ANTIOXIDANT ACTIVITY OF CYCLOLINOPEPTIDES

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Department of Food and Bioproduct Sciences

University of Saskatchewan

Saskatoon

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ABSTRACT

Cyclolinopeptides (CLs) are hydrophobic cyclic peptides found in flaxseed. They show immunosuppressive activity, but the biological function of these compounds is largely unknown. This thesis presents the results of studies that were conducted to determine whether CLs could act as antioxidants. In the first study, flaxseed oil was passed over a silica adsorbent column to remove polar compounds. The polar compounds were then eluted from the silica absorbant using a series of increasingly polar solvents. Individual polar fractions were then added back to the silica-treated flaxseed oil and the oxidative stability index of these samples was determined at 100 °C. A polar fraction containing mainly CLA, β/γ- and δ-tocopherol increased the induction time of silica-treated flaxseed oil from 2.3 ± 0.28 h to 3.2 ± 0.41 h. A positive effect of the polar fraction containing a mixture of CLA and CLD-CLG on the oxidative stability of oil was also observed. The antioxidant mechanism of CLs was investigated in several model systems using electron spin resonance spectroscopy. The concentration of radicals in a DMPO (5,5-dimethyl-1-pyrroline-N-oxide) radical-CLs reaction mixture was monitored. All CLs exhibited dose dependent scavenging activities. CLA–CLC reactions with DMPO-OH at a concentration of 5 mM resulted in a 24–30% decrease in electron paramagnetic resonance (EPR) signal intensity. The reaction of CLs and the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) revealed a more complex interaction than simple radical scavenging. Peptides (CLG and CLG") that contained both tryptophan and methionine showed stronger radical scavenging activity than did CLs containing methionine or methionine sulfoxide but not tryptophan (CLB and CLC). Irradiation of the reaction mixture of DPPH$^\bullet$ and peptide with UV light also affected the radical scavenging behaviour. Scavenging activities of DPPH$^\bullet$ by CLB, CLC and CLA were enhanced by light, whereas scavenging of DPPH$^\bullet$ by the tryptophan containing peptides CLG and CLG″ was not affected. High-performance liquid chromatography with mass spectrometry (HPLC-MS) analysis of the reaction mixtures after a radical scavenging reaction was used to determine the impact of radical scavenging on the peptides. These reactions revealed new masses that were identified and characterized. It was established that DPPH$^\bullet$ reacted with the methionine of CLB and with tryptophan in CLG and CLG″, by formation of a new covalently-bonded species. Covalent linkages between these amino acids (alone or in peptides) and DPPH$^\bullet$ have not been reported previously.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

AA   Antamanide

ALA   Alpha-linolenic acid

AOM   Active oxygen method

BHA   Butylated hydroxyanisole

BHT   Butylated hydroxyl toluene

CD   Circular dichroism

CL   Cyclolinopeptide

CsA   Cyclosporin A

CypA   Cyclophilin A

DAD   Diode array detector

DCM   Dichloromethane

DMPO   5,5-dimethyl-1-pyrroline-N-oxide

DPPH$^{\bullet}$ 2,2-diphenyl-1-picrylhydrazyl

EDTA   Ethylenediaminetetraacetic acid

EPR   Electron paramagnetic resonance

ESI-MS   Electrospray ionization-mass spectrometry

ESI-MS/MS   Electrospray ionization tandem mass spectrometry

ESI-TOF-MS   Electrospray ionization-time of flight-mass spectrometry

HPLC   High-performance liquid chromatography

GC   Gas chromatography

LA   Linoleic acid

NMR   Nuclear magnetic resonance

OSI   Oxidative stability index

PG   Propyl gallate

R$^{\bullet}$ Radical

ROS   Reactive oxygen species

SD   Standard deviation

SEM   Standard error of the mean

CHAPTER 1

INTRODUCTION

Flaxseed (*Linum usitatissimum* L.) is an important oilseed crop in Canada. Canadian researchers are developing higher-value, flaxseed-based products that enhance the benefits of flaxseed for human health, animal productivity and nutrition. In addition, researchers are improving flaxseed and flaxseed processing for improved fibre and industrial uses (Ashley and Sullivan, 2006).

Cyclolinopeptides (CLs) are hydrophobic constituents of flaxseed. CLA, the first reported CL (Kaufmann and Tobschirble, 1959), suppresses a wide range of immunological responses including: delayed-type hypersensitivity response, skin allograph rejection, graft versus host reaction, post adjuvant arthritisand haemolytic anemia of New Zealand black mice (Wieczorek et al., 1990). Eleven CLs have been isolated and characterized from flaxseed and the content of these peptides can range from 81–302 µg/g depending on genotype and environmental conditions (Gui et al., 2012a).

On the other hand, flaxseed oil is greatly valued for its high content of health-supporting unsaturated fatty acids, linoleic acid and linolenic acid, which make it prone to oxidation. To protect its oil, flaxseed has a very active and stable antioxidant system. However, this antioxidant system is not fully understood. For instance, milled flaxseed could be stored for 28 months at ambient temperatures without a noticeable increase in oxidation products. This stability could be caused by the presence of antioxidants other than tocopherols in the seed (Przybylski et al., 2005). The presence of cyclic peptides is not known in any commercial oilseeds other than flaxseed, and it is possible that CLs are an integral element of the flax antioxidant system.

Hypothesis 1: The stability of crude flaxseed oil is determined not only by its content of the fat-soluble antioxidant tocopherols, but also by polar compounds present in the oil, including CLs.

1

Objectives:

- To remove polar compounds from the oil and determine which fractions affect the oxidative stability of oil stripped of minor compounds.
- To identify CLs in antioxidant fractions.
- To examine the effect of CLs on the oxidation of flaxseed oil induced by metal cations.

Hypothesis 2: CLs are chain-breaking antioxidants which are able to trap free radicals.

Objectives:

- To investigate scavenging of hydroxyl radicals, generated by the Fenton system, by CLs.
- To examine interactions between CLs and the stable free radical (2,2-diphenyl-1-picrylhydrazyl, DPPH$^{\bullet}$).
- To explore reaction products of free radicals and CLs by HPLC-MS/MS.

CHAPTER 2
LITERATURE REVIEW

2.1   Flaxseed and Its Oil Content

2.1.1   General Description

Flaxseed (*Linum usitatissmum* L.) grows in the cool climate of Western Canada. It is an annual plant, which was first domesticated around 5000 B.C. in western Asia. Traditionally it has been cultivated for food and for folk medicine ingredients, but today it is grown primarily for its oil (Oomah, 2001). The seed of flax is smooth and oval with a pointed tip and varies in color from dark brown to yellow (Freeman, 1995). Canadian flaxseed typically contains 41% fat, 20% protein, 28% dietary fibre, 7.7% moisture and 3.4% ash, although this amount can vary depending on cultivar, growing conditions and seed processing (DeClercq, 2012). In 2009, Canada produced 1,000,000 tonnes of flaxseed for oil, meal and fibre, representing 43% of world production (Weber, 2009). Flaxseed is currently the second most grown oilseed crop in Western Canada and it is planted in the grassland areas of Saskatchewan (70%), Manitoba (26%) and Alberta (4%) (Touré, and Xueming, 2010).

2.1.2   Flaxseed Oil Content And Stability

Flaxseed oil contains 90–96% triacylglycerols as well as up to 6% glycolipids, 4–6% phospholipids and 0.4–1.3% non-saponifiable matter (Choo et al., 2007). The oil contains high levels of unsaturated fatty acids, especially the essential omega-3 fatty acid, alpha-linoleic acid (ALA). Typical flaxseed varieties grown in Western Canada contain 50–60% linolenic acid. Furthermore, the oleic acid and linoleic acid (LA), the essential omega-6 fatty acid, contents are usually 11%–29% and 9%–21%, respectively (Verasco and Goffman, 2000). The high content of unsaturated fatty acids in flaxseed oil leads to its poor oxidative stability in spite of its relatively high content of antioxidants like tocopherol (Table 2-1). Although flaxseed oil is prone to

Table 2-1   Total tocopherol (mg/100 g), α-tocopherol (mg/100 g), γ-tocopherol (mg/100 g), polyunsaturated fatty acids (PUFA, %) and oxidative stability values of seed oils (Bozan and Temelli, 2008)

Seed oil	Total tocopherol	α-tocopherol	γ-tocopherol	PUFA	Induction time (h)
Flaxseed oil	79.41	0.59	75.67	72.27	1.57 ± 0.20
Poppyseed oil	30.94	5.53	21.74	75.07	5.56 ± 0.80
Safflowerseed oil	53.20	44.09	ND[1]	71.05	2.87 ± 0.50

[1]ND: not detected.

oxidation, milled flaxseed could be stored for four months.

Flaxseed likely contains a number of endogenous antioxidants that protect the oil from oxidation. The total tocopherol (a significant antioxidant present in plant oils) content of flaxseed is 9.3–16.9 mg/100 g of which 96–100% of total tocopherol is γ-tocopherol (Oomah et al., 1997, Verasco and Goffman, 2000) resulting in the concentration of γ-tocopherol in flaxseed oil being 11.2–15.0 mg/100 g. Moreover, plastochromanol-8, α-tocopherol analog that is a stronger antioxidant than α-tocopherol, is present at a concentration of 3.4–5.5 mg/100 g (Choo et al., 2007, Olejink et al., 1997). Phenolic compounds also have been reported as important mediators of oil oxidative stability. Choo et al. (2007) reported total phenolic acids of 76.8–307.3 mg/100 g and 12.7–25.6 mg/100 g of flavonoid (luteolin equivalents) in samples of cold-pressed flaxseed oil. The extraction procedure for separation of seed solids from oil and the treatment of the oil after extraction determines the concentration of antioxidants in flaxseed oil. For instance, refining procedures, such as filtration, degumming, neutralization, bleaching and deodorization remove solid particles, phospholipids, proteins, carbohydrates, free fatty acids and waxes from the oil (Przybylski et al., 2005). Moreover, these procedures may also reduce minor compounds that affect oil shelf life (Siger et al., 2008). Commercially available edible flaxseed oil is mainly prepared by expeller pressing. Most manufacturers claim to release seed oil using a cold-press method in which the seed is pressed at a temperature lower than 50 ºC. Oil processed by this method is typically not refined before sale.

2.2   Cyclolinopeptides in Flaxseed Oil

Cyclolinopeptides (CLs) are hydrophobic compounds present in flaxseed oil. The highest concentration of these peptides was found in flaxseed oil after cold-pressing (Gui et al., 2012b). CLA was the first isolated in 1959 (Kaufmann and Tobschirbel, 1959). In the 1960s, the amino acid sequences of the oligopeptides from linseed were determined by GC-mass spectrometry. The conformation of synthetic CLA was extensively studied by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy (Naider et al., 1971). After the first disovery of CLA, ten additional CLs were identified and at least partially characterized (Table 2-2, Figure 2-1).

Table 2-2   Primary structures of CLs

Type	Primary structure (*cyclo*-)[1]	Chemical Formula (MW)[2]	References
CLA	Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile	$C_{57}H_{85}N_9O_9$ (1040)	Kaufmann and Tobschirbel, 1959
CLB	Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_9S$ (1058)	Morita et al., 1997
CLC	Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{10}S$ (1074)	Morita et al., 1999
CLD	Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile	$C_{57}H_{77}N_9O_9S$ (1064)	Morita et al., 1999
CLE	Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_9S$ (977)	Brühl et al., 2007
CLF	Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_{10}S_2$ (1084)	Stefanowicz, 2001
CLG	Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_{10}S_2$ (1098)	Stefanowicz, 2001
CLH	Mso-Leu-Met-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_9S_2$ (1082)	Matsumoto et al., 2001
CLI	Met-Leu-Mso-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_9S_2$ (1068)	Matsumoto et al., 2001
CLJ	Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_{10}S$ (993)	Morita and Takeya, 2010
CLK	Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{11}S$ (1090)	Morita and Takeya, 2010

[1]The first and/or third positions of amino acid sequences are displayed in Figure 2-1. Abbreviations are Met for methionine and Mso for methionine sulfoxide.

[2]ESI-MS data obtained as described in Figure 2-1. Modified from Gui et al. (2012a).
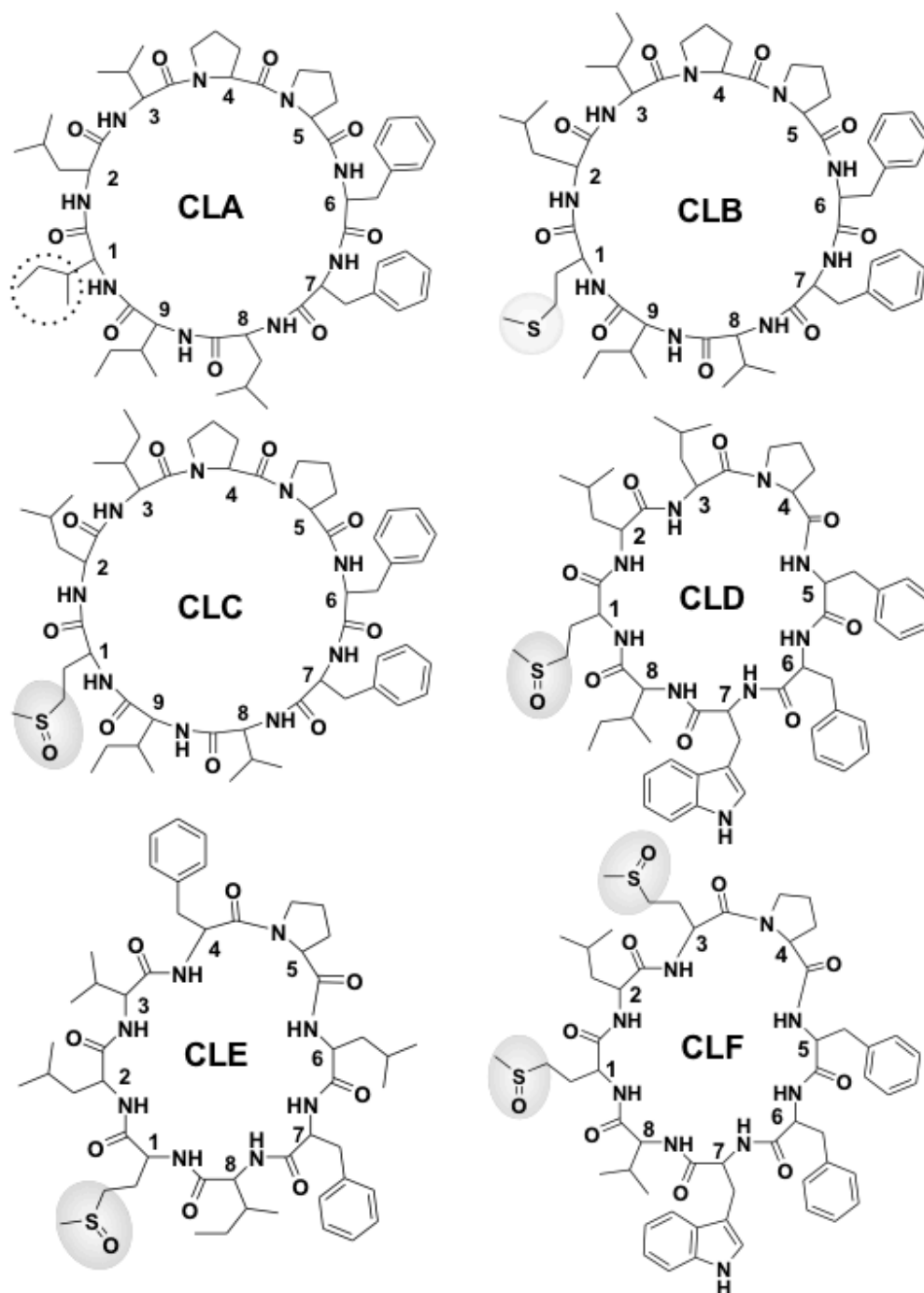
Figure 2-1 Primary structures of CLs from the seeds of *Linum usitatissimum* L. (Kaufmann and Tobschirbel, 1959; Morita et al., 1997; Morita *et al.*, 1999; Matsumoto et al., 2001, 2002).
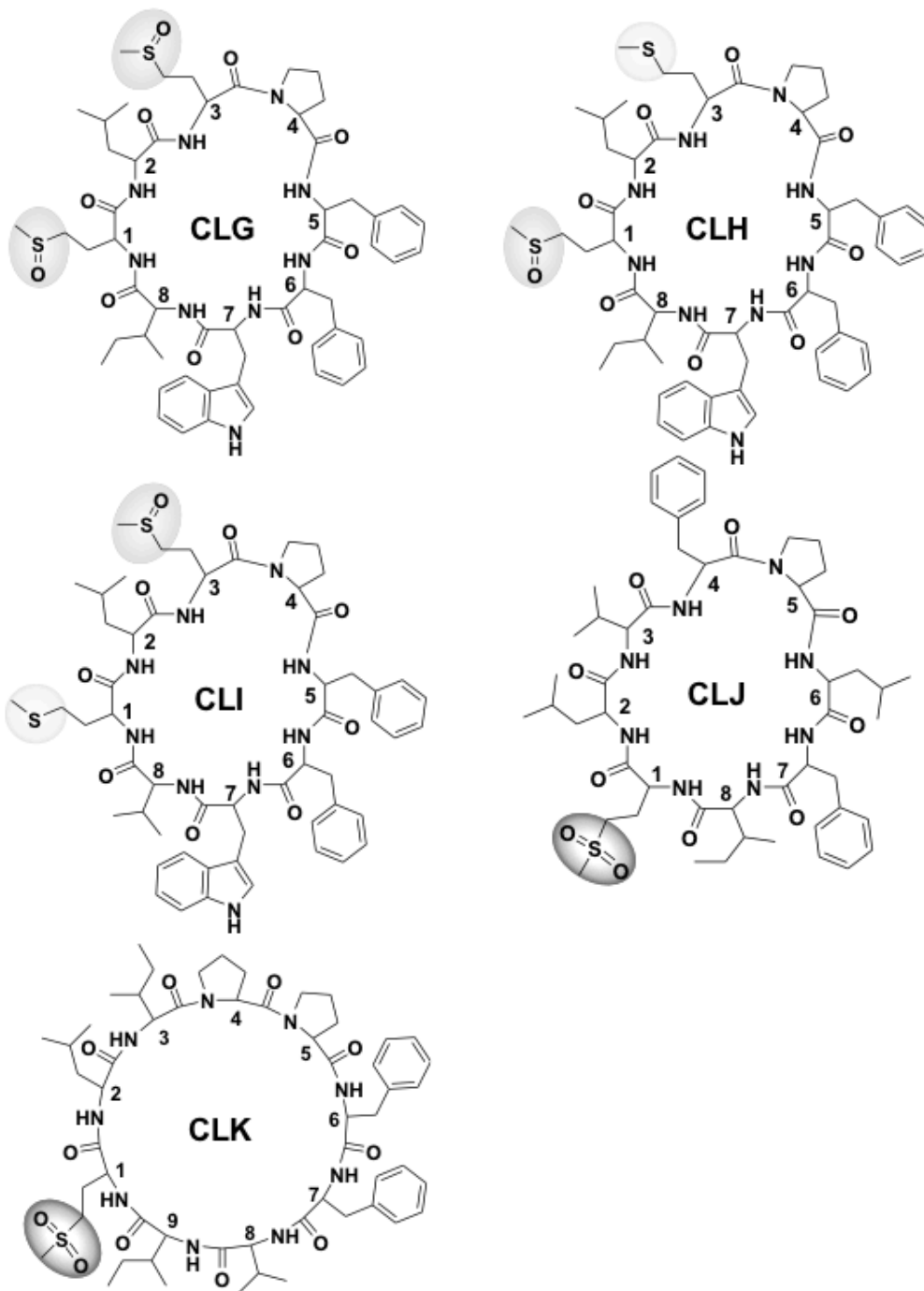
Figure 2-1 Primary structures of CLs from the seeds of *Linum usitatissimum* L.(Kaufmann and Tobschirbel, 1959; Morita et al., 1997; Morita *et al.*, 1999; Matsumoto et al., 2001, 2002) (cont'd).

2.2.1 Biological Activity of CLs

CLs as an Immunosuppressor

CLA inhibits interleukin-1 and interleukin-2 action with a similar activity to cyclosporin, an immunosuppressive agent (Górski et al., 2001, Wieczorek et al., 1991). CLA inhibits T-lymphocyte activation, which modulates the immune response, as occurs with cyclosporin A CsA) or the macrolide FK 506 (Gaymes et al., 1997). The 3-D structure of CLA revealed that its Pro-Pro-Phe-Phe sequence is similar to antamanide (AA), a cyclic decapeptide (Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-Phe). It has been suggested that the -Pro-Xxx-Phe- fragment, where Xxx is a hydrophobic or aromatic amino acid residue, is responsible for the immunosuppression activity of both AA and CLs (Siemion et al., 1999). Other CLs also suppressed immunity. For instance, Morita et al. (1997) showed that CLB inhibits concanavalin-A induced proliferation of human peripheral blood lymphocytes at treatment levels comparable to that of CsA. CLB and CLE inhibited concanavalin-A induced mouse lymphocyte proliferation (Morita et al., 1999). Recently, the effect of these peptides on regulation of apoptosis was investigated (Reaney et al., 2011). In folk medicine, flaxseed has been used to reduce the pain caused by traumatic injuries and arthritis or skin inflammations. As CLA and other CLs are present at a significant amount in flaxseed, the investigation of CLs as biologically active agents from flaxseed is warranted (Benedetti and Pedone, 2005).

2.2.2 Ion Binding Properties of CLA

Siemion (1977) reported that CLA binds K^+ ions, producing a small conformational change in the molecule. Tancredi et al. (1991) concluded that CLA binds Ba^{2+} more tightly than K^+, Na^+, Mg^{2+} and Ca^{2+}. CD spectra indicate that Ba^{2+} ion binding results in the formation of both 1:2 (sandwich) and 1:1 (equimolar) type complexes, depending on the Ba^{2+} ion concentration. The global shape of the complexed peptide can be described as a bowl, with the concave (polar) side hosting Ba^{2+} and the convex side interacting mainly with nonpolar residues (Tancredi et al., 1991). Moreover, Chatterji et al. (1987) identified the formation of a weak complex of CLA with Ca^{+2} mimic cations Tb^{3+} and Pr^{3+}. Based on shifts in NMR spectral peaks, these ions interacted with the Phe residues of the peptide (Chatterji et al., 1987).

2.2.3 Interaction of CLA with Other Molecules

Like cyclosporin A, CLA has the capacity to bind with proteins. Gallo and Pedone (1998) studied the interaction of CLA and its synthetic analogs with bovine cyclophilin A (CypA). The sequence Val-Pro-Pro-Phe participated in cyclophilin binding (Gallo and Pedone, 1998). Moreover, the binding of complex CLA with Human Serum Albumin was investigated with surface plasmon resonance and CD (Rempel et al., 2010).

2.2.4 Edge-To-Face Interaction

Interaction of adjacent aromatic amino acids where positively charged hydrogen atoms of one aromatic group and the π–electron cloud of the adjacent moiety is called an edge-to-face interaction. Experiments show that even weak intermolecular edge-to-face interactions between aromatic rings can impact the conformation of organic molecules in solid state and in solution. Cox et al. (1958) observed this interaction in single crystals of benzene (Brain et al., 2001). In 1985–1988, Burkey and Petsko revealed the same orientation in proteins, oligopeptide crystals and their derivatives. Proteins with aromatic side chains including phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), may all participate in edge-to-face interactions. Siemion et al. (1994) suggested that the immunosuppressive activity of CLA and its analogs was, in part, related to edge-to-face, interactions. Their research showed that CLA is less flexible than a linear peptide A because of its edge-to-face interactions. They proposed that an edge-to-face interaction between the aromatic rings in the cyclic peptides (*cyclo*-(Leu-Ile-Ile-Leu-Val-Pro-Pro-Tyr-Phe)([Tyr^8]CLA) and *cyclo*-(Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Tyr)([Tyr^9]CLA) and *cyclo*-(Leu-Ile-Ile-Leu-Val-Pro-Pro-Tyr-Tyr)([Tyr^{8,9}]CLA) was influential in determining their three dimensional structures (Siemion et al., 1994).

2.3 Lipid Oxidation

Oxidation of food is a process that changes its chemical composition and typically leads to a loss of nutritional quality. The oxidation of fats and oils similarly leads to rancidity and deterioration of quality. The main mechanism of lipid oxidation in foods is a free radical chain reaction known as auto-oxidation. This process is divided into three phases: initiation, propagationand termination.

2.3.1 Initiation Phase

The direct reaction of lipids with oxygen molecules is slow and not favoured. UV light, irradiation, heat, pro-oxidant metal ions, enzymes or photosensitizers can initiate the lipid oxidation process (Koleva, 2007). These initiators promote the formation of reactive oxygen species (ROS). During the initiation phase, oxidation is slow and the radical concentration is low. ROS are mainly responsible for most oxidation reactions in foods which occur after initiation. ROS include oxygen radicals and non-radical derivatives of oxygen. The oxygen radicals are superoxide ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$), peroxy ($ROO^{\bullet}$), alkoxy ($RO^{\bullet}$) and hydroperoxy ($HOO^{\bullet}$) radicals, and non-radical species are hydrogen peroxide (H_2O_2), ozone (O_3) and singlet oxygen ($^1O_2$) (Korycka-Dahla and Richardson, 1978). Table 2-3 shows oxidation capacity of these species. The higher the reduction potential of the ROS, the greater the oxidizing capacity of the compounds. In the case of unsaturated lipids, the reduction potential of an unsaturated fatty acid is 600 mV (Min and Boff, 2002), so ROS which have higher reduction potential are able to remove the hydrogen atom from lipids and initiate auto-oxidation.

In food systems, ROS produce harmful volatile compounds and destroy essential fatty acids, amino acids and vitamins by reacting with them. Furthermore, ROS oxidize proteins, lipids and carbohydrates and change their functionalities. In biological systems, ROS are involved in many deleterious processes, including mutation, carcinogenesis, degenerative diseases, inflammation and aging (Ron and Abraham, 2002). Superoxide anion is generated from triplet oxygen *via* enzymatic and chemical mechanisms (Figure 2-2). To produce superoxide anion, a single electron should be added to the antibonding π^* orbitals of the triplet oxygen. Gamma radiation, pulsed electric field, microwave and ohmic processing of foods can produce superoxide anions. For example, during radiolysis, the water molecule may be converted to ionized water (H_2O^+) within 10^{-16}s and the hydrated electrons (e_{aq}^-) may then reduce the oxygen molecule to superoxide anion.

$$2H_2O \rightarrow H_2O^+ + H_2O^{\bullet} + e_{aq}^- \hspace{4cm} [2\text{-}1]$$

$$^3O_2 + e_{aq} \rightarrow O_2^{\bullet-} \hspace{5cm} [2\text{-}2]$$

(Halliwell and Gutteridge, 1999)

Furthermore, during pulsed electric field processing, both $HOO^{\bullet}$ and $OH^{\bullet}$ radicals may be formed by the following reaction.

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Table 2-3   Standard reduction potential of ROS. Modified from Min and Boff (2002)

Half-cell	Standard reduction potential (mV)
$O_2, H^+/HO_2^{\bullet}$	−460
$O_2/O_2^{-\bullet}$	−330
$H_2O_2, H^+/H_2O, HO^{\bullet}$	320
$O_2^{-\bullet}, 2H^+/H_2O_2$	940
$ROO^{\bullet}, H^+/ROOH$	1000
$HO_2^{\bullet}, H^+/H_2O_2$	1060
$RO^{\bullet}, H^+/ROH$	1600
$HO^{\bullet}, H^+/H_2O$	2310

Table 2-4   Copper and iron contents on edible oils. Adapted from Choe (2008)

Oil	Copper (ppb)	Iron (ppm)
Crude soybean oil	13.2	2.8
Refined soybean oil	2.5	0.2
Cold-pressed seasame oil	16.0	1.2
Virgin olive oil	9.8	0.7
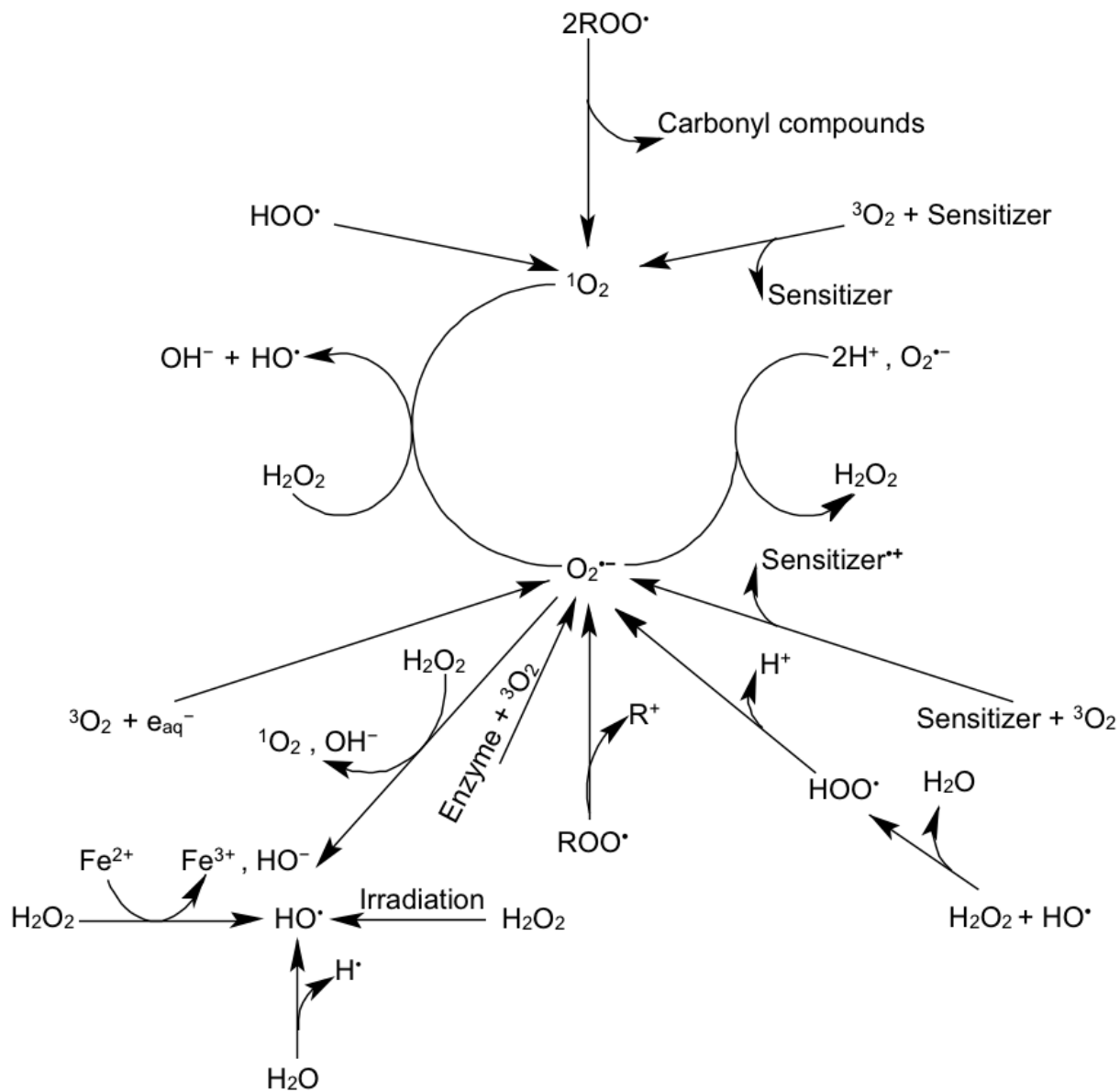
Figure 2-2   Interrelationships among methods of formation of ROS in foods (summarized by Choe and Min, 2006).

$$H_2O^* \rightarrow H^\bullet + OH^\bullet \qquad\qquad [2\text{-}3]$$

$$H^\bullet + O_2 \rightarrow HOO^\bullet \rightarrow H^+ + O_2^{\bullet-} \qquad\qquad [2\text{-}4]$$

<div align="right">(Jacobien et al., 1996)</div>

Additionally, high-energy γ-rays that interact with water can generate hydroxyl radicals

$$2H_2O \rightarrow H_2O^{+\bullet} + H_2O^* + e_{aq}^- \qquad\qquad [2\text{-}5]$$

$$H_2O^* \rightarrow H^\bullet + OH^\bullet \qquad\qquad [2\text{-}6]$$

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH^\bullet \qquad\qquad [2\text{-}7]$$
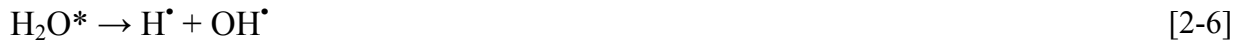
<div align="right">(Jacobien et al., 1996)</div>

Also, some enzymes participate in processes that generate ROS. For instance, xanthine in foods can produce the superoxide anion.

$$\text{Xanthine} + H_2O + 2O_2 \xrightarrow{\text{Xanthine oxidase}} \text{Uric acid} + 2O_2^{\bullet-} + 2H^+ \qquad [2\text{-}8]$$

<div align="right">(Halliwell and Gutteridge, 1999)</div>
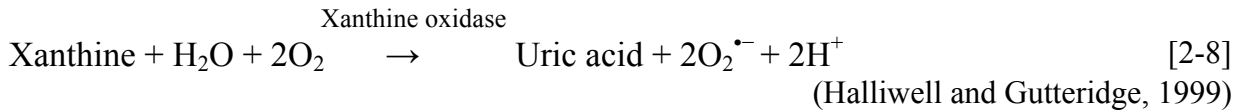
Other influential catalysts of lipid oxidation are metals. Traces of metals are always present in food as a consequence of processing or food composition. Concentrations of metals below 0.5 ppm are sufficient to initiate lipid oxidation (Anwar et al., 2004; Pehlivan et al., 2008; Koleva, 2007). Since transition metal ions, except zinc, contain unpaired electrons in their d orbitals they can enable the formation of radicals (Ron and Abraham, 2002). Among them, copper and iron are most abundant and play a key role in converting oxidants into radicals. Table 2-4 shows the content of copper and iron in some vegetable oils.

Metals can react with lipids and generate lipid radicals or catalyze the production of ROS. For example, iron acts as a catalyst in the metal-catalyzed Haber-Weiss and Fenton reactions to produce hydroxyl radicals. The rate of hydrogen peroxide decomposition is increased when both iron and copper are present (Choe, 2008).

$$Fe^{3+} + O_2^\bullet \rightarrow Fe^{2+} + O_2 \qquad\qquad [2\text{-}9]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^\bullet + HO^- \qquad\qquad [2\text{-}10]$$

$$H_2O_2 + O_2 \rightarrow O_2 + HO^\bullet + HO^- \qquad\qquad [2\text{-}11]$$

$$H_2O_2 + O_2^- \rightarrow HO^\bullet + {}^1O_2 \qquad\qquad [2\text{-}12]$$

<div align="right">(Haber and Weiss, 1934)</div>

When reactive species are formed in food, they react with food components and make a wide

range of radicals, particularly lipid radicals. Depending on reduction potential and characteristics each species acts differently in these reactions (Figures 2-3, 2-4, 2-5).

