Evaluation of some food processing by-products as sources for natural antioxidants

Untersuchung und Charakterisierung von Nebenprodukten der Lebensmittelindustrie als Quelle für natürliche Antioxidantien

vorgelegt von

Adel Abdelrazek Abdelazim Mohdali, M.Sc.

aus El Fayoum (Ägypten)

von der Fakultät III - Prozesswissenschaften

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Promotionsausschuss:

Vorsitzender: Prof. Dr. Dipl.-Ing. Dietrich Knorr

Berichter : Prof. Dr. Dipl.-Ing. Iryna Smetanska

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Evaluation of some food processing by-products as sources for natural antioxidants

Submission from

Adel Abdelrazek Abdelazim Mohdali, M.Sc.

from El Fayoum (Egypt)

Faculty III - Process Science, Institute of Food Technology and Food Chemistry,

Technical University of Berlin,

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This work has been approved by:

Vorsitzender: Prof. Dr. Dipl.-Ing. Dietrich Knorr

Berichter : Prof. Dr. Dipl.-Ing. Iryna Smetanska

Berichter : Prof. Dr. Monika Schreiner

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Zusammenfassung

Synthetische Antioxidationsmittel in Nahrungsmitteln sollen künftig durch natürliche Stoffe mit antioxidativen Eigenschaften ersetzt werden, was durch die Erforschung an verschiedenen Gemüsen sowie an Rohstoffen mit neu identifizierten Antioxidantien gefördert wird.
Die Lebensmittelindustrie erzeugt wesentliche Mengen an phenolreichen Nebenprodukten, welche wertvolle Quellen für Antioxidantien darstellen. Im Rahmen dieser Untersuchungen werden die antioxidativen Eigenschaften sowie die Gesamtgehalte an phenolischen Verbindungen (Flavonoide, Flavonole) von drei industriellen Nebenprodukten untersucht. Dazu wurden Kartoffelschalen, Fruchtfleisch der Zuckerrüben und Sesamkuchen mit verschiedenen Extraktionsmitteln behandelt und später untersucht. Die antioxidativen Verbindungen haben unterschiedliche Aktionsmechanismen, so dass es notwendig war, verschiedenste Methoden zur Beurteilung der antioxidativen Effizienz der Extrakte vorzunehmen. Unter den sechs getesteten Lösungsmitteln stellte sich Methanol als das optimalste Extraktionsmittel mit den höchsten Extraktausbeuten bei Kartoffelschalen und Zuckerrüben heraus. Dagegen erbrachten die Extraktionen von Sesamkuchen mit Diethylether die besten Ergebnisse. Methanol besitzt für phenolische Verbindungen das höchste Extraktionspotential mit Gesamtphenolgehalten von 2.91, 1.79 und 0.81 mg Gallussäure / g Trockengewicht in Kartoffelschalen, Zuckerrüben und Sesamkuchen sowie die höchsten antioxidativen Fähigkeiten der drei genannten Proben. Alle drei Methoden zeigen, dass Kartoffelschalen die höchsten antioxidativen Aktivitäten auf grund ihrer hohen Gehalte an phenolischen Verbindungen und Flavonoiden aufweisen. Die bioaktiven Komponenten, welche mit Methanol bzw. Ethanol extrahiert wurden, konnten in weiteren Analysen auf ihre antioxidativen Fähigkeiten untersucht werden und mit synthetischen Antioxidantien verglichen werden. Dieses Experiment wurde modellhaft unter besonderen beschleunigten Oxidationsbedingungen unter Verwendung von Sonnenblumen- und Sojabohnenöl als Oxidationssubstrat in unterschiedlichen Konzentrationen bei 70°C für 72 Stunden duchgeführt. Es wurden inverse Verhältnisse zwischen Peroxidwerten und oxidativen Stabilitäten sowie zwischen sekundären Oxidationsprodukten, gemessen durch p- Anisidin und den Stabilitäten nach beendeten Lagerungen, festgestellt. Die Absorptionsfähigkeit bei 232 nm und 270 nm ist gleichmäßig mit zunehmender Zeit gestiegen, welches durch die Formation der konjugierten Diene und Polyene verursacht wird. Die Reihenfolge der

oxidativen Stabilitäten sieht wie folgt aus: TBHQ > Kartoffelschalen > BHT = Fruchtfleisch der Zuckerrüben > BHA > Sesamkuchen. Die HPLC Analysen der Kartoffelschalen-, Zuckerrüben- und Sesamkuchenextrakte spiegeln das Vorhandensein der phenolischen Verbindungen wieder. Auf Basis der erhaltenen Ergebnisse stellen Kartoffelschalen, Zuckerrübenfruchtfleisch und Sesamkuchen wertvolle Quellen natürlicher Antioxidantien wieder. Diese antioxidativen Aktivitäten können sinnvoll in der Lebensmittelindustrie verwendet werden, um der Oxidation von Ölen vorzubeugen. Ausserdem könnten sie Verwendung als Konservierungsstoff in der Lebensmittel- und Pharmaindustrie finden.

Schlüsselwörter: beschleunigte Lagerung, antioxidative Aktivität, Nebenprodukt, oxidative Stabilität, Kartoffelschalen, Sesamkuchen, Lösungsmittelpolarität, Sojabohnenöl, Fruchtfleisch der Zuckerrüben, Sonnenblumenöl, Gesamtgehalte Flavonoide und Phenole

ABSTRACT

Growing interest in the replacement of synthetic food antioxidants by natural ones has fostered research on vegetable sources and screening of raw materials to identify new antioxidants. The food-processing industry generates substantial quantities of phenolic-rich by-products that could be valuable natural sources of antioxidants. In this study the antioxidant properties and total phenolic, flavonoid, and flavonol contents of three industrial by-products; potato peels, sugar beet pulp, and sesame cake, extracted with various solvents were examined. Since the antioxidant compounds have different mechanisms of action, several methods were used to assess the antioxidant efficacy of extracts. Among the six solvents tested, methanol gave the highest extract yield of potato peels and sugar beet pulp, while diethyl ether gave the highest extract yield of sesame cake. Methanol exhibited the highest extraction ability for phenolic compound, with total phenolics amounting to 2.91, 1.79, and 0.81 mg gallic acid equivalent g^{-1} dry weight in potato peels, sugar beet pulp, and sesame cake extracts respectively, and also showed the strongest antioxidant capacity in the three assays used. All three methods proved that potato peels extract had the highest antioxidant activity owing to its high content of phenolic compounds and flavonoids. The bioactive materials which extracted with methanol and ethanol were further examined for their antioxidant activity in comparison with synthetic antioxidants under accelerated oxidation conditions using sunflower and soybean oils as oxidation substrates at different concentrations for 72 h at 70 °C. Inverse relationships were noted between peroxide values and oxidative stabilities and also between secondary oxidation products, measured by *p*-anisidine value and stabilities at termination of the storage. Absorptivity at 232 nm and 270 nm increased gradually with the increase in time, due to the formation of conjugated dienes and polyenes. The order of oxidative stability was as follow: TBHQ > potato peels > BHT = sugar beet pulp > BHA > sesame cake. The HPLC analysis of potato peels, sugar beet pulp, and sesame cake extracts revealed the presence of phenolic compounds. On the basis of the results obtained, potato peels, sugar beet pulp, and sesame cake extracts could serve as natural antioxidants, owing to their significant antioxidant activity, that might be explored to prevent oxidation of vegetable oils. Therefore they could be used as preservative ingredients in the food and/or pharmaceutical industries.

Keywords: Accelerated storage, antioxidant activity, by-products, oxidative stability, potato peels, sesame cake, solvent polarity, soybean oil, sugar beet pulp, sunflower oil, total flavonoids, total phenolics content

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Adel Abdel razek Abdel azim Mohdaly

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LIST OF ABBREVIATIONS

TPC	Total phenolic contents
FCR	Folin-Ciocalteu reagent
GAE	Gallic acid equivalent
DW	Dry weight
QE	Quercetain equivalent
PV	Peroxide value
PPE	Potato peel extracts
SCE	Sesame cake extracts
SBP	Sugar beet pulp
AV	*p*-anisidine value
CD	Conjugated dienes
CT	Conjugated trienes
SFO	Sunflower oil
SBO	Soybean oil
TLC	Thin layer chromatography
HPLC	High-performance liquid chromatography
GC	Gas chromatography
MS	Mass spectrometry
UV/Vis	Ultraviolet-visible light
NMR	Nuclear magnetic resonance
AOA	Antioxidant activity
ROOH	Hydroperoxide
PUFA	Polyunsaturated fatty acid

AE	Antiradical efficiency
$^1O_2$	Singlet oxygen
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
PG	Propyl gallate
DG	Dodecyl gallate
TBHQ	Tertiary butyl hydroquinone
NDGA	Nordihydroguaiaretic acid
AOM	Active oxygen method
OS	Oxidative stability
ORAC	Oxygen radical absorbance capacity
TEAC	Trolox equivalent antioxidant capacity
DPPH	1,1-diphenyl-2-picrylhydrazyl
ABTS	2, 2′-azinobis (3-ethylbenzthiazoline- sulphonic acid)
TBA	Thiobarbituric acid value
PAL	Phenylalanine ammonia lyase
PAF	Platelet activating factor
ROS	Reactive oxygen species
RCS	Reactive chlorine species
RNS	Reactive nitrogen species
GRAS	Generally recognized as safe
GAC	Granulated activated carbon
CME	Crude methanolic extract
RSA	Radical scavenging activities
FDA	Food and drug administration

1 INTRODUCTION

One of the principal causes of food quality deterioration is lipid peroxidation (Gordon, 1991). Lipid peroxidation results in formation of reactive oxygen species and free radicals; which are purportedly associated with carcinogenesis, mutagenesis, inflammation, DNA changes, aging, and cardiovascular diseases (Shahid *et al*., 2008). Food lipids undergo a variety of chemical reactions such as accelerated oxidation, thermolysis, and polymerization under heat exposure (Jinyoung *et al*., 2008). Oxidation of oils modifies their organoleptic properties and affecting the shelf life of the product. It results in the loss of nutritional value of food as well as changes in colour, texture, sensory and other physiological properties (Iqbal and Bhanger, 2007). Due to these changes, consumers do not accept oxidized products and industries suffer from economic losses. The oil industry has to pay special attention in this context, as oils, fats and fatty foods suffer stability problems (Valenzuela *et al*., 2003). The oils with higher contents of unsaturated fatty acids, especially polyunsaturated fatty acids, are more susceptible to oxidation. It is, therefore, important to evaluate the oxidative stability (OS) of oils as affected by processing and storage conditions.

Several methods have been reported to measure the OS of edible oils. The oxidative stability of oils and fats with added antioxidants can be determined during storage under normal ambient conditions and packing. However, in general, oxidation can take a long time to occur, e.g. a few days to a few months, which is impractical for routine analysis. For this reason, accelerated oxidation or aging tests are conducted. Normally Schaal oven test is used for determination of oxidation of oils (Mahuya *et al*., 2008). Storage of oil samples at high temperatures (oven test) was employed for monitoring OS of oils and for antioxidant choice. The extent of oxidation in oils has been frequently evaluated by measuring the peroxide value (PV). This index is related to the hydroperoxides, the primary oxidation products, which are unstable and readily decompose to form mainly mixtures of volatile aldehyde compounds. The oxidative degradation compounds that derived from degradation of hydroperoxides are generally termed secondary oxidative products which are determined in oils and fats by the *p*-anisidine (AV) method (Ramadan and Mörsel, 2004).

In order to overcome the stability problems of oils and fats, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ) have been used as food additives. However, recent reports reveal that these compounds may be implicated in many health risks, including cancer and carcinogenesis

(Prior, 2004). Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which are supposed to be safer (Yanishlieva and Marinova, 2001). Natural antioxidants such as flavonoids, tannins, coumarins, curcumanoids, xanthons, phenolics, lignans and terpenoids are found in various plant products (such as fruits, leaves, seeds, and oils) (Jeong *et al*., 2004) and they are known to protect easily-oxidizable constituents of food from oxidation.

Processing of fruits, vegetables, and oilseeds result in large amounts of waste materials such as peels, seeds and stones. Disposal of these materials usually represents a problem that is further aggravated by legal restrictions. Thus, new aspects concerning the use of these wastes as by-products for further production of food additives or supplements with high nutritional value have gained increasing interest because these bioactives are high-value products and their recovery may be economically attractive (Vasso and Constantina, 2007).

Different solvent systems have been used for the extraction of polyphenols from plant material (Pinelo *et al*., 2004). Extraction yield is dependent on the solvent and the method of extraction (Goli *et al*, 2004). Wang and Helliwell (2001) reported that aqueous ethanol was superior to methanol and acetone for extracting flavonoids from tea. However, in another work, water was found to be a better solvent, for extracting tea catechins, than were 80% methanol or 70% ethanol (Khokhar and Magnusdotti, 2002).

Potato (*Solanum tuberosum* L., Solanaceae) is one of the most important staple crops grown worldwide. Because of its low cost, low fat content and a good source of carbohydrates, high quality protein, fibre and vitamins, it plays an important role in human nutrition. Although potatoes are consumed directly, processed potato products such as French fries, chips, mashed potato, crisps, starch, potato flakes, flour, and puree represent majority of the consumption (Amir and Venket, 2009). In processing, 10-25% of the raw product is discharged as waste. Peels are the major waste of potato processing that is perishable and cause many management problems in terms of disposal and sanitation.

Sugar is a strategic commodity to many countries of the world, since it comes right after wheat from the importance point of view to many countries in Europe, Africa, North and South America and Australia. In Egypt, sugar cane was considered to be the main source for sugar industry up to 1981 season and the cultivation of sugar beet did not known economically before 1982 season. Nowadays, sugar beet (*Beta vulgaris* L., Chenopodiaceae) becomes an important crop for sugar in Egypt. Beet pulp is a sugar-depleted and highly fibrous material that is produced after sugar is extracted from sugar beet. Pulp is a valuable

cattle feed and supplies carbohydrates, proteins, and minerals. The pulp content of sugar beet ranges from 4 to 6% (Mosen asadi, 2007).

Sesame (*Sesamum indicum* L., Pedaliaceae) is one of the most important oilseed crops (because of its high content of lipid) in the world (Shyu and Hwang, 2002). It is not only a source of edible oil, but also widely used in baked goods and confectionery products (Namiki, 1995). In Egypt, the major part of the imported sesame is essentially transformed to Halaweh. This food product is obtained after mixing the white tehineh (white sesame seed dehulled, roasted and grinded), saponin (Saponaria officinalis) and Nougat (heat-treated sucrose) (Abu-Jdayil *et al*., 2002). The sesame cake is a by-product of the oil industry which could be recovered and used as a value added product. However, in some sesame processing countries, this by-product is generally discarded, or used in animal feeding.

Due to this fact we investigated the possibilities of obtaining natural antioxidant compounds from inexpensive residual sources. Therefore, the objectives of this study were to evaluate potato peels, sesame cake, and sugar beet pulp as sources of natural antioxidant using different extracting solvents to determine their antioxidant capacities. In the study, the content of total phenols, flavonoids, and flavonols were determined. Since different antioxidant compounds have different mechanisms of action, different methods have been used to assess the antioxidant efficacy of extracts. The aim of this work was also to evaluate the antioxidant effectiveness of potato peels, sesame cake, and sugar beet pulp extracts during oxidation of sunflower and soybean oils by measuring both primary (hydroperoxides) and secondary oxidation products and to compare its antioxidant activity with commercially antioxidants. The study also attempts to identify the antioxidant compounds present in the extracts using chromatographic and spectroscopic techniques.

2 REVIEW OF LITERATURE

2.1 General

Lipids are broadly defined as any fat-soluble (lipophilic), naturally-occurring molecule, such as fats, oils, waxes, cholesterol, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, phospholipids, and others. The main biological functions of lipids include energy storage, acting as structural components of cell membranes, and participating as important signaling molecules (Maton *et al*., 1993). Chemically, fats are generally triesters of glycerol and fatty acids. Fats may be solid or semi-sold at normal room temperature, depending on their chemical composition. Although the words "oils", "fats", and "lipids" are all used to refer to fats, "oils" is usually used to refer to fats that are liquids at room temperature, while "fats" is usually used to refer to fats that are solids at normal room temperature. "Lipids" is used to refer to both liquid and solid fats. Examples of animal fats are lard (pig fat), fish oil, and butter or ghee. Examples of edible plant fats are peanut, soya bean, sunflower, sesame, coconut, olive, and other vegetable oils. (Maton *et al.,* 1993).

Fats, oils and lipid-based foods deteriorate through several degradation reactions both on heating and on long term storage. The main deterioration processes are oxidation reactions and the decomposition of oxidation products which result in decreased nutritional value and sensory quality. The retardation of these oxidation processes is important for the food producer and, indeed, for all persons involved in the entire food chain from the factory to the consumer. Oxidation may be inhibited by various methods including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalysing oxidation, reduction of oxygen pressure, and the use of suitable packaging. Another method of protection against oxidation is to use specific additives which inhibit oxidation. These are correctly called oxidation inhibitors, but nowadays are mostly called antioxidants. These inhibitors represent a class of substances that vary widely in chemical structure, and have diverse mechanisms of action.

2.2 Antioxidant activity

2.2.1 Free radicals

Lipid oxidation is a process with detrimental effects occurring in foods and the metabolically active cells of the body. In foods it can lead to rancidity and loss of nutritional value, flavor, odor, spoilage and potential toxicity. In the cell, however lipid oxidation and products of lipid oxidation are associated with many conditions of cellular damage and cytotoxicity. This is due to changes in membrane structure and fluidity, increased permeability of membranes and damage to biologically important molecules such as DNA and proteins, resulting in chronic diseases such as a herosclerosis and cancer. (De Beer *et al*., 2002).

Lipid oxidation leads to free radical generation. A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital (Young and Woodside, 2001). Radicals are weakly attracted to a magnetic field and are said to be paramagnetic. There are several processes that lead to lipid oxidation, including autoxidation, photoxidation and lipolysis.

2.2.2 Lipid oxidation

2.2.2.1 Lipid autoxidation

Autoxidation is a natural process that takes place between molecular oxygen and unsaturated lipids in the environment. The process of autoxidation of polyunsaturated lipids in food involves a free radical chain reaction that is generally initiated by exposure of lipids to light, heat, ionizing radiation, metal ions or metalloprotein catalysts. Enzyme lipoxygenase can also initiate oxidation. The classical route of autoxidation includes initiation (production of lipid free radicals), propagation and termination (production of nonradical products) reactions (Reaction 2.1 to Reaction 2.6) (Sanchez-Moreno *et al*., 1998).

Initiation: $RH \longrightarrow R^{\bullet} + H^{\bullet}$ (2.1)

Propagation: $R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$ (2.2)

$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$ (2.3)

Termination: $R^{\bullet} + R^{\bullet} \longrightarrow RR$ (2.4)

$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR$ (2.5)

$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$ (2.6)

Where:
RH = Unsaturated fatty acid
$R^{\bullet}$, $ROO^{\bullet}$ = Free radicals
ROOH = Hydroperoxides

The initiation step occurs when an unsaturated lipid (RH) loses a hydrogen atom, forming a free radical ($R^{\bullet}$). The free radical thus formed reacts with oxygen in the propagation step to form an unstable peroxyl radical ($ROO^{\bullet}$), and this last in turn abstract an hydrogen from another lipid (RH) to yield unstable hydroperoxide (ROOH), the primary product of autoxidation, and another free radical ($R^{\bullet}$). The new ($R^{\bullet}$) groups react with oxygen, and the sequence of reactions just described is repeated. This chain reaction continues until either the unsaturated compound has been exhausted or the free radicals have inactivated each other. The propagation reaction becomes a continuous process as long as unsaturated fatty acids are available (German and Dillard, 1998). Transition metals speed up deterioration in food lipids where they are powerful catalysts of autoxidation and initiate free radical chain reaction by electron transfer (Bors *et al*., 1996).

2.2.2.2 Photosensitised oxidation

In food, photosensitized oxidation occurs in the presence of photosensitisers. Natural pigments in foods such as chlorophyll, flavins, protoporphyrins, and riboflavins are efficient photosensitisers (Bradely *et al*., 1992). These sensitisers absorb energy in the visible or near ultraviolet regions. This renders them excited and more efficient to generate singlet oxygen in foods. As singlet oxygen ($^1O_2$) is highly electrophilic, it can react rapidly with unsaturated lipids and initiates lipid oxidation by the ene-reaction (Figure 2.1) to yield lipid hydroperoxides.

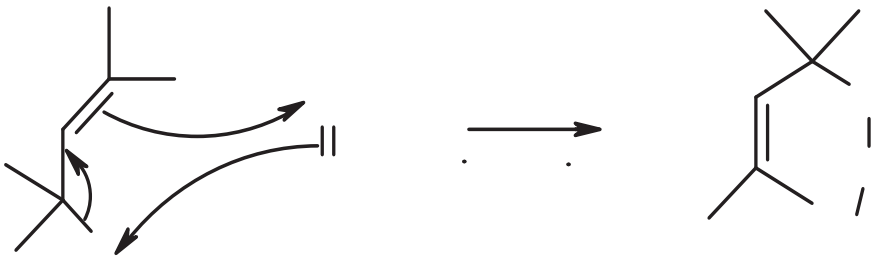


Figure 2.1. The ene reaction (Bradely *et al*., 1992).

Photosensitised oxidation generally involves light excitation of the electrons in the outer orbital of the sensitiser molecule to the singlet state (^{1}Sensitiser*), followed by intersystem crossing to the triplet state (^{3}Sensitiser*).

^{1}Sensitiser + hv ⟶ ^{1}Sensitiser* ⟶ ^{3}Sensitiser*

Two pathways have been proposed for photosensitized oxidation (Chen, 1997):

a) The sensitiser presumably reacts, after absorption of light, with substrate (A) to form intermediates which then react with triplet oxygen and yield the oxidation products.

^{3}Sensitiser* + A ⟶ (Sensitiser - A)

(Sensitiser - A) + $^3O_2$ ⟶ AO_2 + ^{1}Sensitiser

b) Molecular oxygen rather than the substrate is presumably the species that reacts with the sensitiser upon light absorption.

^{3}Sensitizer* + $^3O_2$ ⟶ $^1O_2$* + ^{1}Sensitizer

$^1O_2$* + RH ⟶ ROOH ⟶ Free radicals

Where:

^{1}Sensitizer*: Activated singlet state sensitiser

^{3}Sensitizer*: Activated triplet state sensitiser

$^1O_2$*: Activated singlet state oxygen

$^3O_2$: Activated triple state oxygen

The lifetime of singlet O_2 in the hydrophobic cell membrane is greater than in aqueous solution. Furthermore, photo-oxidation is a quicker reaction than autoxidation since it was demonstrated that photo-oxidation of oleic acid can be 30 000 times quicker than autoxidation and for polyenes photo-oxidation can be 1000-1500 times quicker. Similar effects have been described in liposomes and in intact membranes.

The inhibition of photosensitised oxidation by carotenoids is complicated because they are very susceptible to autoxidation, and are quickly destroyed during the free-radical oxidation process. To be effective in unsaturated lipids exposed to light irradiation, carotenoids must be protected by an antioxidant (Haila *et al*., 1996; Yanishlieva *et al*., 2001). Without antioxidant protection, e.g. in pure triacylglycerols of rapeseed (Haila *et al*., 1996) and of sunflower (Yanishlieva *et al*., 2001) oils, they exert a pro-oxidative effect. Photosensitised oxidation can be also quenched by tocopherols (Edwin, 2006) and flavonoid substances (Criado *et al*., 1995).

2.2.3 Antioxidants

An antioxidant is defined as: "any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate" (Young and Woodside, 2001). The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside, 2001). Antioxidants protect key cell components from damage by neutralizing or preventing the free radicals. They do this by giving up electrons to the free radicals or by removing or inactivating chemical intermediates that produce free radicals. Antioxidants can interfere with the oxidation process by reacting with free radicals in one or more of the following ways: (I) as reducing agents, (II) as free radical scavengers, (III) as complexers of prooxidant metals and (IV) as singlet oxygen quenchers (Pratt and Hudsom, 1990).

Since there are many different oxidation reactions, from the oxidation of iron to rust, to the transformation of lipids to peroxide, there is no single substance that is a universal antioxidant, and there can be no standard antioxidant test because antioxidants are situation

dependent. Each different oxidation reaction will have particular requirements to stop the reaction and respond to specific antioxidant compounds.

Antioxidants are provided through the diet or are produced by the body (Papas, 1999). Antioxidants cannot reverse damage, but they can retard its progress. Damage that leads to chronic diseases is cumulative, usually occurring over decades. It is very important that antioxidant requirements should be met on a daily basis to slow this cumulative damage that builds up over the course of a lifetime (Madhavi and Salunkhe, 1995).

2.2.3.1 Classification of antioxidants

Antioxidants can be classified into primary antioxidants (chain-breaking) and secondary (preventive) antioxidants, according to the mechanism by which they prevent or retard oxidation (Koleva *et al*., 2002). Primary antioxidants interrupt oxidation by donation of hydrogens or electrons to free radicals ($R^{\bullet}$, $ROO^{\bullet}$, $RO^{\bullet}$), resulting in the formation of more stable products such as antioxidant free radicals ($A^{\bullet}$). Secondary antioxidants inhibit the oxidation of lipids by delaying and retarding the rate of oxidation rather than by stopping the radical chain reaction. Some antioxidants may exhibit more than one mechanism, and are therefore called multiple-function antioxidants (Madhavi and Salunkhe, 1995).

Along with specific antioxidant enzymes, antioxidant compounds are physically classified according to their solubility into two major groups: water soluble (hydrophilic) antioxidants (ascorbic acid, polyphenolic compounds as flavonoids, uric acid and thiols) and lipid soluble (lipophilic) antioxidants (vitamin E, carotenoids, ubiquinols) (Arnao *et al*., 2001).

2.2.3.2 Mechanisms of action

Scavenging of free radicals: The reaction of the scavenger with an intermediary free radical and the formation of more or less stable secondary radicals usually lead to final products being different from the products in the absence of the scavenger. Radical scavengers usually donate one electron to the unpaired electron of the free radical, and thus reduce it. Polyphenols are very active in this respect and the radical scavenging activities of propylgallate, nordihydroguaiaretic acid, ellagic acid, flavonoids, ascorbic acid, and tocopherol are due to this function (Kochhar and Rassell, 1990).

Quenching of singlet excited states: The physical transfer of energy from the primarily excited molecule to the quencher results in energy dissipation by light emission or as heat. It thus avoids single electron transfer reactions or direct reaction of the primarily excited molecule with critical targets. Tocopherol and carotenes are physical quenchers of excited states of pigment molecules, as well as of singlet oxygen (Eriksson and Na, 1995).

Chelating of transition metals: Certain oxidation reactions depend on the availability of metals. Interference with this process of catalysis by chelating would have a strong effect on the progress of the radical reaction. Metal chelating agents have effect on increasing the oxidation stability through blocking the pro-oxidant metal ions, and thus limiting the formation of chain initiators by preventing metal-assisted homolysis of hydroperoxides. Many metal chelating substances are present in foods, especially in plant materials. The salts of phytic acid, phospholipids, and oxalates are the most common representatives of this group. Phosphoric, citric, tartaric, quercetin, caffeic, malic and ascorbic acids also possess pronounced chelating activities. Polyphosphates are added to inactivate iron, for example, in meat products (Chen and Ahn, 1998).

Inactivation/ Activation of enzymes: Some enzymes can catalyse highly oxidative species to more stable species; for instance, the enzyme superoxide dismutase can reduce the superoxide radicals $O_2^{\bullet}$, which are produced from hydrogen peroxide, into triplet oxygen

Synergism: Synergism plays an important role in the efficiency of antioxidants. Synergist antioxidants can be classified as oxygen scavengers or chelators. They function by the combination of different mechanisms. In this case, they may be more active than if used alone. This synergistic effect is significant for the reduction in the level of antioxidants added to food, thereby minimising the undesirable side effects of antioxidants, as well as production costs. Pronounced synergistic effects occur between phenolic compounds and certain acidic substances such as ascorbic acid, citric acid and phosphoric acid (Donnelly and Robinson, 1991). The synergistic effect of citric acid is attributed to metal chelation (Frankel, 1998). Other polyvalent acids such as tartaric, malic, gluconic, oxalic, succinic and hydroxyglutaric acids, as well as sodium triphosphate and pyrophosphate also possess synergistic properties similar to those of citric acid. Another chelator, phytic acid (inositol hexaphosphate), has been also reported to be a synergist in lipid oxidation (St Angelo *et al*., 1990). Ascorbic acid can

act as a synergist with tocopherols by regenerating or restoring their antioxidant properties (Niki, 1987).

2.2.3.3 Natural antioxidants

Antioxidants in foods may originate from compounds that occur naturally in the foodstuff or from substances formed during its processing (Shahidi, 1997; Shahidi and Wanasundara, 1992). Natural antioxidants are primarily phenolic and polyphenolic compounds. They are multifunctional and can act as reducing agents (free radical terminators), metal chelators, and singlet oxygen quenchers. Examples of common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acids (Pratt and Hudson, 1990). Several studies have been carried out in order to identify natural phenolics that possess antioxidant activity. Some natural antioxidants have already been extracted from plant sources and are produced commercially (Schuler, 1990).

Tocopherols and their antioxidant activity: Monophenolic antioxidants as tocopherols stabilize most vegetable oils. Tocopherols are composed of eight different compounds belonging to two families, namely, tocols and tocotrienols, referred to as α, β, γ, or δ, depending on the number and position of methyl groups attached to the chromane rings. Tocopherols also possess vitamin E activity. In tocols, the side chain is saturated, while in tocotrienols it is unsaturated. With regard to vitamin E activity, α-tocopherol is the most potent member of this family (Figure 2.2).

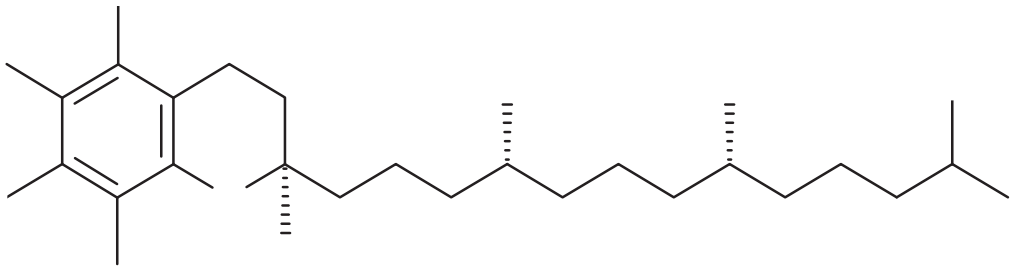


Figure 2.2. Structure of alpha tocopherol (Schuler, 1990).

The antioxidant activity decreases from δ to α (Dziezak, 1986). Vegetable foods contain considerable amounts of different tocopherols and tocotrienols in their lipid fraction. Cereals and cereal products, oilseeds, nuts and vegetables are rich sources of tocopherols; however, in the animal kingdom, tocopherols are only found in trace quantities. During manufacturing of oils, 30 to 40% of tocols and tocotrienols are lost (Dziezak, 1986; Schuler, 1990). Tocopherols are important biological antioxidants. Alpha-tocopherol, or vitamin E, prevents oxidation of body lipids including polyunsaturated fatty acids and lipid components of cells and organelle membranes. Tocopherols are produced commercially and used as food antioxidants. The antioxidant activity of tocopherol is based mainly on the tocopherol–tocopheryl quinone redox system.

Tocopherols (AH_2) are radical scavengers and quench lipid radicals ($R^{\bullet}$), thus regenerating RH molecules as well as producing a tocopheryl semiquinone radical. Two tocopheryl semiquinone radicals (AH) may form a molecule of tocopheryl quinone (A) and a regenerated molecule of tocopherol, as can be seen in Reaction 2.7 and Reaction 2.8:

$$R^{\bullet} + AH_2 \rightarrow RH + AH^{\bullet} \quad (2.7)$$

$$2AH^{\bullet} \rightarrow A^{\bullet} + AH_2 \quad (2.8)$$

The mechanism of oxidation of α-tocopherol with linoleate hydroperoxides has been studied in details (Tappel, 1972). After releasing one H atom, the α-tocopheryl radical formed releases another H atom to produce methyl tocopheryl quinone, which is unstable and gives rise to α-tocopheryl quinone as its main product. The reaction between two semiquinoid radicals may also lead to the formation of α-tocopherol dimer, which possesses antioxidant properties (Schuler, 1990). Ishikawa and Yuki (1975) described the antioxidant effect of the products of tocopherols as oxidized α-, γ-, and δ-tocopherol with trimethylamine oxide. Some of the oxidation products formed were isolated and tested for their antioxidant activity.

Tocopherols are commercially extracted from deodorizer sludge obtained in the deodorization of vegetable oils. Various tocols and tocotrienols of such extracts contain sterols, esters of sterols, free fatty acids and triacylglycerols. The separation of tocopherols from other compounds is possible by esterification with lower alcohol, washing and vacuum distillation or by saponification or fractional liquid–liquid extraction. Further purification may be achieved using molecular distillation, extraction, crystallization or a combination of these processes (Schuler, 1990). The total tocopherol content of the extracts is usually between 30

and 80%, but higher in γ- and δ-tocopherols. The α-tocopheryl acetate is the commercially available form of vitamin E, which is not an antioxidant because its active OH group is blocked. However, under acidic aqueous conditions, tocopherol is released by hydrolysis and the released tocopherol may then act as an antioxidant (Schuler, 1990).

The commercial synthesis of α-tocopherol involves condensation of 2,3,5-trimethyl hydroquinone with phytol, isophytol or phytol halogenides. The crude tocopherol product can be purified by vacuum distillation (Schuler, 1990). Because use of isophytol is preferred, the distillation produces a racemic mixture of all possible tocopherol isomers. Products intended for antioxidant applications are generally marketed in oil forms. Pure, all-racemic α-tocopherol, mixed tocopherols having various contents of α-, γ- and/or δ-tocopherols; synergistic mixtures composed of tocopherols, ascorbyl palmitate or other antioxidants, and synergists such as lecithin, citric acid and carriers are also available (Schuler, 1990).

Flavonoids and their antioxidant activity: Flavonoids constitute a large group of naturally occurring plant phenolics. They are characterised by the carbon skeleton C_6–C_3–C_6. The basic structure of these compounds consists of two aromatic rings linked by a three-carbon aliphatic chain which normally has been condensed to form a pyran or, less commonly, a furan ring (Figure 2.3).

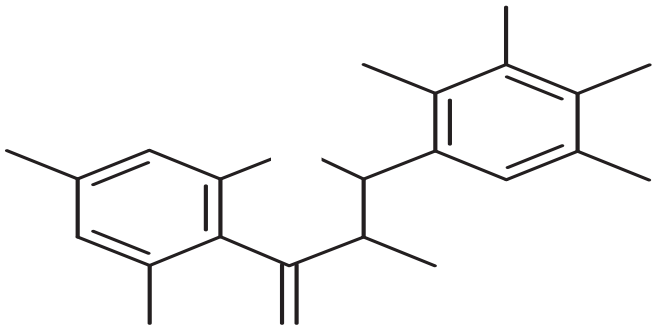


Figure 2.3. Basic structure of flavonoids (Hopia and Heinonen, 1999).

Flavonoids, including flavones, flavonols, isoflavones, flavonones and chalcones occur in all types of higher plant tissues. Flavones and flavonols are found in almost every plant, particularly in the leaves and petals, with flavonols occurring more frequently than flavones

(Hopia and Heinonen, 1999). Approximately 90 % of the flavonoids in plants occur as glycosides. Many of flavonoids and related phenolic acids have shown marked antioxidant characteristics (see Table 2.1) (Pratt and Hudson, 1990).

Table 2.1. Antioxidant activity of flavones.

Compound	**Time to Reach Peroxide Value of 50 (h)[a]**	**Induction Period by Rancimat (h)[b]**	
Control			
Stripped corn oil	110	A	B
Lard	—	1.3	0.35
Isoflavones			
Daidzein (7,4′-dihydroxy)	—	1.4	—
Genistein (5,7,4′-trihydroxy)	—	2.6	—
Chalcones			
Butein (2′,4′,3,4-tetrahydroxy)	—	94.0	—
Okanin (2′,3′,4′,3,4-pentahydroxy)	—	97.0	—
Phenolic acids			
Protocatechuic acid (3,4-dihydroxybenzoic acid)	—	—	4.8
Gallic acid (3,4,5-trihydroxybenzoic acid)	—	—	28.6
Coumaric acid (*p*-hydroxycinnamic acid)	—	120	0.8
Ferulic acid (4-hydroxy-3-methoxy-cinnamic acid)	—	145	2.0
Caffeic acid (3,4-dihydroxycinnamic acid)	—	495	23.3
Dihydrocaffeic acid (3,4 Dihydroxyphenylpropionic acid)	—	—	31.4
Chlorogenic acid (caffeoyl quinic ester)	—	505	—
Quinic acid	—	105	—
Phenolic ester			
Propyl gallate	—	435	21.8

Note: A and B denote different batches of oil.
[a] 5×10^{-4} M in stripped corn oil.
[b] 2.3×10^{-4} M in lard.
Source: Adapted from Pratt and Hudson, 1990 in Food Antioxidants, Hudson, Ed., Elsevier Applied Science, London, 171–192.

The ability of flavonoids to inhibit lipid oxidation is well documented, both for natural lipid products and for model lipids. Flavonoids may act as antioxidants by scavenging radicals that include superoxide anions, lipid peroxyl radicals, and hydroxyl radicals. Other mechanisms of

action of selected flavonoids include singlet oxygen quenching, metal chelation, as well as lipoxygenases inhibition (Bors *et al*., 1996).

Flavonoids and cinnamic acids are known as primary antioxidants and act as free radical acceptors and chain breakers. Flavonols are known to chelate metal ions at the 3-hydroxy-4-keto group and/or 5-hydroxy-4-keto group (when the A-ring is hydroxylated at the fifth position). An *o*-quinol group at the B-ring can also demonstrate metal chelating activity (Pratt and Hudson, 1990).

It has been established that the position and degree of hydroxylation are of primary importance in determining antioxidant activity of flavonoids. The *o*-dihydroxylation of the B-ring contributes to the antioxidant activity. The *p*-quinol structure of the B-ring has been shown to impart an even greater activity than *o*-quinol; however, *para* and *meta* hydroxylation of the B-ring do not occur naturally (Pratt and Hudson, 1990). All flavonoids with 3′,4′-dihydroxy configuration possess antioxidant activity (Dziedzic and Hudson, 1983). Robinetin and myricetin have an additional hydroxy group at their 5′ position, thus leading to enhanced antioxidant activities over those of their corresponding flavones that do not possess the 5′ hydroxy group, namely, fisetin and quercetin. Two flavanones (naringenin and hesperitin) have only one hydroxy group on the B-ring and possess little antioxidant activity. Hydroxylation of the B-ring is the major consideration for antioxidant activity (Pratt and Hudson, 1990). Other important features include a carbonyl group at position 4 and a free hydroxy group at position 3 and/or 5 (Dziedzic and Hudson, 1983).

In addition, it was found that a double bond between the 2 and 3 poisitions on the C ring contributed to antioxidant activity. Also found that the aglycones were stronger antioxidants than their corresponding glucosides. Possibly due to the lack of a free 3-hydroxy substitution in the C ring. The isoflavone aglycones from soybeans exhibited greater activity than their glucosides counterparts.

Bors *et al*. (1996) has investigated the importance of other sites of hydroxylation. It has been shown that the *o*-dihydroxy grouping on one ring and *p*-dihydroxy grouping on the other (e.g., 3,5,8,3′,4′- and 3,7,8,2′,5′ pentahydroxy flavones) produce very potent antioxidants, while 5,7 hydroxylation of the A-ring apparently has little influence on the antioxidant activity of the compounds (Pratt and Hudson, 1990). Thus, quercetin and fisetin have almost the same activity, while myricetin possesses an activity similar to that of robetin. The 3-glycosylation

of flavonoids with monosaccharides/disaccharides reduces their activity compared with that of the corresponding aglycones (e.g., rutin is less active than quercetin).

