# STABILIZATION OF CANOLA OIL BY NATURALLY-OCCURING ANTIOXIDANTS



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# STABILIZATION OF CANOLA OIL BY NATURALLY-OCCURRING ANTIOXIDANTS

BY

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## ABSTRACT

The antioxidant activity of ethanolic extract of canola meal at 100, 200, 500 and 1000 ppm and commercially-available flavonoids at 200 ppm level on refined-bleached canola oil was examined and compared with commonly used synthetic antioxidants such as BHA/BHT/CA (butylated hydroxyanisole/butylated hydroxytoluene/citric acid) at 250 ppm and TBHQ (tert-butylhydroxyquinone) at 200 ppm levels. Accelerated oxidation under Schaal oven test conditions at 65°C was investigated over a 17 day period. Progression of oxidation was monitored by employing weight gain, peroxide value, conjugated diene value, TOTOX value and 2-thiobarbituric acid reactive substances (TBARS) tests. Canola extracts at 500 and 1000 ppm levels were better than BHA/BHT/CA, but less effective than TBHQ. Among the flavonoids tested, myricetin, quercetin, rutin and (-)epicatechin were more effective than BHA/BHT/CA in retarding the formation of primary and secondary oxidation products. Myricetin was the most effective flavonoid tested.

In order to identify the most active antioxidative compound in the ethanolic extract of canola meal, the extract was passed through a Sephadex LH-20 column and fractionated into seven major fractions according to UV absorbance, phenolic and sugar contents. Fraction IV showed the best antioxidative activity as evaluated in a β-carotene-linoleate model system. Further separation of fraction IV by thin layer chromatographic techniques indicated that the compound responsible for strong antioxidative activity of fraction IV was phenolic in nature. Spectroscopic studies

indicated that this compound was 1-O-β-D-glucopyranosyl sinapate (1-O-β-D-glucopyranosyl 3,5-dimethoxy-4-hydroxy cinnamate).

A novel nuclear magnetic resonance (NMR) spectroscopic method was also used to monitor oxidation of canola and soybean oils. The ratios of aliphatic to olefinic and aliphatic to diallylmethylene protons in both oils indicated a steady increase in their numerical values with increasing the length of storage period. A highly significant correlation was found between these ratios and TOTOX values of both oils, thus suggesting that NMR methodology can be used as an effective means to simultaneously estimate both primary and secondary oxidation changes in both canola and soybean oils.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACS | - American Chemical Society |
| ADI | - Allowable daily intake |
| AnV | - p-Anisidine value |
| AOCS | - American Oil Chemists' Society |
| AOM | - Active oxygen method |
| APT | - Attached proton test |
| AV | - Acid value |
| BHA | - Butylated hydroxyanisole |
| BHT | - Butylated hydroxytoluene |
| CA | - Citric acid |
| CD | - Conjugated diene |
| CE | - Canola extract |
| EC | - European Commission |
| ESR | - Electron spin resonance |
| FAME | - Fatty acid methyl ester |
| FDA | - Food and Drug Administration |
| GC | - Gas chromatography |
| GC-MS | - Gas chromatography-Mass spectrometry |
| GL | - Glycolipids |
| HPLC | - High pressure liquid chromatography |

ISC - Inter system crossing

IV - Iodine value

JECFA - Joint expert committee of food additives

MA - Malonaldehyde

NL - Neutral lipids

NMR - Nuclear magnetic resonance

PG - Propyl gallate

PL - Phospholipids

PUFA - Polyunsaturated fatty acids

PV - Peroxide value

PVC - Polyvinyl chloride

RB - Refined bleached

RBD - Refined bleached deodourized

SAS - Statistical analysis system

SCF - Scientific Committee for Food

TBARS - Thiobarbituric acid reactive substances

TBHQ - tert-Butylhydroxyquinone

TLC - Thin layer chromatography

TMS - Tetramethylsilane

USDA - United State Department of Agriculture

UV - Ultraviolet

**DEDICATED**

**TO**

**MY LOVING PARENTS**

# CHAPTER 1
# INTRODUCTION

Canola is the most important vegetable oil for the Canadian edible oil industry. The development of genetically improved canola varieties boosted the use of canola oil in food applications. The content of saturated fatty acids in canola oil is the lowest among all common sources of edible vegetable oils. The ratio of linolenic to linoleic acid in the oil is approximately 1:2 and this is considered to be nutritionally favourable (Ackman, 1990). However, development of off-flavours, due to the autoxidation of unsaturated fatty acids in canola oil, especially linolenic acid, is often criticised (Tokarska *et al.*, 1986). The oxidative deterioration of canola is similar to that of other vegetable oils (Hawrysh, 1990) and involves primarily autoxidative reactions which are accompanied by various reactions having oxidative and non-oxidative characters (Gray, 1978).

Antioxidants are major ingredients which protect the quality of oils by retarding oxidation. In the edible oil industry synthetic antioxidants and chelating agents are often used because they are effective and inexpensive. Currently BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and a mixture of BHA/BHT/CA (citric acid) are used as antioxidants in the canola oil industry. However, the increased popularity of natural food additives may prompt more food manufacturers to replace synthetic antioxidants with ingredients containing natural antioxidative compounds (Marshell, 1974). Therefore, research on natural additives has gained momentum as they are considered to pose no health risk to the consumers.

Most naturally-occurring antioxidative compounds are flavonoids, phenolic acids, lignans, terpenes, tocopherols, phospholipids and polyfunctional organic acids (Dugan, 1980). Canola meal has been reported to have a high content of phenolic compounds (1-2% of defatted meal) (Dabrowski and Sosulski, 1984; Kozlowska *et al.*, 1990; Shahidi and Naczk, 1992). These compounds include phenolic acids (Kozlowska *et al.* 1983; Zadernowski, 1987), flavonoids (Zadernowski *et al.*, 1991) and condensed tannins (Shahidi and Naczk, 1989). However, the presence of high levels of phenolic compounds in canola meal is undesirable due to adverse effects on the nutritional and organoleptic properties of the meal (Clandinin and Robblee, 1981; Kozlowska *et al.*, 1990). Therefore, removal of phenolic compounds from canola meal and proper utilization of phenolics would present new opportunities for the canola meal industry.

In order to evaluate the oxidative deterioration of fats and oils, a number of tests have been developed. Although sensory methods are the most sensitive tests available, they are not always practical because they are time consuming, expensive and often lack reproducibility (Gray, 1978). Many chemical and instrumental methods have therefore been developed to assess oxidative deterioration of fats and oils. These methods detect either primary or secondary changes during lipid oxidation and have been found to correlate with subjective tests or descriptive sensory analysis. However, methods to determine both the primary and secondary products of lipid oxidation simultaneously have not been developed. Therefore, it is of interest to the edible oil industry to search for methods which may fulfil this requirement. Nuclear

Magnetic Resonance (NMR) spectroscopy is considered to have the potential for quantitation of the extent of lipid oxidation (Shahidi, 1992). This method measures the relative changes that occur in NMR absorption pattern of lipid fatty acids during oxidation.

The first objective of this study was to extract and identify potent antioxidative canola phenolics, and to compare their antioxidant activity with the synthetic antioxidants in canola oil. Commercially available flavonoids were also investigated for their antioxidative efficiency in canola oil. In addition to commonly used methods of analysis, proton NMR was also used for monitoring the oxidative deterioration of canola and soybean oils.

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Production of canola and its products (oil and meal) in Canada

Rapeseed is grown around the world for its oil. The word "canola" is used for genetically modified Canadian rapeseed varieties that are low in both erucic acid and glucosinolates. According to the most recent definition, canola is referred to as a rapeseed cultivar that contains less than 2% erucic acid in its oil and less than 30 μmol/g of one or any combination of four of the known aliphatic glucosinolates (gluconapin, progoitrin, glucobrassicanapin and napoleiferin) in the defatted meal (Shahidi, 1990). These "double low" cultivars belong to both species of *Brassica campestris* and *Brassica napus*.

Canola is the most important oilseed crop in Canada. Its production in the 1989/90 crop year was 3.06 million metric tonnes (Anon., 1990) and was larger than the production volume of soybean (1.18 million metric tonnes). The production of canola oil in Canada in 1989/90 was 1.29 million metric tonnes. The export of canola is mainly in the form of seed, oil and meal. The oil is mainly used for cooking or as salad oil (50%) while 16% of it is used for margarine and 34% for shortening production (Statistics Canada, 1991).

## 2.2 Chemistry of fats and oils

Fats and oils are essential nutrients for humans and animals. They are the most concentrated source of energy by contributing 9 kcal/g of lipid (Nawar, 1985), serve as carriers of fat-soluble vitamins (A, D, E, and K) and also provide essential fatty acids. Fats and oils give flavour and taste to the foods, and also serve as a processing medium (Sherwin, 1990).

Neutral lipids, known as triacylglycerols, are the main component of vegetable oils and consist of a molecule of glycerol esterified with three molecules of fatty acids. A liquid lipid at ambient temperature is referred to as oil and the solid lipid is known as a fat. However, the same material may be referred to as an oil or a fat in different regions of the world, depending on the climate (Patterson, 1989). In a triacylglycerol molecule, the three fatty acids may be of the same (simple triacylglycerol) or of different kind (mixed triacylglycerol).

Natural fatty acids are alkyl carboxylic acids and generally contain an even number of carbon atoms and are usually unbranched, straight chain molecules (Taylor, 1973). However, fatty acids of branched chain and uneven number of carbon atoms are also found in nature, but in small quantities (Patterson, 1989). When the carbon atoms in the hydrocarbon chain of a fatty acid hold their full complement of hydrogen they are defined as saturated fatty acids. Saturated fatty acids are most stable either in the free state or in the triacylglycerol form and pack together more easily in the solid state due to their contour arrangement. This behaviour favours a higher melting

point of the fat. As the chain length of fatty acid increases the melting point of the material rises steadily (Patterson, 1989).

Fatty acids with two or more carbon-carbon double bonds are referred as polyunsaturated fatty acids (PUFA). The double bonds in natural unsaturated fatty acids exist in the *cis* form which is associated with softness and liquidity. The double bonds and the methylene ($-CH_2-$) group immediately adjoining them ($\alpha$ methylene group) are notably reactive. When the methylene group lies between two double bonds (eg. linoleic acid, C18:2) the activity is further enhanced (Patterson, 1989).

The kind of fatty acids present in a triacylglycerol has a marked effect upon its physical and chemical behaviour. The fatty acid arrangement in the triacylglycerol molecule affects the texture and melting behaviour of the fat. Therefore, changing of the distribution of fatty acids in the triacylglycerol molecule provides an important way of lipid modification (Taylor, 1973; Patterson, 1989).

Oilseeds are generally processed by using expeller, screw, or hydraulic presses, solvent extraction, or cold pressing. The oil so obtained contains neutral (triacylglycerols) and polar lipids, especially phospholipids, free fatty acids, sterols and sterol esters, waxes, pigments, phenolic compounds and contaminants. Refining removes polar compounds, pigments and contaminants from the oil (Vaisey-Genser and Eskin, 1982). The refined oils contain predominantly triacylglycerols, usually in the range of 98-99 % of the total lipids.

## 2.3 Oxidation of fats and oils

Lipid oxidation, leading to rancidity, has been recognized since antiquity as a problem in the storage of fats and oils and also of lipid-containing foods. Characteristic changes associated with the oxidative deterioration of vegetable oils and animal fats include the development of unpleasant tastes and odours as well as changes in colour, viscosity, specific gravity and solubility (Labuza, 1971; Enser, 1987). Several authors (Labuza, 1971; Frankel, 1980, 1982; Kanner *et al.*, 1987; Hsieh and Kinsella, 1989; Min *et al.*, 1989; Bradley and Min, 1992) have reviewed the mechanisms of lipid oxidation and the subsequent effects of lipid oxidation in foods. Since this study is limited to vegetable oils, the oxidation of vegetable oils will be discussed in terms of autoxidation, photooxidation and thermal oxidation.

### 2.3.1 Autoxidation

Autoxidation is a natural process that takes place between molecular oxygen and unsaturated fatty acids in the environment. Autoxidation of unsaturated fatty acids occurs via a free radical mechanism that consists of basic steps of initiation, propagation and termination. Initiation of this process may happen due to the abstraction of a hydrogen atom adjacent to the double bond in the fatty acid (RH) and this may be catalyzed by light, heat or metal catalysts to form a free radical (Reaction 1). The resultant alkyl free radical ($R^{\bullet}$) reacts with atmospheric oxygen to form an unstable peroxy free radical (Reaction 2) which may in turn abstract a hydrogen atom

from another unsaturated fatty acid to form a hydroperoxide (ROOH) and a new alkyl free radical (Reaction 3). The new alkyl free radical initiates further oxidation and contributes to the chain reaction. The chain reaction (or propagation) may be terminated by formation of non-radical products (Reactions 4-6).

Initiation:

$$RH \xrightarrow{\text{initiator}} R^{\bullet} + H^{\bullet} \qquad (1)$$

Propagation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \qquad (2)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \qquad (3)$$

Termination:

$$R^{\bullet} + R^{\bullet} \rightarrow RR \qquad (4)$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \qquad (5)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \qquad (6)$$

The mechanism of autoxidation in lipids has been postulated by Farmer *et al.* (1942), Boland and Gee (1946) and Bateman *et al.* (1953). The propagation step of the autoxidative process includes an induction period when hydroperoxide formation is minimal (Labuza, 1971; Hawrysh, 1990). The rate of oxidation of fatty acids increases in relation to their degree of unsaturation. The relative rate of autoxidation of oleate, linoleate and linolinate was reported to be in the order of 1:40-50:100 on

the basis of oxygen uptake and in the order of 1:12:25 on the basis of peroxide formation (Hsieh and Kinsella, 1989). Therefore, oils that contain relatively higher amounts of PUFA possess stability problems. Also the breakdown products (alcohols, aldehydes, ketones, hydrocarbons, etc.) of primary lipid oxidation products (hydroperoxides) are sources of off-flavours. These compounds may also interact with other components of the food and cause several other functional and nutritional changes (Sherwin, 1978). A generalized scheme for autoxidation and its possible consequences is illustrated in Figure 2.1.

### 2.3.2. Photooxidation

The degradation of fats and oils during exposure to light is an important factor influencing their flavour stability. Photooxidation or photosensitized oxidation occurs in the presence of photosensitizer and visible light, and proceeds faster than autoxidation (Hawrysh, 1990). During photooxidation, singlet oxygen ($^1O_2$) is generated by interaction of light and a photosensitizer (Gunstone, 1984) which then reacts with the methylene group adjacent to the double bonds of the unsaturated fatty acids to form hydroperoxides (Chan, 1977: Frankel, 1985). Photosensitizers can be dyes (eosin, erythrosine, methylene blue, rose bengal), natural pigments (chlorophyll, riboflavin, hematoporphyrin), metallic salts (cadmium sulphide, zinc oxides, zinc sulphide), transition metal complexes and polycyclic aromatic hydrocarbons such as anthracene (Min *et al.*, 1989).

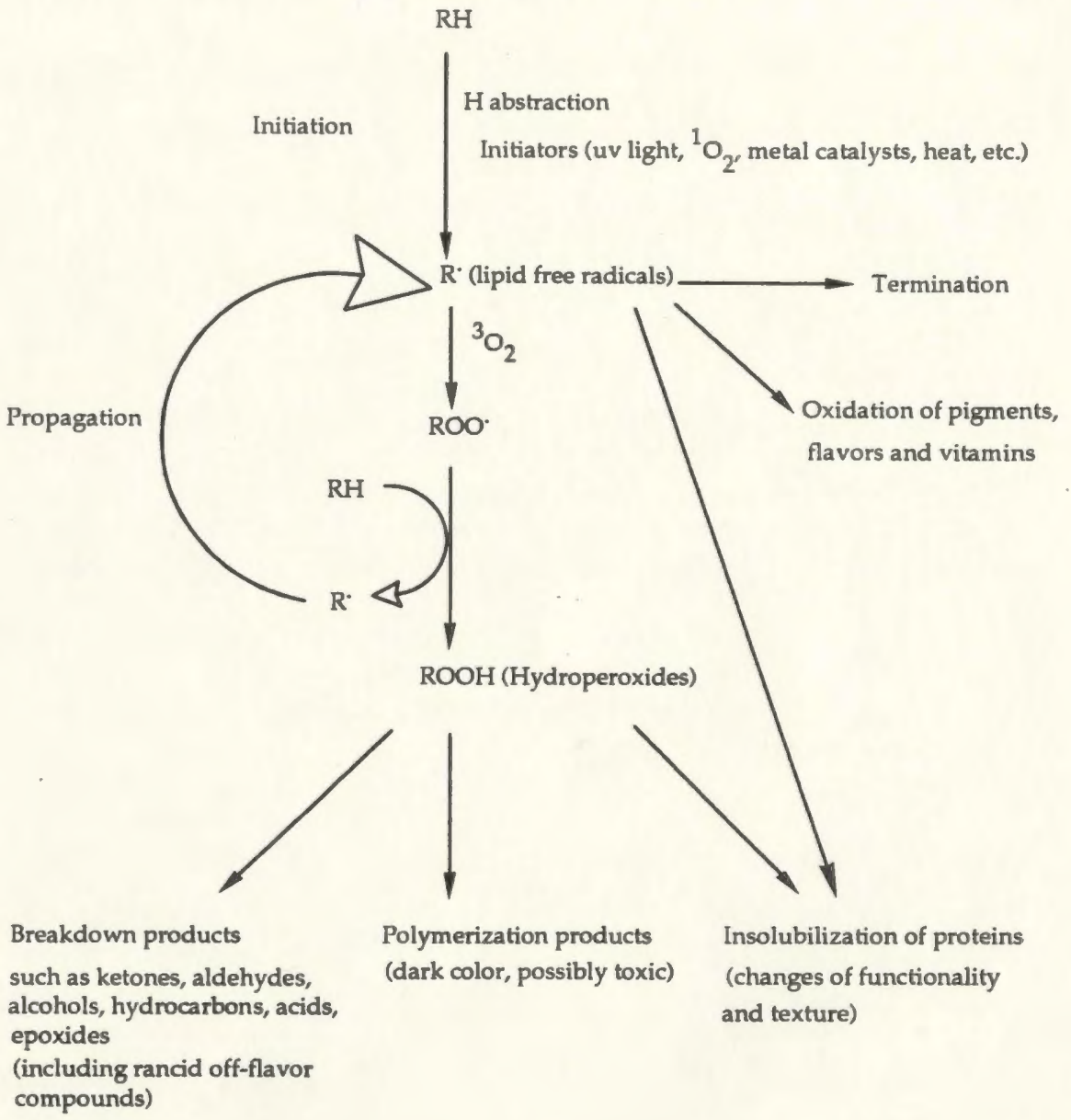



Figure 2.1 General scheme for autoxidation of polyunsaturated fatty acids of lipids and their consequences (Shahidi and Wanasundara, 1992).

The ground state photosensitizer (Sen) absorbs visible or near ultraviolet light and becomes the excited singlet state photosensitizer ($^1Sen^*$) which has a short life time. $^1Sen^*$ rapidly converts into the ground state by emitting fluorescent light or to the excited triplet state sensitizer ($^3Sen^*$) by intersystem crossing (ISC). $^3Sen^*$ has a much longer life time than $^1Sen^*$ and decays to ground state slowly by emitting the phosphorescent light. Efficient sensitizers for the generation of singlet oxygen are long lived $^3Sen^*$ (Min *et al.*, 1989; Bradley and Min, 1992). The triplet excited state sensitizer ($^3Sen^*$) takes two major reaction pathways of Type I and Type II to accomplish photosensitized oxidation of unsaturated lipids (Figure 2.2).

In the Type I mechanism (sensitizer-substrate) pathway, $^3Sen^*$ serves as a photochemically-activated free radical initiator and reacts with substrate (RH) to produce free radicals by hydrogen transfer or electron transfer. The produced free radicals react with triplet state oxygen ($^3O_2$) to produce the oxidized products that readily breakdown to form free radicals that can initiate free radical chain reactions. The rate of type I reaction is dependant on the type and concentration of the sensitizer and substrate (Bradley and Min, 1992).

In the Type II mechanism (singlet oxygen), $^3Sen^*$ reacts with triplet oxygen ($^3O_2$) to generate singlet oxygen ($^1O_2$). The singlet oxygen so produced reacts with the substrate (RH) to give ROOH. There is also an electron transfer from $^3Sen^*$ to triplet oxygen to produce superoxide radical anion ($O_2^{\bullet-}$) and sensitizer radical cation ($Sen^{\bullet+}$) with a chance of less than 1% (Min *et al.*, 1989). Electron-rich compounds

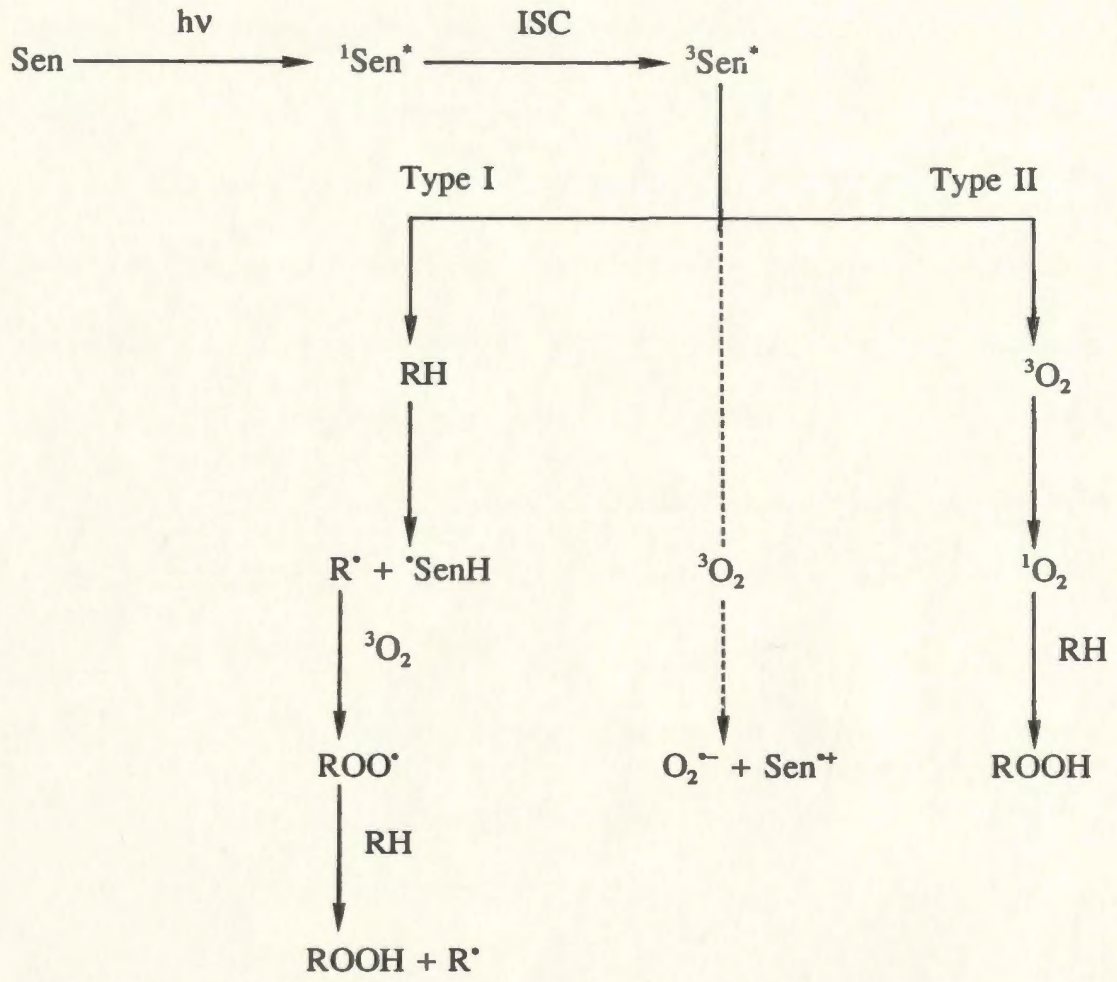



Figure 2.2 Photosensitized oxidation process.

such as simple olefins, dienes and aromatic compounds favour the type II pathway. The rate of the type II reaction mainly depends on the solubility and concentration of oxygen present in the food system. Traces of the sensitizer chlorophyll in vegetable oils would tend to promote photosensitized oxidation by type II pathway because oxygen is more soluble in lipids and non-polar solvents than in water (Bradley and Min, 1992). The involvement of singlet oxygen in the photosensitized oxidation is of Type II which occurs rapidly and thus accounts for almost all photosensitized oxidation reactions (Min *et al.*, 1989).

Photooxidation of vegetable oils is a major concern in the food industry as they contain natural protosensitizers and are commercially sold under light (Labuza, 1971; Frankel, 1980; Simic, 1980). The prooxidant effect of chlorophyll, pheophytin and pheophorbide on the light-induced oxidation in soybean oil is due to the photosensitizing activity of these pigments on singlet oxygen generation (Frankel *et al.*, 1979; Endo *et al.*, 1985). The products of photosensitized oxidation includes both non-conjugated and conjugated diene hydroperoxides, compared to free radical oxidation which produces conjugated diene hydroperoxides only (Rawls and Van Santen, 1970). Carotenoids have been known to act as quenchers for either singlet oxygen or triplet sensitizer in singlet oxygen lipid oxidation. Tocopherols are also known to serve as free radical scavengers and singlet oxygen quenchers (Min *et al.*, 1989).

### 2.3.3 Thermal oxidation

During heating (as in deep fat frying), the oil is subjected to high temperatures (180-190°C) in the presence of air and moisture. Under such conditions, oxidation, hydrolysis and thermal degradation are relatively rapid and no induction period is detected (Hawrysh, 1990). As the oxidation, hydrolysis and thermal reactions proceed, the functional, sensory and nutritional quality of the frying oils are changed (Stevenson *et al.*, 1984). The breakdown products formed from oil during heating (at frying temperature) include volatile and non-volatile decomposition products. Formation of volatile decomposition products, changes the flavour of oil itself and also the flavours of the fried foods (Chang *et al.*, 1978). Formation of non-volatile decomposition products is largely due to thermal oxidation and polymerization of the unsaturated fatty acids in the fried fat. These products cause physical changes such as darkening of colour, increase in viscosity and decrease in the smoke point of the fat as well as chemical changes such as increase in the content of free fatty acids and carbonyl value (Hawrysh, 1990).

### 2.3.4 Factors affecting lipid oxidation

Food systems contain numerous non-lipid components that affect the oxidation of lipids; while some enhance the rate of lipid oxidation, some tend to suppress it (Love, 1985). The number, position and geometry of the double bonds of unsaturated fatty acids affect the rate of lipid oxidation. The *cis* isomers oxidize more rapidly

than the *trans* isomers and conjugated double bonds are more reactive than non-conjugated olefines (Nawar, 1985).

The oxidation rate of lipids is independent of oxygen pressure when the oxygen supply is unlimited. At low oxygen pressure, however, the rate is approximately proportional to the oxygen pressure (Korycka-Daht and Richardson, 1978; Sherwin, 1978). Temperature and surface area also affect the partial pressure of oxygen (when temperature increases, oxygen becomes less soluble) (Nawar, 1985). In general, the rate of oxidation increases as the temperature is increased (Erickson and List, 1985). Ultraviolet and near ultraviolet lights have strong accelerating effect on fat and oil oxidation (Sherwin, 1978). This may be due to photosensitized oxidation as discussed earlier.

Transition metals, particularly those possessing two or more valency states with a suitable oxidation-reduction potential between them (eg. Co, Cu, Fe, Mn, Ni), are major pro-oxidants (Gordon, 1990). At concentrations as low as 0.1 ppm, they can decrease the length of the induction period and increase the rate of oxidation (El-Zeany *et al.*, 1974). Trace amounts of heavy metals are encountered in most edible oils. They may be originated from the soil when plant is grown or from equipment used in processing and storage. Trace metals are also naturally present in all food tissues and fluids of biological origin and are present in both bound and free forms (Nawar, 1985). Contribution of metals ions to lipid oxidation involves two radical producing reactions, one (Reaction 7) involves the metal in its lower oxidation state,

and the other (Reaction 8) in its higher oxidation state (Hiatt *et al.*, 1968).

$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + OH^- + RO^{\bullet} \quad (7)$$

$$M^{(n+1)+} + ROOH \rightarrow M^{n+} + ROO^{\bullet} + H^+ \quad (8)$$

The relative importance of these reactions varies with the type of metal and other factors (eg. coordination, solvent, substrate), but the rate of reaction (7) is generally much faster than that of reaction (8). The two reactions can operate as a cycle so that the overall effect of the metal ion would be to produce more radicals.

The enzyme lipoxygenase, present in most plants tissues, specifically oxygenates PUFA and PUFA esters containing a *cis, cis*-1,4-pentadiene moiety located between carbon 6 and 10 from the methyl terminus. Off flavour development in soybean and soybean products is highly dependant on the action of various endogenous lypoxygenases as subsequent decomposition of the resulting hydroperoxides yields rancid flavours (Richardson and Hyslop, 1985).

## 2.4 Control of lipid oxidation

Since oxidation of lipids containing unsaturated fatty acids can proceed via different mechanisms, several strategies are possible to minimize oxidation. Knowledge of key mechanism(s) for the initiation of lipid oxidation facilitates devising methodologies to control lipid oxidation.

