MODIFICATION OF MARINE OILS AND THEIR BIOLOGICAL SIGNIFICANCE

By

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

Memorial University of Newfoundland

October 2012

St. John's Newfoundland and Labrador Canada

ABSTRACT

Marine oils, such as seal blubber and menhaden oils offer a wide range of health benefits and play a critical role in many functions in the human body. Their effects in alleviating cancer cardiovascular disease, psychiatric disorders, Parkinson's disease and inflammatory ailments have been well demonstrated in a large body of literature. However, these oils are highly vulnerable to oxidation due to their high content of polyunsaturated fatty acids (PUFA). There are a number of means to modify these marine omega-3 oils in order to change their chemical and physical properties including randomization, blending and acidolysis. In this study, the effect of randomization using both chemical and enzymatic catalysts, as well as blending with antioxidant rich-wheat germ oil on the oxidative stability of seal blubber and menhaden oils was investigated. Meanwhile, lipase-catalysed acidolysis of *p*-coumaric acid with triolein, seal blubber and menhaden oils was carried out in this work, followed by examination of the antioxidant activities of the synthesized phenolic lipids in *in vitro* assays, food and biological systems. The results indicated that both chemical and enzymatic randomization lead to the redistribution of fatty acids among the stereoisomeric *sn*-1,3 and *sn*-2 positions of the glycerol moiety of triaclyglycerols of seal blubber and menhaden oils. The changes in oxidative stability were due to the loss of tocopherol and positional redistribution of fatty acids, especially the unsaturated fatty acids. Blending with wheat germ oil modified fatty acid composition and increased the tocopherol content of seal blubber and menhaden oils, hence improving the oxidative stability of blended seal blubber and menhaden oils, mainly due to the increased content of tocopherols, especially gamma- and delta-

tocopheols as the total content of unsaturated fatty acids before and after blending was similar. Therefore, wheat germ oil served as a good stabilizer for marine oils tested. Phenolic lipids derived from triolein, seal blubber oil and menhaden oil showed good antioxidant potential in systems tested. The prepared phenolic lipids exhibited high scavenging capacity towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxyl radicals, and displayed reducing power, strong inhibitory effect in bleaching β-carotene, human low-density lipoprotein (LDL) cholesterol oxidation as well as radical-induced DNA cleavage, which suggests that phenolic lipids derived from seal blubber and menhaden oils may be used as potential stable marine oils for health promotion and disease risk reduction.

ACKNOWLEDGEMENTS

My sincere appreciation is conveyed to my supervisor Dr. F. Shahidi for offering the opportunity and financial support to carry out graduate studies under his supervision. His encouragement and constructive suggestions throughout the course of this program and also in preparation of this thesis as well as other manuscripts are gratefully acknowledged. I would like to extend my thanks to Dr. R. Hooper and Dr. A. M. Martin for serving as my supervisory committee members and their encouragement. Technical assistance provided by Ms. Linda Winsor at the Centre for Chemical Analysis Research and Training (C-CART) and Mr. Craig Skinner of the Department of Biochemistry as well as statistical analysis assistance provided by Mr. Peter Earle from the Department of Biology are gratefully acknowledged. Special appreciation is extended to Dr. E. Reyes Suárez and Dr. J. Kralovec from Ocean Nutrition Canada Ltd. for their generous help for NMR analysis. I would also like to extend my appreciation to the Department of Biology and the School of Grduate Studies at Memorial University of Newfoundland. I am thankful to the fellow students in Dr. Shahidi's laboratory for creating a pleasant work environment. Special thanks to Ms. Shirley Kenny, Ms. Chris Everson and Ms. Shena Quiton from the general office of the Department of Biology and my friends from St. John's for their help and making this my second home. My sincere thanks are extended to my parents, sisters and brothers for their unconditional support, motivation and encouragement in all possible ways that helped me succeed.

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IMPORTANT NOTE

This thesis was prepared in the manuscript format, in which each chapter (Chapters 2-7) is an independent manuscript addressing a common issue. Repetition may occasionally exist, especially in the introduction sections, where the same background information may appear in each chapter. It is hoped that any repetition of this nature is not a cause of boredom as the results reported may compensate for it.

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LIST OF ABBREVIATIONS

AAPH	2,2′-azobis(2-aminopropane) dihydrochloride
ALA	Alpha-linolenic acid
APCI	Atmospheric pressure chemical ionization
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CCF	Central composite face-centred
CD	Conjugated dienes
CRMHO	Chemically randomized menhaden oil
CRSBO	Chemically randomized seal blubber oil
DHA	Docosahexaenoic acid
DHASCO	DHA single cell oil
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPA	Docosapentaenoic acid
DNA	Deoxyribonuclei acid
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
ERMHO	Enzymatically randomized menhaden oil
ERSBO	Enzymatically randomized seal blubber oil
FAMEs	Fatty acid methyl esters

GC	Gas chromatography
HNF	Hepatocyte nuclear factor
HPLC-MS	High performance liquid chromatography-Mass spectrometry
HUFA	Highly unsaturated fatty acids
LDL	Low-density lipoprotein
MAG	Monoaclygylcerols
MCFA	Medium-chain fatty acids
MDA	malondialdehyde
MHO	Menhaden oil
MUFA	Monounsaturated fatty acids
NF	Nuclear factor
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline
PG	Propyl gallate
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
RMCD	Randomly methylated β-cyclodextrin
ROS	Reactive oxygen species
RSM	Response surface methodology
SBO	Seal blubber oil
SREBP	Sterol-regulatory element binding protein

STD	Stearidonic acid
TAE	Tris-acetic acid–ethylenediaminetetraacetic acid
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tertiary-butylhydroquinone
TCA	Trichloroacetic acid
TE	Trolox equivalent
TLC	Thin-layer chromatographic plates
TMP	1,1,3,3-tetramethoxypropane
UV	Ultraviolet
UV-DAD	UV-diode array detector
WGO	Wheat germ oil

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CHAPTER 1

Introduction and overview

1. Marine oils

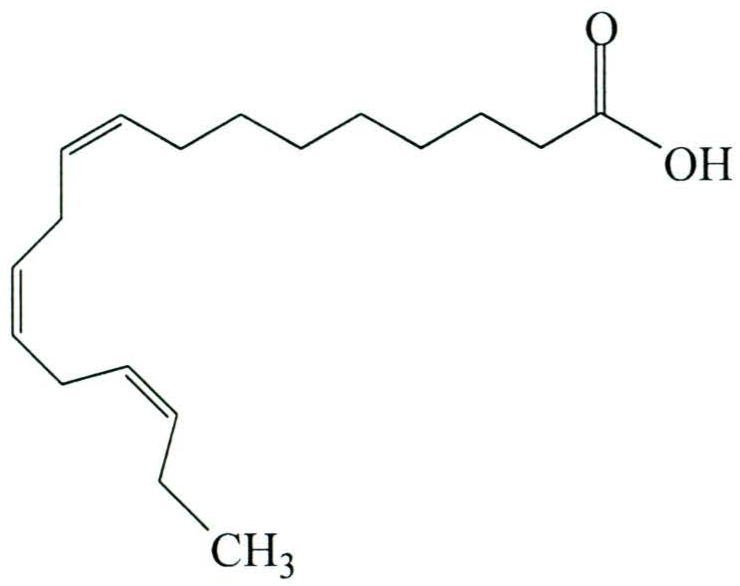
1.1 Chemistry and sources

Lipids perform important biological functions in the body, including serving as structural components in cell membranes, acting as storage and transport forms of metabolic fuel, serving as the protective coating on the surface of many organisms, and acting as cell signaling components (Christie 1982). In foods, they determine the sensory attributes such as flavour and texture properties and also contribute to their nutritional value. Omega-3 polyunsaturated fatty acids (PUFA) have their first carbon-carbon double bond located at the third carbon from the methyl end group of the molecule and subsequent double bonds are positionally located in a methylene-interrupted manner to the first double bond (Senanayake & Shahidi 2002). The chemical structures of naturally occurring omega-3 fatty acids are listed in Figure 1.1.

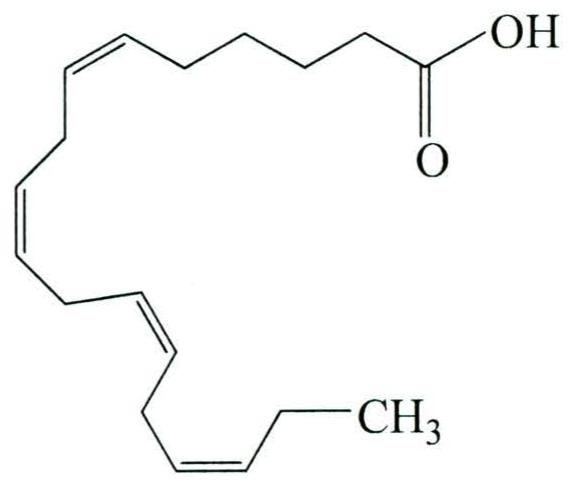
The commonly occurring omega-3 fatty acids include alpha-linolenic acid (ALA, C18:3ω3), stearidonic acid (STD; C18:4 ω3), eicosapentaenoic acid (EPA; C20:5 ω3), docosapentaenoic acid (DPA; C22:5 ω3) and docosahexaenoic acid (DHA; C22:6 ω3) (Brenna 2002). Among the omega-3 fatty acid series, ALA is considered as the parent molecule of STD, EPA, DPA and DHA. Omega-3 fatty acids are considered as essential fatty acids under certain conditions as they cannot be synthesized in the human body. ALA may be used to generate other long chain omega-3 fatty acids including STD, EPA,

DPA and DHA through a series of desaturation and elongation reactions. However, the conversion rate of ALA to other omega-3 fatty acids is limited in the human body, which is below 5 % (Brenna 2002). Therefore, supplementation of long chain omega-3 fatty acids, especially EPA and DHA, in the diet is recommended to maintain health and reduce disease risk.

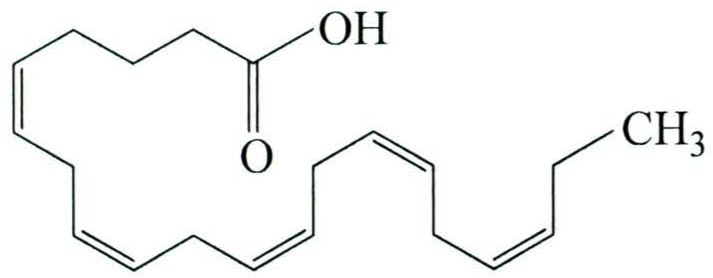
Omega-3 fatty acids can be obtained from plant, algal and marine animal sources. For instance, ALA is mainly found in certain plant seeds such as flaxseed (*Linum usitatissimum*). Flaxseed contains 32–45% (w/w) of oil, in which 51–55% (w/w) of its fatty acids occur as ALA. DHA has been found in algal oils such as DHA single cell oil (DHASCO) derived from microalgae-*Crypthecodinium cohnii*, and the total DHA is about 40% (w/w) of the single cell oil (Hamam & Shahidi 2004). The most common animal sources of omega-3 PUFA are the liver of white lean fish such as cod and halibut, the flesh of fatty fish such as menhaden, tuna and salmon, and the blubber of marine mammals such as seals and whales (Nwosu & Boyd 1996; Shahidi 2009; Wanasundara & Shahidi 1997). Seal blubber oil (SBO) contains typically 5.4-6.4% of EPA, 4.6-4.9% of DPA and 7.6-7.9% of DHA, while menhaden oil (MHO) contains 10.4-13.2% of EPA, 2.0-2.4% of DPA, and 10.1-11.5% of DHA (Wanasundra & Shahidi 1997; Senanayake & Shahidi 2002; In-Hwan Kim *et al.* 2006).



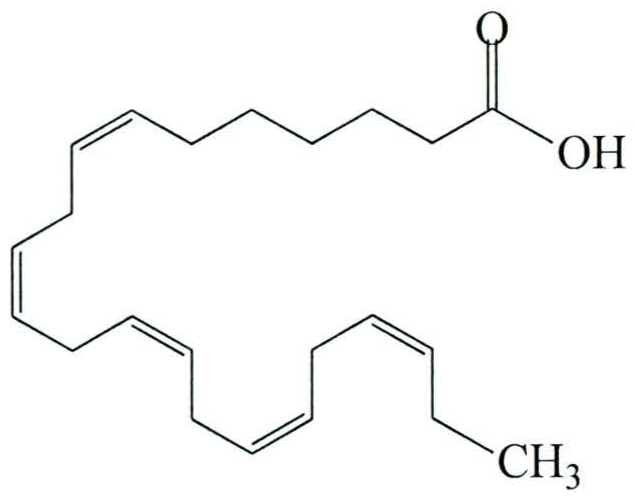
ALA



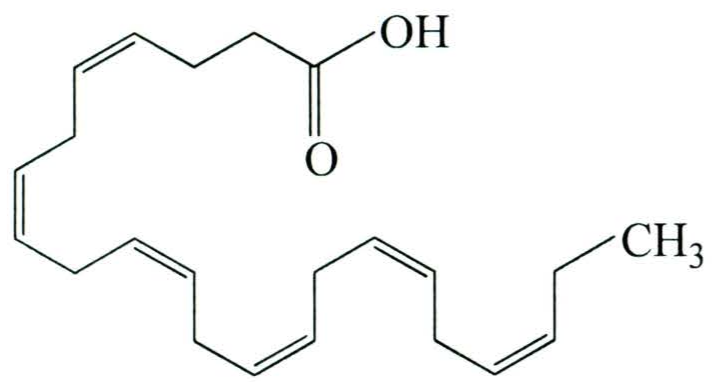
STD



EPA



DPA



DHA

Figure 1.1. Chemical structures of alpha-linolenic acid (ALA), stearidonic acid (STD), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).

1.2 Health benefits and biological significance

The omega-3 PUFA, especially EPA and DHA offer a wide range of health benefits and play a critical role in many functions in the human body. Their role in alleviating cancer (De Deckere 1999; Chen 2007), cardiovascular disease (Marchioli 2002; Wang *et al.* 2006), psychiatric disorders (Puri 2006; Nemets *et al.* 2006), Parkinson's disease (Bousquet 2008) and inflammatory ailments (Chandrasekara *et al.* 1995; Fisher *et al.* 1990; De Cateria & Zampolli 2001; Shearer *et al.* 2010) has been reported in the literature during the past three decades. For instance, DHA and its biological significance have been well recognized in supporting the normal development of the brain, eyes and nerves, especially for infants and fetuses. Therefore, addition of DHA in the diet of pregnant and lactating women as well as infant formulas is strongly recommended (Simopoulos 1991; Denomme 2005; Craig-Schmidt *et al.* 1996).

Omega-3 PUFA function mainly by exerting changes in membrane composition and eicosanoid production and in regulating gene expression (Sampath, 2005; Desvergne & Wahli, 1999). Omega-3 PUFA can profoundly alter the biochemical makeup of lipid rafts and caveolae membrane microdomains, thereby influencing cellular signaling (Ma et al, 2004). EPA serves as a precursor of secondary signaling intermediate-eicosanoids such as thromboxanes, prostacyclins and leukotrienes that are hormone-like substances and play a vital role in inflammation, regulating blood flow, immune response and ion transport, among others. In addition, EPA and DHA can be converted to other anti-inflammatory molecules, such as protectins, resolvins, maresins and omega-3-oxylipins, which may also explain the versatile health benefits of omega-3 oils (Serhan *et al.* 2008). Furthermore,

PUFA can act at the nuclear level, in conjunction with nuclear receptors and transcription factors, to affect the transcription of genes (Desvergne & Wahli, 1999). Several of these transcription mediators have been identified and these include nuclear receptors such as peroxisome proliferator-activated receptor (PPAR), hepatocyte nuclear factor (HNF)-4α, and liver X receptor (LXR) as well as transcription factors such as sterol-regulatory element binding protein (SREBP) and nuclear factor-κB (NFκB). Their interaction with PUFA has been shown to be critical in the regulation of several key genes in lipid metabolism. For instance, as one of PPAR isoforms, PPAR-a is the first transcription factor identified as a prospective fatty acid receptor and is expressed in hepatocytes, cardiomyocytes, renal proximal tubule cells, and enterocytes (Desvergne & Wahli, 1999). PPAR-a plays a role in the regulation of an extensive network of genes involved in glucose and lipid metabolism including fatty acid transport, fatty acid-binding proteins, fatty acyl-CoA synthesis, microsomal, peroxisomal, mitochondrial oxidation and ketogenesis. Therefore, PUFA are potent inducers of fatty acid oxidation and suppressors of fatty acid and TAG synthesis (Takada *et al.* 1994; Power & Newsholme. 1997; Clarke & Jump 1992).

1.3 Application in foods

Food lipids in the unprocessed form are principally triacylglycerols (TAG), which are found naturally in most biological materials. Fats and oils are one of the most useful functional ingredients and processing media (especially as cooking oil) in the food supply of the modern food industry. As nutrients, lipids, especially TAG, are a concentrated

caloric source providing metabolic energy, serve as an important source of essential fatty acids, and act as a solvent and absorption carrier for fat-soluble vitamins (A, D, E and K), carotenoids and organic contaminants (Kinsella 1988). Other than that, lipids occur as emulsion or as free oil/fat dispersed in a solid matrix, and contribute many desirable qualities, including flavour and texture to foods (Gunstone & Norris 1983; Gurr 1984).

The increasing awareness of the health benefits of omega-3 fatty acids has been reflected in the increased consumption of omega-3 oils, especially those from marine sources. More manufacturers have introduced products fortified with omega-3 oils into the market; menhaden oil was first approved as generally recognized as safe (GRAS) food ingredient in 1997 by the US government (Hernadez & Jong 2010). Later on, the use of omega-3 oils from other fish species, single cell algae and marine mammals in foods has been further extended. For instance, fish oil can be used in baked goods, snacks and cereals (Kadam & Probhasanka 2010). The low oxidative stability of marine omega-3 oils has been a major challenge for their application in foods. Therefore, food products fortified with omega-3 oils require low storage temperature, either refrigerated or frozen and short period of storage prior to use. Consequently, addition of antioxidants is required in order to stabilize the omega-3 fatty acids and extend the shelf-life of foods enriched with omega-3 oils (Kadam & Probhasanka 2010).

2. Lipid oxidation

Lipids, especially those possessing high levels of unsaturated fatty acids such as omega-3 oils, are unstable in food and biological systems and may readily undergo autoxidation as

well as photosensitized and enzymatic oxidation, all of which lead to their deterioration and produce oxidative compounds (Erickson 2002). The rate of lipid oxidation is dependent on several factors, including temperature, presence of inhibitors, catalysts, and the nature of the substrate, such as unsaturation level of fatty acids as well as presence of minor components (e.g. tocopherols) and storage conditions. One of the structural characteristics that make PUFA vulnerable to oxidation is that they possess at least one bisallylic methylene group. Carbon-hydrogen bond dissociation energies of fatty acids are lowest at bisallylic methylene positions, which are positions between adjacent double bonds (Koppenol 1990; Gardner 1989). A linear correlation exists between the number of bisallylic methylene positions and the oxidizability of lipids (Senanayake & Shahidi 2002; Cosgrove *et al.* 1987). Lipid oxidation occurring in food systems leads to rancidity as well as protein and pigment degradation. In biological systems, lipid oxidation changes membrane fluidity, and thus causes abnormal function and pathological processes in the cells (Choe & Min 2006; Jadhave *et al.* 1996).