The ability of flavonoids to form complexes with cupric ion has also been demonstrated by UV spectral studies. Such complexations may contribute to the antioxidative action of flavonoids (Hudson and Lewis, 1983). Chelation of metal ions renders them catalytically inactive. Chalcones, the natural precursors of flavones and flavanones, are readily cyclized under acidic conditions and have been shown to possess potent antioxidant activity. The 3,4-dihydroxychalcones are particularly effective and chalcones are more effective than their corresponding flavanones. Effectiveness of 3,4-dihydroxychalcones, namely, butein and okanin, depends on the formation of resonance-stabilized free radicals (Dziedzic and Hudson, 1983). In the isoflavone, it is clear that both hydroxyl groups in 4′ and 5 positions are needed for significant antioxidant activity as genistein. Even 6,7,4′- trihydroxyisoflavone is marginally active when compared to analogous flavon apigenin, which is inactive as an antioxidant. Genistein is particularly active. The resonance-stabilized quinoid structures show that for isoflavone the carbonyl group at position four remains intact and can interact with the 5-hydroxy group, if present; however, in flavone, the carbonyl group at position four loses its functionality. This may explain the superior antioxidant activity of genistein compared with that of apigenin (Dziedzic and Hudson, 1983).

The results of the study led to several conclusions relative to the structural features which affect the antioxidant activity (AOA) of the flavonoids. First, it was noted that the molecule needed multiple hydroxyl group; 3', 4' dihydroxy configuration gave strong antioxidant activity. Second, the molecule must have a 4- carbonyl group for activity. Third, the molecule needed a free 3- hydroxyl group as opposed to a 5- hydroxyl group, but the presence of both 3- and 5- hydroxyl groups was also effective. They projected that there was cooperation between the 4- carbonyl and the 3- or 5- hydroxyl groups which acted to chelate the copper ion.

Phenolic acids and their antioxidant activity: The antioxidant activity of phenolic acids and their esters depends on the number of hydroxy groups in the molecule; this will be strengthened by steric hindrance (Dziedzic and Hudson, 1983). Hydroxylated cinnamic acids have been found to be more effective than their benzoic acid counterparts. It has been mentioned that at least two and, even better, three neighboring phenolic hydroxy groups

(catechol or pyrogallol structure) and a carbonyl group in the form of an aromatic ester or lactone or a chalcone, flavonone or flavone are the essential molecular features required to achieve a high level of antioxidant activity (Dziedzic and Hudson, 1983).

Phenolic antioxidants act to inhibit lipid oxidation by trapping the peroxy radical. This can be accomplished in one of two ways:

$$LOO^{\bullet} + ArOH \longrightarrow LOOH + ArO^{\bullet}$$

$$LO^{\bullet} + ArO^{\bullet} \longrightarrow LOOAr$$

In the first mechanism, the peroxy radical (LOO•) abstracts a hydrogen proton from the antioxidant (ArOH) to yield an aroxyl radical (ArO•) and the hydroperoxide (LOOH). In the second mechanism, a peroxy and an aroxyl react by radical- radical coupling to form a nonradical product.

The aroxyl radical formed from the oxidation of an antioxidant can further react and can, in some instances, contribute to the production of free radicals.

$$ArO^{\bullet} + LOOH \longrightarrow LOO^{\bullet} + ArOH$$

$$ArO^{\bullet} + LH \longrightarrow ArOH + L^{\bullet}$$

Vitamin C: Essential for the production of collagen, the cellular glue that keeps cells attached together. It strengthens the connective tissue. It is thought to protect against cataract, against lipid oxidation, and essential for immune system health. Has the important job of recharging fat-soluble vitamin E when it becomes a free radical itself. The pharmacophore of vitamin C is the ascorbate ion. In living organisms, ascorbate is an antioxidant, since it protects the body against oxidative stress, and is a cofactor in several vital enzymatic reactions (Higdon and Frei, 2004).

Sesamol: Sesamol seed oil exhibits antioxidant property when added to other fats. The active antioxidant of oils is sesamol which is present in unsaponifiable matter of sesamol seed oil (Ohsawa, 1991). Sesamol not only occurs in varying amounts in different sesame oils, but the amount present in a given oil has been shown to be markedly affected by different processing conditions. Furthermore, it may exist in either a free or a bound form, each of which has different properties (Namiki, 1995). Four crude oils obtained from different varieties of

sesame seed were reported to contain 0.7-1.5 % sesamolin, with only traces of free sesamol (Namiki, 1995). Bleached and hydrogenated sesame oils, however, may contain over 0.9 % free sesamol, because of the splitting of sesamolin during bleaching and hydrogenation (Visavadiya and Narasimhacharya, 2008).

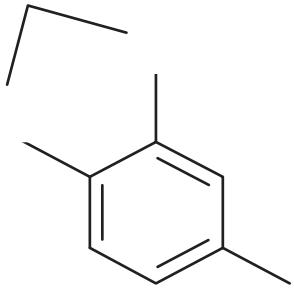


Figure 2.4. Structure of sesamol (Ohsawa, 1991).

Lecithin: Lecithin is one of the first antioxidant to receive serious consideration in the united state for use in edible oils. Commercial lecithin preparation have been found to be somewhat effective in vegetables oils like cotton seed oil but are relatively ineffective in lard (Iwata *et al*., 1993). Sunflower seed can be considered a potential source of lecithin due to the distribution of the main phospholipid components, which appear to be similar to soybean lecithin (Holló *et al*., 1993). Lecithin exhibits emulsification properties in numerous applications in food and pharmaceutical industries (Nieuwenhuyzen, 1981). By changing the hydrophilic/lipophilic balance of phospholipids, it is possible to produce lecithin for different applications (Mustranta *et al*., 1995). Fatty-acid profiles of phospholipids depend on the lecithin source and may be modified by several enzymatic methods, such as hydrolysis, transesterification or alcoholysis, in order to enhance lecithin functional properties (Ghosh and Bhattacharyya, 1997).

2.2.3.4. Synthetic antioxidants

The application of antioxidants in foods is governed by federal regulations. Food and drug administration (FDA) regulations require that the ingredient labels of products declare antioxidants and their carriers followed by an explanation of their intended purpose (Dziezak,

1986). Table 2.2 summarizes the permitted food phenolic antioxidants, some of their properties. Synthetic food antioxidants currently permitted for use in foods are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), dodecyl gallate (DG) and tertiarybutylhydroquinone (TBHQ).

Table 2.2. Synthetic food antioxidants and their properties.

			Gallates		
Characteristic	**BHA**	**BHT**	**Propyl**	**Dodecyl**	**TBHQ**
Melting point °C	50–52	69–70	146–148	95–98	126–128
Carry-through properties	Very good	Fair–good	Poor	Fair–good	Good
Synergism	BHT and gallates	BHA	BHA	BHA	—
Solubility (w/w%)					
Water	0	0	<1	<1	<1
Propylene glycol	50	0	6.5	4	30
Lard	30–40	50	1	—	05–10
Corn oil	30	40	0	0	10
Glycerol	1	0	25	—	<1
Methyl linoleate	Very soluble	Very soluble	1	1	>10

Sources: Adapted from Coppen, 1983, in Rancidity in Foods, Allen and Hamilton, Eds., Applied Science Publishing Company, London, 67–87 and Dziezak, 1986, Food Technol., 9, 94–102.

BHA and BHT are monohydric phenolic antioxidants (Figure 2.5). Chemically, BHA is a mixture of 3-tertiary-butyl-4-hydroxyanisole (90%) and 2-tertiary-butyl-4-hydroxyanisole (10%) (Porter, 1980). BHA is commercially available as white waxy flakes and BHT is available as a white crystalline compound; both are extremely soluble in fats and insoluble in water. Furthermore, both assert a good carry-through effect; however, BHA is slightly better than BHT in this respect (Dziezak, 1986).

BHT is more effective in suppressing oxidation of animal fats than vegetable oils. Among its multiple applications, BHA is particularly useful in protecting the flavour and colour of essential oils and is considered the most effective of all food-approved antioxidants for this application (Stuckey, 1972). BHA is particularly effective in controlling the oxidation of

short-chain fatty acids such as those found in the coconut and palm kernel oils typically used in cereal and confectionary products (Dziezak, 1986).

BHA BHT

Figure 2.5. Chemical structures of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Porter, 1980).

As a monophenol, BHT can produce radical intermediates with moderate resonance delocalization. The tertiary butyl groups of BHT do not generally allow involvement of the radical formed from it after hydrogen abstraction in other reactions. Thus, a lipid peroxy radical may join the molecule of BHT in the *para* position to the phenoxy group (Dziezak, 1986).

The volatile nature of BHA and BHT makes them important additives in packaging materials because they can migrate into foods. For this purpose, these antioxidants are added directly to the wax used in making inner liners or applied to the packaging board as an emulsion (Porter, 1980; Dziezak, 1986). A synergistic effect has been shown to exist when BHT and BHA are used in combination. The oxidative reactions of nut and nut products are very responsive to the combination of these two antioxidants (Dziezak, 1986).

Tertiary-butylhydroquinone is regarded as the best antioxidant for protecting frying oils against oxidation (Khan and Shahidi, 2001) and provides good carry-through protection to fried products similar to those of BHA and BHT. TBHQ may be considered as an alternative to hydrogenation for increasing oxidative stability (Dziezak, 1986). TBHQ is adequately soluble in fats and does not complex with iron or copper; therefore, it does not discolor the treated products. TBHQ is available as a beige-colored powder to be used alone or in

combination with BHA or BHT at a maximum amount of 0.02% or 200 mg/kg, based on the fat content of foods, including essential oils. TBHQ is not permitted in combination with propyl gallate (Dziezak, 1986). Coppen (1983) has reported that TBHQ shows good performance in stabilizing crude oils.

Chelating agents such as citric acid and monoacylglycerol citrate can further enhance lipid-stabilizing properties of TBHQ. This combination is primarily used in vegetable oils and shortenings but not extensively for animal fats. Confectioneries, including nuts and candies, also benefit from the use of TBHQ or its mixtures (Buck, 1984). As a diphenolic antioxidant, TBHQ reacts with peroxy radicals to form a semiquinone resonance hybrid. The semiquinone radical intermediates may undergo different reactions to form more stable products. They can also react with one another to produce dimmers or react with one another to produce a quinone and a hydroquinone molecule or add to a lipid peroxy radical to produce a semiquinone.

Propyl gallate is commercially prepared by esterification of gallic acid with propyl alcohol followed by distillation to remove the excess alcohol. PG is available as a white crystalline powder and is sparingly soluble in water; it functions particularly well in stabilizing animal fats and vegetable oils. With a melting point of 148°C, PG loses its effectiveness during heat processing and is therefore not suitable in frying applications that involve temperatures exceeding 190°C. PG chelates iron ions and forms an unappealing blue–black complex (Dziezak, 1986). Hence, PG is always used with chelators such as citric acid to eliminate the pro-oxidative iron and copper catalysts. Good synergism is obtained with BHA and BHT; however, their coapplication with TBHQ is not permitted (Buck, 1984). PG may be used to inhibit the oxidation of vegetable oils, animal fats and meat products, including fresh and frozen sausages and snacks. Its usage has been permitted in chewing gum base at <0.1% and with BHA and/or BHT at a total concentration of <0.1%. Moreover, the amphiphilic nature of PG makes it a very effective antioxidant for dry vegetable oils. Gallates have lower volatility and thus have less phenolic odor than monohydric phenols such as BHA and BHT (Dziezak, 1986).

Nordihydroguaiaretic acid (NDGA) is a grayish-white crystalline compound that was widely used as an antioxidant in animal fats in the 1950s and 1960s. It possesses phenolic properties similar to gallates, including their advantages and disadvantages. Besides the isolation of natural material (resinous exudate of creosote bush), NDGA has also been chemically

synthesized. Due to unfavorable toxicological findings, NDGA is no longer of practical importance in the food industry (Gordon, 1990; Schuler, 1990).

2.2.3.5 Assays involved in the determination of antioxidant activity

Methods for measuring antioxidant activity are generally based on the inhibition of reactions in the presence of antioxidants. The most widely used methods are those that involve the generation of radical species, where the presence of antioxidant determines the disappearance of these radicals. This approach has been applied to the estimation of antioxidant activity in aqueous systems, of both pure compounds and biological samples (Arnao *et al*., 1998).

The activity of an antioxidant can be estimated by quantitatively determining primary or secondary products of autoxidation of lipids or by monitoring other variables. Generally, the delay in hydroperoxide formation or production of secondary products of autoxidation by chemical or sensory methods can be used. These procedures can be applied to intact foods, their extracts or to model systems. Studies on foods can be performed under normal storage conditions or under accelerated oxidation such as active oxygen method (AOM), Schaal oven test, oxygen uptake/absorption, and oxygen bomb calorimetry, or by using a fully automated oxidative stability instrument (OSI), a Rancimat apparatus, or an oxidograph, among others (Kochhar and Rossell, 1990). The extension of the induction period by addition of an antioxidant has been related to antioxidant efficacy, which is sometimes expressed as antioxidant index or protection factor. It is also possible to use a luminescence apparatus, also known as Photochem, which measures antioxidant activity of hydrophilic and lipophilic compounds (Amarowicz *et al*., 2003). ORAC (oxygen radical absorbance capacity) and TEAC (Trolox equivalent antioxidant capacity) tests have also been used in the recent literatures; artificial radicals such as DPPH (1,1-diphenyl-2-picrylhydrazyl) radical have been employed. All of these offer means of evaluating antioxidant activity of food phenolics and other constituents.

Radical-scavenging methods:

Radical scavenging is the main mechanism by which antioxidants act in foods. Several methods have been developed in which the antioxidant activity is assessed by the scavenging of synthetic radicals in polar organic solvents, e.g. methanol, at room temperature. Those used

include 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzthiazoline-sulphonic acid) (ABTS) radicals.

Screening of antioxidant activity using the DPPH method: The stable organic radical DPPH$^{\bullet}$ (Figure 2.6) has been widely used in antioxidant activity (AOA) studies of single compounds (Sanchez Moreno *et al.,* 1998), plant extracts and foods, etc. (Koleva *et al.,* 2002). DPPH$^{\bullet}$ has an unpaired valence electron at one atom of nitrogen bridge (Eklund *et al*., 2005). As this electron is paired off in the presence of a free radical scavenger, absorption vanishes and the resulting decolouration is stoichiometric with respect to the number of electrons taken up. This bleaching of DPPH, which occurs when the odd electron of the radical is paired, is thus representative of the capacity of the compounds to scavenge free radicals independently on any enzymic activity (Fauconneau *et al*., 1997).

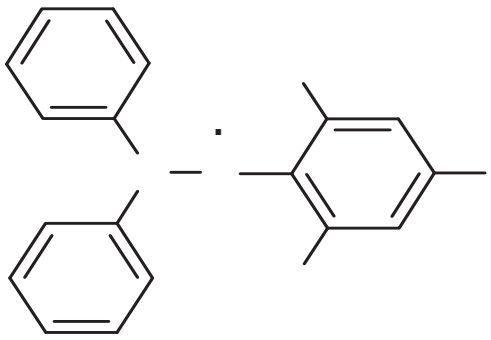


Figure 2.6. Structure of 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) (Eklund *et al*., 2005).

Yamaguchi *et al* (1998) reported the mechanism of the reaction of antioxidants with the DPPH radical:

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet} \quad (2.9)$$

$$A^{\bullet} + DPPH^{\bullet} \longrightarrow DPPH\text{-}A \quad (2.10)$$

$$A^{\bullet} + A^{\bullet} \longrightarrow A\text{-}A \quad (2.11)$$

The newly formed radical (A•) can give radical-radical interactions (Reactions 2.9 and 2.10) to form more stable molecules (Aruoma, 1998). The reduction in DPPH• is measured by the decrease in absorbency at 515 nm as the radical is scavenged by the antioxidant until the reaction reaches a plateau (Figure 2.7) (Espin *et al*., 2000).

Fast reaction of DPPH radicals occurs with some phenols e.g. α-tocopherol, but slow secondary reactions may cause a progressive decrease in absorbance, so that the steady state may not be reached for several hours. Most papers in which the DPPH method has been used report the scavenging after 15 or 30 min reaction time. The antioxidant activity can be expressed as EC_{50} (efficient concentration) and AE (antiradical efficiency). EC_{50} is the concentration of the antioxidant necessary to decrease the initial DPPH• concentration by 50%. Antiradical efficiency (AE) is (1/ EC_{50}). EC_{50} is directly calculated from the curve resulting after plotting DPPH concentration *vs* concentration of the antioxidant.

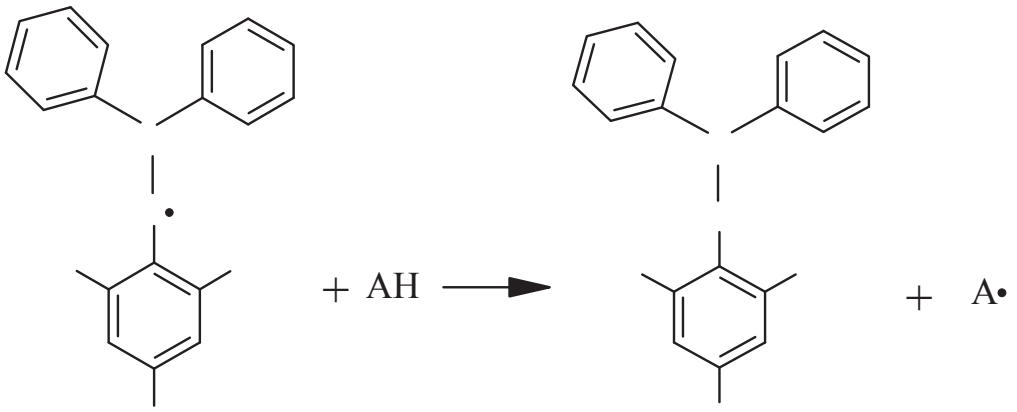


Figure 2.7. Scavenging of free DPPH by antiradical species (Espin *et al*., 2000).

ABTS (2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid)) assay: The ABTS assay, which is also called the ABTS radical assay, has been widely used to evaluate antioxidant activities of components in foods and beverages due to its applicability in aqueous and lipid phases (MacDonald-Wicks, 2006). The original ABTS assay was based on the activation of metmyoglobin by hydrogen peroxide in the presence of ABTS (Miller *et al*., 1993). In the improved version of this assay, a stable ABTS radical cation, which has a blue-green

chromophore absorption, was produced by oxidation of ABTS with potassium persulfate prior to the addition of antioxidants as shown in Figure 2.8 (Re *et al*., 1999). The ABTS radical cation is more reactive than the DPPH radical, and reaction of the ABTS radical cation with an antioxidant is taken as complete within 1 min (Michael, 2001).

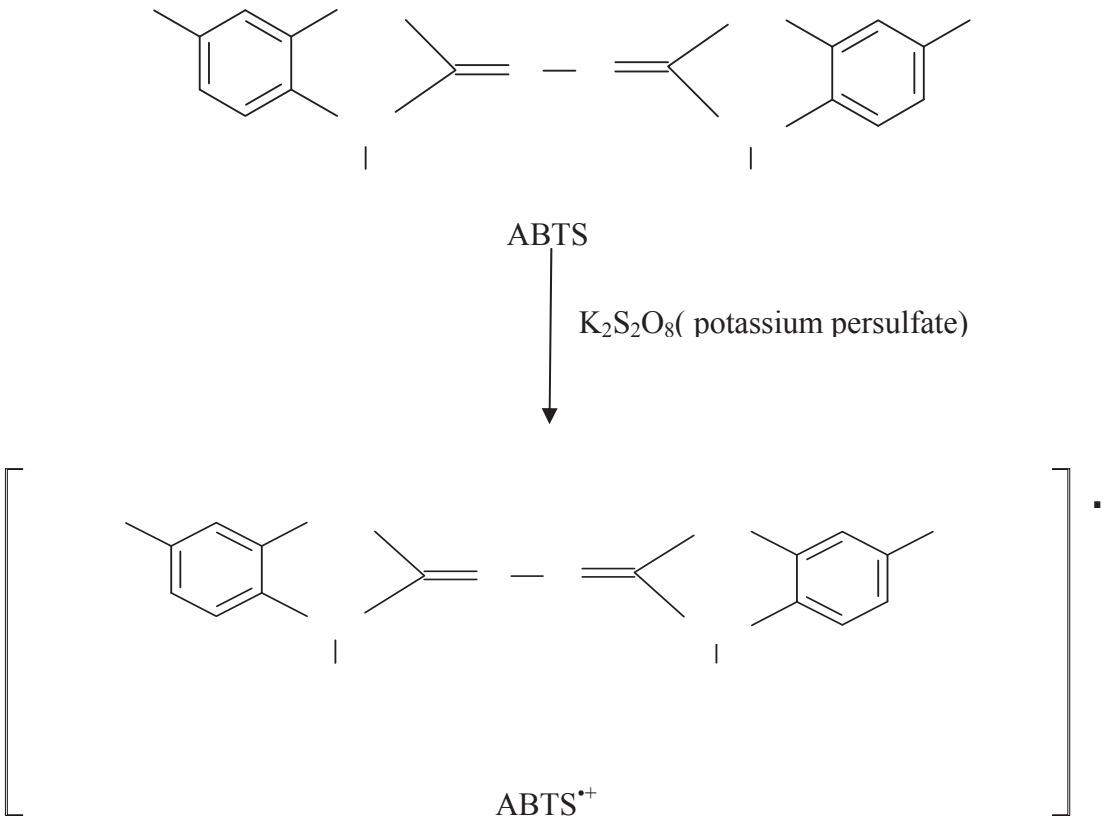


Figure 2.8. Formation of stable ABTS radical from ABTS with potassium persulfate (Re *et al*., 1999).

The antioxidant activity of the natural products, including carotenoids, phenolic compounds, and some plasma antioxidants, is determined by the decolorization of the ABTS, by measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm (Biglari *et al*., 2008). This method has been applied to investigating the antioxidant activities of many natural products including fruits and vegetables (Tachakittirungrod *et al*., 2007; Sun *et al*., 2007), medicinal plants (Surveswaran *et al*., 2007; Kawaree *et al*., 2008),

wines and grapes (Rivero-Perez *et al*., 2008), cereals (Abdel-Aal and Rabalski, 2008; Hu *et al*., 2007), beverages (Gomez-Ruiz *et al*., 2007), and essential oils (Zhao *et al*., 2008; Erkan *et al*., 2008), but antioxidant effectiveness in foods must always be studied by other methods because their activity in foods is dependent on a variety of factors including polarity, solubility, and metal-chelating activity.

Methods for measuring the current state of an oil or food sample:

Some methods can be applied to assessing the current state of an oil or food sample. In order to be applied in assessment of antioxidant effectiveness, an experiment must be designed in which the antioxidant is incorporated into the food and the food is stored under controlled conditions. The principles of these methods are described below.

Sensory analysis: For the food industry, the detection of oxidative off-flavours by taste or smell is the main method of deciding when a lipid-containing food is no longer fit for consumption. Consequently, any antioxidant used in the food will ultimately be evaluated by its potential for extending the time before this off-flavour can be detected. The ability of individuals to describe the nature of the aroma is useful, and the sensitivity of a trained panel to oxidative off-flavours may allow detection of oxidative deterioration at a stage when common chemical methods, e.g. peroxide value measurements, are unable to detect any deterioration. The main problems with sensory evaluation are that different individuals vary in their sensitivity to these off-flavours, and their performance may vary depending on their state of health and other variables. Trained panellists are much more reliable than untrained panellists, but the reproducibility of sensory analysis is normally worse than that of chemical or instrumental methods (Michael, 2001).

Peroxide value (PV): The PV is still the most common chemical method of measuring oxidative deterioration of oils. Although hydroperoxides decompose to a mixture of volatile and non-volatile products and they also react further to endoperoxides and other products, the PV measurement is a useful method of monitoring oxidative deterioration of oils, although it should normally be combined with a method of monitoring secondary oxidation products to provide a fuller picture of the progress of oxidation. Huang *et al*. (1995) showed that increased addition of a-tocopherol to oil may increase the PV whilst reducing hexanal formation. This suggests that a high PV value may reflect either increased formation of

hydroperoxides or reduced decomposition. Consequently, antioxidants may improve the flavour stability of oil without it being evident from PV measurements.

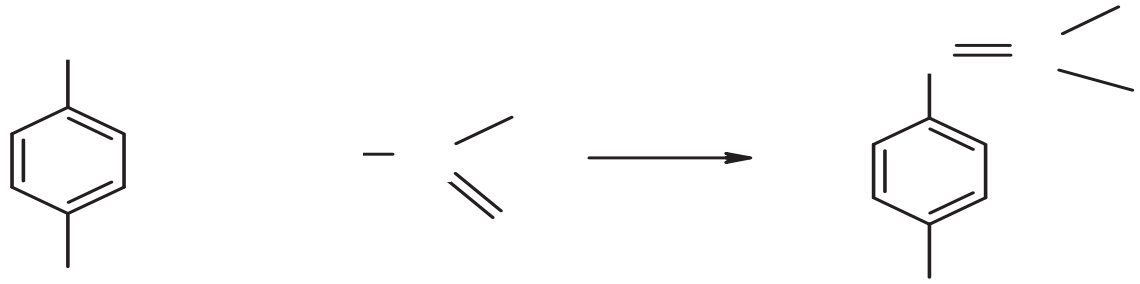
The traditional method of determining PV involves a titration of the oil containing potassium iodide in a chloroform–acetic acid mixture. The hydroperoxides oxidise the iodide to iodine, which is determined by titration with sodium thiosulphate. In order to avoid the use of chloroform, the AOCS has developed an alternative method which uses isooctane as solvent, although the method is limited to PV < 70meq kg^{-1}, as described in the AOCS guidelines (1998). The PV determination should not be used as a method of assessing the deterioration of oils used for frying, since hydroperoxides decompose spontaneously above 150°C, and the measured PV can be more an indication of the cooling and storage conditions after frying than of oxidation products formed at frying temperatures. Even at temperatures of 80 to 90°C, formation of hydroperoxides is accompanied by decomposition at a significant rate.

The PV at which oxidation of oils can be detected as an off-flavour varies widely depending on the nature of the oil. Samples of olive oil may not be perceived as rancid till the PV reaches 20meq kg^{-1} whereas fish oil may develop off-flavours at PV < 1meq kg^{-1}.

Conjugated diene assay: The term conjugated diene is defined as a moiety with two double bonds separated by a single bond. This kind of moiety does not normally occur in unsaturated fatty acids. However, a conjugated diene is readily formed from a moiety with two double bonds separated by a single methylene group, which occurs most commonly in polyunsaturated fatty acids, by the action of ROS and oxygen (formation of monohydroperoxide). Once a conjugated diene is formed, it can be monitored spectrophotometrically using its characteristic absorption at 234 nm (Moore and Roberts, 1998). This represents a simple and rapid method of monitoring oxidative deterioration of oil. The antioxidant effect of test substances can be evaluated by monitoring the conjugated diene formation. The major drawback of this method is that many biological and natural compounds have significant absorbance around 234 nm, which, consequently, interferes with absorption by a conjugated diene. Therefore, this method has not been applied in studies of natural or biological substances as frequently as the β-carotene bleaching assay. However, if a simple fatty acid, such as linoleic acid, is used, this method is useful because of its simplicity. Also, this method can be used for investigation of the early stage of lipid peroxidation. This assay has been used with various other antioxidant assays (TBA, DPPH, and ABTS) for studies of

natural food sources including fruits and vegetables (Chirinos *et al*., 2008), herbs and spices (Lee and Shibamoto, 2002), teas (Yanagimoto *et al*., 2003), and honeys (Gheldof and Engeseth, 2002). The antioxidant activity of an extract of an edible marine red alga was evaluated by conjugated diene assay using linoleic acid and fish oil (Athukorala *et al*., 2003). This method tends to be used in combination with a nonlipid system, such as DPPH and ABTS assays.

***Para*-anisidine value:** *Para*-anisidine is a reagent that reacts with aldehydes to give products that absorb at 350 nm (Figure 2.9). The *p*-anisidine value is defined as the absorbance of a solution resulting from the reaction of 1 g fat in isooctane solution (100 mL) with *p*-anisidine (0.25 % in glacial acetic acid). The products formed by reaction with unsaturated aldehydes (2-alkenals) absorb more strongly at this wavelength, and consequently the test is particularly sensitive to these oxidation products. Although the test does not distinguish between volatile and non-volatile products, the palate is generally more sensitive to unsaturated volatile aldehydes than to saturated volatile aldehydes, so the test is a reasonable way to assess secondary oxidation products (Michael, 2001).



p-anisidine reagent aldehydes (2-alkenals) Absorbs at 350nm

Figure 2.9. The reaction of *p*-anisidine with alkenals (Michael, 2001).

Measurements of *p*-anisidine value are commonly used together with peroxide value measurements in describing the total extent of oxidation by the Totox value, which equals the sum of the *p*-anisidine value plus twice the peroxide value. However, the Totox value is an empirical parameter since it corresponds to the addition of two parameters with different units (Michael, 2001).

***β*-Carotene Bleaching Assay:** It has long been known that *β*-carotene reacts with the peroxyl radical to produce *β*-carotene epoxides (Kennedy and Liebler, 1991). Therefore, *β*-carotene has received attention as a radical scavenger or antioxidant (Tsuchihashi, 1995). Later, an antioxidant assay using *β*-carotene combined with lipids, such as linoleic acid, was established. Lipids, such as linoleic acid, form a peroxyl radical ($LOO^{\bullet}$) in the presence of ROS and O_2. This peroxyl radical reacts with *β*-carotene to form a stable *β*-carotene radical as shown in Figure 2.10; subsequently, the amount of *β*-carotene reduces in a testing solution (Tsuchihashi, 1995). If an antioxidant is present in a testing solution, it reacts competitively with the peroxyl radical (Takada *et al*., 2006). Therefore, antioxidant effects are easily monitored by bleaching the color of a test solution with a spectrophotometer at 470 nm, which is the typical absorbance by *β*-carotene.

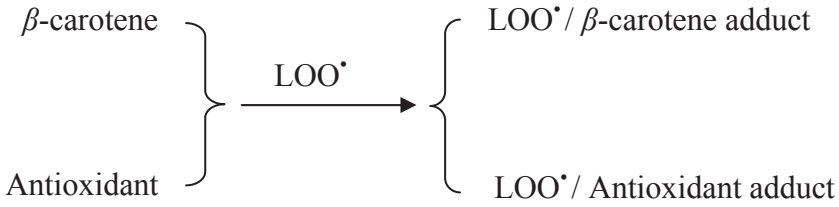


Figure 2.10. Formation of adducts from *β*-carotene and antioxidant with a lipid peroxide radical (Tsuchihashi, 1995).

Chaillou and Nazareno (2006) measured the antioxidant activities of various phenolic compounds using the *β*-carotene bleaching assay. In this study, linoleic acid was selectively oxidized with lipoxygenase. Among 18 phenolic compounds tested by this method, quercetin exhibited the greatest antioxidant activity, which was confirmed by the DPPH assay. A more comprehensive study of the antioxidant activities of 42 flavonoids was reported using the *β*-carotene bleaching method, in which linoleic acid was oxidized by heat treatment (Burda and Oleszek, 2001). Quercetin also exhibited strong antioxidant activity by this method, and the result was consistent with the one obtained by DPPH assay. In addition to studies on phenolic compounds, this method has been used for antioxidant studies on various plants and their components.

Thiobarbituric acid value (TBA): Malonaldehyde may be formed from polyunsaturated fatty acids with at least three double bonds. The concentration of this product may be assessed by reaction with thiobarbituric acid which reacts with malonaldehyde to form red condensation products (Figure 2.11) that absorb at 532–535nm with molar absorptivity of 27.5 absorbance units/µmol (Michael, 2001).

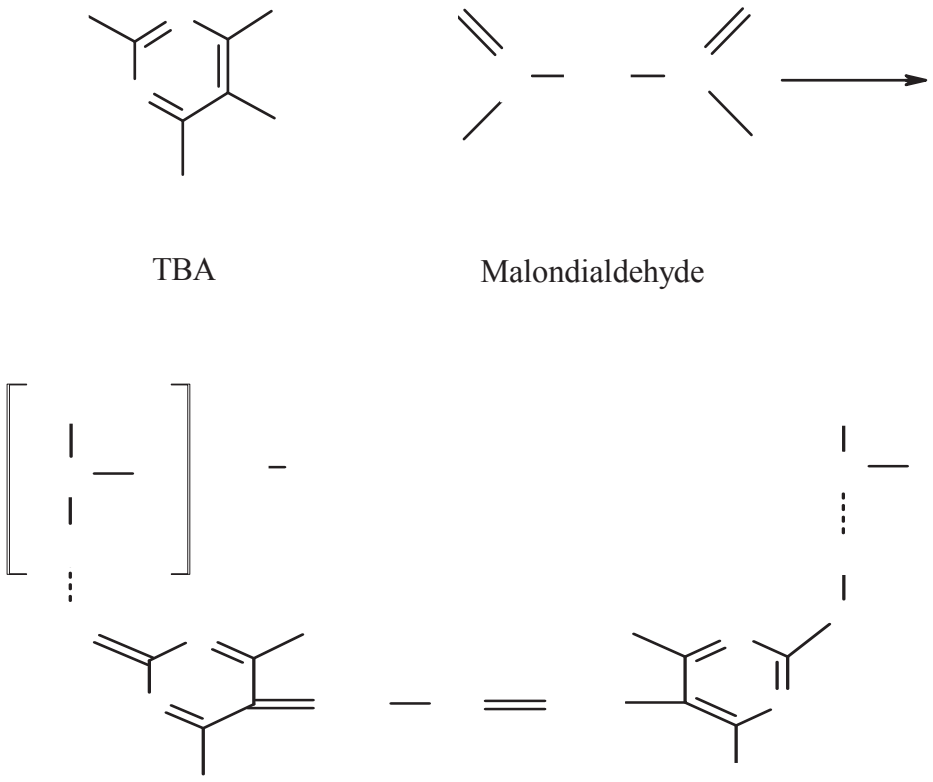


Figure 2.11. Formation of a chromogen by reaction of TBA with malonaldehyde (Michael, 2001).

However, the reaction is not specific, and reaction with a wide variety of other products may contribute to the absorbance. 2, 4-Alkadienals such as 2, 4-decadienal also react with TBA to show strong absorption at 532nm. Saturated aldehydes normally absorb at lower wavelengths after reaction with TBA. Several food components including proteins, Maillard browning products and sugar degradation products affect the determination. In order to emphasise the

lack of specificity, the values obtained in the test are commonly described as TBARS (TBA reactive substances) (Guillen and Guzman, 1998).

Conjugable oxidation products: Analysis of conjugable oxidation products is based on the fact that hydroperoxides from polyunsaturated fatty acids and some decomposition products may be reduced with sodium borohydride and dehydrated to give conjugated trienes and tetraenes (Figure 2.12) (Parr and Swoboda, 1976). Triene and tetraene concentrations are determined from the absorbance values at 268nm and 301nm respectively.

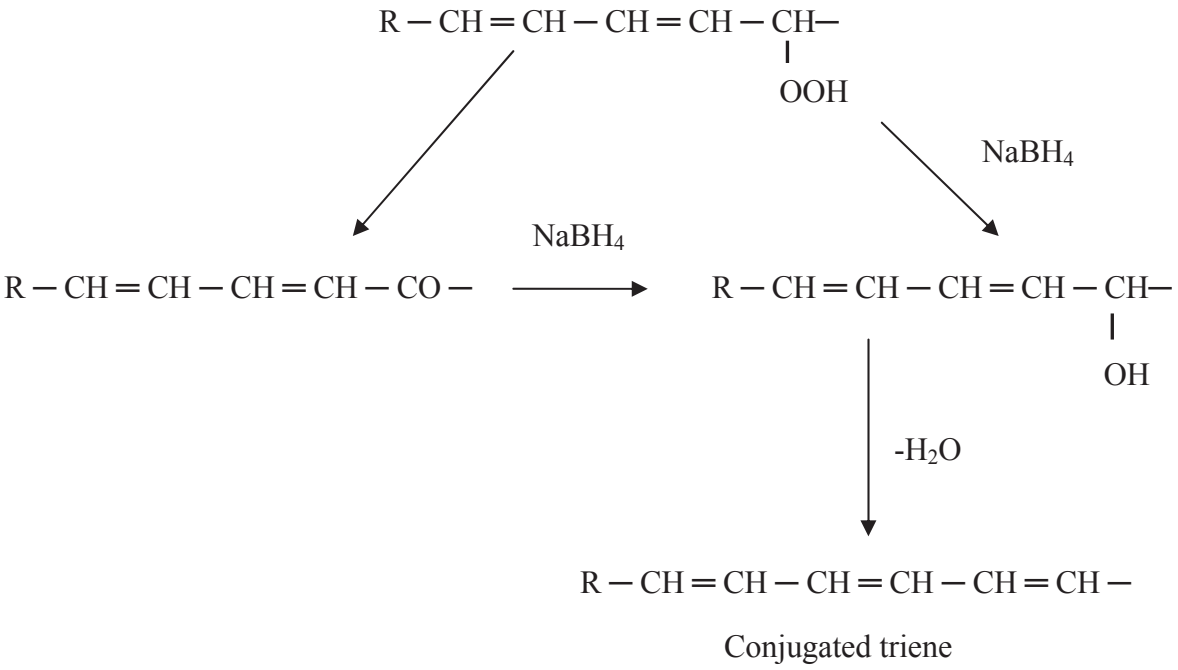


Figure 2.12. Formation of conjugable oxidation products (Michael, 2001).

2.3 Phenolics and their importance as natural products

The plant phenols are secondary metabolites or products that embrace a considerable range of substances that possess one or more aromatic rings bearing one or more hydroxyl constituents. This definition is not entirely satisfactory since it inevitably includes compounds such as oestrone, the female sex hormone (which is principally terpenoid in origin), which is why a definition based on the metabolic origin is preferable. For this reason, plant phenols are

being regarded as those substances derived from the shikimate pathway and the phenyl propanoid pathway (Figure 2.13) (Ryan and Robards, 1998).

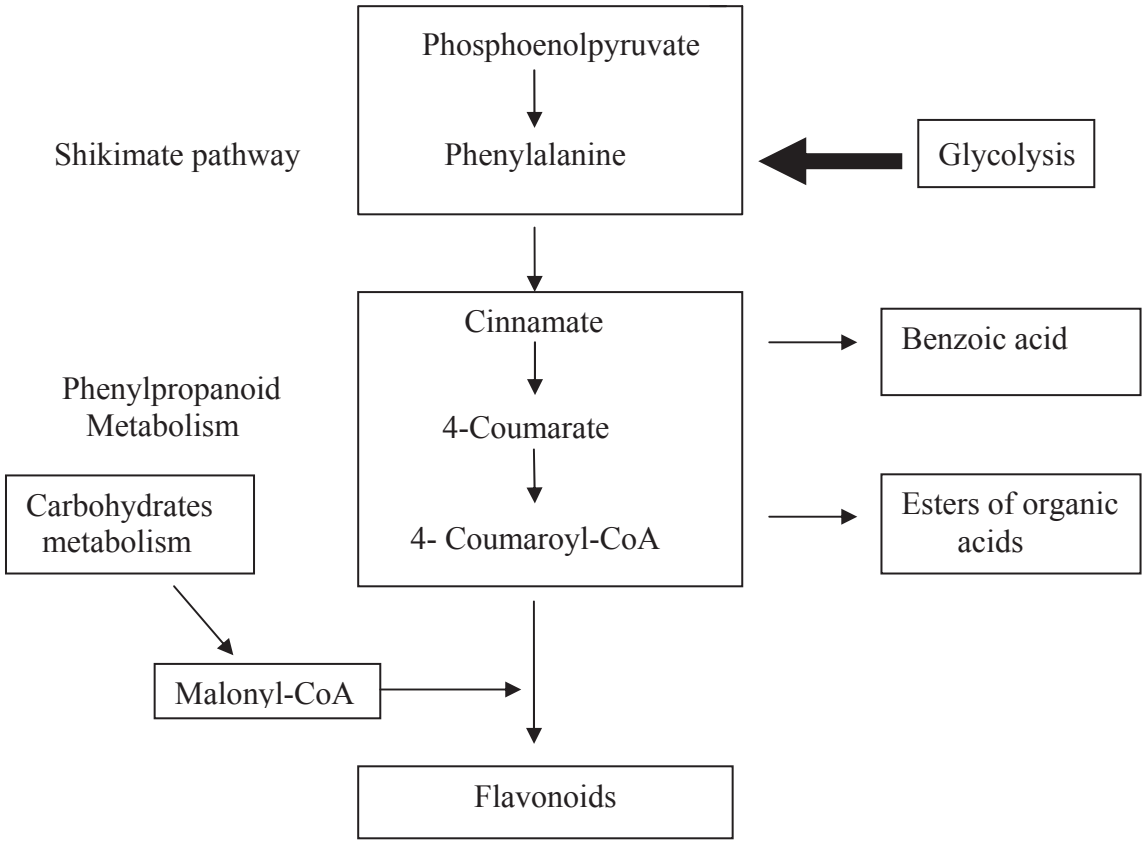
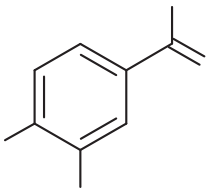


Figure 2.13. Shikimate pathway and phenyl propanoid pathway (Macheix *et al*., 1990).