### 2.4.1 Removal of oxygen

Since oxygen is an essential reactant in lipid oxidation, control of oxygen availability is a critical variable in minimizing oxidation of unsaturated fatty acids. As discussed earlier, the rate of oxidation of unsaturated fatty acids is affected by oxygen pressure when the partial pressure of oxygen in the reaction system is less than 100 mm (Pryor, 1973; Sherwin, 1978). The level of available oxygen may be controlled by vacuum packaging (Lindsay, 1977; Josepson *et al.*, 1985) and by using oxygen scavengers such as glucose oxidase and ascorbic acid oxidase (Hsieh and Kinsella, 1989). These precautions reduce the rate and extent of lipid oxidation, especially when combined with antioxidants and low temperature storage in dark.

### 2.4.2 Hydrogenation

Hydrogenation of vegetable oils has been patented as a method of stabilizing them. Selective hydrogenation of soybean oil is practiced in the United States. This process reduces the linolenic acid content of the oil to a minimum and products so obtained are used in the production of margarines and shortenings (Cowan and Evans, 1962; Patterson, 1989). However, hydrogenation reduces the degree of unsaturation of fatty acids and lowers the nutritional value of PUFA-containing fats.

### 2.4.3 Use of antioxidants and synergists

Antioxidants are added to fats and oils to retard oxidation and to reduce development of rancidity. However, antioxidants cannot improve the quality of an already oxidized food products. According to the USDA Code of Federal Regulations [21 CFR 170.3 (0) (3)], "antioxidants are substances used to preserve food by retarding deterioration, rancidity or discolouration due to oxidation" (Dziezak, 1986). Synergists are substances that enhance the activity of antioxidants without having their own antioxidant effect (Nawar, 1985). Ideal food-grade antioxidants in addition to being safe, should not affect the colour and flavour of food and must be effective at low concentrations, easy to incorporate, survive after processing and be stable in the finished product (carry-through properties) as well as being available at a low cost (Coppen, 1983).

#### 2.4.3.1 Mechanism of action of antioxidants

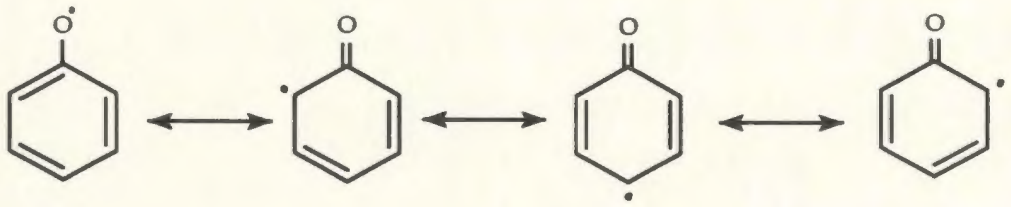
On the basis of lipid oxidation processes, antioxidants can be grouped into two mechanistically distinct classes. One group of antioxidants can inactivate two important radical species, involved in chain propagation steps, alkyl peroxy ($ROO^{\bullet}$) and alkyl ($R^{\bullet}$) radicals, and they can be grouped as chain breakers or primary antioxidants (Heish and Kisella, 1989; Gordon, 1990). This group includes the most common food antioxidants (AH) which interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals according to reactions (9) and (10).

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \qquad (9)$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \qquad (10)$$

Primary antioxidants are able to donate a hydrogen atom to a lipid radical and produce a radical from the antioxidant which is more stable than the lipid radical or is converted to other stable products (Gordon, 1990). Even though phenol itself is inactive as an antioxidant, alkyl substituted phenolic compounds are the most effective antioxidants used in foods (Uri, 1961; Sherwin, 1990). Substitution of alkyl groups in the 2,4 or 6 position increases the electron density on the hydroxy group by an inductive effect and thus increases their reactivity with lipid radicals. Substitution at the 4th position with an ethyl or n-butyl group rather than a methyl group improves the activity of a phenolic antioxidants, however, longer chain or branched alkyl groups in this position decrease the activity (Ingold, 1960). The strong electron donating effect of a methoxy substituent is an important contributor to the effectiveness of 2-tert-butyl-4-methoxyphenol (BHA) as an antioxidant.

The radical formed from the reaction of a phenol with a lipid radical is stabilized by delocalization of the unpaired electron around the aromatic ring as indicated by the valence bond isomers (Reaction 11). The stability of the phenoxy



(11)

radical (A•) reduces the rate of propagation of the autoxidation chain reaction since propagation reactions such as (12)-(14) are very slow as compared with reactions (2) and (3).

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \qquad (12)$$

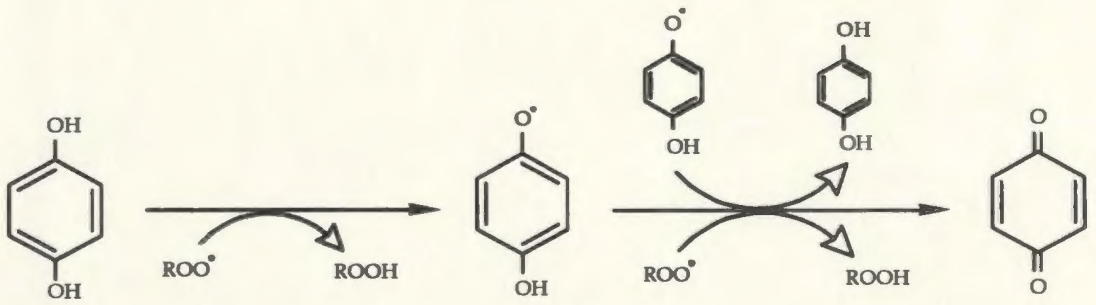
$$AOO^{\bullet} + RH \rightarrow AOOH + R^{\bullet} \qquad (13)$$

$$A^{\bullet} + RH \rightarrow AH + R^{\bullet} \qquad (14)$$

Stability of the phenoxy radical is further increased by the presence of bulky groups in the 2 and 6 positions as in 2,6-di-tert-butyl-4-methylphenol, BHT (Gordon, 1990). However, the presence of bulky substituents in the 2 and 6 positions also reduces the rate of reaction of the phenol with lipid radicals (Reactions 9 and 10). The presence of second hydroxy group at the 2 or the 4 position of a phenol increases the antioxidant activity. The effectiveness of a 1,2-dihydroxybenzene derivative is increased by the stabilization of a phenoxy radical by an intramolecular hydrogen

transfer (Reaction 15). The antioxidant activity of dihydroxybenzene derivatives is partly due to the fact that the semiquinonoid radical produced initially can further oxidize to a quinone via reaction with another lipid radical or may disproportionate to a quinone and a hydroquinone molecule (Reaction 16).

(15)




(16)

The effect of antioxidant concentration on the rate of autoxidation depends on several factors including antioxidant structure, oxidation conditions and the substrate. Often the antioxidant activity of phenolic compounds is lost at high concentrations and they may act as prooxidants due to involvement in initiation reactions (Cillard *et al.*, 1980; Lundberg *et al.*, 1947).

Secondary antioxidants prevent the introduction of chain initiation radicals into the system (Heish and Kinsella, 1989). These may operate by a variety of mechanisms including binding of metal ions, scavenging of oxygen, decomposition of hydroperoxides to non-radical species, absorption of UV radiation or deactivation of singlet oxygen. Secondary antioxidants usually show antioxidant activity if a second minor component is present in the system (Gordon, 1990). Citric acid, ethylenediaminetetraacetic acid (EDTA) and phosphoric acid derivatives (polyphosphates) may extend the shelf life of lipid-containing foods by chelation of metal ions which act as prooxidants. Ascorbic acid, ascorbyl palmitate, erythrobic acid (isoascorbic acid) or sodium erythrobate are also used to stabilize fatty foods. Ascorbic acid is oxidized to dehydroascorbic acid when it functions as an oxygen scavenger, its activity is enhanced in the presence of tocopherols. Ascorbyl palmitate is more effective as an antioxidant because of its increased solubility in the fat phase (Cort, 1974a). It has been shown that enzymes like superoxide dismutase and catalase can remove formed superoxide radical anion ($O_2^{\bullet -}$) and hydrogen peroxide, respectively, which are important in lipid oxidation of biological system (Kellog and Fridovich, 1975). Similarly β-carotene can inhibit lipid oxidation initiated by xanthine oxidase, perhaps due to its quenching effect of singlet oxygen. Amino acids have also been implicated as having some chelating ability, however, their application in oils is limited due to solubility problems (Labuza, 1971).

Many of the flavonoids and related phenolic compounds show marked antioxidant properties (Mehta and Seshadri, 1959). Structures of these flavonoids and related compounds are given in Figure 2.3. Flavonoids and related compounds are known as primary antioxidants and act as free radical acceptors and chain breakers. Flavonoids are known to chelate metal ions at the 3-hydroxy-4-keto group and/or the 5-hydroxy-4-keto group (when the A ring is hydroxylated at position 5; Shahidi *et al.*, 1991). An o-quinol group at the B ring can also demonstrate metal chelating activity (Pratt and Hudson, 1990). Hudson and Lewis (1983) have demonstrated the ability of flavonoids to form complexes with a cupric ion (Figure 2.4) using UV spectral studies. Such complexation may contribute to the antioxidative action of flavonoids. Chelation of metal ions renders them catalytically inactive.

### 2.4.3.2 Synthetic antioxidants

Use of synthetic antioxidants, mainly phenolic compounds, in foods has been in practice since the late 1940's (Sherwin, 1990). The application of antioxidants to foods is governed by Federal regulations. Food and Drug Administration (FDA) regulations require that antioxidants and their carriers be declared on the ingredient labels of products and should be followed by an explanation of their intended purpose (Dziezak, 1986). Synthetic food antioxidants currently permitted for use in foods are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ). In Canada, BHA, BHT and PG are allowed for

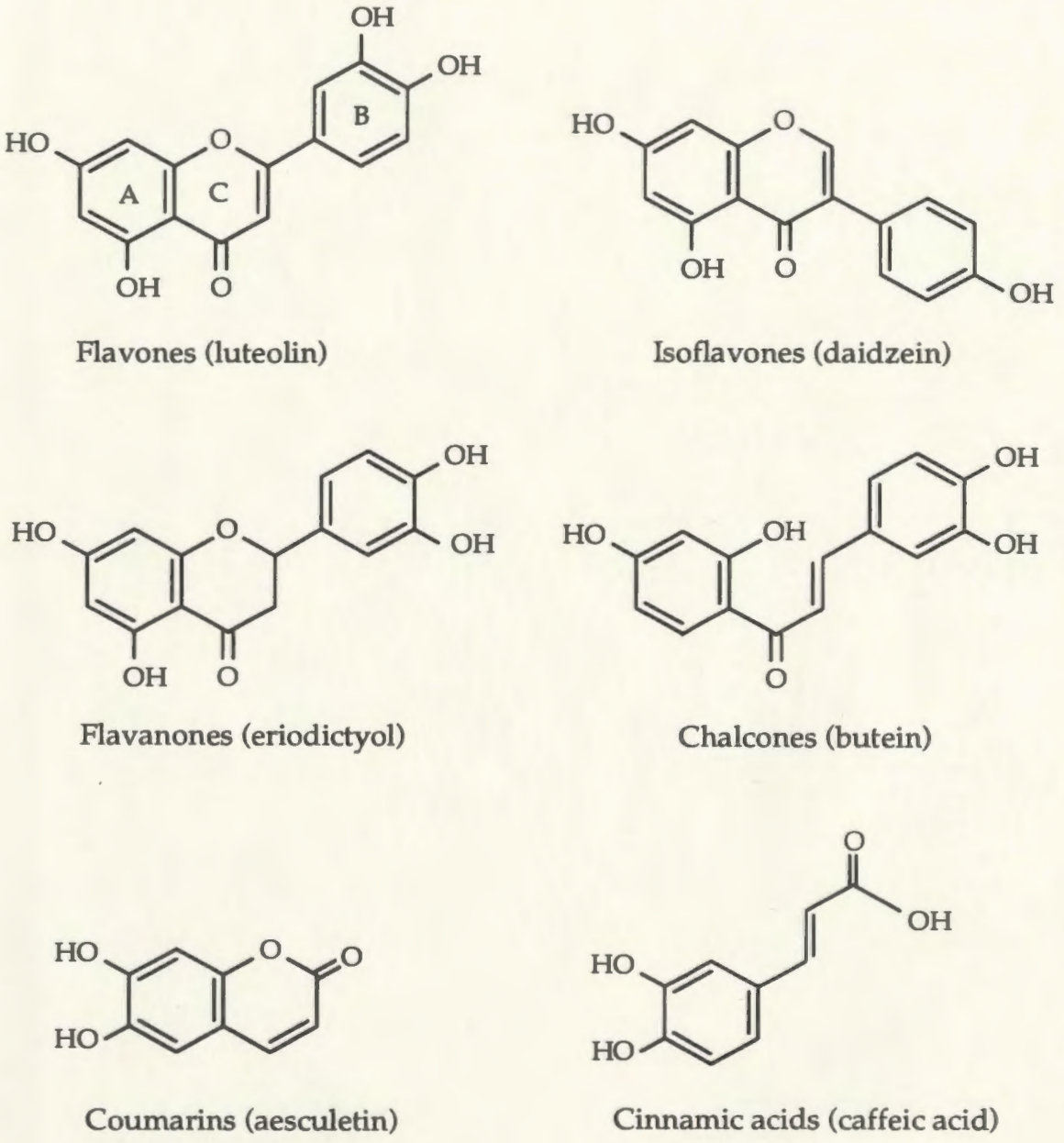
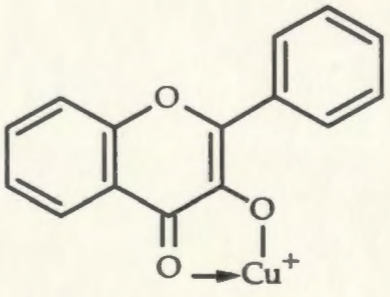
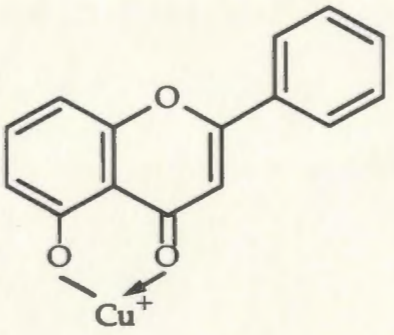


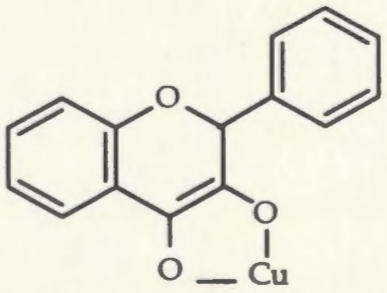

Figure 2.3 Structure of flavonoids and related compounds (Shahidi and Wanasundara, 1992).



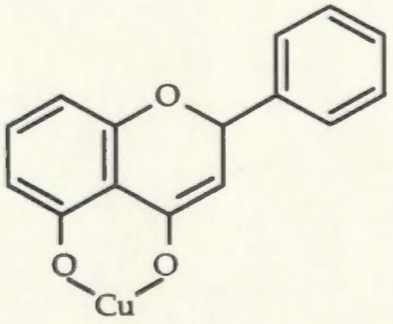

3-Hydroxyflavone




5-Hydroxyflavone




3-Hydroxyflavanone




5-Hydroxyflavanone

Figure 2.4 Forms of copper complexes with flavones and flavanones (Hudson and Lewis, 1983).

food use but not TBHQ. Tables 2.1 and 2.2 summarize the permissable synthetic food antioxidants and some of their properties and levels of allowable use.

The mode of action of phenolic antioxidants as free radical acceptors has been discussed previously. Degradation of phenolic antioxidants (mono-, di-, or tri-phenolic antioxidants) during the course of oxidations of fats and oils has been demonstrated. The formation of antioxidant dimers is the most common feature of the degradation of antioxidants. These are produced by the formation of phenoxy radicals followed by radical rearrangement and coupling reactions (Kikugawa *et al.*, 1990). Degradation products of BHT, BHA (Kikugawa *et al.*, 1990), PG (Kurechi and Kunugi, 1983a) and TBHQ (Kurechi *et al.*, 1983; Kurechi and Kunugi, 1983b) show their own antioxidant activity in model systems.

The toxicology of synthetic antioxidants has become one of the most controversial areas in the continuing debate on the safety of food additives. Several studies on laboratory animals have been carried out to address safety issues related to the use of synthetic antioxidants. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Commission (EC) Scientific Committee for Food (SCF) have established and acceptable daily intake (ADI) for these antioxidants rather than expressing whether particular additives are acceptable or not. ADI values reported so far are summarized in Table 2.3.

Table 2.1 Commonly used synthetic food antioxidants.

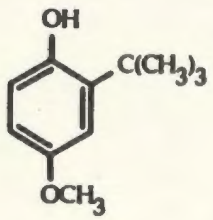
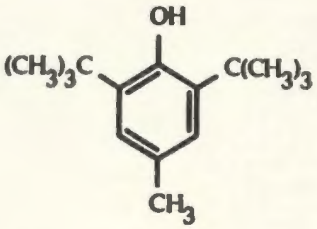
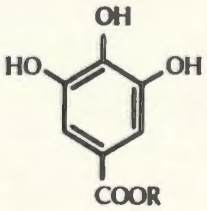
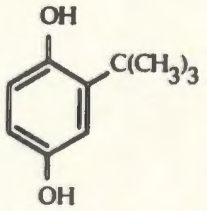
| | BHA | BHT | Gallates | TBHQ |
|---|---|---|---|---|
| Chemical Structure |  |  |  |  |
| | | | R=Propyl or Dodecyl | |
| Carry-through properties | Very good | Fair-good | Propyl: Poor<br>Dodecyl: Fair-good | Good |
| Synergism | With BHT and gallates | With BHA | With BHA | -- |
| Solubilities (% w/v) | | | Propyl Dodecyl | |
| Water | 0 | 0 | 0.35 0.0001 | 1 |
| Animal fat | 30-40 | 20-30 | 1 - | 5-10 |
| Vegetable fat | 40 | 20-30 | 1 1 | 5 |

Table 2.2 Maximum usage levels (ppm) permitted by US FDA in specific application of antioxidants, from Code of Federal Regulations (Dziezak, 1986).

| Food | BHA[a,b] | BHT[b,c] | PG[d] | TBHQ[e] |
|---|---|---|---|---|
| Dehydrated potato shreds | 50 | 50 | -- | -- |
| Active dry yeast | 1000[f] | -- | -- | -- |
| Dry breakfast cereals | 50[f] | 50 | -- | -- |
| Potato flakes | 50 | 50 | -- | -- |
| Potato granule | 10 | 10 | -- | -- |
| Poultry products[g] | 100[f] | 100[i] | 100 | 100 |
| Dry sausages[h] | 30[f] | 30[i] | 30 | 30 |
| Fresh sausages[h] | 100[f] | 100[i] | 100 | 100 |
| Dried meat[h] | 100[f] | 100[i] | 100 | 100 |

[a] 21 CFR 172.110
[b] Given levels are for total BHT and BHA
[c] 21 CFR 172.115
[d] 21 CFR 184.1660
[e] 21 CFR 172.185
[f] BHA only
[g] 9 CFR 381.147(f)(3)
[h] CFR 318.7(c)(4)
[i] BHT only

Table 2.3 Allowable daily intakes (ADI) of synthetic antioxidants[a].

| Antioxidant | ADI mg/kg body weight |
|---|---|
| PG | 0 - 25 |
| TBHQ | 0 - 0.2 |
| BHA | 0 - 0.5 |
| BHT | 0 - 0.125 |

[a] Barlow (1990)

### 2.4.3.3 Natural antioxidants

During the last few decades natural alternatives for synthetic antioxidants have been studied. Food manufacturers as well as consumers prefer natural food additives although possible beneficial effects of those antioxidants can not always be substantiated (Marshell, 1974). Many foods contain compounds with antioxidative activity but some foods are of limited use as additives because they impart specific flavour, aroma or colour to the finished product. Natural antioxidants can be extracted from these foods and also from other materials that are not generally used as food. Sources of natural antioxidative compounds and their main active components reported in the literature are listed in Table 2.4.

Antioxidative compounds of oilseeds like soybean, cottonseed, peanut and sesame have been investigated by some research workers. Most of the work come into the conclusion that the antioxidant activity is not due to a single compound; it is usually due to a group of compounds and also due to the synergistic activity of phospholipids and amino acids (Hayes *et al.*, 1977; Hudson and Ghavami, 1984). However, it is interesting to find the most active antioxidative compound from the natural sources. Research on soybean has concluded that its antioxidative activity is due to isoflavone glycosides, phenolic acids, amino acids, peptides and tocopherols (Hayes *et al.*, 1977; Pratt *et al.*, 1981). In peanuts, dihydroquercetin and taxifolin were identified as antioxidative flavonoids (Pratt and Miller, 1984). Quercetin and rutin were found to be the main antioxidative compounds in cottonseed (Whittern *et*

Table 2.4 Sources of natural antioxidative compounds.

| Source | Antioxidative compounds | References |
|---|---|---|
| Oils and oilseeds | Tocopherols and tocotrienols | Schuler (1990) |
| | Lignans (eg. sesamol in sesame) | Namiki (1990), Fukuda *et al.* (1985) |
| | Phospholipids, isoflavones (eg. soybean) | Rackis (1972), Hayes *et al.* (1977), Pratt *et al.* (1981), Naim *et al.* (1976) |
| | Phenolic acids and condensed tannins, flavonoids (eg. dihydro quercetin and taxifolin in Peanut) | Namiki (1990), Shahidi and Wanasundara (1992), Pratt and Miller (1984), Whittern *et al.* (1984), Rhee *et al.* (1979) |
| Cereal grains | C-glycosyl flavonoids (eg. isovitexin in rice) | Osawa *et al.* (1985), Ramarathnam *et al.* (1986, 1988, 1989) |
| | Phenolic acids, phospholipids (eg. oats) | Schuler (1990) |
| Fruits and vegetables | Ascorbic acid, hydroxy carboxylic acids, flavonoids, carotenoids | Pokorny (1991), Namiki (1990) |
| Fungi | Phenolic compounds | Aoyama *et al.* (1982) |

Continued....

Table 2.4 (continued)

| Sources | Antioxidative compounds | References |
|---|---|---|
| Spices and herbs | Di-terpene lactones (eg. rosamanol in rosemary and sage), di-phenol glycoside (eg. rosemaric acid in rosemary), allyl phenol and lignans (eg. mace), flavonoids (eg. perilla plant), capsicin (eg. capsicum and black pepper), tetrahydro curcumin (curcumin) | Inatani, *et al.* (1983), Namiki (1990), Nakatani and Kukuzaki (1987), Kikuzaki and Nakatani (1989), Nakatani and Inatani (1984), Jitoe *et al*, (1992) |
| Leaves and leaf waxes and medicinal plants | Catechins (eg. tea), tannins, β-diketo compounds, nordihydroguaiaretic acid (NDGA) | Matsuzaki and Hara (1985), Su *et al.* (1988), Shahidi, *et al.* (1992), Namiki (1990) |
| Proteins and protein hydrolyzates | Amino acids, dihydropyridins, maillard reaction products | Kirigaya *et al.* (1968), Namiki (1990) |
| Woodsmoke | Phenolic compounds | Maga (1988), Barylko-Pikielna (1977) |

*al.*, 1984). Sesame seeds are found to contain sesamolinol, sesamol and sesamolin which are attributed to the superior oxidative stability of the oil (Fukuda *et al.* 1985). Both the flour and methanolic extracts of mustard and canola seeds have shown antioxidative activities in meat model systems (Shahidi, 1988; Shahidi and Wanasundara, 1992). However, the chemical nature of the active compounds of mustard or canola seeds were not elucidated.

Recently published review articles (Namiki, 1990; Pokorny, 1991; Shahidi and Wanasundara, 1992) and books (Hudson, 1990) have extensively discussed the occurrence, active ingredients and several other aspects of natural antioxidants. Most of these compounds have been shown to be as effective as BHA and BHT in model system studies, and those from established food sources such as sesame are expected to be safe as food additives. However, the development of antioxidants of natural origin compared to synthetic antioxidants will not be easy with respect to applicability to different food varieties, stability, solubility, ease of manufacture, cost, etc. It is also important to evaluate the safety of these natural additives due to the fact that there is no assurance that natural substances are non-toxic and safe.

## 2.5 Measurements of lipid oxidation

The available methods to monitor and measure the extent of lipid oxidation in lipid-containing foods and biological systems may be divided into two groups. The first group measures primary changes and the second group measures secondary

changes that occur in each system (Coxon, 1987; Gray and Monahan, 1992). Primary changes are generally measured by (a) oxygen uptake, (b) loss of unsaturated fatty acids and (c) hydroperoxide values (PV). Secondary changes are monitored by using the content of (a) carbonyls (as dinitrophenyl hydrazone or by gas chromatography), (b) malonaldehyde and other aldehydes (TBA test), (c) hydrocarbons (pentane value), and (d) fluorescence products (1-amino-3-iminopropane structure). However, the appropriate method to measure the extent of oxidation in a system depends on the particular food product and the way it has been processed and stored.

### 2.5.1 Primary changes

#### 2.5.1.1 Measurement of changes of reactants

Methods that measure primary changes of lipids may be classified as those that quantify loss of reactants (unsaturated fatty acids, oxygen) or formation of primary lipid oxidation products (hydroperoxides). These methods are more suitable to measure low levels of oxidation in uncooked products at low temperatures (Coxon, 1987). Measurement of changes in fatty acid composition is not widely used in assessing lipid oxidation since it requires total lipid extraction, separation into lipid classes and conversion into derivatives suitable for gas chromatographic analysis. However, it has been proven that this method serves as a useful technique to identify which class of lipids and fatty acids are involved in the oxidative changes (Coxon, 1987; Gray and Monahan, 1992) and also to assess lipid oxidation simulated by

different metal complexes that give different products (Gutteridge and Halliwell, 1990). Changes in iodine value due to loss of unsaturation during accelerated oxidation studies may also be used (Hudson, 1983).

During oxidation of lipids the oxygen in the air above the lipid surface slowly reacts with it and changes in the oxygen pressure may be measured quantitatively. This is the principle used in the Sylvester test. The principle used in the Sylvester test is that oil is subjected to high temperature (100°C) in a closed system and the drop of pressure in the system is quantified as oxidation proceeds. Increase in weight of fat due to absorption of oxygen during accelerated oxidation is measured under Schaal oven test conditions. This is a convenient method for measuring the length of the induction period (Olcott and Einest, 1958; Rossell, 1983).

### 2.5.1.2 Measurement of hydroperoxides

The classical method for quantification of hydroperoxides is the determination of "peroxide value" (PV). The hydroperoxide content which is generally referred to as peroxide content is determined by an iodometric method. This is based on the measurement of the iodine released from potassium iodide by the peroxides present in lipids. Drawbacks of this method are absorption of iodine at unsaturated bonds of the fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be titrated (Gray, 1978). Several other chemical methods have been suggested to measure PV. Colorimetric methods based on the oxidation of $Fe^{2+}$ to

$Fe^{3+}$ and the determination of $Fe^{3+}$ as ferric thiocyanate, and a 2,6-dichlorophenol-indophenol procedures are reported in the literature (Gray and Monahan, 1992). In studies on the oxidation of biological tissues and fluids, measurement of fatty acid hydroperoxides is more common than measuring their decomposition products. Fatty acid hydroperoxides can be analyzed by high performance liquid chromatography (HPLC) or their corresponding hydroperoxy acid reduction products may be determined by gas chromatography-mass spectrometry (GC-MS) (Hughes *et al.*, 1983, 1986). Fluorescence methods have been developed to determine hydroperoxides by allowing them to react with substances that form fluorescent products; eg. luminol and dichlorofluorescein (Gray, 1978). Although determination of peroxide value is a common measurement of lipid oxidation, its use is limited to the initial stages of lipid oxidation.

The Active Oxygen Method (AOM), also referred to as Swift test of the American Oil Chemist's Society, is the most commonly used accelerated method for assessing the oxidative stability of fats. This method is based on the principle that aging and rancidification of a fat is greatly accelerated by aeration in a tube held at a constant elevated temperature (Rossell, 1983). The peroxide value reached by the AOM at which a fat will be rancid by organoleptic evaluation varies with the nature of the fat (Dugan, 1955). An automated version of the AOM apparatus, known as Rancimat, is now available. Laubi and Bruttel (1986) compared the AOM and the Rancimat methods for evaluation of PV and found that the Rancimat method yielded

results equivalent to the AOM. Rancimat plots the conductometric determination of volatile degradation products by measuring conductivity against time. Despite the high cost of the apparatus, Rancimat is currently regarded as the method of choice for fat stability studies.

### 2.5.1.3 Measurement of conjugated dienes

Oxidation of polyunsaturated fatty acids is accompanied by an increase in the ultraviolet absorption of the product. Lipids containing methylene interrupted dienes or polyenes show a shift in double bond position during oxidation due to isomerization and conjugation formation (Logani and Davies, 1980). The resulting conjugated dienes show intense absorption at 234 nm and similarly conjugated trienes show absorption at 268 nm. Farmer and Sutton (1946) indicated that the absorption increase is proportionate to the uptake of oxygen and to the formation of peroxides in the early stages of oxidation. St. Angelo *et al.* (1972 and 1975) studied the autoxidation of peanut butter by measuring the PV and the increase in absorbance at 234 nm due to the formation of conjugated dienes. They concluded that the conjugated diene method can be used as an index of stability of peanut butter in place of, or in addition to the PV. The conjugated diene method is faster than the PV determination, is much simpler, does not depend upon chemical reaction or colour development, and requires a smaller samples size.

