ALMOND AS A SOURCE OF NATURAL ANTIOXIDANTS

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BY

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ABSTRACT

Antioxidant efficacy of defatted almond whole seed, brown skin and green shell cover were investigated using a cooked comminuted pork model system. The inhibition of 2-thiobarbituric acid reactive substances (TBARS), total volatiles and hexanal by defatted almond at 2% (w/w) during a 7- day storage period ranged from 8-88, 38-90 and 57-90%, respectively. Since defatted almond products exhibited antioxidant properties, their crude extracts in ethanol were prepared at 70°C for 30 min. The total phenolic contents of ethanolic extracts of brown skin and green shell extracts were 10- and 9-times higher than that of the whole seed. Antioxidant activities of whole seed extract (WSE), brown skin extract (BSE) and green shell extract (GSE) of almond were evaluated using Trolox equivalent antioxidant capacity (TEAC) assay, β-carotene-linoleate model and bulk stripped corn oil systems. Furthermore, inhibition of DNA scission and human low-density lipoprotein (LDL) oxidation by WSE, BSE and GSE was monitored. Different free radical trapping assays were used to investigate the free radical-scavenging activity of the extracts. TEAC assay revealed that the antioxidant capacities of BSE and GSE were 13- and 10- times greater than that of seed extracts at the same extract concentration. These extracts were then tested at 100 and 200 ppm phenolics as quercetin equivalents. Retention of β-carotene in a β-carotene-linoleate model system by WSE, BSE and GSE was 84-96, 74-83 and 71-93% respectively, as compared to 2% retention in the control. In a bulk stripped corn oil system, GSE performed better than BSE and WSE in inhibiting the formation of both primary and secondary oxidation products. In a cooked comminuted pork model system GSE and BSE inhibited formation of TBARS, total volatiles and hexanal more effectively than WSE. The scavenging activity of the superoxide radical by almond seed, skin and shell

cover extracts at 100 and 200 ppm was 76, 89 and 97% (100ppm), and 85, 95 and 99% (200 ppm), respectively. The corresponding reduction of hydrogen peroxide concentration was 59, 63 and 66% (100 ppm), and 86, 91 and 91% (200 ppm), respectively. The hydroxyl radical-scavenging capacities at 100 and 200 ppm were 16 and 42% for WSE, 57 and 100% for BSE and 40 and 56% for GSE, respectively. A 100% scavenging activity of the DPPH radical was achieved by both BSE and GSE at 100 and 200 ppm levels. WSE scavenged 21 and 73% of the DPPH radical at 100 and 200ppm, respectively. A total DNA retention was given by GSE at 50 ppm level against peroxyl-induced strand scission, whereas BSE and WSE reached the same at 100 ppm. On the other hand, for hydroxyl radical induced DNA strand scission, a total protection was exerted by all three almond extracts at 50 ppm level against both non-site specific and site-specific strand scission. BSE performed effectively in preventing copper-induced oxidation of human LDL cholesterol compared to WSE and GSE. All three almond extracts exhibited excellent metal chelating efficacies. High performance liquid chromatographic (HPLC) analysis revealed the presence of quercetin, isorhamnetin, quercitrin, astragalin, kaempferol-3-O-rutinoside, isorhamnetin-3-O-β-D-glucoside and morin as the major flovonoids, and caffeic, ferulic, *p*-coumaric, sinapic and gallic acids as the major phenolic acids in all three almond extracts.

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LIST OF ABBREVIATIONS

ANOVA	-Analysis of variance
AOAC	-Association of Official Analytical Chemists'
AOCS	-American Oil Chemists' Society
BHA	-Butylated hydroxyanisole
BHT	-Butylated hydroxytoluene
CD	-Conjugated dienes
DMPO	-5,5-Dimethyl-1-pyrrole-N-oxide
DNA	-Deoxyribosenucleic acid
DPPH	-2,2-Diphenyl-1-picrylhydrazyl
EDTA	-Ethylenediaminetetraacetic acid
EPR	-Electron paramagnetic resonance
FDA	-Food and Drug Administration
FAO	-Food and Agriculture Organization
GC	-Gas chromatography
HPLC	-High performance liquid chromatography
HS	-Headspace
IR	-Infrared
LDL	-Low-density lipoporotein
MA	-Malonaldehyde
MS	-Mass spectrometry
NADPH	-Nicotinamide adenine dinucleotide phosphate
nm	-Nanometer
NMR	-Nuclear magnetic resonance
PG	-Propyl gallate

ppm	-Parts per million
PV	-Peroxide value
R^2	-Coefficient of determination
ROS	-Reactive oxygen species
SAS	-Statistical Analysis System
SOD	-Superoxide dismutase
TBA	-2-Thiobarbituric acid
TBARS	-Thiobarbituric acid-reactive substances
TBHQ	-Tertiarybutyl hydroquinone
TCA	-Trichloroacetic acid
TEAC	-Trolox equivalent antioxidant capacity assay
UV	-Ultraviolet
USDA	-United States Department of Agriculture
V/V	-Volume by volume
WOF	-Warmed-over flavour
W/V	-Weight by volume
W/W	-Weight by weight

CHAPTER 1
INTRODUCTION

Living organisms depend on biological oxidation as a source of energy for survival and activity. However, the same oxygen species involved in biological oxidation can also cause functional damage in the cellular components by triggering mutagenesis, carcinogenesis, circulatory disturbance and aging (Halliwell and Gutteridge, 1999). Biological antioxidative enzymes such as superoxide dismutase, catalase, and glutathione peroxidase neutralize active oxygen species. In addition, food-derived natural antioxidants such as tocopherols, ascorbic acid and carotenes are found to suppress oxidative activities (Halliwell and Gutteridge, 1999; Cadenas, 1998).

Butylated hydroxytoluene, butylated hydroxyanisole, tertiary-butylhydroquinone and propyl gallate are among the most widely used synthetic antioxidants, which are effective in various foods and are highly stable and low in cost. However, the safety concern of consumers regarding the cancer promotion activity of synthetic antioxidants has made the food manufacturers to seek effective natural sources of antioxidants as alternatives (Nakatani, 1997; El- Alim *et al.*, 1999; Wang *et al.*, 1998).

Phenolic compounds, commonly found in both edible and non-edible plant parts, have multiple biological effects, including antioxidant activity (Lai *et al.*, 1991; El- Alim *et al.*, 1999). Crude extracts of fruits, herbs, nuts, vegetables, cereals, and other plant materials rich in phenolic compounds have been reported to possess beneficial health effects and are known to retard lipid oxidation in order to improve nutritional value of food (Kikuzaki and Nakatani, 1989; Cuvelier *et al.*, 1994; Kahkonen *et al.*, 1999). The

importance of antioxidant constituents of plant materials in protection against coronary heart disease and cancer has received much attention in recent years (Iwamoto *et al.*, 2000; Edwards *et al.*, 1999; Craig, 1997; Ito *et al.*, 1998; Ide and Lau, 2001; Dong *et al.*, 2001).

Nuts are known as a source of nutritious food with high lipid content. Recent studies have revealed the presence of beneficial bioactive compounds in nuts (Spiller *et al.*, 1998; Iwamoto *et al.*, 2000). It has been demonstrated that various nuts and hulls possess antioxidant properties (Yurttas *et al.*, 2000; Moure *et al.*, 2001; Quinn and Tang 1996) but very little is known about the antioxidant activity of almond and its constituents. Almond, scientifically known as *Prunus dulcis* (Mill.) D.A. Webb, belongs to the family *Rosaceae* and is related to stone fruits such as peaches, plums and cherries. The world production of almond is 1,447,378 MT (FAO, 2000) and California is the main supplier at present. Canada imported 6.95 million kg of almond (fresh or dried, shelled, and peeled), during the period of 1993-1997 (Global Agribusiness information Network, 2000). Almond seed with or without its brown skin is consumed as the whole nut or used in various confectioneries and chocolates while its inedible counterparts are discarded or used as livestock feed (Takeoka, 2000). Apart from its nutritional value, almond is reported to have beneficial effects on blood cholesterol and lipoprotein profiles (Spiller *et al.*, 1998). It is evident that almond and its components would be worth investigating, as there is little information available on the properties of their phytochemicals and antioxidative activities. Studies on the evaluation of the antioxidant efficacies of almond components would pave the way for further investigations of their use in different food and specialty applications.

The objectives of this study were to investigate the antioxidative capacities of almond seed as such and its components, brown skin, hard shell and outer green shell cover. This would be achieved by employing free radical trapping-assays and other model systems such as bulk oil, cooked meat and β-carotene linoleate model systems; testing the activities in preventing DNA damage and LDL oxidation to gain more insight to the behaviour of these components in biological systems; examining and identifying the phenolic compounds present in the extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipid oxidation in biological systems

Oxidation of lipids is a common and undesirable chemical change that may impact flavour, aroma, nutritional quality and texture of the foods, and may also lead to the production of toxic compounds. Products of lipid oxidation are found to disturb many vital biological reactions, and it is proven that free radicals and reactive oxygen species (ROS) participate in tissue injuries and the onset and progression of degenerative diseases in human.

The lipids involved in oxidation are primarily unsaturated fatty acids such as oleic, linoleic, linolenic as well as other long-chain polyunsaturated fatty acids. The rate of oxidation of these fatty acids increases with the degree of unsaturation. The cell membranes contain polyunsaturated fatty acids, which are highly susceptible to oxidation (Halliwell and Gutteridge, 1999). Lipid oxidation could take place *via* photosensitised oxidation, hydrolytic oxidation, both enzymatic and non-enzymatic, thermal oxidation, and autoxidation as discussed in the following sections.

2.1.1 Thermal oxidation

This type of oxidation takes place when fats and oils are subjected to high temperatures (as in deep fat frying). The breakdown products during thermal oxidation include volatile and non-volatile decomposition products, which affect the flavour of the oil and fried foods (Chang *et al.*, 1978). The presence of low-molecular-weight materials

such as aldehydes, lactones and pyrazines influence the flavour of deep fried foods but the presence of high-molecular-weight materials contribute to the overall deterioration of both the frying fat and the fried product (Perkins, 1992).

2.1.2 Hydrolytic oxidation

Hydrolytic oxidation or rancidity is due to the reaction of lipid and water in the presence of a catalyst or by the action of enzymes that gives rise to free fatty acids and salts of free fatty acids. This is commonly found in fats containing short and medium chain fatty acids.

2.1.3 Photosensitised oxidation

Photosensitised reaction, which is initiated by a sensitiser and light, is another common pathway of oxidation. Singlet oxygen can be generated mainly by photosensitization of natural pigments such as chlorophyll-a, pheophytin-a and heme compounds (Nawar, 1996). Two pathways have been proposed for photosensitised oxidation (Chan, 1977). In type I, the sensitiser after absorbing light, reacts with the substrate (A) to form intermediates that may thereafter react with ground state (triplet) oxygen to yield oxidation products (Reaction 1 and 2). In type II, molecular oxygen reacts with the sensitiser after light absorption and gives rise to oxidation products (Reaction 3 and 4). The products of photosensitised oxidation include both non-conjugated and conjugated diene hydroperoxides, compared to free radical oxidation which produces only conjugated diene hydroperoxides (Rawls and van Saten 1970).

Type I

$$\text{Sens} + A + h\nu \longrightarrow \text{Intermediate-1} \quad (1)$$

$$\text{Intermediate-1} + O_2 \longrightarrow \text{Products} + \text{Sens} \quad (2)$$

Type II

$$\text{Sens} + O_2 + h\nu \longrightarrow \text{Intermediate-2} \quad (3)$$

$$\text{Intermediate-2} + A + O_2 \longrightarrow \text{Products} + \text{Sens} \quad (4)$$

2.1.4 Autoxidation

Autoxidation is the natural process that takes place between molecular oxygen and unsaturated fatty acids in the environment and involves three steps of initiation, propagation and termination. The production of the first free radicals in the initiation step needs an activation energy of about 35 kcal, hence initiators such as metal catalysts, exposure to light, singlet oxygen, pigments such as chlorophylls and myoglobin (Nawar, 1996), heat (Min, 1998) and UV radiation (Schaich, 1980) are required (Reaction 5). Once the reaction is initiated, a hydrogen atom is removed from the methylene group next to the double bond of allylic position of an unsaturated fatty acid to form free radicals. Thereafter, the reaction is propagated by further abstraction of hydrogen atoms from other unsaturated fatty acids (Reactions 6 and 7). Although the reaction of triplet state oxygen with single state lipid (RH) is thermodynamically unfavourable, the free radicals ($R^{\bullet}$) can react with it to form peroxyl radicals ($ROO^{\bullet}$) (Min, 1998). During the formation of hydroperoxides, a shift in the position of double bond could be observed for resonance stabilization. Moreover, the number of double bonds has an effect on the removal of hydrogen from the unsaturated fatty acids and the formation of hydroperoxide isomers (Min, 1998). Two pathways are known to form lipid peroxides *in vivo*. One

occurs through autoxidation of catecholamine, thiols, quinones and redox reactions of oxyhemoglobin and myoglobin, and other by the action of xanthine oxidase, NADPH oxidase, and other enzymes (Namiki, 1990). The chain reactions may be terminated by formation of non-radical products (Reactions 8-10) and the termination steps are found to be active in low oxygen concentrations.

Initiation:

$$RH \xrightarrow{\text{Initiator}} R^{\bullet} + H^{\bullet} \qquad (5)$$

Propagation:

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \qquad (6)$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \qquad (7)$$

Termination:

$$R^{\bullet} + R^{\bullet} \longrightarrow RR \qquad (8)$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR \qquad (9)$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \qquad (10)$$

2.2 Factors affecting lipid oxidation

Food systems contain numerous non-lipid components that affect oxidation of their lipids components. The number, position and geometry of the double bonds of unsaturated fatty acids affect the rate of lipid oxidation (Nawar, 1996). Apart from this, the type of food system, moisture content, fatty acid composition, partial pressure of

oxygen, storage conditions and molecular orientation affect the rate of lipid oxidation (Belitz and Grosch, 1987). The major factors will be discussed in the following sections.

2.2.1 Role of enzymes in oxidation of lipids

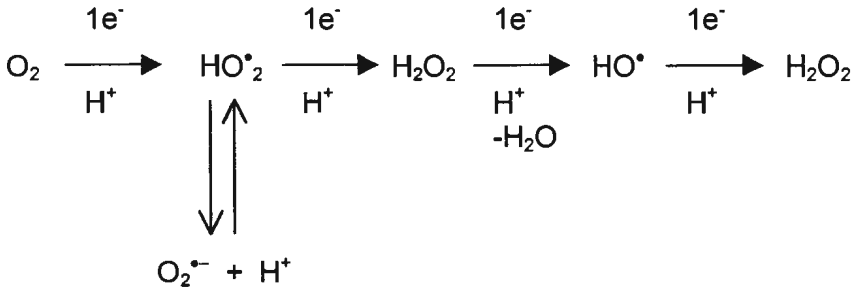
Enzyme activity commences with the lipolysis reaction; the released unsaturated fatty acids are oxidized by lipoxygenases (LO) (Gardner, 1980). Lipoxygenases that are a group of non-heme iron deoxygenases catalyse the formation of lipid hydroperoxides from unsaturated fatty acids (Hammarberg, 2001). Lipoxygenases are present in both plants and animals and have shown to be both regiospecific and stereospecific (Nawar, 1996). These enzymes initiate lipid oxidation even under low humidity conditions (Gardner, 1998). Selenide has shown strong inhibitory effects on the enzyme due to its ability to change its active site (Hammarberg, 2001). The enzyme 15-LO is considered to contribute to the formation of oxidized lipids in atherosclerotic lesions (Cathcart and Folick, 2000). These results gain further support from the studies of Harats *et al.* (2000) on over-expression of 15-LO in vascular walls showing acceleration of atherogenesis.

2.2.2 Role of oxygen and oxygen species in lipid oxidation

Triplet oxygen can give rise to singlet oxygen, which is more electrophilic than triplet state oxygen and hence reacts rapidly with unsaturated compounds. Singlet oxygen may be formed by chemical, enzymatic, photochemical and physical methods, and could initiate the oxidation of food lipids. Singlet oxygen is known to react with lipids according to an "ene" addition mechanism to produce hydroperoxides (Foote, 1976), whereas this reaction is forbidden for triplet oxygen (Rawls and van Santen, 1970). β-Carotene is a potent quencher of singlet oxygen (Krinsky, 1989; Yanishlieva *et al.*,

1998). Aerobic organisms are susceptible to the damaging actions of superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) that are formed during the metabolism of oxygen, especially during the reduction of triplet oxygen by the electron transfer system of mitochondria. The above three species and, nitric oxide ($NO^{\bullet}$), ferryl ion ($Fe^{4+}O$), periferryl ion ($Fe^{5+}O$), hypochlorite ion (ClO^-) together with unstable intermediates in the oxidation of lipids, are referred to as reactive oxygen species (ROS).

Cells generate energy aerobically by reducing molecular oxygen (O_2) to water in which the cytochrome c oxidase-catalysed reactions and flavin enzymes are involved. Transfer of four electrons to oxygen (Reaction 11) is the mechanism by which $O_2^{\bullet-}$ is generated. This is an important protective mechanism in activated phagocytes in animals, which results in killing of bacteria (Halliwell and Gutteridge, 1999). Superoxide dismutase (SOD) is responsible for the removal of $O_2^{\bullet-}$ by converting it to H_2O_2 (Reaction 12) that is less toxic to tissues compared to $O_2^{\bullet-}$.



(11)

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (12)$$

Hydrogen peroxide, present in aerobic cells as a metabolite in low concentrations, is generated by non-enzymatic and superoxide dismutase-catalysed

dismutation reactions (Kanner, 1992). Hydrogen peroxide is also the substrate for two enzymes; catalase and glutathione peroxidase, that catalyse the conversion of H_2O_2 to water and oxygen, thus presumably serves as a detoxification mechanism. Moreover, H_2O_2 is also converted by myeloperoxidase in neutrophils to hypochlorous acid (Halliwell and Gutteridge, 1999). This appears to be a mechanism for a physiological toxic agent, since hypochlorous acid is a strong oxidant that acts as a bactericidal agent in phagocytic cells. The biologically significant reaction of H_2O_2 is its spontaneous conversion, catalysed by Fe^{2+} (Fenton reaction), to the highly reactive $HO^{\bullet}$ that reacts instantaneously with any biological molecule (BH) from which it can abstract a hydrogen atom. The resulting free radical ($B^{\bullet}$) is more stable and hence longer-lived than $HO^{\bullet}$. Hydroxyl radical could also be generated by exposing water to high-energy ionising radiation (Halliwell and Gutteridge, 1999).

2.2.3 Role of metal ions in lipid oxidation

Transition metal ions (iron, cobalt, manganese, copper and nickel) having two or more valency states are found to act as pro-oxidants even at 0.1 ppm concentration (Nawar, 1996). Zinc does not promote radical reactions due to its mono valency state but rather acts as an inhibitor by displacing other transition metal ions from their binding sites (Halliwell and Gutteridge, 1999). Metal catalysed oxidation can take place by acceleration of hydroperoxide decomposition, direct reaction with the unoxidized substrate and activation of molecular oxygen to give rise to singlet oxygen and peroxyl radical (Nawar, 1996) as given in Reactions (13-16). The metal activity is enhanced in organic media (Uri, 1961).

Acceleration of hydroperoxide decomposition

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + OH^- \quad (13)$$

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + H^+ + ROO^\bullet \quad (14)$$

Direct reaction with the unoxidized substrate:

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + H^+ + R^\bullet \quad (15)$$

Activation of molecular oxygen to give singlet oxygen and peroxyl radical:

$$M^{n+} + RH \longrightarrow M^{(n+1)+} + O_2^- \begin{cases} \nearrow {}^1O_2 \\ \searrow {}^\bullet HO_2 \end{cases} \quad (16)$$

Iron is one of the most important biocatalysts, and free iron found in cells originates from ferritin, which is the major iron storage protein in cells (Kanner, 1992). This iron, together with what is present in myoglobin, hemoglobin and transferrin may cause lipid oxidation in muscle tissues (Love, 1987; Wettasinghe and Shahidi, 1996). Ferrous ions in aerobic aqueous solutions could produce $O_2^{\bullet-}$, H_2O_2 and $HO^\bullet$ commonly known as "Fenton" reaction (Reactions17-19). These reactions when cycled by $O_2^{\bullet-}$ and reducing agents are called as "Haber-Weiss" reactions and redox-cycle, respectively. Ferrous ion is found to stimulate lipid peroxidation by generating $HO^\bullet$ and by breaking down lipid peroxides to form alkoxyl radicals ($RO^\bullet$) (Kanner, 1992).

$$Fe^{+2} + O_2^{\bullet-} \longrightarrow Fe^{+3} + O_2 \quad (17)$$

$$2O_2 + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (18)$$

$$Fe^{+2} + H_2O_2 \longrightarrow HO^\bullet + HO^- + Fe^{+3} \quad (19)$$

2.2.4 Role of ionising radiation in lipid oxidation

Ionising radiation (X- and γ-rays, high energy electrons and particles) generates free radicals *via* ionisation of molecules, and the distribution of ionising radiation interaction within a system is proportionate to the electron density fraction of constituent molecules (Simic *et al.*, 1992). Ionised radiation leads to radiolysis of water yielding hydrogen atoms, hydroxyl radicals and hydrated electrons that can accelerate lipid oxidation (Scott, 1997).

2.3 Products of lipid oxidation

Off-flavour development due to lipid oxidation can take place in all types foods such as raw, cooked, cured, uncured, frozen meat and meat products (Dupuy *et al.*, 1987; St. Angelo *et al.*, 1987; Ang and Lyon, 1990; Shahidi and Pegg, 1994a), milk (Neilsen *et al.*, 1985), or edible oils (van Ruth *et al.*, 1999, Frankel and Huang, 1996a). Studies have been conducted to identify the compounds responsible for these rancid odours.

Lipid oxidation in muscle foods has attracted much attention due to the rapid deterioration of sensory qualities during refrigeration of cooked meat (Bailey, 1988). The development of characteristic warmed-over flavour (WOF) is the immediate result in lipid oxidation in cooked meat (Tim and Watts, 1958). The main components responsible for the WOF in cooked meat were reported by Dupuy *et al.* (1987) as propanal, pentanal, hexanal, 2,3-octanedione, nonal, heptanal, octanal, tetradecane and phenyl acetaldehyde. Oxidation of lipids in Milano salami produced hexanal, heptanal, 1-octen-3-ol and decanal which produced off flavour notes (Meynier *et al.*, 1999). Kansci *et al.*

(1999) identified hexanol, 2-alkenals and *t*-2-undecenal in oxidation of a meat model system. Analysis of the volatile compounds formed during storage of fish oil enriched mayonnaise showed that 28 out of 78 compounds detected exerted fishy odour, and 27 of these including 1-penten-3-one, *cis*-2-penten-1-ol, *cis*-3-hexenal, *cis*-4-heptanal, 1-octen-3-one, 1,*cis*-5-octadien-3-one, 1-octen-3-ol, *trans*,*cis*-2,4-heptadienal and *trans*,*cis*-2,6-nonadienal contributed to unpleasant fishy and rancid off flavours (Hartvigen, 2000). Major volatile compound formed during copper and α-tocopherol induced oxidation in butter fat was *trans*-4,5-epoxyhept-*trans*-2-enol (Swoboda and Peers 1978).

Oxidation of pure oleic acid at 100-120°C for long periods was shown to give rise to 9,10-dihydroxystearic and monohydroxystearic acids, other oxiranes, unsaturated carbonyls and dimers. Moreover, epoxides and cleavage products such as octanoic, suberic, azelaic, nonanoic, oxalic acids and carbon dioxide and water were formed when catalysts were used (Swern, 1961). Furthermore, it was reported that uncatalysed autoxidation of methyl oleate resulted in the formation of 30-35% peroxides, 25-30% hydroxyl compounds, 20-25% oxirane compounds, 15% α,β-unsaturated carbonyl compounds, cleavage products and polymers, among others. Formation of conjugated dienes and trienes during oxidation of unsaturated fatty acids was recognized by the increase in absorbance at 234 and 268 nm, respectively (Shahidi *et al.*, 1994; Wanasundara *et al.*, 1995). The secondary oxidation products, which are formed during the breakdown of primary products, include aldehydes, ketones, hydrocarbons and alcohols. Hexanal is a major product formed during ω-6 fatty acid oxidation (Shahidi and Pegg, 1994b). The cleavage of alkoxyl radicals, formed by hydroperoxides was

described by Min (1998). The scission on the acid side gives rise to unsaturated oxo compounds and an alkyl radical while the scission of the carbon-carbon bond between the double bond and the carbon atom bearing the oxygen will form an 1-olefin radical and an alkyl oxo compound. Frankel (1985) reported the presence of hydrocarbons, furans, vinyl alcohols, 1-alkenes, 2-alkenals, alkanals, alkadienals and vinyl ketones in oxidized oils. Hexanal and pentanal have been found in oxidized vegetable oils (Abdalla and Roozen, 1999) whereas, propanal and pentenal were dominant in fish oils (Frankel and Huang, 1996b). In sunflower oil-in-water emulsions, during lipid oxidation, hexanal and conjugated hydroperoxide dienes, pentanal, 3-pentanol and 1-octen-3-one were detected (van Ruth *et al.*, 1999). In full fat and defatted soybean flakes, the major volatile compounds were identified as n-hexanal, acetaldehyde and acetone (Sessa, 1969). Polyunsaturated fatty acids in steak were found to give rise to n-alkanals, 2-alkenals, 1-alkanols and alkylfurans after cooking (Elmore *et al.*, 1999).

Carbonyl compounds formed during lipid oxidation are found to react with cellular macromolecules like proteins, DNA and RNA (Esterbauer *et al.*, 1991; Pearson and Gray, 1983). The significance of products of lipid oxidation on cellular components of living organisms and their contribution to the development of various diseases will be discussed in the following section.

2.4 Reactive oxygen species in human health

Reactive oxygen species (ROS) and other oxidants are by-products of electron transport, oxygen-utilizing systems, peroxisomes and other processes involved in normal aerobic metabolism as well as in lipid peroxidation (Ames and Shegenaga, 1993).

Although free radicals are essential in the human body at normal rate of generation, excessive production may lead to oxidative stress, which in turn would cause tissue damage (Yoshikawa *et al.*, 1997). Inflammatory cells like eosinophils, neutrophils, monocytes and macrophages become activated during inflammation and produce oxygen radicals (Granger, 1988). These are found to play an important role in asthma and bronchial hyper-responsiveness (Yoshikawa *et al.*, 1997). Rheumatoid arthritis is also believed to be initiated by these free radicals (Antila *et al.*, 1997; Lunec *et al.*, 1986).

Mutagenesis is the best-known biological effect of radiation and occurs mainly through damage of DNA either by $HO^{\bullet}$ and other species produced by radiolysis or by direct radiation effect on DNA (Namiki, 1990). Mitogenesis (induced cell division) is a major multiplier of DNA damage leading to mutation and cancer (Ames and Shigenaga, 1992). Hydroxyl, alkoxyl and singlet oxygen are the most harmful species to attack DNA while hydrogen peroxide has been found to break DNA strands (Meneghini and Martins, 1993). These authors also reported that, as Fe^{2+} is present in DNA as a counter ion, and H_2O_2, if reach the nucleus, can react with it and form $HO^{\bullet}$, which would attack the sugar or the base. Changes that take place in DNA by $HO^{\bullet}$ are mainly addition to double bond of pyrimidine base and abstraction of hydrogen from the sugar moiety resulting in scission of DNA (Namiki, 1990; Dizdaroglu, 1993). Products that arise from DNA damage are thymine glycol, 5-hydroxymethyluracil (Ames and Shigenaga, 1993), cytosine glycol, 8-hydroxypurines (Hems, 1990) and 7-hydroxypurines (Steenken, 1989). The carcinogens that are found to interact with DNA are electrophilic, thus radical intermediaries and aldehyde products of lipid oxidation are suggested to be involved in

cancer initiation (Cheeseman, 1993). The ROS not only act as promoters of carcinogenesis but also their ability to damage DNA and produce changes in gene expression imply that they could be involved in all stages of carcinogenesis (Halliwell and Aruoma, 1992).

Oxidation of proteins takes place first by the OH-dependant removal of α-hydrogen atom from any amino acid residue and then by addition of oxygen to produce alkyl peroxide and other radicals (Stadtman, 1998). Free amino acids having sulphydryl groups also can react with 2,3-unsaturated aldehydes of lipid peroxidation products (Uchida and Stadtman, 1998). Free radicals can cause polypeptide chain scission, cross-linking, oxidation and modification of amino acids, which leads to loss of biological function (Niki, 1997).

In studies conducted on human alcoholic liver disease (Peters *et al.*, 1986) it was revealed that free radical effects are not of major importance in the early and reversible stages of alcoholic liver disease though it may be of importance in the pathogenesis of alcoholic cirrhosis. Das (1997) reported that $HO^{\bullet}$ generated through the metal-catalysed Haber-Weiss reaction causes oxidative damage of the gastric mucosa, which resulted in gastric ulceration in rats. The other implications that are arising due to ROS are conditions such as smoking-related lung diseases, reperfusion injuries following surgery and organ transplantation, neurodegenerative diseases like Parkinson's disease, and radiation-induced diseases (Muggli, 1993).

2.5 Role of antioxidants in preventing ROS injury

To balance the levels of ROS and to defend the cellular components from the damage, enzymes such as superoxide dismutase, catalase and glutathione peroxidase play a major role in defence mechanisms (Ames and Shigenaga, 1992). These enzymes detoxify hydrogen peroxide and hydroperoxides as summarized in Table 2.1 to water or their corresponding alcohols. The inhibitory effects in scavenging hydroxyl, alkoxyl and specially peroxyl radical of α-tocopherol (Vitamin E) have been most extensively studied (Niki, 1997). The effectiveness of its activity depends on the trapping of peroxyl radical by α-tocopherol and, the trapping of additional peroxyl radicals by α-tocopheroxy radical (Burton and Ingold, 1986). β-Carotene is well known for its singlet oxygen scavenging capacity. Transfer of energy to β-carotene converts it to excited triplet-state quencher and singlet oxygen is converted to triplet-state oxygen, and this phenomenon is known as triplet-sensitiser quenching (Bradley and Min, 1992). Reduced form of ascorbic acid has the ability to scavenge $O^{\bullet -}$ and $HO^{\bullet}$ and, ascorbic acid is capable of regenerating α-tocopherol from α-tocopheroxy radical (Winyard *et al.*, 1994). Among the plant-derived antioxidants, flavonoids have been found to possess potent activities in scavenging ROS and reducing the risk associated with cardiovascular diseases (Blot *et al.*, 1997; Tijburg *et al.*, 1997). Plant phenolics were shown to enhance the *in vivo* activity of liver glutathione-S-transferase and NAD(P)H-quinone reductase, which are enzymes involved in detoxification of carcinogens and xenobiotics in the body (Singletary, 1996).

Table 2.1 Detoxification activities of antioxidant enzymes in vivo (Adapted from Niki, 1997)

Enzyme	Activity
Superoxide dismutase	Dismutation of $O^{\bullet -}$ to H_2O_2
Glutathione peroxidase (cellular)	Decomposition of H_2O_2 and free acid hydroperoxide
Glutahione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipid hydroperoxide
Phospholipid hydroperxide glutathione peroxidase	Decomposition of phospholipid hydroperoxide
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxide
Glutathione-S-transferase	Decomposition of lipid hydroperoxide

2.6 Control of oxidation in food systems

When the human body fails to counteract the effects of free radicals through the prevention mechanisms and antioxidant systems acting in the body, it may result in carcinogenic, atherosclerotic and other physiologically unfavourable conditions. Antioxidative compounds that are present in food or that are sometimes ingested in the tablet or capsule forms (neutraceuticals) are used to fight the effects of free radicals in the body.

To inhibit lipid oxidation in food systems, various methods have been used from antiquity. The practice of hydrogenation of unsaturated fatty acids (Nawar, 1996), removal of oxygen through vacuum packaging (Ahn *et al.*, 2000), use of superoxide scavengers such as glucose oxidase and ascorbic acid oxidase (Hsieh and Kinsella, 1989), removal or sequestering of metal ions (Kanner, 1994), application of high hydrostatic pressure (Hoover *et al.*, 1989), irradiation (Ahn *et al.*, 2001), refrigeration and freezing (Fernandez-Fernandez, 2001) and use of antioxidants are common-place (Reische *et al.*, 1998). Addition of spices or plant constituents in the powder form or as whole leaves or stems has been practiced from the very early days, though the mechanisms involved in increasing the shelf-life of foods were unknown. Research efforts directed in investigating these bioactive compounds led to the identification of their modes of action in inhibiting lipid oxidation and shelf life extension. These compounds, known as antioxidants, are defined as substances that when present at low concentrations compared to that of an oxidisable substrate, significantly delay or inhibit oxidation of that substrate (Halliwell and Gutteridge, 1999). These antioxidants are now added intentionally to foods to prevent lipid oxidation and are either synthetic or natural

in their origin. Synthetic antioxidants, which are approved for use in food, include phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butylhydroquinone (TBHQ) and non-phenolics such as erythorbic acid, ascorbic acid and ascorbyl palmitate (Frankel, 1996; Reische *et al.*,1998). Natural antioxidants include carotenoids (Wolf, 1982; Erickson, 1998), naturally occurring ascorbic acid, amino acids and dipeptides (Chen *et al.*, 1995; Erickson, 1998), protein hydrolysates (Amarowicz and Shahidi, 1997), water soluble proteins (Okada and Okada, 1998), phospholipids (Bandarra *et al.*, 1999), inorganic salts (Wettasinghe and Shahidi, 1996), tocopherols and their derivatives (Lampi *et al.*, 1997; Sies and Stahl, 1995; Byres, 1993), antioxidant enzymes (Lee *et al.*, 1996; Jarvinen *et al.*, 2000) and a large number of phenolic compounds of edible and non-edible plant parts.

Antioxidants present in food commodities provide protection against oxidative attack by decreasing oxygen concentration, intercepting singlet oxygen, preventing first-chain initiation by scavenging initial radicals, binding metal ion catalysts, decomposing primary products to non-radical compounds, and chain breaking to prevent continuous hydrogen removal from substrates (Shahidi, 1997). Natural antioxidants maybe depleted during processing of foods. Food producers use antioxidants derived from natural or synthetic sources in order to extend shelf-life and improve the quality of processed foods. The antioxidants used in foods should be non-toxic, inexpensive, effective at low concentrations and stable through various processing conditions and impart a minimum effect on colour, odour and flavour of the food (Reische *et al.*, 1998).

2.6.1 Mechanisms of antioxidant action

Antioxidants can be classified as primary or chain breaking antioxidants, secondary or preventive antioxidants and multiple-function antioxidants having more than one mechanism of activity. These are discussed in the following sections.

2.6.1.1 Primary antioxidants

Primary antioxidants could react with lipid ($R^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals and convert them to more stable, antiradical products. This is due to the ability to donate hydrogen atoms to lipid radicals and produce lipid derivatives and antioxidant radicals ($A^{\bullet}$). The antioxidant radicals also participate in termination reactions of $ROO^{\bullet}$, $RO^{\bullet}$ and other antioxidant radicals, thus preventing propagation of the chain reactions. The interactions, which could take place between antioxidants and free radicals, are shown in Reactions 20-25. These reactions are exothermic in nature and therefore, the activation energy would increase with increasing A-H bond dissociation energy (Uri, 1961). The stability of $A^{\bullet}$ is brought about by the delocalisation of the unpaired electron around the phenol ring to form stable resonance hybrids (Decker, 1998). The effectiveness of hydrogen donating ability of phenolic antioxidants is enhanced when alkyl groups are present in the *ortho* and *para* positions. Moreover, presence of another hydroxyl group in any of these positions stabilizes the phenoxy radical through an intramolecular hydrogen bond (Shahidi and Wanasundara, 1992).

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet} \qquad (20)$$

$$RO^{\bullet} + AH \longrightarrow ROH + A^{\bullet} \qquad (21)$$

$$R^{\bullet} + AH \longrightarrow RH + A^{\bullet} \qquad (22)$$

$$ROO^{\bullet} + A^{\bullet} \longrightarrow ROOA \quad (23)$$

$$RO^{\bullet} + A^{\bullet} \longrightarrow ROA \quad (24)$$

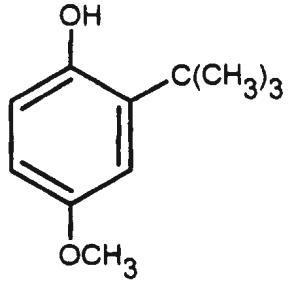
$$R^{\bullet} + A^{\bullet} \longrightarrow AA \quad (25)$$

In order to obtain the highest efficacy, the antioxidant should be added during the induction and initiation stage of oxidation. Though the primary antioxidants are capable of reducing hydroperoxides to hydroxyl compounds, the main mechanism is radical scavenging (Reische *et al.*, 1998). The effectiveness of an antioxidant depends on the activation energy, rate constants, oxidation-reduction potential, ease of antioxidant loss or destruction, and solubility properties (Nawar, 1996). The most commonly used primary antioxidants in foods are synthetically derived BHA, BHT, PG and TBHQ. BHA is a 9:1 mixture of 2- and 3- isomers (2-BHA and 3-BHA) (Kikugawa *et al.*, 1990) and 3-BHA is a better antioxidant than 2-BHA (Shahidi and Naczk, 1995). Figures 2.1 and 2.2 depict the structures of these antioxidants and the generalized mechanism of their action, respectively. Tocopherols (Figure 2.3) are the most commonly used naturally occurring primary antioxidants.

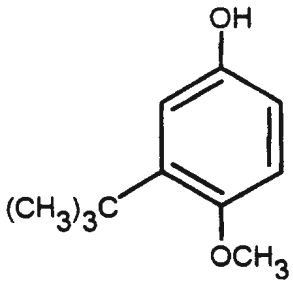
2.6.1.2 Secondary antioxidants

Secondary antioxidants, also known as synergists, enhance the activity of primary antioxidants. They do not convert free radicals to more stable products, but act through other mechanisms to provide protection against oxidation. Chelation of prooxidant metal ions, decomposition of hydroperoxides to non-radical species, deactivation of singlet oxygen, absorption of ultraviolet radiation and scavenging of oxygen are among possible mechanisms. Metals are known to interact with

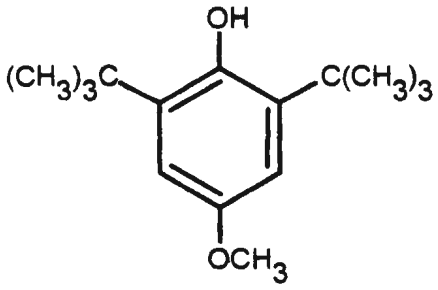
Figure 2.1 Chemical structures of synthetic antioxidants



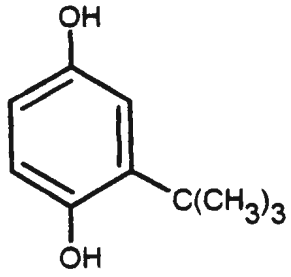
2-BHA (Butylated hydroxyanisole)



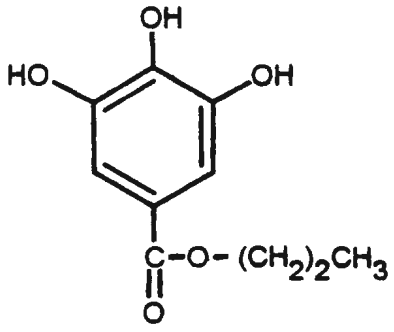
3-BHA (Butylated hydroxyanisole)



BHT (Butylated hydroxytoluene)



(TBHQ) Tertiary-butylhydroquinone



(PG) Propyl gallate

Figure 2.2 Mechanism of action of phenolic antoxidants

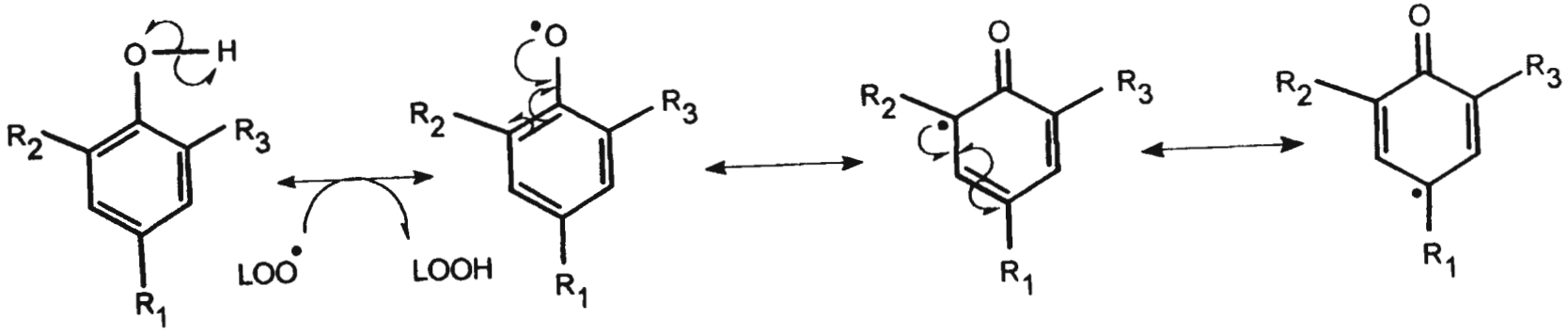
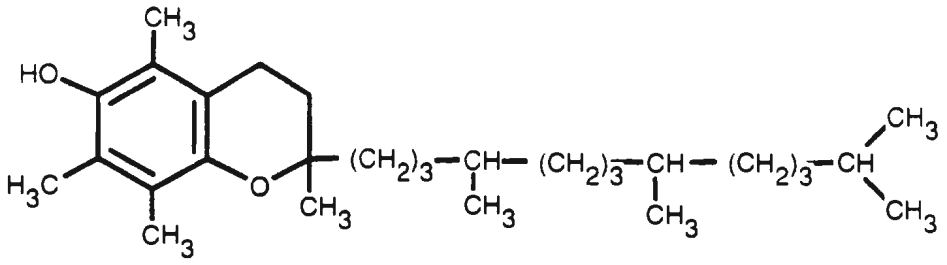
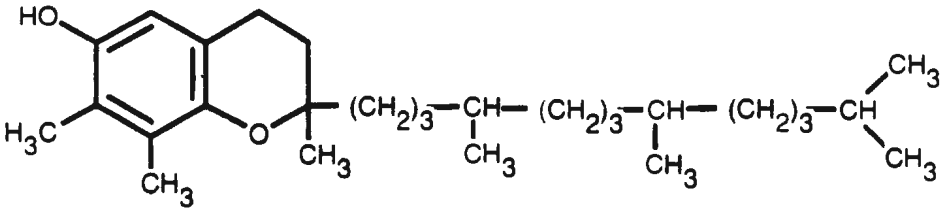

O
H
R2
R3
R1
LOO•
LOOH
•O
O
O
O

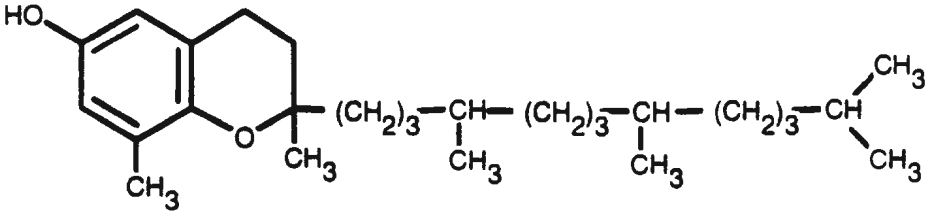
Figure 2.3 Chemical structures of tocopherols



α-Tocopherol
(5,7,8-Trimethyltocol)



γ-Tocopherol
(7,8-Dimethyltocol)



δ-Tocopherol
(8-Methyltocol)

hydroperoxides and promote oxidation, while chelators decrease the prooxidant activities of metals by reducing their redox potential and hence, stabilizing the oxidized form of metal (Reische *et al.*, 1998). Citric, malic, phosphoric, tartaric, and ethylenediaminetetraacetic (EDTA) acids are found to be good metal chelators. Carotenoids are well known for quenching singlet oxygen; other compounds that are having similar capabilities are amino acids, peptides, proteins, phenolics, urate and ascorbate (Bradley and Min, 1992). In complex food and biological systems, the antioxidants function *via* one or more of the mechanisms described above (Kinsella *et al.*, 1993).

2.6.2 Natural versus synthetic antioxidants

Butylated hydroxyanisole is a synthetic antioxidant that has been subjected to extensive testing and research to determine its potential harmful effects. It has been suggested that the sequence of administration determines the behaviour of BHA as an anticarcinogen or a carcinogen (Iverson, 1999). If administered prior to the carcinogenic initiator, it would be effective whereas, administration after the carcinogen makes it a cancer promoter. BHA has now been ruled out as a GRAS (generally recognized as safe) substance by the Food and Drug Administration (FDA) of the United States (Nieto *et al.*, 1993) though it is still permitted as a food additive (Williams *et al.*, 1999) and Canada accepts it at a maximum of 200 ppm based on the lipid content of the foods (Iverson, 1999). BHA, BHT, TBHQ and PG are permitted in foods in Canada at levels not to exceed FDA- and United States Department of Agriculture (USDA)-permitted levels resulting in a maximum total antioxidant content of 0.02% (200 ppm) of the total fat or oil content. These compounds can be used singly or in combination except TBHQ,

which is not lawful for use in combination with PG. Williams *et al.* (1999) report that BHA and BHT do not pose any cancer hazard at current levels of food additive usage and these findings are comparable to the evaluations by The International Agency for Research on Cancer (IARC). However, there is always a need for research to explore dose-response relationships of these additives in response to human health concerns (Aruoma, 1994). Despite their stability and effectiveness, the use of synthetic antioxidants as food additives is restricted in several countries due to the possibility of causing undesirable effects on enzymes in biological systems (Nakatani, 1997). This concern about safety resulted in the increased research on natural antioxidants (Stoick *et al.*, 1991; Liu *et al.*, 1992; El-Alim *et al.*, 1999). The suitability of natural antioxidants can be related to their enhanced masking of off-flavours and off-odours resulting from oxidation and, better consumer acceptability of natural ingredients (Chang *et al.*, 1977). The solubility of most of the natural antioxidants in both oil and water makes them more suitable for multicomponent food systems. However, some antioxidants impart undesirable flavours to foods, which limits their application in spite of their high antioxidant activity. Furthermore, natural is not synonymous with safety and an antioxidant from a natural source does not automatically makes it safe for consumption. The antioxidative properties of various plant materials are related to their phenolic and polyphenolic constituents (Lai *et al.*, 1991; El-Alim *et al.*, 1999). A range of low- and high-molecular-weight plant phenolics showing antioxidant properties has been proposed to retard lipid oxidation (Hagerman *et al.*, 1998). Foods rich in tannins, which are also known as polyphenols, are considered to be of low nutritional value as they have been reported to be responsible for decreases in feed intake, growth rate, feed efficiency, net metabolisable energy, and protein digestibility in experimental animals

(Serra and Ventura, 1997). However, it has also been reported that tannins exert anticarcinogenic, antimoicrobial, antioxidative and physiological effects such as acceleration of blood clotting, decrease in blood pressure and lipid serum levels, and modulation of immunoresponses (Chung *et al.*, 1998). Several studies have revealed that phenolic compounds reduce *in vitro* oxidation of low-density lipoprotein (LDL) and thereby lower atherogenesis (Meyer *et al.*, 1998; Moon and Terao, 1998). Scientific studies have proved the capabilities of antioxidants to protect cells against free radical damage (Saint-Cricq de Gaulejac *et al.*, 1999). Other beneficial activities of natural antioxidants from plant extracts are: antimicrobial (Takenaka *et al.*, 1997; Sivroupoulou *et al.*, 1996; Paster *et al.*, 1995), anti-carcinogenic (Singletary *et al.*, 1996; Offord *et al.*, 1997), anti-inflammatory (Chan *et al.*, 1996), anti-mutagenic (Kanazawa *et al.*, 1995), anti HIV-1 (Paris *et al.*, 1993; Yamasaki *et al.*, 1998), anti-ulcer (Saito *et al.*, 1998), anti-multi drug resistance (Plouzek *et al.*, 1999), anti-allergic (Noguchi *et al.*, 1999) and anti-metastasis (Maeda-Yamamoto *et al.*, 1999) activities.

2.7 Sources of natural antioxidants

The growing interest in replacing synthetic antioxidants by natural ones has brought about research on plant parts, both edible and non-edible, for identifying new antioxidants. These sources, for instance, herbs, spices, fruits, vegetables, nuts, legumes and cereals will be discussed in the following sections.

2.7.1 Herbs and spices

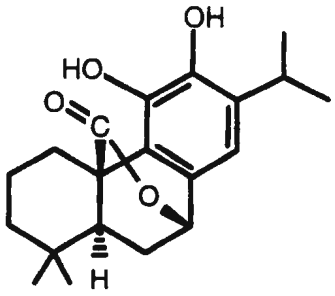
The term 'spice' is used to include all seasonings while herbs are succulent plants, which are also used in cooking, garnishing and medicinal purposes. The first

scientific study on antioxidative properties of spices was conducted by Dubios and Tressler (1943) and thereafter it has been a continuous effort in the screening of compounds having physiologically favourable effects.