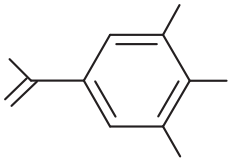
Phenylalanine ammonia lyase (PAL) is the key enzyme in the biosynthetic pathway of most phenolic compounds. PAL catalyses the elimination of ammonium from L-phenylalanine giving rise to trans-cinnamate (Vinson *et al*., 1998), which is the first step in plant phenylpropanoid biosynthesis as lignin, suberin, flavonoids, coumarins and amides (Sanchez-Moreno *et al*., 1998). Plant phenols have been classified into fifteen major groupings distinguished by the number of constitutive carbon atoms in conjugation with the structure of the basic phenolics skeleton. The range of known phenolics is vast.

Phenolic compounds can be simple phenols containing a single aromatic ring, such as vanillin or gallic acid (Figure 2.14), or polyphenols containing more than one ring as flavonoids

(Macheix *et al*., 1990). Polyphenolic components of higher plants manifest antioxidant, antimutagenic, anti-inflammatory, and antimicrobial activities (Sanchez-Moreno *et al*., 1998). Additional structural complexity is introduced by the common occurrence of certain phenolics as O-glycosides in which one or more of the phenolic hydroxyl groups are bound to a sugar or sugars by glycosidic bonds. Glucose is the most commonly encountered sugar, with rhamnose and the disaccharide, rutinose (6-O-α-L-rhamnosyl-D-glucose) also being encountered. Acylation of the glycosides, in which one or more of the sugar hydroxyls are derivatised with an acid, such as acetic or ferulic acid, is occasionally observed (Ryan and Robards, 1998).



Vanillin



Gallic acid

Figure 2.14. Chemical structure of vanillin and gallic acid (Macheix *et al*., 1990).

2.3.1 Classes of phenolic compounds

2.3.1.1 Simple phenols and phenolic acids

The simple phenols include monophenols such as *p*-cresol isolated from several fruits and diphenols. Diphenols such as hydroquinones are probably the most widespread simple phenols (Van Sumere, 1989).

Major types of natural antioxidants are the phenolic acids. There are two classes of phenolic acids, hydroxybenzoic acids and hydroxycinnamic acids. Except in certain red fruits and onions, the content of hydroxybenzoic acids in edible plants is usually very low (Shahidi and Naczk, 2004). Tea can be an important source of gallic acid which is 3,4,5-trihydroxybenzoic acid (Tomas-Barberan and Clifford, 2000). Gallic acid usually occurs in plants in souble form as catechin esters, quinic acid ester or hydrolyzable tannins.

The hydroxycinnamic acids and their derivatives are more common than are the hydroxybenzoic acids in foods. Hydroxycinnamic acids and their derivatives are almost exclusively derived from *p*-coumaric acid, caffeic acid, and ferulic acid whereas sinapic acid is, in general, less encountered (Figure 2.15). The occurrence of hydroxycinnamic acids in food has been reviewed by Herrmann (1989). Caffeic acid and its esterified derivatives are the most abundant hydroxycinnamic acids in a variety of fruits. On the other hand, ferulic acid and its derivatives are the most abundant hydroxycinnamic acids found in cereal grain (Manach *et al.*, 2004).

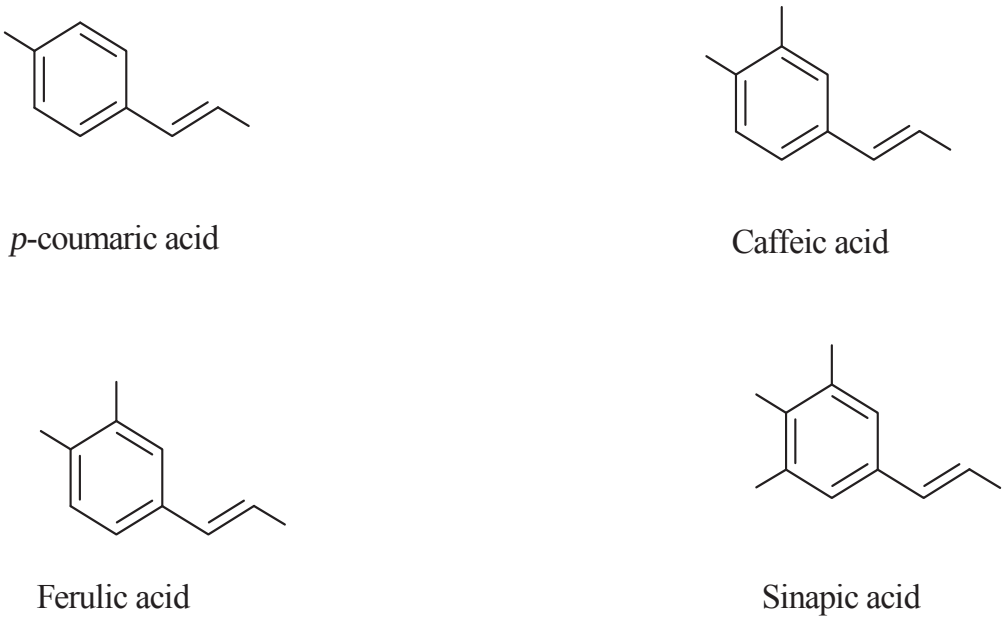


Figure 2.15. Chemical structure of hydroxylated derivatives of some phenolics (Macheix *et al*., 1990).

2.3.1.2 Flavonoids

Flavonoids are ubiquitous in plants; almost all plant tissues are able to synthesize flavonoids. There are also a wide variety of types at least 2000 naturally occurring flavonoids. Flavonoids are present in edible fruits, leafy vegetables, roots, tubers bulbs, herbs, spices, legumes, tea, coffee, and red wine (Shahidi and Naczk, 2004).

In general, the leaves, flowers, and fruits or the plant contain flavonoid glycosides; woody tissues contain aglycones, and seeds may contain both. Flavonoids can be classified into seven groups: flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols (catechins), and anthocyanidins (Table 2.3).

Table 2.3. Different classes of flavonoids, their substitution patterns and dietary sources (Shahidi and Naczk, 2004).

Class	Name	Substitution	Dietary Source
Flavone	Apigenin	5,7-OH	Parsley, celery
	Rutin	5,7,3',4'-OH, 3-O-rutinose,	Buckwheat, citrus
	Tangeretin	4,5,6,7,4'- OCH_3	Citrus
Flavanone	Naringin	5,4' - OH	Citrus
	Naringenin	5,7,4' - OH	Orange peel
Flavonol	Kaempferol	3,5,7,4' - OH	Broccoli, tea
	Quereetin	3,5,7,3',4'-OH	Onion, broccoli, apples, berries
Flavononol	Taxifolin	3,5,7,3',4' -OH	Fruits
Isoflavone	Genistein	5,7,4' - OH	Soybean
	Daidzein	4' - OH, 7- O- glucose	Soybean
	Puerarin	7,4'-OH, 8- C-glucose	Kudzu
Flavanol (catechin)	(-)-Epicatechin	3,5,7, 3',4' -OH	Tea
	(-)-epigallocatechin	3,5,7, 3',4',5'- OH	Tea
	(-)-epigallocatechin gallate	5,7, 3',4',5'- OH, 3-gallate	Tea
Anthocyanidin	Cyanidin	3,5,7,3',4'-OH	Cherry, strawberry
	Delphinidin	3,5,7, 3',4',5'- OH	Dark fruits

2.3.1.3 Stilbenes

Stilbenes are phenolic compounds which contain two benzene rings separated by an ethane bridge. They are widely distributed in higher plants and their main physiological roles relate to their action as phytoalexins and growth regulators (Gotham, 1989). Stilbenes had not caught the attention of food and nutritionists until one of its family members, resveratrol

(3,5,4'-trihydroxystilbene) was reported to demonstrate a preventing effect on cancer (Savouret and Quesne, 2002).

2.3.1.4 Lignans

Lignans are dimers of phenylpropanoid units linked by the central carbons of their side chains. In plants, lignans and their higher oligomers act as defensive substances (Shahidi and Naczk, 2004). Lignan-rich plant products were found to be active ingredients in the treatment of disease in Chinese folk medicine. Unfortunately, many of the active ingredients of these plant products have not been scientifically tested as therapeutic agents (Ayres and Loike, 1990). However, flax and sesame lignans have been considered as important components with health benefits (Spence *et al*., 2003)

Lignans represent a vast and rather diverse group of phenylpropanoidic secondary metabolites found throughout the plant kingdom, including species with a renowned food and dietary use. Their presence is recurrent in a large number of fruits, seeds, vegetables and beverages like juices, beer, coffee and wine (Kuhnle *et al*., 2008; Bonzanini *et al*., 2009). If assumed in nutritionally relevant amounts, lignans are reputed beneficial for preventing the onset of various diseases and disorders; on this regard specific evidences are available for atherosclerosis and cancer prevention, inhibition of platelet activating factor (PAF), reduction of inflammation, risk factors for stroke and oxidative stress (Pradash, 2005). Some of these properties are closely related to a remarkable antioxidant activity exerted by the majority of lignans (Niemeyer and Metzler, 2003).

2.3.1.5 Tannins

Depending on their structures, tannins are defined as hydrolyzable or condensed. Hydrolyzable tannins are glycosylated gallic acids (Ho, 1993). Condensed tannins also known as proanthocyanidins and are linear polymers of flavan-3-ol (catechin and gallocatechin), and flavan-3,4-diol units. The consecutive units of condensed tannins are linked through the interflavonoid bond between C-4 and C-8 or C-6 (Hemingway, 1989). Tannins occur widely in different foods and are often concentrated in the skin of fruits and seed coats, among others.

2.3.2 Antioxidant properties of food phenolics

Antioxidants markedly delay or prevent oxidation of the substrate (Halliwell, 1999; Shahidi, 2000), when they are present in foods or in the body at low concentrations compared to that of an oxidizable substrate. Food manufacturers have used food-grade antioxidants, mainly of a phenolic nature, to prevent quality deterioration of products and to maintain their nutritional value. Antioxidants have also been of interest to health professionals because they help the body to protect itself against damage caused by reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) and reactive chlorine species (RCS) associated with degenerative diseases (Figure 2.16).

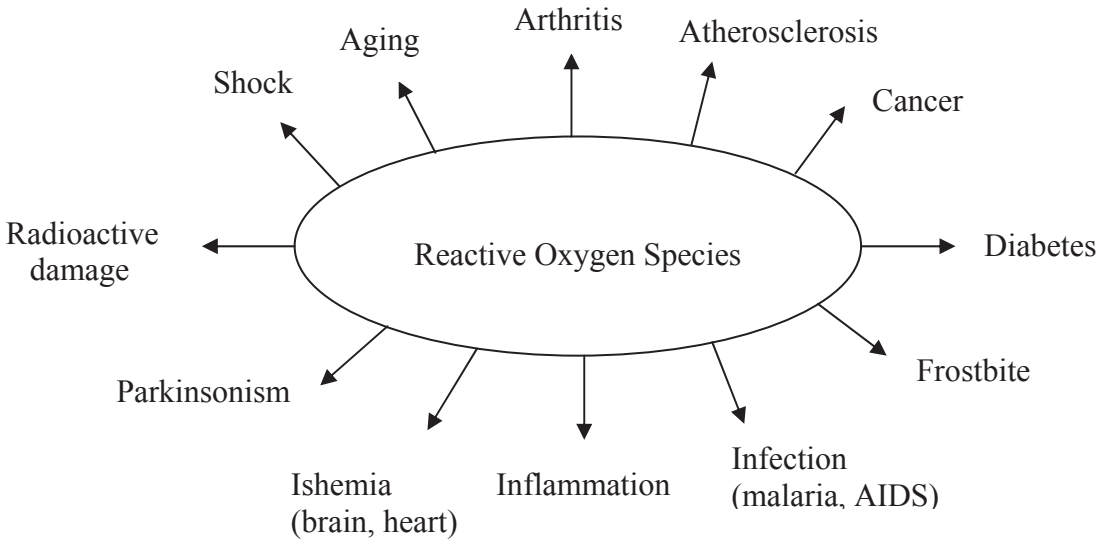


Figure 2.16. Diseases and damages caused by reactive oxygen species (Shahidi and Naczk, 2004).

Antioxidants act at different levels in the oxidative sequence involving lipid molecules. They may decrease oxygen concentration, intercept singlet oxygen, prevent first-chain initiation by scavenging initial radicals such as hydroxyl radicals, bind metal ion catalysts, decompose primary products of oxidation to nonradical species and break chains to prevent continued hydrogen abstraction from substrates (Shahidi, 2000, 2002).

Natural antioxidants from dietary sources include phenolic and polyphenolic compounds, among others. The mechanism by which these antioxidants exert their effects may vary depending on the compositional characteristics of the food, including its minor components. Furthermore, the beneficial health effects of consuming plant foods have been ascribed, in part, to the presence of phenolics, which are associated with counteracting the risk of cardiovascular diseases, cancer and cataract as well as a number of other degenerative diseases. This is achieved by preventing lipid oxidation, protein cross linking and DNA mutation and, at later stages, tissue damage (Figure 2.17).

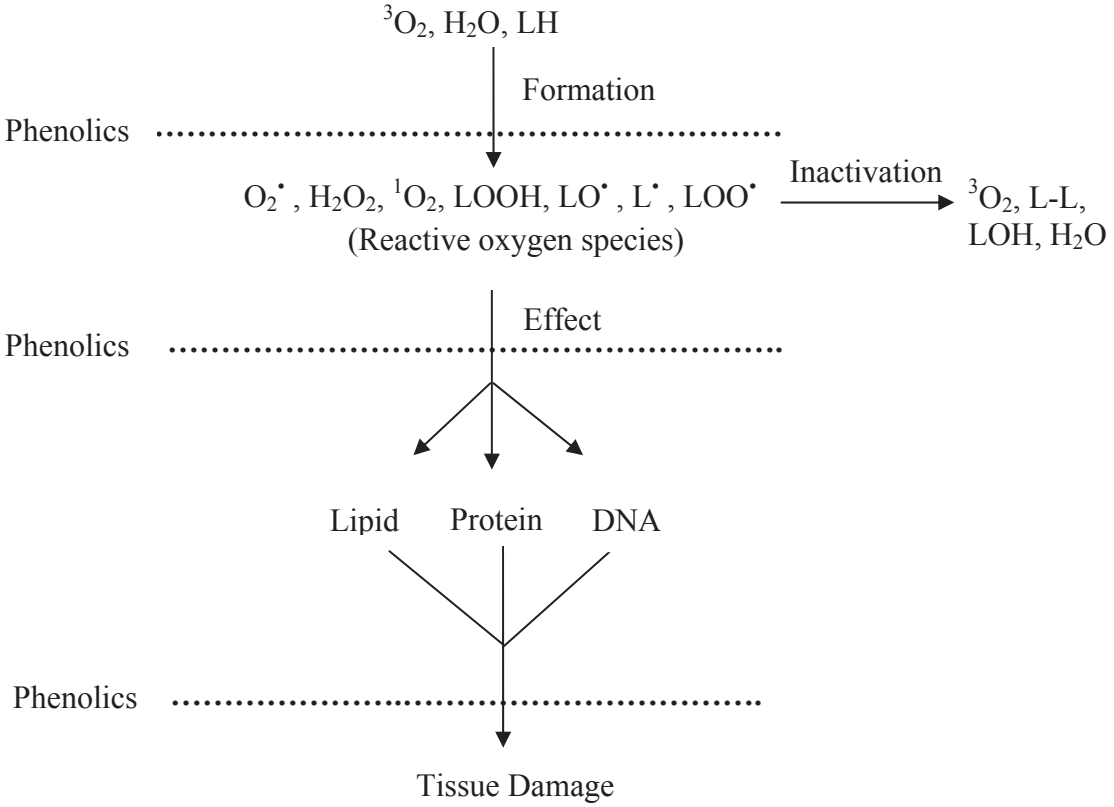


Figure 2.17. Consequences of reactive oxygen species in diseases and preventive role of phenolics (Shahidi and Naczk, 2004).

Although phenolic compounds and some of their derivatives are very efficient in preventing autoxidation, only a few phenolic compounds are currently allowed as food antioxidants. The major considerations for acceptability of such antioxidants are their activity and potential

toxicity and/or carcinogenicity. The approved phenolic antioxidants have been extensively studied, but the toxicology of their degradation products is still not clear. Figure 2.18 represents a general scheme for autoxidation of polyunsaturated lipids and their consequence in quality deterioration of food.

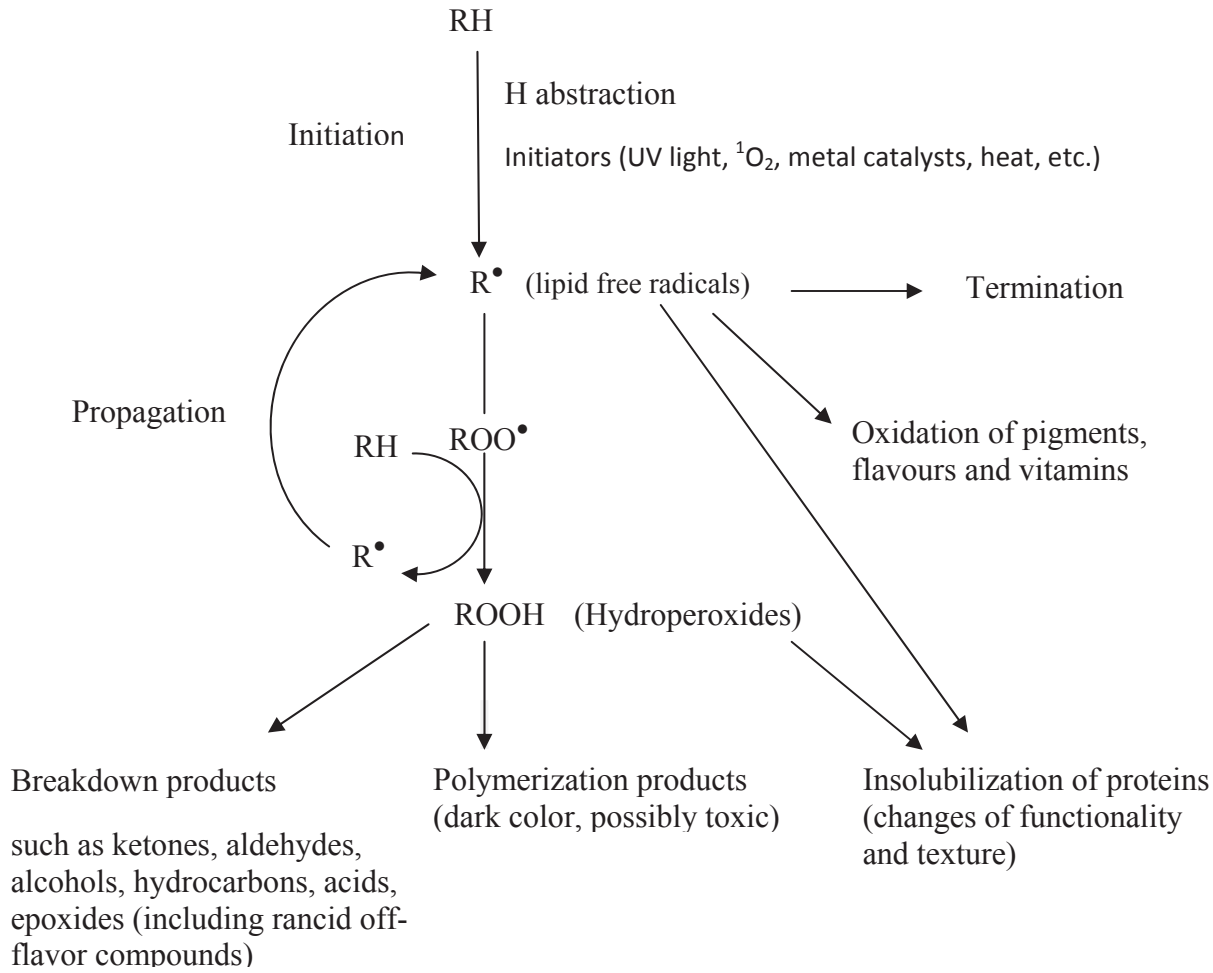


Figure 2.18. General scheme for autoxidation of lipids containing polyunsaturated fatty acids (RH) and their consequences (Shahidi and Naczk, 2004).

2.3.3 Mechanism of action of phenolic antioxidants

The first detailed kinetic study of antioxidant activity was conducted by Boland and ten-Have (1947) who postulated Reaction 2.12 and Reaction 2.13 for free radical terminators. Phenolic

antioxidants (AH) interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals (Reaction 2.12 and Reaction 2.13). The latter reactions compete with chain propagation Reaction 2.4 to Reaction 2.6 and Reaction 2.16:

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (2.12)$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \quad (2.13)$$

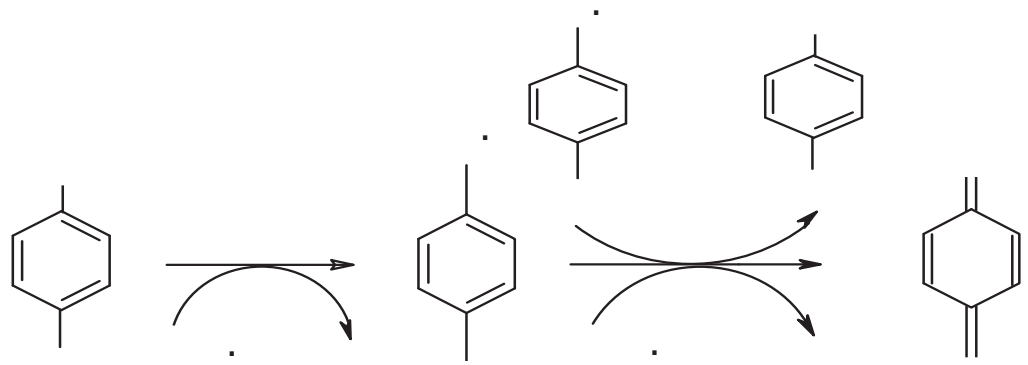
$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \quad (2.14)$$

$$RO^{\bullet} + A^{\bullet} \rightarrow ROA \quad (2.15)$$

$$RO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (2.16)$$

These reactions are exothermic in nature. The activation energy increases with increasing A–H and R–H bond dissociation energy. Therefore, the efficiency of the antioxidants (AH) increases with decreasing A–H bond strength. The resulting phenoxy radical must not initiate a new free radical reaction or be subject to rapid oxidation by a chain reaction. In this regard, phenolic antioxidants are excellent hydrogen or electron donors; in addition, their radical intermediates are relatively stable due to resonance delocalization and lack of suitable sites for attack by molecular oxygen (Belitz and Grosch, 1987).

In the body, free radicals may be involved in a number of diseases and tissue injuries such as those of the lung, heart, cardiovascular system, kidneys, liver, eye, skin, muscle and brain, as well as the process of ageing. Oxidants and radicals are known to mediate such disorders, but are generally neutralized by antioxidant enzymes in healthy individuals. However, with age and in individuals with certain ailments, the endogenous antioxidants may require exogenous assistance from dietary antioxidants in order to maintain the integrity of cell membranes. The phenoxy radical formed by reaction of a phenol with a lipid radical is stabilized by delocalization of unpaired electrons around the aromatic ring as indicated by the valence bond isomers in Reaction 2.17.



(2.17)

However, phenol is inactive as an antioxidant. Substitution of the hydrogen atoms in the *ortho* and *para* positions with alkyl groups increases the electron density of the OH moiety by an inductive effect and thus enhances its reactivity toward lipid radicals. Substitution at the *para* position with an ethyl or *n*-butyl group rather than a methyl group improves the activity of the phenolic antioxidant; however, presence of chain or branched alkyl groups in this position decreases the antioxidant activity (Gordon, 1990).

The stability of the phenoxy radical is increased by bulky groups at the *ortho* positions as in 2, 6-di-tertiary-butyl, 4-methoxyphenol or butylated hydroxyanisole (BHA). Because these substituents increase the steric hindrance in the region of the radicals, they further reduce the rate of possible propagation reactions that may occur, as in Reaction 2.18 to Reaction 2.20, involving antioxidant free radicals (Gordon, 1990):

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \quad (2.18)$$

$$AOO^{\bullet} + RH \rightarrow AOOH + R^{\bullet} \quad (2.19)$$

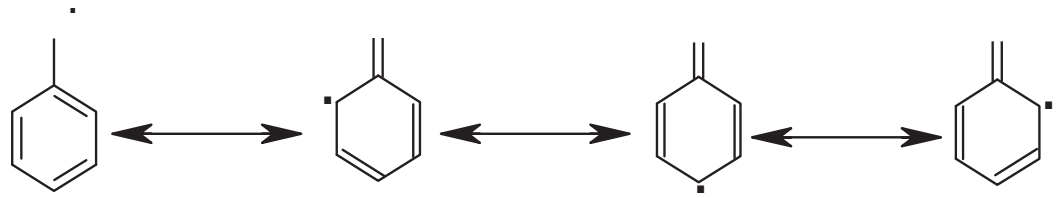
$$A^{\bullet} + RH \rightarrow AH + R^{\bullet} \quad (2.20)$$

The introduction of a second hydroxy group at the *ortho* or *para* position of the hydroxy group of a phenol increases its antioxidant activity. The effectiveness of a 1,2-dihydroxybenzene derivative is increased by the stabilization of the phenoxy radical through intramolecular hydrogen bond. Thus, catechol and hydroquinone are much more effective in their peroxynitrite scavenging activity than phenol (Heignen *et al*., 2001). Similarly, flavonols containing a catechol moiety (3′- and 4′-OH) in ring B (rutin and monohydroxyethyl

rutinoside) or an AC-ring with three OH groups (3-, 5-, and 7-OH) are potent scavengers. The 3-OH group is found to be the active center, its activity influenced by electron-donating groups at the 5- and 7-positions (galangin, kaempferol, and trihydroxyethyl quercetin). In another study, Heim *et al*. (2002) found that multiple hydroxyl groups conferred substantial antioxidant, chelating, and, in some cases, pro-oxidant activity to the molecule. Methoxy groups introduce unfavorable steric effects, but presence of a double band and carbonyl functionality in the C ring increases the activity by affording a more stable flavonoid radical through conjugation and electron delocalization.

Finally, the antioxidant activity of hydroxyflavones is influenced by pH. The antioxidant potential increases, as determined by the TEAC assay, upon deprotonation of the hydroxyl group. This indicates that the mechanism of action of flavonoids is variable and, although abstraction of the hydrogen atom is involved for underprotonated species, electron (not hydrogen) atom donation is involved in the deprotonated species (Lemanska *et al*., 2001). Furthermore, the hydroxyl radical scavenging activity of phenolics involves multiple mechanisms, including hydroxyl bond strength, electron donating ability, enthalpy of single electron transfer and spin distribution of the phenoxy radical after hydrogen abstraction (Cheng *et al*., 2002). Arts *et al*. (2003) have reported a critical evolution of the use of antioxidant capacity in defining optimal antioxidant structures.

The antioxidant activity of dihydroxybenzene derivatives is partly due to the fact that the semiquinoid radical produced initially can be further oxidized to a quinine by reaction with another lipid radical. It can also form into a quinone or hydroquinone molecule, as in Reaction 2.21:



(2.21)

The activity of 2-methoxyphenol is, in general, much lower than that of catechol, which possesses two free hydroxy groups, because 2-methoxyphenols are unable to stabilize the phenoxy radical by hydrogen bonding as in Reaction 2.21 (Gordon, 1990). The effect of antioxidant concentration on autoxidation rates depends on many factors, including the

structure of the antioxidant, oxidation conditions, and nature of the sample oxidized. Often phenolic antioxidants lose their activity at high concentrations and behave as pro-oxidants (Cillard *et al*., 1980) by involvement in initiation reactions such as those in Reaction 2.18 to Reaction 2.20 (Gordon, 1990). Antioxidant activity by donation of a hydrogen atom is unlikely to be limited to phenols. Endo *et al*. (1985) have suggested that the antioxidant effect of chlorophyll in the dark occurs by the same mechanism as phenolic antioxidants.

Phenolic antioxidants are more effective in extending the induction period when added to oil that has not deteriorated to any great extent. However, they are ineffective in retarding decomposition of already deteriorated lipids. Thus, antioxidants should be added to foodstuffs as early as possible to achieve maximum protection against oxidation (Coppen, 1983).

The oils with higher content of unsaturated fatty acids, especially polyunsaturated FA, are most susceptible to oxidation. In order to overcome the stability problems of oils and fats synthetic antioxidants, such as butylate hydroxyanisole (BHA), butylated hydroxy tolune (BHT), tertiary butyl hydroquinone (TBHQ) have been used as food additives. But resent reports reveal that these compounds may be implicated by health risks, including cancer and carcinogenesis (Prior, 2004). Therefore the most powerful synthetic antioxidant (TBHQ) is not allowed for food application in Japan, Canada and Europe. Similarly, BHA has also been removed from the generally recognized as safe (GRAS) list of compounds (Farag *et al*., 1989). Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which in general are supposed to be safer.

2.4 Main sources of natural antioxidants from foods

Plants provide a rich of natural antioxidants. These include tocopherols, vitamin C, carotenoids, and phenolic compounds. There are wide variations between the total phenolics contents of the different fruits or vegetables, or even for the same fruits or vegetables reported by different authors. These differences may be due to the complexity of these groups of compounds, and the methods of extraction and analysis (Kalt *et al*., 2001). For example, phenolic compounds present in fruits are found in both free and bound forms (mainly as *β*-glycosides), but as the latter are often excluded from analyses, the total phenolics contents of fruits are often underestimated (Sun *et al*., 2007). Besides, phenolics contents of plant foods

depend on a number of intrinsic (genus, species, cultivars) and extrinsic (agronomic, environmental, handling and storage) factors (Rapisarda *et al*., 2000). Beverages such as fruit juices, tea and wines are important sources of phenolics in the human diet. Reductions or losses of phenolic compounds have been reported in commercial juices, and these have been attributed to commercial processing procedures (Nagendran *et al*., 2006). Table 2.4 summarizes some of the natural antioxidants and their source in plant materials.

Table 2.4. Natural food sources of some antioxidants (Nagendran *et al.,* 2006).

Compounds	Example of source
Vitamin E (tocopherols)	Oilseed, palm oil, nuts, eggs, dairy products, whole grains, vegetables, cereals, margarine, etc.
Vitamin C	Fruits and vegetables, berries, citrus fruits, sporouts, green peppers, potatoes.
Carotenoids	Dark leafy vegetables, carrots, sweet potatoes, yams, tomatoes, cantaloupes, apricots, citrus fruits, kale, turnip greens, palm oil.
Flavonoids \isoflavones	Fruits and vegetables, oilseeds, berries, eggplants, peppers, citrus fruits, cruciferous vegetables, yams tomatoes, onions,
Phenolic acids	Oilseeds and certain oils, cereals, grains.
Catechins	Green tea, berries, certain oilseeds.
Extracts	Extracts from green tea, rosemary, sage, clove, oregano, thyme, oat, rice bran.

Plant phenolics are thought to protect the plants against tissue injuries as they oxidize and combine with proteins and other components, In addition, phenolic compounds in plants may serve as defense system against herbivory. By products of photosynthesis may also produce high levels of oxygen, free radical, and reactive oxygen species (ROS) in profusion. Thus, plants use a myriad of antioxidant compounds to deal with these in order to survive.

2.4.1 By-products of plant food processing as a source of functional compounds

There is a rapidly growing body of literature covering the role of plant secondary metabolites in food and their potential effects on human health. Furthermore, consumers are increasingly aware of diet related health problems, therefore demanding natural ingredients which are expected to be safe and health-promoting (Vasso and Constantina, 2007).

Processing of fruits, vegetables, and oilseeds result in high amounts of waste materials such as peels, seeds, stones, and oilseed meals. Disposal of these materials usually represents a problem that is further aggravated by legal restrictions. Plant waste is prone to microbial spoilage; therefore, drying is necessary before further exploitation. The cost of drying, storage, and transport poses additional economical limitations to waste utilization. Therefore, agro-industrial waste is often utilized as feed or fertilizer. However, demand for feed or fertilizer varies and depends on agricultural production. Moreover, valuable nutrients contained in agro-industrial wastes are lost. Thus new aspects concerning the use of these wastes as by-products for further exploitation on the production of food additives or supplements with high nutritional value have gained increasing interest because these are high-value products and their recovery may be economically attractive (Vasso and Constantina, 2007). There exist precedents for the recovery of antioxidant substances from herbs, citrus peel, shrimps, grape skins etc. (Bocco *et al*., 1998).

Agricultural and industrial residues are attractive sources of natural antioxidants (Moure *et al*., 2001). By-products, which remain after processing of fruit and vegetable in food processing industry, still contain a huge amount of phenolic compounds. Some studies have already been done on by-products, which could be potential sources of antioxidants. Visoli *et al*. (1999) studied olive oil waste, Sreenath *et al*. (1995) studied citrus by-products, while by-products obtained after artichoke, cauliflower, carrot, celery, and onion processing were investigated by Larrosa *et al*. (2002). There were also many researches done on the polyphenols acquiring from grape marc. Loo and Foo (1999) made researches on grape seeds and grape pomace peels. Bonilla *et al*. (1999) explored the possibilities of using phenolic compounds from grape marc for use as food lipid antioxidants. Louli *et al*. (2004) investigated the effect of various process parameters; solvent type, and feed pretreatment (crushing, removal of stems), on the extraction efficiency of phenolic antioxidants from grape marc, whereas Negro *et al*. (2003) investigated the content of total polyphenols and

antioxidant activity of grape marc extracts. Abdalla *et al*. (2007) collected Egyptian mango seeds as wastes from local fruit processing units and checked their antioxidant potential. Astadi *et al*., (2009) measured antioxidant activity of anthocyanins of black soybean seed coat in human low density lipoprotein (LDL). Maier *et al*. (2009) observed antioxidant potential of seven grape seed samples originating from mechanical seed oil extraction. Besides these, search of newer sources of natural antioxidants from economical materials, agricultural wastes is hot area of research in recent years. As a step towards series of investigations in the said dimension, antioxidant potential of potato peels, sugar beet pulp, and sesame cake have been studied in this study.

2.4.1.1 By-products of potato

Due to its resemblance to a truffle, herbalists in sixteenth century Europe called it by this name for a time. It took a long time for the potato to be accepted as an important part of the diet in Europe, due in part to its similarity to the nightshade, which was known to be poisonous. Others resisted the crop's acceptance because the phallic shape of the tuber caused it to be labeled as an aphrodisiac, casting shame upon anyone who showed interest in it. Furthermore, due to the appearance of the skin, it was suspected of causing leprosy (Brown 1993). The upper-class population at the time deemed potato an inferior dish suitable only for those who could not afford something better (Niederhauser 1993).

Potato (*Solanum tuberosum* L., Solanaceae) is one of the most important staple crops grown worldwide. Because of its low cost, low fat content and a good source of carbohydrates, high quality protein, fibre and vitamins, it plays an important role in human nutrition. Potatoes provide an excellent source of lysine (Freidman 1996), making them superior to cereal proteins, which lack this important amino acid. In addition to high quality proteins, potatoes contain substantial levels of vitamins and minerals, including vitamins C, and B (Niederhauser 1993).

Although potatoes are consumed directly, processed potato products such as French fries, chips, mashed potato, crisps, starch, potato flakes, flour, and puree represent majority of the consumption (Amir and Venket, 2009). Most of the wastes from potato plants arise from peeling, trimming, slicing, cleaning, and rinsing operations, and the discharge of these liquid and solid wastes creates a pollution problem. According to Schieber *et al*. (2001), losses caused by peeling range from 10 to 25%, their amount depending on the procedure applied,

i.e. steam, abrasion or lye peeling. Potato peels, however, contains several beneficial phytochemicals such as the polyphenols and carotenoids that can have potential applications in the formulation of functional foods. Like citrus waste, the use of potato waste has been directed toward its upgrading to single-cell protein. (Mahmood *et al*., 1998) and could be considered as a new source of natural antioxidant. Aqueous peel extracts were shown to be a source of phenolic acids, especially of chlorogenic, gallic, protocatechuic and caffeic acids (Onyeneho and Hettiarachchy, 1993).

A number of byproducts from vegetable processing industry have been previously studied as potential sources of antioxidants (Azizaha *et al*., 1999; Lu and Foo, 2000). However, little work is reported relating to the utilization of the potato peel for the recovery of phenolic compounds.

2.4.1.2 By-products of sugar production

Sugar is a strategic commodity to many countries of the world, since it comes right after wheat from the importance point of view to many countries in Europe, Africa, North and South America and Australia. Whereas, it occupies the second position after rice in Asian countries. Sugar differs than any other food commodity in being consumed daily at different rates by all people regardless of their standards or classes. In addition to the direct consumption of sugar, it is also, used in many industries whether foods, chemicals, cosmetics, painting materials … etc. There are also many industries which are based on the by-products of sugar industry. The production of sugar in the world depends on two main crops namely sugar cane (*Saccharum officinarum* L., Poaceae) and sugar beet (*Beta vulgaris* L. ssp. *Vulgaris* var. *altissima* DÖLL, Chenopodiaceae). In Egypt, sugar cane was considered to be the main source for sugar industry up to 1981 season and the cultivation of sugar beet did not known economically before 1982 season. Nowadays, sugar beet becomes an important crop for sugar in Egypt. Sugar beet contribution to sugar production increased largely from 2.4 % in 1982 season to about 32 % of the total sugar production in 2006 season. At Fayoum province, the cultivation of sugar beet started in 1989/1990 season, so sugar beet considers a recent crop introduced to Fayoum agricultural (Proc. 37th conference of the Egyptian Society of sugar Technologist, 2006).

The beet-sugar factory produces waste products such as pulp, fibrous, and molasses. If beet-sugar producers are not diligent in caring and disposing of waste products, the wastes can

pollute the natural environment significantly. It makes good business sense for producers to reduce pollution and comply with all legally required actions and limitations with regard to the creation, storage, treatment, and disposal of pollutants, wastes, and hazardous compounds. Molasses represents the runoff syrup from the final stage of crystallization. It mainly consists of fermentable carbohydrates (sucrose, glucose, fructose), and of non-sugar compounds which were not precipitated during juice purification. Furthermore, molasses contains substances formed chemically or enzymatically during processing and storage (betaine and other amino acids, *Maillard* products, *Strecker* decomposition products, lactic acid, mineral and trace elements, and vitamins especially of the B-group). Molasses is used as feed and as a source of carbon in fermentation processes, e.g. for the production of alcohol, citric acid, L-lysine and L-glutamic acid (Higginbotham and McCarthy, 1998). In volume, bagasse is the by-product of highest relevance. The fibrous residue from the extraction process is utilized as fuel and as a source of pentosans, for the production of furfural from pentosan-rich raw material, and for the recovery of fibrous products (Delavier, 1998).