2.1 Mechanisms of lipid oxidation

2.1.1 Autoxidation

Autoxidation is a free radical-induced process that takes place between molecular oxygen and unsaturated fatty acids. Autoxidation of unsaturated fatty acids occurs via a free radical chain mechanism consisting of basic steps of initiation, propagation and termination (Figure 1.2). The initiation step starts with abstraction of a hydrogen atom adjacent to a double bond in a fatty acid (RH) molecule/moiety, and this may be catalyzed

by light, or metal ions to form a lipid free radical, also named alkyl radical (Frank 1985). The resultant alkyl radical ($R^{\bullet}$) reacts with atmospheric oxygen to form an unstable lipid peroxyl radical ($ROO^{\bullet}$), which may in turn abstract a hydrogen atom from another unsaturated fatty acid to form lipid hydroperoxides (ROOH), including conjugated and nonconjugated hydroperxides and a new alkyl free radical. The new alkyl free radical initiates further oxidation and contributes to the chain reaction. The chain reaction (or propagation) may be terminated by the formation of non-radical products resulting from a combination of two radical species (Farmer et al, 1942). Transition metals and reactive oxygen species (ROS) are thought to play an important role in initiating free radical autoxidation.

Transition metals, such as iron and copper, are capable of initiating lipid oxidation through different mechanisms by either donating an electron or abstracting a hydrogen atom to generate alkyl radicals by reaction with unsaturated fatty acids. Transition metals are also involved indirectly with triplet oxygen (the normal general ground state of oxygen molecule) to generate superoxide radical, which leads the formation of more reactive oxygen species, such as peroxyl radical anion (Choe & Min 2006). In the other pathway, ferrous ions catalyze the conversion of hydrogen peroxide to hydroxyl anion and hydroxyl radical with the production of ferric ion (Ladikos & Lougovois 1990).

ROS, such as hydrogen peroxide, and hydroxyl radical, also participate in initiating lipid oxidation. Hydrogen peroxide is normally present as a metabolite at low concentrations in aerobic cells and is produced by mitochondia, microsomes, peroxisomes, and cytosolic enzymes (Tyler 1975; Oshio *et al.* 1973). Although it is not capable of reacting directly with unsaturated fatty acids since it is not a strong oxidant, it can be converted to a

hydroxyl radical, which attacks unsaturated fatty acids to form alkyl free radicals and initiates lipid oxidation (Koppenol & Liebman 1984).

2.1.2 Photosensitized oxidation

Photosensitizers such as chlorophylls, phenophytins, riboflavin, and myoglobin in foods and biological systems are capable of absorbing energy from light and transfer it to triplet oxygen to form singlet oxygen (Jung *et al.* 1995). Singlet oxygen is an electrophilic molecule and seeks electrons to fill its vacant molecular orbital, thus reacting with electron-rich double bonds of unsaturated lipid molecules, and forming conjugated and nonconjugated diene hydroperoxides (Figure 1.3) (Min & Lee 1999; Choe & Min 2006).

2.1.3 Enzymatic oxidation

Enzymes that catalyze the conversion of PUFA to fatty acid hydroperoxides are referred to as lipoxygenases. Oxidation products of these enzymes are similar to the hydroperoxides formed in the autoxidation of fatty acids (Figure 1.4). For instance, soybean lipoxyganese generates 13-octadecadienoate from linoleic acid and 15-hydroperoxyeicosatetraenoate from arachidonic acid. The oxidation of PUFA to the conjugated diene hydroperoxides includes two steps, of abstracting a hydrogen atom from a bisallylic group and subsequent addition of molecular oxygen, which may take place stereospecifically (Brash *et al.* 1987; Kernal *et al.* 1987).

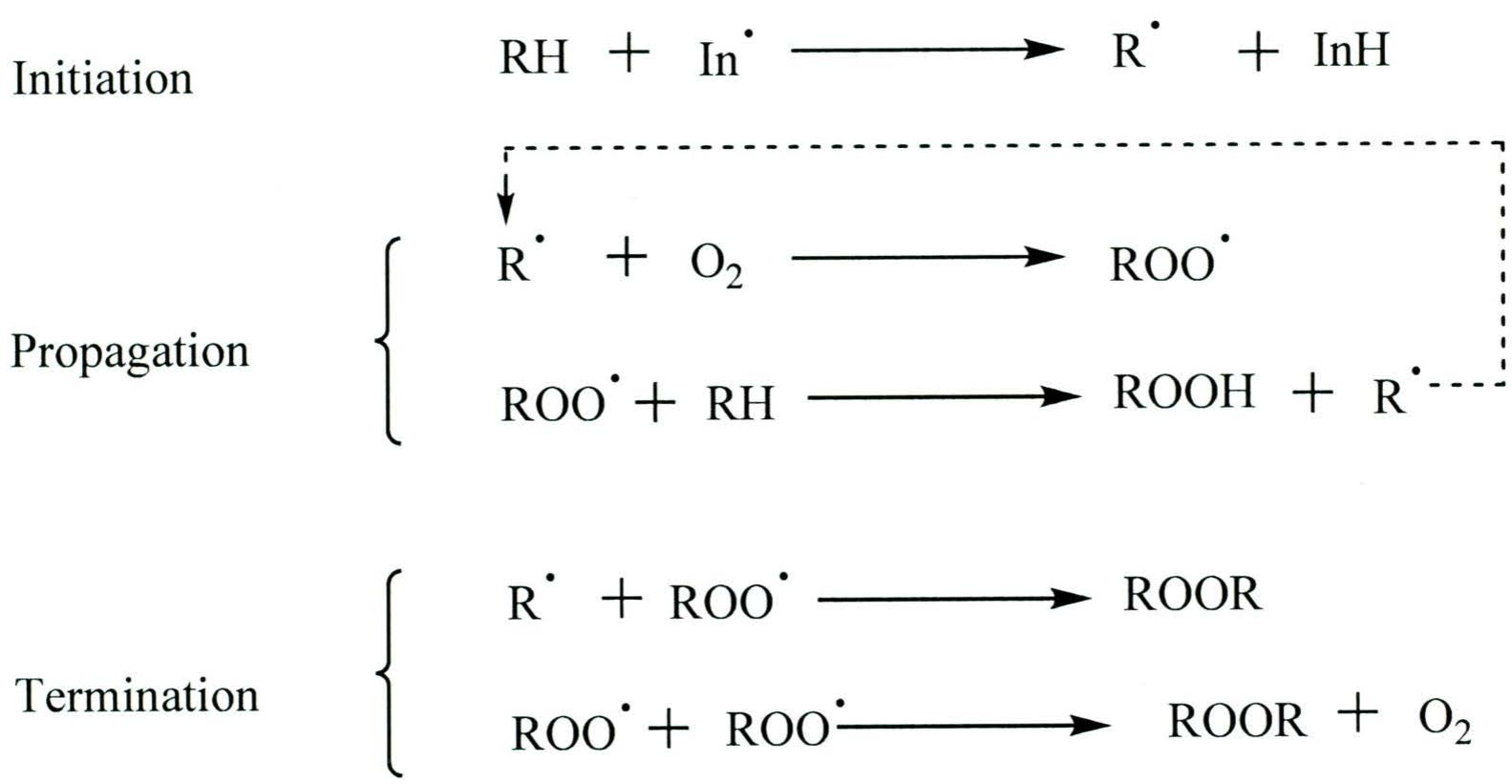


Figure 1.2. Schematic representation of free radical autoxidation of lipids; RH: fatty acid molecule; In: initiator; R•: alkyl radical; ROO•: lipid peroxyl radical; ROOH: lipid hydroperoxide; and ROOR: non-radical product.

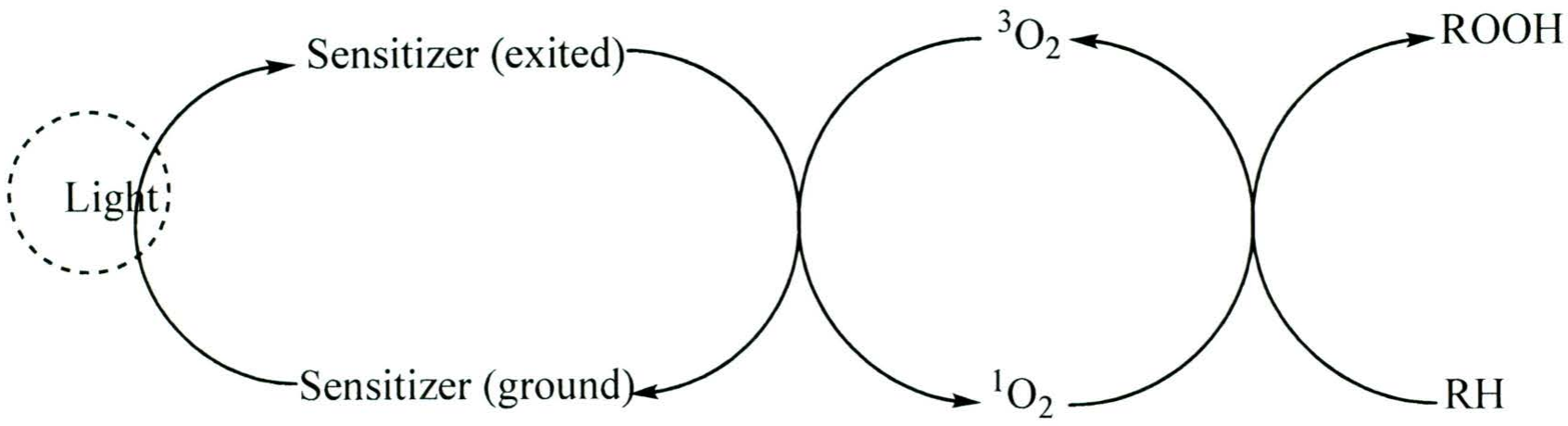


Figure 1.3. Schematic representation of photosensitized oxidation; $^3O_2$: ground state oxygen molecule; $^1O_2$: singlet oxygen molecule; RH: fatty acid molecule; and ROOH: lipid hydroperoxide.

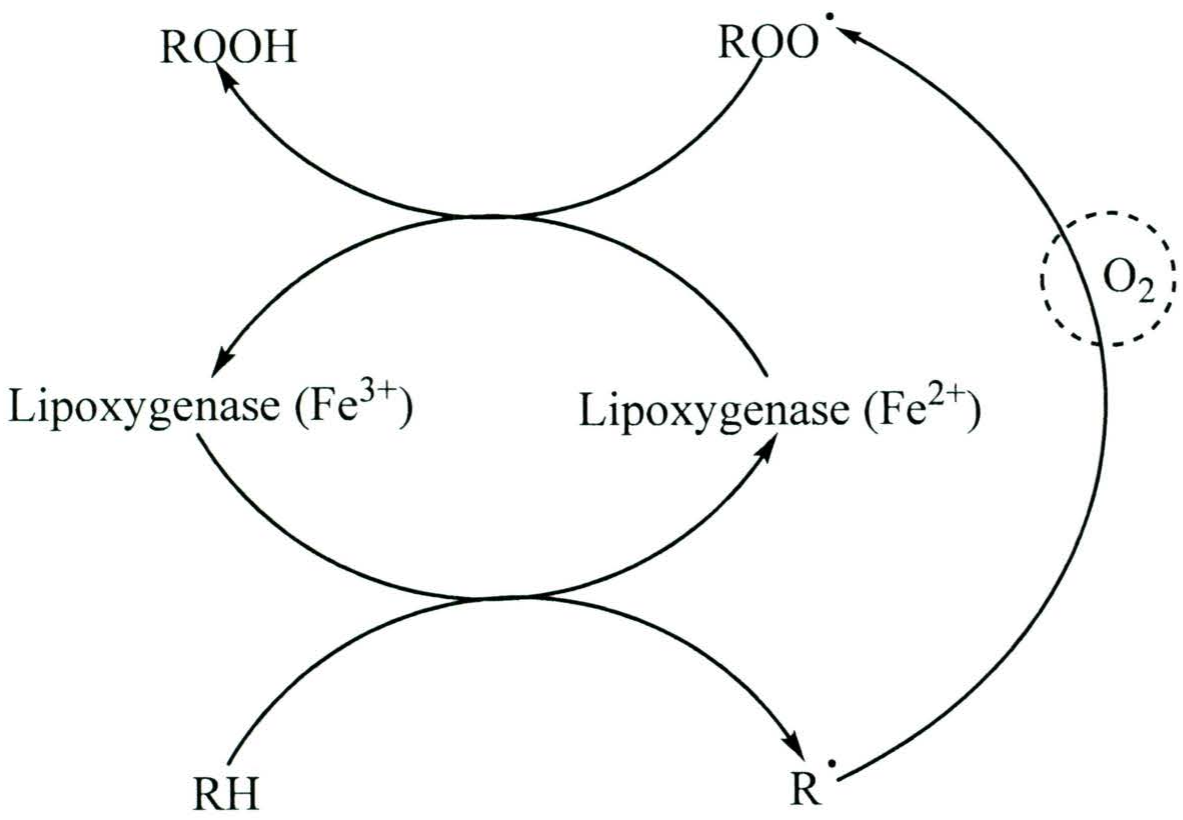


Figure 1.4. Schematic representation of enzymatic oxidation; RH: fatty acid molecule; $R^{\bullet}$: alkyl radical; $ROO^{\bullet}$: lipid peroxyl radical; and ROOH: lipid hydroperoxide.

2.2 Effect of lipid oxidation on food system

Oxidation can alter the flavour, colour, and nutritional quality of foods and produce toxic oxidation products, all of which can make food less acceptable or unacceptable to consumers (Min & Lee 1999). Primary lipid oxidation products, hydroperoxides, do not affect food flavour quality. However, they are unstable, and further degrade to secondary oxidation products. The breakdown products of lipid hydroperoxides, including alcohols, aldehydes, ketones, and hydrocarbons, are volatile, and generally possess off-flavours (Jung *et al.* 1995). These secondary oxidation compounds may interact with other food components and change their functional and nutritional properties (Choe & Min 2006). For instance, proteins undergo substantial oxidation in the presence lipid hydroperoxides and secondary oxidation products. Oxidation of proteins leads to changes in colour (browning), nutritive value (loss of essential amino acids), and food texture (decreased protein solubility) (Jadhave *et al.* 1996). Lipid oxidation products also react with natural food pigments, such as carotenoids, and cause discolouration of foods. In food of plant origin, oxidative browning of pigments is particularly undesirable as plant tissues are generally rich in natural pigments (Bak *et al.* 1999).

2.3 Control of lipid oxidation

Oxidation of lipid can degrade the quality of foods and produces toxic oxidation products (Min & Lee 1999; Choe & Min 2006). For many lipid-containing foods, special measures are taken to reduce or prevent oxidation, including removal of oxygen as well as photosensitizers, and addition of natural and/or synthetic antioxidants (Min & Boff 2002).

2.3.1 Removal of oxygen

The level of available oxygen that is a necessary substrate in the oxidation reaction of lipids may be controlled in foods by specific processing, such as using air-tight containers and as barrier packaging materials or modified atmosphere packaging that improves the stability of many lipid-containing foods. Oxygen scavengers, such as the glucose/glucose-oxidase/catalase system may be employed in foods as they are capable of reducing oxygen by converting it to water (Choe & Min 2006).

2.3.2 Removal of catalysts

As lipid oxidation catalysts, transition metal ions are present not only in raw food materials such as myoglobin and haemoglobin in muscle tissue (Livingston & Brown 1981), but also in potential additives from various exogenous sources including processing equipment, flavourants, colourants and packing materials. Metal contaminants must be complexed with effective chelating agents such as ethylenediaminetetraacetic acid (EDTA), desferrioxamine, diethylenetriaminepentaacetic acid (DTPA), and citric acid or polyphosphates which can significantly affect the kinetics of lipid oxidation catalyzed by transition metal ions (Choe & Min 2006; Gutterdige 1983; Hochstein 1981).

2.3.3 Addition of antioxidants

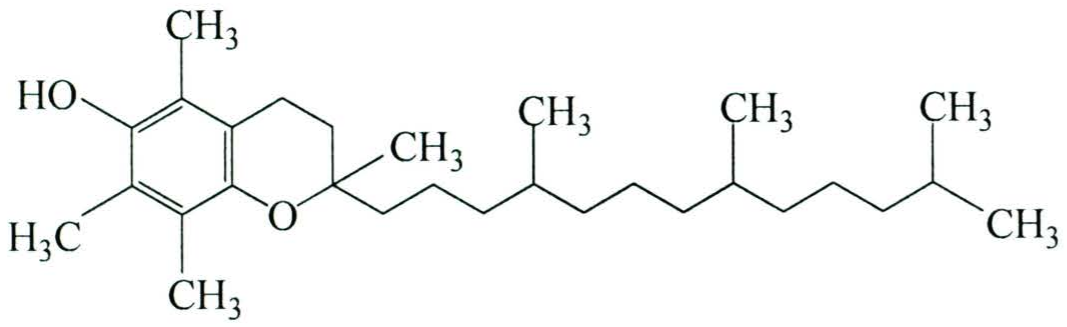
Natural antioxidants (Figure 1.5) such as ascorbic acid and tocopherols have commonly been employed as food additives to inhibit lipid oxidation in lipid-containing foods

(Jadhave 1996). Among these, ascorbic acid and tocopherols are the most important commercial natural antioxidants. However, these natural antioxidants may have several drawbacks, including high use level, undesirable flavour and/or colour contributions, and lower antioxidant efficiency compared to synthetic antioxidants (Reische *et al.* 2002).

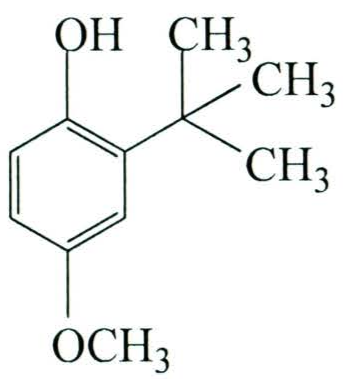
Synthetic antioxidants (Figure 1.5) such as tertiary-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), are commonly used to stabilize vegetable oils, as well as baked and fried foods. BHA and BHT are strong peroxyl radical quenchers and act as oxidation chain terminators and provide greater antioxidant activity when their mixture is used in food systems (Beilitz & Grosch 1987). BHA is effective in preventing oxidation of animal fats, but ineffective for vegetable oils. It also has good carry-through properties in baking but volatilizes during frying. It is commonly added to packaging materials (Rajalakshmi & Narasimhan 1996). TBHQ is generally considered to have higher efficiency in vegetable oils than BHA or BHT and is used in frying applications with highly unsaturated vegetable oils (Buck 1991). PG is a slightly water-soluble powder that is used in foods for which fat-soluble BHA, BHT, and TBHQ are not suitable. However, PG does not have good stability at high temperatures, and is not suitable for frying applications (Coppen 1983).