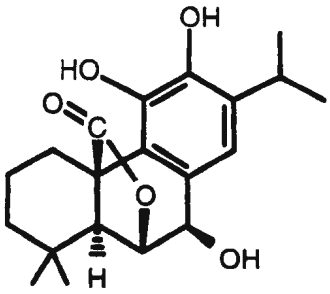
The extract of rosemary leaves has shown to possess the most effective antioxidative activity among the herbs (Chang *et al.*, 1977; Hopia *et al.*, 1996) and the main components responsible for its activity were identified as carnosic and carnosol (Bracco *et al.*, 1981). Epirosmanol and isorosmanol from rosemary extracts were found to be about four times more active than BHT and BHA in oil systems (Nakatani and Inatani, 1984). Similar effects were observed with rosmaridiphenol as well (Houlihan *et al.*, 1984), whereas carnosol and carnosic acid have shown activities higher than α-tocopherol in a bulk methyl linoleate system (Hopia *et al.*, 1996). Chemical structures of the compounds isolated from rosemary are depicted in Figure 2.4.

Carnosic acid, carnosol, rosmadial, rosmanol, epirosmanol and methyl carnosate have been found in *Salvia* or sage (Cuvelier *et al.*, 1994). Moreover, a derivative of carnosic acid (carnosic12-methyl ether-γ-lactone), rosmanol-7-ethyl ether and oleanic acid have been isolated from aerial parts of sage (Djarmati *et al.*, 1992) and 1,8-cineole, thujone, isothujone and camphor were present in sage oil (Wang *et al.*, 1998). A novel compound royleanonic acid, together with hispidulin and eupatorin, was isolated from *Salvia plebeia* R. Br., which exerted a high antioxidative activity when present in the crude extracts (Gu and Weng, 2001). The most effective compounds exerting antioxidative activities in sage are: carnosol, carnosic acid, rosmarinic acid, caffeic acid,

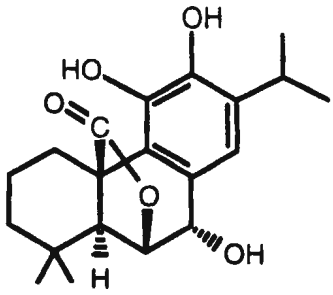
Figure 2.4 Chemical structures of rosemary antioxidants



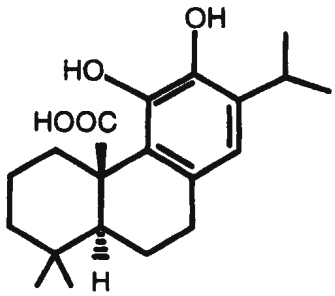
Carnosol



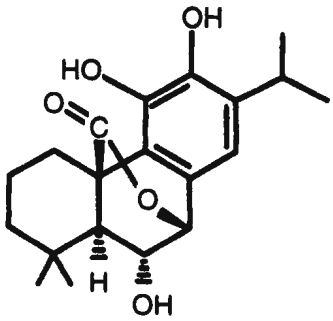
Epirosmanol



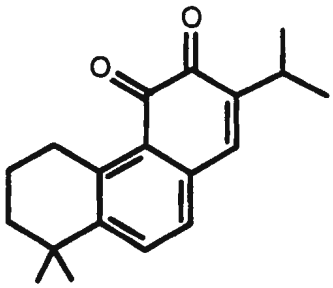
Rosmanol



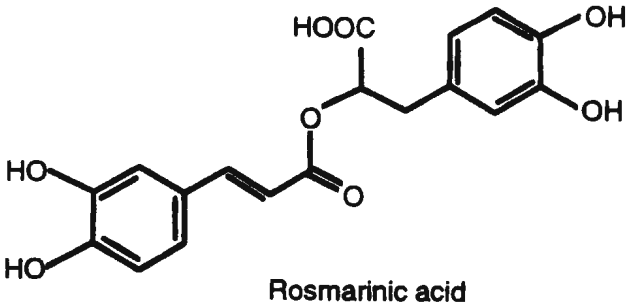
Carnosic acid



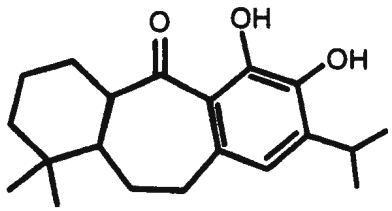
Isorosmanol



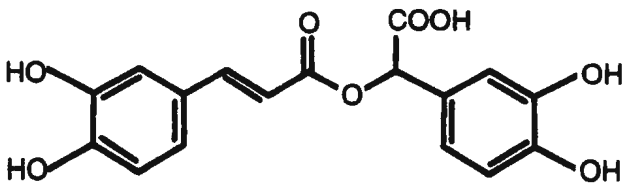
Rosmariquinone



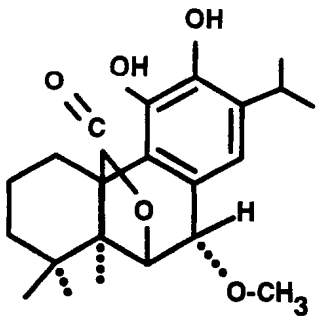
Rosmarinic acid



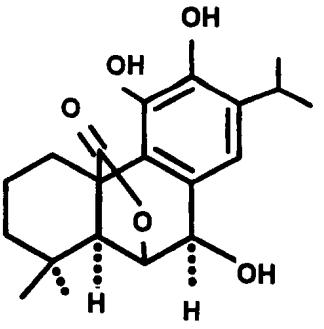
Rosmaridiphenol



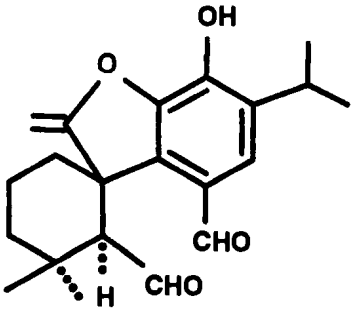
Rosmaric acid



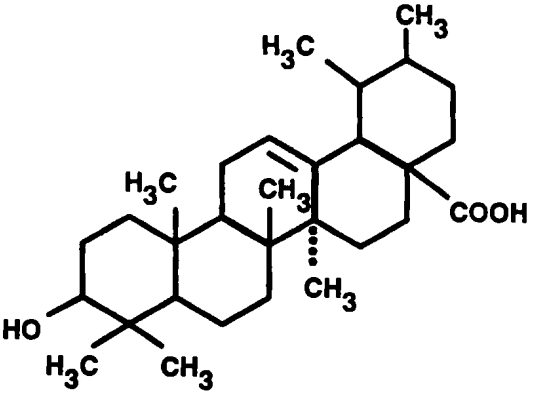
7-Methoxyrosmanol



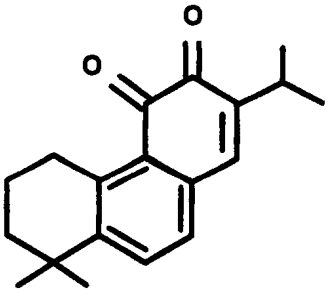
Epirosmanol



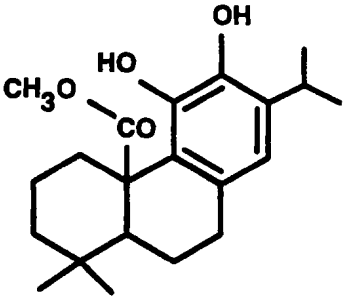
Rosmadial



Ursolic acid



Militrone



Methyl Carnosic acid

rosmanol, rosmadial, genkwanin, cirsimaritin (Cuvelier *et al.*, 1996) and luteolin-7-O-β-D-glucopyranoside (Wang *et al.*, 1998). These compounds are shown in Figure 2.5-A.

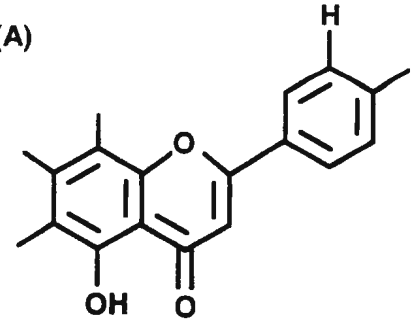
Thyme, mainly contains thymol and carvacrol in its essential oil (Rhyu, 1979; Yamaura, *et al.*, 1989). In addition, thyme essential oil contains flavonoids (Miura and Nakatani, 1989; Haraguchi *et al.*, 1996), biphenyl compounds (Nakatani *et al.*, 1989, Haraguchi *et al.*, 1996) and acetophenone glucosides (Wang *et al.*, 1999a). These compounds are depicted in Figure 2.5-B. It has been observed that thyme increased the activity of antioxidant enzymes in experimental rats (Youdim and Deans, 1999). The flavonoids of thyme showed strong antioxidative activity which was comparable to BHA (Miura and Nakatani, 1989) and p-cymene-2,3-diol was more effective than α-tocopherol and BHA in inhibiting lard oxidation (Schwarz and Ernst, 1996).

Ocimum or basil has been used in folk medicine and has been reported to possess many physiologically favourable effects. Many monoterpenoids, sesquiterpenoids and phenyl proponoids have been identified from basil extracts and oil. Linalool, methyl chavicol, and eugenol (Figure 2.6-A) have been identified as its major constituents (Karawya *et al.*, 1974; Fleisher, 1981; Grayer *et al.*, 1996)

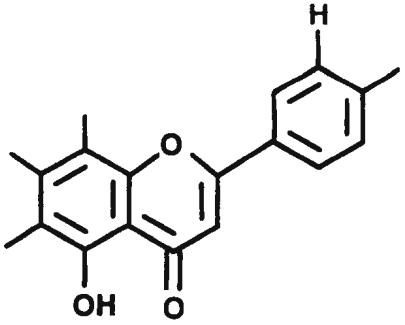
Mints, which are of the genus *Mentha*, have been shown to contain menthone, menthol, pulegeone, neoisomenthol, menthefuran, linalool, linalylacetate, carvone and dihydrocarveol (Kawabe *et al.*, 1993; Hilton *et al.*, 1995; Sivroupoulou *et al.*, 1995). Many flavonoids have been identified from dried mint leaves that exhibited antioxidant

Figure 2.5 Chemical structures of sage (A) and thyme (B) antioxidants

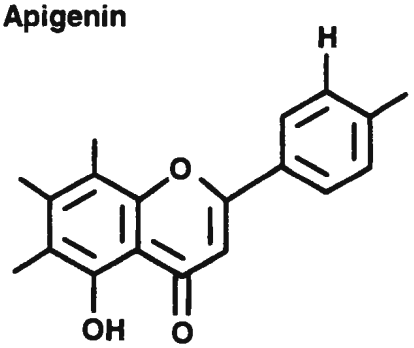
(A)



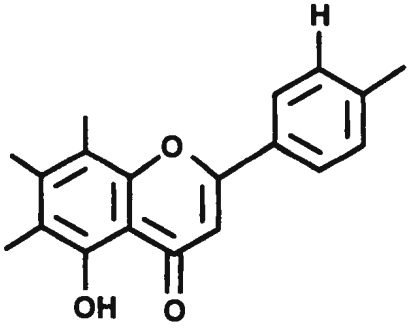
Apigenin



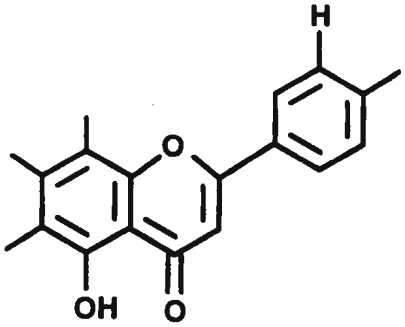
Genkwanin



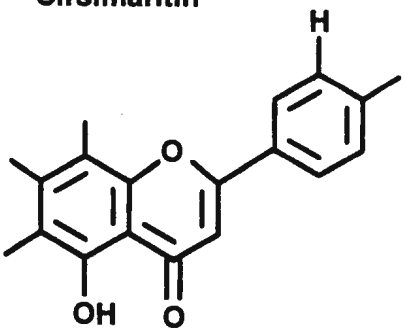
4'-Methoxytectochrysin



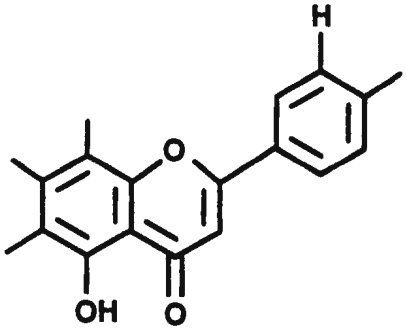
Cirsimaritin



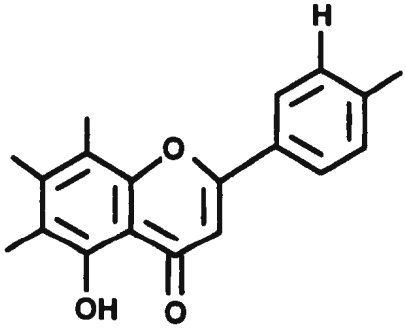
Scutellarein



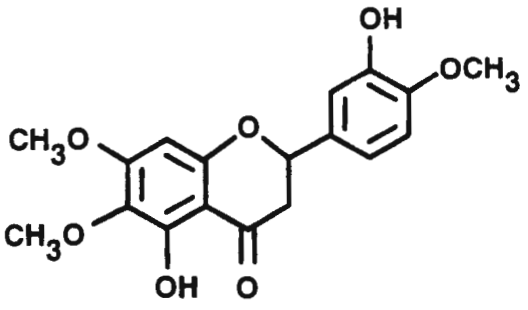
Homoplantagenin



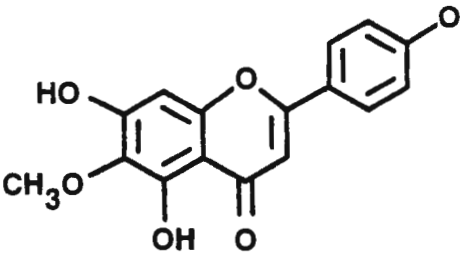
6-OH-Luteolin-7-glucosidase



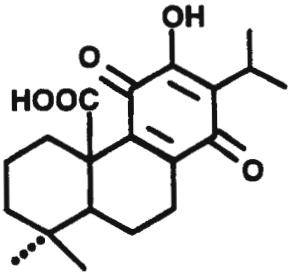
Hesperitin



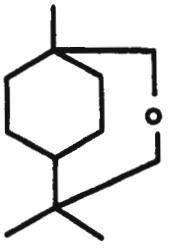
Eupatorin



Hispidulin

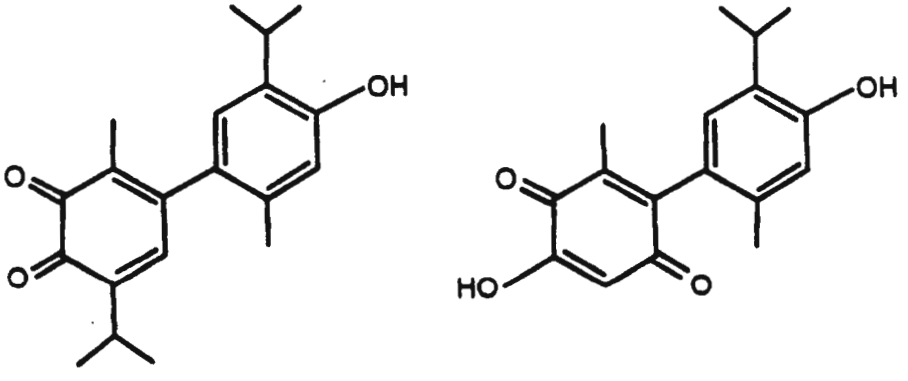


Royleanonic acid



1,8-Cineole

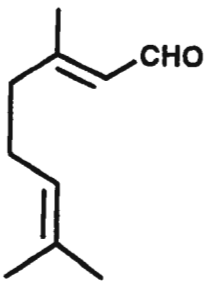
(B)



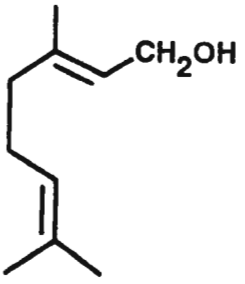
Thymols

Figure 2.6 Chemical structures of basil (A) and mint (B) antioxidants

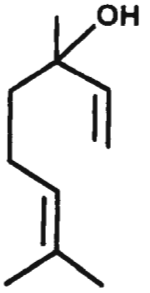
(A)



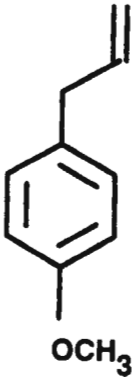
Geranial



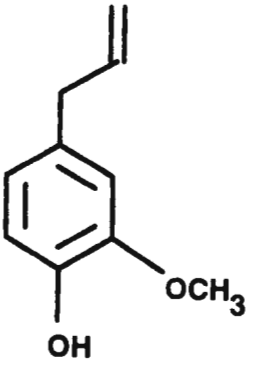
Geraniol



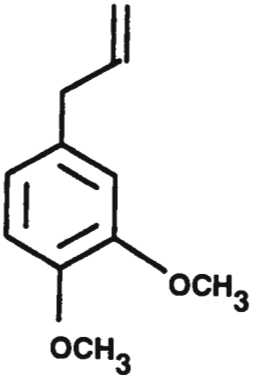
Linalool



Methyl chavicol

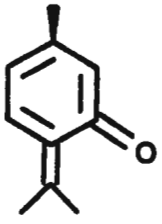


Eugenol

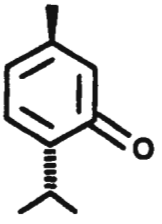


Methyl eugenol

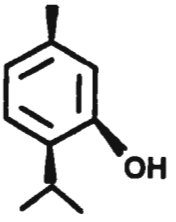
(B)



Pulegone



Menthone



Menthol

activities (Voirin *et al.*, 1999). The chemical structures of these antioxidants are depicted in Figure 2.6-B.

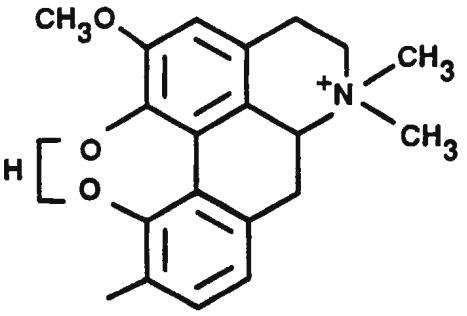
Garlic is well known for its ability in reducing the risk of cancer and heart diseases due to its antioxidative properties (Ide and Lau, 1999; Ide and Lau, 2001; Dong *et al.*, 2001). Flavonoids, kaempferol-3-*O*-β-D-glucopyranose and isorhamnetin-3-*O*-β-D-glucopyranose have been identified in garlic shoots (Mi *et al.*, 2000). Kim *et al.* (1997) reported high antioxidative activities of allicin, diallyl disulphide and diallyl trisulphide, which are sulphur-containing flavour compounds, and Sheen *et al.* (1996) demonstrated that diallyl sulphide was the prime active compound in garlic responsible for anti-cancer activities.

Arbutin, magnoflavine, α-tocopherol and γ-tocopherol have been identified as compounds responsible for antioxidative activity in pericarp and seeds of Japanese pepper (*Xanthuxylum piperitum* DC) (Hisatomi *et al.*, 2000). These compounds are shown in Figure 2.7-A.

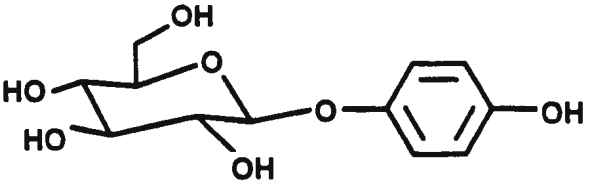
Garcinia indica is used as a garnish for curry and also in folklore medicine in India. Garcinol (Figure 2.7-B), which is a polyisoprenylated benzophenone derivative, was isolated from *Garcinia indica* fruit rind has been shown to possess good free radical scavenging activities (Yamaguchi *et al.*, 2000). Ethanolic extracts of low pungency mustard flour have shown the presence of trihydroxyphenolic compounds and phenolic acids (Shahidi *et al.*, 1994). Diethyl ether extracts of *Aframomum danielle*,

Figure 2.7 **Chemical structures of Japanese pepper (A), Garcinia (B) and oregano (C) antioxidants**

(A)

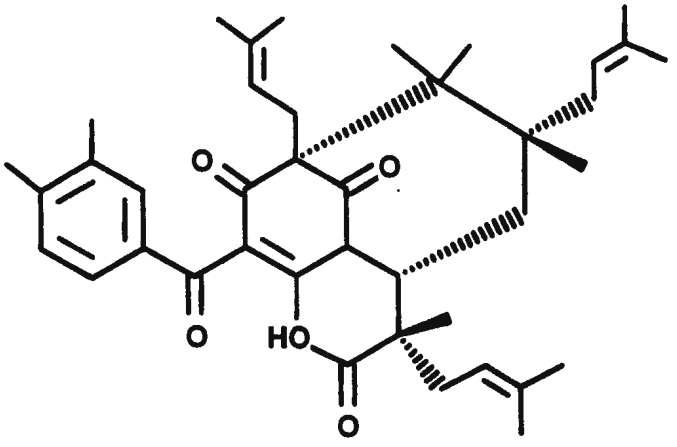


Magnoflorine



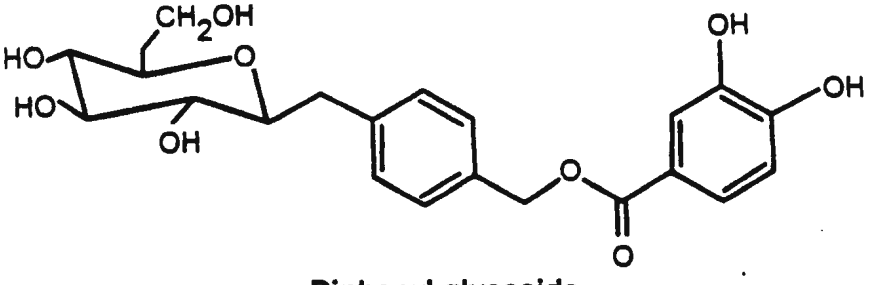
Arbutin

(B)



Garcinol

(C)



Diphenyl glycoside

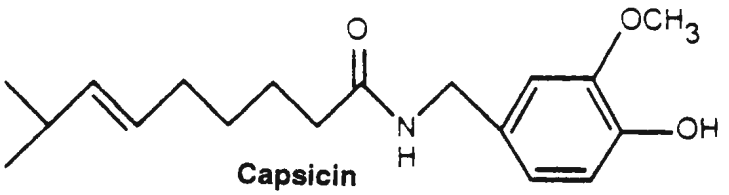
belonging to the family *Zingiberaceae* have shown antioxidative effects due to the presence of trihydroxy type phenolic compounds (Adegoke and Gopala Krishna, 1998).

Oregano, which is also known as marjoram, has been reported to possess potent antioxidant activities, even greater than that of BHA (Nakatani, 1997). Antioxidative phenolic acids and phenyl glucosides (Figure 2.7-C) have been isolated from marjoram (Nakatani and Kikuzaki, 1987). Thyme and carvacrol were found to be the most abundant antioxidant components in the essential oils of marjoram.

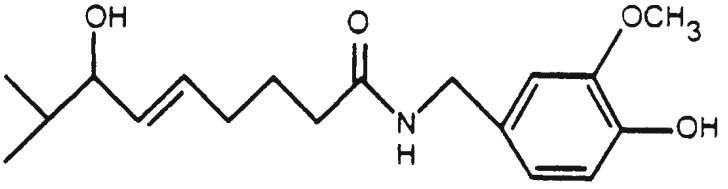
Flavour compounds capsaicin and dihydrocapsaicin extracted from *Capsicum annum* (paprika) powder exhibited a high degree of antioxidative activity (Semwal *et al.*, 1999). Capsanthin isolated from paprika was effective in suppressing hydroperoxide formation, which was comparable to that of β-carotene, lutein and zeaxanthin (Matsufuji *et al.*, 1998). Lee *et al.* (1995) reported the presence of quercetin and luteolin in paprika. An analogue of capsaicin named (-)capsaicinol (Figure 2.8) having strong antioxidative activity was isolated from *Capsicum frutescens* (Masuda and Nakatani, 1991).

Six phenolic compounds (Figure 2.9) isolated from *Dalbergia odorifera T.Chen*, a Chinese traditional medicine, were found to possess strong antioxidative properties when tested in lard (Wang *et al.*, 2000). A native herb *Polygonum multiflorum* Thunb. used in the Orient was found to contain gallic acid and catechin, and the major active component was found to be 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (Chen *et al.*,1999).

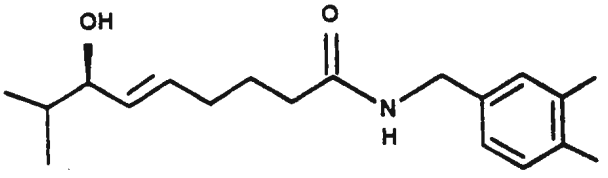
Figure 2.8 Chemical structures of capsicum antioxidants



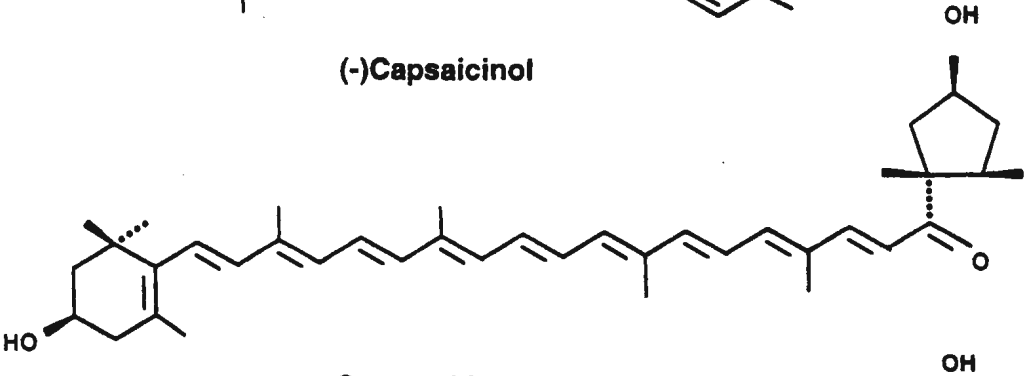
Capsicin



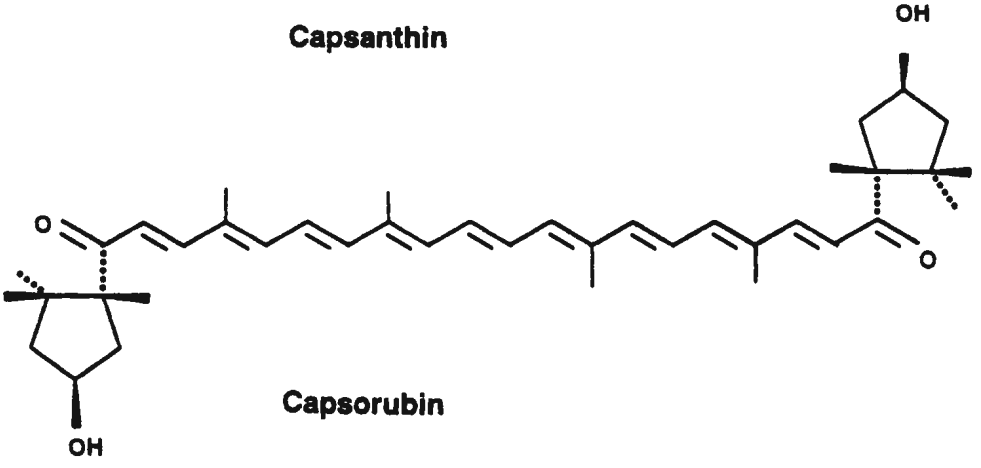
Hydroxy capsicin



(-)Capsaicinol

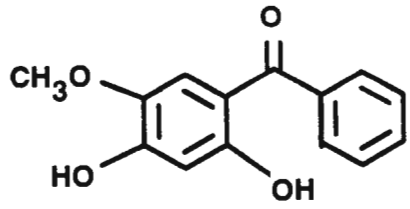


Capsanthin

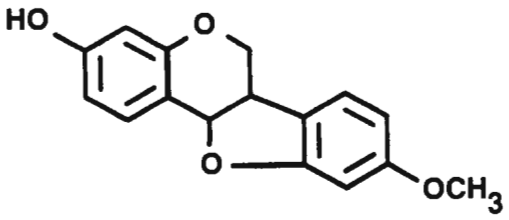


Capsorubin

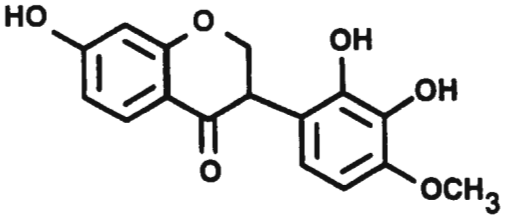
Figure 2.9 Chemical structures of Dalbergia antioxidants



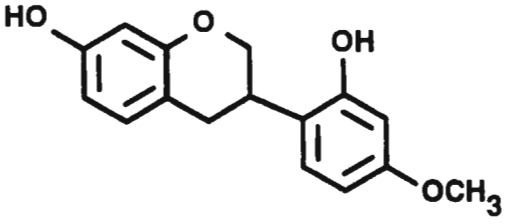
2,4-Dihydroxy-5-methoxybenzophenone



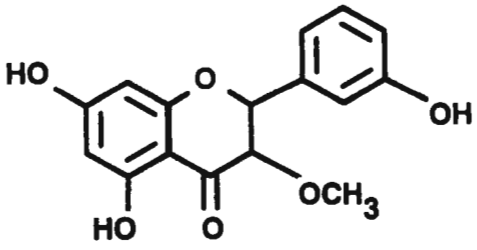
Medicarpin



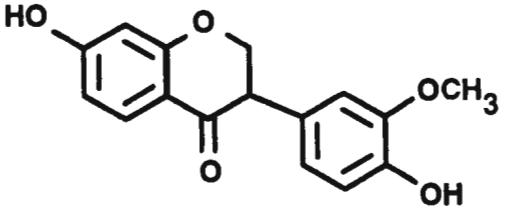
2',3',7-Trihydroxy-4'-methoxyisoflavonone



Versitol



4',5,7-Trihydroxy-3-methoxyflavone



3'-Methoxydaidzein

2.7.2 Oilseeds

Methanolic extracts of peanut hull exhibited a strong antioxidant activity (Yen and Duh, 1994, 1995; Duh and Yen, 1994, 1995) that was attributed to the luteolin content of the methanolic extracts. An antioxidant flavonol dihydroxyquercetin was also identified in the extract (Pratt and Miller, 1984). Apart from the phenolic compounds, protein fractions of peanut have shown to inhibit lipid oxidation in model systems containing metmyoglobin and Fe^{2-}-EDTA as catalysts (Rhee *et al.*, 1979).

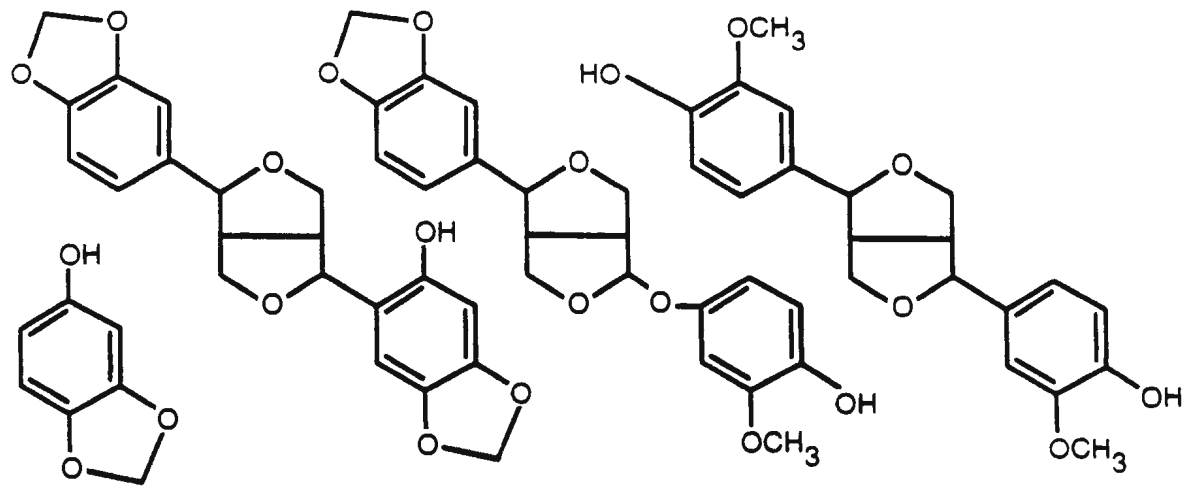
Phenolic acids, flavones and flavonols were identified in canola meal extracts showing potent antioxidative properties (Wanasundara *et al.*, 1996). The most active component was identified to be 1-*O*-β-D-glucopyranosylsinapate (Wanasundara *et al.*, 1994).

Sesame lignans sesamin and episesamin, commonly found in refined sesame seed oil, showed strong antioxidative activities (Umeda-Sawada *et al.*, 1999). Sesaminol and sesamol in sesame oil inhibited LDL oxidation (Kang *et al.*, 1998). The presence of sesamolin, sesaminol glucosides, sesamolinol (Ryu *et al.*, 1998), sesangolin and 2-episesalatin (Kamal-Eldin, *et al.*, 1994) possessing antioxidative capacities have been identified in sesame. The chemical structures of compounds isolated from sesame are shown in Figure 2.10-A.

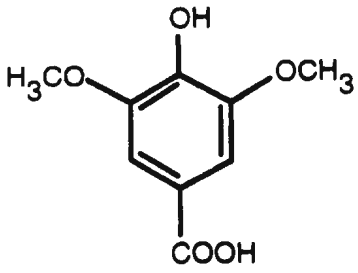
Soybean flour was found to contain antioxidant compounds such as isoflavone glycosides and their derivatives, phospholipids, tocopherols, amino acids and peptides (Hayes *et al.*, 1977). Rackis *et al.* (1972) have identified isoflavone glucosides of

Figure 2.10 Chemical structures of sesame (A) and soybean (B) antioxidants

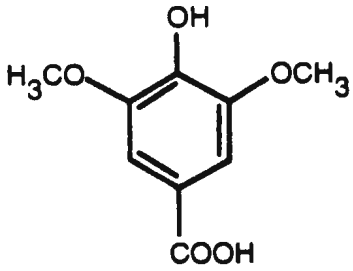
(A)



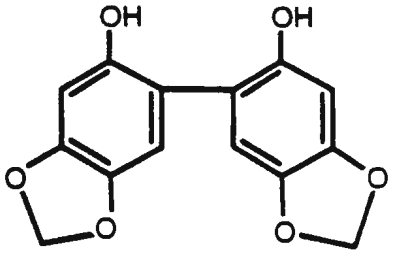
Sesamol **Sesaminol** **Sesamolinol** **Pinoresinol**



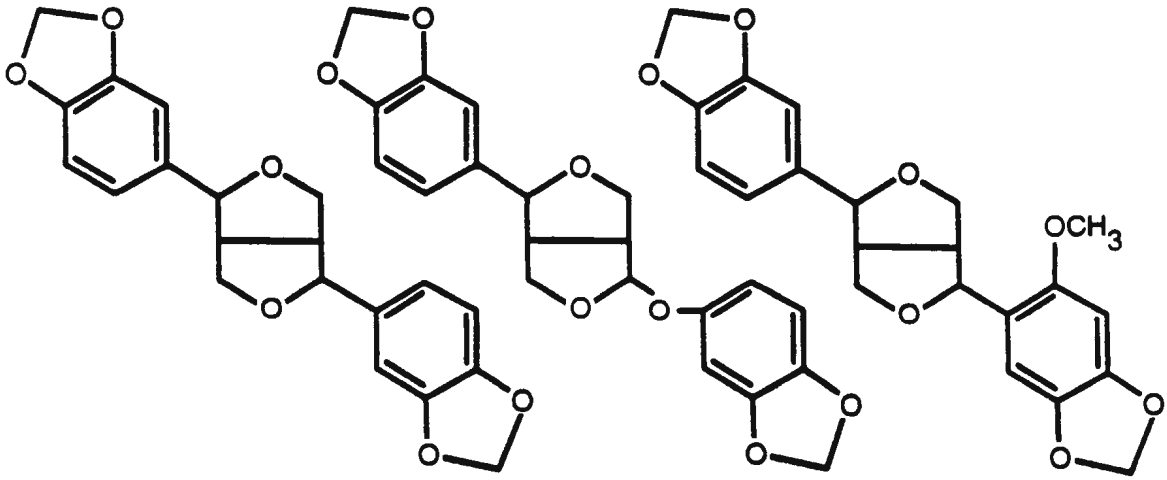
Syringic acid



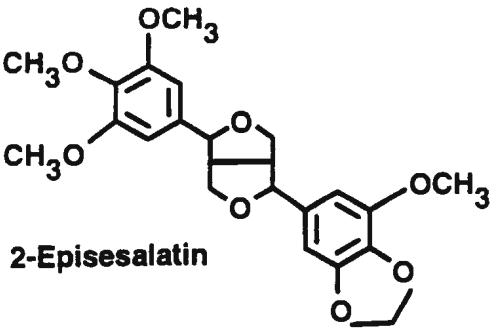
Ferulic acid



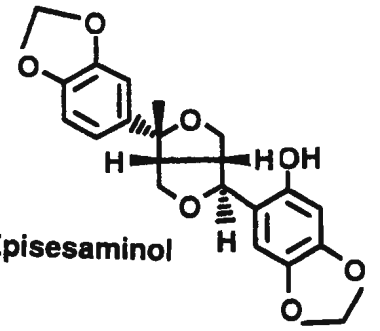
Sesamol dimer



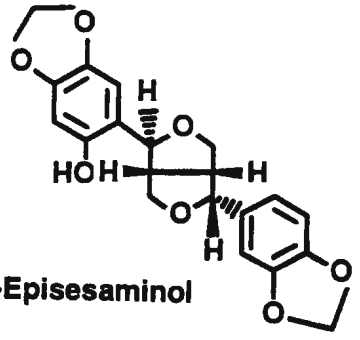
Sesamin **Sesamolin** **Sesangolin**



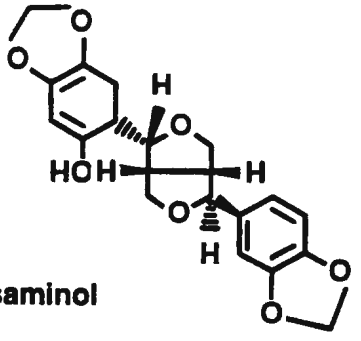
2-Episesalatin



6-Episesaminol

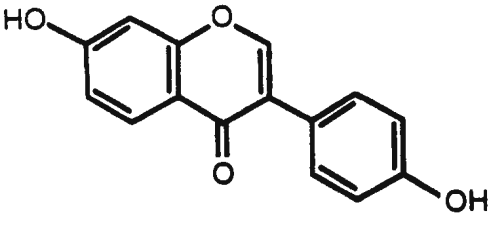


2-Episesaminol

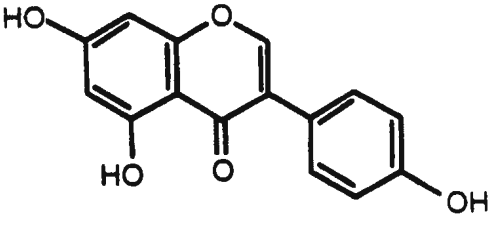


(+)Diasesaminol

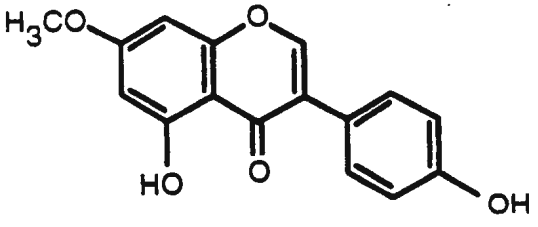
(B)



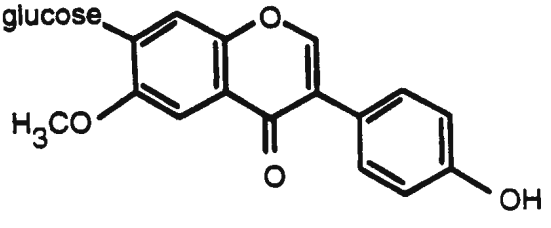
Daidzein



Genistein



Prunetin



Glycitein

soybean meal to be genistein, daidzein, glucitein, prunetin, formononetin, and 4',6',7'-trihydroxyflavone. Inhibition of LDL oxidation by genistein and daidzein (Figure 2.10-B) has been reported by Tikkanen *et al.* (1998).

Cottonseed methanolic extracts contain quercetin glycosides (Whittern *et al.*, 1984) and the main compound was rutin that is reported to be inferior to that of quercetin with regard to its antioxidative activities (Yousseff and Rahman, 1985). Inhibition of oxidation in cooked meat by gossypol present in cottonseed meal was reported by Rhee *et al.* (2001).

2.7.3 Cereals

Two novel tocotrienols, desmethyltocotrienol and didesmethyl tocotrienol, isolated from rice bran, were shown to possess hypocholesterolemic, antioxidative and anti-tumour properties (Qureshi *et al.*, 2000). Wild rice hull exhibited strong antioxidative activities attributed to its content of anisole, vanillin and syringaldehyde. (Asamarai *et al.*, 1996). Ramarathnam *et al.* (1989) reported the presence of isovitexin, a flavonoid in rice hull, that exhibited antioxidant properties as strong as α-tocopherol. Presence of orizanol in rice bran oil was reported by Akiya *et al.* (1970). A rice bran component containing gamma-orizanol was shown to form complexes with cholesterol, making it nonbioavailable that resulted in reduction in blood cholesterol levels (Imai *et al.*, 1994).

Onyeneho and Hettiarachchi (1992) analysed the extracts from durum wheat bran and revealed the presence of protocatechuic, *p*-hydrobenzoic, gentisic, caffeic, vanillic, chlorogenic, syringic, *p*-coumaric and ferulic acids. Three phenolic antioxidative

compounds, isolated from Japanese barnyard millet, exerted antioxidative activities comparable to BHA (Watanabe, 1999). Maillard and Berset (1995) reported that *trans*-ferulic, *trans*-*p*-coumaric and *cis*-ferulic acids were the main compounds responsible for the antioxidative activities in germinated barley and malt extracts. A novel antioxidant was isolated from young green barley leaves and identified as 2'(3')-O-glycosylisovitexin (Osawa *et al.*, 1992).

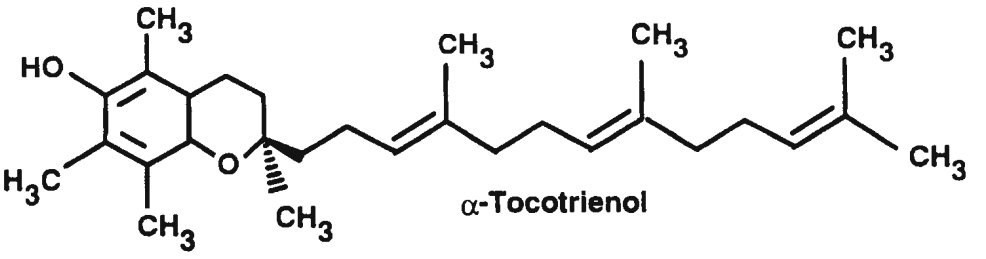
Catechin and its derivatives, tocopherols and carotenoids have been reported as the main active constituents in barley (Goupy *et al.*, 1999). Eight phenolic acids; caffeic, *o*-coumaric, *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, syringic and vanillic acids were identified in buckwheat extracts, and rutin was found to be the major flavonoid followed by quercetin and quercitrin (Przybylski *et al.*, 1998). Chemical structures of antioxidative compounds isolated from cereals are depicted in Figure 2.11.

2.7.4 Tea, coca and coffee

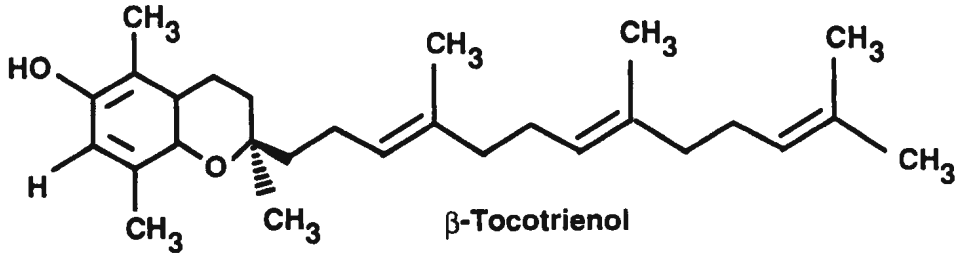
Tea is the brew prepared from the leaves of *Camellia sinensis*. Depending on the preparation process, the tea leaves could be classified as black, green and oolong. Black tea is produced by withering, rolling, sifting and fermenting the tea leaves. Oolong tea is produced in the same manner except that it is subjected to semi-fermentation. In green tea, fermentation is prevented by subjecting the leaves to a firing process after harvesting, to yield a greenish gold colour product. Due to these differences, the compounds present in tea differ (Balentine *et al.*, 1997). Major compounds in green tea are epicatechins such as (-)epigallocatechin-3-gallate (EGCG), (-)epigallocatechin (EGC), (-)epicatechin (EC) and (-)epicatechin-3-gallate (ECG). Due to the fermentation

Figure 2.11 Chemical structures of rice (A), barley (B) and buckwheat (C) antioxidants

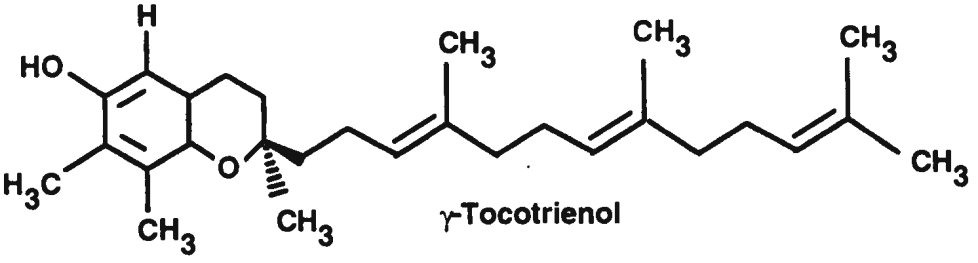
(A)



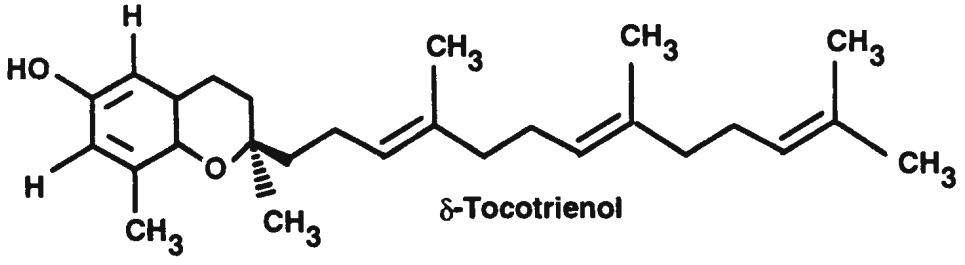
α-Tocotrienol



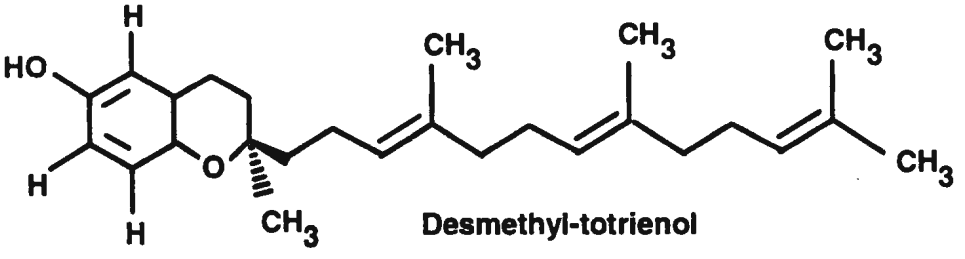
β-Tocotrienol



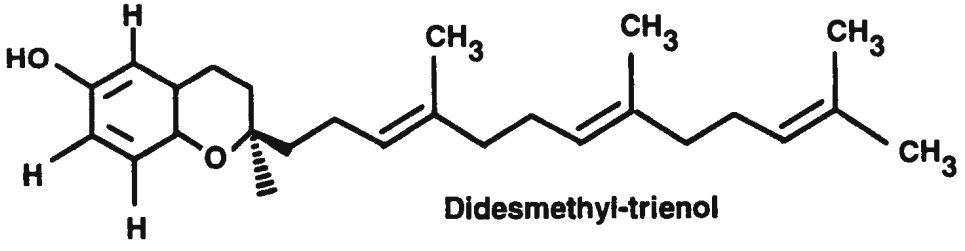
γ-Tocotrienol



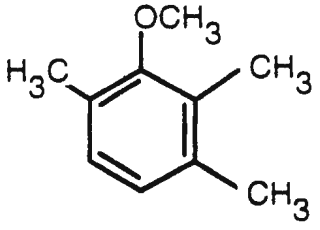
δ-Tocotrienol



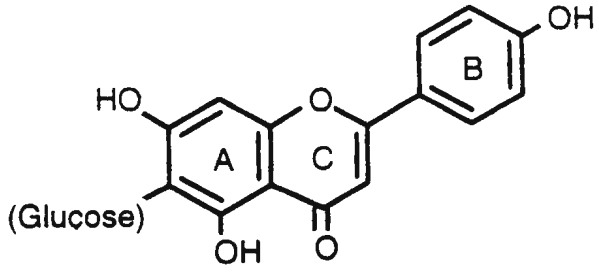
Desmethyl-totrienol



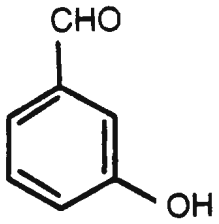
Didesmethyl-trienol



2,3,6-Trimethylanisole

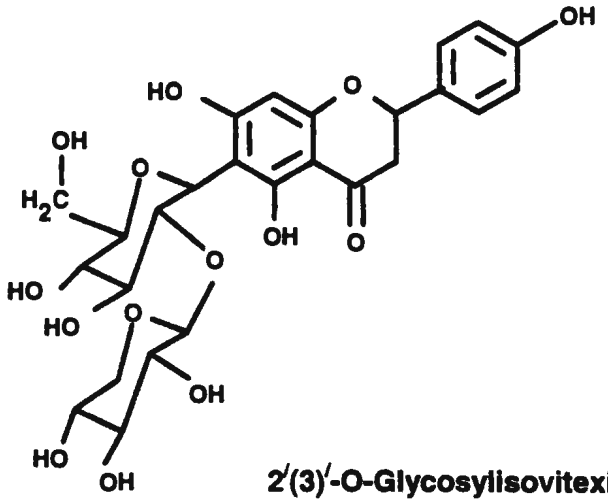


Isovitexin



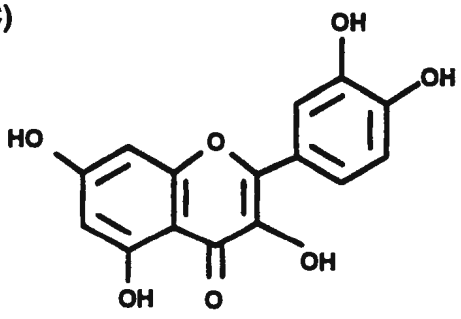
m-Hyrdoxybenzaldehyde

(B)

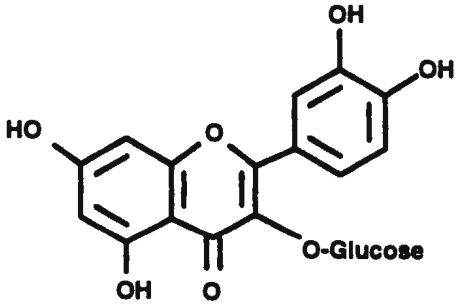


2′(3)′-O-Glycosylisovitexin

(C)



Quercertin



Quercitrin

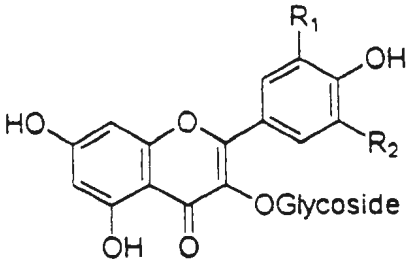
process, black tea contains oxidation and condensation products of catechins that include theaflavins and thearubigens (Balentine *et al.*, 1997). Both green and black teas contain caffeine and flavonoids such as quercetin, rutin, kaempferol and myricitin (Yang *et al.*, 2000). Compounds present in green and black tea are depicted in Figure 2.12.