### 2.5.2 Secondary changes

The primary products (peroxides) of oxidized fats are transitionary intermediates which decompose into various secondary products and measurement of these secondary products as an index of lipid oxidation is more appropriate. This is due to the fact that while primary oxidation products are colourless and flavourless, secondary products of oxidation are generally flavour-active. Secondary oxidation products include carbonyls (ketones and aldehydes), hydrocarbons, etc.

#### 2.5.2.1 Thiobarbituric acid value

One of the oldest and the most frequently used test for assessing lipid oxidation in foods and other biological systems is the 2-thiobarbituric acid (TBA) test. The extent of lipid oxidation is reported as the TBA number and is expressed as milligrams of malonaldehyde (MA) equivalents per kilogram of sample or μmoles of MA equivalents per gram of sample. Malonaldehyde is a relatively minor lipid oxidation product that is formed during the oxidation of polyunsaturated fatty acids and reacts with TBA to produce a coloured complex with an absorption maximum at 530-532 nm (Tarladgis *et al.*, 1964; Gray, 1978). The adduct formed by condensation of 2 moles of TBA with 1 mole of MA is shown in Figure 2.5. Dahle *et al.* (1962) postulated a mechanism for formation of MA and indicated that only peroxides which possess unsaturation, β, γ to the peroxide group are capable of undergoing cyclization with the ultimate formation of MA. Such peroxides could be produced from fatty

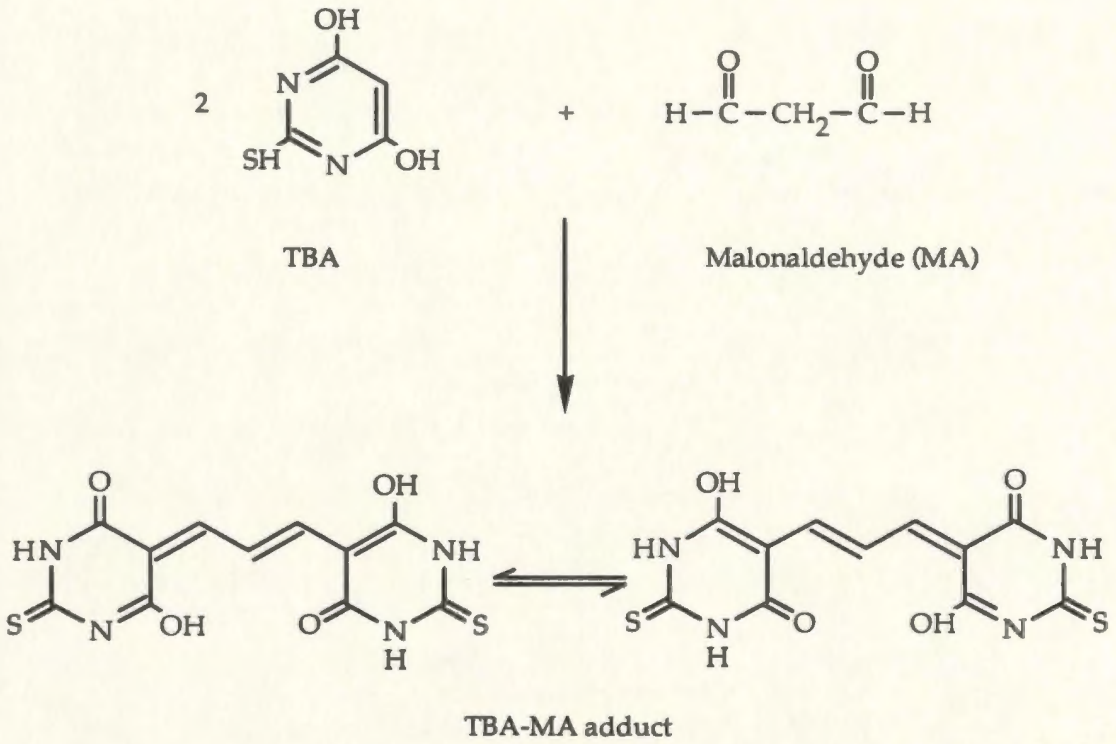



Figure 2.5 Possible reaction between TBA and malonaldehyde.

acids containing three or more double bonds (Dahle *et al.*, 1962; Coxon, 1987). The other products of lipid oxidation, namely other aldehydes and dienals (alka-2,4-dienals) may also react with the TBA reagent to form a pink-coloured complex with the same absorption maximum as the MA-TBA complex (Marcuse and Johansson, 1973). Therefore, the term "thiobarbituric acid-reactive substances" (TBARS) is now commonly used in place of TBA number or value (Ke *et al.*, 1984; Gray and Pearson, 1987). There are certain limitations when using the TBA test for evaluation of the oxidative state of foods and biological systems because of the chemical complexity of these systems. Dugan (1955) has reported that sucrose and some compounds in woodsmoke react with the TBA reagent to give a red colour. Baumgartner *et al.* (1975) have also found that a mixture of acetaldehyde and sucrose when subjected to the TBA test produced a 532 nm absorbing pigment identical to that produced by MA and TBA. Modification of the original TBA test have been reported by Marcuse and Johansson, (1973), Ke and Woyewoda, (1979), Robbles-Martinez *et al.* (1982), Pokorny *et al.* (1985), Shahidi *et al.* (1987), Thomas and Funes (1987) and Schmedes and Holmer (1989). However, it has been suggested that changes in TBARS for a particular situation can show the relative amount of lipid oxidation occurring in the food system during storage or processing. It is always preferable to quantitate the extent of lipid oxidation by a complementary analytical procedure to verify the results.

Several attempts have been made to establish a relationship between TBA values and the development of undesirable flavour characteristics of fats. It has been

shown that flavour thresh holds have a good correlation between the TBA values of vegetable oils including soybean, cottonseed, corn, safflower (Gray, 1978) and canola oil (Hawrysh, 1990).

### 2.5.2.2 p-Anisidine value

p-Anisidine value is used to measure secondary products of lipid oxidation. This method determines the amount of aldehydes (principally 2-alkenals and 2,4-dienals) in animal and vegetable fats and oils. Aldehydes in an oil and the p-anisidine reagent are reacted in an acidic solution (AOCS, 1990). The proposed reaction of p-anisidine with aldehydes to form yellowish products is shown in Figure 2.6. List *et al.* (1974) reported a highly significant correlation between anisidine values of salad oils processed from undamaged soybeans and their flavour acceptability scores.

### 2.5.2.3. Measurement of carbonyls

An alternative approach to determine extent of lipid oxidation is to measure the total or individual volatile carbonyl compounds formed by degradation of hydroperoxides. Hexanal, one of the major secondary products formed during the oxidation of linoleic acid in oils (Frankel *et al.*, 1981) and other aldehydes have been used to follow lipid oxidation in meat products. Shahidi *et al.* (1987) reported a linear relationship between hexanal content, sensory scores and TBA numbers of cooked ground pork, while St. Angelo *et al.* (1987) established a similar correlation

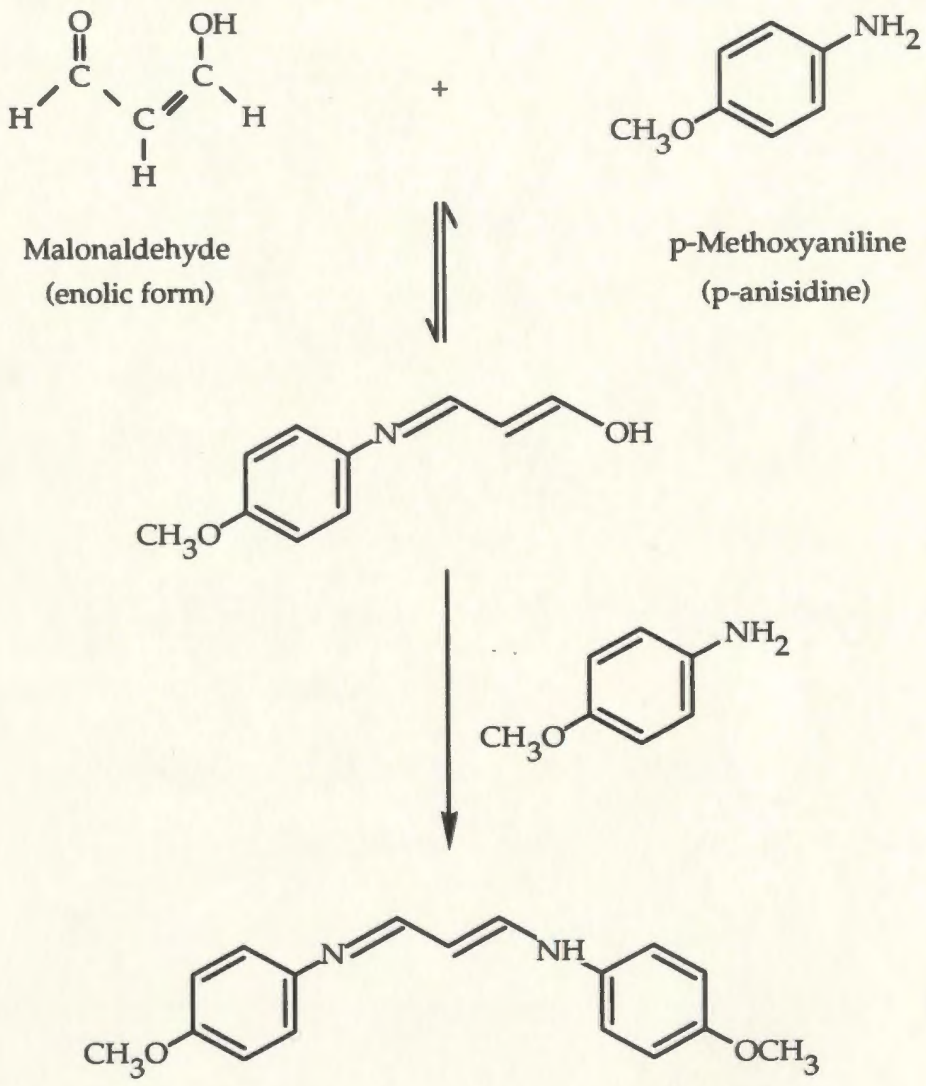



Figure 2.6 Possible reactions between p-anisidine and malonaldehyde.

for cooked beef. These studies suggested that compounds usually associated with lipid oxidation could be used as marker compounds to follow development of off-flavours in lipid-containing foods.

#### 2.5.2.4 Measurement of hydrocarbon and fluorescent products

Oxidative studies with methyl linoleate and soybean oil (Selke *et al.*, 1970) revealed that saturated hydrocarbons could be detected when aldehydes are either absent or undetectable. Evans *et al.* (1967) have reported that pentane is the predominant short chain hydrocarbon formed through thermal decomposition of linoleic acid. Correlation of flavour acceptability scores and pentane formation have been used to determine rancidity of oils by gas chromatographic techniques (Gray, 1978). Significant correlations were obtained between the amount of pentane produced and the number of rancid descriptions of aged vegetable oils and potato chips (Warner *et al.*, 1974). Headspace pentane concentration and sensory scores for rancidity development in stored freeze-dried pork samples was reported by Coxon (1987).

Another secondary change that occurs in biological materials during autoxidation is the formation of fluorescent products from the reaction of MA with amino compounds such as proteins and nucleotides (Gray, 1978; Coxon, 1987). Advantages of the fluorescence method as a mean of measuring lipid oxidation have been reported (Dillard and Tappet, 1971; 1973). The method which is very sensitive

can detect fluorescent compounds at a level of one part per billion and was found to be 10 to 100 times more sensitive than the TBA assay.

### 2.5.3 Recent developments in measurement of lipid oxidation

Lipid oxidation in foods and biological systems has conventionally been studied by monitoring the primary and secondary lipid oxidation products. On the last twenty years advances in pulse radiolysis (Simic, 1980) and electron spin resonance or ESR (Schaich and Borgi, 1980) techniques have facilitated the detection and study of short-lived free radical intermediates. Use of ESR to detect free radicals (ESR detects species with unpaired electrons only) requires rapid freezing, lyophilization or spin trapping (Davies, 1987). Although the application of ESR is precise to study lipid oxidation in animal tissues and other biological model systems its application to food systems is relatively new. However, a method to determine both the primary and secondary products of lipid oxidation simultaneously has not been developed. Due to the differences in chemical nature of these products it is not possible to postulate a chemical method. The solution to this problem may be obtained when changes in the protons in an oxidizing molecule are considered carefully. A possibility of use of proton Nuclear Magnetic Resonance ($^1$H NMR) to evaluate oxidative changes of fatty acid molecule has been described by Saito and Udagawa (1992) and Shahidi (1992).

## 2.6 Problems related to oxidation of canola oil

Canola oil has a unique fatty acid composition which differentiates it from other edible vegetable oils. In general, canola oil has a higher oleic acid (C18:1) content (56-63%) and lower linoleic acid (C18:2) content (19-22%) than most other edible vegetable oils (Table 2.5). Canola oil has a high content (8-14%) of linolenic acid (C18:3) as compared to other vegetable oils such as soybean, sunflower, olive and corn. The higher unsaturated fatty acid content, especially C18:3, in canola influences oil quality and stability. Fresh canola oil is odourless, bland and light coloured. During storage, canola oil develops off-flavours and its quality deteriorates due to autoxidation (Hawrysh, 1990).

According to Canadian canola oil standards (Section 43, Schedule II, Processed Products Standards, 1987), a high quality canola oil should have a PV below 2.0 meq/kg. For fresh commercially processed canola oils, a PV of less than 2.0 meq/kg is associated with sensory scores indicative of oils that are bland (Hawrysh *et al.*, 1988; 1990).

Stability of canola oil with respect to its flavour deterioration has been investigated in the past several years. Studies done by Hawrysh and co-workers (1989) concluded that container material and light play an important role in determining oxidative stability of canola oil. Storage of canola oil in amber coloured bottles under simulated supermarket conditions lowered peroxide formation and delayed flavour deterioration as compared to when it was stored in clear glass bottles.

Table 2.5 Comparison of major fatty acids of some edible vegetable oils (w/w% fatty acids)[a].

| Fatty acid | Canola | Soybean | Corn | Safflower | Sunflower | Peanut | Olive |
|---|---|---|---|---|---|---|---|
| 14:0 | 0.05 | 0.1 | - | 0.1 | - | 0.1 | - |
| 16:0 | 3.55 | 10.8 | 11.4 | 6.5 | 6.2 | 10.0 | 11.0 |
| 16:1 | 0.28 | 0.2 | - | 0.4 | - | 0.1 | 0.8 |
| 18:0 | 1.38 | 4.0 | 1.9 | 2.3 | 4.7 | 2.3 | 2.2 |
| 18:1 | 55.58 | 23.8 | 25.3 | 12.2 | 20.4 | 47.1 | 75.8 |
| 18:2 | 21.87 | 53.3 | 60.7 | 77.4 | 68.8 | 33.6 | 8.3 |
| 18:3 | 12.99 | 7.1 | 0.7 | 0.4 | - | - | 0.6 |
| 20:0 | 0.43 | - | - | - | - | - | - |
| 20:1 | 1.78 | 0.2 | - | - | - | 1.4 | 0.3 |
| 22:0 | 0.20 | - | - | - | - | - | - |
| 22:1 | 1.63 | - | - | - | - | - | _ |

[a] Ackman (1990)

McMullen (1988) and Hawrysh (1989) showed that packaging of canola oil in polyvinyl chloride (PVC) bottles is not suitable for prolonged storage because of permeability of PVC to oxygen. Warner *et al.* (1989) showed that the exposure of low erucic acid rapeseed oil to fluorescent light (7535 lux at 30°C) increased PV and the flavour scores of the oil decreased. It has been concluded that to retard oxidative deterioration and to minimize off-flavour development, packaging materials selected for canola oils should be amber in colour (Hawrysh *et al.*, 1989) or impervious to light of low wavelengths (Sattar *et al.*, 1976a,b). Heating of canola oil to frying temperature develops an unpleasant room odour (Dobbs *et al.*, 1978; Eskin, 1989) that has been described as painty with buttery, sweet, sulphur-like and possessing fishy notes. Niewieadomski (1970) attributed this unpleasant odour to the oxidation of unsaturated fatty acids and later McKeag (1977) suggested that the heated room odour of canola oil may be due to the oxidation of linolenic acid. Hydrogenation reduces the linolenic acid content of canola oil and tends to decrease heated room odour development in the product (Dobbs *et al,*. 1978; Eskin *et al.*, 1989a). Comparison of the flavour and oxidative stability of low- and high-linolinate canola oils showed that low-linolinate canola oil had improved flavour quality and storage stability (Eskin *et al.*, 1989b).

# CHAPTER 3
# MATERIAL AND METHODS

## 3.1 Materials

Canola seeds and oils, either fresh, refined bleached (RB) or refined bleached deodourized (RBD) containing no antioxidants were obtained from CSP Foods (now CanAmera), Saskatoon, Saskatchewan. RBD soybean oil was obtained from CanAmera Foods, Hamilton, Ontario. Synthetic antioxidants namely tert-butylhydroquinone (TBHQ), butylated hydoxyanisole (BHA), butylated hydoxytoluene (BHT) and commercially available flavonoids (apigenin, chrysin, [-]epicatechin, kaempferol, morin, myricetin, naringenin, naringin, quercetin, rutin and taxifolin) were obtained from either Sigma (St. Louis, Missouri) or Aldrich Chemical Company (Milawaukee, Wisconsin). Monoglyceride citrate (CA) was obtained from Griffiths Laboratory, Scarborough, Ontario. All other chemicals used in this study were ACS grade or better.

## 3.2 Preparation of canola extract

Canola seeds were first ground using a Moulinex coffee grinder, then defatted with hexenes using a Soxhlet apparatus and air dried overnight. Defatted canola meal (6.0 g) was extracted with 100 ml of 95% ethanol for 20 min at 80°C. The extraction was repeated two times and residual meal was separated by centrifugation (10 min at 5000 x g). Ethanolic extracts were combined and evaporated to dryness under vacuum at 40°C. This procedure was repeated to obtain a sufficient amount of canola

extract. The dried extract so obtained from several 6.0 g lots of defatted canola meals was transferred into air-tight glass vials and stored at -20°C until use.

### 3.3 Preparation of oil for accelerated oxidation studies

The dried canola extract, synthetic antioxidants and flavonoids were applied to RB canola oil at levels indicated in Table 3.1. The additives were dissolved in a minimum amount of absolute ethanol, then added to the oil (200 g) and mixed thoroughly for 10 min using an ultrasonic water bath. Control samples of oil contained the same minimum amount of ethanol used to dissolve additives. Since the oil samples were cloudy and turbid when 500 or 1000 ppm of canola extract was used, another set of experiments were carried out using 500 and 1000 ppm levels of extracts followed by centrifugation (15 min at 5000 x g) to remove undissolved matters.

The weight gain of 2.0 g of canola oil, as such or treated with different additives, (in triplicate) in glass petri dishes (60 mm diameter and 15 mm height), was carried out after removing traces of water in a vacuum oven set at 35°C overnight. Each sample was reweighed and stored in a forced-air oven (Thelco, Model 2, Precision Scientific Co. Chicago) at 65°C. The rate of oxidation in terms of weight increase was recorded at 24 h intervals over a period of 20 days. The time required for a 0.5% weight increase for each oil sample was taken as the index of stability (Olcott and Einest, 1958).

Table 3.1 Types and levels of additives used in RB canola oil for accelerated oxidation studies.

| Additives | Level |
|---|---|
| Canola extract | 100 ppm (CE-100 ppm) |
| | 200 ppm (CE-200 ppm) |
| | 500 ppm (CE-500 ppm) |
| | 500 ppm (CE-500 ppm, centrifuged) |
| | 1000 ppm (CE-1000 ppm) |
| | 1000 ppm (CE-1000 ppm, centrifuged) |
| BHA/BHT/CA[a] | 100/100/50 ppm (BHA/BHT/CA-250 ppm) |
| TBHQ | 200 ppm |
| Apigenin | 200 ppm |
| Chrysin | 200 ppm |
| (-)Epicatechin | 200 ppm |
| Kaempferol | 200 ppm |
| Morin | 200 ppm |
| Myricetin | 200 ppm |
| Naringenin | 200 ppm |
| Naringin | 200 ppm |
| Quercetin | 200 ppm |
| Rutin | 200 ppm |
| Taxifolin | 200 ppm |

[a] citric acid as its monoglyceride citrate derivative

A 25 ml sample of each treated oil was stored separately under the same conditions in open glass containers (30 mm diameter and 60 mm height) for other chemical analysis. Samples from each treatment were removed on days 0, 2, 5, 9, 13, and 17, flushed with nitrogen, sealed with parafilm and stored at -20°C until analysed.

For nuclear magnetic resonance (NMR) studies samples (25 ml, RBD canola and soybean oils) were stored under the same accelerated oxidation conditions described above for up to 30 days. Sampling was done at 5 day intervals.

### 3.4 Separation of RB canola oil into neutral lipids (NL), glycolipids (GL) and phospholipids (PL)

Separation of lipid classes was done according to the method described by Christie (1982). Samples of the RB canola oil (1.75 g) were applied to a silicic acid column (1.25 cm internal diameter and 20 cm height; 100 mesh silicic acid powder, Mallinckrodt Canada Inc. Pointe-Claire, Quebec). First the neutral lipid (NL) fraction of oil was eluted with chloroform (1200 ml), then monogalactosyl diacylglycerol and digalactocyl diacylglycerol were eluted with chloroform-acetone (50:50 v/v, 900 ml) and acetone (1200 ml), respectively. Finally methanol (1200 ml) was used to elute the phospholipid (PL) fraction. Solvents were removed under vacuum using a rotary evaporator at 40°C. All fractions were weighed and the weight percentage of NL, GL (both mono and digalactocyl diacylglycerol) and PL was calculated.

## 3.5 Fractionation of canola meal extract

Fractionation of ethanolic extract of canola meal was carried out according to the scheme given in Figure 3.1.

### 3.5.1 Sephadex column chromatography

A column (1.5 cm diameter and 77 cm height) was packed with Sephadex LH-20 (particle size 25-100 μm, Pharmacia, Uppsala, Sweden). A 0.5 g sample of dried canola meal extract was dissolved in 3 ml of methanol and was subsequently introduced to the top of the column. The same solvent was used for elution and 8 ml fractions were collected using a LKB Bromma 2112 redirac fraction collector (Phamacia, Uppsala, Sweden).

According to UV absorbance values and contents of phenolics and sugars, samples were separated into seven major fractions (I, II, III, IV, V, VI and VII) and antioxidant activity of each fraction was evaluated in a β-carotene-linoleate model system. Fraction number IV showed the highest antioxidant activity and was further separated by thin layer chromatography (TLC). Antioxidant activity of separated bands were determined using a β-carotene-linoleate spray as given in Section 3.8. The same fraction (fraction IV) was also loaded onto preparative TLC plates and the band corresponding to the $R_f$ value of the most active antioxidative compound (determined by cochromatography) was isolated. Further purification of this compound is described in Section 3.5.2.

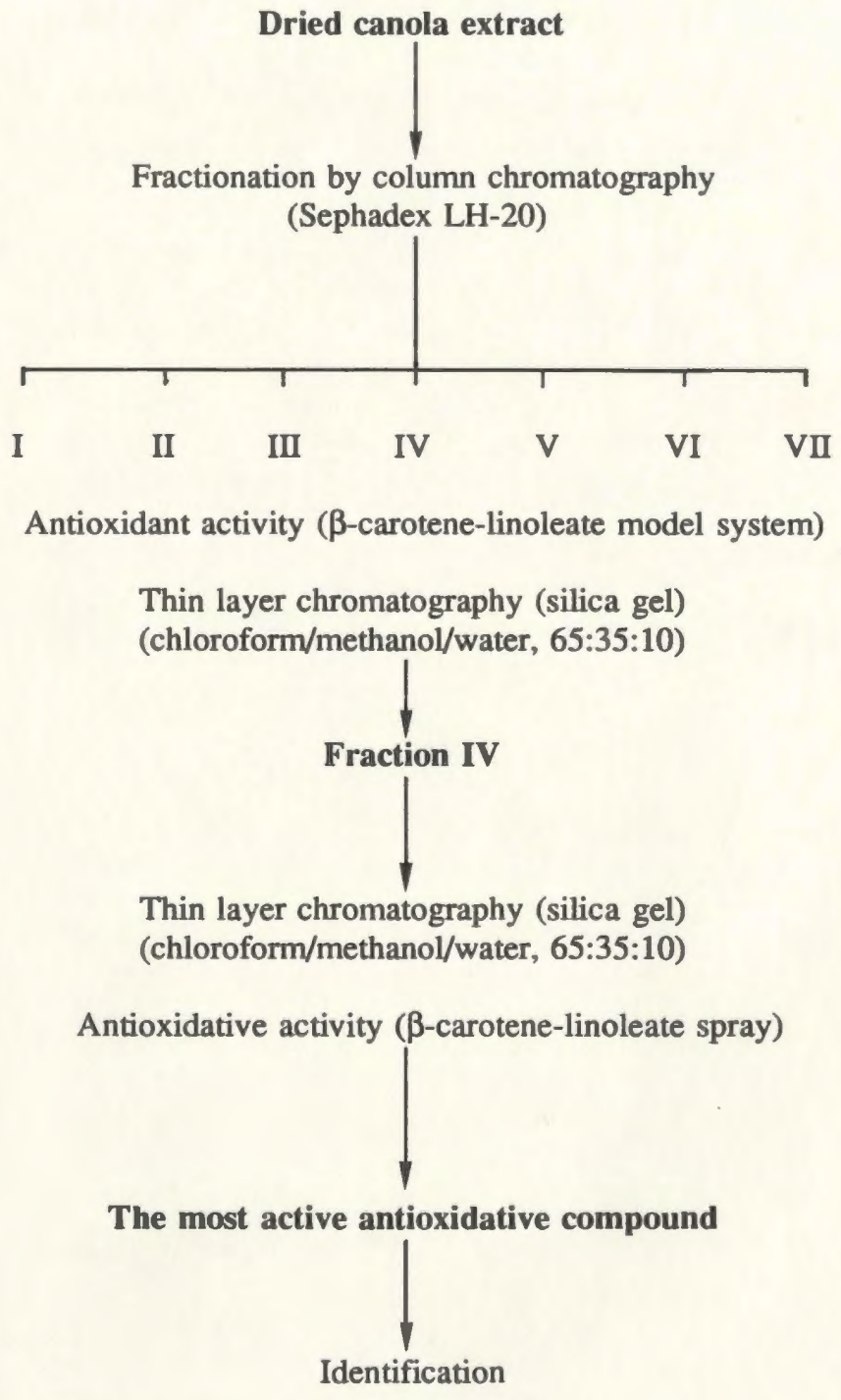



Figure 3.1 Fractionation of ethanolic canola meal extract

### 3.5.2 Thin layer chromatography (TLC)

Different fractions separated by Sephadex LH-20 chromatography (Figure 3.1) were loaded onto thin layer chromatographic (TLC) plates (Silica gel, 60 Å mean pore diameter, 2-25 µm mean particle size, 250 µm thickness, Sigma Chem. Co., St. Louis, Missouri). Chromatograms were developed in a glass chamber 22 cm x 22 cm x 10 cm (Fisher Scientific Ltd. Toronto, Ontario) 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. Westbury, New York). The following sprays (spray 1 and 2) were used to tentatively identify chemical classes of compounds.

Spray 1. 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 solution). Phenols give blue colour with this reagent immediately (Barton *et al.*, 1952).

Spray 2. Ferric chloride in alcohol: $FeCl_3$

A 2% (w/v) solution of $FeCl_3$ in absolute ethanol was prepared. Phenolics with trihydroxy and dihydroxy groups give distinct blue and green colour, with this reagent, respectively. Other phenolics give a red or brown colour (Reio, 1958).

The fraction with highest antioxidative activity in a β-carotene-linoleate model system (fraction IV) was loaded onto a similar TLC plate and chromatogram was developed using chloroform/methanol/water (65:35:10, v/v/v). The antioxidant activity of separated bands was determined according to the procedure given in Section 3.8.

To obtain sufficient quantities of the most active antioxidative compound, fraction IV was subjected to preparative TLC separation. Band with the same $R_f$ value of the most active antioxidative compound (seen in analytical TLC) was scraped and extracted with spectral grade methanol. The mixture was centrifuged (3 min at 5000 x g) and the supernatant was evaporated to dryness under vacuum at 40°C. The dried residue was used for further analysis.

## 3.6 Chemical analyses

### 3.6.1 Analysis of fatty acids

Fatty acid composition of RBD soybean and canola oils and RB canola oil was determined. Fatty acid methyl esters (FAME) were prepared by transmethylation of the lipid fatty acids in 6% $H_2SO_4$ in 99.9 mole% of methanol at 65-70°C for 15 h (Keough and Kariel, 1987). After extraction of the methyl esters into hexane, they were analyzed using a Perkin-Elmer 8310 GC equipped with a 30m x 0.25 mm column (SP 2330, Supelco, Oakville, Ontario). Oven temperature was initially 180°C for 12 min and was ramped to 200°C at 20°C/min and held there for 8 min. The

injection port and flame ionisation detector temperatures were 230°C and 250°C, respectively. The flow rate of the helium carrier gas was 25 ml/min. Identification of FAME was based on the comparison of their retention times with those of FAME standard mixture (Supelco INC, Oakvill, Ontario). Quantification was performed by the computer control using area normalization.

