the production rate of $OH^•$. The OH• reaction with molecules such as ethanol produces secondary radicals being able to reduce or oxidize iron. It seems that ascorbic acid in the reaction mixture can resolve this problem. For example, the reaction rate constants of ethanol and propane-2-ol with $OH^•$ is in the expected range, although by adding Fe^{2+} to the reaction mixture in the absence of ascorbate, ethanol and propane-2-ol, the inhibitory effects are much less than expected which is probably because of radicals derived from alcohol that can reduce Fe^{3+} and increase the $OH^•$ production rate. The absorption values and reaction rate constant are calculated by equation below:

$$\frac{1}{A} = \frac{1}{A^0} \frac{\{1 + KS\,[S]\}}{K_{DR}\,[DR]}$$

where A and A_0 are absorption intensities in the presence of antioxidant and control solution without antioxidants, K_S and K_{DR} are sample and deoxyribose reaction rate constants, [S] and [DR] are sample and deoxyribose concentrations. The curve below represents the relationship between the absorption intensity and the sample concentration and its slope is calculated by the following equation [52]:

Fenton Reaction Mechanism

The Fenton process is an electrochemical system in which Fe^{2+} ions and H_2O_2 molecules are reducing and oxidizing agents, respectively. Because of the electrochemical nature of the Fenton system, it acts as a cell. H_2O_2 molecules reduce and produce active hydroxyl radicals (reaction object). The Fenton method is divided into several types including photo Fenton, electro Fenton, dark Fenton, semi Fenton, etc., which only differ over reaction conditions, but the reactions are basically the same. The reactive substances used in this process are available, cheap and environmentally safe.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$OH^{\bullet} + H_2O_2 \rightarrow H_2O_2 + OH_2^{\bullet}$$

$$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + H^{+} + O_2$$

$$Fe^{2+} + HO_2^{\bullet} \rightarrow Fe^{3+} + HO_2^{-}$$

$$Fe^{2+} + OH^{\bullet} \rightarrow Fe^{3+} + OH^{-}$$

The reaction between Fe^{3+} and H_2O_2 is the so called semi Fenton process, which is as follow [53]:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H_2O^{\bullet} + H^{+}$$

6.20. Tocopheroxyl Radical Attenuating Ability Method (TRAA)

TRAA (tocopheroxyl radical attenuating ability) is a quick and easy method for screening inhibitor's ability to prevent low-density lipoprotein peroxidation (LDL). The method is based on the antioxidant capacity to reduce α-tocopheroxyl radicals' contents. α-tocopherols which are produced by UV light and direct declines with electron spin resonance (ESR) spectroscopy are measured. The TRAA method uses positively or negatively charged micelles by adding α-tocopherol. The TRAA method using $CCl_3O_2^{\bullet}$ is expensive because it needs electron spin resonance machine [23].

6.21. Rancimat Method

An antioxidant extract at the concentration of 1500 ppm is prepared in the refined and bleached vegetable oil without antioxidants. A steady air flow from environment with the speed of 15 l/h is transferred through heating oil samples (110°C) which results in the transmission of volatile compounds from to the water container and increases the electrical conductivity of water. The water electrical conductivity evaluation continues to its highest level. The time duration from the initiating time until sudden electrical conductivity ascent in distilled water is defined as the induction period of oil samples (IP). This index is calculated by the equation below:

Functionality index (F) = the induction period in the presence of inhibitors/induction period in the absence of inhibitors [54].

6.22. Metals Chelating Method

Food contains transition metals or become contaminated by them during production processes. Bivalent transition metals such as iron and copper play an important role as catalysts of oxidative processes and lead to the formation of hydroxyl radicals and hydroperoxide destructive reactions through Fenton phenomena. This process is performed by iron ions chelated in the Denis method. Ferrosin 5 mM and ferrous chloride (II) 2 mM solutions are added to extract and after 10 min incubation at ambient temperature, the absorption is measured at 562 nm. The inhibition percentage of Fe–ferrosin complex formation is calculated with the following equation:

% Inhibition = 100 * [A_0-A_S) / A_S)]

A_0 and A_S are the absorptions of control and sample, respectively. EDTA is used as the control and the Fe chelating activity is expressed as Na_2EDTA equivalent per gram of sample [46].