Depending on the process, exhausted beet pulp has a dry matter content of 8–15%. Therefore, its economic utilization requires dewatering which is mostly performed by mechanical pressing (pressed pulp), followed by thermal drying. Pressed pulp is an energy-rich animal feed the shelf-life of which can be extended by ensiling (Harland, 1998). Enzymatic release of ferulic acid from sugar beet pulp and subsequent bioconversion to vanillin in a two-step process has been demonstrated (Thibault *et al.,* 1998). A freeze-dried arabinan substitute for gum arabicum and a fat replacer based on linear arabinan were also obtained from sugar beet pulp (Broughton *et al.,* 1995). Addition of sugar beet fiber to semolina increased dietary fiber content but adversely affected colour and cooking loss of spaghetti (Ozboy and Koksel, 2000). Owing to its high content of acetyl groups and its low molecular weight, beet pectin has poor gelling properties and is of very limited commercial value (Broughton *et al.,* 1995).

2.4.1.3 By-products of sesame

Sesame (*Sesamum indicum* L.) is one of the most important oilseed crops (because of its high content of lipid) in the world (Shyu and Hwang, 2002). It is not only a source of edible oil, but also widely used in baked goods and confectionery products (Namiki, 1995). It is also consumed as a nutritious food, beneficial to health in oriental countries. Several studies have reported the health-promoting properties of sesame (Shyu and Hwang, 2002). Sesame is

cultivated on a worldwide basis for both oil and protein and the seed is composed of 55% lipid and 20% protein (Abou-Gharbia *et al.,* 1997). Elleuch *et al.* (2007) noted that sesame oil was highly stable to oxidation compared with other plant oils. The stability of sesame oil is due to the presence of endogenous antioxidants, sesamin, sesamolin and sesamol. Sesamol is usually present in traces, but may also be released from sesamolin by hydrogenation, bleaching earth, or other conditions of processing. Sesame seed contains 0.4 to 1.1% sesamin, 0.3 to 0.65% sesamolin and traces of sesamol. Sesamol is a free 3,4-methylenediphenoxy phenol; sesamolin is an acetal type derivative of sesamol and sesamin. The 3,4-methylenediphenoxy phenol is attached to the 2,7-dioxabicyclo-(3,3,0)- octane nucleus directly. Sesamol is as effective as BHT and BHA and more effective than PG (Lyon, 1972).

Sesame is cultivated in several countries such as India, Sudan, China and Burma which are considered as the major producers (60% of its total world production) (Abou-Gharbia *et al.,* 2000). In Tunisia, 80% of the needed sesame seed is imported from Sudan and 20% from Egypt (Elleuch *et al.,* 2007). Its seeds are used essentially for the production of oil, but also in the production of the paste (tehineh) and in food formulations such as Halaweh (sweetened tehineh), java beans and salads (Abou-Gharbia *et al.,* 2000; Abu-Jdayil *et al.,* 2002; Namiki, 1995).

In Egypt, the major part of the imported sesame is essentially transformed to Halaweh. This food product is obtained after mixing the white tehineh (white sesame seed dehulled, roasted and grinded), saponin (*Saponaria officinalis*) and Nougat (heat-treated sucrose) (Abu-Jdayil *et al.,* 2002). The sesame cake is a by-product of the oil industry which could be recovered and used as a value added product. However, in some sesame processing countries, this by-product is generally discarded, or used in animal feeding. Preliminary studies showed that an appreciable amount of antioxidants was still present in sesame cake.

2.5. Aims and objectives of work

With the advent of industrial revolution, environmental pollution has increased significantly. As a result, chances of cardiovascular diseases and cancer have increased. Many epidemiological studies have suggested that antioxidative compounds from different plant sources are useful in the control of these diseases. Many plant polyphenols, such as ellagic acid, catechins, chlorogenic, caffeic and ferulic acids, as well as their dietary sources, such as

tea, have been shown to act as potent antimutagenic and anticarcinogenic agents (Ayrton *et al*., 1992).

Vegetable oils nowadays are a great source of balancing oil consumption in families and because of consumers concern with the saturated/unsaturated fatty acid ratio in the diet, the lipid composition of fruit and vegetable has lately received particular attention. Consumers are especially interested in essential fatty acids, with emphasis on the health potential of polyunsaturated fatty acids. It is considered that these fatty acids play a natural preventive role in cardiovascular diseases and in alleviation of some other health problem, because they promote the reduction of both total and HDL cholesterol (Melgarejo and Artes, 2000)**.** But these fatty acids are damaged by oxidation process and shelf life of oils is decreased due to lipid oxidation/rancidity. Synthetic antioxidants as additives into the oils are not only expensive, but also carcinogenic. So nowadays focus of research is to find antioxidants from natural sources.

In the last few years, an increased attention has been focused on the industrial wastes, especially those containing residual phenols from the plant raw material used (Shaker, 2006)**.** As a step towards series of investigations in the said dimension, this research project follows a line of investigation on the antioxidative potential from potato peels, sugar beet pulp, and sesame cake and their efficacy in stabilization of sunflower and soybean oils under accelerated storage conditions.

The current study covered the following points:

(1) Determining the chemical composition of sesame cake, sugar beet pulp, and potato peels wastes.

(2) Extraction and determination of total phenolic compounds from sesame cake, sugar beet pulp, and potato peels using different extracting solvents as well as measurement of flavonoid and flavonol contents.

(3) Antioxidant activities of sesame cake, sugar beet pulp, and potato peels extracts were evaluated by 2,2-azinobis (3- ethylbenzthiazoline sulphonate) (ABTS) radical scavenging activity, 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging capacity, and *β*-carotene/linoleic acid test system for total antioxidant activity, and compared with that of butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tert-butyl hydroquinone (TBHQ).

(4) Investigation the antioxidant properties of sesame cake, sugar beet pulp, and potato peels in comparison with synthetic antioxidant under accelerated oxidation conditions using sunflower and soybean oils as oxidation substrates at different concentrations for 72 h at 70°C. For this purpose, peroxide value (PV), *p*-Anisidine value (AV), conjugated dienes (CD), and conjugated trienes (CT) of treated oils were determined.

(5) Identification the antioxidant compounds present in sesame cake, sugar beet pulp, and potato peels extracts using high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and UV scan techniques.

3 MATERIALS AND METHODS

3.1 Materials and reagents

Potato peels *(Solanum tuberosum* cv Diamond*)* obtained from a local potato chip manufacturer (6 October, Egypt) and sesame cake *(Sesamum indicum* cv Shandweel -3*)* purchased from Hamada market (Giza, Egypt) in November 2007 and stored in a deep freeze at -20°C until use. Sugar beet pulp *(Beta vulgaris* cv Gloriato*)* was supplied by a local sugar manufacturer (Fayoum, Egypt). BHA, BHT, hydroxybenzoic acid, chlorogenic acid, vanillic acid, and 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) were purchased from Sigma (St Louis, MO, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), *p*-coumaric acid, sinapic acid, caffeic acid, TBHQ, *p*-Anisidine (4-Amino-anisol; 4-Methoxy-anilin), cinnamic acid, and *β* carotene were obtained from Fluka (Buchs, Switzerland). All other chemicals used were analytical grade.

Sunflower oil was produced by Brökelmann-Oelmühle GmbH (Hamm, Germany) obtained from Aldi supermarket (Berlin, Germany), while soybean oil which obtained from Edeka aktivmarkt (Berlin, Germany) was produced by Kunella Feinkost GmbH (Cottbus, Germany) and the oils were free of any synthetic antioxidant.

3.2 Sample preparation

Potato peels, sesame cake and sugar beet pulp were washed, dried in hot air at 40º C and ground to fine powder in a mill. Ground materials (10 g) were extracted with organic solvents (100 mL) methanol, ethanol, acetone, hexane, petroleum ether, and diethyl ether overnight in a shaker at room temperature followed by filtration through Whatman No.1 filter paper. The residues were re-extracted under the same conditions. The combined filtrates were evaporated in a rotary evaporator below 40ºC. The extracts obtained after evaporation of organic solvents were weighted to determine the extract yield and stored at -20°C until further use.

3.3 Proximate analysis of potato peels, sesame cake, and sugar beet pulp

The major chemical constituent, moisture, ash, crude fat, crude fiber, and crude protein were determined in triplicate according to AOAC standard methods (1990). Carbohydrate content was calculated by difference.

3.4 Determination of total phenolics

Total phenolic content of each extract was determined by the Folin–Ciocalteu micro-method (Saeedeh and Asna, 2007). A 20 µL aliquot of extract solution was mixed with 1.16 mL distilled water and 100 µL of Folin–Ciocalteu reagent, followed by addition of 300 µL of Na_2CO_3 solution (20%). Subsequently, the mixture was incubated in a shaking incubator at 40°C for 30 min and its absorbance at 760 nm was measured. Gallic acid was used as a standard for calibration curve. Total phenolic content expressed as gallic acid equivalent (GAE) was calculated using the following linear equation based on the calibration curve:

$A = 0.98C + 9.925 \times 10^{-3}$ $(R^2 = 0.9996)$,

where A is the absorbance and C is the concentration (mg GAE g^{-1} dry weight (DW)).

3.5 Determination of total flavonoids

Total flavonoid content was determined by the method of Ordon *et al*. (2006). A 0.5 mL aliquot of 2% $AlCl_3$ ethanolic solution was added to 0.5 mL of extract solution. After 1 h at room temperature the absorbance at 420 nm was measured. A yellow color indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg mL^{-1}. Total flavonoid content expressed as quercetin equivalent (QE) was calculated using the following equation based on the calibration curve:

$y = 0.0255x$ $(R^2 = 0.9812)$,

where x is the absorbance and y is the concentration (mg QE g^{-1} DW).

3.6 Determination of total flavonols

Total flavonol content was determined by the method of Kumaran and Karunakaran (2007). To 2.0 mL of extract solution, 2.0 mL of 2% $AlCl_3$ ethanol and 3.0 mL (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h at 20°C. Extract samples were evaluated at a final concentration of 0.1 mg mL^{-1}. Total flavonol content expressed as QE was calculated using the following equation based on the calibration curve:

$y = 0.0232x$ $(R^2 = 0.9752)$,

where x is the absorbance and y is the concentration (mg QE g^{-1} DW).

3.7 Antioxidant activity of extracts

Because of the differences among the various test systems available, the results of a single method can provide only a limited assessment of the antioxidant properties of a substance (Gianni *et al*., 2005). For that reason, in this study the antioxidant capacity of each extract was determined through three complementary assay procedures.

3.7.1 Determination of DPPH· radical scavenging capacity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Lee *et al*., 2003) was utilised with some modifications. The stock reagent solution (1 x 10^{-3}M) was prepared by dissolving 22 mg of DPPH in 50 mL of methanol and stored at -20°C until use. The working solution (6 x 10^{-5} M) was prepared by mixing 6 mL of the stock solution with 100 mL of methanol to obtain an absorbance value of 0.8 ± 0.02 at 515 nm, as measured using a spectrophotometer. Extract and synthetic antioxidants (TBHQ, BHA, and BHT in ethanol) solutions of different concentrations (0.1 mL of each) were vortexed for 30 s with 3.9 mL of DPPH solution and left to react for 30 min, after which the absorbance at 515 nm was recorded. A control with no added extract was also analysed. Scavenging activity was calculated as follows:

DPPH radical-scavenging activity (%) = $[(A_{control} - A_{sample})] / (A_{control})] \times 100$,

where A is the absorbance at 515 nm.

3.7.2 ABTS radical scavenging assay

For the ABTS assay the method of Re *et al*. (1999) was adopted. The stock solutions were 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12-16 h at room temperature in the dark. Then 1 mL of the resulting $ABTS^{\bullet+}$ solution was diluted with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm, as measured using a spectrophotometer. $ABTS^{\bullet+}$ solution was freshly prepared for each assay. Extract and synthetic antioxidants (TBHQ, BHA, and BHT in ethanol) solutions of different concentrations (0.1 mL of each) were allowed to react with 1 mL of $ABTS^{\bullet+}$ solution for min, after which the absorbance at 734 nm was recorded. A control with no added extract was also analysed. Scavenging activity was calculated as follows:

ABTS radical-scavenging activity (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control})] \times 100$,

where $Abs_{control}$ is the absorbance of ABTS radical + methanol and Abs_{sample} is the absorbance of ABTS radical + extract/synthetic antioxidants.

3.7.3 ß-Carotene/linoleic acid bleaching

The ability of extracts and synthetic antioxidants to prevent the bleaching of ß-carotene was assessed as described by Keyvan *et al*. (2007). In brief, 0.2 mg ß-carotene in 1mL chloroform, 20 mg of linoleic acid and 200 mg of Tween 20 were placed in a round-bottom flask. After removal of the chloroform, 50 mL of distilled water was added and the resulting mixture was stirred vigorously. Aliquots (6 mL) of the emulsion were transferred to tubes containing different concentrations of extracts/synthetic antioxidants. Immediately after mixing, an aliquot from each tube was transferred into a cuvette and the absorbance (Abs^0) at 470nm was recorded. The remaining samples were placed in a water bath at 50 °C for 2 h, after which the absorbance at 470nm was recorded (Abs^{120}). A control with no added extract was also analysed. Antioxidant activity was calculated as follows:

$$\text{antioxidant activity (\%)} = [1 - (Ab^0_{sample} - Abs^{120}_{sample}) / (Ab^0_{control} - Abs^{120}_{control})] \times 100$$

3.8 Storage studies

3.8.1 Selection of oils

The selection of test oils was based on the presence of varying polyunsaturated fatty acids (PUFA) composition. Thus, sunflower oil (SFO) which includes high level of linoleic acid (18:2n - 6) and soybean oil (SBO) which is rich in α-linolenic acid (18:3n - 3) in addition to linoleic acid were used in this investigation. Schaal oven test (Fennema, 1976) was conducted to evaluate the effect of antioxidants against oxidation during the accelerated oxidative storage of oils.

3.8.2 Sample preparation for oxidative stability determination

Since preliminary evaluation showed that methanolic and ethanolic extracts contained higher antioxidant potential, the methanolic and ethanolic extracts of potato peels, sesame cake, and sugar beet pulp were applied to commercial edible sunflower and soybean oils obtained from local market (free of any antioxidant) at different concentrations (5, 10, 50, 100 and 200 ppm, based on extract weight), in a series of transparent glass bottles having a volume of 20 mL

each, to examine their antioxidative activity. TBHQ, BHA and BHT at a level of 200 ppm were also applied for comparison. The bottles were completely filled with oil and sealed. A control sample was prepared by using the same amount of methanol used to dissolve the antioxidant and the extracts (Moure *et al*., 2000). The antioxidant-enriched oil samples were evaporated in a vacuum evaporator below 40 ºC to evaporate the solvent and subjected to accelerated oxidation in the dark in an oven at 70 °C for 72 h. Aliquot (20 g) were removed periodically every 4, 8, 24, 32, 36, 48, 56, and 72 h for analysis. Immediately after storage period, oil samples were withdrawn for triplicate analyses. The oils were sampled for each measurement from separate bottles.

3.8.3 Analytical procedures

3.8.3.1 Peroxide value (PV)

Peroxide value of samples was measured according to Kirk and Sawyer (1991). The method is based on iodometric titration, which measures the iodine produced from potassium iodide by the peroxides present in the oil. In a stopper conical flask (250 mL) vegetable oils sample (4 ± 0.5 g) was taken along with chloroform (10 mL), glacial acetic acid (15 mL) and fresh saturated aqueous potassium iodide solution (1 mL). The flask was stoppered and shaken vigorously for 1 min and then kept in the dark for further 5 min (IUPAC, 1987). In the next step, double distilled water (10 mL) was mixed thoroughly with the solution and titrated against 0.002 N sodium thiosulphate solution until the yellow colour almost disappeared. Then about 0.5 mL of soluble starch indicator (1%) solution was added. Titration was continued until the blue colour just disappeared. One blank reagent (without sample) was prepared.

$$\text{Peroxide value (meq kg}^{-1}) = \frac{(V - V_0) \times N \times 10^3}{W},$$

where V is the titre value (mL) of sodium thiosulphate solution for sample, V_0 the titre value (mL) of sodium thiosulphate solution for blank, N the normality of sodium thiosulphate solution and W the weight of sample in gram.

3.8.3.2 *p*-Anisidine value (AV)

The *p*-anisidine value (AV) was determined according to Cd 18-90 method (AOCS, 1995). The method is based on the spectrophotometric determination of products formed in the

reaction between aldehydic compounds in the oil and *p*-anisidine. Oil samples (0.5-2.0 g) were dissolved in 25 mL iso-octane and absorbance of this fat solution was measured at 350 nm using a spectrophotometer (Hitachi U-3000, Tokyo, Japan). Five millilitres of the above mixture was mixed with 1 mL 0.25% *p*-anisidine in glacial acetic acid (w/v) and after 10 min standing, absorbance was read at 350 nm using a spectrophotometer. The *p*-anisidine value (AV) was calculated according to the equation:

$AV = 25 \times (1.2\ As - Ab) / m$

where As is the absorbance of the fat solution after reaction with the *p*-anisidine reagent; Ab is the absorbance of the fat solution and m is the mass of oil sample (g).

3.8.3.3 Conjugated dienes (CD) and conjugated trienes (CT)

Specific extinctions at 232 and 270 nm (i.e., conjugated dienes and conjugated trienes) were determined using a spectrophotometer. Oil samples were diluted with iso-octane to bring the absorbance within limits following the standard method of IUPAC method II. D. 23 (IUPAC, 1979).

3.9 Thin-layer chromatography and UV scan of extracts

An aliquot of extracts (10 μL) was spotted on a precoated silica gel plate (F_{254}, 0.25 mm, Merck, Darmstadt, Germany). The plates were developed in the ascending direction for 17 cm with chloroform: methanol (1:1, v/v) then sprayed with 10^{-4} M DPPH in methanol. UV spectrum (200-400 nm) of diluted extracts in methanol (1%) was recorded by spectrophotometry (Shimadzu UV-260 visible recording spectrophotometer; Kyoto, Japan).

3.10 HPLC analysis

The ground materials of potato peels, sesame cake, and sugar beet pulp were used to extract the phenolic acids with 70% methanol (pH 4.0) and placed in ultrasonic water bath with ice for 15 minutes for the better cell wall disintegration. The pellet was re-extracted two times after centrifugation. The supernatant were collected and concentrated in a vacuum concentrator for about 2 hours to a certain volume and then dissolved in 40 % acetonitrile. The samples were filtrated and injected into the HPLC.

Instrumentation and chromatographic conditions: The separation of phenolic compounds was performed on HPLC (Dionex Summit P680A HPLC-System, USA), equipped with P680

pump, ASI-100 automated sample injector, a Narrow-Bore AcclaimPA C16-column (3 µm, 2.1 × 150 mm, Dionex) and PSA-100 photodiode array detector (Dionex) and software Chromeleon 6.8 (Dionex, USA). The column was operated at a temperature of 35 °C. The mobile phase consisted of 0.1% (v/v) phosphoric acid in ultrapure water (eluent A) and of 40% (v/v) acetonitrile in ultrapure water (eluent B). A multistep gradient was used for all separations with an initial injection volume of 40 µL and a flow rate of 0.4 mL/min. The multistep gradient was as follows: 0-1 min: 0.5% (v/v) B; 1-10 min: 0.5-40% B; 10-12 min: 40% B; 12-18 min: 40-80% B; 18-20 min: 80% B; 20-24 min: 80-99% B; 24-30 min: 99-100% B; 30-34 min: 100-0.5% B; 34-39 min: 0.5% B. Simultaneous monitoring was performed at 290, 330 and 254 nm at a flow rate of 0.4 mL/min

3.11 Statistical analysis

Statistical analyses were conducted using SPSS (Statistical Programme for Social Sciences, SPSS Corporation, Chicago, IL, USA) version 16.0 for Windows. All analyses were performed in triplicate and data reported as means ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA). The confidence limits used in this study were based on 95% ($P < 0.05$).

4 RESULTS AND DISCUSSION

4.1 Proximate composition of materials

The results in Table 4.1 show the proximate analysis of potato peels, sesame cake, and sugar beet pulp. This indicates that these by-products could be used as a source of carbohydrate, fat and protein. The lipid content in sesame cake was lower than that reported by Suja *et al*. (2004).

Table 4.1. Chemical composition of potato peels, sugar beet pulp, and sesame cake extracts (% dry weight basis).

Parameter	Moisture	Ash	Crude fat	Crude protein	Crude fiber	Carbohydrate*
potato peels	6.55±0.34	8.48±0.34	8.46±0.36	13.85±0.36	12.98±0.34	56.23±0.36
sugar beet pulp	6.90±0.36	6.64±0.20	6.92±0.34	10.84±0.27	17.82±0.20	57.78±0.27
sesame cake	5.71±0.20	6.12±0.36	30.39±0.27	23.76±0.34	10.86±0.27	28.87±0.34

* by difference

4.2 Extract yields

Solvent extraction is more frequently used for isolation of antioxidants and both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity (Marinova and Yanishlieva, 1997). Ethanol and methanol are the most widely employed solvents for hygienic and abundance reasons, respectively. Since the activity depends on the polyphenol compounds and the antioxidant assay, comparative studies for selecting the optimal solvent providing maximum antioxidant activity are required for each substrate.

The extract yields of potato peels, sugar beet pulp and sesame cake extracts with different solvents varied from 15.9 to 203.1 g kg^{-1} dry weight (Table 4.2). A significant difference ($p < 0.05$) in the yield of extracts with different solvents was observed. For potato peels and sugar beet pulp the highest yield was observed with methanol followed by ethanol, acetone, diethyl ether, petroleum ether, and hexane, while for sesame cake, owing to its high lipid content, the highest yield was observed with diethyl ether followed by ethanol, hexane, petroleum ether, acetone, and methanol. Variation in the yields of various extract is attributed to differences in

polarity of compounds present in plants, and such differences have been reported in the literature on fruit seeds (Jayaprakasha *et al*., 2001).

The amount of materials that can be extracted from a plant depends on the nature and amount of solvent used, mixing during extraction procedure and it is possible sample-to-sample variation in extracted materials (Hsu *et al*., 2006). Therefore, an appropriate extraction method should be developed to extract maximum quantity of antioxidative compounds before its exploration for the possible application in food industry.

Table 4.2. Extract yield and total phenolic content of potato peels, sugar beet pulp and sesame cake extracts.

Sample	Solvent	Extract yield (g kg^{-1} DW)	Total phenolics (mg GAE g^{-1} DW)
Potato peels	Methanol	125.83±0.36	2.91±0.02
	Ethanol	101.50±0.33	2.74±0.03
	Acetone	45.37±0.27	2.39±0.04
	Hexane	16.83±0.15	1.12±0.04
	Diethyl ether	42.50±0.15	1.12±0.03
	Petroleum ether	37.0±0.15	1.08±0.04
Sugar beet pulp	Methanol	108.2±0.34	1.79±0.01
	Ethanol	87.87±0.10	1.52±0.05
	Acetone	40.90±0.17	0.83±0.04
	Hexane	15.93±0.14	0.11±0.05
	Diethyl ether	38.77±0.03	0.12±0.06
	Petroleum ether	36.23±0.09	0.08±0.03
Sesame cake	Methanol	150.03±0.21	0.81±0.02
	Ethanol	192.10±0.14	0.55±0.06
	Acetone	166.23±0.20	0.24±0.04
	Hexane	186.67±0.10	0.12±0.05
	Diethyl ether	203.10±0.15	0.13±0.03
	Petroleum ether	186.07±0.15	0.12±0.02

4.3 Total phenolic compounds as affected by solvent used

Total phenolic contents **(**TPC) were determined by using Folin-Ciocalteu reagent (FCR). Folin-Ciocalteu reagent react nonspecifically with phenolic compounds as it can be reduced by a number of nonphenolic compounds e.g., vitamin C, Cu (II), etc. Although exact reaction of the reagent with reducing species is not known, but it is considered that a complex is formed between phospho-molybdic tungstate and reducing species, phenolate ion, changing color from yellow to blue where absorbance at 760 nm is measured against gallic acid as a standard (Haung *et al*., 2005). The amount of total phenolics determined in different solvent extracts of potato peels, sugar beet pulp, and sesame cake is shown in Table 4.2.The contents of total phenolics varied in the different extracts, ranging from 0.08 to 2.91 mg gallic acid

equivalent (GAE) g^{-1} DW. The results revealed that methanol and ethanol were better than the other solvents at extracting phenolic compounds owing to their higher polarity and good solubility for phenolic components from plant materials (Wieland *et al*., 2006). The data in Table 4.2 show that methanol was the best solvent for extracting phenolic compounds, followed by ethanol and acetone, with respective values of 2.91, 2.74, 2.39 mg GAE g^{-1} DW for potato peels, 1.79, 1.52, 0.83 mg GAE g^{-1} DW for sugar beet pulp, and 0.81, 0.55, 0.24 mg GAE g^{-1} DW for sesame cake. The lower-polarity solvents, particularly hexane, petroleum ether, and diethyl ether, showed much lower ability to extract phenolic compounds compared with the higher-polarity solvents. Singh *et al*. (2002) extracted antioxidative compounds from pomegranate peels and seeds and found that methanol gave maximum antioxidant yield. Similar results were observed in the present investigations as the most effective antioxidative compounds were extracted with methanol.

Potato peels extract had a higher phenolic compound (2.91 mg GAE g^{-1} DW) determined in the methanolic extract in the present study was found to be lower than tomato (2.92-5 mg GAE g^{-1} DW) (Kequan and Liangli, 2006) and apple peels (33 mg GAE g^{-1} DW) (Wolfe and Liu, 2003), but greater than carrot (1.52 mg GAE g^{-1} DW) (Kequan and Liangli, 2006), banana (2.32 mg GAE g^{-1} DW) (Nagendran *et al*., 2006), wheat bran, and onion (1.0 and 2.5 mg GAE g^{-1} DW), respectively (Kähkönen *et al*., 1999).

4.4 Amount of flavonoids and flavonols

Table 4.3 shows the flavonoid and flavonol contents of extracts. Because antioxidant activity does not always correlate with the presence of large quantities of polyphenolic compounds, the two sets of data need to be examined together. Flavonoids possess a broad spectrum of chemical and biological activities including radical scavenging properties. Such properties are especially distinct for flavonols. For this reason, all extracts were analyzed for total phenolic, flavonoid and flavonol contents.

Sugar beet pulp extracts had the highest total flavonoid and flavonol contents, with values ranging from 0.46 to 1.24 (mg quercetain equivalent (QE) g^{-1} dry weight (DW)) and from 0.17 to 0.85, respectively followed by potato peels and sesame cake extracts, depending on the solvent used for extraction. The highest flavonoid and flavonol contents were observed in extracts with methanol, while the lowest levels were found in extracts with petroleum ether. Statistical analysis allowed the detection of significant differences in flavonoid and flavonol contents in all samples ($p < 0.05$). The results confirmed a previous report that flavonoids represent the main group of phenolic compounds in white onion (Yang *et al*., 2004).

Table 4.3. Total flavonoid and flavonol contents of potato peels, sugar beet pulp and sesame cake extracts.

Sample	Solvent	Total flavonoids (mg QE g^{-1} DW)	Total flavonols (mg QE g^{-1} DW)
Potato peels	Methanol	0.96±0.03	0.41±0.02
	Ethanol	0.81±0.04	0.38±0.03
	Acetone	0.65±0.02	0.26±0.01
	Hexane	0.56±0.01	0.22±0.04
	Diethyl ether	0.72±0.02	0.25±0.02
	Petroleum ether	0.51±0.01	0.19±0.01
Sugar beet pulp	Methanol	1.24±0.03	0.85±0.01
	Ethanol	0.91±0.02	0.77±0.04
	Acetone	0.77±0.01	0.65±0.06
	Hexane	0.71±0.05	0.42±0.05
	Diethyl ether	0.76±0.03	0.44±0.06
	Petroleum ether	0.46±0.02	0.17±0.03
Sesame cake	Methanol	0.4±0.02	0.16±0.02
	Ethanol	0.29±0.01	0.14±0.01
	Acetone	0.19±0.02	0.12±0.02
	Hexane	0.06±0,01	0.11±0.01
	Diethyl ether	0.12±0.02	0.10±0.01
	Petroleum ether	0.01±0.03	0.09±0.02

4.5. Antioxidant activity

4.5. 1 General

The antioxidant activity depends on the type and polarity of the extracting solvent, the isolation procedures, purity of active compounds, as well as the test system and substrate to be protected by the antioxidant (Meyer *et al*., 1998). It has been suggested that the determining factor for the antioxidant activity is the lipophilic nature of the molecules and the affinity of the antioxidant for the lipid (Brand-Williams *et al*., 1995; von Gadow *et al*., 1997). A close dependency on the antioxidant activity of phenolic acids (Pekkarinen *et al*., 1999; von Gadow *et al*., 1997) has been reported for phenolic compounds, and even the recommended concentration of synthetic antioxidants has been indicated for some tests (Karamac and Amarovicz, 1997). The antioxidant potential of a compound is different according to different antioxidant assays or, for the same assay when the polarity of the medium differs, since the interaction of the antioxidant with other compounds plays an important role in the activity (Pekkarinen *et al*., 1999). Dramatic differences in the relative antioxidant potential of model compounds were observed when one model compound is strongly antioxidant with one method and prooxidant with another (von Gadow *et al*., 1997). A phenomenon known as `Polar paradox' has been repeatedly reported; hydrophilic antioxidants are more effective than

lipophilic antioxidants in bulk oil, whereas lipophilic antioxidants present greater activity in emulsions.

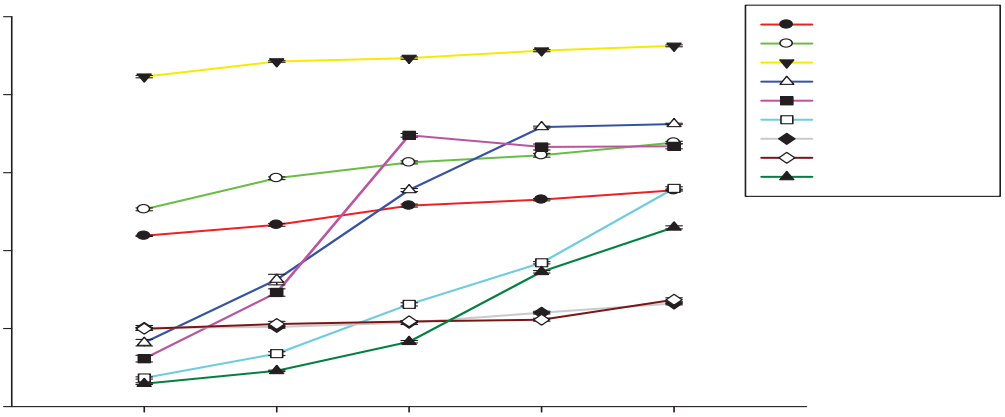
As stressed by Huang *et al*. (2005), no single method is adequate for evaluating the antioxidant capacity of foods, since different methods can yield widely diverging results. Several methods based on different mechanisms must be used. Here we applied assays of ABTS radical-scavenging activity, DPPH radical-scavenging activity and *β*-carotene/linoleic acid bleaching to each extract.

4.5.2 DPPH radical-scavenging activity

Free radicals involved in the process of lipid peroxidation are considered to play a major role in numerous chronic pathologies such as cancer and cardiovascular diseases (Dorman *et al*., 2003). DPPH• is considered to be a model of a stable lipophilic radical with deep violet colour. A chain reaction of lipophilic radicals is initiated by lipid autoxidation. Antioxidants react with DPPH•, reducing the number of DPPH• free radicals equal to the number of their available hydroxyl groups. Therefore, the absorption at 515 nm is proportional to the amount of residual DPPH• (Juan *et al*., 2005). It is visually noticeable as a discoloration from purple to yellow. Because these radicals are very sensitive to the presence of hydrogen donors, the whole system operates at very low concentration; with it, it can allow a large number of samples to be tested in a short time (Iqbal *et al*., 2006; Zhou and Yu, 2004).

The scavenging activity of extracts against $DPPH^{\bullet}$ was concentration-dependent (Figure 4.1). Significant ($p < 0.05$) differences between different extracts were observed, but the results clearly indicate that all extracts exhibited antioxidant activity. The extracts that showed relatively high antioxidant activity (those with methanol and ethanol), as strong as that of BHA and BHT but weaker than that of TBHQ, contained the highest amount of total phenolic compounds (Table 4.2). These findings are in close agreement with previous findings of Singh *et al*. (2002) who found a strong correlation between the contents of TPC and $DPPH^{\bullet}$ scavenging activity of methanolic extract from pomegranate peels.

It has been proven that the antioxidant activity of plant extracts is mainly ascribed to the concentration of the phenolic compounds present in the plants (Heim *et al*., 2002). The scavenging activity of potato peels methanolic extract was far superior to that of any of the other extracts investigated as well as that of green and black tea extracts, which ranged from 49 to 66% according to Yen and Chen (1995).



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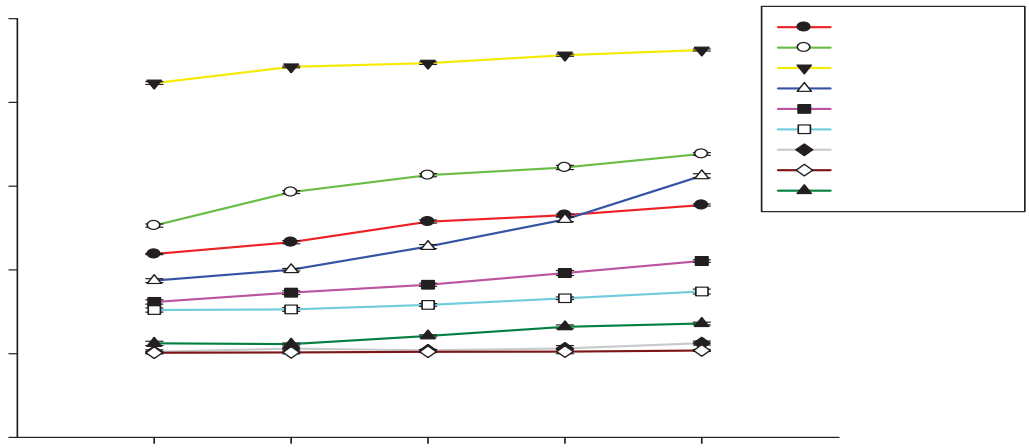

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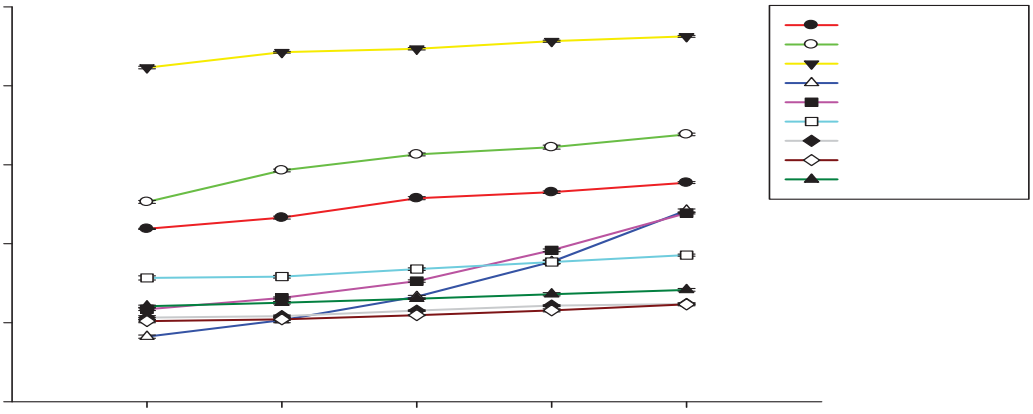


(c)

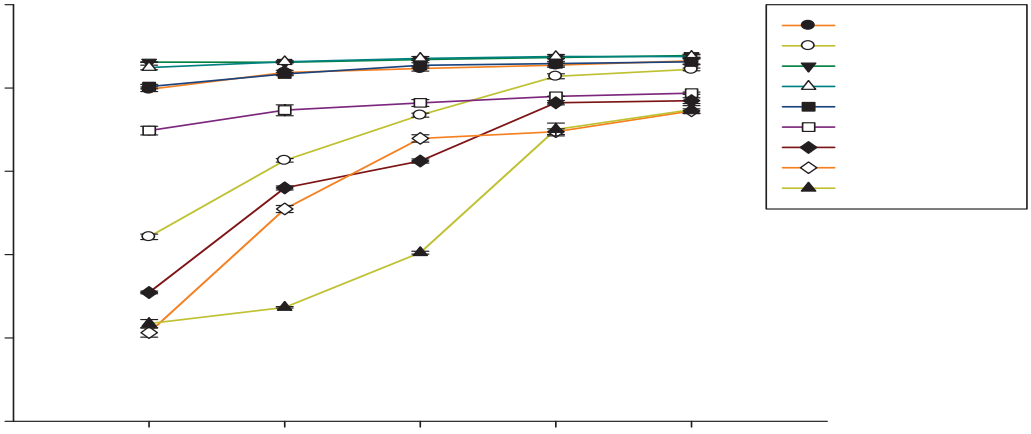
Figure 4.1. Scavenging activity of **(a)** potato peels, **(b)** sugar beet pulp, and **(c)** sesame cake extracts against DPPH radical compared with that of BHA, BHT, and TBHQ at different concentrations. Data are mean ±SD of three determinations.

The results of the $DPPH^{\bullet}$ free radical scavenging assay suggest that components within the extracts are capable of scavenging free radicals via electron- or hydrogen-donating mechanisms and thus should be able to prevent the initiation of deleterious free radical mediated chain reactions in susceptible matrices, e.g. biological membranes. This further shows the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage.

4.5.3 ABTS radical-scavenging activity

Although the $DPPH^{\bullet}$ free radical is ubiquitously used to estimate the potential free radical-scavenging activity of natural products, the $ABTS^{\bullet+}$ free radical is commonly used when issues of solubility or interference arise and the use of $DPPH^{\bullet}$-based assays becomes inappropriate (Dorman and Hiltunen, 2004). Having considered the solubility of the test samples and the advantages and disadvantages of the use of the $DPPH^{\bullet}$ free radical, it was considered necessary to further assess the extracts against the $ABTS^{\bullet+}$ free radical. Proton radical scavenging is an important attribute of antioxidants. $ABTS^{\bullet+}$, a protonated radical, has characteristic absorbance maxima at 734 nm that decreases with the scavenging of the proton radicals (Mathew and Abraham, 2006). The extracts demonstrated a wide range of $ABTS^{\bullet+}$ scavenging activities and could be ranked from 36% to 86% (Figure 4.2). In our study, all extracts exhibited significant antioxidant activity. Extracts with methanol were the most efficient $ABTS^{\bullet+}$ scavenger followed by those with ethanol and acetone. However, extracts with hexane, petroleum ether and diethyl ether were less effective in minimizing the oxidation of lipids. Similar results were found by Djeridane *et al*. (2006) from some medicinal plants.

It can be concluded that the extracts obtained using high polarity solvents were considerably more effective radical-scavengers than those obtained using low-polarity solvents, indicating that antioxidants or active compounds of different polarity could be present in the extracts with high antioxidant capacity. Change in the polarity of a solvent alters its ability to dissolve a selected group of antioxidant compounds and influences activity estimation (Kequan and Liangli, 2004). Scavenging of the $ABTS^{+}$ radical by the extracts was found to be higher than that of DPPH radical. Factors such as stereoselectivity of the radicals and the solubility of extracts in different test systems have been reported to affect the capacity of extracts to react with and quench different radicals (Yu *et al*., 2002).