### 3.6.2 Acid value (AV)

Acid value of oil samples was determined according to the AOCS (1990) method. Samples (15-20 g) were weighed into a 250 ml glass Erlenmeyer flask and 50 ml of 95% (v/v) ethanol (neutralized with 0.5N KOH) and 2 ml of 1% phenolphthalein indicator solution were added to it. The contents were mixed and heated until the temperature was reached to 70°C. The mixture was then titrated with a standardized 0.1N potassium hydroxide (KOH) solution until a permanent pink colour appeared. A blank titration was conducted each time. The AV was expressed as the number of milligrams of KOH required to neutralize the free fatty acids in a gram of oil.

$$AV = \frac{(V_{Sample} - V_{Blank}) \times N_{KOH} \times 56.1}{\text{Mass of sample (g)}}$$

Where V = volume of potassium hydroxide (ml) and N = normality of potassium hydroxide.

### 3.6.3 Iodine value (IV)

Iodine value of oil samples was determined according to the AOCS (1990) method. Samples (0.1-0.2 g) were weighed into a 250 ml glass-stoppered Erlenmeyer flask and 10 ml of chloroform was added to it. After thorough mixing, the flask was wrapped in aluminium foil and 25 ml of Hanus iodine solution was added to it and the mixture was allowed to stand for 30 min in dark. After this period, a 10 ml solution of 15% potassium iodide (KI) and 100 ml of distilled water were added to the sample. The mixture was then titrated with a standardized 0.1N solution of sodium thiosulphate ($Na_2S_2O_3$) with constant shaking until the yellow colour had almost disappeared. Then 0.5 ml of a solution of starch indicator (1% w/v) was added to the mixture and titration was continued until the blue colour of starch-iodine complex disappeared. A blank titration was conducted each time. The IV was expressed as the uptake of iodine in grams by 100 g of oil.

$$IV = \frac{(V_{Blank} - V_{Sample}) \times N_{Na_2S_2O_3} \times 12.692}{\text{Mass of sample (g)}}$$

Where V = volume of sodium thiosulphate solution (ml) and N = normality of sodium thiosulphate solution.

### 3.6.4 Peroxide value (PV)

The method described by AOCS (1990) was used to determine PV of the oil samples. Samples (2.0-4.0 g) were weighed into 250 ml glass-stoppered Erlenmeyer flasks and 30 ml of acetic acid-chloroform (3:2, v/v) solution was then added to each. The contents were mixed until oil was dissolved and then 0.5 ml of saturated potassium iodide (KI) solution was added to it. The mixture was allowed to stand in stoppered flasks with occasional shaking for exactly 1 min and then 30 ml of distilled water was added to it. The liberated iodine was titrated with standardized 0.01N sodium thiosulphate ($Na_2S_2O_3$) solution, with constant shaking, until the yellow colour had almost disappeared. About 0.5 ml of starch indicator solution (1% w/v) was then added to the flask and titration was continued with vigorous shacking until the blue colour of the solution had disappeared. A blank titration was conducted each time. PV was expressed as the uptake of milliequivalents of active oxygen (i.e. peroxide) per kilogram of oil.

$$PV = \frac{(V_{Sample} - V_{Blank}) \times N_{Na_2S_2O_3} \times 1000}{\text{Mass of sample (g)}}$$

Where V = volume of sodium thiosulphate solution (ml) and N = normality of sodium thiosulphate solution.

### 3.6.5 Conjugated diene value (CD)

Conjugated diene value of oil samples was measured by the method of IUPAC (1987). Oil samples (0.02-0.04 g) were weighed into 25 ml volumetric flask, dissolved in isooctane (2,2,4-trimethylpentane) 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 8452A diode array spectrophotometer. Pure isooctane was used as the reference. Conjugated diene value was calculated as:

$$CD = \frac{A}{(c \times d)}$$

Where A = absorbance of the solution at 234 nm, c = concentration of the solution in g/100 ml solution and d = length of the cell (cm).

### 3.6.6 2-Thiobarbituric acid reactive substances (TBARS)

The direct method of determining TBARS value by AOCS (1990) was used. Oil (50-200 mg) was accurately weighed into a 25 ml volumetric flask and dissolved in a small volume of 1-butanol and made up to volume with same solvent. Five millilitres of this solution was transferred into a dry test tube to which 5 ml of fresh TBA reagent (200 mg TBA in 100 ml 1-butanol) was added. The contents were thoroughly mixed and heated in a water bath at 95°C for 120 min. Heated samples were cooled and the absorbance of the resultant coloured complex was read at 532

nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (TMP) as malonaldehyde (MA) precursor (Yu and Sinnhuber, 1967). Micromoles of MA equivalents in a gram of oil, expressed as TBARS value, was calculated using the equation $C = (0.355A_{532})/w$, $r = 0.995$ (for details see Figure A.1 in the Appendix).

### 3.6.7 p-Anisidine value (AnV)

The p-Anisidine value is defined as the optical density measured at 350 nm in a 1.0 cm cell of a solution containing 1.0 g of the oil in 100 ml of a mixture of solvent and reagent according to the method described in AOCS (1990) multiplied by 100. A 0.5-2.0 g of oil was weighed into a 25 ml volumetric flask and dissolved in isooctane (2,2,4-trimethylpentane), to the mark. Absorbance of the solution at 350 nm was read as soon as possible using a reference cell filled with the solvent. Five millilitres of the solution was then transferred into a dry test tube and 1.0 ml of p-anisidine reagent (0.25 g p-anisidine/100ml of glacial acetic acid) was added to it. The absorbance of the solution was then measured at 350 nm after exactly 10 min. A solution containing five millilitres of isooctane and 1.0 ml p-anisidine reagent was used as the blank. The AnV was calculated by the following formula.

$$AnV = \frac{25 \times (1.2A_S - A_B)}{m}$$

Where $A_S$ = absorbance of the solution containing oil after reaction with the p-

anisidine reagent, $A_B$ = absorbance of the solution containing oil and m = mass of the oil sample (g).

### 3.6.8 TOTOX value

The total oxidation (TOTOX) value was calculated from PV and AnV data for each sample according to the following equation.

$$\text{TOTOX value} = 2PV + AnV$$

### 3.6.9 Determination of phenolic content of fractions separated by column chromatography

Concentration of total phenolics of each fraction was estimated colorimetrically using the Folin-Denis reagent as described by Swain and Hillis (1959). To a 0.1 ml of the test solution 0.25 ml Folin-Denis reagent, 0.5 ml saturated $Na_2CO_3$ and 2 ml distilled water were added and mixed thoroughly. After 30 min standing at room temperature, the mixtures were centrifuged and absorbance values at 725 nm were recorded.

### 3.6.10 Determination of sugar content of fractions separated by column chromatography

The sugar content of each fraction was estimated by mixing 0.1 ml of sample with 0.05 ml of 80% (v/v) phenol solution and 5 ml concentrated $H_2SO_4$ (Dubois *et*

*al.*, 1956). Absorbance of the samples at 490 nm was read after 30 min standing at room temperature.

### 3.7 UV absorbance of column chromatographic fractions

A 0.1 ml of each sample fraction was mixed with 2 ml of spectral grade methanol. Absorbance of each sample at 280 nm was measured using a Hewlett-Packard 8452A diode array spectrophotometer.

### 3.8 Evaluation of antioxidant activity

Antioxidant activity of the isolated fractions was evaluated using a β-carotene-linoleate model system (Miller, 1971). A solution of β-carotene was prepared by dissolving 2.0 mg of β-carotene in 10 ml of chloroform. One millilitre of this solution was then pipetted into a round-bottom flask. After removing chloroform under vacuum using a rotary evaporator at 40°C, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier and 50 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquotes (5 ml) of this prepared emulsion were transferred into a series of tubes containing 2 mg of each fraction (fractions I to VII) or BHA for comparative purposes. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 15 min intervals by keeping the samples in a water bath at 50°C until the colour of β-carotene had disappeared (about 120 min).

Relative antioxidant activity of band components on the developed TLC plates were detected using the β-carotene spray method described by Pratt and Miller (1984). Nine milligrams of β-carotene was dissolved in 30 ml chloroform. Two drops of linoleic acid and 60 ml of ethanol were added to the β-carotene chloroform solution. This solution was sprayed on TLC plates which were then exposed to day-light for 2-3 h or until the background colour was bleached. Bands in which the yellow colour persisted were considered as having antioxidant activity and the colour intensity was related to their activity strength.

## 3.9 Instrumental analysis of the most active antioxidative compound of canola extract

### 3.9.1 UV Spectrophotometry

UV absorption spectrum of the most active antioxidative compound in methanol after purification, was recorded using a Hewlett-Packard 8452A diode array spectrophotometer.

### 3.9.2 Mass spectrometry (MS)

All mass spectra were recorded using an electron ionization (EI) mode at 70 eV with a 7070 HS Micromass double focusing mass spectrometer. The temperatures used were source at 200°C, probe at 100-300°C and scanning at 20-25°C.

### 3.9.3 Proton and carbon nuclear magnetic resonance spectrometry

NMR spectra were recorded on a General Electronic 300-NB spectrometer. $^{1}H$ (at 300 MHz) and $^{13}C\{^{1}H\}$ (at 75.5 MHz) NMR data were collected at room temperature in $CD_3OD$. Chemical shifts were reported relative to tetramethylsilane (TMS) internal standard.

## 3.10 NMR spectrometry of oil samples

Proton NMR ($^{1}H$ NMR) spectra of the oil samples, subjected to accelerated oxidation, were recorded using a 300 MHz nuclear magnetic resonance spectrometer (General Electric GN-300) in $CDCl_3$ solvent. Tetramethylsilane (TMS) was used as the internal standard. Solutions containing approximately 35 mg oil in $CDCl_3$ were placed in NMR tubes and the spectra were recorded. The total number of protons under each peak was calculated on the basis of integration of methylene protons of the triacylglycerol backbone.

## 3.11 Statistical analysis

All experiments and/or measurements were replicated 3 times. Mean values ± standard deviation were reported for each case. Analysis of variance and Tukey's studentized range test (Snedecor and Cochran, 1980) were performed on Statistical Analysis System (SAS Inc. 1990, North Carolina, USA) to evaluate the significance of differences between different mean values. Relationships of parameters were established using a linear regression method.

# CHAPTER 4
# RESULTS AND DISCUSSION

## 4.1 Chemical properties of refined-bleached canola oil

Fresh refined-bleached (RB) canola oil was used in this study. It had low acid, iodine and peroxide values as summarized in Table 4.1. The Canadian standards (Canada Agriculture Products Act. 1955) require that high quality canola oil have an acid value of less than 0.6 mg KOH/g oil, an iodine value between 110 and 126 g iodine/100 g oil and a peroxide value below 2 meq/kg oil.

Small amounts of glycolipids (1.38%) and phospholipids (1.02%) were present in this RB oil and the rest were neutral lipids (97.60%). The low content of polar lipids may be due to their partial removal during the degumming process. The total fatty acid composition of RB canola oil indicated that it contained approximately 58% oleic, 23.50% linoleic, 9.37% linolenic acids and a small amount of erucic acid (0.27%) (Table 4.2). According to the Canadian standards, erucic acid content of canola oil should not exceed 2% of its total fatty acids. The fatty acid composition of the oil used in this study showed the typical fatty acid profile of canola oil as reported by Hawrysh *et al.* (1988) and McMullen *et al.* (1991).

Table 4.1 Acid value, iodine value, peroxide value and lipid classes of refined-bleached canola oil (without additives).

| Parameter | Content |
|---|---|
| Acid value (mg KOH/g oil) | 0.05 ± 0.00 |
| Iodine value (g iodine/100 g oil) | 112.0 ± 1.9 |
| Peroxide value (meq/kg oil) | 0.20 ± 0.01 |
| Lipid classes (weight %): | |
| Neutral lipids | 97.6 ± 0.8 |
| Glycolipids | 1.38 ± 0.21 |
| Phospholipids | 1.02 ± 0.16 |

Table 4.2 Fatty acid composition (area %) of refined-bleached canola oil (without additives).

| Fatty acid | Content |
|---|---|
| C16:0 | 4.23 ± 0.00 |
| C16:1 | 0.23 ± 0.00 |
| C18:0 | 1.89 ± 0.01 |
| C18:1 | 57.70 ± 0.10 |
| C18:2 | 23.50 ± 0.10 |
| C18:3 | 9.37 ± 0.03 |
| C20:0 | 0.63 ± 0.00 |
| C20:1 | 1.84 ± 0.01 |
| C22:0 | 0.33 ± 0.01 |
| C22:1 | 0.27 ± 0.01 |

## 4.2 Stability of refined-bleached canola oil as affected by the addition of canola extract (CE), flavonoids and synthetic antioxidants measured by various physical and chemical indices

### 4.2.1 Effect on weight gain, peroxide and conjugated diene (CD) values

Effect of added CE, BHA/BHT/CA and TBHQ, at levels of 100-1000 ppm, on weight gain of canola oil during accelerated oxidation is presented in Figure 4.1. The time required for a 0.5% weight increase (Olcott and Einset, 1958) of oil sample was taken as the length of induction period. It was 5.0, 6.0, 6.3, 6.5, 7.8 and 8.5 days for oils to which CE-100, CE-200, BHA/BHT/CA-250, CE-500, CE-1000 and TBHQ-200 were added, respectively. Weight gain data of canola oil treated with different flavonoids as compared with BHA/BHT/CA and TBHQ are shown in Figure 4.2. All flavonoid-treated samples showed a delayed induction period compared to the control. The time required to achieve a 0.5% weight increase by the sample was 3.5 days for apigenin, kaempferol, chrysin and naringenin, 4.0 days for taxifolin, 5.5 days for naringin and morin, 5.8 days for quercetin, 6.0 days for rutin, 7.0 days for (-)epicatechin and 15 days for myricetin. The corresponding value for the control sample was 3.2 days.

The extension of the induction period of the oil by using CE-500, CE-1000 and TBHQ was 2, 2.5 and 2.7 times that of the control, respectively. Furthermore, samples containing CE-500 and CE-1000 had a delayed induction period compared to that of BHA/BHT/CA which is commonly used in canola oil. Extension of the induction period by (-)epicatechin and myricetin was 2 and 5 times that of the control,

Figure 4.1 Effect of added canola extract and synthetic antioxidants on the weight gain of refined-bleached canola oil stored at 65°C.

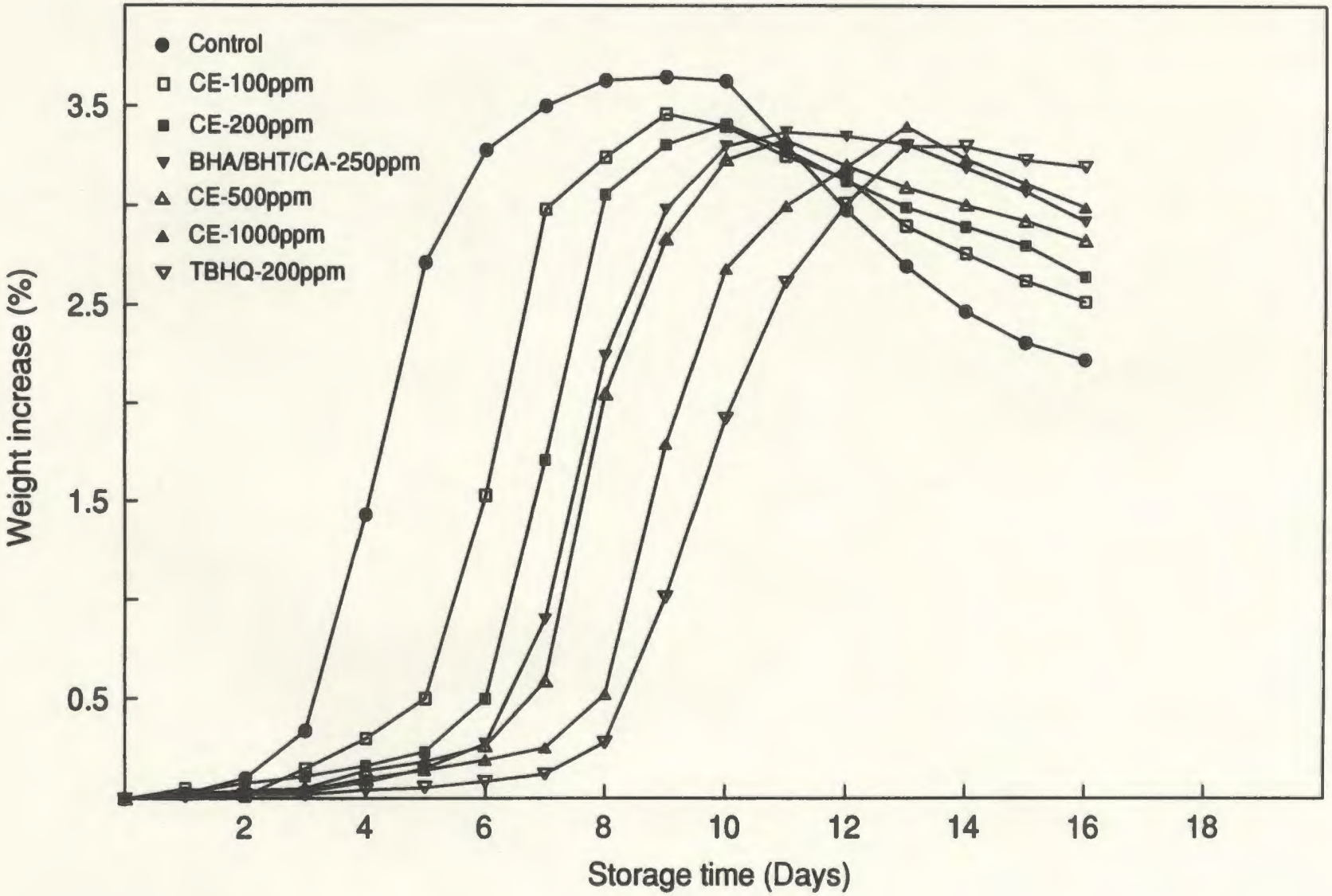
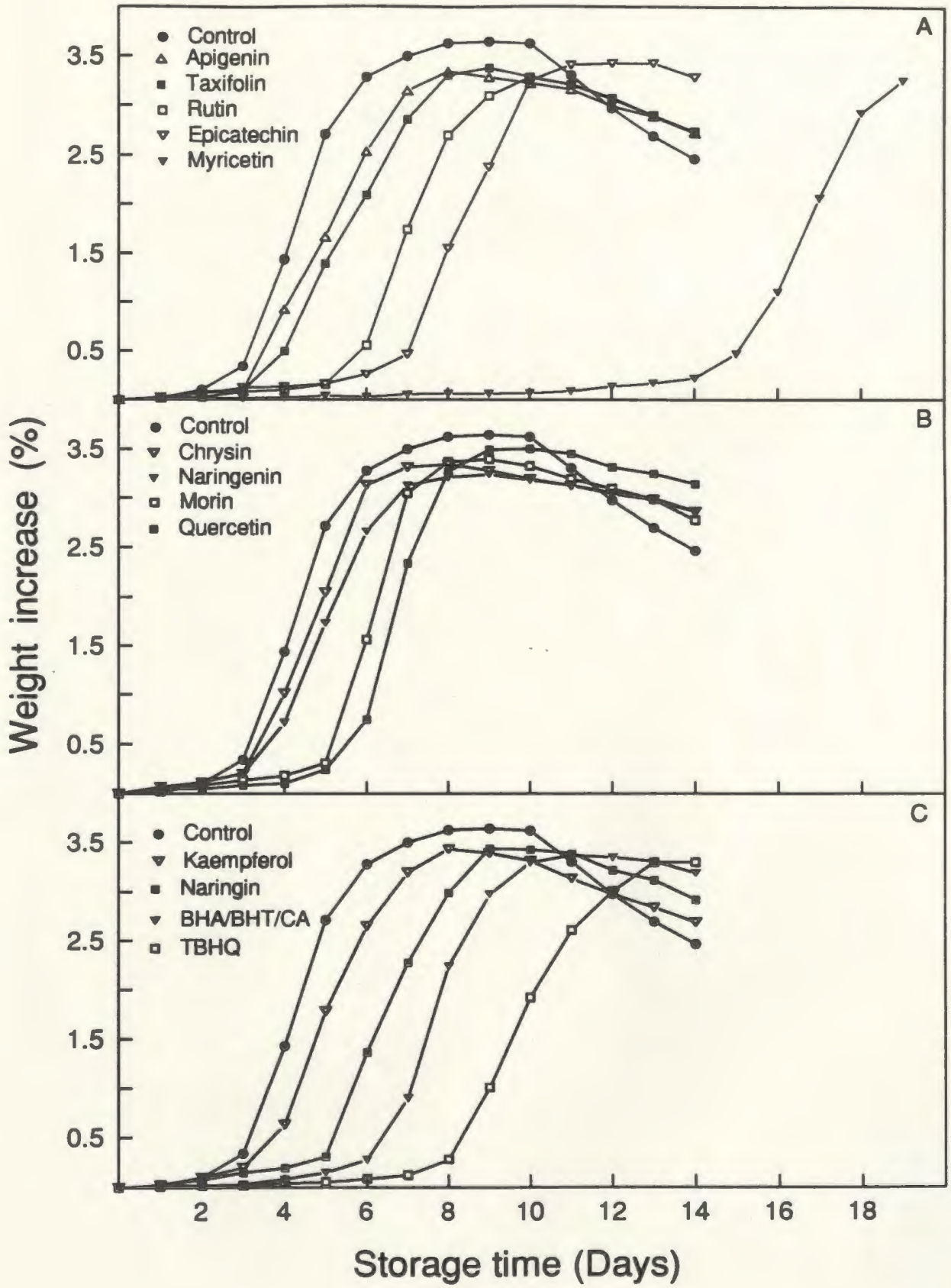

Control
CE-100ppm
CE-200ppm
BHA/BHT/CA-250ppm
CE-500ppm
CE-1000ppm
TBHQ-200ppm
Weight increase (%)
3.5
2.5
1.5
0.5
2
4
6
8
10
12
14
16
18
Storage time (Days)

Figure 4.2 Effect of flavonoids and synthetic antioxidants on the weight gain of refined-bleached canola oil stored at 65°C.


A
Control
Apigenin
Taxifolin
Rutin
Epicatechin
Myricetin
B
Control
Chrysin
Naringenin
Morin
Quercetin
C
Control
Kaempferol
Naringin
BHA/BHT/CA
TBHQ
Weight increase (%)
0.5
1.5
2.5
3.5
2
4
6
8
10
12
14
16
18
Storage time (Days)

respectively. Furthermore, (-)epicatechin and myricetin were more effective than BHA/BHT/CA; however, myricetin was even more effective than TBHQ, the strongest synthetic antioxidant used by the food industry.

A gradual increase in the percent weight gain of all oil samples towards a maximum value with a subsequent decrease during the extended storage period was noticed. The increase in the weight gain may be due to the addition of oxygen to lipid molecules to form hydroperoxides during primary stages of oxidation. The decrease in weight gain in later stages of oxidation may be due to volatile nature of breakdown products of lipid hydroperoxides. Farmer *et al.* (1942, 1943) and Privett and Nickell (1956) have reported that addition of oxygen to lipid to form peroxides is reasonably quantitative during the initial stages of autoxidation. Olcott and Einset (1958) have reported that the weight gain serves as a useful technique to evaluate the effect of antioxidants on the oxidative stability of edible vegetable oils. Ke and Ackman (1976) reported that the method is simple, has a satisfactory reproducibility and can be used to compare oxidation of lipids obtained from different parts of fish. However, surface exposure of the sample to air is an important variable in determining the rate of oxidation, therefore, use of equal size containers to store sample is very important in carrying out the experiments (Kwon *et al.*, 1984).

Peroxide and conjugated diene (CD) values of canola oil samples containing CE, flavonoids, BHA/BHT/CA and TBHQ are presented in Tables 4.3, 4.4, 4.5 and 4.6. Both of these indices measure primary products of lipid oxidation. Addition of

Table 4.3 Effect of CE, BHA/BHT/CA and TBHQ on peroxide value (meq/kg oil) of refined-bleached canola oil stored at 65°C[1].

| Treatment | Storage period, Days | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 | 17 |
| Control | 0.37±0.01[a] | 22.5±0.7[a] | 83.6±0.5[a] | 125.0±4.0[a] | 159.0±3.0[a] | 183.4±2.1[a] |
| CE-100ppm | 0.32±0.02[a] | 7.86±1.25[b] | 42.8±0.6[b] | 63.3±0.3[b] | 94.6±1.5[b] | 107.3±2.2[b] |
| CE-200ppm | 0.31±0.03[a] | 1.92±0.11[d] | 38.8±0.3[c] | 62.2±0.9[b] | 92.0±0.6[c] | 102.5±1.2[c] |
| CE-500ppm | 0.30±0.00[a] | 1.47±0.15[d] | 32.9±1.0[e] | 53.1±1.1[c] | 87.2±1.2[d] | 102.1±2.0[c] |
| CE-500ppm (centrifuged) | 0.33±0.01[a] | 1.62±0.05[d] | 33.1±0.7[e] | 54.4±0.5[c] | 91.9±0.8[c] | 108.2±3.1[b] |
| CE-1000ppm | 0.32±0.03[a] | 1.31±0.02[d] | 22.7±0.4[g] | 43.2±0.7[e] | 80.0±0.9[e] | 93.5±0.6[d] |
| CE-1000ppm (centrifuged) | 0.34±0.01[a] | 1.45±0.01[d] | 28.7±0.7[f] | 48.2±0.7[d] | 78.0±1.0[e] | 102.2±3.1[c] |
| BHA/BHT/CA-250ppm | 0.31±0.05[a] | 3.03±0.12[c] | 36.5±1.4[d] | 55.1±1.0[c] | 62.8±0.7[f] | 78.4±0.3[e] |
| TBHQ-200ppm | 0.32±0.04[a] | 1.13±0.11[d] | 2.02±0.23[h] | 3.77±0.28[f] | 5.37±0.48[g] | 64.0±0.6[f] |

[1] Values in the same column bearing different superscripts are significantly (P>0.05) different.

Table 4.4 Effect of flavonoids, BHA/BHT/CA and TBHQ on peroxide value (meq/kg oil) of refined-bleached canola oil stored at 65°C[1].

| Treatment | Storage period, Days | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 |
| Control | 0.37±0.01$^{a}$ | 22.5±0.7$^{a}$ | 83.6±0.5$^{a}$ | 125.0±4.0$^{a}$ | 159.0±3.0$^{a}$ |
| Flavones: | | | | | |
| Apigenin | 0.37±0.01$^{a}$ | 20.1±0.1$^{b}$ | 63.4±0.1$^{b}$ | 107.2±1.5$^{b}$ | 153.3±1.2$^{b}$ |
| Chrysin | 0.35±0.01$^{a}$ | 22.2±0.2$^{a}$ | 65.8±0.3$^{b}$ | 93.1±2.3$^{d}$ | 145.4±2.7$^{d}$ |
| Flavonols: | | | | | |
| Kaempferol | 0.37±0.01$^{a}$ | 12.0±0.4$^{e}$ | 47.1±1.4$^{de}$ | 96.9±1.2$^{c}$ | 145.2±1.7$^{d}$ |
| Morin | 0.36±0.04$^{a}$ | 9.41±0.51$^{f}$ | 38.4±1.4$^{gh}$ | 64.4±0.7$^{f}$ | 130.3±2.5$^{e}$ |
| Myricetin | 0.36±0.01$^{a}$ | 2.62±0.11$^{i}$ | 10.2±0.4$^{j}$ | 12.0±0.3$^{j}$ | 27.8±0.1$^{j}$ |
| Quercetin | 0.34±0.03$^{a}$ | 7.41±0.21$^{g}$ | 31.3±0.3$^{i}$ | 54.7±0.8$^{gh}$ | 100.7±1.3$^{f}$ |
| Rutin | 0.37±0.00$^{a}$ | 4.63±0.12$^{h}$ | 45.2±0.6$^{e}$ | 64.2±1.1$^{f}$ | 133.3±1.4$^{e}$ |
| Flavanones: | | | | | |
| Naringenin | 0.35±0.01$^{a}$ | 14.6±0.1$^{c}$ | 40.4±1.2$^{fg}$ | 52.5±0.7$^{h}$ | 85.5±1.0$^{h}$ |
| Naringin | 0.36±0.01$^{a}$ | 13.6±0.3$^{d}$ | 48.0±0.4$^{d}$ | 57.5±0.1$^{g}$ | 85.9±0.3$^{h}$ |
| Flavononols: | | | | | |
| Taxifolin | 0.38±0.02$^{a}$ | 21.9±0.2$^{a}$ | 55.5±0.4$^{c}$ | 76.9±0.7$^{e}$ | 149.3±1.2$^{c}$ |
| Flavan-3-ol: | | | | | |
| (-)Epicatechin | 0.36±0.01$^{a}$ | 8.00±0.10$^{g}$ | 41.1±0.3$^{f}$ | 46.9±0.5$^{i}$ | 90.9±1.3$^{g}$ |
| BHA/BHT/CA | 0.31±0.05$^{a}$ | 3.02±0.10$^{i}$ | 36.5±1.4$^{h}$ | 55.1±1.0$^{gh}$ | 62.8±0.7$^{i}$ |
| TBHQ | 0.32±0.04$^{a}$ | 1.13±0.12$^{j}$ | 2.02±0.23$^{k}$ | 3.77±0.28$^{k}$ | 5.37±0.48$^{k}$ |

[1] Values in the same column bearing different superscripts are significantly (P>0.05) different.