Ferreira *et al.* (1998) reported the presence of 4-hydroxycinnamic acid, isoflavones, flavonones, coumestans, xanthones and flavones in processed leaves and stems of honeybush tea, which is a traditional herbal tea in South Africa. Their chemical structures are illustrated in Figure 2.13.

Epicatechin and catechin were found in cocoa as well as dark and milk chocolates (Osakabe *et al.*, 1998; Vinson *et al.*, 1999). It was reported that the phenolic compounds quercetin, clovamide and deoxyclovamide (Figure 2.14-A) isolated from coca liquor were responsible for the stability of chocolate against oxidation.

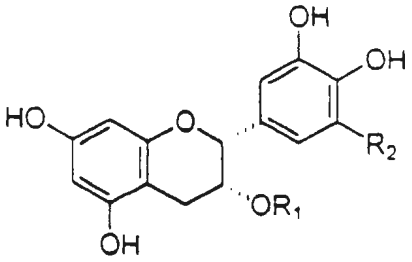
Chlorogenic, caffeic and quinic are the major phenolic acids in coffee that have shown potent antioxidative activities *in vitro* (Olthof *et al.*, 2001). Brewed coffee contained pyrroles, furans, and thiopenes, which exhibited antioxidative activities comparable to that of BHT (Fuster *et al.*, 2000). Caffeoyltryptophan isolated from robusta coffee beans exhibited potent radical scavenging properties (Ohnishi *et al.*, 1998). Caffeine (Figure 2.14-B) has shown activities similar to that of glutathione, which is a biological antioxidant (Devasagayam *et al.*, 1998).

Figure 2.12 Chemical structures of tea antioxidants



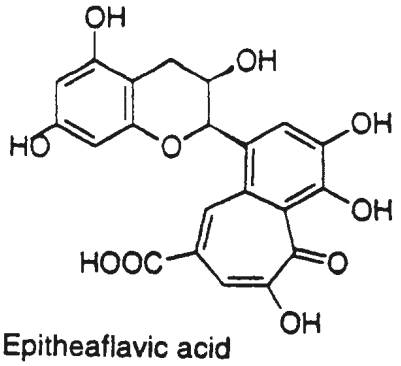
Major tea flavonols

		R_1	R_2
Kaempferol glycoside	KaG	H	H
Quercitin glycoside	QuG	OH	H
Myricitin glycoside	MyG	OH	OH

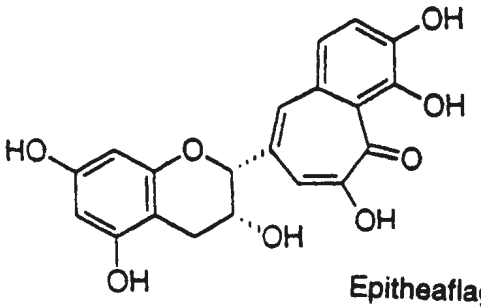


Major tea catechins

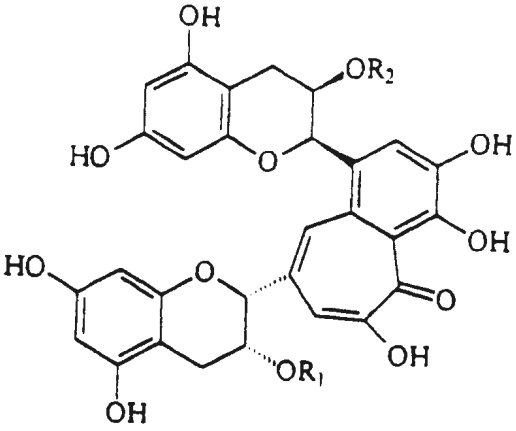
		R_1	R_2
Epicatechin	EC	H	H
Epicatechin gallate	ECG	Gallate	H
Epigallocatechin	EGC	H	OH
Epigallocatechin gallate	EGCG	Gallate	OH



Epitheaflavic acid

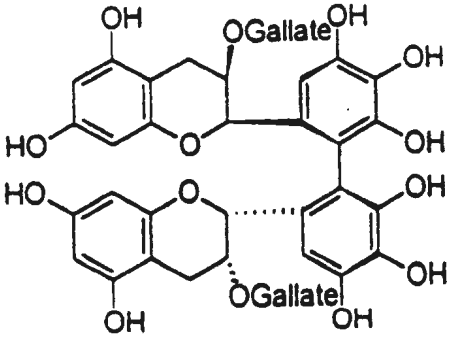


Epitheaflagallin

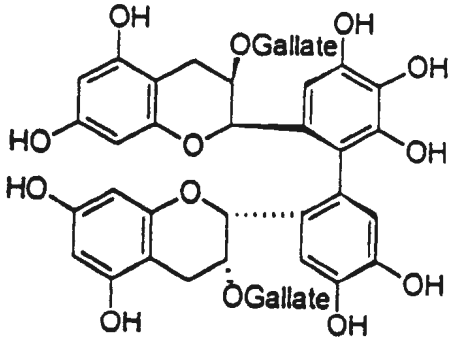


Theaflavins

		R_1	R_2
Theaflavin	TF	H	H
Theaflavin 3-gallate	TF3G	Gallate	H
Theaflavin 3′-gallate	TF3′G	H	Gallate
Theaflavin 3,3′-digallate	TFDG	Gallate	Gallate

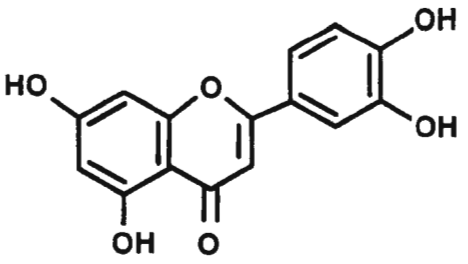


Theasinensin A

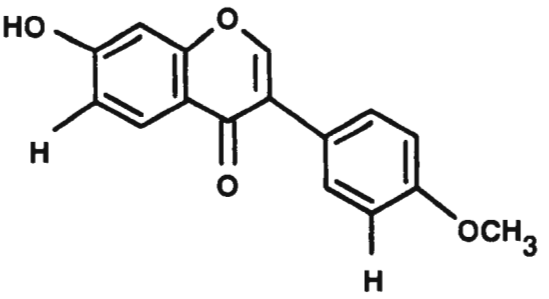


Theasinensin F

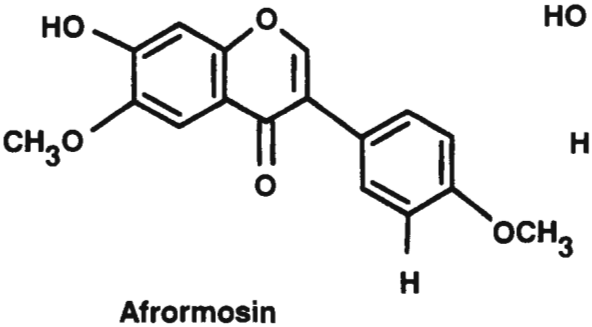
Figure 2.13 Chemical structures of honeybush antioxidants



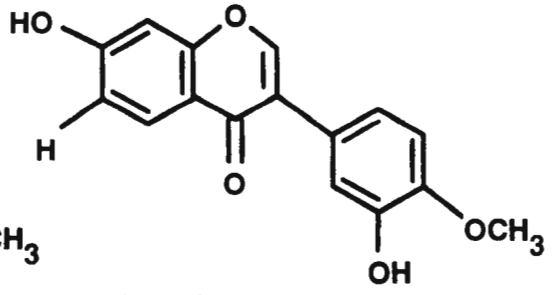
Luteolin



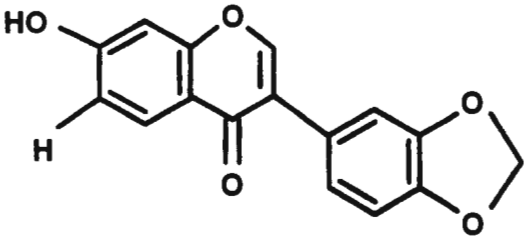
Formononetin



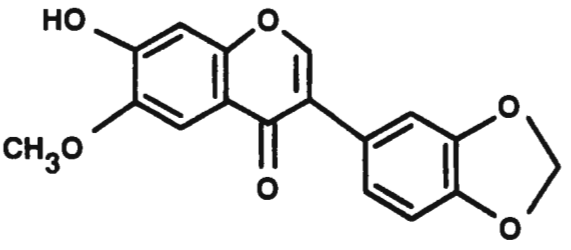
Afrormosin



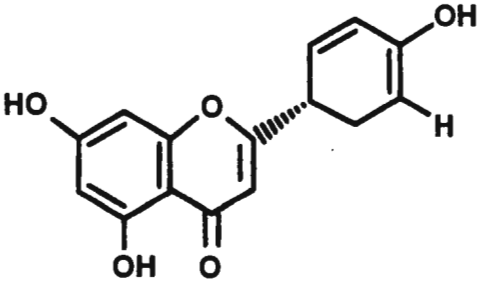
Calycosin



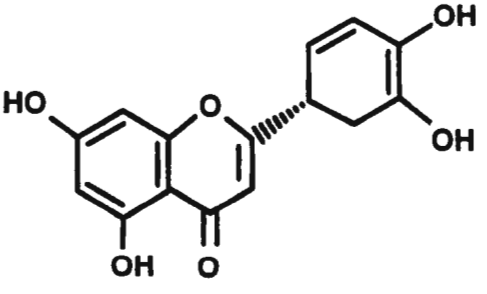
Pseudobaptigen



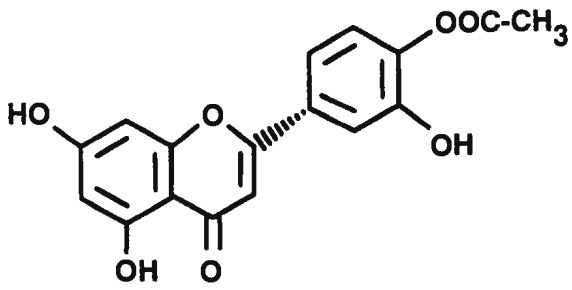
Fujikinetin



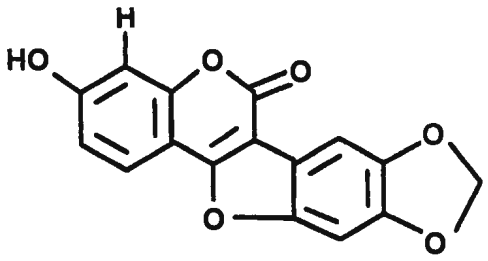
Naringin



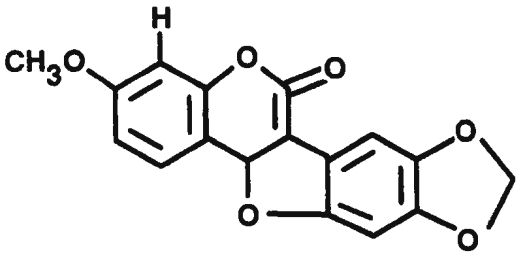
Eriodictoyl



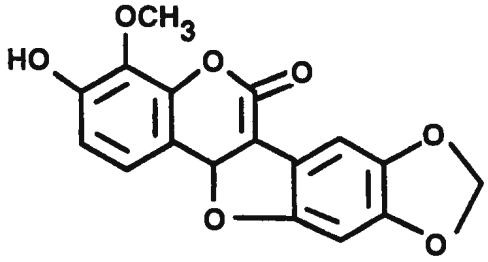
Hesperitn



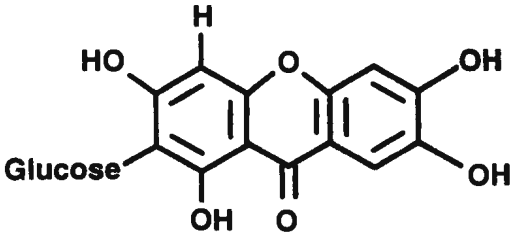
Medicagol



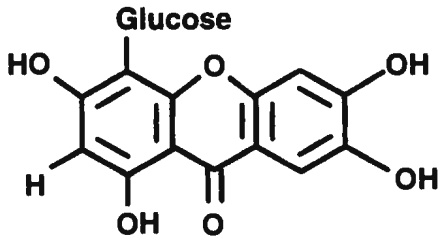
Flemichapparin



Sophoracoumestan



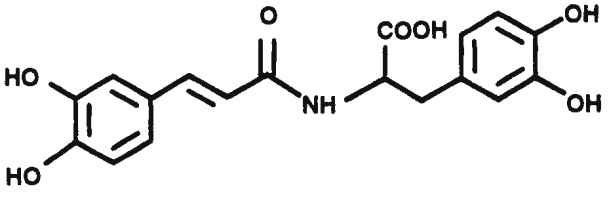
Mangiferin



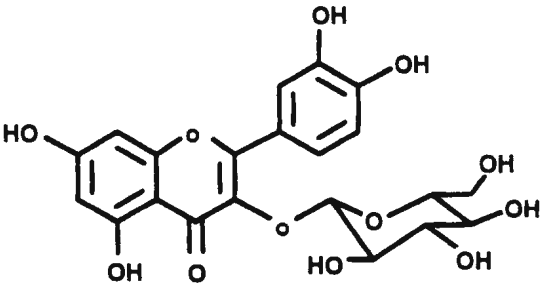
Isomangiferin

Figure 2.14 Chemical structures of cocoa (A) and coffee (B) antioxidants

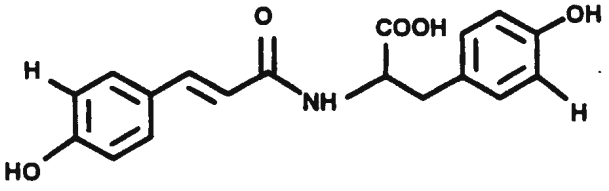
(A)



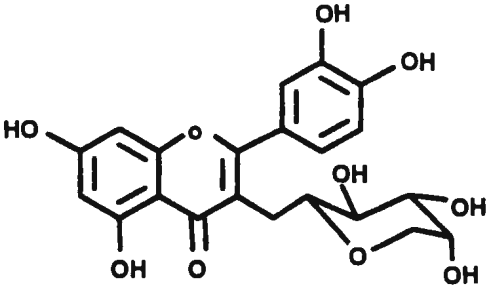
Clovamide



Quercetin-3-O-β-D-glucopyranoside

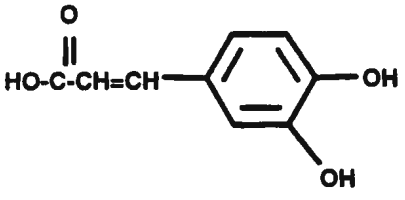


Dideoxy clovamide

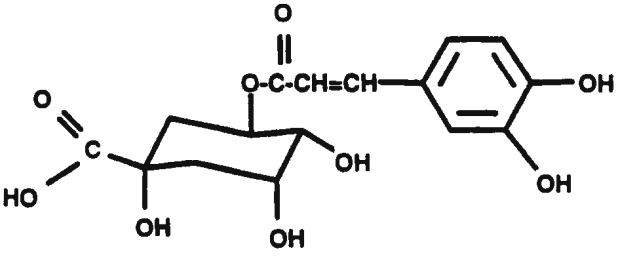


Quercetin-3-O-α-D-arabinopyranoside

(B)



Caffeic acid



Chlorogenic acid

2.7.5 Fruits and vegetables

Consumption of fruits and vegetables provides protection against cancers that involve epithelial cells such as cancers of the lung, oesophagus, stomach, colon, pancreas and cervix (Tavani and La Vecchia, 1995). A variety of phenolic compounds, in addition to flavonoids, widely distributed in fruits and vegetables, are responsible for their anti-carcinogenic effects (Craig, 1997). The antioxidant capacity of citrus fruit juices is attributed to their vitamin C content and total phenolics (Gardner *et al.*, 2000). Catechin, epicatechin, *trans*-caffeoyl tartrate, *cis*-*p*-coumaroyl tartrate, *trans*-*p*-coumaroyl tartrate and procyanidins were identified in grape juice (Jaworski and Lee, 1987). Grape seeds contain catechin, (-)epicatechin, (-)epicatechin-3-*O*-gallate, and procyanidins such as 14 dimeric-, 11 trimeric- and tetrameric procyanidins (Fuleki and da Silva., 1997). Procyanidins and (-)epicatechin-3-*O*-gallate exhibited potent hydroxyl and superoxide radical scavenging activities in aqueous model systems (da Silva *et al.*, 1991). Flavonols such as quercetin, kaempferol, myricetin and phenolic acids, namely, caffeic, ferulic, *p*-hydroxybenzoic, *p*-coumaric and ellagic acids are present in various berries (Torronen *et al.*, 1997; Hakkinen *et al.*, 1999). In tart cherries (*Prunus cerasus*), the most effective phenolic compound responsible for inhibition of liposome oxidation is 7-dimethoxy-5,8,4'-trihydroxyflavone, followed by quercetin-3-rhamnoside, genistein, chlorogenic acid, naringenin and genistin (Wang *et al.*, 1999b). Avacado contains gallic, vanillic, *o*-, *m*-, and *p*-coumaric, caffeic, ferulic, sinapic, parotocatechuic, β- and γ-resorcylic, *p*-hydroxybenzoic and syringic acids (Torres *et al.*, 1987).

2.7.6 Nuts

Nuts belong to a diverse group of plants belonging to several families. Peanuts belongs to legume family; hazelnut in birch family; macadamias in proteaceae and almonds in rosaceae. In spite of their diversity, they are grouped together due to the similarities in their biochemistry and nutritional make up. Nuts provide energy, fat, protein, dietary fibre, essential fatty acids, vitamins and minerals and considered as a staple food for humans (Dreher *et al.*, 1996). Although about 80% of the calories in nuts come from fat, most of the fat is monounsaturated and polyunsaturated fatty acids, which may help in lowering blood cholesterol level and improve cardiovascular health. Most of the nuts are rich in oleic acid, a monounsaturated fatty acid, with the exception of walnuts, which are high in linoleic and α-linolenic acids that are polyunsaturated fatty acids (Dreher *et al.*, 1996). Nuts have also shown capacities to reduce risks of cancer, diabetes and other chronic diseases (Pszczola, 2000). In addition, nuts are found to contain bioactive compounds such as flavonoids, phenolic components and isoflavones (Dreher *et al.*, 1996).

Methanolic extract of hazelnuts contain gallic acid, *p*-hydroxylbenzoic acid, caffeic acid, epicatechin, sinapic acid and quercetin (Yurttas *et al.*, 2000). These authors reported that the antioxidative activities of nonhydrolysed hazelnut extracts were greater than their hydrolysed counterparts. This difference may be due to the destruction of phenolics during hydrolysis. Chilean hazelnut extracts showed strong antioxidant capacities comparable to those of BHA and BHT in β-carotene bleaching, accelerated oxidation of soybean oil and DPPH radical scavenging assays (Moure *et al.*, 2000, 2001).

Phenolic compounds from macadamia nuts and shells were 2,6-dihdroxybenzoic acid, 2'-hydroxy-4'-methoxyacetophenone, 3',5'-dimethoxy-4-hydroxy-acetophenone and 3',5'-dimethoxy-4-hydroxycinnamic acid and were effective in reducing oxidation in macadamia nut oil (Quinn and Tang 1996). Incorporation of pistachio nuts in a diet to provide 20% of the daily caloric intake improved lipid profiles in humans suffering from hypercholesterolemia (Edwards *et al.*, 1999). Chemical structures of antioxidative compounds isolated from nuts are shown in Figure 2.15.

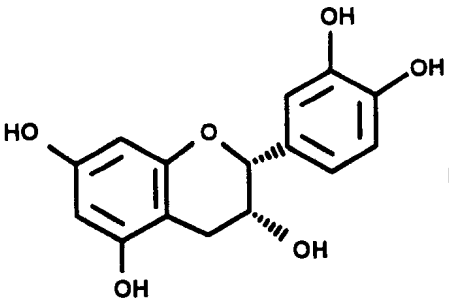
Methanol-soluble fraction of walnut oil showed strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities compared to the insoluble fraction in methanol, due to the presence of phenolic compounds in the former (Espin *et al.*, 2000). Incorporation of moderate quantities of walnuts into the diet decreased serum cholesterol levels and modified lipoprotein profiles in Japanese women (Iwamoto *et al.*, 2000). Walnut contains high amounts of α-tocopherol in its kernel, and ellagic acid in the thin skin covering the kernel (Ravai, 1995).

2.7.7 Potential of almond as a source of natural antioxidants

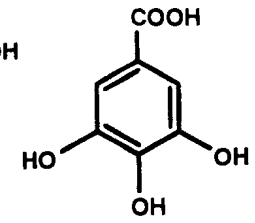
World production of almond is 1,447,378 MT and the major producer is USA with an annual production of 485,000 MT (FAO, 2000). England, Canada, India, Spain, France, Germany and the Netherlands are the main importers of almonds. Canada imported 38.5 million kilograms of almond, fresh or dried, shelled, peeled during the period of 1993-1997 (Global Agribusiness information Network, 2000). Outermost covering of the almond fruit is green and turns into a leathery brown material upon drying. The innermost seed or the nut is covered with a hard shell and in between there

Figure 2.15 Chemical structures of hazelnut (A) and almond (B) antioxidants

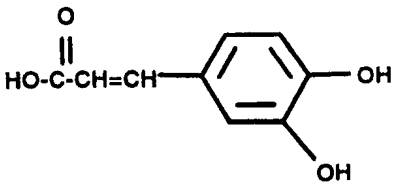
(A)



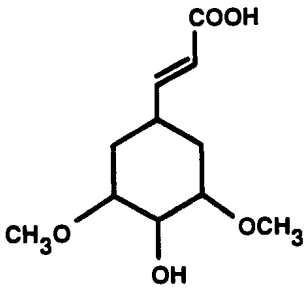
Epicatechin



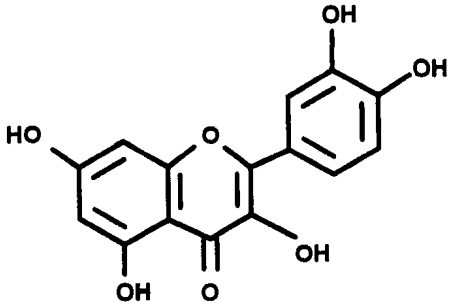
Gallic acid



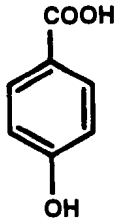
Caffeic acid



Sinapic acid

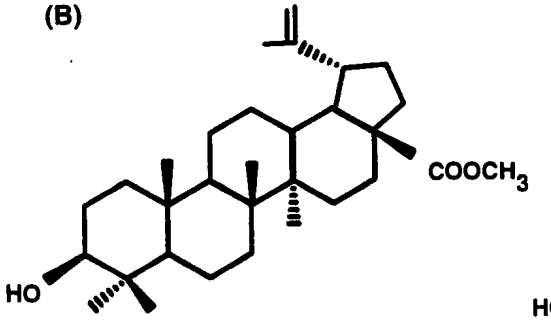


Quercetin

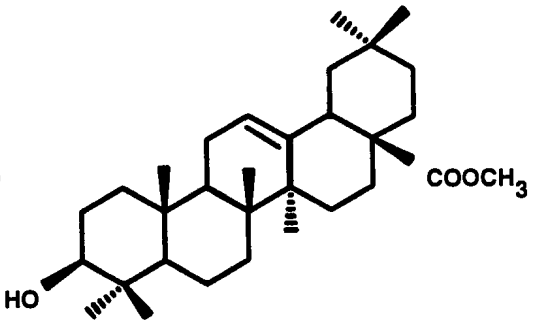


p-Hydroxybenzoic acid

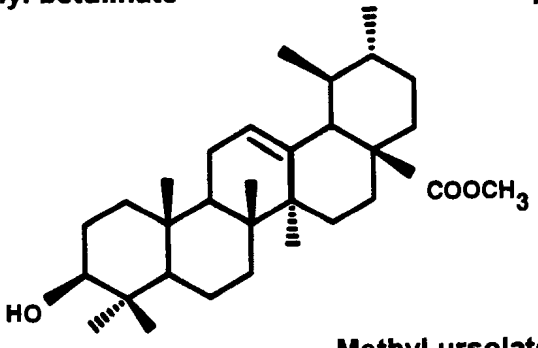
(B)



Methyl betulinate



Methyl oleanolate



Methyl ursolate

is a flimsy hull that becomes brown once dried. Almond fruit with or without the brown skin is consumed as the whole nut or used in salads, various dishes, confectioneries and chocolates. Almond oil extracted mainly from bitter almond (*Prunus amygdalus*), is an important flavouring ingredient in the food industry, and consists mainly of benzaldehyde (Remaud *et al.*, 1997). Apart from its nutritional value, almond based diets lower blood cholesterol levels in human (Spiller *et al.*, 1998) and decrease the risk of colon cancer in experimental rat model systems (Davis *et al.*, 2001). The presence of triterpenoids; methyl betulinate, methyl oleanolate and methyl ursolate in hulls has been reported recently, and the annual production of hulls is estimated to exceed 6 million tonnes that is used as a livestock feed (Takeoka, 2000). It is evident that these components would be worth investigating, as there is little information on antioxidative activities of almond as such and it's inedible by products. Extraction of antioxidative compounds, evaluation of their antioxidative capacities, and identification of the active compounds involved are discussed in the following sections.

2.8 Extraction of antioxidant compounds

Extraction techniques should afford antioxidants that are effective at low concentrations and have little or no effect on aroma and colour (Sebestian *et al.*, 1998). Bracco *et al.* (1981) proposed a technique that could commonly be used for spices, herbs and vegetables to recover natural antioxidants. This method is based on micronization (rupture of cell wall to free antioxidants from protoplasm) of antioxidant-bearing material to transfer the antioxidant to a liquid phase, cleaning the liquid phase by centrifuging or filtering and molecular distillation to collect active components. Wu *et al.* (1982) described a method to extract antioxidants from rosemary leaves. In this system,

a methanolic extract of ground rosemary leaves was obtained and then directly bleached with activated carbon. Activated carbon was then separated by filtering and the antioxidative compounds were isolated after solvent removal. A two-stage process for isolating antioxidants from rosemary and sage using supercritical carbon dioxide as a solvent was developed by Bauman *et al.* (1999). This method is suitable for isolation of thermolabile natural substances. The optimal process parameters were defined as 25 Mpa pressure at 100°C for the first stage in which essential oils were extracted and 47.5 Mpa and 100°C for the second stage to isolate antioxidant components. Weinberg *et al.* (1999) proposed an enzyme-assisted ensiling (ENLAC) to improve recovery of polyphenols from rosemary and sage. However, at the present, solvent extraction seems to be the widely used method. This is a simple and easy way of extracting antioxidants in which the plant part is first crushed or ground, and if necessary, defatted and extracted under reflux conditions at elevated temperatures for varying time periods.

Antioxidant activity and the yield depend on the solvent due to the polarity differences of various compounds (Marinova and Yanishlieva, 1997). Ethanol and water are preferred solvents because of their safety and abundance. Use of hexane, diethyl ether, ethyl acetate, acetone and methanol extracts of buckwheat groats and hulls revealed that antioxidative and DPPH radical activities of the extract increased with increasing solvent polarity, and highest values were obtained using methanolic extracts (Przybylski *et al.*, 1998). Similar results were obtained when methanol, diethyl ether and petroleum ether extracts of *Aframomun danielli* were tested for their antioxidative efficacies (Adegoke and Gopala Krishna, 1998). However, when antioxidants of evening primrose meal were extracted using ethanol, methanol and acetone at different

concentrations and tested for their antioxidative capacities, the highest activity was observed for 71% acetone extracts (Wettasinghe and Shahidi, 1999). Phenolics from berries extracted with acetone possessed potent activities in inhibiting LDL and liposome oxidation (Heinonen *et al.*, 1998). Methanolic extracts of cereals have been shown to perform better than acetone, water or ethanolic extracts (Zielinski and Kozlowska, 2000). Methanol has also been used to extract active components from peanut hull powder (Duh and Yen, 1994; Yen and Duh, 1995), oat groats and hulls (Xing and White, 1997), grape seeds (Castillo *et al.*, 2000), sage and rosemary (Madsen *et al.*, 1998) and mung bean hulls (Duh *et al.*, 1997).

Use of hexane to extract antioxidants has been reported for Japanese pepper (Hisatomi *et al.*, 2000). Shahidi *et al.* (1994) reported the use of ethanol to extract active components from mustard flour. Boling water extracts of sage, linden flowers, black tea (Yildirim *et al.*, 2000), zu-zhong tea (Yen and Hsieh, 1998), *Cassia tora* (Yen and Chuand, 2000) and barley (Duh *et al.*, 2001) possessed high antioxidative capacities.

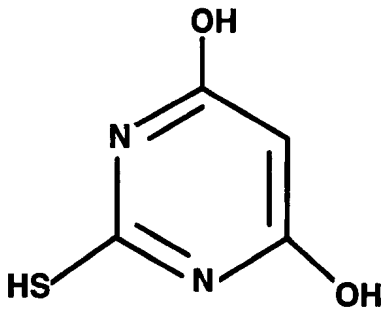
2.9 Evaluation of antioxidative capacities

2.9.1 Meat model systems

Lipids present in muscle tissue can easily undergo oxidation, giving rise to off flavours and off odours, which in turn affect the palatability, nutritional quality and functionality of meat constituents. This problem is even greater with manufacturing of convenience food items such a pre-cooked and restructured meat products where loss of desirable flavour is due to the formation of warmed-over flavours (WOF). The WOF, which causes the stale flavour of meat, develops rapidly in refrigerated cooked meat,

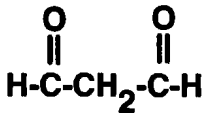
especially poultry, and becomes apparent within 48 h (Tim and Watts, 1958). Therefore, the use of novel antioxidants during processing to counteract the oxidative effects has been tested using different meat systems such as beef (Murphy *et al.*, 1998; Wong *et al.*, 1995), chicken (El-Alim *et al.*, 1999; Ang and Lyon, 1990), turkey (Lee *et al.*, 1996), pork (Kansci *et al.*, 1997; El-Alim *et al.*, 1999; Wettasinghe and Shahidi, 1996; Shahidi *et al.*, 1987), mutton (Wilson *et al.*, 1976; Mendiratta *et al.*, 2000) and also in fish model systems (Boyd *et al.*, 1993). The 2-thiobarbituric acid (TBA) test is one of the most frequently used methods for assessing lipid peroxidation both *in vitro* and *in vivo* (Gray, 1978). It is a measurement of a pink chromogen formed (Figure 2.16) between TBA and malonaldehyde (MA), which is an end product of lipid peroxide decomposition (Shahidi and Hong, 1991). However, non-lipid materials such as sugars, amino acids, urea, biliverdin, glyoxal and furfuraldehyde may also react with TBA to form coloured complexes that absorb at the same wavelength range (530-535 nm) in which the TBA-MA adduct is measured (Hoyland and Taylor, 1991; Shahidi and Wanasundara, 1998). The measurement of MA in meat model systems is carried out mainly by distillation (Tarladgis, 1960) or filtration methods (Tarladgis, 1964). Siu and Draper (1978) later used the filtration method with slight modifications. Briefly, in the modified method, first the meat sample is homogenized after mixing with distilled water and trichloroacetic acid (TCA). Then the TCA extract containing MA and other TBA reactive substances is reacted with the TBA reagent at 80°C for 90 min and the absorbance of the resulted solution is then read at 532 nm. In the distillation method, the meat-water-hydrochloric acid homogenate is distilled and the collected distillate is reacted with TBA as described earlier. The Filtration method is favoured, due to its simplicity and milder operating

Figure 2.16 Steps involved in formation of thiobarbituric acid-malonaldehyde (TBA-MA) adduct

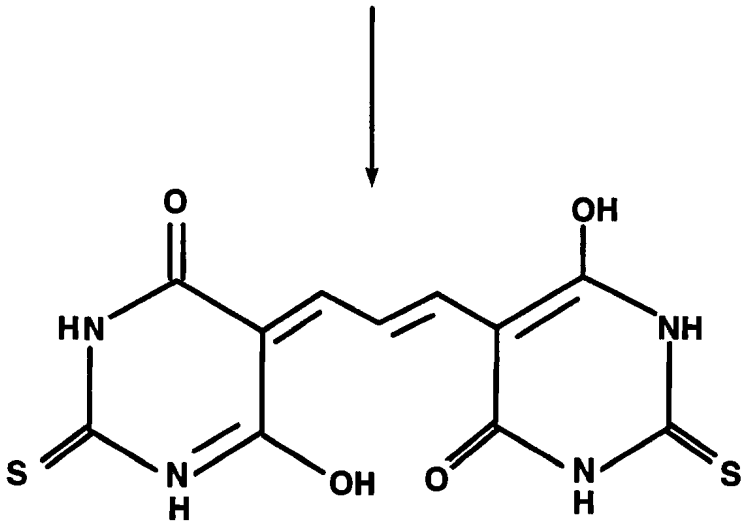


(TBA)

+



(MA)

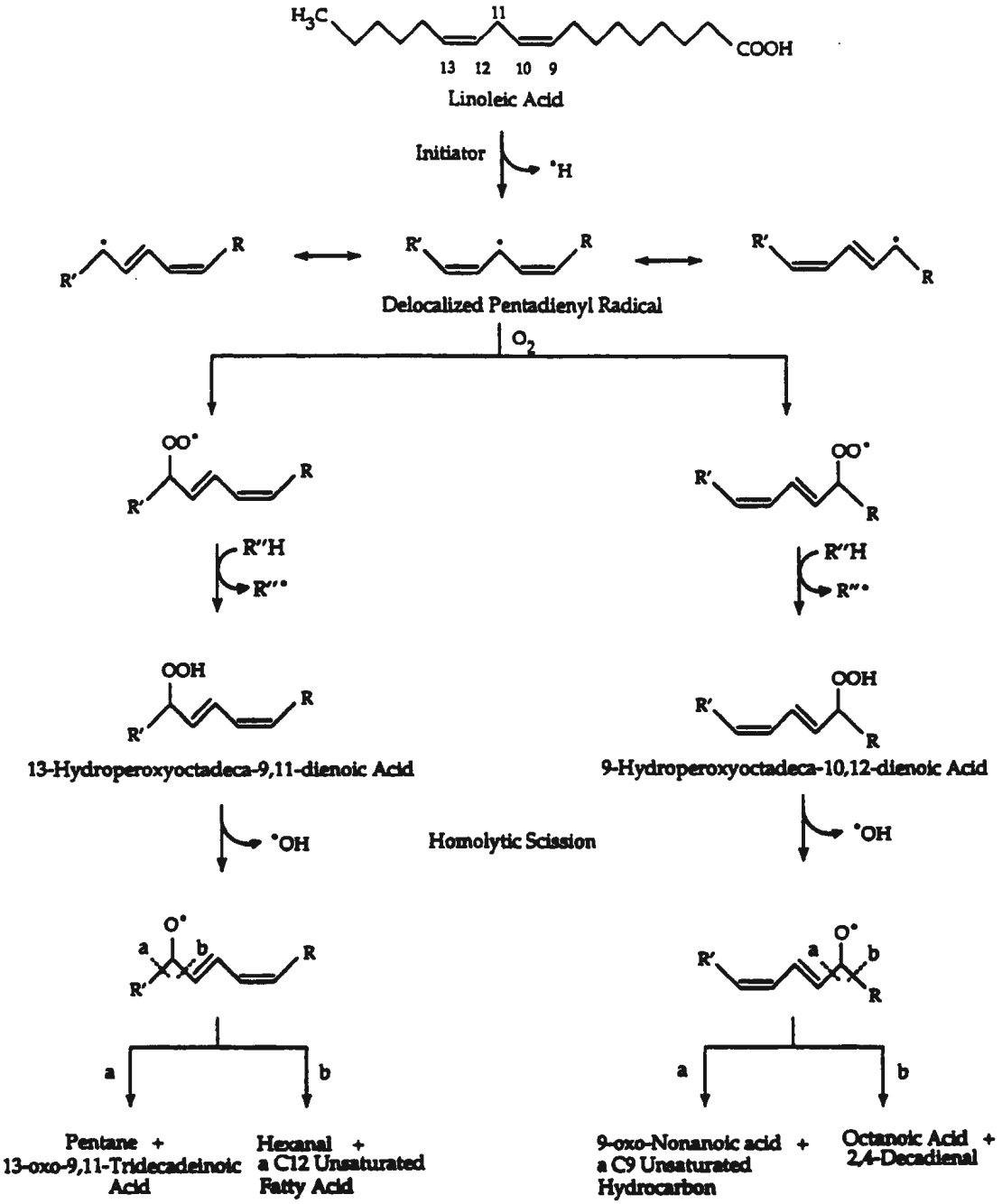


TBA-MA adduct

conditions as compared to the distillation method (Shahidi and Hong, 1991). However, the distillation method has been more suitable for samples with high fat content due to the turbidity that could exist in the TCA solution (Siu and Draper, 1978; Shahidi and Hong, 1991) or for samples that are highly coloured (Shahidi and Wanasundara, 1998; Shahidi and Hong, 1991). Malonaldehyde can also be measured using gas chromatographic (Kansci *et al.*, 1997) or high performance liquid-chromatographic (HPLC) techniques (Bergamo *et al.*, 1998). The extent of lipid oxidation in meat depends both on the temperature at which the cooked meat is held and the intensity of the heat treatment. The higher the temperature and longer the duration of heat application, the lower would be the development of rancidity and hence, cooking temperatures of less than 80°C would be suitable in model systems intended for antioxidant activity evaluations (Cross *et al.*, 1987).

Another approach to monitor lipid oxidation is to measure the carbonyl compounds formed upon decomposition of fatty acid hydroperoxides. Foods containing high linoleic acid contents give rise to hexanal (Figure 2.17), thus hexanal serves as an important index of meat oxidation and flavour quality deterioration (Shahidi and Pegg, 1994a, 1994b). In contrast, propanal and pentenal are dominant in oxidative products of fish systems (Frankel and Huang, 1996a). Headspace gas chromatographic technique may therefore be used to monitor hexanal and other volatiles formed during lipid oxidation (Ang and Liu, 1996; Wittkowski *et al.*, 1990; Shahidi and Pegg, 1994a, 1994b). This method is highly sensitive as it could detect hexanal concentrations below 1 ppm, which is the level when rancidity, based on sensory evaluation, is detected (Robards *et*

Figure 2.17 Mechanism of hexanal formation (Adapted from Frankel *et al.*, 1984)


H3C
11
COOH
13 12 10 9
Linoleic Acid
Initiator
•H
R
R'
Delocalized Pentadienyl Radical
O2
OO•
R''H
R''•
OOH
13-Hydroperoxyoctadeca-9,11-dienoic Acid
9-Hydroperoxyoctadeca-10,12-dienoic Acid
•OH
Homolytic Scission
O•
a
b
Pentane +
13-oxo-9,11-Tridecadeinoic
Acid
Hexanal +
a C12 Unsaturated
Fatty Acid
9-oxo-Nonanoic acid +
a C9 Unsaturated
Hydrocarbon
Octanoic Acid +
2,4-Decadienal

al.,1988). Moreover, it is ideal for routine analysis as a rapid procedure requiring no cleaning between sample injections (Snyder *et al.*, 1988).

2.9.2 Bulk oil and oil-in-water emulsion systems

The ability of different compounds to act as prooxidants or antioxidants as well as the antioxidative properties of compounds are system dependant and affected by the concentration of the compound, type of substrate, method and duration of oxidation as well as the other interacting compounds present in the system (Huang *et al.*, 1994; Hopia *et al.*, 1996; Frankel and Meyer, 2000). Therefore, testing the activity of a compound in several systems is essential in order to obtain a better understanding of the antioxidant mechanisms and prediction of the activity of the constituents in food and biological systems. Bulk oil and oil-in-water emulsion systems have commonly been used in evaluation of antioxidant efficacies of various compounds and antioxidant mixtures. Hydrophilic compounds were less active in emulsion systems compared to bulk oil systems, whereas hydrophobic compounds performed better in emulsion systems than in bulk oils (Frankel *et al.*, 1996a, 1996b; Huang and Frankel 1997; Huang *et al.*, 1994; Huang *et al.*, 1996a, 1996b; Hopia *et al.*, 1996). This was explained by Porter *et al.* (1989) as the 'polar paradox theory' indicating that polar antioxidants were more effective in non-polar lipids, whereas non-polar antioxidants were more effective in polar lipid emulsions. Partitioning of antioxidants into different phases according to their affinities towards the systems was responsible for this behaviour (Huang *et al.*, 1996). The use of corn (Huang *et al.*, 1996a,b; Hopia *et al.*, 1996; Huang *et al.*, 1994), olive (Marinova and Yanishlieva , 1997), sunflower (Yanishlieva and Marinova, 1995; Abdalla and Roozen, 1999), flax (Chen and Ahn, 1998), soybean, peanut and fish oils (Frankel

et al., 1996b) as bulk or emulsions has been reported for evaluation of the antioxidant activities of different compounds. The oxidation of linoleic acid in a micellar system with a surfactant such as Tween 20 (Huang *et al.*, 1996b) or sodium dodecyl sulphate (Foti *et al.*, 1996) has also been monitored, but was found to be less suitable as a substrate for evaluating food antioxidants. This is due to the unique nature of linoleic acid, which forms mixed micelles with surfactants. This would allow it to move freely between the three states in aqueous dispersion as emulsion particles, mixed micelles and as free molecules in the solution. In contrast, a non-polar lipid would remain within the oil droplet of an emulsion. Therefore, the interaction of antioxidants in linoleic acid systems is different than those of triacylglycerols (Hopia *et al.*, 1996). The β-carotene-linoleate model system is another model system, which has been used widely in evaluating antioxidant capacities. It is based on minimizing β-carotene loss in the coupled oxidation of linoleic acid and β-carotene using an emulsified, aqueous system. An emulsion containing the antioxidant, β-carotene and lipid is prepared using aerated distilled water and subjected to oxidation at 50°C. The destruction of carotene in the system is measured spectrophotometrically at 470 nm and the rate of decolourisation is used to evaluate the antioxidant capacity (Miller, 1971).

A variety of methods have been used to measure the oxidation in bulk oil and oil emulsion systems. Detection of the formation of hydroperoxides as the peroxide value (Marinova and Yanishlieva , 1997; Yanishlieva and Marinova, 1995) or conjugated dienes (Huang and Frankel, 1997; Abdalla and Roozen, 1999; Frankel *et al.*, 1996b), and decomposition products of hydroperoxides by thiobarbituric acid reactive substances (TBARS) (Kansci *et al.*, 1997; Shahidi *et al.*, 1994) or volatile aldehydes

(Abdalla and Roozen, 1999; Frankel *et al.*, 1996b) has been reported. When measuring these indices in an oil-in-water emulsion system, it is necessary to first extract the oil from the emulsion. This could be achieved by first extracting the oil using a methanol-hexane (1:1, v/v) mixture, then washing the methanol layer with hexane, combining the hexane extracts and finally evaporating the solvent under a stream of nitrogen (Frankel *et al.*, 1996b).

Peroxide value is determined based on the liberation of iodine from potassium iodide or conversion of ferrous to ferric ions (thiocyanate method) by peroxides, which are the main initial products of lipid oxidation of fats. It is expressed as milliequivalents (meq) of oxygen per kilogram of sample (AOCS, 1990). As oxidation proceeds, the peroxide value first increases and after reaching a maximum point starts to decline. Therefore, it does not always correlate with the extent of oxidation activities (Saito and Udagawa, 1992). It simply measures the level of peroxides in a food sample but does not evaluate fat stability under storage conditions.

Unsaturated fatty acids during oxidation, upon reaction with molecular oxygen, produce conjugated dienes (absorption maximum λ_{max} at 234 nm) and trienes (λ_{max} at 268 nm) (Shahidi and Wanasundara, 1998). Measurement of these compounds at their λ_{max} after dissolving the oil in isooctane is a simple and rapid method for assessing lipid oxidation. Weight gain of the oil due to the uptake of oxygen and formation hydroperoxides has provided a simple method to measure lipid oxidation (Wanasundara and Shahidi, 1996). The TBA test used for evaluation of oxidation in oil is the same as that explained in the meat model system with slight modifications in the methodology.

For an oil system, a known quantity of oil is dissolved in butanol and then mixed with the TBA reagent, which is also prepared using butanol and incubated at 95°C for 2 h. The absorbance of the resultant coloured complex is read at 532 nm. The headspace gas chromatographic technique used in oil systems is also similar to that of the meat model system except that a lesser amount of sample is used.

The active oxygen method (AOM), also known as the Swift test, uses accelerated oxidation conditions (passing a constant stream of air through fat, which is heated at 100°C) for evaluating fat stability. AOM determines the time required for obtaining a certain peroxide value expressed in meq O_2/ kg sample, under these experimental conditions (deMan *et al.*, 1987). The larger the AOM value, the better the flavour stability of the oil. AOM gives an evaluation of fat stability and its resistance to peroxide formation. Rancimat method provides a more rapid alternative method of evaluation in which the volatile products formed during oxidation are recorded by means of conductivity (Nawar, 1996). Formic acid produced during oxidation conditions of the Rancimat method is the main compound responsible for the conductivity change (deMan *et al.*, 1987). This method has certain drawbacks as it is continuously being aerated by air, both the anti- and pro-oxidant volatile components could be evaporated and lost. This means that the sample will change while the analysis is being carried out (Kochhar and Rossell, 1990). However, it has been shown that Rancimat results closely correlate with stability under ambient storage conditions in a wide range of vegetable oils (Gordon and Mursi, 1994).

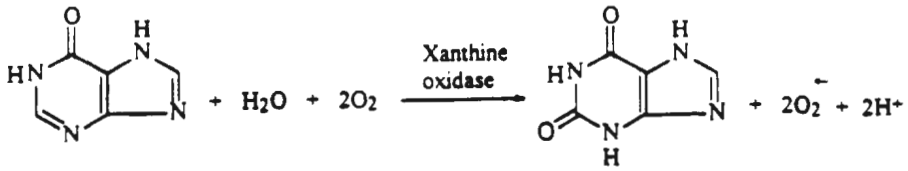
The use of nuclear magnetic resonance (NMR) to evaluate the oxidative stability of oils has been reported (Saito and Udagawa, 1992; Wanasundara and Shahidi, 1993; Senanayake and Shahidi, 1999). NMR may be used to monitor the oxidation process by measuring the ratios of aliphatic protons to that of olefinic (R_{ao}) or diallylmethylene (R_{ad}) protons (Saito and Udagawa, 1992; Wanasundara and Shahidi, 1993). These ratios were shown to increase continuously with the deterioration process (Saito and Udagawa, 1992: Wanasundara and Shahidi, 1993). Senanayake and Shahidi (1999), reported a high level of correlation between both conjugated diene values and changes in R_{ao} and R_{ad}, and TBARS and changes in R_{ao} and R_{ad}.

