CHARACTERIZATION OF NATURAL ANTIOXIDANTS OF MEALS OF BORAGE AND EVENING PRIMROSE

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BY

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THIS WORK IS DEDICATED TO

MY LOVING PARENTS, WIFE & SON

ABSTRACT

Antioxidant efficacy of borage and evening primrose meals in a cooked comminuted pork model system was investigated. Borage meal, at 2% (w/w), reduced the formation of thiobarbituric acid-reactive substances (TBARS), hexanal and total volatiles in treated samples by 27, 30 and 19%, respectively. Formation of TBARS, hexanal and total volatiles in samples containing 2% (w/w) evening primrose meal was reduced on day-7 of the storage by 44, 73 and 63%, respectively. Since meals demonstrated antioxidant properties, their crude extracts were prepared under optimum extraction conditions which were determined by employing response surface methodology (RSM). Effects of three variables, namely the solvent content in the aqueous extraction medium (x_1, %, v/v), extraction temperature (x_2, °C) and time (x_3, min) on antioxidant efficacy (Y) of the extracts were investigated. RSM predicted that the maximum antioxidant activity of borage extract was reached when x_1, x_2 and x_3 were 52% ethanol (v/v), 72°C and 62 min, respectively. The corresponding optimum extraction conditions for evening primrose were predicted to be 56% acetone (v/v), 71°C and 47 min, respectively. Fitted polynomial models for borage and evening primrose were significant ($p \leq 0.05$) and reproducible (CV < 5%). Verification experiments carried out to determine the adequacy of the models showed that the predicted response values were well in agreement with the observed values ($r > 0.95$). Crude extracts prepared under

optimum extraction conditions, were subjected to Sephadex LH-20 column chromatography. For both types of extracts, six fractions (I-VI) were obtained and their contents of total, hydrophobic and hydrophillic phenolics determined. Borage fractions I-VI consisted of 283, 129, 140, 366, 280 and 347 mg of phenolics as sinapic acid equivalents, respectively, while evening primrose fractions I-VI contained 158, 313, 369, 402, 279 and 445 mg of phenolics as catechin equivalents, respectively. Borage fractions contained more of hydrophillic than hydrophobic phenolics whereas evening primrose fractions contained high amounts of both types of phenolics. A qualitative vanillin test was employed to determine the presence or absence of condensed tannins in borage and evening primrose fractions. Borage fractions did not contain condensed tannins as evidenced by a negative vanillin test, but fractions III-VI of evening primrose did. Ultraviolet (UV) spectra of borage fractions indicated possible presence of phenolic acids while those for evening primrose fractions suggested possible presence of procyanidins.

Antioxidant efficacies of borage and evening primrose crude extracts and their fractions (additives) were investigated in β-carotene-linoleate, cooked comminuted pork, bulk stripped corn oil and stripped corn oil-in-water emulsion systems. In general, all additives exhibited varying antioxidant activities in all four types of model systems investigated. After a 2 h assay period, borage and evening primrose additives were able to retain 27-79% and 37-84% of initial content of β-carotene, respectively, as compared to 10% retention in the control. In cooked comminuted pork model systems, borage and

evening primrose additives inhibited the formation of TBARS, hexanal and total volatiles (on day-3 of storage) to varying degrees (19-97%) as compared to the control. On day-3 of storage, 200 ppm (as sinspic acid equivalents) of borage additives inhibited the formation of conjugated dienes, hexanal and total volatiles in bulk stripped-corn oil and its oil-in-water emulsions by 21-95% as compared to the control. Inhibition of formation of oxidation products in samples treated with 200 ppm (as catechin equivalents) of evening primrose additives, on day-3, ranged from 17 to 94% as compared to the control.

In general, borage additives were better antioxidants in bulk stripped corn oil systems than their emulsion counterparts. Evening primrose additives performed well in almost all systems examined. These effects were attributed to the different affinities of active compounds to various phases and interfaces of the model systems. In an attempt to investigate the antioxidant mechanisms of additives, iron (II) chelating, reactive-oxygen species (ROS) scavenging and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of borage and evening primrose additives were also determined. Iron (II) binding capacities of borage and evening primrose additives ranged from 33 to 100%, depending upon the type and concentration of additives investigated. In general, borage and evening primrose additives exerted strong ROS scavenging properties of 100% in most cases. Borage and evening primrose additives also exhibited strong DPPH radical scavenging activities. An attempt was made to correlate iron (II) chelating, ROS

and DPPH scavenging activities of the additives to the antioxidant activities brought about by respective additives in model systems: correlations ranged from weak ($r < 0.6$) to very strong ($r > 0.9$), depending mainly upon the nature of the model system involved, as determined by linear regression analysis.

For the first time, major phenolic antioxidants present in the borage and evening primrose extracts were identified. Presence of rosmarinic, syringic and sinapic acids in borage extract was confirmed by chromatographic as well as UV, mass and nuclear magnetic resonance (NMR) spectroscopic data. Instrumental analysis of isolated evening primrose phenolics allowed identification of (+)catechin, (-)epicatechin and gallic acid as the major active compounds responsible for antioxidant activity of its extracts.

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LIST OF ABBREVIATIONS

ANOVA	-Analysis of variance
AOAC	-Association of Official Analytical Chemists'
AOCS	-American Oil Chemists' Society
ATP	-Adenosintriphosphate
BHA	-Butylated hydroxyanisole
BHT	-Butylated hydroxytoluene
CD	-Conjugated dienes
COSY	-Correlation spectroscopy
CRD	-Completely randomized block design
CV	-coefficient of variance
DMPO	-5,5-Dimethyl-1-pyrrole-N-oxide
DNA	-Deoxyribonucleic acid
DHA	-Docosahexaenoic acid
DPPH	-2,2-Diphenyl-1-picrylhydrazyl
EDTA	-Ethylenediaminetetraacetic acid
EPA	-Eicosapentaenoic acid
EPR	-Electron paramagnetic resonance
FAME	-Fatty acid methyl esters
FID	-Flame ionization detector
FDA	-Food and Drug Administration

GC	-Gas chromatography
GLM	-General linear model
HPLC	-High performance liquid chromatography
HS	-Headspace
IR	-Infrared
LD	-Lethal dose
LDL	-Low-density lipoprotein
LM	-Linear model
MA	-Malonaldehyde
MS	-Mass spectrometry
m/z	-mass to charge ratio
NADPH	-Nicotimamide adenine dinucleotide phosphate
ND	-Not detected
nm	-nanometre
NMR	-Nuclear magnetic resonance
OSI	-Oil Stability Instrument
PG	-Propyl gallate
ppm	-Parts per million
PUFA	-Polyunsaturated fatty acids
PV	-Peroxide value
QM	-Quadratic model

r	-Correlation coefficient
R^2	-Coefficient of determination
RNA	-Ribonucleic acid
ROS	-Reactive-oxygen species
RSM	-Response surface methodology
RSREG	-Response surface regression
SAS	-Statistical Analysis System
SOD	-Superoxide dismutase
TBA	-2-thiobarbituric acid
TBARS	-Thiobarbituric acid-reactive substances
TBHQ	-*tertiary*-Butyl hydroquinone
TCA	-Trichloroacetic acid
TLC	-Thin-layer chromatography
TMS	-Tetramethylsilane
UV	-Ultraviolet
USDA	-United States Department of Agriculture
UHP	-Ultra high purity
v/v	-Volume by volume
WOF	-Warmed-over flavour
w/v	-Weight by volume
w/w	-Weight by weight

CHAPTER 1

INTRODUCTION

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavours and potentially toxic reaction products (Maillard et al., 1996; Shahidi and Wanasundara, 1992). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tertiary*-butylhydroquinone (TBHQ) and propyl gallate (PG) may be added to food products to retard lipid oxidation (Winata and Lorenz, 1996). However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (Hettiarachchy et al., 1996). Therefore, search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Several sources of natural antioxidants are known and some of them are currently used in a variety of food products (Metha et al., 1994; Inatani et al., 1982). Extracts of herbs such as rosemary (Bracco et al., 1981), thyme (Inatani et al., 1982) and sage (Pizzocaro et al., 1994), oilseeds such as sesame (Fukuda et al., 1985; Osawa et al., 1985), canola (Wanasundara et al., 1994), flax (Amarowicz et al., 1990; Oomah et al., 1995), soybean (Chen et al., 1995) and peanut (Duh and Yen, 1995), cereals such as rice (Asamarai et al., 1996) and barley (Maillard et al., 1996), spices such as clove (Kramer, 1985), mustard (Shahidi et al., 1994a), turmeric (Chipault et al., 1955), ginger rhizome (Lee et al., 1986) and fenugreek (Hettiarachchy et al., 1996), and beverages such as black tea (Ruch et al., 1989) and green tea (Amarowicz and Shahidi, 1996; Ruch et al., 1989) have been reported to be antioxidative in

various model systems. The antioxidant activity of these extracts has always been attributed to their phenolic constituents. For instance, the antioxidants in rosemary extracts have been identified as phenolics such as rosmarinic acid, rosemary diphenol and rosmanol (Houlihan et al., 1984; Nakatani and Inatani, 1984). Several authors have reported the presence of flavonoids such as catechins in extracts of green and black teas. These phenolic compounds can retard lipid oxidation by donating a hydrogen atom or an electron to chain initiating free radicals such as the hydroxyl and superoxide radicals (Cao et al., 1997; Shahidi and Wanasundara, 1992). They can also neutralize the substrate-derived free radicals such as the fatty acid free radicals and alkoxy radicals (Cao et al., 1997; Packer and Glazer, 1990). Ruch et al. (1989) reported that tea extracts were capable of scavenging reactive oxygen species (ROS), namely hydrogen peroxide, superoxide and hydroxyl radicals. This property of plant extracts has an important role in retarding lipid oxidation in food products and living tissues. Incorporation of such extracts in human foods not only preserves their wholesomeness, but also reduces the risk of developing artherosclerosis and cancer (Ames, 1983; Namiki, 1990; Ramarathnam et al., 1995). Plant extracts, when added to lipid-containing foods, can also reduce the loss of α-tocopherol (Rice-Evans et al., 1996). Plant phenolics can regenerate α-tocopherol from tocopheryl free radical by donating an electron or a hydrogen atom. Some phenolic compounds present in plant extracts are reported to retard lipid oxidation through chelation of transition metal ions such as those of iron, copper and manganese (Rice-Evans et al., 1996).

As already mentioned, natural antioxidants in the meals of oilseeds are easily and abundantly available. Borage (*Borago officinalis* L.) and evening primrose (*Oinothera biennis*), oilseed crops grown mainly in North America, Europe and Australia, have earned an important place in the pharmaceutical industry due to the high content [19-22% and 9 - 10% (w/w), in borage and evening primrose seeds, respectively] of γ-linolenic acid (GLA) in their seed oils (Gibson et al., 1992; Rahmatulla et al., 1994; Redden et al., 1995). Borage and evening primrose oils have been used for treating several skin disorders (Chapkin and Charmicheal, 1990; Engler et al., 1991, 1992). The meals after oil removal may retain a substantial amount of phenolic antioxidants (Lu and Foo, 1995) which may be extracted by employing a proper extraction technique. It may be necessary to evaluate the extract in several model systems using different analytical techniques in order to draw a valid conclusion on their antioxidant efficacy. Several authors have reported that the antioxidant activity of plant phenolics differs greatly according to the physical and chemical properties of the model system in which they are evaluated. Frankel et al. (1994) reported that hydrophilic antioxidants such as Trolox (an α-tocopherol without its long chain hydrocarbon) are more effective in bulk oil systems whereas hydrophobic antioxidants, such as α-tocopherol, are more effective in oil-in-water emulsions. This phenomenon is due primarily to the differential affinities of the antioxidant compounds for oil-air and oil-water interfaces in bulk oil and oil-in-water emulsions, respectively. This property is greatly affected by the chemical nature of the antioxidative compounds involved (Frankel , 1996).

One of the important aspects of the extraction of antioxidative compounds from plant materials is the selection of appropriate extraction conditions. It is not advisable to apply the conditions used for one kind of plant material to another because the diverse nature of natural antioxidants makes the generalized extraction conditions inefficient. However, a set of optimum extraction conditions for a particular material can be obtained by employing response surface methodology (RSM), a tool used by many researchers to predict optimum experimental conditions to maximize various responses (Gao and Mazza, 1996; Wanasundara and Shahidi, 1996).

Based upon the literature evidences for antioxidant and radical intercepting properties of plant extracts, an hypothesis was made that borage and evening primrose extracts might also possess similar activities due to the presence of phenolic antioxidants. Also based upon literature evidences for the involvement of various phenolic compounds in antioxidant mechanisms, this hypothesis was further extended that borage and evening primrose phenolics might participate in similar mechanisms as other phenolics. Since antioxidant activities of phenolic compounds largely depend upon their affinities to different phases and interfaces of model systems being employed to evaluate them, it was thought that crude plant antioxidants might also act in a similar fashion. Therefore, in order to investigate these hypotheses, several objectives were considered. These objectives were : (1) to evaluate the antioxidant efficacies of borage and evening primrose meals in a meat model system, (2) to optimize extraction conditions for both borage and evening primrose to obtain extracts with high antioxidant activity, (3) to evaluate the

antioxidant efficacies of crude extracts as well as their fractions in different model systems, (4) to examine the metal chelating and reactive oxygen species (ROS) as well as organic free radical-scavenging activities of borage and evening primrose crude extracts and their fractions in order to understand the antioxidant mechanisms involved, (5) to elucidate the chemical structures of active compounds present in the extracts of borage and evening primrose meals and (6) to quantify the identified compounds in the extracts as well as in the source materials.

CHAPTER 2

LITERATURE REVIEW

Lipids are heterogeneous compounds which primarily serve as a source of fuel for both plants and animals. Apart from providing a condensed source of energy for living organisms (9 kCal/g), they play several important functions in foods. Lipids contribute to food quality by providing organoleptic character notes that make them appealing for consumption. These properties include flavour, colour, texture and mouthfeel. In human nutrition, food lipids not only provide the essential fatty acids such as linoleic and linolenic acids, but also serve as a source and a carrier for fat-soluble vitamins such as A, D, E and K (St. Angelo, 1996). Lipids, however, undergo oxidation reactions which shorten the shelf-life of lipid-containing foods. During production, distribution, and storage preceding actual consumption, food lipids undergo various deterioration processes that involve microbial, physical and most importantly, chemical modes. The latter is characterized by enzymatic and nonenzymatic oxidation of lipids which cause undesirable changes in flavour, colour, and nutritional value. Deoxygenation, airtight packaging and other techniques have solved some of these problems, but the role of antioxidants cannot be overlooked (Namiki, 1990). In this chapter, the mechanism of lipid oxidation, toxicity and diseases associated with the consumption of oxidized lipids, methods of assessing lipid oxidation and role of antioxidants in lipid-containing foods will be discussed.

2.1 Mechanism of lipid oxidation: initiation and propagation

When the oxidation of a lipid is monitored experimentally (by measuring the oxygen uptake or peroxide value) it is found that the course of the oxidation shows three distinct phases. During the first phase (induction period), the oxidation takes place slowly, but at a uniform rate. After oxidation reaches a certain point the reaction enters a second phase (propagation) with a sharp accelerating rate, and the eventual rate is many times greater than that observed in the initial phase. The third phase (termination) is characterized by a decrease in the rate of oxidation (Figure 2.1).

Figure 2.2(A) outlines the initiation and propagation steps of the classical lipid oxidation mechanism. In a peroxide-free system, lipid peroxidation is initiated when a hydrogen atom is abstracted from a methylene group (-CH_2 group) adjacent to a double bond of an unsaturated fatty acid (Halliwell and Gutteridge, 1985). Free radicals can be defined as species with an unpaired electron, and the reactivity of oxygen-derived free radicals varies from relatively low, as in the case of the oxygen molecule itself, to very high, as in the case of the short-lived and highly reactive hydroxyl radical ($^{\bullet}OH$) (Packer and Glazer, 1990; Packer, 1994). Hydrogen is abstracted from fatty acids by highly reactive oxygen species (ROS) such as $^{\bullet}OH$, hence any reaction or process which forms ROS would definitely stimulate lipid oxidation. Hydrogen abstraction is easier in unsaturated fatty acids than in their saturated counterparts, thus making them more susceptible to ROS attack.

Upon abstraction of a single hydrogen atom from a fatty acid, the fatty acid carbon

Figure 2.1 Graphical representation of the steps involved in lipid oxidation.

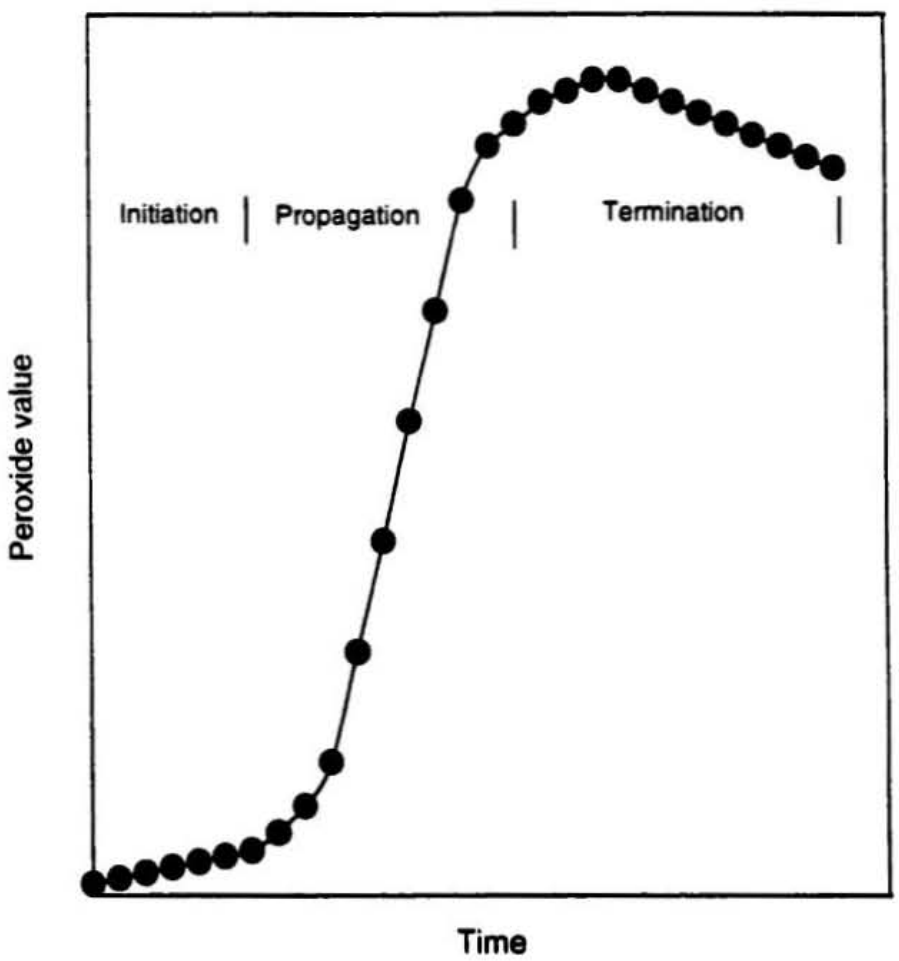
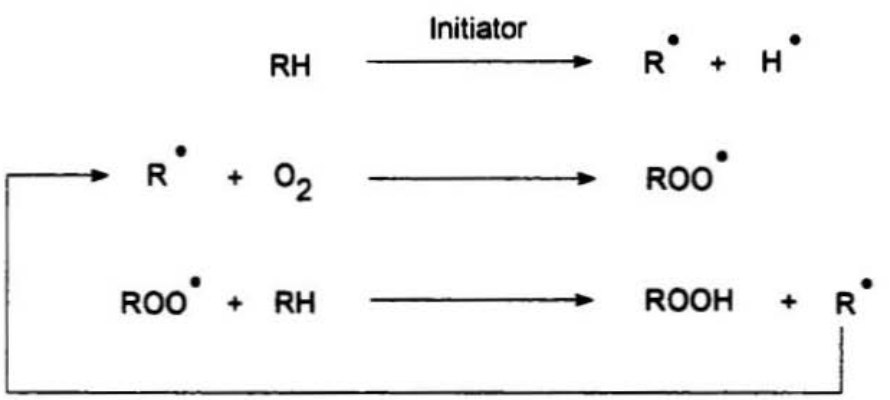
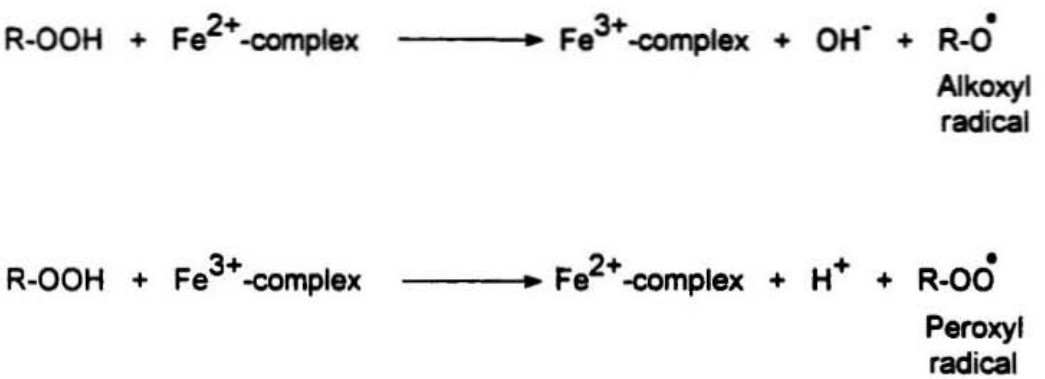

Initiation
Propagation
Termination
Peroxide value
Time

Figure 2.2 Initiation and propagation steps of the classical lipid autoxidation mechanism (A) and iron induced decomposition of hydroperoxides (B).

(A)


Initiator
RH
R• + H•
R• + O2
ROO•
ROO• + RH
ROOH + R•

(B)


R-OOH + Fe2+-complex
Fe3+-complex + OH- + R-O•
Alkoxyl radical
R-OOH + Fe3+-complex
Fe2+-complex + H+ + R-OO•
Peroxyl radical

is left with an unpaired electron, i.e. a fatty acid free radical is formed. This radical is stabilized by a molecular rearrangement into a conjugated diene (Esterbauer *et al.*, 1991) which can undergo reactions such as cross-linking with fatty acid molecules. Under aerobic conditions, however, the most likely reaction is with oxygen and the product is a peroxyl radical (Halliwell and Gutteridge, 1985). The peroxyl radicals in turn form cyclic peroxides and are sufficiently reactive to abstract a hydrogen atom from fatty acid chains of other lipid molecules, forming a lipid hydroperoxide and a new fatty acid free radical. Thus, the process of lipid oxidation is propagated by a free radical chain reaction, one initial hydrogen abstraction potentially leading to the formation of many lipid hydroperoxides and cyclic peroxides, collectively known as lipid peroxides. Pure lipid peroxides are reported to be stable at physiological temperatures, but rapidly decompose in the presence of transition metal complexes, especially iron salts (Packer and Glazer, 1990). Ferrous ions reduce lipid peroxides to alkoxyl radicals, while ferric ions can form both alkoxy and peroxy radicals with lipid peroxides [Figure 2.2.(B)]. Final decomposition products of the reaction between lipid peroxides and iron or copper complexes include hydrocarbon gases such as ethane, ethylene and pentane, carbonyl compounds such as aldehydes and ketones as well as alcohols (Esterbauer *et al.*, 1991; Wettasinghe and Shahidi, 1996). Mechanisms involved in the decomposition of hydroperoxides will be detailed in a later section.

2.1.1 Factors that affect lipid oxidation

The major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* have been identified as oxygen and oxygen species, transition metal ions and their complexes (haem proteins), electromagnetic radiation and enzymes. The contribution of these factors to lipid oxidation will be reviewed in the following sections.

2.1.1.1 Role of oxygen and oxygen species

The electronic structure of oxygen has two unpaired electrons at energy levels of p antibonding, in triplet state ($^3\Sigma_g$, Korycka-Dhal and Richardson, 1978). The reaction of oxygen with ground state molecules of singlet multiplicity (i.e., polyunsaturated fatty acids, PUFA) is spin forbidden. However, this barrier does not apply to reactions which involve single electrons, hydrogen atoms, and molecules containing unpaired electrons such as transition metal complexes and free radicals. Therefore, the triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$) (Kanner *et al.*, 1987). Triplet oxygen can also undergo energy transition to generate singlet oxygen ($^1\Sigma_g$ and $^1\Delta_g$) (Simic and Taylor, 1988). Photosensitizers such as natural pigments (chlorophylls, flavins and haem pigments) can generate singlet oxygen from triplet oxygen. Photosensitizers absorb visible or near-UV light to become electronically excited. They can transfer energy (photons) onto triplet state oxygen molecules upon their returning to ground state which generates singlet oxygen (Krinsky, 1977). Singlet oxygen in turn initiates lipid oxidation by an "ene"

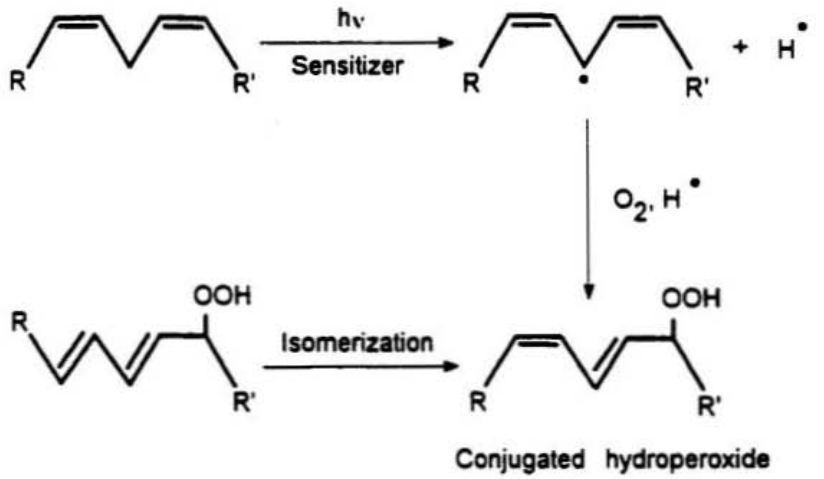
reaction (Figure 2.3) where olefenic groups (–C = C–) of fatty acids are converted to their corresponding allyl hydroperoxides (Adam, 1975).

The singlet oxygen is generated mainly by photoexcitation of triplet oxygen and its reaction with PUFA only forms peroxides which do not trigger a radical chain reaction. It has been reported that singlet oxygen-mediated lipid oxidation is more important in food than in biological systems (Wasserman, 1979). Singlet oxygen, while a reactive oxygen species, lacks an unpaired electron and is therefore not a free radical by definition. Free radical derivatives of oxygen include superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), hydroperoxy radical ($HO_2^{\bullet}$), and nitric oxide ($^{\bullet}NO$) (Halliwell and Gutteridge, 1985; Packer and Glazer, 1990; Borg, 1993; Beckman *et al.*, 1994).

Superoxide radical ($O_2^{\bullet-}$) is generated by four electron reduction of molecular oxygen into water [Figure 2.4(A)]. This radical is also formed in aerobic cells due to electron leakage from the electron transport chain. Superoxide radical ($O_2^{\bullet-}$) is also formed by activated phagocytes (monocytes, macrophages, eosinophils and neutrophils) and the production of $O_2^{\bullet-}$ is an important factor in the killing of bacteria by phagocytes (Halliwell and Gutteridge, 1985; Packer and Glazer, 1990). In living organisms, $O_2^{\bullet-}$ is removed by the enzymes called superoxide dismutases (SOD). The dismutation reaction of $O_2^{\bullet-}$ is shown in Figure 2.4(B).

The H_2O_2, formed due to 4 electron reduction of O_2 into H_2O and dismutation of $O_2^{\bullet-}$, is not a free radical, but an oxidizing agent. It, in the presence of $O_2^{\bullet-}$ and transition

Figure 2.3 Formation of hydroperoxides *via* ene reaction; involvement of triplet and singlet oxygen in this process.


hν
Sensitizer
R
R'
+ H•
O2, H•
OOH
Isomerization
Conjugated hydroperoxide

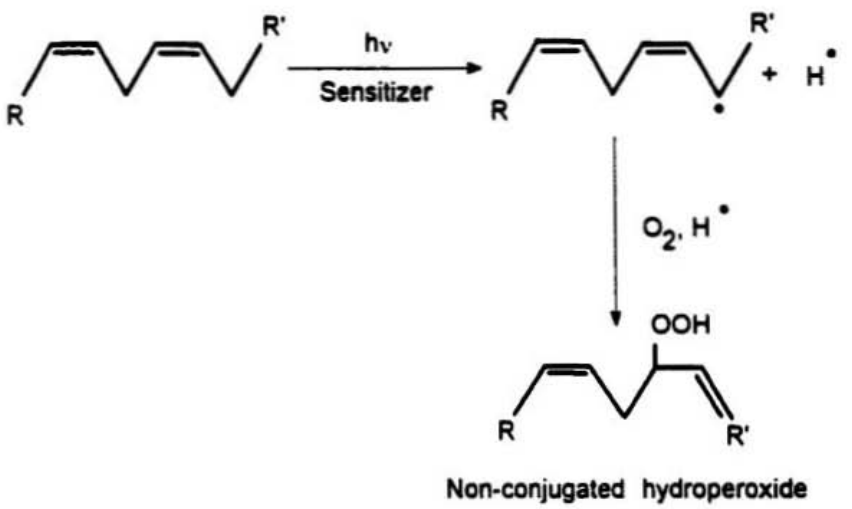
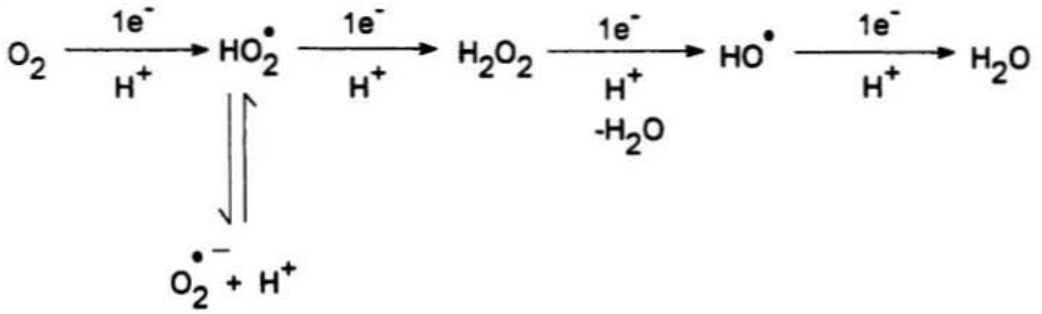
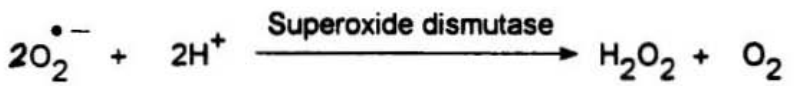

hν
Sensitizer
R
R'
+ H•
O2, H•
OOH
Non-conjugated hydroperoxide

Figure 2.4 Four electron reduction of oxygen into water (A), superoxide dismutation reaction (B) and superoxide driven Fenton reaction (C).

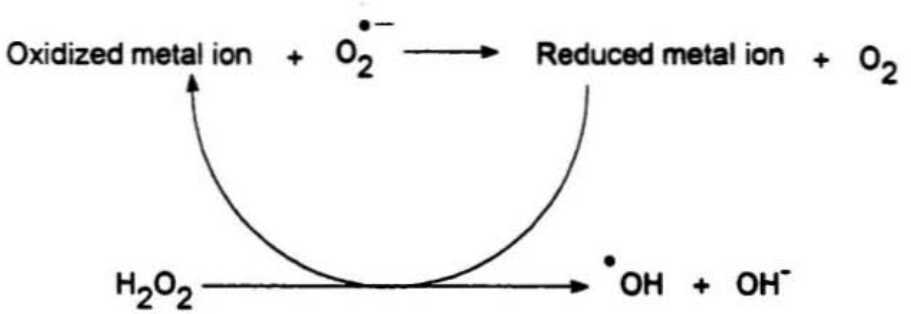
(A)



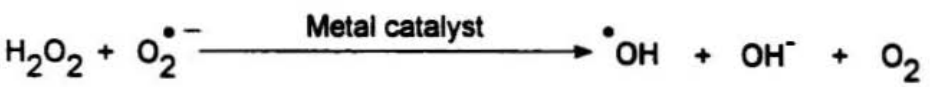
(B)



(C)



Overall reaction



metal ions, can generate $^{\bullet}OH$ via the superoxide-driven Fenton reaction (Halliwell and Gutteridge, 1985). This reaction can be summarized as shown in Figure 2.4(C). The $^{\bullet}OH$, formed by this reaction and 4 e^- reduction of O_2, is highly reactive and causes damage to deoxyribonucleic acid (DNA) and initiates lipid oxidation (Packer and Glazer, 1990).

The hydroperoxy radical ($HO_2^{\bullet}$) is formed by photooxidation of the $O_2^{\bullet-}$. Packer and Glazer (1990) reported that $HO_2^{\bullet}$ is more reactive than $O_2^{\bullet-}$ and readily difusable across biological membranes where it attacks unsaturated fatty acids. Nitric oxide ($^{\bullet}NO$), a vasodialating factor, is released by the endothelium in response to stimulation with a variety of substances. It is also produced by platelets in which it inhibits platelet aggregation. The biological activity of $NO^{\bullet}$ is limited by the concurrent presence of $O_2^{\bullet-}$, which reacts rapidly to form the peroxynitrite anion ($OONO^-$) (Iuliano *et al.*, 1997). However, protonated $OONO^-$ can decompose into $^{\bullet}OH$ which in turn initiates lipid oxidation (Beckman *et al.*, 1994).

The occurrence of ROS in foods is inevitable due to their biological nature. Kanner *et al.* (1986) reported that muscle lipid oxidation is initiated by ROS and haem proteins. ROS also initiate lipid peroxidation in vegetable and animal fats and oils (Bradley and Min, 1992; Rawls and van Santen, 1970). Free radical species of O_2 can directly abstract hydrogen atoms from methylene groups adjucent to olefenic groups of fatty acids resulting in the formation of fatty acid free radicals. In meats, H_2O_2, as described elsewhere, can generate $^{\bullet}OH$ in the presence of Fe^{2+} via Fenton reaction (Kanner

et al., 1987; Kanner and Doll, 1991; Kanner, 1994).

2.1.1.2 Role of transition metals

Transition metal ions such as those of iron, copper, magnesium, manganese and zinc are abundantly present in both living organisms and foods of both plant and animal origin (Schaich, 1980; Tichivangana Morrissey, 1985). They participate in direct and indirect initiation of lipid oxidation (Schaich, 1980). Higher valence state metals such as iron, copper, manganese, nickel and cobalt are known to participate in direct initiation of lipid oxidation via electron transfer and lipid alkyl radical formation.

Lower valence state metals can directly initiate lipid oxidation via formation of ROS which can abstract a hydrogen atom from methylene groups adjacent to double bonds of unsaturated fatty acids leading to a free radical chain reaction (Kanner *et al.*, 1987, 1986). Fe^{2+} can be oxidized to Fe^{3+} while reducing O_2 to $O_2^{\bullet-}$; $O_2^{\bullet-}$ in turn can generate $^{\bullet}OH$ via superoxide-driven Fenton reaction.

Indirect initiation of lipid oxidation by metal ions occurs when preformed hydroperoxides (LOOH) are oxidized or reduced to form radicals such as $LO^{\bullet}$ and $LOO^{\bullet}$ (Minotti and Aust, 1992). $LO^{\bullet}$ and $LOO^{\bullet}$ can increase the rate of initiation by abstracting hydrogen atoms from methylene groups adjacent to double bonds of unsaturated fatty acids. The redox potential of other metals such as manganese and cobalt are too low to cause hydroperoxide decomposition in aqueous systems, but they may catalyze hydroperoxide decomposition, especially in non-polar media, by formation of metal-

hydroperoxide complexes (Kanner *et al.*, 1986, 1987).

One of the major problems encountered by the meat industry is the rapid deterioration of cooked meat quality due to lipid oxidation (Decker and Xu, 1998; Kanner *et al.*, 1986). In muscle tissue, iron exists in a protein-bound form in myoglobin, heamoglobin, ferritin and transferrin. All of these haem proteins have been shown to catalyze lipid oxidation (Decker and Welch, 1990; Love and Pearson, 1971; Love, 1987, 1988; Wettasinghe and Shahidi, 1996). Several authors reported that the nonhaem iron, as opposed to haem iron, is the principle prooxidant in muscle (Kanner *et al.*, 1987), but this idea is not widely accepted. Several other authors have claimed that alkali and alkali-earth metals also contribute to muscle lipid peroxidation by displacing catalytic iron ions from haem compounds (Rhee *et al.*, 1983; Wettasinghe and Shahidi, 1996).

2.1.1.3 Role of ionizing radiation

The ionizing radiation of principle concern in chemical and biological systems are charged particles such as electrons (β-particles and δ-rays), protons and α-particles, and electromagnetic waves or photons such as X-rays and γ-rays (Bielski, 1976). These particles and electromagnetic waves can ionize atoms and molecules by ejecting electrons from them, thus forming a positively charged species in the parent material (Schaich, 1980). Electrons ejected in the ionization process may themselves be sufficiently energetic to produce further ionization and excitation. If their energy is less than 100 eV, their range is short, and resulting secondary ionizations will be close to the primary ionization

site, thus forming small clusters or spurs of excited and ionized species. More energetic electrons, ie., δ-rays, travel further from the initial site and make tracks of their own, similar to those of β-particles or other electrons with the same energy (Spinks and Woods, 1961; Bielski, 1976).

The major immediate consequence of the absorption of high-energy radiation is the production of free radicals. In biological systems, this may occur directly by deposition of energy within the molecule itself or indirectly through reactive species produced from the radiolysis of water. Since water constitutes 80 - 90% of most cells (or even a greater percentage of aqueous model systems), indirect effects are often considered to provide major contribution to molecular damage (Schaich, 1980). Reactive oxygen species (ROS), such as H_2O_2, $HOO^{\bullet}$ and $^{\bullet}OH$ radicals, formed during radiolysis of water, may initiate many toxic reactions in biological systems through hydrogen abstraction reactions (Simic and Hunter, 1984).

2.1.1.4 Role of ultraviolet (UV) radiation and visible light

The reactions which involve UV and visible light are referred to as photochemical reactions. In this type of reactions energy is selectively absorbed and electronic excitation is the major consequence. This process differs distinctly from ionizing radiations where molecules are non-selectively ionized or excited along particle tracks. Ionization of molecules in photochemical reactions is a rare event. The solvated electrons, produced during photolysis, are not sufficiently energetic to cause a harmful effect (Dainton, 1967).

UV radiation (λ = 180 to 400 nm) is more energetic than visible light (λ = 400 to 800 nm). Some frequencies in UV radiation oscillate in resonance with covalently bond electrons. In molecules with such electrons, photons are easily taken up, causing weakening of the molecular structure, disruption of bonds, creation of free radicals , and changes in chemical reactivity. Thus, UV radiation, rather than visible light, is responsible for most direct photochemical reactions (Decuyper *et al.*, 1984).

The excitation of molecules following UV radiation and visible light absorption involves transition of electrons from one orbital to another. Two basic types of transition occur depending upon the orbital origin of the excited electron. They are the bonding and antibonding electronic transitions (Neckers, 1969). When an electron is promoted from a bonding p orbital of a carbon covalent bond to an excited antibonding π^* state, the transition is called (π - π^*). This type of transitions are important in olefins (eg. fatty acids). π - π^* transitions occur at shorter wavelengths (<270 nm) and the resulting antibonding π^* state may be considered as a biradical (e.g. $^{\bullet}CH_2$–$^{\bullet}CH_2$), but excess spin density is distributed throughout the π electron system of the molecule, thus diminishing any free radical character (Schaich, 1980).

In molecules containing heteroatoms, such as oxygen, nitrogen and sulphur, transitions arise from the excitation of an electron in the *2p* nonbonding orbital of the heteroatom to the π^* antibonding orbital (n - π^*). Carbonyl groups (–C=O) are the most typical of the compounds with these characteristics. The n–π^* transitions require a small amount of energy and therefore occur at longer wavelengths (λ > 300 nm). The

localization of the excited electrons on the heteroatom results in stretching of the π bond, with the following consequences: (1) charge densities on each of the bonded atoms are diminished, thus decreasing net molecular polarity (Schaich, 1980), and (2) the $n-\pi^*$ state configuration and subsequent behaviour are analogous to that of an electron-deficient π system (Schaich, 1980). Therefore, $n-\pi^*$ states are highly reactive in hydrogen abstraction with macromolecules such as lipids, proteins and nucleic acids.

2.1.1.5 Role of enzymes

The enzyme lipoxygenase is widely distributed throughout the plant and animal kingdoms (German and Kinsella, 1985). This enzyme is highly substrate-specific that it oxygenates the polyenoic fatty acids containing a doubly allylic methylene to form conjugated dienoic hydroperoxides. For example, Roozen *et al.* (1994) have reported that soya bean lipoxygenase-1, incorporated oxygen into linoleic acid resulting in 13-hydroperoxide.

Lipoxygenase prefers free fatty acids as substrates, though there are some lipoxygenase isozymes which can react with triacylglycerols. The regiospecificity of the reaction, i.e. the preference for certain positions on the carbon chain, and the stereospecificity, i.e. the preference for one of the possible two methylenic hydrogens at specific carbon atom [Figure 2.5(A)].

While several enzymatic (e.g. xanthine oxidase and peroxidase) and non-enzymatic mechanisms (e.g. activated haem proteins, catalytic non-haem iron) exist in biological

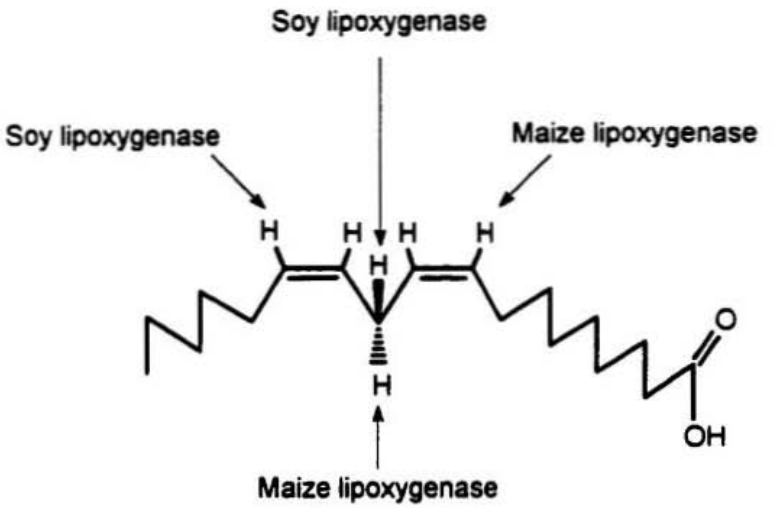
tissues capable of generating reactive oxygen species, the postmortem release of lipoxygenase from certain tissues, i.e. gills in fish (German and Kinsella, 1985), can generate significant quantities of reactive lipid hydroperoxides. These, in conjunction with metal catalysts, serve as potent agents of generating free radicals which can eventually initiate membrane lipid oxidation [Figure 2.5(B)].

2.2 Mechanism of lipid oxidation: hydroperoxide decomposition and termination of chain reactions

Lipid hydroperoxides are highly labile and undergo decomposition, mainly by cleavage on either side of the carbon atom bearing the oxygen atom. As shown in Figure 2.6, the first step involves a homolytic cleavage of the oxygen-oxygen bond to yield alkoxy and hydroxy free radicals. The alkyl radical can then react to form an aldehyde and a new free radical. This reaction involves C–C bond scission which can occur on either side of the radical. The aldehyde which is formed due to scission, can either a short chain volatile compound or it can remain attached to the acylglycerol part of the molecule as a non-volatile product (Grosch, 1987). The volatile aldehydes are known to play an important role in the oxidized flavour of fats (Ho *et al.*, 1987). Abstraction of a hydrogen atom from another molecule can yield an alcohol and a new free radical. The free radicals,

Figure 2.5 Regiospecificity of lipoxygenase (A) and involvement of lipoxygenase in the generation of free radicals (B).

(A)


Soy lipoxygenase
Soy lipoxygenase
Maize lipoxygenase
H
H
H
H
H
H
O
OH
Maize lipoxygenase

(B)

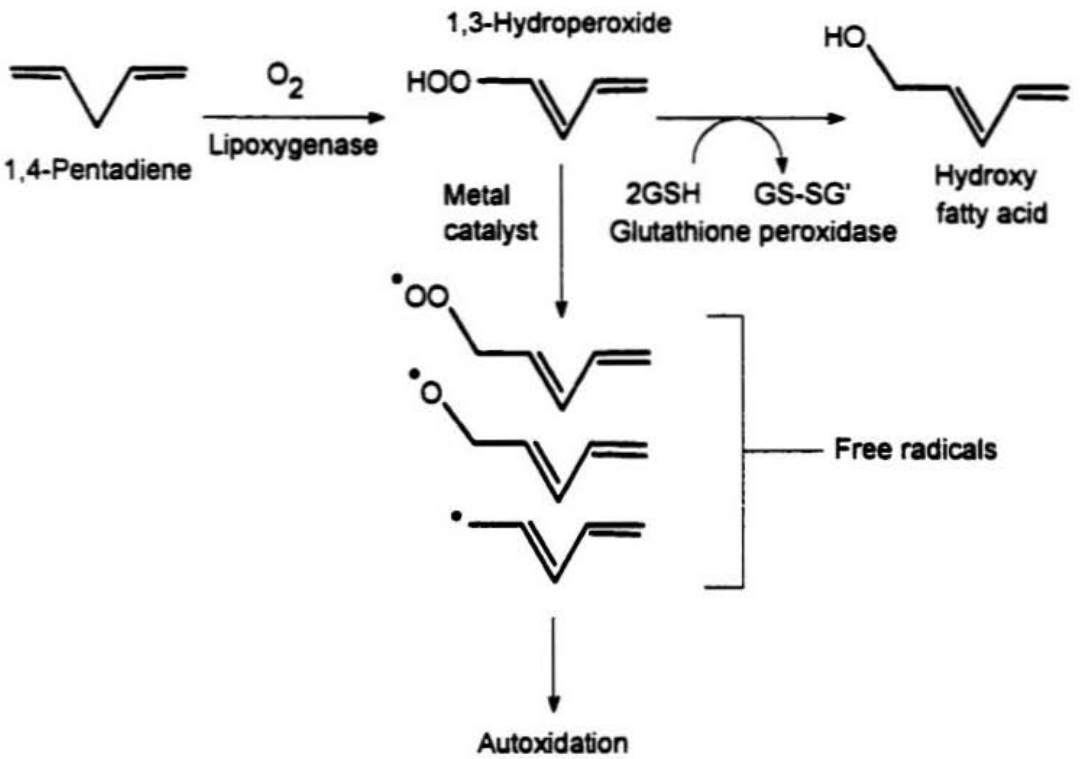
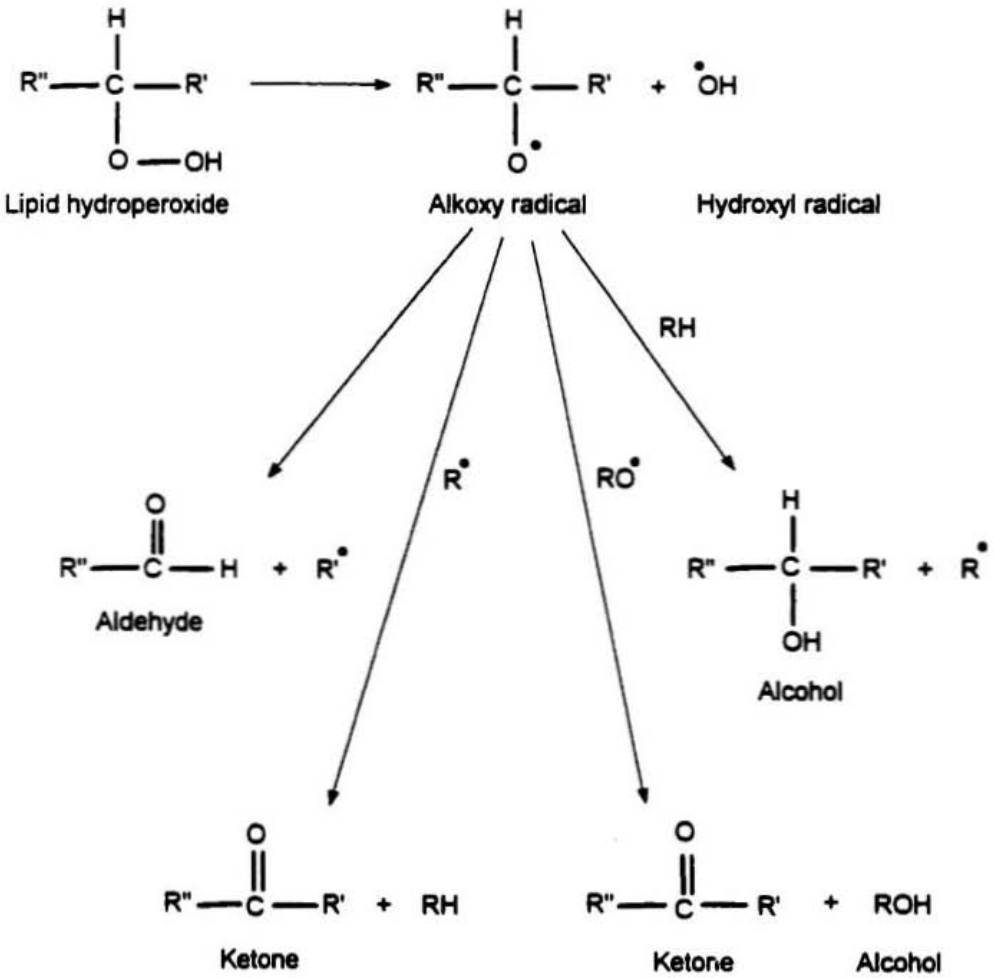

1,3-Hydroperoxide
HO
O_2
HOO
1,4-Pentadiene
Lipoxygenase
Metal
catalyst
2GSH
GS-SG'
Glutathione peroxidase
Hydroxy
fatty acid
•OO
•O
•
Free radicals
Autoxidation

Figure 2.6 Decomposition of hydroperoxides and subsequent termination of chain reactions.

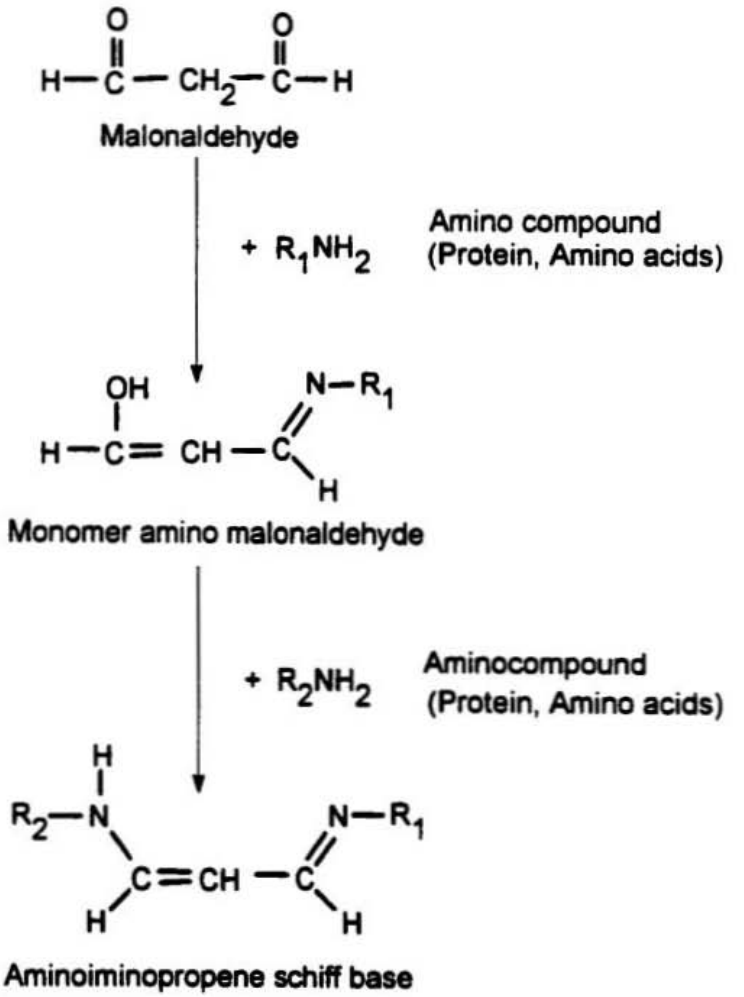

Lipid hydroperoxide
Alkoxy radical
Hydroxyl radical
RH
R•
RO•
Aldehyde
Alcohol
Ketone
Ketone
Alcohol

formed during these reactions, may participate in propagation of the chain reactions, while interaction of two free radicals can yield non-radical products and thus terminate the chain, leading to the formation of ketones (Paquette *et al.*, 1985). These secondary oxidation products not only impart off flavours to food, but also could react with cellular macromolecules (through Schiff base formation) such as proteins, DNA and RNA (Figure 2.7; Esterbauer *et al.*, 1991).

2.3 Effects of lipid oxidation on food quality

Carbonyl compounds, particularly volatile aldehydes formed during oxidation of lipids, play both positive and negative roles in quality of lipid-containing foods (Yamamoto *et al.*, 1970). Several authors have reported the presence of various aldehydes, ketones, alcohols and hydrocarbons in oxidized meat (Buttery *et al.*, 1977; Hayashi *et al.*, 1986; Shahidi, 1989; Tang *et al.*, 1983), milk (Chang *et al.*, 1966; Langler and Day, 1964; Shibamoto *et al.*, 1980), bulk oil (Krishnamurthy and Chang, 1967; Chang *et al.*, 1978; Snyder *et al.*, 1985) and emulsions (Mick and Schreier, 1984). Ramarathnam *et al.* (1991a,b) have reported and identified the volatile constituents of uncured and cured beef, chicken and pork using a GC-MS technique. They have identified four common volatiles, namely 4-methyl-2-pentanone, 2,2,4-trimethylhexane, 1,2,4-trimethylcyclohexane, and 1,3-dimethylbenzene, in the three meat species as key flavour-active compounds in cured-meat aroma. They reported the presence of above compounds in low concentrations in cured meat while cooked uncured meats were devoide of those

Figure 2.7 Reaction of malonaldehyde, a secondary oxidation product, with amino compounds to produce an aminoiminopropene schiff base.


Malonaldehyde
+ R_1NH_2
Amino compound
(Protein, Amino acids)
Monomer amino malonaldehyde
+ R_2NH_2
Aminocompound
(Protein, Amino acids)
Aminoiminopropene schiff base

four constituents. They also reported that the characteristic pork flavour is attributable to 16-octadecenal, benzaldehyde, 2,3-octanedione and 2,4-decadienal, while 2-hexanone and 3,3-dimethylhexanal have been identified in beef. The characteristic chicken-like flavour includes a mixture of 3-hexanone, 2-hexanal, 3-methyl-4-heptanone, 3-methylhexanal, 2-heptenal, octanal, 2-octenal, nonanal, 16-octadecenal, 4-ethylbenzaldehyde, and decanal.

Hydrocarbons are also derived from thermal oxidative decomposition of lipids, a reaction catalyzed by haem compounds such as haemoglobin and myoglobin (Ben-Aziz *et al.*, 1970). It is believed that they do not contribute significantly to the flavour of meat. Min *et al.* (1979) have reported that saturated and unsaturated hydrocarbons do not play a role in roast beef flavour since they possess weak "non-beeflike" odours. A large number of alkanes and alkenes have been identified in beef, pork, chicken and mutton (Shahidi *et al.*, 1986).

Lipid oxidation products, when present at above their threshold values, can adversely affect the flavour of meat (Drumm and Spanier, 1991). Off-flavour development due to lipid oxidation can take place in all types of raw, cooked, cured, uncured, canned and frozen meats and meat products (Ang and Lyon, 1990; Dupuy *et al.*, 1987; Gray and Monahan, 1992; Pearson *et al.*, 1977; Ramarathnam *et al.*, 1991a, b; Ramarathnam and Rubbin, 1994; Shahidi, 1992a,b; St. Angelo *et al.*, 1987, 1988). For example, Bailey *et al.* (1980) have reported volatile compounds of roasted beef, that increased most rapidly during storage at 4°C, include hexanal and 2-pentylfuran as well as pentanal. The content of carbonyl compounds increased as off-flavour developed in

precooked-refrigerated turkey and these were detected within hours after cooking (Wu and Sheldon, 1988). The total volatiles of ground roasted beef increased appreciably within hours after storage at 4°C; hexanal increased most rapidly. Increases were also observed in other compounds, e.g. propanal, pentanal, 2,3-octanedione, nonanal and 2-pentylfuran (Dupuy *et al.*, 1987; St. Angelo *et al.*, 1987).

Shibamoto and Macku (1991) reported the presence of 18 aldehydes, 13 hydrocarbons, 11 ketones, 4 alcohols, 3 esters, 3 furans, 2 aromatic compounds and 1 lactone in a headspace sample of an oxidized corn oil sample. Among these 55 compounds, aldehydes were the major components present. Similar oxidation products have been reported in other vegetable oils such as soybean and peanuts. Lipoxygenase-mediated lipid oxidation in soybean oil leads to the development of a beany flavour which is known as flavour reversion (Chang *et al.*, 1966). These authors have reported 2-pentylfuran as being one of the contributing factors responsible for flavour reversion of soybean oil. Vegetable oils such as corn oil, soybean oil and peanut oil contain a substantial amount of ω-6 fatty acids; mainly linoleic acid. Oxidation of ω-6 fatty acids can produce hexanal which is the predominant aldehyde formed during the oxidation of terrestrial animal and plant lipids (Frankel, 1993a,b; Frankel *et al.*, 1989). Figure 2.8 depicts the mechanism of hexanal formation from linoleic acid. The characteristic flavour note of this aldehyde has been explained as an intense grassy flavour (Shahidi and Pegg, 1992). In marine lipids, however, the predominant PUFA are of ω-3 type, thus the major aldehyde formed during oxidation is propanal (Figure 2.9, Frankel *et al.*, 1993a,b). The

Figure 2.8 Mechanism of hexanal formation.

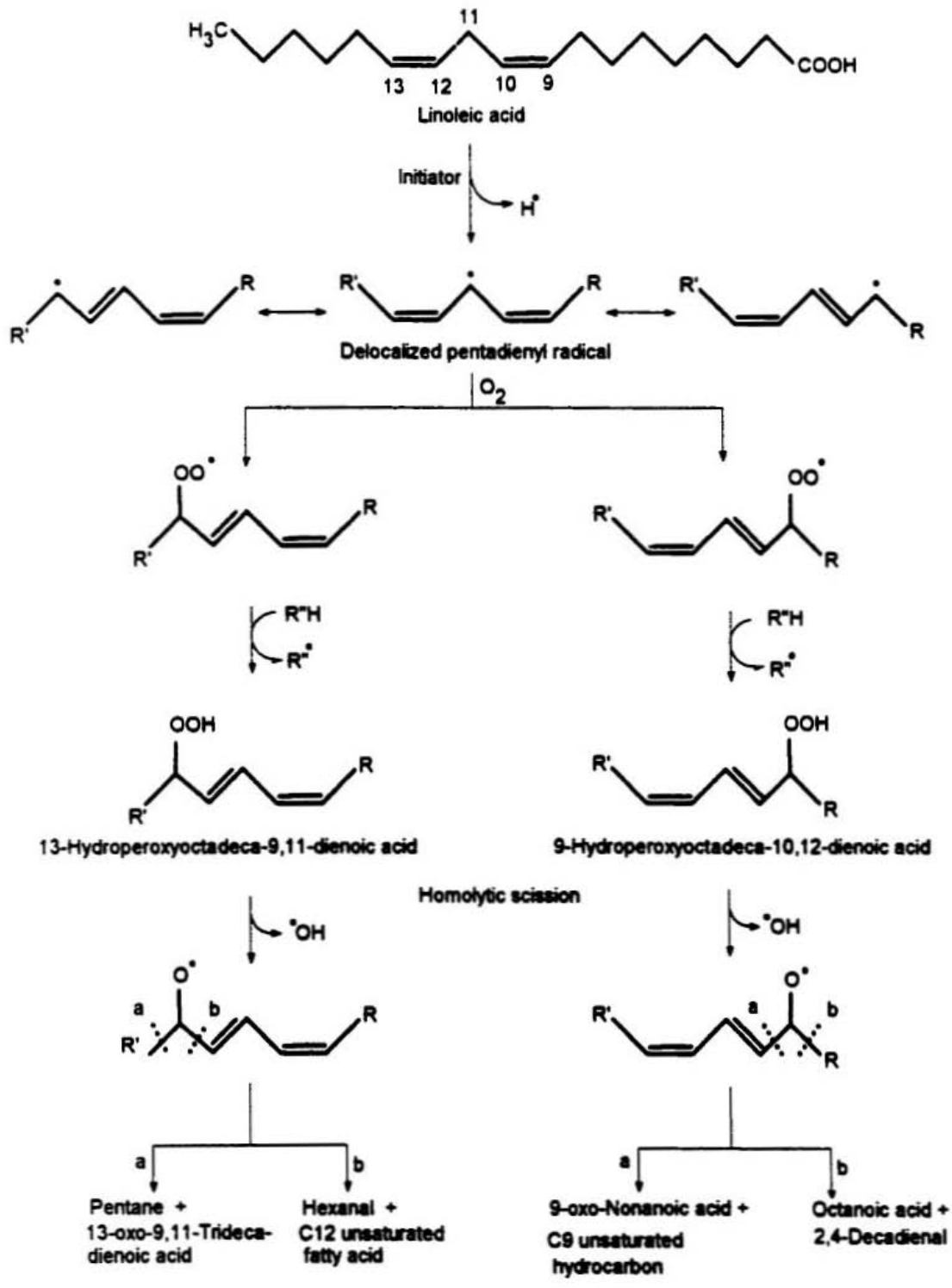
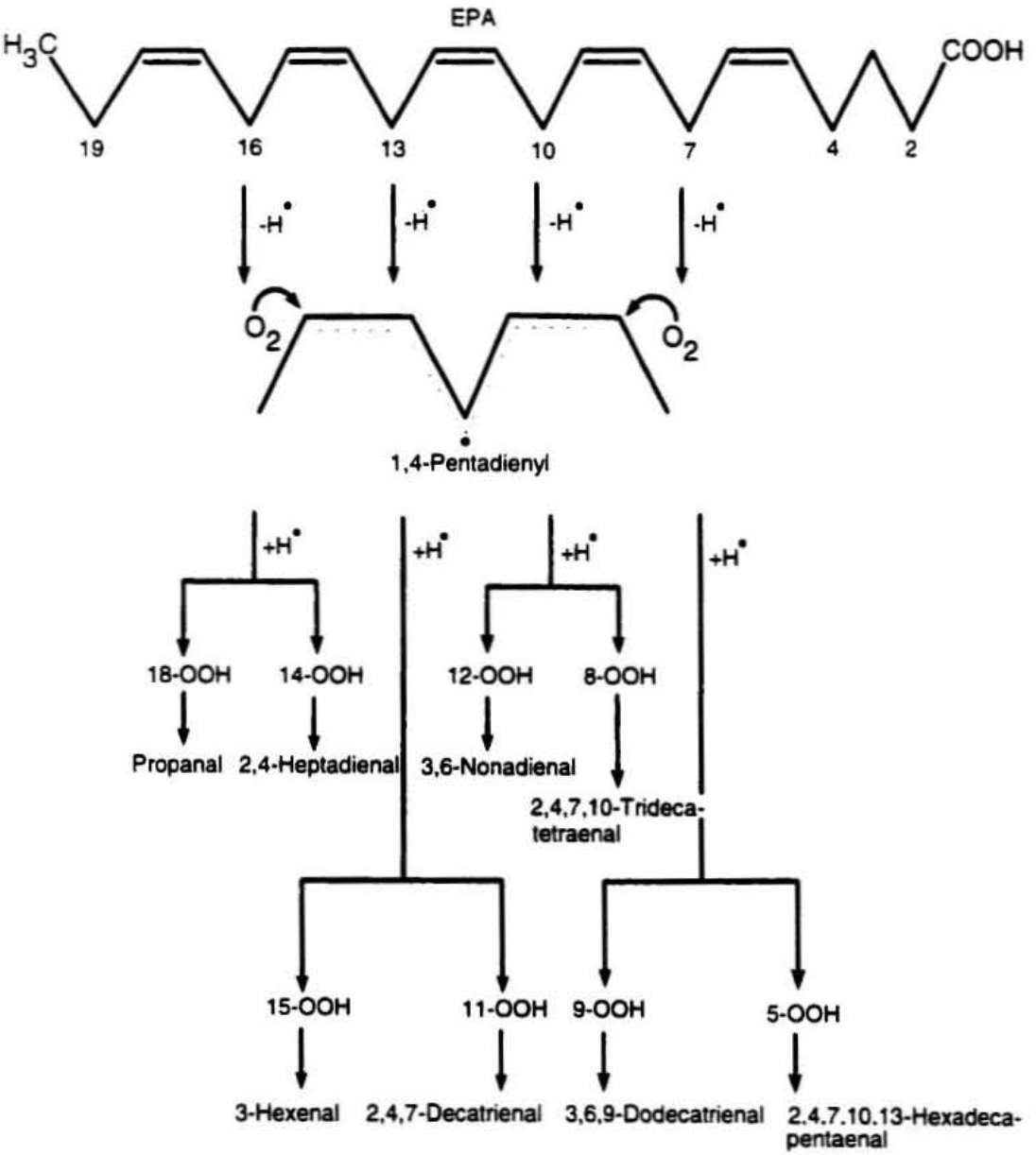

H3C
11
13
12
10
9
COOH
Linoleic acid
Initiator
H•
R
R'
Delocalized pentadienyl radical
O2
OO•
R"H
R"•
OOH
13-Hydroperoxyoctadeca-9,11-dienoic acid
9-Hydroperoxyoctadeca-10,12-dienoic acid
Homolytic scission
•OH
O•
a
b
Pentane +
13-oxo-9,11-Trideca-
dienoic acid
Hexanal +
C12 unsaturated
fatty acid
9-oxo-Nonanoic acid +
C9 unsaturated
hydrocarbon
Octanoic acid +
2,4-Decadienal

Figure 2.9 Mechanism of propanal formation.


EPA
H3C
COOH
19
16
13
10
7
4
2
-H•
O2
1,4-Pentadienyl
+H•
18-OOH
14-OOH
12-OOH
8-OOH
Propanal
2,4-Heptadienal
3,6-Nonadienal
2,4,7,10-Trideca-tetraenal
15-OOH
11-OOH
9-OOH
5-OOH
3-Hexenal
2,4,7-Decatrienal
3,6,9-Dodecatrienal
2,4,7,10,13-Hexadeca-pentaenal

characteristic off-flavour of propanal has been reported as nut-like.

In raw milk, lypolysis is the major cause of rancid flavour development. The hydrolytic action of lipase on milkfat causes the liberation of short chain fatty acids such as butyric acid. The liberation of butyric acid causes the development of characteristic rancid odour in milk and milk products rendering them unappealing for consumption (Nawar, 1985).

Lipid oxidation in muscle foods has been studied extensively because of the rapid deterioration of sensory quality of refrigerated cooked meat (Bailey, 1988; Ladikos and Langovois, 1990). The immediate consequence of lipid oxidation in cooked meat is the development of a characteristic off-flavour known as warmed-over flavour (WOF, Igene and Pearson, 1979; Love, 1971, 1987, 1988).

Apart from the development of off-flavours in foods, upon consumption and absorption, carbonyl compounds formed during lipid oxidation, may react with cellular macromolecules such as proteins (Esterbauer *et al.*, 1991; Nair *et al.*, 1986), DNA and RNA (Pearson and Gray, 1983). Malonaldehyde, a dialdehyde formed during the oxidation of PUFA reacts with these macromolecules. 4-Hydroxynonenal has also been reported to react with cellular macromolecules (Esterbauer *et al.*, 1991). However, the major source of these potentially toxic compounds is not dietary, but formed *in vivo* during cellular lipid peroxidation (Benzie, 1996). The following section will focus on the interaction of lipid oxidation products with cellular components in living organisms and their contribution to the development of various diseases.

2.4. Link between lipid oxidation products and human disease conditions

The lipid oxidation products may be classified broadly as primary and secondary (Wong, 1989). Primary products are lipid hydroperoxides while the secondary products are aldehydes, ketones, alcohols, hydrocarbons and acids, among others (Wong, 1989). There are no direct evidence that lipid hydroperoxides are involved in human disease, but animal studies involving lipid hydroperoxides or oxidized fats have shown the potential health risk of these compounds (Cortesi and Privett, 1972). It is unlikely, however, that the dietary hydroperoxides, as such, pose a health problem because they readily decompose in the presence of heat. The major potentially toxic compounds such as malonaldehyde and 4-hydroxynonenal are formed due to decomposition of lipid hydroperoxides (Dahle *et al.*, 1962; Pryor *et al.*, 1976a,b).

The presence of malonaldehyde in foods and other biological systems has prompted investigation into its possible harmful effects in animals. Crawford *et al.* (1965) showed that malonaldehyde, as its enolic sodium salt (sodium β-oxyacrolein) and as its acetal (tetraethoxypropane), was toxic in rats with LD_{50} levels of 632 and 527 mg/kg, respectively. They have reported that malonaldehyde reacts with DNA through cross linking with the amino groups of guanine and cytosine bases of DNA molecules. Reiss *et al.* (1972) have reported that structural changes in DNA, caused by malonaldehyde, eventually leads to the loss of template activity of DNA. Malonaldehyde has been shown to cause mutagenesis in some strains of *Salmonella*. However, according to Marnett and Tuttle (1980), the mutagenic activity of earlier malonaldehyde preparations was generally

due to contaminants formed during the generation of malonaldehyde from tetraethoxypropane. These authors have isolated two intermediates, namely β-alkoxyacrolein and 3,3-dialkoxy propionaldehyde, formed during the hydrolysis of tetraethoxypropane, and shown that they were more mutagenic than malonaldehyde itself.

Lipid oxidation products can react with proteins and amino acids to cause damage in foods and living tissues (Gardner, 1979). Andrews *et al.* (1965) showed that the reaction between autoxidizing linoleate and two different proteins, namely insulin and gelatin, in model systems resulted in modification of the proteins. Chio and Tappel (1969) characterized compounds formed by the reaction of leucine, valine and glycine with malonaldehyde. They reported that two moles of amino acids react with one mole of malonaldehyde to form one mole of conjugated Schiff base.

Malonaldehyde-amine reaction products have been shown to occur in certain biological processes. It is thought that pigmentation of brain, heart and testes with age in animals is due to the formation of conjugated Schiff bases such as the 1-amino-3-iminopropane (Tapel, 1973).

Bosch-Morell *et al.* (1996) reported that human retina is susceptible to oxidative damage. They proposed that lipid oxidation products found in subretinal fluid originate from peroxidation of membrane structural lipids of rod cells. Lipid-derived intermediates, such as malonaldehyde and 4-hydroxy-2,3-trans-alkadienals, are of prime importance because the former is directly related to the extent of damage, and the latter shows toxic effect (Esterbauer, 1991). Puertas *et al.* (1994) demonstrated the inflammatory effect of

4-hydroxynonenal on eyes of rabbits. They also observed the impairment of the blood-retina in rabbit eye.

4-Hydroxynonenal, formed during the oxidation of PUFA, is strongly electrophilic and reacts with nucleophiles in tissues, preferentially with sulphydryl (SH)-containing molecules such as cysteine, glutathione, and proteins (Siems *et al.*, 1996). It has been proposed that most biological effects of 4-hydroxyalkenal is based on its reaction with suphydryl and amine groups of amino acids (Winter *et al.*, 1986). 4-Hydroxyalkenals inhibit the biosynthesis of proteins, DNA, RNA, glucose (glycolysis) and triacylglycerols due to inhibition of various enzymes involved in the biosynthetic pathways (Winter *et al.*, 1986). As reported by Siems *et al.* (1996), 4-hydroxynonenal inhibits Na^+-K^+-ATPase due to its binding to cysteine and lysine residues. The inactivation of Na^+-K^+-ATPase can seriously affect sodium and potassium transport through the plasma membrane causing electrolyte imbalance in cells (Skou and Hilberg, 1965; Pedemonte and Kaplan, 1990).

The diabetes in humans and animals is closely associated with lipid peroxidation (Nickander *et al.*, 1996). Membrane lipid peroxidation in nerve tissues has been suggested as one of the causes of diabetic neuronal damage (Low and Nickander, 1991; Nickander *et al.*, 1994).

Artherosclerotic vascular disease is the leading cause of death among persons with a Western life style. Although an elevated plasma level of low-density lipoprotein (LDL) is clearly a major risk factor for cardiovascular disease, the mechanisms involved are unclear (Esterbaur, 1992). Several lines of evidence implicate oxidatively damaged LDL

as an artherogenic agent. One such important evidences is that the immunohistochemical detection of malonaldehyde in human and animal artherosclerotic lesions (Esterbauer, 1992). Lipoprotein oxidation is unlikely to occur in plasma because of the presence of high concentrations of antioxidants and proteins that chelate metal ions (Henning and Chow, 1988), but may occur in an environment where antioxidants can become depleted and lipoproteins are exposed to oxidative stress. The artery wall might represent one such environment, and *in vitro* studies have clearly demonstrated that vascular cells accelerate LDL oxidation. Several pathways involved in LDL oxidation have been identified in *in vitro* experiments. Metal-catalyzed oxidation of LDL is known to take place *in vitro* in cultured smooth muscle cells, but the role of extracellular free metal ions in LDL oxidation *in vivo* is not clear (Heinecke, 1987). Protein-bound metal ions also play a major role in LDL oxidation. Ceruloplasmin, the major carrier of plasma copper, oxidizes LDL *in vitro* (Henning and Chow, 1988) and has been isolated from artherosclerotic lesions. Hemin, a low molecular weight chelate of iron, formed during heam protein metabolism, is also a potent catalyst for LDL oxidation. Factors such as thiol (Tien *et al.*, 1982), superoxide (Cathcart *et al.*, 1989), nitric oxide (Radi *et al.*, 1991) and lipoxygenase (Kuhn *et al.*, 1994) have been reported to promote the oxidation of LDL. Sparrow and Olszewski (1993) have reported that cultured macrophages and endothelial cells use an L-cystine-dependent pathway to generate extracellular thiol, which causes LDL oxidation in media containing metal ions. Thiols (L-cysteine) autoxidize with the concomitant production of reduced metal ions, reactive-oxygen species (ROS) and sulphur-centred radicals ($RS^{\bullet}$,

$RSSR^{\bullet-}$), all of which oxidize LDL. Lipoxygenase oxidizes fatty acids bound to phospholipids (Yamamoto, 1992; Belkner *et al.*, 1993) and LDL (Kuhn *et al.*, 1994). Free radicals such as $O_2^{\bullet-}$, $^{\bullet}OH$ and $NO^{\bullet}$ initiate LDL oxidation by abstracting hydrogen atoms from PUFA (Jessup *et al.*, 1993). Although LDL oxidation is not the only factor which triggers the initiation of artherosclerosis, evidence gathered from both *in vitro* and *in vivo* studies suggests that oxidized LDL is likely to play an important role in the development of artherosclerosis.

2.5. Role of reactive-oxygen species (ROS) in development of disease conditions

The intracellular generation of reactive-oxygen species (ROS) can damage the cellular components such as enzymes, nucleic acids, membrane lipids and proteins (Shen *et al.*, 1996). ROS, such as H_2O_2, $O_2^{\bullet-}$ and $^{\bullet}OH$, are generated by several pathways involving the aerobic cellular metabolism (Ames, 1988; Frankel and Chrzan, 1987; Babior, 1993).

ROS have been implicated in the development and progression of cancer (Slaga *et al.*, 1978), inflammation and aging (Rohrdanz and Kahl, 1998). Frankel and Chrzan (1987) have reported the DNA base modification by hydrogen peroxide in polymorphonuclear leukocytes. Both DNA damaging agents (initiating mutagens) and promoters play important roles in carcinogenesis. The toxicity of $O_2^{\bullet-}$ and H_2O_2 in living organisms, however, is due to their conversion into $^{\bullet}OH$ and reactive-radical metal complexes. These processes are often referred to as iron-catalyzed Haber-Weiss reaction.

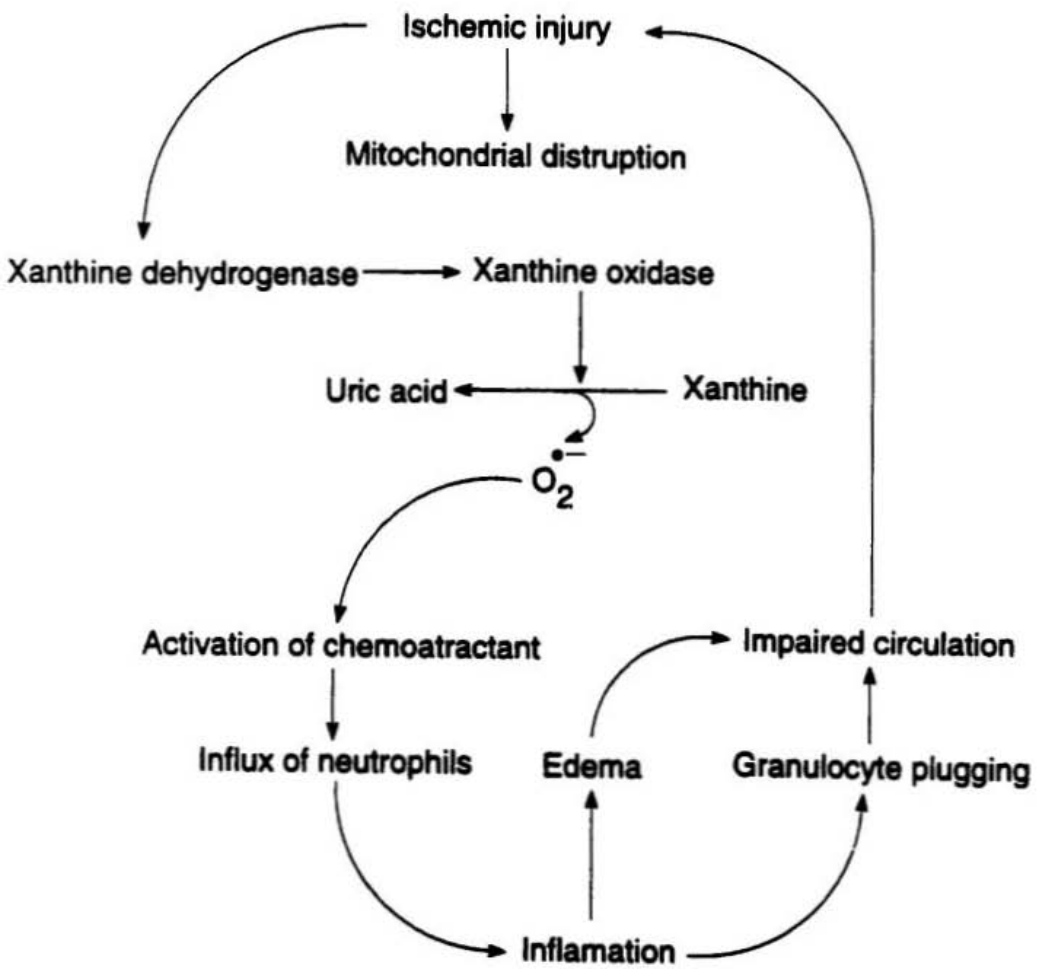
On production *in vivo*, $^{\bullet}OH$ reacts at its site of formation, thus it has an estimated half-life in cells of only 10^{-9} s (Halliwell and Gutteridge, 1985). Reaction of $^{\bullet}OH$ can be classified into three main types: (a) hydrogen abstraction; (b) addition; and (c) electron transfer. *In vivo* $^{\bullet}OH$ generation involves radiolysis of water, photolysis of H_2O_2, Fenton reaction and electron transfer mechanisms (Korycka-Dahl and Richardson, 1978). Many biological effects may result from the reaction of $^{\bullet}OH$ with membranes, enzymes, nucleic acids and polysaccharides (Willson, 1979). Damage may result from the direct action of the radicals themselves or from the action of toxic products. If damage is extensive, the cell may die. If damage is slight, the cell may survive, but may undergo some inheritable changes which lead to uncontrolled proliferation, hence cancer. Damage to a nucleic acid base may be direct or indirect or through the covalent grafting of a foreign chemical which has also undergone free radical attack (Fong *et al.*, 1973).

ROS produced by macrophages, monocytes and neutrophils are important in mediating inflammation. As an important part of their antibacterial defense, neutrophils possess a membrane-bound NADPH oxidase (Fantone and Ward, 1982). This enzyme produces $O_2^{\bullet-}$ as a kind of broad spectrum antibiotic, which aids in the killing of any microorganism engulfed by these cells. Furthermore, $O_2^{\bullet-}$ released by metabolically-activated neutrophils (even in the absence of infection) serves to amplify the inflammatory response by activation of a latent chemoattractant present in extracellular fluid (Heinecke, 1987). *In vivo*, oxidative tissue damage (ischemic injury) and inflammation may be synergistic (Ferrari *et al.*, 1991; Weiss, 1986) and Figure 2.10 depicts the sequence of

events whereby oxidative injury may lead to inflammation.

Vascular endothelial cells form a border separating deeper layers of the blood vessel wall and cellular interstitial space from the blood and circulating cells (Hammerson and Hammerson, 1985). Cell injury, or any event which may disrupt endothelial integrity and permeability, may be involved in the early events leading to the formation of artherosclerotic lesion by allowing increased uptake of cholesterol-rich lipoproteins into the arterial wall (Ross and Hakker, 1976; Ross, 1986). Among factors which may contribute to endothelial cell injury or dysfunction are free radical-mediated reactions, including lipid oxidation (Trillo and Prichard, 1979). Endothelial cells of blood vessels possess both xanthine oxidase activity and purine nucleotide phosphorylase activity. The latter enzyme catalyses the formation of the major xanthine oxidase substrate, hypoxanthine (Henning and Chow, 1988). Therefore, oxidation of hypoxanthine by xanthine oxidase, with concomitant formation of the superoxide radical, is an important mechanism of vascular endothelial cell injury (Rubanyi, 1988). The dismutation of superoxide radicals can lead to the production of hydrogen peroxide (Bielski and Allen, 1977). The later can be converted to hydroxyl radicals *via* Fenton reaction (Fridovich, 1977). All these ROS, in turn, can initiate lipid oxidation in endothelial cell membranes, leading to cell injury (McCord, 1988).

Figure 2.10 Cycle of events associated with free radical-mediated inflammation and subsequent schemic injury.


Ischemic injury
Mitochondrial distruption
Xanthine dehydrogenase
Xanthine oxidase
Uric acid
Xanthine
$O_2^{\bullet-}$
Activation of chemoatractant
Impaired circulation
Influx of neutrophils
Edema
Granulocyte plugging
Inflamation

2.5.1 Role of antioxidant enzymes in prevention of ROS injury

Various enzyme systems have been evolved in biological systems to deal with the ROS formed due to various biochemical and physical mechanisms. Superoxide dismutase (SOD), glutathione peroxidase and catalase are free radical-scavenging enzymes that defend cells from ROS stress by distorting $O_2^{\bullet-}$ and H_2O_2. Glutathione peroxidase and catalase are the major antioxidant enzymes responsible for H_2O_2 detoxification in cytosol (Ewing and Jones, 1987). Manganese-superoxide dismutase (MnSOD) exists predominantly in the mitochondria, which is the dominant site for ROS generation, and copper-superoxide dismutase (CuSOD) is found primarily in the cytoplasm (Sun *et al.*, 1998).

2.5.2 Role of dietary antioxidants in prevention of ROS injury

Many studies have been carried out on biological systems to investigate the preventive action of vitamins E (α-tocopherol), C (L-ascorbic acid) and A on ROS injury. In this respect, vitamin E is most important since it is oxidized by free radicals and regenerated by vitamin C and glutathione (Namiki, 1990). Vitamin E reacts with the peroxy radicals and also with singlet oxygen, although reaction of β-carotene with singlet oxygen is more rapid and efficient (Anderson and Krinsky, 1973). Both vitamins E and C have been reported to deactivate singlet oxygen, $O_2^{\bullet-}$ and $^{\bullet}OH$ *in vitro* (Bielski *et al.*, 1976). Studies in animals and *in vitro* systems have shown that phenolic antioxidants can inhibit free radical-induced damage to macromolecules. Green tea catechins are the most

investigated phenolic antioxidants in this regard (Zhao *et al.*, 1989).

2.6 Control of lipid oxidation in Food

Over the decades, researchers have developed a number of chemical and physical means to prevent or retard lipid oxidation in foods. For instance, hydrogenation of unsaturated fatty acids (Cowan and Evans, 1962; Patterson, 1989), removal of oxygen through vacuum packaging (Hwang, *et al.*, 1990; Lindsay, 1977), use of superoxide scavengers such as glucose oxidase and ascorbic acid oxidase (Hsieh and Kinsella, 1989), removal of metal ions (Kanner, 1994; Moledina *et al.*, 1977), refrigeration and freezing (Kanner, 1994), and addition of synthetic and natural antioxidants (Johnson *et al.*, 1996; Torel *et al.*, 1986) are known to retard lipid oxidation in foods. All these preventative measures are not always applicable in actual systems. The use of antioxidants, however, has become more acceptable, probably due to their high efficiency and multifunctional nature (Giese, 1996).

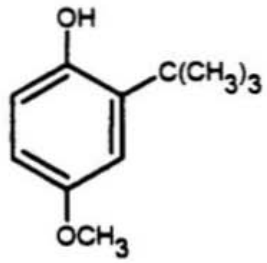
The food antioxidants can be classified into two broad categories, namely synthetic and natural. Synthetic antioxidants include phenolics such as BHA, BHT, TBHQ and PG, and non-phenolics such as ascorbic acid, erythorbic acid and ascorbyl palmitate (Frankel, 1996; Shahidi *et al.*, 1987; Sherwin, 1976). Natural antioxidants include β-carotene (Burton and Ingold, 1984), naturally occurring ascorbic acid, citric acid, certain nucleotides and dipeptides (Chen *et al.*, 1995), inorganic salts (Wettasinghe and Shahidi, 1996), tocopherols and their derivatives (Jain and Palmer, 1997; Murase *et al.*, 1998;

Lampi *et al.*, 1997; Martin *et al.*, 1996), antioxidant enzymes (Jung *et al.*, 1997) and a large number of various phenolic compounds of mainly plant origin. The following sections will focus on the role of phenolic antioxidants (both synthetic and natural) in food.

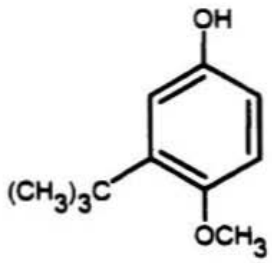
2.6.1. Synthetic antioxidants

BHA, BHT, TBHQ and PG are the commonly used synthetic antioxidants in foods and non-food products (Bishov *et al*, 1977; Kramer, 1985; Duh and Yen, 1995). These compounds possess the ability to donate a hydrogen atom or an electron to free radicals and therefore are known as chain-breaking antioxidants (Cort, 1974). Figure 2.11 depicts the chemical structures of BHA, BHT, TBHQ and PG while Figure 2.12 depicts the generalized mechanism of their action. BHA is a mixture of two isomers: 2-BHA and 3-BHA. The 3-isomer is the better antioxidant; commercial BHA generally has 90% 3-isomer (Shahidi and Naczk, 1995). BHA is an effective antioxidant in animal fats, but there are evidences that BHA is inefficient in vegetable oils (Sherwin, 1976). BHA is used in granola bars, breakfast cereals, vegetable oils, animal fats, potato chips, sausages, snack foods and chewing gum (Buford, 1988). BHA and BHT have similar properties and applications and together they act synergistically to protect lipids from oxidation (Kurechi and Kato, 1980). TBHQ is the most effective synthetic antioxidant for most fats and oils, especially vegetable oils (Sherwin, 1976). BHA, BHT and TBHQ have good "carry through" capacities in frying applications. PG is also used to stabilize animal fats and

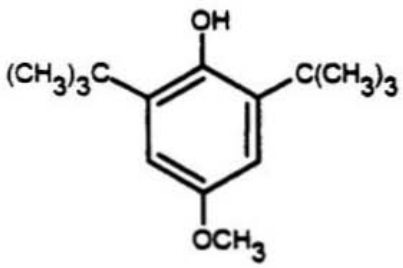
Figure 2.11 Chemical structures of synthetic antioxidants.



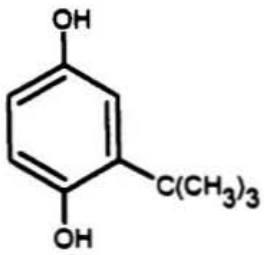
2-BHA (Butylated hydroxyanisole)



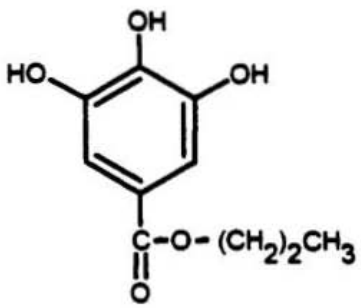
3-BHA (Butylated hydroxyanisole)



BHT (Butylated hydroxytoluene)

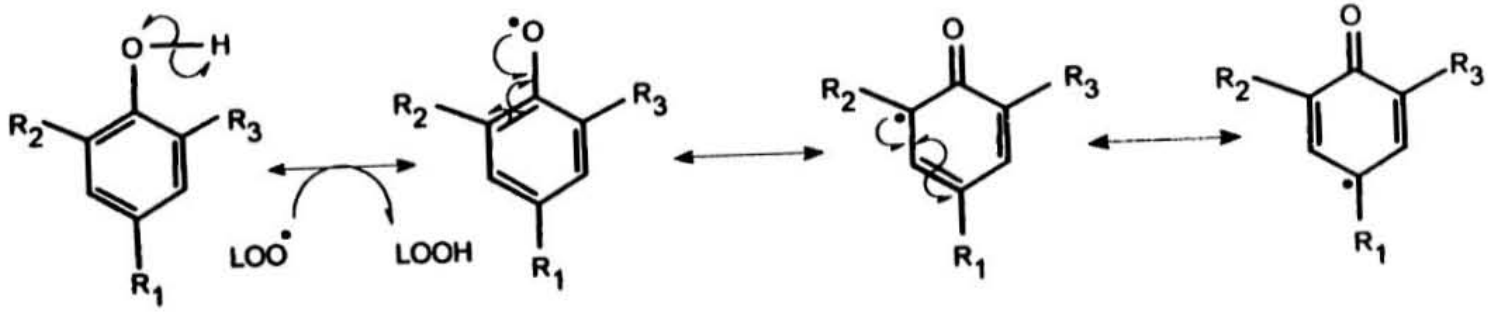


(TBHQ) Tertiary-butylhydroquinone



(PG) Propyl gallate

Figure 2.12 Mechanism of action of phenolic antioxidants.


O
H
R2
R3
R1
LOO•
LOOH
•O
O
O
O

vegetable oils, but its "carry through" capacity in frying applications is poor. PG also imparts a blue-black colour to products due to complexation with iron ions. Therefore, PG is always used with a metal chelator such as citric acid (Shahidi and Wanasundara, 1992).

BHA, BHT, TBHQ and PG are permitted in foods at levels not to exceed FDA- and USDA-permitted levels resulting in a maximum total antioxidant content of 0.02% (200 ppm) of the total fat or oil content. These compounds may be used singly or in combination except TBHQ which is not lawful for use in combination with propyl gallate. Furthermore, TBHQ is not approved in Canada.

2.6.2. Natural antioxidants

The investigation of natural antioxidants for food preservation has received much attention due to the general resistance to synthetic food additives by consumers and manufacturers. This necessity was further intensified by substantial number of scientific evidences showing the possible carcinogenic effects of the most commonly used synthetic antioxidants, BHA and BHT (Namiki, 1990).

It is well known that diets rich in fruits and vegetables are protective against several diseases. These protective effects have been largely attributed to the antioxidants present, including antioxidant nutrients, vitamin C and β-carotene, and also to minor carotenoids, and plant phenolics such as flavonoids. The polyphenolic compounds of higher plants may act as antioxidants or as agents of other mechanisms contributing to

anticarcinogenic or cardioprotective action (Tijburg *et al.*, 1997).

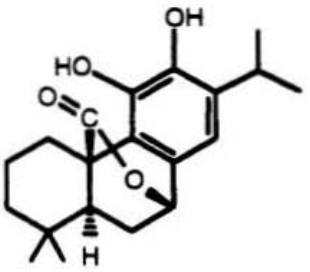
2.6.2.1 Sources of natural antioxidants

Antioxidant activity of extracts prepared from various plant species has been reported in the literature. For instance, spices, oilseeds, cereals, legumes, teas and herbs have been reported to contain antioxidant compounds of phenolic nature. These sources will be discussed in the following sections.

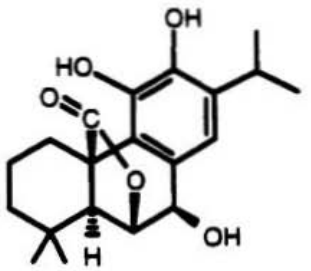
2.6.2.1.1 Spices

Since ancient times, spices have been added to different types of food to improve their flavour. For example, garlic and red chili were added to butterfat (ghee) and red chili, fennel or clove were often used in the preparation of pickles (Madsen and Bertelsen, 1995). Dubois and Tressler (1943) showed antioxidative action of sage, mace, and black pepper on frozen meat and this study was the first such scientific study on antioxidative properties of spices. Chipault *et al.* (1952, 1956) also showed the ability of extracts of rosemary, sage and thyme to retard lipid oxidation in a lard model system. Kramer (1985) reported that clove extracts contained both eugenol and gallic acid. Ethanolic and ether extracts of clove have been shown to retard lipid oxidation in various model systems (Kramer, 1985). Nakatani and Inatani (1984) have isolated antioxidative compounds from extracts of dried rosemary leaves; these were carnosol, rosmanol (7α-), epirosmanol (7β-) and isorosmanol (Figure 2.13). Among these compounds, rosmanol has shown a stronger

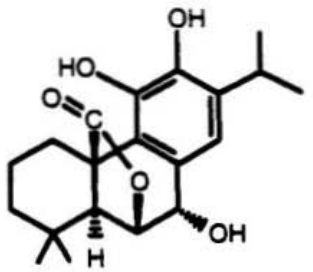
Figure 2.13 Chemical structures of rosemary antioxidants.



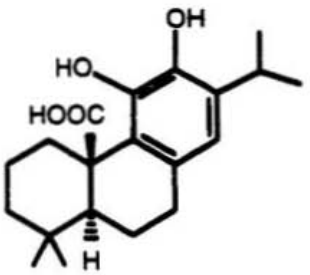
Carnosol



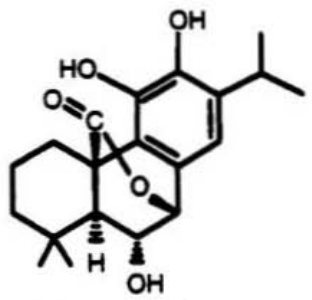
Epirosmanol



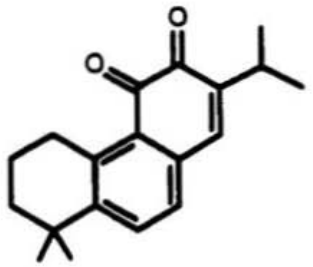
Rosmanol



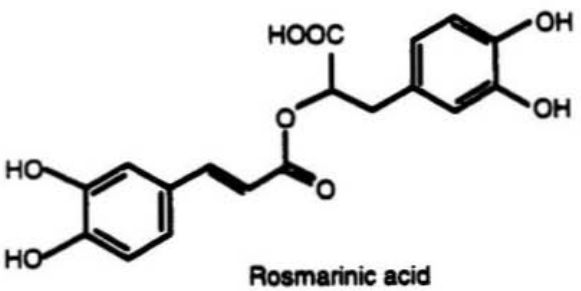
Carnosic acid



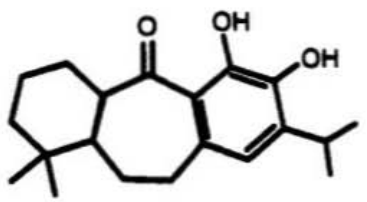
Isorosmanol



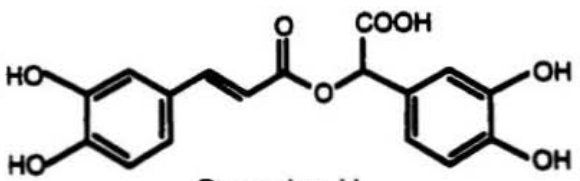
Rosmariquinone



Rosmarinic acid



Rosmaridiphenol



Rosmaric acid

antioxidant activity than BHA, at the same concentration, in lard and water-in-oil emulsion systems (Inatani *et al.*, 1982, 1983). Houlihan *et al.* (1984, 1985) have reported the presence of two novel compounds in rosemary extracts namely rosemarydiphenol and rosmaryquinone (Figure 2.13).