Phenolic acids have been extensively examined for their antioxidant activity and inhibition of lipid oxidation in foods (Ferguson *et al.* 2005; Kikugawa *et al.* 1983). The great antioxidant activity of *p*-coumaric acid (Figure 1.5), a common phenolic acid occurring in various food sources and beverages, including tomatoes, carrots, garlic as well as wines and vinegars in *in vitro* assays as well as in food and biological model systems has been widely reported (Andreasen *et al.*2001; Dávalos *et al.* 2004; Fukumoto

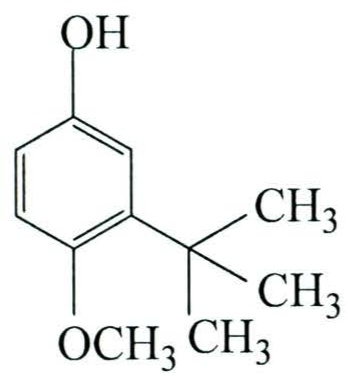
& Mazza 2000; Gadow *et al.* 1997; Kikugawa *et al.* 1983; Medina *et al.* 2007; Shahidi *et al.* 2012).



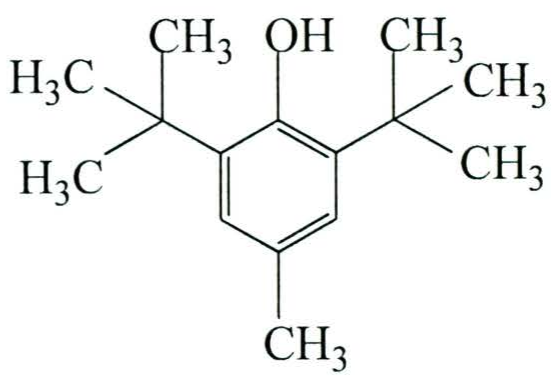
α-Tocopherol



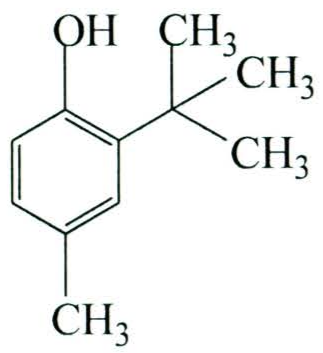
2-BHA



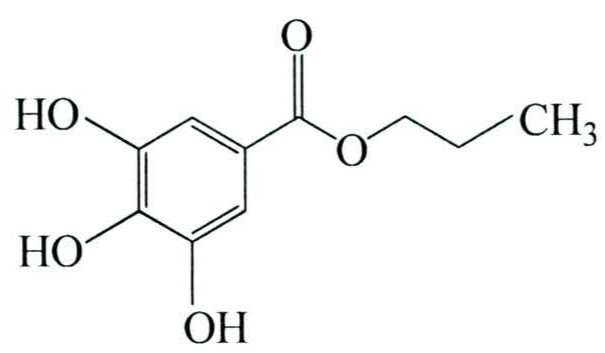
3-BHA



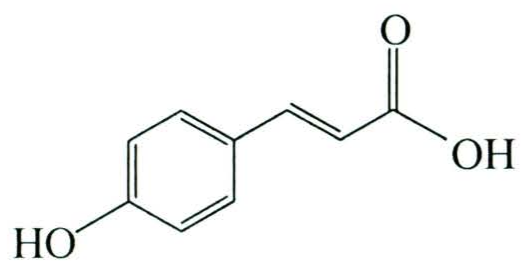
BHT



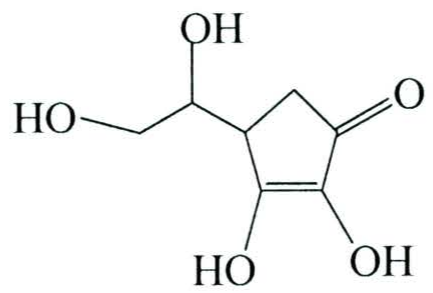
TBHQ



PG



p-Coumaric acid



Ascorbic acid

Figure 1.5. Chemical structures of natural and synthetic antioxidants, including α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butylhydroquinone (TBHQ), propyl gallate (PG), *p*-coumaric acid and ascorbic acid.

3. Modification of marine oils

Modified lipids are TAG modified to alter their fatty acid distribution on the glycerol backbone using a chemical or enzymatic process, including randomization (interesterification) and acidolysis. In addition, oil blending has also been employed to modify commonly used edible oils as well as marine omega-3 oils in the food and pharmaceutical industries to achieve desired chemical and physical properties as well as health benefits.

3.1 Randomization

Randomization is generally catalyzed by chemical catalysts such as sodium methoxide or enzymatic catalysts such as Novozyme 435 (Marangoni & Rousseau 1995; Rousseau & Marangoni 1997). Both chemical and enzymatic randomizations lead to positional redistribution of fatty acids. Positional redistribution of fatty acids in TAG occurs during intermolecular ester-ester interchange (Sreenivasan 1978). For instance, the chemical catalyst, sodium methoxide reacts with a TAG molecule to form a reaction complex (the true catalyst), enolate ion, which will further react with another TAG molecule with the same or different fatty acid composition to form a beta keto ester. Then, two TAG molecules with new fatty acid chains will be formed from the breakdown of the beta keto ester when the two fatty acids finish positional exchange from their joined structure (containing two TAG moieties). The interchanging process may continue until all fatty acids have changed positions and reaction reaches an equilibrium composition. However, the pathway of sodium methoxide catalyzed randomization is only a postulate without

solid experimental support (Sreenivasan 1978). The effect of chemical and enzymatic randomization on compositional distribution of fatty acids and oxidative stability in vegetable oils was studied by Tautorus and MacCurdy (1990). Both chemical and enzymatic randomization altered the positional distribution of determined fatty acids at different levels in canola, corn, linseed, soybean and sunflower oils, which caused slight changes of their oxidative stability. The insignificant changes of their oxidative stability were explained by the lower ratio of unsaturated to saturated fatty acids in these tested oils (Tautorus & MacCurdy 1990). Marine oils such as SBO and MHO present a high ratio of unsaturated fatty acids to saturated fatty acids; therefore, relocation of unsaturated fatty acids, especially omega-3 fatty acids in the TAG molecules caused by randomization may exert greater impact on their oxidative stability, as well as their absorption and deposition in the human body compared to vegetable oil containing lower unsaturated fatty acids. Effect of chemical and enzymatic randomization on positional distribution and stability of SBO and MHO will be explained in Chapter 2 and 3, respectively.

3.2 Blending

Oil blending has commonly been employed in the pharmaceutical and food industries to obtain desired functionality and oxidative stability of selected oils (Chu & Kung 1998). Omega-3 oils have been added to other oils in order to maximize the health benefits of the resultant formulations. For instance, blended oils from fish oil and flax seed oil are considered as new pharmaceutical products with maximized benefits from both (Harper

& Jacobson 2005). Palm oil has been used to enhance the stability of cooking oils such as canola oil, during food processing and high temperature deep-fat frying. In addition, oils with high PUFA content such as sunflower oil have been stabilized by blending them with tocopherol rich-oils such as rice bran oil (Mezouari & Eichner 2007). Omega-3 oils are vulnerable to oxidation due to their high PUFA content; however, their oxidative stability may be improved when blending with stable oils such as wheat germ oil (WGO). WGO is well known as a tocopherol-rich food lipid, which is procured from the germ of wheat kernel (Wang & Johnson 2001). Octacosanol, a 28 carbon long-chain saturated primary alcohol, has also been found as one of the minor components of wheat germ oil, which is believed to serve as a physical performance enhancing agent as well as for improving Parkinson's disease and lowering plasma cholesterol (Hargrove *et al.* 2004; Taylor & Rapport 2003). Addition of wheat germ oil to omega-3 oils such as SBO and MHO would be expected to improve their oxidative stability with the increased content of tocopherols, and it will be illustrated in Chapter 4.

3.3 Acidolysis

Modification of the structure of triacylglycerols can be used as an effective means to improve their nutritional and health benefits. Acidolysis is a process, which incorporates desired fatty acids or other forms of organic acids, such as phenolic acids, into triacylglycerols by using enzyme catalysts (Senanayake & Shahidi 2002). For instance, medium-chain fatty acids (MCFA) are incorporated into fish and seal blubber oils to obtain the structured lipid with desired functionality. MCFA esterified on terminal

positions of the glycerol backbone may offer protection to PUFA that are located on the middle position of the glycerol backbone, so these monoacylgylcerols (MAG) with PUFA can effectively be absorbed in the small intestine and transported to other parts of the body upon hydrolysis. Meanwhile, MCFA can be absorbed and used as a fast source of energy without being accumulated in the adipose tissues (Hamam & Shahidi 2004). In addition, lipase-catalyzed acidolysis of phenolic acids and triacylglycerols has been successfully conducted and reported in several studies (Safari *et al.* 2006; Sabally *et al.* 2006; Sabally *et al.* 2007).

Phenolic acids have been extensively examined for their antioxidant activity and inhibition of lipid oxidation in foods (Ferguson *et al.* 2005; Kikugawa *et al.* 1983). The antioxidant efficacy of phenolic acid such as *p*-coumaric acid and dihydrocaffeic acid has been demonstrated in *in vitro* assays as well as in food and biological model systems (Shahidi *et al.* 2010; Kikugawa *et al.* 1983; Fukumoto & Mazza 2000; Dávalos *et al.* 2004; Medina *et al.* 2006; Gadow *et al.* 1997; Andreasen *et al.* 2001; Sabally *et al.* 2007). Phenolic lipids, including monopalmityl dihydocaffeate, monoleyl dihydocaffeate, dieicosapentaenoyl dihydrocaffeate, eicosapentaenoyl dihydrocaffeate and docosahexaenoyl dihydrocaffeate, obtained from acidolysis of dihydrocaffeic acid with fish liver oil, showed strong radical scavenging ability, which is 50% of that of α-tocopherol (Sabally *et al.* 2007). Furthermore, phenolic lipids present better lipophilicity compared to that of phenolic acids and improved hydrophilicity compared to that of TAG, and such functionality may lead to strong antioxidant activity in food and biological systems. Thus phenolic lipids containing omega-3 fatty acids produced from acidolysis of phenolic acid with SBO and MHO would be ideal lipids with great antioxidant activity

and health benefits. These lipids may be used as potential ingredients in functional foods and supplements for health promotion and disease risk reduction. A model study using *p*-coumaric acid and triolein was first conducted in order to investigate the effects of various of experimental conditions on the degree of esterification of *p*-coumaric acid and to determine the proper synthesis conditions to be further employed in natural oils (Chapter 5), followed by the investigation of the antioxidant activity of synthesized phenolic lipids in *in vitro* tests, food and biological model systems (Chapter 6). Acidolysis of *p*-coumaric acid with SBO and MHO, followed by assessment of antioxidant activity of obtained phenolic lipid derivatives in selected systems will be explained in Chapter 7.

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CHAPTER 2

Effect of chemical randomization on positional distribution and stability of seal blubber and menhaden oils

Introduction

The omega-3 polyunsaturated fatty acids (PUFA), especially all cis-5,8,11,15,17-eicosapentaenoic acid (EPA) and all cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) offer a wide range of health benefits and play a critical role in many functions in the human body. Their role in alleviating cancer (De Deckere 1999; Chen 2007), cardiovascular disease (Marchioli 2002; Wang *et al.* 2006), psychiatric disorders (Puri 2006; Nemets *et al.* 2006), Parkinson's disease (Bousquet 2008) and inflammatory ailments (Chandrasekara *et al.* 1995; Fisher *et al.* 1990; De Cateria & Zampolli 2001; Shearer *et al.* 2010) have been well demonstrated in the literature during the past three decades. DHA and its biological significance have been well recognized in supporting the normal development of the brain, eyes and nerves, especially for infants and fetuses. Therefore, addition of DHA in the diet of pregnant and lactating women as well as infant formula is strongly recommended (Simopoulos 1991; Denomme 2005; Haag 2003; Craig-Schmidt *et al.* 1996). Furthermore, EPA serves as a precursor of eicosanoids, including thromboxanes, prostacyclins and leukotrienes that play a vital role in inflammation, regulating blood flow, immune response and ion transport, among others. In addition, omega-3 PUFA, especially EPA and DHA can be converted to other anti-inflammatory

molecules, such as protectins, resolvins, maresins and omega-3-oxylipins, which may also explain the versatile health benefits of omega-3 oils (Serhan *et al.* 2008).

The most common sources of omega-3 PUFA are the liver of white lean fish such as cod and halibut, the flesh of fatty fish such as menhaden, tuna and salmon, and the blubber of marine mammals such as seals and whales (Nwosu & Boyd 1996; Shahidi 2009; Wanasundara & Shahidi 1997). Seal blubber oil (SBO) contains typically between 5.4 and 6.4% EPA, 4.6-4.9% docosapentaenoic acid (DPA) and 7.6-7.9% DHA, while menhaden oil (MHO) contains 10.4-13.2% EPA, 2.0-2.4% DPA, and 10.1-11.5% DHA (Wanasundra & Shahidi 1997; Senanayake & Shahidi 2002; In-Hwan Kim 2006). The positional distribution of fatty acids, especially omega-3 PUFA in triacylglycerols (TAG) may affect their absorption and deposition in the human body, as well as influence their oxidative stability (Ando *et al.* 1992; Gunstone & Seth 1994). Omega-3 PUFA are primarily located at the terminal *sn*-1,3 positions of TAGs of marine mammals, including whale and seal oils. In fish oil, DHA is strongly enriched at the *sn*-2 position, whereas EPA varies. In fish oils with higher levels of EPA, the proportion of *sn*-2 is low (13-40%), whereas higher levels of *sn*-2 (up to 60%) are found for low EPA (<6%)-containing oils (Cho *et al.* 1987; Hsieh & Kinsella 1989).

Chemical redistribution of fatty acids in TAGs is a common means to change chemical and physical properties of the oils (Marangoni & Rousseau 1995; Rousseau & Marangoni 1997). The purpose of the present study was to study the effect of chemical redistribution on positional distribution of fatty acids in SBO and MHO, using gas chromatography (GC) and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. In addition, the effect of chemical randomization on the oxidative stability of the resultant oils was examined by

comparing the content of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) in the oils following accelerated oxidation at 60 °C for 3 days.

Materials and methods

Materials. Sodium methoxide, 2-thiobarbituric acid, $CDCl_3$, and tocopherol standards were obtained from Sigma-Aldrich (Mississauga, ON, Canada). Tocopherol standards, and standards of fatty acid methyl esters (FAMEs; GLC-461) were purchased from Nu-Check (Elysian, MN, USA). Porcine pancreatic lipase (EC 3.11.3), sodium taurocholate, and silica gel thin-layer chromatographic plates (TLC; 20 x 20 cm; 60 Å mean pore diameter, 2-25 μm mean particle size, 500 μm thickness, with dichlorofluorescein) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents used were analytical grade and were purchased from Fisher Scientific (Nepean, ON, Canada).

Sodium methoxide-catalyzed chemical redistribution of SBO and MHO. Chemical redistribution was carried out following the method of Rousseau *et al.* (1996) with minor modifications. The starting oil (25 g) was heated under a nitrogen blanket in a 250-ml round-bottom flask at 85 °C. Sodium methoxide (0.5%; w/w of oil sample) was added to the oil to initiate the reaction. The temperature of the oil was increased to 88 °C ± 1 under a nitrogen blanket, and kept there for 60 min. Sodium methoxide was removed after acidification with citric acid (20%, w/v), and then the mixture was washed with a sodium bicarbonate (0.05 M) solution to neutralize the residual acids. The soaps so produced were removed by hot water. Residual water and colorants were removed by adding

bentonite clay (1.5 %, w/w), and the recovered oils were stored at -20 °C until use within a month.

Positional distribution of fatty acids by GC analysis and ^{13}C NMR spectroscopy.

(a) Selective hydrolysis using pancreatic lipase. The oil samples were hydrolyzed using pancreatic lipase as described by Christie (1982), with minor modifications. The oil (25 mg) was weighed into a glass tube, and then 5.0 ml Tris-HCl buffer (1.0 M, pH 8.0), 0.5 ml of calcium chloride (2.2%) and 1.25 ml of sodium taurocholate (0.05%) were added. Porcine pancreatic lipase (5.0 mg; EC 3.11.3) was added to the mixture after it was kept in a water bath for 5.0 min at 40°C. The glass tube was subsequently placed in a gyratory water bath shaker at 250 rpm under a blanket of nitrogen for 1 h at 40°C. The enzymatic reaction was stopped by adding 5.0 ml of ethanol, followed by the addition of 5.0 ml of 6.0 M HCl. At the end of the reaction, fatty acids from terminal positions are hydrolyzed, and in monoacylglycerols and free fatty acids are produced.

(b) Extraction and separation of hydrolytic products. Diethyl ether (50 ml in total) was used to extract the hydrolytic products three times, which were then washed twice with distilled water and dried over anhydrous sodium sulphate followed by removal of the solvent under reduced pressure at 30°C. The hydrolytic products were separated on silica gel TLC (thin layer chromatography) plates (20×20 cm, 60Å mean pore diameter, 2–25 μm mean particle size, 500 μm thickness, with dichlorofluorescein, Sigma, Mississauga, ON, Canada). The plates were developed using a mixture of hexane-diethyl ether-acetic acid (70:30:1, v/v/v) for 45-55 min. The bands were located by viewing under short

wavelength (254 nm) ultraviolet (UV) light (Spectraline, Model ENF-240C, Spectronics Co., Westbury, NY, USA). The free fatty acid bands were scraped off and lipids extracted into diethyl ether, which were subsequently used for fatty acid analysis as described by Senanayake & Shahidi (2000).

(c) Fatty acid compositional analysis of hydrolytic products by GC. Fatty acid composition and positional distribution of the products were determined by their conversion to the corresponding methyl esters. The transmethylation reagent (2.0 ml) consisting of freshly prepared 6% sulphuric acid in methanol containing 15 mg of hydroquinone as an antioxidant was added to the sample vial, followed by vortexing. The mixture was incubated for 24 h at 60°C and subsequently cooled to ambient temperature. Distilled water (1.0 ml) was then added to the mixture, after thorough mixing, and hydroquinone was added to each vial to prevent oxidation. The FAMEs were extracted three times, each with 1.5 ml of high performance liquid chromatography (HPLC)-grade hexane. The combined hexane layers were then transferred to the test tube and washed twice with 1.0 ml distilled water. The hexane was evaporated under a stream of nitrogen, and the extracted FAMEs were then dissolved in 1.0 ml of carbon disulphide and used for subsequent GC analysis. A Hewlett-Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a SUPELCOWAX-10 column (30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON, Canada) was used to analyze the FAMEs. The oven temperature was first raised to 220°C and kept there for 10.25 min and then raised up to 240°C at 30°C/min and held there for 15 min. The injector and FID temperatures were 250°C. Ultra high purity helium was used as a

carrier gas at a flow rate of 15 ml/min. A Hewlett-Packard 3365 Series II ChemStation Software (Agilent) was used for data handling and processing. The FAMEs were identified by comparing their retention times with those of a known standard mixture. Positional distribution of fatty acids at *sn*-1, 3 positions was calculated as: (% fatty acid at *sn*-1, 3 positions/% fatty acid in triacylglycerols)*100.