2.9.3 Free radical scavenging assays

Free radicals are compounds possessing an unpaired electron, which makes them highly reactive. Antioxidants neutralize free radicals by donating hydrogen atoms to them. Attempts have been made to evaluate the effectiveness of antioxidants in neutralising the free radical activity by using free radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), hydroxyl (HO$^{\bullet}$), superoxide ($O_2^{\bullet -}$) and nitric oxide (NO$^{\bullet}$) radicals under experimental conditions. In most of these methods antioxidant efficacies are measured at room temperature and it eliminates any risk of thermal degradation of the molecules tested. The radicals are detected by spectrophotometric or electron paramagnetic resonance (EPR) techniques. Spectrophotometric methods are rapid, simple and easy to handle. DPPH radical has a λ_{max} at 515-520 nm and the absorbance is decreased over a time period in the presence of radical scavengers (Bondet *et al.*, 1997; Amarowicz *et al.*, 2000; Yen and Duh, 1994; Moure *et al.*, 2000; Chu *et al.*, 2000). Superoxide radical anion can be generated enzymatically using hypoxanthine/ xanthine

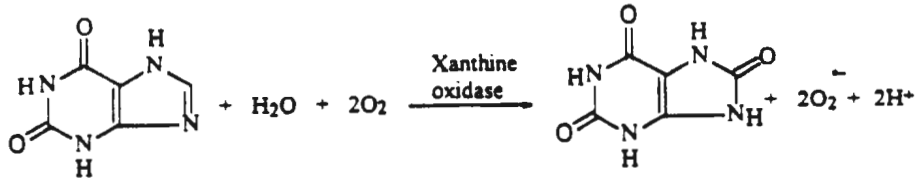
oxidase (Silva *et al.*, 1991; Wettasinghe and Shahidi, 1999) or non-enzymatically using phenazine methasulphate/ NADH (Nishikimi *et al.*, 1972) systems. The anion thus produced could reduce nitro blue tetrazolium added to the system. The reduction of the ink blue colour (λ_{max} 560 nm) of the reduced nitro blue tetrazolium in the presence of free radical scavengers directly relates to the radical scavenging capacity. The mechanism by which $O_2^{\bullet-}$ is generated is illustrated in Figure 2.18. Presence of these radicals can also be detected by EPR, which provides information about the behaviour and local environment of unpaired electrons in the system under investigation. A sample is placed in a magnetic field and subjected to microwave or infrared radiation. Unpaired electrons, if present in the sample, would orient themselves parallel or anti-parallel to the applied field having either a low or high energy level. When exposed to radiation at a frequency that matches the energy difference, it absorbs energy and moves to the higher energy level. The absorption of this energy is detected by a diode giving an electron paramagnetic spectrum. The intensity of the signal is proportional to the concentration of free radicals in the system (Mason and Chignell, 1994). The highly reactive state of the radicals makes them unstable and short lived and therefore should be trapped into a stable form in order to be detected by the EPR. Spin trapping technique is widely used in which diamagnetic compounds, usually nitroso or nitrone derivatives, and react with radicals and convert them to a more stable form (Kristensen and Skibsted, 1999). Some commonly used spin traps are 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), nitrobenzene, *N-tert*-butyl-α-phenylnitrone (PBN), α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN), 2-methyl-2-nitrosopropane, 3,3,5,5-tetramethyl-pyrriline-*N*-oxide, and 2,4,6-tri-*tert*-butyl-nitrosobenzene.

Figure 2.18 **Mechanism of which the superoxide radicals are generated and subsequent reaction of superoxide with nitro blue tetrazolium indicator (Source: Halliwell and Gutteridge, 1999)**



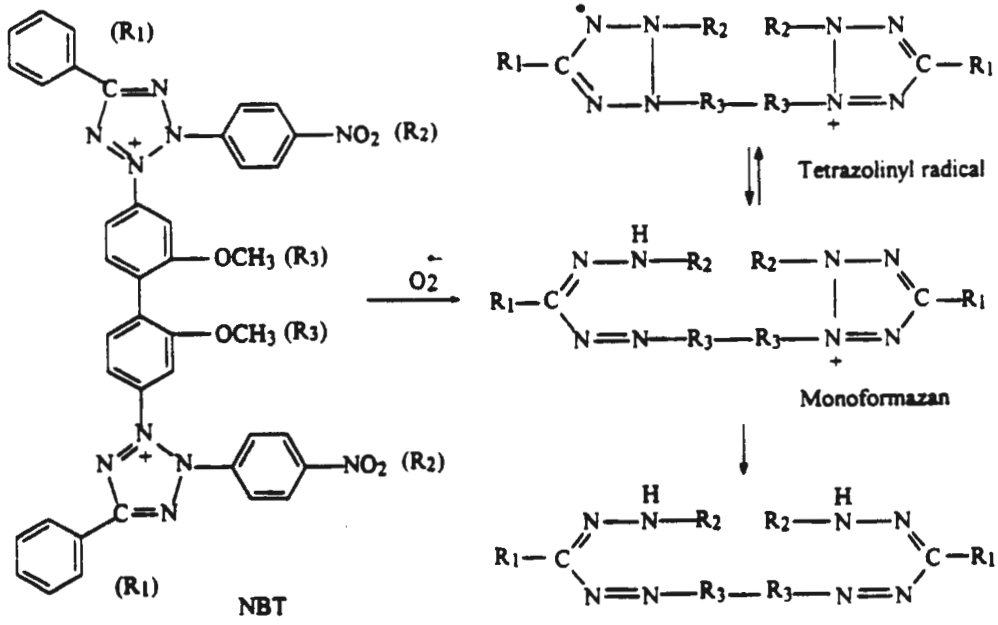
Hypoxanthine

Xanthine



Xanthine

Uric acid



NBT

Among free radical trapping assays, the total radical-trapping parameter (TRAP) assay uses the peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) to oxidise plasma antioxidants and thereby measures the total antioxidant capacity of plasma and serum (Wayner *et al.*, 1985). Trolox equivalent antioxidant capacity (TEAC) assay is another frequently used method in which 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) is first produced by the reaction between ferrymyoglobin-radical generated from metmyoglobin, and hydrogen peroxide and peroxidase; the antioxidant activity is then measured by the decrease in its absorbance at 734 nm (Miller *et al.*, 1995). TEAC reflects the amount of Trolox (mM) required in producing the same activity as 1 mM amount of a test compound (Strube *et al.*, 1997). TEAC assay was later modified by generation of $ABTS^{\bullet+}$ by the direct oxidation of ABTS with potassium persulphate (Re *et al.*, 1999) or by producing ABTS radical anion ($ABTS^{\bullet-}$) by the reaction between ABTS and AAPH (van den Berg *et al.*, 1999). The possibility to use this assay to evaluate antioxidant mixtures and unknown compounds has been demonstrated (van den Berg *et al.*, 1999). However, the ability of an antioxidant to scavenge an artificial radical may not always reflect its performance in a complex physiological system. The ferric-reducing antioxidant power (FRAP) assay directly measures the ability of antioxidants to reduce a ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to its ferrous complex (Fe^{2+}-TPTZ) at low pH. The resulting blue colour is then measured at 593 nm, which is directly proportional to the total reducing capacity of the antioxidant (Benzie and Strain, 1996). The oxygen radical absorbance capacity (ORAC) assay is another frequently used method in antioxidant assessments. It measures the fluorescence during AAPH-induced oxidation of porphyridium cruentum β-phycoerythrin (β-PE) and the antioxidant capacity is

quantified by calculating the net protection as the area under the fluorescence decay curve of β-PE in the presence of an antioxidant or serum (Cao *et al.*, 1993).

2.9.4 DNA scission studies

Protective effects of antioxidants against oxidative damage to DNA and proteins are important because compounds that protect lipids against oxidation may not necessarily protect biomolecules, and some may indirectly enhance damage to DNA (Aruoma *et al.*, 1990). Models similar to the 'test tube' assay proposed by Halliwell *et al.* (1987) to determine rate constants for reactions of hydroxyl radical have been used extensively to study the protection of antioxidants against free radical-induced DNA damage. The system may use a Fe^{2+}-EDTA chelate incubated with deoxyribose and hydrogen peroxide to induce degradation of deoxyribose in order to study the non-site specific and site specific DNA damage by hydroxyl radical (Hu and Kitts 2001). Protective effects of phenolic compounds against DNA damage, induced by peroxyl radical (Hu *et al.*, 2000) peroxynitrite (Ohshima *et al.*, 1998; Grace *et al.*, 1998), hydrogen peroxide (Jhonson and Loo, 2000), and superoxide and metals (Li *et al.*, 2000) have also been investigated.

2.9.5 LDL oxidation studies

It has been clinically proven that oxidation of low-density lipoprotein (LDL) leads to atherogenesis (Thomas and Stocker, 2000), thus protection against LDL oxidation is a vital factor in reducing the risk of cardiovascular diseases. Many methods have been developed in order to determine the inhibitory capacities of various antioxidants as well as food components against LDL oxidation. AAPH- mediated and copper-catalysed

oxidations have extensively been used in this context. The inhibitory activities have been assayed by measuring the oxidation products such as hexanal (Heinonen *et al.*, 1998; Meyer *et al.*, 1998), conjugated dienes (Kontush *et al.*, 1996; Abuja *et al.*, 1998; Hu and Kitts, 2001) and cholesteryl ester hydroperoxides (Moon and Terao, 1998). In the copper catalysed system, the antioxidant properties may also be attributed to the metal chelation activity. The ability of phenolic compounds to form complexes with proteins may provide a mechanism for the antioxidant activity by blocking copper catalysts for the binding sites of LDL and favouring the access of the antioxidant to the lipids and thereby protect them against oxidation (Satue-Gracia *et al.*, 1997).

2.10 Identification of active compounds

Once the antioxidative activity of an extract is determined the next step is to identify the components responsible for the particular activities. Identification of the compounds would provide more insight to the behaviour of extracts and the possible mechanisms of protecting different food systems.

Thin layer chromatography (TLC) and column chromatography could be used in separating antioxidant fractions for subsequent identification (Amarowicz *et al.*, 1994). This procedure has been reported for identifying compounds in sweet potato roots (Kawanishi *et al.*, 1990), flaxseed extracts (Amarowicz *et al.*, 1994), echinea, senega and wild licorice roots, bearberry leaves and horsetail aerial parts (Amarowicz *et al.*, 1999) evening primrose meals (Shahidi *et al.*, 2000), ginger (Kang-Jin-Cho *et al.*, 2001), and Moldavian dragonhead leaves and flowering parts (Povilaityte *et al.*, 2001)

High-performance liquid chromatography (HPLC) is a widely used method in detecting phenolic substances. The crude extract could be injected after filtration, as such, or fractionated and then used for identification of compounds on the basis of their retention times and UV spectrums. As many phenolics show similar ultraviolet absorption spectra with maxima in a narrow range of 280-320 nm, the quantification of phenolics by HPLC has been problematic (Jaworski and Lee, 1987). These authors proposed a method, in which first fractionation of phenolic compounds into acidic and neutral groups was done by passing the material through a preconditioned C18 SEP-PAK cartridge and then sequential injection of fractions into a HPLC column. Abou-Zaid *et al.* (2000) described a gradient chromatographic technique using HPLC in identifying phenolics compounds from deciduous leaves and coniferous needles. Identification of antioxidant compounds using HPLC analysis has been reported for rapeseed (Amarowicz *et al.*, 2001; Amarowicz and Kolodziejczyk, 2001), Moldavian dragonhead leaves and flowering parts (Povilaityte *et al.*, 2001), black currant (Mikkonen *et al.*, 2001), wines (Baderschneider and Winterhalter, 2001; Landrault *et al.*, 2001), ginger (Kang-Jin-Cho *et al.*, 2001), grape seeds (Jayaprakash *et al.*, 2001), rice bran oil (Singh *et al.*, 2000), beach pea (Shahidi *et al.*, 2001), olives (McDonald *et al.*, 2001), and prunes (Nakatani *et al.*, 2001).

An HPLC method coupled with on-line mass spectrometry (MS) analysis using an atmospheric pressured ionisation electrospray chamber has been reported for identifying proanthocyanidins in foods and beverages (Lazarus *et al.*, 1999). Singh *et al.* (1999) reported the synthesis of a diazotized sulphanilamide reagent, which is highly specific and sensitive in identifying tea catechins on paper chromatograms as well as in

spectrophotometric quantification. Gel permeation chromatographic (GPC) technique is used to estimate the molecular size of a compound and has shown good correlations with mass spectrometry data (Masuda *et al.*, 1999). They used an HPLC equipped with a GPC column to study the antioxidant mechanism and radical reaction products of curcumin. Nuclear magnetic resonance (NMR) spectral studies have been used to determine the structure of new compounds and assign structure and stereochemistry of the known compounds (Nawwar, 1994; Servili *et al.*, 1999; Hisatomi *et al.*, 2000; Baderschneider and Winterhalter, 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Almond seeds, skin, shells and shell covers were obtained from Almond Board of California (Modesto, CA). Corn oil stripped of its natural antioxidants and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Acros Organics (New Jersey, USA). Reagents sodium carbonate, sodium chloride, hexane, ethanol, methanol, trichloroacetic acid, sulphuric acid, hydrochloric acid, glacial acetic acid, butanol, isooctane and chloroform were purchased form Fisher Scientific Co. (Nepean, ON). Reagents nitro blue tetrazolium, α-tocopherol, hydrogen peroxide, 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), Trizma base, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ethidium bromide, sodium hydroxide, boric acid, butylated hydroxyanisole (BHA), 2-thiobarbutiric acid (2-TBA), 1,1,3,3-teramethoxyparopane, xanthine oxidase, hypoxanthine, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid, Tween 40 (polyoxyethylene sorbitan monopalmitate), butylated hydroxyanisole (BHA), quercetin, ferrous sulphate, Folin and Ciocalteu's phenol reagent, ascorbic acid, mono- and dibasic sodium phosphate, vanillin, tetramethylmurexide, copper sulphate, ferric chloride, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid, human low-density lipoprotein (LDL) and DNA of pBR322 (*E. coli* strain RRI) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Aldehyde standards pentenal, hexanal, heptanal and 2-heptanone were obtained from Aldrich Chemical Company (St. Louis, MO). Hexamethylenetetramine was purchased from J.T. Baker Inc. (Phillipsburg, NJ).

3.2 Methods

3.2.1 Determination of proximate composition of almond nuts

3.2.1.1 Moisture content

Approximately 3-4 g of finely powdered sample were accurately weighed into a preweighed, dry aluminium dish and placed in a forced-air convection oven (Fisher Isotemp 300, Fisher Scientific, Fair Lawn, NJ), which was preheated to 105 ± 1°C. Samples were held at this temperature overnight or until a constant mass was obtained. The moisture content was then calculated as the percent ratio of the weight difference of the sample before and after drying to that of the original material.

3.2.1.2 Protein content

Approximately 0.3-0.4 g of the finely powdered sample were accurately weighed on a nitrogen-free paper and placed in a digestion tube of Büchi digester (Büchi 321, (Büchi Laboratories, Flawil, Switzerland). The sample was digested using 20 mL of concentrated sulphuric acid in the presence of two Kjeltab catalyst tablets (Profamo, Dorval, PQ) in the digester until a clear solution was obtained. Digested samples were diluted with 50 mL of distilled water followed by addition of 150 mL of a 25% (w/v) solution of sodium hydroxide. Nitrogen in the sample was converted to ammonia, which was steam-distilled (Büchi 321, Büchi Laboratories, Flawil, Switzerland) into a 50 mL solution of 4% boric acid containing the end point indicator (EM Science, Gibbstown, NL). Approximately 200 mL of distillate were collected and the content of ammonia in the distillate was determined by titrating it against 0.1 N standard solution of sulphuric acid (AOCS, 1990). The crude protein content (%) in the sample was calculated as N% × 6.25.

3.2.1.3. Total lipid content

Total lipids were extracted into a mixture of chloroform and methanol as described by Bligh and Dyer (1959). Approximately 25 g of the sample were accurately weighed and then extracted with a mixture of 25 mL of chloroform and 50 mL of methanol (1:2, v/v) by homogenizing for 3 min with a Polytron homogeniser (Brinkmann Instruments, Rexdale, ON). A further extraction was done with the addition of 25 mL of chloroform followed by homogenisation. Approximately 25 mL distilled water were added and the mixture was then filtered through a Buchner funnel using a Whatman No. 2 filter paper. The filtrate was allowed to separate overnight in a separatory funnel. Dilution with chloroform and water resulted in separation of homogenate layers and inclusion of lipids in the chloroform. Ten-millilitre aliquots of the lipid extract in chloroform were transferred into a round bottom flask and the solvent was removed under vacuum using a Büchi RE 111 rotovapor (Büchi Laboratories, Flawil, Switzerland). The flask was then placed in a forced-air convection oven at 80°C for 1 h. After cooling in a desiccator, the lipid content was determined gravimetrically.

3.2.1.4 Ash content

Approximately 3-4 g of the sample were weighed into a clean dry porcelain crucible and charred over a Bunsen burner and subsequently placed in a temperature-controlled muffle furnace (Blue M Electric Co., Blue Island, IL), which was preheated to 550°C. Samples were held at this temperature until a grey ash was produced. It was then cooled in a desiccator and weighed. Ash content was calculated as percent ratio of the mass of the ash obtained after ignition, to that of the original material (AOAC, 1990).

3.2.2 Preparation of almond seed, skin, and outer shell cover samples

Almond whole seed, brown skin and green shell covers were ground in a coffee bean grinder (Black and Decker Canada Inc. Brockville, ON) for 10 min and then defatted by blending with hexane (1:5 w/v, 5 min × 3) in a Waring blender (Model 33BL73, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted powders were air dried for 12 h and stored in vacuum packaged polythene pouches at -20°C until further use.

3.2.3 Preparation of crude phenolic extracts

Phenolic compounds present in defatted almond samples were extracted using 80% ethanol (6g/100mL) under reflux condition in a thermostated water bath at 70°C for 30 min. The resulting slurries were centrifuged for 5 min at 4000 × g (ICE Centra MS, International Equipment Co., Needham Heights, MA) and the supernatants were collected. The residue was reextracted under the same condition, and the supernatants collected. The solvent from the combined supernatants was removed under vacuum at 40°C and the resulting concentrated solutions were lyophilised for 72h at –48°C and 46 $\times 10^{-3}$ mbar (Freezone 6, Model 77530, Labanco Co., Kansas City, MO).

3.2.4 Qualitative detection of vanillin positive compounds in almond extracts

To 1 mL of methanolic solution of crude extracts 5 mL of a freshly prepared 0.5% vanillin solution in 4% hydrochloric acid were added, mixed and allowed to stand for 20 min at 30°C. A positive test was indicated by a characteristic pink colour in the solution (Price and Butler, 1977).

3.2.5 Determination of total phenolics content

Extracts were dissolved in methanol to obtain a concentration of 1 mg/mL for seed extract and 0.5 mg/mL for skin and shell cover extracts. The content of total phenolics was determined according to a modified version of the procedure described by Singleton and Rossi (1965). Folin and Ciocalteu's reagent (0.5 mL) was added to centrifuge tubes containing 0.5 mL of the extracts. Contents were mixed and 1 mL of a saturated sodium carbonate solution was added to each tube. Volume was adjusted to 10 mL with distilled water and the contents were thoroughly mixed. Tubes were allowed to stand at ambient temperature for 45 min and then centrifuged for 5 min at 4000 × g (ICE Centra MS, International Equipment Co., Needham Heights, MA). Absorbance of the supernatants was read at 725 nm. A blank sample for each extract was used for background subtraction. Content of total phenolics in each extract was determined using a standard curve prepared for quercetin. Total extracted phenolics were expressed as mg of quercetin equivalents per g extract.

3.2.6 Determination of the content of hydrophilic and hydrophobic phenolics

The crude extract was fractionated into its hydrophilic and hydrophobic components by mixing 5 g of it with 100 mL water and 100 mL butanol in a 250 mL separatory funnel. The mixture was allowed to stand at 4°C for 12 h. The separated layers were removed and desolventised using a rotary evaporator (Büchi, Flawil, Switzerland) set at 40°C. The resulting concentrated solution was lyophilised for 72 h at –48°C and 46 $\times 10^{-3}$ mbar (Freezone 6, Model 77530, Labanco Co., Kansas City, MO). Weight of each fraction was recorded and the content of phenolics determined as explained in **Section 3.2.5.**

3.2.7 Evaluation of antioxidative activity of crude extracts

Different model systems were used for evaluating the antioxidative properties of crude extracts. The TEAC (Trolox equivalent antioxidant capacity) assay was used to evaluate the relative antioxidant capacities of crude extracts as such, and thereafter the extracts were tested at concentrations of 100 and 200 ppm phenolics as quercetin equivalents.

3.2.7.1 Measurement of total antioxidant capacity by Trolox equivalent antioxidant capacity (TEAC) assay

Total antioxidant capacity was determined according to the method described by van den Berg *et al.* (1999). All solutions were prepared in a 0.1 M phosphate buffer (pH 7.4) solution containing 0.15 M sodium chloride (PBS). A solution of 2,2'-azinobis-(3-ethlbenzthiazoline-6-sulphonate) radical anion ($ABTS^{\bullet-}$) was prepared by mixing 2.5 mM 2,2'-azobis-(2-methylpropionamidine)dihydrochloride (AAPH) with 2.0 mM $ABTS^{2-}$ stock solution. The solution was heated for 12 min at 60°C, protected from light and stored at room temperature. Blank measurements (decrease in the absorption at 734 nm due to the solvent without any compound added) were made and values recorded each time. To measure the antioxidant capacity, almond extracts were dissolved in PBS solution at a concentration of 2 mg/mL and diluted accordingly to have it fit in the range of the assay values. A standard curve was prepared by measuring the reduction in absorbance of the $ABTS^{\bullet-}$ solution at different concentrations of Trolox over a period of 6 min as the change in absorbance after 6 min of assay was marginal. The absorbance values were corrected for the solvent. TEAC values for the almond extracts were determined in the same way; a 40 μL solution of the extract were mixed with 1960 μL of

the radical solution and the absorbance was monitored for 6 min. The TEAC of an extract represents the concentration of a Trolox solution that has the same antioxidant capacity as the extract. TEAC values were determined as follows:

$$\Delta A_{Trolox} = (A_{t=0\ Trolox} - A_{t=6min\ Trolox}) - \Delta A_{solvent\ (0\text{-}6min)}$$

$$\Delta A_{Trolox} = m * [Trolox]$$

$$TEAC_{extract} = (\Delta A_{extract} / m) * d$$

Where, ΔA = reduction of absorbance, A = absorbance at a given time, m = slope of the standard curve, [Trolox] = concentration of Trolox, d = dilution factor.

3.2.7.2 β-Carotene-linoleate model system

A solution of 5 mg/10 mL β-carotene was prepared in chloroform and 2 mL of this solution were pipetted into a 100 mL round bottom flask. After chloroform was removed under vacuum using a rotary evaporator at 40°C, 40 mg of linoleic acid, 400 mg of Tween 40 emulsifier, and 100 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots of 4.8 mL of this emulsion were transferred into a series of tubes containing 100 or 200 μL of the extracts (in methanol) so that the final concentration of phenolics in the assay media was 100 or 200 ppm. The total volume was adjusted to 5 mL with methanol. BHA and quercetin were used for comparative purposes. Immediately after the addition of the emulsion to each tube, the zero time absorbance was measured at 470 nm using a Hewlett Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). Subsequent absorbance readings were recorded over a two-hour period at 15-min intervals by keeping the samples in a water bath at 50°C. Blank samples devoid of β-carotene were

prepared for background subtraction (Miller, 1971). The content of β-carotene in assay media was determined using a standard curve prepared using β-carotene.

3.2.7.3 Cooked comminuted pork model system

Ground pork (1.5 kg) was mixed with 20% (w/w) deionised water in Mason jars (height 10 cm, internal diameter 6 cm). Almond meals (1 and 2%) as well as BHA, α-tocopherol, quercetin and almond extracts (100 and 200 ppm based on phenolics) were added separately to meat, and thoroughly homogenized. A control sample containing no meal/extract was also prepared. Samples were cooked in a thermostated water bath at 80 ± 2°C (internal temperature of 72 ± 2°C) for 40 min while stirring every 5 min with a glass rod. After cooling to room temperature, the meat systems were homogenized in a Waring blender for 30 s, transferred into plastic bags and then stored in a refrigerator at 4°C for 7 days (Wettasinghe and Shahidi, 1996). Samples for the analyses of headspace gases and thiobarbituric reactive substances (TBARS) were drawn on days 0, 1, 3, 5 and 7.

3.2.7.3.1 Proximate composition of pork

Proximate composition of pork was determined using the methods discussed in section **3.2.1.**

3.2.7.3.2 Determination of TBARS in meat

Samples were analysed for TBARS over a period of 7-days, according to the method of Siu and Draper (1978) with modifications. Two grams of each sample were weighed in a centrifuge tube to which 5 mL of a 10% (w/v) solution of trichloroacetic acid

(TCA) were added and vortexed (Vortex Genie 2, Fisher Scientific, Nepean, ON) at high speed for 2 min. Aqueous solution (0.02M) of 2-thiobarbituric acid (5 mL) was then added to each centrifuge tube that was further vortexed for 30 s. The samples were then centrifuged at 3000 × g for 10 min and the supernatants were filtered through a Whatman No.3 filter paper. Filtrates were heated in a boiling water bath for 45 min, cooled to room temperature in ice, and the absorbance of the resultant pink-coloured chromogen was read at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane as the malonaldehyde (MA) precursor. TBARS values were calculated using the standard curve and expressed as mg MA equivalents /kg sample.

3.2.7.3.3 Static headspace gas chromatographic analysis

A Perkin-Elmer 8500 gas chromatograph and an HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) were used for volatile analysis of cooked comminuted lean pork samples. A high polarity Supelcowax 10 fused silica capillary column (30 m × 0.32 mm internal diameter, 0.10 mm film thickness, Supelco Canada Ltd., Oakville, ON) was used. The carrier gas helium was employed at an inlet column pressure of 17.5 psig with a split ratio of 7:1. The oven temperature was maintained at 40°C for 5 min and then ramped to 100°C at 20°C/min and held there for 5 min. The injector and flame ionisation detector (FID) temperatures were adjusted to 280°C and held at there throughout the analysis (Wettasinghe and Shahidi, 1996).

For headspace analysis, 4.0 g of homogenized meat were transferred to 5 mL glass vials. The vials were then capped with teflon-lined septa, crimped and kept at -60°C (Ultra Low, Revco, Inc., West Columbia, SC) until used. To avoid heat shock after

removal from storage, frozen vials were tempered at room temperature for 30 min and then preheated in the HS-6 magazine assembly at 90°C for 45 min equilibrium period. Pressurization time of the vial was 6 s, and the volume of the vapour phase drawn was approximately 1.5 mL. Chromatographic peak areas were expressed as integrator count units. Individual volatile compounds were identified by comparing relative retention times of GC peaks with commercially available standards. Quantitative determination of dominant aldehydes was accomplished using 2-heptanone as an internal standard (Wettasinghe and Shahidi, 1996).

3.2.7.4 Bulk stripped corn oil model system

Extracts and standards (BHA, α-tocopherol, quercetin) and oil (5 g) were mixed well in 30 mL capped glass tubes (13 cm × 2 cm internal diameter) so that the final concentrations of phenolics were 100 or 200 ppm. Samples were placed in a forced-air convection oven (Thelco, Model 2, Precision Scientific Co., Chicago, IL) at 60°C for seven days. Samples for analyses of conjugated dienes (CD), TBARS analysis were drawn on days 0,1,3,5 and 7. Method for determining volatiles was the same as that for the meat model system, but only 200 mg of oil were used.

3.2.7.4.1 Determination of conjugated dienes

Conjugated dienes of samples were measured according to the IUPAC (1987) method. Oil was weighed (0.02-0.04 g) into a 25 mL volumetric flask, diluted and made up to the mark with isooctane (2,2,4-trimethylpentane). The solution was thoroughly mixed and its absorbance read at 243 nm using a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). Pure

isooctane was used as the reference. Conjugated dienes were calculated as, CD = A/(c*d) where, A = absorbance of the solution at 234 nm; c = concentration of the oil in g/100 mL; and d = length of the cell (cm).

3.2.7.4.2 Determination of 2-thiobarbituric acid reactive substances (TBARS)

A direct method for determination of TBARS was employed using the AOCS (1990) procedure. Oil (50-200 mg) was accurately weighed into 25 mL volumetric flask and dissolved in a small quantity of 1-butanol and made up to volume with the same solvent. Five millilitres of this solution were transferred into a dry test tube to which fresh TBA (5 mL) reagent (200 mg TBA in 100 mL butanol) was added. Contents were thoroughly mixed and heated in a water bath at 95°C for 120 min. Heated samples were cooled in an ice bath and the absorbance of the resultant coloured complex was read at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane as the malonaldehyde (MA) precursor. TBARS were calculated using the standard curve and values expressed as μmoles MA equivalents/g oil.

3.2.8 Evaluation of reactive oxygen species- and DPPH free radical-scavenging efficacies of almond components

3.2.8.1 Hydrogen peroxide scavenging assay

Almond seed, skin and shell cover extracts were dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) solution and mixed with 600 mL of 43 mM solution of hydrogen peroxide (prepared in the same buffer). Quercetin was used as the reference compound. Final concentrations of extracts and standards were 100 or 200 ppm. The absorbance values (at 230 nm) of the reaction mixtures were recorded initially at 0 min,

and then for 40 min at every 10 min. For each concentration, a separate blank sample devoid of hydrogen peroxide was used for background subtraction (Ruch *et al.*, 1989). The concentration of hydrogen peroxide in the assay media was determined using a standard curve and hydrogen peroxide scavenging capacities of the extracts were calculated using the following equation.

Hydrogen peroxide scavenging capacity, % = 100 - (hydrogen peroxide concentration of medium containing the additive of concern / hydrogen peroxide concentration of the control medium) * 100

3.2.8.2 Superoxide radical-scavenging assay

A modified version of the method explained by Nishikimi *et al.* (1972) was employed. Superoxide radicals were generated via an enzymatic reaction. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of 100 mIU xanthine oxidase, 1 mL of 12 mM diethylenetriaminepentaacetic acid, 1 mL of 178 μM nitro blue tetrazolium and 1 mL of the extracts (final concentration of the phenolics in the reaction mixture was 100 or 200 ppm as quercetin equivalents). The reference antioxidant was quercetin. All solutions were prepared in a 0.1 M phosphate buffer (pH 7.4) solution. The absorbance values of the mixtures at 560 nm were recorded initially at 0 min, and thereafter every 10 min for up to 60 min. Superoxide radical-scavenging capacities (after 10 min of assay) of the additives were calculated using the following equation.

Superoxide radical scavenging capacity, % = 100 - (absorbance of medium containing the additive of concern / absorbance of the control medium) *100

3.2.8.3 Hydroxyl radical-scavenging assay

A modified version of a method described by Shi *et al.* (1991) was used to determine the hydroxyl radical-scavenging ability of almond extracts. The hydroxyl radicals were generated via iron-catalysed Haber-Weiss reaction and spin trapped with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The resultant DMPO-OH adduct was detected using an electron paramagnetic resonance (EPR) spectrometer (Bruker ESP 300, Bruker Instruments, Inc., Billeria, MA). Almond extracts and quercetin were dissolved in 0.1M-phosphate buffer (pH 7.4) so that a 200 μL aliquot would afford 100 or 200 ppm of phenolics in the final assay medium (final volume was 800 μL). Extracts (200 μL) were mixed with 200 μL of 0.075 mM DMPO, 200 μL of 2.5 mM ferrous sulphate and 200 μL of 2.5 mM hydrogen peroxide. All solutions were prepared in a 0.1 M phosphate buffer (pH 7.4) solution. After 3 min, 100 μL of the mixture were taken into a syringe and transferred into a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at 8×10^5 receiver gain, 1.0 G modulation amplitude, 167.77 s sweep time, 3460 G centre field, 100 G sweep width and 0.655 s time constant. Hydroxyl radical scavenging capacities of the additives were calculated using the following equation.

Hydroxyl radical scavenging capacity, % = 100 - (EPR signal intensity for the medium containing the additive of concern / EPR signal intensity for the control medium) * 100

3.2.8.4 DPPH (2,2-diphenyl-1-picrylhydazyl) radical-scavenging assay

DPPH radical-scavenging assay was performed using a modified version of the method described by Santiago *et al.* (1992). A 100 μL of a 0.5 mM solution of DPPH in ethanol was added to a 100 μL of a solution containing almond extracts so that the concentration of phenolics in the final assay medium was either 100 or 200 ppm as quercetin equivalents. Contents were mixed and after 1 min 100 μL of the mixture was drawn into a syringe and transferred to a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at 2×10^5 receiver gain, 1.0 G modulation amplitude, 167.77 s sweep time, 3460 G centre field, 100 G sweep width and 0.655 s time constant. The reference antioxidant was quercetin. DPPH radical-scavenging capacities of the additives were calculated using the following equation.

DPPH radical scavenging capacity, % = 100 - (EPR signal intensity for the medium containing the additive of concern / EPR signal intensity for the control medium) *100

3.2.9 Evaluation of iron (Fe^{2+}) chelating activity of almond extracts

Ferrous sulphate (400 μM), almond extracts, quercetin and 1 mM tetramethylmurexide were respectively dissolved in a hexamine-HCl buffer (10 mM, pH 5.0) containing 10 mM potassium chloride. Solutions of 1 mL ferrous sulphate and 1 mL extracts were mixed followed by the addition of 0.1 mL of 1 mM tetramethylmurexide. The final concentration of quercetin and almond extracts was 100 or 200 ppm (as quercetin equivalents). Absorbance at 460 nm (A_{460nm}) and 530 nm (A_{530nm}) were read and the ratio of A_{460nm} to A_{530nm} calculated. A standard curve was used to determine the

amount of free Fe^{2+} left after chelation by the extracts (Terasawa *et al.*, 1991). Iron chelation capacity of additives was calculated using following equation.

Iron (Fe^{2+}) chelation capacity, % = 100- (Absorbance ratio for medium containing the additive of concern/ absorbance ratio for the control) * 100

3.2.10 Supercoiled DNA strand scission by peroxyl and hydroxyl radicals

Plasmid DNA (pBR322) was dissolved in a 10 mM phosphate buffered saline (PBS, pH 7.4, 0.15 mM sodium chloride). DNA (150 ng) was mixed with quercetin and almond extracts dissolved in the same buffer to attain final concentrations of 2, 5, 10, 50 and 100 ppm. Peroxyl radical was generated using AAPH at a final concentration of 9 mM and reactants (total volume 12 μL) were incubated at 37°C for 2 h in the dark. (Hu *et al.*, 2000). The hydroxyl radical was generated using 100 μM EDTA, 100 μM ferric chloride, 100 μM ascorbic acid and 1 mM hydrogen peroxide for non site-specific hydroxyl radical generation (Halliwell *et al.*, 1987). For site-specific hydroxyl radical generation EDTA was replaced with PBS (Hu and Kitts, 2001). Ferric chloride and ascorbic acid were dissolved in deionised water immediately before use. Reaction mixtures were incubated at 37°C for 1 h.

After incubation 2 μL of the loading dye (consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose in distilled water) were added to the sample and loaded to a 0.8% (w/v) agarose gel prepared in 40 mM Tris-acetate/ 2 mM EDTA buffer (pH 8.5). Gel electrophoresis was performed in the same buffer using a horizontal gel electrophoresis apparatus at 60 volts for 3 h. The gels were stained by ethidium bromide

(0.5 μg/mL) and visualized under ultraviolet light. Images were taken and analysed using AlphaEase™ Stand Alone software (Alpha Innotech Corporation, San Leandro, CA). The protective effect of the additives was measured using the retention percentage of supercoiled DNA as given below.

$$\textit{DNA retention, \%} = \frac{\textit{DNA content with the oxidative radical and extract}}{\textit{DNA content without the oxidative radical}} \textit{ X 100\%}$$

3.2.11 Cupric ion induced human low density lipoprotein (LDL) oxidation

Human LDL in PBS (pH 7.4, 0.01% EDTA) was dialyzed against 10 mM PBS (pH 7.4, 0.15 M NaCl) at 4°C for 24 h. The obtained EDTA-free human LDL was further diluted to obtain a concentration of 0.2 mg of protein/mL, and mixed with quercetin or almond extracts at 10, 50, 100 and 200-ppm levels. Oxidation was initiated by adding 10 μM copper sulphate and incubating at 37°C for 20 h (Hu *et al.*, 2000). The formed conjugated dienes due to oxidation of human LDL were measured by reading the absorbance at 234 nm. The inhibitory effects of the extracts on the formation of conjugated dienes were calculated according to the equation given below.

$$\textit{\% Inhibition of conjugated dienes} = \frac{Abs_{oxidative} - Abs_{sample}}{Abs_{oxidation} - Abs_{native}} \textit{ X 100\%}$$

Where, $Abs_{oxidative}$= Absorbance at 234 nm of human LDL with copper sulphate; Abs_{sample} = Absorbance at 234 nm of human LDL with copper sulphate and extracts; and Abs_{native} = Absorbance at 234 nm of human LDL without copper sulphate.

3.2.12 Identification of active compounds

3.2.12.1 Preparation of samples for analysis

Native and hydrolysed extracts were used for high performance liquid chromatographic (HPLC) analysis. Crude native almond extracts were dissolved in methanol (10 mg/mL) and filtered through a 0.45 μM nylon filter. For hydrolysis, 50 mg of extract were dissolved in 10 mL of 1.2 M HCl in 50% (v/v) aqueous methanol containing 25 mg of TBHQ as an antioxidant. The sample was hydrolysed at 90°C for 2 h and then filtered through a 0.45 μM nylon filter (Crozier *et al.*, 1997).

3.2.12.2 Analysis of flavonoids

A Shimadzu HPLC system (Mandel Scientific Co., Ltd., Guelph, ON) was employed (two LC 10AD pumps, SPD M10A diode array detector, SACL AA system controller, CTO 10AS column oven). The conditions for separation were as follows: pre-packed LUNA C18 column (5 μM, 4.6 × 250 mm; Phnomenex); mobile phase: A – 15% (v/v) acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid (TFA), B- 35% (v/v) acetonitrile with water adjusted to pH 2.5 with TFA, gradient 0-100% B, 0-35 min, 100% B, 35-45 min, 100% A 45-55 min; flow rate 1 mL/ min, injection volume 20 μL, the detector was set at 320 nm, column temperature 30°C (Crozier *et al.*, 1997).

3.2.12.3 Analysis of phenolic acids

Free phenolic acids and those liberated from soluble esters and glycosides were isolated from the extract according to the method described by Weidner *et al.* (1999). An aqueous suspension of the extract (800 mg in 20 mL water) was adjusted to pH 2 (6 M HCl), and free phenolic acids were extracted 5 times into 20 mL diethyl ether using a

separatory funnel. The ether extract was evaporated to dryness under vacuum at room temperature. The aqueous solution was neutralized and then lyophilised. The residue was dissolved in 20 mL of 2 M NaOH and hydrolysed for 4 h under a nitrogen atmosphere at room temperature. After acidification to pH 2 using 6 M HCl, phenolic acids released form soluble esters were extracted from the hydrolysate 5 times into 30 mL diethyl ether using a separatory funnel. To the water solution, 15 mL of 6 M HCl were added, and the solution obtained was placed under a nitrogen atmosphere and hydrolysed for 1 h in a water bath at 100°C. Phenolic acids released from soluble glycosides were separated from the hydrolysate 5 times into 45 mL diethyl ether. After ether evaporation, the dry residue was dissolved in 10 mL methanol and filtered through 0.45 μM nylon filter. A Shimadzu HPLC system was employed as described in section **3.2.12.2.** The separation conditions were as follows: pre-packed LiChrosper 100 RP-18 column (5 μM, 4 × 250 mm; Merck); mobile phase, water-acetonitrile-acetic acid (88:10:2, v/v); flow rate, 1 mL/min: injection volume, 20 μL; detector was set at 320 nm, column temperature 30°C

3.2.13 Statistical analysis

All experiments were carried out in triplicates. The significance of differences among mean values were determined at $p \leq 0.05$ using analysis of variance (ANOVA) followed by Tukey's least honest significant difference (HSD) test (Snedecor and Cochran, 1980).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Proximate composition of almond

The contents of moisture, ash, crude protein and total lipids of almond were 2.7±0.2, 2.9±0.1, 25.3±0.9 and 48.8±0.9% (w/w%), respectively. These values are comparable with published data (USDA, 2001).

4.2 Antioxidative activity of almond meals

A cooked comminuted pork model system was first used to evaluate the potential of defatted almond meals as inhibitors of lipid oxidation. The moisture content, ash, crude protein and total lipids of pork used in this study were 72.3 ± 0.3, 1.70 ± 0.04, 19.8 ± 0.2 and 7.6 ± 0.3% (w/w%), respectively. Defatted almond meals were added at 1 and 2% (weight of sample/weight of pork) levels. The oxidation of the ground cooked pork samples, stored at 4°C for 7 days, was monitored using 2-thiobarbituric acid (TBA) test and static headspace chromatographic analysis. The results for TBARS, hexanal and total volatiles are given in Tables 4.1, 4.2 and 4.3, respectively. Defatted almond whole seed, brown skin and green shell meals exhibited a significant ($p < 0.05$) inhibitory effect on the formation of TBARS, hexanal and total volatiles when employed at 1 and 2% levels. The inhibitory effect of extracts was concentration-dependant as evidenced by the results displayed in Figures 4.1 and Figure 4.2; samples containing 2% (w/w) of the meals had lower values. Among the three meals, TBARS and hexanal formation of the brown skin and green shell cover meals were significantly lower ($p \leq 0.05$) than those observed for the whole seed meal at both 1 and 2% levels. The results

Table 4.1 Thiobarbituric acid reactive substances (TBARS) (μg malonaldehyde equivalents / kg sample) in a meat model system in the presence of almond meals at 1 and 2% levels over a 7-day storage period at 4°C[1]

Additive	Storage time (days)				
	0	1	3	5	7
Control	1.2 ± 0.1[a]	8.1 ± 0.1[c]	10.8 ± 0.6[b]	18.4 ± 0.4[c]	23.2 ± 1.2[c]
WSE 1%	1.1 ± 0.1[a]	8.0 ± 0.3[d]	10.2 ± 0.6[b]	16.9 ± 0.4[b]	20.9 ± 0.6[b]
BSE 1%	1.0 ± 0.1[a]	2.2 ± 0.1[a]	2.8 ± 0.1[a]	3.1 ± 0.1[a]	3.5 ± 0.1[a]
GSE 1%	1.1 ± 0.1[a]	2.9 ± 0.1[b]	3.1 ± 0.1[a]	3.2 ± 0.1[a]	3.5 ± 0.1[a]
Control	1.2 ± 0.1[a]	8.1 ± 0.1[c]	10.8 ± 0.6[c]	18.4 ± 0.4[c]	23.2 ± 1.2[c]
WSE 2%	1.0± 0.1[a]	6.6 ± 0.4[b]	9.9 ± 0.5[b]	12.3 ± 0.7[b]	14.7 ± 0.8[b]
BSE 2%	1.0 ± 0.1[a]	2.0 ± 0.1[a]	2.1 ± 0.1[a]	2.3 ± 0.1[a]	2.6 ± 0.1[a]
GSE 2%	1.1 ± 0.1[a]	2.5 ± 0.1[a]	2.6 ± 0.1[a]	2.8 ± 0.1[a]	3.0 ± 0.1[a]

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript within a level are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

Table 4.2 Hexanal content (mg/ kg sample) produced in a meat model system in the presence of almond meals at 1 and 2% levels over a 7-day storage period at 4°C[1]

Additive	Storage Time (days)				
	0	1	3	5	7
Control	6.3 ± 0.2[b]	9.9 ± 0.4[b]	15.3 ± 0.9[c]	19.4 ± 1.1[c]	25.7 ± 1.4[c]
WSE 1%	3.0 ± 0.1[a]	3.3 ± 0.1[a]	5.7 ± 0.1[b]	6.6 ± 0.2[b]	11.8 ± 0.4[b]
BSE 1%	2.9 ± 0.1[a]	3.2 ± 0.2[a]	3.4 ± 0.2[a]	3.5 ± 0.4[a]	3.6 ± 0.1[a]
GSE 1%	2.9 ± 0.1[a]	3.2 ± 0.1[a]	3.4 ± 0.1[a]	3.5 ± 0.1[a]	3.6 ± 0.1[a]
Control	6.3 ± 0.2[b]	9.9 ± 0.4[c]	15.3 ± 0.9[c]	19.4 ± 1.1[c]	25.7 ± 1.4[c]
WSE 2%	2.1 ± 0.1[a]	2.5 ± 0.2[b]	6.5 ± 0.4[b]	7.2 ± 0.4[b]	7.9 ± 0.4[b]
BSE 2%	2.0 ± 0.1[a]	2.4 ± 0.1[b]	2.8 ± 0.1[a]	2.8 ± 0.1[a]	2.8 ± 0.1[a]
GSE 2%	1.8 ± 0.1[a]	1.8 ± 0.1[a]	2.5 ± 0.1[a]	2.5 ± 0.1[a]	2.6 ± 0.1[a]

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript within a level are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

Table 4.3 Total volatiles (mg/ kg sample) produced in a meat model system in the presence of almond meals at 1 and 2% levels over a 7-day storage period at 4°C[1]

Additive	Storage Time (days)				
	0	1	3	5	7
Control	25.3 ± 1.1^{c}	40.8 ± 2.2^{d}	42.5 ± 2.5^{c}	51.6 ± 1.6^{d}	64.2 ± 2.3^{d}
WSE 1%	20.1± 1.1^{b}	22.9 ± 1.1^{c}	27.2 ± 1.4^{b}	35.3 ± 1.4^{c}	43.4 ± 1.7^{c}
BSE 1%	11.3 ± 0.9^{a}	15.1 ± 0.8^{b}	18.2 ± 0.9^{a}	30.1 ± 2.1^{b}	30.6 ± 1.9^{b}
GSE 1%	10.1 ± 0.5^{a}	12.2 ± 0.6^{a}	17.9 ± 1.1^{a}	20.6 ± 1.6^{a}	22.6 ± 1.8^{a}
Control	25.3 ± 1.1^{d}	40.8 ± 2.2^{d}	42.5 ± 2.5^{d}	51.6 ± 1.6^{d}	64.2 ± 2.3^{d}
WSE 2%	15.4 ± 0.7^{c}	21.9 ± 1.3^{c}	26.2 ± 1.4^{c}	30.9 ± 1.6^{c}	34.6 ± 1.6^{c}
BSE 2%	7.7 ± 0.3^{b}	8.5 ± 0.4^{b}	18.1 ± 1.1^{b}	22.8 ± 1.5^{b}	29.2 ± 1.6^{b}
GSE 2%	4.0 ± 0.2^{a}	4.2 ± 0.3^{a}	14.1 ± 1.0^{a}	15.9 ± 1.1^{a}	16.7 ± 1.1^{a}

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript within a level are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract

Figure 4.1 **Effect of almond meals at 1% (w/w) level on the formation of thiobarbituric acid reactive substances (TBARS), hexanal and total volatiles in a cooked comminuted meat model system stored at 4°C for 7 days.**

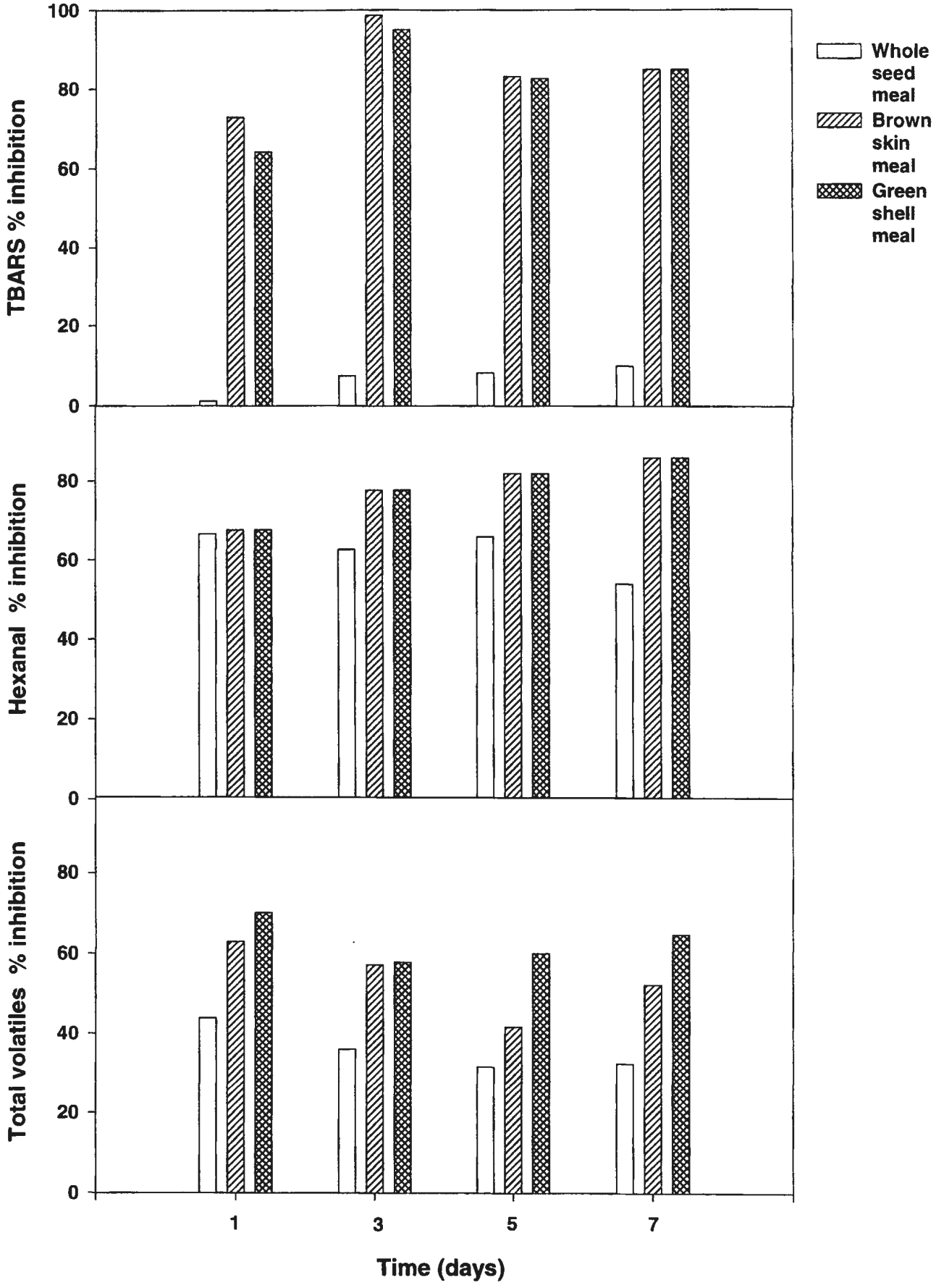
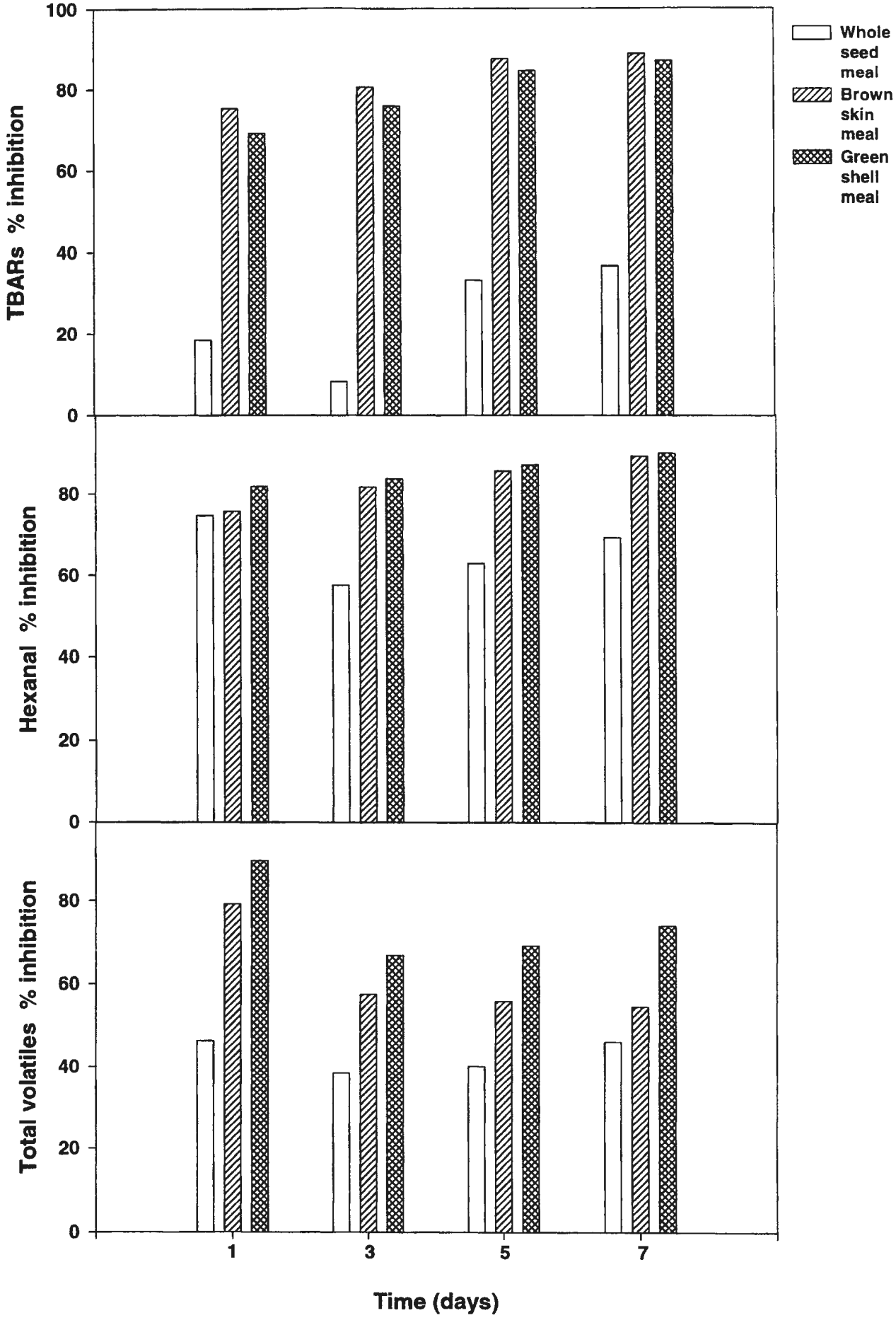

TBARS % inhibition
Hexanal % inhibition
Total volatiles % inhibition
0
20
40
60
80
100
1
3
5
7
Time (days)
Whole seed meal
Brown skin meal
Green shell meal

Figure 4.2 **Effect of almond meals at 2% (w/w) level on the formation of thiobarbituric acid reactive substances (TBARS), hexanal and total volatiles in a cooked comminuted meat model system stored at 4°C for 7 days.**


TBARs % inhibition
Hexanal % inhibition
Total volatiles % inhibition
0
20
40
60
80
100
1
3
5
7
Time (days)
Whole seed meal
Brown skin meal
Green shell meal

for total volatile contents were significantly ($p < 0.05$) different from one another for different meals at both concentrations. At 2% level, the green shell cover meal had a more pronounced effect in inhibiting the formation of total volatiles by 74%, whereas the brown skin and the whole seed meals inhibited the formation of total volatiles by 54 and 36%, respectively.