2.3.2 Propagation Phase

Propagation occurs when radicals produced during initiation react with triplet state oxygen, producing peroxy radicals ($ROO^{\bullet}$) (Figure 2-6). The standard one-electron reduction potential of a peroxyl radical is 1000 mV, thus it easily binds hydrogen from fatty acids and produces hydroperoxide and other alkyl radicals. In this phase, hydroperoxide and alkyl radicals play main roles because they stimulate oxidation by producing additional radicals and maintain a free radical chain reaction. $ROO^{\bullet}$ activity is considered as selective because it reacts with the most weakly bound hydrogen in the saturated lipid. Hydroperoxides, the primary oxidation products, are comparatively stable at room temperature. The presence of metal or elevated temperature can cause the decomposition of hydroperoxides and homolytic mechanisms are mainly involved in their decomposition. Alkoxyl and hydroxyl radicals are major reaction products since the oxygen-oxygen bond (44 kcal/mole) has lower energy than the oxygen-hydrogen bond (90 kcal/mole) of the hydroperoxide molecule (Choe, 2008; Choe and Min, 2006; Gardner, 1975).

$$ROOH + Me^{n+} \rightarrow RO^{\bullet} + Me^{(n+1)+} + OH^{-} \qquad \text{[2-13]}$$

$$ROOH + Me^{(n+1)+} \rightarrow ROO^{\bullet} + Me^{n+} \qquad \text{[2-14]}$$

(Gardner, 1975)

Alkoxyl (standard reduction potential of 1600 mV) and hydroxyl radicals (standard reduction potential of 2300 mV) have a strong influence on producing new radicals, chain branching and formation of secondary products. For instance, homolytic β-scission the alkoxyl radical's C-C bond produces oxo-compounds, aldehydes, acids, alcohols and short-chain hydrocarbons (Lee et al., 2003) (Figure 2-7). Hydroxyl radicals are especially reactive and able to abstract hydrogen from many species and make different radical species. In fact, the rate of hydrogen atom abstraction from lipid molecules by $OH^{\bullet}$ is 109 times more rapid than $ROO^{\bullet}$ (Koleva, 2007). In this stage, group transfer, fragmentation, rearrangement and cyclization processes dominate other individual chain reaction processes.

H₃C(CH₂)₄−HC=CH−CH₂−CH=CH−(CH₂)₇COOH   +   HOO•

↓ → H₂O₂

H₃C(CH₂)₄−HĊ−CH=CH−CH=CH− (CH₂)₇COOH   +   H₃C(CH₂)₄−HC=CH−CH=CH−ĊH−(CH₂)₇COOH

13-radical   9-radical
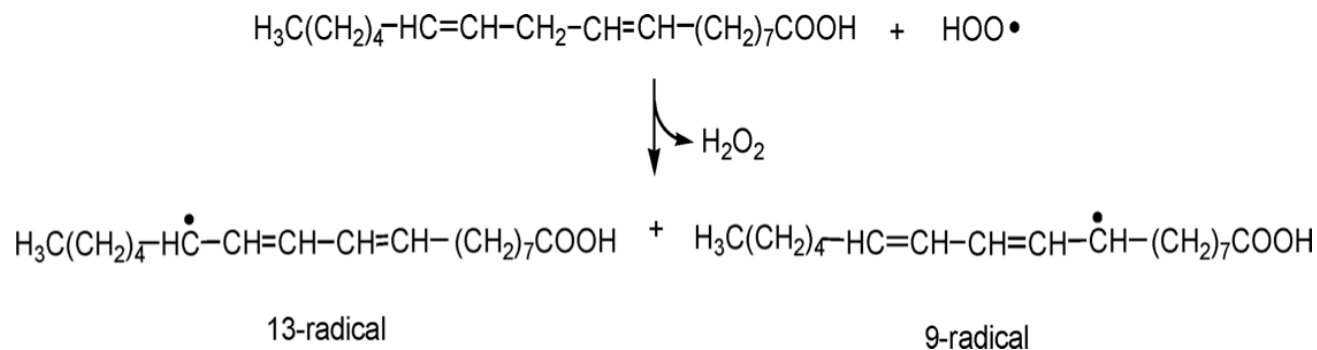
Figure 2-3   Linoleic acid and hydrogen peroxide reaction producing alkyl radicals (Choe and Min, 2006).

H₃C(CH₂)₇−HC=CH−(CH₂)₇COOH

↓ + HO•

H₃C(CH₂)₇−HĊ−CH−(CH₂)₇COOH   +   H₃C(CH₂)₇−HC−ĊH−(CH₂)₇COOH
$\qquad$ OH $\qquad\qquad\qquad\qquad\qquad\qquad$ OH

↓ + ³O₂

OO•$\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ OO•
H₃C(CH₂)₇−HC−CH−(CH₂)₇COOH   +   H₃C(CH₂)₇−HC−CH−(CH₂)₇COOH
$\qquad$ OH $\qquad\qquad\qquad\qquad\qquad\qquad$ OH
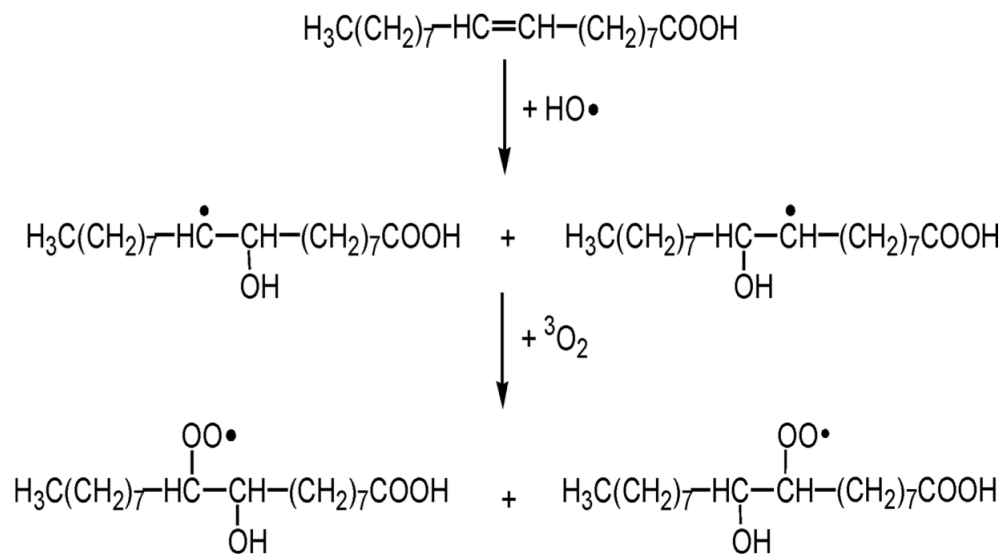
Figure 2-4   Addition of electrophilic hydroxyl radical to oleic acid (Choe and Min, 2006).

H3C(CH2)4−HC=CH−CH2-CH=CH−(CH2)7COOH   +   HO•

$$\downarrow$$

$$\left[H_3C(CH_2)_4{-}HC{=}CH{-}\overset{\bullet}{C}H{-}CH{=}CH{-}(CH_2)_7COOH \right]$$

$$\downarrow$$

H3C(CH2)4−H$\overset{\bullet}{C}$−CH=CH−CH=CH−(CH2)7COOH   +   H3C(CH2)4−HC=CH−CH=CH−$\overset{\bullet}{C}$H−(CH2)7COOH

$$\downarrow + {}^3O_2$$

H3C(CH2)4−HC−CH=CH−CH=CH−(CH2)7COOH   +   H3C(CH2)4−HC=CH−CH=CH−CH−(CH2)7COOH
$\quad\quad\quad$ $\overset{|}{OO\bullet}$ $\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ $\overset{|}{OO\bullet}$

$\quad\quad\quad\quad$ 13-OO• $\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ 9-OO•
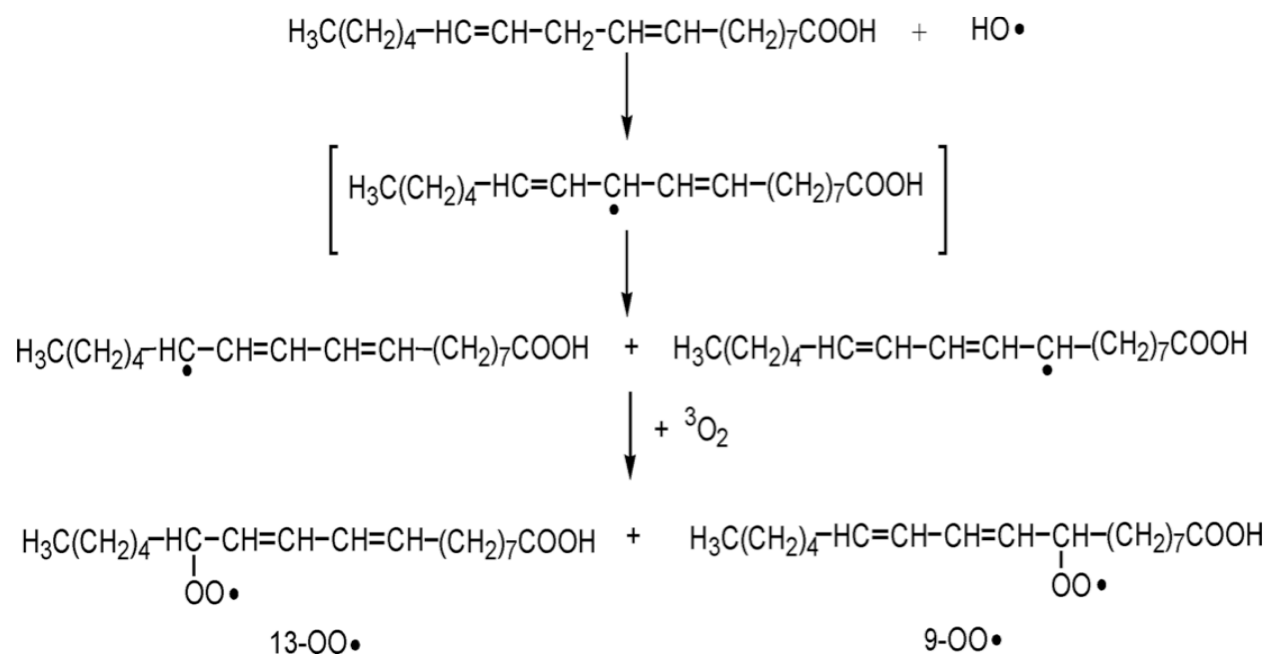
Figure 2-5   Hydroxyl radical initiation of linoleic acid oxidation (Choe and Min, 2006)

CH_3-$(CH_2)_4$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_6$-COOH

$$\downarrow -H^\bullet$$

CH_3-$(CH_2)_4$-CH_2-$CH^\bullet$-CH=CH-CH=CH-CH_2-$(CH_2)_6$-COOH

$$\text{Initiation} \downarrow +O_2$$

CH_3-$(CH_2)_4$-CH_2-$CHOO^\bullet$-CH=CH-CH=CH-CH_2-$(CH_2)_6$-COOH
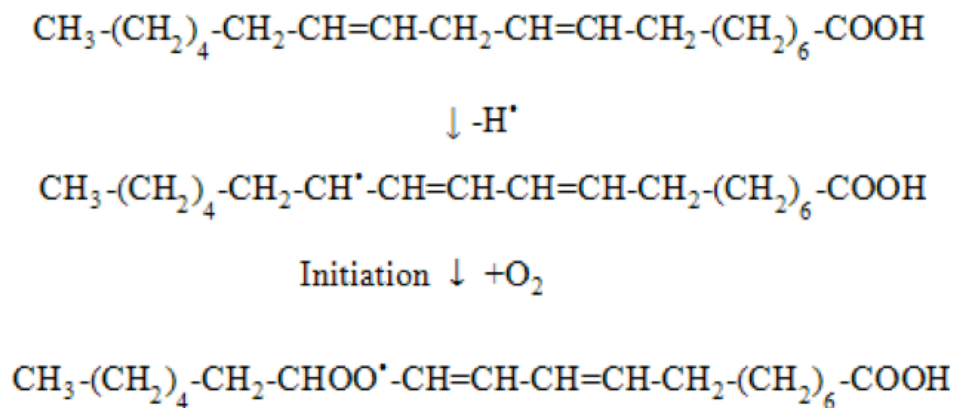
Figure 2-6   Formation of hydroperoxy radicals by triplet oxygen oxidation of linoleic acid (Koleva, 2007).
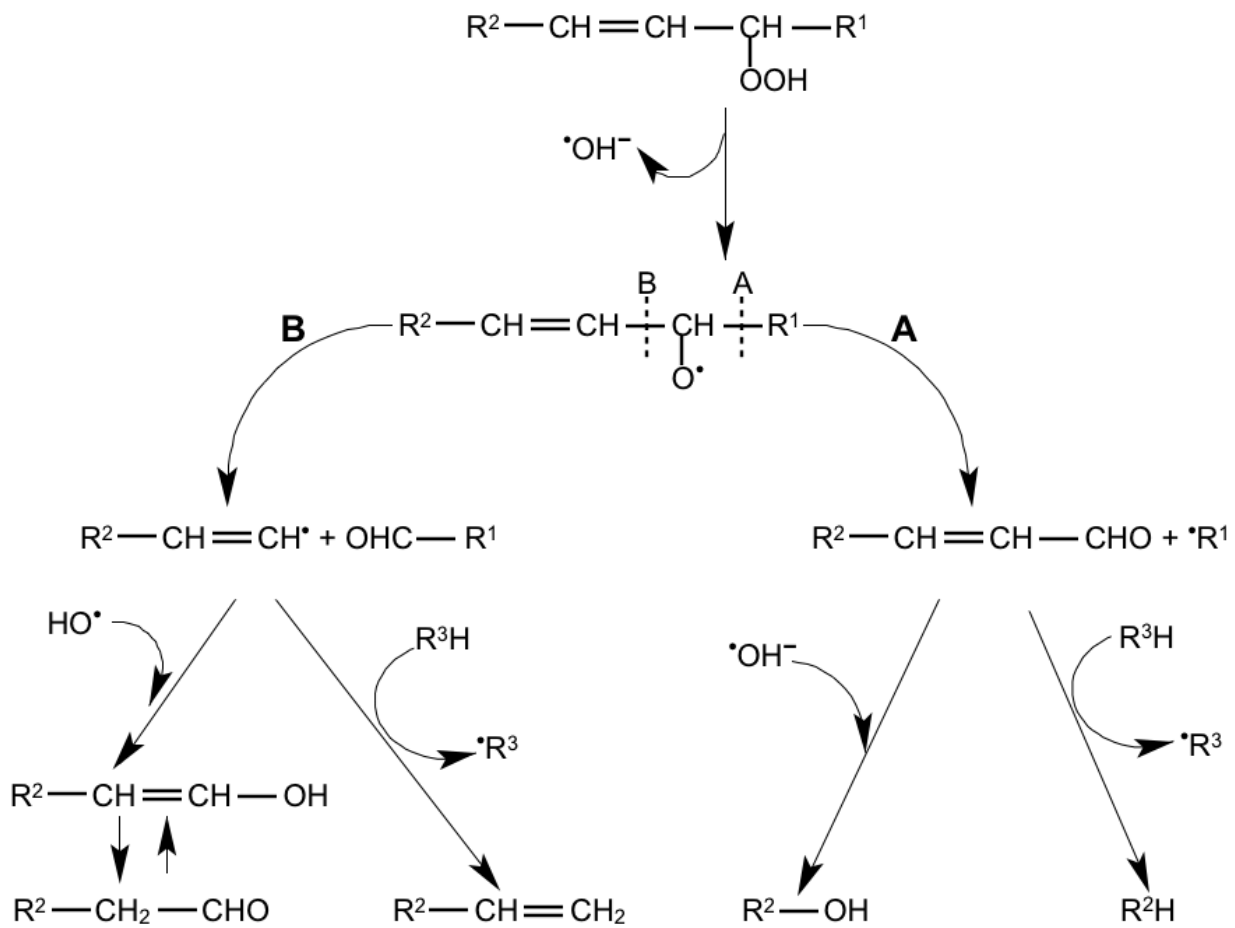
Figure 2-7   Decomposition of lipid peroxides. Modified from Choe and Min (2006).

2.3.3　Termination Phase

Termination phase starts when the concentration of radicals is high enough for there to be a high probability of reaction of two radicals. Two types of reaction can terminate chain reactions produced during the propagation stage, radical-radical coupling and radical-radical disproportionation, a process in which two non-radical compounds are produced from radicals by atom or group transfer process (Koleva, 2007) (Figure 2-8).

2.4　Antioxidants

An antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. In lipid-containing foods, antioxidants can occur in the form of natural compounds, or they can be formed during processing. In addition, synthetic and natural antioxidants can be added to products (Koleva, 2007).

Antioxidants can be divided into two groups according to their mechanism of action. Primary or chain-breaking antioxidants can delay or inhibit the initiation process or disrupt the propagation step of autoxidation. The basic mechanism of these antioxidants is free-radical scavenging. They quench free-radicals of foods by giving them hydrogen and producing comparatively stable antioxidant radicals with standard reduction potentials less than 500 mV. Thus, the standard reduction potential of antioxidant radicals should be lower than food radicals or oxygen-related radicals. The reduction potentials of hydroxyl, alkyl, alkoxyl, alkyl peroxyl and superoxide anion radicals are 2300, 600, 1600, 1000 and 940 mV, respectively. Tocopherol, ascorbic acidand quercetin radicals have reduction potentials of 500, 330 and 330 mV, respectively (Choe and Min, 2006).

Moreover, phenolic ring structures of antioxidants are able to stabilize antioxidant radicals by resonance delocalization (Reische et al., 2002). For instance, the antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and a few natural tocopherols and carotenoids contain mono- or polyhydroxy phenols with various ring substitutions. These are grouped as primary antioxidants due to their direct reaction with ROS. The bond dissociation energy between oxygen and phenolic hydrogen, which strongly depends on molecular structure and the intramolecular hydrogen bond between phenolic hydrogen and the
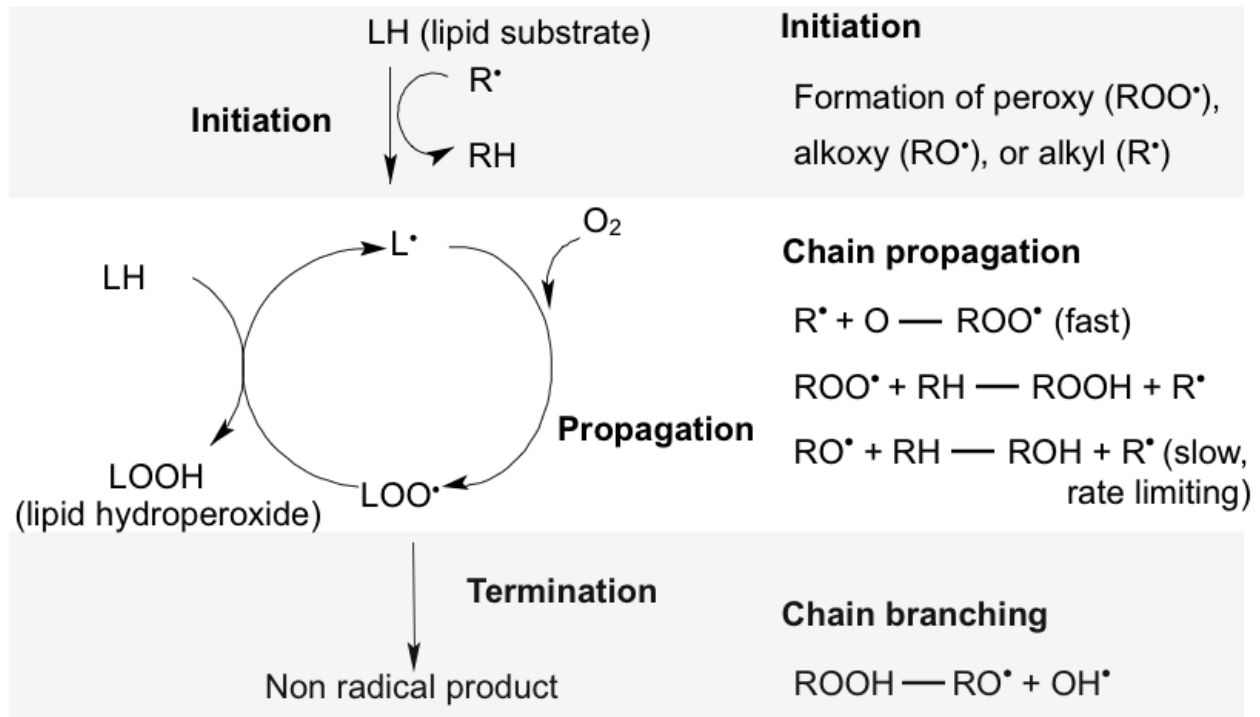
Figure 2-8   Oxidation overview of unsaturated lipid. Modified from Koleva (2007).

oxygen-containing substituent of antioxidants, can determine antioxidant properties. If the antioxidant bond dissociation energy is low, then hydrogen can easily transfer to free radicals. Moreover, antioxidant radicals arising from species with lower bond dissociation energies are more stable. In fact, bond dissociation energies for O-H of tocopherol homologs decrease in order from $\delta > \gamma > \beta > \alpha$-tocopherols. This trend is consistent with antioxidant activity (Table 2-5 and Figure 2-9) (Choe and Min, 2009).

The pH of the reaction system has a strong effect on the antioxidant activity of some phenolic acids like caffeic acid or protocatechuic acid. These radical scavengers are ineffective at acidic pH, but above pH 7–8 show antioxidant activity. Electron transfer from the anion of phenolic acids to lipid radicals can explain this. This reaction is not possible with the protonated phenolic acid (Figure 2-10) (Amorati and Pedulli, 2006). In addition, polar solvents like acetonitrile are able to interact with antioxidants through intermolecular hydrogen bonds and increase the bond dissociation energy of OH groups in phenolic compounds (Mitroka et al., 2010).

Antioxidants may also involve termination reactions by reacting with lipid radicals or forming an antioxidant radical dimer. For example, at high concentrations, tocopherol radicals can react with lipid peroxy radicals to produce tocopherol peroxide (Figure 2-11). Also, tocopherol radicals are able to abstract hydrogen from lipids and form tocopherol and lipid radicals, when the concentration of tocopherol radical is high and the concentration of lipid peroxyl radical is low (Koleva, 2007; Kim et al., 2007).

Secondary-preventive antioxidants slow the rate of oxidation by numerous mechanisms. Unlike primary antioxidants, secondary antioxidants do not convert free radicals to more stable products. Instead, they can chelate pro-oxidant metals, refill hydrogen to primary antioxidants, decompose hydroperoxides to non-radical species, deactivate singlet oxygen, scavenge molecular oxygen, absorb ultraviolet radiation, or inhibit pro-oxidative enzymes. Synergists are recognized as secondary antioxidants due to their ability to work with primary antioxidants, even though they do not show antioxidant activity when used alone (Reische et al., 2002; Koleva, 2007). Metal-deactivating antioxidants reduce the rate of oxidation by metals ions. They react with metals by forming insoluble metal complexes or providing stearic barriers between metals and food components or their oxidation intermediates. Ethylenediamine tetraacidic acid (EDTA) and citric acid are common metal chelating compounds present in food systems.

Table 2-5   Chemical structure and antioxidant activity of tocopherols

Tocopherols	R_1	R_2	R_3	Antioxidant activity (with α-tocopherol as 100%)
α-tocopherol	CH_3	CH_3	CH_3	100
β-tocopherol	CH_3	H	CH_3	130
γ-tocopherol	H	CH_3	CH_3	200
δ-tocopherol	H	H	CH_3	300–500

Sorce: www.rikenvitamin.jp/int/tocopherol/toc1.html, 2002.
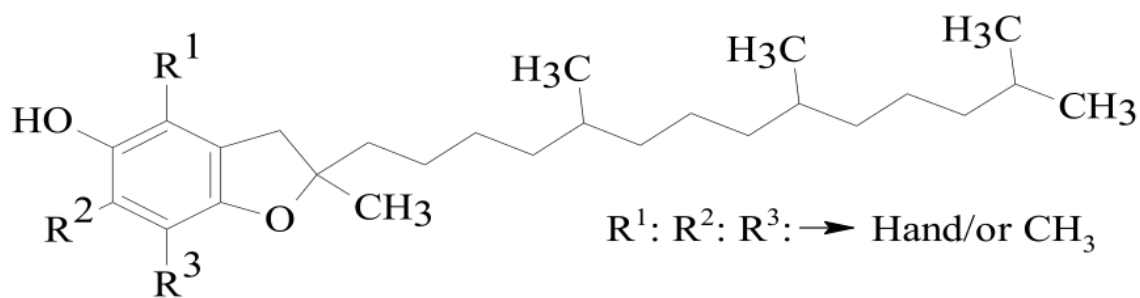
Figure 2-9   Structure of tocopherols (www.rikenvitamin.jp/int/tocopherol/toc1.html, 2002).
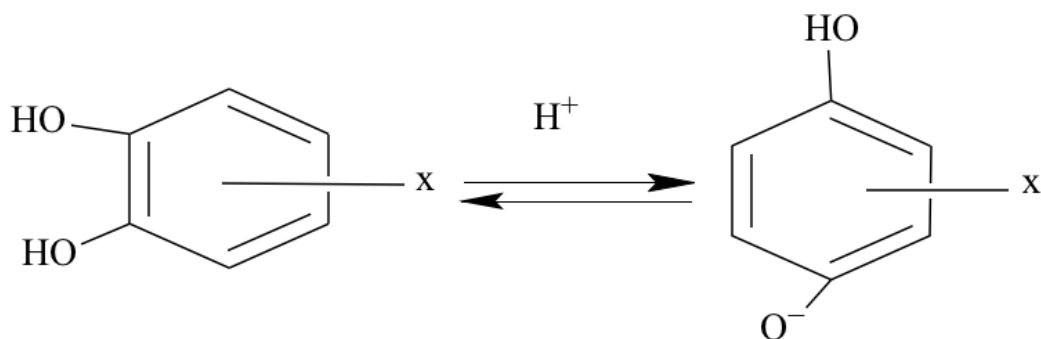
Figure 2-10 Formation of phenolic acid anion (Amorati and Pedulli, 2006).
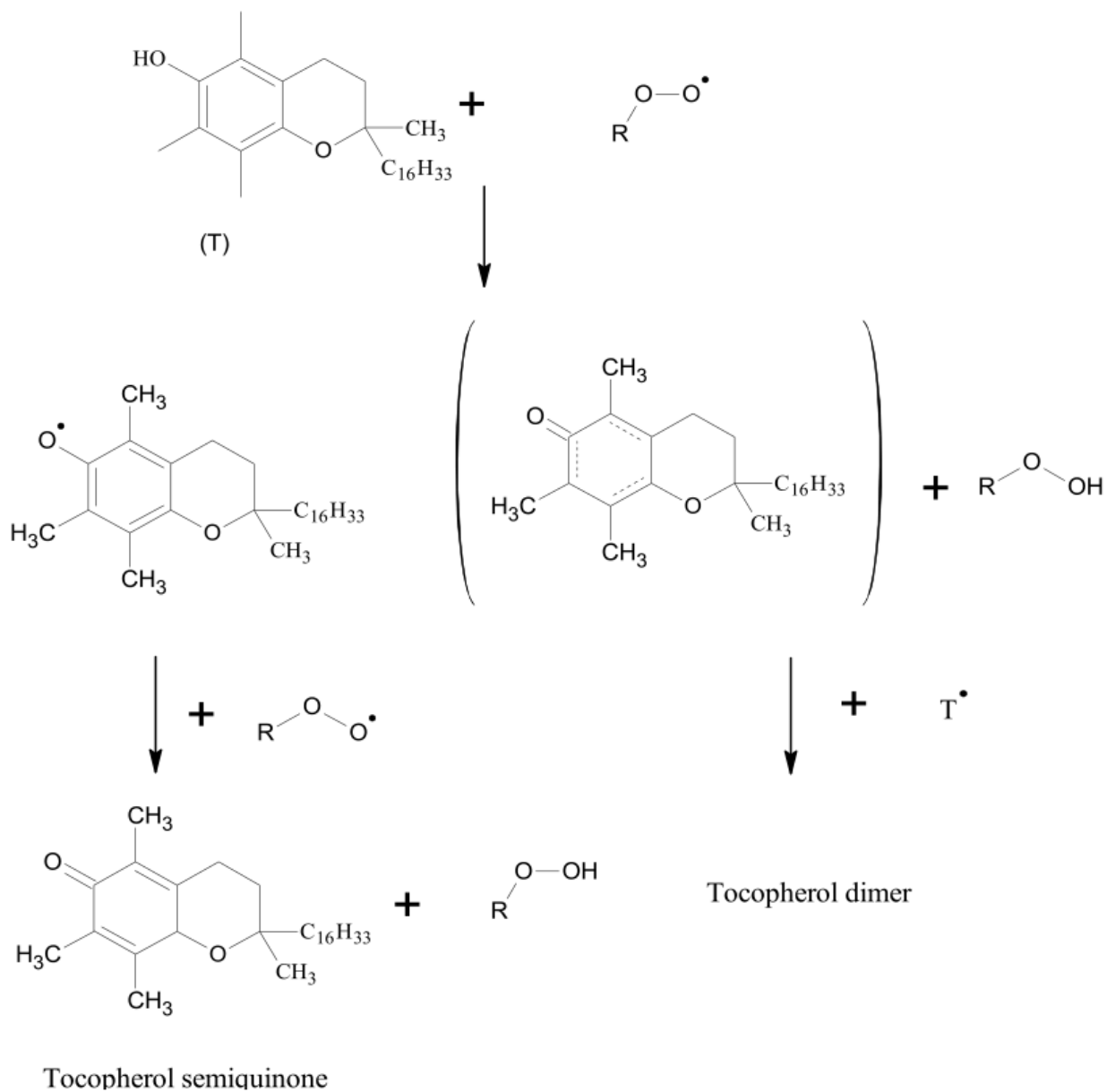
Figure 2-11 Reaction of α-tocopherol with lipid peroxy radicals (R-alkyl group). Modified from Choe and Min (2006).

Moreover, phospholipids, flavonoids, lignans, polyphenols, ascorbic acid and some amino acids (carnosine and histidine) are able to bind metals (Choe, 2008; Choe and Min, 2009).

Headspace oxygen or dissolved oxygen can enhance the oxidation process. In fact, when oil oxidizes, polar groups like hydroxyl and/or carbonyl are produced from oxygen present in the headspace. These polar groups are less soluble in oil, and thereby decrease the surface tension between oil and air. Under these conditions, oxygen molecules transfer more easily from air to oil (Kim and Min, 2007). Ascorbic acid, ascorbyl palmitate, erythorbic acid, sodium erythorbate and sulfites can inhibit oxidation, either by scavenging oxygen or changing oxygen molecules by electron or hydrogen transfers (Reische et al., 2002). Tocopherols, carotenoids, curcumin, phenolics, urate and ascorbate can quench singlet oxygen. These antioxidants scavenge the singlet oxygen through either physical or chemical mechanisms. During physical quenching, the singlet oxygen returns to the triplet oxygen by either energy transfer or charge transfer. If a quencher's (Q) energy level is near or lower than that of singlet oxygen, it transfers its energy to the singlet oxygen (Equitions 2-15 and 2-16).

$$^1O_2 + Q \rightarrow {}^3O_2 + {}^3Q \qquad\qquad\qquad [2\text{-}15]$$

$$^3Q \rightarrow {}^1Q \text{ (no radiation)} \qquad\qquad\qquad [2\text{-}16]$$
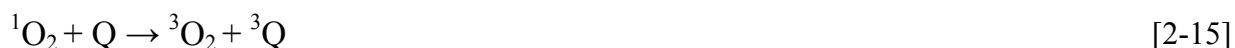
(Joseph, 2006)

Carotenoids, with nine or more conjugated double bonds, are able to quench singlet oxygen by energy transfer. Conjugated double bonds and oxo- or conjugated keto-groups enhance quenching ability (Equition 2-17) (Joseph, 2006).

$$^1O_2 + Q \rightarrow [O_2^- \text{ - - - } Q^+]^1 \rightarrow [O_2^- \text{ - - - } Q^+]^3 \rightarrow {}^3O_2 + Q \qquad\qquad [2\text{-}17]$$
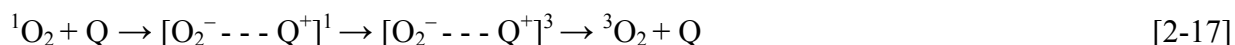
Compounds like amines, phenols, sulfides, iodides and azides, which have many electrons or high-reduction potential and lower triplet energy states, can donate electrons to quench singlet oxygen. By accepting electrons, the singlet oxygen forms a singlet state charge transfer complex, which transfers to triplet state complex by intersystem crossing. In the end, the latter complex generates triplet oxygen (Joseph, 2006).

Chemical quenching involves scavenger reactions with singlet oxygen to form oxidized products. β-Carotene, tocopherols, ascorbic acid, amino acids (histidine, tryptophan, cysteine and methionine), peptides and phenolics can react with singlet oxygen (Min and Boff, 2002; Reische

et al., 2002; Choe, 2008).

Photosensitizer inactivators can draw energy from photosensitizors instead of transferring it to triplet oxygen. For example, the singlet state of carotenoids with less than nine conjugated double bonds receive energy from photosensitizers and form triplet state cartenoids, which return to the singlet state by transferring energy to the environment or emitting photons (Choe and Min, 2009).

Antioxidants may interact with each other in inhibition of food oxidation. The interaction may increase (synergism) or decrease (antagonism) the antioxidant potential of individual antioxidants. If there are two or more free radical scavengers, the one having a higher reduction potential plays the primary role in antioxidant activity and the other scavenger regenerates the primary antioxidant. In case of the interaction between tocopherol and carotenoids, they can regenerate each other, but carotenoid regeneration by tocopherol is more favorable. Another common synergism is the combination of metal chelators and free radical scavengers. Chelators can reduce free radical production in the initiation step and enhance the effectiveness of free radical scavengers by binding pro-oxidant metals. In contrast, antagonism occurs when a strong antioxidant is spent on regeneration of the weak antioxidant, or antioxidant radicals oxidize more effective antioxidants (Choe and Min, 2009).

2.4.1   Antioxidant Activity of Proteins and Protein Related Substances

Numerous amines, amino acids, peptides and protein hydrolysates have antioxidant activity. Amines such as hypoxanthine and xathine showed antioxidant activity (Sasaki et al., 1996). Also, the amino acids glycine, methionine, histidine, tryptophan, proline and lysine are valued antioxidants in oil. The ability of amino acids to chelate metals is proposed as their main antioxidant mechanism (Ahmad et al., 1983). Elias et al. (2008) summarized studies on the antioxidant properties of proteins describing the antioxidant activity of milk (Taylor and Richardson, 1980), blood plasma (Faraji et al., 1991) and soy protein (Pena-ramos and Xiong, 2003; Park et al., 2010) in the lipid component of muscle foods and whey protein concentrate (Shantha and Decker, 1995). Moreover, specific milk proteins that exhibit antioxidant activity include casein, β-lactoglobulin, transferrin and lactoferrin. Proteins differ from other food antioxidants as they can reduce lipid oxidation by multiple mechanisms. In general, proteins can inactivate ROS, quench free radicals, bind transition metals, reduce hydroperoxides,

enzymatically reduce oxidants (superoxide dismutase, peroxidases) and split up reactive species by altering the physical properties of food systems. In addition to proteins that bind metals as part of their biological function (ferritin, transferrin, lactoferrin, haptoglobins, hemopexin, albumin and ceruloplasmin), other proteins with surface-exposed amino acid residues, including histidine, glutamic acid, aspartic acid, phosphorylated serine or threonine, are able to chelate metals. The chelating power of these proteins depends on pH, as this determins the electrostatic interaction between the protein and cationic transition metals.

There are many studies that have elucidacted the biological activity of peptides, including their anti-cancer, antimicrobial and immunomodulatory effects. Food protein hydrolysates are being studied extensively as natural antioxidants, for examples, casein (Cervato et al., 1999), whey proteins (Faraji et al., 2004) and bovine serum albumin (Villiere et al., 2005). In contrast to the use of synthetic antioxidants (Becker, 1993), the addition of peptides derived from food products to food is considered safe. Additionally, this practise may confer positive nutritional and functional value (Bamdad et al., 2011; Chen et al., 1998). For instance, enzymatically-derived soybean peptides can act as potent antioxidants against lipid peroxidation and the primary structure of these peptides is critical for their activity (Chen et al., 1995). Similarly, peptides generated from zein protein (Kong and Xiong, 2006), egg yolk (Sakanaka et al., 2004), porcine haemoglobin (Chang et al., 2007), fish protein (Thiansilakul et al., 2007), canola protein (Cumby et al., 2008) and whey protein (Peng et al., 2009) also are effective antioxidants. Recently, other potent antioxidants and radical-scavenging peptides have been identified from porcine muscle and milk casein hydrolysates (Kim et al., 2001).