Oregano, a plant belonging to the family *Perilla*, has been reported to possess excellent antioxidant properties which were greater than those of BHA (Nakatani, 1997). Nakatani and Kikuzaki (1987) have isolated a new antioxidative glycoside from oregano which was confirmed to be diphenol glycoside [Figure 2.14(A)]. Nakatani and Kikuzaki (1987) reported the presence of a caffeic acid derivative in oregano which was later named rosmaric acid [Figure 2.14(A)].

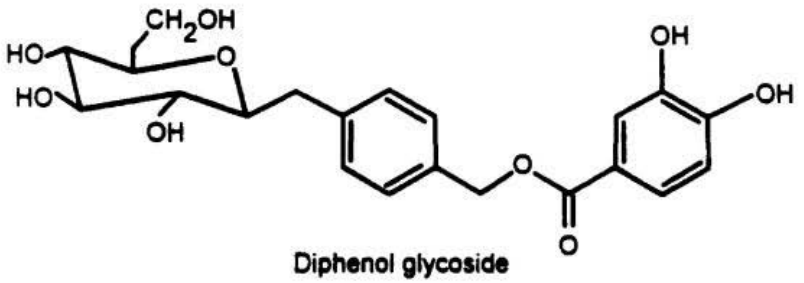
Thyme, another *Perilla* plant, has been reported to contain several biphenyl derivatives [Figure 2.14(B)]. Their activities were reported to be comparable to those of BHT (Nakatani *et al.*, 1989; Miura and Nakatani, 1989).

Capsicin and dihydrocapsicin [Figure 2.15(A)], hot-tasting components of *Capsicum* fruits, are antioxidative, but not suitable for general food use. However, a new antioxidant without hot taste [Figure 2.15(B)] has been isolated from *Capsicum frutescens* by Nakatani *et al.* (1988).

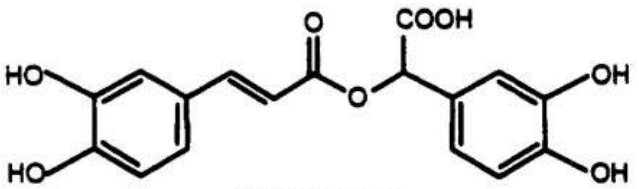
Nakatani *et al.* (1986) have isolated five antioxidative amides of phenolic acids [Figure 2.15(C)] from a black pepper extract. The ferulic acid amide of thyramine and a piperine-related compound with an open methylenedioxy ring have been reported to be tasteless, odourless and fat soluble (Nakatani *et al.*, 1986).

Figure 2.14 Chemical structures of oregano (A) and thyme (B) antioxidants.

(a)

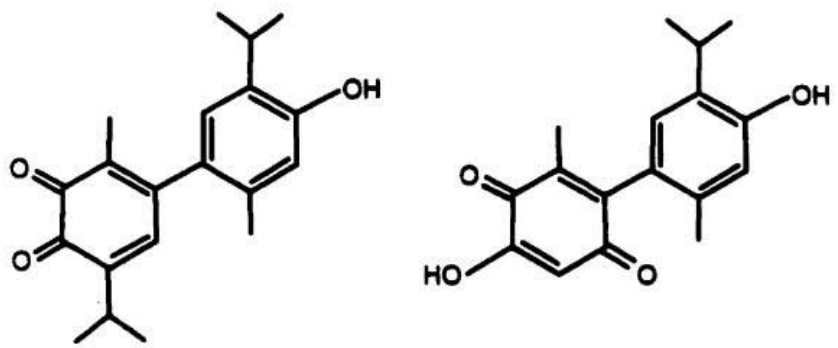


Diphenol glycoside



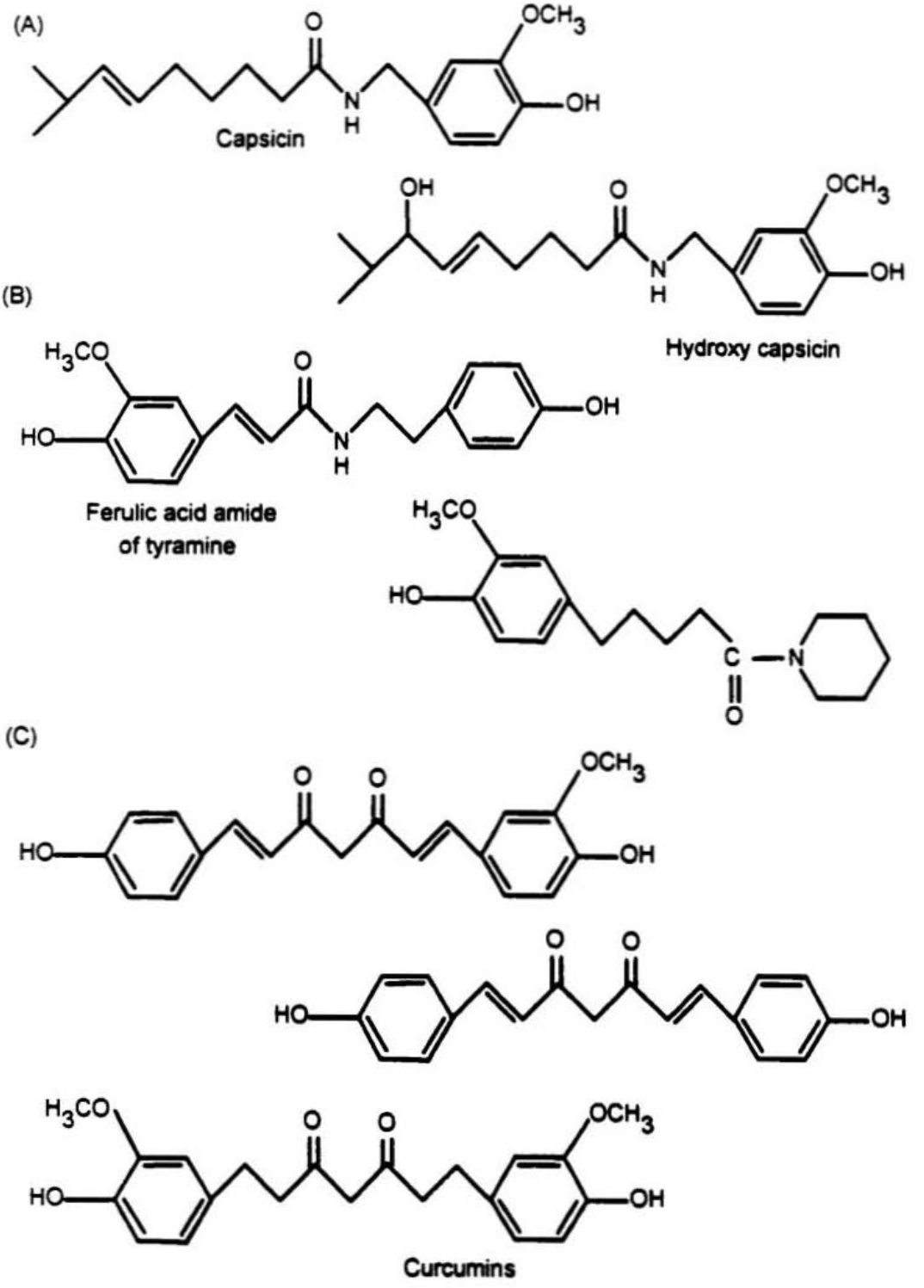
Rosmaric acid

(b)



Thymols

Figure 2.15 Antioxidants of *Capsicum frutescens* (A), black pepper (B) and turmeric (C).


(A)
O
OCH3
N
H
OH
Capsicin
OH
O
OCH3
N
H
OH
(B)
Hydroxy capsicin
H3CO
O
HO
OH
N
H
Ferulic acid amide
of tyramine
H3CO
HO
C
N
O
(C)
O
O
OCH3
HO
OH
O
O
HO
OH
H3CO
O
O
OCH3
HO
OH
Curcumins

The antioxidant compounds present in turmeric (*Curcumin longa* L.) have been reported to have structures similar to the yellow pigment curcumin (Toda *et al.*, 1985). Tetrahydrocurcumin is colourless, heat resistant, and antioxidative (Osawa *et al.*, 1989). These compounds have phenolic β-diketone groups. Tetrahydrocucumin has also been isolated from the antioxidative fractions of *Eucaliptus* leaf wax [Figure 2.15(C); Osawa *et al.*, 1989].

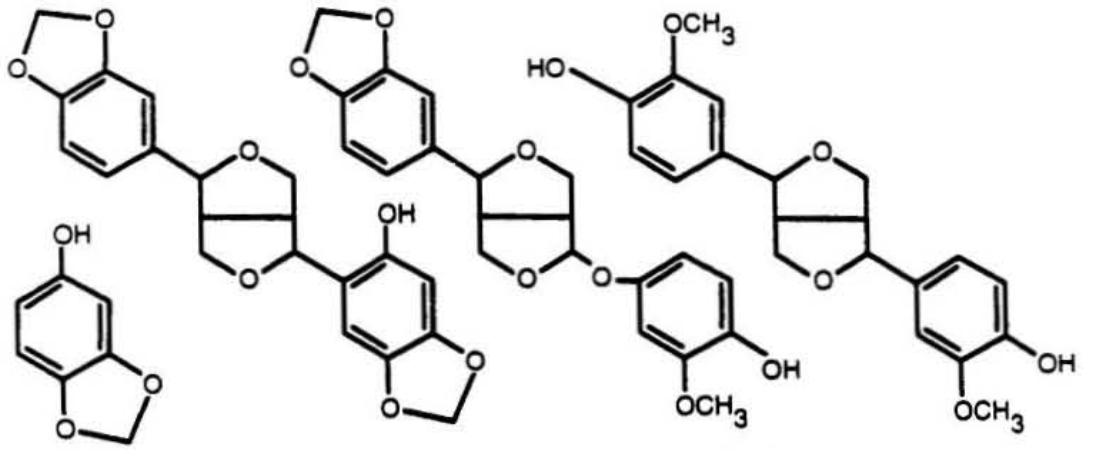
2.6.2.1.2 Oilseeds

Several antioxidative compounds have been isolated from different oilseeds. One of the widely studied oilseeds is sesame. Its oil is commercially produced from non-roasted and roasted seeds (Fukuda and Namiki, 1988). The latter has a higher oxidative stability than the former (Fukuda *et al.*, 1985). It has been reported that the antioxidant compounds of sesame are tocopherols, sesamol, sesamolinol, sesaminol, pinoresinol, syringic acid, ferulic acid and sesamol dimmer [Figure 2.16(A)]. The high oxidative stability of roasted sesame oil has been attributed to the γ-tocopherol, sesamol and Maillard reaction products (Fukuda *et al.*, 1986). It has been reported that sesame lignans [Figure 2.16(B)] are hydrolyzed during roasting to produce sesamol which imparts a high stability to the roasted sesame oil.

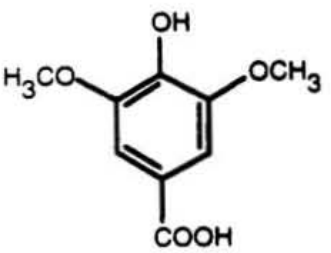
Shahidi *et al.* (1994a) have reported that ethanolic extracts of mustard flour exhibit antioxidant properties in a β-carotene-linoleate model system. These authors attributed this antioxidant effect to polyhydroxyphenols such as flavones and flavonols present in the

Figure 2.16 Chemical structures of sesame antioxidants (A) and lignans (B).

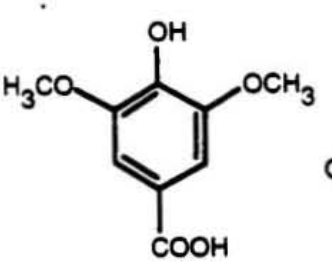
(A)



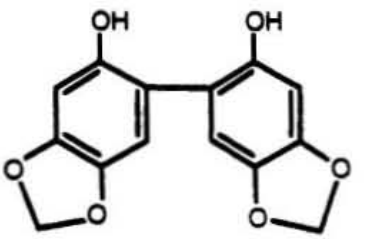
Sesamol Sesaminol Sesamolinol Pinoresinol



Syringic acid

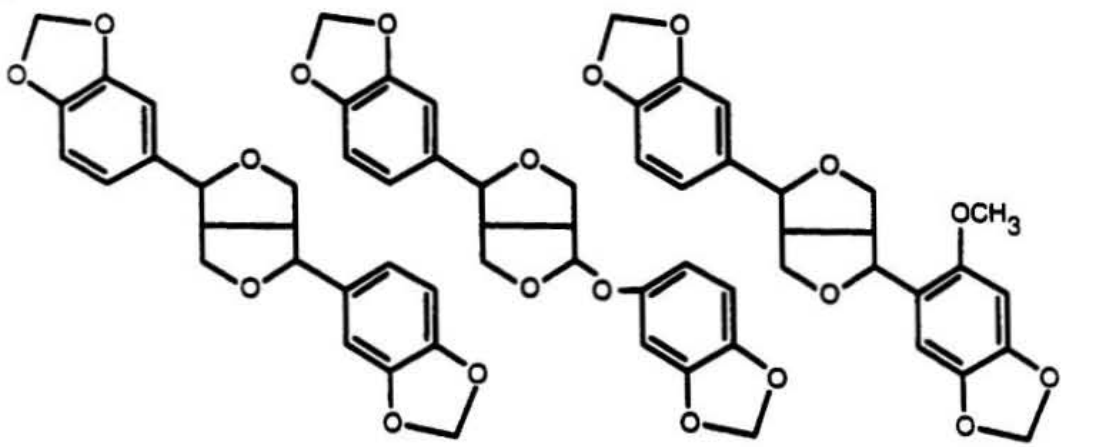


Ferulic acid



Sesamol dimer

(B)



Sesamin Sesamolin Sesangolin

extracts. Wanasundara *et al.* (1994) were able to identify the most active phenolic antioxidant in canola meal as 1-O-β-D-glucopyranosyl sinapate [Figure 2.17(A)].

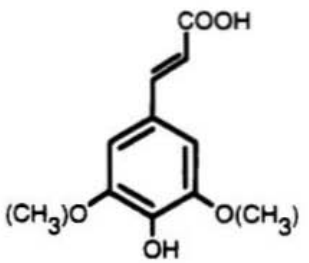
The antioxidants present in soybean oil are α-tocopherol, γ-tocopherol and δ-tocopherol [Figure 2.17(B); Gyorgy *et al.*, 1964]. The main antioxidative compounds in soybean meal have been identified to be isoflavones, glycosides and their derivatives, tocopherols, amino acids, and peptides (Gyorgy *et al.*, 1964; Hayes *et al.*, 1977; Naim *et al.*, 1973, 1974, 1976). Some of the isoflavone glycosides of soybean meal have been identified as genistein, daidzein, glycitein, prunetin, formononetin and 4',6',7'-trihydroxyisoflavone [Figure 2.18(A); Rackis, 1972].

An antioxidative flavonol, dihydroquercetin , has been isolated from methanolic extracts of peanut (Pratt and Miller, 1984). The chemical structures of antioxidants present in peanut meal are shown in Figure 2.18(B). Apart from phenolic antioxidants, protein fractions of peanut have been shown to inhibit lipid oxidation in model systems containing metmyoglobin and Fe^{2+}-EDTA as catalysts (Rhee *et al.*, 1979). Antioxidant activity of protein fractions of peanuts in fresh beef homogenates and beef patties has also been reported (Ziprin *et al.*, 1981).

The major phenolic compounds present in the methanolic extracts of cottonseeds were quercetin glycosides (Whiltern *et al.*, 1984). The main quercetin glycoside of cottonseed is rutin [Figure 2.18(C)] and its antioxidant activity is reported to be inferior to that of quercetin (Yousseff and Rahman, 1985).

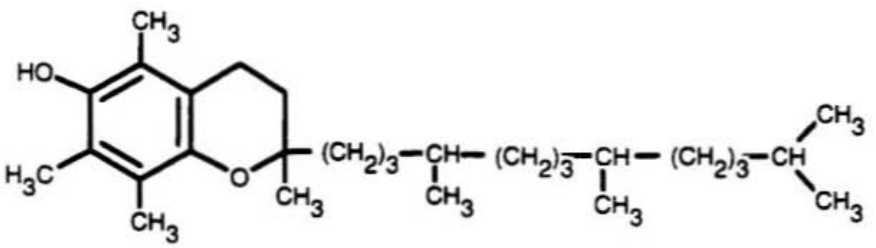
Figure 2.17 Chemical structures of the most active phenolic antioxidant in canola meal (A) and tocopherols (B).

(A)

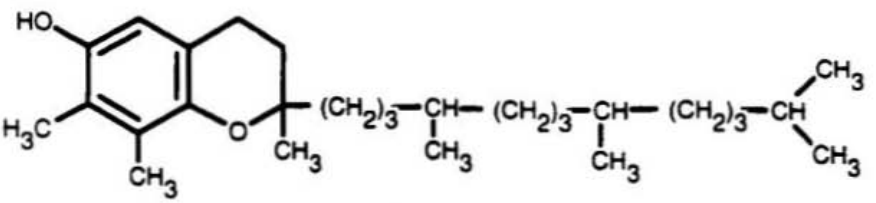


Sinapic acid

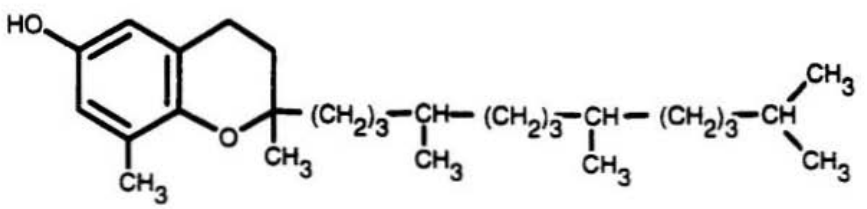
(B)



α-Tocopherol
(5,7,8-Trimethyltocol)



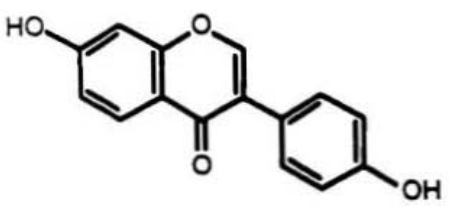
γ-Tocopherol
(7,8-Dimethyltocol)



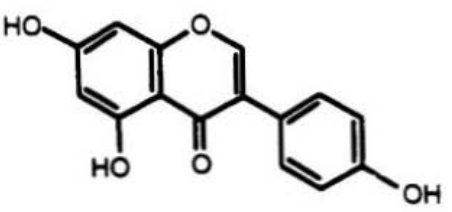
δ-Tocopherol
(8-Methyltocol)

Figure 2.18 Chemical structures of soybean (A) and peanut (B) antioxidants.

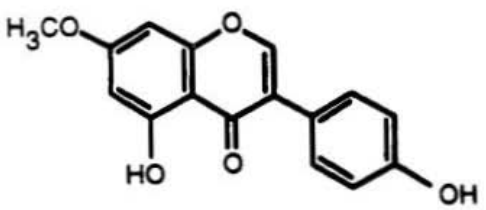
(A)



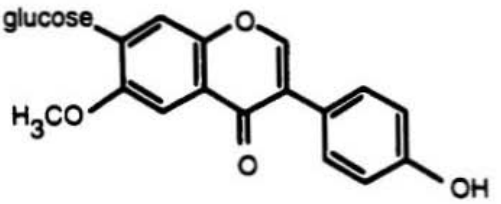
Daidzein



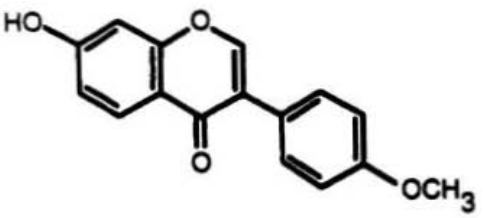
Genistein



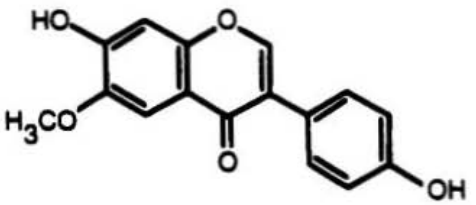
Prunetin



Glycitein

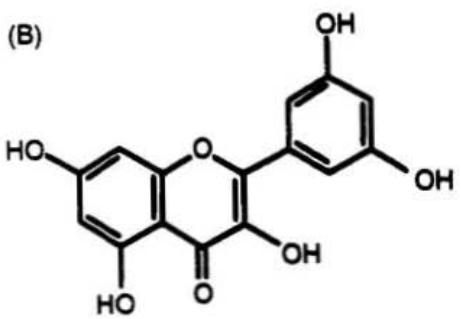


Formononetin



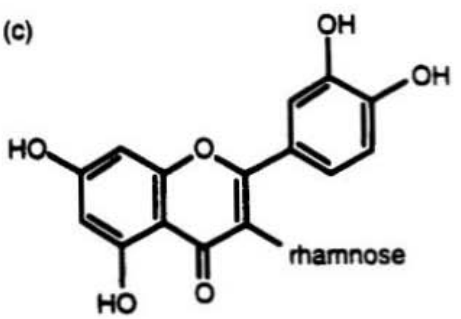
4',6',7'-Trihydroxyisoflavone

(B)



Taxifolin

(c)



Rutin

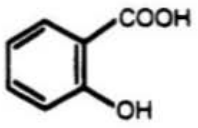
Rapeseed and its genetically improved version canola, contain several phenolic compounds which exist as phenolic acids, their derivatives and condensed tannins (Kozlowska *et al.*, 1990). Rapeseed meal contains phenolic acids namely, salicylic, cinnamic, *p*-hydroxybenzoic, veratric, vanillic, gentisic, protocatechuic, syringic, caffeic, sinapic and ferulic acids (Figure 2.19)(Kozlowska *et al.*, 1990). The major condensed tannins of rapseed meal are cyanidin, pelargonidin and kaempferol and their derivatives (Figure 2.19).

2.6.2.1.3 Cereals

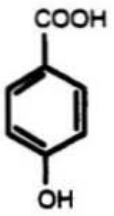
Asamarai *et al.* (1996) have shown the antioxidant activity of methanolic extracts of wild rice hulls. They have identified the active compounds as 2,3,6-trimethylanisole, *m*-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde). Presence of tocopherols and isovitexin, a glycosyl flavonoid, in a long-lived rice variety (Katakutara) has also been documented (Ramarathnam *et al.*, 1989). The chemical structures of rice antioxidants are shown in Figure 2.20(A)

Tian and White (1994) have reported that the methanolic extracts of oat groats exhibited antioxidant properties in soybean and cottonseed oil systems. The antioxidant activity of oat extract has been attributed to its high contents of dihydrocaffeic acid and phospholipids [Schuler, 1990, Figure 2.20(B)]. Iwami *et al.* (1987) showed the antioxidant effect of wheat gliadin in a powder model system consisting of linoleic acid,

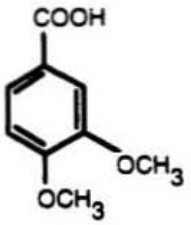
Figure 2.19 Chemical structures of antioxidants of rapseed meal.



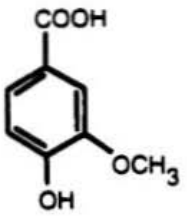
Salicylic acid



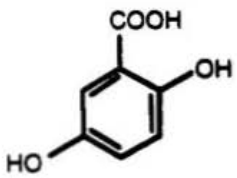
p-Hydroxybenzoic acid



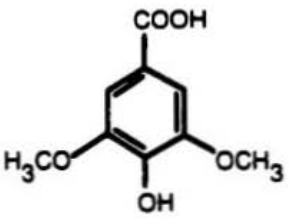
Veratric acid



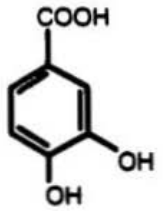
Vanillic acid



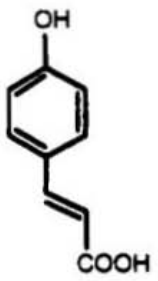
Gentisic acid



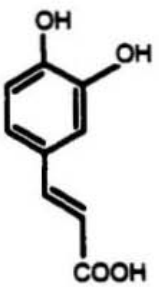
Syringic acid



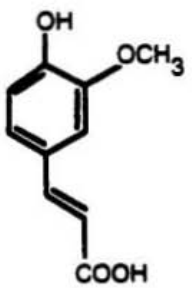
Protocatechuic acid



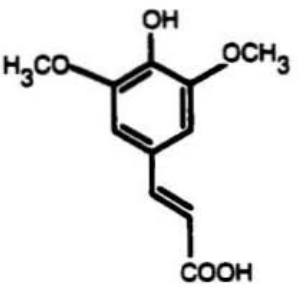
Cinnamic acid



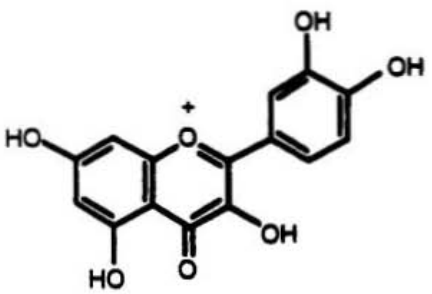
Caffeic acid



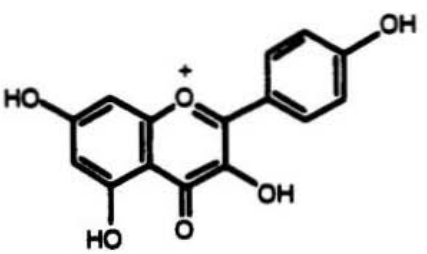
Ferulic acid



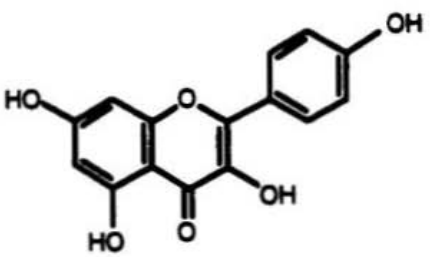
Sinapic acid



Cyanidin



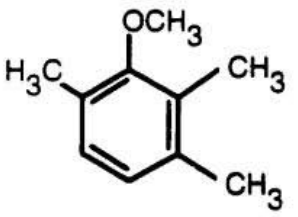
Pelargonidin



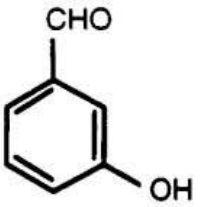
Kaempferol

Figure 2.20 Chemical structures of rice (A) and oat (B) antioxidants.

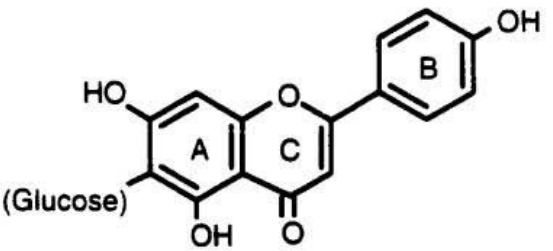
(A)



2,3,6-Trimethylanisole

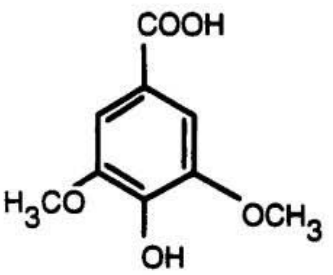


m-Hydroxybenzaldehyde

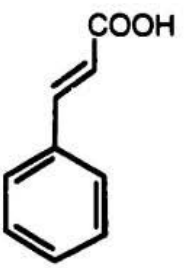


Isovitexin

(B)



Syringaldehyde



Dihydrocaffeic acid

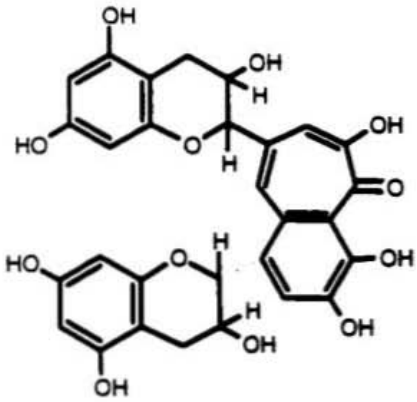
palmitic acid, starch, cellulose, β-cyclodextrin and quartz sand.

2.6.2.1.4 Beverages and herbs

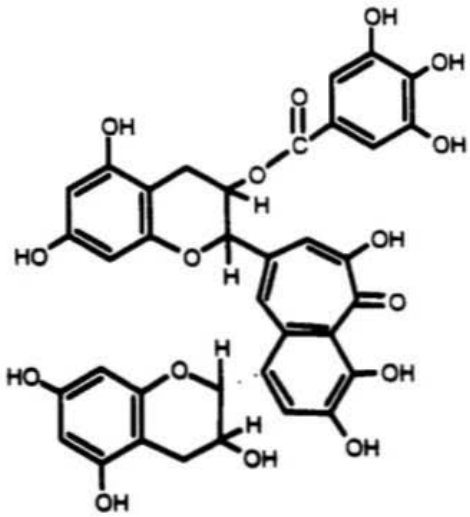
Leafy materials are well known as rich sources of flavonoids and phenolic acids and are recognized as a major source of flavonoids in the human diet (Hertog *et al.*, 1993; Tijburg *et al.*, 1997). Leaves of *Camellia sinensis* (tea) is one of the extensively studied plant materials for natural antioxidants. Tea beverage is prepared, mainly, from two types of manufacturing processes. Black tea, fermented tea leaves, typically contains approximately 31% (w/w) flavonoids whose chemical structures are depicted in Figure 2.21. These flavonoids consist of a mixture of catechins (9%), theaflavin (4%), flavonols (3%) and unidentified catechin condensation products (15%) known as thearubigins. Oolong tea is a semi-fermented tea which is also used extensively. Green tea, a non-fermented dried tea leaves, contains approximately 33% (w/w) flavonoids with flavonols and catechins accounting for about 3 and 30%, respectively (Tijburg *et al.*, 1997; Wiseman *et al.*, 1997).

von Gadow *et al.* (1997) have compared four different types of teas, namely rooibos (*Aspalathus linearis*), green, oolong and black tea for their free radical scavenging properties in an assay medium containing DPPH free radicals. The antioxidant activity assessed by this method decreased in the order of green> unfermented rooibos> fermented rooibos> semi-fermented rooibos> black> oolong. von Gadow *et al.* (1997) have also employed a β-carotene-linoleate model system to assess the antioxidant activity of the four

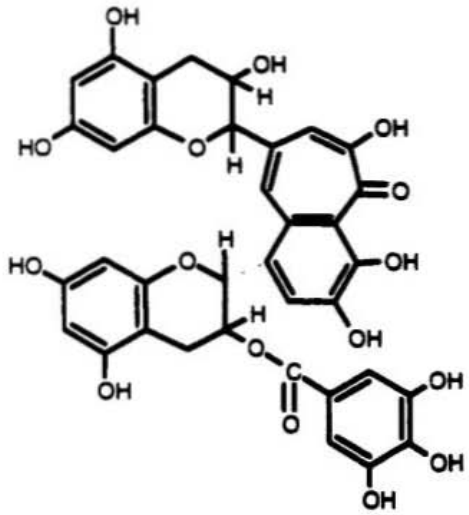
Figure 2.21 **Chemical structures of black tea antioxidants.**



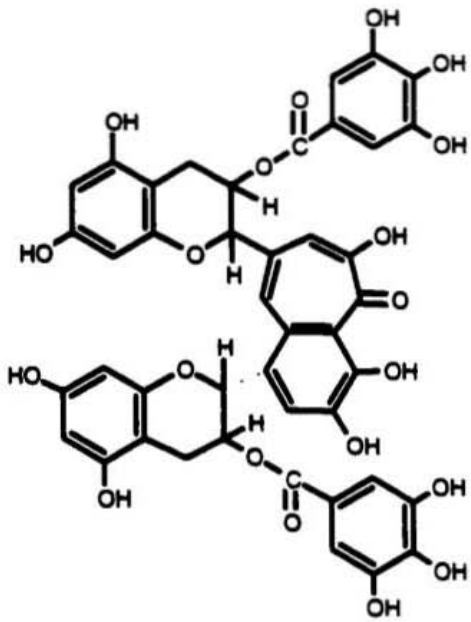
Theaflavin



Theaflavin monogallate A



Theaflavin monogallate B



Theaflavin digallate

types of teas. In this system the activity decreased in the order of green> black> oolong> fermented rooibos> unfermented rooibos> semi-fermented rooibos. Several authors have reported that the tea extracts are antimutagenic (Yen and Chen, 1995) and anticarcinogenic (Blot *et al.*, 1997; Dreosti *et al.*, 1997; Hollman *et al.*, 1997; Ruch *et al.*, 1989).

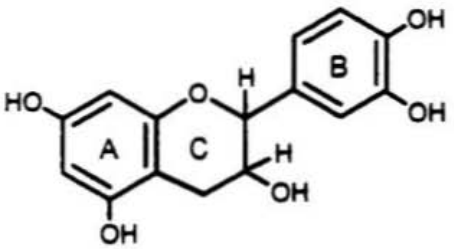
Amarowicz and Shahidi (1996) have isolated individual catechins from green tea. They reported that tea catechins were composed of a mixture of (-)epicatechin, (+)epigallocatechin, (-)epicatechin-3-gallate and (-)epigallocatechin-3-gallate (Figure 2.22). He and Shahidi (1997) showed the antioxidant activity of different catechins in a fish meat model system. According to these authors, the potency of catechins in the prevention of oxidation in fish meat was in the decreasing order of (-)epigallocatechin gallate = (-)epicatechin-3-gallate > (-)epigallocatechin > (-)epicatechin.

Fructus schisandrae, a traditional Chinese herb, has been used for centuries as a sedative beverage or tonic. It has been used for the treatment of chronic viral hepatitis in China since the1970's. More than 20 dibenzocyclooctene lignans have been isolated from *Schisandraceae* (Liu, 1996). Figure 2.23 depicts the chemical structures of *Schisandraceae* antioxidants.

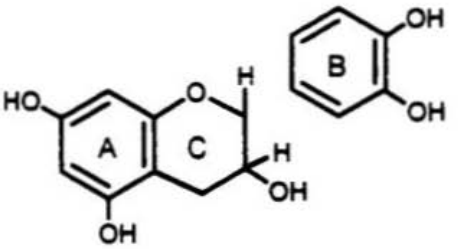
2.6.6.2 Extraction techniques

A variety of techniques for the extraction of antioxidative compounds from plant material is available. The simplest procedure involves the heating of the fresh or dry plant

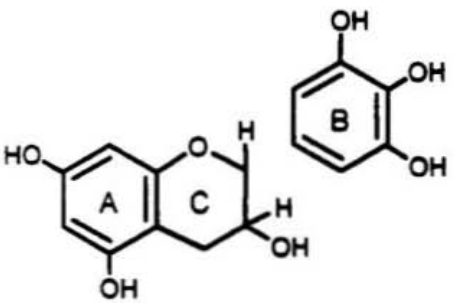
Figure 2.22 Chemical structures of green tea antioxidants.



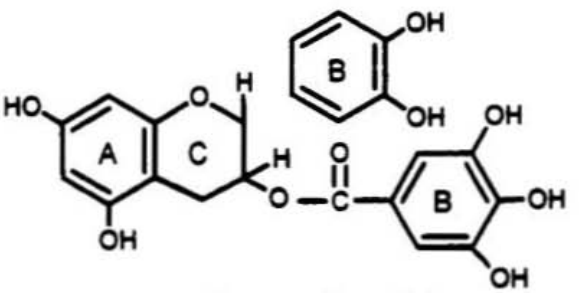
(+)Catechin



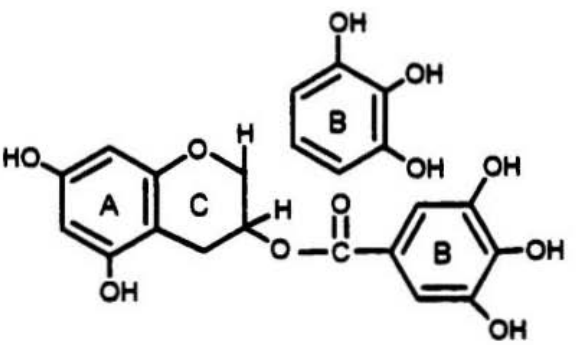
(-)Epicatechin



(-)Epigallocatechin

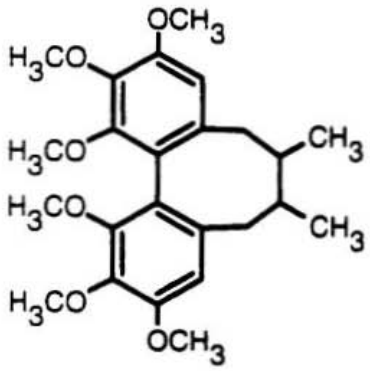


(-)Epicatechin gallate

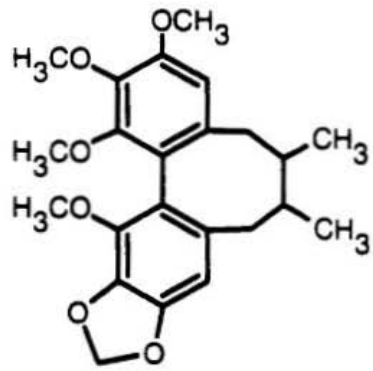


(-)Epigallocatechin gallate

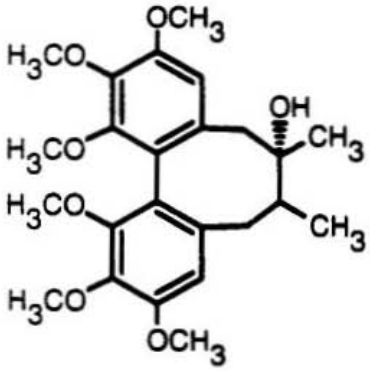
Figure 2.23 Chemical structures of *Schisandraceae* antioxidants.



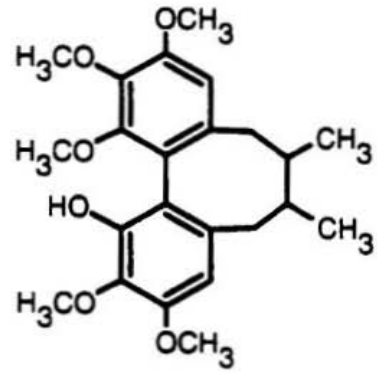
Schizandrin A



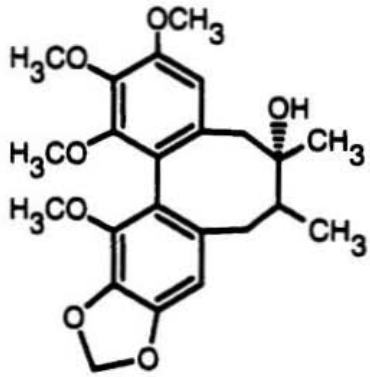
Schizandren B



Schizandrol A



Schizanhenol



Schizandrol

parts in water to boil followed by filtration or centrifugation to recover the liquid portion. This extraction procedure has been employed for green tea and black tea. Lee *et al.* (1986) have used a direct extraction method for ginger rhizomes which involves the squeezing of the peeled, sliced and ground ginger rhizomes through four layers of cheese cloth followed by centrifugation. Organic solvents such as methanol, ethanol, acetone, hexane, ethyl ether, dimethyl sulphoxide as well as the aqueous solutions of polar solvents have been used for the extraction of antioxidants from various plant parts. For example, Inatani *et al.* (1982) have extracted rosemary antioxidants into hexane. Mizuno *et al.* (1987) have also used hexane and methanol to extract phenolic compounds from seeds of *Coptis japonica* var. *Dissecta.* Jaworski and Lee (1987) extracted phenolics from crushed grapes with absolute ethanol. Licorice roots extracts have been prepared in acetone and absolute ethanol (Gordon and An, 1995; Vaya *et al.*, 1997). Wanasundara *et al.* (1994) have extracted antioxidants of canola meal into 95% (v/v) aqueous ethanol and Shahidi *et al.* (1994a) have also used the same medium to extract antioxidants from low-pungency mustard flour. Aqueous methanol (75%, v/v) has been used to extract antioxidants from wild rice hulls (Asamarai *et al.*, 1996) and fruit and vegetable juices (Chambers *et al.*, 1996). Plumb *et al.* (1997) employed 70% (v/v) aqueous methanol to extract phenolics from lyophilized-powdered fruit and vegetable extracts. Oomah *et al.* (1995) employed absolute methanol to extract phenolic acids from flaxseeds. Use of ethyl acetate to extract antioxidants from highbush blueberries has been reported by Kader *et al.* (1996). Hettiarachchy *et al.* (1996) have extracted antioxidants from fenugreek with 60% (v/v)

aqueous ethanol. Both methanol and ether have been used for the extraction of phenolic compounds from lyophilized avocado powder (Torrel *et al.*, 1986).

All of the extraction media, discussed so far, have been applied in a simple mixing of ground plant parts with solvent followed by extraction under reflux conditions at elevated or less commonly at room temperature for varying time courses. A completely different approach to extract antioxidants from rosemary, sage, paprika, nutmeg and cocoa shells has been adopted by Bracco *et al.* (1981). These authors have mixed crushed plant parts with an edible oil, such as peanut oil, and subjected to molecular distillation in order to recover phenolic compounds. Schwarz and Ernst (1996) have used lyophilized thyme leaves to prepare a hexane extract in a Soxhlet extraction apparatus similar to the one used for crude lipid determination. A stepwise solvent extraction procedure for the extraction of antioxidants from isubgol was adopted by Metha *et al.* (1994). A dried isubgol sample was repeatedly extracted with ethyl ether, petroleum ether, chloroform-ether, chloroform-ether-dichloroethane, methanol, and chloroform-methanol-dichloroethane in a stepwise manner.

Recently, a novel technique, which employs supercritical fluids, such as liquid carbon dioxide, has been adopted to extract antioxidants from various plant and animal samples. The main advantage of this technique over others is the possibility of extracting labile antioxidants without incurring any structural changes to the molecules of interest (Rizvi *et al.*, 1986). A number of gases are known to have good selective solvent properties when raised to pressures above their critical values. These pressures are very

high, in the order of 1000-2000 psig. For food commodities, CO_2 is the solvent of choice because it has moderate critical temperature and pressure (31.1°C , 1070 psig) and is inert, inexpensive, non-flammable, environmentally acceptable, readily available and safe (Mishra *et al.* 1993). Supercritical fluid extraction has been effectively used to extract tocopherols from fish oil (Rizvi *et al.* 1986) and caffeine from coffee.

Other important parameters that determine the efficacy of extraction as well as the integrity of antioxidant molecules are the extraction temperature and time courses. Varying extraction temperatures and time courses have been reported in the literature. Unfortunately, most authors have not provided a justification as to why a specific set of conditions had been employed. Duh and Yen (1995) used methanol at room temperature over a 12 h time course to extract antioxidants from irradiated peanut hulls. Maillard *et al.* (1996) extracted phenolics from barley and malt into methanol at room temperature over an 80 min time course. Extraction times as short as 20 - 30 min have also been reported (Oomah *et al.*, 1995). Temperatures ranging from 40 to 100°C have been applied in several studies which involved various plant materials (Asamarai *et al.*, 1996; Chambers *et al.*, 1996; Shahidi *et al.*, 1994a; Wanasundara *et al.*, 1994).

In a majority of studies, arbitrarily selected set of extraction conditions have been employed to extract antioxidants from plant materials. Use of a multivariate approach such as response surface methodology (RSM) to locate the optimum extraction conditions for phenolic antioxidants has not been reported in the literature. RSM, however, has been successfully adapted to locate optimum experimental conditions for isolation of proteins

and pigments (Wanasundara and Shahidi, 1996; Gao and Mazza, 1996). This suggests that the same technique may be used to set optimum conditions for the extraction of plant antioxidants. The following section deals with the theory of RSM.

2.6.2.2.1 Polynomial models and response surface methodology (RSM)

Polynomial models provide sufficient flexibility to adequately approximate many complicated, but unknown, relationships between a response and one or more predictor variables. In many important problems in science the underlying mechanism that generates the data is not well understood, due to the complexity of the problem and lack of sufficient theory. In these cases polynomial models can provide adequate approximations to the unknown functional relationship (Mason *et al.*, 1989). One should start with the simplest model warranted by what is known about the response under investigation. If a lack-of-fit test indicates that the proposed model is an inadequate approximation to the observed responses, one can either add the next higher-order terms into the model or investigate nonlinear models. In many experimental situations, a first- or second-order polynomial is adequate to describe a response (Mason *et al.*, 1989).

A response surface model represents the functional form of a response and can be based on either theoretical or empirical considerations. When a theoretical model cannot be specified in an experimental investigation, polynomial models often are used to approximate the response surface. A quadratic polynomial can provide a useful approximation for a broad range of application. When a polynomial representation of a

response is obtained, the factor levels that determine the optimum response can be obtained graphically by contour plotting or algebraically by canonical analysis. When second order regression models are used to characterize a response surface, a canonical analysis must be used to identify the location of the optimum response (Mason *et al.*, 1989). Since the antioxidant activity of a particular plant extract is a function of many variables, RSM can be employed to provide an adequate approximation to the unknown functional relationship and also to locate the optimum extraction conditions.

2.6.3 Evaluation of antioxidants

Plant extracts and their active compounds have been evaluated in a variety of model systems using various analytical techniques. The choice for the model system depends mainly upon the chemical nature of substances to be investigated. The following sections will focus on widely used model systems and associated analytical techniques.

2.6.3.1 Meat model systems

Due to the importance of antioxidants in processed meat industry, different meat types, such as chicken and turkey (Saligh *et al.*, 1989), beef (Morrissey and Tichivangana, 1985; Roozen, 1987), pork (Decker and Crum, 1993; Rhee *et al.*, 1983; Shahidi *et al.*, 1987; Wettasinghe and Shahidi, 1997; Wettasinghe and Shahidi, 1996) and mutton (Wilson *et al.*, 1976) have been used as models to evaluate the efficacy of antioxidants. The use of fish meat model systems to evaluate antioxidants has also been reported

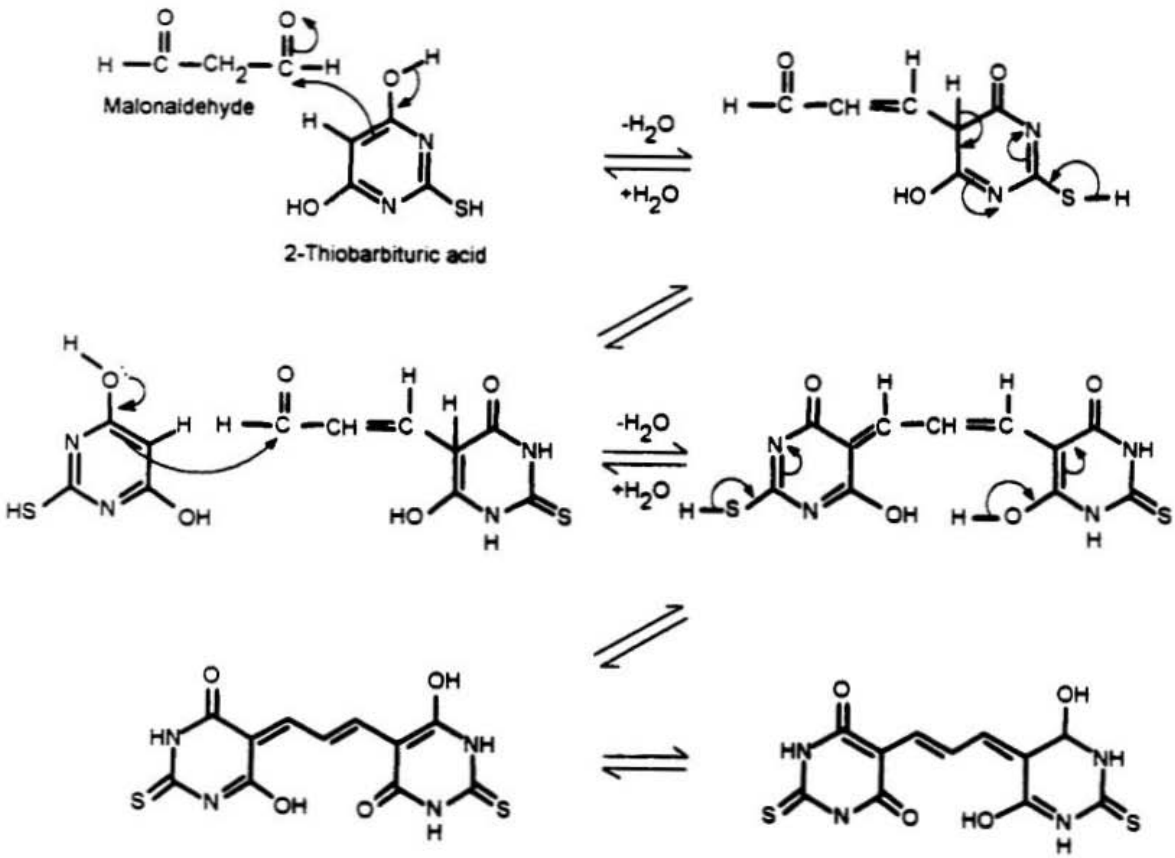
(Castell *et al.*, 1965; Castell and Spears, 1968; He and Shahidi, 1997). As discussed earlier, cooked meat undergoes rapid deterioration causing off-flavour development known as warmed-over flavour (WOF) (Tims and Watts, 1958). WOF also develops rapidly in raw meat that has been ground and exposed to air (Sato and Hegarty, 1971). The origin of WOF is the oxidizing lipids which produce a mixture of aldehydes, ketones, alcohols and hydrocarbons. The content of these oxidation products increases with storage at refrigeration temperatures, but is reduced in the presence of an antioxidant. The formation of off-flavours may be monitored quantitatively using chemical analyses, such as the 2-thiobarbituric acid (TBA) test (Janero and Burghardt, 1989; Lai *et al.*, 1995) and physical techniques, such as static headspace gas chromatography (Frankel *et al.*, 1981; Macku and Shibamoto, 1991a,b; Tamura *et al.*, 1991). In the TBA test, the TBARS formed during the storage of meat at 4°C are measured and expressed as mg malonaldehyde equivalents/kg sample (Halliwell and Gutteridge, 1985; Packer and Glazer, 1990; Esterbauer *et al.*, 1991). Although there are many variations of the TBA test, the two of which applicable to meat are the distillation (Tarladgis *et al.*, 1964) and extraction (Siu and Draper, 1978) methods. A special glass distillation apparatus is used in the distillation method. The sample is heated in a dilute acid medium and the distillate is collected for subsequent analysis (Tarladgis *et al.*, 1964). The extraction method, which is more simple than the distillation method, involves the extraction of bound malonaldehyde into a dilute trichloroacetic acid solution, mixing with TBA reagent followed by centrifugation to separate the liquid fraction. This liquid fraction or supernatant is then

heated in a boiling water bath for 45 min followed by measurement of the absorbance at 532 nm (Siu and Drapper, 1978).

The principle involved in the TBA test is the formation of a pink coloured adduct when animal or plant tissues are incubated with 2-thiobarbituric acid (Kohn and Liversedge, 1944). Figure 2.24 depicts the steps involved in the formation of the pink-coloured TBA-MA adduct which has an absorption maximum at 532 nm (Kosugi *et al.*, 1989).

The most widely used physical methods to monitor lipid oxidation in meat are the headspace gas chromatographic analysis and high performance liquid chromatography (HPLC). Direct gas chromatographic analysis of an extract of meat sample is a time consuming process whereas gas chromatography coupled with a headspace sampler provides a relatively simple alternative to the time consuming extraction procedures. HPLC is more suitable for isolation and quantification of non-volatile polymeric decomposition products and thermally labile peroxides and hydroperoxides (Robards *et al.*, 1988). A distinctive feature of the headspace gas chromatographic analysis is that the volatile composition of the gas phase in a sealed environment is used to determine the nature and composition of volatile oxidation products in the condensed phase with which the gas phase is in contact (Ioffe and Vitenberg, 1984). The main drawback associated with this technique is the size of sample that can be injected. Only those component that, by virtue of their concentration and relative volatility, are present in quantities sufficient to activate the detector will be detected. Relatively low-molecular-weight and highly volatile

Figure 2.24 Steps involved in the formation of thiobarbituric acid-malonaldehyde (TBA-MA) adduct.


Malonaldehyde
2-Thiobarbituric acid
-H2O
+H2O
-H2O
+H2O

compounds, such as 2 - 8 carbon esters, aldehydes and ketones can be readily detected by the direct injection of a restricted quantity of headspace gas (Jennings, 1979). The aldehydes used to monitor lipid oxidation in meat and fish are hexanal and propanal, respectively. Hexanal is the dominant aldehyde formed during the oxidation of lipids of terrestrial origin whereas propanal is the dominant aldehyde formed in the marine lipids (Frankel *et al.*, 1989). The fatty acids composition of a particular lipid determines the nature of dominant aldehydes formed during oxidation. For instance, lipids of terrestrial origin contain more of ω-6 fatty acids (i.e. linolenic acid) and their oxidative cleavage gives rise to hexanal. Marine lipids, on the other hand, are rich in ω-3 fatty acids [eg. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] which, upon oxidative cleavage, result in propanal (Frankel *et al.*, 1993b). A well established fact is that the concentration of these aldehydes highly correlates with other measurements of lipid oxidation, such as TBARS values (Wettasinghe and Shahidi, 1996).

2.6.3.2 Bulk oil and oil-in-water emulsion systems

Different lipid systems have been employed to investigate antioxidant properties of various substances. The effectiveness of antioxidants is dependent upon the nature of lipid substrate, concentration of antioxidants, length of storage period, and the analytical protocols employed to monitor lipid oxidation (Chipault *et al.*, 1955, 1956; Frankel *et al.*, 1994; Huang *et al.*, 1994; Huang *et al.*, 1996; Lea and Ward, 1959; Porter, 1980; Pryor *et al.*, 1988). Natural antioxidants have been difficult to evaluate in view of the complex

interfacial affinities between air-oil and oil-water interfaces involved (Porter, 1980). In bulk oils, polar antioxidants such as propyl gallate, TBHQ, and Trolox C (a hydrophilic carboxylic acid derivative of α-tocopherol: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) are more effective than nonpolar antioxidants, such as BHA, BHT and tocopherols. In contrast, lipophilic antioxidants are strongly favoured in foods of high surface-to-volume ratio (eg. emulsified oils) (Coupland and McClements, 1996; Frankel *et al.*, 1994).

Corn oil (stripped of its endogenous antioxidants) and its oil-in-water emulsions have been used to evaluate antioxidant activity of Trolox C, L-ascorbic acid, α-tocopherol and L-ascorbic acid 6-palmitate (Frankel *et al.*, 1994). Yamauchi *et al.* (1995) have used bulk methyl linoleate as a model system to evaluate antioxidant efficacy of α-tocopherol. Koga and Terao (1995) have used a mixture of methyl linoleate and methyl laurate as a model for edible oil in bulk phase to investigate the synergism between phosphoplipids and α-tocopherol. Marinova and Yanishlieva (1996) employed triacylglycerols and fatty acid methyl esters from olive oil to evaluate 4-hydroxycinnamic (*p*-coumaric) acid, 3-methoxy-4-hydroxycinnamic (ferulic) acid and 3,4-dihydroxycinnamic (caffeic) acid. Hsieh and Kinsella (1986) have used polyunsaturated fatty acids namely, linolenic acid (18:3, ω3), arachidonic acid (20:5, ω6), EPA (20:5, ω3), and DHA (22:6, ω3) to investigate the catalytic nature of lipoxyganase.

A variety of analytical techniques have been developed to assess the oxidative status of bulk fats and oils (Shahidi *et al.*, 1994b). Many of these techniques can also be

used to monitor lipid oxidation in emulsions, although the extraction of oil phase before analysis is necessary (Frankel *et al.*, 1994; Huang and Frankel, 1996). These techniques measure changes in the concentration of molecules within a system that are indicative of the degree of lipid oxidation. The loss of reactants (eg. oxygen or lipid), formation of intermediates (eg. hydroperoxides or conjugated dienes) and degradation products (eg. alcohols, aldehydes, ketones and hydrocarbons) have often been studied (Coupland and McClements, 1996).

Oxidation of bulk oil can be monitored by gravimetric measurement of the oxygen uptake. This is usually achieved by recording the weight gain of oil, placed in open containers as a thin layer, at several points of storage in an oven set at 60 - 100°C (Olcott and Einest, 1958; Rossell, 1983). This method is not suitable for emulsions as they lose water due to evaporation. Oxygen uptake by lipid phase of emulsion systems can be measured with the help of an oxygen electrode. Zhuang *et al.* (1991) have used an oxygen electrode to monitor oxygen uptake by an emulsion system made of methyl linoleate, phosphate buffer and antioxidants. The experiment has been carried out at 25°C for 30 min.

The active oxygen method, also known as the Swift test, is a commonly used accelerated method for assessing oxidative stability of fats and oils, which has also been adapted to evaluate antioxidants. This method is based on the principle that oxidation of lipids is accelerated by aeration in a tube held at a constant elevated temperature. The peroxide value reached by the active oxygen method at which a fat will be rancid by

organoleptic evaluation varies with the nature of the fat. Even though this method has been used extensively over the years, its inherent deficiencies are determined by the amount of peroxides in the oxidized oil; peroxides are unstable and decompose readily to more stable secondary products and during the rapid oxidation phase, the reaction is extremely susceptible to variations in the oxygen supply.

Automated versions of the active-oxygen apparatus, known as the Oil Stability Instrument (OSI), rancimat and oxidograph are now available for monitoring the oxidative stability of oils. These methods may be considered as automated active-oxygen methods because they employ the principle of accelerated oxidation. However, the OSI and Rancimat tests measure the changes in conductivity caused by ionic volatile organic acids, mainly formic acids, automatically and continuously, whereas in the active-oxygen method, peroxide values are determined. Rancimat tests proceed slowly at first because during the induction period little acid is released. The end point is selected as the point at which the rapid rise in conductance begins. The Rancimat is capable of running six samples simultaneously, however, OSI is capable of running up to 22 samples at a time (Shahidi and Wanasundara, 1997).

Proton Nuclear magnetic resonance (H^1NMR) spectroscopy has been employed to assess oxidative state of edible oils treated with different antioxidants (Shahidi, 1992a; Wanasundara and Shahidi, 1993). NMR absorption peaks of olefinic and aliphatic protons of triacylglycerols were found to appear at δ 5.1 - 5.6 and δ 0.6 - 2.5, respectively. The number of protons under each peak is estimated by integration. Therefore, it has been

suggested that the NMR technique may offer a useful means for measuring the oxidative deterioration of lipids (Shahidi *et al.*, 1994b). This method may be used to measure the relative changes in the NMR absorption pattern of lipid fatty acids and formation of both primary and secondary oxidation products.

Since hydroperoxides are the primary products of lipid oxidation, their content is often used as an indicator for the initial stages of oxidation. The hydroperoxides content is determined quantitatively by means of an iodometric titration and expressed as peroxide value (PV). However, peroxide value may not necessarily be indicative of the actual extent of lipid oxidation (Sherwin, 1976). Measurement of the conjugated dienes [UV absorption maximum (λ_{max}) at 234 nm] and trienes (λ_{max} at 268 nm) is a simple physical technique which has also been employed to assess the oxidative stability of lipids. Both linoleic acid and α-linolenic acid form conjugated dienes upon oxidation which have absorption maxima at 234 nm, whereas secondary oxidative products, especially diketones, have absorption maxima at 268 nm (Patterson, 1989). The 2-thiobarbituric acid test and headspace volatile analysis have also been employed to monitor oxidative stability of antioxidant-treated fats and oils (Asamarai *et al.*, 1996; Frankel *et al.*, 1994; Kansci *et al.*, 1997; Shahidi *et al.*, 1994b). The 2-thiobarbituric acid test adopted for fats and oils is somewhat different from that used for meat samples whereas the headspace volatile analysis is the same as that used for meat samples. For the determination of TBARS, a known quantity of oil is mixed with TBA reagent and heated in a water bath set at 50°C for 45 min. The absorbance of the pink-coloured TBA-MA adduct is then

measured at 532 nm.

2.6.3.3 Free radical and hydrogen peroxide-scavenging assays

The antioxidant activity of phenolics is attributed to their ability to quench free radicals and peroxides. Therefore, their efficacy can be determined based upon the free radical or hydrogen peroxide-scavenging activities. Generally, free radical methods involve either experimentally generated inorganic free radicals such as superoxide and hydroxyl radicals or stable organic free radicals such as 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$, Brand-Williams *et al.*, 1995; Cotelle *et al.*, 1996; Haseloff *et al.*, 1990; Halliwell *et al.*, 1995; Murase *et al.*, 1998; Rahman and McNee, 1996; Thomas, 1995; Yamauchi *et al.*, 1995; Yuting *et al.*, 1990). $DPPH^{\bullet}$, in methanol has been used to evaluate antioxidants (Brand-Williams *et al.*, 1995; Jumenez *et al.*, 1993; Shimada *et al.*, 1992). The reduction in the intensity of characteristic ink blue colour of $DPPH^{\bullet}$ upon its reaction with a phenolic antioxidant can be monitored spectophotometrically by measuring the absorbance at 515 nm. Disappearance of $DPPH^{\bullet}$ in the presence of an antioxidant may also be monitored using electron paramagnetic resonance (EPR) spectroscopy. Free radicals are unique as they possess an unpaired electron which when placed in an external magnetic field can orient either parallel or antiparallel to that field, and thus can have two possible energy levels. If electromagnetic radiation of the correct energy is applied, it will be absorbed and used to move the electron from the lower energy level to the upper one. Thus, an absorption spectrum is obtained, usually in the microwave region of the

electromagnetic spectrum (Halliwell and Gutteridge, 1985). The intensity of the EPR signal is dependent upon the concentration of the free radical being measured.

Oxygen-derived free radicals such as $O_2^{\bullet-}$, and $^{\bullet}OH$ have also been used to assess antioxidant properties of various compounds. Superoxide free radical ($O_2^{\bullet-}$) in aqueous solution is a reducing agent, i.e. a donor of electrons. For example it reduces cytochrome C, a haem protein. It can reduce the yellow dye, nitro blue tetrazolium to produce an ink blue colour compound known as formazan (Halliwell and Gutteridge, 1985; Rohrdanz and Kahl, 1998). The extent of the dye reduction can be monitored spectrophotometrically (Oberley and Spitz, 1985). Hypoxanthine/xanthine oxidase system is widely used to generate $O_2^{\bullet-}$ (Scherer *et al.*, 1997; Aitken *et al.*, 1996). Antioxidant compounds are added to the medium and the extent of dye reduction is used as a measurement of antioxidant activity of a given compound. For instance, compounds with high antioxidant activity neutralize the $O_2^{\bullet-}$, thus the extent of dye reduction is minimal or none. Both $O_2^{\bullet-}$ and $^{\bullet}OH$ can be detected by EPR spectroscopy. However, these radicals, as such, are extremely short-lived and thus unable to detect by EPR spectroscopy. Trapping of the radicals with a spin trap produces relatively long-lived adducts which are readily detectable in an EPR spectrometer (Ozaki *et al.*, 1988; Zhang *et al.*, 1996). Several nitrone compounds, such as 5,5-dimethyl-1-pyrrole-N-oxide (DMPO), N-*tert*-butyl-α-phenylnitrone, 2-methyl-2-nitrosopropane, nitrozobenzene, α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone, 3,3,5,5-tetramethyl pyrroline-N-oxide, and 2,4,6-tri-*tirt*-butyl-nitrosobenzene, have been used to spin trap free radicals.

2.6.4 Identification of active compounds

Active compounds present in plant extracts have been isolated on silica gel thin-layer chromatographic plates (TLC, Amarowicz *et al.*, 1992). Fractionation of crude plant extracts on a Sephadex LH-20 column prior to TLC makes isolation of active compounds somewhat easier. Solvent systems (mobile phases) employed to develop TLC plates vary widely. Use of a mixture of solvents/weak organic acids occasionally provides a better separation of spots on TLC plates (Amarowicz *et al.*, 1990). Spots on TLC plates are visualized by spraying the plates with a solution of ferric chloride ($FeCl_3$), $KFeCN_6$, vanillin (for condensed tannins) or concentrated sulphuric acid. Spots can also be located by observing the plates under UV light (254 nm and 365 nm). Tentative identification of compounds can be achieved by comparing the R_f values of spots with those of authentic standards.

Different instrumental techniques have been employed to elucidate the exact chemical identity of isolated compounds. Use of instrumental techniques, such as UV-visible, infrared (IR; Pomeranz and Meloan, 1982), nuclear magnetic resonance (NMR; Inatani *et al.*, 1982), and mass (MS; Mizuno *et al.*, 1987) spectroscopies is commonplace.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Evening primrose and borage seeds were obtained from Scotia Pharmaceuticals Ltd., Kentville, NS, and Bioregional Food Co. Ltd., Saskatoon, SK, respectively. Three 1 kg packages of each type of seeds were received and stored at -20°C until used. Three fresh pork shoulders (one day after slaughter) were acquired from a local supermarket. The meat, after removal of any surface fat, was ground twice in a meat grinder (Omega, Type 12, Larry Sommers Ltd., Toronto, ON) using a 0.79 and then a 0.48 cm plate. Ground pork was vacuum packaged in polyethylene pouches and stored in a freezer (Ultra Low, Revco, Inc., West Columbia, SC) at -60°C until used. Bulk corn oil stripped of its natural antioxidants was purchased from Fisher Scientific Co. (Nepean, ON).

Reagents 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, β-carotene, linoleic acid, Tween 40 (polyoxyethylene sorbitan monopalmitate), butylated hydroxyanisole (BHA), α-tocopherol, sodium carbonate, mono- and dibasic sodium phosphate, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), nitro blue tetrazolium (NBT), hypoxanthine, xanthine oxidase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), tetramethylmurexide, hexamine, hexanal, (+)catechin, sinapic acid, gallic acid, syringic acid, vanillin, Folin-Denis reagent, methyl triecosanoate, hydroquinone and Sephadex LH-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Deuterated methanol

(methanol d-4) and acetone (acetone d-6) were obtained from Cambridge Isotope Laboratories (Andover, MA). Hexane, methanol, ethanol, butanol, acetone, chloroform, toluene, hydrogen peroxide, ferrous sulphate, ferric chloride, hydrochloric acid, sulphuric acid and trichloroacetic acid (TCA) were obtained from Fisher Scientific (Nepean, ON). Helium, hydrogen, nitrogen and compressed air were obtained from Canadian Liquid Air Ltd. (St John's, NF).

3.2 Methods

3.2.1 Preparation of borage and evening primrose meals

Seeds were ground in an electric grinder (Black & Decker Canada Inc., Brockville, ON) for 15 min and then defatted by blending ground seeds with hexane (1:5 w/v, 5 min, x3) in a Waring Blendor (Model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted seeds were air dried for 12 h and stored in vacuum packaged polyethylene pouches at -20°C until used.

3.2.2 Assessment of antioxidant activity of borage and evening primrose meals

Meals were examined for their effects on oxidative stability of cooked comminuted lean pork stored at 4°C for 7 days. Oxidative stability of cooked comminuted lean pork was monitored using TBA test and static headspace gas chromatographic analysis. Methods for preparation of meat model systems, TBA test and static headspace analysis are given in details in sections **3.2.8.2**, **3.2.8.2.1** and **3.2.8.2.2**, respectively.

3.2.3 Preparation of the crude extracts

Extraction of phenolic compounds present in the meals was carried out under reflux conditions in a thermostated water bath at varying temperatures for a series of time courses. The antioxidant compounds present in the meals (6 g) were extracted into different aqueous organic solvents (100 mL of 0 -100%, v/v ethanol, methanol or acetone) at different temperatures (25 - 80°C) and time courses (10 -105 min). The resulting slurries were centrifuged for 5 min at 4000xg (ICE Centra M5, International Equipment Co., Needham Heights, MA) and the supernatants were collected. The solvent was removed under vacuum at 40°C and the resulting concentrated solutions were lyophilized for 72 h at -49°C and $62*10^{-3}$ mbar (Freezone 6, Model 77530, Labconco Co., Kansas City, MO). Yields (g/100 g meal) and total phenolics in the extracts as mg catechin (for evening primrose) or sinapic acid (for borage) equivalents/g extract were determined (method for total phenolics determination is given in the section **3.2.4**). Optimum extraction conditions were established using response surface methodology (RSM) as explained in section **3.2.5**.

3.2.4 Determination of the content of total phenolics

Extracts were dissolved in methanol to obtain a concentration of 0.5 mg/mL. Folin-Denis 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 into each tube. Volume was then adjusted to 10 mL by the addition of 8 mL of

deionized water and the contents were mixed vigorously. Tubes were allowed to stand at ambient temperature for 25 min and then centrifuged for 5 min at 4000xg. Absorbance of the supernatants was measured 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 (+)catechin (Figure A.1) or sinapic acid (Figure A.2) (Swain and Hillis, 1959). Total extracted phenolics were expressed as mg (+)catechin (for evening primrose) or *trans*-sinapic acid (for borage) equivalents/g extract.

3.2.5 Response surface methodology (RSM)

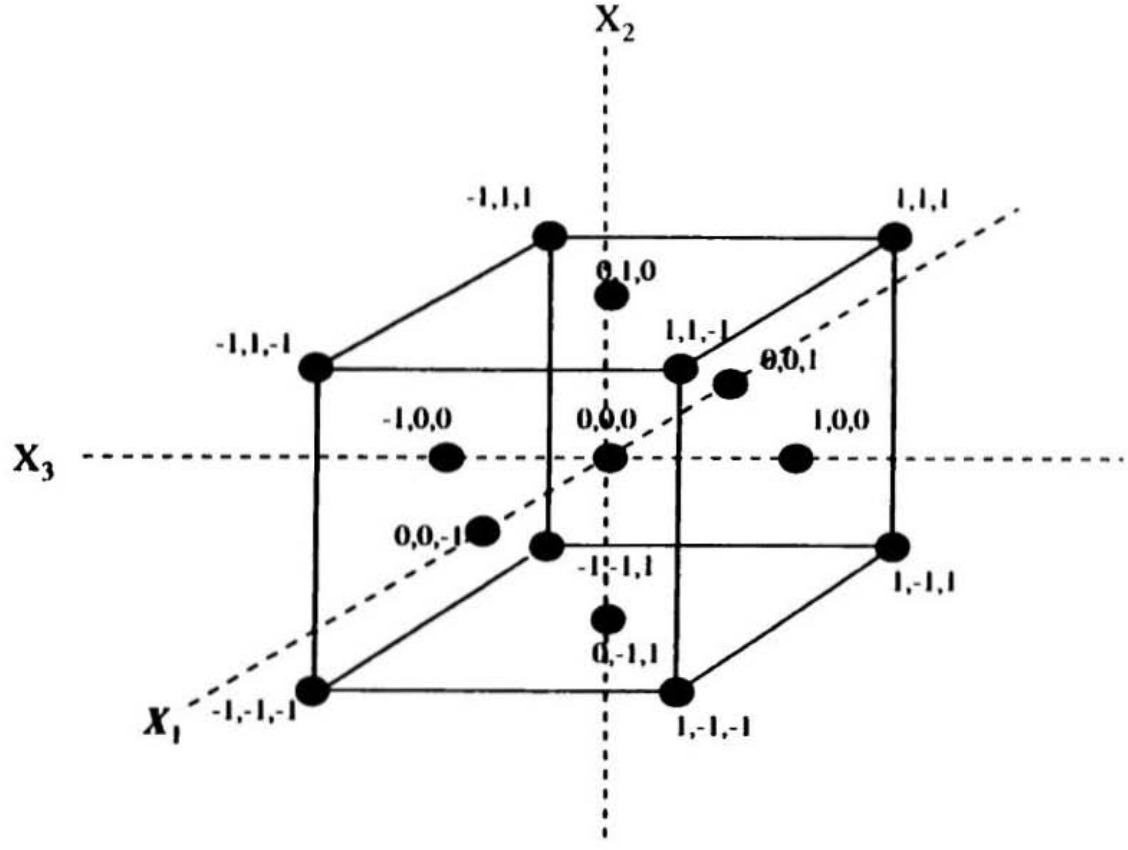
The experimental design adapted for RSM was a three-factor, three-level face-centred cube design with 15 different design points (Table 3.1 and Figure 3.1) (Gao and Mazza, 1996; Mason *et al.*, 1989; Snedecor and Cochran, 1980). Three independent variables or factors studied were organic solvent content in the extraction medium (%, v/v, x_1), extraction temperature ($^{\circ}C$, x_2), and extraction time (min, x_3) (Table 3.1). Response (Y) studied was the antioxidant index calculated with data obtained from a β-carotene-linoleate model system (see section **3.2.8.1** for the method and formula for antioxidant index calculation). The antioxidant index of extracts, prepared at each design point, was recorded. Duplicate extractions were carried out at all design points except for the centre point (0,0,0) where triplicate extractions were carried out.

Table 3.1 Face-centred cube design.

Design point	X_1 (%, v/v)	X_2 (°C)	X_3 (min)
1	(−1)*	(−1)	(−1)
2	(−1)	(−1)	(+1)
3	(−1)	(+1)	(−1)
4	(−1)	(+1)	(+1)
5	(+1)	(−1)	(−1)
6	(+1)	(−1)	(+1)
7	(+1)	(+1)	(−1)
8	(+1)	(+1)	(+1)
9	(−1)	(0)	(0)
10	(+1)	(0)	(0)
11	(0)	(−1)	(0)
12	(0)	(+1)	(0)
13	(0)	(0)	(+1)
14	(0)	(0)	(−1)
15	(0)	(0)	(0)
16	(0)	(0)	(0)
17	(0)	(0)	(0)

*Coded variable levels. Coded value = (Original value − M)/S, where, M and S are the average of the highest and lowest variable levels, and half their difference.

Figure 3.1 Graphical representation of the face-centred cube design.


X2
X3
X1
-1,1,1
1,1,1
0,1,0
-1,1,-1
1,1,-1
0,0,1
-1,0,0
0,0,0
1,0,0
0,0,-1
-1,-1,1
1,-1,1
0,-1,1
-1,-1,-1
1,-1,-1

The generalized second order polynomial model, used in the RSM, was:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i<j=1}^{2}\sum\beta_{ij} x_i x_j$$

where b_0, b_i, b_{ii} and b_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively; x_i and x_j are the independent variables. Antioxidant index data were analyzed using general linear model (GLM) and response surface regression (RSREG) procedures of SAS Institute, Inc. (1990) and the estimated regression coefficients were substituted in the quadratic polynomial equation. Response surfaces and contour plots were obtained using the fitted model. Verification experiments were carried out using combinations of variables at different levels (within the experimental range) to determine the adequacy of the model. For both borage and evening primrose, about 100 g of extracts were prepared at critical extraction conditions predicted by RSM.

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 with 100 mL of deionized water and 100 mL of butanol in a 250 mL separatory funnel. The mixture was allowed to stand at 4°C for 12 h; separated layers were removed and desolventized using a Rotavapor (Buchi, Flawil, Switzerland) set at 40°C. The resulting concentrated solution was lyophilized for 72 h at -49°C and $62*10^{-3}$ mbar. Weight of each fraction was recorded and the content of phenolics determined as explained in section **3.2.4**.

3.2.7 Qualitative detection of vanillin positive compounds in borage and evening primrose crude extracts

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

3.2.8 Evaluation of antioxidant activity of the crude extracts

Four different model systems were used for evaluating antioxidative properties of crude extracts. The concentrations of the extracts tested were 100 and 200 ppm phenolics as sinapic acid and catechin equivalents for evening primrose and borage, respectively. For the convenience of discussion, these concentrations will be referred to, in the text, as 100 and 200 ppm. Following sections will focus on methodologies employed for evaluation of these model systems.

3.2.8.1 β-carotene-linoleate model system

A solution of β-carotene was prepared by dissolving 3.0 mg of β-carotene in 10 mL of chloroform. Two millilitres 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 (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 concentrations of phenolics in the assay media were 100 and 200 ppm. The total volume of the systems was adjusted to 5.0 mL with methanol. BHA, sinapic acid and authentic catechin were used for comparative purposes. As soon as the emulsion was added 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 2-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). Content of β-carotene in assay media was determined using a standard curve (Figure A.3). Antioxidant index (AI) was calculated using the following equation:

AI = (β-carotene content after 2 h of assay/Initial β-carotene content)*100

3.2.8.2 Cooked comminuted pork model system

Ground pork was mixed with 20% by weight of deionized water in Mason jars (height 10 cm, internal diameter 6 cm). Seed meals (1 and 2%, w/w) as well as BHA, α-tocopherol, sinapic acid, catechin and extracts (100 and 200 ppm based on phenolics) were added separately to meat and were thoroughly homogenized. A control sample containing no meal/extract was also prepared. Samples were cooked in a thermostated water bath at 85 ± 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 Blendor

(Model 33BL73) for 30 s, transferred into plastic bags and then stored in a cold room for 7 days at 4 °C (Wettasinghe and Shahidi, 1996).

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

Samples were analyzed for TBARS over a 7-day period according to the method of Siu and Draper (1978). Two grams of each sample were placed in a centrifuge tube to which 5 mL of a 10% (w/v) solution of TCA were added and vortexed (Fisher Vortex Genie 2, Fisher Scientific, Nepean, ON) at high speed for 2 min. Five millilitres of a 0.02 M aqueous solution of 2-thiobarbituric acid was then added to each centrifuge tube which was further vortexed for 30 s. The samples were then centrifuged at 3000xg 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 resulting pigment was read at 532 nm using a Hewlett Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). TBARS values were calculated by multiplying the absorbance readings by a factor of 3.4 which was obtained from a standard line prepared with 1,1,3,3-tetramethoxypropane as a precursor of malonaldehyde (Figure A.4)

3.2.8.2.2 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. Helium was the carrier gas 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 200 °C at 20 °C/min and held there for 5 min. The injector and flame ionization detector (FID) temperatures were adjusted to 280 °C and held at this temperature throughout the analysis (Wettasinghe and Shahidi, 1996).

For headspace (HS) analysis, 4.0 g portions of homogenized pork samples were transferred to 5 mL glass vials. The vials were capped with teflon-lined septa, crimped and then frozen 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 equilibration period. Pressurization time of the vial was 6 s, and the volume of the vapour phase drawn was approximately 1.5 mL. Chromatograph peak areas were expressed as integrator count units. Individual volatile compounds were tentatively identified by comparing relative retention times of GC peaks with those of commercially available standards. Quantitative determination of dominant aldehydes was accomplished using 2-heptanone as an internal standard (Wettasinghe and Shahidi, 1996).

3.2.8.2.3 Determination of moisture content of pork

Approximately 3-4 g of comminuted pork were accurately weighed into a preweighed aluminium dish and placed in a forced-air convection oven (Fisher Isotemp 300, 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 samples before and after drying to that of the original material (AOAC, 1990).

3.2.8.2.4 Determination of crude protein content of pork

Approximately 0.3 - 0.4 g of comminuted pork were accurately weighed on a nitrogen-free paper and placed in a digestion tube of a Buchi 430 digester (Buchi Laboratories, Flawil, Switzerland). The nitrogen content in different samples was determined by digestion in 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 (Buchi 321, Buchi Laboratories, Flawil, Switzerland) into a 50 mL solution of 4% (w/v) boric acid containing a few drops of end point indicator (EM Science, Gibbstown, NJ). Approximately 200 mL of distillate were collected and the content of ammonia in the distillate was determined by titrating it against 0.1N standard solution of sulphuric acid (AOCS, 1990). The crude protein was calculated as N%*6.25.

3.2.8.2.5 Determination of total lipid content of pork

Total lipids were extracted into a mixture of chloroform and methanol as described by Bligh and Dyer (1959). Approximately 25 g of samples 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 homogenizer (Brinkmann Instruments, Rexdale, ON) at a speed of 4. A further extraction was done with the addition of 25 mL of chloroform followed by homogenization. About 25 mL of distilled water was added and the mixture was then filtered through a Buchner funnel using a Whatman No.3 filter paper (Fisher Scientific, Nepean, ON). 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. A 10 mL aliquots of the lipid extract in chloroform, after drying over anhydrous sodium sulphate, was transferred into a tarred 50 mL round bottom flask and the solvent was removed under vacuum using Buchi RE 111 rotovapor (Buchi Laboratories, Flawil, Switzerland). The flask was then placed in a forced-air convection oven (Fisher Isotemp 300, Fairlawn, NJ) at 80°C for 1 h. After cooling in a desiccator, the lipid content was determined gravimetrically.

3.2.8.2.6 Determination of ash content of pork

Approximately 3 - 4 g of pork were weighed into a cleaned porcelain crucible and then charred over a Bunsen burner and subsequently placed in a temperature controlled muffle furnance (blue M Electric Co., Blue Island, IL) which was preheated to 550°C. Samples were

held at this temperature until a gray ash was produced and then cooled in a desiccator and weighed immediately. Ash content was calculated as percent ratio of the mass of the ash, obtained after ignition, to that of the original material (AOAC, 1990).

3.2.8.2.7 Analysis of fatty acid composition of lipids

Fatty acid composition of lipids was determined after their conversion to corresponding methyl esters (FAMEs). About 10 mg of each oil were weighed into a 6 mL well-cleaned Teflon-lined, screw capped conical vials. The internal standard [250 mg of methyl treicosanoate (C 23:0)/100 mL chloroform] was added to the vial and the solvent in the oil-internal standard mixture was evaporated under a stream of nitrogen. Transmethylation reagent (2 mL, freshly prepared 6 mL of concentrated sulphuric acid made up to 100 mL with spectral grade methanol and 15 mg of hydroquinone as an antioxidant) was added to the sample vial and mixed by vortexing. The mixture was incubated overnight at 60°C and subsequently cooled (Keough and Kariel, 1987). Distilled water (1 mL) was added to the mixture and after thorough mixing, extracted three times with 1 - 5 mL of pesticide grade hexane. A few crystals of hydroquinone were added to each vial prior to extraction with hexane. Hexane layers were separated, combined and transferred to a clean tube and then washed two times with 1- 5 mL of distilled water. In the first wash, the aqueous layer was removed and in the second wash, the hexane layer was separated and evaporated under a stream of nitrogen. FAMEs were then dissolved in 1 mL of carbon disulphide and used for gas chromatographic analysis. A Hewlett-Packard 5890 Series II gas chromatograph (Hewlett

Packard, Toronto, ON) equipped with a Supelcowax-10 column (0.25 mm diameter, 30 m length, 0.25 mm film thickness, Supelco Canada Ltd., Oakville, ON) was used for analyzing FAMEs. The oven temperature was initially 220°C for 10.25 min and then ramped to 240°C at 30°C/min and then held for 9 min. The injector and flame ionization detector (FID) temperatures were both at 270°C. Ultra high purity (UHP) helium was used as the carrier gas (15 mL/min). HP 3365 Series II ChemStation software (Hewlett-Packard, Toronto, ON) was used for data handling. The FAMEs were tentatively identified by comparison of their retention times with those of authentic standard mixtures (PUFA 1, Supelco Canada Ltd., Oakville, ON and GLC-416, Nu-Check). The area under each peak was calculated on a weight percentage basis using methyl tricosanoate (C23:0) as an internal standard.

3.2.8.3 Bulk stripped corn oil model system

Extracts or standards (BHA, α-tocopherol, sinapic acid or catechin) and oil (5 g) were mixed well in 70 mL capped glass tubes (19 cm x 2 cm internal diameter) so that the final concentration of phenolics was 100 or 200 ppm. Systems were stored in a forced-air convection oven set at 60 °C for seven days. Samples for conjugated dienes (0.2 g) and headspace (0.2 g) analyses were drawn on days 0, 1, 3, 5 and 7. Method for determining volatiles was the same as for the meat model systems (see section **3.2.8.2.2** for detailed methodology), but only 200 mg of oil was used.

3.2.8.3.1 Determination of conjugated dienes (CD)

Oil (0.02 - 0.04 g) was weighed into a 25 mL volumetric flask, dissolved in isooctane and made up to the mark with the same solvent. The solution was thoroughly mixed and the absorbance was read at 234 nm using a Hewlett-Packard 8452 A diode array spectrophotometer. Pure isooctane was used as the reagent blank. CD value was calculated using the following equation:

CD value = Absorbance at 234 nm /(concentration of oil in g/100 mL*length of the cell in cm)

3.2.8.3.2 Determination of fatty acid composition of stripped-corn oil

Fatty acid composition of stripped corn oil was determined using the method given in section **3.2.8.2.7**.

3.2.8.4 Stripped corn oil-in-water emulsion system

Corn oil (10%, w/w), deionized water (88%, w/w) and Tween 40 emulsifier (2%, w/w) were mixed in a beaker and sonicated for 30 min. The resulting emulsion (20 mL) was transferred into 70 mL capped glass tubes (19 cm x 2 cm internal diameter) containing extract or standards (BHA, α-tocopherol, sinapic acid or catechin) to produce a concentration of 100 or 200 ppm of phenolics in the final emulsion. Emulsions were stored in a forced-air convection oven at 60 °C. Aliquots for conjugated dienes (2 mL) and headspace (2 mL) analyses were drawn on days 0, 1, 3, 5 and 7. Oil was extracted with hexane and desolventised

under nitrogen. The weight of the retrieved oil was recorded. Methods for headspace volatiles and conjugated diene analyses are given in sections **3.2.8.2.2** and **3.2.8.3.1**, respectively.

3.2.9 Evaluation of Iron (II) chelating activities of borage and evening primrose crude extracts

Ferrous sulphate (400 μM), extracts, standards (sinapic acid and catechin) and tetramethylmurexide (1 mM) were respectively dissolved in a 10 mM hexamine-HCl buffer containing 10 mM KCl (pH 5.0). Solutions of ferrous sulphate (1 mL) and extracts (1 mL) were mixed followed by the addition of 0.1 mL of a 1 mM solution of tetramethylmurexide. The final concentration of extracts and standards was 100 or 200 ppm (based on phenolics). Absorbance of the reaction mixtures was recorded at 460 nm and 530 nm and the ratio of A_{460} to $A_{530\ nm}$ calculated. A standard curve (Figure A.5) was used to determine the free iron (II) left after chelation by the extracts (Terasawa *et al.*, 1991). Iron (II) chelation capacities of additives were calculated using the following equation:

Iron (II) chelation capacity, % = 100 - (Absorbance ratio for medium containing the additive of concern/absorbance ratio for the control)*100

3.2.10. Evaluation of reactive-oxygen species (ROS) and DPPH free radical-scavenging efficacies of borage and evening primrose crude extracts

3.2.10.1 Hydrogen peroxide-scavenging assay

Borage and evening primrose crude extracts were dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 mL of 43 mM solution of hydrogen peroxide (prepared in the same buffer). Authentic sinapic acid and (+)catechin were used as the reference antioxidants. Final concentration of extracts and standards was 100 or 200 ppm. The absorbance value (at 230 nm) of the reaction mixtures were recorded at 0 min and then at every 10 min up to 40 min. For each concentration, a separate blank sample (devoid of hydrogen peroxide) was used for background subtraction (Ruch et al., 1989). The concentration (mM) of hydrogen peroxide in the assay medium was determined using a standard curve (Figure A.6) and hydrogen peroxide-scavenging capacities of additives 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

3.2.10.2 Hydroxyl radical-scavenging assay

The hydroxyl radicals were generated *via* iron-catalyzed Haber-Weiss reaction (Fenton driven 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). Borage and evening primrose crude extracts as well as authentic sinapic acid and catechin were dissolved in 0.1 M phosphate buffer (pH 7.4) so that a 200 mL aliquot will result in 200 ppm of phenolics in the final assay medium (final volume was 800 mL). For 100 ppm concentration, 100 μL of the same extract stock solution was used, but the volume was adjusted to 200 μL by adding 100 μL of the buffer. Extracts (200 μL) were mixed with 200 μL of 100 μM DMPO, 200 μL of 10 μM $FeSO_4$ and 200 mL of 10 μM hydrogen peroxide. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). After 3 min, 10 μL of the mixture were drawn into a syringe and transferred into a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at 2×10^5 receiver gain, 1.0 G modulation amplitude, 200 s scan time, 3460 G center field, 100 G sweep width and 0.5 s time constant (Shi et al., 1991). Hydroxyl radical-scavenging capacities of the additives were calculated using the following equation:

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

3.2.10.3 Superoxide radical-scavenging assay

3.2.10.3 Superoxide radical-scavenging assay

A modified version of the method described by Nishikimi et al. (1972) was employed. Superoxide radicals were generated with an enzymatic reaction. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of xanthine oxidase (100 mIU), 1 mL of 12 mM diethylenetriaminepentaacetic acid, 1mL of 178 mM nitro blue tetrazolium and 1 mL of the extracts (final concentration of the phenolics in the reaction mixture was 200 ppm). For 100 ppm concentration, 0.5 mL of the stock extract solution was diluted with 0.5 mL of the buffer. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). Authentic sinapic acid and catechin (100 and 200 ppm) were used as the reference antioxidants. The absorbance values (at 560 nm) of systems were recorded at 0 min and then after every 10 min up to 60 min. For each system, the absorbance values were corrected by subtracting 0 min readings from subsequent readings. 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

3.2.10.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

One hundred microlitres of a 30 μM solution of DPPH in toluene were added to 100 μL of a solution containing borage or evening primrose crude extracts in toluene so that the concentration of phenolics in the final assay media was either 100 or 200 ppm. Contents were

mixed and transferred into an EPR cell. After 60 s, 10 μL of the mixture were drawn into a syringe and transferred into a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at 2 x 10^5 receiver gain, 1.0 G modulation amplitude, 200 s scan time, 3460 G center field, 100 G sweep width and 0.5 s time constant (Santiago *et al.*, 1992). Authentic sinapic acid and catechin (100 and 200 ppm) were used as reference antioxidants. DPPH radical-scavenging capacities of the additives were calculated using the following equation:

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

3.2.11 Column chromatographic fractionation of crude extracts

A 1 g portion of crude extracts was dissolved in 10 mL of HPLC grade methanol and applied to a column (1.5 cm diameter and 77 cm height) filled with Sephadex LH-20 (particle size 25 - 100 mm, Sigma Chemical Co., Nepean, ON) and eluted with methanol. Methanolic fractions (8 mL each) were collected in test tubes placed in a LKB Bromma 2112 redirac fraction collector (Pharmacia, Uppsala, Sweden) and their absorbance was measured at 280 nm. Eluates were then pooled into fractions I - VI. Solvent was evaporated under vacuum at 40°C. Dried fractions were stored in tinted glass bottles at -18°C until used. The total phenolics of each fraction were determined as explained in section **3.2.4**. The content of hydrophilic and hydrophobic phenolics of each fraction was also determined according to the method given in section **3.2.6**. Results were tabulated along with those for crude extracts.

Presence or absence of vanillin-positive compounds in evening primrose fractions was determined as explained in section **3.2.7**.

3.2.11.1 Evaluation of antioxidant activity of column chromatographic fractions

All fractions were investigated for their antioxidative activities in a β-carotene-linoleate, cooked comminuted pork, bulk stripped-corn oil and stripped corn oil-in-water emulsions as described in sections **3.2.8.1**, **3.2.8.2**, **3.2.8.3** and **3.2.8.4**, respectively. Results were tabulated along with those for crude extracts.

3.2.11.2 Evaluation of iron (II) chelating activities and reactive-oxygen species as well as DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging activities of fractions

Iron (II) chelating activities of borage and evening primrose fractions were determined as explained in section **3.2.9**. Hydrogen peroxide-, hydroxyl radical-, superoxide radical- and DPPH-scavenging activity of fractions were evaluated according to the methods given in sections **3.2.10.1**, **3.2.10.2**, **3.2.10.3** and **3.2.10.4**, respectively. Results were tabulated along with those for crude extracts.

3.2.12 Thin-layer chromatography (TLC)

Column chromatographic fraction (I - VI) were loaded onto analytical TLC plates (Silica gel, 60 A mean pore diameter, 2 - 25 mm mean particle size, 250 mm thickness, Sigma

Chemical Co., St. Louis, MO). Chromatograms were developed in a glass chamber (22 cm x 22 cm x 10 cm, Fisher Scientific Ltd., Nepean, ON) using chloroform-methanol-water (65:35:10, v/v/v) as the mobile phase (Amarowicz *et al.*, 1992). After drying, bands were located by viewing under short (254 nm) and long (365 nm) UV radiation (Spectraline, Model ENF-240C, Spectronics Co., Westburg, NY). The following sprays were used to locate phenolic compounds (spray A) and to examine their antioxidant properties (spray B):

Spray A. Ferric chloride-potassium ferricyanide [$FeCl_3$–$K_2Fe(CN)_6$]

Equal volumes of 1% (w/v) aqueous solutions of each salt were freshly mixed (producing an orange-brown reagent). Phonolics produce blue colour with this reagent (Barton *et al.*, 1952).

Spray B. β-carotene-linoleate spray

The relative antioxidant activity of band components on the developed TLC plates was determined using the β-carotene-linoleate spray method (Pratt and Miller, 1984). β-carotene (9 mg) was dissolved in 30 mL of chloroform. Two drops of linoleic acid and 60 mL of ethanol were added to the β-carotene-chloroform solution. The plates were sprayed with this solution followed by their exposure to fluorescent light for 3 h or until the background orange colour disappeared. Bands with persisting orange colour were considered as possessing antioxidant activity. The colour intensity, as judged by the naked eye, was related to their antioxidant strength.

The fractions containing compounds with high antioxidant activity (as determined by β-carotene-linoleate spray) were scraped and extracted into spectral grade methanol. The slurry was centrifuged (3 min at 5000xg) and the supernatant was evaporated to dryness under a stream of nitrogen. The dried residues of active components were used for further analysis.

3.2.13 High performance liquid chromatographic (HPLC) analysis of active compounds

A Shimadzu HPLC chromatograph (Kyoto, Japan) equipped with LC-6A pump, SPD-6A V UV-VIS spectrophotometric detector, SCL-6B system controller and CR 501 Chromatopac was used for analytical and preparative HPLC of isolated compounds. Conditions for preparative HPLC were: Hilber pre-packed column RT (10 x 250 mm) with Lichrosorb RP-18 (7 μm, Merck, Darmstad, Germany); water-acetonitrile-methanol-acetic acid (79.5:18:2:0.5, v/v/v/v) mobile phase; 3 mL/min flow rate; 500 μL injection volume. Pure compounds so obtained were also examined by HPLC separation at ambient temperatures of 20 - 22°C on an analytical column. Analytical CWSL column (4.5 x 250 mm) with Spherisorb-ODS-2 (10 μm, Chromatography Sciences Co. Inc., Montreal, PQ) was used for this purpose. Flow rate was 0.8 mL/min, injection volume was 20 μL. For both preparative and analytical HPLC, the detector was preset at 280 nm (Amarowicz and Shahidi, 1996).

3.2.14 Ultraviolet (UV) spectroscopy of purified compounds

UV absorption spectra (200 - 400 nm) of purified compounds (in methanol) were recorded using a Hewlett-Packard 8452A (Hewlett Packard Co., Mississauga, ON).

3.2.15 Mass spectroscopy of purified compounds

All mass spectra of purified compounds (in methanol) were recorded using an electron ionization (EI) mode at 70 eV in a 7070 HS Micromass double focusing mass spectrometer (V.G. Micromass Ltd., UK). The source, probe and scanning temperatures, used in this study, were 200, 100 -300, and 20 - 25°C, respectively.