Results indicate the potential of defatted almond whole seed, brown skin and green shell cover meals as inhibitors of oxidation in cooked comminuted pork. Since these almond components exhibited antioxidant activities, preparation of extracts was carried out subsequently.

4.3 Yield and phenolic contents of almond extracts

Preliminary experiments and close scrutiny of literature data indicated that extraction conditions were as best when carried out in 80% ethanol, at 70°C for 40 min to obtain the highest amounts of phenolic extracts from almond meals. All almond extracts gave a positive vanillin test that indicated the presence of condensed tannins. Furthermore, preliminary studies showed the presence of quercetin or structurally similar compounds in the extracts. Therefore, the total phenolic contents were reported as quercetin equivalents and the antioxidative activities of extracts were compared with it. Other antioxidants were also used depending on the model system employed in testing.

The highest yield of the ethanolic extract, 41±3% (w/w), was obtained from the green shell and the lowest, 8±1% (w/w), from brown skin. However, despite its lowest

yield, the brown skin had the highest content of phenolics, and the brown skin and green shell covers contained 11- and 9- times more total phenolic compounds than that of the defatted whole seed extracts (Table 4.4). The almond seed alone without its brown skin contained only 0.31±0.03 mg total phenolics/ g of defatted meal. The inner hard shell of almond did not produce a sufficient quantity of ethanolic extract, thus these two components: almond seed alone without its brown skin and the inner hard shell were excluded from subsequent testing. Further investigations were focussed on the antioxidant capacities of the whole almond seed extract (WSE) with the brown skin, the brown skin extract (BSE) and outer green shell extract (GSE).

The ratio of hydrophilic to hydrophobic phenolics was 1:2, 3:8 and 2:5 for WSE, BSE and GSE, respectively. Generally, the hydrophobic phenolic contents of the phenolic extracts were 2-3 times higher than those of their hydrophilic counterparts. The antioxidant activity of the extracts depends on the type of oxidisable substrate and the composition of the model system (Frankel and Meyer, 2000). Activity in a particular model system is influenced by the partitioning properties of the antioxidants between the lipid and aqueous phases (Frankel *et al.*, 1994, 1996a,b). The hydrophobic and hydrophilic compounds present in a mixture of antioxidants are responsible for the strength of their antioxidant activities (Huang *et al.*, 1994). Since a crude plant extract contains a mixture of compounds having different polarities it could be expected that they exhibit varying antioxidant capacities depending on the model system used. Therefore, to gain a better understanding of the mechanisms involved in their antioxidant activities, various model systems, free radical scavenging assays, DNA scission studies and human LDL oxidation model systems were employed to evaluate

Table 4.4 Yield and total phenolic contents of almond extracts[1]

Extract	Yield of extract (g/ 100g of defatted powder)	Phenolics (mg quercetin equivalents / g ethanolic extract)		
		Total	Hydrophilic	Hydrophobic
WSE	18.63 ± 1.55[b]	8.05 ± 0.87[a]	3.11 ± 1.13[a]	6.23 ± 1.32[a]
BSE	8.28 ± 0.92[a]	87.76 ± 1.75[c]	24.42 ± 1.63[c]	65.40 ± 2.14[c]
GSE	40.54 ± 2.52[c]	71.03 ± 1.74[b]	21.31 ± 1.42[b]	51.73 ± 2.42[b]

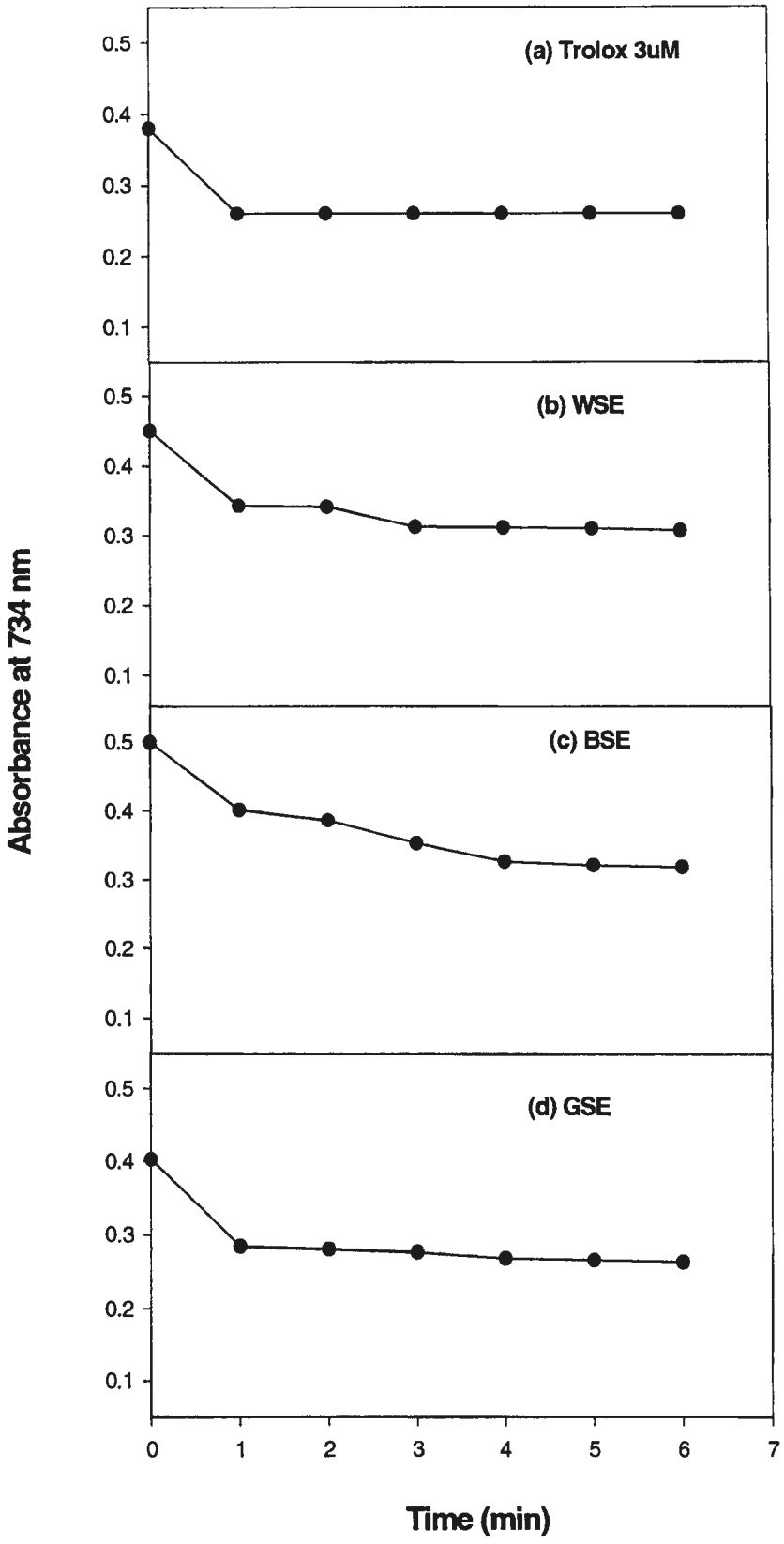
[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

the efficacy of almond WSE, BSE and GSE. The results are presented and discussed in the following sections.

4.4 Total antioxidant capacity of almond extracts

Trolox equivalent antioxidant capacity (TEAC) assay was employed to evaluate the antioxidant capacities of water-soluble compounds in a preparation as the experiment is performed in an aqueous buffer. The antioxidant capacity of the extracts is determined through the measurement of the absorbance of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid radical anion ($ABTS^{\bullet-}$) at 734 nm in the presence of the extracts, and compared with that of Trolox, a water-soluble vitamin E analogue. The TEAC of a compound determines its antioxidant capacity relative to that of Trolox on a mole basis. TEAC of an unknown mixture represents the concentration of a Trolox solution that has the same activity as the unknown mixture under investigation. The unknown compounds are tested at a concentration in which they fit in the range of the absorbance (0.5 - 0.1) (van den Berg *et al.* 1999). The almond extracts were tested at a concentration of 2 mg/mL and diluted accordingly to achieve the best fit. Trolox reacts instantaneously with $ABTS^{\bullet-}$ and the reaction is completed within one minute (Figure 4.3). On the other hand, almond extracts show a biphasic reaction in which the reaction is completed after 6 min. This type of reaction has also been reported by van den Berg *et al.* (2000) for a series of flavonoids tested. These authors suggested that total TEAC (TEAC after 6 min) could be used in predicting antioxidative capacities of structurally related compounds as it correlates positively with the inhibition of lipid peroxidation and thus protection against doxorubicin-induced cardiotoxicity (van den Berg *et al.*, 2000). TEAC after 6 min, therefore, was used to evaluate the antioxidant capacities of almond

Figure 4.3 **Reaction of Trolox (3μM) and almond extracts at a concentration of 2mg/ mL with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical anion ($ABTS^{\bullet-}$)**


(a) Trolox 3uM
(b) WSE
(c) BSE
(d) GSE
Absorbance at 734 nm
Time (min)
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7

extracts (Table 4.5). As TEAC can be used to provide a ranking order of the antioxidant capacity of unknown mixtures (van den Berg *et al.*, 1999), the capacities of almond extracts at the same extract concentration were in the increasing order of WSE < GSE < BSE. Moreover, it could be stated that the TEAC of BSE and GSE are 13- and 10-times greater than that of WSE. The total phenolic content of the extracts showed a similar pattern hence it could be suggested that at a given extract concentration BSE and GSE would exhibit more effective antioxidative activities compared to that of WSE.

4.5 Antioxidant activity of crude almond extracts

In the following model systems and free radical scavenging assays, concentrations of the extracts tested were 100 and 200 ppm phenolics as quercetin equivalents and in the text are referred to as 100 and 200 ppm for convenience.

4.5.1 β-Carotene-linoleate model system

In this model system, β-carotene is subjected to decolourisation due to oxidation by the free radicals formed from linoleic acid. The presence of a phenolic antioxidant, by neutralizing the linoleate free radical, hinders the extent of β-carotene decolourisation. As the change in β-carotene content after 120 min was marginal, the retention of β-carotene over 120 min of assay was used to evaluate the efficacies of the extracts (Table 4.6). The initial mass of β-carotene in the assay media (5 mL) was 75 ± 2μg. The control sample devoid of any additive lost 98% of its initial β-carotene after 120 min of assay. As shown in Figures 4.4 and 4.5 the amount of β-carotene in the control, as measured by the absorbance at 470 nm, was reduced exponentially. In contrast in the presence of additives this reduction followed a second order polynomial

Table 4.5 Trolox equivalent antioxidant capacity (TEAC) values of almond extracts[1]

Extract	Concentration (mg/mL)	Dilution factor	TEAC
WSE	2	1	4.21 ± 0.24^{a}
BSE	2	10	52.9 ± 0.88^{c}
GSE	2	10	41.05 ± 0.65^{b}

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

Table 4.6 Retention of β-carotene after 2 h assay in a β-carotene-linoleate model system at 100 and 200 ppm[1]

Additive	Retained quantity of β-carotene (μg) (Initial content 75 ± 2 μg)	
	100	200
WSE	62.98 ± 0.24[c]	71.96 ± 0.14[d]
BSE	55.71 ± 0.18[b]	62.39 ± 0.29[b]
GSE	57.49 ± 0.15[b]	69.49 ± 0.17[c]
Quercetin[2]	68.44 ± 0.12[d]	73.24 ± 0.15[d]
BHA[2]	67.75 ± 0.14[d]	71.26 ± 0.12[d]
Control	1.79 ± 0.41[a]	1.79 ± 0.41[a]

[1]Results are mean values of three determinations ± standard deviation.
[2]Refernce antioxidants.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, butylated hydroxyanisole.

Figure 4.4 **Effect of almond extracts at 100 ppm, as quercetin equivalents, on the retention of β-carotene in a β-carotene-linoleate model system at 50°C for 120 min**

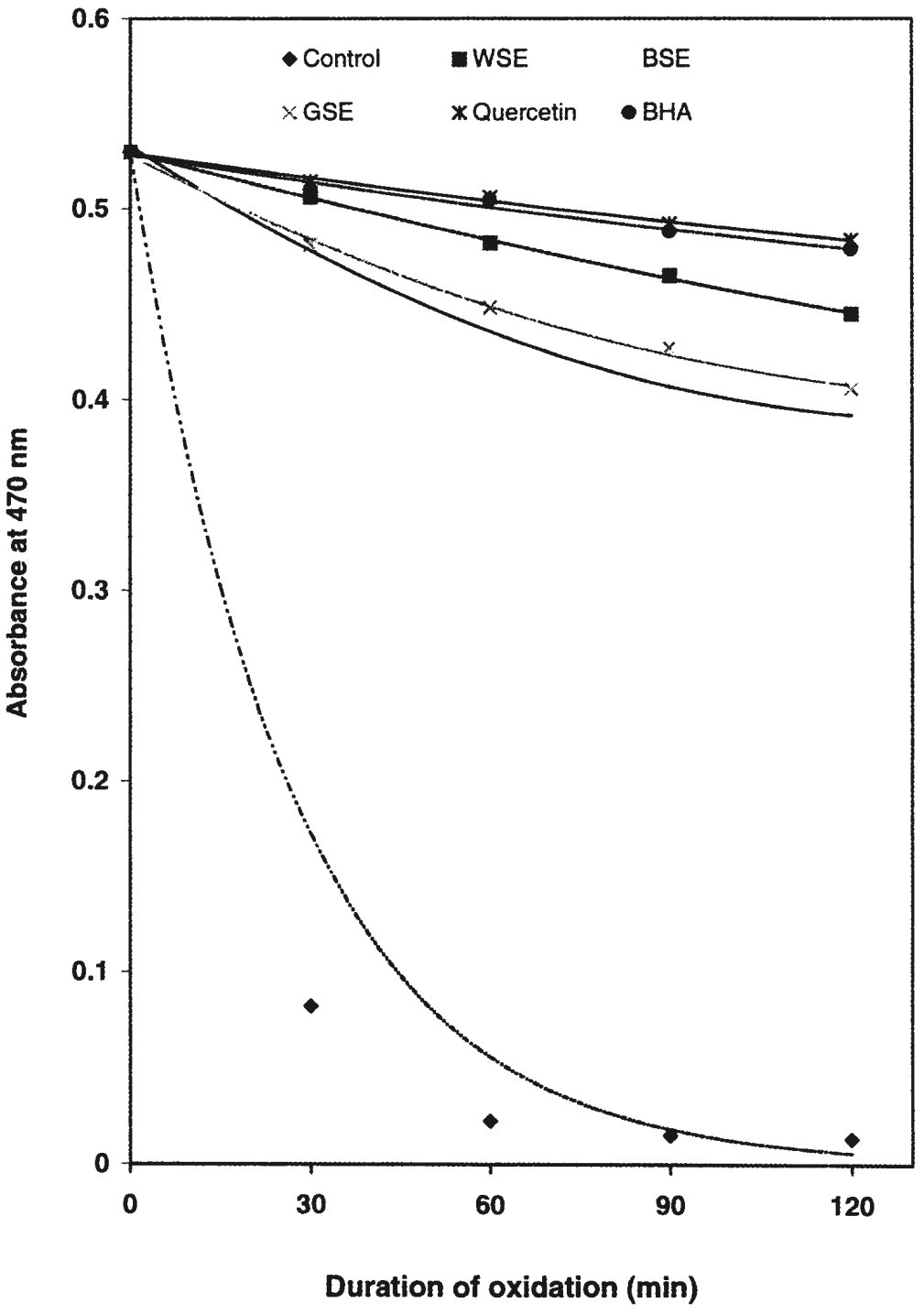
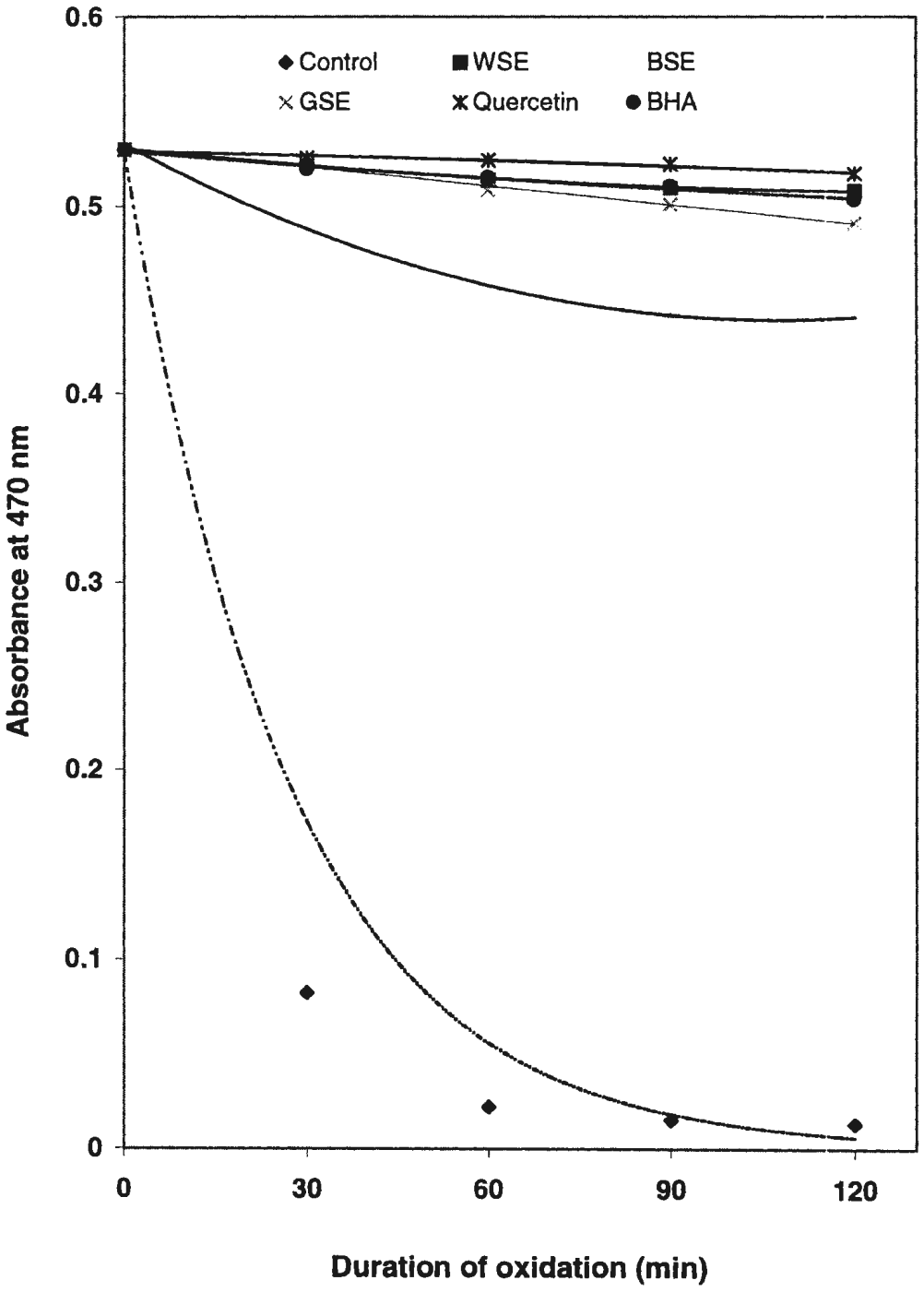

Control
WSE
BSE
GSE
Quercetin
BHA
Absorbance at 470 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
30
60
90
120
Duration of oxidation (min)

Figure 4.5 **Effect of almond extracts at 200 ppm, as quercetin equivalents, on the retention of β-carotene in a β-carotene-linoleate model system at 50°C for 120 min**


0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 470 nm
Control
WSE
BSE
GSE
Quercetin
BHA
0
30
60
90
120
Duration of oxidation (min)

pattern showing a significantly ($p \leq 0.05$) high retention of β-carotene compared to the control. The retention was more pronounced at the 200 ppm level compared to that of the 100 ppm. The highest retention (98% of the initial content) of β-carotene was observed for quercetin at 200 ppm and the activity of WSE and BHA at the same concentration was comparable to that of quercetin. BSE, which had the lowest activity, was still able to retain 83% of the initial β-carotene content.

The β-carotene-linoleate model is similar to an oil-in-water emulsion system and variations in activities could be attributed to the differences in the proportion of hydrophobic and hydrophilic compounds present in each extract. The hydrophobic antioxidants are likely to perform more efficiently than hydrophilic antioxidants in preventing oxidation in oil-in-water emulsion systems by orienting in the oil, and oil-water interface (Frankel and Meyer, 2000). This is true when considering the effectiveness of β-carotene retention by quercetin and BHA, which are lipophilic in nature. However, this pattern was not observed with the almond extracts. WSE with the highest proportion of hydrophilic compounds compared to BSE and GSE was most effective, whereas BSE having the highest proportion of hydrophobic compounds was least effective in preventing β-carotene bleaching. Even though the hydrophilic compounds are diluted in the aqueous phase, if present in high quantities, they still can reduce the oxygen-derived free radicals in the aqueous phase. Some of the phenolic compounds may exist in their glycosylated form, which would be measured as hydrophilic compounds, but the glycosylated phenolic components have not always been effective in antioxidative activity (Bocco *et al.*, 1998). Another possibility is the presence of potent hydrophobic antioxidants in the extract, which even at small concentrations could effectively inhibit

lipid oxidation. The variation may also be due to linoleic acid, which behaves in a different manner when compared to other lipids. Though non-polar compounds generally tend to remain within the oil phase in an emulsion, it has been observed that linoleic acid could form micelles which would allow it to move among different phases in an emulsion system (Hopia *et al.*, 1996). As such, hydrophobic as well as hydrophilic groups could exert protective effects against linoleate oxidation and thereby prevent β-carotene decolourisation.

4.5.2 Cooked comminuted pork model system

This model system which was first used to evaluate the antioxidant potential of almond meals at 1-2 % levels was also used to examine the behaviour of the crude extracts at 100 and 200 ppm as quercetin equivalents. Standards used for comparative purposes were quercetin, α-tocopherol and BHA. The antioxidant effects of the crude extracts were less than those observed for their corresponding meals at 1 and 2% (w/w) levels. All three extracts showed a concentration-dependant activity in lowering TBARS (Tables A.7 and A.8), hexanal (Tables A.9 and A.10) and total volatiles (Tables A.11 and A.12). The control with no additives showed the highest values for all three indices. The trends in inhibition of oxidation by the additives, as evaluated by the inhibition of formation of TBARS, hexanal and total volatiles, slightly varied on different days of storage and therefore, results at the end of the storage period were used to elaborate the differences of the activity exerted by the additives. Almond extracts at 100 and 200 ppm levels inhibited the formation of TBARS, hexanal and total volatiles by 2-36 and 22-74%, 20-44 and 54-76%, and 1-23 and 42-70%, respectively (Figures 4.6 and 4.7). Inhibition of TBARS formation by BSE was significantly ($p \leq 0.05$) higher than those of

Figure 4.6 **Effect of almond extracts at 100 ppm, as quercetin equivalents, on the formation of thiobarbituric acid reactive substances (TBARS), hexanal and total volatiles in a cooked comminuted meat model system at 4°C for 7 days. Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, butylated hydroxyanisole.**

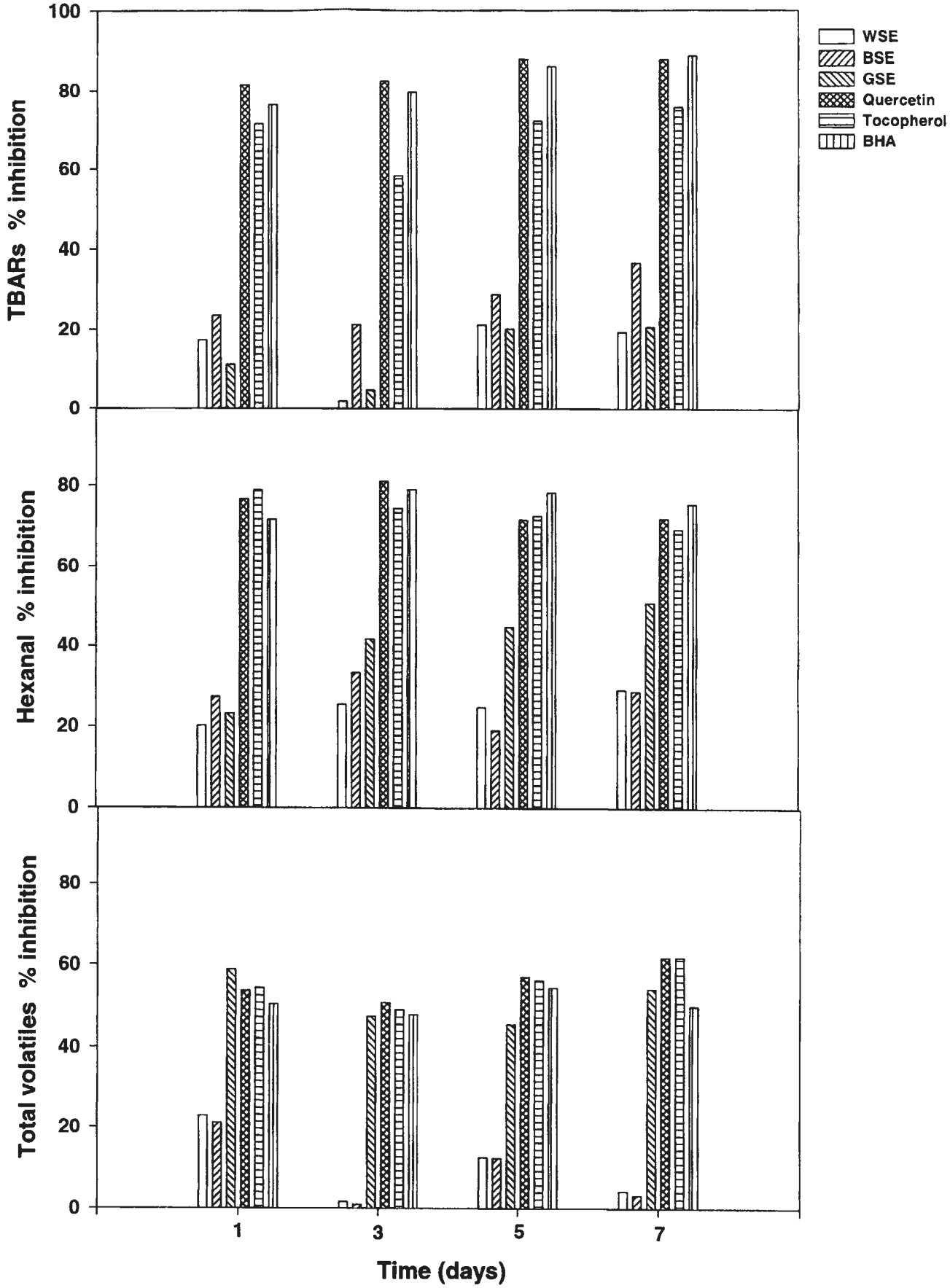
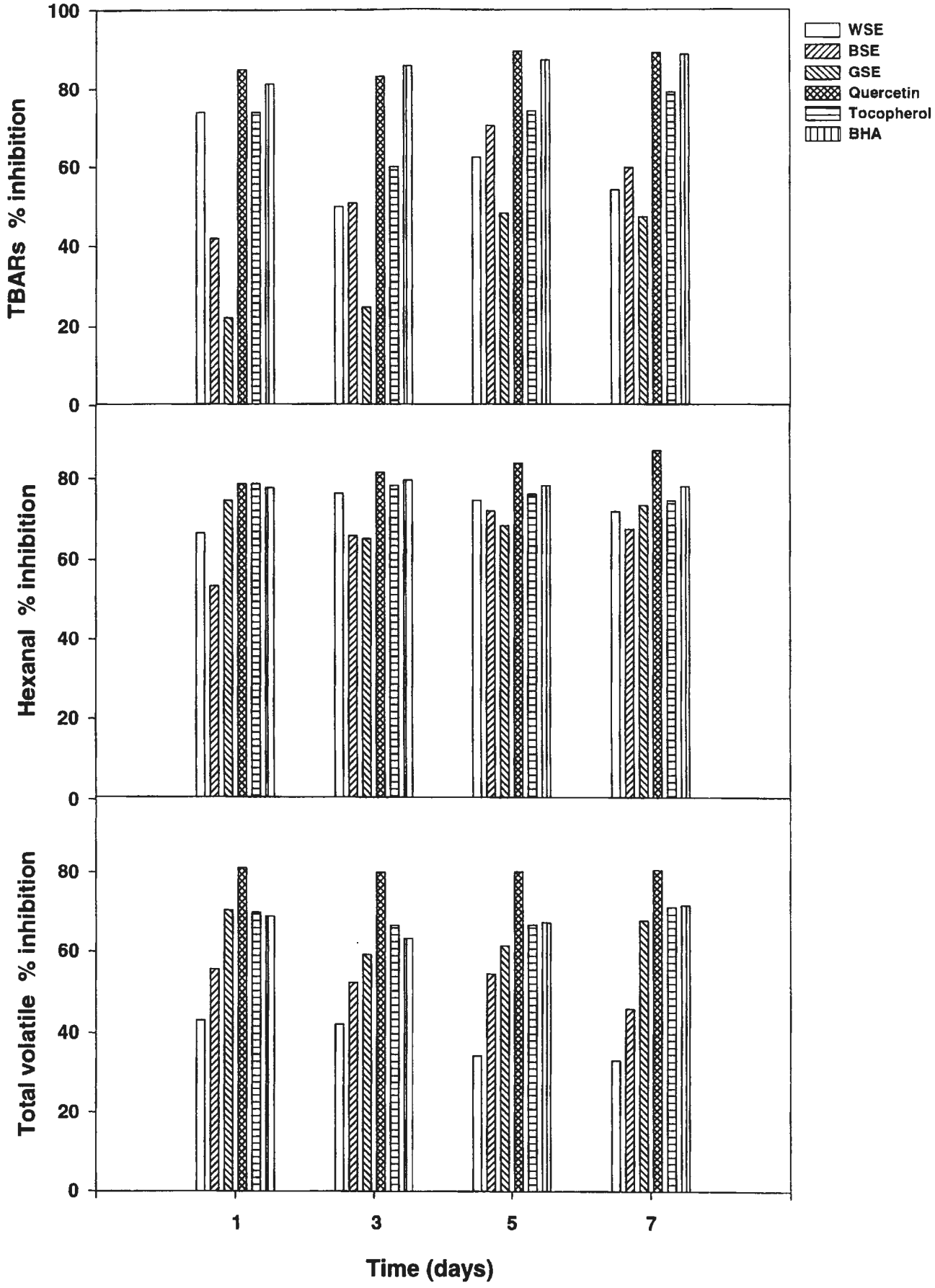

WSE
BSE
GSE
Quercetin
Tocopherol
BHA
TBARs % inhibition
Hexanal % inhibition
Total volatiles % inhibition
0
20
40
60
80
100
1
3
5
7
Time (days)

Figure 4.7 **Effect of almond extracts at 200 ppm as quercetin equivalents on the formation of thiobarbituric acid reactive substances (TBARS), hexanal and total volatiles in a cooked comminuted meat model system maintained at 4°C for 7 days. Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, butylated hydroxyanisole.**


WSE
BSE
GSE
Quercetin
Tocopherol
BHA
TBARs % inhibition
Hexanal % inhibition
Total volatile % inhibition
0
20
40
60
80
100
1
3
5
7
Time (days)

WSE and GSE, but significantly ($p \leq 0.05$) lower than those of the reference antioxidants, quercetin, α-tocopherol and BHA. For hexanal and total volatile formation, the inhibition by GSE was not significantly ($p > 0.05$) different from those of BHA and α-tocopherol. The inhibition of hexanal formation at 200 ppm at the end of storage period was in the order: quercetin > α-tocopherol ~ BHA ~ GSE > BSE > WSE. The inhibition of formation of total volatiles by the additives followed the same order but the observed differences between GSE and BSE, and BSE and WSE were not significantly ($p > 0.05$) different, whereas values for GSE and WSE were significantly ($p < 0.05$) different from one another. Thus, it may be concluded that GSE and BSE are more efficient than WSE in inhibiting lipid oxidation in a pork model system. As both hydrophobic and hydrophilic compounds are present in substantial amounts in the extracts they could effectively inhibit lipid oxidation. The mechanisms by which the extracts contribute to antioxidative activities in cooked comminuted pork could be due to their free radical scavenging, hydrogen peroxide decomposing, as well as metal chelating abilities.

4.5.3 Bulk corn oil system

Commercially available corn oil, stripped of its endogenous antioxidants, was used to evaluate the efficacy of different extracts on its oxidative stability. Inhibition of the formation of conjugated dienes (CD) (primary oxidation products), hexanal and TBARS (secondary oxidation products) in treated oil samples was used as indicators of antioxidant activity of the additives of concern.

The conjugated diene, hexanal and TBARS values of bulk stripped corn oil containing almond extracts at 100 and 200 ppm are given in Tables A.1 to A.6 in the

Appendix. Figures 4.8 and 4.9 depict the % inhibition of formation of CD, hexanal and TBARS at 100 and 200 ppm levels, respectively. The most effective additive in reducing the oxidation level was quercetin and the least active one was α-tocopherol. The conjugated diene values of bulk stripped corn oil increased by 4-5 fold at the end of the 7-day storage period, whereas the control samples showed a 6-fold increase. At 100 and 200 ppm levels the additives inhibited the formation of conjugated dienes by 0-38% and 3-55%, respectively. This oxidation inhibitory activity of extracts and standards decreased in the order: quercetin > GSE > BHA > WSE > BSE > α-tocopherol. Hexanal content in oil samples increased throughout the storage period but there was a significant ($p \leq 0.05$) decrease in their formation when additives were used, compared with those of the control. At the higher concentration level almond extracts were able to reduce the formation of hexanal in corn oil by 82-93%. The inhibition of hexanal formation by the additives used decreased in the order of quercetin > GSE ~ BSE ~ WSE ~ BHA > α-tocopherol. TBARS values of the control showed a 5-fold increase at the end of a 7-day storage period; with the addition of almond extracts there was a 2-3-fold increase at 200 ppm. On the 7^{th} day of storage at 100 and 200 ppm levels the additives inhibited TBARS by 28-55% and 19-74%, respectively. The order of the activity followed a similar trend to that observed for conjugated dienes but BHA showed a significantly ($p \leq 0.05$) higher inhibition than that of GSE. These results show that GSE was more effective than WSE and BSE in reducing primary as well as secondary oxidation in a bulk oil system. α-Tocopherol, a lipophilic antioxidant, exerted the least protection against oxidation of bulk corn oil, which could be attributed to its partitioning in the bulk lipid phase than orienting at the oil-air interface where a better protection could have been gained. This concept, however, is not applicable with the crude

Figure 4.8 **Effect of almond extracts at 100 ppm, as quercetin equivalents, on the formation of conjugated dienes (CD), hexanal and thiobarbituric acid reactive substances (TBARS), in a bulk corn oil system at 60°C for 7 days. Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, butylated hydroxyanisole.**

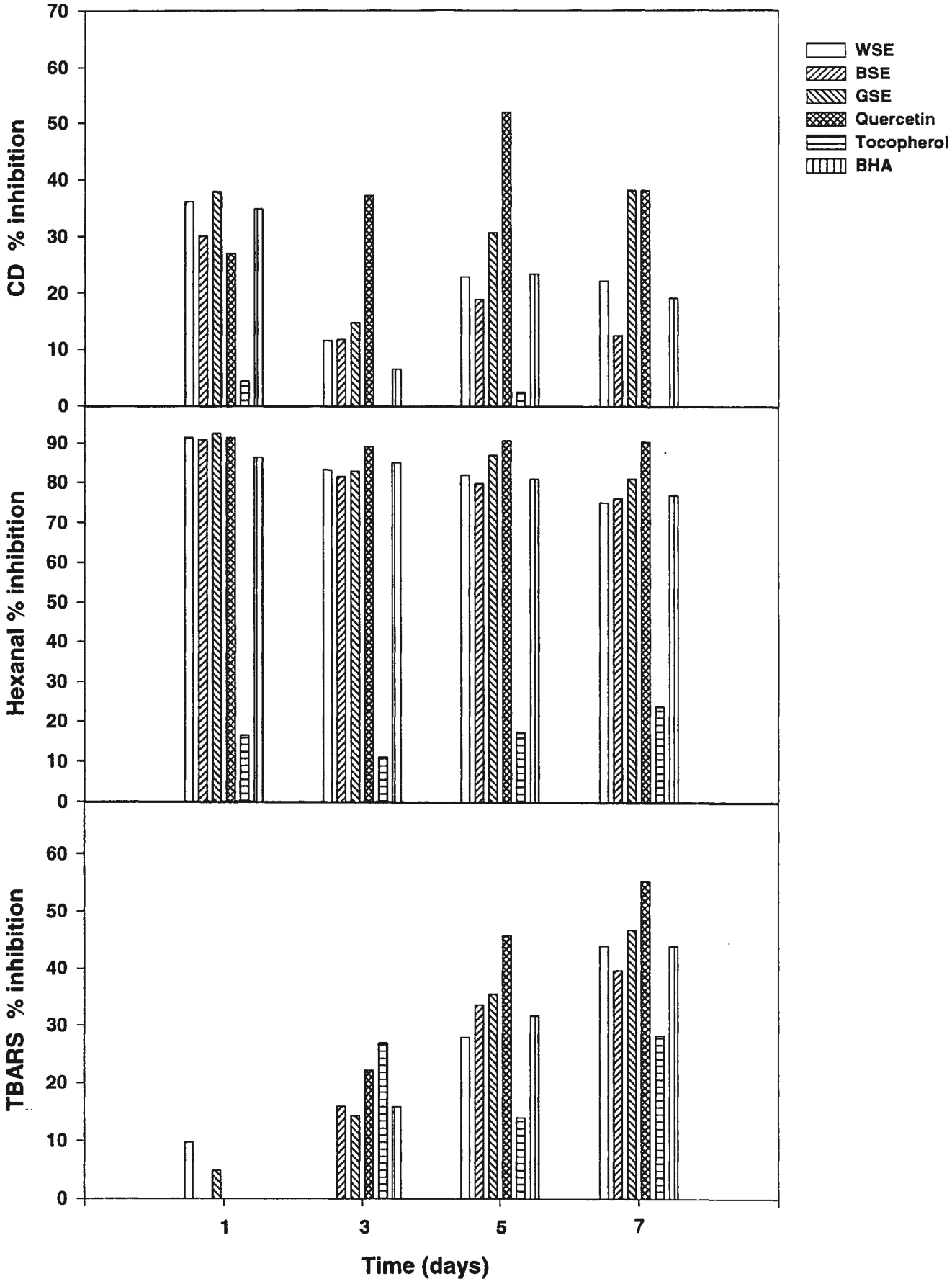
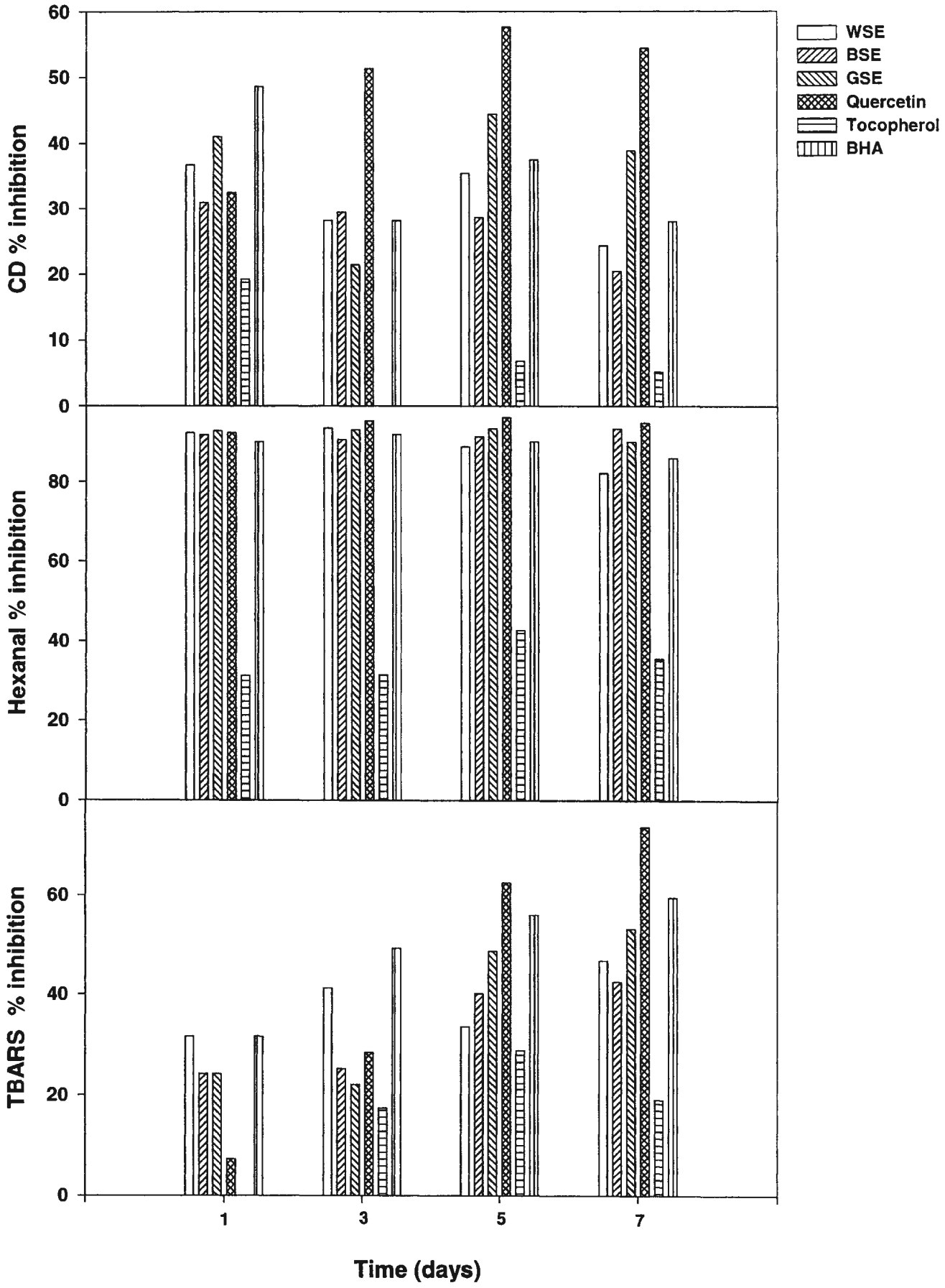

WSE
BSE
GSE
Quercetin
Tocopherol
BHA
CD % inhibition
Hexanal % inhibition
TBARS % inhibition
Time (days)
0
10
20
30
40
50
60
70
80
90
1
3
5
7

Figure 4.9 **Effect of almond extracts at 200 ppm, as quercetin equivalents, on the formation of conjugated dienes (CD), hexanal and thiobarbituric acid reactive substances (TBARS), in a bulk corn oil system at 60°C for 7 days. Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, butylated hydroxyanisole.**


WSE
BSE
GSE
Quercetin
Tocopherol
BHA
CD % inhibition
Hexanal % inhibition
TBARS % inhibition
0
10
20
30
40
50
60
80
1
3
5
7
Time (days)

extracts containing a mixture of hydrophobic and hydrophilic compounds. The overall activity of almond extracts will not only depend on the ratio of these two but also on the activity of individual compounds in each fraction.