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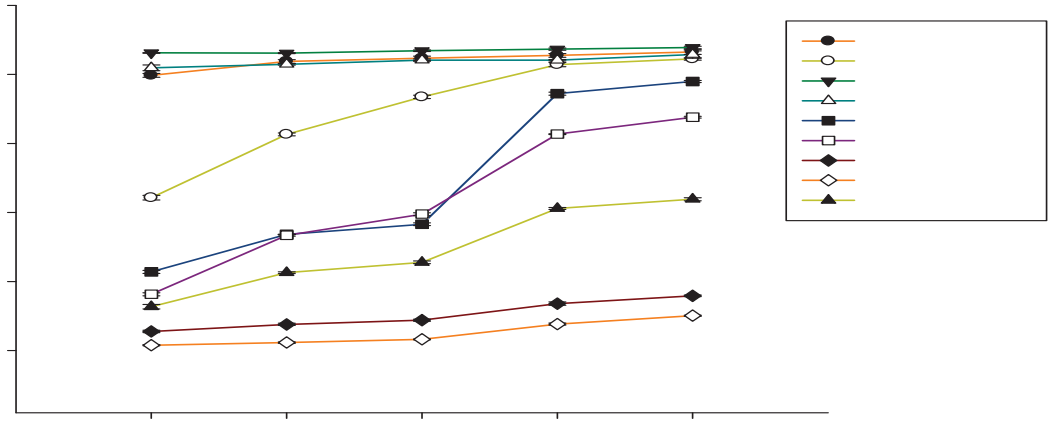

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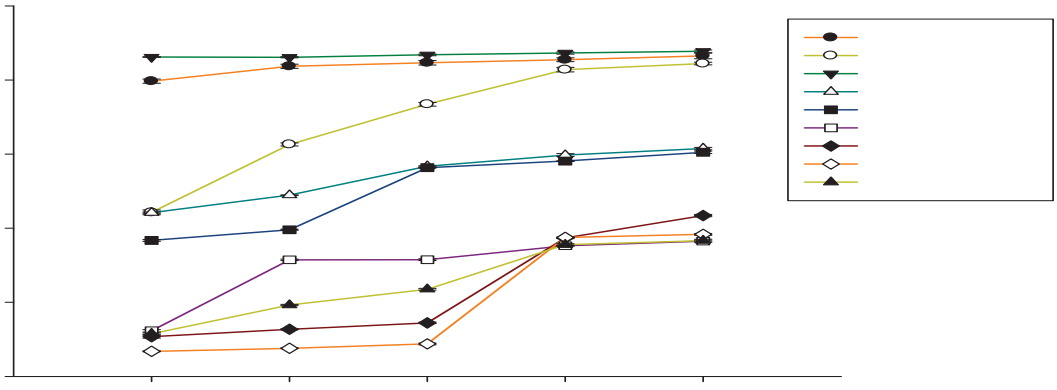


(c)

Figure 4.2. Scavenging activity of **(a)** potato peels, **(b)** sugar beet pulp, and **(c)** sesame cake extracts against ABTS radical compared with that of BHA, BHT, and TBHQ at different concentrations. Data are mean ±SD of three determinations.

Wang *et al*. (1998) found that some compounds possessing $ABTS^{+}$ -scavenging activity did not show DPPH -scavenging activity. This was not the case in the present study.

The $ABTS^{\bullet+}$ scavenging data suggest that components within the extracts are capable of scavenging free radicals via a mechanism of electron/hydrogen donation and should be able to protect susceptible matrices from free radical-mediated oxidative degradation.

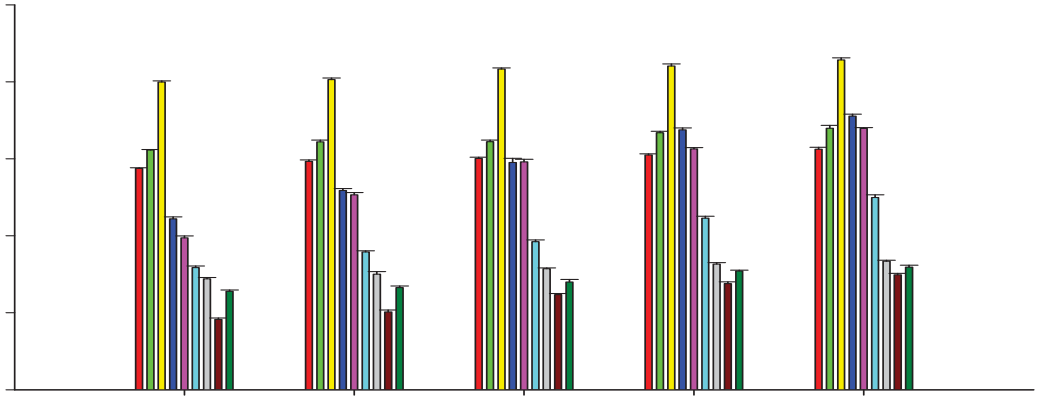
4.5.4 ß-carotene/linoleic acid bleaching

Synthetic free radical-scavenging (ABTS and DPPH) models are valuable tools to indicate the potential antioxidant activity of plant extracts; however, these systems do not use a food or biologically relevant oxidisable substrate so no direct information on an extract's protective action can be obtained (Dorman *et al*., 2003). Therefore, it was considered important to assess the extracts in a ß-carotene/linoleic acid lipid-water emulsion assay despite its reported limitations (Koleva *et al*., 2002; Ley and Bertram, 2003). In this assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals that attack the chromophore of ß-carotene, resulting in bleaching of the reaction emulsion. An extract capable of retarding/ inhibiting the oxidation of ß-carotene may be described as a free radical scavenger and primary antioxidant (Liyana-Pathirana and Shahidi, 2006).

As can be seen in Figure 4.3, all extracts were capable of inhibiting the bleaching of ß-carotene by scavenging linoleate-derived free radicals. The order of decreasing efficacy at a dose of 200 µg mL^{-1} was TBHQ > methanol extracts > BHT = ethanol extracts > BHA > acetone extracts > diethyl ether extracts > hexane extracts = petroleum ether extracts. The results reveal that, overall, methanolic and ethanolic extracts had comparable scavenging ability to the synthetic antioxidants BHA, BHT, and TBHQ.

It has been suggested that the polarity of an extract is important in water-oil emulsions, in that non-polar extracts are more effective antioxidants than polar extracts owing to a 'concentrating effect' within the lipid phase (Koleva *et al*., 2002). Thus it would be expected that the less polar extracts would be more potent. This phenomenon was not observed in the case of all extracts studied here, a finding which has been reported previously (Koleva *et al*., 2003).

According to the ß-carotene/linoleic acid bleaching data, the extracts are capable of scavenging free radicals in a complex heterogenous medium. This suggests that the extracts may have potential use as antioxidative preservatives in emulsion-type systems.



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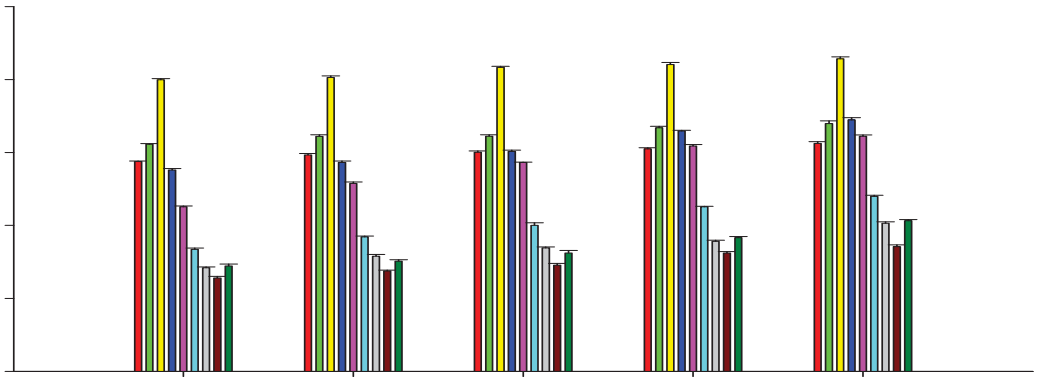

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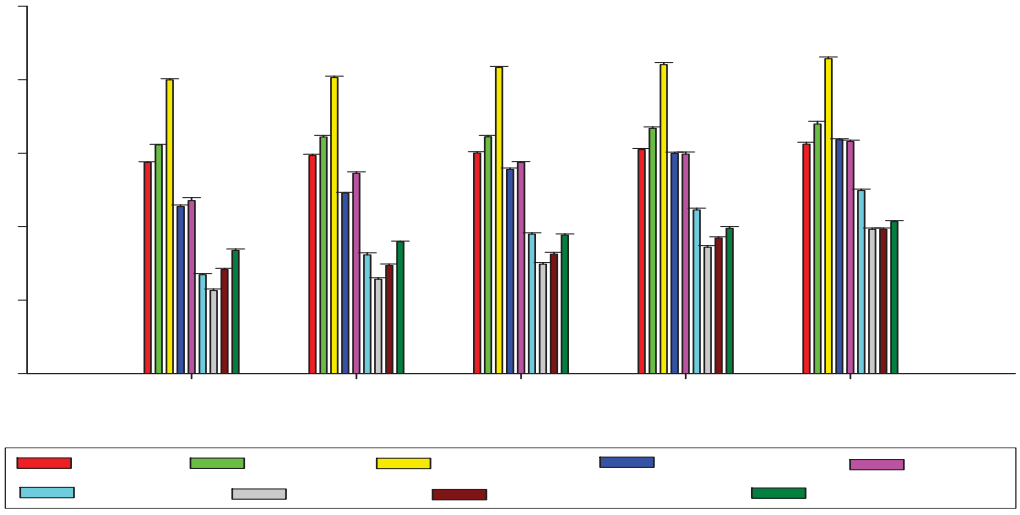

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Figure 4.3. Antioxidant activity of **(a)** potato peels, **(b)** sugar beet pulp, and **(c)** sesame cake extracts in ß-carotene/linoleic acid system compared with that of BHA, BHT, and TBHQ at different concentrations. Data are mean ±SD of three determinations.

Considering the results of all three assays, the extracts prepared from potato peels had the highest antioxidant activity, followed by those prepared from sugar beet pulp, while sesame cake extracts were less effective. This may be due to the high soluble sugar and protein contents of sesame cake, which could produce considerable interference in these antioxidant capacity assays. Phenolic compounds can explain high antioxidant capacity, (Fernandez-Pachon *et al*., 2006; Kevers *et al*., 2007; Mullen *et al*., 2007) although some authors have reported that there is no correlation between the content of these main antioxidant compounds and radical-scavenging capacity (Yu et *al*., 2002). The results obtained by us do not support these claim. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994).

4.6 Stability of oils as affected by addition of potato peels, sugar beet pulp, and sesame cake extracts

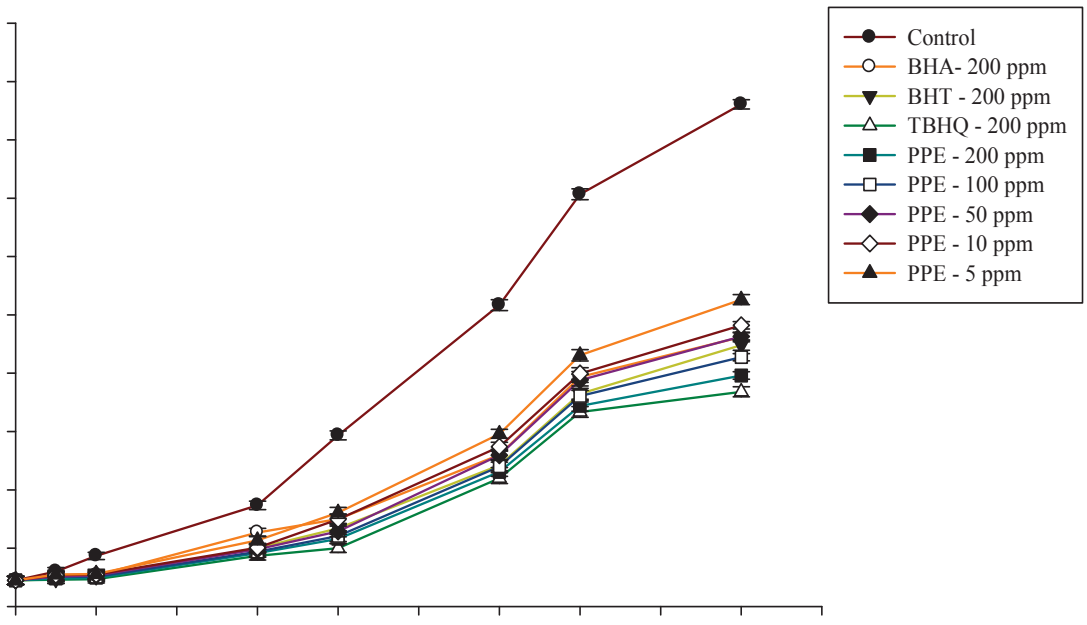
4.6.1 General

On the basis of preliminary evaluation of antioxidant activity of extracts of potato peels, sugar beet pulp and sesame cake from different solvents, methanolic and ethanolic extracts showing higher antioxidant activity were further evaluated towards stabilization of sunflower and soybean oils. Oil stability is usually determined under accelerated oxidation conditions (60 °C or more) because ambient conditions demand an excessively long period. Similar accelerated storage tests have been used by other authors to evaluate the efficacy of antioxidants (Mariod *et al*., 2006, 2008). To evaluate the antioxidant efficacies of methanolic and ethanolic extracts in soybean and sunflower oils, PV, AV and UV absorptivity were determined as indices of lipid oxidation. The oxidative stability studies were carried out at 70 °C in an oven. This temperature was ideal, because at higher temperatures the peroxides will decompose very fast (Mariod *et al*., 2010).

4.6.2 Effect of methanolic extracts on sunflower oil oxidation

Peroxide value (PV) is a measure of the concentration of peroxides and hydroperoxides formed in the initial stages of lipid oxidation. Peroxide value is one of the most widely-used tests for the measurement of oxidative rancidity in oils and fats. A continuous increase in PV

with the increase in storage period was observed for all the samples (Figure 4.4). Initially rate in PV was very slow, but it started to increase after 32 h of storage and went on increasing further with the increase in storage period. Peroxide value was in the range of 1.34 ± 0.21–15.78 ± 0.26 meq kg^{-1} for potato peels extracts (PPE), 1.34 ± 0.21–16.94 ± 0.24 meq kg^{-1} for sesame cake extracts (SCE), while it was 1.34 ± 0.21–16.88 ± 0.24 meq kg^{-1} for sugar beet pulp ones (SBP). Sunflower oil samples without antioxidant (control) reached a maximum PV of 25.82 ± 0.24 meq kg^{-1} after 72 h of storage. A significant difference ($p < 0.05$) in PV was observed between the control and sunflower oil samples containing extracts and synthetic antioxidants, which slowed the rate of peroxide formation. The PV of sunflower oil containing 200 ppm of potato peels, sesame cake, sugar beet pulp, TBHQ, BHT, and BHA were found to be 11.88 ± 0.19, 13.86 ± 0.26, 13.8 ± 0.26, 11.04 ± 0.26, 13.44 ± 0.26 and 13.86 ± 0.24 meq kg^{-1} after 72 h of storage, respectively. These data suggest the superiority of antioxidant activity of potato peels, sesame cake, and sugar beet pulp extracts over synthetic antioxidants. However, among these treatments, TBHQ remained the most effective and gave the lowest PV (Ying *et al*., 2010).



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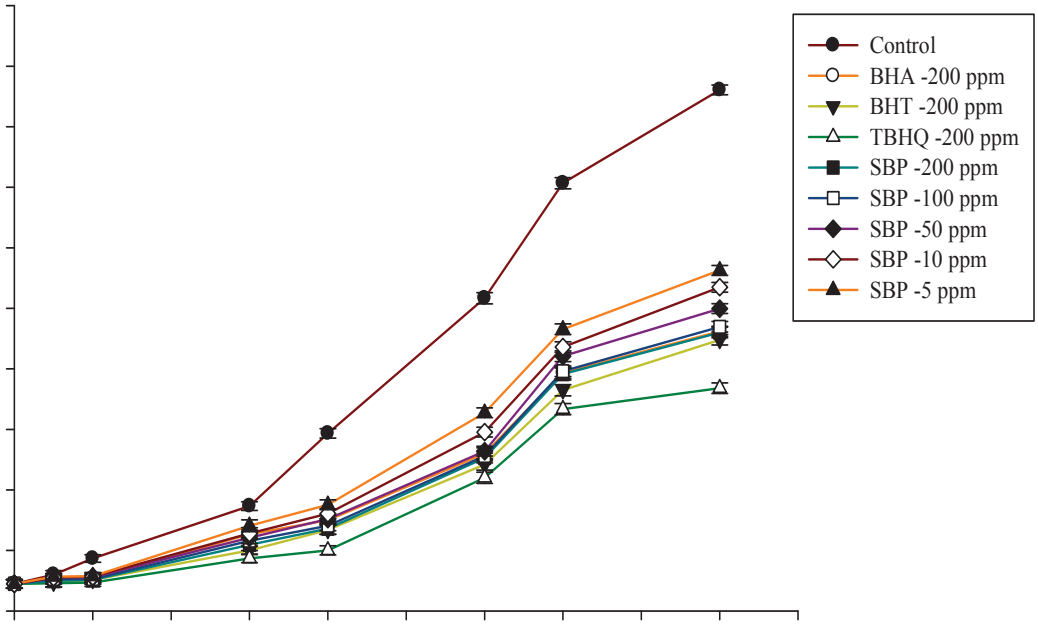
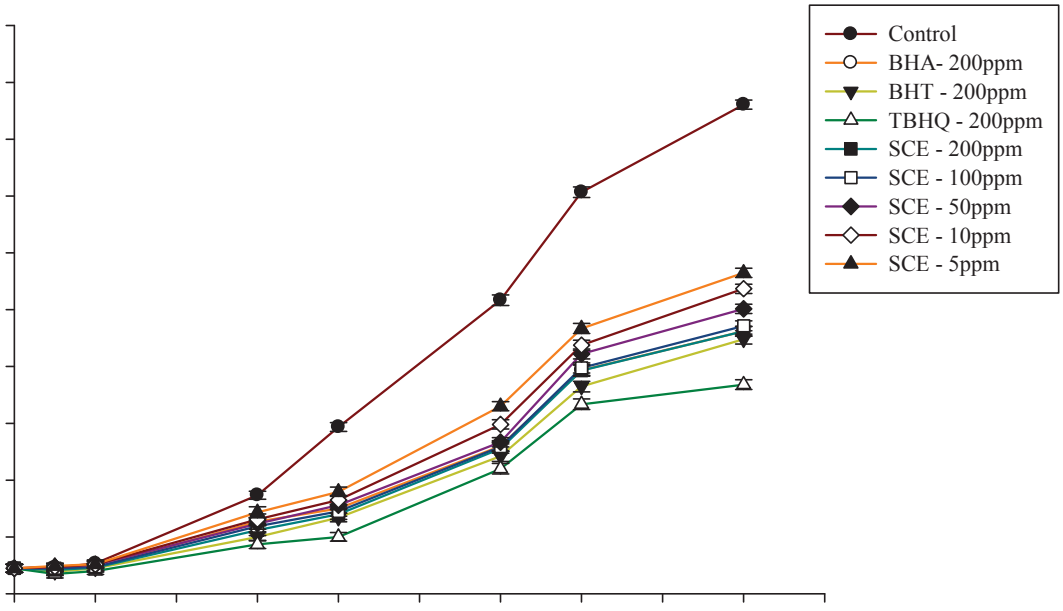


(b)

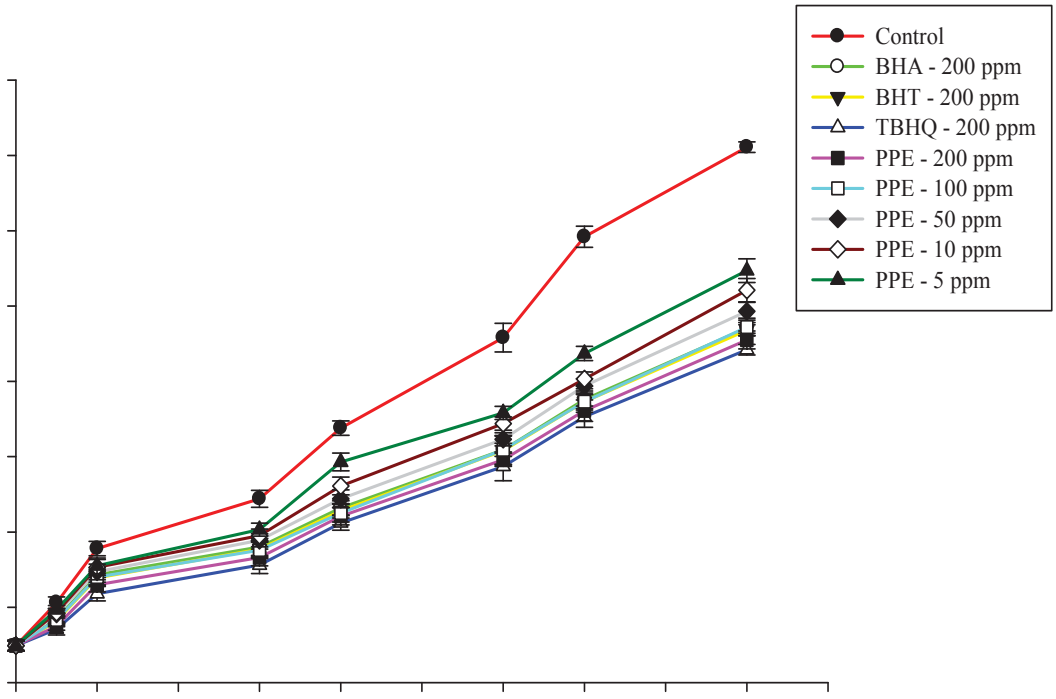


(c)

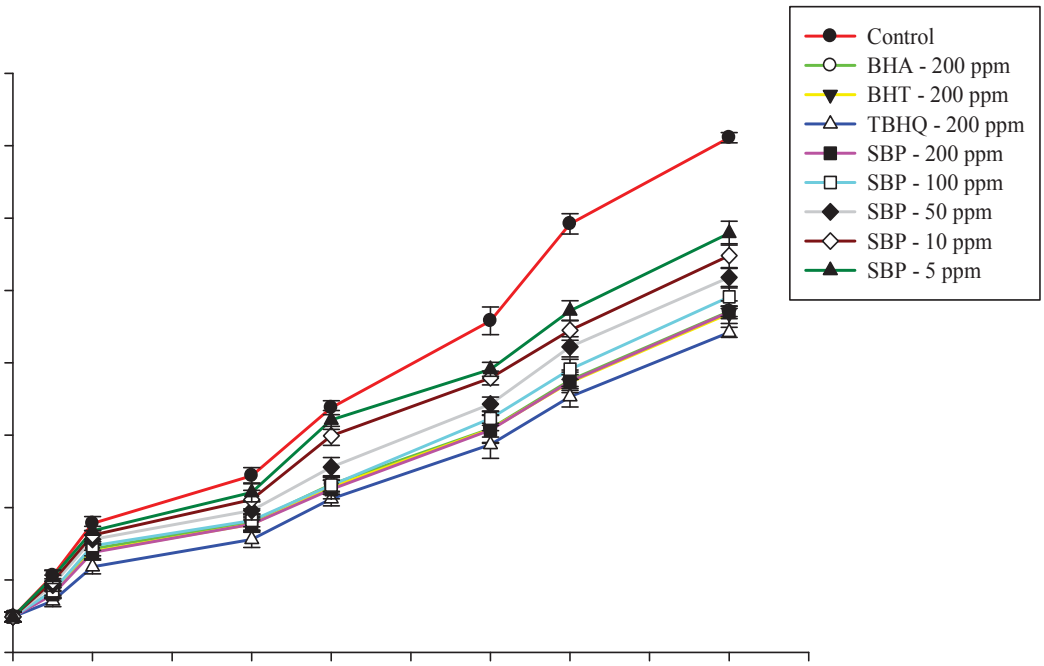
Figure 4.4. Relative increase in peroxide value (PV) of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

Antioxidants are mainly used in lipids to delay the accumulation of primary oxidation products and thus to improve the oxidative stability. The primary products of lipid peroxidation are hydroperoxides, which are generally referred to as peroxides. Therefore the results of PV estimation give a clear indication of lipid autoxidation. For further confirmation of these results, other oxidation parameters, such as conjugated dienes, conjugated trienes and *p*-anisidine values were also measured.

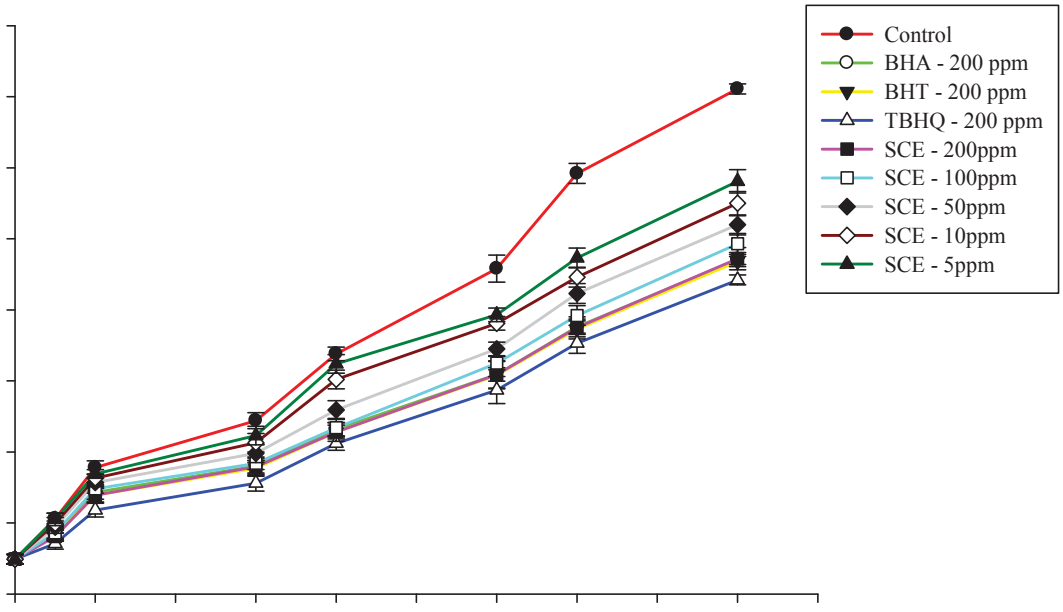
The *p*-anisidine value (AV), which measures the secondary oxidation products produced during the oxidative degradation of oil, was determined by reacting *p*-anisidine with the oil in iso-octane and the resultant color was measured at 350 nm. Figure 4.5 depicts the *p*-ansidine values for sunflower oil samples stabilized with methanolic extracts, TBHQ, BHT, BHA, and control.



(a)



(b)

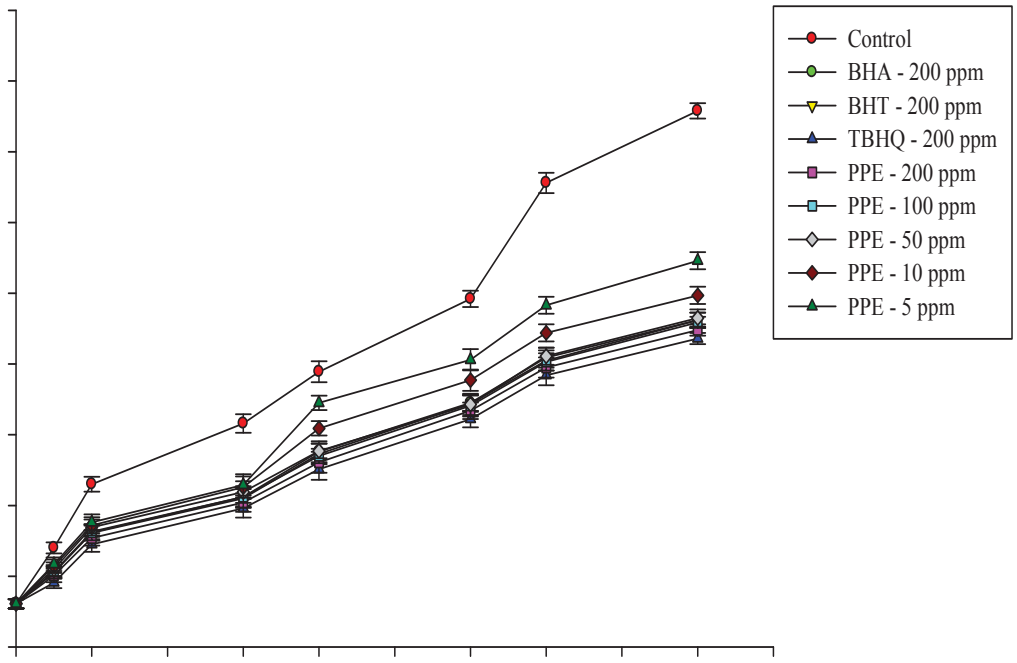


(c)

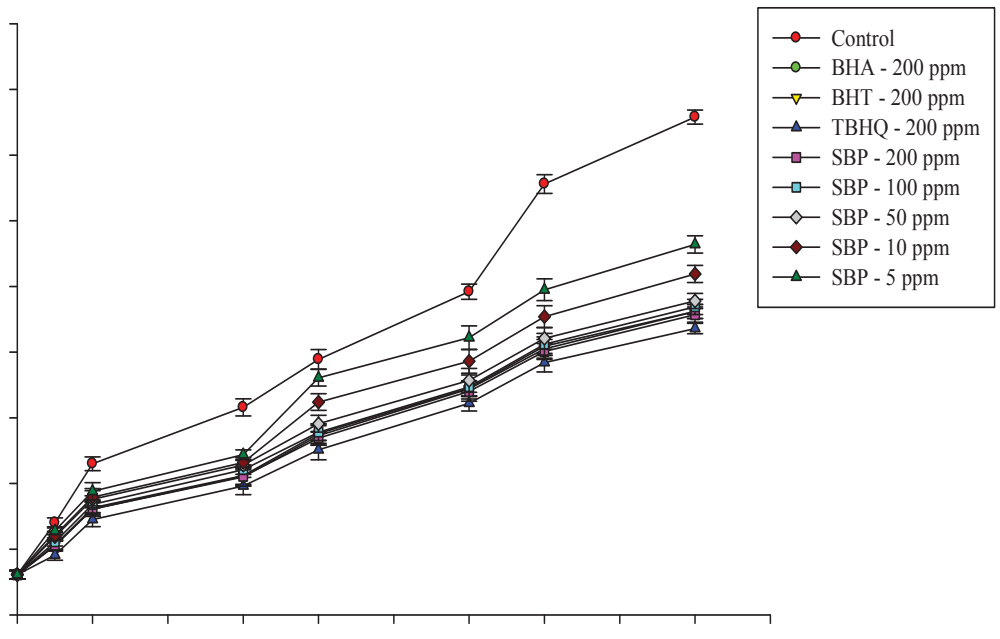
Figure 4.5. Relative increase in *p*-anisidine value (AV) of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

The *p*-anisidine value of control reached a maximum of 14.22 ± 0.14 from an initial value of 0.98 ± 0.13 after 72 h of storage. The values for potato peels, sesame cake, sugar beet pulp, BHA, BHT and TBHQ at 200 ppm were 9.10 ± 0.24, 9.44 ± 0.31, 9.40 ± 0.31, 9.42 ± 0.14, 9.36 ± 0.14, and 8.84 ± 0.14, respectively. A significant difference was noted between the values for control and experimental samples. The results demonstrated that the extracts prepared from potato peels had higher antioxidant activity than BHT, sugar beet pulp extracts, BHA, and sesame cake extracts but lower than TBHQ. The results confirmed our pervious experiments, wherein phenolic compounds can explain higher antioxidant capacity of potato peels (2.91 ± 0.02 mg GAE g^{-1} DW) than sugar beet pulp (1.79 ± 0.01 mg GAE g^{-1} DW) and sesame cake (0.81 ± 0.02 mg GAE g^{-1} DW).

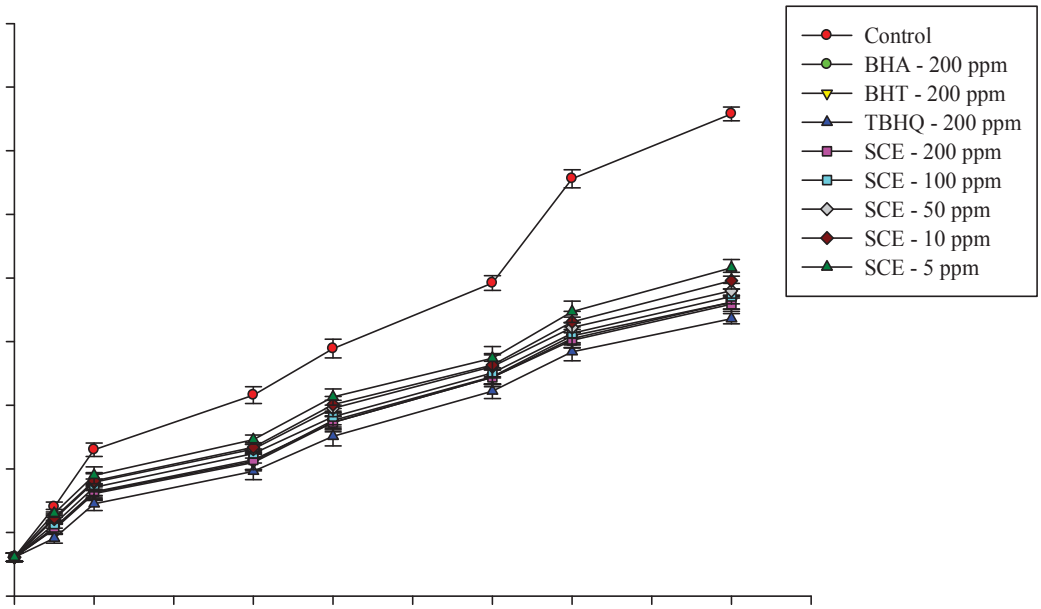
Figures 4.6 and 4.7 show the relative increase in conjugated dienes (CD) and trienes (CT) contents of sunflower oil under accelerated storage as function of storage time. The assessment of CD and CT is a good parameter for the measurement of oxidative deterioration of oils, hence indicates the effectiveness of antioxidants in oils (Shahidi and Wanasundara, 1997). Initially, rate of formation of CD was higher, and went on decreasing with the increase in storage time, while the reverse behavior was observed for CT content, i.e., initial rate was lower, and went on increasing with the storage time. Formation of high contents of CD may be related to the presence of higher contents of polyunsaturated fatty acids (Liu and White, 1992) in sunflower oil. Conjugated trienes may be produced by dehydration of conjugated diene hydroperoxides (Fishwick and Swoboda, 1977). Highest contents were observed for control, indicating greater intensity of oxidation, followed by BHA = BHT, SCE, SBP, PPE and TBHQ.


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

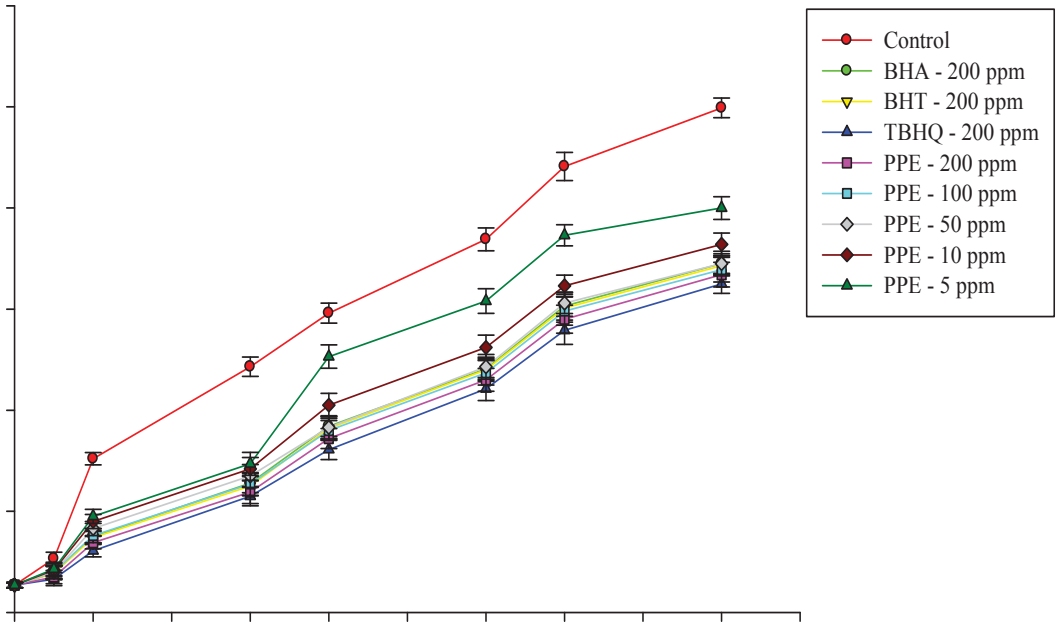
(b)



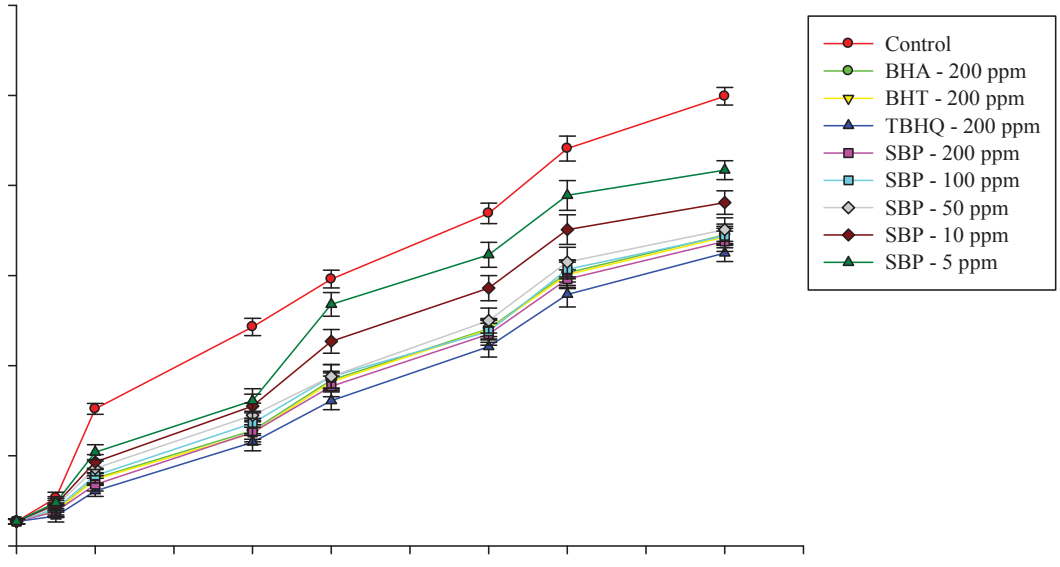
(c)

Figure 4.6. Absorptivity at 232 nm of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

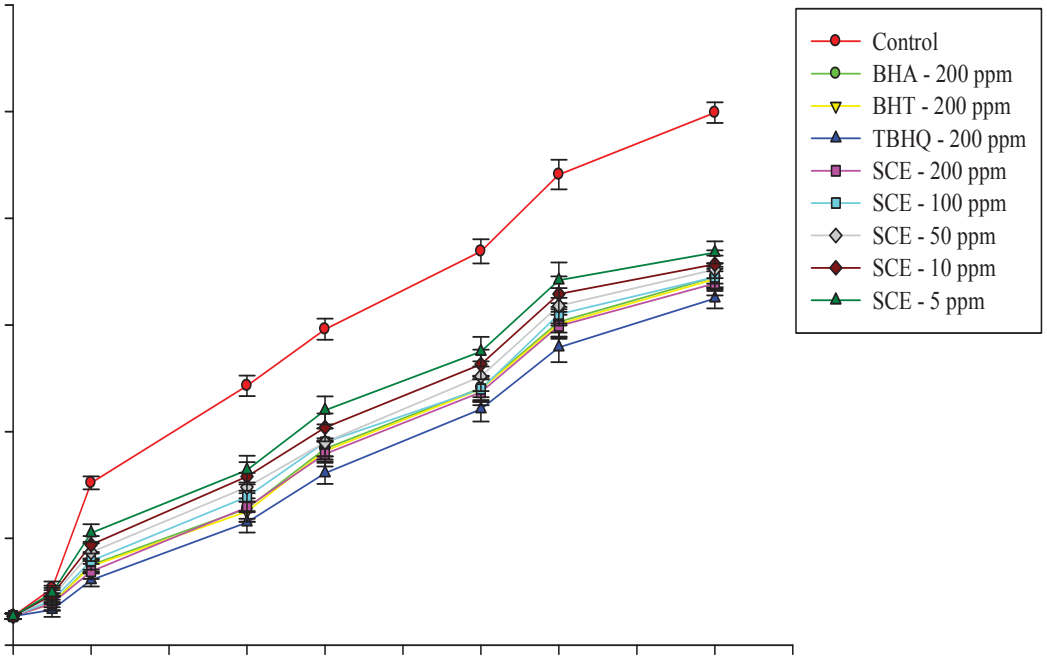
The increase in CD and CT contents is proportional to the uptake of oxygen. The greater the levels of CD and CT the lower the oxidative stability of the oils will be (Chatha *et al*., 2006). Iqbal and Bhanger (2007) described the antioxidant activity of garlic extracts in sunflower oil, assessed under accelerated conditions, using CD and CT as indicators of oxidative degradation. Siddiq *et al*. (2005) also investigated the antioxidant efficacy of methanolic extract of *Moringa oleifera* leave indicating antioxidant potential of stabilization of sunflower oil under accelerated aging by measuring of CD and CT contents.