Udenigwe et al. (2009) hydrolyzed flaxseed protein using seven proteases to derive peptides. Subsequently, the peptides were fractionated *via* ultrafiltration (3–5, 1–3 and < 1 kDa). DPPH assays showed that fractions of intermediate molecular weight (1–3 kDa) had the highest scavenging capacity. Moreover, enzymatic digests of flaxseed protein also quench hydroxyl radicals and higher scavenging activities are correlated to longer chain peptides or lower degree of hydrolysis (Marambe et al., 2008).

The anti-oxidative mechanism of peptides is not well understood. Peptide moieties contain aromatic residues (tyrosine, histidine, tryptophan and phenylalanine) that can stabilize ROS through electron transfer (Jung et al., 2005). Moreover, peptides are well known metal

chelators and this property may contribute to antioxidant properties. However, many peptides like zein protein hydrolysates (Kong and Xiong, 2006), peanut peptides (Zhang et al., 2011), soybean peptide (Chen et al., 1998) and yoghurt peptide (Farvin et al., 2010) have been reported as metal chelators but the correlation between metal-ion chelating activity and antioxidant activity is poor (Chen et al., 1998; Zhang et al., 2008).

It is believed that the biological activity of peptides is generally determined by the amino acid composition. Histidine-containing peptides, for example, commonly possess antioxidant activity (Chen et al., 1995; Chen et al., 1998; Je et al., 2005; Jung et al., 2005). In addition, proline, phenylalanine, tyrosine, cystein methionine and tryptophan residues are present in many antioxidant peptides (Zhang et al., 2008; Farvin et al., 2010).

2.5   Antioxidant Assays

A kinetic assay of antioxidant reactions of natural antioxidants is vital for understanding the mechanism of action. Many different assays are available for determining antioxidant activity. Assays vary based on the structure of the substrate being oxidized. Often, the substrate comprises mixtures of several of compounds with different functional groups, polarity and chemical properties. Antioxidant assays can be divided into two types. The first type comprises assays based on lipid/substrate oxidation, which measure, for example, changes in mass or accumulation of oxidized products, including hydroperoxides, aldehydes and conjugated dienes (active oxygen method, oven oxygen method and shelf life test) (Koleva, 2007).

Antioxidant activity can also be determined based on radical scavenging ability. These methods are designed to measure the ability of the antioxidant to quench free radicals in a model system. Examples of these assays are exemplified by the DPPH$^{\bullet}$ quenching assay, hydroxyl radical quenching assay, superoxide radical quenching assay, EPR spin-trap tests, ferric reducing antioxidant power, oxygen radical absorption capacity, total radical-trapping antioxidant parameter and trolox equivalent antioxidant capacity (Wanasundara and Shahidi, 2005).

2.5.1   Active Oxygen Method

Both the Active Oxygen Method (AOM) and the The oxidative stability index (OSI) assays involve passing air through a heated lipid sample to accelerate oxidation. The air that has passed through the sample is then bubbled through distilled water in a test tube. In both methods,

the instrument determines the change in conductivity of the distilled water. Volatile organic acids, mainly formic acid, produce ions that increase water conductivity. These methods determine the induction period or the OSI that may be used as a measure of stability of antioxidants-containing lipid (Shahidi and Wanasundara, 2007).

2.5.2 Electron Paramagnetic Resonance Spectroscopy

Electron Paramagnetic Resonance (EPR) spectroscopy can detect the presence of unpaired electrons in a chemical system (free radicals, odd electron molecules, transition-metal complexes, lanthanide ions, triplet-state molecules). In strong magnetic fields, unpaired electrons absorb microwave radiation, which may be detected by EPR. EPR spectra provide meaningful structural and dynamic information. Data may be collected from chemical or physical processes without influencing the process itself. In this way, the analysis can be more selective than other routinely used analytical techniques. In addition, there is a direct correlation between the integrated intensity of an EPR signal and the concentration of unpaired electrons in the sample. However, the signal intensity depends on both concentration and microwave power, so proper operating conditions are essential. With low levels of microwave power, the signal intensity increases linearly with the square root of the applied energy. At higher power levels, the signal grows more slowly and saturation may occur. For studies of chemical kinetics, mechanism of catalysis and protein folding, rapid mixing of reactants is crucial for the generation of useful kinetic data. Such experiments are often described as either continuous-flow (CF) or stopped-flow (SF) methods (Ramachandra et al., 1998; Grigoryants et al., 2000).

2.5.2.1 DPPH˙ Assay

DPPH˙ contains an unpaired electron that is delocalized over the whole molecule through resonance to produce a stable free radical compound. The DPPH˙ is paramagnetic but can become a stable diamagnetic molecule when it accepts an electron or hydrogen radical (Figure 2-12). This characteristic enables the use of electron paramagnetic spectroscopy for detecting DPPH˙ signal intensity, which is closely related to the antioxidant concentration and reaction time. Another routinely used technique is the decoloration assay, based on an absorbance decrease at 515–528 nm when DPPH˙ adduct is formed in ethanol or methanol solution (Sanchez-Moreno et al., 1998). The DPPH˙ quenching process can be affected by light, medium pH and dissolved compounds. DPPH˙ assays are easily conducted, accurate and reproducible.

28
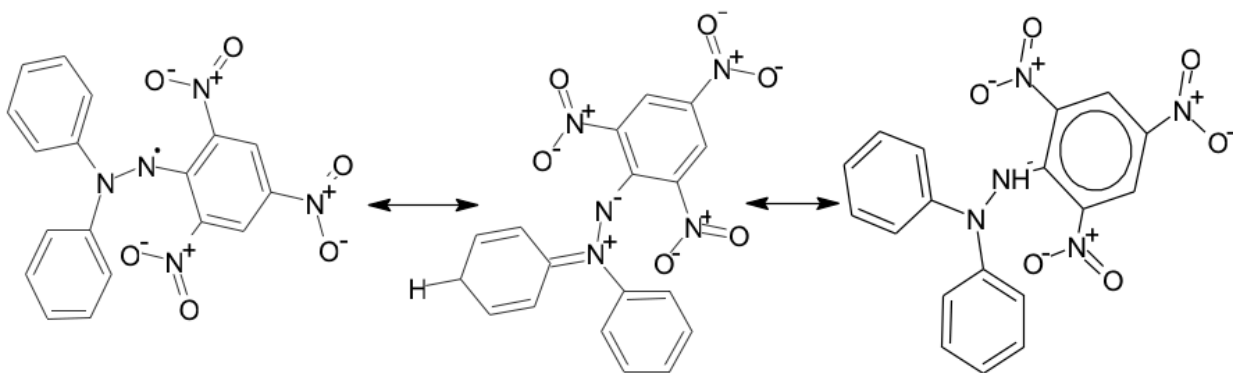
Figure 2-12 Structure of the stable free radical DPPH$^\bullet$. Adapted from Ionha (2005).

Although the DPPH assay is widely used for evaluating antioxidant activity, there is no standard protocol for this assay. Therefore, different authors apply a variety of radical concentrations and reaction times. Sharma et al. (2009) compared a number of decoloration assays. They noted significant differences in reaction conditions, including radical concentration (22.5–250.0 µM), incubation time (5 min–1 h) and media. Such results (IC$_{50}$ of BHT 5.4–86.6) are not readily comparable among the various assays (Sharma and Bhat, 2009). The parameter IC$_{50}$ or EC$_{50}$ "efficient concentration" value, introduced by Brand-Williams and his colleagues, is itself very questionable (Brand-Williams et al., 1995). Efficient concentration is the substrate concentration that causes 50% loss of the DPPH$^{\bullet}$. At this point, there should be a clear understanding of "the end" point of the titration since any residual (yellow) colour could form from reduced DPPH$^{\bullet}$ or non-specific absorbance of the sample (Molyneux, 2004). Also, Brand-Williams et al., (1995) reported that certain analytes (e.g. coumaric acid and vanillin) never quench more than 75% of the initial DPPH$^{\bullet}$ when applied at high concentration over seven hours. Finally, a reversible reaction of DPPH$^{\bullet}$ with eugenol has been reported (Bonder et al., 1997). Thus, chemical reactions can restore the DPPH$^{\bullet}$.

Furthermore, since IC$_{50}$, is a concept arising from dose-response curves, reaction time may be neglected. To correct this gap, some authors use antiradical activity, the percentage of the DPPH$^{\bullet}$ remaining when the kinetics reached a plateau and classified antioxidants into three classes according to their kinetic behaviour: quick [less than 5 min; intermediate (5–30 min) and slow (1–6 h)] (Brand-Williams et al., 1995). Sanchez-Moreno et al. (1998) proposed a new term, antioxidant efficiency (AE), to evaluate antioxidant power. This parameter is inversely proportional to the concentration at EC$_{50}$ and the time needed to reach the steady state (Tec$_{50}$) (Sanchez-Moreno et al., 1998).

The reaction between an antioxidant and DPPH$^{\bullet}$ depends on the structural conformation of the antioxidant (Huang et al., 2005). Some compounds react rapidly with DPPH$^{\bullet}$, while others are slow to react. For instance, a study of reactions of the known natural product antioxidants eugenol and isoeugenol and the synthetic antioxidant, butylated hydroxytoluene (BHT), revealed different reaction behaviours despite their structural similarities. The synthetic antioxidant BHT (stoichiometic ratio 2.8) reacted with DPPH$^{\bullet}$ over a period of 5 h. On the other hand, the natural product antioxidant eugenol (stoichiometic ratio 1.9) reacted with DPPH$^{\bullet}$ within 2 h. The isoeugenol reaction with DPPH$^{\bullet}$ was, however, much faster but at the same time reversible

(Bonder et al., 1997). Similar unexpected effects of media on reaction rates are reported in other studies (Abe et al., 2000; Foti et al., 2004; Litwinienko and Ingold, 2007; Musialik et al., 2009). These controversial results could not be explained by single-step hydrogen atom transfer (HAT) in which the reaction rate is strongly dependent on bond dissociation enthalpy of X-H and the magnitude of the kinetic solvent effect (Musialik et al., 2009). Recently, a range of proton and electron transfer mechanisms have been employed to explain antioxidant quenching. Foti et al. (2004) compared the reaction rate of cinnamic acids and their methyl esters with DPPH$^\bullet$ in polar and nonpolar solvents and concluded that the mechanism of these reactions in alcoholic solvents is a fast electron transfer process from phenoxide anions to DPPH$^\bullet$. Studies of solvent effects on the rates and mechanism of reaction of phenols and flavonoids with free radicals suggest that the mechanism could be changed from HAT to sequential proton loss-electron transfer depending on the medium (Litwinienko and Ingold, 2007; Musialik et al., 2009).

Most experimental data show that DPPH$^\bullet$ reacts mainly with another radicals by coupling in the para-position on the phenyl ring (Constantinescu et al., 1996; Ionha, 2005; Hristea et al., 2006). Nevertheless, the DPPH$^\bullet$ has different reaction centres for other free radicals; these include a nitrogen-centred radical and the picryl moiety.

The DPPH$^\bullet$ radical is capable of abstracting hydrogen atoms from mercaptans, hydroaromatic compounds, secondary amines, phenols and hydrogen halides (Hazell and Russel, 1958). The study of the reaction mechanisms and products of DPPH$^\bullet$ scavenging processes is rare and most studies describe reactions with compounds with high redox-potentials such as quinines, flavanoids, lignans, phenols and acids (Foti et al., 2004; Xie et al., 2005; Ordoudi et al., 2006; Takebayashi et al., 2007; Litwinienko and Ingold, 2007; Musialik et al., 2009).

2.5.2.2 Hydroxyl Radical Assay

Hydroxyl radical generated from a Fenton reaction in a buffered system can be used to elucidate the hydroxyl radical scavenging properties of an antioxidant (Halliwell and Gutteridge, 1981; Yamazaki and Piette, 1990; Burkitt, 1993). The principal mechanism proposed involves the reaction of H_2O_2 in the presence of an excess of Fe^{+2} ions as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^\bullet OH + OH^- \tag{2-18}$$

$$^\bullet OH + Fe^{2+} \rightarrow Fe^{3+} + OH^- \, (k = 10^7 - 10^{10}\, M^{-1}s^{-1}) \tag{2-19}$$
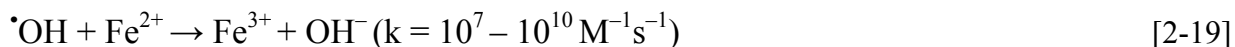
In the reaction mixture, Fe^{2+}-EDTA is generated from the reaction of ferric chloride

(FeCl$_3$) with EDTA in the presence of ascorbate. Then, Fe (II)-EDTA is oxidized into Fe (III)-EDTA by hydrogen peroxide and a hydroxyl radical. Furthermore, the activity of hydroxyl radicals is determined by the rate of the degradation of deoxyribose. Some of the degradation products are able to react with thiobarbituric acid at low pH to produce a pink chromogen (Halliwell et al., 1995). The Fenton system is affected by many parameters, including pH, the ratio of Fe^{2+}/H$_2$O$_2$, temperature and dissolved oxygen. EDTA also may conceal the antioxidant capacity of compounds that bind metals (Hagerman et al., 1998). The Fenton system produces high-valent iron-oxo species including FeO$^{2+.}$ This species achieves comparable kinetic characteristics to hydroxyl radicals (Equation 2-20), (Yamazaki and Piette, 1990; Burkitt, 1993; Dufield et al., 2004).

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \qquad\qquad [2\text{-}20]$$

Moore et al. (2006) categorized hydroxyl radical generating systems into five classes based on the nature of the radicals and chemical reactions occurring in the Fenton system: the classic Fenton reaction, the superoxide-driven Fenton reaction, the Fenton-like system, pulse radiolysis of water, and the photo-Fenton reaction (Moore et al., 2006).

EPR is commonly used because of its high sensitivity and direct detection of free radicals. DMPO (5,5-dimethyl-1-proline-*N*-oxide) is the main spin trap for the HO$^{\bullet}$ monitoring. The spin trap traps the hydroxyl radical as a stable DMPO adduct (DMPO/OH$^{\bullet}$) that is readily observed by EPR.

$$[2\text{-}21]$$
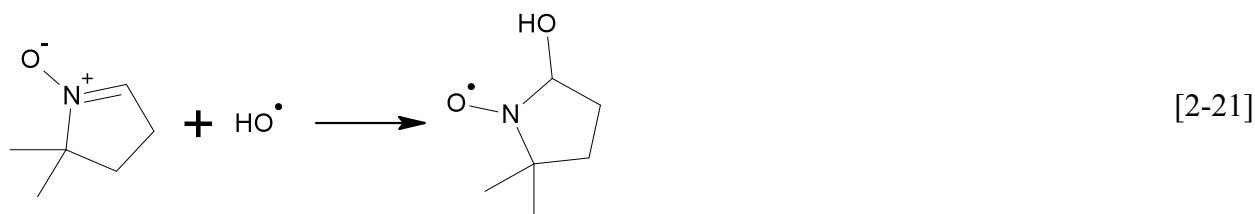
The DMPO adduct is formed at a very high rate (k = 3.4 × 10^9 M^{-1}S^{-1}) and after 25–100 s the product concentration reaches a peak (Burkitt, 1993). Researchers noticed some difficulties when DMPO is used as a quencher of hydroxyl radicals. In fact, not only hydroxyl radicals but also superoxide radicals can form covalent adducts with DMPO (DMPO/OOH$^{\bullet}$). Moreover, Yamazaki et al. (1990) reported that DMPO also produces a carbon-centred adduct with ethanol during the Fenton reaction. In addition, DMPO/OH$^{\bullet}$ adducts are not always generated directly from oxidation of the trap. For example

$$FeO^{2+} + DMPO + H^+ \rightarrow Fe^{3+} + DMPO^+OH^-$$ [2-22]

$$DMPO^+ + H_2O \rightarrow DMPO/OH + H^+$$ [2-23]

Therefore, a variety of active species could react with the spin trap. The rate, yield and pathway of the Fenton reaction was strongly affected by the iron chelators present in this system. Indeed, the intensity of the EPR signals of DMPO/OH$^\bullet$ is reduced in the following order DETAPAC (diethylenetriaminepentaaceticacid); EDTA; phosphate (Yamazaki & Piette, 1990; Burkitt, 1993). Zhu et al. (2000) reported that a reaction between tetrachlorohydro-quinone (TCHQ), a main metabolite of the biocide pentachlorophenol, and H_2O_2 generates hydroxyl radical. This is, possibly a metal independent variant of the classical Fenton reaction. Furthermore, the yield of 2,3- and 2,5-dihydroxybenzoic acid (DHBA), products of hydroxyl radical and salicylic acid, were inhibited by known hydroxyl radical quenchers and were not affected by metal chelators (Zhu et al., 2000).

The Fenton reaction is suited for producing OH$^\bullet$ radicals in aqueous solution. Many antioxidants are isolated using organic solvents. Additionally, inclusion of organic solvents in the antioxidant assay mixture may be necessary due to the low solubility of the assay reactants in water. As the hydroxyl radical is generated in the aqueous phase, these organic solvents may suppress the diffusion of the lipophilic antioxidant molecules, or may produce carbon or oxygen centred radicals, which could interfere with analysis or interpretation of the spectra. Cheng (2007) reported that EPR signal intensities of DMPO/OH$^\bullet$ generated in the Fe^{2+}/H_2O_2 system with water immiscible organic solvents (chloroform, hexane, benzene, ethyl acetate, n-butane) were far less intense than those measured in aqueous solution. Moreover, some strong antioxidants, for instance α-tocopherol, do not quench hydroxyl radicals when dissolved in an organic solution that is not miscible with water. Time and dose dependent EPR spectra revealed the water miscible organic solvents (DMSO, ethanol and methanol) also are able to interact strongly with hydroxyl radicals, inhibiting the signal intensity. Among studied solvents, acetone and acetonitrile have the lowest rate constant for reacting with OH$^\bullet$ (Cheng et al., 2007).

CHAPTER 3

EFFECT OF CYCLOLINOPEPTIDES ON THE OXIDATIVE STABILITY OF FLAXSEED OIL

3.1   Abstract

Polar compounds present in flaxseed oil increase its oxidative stability. Flaxseed oil becomes less stable to oxidation when filtered with silica. This observation may be linked to antioxidant compounds present in flaxseed oil. Flaxseed oil was passed over a silica adsorbent column to remove polar compounds. The polar compounds were then eluted from the silica absorbant using a series of increasingly polar solvents. The polar fractions from flaxseed oil were then added back to silica-treated flaxseed oil to determine the impact of the fractions containing polar compounds on oxidative stability (induction time) at 100 °C. A polar fraction containing mainly CLA, but also containing β/γ- and δ-tocopherol increased the induction time of silica-treated flaxseed oil from 2.3 ± 0.28 h to 3.2 ± 0.41 h. Other flaxseed fractions and solvent controls did not affect oil stability when oxidative stability was determined immediately after addition of the polar fractions. However, when the OSI test was delayed for three days after the addition of the polar fractions to the flaxseed oil, it was observed that the control oil treated with silica had become highly sensitive to oxidation. A polar fraction containing a mixture of CLs (CLA and CLD–CLG), improved the oxidative stability of peptid-–free oil with respect to the control when the OSI measurement was made three days after adding the fraction. In addition, effects of CLA on the oxidative stability of peptide free oil containing divalent cations was investigated.

3.2   Hypothesis

Flaxseed oil loses oxidative stability when treated with the polar adsorbent silica gel. It is proposed that antioxidants removed from flaxseed oil and trapped on silica gel can be recovered and added back to the oil to restore part or all of the lost oxidative stability. Analysis of the

compounds present in fractions that contribute to oxidative stability will reveal compound contributing to the antioxidant mechanism in flaxseed oil. The mode of action of cyclic peptide fractions may depend on their interaction with metals present in the oil.

3.3   Introduction

Flaxseed is an oilseed adapted to cool northern climates. Whole flaxseed contains 41% fat, 28% dietary fibre and 21% protein having an amino acid profile comparable to that of soybean meal. Flaxseed is one of the richest dietary sources of ALA, having high levels of polyunsaturated fatty acids (73%). Flaxseed was used for herbal medical applications in ancient Greece and Rome, including use as a laxative and remedy for gastric distress. Today, flaxseed is valued for its health benefits. Consumption of flaxseed is associated with lowering blood cholesterol; decreasing heart disease and stroke, decreasing the progress of certain cancers, and enhancing the immune response (Oomah and Mazza, 1999; Tolkachev and Zhuchenko, 2000). Most of these health effects are attributed to the content of ALA, lignan and/or fibre components of flaxseed (Diane, 2001).

While the high content of unsaturated fatty acid present in flaxseed is important to its purported health benefits, its chemical structure makes it vulnerable to oxidation. Oxidation of flaxseed oil during improper processing and storage reduces its nutritional value and causes the development of undesirable flavours. In spite of its distinctive unstable oil content, flaxseed produces active and stable antioxidants that protect the oil in storage and during germination. Flaxseed contains several known bioactive compounds, including lignans, phenolic acids, anthocyanin pigments, flavonols, flavones and phytic acid, all of which contribute to the seed's antioxidant capacity (Westcott and Muir, 2003). The concentration of these antioxidants in flaxseed oil depends on the extraction procedure and the treatment of the oil. For instance, refining procedures, such as filtration, degumming, neutralization, bleaching and deodorization, remove solid particles, phospholipids, proteins, carbohydrates, free fatty acids and waxes from the oil (Przybylski et al., 2005). Moreover, refining may also reduce minor compounds that have an effect on oil shelf-life. The unrefined oil may have higher nutritional value and longer shelf-life than refined flaxseed oil (Siger et al., 2008). On the other hand, cold-pressing procedures do not involve heat or chemical treatments and the oil is extracted under mild conditions (Choo et al., 2007). The amount of antioxidants and nutrients recovered by this mild treatment may be less

than achieved by a more vigorous extraction. Siger et al. (2008) reported several antioxidant compounds in cold-pressed flaxseed oil, including 12.7–25.6 mg/100 g flavonoids, 76.8–307.3 mg/100 g phenolic acids, 0.55–9.11 mg/100 g α-tocopherol, γ-tocopherol (10.56–15.00 mg/100 g) and 3.37–5.53 mg/100 g plastochromanol-8. However, tocopherols are considered the main antioxidants in flaxseed oil. Some authors report weak (Oomah et al., 1997) or no relationship between flaxseed oil oxidative stability and the content of tocopherols or phenol compounds in the oil (Bozan and Temelli, 2008). Therefore, it is possible that other minor oil soluble compounds play a role in slowing the oxidation of flaxseed oil. For example, the oxidative stability of meadowfoam crude oil may be reduced from 246.9 to 67.3 h at 110 $^{\circ}$C by refining, even though the concentration of tocopherols remains relatively constant after the refining process (Isbell et al., 1999). An acetonitrile extract of meadowfoam crude oil could be added to the refined oil and restore its oxidative stability. Analysis of the extract showed the presence of tocopherols as well as 1,3-di-(3-methoxybenzyl) thiourea and related degradation products of glucosinolates in the extract. Interestingly, the thiourea compounds and their mixtures were strong oil antioxidants (Abbott et al., 2002).

CLs are hydrophobic compounds present in flaxseed oil with comparable polarity to known natural antioxidants but the antioxidant activity of these compounds has not been reported previously. The first discovered CLA was characterized in 1959 (Kaufmann and Tobschirbel, 1959). Its unique structure induced researchers to investigate its bioactivity. Up to 11 CLs have been identified and isolated from flaxseed since the original discovery (Picur et al., 2006). Researchers have discovered that CLA possesses immunosuppressive activity comparable to that of cyclosporin A, a fungal compound that is useful for preventing tissue rejection.

Recent studies show that peptides possess a wide range of biological activities and can act as anti-cancer and antimicrobial agents. Peptides have also demonstrated immunomodulatory and antioxidant activities. Moreover, some characteristics of peptides, including low molecular weight, simple structure, low antigenicity, easy absorption and low toxicity, increase the potential for inclusion of peptides in pharmaceutical and foods (Ma et al., 2006).

Some peptides produced in living cells act as antioxidants. Well-known examples of such antioxidants include hormones [melatonin (Zang et al 1998), angiotensin, oxytocin, enkephalin (Moosmann and Behl, 2002)] and skeletal muscle peptides [carnosine and anserine (Chan and Decker, 1994)]. Moreover, peptides derived by hydrolysis of protein from egg yolk (Senji and

Yumi, 2006), porcine haemoglobin (Chang, 2007), fish (Thiansilakul, 2007), canola meal (Cumby, 2008) and whey (Peng et al., 2009) are also effective antioxidants. The anti-oxidative mechanism of peptides is not well understood. Some authors suggest that peptides that contain aromatic residues (tyrosine, histidine, tryptophan and phenylalanine) are able to stabilize ROS through electron transfer (Jung, 2005). Moreover, peptides are well known metal chelators and this property may contribute to antioxidant properties. The antioxidant activity of peptides produced by protein digestion is often greater than that of the intact proteins and the amino acid, order and structure both affect antioxidant activity (Chen et al., 1995).

In accelerated oxidation tests on lipids, the oxidation rate is normally low until natural antioxidants are consumed and then the rate of oxidation increases. The period of the time until this change in oxidation rate is called the induction time. The active oxygen method (AOM) accelerates lipid oxidation by applying both elevated temperatures and oxygen to an oil. The OSI is an accelerated oxidation assay that determines production of volatile organic compounds produced during oil oxidation. The main criticism of this assay is that the oxidation mechanism at high temperatures is not similar to the mechanism encountered under normal storage conditions. Nevertheless, it is reported that the shelf life of the sample at ambient conditions can be predicted based on OSI at elevated temperature (Farhoosh, 2007; Koleva, 2007). In general, lipid oxidation is the result of many complex chemical processes and interactions. The reaction between unsaturated fatty acid groups in lipids and ROS created in different ways is important to the stability of lipids containing unsaturated fats (Min and Boff, 2002).

3.4 Methods

3.4.1 Chemicals and Instruments

Cold-pressed flaxseed oil was donated by Biorginal Food and Science, Inc., Saskatoon, SK, and stored at 4 °C. Canola oil was purchased from a retail supplier (Superstore, Saskatoon, SK). Silica gel 60 (particle size, 0.040–0.063 mm, 230–400 mesh) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). High-pressure liquid chromotograph (HPLC) and column chromotography solvents were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted. A Milli-Q system (Millipore, Bedford, MA) was used to prepare deionized water for all mobile phases. Industrial grade metal stearates (Zn^{2+}, Ni^{2+} and Co^{2+}) were obtained from Sigma-Aldrich Canada LTD (Oakville, ON). CLA was prepared by reverse

phase chromatography of a silica extract of flaxseed oil according to Reaney et al. (2009).

3.4.2 Silica Gel Flash Column Chromatography

Before dry packing a 2.5-cm i.d. glass column (vertical) with silica gel, a cotton ball was placed in the bottom of the glass column. Then a 1-cm sand (50–70 mesh) layer was placed on top. Subsequently, 80 cm^3 of silica gel (grade 60, 230–400 mesh) was added to the column and this layer was covered by a 1-cm layer of sand (Figure 3-1).

Flaxseed oil (400 mL) was introduced into the column at a ratio of 5:1 (v/v, oil to silica gel) and was allowed to elute through the column under gravity to yield peptide-free oil (Figure 3-1). The column was eluted with 400 mL of 100% n-hexane (A), 250 mL of 20% ethyl acetate (EtOAc) in hexane (B), 250 mL of 50% EtOAc in hexane (C), 250 mL of 100% EtOAc (D) and finally 250 mL of 10% MeOH in dichloromethane (DCM, E) (Figure 3-2). Each of the fractions B to E (approximately 230 mL each) was collected in volumetric flask and diluted to a final volume of 250 mL. Samples of each fraction (B to E; 20 mL) were taken and concentrated under vacuum using a rotary evaporator (9,000 Pa, 40 ºC water bath, Rotavapor R-200 Buchi, Westbury, NY) and the resulting residue was subjected to HPLC and HPLC-MS analysis.

3.4.3 HPLC Analysis

HPLC analysis was conducted using an Agilent 1200 series HPLC systems equipped with a quaternary pump (G1311A), auto sampler (G1316A) and diode array detector (DAD) (G1315D; wavelength range 190–600 nm) and a ZORBAX Eclipse XDB-C18™ column (3 µm particle size silica, 50 × 4.6 mm i.d.), equipped with an in-line filter. The mobile phase consisted of a linear gradient of water-acetonitrile (Table 3-1) and a flow rate of 2 mL/min.

3.4.4 Mass Spectrometry

Tocopherols in fraction D were separated using a Chromolith FastGradient RP-18e column (3 µm particle size silica, 50 mm × 2.0 mm i.d.). Use was made of an Agilent HPLC (1200 series) equipped with a quaternary pump, autosampler, DAD (wavelength range 190–300 nm) and a degasser directly connected to a Bruker microOTOF-Q II Mass Spectrometer (Hybrid Quadrupole-TOF MS/MS; Bruker, Bremen, Germany) with an Apollo II ESI ion source operated with Nebulizer gas at 4.0 bar and dry gas temperature held at 200 °C. The mobile phase consisted of a linear gradient of 0.1% formic acid in water and 0.1% formic in acetonitrile
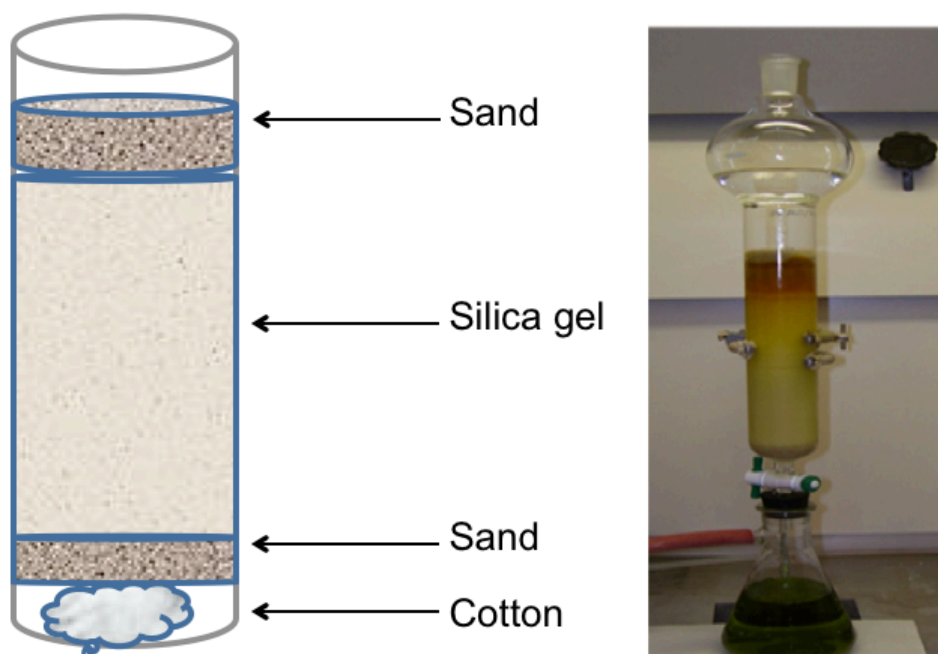
Figure 3-1   Silica gel flash chromatography
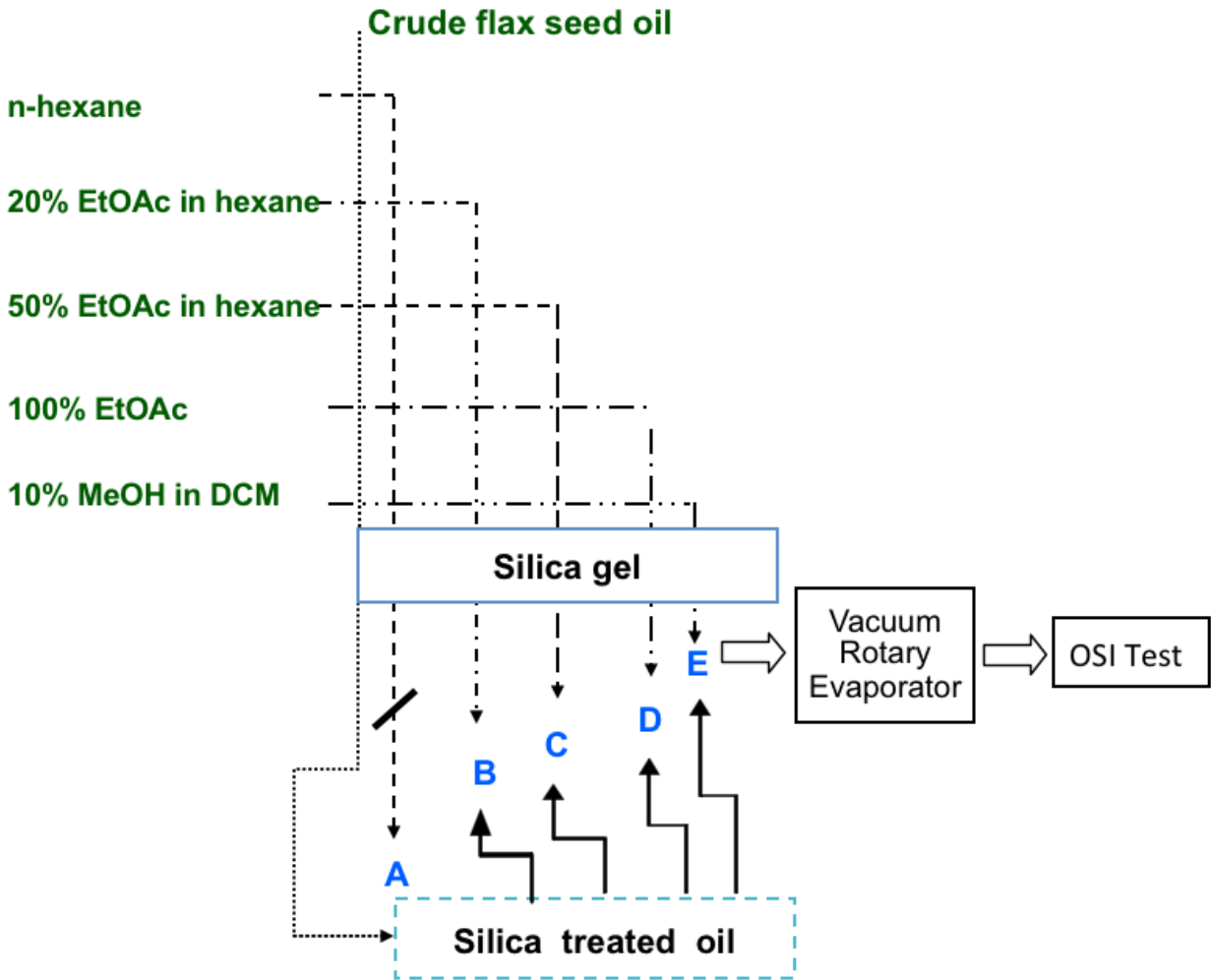
Figure 3-2 Flow chart for isolation of CL containing fractions and sample preparation for OSI.

Table 3-1   Solvent program for CL identification and quantification by HPLC

Time (min)	Solvent composition (% acetonitrile)	Flow rate (mL/min)
0	30	0.5
3	40	0.5
6	45	0.5
7	65	0.5
19	65	0.5
22	66	0.5
23	70	1.0
24	100	1.0
26	100	1.0
31	30	1.0

(60:40 for 2 min, to 10:90 in 8 min, to 60:40 in 0.5 min, to equilibration for 5.5 min) and a flow rate of 0.40 mL/min.