(d) Fatty acid compositional analysis of hydrolytic products by ^{13}C NMR spectroscopy. Quantitative ^{13}C NMR spectra of natural oils and their chemically randomized counterparts were recorded under continuous ^{1}H decoupling at 20 ^{o}C at a ^{13}C frequency of 125 MHz using a Bruker Avance-500 MHz spectrometer on 100 mg samples dissolved in 0.7 ml 99.8% $CDCl_3$. Experimental parameters were: 128K complex data points, spectral width 33,784 Hz (268 ppm), 15s relaxation delay with collection of 1000 scans. The spectra were Fourier transformed and processed using the standard procedures of resolution and sensitivity enhancement. Carbonyl peak integrals were measured accurately using the standard deconvolution algorithm from TopSpin V 2.1 software, assuming a pure Lorentzian lineshape. In all spectra, ^{13}C chemical shifts were expressed in parts per million (ppm) relative to $CDCl_3$ at 77.16 ppm (Gottlieb 1997).

(f) Determination of tocopherol content by HPLC-MS. Seal blubber oil and menhaden oil as well as their randomized counterparts were saponified according to the procedure described by Maguire *et al.* (2004), with some modification (a method described in Chapter 4 allows direct determination tocopherol contents in seal blubber oil and menhaden oil without saponification, which indicates higher accuracy). The oil (0.5 g)

was mixed thoroughly with 1 ml of 60% KOH (w/v) and 4 ml of ethanolic pyrogallol (w/v) in screw-capped tubes fitted with Teflon lined caps. Saponification progressed at 70°C in a water bath for 45 min. The tubes were then cooled in ice and unsaponified components were extracted three times with 2 ml of hexane. Hexane extracts were evaporated under nitrogen flush and redissolved in 2 ml hexane. Tocopherol standards were prepared by dissolving a known tocopherol mixture in hexane and then by diluting to different concentrations. Prior to HPLC analysis, both samples and standards were filtered using a 0.45 μm syringe-filter (Whatman, Clifton, NJ, USA).

Tocopherol content in prepared samples and standards was determined by normal phase high performance liquid chromatography (HPLC) – mass spectrometry (MS). The analysis was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) with a UV-diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm × 4.6 mm i.d., 5 μm, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) coupled with a Supelcosil LC-Si guard column. Tocopherols were eluted using an isocratic solvent system containing hexane-diethyl ether (99:1, v/v) at a flow of 1.0 ml/min. Each tocopherol standard and sample (80 μl) were injected using an auto-sampler. Tocopherols were detected at 295 nm by a UV detector and identified by comparing their retention times with those of known tocopherol standards. LC flow was analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) using positive ion APCI (atmospheric pressure chemical ionization). The operating conditions were 121 V for the fragmentor voltage, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, drying

gas flow of 7 l/min, A standard curve was constructed for each tocopherol homologue (peak area versus concentration). Tocopherol concentrations in samples were obtained from the standard curve, and expressed as mg alpha-tocopherol per 100 g oil.

Accelerated oxidation of SBO and MHO. Oxidative stability of randomized SBO and MHO as well as their original counterparts was determined under Schaal oven conditions at 60°C for 72 h. Each day (24 h) of storage under such conditions is equaivalent to 1 month of storage at ambient temperatures. Oils (0.4–0.5 g) were placed in loosely capped test tubes (10 mm diameter and 4.0 cm height) and stored at 60°C in a forced-air oven (Thelco, Model 2; Precision Scientific Co., Chicago, IL, USA). Samples were removed from the oven at 0, 6, 12, 24, 48, 72 h, cooled to room temperature, flushed with nitrogen, capped, and stored at −20°C until analyzed.

Oxidative stability assays

(a) Conjugated dienes (CD) test. The experiments were carried out in triplicate. CD in the oils was determined according to the IUPAC (1987) method 20505. Oil samples (0.02–0.04 g) were weighed into 50-ml volumetric flasks, dissolved in iso-octane, and made up to the mark with the same solvent. The contents were thoroughly mixed, and the absorbance was read at 234 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent).

(b) TBA test. The determination of TBARS was carried out as described by the AOCS (1990) method Cd 19-90. A series of 1,1,3,3-tetramethoxypropane (TMP) standard

solution with different concentrations was prepared and then transferred to the screw-capped tubes that contain 5 ml of 2-thiobarbituric acid (TBA) reagent. The screw-capped tubes were then kept in hot water bath (95°C) for 2 h. After cooling screw-capped tubes in the ice bath, the absorbance of solutions was read at 532 nm using a Hewlett-Packard diode array Model 8452 A spectrophotometer (Agilent), which was used to construct a standard curve. Samples (0.05–0.10 g) were weighed into 25-ml volumetric flasks, dissolved in a small volume of 1-butanol, and then made up to the mark with the same solvent. This solution (5 ml) was transferred into a screw-capped test tube, and then 5 ml of freshly prepared TBA reagent (0.5 g TBA in 250 ml n-butanol) were added. The mixture was thoroughly mixed, and then the test tube was placed in a water bath at 95 °C for 2 h. Heated samples were cooled in an ice bath, and the absorbance of the resultant coloured complex was read at 532 nm. TBARS values in oils were obtained using the standard curve as μmol malonaldehyde equivalent/g of oil.

Statistical analysis and Data Interpretation. Paired-samples *t*-tests were performed at a $P<0.05$ level using Statistical Package for the Social Sciences (SPSS) to assess for the significant differences. Data were expressed as mean ± s.d., (n = 3).

Results and discussion

Fatty acid positional distributions by GC analyses. The positional distribution of the major fatty acids of SBO, MHO and their chemically randomized counterparts CRSBO

and CRMHO, respectively, calculated from the fatty acid profiles of hydrolytic products, are shown in Tables 2.1 and 2.2, respectively.

Fatty acid positional distributions of seal bubbler oil before and after chemical randomization. In the original SBO, unsaturated fatty acids, especially PUFA such as C18:2ω6, and those of the omega-3 class C18:3, C18:4, C22:5 and C22:6 were preferentially located at the *sn*-1,3 positions (Table 2.1). The important omega-3 fatty acid C20:5 was distributed nearly evenly among *sn*-1,3 (68%) and *sn*-2 positions (32%, Table 2.1). It is worth noticing that equal distribution in this work is defined as a sn-1,3 regiospecificity of 66.67 mole %, and at a sn-2 regiospecificity of 33.33 mol%.

Saturated residues are assembled differently in TAG of the original SBO; for instance, C18:0 residues reside mostly at the terminal *sn*-1,3 positions (93%), C16:0 appears to be close to equally distributed, whereas C14:0 showed a slight preference for the *sn*-2 position (41%).

The monounsaturated fatty acids C17:1 and C18:1n-9 appeared to be nearly equally distributed (66 and 64% at *sn*-1,3 positions) at terminal positions and the middle position. The other monounsaturated residues C14:1ω9, C16:1ω7 and C18:1ω11 were in slight excess on the *sn*-2 positions, judging from the *sn*-2 area percentages which were close to 43% in each case (Table 2.1).

PUFA were more equally distributed in the glycerol backbone of the TAGs of chemically randomized seal blubber oil, as a result of relocation from the *sn*-1,3 positions to the *sn*-2 position (Table 2.1). For instance, 9% of each of C18:4ω3 and C22:5ω3 chains were relocated from the *sn*-1,3 positions to the *sn*-2 position (Table 1). The proportion of *sn*-1,3 chains of C18:3ω3 and C22:6ω3 decreased 3 and 5%, respectively, and from the

resulting *sn*-1,3 area percentages of 76 and 71%, respectively, it was concluded that these fatty acids remained attached preferentially to the *sn*-1,3 positions in the chemically randomized seal blubber oil (CRSBO, Table 2.1).

The chemical randomization also redistributed C18:2ω-6 chains, relocating them from mostly at the *sn*-1,3 positions (77%) in natural SBO to equally distributed in CRSBO (67% *sn*-1,3). Meanwhile, C20:5ω3 moieties that were distributed evenly among *sn*-1,3 (67%) and *sn*-2 (33%) positions in natural SBO were relocated (12%) to the *sn*-2 position (44% area *sn*-2) upon chemical randomization (CRSBO, Table 2.1.).

The positional distributions of saturated residues were affected in different fashions upon chemical randomization. For instance, palmitic acid (C16:0) residues had significant increase at the terminal positions in CRSBO (Table 2.1); C14:0 residues increased (14%) at the terminal *sn*-1,3 positions (to 63% area), whereas the proportion of *sn*-1,3 chains of C18:0 decreased 15%, although from the resultant area% (78%), it remained attached preferentially to the *sn*-1,3 positions.

For the most part, monounsaturated residues increased by 5-8% at the terminal *sn*-1,3 positions, with the exception of C18:1ω9 which remained unaffected, and of C17:1 that was relocated (4%) from the terminal *sn*-1,3 positions to the *sn*-2 position.

All of these changes on the positional distribution of fatty acids in SBO resulted from intermolecular ester-ester interchange (Sreenivasan 1978). During the randomization process, the chemical catalyst, sodium methoxide reacts with a triacylglycerol molecule to form a reaction complex (the true catalyst), enolate ion, which will further react with another triacylglycerol molecule with the same or different fatty acid compositions to form a beta keto ester. Then, two triacyglycerol molecules with a new fatty acid chain

will be formed from the breakdown of the beta keto ester when the two fatty acids finish their positional exchange from their joined structure. The interchanging process may continue until all fatty acids have changed positions and the reaction has reached an equilibrium composition. However, the pathway of sodium methoxide catalyzed randomization is only a postulate without solid experimental support (Sreenivasan 1978).

Table 2.1. Percent fatty acid distribution of total triacylglycerols located at *sn*-1,3 and *sn*-2 positions of the original seal blubber oil and its chemically randomized counterpart calculated by GC analysis.

Fatty acid	*sn*-1,3[A]		*sn*-2[B]	
	SBO	CRSBO	SBO	CRSBO
C14:0	58.70 ± 0.51[a]	62.56 ± 0.19[b]	41.30 ± 0.51	37.44 ± 0.19
C14:1ω9	58.44 ± 0.40[a]	63.03 ± 0.16[b]	41.56 ± 0.40	36.97 ± 0.16
C16:0	64.16 ± 0.35[a]	66.25 ± 0.06[b]	35.84 ± 0.35	33.75 ± 0.06
C16:1ω7	57.38 ± 0.78[a]	64.69 ± 0.38[b]	42.62 ± 0.78	35.31 ± 0.38
C17:1	66.41 ± 2.22	62.06 ± 0.21	33.59 ± 2.22	37.94 ± 0.21
C18:0	93.00 ± 1.31[a]	77.63 ± 0.02[b]	7.00 ± 1.31	22.37 ± 0.02
C18:1ω9	63.80 ± 0.56[a]	65.84 ± 0.07[b]	36.20 ± 0.56	34.16 ± 0.07
C18:1ω11	56.61 ±1.76[a]	62.20 ± 0.06[b]	43.39 ± 1.76	37.80 ± 0.06
C18:2ω6	77.17 ± 1.48	67.41 ± 0.88	22.83 ± 1.48	32.59 ± 0.88
C18:3ω3	79.26 ± 2.56	75.76 ± 1.46	20.74 ± 2.56	24.24 ± 1.46
C18:4 ω3	70.58 ± 0.89	62.07 ± 0.64	29.42 ± 0.89	37.93 ± 0.64
C20:1ω9	68.50 ± 0.28	68.49 ± 0.23	31.50 ± 0.28	31.51 ± 0.23
C20:5ω3	68.44 ± 1.09[a]	55.98 ± 0.13[b]	31.56 ± 1.09	44.02 ± 0.13
C22:1ω11	67.34 ± 2.30	80.09 ± 0.21	32.66 ± 2.30	19.91 ± 0.21
C22:5ω3	90.16 ± 2.48[a]	80.51 ± 0.01[b]	9.84 ± 2.48	19.49 ± 0.01
C22:6ω3	76.11 ± 1.06	70.98 ± 0.15	23.89 ± 1.06	29.02 ± 0.15

SBO: seal blubber oil before randomization

CRSBO: seal blubber oil after randomization

[A]: (% fatty acid at the *sn*-1,3 positions/% fatty acid in triacylglycerols)*100

[B]: (% fatty acid at the *sn*-2 position/% fatty acid in triacylglycerols)*100

Values across a row not sharing a common superscript letter are significantly different from one another ($P<0.05$)

Fatty acid positional distributions of menhaden oil before and after chemical randomization. In the original MHO, the PUFA C18:2ω6 and C18:3ω3 were esterified preferentially at the *sn*-1,3 positions of glycerol, as judged from the *sn*-1,3 area percentages of 83 and 75%, respectively. The other omega-3 PUFA, C18:4ω3, C20:5ω3, C22:5ω3 and C22:6ω3 were attached preferentially at the *sn*-2 position (Table 2.2).

Of the three major saturated fatty acids of MHO, C14:0 and C16:0 were equally distributed among the middle and terminal positions, and the third one C18:0 resided mostly at the *sn*-1,3 positions (Table 2.2). Of the six monounsaturated fatty acids analyzed, five displayed *sn*-1,3 area percentages of 70% or higher and the sixth one (C17:1) was close to evenly distributed (65% *sn*-1,3).

Although the PUFA distribution was not largely affected by the randomization process, higher changes were observed for C18:3ω3, C18:2ω6 and C22:6ω3. In the first two, their proportions at *sn*-1,3 positions decreased by 7 and 5%, respectively. For C22:6ω3, its proportion at *sn*-1,3 positions increased by 5%. Relocation of C18:3ω3 residues from the *sn*-1,3 positions to the *sn*-2 position resulted in an equal distribution; C18:2 remained mostly attached to the *sn*-1,3 positions (79%), whereas C22:6ω3 residues increased to 55.6% at the *sn*-1,3 positions upon redistribution. With the exception of C18:2ω6, the *sn*-1,3 area% decreased by 4%; the distribution of the remaining omega-3 acids C18:4ω3, C20:5ω3 and C22:5ω3 remained almost unaffected upon chemical randomization.

The equal distribution of the saturated chains C14:0 and C16:0 in original oil was changed upon chemical randomization to a distribution of a slight (5%) higher at the *sn*-1,3 positions in randomized oil. Meanwhile, 6% of C18:0 residues that resided mostly at the *sn*-1,3 positions of MHO were relocated to the *sn*-2 position, but the overall statistical

distribution was unchanged. On chemical randomization, almost 20% of the monounsaturated residue C20:1ω9 were relocated from the *sn*-1,3 positions to the *sn*-2 position, although from the resultant *sn*-1,3 area percentage of 70, this fatty acid remained attached mostly to the terminal *sn*-1,3 positions. The positional distribution of the remaining monounsaturated fatty acids remained almost unchanged.

Table 2.2. Percent fatty acid distribution of total triacylglycerols located on *sn*-1,3 and *sn*-2 positions of the original menhaden oil and its chemically randomized counterpart calculated by GC analysis.

Fatty acid	*sn*-1,3[A]		sn-2[B]	
	MHO	CRMHO	MHO	CRMHO
C14:0	67.97 ± 0.67[a]	73.16 ± 0.26[b]	32.03 ± 0.67	26.84 ± 0.26
C14:1ω9	69.88 ± 0.71	71.9 ± 0.91	30.12 ± 0.71	28.1 ± 0.91
C16:0	67.22 ± 0.64[a]	71.52 ± 0.24[b]	32.78 ± 0.64	28.48 ± 0.24
C16:1ω7	69.68 ± 0.65	73.3 ± 0.16	30.32 ± 0.65	26.7 ± 0.16
C17:1	65.26 ± 0.30	64.16 ± 0.29	34.74 ± 0.30	35.84 ± 0.29
C18:0	78.78 ± 0.34	73.46 ± 0.39	21.22 ± 0.34	26.54 ± 0.39
C18:1ω9	76.37 ± 0.28	73.92 ± 0.04	23.63 ± 0.28	26.08 ± 0.04
C18:1ω11	76.07 ± 1.47	76.03 ± 0.82	23.93 ± 1.47	23.97 ± 0.82
C18:2ω6	83.35 ± 0.47	78.67 ± 0.03	16.65 ± 0.47	21.33 ± 0.03
C18:3ω3	74.69 ± 1.33	67.6 ± 0.83	25.31 ± 1.33	32.4 ± 0.83
C18:4ω3	60.27 ± 0.61	57.83 ± 0.32	39.73 ± 0.61	42.17 ± 0.32
C20:1ω9	88.6 ± 1.69[a]	70.16 ± 0.42[b]	11.4 ± 1.69	29.84 ± 0.42
C20:5ω3	49.46 ± 0.13	47.58 ± 0.13	50.54 ± 0.13	52.42 ± 0.13
C22:5ω3	56.81 ± 1.49	55.24 ± 0.82	43.19 ± 1.49	44.76 ± 0.82
C22:6ω3	50.97 ± 1.06[a]	55.61± 0.92[b]	49.03 ± 1.06	44.39 ± 0.92

MHO: menhaden oil before randomization

CRMHO: menhden oil after randomization

[A]: (% fatty acid at the *sn*-1,3 positions/% fatty acid in triacylglycerols)*100

[B]: (% fatty acid at the *sn*-2 position/% fatty acid in triacylglycerols)*100

Values across a row not sharing a common superscript letter are significantly different from one another ($P<0.05$)

Fatty acid positional distributions by ^{13}C NMR analyses.

Fatty acid positional distributions of seal blubber oil before and after chemical randomization. The NMR spectral analysis of original SBO indicated that the omega-3 PUFA C18:4, C22:5 and C22:6 were attached only to the terminal *sn*-1,3 positions and none of them were found at the middle *sn*-2 position (Figure 2.1). These results differ from those obtained by GC analysis that revealed that these omega-3 PUFA were preferentially, but not uniquely located in the terminal *sn*-1,3 positions (Table 2.1). From GC analysis it was established that the omega-3 acid C20:5 was evenly distributed among terminal and middle positions (Table 2.1). However, NMR spectral data revealed that C20:5 was attached only to the *sn*-1,3 positions of MHO (Figure 2.1).

In chemically randomized SBO, both GC and NMR spectral analysis revealed that the omega-3 acids C18:4, C20:5, C22:5 and C22:6 were distributed more equally in the middle and terminal positions (Figure 2.1 and Table 2.1). In fact, NMR spectral analysis indicated that, with the exception of C22:6, half of the total amount of these acids was relocated to the middle *sn*-2 position on chemical randomization (Figure2.1). For C22:6, the amounts of *sn*-1,3 moieties relocated to the *sn*-2 position were lower (37%) comparing to that of other fatty acids. With the exception of C22:6, the omega-3 PUFA in CRSBO displayed a preference for the *sn*-2 position; C22:6 was nearly equally distributed (Figure 2.1).