6.23. Reducing Power Assay

Reducing Fe (III) is often used as an electron donating criterion, which forms an important mechanism in the phenolic compounds oxidation process. Different amounts of extracts in water are mixed with phosphate buffer solution 0.2 M and potassium ferry cyanide. The mixture is incubated at 50°C for 20 min. 2.5 mL of 1% trichloroacetic acid is added to the mixture and then centrifuged at 3000 rpm for 10 min. The upper phase is mixed with water and ferric chloride 0.1 M, and finally, the absorbance is measured at 700 nm against the blank. The absorbance increasing in the mixture reaction is an indication of greater reducing power (antioxidant). Ascorbic acid is used as the positive control for comparison [46].

6.24. Ferric Thiocyanate Method (FTC)

This method measures the amount of peroxide at the initiation of the oil peroxidation. Peroxide reacts with ferrous-chloride and ferric ions. Then ferric ions combine ammonium thiocyanate and produce red ferric thiocyanate with absorption maxima at 500 nm. By adding antioxidants, the peroxide concentration decreases, and thereby the red color intensity and absorbance reduce as well. A lower absorbance rate indicates further antioxidant activity. In most studies, linoleic acid is used as the hydro-peroxide source. The antioxidants inhibition effect is assessed by preventing ferrous oxidation to ferric ion and monitoring the formation of ferric thiocyanate complex. The disadvantage of this method is the presence of compounds with similar absorbing wavelengths.

6.25. TBARS Method

During the oxidation process and the final oxidation stage, peroxide gradually decomposes to carbonyl compounds with lower molecular weights such as malone aldehyde. In the TBA method, malone aldehyde links to thiobarbitoric acid and produces a red complex with maximum absorption at 532 nm. In the presence of an antioxidant, the red complex concentration and absorption intensity decrease and later, the inhibition percentage is calculated by the following relationship:

$$IP = (A_T/A^0_T) * 100$$

Where A_T and A^0_T represent the sample and control absorption values after the incubation time, respectively.

Short TBARS

To prepare the TBARS solution; TBA/TCA (20 mM thiobarbitoric acid in tricholoroacetic acid 15%) and ethanolic BHT solution 0.01% are necessary. The Moller *et al.* (2002) procedure is applied in the antioxidant activity assay for meat system. Homogeneous fish meat (10% w/v in distilled water) and an aqueous extract solution 0.1 and 5% (w/v) are prepared. At first, 4 mL homogeneous flesh is added to all test tubes. Then 500 µL extract is added to each test tube and 500µL distilled water is added to the negative control tubes. The tubes are placed at a 37 °C water bath for 0, 30, 60 and 90 min. Then 4 mL TBA/TCA and 50 µL ethanolic BHT 0.01% solutions are added to all tubes and placed in a boiling water bath for 15 min. The tubes are then centrifuged for 15 min at 3000 rpm and the supernatant absorbance versus control at 532 nm is measured by spectrophotometry.

Long TBARS

At first, all 4 mL homogeneous meat is added to all test tubes. Half of the test tubes are put for 15 min in a boiling water bath. Then 500 µL extract is added to the test tubes, 500 µL distilled water to the negative control tubes and 500 µL ethanolic BHT solution 0.01% is added to the positive control tubes. All tubes are placed in the dark and refrigerator conditions until analysis. The tubes are tested at days (0, 3, 7 and 14). After removing the tubes from refrigerator, they are allowed to reach the ambient temperature. Then 4 mL TBA/TCA and 50 µL ethanolic BHT solutions 0.01% are added to each tube. The tubes are then placed in the boiling water bath for 15 min and, next they are centrifuged for 15 min at 3000 rpm and the supernatant absorption is read at the 532 nm by spectrophotometer against control. The antioxidant activity decrease is reported over time and with increasing temperature. By passing time, the polyphenols and vitamin C contents antioxidant capacity will decrease. During the process, the antioxidant characteristics of foods stuff can remain unchanged or change. These changes are attributed to the formation of new compounds with more antioxidant or pro-oxidant ability. The antioxidant activity of citrus fruit extracts is due to the presence of flavonoids, carotenoids and ascorbic acid. Also, in non-volatile components of methanol extracted citrus peel, flavonoids and phenolic antioxidant compounds have been identified. The antioxidant effect or mechanisms are dependent on the real or model agents such as medium polarity, temperature, substrate, oxidation conditions, and oxidation environment physical state (emulsion, liquid, solid) which may cause differences in the antioxidant power of a molecule in various experiments. Ahn *et al*. (2006) found that the TBARS values increase over time (up to 8 days). Lipid oxidation increase by time can cause further release of free iron and other pro-oxidants from further muscle degradation during the storage. Generally speaking, with increasing the storage time, the hydrolysis and the oxidation of lipids increase, hydro-peroxides and conjugated DNA contents raise and the reagent increases. However, cooking method also is effective on the TBARS values. Boiling in water and baking increase the TBARS values, whereas broiling and frying do not cause changes in its values. Cooking changes antioxidant compounds, damages cellular structure and exposes membrane lipid to the environment. Raw meat has antioxidant effects, unless heated, denatured or pro-oxidants are added. Increasing the TBARS values of cooked meat can be explained by production of oxidized myoglobin that is more susceptible to oxidation. Fatty acids with several double bonds, such as EPA (eicosapentaenoic) and DHA (decosahexaenoic) during heating are susceptible to oxidation [55].