3.4.5 OSI Analysis

3.4.5.1 Effect of Polar Fractions on Oxidation of Silica-Treated Flaxseed Oil

Eluate from each fraction (B–E; 30 mL) was added to 30 mL of silica-treated oil and the mixture was concentrated under vacuum using a rotary evaporator (approximately 9,000 Pa, 40 °C water bath, Rotavapor R-200, Buchi) to remove organic solvent. The same procedure was applied for control samples, where the solvent from the same reagent bottles used to elute the column was added instead of the column eluate. Crude and silica-treated flaxseed oil samples were kept under vacuum in a desiccator for 24 h prior to analysis by OSI. The OSI of these samples was determined as described below.

3.4.5.2 Effect of the Concentration of Polar Fraction on the OSI of Silica-Treated Flaxseed Oil

Fractions D and E (200 mL) were collected from a silica gel flash column and concentrated as described above. The concentrate was added to 30 mL silica-treated oil and stored in a desiccator that was sealed and connected with a vacuum system for 24 h. The OSI of these samples was determined as described below.

3.4.5.3 Effect of CLA and Divalent Metal Cations on the OSI Of Flaxseed Oil

Stock solutions of metal stearates (Ni^{2+}, Zn^{2+}; 1.25 mM) and CLA (2.5 mM) were prepared in silica-treated flaxseed oil. The stock solutions were then added to silica-treated flaxseed oil to achieve desired final concentrations of metal stearate (0.125 mM) and CLA (0.25 mM). The OSI of these samples was determined as described below. Stock solutions of zinc stearate (1 mM) and CLA (1 mM) were prepared in canola oil. Subsequently, CLA (1 mL; 2 mL; 3 mL) and zinc stearate (4 mL) stock solutions were added to 23 mL silica-treated oil. The mixture volume was increased to 30 mL by the addition of canola oil. The final concentration of CLA was 0.03–0.09 mM and that of Zn^{2+} was 0.12 mM. The OSI of these samples was determined as described below. A stock solution of CLA (0.625 mM) and cobalt stearate (0.625 mM) were prepared in canola oil. The canola oil along with metal-stearate-treated canola oil were added to 25 mL of silica-treated flaxseed oil until the volume of the oil was 32 mL. The

addition of treated canola oil produced mixed oils with final concentrations of 0.04 or 0.12 mM CLA and/or 0.02 mM cobalt stearate. The OSI of these samples was determined as described below.

3.4.5.4 Measurement of OSI

The oxidative stability index was measured with an OSI instrument (Omnion, ADM, Rockville, MD) (Figure 3-3) using AOCS method Cd 12b-92 (Firestone, 1993). Samples (5.0 ± 0.05 g) were taken in a glass test tube and then placed in the preheated (100 °C) OSI instrument. Air was bubbled through each sample (130 mL/min flow rate) during heating. Replicate samples (3–4) were analyzed in each experiment. Crude oil and silica-treated oil were taken as reference samples. OSI values were calculated using the Omnion OSI v 8.18 software, which detects the end point mathematically by determining the maximum of the second derivative of conductivity plotted against time.

3.5   Results and Discussion

3.5.1   Silica Gel Flash Column Chromatography

Silica-treated flaxseed oil, with a clear yellow color, was obtained by passing crude oil through a silica gel column. Using an approach analogous to that applied in this study, Abuzaytoun and Shahidi (2006) analyzed fractions obtained by passing cold-pressed hempseed and flaxseed oil through a column packed with two adsorbents (activated silicic acid and activated charcoal) and eluting the compounds with a series of organic solvents. They observed that after absorption on silica and carbon, the oil was essentially free of tocopherols and lacked light absorbance at 550–710 nm, which would indicate a loss of chlorophyll molecules. UV absorbance indicated the presence of carotenoids in the oil. In the present study, polar fractions eluted from silica containing CLB–CLE were separated by flash column chromatography from cold-pressed flaxseed oil and the solvent was removed from the fractions by concentrating under vacuum. The yields of the extracts obtained by silica gel flash chromatography are presented in units of g/100 mL of flaxseed oil (Table 3-2). CLs were identified by their standard HPLC retention times, which were assigned by Gui et al. (2012a). As presented in Table 3-3 and Figure 3-4, the crude flaxseed oil was rich in CLs A, F, G, C, E, D, F″ and E″. The analyses of fractions B through E using HPLC revealed the presence of CLs in fractions D and E. Fraction D contained

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Table 3-2 Yield of flaxseed oil extracts in different solvents (g/100 mL oil; 4 samples per measurement; $n = 4$)

Fraction	Yield g/100 mL	Details of content
B: 20% EtOAc in hexane[1]	24.96 ± 0.12	ND[2]
C: 50% EtOAc in hexane[1]	2.29 ± 0.28	ND
D: 100% EtOAc	1.11 ± 0.98	CLA and β, γ and δ-tocopherols
E: 10% MeOH in DCM	1.09 ± 0.34	CLA, CLC-CLG

[1]Oil was a major component of these fractions.

[2]ND: not detected.

Table 3-3 HPLC profile of crude flaxseed oil and its fractions

CL	Retention time (min)[1]	Fraction E		Fraction D		Crude flaxseed oil		Conversion ratio[3]
		Retention time (min)	Area[2] (mAU × s)	Retention time (min)	Area[2] (mAU × s)	Retention time	Area[2] (mAU × s)	
CLF	ND[4]	12.36	4550	ND	ND	12.35	736	6.18
CLG	12.83	12.85	16993	ND	ND	12.83	2467	6.89
CLC	13.61	13.78	39923	13.77	177	13.47	5147	7.79
CLE	15.72	15.77	74445	ND	ND	15.70	9679	7.69
CLD	17.22	17.93	25428	ND	ND	17.81	3950	6.44
CLA	24.05	25.30	1821	25.28	17650	24.23	2434	7.99

[1]Retention time assigned by Gui et al. (2012a).

[2]Results of a single measurement.

[3]Conversion ratio = CLs peak area of crude oil/CLs peak area of fractions.
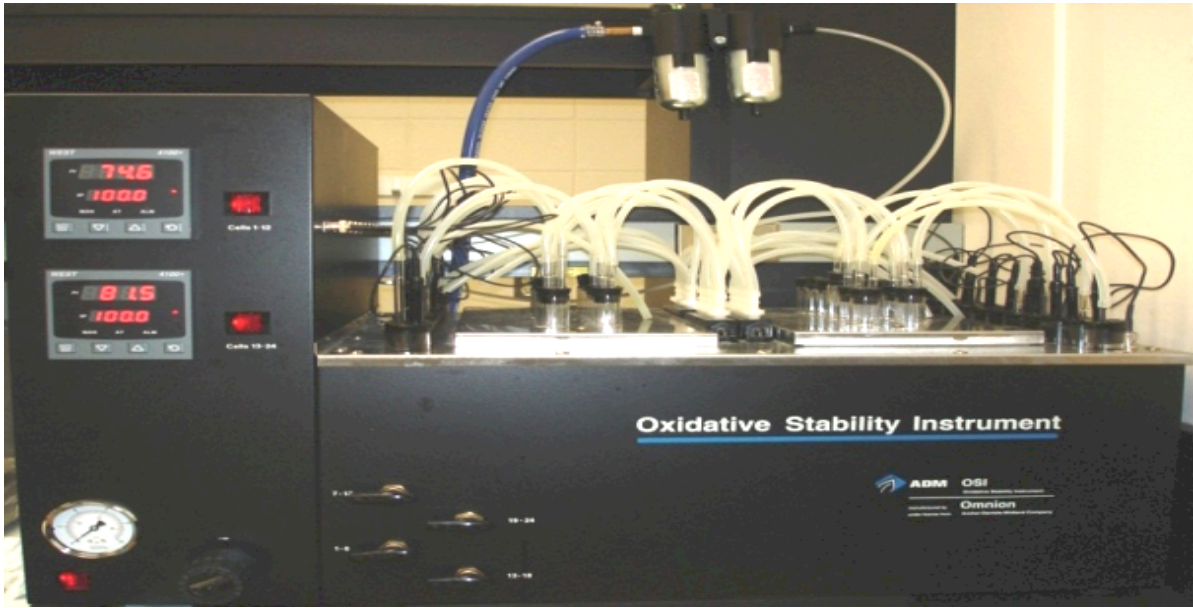
[4]ND: not detedcted.
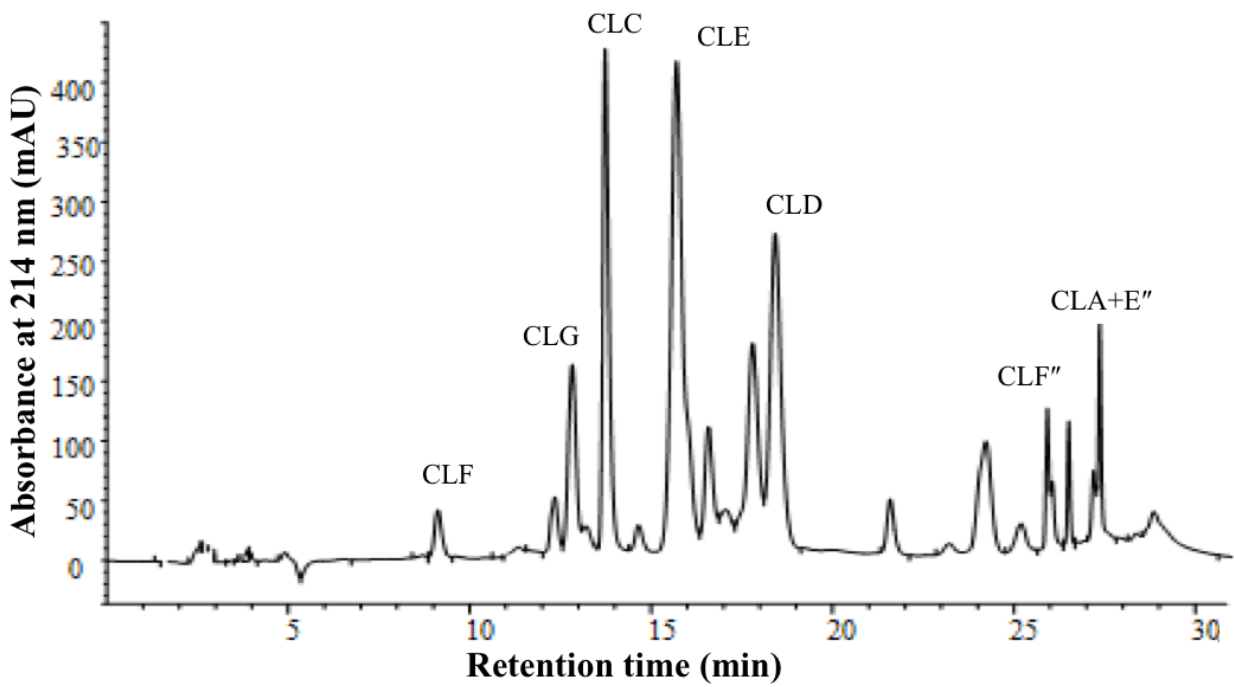
Figure 3-3   The OSI instrument.

Figure 3-4   HPLC profile of crude flaxseed oil.

CLA, whereas fraction E contained a mixture of CLs (CLA and CLD–CLG) (Figure 3-5). Peptides containing methionine (CLH, CLI, CLB, CLF″ and CLE″) were not apparent in the HPLC chromatograms of polar compounds isolated from flaxseed oil. Therefore, peptides containing methionine sulfoxide, (CLF, CLG, CLC and CLE) are presented (Table 3-3). Oxidation of the methionine group of CLs into methionine sulfoxide is observed regularly with the exposure of flaxseed oil to heat and oxygen during oil extraction (Gui, 2011). Most of the peptides were observed in fractions D and E. In addition, mass spectrometric analysis of fraction D indicated the presence of β-, γ- and δ-tocopherols (Figure 3-6).

3.5.2 Oxidative Stability Test

The OSI values were determined at 100 ℃ on crude, pressed flaxseed oil, silica-treated flaxseed oil and silica-treated flaxseed oil with polar fractions (B through E) added to determine the presence of antioxidant substances (Tables 3-4, 3-5 and 3-6). Crude flaxseed oil had higher oxidative stability than the silica-treated peptide-free oil, indicating that some of the chemical constituents that were removed when treating the flaxseed oil with silica gel play a significant role in reducing the rate of flaxseed oil oxidation. OSI times of crude and silica-treated flaxseed oil varied from 3.0–4.3 h and 1.5–2.7 h, respectively. Similarly, Abuzaytoun and Shahidi (2006) observed that after absorption of flaxseed on a silicic acid/charcoal column, the TBARS (thiobarbituric acid-reactive substances) value of cold-pressed flaxseed oil decreased from 6.01 to 4.54 μmol/g and conjugated dienes from 1.65 to 1.09 μmol/g. Several natural antioxidant compounds have been determined in flaxseed oil and the quantity of these compounds depends on cultivar, growing conditions and processing method. According to the study of Choo et al. (2007), cold-pressed flaxseed oil, taken from flaxseed oil purchased in New Zealand and including locally-produced and imported oil, contained 76.8–307 mg/100 g phenolic acid, 12.7–25.6 mg/100 g flavanoids, 3.37–5.53 mg/100 g plastochromanol-8 and 11.07–24.47 mg/100 g tocopherols. In addition, cold-pressed flaxseed oil contains up to 150 mg/100 g CLs (Gui et al., 2012). Therefore, reduced oxidative stability observed with silica-treatment may be related to the removal of any or all of these polar compounds. The presence of other minor compounds and synergistic interaction among substances should be considered (Abuzaytoun and Shahidi, 2006; Choo et al., 2007). Moreover, addition of fraction D to silica-treated flaxseed oil increased its stability to oxidation when compared to oil treated with fractions B, C or E (Figure 3-7).
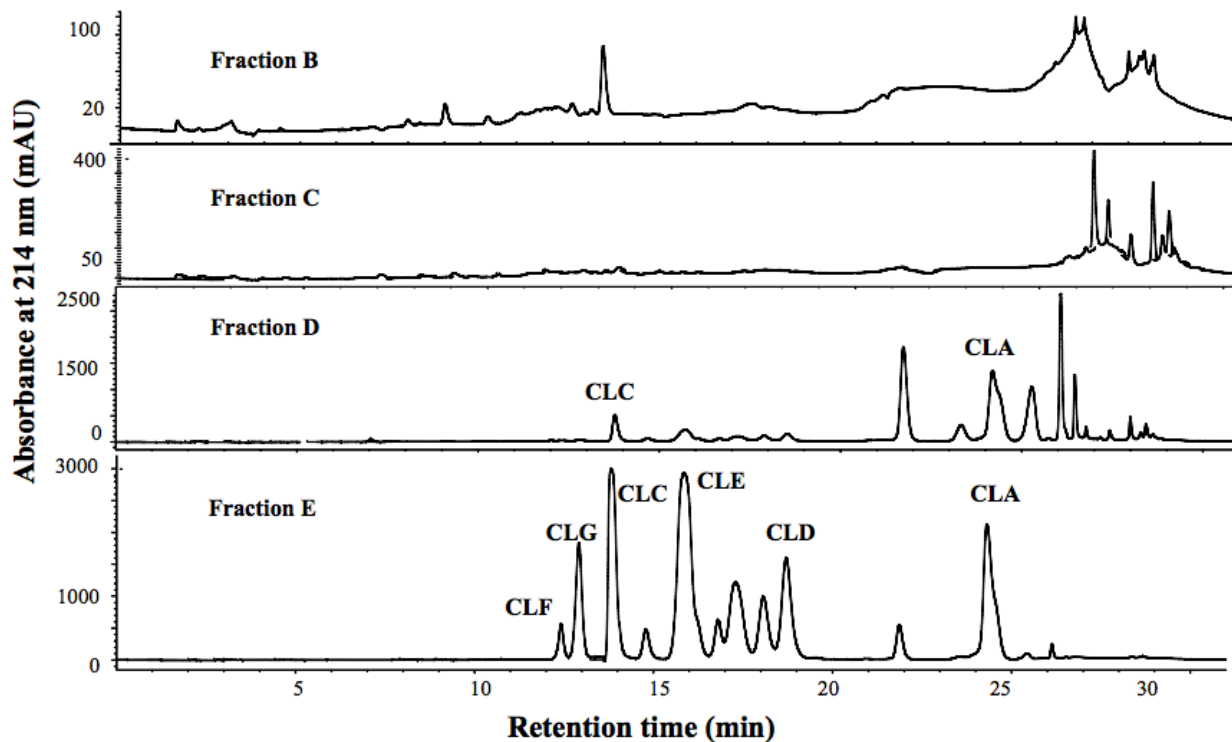
Figure 3-5 HPLC profile of polar fractions obtained by silica absorption of flaxseed oil.
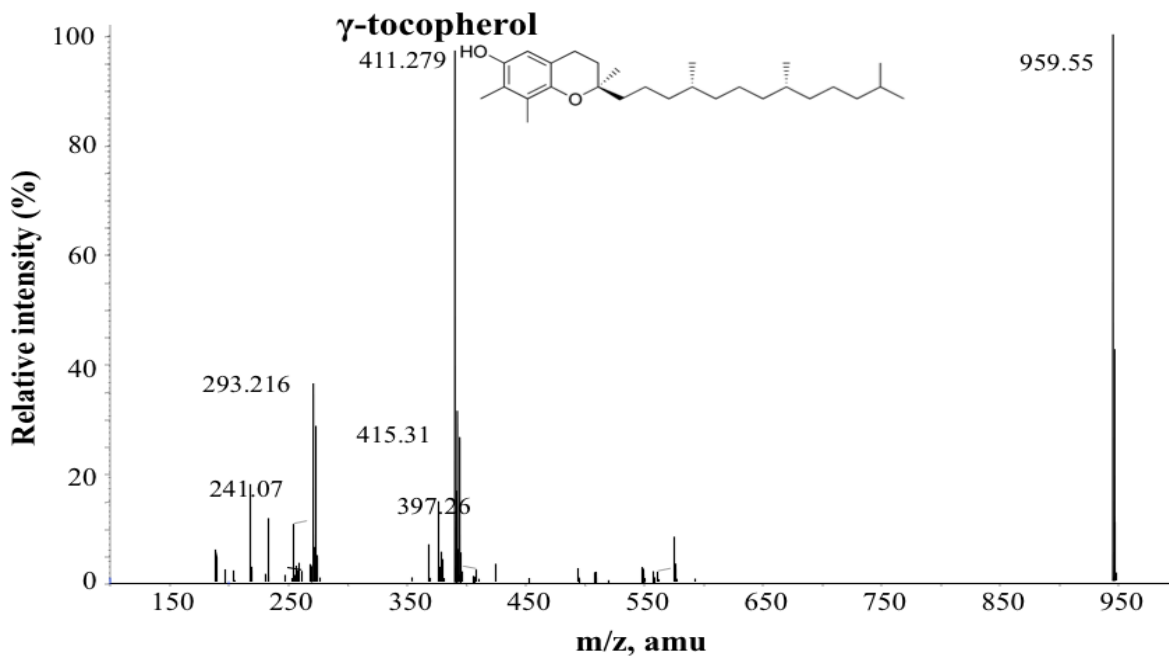
Figure 3-6 Mass spectrum of fraction D containing tocopherols.

Table 3-4   Oxidative stability of flaxseed oil and CLs-containing fractions

Fraction	OSI index (h)[1]	
	Reference (solvent)	Sample
B (20% EtOAc in hexane)	2.65 ± 0.41	2.78 ± 0.57
C (50% EtOAc in hexane)	2.54 ± 0.26	2.41 ± 0.50
D (100% EtOAc)	2.03 ± 0.03	3.20 ± 0.41
E (10% MeOH in DCM)	2.34 ± 0.30	2.77 ± 0.48
Crude oil	ND[2]	4.30 ± 0.56
Silica-treated oil	ND	2.36 ± 0.28

[1]Average of 4 replicates.

[2]Not detected.
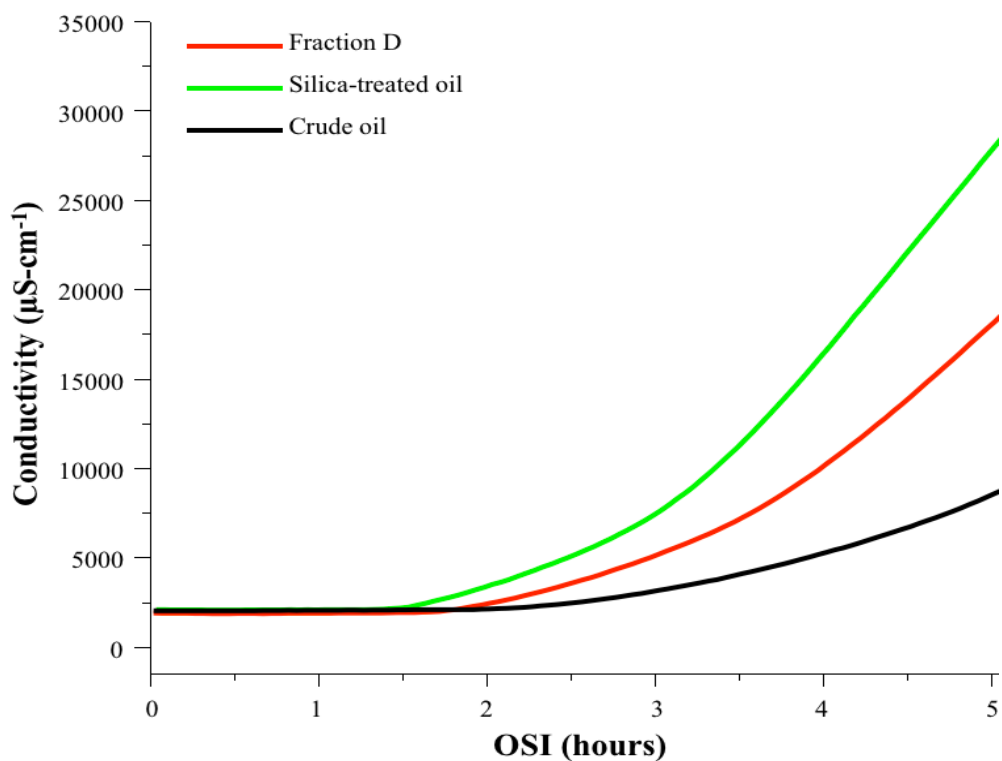
Figure 3-7   The OSI of flax oil and CLs-containing fractions.

This fraction contained CLA and trace amounts of γ-, β-, and δ-tocopherols. These compounds could potentially contribute to the observed increase in oxidative stability. Fractions B and C did not affect oxidative stability of silica-treated flaxseed oil as controls of silica-treated oil with added then evaporated solvents showed comparable OSIs to samples treated with fractions B and C. Fraction E contained more polar CLs than Fraction D (Table 3-4). Possibly, other compounds such as phenolics and flavonoids with known antioxidant properties (Panagiotopoulou and Tsimidou, 2002; Bukhari et al., 2008) did not significantly increase the induction time of silica-treated oil.

Reports of the presence of phenolic compounds in flaxseed (Oomah et al., 1996; Choo et al., 2007; Siger et al., 2008) are based on the use of colorimetric methods that employ low specificity reagents. For instance, the Folin-Ciocaltue reagent was used to determine the flavonoid content of cold-pressed flaxseed oil (Choo et al., 2007) but this reagent may also react with a broad range of reducing substances including nitrogen containing compounds such as indole derivatives (Ikawa et al., 2003). As indicated in Figure 3-4, fraction E contains CLF, CLG and CLD. Each of these peptides contains tryptophan and methionine residues. It is possible that these substances reduce the Folin-Ciocaltue reagent, giving a false indication of the presence of phenolic compounds in flaxseed oil.

Many studies have revealed that polar fractions obtained from various vegetable oils contain phenolic compounds that contribute antioxidant activity. For instance, Zhang et al., (2006) contacted parsley essential oil with silica gel and conducted a stepwise solvent elution of the silica with ethyl acetate, ethyl acetate/methanol (1:1, v/v) and methanol. The study revealed that the antioxidant activity of the fractions increased with the polarity of the solvent used to elute that fraction from the silica. Phenolic compounds are also reported in fractions exhibiting the strongest antioxidant activity (Zhang et al., 2006). In comparison to fraction D, fraction E has little or no effect on flaxseed oil oxidation in spite of its higher polarity (Table 3-3). It is possible that fraction E does not contains phenolic compounds as indicated by previous researchers, but that the elevated induction time may be linked to specific CLs abundant in fraction D but that are absent from fraction E, or synergic activity of the peptides with tocopherols detected in fraction D. The dose-response and time-dependent activity of fractions D and E were studied after 10 months storage at 4 °C in a refrigerator (Table 3-5 and Figure 3-8). As shown in Table 3-5, the induction time of crude oil decreased from 4.3 to 3 h during the 10-month storage period.

Table 3-5   Antioxidant activity of CLs fractions

Sample	OSI (h) 0 month	OSI (h) 10 months	
	1st day	1st day	3rd day
Crude oil	4.30 ± 0.56	3.00 ± 0.10	3.07 ± 0.47
Fraction D[1]	3.20 ± 0.41	3.56 ± 0.68	2.86 ± 0.21
Fraction E[2]	2.77 ± 0.48	3.02 ± 0.45	2.35 ± 0.27
Tocopherol	ND[3]	3.51 ± 0.90	3.00 ± 0.43
Silica-treated oil	2.36 ± 0.28	2.75 ± 0.20	1.15 ± 0.07

[1]Fraction D: 100% EtOAc (v/v).

[2]Fraction E: 10% MeOH in DCM (v/v).

[3]Not detected.
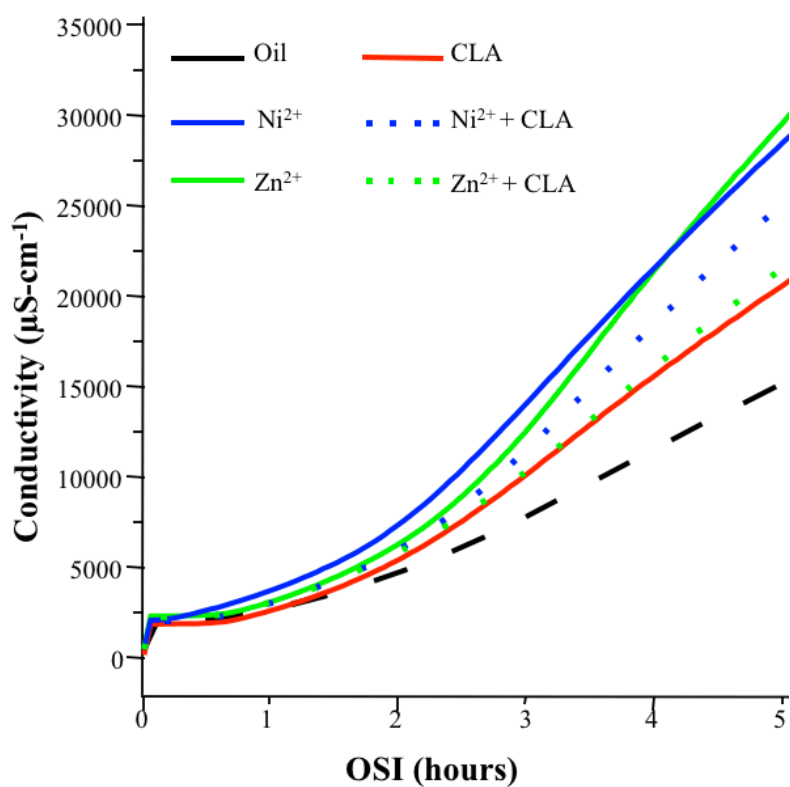
Figure 3-8   The OSI of flaxseed oil and CLA + M^{2+}.

Gusevaa et al., (2010) observed that the peroxide value of cold-pressed flaxseed oil increased from 1.8 to 3.9 after six months storage at 10 ºC, while Yildirim (2009) reported that over 126 days storage at 4 ºC, the PV (peroxide value) increased from 3.01 to 6.73. In addition, the fatty acid and antioxidant contents in crude flaxseed oil changed significantly over the first seven months of storage after which no further changes were observed (Yildirim, 2009). In this study the OSI times were measured over three days (Table 3-5) after removal from storage. The oxidative stability of silica-treated oil decreased significantly, by over 50%, after removal from storage, while oil subjected to other treatments was more stable. Silica-treated oil with added fractions D and E decreased in induction period by 20% after 3 days removal from storage. Furthermore, the study indicated that these both fractions D and E have lipid antioxidant properties and the activity may be effective for longer periods. It is important to note that the largest impact of fractions D and E was observed only after long storage followed by removal from storage for three days (Table 3-5).

3.5.3 Influence of CLA on Metal Cation Induced Oxidation of Flaxseed Oil

Crude, cold-pressed vegetable oil contains small amount of metals. The quantity of trace metals varies depending on genetics, environmental conditions during plant growth, and processing methods (Pehlivan, et al., 2008, Anwar, et al., 2004). These metals may potentially act as catalysts during lipid oxidation, In this study, transition metal ions (Zn^{2+}, Co^{2+} and Ni^{2+}) were added to flaxseed oil to determine their effects on the rate of oil oxidation. Also, as CLs potentially bind to metals possible interactions between the addition of CLA on flaxseed oil oxidation and transition metal ions were explored. Results of this study are presented in Tables 3-7, 3-8 and 3-9. Transition metals, including two redox-active metals, iron and cobalt, are components of many biological processes. Metal ion binding and interaction with CLA have been reported. Balasubramanian et al. (1976) reported that CLA binds weakly to Ca^{+2} ions in methanol. Moreover, Chatterji et al. (1987) detected CLA: Tb^{+3} complexes stabilized by the f orbital of the lanthanide. Although binding of both ions with CLA was stabilized by Phe residues, the interaction with Ca^2 did not involve charge electrons (Chatterji et al., 1987).

Silica gel adsorption readily removes divalent metal ions with high efficiency from different substrates (Goswami and Singh, 2002; Baytak et al., 2005; Prado et al., 2005). Hence, silica-treated flaxseed oil is likely greatly reduced in, or free from, divalent metal ions. We

observed that transition metal ions Zn^{2+} and Ni^{2+} accelerated the oxidation rate of silica-treated flaxseed oil (Table 3-6). Induction times of silica-treated flaxseed oil in the presence of these metals at 0.25 mM concentration were reduced by over 0.8 h when compared with controls without added metal. The potential antioxidant property of CLA against oxidation induced by metal ions was explored (Figure 3-8). CLA slowed oxidation by Ni^{2+} and Zn^{2+} by 17 and 37 percent, respectively (Table 3-6). The current study is the first published data describing the impact of CLs on the rate of oil oxidation in the presence of a transition metal (Zn^{2+}, Ni^{2+} and Co^{2+}). The observed results could be attributed to CLA binding with metal soaps, making them unavailable for oil oxidation. In addition, Zn^{2+} ion is abundant in flaxseed (4 mg/100 g) and this study revealed that CLA could potentially interact with zinc. Additional experiments were focused on the dose-dependent interaction of zinc with CLA.

Table 3-7 presents the effect of CLA and Zinc ion on oxidation of silica-treated flaxseed oil when applied at a mole ratio of 1:4, 1:2 and 1:1.3 of CLA to Zn^{2+}. In this study, canola oil was used to dissolve metal stearates and CLA, Hence OSI times were not comparable to the previous study. Addition of canola oil significantly improved the OSI time of silica-treated flaxseed (from 2.35 h up to 3.5–8 h). Blends of flaxseed oil with sesame, milk thistle (Gusevaa et al., 2010) and olive oil (Yildirim, 2009) have superior oxidative stability when compared to unblended flaxseed oil. This phenomenon occurs with other oils. For example, Isbell et al. (1999) reported that adding just 5% of crude meadow foam oil to triolein enhanced its stability by 35%.

The addition of canola oil modified the impact of zinc on oil OSI. Zinc ions did not lower the OSI of blends of canola and flaxseed oil when canola oil was also added. It is possible that antioxidant compounds are present in canola oil that inhibits oxidation. Canola oil is rich in tocopherols; commercial canola oil contains up to 700 mg/kg tocophenols. Moreover during refining processes, especially after bleaching and deodorizing, metal cations would have been almost cleared from the commercial oil used in this study (Przybylski et al., 2005).

CLA improved the oxidative stability of the blends when added alone to the oil. However, the presence of zinc reduced the antioxidant effect of CLA (Table 3-7). At 0.12 mM concentration, CLA enhanced the induction time of oxidation oil by 11.4% and 6.7% when canola oil was present at 26.0% and 16.6%, respectively. An interaction of CLA with zinc on oxidative stability was also observed in this study. As shown in Table 3-7, the OSI times of a sample mixed with CLA is close to that of the sample with CLA: Zn^{2+} (1:1.3). Therefore, it is

Table 3-6   The OSI of flaxseed oil with CLA and Zn^{2+} and Ni^{2+} stearates

Silica-treated crude flaxseed oil	CLA (0.125 mM)	Zn^{2+} (0.25 mM)	CLA + Zn^{2+} (1:2)	Ni^{2+} (0.25 mM)	CLA + Ni^{2+} (1:2)
2.36 ± 0.20	2.21 ± 0.40	1.50 ± 0.20	2.05 ± 0.25	1.45 ± 0.15	1.71 ± 0.13

Table 3-7   The OSI of flaxseed oil with CLA and Zn^{2+} stearates

Peptide free flaxseed oil	CLA (0.12 mM)	Zn^{2+} (0.12 mM)	CLA: Zn^{2+}		
			1:4	1:2	1:1.3
3.50 ± 0.33[1]	3.90 ± 0.30	3.50 ± 0.31	3.58 ± 0.17	3.65 ± 0.28	3.75 ± 0.13
8.07 ± 0.33[2]	8.61 ± 0.41	8.25 ± 0.38	8.08 ± 0.02	8.20 ± 0.10	8.77 ± 0.23

[1]Content of canola oil is 26.0% by volume of blend.

[2]Content of canola oil is 16.6% by volume of blend.

Table 3-8   The OSI of flaxseed oil with CLA and Co^{2+} stearates

Peptide free flaxseed oil	CLA (0.08 mM)	Co^{2+} (0.02 mM)	CLA: Co^{2+}	
			2:1	6:1
3.41 ± 0.13[1]	3.55 ± 0.16	3.05 ± 0.15	3.10 ± 0.14	3.11 ± 0.15

[1]Content of canola oil is 28.5% for volume canola oil.

possible that CLA almost completely binds Zn^{2+} at this ratio. In contrast at a ratio 1:4, oil oxidizes at the same rate as oil in the presence of metal cations but no CLA. This indicates that the presence of excess amounts of metal ion exceeds the metal binding capacity of CLA.

In addition, the effect of Co^{2+} ions on the oxidation of silica-treated flax oil was studied. However, CLA had no effect on the rate of oil the oxidation in the presence of cobalt as there was no influence of CLA on OSI in the presence of cobalt stearate. Since cobalt has a strong redox potential it may be able to initiate oxidation. It cannot be determined if binding to CLA has occurred (Table 3-8).

3.6   Conclusions

- The crude oil showed higher oxidative stability than the peptide-free oil, indicating that some of the chemical constituents removed upon treatment with silica gel play a significant role in reducing the oxidation of flaxseed oil.

- Fraction D containing CLA and tocopherols, and fraction E, containing a mixture of CLA, CLD, CLE, CLF and CLG, improved the oxidative stability of peptide-free oil, suggesting the significant role played by these compounds in protecting oil from oxidation.

- CLs have their own role in the strong antioxidant system of flaxseed oil. They may behave as antioxidants or pro-oxidants depending on concentration and environment. Dose and time-dependent antioxidant properties of these peptides were exposed.

- CLA is able to interact with metals selectively. Hence, it can inhibit oxidation process by binding metals.

3.7   Connection to the Next Study

At present, oil seed flax is the main commercially-produced flaxseed in Canada. Consequently, to improve the variety of uses for flaxseed a national program was established called Flax 2015 (Flax Council of Canada, 2007). A main strategy of this initiative is to enhance value-added opportunities for flaxseed and one branch of this plan could be phytochemicals in flaxseed (Ashley and Sullivan, 2006). One of the groups of phytochemicals in flax is the CLs, which are hydrophobic compounds present in flaxseed. The effect of polar extracts containing CLs of cold-pressed flaxseed oil on oxidation was tested and it was revealed that extracts that

contained peptides were also antioxidants. Although, the OSI test was able to illuminate an approximate picture of oxidation in the presence of CLs, possible trace compounds in the studied fractions rendered these results inconclusive. To clarify the obtained findings, further research should be conducted with simplified model systems. On the other hand, an inadequate availability of CLs demands more sensitive analytical methods.