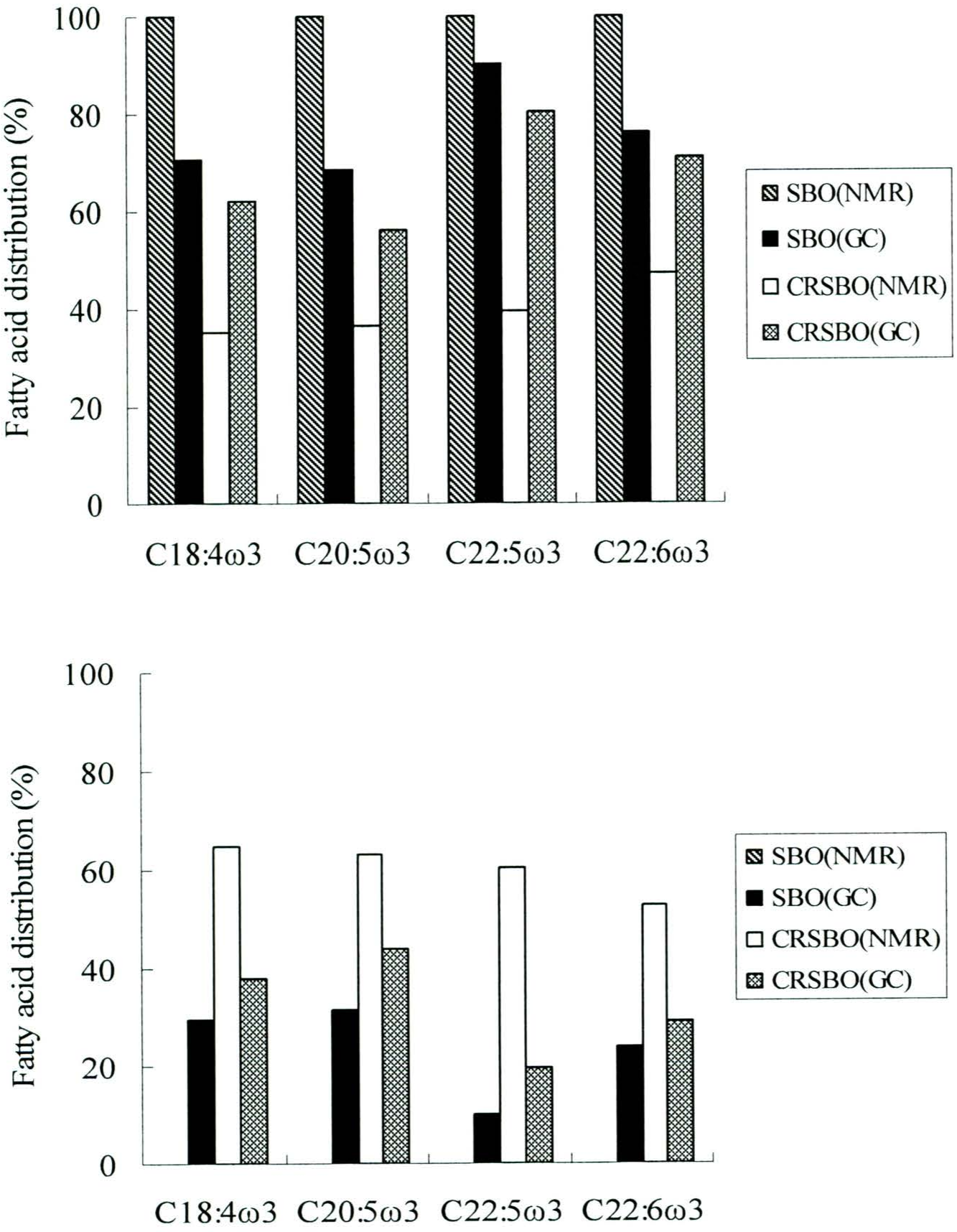

Figure 2.1. Percent fatty acid distribution of total TAG located at the *sn*-1,3 positions (top) and the *sn*-2 position (bottom) of the orginal seal blubber oil and its chemically randomized counterpart; SBO(NMR) indicates data obtained for SBO using ^{13}C NMR; SBO(GC) data obtained for SBO using GC.; CRSBO(NMR) data obtained for CRSBO using ^{13}C NMR; while CRSBO(GC) data obtained for CRSBO using GC. Note that the % *sn*-2 distribution of omega-3 PUFA in SBO was zero, as determined by ^{13}C NMR.

Fatty acid positional distributions of menhaden oil before and after chemical randomization. For the most part, NMR spectral analysis of omega-3 PUFA distribution in MHO was in agreement with the GC analysis. For instance, C22:5ω3 and C22:6ω3 were preferentially esterified to the *sn*-2 position (62 and 66%, respectively); C18:4ω3 moieties were also attached primarily to the *sn*-2 position, but in lesser amounts (46%) (Figure 2.2). However, C20:5ω3 was the exception; while, GC analysis indicated a preference for the middle *sn*-2 position (51% area; Table 2.2), NMR spectral analysis suggested a slight preference for the *sn*-1,3 positions (71% area; Figure. 2.2).

For CRMHO, the NMR spectral data agree only partially with that from GC analysis. For instance, while GC analysis suggested that the PUFA distribution was almost unaltered upon chemical randomization (Table 2.2); NMR analysis indicated that this occurred only for C18:4ω3 and C20:5ω3 residues (Figure 2.2). For the omega-3 PUFA C22:5 and C22:6, drastic changes were observed. The former was relocated from being primarily at the *sn*-2 position (62%) in original MHO, to almost equally distributed in chemically randomized MHO (35% *sn*-2; Figure 2.2). The latter was relocated from being primarily at the *sn*-2 position (66%) of MHO, to a slight excess at the *sn*-1,3 positions (70%) in CRMHO (Figure 2.2).

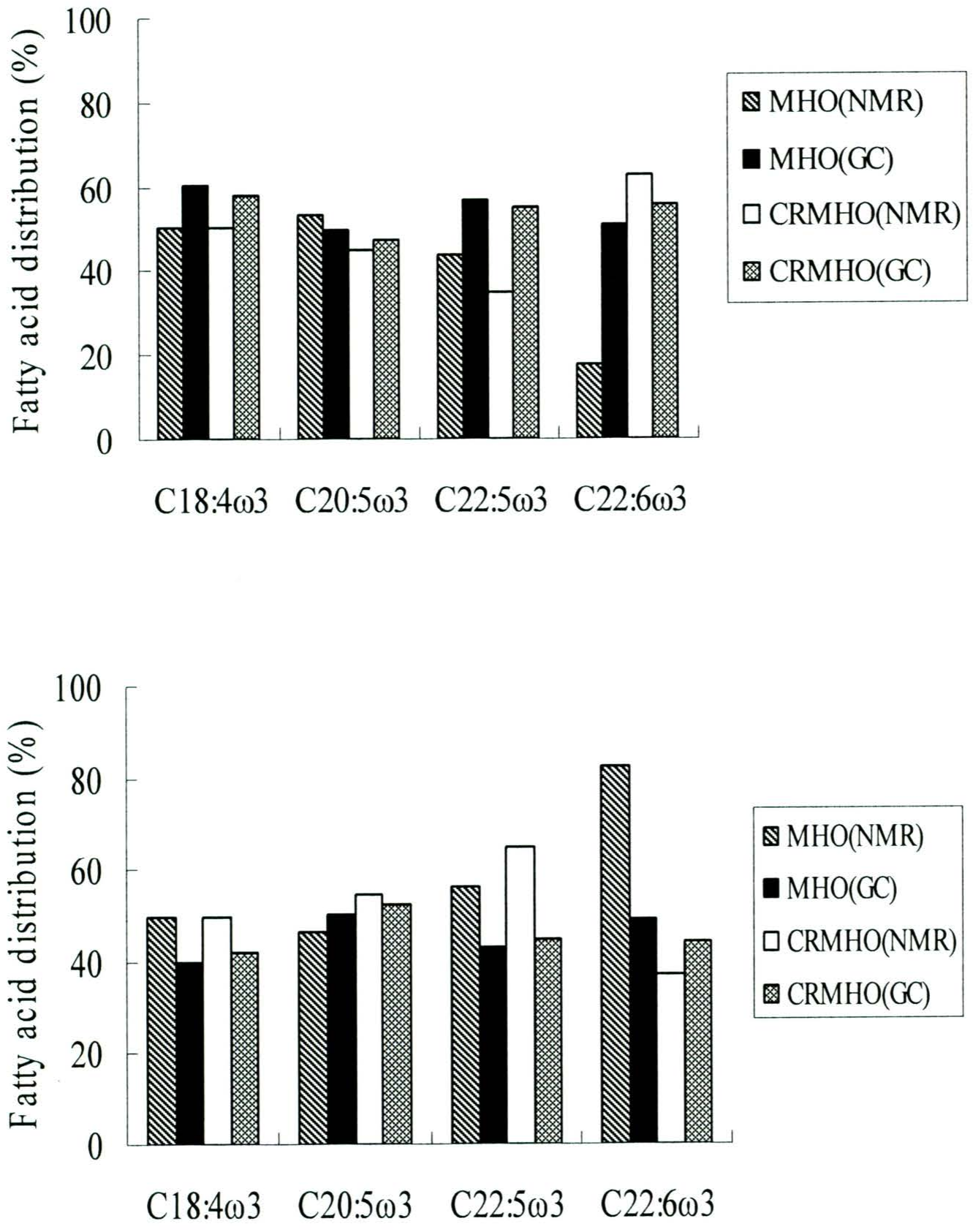

Figure 2.2. Percent fatty acid distribution of total TAG located at the *sn*-1,3 positions (top) and the *sn*-2 position (bottom) of the original menhaden oil and its chemically randomized counterpart; MHO(NMR) indicates data are obtained for MHBO using ^{13}C NMR; MHO(GC) indicates data obtained for MHO using GC; CRMHO(NMR) indicates data obtained for MHOBO using ^{13}C NMR; and CRMHO(GC) indicates data obtained for MHO using GC.

Changes of tocopherol content in original oils and their randomized counterparts. Alpha-tocopherol is the natural antioxidant in marine oils, and it is the dominant tocopherol homologue detected in SBO and MHO (Logani & Davies. 1980). Figure 2.3 shows changes in the content of alpha-tocopherol following the randomization process. A significant ($P<0.05$) decrease in the levels of alpha-tocopherol was noted in the randomized oils; 51% for SBO and 25% for MHO.

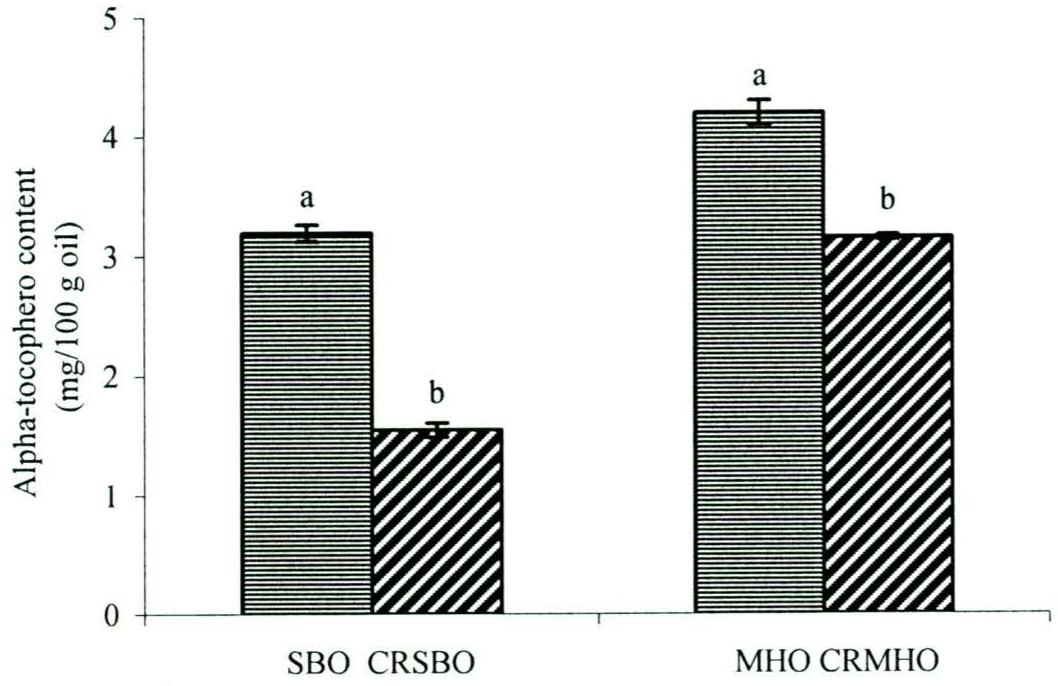

Figure 2.3. Alpha-tocopherol content (mg/100 g oil) in seal blubber oil (SBO), menhaden oil (MHO), chemically randomized seal blubber oil (CRSBO) and menhaden oil (CRMHO); Different letters within same group indicates significant difference ($P<0.05$) between alpha-tocopherol levels

Oxidative stability

CD test. The CD value of lipids, reflected in their absorption at 234 nm, is an indicator of primary oxidation products, conjugated dienes, are formed due to a shift in the double bond positions upon oxidation of lipids that contain dienes or polyenes (Logani & Davies 1980). Figure 2.4 shows the CD contents of randomized SBO and its unrandomized counterpart under accelerated oxidation conditions at 60°C. Both randomized and original SBO followed an increasing trend in their CD levels throughout the experimental period due to the formation and accumulation of lipid hydroperoxides as primary oxidation products (Hamam & Shahidi 2004). Formation of lipid hydroperoxides coincides with that of CD upon oxidation (Hamam & Shahidi 2006; Farmer & Sutton 1946). CD values increased slowly from 0 to 24 h when primary oxidation products, hydroperoxides, were generated and released during lipid oxidation. However, the amount of hydroperoxide formed was fairly low at the beginning of oxidation chain reaction. As the storage time was extended to 72 h, large amounts of primary oxidation products were produced due the abundance of free racicals and the oxidation chain reaction, and this sharply increased CD values. CD values reached a peak within 72 h. Randomized SBO had a significantly higher CD value on each tested point except at the beginning point than its unrandomized counterpart. Positional distribution of fatty acids, especially unsaturated fatty acids, and the level of alpha-tocopherol (major antioxidant in fish and seal blubber oils) are thought to influence the oxidative stability. Less PUFA, including C18:2, C18:3, C18:4, C20:5, C22:5 and C22:6, were located on the terminal positions in the randomized oil, and this should contribute better to the oxidative stability of the oil.

However, the decreased oxidative stability shown in the tested randomized oil was due to the loss of alpha-tocopherol, the main natural antioxidant (strong free radical quencher) in seal blubber oil, caused by oxidation and formation of tocopheoryl esters in randomized oil during chemical randomization (Hamam & Shahidi 2006; Jackson 1982*)*. In addition, monounsaturated fatty acids (MUFA), such as C14:1, C16:1, C18:1 and C22:1 on the terminal positions of TAGs increased at different levels after chemical randomization. Together, C14:1, C16:1, C18:1 and C22:1 accounted for more than 50% (wt) of total fatty acids in seal blubber oil. Therefore, redistribution of these MUFA may also account for deceased oxidative of resultant oils since unsaturated fatty acids located on the terminal positions are more easily attacked by free radicals (Lau *et al*. 1982).

Results also show an increasing trend in CD values of both original MHO and its randomized counterpart, except that the peak value of randomized MHO appeared at 72 h. Comparing each tested point with the original oil, randomized MHO had generally higher CD values, brought about by redistribution of unsaturated fatty acids and loss of alpha-tocopherol. The reason for the differences of CD value between the two groups of oil samples may possibly be due to the fact that alpha-tocopherol exerts a stronger influence on oxidative stability of MHO than that of positional distribution of unsaturated fatty acids. The loss of alpha-tocopherol directly led to decreased oxidative stability of randomized oil although there were more unsaturated fatty acids located on the middle position which are expected to be better protected from oxidation.

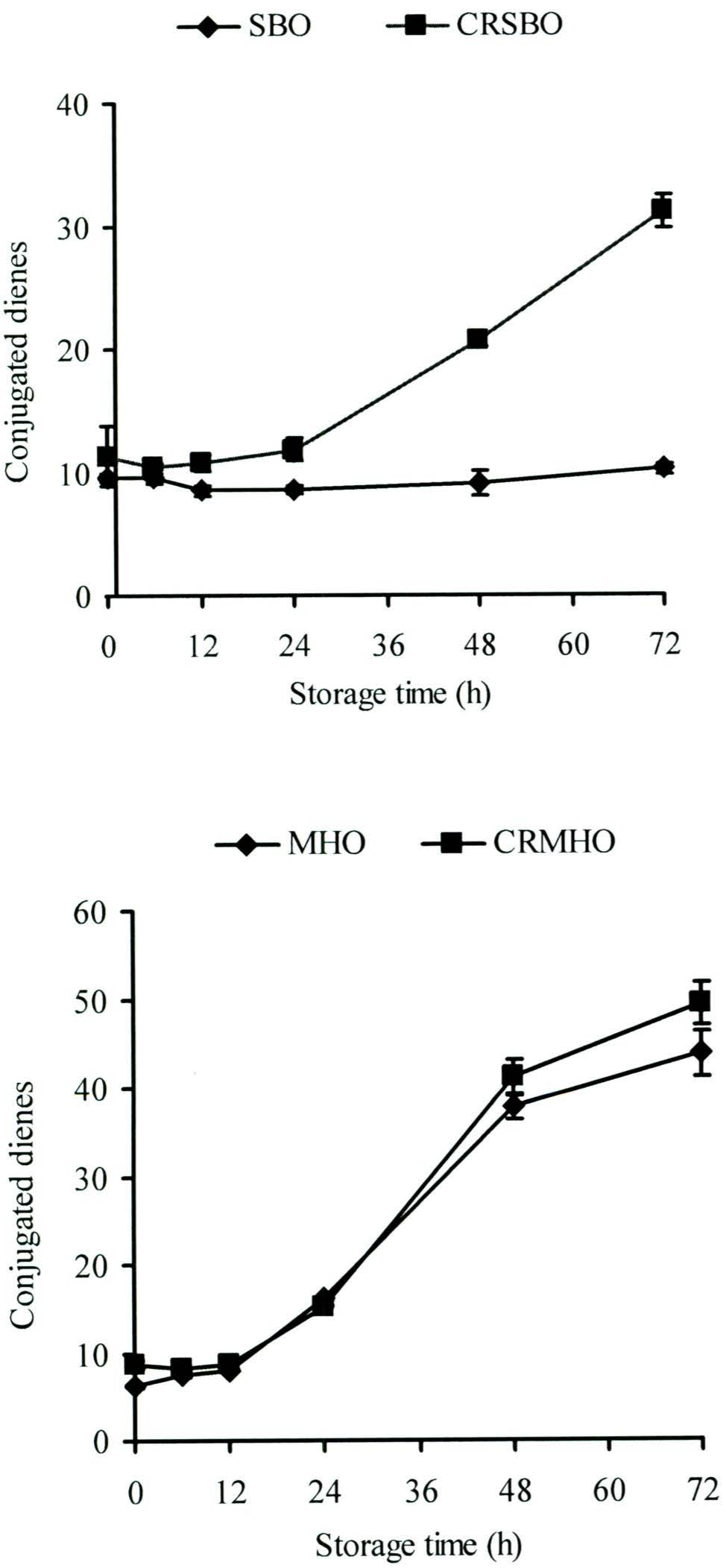

Figure 2.4. Conjugated dienes values of SBO (top) and MHO (bottom) before and after chemical randomization and stored under Schaal oven conditions at 60°C; SBO: seal blubber oil before randomization; CRSBO: seal blubber oil after randomization; MHO: menhaden oil before randomization; and CRMHO: menhaden oil after randomization.