Table 4.5 Effect of CE, BHA/BHT/CA and TBHQ on conjugated diene value[1] of refined-bleached canola oil stored at 65°C[2].

| Treatment | Storage period, Days | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 | 17 |
| Control | 2.19±0.02$^{a}$ | 4.72±0.02$^{a}$ | 8.80±0.49$^{a}$ | 13.11±0.61$^{a}$ | 19.8±0.3$^{a}$ | 21.7±0.1$^{a}$ |
| CE-100ppm | 2.18±0.09$^{a}$ | 3.18±0.06$^{b}$ | 5.48±0.29$^{b}$ | 8.51±0.19$^{b}$ | 13.3±0.0$^{b}$ | 14.9±0.1$^{b}$ |
| CE-200ppm | 2.19±0.01$^{a}$ | 2.61±0.01$^{c}$ | 5.15±0.72$^{b}$ | 8.32±0.02$^{bc}$ | 12.6±0.0$^{c}$ | 14.6±0.1$^{b}$ |
| CE-500ppm | 2.17±0.00$^{a}$ | 2.57±0.02$^{c}$ | 4.82±0.26$^{b}$ | 8.16±0.12$^{bc}$ | 11.5±0.0$^{d}$ | 14.5±0.1$^{b}$ |
| CE-500ppm (centrifuged) | 2.16±0.01$^{a}$ | 2.59±0.01$^{c}$ | 4.77±0.20$^{b}$ | 7.96±0.01$^{bc}$ | 12.5±0.2$^{c}$ | 14.8±0.1$^{b}$ |
| CE-1000ppm | 2.14±0.01$^{a}$ | 2.29±0.10$^{d}$ | 4.23±0.60$^{b}$ | 7.72±0.01$^{bc}$ | 10.7±0.1$^{e}$ | 13.8±0.1$^{c}$ |
| CE-1000ppm (centrifuged) | 2.18±0.02$^{a}$ | 2.61±0.00$^{c}$ | 4.50±0.01$^{b}$ | 7.61±0.55$^{c}$ | 11.4±0.1$^{d}$ | 13.9±0.2$^{c}$ |
| BHA/BHT/CA-250ppm | 2.15±0.01$^{a}$ | 3.14±0.01$^{b}$ | 5.04±0.04$^{b}$ | 8.20±0.03$^{bc}$ | 10.8±0.2$^{e}$ | 13.7±0.1$^{c}$ |
| TBHQ-200ppm | 2.17±0.12$^{a}$ | 2.31±0.00$^{d}$ | 2.43±0.00$^{c}$ | 2.55±0.11$^{d}$ | 3.41±0.11$^{f}$ | 9.93±0.03$^{d}$ |

[1] Conjugated diene value = A/(c x d) where, A = absorbance of the solution at 234nm, c = concentration of the solution in g/100ml of solvent, and d = length of the cell in cm.
[2] Values in the same column bearing different supescripts are significantly (P>0.05) different.

Table 4.6. Effect of flavonoids, BHA/BHT/CA and TBHQ on conjugated diene value[1] of refined-bleached canola oil stored at 65°C[2].

| Treatment | Storage period, Days | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 |
| Control | 2.19±0.02$^{a}$ | 4.72±0.02$^{a}$ | 8.80±0.43$^{a}$ | 13.1±0.6$^{a}$ | 19.8±0.3$^{a}$ |
| Flavones: | | | | | |
| Apigenin | 2.24±0.00$^{a}$ | 4.68±0.01$^{a}$ | 8.72±0.07$^{a}$ | 12.2±0.1$^{a}$ | 18.4±0.5$^{ab}$ |
| Chrysin | 2.18±0.06$^{a}$ | 4.57±0.02$^{a}$ | 7.74±0.08$^{a}$ | 10.9±0.1$^{b}$ | 15.5±0.1$^{cd}$ |
| Flavonols: | | | | | |
| Kaempferol | 2.23±0.01$^{a}$ | 3.54±0.03$^{ab}$ | 5.44±0.02$^{abc}$ | 9.16±0.08$^{cd}$ | 17.5±0.5$^{abc}$ |
| Morin | 2.14±0.02$^{a}$ | 2.51±0.02$^{b}$ | 6.53±0.83$^{ab}$ | 9.07±0.07$^{cd}$ | 13.8±0.1$^{de}$ |
| Myricetin | 2.24±0.04$^{a}$ | 2.69±0.02$^{b}$ | 2.71±0.01$^{bc}$ | 2.72±0.01$^{g}$ | 7.64±0.07$^{g}$ |
| Quercetin | 2.20±0.02$^{a}$ | 3.94±0.11$^{ab}$ | 5.88±0.11$^{abc}$ | 9.10±0.19$^{cd}$ | 11.8±0.1$^{ef}$ |
| Rutin | 2.22±0.03$^{a}$ | 3.45±0.67$^{ab}$ | 6.57±0.01$^{ab}$ | 8.80±0.08$^{cd}$ | 16.0±2.6$^{bcd}$ |
| Flavanones: | | | | | |
| Naringenin | 2.14±0.05$^{a}$ | 3.08±0.66$^{ab}$ | 5.23±0.61$^{abc}$ | 8.39±0.08$^{cde}$ | 10.2±0.1$^{fg}$ |
| Naringin | 2.23±0.00$^{a}$ | 3.49±0.06$^{ab}$ | 6.33±0.42$^{ab}$ | 7.57±0.03$^{ef}$ | 11.7±0.1$^{ef}$ |
| Flavononols: | | | | | |
| Taxifolin | 2.22±0.01$^{a}$ | 4.62±0.02$^{a}$ | 7.10±0.52$^{a}$ | 9.30±0.09$^{c}$ | 18.2±0.5$^{abc}$ |
| Flavan-3-ol: | | | | | |
| (-)Epicatechin | 2.23±0.05$^{a}$ | 3.13±0.01$^{ab}$ | 6.61±0.17$^{ab}$ | 6.96±0.02$^{f}$ | 12.1±0.1$^{ef}$ |
| BHA/BHT/CA | 2.15±0.01$^{a}$ | 3.14±0.01$^{ab}$ | 5.04±0.04$^{abc}$ | 8.20±0.03$^{de}$ | 10.8±0.2$^{f}$ |
| TBHQ | 2.17±0.12$^{a}$ | 2.31±0.00$^{c}$ | 2.43±0.00$^{c}$ | 2.55±0.11$^{g}$ | 3.41±0.11$^{g}$ |

[1] Conjugated diene value = A/(c x d) where, A = absorbance of the solution at 234nm, c = concentration of the solution in g/100ml of solvent, and d = length of the cell in cm.
[2] Values in the same column bearing different superscripts are significantly (P>0.05) different.

CE at 100 to 1000 ppm levels to canola oil significantly (P>0.05) decreased the peroxide values during accelerated oxidation. These data indicated that an increase in the addition level of CE paralleled a decrease in the formation of peroxides. However, peroxide values of canola oil samples treated with 200, 500 and 1000 ppm of CE were similar for up to day-2. After day-5, peroxide values of the samples containing varying concentration of CE were significantly (P>0.05) different. It was also observed that centrifugation of canola oil samples treated with CE at 500 and 1000 ppm levels to remove insoluble residues did not affect the decrease of peroxide value of the oil. For up to 17 days, the peroxide values of the control sample increased from 0.37 meq/kg (fresh oil) to 183.4 meq/kg (oxidized oil) but the corresponding values for oil treated with CE-500 and CE-1000 were smaller. It changed from 0.30 to 102.1 and from 0.32 to 93.5 meq/kg, respectively. CE was most effective at 1000 ppm level and gave much lower peroxide values than the control, BHA/BHT/CA and other CE-treated oil samples (Table 4.3). Peroxide values of canola oil treated with 200 ppm TBHQ were lowest throughout the period studied as compared with other additives (CE and BHA/BHT/CA).

Consideration of the change of CD values of the samples indicates small differences in this parameter during the initial stages of oxidation. However, during later stages, TBHQ and CE-1000-treated samples had a significant effect on lowering CD formation. Removal of any undissolved residues in the oil by centrifugation had no significant effect on the lowering of the CD values of the samples. The

effectiveness of CE at 500 and 1000 ppm was superior to that of BHA/BHT/CA. The CD values of samples treated with 1000 ppm of CE were the lowest of all CE-treated samples. A similar trend was observed in corresponding peroxide values.

Addition of flavonoids to canola oil resulted in the reduction of peroxide as well as CD values compared to the control sample (Tables 4.4 and 4.6). When different groups of flavonoids are compared, flavonols showed lower peroxide and CD values. In general, the effect of flavones, flavanones and flavonols on primary oxidation product formation was almost similar. Among the flavonols tested, myricetin gave rise to the lowest peroxide and CD values throughout the storage period. The effect of myricertin was far better than BHA/BHT/CA mixture as indicated by lower peroxide and CD values. However, TBHQ maintained the strongest inhibitory activity on the formation of primary lipid oxidation products throughout the length of storage period. Furthermore, it was noted that the ability of these additives (CE, flavonoids and synthetic antioxidants) to suppress peroxide and CD formation was decreased with increasing the length of storage period.

Since hydroperoxides are the primary products of lipid oxidation (Labuza, 1971), peroxide value provides a clear indication of the oxidative state of vegetable oils. However, due to instability of peroxides in the oxidation pathway, measurement of peroxide value provides only information about the initial oxidation potential of the oil. Conjugated diene value is also a measure of the degree of formation of primary products of lipid oxidation. It has been observed that conjugated dienes are formed

due to the shift in double bond position upon oxidation of lipids containing dienes or polyenes (Logani and Davies, 1980). St. Angelo *et al.* (1975) have suggested that CD values can be used as an index of stability for lipid-containing foods. Farmer and Sutton (1943) indicated that CD method can be used as a measurement of primary oxidation since it correlates well with hydroperoxide value.

Hawrysh *et al.* (1988) and McMullen *et al.* (1991) have reported that TBHQ at 200 ppm level of addition markedly reduced the formation of peroxides but BHA/BHT/CA at 250 ppm level was less effective in retarding the formation of peroxides in canola oil under accelerated oxidation conditions. According to the results obtained from this experiment CE (>200 ppm) and myricertin were found to be more effective in lowering peroxide and CD formation in canola oil than BHA/BHT/CA (250 ppm) but were less potent than TBHQ (200 ppm).

### 4.2.2 Effect on 2-thiobarbituric acid reactive substances (TBARS) formation

TBARS value measure secondary products of lipid oxidation and it is the most frequently used indicator to monitor oxidation of vegetable oils. In this experiment addition of CE, flavonoids, BHA/BHT/CA and TBHQ to canola oil showed a significant ($P>0.05$) effect on reducing TBARS formation as compared with the control sample (Tables 4.7 and 4.8). Among these additives, TBHQ was most effective in retarding TBARS formation at 200 ppm level. Ability of TBHQ (200 ppm) to lower TBARS values of stored canola oil has been reported in the literature

Table 4.7 Effect of CE, BHA/BHT/CA and TBHQ on TBARS value (μmol/g oil) of refined-bleached canola oil stored at 65°C[1].

| Treatment | Storage period, days | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 | 17 |
| Control | 0.30±0.02$^{a}$ | 0.68±0.03$^{a}$ | 1.25±0.03$^{a}$ | 1.82±0.13$^{a}$ | 2.72±0.01$^{a}$ | 3.57±0.03$^{a}$ |
| CE-100ppm | 0.29±0.04$^{a}$ | 0.44±0.03$^{b}$ | 0.97±0.02$^{b}$ | 1.29±0.03$^{b}$ | 2.05±0.02$^{b}$ | 2.39±0.14$^{b}$ |
| CE-200ppm | 0.28±0.04$^{a}$ | 0.41±0.04$^{bc}$ | 0.85±0.01$^{bc}$ | 1.28±0.01$^{b}$ | 1.94±0.02$^{c}$ | 2.10±0.03$^{c}$ |
| CE-500ppm | 0.27±0.02$^{a}$ | 0.40±0.01$^{bc}$ | 0.78±0.04$^{c}$ | 1.20±0.03$^{bc}$ | 1.84±0.02$^{d}$ | 1.99±0.02$^{c}$ |
| CE-500ppm (centrifuged) | 0.27±0.01$^{a}$ | 0.40±0.02$^{bc}$ | 0.88±0.01$^{bc}$ | 1.22±0.02$^{bc}$ | 1.90±0.03$^{cd}$ | 2.15±0.07$^{c}$ |
| CE-1000ppm | 0.26±0.03$^{a}$ | 0.37±0.03$^{bc}$ | 0.60±0.02$^{d}$ | 1.09±0.01$^{c}$ | 1.39±0.01$^{f}$ | 1.59±0.09$^{de}$ |
| CE-1000ppm (centrifuged) | 0.25±0.01$^{a}$ | 0.38±0.05$^{bc}$ | 0.72±0.13$^{cd}$ | 1.17±0.02$^{bc}$ | 1.43±0.01$^{f}$ | 1.66±0.04$^{d}$ |
| BHA/BHT/CA-250ppm | 0.25±0.01$^{a}$ | 0.42±0.01$^{b}$ | 0.96±0.07$^{b}$ | 1.19±0.01$^{bc}$ | 1.51±0.02$^{e}$ | 1.77±0.06$^{d}$ |
| TBHQ-200ppm | 0.27±0.02$^{a}$ | 0.33±0.02$^{c}$ | 0.36±0.04$^{e}$ | 0.39±0.06$^{d}$ | 0.58±0.04$^{g}$ | 1.42±0.03$^{e}$ |

[1] Values in the same column bearing dufferent superscripts are significantly (P>0.05) different.

Table 4.8 Effect of flavonoids, BHA/BHT/CA and TBHQ on TBARS value (μmol/g oil) of refined-bleached canola oil stored at 65°C[1].

| Treatment | Storage period, Days | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 |
| Control | 0.25±0.01$^{a}$ | 0.68±0.01$^{a}$ | 1.25±0.03$^{a}$ | 1.82±0.10$^{a}$ | 2.71±0.24$^{a}$ |
| Flavones: | | | | | |
| Apigenin | 0.23±0.00$^{a}$ | 0.59±0.02$^{b}$ | 1.15±0.04$^{abc}$ | 1.56±0.05$^{ab}$ | 2.20±0.06$^{bc}$ |
| Chrysin | 0.30±0.01$^{a}$ | 0.64±0.01$^{ab}$ | 1.08±0.04$^{bcd}$ | 1.39±0.10$^{bc}$ | 2.20±0.09$^{bc}$ |
| Flavonols: | | | | | |
| Kaempferol | 0.30±0.03$^{a}$ | 0.45±0.42$^{cd}$ | 0.64±0.03$^{i}$ | 1.39±0.05$^{bc}$ | 2.12±0.11$^{cd}$ |
| Morin | 0.29±0.02$^{a}$ | 0.42±0.05$^{de}$ | 0.99±0.02$^{cde}$ | 1.19±0.03$^{cde}$ | 2.22±0.11$^{bc}$ |
| Myricetin | 0.23±0.04$^{a}$ | 0.34±0.04$^{f}$ | 0.36±0.02$^{j}$ | 0.40±0.03$^{g}$ | 0.76±0.02$^{h}$ |
| Quercetin | 0.27±0.02$^{a}$ | 0.40±0.02$^{def}$ | 0.68±0.07$^{hi}$ | 1.22±0.41$^{bcde}$ | 1.81±0.08$^{def}$ |
| Rutin | 0.30±0.01$^{a}$ | 0.36±0.03$^{ef}$ | 0.75±0.04$^{ghi}$ | 1.15±0.06$^{cde}$ | 1.94±0.05$^{cde}$ |
| Flavanones: | | | | | |
| Naringenin | 0.24±0.00$^{a}$ | 0.47±0.02$^{cd}$ | 0.83±0.02$^{fgh}$ | 0.99±0.06$^{def}$ | 1.44±0.07$^{fg}$ |
| Naringin | 0.30±0.02$^{a}$ | 0.50±0.01$^{c}$ | 0.74±0.48$^{ghi}$ | 0.89±0.05$^{ef}$ | 1.36±0.03$^{g}$ |
| Flavononols: | | | | | |
| Taxifolin | 0.27±0.02$^{a}$ | 0.58±0.04$^{b}$ | 0.84±0.03$^{efg}$ | 1.27±0.05$^{bcd}$ | 1.95±0.08$^{cde}$ |
| Flavan-3-ol: | | | | | |
| (-)Epicatechin | 0.26±0.00$^{a}$ | 0.35±0.03$^{f}$ | 0.65±0.03$^{i}$ | 0.76±0.02$^{f}$ | 1.68±0.11$^{efg}$ |
| BHA/BHT/CA | 0.25±0.01$^{a}$ | 0.42±0.01$^{de}$ | 0.96±0.07$^{def}$ | 1.19±0.01$^{bcde}$ | 1.51±0.02$^{fg}$ |
| TBHQ | 0.27±0.02$^{a}$ | 0.33±0.02$^{f}$ | 0.36±0.04$^{j}$ | 0.39±0.06$^{g}$ | 0.58±0.04$^{h}$ |

[1] Values in the same column bearing different superscripts are significantly (P>0.05) different.

(Hawrysh *et al.* 1988, 1990). Addition of CE (>200 ppm) was equivalent or slightly better than that of BHA/BHT/CA. At 1000 ppm level, CE was able to lower TBARS values more effectively than BHA/BHT/CA even at day 17. Oil treated with CE-1000 showed 46, 52, 40, 49 and 56% reduction in TBARS on days 2, 5, 9, 13 and 17, respectively, while BHA/BHT/CA-treated samples showed 38, 23, 34, 44 and 50% reduction (Table 4.7). The general trend of flavonoids on TBARS formation, showed that flavonols and flavanones were more effective than other types of flavonoids. Among flavonoids tested, myricetin, quercetin, (-)epicatechin were able to maintain >40% inhibition of TBARS formation for up to 9 days of storage (Table 4.8). Rutin was able to inhibit TBARS formation by 47 and 40% at days 2 and 5, respectively, however, its effectiveness was less after 9 days of storage. It was clear that the effect of myricetin was the strongest among flavonoids and it was similar to that of TBHQ in reducing TBARS values of oil samples throughout the storage period. The overall order of potency of flavonoids on inhibition of TBARS formation was as follows:

Myricetin > (-)epicatechin > naringin > naringenin > quercetin >

rutin > morin > kaempferol > taxifolin > apigenin > chrysin.

The 2-Thiobarbituric acid (TBA) test measures the secondary oxidation products of lipids mainly aldehydes (or carbonyls) which may contribute to off-flavour of oxidized foods. Results of this study indicate that CE and some of the flavonoids tested have a marked effect on the inhibition of the formation of TBARS of canola oil. The effect of CE in suppressing TBARS formation was better than

BHA/BHT/CA but less than that of TBHQ. Effectiveness of myricetin was similar to that of TBHQ. This findings lends support to previous reports (Vaisey-Genser and Ylimaki, 1985; Hawrysh *et al.*, 1988; McMullen *et al.*, 1991) that BHA/BHT/CA is less effective than TBHQ in reducing TBARS formation of canola oil and further concluded that this mixture is not the best antioxidant for improving the storage stability of canola oil despite its common use by the oil industry. Furthermore, results of this study confirms that BHA/BHT/CA is not as effective as CE (at ≥200 ppm) or some of the flavonoids and TBHQ at 200 ppm level of addition.

### 4.2.3 Effect on TOTOX value

The total oxidation or TOTOX value of treated (CE, BHA/BHT/CA and TBHQ) oil samples are given in Table 4.9. Oil samples treated with CE (at ≥200 ppm) had comparatively low TOTOX values. Oil samples treated with 200 ppm CE had 50% lower TOTOX values up to 13 days of storage as compared with those of the control sample. The lowest TOTOX values were noticed for 1000 ppm level of addition of CE resulting in 90, 73, 65, 52 and 50% inhibition on days 2, 5, 9, 13 and 17 of storage, respectively. However, as compared with the BHA/BHT/CA after 9 days, CE-1000-treated oil had a slightly higher TOTOX values perhaps due to higher peroxide values (Table 4.5) in the CE-1000-treated oil during later stages of storage.

Most of the flavonoids tested, except chrysin, were effective in reducing TOTOX values. Myricetin, quercetin, morin, kaempferol, rutin and (-)epicatechin

Table 4.9 Effect of CE, BHA/BHT/CA and TBHQ on TOTOX value[1] of refined-bleached canola oil stored at 65°C[2].

| Treatment | Storage period, Days | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 | 17 |
| Control | 2.15±0.21$^{a}$ | 50.5±1.1$^{a}$ | 186.4±2.3$^{a}$ | 287.3±5.5$^{a}$ | 362.3±8.9$^{a}$ | 423.3±4.8$^{a}$ |
| CE-100ppm | 1.85±0.03$^{a}$ | 19.5±1.2$^{b}$ | 95.5±0.2$^{b}$ | 145.2±4.4$^{b}$ | 210.3±4.9$^{b}$ | 242.3±5.7$^{bc}$ |
| CE-200ppm | 1.90±0.06$^{a}$ | 5.78±0.41$^{cd}$ | 84.5±0.3$^{c}$ | 141.2±1.4$^{b}$ | 203.4±2.9$^{bc}$ | 231.3±2.8$^{cd}$ |
| CE-500ppm | 1.81±0.02$^{a}$ | 4.66±0.38$^{d}$ | 71.8±2.7$^{d}$ | 124.2±2.1$^{d}$ | 196.4±3.9$^{c}$ | 230.4±7.6$^{cd}$ |
| CE-500ppm (centrifuged) | 2.03±0.09$^{a}$ | 4.91±0.06$^{d}$ | 74.4±1.9$^{d}$ | 126.4±2.5$^{c}$ | 207.6±3.6$^{bc}$ | 246.3±4.5$^{b}$ |
| CE-1000ppm | 1.92±0.06$^{a}$ | 4.02±0.12$^{d}$ | 50.2±1.3$^{f}$ | 98.2±1.8$^{e}$ | 175.4±3.4$^{d}$ | 209.4±4.6$^{e}$ |
| CE-1000ppm (centrifuged) | 1.89±0.02$^{a}$ | 4.43±0.30$^{d}$ | 62.9±2.1$^{e}$ | 111.1±3.7$^{d}$ | 175.4±5.4$^{d}$ | 225.2±9.6$^{d}$ |
| BHA/BHT/CA-250ppm | 1.89±0.42$^{a}$ | 7.75±0.28$^{c}$ | 80.9±1.2$^{c}$ | 129.5±2.6$^{c}$ | 146.3±2.7$^{e}$ | 183.4±2.6$^{f}$ |
| TBHQ-200ppm | 1.78±0.10$^{a}$ | 3.64±0.10$^{d}$ | 5.26±0.37$^{g}$ | 10.6±0.4$^{f}$ | 13.3±1.3$^{f}$ | 143.1±2.5$^{g}$ |

[1] TOTOX value = 2PV + AnV

[2] Values in the same column bearing different superscripts are significantly (P>0.05) different.

served best in lowering TOTOX values (<45%) as compared with the control sample for up to 5 days of storage (Table 4.10). Among the flavonoids tested myricetin was the most effective flavonol which was also superior to BHA/BHT/CA. Myricetin-treated oils had TOTOX values which were 6.7, 22.0, 27.7, and 64.7 on days 2, 5, 9 and 13, respectively. Corresponding values for BHA/BHT/CA-treated oils were 7.75, 80.9, 129.5 and 146.3 and those for the control sample were 50.5, 186.4, 287.3 and 362.3, respectively. However, TBHQ lowered TOTOX values better than all other antioxidants used in this study.

Changes in TOTOX values provide information regarding progression of formation of primary and secondary oxidation products. CE had a marked effect in lowering the TOTOX values of canola oil when compared with the commonly used synthetic antioxidant mixture, BHA/BHT/CA. Among the flavonoids tested, myricetin exerted the strongest effect in retarding oxidation of canola oil and lowering the TOTOX values. TOTOX value is often considered as a useful indicator of oxidation of oils because it combines evidences about the past history (p-anisidine value) with the present state of the oil (peroxide value) of the oil (Rossel, 1983).

The crude canola extract possesses good antioxidative properties as evidenced by weight gain, peroxide, CD, TBARS and TOTOX values of the oil. Development of oxidative rancidity during accelerated oxidation at 65°C may be pursued, under these conditions, 1 day is equivalent to that aged for 1 month at ambient temperatures (Evans *et al.*, 1973). Effectiveness of CE at 500 and 1000 ppm levels was better than

Table 4.10 Effect of flavonoids, BHA/BHT/CA and TBHQ on TOTOX value[1] of refined-bleached canola oil stored at 65°C[2].

| Treatment | Storage period, Days | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 5 | 9 | 13 |
| Control | 2.15±0.21$^{a}$ | 50.5±1.1$^{a}$ | 186.4±2.3$^{a}$ | 287.3±5.5$^{a}$ | 362.3±8.9$^{a}$ |
| Flavones: | | | | | |
| Apigenin | 2.13±0.07$^{a}$ | 43.9±0.2$^{c}$ | 143.3±2.5$^{b}$ | 249.4±4.6$^{b}$ | 336.4±2.6$^{b}$ |
| Chrysin | 2.05±0.07$^{a}$ | 48.7±1.8$^{ab}$ | 143.5±5.7$^{b}$ | 210.4±5.5$^{c}$ | 333.3±7.3$^{b}$ |
| Flavonols: | | | | | |
| Kaempferol | 2.01±0.04$^{a}$ | 25.9±1.0$^{f}$ | 97.8±1.9$^{de}$ | 229.4±7.3$^{bc}$ | 326.4±2.9$^{b}$ |
| Morin | 1.98±0.03$^{a}$ | 20.7±1.2$^{g}$ | 83.5±4.5$^{g}$ | 152.2±2.7$^{bc}$ | 301.3±7.7$^{c}$ |
| Myricetin | 1.95±0.07$^{a}$ | 6.70±0.20$^{j}$ | 22.0±1.1$^{i}$ | 27.7±3.4$^{h}$ | 64.7±2.1$^{g}$ |
| Quercetin | 1.89±0.01$^{a}$ | 15.1±1.6$^{h}$ | 69.3±1.4$^{h}$ | 124.3±3.8$^{fg}$ | 225.5±2.5$^{d}$ |
| Rutin | 1.95±0.02$^{a}$ | 11.1±0.8$^{i}$ | 98.8±5.5$^{de}$ | 143.5±3.6$^{ef}$ | 295.8±3.3$^{c}$ |
| Flavanones: | | | | | |
| Naringenin | 2.10±0.07$^{a}$ | 33.5±0.3$^{d}$ | 94.9±3.7$^{ef}$ | 136.3±8.4$^{ef}$ | 198.2±3.8$^{e}$ |
| Naringin | 1.99±0.10$^{a}$ | 29.5±0.4$^{e}$ | 109.4±4.5$^{d}$ | 134.4±5.8$^{ef}$ | 191.1±3.2$^{e}$ |
| Flavononols: | | | | | |
| Taxifolin | 2.06±0.14$^{a}$ | 47.3±2.7$^{b}$ | 119.0±2.6$^{c}$ | 173.6±3.8$^{d}$ | 334.4±2.4$^{b}$ |
| Flavan-3-ol: | | | | | |
| (-)Epicatechin | 2.13±0.13$^{a}$ | 23.7±0.2$^{f}$ | 87.6±2.3$^{fg}$ | 103.5±2.2$^{g}$ | 199.1±4.4$^{e}$ |
| BHA/BHT/CA | 1.89±0.42$^{a}$ | 7.75±0.28$^{j}$ | 80.9±1.2$^{g}$ | 129.2±2.7$^{ef}$ | 146.3±2.4$^{f}$ |
| TBHQ | 1.78±0.10$^{a}$ | 3.64±0.10$^{k}$ | 5.26±0.37$^{j}$ | 10.6±0.4$^{h}$ | 13.3±1.3$^{h}$ |

[1] TOTOX value = 2PV + AnV.
[2] Values in the same column bearing different superscripts are significantly (P>0.05) different.

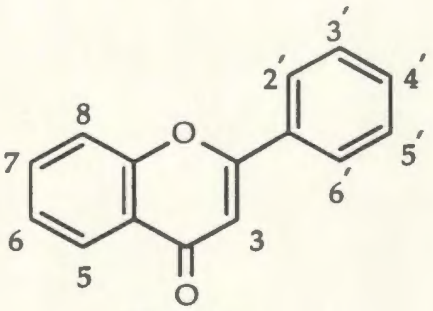
that of BHA/BHT/CA (250 ppm) but less than that of TBHQ (200 ppm). Apart from being a stronger antioxidant, CE did not impart any visible colour (at higher concentrations after removing residual extract) or perceivable odour to the treated canola oil. These qualities suggest the potential use of ethanolic extracts of canola meal as a natural antioxidant for use in canola oil, and possibly other edible oils.

The scientific literature in the past two decades is replete with reports on antioxidative activity of natural sources. Different kinds of seed hulls and meals have been studied for their antioxidative properties. Ethanolic extracts of navy bean hull (Onyenebo and Hettiarachchy, 1991), oat hull (Duve and White, 1991), methanolic extracts of cottonseed (Whittern *et al.*, 1984), peanut hull (Duh *et al.*, 1992), and aqueous extract of soybean flour (Rhee *et al.*, 1979; Ziprin *et al.*, 1981) show strong antioxidative effect on vegetable oils, β-carotene-linoleate and meat model systems. Most of these antioxidative compounds are of phenolic nature. However, only a few reports are available on antioxidative ability of *Brassica* seed meals. It is well known that the phenolic content of *Brassica* seed meals is quiet high (1-2%) compared to other oilseeds (Kozlowska *et al.*, 1983; Dabrowski and Sosulski, 1984, Shahidi and Naczk, 1992). Shahidi *et al.* (1991) have reported that low pungency mustard flour extracts effectively reduced warmed over flavour (WOF) developed in meat model systems. They have indicated that the antioxidative efficacy of these extracts was directly proportional to their total content of phenolics. Phenolic compounds of *Brassica* (especially rapeseed or canola) include phenolic acids (free, esterified and

insoluble bound) (Zadernowski, 1987, Shahidi and Naczk, 1992), flavonoids (Zadernowski *et al.*, 1991) and condensed tannins (Shahidi and Naczk, 1989). Antioxidative activity of separated rapeseed phenolic acids examined in a β-carotene-linoleate model system has also been reported by Nowak *et al.* (1992).