6.26. DMPD (N, N-dimethyl-P-phenilindiamine) Method

This method is based on the fact that in acidic pH and in the presence of appropriate oxidant solution, DMPD is able to form the stable colored radicals $DMPD^{\bullet+}$. $DMPD^{\bullet+}$ shows maximum absorption at 505 nm UV-VIS spectrum. Antioxidant compounds that can transfer hydrogen atoms to $DMPD^{\bullet+}$, bleach color and produce colorless solution corresponding to its values.

This reaction is fast and lasts less than 10 min and its fixed endpoint is the antioxidant criterion. Therefore, this method reflects a radical hydrogen donating ability to remove the single electron of $DMPD^{\bullet+}$. The selection of oxidant solution and the ratio between $DMPD^{\bullet+}$ concentration and oxidant composition are important for method's effectiveness. Hydrogen peroxide produces a colored solution with little stability. Sodium hypochlorite with moderate stability can solve this problem. However, the results of this compound have also failed commercially due to the changes in the concentration and low reproducibility. Using Cu^{2+} and ABAP produce radicals very slowly and the absorption increases continuously. The best results are obtained with $FeCl_3$ that is a colored stable solution up to 0.1 mM concentration, with low cost and high reproducibility. The absorption initiating point is between 0.8-1 at 505 nm for high sensitivity and sufficient inhibition. This value is obtained by 1 mM chromophore. Higher concentrations of DMPD do not produce more color. Different amounts of $FeCl_3$ are examined by using DMPD 1 mM concentration: the final concentration 0.1 mM is allowed to form enough color and prevent the formation of ferric ion residues in the colored solution. The best results are obtained in the molar ratio DMPD: Fe^{3+} of 10:1. Different concentrations of ascorbic acid and trolox are used to assess the method sensitivity. Standard solutions of these two antioxidants are stable for a week at 4 °C and at least for six months at 20 °C. the DMPD 100 mM solution is prepared by dissolving 209 mg DMPD in 10 mL deionized water; 1 mL of this solution is added to 100 mL acetate buffer 0.1 M and pH=5.25 and colored $DMPD^{\bullet+}$ cation radicals are obtain by adding 0.2 mL ferric choloride solution 0.05 M (final concentration 0.1 mM). 1 mL of this solution is directly added into a plastic cuvettes and the absorption is read at 505 nm. Optical density shows 0.9 ± 0.1 absorption units. The optical density of the fresh and daily prepared solution is constant up to 12 hours at room temperature. Standard solutions of different antioxidant compounds were produced as follow:

1 mg/mL ascorbic acid is prepared by dissolving 0.1 g ascorbic acid in 100 mL deionized water and trolox 1 mg/mL by dissolving 0.1 g trolox in 100 mL methanol. 50 µl standards antioxidants are added to cuvettes and the absorption is measured at 505 nm after 10 minutes at 25 °C.

The control cuvette contained the buffer solution is only added the solvent cuvettes:

Inhibition of A_{505} (%) = $(1 - A_f/A_0) \times 100$

A_0 = not inhibited radical cation absorption A_f = absorption 10 min after adding antioxidants.

$$\text{DMPD (uncolored)} + \text{Oxidant } (Fe^{3+}) + H^+ \rightarrow DMPD^{\bullet+} \text{ (purple)}$$

$$DMPD^{\bullet+} \text{ (purple)} + AOH \rightarrow DMPD^{+} \text{(uncolored)} + AO$$

This method is based on expanding the radical reduction in a specified time and on reduction rate. In contrast to the ABTS methods, the DMPD method ensures a very stable endpoint. In this method, time change is not as important as in the ABTS colorless evaluation. The main disadvantage of DMPD is that the sensitivity and reproducibility in the case of using hydrophobic antioxidants such as α-tocopherol and BHT or application of methanol as a solvent decline considerably [56].