TBA test. The TBA test has been widely used for assessing the content of secondary oxidation products of lipids. The formation rate of secondary oxidation products is not only affected by the amount of primary oxidation products, hydroperoxides, and the level of alpha-tocopherol, but is also influenced by the nature of hydroperoxides as different hydroperoxides may not be equally decomposed to secondary oxidation products under the same conditions (Nevdakh *et al.* 1984*)*. The type of hydroperoxide is determined by the nature of fatty acid oxidized. The results given in Figure 2.5 summarize TBARS values and demonstrate that their content increases progressively until reaching peak values during the storage period in both SBO and its randomized counterpart. Compared to SBO, randomized SBO had lower TBARS values at 0, 6 and 12 h, but higher at other tested points. TBARS values of randomized SBO increased sharply after 24 h and reached a peak value at 72 h since more primary oxidation products were broke down to secondary oxidation products (Strange *et al.* 1997; Senanyake & Shahidi 2001*)*. Meanwhile, TBARS values of unrandomized SBO started to increase dramatically after 72 h. Thus, randomized SBO was more stable at the early storage period and less stable afterwards. The difference of TABRS values between the original and the randomized oil was possibly caused by a combination of loss of alpha-tocopherol and changes in positional distribution of fatty acids. The superior influence of alpha-tocopherol to positional distribution of unsaturated fatty acid on oxidative stability in oils is proved as original oil is considered to be more stable than it randomized counterpart as evidenced by comparing levels of the difference of TBARS values between early storage period and later period. The differences of TBARS values between randomized and original oils are much higher at the later storage period compared to those at the early period. The results

also show that both randomized MHO and its original counterpart followed an increasing trend for TBARS values. Randomized MHO had generally lower TBARS values throughout the entire experimental period, thus indicating its better oxidative stability during the 72 h of storage, and such results are also caused by loss of alpha-tocopherol and redistribution of unsaturated fatty acids.

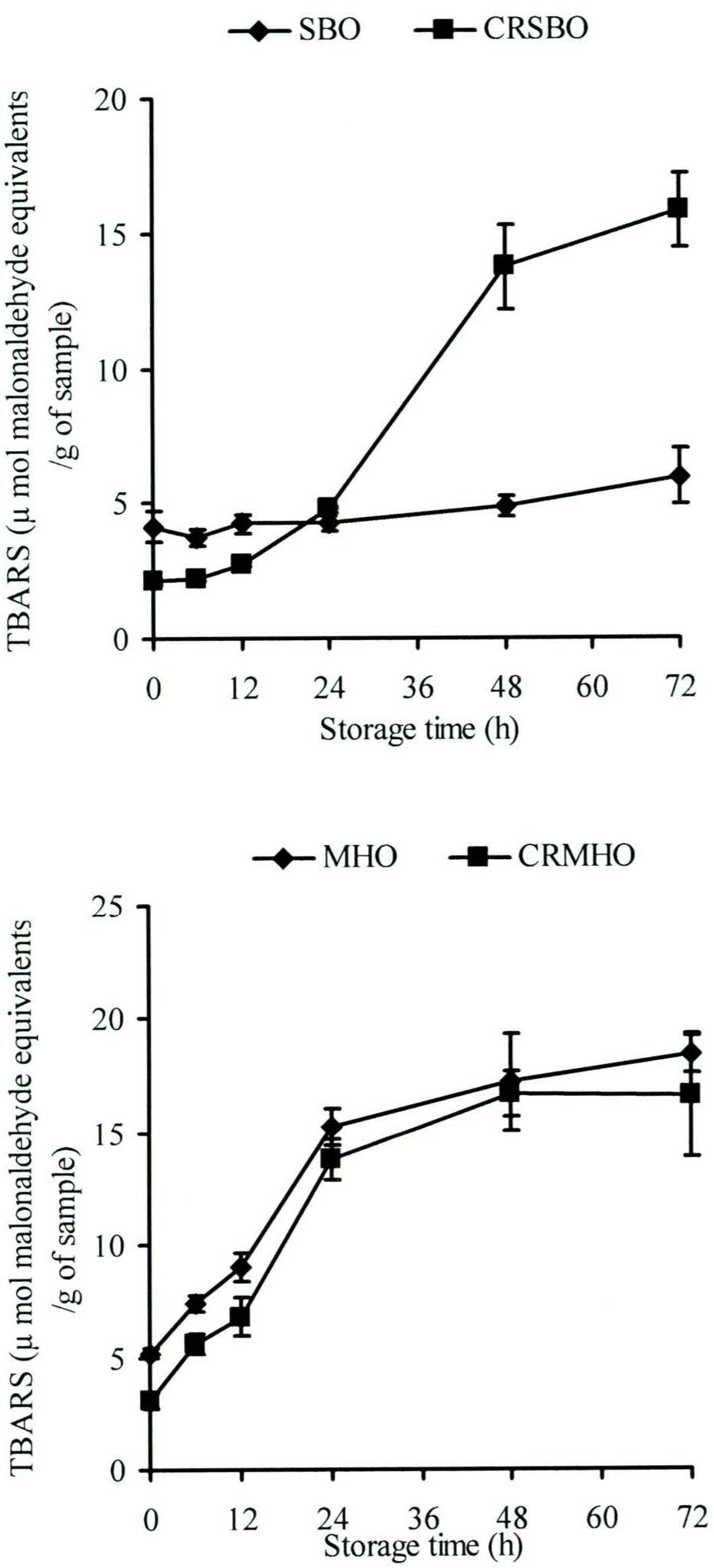

Figure 2.5. TBARS values of SBO (top) and MHO (bottom) before and after chemical randomization and stored under Schaal oven condition at 60 °C; SBO: seal blubber oil before randomization; CRSBO: seal blubber oil after randomization; MHO: menhaden oil before randomization; and CRMHO: menhaden oil after randomization.

Conclusions

This study demonstrated that chemical randomization leads to the redistribution of fatty acids among the stereoisomeric *sn*-1,3 and *sn*-2 positions of the glycerol moiety of TAGs of SBO and MHO. However, the effect of randomization on positional distribution of each fatty acid occurred at different levels in the oils tested. Furthermore, while results obtained from GC and ^{13}C NMR spectral analysis were different when comparing positional distributions of C18:4, C20:5, C22:5 and C22:6 in seal blubber and menhaden oils before and after randomization, they followed a similar trend. Redistribution of fatty acids, especially unsaturated fatty acids, is expected to lead to changes in the chemical and physical properties of the oils and hence in their oxidative stability. The changes in stability of the oils are also partially due to the loss of some alpha-tocopherol upon randomization, which is a subject of interest for future research.

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CHAPTER 3

Effect of enzymatic randomization on positional distribution and stability of seal blubber and menhaden oils

Introduction

The omega 3 oils from marine origin contain high levels of polyunsaturated fatty acids (PUFA). For instance, seal blubber oil (SBO) typically contains more than 0.4% α-linolenic acid (ALA), 5% eicosapentaenoic acid (EPA), 4% docosapentaenoic acid (DPA) and 7% docosahexaenoic acid (DHA), while menhaden oil (MHO) contains 0.6 % ALA, 10% EPA, 2% DPA, and 10% DHA (Wanasundara & Shahidi 2007; Senanyake & Shahidi 2002; Kim *et al*. 2006). The importance of PUFA in nutrition and health is well recognized. Studies have shown the biological significance of DHA in supporting normal development of the brain, the eyes and the nervous system, especially for infants and fetuses, and thus aaddition of DHA in the diet of pregnant and lactating women as well as infant formula is strongly recommended (Simopoulos 1991; Denomme *et al*. 2005; Haag 2003; Craig-Schmidt *et al*. 1996). Long chain omega-3 PUFA, can be converted to other anti-inflammatory molecules, such as protectins, resolvins, maresins and omega-3-oxylipins, which may also explain the versatile health benefits of omega-3 oils (Serhan *et al*. 2008). The role of omega-3 oils in alleviating cancer (De Deckere 1999; Chen 2007), cardiovascular disease (Marchioli 2002; Wang *et al*. 2006), psychiatric disorders (Puri 2006; Nemets *et al*. 2006), Parkinson's disease (Bousquet et al. 2008) and inflammatory

disorders (Chandrasekara *et al.* 1995; Fisher *et al.* 1990; De Caterina & Zampolli 2001) have been widely studied and confirmed in the recent literature.

Chemical and enzymatic randomizations are common means to modify the chemical and physical properties of the oils (Marangoni & Rousseau 1995; Rousseau & Maragoni 1997). The effect of chemical randomization on positional distribution and stability of seal blubber and menhaden oils has already been studied (Wang *et al.* 2010). The results demonstrated that chemical randomization leads to the redistribution of fatty acids among the stereoisomeric *sn*-1,3 and *sn*-2 positions of the glycerol moiety of triacylglycerols (TAGs) of SBO and MHO, and redistribution of fatty acids, especially unsaturated fatty acids, is expected to lead to changes in the chemical and physical properties of the oils and hence in their oxidative stability. The changes in stability of the oils are also partially due to the loss of some of the α-tocopherol present upon randomization due to esterification with fatty acids present (Hamam & Shahidi 200; Criado *et al.* 2007). The contribution of the latter effect in previous studies was not eliminated, and hence results on the effect of positional distribution of fatty acids were not independent of this factor (Christie 1982).

The purpose of the present study was not only to examine the effect of enzymatic randomization on positional distribution of fatty acids in SBO and MHO, using gas chromatography (GC), but also to investigate the effect of positional distribution of fatty acids on the oxidative stability of the resultant oils by comparing conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) values following accelerated

oxidation at 60°C for 3 days after the addition of a sufficient amount of α-tocopherol to reach its original level after randomization.

Materials and methods

Materials. Novozyme 435, 2-thiobarbituric acid, porcine pancreatic lipase (EC 3.11.3), tocopherol standards, sodium taurocholate, and silica gel thin-layer chromatographic plates (TLC; 20 x 20 cm; 60 Å mean pore diameter, 2-25 μm mean particle size, 500 μm thickness, with dichlorofluorescein) were purchased from Sigma-Aldrich (Mississauga, ON, Canada). A standard mixture of fatty acid methyl esters (FAMEs; GLC-461) was purchased from Nu-Check (Elysian, MN, USA). All solvents used were of analytical grade and purchased from Fisher Scientific (Nepean, ON, Canada).

Enzymatic randomization of SBO and MHO catalyzed by Novozyme 435 (Lipase acrylic resin from *Candida antarctica*). Enzymatic randomization was carried out following the method of Criado *et al.* (2007) with minor modification. SBO and MHO (25 g) were heated under a nitrogen blanket in 250-ml round-bottom flasks at 75°C. The randomization reaction was initiated with the addition of 10% (w/w of oil sample) Novozyme 435 (Sigma-Aldrich, Mississauga, ON, Canada), and maintained for 3 h at 75°C ± 1 under a nitrogen blanket. The resultant mixtures were washed with a sodium bicarbonate (0.05 M) solution to neutralize free fatty acids and the soaps so produced were removed with hot water. Bentonite clay (Sigma-Aldrich, Mississauga, ON, Canada) (1.5%, w/w) was subsequently added to remove colourants and residual water, followed by vacuum filtration. The resultant oils were stored at -20°C until use within 2 weeks.

Positional distribution of fatty acids by GC analysis

(a) Selective hydrolysis using pancreatic lipase. The oil samples were hydrolyzed using pancreatic lipase as described by Christie (1982) with minor modifications. The oil (25 mg) was weighed into a glass tube, and then 5.0 ml Tris-HCl buffer (1.0 M, pH 8.0), 0.5 ml of calcium chloride (2.2%) and 1.25 ml of sodium taurocholate (0.05%) were added. Porcine pancreatic lipase (5.0 mg; EC 3.11.3) was added to the mixture after it was kept in a water bath for 5.0 min at 40°C. The glass tube was subsequently placed in a gyratory water bath shaker (New Brunswick Scientific, New Brunswick, NJ, USA) at 250 rpm under a blanket of nitrogen for 1 h at 40°C. The enzymatic reaction was stopped by adding 5.0 ml of ethanol, followed by the addition of 5.0 ml of 6.0 M HCl.

(b) Extraction and separation of hydrolytic products. Diethyl ether (50 ml in total) was used to extract the hydrolytic products three times, and then the extract was washed twice with distilled water and dried over anhydrous sodium sulphate followed by the removal of the solvent under reduced pressure at 30°C. The hydrolytic products were separated on silica gel TLC plates. The plates were developed using a mixture of hexane-diethyl ether-acetic acid (70:30:1, v/v/v) for 45-55 min. The bands were located by viewing under a short wavelength (254 nm) UV light (Spectraline, Model ENF-240C, Spectronics Co., Westbury, NY, USA). The free fatty acid bands were scraped off and lipids extracted into diethyl ether, which were then used for fatty acid analysis as described by Senanayake & Shahidi (2000).

(c) Fatty acid compositional analysis of hydrolytic products. Fatty acid composition and positional distribution of the products were determined following their conversion to the corresponding methyl esters. The transmethylation reagent (2.0 ml) consisting of freshly prepared 6% sulphuric acid in methanol containing 15 mg of hydroquinone as an antioxidant was added to the sample vial, followed by vortexing. The mixture was incubated for 24 h at 60°C and subsequently cooled to ambient temperature. Distilled water (1.0 ml) was then added to the mixture, after thorough mixing, and hydroquinone was added again to each vial to prevent oxidation. The FAMEs were extracted three times, each with 1.5 ml of HPLC grade hexane. The combined hexane layers were then transferred to the test tube and washed twice with 1.0 ml distilled water. The hexane was evaporated under a stream of nitrogen, and the extracted FAMEs were then dissolved in 1.0 ml of carbon disulphide and used for subsequent GC analysis. A Hewlett-Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a SUPELCOWAX-10 column (30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON, Canada) was used to analyze the FAMEs. The oven temperature was first raised to 220°C and kept there for 10.25 min and then raised up to 240°C at 30°C/min and held there for 15 min. The injector and FID temperatures were 250°C. Ultra high purity helium was used as a carrier gas at a flow rate of 15 ml/min. A Hewlett-Packard 3365 Series II ChemStation Software (Agilent) was used for data handling and processing. The FAMEs were identified by comparing their retention times with those of a known standard mixture. Positional distribution of fatty acids at *sn*-1, 3 positions was calculated as (% fatty acid at *sn*-1,3 positions / % fatty acid in triacylglycerols)*100.

(d) Determination of tocopherol content by HPLC-MS. Seal blubber oil and menhaden oil as well as their randomized counterparts were saponified according to the procedure described by Zhong & Shahidi (2007) with some modifications. The oil (0.5 g) was mixed thoroughly with 1ml of 60% KOH (w/v) and 4 ml of 0.25% (w/v) ethanolic pyrogallol in screw-capped tubes fitted with Teflon lined caps. Saponification progressed at 70°C in a water bath for 45 min. The tubes were then cooled in ice and unsaponified components were extracted three times with 2 ml of hexane. Hexane extracts were combined and evaporated under a stream of nitrogen and redissolved in 2 ml hexane. Standard solutions of tocopherols standards were prepared by dissolving a known tocopherol mixture (consisting of 4 tocopherol analogs) in hexane and then by diluting to different concentrations. Prior to HPLC analysis, both samples and standards were filtered using a 0.45 μm syringe-filter (Whatman, Clifton, NJ, USA).

Tocopherol content in prepared samples and standards was determined by normal phase high performance liquid chromatography (HPLC) – mass spectrometry (MS). The analysis was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) with a UV-diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm × 4.6 mm i.d., 5 μm, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) coupled with a Supelcosil LC-Si guard column. Tocopherols were eluted using an isocratic solvent system containing hexane-diethyl ether (99:1, v/v) at a flow of 1.0 ml/min. Each tocopherol standard and sample (80 μl) were injected using an autosampler. Tocopherols were detected at 295 nm by a UV detector and identified by comparing their retention times with those of known tocopherol standards. LC flow was

analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) using positive ion APCI (atmospheric pressure chemical ionization). The operating conditions used were 121 V for the fragmentor voltage, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, drying gas flow of 7 l/min, A standard curve was constructed for each tocopherol homologue (peak area versus concentration). Tocopherol concentrations in samples were obtained from the standard curve, and expressed as mg α-tocopherol per 100 g oil.

Recovery of α-tocopherol in randomized oils. The standard α-tocopherol was dissolved in hexane and added to randomized SBO and MHO in order to replenish α-tocopherol contents to their initial levels. Hexane was removed from samples under a flow of nitrogen, and oils were stored under -20°C for up to 2 weeks for oxidative stability tests.

Oxidative stability assays

(a) CD test. Oxidative stability of randomized SBO and MHO as well as their original counterparts was determined under Schaal oven conditions at 60°C for 72 h. Each day (24 h) of storage under such conditions is equaivalent to 1 month of storage at ambient temperatures. Oils (0.4–0.5 g) were placed in loosely capped test tubes (10 mm diameter and 4.0 cm height) and stored at 60 °C in a forced-air oven (Thelco, Model 2; Precision Scientific Co., Chicago, IL, USA). Samples were removed from the oven at 0, 6, 12, 24, 48 and 72 h, cooled down to room temperature, flushed with nitrogen, capped, and stored at −20°C for up to 7 days until analyzed. The experiments were carried out in triplicate. CD in the oils was determined according to the IUPAC (1987) method 20505. Oil

samples (0.02–0.04 g) were weighed into 50-ml volumetric flasks, dissolved in iso-octane, and made up to the mark with the same solvent. The contents were thoroughly mixed, and the absorbance was read at 234 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent).

(b) TBA test. The determination of TBARS was carried out as described by the AOCS (1990*)* method Cd 19-90. Oil samples (0.05–0.10 g) were weighed into 25-ml volumetric flasks, dissolved in a small volume of 1-butanol, and then made up to the mark with the same solvent. This solution (5 ml) was transferred into a screw-capped test tube, and then 5 ml of freshly prepared TBA reagent (0.5 g TBA in 250 ml n-butanol) were added. The mixture was thoroughly mixed, and then the test tube was placed in a water bath at 95 °C for 2 h. Heated samples were cooled in an ice bath, and the absorbance of the resultant coloured complex was read at 532 nm using a Hewlett-Packard diode array Model 8452 A spectrophotometer (Agilent).