Among the flavonoids tested in this study, flavonols namely myricetin, quercetin and rutin and (-)epicatechin (flavan-3-ol; Figure 4.3) were found to have the strongest antioxidant properties and stabilizing effect on canola oil. The flavonones, naringenin and naringin were (Figure 4.3) also effective but were less potent than those of flavonols or flavan-3-ol. Flavonoids may act as primary antioxidants by donating a hydrogen atom to the peroxy radicals derived from oxidizing fatty acids (Torel *et al.*, 1986), may also function as free radical acceptors or chain breakers, and may serve as metal chelators (Hudson and Lewis, 1983). Larson (1988) has reported that quercetin and some flavonoids are also potent quenchers of singlet oxygen.

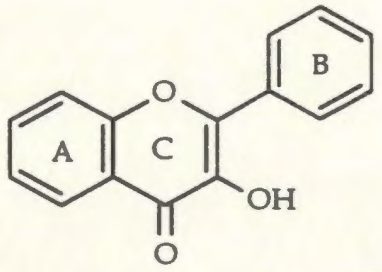
It has been found that the antioxidative activity of flavonoids is generally governed by their chemical structure (Shahidi *et al.*, 1991). All flavonoids possessing a 3′, 4′-dihydroxy configuration have antioxidant activity. Myricetin with an additional hydroxy group at the 5′ position shows a better antioxidant activity than that of its corresponding flavonol devoid of a 5′-hydroxy group, i.e. quercetin (Figure 4.3). Naringin and naringenin with a single hydroxyl group on the B-ring possess only slight antioxidative activity. Therefore, hydroxylation of the B-ring is the major consideration for antioxidant activity of flavonoids. Rutin with a etherified sugar




Flavones

Apigenin: 5,7,4′-tri-OH

Chrysin: 5,7-di-OH




Flavonols

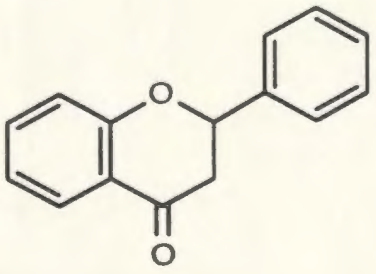
Kaempferol: 3,5,7,4′-tetra-OH

Quercetin: 3,5,7,3′,4′-penta-OH

Myricetin: 3,5,7,3′,4′,5′-hexa-OH

Morin: 3,5,7,2′,4′-penta-OH

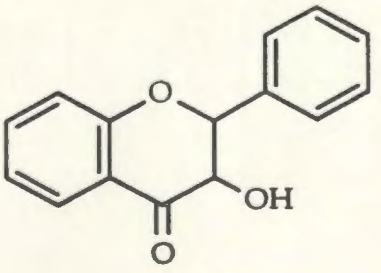
Rutin: 3-O-rutinose,5,7,3′,4′-tetra-OH




Flavanones

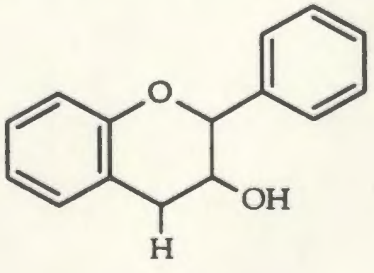
Naringinin: 5,7,4′-tri-OH

Naringin: 7-rhamnoglucose,5,4′-di-OH




Flavanonols

Taxifolin: 3,5,7,3′,4′-penta-OH




Flavan-3-ol

(-)Epicatechin: 3,5,7,3′,4′-penta-OH

Figure 4.3 Structures of some flavonoids.

moiety at its 3rd (C-ring) position showed a lower antioxidant activity than quercetin which has a hydroxyl group at 3rd position. Therefore, glycosyl substitution of flavonoids reduces their antioxidant activity, perhaps due to lack of their ability to donate a hydrogen atom to lipid free radicals. In addition, (-)epicatechin which is a flavan-3-ol showed an antioxidant activity similar or superior to that of quercetin. The relationship between hydroxylation of flavonoids and antioxidant activity has been well documented (Hudson and Lewis, 1983; Pratt and Hudson, 1990; Shahidi and Wanasundara, 1992). Myricetin possessing the largest number of hydroxyl groups in its structure was the most active flavonoid (see Figure 4.3). Similar results were obtained by Das and Pereira (1990) for palm oil and by Ramanathan and Das (1992) for ground fish samples. Our study also revealed that several flavonoids especially flavonols, flavan-3-ol and flavanones may be considered as potential antioxidants for the stabilization of canola oil. These flavonoids are naturally present in many plant products which have been consumed. The comsumption of food flavonoids amounts to 1 g/day for human being (Kuhnau, 1976) safely since antiquity (Bete-Smith, 1954; Pokorny, 1991). In addition, food-derived flavonoids such as quercetin, kaempferol and myricetin have been shown to have anticarcinogenic and antimutagenic effects in both *in vitro* and *in vivo* studies (Kato *et al.*, 1983; Huang *et al.*, 1983; Mukhtar *et al.*, 1988; Francis *et al.*, 1989; Deschner *et al.*, 1991).

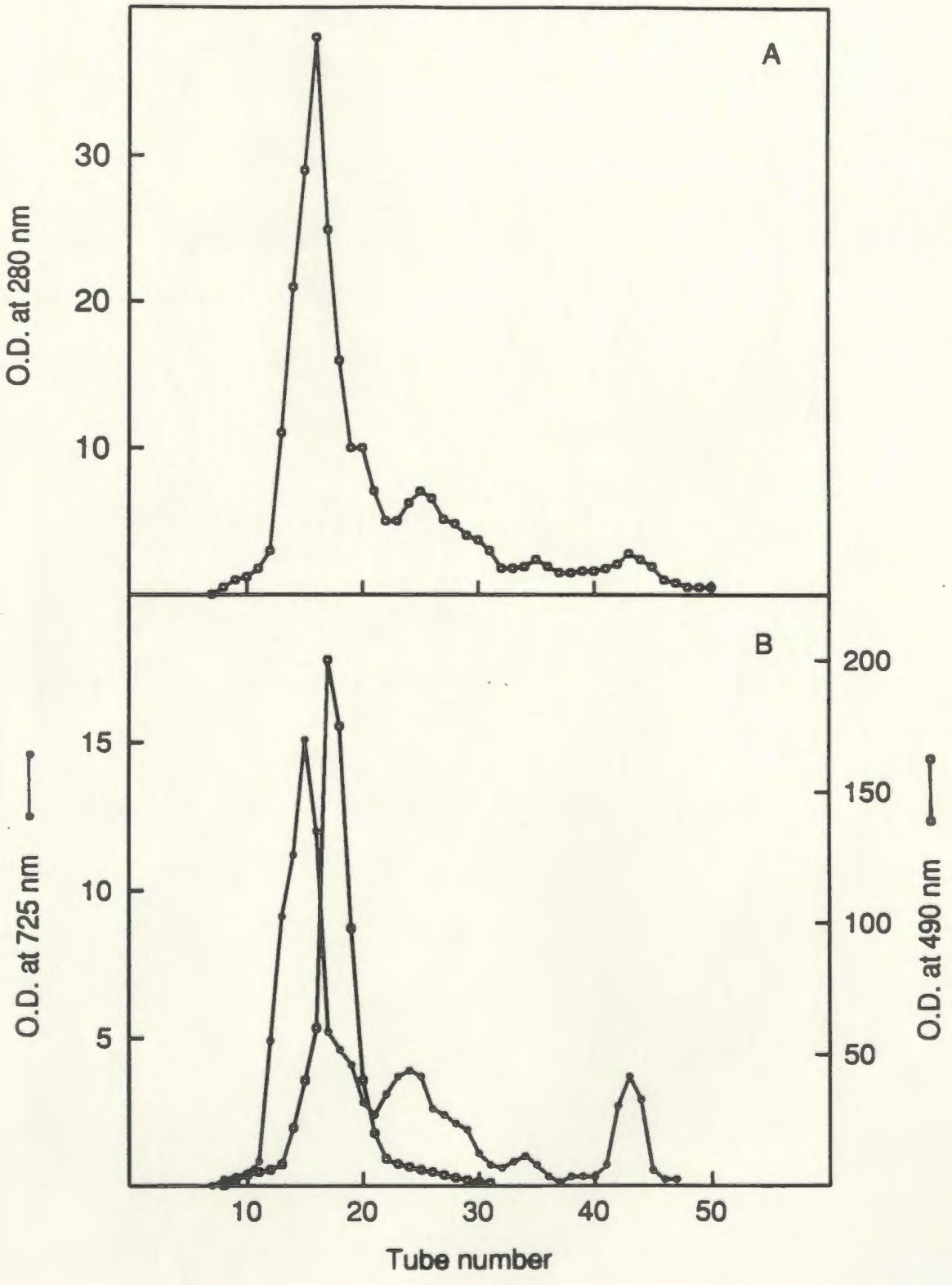
## 4.3 Screening of the most active antioxidative compound of CE

Results of the Schaal oven test showed that addition of CE was able to retard oxidative deterioration of refined-bleached canola oil as evidenced by reduced peroxide, conjugated diene, TBARS and TOTOX values of the treated samples. Its effectiveness at >200 ppm level was better than BHA/BHT/CA (250 ppm). Therefore, the fractionation, isolation and identification of its most active antioxidative component was carried out (see below).

### 4.3.1 Fractionation of the crude canola extract

It was assumed that phenolic compounds present in canola seed may be responsible for its antioxidative activity. The extract was fractionated, using a Sephadex LH-20 column, according to molecular weight and polarity. Sephadex LH-20 is regarded as an efficient medium for separation of plant phenolics (Johnston *et al.*, 1968; Amarowicz, *et al.*, 1992). The UV absorbance (at 280 nm) of different fractions of CE and the content of their phenolic compounds and sugars are presented in Figure 4.4. Five peaks were clearly defined according to the absorbance reading at 280 nm and the phenolic content of samples. A large peak was given for sugars, probably from glucosinolates and soluble sugars present in the canola extracts as reported by Amarowicz *et al.* (1990). Based on these data, samples were separated into seven major fractions (I, II, III, IV, V, VI and VII).

Figure 4.4 UV absorbance (A), phenolic, • and sugar, ○ contents (B) of eluates from Sephadex LH-20 column.


A
B
O.D. at 280 nm
O.D. at 725 nm
O.D. at 490 nm
30
20
10
15
10
5
200
150
100
50
10
20
30
40
50
Tube number
Fractions: I II III IV V VI VII

### 4.3.2 Antioxidant activity of fractions following column chromatographic separation

Antioxidative activity of equal weights of each of the separated major fractions was determined in a β-carotene-linoleate model system as illustrated in Figure 4.5. It can be seen that among the seven major fractions, fraction number IV possessed the strong antioxidative activity as it showed the best activity against bleaching of β-carotene. All fractions showed lower antioxidant activity than BHA. However, the crude canola extract showed a higher antioxidant activity than BHA in canola oil. This slightly inferior performance of the antioxidant activity in the fractions may be due to possible removal of other compounds which may have imparted synergistic activity or the combined synergistic effect of components present in the extract.

### 4.3.3 Characterization of the column chromatographic fractions

The thin layer chromatograms of the seven fractions under UV light or after spraying different reagents on them are given in Figure 4.6. It was observed that these fractions contain a mixture of compounds having different $R_f$ values. Fraction number IV which had the highest antioxidant activity yielded nine fluorescent bands with $R_f$ values of 0.20, 0.30, 0.40, 0.48, 0.53, 0.60, 0.64, 0.70 and 0.77 at 254 nm (plate A Figure 4.6) and five bands with $R_f$ values of 0.40, 0.65, 0.78, 0.84 and 0.90 at 365 nm (plate B Figure 4.6), respectively. This fraction contained eleven different phenolic compounds which produced a blue colour with ferric chloride-potassium

Figure 4.5 Antioxidant activity of fractions separated on a Sepadex LH-20 column.

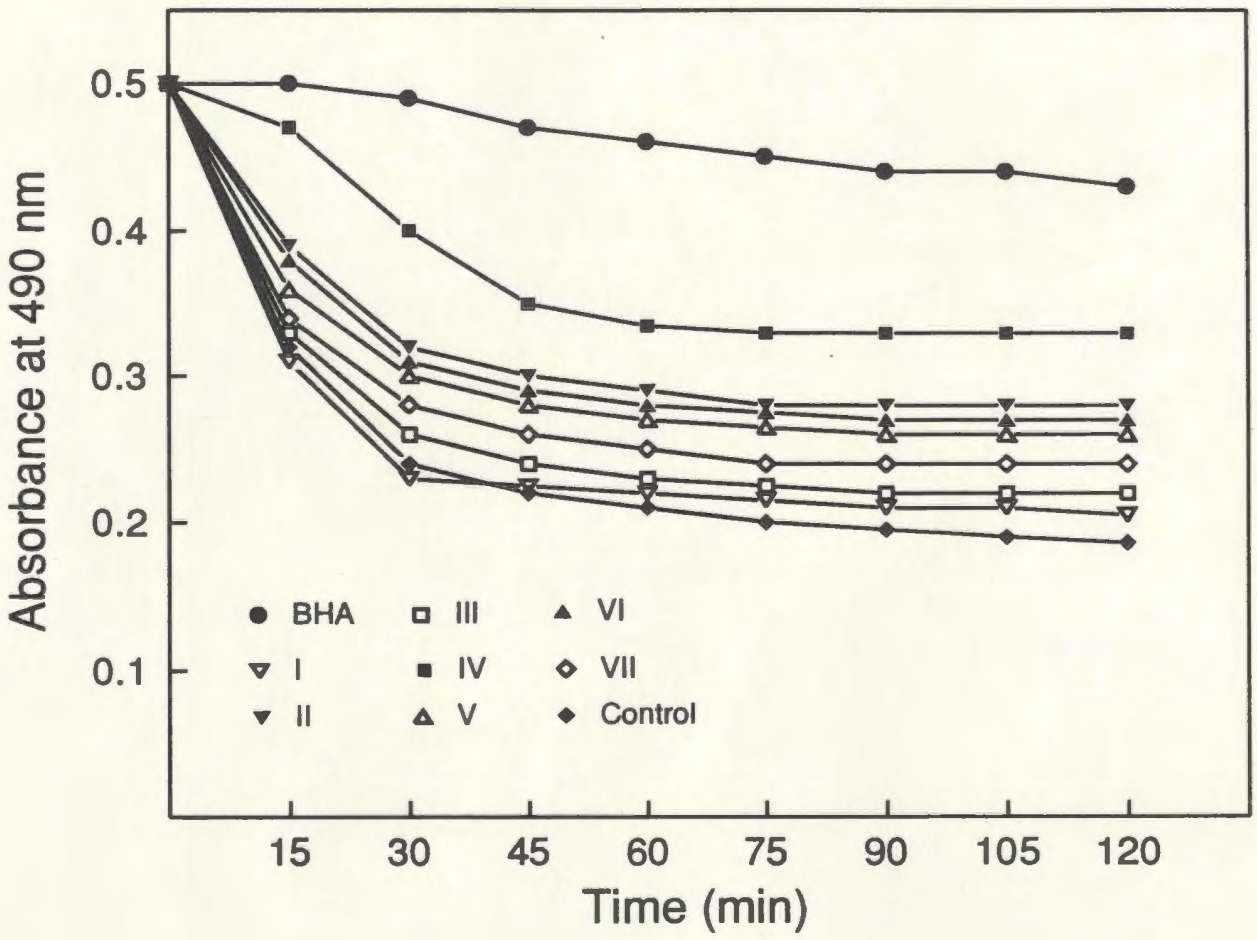
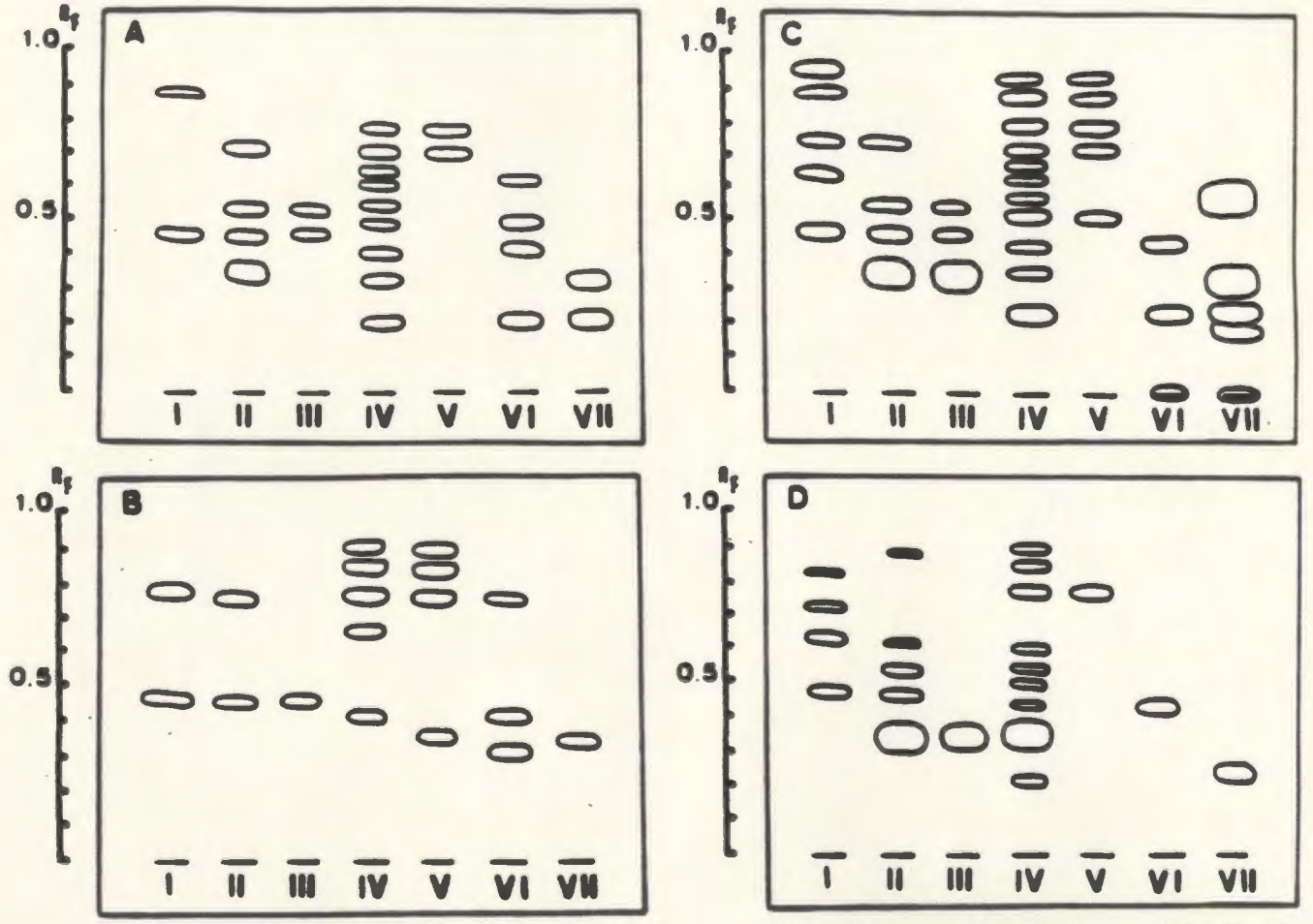

Absorbance at 490 nm
0.5
0.4
0.3
0.2
0.1
BHA
I
II
III
IV
V
VI
VII
Control
15
30
45
60
75
90
105
120
Time (min)

Figure 4.6 TLC chromatograms of fractions separated on a Sepadex LH-20 column.




A - Under UV light (254nm)
B - Under UV light (365nm)
C - Spray with $FeCl_3$ - $K_3Fe(CN)_6$
D - Spray with $FeCl_3$

ferricyanide reagent (plate C Figure 4.6). Most of these bands detected with ferric chloride-potassium ferricyanide spray had $R_f$ values similar to the bands observed under UV light. Chromatogram sprayed with ferric chloride showed that fraction number IV had eight different phenolic compounds with $R_f$ values of 0.30, 0.40, 0.53, 0.60, 0.78, 0.84 and 0.90. These phenolic compounds may contain trihydroxy substituents. It is reported that compounds with trihydroxy substituent give blue colouration with ferric chloride (Reio, 1958). Due to antioxidant activity of fraction number IV, further separation of this fraction by TLC was carried out.

Antioxidant activity of individual components of fraction number IV was estimated using a β-carotene-linoleate spray (Figure 4.7). This fraction contained four major antioxidative compounds with $R_f$ values of 0.20, 0.50, 0.80 and 0.90. Among the four major bands, the band with an $R_f$ value of 0.50 was found to be most effective in preventing oxidation of β-carotene. This band was isolated and used for structural elucidation.

### 4.3.4 Structural analysis of the most active antioxidative compound of canola extract

The UV spectrum of the component with most antioxidative effect showed an absorption maximum at 330 nm (Figure 4.8). This absorption band may be due to the presence of sinapic acid or its derivatives as reported by Kozlowska *et al.* (1983), Dabrowski and Sosulski (1984) and Naczk *et al.* (1992).

Figure 4.7 TLC chromatogram of fraction number IV after β-carotene-linoleate spray.

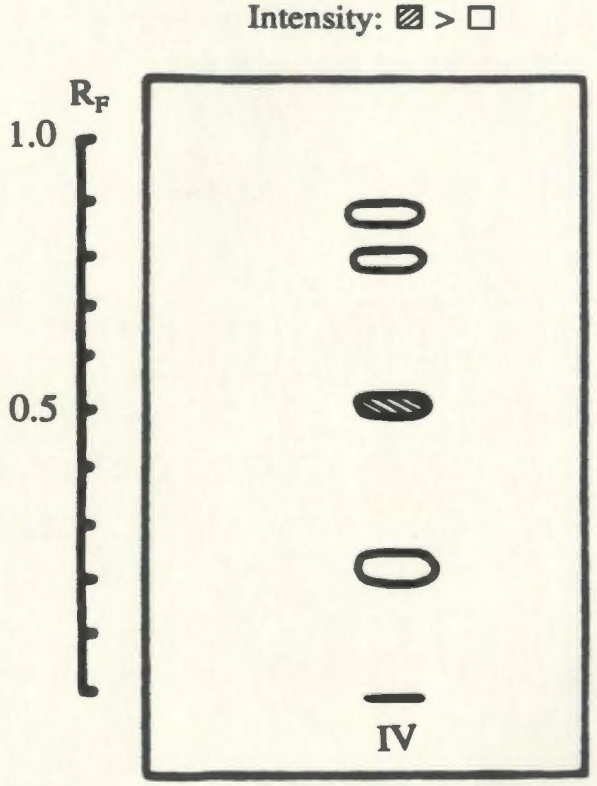
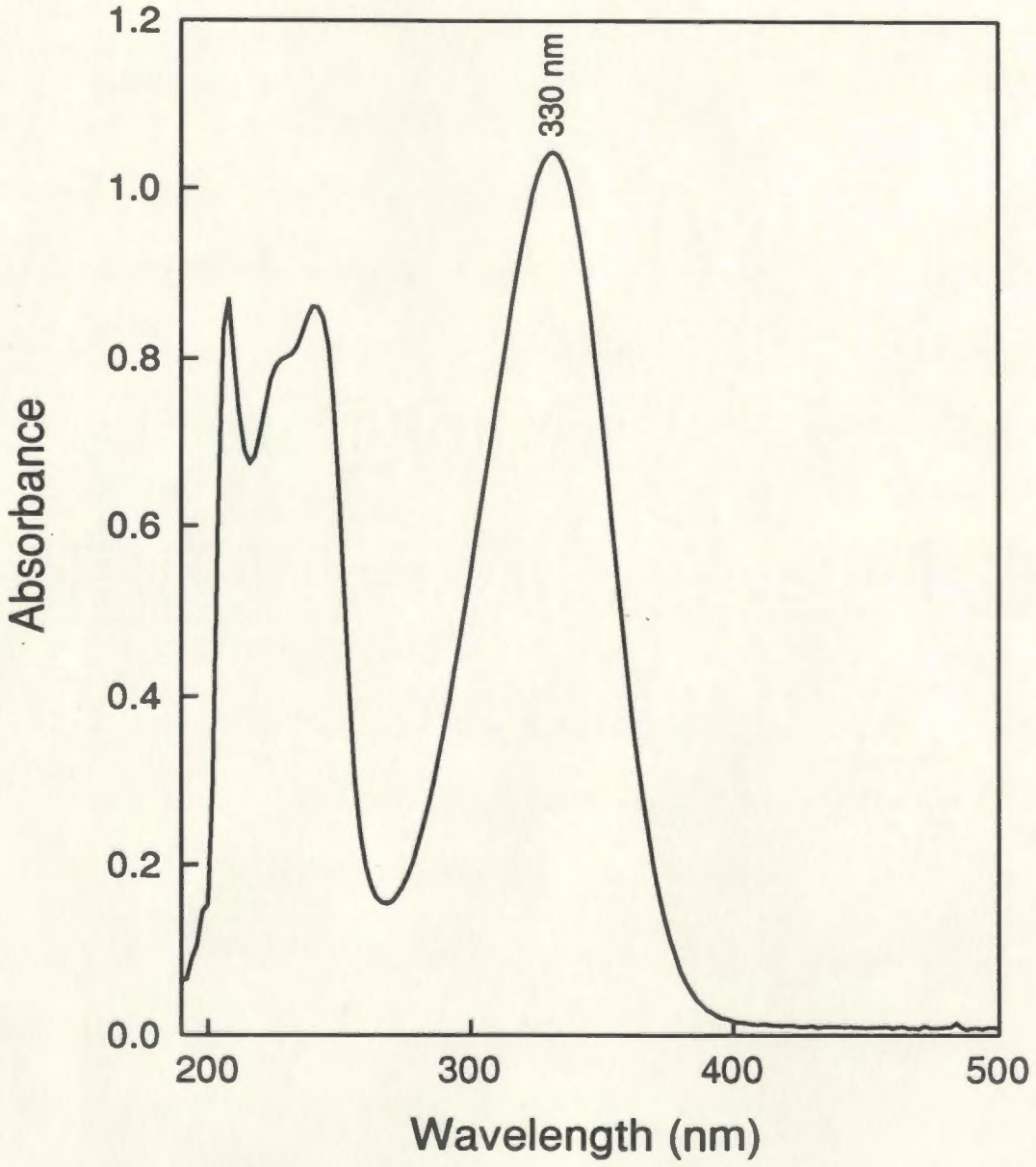

Intensity: ▨ > □
$R_F$
1.0
0.5
IV

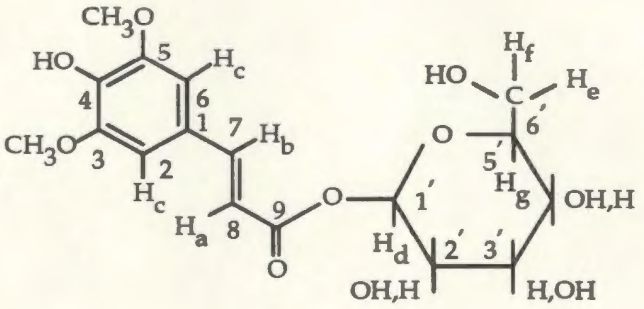
Figure 4.8 UV spectrum of the most active antioxidative compound of canola extract


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
330 nm
200
300
400
500
Wavelength (nm)

The mass spectral analyses showed two major fragment ions with m/z of 180 and 206 (see Figure A.2). The m/z 180 fragment suggested the presence of a six carbon sugar moiety in the molecule. The other fragment may represent sinapic acid. The total molecular mass of the suggested compound was 386, and corresponded with these and other fragment ions present in the mass spectrum of the sample.

Further characterization of the chemical nature of this compound, using the NMR facilities of the Department Chemistry, Memorial University of Newfoundland was carried out. The $^1H$ NMR spectrum of this compound in deuterated methanol, $CD_3OD$, displayed eight resonances (see Figure A.3). Two doublet-doublets centered at $\delta$ 3.85 ($J_{ef}$ = 12Hz, $J_{eg}$ = 1.8Hz) and 3.68 ($J_{ef}$ = 12Hz, $J_{fg}$ = 4.8Hz), with a relative integration of each equivalent to one proton, were diagnostic of the $H_e$ and $H_f$ protons of the sugar moiety, respectively (Table 4.11). Two doublets at $\delta$ 7.72 ($J_{ab}$ = 16Hz) and 6.43 ($J_{ab}$ = 16Hz), with a relative integration of each equivalent to one proton, were assigned to the $H_b$ and $H_a$ olefinic protons of the phenolic acid moiety, respectively. The assignment of the resonance due to these olefinic protons is based on the chemical shifts predicted by using the additivity rule (Pretsch *et al.*, 1989). A singlet at $\delta$ 6.93 with relative integration equivalent to two protons was assigned to the $H_c$ protons of the phenolic ring. The relative integration equivalent to six protons was observed for a singlet at $\delta$ 3.87 which is diagnostic of two $CH_3O$ groups attached to the phenolic ring. The multiplet at $\delta$ 3.41 represented all other protons of the sugar and phenolic acid moieties. An ester linkage in the molecule may be assigned on the basis of the chemical shift of $H_d$ of the sugar moiety at $\delta$ 5.57.