CHAPTER 4

FREE RADICAL SCAVENGING ACTIVITY OF CYCLOLINOPEPTIDES

4.1   Abstract

The antioxidant properties of three cyclolinopeptides (CLs) isolated from flaxseed were explored using EPR spectroscopy. EPR signal intensities were monitored by varying the concentration of a radical in radical-CLs reaction mixtures. All three CLs exhibited dose-dependent scavenging activities. CLA, B and C reactions with DMPO-OH (5 mM) resulted in a 24–30% decrease in EPR signal intensity. The reaction of CLs with the more stable DPPH$^\bullet$ radicals revealed a more complex chemistry than simple scavenging. Tryptophan-containing peptides (CLG and CLG") showed stronger activity than those containing methionine and methionine sulfoxide (CLB and CLC). Activation of residues with light also contributed to scavenging activity.

4.2   Hypothesis

The presence of low molecular weight hydrophobic amino acids, including methionine, tryptophan, proline and leucine moieties, typically contribute to the antioxidant activity of linear peptides. It is the hypothesis of the current study that the amino acid composition of cyclic peptides from flaxseed will afford some antioxidant activity.

4.3   Introduction

Molecules with unpaired electrons are paramagnetic and can be loosely referred to as free radicals. Generally, radicals are unstable and most often transient since they possess higher potential energy than non-radicals of similar molecular mass and they are invariably produced *via* bimolecular collisions, rearrangements or elimination reactions. They are highly reactive and form new and complex molecules in their reactions. ROS, oxygen radicals and non-radical

derivatives of oxygen are unavoidably produced during metabolic processes like respiration and photosynthesis. ROS are naturally occurring products of metabolism but overproduction can cause cell damage. Molecules such as lipids, sugars, proteins and DNA react with ROS and the reaction of these species with ROS can induce further oxidative stress (Halliwell, 1992; Apel and Hirt, 2004). ROS are implicated in biological processes including mutation, carcinogenesis, degenerative diseases, inflammation and aging (Ron and Abraham, 2002; Evgeny and Afanas'ev, 2005). Aerobic organisms have developed a range of defense mechanisms, including the production of antioxidants, to minimize the deleterious effects of ROS. Typical antioxidants are present at low concentrations compared to oxidizable substrates. In spite of their low relative concentrations, these compounds significantly delay or prevent oxidation of substrates. Antioxidants such as uric acid, flavonoids, ascorbic acid (vitamin C) and tocopherols (vitamin E) are good radical scavengers (Koleva, 2007). In addition to the natural sources of ROS outlined above, abiotic factors such as heat, light and metals in the environment have been implicated in ROS production in organisms and in the surrounding environment as well. ROS present in food cause lipid oxidation that, in turn, leads to toxic reaction products, undesirable off-flavours and reduced nutritional value. Synthetic or natural antioxidants are, therefore, added to food products to prevent or delay their deterioration. Natural antioxidants such as ascorbic acid and tocopherols are not very effective in many systems because their activity is short lived. Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), among others have found widespread application in the food industry (Williams et al., 1999), although the use of these compounds is regulated due to the potential health hazards of long-term consumption (Clapp et al., 1973, Williams, 1986). There is growing interest in discovering new antioxidants from natural sources that have lower health risks and are as effective as synthetic antioxidants. At the same time, protein hydrolysates from animal and plant sources have been studied widely and are regarded as safe, available, nutritional and functional antioxidants in food products (Elias et al., 2008). Examples of these are enzymic hydrolyzates derived from flaxseed (Marambe et al., 2008), whey (Peng et al., 2009), blue mussel (Jung et al., 2005), Alaska pollack (Je et al., 2005), silver carp (Marambe et al., 2008) bean seed (Comfort et al., 2011), chickpea (Zhang et al., 2011), goat placenta (Teng et al., 2011), hemp seed (Girgih et al., 2011), horse mackerel viscera (Sampath Kumar et al., 2011), barley hordein (Bamdad et al., 2011), zein (Kong and Xiong, 2006), egg yolk (Senji and Yumi, 2006), porcine haemoglobin (Chang, 2007),

fish (Thiansilakul, 2007), canola (Cumby, et al., 2008), porcine muscle and milk casein (Kim et al., 2001). Thus, the antioxidant activity of peptides is predictable since they are composed of the same amino acid residues in the proteins outlined above. Indeed, peptides containing histidine, proline, phenylalanine, tyrosine, cysteine, methionine and tryptophan are present in many antioxidant peptides (Chen et al., 1995, Chen et al., 1998; Je et al., 2005; Jung et al., 2005; Zhang et al., 2008; Farvin et al., 2010). In addition to the effects of amino acid composition, amino acid sequences and configuration also determine peptide ROS scavenging properties (Chen et al., 1995; Chen et al., 1996).

There is no universally accepted hypothesis describing the antioxidant mechanism of peptides. Scavenging properties of aromatic residues (tyrosine, histidine, tryptophan and phenylalanine) may be attributed to the ability of these moieties to donate electrons to ROS and stabilize them (Jung, 2005). However, metal chelating assays show that peptides are able to bind metals (Chen et al., 1998; Kong and Xiong, 2006; Farvin et al., 2010; Zhang et al., 2011), but a good correlation between metal-ion chelating activity and antioxidant activity has not been established (Chen et al., 1998; Zhang et al., 2008). Also, the antioxidant potential of compounds is dependent on the nature of the other compounds present. For instance, vitamin E is able to quench lipid peroxyl radicals in lipid or membranous systems but not in aqueous solutions (Packer et al., 1995). However, the higher the number of hydrophobic amino acid residues in a peptide correlates with antioxidant activity and the degree of hydrolysis (DH). It appears that both trends play a role in the ability of peptides to scavage free radicals (Pownall et al., 2010; Zhong et al., 2011; Ajibola et al., 2011). Therefore, hydrophobic antioxidant peptides are able to access hydrophobic targets in cell membranes, protecting them from oxidative damage (Szeto, 2006). Table 4-1 shows a summary of EPR radical scavenging activity studies of peptides derived from animal sources as by-products of the fish industry. These are considered as cheap and safe antioxidant sources for food application.

Due to their short lifetime and unstable characteristics it is a challenge to directly study free radicals in biological systems. In research practice, synthetic stable radicals or simple radical generating systems are used to mimic free radicals produced in natural oxidation processes. The DPPH˙ radical is commonly used in studies of antioxidants due to its stability and simple ESR signal. The DPPH˙ radical is paramagnetic and it can react with radicals or other molecules to become part of a stable diamagnetic molecule by accepting an electron or hydrogen radical

Table 4-1   Inhibition of EPR signal intensity of DMPO/OH$^\bullet$ by peptides

Source	Peptides sequences	MW (Da)	Hydroxyl radical scavenging activity (%)	Reference
Fermented blue mussel	Phe-Gly-His-Pro-Tyr	620	89.5% at 64.8 µM	Jung et al., 2005
Fermented mussel sauce	His-Phe-Gly-Asp-Pro-Phe-His	962	96.0% at 200 µg/mL	Rajapakse et al., 2005
Alaska Pollack (*Theragra chalcogramma*) frame	Leu-Pro-His-Ser-Gly-Tyr	672	35.0% at 53.6 µM	Je et al., 2005
Jumbo squid (*Dosidcus gigas*) skin gelatin	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu	880	50.0% at 90.9 µM	Mendis et al., 2005
	Asp-Gly-Pro-Leu-Glu-Gin-Pro-Gly-Glu-Arg	1241	50.0% at 100.72 µM	
Horse mackerel (*Magalaspis cordyla*) viscera	Ala-Cys-Phe-Leu	518	59.1% at 0.2 mg/mL	Sampath Kumar et al., 2011

or undergo a bond forming reaction. The reaction mechanism between an antioxidant and DPPH$^\bullet$ varies depending on the antioxidant structure and the reaction media. In most cases, the reaction rate is slow and the mechanism is complex (Bonder et al., 1997). Multiple pathways are involved in DPPH$^\bullet$ reactions with antioxidants. Different reaction products and covalent adducts have been reported (Abe et al., 2000; Takebayashi et al., 2007; Osman, 2011).

On the other hand, the hydroxyl radical is very short lived, highly reactive and it is extremely toxic to an organism. It can damage all types of macromolecules. Hydroxyl radicals may be reliably generated using the Fenton reaction, which is the degradation of H_2O_2 in the presence of an excess of Fe^{2+}. This reaction may be conducted both *in vivo* and *in vitro* (Kehrer, 2000). The Fenton reaction also produces high valent iron-oxo species like FeO^{2+}, which have comparable reaction kinetics characteristics when compared with hydroxyl radicals (Yamazaki and Piette, 1990; Burkitt, 1993; Dufield et al., 2004). These ferryl ions ($FeOH^{3+}$) are less diffusible than free hydroxyl radicals and more likely to engage in site-specific and selective oxidation of proteins (Itakura et al., 1994; Dufield et al., 2004). Ferryl ions are able to conjugate to peptides and their stability depends on functional groups. A study of oxidation of natural amino acids by a ferryl complex revealed that thiol-, phenol-, indole- and sulfur-containing side chains are highly reactive with ferryl species (Jabre et al., 2009). Spin traps are molecules that can react with radicals and produce stable radical adducts. The development of spin traps such as DMPO, DEMPO, TMPO, TEMPO, etc have made it possible to trap and quantify ROS in cells. DMPO is the most common spin trap used in testing the antioxidant ability of molecules.

4.4   Methods

4.4.1   Chemicals and Instruments

All solvents were HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted. Analytical grade chemicals, including DPPH$^\bullet$ and DMPO, iron (II) sulfate hydrate ($FeSO_4$) and hydrogen peroxide (H_2O_2), were purchased from Sigma-Aldrich (Oakville, ON). CLs were prepared by reverse phase chromatography of a silica extract of flaxseed oil according to Reaney et al. (2009). EPR was recorded on a Bruker EMX X-band EPR spectrometer (Bremen, Germany). Typical operating parameters for hydroxyl radical quenching assays were as follows: microwave power 1.00 mW, center field 3348.8 G, sweep width 100 G,

conversion time (20.48 ms), time constant 10.240 ms, frequency 9.3 GHz, modulation amplitude 1.5 G, receiver gain 1.42 x10^5, number of scans 7. Each experiment was done in triplicate. A UVC 500 UV cross-linker (Hoefer, San Francisco, CA), which illuminates with 254 nm UV radiation and an energy density of 100 µJ/cm^2, was used for this experiment. CLs, DPPH$^•$ and its products were separated using a Chromolith Fast Gradient RP-18e column (3 µm particle size silica, 50 × 2.0 mm i.d.; EMD Millipore, Bellerica, MA). Mass spectra were recorded using an Agilent HPLC 1200 series directly connected to a Bruker microQTOF-Q II Mass Spectrometer (Hybrid Quadrupole-TOF MS/MS; Bruker) with an Apollo II ESI ion source. The mobile phase consisted of a linear gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (60:40 for 2 min, to 10:90 in 8 min, to 60:40 in 0.5 min, with a hold time of 5.5 min for equilibration prior to the next injection) and a flow rate of 0.40 mL/min.

4.4.2 Hydroxyl Radical Assay

ROS scavenging was measured for CLA, CLB and CLC. The maximum quantity of ROS generated by the Fenton reaction was measured by spin trapping with DMPO. The anti-oxidant abilities of the CLs were measured from a competitive reaction between CLs and DMPO. The first challenges in this experiment were to develop both a protocol for keeping both CLs and DMPO in solution and also to prevent side reactions so that a bimolecular reaction between ROS and DMPO or CLs was the major reaction in the system. The Fenton reaction is typically buffered but due to low solubility of CLs in aqueous solution, a buffered reaction was not used. As shown in Figure 4.1, A is a buffered reaction whereas B is not buffered.

The dissolution of highly hydrophobic CLs was challenging due to their low solubility in the buffered aqueous ROS generating system of the Fenton reaction. CL solutions were prepared at a concentration of 40 µM in acetonitrile solution without any precipitation. In the first experiment, 20 µL of each of the three solutions of 20 mM CLA/acetonitrile, 0.3 M DMPO and 10 mM H_2O_2 were mixed and then added to the same amount of 10 mM $FeSO_4$/H_2O. The EPR spectrum of the DMPO product in 20% acetonitrile solution was obtained. However, some precipitation and blockage was observed in the pipette. Also, the observed EPR intensity of the trapped DMPO was higher than anticipated from the reference. After an optimization process, it was determined that unbuffered solution could be used with a water content that should not exceed 20%.

Hydroxyl radicals (HO$^•$) were generated *via* the Fenton reaction where ferrous sulphate reacts with hydrogen peroxide to produce the hydroxyl radical.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^•OH + OH^- \hspace{3cm} [4\text{-}1]$$

The antioxidant ability of CLs was evaluated by mixing CLs into the Fenton solution. The hydroxyl radical produced rapidly reacts with either the added CLs or DMPO. DMPO traps the hydroxyl radical and forms relatively stable DMPO/OH$^•$ adducts which are detectable by EPR (Harbour et al., 1974). CLs and DMPO solutions were prepared in acetonitrile while H_2O_2 and ferrous sulphate were dissolved in distilled water. The control reaction mixture consisted of 20 µL of 10 mM DMPO, 10 µL of 10 mM H_2O_2, 5 µL of 5 mM ferrous sulphate and 20 µL of acetonitrile, so as to give final concentrations of 3.6 mM DMPO, 1.8 mM H_2O_2 and 0.2 mM ferrous sulphate. Acetonitrile was replaced by 20 µL of CLs solution to provide final concentrations of 1.1 mM, 2.2 mM, 4.4 mM and 8.8 mM in the test sample. The sample (40 µL) was transferred to a capillary tube (60 mm length, 1.1 mm o.d., 0.8 mm i.d. KIMAX-51), then the capillary containing the sample was sealed with Teflon tape (Teflon type-20D01-102, Villa Kuala Lumpur, Malaysia), put into a 0.3 mm quartz standard EPR tube and then scanned by the EPR spectrometer exactly 3 min after ferrous sulphate had been added to the sample. This procedure was applied in all of the following experiments unless otherwise noted.

4.4.3 DPPH$^•$ Scavenging Assay

4.4.3.1 Concentration Dependent Scavenging Activity of Individual CLs

DPPH$^•$ disappearance was determined by measuring the ESR intensity of the DPPH$^•$ reaction mixture relative to a standard DPPH$^•$ solution. The reaction samples were prepared from 40 mM CLs (CLA–CLC) and 1.6 mM DPPH$^•$ stock solutions in acetonitrile. In the reference samples 10 µL of DPPH$^•$ solution was diluted five-fold by acetonitrile, resulting in a final concentration 0.32 mM. The test mixtures were prepared by replacing 10-µL acetonitrile with 0.32–10 µL CLs solution. The final concentrations of CLs were 0.32 mM; 0.64 mM; 1 mM; 2 mM; 4 mM; 8 mM and 10 mM. An aliquot of 40 µL of the test samples was transferred into a capillary tube and sealed with Teflon tape. The EPR spectral intensities of the DPPH$^•$ signal were determined by double integration of the entire spectrum.

4.4.3.2 CLs DPPH$^•$ Radical Scavenging Activity in UV Light

The impact of UV light on radical quenching was investigated by using CLs that contain the UV absorbing residue, tryptophan (CLG and CLG″). Reference and test samples were prepared by the procedure described in the previous section. The final concentration of the reagents CLs and DPPH⁻ were 8 mM and 0.32 mM (molar ratio 25:1), respectively. Six out of 12 samples were placed in a UVC 500 UV cross linker and irradiated for 1 and 3 min, while the remainders of the samples were kept in a dark room for 30 minutes. The DPPH⁻ EPR signal intensities in the mixture were measured 1, 4 and 72 h after UV irradiation.

4.4.4   Data Analysis

WinEPR software (Bruker) was used for EPR data processing. All spectra were baseline corrected and double integrated to obtain areas and intensities that were assumed to be proportional to concentration (Appendix D). The scavenging activities of CLs were calculated using

$$\text{Activity} = [(Ac - As) / Ac] \times 100\%$$

Where: As = double integrated EPR spectra area (D/N) of the reaction mixture; Ac = Control experiment's area (DPPH⁻). The average of triplicate samples is presented.

4.5   Results and Discussion

4.5.1   Hydroxyl Radical Assay

EPR spectroscopy directly detects paramagnetic species. When a transient free radical is present in the system, it reacts with a diamagnetic molecule (such as the spin trap) forming a stable free radical product (the spin adduct). Hence the spin adduct is paramagnetic and EPR active. In the presence of the DMPO spin trap the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$) produces a spin adduct with an EPR signal that is a 1:2:2:1 quartet with hyperfine coupling parameters $a(^{14}N) = a(^{1}H) = 14.9$ G (Burkitt, 1993) (Figure 4-1). The effect of antioxidants on the EPR signal intensity of the DMPO/HO⁻ can be revealed by a dose-dependent or time dependent decrease in the signal. They may donate an electron or hydrogen to the DMPO/HO⁻ spin adducts or scavenges hydroxyl radicals by competing with the spin trap (Figure 4-2).
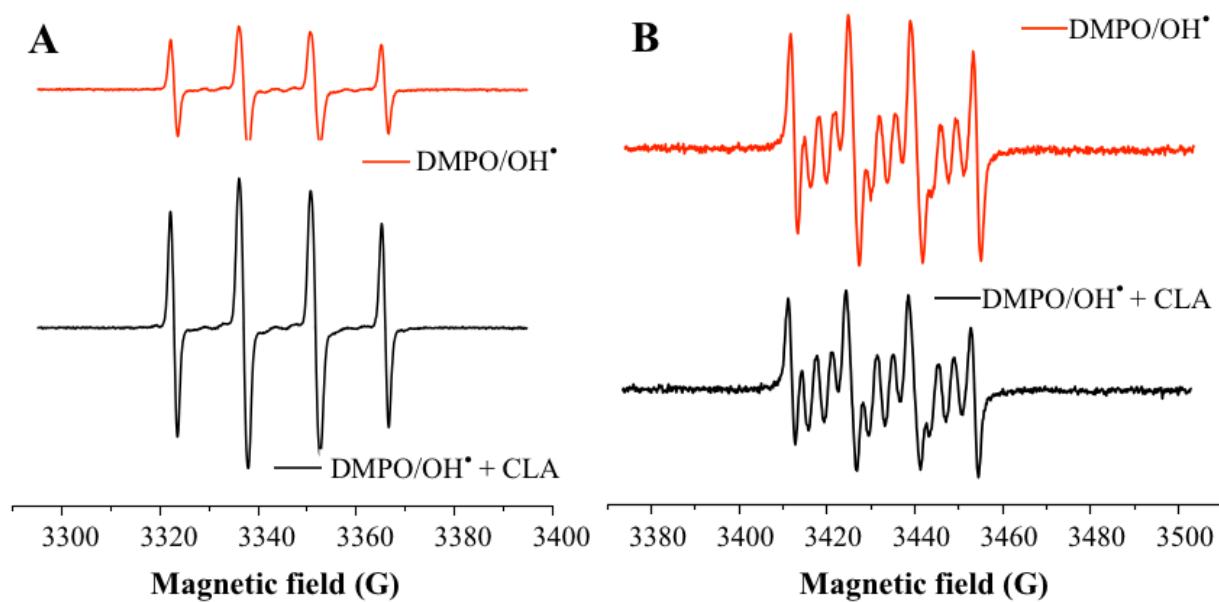
Figure 4-1 Representative EPR spectra of free radicals formed by the Fenton reaction. A, with buffer solution (0.075 M DMPO, 2.5 mM H_2O_2 and 2.5 mM ferrous sulphate in air at room temperature), B, without buffer solution. g = 2.005 and the hyperfine of the main components remained at 14.9 Gauss.
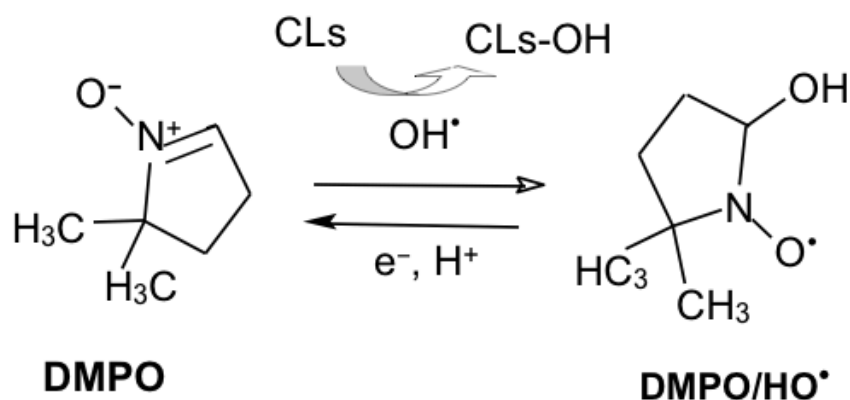

Figure 4-2 Possible effects of CLs on EPR signal intensity of DMPO/HO.

On the other hand, they may form stable radicals or affect the production of hydroxyl radicals by reacting with reagents in the Fenton reaction (Burkitt, 1993). The data presented in Table 4-2 show that CLs reduced the observed EPR signal intensity of DMPO/HO$^{\bullet}$ in a dose-dependent manner. Overall, the CLs were able to affect DMPO/HO$^{\bullet}$ production up to 38% depending on peptide concentration and reactivity.

The effect of linear peptides on hydroxyl radical production by the Fenton reaction has been studied previously. However, data regarding the scavenging activities of plant-derived purified peptides against HO$^{\bullet}$ are quite rare and no report of the activity of cyclic peptides has been reported. Most published studies to date discuss the activity of hydrolysate fractions. Overall, compared to those reports, scavenging properties of CLs against hydroxyl radicals are low. For instance, a hempseed fraction, having 20% scavenging at a concentration of 1 mg/mL, was considered a poor scavenger of hydroxyl radicals compared to an alfalfa leaf protein fraction having 80% scavenging activity at 1.2 mg/mL. In the present work, addition of 5 mg/mL of CLs reduced the EPR signal of DMPO/HO$^{\bullet}$ adduct by 20–30%. Comparison of plant-derived protein fractions with CLs is further complicated as these fractions may contain other plant antioxidants. In addition, most of studies utilized spectrophotometric assays, whereas the current study describes the use of EPR to determine the hydroxyl scavenging activity (Zhang et al., 2007; Sampath Kumar et al., 2011; Comfort et al., 2011).

The CLs amino acid profiles for CLA [*cyclo*-(Pro-Pro-Phe-Phe-Leu-Ile-Leu-Val)], CLB [*cyclo*-(Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile)] and CLC [*cyclo*-(Pro-Pro-Phe-Phe-Val-Ile-Mso-Leu-Ile)] are well established. Suetsuna (2000) reported that leucine at the C-terminal of a linear amino acid is important for antioxidant activity. C-terminal Phe and Tyr also supported antioxidant activity. Moreover, N-terminal hydrophobic amino acid residues such as Val, Leu, Ile, Ala and Phe enhanced hydroxyl-scavenging activity (Kawashima et al., 1979; Suetsuna et al., 2000; Guo et al., 2009). Kawashima et al. (1979) clarified the importance of branched chain amino acids such as Ile, Leu, Val and Met in antioxidant activity of peptides and also stressed the importance of their location at the N terminal position. However, they reported that a carboxylic acid group is not essential for antioxidant activity. Moreover, protecting the N terminal of the peptide using an amidation reagent had a strong negative effect on its antioxidant activity (Kawashima et al., 1979). Although CLs have a high content of branched-chain amino acid residues, including Leu, Ile and Val, their lower ability to inhibit hydroxyl radicals may be due to

their cyclic structure. There is no free N-terminal hydrogen. In addition, the charged acidic amino acid residue is considered to be important for hydroxyl radical scavenging in the synthetic antioxidant peptide Tyr-Phe-Tyr-Pro-Glu-Leu (Suetsuna et al., 2000). The lack of charged residues may also limit the activity of CLs toward hydroxyl radicals.

Proline is one of the more active residues in antioxidant peptides. The pyrrolidine ring protects the secondary structure of the peptide, imposing conformational constraints and making amino acids more available to interact with free radicals (Rajapakse et al., 2005; Alemán et al., 2011). Specific amino acid sequences have shown higher antioxidant activity than would be expected from the constituent amino acids alone (Elias et al., 2008). Therefore, the secondary structure of CLs is another factor that likely determines antioxidant activity.

Furthermore, just as hydrophobicity is important in the antioxidant activity of linear peptides, molecular weight and degree of hydrolyses (DH) also affect peptide antioxidant activity. Superior antioxidant peptides typically contain 4-16 amino acid residues and molecular weight ranges between 0.5 and 1.5 kDa (Pownall et al., 2010; Zhong et al., 2011; Sampath Kumar et al., 2011; Ajibola et al., 2011; Comfort et al., 2011). For instance, digestion of silver carp protein followed by separation based on molecular size generates antioxidant peptide fractions (>10 kDa; 5–10 kDa; 3–5 kDa; 1–3 kDa and < 1 kDa), which are able to diminish the spin intensity of the signal due to HO$^{\bullet}$ by 15.6%, 25.1%, 50.4%, 55.7% and 75.4%, respectively. However, amino acid profiles of these fractions were similar, but the strongest anti-free radical fraction (< 1 kDa) had higher contents of Leu, Tyr and Pro than the other four fractions (Zhong et al., 2011). Molecular size of the CLs in this study is favorable for antioxidant activity. The bulky side chain groups of the peptides are believed to enhance radical scavenging ability. His (imidazole group), Trp (indole group) and Tyr (hydroxyl group) act as hydrogen donors when present as amino acid residues. Aromatic amino acid residues (Tyr and Phe) are, however, able to provide protons to electron deficient radicals and keep their structure by resonance (Jung et al., 2005). According to these statements, Pro-Pro-Phe-Phe sequences of CLs could be involved actively in the interaction with the Fenton system. In addition, Hawkins and Davies (1998) noticed that hydroxyl radical (Ti^{3+}/H$_2$O$_2$) preferably attacks amino acid residues with larger chains due to steric hindrance.

Transition metal ions that have unpaired electrons can initiate and accelerate lipid oxidation. Peptides are known metal chelaters, thus when evaluating antioxidant properties of

peptides, their metal chelating activity should also be measured in parallel. Zein protein hydrolysates (Kong and Xiong, 2006), peanut peptides (Zhang et al., 2011), soybean peptides (Chen et al., 1998) and yoghurt peptides (Farvin et al., 2010) may chelate metals. Nevertheless, the correlation between metal-ion chelating activity and antioxidant activity is poor (Chen et al., 1998; Zhang et al., 2008). Therefore, a decrease in the intensity of the observed EPR spectrum for DMPO/HO$^\bullet$ may due to ferrous ion interaction with antioxidant peptides but it is unlikely to be a major effect. In this study the metal chelating activities of given peptides were not tested, although CLA is known to bind the divalent metals Ba^{2+}, Mg^{2+} and Ca^{2+} as well as others including K^+, Na^+, Tb^{3+} and Pr^{3+} (Siemion 1977; Tancredi et al., 1991; Chatterji et al., 1987). In addition, Phe residue participation in metal binding was revealed by NMR (Chatterji, et al., 1987). CLB (CLC was not tested at lower than a concentration of 2 mM) was able to interact with the Fenton system at a lower concentration than CLA, indicating participation of the methionine group in inhibition of DMPO/HO$^\bullet$ signal intensity. Moreover, there was a slight difference between methionine (CLB) and methionine sulfoxide (CLC) containing peptides in hydroxyl radical scavenging activity. This trend was consistent with a study that compared methionine to methionine sulfoxide (Table 4-3) (Unnikrishnan and Rao, 1990).

Levine et al. (1996) proposed that methionine acts as an endogenous antioxidant that protects structural amino acid residues from oxidation. Methionine residues in peptides may be selectively attacked by ROS and converted to methionine-sulfoxide, often with little change in structure and activity. The oxidation product methionine-sulfoxide can be reduced by the methionine sulfoxide reductase (Shechter et al., 1975; Levine et al., 1996). Overall, scavenging activity of given CLs against hydroxyl radicals is not strong compared to other compounds. Furthermore, the reaction mixture of the Fenton reaction and CLs was directly injected into an ESI-MS mass spectrometer. The spectra did not show any new mass fragments in addition to the CLs (Figure 4-3).

Thus, the decrease in EPR signal intensity of DMPO/HO$^\bullet$ may be due to weak interactions between one of the reagents of the Fenton reaction with the CLs. These complexes are likely decomposed during ESI-MS. On the other hand, oxo-iron species could interact with CLs.   During the Fenton reaction metal-bonded hydroxyl radical is formed in addition to free hydroxyl radicals (Equations 4-1 and 4-2) (Koppenol, 1985).

Table 4-2   Concentration-dependent quenching activity of CLs against OH$^{\bullet}$

CL	Hydroxyl scavenging activity (%) ± SEM		
	Concentration mM		
	2.2	4.4	8.8
CLA	12.2 ± 0.2	30.0 ± 1.2	29.6 ± 2.4
CLB (methionine)	ND[1]	24.1 ± 0.9	38.1 ± 0.8
CLC (methionine sulfoxide)	19.4 ± 0.2	26.0 ± 0.6	38.4 ± 0.7

[1]ND: not detected.

Table 4-3   Hydroxyl radical scavenging activity of methionine and its oxidation products

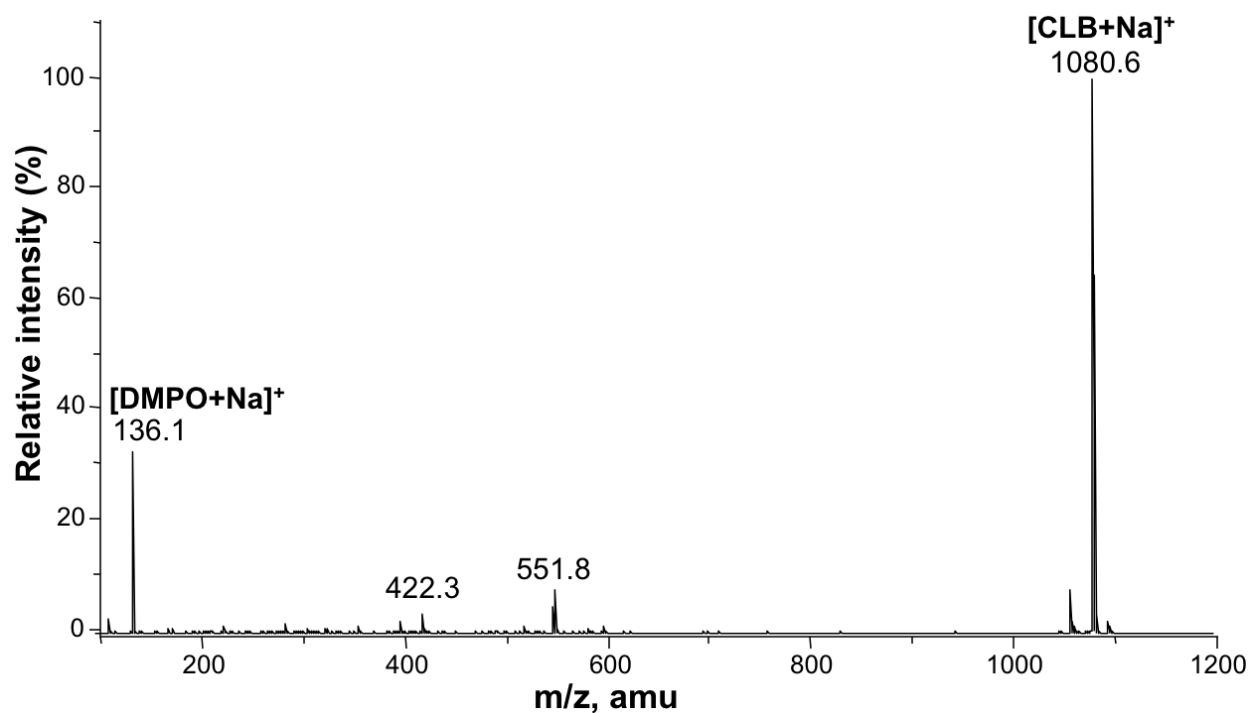| Amino acid	Hydroxyl scavenging activity (%) ± SEM		
	Concentration (mg/mL)		
	0.2	0.4	0.8
Methionine	42.7 ± 0.2	57.1 ± 0.9	87.6 ± 1.4
Methionine sulfoxide	36.8 ± 0.2	57.2 ± 0.6	84.4 ± 0.7

Figure 4-3　Electrospray mass spectrum of acetonitrile solution of Fe^{2+}, H_2O_2, DMPO and CLB.

Table 4-4　Summary of some amino acids composition of various protein products showing antioxidant activity

Source	Fraction	Amino acid content (%)							Quenching%	
		Phe	Trp	Pro	Met	Cys	His	HAA[1]	OH•	DPPH•
Rapeseed[2]	RP55	8.3	2.0	10.7	2.0	2.2	2.2	47.2	39.1	89.9
	RP25	4.1	1.4	6.6	2.7	1.0	2.6	32.5	45.3	25.2
African yam bean seed[3]	< 1 kDa	7.3	1.2	0.5	1.1	0.3	3.0	42.9	28.2	40.3
	1–3 kDa	6.3	0.8	1.4	0.9	0.5	3.3	40.0	20.2	38.1
Pea seed[4]	F3	8.7	0.7	5.1	0.9	0.4	1.9	48.9	12.5	13.1
	F4	12.1	1.4	5.4	1.1	0.3	1.9	55.0	15.0	17.6

[1]HAA- hydrophobic amino acids

[2-4]Referenes; [2]Zhang et al. (2008), [3]Ajibola et al. (2011), [4]Pownall et al. (2010).

$$Fe^{2+} + H_2O_2 = Fe^{3+} + OH^\bullet + OH^-$$ [4-2]

$$Fe^{2+} + H_2O_2 = FeOH^{3+} + OH^\bullet$$ [4-3]

Ferryl ions ($FeOH^{3+}$) diffuse more slowly than free hydroxyl radicals and are more active in site-specific and selective protein oxidation (Itakura et al., 1994; Dufield et al., 2004). Ferryl ions are able to conjugate to peptides and their stability depends on functional groups (Jabre et al., 2009). The high-valent iron (IV)-oxo species, metal-based oxidants play a key role in biological oxidation reactions including the catalytic cycle of heme iron enzymes such as cytochrome P450. Iron (IV)-oxo species also have been identified as intermediates in several catalytic cycles. Studies of oxidation of natural amino acids by a ferryl complex revealed that thiol-, phenol-, indole- and sulfur-containing side chains react with ferryl species. Moreover, the ferryl complex reacts more rapidly with the amino acid side chain than the amide backbone. The oxidation of Cys, Tyr, Trp and Gly by ferryl complex involves the loss of hydrogen atoms, whereas, oxidation of Met was associated with O atom transfer (Ahmed et al., 2009). Matrix-assisted laser desorption ionization (MALDI) mass spectrometry revealed that CLA was able to weakly bind Fe^{2+} while CLC cannot bind with ferric ions (unpublished data). The enhanced scavenging activity of CLA over CLB and CLC and concentration-dependent scavenging activity of CLB and CLC might be explained by their ability to coordinate oxo-iron species in the Fenton system.