3.2.16 Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded using a General Electric 300-NB spectrometer. ^{1}H (at 300 MHz), (^{1}H) correlation spectroscopy (COSY, at 300 MHz) and ^{13}C (at 75.5 MHz) NMR data were collected at room temperature in methanol d-4 (CD_3OD) or acetone d-6 (CD_3COCD_3). Chemical shifts (δ, ppm) were reported relative to tetramethylsilane (TMS) internal standard. Data were analyzed using NUTS software (NMR Data Processing Program, Acron NMR Inc., Fremont, CA).

3.2.17 Tukey's studentized range test

All experiments, except RSM, used completely randomized block designs (CRD) and analyses were carried out in triplicate. RSM used a 3-factor, 3-level, face-centred cube design with triplicate determinations at the center point. The significance of differences among mean values was determined at $p \leq 0.05$ using analysis of variance (ANOVA) followed by Tukey's multiple range test (Snedecor and Cochran, 1980).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Antioxidant activity of meals of borage and evening primrose

Cooked comminuted pork model systems were used for evaluation of antioxidant activity of meals of borage and evening primrose. The moisture, crude protein, crude lipid and ash contents of the pork used in this study, were 71.0, 20.5, 7.4 and 1.1% (w/w), respectively. Oxidative stability of samples, which were stored at 4°C for 7 days, was monitored using 2-thiobarbaturic acid (TBA) test and static headspace gas chromatographic analysis. The TBA test measured carbonyl compounds, including malonaldehyde, which formed during oxidation of lipids in pork (Siu and Draper, 1978). Static headspace gas chromatographic analysis measured volatile aldehydes and ketones which were formed in detectable quantities during the oxidation process (Frankel, 1993a,b). The major aldehyde formed in lipids of land animal and plants is hexanal (Frankel, 1993a,b) while that for marine lipids is propanal (He and Shahidi, 1997). Figures 4.1and 4.2 show the dominance of hexanal among the series of volatiles formed in cooked comminuted pork. Fatty acid analysis of lard, extracted from pork samples, showed the presence of a substantial amount of omega-6 fatty acids such as 18:2 (Table 4.1) which could degrade to hexanal upon oxidative cleavage. The content of ω-3 fatty acids, which give rise to propanal, was lower in lard, thus the concentration of propanal in the oxidized samples was low. A close scrutiny of the existing literature showed that there

Figure 4.1 Day-3 gas chromatograms of cooked comminuted pork treated with borage meal. (A) Control, (B) 1%, w/w meal, (C) 2%, w/w meal.

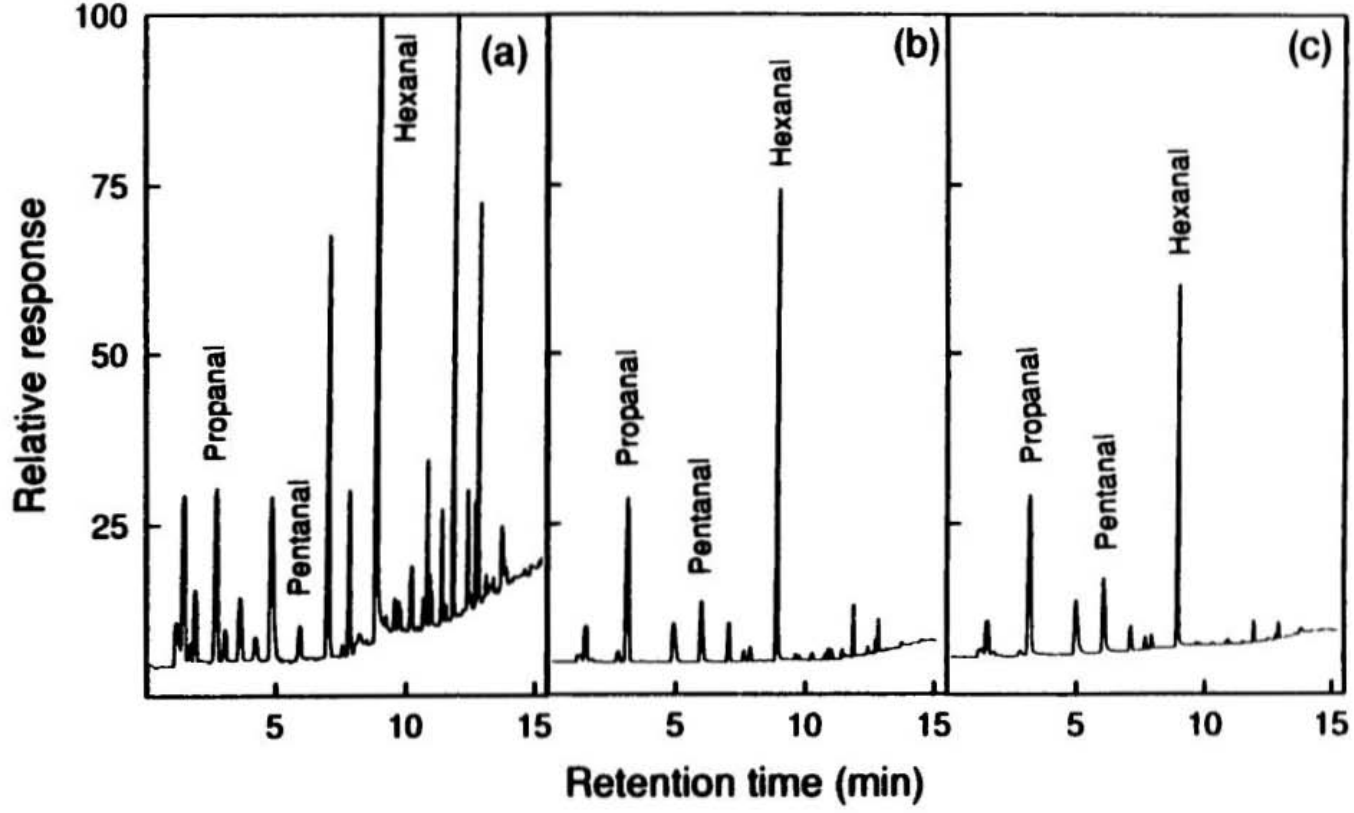

Relative response
100
75
50
25
(a)
(b)
(c)
Propanal
Pentanal
Hexanal
5
10
15
Retention time (min)

Figure 4.2 Day-3 gas chromatograms of cooked comminuted pork treated with evening primrose meal. (A) Control, (B) 1%, w/w meal, (C) 2%, w/w meal.

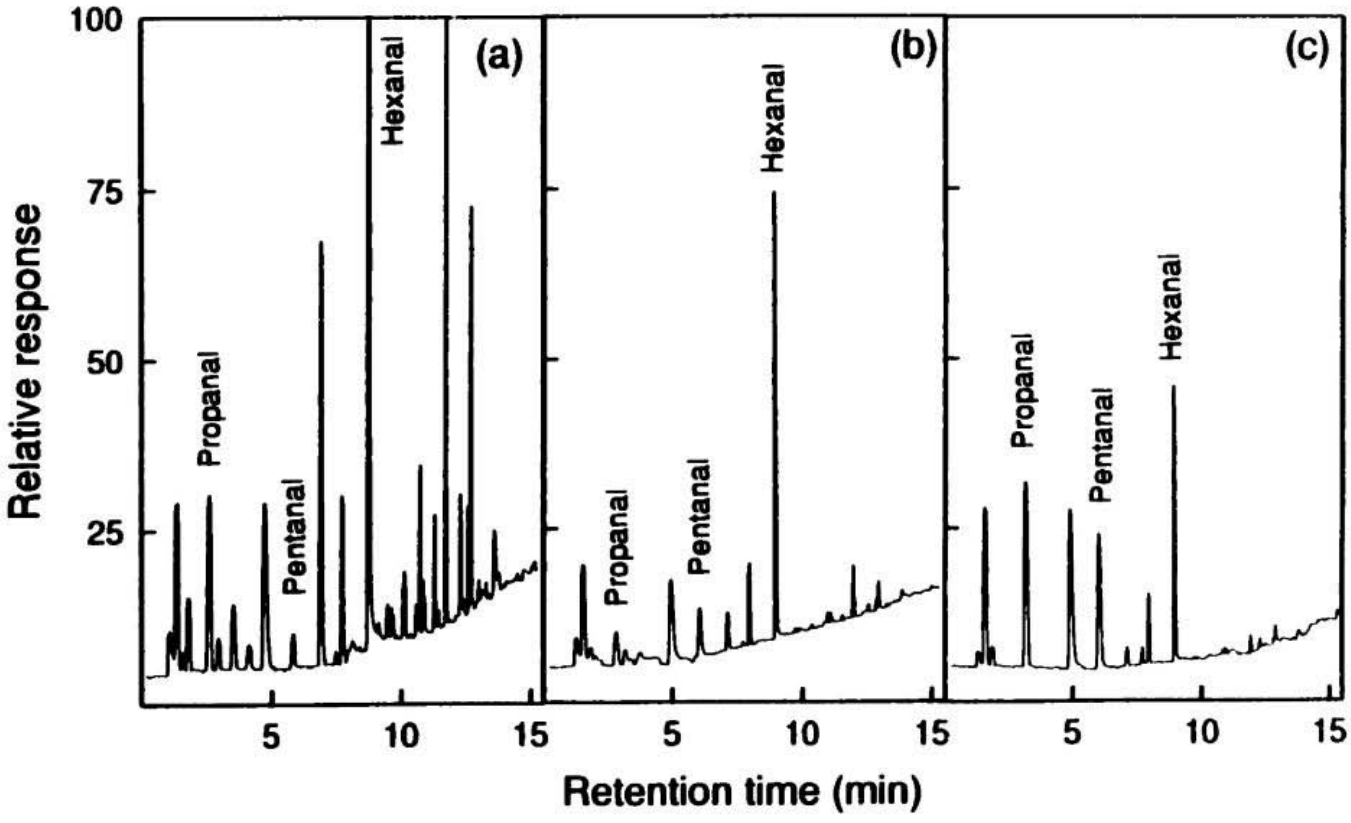

100
75
50
25
Relative response
(a)
(b)
(c)
Propanal
Pentanal
Hexanal
5
10
15
Retention time (min)

Table 4.1 Fatty acid composition of lard and stripped corn oil[1].

Fatty acid	Retention time (min)	GC peak area (%)	
		Lard	Stripped corn oil
10:0	1.810	0.0486	ND
12:0	2.070	0.0531	ND
14:0	2.543	1.0391	ND
14:1	2.691	0.0405	ND
15:0	2.908	0.0537	ND
16:0	3.408	19.410	8.4520
16:1	3.593	2.3544	0.1291
17:0	4.057	0.2742	0.0644
17:1	4.284	0.3407	0.3168
18:0	4.953	10.145	1.6738
18:1	5.249	37.575	24.528
18:2	5.782	10.238	46.411
18:3	6.670	0.6146	0.7522
20:0	7.691	0.1909	0.4186
20:1	8.134	0.9031	0.3145

....continued on next page.

Table 4.1 ....continued.

20:2	9.146	0.4532	ND
20:3	9.806	0.0830	ND
20:4	10.677	0.0889	ND
22:0	11.727	ND	0.1272
22:4	14.317	0.0847	ND
24:0	16.112	ND	0.1634
Unknown	--	15.747	16.648

ND = not detected

is moderate to strong correlations between TBARS (thiobarbituric acid reactive substances) values and hexanal contents of various types of oxidizing samples such as meat, bulk oil and emulsions (St. Angelo *et al*, 1988, 1987). Figures 4.3 and 4.4 depict the effect of addition of 1 and 2% (w/w) meals of borage and evening primrose on the formation of TBARS and volatiles, respectively. For both types of meals, TBARS (Figures 4.3A and 4.4A), hexanal (Figures 4.3B and 4.4B) and total volatiles (4.3C and 4.4C) increased over a seven-day storage period. TBARS values of meat treated with borage meal at 1% (w/w) were not significantly different ($p > 0.05$) from those for the control. Significantly lower ($p \leq 0.05$) TBARS values were evident for samples treated with borage meal at 2% (w/w). For samples treated with evening primrose meal at both levels, TBARS formation was significantly lower than that in the control sample throughout the entire storage period. The differences in TBARS values of samples containing 1 and 2% (w/w) evening primrose meal were also significant ($p \leq 0.05$). Lower TBARS values were evident for samples containing 2% (w/w) evening primrose meal, possibly due to a concentration effect. Hexanal contents of samples treated with borage meal at 1% (w/w) were not significantly different ($p > 0.05$) from those for the control. However, lower hexanal contents were evident for samples containing 2% (w/w) borage meal. Evening primrose meal at 1 and 2% (w/w) addition levels, exerted an inhibitory effect on hexanal formation in treated samples as shown by their lower hexanal contents as compared to those for the control. The inhibition of hexanal formation was more pronounced in samples treated with evening primrose meal at 2 % (w/w) than those

Figure 4.3 Effect of borage meal on TBARS (A), hexanal (B) and total volatiles (C) formation in a cooked comminuted pork model system. Each bar represents mean ± standard deviation of triplicate analyses. In each pannel, bars sharing the same letter in a group of bars are not significantly different ($P > 0.05$) from one another.

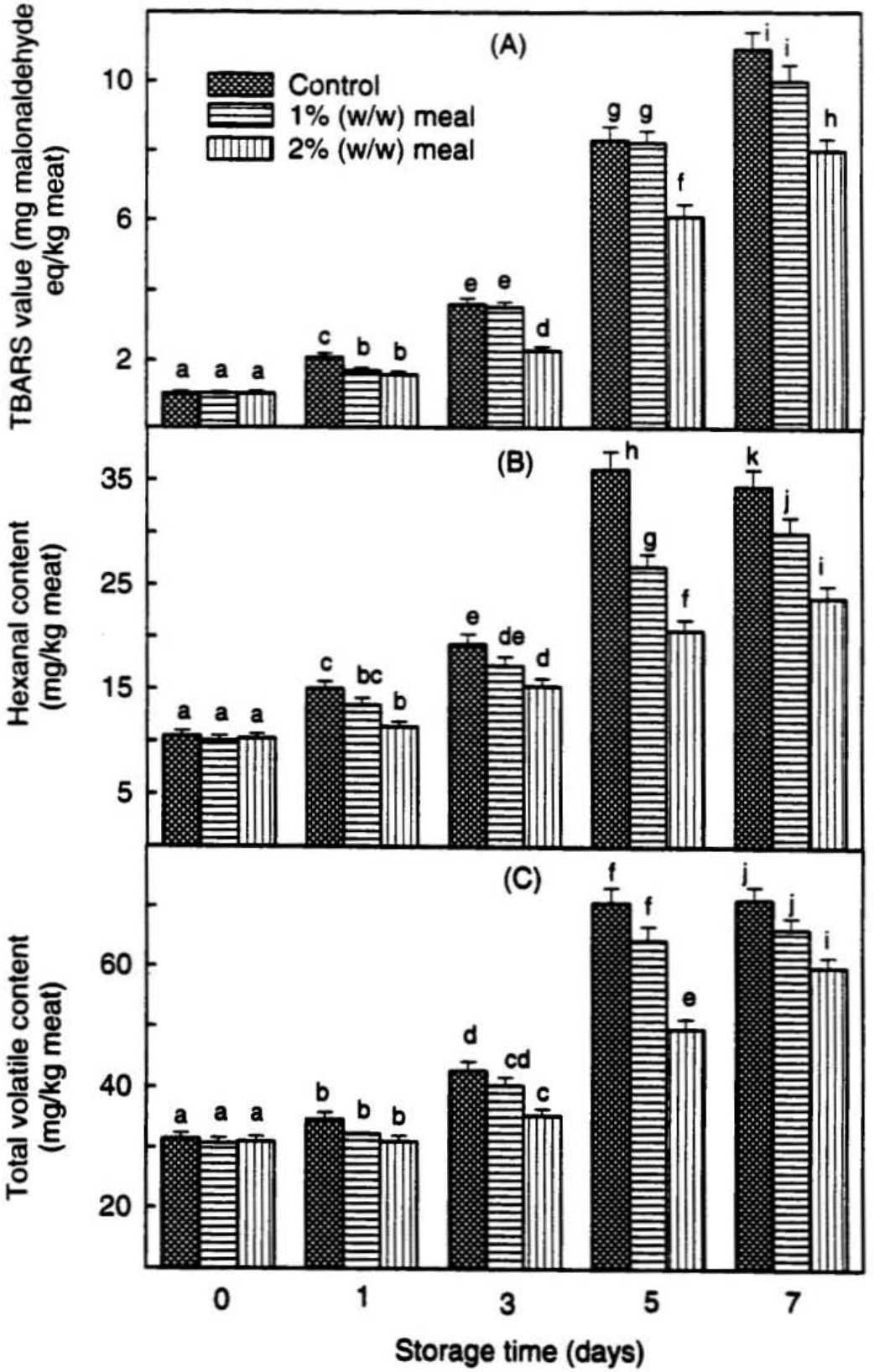
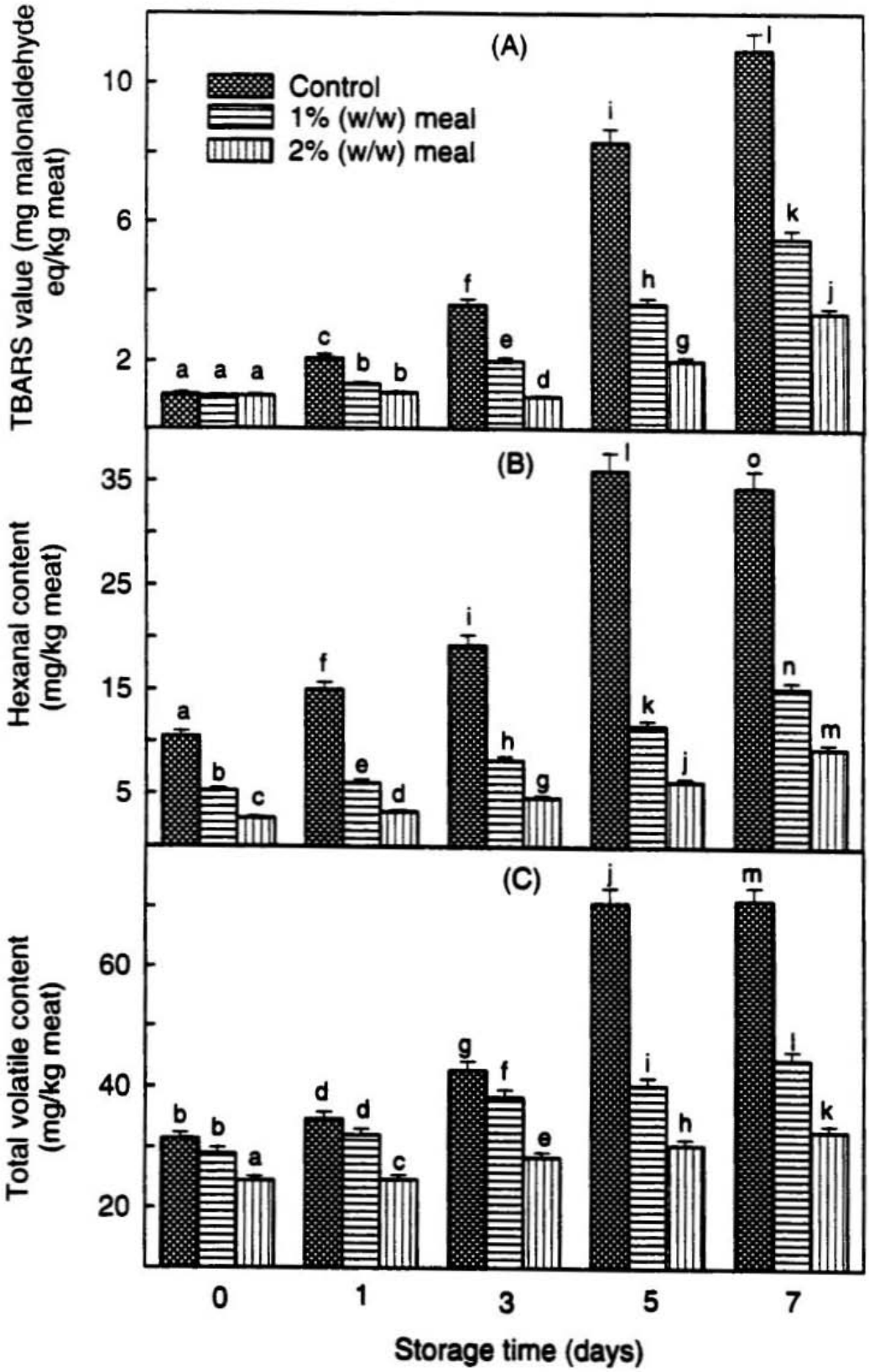

(A)
Control
1% (w/w) meal
2% (w/w) meal
TBARS value (mg malonaldehyde eq/kg meat)
10
6
2
a a a
c b b
e e d
g g f
i i h
(B)
Hexanal content (mg/kg meat)
35
25
15
5
a a a
c bc b
e de d
h g f
k j i
(C)
Total volatile content (mg/kg meat)
60
40
20
a a a
b b b
d cd c
f f e
j j i
0
1
3
5
7
Storage time (days)

Figure 4.4 Effect of evening primrose meal on TBARS (A), hexanal (B) and total volatiles (C) formation in a cooked comminuted pork model system. Each bar represents mean ± standard deviation of triplicate analyses. In each pannel, bars sharing the same letter in a group of bars are not significantly different ($P > 0.05$) from one another.


(A)
Control
1% (w/w) meal
2% (w/w) meal
TBARS value (mg malonaldehyde eq/kg meat)
10
6
2
a a a
c b b
f e d
i h g
l k j
(B)
Hexanal content (mg/kg meat)
35
25
15
5
a b c
f e d
i h g
l k j
o n m
(C)
Total volatile content (mg/kg meat)
60
40
20
b b a
d d c
g f e
j i h
m l k
0
1
3
5
7
Storage time (days)

treated with 1% (w/w). Borage meal at 1% (w/w) had no significant effect ($p > 0.05$) on total volatiles formation, but at 2% (w/w) it exhibited an inhibitory effect on total volatiles formation. Total volatiles formation in samples treated with evening primrose meal at 1 and 2% (w/w) were significantly lower ($p \leq 0.05$) after day three of the storage and the inhibitory effect was concentration-dependent as evidenced by lower total volatiles formation in samples containing 2% (w/w) evening primrose meal. Results for meat model systems suggested that meals of borage and evening primrose have the ability to improve oxidative stability of cooked comminuted pork due to inhibition of lipid oxidation by these additives.

Since the meals of both borage and evening primrose demonstrated antioxidant effects, preparation of extracts was carried out. One of the major obstacles one would encounter in the preparation of such extracts is the selection of appropriate extraction medium, temperature and time course. Response surface methodology (RSM), a technique to locate optimal process conditions, has been widely used in many areas of research (Mason *et al.*, 1989). A survey of recent literature showed the adaptation of this technique in the field of food research (Gao and Mazza, 1996; Wanasundara and Shahidi, 1996). RSM in conjunction with an appropriate experimental design could provide optimum process conditions that would yield a product which could deliver the highest performance. Therefore, RSM was adapted to optimize the process parameters for extraction of antioxidants from borage and evening primrose meals.

4.2 Locating an appropriate experimental region for RSM

As mentioned in the previous section, the most important parameters that affect the antioxidant activity of final extracts are the composition of the extraction medium, temperature and the time course. Three organic solvents, namely methanol (CH_3OH), ethanol (CH_3CH_2OH) and acetone (CH_3COCH_3), were selected for priliminary experiments involving the selection of an appropriate extraction medium. For each of the three organic solvents selected, a series of aqueous extraction media consisting of 0-100% (v/v) organic solvent was prepared. To determine the effect of extraction media on the antioxidant activity of extracts, extractions were carried out in varying compositions of the extraction media while holding the temperature and time at 80°C and 60 min, respectively. The effect of various extraction media on the antioxidant activity of extracts of borage meal in a β-carotene-linoleate model system is depicted in Figure 4.5A (for methanol), B (for ethanol) and C (for acetone) while those for extracts of evening primrose are presented in Figure 4.6A, B and C. The antioxidant activity of extracts first increased as the proportion of organic solvent in the extraction media increased, reached a maximum and then started to decrease. This trend was common for all three organic solvents employed. This suggested that the extraction of antioxidant components of meals of borage and evening primrose was dependent upon the proportion of organic solvent in the extraction medium. Furthermore, linear and quadratic regression analyses showed that the antioxidant activity of the prepared extracts behaved more as a quadratic than a linear function of the proportion of organic solvent in the extraction media. Correlation

Figure 4.5 Effect of varying extraction conditions on the antioxidant activity of crude extracts of borage meal in a β-carotene-linoleate model system. Each data point represents the average of two determinations. LM and QM denote linear and quadratic models, respectively.

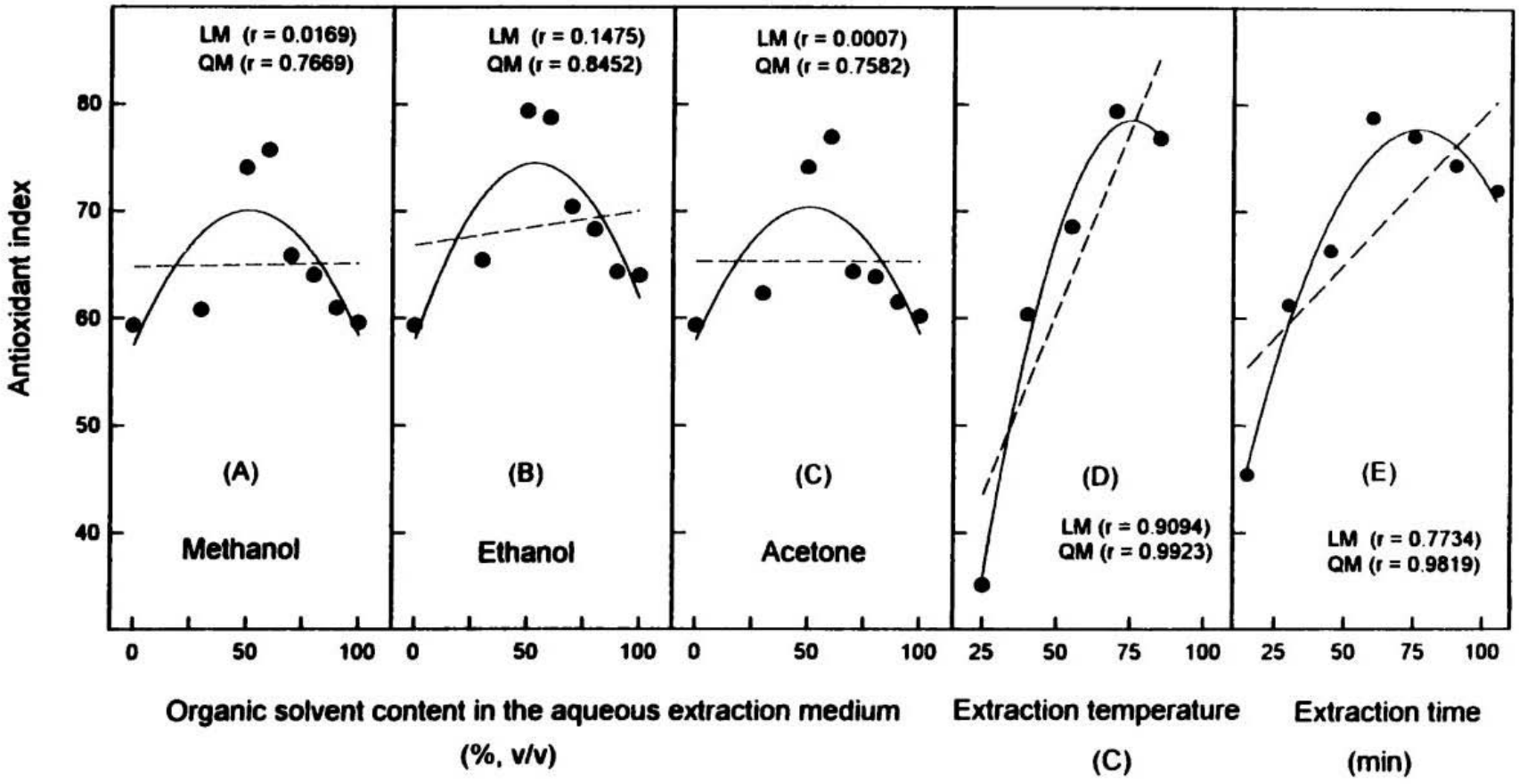
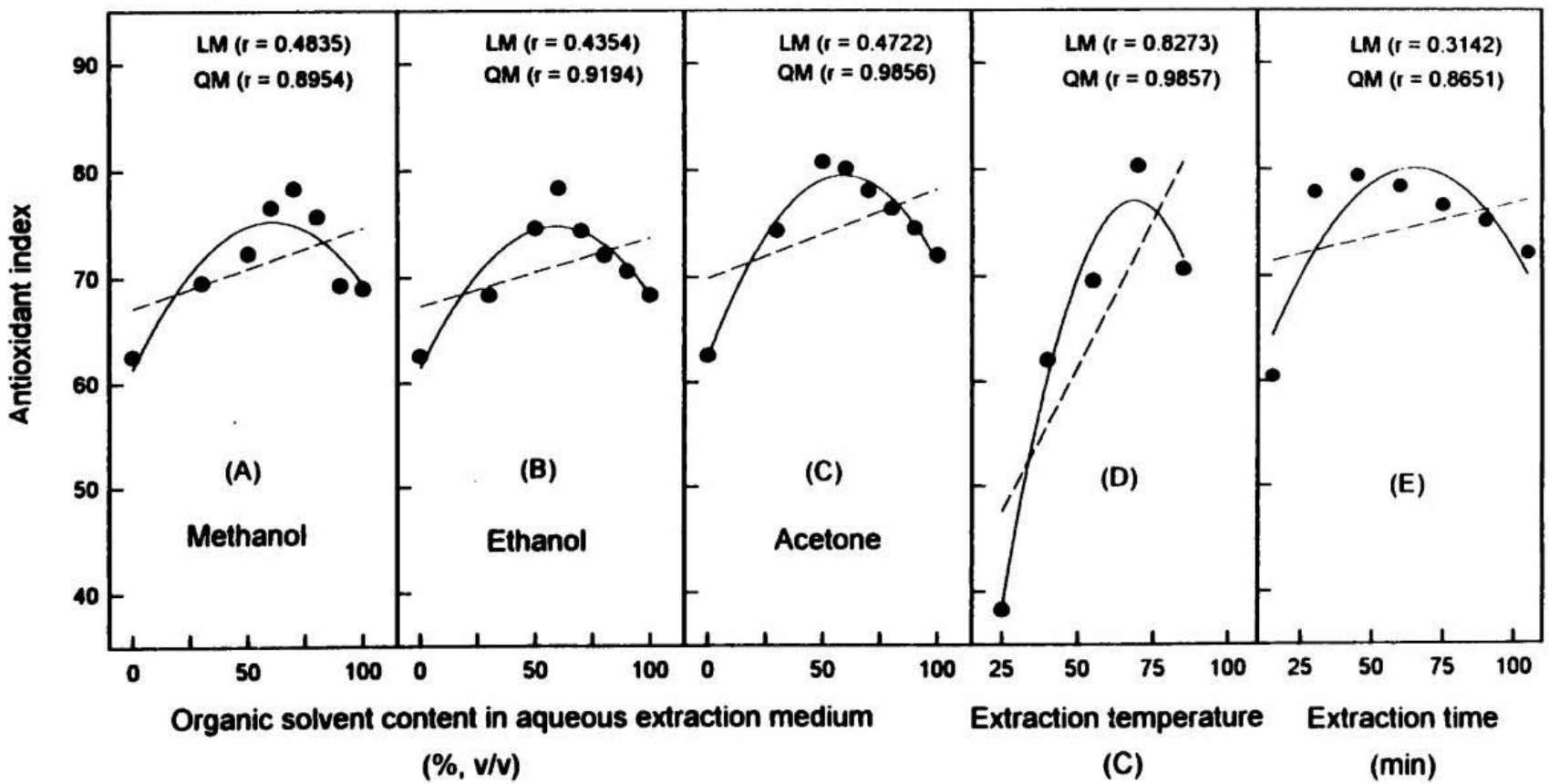

Antioxidant index
LM (r = 0.0169)
QM (r = 0.7669)
(A)
Methanol
LM (r = 0.1475)
QM (r = 0.8452)
(B)
Ethanol
LM (r = 0.0007)
QM (r = 0.7582)
(C)
Acetone
LM (r = 0.9094)
QM (r = 0.9923)
(D)
LM (r = 0.7734)
QM (r = 0.9819)
(E)
80
70
60
50
40
0
50
100
25
50
75
100
Organic solvent content in the aqueous extraction medium
(%, v/v)
Extraction temperature
(C)
Extraction time
(min)

Figure 4.6 Effect of varying extraction conditions on the antioxidant activity of crude extracts of evening primrose meal in a β-carotene-linoleate model system. Each data point represents the average of two determinations. LM and QM denote linear and quadratic models, respectively.


Antioxidant index
90
80
70
60
50
40
LM (r = 0.4835)
QM (r = 0.8954)
(A)
Methanol
LM (r = 0.4354)
QM (r = 0.9194)
(B)
Ethanol
LM (r = 0.4722)
QM (r = 0.9856)
(C)
Acetone
LM (r = 0.8273)
QM (r = 0.9857)
(D)
LM (r = 0.3142)
QM (r = 0.8651)
(E)
0
50
100
25
50
75
100
Organic solvent content in aqueous extraction medium
(%, v/v)
Extraction temperature
(C)
Extraction time
(min)

coefficients (r) for linear regression models varied from 0.0007 to 0.4835, depending upon the type of organic solvent used, whereas those for quadratic models ranged from 0.7582 to 0.9856 (Figures 4.5A, B, C and 4.6A, B, C). In general, aqueous ethanol yielded extracts with slightly higher antioxidant activity from borage meal whereas aqueous acetone produced better extracts from evening primrose meal. Based upon these observations, three solvent concentrations selected for RSM were 30, 50 and 70% (v/v) ethanol for borage meal and 50, 70, and 90% (v/v) acetone for evening primrose meal.

The second factor investigated in the preliminary study was the extraction temperature. For these experiments, extractions were carried out in 50% (v/v) ethanol for borage and 70% (v/v) for evening primrose for a 60 min total extraction time while changing the extraction temperature. Figures 4.5D and 4.6D depict the results for borage and evening primrose, respectively. For both cases, antioxidant activity of extracts increased with increasing temperature, reached a maximum and then decreased. This trend suggested that the high temperatures were able to mobilize certain antioxidants while promoting the concurrent decomposition of the antioxidants which were quickly mobilized at lower temperatures. However, the rate of extraction of thermally stable antioxidants at elevated temperatures was higher than the rate of decomposition of less heat resistant antioxidants as evidenced by relatively high antioxidant activities of extracts prepared at higher temperatures. Antioxidant activities of both borage and evening primrose extracts did not show adequate linear relationships with the extraction temperatures at which they were prepared and this was confirmed by low correlation

coefficients (r = 0.9094 and 0.8273 for borage and evening primrose, respectively). High correlation coefficients (r = 0.9923 and 0.9857 for borage and evening primrose, respectively) were evident for quadratic regression analyses which clearly demonstrated that the relationships were more of a quadratic nature than a linear one. Based upon the results, the three temperatures chosen for RSM of both borage and evening primrose were 40, 60 and 80°C.

The last factor investigated was the time course of the extraction. For this set of experiments, temperature was held at 70°C while the extraction media for borage and evening primrose were 50% (v/v) ethanol and 70% (v/v) acetone, respectively. The effects of varying time courses on antioxidant activity of extracts are shown in Figures 4.5E and 4.6E. Relationships between antioxidant activities of extracts of borage and evening primrose meals followed trends which were similar to those for the other two factors investigated. Short extraction times yielded extracts with lower antioxidant activity whereas prolonged extraction times also had a negative effect on the antioxidant activity of extracts. Low antioxidant activities of extracts prepared by employing shorter time courses may be attributed to inadequate extraction of active compounds. On the other hand, prolonged extraction times certainly increased the chance of exposure of antioxidants to prolonged heating, thus their decomposition. Based upon these results, extraction times of 30, 60 and 90 min were selected for RSM of both borage and evening primrose.

4.3 Experimental design for RSM

Several different types of designs are available to fit a response surface to a set of experimental data (Hills and Hunter, 1966; Mason *et al.*, 1989). Complete and fractional factorial experiments in completely randomized designs are extremely useful when one is exploring the factor space in order to identify the region where the optimum response is located (Mason *et al.* 1989). A special form of central composite design, in that the star (*) points are on the face of the cube formed from the 2^3 factorial, was chosen. This design is called a face-centred cube design and was chosen over alternatives such as a rotatable design because the face-centred design only uses three levels of each factor, whereas other central composite designs would require five levels of each (0, ± 1, ± star point). Having three levels instead of five was cited as desirable because it reduces the preparation time and lessens the potential for mistakes in preparing the extracts (Mason *et al.*, 1989). Three replicates were taken at the centre, so that the total number of observations was n = 8 + 6 + 3 = 17. This is slightly over half the number of observations that would be require for a three-level factorial without repeats ($3^3 = 27$). Tables 4.2 and 4.3 show the response values observed for various points of the designs for borage and evening primrose, respectively.

Table 4.2 Face-centred cube design and experimental response values for borage.

Design point	X_1 (%, v/v)	X_2 (°C)	X_3 (min)	Y (Antioxidant index)[1]
1	30* (−1)**	40 (−1)	30 (−1)	60.4
2	30 (−1)	40 (−1)	90 (+1)	62.3
3	30 (−1)	80 (+1)	30 (−1)	65.6
4	30 (−1)	80 (+1)	90 (+1)	68.7
5	70 (+1)	40 (−1)	30 (−1)	66.4
6	70 (+1)	40 (−1)	90 (+1)	65.6
7	70 (+1)	80 (+1)	30 (−1)	70.8
8	70 (+1)	80 (+1)	90 (+1)	70.0
9	30 (−1)	60 (0)	60 (0)	68.3
10	70 (+1)	60 (0)	60 (0)	71.4
11	50 (0)	40 (−1)	60 (0)	79.3
12	50 (0)	80 (+1)	60 (0)	77.2
13	50 (0)	60 (0)	90 (+1)	74.8
14	50 (0)	60 (0)	30 (−1)	79.7
15	50 (0)	60 (0)	60 (0)	80.3
16	50 (0)	60 (0)	60 (0)	81.2
17	50 (0)	60 (0)	60 (0)	79.8

[1]All Response values are averages of two determinations except for the middle point where triplicate determinations were carried out.
*Uncoded variable levels.
**Coded variable levels. Coded value = (Original value − M)/S, where, M and S are the average of the highest and lowest variable levels, and half their difference, respectively.

Table 4.3 Face-centred cube design and experimental response values for evening primrose.

Design point	X_1 (%, v/v)	X_2 (°C)	X_3 (min)	Y (Antioxidant index)[1]
1	50* (−1)**	40 (−1)	30 (−1)	65.8
2	50 (−1)	40 (−1)	90 (+1)	60.6
3	50 (−1)	80 (+1)	30 (−1)	77.8
4	50 (−1)	80 (+1)	90 (+1)	68.4
5	90 (+1)	40 (−1)	30 (−1)	55.1
6	90 (+1)	40 (−1)	90 (+1)	58.1
7	90 (+1)	80 (+1)	30 (−1)	65.2
8	90 (+1)	80 (+1)	90 (+1)	64.7
9	50 (−1)	60 (0)	60 (0)	80.8
10	90 (+1)	60 (0)	60 (0)	62.8
11	70 (0)	40 (−1)	60 (0)	72.3
12	70 (0)	80 (+1)	60 (0)	74.0
13	70 (0)	60 (0)	90 (+1)	68.8
14	70 (0)	60 (0)	30 (−1)	76.3
15	70 (0)	60 (0)	60 (0)	79.5
16	70 (0)	60 (0)	60 (0)	78.1
17	70 (0)	60 (0)	60 (0)	76.2

[1]All Response values are averages of two determinations except for the middle point where triplicate determinations were carried out.
*Uncoded variable levels.
**Coded variable levels. Coded value = (Original value − M)/S, where, M and S are the average of the highest and lowest variable levels, and half their difference.

4.4 Location of critical (optimum) extraction conditions

As previously explained in section **4.2**, the antioxidant activity of extracts of borage and evening primrose was a quadratic function of the organic solvent concentration in the extraction medium, extraction temperature and time. Therefore, a quadratic polynomial model was selected for RSM (generalized function is given in section **3.2.5**). This model was fitted to data presented in Tables 4.2 and 4.3. Response surfaces were generated as a function of two factors while holding the third factor at a fixed level. Generated response surfaces were explored to determine the factor levels that would produce the maximum response. Figures 4.7 - 4.9 show the response surfaces obtained for borage and Figures 4.10 - 4.12 are those obtained for evening primrose. An alternative approach to plotting the two-factor response surface is to plot contours of constant response as a function of the two factors. These are similar to the contours of equal elevation in a topographical map (Mason *et al.*, 1989). Values for response variables in contour plots were calculated for a grid of values of the two factors while holding the third factor at a fixed level. The numerical values of the response were plotted on a graph as a function of the two factors; i.e., the two axes represent the factor values and the numerical values of the calculated response was placed on the grid at the intersection of the two factor values used to calculate it. Figures 4.7 - 4.9 and 4.10 - 4.12 also depict the contour plots generated for borage and evening primrose, respectively. These plots showed the location of a maximum response within the experimental region for both borage and evening primrose.

Figure 4.7 Response surface and its contour plot depicting the dependence of antioxidant activity of crude extracts of borage meal in a β-carotene-linoleate model system on combined effect of extraction medium and temperature. Extraction time was held at a fixed level.

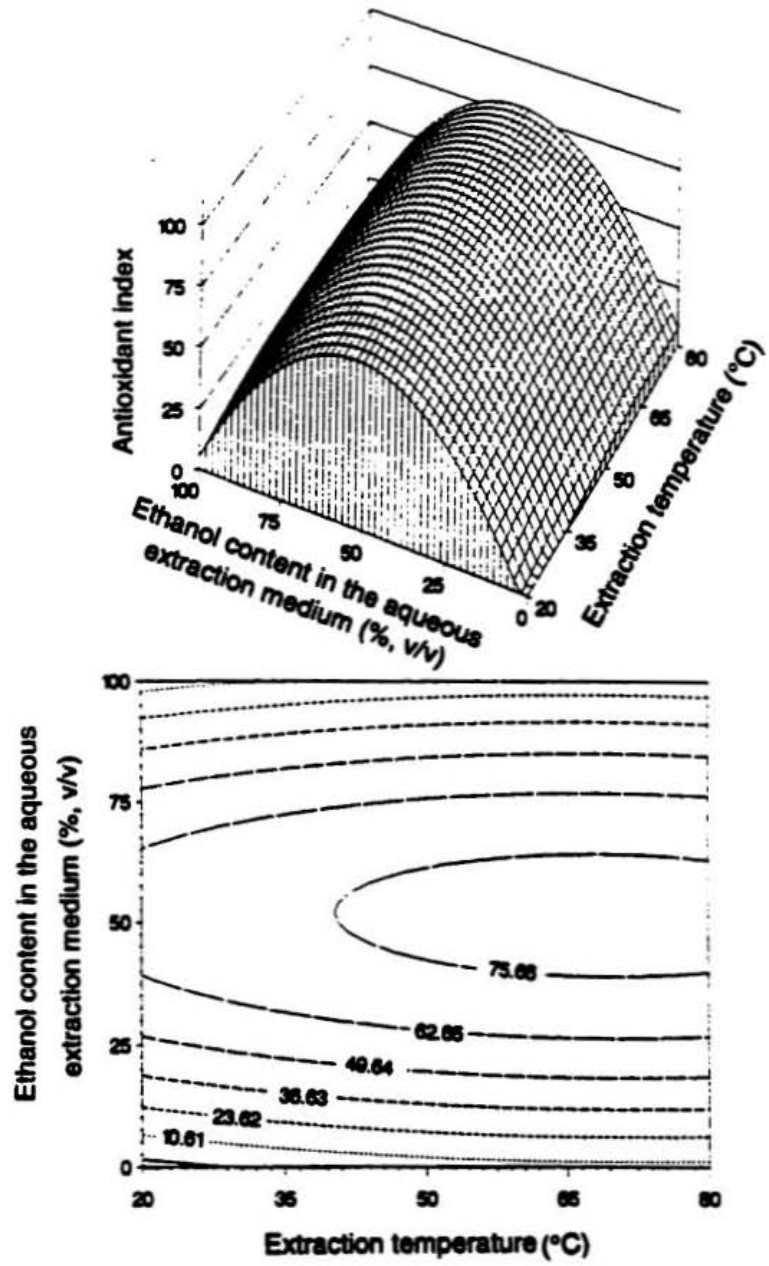

Antioxidant index
0
25
50
75
100
Ethanol content in the aqueous extraction medium (%, v/v)
100
75
50
25
0
Extraction temperature (°C)
20
35
50
65
80
Ethanol content in the aqueous extraction medium (%, v/v)
100
75
50
25
0
75.66
62.65
49.64
36.63
23.62
10.61
20
35
50
65
80
Extraction temperature (°C)

Figure 4.8 Response surface and its contour plot depicting the dependence of antioxidant activity of crude extracts of borage meal in a β-carotene-linoleate model system on combined effect of extraction medium and time. Extraction temperature was held at a fixed level.

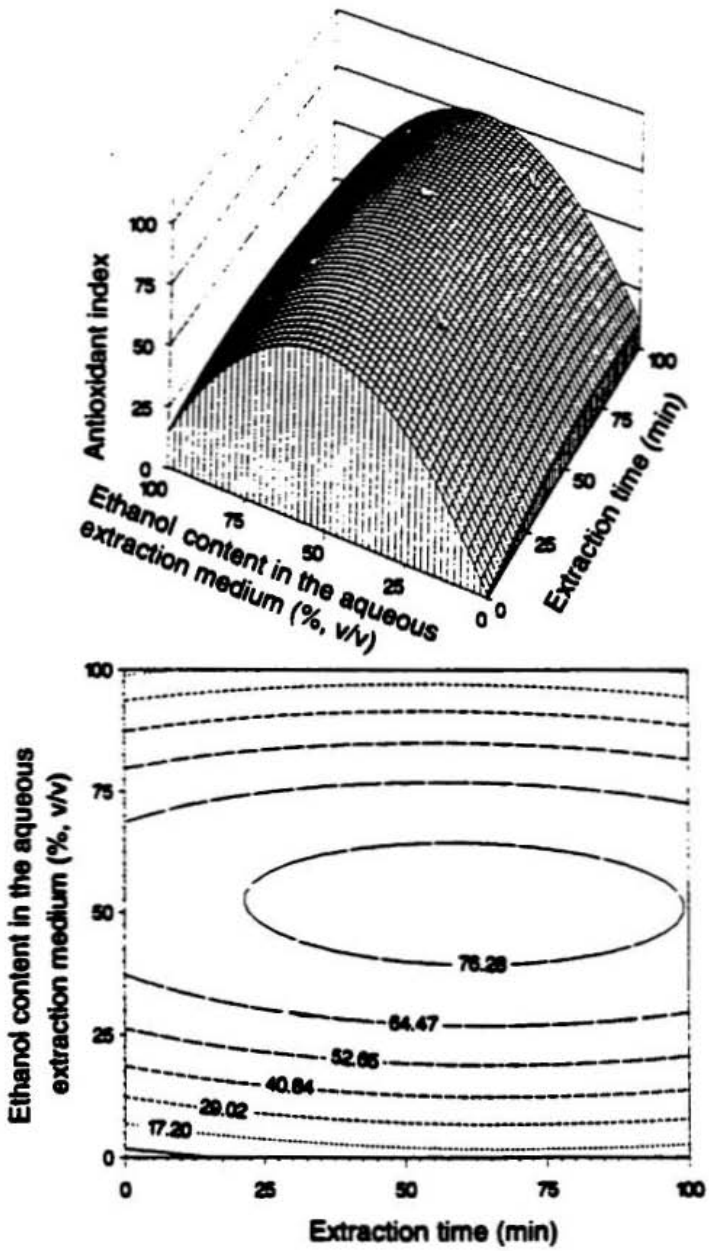

Antioxidant index
0
25
50
75
100
Ethanol content in the aqueous extraction medium (%, v/v)
100
75
50
25
0
Extraction time (min)
0
25
50
75
100
Ethanol content in the aqueous extraction medium (%, v/v)
76.28
64.47
52.65
40.84
29.02
17.20
Extraction time (min)

Figure 4.9 Response surface and its contour plot depicting the dependence of antioxidant activity of crude extracts of borage meal in a β-carotene-linoleate model system on combined effect of extraction temperature and time. Ethanol content in the extraction medium was held at a fixed level.

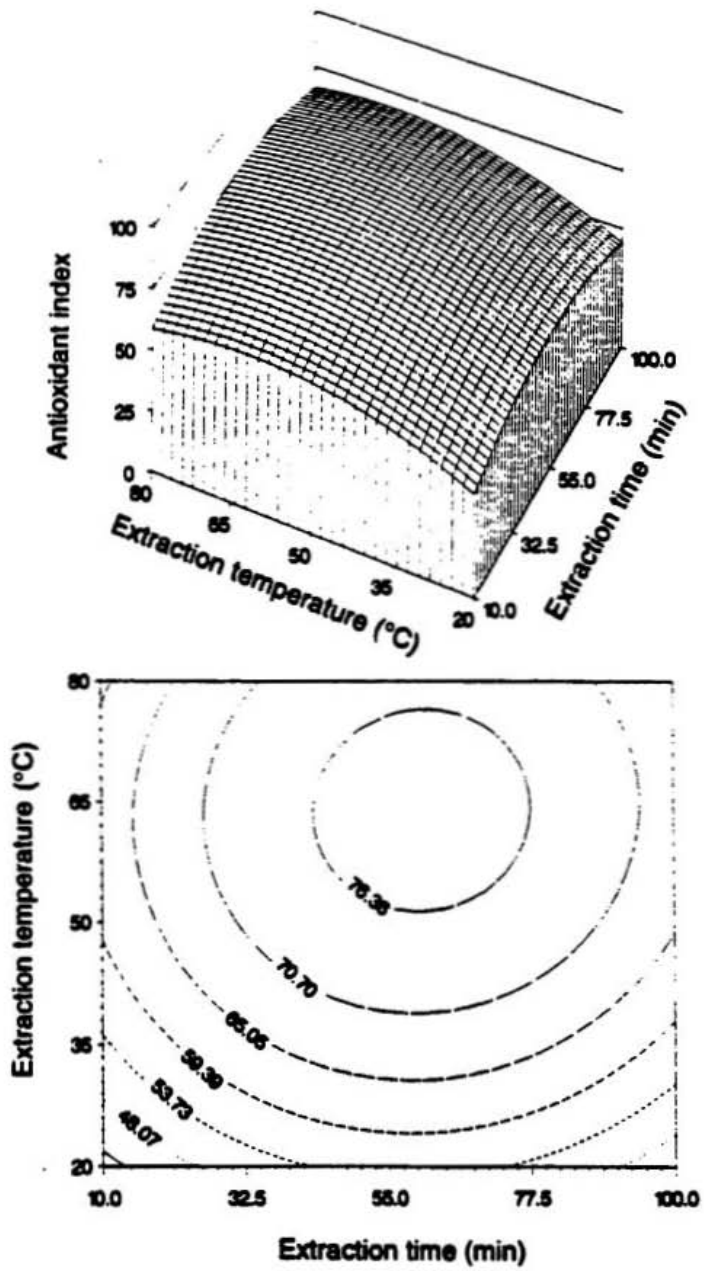

Antioxidant index
100
75
50
25
0
80
65
50
35
20
Extraction temperature (°C)
10.0
32.5
55.0
77.5
100.0
Extraction time (min)
80
65
50
35
20
Extraction temperature (°C)
76.38
70.70
65.05
59.39
53.73
48.07
10.0
32.5
55.0
77.5
100.0
Extraction time (min)

Figure 4.10 Response surface and its contour plot depicting the dependence of antioxidant activity of crude extracts of evening primrose meal in a β-carotene-linoleate model system on combined effect of extraction medium and temperature. Extraction time was held at a fixed level.

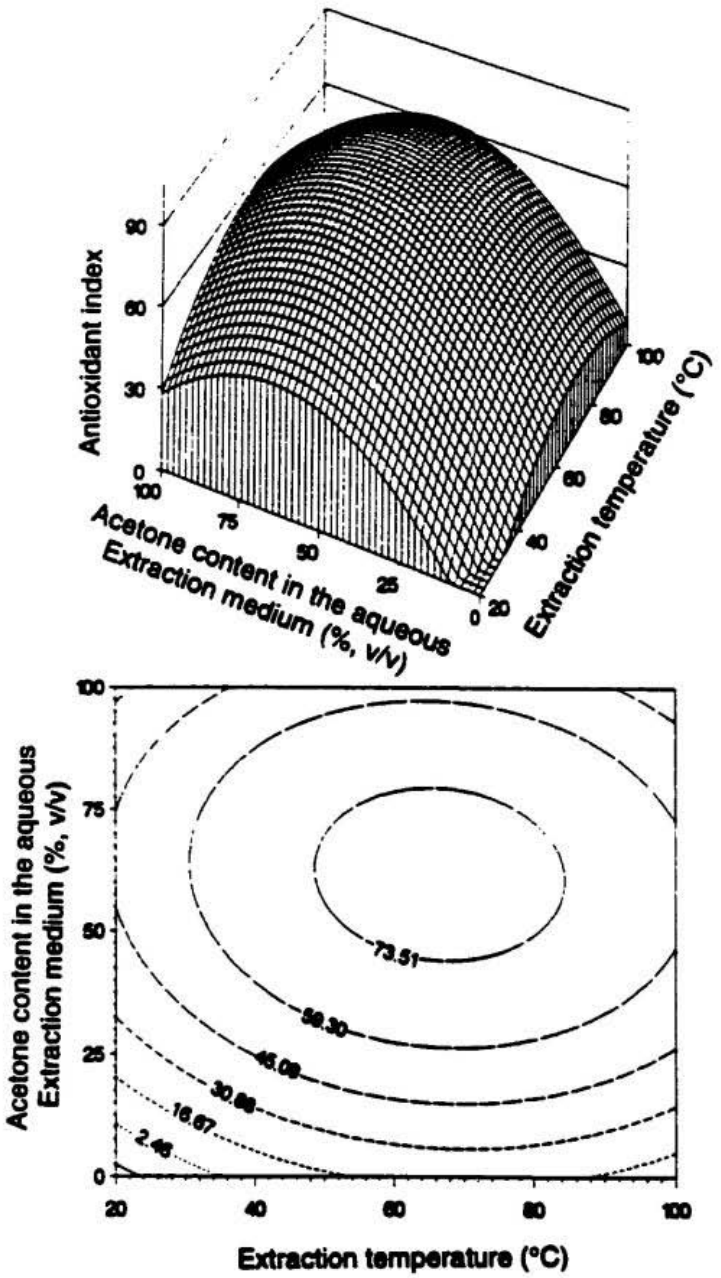

Antioxidant index
0
30
60
90
100
75
50
25
0
Acetone content in the aqueous
Extraction medium (%, v/v)
20
40
60
80
100
Extraction temperature (°C)
Acetone content in the aqueous
Extraction medium (%, v/v)
73.51
59.30
45.09
30.88
16.67
2.46
Extraction temperature (°C)

Figure 4.11 Response surface and its contour plot depicting the dependence of antioxidant activity of crude extracts of evening primrose meal in a β-carotene-linoleate model system on combined effect of extraction medium and time. Extraction temperature was held at a fixed level.

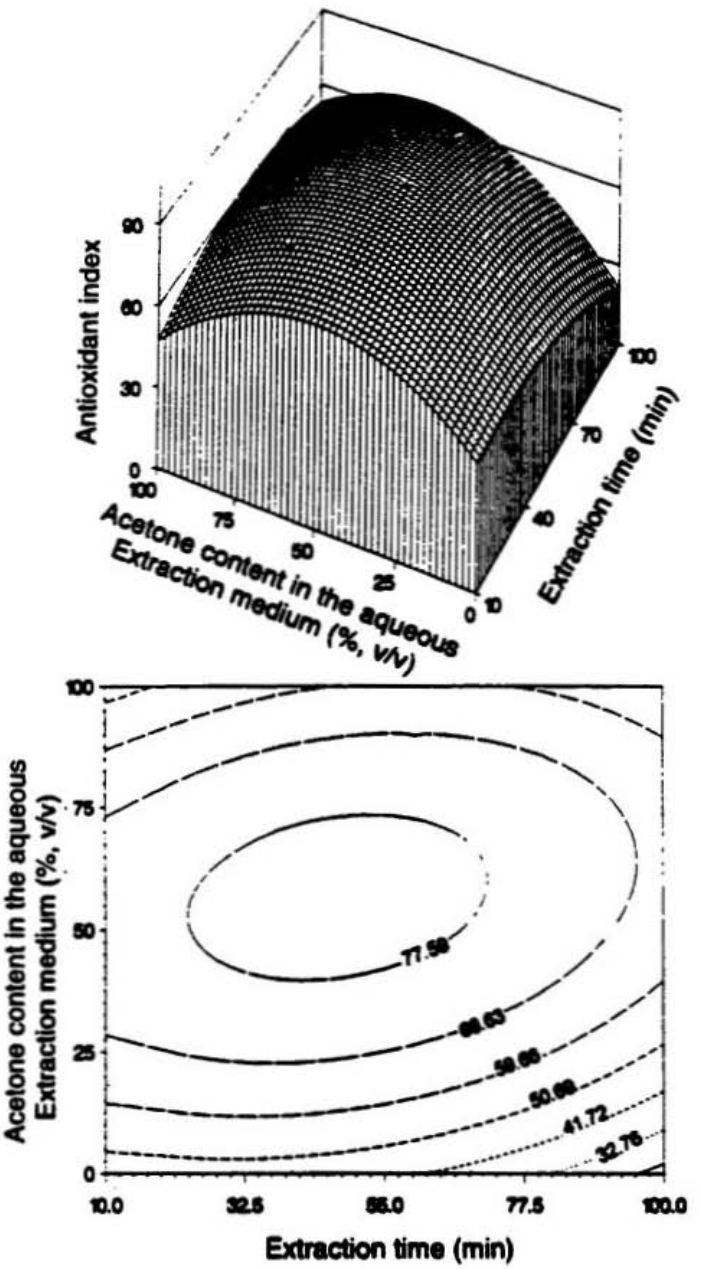

Antioxidant index
0
30
60
90
100
75
50
25
0
Acetone content in the aqueous
Extraction medium (%, v/v)
10
40
70
100
Extraction time (min)
Acetone content in the aqueous
Extraction medium (%, v/v)
100
75
50
25
0
77.59
68.63
59.66
50.69
41.72
32.76
10.0
32.5
55.0
77.5
100.0
Extraction time (min)

Figure 4.12 Response surface and its contour plot depicting the dependence of antioxidant activity of crude extracts of evening primrose meal in a β-carotene-linoleate model system on combined effect of extraction temperature and time. Acetone content in the extraction medium was held at a fixed level.

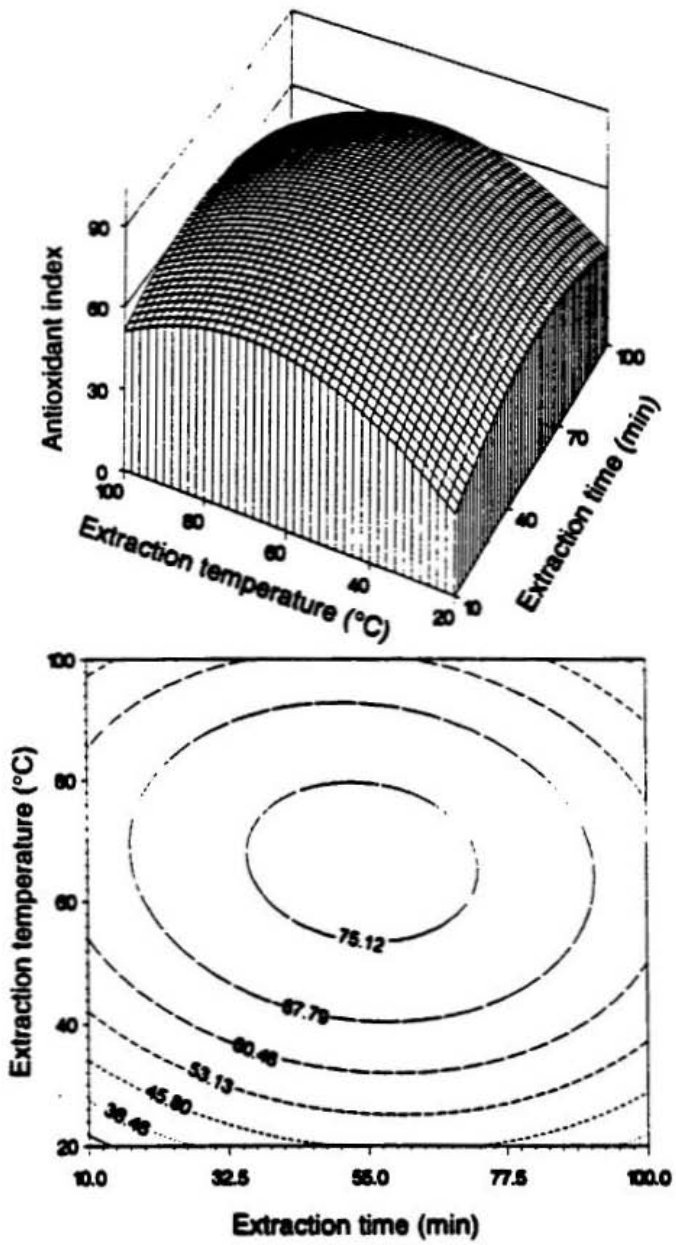

Antioxidant index
0
30
60
90
Extraction temperature (°C)
100
80
60
40
20
Extraction time (min)
10
40
70
100
Extraction temperature (°C)
100
80
60
40
20
75.12
67.79
60.46
53.13
45.80
38.46
10.0
32.5
55.0
77.5
100.0
Extraction time (min)

Table 4.4 shows the results of the analysis of variance and multivariate regression (which employed to determine the regression coefficients and their significance) for both borage and evening primrose. Regression coefficients of intercept, linear, quadratic, and interaction terms of the model were calculated using least square techniques and their significance was determined using t-test. Among the linear effects, the linear effect of the content of organic solvent in the extraction medium (x_1) and extraction temperature (x_2) were significant ($p \leq 0.05$) for borage and evening primrose, respectively. For borage, the quadratic effect of the content of organic solvent in the extraction medium (x_1) was significant ($p \leq 0.05$). All three quadratic effects of the model, fitted to data for evening primrose, were significant ($p \leq 0.05$). For borage, all interaction effects were insignificant ($p > 0.05$). For evening primrose, the interaction effect of the content of organic solvent in the extraction medium (x_1) and extraction temperature (x_2) was significant ($p \leq 0.05$). The polynomial models, fitted tc experimental data for both borage and evening primrose were highly significant ($p \leq 0.05$) and the coefficients of determination (R^2) were 0.9439 and 0.9443 for borage and evening primrose, respectively. This indicated that the most variation observed for design points was explainable by the predicted polynomial models. Furthermore, results of the error analysis indicated that the lack of fit was insignificant ($p > 0.05$), thus the response function was adequately explained by the proposed polynomial models. Coefficient of variation (CV) of less than 5% for both cases, indicated that the models were reproducible (Mason *et al.*, 1989). The fitted second-order polynomial models were:

Table 4.4 Estimeted regression coefficients of the quadratic polynomial models for borage and evening primrose.

Parameter[a]	Borage		Evening primrose	
	Estimated coefficient	Standard error	Estimeted coefficient	Standard error
Intercept				
β_0	−17.1765	14.3677	−24.4791	22.9280
Linear				
β_1	2.7029***	0.4031	1.2096	0.6300
β_2	0.4910	0.4667	1.6981**	0.5556
β_3	0.3188	0.2285	0.4600	0.2773
Quadratic				
β_{11}	−0.0251***	0.0037	−0.0110*	0.0043
β_{22}	−0.0034	0.0037	−0.0106*	0.0043
β_{33}	−0.0026	0.0016	−0.0016*	0.0019
Interaction				
β_{12}	−0.0007	0.0021	−0.0019*	0.0025
β_{13}	−0.0009	0.0014	0.0034	0.0017
β_{23}	−0.0007	0.0014	−0.0020	0.0017
β_{123}	—		—	
R^2	0.9439		0.9443	
F ratio	13.091		13.177	
p value	0.0013		0.0013	
CV, %	3.3737		4.0405	

[a]Coefficients refer to the general model.
***Significant at 0.001 level, **Significant at 0.01 level, *Significant at 0.05 level.

$$Y_1 = -17.1765 + 2.7029x_1 + 0.4910x_2 + 0.3188x_3 - 0.0251x_1^2 - 0.0034x_2^2 - 0.0026x_3^2 - 0.0007x_1x_2 - 0.0009x_1x_3 - 0.0007x_2x_3$$

$$Y_2 = -24.4791 + 1.2096x_1 + 1.6981x_2 + 0.4600x_3 - 0.0110x_1^2 - 0.0106x_2^2 - 0.0016x_3^2 - 0.0019x_1x_2 - 0.0034x_1x_3 - 0.0020x_2x_3$$

where Y_1, Y_2 , x_1, x_2, and x_3 are response for borage, response for evening primrose, content of organic solvent in the aqueous extraction medium (%, v/v), extraction temperature ($^{\circ}C$) and time (min). These two models were used to generate response surfaces for borage and evening primrose. Once the responses were generated, canonical analyses were performed in order to examine the nature of the critical point (stationary point) and to obtain the critical values of the independent variables at the stationary point (Box and Wilson, 1951; Mead 1988). Since all eigen values at stationary point were negative, the nature of the response for both cases was maximum (antioxidant index of 80.7 and 80.6 for borage and evening primrose, respectively). As given in Table 4.5, critical values were located within the experimental region. As shown in Figures 4.13 and 4.14, results for verification experiments were in agreement with the predicted response data. Once the critical extraction conditions were located, sufficient amounts of extracts of both borage and evening primrose meals were prepared and used in subsequent studies.

Table 4.5 Critical factor levels and response at maximum point.

Parameter	Borage	Evening primrose
x_1^*, (%, v/v)	52	56
x_2^{**}, (°C)	74	71
x_3^{***}, (min)	62	47
Y (Antioxidant index)	80.7	80.6

*Ethanol content in the extraction medium for borage; acetone content in the extraction medium for evening primrose.
**Extraction temperature.
***Extraction time.

Figure 4.13 Relationship between predicted and observed responses for borage.

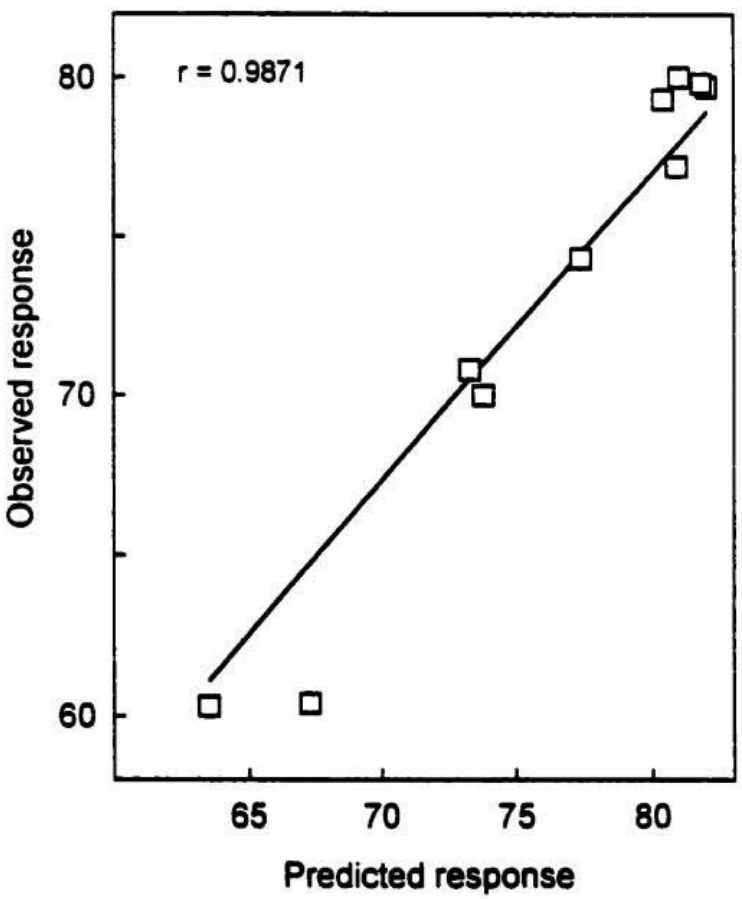
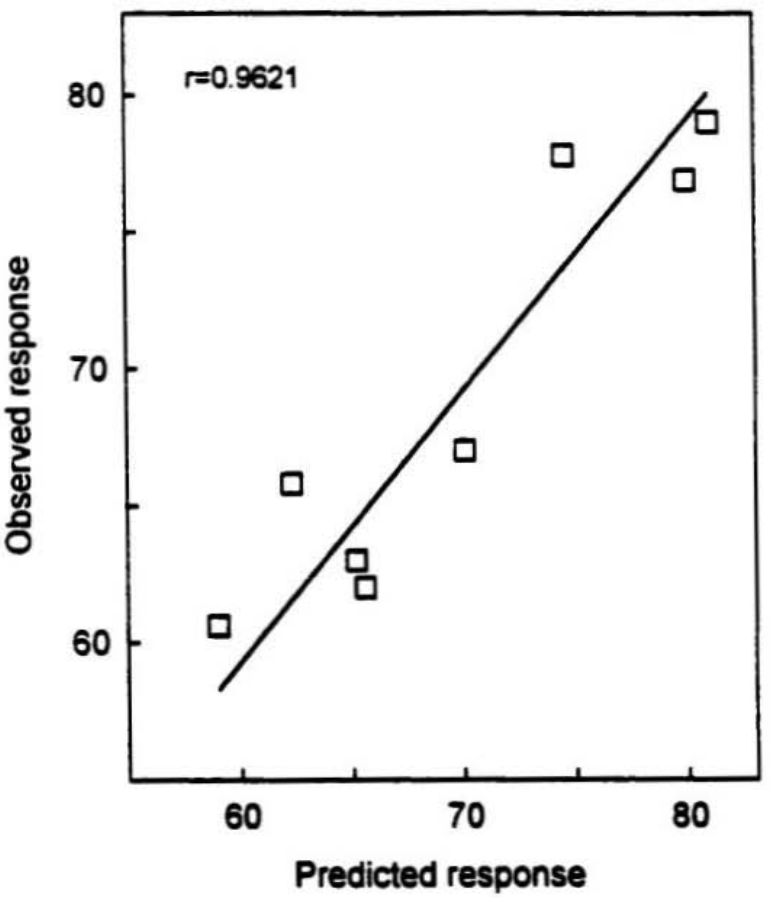

r = 0.9871
80
70
60
65
70
75
80
Observed response
Predicted response

Figure 4.14 Relationship between predicted and observed responses for evening primrose.


r=0.9621
80
70
60
Observed response
60
70
80
Predicted response

4.5 Sephadex LH-20 column chromatography of crude extracts of borage and evening primrose meals

A portion of crude extracts of borage and evening primrose meals was subjected to column chromatography as the first step towards the identification of the active components in the extracts. Sephadex LH-20 column chromatography has been used by many researchers to fractionate various plant extracts (Amarowicz *et al.*, 1996; Amarowicz *et al.*, 1992, 1993). Sephadex LH-20 is probably one of the best stationary phases available for separation of phenolics because of the faster, yet satisfactory separation of phenolics on the column (Wanasundara *et al.*, 1994). Figure 4.15A shows the fraction profile for borage. Even though the profile was continuous, six major fractions were clearly identifiable. These fractions were labled I-VI. Yield of individual fractions as a relative fraction of the crude extract is given in Table 4.6. As depicted in Figure 4.15B, evening primrose crude extract was also separated into six major fractions (I-VI) with varying relative yields as given in Table 4.6.

UV spectra of all column chromatographic fractions were recorded and shown in Figures 4.16 and 4.17 for borage and evening primrose, respectively. UV spectral data for borage and evning primrose crude extracts and their fractions are summarized in Table 4.7. Borage fractions I-VI showed possible presence of phenolic acids. Spectral data for primrose fractions I-VI suggested possible presence of flavonoid class of compounds. Presence of catechin or similar compounds in evening primrose fractions III-

Figure 4.15 Column chromatographic fraction profile of borage (A) and evening primrose (B) crude extracts.

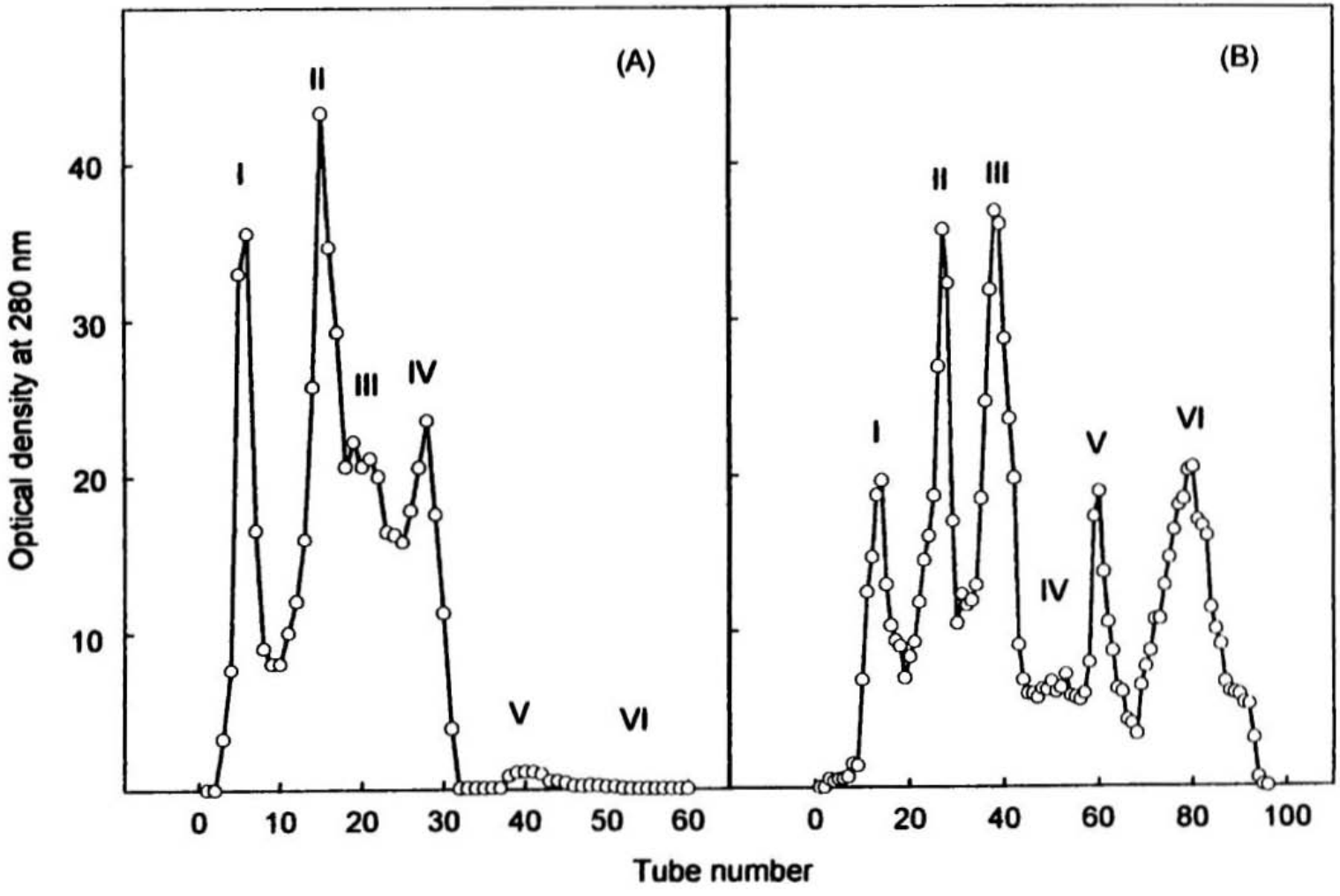

(A)
(B)
I
II
III
IV
V
VI
Optical density at 280 nm
Tube number
0
10
20
30
40
50
60
80
100

Table 4.6 Column chromatoraphic data for borage and evening primrose[1].

Fraction	Borage[2]		Evening primrose[3]	
	Weight (mg)	Relative fraction (%, w/w)	Weight (mg)	Relative fraction (%, w/w)
I	168	19.6	162	17.3
II	309	36.2	218	23.2
III	244	28.4	287	30.6
IV	65.0	7.57	70.2	7.46
V	26.4	3.07	65.3	6.93
VI	46.0	5.35	136	14.5

[1] Results are averages of two runs (within 7%).
[2] Weight of crude extract used = 1 g; recovery = 86%.
[3] Weight of crude extract used = 1 g; recovery = 94%.

Figure 4.16 Ultraviolet (UV) spectra of borage crude extract and its fractions.

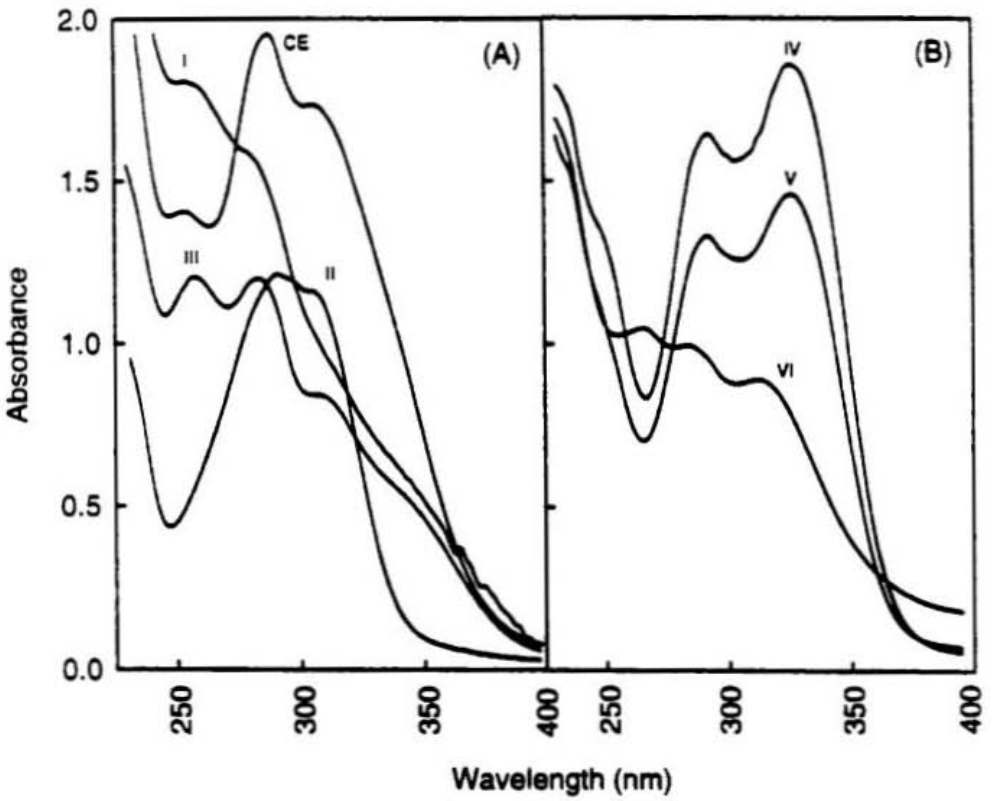

2.0
1.5
1.0
0.5
0.0
Absorbance
CE
I
II
III
(A)
IV
V
VI
(B)
250
300
350
400
Wavelength (nm)

Figure 4.17 Ultraviolet (UV) spectra of evening primrose crude extract and its fractions.

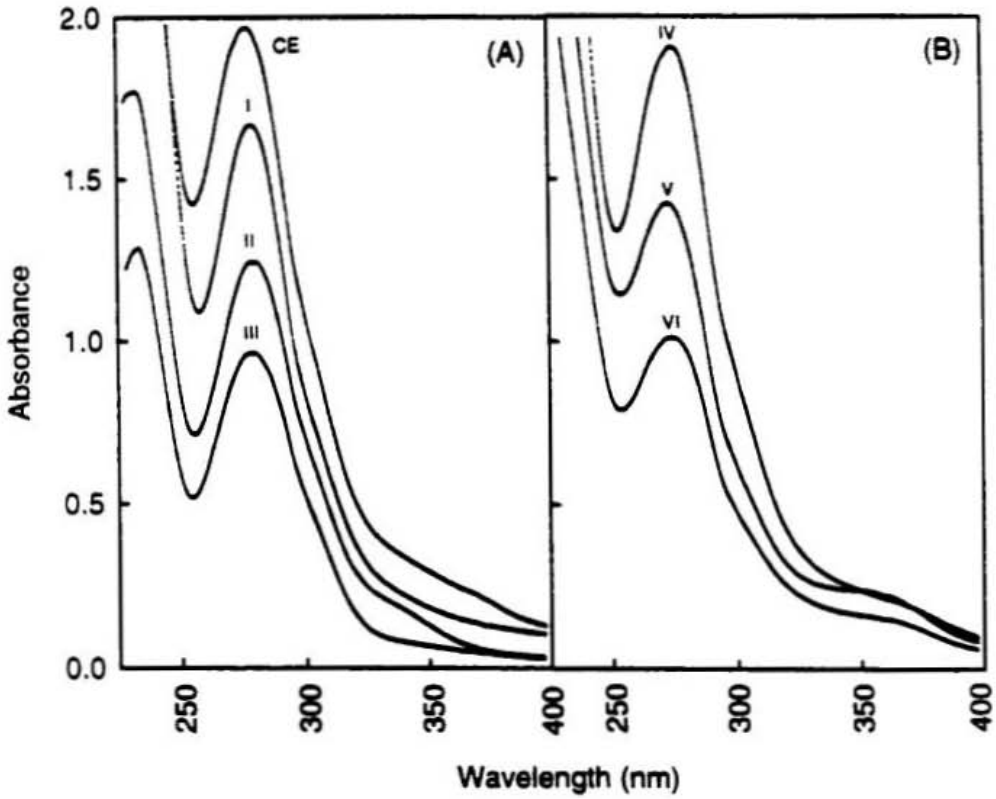

2.0
1.5
1.0
0.5
0.0
Absorbance
CE
(A)
I
II
III
(B)
IV
V
VI
250
300
350
400
Wavelength (nm)

Table 4.7 Ultraviolet (UV) spectral data for borage and evening primrose crude extracts and their fractions[1].

Extract/fraction	Absorbance maxima (λ_{max},nm)	Shoulder (λ_{sho}, nm)
Borage		
Crude extract	256, 286	306
Fraction I	298, 306	--
Fraction II	270	306
Fraction III	260, 270, 312	--
Fraction IV	292, 328	--
Fraction V	256, 282, 324	--
Fraction VI	256, 282, 314	--
Evening primrose		
Crude extract	280	--
Fraction I	280	--
Fraction II	280	--
Fraction III	280	--
Fraction IV	280	--
Fraction V	280	--
Fraction VI	280	--

[1]Spectra were recorded in methanol.
--No shoulder.

VI was confirmed by a positive vanillin test. Also the absence of such compounds in all borage fractions was confirmed by a negative vanillin test.

Table 4.8 shows the total, hydrophilic and hydrophobic phenolics contents of borage crude extract and its fractions. The total phenolics content of borage crude extract was 413 mg as sinapic acid equivalents/g and it consisted of 89% (w/w) and 11% (w/w) of hydrophilic and hydrophobic phenolics, respectively. The hydrophilic phenolics contents of borage fractions were 2 - 5 times higher than their hydrophobic counterparts. Evening primrose crude extract contained 304 mg of phenolics as catechin equivalents/g and its hydrophilic and hydrophobic phenolics were present at a 3:2 (w/w) ratio. Almost equal amounts of hydrophilic and hydrophobic phenolics were present in evening primrose fractions I and V. Fraction II had somewhat a higher content of hydrophobic phenolics as compared to its hydrophilic counterpart. Fraction III had two times more hydrophobic phenolics than hydrophilic, and the reverse was evident for fraction VI. The hydrophobic phenolics of fraction IV were 32-times higher than hydrophilic phenolics (Table 4.9). These results show the presence of varying amounts of both hydrophilic and hydrophobic phenolics in borage and evening primrose crude extracts and their fractions. Studies using purified phenolics have shown that the partition of hydrophilic and hydrophobic compounds of a mixture of phenolics into different phases of food systems plays a major role in determining the antioxidant efficacy of natural antioxidants (Porter, 1980; Huang *et al.*, 1994). Thus, the ratio of hydrophilic to hydrophobic phenolics may be useful to predict the efficacy of a given crude extract in a specified system. An attempt will be

Table 4.8 Contents of total, hydrophillic and hydrophobic phenolics of borage crude extract and its fractions[1].

Sample	Phenolics as mg sinapic acid equivalents/g sample		
	Total	Hydrophillic	Hydrophobic
Crude extract	413 ± 18	367 ± 16 (89%)	46 ± 2 (11%)
Fraction I	283 ± 16	200 ± 11 (70%)	83 ± 4 (30%)
Fraction II	129 ± 5	111 ± 5 (86%)	18 ± 2 (14%)
Fraction III	140 ± 8	106 ± 7 (76%)	34 ± 2 (24%)
Fraction IV	366 ± 14	300 ± 12 (82%)	66 ± 3 (18%)
Fraction V	280 ± 10	220 ± 10 (78%)	60 ± 3 (22%)
Fraction VI	347 ± 15	290 ± 15 (83%)	57 ± 3 (17%)

[1] Results are mean values of three determinations ± standard deviation.
* Values in parenthesis show the % (w/w) contents of hydrophillic and hydrophobic phenolics in the total phenolics.

Table 4.9 Contents of total, hydrophillic and hydrophobic phenolic of evening primrose crude extract and its fractions[1].

Sample	Phenolics as mg catechin equivalents/g sample		
	Total	Hydrophillic	Hydrophobic
Crude extract	304 ± 14	185 ± 7 (61%)*	121 ± 6 (39%)
Fraction I	158 ± 10	78 ± 5 (49%)	80 ± 6 (51%)
Fraction II	313 ± 18	128 ± 10 (41%)	185 ± 10 (59%)
Fraction III	369 ± 20	123 ± 9 (33%)	246 ± 12 (67%)
Fraction IV	402 ± 26	11 ± 1 (3%)	391 ± 23 (97%)
Fraction V	279 ± 10	136 ± 8 (49%)	143 ± 4 (51%)
Fraction VI	445 ± 25	300 ± 15 (67%)	145 ± 8 (33%)

[1] Results are mean values of three determinations ± standard deviation.
* Values in parenthesis show the % (w/w) contents of hydrophillic and hydrophobic phenolics in the total phenolics.

made, in the following sections, to link the composition of borage and evening primrose crude extracts and fractions to their antioxidant activities in various model systems.

4.6 Oxidative stability of model systems as affected by borage and evening primrose crude extracts and their fractions

Although a variety of model systems are available for evaluation of the efficacy of antioxidants, the choice of a particular system depends mainly upon the chemical nature of antioxidants to be evaluated. There is evidence for discrepancies in antioxidant efficacies of substances when evaluated in different model systems (Decker, 1998). For example, Trolox (a hydrophilic analogue of α-tocopherol) was reported to be more effective than its lipophilic counterpart, α-tocopherol, in bulk oil, but less active in oil-in-water emulsions (Huang *et al.*, 1996). Furthermore, a particular antioxidant can promote the formation of hydroperoxides in the early stages of oxidation and the same antioxidant may inhibit the formation of secondary oxidation products (aldehydes, ketones, alcohols and hydrocarbons) at later stages (Frankel, 1996). Therefore, the antioxidant efficacies of borage and evening primrose extracts were evaluated in different model systems using a variety of analytical techniques. Results for the model systems studies will be discussed in the following sections.

4.6.1 Antioxidant efficacy of borage and evening primrose crude extracts and their fractions in a β-carotene-linoleate model system

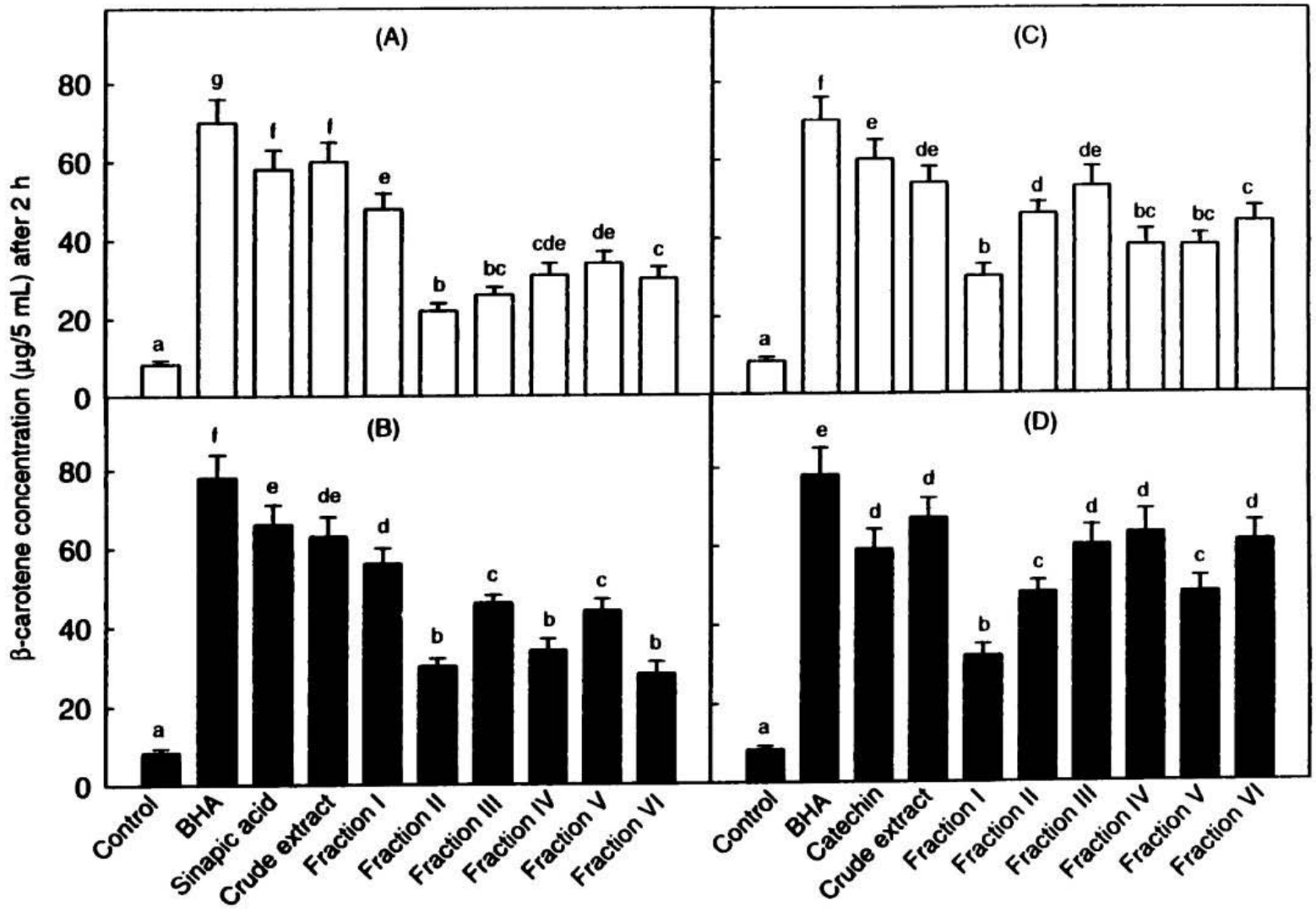
The basis of this system is that the rapid discoloration of β-carotene in the absence of an antioxidant could be slowed down by addition of an antioxidant. The discoloration occurs due to the coupled oxidation of β-carotene and linoleic acid which generates free radicals. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its allylic methylene groups, attacks the highly conjugated β-carotene molecules and so does the oxygen-derived free radicals. As more and more β-carotene molecules loose their double bonds, the system loses its characteristic orange colour which can be monitored spectrophotometrically. On the other hand, presence of an antioxidant can hinder the extent of discoloration by breaking the free radical chain reactions which consequently reduces the concentration of substrate-derived and oxygen-derived free radicals in the system (Benzie, 1996). This system serves as a convenient means of determining antioxidant activity of plant extracts, but the effect seen in this system for a given antioxidant may not always reflect its activity in other systems such as meats and vegetable oils.

Effects of borage and evening primrose crude extract and their fractions at 100 and 200 ppm, in a β-carotene-linoleate model system are shown in Table A.1-A.4. Concentration of β-carotene in the assay media containing additives decreased over 2-h assay period. For the convenience of discussion, β-carotene concentrations after 2 h assay period were selected as an indicator of antioxidant efficacy of the extracts and

fractions. The initial mass of β-carotene in the assay media (5 mL) was 80 ± 6 μg. As depicted in Figure 4.18A and B, control sample, devoid of antioxidants, lost almost 90% of its initial β-carotene after 2 h of assay. Borage crude extracts/fractions at both 100 and 200 ppm resulted in a significantly ($p \leq 0.05$) high retention of β-carotene in assay media with marginal differences between lower and higher concentrations (except for fraction III which had a clear concentration effect). As expected, BHA exerted the strongest antioxidant effect at both concentrations. Crude borage extract, at both concentrations, exhibited an effect similar to that exerted by sinapic acid at the same concentration. Furthermore, the crude borage extract, as such, helped retain more β-carotene than did any of its fractions. Among fractions, fraction I more effectively protected β-carotene from oxidation while fractions II, IV and VI (at 200 ppm) were least effective. Antioxidant activity of borage fractions III and V were intermediary.

The varying antioxidant effects of borage crude extract and its fractions in the β-carotene-linoleate model system may be attributed to structural differences in active components which determine their affinity to different phases of the model system. Partitioning of active components into aqueous and lipid phases of the model system has shown to play a major role in determining antioxidant efficacy of natural antioxidants (Frankel *et al.*, 1994). For fractions I-VI, the antioxidant activity increased as the content of hydrophobic phenolics increased. At 200 ppm, the antioxidant activity of fractions decreased in the order of I > III = V > II = IV = VI (Figure 4.18B). Despite its low content of hydrophobic phenolics (11%, w/w), borage crude extract, as such, was the

Figure 4.18 Effect of borage and evening primrose additives on oxidative stability of β-carotene (after 2h) in a β-carotene-linoleate model system. (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.1 - A.4.


(A)
(B)
(C)
(D)
β-carotene concentration (μg/5 mL) after 2 h
0
20
40
60
80
Control
BHA
Sinapic acid
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

most active antioxidant in the β-carotene-linoleate model system. This could be attributed to the greater possibility of synergism among the active components in the crude extract. Most of the synergistic interactions were certainly disturbed during fractionation, thus the resulting fractions had reduced antioxidant activity.

Figure 4.18C and D depict the antioxidant activity of evening primrose crude extract and its fractions in a β-carotene-linoleate model system. Assay media containing additives at 100 ppm retained more of β-carotene (30 - 54 μg of the initial 80 μg) as compared to very low retention in the medium devoid of additives. Even greater retention of β-carotene was evident when the additives were present at 200 ppm. At 200 ppm, fractions III, IV and VI, exerted an effect similar to that of the authentic catechin at the same concentration while fraction I was least effective.