Table 4.11 $^1$H NMR assignment of the most active antioxidative compound of canola.




| δ(ppm) | Multiplicity | J | Integration | Assignment |
|---|---|---|---|---|
| 7.72 | doublet | $J_{ab}$ = 16Hz | 1H | $H_b$ |
| 6.93 | singlet | | 2H | $H_c$ |
| 6.43 | doublet | $J_{ab}$ = 16Hz | 1H | $H_a$ |
| 5.57 | doublet | J = 7.8Hz | 1H | $H_d$ |
| 3.87 | singlet | | 6H | $CH_3O$ |
| 3.85 | doublet-doublet | $J_{ef}$ = 12Hz, $J_{eg}$ = 1.8Hz | 1H | $H_e$ |
| 3.68 | doublet-doublet | $J_{ef}$ = 12Hz, $J_{fg}$ = 4.8Hz | 1H | $H_f$ |
| 3.41 | multiplet | | | other CH's from sugar and phenolic acid |

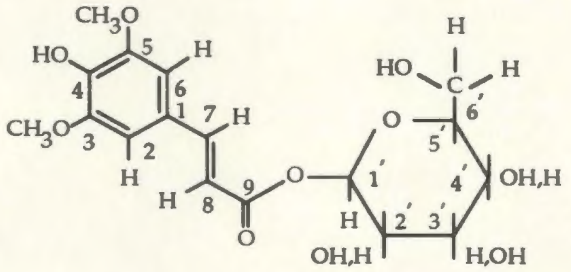
Note - Assignment of coupling interactions (J) confirmed by a COSY experiment

The $^{13}C\{^1H\}$ NMR spectral data in $CD_3OD$ revealed 13 resonances from the proposed 17 carbon adduct suggest considerable symmetry in the molecule (see Figure A.4). However, the signal for C-1 was obscured in the noise even after overnight scanning. Assignment for the $^{13}C$ NMR spectrum was aided by attached proton tests (APT) data which showed the presence of three or four ipso carbons (at δ 126.4, 149.5 and 167.6), one methylene carbon (at δ 62.3) and one or two methyl carbons (at δ 56.8). The vinyl protons (at δ 6.43 and 7.72) and other methine protons of the phenolic acid and sugar moieties were correlated with their corresponding $^{13}C$ resonances. The chemical shifts of all carbon atoms of the sugar moiety were in agreement with the standard values of β-D-glucopyranose (Pretsch *et al.*, 1989). Thin layer chromatography of the compound upon hydrolysis also indicated the presence of β-D-glucopyranose using a set of monosaccharide standards (see Figure A.6) A summary of the $^{13}C\{^1H\}$ NMR assignments is shown in Table 4.12.

Based on the UV, MS as well as $^1H$ and $^{13}C\{^1H\}$ NMR spectral data, the most active antioxidative compound isolated from canola extract was identified. Its chemical name is 1-O-β-D-glucopyranosyl sinapate (1-O-β-D-glucopyranosyl 3,5-dimethoxy-4-hydroxy cinnamate).

Phenolic acids of canola and/or rapeseed include sinapic, p-hydroxybenzoic, vanillic, gentisic, procatechuic, syringic, p-coumaric and ferulic acids. (Kozlowska *et al.*, 1990). However, sinapic acid is the major phenolic acid present constituting over 73% of free phenolic acids and about 99% of phenolic acids released from esters and

Table 4.12 $^{13}C\{^{1}H\}$ NMR assignment of the most active antioxidative compound of canola.



| δ (ppm) | Assignment |
|---|---|
| 167.6 | COO |
| 149.5 | C-3,5 |
| 148.4 | C-7 |
| 126.4 | C-4 |
| 115.2 | C-8 |
| 107.0 | C-2,6 |
| 95.8 | C-1′ |
| 78.8 | C-2′ |
| 78.0 | C-3′ |
| 74.1 | C-4′ |
| 71.1 | C-5′ |
| 62.3 | C-6′ |
| 56.8 | $CH_3O$ |

Note - Signal from C-1 was obscured in the noise even after overnight scanning.

glycosides (Kozlowska *et al.*, 1983). Antioxidant activity of canola sinapic acid has recently been reported by Nowak, *et al.* (1992). Sinapic acid is a derivative of cinnamic acid and presence of a -CH=CH-COOH group in the molecule ensures a better antioxidant activity for the molecule as compared with benzoic acid derivatives possessing only a -COOH group (Cuvelier *et al.*, 1992). In fact, sinapic, ferulic and p-coumaric acids, all cinnamic acid derivatives, were found to be more active than benzoic acid derivatives such as procatechuic, syringic, vanillic and p-hydroxy benzoic acids. This may be due to participation of the double bond (-HC=CH-) in stabilizing the aryloxy radical by resonance. Several authors (Cort, 1974b; Pokorny, 1987) have shown that the antioxidant efficiency of monophenolic compounds was increased substantially by substitution of electron donor alkyl or methoxy groups which stabilized the aryloxyl radical. In the molecular structure of sinapic acid two methoxy groups are substituted at the *ortho* position relative to the hydroxyl group. Therefore, a greater antioxidant activity is expected for the molecule. Esterification of the acid group with another bulky compound may further enhance its activity. Accordingly, the most active antioxidative compound of canola extract was identified as 1-O-β-D-glucopyranosyl 3,5-dimethoxy-4-hydroxy cinnamate (Tables 4.11 and 4.12). However, the other chemical constituents (phenolic acids, flavonoids, tannins, etc.) in the crude extract may also possess either antioxidative or synergistic effects. Therefore, the overall antioxidant activity of canola extract is probably due to a combined effect of its most active component and other active constituents.

## 4.4 Proton NMR study of canola and soybean oils during accelerated oxidation

### 4.4.1 Chemical properties of refined-bleached-deodourized (RBD) canola and soybean oils used for proton NMR studies

Both RBD canola and soybean oils used for NMR studies had good initial qualities, i.e. having iodine values of 111 and 126 g iodine/100g oil and peroxide values of 0.91 and 0.54 meq/kg oil, respectively (Table 4.13). Fatty acid composition of both oils showed that canola oil contains a reasonably high content of monoenes as compared with soybean oil, but the latter had a higher total content of polyunsaturated fatty acids.

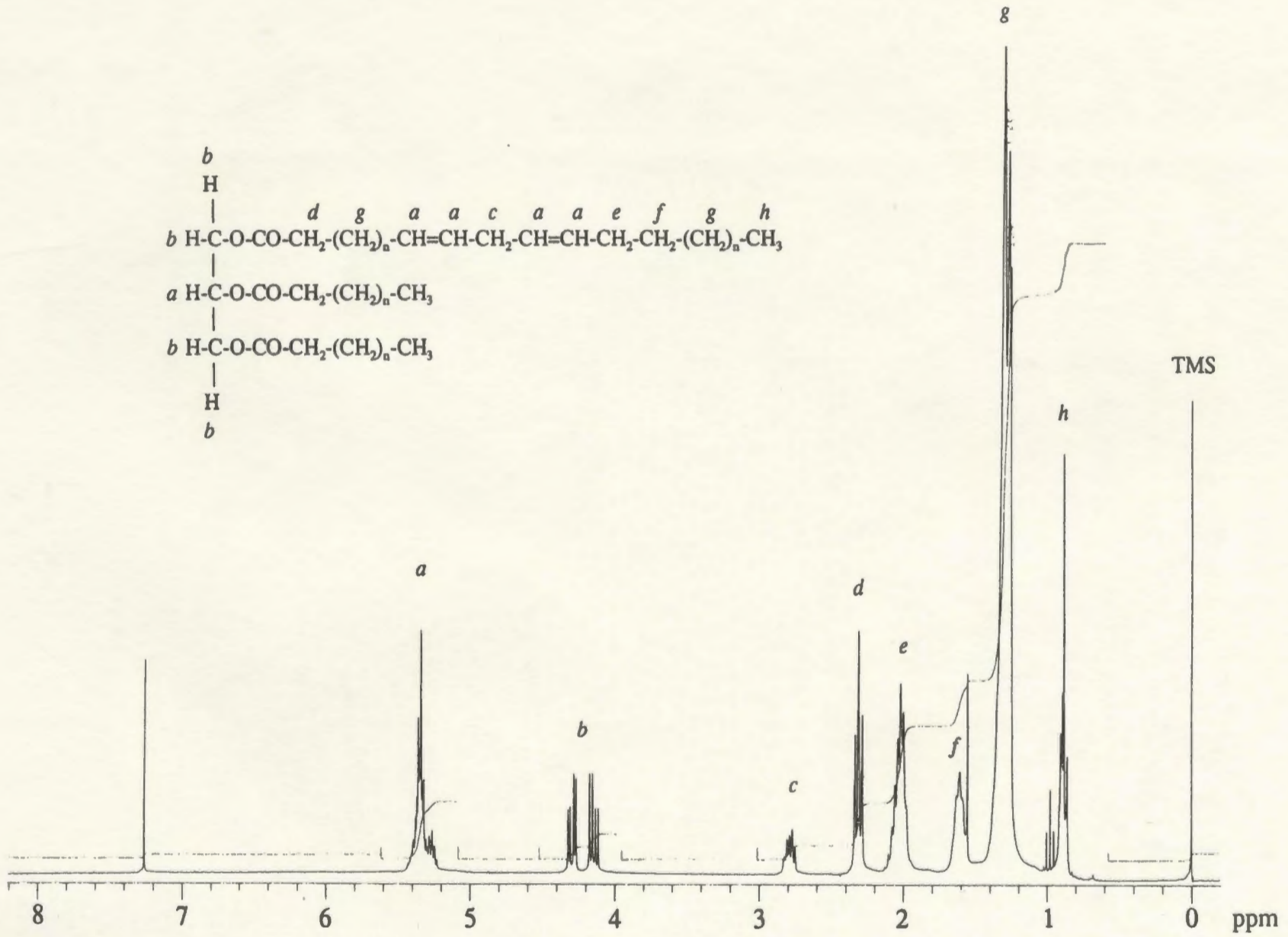
### 4.4.2 Proton NMR spectra of RBD canola and soybean oils

The spectrum of RBD canola oil is shown in Figure 4.9. The spectrum shows eight groups of signals labelled *a* to *h*. These signals are assigned as follows: *a*, hydrogen directly attached to double-bonded carbons (olefinic protons) and the methine proton in the glyceryl moiety (δ 5.1 ~ 5.4 ppm); *b*, two methylene groups in the glyceryl moiety (δ 4.0 ~ 4.4 ppm); *c*, $CH_2$ groups attached to two double-bonded carbon atoms (diallylmethylene protons) (=HC-$CH_2$-CH=; δ 2.6 ~ 2.9 ppm); *d*, three $CH_2$ groups alpha to carboxyl groups (α-$CH_2$; δ 2.2 ~ 2.4 ppm); *e*, $CH_2$ groups attached to saturated carbons and double-bonded carbon atoms (-$CH_2$-C=; δ 1.8 ~ 2.2 ppm); *f*, $CH_2$ groups attached to saturated carbon atoms (=C-$CH_2$-$\underline{CH_2}$; δ 1.45 ~ 1.8 ppm); *g*, $CH_2$ groups bonded to two saturated carbon atoms ($[CH_2]_n$; δ 1.1 ~ 1.45

Table 4.13 Chemical properties of refined, bleached and deodorized (RBD) canola and soybean oils used for $^{1}H$ NMR study.

| Parameter | Canola | Soybean |
|---|---|---|
| Iodine value (g iodine/100g oil) | 111.0 ± 2.01 | 126.0 ± 3.20 |
| Peroxide value (meq/kg oil) | 0.91 ± 0.02 | 0.54 ± 0.01 |
| Fatty acid composition (area %) | | |
| C16:0 | 4.20 ± 0.02 | 10.20 ± 0.04 |
| C16:1 | 0.26 ± 0.01 | -- |
| C18:0 | 1.91 ± 0.02 | 4.22 ± 0.20 |
| C18:1 | 57.60 ± 1.51 | 24.40 ± 0.11 |
| C18:2 | 23.40 ± 1.00 | 52.00 ± 0.22 |
| C18:3 | 9.10 ± 0.11 | 7.70 ± 0.20 |
| C20:0 | 0.81 ± 0.01 | 0.88 ± 0.01 |
| C20:1 | 2.00 ± 0.10 | 0.30 ± 0.04 |
| C22:0 | 0.34 ± 0.00 | 0.30 ± 0.05 |
| C22:1 | 0.38 ± 0.00 | -- |

Figure 4.9 $^{1}H$ NMR spectrum of RBD canola oil (peaks at 0.00 ppm and 7.26 ppm for TMS and $CHCl_3$ protons, respectively).


b
H
d g a a c a a e f g h
b H-C-O-CO-CH2-(CH2)n-CH=CH-CH2-CH=CH-CH2-CH2-(CH2)n-CH3
a H-C-O-CO-CH2-(CH2)n-CH3
b H-C-O-CO-CH2-(CH2)n-CH3
H
b
g
TMS
h
a
d
e
b
f
c
8
7
6
5
4
3
2
1
0
ppm

ppm) and *h*, three terminal $CH_3$ groups (δ 0.7 ~ 1.0 ppm). Quantitative determination of protons in each group was calculated based on the integration of methylene protons (δ 4.0 ~ 4.4 ppm) of triacylglycerol backbone (4 protons in the two methylene groups in the triacylglycerol moiety).

### 4.4.3 Relative changes in the proton NMR absorption pattern of RBD canola and soybean oils during accelerated oxidation

Relative changes of total aliphatic ($CH_3$ + $[CH_2]_n$ + $CH_2$-$CH_2$-C= + $CH_2$-C= + α-$CH_2$; 0.6 ~ 2.5 ppm), olefinic (-HC=CH-; 5.1 ~ 5.4 ppm) and diallylmethylene (=C-$CH_2$-C=; 2.6 ~ 2.9 ppm) protons of canola and soybean oils during oxidation is shown in Table 4.14. It was found that during a 30-day storage, the relative number of olefinic (from 7.12 to 5.21 ppm for canola oil and from 8.12 to 5.88 ppm for soybean oil) and diallylmethylene (from 2.18 to 0.99 ppm for canola oil and from 4.00 to 2.60 ppm for soybean oil) protons decreased and the total number of aliphatic (from 79.3 to 88.2 ppm for canola oil and from 75.6 to 86.0 ppm for soybean oil) protons increased. During the oxidation process, initially diallylmethylene protons are attacked by free radicals and then intramolecular rearrangement of the olefinic protons occurs (Saito and Udagawa, 1992). Therefore, the relative number of diallylmethylene and olefinic protons is decreased. Considering diallylmethylene and olefinic protons of both oils, soybean oil had a relatively higher proportion of both types of protons than canola oil (Table 4.14). This may be due to a higher content of polyunsaturated fatty acids in soybean oil as compared with canola oil (Table 4.13).

Table 4.14 Total olefinic, diallylmethylene and aliphatic protons of RBD canola and soybean oils during accelerated oxidation at 65°C[1].

| Storage time (Days) | Olefinic protons | | Diallylmethylene protons | | Aliphatic protons | |
|---|---|---|---|---|---|---|
| | Canola | Soybean | Canola | Soybean | Canola | Soybean |
| 0 | 7.12 | 8.12 | 2.18 | 4.00 | 79.28 | 75.64 |
| 5 | 6.76 | 8.04 | 1.90 | 3.92 | 80.94 | 79.44 |
| 10 | 6.37 | 7.60 | 1.86 | 3.80 | 83.32 | 80.48 |
| 15 | 6.00 | 7.24 | 1.54 | 3.40 | 84.19 | 80.64 |
| 20 | 5.57 | 7.08 | 1.36 | 3.36 | 85.62 | 84.89 |
| 25 | 5.36 | 6.52 | 1.12 | 2.84 | 86.88 | 85.24 |
| 30 | 5.21 | 5.88 | 0.99 | 2.60 | 88.18 | 86.00 |

[1]Calculated on the basis of integration of methylene protons of the triacylglycerol backbone.

### 4.4.4 The relationship between TOTOX value and the ratios of aliphatic to olefinic and aliphatic to diallylmethylene protons during accelerated oxidation of RBD canola and soybean oils

The ratio of aliphatic to olefinic protons ($R_{ao}$) and aliphatic to diallylmethylene protons ($R_{ad}$) were calculated. These ratios were increased steadily during the storage of both oils. The numerical values of $R_{ao}$ and $R_{ad}$ were plotted against the corresponding TOTOX values for both canola and soybean oils (Figures 4.10 and 4.11, respectively). A highly significant correlation existed between both $R_{ao}$ and $R_{ad}$ and TOTOX values; correlation coefficients were 0.984 for $R_{ao}$ and 0.933 for $R_{ad}$ for canola oil and 0.985 for $R_{ao}$ and 0.969 for $R_{ad}$ for soybean oil. The linear regression equations of these relationships were as follows:

$$TOTOX_{Canola} = 155.0R_{ao} - 1662.0$$

$$TOTOX_{Canola} = 16.4R_{ad} - 433.3$$

$$TOTOX_{Soybean} = 208.9R_{ao} - 1918.0$$

$$TOTOX_{Soybean} = 72.8R_{ad} - 1297.0$$

Therefore, it is clear that TOTOX values correlated well with NMR results, thus reflecting the applicability of this method to estimate formation of both primary and secondary products during oxidation of canola and soybean oils. Saito and Udagawa (1992) have recently used the NMR method for estimation of oxidative deterioration of brown fish meal. These authors correlated peroxide values with the NMR results. Correlation coefficients between peroxide values and $R_{ao}$ and $R_{ad}$ values

Figure 4.10 Relationship between TOTOX values and the ratio of aliphatic to olefinic protons ($R_{ao}$) of oxidized RBD canola and soybean oils.

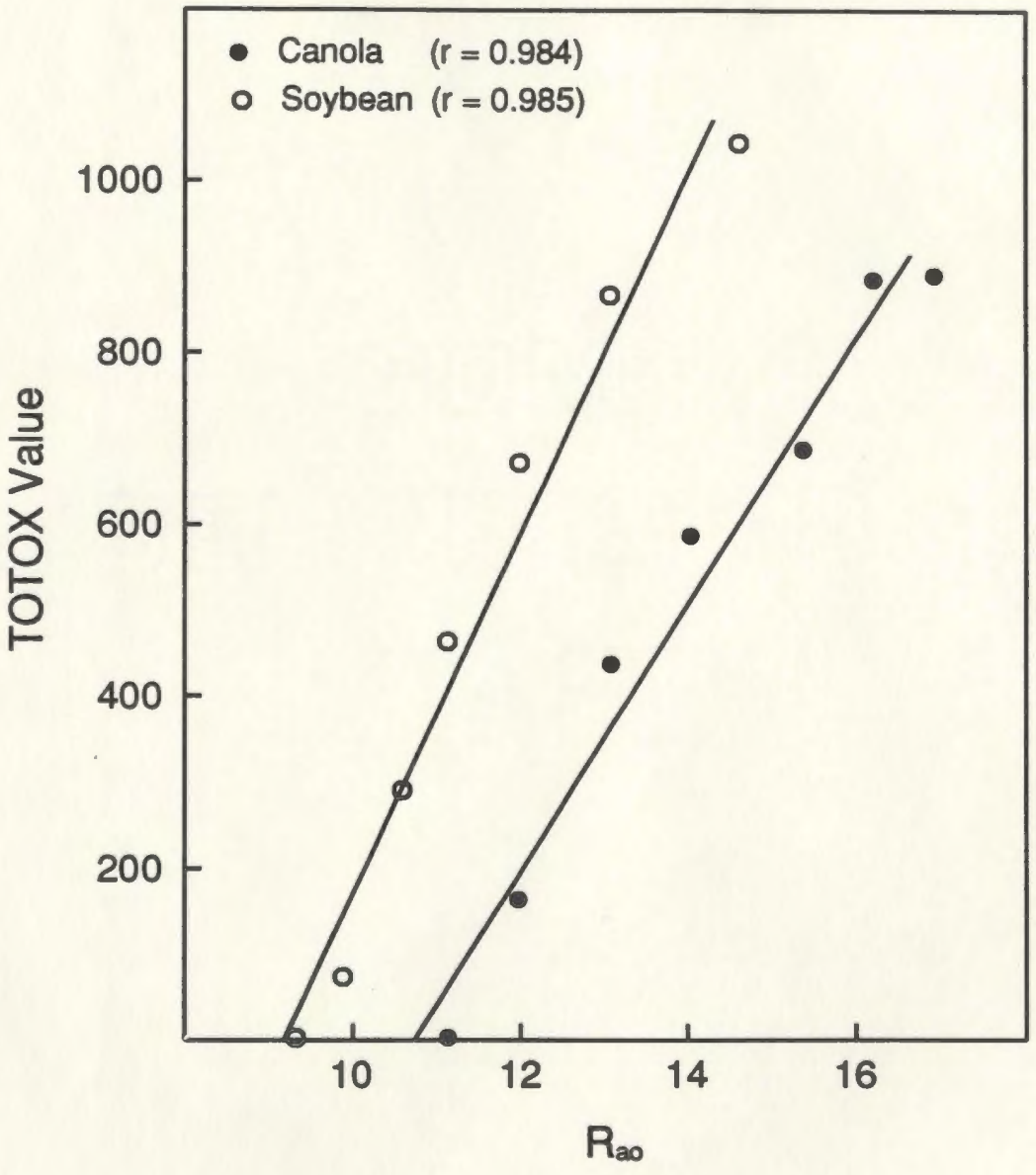
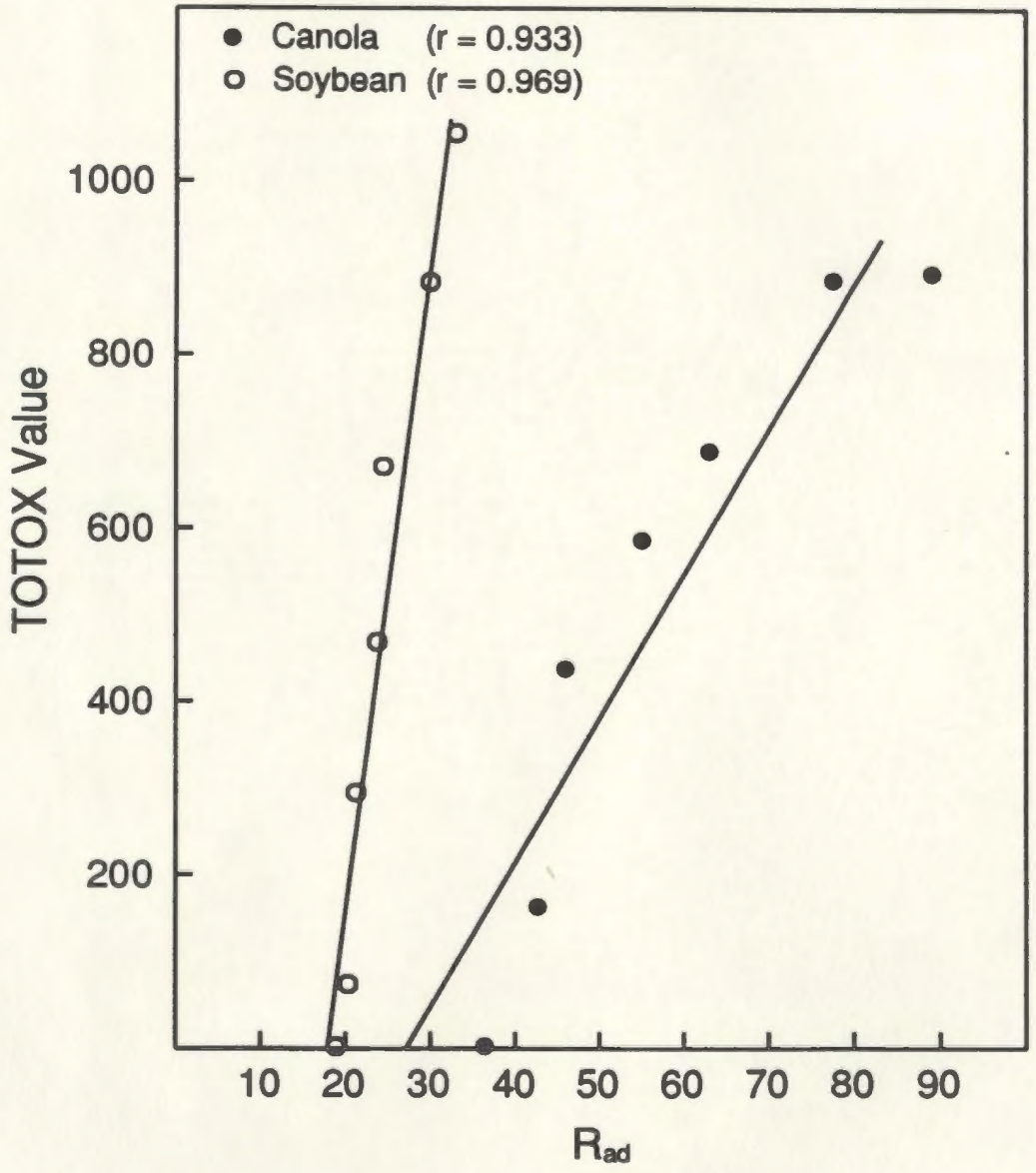

Canola (r = 0.984)
Soybean (r = 0.985)
TOTOX Value
1000
800
600
400
200
10
12
14
16
Rao

Figure 4.11 Relationship between TOTOX values and the ratio of aliphatic to diallylmethylene protons ($R_{ad}$) of oxidized RBD canola and soybean oils.


Canola (r = 0.933)
Soybean (r = 0.969)
TOTOX Value
1000
800
600
400
200
10
20
30
40
50
60
70
80
90
$R_{ad}$

in this study were, 0.848, 0.623, 0.880 and 0.820 for canola and soybean oils, respectively (Figures 4.12 and 4.13). It was also noted that better non-linear correlations may exist between these variables. It is obvious that TOTOX values correlated better with $R_{ao}$ and $R_{ad}$ than peroxide values did. This is not surprising since both TOTOX values and NMR methodology estimate the overall changes in fatty acid profile and include both primary and secondary changes in canola and soybean oils during oxidation. The simplicity of the NMR analysis provides a rapid, non-destructive procedure for analysis of edible oils with respect to oxidative changes occurring during storage.

Figure 4.12 Relationship between peroxide values and the ratio of aliphatic to olefinic protons ($R_{ao}$) of oxidized RBD canola and soybean oils.