4.6 Evaluation of the reactive oxygen species scavenging activities of almond extracts

4.6.1 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the extracts was concentrate-dependent (Figure 4.10). Within the first 10 min most of the hydrogen peroxide was scavenged as shown in Table 4.7. At 100 ppm level, all additives showed significantly ($p < 0.05$) different scavenging capacities, but at 200 ppm both BSE and GSE showed a similar 91% scavenging activity, which was not significantly ($p \geq 0.05$) different from that of quercetin. The hydrogen peroxide scavenging activity of WSE was inferior to other extracts. Although hydrogen peroxide is a weak initiator of lipid peroxidation, its potential to produce highly reactive oxygen species, such as hydroxyl radical through Fenton reaction is high (Yoshikawa *et al.*, 1997). Hydrogen peroxide is found to be poorly reactive in aqueous solutions and is toxic to cells at 10-100 μM levels and can cross biological membranes rapidly to form cytotoxic hydroxyl radicals (Halliwell and Gutteridge, 1999). Thus, hydrogen peroxide-scavenging activity of almond extracts may contribute to the inhibition of lipid peroxidation and protection of cells from oxidative damage.

4.6.2 Superoxide radical-scavenging activity

The superoxide radical is a powerful oxidizing agent that could react with

Figure 4.10 **Hydrogen peroxide scavenging activity of almond extracts as exhibited by the reduction of absorbance of hydrogen peroxide at 230 nm**

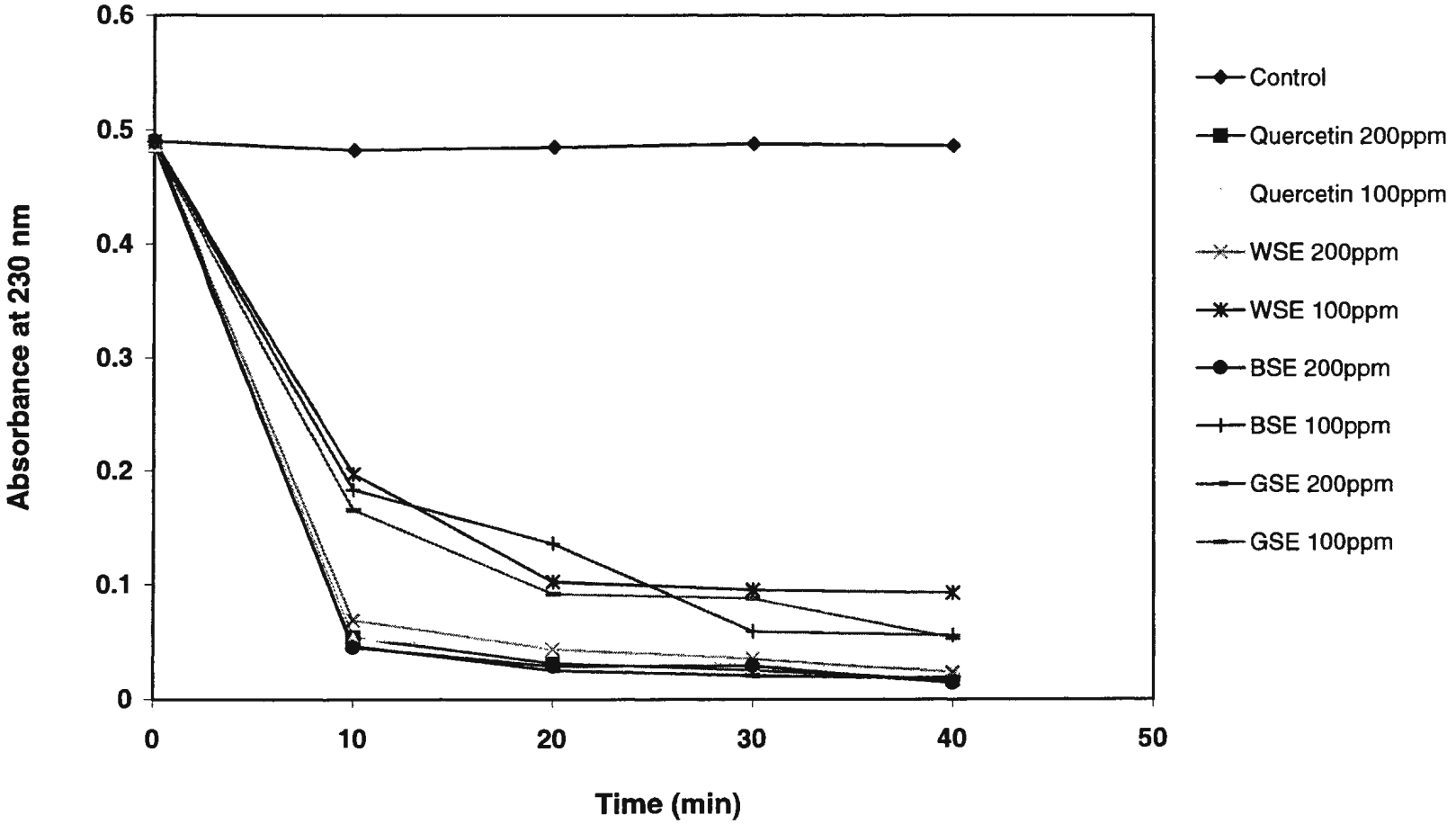

0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 230 nm
0
10
20
30
40
50
Time (min)
Control
Quercetin 200ppm
Quercetin 100ppm
WSE 200ppm
WSE 100ppm
BSE 200ppm
BSE 100ppm
GSE 200ppm
GSE 100ppm

Table 4.7 Hydrogen peroxide scavenging activity of almond extracts at 100 and 200 ppm (as quercetin equivalents)[1]

Extract	H_2O_2 scavenging capacity, %	
	100	200
Quercetin	89 ± 2^{c}	89 ± 1^{b}
WSE	59 ± 2^{a}	86 ± 1^{a}
BSE	$63 \pm 1^{a,b}$	91 ± 1^{b}
GSE	66 ± 1^{b}	91 ± 1^{b}

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

biological membranes and induce tissue damage (Yoshikawa *et al.*, 1997). It also decomposes to singlet oxygen, hydroxyl radical or hydrogen peroxide (Niki, 1997). In this study, superoxide radical was enzymatically generated using a hypoxanthine/ xanthine oxidase system. The spectrophotometric measurement of the ink blue colour of reduced nitro blue tetrazolium in the presence of superoxide anion was used as a means of evaluating the scavenging activity of the extracts. The continuous development of the ink blue colour in the control showed the generation of superoxide radical. All three almond extracts showed a dose-dependent scavenging activity (Figure 4.11). GSE gave an outstanding 99% radical scavenging activity, which was comparable with that of quercetin after 10 min assay (Table 4.8). BSE and WSE, at 200 ppm level, inhibited superoxide radical by 95 and 85%, respectively. This superoxide radical scavenging potential of extracts, especially of the BSE and GSE, is one of the major mechanisms by which the extracts exert their antioxidant capacities.

4.6.3 Hydroxyl radical-scavenging activity

Hydroxyl radicals were generated by Fenton driven Haber Weiss reactions. The formed hydroxyl radicals were then spin trapped with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) to form DMPO-OH adduct due to the short lifetime of the hydroxyl radicals. The EPR spectrum of the DMPO-OH adduct, after the addition of extracts is presented in Figure 4.12. The % reduction of the intensity of the signals is presented in Table 4.9. The intensity of the signal was reduced but did not completely disappear at 100 ppm for any of the extracts. The minimum concentration required for a 100% inhibition was exhibited by BSE and quercetin at 200 ppm and 300 ppm, respectively. Intensity reduction at higher concentrations of WSE and GSE extracts could not be measured

Figure 4.11 **Superoxide radical-scavenging activity of almond extracts as exhibited by the reduction of the intensity of the reduced nitro blue tetrazolium indicator in a hypoxanthine/xanthine oxidase enzymatic system**

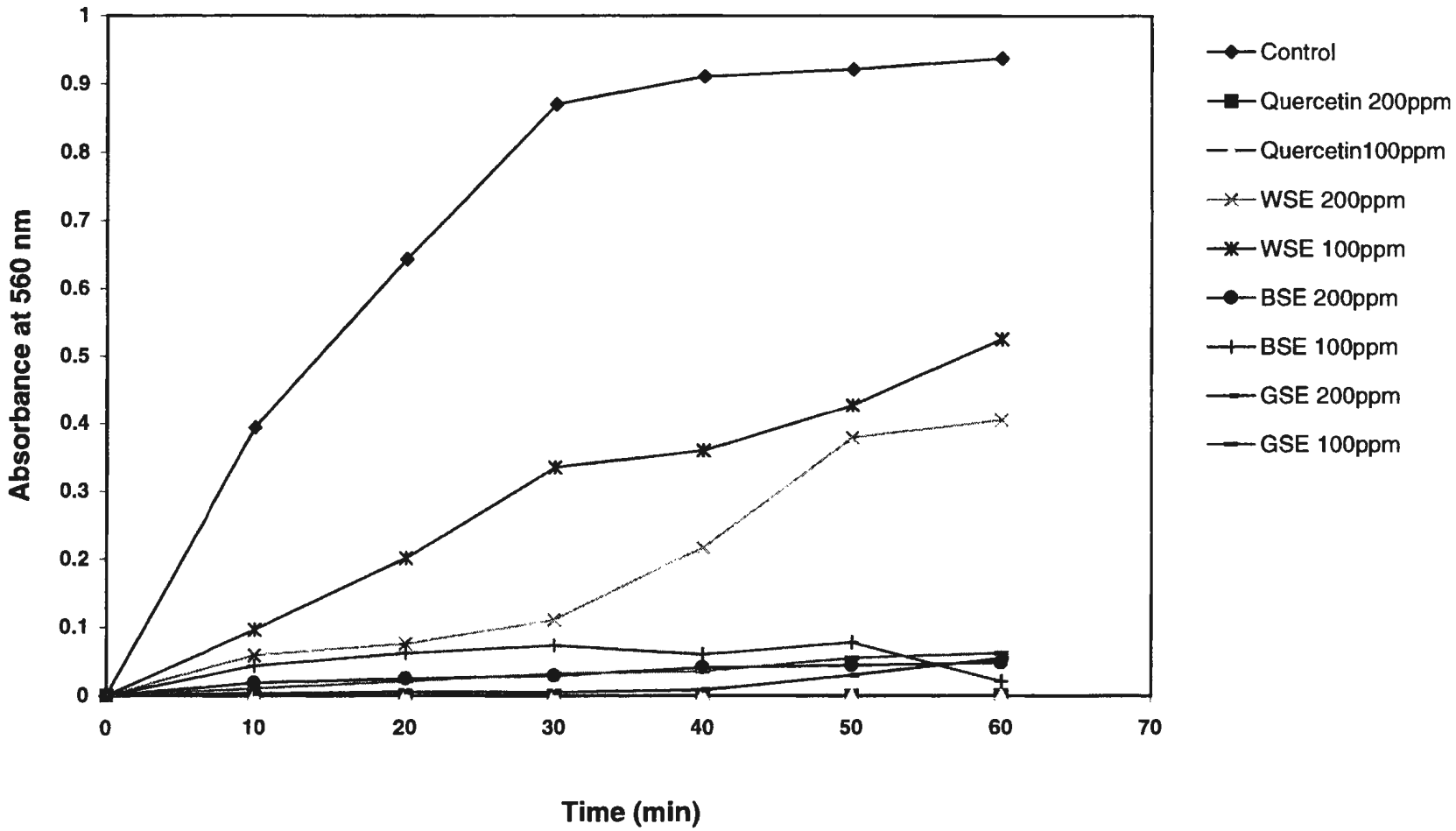
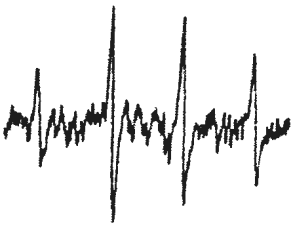

Absorbance at 560 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Time (min)
Control
Quercetin 200ppm
Quercetin100ppm
WSE 200ppm
WSE 100ppm
BSE 200ppm
BSE 100ppm
GSE 200ppm
GSE 100ppm

Table 4.8 Superoxide radical-scavenging activity of the almond extracts at 100 and 200 ppm (as quercetin equivalents)[1]

Extract	Superoxide radical-scavenging capacity, %	
	100	200
Quercetin	99 ± 1[c]	99 ± 1[c]
WSE	76 ± 1[a]	85 ± 1[a]
BSE	89 ± 1[b]	95 ± 1[b]
GSE	97 ± 1[c]	99 ± 1[c]

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

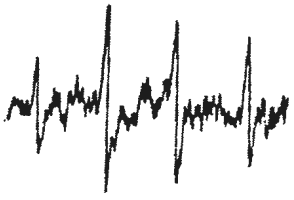
Figure 4.12 **Electron paramagnetic resonance (EPR) spectra of 5,5-dimethyl-1-pyrroline-N-oxide-OH (DMPO-OH) adduct as affected by almond extracts. Columns A and B depict the effects at 100 and 200 ppm (as quercetin equivalents), respectively**

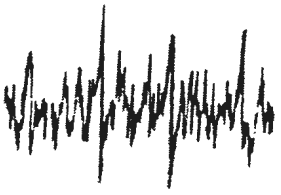


Control

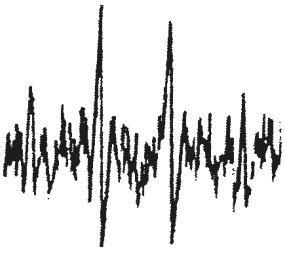
(A)

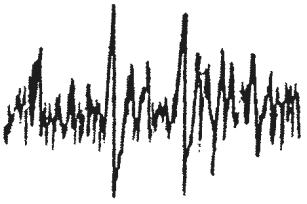
(B)



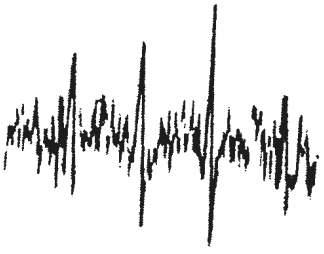


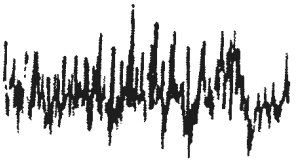
Quercetin



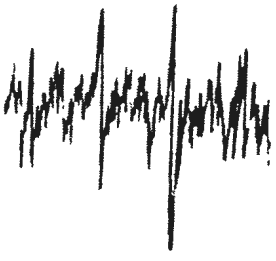


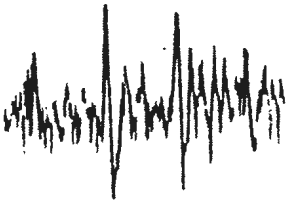
WSE





BSE





GSE

Table 4.9 Hydroxyl radical-scavenging activity of almond extracts at 100 and 200 ppm (as quercetin equivalents)[1]

Extract	Hydroxyl radical-scavenging capacity, %	
	100	200
Quercetin	40 ± 2[b]	60 ± 3[c]
WSE	16 ± 2[a]	42 ± 2[a]
BSE	57 ± 3[c]	100 ± 0[d]
GSE	40 ± 2[b]	56 ± 1[b]

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

due to the presence of colloidal particles at higher levels. However, WSE showed the lowest activity while BSE showed the highest.

4.6.4 DPPH radical-scavenging activity

The use of DPPH free radical in evaluating the antioxidant efficacies is advantageous because it is more stable than the laboratory generated hydroxyl and superoxide radicals. The EPR spectrum of the DPPH radical and the resulting spectrum after addition of extracts are shown in Figure 4.13. Quercetin and almond skin extract at 100 ppm and shell cover extract at 200 ppm showed a 100% scavenging of DPPH radical (Table 4.10). The lowest concentration required for complete scavenging of DPPH radical by quercetin, WSE, BSE, and GSE was 40, 210, 50 and 120 ppm, respectively. These results illustrate that BSE efficiently scavenges organic free radicals. As proposed by Blois (1958), the hydrogen donation from an antioxidant involves the decolourisation of DPPH radicals as shown below.

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

In the above equation A-H is the antioxidant and $A^{\bullet}$ is the antioxidant radical. This confirmed the hydrogen-donating ability of almond extracts.

4.7 Iron chelating capacity of almond crude extracts

In iron chelating experiments, tetramethylmurexide was used to quantitatively determine ferrous ion (Fe^{2+}) chelating capacity of almond extracts. Tetramethylmurexide is a chelating agent that shows a maximum absorption at 530 nm.

Figure 4.13 **Electron paramagnetic resonance (EPR) spectra of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as affected by almond extracts. Columns A and B depict the effects of 100 and 200 ppm (as quercetin equivalents), respectively**

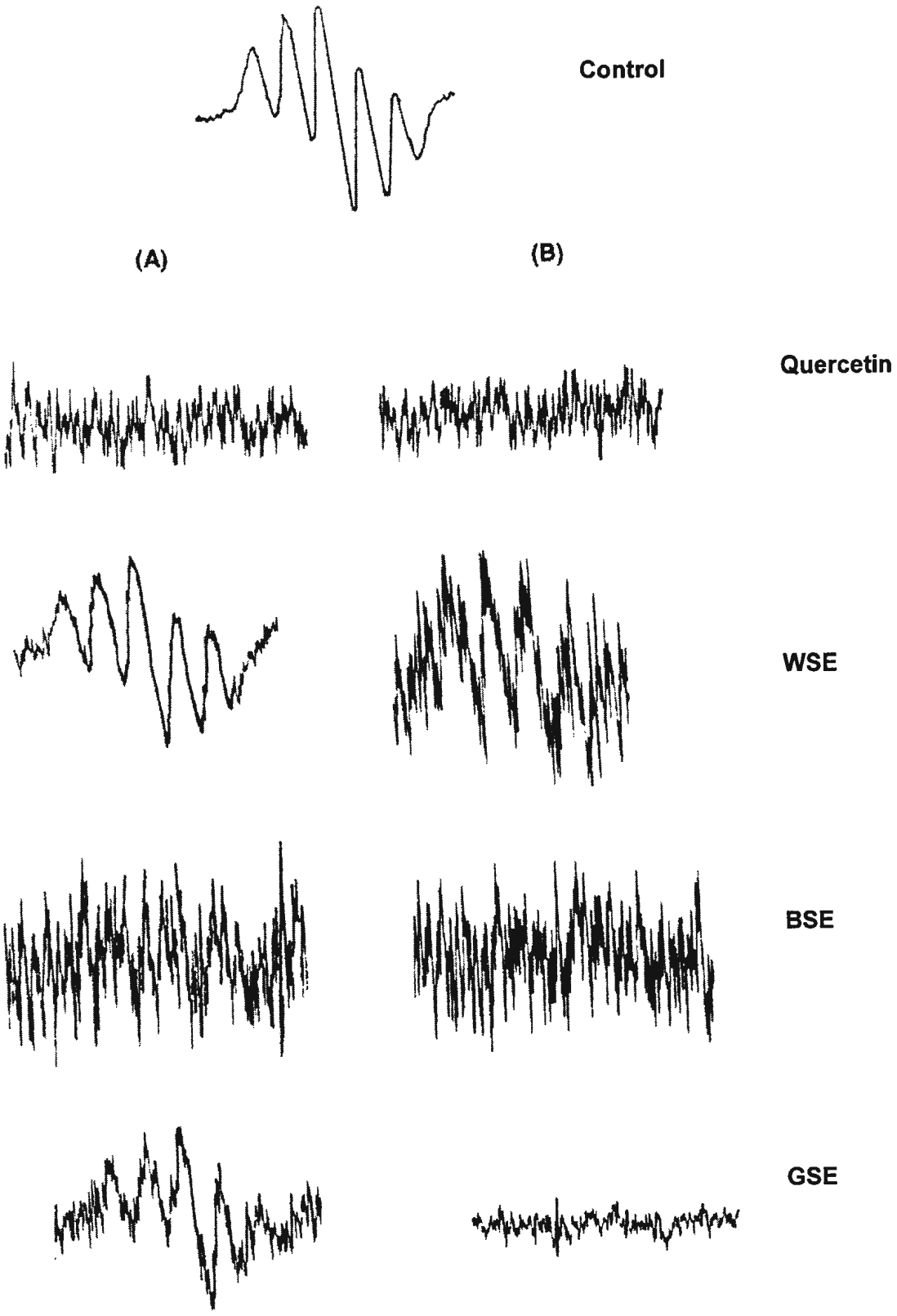

Control
(A)
(B)
Quercetin
WSE
BSE
GSE

Table 4.10 DPPH radical-scavenging activity of the almond extracts at 100 and 200 ppm (as quercetin equivalents)[1]

Extract	DPPH radical-scavenging capacity, %	
	100	200
Quercetin	100 ± 0^{c}	100 ± 0^{b}
WSE	21 ± 2^{a}	73 ± 2^{a}
BSE	100 ± 0^{c}	100 ± 0^{b}
GSE	35 ± 2^{b}	100 ± 0^{b}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: DPPH, 2,2-diphenyl-1-picrylhydrazyl; WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

However, shifting of the absorption maximum to 460 nm occurs when a metal ion chelates with tetramethylmurexide. The ratio of absorbance at 460 to 560 nm is linearly correlated with the metal ion concentration (Asakura *et al.*, 1990). When a known concentration of Fe^{2+} is added to a buffered solution of additives, some Fe^{2+} chelates with additives while leaving unreacted or free Fe^{2+} in the solution. When tetramethylmurexide is added to the solution, it chelates the remaining Fe^{2+} and the absorption maximum shifts from 530 to 460 nm. Then the unreacted free Fe^{2+} can be determined from a calibration line and the concentration of the chelated Fe^{2+} by additives calculated by subtracting free Fe^{2+} concentration from that initially present.

As shown in Table 4.11, almond extracts exhibited strong metal chelating capacities. Almond extracts at 100 and 200 ppm chelated 95-98 and 97-100 %, respectively. As a reference antioxidant, quercetin chelated 96% at both 100 and 200 ppm levels. The Fe^{2+} chelating capacities of extracts may be attributed to metal chelating agents, mainly phenolics, present in additives. Flavonoids are capable of chelating metal ions depending on their structural features (van Acker *et al.*, 1996; Arora *et al.*, 1998). Thompson *et al.* (1976) revealed that the stability of the metal-antioxidant complex is higher in six-membered ring than five-membered ring complexes. The iron chelating capacities of the additives and quercetin could arise from their formation of six-membered complexes with iron ions.

4.8 DNA scission studies

Oxidative damage of DNA results in strand breakage and sister chromatid exchange, DNA-DNA and DNA-Protein cross links, and base modifications (Dizdaroglu,

Table 4.11 Concentration (μM) and proportion (%) of chelated ferrous ions[1] by almond crude extracts at 100 and 200 ppm[2]

Additive	100		200	
	μM	%	μM	%
No additive	0 ± 0^{a}	0	0 ± 0^{a}	0
WSE	380 ± 3^{b}	95	387 ± 3^{b}	97
BSE	$385 \pm 3^{b,c}$	96	390 ± 2^{b}	98
GSE	390 ± 2^{c}	98	399 ± 1^{c}	100
Quercetin	383 ± 3^{b}	96	385 ± 3^{b}	96

[1]The initial ferrous ion concentration is 400 μM.
[2]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract

1993; Halliwell and Aruoma 1992). All three almond extracts showed a reduction in the three types of free radical induced strand scissions in a concentration-dependent manner. Figures A.7 to A.15 show the activity of almond extracts and quercetin on intact DNA retention and Table 4.12 summarises the % DNA retention values deduced from these figures. GSE showed the highest activity at 2, 5, 10, 50 and 100 ppm levels against peroxyl induced strand scission, which was superior even to that of quercetin. Total DNA retention was achieved by GSE at 50 ppm level, whereas other extracts showed the same activity at 100 ppm. On the other hand, for hydroxyl radical induced DNA strand scission, all three almond extracts exerted a total protection at 50 ppm against both non-site specific and site-specific strand scissions. However, the activity of WSE against strand scission by hydroxyl radical was significantly ($p \leq 0.05$) higher than both BSE and GSE at 2, 5 and 10 ppm. It has been observed that compounds such as mannitol, glucose and thiourea exhibit strong hydroxyl radical scavenging activity (Gutteridge, 1984) and this might explain the effective antioxidative properties of WSE despite their low phenolic contents. Due to the low concentration of phenolics in WSE, a higher weight of it had to be used to obtain the required phenolic levels. This inadvertently caused extraction of non-phenolic compounds, such as free amino acids and soluble carbohydrates into ethanol, which may possess antioxidant properties. As discussed in section 4.10 all extracts possess strong metal chelating capacities. If proteins capable of iron binding were present in WSE, in addition to phenolic compounds, they could 'wrap around' the iron ion and intercept a higher percentage of any $OH^{\bullet}$ generated than Fe^{2+}-EDTA complexes (Halliwell and Gutteridge, 1986). The concept of site-specific toxicity of hydroxyl radicals was first described by Gutteridge (1984). He observed that in the absence of EDTA iron ions bind to deoxyribose

Table 4.12 Retention (%) of supercoiled DNA by almond extracts and quercetin (at 2-100 ppm) in free radical induced strand scission[1]

Radical	Extract	2	5	10	50	100
Peroxyl	WSE	67.4 ± 3.2 [b]	73.9 ± 2.2 [a,b]	86.6 ± 2.1 [b]	94.2 ± 0.5 [b]	96.3 ± 0.1 [b]
	BSE	55.1 ± 2.4 [a]	68.2 ± 3.5 [a]	77.6 ± 3.2 [a]	87.1 ± 1.1 [a]	94.9 ± 0.3 [a]
	GSE	74.3 ± 3.3 [b]	87.7 ± 2.3 [c]	94.7 ± 0.8 [c]	99.3 ± 0.1 [d]	99.5 ± 0.1 [c]
	Quercetin	68.6 ± 2.1 [b]	79.2 ± 2.1 [b]	92.6 ± 0.7 [c]	97.1 ± 0.2 [c]	99.9 ± 0.1 [c]
Site-specific hydroxyl	WSE	36.1 ± 3.2 [b]	85.3 ± 2.2 [b]	90.9 ± 0.1 [c]	99.9 ± 0.1 [a]	99.9 ± 0.1 [a]
	BSE	4.9 ± 1.5 [a]	13.8 ± 5.4 [a]	38.4 ± 3.8 [a]	99.9 ± 0.1 [a]	99.9 ± 0.1 [a]
	GSE	8.5 ± 1.9 [a]	36.5 ± 4.7 [c]	62.8 ± 3.6 [b]	99.9 ± 0.1 [a]	99.9 ± 0.1 [a]
	Quercetin	0	0	0	0	0
Non site- specific hydroxyl	WSE	70.3 ± 4.6[b]	89.9 ± 0.1[c]	93.1± 0.1[d]	99.9 ± 0.1[b]	99.9 ± 0.1[b]
	BSE	0	27.5 ± 3.4 [a]	78.9 ± 0.1 [a]	99.9 ± 0.1 [b]	99.9 ± 0.1 [b]
	GSE	4.5 ± 1.2 [a]	86.2 ± 0.5 [c]	88.9 ± 0.1 [c]	99.9 ± 0.1 [b]	99.9 ± 0.1 [b]
	Quercetin	8.2 ± 1.6 [a]	80.4 ± 1.8 [b]	83.9 ± 1.1 [b]	84.9 ± 2.5[a]	85.9 ± 2.3 [a]

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript for a particular radical are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

molecules and bring about a site-specific reaction on the molecule. However, in the presence of EDTA, which competes with deoxyribose for transition metal ions, iron is removed form the binding site to form Fe^{2+}-EDTA complex and produce $OH^{\bullet}$ that could be readily removed by $OH^{\bullet}$ scavengers. It was further explained that most $OH^{\bullet}$ scavengers show poor inhibitory capacities for site-specific hydroxyl reaction. This was observed with almond extracts and quercetin as well. In general, almond extracts performed less effectively against site-specific hydroxyl radical reaction, whereas quercetin did not exert any protection towards site-specific hydroxyl radical scission perhaps due to a pro-oxidant activity in the absence of EDTA. In contrast, the presence of EDTA, which is a strong metal chelator, reversed the activity of quercetin to an antioxidant, which inhibited oxidative activity of hydroxyl radical. DNA damage was not observed when DNA was incubated with quercetin in the presence of diethylenetriaminepenataacetic acid (Jhonson and Loo, 2000), whereas in the presence of metal ions, quercetin at higher concentrations was capable of exerting pro-oxidant effects and bringing about DNA damage in rat liver nuclei (Sahu and Washington, 1991). Though plant-derived phenolic compounds could act as pro-oxidants and damage biomolecules (Aruoma *et al.*, 1998), the three almond extracts tested showed protective effects even up to 100 ppm level.

4.9 Effect of almond extracts on preventing cupric ion induced human low-density lipoprotein (LDL) oxidation

Oxidation of human LDL by free radicals lipid oxidation products may be involved in the pathogenesis of atherosclerosis, and transition metal ions could promote oxidative modification through interaction with lipid hydroperoxides (Decker *et al.*, 2001).

Breakdown products of hydroperoxides may modify proteins and nucleic acids *via* Schiff base reactions (Esterbauer *et al.*, 1987). Cupric ion has been shown to be a strong oxidizing agent of EDTA-free human LDL and hence, has been used extensively to evaluate oxidation inhibitory effects of various compounds in *in vitro* models (Hu and Kitts, 2001). The three almond extracts showed a concentration dependent protection against human LDL oxidation which was also significantly ($p \leq 0.05$) higher than that observed for quercetin (Table 4.13). When a series of 3,5,7,3',4'-pentahydroxy phenols having identical arrangements around their A and B rings were examined for their inhibitory effects on LDL oxidation, it was revealed that the presence of the double bond in the C ring of quercetin, in addition to the 4-oxo group, contributed to a lower antioxidant activity (Meyer *et al.*, 1998).

The highest activity among the almond extracts was exerted by BSE and the lowest by GSE up to 100 ppm, whereas at 200 ppm the inhibitory effects were not significantly ($p > 0.05$) different from one another. It is noteworthy that the extracts did not exhibit any prooxidant activity towards human LDL within the range of concentrations tested. The differences in antioxidative activities by the extracts towards LDL oxidation observed could be ascribed to factors including differences in the solubility and partitioning behaviour between aqueous and lipid phases in the LDL system. It is well known that lipophilic antioxidants such as α-tocopherol provide a greater protection against LDL oxidation than hydrophilic antioxidants (Ziouzenkova *et al.*, 1996; Thomas and Stocker, 2000). However, hydrophilic antioxidants such as ascorbic acid are known to exert a high protection against cupric ion induced LDL oxidation (Hu *et al.*, 2000).

Table 4.13 Inhibition (%) of conjugated diene formation by almond extracts and quercetin (at 10-200 ppm) during copper-induced LDL oxidation[1]

Extract	10	50	100	200
WSE	53.9 ± 2.2^{c}	88.2 ± 1.2^{d}	93.5 ± 0.9^{a}	99.9 ± 0.1^{b}
BSE	71.9 ± 1.4^{d}	$90.2 \pm 0.8^{c,d}$	99.1 ± 0.2^{b}	99.6 ± 0.2^{b}
GSE	36.2 ± 2.2^{b}	64.7 ± 2.6^{b}	90.1 ± 0.4^{c}	98.9 ± 0.5^{b}
Quercetin	28.6 ± 4.1^{a}	53.1 ± 3.5^{a}	83.7 ± 1.9^{d}	94.8 ± 0.7^{a}

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

It has been reported that copper-mediated oxidation of tryptophan residues in the LDL-aplolipoprotein B is responsible for lipid oxidation in LDL particles (Giessauf *et al.*, 1995) and that inhibition of cupric ion-dependent LDL oxidation can occur by free radical scavenging and/or copper chelation activity (Decker *et al.*, 2001). This further confirms the metal chelating capacities of almond extracts. It has been revealed that structural features and differences in protein binding ability may affect antioxidative activities of phenolic compounds (Teissedre *et al.*, 1996). This might be attributed to the differences in activity observed among almond extracts in inhibiting oxidation of LDL.

4.10 Active compounds in almond extracts

HPLC analysis of almond extract showed the presence of various flavonols and phenolic acids as shown in Table 4.14. Each compound was tentatively identified by its retention time and comparing with standards under the same condition. The chromatograms obtained for HPLC analysis are given in the Appendix (Figures A-17 to A-22).

4.10.1 Flavonoids

Two flavonols; quercetin (3,5,7,3',4'-pentahydroxyflavone) and isorhamnetin (3,4',5,7-tetrahydroxy-3'-methoxyflavone), were identified in all three almond extracts. The retention times for quercetin and isorhamnetin were 33.4 and 42.7 min, respectively. The quantity of these flavonols in all three extracts after hydrolysis was significantly higher ($p \leq 0.05$) than those of native samples. Hydrolysed BSE afforded the highest quantities of quercetin and isorhamnetin, whereas native GSE contained the lowest.

Table 4.14 Flavonol and phenolic acid content of almond extracts[1]

Compound	Quantity of flavonols in almond extracts (μg/g extract)					
	WSE		BSE		GSE	
	Native	Hydrolysed	Native	Hydrolysed	Native	Hydrolysed
Flavonols						
Quercetin	0.013 ± 0.002[a]	0.074± 0.003[a]	0.12 ± 0.002[a]	0.309± 0.004[a]	0.013± 0.001[a]	0.152± 0.003[b]
Isorhamnetin	0.039 ± 0.003[b]	0.324± 0.003[b]	0.155± 0.003[b]	1.069± 0.005[b]	0.019± 0.002[b]	0.127± 0.004[a]

	Quantity of phenolic acids in almond extracts (μg/g extract)					
	WSE		BSE		GSE	
	Free	Esterified	Free	Esterified	Free	Esterified
Phenolic acids						
Caffeic	trace	4.34± 0.01[b]	trace	160.09± 0.95[d]	trace	653.52± 1.25[d]
p-Coumaric	trace	3.09± 0.01[a]	4.55± 0.02[b]	64.17± 0.35[c]	1.34± 0.01[a]	195.56± 1.22[c]
Ferulic	trace	23.88± 0.05[d]	2.19± 0.01[a]	31.92± 0.15[b]	2.71± 0.02[b]	76.27± 0.56[b]
Sinapic	trace	8.98± 0.03[c]	9.51± 0.03[c]	22.36± 0.56[a]	9.92± 0.02[c]	40.01± 0.44[a]

[1]Results are mean values of three determinations ± standard deviation.
Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract and GSE, green shell extract.

Quercetin is the aglycone of a number of other flavonoids such as rutin, quercitrin, isoquercetin and hyperoside. Methylation of oxygen at the 3′ position of quercetin gives rise to isorhamnetin (Hosny *et al.*, 2001). Studies on inhibitory effects of quercetin and isorhamnetin on copper-induced lipid peroxidation in human LDL revealed that isorhamnetin was less effective than quercetin which implies that introduction of a conjugated group to the position of the dihydroxyl group in the B ring markedly decreases the Inhibition of LDL oxidation (Yamamoto *et al.*, 1999). The sugar molecule, when replacing one of the hydroxyl groups on the C ring, changes the activity of the molecule (Schuler, 1990). Ioku *et al.* (1995) reported that flavonoid glucosides possess a lower peroxyl radical-scavenging activity than corresponding aglycones. The antioxidant activity of flavonols, such as quercetin and isorhamnetin, is mainly based on the hydroxyl group at C-3 and the double bond between C-2 and C-3 atoms, whereas the metal chelating capacity is centred around the C-3′ and C-4′ atoms (Schuler, 1990). Chelation occurs at the 3-hydroxy, 4-keto grouping and/or at 5-hydroxy, 4-keto group of the A ring which is hydroxylated in the 5 position (Hudson and Lewis 1983). The 5,7-hydroxylation on A ring has shown little effect on antioxidant activity (Pratt and Hudson, 1990).

Studies conducted by Abou-Zaid and Amarowicz (unpublished data) further revealed the presence of quercetin-3-*O*-β-L-rhamnoside (quercitrin), kaempferol-3-*O*-β-D-glucoside (astragalin), kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-β-D-glucoside and morin in all three almond extracts. Their results show that the majority of the flovonols are present in their glycosylated forms that would account for the hydrophilic compounds. Quercetin is rather a lipophilic antioxidant compared with its glycosides or

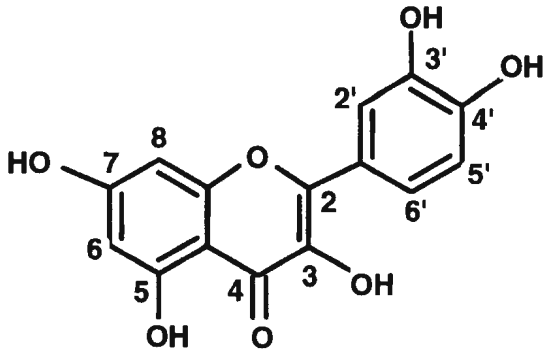
conjugates and seems to interact with the polar head of phospholipid bilayers by locating near the surface of the membrane (De Whalley *et al.*, 1990). This location may be favourable for the trapping of the peroxyl radicals originating from the aqueous phase (Morand, 1998). It has been revealed that quercetin is more powerful than morin in preventing oxidation of lipids, protein and DNA (Makris and Rossiter, 2001). Moreover, quercetin has shown higher iron chelating (van Acker *et al.*, 1996; Brown *et al.*, 1998) and hydroxyl radical-scavenging activity than morin (Husain *et al.*, 1987). The difference in activity has been attributed to the *ortho*-diphenol B-ring structure in quercetin. The structures of the above flavonols identified in almond extracts are given in Figure 4.14.

Quercetin appears to have many beneficial effects on human health including cardiovascular protection (Pace-Ascial *et al.*, 1995), anti-cancer activity (Scambia *et al.*, 1992), anti-ulcer activity (Alarcon de la Lastra *et al.*, 1994), anti-inflammatory activity (Bronner *et al.*, 1985), cataract prevention (Chaudry *et al.*, 1983), antiviral activity (Kaul *et al.*, 1985) and anti-inflammatory effects (Della Loggia *et al.*, 1988). These beneficial effects may be due to its antioxidant activity. Quercetin scavenges oxygen radicals (Saija *et al.*, 1995), inhibits xanthine oxidase (Chang *et al.*, 1993), and inhibits lipid peroxidation (Chen *et al.*, 1990). Quercetin also inhibits oxidation of human LDL by protecting vitamin E in LDL from being oxidized or by regenerating oxidized vitamin E (DeWhalley, 1990). Isorhamnetin has been reported to possess antimicrobial activity (Omidiji, 1990) and inhibitory effects on xanthine oxidase (Nagao *et al.*, 1999).

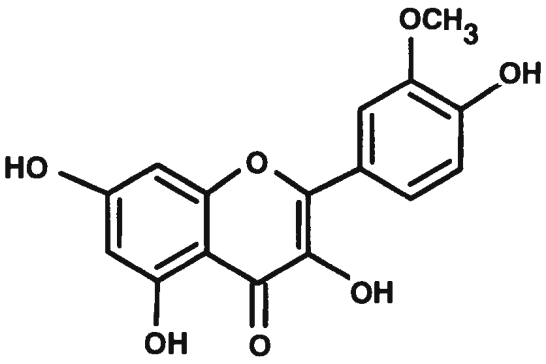
4.10.2 Phenolic acids

In general, all almond extracts had the same array of phenolic acids: caffeic, *p*-

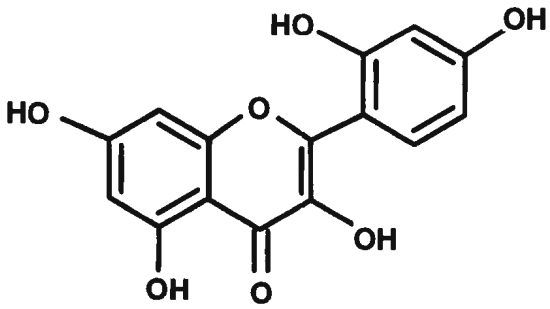
Figure 4.14 Flavonoids identified in almond by high performance liquid chromatographic analysis



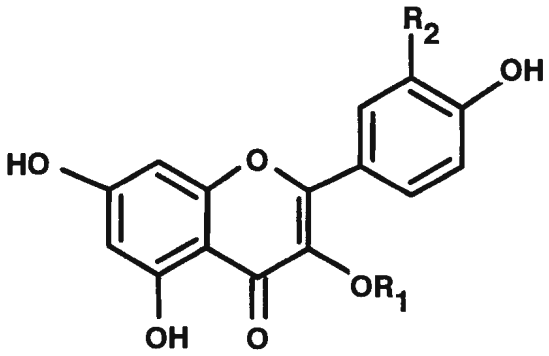
Quercetin



Isorhamnetin



Morin



R_1 = Glucosyl, R_2 = H - Kaempferol-3-*O*-glucoside

R_1 = Rhamnoglucosyl, R_2 = H - Kaempferol-3-*O*-rhamnoglucoside

R_1 = Rhamnosyl, R_2 = OH - Quercetin-3-*O*-glucoside

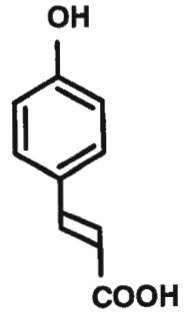
R_1 = Glucosyl, R_2 = OCH_3 - Isorhamnetin-3-*O*-glucoside

coumaric, ferulic and sinapic acids with retention times of 12.7, 25.3, 36.8 and 40.7 min, respectively. Substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the predominant phenolic acids present in foods of plant origin and the derivatives differ in hydroxylation and methoxylation patterns of their aromatic rings (Shahidi and Naczk, 1995). The identified phenolic acids in almonds are derivatives of cinnamic acid.

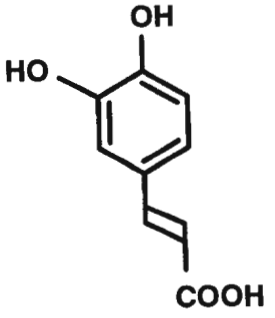
The majority of these phenolic acids, also called as phenyl proponoids were found in the form of soluble esters as shown in Table 4.14. Total amounts of the identified free phenolic acids in BSE and GSE were 16.28 ± 0.03 and 13.99 ± 0.03 μg/g extract, respectively, whereas WSE contained only trace amounts. Identified total esterified phenolic acids in WSE, BSE and GSE were 40.34 ± 0.05, 279.55 ± 1.01 and 967.10 ± 1.74 μg/g extract, respectively. GSE served as a better source of phenolic acids compared to WSE and BSE. Caffeic acid was the predominant phenolic acid present in BSE and GSE in the bound form, but was present only in trace amounts in the free form; a pattern observed also for fruits (Shahidi and Naczk, 1995). Abou-Zaid (ongoing experiments) has further identified gallic acid in all three extracts. The structures of the phenolic acids identified in almond are depicted in Figure 4.15.

The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule (Dziedzic and Hudson 1983). Hydroxylated cinnamic acids were found to be more effective than their benzoic acid counterparts (Shahidi and Naczk, 1995). Caffeic acid, which has two hydroxyl groups, was more effective than *p*-coumaric acid that has only one hydroxyl group, in preventing oxidation in a stripped corn oil system (Pratt and Hudson, 1990). The electron withdrawing effect

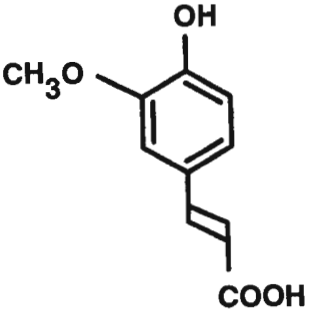
Figure 4.15 Phenolic acids identified in almond by high performance liquid chromatographic analysis



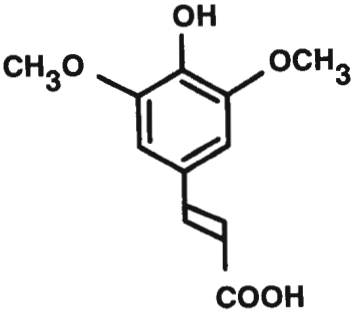
p-Coumaric acid



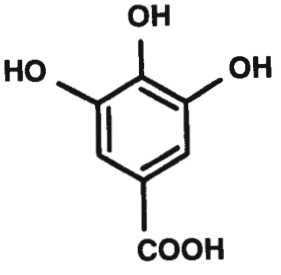
Caffeic acid



Ferulic acid



Sinapic acid



Gallic acid

of the carboxylic acid group is greatly reduced due to the presence of a -CH=CH- moiety in these phenolic acids. However, the -CH=CH- moiety contributes to the radical stabilization of these acids through resonance which has a positive influence on their overall antioxidant efficacy (Foti *et al.*, 1996). Ferulic acid has shown lower antioxidant activity compared to caffeic acid (Hudson and Mahgoub, 1980), possibly due to its methoxylation in the *ortho* position, which could result in a decrease in radical scavenging ability. Hydroxylation, as in caffeic acid, in place of methoxylation is substantially more effective (Bors *et al.*, 1990; Chimi *et al.*, 1991). However, the antioxidant activity increased in the order: caffeic < *p*-coumaric < ferulic < sinapic, in preventing accelerated autoxidation of methyl linoleate, indicating an increased antioxidant efficacy of phenolic acids with methoxy substitutions (Cuvelier *et al.*, 1992). As for sinapic acid, the methoxy substitutions in 3- and 5- positions may impose a negative effect on its hydrogen donating ability. However, the orientation of functional groups in 3-, 4- and 5- positions enhances the antioxidant efficacy of the molecules as metal chelators (Shahidi and Naczk, 1995). A study conducted to investigate the protection exerted by monomeric hydroxycinnamates against oxidation of human LDL revealed that antioxidant activity decreased in the order of: caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid (Andreasen *et al.*, 2001). In studies related to *in vitro* human LDL oxidation, the antioxidant activity was improved as the number of hydroxyl and methoxyl groups increased, and the presence of the *o*-dihydroxy group in the phenolic ring, as in caffeic acid, enhanced the antioxidant activity (Natella *et al.*, 1999; Meyer *et al.*, 1998).

SUMMARY AND RECOMMENDATIONS

Almond whole seed, brown skin and green shell meals reduced the formation of TBARS, hexanal and total volatiles effectively in a pork model system in a concentration-dependent manner. Since the meals exerted antioxidative effects, their crude extracts were then prepared using 80% ethanol at 70°C for 30 min. The total antioxidant capacity of almond whole seed extract (WSE), brown skin extract (BSE) and green shell extract (GSE), evaluated using the Trolox equivalent antioxidant capacity (TEAC) assay, showed that BSE and GSE possessed greater antioxidant capacities than WSE at the same concentration. The antioxidant efficacies of extracts were tested at 100 and 200 ppm based on their total phenolics as quercetin equivalents in several model systems. In general, almond extracts exerted antioxidative effects comparable to or better than BHA, at the same concentration, in β-carotene-linoleate, bulk corn oil and cooked comminuted pork model systems.

Among the three almond extracts, BSE and GSE exhibited excellent hydrogen peroxide-scavenging and free radical-scavenging capacities in an aqueous medium. The variations of activities observed among the extracts may possibly be due to their varying degrees of partitioning into lipid and aqueous phases of the model systems. Therefore, the hydrogen peroxide-scavenging and free radical-scavenging activity of almond extracts seen in monophasic assay media may not always reflect their behaviour in complex multiphasic model systems. In protecting DNA against free radical-induced strand scission, WSE and GSE exhibited better protective effects than quercetin at the same concentration. All three extracts showed strong antioxidative

effects against copper induced human LDL oxidation. Studies on metal chelating properties showed that all three extracts possessed strong metal scavenging activities.

Based upon high performance liquid chromatographic (HPLC) data, the major flavonoids present in all three almond extracts were identified as quercetin, isorhamnetin, quercitrin, astragalin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-β-D-glucoside and morin. Caffeic, ferulic, *p*-coumaric, sinapic and gallic were the major phenolic acids identified in the extracts. However, more research is required to identify and quantify minor antioxidant compounds in almond.

Almonds, when added to sweets, confectionaries, salads and cooked rice and meat may improve their oxidative stability and may also be incorporated in bulk oils and food emulsions to extend their shelf life. As demonstrated by DNA scission studies and LDL-oxidation model systems, incorporation of almond and possibly its by-products, brown skin and green shell cover, into foods may reduce the risk of developing certain disease conditions in humans. Therefore, further studies using animal models, cell culture lines and clinical trials are necessary to shed further light on the beneficial effects of almond in *in vivo* systems. Isolating the active compounds and identifying their individual contribution to the biological activity would be of paramount importance in controlling various disease and physiological conditions in humans. The mechanisms by which these compounds act at target sites in human to arrest a particular disease and whether it is a single compound responsible for the observed effects, or is it due to a synergistic action between two or more compounds would be worth investigating. Future research should also focus on the economic feasibility of large-scale production

of the bioactive compounds. The importance of research on the possible cytotoxic, genotoxic and allergic potencies of these compounds are unquestionable. In summary, it is imperative to conduct more research concerning not only the chemistry of the constituents but also their absorption, metabolism, excretion and behaviour in experimental models and human.