Since β-carotene-linoleate system is a type of lipid-in-water emulsion, one would expect the hydrophilic antioxidants to be less effective due to a dilution effect. On the other hand hydrophobic antioxidants could partition into the lipid phase, thus more effectively inhibit lipid oxidation. This, however, was not the case for evening primrose crude extract and its fractions. Evening primrose crude extract and fraction VI (content of hydrophobic phenolics was 39 and 33%, respectively) exerted the most antioxidant effect when compared to the other fractions with higher proportions of hydrophobic antioxidants. As was the case for borage, the high antioxidant activity of evening primrose crude extract could arise from many synergistic interactions operative among various antioxidant components. Separation of different phenolics in fractions due to their

different polarities and molecular size did upset the synergistic interactions among them which eventually resulted in a decrease in antioxidant activity of some fractions. Even though the dissolution of hydrophilic antioxidants in the aqueous phase had a negative effect on overall antioxidant activity, presence of these compounds in high concentration could reduce the concentration of oxygen-derived free radicals in the aqueous phase. This could be the reason for high antioxidant activity of fraction VI which had more hydrophilic than hydrophobic phenolics. Another possibility is the presence of highly efficient hydrophobic antioxidants in fraction VI. Antioxidant effects of additives in this model system will be further discussed with respect to their metal chelating, hydrogen peroxide-scavenging and free radical-scavenging capacities in sections **4.7.1, 4.8.1** and **4.9.1,** respectively.

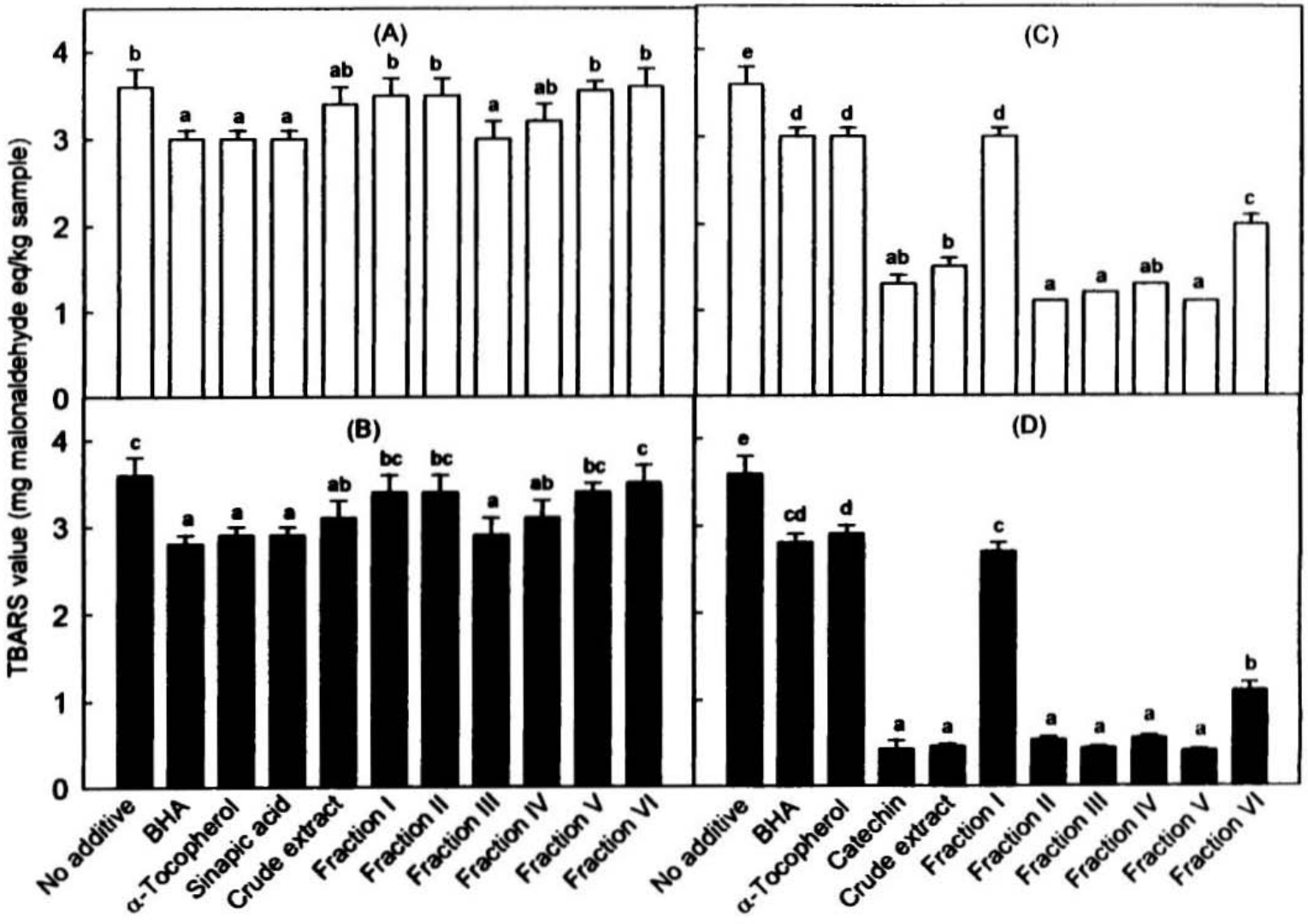
4.6.2 Antioxidant efficacy of borage and evening primrose crude extracts and their fractions in a cooked comminuted pork model system

Different types of meats have been used as models for evaluation of antioxidants, but research with pork systems represents the majority. As explained in **Chapter 2**, cooked meats regardless of their origin undergo rapid oxidative deterioration at refrigeration temperatures. Incorporation of an antioxidant into processed meats or even glazing of chunked meats could slow down their deterioration, thus extending shelf-life of products (Hwang *et al.*, 1990). This fact provides the basis for use of meat model systems for evaluation of antioxidant activity of various substances.

Oxidative stability of cooked comminuted pork (composition is given in section 4.1), stored at 4°C was monitored by TBARS and headspace volatile analyses. TBARS values of samples containing borage crude extract and its fractions are given in Tables A.5 and A.6. As shown, TBARS values of samples containing borage extract/fractions increased over time, but at slightly slower rates as compared to that of the control. The differences in TBARS values of samples containing borage crude extract and fractions were either marginal or insignificant ($p > 0.05$) over the entire storage period. At day-3 of storage, TBARS values of samples containing BHA, α–tocopherol and sinapic acid (at both concentrations) were about 16 - 22 % lower than that of the control (Figure 4.19A and B). At 100 ppm, only fraction III-treated sample had very low TBARS values as compared to the control. All other additives were ineffective, possibly due to their inadequate concentration in the samples. The TBARS values of samples treated with 200 ppm of BHA, α-tocopherol and sinapic acid were not different from those for 100 ppm. Borage crude extract, fractions III and IV, at 200 ppm, marginally reduced (14 - 19%) the TBARS formation in treated samples whereas the effects of other fractions were not significant ($p > 0.05$) as compared to that of the control.

Tables A.7 and A.8 show the TBARS values of cooked comminuted pork samples containing evening primrose crude extract and its fractions at 100 or 200 ppm. At both concentrations, all additives significantly ($p \leq 0.05$) reduced the formation of TBARS throughout the entire storage period and the effects were more pronounced at 200 ppm addition level. The crude extract of evening primrose and its fractions generally lowered

Figure 4.19 Effect of borage and evening primrose additives on formation of TBARS in a cooked comminuted pork model system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.5 - A.8.

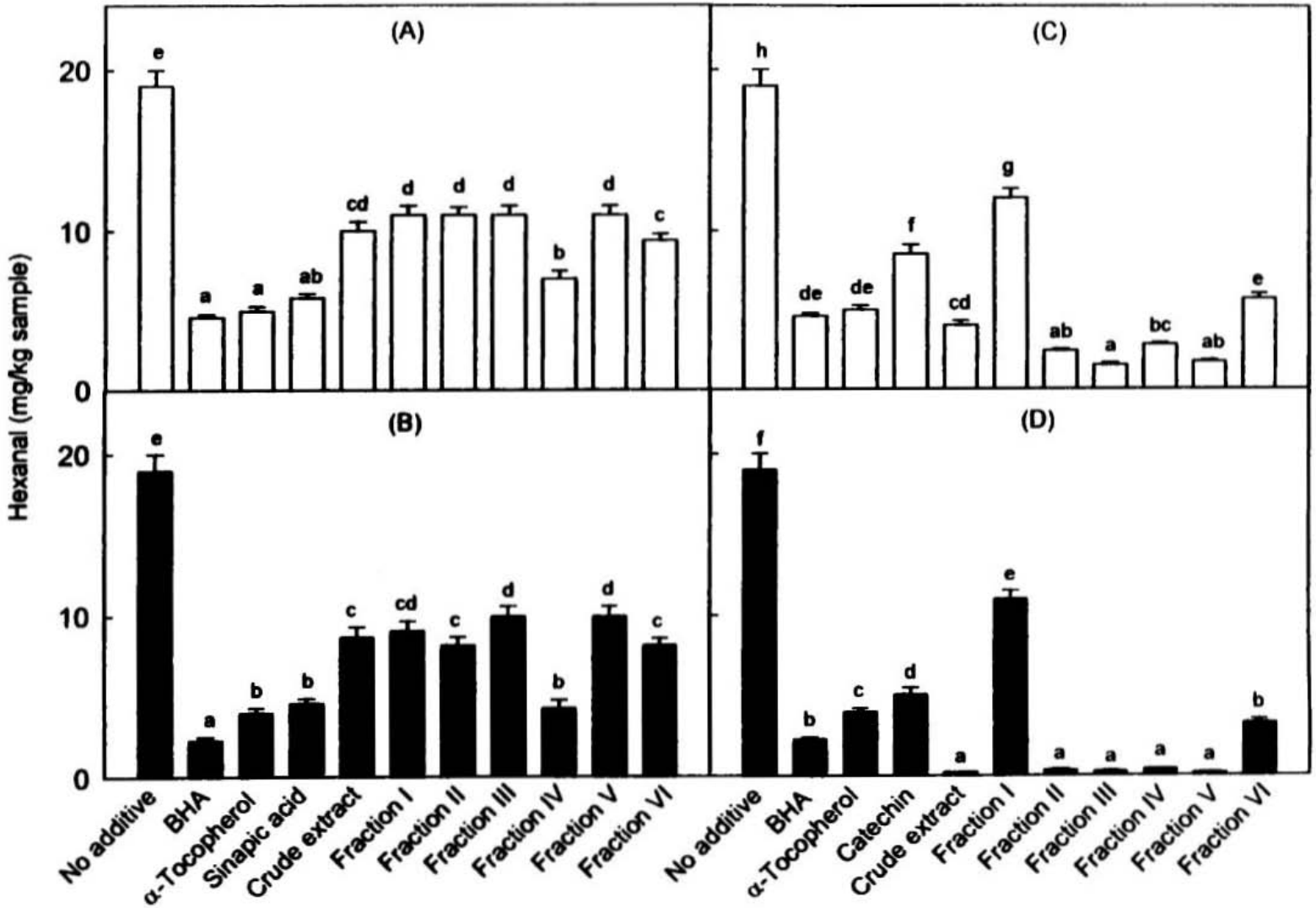

(A)
(B)
(C)
(D)
TBARS value (mg malonaldehyde eq/kg sample)
0
1
2
3
4
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
No additive
BHA
α-Tocopherol
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

the TBARS of treated samples by 44 - 89%. As depicted in Figure 4.19C and D, TBARS values of samples on day-3 of storage reflected that evening primrose crude extract and fractions II-IV were better than BHA at the same concentration. The antioxidant activity of additives, at 200 ppm, decreased in the order of: catechin = evening primrose crude extract = II = III = IV = V > VI > I = BHA > α-tocopherol. On day-3 of storage, the reduction in TBARS values of cooked comminuted pork, treated with 200 ppm of evening primrose crude extract and its fractions, were in the range of 25 - 89% as compared to the control. Fraction I of evening primrose extract reduced the TBARS formation by about 25%.

Hexanal contents of cooked comminuted pork samples treated with borage crude extract and its fractions at 100 and 200 ppm are presented in Tables A.9 and A.10, respectively. Hexanal content in the control as well as treated samples increased during the entire storage period. Formation of hexanal in samples treated with BHA, α-tocopherol and sinapic acid as well as borage crude extract and its fractions (at both concentrations) were significantly lower as compared to that of the control. Figure 4.20A and B depicts the effect of additives on hexanal formation in treated samples on day-3 of the storage. On day-3, hexanal formation in samples treated with 200 ppm of reference antioxidants and borage additives was reduced by 47 - 88% as compared to that of the control.

Evening primrose crude extract and its fractions, at both concentrations, reduced the formation of hexanal in treated samples throughout the entire storage period (Tables

Figure 4.20 Effect of borage and evening primrose additives on formation of hexanal in a cooked comminuted pork model system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Table 4.9 - 4.12.

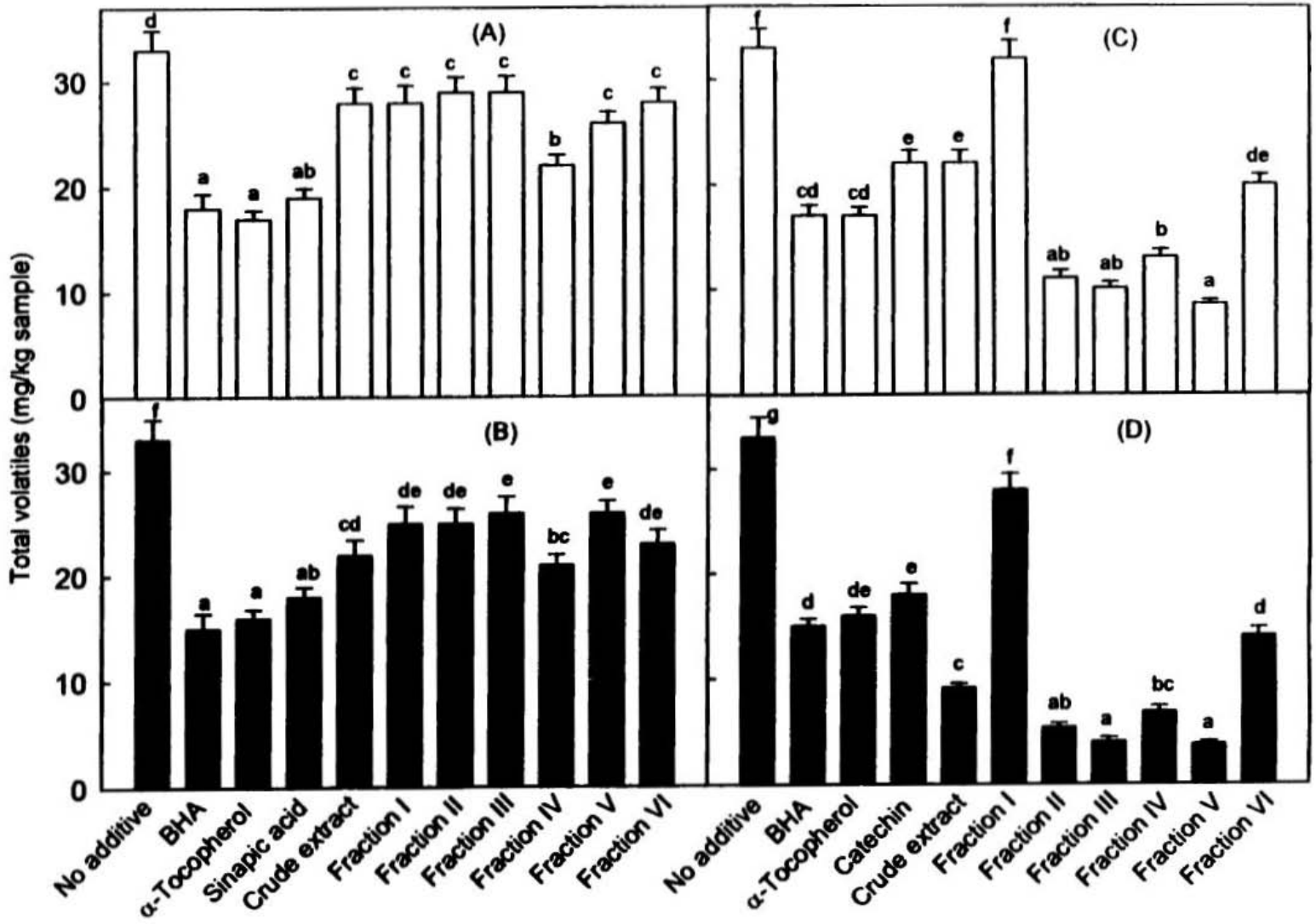

(A)
(B)
(C)
(D)
Hexanal (mg/kg sample)
0
10
20
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin

A.11 and A.12 show results for additives at 100 ppm and 200 ppm, respectively) as compared to that of the control. On day-3 of storage, hexanal contents of treated samples was significantly lower ($p \leq 0.05$) than that of the control. Furthermore, fractions II-V, at 100 ppm, delivered antioxidant effects which were greater than those for reference antioxidants at the same concentration. On day-3 of storage, hexanal formation in meat samples containing evening primrose crude extract and fraction II-V was similar. On day-3 of storage, inhibition of hexanal formation due to the addition of 200 ppm of catechin and evening primrose additives to meat were in the range of 42 to 99% (Figure 4.20C and D).

Total volatiles formation in cooked comminuted pork was reduced (21 -74%) by the addition of borage crude extracts and its fractions (results for 100 and 200 ppm are shown in Tables A.13 and A.14, respectively). As seen for day-3 results, borage crude extract or its fraction IV failed to deliver effects greater than those for BHA and α-tocopherol. As shown in Figure 4.21A and B, total volatile formation in samples containing fraction IV and authentic sinapic acid at both 100 and 200 ppm, was not different ($p > 0.05$).

Evening primrose crude extract and its fractions (except fraction I) at 100 and 200 ppm, reduced the total volatiles contents in treated samples during the entire storage period (results for 100 and 200 ppm are given in Tables A.15 and A.16, respectively) as compared to the control. Evening primrose crude extract was less efficient at 100 ppm, but its activity was still comparable to that of catechin at the same concentration.

Figure 4.21 Effect of borage and evening primrose additives on formation of total volatiles in a cooked comminuted pork model system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.13 - A.16.


(A)
(B)
(C)
(D)
Total volatiles (mg/kg sample)
0
10
20
30
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
No additive
BHA
α-Tocopherol
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

Inhibition of total volatiles formation due to the incorporation of 200 ppm evening primrose crude extract and fractions II-V exceeded that of the reference antioxidants at the same concentration (Figure 4.21C and D).

Antioxidant activity of borage and evening primrose crude extracts and their fractions in cooked comminuted pork may be attributed to their metal-binding activity and free radical-scavenging properties (Kanner *et al.*, 1986). The ability of borage and evening primrose additives to decompose hydrogen peroxide could also contribute to their antioxidant effects. Relationships between antioxidant activity of additives and their metal chelating, hydrogen peroxide- and free radical-scavenging capacities will be discussed in sections **4.7.1**, **4.8.1** and **4.9.1**, respectively.

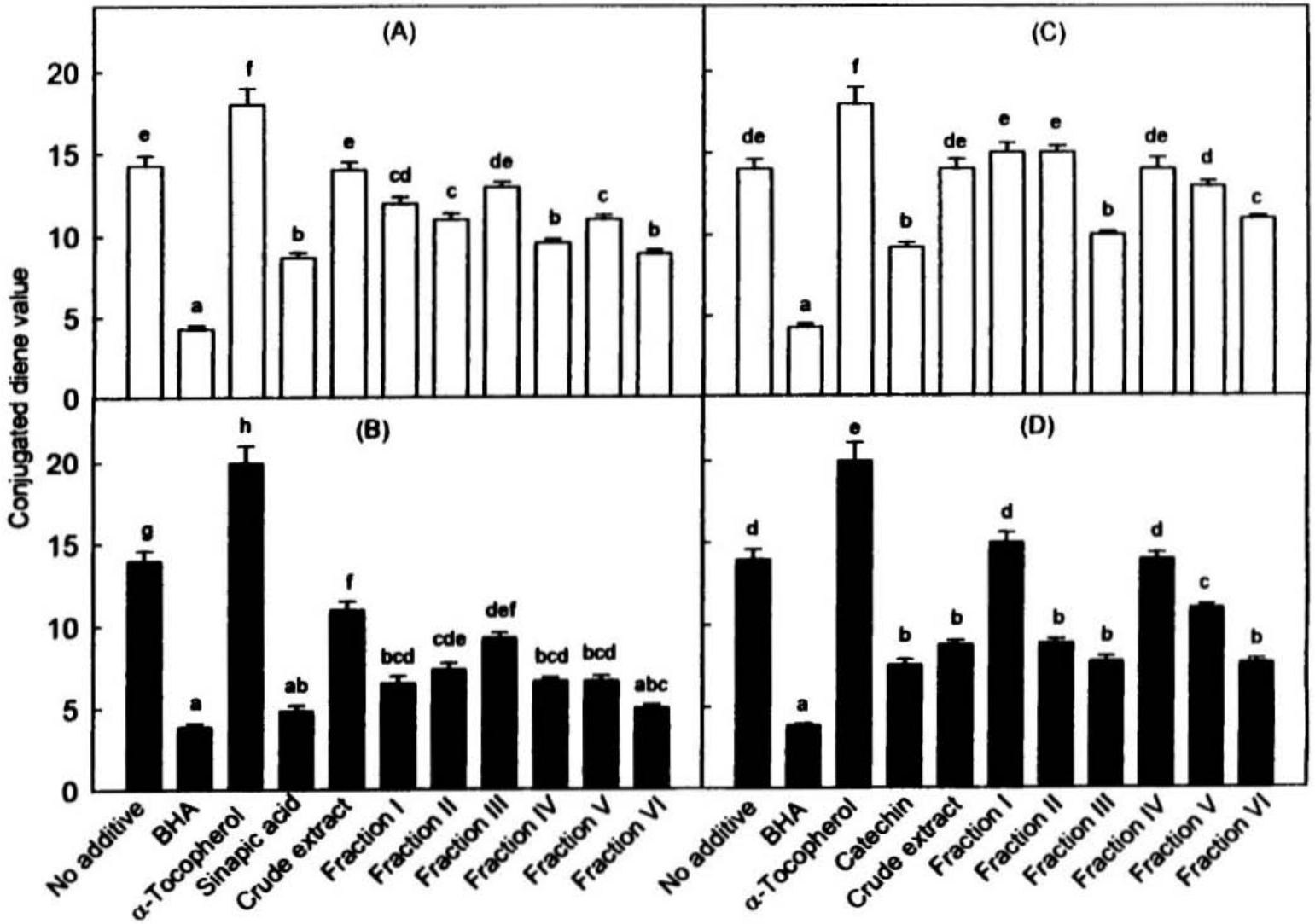
4.6.3 Antioxidant efficacy of borage and evening primrose crude extracts and their fractions in a bulk stripped corn oil model system

Commercial corn oil contains naturally occurring antioxidants, mainly tocopherols. Stripping corn oil of its natural antioxidants provides a better medium to evaluate the effects of added antioxidants without the interference from endogenous antioxidants (Frankel *el al.*, 1994). Regular corn oil may be stripped of its antioxidants by passing it through a column packed with silicic acid and charcoal. Commercially available stripped-corn oil was used in this study and its oxidative stability in the presence and absence of additives was assessed. Inhibition of formation of conjugated dienes (primary oxidation products) and volatiles (secondary oxidation products) in treated samples was used as an

indication of antioxidant activity of the additives being investigated. The dominant volatile formed in corn oil, like in lard, was hexanal, thus it was chosen as an indicator of oxidation in this system.

Conjugated diene values of bulk stripped-corn oil (fatty acid composition is given in Table 4.1) containing borage crude extract and its fractions at 100 and 200 ppm, are given in Tables A.17 and A.18, respectively. Conjugated diene values of treated oil increased by about 7 - 10-fold at the end of a 7-day storage period, whereas the increase for the control was about 11-fold. BHA exerted the greatest inhibition on conjugated diene formation with only 4-fold increase for the entire storage period. To simplify the comparison between different additives, results for day-3 of storage (Figure 4.22A and B) were chosen as there were differences in the trends for some additives at different days of storage. Borage crude extract and fraction III, at 100 ppm, were unable to inhibit the formation of conjugated dienes as there were no significant differences ($p > 0.05$) among results for treated and control samples, but the other fractions, at the same concentration, resulted in 21 -36% lower conjugated diene values as compared to the control. Both concentrations of α-tocopherol were inefficient as day-3 conjugated diene value of treated oil was greater than that of the control on the same day. Regardless of the concentration used, conjugated diene formation in oil samples treated with BHA and sinapic acid was reduced by 38 - 72% as compared to the control. At higher concentration, borage crude extract and its fractions were able to significantly ($p \leq 0.05$) reduce the formation of conjugated dienes in the treated oil by 21 - 64%.

Figure 4.22 Effect of borage and evening primrose additives on formation of conjugated dienes in a bulk stripped corn oil model system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.17 - A.20.

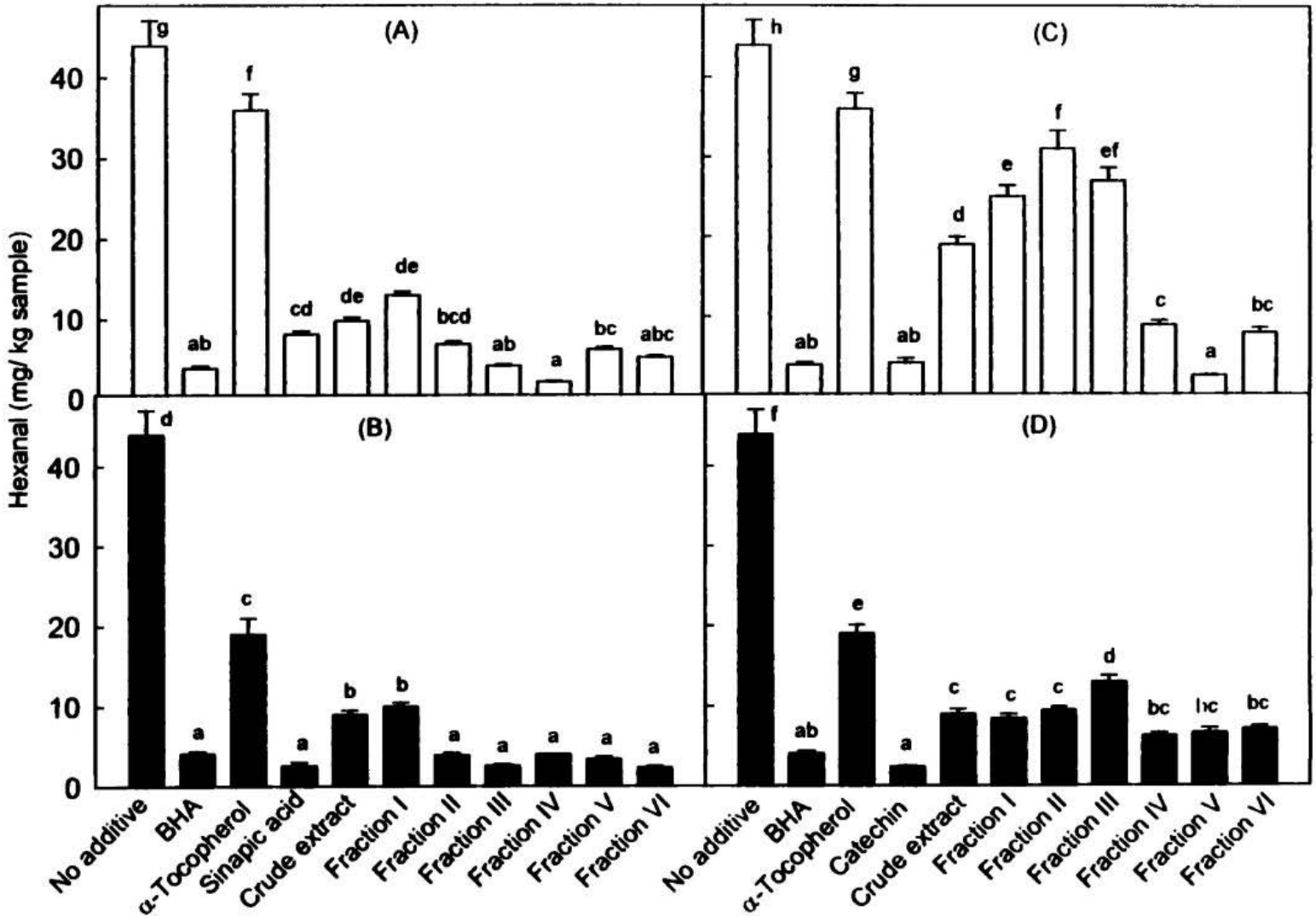

(A)
(B)
(C)
(D)
Conjugated diene value
0
5
10
15
20
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin

At 100 ppm, evening primrose crude extract and its fractions I, II, IV and V were unable to inhibit the conjugated diene formation in bulk oil to a greater extent. Only fractions III and VI performed well at both concentrations (Tables A.19 and A.20). When added to oil at 200 ppm, evening primrose crude extract and its fractions, except fractions I and IV, reduced the formation of conjugated dienes (on day-3) by 21 - 45% as compared to the control. Fractions I and IV had very little or no effect on the inhibition of formation of conjugated dienes in bulk oil even at 200 ppm addition level (Figure 4.22C and D).

Hexanal contents of bulk stripped-corn oil treated with borage crude extract and its fractions at 100 and 200 ppm are presented in Tables A.21 and A.22, respectively. Hexanal content of the control and treated samples increased throughout the entire storage period. On day-3 of storage, samples containing 100 (Figure 4.23A) and 200 ppm (Figure 4.23B) of borage crude extract and its fractions had 70 - 95% lower hexanal contents as compared to the control. Among reference antioxidants used, α-tocopherol was least effective while BHA and authentic sinapic acid exhibited strong antioxidant effects as shown by 81 - 95% lower hexanal contents of the treated oils. Inhibition of hexanal formation in samples treated with 200 ppm of borage fractions II-VI was comparable to those of BHA and sinapic acid.

Figure 4.23C and D depicts the day-3 hexanal contents of bulk stripped-corn oil samples containing 100 and 200 ppm of evening primrose crude extract and its fractions (data were taken from Tables A.23 and A.24). Evening primrose additives at both concentrations, reduced the hexanal formation in oil by 30 - 86% and the effects were

Figure 4.23 Effect of borage and evening primrose additives on formation of hexanal in a bulk stripped corn oil model system (at day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.21 - A.24.

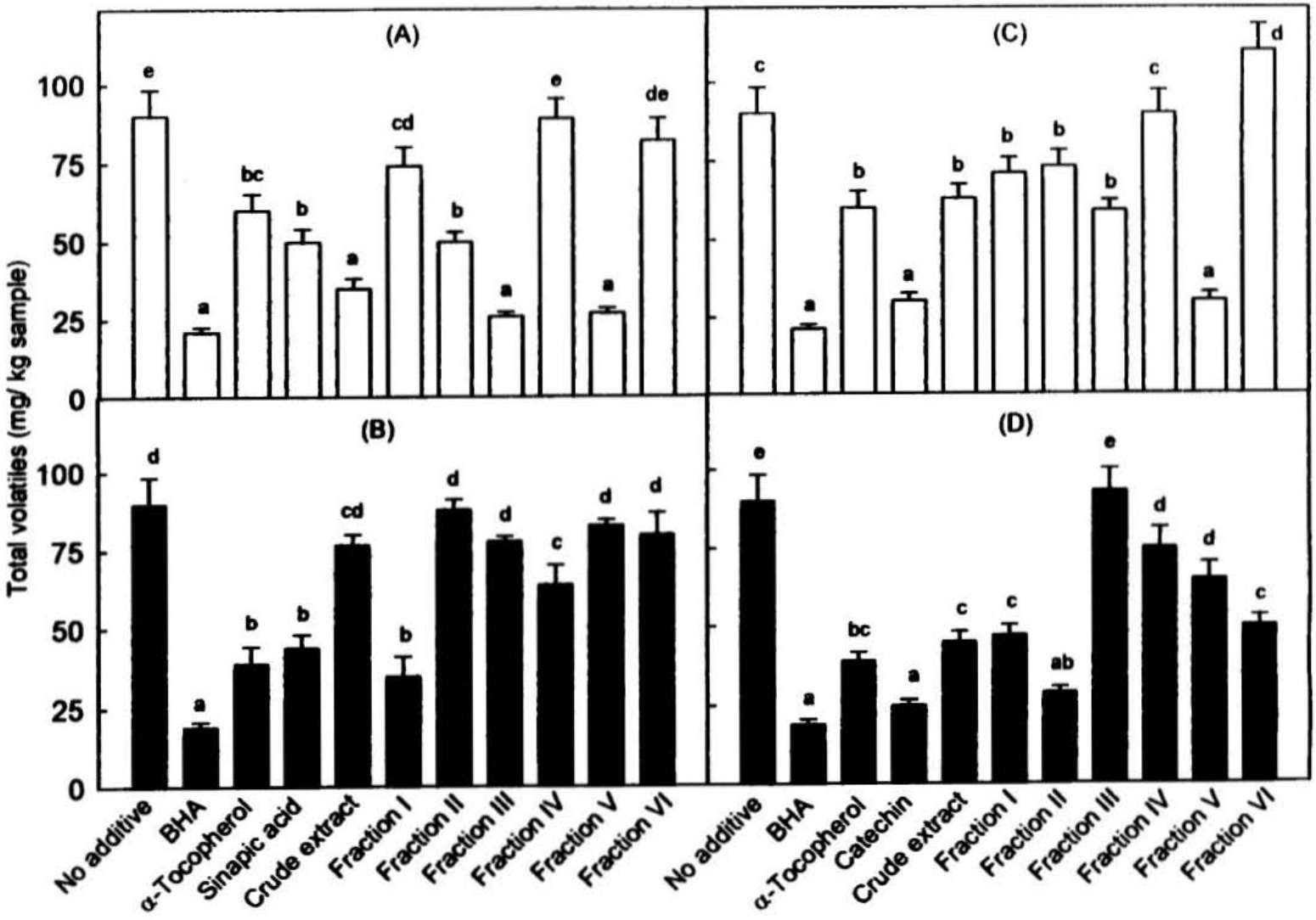

Hexanal (mg/ kg sample)
(A)
(B)
(C)
(D)
0
10
20
30
40
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin

more pronounced when the concentration of additives was 200 ppm. Among reference antioxidants used, authentic catechin exerted the strongest antioxidant effect (>90% inhibition) on hexanal formation.

Content of total volatiles in bulk stripped corn oil containing 100 and 200 ppm of crude borage extract and its fractions are given in Tables A.25 and A.26, respectively. Total volatiles formation in the control as well as treated samples increased as the oxidation of oil progressed. For some treatments, content of total volatiles first increased, peaked between day-3 and -5, and finally decreased. This may be due to the loss of low-molecular-weight volatiles towards the end of the storage period. In general, samples treated with borage additives, regardless of the concentration used, had lower contents of total volatiles throughout the entire storage period. As shown in Figure 4.24A, oil treated with 100 ppm of borage additives (except borage fraction IV and VI), had significantly ($p \leq 0.05$) lower contents (44 -71%) of total volatiles on day-3 as compared to the control on the same day. On day-3, total volatiles of samples treated with 200 ppm of fractions I and IV were reduced by 29 and 61%, respectively. Among reference antioxidants, BHA exhibited the greatest antioxidant effect (>75% inhibition) on the formation of volatiles. Reduction in total volatile contents of samples treated with 100 and 200 ppm of α-tocopherol and sinapic acid was in the range of 33 to 56%.

As presented in Tables A.27 and A.28, content of total volatiles in the control and bulk stripped corn oil, treated with evening primrose additives, increased over a 7-day storage period. Comparison of data for day-3 showed BHA at both concentrations was

Figure 4.24 Effect of borage and evening primrose additives on formation of total volatiles in a bulk stripped corn oil model system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.25 - A.28.


(A)
(B)
(C)
(D)
Total volatiles (mg/ kg sample)
0
25
50
75
100
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
No additive
BHA
α-Tocopherol
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

the most efficient inhibitor of total volatile formation (Figures 4.24C and D). Contents of total volatiles in bulk oil treated with 100 and 200 ppm of BHA was reduced by 77 and 79%, respectively. Alpha-tocopherol and sinapic acid at 100 ppm reduced the formation of total volatiles by 33 and 67%, respectively. At 200 ppm, α-tocopherol and sinapic acid reduced the total volatiles formation by approximately 57 and 72%, respectively. On day-3, contents of total volatiles in samples containing 100 ppm evening primrose crude extract and fractions I, II, III and V, were 18 - 67% lower as compared to the control. Evening primrose fraction IV did not have any effect on total volatile content on day-3 of storage. Furthermore, bulk oil treated with 100 ppm of evening primrose fraction VI had the highest total volatiles content on day-3. Day-3 data for bulk oil treated with 200 ppm of evening primrose additives showed that the formation of total volatiles was reduced by 17 - 72% due to the presence of additives. On day-3, total volatiles content of bulk oil containing 200 ppm of fraction III was not significantly ($p > 0.05$) different from that of the control. With 200 ppm, the effect of evening primrose crude extract and its fraction I on volatile formation in bulk oil was not different.

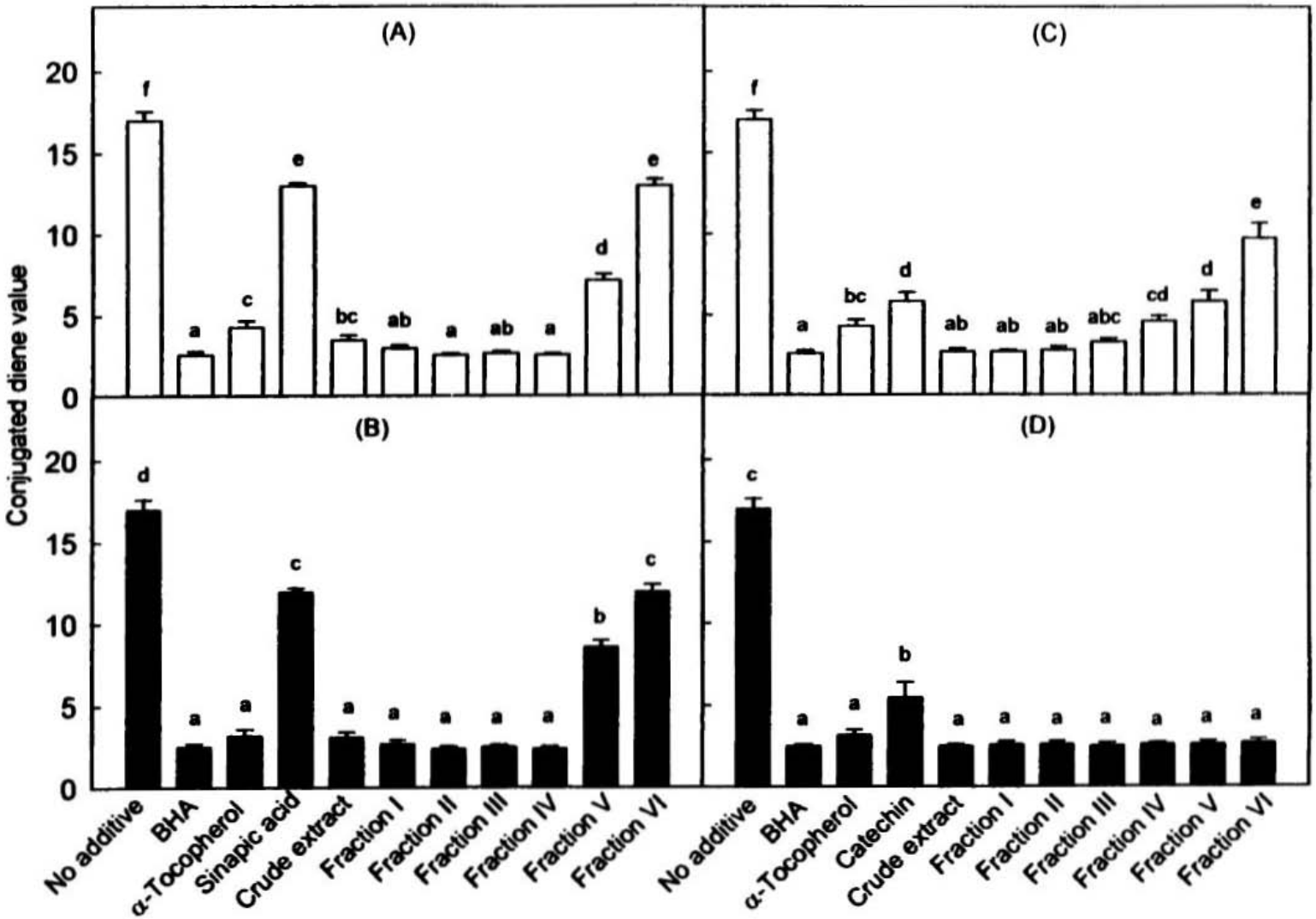
The antioxidant effect of both borage and evening primrose additives in bulk stripped corn oil could arise from their ability to chelate metal ions and also to scavenge free radicals. Partition of hydrophilic phenolics of additives into oil-air interface of the bulk oil may have also played a role in determining the efficacy of additives. Some additives even with low amount of hydrophilic phenolics were able to act as efficient antioxidants in bulk oil. This effect could be attributed to the structural features of their

phenolic constituents which made them highly efficient regardless of the physical nature of the system in which they were evaluated. Results for α-tocopherol, sinapic acid and catechin supported the hypothesis that hydrophilic antioxidants were more efficient in bulk oil. However, this hypothesis is not always applicable to crude antioxidants such as the ones used in this study because all of them contained substantial amounts of both hydrophilic and hydrophobic phenolics which could effectively inhibit lipid oxidation in bulk phase as well as at the interfaces. Relationships between antioxidant activities of additives in bulk oil and their metal chelating capacities, hydrogen peroxide scavenging and free radical scavenging capacities will be discussed in sections **4.7.1**, **4.8.1** and **4.9.1**, respectively).

4.6.4 Antioxidant efficacy of borage and evening primrose crude extracts and their fractions in a stripped corn oil-in-water emulsion system

In order to investigate the antioxidant efficacy of borage and evening primrose extracts and their fractions in a biphasic system, an oil-in-water emulsion of stripped-corn oil was used. Oxidative stability of the emulsions was assessed by quantitating conjugated dienes, hexanal and total volatiles in the samples. Conjugated diene values of oil extracted from emulsions containing 100 and 200 ppm of borage crude extract and its fractions are given in Tables A.29 and A.30, respectively. In general, conjugated dienes in treated as well as non-treated emulsions increased by 4-11 folds during the 7-day storage. Day-3 results, as depicted in Figure 4.25A and B, will be used to compare the antioxidant

Figure 4.25 Effect of borage and evening primrose additives on formation of conjugated dienes in a stripped corn oil-in-water emulsion system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.29 - A.32.


(A)
(B)
(C)
(D)
Conjugated diene value
0
5
10
15
20
No additive
BHA
α-Tocopherol
Sinapic acid
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

efficacies of the additives. On day-3, 100 ppm of reference antioxidants, BHA, α-tocopherol, and sinapic acid, reduced the formation of conjugated dienes by approximately 85, 75 and 23%, respectively. The formation of conjugated dienes in samples containing 200 ppm of BHA, and sinapic acid were similar to those of samples treated with 100 ppm. Conjugated diene value for the sample containing 200 ppm of α-tocopherol, was 81% lower than that for the control. On the other hand, sinapic acid was not as effective as it was in the bulk oil system. This could be because of dilution of sinapic acid in the aqueous phase due to its hydrophilicity. Unlike in bulk oil, α-tocopherol more effectively reduced the formation of conjugated dienes in this model system, perhaps due to its partitioning into lipid phase of the emulsion and orientation at the lipid-water interface, thus effectively preventing lipid oxidation at the interface (Huang *et al.*, 1994). On day-3 conjugated diene values for samples treated with 100 ppm of borage crude extract and its fractions, were 23 - 85% lower than that of the control. The concentration effect of these additives on conjugated diene formation was not profound as the differences between data for 100 and 200 ppm were marginal. Inhibitory effects of borage fractions I-IV, at both concentrations, were comparable to that of BHA.

As reflected by conjugated diene values presented in Tables A.31 and A.32, evening primrose crude extracts and its fractions, at both concentrations, resulted in 43 - 85% lower conjugated diene values as compared to the control. Conjugated diene values of samples containing additives at 200 ppm were 84 - 85% lower than that of the control

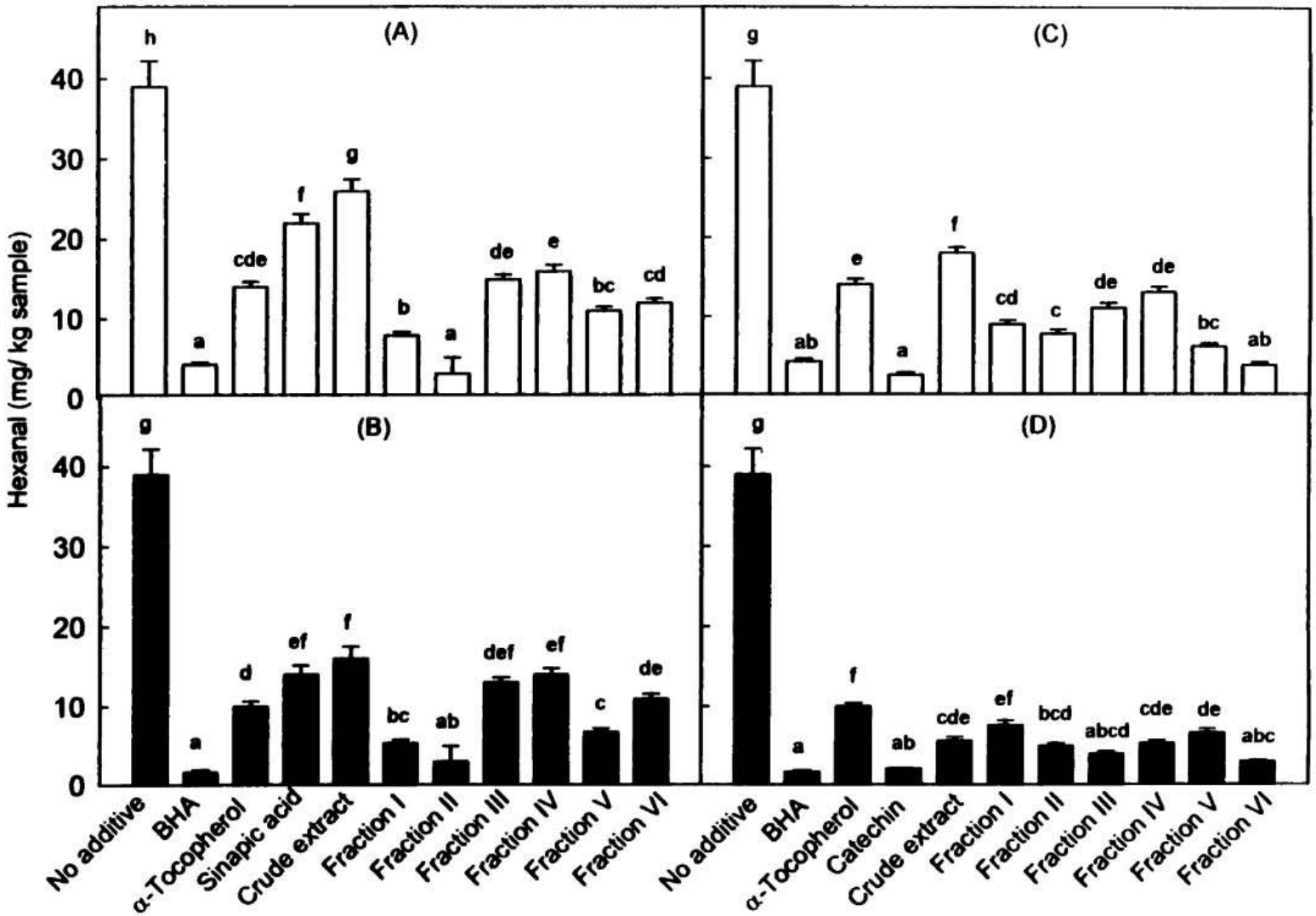
and were comparable to those of BHA and α-tocopherol and better than that of the authentic catechin (Figure 4.25C and D).

Borage crude extract and its fractions at 100 and 200 ppm inhibited the formation of hexanal in samples throughout the entire storage period (Tables A.33 and A.34). On day-3 of storage, hexanal contents in samples treated with borage additives were 33 - 94% lower than that of the control (Figure 4.26A and B). Borage fraction II, at both concentrations, exhibited the strongest inhibition as hexanal contents of treated samples were >92% lower as compared to the control and were comparable to that of BHA. At both concentrations, inhibitory effects of other additives were lower than that of BHA. Hexanal contents of samples treated with 200 ppm of borage additives (except crude extract) were either comparable or lower than those of α-tocopherol- and sinapic acid-treated samples.

As shown in Tables A.35 and A.36, evening primrose additives reduced the formation of hexanal in treated samples throughout the entire storage period. Day-3 hexanal contents of samples containing 100 and 200 ppm of additives are depicted in Figure 4.26C and D, respectively. Evening primrose additives reduced the formation of hexanal in the samples to varying degrees (54 - 95%) and the effects were concentration-dependent. Among reference antioxidants, BHA and catechin exerted strong antioxidant effect while α-tocopherol at both concentrations, was relatively less effective.

Higher contents of total volatiles were present in samples treated with borage additives (Table A.37 and A.38). On day-3 of storage, only fraction III resulted in

Figure 4.26 Effect of the borage and evening primrose additives on formation of hexanal in a stripped corn oil-in-water emulsion system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.33 - A.36.

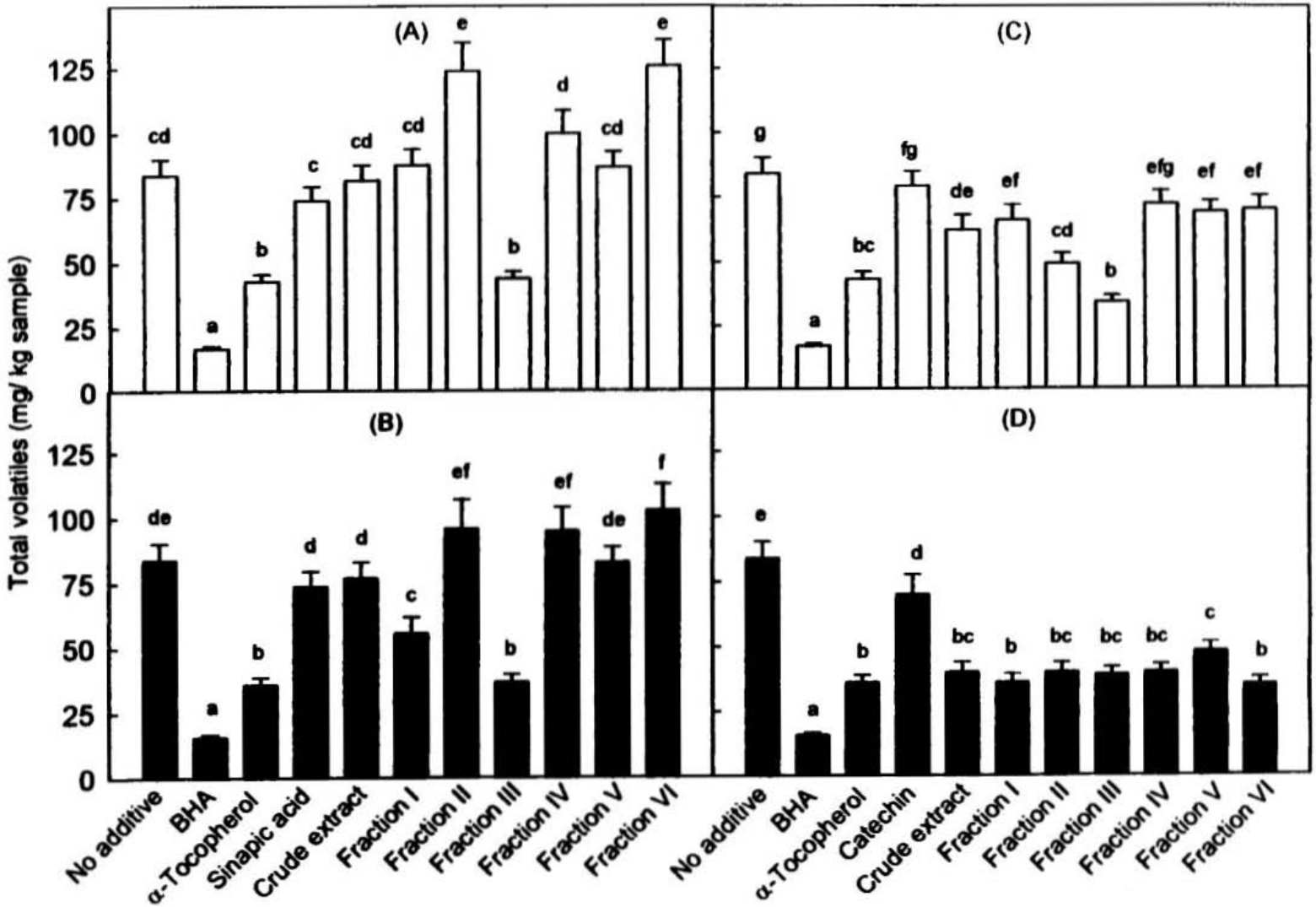

(A)
(B)
(C)
(D)
Hexanal (mg/ kg sample)
0
10
20
30
40
h
a
cde
f
g
b
a
de
e
bc
cd
g
a
d
ef
f
bc
ab
def
ef
c
de
g
ab
e
a
f
cd
c
de
de
bc
ab
g
a
f
ab
cde
ef
bcd
abcd
cde
de
abc
No additive
BHA
α-Tocopherol
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
No additive
BHA
α-Tocopherol
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

significantly ($p \leq 0.05$) lower contents of total volatiles. Contents of total volatiles in samples treated with other additives, except reference antioxidants, BHA and α-tocopherol, were either similar to or significantly ($p \leq 0.05$) higher than that of the control (Figure 4.27A and B).

Contents of total volatiles in emulsions containing evening primrose additives are given in Tables A.39 and A.40. As evidenced by day-3 data, total volatiles formation in samples containing 100 ppm of fraction IV and catechin was not significantly ($p > 0.05$) different from that of the control while all other additives reduced the content of total volatiles in the treated samples (Figure 4.27C). At 200 ppm, all additives reduced (16 - 58%) the content of total volatiles in treated samples as compared to the control (4.27D).

The observed differences in the antioxidant properties of additives in bulk stripped-corn oil and its oil-in-water emulsion may be attributed to the solubility characteristics and surface activities of the antioxidant molecules. Being a strong antioxidant, BHA was able to reduce the extent of lipid oxidation in both bulk oil and emulsions, probably due to the strong ability of BHA to donate hydrogen atoms to free radicals and subsequent formation of a stable BHA free radical. On the other hand, α-tocopherol, a lipophilic antioxidant, exhibited a better antioxidant effect in emulsion than in bulk oil. This was not surprising because α-tocopherol is not only lipophilic, but it also possesses surface activity (Huang *et al.*, 1994). These two properties make α-tocopherol to partition at the oil-water interfaces of an emulsion, thus efficiently protect lipids from oxidation (Huang *et al.*, 1994). In contrast, catechin and sinapic acid are hydrophilic, thus rapidly partitioned at

Figure 4.27 Effect of borage and evening primrose additives on formation of total volatiles in a stripped corn oil-in-water emulsion system (on day 3). (A) and (B) depict the effect of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the effect of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another. Data were taken from Tables A.37 - A.40.


(A)
(B)
(C)
(D)
Total volatiles (mg/ kg sample)
0
25
50
75
100
125
No additive
BHA
α-Tocopherol
Sinapic acid
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

the oil-air interface of the bulk oil where the oxidation reactions are most likely to take place due to high oxygen stress. Dilution of α-tocopherol in the bulk phase reduced its antioxidant efficacy. Similarly, dilution of catechin and sinapic acid in the aqueous phase of an emulsion system made them less effective at these concentrations. Unlike pure reference antioxidants, borage and evening primrose additives contain both hydrophilic and hydrophobic constituents. In general, their antioxidant effects in bulk and emulsion systems cannot be solely explained based upon solubility characteristics of their active components. Other factors, such as the structural features of active components and presence or absence of prooxidants may well contribute to the overall performance of borage and evening primrose additives in both systems. Nonetheless, it was evident that the presence of balanced or near-balanced amounts of hydrophilic and hydrophobic phenolics in most of the evening primrose additives made them efficient in both bulk oil and emulsion systems. In general, borage additives contained a lesser amount of hydrophobic components as compared to hydrophilic, thus rendering them rather inefficient in emulsions than in bulk oil. The antioxidant effects of borage and evening primrose additives in bulk and emulsion systems will be revisited in later sections (**4.7.1**, **4.8.1** and **4.9.1**) with an emphasis on antioxidant mechnisms.

4.7 Iron (II) chelation capacity of borage and evening primrose crude extracts and their fractions

In this series of experiments, tetramethylmurexide was used to quantitatively determine iron (II) chelating capacity of the additives. Tetramethylmurexide is a chelating agent which shows an absorption maximum at 530 nm. However, shifting of the absorption maximum to 460 nm occurs when a metal ion chelates with tetramethyl-murexide. The ratio of absorbance at 460 nm to 530 nm is linearly correlated with the metal ion concentration (Asakura *et al.*, 1990). When a known concentration of iron (II) is added to a buffered solution of additives, some of the iron (II) chelates with additives while leaving unreacted or free iron (II) in the solution. When tetramethylmurexide is added to the solution, it chelates the remaining iron (II) and the absorbance maximum shifts from 530 to 460. Then the unreacted iron (II) can be determined from a calibration line and the concentration of iron (II) chelated by additives calculated by subtracting free iron (II) concentration from that initially present (Asakura *et al.*, 1990).

As shown in Table 4.10, 100 and 200 ppm of borage crude extract chelated 43 and 91% of iron (II), respectively. Iron (II) chelation for 100 ppm of borage fractions ranged from 33 to 62%, whereas 200 ppm of fractions chelated 56 - 100% of iron (II). A complete chelation of added iron (II) was evident for borage fraction III. As a reference antioxidant, sinapic acid also exhibited a strong iron (II) chelation capacity. Evening primrose crude extract at 100 and 200 ppm chelated 63 and 100% of iron (II), respectively. For both concentrations, evening primrose fractions III and V exhibited a

Table 4.10 Concentration (μM) and proportion (%) of chealated iron (II) by borage crude extract and its fractions[1].

Additive	100 ppm		200 ppm	
	μM	%	μM	%
No additive	0 ± 0.00^{a}	0	0 ± 0.00^{a}	0
Sinapic acid	375 ± 28^{e}	74	506 ± 00^{d}	100
Crude extract	220 ± 18^{bc}	43	462 ± 46^{cd}	91
Fraction I	168 ± 15^{b}	33	283 ± 21^{b}	56
Fraction II	215 ± 20^{bc}	42	302 ± 18^{b}	60
Fraction III	312 ± 27^{d}	62	506 ± 00^{d}	100
Fraction IV	235 ± 21^{c}	46	407 ± 30^{c}	80
Fraction V	193 ± 14^{bc}	38	315 ± 24^{b}	62
Fraction VI	200 ± 18^{bc}	39	409 ± 33^{c}	81

[1] Results are mean values of three determinations $\pm$ standard deviation. Values sharing the same superscript in a column are not significantly different at $p > 0.05$.

complete chelation of iron (II). At 200 ppm, all additives, except fraction I, chelated 100% of the added iron (II) (Table 4.11).

The iron (II) chelation properties of the additives may be attributed to metal chelating agents, mainly phenolics, present in the additives. Certain phenolic compounds have properly oriented functional groups which can chelate metal ions (Thompson *et al.*, 1976). For example, van Acker *et al.* (1996) reported that flavonoids chelate iron (II) with chelating capacities ranging from strong to weak depending upon the structural features. Thompson *et al.* (1976) found that the stability of the metal-antioxidant complex is higher in six-membered ring than five-membered ring complexes. The greater iron (II) chelation capacities of catechin and evening primrose additives as compared to those for borage additives could stem from their formation of six-membered complexes with iron (II).

4.7.1 Relationship between iron (II) chelating capacity and antioxidant activity of borage and evening primrose additives in model systems

Strength of the relationships was determined by performing linear regression analysis on data observed for model systems. Correlation coefficients (r) greater than 0.9000 were considered as very strong and those of 0.7500 - 0.9000 were designated as strong. Correlation coefficients ranging from 0.6000 to 0.7500 were designated as good while those less than 0.6000 were considered poor.

Table 4.11 Concentration (μM) and proportion (%) of chealated iron (II) by evening primrose crude extract and its fractions[1].

Additive	100 ppm		200 ppm	
	μM	%	μM	%
No additive	0 ± 0.00[a]	0	0 ± 0.00[a]	0
Catechin	506 ± 00[f]	100	506 ± 00[f]	100
Crude extract	318 ± 29[c]	63	506 ± 00[f]	100
Fraction I	284 ± 23[c]	56	376 ± 34[b]	74
Fraction II	180 ± 16[b]	36	506 ± 00[f]	100
Fraction III	506 ± 00[f]	100	506 ± 00[f]	100
Fraction IV	459 ± 34[d]	91	506 ± 00[f]	100
Fraction V	506 ± 00[f]	100	506 ± 00[f]	100
Fraction VI	308 ± 23[c]	61	506 ± 00[f]	100

[1] Results are mean values of three determinations ± standard deviation. Values sharing the same superscript in a column are not significantly different at $p > 0.05$.

Figure 4.28 depicts the relationship between iron (II) chelation capacities and antioxidant activities of corresponding borage and evening primrose additives in a β-carotene-linoleate model system. Contribution of iron (II) chelating capacities of borage additives to protect β-carotene against bleaching was poor (Figure 4.28A, r = 0.4520) whereas that for evening primrose additives was good (Figure 4.28B, r = 0.7227). This suggested that most of the observed antioxidant activities of borage antioxidants in this model system were due to mechanisms other than metal chelation. For evening primrose additives, metal chelation played a major role in this model system, possibly due to the formation of stable six-membered chelation complexes by their active compounds. For evening primrose additives, partitioning of more hydrophobic phenolics into the lipid phase of the emulsion might have enabled them to chelate more of the prooxidant metal ions in that phase.

Good correlation coefficients were evident for relationships between iron (II) chelation and formation of TBARS (r = 0.7273), hexanal (r = 0.6704) and total volatiles (r = 0.7061) in cooked comminuted pork treated with borage additives (Figure 4.29). As depicted in Figure 4.30, iron (II) chelation capacities of evening primrose additives strongly correlated with TBARS (r = 0.7786) and hexanal (r = 0.8055) formation in the treated samples while total volatiles had a good correlation (r = 0.7479). Free metal irons and heme complexes, especially free iron (II) and ferritin, play a major role in initiating and propagating lipid oxidation in muscle systems (Kanner and Doll, 1991). Calcium (II) is also known to accelerate lipid oxidation in muscle tissues in a pattern similar to that of

Figure 4.28 Relationships between iron (II) chelating capacity of borage (A)/evening primrose (B) additives and stability of β-carotene in a β-carotene-linoleate model system containing the respective additives.

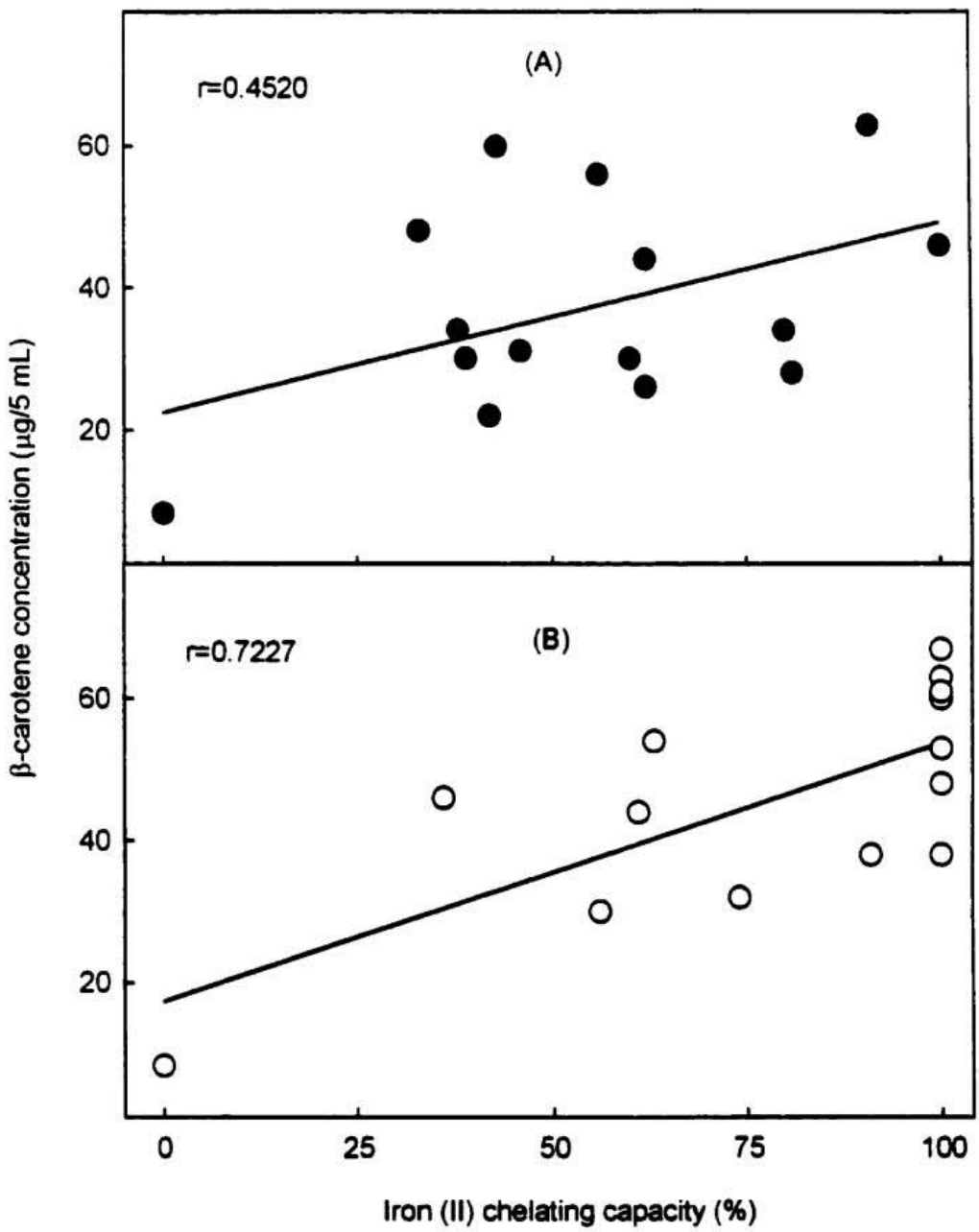

(A)
r=0.4520
60
40
20
(B)
r=0.7227
60
40
20
0
25
50
75
100
β-carotene concentration (μg/5 mL)
Iron (II) chelating capacity (%)

Figure 4.29 Relationships between iron (II) chelating capacity of borage additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.

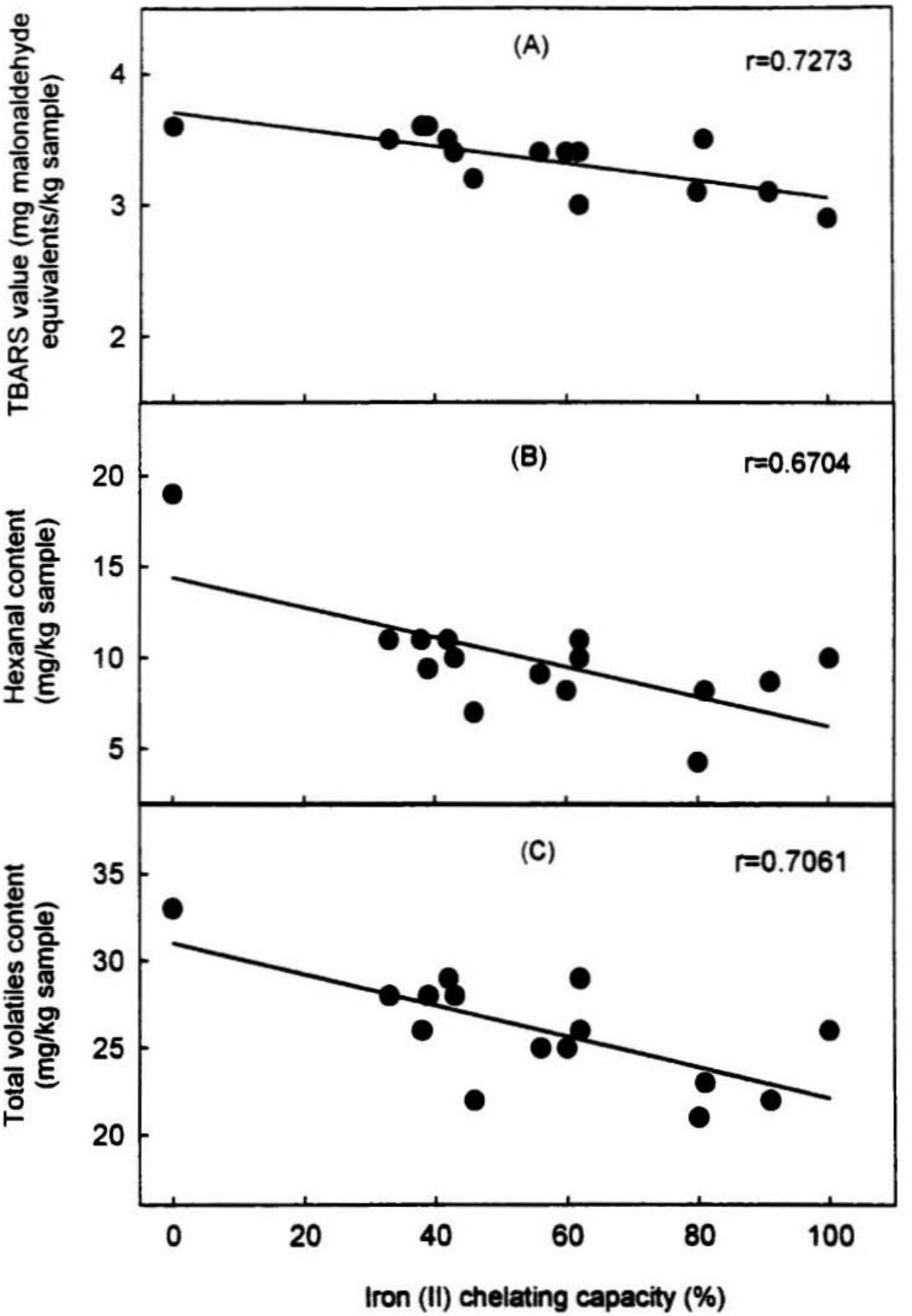
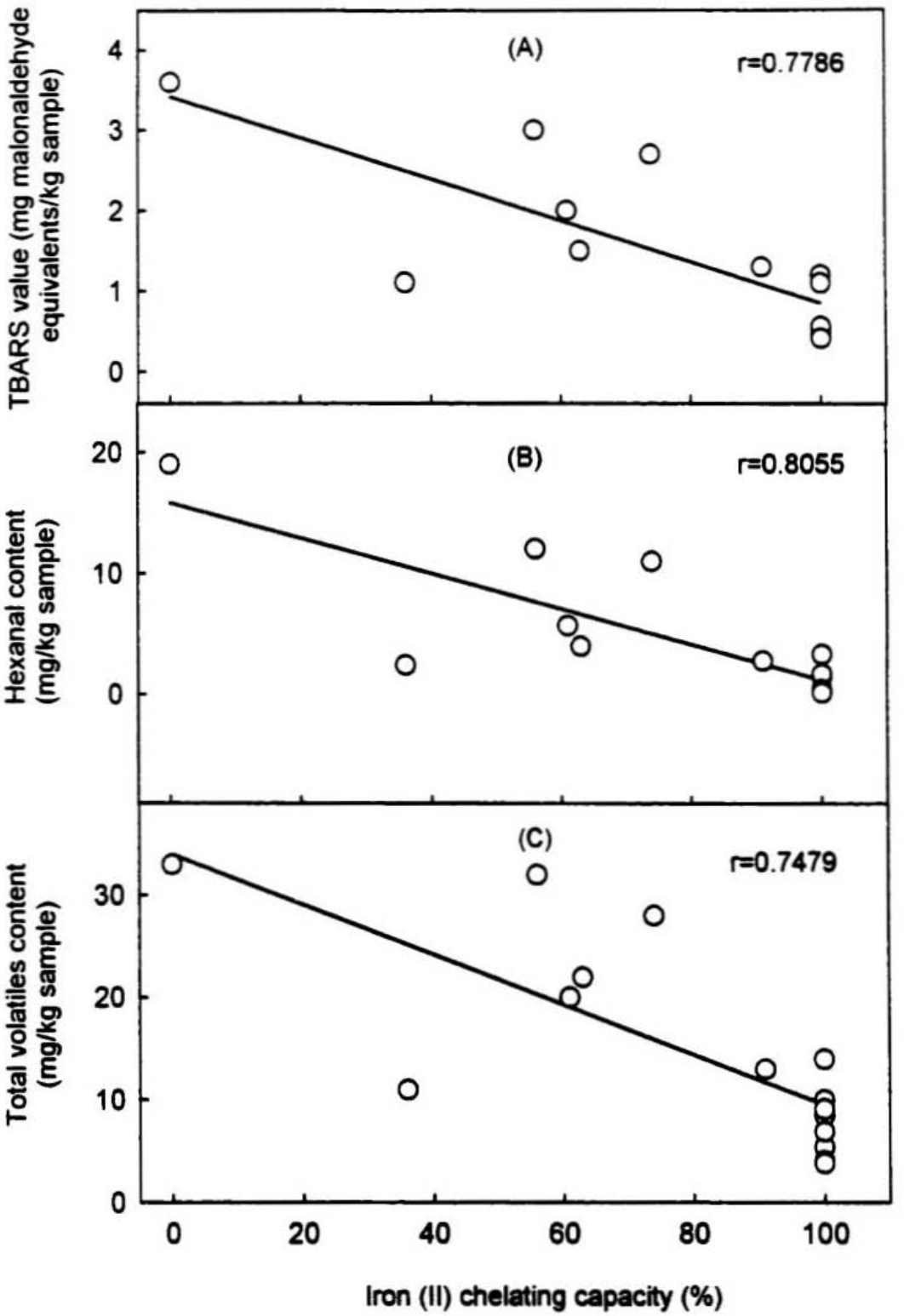

(A)
r=0.7273
TBARS value (mg malonaldehyde equivalents/kg sample)
4
3
2
(B)
r=0.6704
Hexanal content (mg/kg sample)
20
15
10
5
(C)
r=0.7061
Total volatiles content (mg/kg sample)
35
30
25
20
0
20
40
60
80
100
Iron (II) chelating capacity (%)

Figure 4.30 Relationships between iron (II) chelating capacity of evening primrose additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.


TBARS value (mg malonaldehyde equivalents/kg sample)
(A)
r=0.7786
Hexanal content (mg/kg sample)
(B)
r=0.8055
Total volatiles content (mg/kg sample)
(C)
r=0.7479
Iron (II) chelating capacity (%)

iron (II) catalysis, but copper (II) has been reported to be less effective as a prooxidant in muscle systems due to its low availability in tissues (Tichivangana and Morrissey, 1985). Therefore, metal chelating ability of borage and evening primrose additives could improve the oxidative stability of treated samples by rendering metal ions unavailable for catalysis of initiation and propagation reactions of lipid oxidation.

Figure 4.31 depicts the relationships between iron (II) chelating capacities of borage additives and formation of oxidation products in treated bulk stripped-corn oil. Formation of conjugated dienes in samples treated with borage additives was poorly correlated ($r = 0.5327$) with the iron (II) chelating capacities of the corresponding additives. A good correlation ($r = 0.6738$) existed between iron chelation capacities of borage additives and hexanal formation in treated bulk oil, but total volatiles did not show a linear relationship ($r = 0.0529$). Similar relationships were evident for samples treated with evening primrose crude extracts and its fractions, except for a strong relationship ($r = 0.8041$) between hexanal formation and iron (II) chelation (Figure 4.32). These results suggest that the metal ions were involved in the catalysis of pathways which lead to the formation of hexanal in bulk oil, but may not have a profound prooxidant effect at the initiation stages of oxidation. This is, however, dependent upon the type and concentration of metal catalysts present in bulk oil. Furthermore, most of the metal ions present in the original corn oil might have been removed during the strippping process of corn oil, thus leaving behind extremely low concentrations of metal ions in the samples.

Figure 4.31 Relationships between iron (II) chelating capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

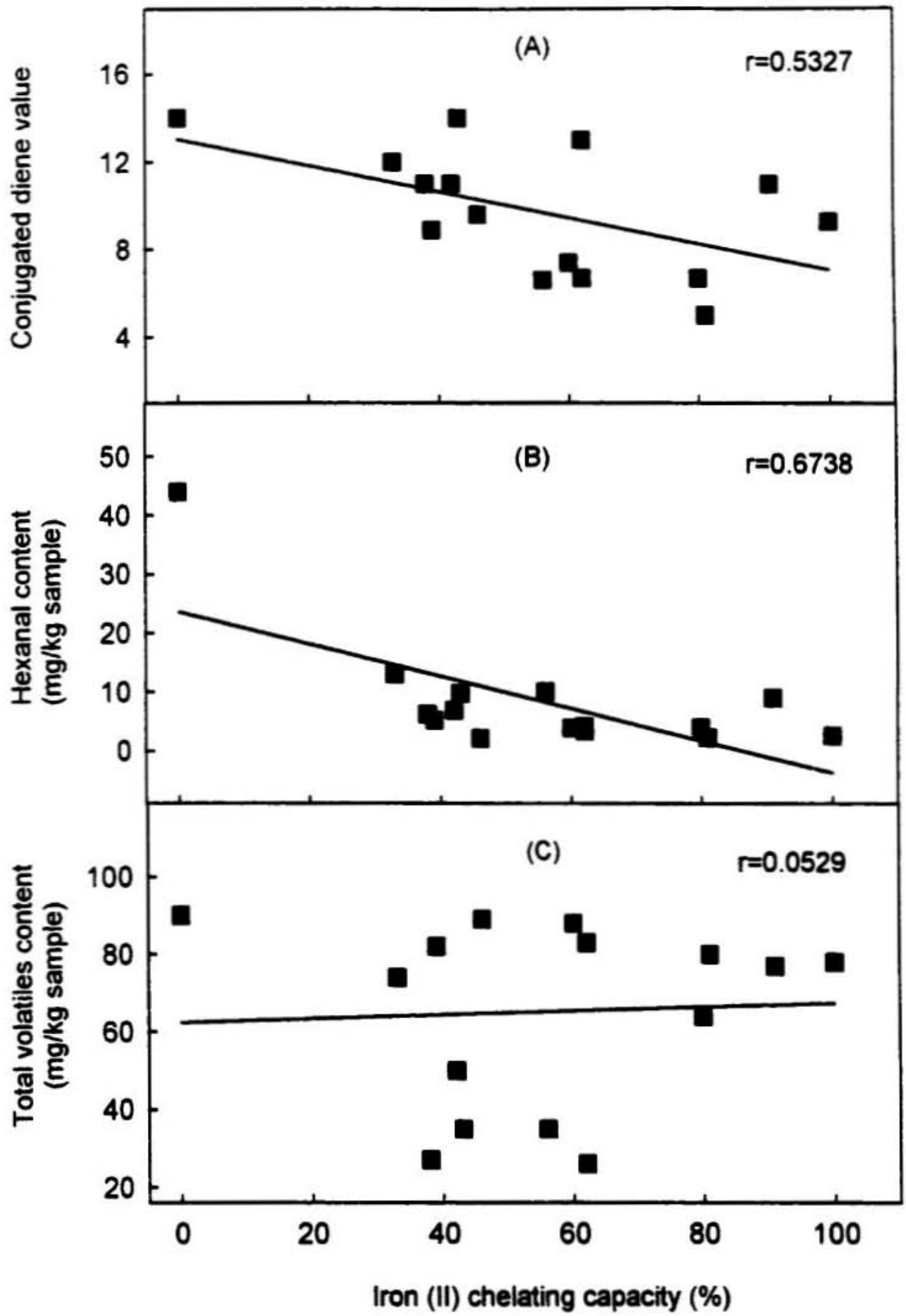
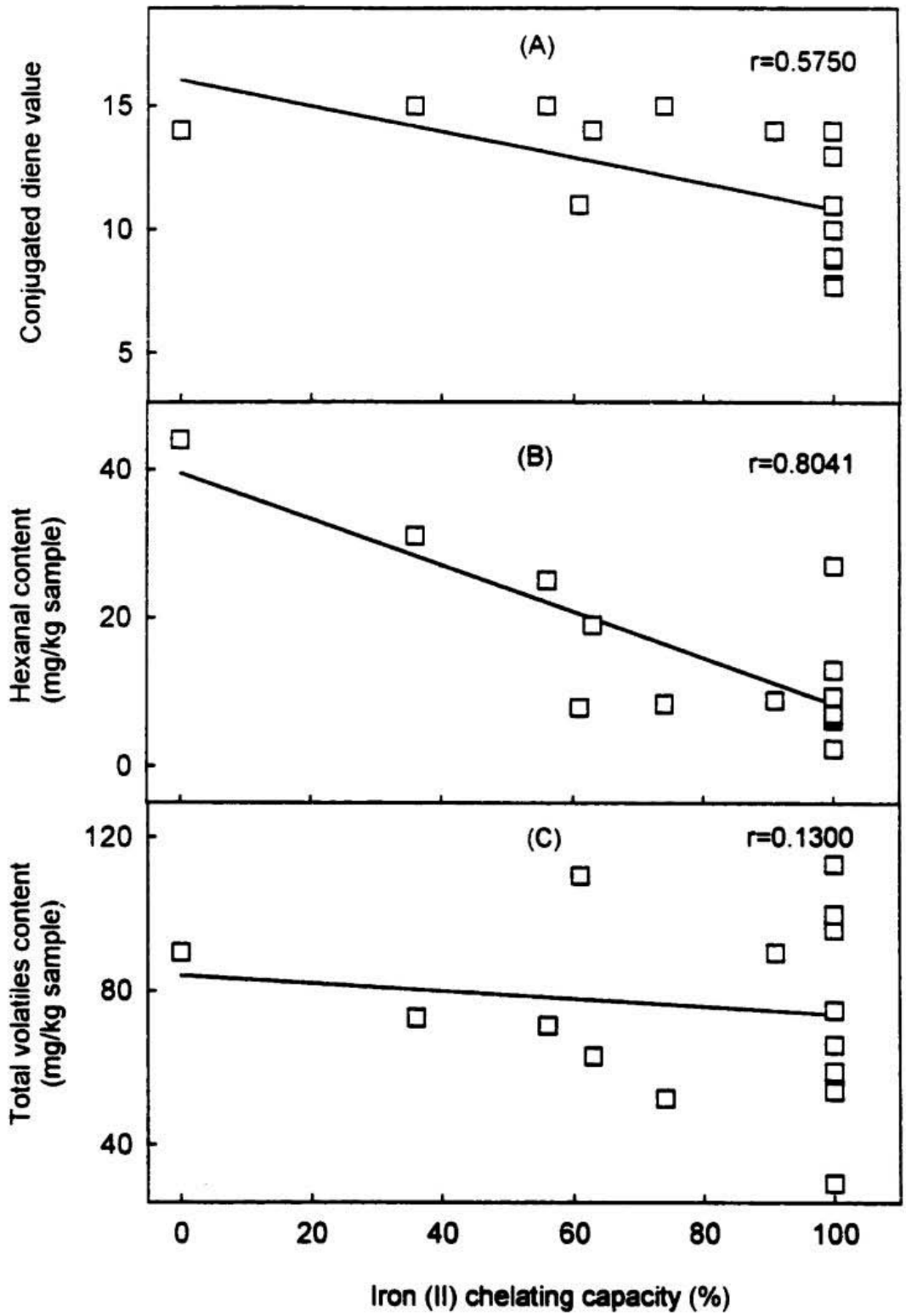

(A)
r=0.5327
Conjugated diene value
16
12
8
4
(B)
r=0.6738
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.0529
Total volatiles content (mg/kg sample)
100
80
60
40
20
0
20
40
60
80
100
Iron (II) chelating capacity (%)

Figure 4.32 Relationships between iron (II) chelating capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.


(A)
r=0.5750
Conjugated diene value
15
10
5
(B)
r=0.8041
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.1300
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Iron (II) chelating capacity (%)

The correlation between iron (II) chelation capacities and formation of oxidation products, conjugated dienes (r = 0.4588), hexanal (r = 0.4007) and total volatiles (r = 0.3669), in emulsions treated with borage additives were poor (Figure 4.33). However, as depicted in Figure 4.34, the iron (II) chelating capacities of evening primrose additives and the formation of oxidation products in emulsions treated with corresponding additives correlated well (r = 0.6227 - 0.7395). Poor correlations observed for borage additives in emulsions may be due to the insufficient distribution of hydrophobic phenolics on the oil-water interfaces where metal ions might have participated in oxidation reactions. On the other hand, evening primrose additives consisted of high amounts of both hydrophilic and hydrophobic phenolics which could partition into both lipid and aqueous phases of the emulsion. Some of these hydrophobic phenolics could have been oriented at the oil-water interfaces due to their surface activity. Therefore, metal chelating capacities of evening primrose additives might have played a significant role in preventing oxidation. Other antioxidant mechanisms might also have played a major role in preventing oxidation of corn oil in bulk phase and emulsion.

4.8 Hydrogen peroxide (H_2O_2)-scavenging capacity of borage and evening primrose crude extracts and their fractions

Figure 4.35 (A, B) depicts the H_2O_2-scavenging activities of 100 and 200 ppm of borage additives; values were lower for 100 ppm as compared to those for 200 ppm level of addition. At 100 ppm, none of the additives could completely remove H_2O_2 from the

Figure 4.33 Relationships between iron (II) chelating capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

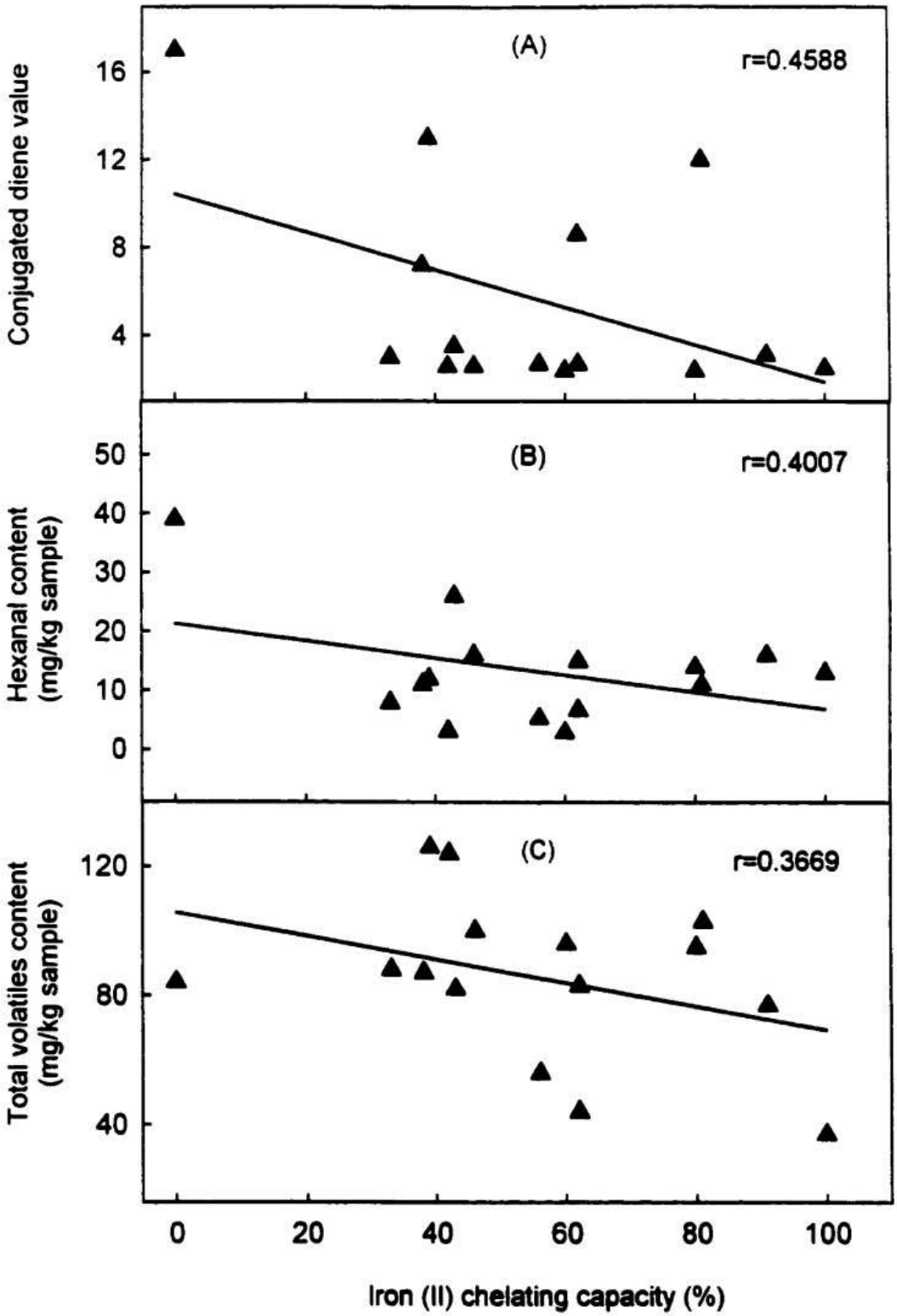

(A)
r=0.4588
Conjugated diene value
16
12
8
4
(B)
r=0.4007
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.3669
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Iron (II) chelating capacity (%)

Figure 4.34 Relationships between iron (II) chelating capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

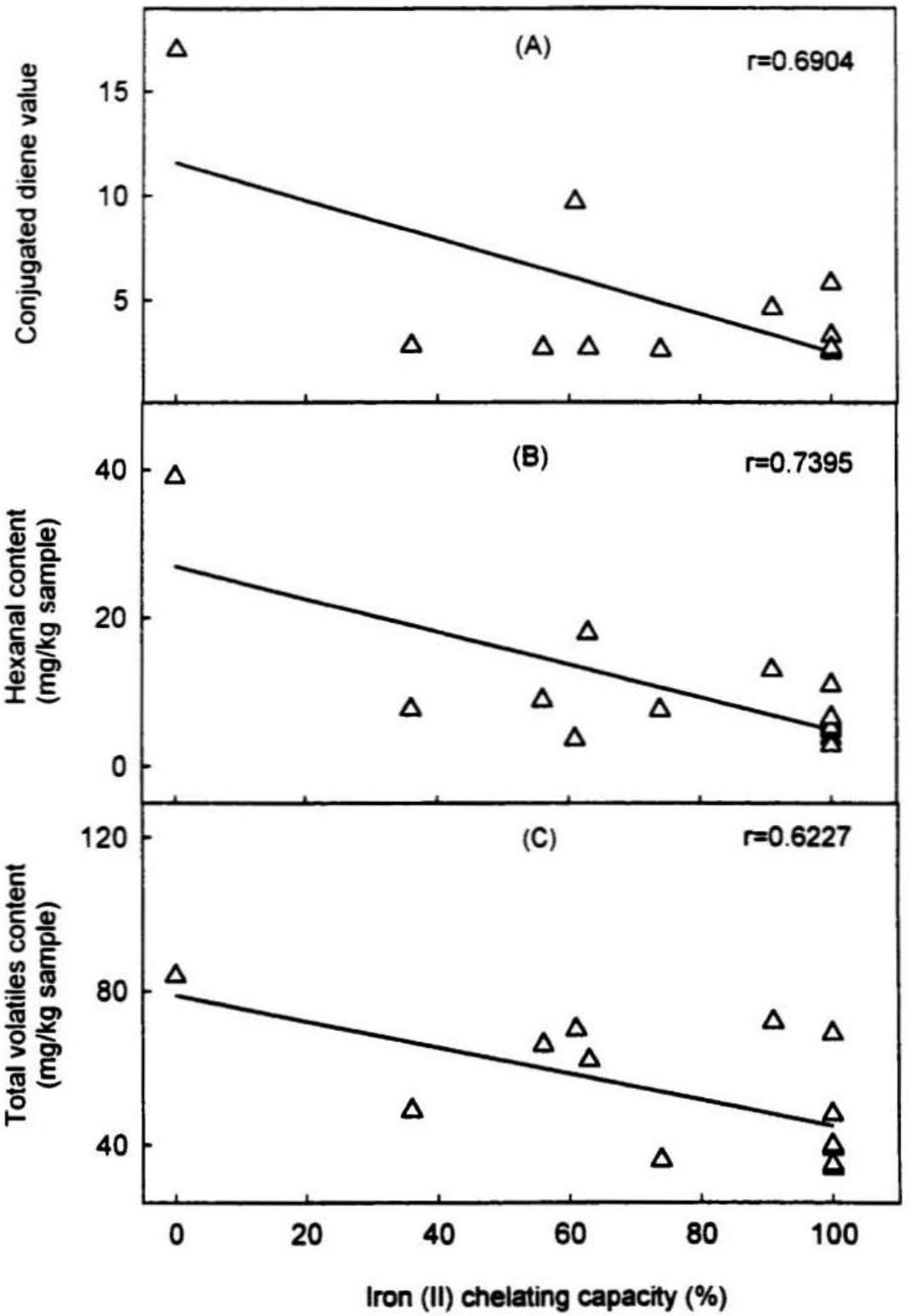
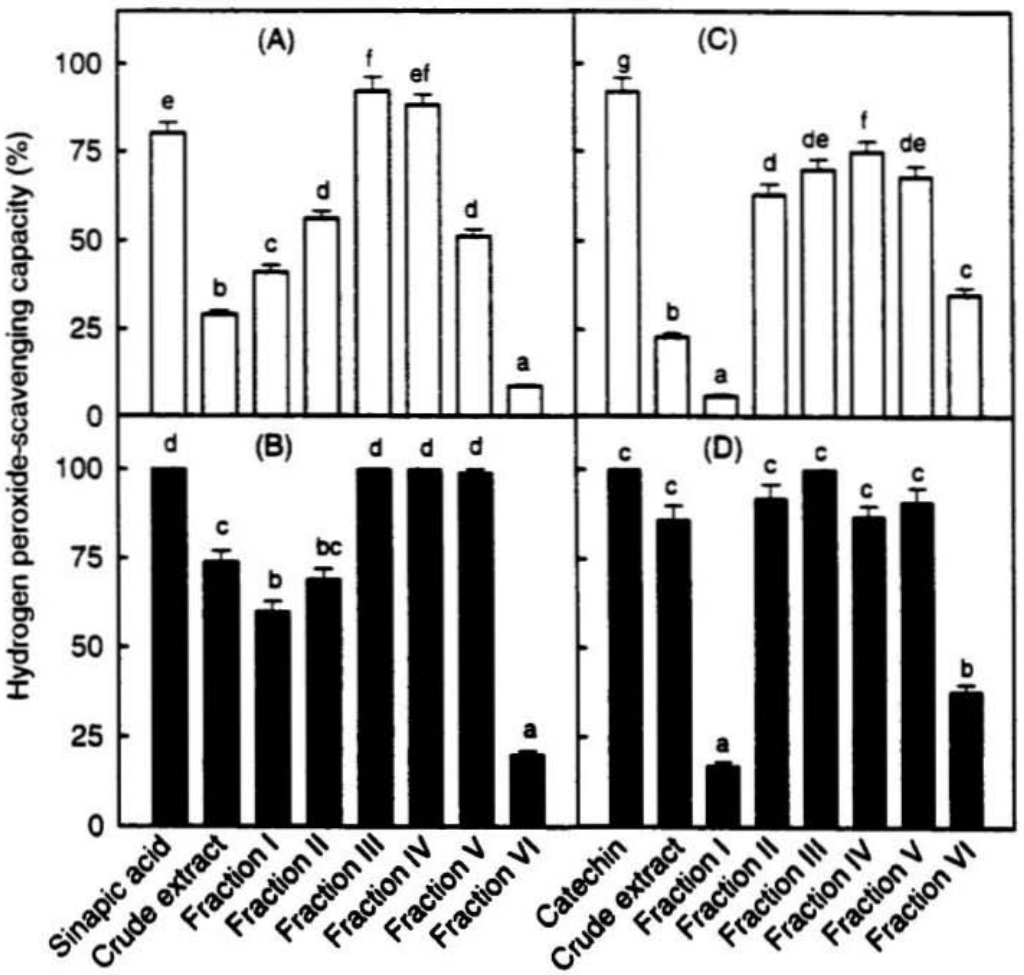

(A)
r=0.6904
Conjugated diene value
15
10
5
(B)
r=0.7395
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.6227
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Iron (II) chelating capacity (%)

Figure 4.35 Hydrogen peroxide-scavenging capacity of borage and evening primrose additives. (A) and (B) depict the scavenging capacities for 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the scavenging capacities for 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another.