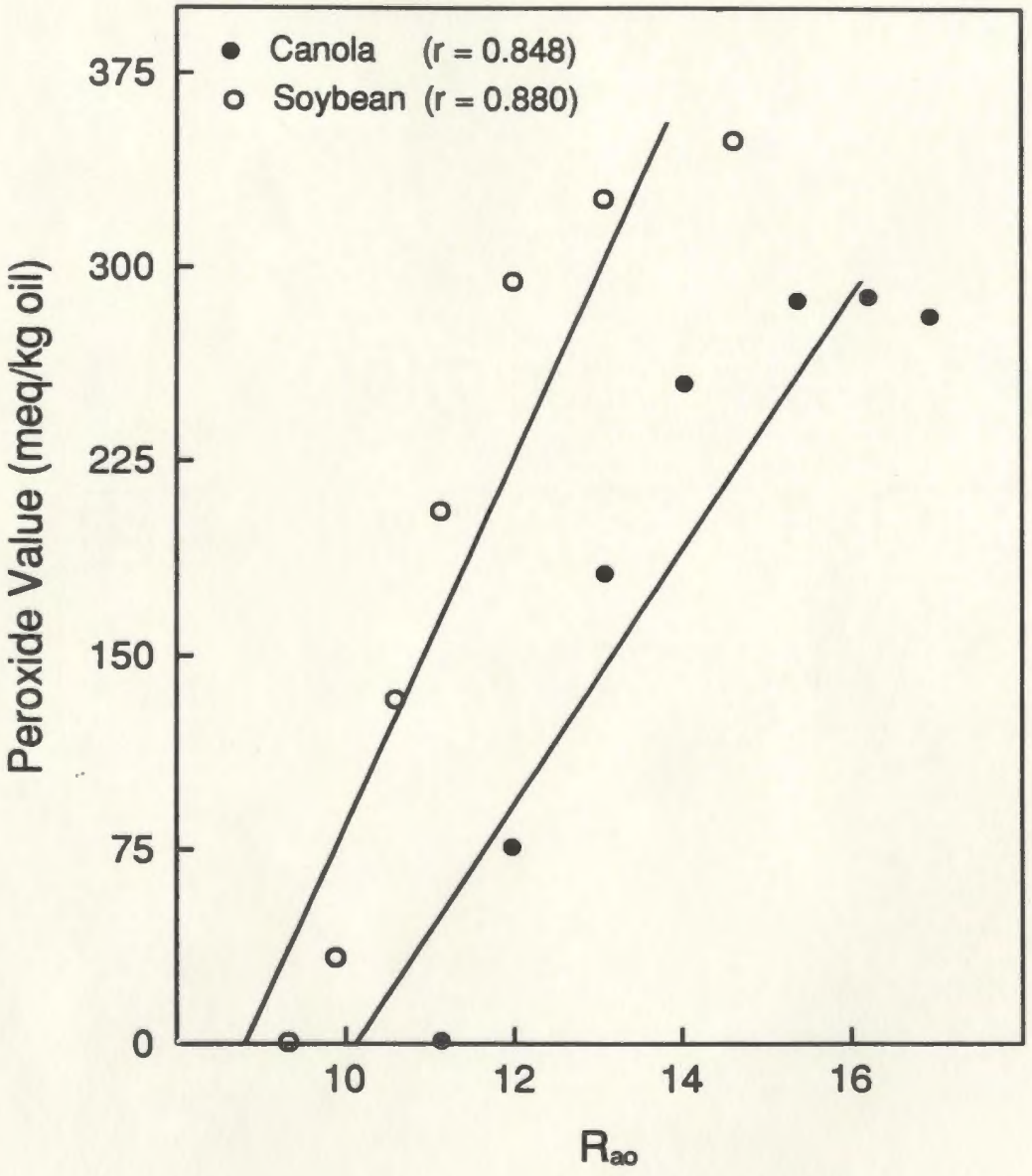
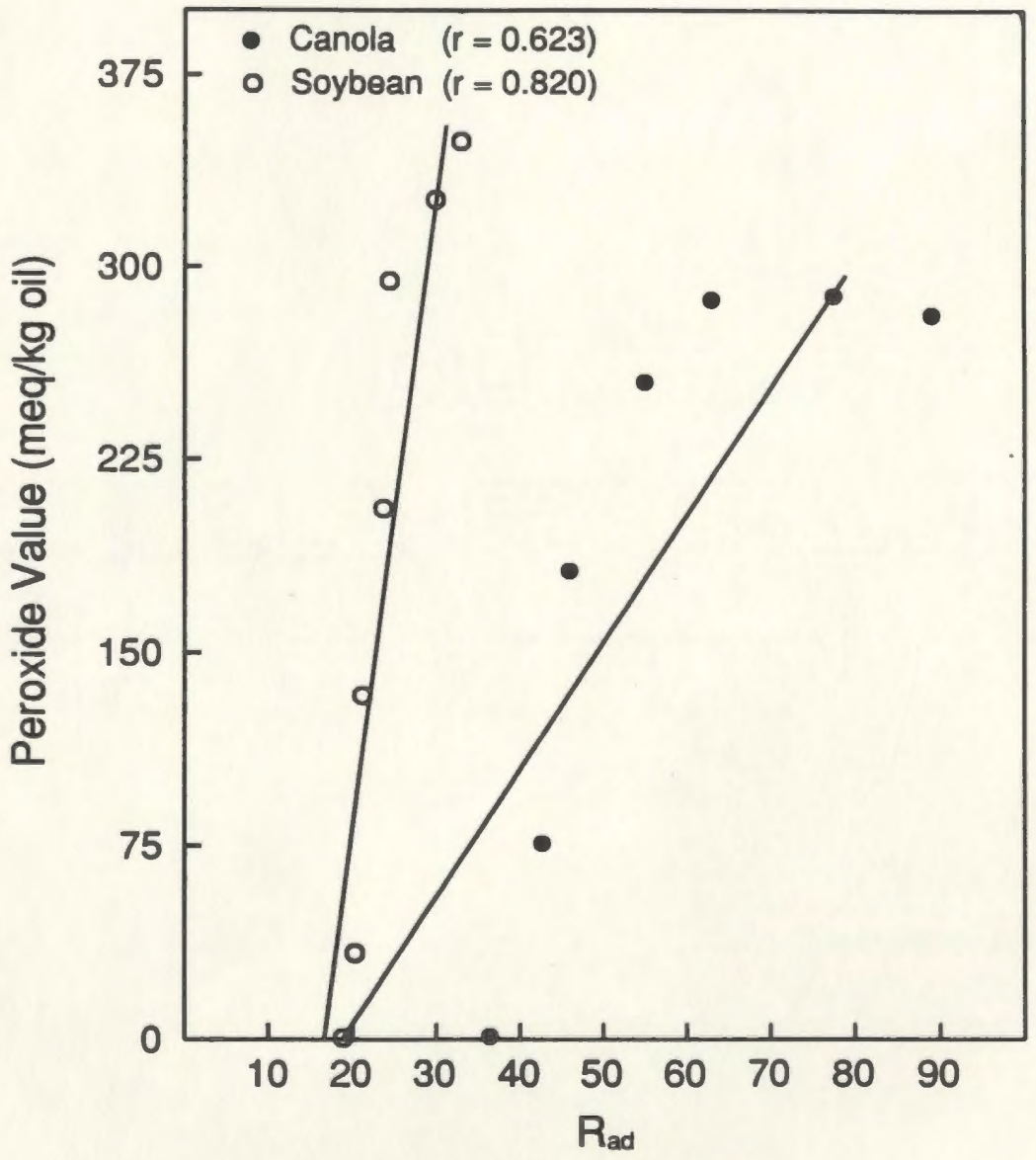

Canola (r = 0.848)
Soybean (r = 0.880)
Peroxide Value (meq/kg oil)
375
300
225
150
75
0
10
12
14
16
$R_{ao}$

Figure 4.13 Relationship between peroxide values and the ratio of aliphatic to diallylmethylene protons ($R_{ad}$) of oxidized RBD canola and soybean oils.


Canola (r = 0.623)
Soybean (r = 0.820)
Peroxide Value (meq/kg oil)
375
300
225
150
75
0
10
20
30
40
50
60
70
80
90
$R_{ad}$

## CONCLUSIONS AND RECOMMENDATIONS

Ethanolic extract of canola meal showed strong antioxidant properties when added to refined-bleached canola oil. At an addition levels of 500 and 1000 ppm, the activity of the extract was better than that of BHA/BHT/CA (250 ppm) which is commonly used in the canola oil industry. Therefore, extracts of canola meal can potentially be used as alternative natural antioxidant system for stabilizing canola oil. The effectiveness of canola extract at these levels was less than that of TBHQ (200 ppm), however, TBHQ is not permitted as a food additive in Canada. Canola extract did not impart any visible colour or perceivable odour to canola oil.

The most active antioxidative component of canola extract, separated by chromatographic techniques, was a phenolic compound. Detailed spectral analyses identified it as 1-O-β-D-glucopyranosyl 3,5-dimethoxy-4-hydroxy cinnamate.

The antioxidant activity of a number of flavonoids was also tested in this study. Results indicated that myricetin, quercetin and rutin, all flavonols and (-)epicatechin a flavan-3-ol, were most effective in stabilizing canola oil. Naringin and narigenin (flavonones) were considerably less effective than flavonols and flavan-3-ol tested.

A novel nuclear magnetic resonance (NMR) spectroscopy was also used to monitor oxidation of canola and soybean oils. The ratios of the integrated areas of aliphatic to olefinic protons and aliphatic to diallylmethylene protons of oils under different stages of oxidation correlated well with TOTOX values. The NMR methodology offers a rapid, non-destructive procedure for evaluation of the oxidative stability of edible oils, as exemplified for canola and soybean oils.

Based on the results obtained in this study it is recommended that:

1. The effectiveness of ethanolic extract of canola in stabilizing other edible oils which are highly prone to oxidation be studied.
2. The toxicological properties of the extract to be studied in order to meet possible legislative requirements for their commercial application in the edible oil industry.
3. The applicability of $^{1}H$ NMR as an indicator of oxidation of lipids in other food systems and relationship of this indicator with sensory properties to explored further.

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# APPENDIX

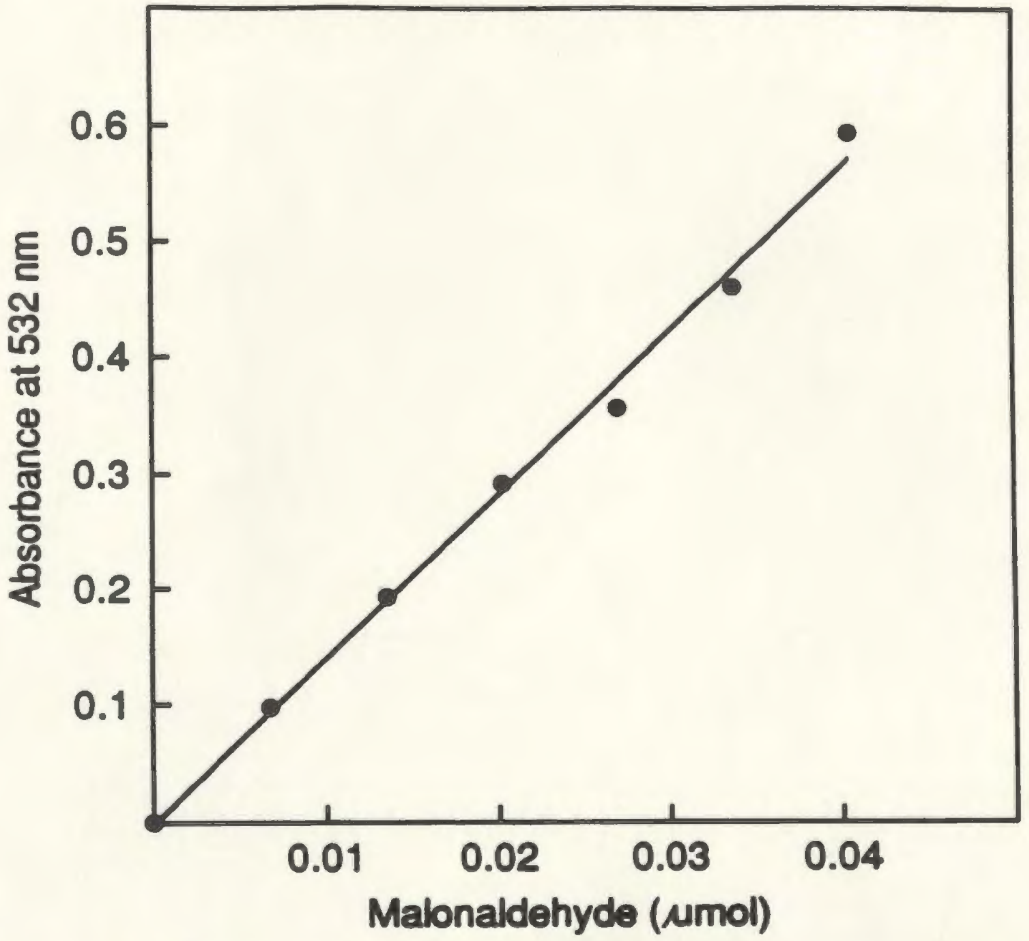



Figure A.1 Standard line of concentration dependance of TBARS as reflected in the absorbance of the TBA-malonaldehyde complex.

Regression coefficient (r) = 0.995
Equation of the line (Y=aX+b) where,

Y = absorbance at 532nm ($A_{532}$)
X = concentration of malonaldehyde (MA) in 5 ml solution, μmole (C)
a = 14.116
b = 0.0

$A_{532} = 14.116*C$

Therefore, $C = 0.071A_{532}$

Since the w grams of oil dissolved in 25 ml of solution, the MA concentration is:

$C = (0.355A_{532})/w$ (μmole of MA/g oil)

Figure A.2 Mass spectrum of the most active antioxidative compound of canola extract (Molecular ion was not observed).

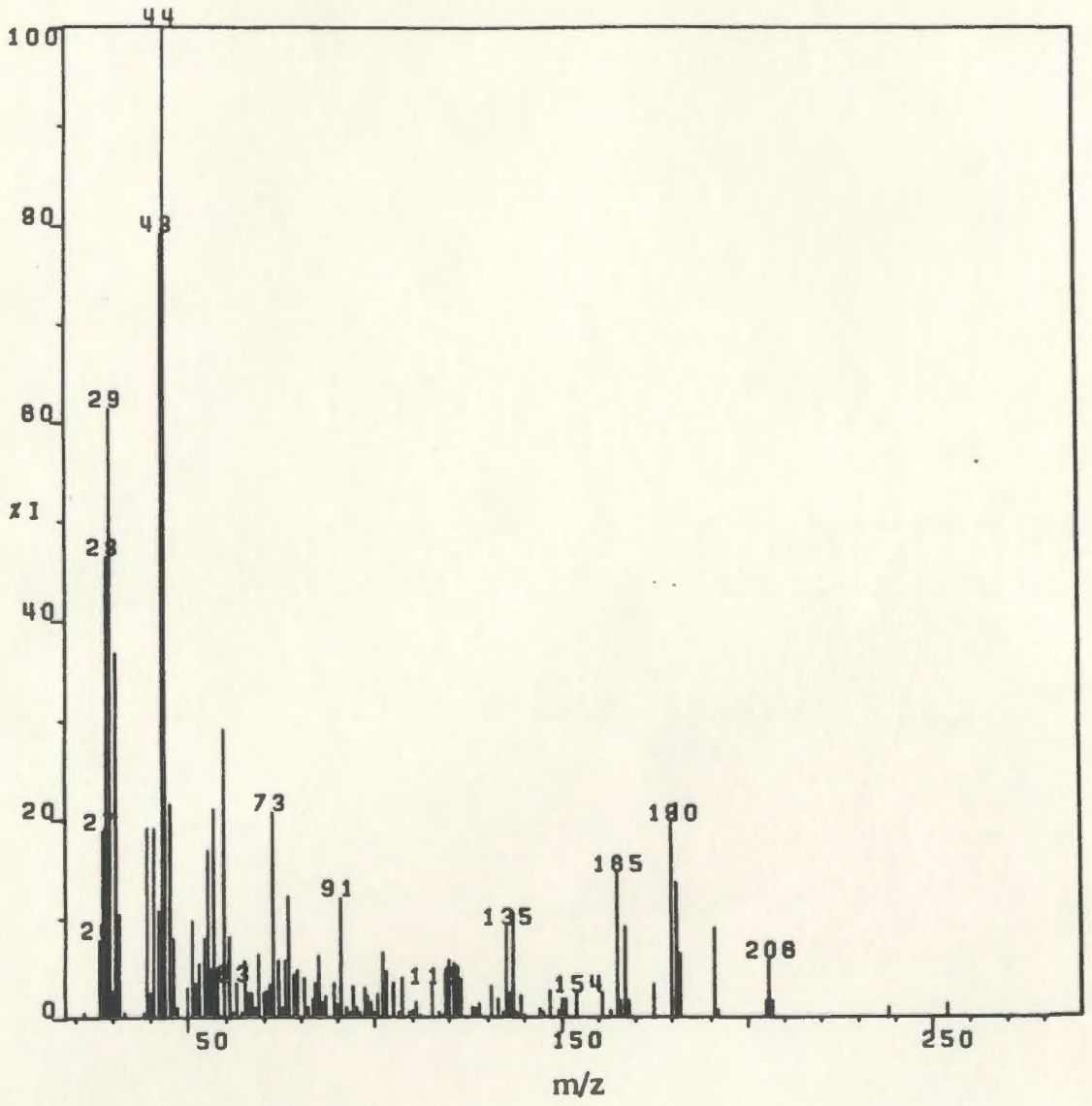

44
100
80
43
29
60
x1
28
40
73
20
27
180
185
91
135
2
208
3
11
154
0
50
150
250
m/z

Figure A.3 $^{1}H$ NMR spectrum of the most active antioxidative compound of canola extract.

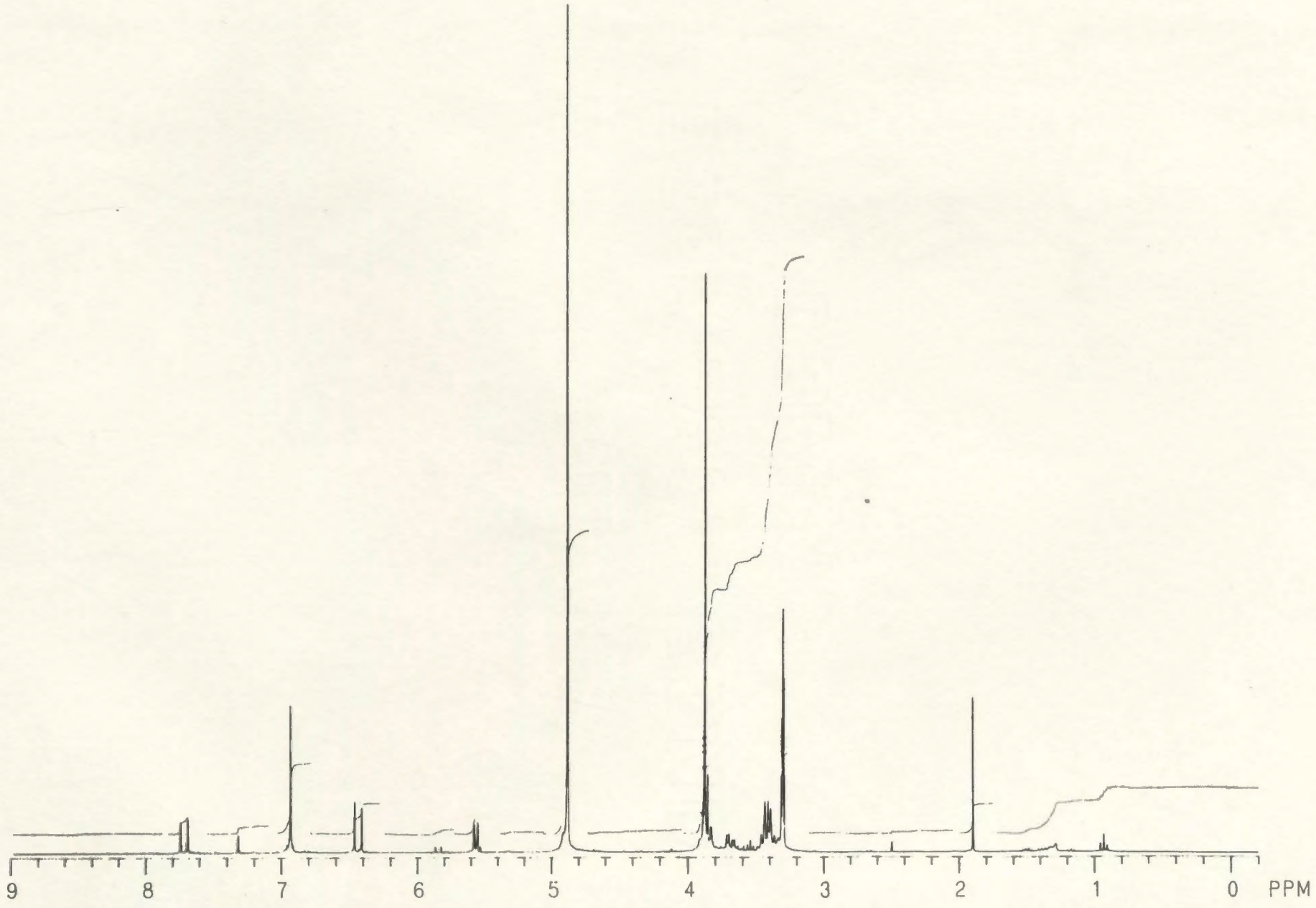

9
8
7
6
5
4
3
2
1
0
PPM

Figure A.4 $^{13}C$ $\{^{1}H\}$ NMR and attached proton spectra of the most active antioxidative compound of canola extract.

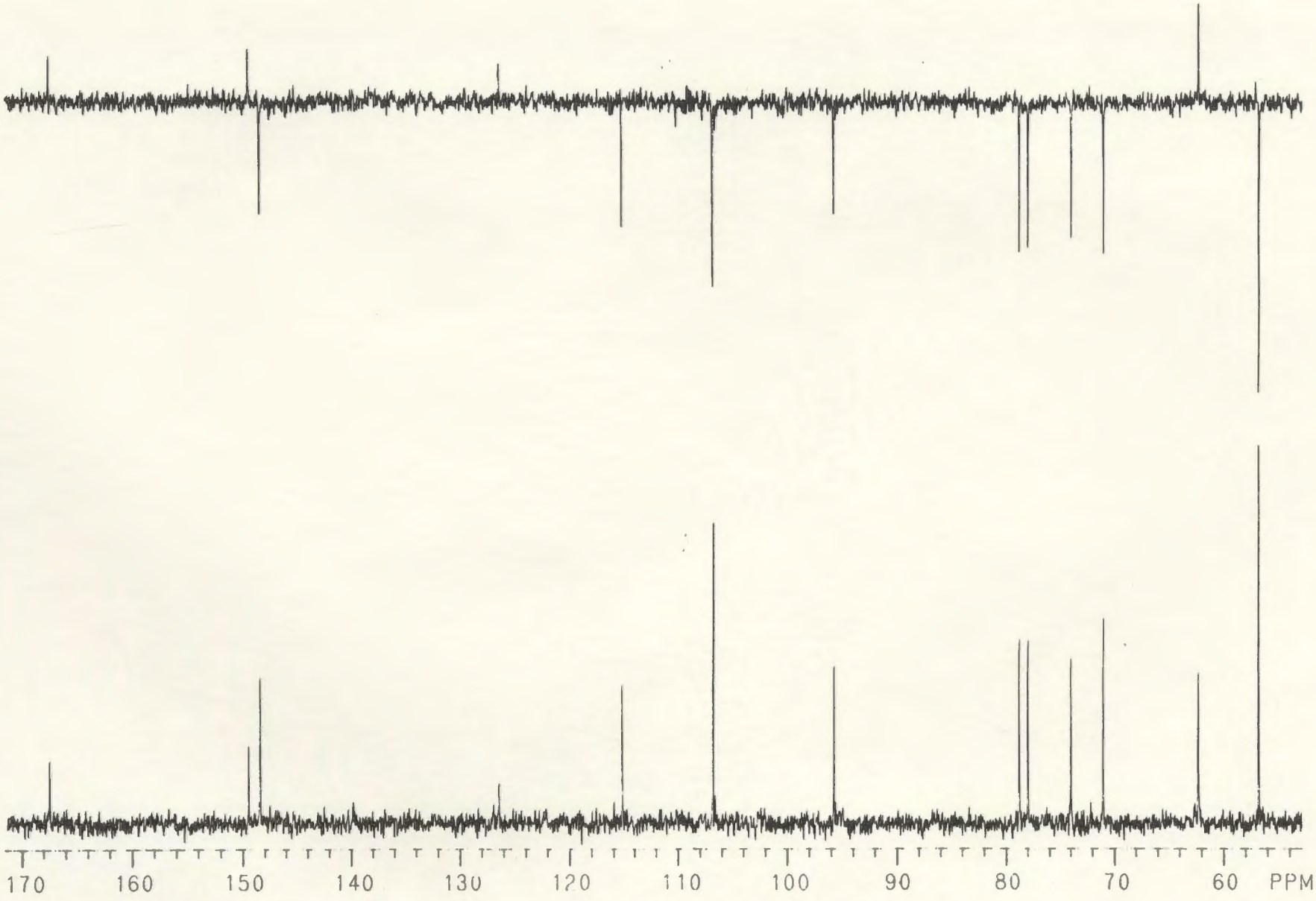
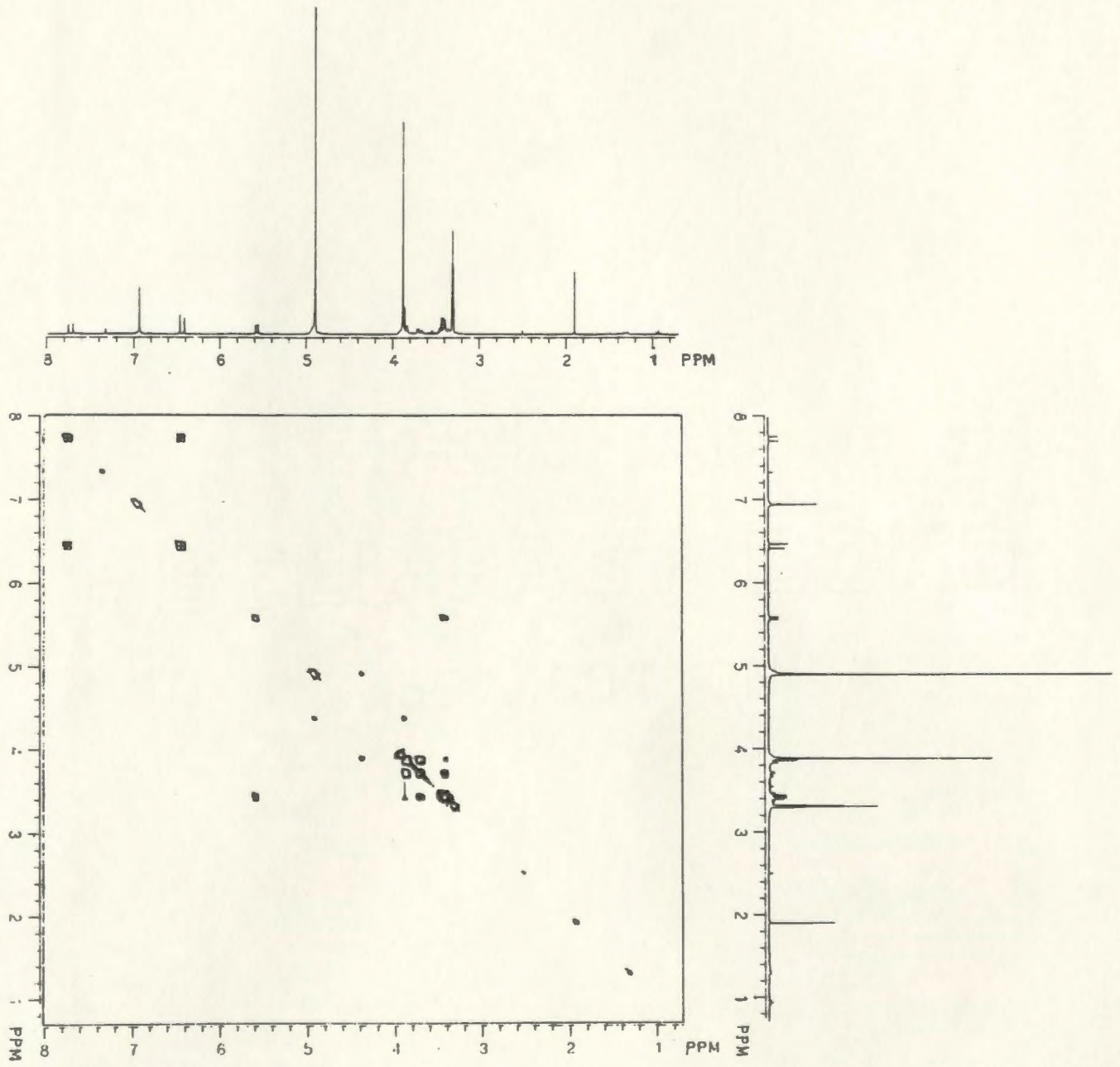

170
160
150
140
130
120
110
100
90
80
70
60
PPM

Figure A.5 $^{1}H$-$^{1}H$ COSY spectrum of the most active antioxidative compound of canola extract.


8
7
6
5
4
3
2
1
PPM
PPM
PPM

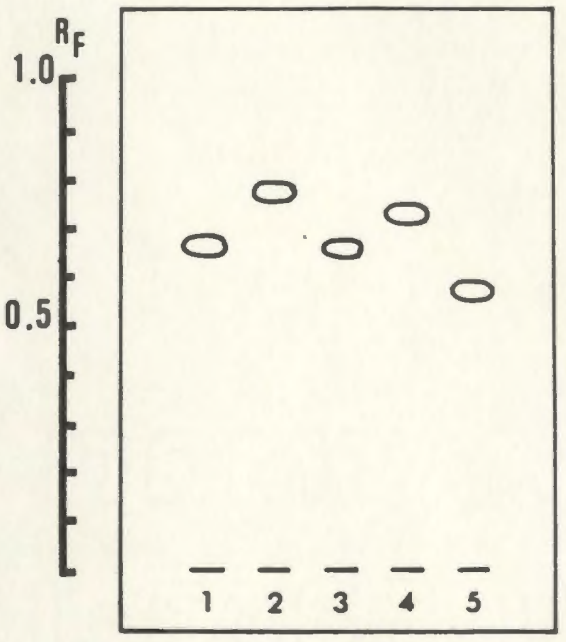



Figure A.4 Chromatogram (TLC) of the sugar component obtained upon hydrolysis of the most active antioxidative compound of canola. (1) sample, (2) rhamnose, (3) glucose, (4) mannose and (5) galactose.

**Identification of sugar of the most active antioxidative compound of canola following acid hydrolysis**

Five milligram of the most active antioxidative compound, isolated as indicated before, was hydrolyzed at 100°C for 1 h using a 1.0 ml concentrated HCl/water/ethanol (6:3:9, v/v/v). After 1 h the sample was cooled and diluted with water (1.0 ml) and the phenolic acid portion of the molecule, was extracted with diethyl ether and removed. The aqueous layer containing the sugar of interest was loaded onto a TLC plate (Silica gel, 60 Å mean pore diameter, 2-25 µm mean particle size, 250 µm thickness, Sigma Chem. Co., St. Louis, Missouri) with standard monosaccharides (Sigma Chem. Co. St. Louis, Missouri). The TLC plate was developed in a glass chamber 22 cm x 22 cm x 10 cm (Fisher Scientific Ltd. Toronto, Ontario) using chloroform/methanol (60:40, v/v) with 0.02M sodium acetate as the mobile phase (Pifferi, 1965). After drying, bands were located and identified spraying with aniline-diphenylamine-phosphoric acid (Pifferi, 1965).

Spray reagent:

Four gram of diphenylamine, 4 ml of aniline and 20 ml of 85% phosphoric acid were dissolved in 200 ml acetone. After spraying, plate was heated at 85°C for 10 min in an oven.

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