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

(b)

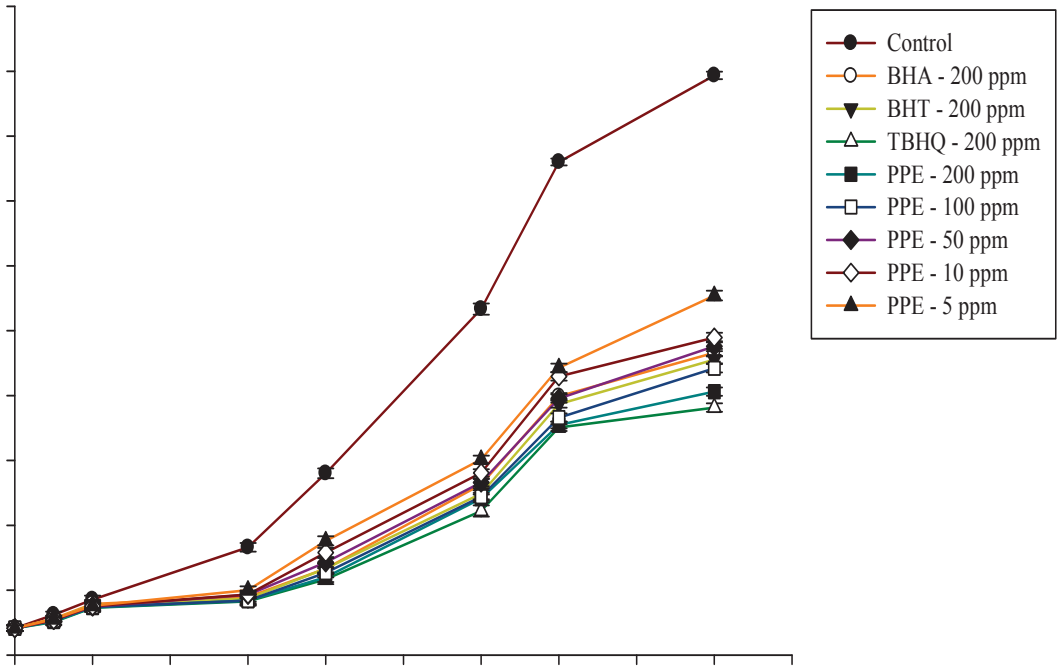


(c)

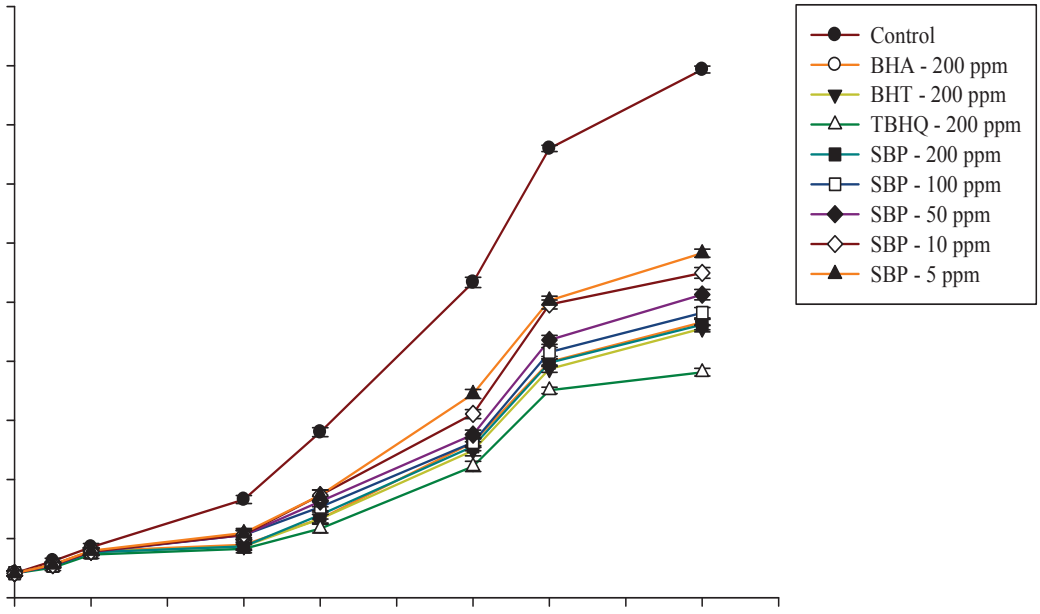
Figure 4.7. Absorptivity at 270 nm of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

4.6.3 Effect of methanolic extracts on soybean oil oxidation

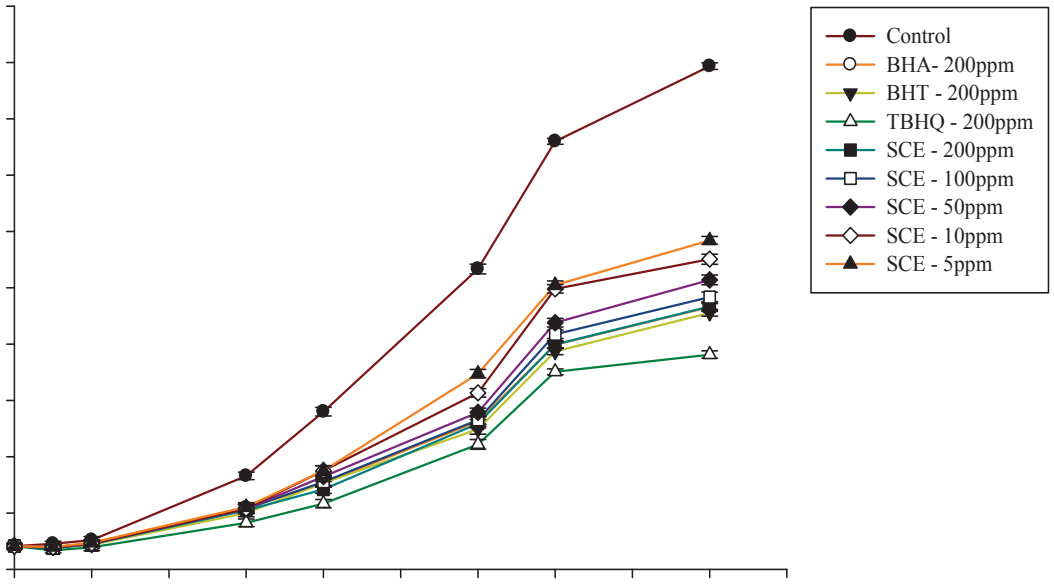
Figure 4.8 shows the PV developments during the storage of soybean oil at 70 °C for 72 h with various concentrations of potato peels, sesame cake, and sugar beet pulp extracts. Additional treatments included TBHQ, BHA, and BHT at 200 ppm and a control without additives. Highest PV was observed for control followed by sesame cake, BHA, sugar beet pulp, BHT, potato peels, and TBHQ at 200 ppm after 72 h of storage. A significant difference (P <0.05) in PV was observed between the control and soybean oil containing extracts, BHT, BHA, and TBHQ. These results indicated that potato peels, sesame cake, and sugar beet pulp extracts inhibited soybean oil oxidation. Further, the antioxidant effect of potato peels extracts was better than BHT and BHA; while sugar beet pulp and sesame cake extracts had comparable scavenging ability to the synthetic antioxidants BHA, BHT. During incubation, TBHQ maintained significantly the lowest PV.


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

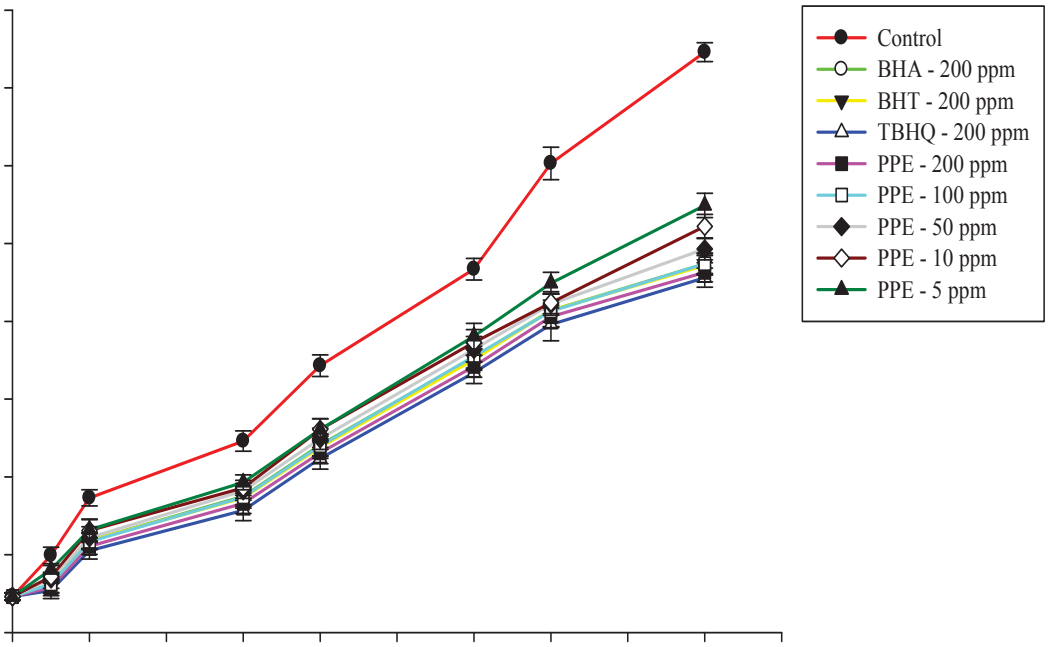
(b)



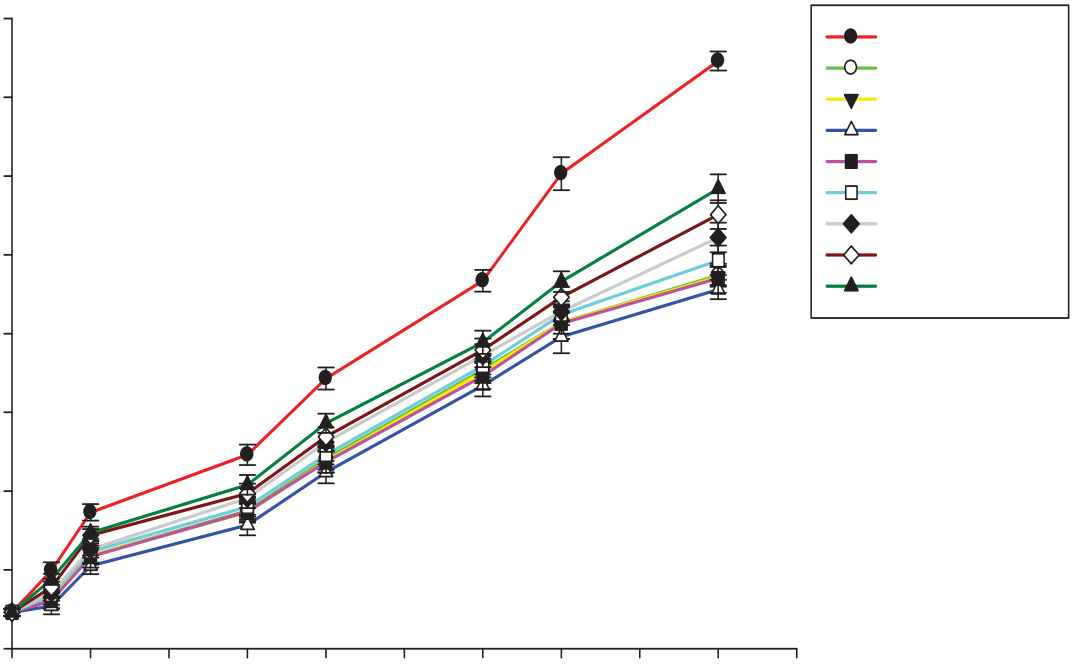
(c)

Figure 4.8. Relative increase in peroxide value (PV) of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

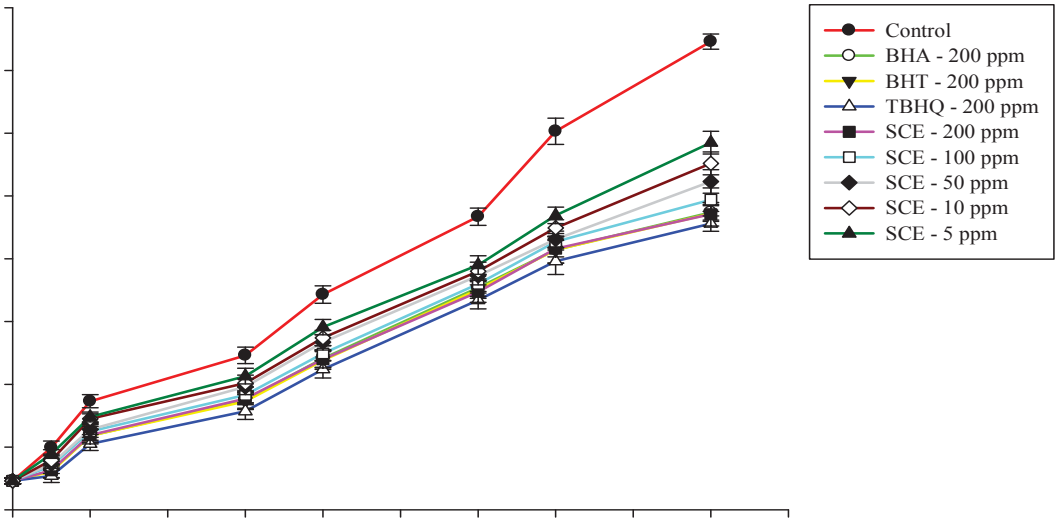
The effect of potato peels, sesame cake, and sugar beet pulp extracts on soybean oil oxidation (measured by *p*-anisidine value) is shown in Figure 4.9 In control sample formation of carbonyls was higher than in samples with added extracts ($P < 0.05$). When compared with the control, potato peels, sugar beet pulp, and sesame cake extracts at 200 ppm were found to be even more effective in retarding the formation of carbonyl components than BHT and BHA, which predicted their high antioxidant potential. However, at all the stages of storage period these extracts were less effective than TBHQ. Utilization of *p*-anisidine measurement to assess potential of natural antioxidants in vegetable oils under accelerated storage conditions is generally accepted. These findings were supported by previous reports of Ying *et al*. (2010), who reported that in soybean oil rosemary extract high in carnosic acid, could significantly ($P<0.05$) lower the peroxide value and *p*-anisidine value of oil during storage at 60°C. The study also indicated a better antioxidant effect for carnosic acid, which separated from rosemary dried leaves, than BHT and BHA, but less active than TBHQ.


Control
BHA - 200 ppm
BHT - 200 ppm
TBHQ - 200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)



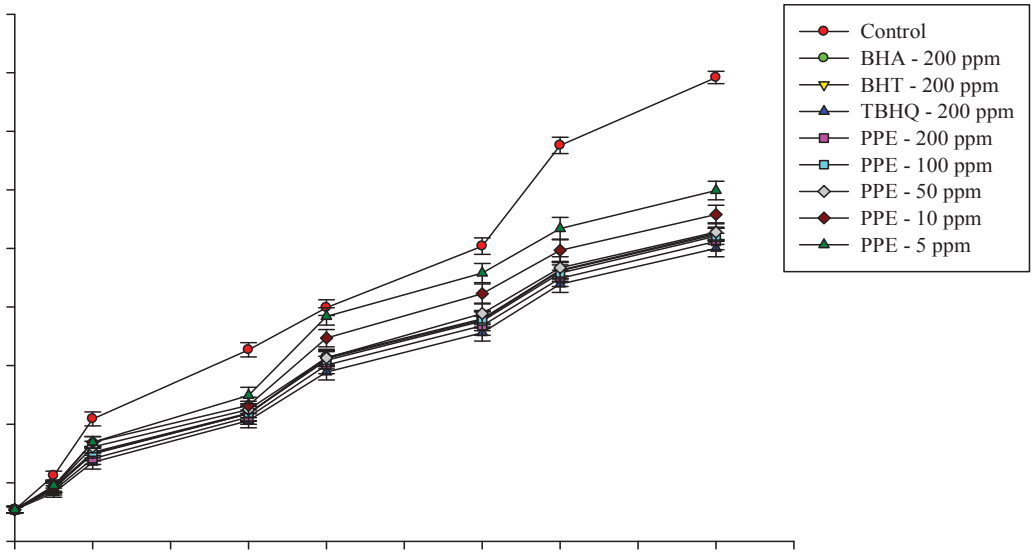
(b)



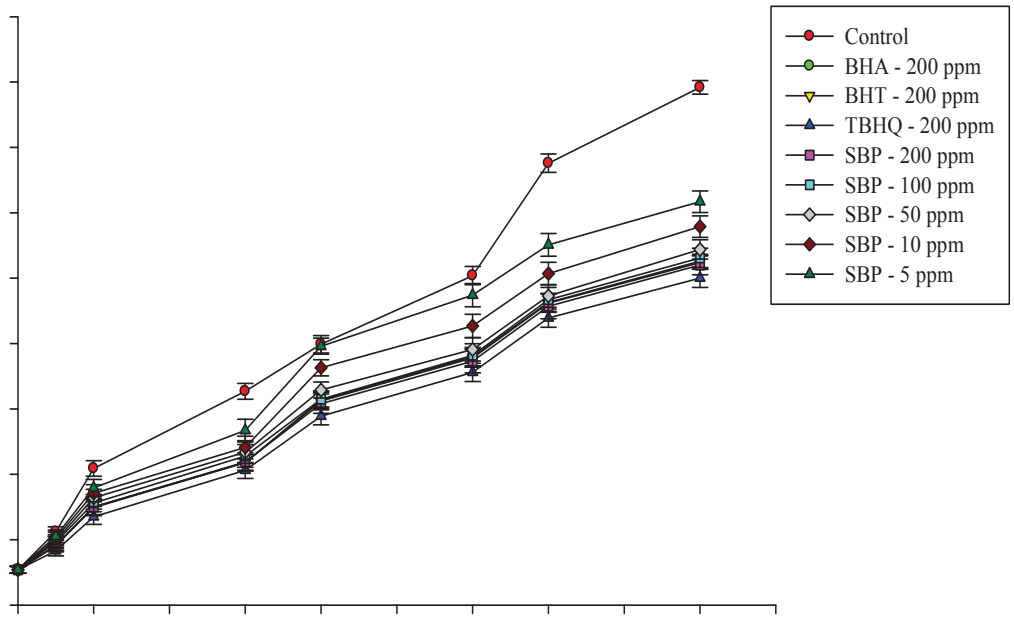
(c)

Figure 4.9. Relative increase in *p*-anisidine value (AV) of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

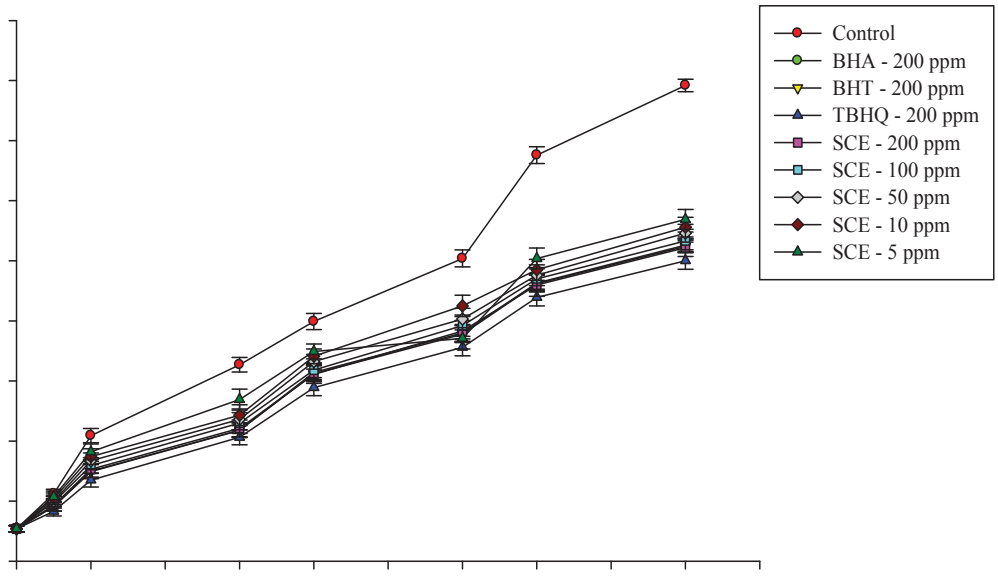
Absorption at 232 nm and 270 nm, due to the formation of primary and secondary compounds of oxidation (Figures 4.10 and 4.11), showed a pattern in good agreement with that of the PV and AV.



(a)



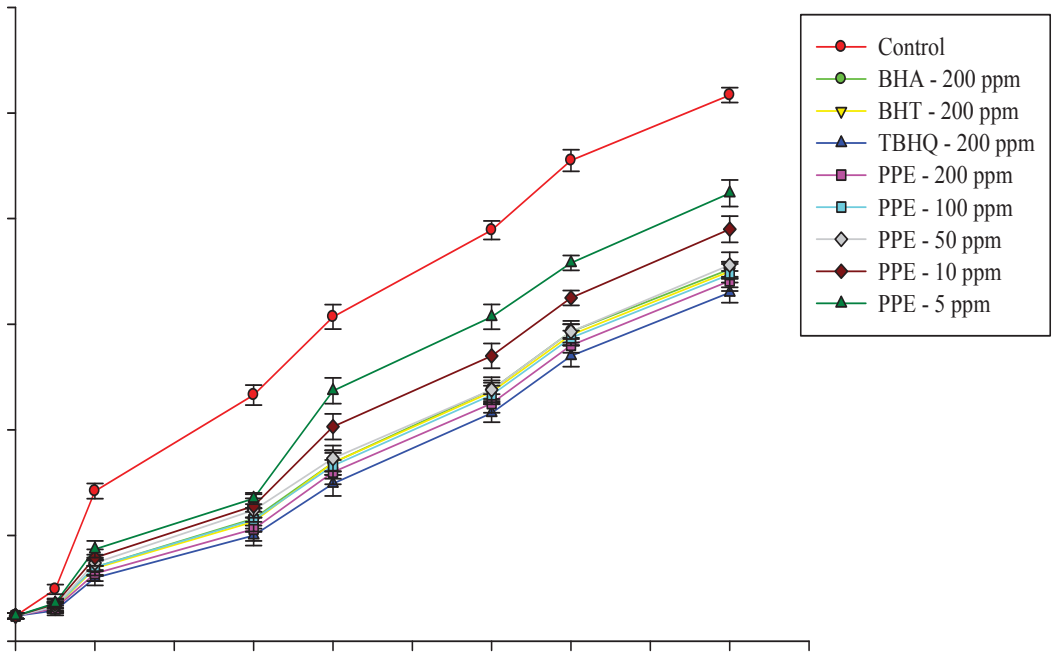
(b)



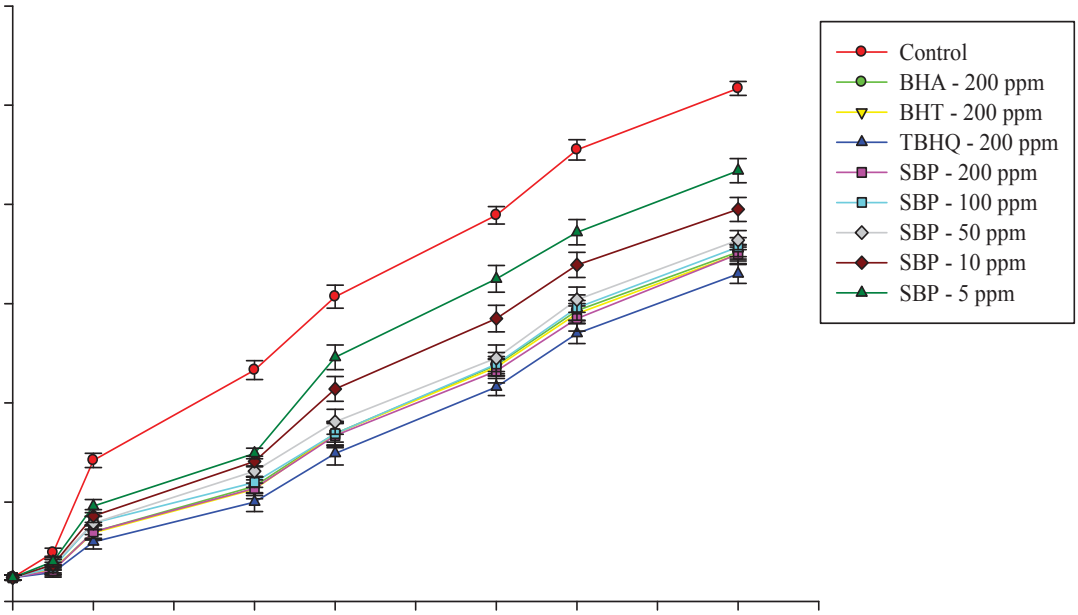
(c)

Figure 4.10. Absorptivity at 232 nm of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

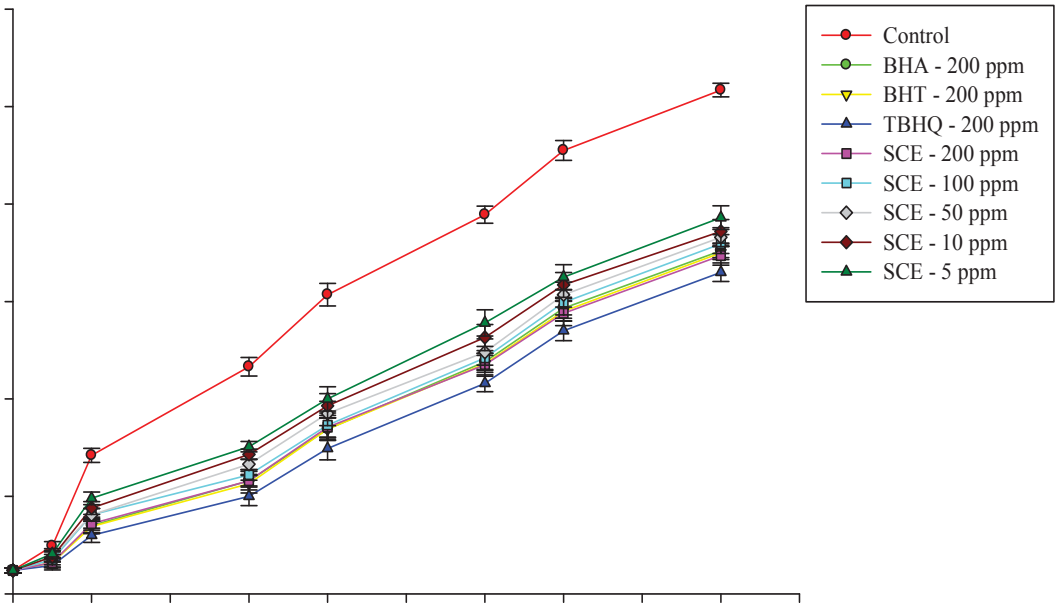
CD and CT contents continued to increase with the increase in storage time. Control exhibited the highest content of CD followed by BHA, BHT, SCE, SBP, PPE and TBHQ. On the other hand, CT levels followed the pattern control > BHA > BHT = SBP > SCE > PPE > TBHQ, respectively. CD and CT of all stabilized samples are quite lower than control; thus indicating that the extracts under investigation had good antioxidant activity. Similar results were found by Iqbal *et al*. (2008), who reported that control exhibited the highest content of CD and CT followed by BHT, and pomegranate peel extract.



(a)



(b)

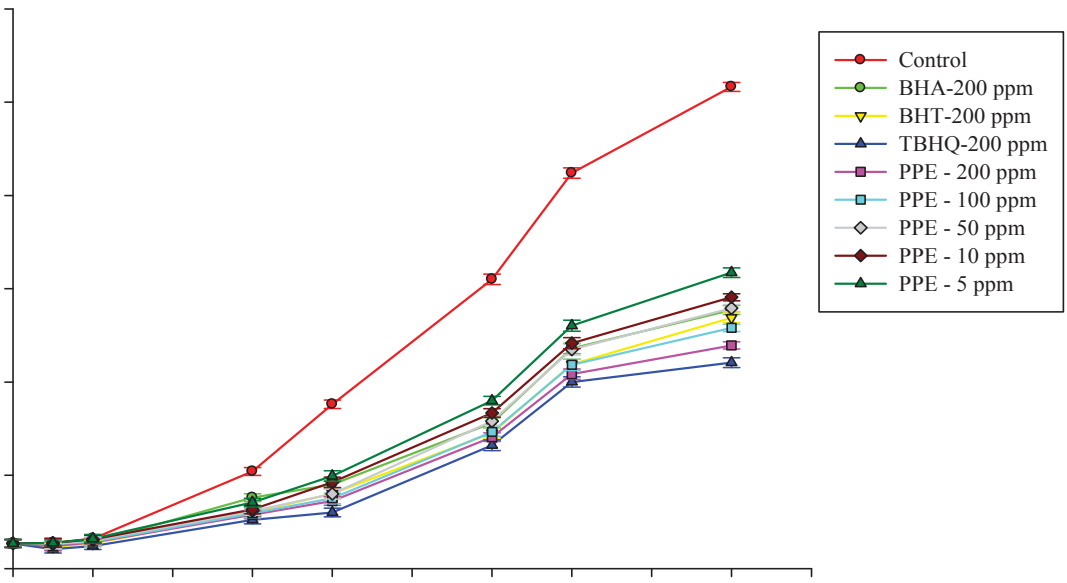


(c)

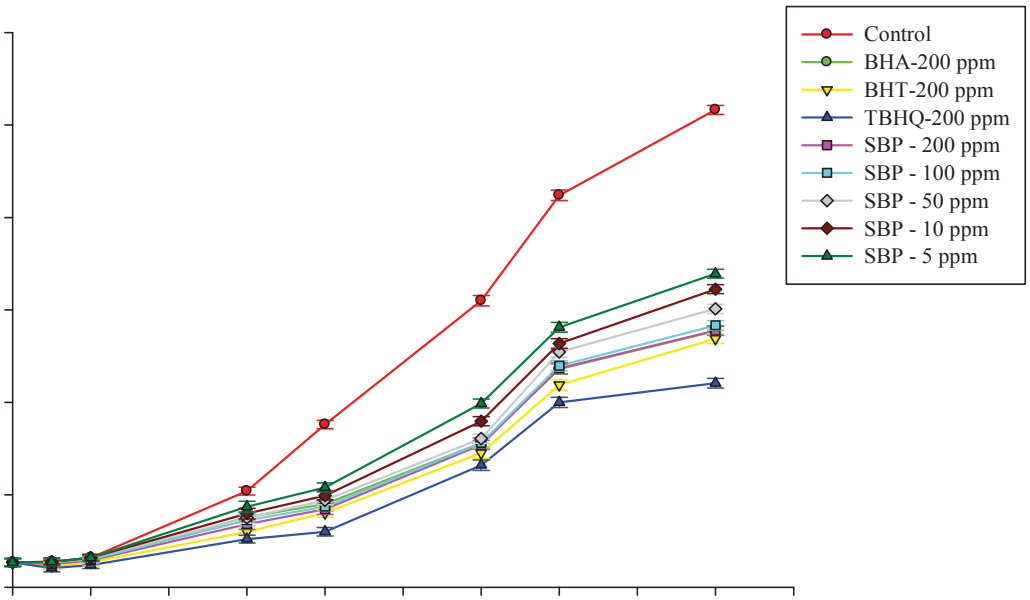
Figure 4.11. Absorptivity at 270 nm of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake methanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

4.6.4 Effect of ethanolic extracts on sunflower oil oxidation

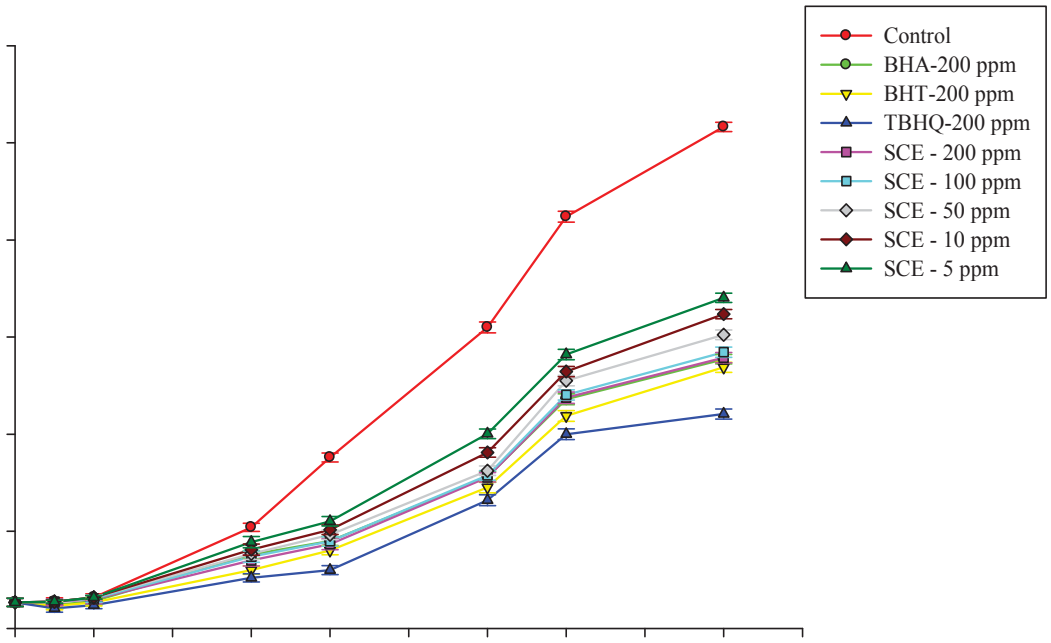
Oxidation degree on sunflower oil samples was determined by measuring PV in the absence and presence of antioxidants at 70 °C for 72 h. The influence of antioxidants on PV of potato peels, sugar beet pulp, and sesame cake ethanolic extracts during storage is shown in Figure 4.12. In general, peroxide value of all sunflower oil samples increased significantly ($p < 0.05$) with increasing storage time. Initially on 0 h, all samples including control indicated similar peroxide value. On 4 and 8 hours of storage, all the antioxidant fortified samples differed significantly ($p < 0.05$) in their peroxide formation levels except potato peels, sugar beet pulp, and sesame cake extracts at 5 and 10 ppm. But from 24 h onwards, all the treatments had significantly ($p < 0.05$) lower peroxide value compared with the control. Ability of antioxidants in preventing peroxide formation in samples decreased in the order of TBHQ> PPE > BHT > BHA = SBP > SCE throughout the storage period. TBHQ showed highest ability in preventing peroxide formation because TBHQ is a strong synthetic antioxidant. BHA and BHT although used frequently in food industry, are not strong antioxidants like TBHQ. Besides, TBHQ is stable at high temperature and less volatile than BHA and BHT (Allen, 1983). Almeida-Doria and Regitano-Darce (2000) observed that natural extracts from rosemary and oregano were as effective as BHA and BHT in controlling oxidation of oil but less effective than TBHQ.



(a)



(b)



(c)

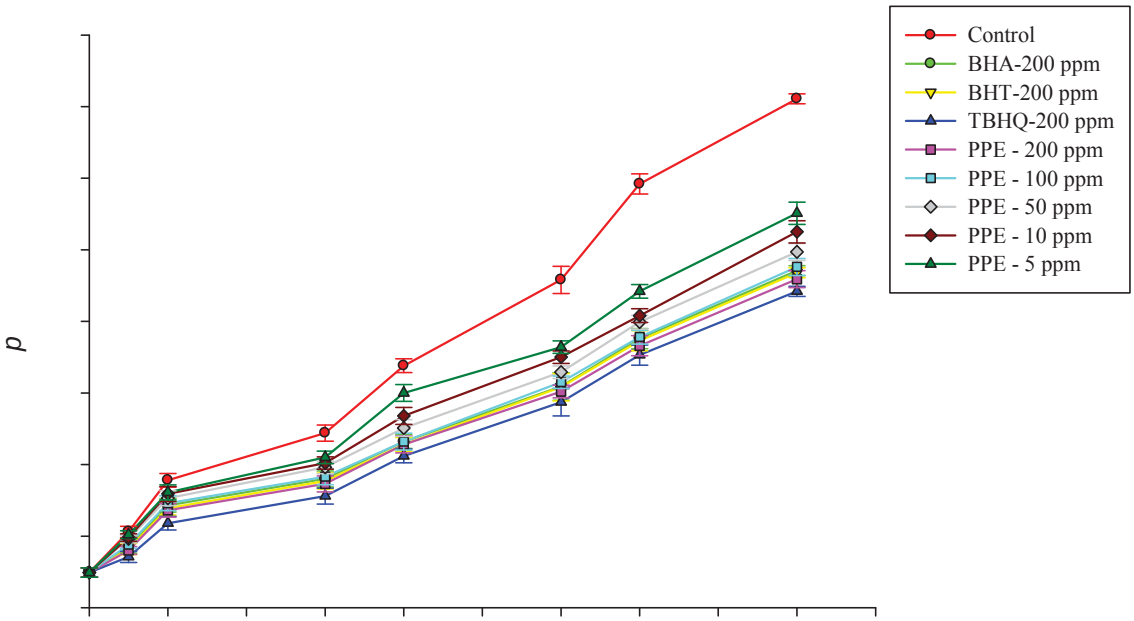
Figure 4.12. Relative increase in peroxide value (PV) of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

Regarding the inhibitory effect of natural antioxidants in peroxide formation, sesame cake extracts showed poor performance than other antioxidants. This is in accordance with our previous finding that sesame cake extract has lower antioxidant activity in all three assays used.