(A)
(B)
(C)
(D)
Hydrogen peroxide-scavenging capacity (%)
0
25
50
75
100
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

assay medium (about 10 - 90% scavenging), but at 200 ppm, borage fractions III, IV and V scavenged H_2O_2 completely. Meanwhile, 200 ppm sinapic acid used as the reference antioxidant, scavenged H_2O_2 completely. For both concentrations, borage fraction VI was the least effective among borage additives while other borage additives, at 200 ppm, scavenged 60 -75% of H_2O_2.

Hydrogen peroxide-scavenging activities of evening primrose additives, at 100 and 200 ppm, are depicted in Figure 4.35C and D. Evening primrose crude extract and its fraction I, at 100 ppm, showed weak H_2O_2-scavenging capacities. At 200 ppm, the efficacy of crude extract was enhanced, but fraction I remained less efficient. Hydrogen peroxide-scavenging capacities of 200 ppm of evening primrose crude extract and fractions II-V were similar. Catechin, at both concentrations, was highly efficient in its H_2O_2 scavenging effect.

Scavenging of H_2O_2 by borage and evening primrose additives may be attributed to their phenolics which could donate electrons to H_2O_2, thus neutralizing it to water, as shown in the following equation (Halliwell and Gutteridge, 1985).

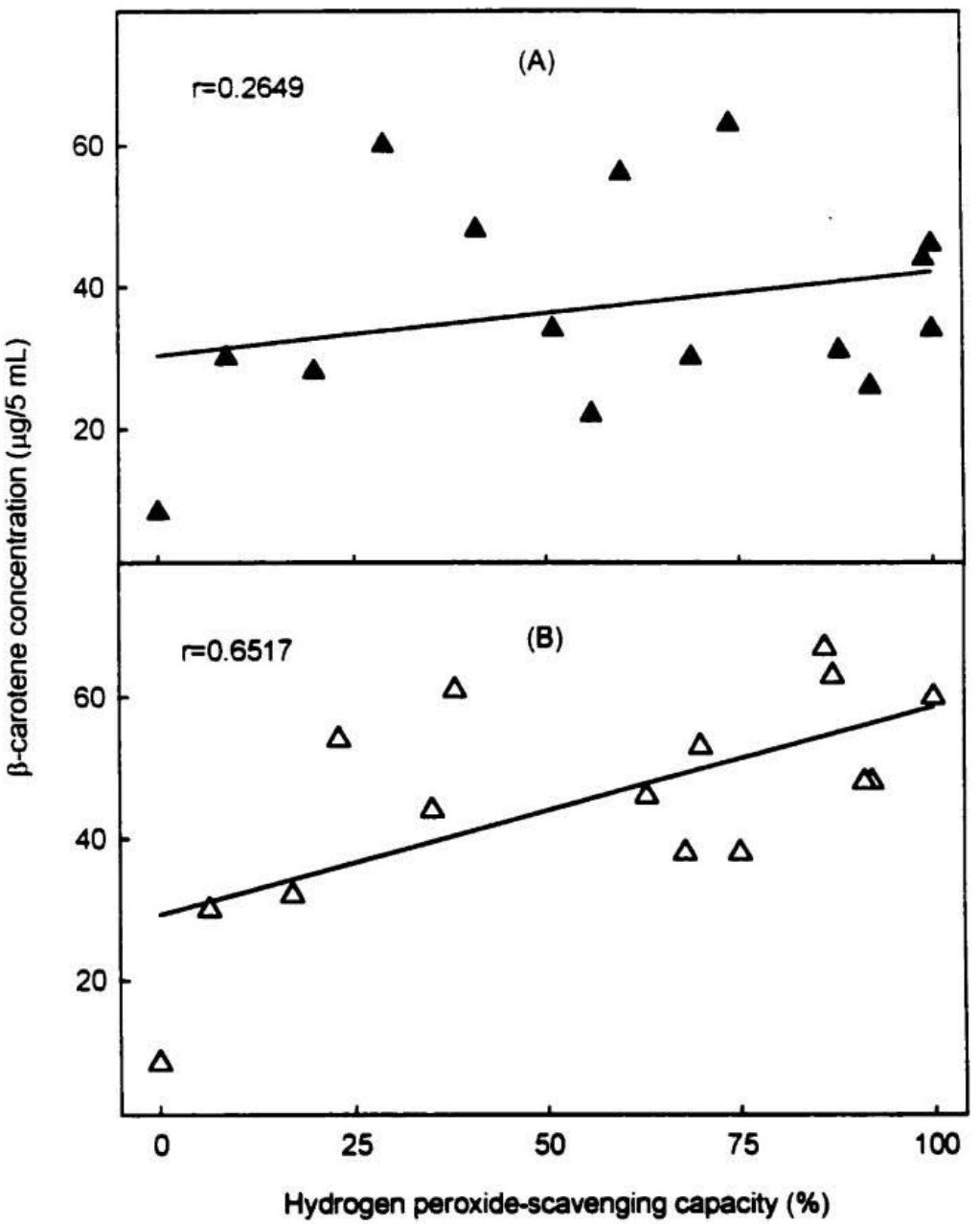
$$H_2O_2 + 2H^- + 2e^- \longrightarrow 2H_2O$$

The differences in H_2O_2-scavenging activities of borage and evening primrose additives may be attributed to the structural features of their active components which determine their electron donating abilities.

4.8.1 Relationship between hydrogen peroxide (H_2O_2)-scavenging capacity and antioxidant activity of borage and evening primrose additives in model systems

Being a dispersion system, β-carotene-linoleate system had the potential to generate H_2O_2 due to photolysis of water and hydroperoxides upon the exposure to fluorescent light (Saran *et al.*, 1983). The quantities formed in this system may be low, yet it could initiate lipid oxidation as H_2O_2 serves as a strong oxidant. On the other hand, H_2O_2 could well be involved in the generation of oxygen-derived free radicals such as hydroxyl radical, which could accelerate the decoloration of β-carotene through hydrogen abstraction. As shown in Figure 4.36A, a poor correlation ($r = 0.2649$) exists between H_2O_2-scavenging capacities of borage additives and antioxidant activities of corresponding additives in a β-carotene-linoleate model system. It is logical to assume that borage additives with a substantial amount of hydrophilic phenolics could scavenge H_2O_2 in the aqueous phase. However, H_2O_2 generated at the interfaces or their vicinity may not be efficiently scavenged due to poor partitioning of borage additives into the lipid phase and interfaces of the emulsion. Unlike borage additives, a good correlation ($r = 0.6517$) existed between H_2O_2-scavenging capacity of evening primrose additives and the antioxidant activities of respective additives in the β-carotene-linoleate systems (Figure 4.36B). This suggests that the active components of evening primrose additives might have scavenged H_2O_2 generated at the interfaces or their vicinities.

Figure 4.36 Relationships between hydrogen peroxide-scavenging capacity of borage (A)/evening primrose (B) additives and stability of β-carotene in a β-carotene-linoleate model system containing the respective additives.


(A)
r=0.2649
(B)
r=0.6517
β-carotene concentration (μg/5 mL)
60
40
20
0
25
50
75
100
Hydrogen peroxide-scavenging capacity (%)

As shown in Figure 4.37A, a strong linear correlation (r = 0.7871) existed between H_2O_2-scavenging capacity of borage additives and the formation of TBARS in a cooked comminuted pork model system containing respective additives. However, the relationships between H_2O_2-scavenging and formation of hexanal (Figure 4.37B) and total volatiles (Figure 4.37C) were poor (r = 0.5329 and 5176, respectively). Formation of TBARS, and total volatiles in cooked comminuted pork treated with evening primrose additives exhibited very strong linear correlations with H_2O_2-scavenging capacities of corresponding additives [r = 0.9205, and 0.9637 for TBARS (Figure 4.38A) and total volatiles (Figure 4.38C), respectively]. Relationship between hexanal formation in treated samples and H_2O_2-scavenging capacities of corresponding additives was strong (r = 0.8770) (Figure 4.38B).

Hydrogen peroxide is present in muscle systems in small quantities (Harel and Kanner, 1985). It could be generated in muscle tissues *via* enzymatic and non-enzymatic reactions with the latter being prominent. Enzymes located in mitochondria, peroxisomes as well as cytosolic enzymes are capable of generating H_2O_2 as a by-product (Harel and Kanner, 1985). However, the major enzymatic pathway of generating H_2O_2 in muscle tissues is the dismutation of $O_2^{\bullet-}$ (Frankel and Chrzan, 1987). Oxidation of oxymyoglobin and oxyhemoglobin is the major non-enzymatic means of H_2O_2 generation (Harel and Kanner, 1985). Therefore, antioxidants, which could scavenge H_2O_2, are likely to increase oxidative stability of muscle systems. As reflected by high correlation coefficients, this study also supports the hypothesis that H_2O_2 is a major prooxidant in muscle systems.

Figure 4.37 Relationships between hydrogen peroxide-scavenging capacity of borage additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.

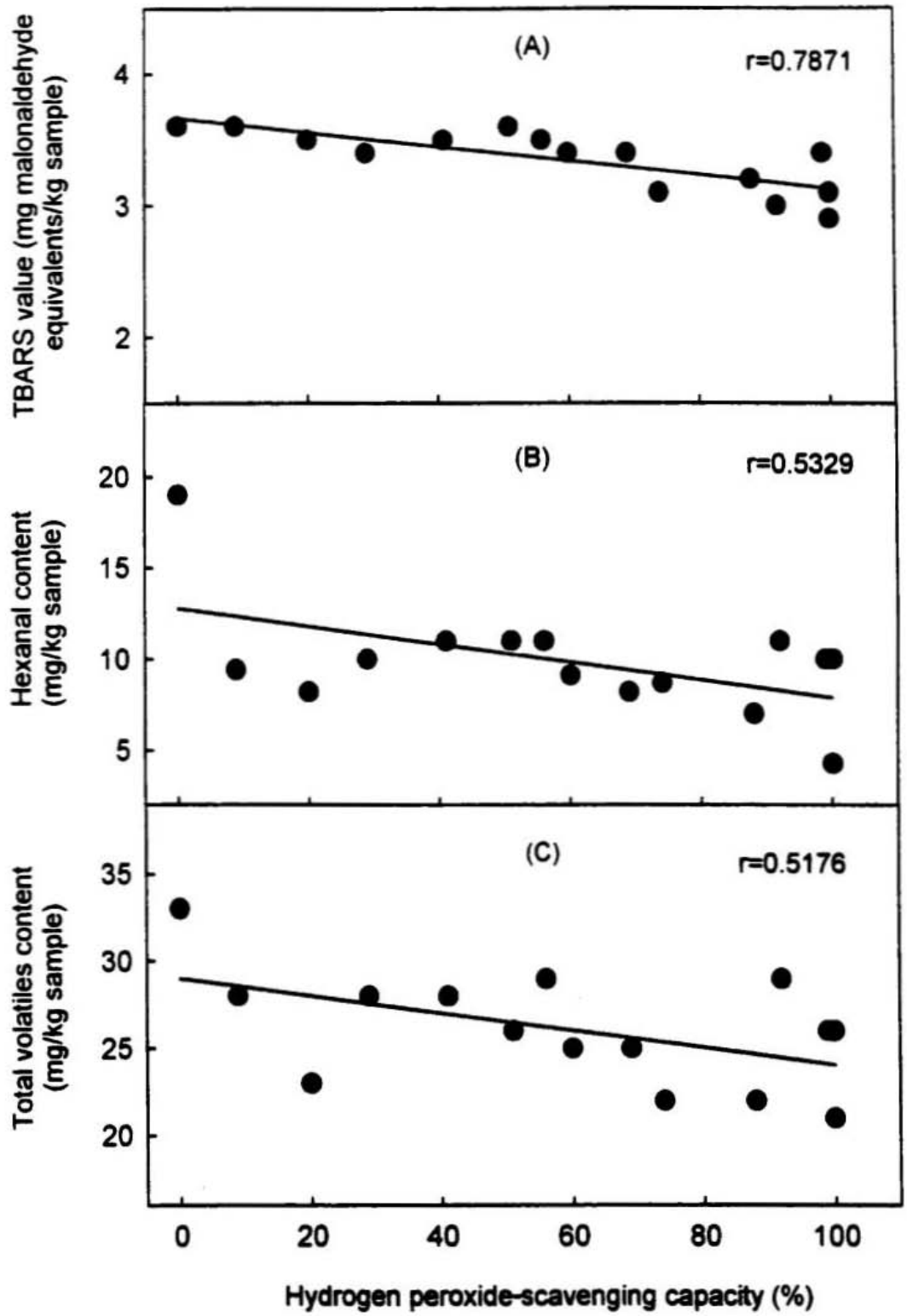
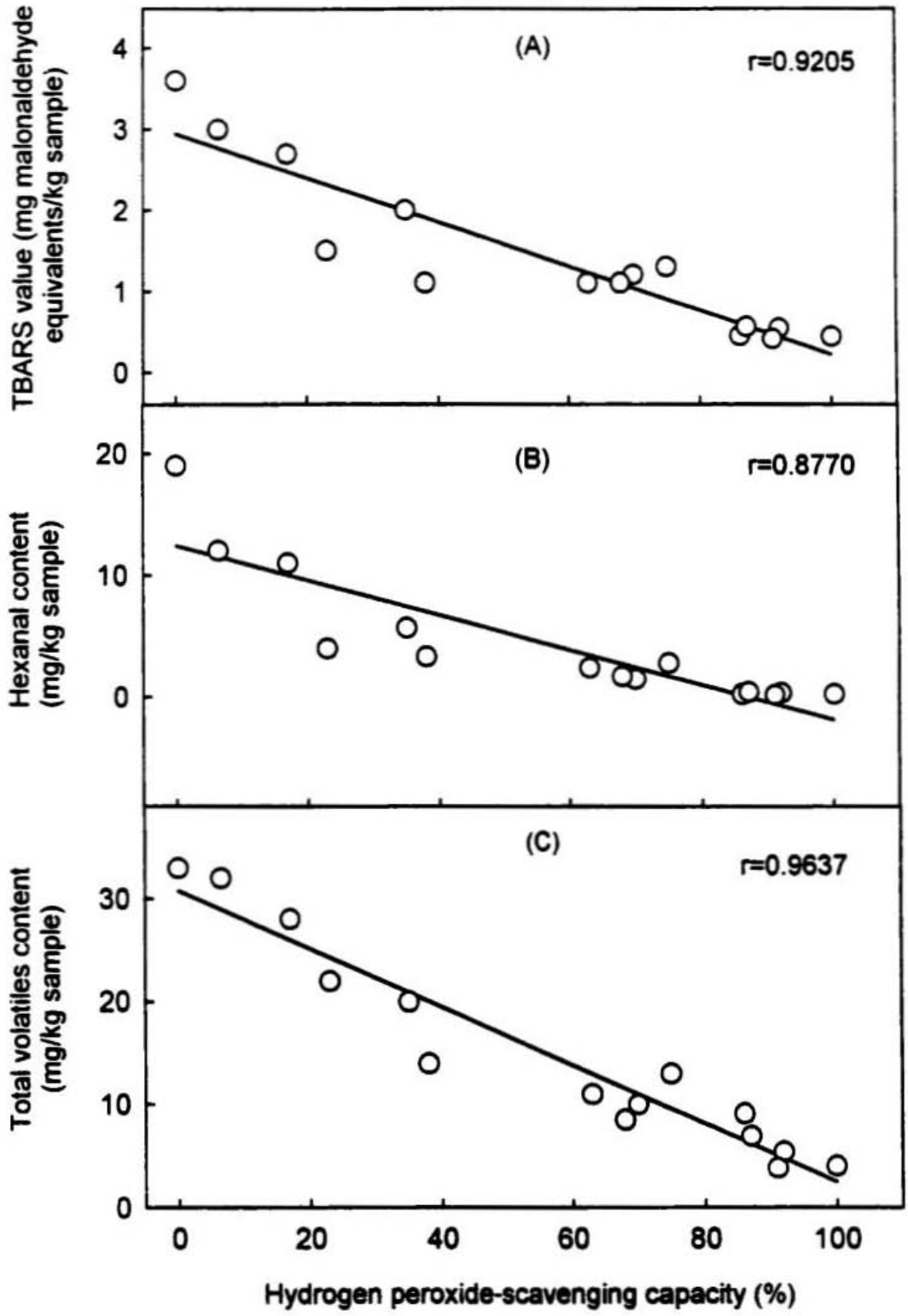

(A)
r=0.7871
TBARS value (mg malonaldehyde equivalents/kg sample)
4
3
2
(B)
r=0.5329
Hexanal content (mg/kg sample)
20
15
10
5
(C)
r=0.5176
Total volatiles content (mg/kg sample)
35
30
25
20
0
20
40
60
80
100
Hydrogen peroxide-scavenging capacity (%)

Figure 4.38 Relationships between hydrogen peroxide-scavenging capacity of evening primrose additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.


TBARS value (mg malonaldehyde equivalents/kg sample)
(A)
r=0.9205
Hexanal content (mg/kg sample)
(B)
r=0.8770
Total volatiles content (mg/kg sample)
(C)
r=0.9637
Hydrogen peroxide-scavenging capacity (%)

As shown in Figure 4.39A and B, the relationship between the formation of TBARS and hexanal in bulk stripped corn oil treated with borage additives and H_2O_2-scavenging capacities of corresponding additives were poor (r = 0.2819 and 0.5664 for TBARS and hexanal, respectively). Formation of total volatiles in bulk oil containing borage additives did not show a linear relationship with corresponding H_2O_2-scavenging capacities at all (r = 0.0000) (Figure 4.39C). Similar trends were observed for bulk oil treated with evening primrose additives (Figure 4.40A-C). These results suggest that hydrogen peroxide might not be a major prooxidant in bulk oil because of lack of stronger relationships. In bulk oil, the chance of H_2O_2 generation may be slim as the system lacks sufficient water. On the other hand, experiments were carried out in the dark which rules out the possibility of H_2O_2 being formed due to photolysis of water and hydroperoxides. Since stripping removes most of the minor components, including enzymes, from oil, enzymatic generation of H_2O_2 is remote.

For stripped corn oil-in-water emulsion, relationships between lipid oxidation indicators and H_2O_2-scavenging capacity of borage additives were poor (r ranged from 0.3375 to 0.4800) (Figure 4.41A-C). The relationships for emulsions treated with evening primrose additives, were also poor (r ranged from 0.4810 to 0.5406) (Figure 4.42A-C). Like in bulk oil, H_2O_2 might not be a major prooxidant in emulsions due to the same reasons as for bulk oil.

Figure 4.39 Relationships between hydrogen peroxide-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

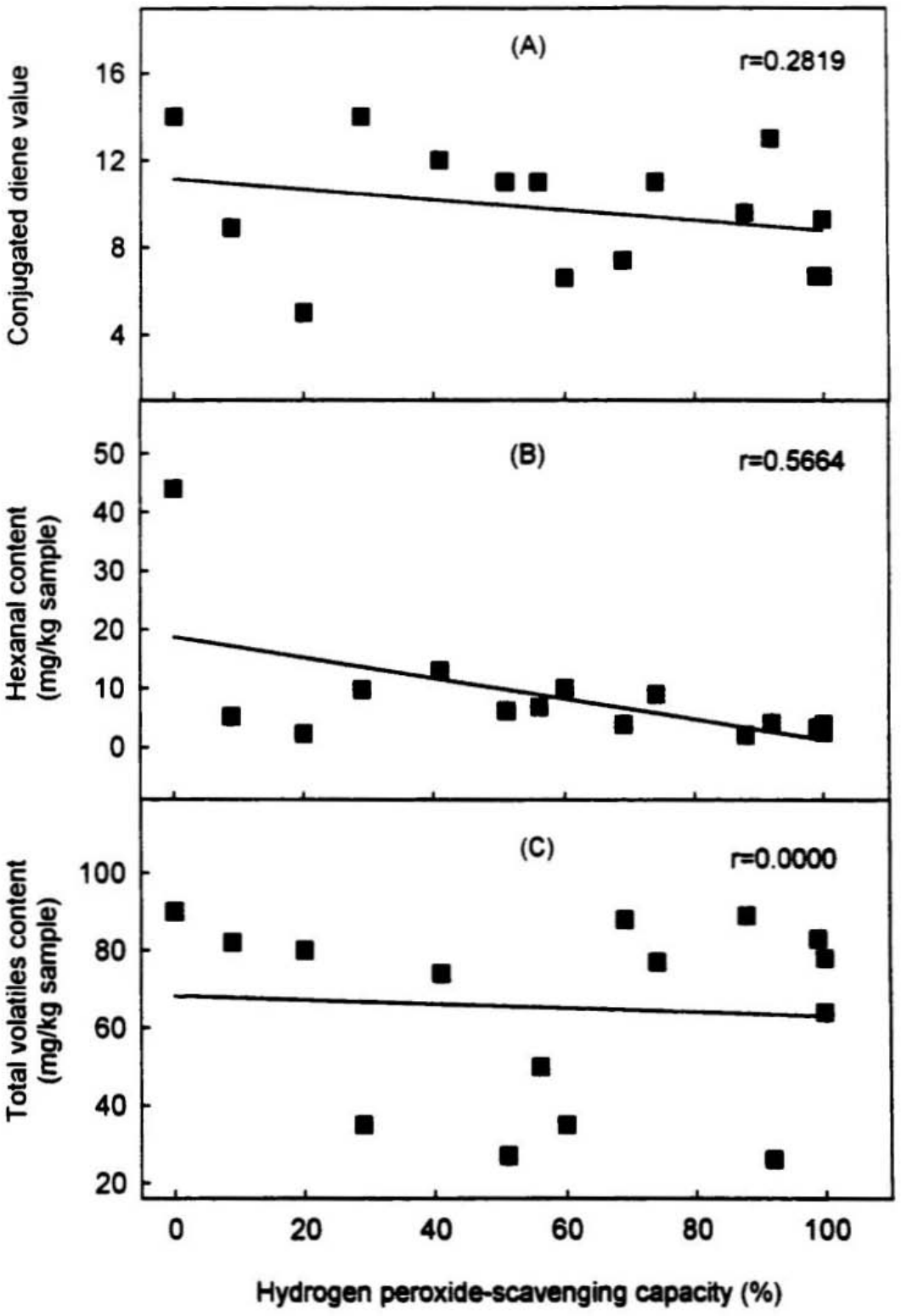

(A)
r=0.2819
Conjugated diene value
16
12
8
4
(B)
r=0.5664
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.0000
Total volatiles content (mg/kg sample)
100
80
60
40
20
0
20
40
60
80
100
Hydrogen peroxide-scavenging capacity (%)

Figure 4.40 Relationships between hydrogen peroxide-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

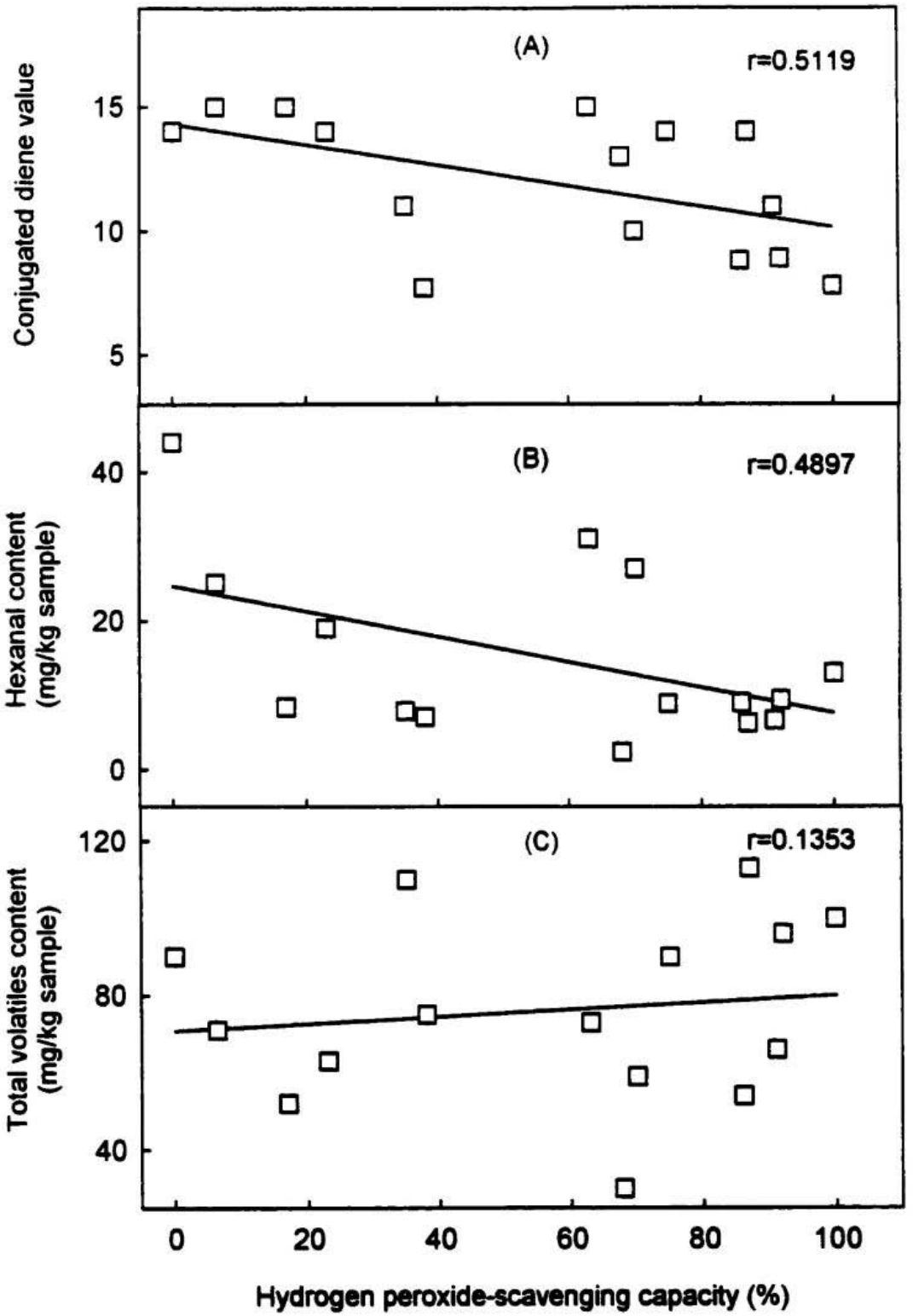

(A)
r=0.5119
Conjugated diene value
15
10
5
(B)
r=0.4897
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.1353
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Hydrogen peroxide-scavenging capacity (%)

Figure 4.41 Relationships between hydrogen peroxide-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

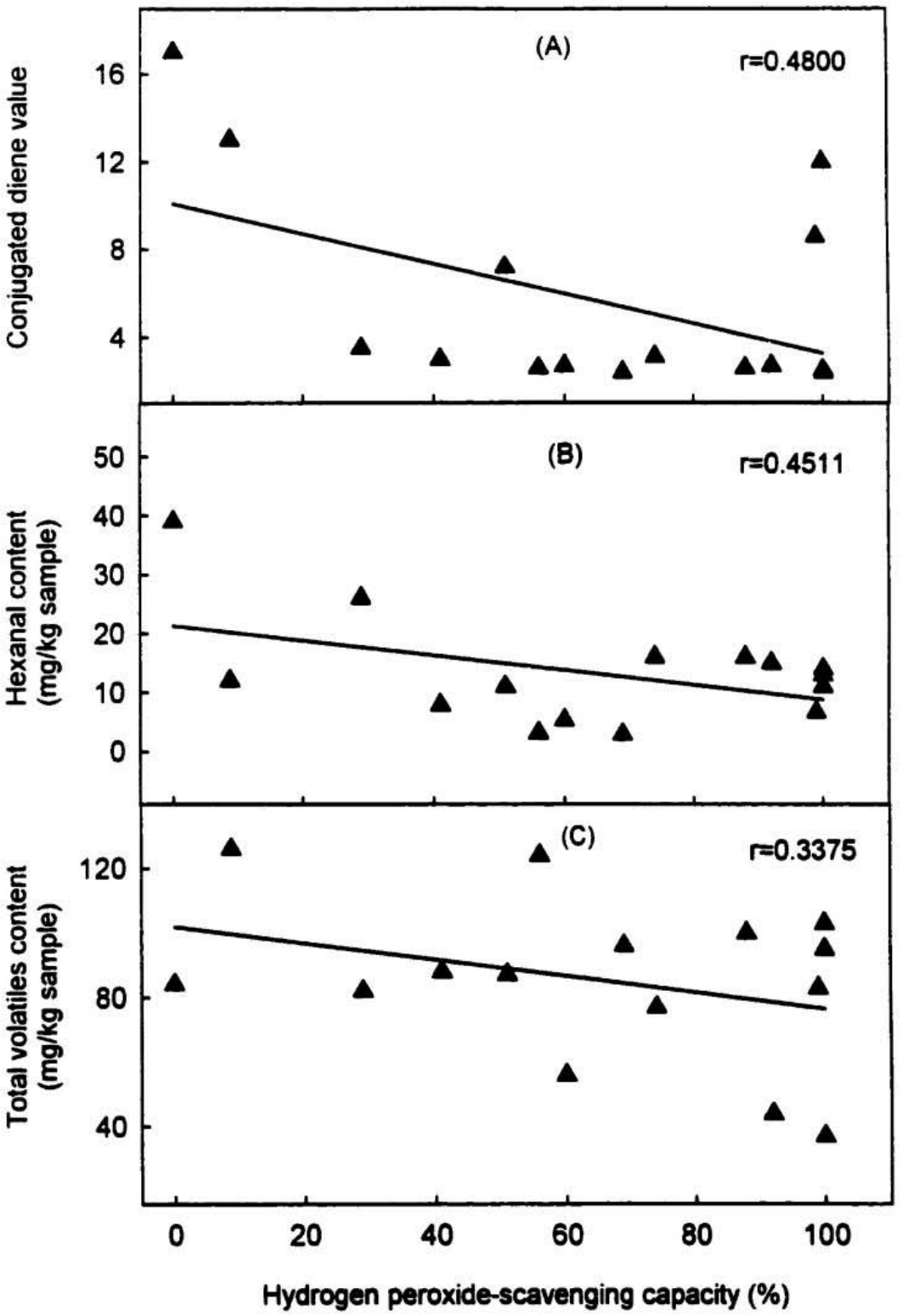
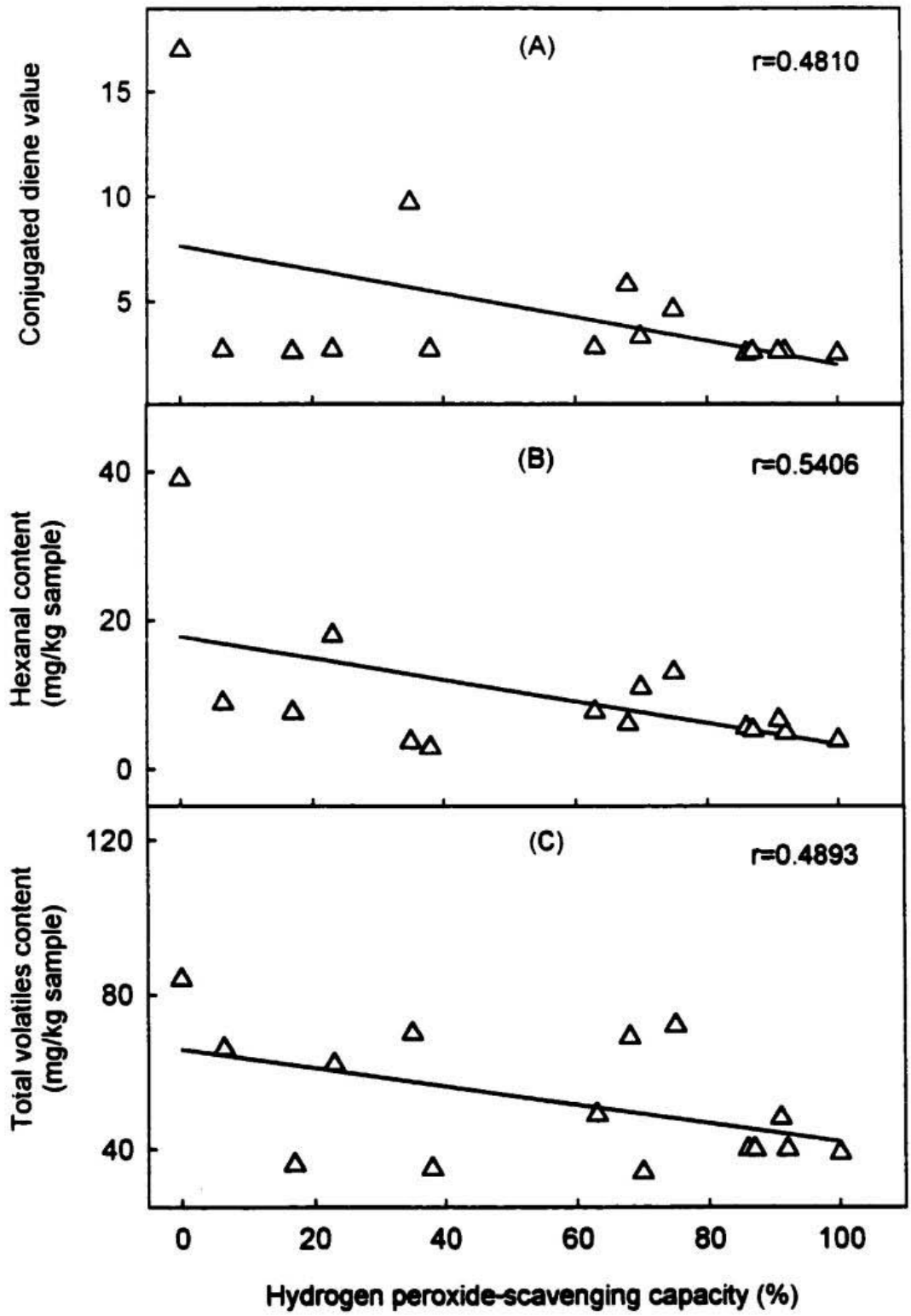

(A)
r=0.4800
Conjugated diene value
16
12
8
4
(B)
r=0.4511
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.3375
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Hydrogen peroxide-scavenging capacity (%)

Figure 4.42 Relationships between hydrogen peroxide-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.


(A)
r=0.4810
Conjugated diene value
15
10
5
(B)
r=0.5406
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.4893
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Hydrogen peroxide-scavenging capacity (%)

4.9 Free radical-scavenging capacity of borage and evening primrose additives

Superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), two oxygen-derived free radical species, and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), an organic free radical, were used to study the free radical-scavenging capacity of borage and evening primrose crude extracts and their fractions. The first species investigated was $O_2^{\bullet-}$ generated in a hypoxantine/xanthine oxidase system. The $O_2^{\bullet-}$ was readily scavenged by borage and evening primrose additives (Figure 4.43). A complete scavenging of $O_2^{\bullet-}$ was evident for assay media containing 200 ppm borage and evening primrose additives. The only exceptions were borage fraction V and evening primrose fraction I which exhibited approximately 75% scavenging of $O_2^{\bullet-}$. For the control and 100 and 200 ppm of some additives, the generation of $O_2^{\bullet-}$ was indicated by the development of an ink-blue colour in the assay media; the mechanism of the colour generation is depicted in Figure 4.44. For the control, intensity of this colour increased with time, but then leveled off. Therefore, the absorbance values after 60 min were used to determine the $O_2^{\bullet-}$-scavenging capacity of the additives. The scavenging of $O_2^{\bullet-}$ by additives may be attributed to their various phenolic constituents. Cotelle *et al.* (1996) reported that flavones, a class of flavonoids, bearing hydroxyl groups at positions 3', 4' or 3', 4', 5' scavenged $O_2^{\bullet-}$. These authors also reported that flavonoids containing one hydroxyl group in the C-7 position of ring A could inhibit generation of $O_2^{\bullet-}$ in the xanthine/xanthine oxidase system by inhibiting the enzyme. Yutig *et al.* (1990) reported that different types of flavonoids namely, rutin,

Figure 4.43 Superoxide radical-scavenging capacity of borage and evening primrose additives. (A) and (B) depict the scavenging capacities for 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the scavenging capacities for 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively. Each bar represents mean ± standard deviation of triplicate determinations. Bars sharing the same letter on each pannel are not significantly different ($p > 0.05$) from one another.

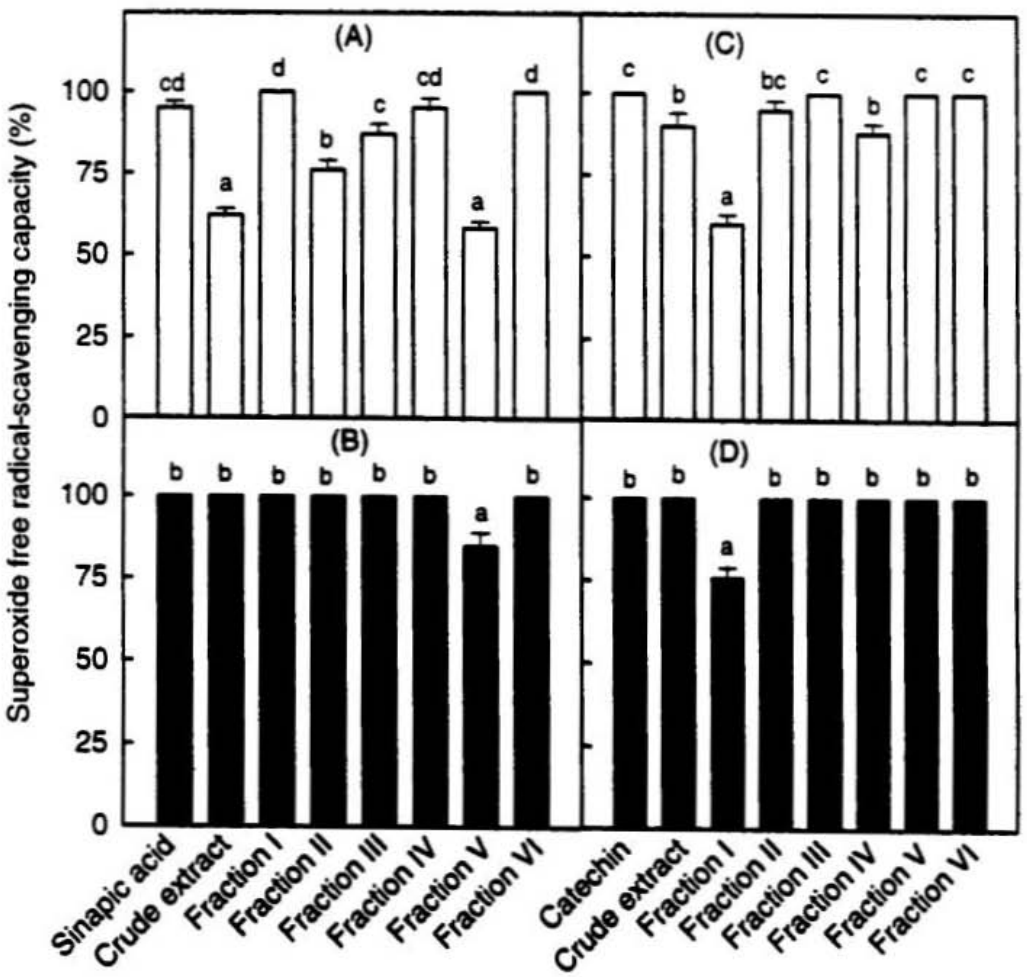
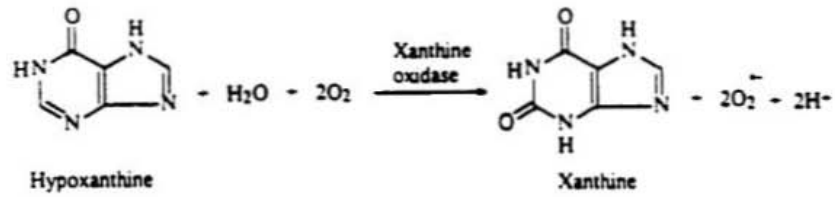
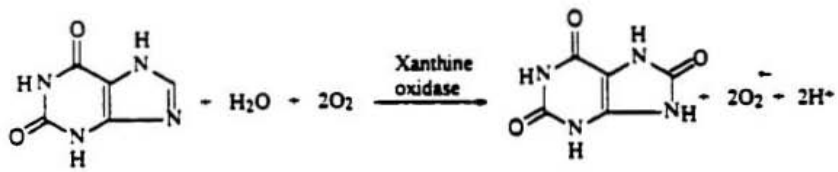

(A)
(B)
(C)
(D)
Superoxide free radical-scavenging capacity (%)
0
25
50
75
100
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

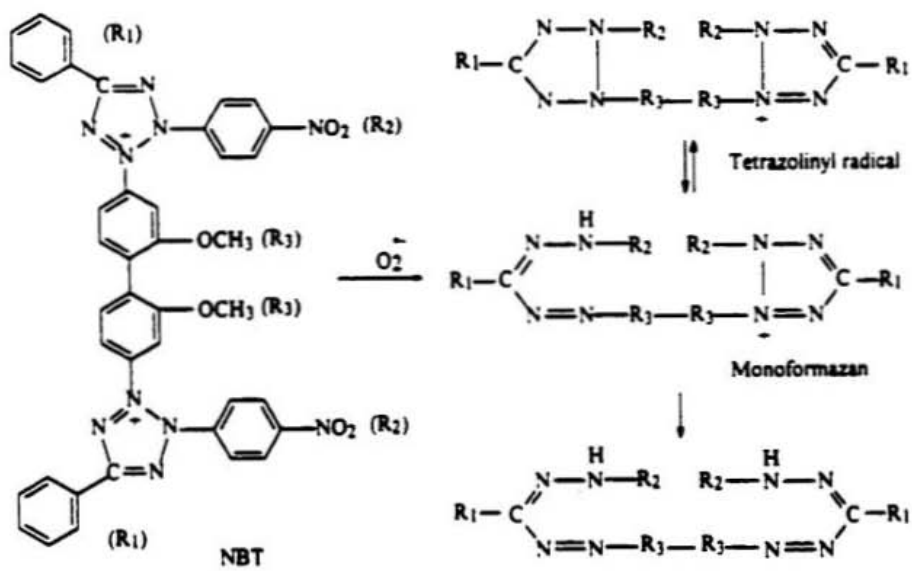
Figure 4.44 Mechanism by which the superoxide radicals are generated and subsequent reaction of superoxide with nitro blue tetrazolium indicator.



Hypoxanthine Xanthine



Xanthine Uric acid



NBT

naringin, quercetin and hispidulin, scavenged $O_2^{\bullet-}$. Several researchers have suggested that 3',4'-diphenolic group on ring B is required for flavonoids to be effective free radical scavengers (Latan, 1966; Younes and Siegers, 1981). Phenolic acids and their derivatives are also reported to be excellent free radical scavengers. Rice-Evans *et al.* (1996) reported that dihydroxybenzoic acids, such as protocatechuic and resorcylic acids, as well as hydroxycinnamic acids, such as caffeic and ferulic acids, were able to quench free radicals, including $O_2^{\bullet-}$, by hydrogen donation. The radical-scavenging ability of phenolic acids is mediated by the number of hydroxyl groups in the molecule (Dziedzic and Hudson, 1983). The electron-withdrawing property of the carboxylic acid group in benzoic acid has a negative influence on hydrogen donating ability of hydroxybenzoates. Hydroxylated cinnamates are more effective than their benzoate counterparts (Rice-Evans *et al*, 1996). Therefore, $O_2^{\bullet-}$-scavenging properties of borage and evening primrose additives may also be attributed to both neutralization of superoxide radicals *via* hydrogen donation and inhibition of xanthine oxidase by various phenolic components present in the additives.

Hydroxyl radicals were generated through an iron-catalyzed Haber-Weiss reaction as shown below.

$$Fe^{2+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2 \qquad \text{(metal reduction)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad \text{(Fenton reaction)}$$

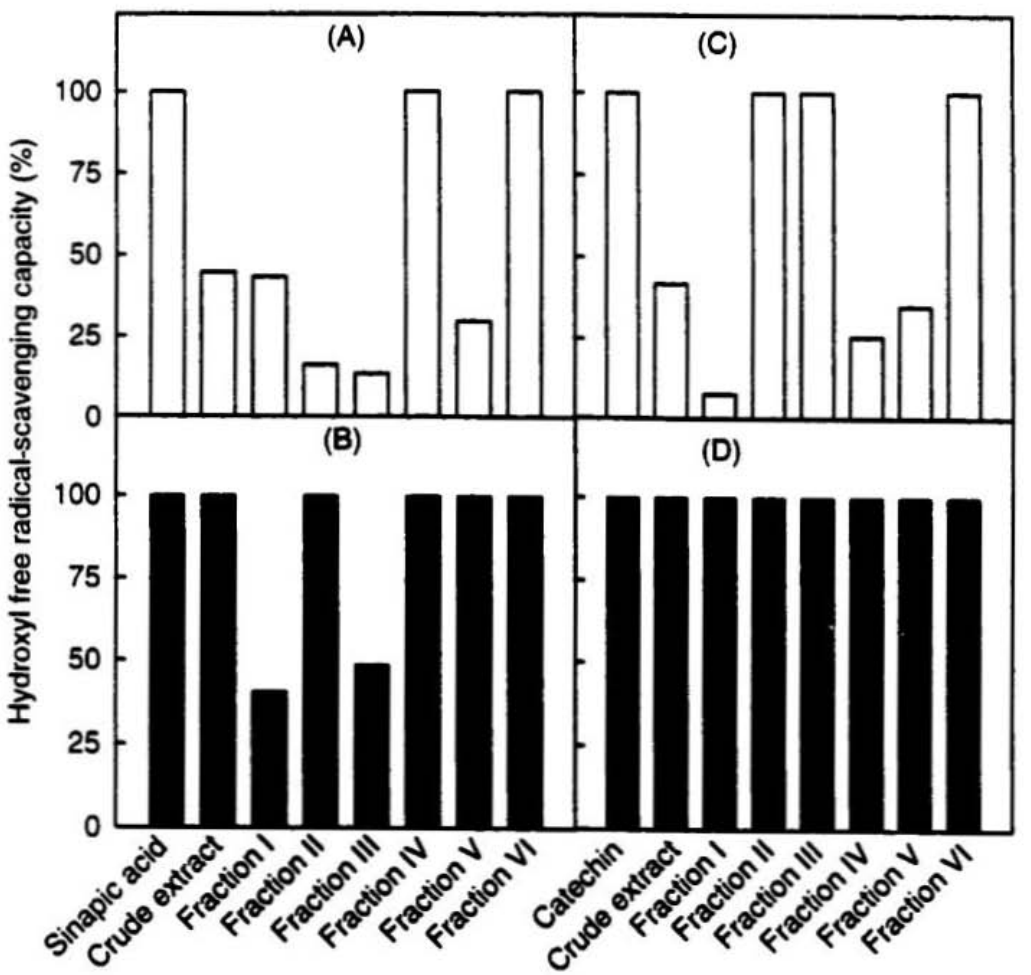
$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + {}^{\bullet}OH + OH^- \qquad \text{(Haber-Weiss reaction)}$$

Hydroxyl radicals, generated *via* these reactions, were spin trapped with DMPO (5,5-dimethyl-1-pyrrole-N-oxide) which formed a DMPO-OH adduct, a relatively stable free radical (Ruch *et al.*, 1989; Halliwell and Gutteridge, 1985). Spin trapping was done because the detection of $^{\bullet}OH$, as such, is extremely difficult due to their very short life time (Ruch *et al.*, 1989). As shown in Figures A.7 and A.8, the DMPO-OH adduct generated a 1:2:2:1 quartet with hyperfine coupling constant of 14.9 G (Yen and Chen, 1995).

As shown in Figure A.7, the characteristic quartet signal for DMPO-OH adduct was not detected in the assay media containing 100 and 200 ppm sinapic acid. One possibility was the quenching of $^{\bullet}OH$ by sinapic acid due to hydrogen donation. The other possibility was inhibition of generation of $^{\bullet}OH$ due to chelation of iron (II) by sinapic acid. For 100 ppm borage fractions IV and VI, the characteristic quartet signal for DMPO-OH adduct was not detected. At 200 ppm, the signal was detected only in assay medium containing borage fraction III while the signal was not detected in assay media containing other additives. Reduction in signal intensity (scavenging capacity) due to the presence of 100 and 200 ppm borage additives in the assay media are depicted in Figure 4.45A and B, respectively. Signal intensity for the control was 100%, thus its scavenging capacity was zero (not shown in Figure 4.45).

EPR signals of DMPO-OH adduct in assay media containing evening primrose additives are shown in Figure A.8. No Signal was detected in assay media containing 100 ppm of catechin and evening primrose (concentrations based upon phenolics as catechin

Figure 4.45 Hydroxyl radical-scavenging capacity of borage and evening primrose additives. (A) and (B) depict the scavenging capacities for 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the scavenging capacities for 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively.


(A)
(C)
(B)
(D)
Hydroxyl free radical-scavenging capacity (%)
100
75
50
25
0
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

equivalents) fractions II, III and VI. No signal was detected when catechin and evening primrose additives were present at 200 ppm concentration. Reduction in signal intensity (scavenging capacity) due to the presence of 100 ppm and 200 ppm evening primrose additives in the assay media are depicted in Figure 4.45C and D, respectively. The disappearance of the EPR signal in assay media containing evening primrose additives may also be attributed to the same reasons as explained for borage additives.

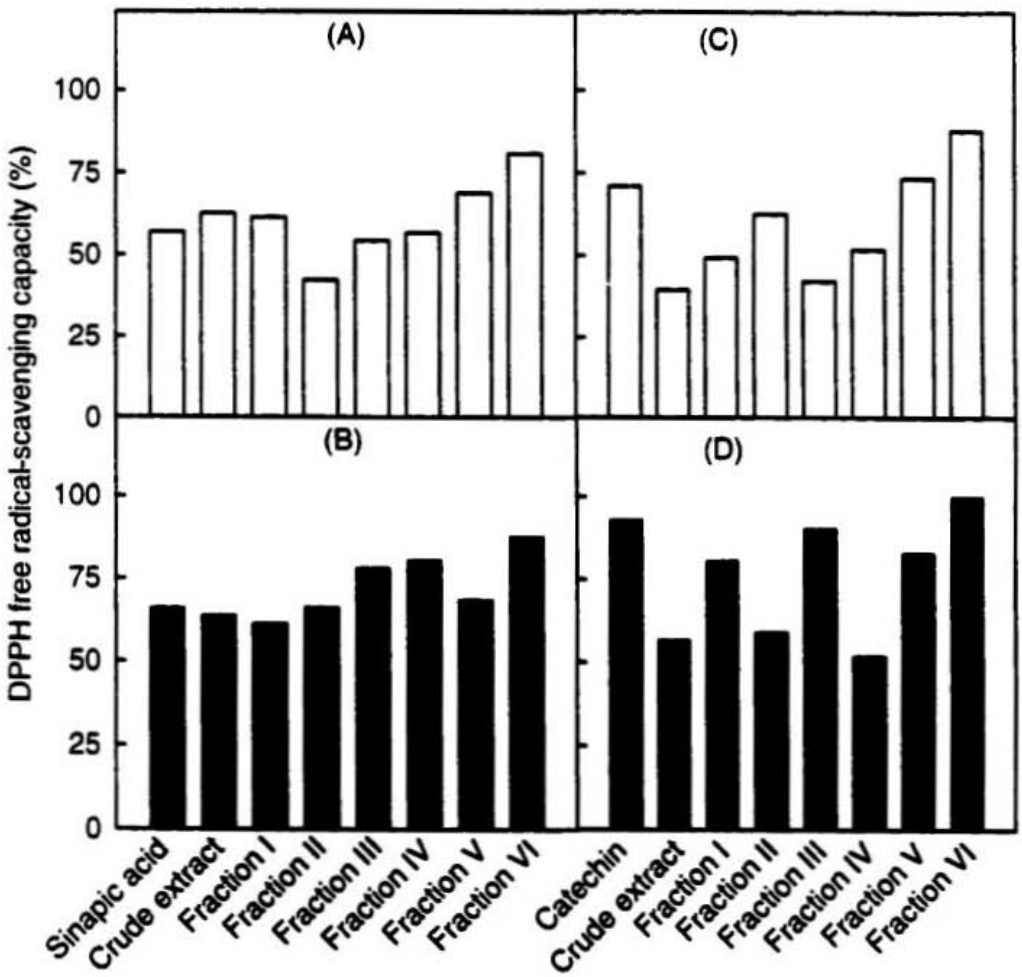
DPPH$^{\bullet}$, a stable free radical, has been used to evaluate free radical scavenging properties of natural antioxidants (Blois, 1958). Unlike the laboratory generated free radicals, such as $O_2^{\bullet-}$ and $^{\bullet}OH$, use of a stable free radical has the advantage of being unaffected by side reactions, such as metal chelation and enzyme inhibition, brought about by the additives. The reduction of DPPH$^{\bullet}$ in the presence of an additive was monitored by measuring the intensity of EPR signals. Generalized reduction reactions between DPPH$^{\bullet}$ and additives may be written as:

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

$$\text{DPPH}^{\bullet} + \text{R}^{\bullet} \longrightarrow \text{DPPH-R}$$

where, AH and R$^{\bullet}$ denote antioxidant components of the additives and radical species, respectively. Figures A.9 and A.10 show the EPR signal for DPPH$^{\bullet}$ as affected by borage and evening primrose additives, respectively. Figure 4.46A-D depicts the reduction in signal intensity (scavenging capacity) in the presence of borage and evening primrose

Figure 4.46 Organic free radical (DPPH)-scavenging capacity of borage and evening primrose additives. (A) and (B) depict the scavenging capacities for 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively. (C) and (D) depict the scavenging capacities of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively.


(A)
(C)
(B)
(D)
DPPH free radical-scavenging capacity (%)
100
75
50
25
0
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

additives. Control exhibited 100% signal intensity, thus its scavenging capacity was zero (not shown in Figure 4.46). Borage additives, at 100 ppm, scavenged DPPH$^{\bullet}$ by about 42 -88%. Scavenging capacity for evening primrose additives ranged from 40 to 100%. These results suggest that both borage and evening primrose additives possess the ability to quench free radicals and the effects may be attributed to the hydrogen and electron donating abilities of their phenolics.

4.9.1 Relationship between free radical-scavenging capacity and antioxidant activity of borage and evening primrose additives in various model systems

Strength of the relationships (discussed in this section) as determined by linear regression, will be designated as poor, good, strong or very strong (definitions are given in section **4.7.1**). In a β-carotene-linoleate system, the relationship between $O_2^{\bullet-}$-scavenging capacity and antioxidant activity of borage additives was poor (r = 0.4540) while that for evening primrose was strong (r = 0.7649) (Figure 4.47). Hydroxyl radical-scavenging capacity of borage additives did not linearly correlate (r = 0.2443) with their protective effects on β-carotene in a β-carotene-linoleate model system, but a stronger relationship (r = 0.7032) existed for evening primrose additives (Figure 4.48). Both $O_2^{\bullet-}$ and $^{\bullet}OH$ may be generated in a β-carotene-linoleate system due to photosensitizing property of β-carotene and photolysis of water/hydroperoxides, respectively. Results suggest that evening primrose additives were better free radical scavengers than borage additives in this

Figure 4.47 Relationships between superoxide radical-scavenging capacity of borage (A)/evening primrose (B) additives and stability of β-carotene in a β-carotene-linoleate model system containing the respective additives.

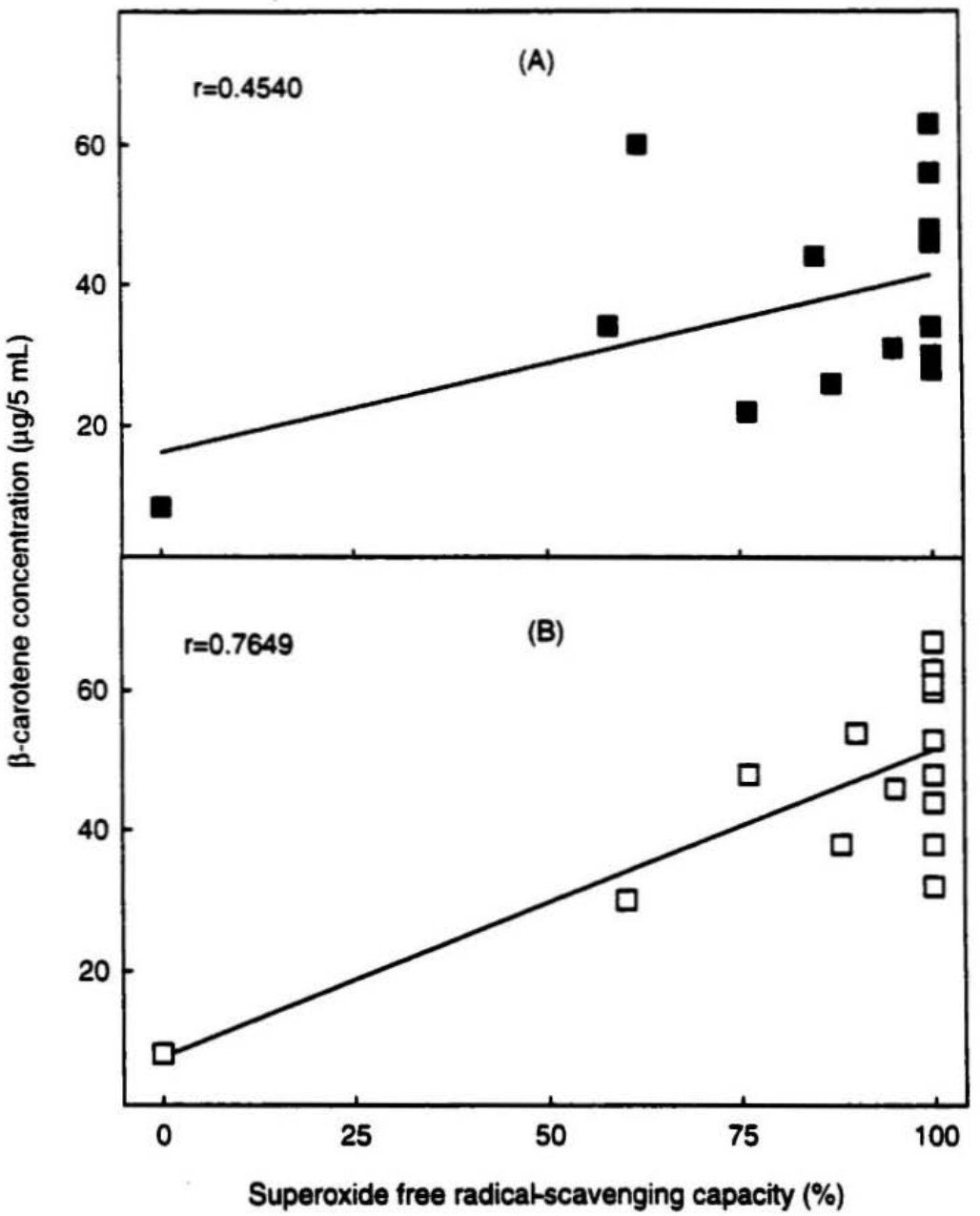
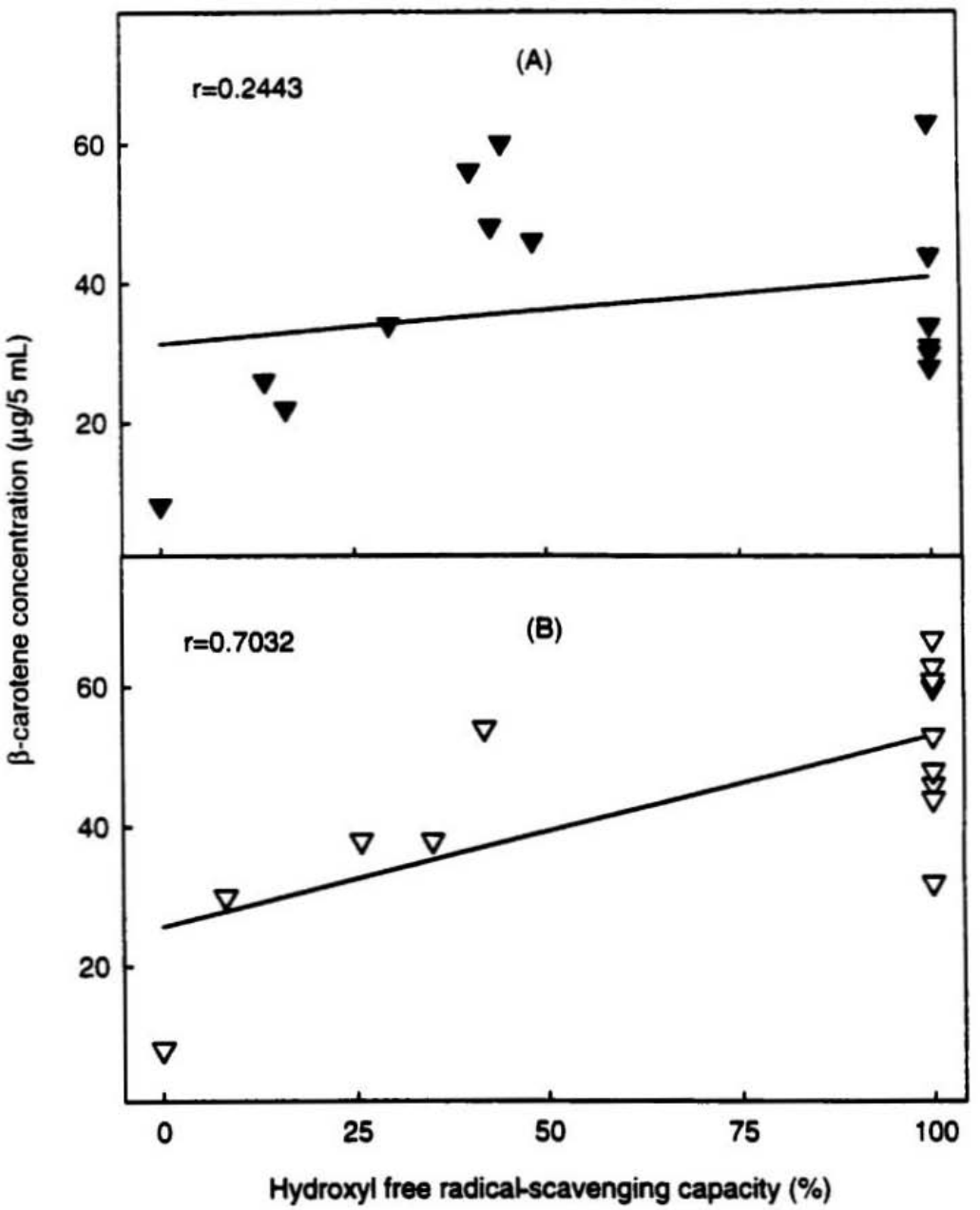

(A)
r=0.4540
60
40
20
(B)
r=0.7649
60
40
20
0
25
50
75
100
β-carotene concentration (μg/5 mL)
Superoxide free radical-scavenging capacity (%)

Figure 4.48 Relationships between hydroxyl radical-scavenging capacity of borage (A)/evening primrose (B) additives and stability of β-carotene in a β-carotene-linoleate model system containing the respective additives.


(A)
r=0.2443
60
40
20
(B)
r=0.7032
60
40
20
0
25
50
75
100
β-carotene concentration (μg/5 mL)
Hydroxyl free radical-scavenging capacity (%)

system, probably due to partitioning of more of active components into lipid phase and interfaces.

Since DPPH$^{\bullet}$ is a synthetic radical, it was assumed that DPPH$^{\bullet}$-scavenging capacity represents the general organic free radical-scavenging capacity of borage and evening primrose additives. As evident by poor correlation coefficients, organic free radical-scavenging capacity of borage and evening primrose additives did not have a linear relationship with their corresponding antioxidant activity in a β-carotene-linoleate model system (Figure 4.49).

Among the secondary oxidation products used to assess oxidative stability of cooked comminuted pork containing borage additives, hexanal contents correlated strongly with $O_2^{\bullet-}$-scavenging capacity of the respective additives (r = 0.8375) while the content of total volatiles exhibited a good correlation (r = 0.6866) (Figure 4.50). Meanwhile, good correlations existed between $O_2^{\bullet-}$-scavenging capacity of evening primrose additives and the TBARS values as well as the content of total volatiles in samples treated with the corresponding additives (r = 0.6981 and 0.6264, respectively); hexanal content showed a strong correlation (r = 0.7997) (Figure 4.51).

As shown in Figure 4.52, $^{\bullet}$OH-scavenging capacity of borage additives did not linearly correlate (r = 0.1446) with TBARS values of cooked comminuted pork model systems containing the respective additives, but hexanal content did (r = 0.7293). The content of total volatiles exhibited a strong correlation (r = 0.7665). Good correlations existed between $^{\bullet}$OH-scavenging capacity of evening primrose additives and the TBARS

Figure 4.49 Relationships between organic free radical (DPPH)-scavenging capacity of borage (A)/evening primrose (B) additives and stability of a β-carotene in β-carotene-linoleate model system containing the respective additives.

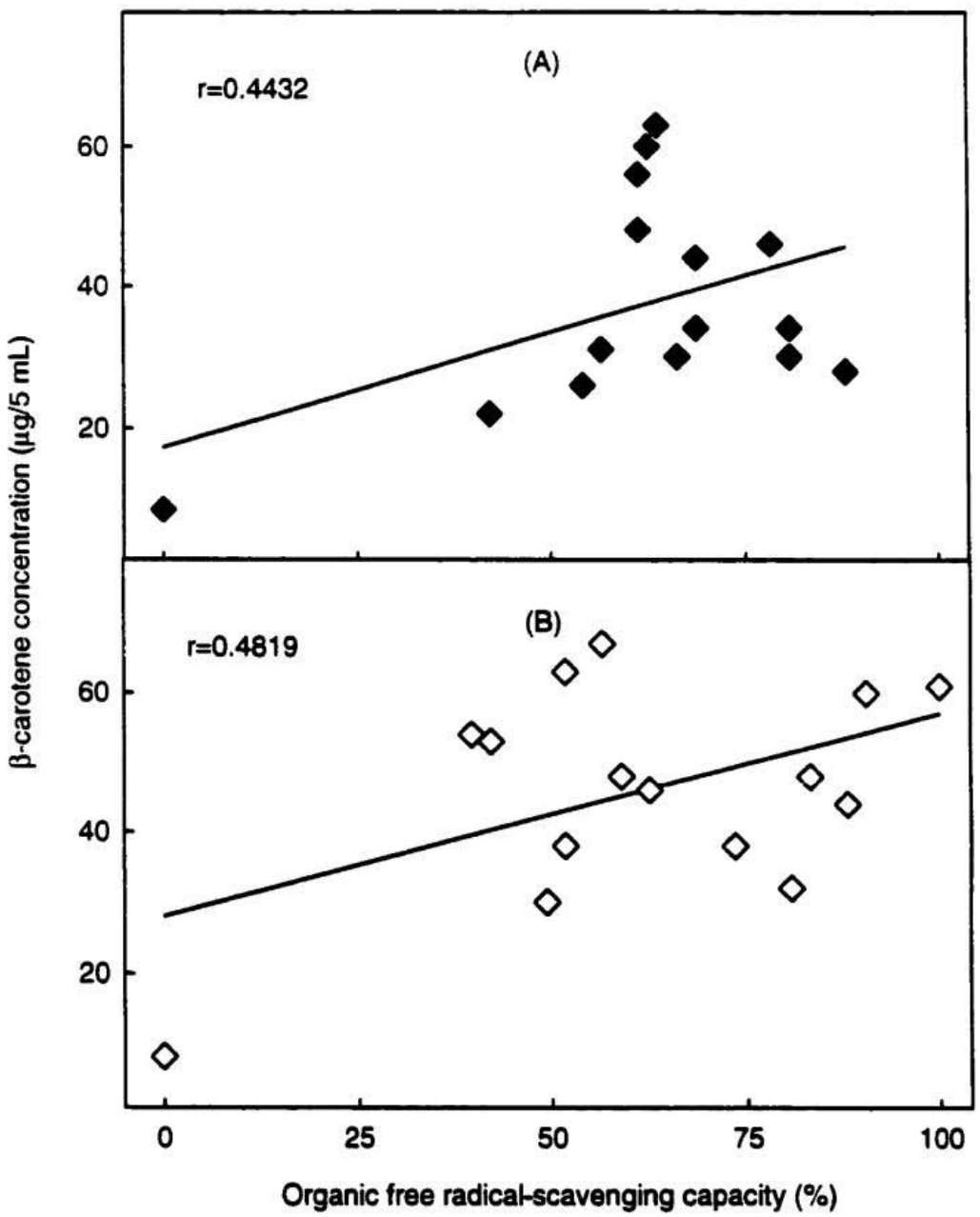

(A)
r=0.4432
(B)
r=0.4819
β-carotene concentration (μg/5 mL)
60
40
20
0
25
50
75
100
Organic free radical-scavenging capacity (%)

Figure 4.50 Relationships between superoxide radical-scavenging capacity of borage additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.

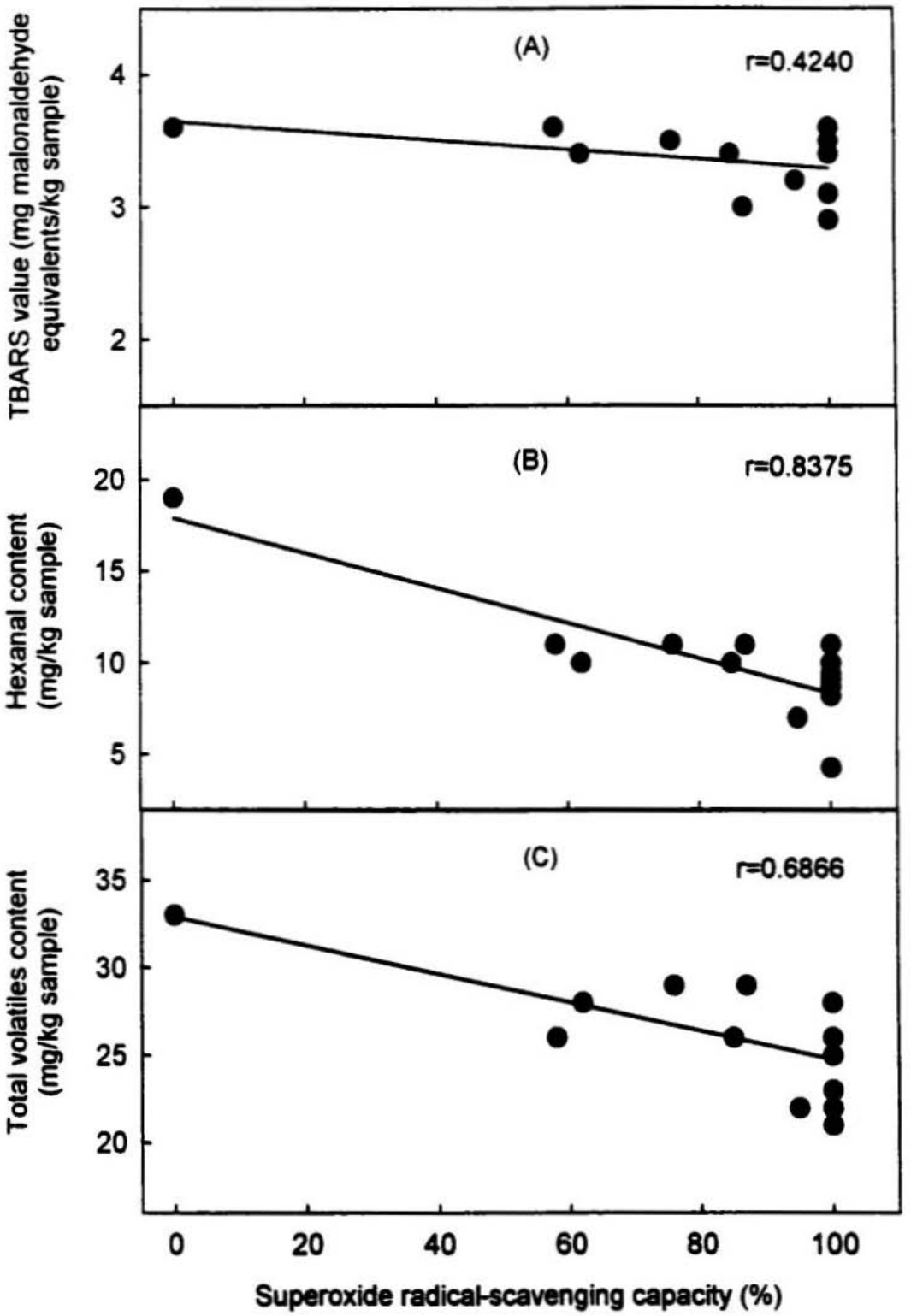

(A)
r=0.4240
TBARS value (mg malonaldehyde equivalents/kg sample)
4
3
2
(B)
r=0.8375
Hexanal content (mg/kg sample)
20
15
10
5
(C)
r=0.6866
Total volatiles content (mg/kg sample)
35
30
25
20
0
20
40
60
80
100
Superoxide radical-scavenging capacity (%)

Figure 4.51 Relationships between superoxide radical-scavenging capacity of evening primrose additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.

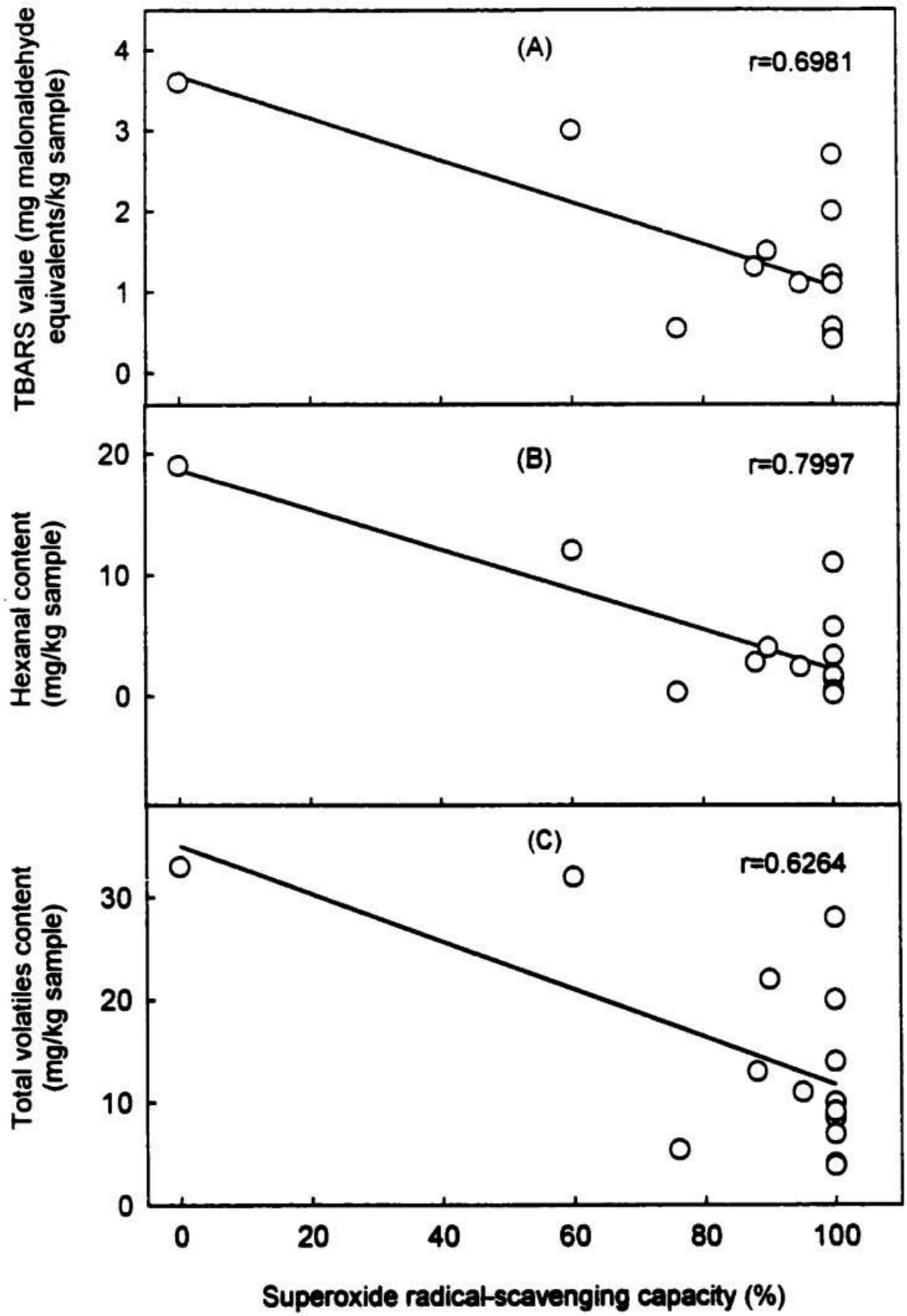
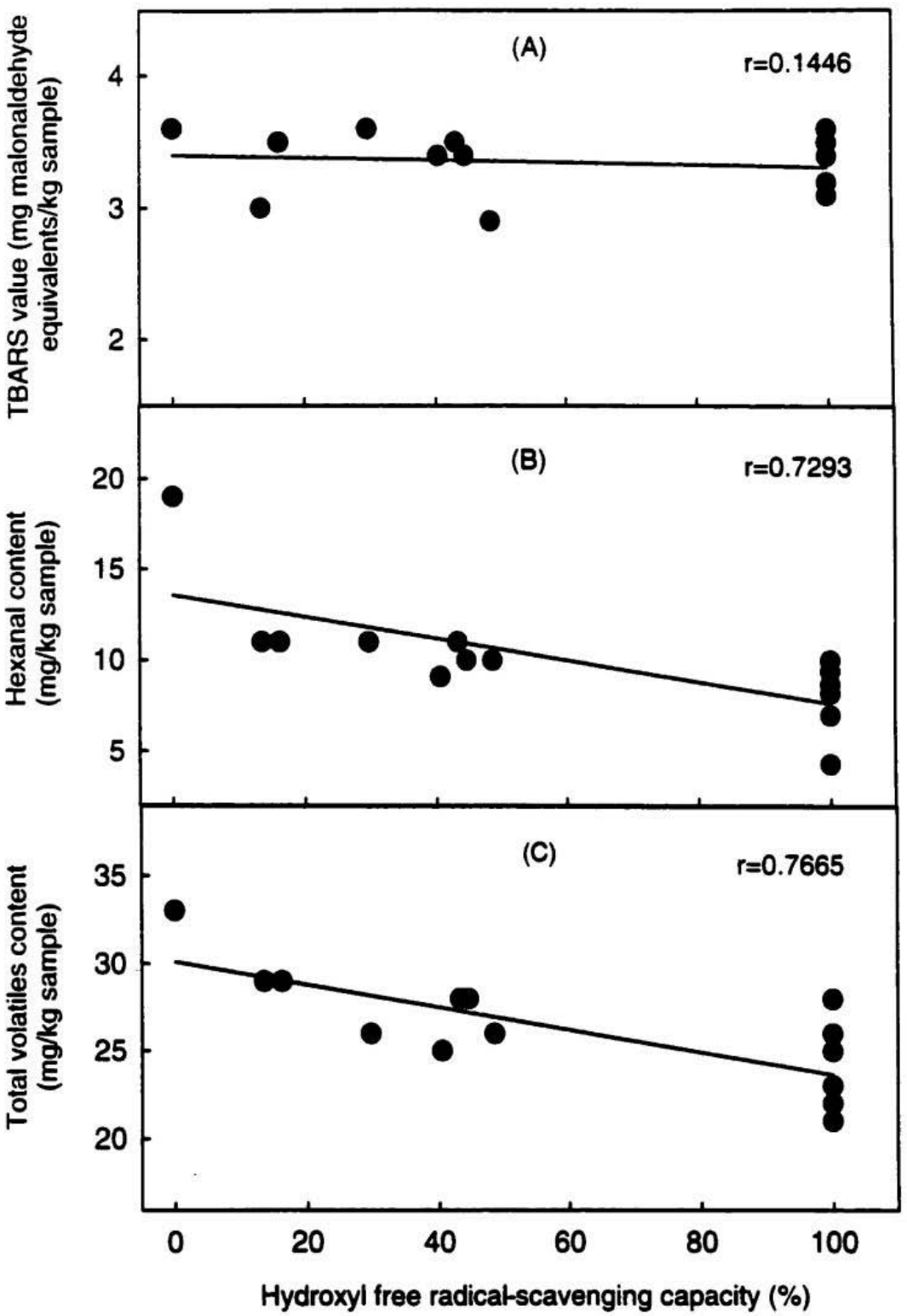

TBARS value (mg malonaldehyde equivalents/kg sample)
(A)
r=0.6981
Hexanal content (mg/kg sample)
(B)
r=0.7997
Total volatiles content (mg/kg sample)
(C)
r=0.6264
Superoxide radical-scavenging capacity (%)

Figure 4.52 Relationships between hydroxyl radical-scavenging capacity of borage additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.


(A)
r=0.1446
TBARS value (mg malonaldehyde equivalents/kg sample)
4
3
2
(B)
r=0.7293
Hexanal content (mg/kg sample)
20
15
10
5
(C)
r=0.7665
Total volatiles content (mg/kg sample)
35
30
25
20
0
20
40
60
80
100
Hydroxyl free radical-scavenging capacity (%)

values (r = 0.6305) as well as hexanal content (r = 0.6202) and total volatiles content (r = 0.6079) in meat samples (Figure 4.53).

When DPPH$^{\bullet}$-scavenging capacity of borage additives was related to oxidation indicators of cooked comminuted pork containing respective additives, only haxanal content exibited a good linear relationship (r = 0.6604) (Figure 4.54). There was no linear correlation between the formation of oxidation products in meat systems containing evening primrose additives and the DPPH$^{\bullet}$-scavenging capacity of the respective additives (Figure 4.55).

In muscle systems, $O_2^{\bullet-}$ may be generated by leukocytes and undergoes dismutation reactions which first produce hydrogen peroxide and then hydroxyl radicals (Badwers and Karnovsky, 1980). Superoxide radical may also be generated in muscle systems through metal reduction. Hydroxyl radicals may be generated in muscle tissues due to Fenton reaction which uses hydrogen peroxide and iron ions as reactants (Kanner *et al.*, 1987). These reactive-oxygen species have been known to initiate lipid oxidation in meat (McCay and Poyer, 1985). In general, free radical-scavenging (as reflected by scavenging of $O_2^{\bullet-}$, $^{\bullet}OH$ or DPPH$^{\bullet}$) capacity of both borage and evening primrose additives played a prominent antioxidant role in meat as reflected by their better correlations with the content of oxidation products.

The relationship between $O_2^{\bullet-}$-scavenging capacity of borage additives and the conjugated diene values of the treated bulk stripped corn oil was good (r = 0.6095) while that with hexanal content was strong (r = 0.8356). Content of total volatiles in samples

Figure 4.53 Relationships between hydroxyl radical-scavenging capacity of evening primrose additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.

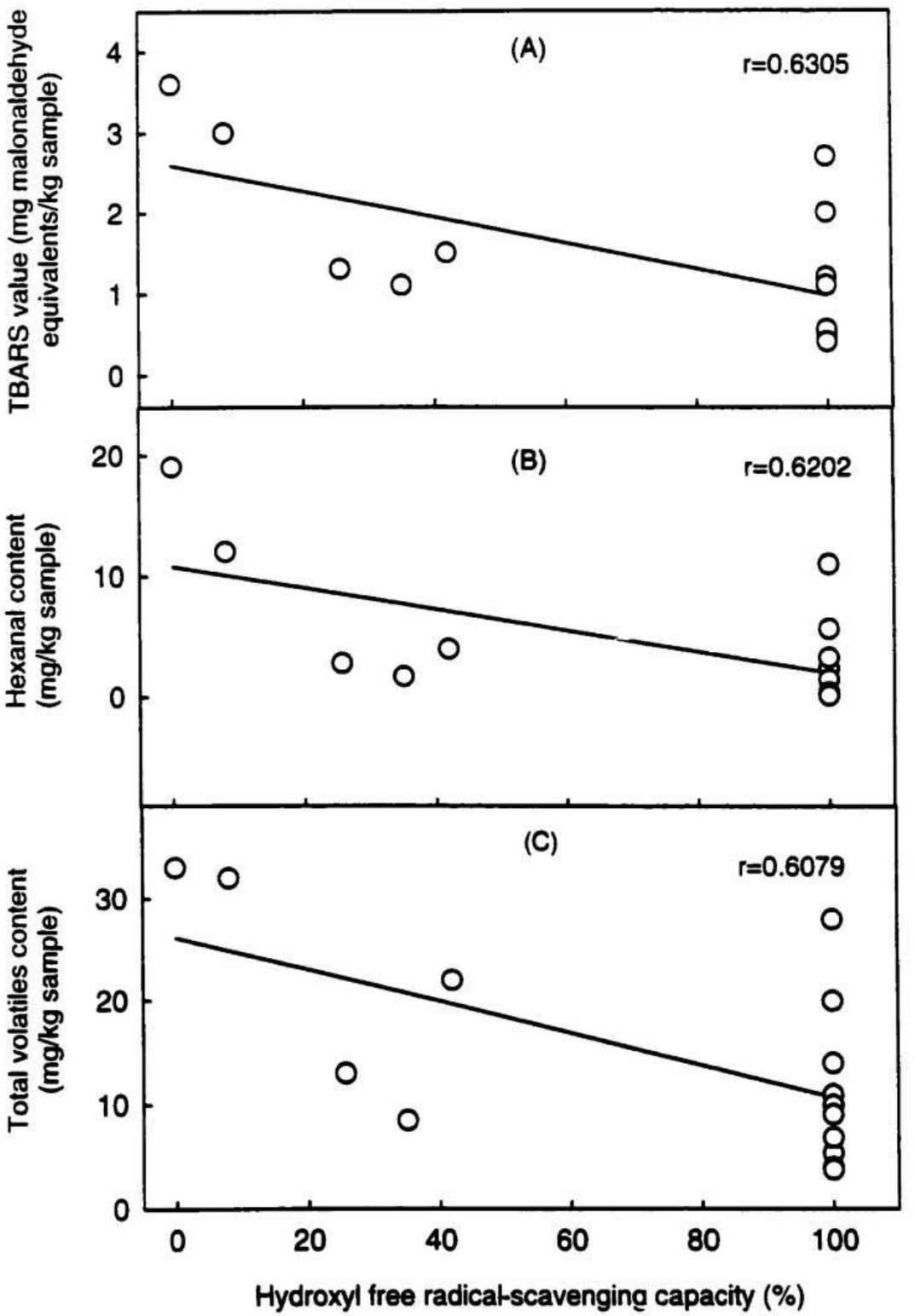

TBARS value (mg malonaldehyde equivalents/kg sample)
(A)
r=0.6305
Hexanal content (mg/kg sample)
(B)
r=0.6202
Total volatiles content (mg/kg sample)
(C)
r=0.6079
Hydroxyl free radical-scavenging capacity (%)

Figure 4.54 Relationships between organic free radical (DPPH)-scavenging capacity of borage additives and formation of TBARS (A), hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.

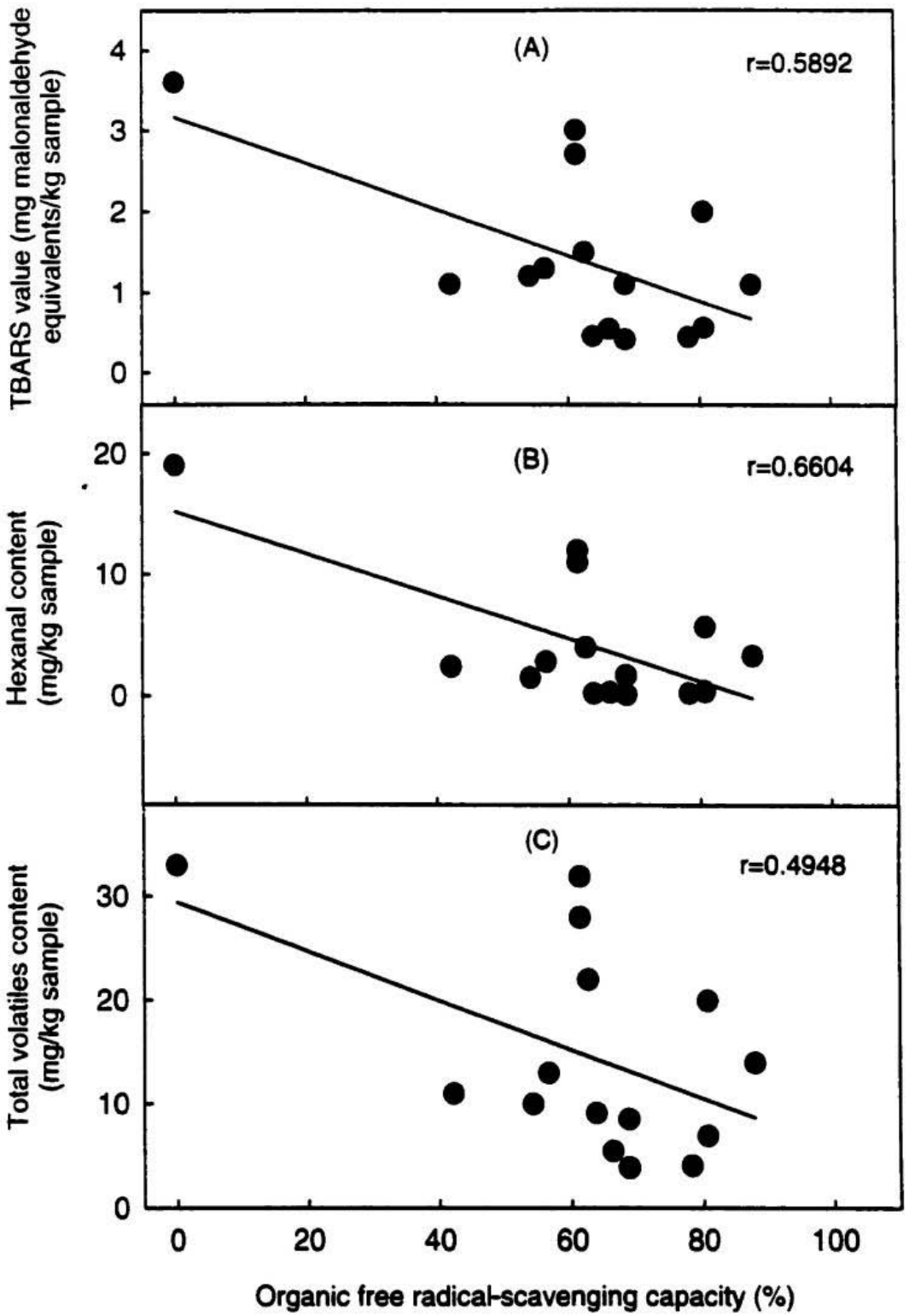
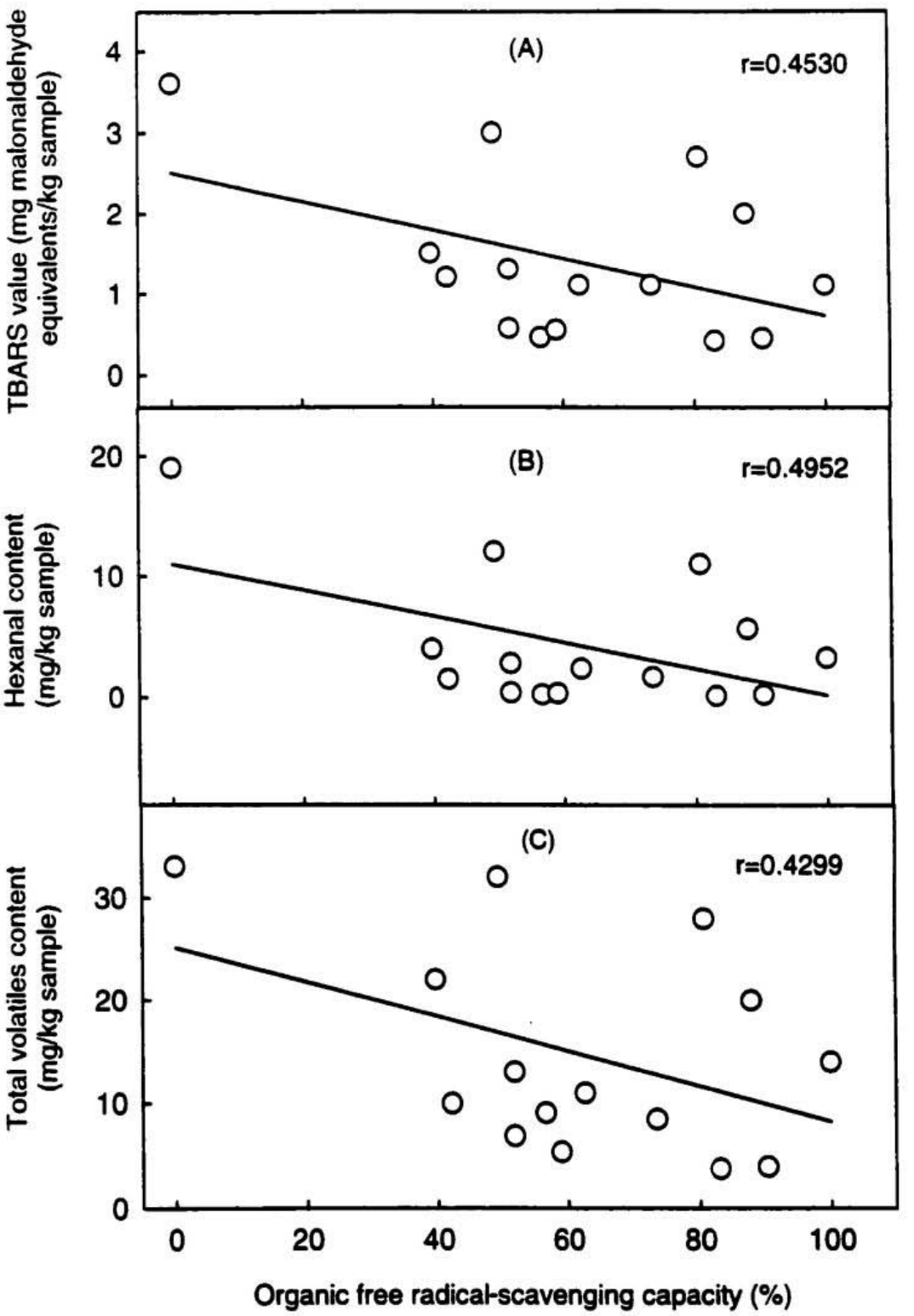

TBARS value (mg malonaldehyde equivalents/kg sample)
(A)
r=0.5892
Hexanal content (mg/kg sample)
(B)
r=0.6604
Total volatiles content (mg/kg sample)
(C)
r=0.4948
Organic free radical-scavenging capacity (%)

Figure 4.55 Relationships between organic free radical (DPPH)-scavenging capacity of evening primrose additives and formation of TBARS (A). hexanal (B) and total volatiles (C) in a cooked comminuted pork model system containing the respective additives.


TBARS value (mg malonaldehyde equivalents/kg sample)
(A)
r=0.4530
Hexanal content (mg/kg sample)
(B)
r=0.4952
Total volatiles content (mg/kg sample)
(C)
r=0.4299
Organic free radical-scavenging capacity (%)

treated with borage additives did not linearly correlate (r = 0.0830) with the $O_2^{\bullet-}$-scavenging capacity of the respective additives (Figure 4.56). For samples treated with evening primrose additives, only hexanal content showed a good corrrelation (r = 0.7314) with $O_2^{\bullet-}$-scavenging capacity of the respective additives (Figure 4.57).

The relationships between $^{\bullet}OH$-scavenging capacity of both borage and evening primrose additives and the content of oxidation products in bulk stripped corn oil were poor (Figures 4.58 and 4.59; r ranged from 0.5592 to 0.1414) except for the conjugated diene value of samples containing borage additives (r = 0.6850).