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APPENDIX

Figure A.1 **Dependence of the absorbance of quercetin-metal complex at 725 nm on the content of quercetin in the medium**

Correlation coefficient (r) = 0.9936
Equation of the line was Y= aX where,
Y = absorbance at 725nm (A_{725nm})
X = Content of quercetin in μg/10mL assay media (C)
a = 0.013
Therefore, C = 76.92* A_{725nm}

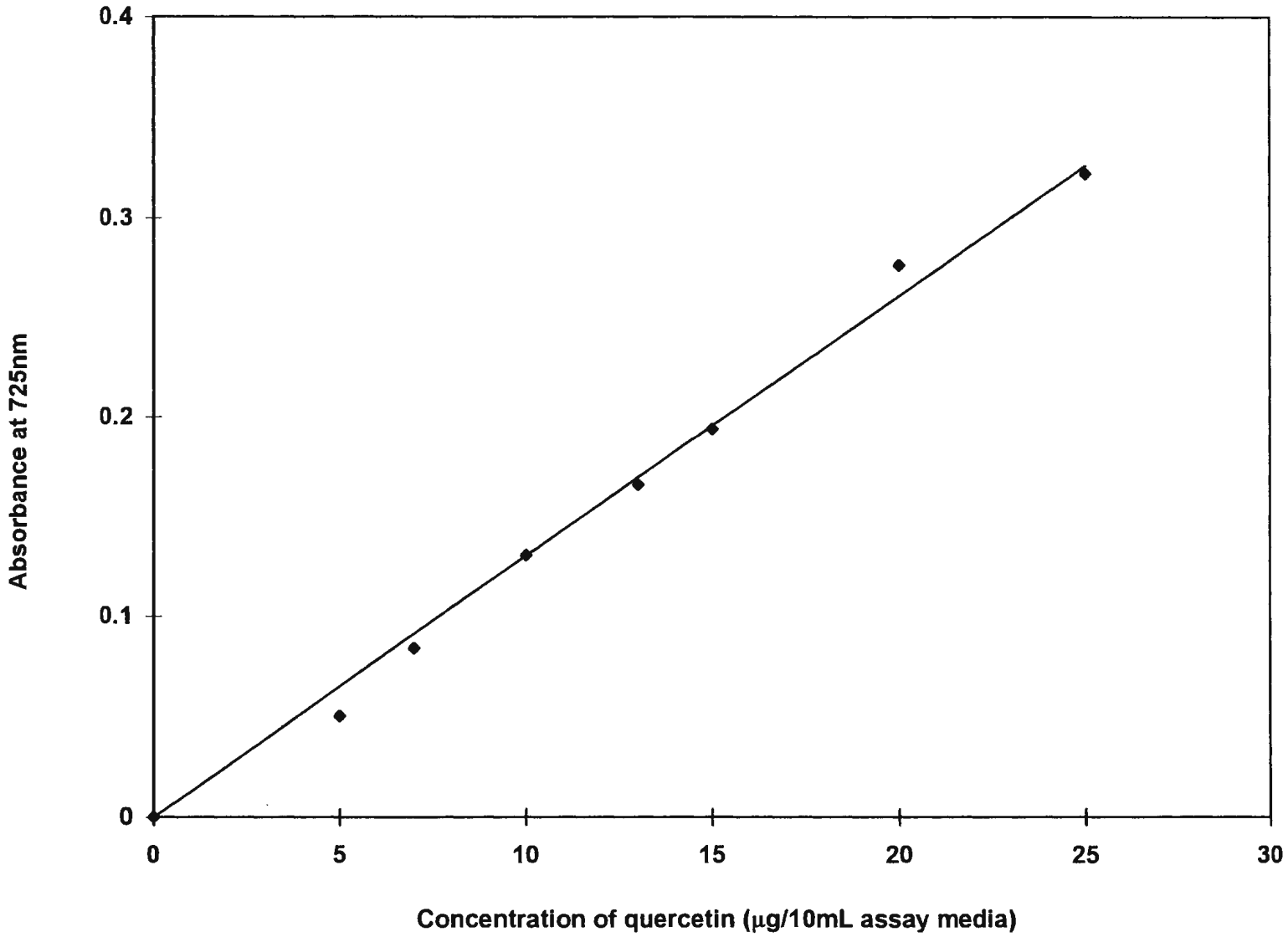

0.4
0.3
0.2
0.1
0
Absorbance at 725nm
0
5
10
15
20
25
30
Concentration of quercetin (µg/10mL assay media)

Figure A.2 **Dependence of the absorbance of malonaldehyde (MA)-TBA complex at 532 nm on the content of malonaldehyde and related compounds in a meat model system**

Correlation coefficient (r) = 0.9758
Equation of the line was Y= aX where,
Y = absorbance at 532nm (A_{532nm})
X = Content of malonaldehyde (μg/10 mL) in the assay media (C)
a = 0.0284
Therefore, C = 35.25* A_{532nm}

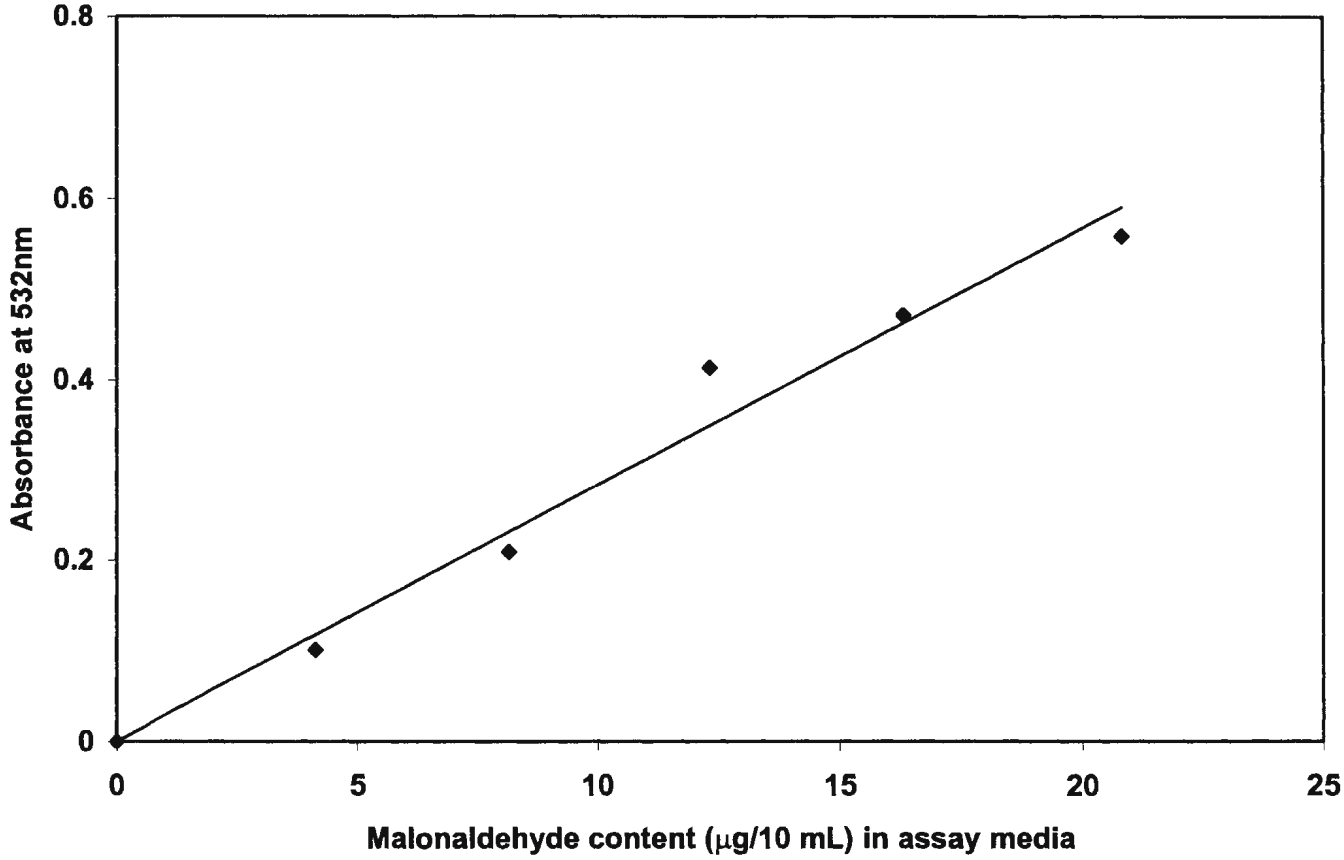

0.8
0.6
0.4
0.2
0
Absorbance at 532nm
0
5
10
15
20
25
Malonaldehyde content (μg/10 mL) in assay media

Figure A.3 **Dependence of the absorbance of malonaldehyde (MA)-TBA complex at 532 nm on the concentration of malonaldehyde**

Correlation coefficient (r) = 0.9758
Equation of the line was Y= aX where,
Y = absorbance at 532nm (A_{532nm})
X = Content of malonaldehyde μmol/10mL assay media (C)
a = 0.0284
Therefore, C = 35.25* A_{532nm}

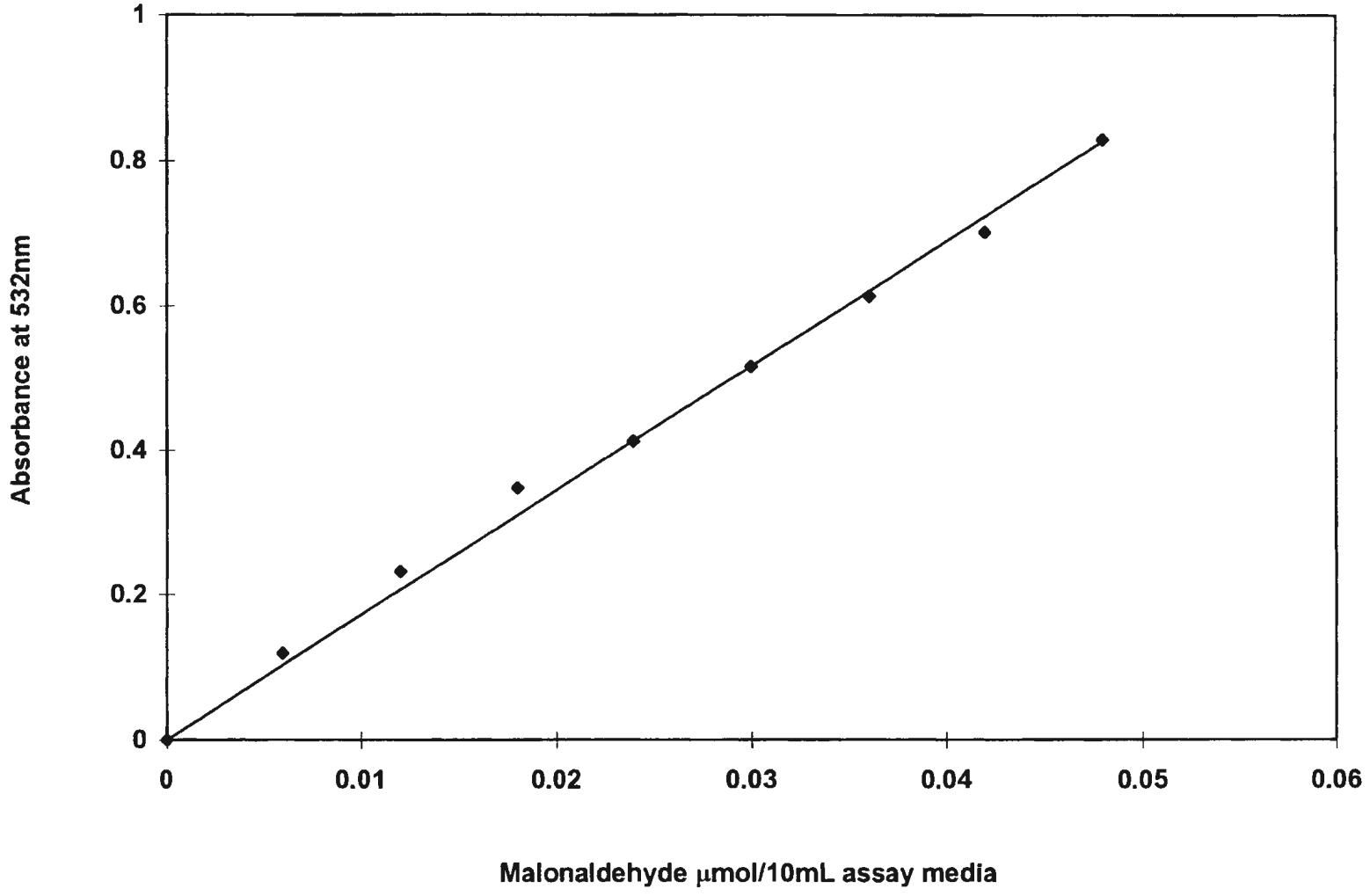

Absorbance at 532nm
1
0.8
0.6
0.4
0.2
0
0
0.01
0.02
0.03
0.04
0.05
0.06
Malonaldehyde μmol/10mL assay media

Figure A.4 **Dependence of the decrease in absorbance of 2,2′-azinobis-(3-ethlbenzthiazoline-6-sulphonate) at 734 nm radical anion ($ABTS^{\bullet-}$) solution on the concentration of Trolox**

Correlation coefficient (r) = 0.9924
Equation of the line was Y= aX where,
Y = absorbance at 734nm (A_{734nm})
X = Concentration (μM) of Trolox (C)
a = 0.0342
Therefore, C = 29.24* A_{734nm}

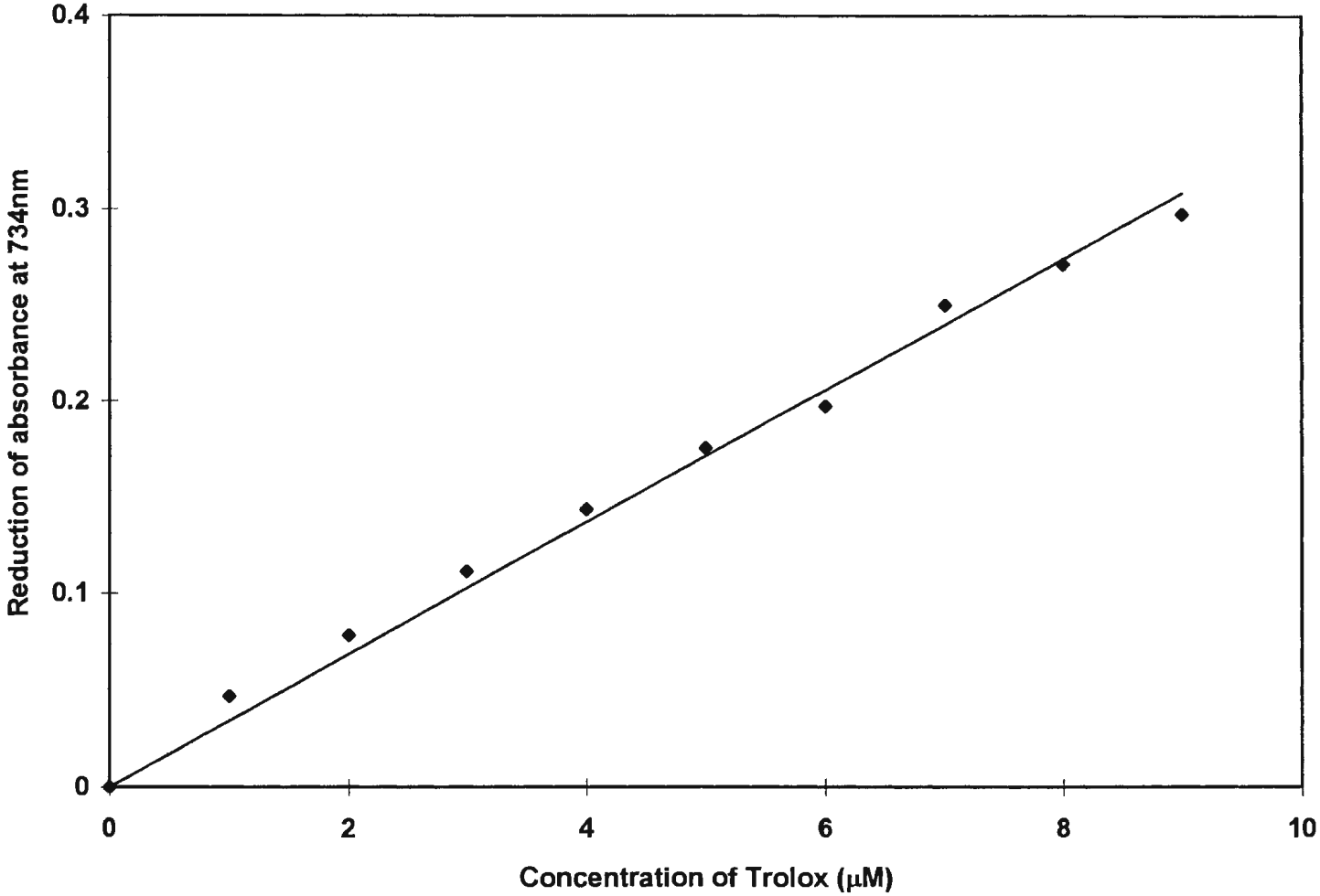

0.4
0.3
0.2
0.1
0
Reduction of absorbance at 734nm
0
2
4
6
8
10
Concentration of Trolox (μM)

Figure A.5 **Dependence of the absorbance of β-carotene at 470 nm on the concentration of β-carotene**

Correlation coefficient (r) = 0.997
Equation of the line was Y= aX where,
Y = absorbance at 470nm (A_{470nm})
X = Concentration of β-carotene (μg/10mL)
a = 0.0069
Therefore, C = 144.9* A_{470nm}

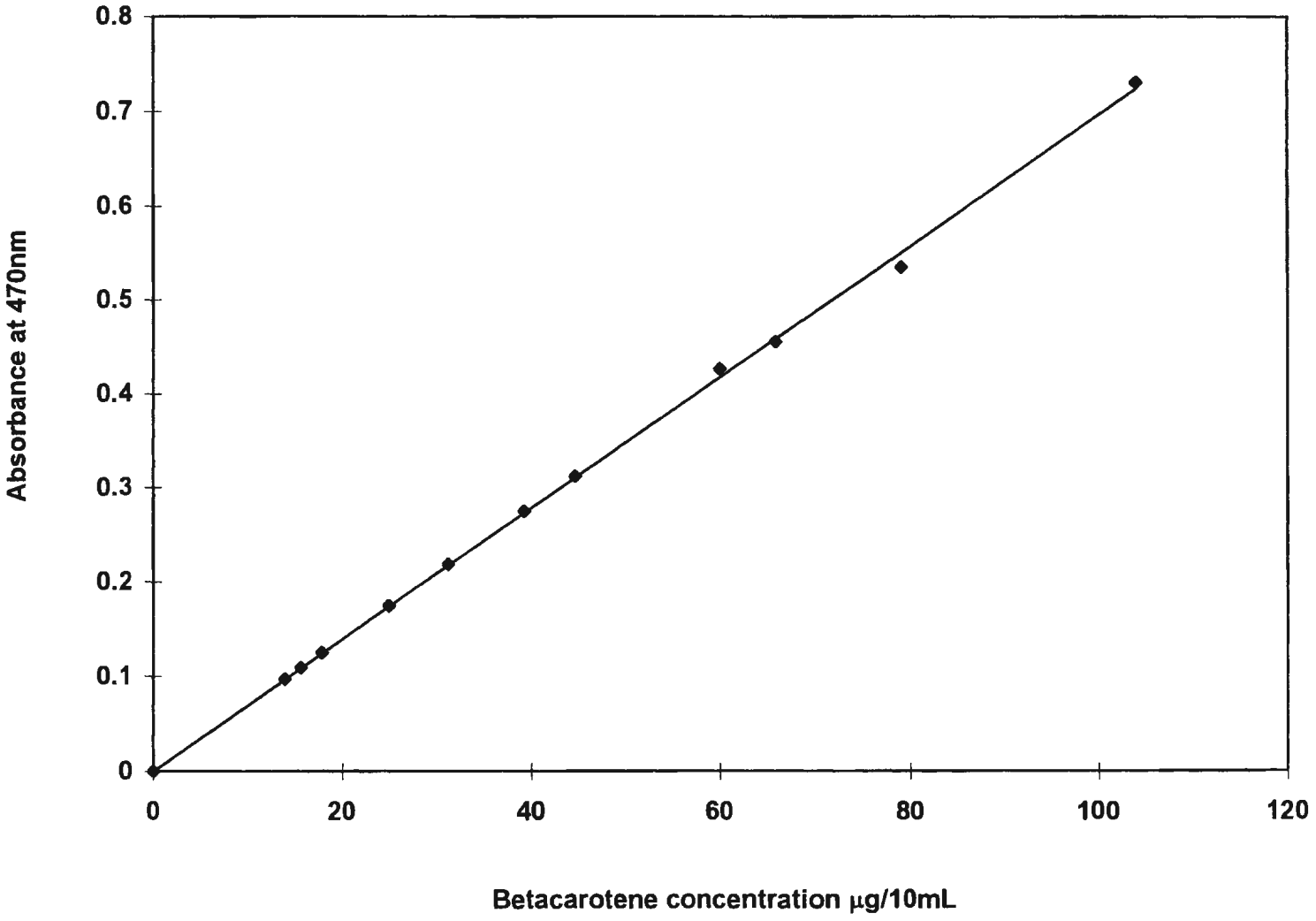

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 470nm
0
20
40
60
80
100
120
Betacarotene concentration µg/10mL

Figure A.6 **Dependence of the absorbance ratio (460 nm/ 530nm) on the concentration of free ferrous ions in μM**

Correlation coefficient (r) = 0.997
Equation of the line was Y= aX where,
Y = absorbance ratio ($A_{460nm/\ 530nm}$)
X = Concentration of free ferrous ions (μM)
a = 0.0007
Therefore, C = 1428.57 * $A_{460nm/\ 530nm}$

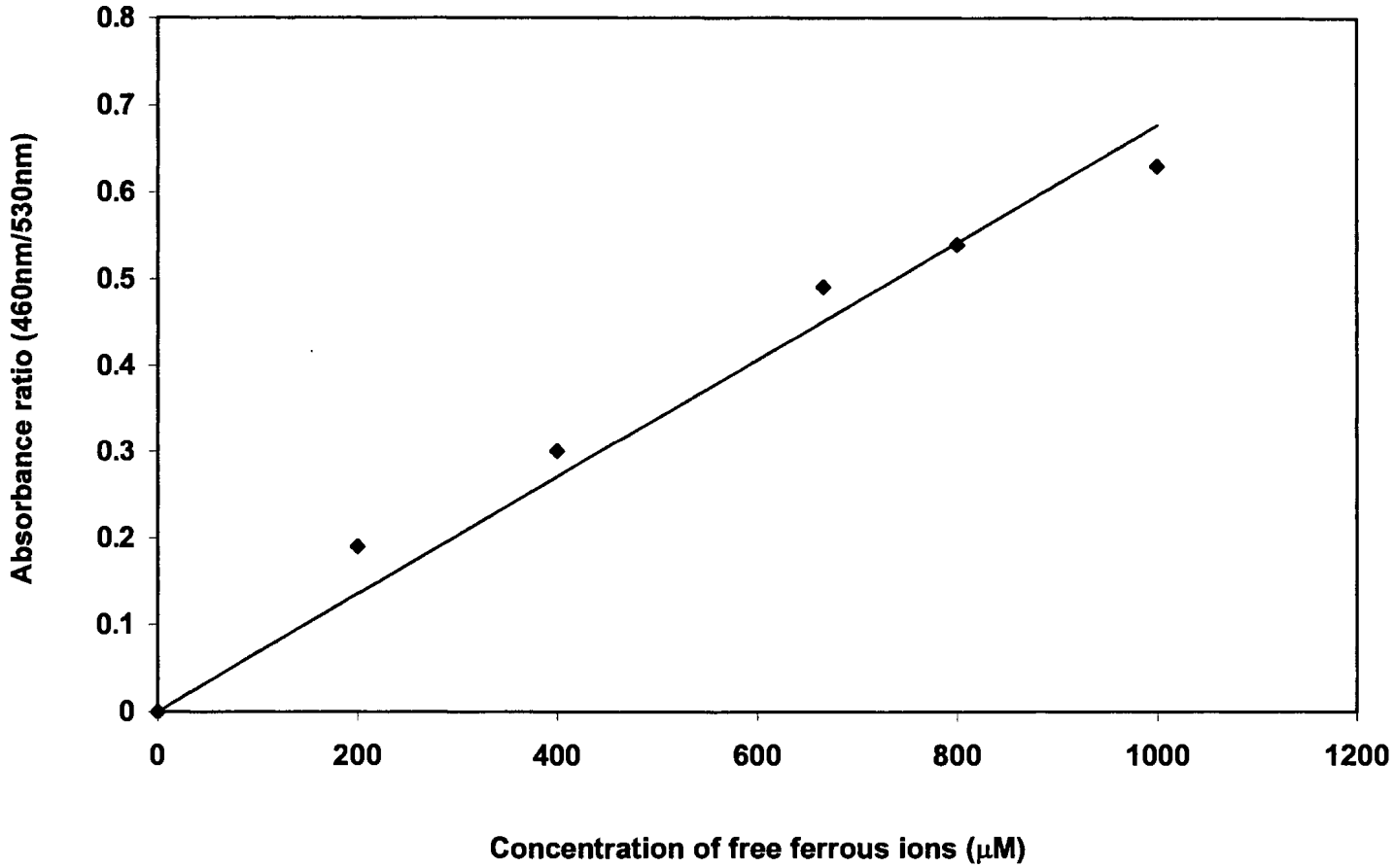

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance ratio (460nm/530nm)
0
200
400
600
800
1000
1200
Concentration of free ferrous ions (μM)

Figure A.7 **Dependence of the absorbance of hydrogen peroxide 230 nm on the concentration of hydrogen peroxide**

Correlation coefficient (r) = 0.9971
Equation of the line was Y= aX where,
Y = absorbance at 230nm (A_{230nm})
X = Concentration (mM) hydrogen peroxide
a = 0.078
Therefore, C = 12.82* A_{230nm}

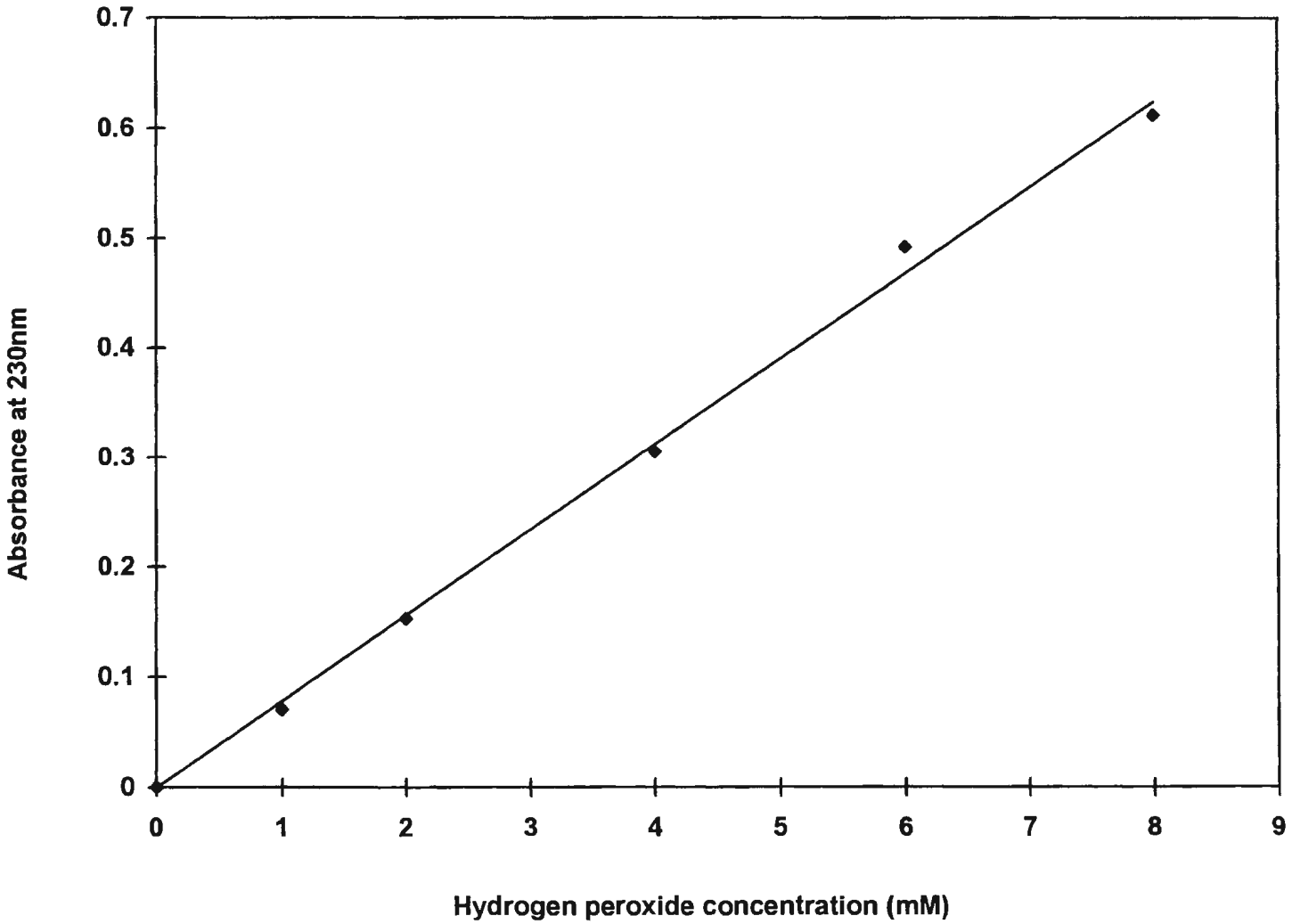

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 230nm
0
1
2
3
4
5
6
7
8
9
Hydrogen peroxide concentration (mM)

Figure A-8 **Effect of quercetin in preventing peroxyl radical induced DNA scission at 37°C.**
Lane 1= DNA + 9 mM AAPH; Lane 2= DNA + quercetin 2μg/mL + 9 mM AAPH; Lane 3= DNA + quercetin 5μg/mL + 9 mM AAPH; Lane 4= DNA + quercetin 10 μg/mL + 9 mM AAPH; Lane 5= DNA + quercetin 50 μg/mL + 9 mM AAPH; Lane 6= DNA + quercetin 100 μg/mL + 9 mM AAPH; Lane 7= DNA + PBS.
Abbreviations are: AAPH, 2,2′-azobis-(2-methylptopionamidine) dihydrochloride; S, Supercoiled DNA strand; N, Nicked DNA strand.

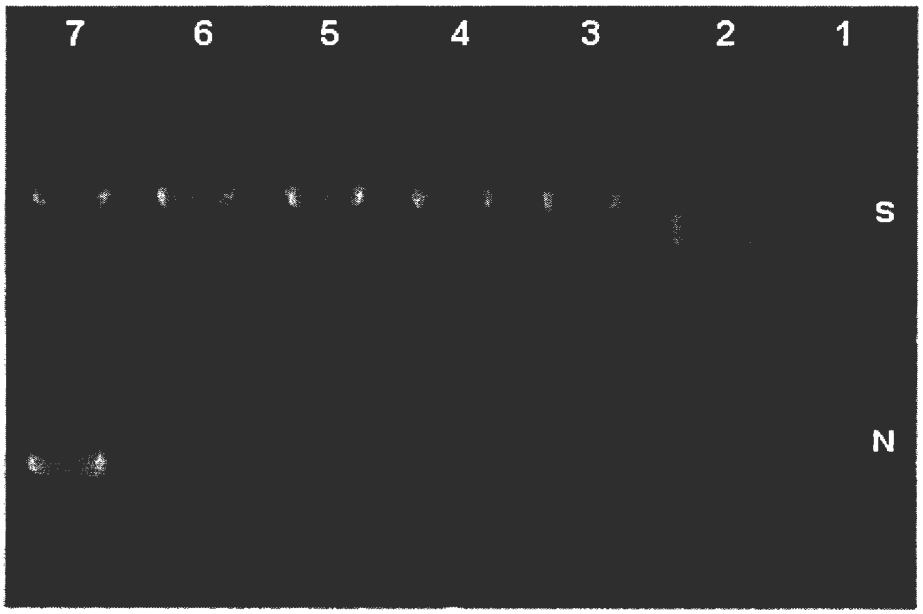

7
6
5
4
3
2
1
S
N

Figure A-9 **Effect of almond whole seed extract (WSE) in preventing peroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + PBS; Lane 2= DNA + 9 mM AAPH; Lane 3= DNA + WSE 2μg/mL + 9 mM AAPH; Lane 4= DNA + WSE 5 μg/mL + 9 mM AAPH; Lane 5= DNA + WSE 10 μg/mL + 9 mM AAPH; Lane 6= DNA + WSE 50μg/mL + 9 mM AAPH; Lane 7= DNA + WSE 100 μg/mL + 9 mM AAPH.
Abbreviations are: WSE, whole seed extract; AAPH, 2,2′-azobis-(2-methylpropionamidine)dihydrochloride; S, Supercoiled DNA strand; N, Nicked DNA strand.

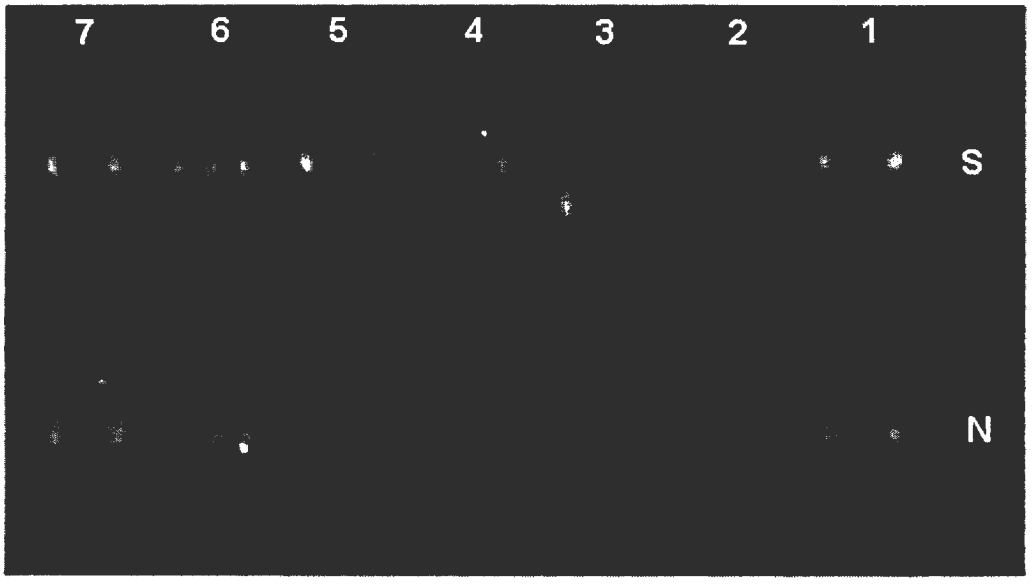

7
6
5
4
3
2
1
S
N

Figure A-10 **Effect of almond brown skin extract (BSE) in preventing peroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + PBS; Lane 2= DNA + 9 mM AAPH; Lane 3= DNA + BSE 2μg/mL + 9 mM AAPH; Lane 4= DNA + BSE 5 μg/mL + 9 mM AAPH; Lane 5= DNA + BSE 10 μg/mL + 9 mM AAPH; Lane 6= DNA + BSE 50μg/mL + 9 mM AAPH; Lane 7= DNA + BSE 100 μg/mL + 9 mM AAPH.
Abbreviations are: BSE, brown skin extract; AAPH, 2,2′-azobis-(2-methylpropionamidine)dihydrochloride; S, Supercoiled DNA strand; N, Nicked DNA strand.

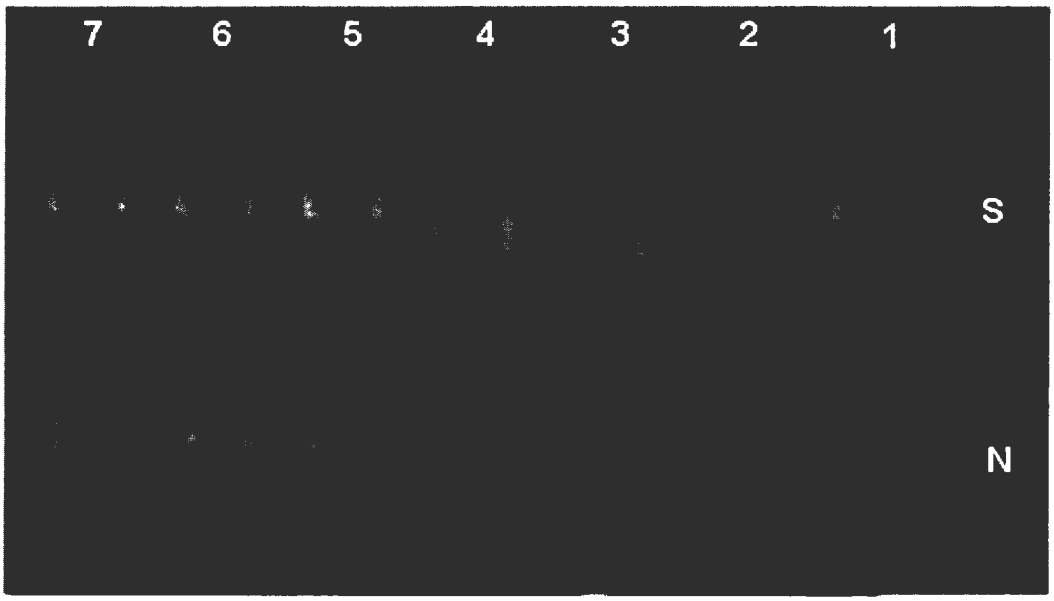

7
6
5
4
3
2
1
S
N

Figure A-11 **Effect of almond green shell extract (GSE) in preventing peroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + 9 mM AAPH; Lane 2= DNA + GSE 2μg/mL + 9 mM AAPH; Lane 3= DNA + GSE 5 μg/mL + 9mM AAPH; Lane 4= DNA + GSE 10 μg/mL + 9 mM AAPH; Lane 5= DNA + GSE 50μg/mL + 9 mM AAPH; Lane 6= DNA + GSE 100 μg/mL + 9 mM AAPH; Lane 7= DNA + PBS.
Abbreviations are: GSE, green shell extract; AAPH, 2,2′-azobis-(2-methylpropionamidine)dihydrochloride; S, Supercoiled DNA strand; N, Nicked DNA strand.

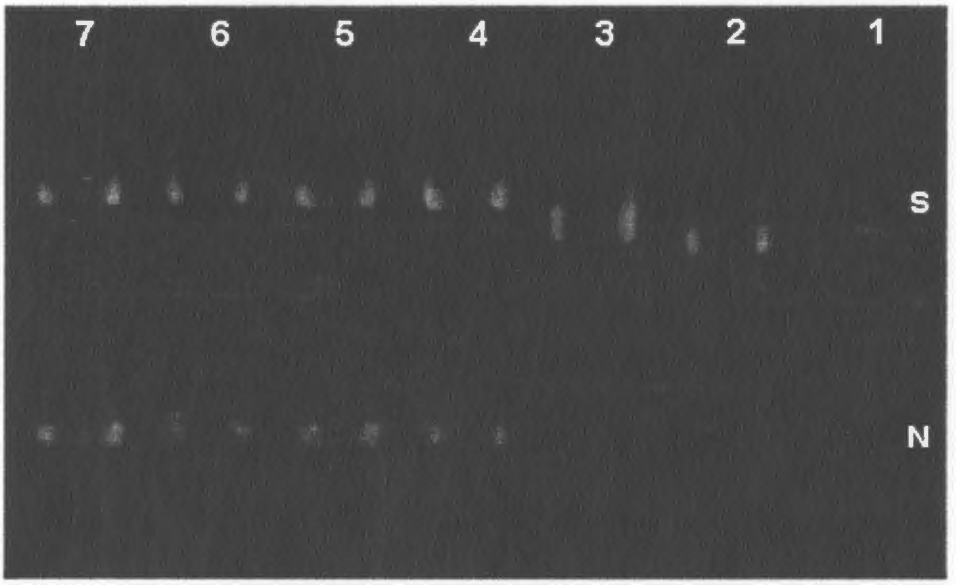

7
6
5
4
3
2
1
S
N

Figure A-12 **Effect of quercetin in preventing non site-specific hydroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + hydroxyl radical; Lane 2= DNA + PBS; Lane 3= DNA + quercetin 2μg/mL + hydroxyl radical; Lane 4= DNA + quercetin 5μg/mL + hydroxyl radical; Lane 5= DNA + quercetin 10 μg/mL + hydroxyl radical ; Lane 6= DNA + quercetin 50 μg/mL + hydroxyl radical ; Lane 7= DNA + quercetin 100 μg/mL + hydroxyl radical.
Abbreviations are: S, Supercoiled DNA strand; N, Nicked DNA strand.

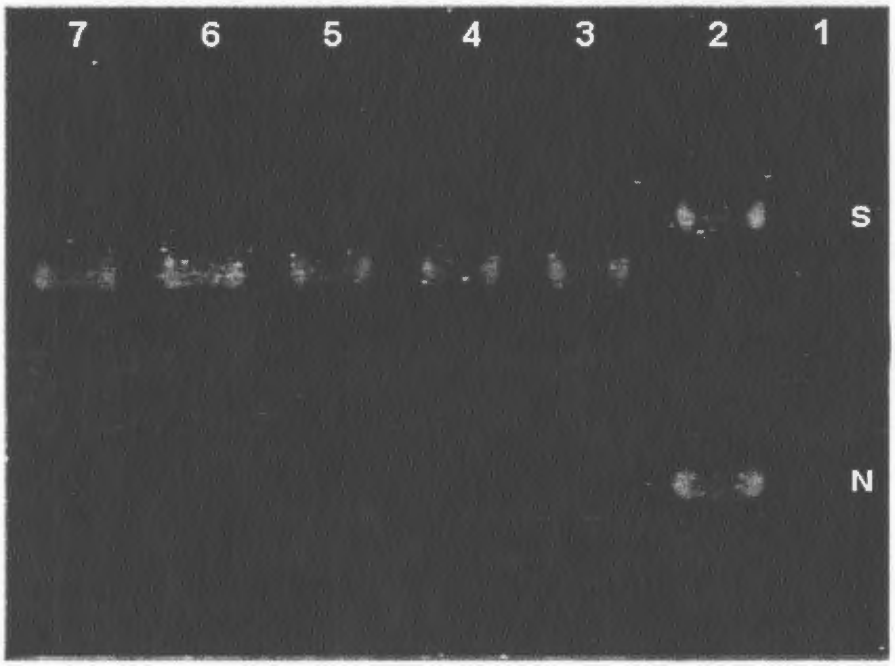

7
6
5
4
3
2
1
S
N

Figure A-13 **Effect of almond whole seed extract (WSE) in preventing non site-specific hydroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + PBS; Lane 2= DNA + hydroxyl radical; Lane 3= DNA + WSE 2μg/mL + hydroxyl radical; Lane 4= DNA + WSE 5μg/mL + hydroxyl radical; Lane 5= DNA + WSE 10 μg/mL + hydroxyl radical; Lane 6= DNA + WSE 50 μg/mL + hydroxyl radical ; Lane 7= DNA + WSE 100 μg/mL + hydroxyl radical.
Abbreviations are: WSE, whole seed extract; S, Supercoiled DNA strand; N, Nicked DNA strand.

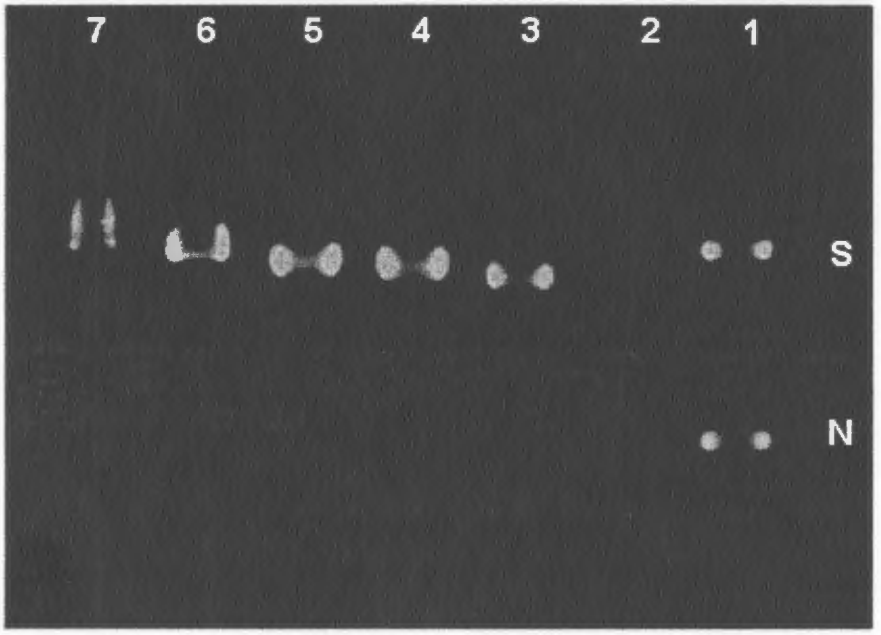

7
6
5
4
3
2
1
S
N

Figure A-14 **Effect of almond brown skin (BSE) and green shell (GSE) extracts in preventing non site-specific hydroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + PBS; Lane 2= DNA + hydroxyl radical; Lane 3= DNA + BSE 2μg/mL + hydroxyl radical; Lane 4= DNA + BSE 5μg/mL + hydroxyl radical; Lane 5= DNA + BSE 10 μg/mL + hydroxyl radical; Lane 6= DNA + BSE 50 μg/mL + hydroxyl radical; Lane 7= DNA + BSE 100 μg/mL + hydroxyl radical; Lane 8= DNA + GSE 2μg/mL Lane 9= DNA + GSE 5μg/mL + hydroxyl radical; Lane 10= DNA + GSE 10 μg/mL + hydroxyl radical; Lane 11= DNA + GSE 50 μg/mL + hydroxyl radical ; Lane 12= DNA + GSE 100 μg/mL + hydroxyl radical.
Abbreviations are: BSE, brown skin extract; GSE, green shell extract; S, Supercoiled DNA strand; N, Nicked DNA strand.

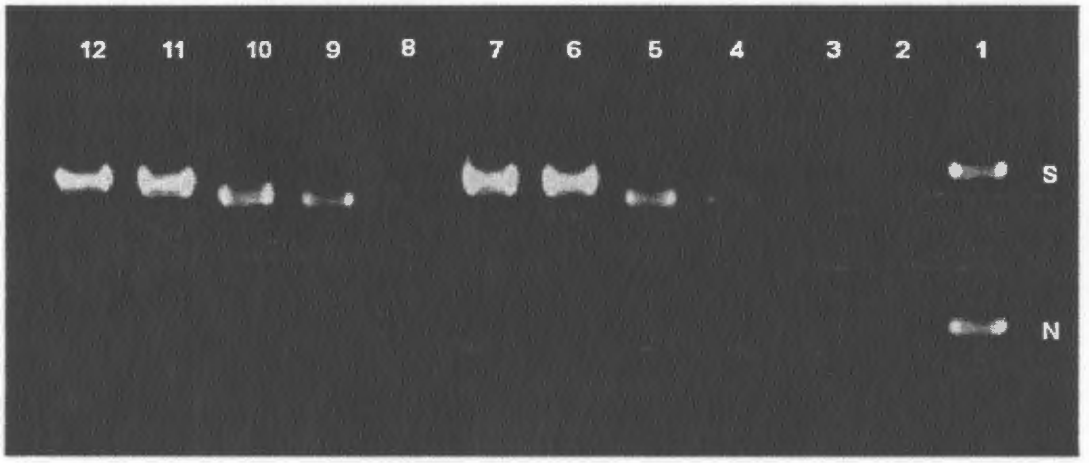

12
11
10
9
8
7
6
5
4
3
2
1
S
N

Figure A-15 **Effect of almond whole seed extract (WSE) in preventing site-specific hydroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + PBS; Lane 2= DNA + hydroxyl radical; Lane 3= DNA + WSE 2μg/mL + hydroxyl radical; Lane 4= DNA + WSE 5μg/mL + hydroxyl radical; Lane 5= DNA + WSE 10 μg/mL + hydroxyl radical; Lane 6= DNA + WSE 50 μg/mL + hydroxyl radical ; Lane 7= DNA + WSE 100 μg/mL + hydroxyl radical.
Abbreviations are: WSE, whole seed extract; S, Supercoiled DNA strand; N, Nicked DNA strand.