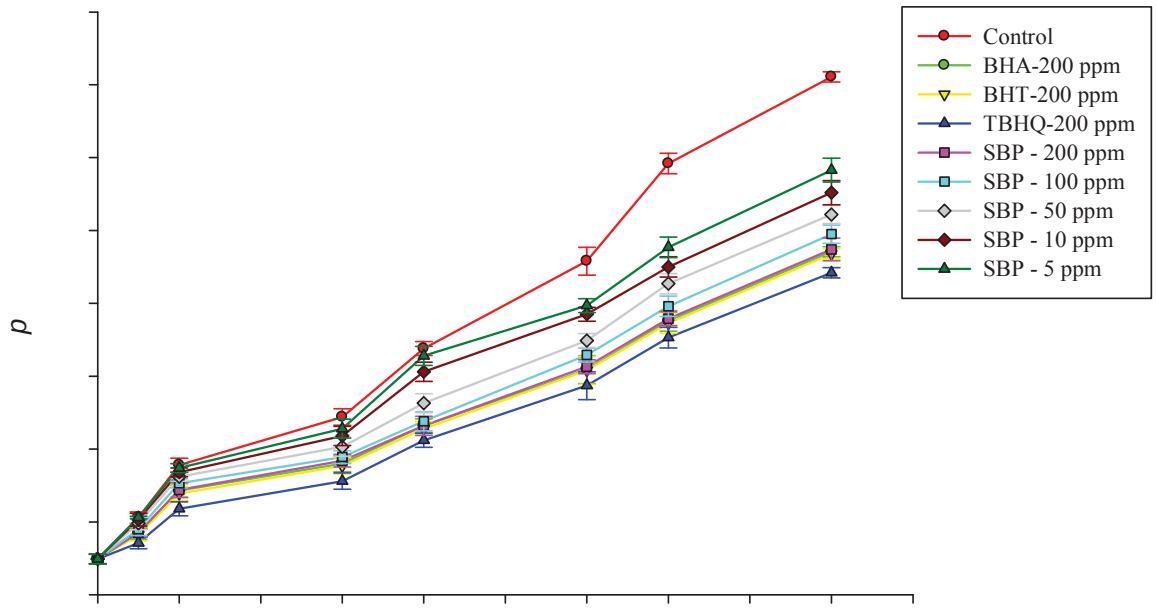
p-Anisidine value (AV) plays an important role in the oxidation process of edible oil and edible fats. Calculating AV is one of the oldest methods for evaluating secondary lipid oxidation. It is based on the reactiveness of the aldehyde carbonyl bond on the *p*-anisidine amine group, leading to the formation of a Schiff base that absorbs at 350 nm (Ying *et al*., 2010):

$$R{=}CH{=}O + H_2N{-}\Phi{-}OMe \rightarrow R{-}CH{=}N{-}\Phi{-}OMe \text{ (Schiff base)} + H_2O$$

Figure 4.13 depicts the *p*-ansidine values for sunflower oil samples stabilized with PPE, SBP, SCE extracts, TBHQ, BHT, BHA, and control. We can see AV increased significantly throughout the storage time, which increased in acceleration after 32 h. The *p*-anisidine value of control reached a maximum of 14.22 ± 0.14 from an initial value of 0.98 ± 0.13 after 72 h of storage. The difference in antioxidant activity may be accounted for on the basis of chemical structures. The stability of phenoxy radicals reduces the rate of propagation and further reactions and thus increases the oxidative stability of lipids (Ying *et al*., 2010).



(a)



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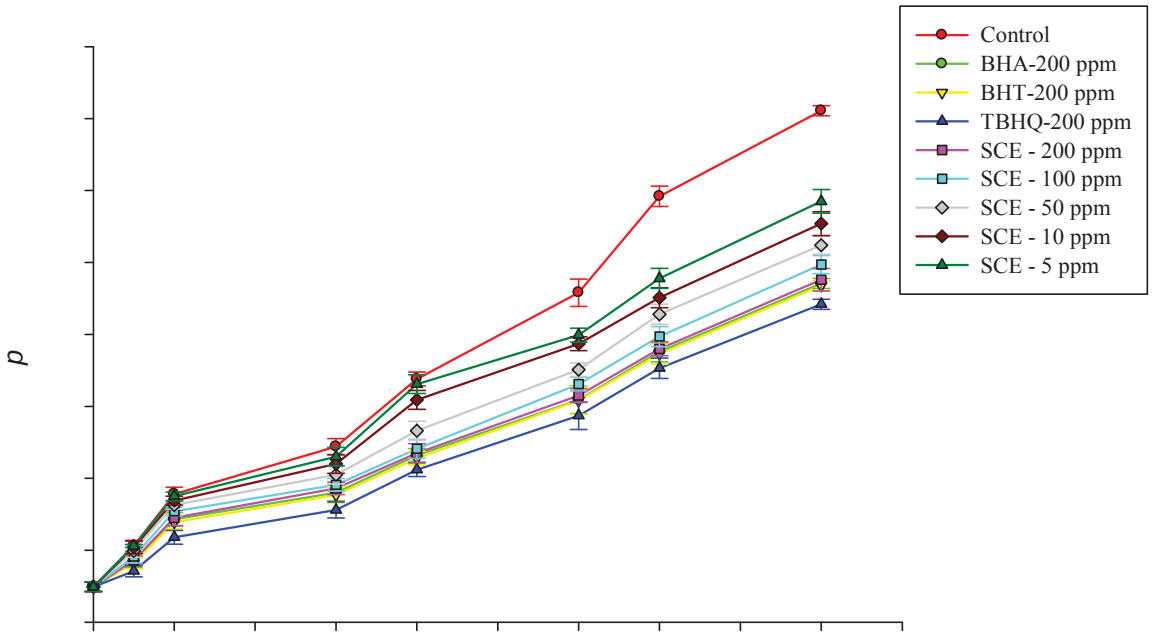
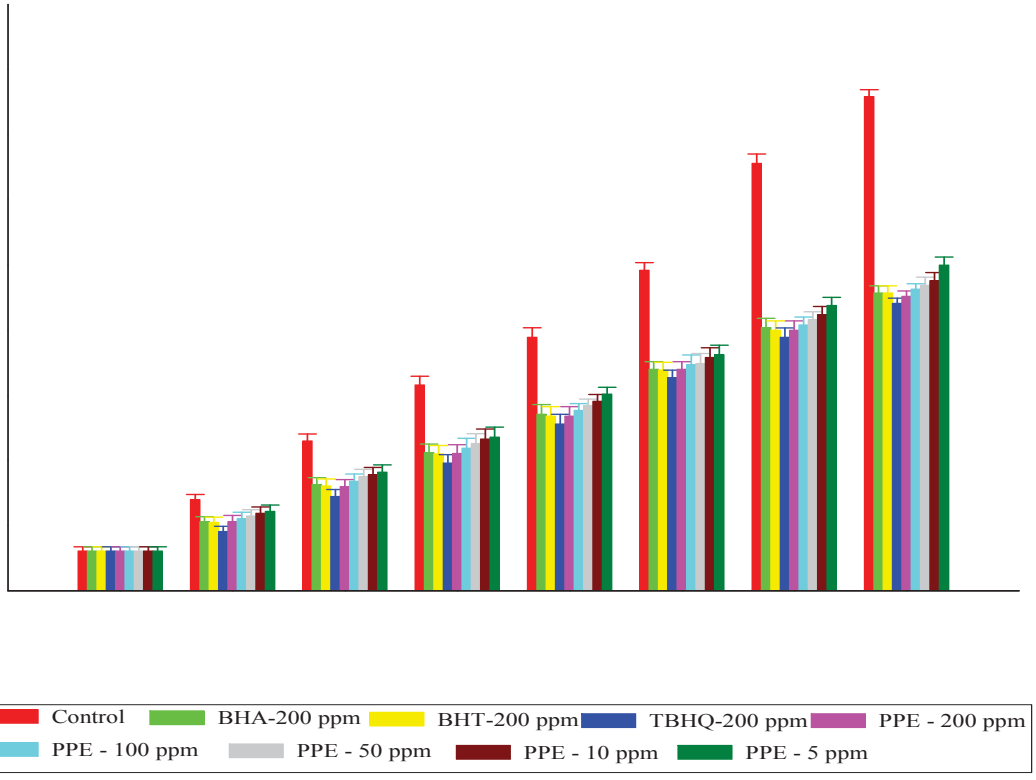


(c)

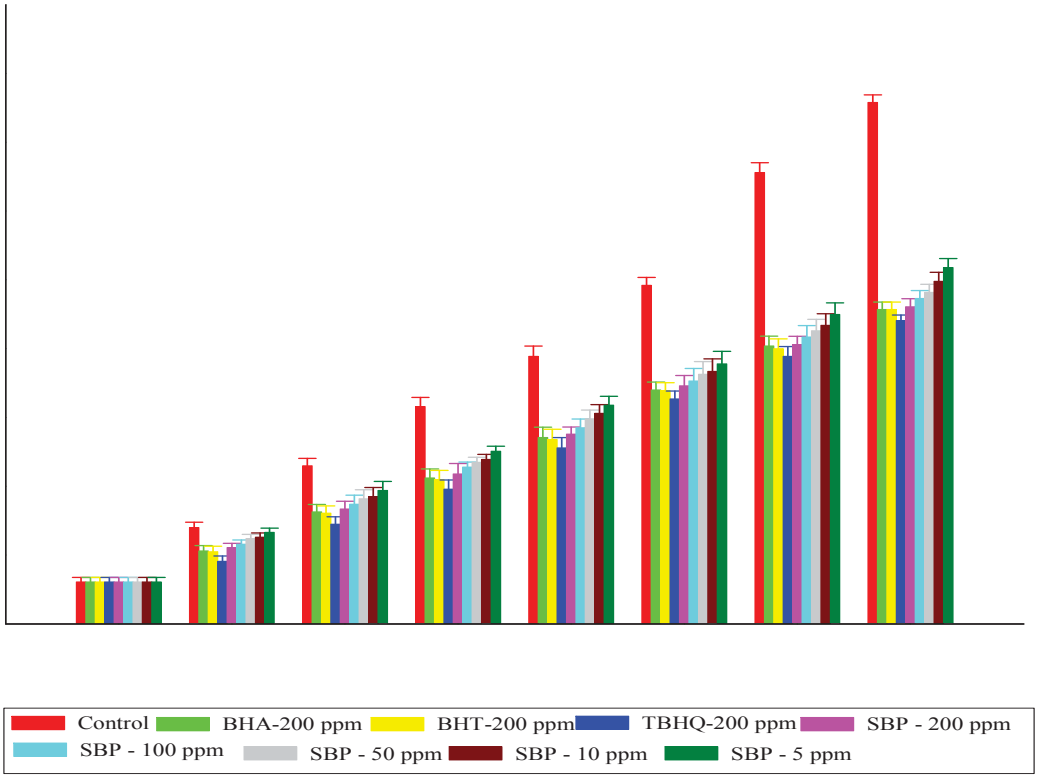
Figure 4.13. Relative increase in *p*-anisidine value (AV) of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

The results demonstrated that potato peels extract had higher antioxidant activity than BHA and BHT but lower than TBHQ. In addition, in most cases, the synthetic antioxidant TBHQ which has two *para*-hydroxyl groups can make the phenols more easily donate hydrogen atoms to active free radicals to interrupt the chain reaction of oxidation (Jiang and Wang, 2006) and this is responsible for superior antioxidant activity in various edible oils (Ying *et al*., 2010). In this study, in comparison with synthetic antioxidants, effects of potato peels, sugar beet pulp, and sesame cake extracts may also play an important role in the observed trends.

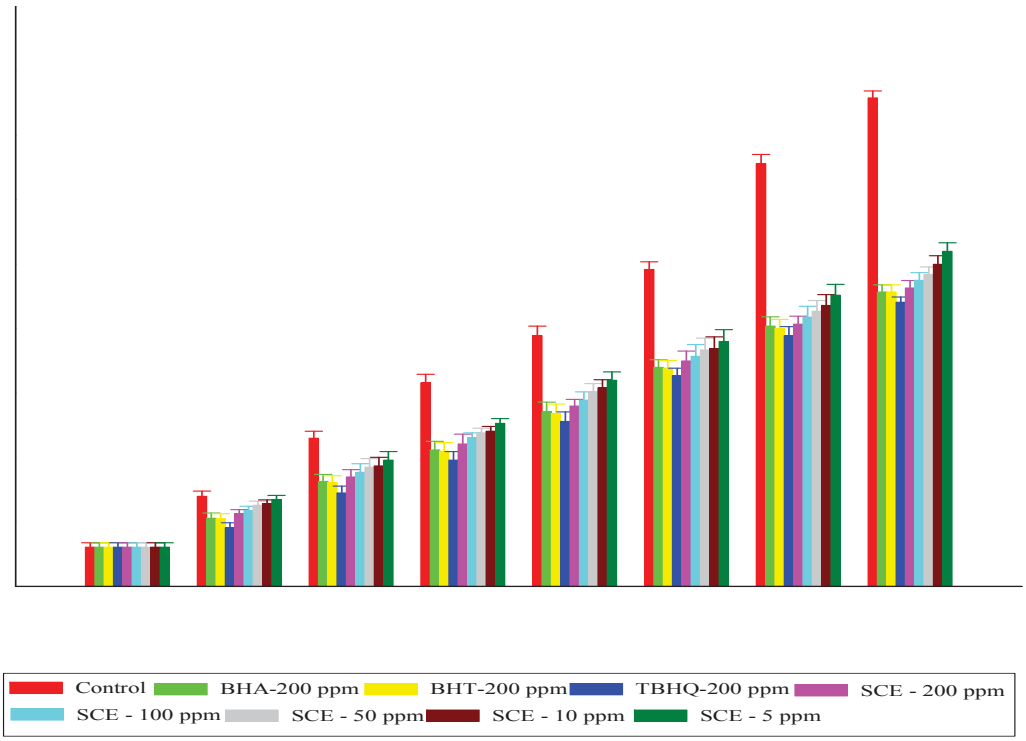
Conjugated dienes (CD) and trienes (CT) contents of sunflower oil samples stabilized with natural extracts, BHT, BHA, TBHQ, and control are shown in Figures 4.14 and 4.15. The CD and CT contents increased parallely to increase of storage time with greater rate for control. The oil samples stabilized with PPE, SBP, and SCE extracts showed lower levels of CD and CT compared to control, indicating antioxidant potential of the potato peels, sugar beet pulp, and sesame cake extracted components. The results of the CD and CT in present study revealed the antioxidant activity of extracts applied at 200 ppm as strong as that of BHA and BHT but weaker than that of TBHQ at their legal amounts.



(a)



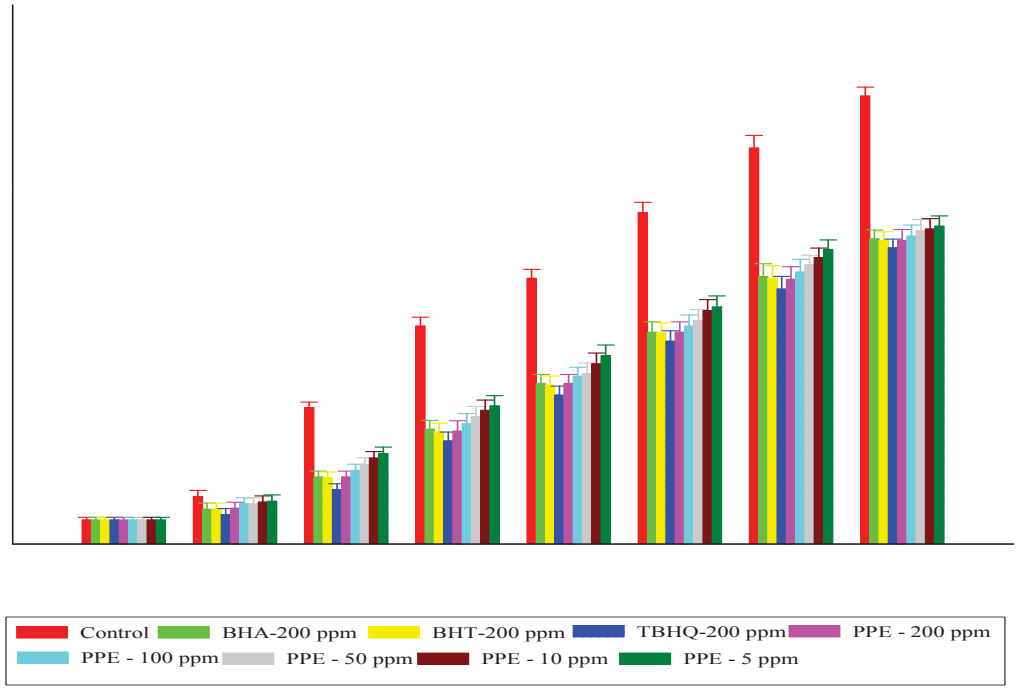
(b)



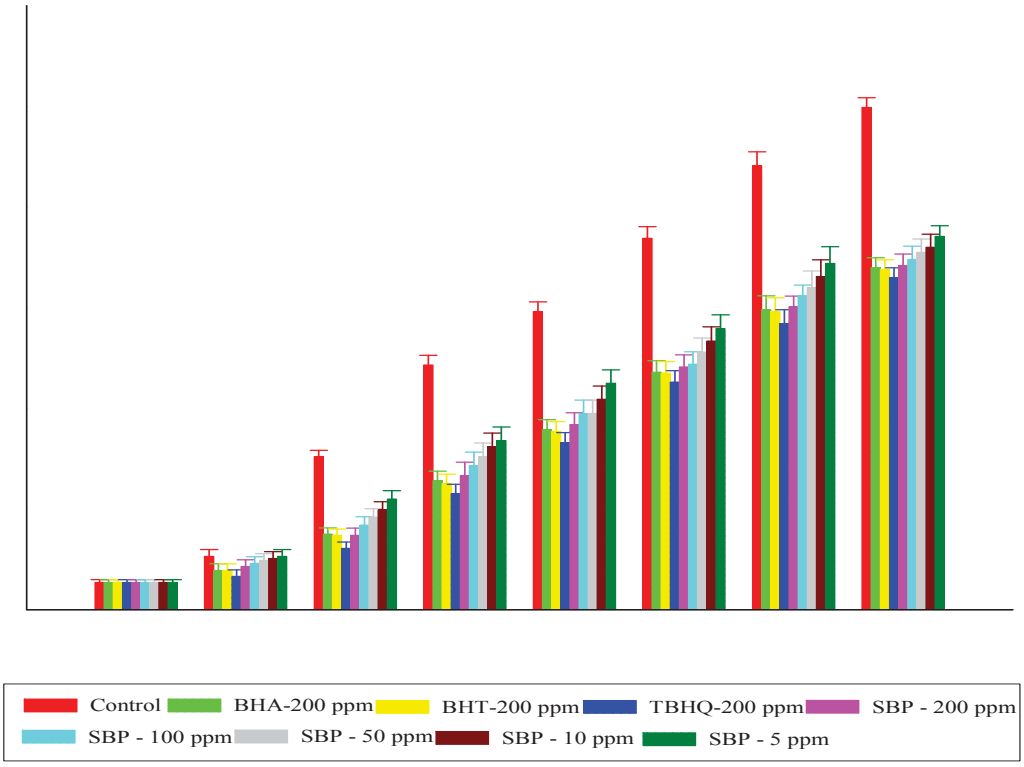
(c)

Figure 4.14. Absorptivity at 232 nm of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

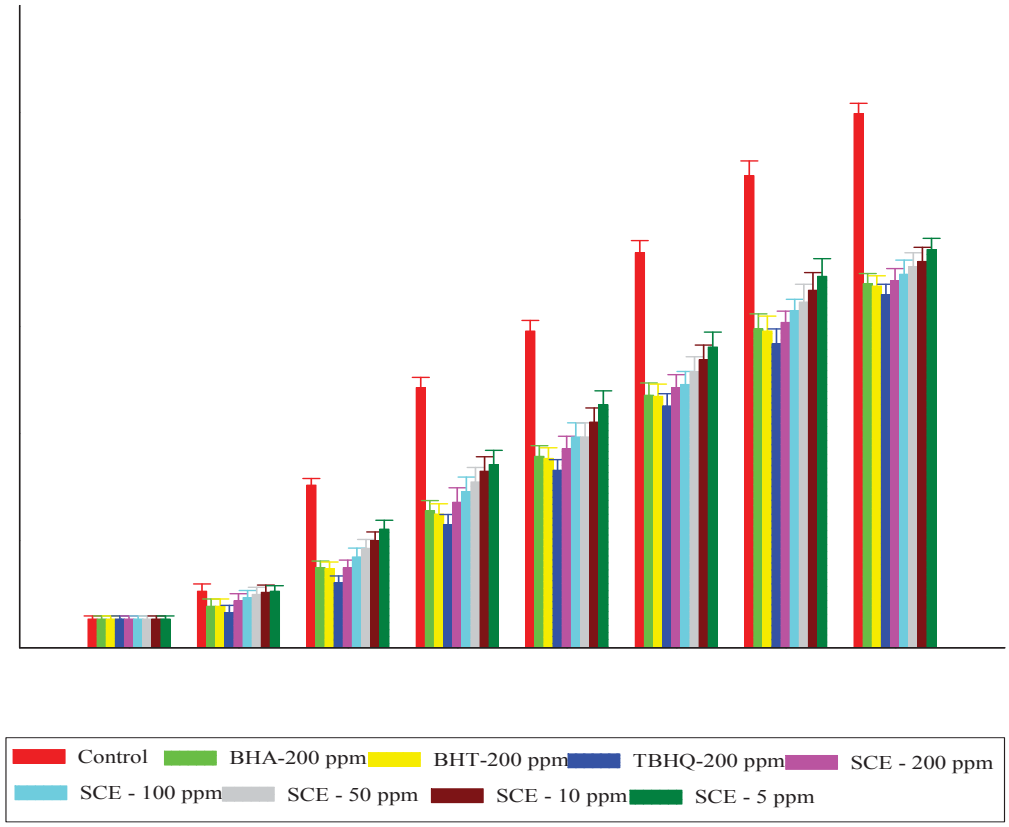
Measurement of CD and CT is a good parameter for the determination of oxidative stability of the oils. Lipids containing methylene-intrupted dienes or polyenes show a shift in their double bond position during oxidation. The resulting conjugated dienes exhibit intense absorption at 232 nm; similarly conjugated trienes absorb at 270 nm.



(a)



(b)

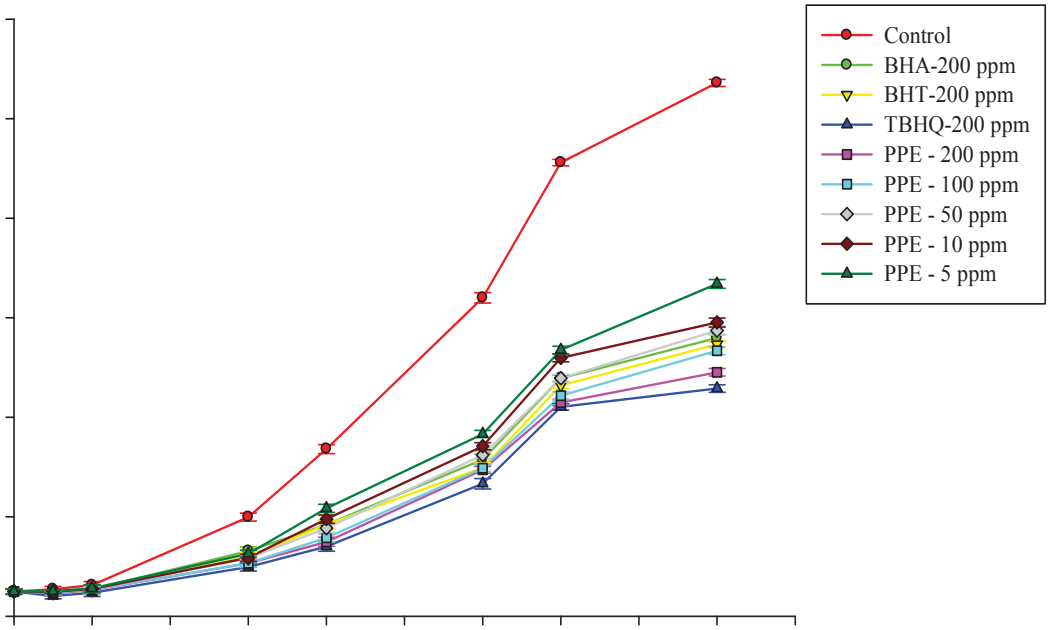


(c)

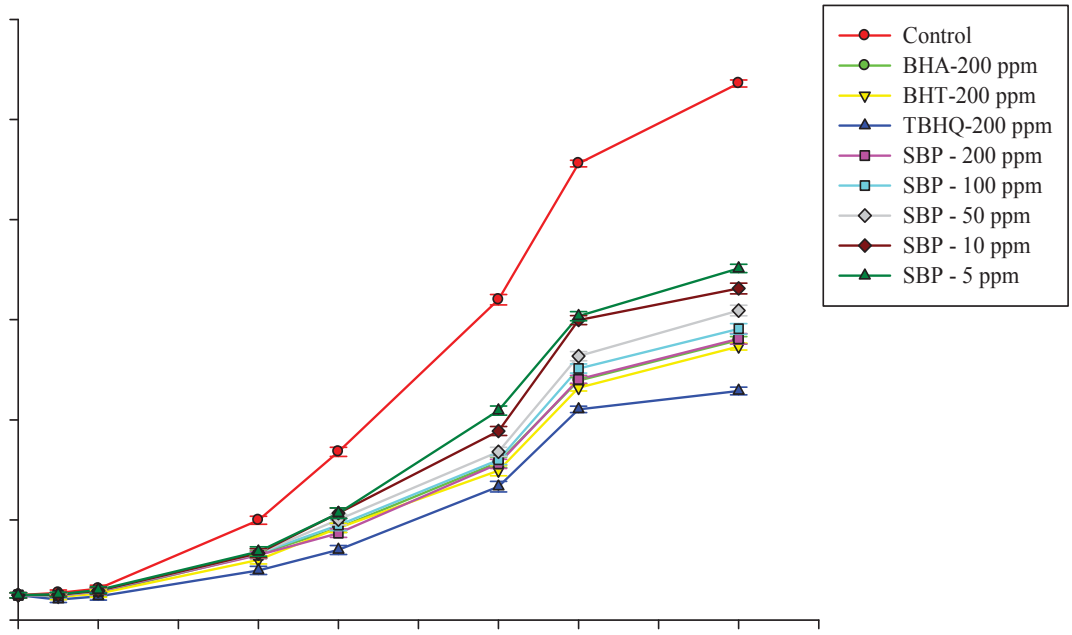
Figure 4.15. Absorptivity at 270 nm of treated sunflower oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

4.6.5 Effect of ethanolic extracts on soybean oil oxidation

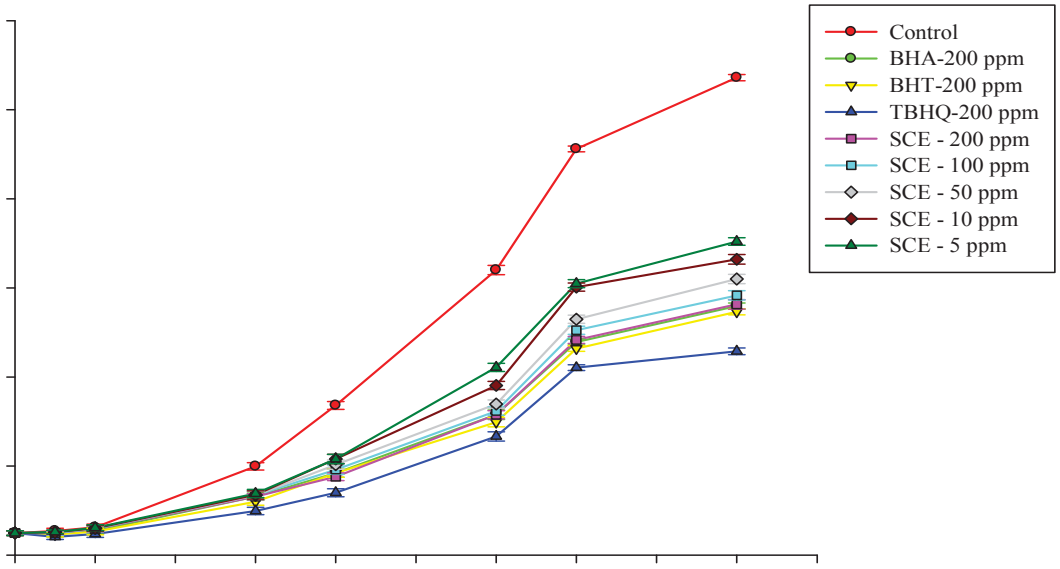
The effect of natural and synthetic antioxidants on peroxide value of potato peels, sugar beet pulp, and sesame cake ethanolic extracts over 72 h of storage in oven is shown in Figure 4.16 Results showed that peroxide value increased linearly with storage time and increased in acceleration after 24 h. PV was in the range 11.44-17.60 meq kg^{-1} for stabilized samples up to 72 h, while maximum value of PV for control sample was 26.80 meq kg^{-1}. At all stages, highest PV was observed for control sample followed by SCE, SBP, BHA, BHT, PPE, and TBHQ, respectively. A significant difference ($p < 0.05$) in PV was observed between the control and soybean oil samples. These results indicated that potato peels, sugar beet pulp, and sesame cake extracts, at all the concentrations, controlled peroxide value appreciably; revealing good antioxidant efficacy in stabilization of soybean oil.


Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

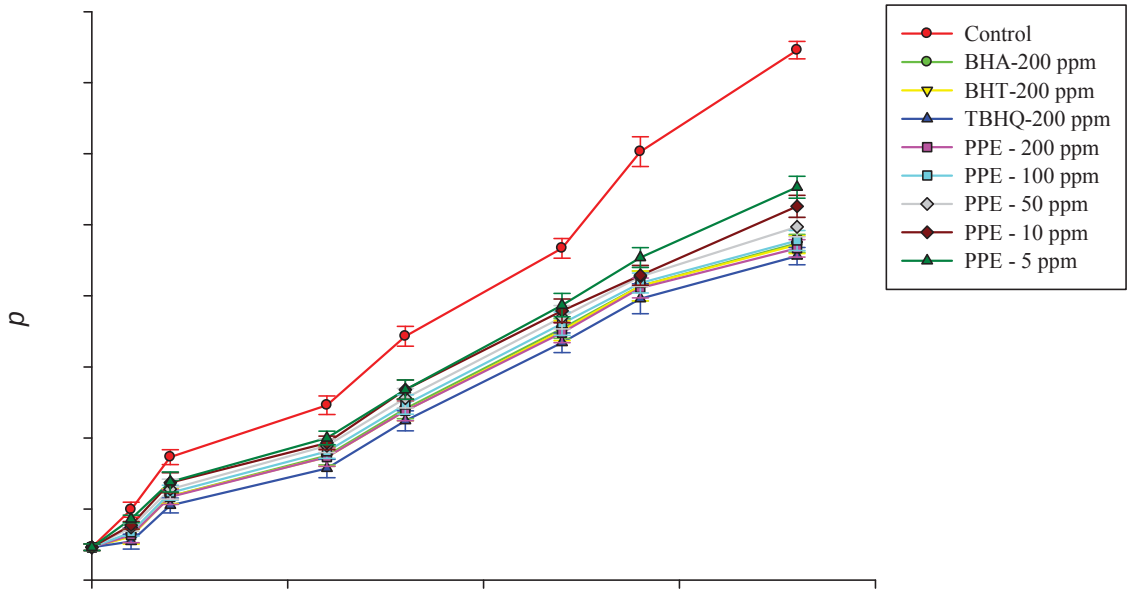
(b)



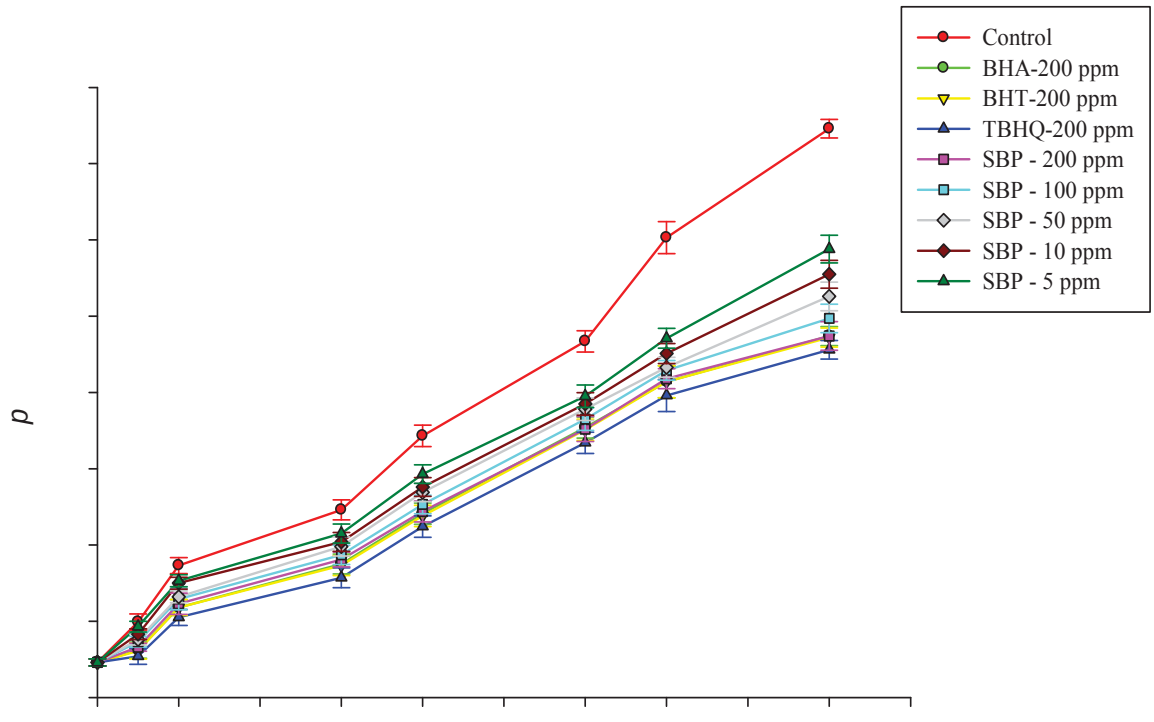
(c)

Figure 4.16. Relative increase in peroxide value (PV) of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

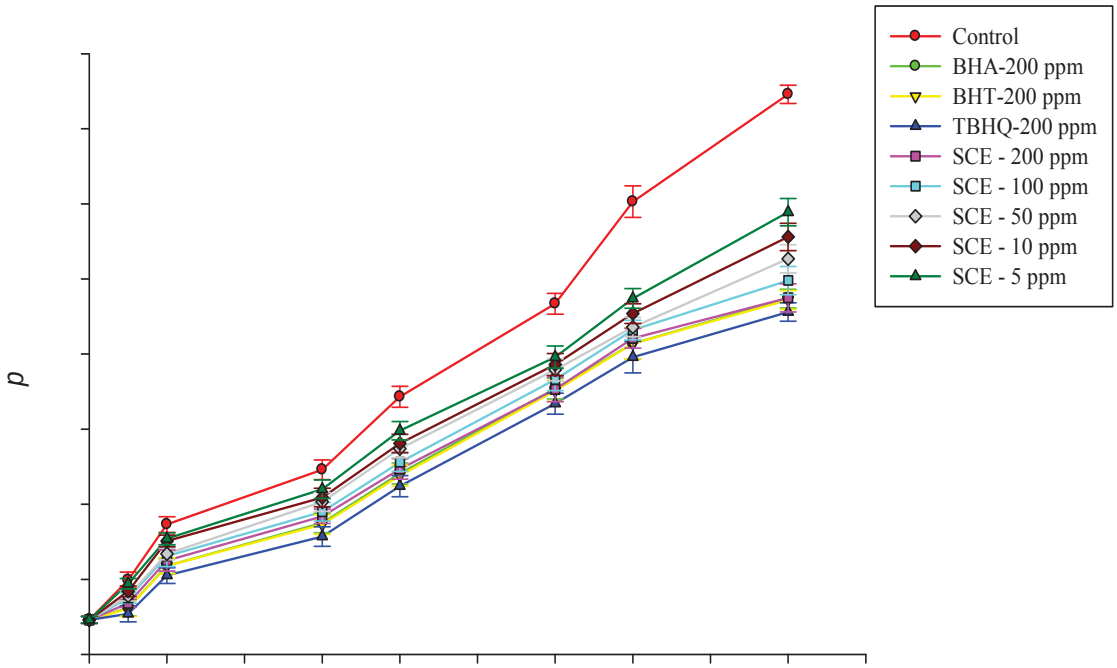
The effect of potato peels, sugar beet pulp, and sesame cake extracts on soybean oil oxidation (measured by *p*-anisidine value) is shown in Figure 4.17 We can see *p*-anisidine value increased significantly throughout the storage time, which increased in acceleration after 24 h. Addition of PPE, SBP, and SCE extracts caused significant reduction in AV of soybean oil during 72 h at 70 °C. It is evident from these results that, the same order of natural extracts efficiency was observed as the content of polyphenol increased, inhibitory effects on AV also increased considerably, better than BHA and BHT but lower than TBHQ.


p
Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


p
Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

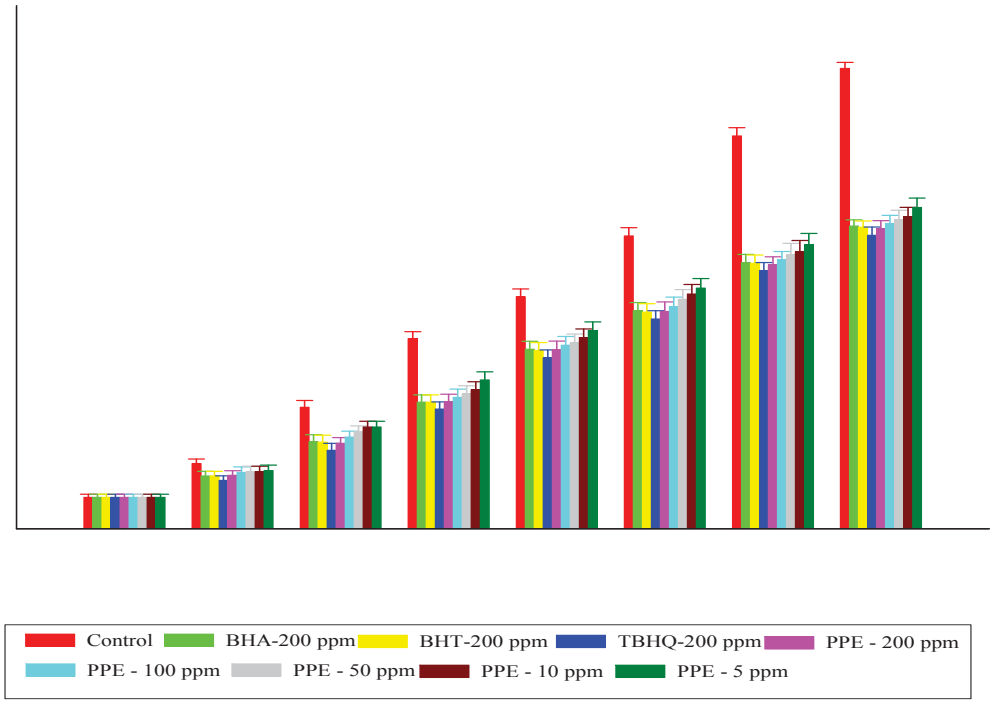
(b)



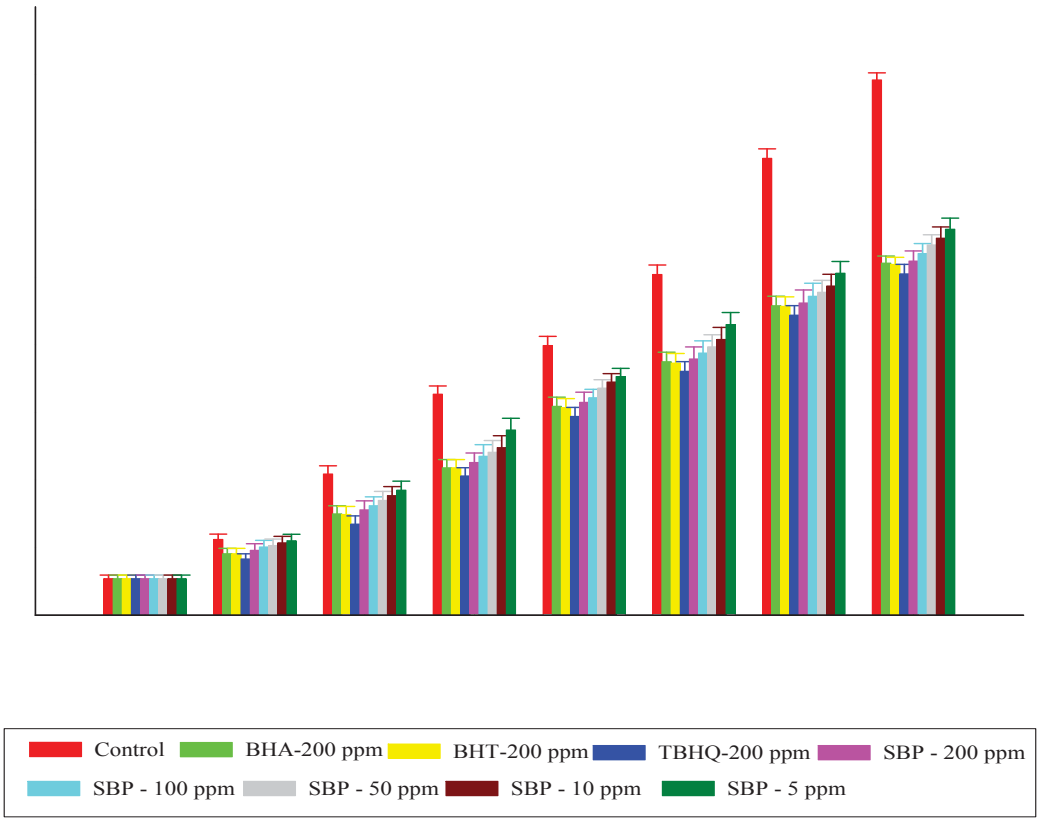
(c)

Figure 4.17. Relative increase in *p*-anisidine value (AV) of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts under accelerated storage. Error bars show the variations of three determinations in terms of standard deviation.