6.27. Scavenging Activity of Superoxide Anion

The phenansin–methosulfate (PMS)-NADH method presented by Robak and Gryglewskiis in 1998 is used to produce O_2^-. Test tubes containing 12 µM, PMS, 100 µM, anion O_2^- and 100 µM tetrazolium nitro blue (NBT) in phosphate buffer 0.1 M (K_2HPO_4-KH_2PO_4) at pH=7.8 are incubated at room temperature for 2 min. Then 100 µL HCl 0.1 M is added to stop the reaction. The spectrophotometric absorption is measured at 560 nm versus blank samples in the absence of PMS. Ascorbic acid and other components are added to the test tubes at a different series of concentrations before adding PMS. In the PMS/NADH-NBT system, a superoxide anion is produced using PMS, which is a non-enzymatic reaction in the presence of NADH and molecular oxygen. In both methods, the superoxide anion reduces NBT to fourmazan at room temperature and pH=7.8 with absorption at 560 nm. The absorption decline at 560 nm with antioxidants is an indication that the superoxide anion is used in the reaction mixture. The superoxide anion radical scavenging activity is likely related to the presence of phenolic compounds. Increase in the activity is due to the increase in the number of phenolic hydroxyl groups in the molecule [10]. Although the superoxide anion is a weak oxidant, yet it produces powerful and dangerous hydroxyl radicals and single oxygen, which contribute to the oxidative damage. Therefore, scavenging superoxide radicals by antioxidants has medical effects [2]. the reaction mixture containing 1.1 mL phosphate buffer 0.1 M (pH=4.7), 0.5 mL NADH (reduced nicotine amide adenine dinucleotide) 6.105 µM, 0.5 ml NBT 66 µM and 0.1 mL of samples in different concentrations is dissolved in methanol. The reaction is initiated by adding 0.5 mL PMS 30 µm in to the mixture. Methanol

can be used as the control. After 10 min when the reaction mixture reached a fixed color, the absorption versus solvent is read at 560 nm. The ability to scavenge superoxide radicals is estimated using the following equation and the results are expressed as mg sample needed to prevent the oxidation of 50% NBT (IC_{50}) [14].

Scavenging Effect (%) = $[(OD_{control} - OD_{sample}) / OD_{control}] \times 100$

6.28. Nitric Oxide Radicals Trapping Method

This method is based on this assumption that sodium nitroprusside which reacts with environmental oxygen to produce nitrite ions in an aqueous solution at physiological pH slowly produces nitric oxide. Nitrite ions are evaluated in the presence of the Grace reagent. Nitric oxide trapping and competing with oxygen decrease the nitrite ions production and in order to do this, sodium nitroprusside is dissolved in phosphate buffer at various concentrations of the extract which were separately dissolved in water. The mixture is incubated at room temperature for 150 min. Then 0.5 mL of the Grace reagent (including sulfanil amide 1%, naftil ethylene diamine dihydrochloride 0.1% in 2% phosphoric acid) is added to the mixture. The absorption at 546 nm is read versus solvent. Quercetin is used as the control for comparison [57]. The reactions between nitric oxide (NO) and superoxide radical ($O_2^{\bullet-}$) result in the production of oxidant peroxy nitrite ($ONOO^-$) with constant rate of 6.7×10^9 M/s. Proxy nitrite can cause lipid peroxidation, oxidation of methionine and sulfhydryl residues in proteins, decrease in antioxidants and damage to DNA. Adding proxy nitrite to bio streams causes the nitration of tyrosine residues which indicates are proxy related injuries in the living organism.

In addition, the excess amounts of 3-nitrotyrosine has been identified in many human diseases such as parkinson's, alzheimer's and asthma. $ONOO^-$ scavenging by antioxidants is based on techniques such as tyrosine nitration and ^{1}α-proteinase deactivation which are used as useful research tools in laboratories and provide information about the antioxidant profile of body guards nervous. Ergothionin is an $ONOO^-$ scavenger [23].