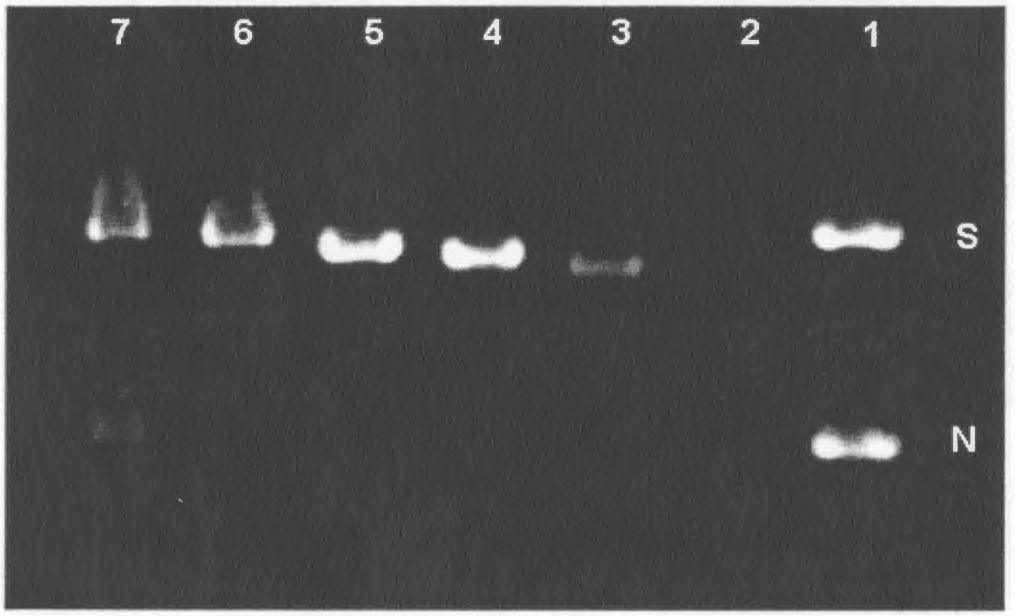

7
6
5
4
3
2
1
S
N

Figure A-16 **Effect of almond brown skin (BSE) and green shell (GSE) extracts in preventing site-specific hydroxyl radical induced DNA scission at 37°C**
Lane 1= DNA + PBS; Lane 2= DNA + hydroxyl radical; Lane 3= DNA + BSE 2μg/mL + hydroxyl radical; Lane 4= DNA + BSE 5μg/mL + hydroxyl radical; Lane 5= DNA + BSE 10 μg/mL + hydroxyl radical; Lane 6= DNA + BSE 50 μg/mL + hydroxyl radical; Lane 7= DNA + BSE 100 μg/mL + hydroxyl radical; Lane 8= DNA + GSE 2μg/mL Lane 9= DNA + GSE 5μg/mL + hydroxyl radical; Lane 10= DNA + GSE 10 μg/mL + hydroxyl radical; Lane 11= DNA + GSE 50 μg/mL + hydroxyl radical; Lane 12= DNA + GSE 100 μg/mL + hydroxyl radical.
Abbreviations are: BSE, brown skin extract; GSE, green shell extract; S, Supercoiled DNA strand; N, Nicked DNA strand.

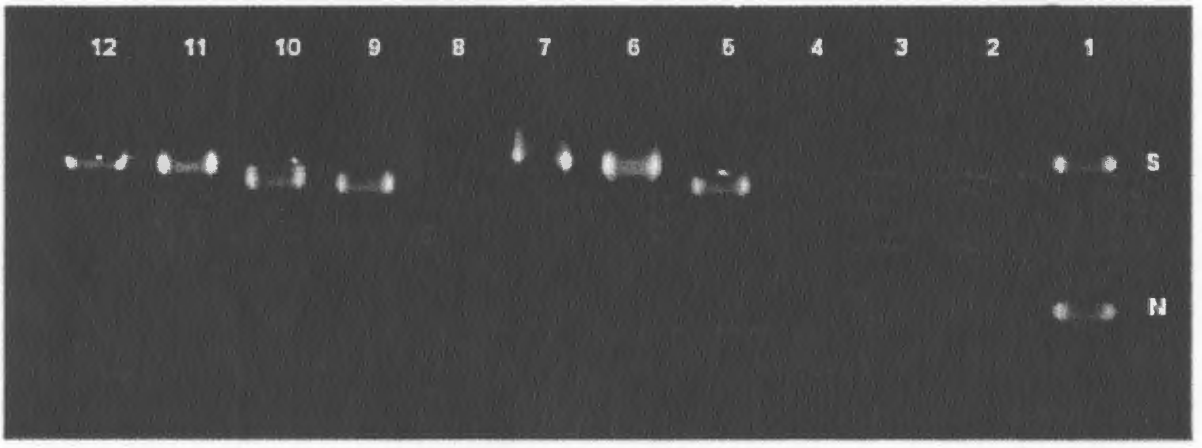
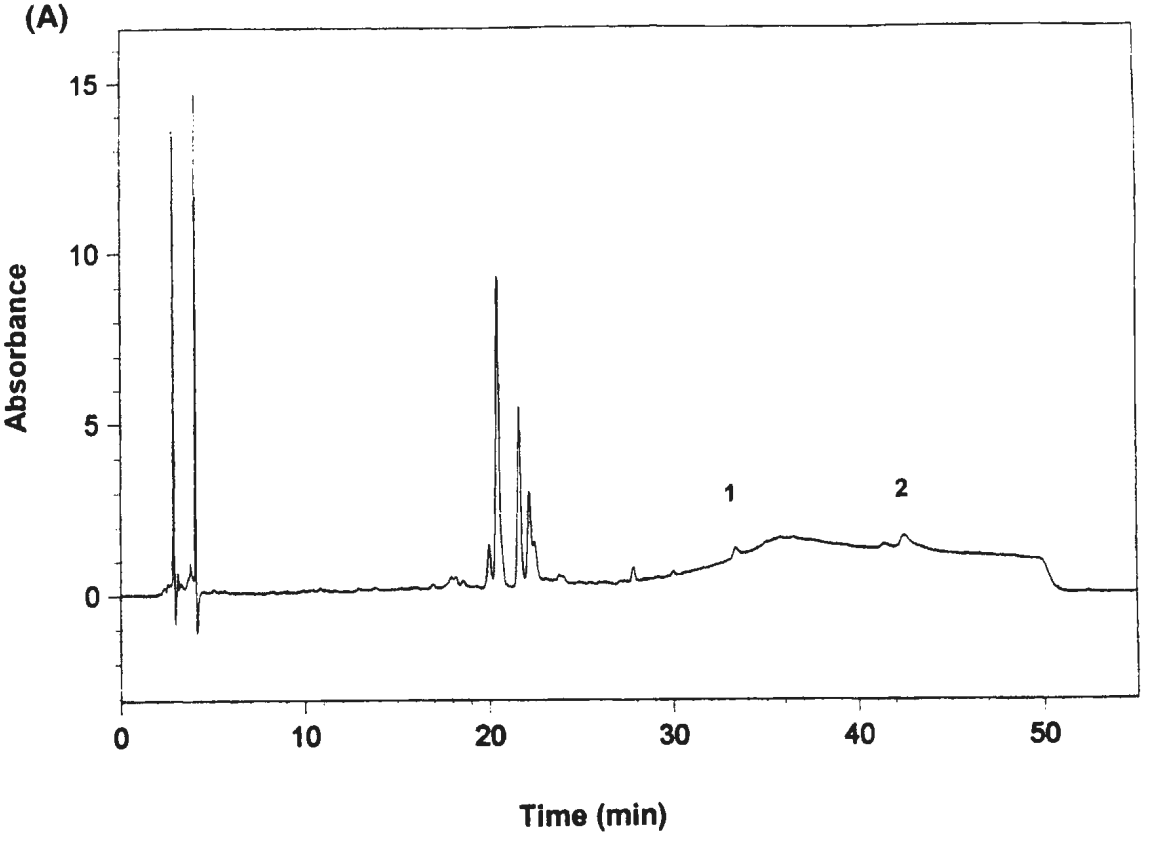

12
11
10
9
8
7
6
5
4
3
2
1
S
N

Figure A-17 **High performance liquid chromatographic profiles of (A) native and (B) hydrolysed almond whole seed extract detected at 365 nm Peaks: 1, quercetin; 2, isorhamnetin**


(A)
Absorbance
15
10
5
0
1
2
0
10
20
30
40
50
Time (min)

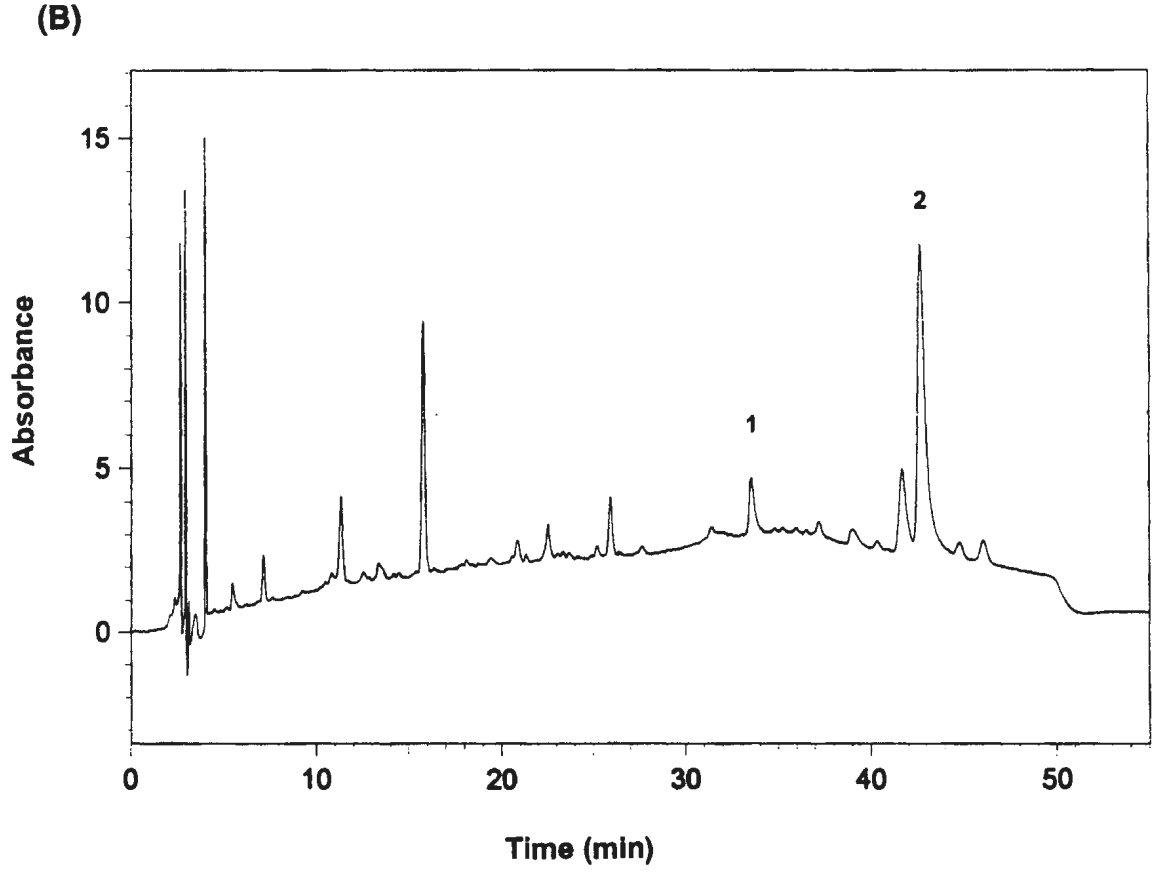

(B)
Absorbance
15
10
5
0
1
2
0
10
20
30
40
50
Time (min)

Figure A-18 **High performance liquid chromatographic profiles of (A) native and (B) hydrolysed almond brown skin extract detected at 365 nm Peaks: 1, quercetin; 2, isorhamnetin**

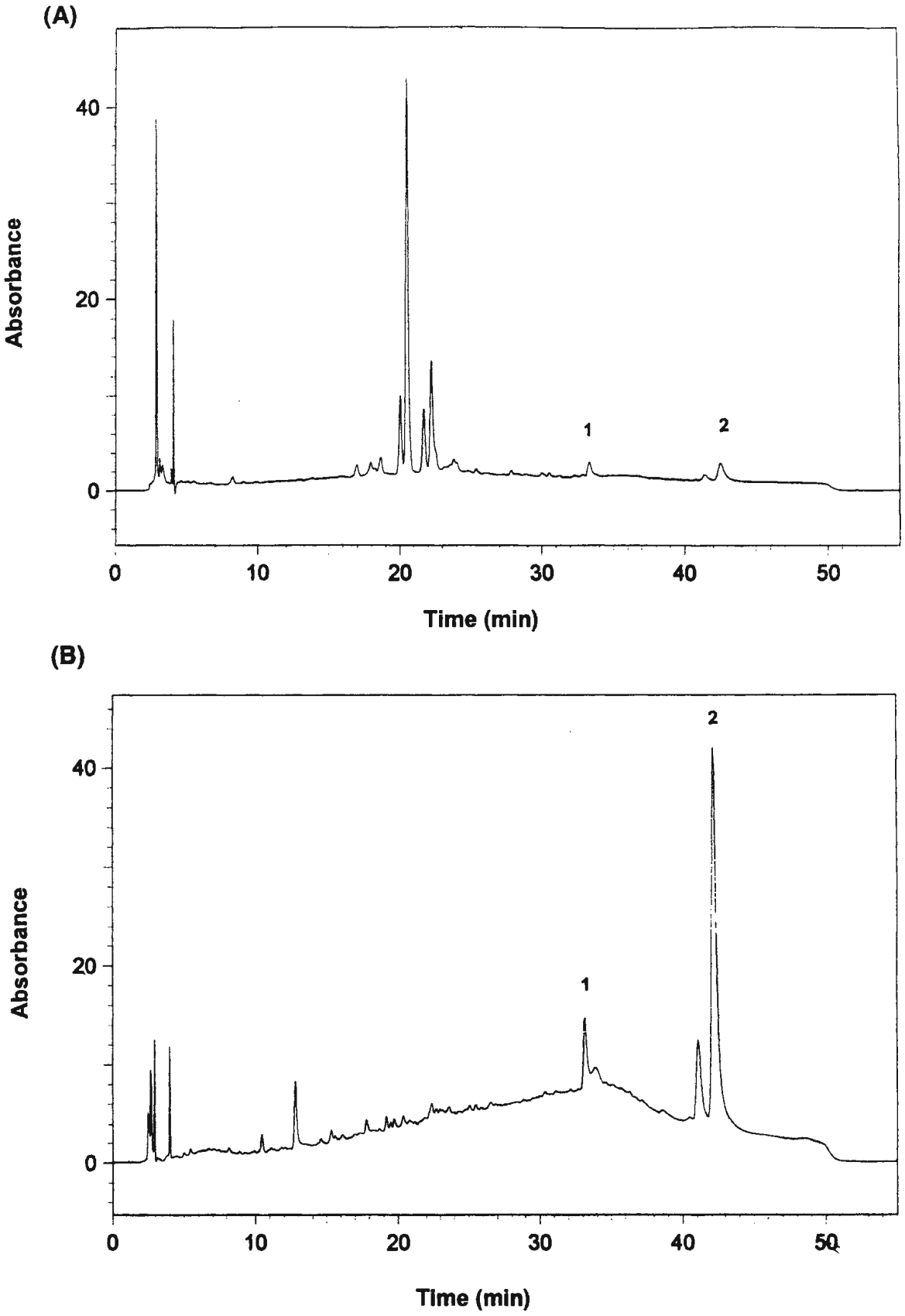

(A)
Absorbance
40
20
0
1
2
0
10
20
30
40
50
Time (min)
(B)
Absorbance
40
20
0
1
2
0
10
20
30
40
50
Time (min)

Figure A-19 **High performance liquid chromatographic profiles of (A) native and (B) hydrolysed almond green shell extract detected at 365 nm Peaks: 1, quercetin; 2, isorhamnetin**

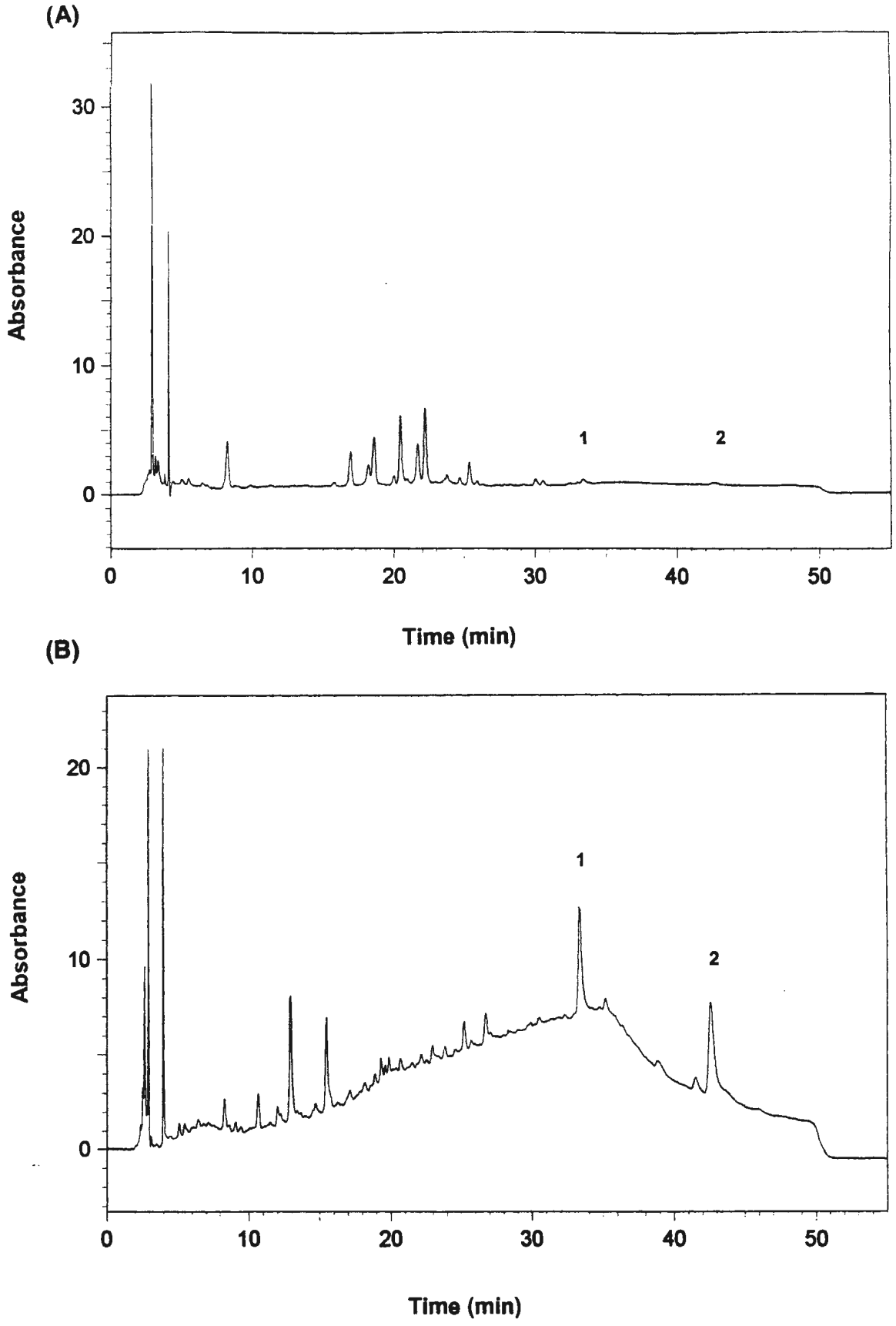

(A)
Absorbance
30
20
10
0
1
2
0
10
20
30
40
50
Time (min)
(B)
Absorbance
20
10
0
1
2
0
10
20
30
40
50
Time (min)

Figure A-20 **High performance liquid chromatographic profiles of phenolics liberated from esters in almond whole seed extract detected at 330 nm**
Peaks: 1, caffeic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, sinapic acid

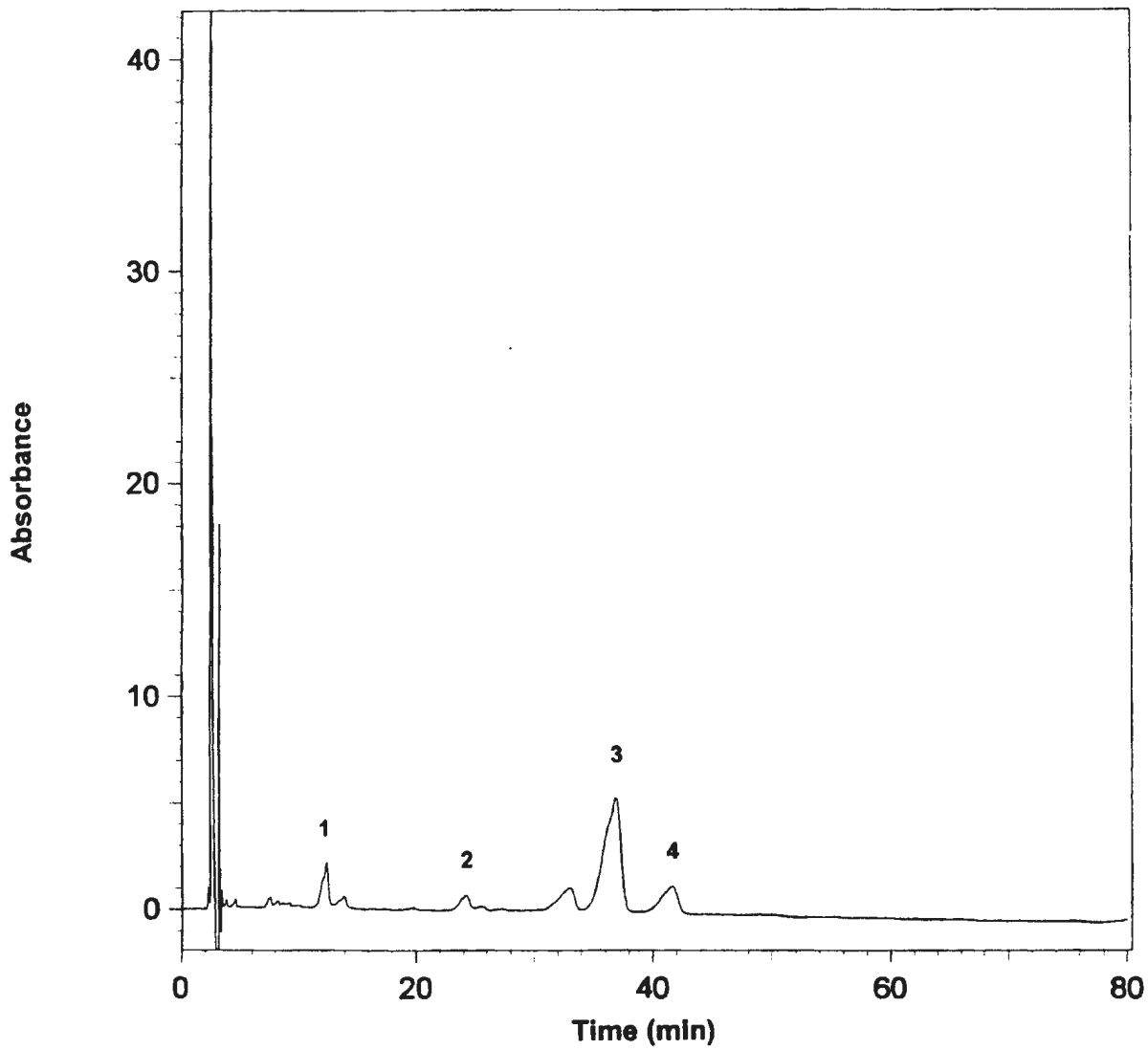

Absorbance
40
30
20
10
0
1
2
3
4
0
20
40
60
80
Time (min)

Figure A-21 **High performance liquid chromatographic profiles of phenolics liberated from esters in almond brown skin extract detected at 330 nm**
Peaks: 1, caffeic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, sinapic acid

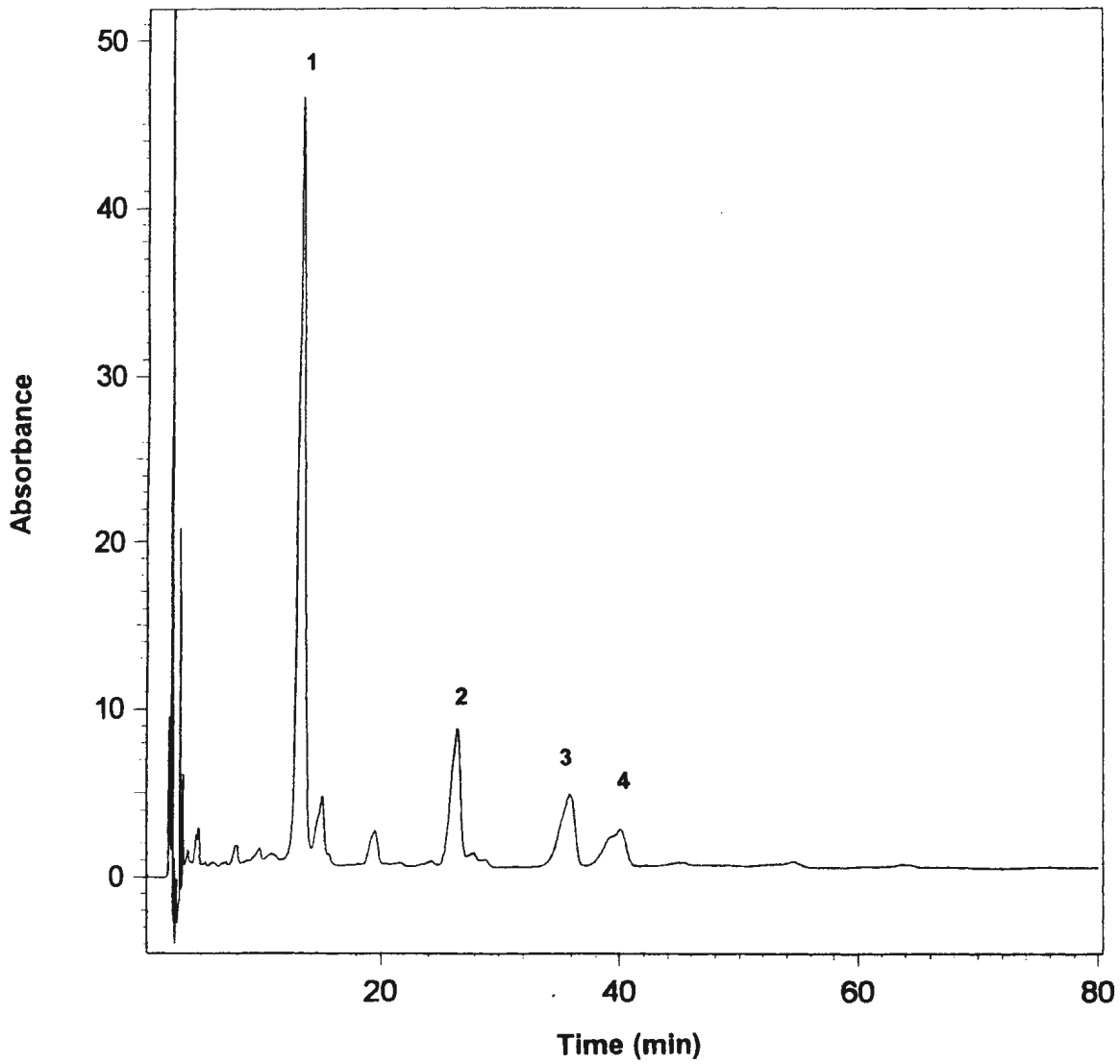

50
40
30
20
10
0
Absorbance
1
2
3
4
20
40
60
80
Time (min)

Figure A-22 **High performance liquid chromatographic profiles of phenolics liberated from esters in almond green shell extract detected at 330 nm**
Peaks: 1, caffeic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, sinapic acid

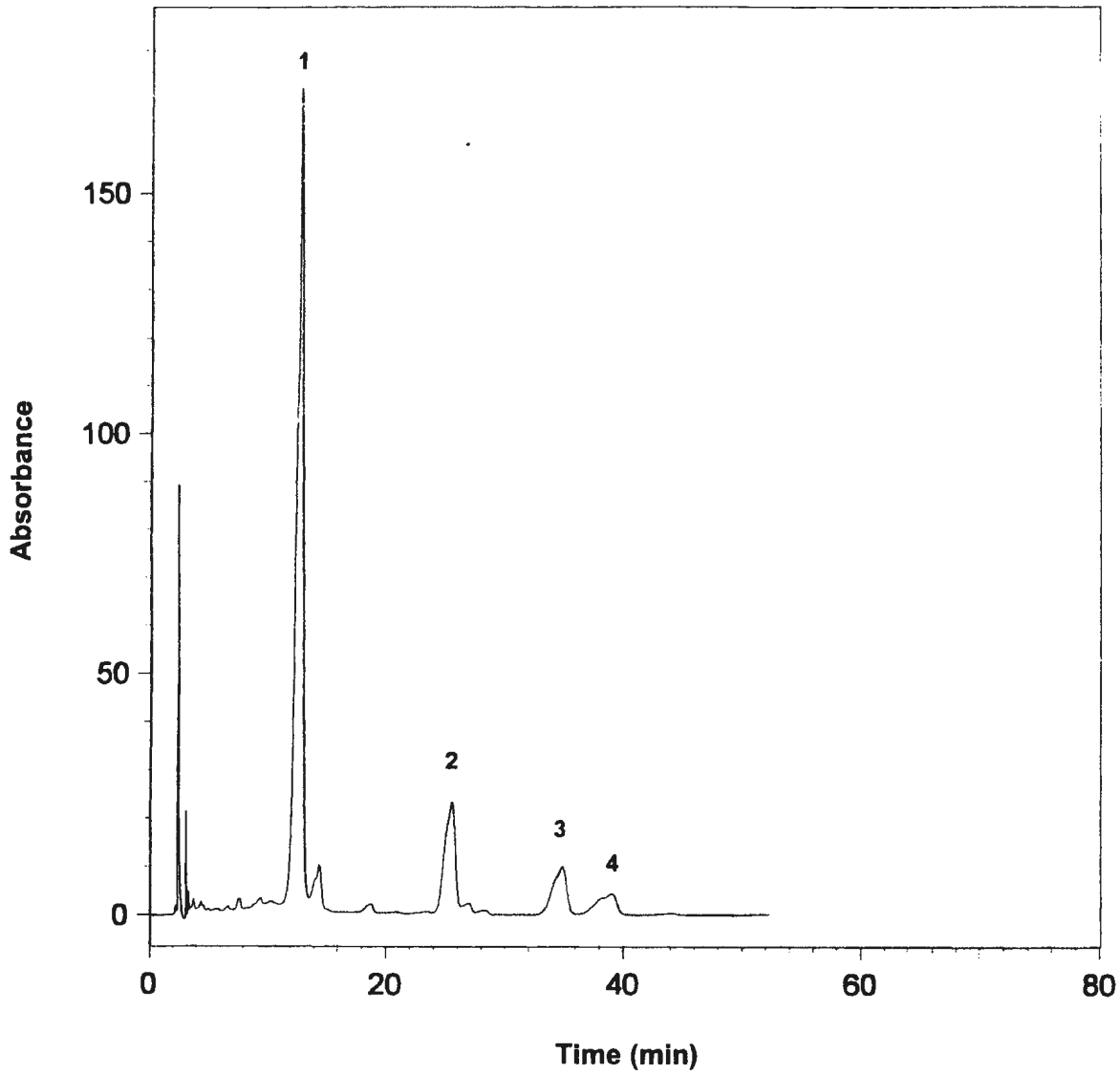

1
2
3
4
150
100
50
0
Absorbance
0
20
40
60
80
Time (min)

Table A-1 Conjugated diene values of almond extracts and reference antioxidants at 100 ppm as quercetin equivalents in a bulk stripped corn oil system during a 7-day storage period at 60°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	$2.24 \pm .12^{a}$	$3.93 \pm .12^{c}$	$5.56 \pm .09^{cd}$	$9.74 \pm .24^{d}$	$13.84 \pm .31^{d}$
WSE	$2.21 \pm .10^{a}$	$2.51 \pm .11^{a}$	$4.92 \pm .36^{bc}$	$7.51 \pm .29^{bc}$	$10.77 \pm .21^{b}$
BSE	$2.33 \pm .15^{a}$	$2.75 \pm .04^{ab}$	$4.91 \pm .21^{bc}$	$7.90 \pm .09^{c}$	$12.12 \pm .28^{c}$
GSE	$2.11 \pm .21^{a}$	$2.44 \pm .09^{a}$	$4.74 \pm .22^{b}$	$6.75 \pm .51^{b}$	$8.56 \pm .35^{a}$
Quercetin[2]	$2.25 \pm .12^{a}$	$2.87 \pm .12^{b}$	$3.49 \pm .27^{a}$	$4.68 \pm .41^{a}$	$8.56 \pm .32^{a}$
α-Tocopherol[2]	$2.32 \pm .18^{a}$	$3.76 \pm .19^{c}$	$6.07 \pm .24^{d}$	$9.50 \pm .31^{d}$	$14.17 \pm .42^{d}$
BHA[2]	$2.25 \pm .12^{a}$	$2.56 \pm .15^{a,b}$	$5.20 \pm .21^{b,c}$	$7.46 \pm .51^{b,c}$	$11.19 \pm .42^{b,c}$

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-2 Conjugated diene values of almond extracts and reference antioxidants at 200 ppm as quercetin equivalents in a bulk stripped corn oil system during a 7-day storage period at 60°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	2.24 ± 0.12^{a}	3.95 ± 0.02^{e}	5.61 ± 0.09^{c}	9.98 ± 0.16^{d}	13.65 ± 0.45^{e}
WSE	2.21 ± 0.10^{a}	2.50 ± 0.20^{bc}	4.03 ± 0.49^{b}	6.45 ± 0.22^{bc}	10.33 ± 0.36^{cd}
BSE	2.33 ± 0.15^{a}	2.73 ± 0.09^{c}	3.96 ± 0.17^{b}	7.13 ± 0.17^{c}	10.86 ± 0.34^{d}
GSE	2.11 ± 0.21^{a}	2.33 ± 0.09^{b}	4.41 ± 0.13^{b}	5.55 ± 0.62^{b}	8.35 ± 0.21^{b}
Quercetin[2]	2.25 ± 0.12^{a}	2.67 ± 0.01^{c}	2.73 ± 0.04^{a}	4.23 ± 0.12^{a}	6.23 ± 0.20^{a}
α-Tocopherol[2]	2.32 ± 0.18^{a}	3.19 ± 0.14^{d}	6.01 ± 0.35^{c}	9.30 ± 0.51^{d}	12.93 ± 0.35^{e}
BHA[2]	2.25 ± 0.12^{a}	2.03 ± 0.02^{a}	4.03 ± 0.29^{b}	$6.24 \pm 0.25^{b,c}$	9.83 ± 0.12^{c}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-3 Hexanal content (mg/kg sample) of almond extracts and reference antioxidants at 100 ppm as quercetin equivalents in a bulk stripped corn oil system during a 7-day storage period at 60°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	1.1 ± 0.1[a]	18.2 ± 1.2[c]	38.7 ± 1.5[d]	85.2 ± 3.9[d]	105.3 ± 6.8[c]
WSE	1.0 ± 0.2[a]	1.6 ± 0.2[a]	6.5 ± 0.5[ab]	15.4 ± 1.7[b]	26.3 ±1.8[b]
BSE	1.0 ± 0.1[a]	1.7 ± 0.2[a]	7.2 ± 0.6[b]	17.2 ± 1.7[b]	25.2 ±1.6[b]
GSE	1.2 ± 0.1[a]	1.4 ± 0.2[a]	6.6 ± 0.5[ab]	11.2 ± 1.2 [ab]	20.1 ± 1.6[ab]
Quercetin[2]	1.1 ± 0.1[a]	1.6 ± 0.1[a]	4.2 ± 0.2[a]	8.1 ± 0.9[a]	10.2 ± 0.9[a]
α-Tocopherol[2]	1.2 ± 0.1[a]	15.2 ± 0.2[b]	34.4 ± 1.6[c]	70.5 ± 2.3[c]	80.1 ± 6.7[c]
BHA[2]	1.3 ± 0.1[a]	2.5 ± 0.3[a]	5.8 ± 0.3[ab]	16.3 ± 1.3[b]	24.3 ± 2.6[b]

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-4 Hexanal content (mg/kg sample) of almond extracts and reference antioxidants at 200 ppm as quercetin equivalents in a bulk stripped corn oil system during a 7-day storage period at 60°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	1.1 ± 0.1[a]	18.2 ± 1.2[c]	38.7 ± 1.5[c]	85.2 ± 3.9[d]	105.3 ± 6.8[d]
WSE	1.0 ± 0.2[a]	1.4 ± 0.1[a]	2.5 ± 0.3[a]	9.5 ± 0.9[b]	18.7 ± 1.1[b]
BSE	1.0 ± 0.1[a]	1.5 ± 0.2[a]	3.6 ± 0.4[a]	7.3 ± 1.7[ab]	16.3 ±.1.9[b]
GSE	1.2 ± 0.1[a]	1.3 ± 0.2[a]	2.5 ± 0.3[a]	5.7 ± 0.5[a]	10.2 ± 1.2[a]
Quercetin[2]	1.1 ± 0.1[a]	1.4 ± 0.2[b]	1.8 ± 0.2[a]	3.2 ± 0.3[ab]	5.4 ± 0.8[ab]
α-Tocopherol[2]	1.2 ± 0.1[a]	12.5 ± 0.5[b]	26.5 ± 1.8[b]	48.6 ± 3.6[c]	67.7 ± 3.5[c]
BHA[2]	1.3 ± 0.1[a]	1.8 ± 0.4[a]	3.1± 0.2[a]	8.4 ± 0.7[ab]	14.7 ± 1.1[b]

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-5 TBARS (as μmoles malonaldehyde equivalents) of almond extracts and reference antioxidants at 100 ppm as quercetin equivalents in a bulk stripped corn oil system during a 7-day storage period at 60°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	0.29 ± 0.02^{a}	0.41± 0.01^{a}	0.63 ± 0.06^{bc}	1.07 ± 0.14^{c}	1.41 ± 0.09^{c}
WSE	0.28 ± 0.03^{a}	0.37 ± 0.05^{a}	0.71 ± 0.02^{c}	0.77 ± 0.04^{ab}	0.79 ± 0.06^{ab}
BSE	0.25 ± 0.02^{a}	0.42 ± 0.01^{a}	0.53 ± 0.04^{ab}	0.71 ± 0.08^{ab}	0.85 ±.0.05^{ab}
GSE	0.29 ± 0.01^{a}	0.39 ± 0.03^{a}	0.54 ± 0.08^{ab}	0.69 ± 0.05^{a}	0.75 ± 0.04^{a}
Quercetin[2]	0.26± 0.02^{a}	0.46 ± 0.03^{a}	0.49 ± 0.02^{ab}	0.58 ± 0.03^{a}	0.63 ± 0.05^{a}
α-Tocopherol[2]	0.27 ± 0.02^{a}	0.48 ± 0.03^{a}	0.46 ± 0.05^{a}	0.92 ± 0.02^{bc}	1.01 ± 0.2^{b}
BHA[2]	0.26 ± 0.01^{a}	0.41 ± 0.09^{a}	0.53 ± 0.06^{ab}	0.73 ± 0.11^{ab}	0.79 ± 0.02^{ab}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-6 TBARS (as μmoles malonaldehyde equivalents) of almond extracts and reference antioxidants at 200 ppm as quercetin equivalents in a bulk stripped corn oil system during a 7-day storage period at 60°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	0.29 ± 0.02^{a}	0.41 ± 0.01^{b}	0.63 ± 0.06^{d}	1.41 ± 0.14^{d}	1.41 ± 0.09^{f}
WSE	0.26 ± 0.03^{a}	0.28 ± 0.01^{a}	0.37 ± 0.02^{ab}	0.71 ± 0.05^{ab}	0.75 ± 0.04^{cd}
BSE	0.28 ± 0.01^{a}	0.31 ± 0.02^{a}	0.47 ± 0.08^{bc}	0.64 ± 0.03^{bc}	$0.81 \pm .0.04^{d}$
GSE	0.27 ± 0.02^{a}	0.31 ± 0.04^{a}	0.49 ± 0.01^{c}	0.55 ± 0.02^{c}	0.66 ± 0.03^{bc}
Quercetin[2]	0.26 ± 0.02^{a}	0.38 ± 0.03^{b}	0.45 ± 0.02^{bc}	0.40 ± 0.03^{bc}	0.37 ± 0.05^{a}
α-Tocopherol[2]	0.26 ± 0.02^{a}	0.43 ± 0.02^{b}	0.52 ± 0.01^{cd}	0.76 ± 0.03^{cd}	1.14 ± 0.05^{e}
BHA[2]	0.26 ± 0.01^{a}	0.28 ± 0.01^{a}	0.32 ± 0.02^{a}	0.47 ± 0.01^{a}	0.57 ± 0.02^{b}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-7 TBARS (as μg malonaldehyde equivalents/kg sample) of almond extracts and reference antioxidants at 100 ppm as quercetin equivalents in a meat model system during a 7-day storage period at 4°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	1.2 ± 0.1^{a}	8.1± 0.1^{e}	10.8 ± 0.6^{d}	18.4 ± 0.4^{d}	23.2 ± 1.2^{e}
WSE	1.0 ± 0.1^{a}	6.7± 0.2^{cd}	10.6 ± 0.9^{d}	14.5 ± 0.6^{c}	18.7 ± 0.8^{d}
BSE	1.1 ± 0.1^{a}	6.2± 0.3^{c}	8.5 ± 0.3^{c}	13.1 ± 0.8^{c}	14.7 ±.0.9^{c}
GSE	1.1 ± 0.1^{a}	7.2 ± 0.4^{d}	10.3 ± 0.5^{d}	14.7 ± 0.4^{c}	18.4 ± 0.8^{d}
Quercetin[2]	1.0± 0.1^{a}	1.5 ± 0.2^{a}	1.9 ± 0.3^{a}	2.2 ± 0.4^{a}	2.8 ± 0.3^{a}
α-Tocopherol[2]	1.0 ± 0.1^{a}	2.3 ± 0.3^{b}	4.5 ± 0.4^{b}	5.1 ± 0.8^{b}	5.6 ± 0.6^{a}
BHA[2]	1.1 ± 0.1^{a}	1.9 ± 0.2^{ab}	2.2 ± 0.5^{a}	2.5 ± 0.6^{a}	2.6 ± 0.4^{a}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-8 TBARS (as μg malonaldehyde equivalents/kg sample) of almond extracts and reference antioxidants at 200 ppm as quercetin equivalents in a meat model system during a 7-day storage period at 4°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	1.2 ± 0.1[a]	8.1 ± 0.1[d]	10.8 ± 0.6[d]	18.5 ± 0.4[e]	23.2 ± 1.2[e]
WSE	1.0 ± 0.1[a]	2.1 ± 0.1[a]	5.4 ± 0.5[b]	6.9 ± 0.3[c]	10.6 ± 0.8[cd]
BSE	1.1 ± 0.1[a]	4.7 ± 0.8[b]	5.3 ± 0.2[b]	5.4 ± 0.7[b]	9.3 ±.0.5[c]
GSE	1.1 ± 0.1[a]	6.3 ± 0.6[c]	8.1 ± 0.4[c]	9.5 ± 0.4[d]	12.2 ± 0.5[d]
Quercetin[2]	1.0± 0.1[a]	1.2 ± 0.2[a]	1.8 ± 0.2[a]	1.9 ± 0.3[a]	2.5 ± 0.3[a]
α-Tocopherol[2]	1.0 ± 0.1[a]	2.1± 0.2[a]	4.3 ± 0.4[b]	4.7 ± 0.3[b]	4.8 ± 0.4[b]
BHA[2]	1.1 ± 0.1[a]	1.5 ± 0.2[a]	1.5 ± 0.3[a]	2.3 ± 0.3[a]	2.6 ± 0.2[a]

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-9 Hexanal content (mg/kg sample) of almond extracts and reference antioxidants at 100 ppm as quercetin equivalents in a meat model system during a 7-day storage period at 4°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	6.3 ± 0.2^{c}	9.9 ± 0.4^{c}	15.3 ± 0.9^{d}	19.4 ± 1.1^{d}	25.7 ± 1.4^{d}
WSE	6.2 ± 0.2^{c}	7.9 ± 0.2^{b}	11.4 ± 0.3^{c}	14.6 ± 0.5^{c}	18.2 ± 0.5^{c}
BSE	5.3 ± 0.1^{b}	7.2 ± 0.3^{b}	10.2 ± 0.7^{c}	15.7 ± 0.5^{c}	18.3 ± 0.9^{c}
GSE	1.9 ± 0.1^{a}	7.6 ± 0.4^{b}	8.9 ± 0.3^{b}	10.7 ± 0.4^{b}	12.6 ± 0.5^{b}
Quercetin[2]	2.0 ± 0.1^{a}	2.3 ± 0.1^{a}	2.9 ± 0.1^{a}	5.5 ± 0.1^{a}	7.2 ± 0.2^{a}
α-Tocopherol[2]	2.0 ± 0.1^{a}	2.1 ± 0.1^{a}	3.9 ± 0.1^{a}	5.3 ± 0.2^{a}	7.9 ± 0.2^{a}
BHA[2]	2.2 ± 0.1^{a}	2.8 ± 0.1^{a}	3.2 ± 0.1^{a}	4.2 ± 0.1^{a}	6.3 ± 0.2^{a}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-10 Hexanal content (mg/kg sample) of almond extracts and reference antioxidants at 200 ppm as quercetin equivalents in a meat model system during a 7-day storage period at 4°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	6.3 ± 0.2^{d}	9.9 ± 0.4^{d}	15.3 ± 0.9^{c}	19.4 ± 1.1^{d}	25.7 ± 1.4^{d}
WSE	3.3 ± 0.2^{b}	3.3 ± 0.1^{b}	3.6 ± 0.2^{a}	4.9 ± 0.1^{bc}	7.2 ± 0.3^{bc}
BSE	4.1 ± 0.1^{c}	4.6 ± 0.2^{c}	5.2 ± 0.2^{b}	$5.4 \pm 0.2^{b,c}$	8.3 ± 0.4^{c}
GSE	1.9 ± 0.1^{a}	2.5 ± 0.1^{a}	5.3 ± 0.2^{b}	6.1 ± 0.2^{c}	6.8 ± 0.2^{bc}
Quercetin[2]	2.0 ± 0.1^{a}	2.1 ± 0.1^{a}	2.8 ± 0.1^{a}	3.1 ± 0.1^{a}	3.3 ± 0.1^{a}
α-Tocopherol[2]	2.0 ± 0.1^{a}	2.1 ± 0.1^{a}	3.3 ± 0.1^{a}	4.6 ± 0.2^{b}	6.5 ± 0.2^{b}
BHA[2]	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}	3.1 ± 0.1^{a}	4.2 ± 0.1^{b}	5.6 ± 0.1^{b}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-11 Total volatile content (mg/kg sample) of almond extracts and reference antioxidants at 100 ppm as quercetin equivalents in a meat model system during a 7-day storage period at 4°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	25.3 ± 1.1[b]	40.8 ± 2.2[c]	42.5 ± 2.5[b]	51.6 ± 1.6[c]	64.2 ± 2.3[c]
WSE	16.2 ± 1.1[a]	31.5 ± 1.8[b]	41.8 ± 1.9[b]	45.2 ± 1.9[b]	61.5 ± 2.1[c]
BSE	17.1 ± 1.2[a]	32.2 ± 1.3[b]	42.1 ± 1.5[b]	45.3 ± 1.6[b]	62.2 ± 1.8[c]
GSE	14.1 ± 1.5[a]	16.8 ± 1.3[a]	22.3 ± 1.4[a]	28.1 ± 1.3[a]	29.4 ± 1.5[b]
Quercetin[2]	15.4 ± 1.1[a]	18.9 ± 1.2[a]	20.9 ± 1.5[a]	22.1 ± 0.9[a]	24.5 ± 1.0[a]
α-Tocopherol[2]	14.1 ± 0.8[a]	18.6 ± 0.9[a]	21.6 ± 1.1[a]	22.6 ± 1.1[a]	24.5 ± 1.2[a]
BHA[2]	16.6 ± 0.9[a]	20.2 ± 0.8[a]	22.1 ± 0.9[a]	23.5 ± 0.9[a]	32.1 ± 1.0[b]

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.

Table A-12 Total volatile content (mg/kg sample) of almond extracts and reference antioxidants at 200 ppm as quercetin equivalents in a meat model system during a 7-day storage period at 4°C[1]

Sample	Storage time (days)				
	0	1	3	5	7
Control	25.3 ± 1.1^{e}	40.8 ± 2.2^{e}	42.5 ± 2.5^{e}	51.6 ± 1.6^{e}	64.2 ± 2.3^{e}
WSE	15.6 ± 0.9^{d}	23.2 ± 1.1^{d}	24.6 ± 1.1^{d}	33.9 ± 1.3^{d}	42.9 ± 1.9^{d}
BSE	12.9 ± 0.9^{c}	18.1 ± 0.9^{c}	20.3 ± 1.0^{c}	23.5 ± 1.2^{c}	34.7 ± 1.6^{c}
GSE	11.9 ± 0.8^{bc}	12.1 ± 0.9^{b}	17.3 ± 1.1^{bc}	19.9 ± 1.2^{b}	20.7 ± 1.5^{b}
Quercetin[2]	6.8 ± 0.7^{a}	7.7 ± 0.7^{a}	8.5 ± 0.7^{a}	10.2 ± 0.8^{a}	12.4 ± 0.8^{a}
α-Tocopherol[2]	10.9 ± 0.9^{bc}	12.3 ± 0.8^{b}	14.2 ± 0.9^{b}	17.2 ± 0.9^{b}	18.5 ± 1.1^{b}
BHA[2]	10.2 ± 0.9^{b}	12.7 ± 0.9^{b}	15.6 ± 1.1^{bc}	16.9 ± 1.5^{b}	18.2 ± 1.0^{b}

[1]Results are mean values of three determinations ± standard deviation. Means in a column sharing the same superscript are not significantly ($p > 0.05$) different from one another.
[2]Reference antioxidants.
Abbreviations are: WSE, whole seed extract; BSE, brown skin extract; GSE, green shell extract and BHA, Butylated hydroxyanisole.