In Table 4-4, the free radical scavenging activities of digested protein fractions are compared. These data indicate that the scavenging properties against hydroxyl and $DPPH^\bullet$ of given peptide fractions were slightly different. However, increased scavenging activities of some fractions against hydroxyl radicals were positively correlated with the content of hydrophobic amino acid (HAA) and inversely correlated with peptide size. Methionine residues strongly influenced the availability of hydroxyl radicals. In contrast, methionine has no effect on $DPPH^\bullet$ indicating that ferryl ions are involved in the oxidation, and not the more diffusible and less selective hydroxyl radicals. Moreover, the interaction of CLA with the Fenton reaction in the presence and absence of phosphate buffer is complex. In phosphate buffer, CLA acted as a catalyst in the Fenton reaction (Figures 4-4 and 4-5). A similar trend was observed with this experiment, enhanced production of hydroxyl radicals, when Fe complex is formed with ATP or pyrophosphate, or citrate or phosphate buffer. In phosphate buffer, the reaction rate was lower than in those with other chelators because of the instability of ferric phosphate (Sutton, 1985).
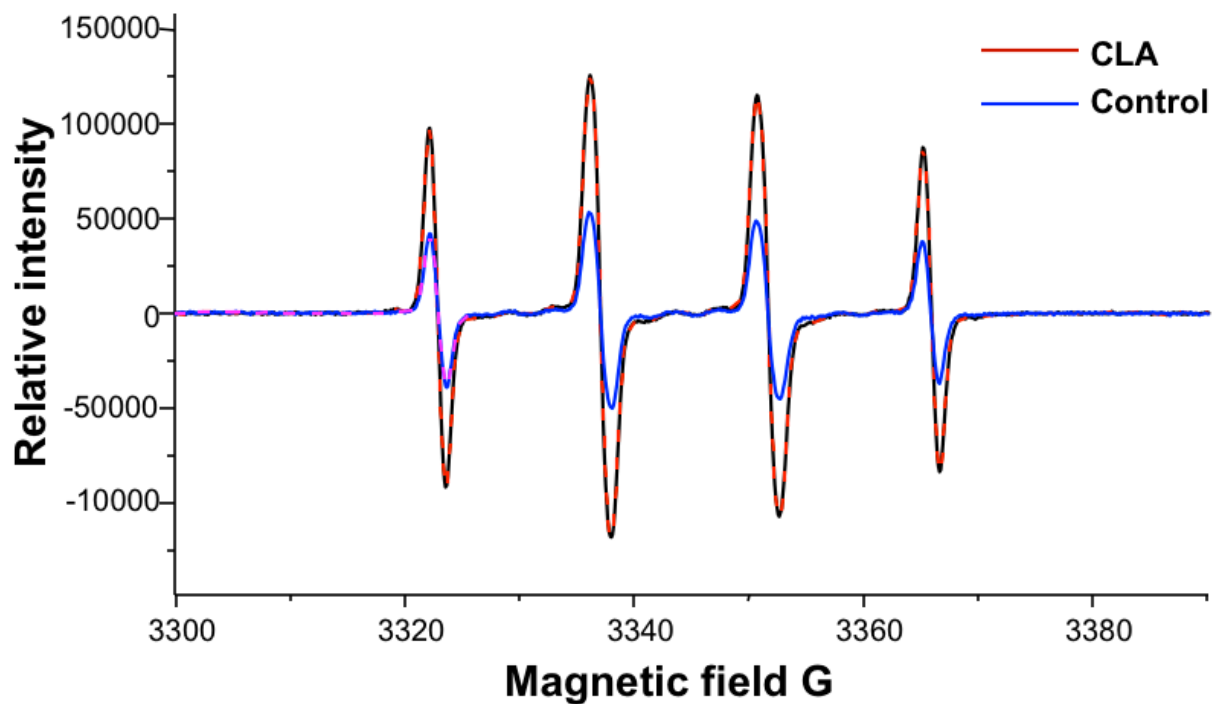
70

Figure 4-4 OH$^\bullet$ production by the Fenton reaction in the presence CLA in phosphate buffer. Condition, center field 3438.24 G, sweep with 100 G, conversion time (327.68 ms), time constant 163.84 ms, MW frequency 96357 GKHz, modulation amplitude 1 G number of scans 1 (DMPO: H$_2$O$_2$: CLA = 4:1:2).
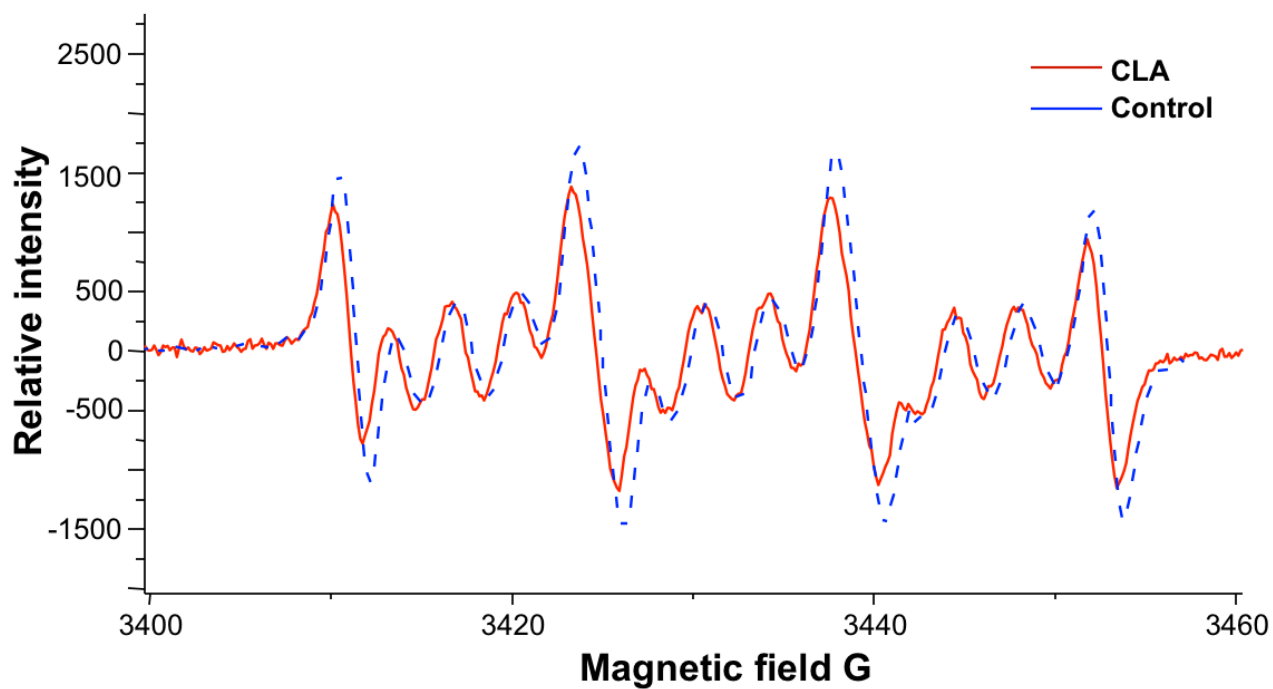
Figure 4-5   OH$^\bullet$ production by the Fenton reaction in presence CLA without the phosphate buffer. Condition, center field 3438.24 G, sweep with 100 G, conversion time (327.68 ms), time constant 163.84 ms, MW frequency 96357 GKHz, modulation amplitude 1 G number of scans 1 (DMPO: H$_2$O$_2$: CLA = 4:1:2).

CLA may increase ferric phosphate stability and diffusibility as the signal intensity of control samples is almost half-fold that of the CLA containing sample. This indicates a strong positive effect of CLA on hydroxyl radical production. Moreover, in comparison with uncomplexed ion, chelating of Fe^{2+} with oxygen donors reduces the redox potential of Fe^{3+}/Fe^{2+} making reactions thermodynamically more favoured. Also, electron transfer may be delayed due to the stearic effect that is forced upon the ferrous ion complex by the ligands and prohibit intermediate or unnecessary side reactions (Winterbournb and Sutton, 1986; Todolini, 1989; Rush et al., 1990). Since CLA is able to bind Na^+ (Tancredi et al., 1991), it might enhance production of a complex of ferric ions and hydroxyl radicals by sodium cation binding. On the other hand, the loss of its quenching properties could be linked to blockage of the ferric ion-binding site by sodium. In addition, cyclic peptides with amino acid residues containing side-chain substituents such as imidazole, carboxylate or thioether groups can coordinate to metal ions in a similar way to the coordination sites in enzymes (Isied et al., 1982; Arena et al., 1987).

4.5.2 DPPH˙ Assay

DPPH˙ is routinely used commercially as a stable radical for free radical research. It produces a characteristic EPR spectrum due to its paramagnetic properties (Figure 4-6). Once free radical scavengers interact with DPPH˙, the EPR signal intensity decreases (Figure 4-7), forming a non-detectable complex.

In the present work, dose dependent interactions of CLA–CLC, CLG and CLG" with DPPH˙ were determined over time. This work was designed to clarify previous findings and possibly determine the mode of action of CLs as free radical scavengers.

4.5.2.1 CLA

When CLA was incubated with DPPH˙ in the dark no quenching was observed (data not shown). Figure 4-8 shows DPPH˙ scavenging of CLA at various concentrations. CLA was able to scavenge DPPH˙ in a dose dependent manner and 50% DPPH˙ present were quenched at 8.0 mM concentration after 20 min UV irradiation.

4.5.2.2 CLB

In contrast to CLA, CLB reacted with DPPH˙ in the dark (Figure 4-9), indicating that CLB is more active than CLA. The rate of reaction was slow and after 48 h only 20% of DPPH˙ was scavenged.
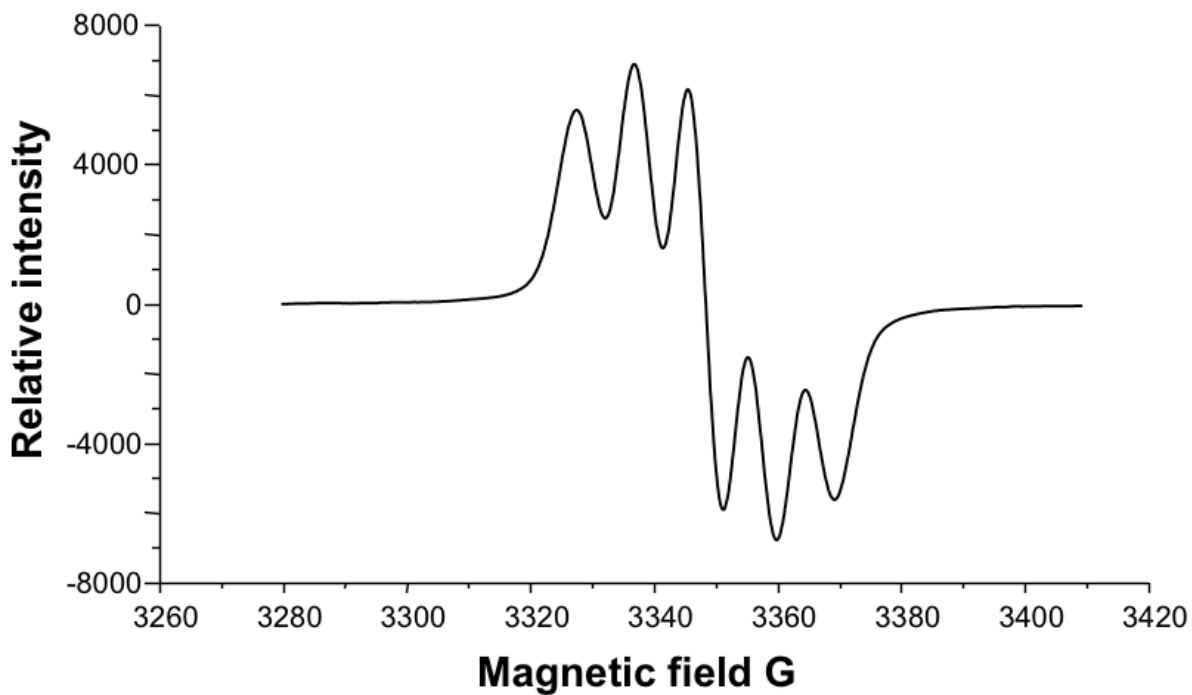
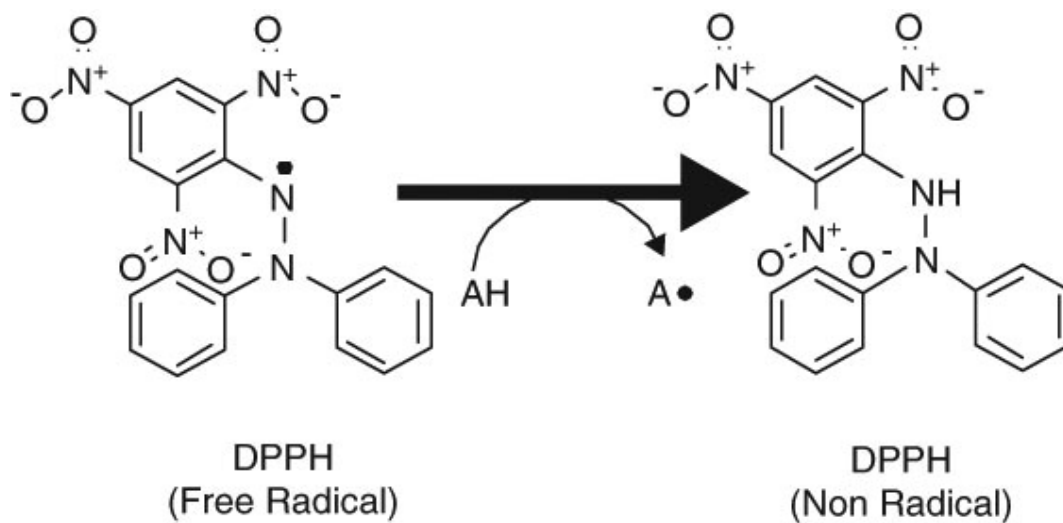**Figure 4-6** EPR spectrum of the DPPH$^\bullet$ in acetonitrile.

DPPH
(Free Radical)

DPPH
(Non Radical)

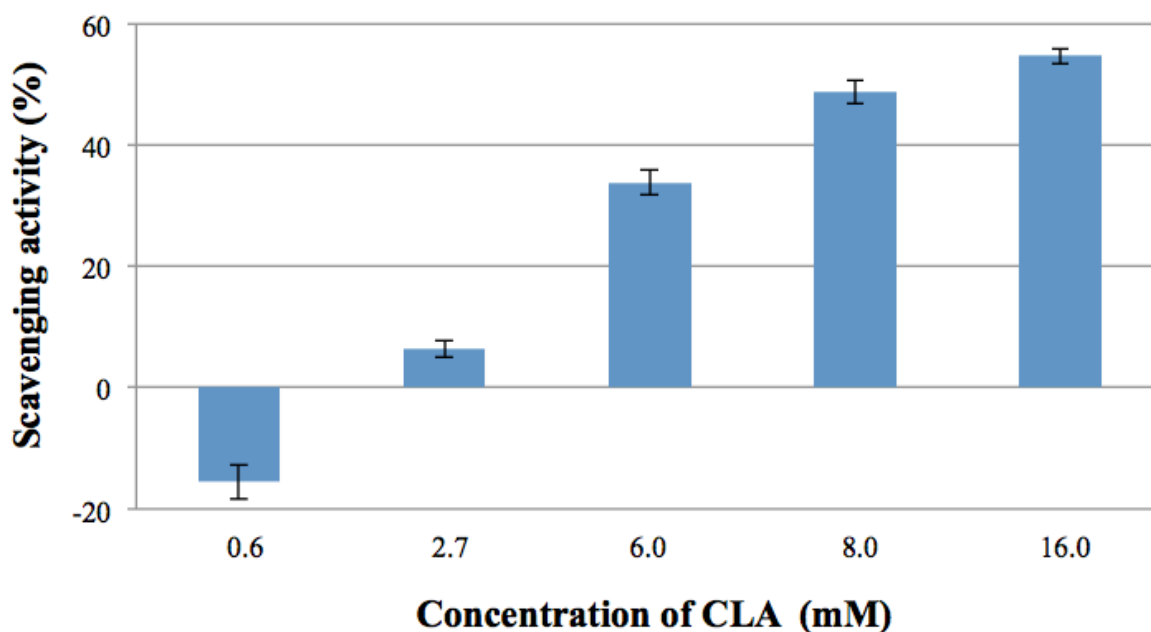**Figure 4-7** DPPH$^\bullet$ antioxidant reaction.

Figure 4-8   Concentration-dependent quenching activity of DPPH$^\bullet$ by CLA in UV light. Values expressed as mean ± standard deviation (SD) of triplicate determinations.
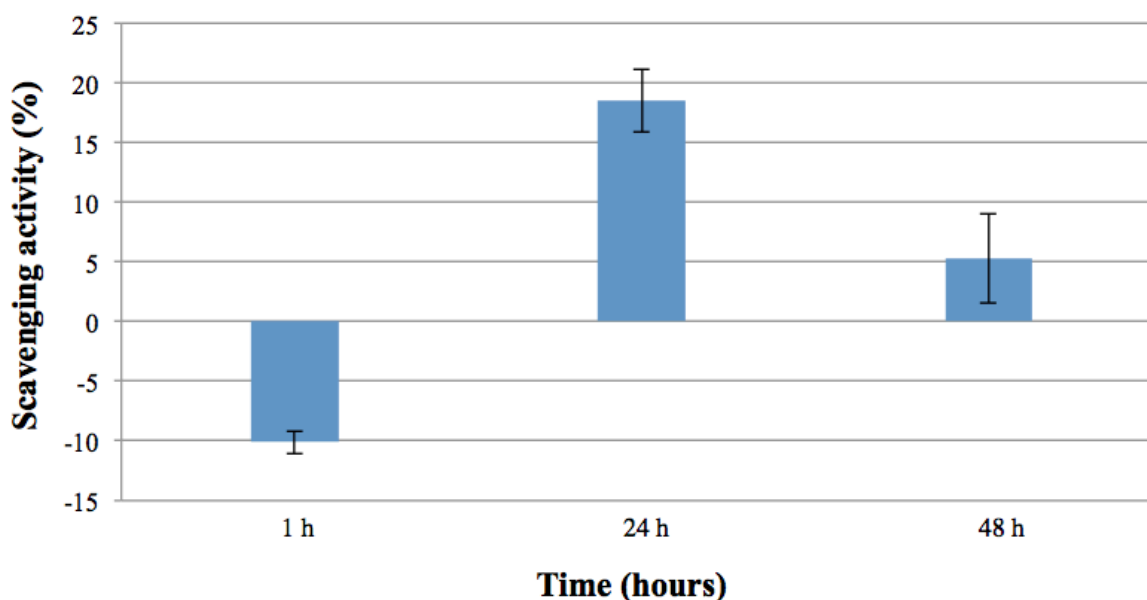

Figure 4-9   Interaction CLB with DPPH$^\bullet$ in the dark. A solution of CLB (4 mM) and DPPH$^\bullet$ (1 mM) were dissolved in CH_3CN solution and stirred (molar ratio 4:1). Values expressed as mean ± SD of triplicate determinations.

To accelerate the scavenging process the reaction mixture was irradiated with UV light for five minutes. The spectra and spectral intensities of this study are presented in Figure 4-10. The EPR signal intensity of the DPPH$^\bullet$ was inhibited in presence of CLB and with 5 min irradiation with UV light in a dose-dependent fashion. Under these conditions, CLB quenched 50% of DPPH$^\bullet$ at a concentration of 4 mM.

4.5.2.3 CLC

CLC, containing methionine sulfoxide, possesses stronger antioxidant activity against DPPH$^\bullet$ compared to the previous two peptides without UV activation (Figure 4-11 and 4-12). However, CLC started to quench DPPH$^\bullet$ after 24 h. The concentration-dependent scavenging effect of DPPH$^\bullet$ was limited (Figure 4-13). At 24 h, a CLC solution (4 mM) scavenged 20% of DPPH$^\bullet$, while CLB showed the same activity after 48 h.

4.5.2.4 CLG

Figure 4-13 shows the interaction of CLG with DPPH$^\bullet$. DPPH$^\bullet$ was quickly quenched at 4 mM concentration one hour after mixing the reagents and holding the samples in the dark. The scavenging rate of CLG increased at concentrations between 1 and 4 mM, but little reaction was observed at higher concentrations.

4.5.2.5 CLG″

CLG″ quenching behaviour was similar to that of CLG, indicating that the Trp residue (indole group) of the peptide is playing a prominent role in the observed scavenging or quenching process (Figure 4-14). The impacts of specific amino acids on CL radical scavenging activities of DPPH$^\bullet$ were investigated. α-Tocopherol, which reacts rapidy with DPPH$^\bullet$,was tested as a positive control under the current reaction conditions. The reaction rate was not observable with the current apparatus. In contrast, CL reactions with DPPH$^\bullet$ were slower and could be activated by UV absorption. This observation is similar to that of Chen et al., who reported that 50 μM δ-tocopherol scavenged 32% of DPPH$^\bullet$, while His-containing antioxidant peptides were mostly inactive in the same assay.

4.5.2.6 Effect of UV Irradiation On CL Scavenging Activity

UV irradiation activated the free radical-scavenging ability of CLA and the methionine containing peptide (CLB), but had no influence on tryptophan containing peptides (CLG and
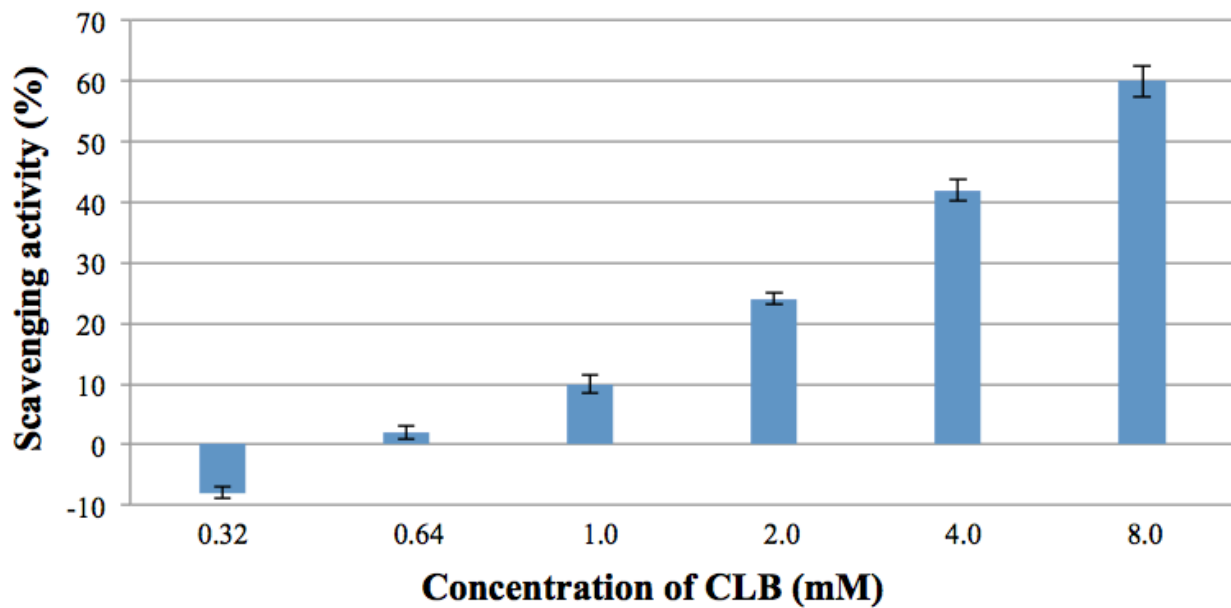
Figure 4-10 Concentration and UV light-dependent CLB scavenging of DPPH$^{\bullet}$. Values expressed as mean ± SD of triplicate determination.
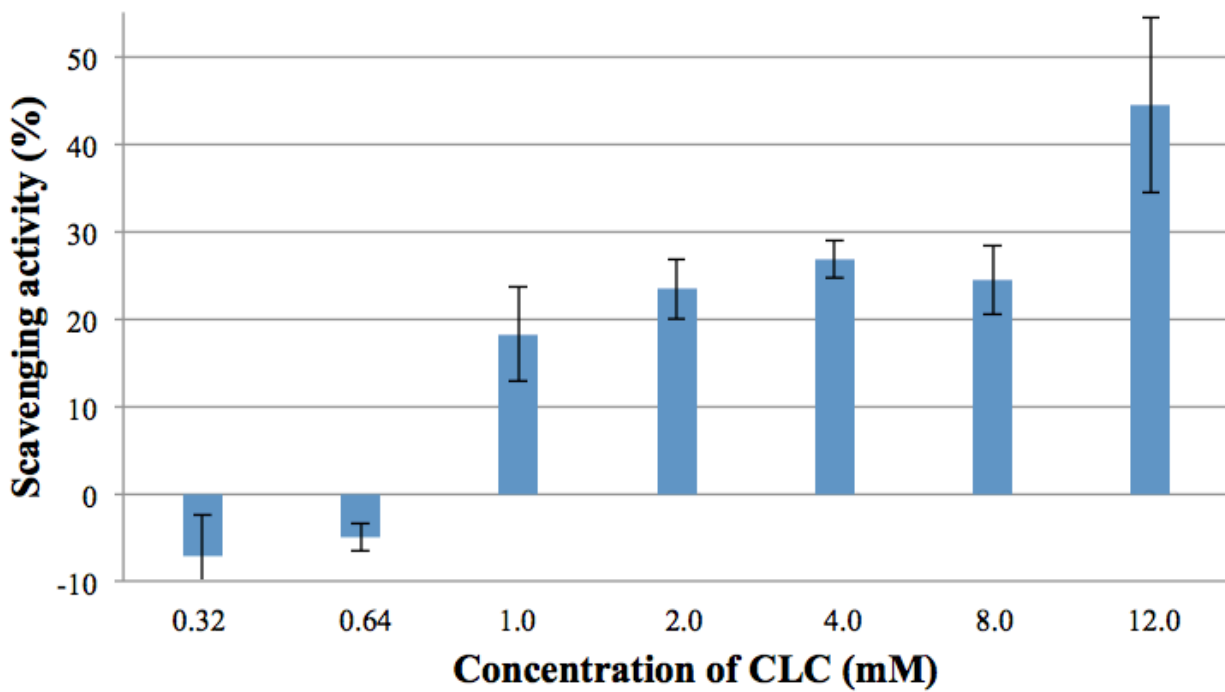

Figure 4-11 CLC scavenging activity against DPPH$^{\bullet}$ after an hour in the dark. Values expressed as mean ± SD of triplicate determinations.
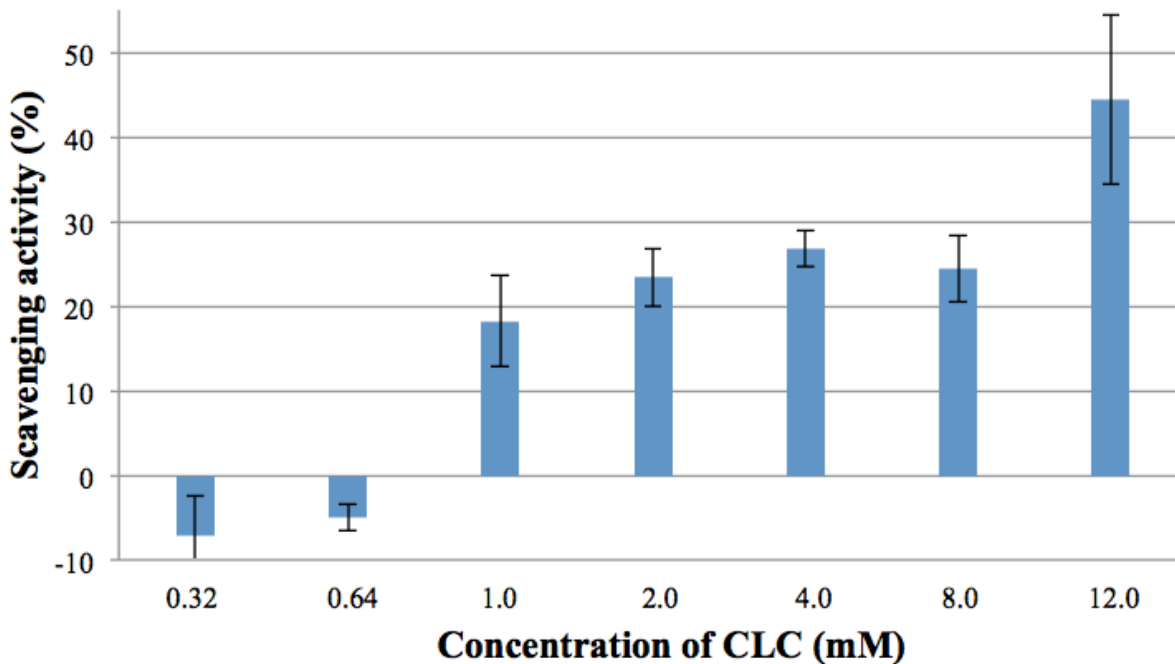
Figure 4-12 CLC scavenging activity against DPPH$^{\bullet}$ after 24 h in the dark. Values expressed as mean ± SD of triplicate determinations.
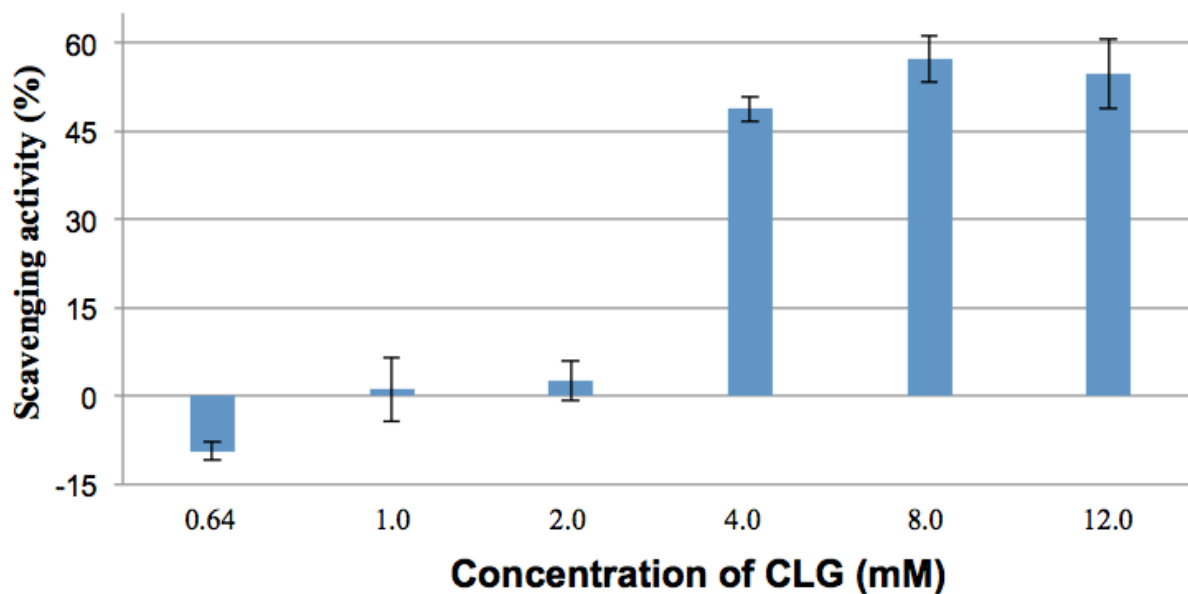

Figure 4-13 DPPH$^{\bullet}$ scavenging properties of CLG. EPR an hour after preparation of the samples. Values expressed as mean ± SD of triplicate determinations
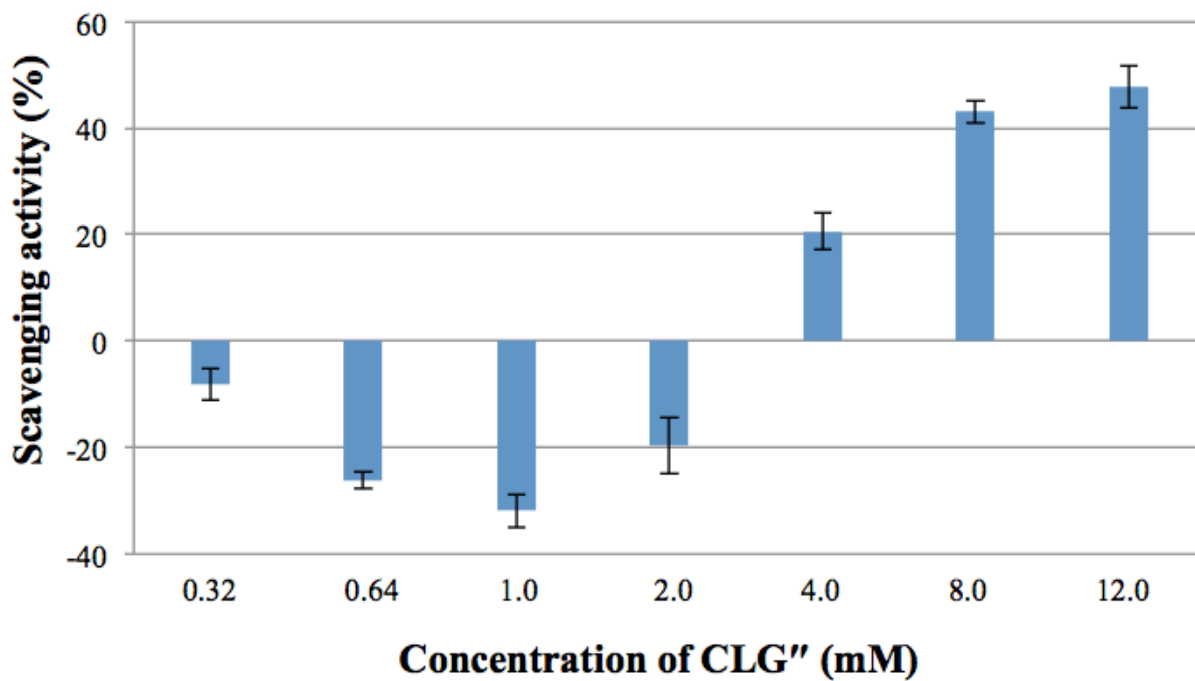
Figure 4-14 Concentration-dependent scavenging activity of CLG″ on DPPH•. The final concentration of DPPH• was 0.32 mM. Spectra were obtained 30 min after preparation of the reaction mixture. Values expressed as mean ± SD of triplicate determinations

CLG″) (Figures 4-15 to 4-19).

Photo-induction of amino acids and energy transfer within molecules has been reported previously (Koch et al., 1962; Casteleijn et al., 1964; Burke and Augenstein 1969). For instance, Burke and Augenstein (1969) determined the effect of UV on the enzymatic activity of trypsin and its constituent amino acids. Results showed that damage to different amino acids in trypsin was dependant on the location within the molecule. In an amino acid mixture, UV irradiation destroys tyrosine, tryptophan and cysteine, whereas in trypsin the indole moiety was not lost even though 90% of enzyme activity was destroyed. Among the six constituent cysteine residues in trypsin, three were destroyed at a higher rate compared to the remaining three (Burke and Augenstein, 1969). The sensitivity of CLB and the stability of indole in CLG″ to UV light irradiation also support these observations. Casteleijn et al. (1964) observed protection of the aliphatic chains by aromatic residues of peptide molecules in the presence of UV radiation, with the indole groups being more active than phenyl groups (Casteleijn et al., 1964). Therefore, the decreased effect of sulfur groups on UV-light-enhanced quenching in peptides containing indole groups (CLG and CLG″) (Figure 4-18 and 4-19) is in agreement with this study.

CLG and CLG″ have two UV chromophores absorbing UV wavelengths longer than 240 nm, namely indole and phenyl. It is likely that most energy is absorbed by indole groups and that methionine groups are protected from activation. Others have reported that energy may be transferred to acetonitrile (solvent), which is able to absorb energy and alter the rate of energy transfer from indole moieties to other structures (Valeur et al., 1992; Eden et al., 2003).

The effect of radiation on amino acids and proteins is well studied, particularly related to the study of damage to proteins by radiation. When peptides are irradiated, they form carbon-centered radicals (Casteleijn et al., 1964; Collins and Grant, 1969; Meybeck and Windle, 1969), as well as various sulfur radicals (Henriksen, 1962; Fessenden and Neta, 1971; Saxebol and Herskedal, 1975).