Results and discussion

Fatty acid positional distributions by GC analysis. The positional distribution of the major fatty acids of SBO, MHO and their enzymatically randomized counterparts ERSBO and ERMHO, respectively, calculated from the fatty acid profiles of hydrolytic products, are shown in Tables 3.1 and 3.2, respectively.

Fatty acid positional distributions of seal bubbler oil before and after enzymatic randomization. In the original SBO, unsaturated fatty acids, especially PUFA such as C18:2ω6, and those of the omega-3 class, including C18:3ω3, C18:4ω3, C22:5ω3 and C22:6ω3, were preferentially located at the *sn*-1,3 positions (terminal positions) (Table 3.1). The important omega-3 fatty acid C20:5 was distributed nearly evenly among *sn*-1,3 (68%) and *sn*-2 (32%) positions (Table 3.1). It is important to understand that equal distribution in this study is defined as a *sn*-1,3 regiospecificity of 66.67 mole %, and at a *sn*-2 regiospecificity of 33.33 mol%.

Saturated fatty acids are located differently in TAG of the original SBO; for instance, C18:0 resides mostly at the terminal *sn*-1,3 positions (93%), C16:0 appears to be fairly equally distributed, while C14:0 showed a slight preference for the *sn*-2 position (41%). The monounsaturated fatty acids C17:1 and C18:1ω9 appeared to be nearly equally distributed (66 and 64%, respectively) at the *sn*-1,3 positions. The other monounsaturated residues C14:1ω9, C16:1ω7 and C18:1ω11 were in slight excess at the *sn*-2 positions, judging from the *sn*-2 area percentages which were close to 43% in each case (Table 3.1). The distribution of PUFA at *sn*-2 position at the glycerol backbone of the TAGs of enzymatically randomized seal blubber oil was recognized except for C18:2ω6 (Table 3.1). For instance, 8% of C18:2ω6 was relocated from the *sn*-1,3 positions to the *sn*-2 position during randomization (Table 3.1). Distribution of C18:4ω3 at *sn*-1,3 positions decreased from 71 to 59% while that of C20:5ω3 decreased from 68 to 53% in enzymatically randomized seal blubber oil (ERSBO). These fatty acids were nearly equally distributed among terminal positions and middle position in the randomized oil. Meanwhile, the proportion of C22:5ω3 and C22:6ω3 moieties decreased about 15 and

13% in ERSBO, respectively. However, C22:5ω3 and C22:6ω3 were still predominantly located at the terminal positions of ERSBO. The C18:3ω3 was the only PUFA that increased its distribution on terminal positions in ERSBO, and 7% of it was relocated from the middle position to the terminal positions.

The positional distribution of saturated fatty acids was affected to different degrees by enzymatic randomization. For instance, both myristic acid (C14:0) and palmitic acid (C16:0) residues increased by 5 and 2% at the terminal positions, respectively (Table 3.1). Although C18:0 remained attached preferentially to the *sn*-1,3 positions of resultant oil, it decreased by 15% at the terminal positions.

Monounsaturated residues, including C16:1, C18:1, C20:1 and C21:1, increased by 1-9% at the terminal positions, with the exception of C17:1 which was relocated (7%) from the *sn*-1,3 positions to the *sn*-2 position in ERSBO.

Table 3.1. Percent fatty acid distribution of total triacylglycerols located at *sn*-1,3 and *sn*-2 positions of the original seal blubber oil and its enzymatically randomized counterpart calculated by GC analysis

Fatty acid	sn-1+sn-3[A]		sn-2[B]	
	SBO	ERSBO	SBO	ERSBO
C14:0	58.70 ± 0.51[a]	63.54 ± 0.28[b]	41.30 ± 0.51	36.46 ± 0.28
C14:1ω9	58.44 ± 0.40[a]	67.73 ± 0.34[b]	41.56 ± 0.40	32.27 ± 0.34
C16:0	64.16 ± 0.35[a]	65.72± 0.23[b]	35.84 ± 0.35	34.28 ± 0.23
C16:1ω7	57.38 ± 0.78[a]	63.29 ± 0.36[b]	42.62 ± 0.78	36.71 ± 0.36
C17:1	66.41 ± 2.22[a]	58.96 ± 1.68[b]	33.59 ± 2.22	41.04 ± 1.68
C18:0	93.00 ± 1.31[a]	78.28 ± 0.83[b]	7.00 ± 1.31	21.72 ± 0.83
C18:1ω9	63.80 ± 0.56[a]	66.18 ± 0.17[b]	36.20 ± 0.56	33.82 ± 0.17
C18:1ω11	56.61 ±1.76[a]	61.43 ± 0.87[b]	43.39 ± 1.76	38.57 ± 0.87
C18:2ω6	77.17 ± 1.48	69.00 ± 5.20	22.83 ± 1.48	31.00 ± 5.20
C18:3ω3	79.26 ± 2.56[a]	86.23 ± 1.03[b]	20.74 ± 2.56	13.77 ± 1.03
C18:4 ω3	70.58 ± 0.89[a]	59.49 ± 1.65[b]	29.42 ± 0.89	40.51 ± 1.65
C20:1ω9	68.50 ± 0.28[a]	69.89 ± 0.11[b]	31.50 ± 0.28	30.11 ± 0.11
C20:5ω3	68.44 ± 1.09[a]	52.98 ± 0.53[b]	31.56 ± 1.09	47.02 ± 0.53
C22:1ω11	67.34 ± 2.30[a]	75.80 ± 2.01[b]	32.66 ± 2.30	24.20 ± 2.01
C22:5ω3	90.16 ± 2.48[a]	75.52 ± 0.23[b]	9.84 ± 2.48	27.48 ± 0.23
C22:6ω3	76.11 ± 1.06[a]	63.80 ± 0.02[b]	23.89 ± 1.06	36.20 ± 0.02

SBO: seal blubber oil before enzymatic randomization

ERSBO: seal blubber oil after enzymatic randomization

[A]: (% fatty acid at the *sn*-1,3 positions/% fatty acid in triacylglycerols)*100

[B]: (% fatty acid at the *sn*-2 position/% fatty acid in triacylglycerols)*100

Values across a row not sharing a common superscript letter are significantly different from one another ($P<0.05$)

Fatty acid positional distributions of menhaden oil before and after enzymatic randomization. In the original MHO, omega-3 PUFA, C18:4, C20:5, C22:5 and C22:6 were attached preferentially at the *sn*-2 position, and other PUFA, including C18:2ω6 and C18:3ω3 were esterified preferentially at the *sn*-1,3 positions of glycerol, as judged from the *sn*-1,3 percentages of 83 and 75%, respectively (Table 3.2).

Of the three major saturated fatty acids of MHO, C14:0 and C16:0 were resided equally among middle position and terminal positions, whereas C18:0 was distributed mostly at the *sn*-1,3 positions (Table 3.2). Of the six monounsaturated fatty acids examined, five of them were present at the *sn*-1,3 positions at ≥70% and the sixth one (C17:1) was nearly evenly distributed among the *sn*-1,2,3 positions (65% s*n*-1,3).

The PUFA were distributed more evenly at the terminal and middle positions of the enzymatically randomized menhaden oil (ERMHO) compared to their positional distribution in the original menhaden oil. For instance, the proportion of *sn*-1,3 moieties of C18:2ω6 decreased by 9% (from 83 to 74%), while that of C18:3ω3 decreased by 1% (from 74 to 73%), and such results indicated a trend of equal distribution (66.67% at the terminal positions) in ERMHO (Table 3.2). The other PUFA, including C18:4ω3, C20:5ω3, C22:5ω3 and C22:6ω3, which were preferentially located at the *sn*-2 position in the original oil, increased their distribution at the terminal positions in the randomized oil. Relocation of C18:4 ω3, C20:5ω3, C22:5ω3 and C22:6ω3 residues from *sn*-2 position to *sn*-1,3 positions resulted in their equal distribution. The C18:4ω3 residues increased to 62% at the *sn*-1,3 positions upon its redistribution, whereas the proportion of C20:5ω3 at the *sn*-1,3 positions increased to 61%, indicating a significant relocation (12%).

Meanwhile, C22:5ω3 and C22:6ω3 residues showed a 15% increase at the *sn*-1,3 positions, and reached a final distribution of 72 and 66%, respectively.

The enzymatic randomization of menhaden oil resulted in a decreased distribution of C14:0, C16:0 and C18:0, at the *sn*-1,3 positions from 4 to 6%. The C14:0 residue at *sn*-1,3 positions decreased from 68 to 62%, while that of C16:0 decreased from 67 to 63%. There was a 6% decrease on the proportion of C18:0 at the *sn*-1,3 positions in ERMHO. Monounsaturated residues, including C16:1, C17:1, and C18:1, decreased by 4-11% on the *sn*-1,3 positions, with the exception of C20:1 which was relocated (2%) from the *sn*-2 positions to the *sn*-1,3 position in ERMHO.

Table 3.2. Percent fatty acid distribution of total triacylglycerols located at *sn*-1,3 and *sn*-2 positions of the original menhaden oil and its enzymatically randomized counterpart calculated by GC analysis.

Fatty acid	*sn*-1+*sn*-3[A]		*sn*-2[B]	
	MHO	ERMHO	MHO	ERMHO
C14:0	67.97 ± 0.67[a]	61.73 ± 0.25[b]	32.03 ± 0.67	38.27 ± 0.25
C14:1ω9	69.88 ± 0.71[a]	65.87 ± 0.44[b]	30.12 ± 0.71	34.13 ± 0.44
C16:0	67.22 ± 0.64[a]	63.01 ± 0.25[b]	32.78 ± 0.64	36.99 ± 0.25
C16:1ω7	69.68 ± 0.65[a]	62.04 ± 0.22[b]	30.32 ± 0.65	37.96 ± 0.22
C17:1	65.26 ± 0.30[a]	60.57 ± 0.51[b]	34.74 ± 0.30	39.43 ± 0.51
C18:0	78.78 ± 0.34[a]	72.69 ± 0.48[b]	21.22 ± 0.34	27.31 ± 0.48
C18:1ω9	76.37 ± 0.28[a]	72.66 ± 0.34[b]	23.63 ± 0.28	27.34 ± 0.34
C18:1ω11	76.07 ± 1.47[a]	65.01 ± 1.53[b]	23.93 ± 1.47	34.99 ± 1.53
C18:2ω6	83.35 ± 0.47[a]	74.76 ± 2.19[b]	16.65 ± 0.47	25.24 ± 2.19
C18:3ω3	74.69 ± 1.33	73.99 ± 0.88	25.31 ± 1.33	26.01 ± 0.88
C18:4ω3	60.27 ± 0.61	62.17 ± 0.45	39.73 ± 0.61	37.83 ± 0.45
C20:1ω9	88.6 ± 1.69	90.75 ± 0.69	11.4 ± 1.69	9.25 ± 0.69
C20:5ω3	49.46 ± 0.13[a]	61.28 ± 0.33[b]	50.54 ± 0.13	38.72 ± 0.33
C22:5ω3	56.81 ± 1.49[a]	71.97 ± 0.51[b]	43.19 ± 1.49	28.03 ± 0.51
C22:6ω3	50.97 ± 1.06[a]	66.07 ± 0.44[b]	49.03 ± 1.06	33.93 ± 0.44

MHO: menhaden oil before enzymatic randomization

ERMHO: menhden oil after enzymatic randomization

[A]: (% fatty acid at the *sn*-1,3 positions/% fatty acid in triacylglycerols)*100

[B]: (% fatty acid at the *sn*-2 position/% fatty acid in triacylglycerols)*100

Values across a row not sharing a common superscript letter are significantly different from one another ($P<0.05$)

Tocopherol content. Alpha-tocopherol is the natural antioxidant in marine oils, and it is the only tocopherol homologue detected in SBO and MHO (Logani & Davies 1980). Figure 3.1 shows changes in the content of α-tocopherol following randomization. A significant ($P<0.05$) decrease in the content of α-tocopherol was noted in randomized oils; 82% for both SBO and MHO. Formation of tocopherol esters in randomized oil during enzymatic randomization as well as their loss or oxidation may be responsible for the decrease in the α-tocopherol content. Alpha-tocopherol acts as a natural antioxidant in fish and seal blubber oils. Thus, commercial α-tocopherol was added in randomized oils to adjust its content to the original levels prior to conducting the oxidative stability tests in order to eliminate the effect of lost α-tocopherol on oxidative stability of the tested oils.

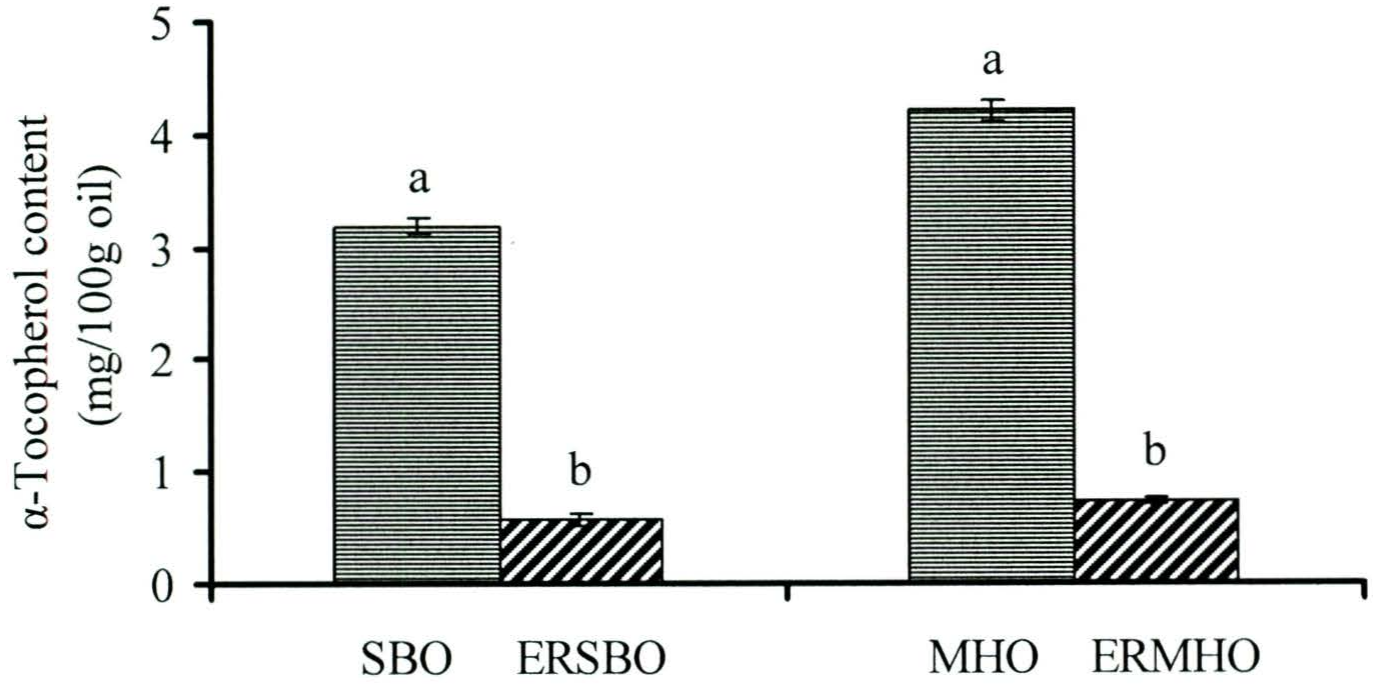

Figure 3.1. Alpha-tocopherol contents in randomized seal blubber and menhaden oils and their counterparts SBO: seal blubber oil before randomization; ERSBO: seal blubber oil after randomization; MHO: menhaden oil before randomization; and ERMHO: menhaden oil after randomization

Oxidative stability

CD test. The CD value of lipids, measured by their absorption at 234 nm, is an indicator of primary oxidation products, conjugated dienes, which are formed due to a shift in the double bond positions upon oxidation of lipids that contain dienes or polyenes (Farmer & Sutton 1946*)*. Fig. 3.2 shows the CD contents of enzymatically randomized SBO and its original counterpart under accelerated oxidation conditions at 60 °C. Both randomized and original SBO followed an increasing trend in their CD levels throughout the experimental period due to the formation and accumulation of lipid hydroperoxides as primary oxidation products (Nevdakh *et al.* 1984*)*. Formation of lipid hydroperoxides coincides with that of CD upon oxidation. CD values increased slowly from 0 to 24 h when primary oxidation products, hydroperoxides, were generated and released during lipid oxidation. However, the amount of hydroperoxide formed was fairly low at the beginning of oxidation chain reaction. As the storage time was extended to 48 h, large amounts of primary oxidation products were produced due the abundance of free radicals and the oxidation chain reaction, and this sharply increased CD values (Figure 3.2). CD values reached the highest values at 72 h tested points. Values obtained from the 0, 6, 12 and 24 h did not show any significant difference between the randomized and the original oils. However, CD values from the original SBO increased sharply from 48 to 72 h, and randomized SBO showed a significantly higher CD value at 48 and 72 h than its unrandomized counterpart, and thus randomized SBO was less stable compared to its unrandomized counterpart. Positional distribution of fatty acids, especially unsaturated fatty acids, is thought to influence the oxidative stability (Lau *et al.* 1982). Less unsaturated fatty acids including C17:1, C18:2, C18:4, C22:5 and C22:6, and more

C14:1, C16:1, C18:1, C18:3, C20:1 and C22:1 (Table 3.1) were located on the terminal positions in the randomized oil, and this contributes to the decreased oxidative stability of the resultant products.

The CD values of both original MHO and its randomized counterpart were decreased during the oxidation period, and the highest values appeared at 72 h. Comparing each tested point with the original oil, randomized MHO had generally lower CD values, which is an indicator of higher oxidative stability. The reason for the differences of CD value between the two groups of oil samples is due to the fact that less C14:1, C16:1, C17:1, C18:1 and C18:3, and more C18:4, C20:1, C20:5, 22:5 and C22:6 (Table 3.2) were located on the terminal positions in the randomized MHO, which are considered to be less protected from oxidation as they are more exposed to the free radicals compared to those located at the middle position (Lau *et al*. 1982).