For bulk oil treated with borage and evening primrose additives, only hexanal content showed a strong correlation (r = 0.8430 and 0.7273 for borage and evening primrose additives, respectively) with DPPH$^{\bullet}$-scavenging capacity of the respective additives (Figures 4.60 and 4.61).

Relationships between antioxidant effects of borage additives in stripped corn oil-in-water emulsions and their $O_2^{\bullet-}$-scavenging capacities followed similar patterns to those observed in bulk oil (Figure 4.62). Relationships for evening primrose additives in emulsions were rather different from those observed in the bulk oil. The conjugated dienes value and hexanal content were strongly affected by the $O_2^{\bullet-}$-scavenging capacity of evening primrose additives (r = 0.7503 and 0.8595, respectively), but not by the content of total volatiles (r = 0.6051) (Figure 4.63). The content of any of the oxidation products in emulsions containing borage additives did not linearly correlate with the $^{\bullet}OH$-scavenging capacity of the corresponding additives (Figure 4.64, r ranged from 0.0412 to

Figure 4.56 Relationships between superoxide radical-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

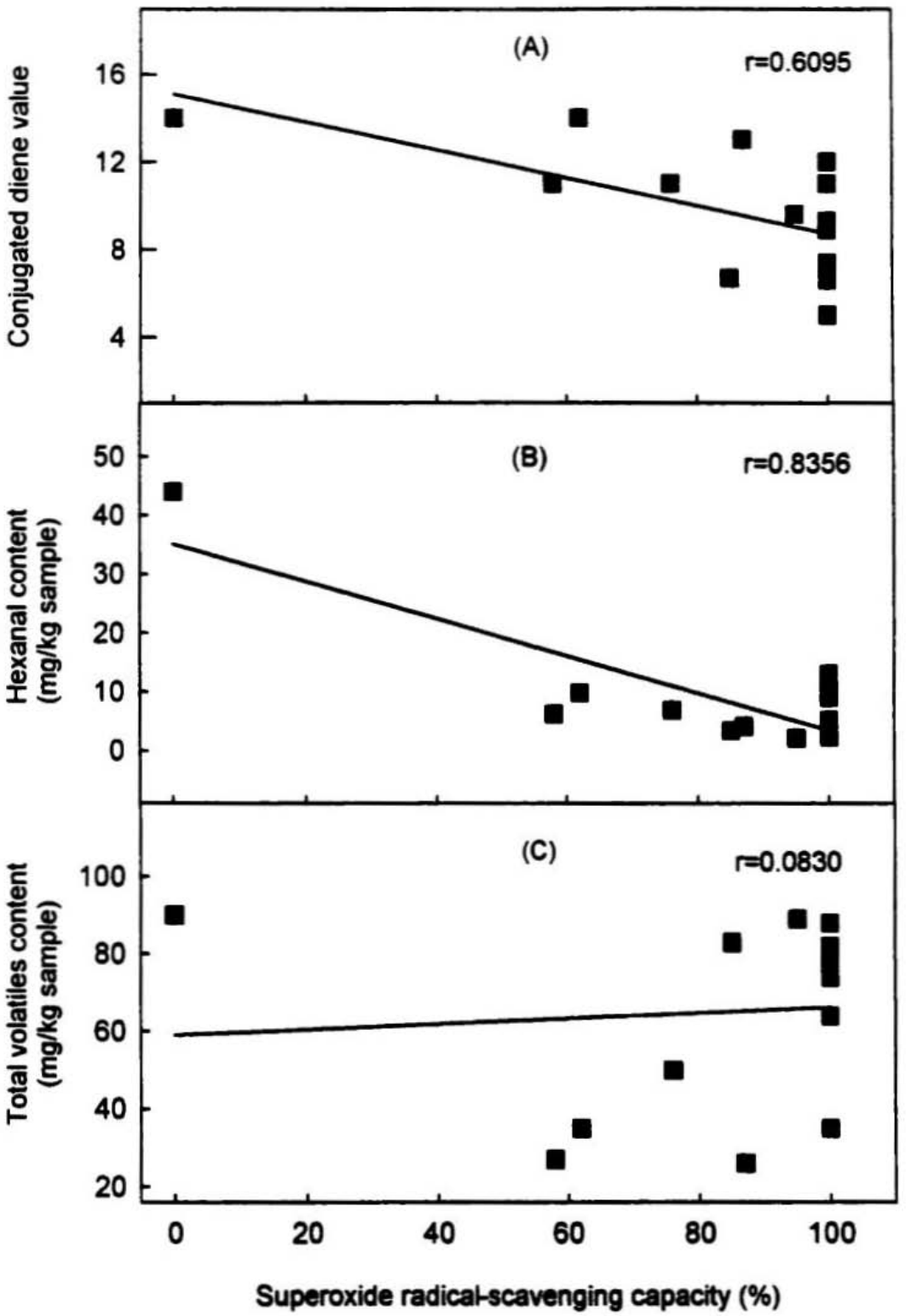

(A)
r=0.6095
Conjugated diene value
16
12
8
4
(B)
r=0.8356
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.0830
Total volatiles content (mg/kg sample)
100
80
60
40
20
0
20
40
60
80
100
Superoxide radical-scavenging capacity (%)

Figure 4.57 Relationships between superoxide-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

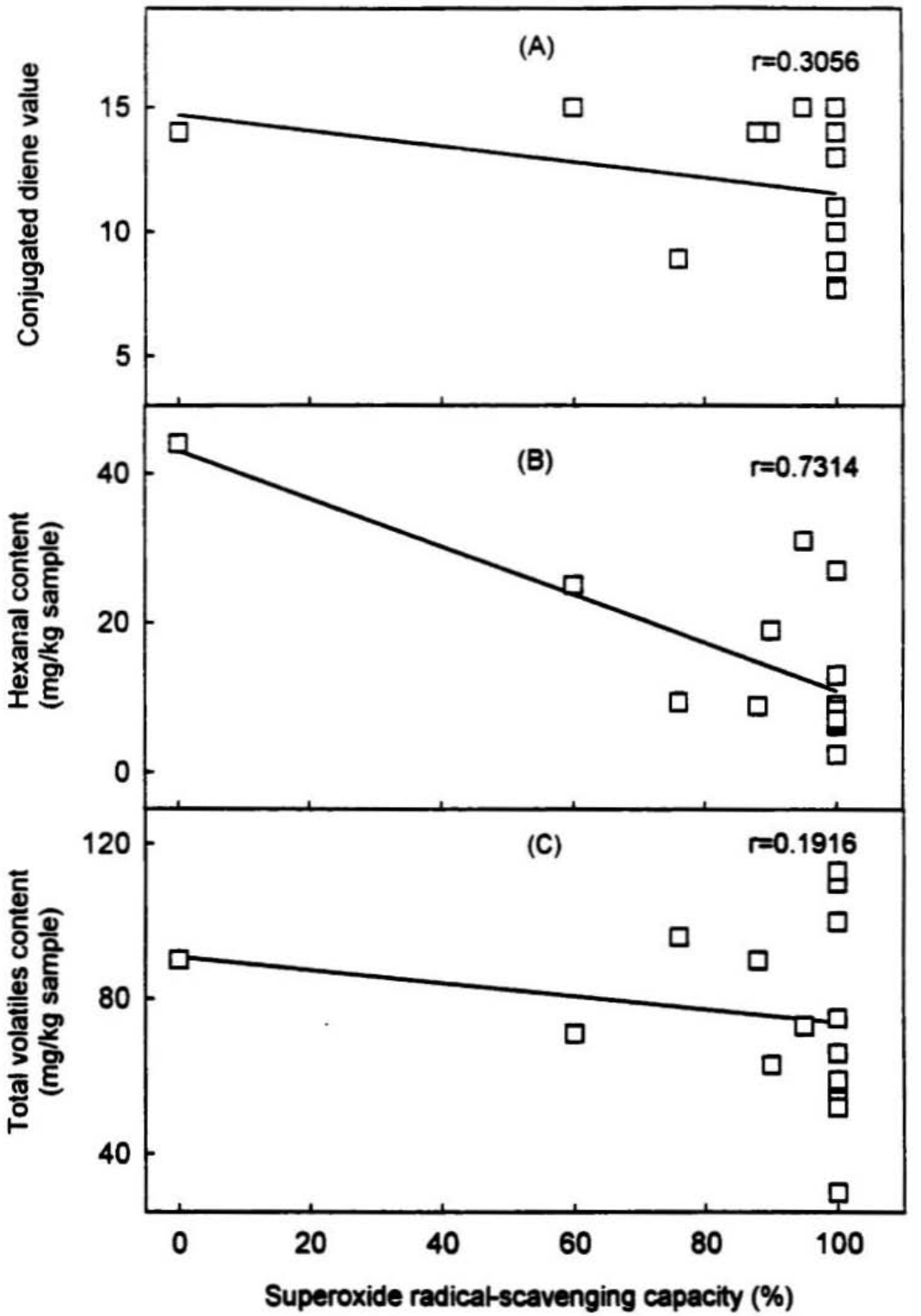

(A)
r=0.3056
Conjugated diene value
15
10
5
(B)
r=0.7314
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.1916
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Superoxide radical-scavenging capacity (%)

Figure 4.58 Relationships between hydroxyl radical-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

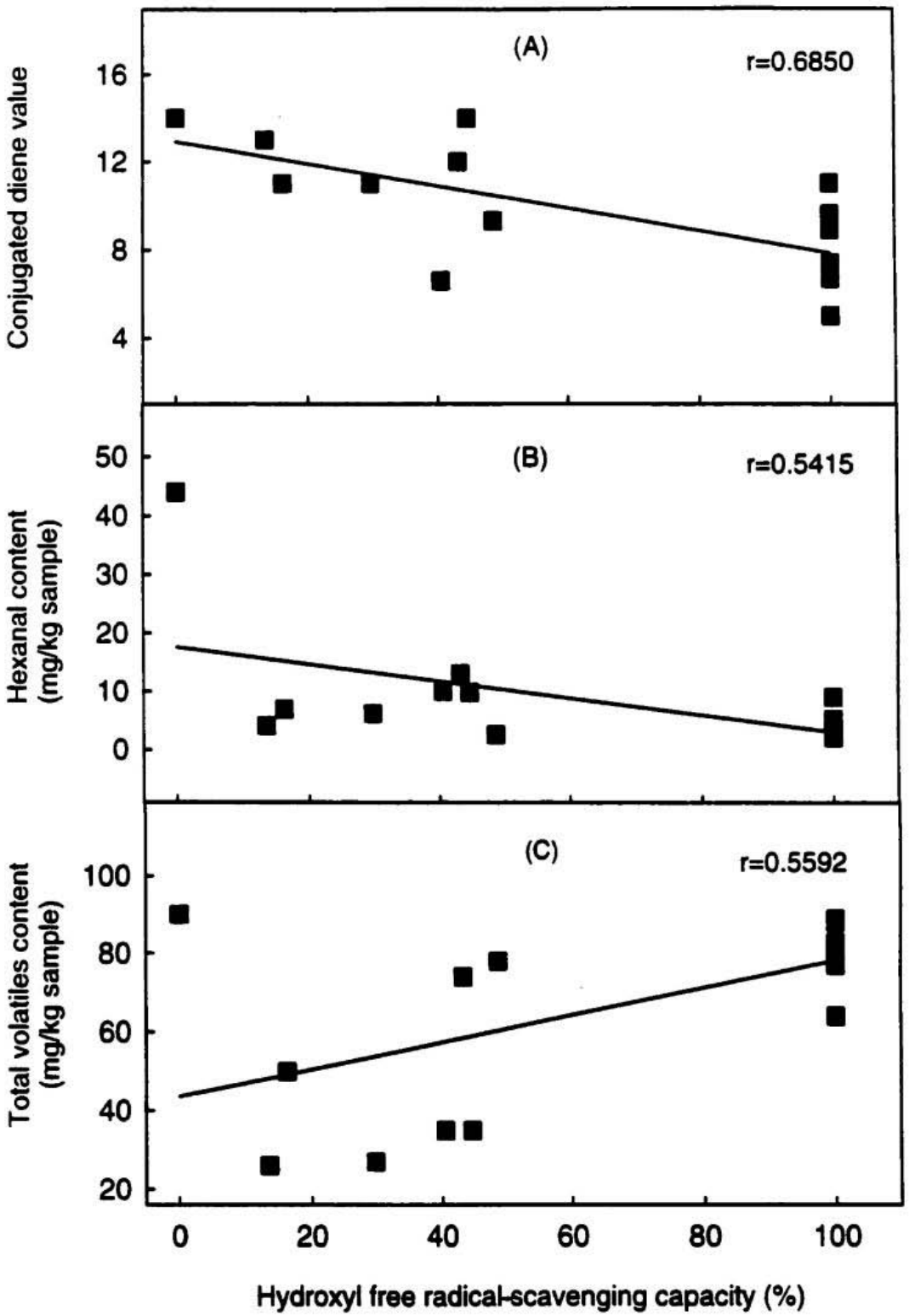

(A)
r=0.6850
Conjugated diene value
16
12
8
4
(B)
r=0.5415
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.5592
Total volatiles content (mg/kg sample)
100
80
60
40
20
0
20
40
60
80
100
Hydroxyl free radical-scavenging capacity (%)

Figure 4.59 Relationships between hydroxyl radical-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

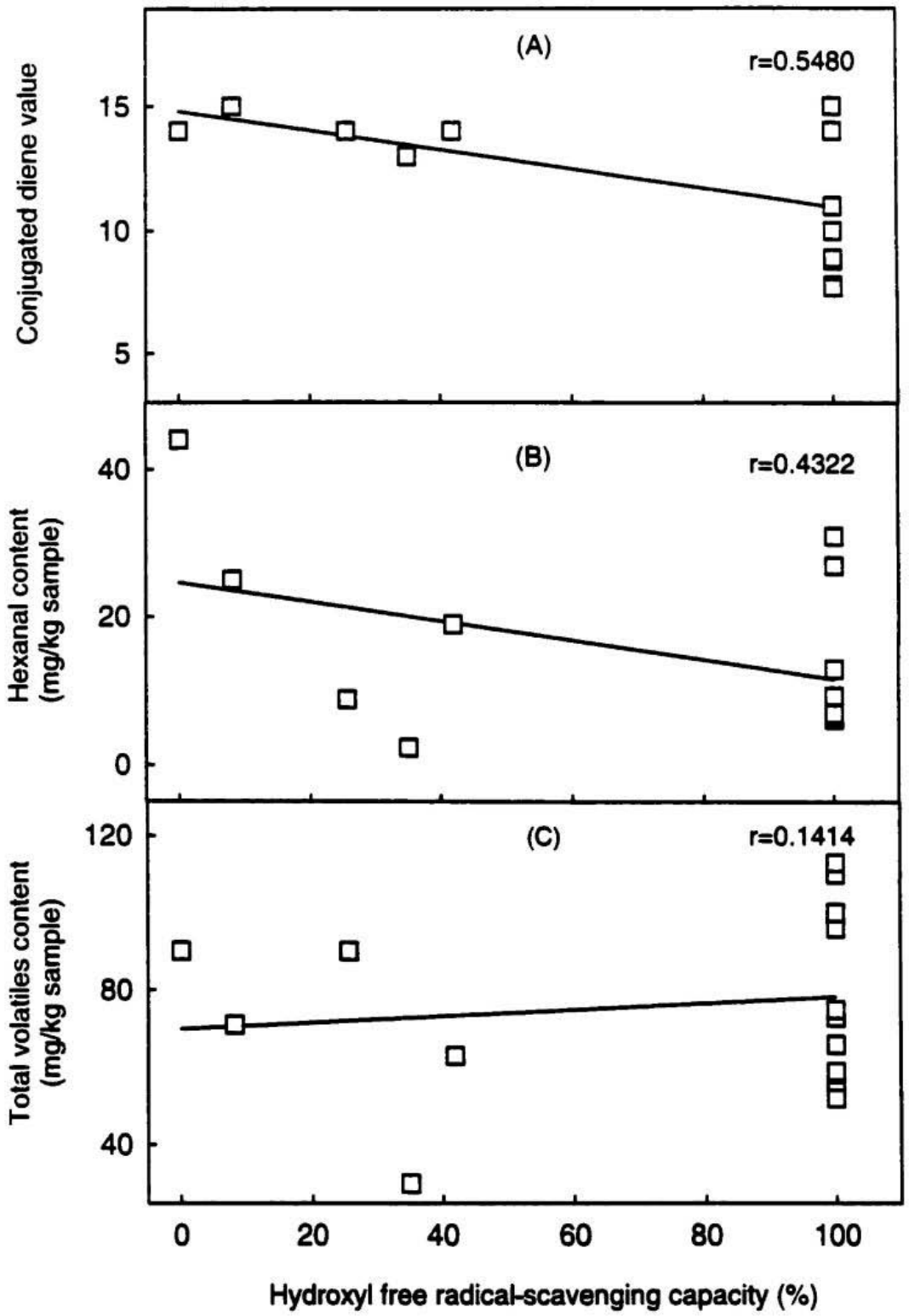

(A)
r=0.5480
Conjugated diene value
15
10
5
(B)
r=0.4322
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.1414
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Hydroxyl free radical-scavenging capacity (%)

Figure 4.60 Relationships between organic free radical (DPPH)-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

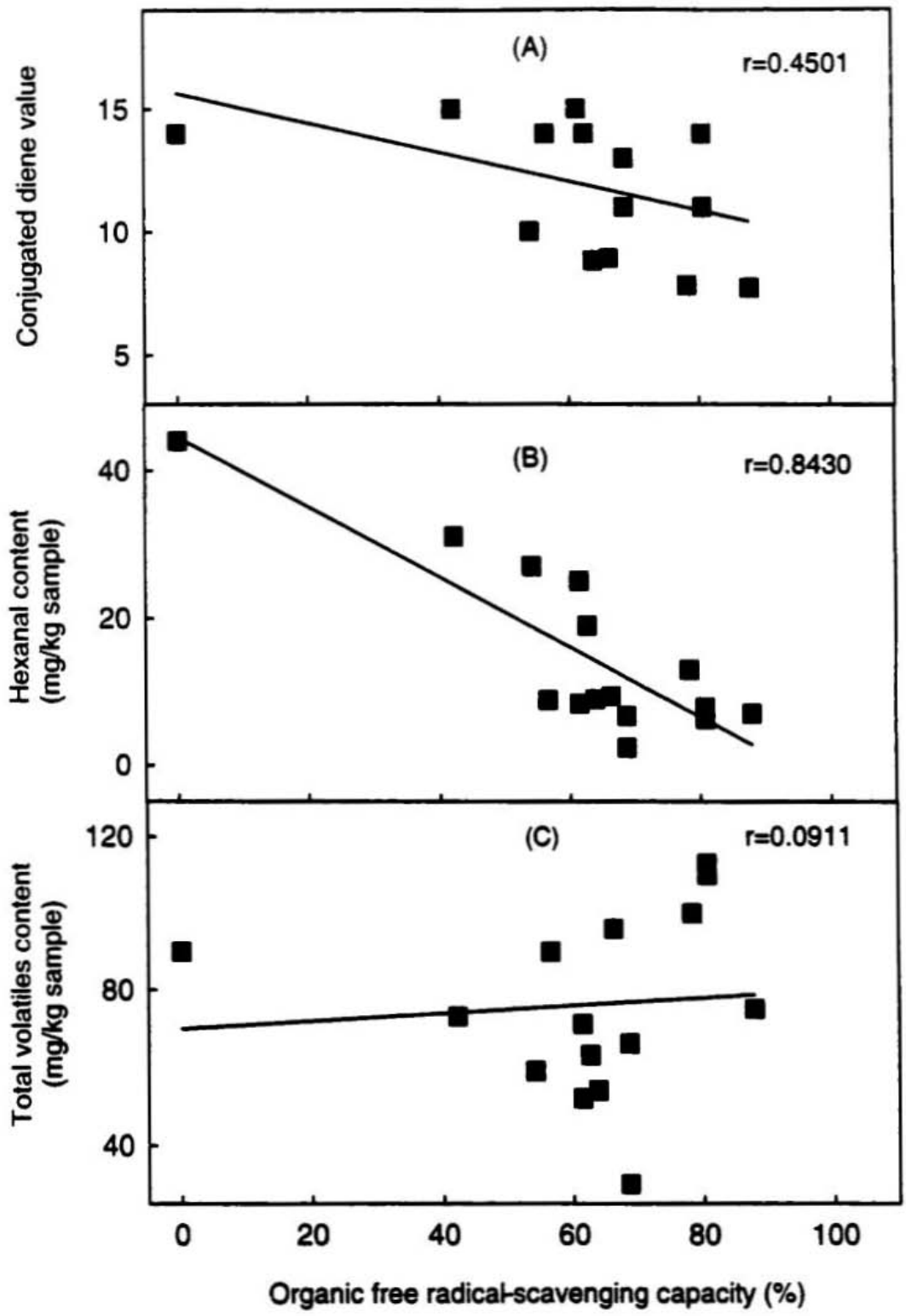

(A)
r=0.4501
Conjugated diene value
15
10
5
(B)
r=0.8430
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.0911
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Organic free radical-scavenging capacity (%)

Figure 4.61 Relationships between organic free radical (DPPH)-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a bulk stripped corn oil model system containing the respective additives.

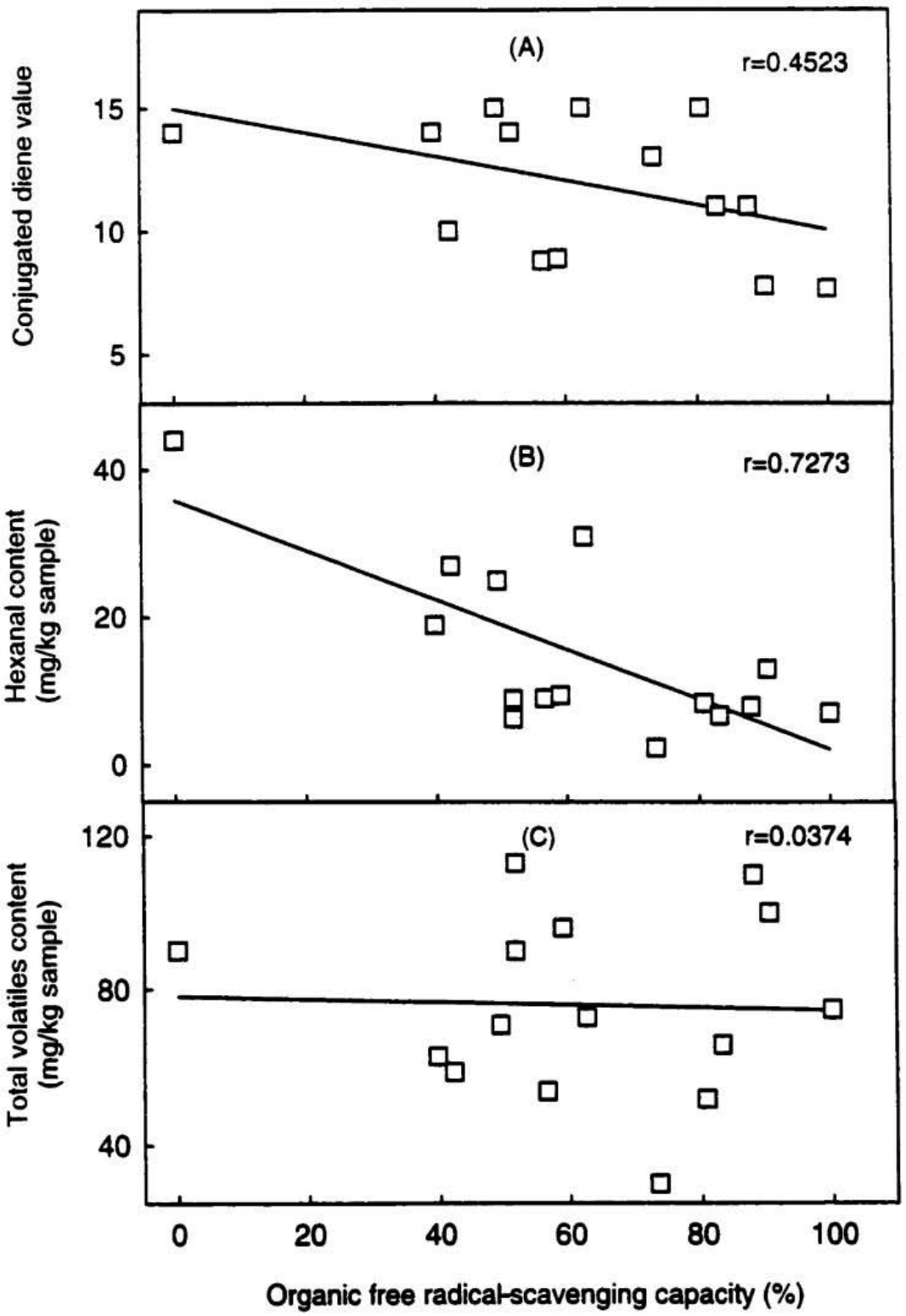

(A)
r=0.4523
Conjugated diene value
15
10
5
(B)
r=0.7273
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.0374
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Organic free radical-scavenging capacity (%)

Figure 4.62 Relationships between superoxide radical-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

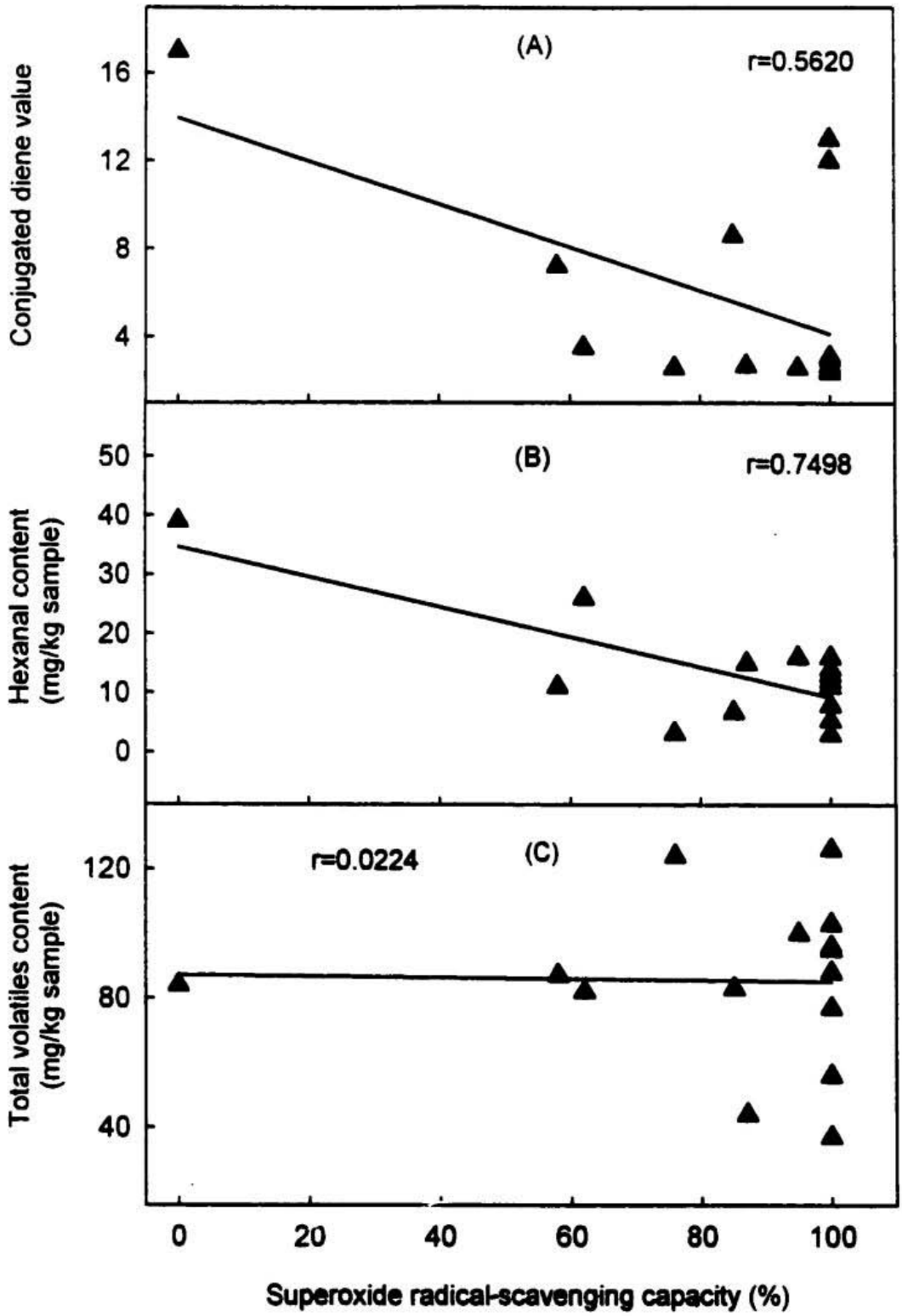

(A)
r=0.5620
Conjugated diene value
16
12
8
4
(B)
r=0.7498
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.0224
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Superoxide radical-scavenging capacity (%)

Figure 4.63 Relationships between superoxide radical-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

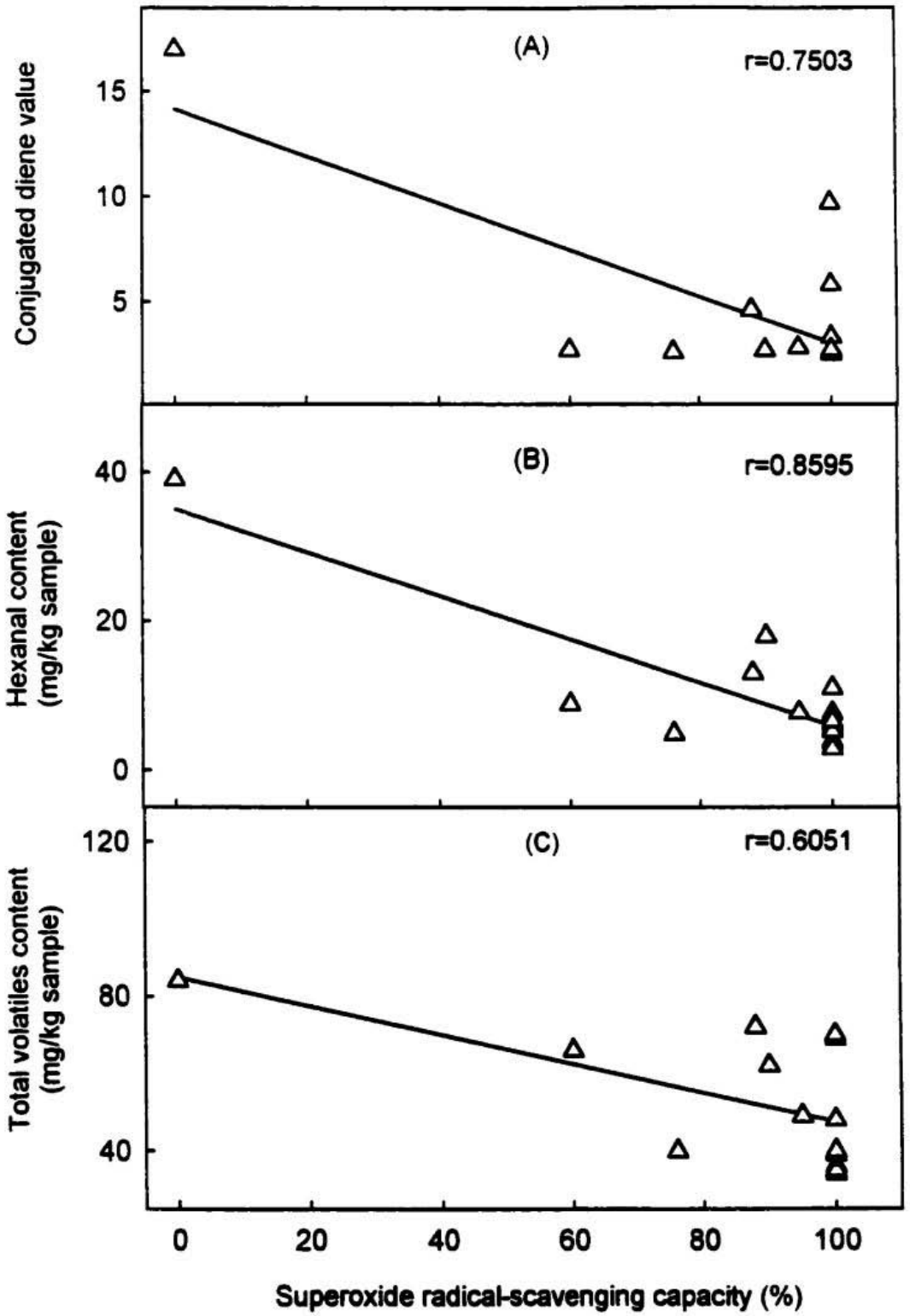
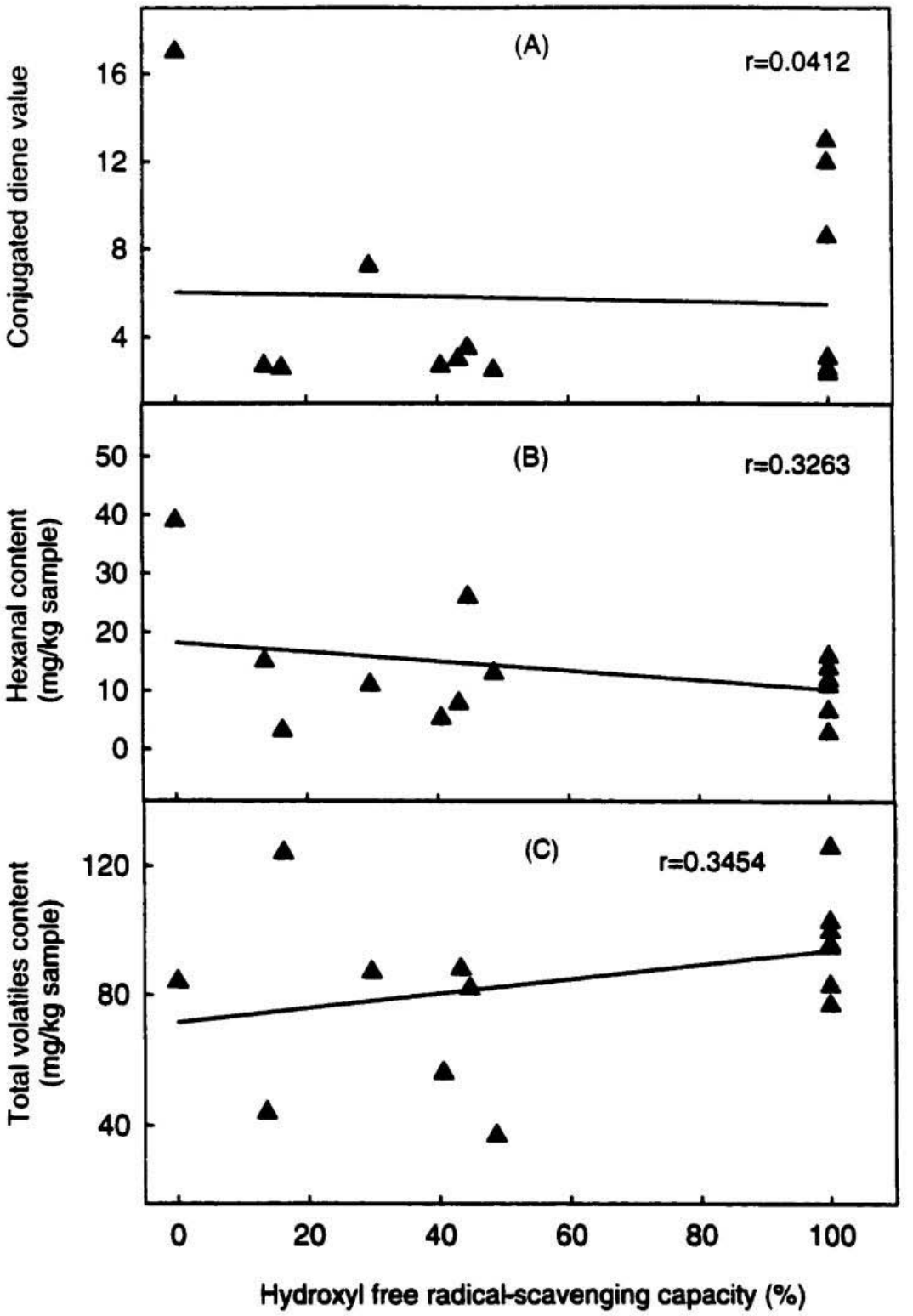

(A)
r=0.7503
Conjugated diene value
15
10
5
(B)
r=0.8595
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.6051
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Superoxide radical-scavenging capacity (%)

Figure 4.64 Relationships between hydroxyl radical-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.


(A)
r=0.0412
Conjugated diene value
16
12
8
4
(B)
r=0.3263
Hexanal content (mg/kg sample)
50
40
30
20
10
0
(C)
r=0.3454
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Hydroxyl free radical-scavenging capacity (%)

0.3454). As depicted in Figure 4.65, good relationships existed between $^{\bullet}OH$-scavenging capacity of evening primrose additives and their effects on the formation of conjugated dienes (r = 0.6305) and hexanal (r = 0.6826), whereas the relationship for total volatiles was strong (r = 0.8335).

As depicted in Figure 4.66, relationships between DPPH$^{\bullet}$-scavenging capacity of borage additives and the conjugated dienes value as well as hexanal content of emulsions containing the respective additives were good (r = 0.6592) and strong (r = 0.8855), respectively, but the correlation (r = 0.5003) with the content of total volatiles was poor. For emulsions containing evening primrose additives, only the content of hexanal showed a strong correlation (r = 0.8390) with DPPH$^{\bullet}$-scavenging capacity of the respective additives (Figure 4.67). This suggests that scavenging of free radicals (as reflected by scavenging of $O_2^{\bullet-}$, $^{\bullet}OH$ or DPPH$^{\bullet}$) by the additives might have contributed greatly to increased oxidative stability of the model systems. Furthermore, better correlations existed between the $O_2^{\bullet-}$-scavenging capacity and antioxidant activity of borage and evening primrose additives in both bulk oil and emulsion systems. Results also suggest that borage additives were inefficient $^{\bullet}OH$ scavengers in both bulk oil and emulsions while evening primrose additives were efficient only in emulsions.

Figure 4.65 Relationships between hydroxyl radical-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

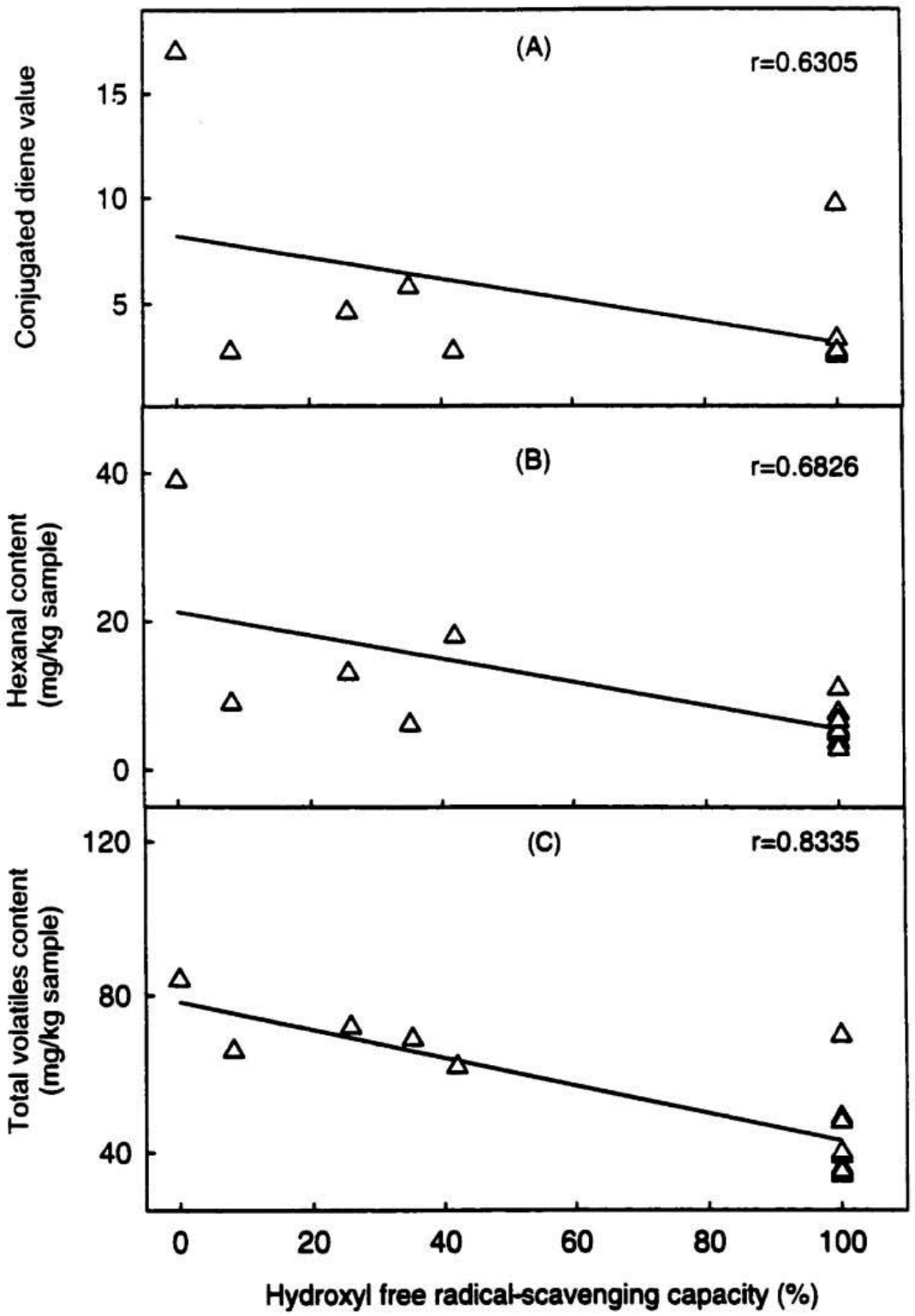

(A)
r=0.6305
Conjugated diene value
15
10
5
(B)
r=0.6826
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.8335
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Hydroxyl free radical-scavenging capacity (%)

Figure 4.66 Relationships between organic free radical (DPPH)-scavenging capacity of borage additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.

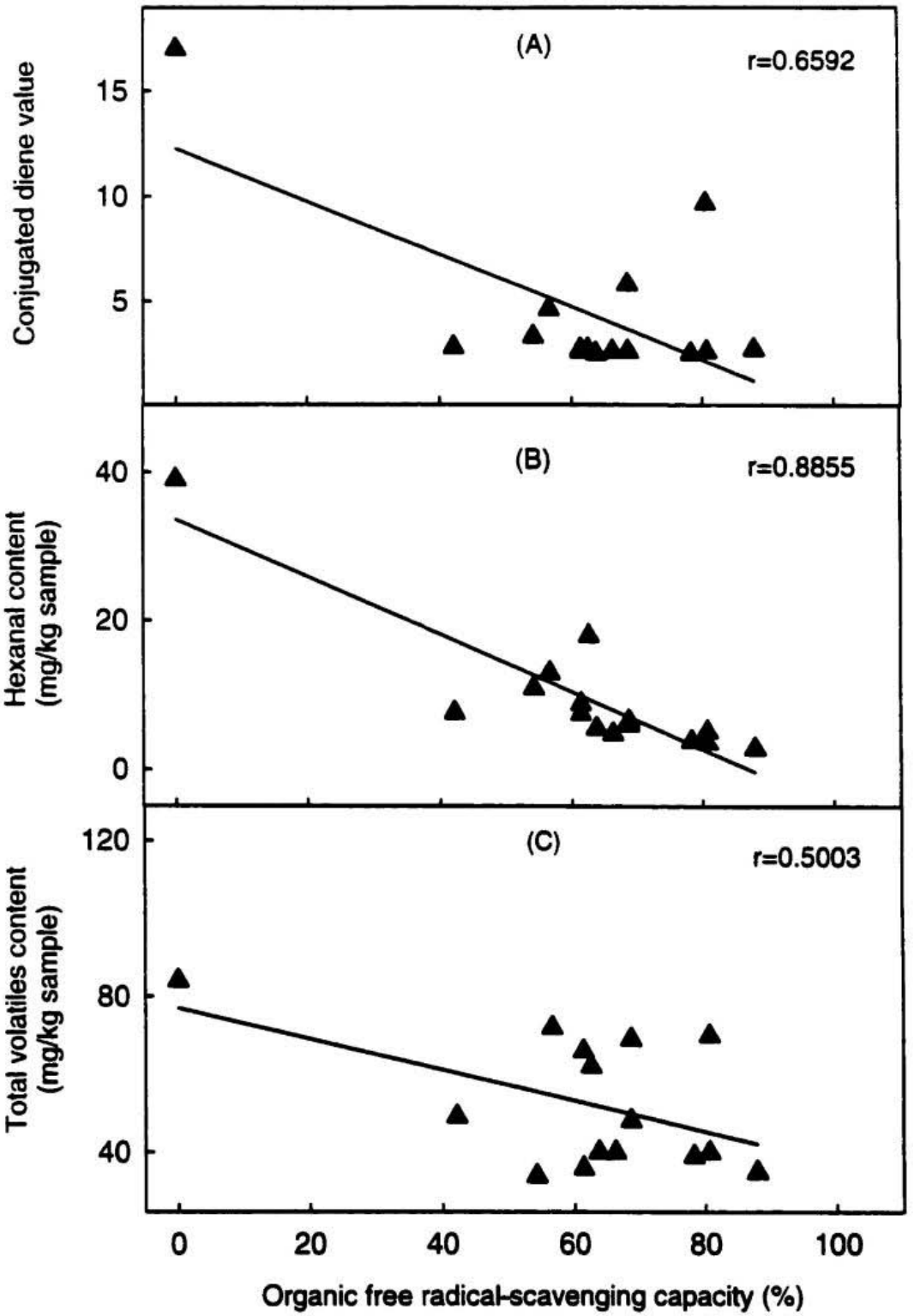
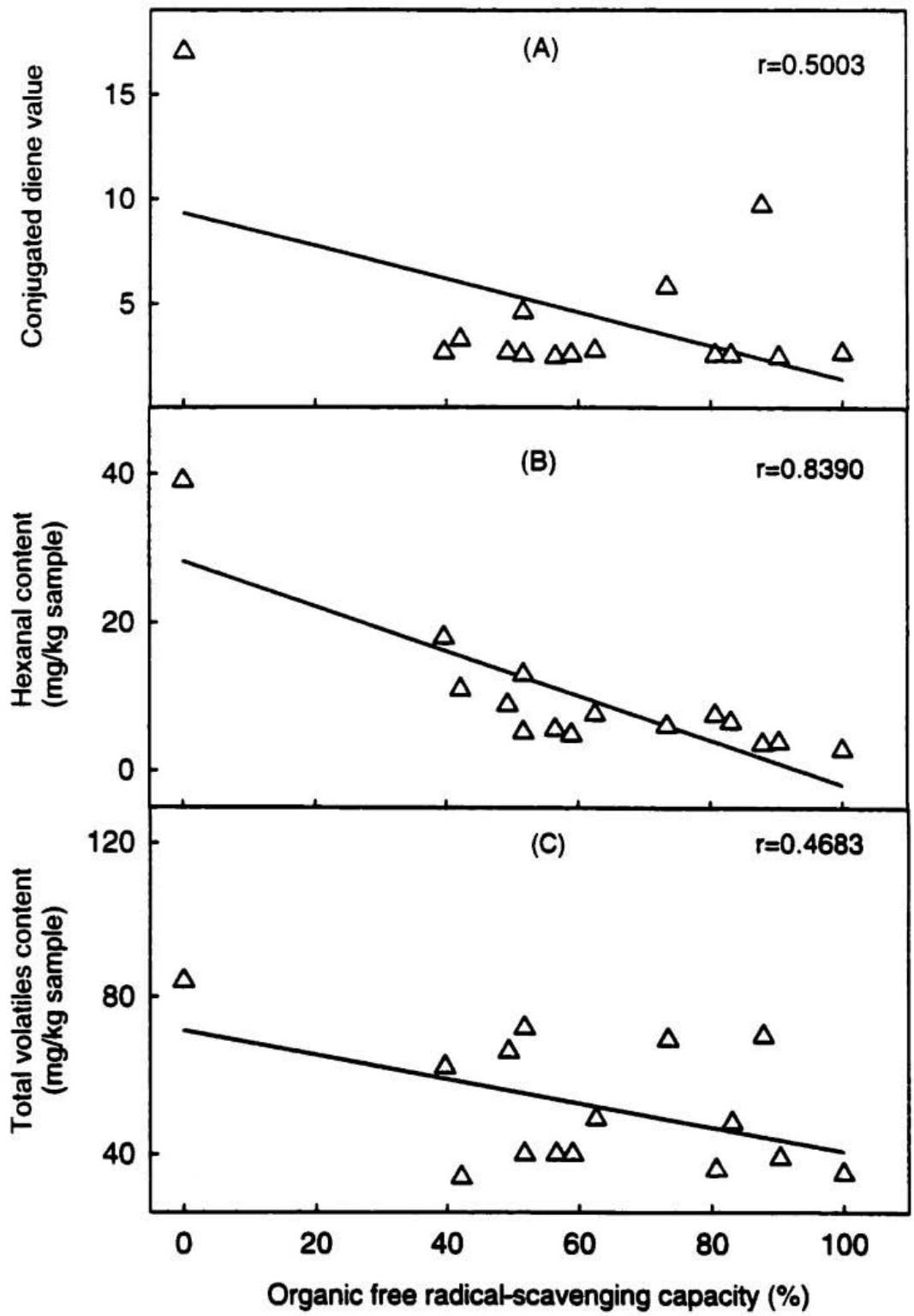

(A)
r=0.6592
Conjugated diene value
15
10
5
(B)
r=0.8855
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.5003
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Organic free radical-scavenging capacity (%)

Figure 4.67 Relationships between organic free radical (DPPH)-scavenging capacity of evening primrose additives and formation of conjugated dienes (A), hexanal (B) and total volatiles (C) in a stripped corn oil-in-water emulsion containing the respective additives.


(A)
r=0.5003
Conjugated diene value
15
10
5
(B)
r=0.8390
Hexanal content (mg/kg sample)
40
20
0
(C)
r=0.4683
Total volatiles content (mg/kg sample)
120
80
40
0
20
40
60
80
100
Organic free radical-scavenging capacity (%)

4.10 Elucidation of chemical structures of active compounds of borage and evening primrose extracts

In an attempt to determine chemical structures of phenolic compounds of borage and evening primrose, crude extracts were subjected to Sephadex LH-20 column chromatography. As discussed in section **4.5**, six fractions from each extract were obtained and the desolventized fractions were then chromatographed on silica gel thin-layer chromatographic (TLC) plates.

4.10.1 Thin-layer chromatography (TLC) of borage fractions and high performance liquid chromatography of TLC spots.

Table 4.12 shows the results for TLC chromatography of borage fractions and standard phenolic compounds. When a developed plate was sprayed with a solution of 1% (w/v) ferric chloride-potassium ferricyanide [$FeCl_3$-$K_2Fe(CN)_6$], several pale blue and intense blue colour spots were visualized for each of the fractions; their R_f values are given in Table 4.12. When sprayed with 1% $FeCl_3$-$K_2Fe(CN)_6$ solution, an intense blue colour was produced by the spot at R_f 0.51 of fraction I. When a second plate (developed in the same solvent system) was sprayed with a β-carotene-linoleate emulsion, the spot at R_f 0.51 of fraction I showed an intense yellow colour even after 1 h exposure to fluorescent light. This was an indication of the presence of one or more phenolic compounds with moderate antioxidant activity. Spot with R_f 0.56 of fraction II gave a pale blue colour with $FeCl_3$-$K_2Fe(CN)_6$ and produced a yellow colour with β-carotene-linoleate emulsion.

Table 4.12 R_f values and antioxidant activities of various borage phenolics and authentic standards resolved on TLC plates.

Fraction	Spray A	Spray B		
	Blue[1]	Orange[2]	Yellow[3]	Pale yellow[4]
I	0.33, 0.51, 0.57, 0.68, 0.76	--	0.51	--
II	0.44, 0.56, 0.78	--	0.56	0.44, 0.78
III	0.44, 0.56, 0.82, 0.90	--	0.56	0.44, 0.82, 0.90
IV	0.57, 0.82	0.82	0.57	--
V	0.30, 0.44, 0.56, 0.84	0.84	0.56	0.30, 0.44
VI	0.32, 0.44	--	--	0.32, 0.44
Gallic acid[5]	0.63	0.63	--	--
(+)Catechin[5]	0.92	0.92	--	--
(-)Epicatechin[5]	0.92	0.92	--	--
Sinapic acid[5]	0.82	0.82	--	--
Syringic acid[5]	0.57	0.57	--	--

[1]Colour produced when Spray A [1% $FeCl_3$-$K_2Fe(CN)_6$] was sprayed.
[2,3,4]Intensity of colour of β-carotene after 1 h from sprying of Spray B (β-carotene-linoleate emulsion).
[5]Concentration of authentic standard solutions was 1 mg/mL.

Other two spots of fraction II had weak antioxidant activities as they could not retain much of the β-carotene during exposure to fluorescent light. Among four spots observed for fraction III, the spot with R_f 0.56 showed moderate antioxidant activity and this spot gave a pale blue colour with 1% solution of $FeCl_3$-$K_2Fe(CN)_6$. Other spots of fraction III exhibited weak antioxidant activities. The spot with R_f 0.82 of fraction IV showed strong antioxidant activity as its colour was intense orange even after 1 h exposure to fluorescent light. The other spot with R_f 0.57 of fraction IV showed moderate antioxidant activity. These two spots produced a highly intense blue colour with $FeCl_3$-$K_2Fe(CN)_6$. Fraction V also contained a spot (R_f = 0.84) with high antioxidant activity. Other three spots had moderate to weak antioxidant activities. However, none of the spots of fraction V contained sufficient phenolics (as judged by the intensity of blue colour) to be considered for further analysis. Spots of fraction VI showed weak antioxidant activities as their colour faded rapidly under fluorescent light. As indicated by R_f values of standards, absence of (+)catechin and (-)epicatechin in any of the fractions was evident. Absence of flavonoid class of compounds in borage crude extract was also confirmed by a negative vanillin test. R_f values of spots of fraction IV matched with those of syringic (0.57) and sinapic (0.82) acids. The spot with R_f 0.51 in fraction I did not correspond with R_f of any of the standards used, but the intense blue colour of the spot indicated that it contained a major phenolic compound(s). After isolating the spots on a preparative TLC plate, they were subjected to preparative HPLC. A sharp single peak produced by analytical HPLC of each of the spot indicated that each of them contained a single compound. Purified

borage compounds were labeled as A, B and C and subjected to instrumental analysis in order to elucidate their exact chemical identities.

4.10.2 Thin-layer chromatography (TLC) of evening primrose fractions and high performance liquid chromatography of TLC spots

Thin-layer chromatography of evening primrose fractions showed the presence of various phenolic compounds (Table 4.13). All fractions contained phenolics which did not move from the origin (R_f = 0) and showed strong antioxidant activities because the corresponding spots retained the orange colour of β-carotene even after 1 h exposure to fluorescent light. Spot with R_f 0.68 of fraction I exerted moderate antioxidant activity while the other two spots contained phenolics with relatively weak antioxidant activities. The spot with R_f of 0.88 of fraction II was unable to retain any β-carotene. Other two spots of fraction II had moderate to weak antioxidant activities. The spot with R_f 0.22 of fraction III did not show antioxidant activity. A spot with R_f 0.92 of fraction III showed strong antioxidant activity and an intense blue colour produced upon spraying with a 1% solution of $FeCl_3$-$K_2Fe(CN)_6$. The other spot (R_f = 0.53) of fraction III exhibited a weak antioxidant activity. Fraction IV contained phenolics with moderate to high antioxidant activities, but these spots did not produce an intense blue colour with $FeCl_3$-$K_2Fe(CN)_6$. Fraction V contained a spot with R_f 0.63 and exhibited a strong antioxidant activity. Fraction VI contained the most number of spots with strong to weak antioxidant activities, but their quantities were very small as reflected by the low intensity of blue colour

Table 4.13 R_f values and antioxidant activities of various evening primrose phenolics and authentic standards resolved on TLC plates.

Fraction	Spray A	Spray B		
	Blue[1]	Orange[2]	Yellow[3]	Pale yellow[4]
I	0.00, 0.68, 0.90, 0.97	0.00	0.68	0.90, 0.97
II	0.00, 0.77, 0.88, 0.95	0.00	0.95	0.77
III	0.00, 0.22, 0.53, 0.92	0.00, 0.92	--	0.53
IV	0.00, 0.22, 0.32	0.00, 0.32	0.22	--
V	0.00, 0.13, 0.63	0.00, 0.63	0.13	—
VI	0.00, 0.03, 0.06, 0.13, 0.22, 0.32, 0.63	0.00, 0.03, 0.06	0.13, 0.22, 0.63	0.32
Gallic acid[5]	0.63	0.63	--	--
(+)Catechin[5]	0.92	0.92	--	--
(-)Epicatechin[5]	0.92	0.92	--	--
Sinapic acid[5]	0.82	0.82	--	--
Syringic acid[5]	0.57	0.57	--	--

[1]Colour produced when Spray A [1% $FeCl_3$-$K_2Fe(CN)_6$] was sprayed.
[2,3,4]Intensity of colour of β-carotene after 1 h from spraying of Spray B (β-carotene-linoleate emulsion).
[5]Concentration of authentic standard solutions was 1 mg/mL.

produced with $FeCl_3$-$K_2Fe(CN)_6$. Therefore, these spots were not considered for further analysis. R_f value of one of the spots (0.92) of fraction III correspond with those of authentic (+)catechin and (-)epicatechin. Presence of flavonoid class of phenolics in evening primrose crude extract and fractions III-VI was also confirmed by a positive vanillin test (which produced a pink colour). The R_f value of one the spots (0.63) of fraction V correspond with that of the authentic gallic acid. Syringic and sinapic acids were not present in any of the evening primrose fractions. Spots corresponding to catechin and gallic acid were isolated on a preparative plate and subjected to analytical HPLC. Analysis of spot with R_f 0.92 of fraction III, using an analytical HPLC column, showed the presence of two closely related compounds and these two compounds resolved very close to each other. When these two compounds were resolved on a preparative HPLC column, they had retention times slightly different from one another. A careful setting of the flow rate permitted the collection of two pure compounds at the outlet port. These two compounds were labeled as D and E. The spot with R_f 0.63 of fraction V was a pure compound as its analytical HPLC chromatogram showed a single sharp peak. This compound was labeled as F. Compounds D, E and F were subjected to instrumental analysis in order to elucidate their exact chemical identities.

4.11 Structural analysis of active components of borage and evening primrose extracts

As discribed in sections **4.10.1** and **4.10.2**, the tentative identities of compound B, C, D, E and F were established as being syringic acid, sinapic acid, (+)catechin, (-)epicatechin and gallic acid, respectively. Tentative identity of compound A was not established. The following sections will focus on the structure elucidation of these compounds by means of UV, mass (MS) and nuclear magnetic resonance (NMR) spectroscopies.

4.11.1 Structural analysis of compound A

As shown in Figure 4.68, compound A had a UV absorbance maximum at 330 nm and a shoulder at 294 nm. This could be due to a phenolic acid which has two aromatic rings (Rice-Evans *et al.*, 1996). The electron-impact mass spectrum of compound A is shown in Figure A.11 and its major fragments are presented in Table 4.14. The fragment ion with m/z of 299 could conceivably be produced *via* losses of CO_2 and OH from the molecular radical ion ($M^{+\bullet}$). As expected, the molecular radical ion ($M^{+\bullet}$) at m/z 360 was not present as it decomposed in the ionization chamber. Fragment ion with m/z of 181 was due to the loss of a $C_9H_7O_4$ moiety from the $M^{+\bullet}$ and further loss of one H from the fragment resulted in an ion with m/z of 180. The fragment ion with m/z of 179 indicated loss of a molecule of hydrogen from the fragment ion at m/z of 181. Losses of OH, H_2O and H_3O respectively from the fragment ion at m/z of 181 resulted in three fragment ions

Figure 4.68 UV spectra of compounds A, B and C.

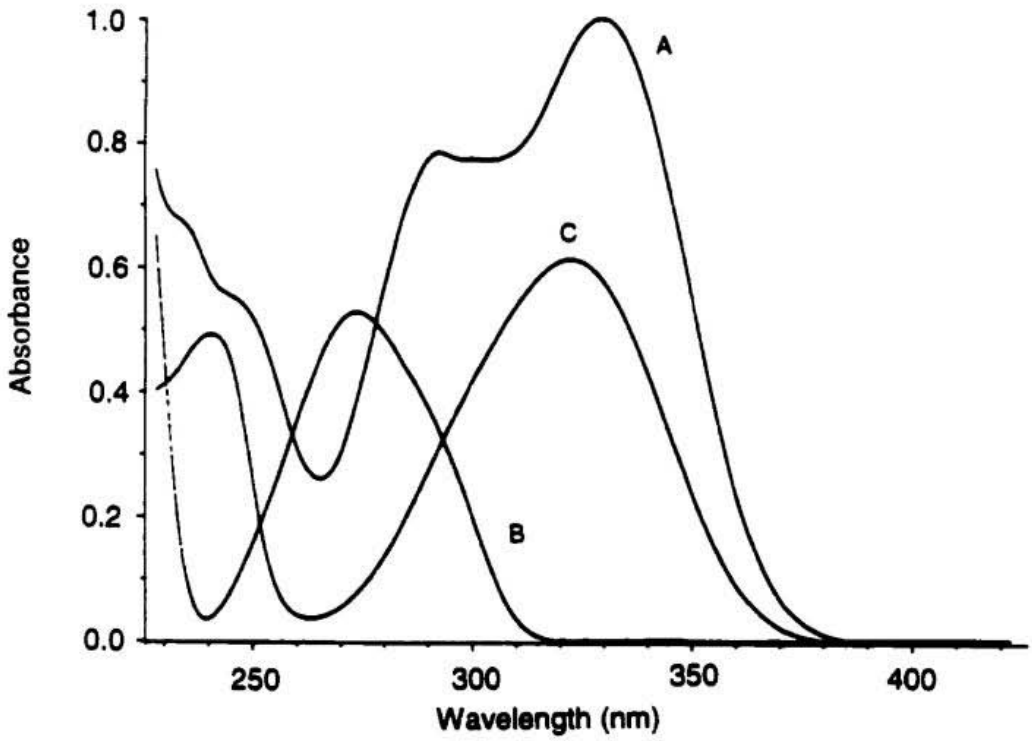
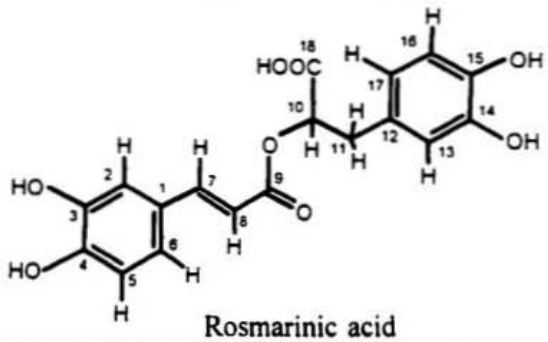

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
250
300
350
400
Wavelength (nm)
A
B
C

Table 4.14 Mass spectral fragmentation pattern of compound A.



Rosmarinic acid

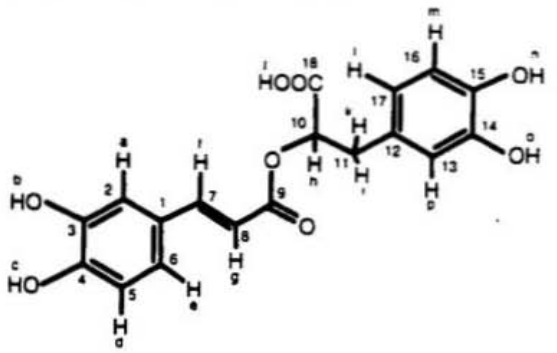
m/z	% Intensity	Fragment ion
360.00	ND	$C_{18}H_{16}O_8^{\cdot}$, ($M^{\cdot\cdot}$)
299.04	1.00	$C_{17}H_{15}O_5^{-}$, ($M^{\cdot\cdot}$ - CO^2 - OH)
181.01	11.00	$C_9H_9O_4^{\cdot}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$)
179.96	38.50	$C_9H_8O_4^{\cdot}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - H)
178.99	4.25	$C_9H_7O_4^{-}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - 2H)
164.06	17.25	$C_9H_8O_3^{\cdot}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - OH)
163.03	70.00	$C_9H_7O_3^{-}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - H_2O)
162.00	23.00	$C_9H_6O_3^{\cdot}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - H_3O)
137.06	8.00	$C_8H_9O_2^{-}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - CO_2)
136.07	25.75	$C_8H_8O_2^{\cdot}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - CO_2 - H)
135.05	14.50	$C_8H_6O_2^{\cdot}$, ($M^{\cdot\cdot}$ - $C_9H_7O_4$ - CO_2 -2H)
124.08	23.00	$C_7H_8O_2^{\cdot}$
123.06	87.00	$C_7H_7O_2^{+}$
109.93	34.00	$C_6H_5H_2^{+}$
44.02	100	$CO_2^{\cdot}$

ND: Not detected

with m/z of 164, 163 and 162. However, it is evident that the loss of water was the major factor in the fragmentaion process because the intensity of fragment with m/z of 163 was 70%. Loss of CO_2 from the fragment ion at m/z of 181 produced a fragment ion with m/z of 137. The fragment ion at m/z of 136 was formed by losses of both H and CO_2 or an acid group from the fragment ion at m/z of 181. Loss of H_2 and CO_2 or H and COOH from the fragment ion at m/z of 180 gave an ion at m/z of 135. This pattern suggests the presence of a carboxylic acid group in the molecule. Two major moieties of the molecule produced a fragment ion ($C_7H_8O_2^-$) at m/z of 124. Further loss of a hydrogen atom from one of the two OH groups of the $C_7H_8O_2$ ion produced an ion at m/z of 123 and its intensity was 87%. The fragment ion at m/z of 110 represented the dihydroxybenzene ion produced from the two major moieties of the molecule. Loss of CO_2 from the molecule was evident by the presence of a fragment with m/z of 44. This ion had a 100% intensity and suggested possible presence of acid groups in the molecules (Pretsch *et al.*, 1983).

^{1}H-NMR spectrum of compound A is shown in Figure A.12 and proton assignments are given in Table 4.15. Signal for proton 'f' appeared at 7.50 ppm and it was split by the magnetic field of the proton 'g'. Proton 'a' also produced a doublet at 7.09 ppm due to coupling with proton 'e'. Coupling of proton 'e' with protons 'a' and 'd' produced a doublet at 6.79 ppm. Proton 'd' produced a doublet at 6.79 ppm due to its coupling with proton 'e'. A doublet signal (6.69 ppm) for proton 'p' also appeared very close to the signal of proton 'd'. Signal for proton 'm' split due to its coupling with proton 'l' while coupling of proton 'i' with protons 'm' and 'p' produced a doublet of

Table 4.15 Proton assignment for compound A.



Rosmarinic acid

ppm	Multiplicity	J (Hz)	Integration	Assignment
7.50	d	$J_{fg}=16$	--	H_f
7.09	d	$J_{ac}=2$	--	H_a
6.96	dd	$J_{ac}=2$, $J_{dc}=8$	--	H_c
6.79	d	$J_{dc}=8$	--	H_d
6.79	d	$J_{pl}=2$	--	H_p
6.69	d	$J_{lm}=8$	--	H_m
6.60	dd	$J_{pl}=2$, $J_{lm}=8$	--	H_l
6.23	d	$J_{fg}=16$	--	H_g
5.17	dd	$J_{hk}=5$, $J_{hi}=8$	--	H_h
3.03	2t	$J_{hk}=5$, $J_{hi}=8$	--	H_i, H_k

Note-Assignment of coupling interactions (J) confirmes by a COSY experiment (Figure A.13).
-- Integrations did not reflect actual number of protons due to insufficient peak intensities.

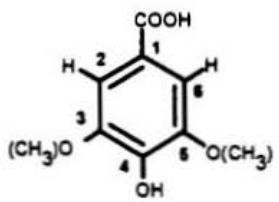
doublet. A doublet for proton 'g' appeared at 6.23 ppm while proton 'h' produced a doublet of doublet at 5.17 ppm. Protons 'I' and 'k' resolved as quartets at 3.03 ppm. These assignments were well in agreement with those reported by Kelly *et al.* (1975) who reported ^{1}H-NMR data for lithospermic acid and rosmarinic acid isolated from *Lithospermum ruderale*, a plant belonging to *Boraginaceae* family.

4.11.2 Structural analysis of compound B

UV spectrum of compound B is shown in Figure 4.68. This compound has an absorbance maximum at 272 nm and also an identical UV spectrum to the authentic syringic acid. The electron impact mass spectrum of compound B is shown in Figure A.14 and its major fragments are listed in Table 4.16. The presence of the molecular radical ion ($M^{+\bullet}$) at m/z 198 suggests that the compound could be syringic acid. The fragment ion at m/z of 184 was formed from molecular radical ion due to the loss of $CH_2^{\bullet}$. Another fragment ion at m/z 183 indicated loss of $CH_3^{\bullet}$ form the molecular radical ion. This demonstrated the presence of methyl groups in the molecule. The fragment at m/z of 181 was due to the loss of $OH^{\bullet}$ from the molecular radical ion and suggested that the compound contained OH group(s). The aromaticity of compound B was confirmed by the formation of fragments at m/z of 79, 67, 51 and 39 (Pretsch *et al.*, 1983). A similar fragmentation pattern was observed for the authentic syringic acid.

Figure A.15 shows the ^{1}H-NMR spectrum of compound B; assignment of protons is given in Table 4.17. A singlet at 7.32 ppm shows the presence of two aromatic protons

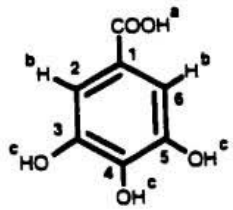
Table 4.16 Mass spectral fragmentation pattern of compound B.



4-Hydroxy-3,5-dimethoxybenzoic acid (Syringic acid)

m/z	% Intensity	Fragment ion
198.05	100	$C_9H_{10}O_5^{\cdot}$. ($M^{\cdot\cdot}$)
184.04	2.82	$C_8H_8O_5^{\cdot}$. ($M^{\cdot\cdot}$+H)-CH_3
183.03	29.12	$C_8H_7O_5^{\cdot}$, ($M^{\cdot\cdot}$-CH_3)
181.01	7.40	$C_9H_9O_4^{\cdot}$, ($M^{\cdot\cdot}$-OH)
78.96	8.53	$C_6H_7^{\cdot}$, (Aromatic)
67.06	10.84	$C_5H_7^{\cdot}$, (Aromatic)
50.97	11.45	$C_4H_3^{\cdot}$, (Aromatic)
38.97	17.98	$C_3H_3^{\cdot}$, (Aromatic)

Table 4.17 Proton assignments for compound B.



4-Hydroxy-3,5-dimethoxybenzoic acid (Syringic acid)

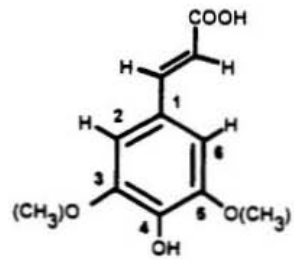
(ppm)	Multiplicity	J (Hz)	Integration	Assignment
7.32	s	—	2H	H_b
4.86	s	—	3H	Solvent
3.87	s	—	6H	CH_3O
3.30	s	—	1H	Solvent

(Pretsch *et al.*, 1983). A sharp singlet at 3.87 is characteristic of methoxy (-O-CH_3) groups and the integration of this peak confirmed the presence of six protons, thus two methoxy groups in the molecule. Other peaks were due to the solvent. Proton of the carboxylate group did not resolve because it exchanged with deuterium. A similar ^{1}H-NMR spectrum was observed for the authentic syringic acid sample. ^{13}C-NMR of compound B was not carried out due to small sample size. However, UV, mass and ^{1}H-NMR spectroscopies provided convincing evidence to identify compound B as 4-hydroxy-3-5-dimethoxybenzoic acid (or syringic acid).

4.11.3 Structural analysis of compound C

As evidenced by UV spectrum of compound C (Figure 4.68), the absorbance maximum of their compound was 322 nm. The shape of the UV spectrum and the absorbance maximum of compound C were identical to those of the authentic sinapic acid. Thus, it was suspected that this compound could well be sinapic acid. Figure A.16 depicts the electron-impact mass spectrum of compound C, major fragments of which are presented in Table 4.18. The molecular radical ion ($M^{+\bullet}$) was noticed at m/z of 224. The fragment ion at m/z of 209 was formed from the loss of a $CH_3^\bullet$ group from $M^{+\bullet}$. Losses of both CO_2 and $CH_3^\bullet$ from $M^{+\bullet}$ produced a fragment ion at m/z of 163. The fragment ion at m/z of 121 suggested the formation of quinone structure during the fragmentation process (Pretsch *et al.*, 1983). The aromaticity of the compound was confirmed by the

Table 4.18 Mass spectral fragmentation pattern of compound C.



4-Hydroxy-3,5-dimethoxycinnamic acid (Sinapic acid)

m/z	% Intensity	Fragment ion
224.08	100	$C_{11}H_{12}O_5^{\cdot}$, ($M^{\cdot}$)
209.02	13.11	$C_{10}H_9O_5^{-}$, ($M^{\cdot}$-CH_3)
163.03	6.59	$C_{10}H_{11}O_3^{-}$, ($M^{\cdot}$-CO_2-OH)
120.97	10.48	$C_7H_5O_2^{-}$
78.97	3.40	$C_7H_7^{-}$, (Aromatic)
65.11	12.38	$C_5H_5^{-}$, (Aromatic)
53.07	8.70	$C_4H_5^{-}$, (Aromatic)
50.97	10.19	$C_4H_3^{-}$, (Aromatic)
38.97	10.64	$C_3H_3^{-}$, (Aromatic)

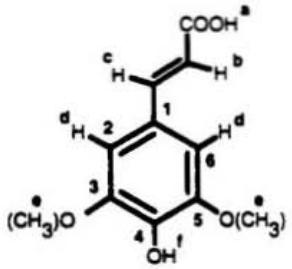
presence of fragments at m/z of 79, 65, 53, 51 and 39 (Pretsch *et al.*, 1983). The fragmentation pattern of compound C was similar to that of the authentic sinapic acid under the same electron-impact MS conditions.

^{1}H-NMR spectrum of compound C is shown in Figure A.17 and proton assignments for it are given in Table 4.19. A doublet at 7.576 ppm was due to coupling interactions of proton 'c' with the adjacent protons 'b'. Integration of the peak showed the presence of a single proton. Since proton c was more deshielded than proton 'b', thus it resolved in the low field while proton 'b' resolved as a doublet at 6.322 ppm. Aromatic protons (d) resolved at 6.878 ppm and integration showed the presence of two chemically equivalent protons. A sharp peak was evident at 3.863 ppm due to methoxy-substituted benzene ring; the integration of this peak showed the presence of two methoxy groups. Protons of the authentic sinapic acid also had similar chemical shifts (ppm) as compound C. ^{13}C-NMR of this compound was not done due to inadequate sample size. Nonetheless, the evidence from UV, mass and ^{1}H-NMR spectroscopic analyses of compound C were convincing enough to propose the structure of the compound as 4-hydroxy-3,5-dimethoxycinnamic acid (or sinapic acid).

4.11.4 Structural analysis of compound D

As shown in Figure 4.69, compound D had a UV absorbance maximum at 282 nm. The shape and absorbance maximum of compound D matched those of the authentic (+)catechin. The electron-impact mass spectrum of compound D is shown in Figure A.18.

Table 4.19 Proton assignments for compound C.

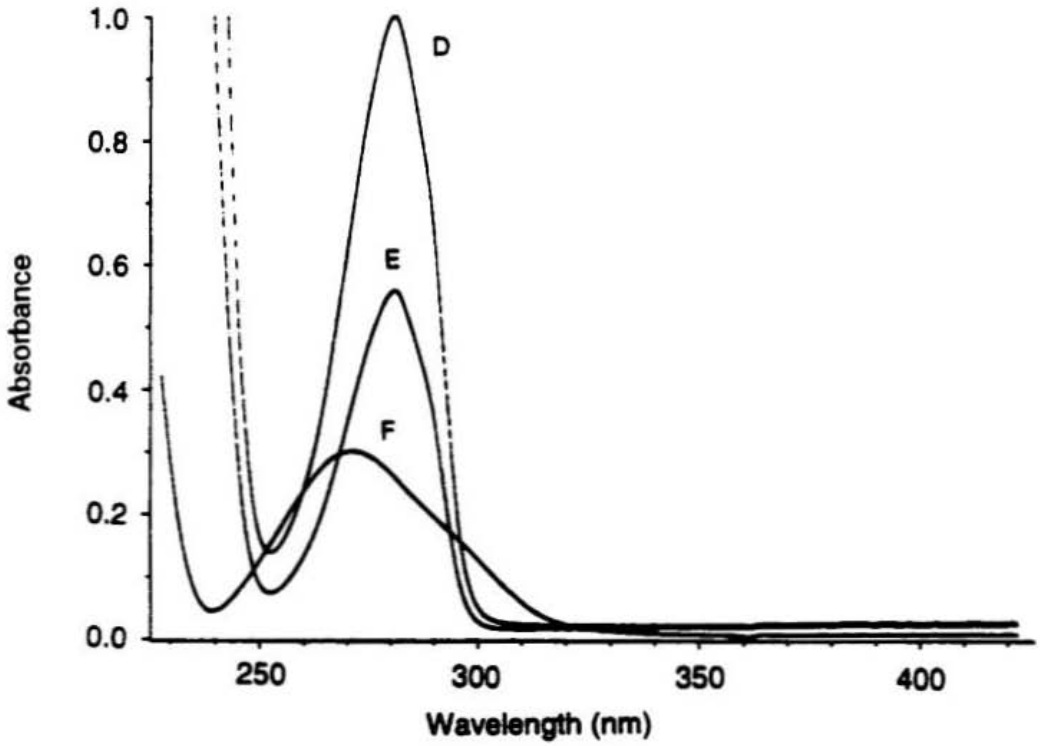


4-Hydroxy-3,5-dimethoxycinnamic acid (Sinapic acid)

(ppm)	Multiplicity	J (Hz)	Integration	Assignment
7.576	d	J_{bc}	1H	H_c
6.878	s	--	2H	H_d
6.322	d	J_{bc}	1H	H_b
4.859	s	—	3H	Solvent
3.863	s	—	6H	CH_3O
3.290	s	—	1H	Solvent

Note-Assignment of coupling interactions (J) confirmed by a COSY experiment (Figure A.18).

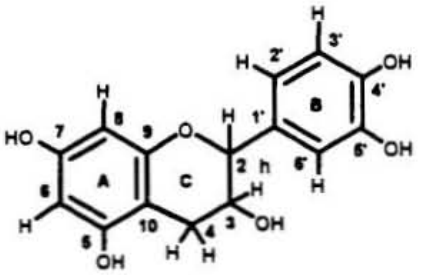
Figure 4.69 UV spectra of compounds D, E and F.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
D
E
F
250
300
350
400
Wavelength (nm)

The molecular radical ion ($M^{+\bullet}$) was at m/z 290. The fragment ion at m/z 167 can be rationalized by the opening up of the ring C of the $M^{+\bullet}$ and the subsequent loss of ring B along with cleavage of C-2 from ring C. The fragment ion at m/z of 152 resulted from the cleavage of the bond between C-9 of ring A and the oxygen of the benzopyran ring, followed by another cleavage of C-C bond between C-2 and C-3 of the benzopyran ring. The fragment ion at m/z of 139 resulted from the formation of semiquinone structure from aromatic A ring followed by the cleavage of C-C bond between C-3 and C-4 of the benzopyran ring. The fragment ion at m/z of 124 could result from the loss of CH_3 from the ion at m/z 139. The fragment ion at m/z of 123 could result from the loss of CH_3 and H from the ion at m/z 139. The fragment ion at m/z of 77 is characteristic of aromatic compounds (Table 4.20). The fragmentation pattern for compound D was identical to that of the authentic (+)catechin under the same electron-impact-MS conditions.

^{1}H-NMR spectrum of compound D is shown in Figure A.19 and the proton assignments are given in Table 4.21. Integration of the broad singlet signal at 8.002 ppm showed the presence of four protons and interpreted as protons of four aromatic hydroxyl groups of rings A and B. Proton 'n' of the aromatic B ring was resolved as a doublet at 6.887 ppm. NMR signal of proton 'n' was split due to its coupling with the magnetic field of proton 'j' of ring B. NMR signal of proton 'k' of ring B was split by the magnetic field of adjacent proton 'j', thus a doublet was observed at 6.801 ppm. A doublet of doublet at 6.762 ppm was produced by proton 'j' due to its coupling interactions with proton 'k' and proton 'n' of ring B. Coupling interaction between protons 'a' and 'c' of ring A resulted

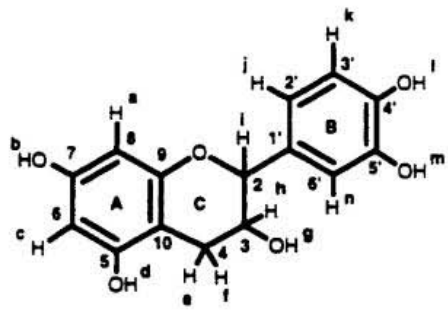
Table 4.20 Mass spectral fragmentation pattern of compound D.



2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol
(+)Catechin

m/z	% Intensity	Fragment ion
290.19	13.82	$C_{15}H_{14}O_6^{\cdot}$, ($M^{\cdot}$)
167.07	4.33	$C_8H_7O_4^{\cdot}$, ($M^{\cdot}$-$C_7H_7O_2$)
152.07	40.30	$C_8H_8O_3^{\cdot}$, ($M^{\cdot}$-$C_7H_6O_3$)
140.01	7.91	$C_7H_8O_3^{\cdot}$, ($P^{\cdot}$+H)
139.02	100.00	$C_7H_7O_3^{\cdot}$, ($P^{\cdot}$)
124.11	17.95	$C_6H_4O_3^{\cdot}$, ($P^{\cdot}$-CH_3)
123.11	36.66	$C_6H_3O_3^{\cdot}$, ($P^{\cdot}$-CH_3-H)
77.08	8.12	$C_6H_5^{\cdot}$, (Aromatic)

Table 4.21 Proton assignments for compound D.



2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol
(+)Catechin

(ppm)	Multiplicity	J (Hz)	Integration	Assignment
8.002	s	—	4H	H_b,H_d,H_l,H_m
6.887	d	J_{jn}=1.9	1H	H_n
6.801	d	J_{jk}=8.1	1H	H_k
6.762	dd	J_{jk}=8.1	1H	H_j
6.019	d	J_{ac}=2.2	1H	H_c
5.871	d	J_{ac}=2.2	1H	H_a
4.561	d	J_{hi}=8.3	1H	H_i
4.001	't'	J_{hi}=8.3, J_{hf}=5.0	2H	H_h,H_g
2.917	dd	J_{eh}=16, J_{fi}=5.0	1H	H_f
2.557	dd	J_{eh}=16, J_{ei}=8.3	1H	H_e
2.028	s	—	6H	Solvent

Note-Assignment of coupling interactions (J) confirmed by a COSY experiment (Figure A. 21).

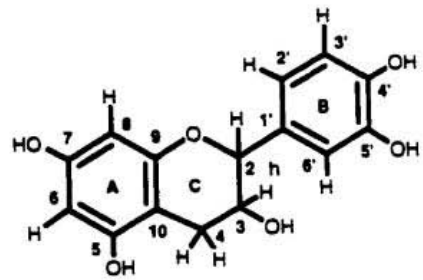
in doublets at 5.871 ppm and 6.019 ppm, respectively. Proton 'i' of ring C coupled with adjacent proton 'h' to split its signal and produced a doublet at 4.561 ppm. A pseudo-triplet at 4.001ppm was evident for proton 'h' of ring C due to its coupling with two adjacent protons, 'i' and 'f'. Resonance of proton 'g' of OH group attached to C-3 of benzopyran ring overlapped with that of proton 'h' (Breitmaier, 1993). Coupling interactions of proton 'f' with proton 'h' and 'i' produced a doublet of doublet at 2.917 ppm. Proton 'e' of ring C also produced a doublet-doublet due to its coupling interactions with proton 'h' and 'i'. Singlet at 2.028 ppm was due to the protons of acetone used as a solvent. Similar ^{1}H-NMR peak patterns were seen for the authentic (+)catechin.

^{13}C-NMR showed resonance of twelve carbons, but signals for C-4, C-10 and C-1' were not detected (Figure A.20). Carbon assignments for compound D are given in Table 4.22. A similar ^{13}C resonating patterns were seen for the authentic (+)catechin. Based upon spectroscopic evidence, compound D was identified as 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol [or (+)catechin].

4.11.5 Structural analysis of compound E

The UV absorbance maximum of compound E (282 nm) was identical to that of compound D (Figure 4.69). The electron-impact mass spectrum of compound E is shown in Figure A.21 and its major fragments are listed in Table 4.23. The molecular radical ion ($M^{+\bullet}$) at m/z 290 was identical to that of compound D. The fragments at m/z of 169, 168 and 167 could originate from loss of ring B along with the C-2 of ring C from M^{+} + 2,

Table 4.22 Carbon assignments for compound D.

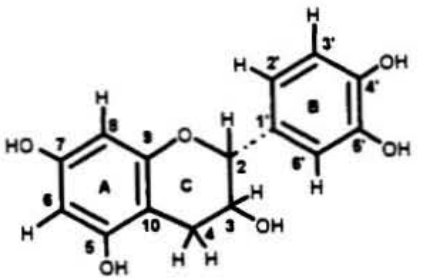


2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol
(+)Catechin

(ppm)	Assignment
202.9	C-9
154.0	C-7
153.5	C-5
142.0	C-4'
141.9	C-3'
116.4	C-5'
112.0	C-6'
111.6	C-2'
92.4	C-6
91.8	C-8
79.0	C-2
64.7	C-3

Note-Signal from C-10 was obscured in the noise.

Table 4.23 Mass spectral fragmentation pattern of compound E.



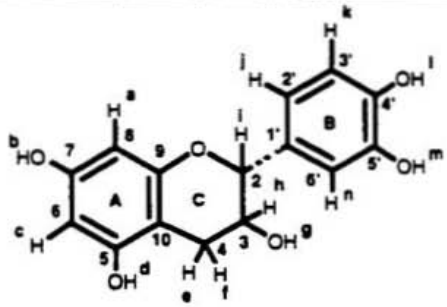
2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol
(-)Epicatechin

m/z	% Intensity	Fragment ion
290.19	0.57	$C_{15}H_{14}O_6^{\cdot}$, ($M^{\cdot}$)
167.05	2.03	$C_8H_7O_4^{\cdot}$, ($M^{\cdot}$-$C_7H_7O_2$)
142.03	68.56	$C_7H_{10}O_3^{\cdot}$, ($P^{\cdot}$+H)
141.01	100.00	$C_7H_9O_3^{\cdot}$, ($P^{\cdot}$)
139.97	71.56	$C_7H_8O_3^{\cdot}$, ($P^{\cdot}$-H)
139.01	24.61	$C_7H_7O_3^{\cdot}$, ($P^{\cdot}$-2H)
124.09	13.11	$C_7H_8O_2^{\cdot}$, ($P^{\cdot}$-OH)
77.08	47.37	$C_6H_5^{\cdot}$, (Aromatic)

$M^{-\bullet} + 1$ and $M^{-\bullet}$ ions, respectively. A fragment ion at m/z of 141 was formed from opening up of the ring C followed by the cleavage at C-C bond between C-4 and C-3. The fragment ions at m/z of 140 and 139 could originate from losses of H from the OH groups of m/z 141. Loss of OH from the ion at 141 gave the ion with at m/z 124. The fragment ion at m/z of 77 is characteristic of aromatic compounds (Pretsch *et al.*, 1983). The fragmentation patterns of compounds D and E were not identical, but the presence of common molecular ion as well as various frgment ions suggests that these two compounds are closely related. Comparison of electon-impact mass spectra of compound E and authentic (-)epicatechin showed similar fragment patterns under the same electron-impact-MS conditions.

As shown in Figure A.22 and listed in Table 4.24, chemical shifts of protons of compound E were slightly different from those of compound D (Figure A.21 and Table 4.23). This was due to the use of methanol d-4 as the solvent for compound E (acetone d-6 was used for compound D as it produced a turbid solution in methanol d-4). However, coupling constants of interacting protons of both compounds D and E, were identical and this certainly, was a strong indication of the structural similarities of two compounds. Another important feature observed for compound E was the absence of signals for protons of aromatic OH groups and OH group of ring C. It is possible that these protons were easily exchanged with deuterium of methanol d-4. Similar deuterium exchanges

Table 4.24 Proton assignments for compound E.



2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol
(-)Epicatechin

(ppm)	Multiplicity	J (Hz)	Integration	Assignment
6.962	d	J_{jn}=1.9	1H	H_n
6.807	d	J_{jk}=8.1	1H	H_k
6.759	dd	J_{jk}=8.1	1H	H_j
5.927	d	J_{ac}=2.2	1H	H_c
5.901	d	J_{ac}=2.2	1H	H_a
4.856	s	—	3H	Solvent
4.165	d	J_{hi}=8.3	1H	H_i
3.562	q		1H	H_h
3.287	s	—	1H	Solvent
2.756	dd	J_{eh}=16, J_{fi}=5.0	1H	H_f
2.747	dd	J_{eh}=16, J_{ei}=8.3	1H	H_e

Note-Assignment of coupling interactions (J) confirmed by a COSY experiment (Figure A.25).

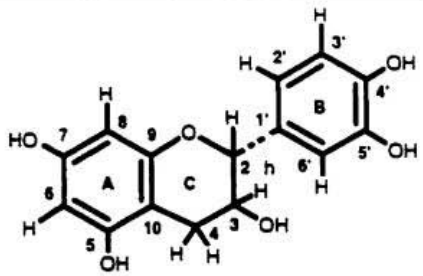
were reported by Breitmaier (1993). Furthermore, ^{1}H-NMR spectrum of the authentic (-)epicatechin matched well with that of compound E.

Unlike compound D, all carbons of compound E were resolved in its ^{13}C-NMR spectrum (Figure A.23). Carbon assignments, given in Table 4.25, were similar to those for compound D and the authentic (-)epicatechin. Based upon all evidences, compound E was identified as (-)epicatechin which is an isomer of (+)catechin.

4.11.6 Structural analysis of compound F

Compound F had UV absorbance maximum of 272 nm (Figure 4.69). Shape of the UV spectrum and absorbance maximum of compound F matched with those of the authentic gallic acid. The electron-impact mass spectrum of compound F is shown in Figure A.24 and its major fragments are summarized in Table 4.26. The molecular radical ion ($M^{-\bullet}$) of compound F was present at m/z of 170. The fragment ion at m/z of 153 was due to the loss of OH from $M^{-\bullet}$. The loss of CO_2 molecule from $M^{-\bullet}$ produced the fragment ion at m/z of 126. The loss of CO_2 molecule confirmed the presence of an acid group in the molecule. A fragment ion at m/z of 109 originated from the loss of $OH^{\bullet}$ from the ion at m/z 126. Another fragment ion at m/z of 108 was produced due to the loss of a molecule of H_2O from the ion at m/z 126. This suggests the presence of adjacent OH groups in the molecules. The formation of a semiquinone structure was evident by the presence of an ion at m/z of 97. The fragment ion at m/z of 79 is characteristics of aromatic compounds substituted with H-containing groups (Pretsch *et al.*, 1983) and m/z

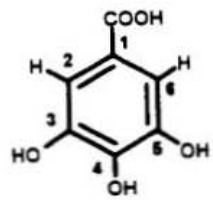
Table 4.25 Carbon assignments for compound E.



2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol
(-)Epicatechin

(ppm)	Assignment
153.8	C-9
153.5	C-7
153.2	C-5
141.7	C-4'
141.6	C-3'
128.1	C-1'
115.2	C-5'
111.1	C-6'
111.7	C-2'
95.8	C-10
92.2	C-6
91.2	C-8
75.6	C-2
63.3	C-3
25.1	C-4

Table 4.26 Mass spectral fragmentation pattern of compound F.



3,4,5-Trihydroxybenzoic acid (Gallic acid)

m/z	% Intensity	Fragment ion
169.98	8.81	$C_7H_6O_5^{\cdot}$, ($M^{\cdot\cdot}$)
153.03	7.77	$C_7H_5O_4^{\cdot}$, ($M^{\cdot\cdot}$-OH)
126.07	100.00	$C_6H_6O_3^{\cdot}$, ($P^{\cdot}$)
108.97	2.44	$C_6H_5O_2^{\cdot}$, ($P^{\cdot}$-OH)
108.00	28.65	$C_6H_4O_2^{\cdot}$, ($P^{\cdot}$-H_2O)
97.03	9.47	$C_6H_9O^{\cdot}$
79.96	38.42	$C_6H_8^{\cdot}$, (Cyclohexene)
79.01	14.73	$C_6H_7^{\cdot}$, (Aromatic)
44.09	92.75	$CO_2^{\cdot}$, (Carboxylic)

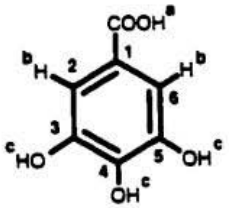
of 44 is due to CO_2 released from the carboxylic acid group. A similar fragmentation pattern was observed for the authentic gallic acid under the same experimental conditions.

The ^{1}H-NMR spectrum of compound F is shown in Figure A.25 and its proton assignments are given in Table 4.27. As evident from the spectrum, protons of aromatic OH groups (H-a) of compound F were not detected due to deuterium exchange. Presence of two aromatic protons (H-b) was indicated by a singlet at 7.044 ppm. ^{1}H-NMR spectrum of compound F was identical to that of the authentic gallic acid. ^{13}C-NMR was not done on this compound due to inadequate sample size. UV, mass and ^{1}H-NMR produced sufficient evidence to propose the structure of 3,4,5-trihydroxybenzoic acid (or gallic acid) for compound F.

4.12 Thin-layer chromatographic (TLC) quantification of isolated compounds

Table 4.28 shows the results for TLC quantification of isolated compounds. Rosmarinic acid, syringic acid and sinapic acid contributed approximately 3.9% (w/w) of the borage crude extract whereas (+)catechin, (-)epicatechin and gallic acid contributed 10.4% (w/w) of the evening primrose crude extract. Content of rosmarinic acid, syringic acid and sinapic acid in borage meal was approximately 0.6% (w/w) while evening primrose meal contained approximately 1.6% (w/w) of (+)catechin, (-)epicatechin and gallic acid.

Table 4.27 **Proton assignments for compound F.**



3,4,5-Trihydroxybenzoic acid (Gallic acid)

(ppm)	Multiplicity	J (Hz)	Integration	Assignment
7.044	s	—	2H	H_b
4.858	s	—	3H	Solvent
3.289	s	—	1H	Solvent

Table 4.28 Contents of isolated crude compounds in fractions, crude extracts and meals[1].

Compound	% (w/w) in each fraction[2]	% (w/w) in crude extract[3]	% (w/w) in meal[4]
Borage			
Rosmarinic acid (I)[5]	15.2	2.55	0.383
Syringic acid (IV)	10.2	0.663	0.113
Sinapic acid (IV)	11.0	0.715	0.121
Evening primrose			
Catechins (III)	35.4	10.1	1.62
Gallic acid (V)	6.33	0.413	0.066

[1] Average of two runs. Twenty milligrams of each fraction were chromatographed.
[2] %, w/w in fraction = (weight of isolated compound in mg/weight of fraction used for TLC in mg)*100
[3] %, w/w in extract = [(weight of isolated compound in mg*fraction yield in mg)/(weight of fraction used for TLC in mg*weight of extract used for column chromatography)]*100
[4] %, w/w in meal = [(weight of isolated compound in mg*fraction yield in mg*extract yield in mg)/(weight of fraction used for TLC in mg*weight of extract used for column chromatography*weight of meal used to prepare extract in mg)]*100
[5] Fraction number.