6.29. HOCl Radicals Scavenging Method

This method according to Ching *et al.* (1994) and Fernandes *et al*. (2003) is based on thionitrobenzoin acid (TNB) oxidation to dithionitrobenzoin acid (DTNB) which is mediated with HOCl. The TNB oxidation is identified by a decline in the absorption at 412 nm which can be inhibited with HOCl scavenger in the reaction. HOCl by adjusting the pH with 2 mM NaOCl solution with H_2SO_4 0.5 M is prepared before using. The HOCl spectrophotometric concentration at 235 nm is 100 M/cm. HOCl will be diluted for each test. TNB is prepared by incubation of DTNB 1 mM in potassium phosphate buffer 50 mM at pH=6.6 (completed with EDTA 5 mM) with $NaBH_4$ 20 mM for 30 min at 37 °C. The TNB concentration by spectrophotometry at 412 nm wavelength is 13600 M/cm. DTNB is diluted with buffer for each assessment. Tests are performed at room temperature and the reaction mixtures (final volume 3 mL) contain the same molar quantities of TNB, HOCl (40 μM) and plant extracts in the suitable concentrations. The absorption is measured at nm 4125 minutes after HOCl is added. The amounts of unchanged (not oxidized) TNB are calculated after the incubation and expressed as initial percentage of the TNB concentration [24]. A review of some previous works is presented in Table **4**.

Table 4: A Review of Some Previous Works

Method	Tested compounds	References
FRAP and TBARS	Caraway	[58]
FCR, FRAP and ORAC	Blackberries	[59]
TEAC and ORAC	Peanuts	[60]
DPPH, TEAC and lipoxygenase inhibition	Walnuts and almonds	[61]
DPPH, TEAC and FRAP	Green tea	[62]
DPPH and β-carotene bleaching	Raw and blanched vegetables	[29]
TEAC	Yellow and purple plum	[63]
TEAC	Mango	[64]
DPPH and FRAP	Pomegranate	[65]
TEAC	Grape seeds oil	[66]

(Table 4). Continued.

Method	Tested compounds	References
DPPH	Sweet cherry	[67]
DPPH	Tomato	[68]
DPPH TEAC) III) and superoxide radicals scavenging	*Ficus microcarpa* L. fil. extract	[14]
DPPH and FCR	Pepper	[30]
TEAC (III), FCR and DPPH	White cabbage	[9]
FRAP	Citrus	[32]
TEAC and FCR	Native Australian fruits	[69]
DPPH ,TEAC (III), FRAP and β-carotene bleaching	Cereals and fruits	[21]
DPPH and TEAC	Persimmons	[70]
Oven test and peroxide value	Natural resins and bioactive triterpenes in oil substrates	[71]
DPPH and β-carotene bleaching	*Artemisia dracunculus* L. and *Matricaria chamomilla* L. essential oils	[72]
Peroxide, thiobarbituric acid (TBA) and free fatty acid values	*Coriandrum sativum L.* essential oil	[73]
Peroxide (PV), conjugate dienes (CD) and conjugate trienes (CT) values.	Satureja hortensis L. and Zataria multiflora Boiss essential oils	[74]
Oven test	Tea seed oil	[75]
DPPH and Peroxide, thiobarbituric acid (TBA) and free fatty acid values	Tea and sesame seed oils	[76]
Peroxide value	Linseed oil	[77]
Peroxide, thiobarbituric acid (TBA) and free fatty acid values	Chamomile essential oil	[78]
Peroxide and thiobarbituric acid values	*Cinnamon verum* essential oil and echinacea extract	[79]
Peroxide and thiobarbituric acid values	Tea and sesame seed oils	[80]
DPPH, ABTS and β-carotene bleaching	Pistachio (Ahmadaghaei variety) green hull extracts (crude and purified extracts)	[81]
Peroxide and thiobarbituric acid values	Extract of aerial parts of *Echinacea purpurea* L.	[82]
DPPH, ABTS and β-carotene bleaching	Leaf extract of *Hyssopus officinalis* L. and extract of aerial parts of Echinacea purpurea L.	[83]
DPPH, ABTS and β-carotene bleaching	Essential oil of *Lavandula angustifolia* and *Malva sylvestris* L. extract	[84, 85]
Peroxide (PV) and conjugate dienes (CD) values.	Zataria multiflora Boiss. and *Satureja hortensis* L. essential oils	[86]
Peroxide and thiobarbituric acid values	Pomegranate peel extracts	[87]
DPPH, ABTS, β-carotene bleaching, hydrogen peroxide, Fe-reducing power, peroxide and thiobarbituric acid values	Zataria multiflora and Bunium persicum essential oils	[88]

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