Sulfur radicals give a very broad spectrum at room temperature. Therefore, it will be difficult to prove the existence of such radicals by EPR. In addition, the principal g of the majority of sulfur radicals is around 2 (Saxebol and Herskedal, 1975) while the g value of DPPH˙ is 2.03. Therefore, peptide-derived radical signals in EPR spectra may be unseen if they overlap with the strong DPPH˙ signal.

The absorption of a photon of light can be used not only for providing activation energy

Figure 4-15 CLA scavenging activity against DPPH$^{\bullet}$: EPR spectra were recorded after UV irradiation. Values expressed as mean ± SD of triplicate determinations (concentrations of the reagents were 8 mM CLs and 0.32 mM DPPH$^{\bullet}$).

Figure 4-16 CLB and CLC scavenging activities against DPPH$^{\bullet}$. EPR spectra were recorded after UV irradiation. Values expressed as mean ± SD of triplicate determinations.

Figure 4-17 Time-dependent scavenging activities of CLB and CLC against DPPH$^\bullet$ in the dark. Values expressed as mean ± SD of triplicate determinations.
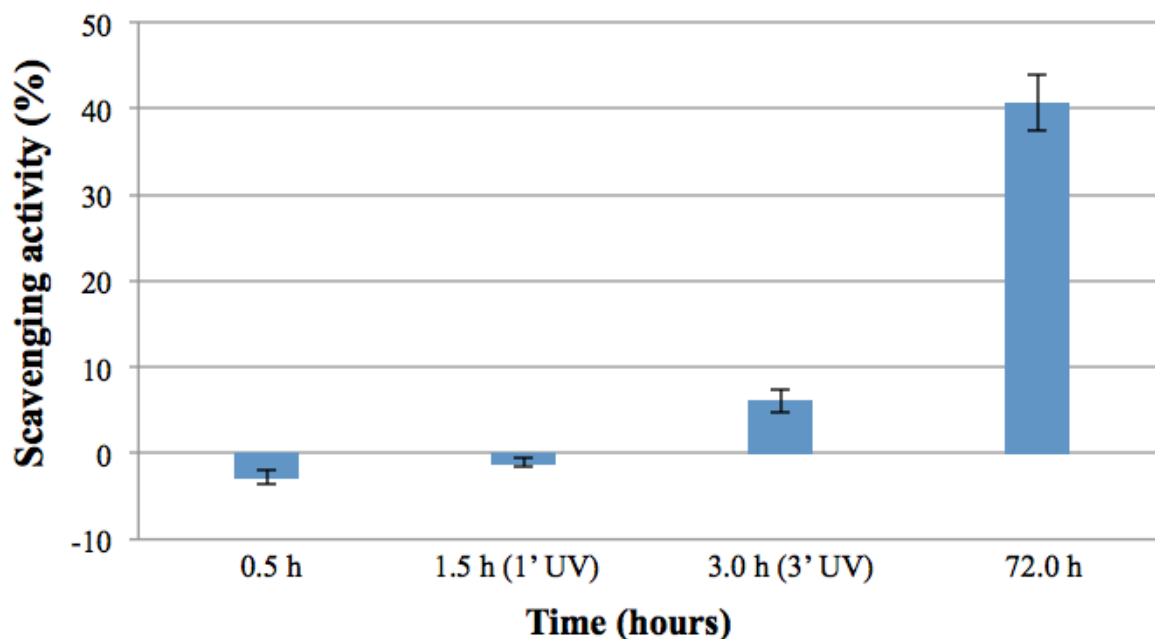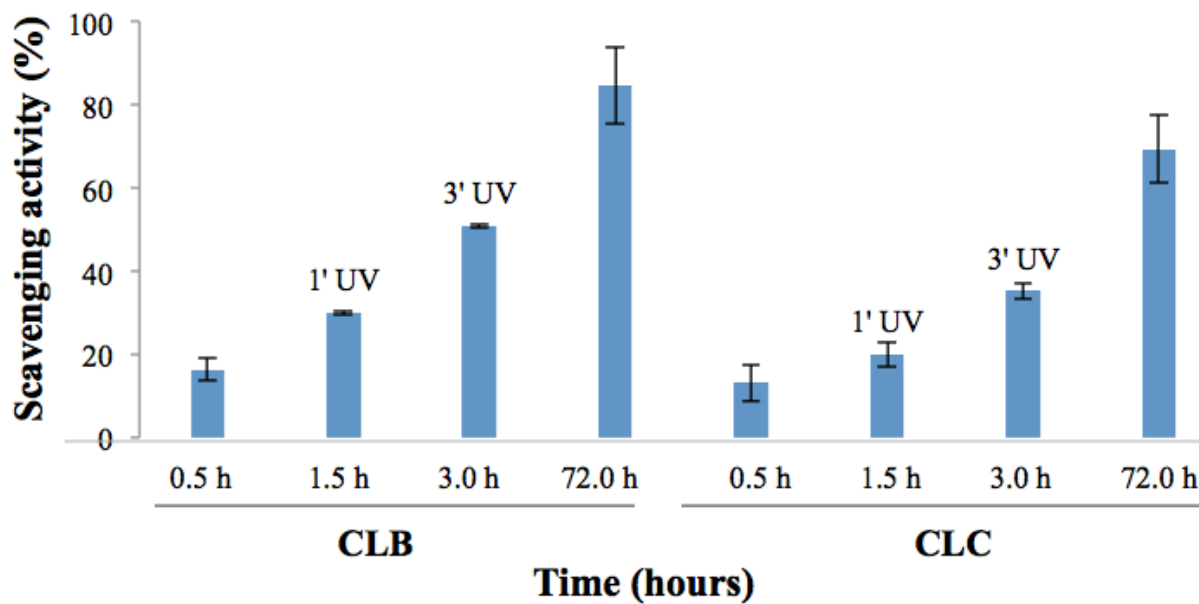

Figure 4-18 CLG and CLG″ scavenging activities against DPPH$^\bullet$. EPR spectra were recorded after UV irradiation. Values expressed as mean ± SD of triplicate determinations.
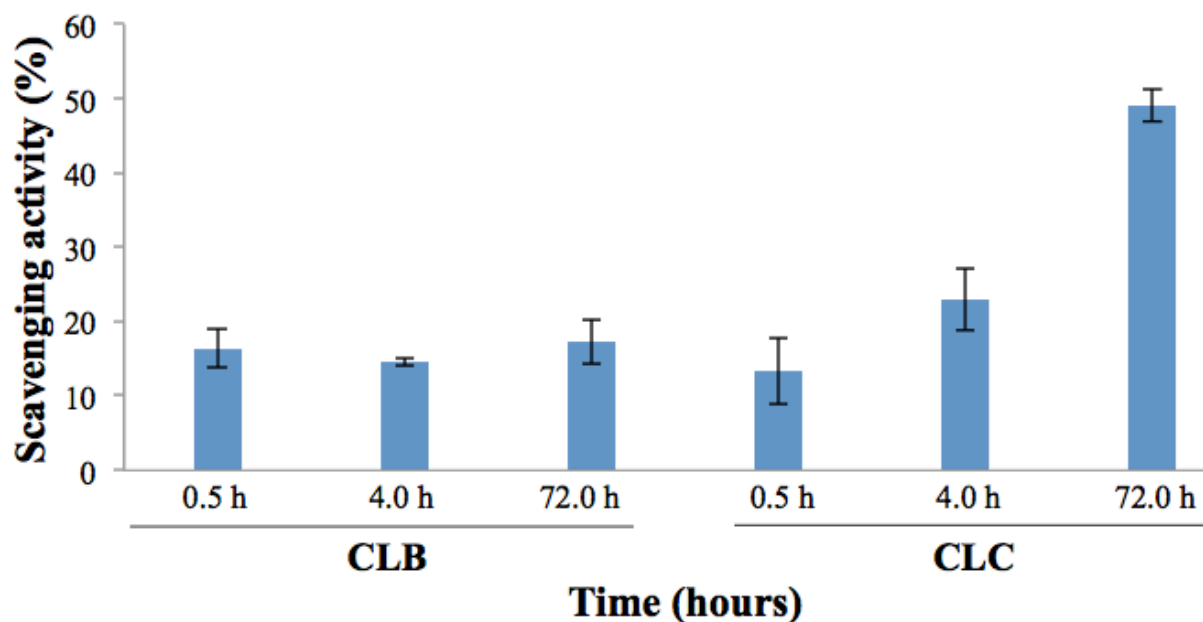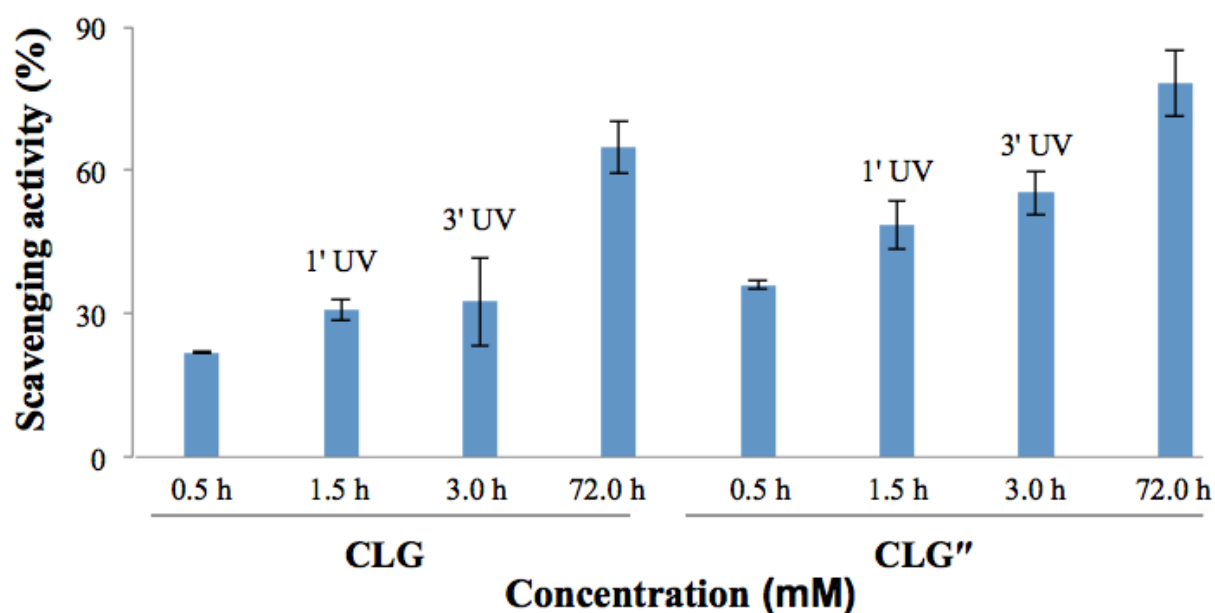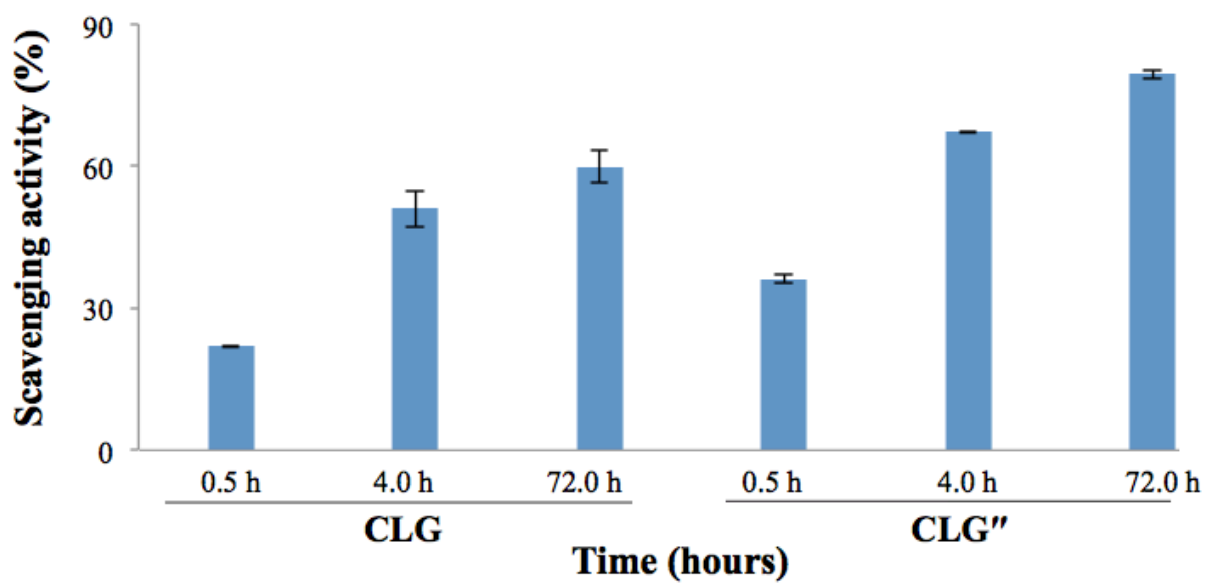
Figure 4-19 Time-dependent scavenging activities of CLG and CLG″ against DPPH$^\bullet$ in the dark. Values expressed as mean ± SD of triplicate determinations.

but also for changing the electron configuration of a molecule. Reaction rates of CLs with DPPH$^\bullet$ were positively related to UV irradiation. These findings suggest that CLs may help plants respond to heat stress and UV damage. In comparison, the DPPH$^\bullet$ scavenging activity for CLs were in the order CLG" (38%) > CLG (22%) > CLC (18%) > CLB (16%) > CLA (–4%) at a molar ratio of CLs: DPPH$^\bullet$ of 25:1, after 30 min incubation in the dark (Figures 4-15 to 4-19).

The DPPH$^\bullet$ scavenging test is extensively used to evaluate the antioxidant activity of peptides and peptide-containing fractions. It is believed that Phe, Trp, His, Pro, Tyr, Lys, Leu and Met are the main amino acid residues that demonstrate antioxidant activity. For example, a peptide from fermented mussel sauce (His-Phe-Gly-Asp-Pro-Phe-His) scavenges 72% of DPPH$^\bullet$, while a peptide from horse mackerel viscera (Ala-Cys-Phe-Leu) achieved 89.2% scavenging activity against DPPH$^\bullet$ (at 200 μM/mL concentrations), respectively (Rajapakse et al. 2005; Sampath et al., 2011). Moreover, Table 4-3 shows a strong positive relation between the content of Phe, Trp and Pro residues in the peptide fractions and the scavenging activity against DPPH$^\bullet$. Thus, Trp, Pro and Phe residues of studied CLs are the main contributors of DPPH$^\bullet$ scavenging activities of these peptides. These amino acids were able to donate either an electron or proton to DPPH$^\bullet$. As the amino acids are hydrophobic they are able to react with hydrophobic targets (Je et al., 2005; Zhang et al., 2008; Farvin et al., 2010; Pownall et al., 2010; Ajibola et al., 2011; Sampath Kumar et al., 2011; Zhang et al., 2011).

New antioxidant discovery requires that researchers investigate the relationship between compound structure and antioxidant activity. Several papers have elucidated the relationship between compound structure and scavenging activity toward DPPH$^\bullet$. These studies considered phenolic compounds (Saito et al., 2005; Ordoudi et al., 2006; Huang et al., 2006; Velkov et al., 2007; Musialik et al., 2009). Overall, the time-dependent scavenging properties of CLs might be related to deprotonation ability. The scavenging activity of CLs increased in the following order CLA < CLB < CLC < CLG < CLG". The acidity of peptides should be dependent on the nature and location of other functional groups present in the peptide molecule. There is very little information about the effect of substituent groups on the acidity of the peptide hydrogen. In comparing CLG and CLG", however, electron-withdrawing sulfoxy (S(O)-CH$_3$) substituents should increase N-H acidity of CLG over the thiol (S-CH$_3$) group of CLG". The observed scavenging activities may be connected to structural and energy transfers among atoms of these compounds.

The hypothesis that scavenging properties of CLs are determined by their deprotonation ability can be supported by the strong UV dependent activity of CLs. UV light induction of CLs affects electronic configuration and acid-base properties. According to Fessenden and Neta (1971), when thiols are irradiated by UV light, RS$^{\bullet}$ radicals are generated as a major product by hydrogen abstraction. Available data showed that carbon radicals are expected instead of S-H (Fessenden and Neta, 1971). In addition, Kurita and Gordy (1961) observed in an EPR study of cysteine dihydrochloride an isotropic interaction of the unpaired electron, mainly with one of the two protons in the CH$_2$ group adjacent to sulfur (HOOC-CH(NH$_2$)-CH$_2$-S$^{\bullet}$). HPLC-MS/MS analysis of the reaction mixture of CLs and DPPH$^{\bullet}$ with UV light revealed that methionine-containing CLB and tryptophan-containing CLG and CLG″ react with DPPH$^{\bullet}$, whereas DPPH$^{\bullet}$ adducts did not form with CLA and CLC.

Mass spectral analyses of the reaction mixture of CLB and DPPH$^{\bullet}$ in positive ion mode revealed two new products at m/z 1437.7 ([M + H]$^+$) and 1405.7 ([M + H]$^+$) amu with molecular formula C$_{73}$H$_{93}$N$_{14}$O$_{15}$S and C$_{73}$H$_{93}$N$_{14}$O$_{15}$, respectively. The reaction mixture was subjected to LC-MS/MS analysis. Both compounds showed fragmentation patterns similar to that of the mother compound, CLB. The product at m/z 1437.7 ([M + H]$^+$) indicated a mass of CLB and DPPH$^{\bullet}$, less 15 amu, suggesting substituion of the methionine methyl by DPPH$^{\bullet}$. The HPLC-MS/MS of 1437.7 showed neutral loss of two 113 amu resulting from consecutive fragmentation of Ile and Leu. Next, the neutral loss of 226 amu suggested fragmentation of a 2,4,6-trinitroariline portion of DPPH$^{\bullet}$, followed by a fragment with mass of 282.2 amu rusulting from loss of diphenylarine attached to homocysteine, followed by sequential loss of 113 (Ile), 99 (Val) and 147 (Phe) amu to yield a fragment with mass of 342.2 amu resulting from Phe (147) and two Pro units (97.5 × 2). The structure of product 1437.7 was therefore characterized by HPLC-MS and is shown in Figure 4-20. The product possibly resulted from loss of methyl of methionine to yield a homoserine free radical that reacted with DPPH$^{\bullet}$.

In addition to products 1437.7 and 1405.7, other products with masses m/z 1010.6 ([M + H]$^+$) and 1012.6 ([M + H]$^+$) were dectected in the mass spectra. The HPLC MS/MS spectra of compound 1010.6 revealed subsequent loss of neutral fragments 113 (Ile), 113 (Leu), 83 (2-amino-2-butenoic acid, Dhb), 113 (Ile), 99 (Val) and 147 (Phe) amu resulting in a fragment with a mass of 342.2 amu comprising Phe (147) and two Pro units (97.5 × 2). The amino acid sequence of compound 1010.6 was therefore established to be *cyclo*-(Ile-Leu-Dhb-Ile-Val-Phe-
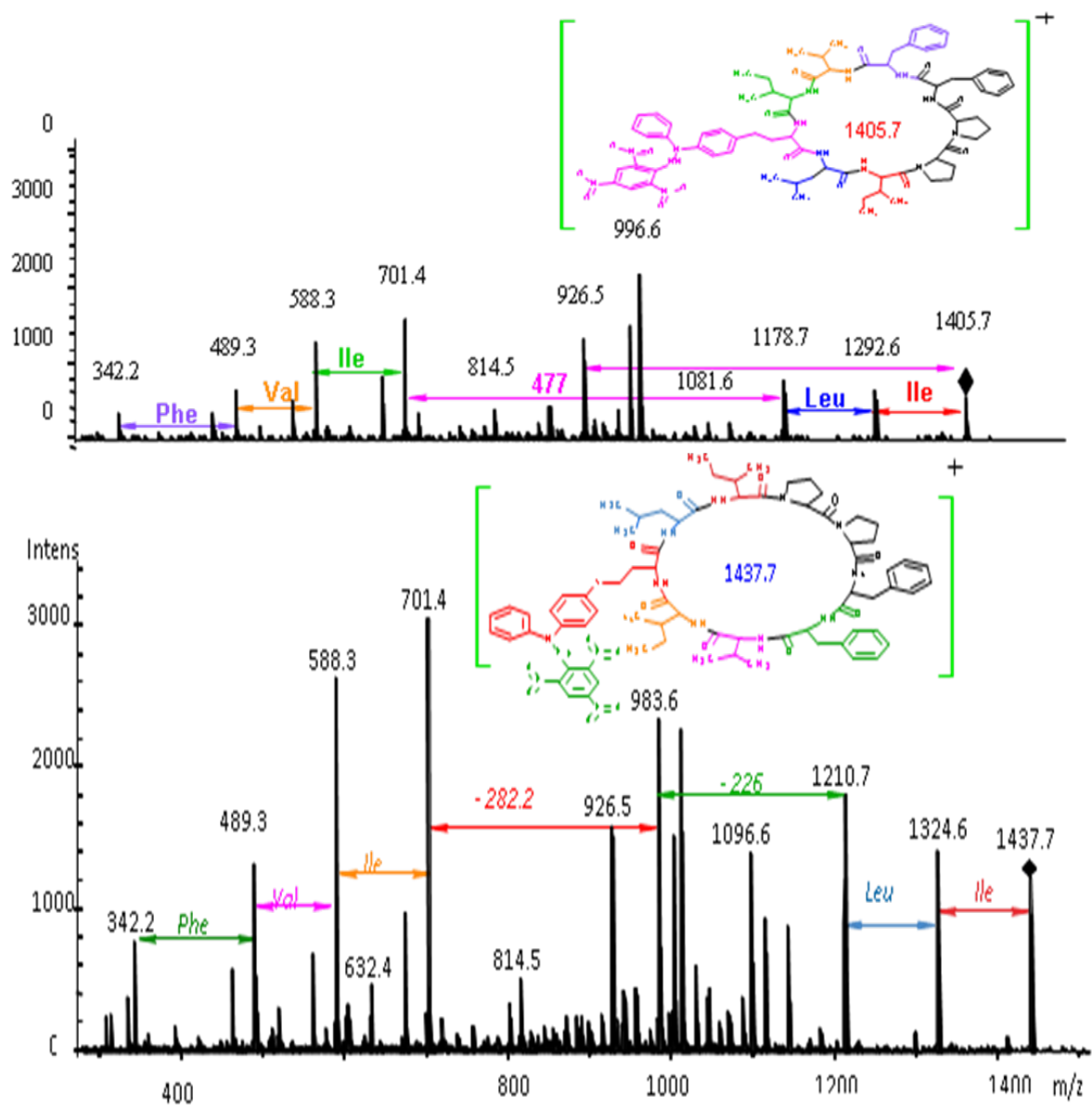
Figure 4-20 HPLC-MS/MS analysis of the two main products of reaction CLB with DPPH•

Phe-Pro-Pro). On the other hand, HPLC MS/MS spectra of compound 1012.6 revealed subsequent loss of neutral fragments 113 (Ile), 113 (Leu), 85 (2-aminobutanoic acid, Abu), 113 (Ile), 99 (Val) and 147 (Phe) amu resulting in a fragment 342.2 amu comprising Phe (147) and two Pro units (97.5 × 2). The amino acid sequence of compound 1012.6 was established to be *cyclo*-(Ile-Leu-Abu-Ile-Val-Phe-Phe-Pro-Pro).

Mass spectral analyses of the reaction mixture of CLG″ and DPPH• in positive ion mode revealed an unidentified product at *m/z* 1475.6 ([M + H]+) amu with a molecular formula of $C_{56}H_{75}N_9O_{10}S_2$. The detected mass suggested that compound 1475.6 could be an oxidized product of a reaction between CLG″ and DPPH• since the detected mass and molecular formula supported those of CLG″, DPPH• and additional 16 amu, suggesting the presence of an additional oxygen (Figure 4-21). Interestingly, the reaction product did not exhibit loss of methyl or thiomethyl groups as was observed with CLB. To fully characterize the product, the CLG″-DPPH• reaction mixture was subjected to extensive LC-MS/MS analyses. The initial fragmentation pattern followed that of the mother compound, CLG″. Thus, compound 1475.6 showed sequential loss of neutral fragments 131 (Met), 113 (Leu), 131 (Met) and 113 (Ile) amu. Further fragmentation showed loss of 226 amu resulting from 2,4,6-trinitroariline from DPPH•, followed by a fragment with mass of 370 amu attributed to loss of diphenylarine attached to oxotryptophan and subsequent loss of 147 (Phe) amu to a base fragment of 245 amu comprising Phe (147) and Pro (97). The amino acid sequence of compound 1475.6 was established to be *cyclo*-(Met-Leu-Met-Ile-(DPPH-*O*-Trp)-Phe-Phe-Pro).

The mass spectral analyses of the reaction mixture of CLG and DPPH• in positive ion mode revealed an unidentified product at *m/z* 1491.6 ([M + H]+) amu with a molecular formula of $C_{56}H_{75}N_9O_{10}S_2$. The detected mass suggested that compound 1491.6 could be a product of a reaction between CLG and DPPH•. Interestingly, the reaction product did not exhibit loss of methyl or thiomethyl groups as was observed with CLB. To fully characterize the product, the CLG-DPPH• reaction mixture was subjected to extensive LC-MS/MS analyses. The initial fragmentation pattern followed that of the mother compound, CLG. Thus, compound 1491.6 showed sequential loss of neutral fragments of 113 (Ile), 113 (Leu), Next, neutral loss of 226 amu suggested fragmentation of a 2,4,6-trinitroariline portion of DPPH•, followed by fragment with mass of 282.2 amu resulting from loss of diphenylarine attached to homocysteine, both
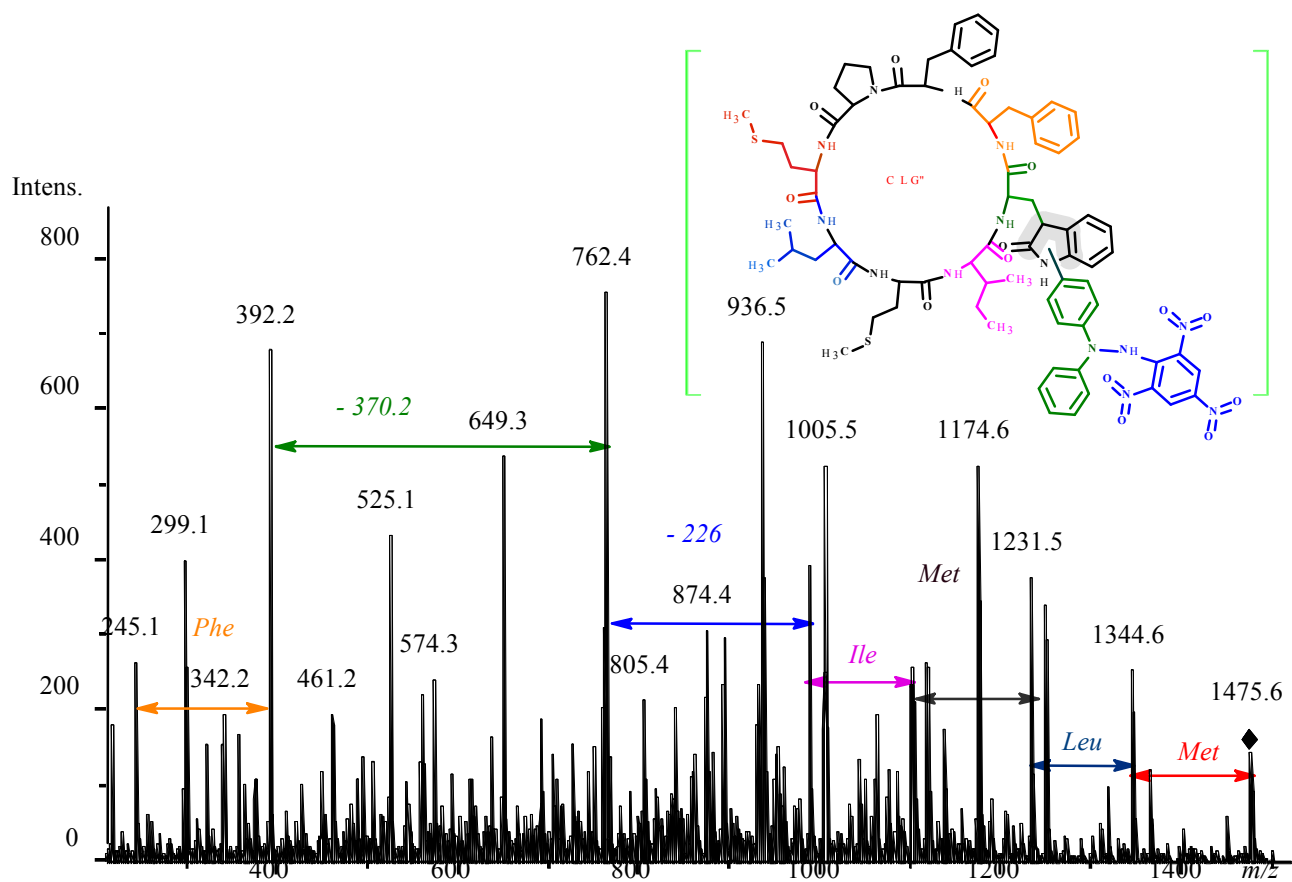
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Figure 4-21 MS/MS spectra of CLG″ + DPPH• and possible product ions.

compounds showed fragmentation patterns similar to that of the mother compound, CLB. The product at m/z 1437.7 ([M + H]$^+$) indicated a mass of CLB and DPPH$^•$, less 15 amu, suggesting substituion of the methionine methyl by DPPH$^•$ (Figure 4-22). An adduct of CLG's with DPPH$^•$ radical (m/z 1491) was found in the MS/MS of CLG reaction mixtures with DPPH$^•$. The fragmentation if this ion was similar to pure CLG (Figure 4-22), but once the ring was opened the MS spectrum revealed oxidation products of CLG through tryptophan residues.

4.6   Conclusions

Flaxseed CLs are free radical scavengers. EPR measurement established the ability of these peptides to quench DPPH nd hydroxyl radicals. The hydroxyl radical assay revealed that CLA, B and C suppress the EPR signal intensity of DMPO/HO$^•$ produced in Fenton reaction and site-specific interaction between CLs and the Fenton system was observed. Moreover, tryptophan containing CLs (CLG and CLG″) scavenged DPPH$^•$ more easily than methionine and methionine sulfioxide containing (CLB and CLC) peptides. In contrast, CLA amino acid residues, Phe, Pro, Leu and Ile were only able to reduce the EPR signal intensity of DPPH$^•$ when activated by UV.
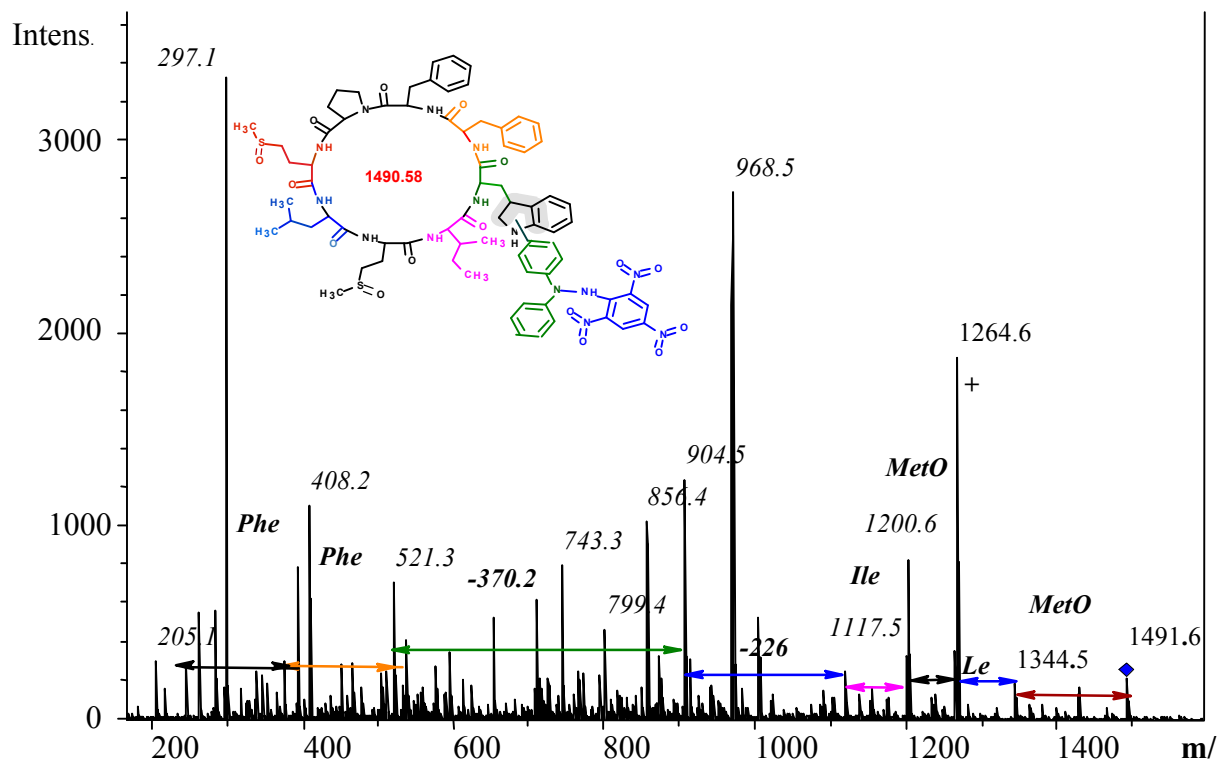
Figure 4-22 MS/MS spectra of CLG + DPPH• and possible product ions.

CHAPTER 5
GENERAL DISCUSSION

The main restriction for increasing flaxseed utilization in the food market is the low stability of this product and its short maximum storage period (Oomah and Mazza, 1999; Tolkachev and Zhuchenko, 2000; Bozan and Temelli, 2008). On the other hand, flaxseed and milled flaxseed can be stored for relatively long periods without significant changes. In this project, antioxidant activities of CLs were investigated in the following ways: 1) the effect of CL-containing fractions obtained from crude flaxseed oil were tested for their effects on the oxidative stability of peptide-free flaxseed oil; 2) the effects of CLs on the oxidative stability of flaxseed oil in the presence of metal cations were determined, and 3) scavenging activities of CLs against free radicals were determined.

Minor compounds present in flaxseed oil, including CLs, were effectively removed from the crude flaxseed oil by silica gel flash column chromatography. Subsequent solvent elution with increasing polarity separated hydrophobic fractions. Fraction D containing CLA and tocopherols, and Fraction E containing a mixture of CLA and CLD–CLG, increased the oxidative stability index of peptide-free oil. In addition, a time-dependent antioxidant activity of peptides mixture was observed in this study.

Others have measured the antioxidant activities of fractions obtained from flaxseed and other oils. Typically these studies have not identified compounds present in the oils directly by mass spectrometry but have, instead measured the compounds using colourimetric assays. For example, reports of the presence of antioxidant phenolic compounds in flaxseed (Oomah et al., 1996; Choo et al., 2007; Siger et al., 2008) are based on the use of colorimetric methods that employ low specificity reagents. For instance, the Folin-Ciocaltue reagent was used to determine the flavonoid content of cold-pressed flaxseed oil (Choo et al. 2007) but this reagent may also react with a broad range of reducing substances including nitrogen containing compounds such as indole derivatives (Ikawa et al., 2003).

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DPPH scavenging activity of extracts of nine vegetable oils have also been reported. Extracts that would be rich in cyclic peptides in flax were reported to have similar reactivity with DPPH to sunflower oil, greater reactivity than peanut oil and less reactivity than all other oils tested (Tuberoso et al., 2007). Using similar extracts and oils, Siger et al. (2008) reported that cold pressed flax oil extracts had comparatively weak antioxidant activity when tested for DPPH scavenging.