4.13 Structure-antioxidant activity relationships of identified compounds

Antioxidant activities of phenolic compounds may be exerted *via* two main mechanisms, namely donation of a hydrogen atom or an electron to free radicals and chelation of transition metal ions (Naim *et al.*, 1974). Structural features of the molecules play a major role in determining the overall antioxidant efficacy of a particular phenolic compound (Rice-Evans *et al.*, 1995, 1996). Therefore, relationships between structural features of the identified compounds and their importance in determining antioxidant activities will be discussed in the following sections.

4.13.1 Rosmarinic acid (Compound A of borage extract)

Being an ester of a hydroxylated cinnamic acid (caffeic acid), rosmarinic acid more effectively donates hydrogen atoms to radical species as compared to the low efficiency of its benzoic acid counterpart (Dziedzic and Hudson, 1983). This is because electron withdrawing effect of the carboxylic acid group of cinnamic acid is less than that of its benzoic acid counterpart. When the strength of electron withdrawing effect of carboxylic acid group is high, the phenolic OH group tends to loose a proton rather than a hydrogen atom. Dihydroxylation in the 3,4-positions of the molecule, as in rosmarinic acid, enhances the efficacy of the cinnamic acid derivatives. Rosmarinic acid with its strong hydrogen donating ability was able to neutralize free radicals formed in food and low density lipoprotein (LDL) models (Castelluccio *et al.*, 1995). Absence of methoxy substitution may make rosmarinic acid a more effective hydrogen donor than its methoxy

substituted derivatives (Shahidi and Wanasundara, 1992). It has been shown that methoxylation in the *ortho* position of diphenolics, as in ferulic acid, results in a decrease in radical scavenging ability. Hydroxylation, as in caffeic acid, in place of methoxylation is substantially more effective (Bors *et al.*, 1990). The presence of a CH=CH-COOH group in position 1 of rosmarinic acid increases the hydrogen donating ability and subsequent radical stabilization. Theoretically, one mole of rosmarinic acid can reduce four moles of free radicals as it has four aromatic hydroxyl groups (which have the potential to donate hydrogen atoms) per molecule (Brand-Williams *et al.*, 1995). On the other hand, rosmarinic acid can actively participate in metal chelation reactions, hence delay the onset of oxidation reactions. Diphenolic structure of rosmarinic acid allows it to make stable five-membered metal complexes.

4.13.2 Syringic acid (compound B of borage extract)

Syringic acid is a methoxylated derivative of hydroxybenzoic acid, thus electron withdrawing effect of carboxylic acid group has a negative influence on its hydrogen donating ability. The substitution of the 3- and 5-hydroxy groups with methoxy groups in syringic acid also has a negative effect on its antioxidant efficacy compared to its trihydroxy derivatives (Rice-Evans *et al.*, 1996). Foti *et al.* (1996) suggested that the greater reactivity of the *ortho* dihydroxy system is possibly due to the smaller dissociation energy of the O-H bonds in comparison with the 1,3-dihydroxy system. However, *ortho* dimethoxylation, as in syringic acid, eliminates that advantage. The other mechanism by

which syringic acid may act as an antioxidant, is its formation of complexes with metal ions. Functional groups at positions 3,4 and 5 can participate in five-membered ring formation with metal ions such as iron (II) and copper (II).

4.13.3 Sinapic acid (compound C of borage extract)

Being a derivative of cinnamic acid, sinapic acid is more efficient antioxidant than its benzoic acid counterpart. This could arise from several structural features of the sinapic acid molecule. Similar to rosmarinic acid, the electron withdrawing effect of carboxylic group of sinapic acid is greatly reduced due to the presence of a -CH=CH- moiety as compared to the acid group of syringic acid. On the other hand, -CH=CH- moiety contributes to the radical stabilization of sinapic acid through resonance which also has a positive influence on its overall antioxidant efficacy (Foti *et al.*, 1996). As was the case for syringic acid, sinapic acid also has methoxy substitution in its positions 3 and 5. This feature, as explained earlier, has a negative effect on hydrogen donating ability of the molecules, thus lowering their free radical scavenging ability. However, presence of properly oriented functional groups in positions 3, 4 and 5 of sinapic acid can enhance its antioxidant efficacy through metal chelation.

4.13.4 (+)Catechin and (-)epicatechin (compounds D and E of evening primrose extract)

(+)catechin and (-)epicatechin are polyphenolic flavonoids with a diphenyl propane ($C_6C_3C_6$) skeleton. This family includes monomeric flavanols, flavanones, anthocyanidins, flavones and flavonols (Herrmann, 1976). In general, the OH substitution on aromatic rings, especially ring B, determines the antioxidant efficacy of flavonoids (Hendrickson *et al.*, 1994a, b). Presence of hydroxyl groups in positions 4' and 5' (or 3') in flavanoids has been shown to enhance hydrogen donating ability and resonance stabilization of flavonoid aroxyl radicals (Bores *et al.*, 1990; Rice-Evans *et al.*, 1995). Both (+)catechin and (-)epicatechin are efficient metal chelators. For chelation of ions, 3-OH group of benzopyran ring is more important than 4' or 5' OH groups because 3-OH can participate in the formation of a highly stable six-membered ring structure with metal ions. Stability of six-membered chelation complexes has been reported to be greater as compared to those of five- and four-membered ring complexes (Thompson *et al.*, 1976). Another mechanism by which flavonoids could inhibit lipid oxidation is inhibition of certain prooxidative enzymes which catalyze the generation of free radicals. However, it has been reported that flavonoids devoid of 5-OH substitution are potent enzyme inhibitors as compared to those possessing 5-OH (Bohmont *et al.*, 1987). With presence of 5-OH substitution, (+)catechin and (-)epicatechin might not be important inhibitors of prooxidant enzymes such as NADH-oxidase and xanthine oxidase.

4.13.5 Gallic acid (compound F of evening primrose extract)

Gallic acid, 3,4,5-trihydroxybenzoic acid, has a greater antioxidant capacity than its 3,5-methoxylated counterpart, syringic acid. High antioxidant activity of gallic acid has been attributed to its three available hydroxyl groups (Rice-Evans *et al.*, 1996). Haseloff *et al.* (1990) reported that gallic acid esters with short hydrocarbon chain lengths are more efficient hydroxyl radical scavengers than trihydroxybenzoic acid. These authors have reported that gallic acid propyl ester (propyl gallate) is one of the most efficient antioxidants among benzoic acid derivatives. Gallic acid esters have been reported to be superior free radical scavengers as compared to BHT and α-tocopherol. However, free radical-scavenging activity of these esters decreases as the length of the hydrocarbon chain increases. The 3,4,5-tri hydroxyl arrangement of gallic acid makes it a strong metal chelating agent, hence greater antioxidant activity.

SUMMARY AND CONCLUSIONS

The defatted meals of boragae and evening primrose (at 1 and 2%, w/w level) exhibited strong antioxidant activity in a meat model system, as reflected in their thiobarbituric acid-reactive substances (TBARS), hexanal and total volatiles, in a concentration-dependent manner. However, the antioxidant efficacy of borage meal in cooked comminuted pork was lower than that of the evening primrose at the same concentration. Since both meals exerted antioxidative effects, their crude extracts were prepared under optimum process conditions, for further investigations using response surface methodology (RSM). The highest antioxidant activity of borage extract was attained when the combination of variables, namely the content of organic solvent in the aqueous extraction medium, extraction temperature and time, was 52% (v/v) ethanol at 72 °C and 62 min; combination of variables for evening primrose was 56% (v/v) acetone, 71 °C extraction temperature and 47 min time course.

The crude extracts of meals of borage and evening primrose were separated by a column chromatographic technique afforded six fractions (I-VI). Several analytical techniques were used to evaluate the antioxidant efficacies of the crude extracts and their fractions at concentrations of 100 and 200 ppm. Borage and evening primrose crude extracts and their fractions exhibited weak to strong, concentration-dependent, antioxidative effects in several model systems, namely β-carotene-linoleate, cooked comminuted pork, bulk corn oil and corn oil-in-water emulsion. In general, evening primrose crude extract and its fractions at 200 ppm (as catechin equivalents) exerted

better antioxidative effects (comparable to or better than BHA and the authentic catechinin at the same concentration) in model systems than those exhibited by 200 ppm (as sinapic acid equivalents) borage crude extract and its fractions. Furthermore, borage crude extract and its fractions were more efficient antioxidants in a bulk stripped corn oil model system than in the other model systems, perhaps due to their high content of hydrophilic phenolics which had the potential to orient at the oil-air interfaces of the bulk oil.

Borage and evening primrose crude extracts and their fractions exhibited excellent metal chelating, hydrogen peroxide-scavenging and free radical-scavenging capacities in aqueous assay media. The relationships among these effects and oxidation indicators in model systems were linear and weak ($r < 0.5$) to strong ($r > 0.9$). The hexanal contents of cooked comminuted pork, bulk corn oil and its oil-in-water emulsion containing borage and evening primrose additives showed high strengths of linear associations with metal chelating, reactive-oxygen species and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging capacities of the additives. This could be due to the better representation of oxidative status of meat and corn oil by a single volatile such as hexanal as opposed to TBARS values. The antioxidative effects of borage and evening primrose crude extracts and their fractions varied in model systems, possibly due to varying degrees of their partitioning into lipid and aqueous phases of the model systems. Therefore, the metal chelating, hydrogen peroxide-scavenging and free radical-scavenging capacities of borage and evening primrose additives seen in monophasic assay media did not always reflect their behaviour in complex multiphasic model systems. Thus, the mode of action of

extracts of borage and evening primrose meals involves several mechanisms that act synergistically to retard lipid oxidation in model systems; these effects may not be easily segregated.

Based upon various chromatographic and spectroscopic data, the major phenolic antioxidants present in crude extracts of borage and evening primrose were identified. The major phenolics of borage crude extract were identified as rosmarinic acid, syringic acid and sinapic acid while those of evening primrose crude extracts were (+)catechin, (-)epicatechin and gallic acid. However, more research is required to identify minor antioxidants in both types of extracts because these may exert important synergistic effects as well as partitioning in the aqueous and lipid systems.

Crude extracts of Borage and evening primrose and their fractions, similar to rosemary and sage extracts, may be incorporated into meat products, bulk oils and food emulsions to improve their oxidative stability. Incorporation of borage and evening primrose crude extracts or their fractions into foods may also reduce the risk of developing certain disease conditions in humans, but such uses need to be justified using evidences gathered from cultured cell line studies, animal studies and clinical trials. Furthermore, the possibility of adding borage and evening primrose extracts into their oils should also be investigated as this could increase their shelf-life and medicinal value. Future research on borage and evening primrose extracts should therefore focus on the identification of their minor antioxidants, including glycosylated phenolics, as well as toxicological studies and unravelling of their medicinal importance and economic feasibility of large scale production.

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APPENDIX 1

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

Correlation coefficient (r) = 0.9920
Equation of the line was Y = aX + b where,
Y = absorbance at 725 nm ($A_{725\ nm}$)
X = content of sinapic acid in μg (C)
a = 0.00482
b = 0.01781
Therefore, C = 207.5*$A_{725\ nm}$-3.695

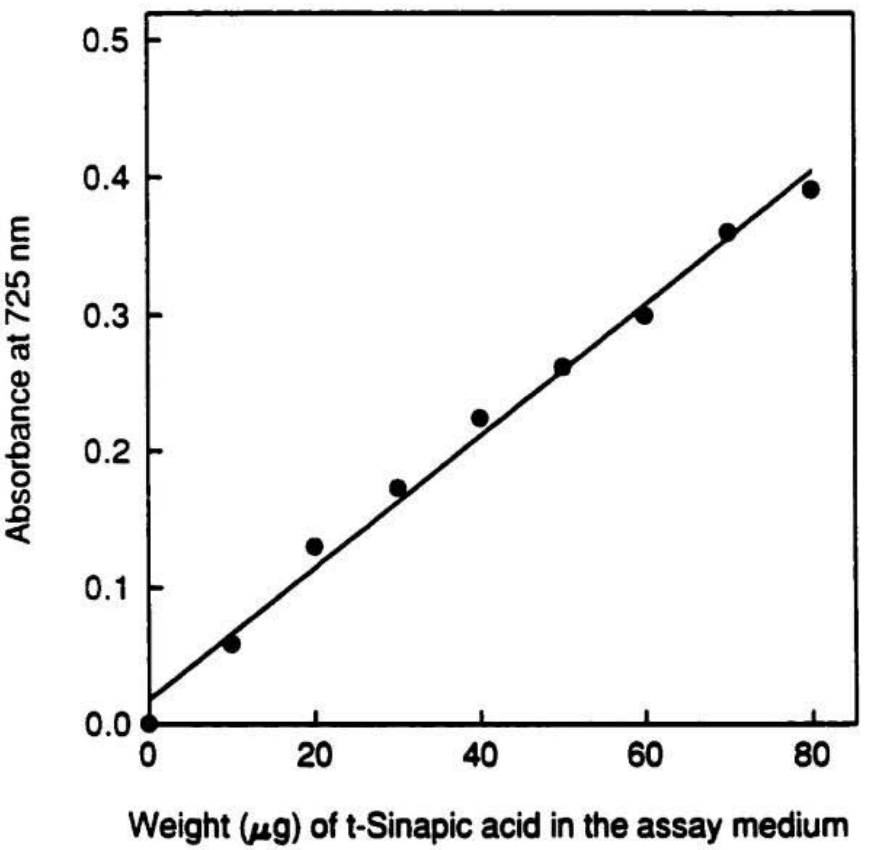
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 725 nm
0
20
40
60
80
Weight (μg) of t-Sinapic acid in the assay medium

Figure A.2 Dependence of the absorbance of catechin-metal complex at 725 nm on the content of catechin in the medium.

Correlation coefficient (r) = 0.9969
Equation of the line was Y = aX + b where,
Y = absorbance at 725 nm ($A_{725\ nm}$)
X = content of catechin in μg (C)
a = 0.00998
b = 0.0
Therefore, C = 100.2*$A_{725\ nm}$

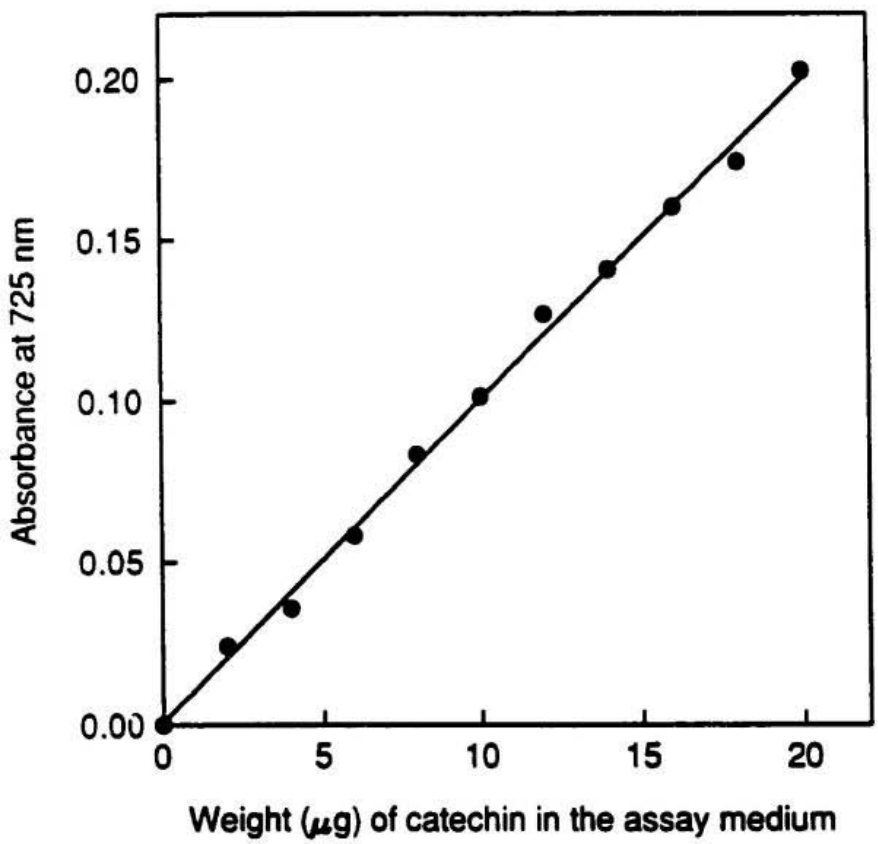
0.20
0.15
0.10
0.05
0.00
Absorbance at 725 nm
0
5
10
15
20
Weight (μg) of catechin in the assay medium

Figure A.3 Dependence of the absorbance of β-carotene at 470 nm on the concentration of β-carotene in the assay medium.

Correlation coefficient (r) = 0.9995
Equation of the line was Y = aX + b where,
Y = absorbance at 470 nm ($A_{470\ nm}$)
X = concentration of β-carotene in μg/5 mL (C)
a = 0.01136
b = 0.0
Therefore, C = 88.03*$A_{470\ nm}$

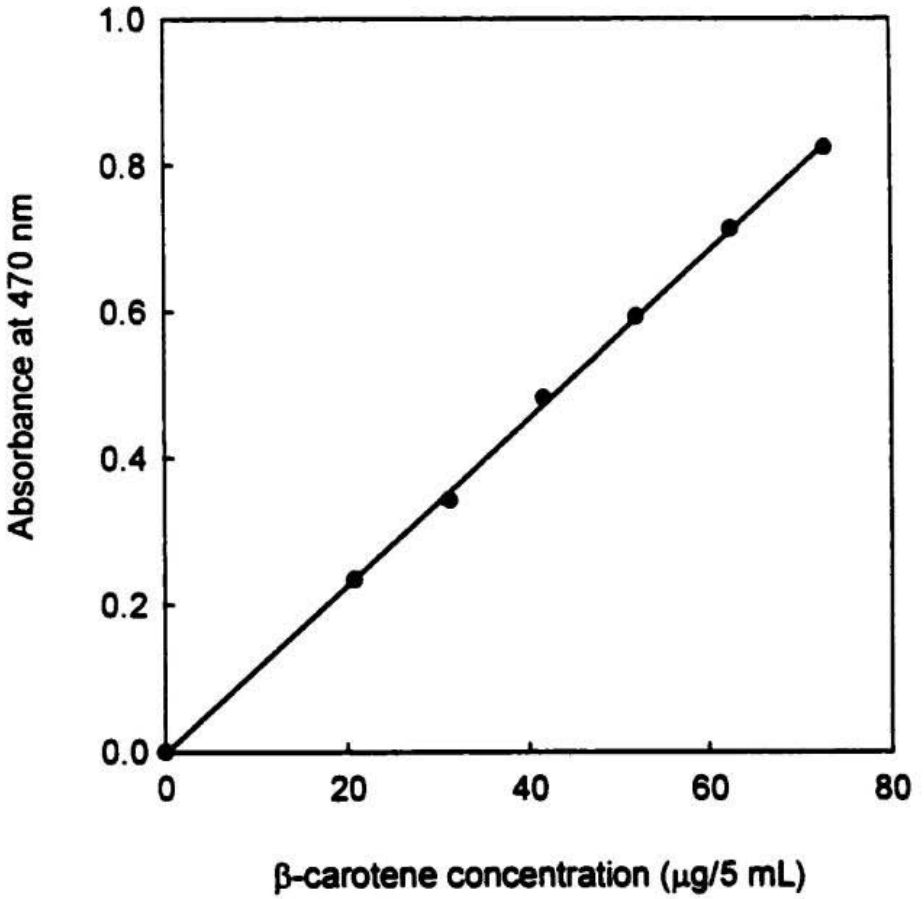
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 470 nm
0
20
40
60
80
β-carotene concentration (μg/5 mL)

Figure A.4 Dependence of the absorbance of malonaldehyde (MA)-TBA complex at 532 nm on the concentration of MA.

Correlation coefficient (r) = 0.9996
Equation of the line was Y = aX + b where,
Y = absorbance at 532 nm ($A_{532\ nm}$)
X = concentration of MA in mg MA equivalents/kg meat (C)
a = 0.293
b = 0
Therefore, C = $3.4*A_{532\ nm}$

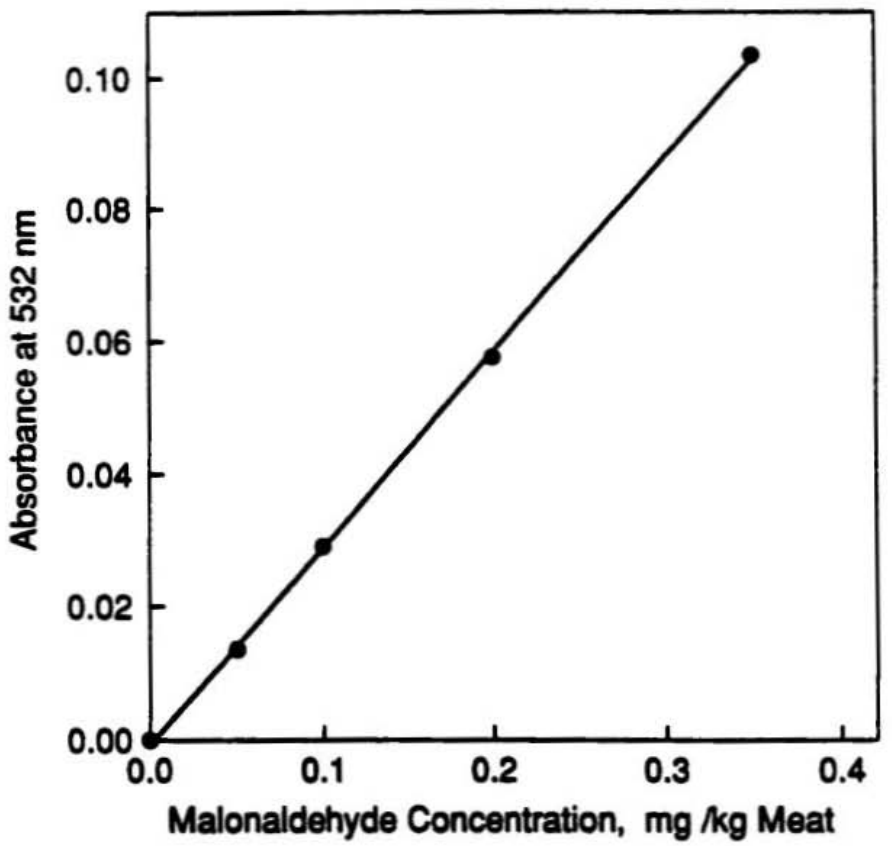
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance at 532 nm
0.0
0.1
0.2
0.3
0.4
Malonaldehyde Concentration, mg /kg Meat

Figure A.5 Dependence of the absorbance ratio (469 nm/530 nm) on the concentration of free Fe^{2+} in μM.

Correlation coefficient (r) = 0.9945
Equation of the line was Y = aX + b where,
Y = absorbance ratio ($A_{469\ nm/530\ nm}$)
X = concentration of free Fe^{2+} in μM (C)
a = 0.0006
b = 0.0
Therefore, $C = 1667 * A_{469\ nm/530\ nm}$

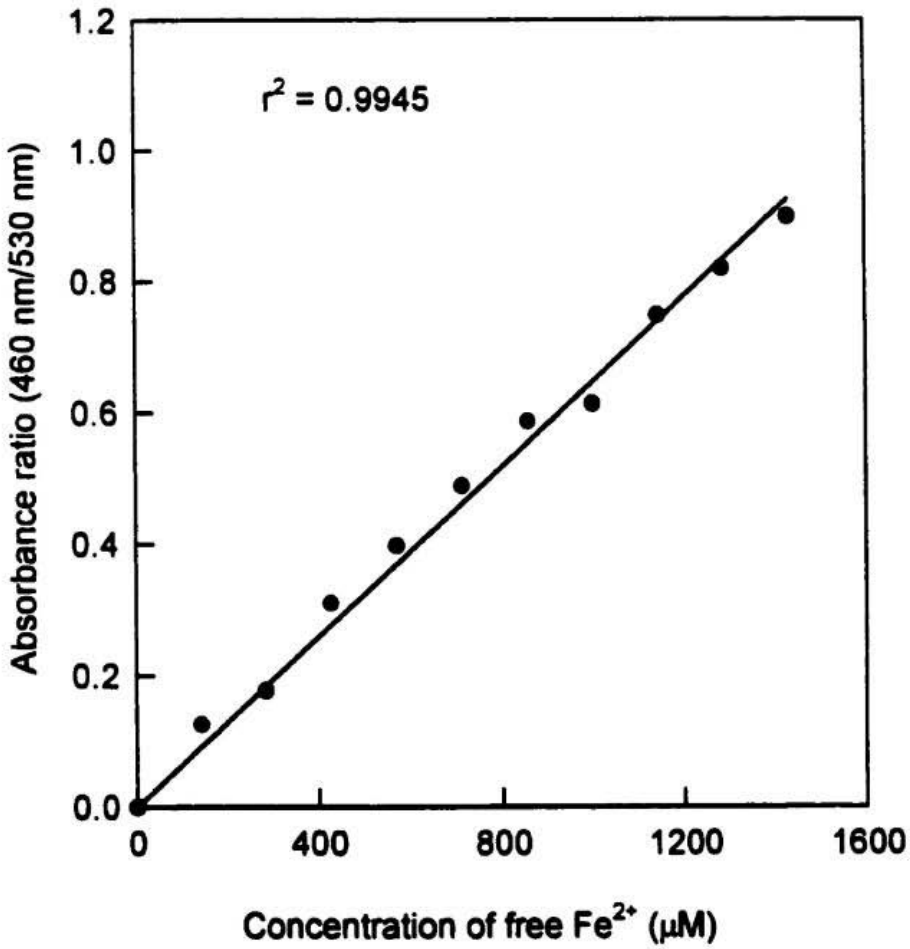
1.2
1.0
0.8
0.6
0.4
0.2
0.0
r^2 = 0.9945
Absorbance ratio (460 nm/530 nm)
0
400
800
1200
1600
Concentration of free Fe^{2+} (μM)

Figure A.6 Dependence of the absorbance of hydrogen peroxide at 230 nm on the concentration of hydrogen peroxide in the assay medium.

Correlation coefficient (r) = 0.9994
Equation of the line was Y = aX + b where,
Y = absorbance at 230 nm ($A_{230\ nm}$)
X = concentration of hydrogen peroxide in mM (C)
a = 0.0798
b = 0.0
Therefore, C = 12.53*$A_{230\ nm}$

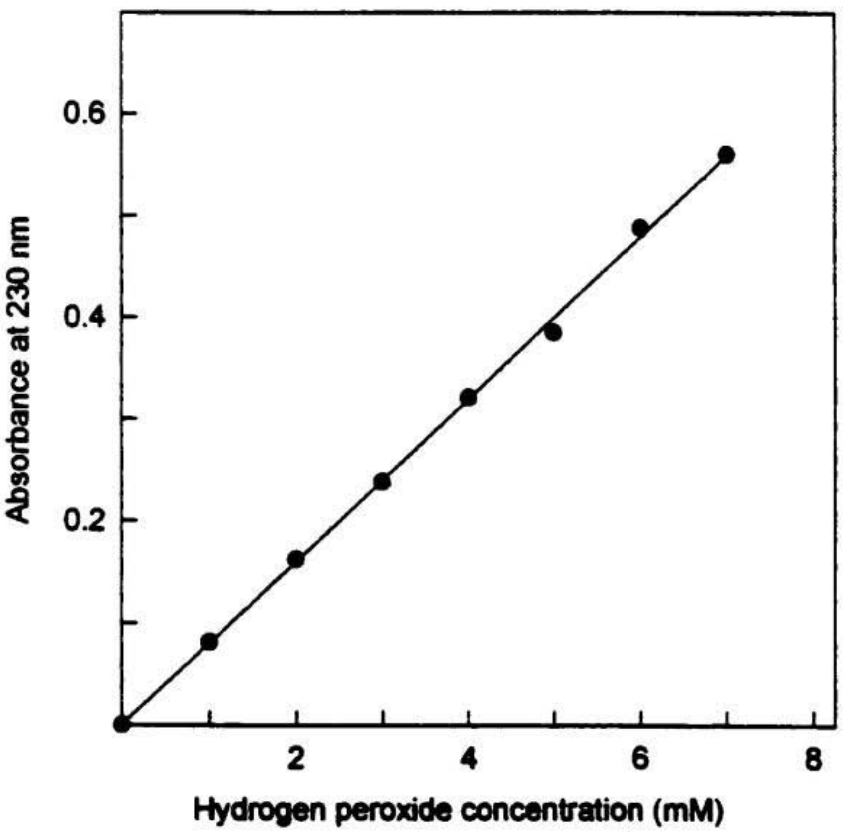
0.6
0.4
0.2
Absorbance at 230 nm
2
4
6
8
Hydrogen peroxide concentration (mM)

Figure A.7 Electron paramagnetic resonance (EPR) spectra of DMPO-OH adduct as affected by borage additives. Column A and B depict the effects of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively.

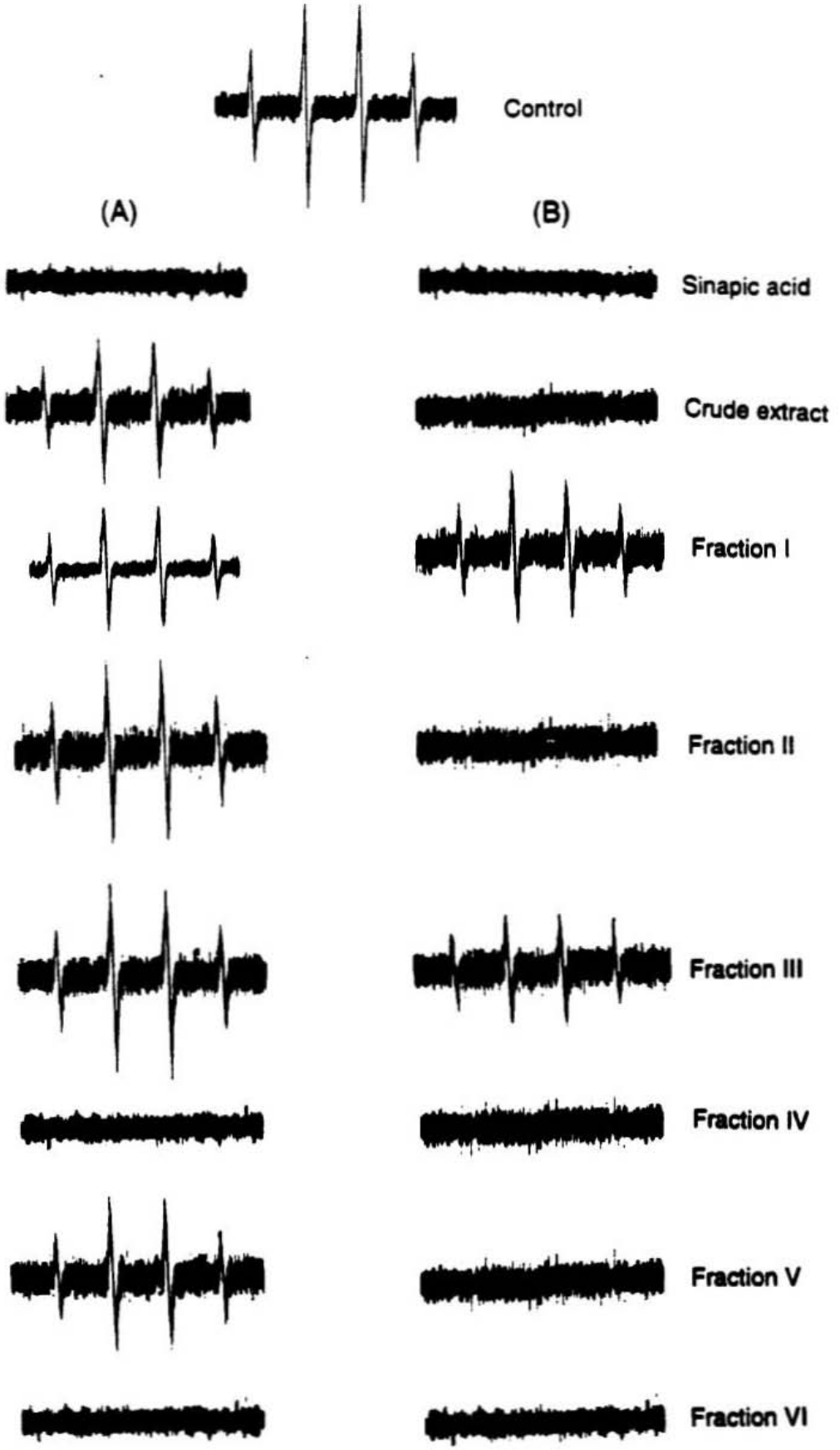
Control
(A)
(B)
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

Figure A.8 Electron paramagnetic resonance (EPR) spectra of DMPO-OH adduct as affected by evening primrose additives. Column A and B depict the effects of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively.

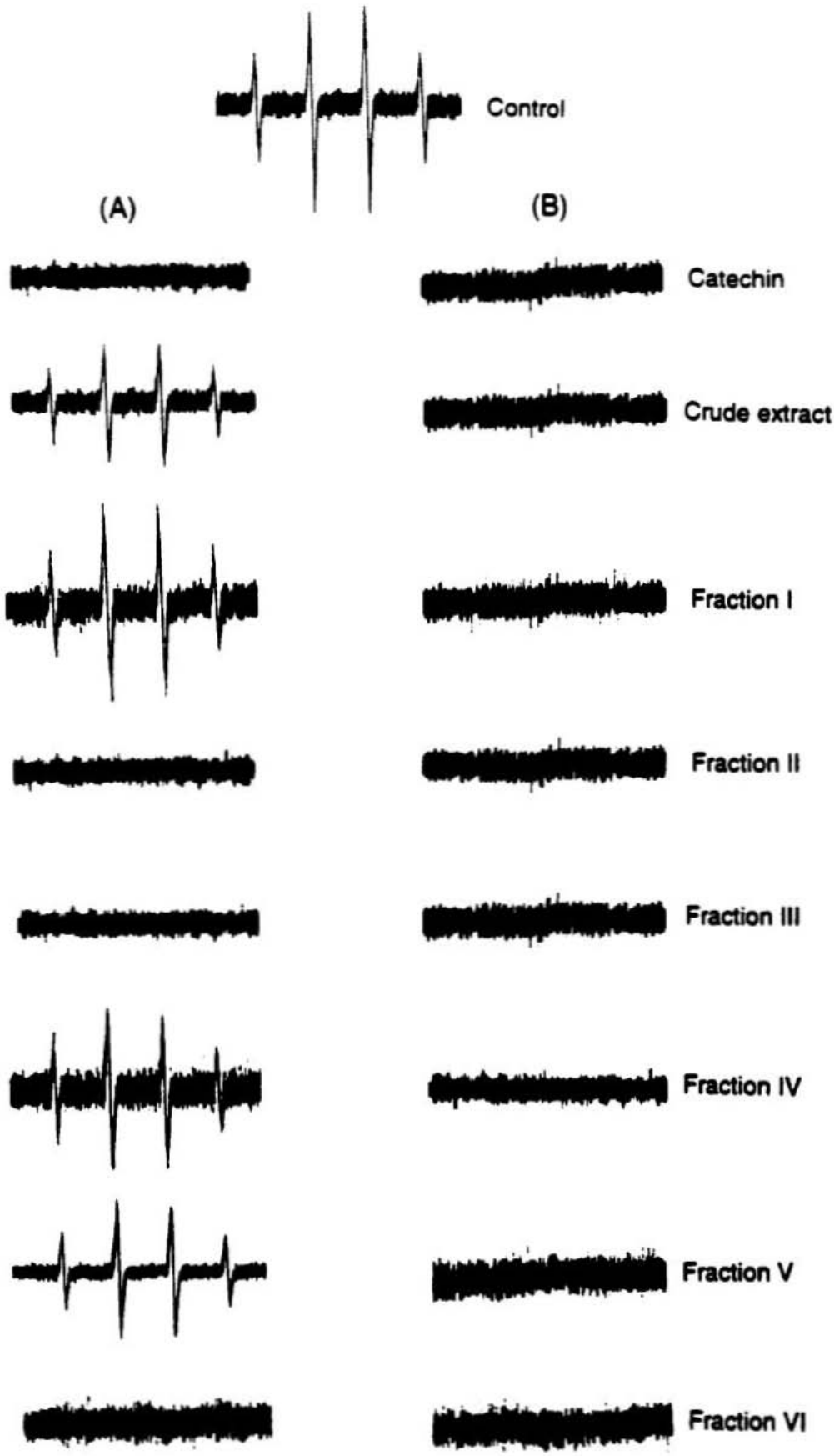
Control
(A)
(B)
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

Figure A.9 Electron paramagnetic resonance (EPR) spectra of DPPH free radicals as affected by borage additives. Column A and B depict the effects of 100 and 200 ppm (as sinapic acid equivalents) of borage additives, respectively.

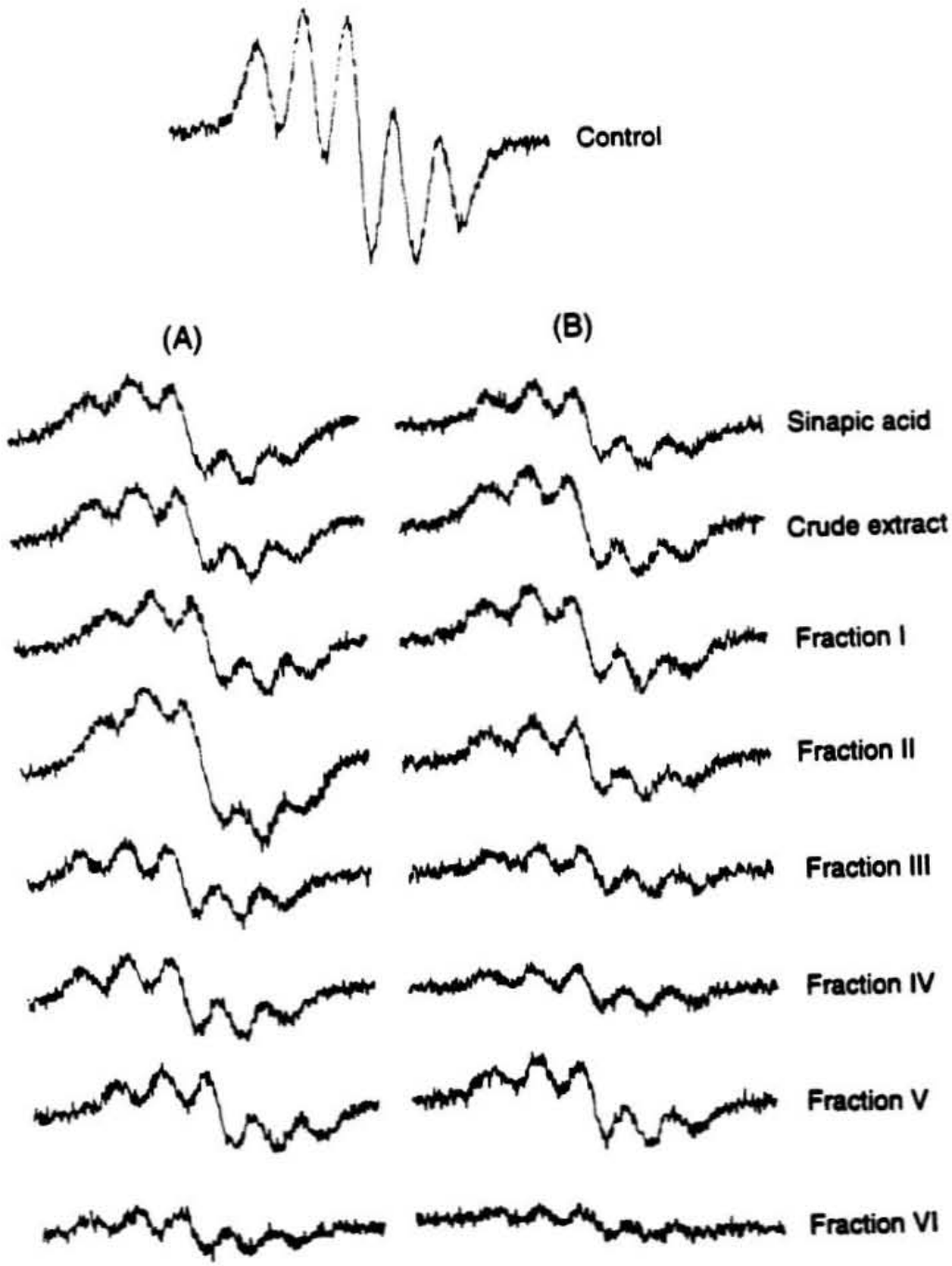
Control
(A)
(B)
Sinapic acid
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

Figure A.10 Electron paramagnetic resonance (EPR) spectra of DPPH free radicals as affected by evening primrose additives. Column A and B depict the effects of 100 and 200 ppm (as catechin equivalents) of evening primrose additives, respectively.

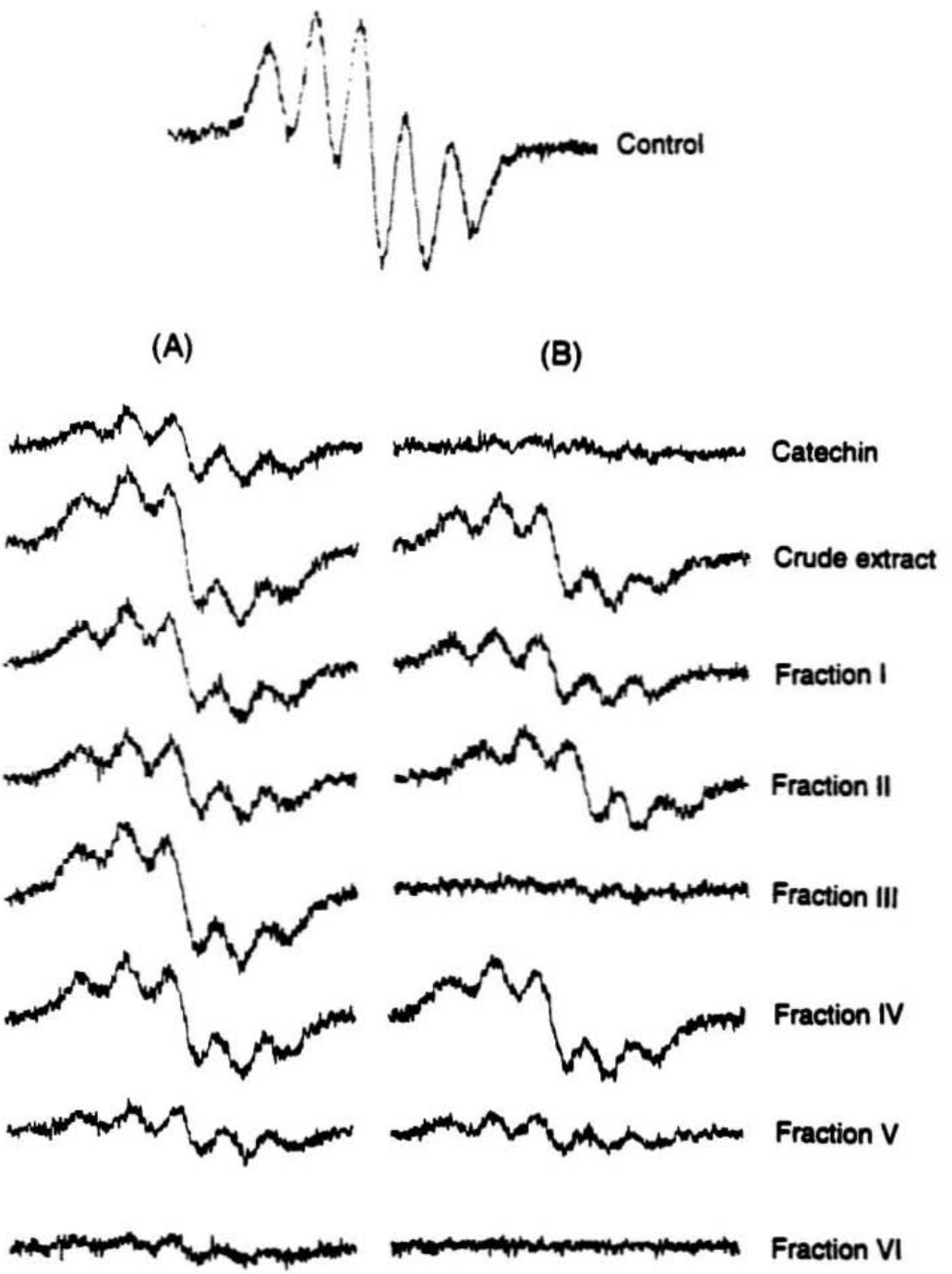
Control
(A)
(B)
Catechin
Crude extract
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI

Figure A.11 Mass spectrum of compound A.

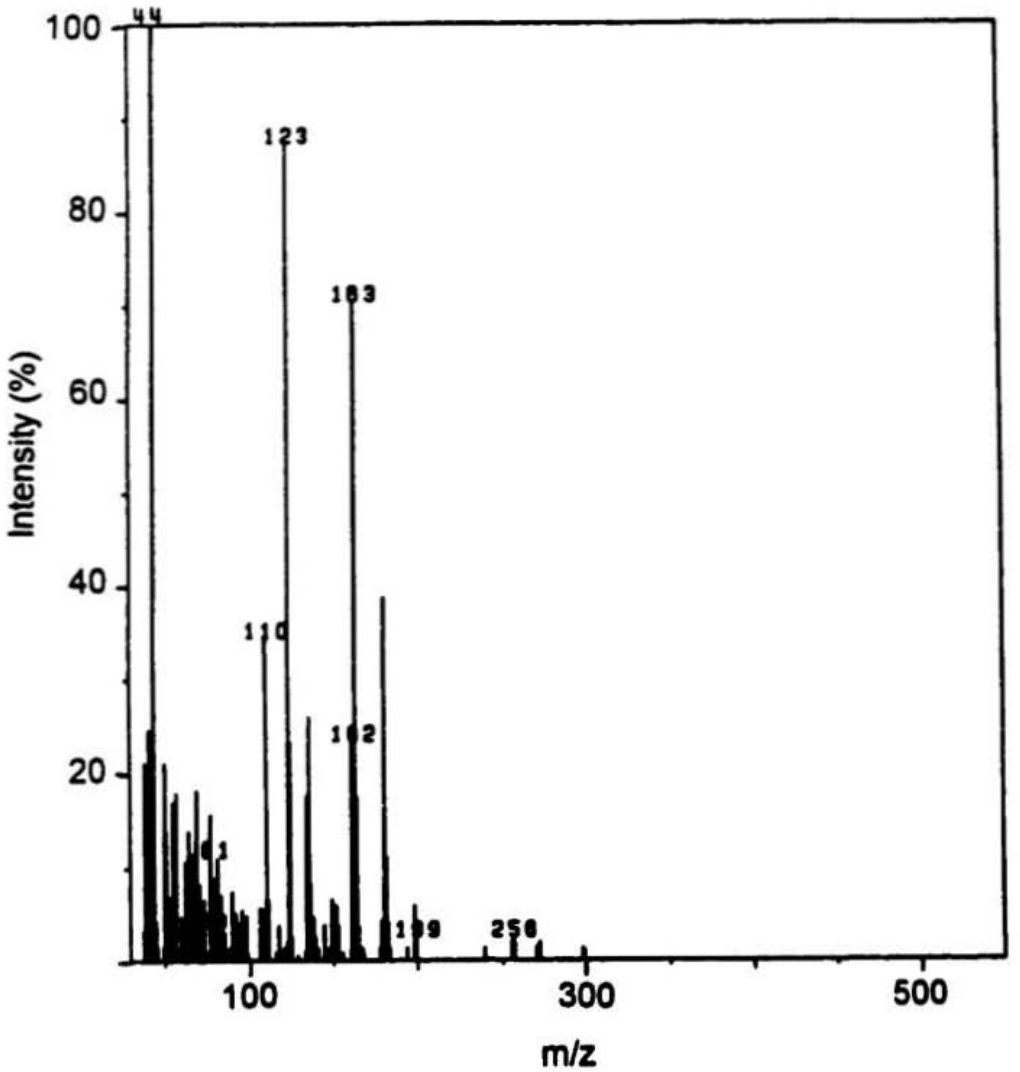
100
80
60
40
20
Intensity (%)
44
123
183
110
182
61
139
258
100
300
500
m/z

Figure A.12 ^{1}H-NMR spectrum of compound A in acetone d-6.

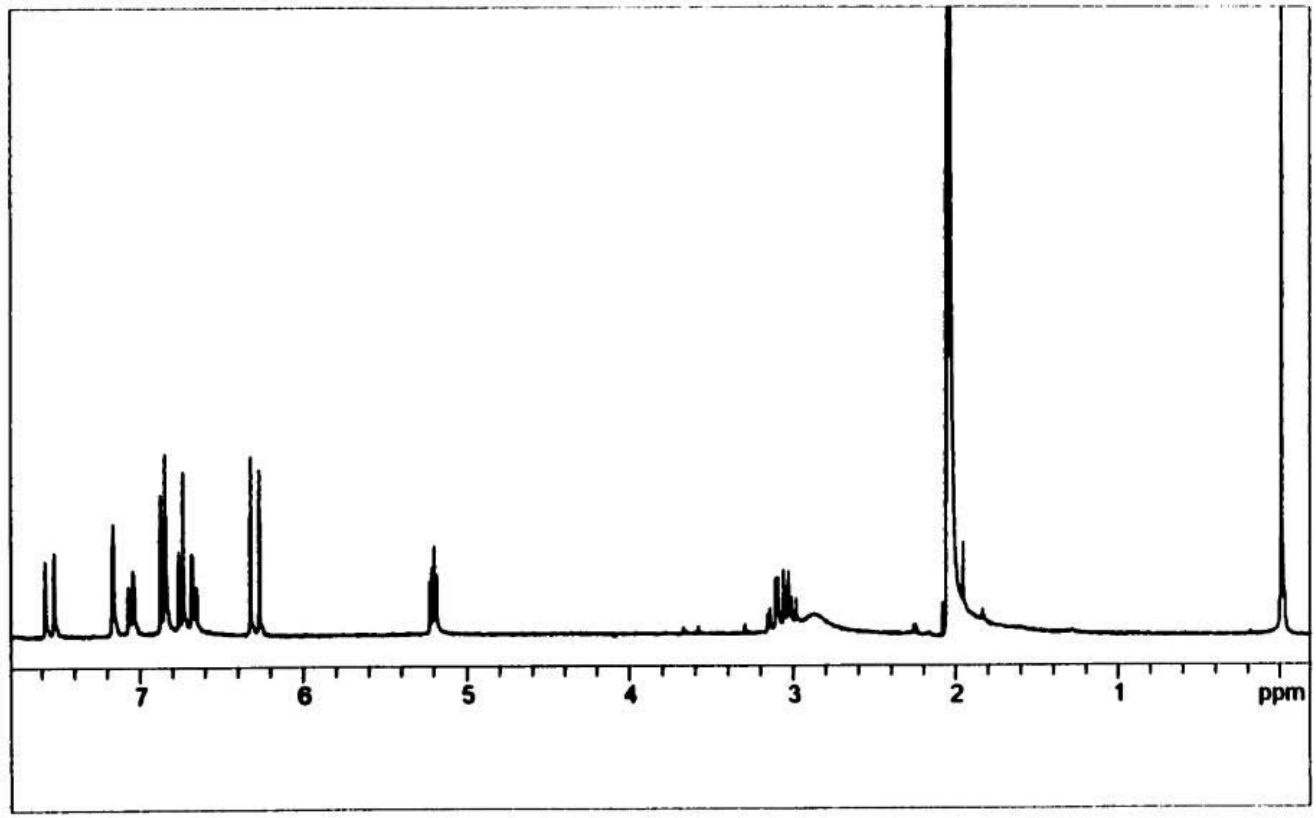
7
6
5
4
3
2
1
ppm

Figure A.13 $^{1}H,^{1}H$-COSY spectrum of compound A in acetone d-6.

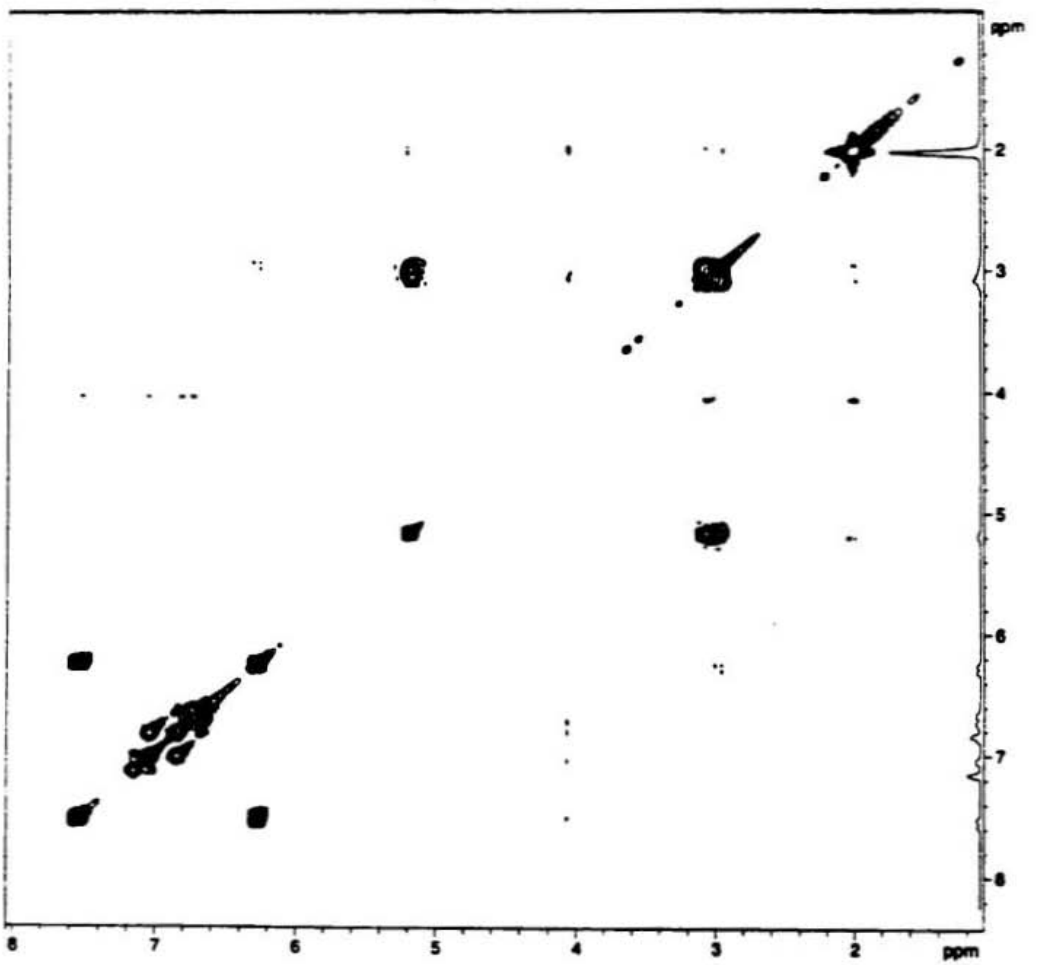
ppm
2
3
4
5
6
7
8
8
7
6
5
4
3
2
ppm

Figure A.14 Mass spectrum of compound B.

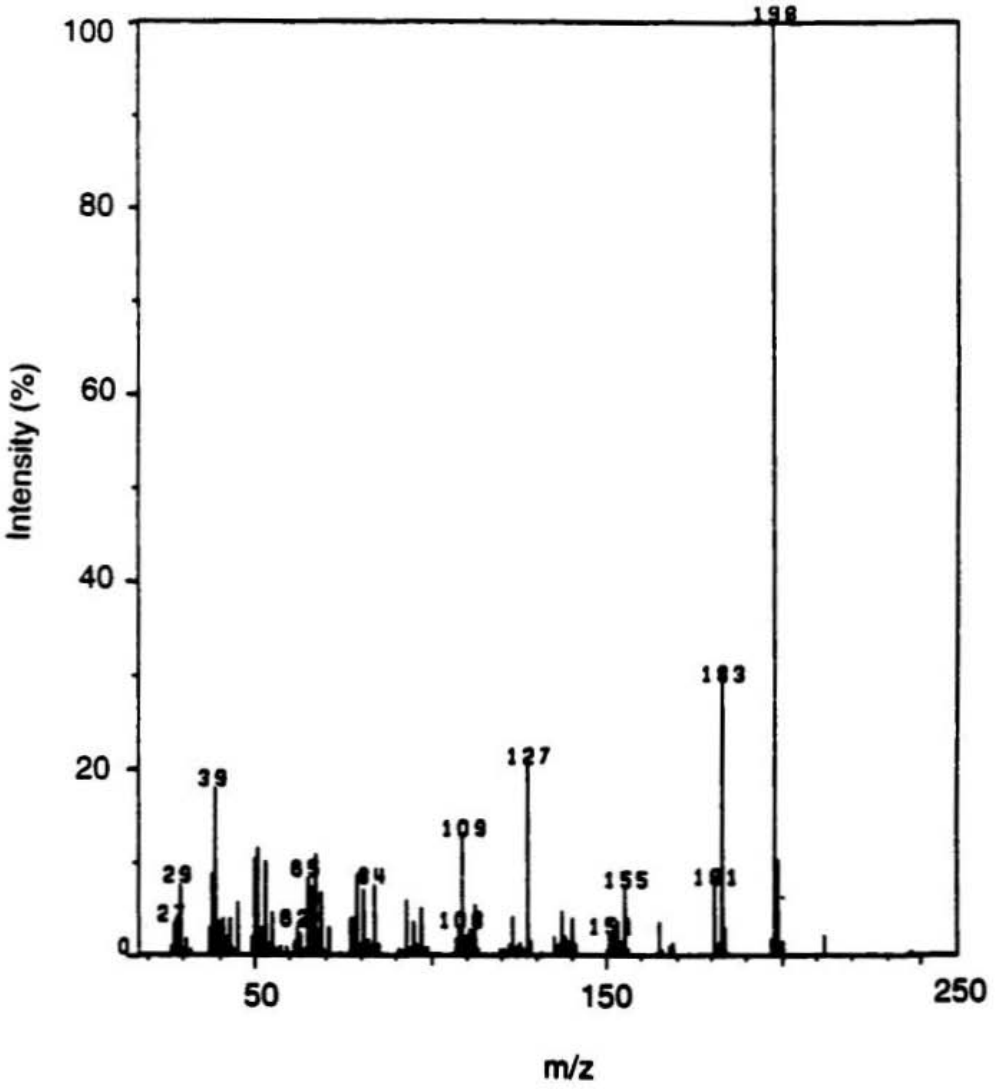
100
80
60
40
20
0
Intensity (%)
198
183
127
39
109
29
155
181
50
150
250
m/z

Figure A.15 ^{1}H-NMR spectrum of compound B in methanol d-4.

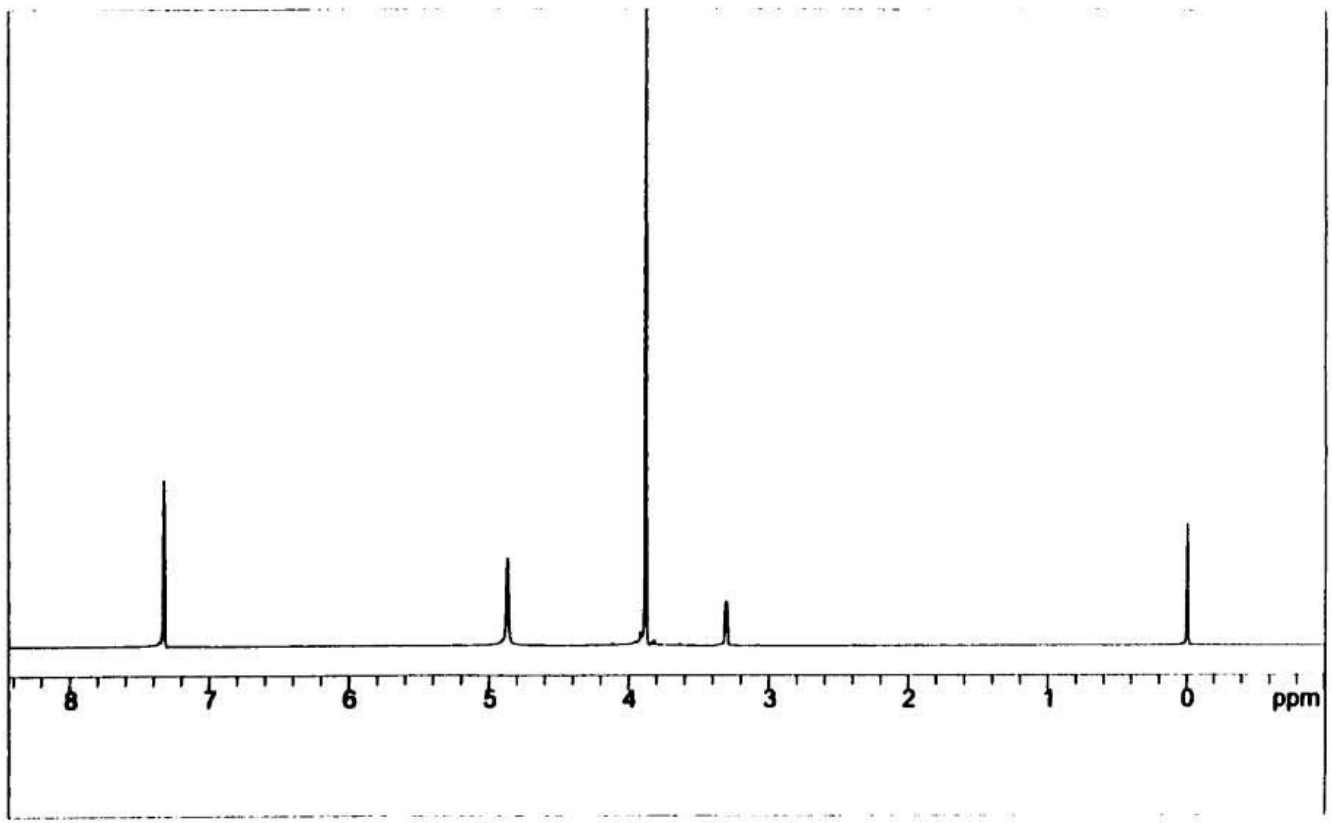
8
7
6
5
4
3
2
1
0
ppm

Figure A.16 Mass spectrum of compound C.

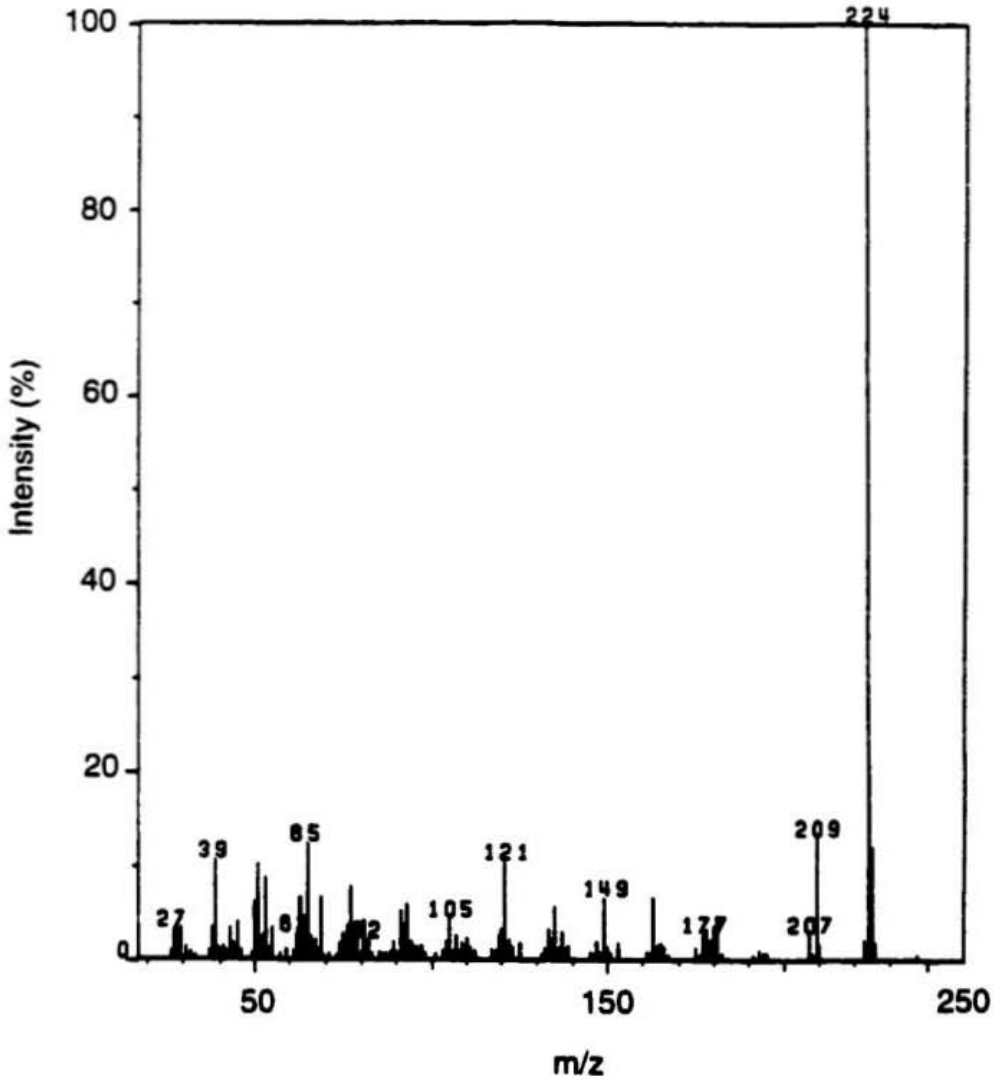
100
80
60
40
20
0
Intensity (%)
224
209
85
39
121
149
105
27
177
207
50
150
250
m/z

Figure A.17 ^{1}H-NMR spectrum of compound C in methanol d-4.

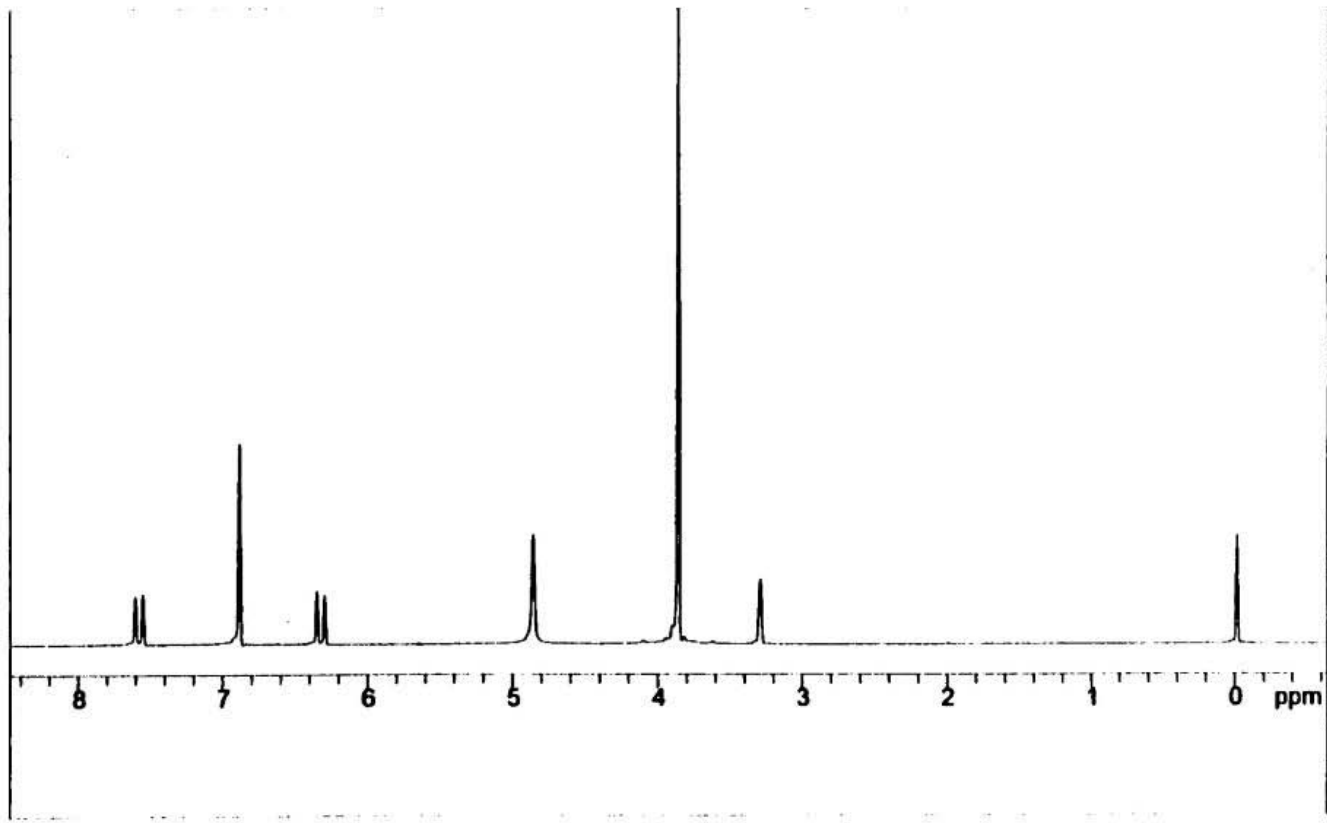
8
7
6
5
4
3
2
1
0
ppm

Figure A.18 $^{1}H,^{1}H$-COSY spectrum of compound C in methanol d-4.

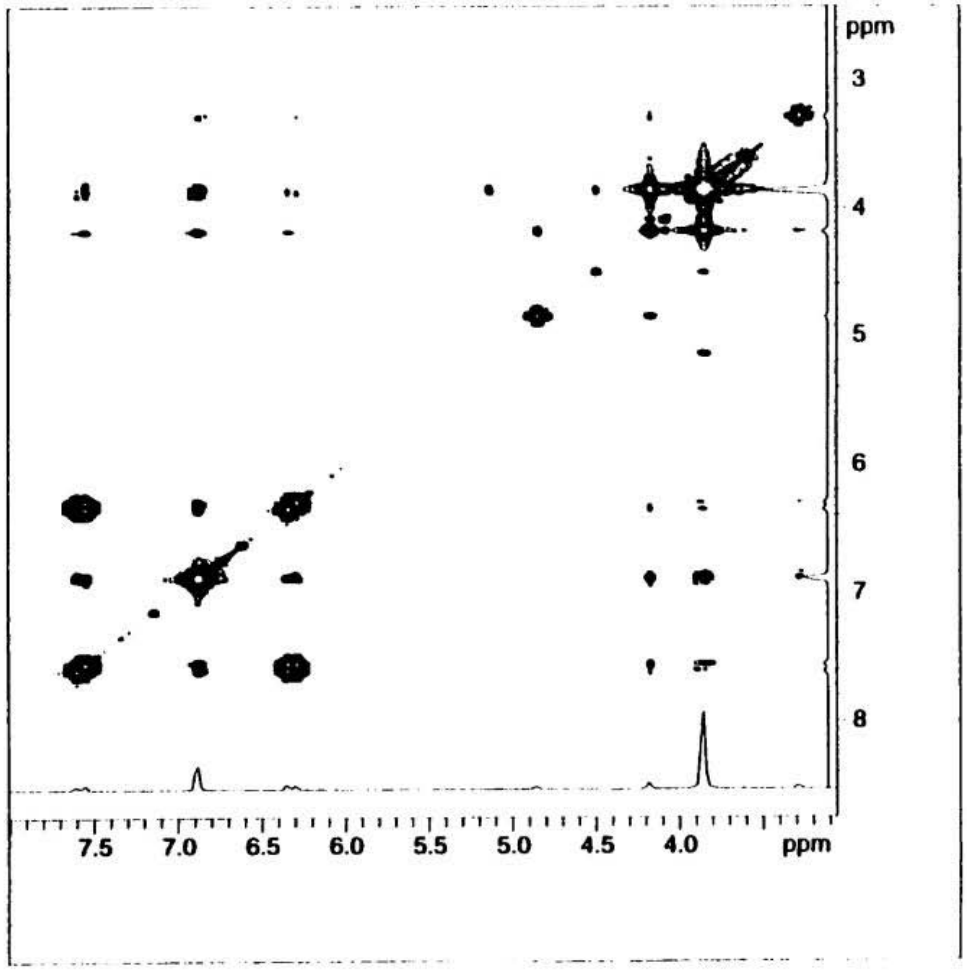
ppm
3
4
5
6
7
8
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
ppm

Figure A.19 Mass spectrum of compound D.

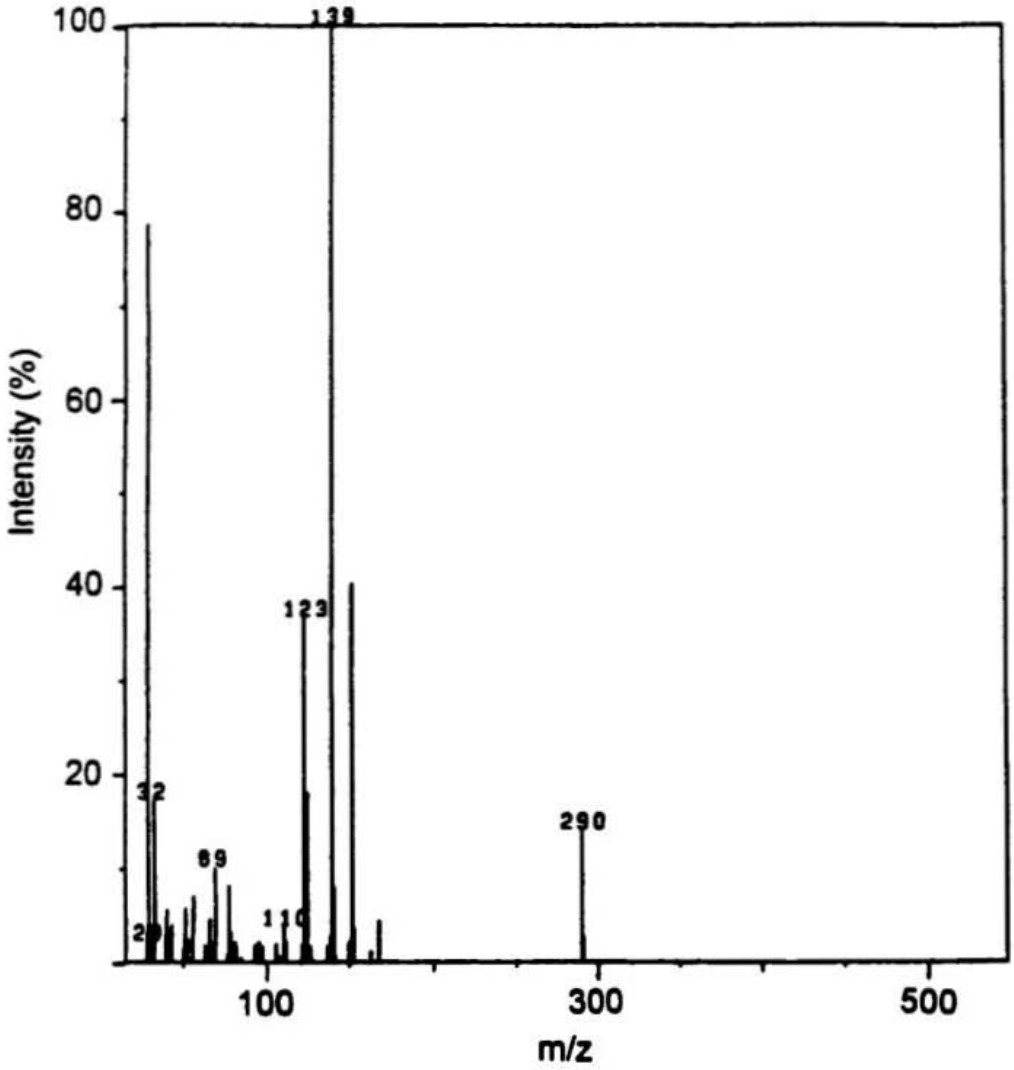
100
80
60
40
20
Intensity (%)
139
123
32
69
110
290
100
300
500
m/z

Figure A.20 ^{1}H-NMR spectrum of compound D in acetone d-6.

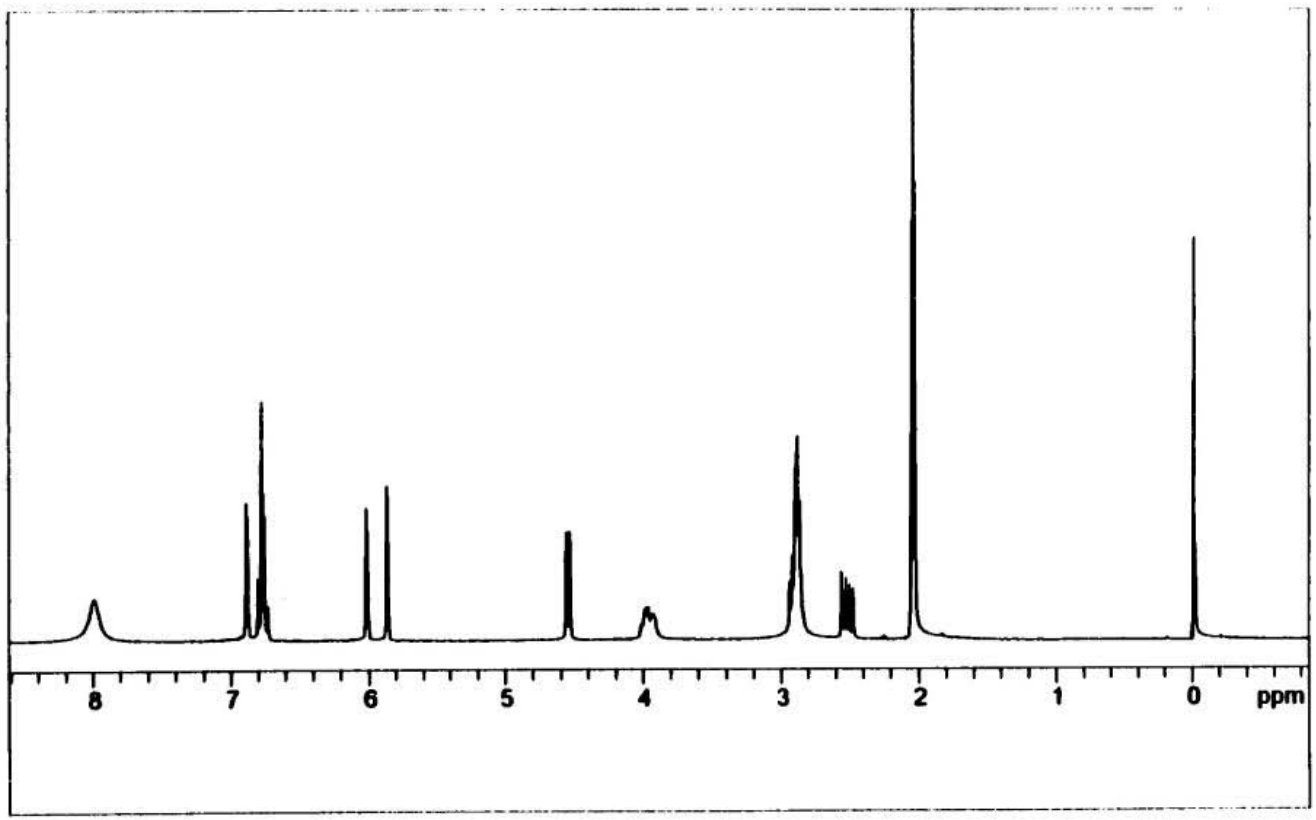
8
7
6
5
4
3
2
1
0
ppm

Figure A.21 $^{1}H,^{1}H$-COSY spectrum of compound D in acetone d-6.

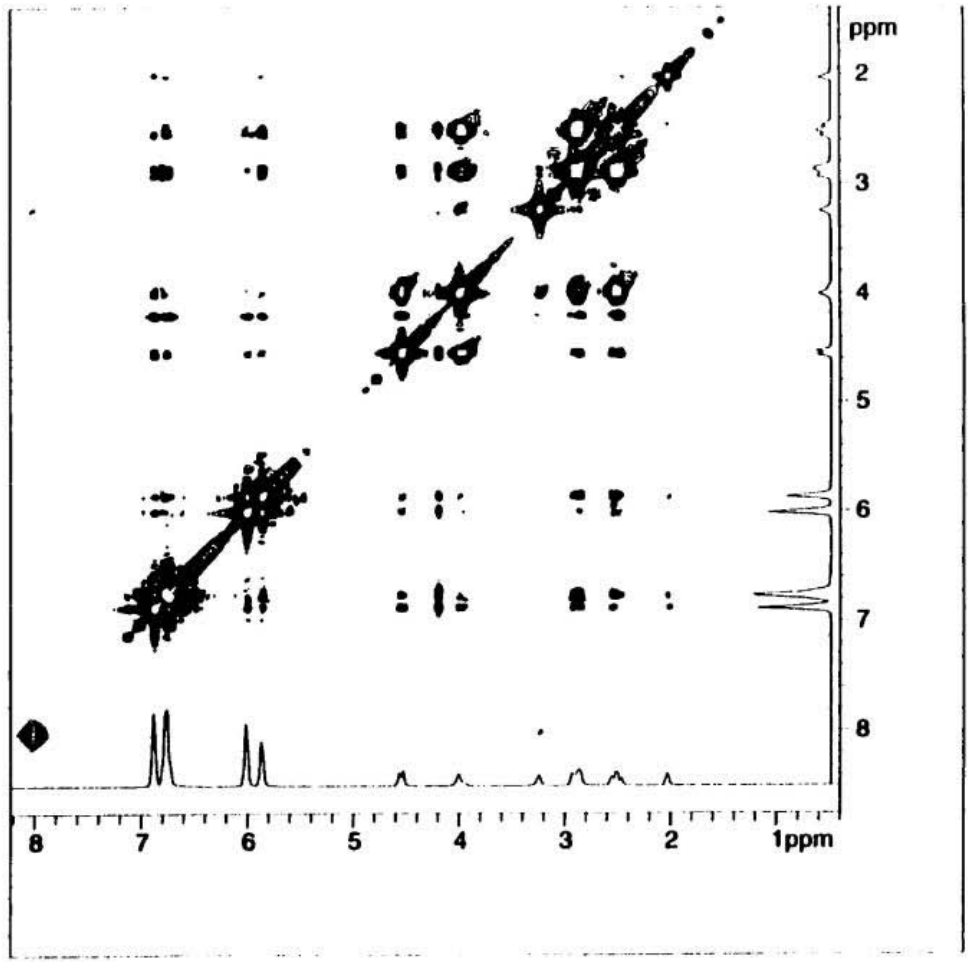
ppm
2
3
4
5
6
7
8
8
7
6
5
4
3
2
1ppm

Figure A.22 ^{13}C-NMR spectrum of compound D in acetone d-6.

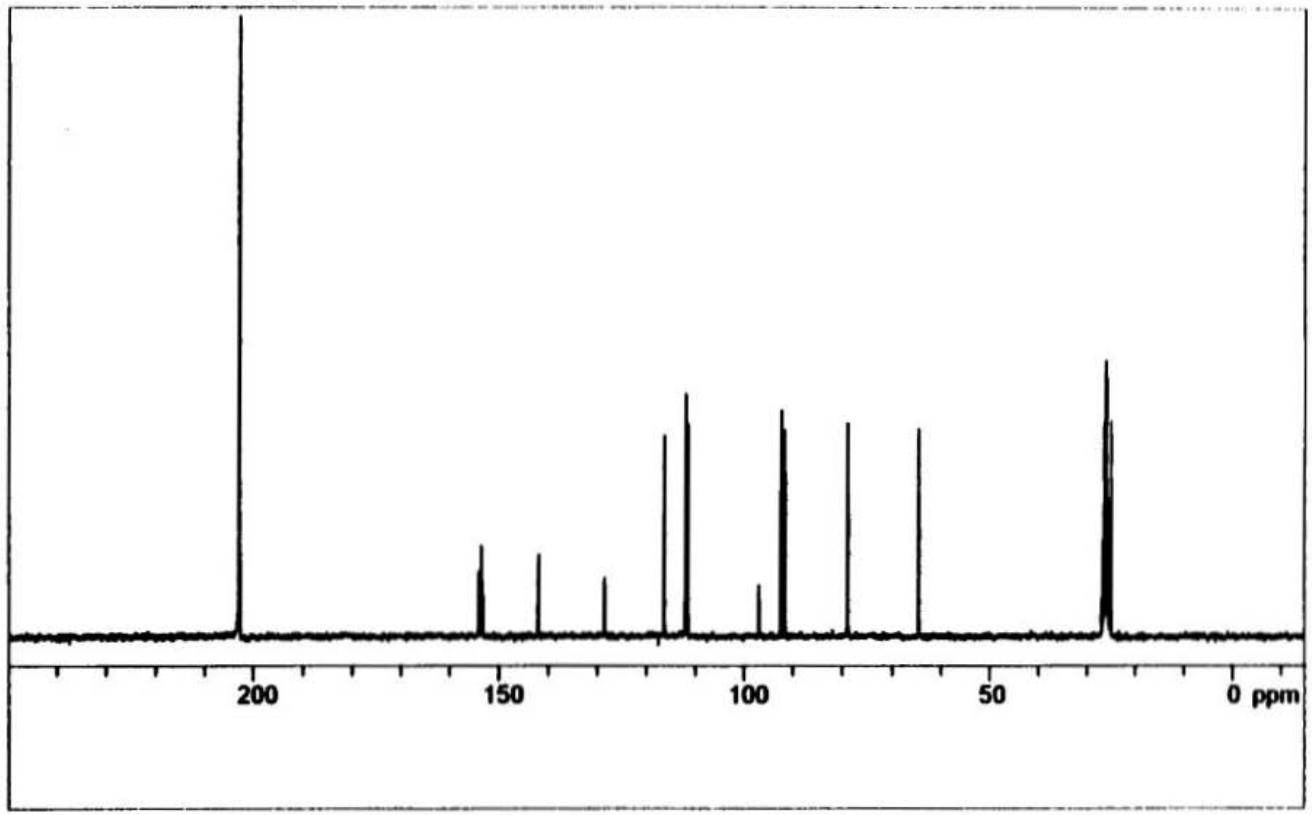
200
150
100
50
0 ppm

Figure A.23 Mass spectrum of compound E.

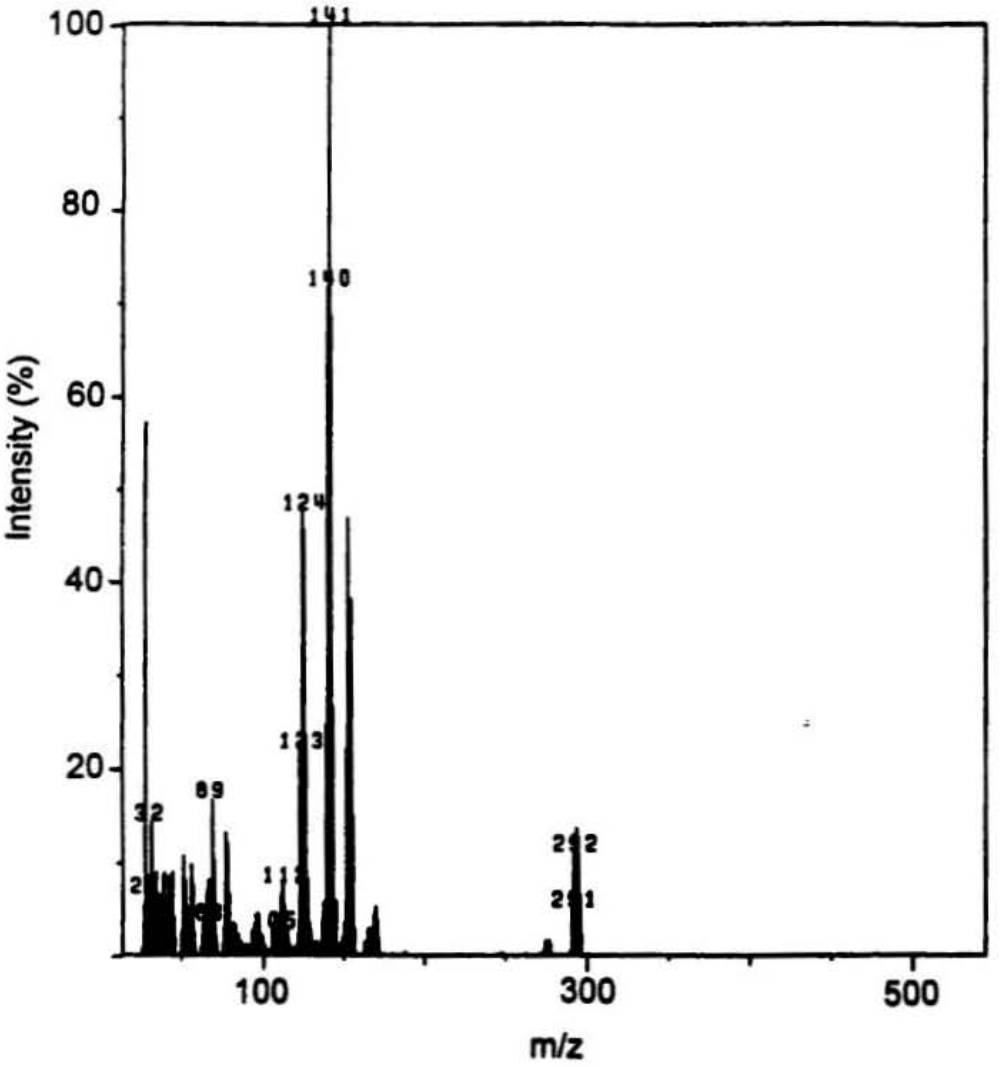
100
80
60
40
20
Intensity (%)
141
124
123
89
32
100
300
500
m/z

Figure A.24 ^{1}H-NMR spectrum of compound E in methanol d-4.

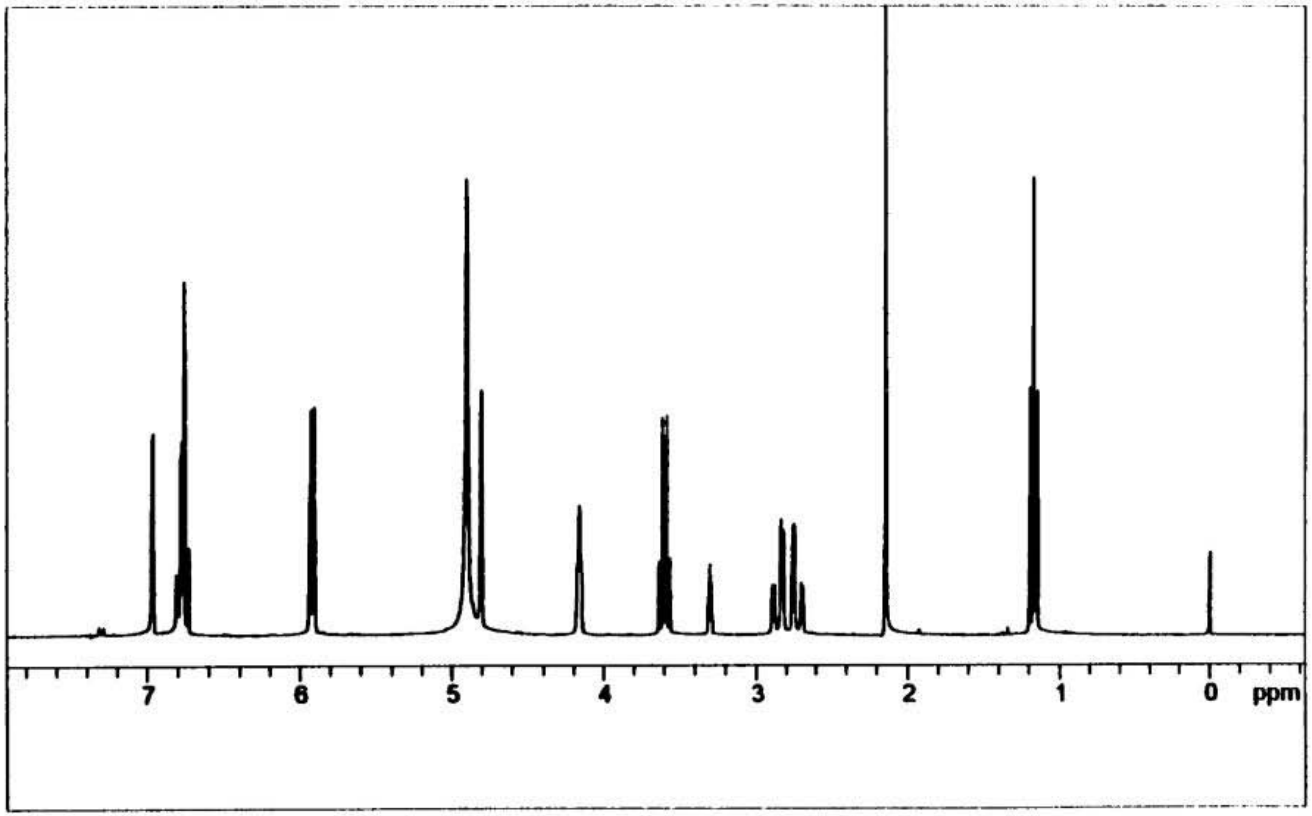
7
6
5
4
3
2
1
0
ppm

Figure A.25 $^{1}H,^{1}H$-COSY spectrum of compound E in methanol d-4.

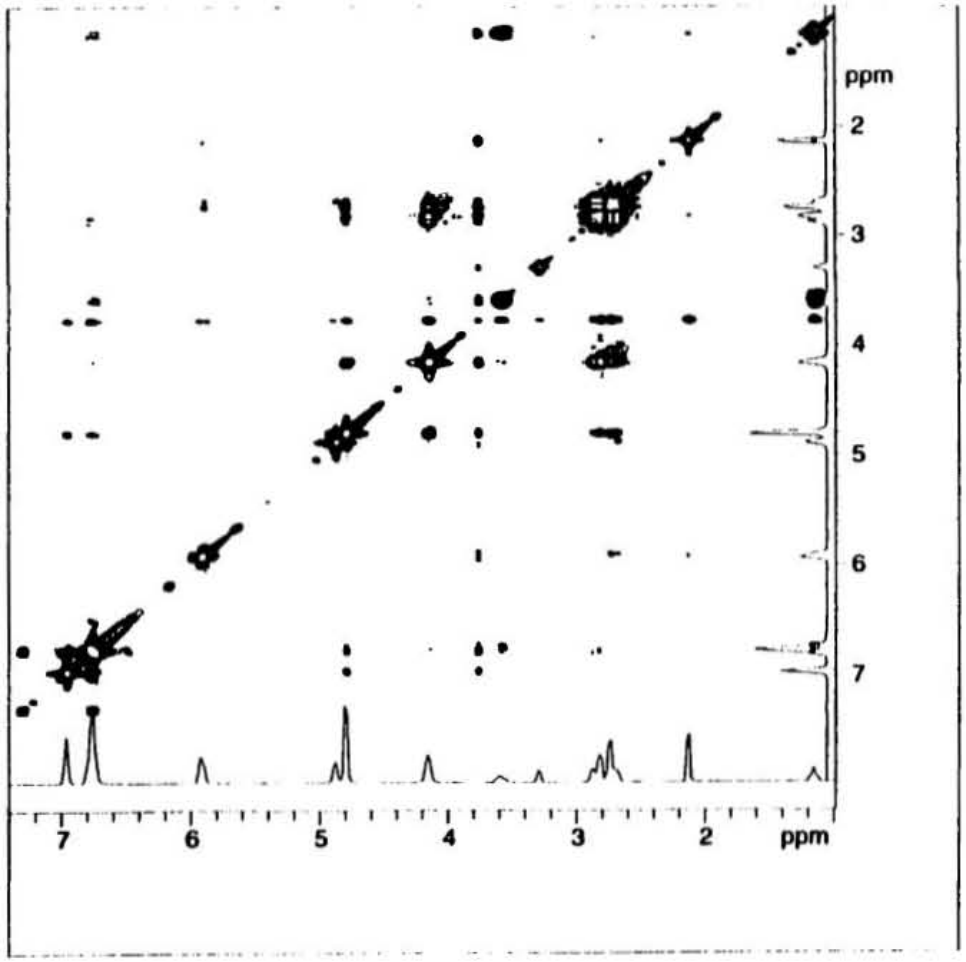
ppm
2
3
4
5
6
7
7
6
5
4
3
2
ppm

Figure A.26 ^{13}C-NMR spectrum of compound E in methanol d-4.