Absorption at 232 nm and 270 nm, due to the formation of primary and secondary compounds of oxidation (Figures 4.18 and 4.19), showed a pattern in good agreement with that of the PV and AV. On the 0 h, although the control did not differ significantly from the treatments but it showed significantly ($P < 0.05$) higher absorptivity value at 232 and 270 nm than the treatments with increasing storage period. Exception is seen in sugar beet pulp and sesame cake extracts at 5 ppm which are very close to the values obtained for the control. According to the ability of antioxidants in reducing absorptivity level at 232 and 270 nm, the antioxidants can be arranged in the following decreasing order TBHQ> PPE > BHT > BHA > SBP > SCE. TBHQ has shown the best ability in reducing the absorptivity. Differences in absorptivity between the natural antioxidant samples were not of large magnitude. However, among them, potato peels showed the best result.


Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


Control
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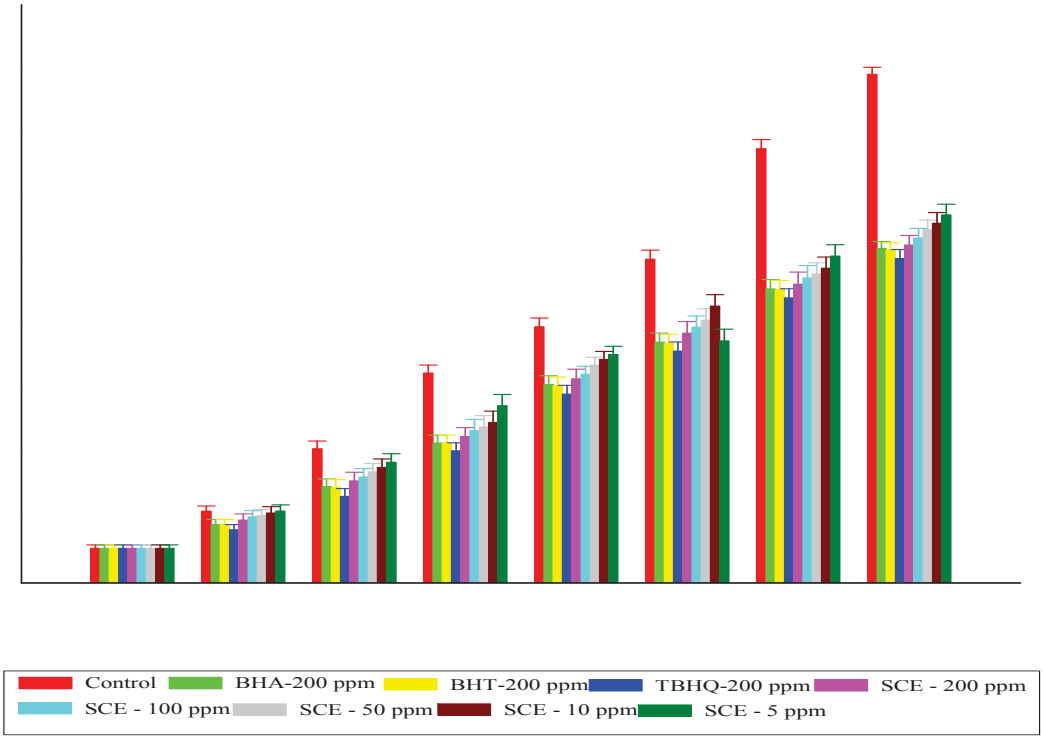
BHT-200 ppm
TBHQ-200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

(b)

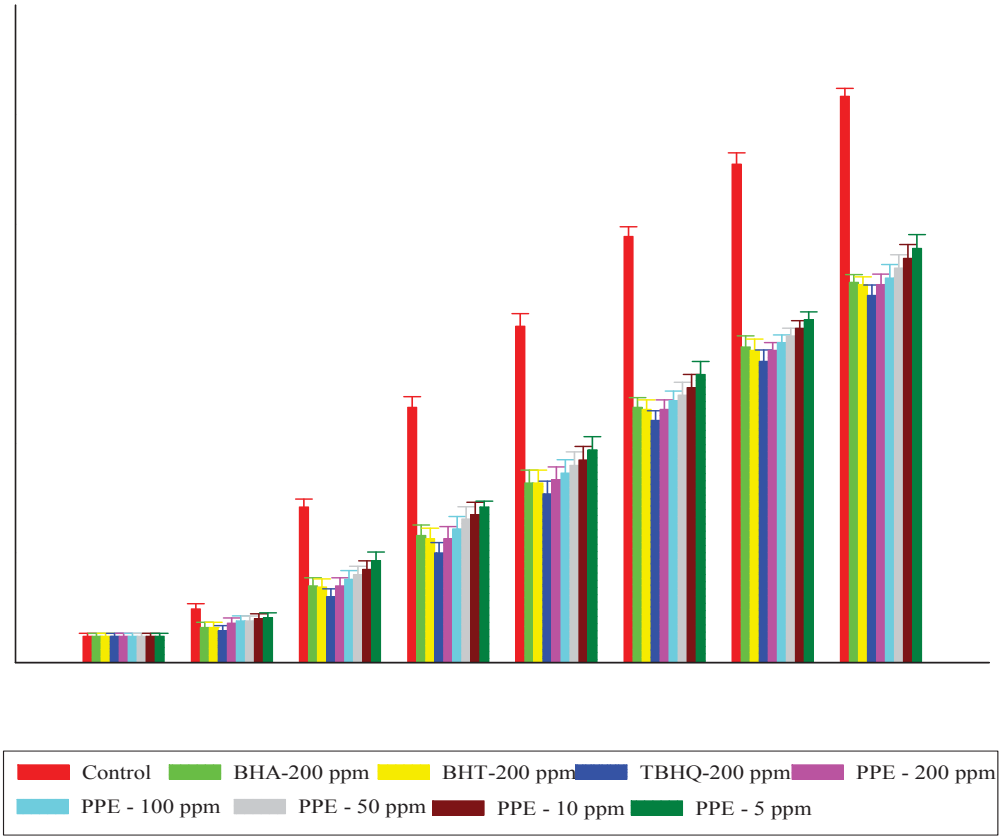


(c)

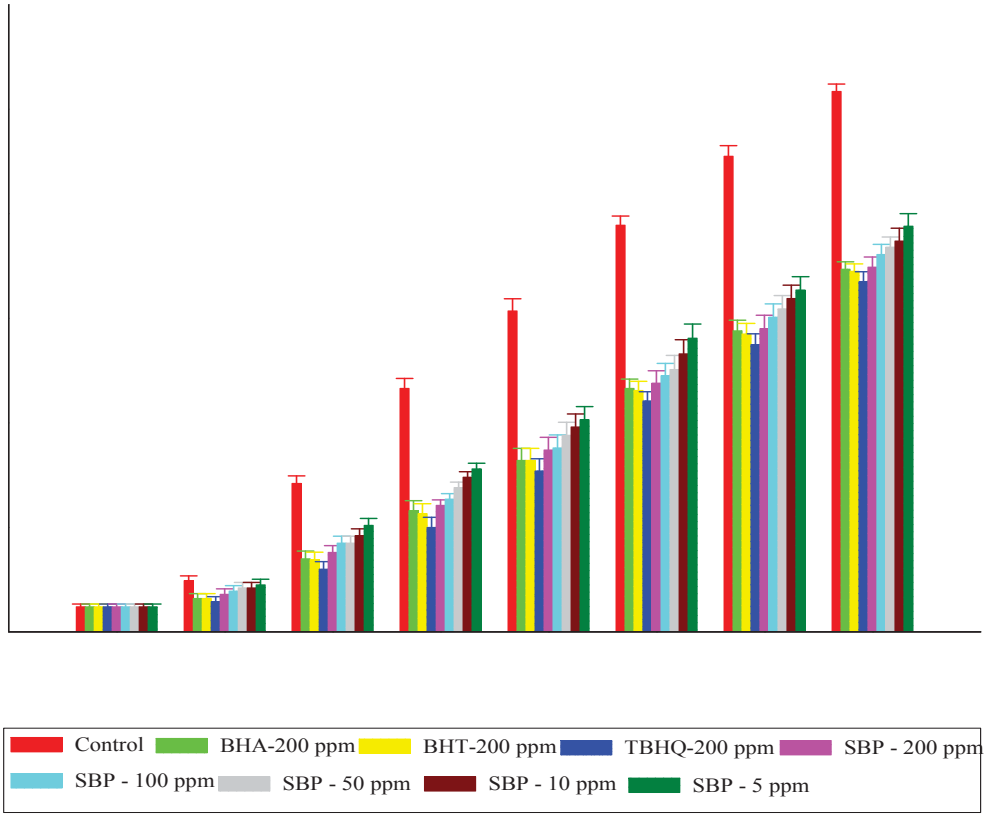
Figure 4.18. Absorptivity at 232 nm of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

Peroxide value (PV), *p*-anisidine value (AV), and UV absorptivity analyses showed that sunflower oil have significantly better oxidative stabilities than soybean oils. This is because sunflower oil (SFO) includes high level of linoleic acid (18:2n - 6) content but soybean oil (SBO) is rich in α-linolenic acid (18:3n -3) in addition to linoleic acid. . These results indicate that oils with high linolenic contents have low oxidative stabilities.

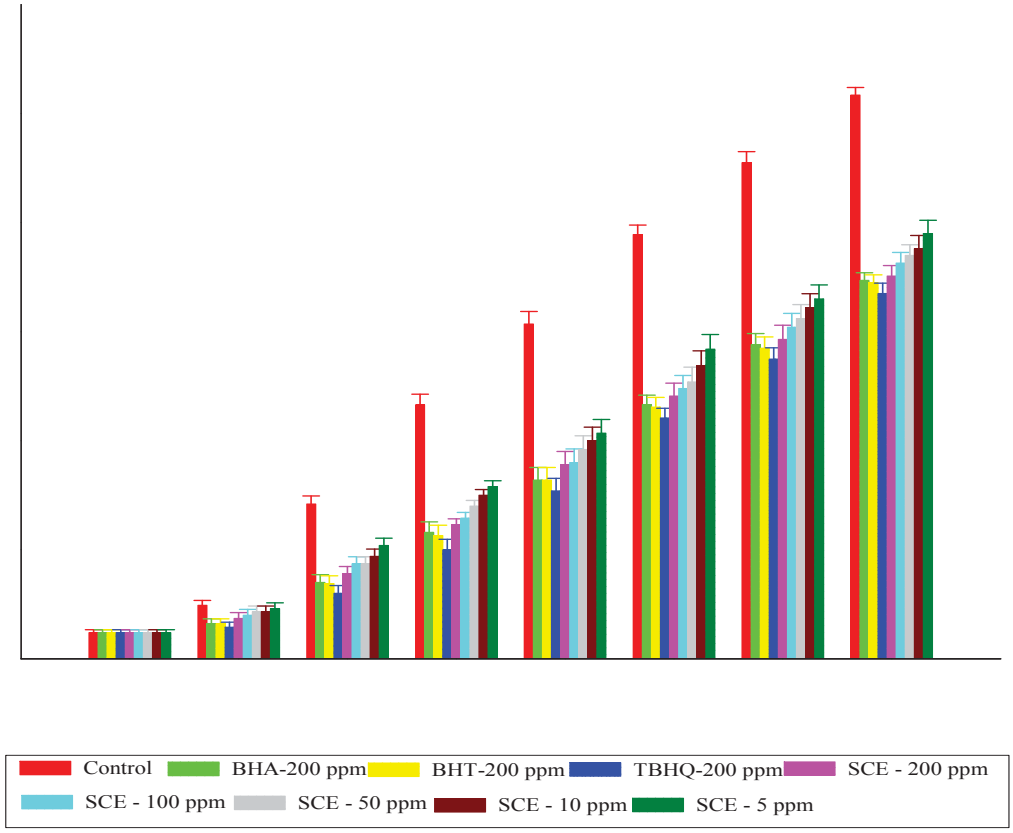
Soybean oil is relatively unstable to oxidation than sunflower oil. The off-flavore that develop are caused by volatile compounds released during the breakdown of hydroperoxides, which are flavourless but unstable compounds formed during the oxidation of unsaturated fats The hydroperoxides are transformed to secondary products such as aldehydes, alcohols, ketones, acids, hydrocarbons, esters and lactones. The degree of unsaturation of a fatty acid has a significant effect on the oxidation rate. The relative reaction rate with oxygen and the hydroperoxide decomposition rate of linolenate (18:3) are much faster than those of linoleate (18:2) and oleate (18:1). Because 18:3 oxidizes much easier than the other fatty acids, it has been considered an important cause of off-flavor development in soybean oil, although it accounts for only 8 % to 10 % of the total fatty acids in soybean oil (Stephanie *et al*., 2007).


Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
PPE - 200 ppm
PPE - 100 ppm
PPE - 50 ppm
PPE - 10 ppm
PPE - 5 ppm

(a)


Control
BHA-200 ppm
BHT-200 ppm
TBHQ-200 ppm
SBP - 200 ppm
SBP - 100 ppm
SBP - 50 ppm
SBP - 10 ppm
SBP - 5 ppm

(b)



(c)

Figure 4.19. Absorptivity at 270 nm of treated soybean oil samples: **(a)** with potato peels, **(b)** with sugar beet pulp, and **(c)** with sesame cake ethanolic extracts during oven test. Error bars show the variations of three determinations in terms of standard deviation.

4.7 Identification of phenolic compounds using TLC and UV spectra

To know what is/are the responsible active ingredient(s) in potato peels, sugar beet pulp, and sesame cake, TLC and absorptivity spectra between 200 and 400 nm were screened. Phenolic compounds exhibit two major absorption bands in the ultraviolet/visible region: a first band in the range between 320 and 380 nm and a second band in the 250 to 285 nm range (Matthäus, 2002). As can be seen from Figures 4.20 and 4.21, methanolic and ethanolic extracts of potato peels, sugar beet pulp, and sesame cake showed maximum absorption in the range between 220 and 380 nm. This absorbance was more accentuated for potato peels extract than sugar beet pulp and sesame cake. These results are in agreement with the peroxide and *p*-anisidine values, which showed that potato peels extract had the highest antioxidant activity followed by sugar beet pulp and sesame cake. Absorption maxima of extracts at 220 nm may be due to the presence of flavone/flavonol derivatives (Chang *et al*., 2002).

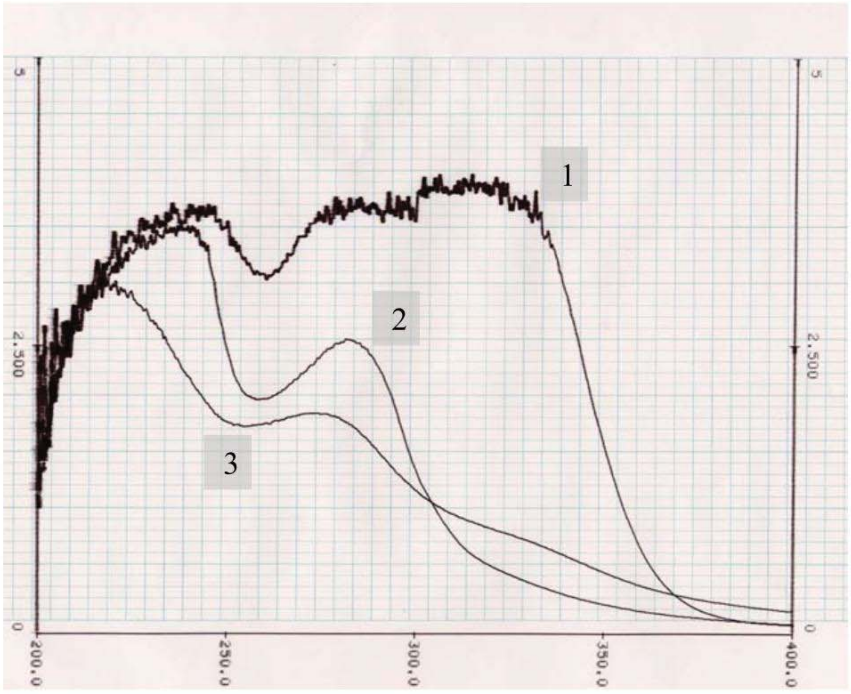


Figure 4.20. UV absorbance scanning between 200 and 400 nm of (1) potato peels, (2) sugar beet pulp, and (3) sesame cake methanolic extracts.

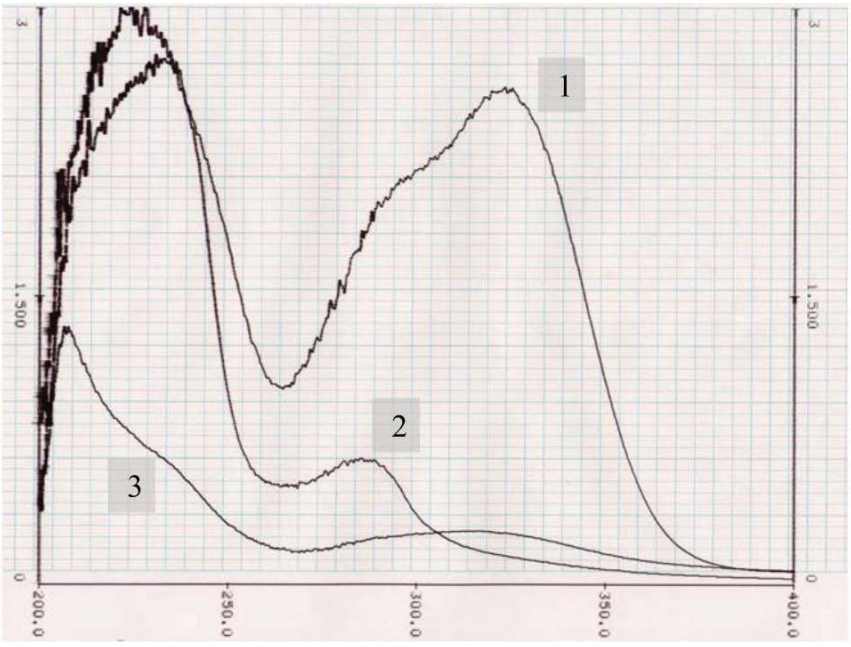


Figure 4.21. UV absorbance scanning between 200 and 400 nm of (1) potato peels, (2) sugar beet pulp, and (3) sesame cake ethanolic extracts.

In the past decades, extensive analytical methods have been developed to separate and determine phenolic compounds in various plant samples. The techniques include thin-layer chromatography (Kader *et al*., 1996), gas chromatography (GC) (Fiamegos *et al*., 2004), high-performance liquid chromatography (HPLC) (Seeram *et al*., 2005; Soong and Barlow, 2005), and capillary electrophoresis (Kronholma *et al*., 2004). Thin layer chromatography (TLC) has its own advantages (e.g., rapidity and inexpensiveness) (Kader *et al*., 1996).

Some authors have reported that the total phenolic content did not correspond well with the antioxidant activity of the extracts. Thus, individual phenolic compounds may provide a better indication of the antioxidant activity of the extracts in vegetable oils (Bin and Clifford, 2008). Figure 4.22 shows a representative TLC profile of the samples and standard. It shows that the methanolic extract of potato peels contains chlorogenic, gallic, *p*-coumaric, and caffeic acids, while in sesame cake extract *p*-coumaric was identified. Chlorogenic, *p*-coumaric, sinabic and gallic acids were detected in sugar beet pulp.

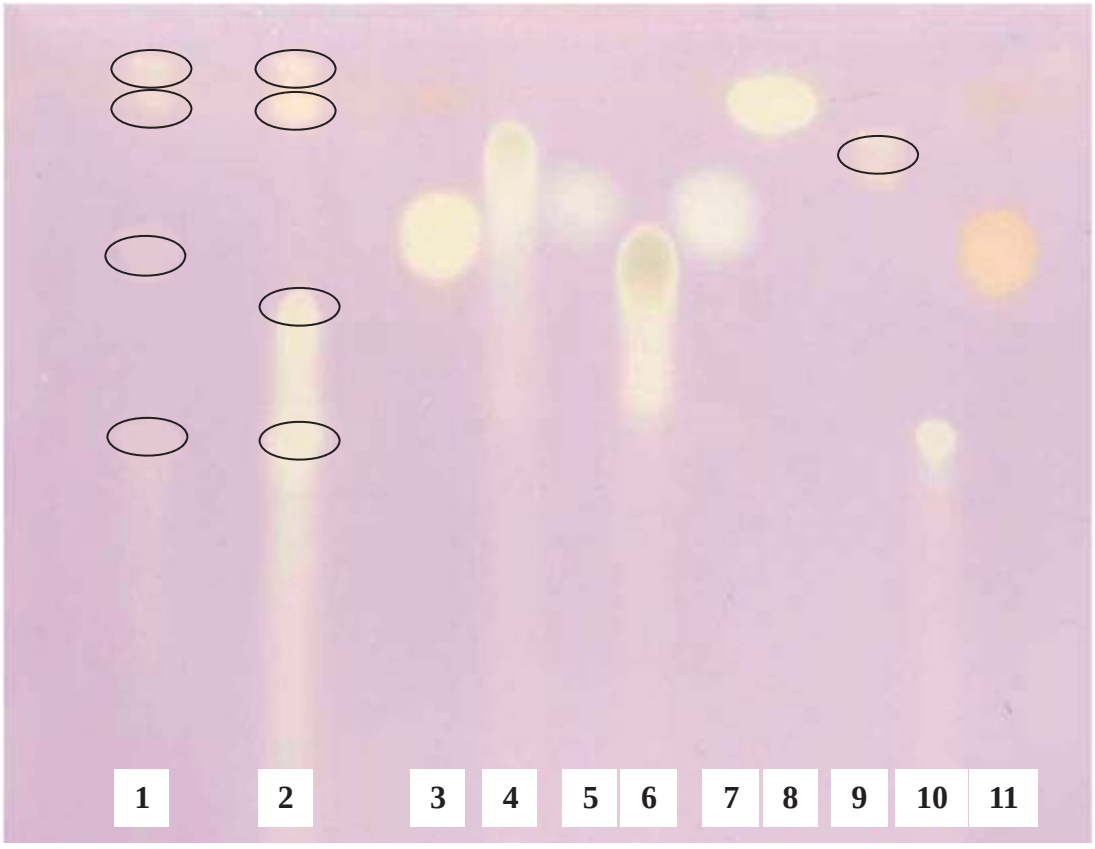


Figure 4.22. Thin layer chromatography profile of the samples and standard. Lanes: 1, sugar beet pulp extract; 2, potato peels extract; 3, ferulic acid; 4, *p*- coumaric acid; 5, *o*-cumaric acid; 6, caffeic acid; 7, qurecetin; 8, gallic acid; 9, sesame cake extract; 10, chlorogenic acid; and 11, sinabic acid.

The results confirmed our pervious experiments, wherein methanolic extract of potato peels showed the highest total phenols followed by sugar beet pulp and sesame cake. Chlorogenic, and caffeic acids may play an important role in the antioxidant activity of potato peels extract. In the present research, phenolic acids are proven, using TLC analysis, to be present in potato peels, sugar beet pulp, and sesame cake and the antioxidant activity of these compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). This finding may indicate that these compounds contribute to the antioxidant activity of potato peels, sugar beet pulp, and sesame cake extracts.

4.8 Identification of phenolic compounds using HPLC analysis.

The analysis of phenolic compounds is very challenging due to the great variety and reactivity of these compounds. On the other hand, polyphenolics are suitable compounds for analysis using modern separation and detection methods, such as hyphenated techniques of high performance liquid chromatography (HPLC) with mass spectrometry (MS), ultraviolet-visible light (UV/Vis), or nuclear magnetic resonance (NMR) spectroscopy (Bronze and Boas, 1998). For the purpose of separation and quantification of individual phenolic compounds, HPLC is most frequently used because of its high-separation capacity and relative simplicity. It does not require sample derivatization prior to analysis (Huang *et al*., 2007).

Of the six solvent extracts, methanolic and ethanolic extracts exhibited high yield, and antioxidant activity (high in TPC and better AOA). Therefore, methanolic and ethanolic extracts were used for further investigations towards identification by HPLC. The HPLC analysis of potato peels, sugar beet pulp, and sesame cake extracts revealed the presence of phenolic compounds. By this means, the crude methanolic and ethanolic extracts in the three samples were analysed.

Chlorogenic, caffeic, 4-hydroxybenzoic, *p*-coumaric, vanillic, trans-*o*-hydroxycinnamic, cinnamic, and sinapic acids were detected in both extracts (methanolic and ethanolic), but slightly higher in methanolic extracts (Figure 4.23A, B, and C) than in ethanolic extracts (Figure 4.24A, B, and C). These compounds have been identified according to their retention time and the spectral characteristics of their peaks compared to those of standards, as well as by spiking the sample with standards. Tentative identification of peaks for which standard compounds were not available was obtained by comparing their retention time with the data from the pervious literatures.

Figure 4.23A, B, and C shows a representative chromatogram of the crude methanolic extracts of potato peels, sugar beet pulp, and sesame cake, respectively.

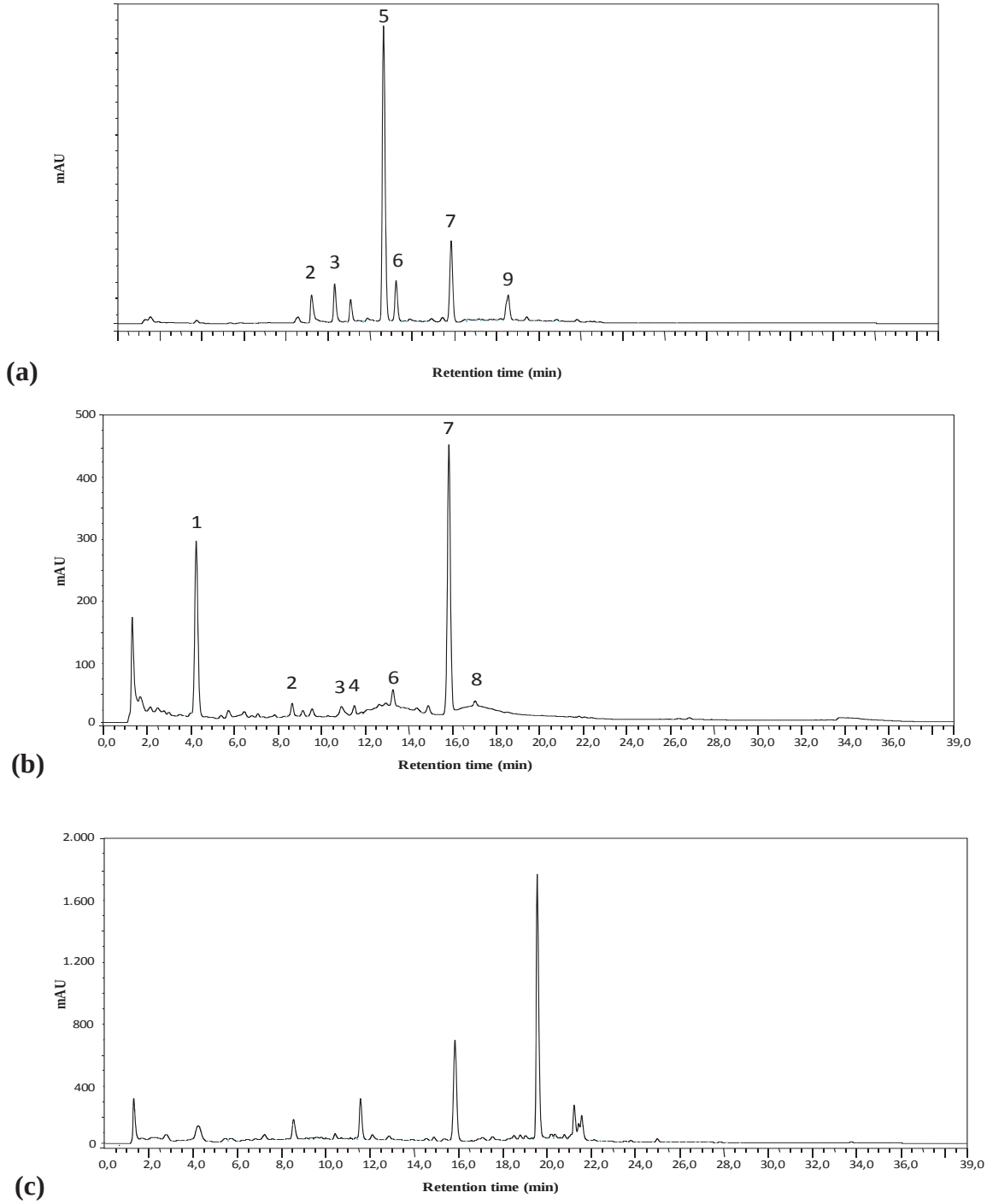


Figure 4.23. HPLC chromatograms of methanolic extract from **(a)** potato peels **(b)** sugar beet pulp and **(c)** sesame cake. Identification: peak 1, unknown; peak 2, unknown; peak 3, hydroxybenzoic acid; peak 4, vanillic acid; peak 5, chlorogenic acid; peak 6, caffeic acid; peak 7, *p*-coumaric acid; peak 8, sinapic acid; peak 9, trans-*o*-hydroxycinnamic acid; peak 10, unknown, and peak 11, cinnamic acid.

Chlorogenic acid was detected to be the major phenolic component in methanolic and ethanolic extracts of potato peels, contributing about 48.65% and 48.34% to the total amount, respectively. Caffeic, 4-hydroxybenzoic, *p*-coumaric, and trans-*o*-hydroxycinnamic acids were also predominant in both methanolic and ethanolic extracts of potato peels. While in methanolic and ethanolic extracts of sugar beet pulp *p*-coumaric acid was detected to be the major phenolic component, contributing about 48.43% and 45.41% to the total amount, respectively. Caffeic, 4-hydroxybenzoic, sinapic, and vanillic acids were also predominant in both methanolic and ethanolic extracts of sugar beet pulp.

Figure 4.23C shows that the crude methanolic extract of sesame cake contains cinnamic, 4-hydroxybenzoic, *p*-coumaric, and vanillic acids with high area in cinnamic acid. Based on their retention time, some peaks were tentatively identified. Three significant peaks (peak 1, 2, and 10) in Figure 4.23, which were tentatively identified as protocatechuic acid, gallic acid, and sesamin respectively (Onyeneho and Hettiarachchy, 1993; Rangkadilok *et al*., 2010).

Results demonstrated that differences in methanolic extracts of potato peels, sugar beet pulp, and sesame cake phenolic composition were significantly quantitative and qualitative, where potato peels showed higher amounts in 4-hydroxybenzoic than sugar beet pulp and sesame cake. While, sugar beet pulp and sesame cake contain sinapic and cinnamic acids, respectively, which were not found in potato peels. Potato peels contains chlorogenic, trans-*o*-hydroxycinnamic acids which were not found in sugar beet pulp and sesame cake.

Methanolic and ethanolic extracts of potato peels, sugar beet pulp, and sesame cake possess similar composition. Therefore, the differences in both extracts in phenolic composition were significantly more quantitative than qualitative, where methanolic extract showed higher amount than ethanol crude extract.

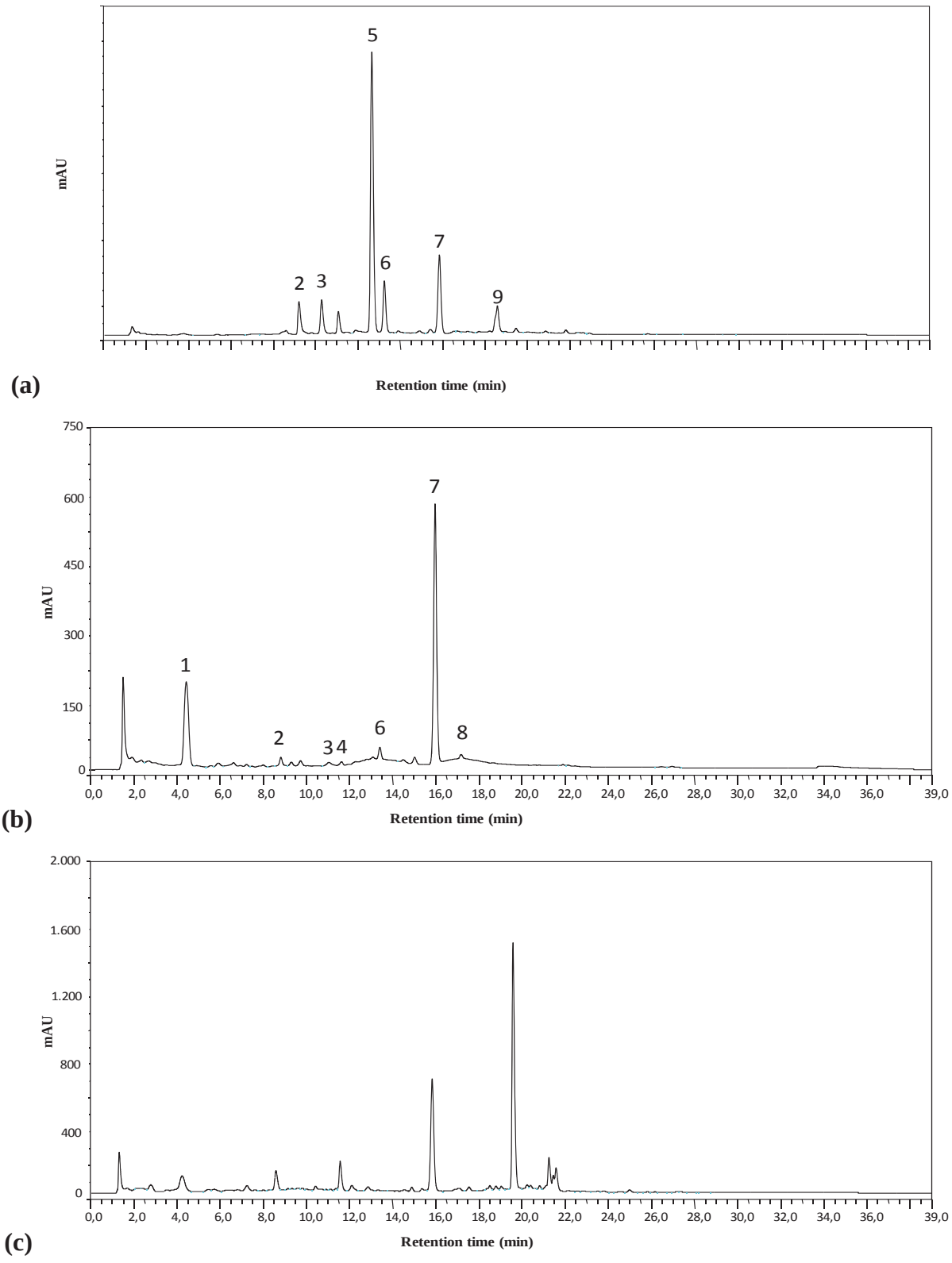


Figure 4.24. HPLC chromatograms of ethanolic extract from **(a)** potato peels **(b)** sugar beet pulp and **(c)** sesame cake. Identification: peak 1, unknown; peak 2, unknown; peak 3, hydroxybenzoic acid; peak 4, vanillic acid; peak 5, chlorogenic acid; peak 6, caffeic acid; peak 7, *p*-coumaric acid; peak 8, sinapic acid; peak 9, trans-*o*-hydroxycinnamic acid; peak 10, unknown, and peak 11, cinnamic acid.

The antioxidant activity of phenolic compounds is affected by their chemical structure. Structure-activity relationships have been used as a theoretical method for predicting antioxidant activity and are studied by Hudson and Lewis (1983), Ogata *et al*. (1997), and Saint-Cricq de Gaulejac *et al*. (1999). Polymeric polyphenols are more potent antioxidants than simple monomeric phenolics. Also the antioxidant effect depends on the number and position of hydroxyl and methoxyl groups in the benzene ring and on the possibility of electron delocalization in the double bonds (Xiang and Ning, 2008). Vanillic acid, which was not found in potato peels, is hindered phenols, since the -OCH_3 group ortho or meta to the hydroxyl group suppresses antioxidant activity. This steric hindrance is likely responsible for the relative ineffectiveness of vanillic. In addition, the caffeic acid, which was found in potato peels and sugar beet pulp but not in sesame cake, having two -OH groups at adjacent positions, acts as a chelator for most of the metal ions that act as pro-oxidants and that may catalyze the reaction even if present in trace amounts. For those reasons, all experiments demonstrated that the extracts prepared from potato peels had the highest antioxidant activity, followed by those prepared from sugar beet pulp, while sesame cake extracts were less effective

Previous studies reported that phenolic compounds such as hydroxybenzoic, chlorogenic, sinapic and *p*-coumaric acids containing significant antioxidant activities (Zhang *et al*., 2009). The above mentioned HPLC results indicate that such phenolic rich extracts from potato peels, sugar beet pulp, and sesame cake may inhibit the oxidation of vegetable oils. Isolation and characterisation of such extracts may be useful in developing natural antioxidants.

5 CONCLUSIONS

As a continuation of our study on potato peels, sugar beet pulp, and sesame cake, extracts of these by-products were prepared using different organic solvents, and *in vitro* antioxidant activity of each extract was investigated. The various extracts showed varying degrees of antioxidant activity in different test systems in a dose-dependent manner. Furthermore, the pattern of activity of the extracts within the assays also differed. As observed, extracts with higher antioxidant capacity also had higher polyphenol content. It can be concluded that the extracts obtained using higher-polarity solvents were more effective radical-scavengers than those obtained using lower-polarity solvents. Methanol showed slightly better characteristics than ethanol as a solvent for phenolic compounds, flavonoids, flavonols, and antioxidant activity but the differences were not large, so, for use in the food industry, ethanol would be a more appropriate solvent. Furthermore, it is notable that potato peels extracts exhibited the strongest antioxidant capacity in all assays used, followed by sugar beet pulp and sesame cake extracts.

From the present study, it can be concluded that potato peels, sugar beet pulp, and sesame cake can stabilize both sunflower and soybean oils very effectively at all concentrations. They inhibit thermal deterioration of oil by improving its hydrolytic stability, inhibiting double bond conjugation and reducing the losses of polyunsaturated fatty acids. Potato peels extract at concentration of 100 and 200 ppm and sugar beet pulp and sesame cake extracts at 200 ppm have stabilization efficiency comparable to commonly-employed synthetic antioxidants BHT and BHA at their legal limit, but less effective than the synthetic antioxidant TBHQ. Potato peels extract has a strong antioxidative effect during initial and final steps of oxidation in the dark in an oven at 70 °C for 72 h followed by sugar beet pulp and sesame cake extracts. Therefore, potato peels, sugar beet pulp and sesame cake could be recommended as potent sources of antioxidants for the stabilization of food systems, especially unsaturated vegetable oils and they could be used as preservative ingredients in the food and/or pharmaceutical industries provided that any resulting organoleptic effects were acceptable. However, further research is required before such use can be proposed with confidence. Eight different individual phenolic compounds were identified in the studied samples by HPLC. The phenolic compounds appear to be responsible for the antioxidant activity of potato peels, sugar beet pulp, and sesame cake, although further studies are required to reveal whether they contain other antioxidative

constituents. In addition, *in vivo* evidence and isolation of antioxidant components in potato peels, sugar beet pulp, and sesame cake merit further investigation to evaluate their potential benefits.

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