Cobalt accelerates the development of peroxide value in flax oil by 100 fold. It has peen reported that the products of both cobalt catalyzed drying and metal free drying of flax oil are the same (Mallégol et al., 2000). Cobalt used as a drier is often in the form of a dimeric cobalt complex (van Gorkem and Bouwman, 2005) Furthermore, the effect of CLA and some transition metals (Zn^{2+}, Ni^{2+} and Co^{2+}) on the oxidative stability of peptide-free flaxseed oil was evaluated. CLA addition was able to selectively inhibit oxidation induced by metal cations. The reaction with Zn^{2+} was stronger than with Ni^{2+}, whereas no effect on Co^{2+} was observed. Moreover, a synergistic effect of CLA and one or more compounds were also observed.

The main difficulties in this experimental part were data interpretation, especially in the first experiment where an effect of polar fractions containing CLs on the oxidative stability of silica treated oil was tested. This weakness revealed to us that we did not recovered the silica treated flax oil with sufficient amount of antioxidant peptide fractions and that the sensitivity of the experimental method, OSI test, is also not adequate for this study. In addition, when silica treated oil was recovered by more concentrated polar fractions and tested time dependent manner we able to get more strong supporting data on antioxidant activity of flax CLs. Based on this observation, systematic and well-designed dose and time dependent experiments of the CLs containing fractions can conceal more hidings of contribution CLs on antioxidant pool of flax oil. Moreover, CLs could possess a variety of biological functions in the plant system. Selective binding metal cation by CLA was one of the results of our research. Therefore, studies of interactions CLs with other compounds in the flax oil, like different metals and other minor and main antioxidants, will be continued.

The free radical scavenging abilities of CLs were evaluated using EPR method. The scavenging effects of CLA, Band C on free radicals formed in an H_2O_2/Fe^{2+}/acetonitrile system were studied. A 24–30% decrease in EPR signal intensity of hydroxyl radical DMPO adducts was observed at a 5 mM concentration of peptides, indicating that the CLs are weak scavengers

compared to reported antioxidant peptides. No new compounds were observed by mass spectrometry analysis of the reaction mixture after irradiation. The relative participation of site-specific oxo-iron species effects of the peptides versus their reaction with hydroxyl radicals in the decrease in signal was not ascertained.

Furthermore, the interaction of CLA–CLC, CLG and CLG″, with the stable free radical DPPH$^\bullet$ was investigated by spin–trapping followed by mass spectroscopy. The study explored scavenging activities of these peptides against DPPH$^\bullet$ in a time and dose dependent manner and active participation of side chain functional groups in these interactions. Tryptophan-containing CLG and CLG″ scavenged DPPH$^\bullet$ more easily than methionine- or methionine sulfoxide-containing CLB and CLC. In addition, scavenging activities of CLB, CLC and CLA against DPPH$^\bullet$ significantly increased after UV exposure. Mass spectrometry investigation of reaction products revealed that CLB, CLG and CLG″ reacted with DPPH$^\bullet$ to form stable products, whereas CLA and CLC did not show any detectable reaction products. Besides these findings, there was weakness in this part research. As stated in the main part of the thesis, up to now there is not perfect methodology to evaluate an antioxidant capacity of compounds. In case of studied CLs, we found relatively low scavenging activity (lower EC$_{50}$ value) against free radicals OH$^\bullet$ and DPPH$^\bullet$ compared to antioxidant peptides listed in the literature. Even some CLs were able to quench only under UV light activation. However it has been found to be significantly less potent that peptides generated by enzymatic digestion, which could be because of structural differences and accessibility to free radicals in these peptides in reaction media, CLs may show higher scavenging effect in active species produced by lipid oxidation. Thus, similarly to this work, interaction CLs with ROS species should be tested in different media and time intervals in designed model system.

Lastly, CLs allow a unique method to study the mechanism of peptide antioxidants due to following characteristics.

• CLs are available that have a singly substituted amino acid.

• CLs do not have an N or C - terminal that might contribute to antioxidant properties.

• CLs have many residues that are likely to resist oxidation.

• CLs are conformationally bound which may help in understanding the mechanism of reaction with ROS.

CHAPTER 6
GENERAL CONCLUSIONS

Polar fractions extracted from flaxseed oil contain CLs can inhibit flaxseed oil oxidation. The polar fractions of flax oil are complex mixtures that contain peptide and non-peptide compounds. Future research is required to determine the most effective antioxidant compounds of the polar fractions. In addition, the potential for synergistic effects of CLs with other antioxidant compounds could be studied to aid in understanding the flax antioxidant system.

Transition metals in oil accelerate the rate of flax oil oxidation while CLs inhibited this effect for Zn and Ni but not for Co. It is possible that metals formed complexes in solution with the peptides that were less active that the metals without peptides. The interaction between CLs with transition metals has not been described previously. When compared to peptides that exhibit strong antioxidant effects CLs are comparatively weaker scavengers of hydroxyl radicals generated by the Fenton reaction. It cannot be concluded that the cyclic peptides are not effective radical scavengers under other conditions such as those that are found in flaxseed products.

This thesis describes several non-radical reaction products of peptide with DPPH radicals. The presence of these reaction products conclusively shows that the amino acids methionine and tryptophan form covalent linkages with DPPH. It cannot be concluded that this is a major reaction that leads to quenching of free radical chain reactions. It was also noted that where both methionine and tryptophan were present in the same peptide that only products containing a single radical with tryptophan were observed. Other radicals adducts may have been formed but their presence was not detected. The formation of non-radical adducts suggests a possible mechanism whereby these peptide species might act as antioxidants.

Taken as a whole, the evidence presented in this thesis shows that flax cyclic peptides act in both flax oil and in model systems to diminish the rate and extent of radical reactions. The antioxidant mechanism may be in part through the ability of methionine sulfide and tryptophan imidazole to react directly with radicals.

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APPENDIX A

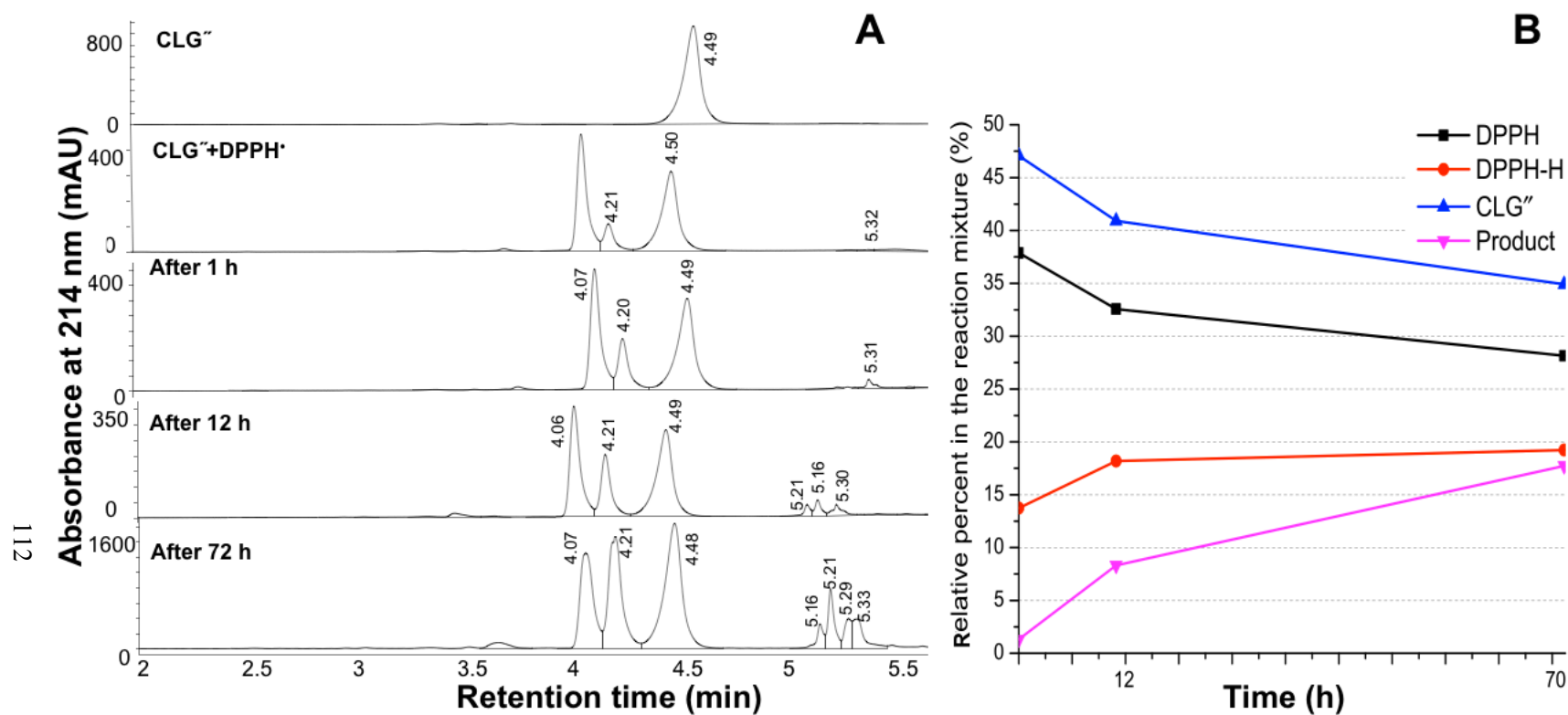## Reaction of 5 mM DPPH$^\bullet$ with 5 mM CLG

Figure A-1 Time-course HPLC chromatogram of the reaction mixture of CLG″ and DPPH• in acetonitrile. The respective peaks were assigned to CLG″ (4.5 min), DPPH + H (4 min) produced by reduction of DPPH•, DPPH• (4.2 min) and unknown compound (5.2–5.5 min) (left, Figure A-1A). DPPH• oxidation and establishing reaction equilibrium also observed from this assignment. HPLC-Ms analysis investigated CLG″–DPPH• adduct from this unknown peaks (right, Figure A-1B).
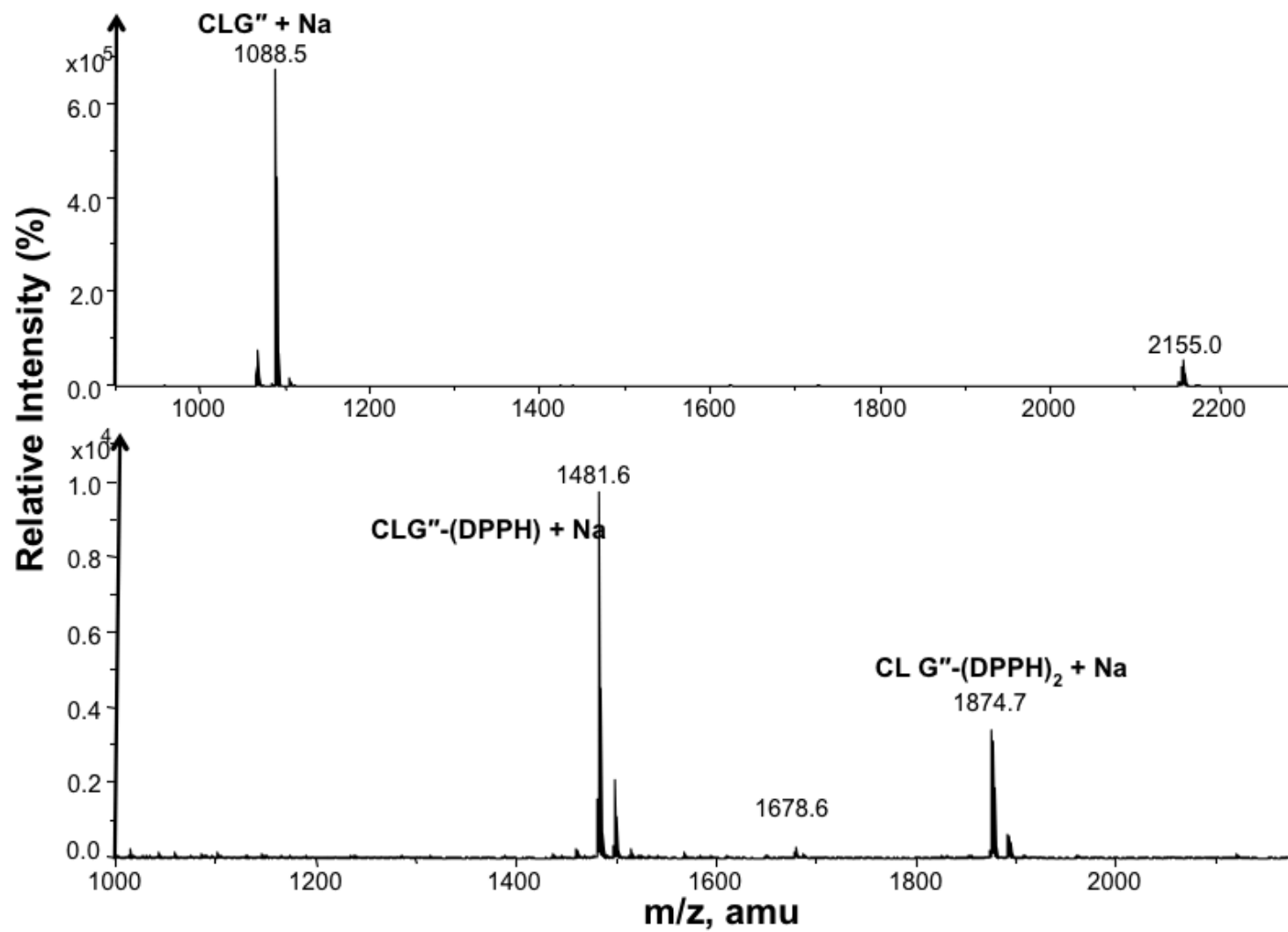
Figure A-2 HPLC-MS spectra of CLG″-DPPH• adducts.

APPENDIX B

PROOXIDANT ACTIVITY DEPENDENING ON SOLVENT CONCENTRATION OR MIXTURE
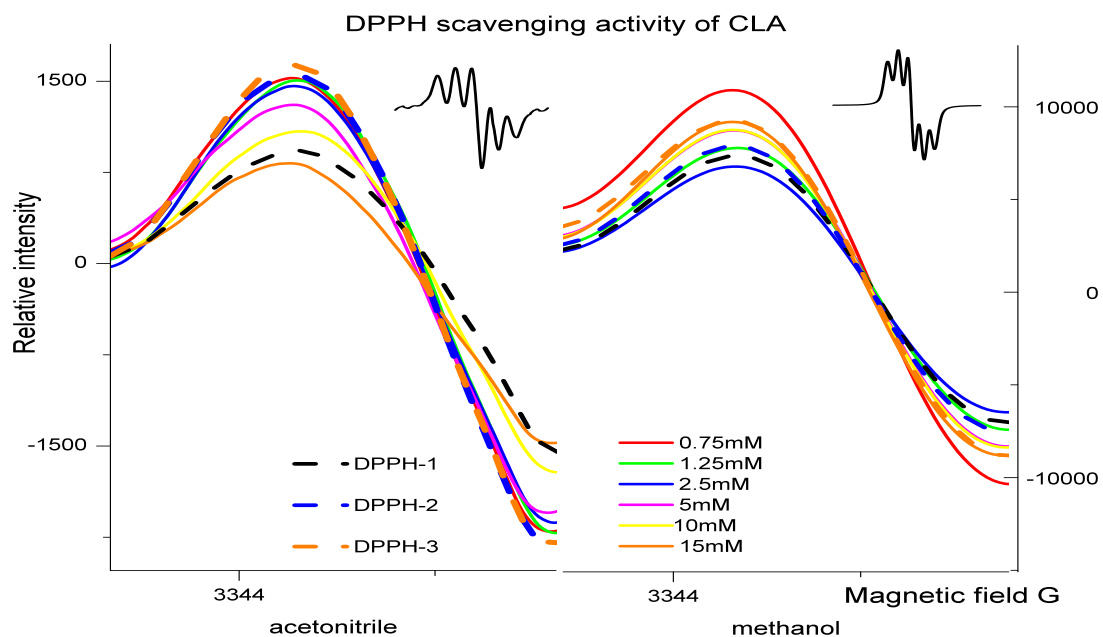
Figure B-1 DPPH⁺ scavenging activity of CLA in different solvents. Experimental condition: microwave power 2.00 mW, center field 3344.44 G, sweep with 129.57 G, conversion time (81.92 ms), time constant 40.96 ms, modulation frequency 100 GKHz, modulation amplitude 0.5 G, receiver gain 1.78×10^4, number of scans 2. UV 3 min. Final concentration of reagents in acetonitrile solution: 1.5 mM (H_2O_2); 5 mM (DMPO); 0.17 mM ($FeSO_4$); 0.84 and 6.6 mM (CLA); average of 3 samples. CLA contained trace amounts of CLI.
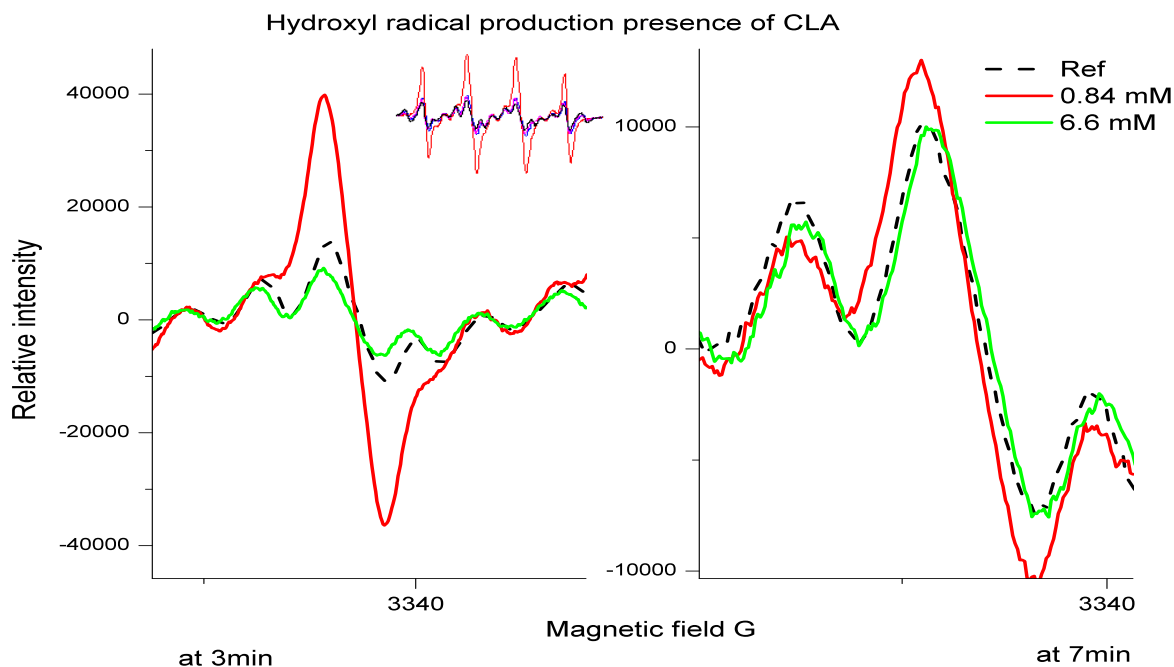
Figure B-2 Hydroxyl radical production presence of CLA. Experimental condition: microwave power 1.00 mW, center field 3345 G, sweep with 60 G, conversion time (163.84 ms), time constant 89.9 ms, modulation frequency 100 GKHz, modulation amplitude 0.5 G, receiver gain 1.42×10^5, number of scans 1. Time is counted after adding FeSO$_4$. Final concentration of reagents: 0.25 mM (DPPH$^\bullet$) and 0.75–15mM (CLA) EPR run after 3 min UV irradiation; single experiment. Experimental condition: microwave power 2.00 mW, center field 3344.44 G, sweep with 129.57 G, conversion time (81.92 ms), time constant 40.96 ms, modulation frequency 100 GKHz, modulation amplitude 0.5 G, receiver gain 1.78×10^4, number of scans 2. UV 3 min. Final concentration of reagents in acetonitrile solution: 1.5 mM (H$_2$O$_2$); 5 mM (DMPO); 0.17 mM (FeSO$_4$); 0.84 and 6.6 mM (CLA); average of 3 samples. CLA contained trace amounts of CLI.

APPENDIX C

Table C-1 Advantages and limitations of methods for estimation of lipid stability and evaluation of antioxidant activity. Summarized by Koleva (2007)

| Methods	Advantages	Limitation
Stability tests of oil/fats		
Storage: Ambient temperature At elevated temperature	+ The most relevant to real-life. + No equipment is needed. + Speeded up in comparison to the above technique. + Common equipment	− Very slow − Not very reproducible − Possible loss of volatile or thermally labile antioxidants − Changed oxidation mechanism − Thermal alterations of lipids that are the real storage conditions may occur
Weight gain	+ Simple performance + Common equipment + Satisfactory reproducibility	− Not very sensitive − Formation of volatile products affect the obtained results. − Accurate control of the experimental parameters to minimize their influence on the results is needed. − Laborious
Rancimat and Active oxygen Method (AOM)	+ Rancimat is automated. + Measure the rate of oxidation	− Questionable end point − Rancimat requires higth levels of oxidation − Loss of volatile or thermally laible antioxidants − Unreliable results due to hydroperoxide decomposition and side reactions of the lipid - not suitable for easily oxidized lipids whose hydroperoxides reaily decompose − Sensitive to small variations in oxygen − Large sample size is needed − Low relevance to flavour scores − No relation to the oxidation under normal storage conditions − Time-consuming − Requires specific equipment (Rancimat)
Schaal oven	+ Relatively mild oxidation and fewer problems in comparison to the other accelerated tests. + Good relevance to the real shelf-life. + No need of special equipment.	− Slow − Large sample size is needed
Oxygen uptake	+ Small samples can be studied. + Measures the rate of oxidation + Good correlation between the rate of oxygen consumption and storage time - can be used for shelf-life	− Require high level of oxidation − Nor very sensitive − Requires special instrumentation

	prediction.	
Oxygen bomb	+ Excellent reproducibility + Measures the rate of oxidation + Smaller sample required	
Other tests		
Sensory panel tests	+ Best related to the quality and consumer acceptability of the food + Sensitive	− Requires specially trained panelists and suitable premises − Results depend on the experience and sensitivities of the panelists − Results are subject of misunderstanding due to the lack of common descriptive vocabulary − Results depend on the threshold value of the analyzed volatiles − Poor reproducibility − Low precision
HPLC analysis of the changes in fatty acid composition or HPLC of formed hydroperoxides	+ Very sensitive ; Reproducible + Provides qualitative and quantitative information for the fatty acids involved in the oxidation or the formed hydroperoxids + Oxidation of eaily oxidized lipids may be monitored + No need of preliminary derivatization fatty acids or sample preparation	− Good knowledge about the nature and retention characteristics of the analyzed compounds is needed − Requires relatively expensive instrumentation
GC analysis of the changes in fatty and composition Headspace GC of secondary volatiles	+ Very sensitive + Reproducible + Provides qualitative and quantitative information for the fatty acids involved in the oxidation. + Oxidation of easily oxidized lipids may be monitored + Oxidation may be followed in static or dynamic model + Very good correlation with the results from sensory analysis	− Requires sophisticated instrumentation, especially for the analysis of volatiles (head space device) − Preliminary sample preparation such as fatty acid devivatization is needed, which may affect the precision and accuracy of the quantitative analysis − Low precision
• Static	+ Relatively rapid - automatic performance- routine analysis of the a large number of samples	− Not suitable for compounds, that decompose during the equilibration step in the static headspace GC
• Dynamic	+ Direct analysis of complex samples without special pretreatments + Capable to detect trace amounts + Sample subjected to lower-temperature heating than in the static version	− Dynamic headspace GC is slow and not suitable for routine analysis → requires much more and labour input

|		
Peroxide value (PV)	+ Provides information about the concentration of the formed hydroperoxides + Suitable for bulk oils	− Not suitable to study the advanced oxidation phase when hydroperoxides start to decompose → not very suitable for easily oxidized lipids − Not suitable for emulsions on biological materials − Not very sensitive and selective − Low results due to possible addition of iodine across unsaturated bonds
• Iodometric titration	+ Routine analytical protocol and common reagents + Relatively sensitive	− Oxidation of iodide by dissolved oxygen − Variations of reactivity of peroxides − Requires relatively high level of oxidation → not suitable for the very first moment of oxidation when the hydroxide concentration is low − The correlation between the PV and the onset of rapcidity is not always good → need cautiousness in shelp-life prediction. − Provides information only for the current oxidative status of the sample but not about its potential to oxidize.
• Ferric thiocyanate colorimetry	+ More sensitive than iodometric titration + Requires smaller size sample than for iodometric titration	− Requires relatively large samples − Experimental conditions (e.g. light; operator's skillfulness; time) may strongly affect the results. − Chemical structure and reactivity of ROOH may influence the results.
Conjugated dienes	+ Sensitive: Reproducible results do not depend on Chemical reactions + Faster than PV; simple + Selective if using HPLC or matrix subtraction using second – derivative spectroscopy + Measures only early stage in the oxidation + Requires small sample size + Uses common spectrophotometric equipment + Suitable for both bulk and emulsions	− Other chromophores absorbing in the same spectral region (e.g. carotenes) present in the sample may interfere − Requires mild oxidation of the sample in order to prevent hydroperoxide decomposition − Not suitable for hydrogenated or deodorized oils since they contain conjugated di- or three- enes − Generic measurement → little info about the structure of the compounds
TBARS. Total or selected carbonyl compounds	+ Simple; Sensitive especially for polyunsaturated lipids + Selective if using HPLC → characterization the individual species + Precise + Uses common spectrophotometric equipment + Good correlation with the sensory analysis of same vegetable oils	− Not very sensitive for mono- and di unsaturated lipids − Not specific as other sample components may form red-colored products with the TBA − Reaction conditions have effected color development − Strong influence of the experimental conditions (light; temperature; time of heating; presence of metal ions or certain compounds such as amines, etc.)

		− Some experimental protocols can be time consuming or provoke formation of artifacts
p-Anisidine value	+ Precise + Both volatile or non-volatile carbonyls can be analyzed + Good correlation with the flavour acceptability scores	Less sensitive than GC − Other samples compounds may interfere either with the reactions or the absorption
TOTOX (2PV + AnV)	+ Present State i.e. PV past history i.e p-AnV	− Lacks sound scientific basis 2PV + TBA
β-Carotene bleaching	+ Simple; Relatively rapid + Sensitive + Uses common spectro-photometric equipment	− Non specific − Strong influence of experimental conditions and other sample components − Oxidizable substrate is not representative for real lipids → the obtained data are not directly realted to the actual oxidation − Mechanism of oxidation is not clear − Reliable results are limited to less polar antioxidants
Fluorescent analysis of ROOH or secondary oxidation products	+ Very sensitive + Suitable for complex food and biological systems	− Not specific − Requires special instrumentation
Other methods for evaluation of antioxidants		
Chemiluminescence	+ Very sensitive; ability to detect low levels of oxidation → oils can be easily studied with no need of elevated temperature + Suitable for complex food and biological systems + Reproducible; The scavenging activity toward radical species and ROS that are generated in real systems can be evaluated + Can be combined on-line with HPLC for large-scale screening of complex samples	− Requires special instrumentation − Uses relatively expensive and not very stable reagents (e.g. enzymes)
Radical- trapping methods employing stable model radicals (e.g. DPPH$^•$; ABTS$^•$; DMPD$^•$; DBO$^•$ etc.)	+ Sensitive; reproducible; + selective; rapid; simple and routinely manageable + Robust; Easy; uses common spectrophotometric instrumentation and easily available reagents + Can be combined on-line with HPLC for a large-scale screening of complex samples + Kinetic studies are possible + High resolution for DBO + DBO provides direct experimental info on the absorbance reactivity or	− Employs model radical species not encountered in real systems→ no direct correlation with the oxidation- antioxidation mechanism in real system − Does not employ lipid substrate → no information about which lipid system a tested antioxidant will be efficient for DPPH$^•$ → depends on compounds' structural conformation − More expensive ABTS – commercial kits

|		
	concentration of antioxidants	
	+ Applicable for heterogeneous biological systems	
Total radical trapping antioxidant parameter (TRAP)	+ Precise if using CL + Automated if using CL	− Oxygen electrode will not maintain its stability till the end-point − Time consuming (up to 2 h/ sample)
Ferric ion reducing antioxidant power (FRAP)	+ Simple + Convenient; + Inexpensive	− Does not measure the SH group-containing antioxidants − Measuring reducing capacity does not necessarily reflect antioxidant activity
ORAC (oxygen radical absorbance capacity)	+ Specific + Responds to numerous antioxidants	− Requires fluorescence detector→ long time of analysis − Not linear with time − Oxygen electrode end point→ imprecision
EPR	+ The only analytical technique that can detect the free radicals involved in autoxidation + Detects and evaluates short- lived radical species + Selective	− Requires sophisticated instrumentation − Specialist nature − Direct application to lipid oxidation system is hampered by the short life of many radical species − Special techniques such as freezing and spin trapping of free radicals − Spin traps can have oxidant and antioxidant-action, while spin adducts can act as antioxidants − Insensitive only fairly uncreative radicals (in bimolecular)
Infrared (IR) spectroscopy	+ Monitors formation/ disappearance of certain oxidation products (ROOH; carbonyls; acids etc.) + Requires very small amount of sample; Simple; Rapid + After calibration it may serve for determination of PV or anisidine value	− Not very specific − Not very sensitive
^{1}H-NMR	+ Non destructive; Requires very small amount of sample + Rapid; Reliable + Data from ^{1}H-NMR correlate with TOTOX	− The changes of fatty- acid profiles reflect both primary and secondary oxidation products − Not very specific − Not very sensitive

APPENDIX D

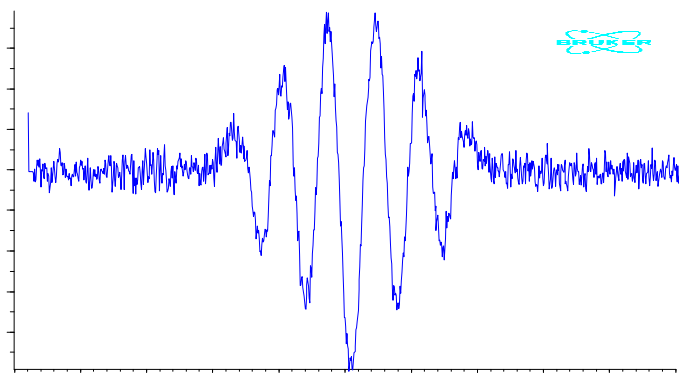# SPECTRUM MANIPULATION
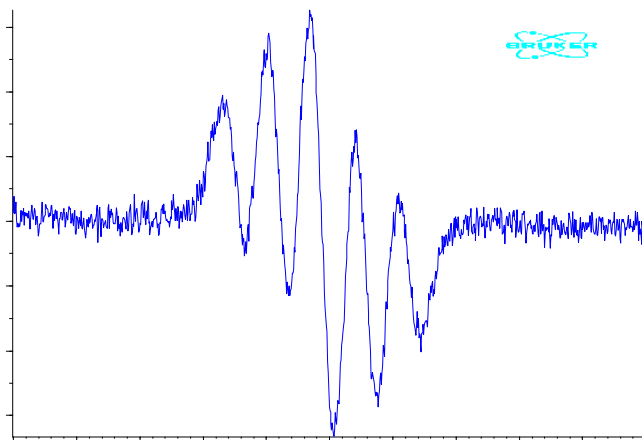
EPR spectrum the DPPH• + CLC
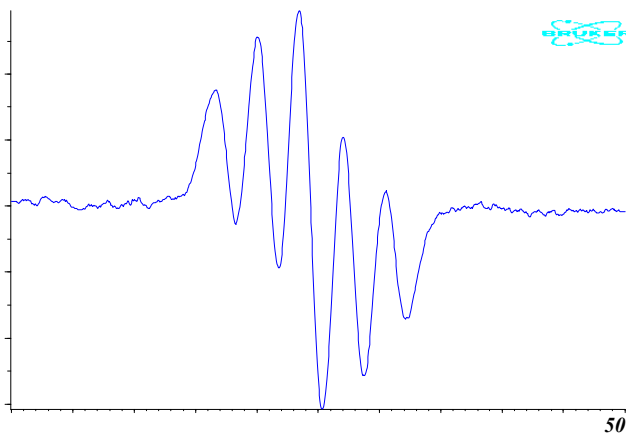
Filtration (moving average x15)
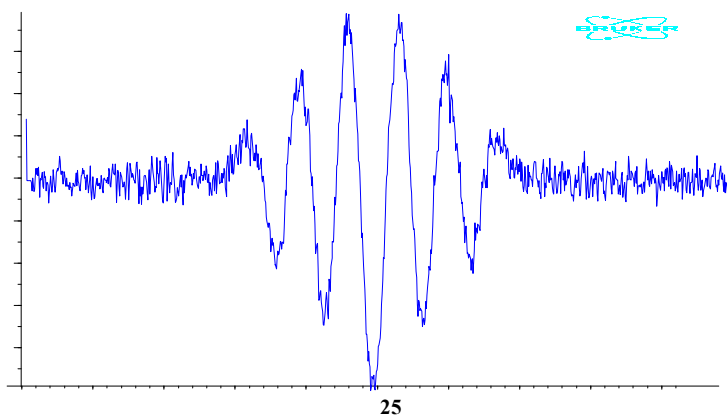
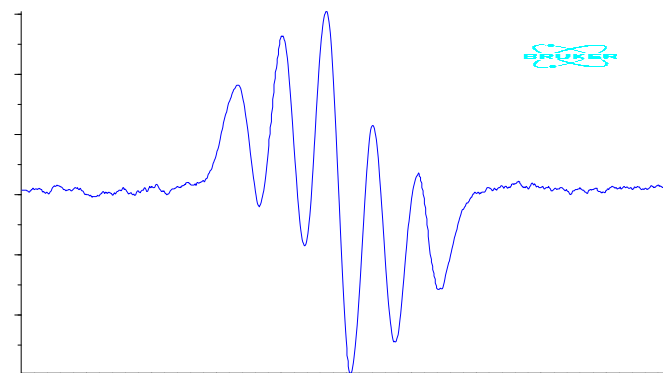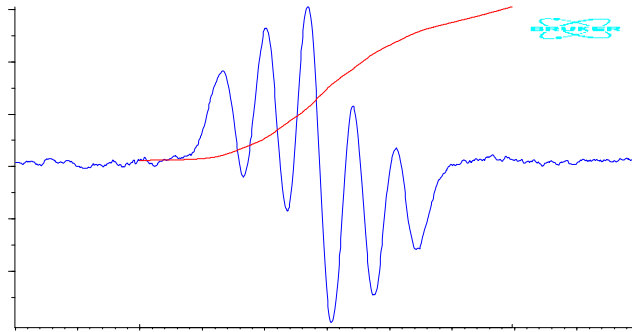**Differentiation**
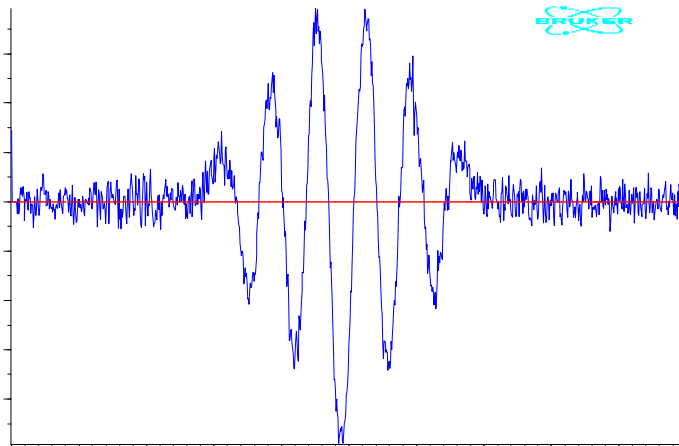
Baseline Correction (constant)

Subtraction

Integration

124

Integration region

Double integration

Integral List
Normalisation value: N = 4.287e+006
Start[]   End[]   DI   DI/N

9.9707 39.7849 1.218e+007 2.841e+0007