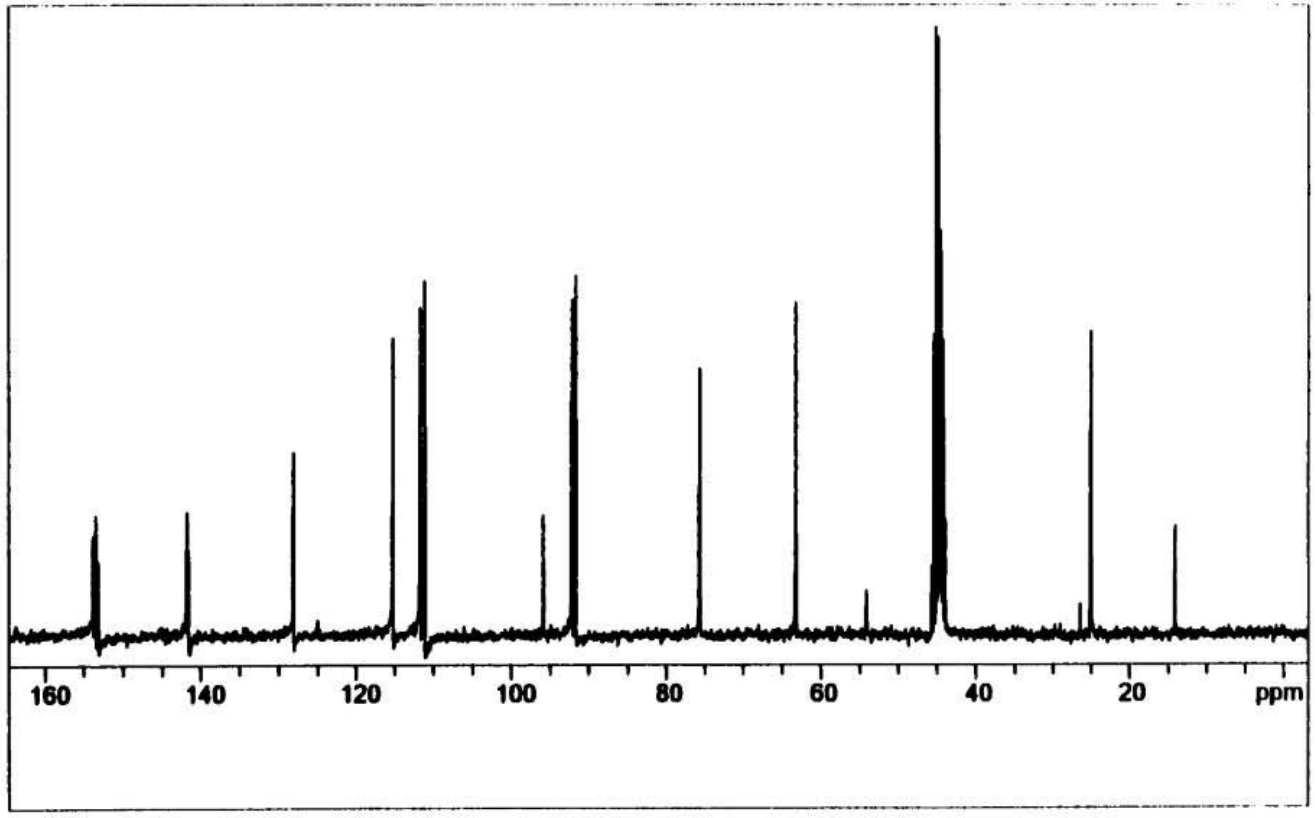
160
140
120
100
80
60
40
20
ppm

Figure A.27 Mass spectrum of compound F.

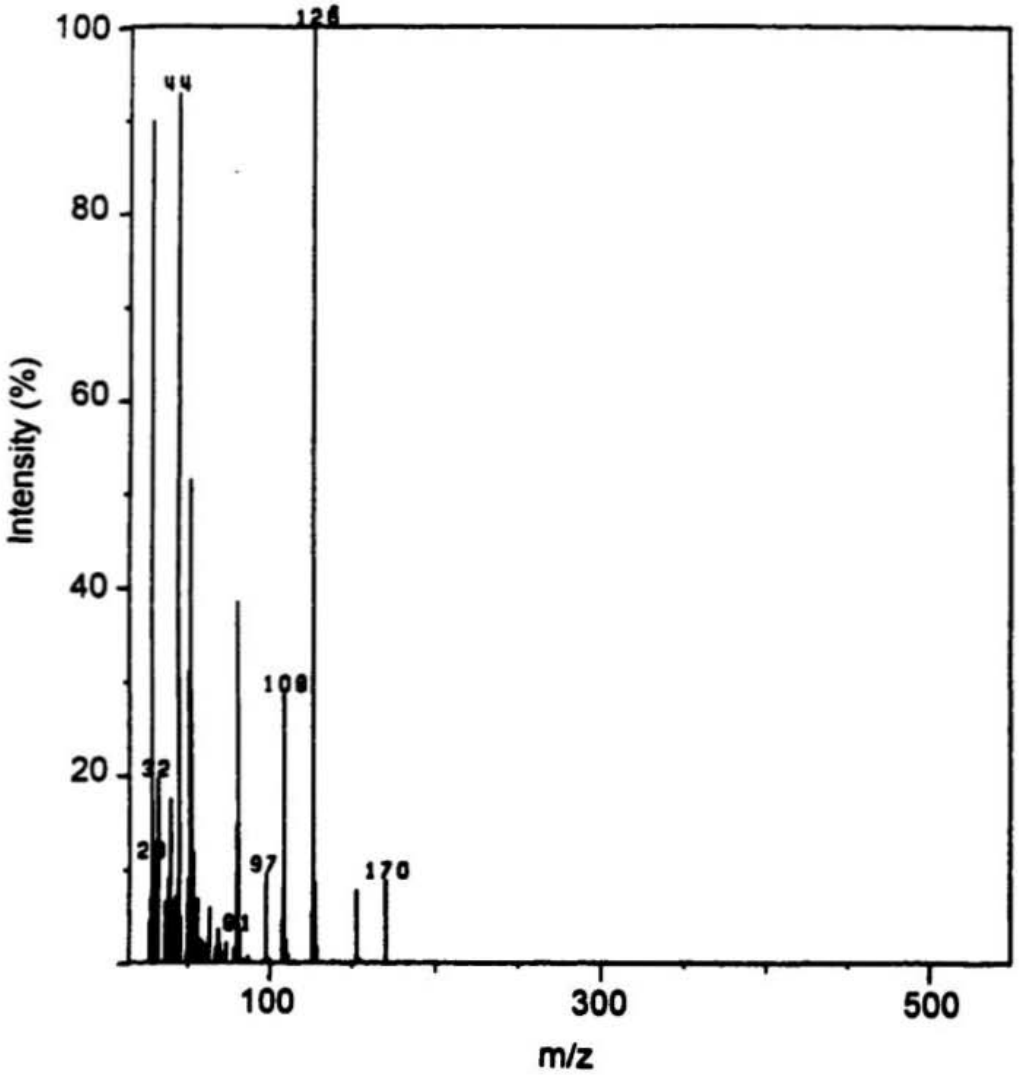
100
80
60
40
20
Intensity (%)
126
44
108
32
28
97
170
61
100
300
500
m/z

Figure A.28 ^{1}H-NMR spectrum of compound F in methanol d-4.

ppm
0
1
2
3
4
5
6
7

Table A.1 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on stability of a β-carotene-linoleate model system maintained at 50°C[1].

Additive	β-carotene concentration (μg/5 mL)				
	0 min	30 min	60 min	90 min	120 min
No additive	80 ± 6^{a}	29 ± 2^{a}	14 ± 2^{a}	8.2 ± 1^{a}	8.3 ± 1^{a}
BHA[2]	80 ± 6^{a}	80 ± 6^{f}	78 ± 7^{g}	74 ± 6^{f}	70 ± 6^{g}
Sinapic acid[2]	80 ± 6^{a}	76 ± 6^{f}	70 ± 6^{fg}	66 ± 6^{ef}	58 ± 5^{f}
Crude extract	80 ± 6^{a}	70 ± 5^{e}	67 ± 5^{f}	62 ± 6^{e}	60 ± 5^{f}
Fraction I	80 ± 6^{a}	73 ± 7^{ef}	53 ± 5^{e}	51 ± 4^{d}	48 ± 4^{e}
Fraction II	80 ± 6^{a}	32 ± 4^{ab}	26 ± 3^{b}	24 ± 2^{b}	22 ± 2^{b}
Fraction III	80 ± 6^{a}	44 ± 3^{cd}	31 ± 3^{bc}	28 ± 2^{bc}	26 ± 2^{bc}
Fraction IV	80 ± 6^{a}	50 ± 5^{d}	38 ± 4^{cd}	35 ± 3^{c}	31 ± 3^{cde}
Fraction V	80 ± 6^{a}	47 ± 5^{cd}	40 ± 4^{d}	36 ± 4^{c}	34 ± 3^{de}
Fraction VI	80 ± 6^{a}	40 ± 4^{bc}	35 ± 3^{cd}	33 ± 3^{c}	30 ± 3^{e}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.2 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on stability of a β-carotene-linoleate model system maintained at 50°C[1].

Additive	β-carotene concentration (μg/5 mL)				
	0 min	30 min	60 min	90 min	120 min
No additive	80 ± 6^{a}	29 ± 2^{a}	14 ± 2^{a}	8.2 ± 1^{a}	8.3 ± 1^{a}
BHA[2]	80 ± 6^{a}	78 ± 6^{e}	78 ± 6^{f}	78 ± 6^{f}	78 ± 6^{f}
Sinapic acid[2]	80 ± 6^{a}	77 ± 7^{e}	74 ± 6^{f}	66 ± 6^{e}	66 ± 6^{e}
Crude extract	80 ± 6^{a}	70 ± 7de	69 ± 6^{f}	63 ± 5de	63 ± 5de
Fraction I	80 ± 6^{a}	65 ± 5de	65 ± 6^{e}	56 ± 5^{d}	56 ± 5^{d}
Fraction II	80 ± 6^{a}	47 ± 4^{bc}	38 ± 4^{bc}	30 ± 3^{b}	30 ± 3^{b}
Fraction III	80 ± 6^{a}	67 ± 4de	53 ± 4^{d}	46 ± 5^{c}	46 ± 5^{c}
Fraction IV	80 ± 6^{a}	42 ± 4^{ab}	40 ± 4^{c}	34 ± 3^{b}	34 ± 3^{b}
Fraction V	80 ± 6^{a}	60 ± 6^{cd}	54 ± 5^{d}	44 ± 4^{c}	44 ± 4^{c}
Fraction VI	80 ± 6^{a}	32 ± 3^{ab}	29 ± 2^{b}	28 ± 2^{b}	28 ± 2^{b}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] reference antioxidants.

Table A.3 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents on stability of a β-carotene-linoleate model system maintained at 50°C[1].

Additive	β-carotene concentration (μg/5 mL)				
	0 min	30 min	60 min	90 min	120 min
No additive	80 ± 6[a]	29 ± 2[a]	14 ± 2[a]	8.2 ± 1[a]	8.3 ± 1[a]
BHA[2]	80 ± 6[a]	80 ± 6[d]	78 ± 7[g]	74 ± 6[f]	70 ± 6[f]
Catechin[2]	80 ± 6[a]	66 ± 5[c]	63 ± 5[f]	60 ± 5[e]	60 ± 5[e]
Crude extract	80 ± 6[a]	70 ± 7[cd]	58 ± 6[ef]	54 ± 4[de]	54 ± 4[de]
Fraction I	80 ± 6[a]	50 ± 5[b]	33 ± 4[b]	30 ± 3[b]	30 ± 3[b]
Fraction II	80 ± 6[a]	54 ± 5[b]	53 ± 5[de]	46 ± 3[d]	46 ± 3[d]
Fraction III	80 ± 6[a]	66 ± 6[c]	62 ± 6[ef]	53 ± 5[de]	53 ± 5[de]
Fraction IV	80 ± 6[a]	53 ± 5[b]	42 ± 5[bc]	38 ± 4[bc]	38 ± 4[bc]
Fraction V	80 ± 6[a]	65 ± 6[c]	43 ± 5[c]	38 ± 3[bc]	38 ± 3[bc]
Fraction VI	80 ± 6[a]	54 ± 5[b]	48 ± 4[cd]	44 ± 4[c]	44 ± 4[c]

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.4 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents) on stability of a β-carotene-linoleate model system maintained at 50°C[1].

Additive	β-carotene concentration (μg/5 mL)				
	0 min	30 min	60 min	90 min	120 min
No additive	80 ± 6[a]	29 ± 2[a]	14 ± 2[a]	8.3 ± 1[a]	8.3 ± 1[a]
BHA[2]	80 ± 6[a]	78 ± 6[c]	78 ± 6[c]	78 ± 6[c]	78 ± 7[c]
Catechin[2]	80 ± 6[a]	70 ± 6[c]	67 ± 5[d]	61 ± 6[d]	59 ± 5[d]
Crude extract	80 ± 6[a]	74 ± 4[c]	70 ± 5[de]	68 ± 6[d]	67 ± 5[d]
Fraction I	80 ± 6[a]	55 ± 4[b]	38 ± 4[b]	36 ± 4[b]	32 ± 3[b]
Fraction II	80 ± 6[a]	70 ± 5[c]	53 ± 4[c]	50 ± 4[c]	48 ± 3[c]
Fraction III	80 ± 6[a]	76 ± 6[c]	69 ± 6[de]	64 ± 6[d]	60 ± 5[d]
Fraction IV	80 ± 6[a]	70 ± 4[c]	69 ± 6[de]	64 ± 5[d]	63 ± 6[d]
Fraction V	80 ± 6[a]	69 ± 4[c]	51 ± 4[c]	49 ± 4[c]	48 ± 4[c]
Fraction VI	80 ± 6[a]	77 ± 6[c]	66 ± 5[d]	62 ± 4[d]	61 ± 5[d]

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.5 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of TBARS in a cooked comminuted pork model system stored at 4°C[1].

Additive	TBARS value (mg malonaldehyde equivalents/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.0 ± 0.1^{a}	2.1 ± 0.1^{c}	3.6 ± 0.2^{b}	8.3 ± 0.4^{b}	11 ± 0.6^{c}
BHA[2]	1.0 ± 0.1^{a}	1.7 ± 0.1^{ab}	3.0 ± 0.1^{a}	3.5 ± 0.1^{a}	4.5 ± 0.2^{a}
α-Tocopherol[2]	1.0 ± 0.1^{a}	1.8 ± 0.1^{ab}	3.0 ± 0.1^{a}	8.0 ± 0.4^{b}	9.1 ± 0.2^{b}
Sinapic acid[2]	1.0 ± 0.1^{a}	1.8 ± 0.1^{ab}	3.0 ± 0.1^{a}	8.2 ± 0.3^{b}	9.0 ± 0.2^{b}
Crude extract	1.0 ± 0.1^{a}	1.9 ± 0.1^{bc}	3.4 ± 0.2^{ab}	8.1 ± 0.4^{b}	9.0 ± 0.5^{b}
Fraction I	1.1 ± 0.0^{a}	2.1 ± 0.1^{c}	3.5 ± 0.2^{b}	8.2 ± 0.3^{b}	11 ± 0.7^{c}
Fraction II	1.0 ± 0.1^{a}	2.1 ± 0.2^{c}	3.5 ± 0.2^{b}	8.4 ± 0.3^{b}	11 ± 0.7^{c}
Fraction III	1.0 ± 0.1^{a}	2.1 ± 0.1^{c}	3.0 ± 0.2^{a}	8.2 ± 0.4^{b}	12 ± 0.6^{cd}
Fraction IV	1.1 ± 0.0^{a}	1.6 ± 0.1^{a}	3.2 ± 0.2^{ab}	8.0 ± 0.4^{b}	11 ± 0.8^{c}
Fraction V	1.0 ± 0.1^{a}	1.8 ± 0.1^{ab}	3.6 ± 0.1^{b}	8.3 ± 0.4^{b}	13 ± 0.9^{d}
Fraction VI	1.1 ± 0.1^{a}	2.1 ± 0.1^{c}	3.6 ± 0.2^{b}	8.4 ± 0.3^{b}	11 ± 0.6^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.6 Effect borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of TBARS in a cooked comminuted pork model system[1].

Additive	TBARS value (mg malonaldehyde equivalents/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.0 ± 0.1^{a}	2.1 ± 0.1^{c}	3.6 ± 0.2^{c}	8.3 ± 0.4^{b}	11 ± 0.6^{de}
BHA[2]	1.0 ± 0.1^{a}	1.4 ± 0.1^{a}	2.8 ± 0.1^{a}	3.1 ± 0.1^{a}	3.4 ± 0.1^{a}
α-Tocopherol[2]	1.0 ± 0.1^{a}	1.4 ± 0.1^{a}	2.9 ± 0.1^{a}	7.8 ± 0.3^{b}	8.1 ± 0.2^{b}
Sinapic acid[2]	1.0 ± 0.1^{a}	1.4 ± 0.1^{a}	2.9 ± 0.1^{a}	7.8 ± 0.4^{b}	8.0 ± 0.2^{b}
Crude extract	1.0 ± 0.1^{a}	1.7 ± 0.1^{b}	3.1 ± 0.1^{b}	8.0 ± 0.3^{b}	8.1 ± 0.3^{b}
Fraction I	1.0 ± 0.1^{a}	2.1 ± 0.1^{c}	3.4 ± 0.1^{c}	7.9 ± 0.3^{b}	10 ± 0.7^{cd}
Fraction II	1.0 ± 0.1^{a}	2.2 ± 0.1^{c}	3.4 ± 0.1^{c}	8.3 ± 0.2^{b}	11 ± 0.7^{de}
Fraction III	1.1 ± 0.1^{a}	2.1 ± 0.1^{c}	2.9 ± 0.1^{a}	8.2 ± 0.3^{b}	9.0 ± 0.4^{bc}
Fraction IV	1.1 ± 0.0^{a}	1.4 ± 0.1^{a}	3.1 ± 0.1^{b}	7.8 ± 0.3^{b}	10 ± 0.6^{cd}
Fraction V	1.0 ± 0.0^{a}	1.7 ± 0.1^{b}	3.4 ± 0.1^{c}	8.3 ± 0.3^{b}	12 ± 0.8^{e}
Fraction VI	1.1 ± 0.1^{a}	2.0 ± 0.1^{c}	3.5 ± 0.1^{c}	8.0 ± 0.3^{b}	9.1 ± 0.7^{bc}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.7 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents on formation of TBARS in a cooked comminuted pork model system[1].

Additive	TBARS value (mg malonaldehyde equivalents/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.0 ± 0.1^{a}	2.1 ± 0.1^{f}	3.6 ± 0.2^{e}	8.3 ± 0.4^{e}	11 ± 0.6^{e}
BHA[2]	1.0 ± 0.1^{a}	1.7 ± 0.1^{de}	3.0 ± 0.1^{d}	3.5 ± 0.1^{c}	4.5 ± 0.2^{c}
α-Tocopherol[2]	1.0 ± 0.16^{a}	1.8 ± 0.1^{e}	3.0 ± 0.1^{d}	8.0 ± 0.4^{e}	9.1 ± 0.2^{d}
Catechin[2]	1.0 ± 0.18^{a}	1.0 ± 0.1^{a}	1.3 ± 0.1^{ab}	1.9 ± 0.1^{a}	3.2 ± 0.2^{ab}
Crude extract	1.1 ± 0.0^{a}	1.4 ± 0.1^{c}	1.5 ± 0.1^{b}	2.4 ± 0.3^{ab}	2.8 ± 0.1^{a}
Fraction I	1.0 ± 0.1^{a}	1.8 ± 0.1^{e}	3.0 ± 0.1^{d}	8.0 ± 0.3^{e}	11 ± 0.6^{e}
Fraction II	1.0 ± 0.1^{a}	1.0 ± 0.0^{a}	1.1 ± 0.0^{a}	2.0 ± 0.1^{a}	3.9 ± 0.1^{bc}
Fraction III	1.0 ± 0.1^{a}	1.2 ± 0.0^{b}	1.2 ± 0.0^{a}	2.0 ± 0.1^{a}	4.1 ± 0.2^{c}
Fraction IV	0.9 ± 0.0^{a}	1.2 ± 0.0^{b}	1.3 ± 0.0^{ab}	3.4 ± 0.2^{c}	4.0 ± 0.2^{bc}
Fraction V	1.0 ± 0.0^{a}	1.0 ± 0.1^{a}	1.1 ± 0.0^{a}	3.0 ± 0.1^{bc}	3.2 ± 0.2^{ab}
Fraction VI	1.1 ± 0.1^{a}	1.6 ± 0.0^{d}	2.0 ± 0.1^{c}	6.0 ± 0.3^{d}	8.6 ± 0.3^{d}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.8 Effect evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of TBARS in a cooked comminuted pork model system[1].

Additive	TBARS value (mg malonaldehyde equivalents/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.0 ± 0.1^{a}	2.1 ± 0.1^{e}	3.6 ± 0.2^{e}	8.3 ± 0.4^{e}	11 ± 0.6^{d}
BHA[2]	1.0 ± 0.1^{a}	1.4 ± 0.1^{c}	2.8 ± 0.1^{cd}	3.1 ± 0.1^{b}	3.4 ± 0.1^{b}
α-Tocopherol[2]	1.0 ± 0.1^{a}	1.4 ± 0.1^{c}	2.9 ± 0.1^{d}	7.8 ± 0.3^{de}	8.1 ± 0.2^{c}
Catechin[2]	1.0 ± 0.0^{a}	0.47 ± 0.03^{a}	0.43 ± 0.03^{a}	0.53 ± 0.02^{a}	1.0 ± 0.1^{a}
Crude extract	1.0 ± 0.0^{a}	0.43 ± 0.04^{a}	0.45 ± 0.05^{a}	0.46 ± 0.04^{a}	1.3 ± 0.1^{a}
Fraction I	1.0 ± 0.1^{a}	1.6 ± 0.1^{d}	2.7 ± 0.1^{c}	7.6 ± 0.3^{d}	11 ± 0.6^{d}
Fraction II	1.0 ± 0.0^{a}	0.46 ± 0.02^{a}	0.54 ± 0.03^{a}	0.43 ± 0.02^{a}	1.7 ± 0.1^{a}
Fraction III	1.0 ± 0.0^{a}	0.43 ± 0.06^{a}	0.44 ± 0.02^{a}	0.42 ± 0.02^{a}	1.1 ± 0.0^{a}
Fraction IV	1.0 ± 0.0^{a}	0.46 ± 0.04^{a}	0.56 ± 0.03^{a}	0.56 ± 0.03^{a}	1.7 ± 0.1^{a}
Fraction V	1.0 ± 0.0^{a}	0.44 ± 0.05^{a}	0.41 ± 0.02^{a}	0.51 ± 0.03^{a}	1.0 ± 0.0^{a}
Fraction VI	1.1 ± 0.0^{a}	0.75 ± 0.02^{b}	1.1 ± 0.1^{b}	4.3 ± 0.2^{c}	7.9 ± 0.3^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.9 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of hexanal in a cooked comminuted pork model system stored at 4°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	10 ± 0.4^{f}	15 ± 0.7^{d}	19 ± 1.0^{e}	36 ± 2.0^{c}	34 ± 2.0^{f}
BHA[2]	4.0 ± 0.2^{a}	4.4 ± 0.3^{a}	4.6 ± 0.2^{a}	5.3 ± 0.3^{a}	5.4 ± 0.3^{a}
α-Tocopherol[2]	4.2 ± 0.2^{ab}	4.4 ± 0.2^{a}	5.0 ± 0.3^{a}	5.6 ± 0.3^{a}	7.0 ± 0.4^{a}
Sinapic acid[2]	5.8 ± 0.3^{cd}	5.4 ± 0.3^{ab}	5.8 ± 0.3^{ab}	7.3 ± 0.4^{a}	10 ± 0.6^{b}
Crude extract	6.6 ± 0.4^{de}	8.2 ± 0.5^{c}	10 ± 0.6^{cd}	11 ± 0.6^{b}	20 ± 1.0^{de}
Fraction I	6.8 ± 0.3^{e}	8.9 ± 0.5^{c}	11 ± 0.6^{d}	12 ± 0.6^{b}	22 ± 1.1^{e}
Fraction II	7.0 ± 0.4^{e}	8.2 ± 0.4^{c}	11 ± 0.5^{d}	13 ± 0.5^{b}	22 ± 1.1^{e}
Fraction III	6.6 ± 0.4^{de}	8.0 ± 0.5^{c}	11 ± 0.6^{d}	13 ± 0.6^{b}	20 ± 1.0^{de}
Fraction IV	5.0 ± 0.3^{bc}	6.3 ± 0.4^{b}	7.0 ± 0.5^{b}	11 ± 0.6^{b}	15 ± 0.7^{c}
Fraction V	7.0 ± 0.4^{e}	8.6 ± 0.6^{c}	11 ± 0.6^{d}	12 ± 0.7^{b}	19 ± 1.0^{d}
Fraction VI	7.0 ± 0.4^{e}	8.1 ± 0.4^{c}	9.4 ± 0.4^{c}	11 ± 0.5^{b}	19 ± 1.1^{d}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.10 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of hexanal in a cooked comminuted pork model system stored at 4°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	10 ± 0.4^{f}	15 ± 0.7^{e}	19 ± 1.0^{e}	36 ± 2.0^{e}	34 ± 2.0^{d}
BHA[2]	2.2 ± 0.1^{a}	2.0 ± 0.1^{a}	2.3 ± 0.1^{a}	4.0 ± 0.2^{a}	4.8 ± 0.3^{a}
α-Tocopherol[2]	3.0 ± 0.2^{b}	3.6 ± 0.1^{b}	4.0 ± 0.2^{b}	4.4 ± 0.3^{a}	6.2 ± 0.4^{a}
Sinapic acid[2]	4.3 ± 0.3^{c}	4.4 ± 0.2^{b}	4.6 ± 0.2^{b}	5.3 ± 0.3^{ab}	5.0 ± 0.3^{a}
Crude extract	4.9 ± 0.2^{cd}	8.7 ± 0.4^{c}	8.7 ± 0.4^{c}	12 ± 0.6^{d}	18 ± 1.0^{c}
Fraction I	5.5 ± 0.3^{d}	9.1 ± 0.6^{cd}	9.1 ± 0.6^{cd}	10 ± 0.5^{cd}	16 ± 0.9^{c}
Fraction II	5.3 ± 0.3^{d}	8.2 ± 0.5^{c}	8.2 ± 0.5^{c}	10 ± 0.5^{cd}	17 ± 0.8^{c}
Fraction III	4.2 ± 0.2^{c}	10 ± 0.6^{d}	10 ± 0.6^{d}	12 ± 0.6^{d}	18 ± 0.7^{c}
Fraction IV	2.4 ± 0.1^{a}	4.3 ± 0.2^{b}	4.3 ± 0.2^{b}	6.6 ± 0.4^{b}	12 ± 0.5^{b}
Fraction V	5.2 ± 0.2^{d}	10 ± 0.5^{d}	10 ± 0.5^{d}	9.0 ± 0.4^{c}	16 ± 0.8^{c}
Fraction VI	6.3 ± 0.4^{e}	8.2 ± 0.4^{c}	8.2 ± 0.4^{c}	10 ± 0.5^{cd}	16 ± 0.6^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.11 Effect of evening primrose additives at a concentration of 100 ppm phenolics as catechin equivalents on formation of hexanal in a cooked comminuted pork model system stored at 4°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	10 ± 0.4^{g}	15 ± 0.7^{g}	19 ± 1.0^{h}	36 ± 2.0^{e}	34 ± 2.0^{g}
BHA[2]	4.0 ± 0.2^{d}	4.4 ± 0.3^{c}	4.6 ± 0.2^{de}	5.3 ± 0.3^{b}	5.4 ± 0.3^{bc}
α-Tocopherol[2]	4.2 ± 0.2^{d}	4.4 ± 0.2^{c}	5.0 ± 0.3^{de}	5.6 ± 0.3^{b}	7.0 ± 0.4^{c}
Catechin[2]	5.2 ± 0.2^{e}	6.2 ± 0.2^{e}	8.5 ± 0.6^{f}	10 ± 0.4^{c}	12 ± 0.5^{d}
Crude extract	1.8 ± 0.1^{b}	2.5 ± 0.1^{b}	4.0 ± 0.3^{cd}	6.0 ± 0.3^{b}	6.3 ± 0.3^{c}
Fraction I	7.0 ± 0.3^{f}	8.5 ± 0.5^{f}	12 ± 0.6^{g}	14 ± 0.7^{d}	24 ± 1.4^{f}
Fraction II	2.0 ± 0.1^{b}	2.0 ± 0.1^{b}	2.4 ± 0.1^{ab}	2.4 ± 0.1^{a}	3.5 ± 0.2^{ab}
Fraction III	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.5 ± 0.1^{a}	1.7 ± 0.1^{a}	1.8 ± 0.1^{a}
Fraction IV	1.9 ± 0.1^{b}	2.5 ± 0.2^{b}	2.8 ± 0.1^{bc}	3.0 ± 0.1^{a}	3.6 ± 0.1^{ab}
Fraction V	1.8 ± 0.1^{a}	1.7 ± 0.1^{ab}	1.7 ± 0.1^{ab}	1.8 ± 0.1^{a}	1.9 ± 0.1^{a}
Fraction VI	3.0 ± 0.2^{c}	5.3 ± 0.4^{d}	5.7 ± 0.3^{e}	9.4 ± 0.5^{c}	16 ± 0.8^{e}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.12 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of hexanal in a cooked comminuted pork model system stored at 4°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	10 ± 0.4^{e}	15 ± 0.7^{f}	19 ± 1.0^{f}	36 ± 2.0^{e}	34 ± 2.0^{e}
BHA[2]	2.2 ± 0.1^{b}	2.0 ± 0.1^{b}	2.3 ± 0.1^{b}	4.0 ± 0.2^{b}	4.8 ± 0.3^{b}
α-Tocopherol[2]	3.0 ± 0.2^{c}	3.6 ± 0.1^{c}	4.0 ± 0.2^{c}	4.4 ± 0.3^{b}	6.2 ± 0.4^{b}
Catechin[2]	3.2 ± 0.2^{c}	4.6 ± 0.2^{d}	5.1 ± 0.4^{d}	6.4 ± 0.4^{c}	9.8 ± 0.4^{c}
Crude extract	0.13 ± 0.02^{a}	0.26 ± 0.01^{a}	0.24 ± 0.05^{a}	0.36 ± 0.04^{a}	0.8 ± 0.0^{a}
Fraction I	5.8 ± 0.4^{d}	7.5 ± 0.5^{e}	11 ± 0.5^{e}	9.9 ± 0.7^{d}	20 ± 1.3^{d}
Fraction II	0.14 ± 0.05^{a}	0.23 ± 0.03^{a}	0.35 ± 0.03^{a}	0.44 ± 0.01^{a}	1.3 ± 0.1^{a}
Fraction III	0.16 ± 0.06^{a}	0.17 ± 0.02^{a}	0.26 ± 0.04^{a}	0.23 ± 0.01^{a}	1.6 ± 0.1^{a}
Fraction IV	0.17 ± 0.03^{a}	0.36 ± 0.04^{a}	0.41 ± 0.00^{a}	0.56 ± 0.02^{a}	1.4 ± 0.1^{a}
Fraction V	0.13 ± 0.02^{a}	0.14 ± 0.05^{a}	0.16 ± 0.02^{a}	0.13 ± 0.03^{a}	0.4 ± 0.0^{a}
Fraction VI	0.54 ± 0.04^{a}	1.3 ± 0.1^{b}	3.3 ± 0.2^{b}	7.0 ± 0.5^{c}	12 ± 0.8^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.13 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of total volatiles in a cooked comminuted pork model system stored at 4°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	21 ± 1.4^{bc}	25 ± 1.5^{d}	33 ± 1.9^{d}	61 ± 4.0^{d}	61 ± 3.8^{g}
BHA[2]	16 ± 1.2^{a}	17 ± 1.1^{a}	18 ± 1.4^{a}	18 ± 0.9^{a}	18 ± 0.9^{a}
α-Tocopherol[2]	16 ± 1.0^{a}	17 ± 1.0^{a}	17 ± 0.8^{a}	18 ± 1.9^{a}	19 ± 1.3^{a}
Sinapic acid[2]	20 ± 1.1^{bc}	19 ± 1.0^{ab}	19 ± 0.9^{ab}	23 ± 1.4^{a}	29 ± 2.0^{b}
Crude extract	20 ± 1.1^{bc}	25 ± 1.4^{d}	28 ± 1.4^{c}	35 ± 1.9^{c}	50 ± 2.4^{def}
Fraction I	23 ± 1.4^{c}	25 ± 1.4^{d}	28 ± 1.6^{c}	34 ± 2.0^{c}	54 ± 2.6^{f}
Fraction II	23 ± 1.3^{c}	24 ± 1.3^{cd}	29 ± 1.4^{c}	34 ± 2.0^{c}	54 ± 2.9^{f}
Fraction III	22 ± 1.4^{bc}	24 ± 1.2^{cd}	29 ± 1.5^{c}	35 ± 1.8^{c}	53 ± 2.3^{ef}
Fraction IV	19 ± 1.0^{ab}	21 ± 1.0^{bc}	22 ± 1.0^{b}	28 ± 1.6^{b}	38 ± 2.0^{c}
Fraction V	20 ± 1.1^{bc}	22 ± 1.0^{bcd}	26 ± 1.1^{c}	35 ± 2.1^{c}	46 ± 2.4^{d}
Fraction VI	21 ± 1.2^{bc}	24 ± 1.4^{cd}	28 ± 1.3^{c}	35 ± 2.1^{c}	47 ± 2.4^{dc}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.14 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of total volatiles in a cooked comminuted pork model system stored at 4°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	21 ± 1.4^{f}	25 ± 1.5^{fg}	33 ± 1.9^{f}	61 ± 4.0^{e}	61 ± 3.8^{g}
BHA[2]	14 ± 0.7^{a}	14 ± 0.6^{a}	15 ± 0.7^{a}	16 ± 0.8^{a}	16 ± 0.8^{a}
α-Tocopherol[2]	14 ± 0.8^{a}	16 ± 0.8^{ab}	16 ± 0.8^{a}	16 ± 0.8^{a}	17 ± 0.8^{a}
Sinapic acid[2]	17 ± 0.9^{bcd}	18 ± 1.1^{bc}	18 ± 0.9^{ab}	19 ± 1.0^{ab}	21 ± 1.0^{abc}
Crude extract	19 ± 1.0^{def}	20 ± 1.0^{cd}	22 ± 0.9^{cd}	26 ± 1.4^{cd}	42 ± 2.2^{ef}
Fraction I	18 ± 0.7^{cde}	25 ± 1.2^{fg}	25 ± 1.3^{de}	26 ± 1.5^{cd}	38 ± 2.0^{de}
Fraction II	17 ± 0.7^{bcd}	28 ± 1.6^{g}	25 ± 1.3^{de}	27 ± 1.3^{cd}	40 ± 2.0^{e}
Fraction III	14 ± 0.8^{a}	24 ± 1.4^{ef}	26 ± 1.2^{e}	28 ± 1.4^{d}	46 ± 1.9^{f}
Fraction IV	15 ± 0.7^{ab}	20 ± 1.0^{cd}	21 ± 1.1^{bc}	19 ± 0.9^{ab}	25 ± 1.2^{c}
Fraction V	16 ± 0.7^{abc}	21 ± 1.1^{cde}	26 ± 1.4^{e}	23 ± 1.1^{bc}	33 ± 1.7^{d}
Fraction VI	20 ± 1.0^{ef}	23 ± 1.1^{def}	23 ± 1.2^{de}	26 ± 1.3^{cd}	22 ± 1.0^{bc}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.15 Effect of evening primrose additives at a concentration of 100 ppm catechin equivalents on formation of total volatiles in a cooked comminuted pork model system stored at 4°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	21 ± 1.4^{c}	25 ± 1.5^{e}	33 ± 1.9^{f}	61 ± 4.0^{h}	61 ± 3.8^{f}
BHA[2]	16 ± 1.2^{b}	17 ± 1.3^{c}	17 ± 1.0^{cd}	18 ± 1.4^{cd}	18 ± 0.9^{h}
α-Tocopherol[2]	16 ± 1.0^{b}	17 ± 1.0^{c}	17 ± 0.8^{cd}	18 ± 1.9^{bcd}	19 ± 1.3^{h}
Catechin[2]	15 ± 0.6^{b}	17 ± 0.9^{c}	22 ± 1.2^{e}	25 ± 1.4^{ef}	30 ± 2.0^{c}
Crude extract	16 ± 0.6^{b}	20 ± 1.1^{d}	22 ± 1.2^{e}	22 ± 1.4^{de}	61 ± 4.0^{f}
Fraction I	23 ± 2.1^{c}	25 ± 1.4^{e}	32 ± 1.7^{f}	37 ± 1.9^{g}	55 ± 3.6^{e}
Fraction II	10 ± 0.7^{a}	9.3 ± 0.5^{ab}	11 ± 0.7^{ab}	14 ± 0.8^{abc}	35 ± 2.4^{cd}
Fraction III	11 ± 0.5^{a}	11 ± 0.7^{ab}	10 ± 0.6^{ab}	9.6 ± 0.8^{a}	10 ± 0.8^{a}
Fraction IV	11 ± 0.6^{a}	12 ± 0.7^{b}	13 ± 0.7^{b}	13 ± 0.6^{a}	13 ± 1.0^{ab}
Fraction V	9.0 ± 0.4^{a}	8.5 ± 0.6^{a}	8.5 ± 0.4^{a}	9.3 ± 0.6^{a}	9.6 ± 0.6^{a}
Fraction VI	11 ± 0.6^{a}	20 ± 1.0^{d}	20 ± 0.9^{de}	28 ± 1.5^{f}	41 ± 2.2^{d}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.16 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of total volatiles in a cooked comminuted pork model system stored at 4°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	21 ± 1.4^{e}	25 ± 1.5^{g}	33 ± 1.9^{g}	61 ± 4.0^{e}	61 ± 3.8^{h}
BHA[2]	14 ± 0.7^{d}	14 ± 0.6^{f}	15 ± 0.7^{d}	16 ± 0.8^{c}	16 ± 0.8^{cd}
α-Tocopherol[2]	14 ± 0.8^{d}	16 ± 0.8^{f}	16 ± 0.8^{de}	16 ± 0.8^{c}	17 ± 0.8^{d}
Catechin[2]	13 ± 0.8^{d}	15 ± 0.6^{f}	18 ± 1.0^{e}	20 ± 0.9^{c}	24 ± 1.6^{e}
Crude extract	10 ± 0.6^{c}	7.8 ± 0.4^{de}	9.1 ± 0.4^{c}	8.1 ± 0.6^{b}	11 ± 0.7^{bc}
Fraction I	21 ± 1.0^{e}	23 ± 1.3^{g}	28 ± 1.5^{f}	26 ± 1.4^{d}	46 ± 3.2^{g}
Fraction II	7.6 ± 0.4^{b}	5.3 ± 0.2^{bc}	5.4 ± 0.4^{ab}	6.4 ± 0.3^{ab}	28 ± 2.6^{e}
Fraction III	7.2 ± 0.4^{b}	4.3 ± 0.2^{ab}	4.0 ± 0.4^{a}	4.5 ± 0.4^{ab}	6.6 ± 0.5^{ab}
Fraction IV	6.9 ± 0.4^{b}	6.5 ± 0.4^{cd}	6.9 ± 0.5^{bc}	5.5 ± 0.4^{ab}	7.1 ± 0.6^{ab}
Fraction V	3.6 ± 0.2^{a}	3.2 ± 0.2^{a}	3.8 ± 0.2^{a}	3.9 ± 0.2^{a}	4.8 ± 0.4^{a}
Fraction VI	8.7 ± 0.5^{bc}	9.4 ± 0.5^{e}	14 ± 0.8^{d}	18 ± 0.9^{c}	34 ± 2.4^{f}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.17 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of conjugated dienes in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	6.7 ± 0.3^{cd}	14 ± 0.6^{e}	23 ± 1.6^{d}	25 ± 1.4^{e}
BHA[2]	2.3 ± 0.1^{a}	2.5 ± 0.1^{a}	4.3 ± 0.2^{a}	7.4 ± 0.3^{a}	8.7 ± 0.3^{a}
α-Tocopherol[2]	2.3 ± 0.1^{a}	9.4 ± 0.4^{e}	18 ± 1.0^{f}	22 ± 1.3^{d}	23 ± 1.2^{de}
Sinapic acid[2]	2.3 ± 0.2^{a}	2.7 ± 0.2^{a}	8.7 ± 0.3^{b}	15 ± 0.6^{b}	17 ± 0.5^{b}
Crude extract	2.3 ± 0.1^{a}	6.8 ± 0.2^{d}	14 ± 0.5^{e}	23 ± 1.4^{d}	23 ± 1.3^{de}
Fraction I	2.3 ± 0.1^{a}	6.7 ± 0.3^{cd}	12 ± 0.4^{cd}	20 ± 1.3^{cd}	21 ± 1.2^{cd}
Fraction II	2.3 ± 0.1^{a}	5.5 ± 0.2^{b}	11 ± 0.4^{c}	18 ± 0.8^{c}	19 ± 1.0^{bc}
Fraction III	2.3 ± 0.1^{a}	6.6 ± 0.3^{cd}	13 ± 0.3^{de}	20 ± 1.4^{cd}	22 ± 1.2^{d}
Fraction IV	2.3 ± 0.1^{a}	5.7 ± 0.1^{b}	9.6 ± 0.2^{b}	16 ± 0.6^{b}	18 ± 0.8^{b}
Fraction V	2.3 ± 0.1^{a}	6.0 ± 0.4^{bc}	11 ± 0.3^{c}	18 ± 0.7^{c}	19 ± 1.1^{bc}
Fraction VI	2.3 ± 0.1^{a}	5.5 ± 0.2^{b}	8.9 ± 0.2^{b}	15 ± 0.4^{b}	17 ± 0.6^{b}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.18 Effect of the borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of conjugated dienes in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	6.7 ± 0.1^{c}	14 ± 0.6^{g}	23 ± 1.6^{gh}	25 ± 1.4^{g}
BHA[2]	2.3 ± 0.1^{a}	2.4 ± 0.1^{a}	3.9 ± 0.1^{a}	4.3 ± 0.1^{a}	5.2 ± 0.2^{a}
α-Tocopherol[2]	2.3 ± 0.0^{a}	9.5 ± 0.4^{d}	20 ± 1.1^{h}	25 ± 2.0^{h}	25 ± 1.4^{g}
Sinapic acid[2]	2.3 ± 0.1^{a}	2.5 ± 0.1^{a}	4.9 ± 0.1^{ab}	10 ± 0.4^{b}	14 ± 0.7^{b}
Crude extract	2.3 ± 0.1^{a}	4.4 ± 0.1^{b}	11 ± 0.4^{f}	22 ± 1.4^{fg}	23 ± 1.2^{fg}
Fraction I	2.3 ± 0.1^{a}	4.6 ± 0.2^{b}	6.6 ± 0.2^{bcd}	19 ± 0.9^{de}	20 ± 1.2^{de}
Fraction II	2.3 ± 0.1^{a}	4.4 ± 0.1^{b}	7.4 ± 0.3^{cde}	17 ± 0.7^{cd}	20 ± 1.0^{de}
Fraction III	2.3 ± 0.2^{a}	4.4 ± 0.2^{b}	9.3 ± 0.2^{def}	20 ± 0.8^{ef}	21 ± 1.3^{ef}
Fraction IV	2.3 ± 0.0^{a}	4.6 ± 0.2^{b}	6.7 ± 0.3^{bcd}	16 ± 0.6^{c}	17 ± 0.6^{c}
Fraction V	2.3 ± 0.1^{a}	4.4 ± 0.3^{b}	6.7 ± 0.4^{bcd}	16 ± 0.6^{c}	17 ± 0.8^{c}
Fraction VI	2.3 ± 0.1^{a}	4.9 ± 0.1^{b}	5.0 ± 0.1^{abc}	15 ± 0.5^{c}	18 ± 1.0^{cd}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table 4.19 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents on formation of conjugated dienes in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	6.7 ± 0.3^{d}	14 ± 0.6^{de}	23 ± 1.3^{e}	25 ± 1.4^{d}
BHA[2]	2.3 ± 0.1^{a}	2.5 ± 0.1^{a}	4.3 ± 0.2^{a}	7.4 ± 0.3^{a}	8.7 ± 0.3^{a}
α-Tocopherol[2]	2.3 ± 0.1^{a}	9.4 ± 0.4^{e}	18 ± 1.0^{f}	22 ± 1.3^{de}	23 ± 1.2^{cd}
Catechin[2]	2.3 ± 0.1^{a}	4.8 ± 0.2^{b}	9.2 ± 0.3^{b}	16 ± 0.8^{b}	18 ± 0.3^{b}
Crude extract	2.3 ± 0.1^{a}	5.2 ± 0.1^{b}	14 ± 0.6^{de}	20 ± 1.3^{cd}	21 ± 1.4^{bc}
Fraction I	2.3 ± 0.1^{a}	5.9 ± 0.2^{c}	15 ± 0.6^{e}	20 ± 1.1^{cd}	21 ± 1.2^{bc}
Fraction II	2.3 ± 0.1^{a}	6.1 ± 0.1^{c}	15 ± 0.4^{e}	21 ± 1.3^{de}	22 ± 1.3^{cd}
Fraction III	2.3 ± 0.1^{a}	4.8 ± 0.2^{b}	10 ± 0.2^{b}	16 ± 0.8^{b}	19 ± 1.0^{bc}
Fraction IV	2.3 ± 0.1^{a}	6.4 ± 0.2^{cd}	14 ± 0.7^{de}	20 ± 0.9^{cd}	22 ± 1.3^{cd}
Fraction V	2.3 ± 0.1^{a}	6.1 ± 0.2^{c}	13 ± 0.3^{d}	18 ± 0.8^{bc}	20 ± 1.4^{bc}
Fraction VI	2.3 ± 0.1^{a}	5.1 ± 0.1^{b}	11 ± 0.2^{c}	16 ± 0.6^{b}	18 ± 0.8^{b}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.20 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of conjugated dienes in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1[a]	6.7 ± 0.3[d]	14 ± 0.6[d]	23 ± 0.6[gh]	25 ± 1.4[d]
BHA[2]	2.3 ± 0.1[a]	2.4 ± 0.1[a]	3.9 ± 0.1[a]	4.3 ± 0.1[a]	5.2 ± 0.1[a]
α-Tocopherol[2]	2.3 ± 0.1[a]	9.5 ± 0.4[e]	20 ± 1.1[e]	25 ± 2.0[h]	25 ± 1.4[d]
Catechin[2]	2.3 ± 0.1[a]	4.5 ± 0.2[b]	7.6 ± 0.3[b]	13 ± 0.2[b]	16 ± 0.8[b]
Crude extract	2.3 ± 0.1[a]	4.4 ± 0.2[b]	8.8 ± 0.2[b]	18 ± 0.8[dc]	20 ± 1.4[c]
Fraction I	2.3 ± 0.1[a]	6.5 ± 0.2[d]	15 ± 0.6[d]	21 ± 0.7[fg]	21 ± 1.4[c]
Fraction II	2.3 ± 0.1[a]	4.7 ± 0.1[b]	8.9 ± 0.2[b]	15 ± 0.4[bc]	19 ± 1.1[c]
Fraction III	2.3 ± 0.1[a]	4.5 ± 0.2[b]	7.8 ± 0.3[b]	14 ± 0.3[b]	16 ± 0.8[b]
Fraction IV	2.3 ± 0.1[a]	6.3 ± 0.2[d]	14 ± 0.4[d]	20 ± 1.3[ef]	21 ± 0.9[c]
Fraction V	2.3 ± 0.1[a]	5.5 ± 0.2[c]	11 ± 0.2[c]	17 ± 0.8[cd]	20 ± 0.8[c]
Fraction VI	2.3 ± 0.1[a]	4.6 ± 0.1[b]	7.7 ± 0.2[b]	13 ± 0.3[b]	16 ± 0.4[b]

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.21 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of hexanal in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.2 ± 0.1^{ab}	24 ± 1.2^{e}	44 ± 3.1^{g}	88 ± 6.0^{f}	107 ± 8.0^{g}
BHA[2]	1.0 ± 0.0^{a}	1.0 ± 0.1^{a}	3.8 ± 0.3^{ab}	6.4 ± 0.4^{a}	13 ± 0.7^{a}
α-Tocopherol[2]	1.0 ± 0.1^{ab}	20 ± 1.0^{d}	36 ± 2.0^{f}	42 ± 3.4^{e}	64 ± 5.1^{f}
Sinapic acid[2]	1.0 ± 0.1^{a}	3.0 ± 0.2^{b}	8.1 ± 0.4^{cd}	16 ± 1.3^{b}	42 ± 4.0^{c}
Crude extract	1.0 ± 0.1^{a}	7.3 ± 0.4^{c}	9.8 ± 0.5^{de}	23 ± 1.2^{c}	57 ± 4.1^{f}
Fraction I	1.3 ± 0.1^{b}	1.5 ± 0.1^{a}	13 ± 0.5^{de}	31 ± 1.3^{d}	37 ± 3.6^{de}
Fraction II	1.4 ± 0.2^{b}	1.9 ± 0.2^{ab}	6.9 ± 0.3^{bcd}	17 ± 1.0^{bc}	26 ± 2.0^{bc}
Fraction III	1.2 ± 0.2^{ab}	1.8 ± 0.1^{a}	4.1 ± 0.2^{ab}	21 ± 1.1^{bc}	30 ± 3.1^{cd}
Fraction IV	1.4 ± 0.1^{b}	1.6 ± 0.1^{a}	2.1 ± 0.1^{a}	7.0 ± 0.6^{a}	21 ± 2.1^{abc}
Fraction V	1.2 ± 0.1^{ab}	1.0 ± 0.1^{a}	6.2 ± 0.3^{bc}	20 ± 1.1^{bc}	18 ± 1.1^{ab}
Fraction VI	1.3 ± 0.1^{b}	1.4 ± 0.2^{a}	5.2 ± 0.2^{abc}	17 ± 0.9^{bc}	17 ± 1.1^{ab}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.22 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of hexanal in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.1 ± 0.1^{a}	24 ± 1.2^{e}	44 ± 3.1^{d}	88 ± 6.0^{e}	107 ± 8.0^{g}
BHA[2]	1.0 ± 0.0^{a}	1.5 ± 0.1^{ab}	4.1 ± 0.3^{a}	7.2 ± 0.6^{a}	14 ± 0.9^{abc}
α-Tocopherol[2]	1.1 ± 0.1^{a}	15 ± 0.8^{d}	19 ± 1.1^{c}	26 ± 2.1^{c}	37 ± 3.2^{e}
Sinapic acid[2]	1.2 ± 0.1^{a}	2.1 ± 0.1^{ab}	2.6 ± 0.1^{a}	10 ± 0.5^{ab}	34 ± 3.0^{e}
Crude extract	1.0 ± 0.1^{a}	7.1 ± 0.5^{c}	9.0 ± 0.4^{b}	34 ± 3.0^{d}	54 ± 4.1^{f}
Fraction I	1.1 ± 0.0^{a}	1.0 ± 0.0^{a}	10 ± 0.5^{b}	30 ± 2.6^{cd}	31 ± 2.6^{d}
Fraction II	1.0 ± 0.1^{a}	1.2 ± 0.0^{ab}	3.9 ± 0.2^{a}	14 ± 0.9^{b}	23 ± 2.1^{cd}
Fraction III	1.3 ± 0.1^{a}	2.3 ± 0.1^{b}	2.6 ± 0.1^{a}	14 ± 0.9^{b}	20 ± 1.4^{bc}
Fraction IV	1.2 ± 0.1^{a}	0.94 ± 0.13^{a}	4.0 ± 0.1^{a}	13 ± 0.6^{ab}	12 ± 0.6^{ab}
Fraction V	1.2 ± 0.1^{a}	1.2 ± 0.1^{ab}	3.4 ± 0.1^{a}	11 ± 0.5^{ab}	12 ± 0.7^{ab}
Fraction VI	1.2 ± 0.1^{a}	2.4 ± 0.1^{b}	2.3 ± 0.1^{a}	14 ± 0.8^{b}	10 ± 0.5^{a}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.23 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents on formation of hexanal in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.2 ± 0.1^{ab}	24 ± 1.2^{f}	44 ± 3.1^{h}	88 ± 6.0^{g}	107 ± 8.0^{f}
BHA[2]	0.96 ± 0.04^{a}	1.0 ± 0.1^{a}	3.8 ± 0.3^{ab}	6.4 ± 0.4^{a}	13 ± 0.7^{a}
α-Tocopherol[2]	1.0 ± 0.1^{a}	20 ± 1.0^{e}	36 ± 2.0^{g}	42 ± 3.4^{f}	64 ± 5.1^{de}
Catechin[2]	1.1 ± 0.1^{a}	1.2 ± 0.1^{a}	4.0 ± 0.6^{ab}	8.9 ± 0.5^{a}	45 ± 3.1^{bc}
Crude extract	1.2 ± 0.1^{ab}	7.6 ± 0.4^{b}	19 ± 0.9^{d}	21 ± 1.1^{cd}	70 ± 4.7^{e}
Fraction I	1.1 ± 0.2^{a}	13 ± 0.7^{c}	25 ± 1.4^{e}	31 ± 1.4^{e}	110 ± 9.6^{f}
Fraction II	1.2 ± 0.1^{ab}	17 ± 0.7^{d}	31 ± 2.3^{f}	40 ± 3.1^{f}	135 ± 11^{g}
Fraction III	1.0 ± 0.1^{a}	21 ± 1.1^{e}	27 ± 1.6^{ef}	29 ± 1.7^{e}	48 ± 3.0^{cd}
Fraction IV	1.1 ± 0.2^{a}	8.9 ± 0.6^{b}	8.9 ± 0.6^{c}	28 ± 1.7^{de}	100 ± 7.4^{f}
Fraction V	1.4 ± 0.1^{b}	1.5 ± 0.1^{a}	2.4 ± 0.1^{a}	11 ± 0.5^{ab}	106 ± 8.1^{f}
Fraction VI	1.1 ± 0.1^{a}	6.9 ± 0.4^{b}	7.9 ± 0.6^{bc}	18 ± 0.8^{bc}	28 ± 1.7^{ab}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.24 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents) on formation of hexanal in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.1 ± 0.1^{a}	24 ± 1.2^{g}	44 ± 3.1^{f}	88 ± 6.0^{e}	107 ± 8.0^{e}
BHA[2]	1.0 ± 0.0^{a}	1.5 ± 0.1^{a}	4.1 ± 0.3^{ab}	7.2 ± 0.6^{a}	14 ± 0.9^{a}
α-Tocopherol[2]	1.1 ± 0.1^{a}	15 ± 0.8^{f}	19 ± 1.1^{e}	26 ± 2.1^{c}	37 ± 3.2^{bc}
Catechin[2]	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	2.4 ± 0.1^{a}	5.6 ± 0.3^{a}	24 ± 1.5^{ab}
Crude extract	1.0 ± 0.0^{a}	1.4 ± 0.1^{a}	9.0 ± 0.6^{c}	11 ± 0.5^{ab}	60 ± 4.1^{d}
Fraction I	1.1 ± 0.1^{a}	8.4 ± 0.4^{d}	8.4 ± 0.6^{c}	36 ± 2.4^{d}	142 ± 11^{f}
Fraction II	1.2 ± 0.1^{a}	3.7 ± 0.2^{b}	9.4 ± 0.5^{c}	14 ± 0.6^{b}	26 ± 1.5^{ab}
Fraction III	1.1 ± 0.1^{a}	13 ± 0.5^{e}	13 ± 0.8^{d}	24 ± 1.5^{c}	37 ± 2.8^{bc}
Fraction IV	1.1 ± 0.1^{a}	7.5 ± 0.3^{cd}	6.3 ± 0.4^{bc}	38 ± 1.8^{d}	45 ± 3.1^{c}
Fraction V	1.1 ± 0.2^{a}	6.6 ± 0.3^{c}	6.7 ± 0.5^{bc}	16 ± 0.7^{b}	40 ± 3.5^{c}
Fraction VI	1.3 ± 0.1^{a}	3.3 ± 0.2^{b}	7.1 ± 0.4^{bc}	14 ± 0.5^{b}	25 ± 1.5^{ab}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.25 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of total volatiles in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	25 ± 1.5^{a}	54 ± 4.5^{ef}	90 ± 8.4^{e}	125 ± 10^{f}	247 ± 20^{f}
BHA[2]	25 ± 1.4^{a}	18 ± 2.1^{a}	21 ± 1.6^{a}	30 ± 2.5^{a}	38 ± 2.5^{a}
α-Tocopherol[2]	25 ± 1.6^{a}	40 ± 4.0^{cd}	60 ± 5.4^{bc}	77 ± 5.6^{de}	80 ± 6.1^{cd}
Sinapic acid[2]	24 ± 1.5^{a}	49 ± 4.2^{de}	50 ± 4.1^{b}	64 ± 4.6^{bcd}	105 ± 8.3^{d}
Crude extract	24 ± 1.3^{a}	36 ± 4.0^{c}	35 ± 3.1^{a}	89 ± 6.4^{e}	180 ± 14^{e}
Fraction I	24 ± 1.6^{a}	63 ± 5.1^{f}	74 ± 6.1^{cd}	73 ± 6.1^{de}	73 ± 5.5^{c}
Fraction II	25 ± 1.1^{a}	30 ± 2.8^{bc}	50 ± 3.2^{b}	65 ± 5.2^{cd}	67 ± 5.0^{bc}
Fraction III	24 ± 1.5^{a}	35 ± 3.0^{c}	26 ± 1.5^{a}	84 ± 6.2^{e}	90 ± 6.1^{cd}
Fraction IV	25 ± 1.3^{a}	40 ± 3.5^{cd}	89 ± 6.3^{e}	87 ± 5.5^{e}	47 ± 3.1^{ab}
Fraction V	26 ± 1.5^{a}	16 ± 1.4^{a}	27 ± 1.7^{a}	55 ± 4.1^{bc}	73 ± 6.1^{c}
Fraction VI	23 ± 1.2^{a}	19 ± 1.4^{ab}	82 ± 7.0^{de}	47 ± 3.8^{ab}	47 ± 2.8^{ab}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.26 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents) on formation of total volatiles in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	25 ± 1.5^{a}	54 ± 4.5^{f}	90 ± 8.4^{d}	125 ± 10^{e}	247 ± 20^{e}
BHA[2]	24 ± 1.4^{a}	14 ± 0.8^{a}	19 ± 1.5^{a}	26 ± 1.7^{a}	30 ± 2.1^{a}
α-Tocopherol[2]	25 ± 1.5^{a}	25 ± 1.4^{bc}	39 ± 2.6^{b}	52 ± 3.4^{b}	64 ± 4.8^{bc}
Sinapic acid[2]	25 ± 1.1^{a}	36 ± 2.5^{e}	44 ± 3.0^{b}	51 ± 3.1^{b}	79 ± 5.1^{c}
Crude extract	26 ± 1.2^{a}	28 ± 2.0^{cd}	77 ± 4.6^{cd}	60 ± 4.0^{bc}	127 ± 10^{d}
Fraction I	25 ± 1.4^{a}	33 ± 2.0^{de}	35 ± 2.8^{b}	81 ± 5.9^{d}	60 ± 3.8^{bc}
Fraction II	25 ± 1.6^{a}	55 ± 3.7^{f}	88 ± 6.4^{d}	51 ± 3.9^{b}	61 ± 3.0^{bc}
Fraction III	25 ± 1.5^{a}	25 ± 1.6^{bc}	78 ± 6.0^{d}	77 ± 5.4^{d}	33 ± 1.8^{a}
Fraction IV	26 ± 1.5^{a}	33 ± 2.4^{de}	64 ± 4.1^{c}	58 ± 3.6^{b}	33 ± 2.2^{a}
Fraction V	23 ± 1.2^{a}	16 ± 1.0^{a}	83 ± 6.1^{d}	83 ± 6.4^{d}	48 ± 2.8^{ab}
Fraction VI	26 ± 1.6^{a}	20 ± 1.2^{ab}	80 ± 5.7^{d}	74 ± 5.9^{cd}	32 ± 1.7^{a}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.27 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents) on formation of total volatiles in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	25 ± 1.5^{a}	54 ± 4.5^{d}	90 ± 8.4^{c}	125 ± 10^{d}	247 ± 20^{e}
BHA[2]	25 ± 1.4^{a}	18 ± 2.1^{a}	21 ± 1.6^{a}	30 ± 2.5^{a}	38 ± 2.5^{a}
α-Tocopherol[2]	25 ± 1.6^{a}	40 ± 4.0^{c}	60 ± 5.4^{b}	77 ± 5.6^{c}	80 ± 6.1^{bc}
Catechin[2]	24 ± 1.5^{a}	28 ± 1.6^{b}	30 ± 2.4^{a}	34 ± 2.6^{a}	43 ± 3.1^{ab}
Crude extract	26 ± 1.5^{a}	33 ± 2.0^{bc}	63 ± 4.2^{b}	81 ± 6.2^{c}	140 ± 12^{d}
Fraction I	24 ± 1.5^{a}	40 ± 2.4^{c}	71 ± 4.7^{b}	70 ± 4.6^{c}	240 ± 20^{e}
Fraction II	25 ± 1.4^{a}	36 ± 2.1^{bc}	73 ± 5.2^{b}	120 ± 10^{d}	260 ± 21^{e}
Fraction III	27 ± 1.6^{a}	52 ± 3.6^{d}	59 ± 3.4^{b}	112 ± 8.6^{d}	116 ± 10^{cd}
Fraction IV	26 ± 1.5^{a}	50 ± 3.5^{d}	90 ± 7.4^{c}	81 ± 6.6^{c}	250 ± 21^{e}
Fraction V	25 ± 1.4^{a}	53 ± 3.1^{d}	30 ± 2.7^{a}	49 ± 3.6^{b}	224 ± 19^{e}
Fraction VI	24 ± 1.3^{a}	53 ± 2.9^{d}	110 ± 8.6^{d}	78 ± 5.4^{c}	86 ± 6.4^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.28 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents) on formation of total volatiles in a bulk stripped corn oil model system stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	25 ± 1.5^{a}	54 ± 4.5^{e}	90 ± 8.4^{e}	125 ± 10^{e}	247 ± 20^{e}
BHA[2]	24 ± 1.4^{a}	14 ± 0.8^{a}	19 ± 1.5^{a}	26 ± 1.7^{a}	30 ± 2.1^{a}
α-Tocopherol[2]	25 ± 1.5^{a}	25 ± 1.4^{bc}	39 ± 2.6^{bc}	52 ± 3.4^{b}	64 ± 4.8^{b}
Catechin[2]	24 ± 1.6^{a}	22 ± 1.2^{b}	25 ± 1.6^{a}	28 ± 1.8^{a}	34 ± 2.5^{a}
Crude extract	23 ± 1.3^{a}	30 ± 1.9^{cd}	45 ± 3.1^{c}	54 ± 3.8^{b}	90 ± 7.2^{bc}
Fraction I	26 ± 1.6^{a}	30 ± 1.7^{cd}	47 ± 3.3^{c}	52 ± 3.7^{b}	165 ± 13^{d}
Fraction II	26 ± 1.6^{a}	33 ± 2.1^{d}	29 ± 1.6^{ab}	96 ± 7.5^{d}	96 ± 6.4^{c}
Fraction III	24 ± 1.6^{a}	53 ± 3.8^{e}	93 ± 7.1^{e}	100 ± 8.0^{d}	94 ± 6.0^{c}
Fraction IV	23 ± 1.7^{a}	30 ± 2.5^{cd}	75 ± 6.1^{d}	113 ± 9.6^{de}	148 ± 13^{d}
Fraction V	24 ± 1.6^{a}	55 ± 4.0^{e}	65 ± 5.0^{d}	66 ± 4.9^{bc}	98 ± 7.2^{c}
Fraction VI	25 ± 1.4^{a}	33 ± 2.1^{d}	50 ± 3.3^{c}	75 ± 5.9^{c}	72 ± 5.6^{bc}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.29 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of conjugated dienes in a stripped corn oil-in-water emulsion system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	2.9 ± 0.1^{e}	17 ± 0.6^{f}	25 ± 1.4^{g}	27 ± 1.4^{e}
BHA[2]	2.3 ± 0.1^{a}	2.2 ± 0.1^{a}	2.6 ± 0.2^{a}	3.4 ± 0.2^{a}	7.8 ± 0.4^{a}
α-Tocopherol[2]	2.2 ± 0.1^{a}	2.3 ± 0.2^{ab}	4.3 ± 0.4^{c}	12 ± 0.4^{c}	20 ± 1.0^{d}
Sinapic acid[2]	2.3 ± 0.1^{a}	2.7 ± 0.1^{de}	13 ± 0.2^{e}	15 ± 0.3^{de}	25 ± 1.0^{e}
Crude extract	2.3 ± 0.1^{a}	2.2 ± 0.1^{a}	3.5 ± 0.3^{bc}	6.1 ± 0.3^{b}	12 ± 0.9^{b}
Fraction I	2.2 ± 0.1^{a}	2.4 ± 0.1^{abc}	3.0 ± 0.2^{ab}	13 ± 0.8^{cd}	14 ± 1.0^{bc}
Fraction II	2.2 ± 0.1^{a}	2.5 ± 0.1^{bcd}	2.6 ± 0.1^{a}	12 ± 0.8^{c}	16 ± 1.0^{c}
Fraction III	2.3 ± 0.1^{a}	2.6 ± 0.1^{cd}	2.7 ± 0.1^{ab}	11 ± 0.6^{c}	24 ± 1.0^{e}
Fraction IV	2.3 ± 0.1^{a}	2.4 ± 0.1^{abc}	2.6 ± 0.1^{a}	15 ± 0.9^{de}	26 ± 1.4^{e}
Fraction V	2.3 ± 0.1^{a}	2.6 ± 0.1^{cd}	7.2 ± 0.4^{d}	17 ± 1.1^{e}	26 ± 1.1^{e}
Fraction VI	2.2 ± 0.1^{a}	2.7 ± 0.1^{de}	13 ± 0.4^{e}	21 ± 1.0^{f}	27 ± 1.4^{e}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.30 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of conjugated dienes in a stripped corn oil-in-water emulsion system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	2.9 ± 0.1^{c}	17 ± 0.6^{d}	25 ± 1.4^{e}	27 ± 1.4^{e}
BHA[2]	2.3 ± 0.1^{a}	2.4 ± 0.1^{ab}	2.5 ± 0.1^{a}	3.0 ± 0.1^{a}	6.2 ± 0.3^{a}
α-Tocopherol[2]	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}	3.2 ± 0.3^{a}	10 ± 0.4^{b}	16 ± 0.9^{b}
Sinapic acid[2]	2.3 ± 0.1^{a}	2.4 ± 0.0^{ab}	12 ± 0.7^{c}	16 ± 1.1^{c}	20 ± 0.9^{c}
Crude extract	2.3 ± 0.1^{a}	2.3 ± 0.1^{ab}	3.1 ± 0.3^{a}	5.3 ± 0.3^{a}	16 ± 0.4^{b}
Fraction I	2.3 ± 0.1^{a}	2.5 ± 0.1^{b}	2.7 ± 0.3^{a}	10 ± 0.8^{b}	15 ± 0.8^{b}
Fraction II	2.3 ± 0.1^{a}	2.4 ± 0.1^{ab}	2.4 ± 0.2^{a}	11 ± 0.6^{b}	17 ± 1.0^{b}
Fraction III	2.3 ± 0.1^{a}	2.4 ± 0.1^{ab}	2.5 ± 0.1^{a}	10 ± 0.7^{b}	20 ± 0.8^{c}
Fraction IV	2.3 ± 0.1^{a}	2.4 ± 0.1^{ab}	2.4 ± 0.1^{a}	17 ± 1.0^{c}	23 ± 1.1^{d}
Fraction V	2.3 ± 0.1^{a}	2.5 ± 0.0^{b}	8.6 ± 0.3^{b}	17 ± 1.1^{c}	23 ± 1.0^{d}
Fraction VI	2.2 ± 0.1^{a}	2.8 ± 0.1^{c}	12 ± 0.7^{c}	21 ± 1.2^{d}	26 ± 1.0^{e}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.31 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents on formation of conjugated dienes in a stripped corn oil-in-water emulsion system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	2.9 ± 0.1^{c}	17 ± 0.6^{f}	25 ± 1.4^{f}	27 ± 1.4^{f}
BHA[2]	2.3 ± 0.1^{a}	2.2 ± 0.1^{a}	2.6 ± 0.2^{a}	3.4 ± 0.2^{a}	7.8 ± 0.4^{a}
α-Tocopherol[2]	2.2 ± 0.1^{a}	2.3 ± 0.2^{ab}	4.3 ± 0.4^{bc}	12 ± 0.4^{c}	20 ± 1.0^{d}
Catechin[2]	2.3 ± 0.1^{a}	2.4 ± 0.2^{ab}	5.8 ± 0.6^{d}	15 ± 1.2^{d}	25 ± 1.4^{ef}
Crude extract	2.3 ± 0.1^{a}	2.4 ± 0.1^{ab}	2.7 ± 0.2^{ab}	4.3 ± 0.3^{a}	10 ± 0.4^{ab}
Fraction I	2.3 ± 0.1^{a}	2.3 ± 0.1^{ab}	2.7 ± 0.1^{ab}	4.9 ± 0.4^{a}	11 ± 0.3^{b}
Fraction II	2.3 ± 0.2^{a}	2.4 ± 0.1^{ab}	2.8 ± 0.2^{ab}	7.8 ± 0.8^{b}	12 ± 0.9^{bc}
Fraction III	2.2 ± 0.1^{a}	2.4 ± 0.2^{ab}	3.3 ± 0.2^{abc}	11 ± 0.5^{c}	14 ± 0.7^{c}
Fraction IV	2.3 ± 0.2^{a}	2.4 ± 0.2^{ab}	4.6 ± 0.3^{cd}	12 ± 1.0^{c}	19 ± 1.0^{d}
Fraction V	2.3 ± 0.1^{a}	2.5 ± 0.1^{ab}	5.8 ± 0.7^{d}	15 ± 1.2^{d}	18 ± 1.1^{d}
Fraction VI	2.2 ± 0.1^{a}	2.6 ± 0.2^{bc}	9.7 ± 0.9^{e}	22 ± 1.3^{e}	24 ± 1.4^{e}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.32 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of conjugated dienes in a stripped corn oil-in-water emulsion system stored at 60°C[1].

Additive	Conjugated diene value				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	2.3 ± 0.1^{a}	2.9 ± 0.1^{b}	17 ± 0.6^{c}	25 ± 1.4^{f}	27 ± 1.4^{i}
BHA[2]	2.3 ± 0.1^{a}	2.4 ± 0.1^{a}	2.5 ± 0.1^{a}	3.0 ± 0.1^{a}	6.2 ± 0.3^{a}
α-Tocopherol[2]	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}	3.2 ± 0.3^{a}	10 ± 0.4^{d}	16 ± 0.9^{g}
Catechin[2]	2.3 ± 0.1^{a}	2.6 ± 0.2^{ab}	5.5 ± 0.9^{b}	13 ± 0.7^{e}	20 ± 1.2^{h}
Crude extract	2.3 ± 0.2^{a}	2.3 ± 0.2^{a}	2.5 ± 0.1^{a}	4.3 ± 0.4^{ab}	8.1 ± 0.2^{ah}
Fraction I	2.3 ± 0.1^{a}	2.3 ± 0.1^{a}	2.6 ± 0.2^{a}	4.3 ± 0.3^{ah}	11 ± 0.4^{cd}
Fraction II	2.3 ± 0.1^{a}	2.4 ± 0.2^{a}	2.6 ± 0.2^{a}	4.5 ± 0.3^{ah}	10 ± 0.4^{bc}
Fraction III	2.3 ± 0.1^{a}	2.3 ± 0.2^{a}	2.5 ± 0.2^{a}	4.7 ± 0.4^{b}	12 ± 0.3^{cde}
Fraction IV	2.3 ± 0.2^{a}	2.3 ± 0.1^{a}	2.6 ± 0.1^{a}	6.2 ± 0.4^{c}	13 ± 0.8^{def}
Fraction V	2.3 ± 0.1^{a}	2.3 ± 0.1^{a}	2.6 ± 0.2^{a}	7.3 ± 0.4^{c}	14 ± 0.7^{edf}
Fraction VI	2.3 ± 0.1^{a}	2.4 ± 0.2^{a}	2.7 ± 0.2^{a}	10 ± 0.4^{d}	15 ± 0.6^{fg}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.33 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of hexanal in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.2 ± 0.1^{ab}	21 ± 1.6^{g}	39 ± 3.2^{h}	80 ± 6.2^{e}	96 ± 6.5^{h}
BHA[2]	1.0 ± 0.1^{a}	1.2 ± 0.1^{a}	4.2 ± 0.3^{a}	5.6 ± 0.5^{a}	7.4 ± 0.4^{ab}
α-Tocopherol[2]	1.1 ± 0.1^{ab}	7.4 ± 0.5^{c}	14 ± 0.7^{cde}	20 ± 1.0^{b}	25 ± 1.6^{c}
Sinapic acid[2]	1.0 ± 0.1^{a}	15 ± 0.8^{e}	22 ± 1.2^{f}	35 ± 2.7^{c}	58 ± 3.7^{f}
Crude extract	1.1 ± 0.1^{ab}	18 ± 0.7^{f}	26 ± 1.5^{g}	44 ± 3.2^{d}	78 ± 5.3^{g}
Fraction I	1.0 ± 0.1^{ab}	4.4 ± 0.3^{b}	7.9 ± 0.4^{b}	6.9 ± 0.5^{a}	6.1 ± 0.4^{a}
Fraction II	1.3 ± 0.1^{ab}	1.3 ± 0.1^{a}	3.1 ± 0.2^{a}	5.2 ± 0.4^{a}	8.3 ± 0.5^{abc}
Fraction III	1.0 ± 0.1^{ab}	11 ± 0.5^{d}	15 ± 0.6^{de}	14 ± 0.8^{b}	17 ± 0.8^{de}
Fraction IV	1.3 ± 0.1^{ab}	22 ± 1.1^{g}	16 ± 0.8^{e}	17 ± 0.8^{b}	16 ± 0.7^{cd}
Fraction V	1.3 ± 0.1^{b}	7.3 ± 0.4^{c}	11 ± 0.5^{bc}	19 ± 0.9^{b}	13 ± 0.5^{abcd}
Fraction VI	1.2 ± 0.1^{ab}	8.9 ± 0.5^{cd}	12 ± 0.6^{cd}	20 ± 1.3^{b}	15 ± 0.7^{bcd}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.34 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of hexanal in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.2 ± 0.1^{a}	21 ± 1.6^{e}	39 ± 3.2^{g}	80 ± 6.2^{g}	96 ± 6.5^{f}
BHA[2]	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.6 ± 0.2^{a}	4.3 ± 0.4^{a}	6.0 ± 0.3^{a}
α-Tocopherol[2]	1.1 ± 0.1^{a}	6.0 ± 0.5^{b}	10 ± 0.4^{d}	18 ± 1.1^{de}	18 ± 0.9^{c}
Sinapic acid[2]	1.4 ± 0.1^{a}	10 ± 0.6^{c}	14 ± 0.7^{ef}	20 ± 1.1^{ef}	40 ± 2.8^{d}
Crude extract	1.3 ± 0.1^{a}	13 ± 0.7^{d}	16 ± 0.8^{f}	25 ± 1.4^{f}	66 ± 4.5^{e}
Fraction I	1.2 ± 0.1^{a}	4.5 ± 0.3^{b}	5.3 ± 0.3^{bc}	5.9 ± 0.3^{ab}	5.3 ± 0.3^{a}
Fraction II	1.3 ± 0.1^{a}	1.5 ± 0.1^{a}	2.9 ± 0.3^{ab}	4.4 ± 0.3^{a}	7.1 ± 0.4^{a}
Fraction III	1.1 ± 0.1^{a}	11 ± 0.6^{c}	13 ± 0.5^{def}	11 ± 0.6^{bc}	18 ± 0.7^{c}
Fraction IV	1.3 ± 0.2^{a}	11 ± 0.5^{c}	14 ± 0.6^{ef}	13 ± 0.5^{cd}	10 ± 0.5^{ab}
Fraction V	1.1 ± 0.1^{a}	2.5 ± 0.2^{a}	6.7 ± 0.4^{c}	8.5 ± 0.5^{abc}	11 ± 0.5^{abc}
Fraction VI	1.3 ± 0.1^{a}	5.7 ± 0.4^{b}	11 ± 0.5^{de}	10 ± 0.6^{abc}	16 ± 0.6^{bc}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.35 Effect of evening primrose additives at a concentration of 100 ppm as catechin equivalents on formation of hexanal in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.2 ± 0.1^{a}	21 ± 1.6^{f}	39 ± 3.2^{g}	80 ± 6.2^{g}	96 ± 6.5^{f}
BHA[2]	1.0 ± 0.1^{a}	1.2 ± 0.1^{a}	4.2 ± 0.3^{ab}	5.6 ± 0.5^{a}	7.4 ± 0.4^{a}
α-Tocopherol[2]	1.1 ± 0.1^{a}	7.4 ± 0.5^{e}	14 ± 0.7^{e}	20 ± 1.0^{f}	25 ± 1.6^{bcd}
Catechin[2]	1.2 ± 0.1^{a}	1.3 ± 0.2^{a}	2.5 ± 0.3^{a}	7.9 ± 0.5^{ab}	30 ± 2.1^{cd}
Crude extract	1.2 ± 0.1^{a}	6.2 ± 0.5^{e}	18 ± 0.7^{f}	19 ± 1.0^{f}	66 ± 4.4^{e}
Fraction I	1.1 ± 0.1^{a}	4.5 ± 0.4^{cd}	8.9 ± 0.5^{cd}	14 ± 0.7^{cde}	29 ± 1.6^{bcd}
Fraction II	1.2 ± 0.1^{a}	3.0 ± 0.2^{bc}	7.7 ± 0.5^{c}	19 ± 0.8^{ef}	29 ± 1.6^{bcd}
Fraction III	1.1 ± 0.2^{a}	2.4 ± 0.2^{ab}	11 ± 0.6^{de}	12 ± 0.5^{bcd}	24 ± 1.6^{bc}
Fraction IV	1.0 ± 0.1^{a}	4.6 ± 0.2^{d}	13 ± 0.7^{de}	16 ± 0.6^{def}	33 ± 1.8^{d}
Fraction V	1.3 ± 0.1^{a}	3.5 ± 0.2^{bcd}	6.1 ± 0.4^{bc}	9.5 ± 0.5^{abc}	21 ± 1.1^{b}
Fraction VI	1.1 ± 0.1^{a}	2.1 ± 0.1^{ab}	3.7 ± 0.4^{ab}	10 ± 0.5^{abc}	31 ± 1.9^{cd}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.36 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of hexanal in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Hexanal (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	1.2 ± 0.1^{a}	21 ± 1.6^{e}	39 ± 3.2^{g}	80 ± 6.2^{e}	96 ± 6.5^{d}
BHA[2]	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.6 ± 0.2^{a}	4.3 ± 0.4^{a}	6.0 ± 0.3^{a}
α-Tocopherol[2]	1.1 ± 0.1^{a}	6.0 ± 0.5^{d}	10 ± 0.4^{f}	18 ± 1.1^{cd}	18 ± 0.9^{b}
Catechin[2]	1.1 ± 0.1^{a}	1.3 ± 0.1^{ab}	2.0 ± 0.1^{ab}	3.4 ± 0.2^{a}	20 ± 0.8^{b}
Crude extract	1.1 ± 0.1^{a}	1.2 ± 0.1^{ab}	5.6 ± 0.5^{cde}	11 ± 0.6^{abc}	33 ± 2.0^{c}
Fraction I	1.2 ± 0.1^{a}	5.4 ± 0.3^{d}	7.6 ± 0.6^{ef}	21 ± 1.1^{d}	33 ± 1.6^{c}
Fraction II	1.1 ± 0.1^{a}	2.7 ± 0.2^{bc}	4.9 ± 0.4^{bcd}	16 ± 0.8^{bcd}	24 ± 1.2^{b}
Fraction III	1.1 ± 0.1^{a}	3.0 ± 0.3^{c}	3.9 ± 0.3^{abcd}	9.5 ± 0.6^{ab}	17 ± 0.7^{b}
Fraction IV	1.0 ± 0.1^{a}	2.4 ± 0.2^{abc}	5.2 ± 0.4^{cde}	13 ± 0.8^{bc}	21 ± 0.8^{b}
Fraction V	1.2 ± 0.1^{a}	2.1 ± 0.2^{abc}	6.6 ± 0.5^{de}	11 ± 0.6^{abc}	19 ± 0.8^{b}
Fraction VI	1.1 ± 0.1^{a}	1.9 ± 0.2^{abc}	2.9 ± 0.2^{abc}	9.4 ± 0.7^{ab}	16 ± 0.6^{b}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.37 Effect of borage additives at a concentration of 100 ppm as sinapic acid equivalents on formation of total volatiles in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	27 ± 1.6^{a}	48 ± 2.6^{cd}	84 ± 6.3^{cd}	118 ± 9.5^{de}	225 ± 19^{e}
BHA[2]	25 ± 1.5^{a}	18 ± 0.8^{a}	17 ± 0.9^{a}	18 ± 0.8^{a}	26 ± 1.5^{a}
α-Tocopherol[2]	25 ± 1.7^{a}	30 ± 1.8^{b}	43 ± 2.8^{b}	76 ± 4.6^{bc}	84 ± 6.0^{b}
Sinapic acid[2]	25 ± 1.5^{a}	38 ± 2.0^{bc}	74 ± 5.6^{c}	88 ± 6.4^{c}	170 ± 14^{d}
Crude extract	26 ± 1.5^{a}	45 ± 3.5^{cd}	82 ± 6.0^{cd}	115 ± 9.3^{d}	212 ± 19^{e}
Fraction I	26 ± 1.5^{a}	69 ± 4.1^{e}	88 ± 6.0^{cd}	143 ± 12^{f}	134 ± 10^{c}
Fraction II	25 ± 1.4^{a}	37 ± 2.1^{bc}	124 ± 11^{e}	135 ± 11^{ef}	140 ± 10^{cd}
Fraction III	26 ± 1.5^{a}	33 ± 1.8^{b}	44 ± 2.8^{b}	59 ± 3.7^{b}	83 ± 6.6^{b}
Fraction IV	25 ± 1.6^{a}	64 ± 4.4^{e}	100 ± 8.9^{d}	165 ± 15^{g}	142 ± 10^{cd}
Fraction V	25 ± 1.6^{a}	52 ± 3.6^{d}	87 ± 5.8^{cd}	168 ± 14^{g}	154 ± 11^{cd}
Fraction VI	24 ± 1.4^{a}	113 ± 9.5^{f}	126 ± 10^{e}	138 ± 11^{f}	124 ± 9.7^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.38 Effect of borage additives at a concentration of 200 ppm as sinapic acid equivalents on formation of total volatiles in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	27 ± 1.6^{a}	48 ± 2.6^{cd}	84 ± 6.3^{de}	118 ± 9.5^{e}	225 ± 19^{e}
BHA[2]	25 ± 1.3^{a}	18 ± 0.9^{a}	16 ± 0.7^{a}	17 ± 0.7^{a}	23 ± 1.3^{a}
α-Tocopherol[2]	25 ± 1.6^{a}	25 ± 1.4^{ab}	36 ± 2.8^{b}	42 ± 3.1^{b}	60 ± 4.1^{b}
Sinapic acid[2]	25 ± 1.5^{a}	38 ± 2.6^{bc}	74 ± 5.0^{d}	86 ± 4.7^{c}	170 ± 15^{d}
Crude extract	26 ± 1.5^{a}	40 ± 3.4^{c}	77 ± 5.6^{d}	96 ± 6.2^{cd}	185 ± 17^{d}
Fraction I	24 ± 1.5^{a}	60 ± 4.8^{d}	56 ± 4.5^{c}	108 ± 7.3^{de}	87 ± 7.5^{bc}
Fraction II	26 ± 1.6^{a}	40 ± 3.6^{c}	96 ± 8.0^{ef}	111 ± 7.8^{de}	98 ± 7.3^{c}
Fraction III	24 ± 1.5^{a}	39 ± 2.5^{c}	37 ± 2.6^{b}	45 ± 3.6^{b}	98 ± 7.5^{c}
Fraction IV	25 ± 1.3^{a}	87 ± 5.9^{e}	95 ± 7.3^{ef}	92 ± 8.8^{cd}	108 ± 8.6^{c}
Fraction V	25 ± 1.4^{a}	40 ± 3.8^{c}	83 ± 8.4^{de}	112 ± 10de	168 ± 14^{d}
Fraction VI	24 ± 1.6^{a}	100 ± 8.5^{e}	103 ± 7.8^{f}	143 ± 12^{f}	118 ± 8.8^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.39 Effect of evening promrose additives at a concentration of 100 ppm as catechin equivalents on formation of total volatiles in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	27 ± 1.6^{a}	48 ± 2.6^{de}	84 ± 6.3^{g}	118 ± 9.5^{f}	225 ± 19^{e}
BHA[2]	25 ± 1.5^{a}	18 ± 0.8^{a}	17 ± 0.9^{a}	18 ± 0.8^{a}	26 ± 1.5^{a}
α-Tocopherol[2]	25 ± 1.7^{a}	30 ± 1.8^{b}	43 ± 2.8^{bc}	76 ± 4.6^{bc}	84 ± 6.0^{b}
Catechin[2]	25 ± 1.6^{a}	40 ± 3.4^{cd}	79 ± 5.8^{fg}	107 ± 11^{ef}	198 ± 16^{e}
Crude extract	26 ± 1.6^{a}	34 ± 2.7^{bc}	62 ± 5.8^{de}	73 ± 6.5^{bc}	136 ± 11^{d}
Fraction I	25 ± 1.4^{a}	40 ± 2.8^{cd}	66 ± 5.8^{ef}	90 ± 8.4^{bcde}	124 ± 11^{cd}
Fraction II	25 ± 1.3^{a}	35 ± 2.4^{bc}	49 ± 4.0^{cd}	80 ± 6.4^{bcd}	117 ± 11^{bcd}
Fraction III	25 ± 1.6^{a}	31 ± 2.1^{bc}	34 ± 2.6^{b}	68 ± 5.1^{b}	91 ± 7.6^{bc}
Fraction IV	25 ± 1.5^{a}	60 ± 4.7^{fg}	72 ± 5.3^{efg}	96 ± 10^{cdef}	90 ± 7.7^{b}
Fraction V	26 ± 1.5^{a}	56 ± 3.8^{ef}	69 ± 4.2^{ef}	100 ± 9.6^{def}	136 ± 11^{d}
Fraction VI	25 ± 1.4^{a}	69 ± 4.8^{g}	70 ± 5.1^{ef}	110 ± 11^{ef}	144 ± 14^{d}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

Table A.40 Effect of evening primrose additives at a concentration of 200 ppm as catechin equivalents on formation of total volatiles in a stripped corn oil-in-water emulsion stored at 60°C[1].

Additive	Total volatiles (mg/kg sample)				
	Day 0	Day 1	Day 3	Day 5	Day 7
No additive	27 ± 1.6^{a}	48 ± 2.6^{d}	84 ± 6.3^{e}	118 ± 9.5^{f}	225 ± 19^{e}
BHA[2]	25 ± 1.3^{a}	18 ± 0.9^{a}	16 ± 0.7^{a}	17 ± 0.7^{a}	23 ± 1.3^{a}
α-Tocopherol[2]	25 ± 1.6^{a}	25 ± 1.4^{b}	36 ± 2.8^{b}	42 ± 3.1^{b}	60 ± 4.1^{b}
Catechin[2]	24 ± 1.4^{a}	37 ± 2.8^{c}	70 ± 7.4^{d}	82 ± 5.8^{e}	156 ± 13^{d}
Crude extract	26 ± 1.6^{a}	28 ± 1.8^{b}	40 ± 3.8^{bc}	47 ± 4.1^{bc}	84 ± 9.1^{bc}
Fraction I	26 ± 1.6^{a}	30 ± 1.7^{b}	36 ± 3.3^{b}	65 ± 4.6^{d}	201 ± 19^{e}
Fraction II	24 ± 1.4^{a}	30 ± 1.8^{b}	40 ± 3.7^{bc}	52 ± 4.2^{bcd}	74 ± 7.5^{bc}
Fraction III	25 ± 1.3^{a}	30 ± 1.8^{b}	39 ± 3.0^{bc}	49 ± 3.6^{bc}	86 ± 6.3^{bc}
Fraction IV	25 ± 1.4^{a}	36 ± 2.1^{c}	40 ± 2.9^{bc}	58 ± 4.7^{cd}	88 ± 7.2^{bc}
Fraction V	25 ± 1.5^{a}	39 ± 2.3^{c}	48 ± 3.2^{c}	44 ± 3.8^{b}	76 ± 6.4^{bc}
Fraction VI	25 ± 1.5^{a}	30 ± 2.0^{b}	35 ± 2.8^{b}	47 ± 3.1^{bc}	96 ± 10^{c}

[1] Results are mean values of three determinations ± standard deviation. Means sharing the same superscript in a column are not significantly ($p > 0.05$) different from one another.
[2] Reference antioxidants.

APPENDIX 2

Y = Antioxidant index
X 1 = Ethanol content in the aqueous extraction medium (%, v/v)
X 2 = Extraction temperature (C)
X 3 = Extraction time (min)

Coding Coefficients for the Independent Variables

Factor	Subtracted off	Divided by
X1	50.000000	20.000000
X2	60.000000	20.000000
X3	60.000000	30.000000

Response Surface for Variable Y: ANT IND

Response Mean	71.870588
Root MSE	2.424728
R-Square	0.9439
Coef. of Variation	3.3737

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	71.491000	0.0974	4.053	0.0580
Quadratic	3	616.739167	0.8404	34.967	0.0001
Crossproduct	3	4.470000	0.0061	0.253	0.8566
Total Regress	9	692.700167	0.9439	13.091	0.0013

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	5	40.148460	8.029692	15.953	0.0600
Pure Error	2	1.006667	0.503333		
Total Error	7	41.155127	5.879304		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|	Parameter Estimate from Coded Data
INTERCEPT	1	-17.176549	14.367737	-1.195	0.2708	79.956338
X1	1	2.702898	0.403118	6.705	0.0003	1.890000
X2	1	0.490952	0.466687	1.052	0.3278	1.890000
X3	1	0.318812	0.228292	1.397	0.2052	0.070000
X1*X1	1	-0.025121	0.003703	-6.783	0.0003	-10.048592
X2*X1	1	-0.000688	0.002143	-0.321	0.7577	-0.275000
X2*X2	1	-0.003371	0.003703	-0.910	0.3929	-1.348592
X3*X1	1	-0.000917	0.001429	-0.642	0.5416	-0.550000
X3*X2	1	0.000708	0.001429	0.496	0.6352	0.425000
X3*X3	1	-0.002610	0.001646	-1.585	0.1569	-2.348592

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F	
X1	4	309.280628	77.320157	13.151	0.0023	SCEM
X2	4	42.643741	10.660935	1.813	0.2309	ET
X3	4	18.692402	4.673100	0.795	0.5645	EP

Canonical Analysis of Response Surface
(based on coded data)

Factor	Critical Value Coded	Uncoded	
X1	0.082537	51.650735	SCEM
X2	0.703166	74.063325	ET
X3	0.068861	62.065815	EP

Predicted value at stationary point 80.701237

Eigenvalues	Eigenvectors X1	X2	X3
-1.301173	-0.021806	0.978670	0.204277
-2.384265	-0.031425	-0.204896	0.978279
-10.060337	0.999268	0.014913	0.035223

Stationary point is a maximum.

Estimated Ridge of Maximum Response for Variable Y: ANT IND

Coded Radius	Estimated Response	Standard Error	Uncoded Factor Values X1	X2	X3
0.0	79.956338	1.037541	50.000000	60.000000	60.000000
0.1	80.178313	1.036445	50.960134	61.753106	60.103530
0.2	80.343193	1.034196	51.339680	63.764238	60.283691
0.3	80.472151	1.034432	51.508169	65.796318	60.536913
0.4	80.570914	1.043367	51.591212	67.819757	60.847793
0.5	80.641475	1.069237	51.632092	69.833218	61.203842
0.6	80.684709	1.121152	51.649081	71.838445	61.595749
0.7	80.701067	1.207322	51.651190	73.837133	62.016546
0.8	80.690805	1.333394	51.643376	75.830592	62.460942
0.9	80.654084	1.501830	51.628601	77.819807	62.924857
1.0	80.591010	1.712506	51.608743	79.805524	63.405091

Y = Antioxidant index
X 1 = Acetonecontent in the aqueous extraction medium (%, v/v)
X 2 = Extraction temperature (C)
X 3 = Extraction time (min)

Coding Coefficients for the Independent Variables

Factor	Subtracted off	Divided by
X1	70.000000	20.000000
X2	60.000000	20.000000
X3	60.000000	30.000000

Response Surface for Variable Y: ANT IND

Response Mean	69.705882
Root MSE	2.816465
R-Square	0.9443
Coef. of Variation	4.0405

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	387.534000	0.3890	16.285	0.0015
Quadratic	3	504.343074	0.5063	21.193	0.0007
Crossproduct	3	48.825000	0.0490	2.052	0.1953
Total Regress	9	940.702074	0.9443	13.177	0.0013

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	5	50.040671	10.008134	3.648	0.2290
Pure Error	2	5.486667	2.743333		
Total Error	7	55.527338	7.932477		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|	Parameter Estimate from Coded Data
INTERCEPT	1	-24.479085	22.928083	-1.068	0.3211	77.619718
X1	1	1.209577	0.629997	1.920	0.0963	-4.700000
X2	1	1.698102	0.555634	3.056	0.0184	3.530000
X3	1	0.460018	0.277359	1.659	0.1412	-2.050000
X1*X1	1	-0.010961	0.004302	-2.548	0.0382	-4.384507
X2*X1	1	-0.001875	0.002489	-0.753	0.4759	-0.750000
X2*X2	1	-0.010586	0.004302	-2.461	0.0434	-4.234507
X3*X1	1	0.003375	0.001660	2.034	0.0815	2.025000
X3*X2	1	-0.002000	0.001660	-1.205	0.2673	-1.200000
X3*X3	1	-0.005372	0.001912	-2.810	0.0262	-4.834507

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F	
X1	4	309.710549	77.427637	9.761	0.0054	SCEM
X2	4	188.670681	47.167670	5.946	0.0208	ET
X3	4	148.970549	37.242637	4.695	0.0370	EP

Canonical Analysis of Response Surface
(based on coded data)

Factor	Critical Value Coded	Uncoded	
X1	-0.679051	56.418986	SCEM
X2	0.536577	70.731542	ET
X3	-0.420826	47.375227	EP

Predicted value at stationary point 80.593892

Eigenvalues	Eigenvectors X1	X2	X3
-3.151765	0.628141	-0.532136	0.567689
-4.611314	0.531000	0.826448	0.187144
-5.690442	-0.566751	0.183890	0.801690

Stationary point is a maximum.

Estimated Ridge of Maximum Response for Variable Y: ANT IND

Coded Radius	Estimated Response	Standard Error	Uncoded Factor Values X1	X2	X3
0.0	77.619718	1.205166	70.000000	60.000000	60.000000
0.1	78.209007	1.203862	68.505018	61.132146	58.957231
0.2	78.732284	1.200410	67.037203	62.260106	57.819186
0.3	79.190113	1.196133	65.592698	63.383735	56.603891
0.4	79.582929	1.193143	64.168191	64.503130	55.325429
0.5	79.911071	1.194286	62.760881	65.618513	53.994852
0.6	80.174806	1.203049	61.368407	66.730163	52.620897
0.7	80.374347	1.223357	59.988788	67.838376	51.210526
0.8	80.509867	1.259249	58.620355	68.943445	49.769331
0.9	80.581506	1.314446	57.261702	70.045646	48.301850
1.0	80.589378	1.391926	55.911639	71.145238	46.811787