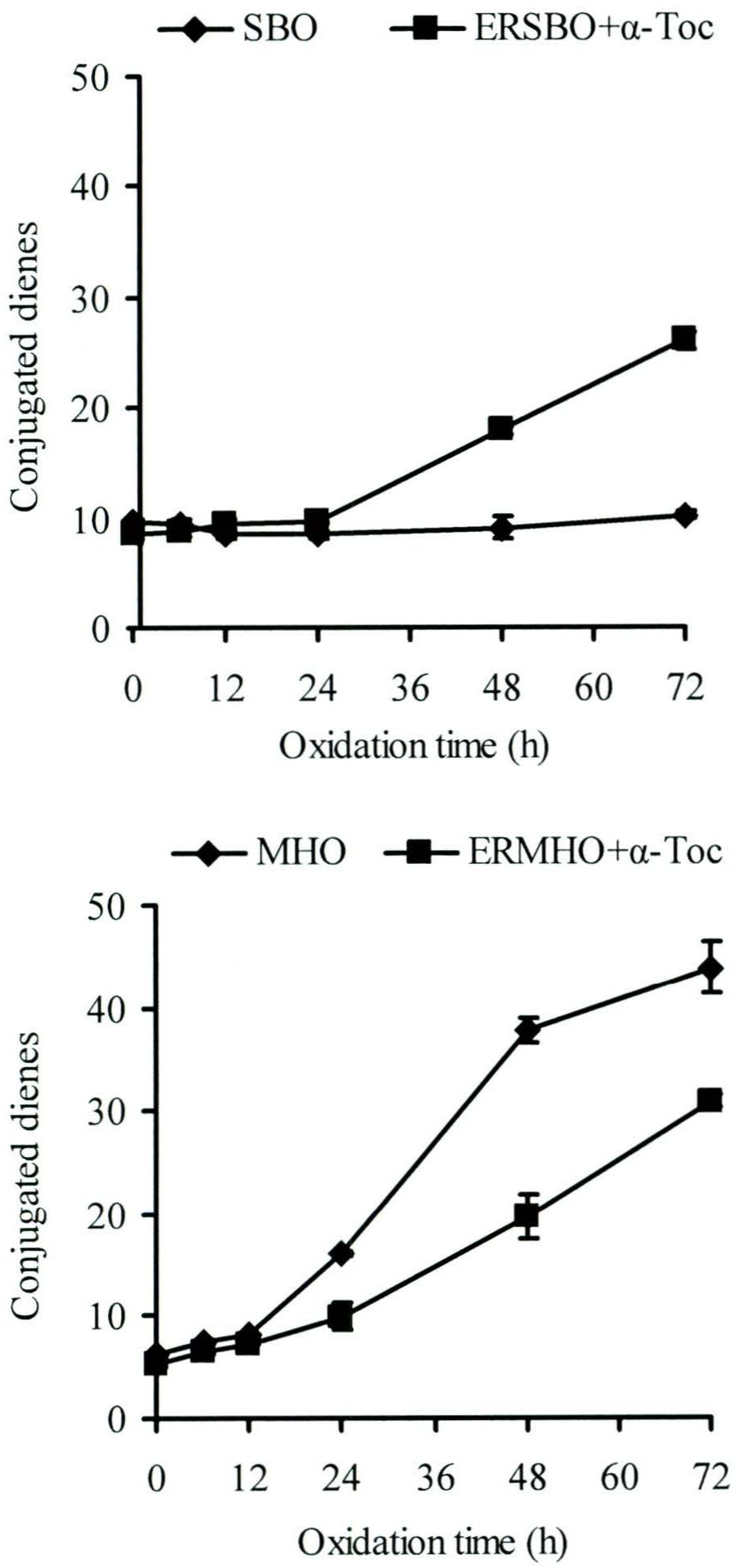

Figure 3.2. Conjugated dienes values of SBO (top) and MHO (bottom) before and after enzymatic randomization and stored under Schaal oven condition at 60 °C; SBO: seal blubber oil before randomization; α-Toc: alpha-tocopherol; ERSBO+α-Toc: seal blubber oil after randomization; MHO: menhaden oil before randomization; and ERMHO+α-Toc: menhaden oil after randomization with added α-tocopherol.

TBA test. The TBA test has been commonly used to assess the degree of lipid oxidation by measuring the content of secondary oxidation products. The rate of formation of secondary oxidation products is mainly affected by three factors, including the amount of hydroperoxides as primary oxidation products, the level of α-tocopherol, and the nature of hydroperoxides which may be decomposed to secondary oxidation products at different rates under the same conditions (Hamam & Shahidi 2006*)*. The type of hydroperoxide is dictated by the nature of the fatty acid being oxidized. The results shown in Figure 3.3 summarize TBARS values and demonstrate their progressively increased content until reaching their highest values during the storage period in both SBO and its randomized counterpart. Compared to SBO, randomized SBO had higher TBARS values from 24 to 72 h, and lower but insignificant values at other tested points. TBARS values of the randomized SBO increased sharply after 12 h and reached the highest value at 72 h since more primary oxidation products were broken down to secondary oxidation products (Strange *et al*. 1997; Senanayake & Shahidi 2001*)*. On the other hand, TBARS values of original SBO increased slowly throughout the entire oxidation period and reached the highest value at 72 h. Comparing these two groups of TBARS values, the original oil was oxidatively more stable than the randomized SBO during the tested period. The decreased oxidative stability in randomized oil was possibly caused by changes in positional distribution of fatty acids, especially unsaturated fatty acids (Table 3.1), as they are more easily attacked by free radicals and generate primary oxidation products such as conjugated dienes (Figure 3.2). In this case, the accumulation trend of TBARS was shown to be positively correlated with that of conjugated dienes in both SBO and its counterpart. The results obtained from MHO samples also showed that both randomized MHO and

original MHO followed an increasing trend for TBARS values during the oxidation period. Randomized MHO had lower TBARS values throughout the entire experimental period, thus indicating its better oxidative stability during the 72 h of storage, and such results are also caused by redistribution of unsaturated faty acids (Table 3.2).

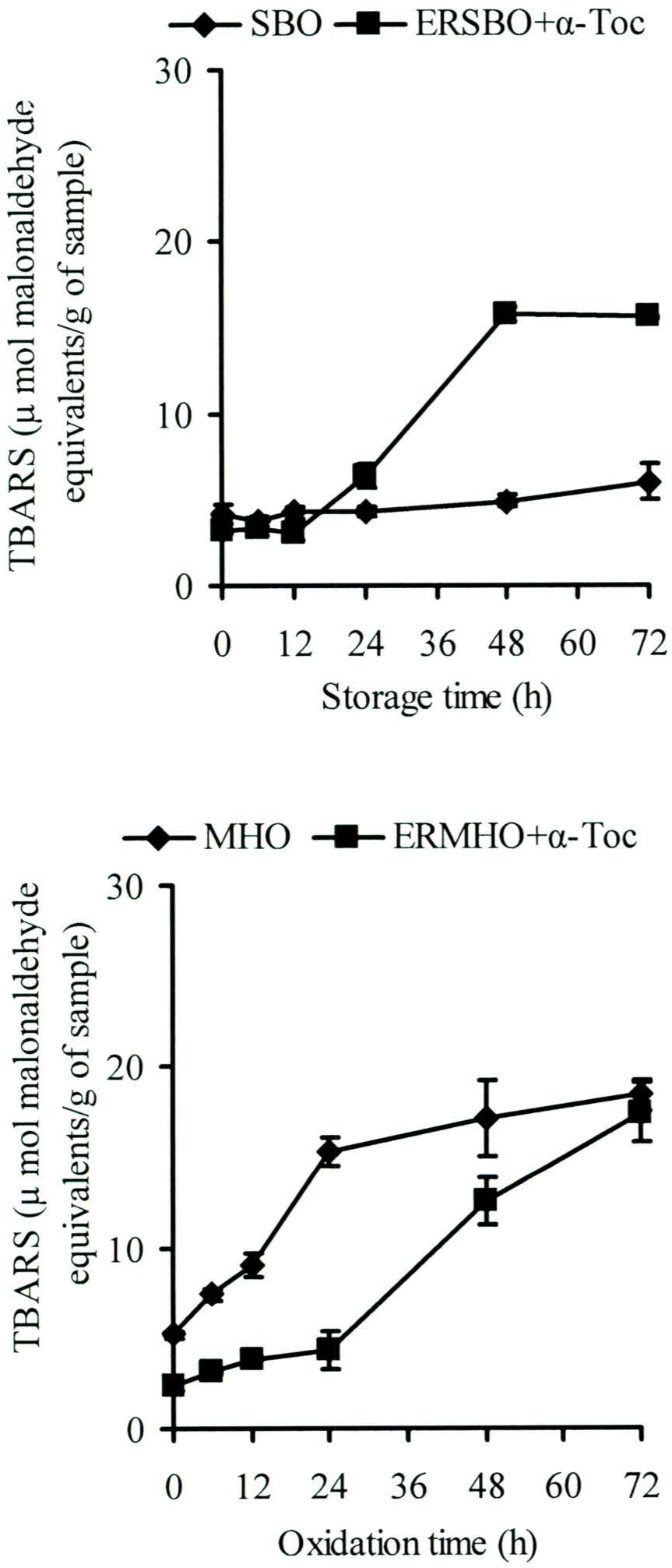

Figure 3.3. TBARS values of SBO (top) and MHO (bottom) before and after enzymatic randomization and stored under Schaal oven condition at 60 °C; SBO: seal blubber oil before randomization; α-Toc: alpha-tocopherol; ERSBO+α-Toc: seal blubber oil after randomization with added α-tocopherol; MHO: menhaden oil before randomization; and ERMHO+α-Toc: menhaden oil after randomization with added α-tocopherol.

Conclusions

Fatty acids were redistributed among the *sn*-1,3 and *sn*-2 positions of the glycerol moiety of TAGs of SBO and MHO at different levels during enzymatic randomization, and this led to the changes of oxidative stability of these omega-3 oils. The oxidative stability of SBO and MHO is determined by three factors, including fatty acid composition, positional distribution of fatty acids and tocopherol content. The fatty acids composition remained the same and the tocopherol content was replenished to its original level in the randomized oils after randomization. Therefore, redistribution of fatty acids, in particular, unsaturated fatty acids, was the major factor affecting the oxidative stability of omega-3 oils.

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CHAPTER 4

Effect of addition of wheat germ oil on the oxidative stability of seal blubber and menhaden oils

Introduction

Oils from marine mammals, fish and microalgae have been well recognized as preferable supplementary sources of long-chain omega-3 polyunsaturated fatty acids (PUFA). For instance, seal blubber oil (SBO) contains high contents of omega-3 fatty acids such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), while all these fatty acids can also be found in menhaden oil (MHO) (Wanasundara & Shahidi 1997; Kim *et al*. 2006). On the other hand, the oil derived from *Crypthecodinium cohnii* microalgae contains about 40% DHA (Hamam & Shahidi 2006). A large body of studies has demonstrated the health benefits of these PUFA in humans. As the main components of membrane lipids, DHA supports normal development of the brain, the eyes and the nervous system, especially for infants and fetuses, and thus addition of DHA in the diet of pregnant and lactating women as well as infant formula is strongly recommended (Simopoulos 1991; Denomme *et al*. 2005; Haag 2003; Craig-Schmidt 1996). Furthermore, PUFA such as EPA play a vital role in inflammation, regulating blood flow, immune response and ion transport by serving as a precursor of signaling eicosanoid molecules. In addition, long chain omega-3 PUFA, can be converted to other anti-inflammatory molecules, such as resolvins, maresins and omega-3-oxylipins

(Serhan 2008), which may also explain the function of omega-3 fatty acids in alleviating cancer (De Deckere 1999; Chen 2007), cardiovascular disease (Marchioli 2002; Wang 2006), psychiatric disorders (Puri 2006; Nemets *et al.* 2006), Parkinson's disease (Bousquet *et al.* 2008) and inflammatory conditions (Chandrasekara *et al.* 1995; Fisher *et al.* 1990).

Wheat germ oil (WGO) is well known as a tocopherol rich food lipid, which is procured from the germ of wheat kernel. It contains more than 55 % polyunsaturated fatty acids, mainly linoleic and linolenic acids (Wang & Johnson 2001). Octacosanol, a 28 carbon long-chain saturated primary alcohol, has also been found as one of the minor components of wheat germ oil, which is believed to serve as a physical performance enhancing agent as well as for improving Parkinson's disease and lowering plasma cholesterol (Hargrove *et al.* 2004; Taylor & Rapport 2003).

Oil blending has commonly been employed in the pharmaceutical industry and food industries (Chu & Kung 1998). For instance, omega 3 oils have been added to other oils in order to maximize the health benefits of the resultant formulations. Blended oils from fish oil and flax seed oil are considered as new pharmaceutical products with maximized benefits from both (Harper & Jacobson 2005). In the food industry, palm oil has been used to enhance the stability of cooking oils such as canola oil, during food processing and high temperature deep-fat frying. Addition of wheat germ oil to seal blubber and menhaden oils would may not only be expected to improve their oxidative stability with the increased content of tocopherols, but also to enhance health benefits contributed from the addition of its minor component, octacosanol.

The purpose of the present study was to examine the effect of blending SBO and MHO with WGO on their oxidative stability. Changes of tocopherol content and fatty acid composition in blended SBO and MHO was assessed by HPLC-MS and GC, respectively. Oxidative stability of blended oils was monitored by comparing conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) values following accelerated oxidation at 60 °C for 72 h with those of the original oils.

Materials and methods

Marerials The compound 2-thiobarbituric acid and standards of tocopherols were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Standard fatty acid methyl esters (FAMEs; GLC-461) were procured from Nu-Check (Elysian, MN, USA). All solvents used were of analytical grade and purchased from Fisher Scientific (Nepan, ON, Canada).

Methods

Blending SBO and MHO with WGO. SBO (19 or 18 g) and MHO (19 or 18 g) were physically blended with 5 (1 g) and 10% (2 g) of WGO, respectively. All the oil samples were flushed with nitrogen gas and stored at -20°C up to 14 days before analysis.

Determination of Fatty acid composition of SBO, MHO, WGO and their blended counterparts by GC analysis. Fatty acid compositions of SBO, MHO, WGO, SBO (5% of WGO), SBO (10% of WGO), MHO (5% of WGO), MHO (10% of WGO) were

determined following their conversion to the corresponding methyl esters (Maguire *et al.* 2004). The transmethylation reagent (2.0 ml) consisting of freshly prepared 6% sulphuric acid in methanol and 15 mg of hydroquinone were added to each sample vial, followed by vortexing. The mixture was incubated for 24 h at 60°C and subsequently cooled to ambient temperature. Distilled water (1.0 ml) and a few crystals of hydroquinone were then added to the mixture followed by thorough mixing. The resultant FAMEs were extracted three times, each with 1.5 ml of HPLC grade hexane. The combined hexane layers were then transferred to a test tube and washed twice with 1.0 ml of distilled water. The hexane was evaporated under a stream of nitrogen, and the extracted FAMEs were then dissolved in 1.0 ml of carbon disulphide which was used for GC analysis. A Hewlett-Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a SUPELCOWAX-10 column (30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON, Canada) was used to analyze the FAMEs. The oven temperature was first raised to 220°C and kept there for 10.25 min and then increased to 240°C at 30°C/min and held there for 15 min. The injector and FID temperatures were set at 250°C. Ultra high purity helium was used as a carrier gas at a flow of 15 ml/min. A Hewlett-Packard 3365 Series II ChemStation Software (Agilent) was used for data handling and processing. The FAMEs were identified by comparing their retention times with those of a known standard mixture, and the results were presented as weight percentage.

Determination of tocopherols content by HPLC-MS. Assessment of tocopherol content in seal blubber oil, menhaden oil, wheat germ oil and their blends was conducted in this

study. The tested oil samples were prepared by dissolving 0.1 g of oil in 2 ml of methonal-acetonitrile-isopropanol (41:59:300, v/v/v). Tocopherol standards were prepared by dissolving a known amount of tocopherol mixture (consisting of 4 tocopherol analogs) in the same solvent followed by dilution to different concentrations. Prior to HPLC analysis, both samples and standards were filtered using a 0.45 μm syringe-filter (Whatman, Clifton, NJ, USA).

Tocopherol contents in the prepared samples and standards were determined by reversed phase HPLC-MS, using an Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA), equipped with a UV–diode array detector (UV-DAD). Separation was achieved on a C-18 column (4.6 mm × 250 mm coupled with a guard column, Agilent) by gradient elution with a methanol-acetonitrile-isopropanol mobile phase at a flow of 0.8 ml/min; and fractions were detected at 295 nm. The mobile phase started with methanol-acetonitrile-isopropanol (41:59:0, v/v/v) and was maintained for 15 min. The mobile phase was gradually changed to methanol-acetonitrile-isopropanol (16.5:23.5:60, v/v/v) from 15 to 25 min, and then it reached 100% of isopropanol from 25 to 35 min, and was kept there for 10 min. The mobile phase was changed to its initial state, methanol-acetonitrile-isopropanol (41:59:0, v/v/v) in 5 min, and it was maintained for 10 min in order to recondition the column. LC flow was analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) using positive ion APCI (atmospheric pressure chemical ionization). The operating conditions used were 121 V for the fragmentor voltage, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, drying gas flow of 7 l/min. Each tocopherol standard

and sample (50 µl) was injected by using an auto-sampler. Tocopherols were detected at 295 nm by a UV detector and identified by comparing their retention times with those of known tocopherol standards. A standard curve was constructed for each tocopherol homologue (peak area versus concentration). Tocopherol concentrations in samples were calculated using the standard curve and expressed as mg tocopherol per 100 g oil.

Oxidative stability tests

(a) CD test. Oxidative stability of SBO, MHO, WGO and their blends was determined under Schaal oven conditions at 60°C for 72 h. Each day (24 h) under these conditions is equivalent to 1 month of storage at ambient temperatures. Oils (0.4–0.5 g) were placed in loosely capped test tubes (10 mm diameter and 4.0 cm height) and stored at 60°C in a forced-air oven (Thelco, Model 2; Precision Scientific Co., Chicago, IL, USA). Samples were removed from the oven at 0, 6, 12, 24, 48 and 72 h, cooled to room temperature, flushed with nitrogen, capped, and stored at -20°C for up to 7 days until analyzed. CD in the oils was determined according to the IUPAC (1987) method 20505. Oil samples (0.02–0.04 g) were weighed into 50-ml volumetric flasks, dissolved in iso-octane, and made up to the mark with the same solvent. The contents were thoroughly mixed, and the absorbance was read at 234 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent).

(b) TBA test. The determination of TBARS was carried out as described by the AOCS (1990) method Cd 19-90. Oil samples (0.05–0.10 g) were weighed into 25-ml volumetric

flasks, dissolved in a small volume of 1-butanol, and then made up to the mark with the same solvent. This solution (5 ml) was transferred into a screw-capped test tube, and then 5 ml of freshly prepared TBA reagent (0.5 g TBA in 250 ml n-butanol) were added. The mixture was thoroughly mixed, and then the test tubes were placed in a water bath at 95°C for 2 h. Heated samples were cooled in an ice bath, and the absorbance of the resultant coloured complex was read at 532 nm using a Hewlett-Packard diode array Model 8452 A spectrophotometer (Agilent).

Results and discussion

The fatty acid composition of SBO, MHO, WGO and their blends. The fatty acid composition of SBO, MHO, WGO and their blends are shown in Tables 4.1 and 4.2. Wheat germ oil is a rich source of unsaturated fatty acids, including the polyunsaturated fatty acid C18:2ω6 and the monounsaturated fatty acid C18:1ω9 (Table 4.1). More than 80% of the total fatty acids present were in a form of C18:2ω6 (55.53%) and C18:1ω-9 (25.4%). Alpha-linolenic acid (C18:3ω3) was the only omega-3 fatty acid that was detected in wheat germ oil at 3.6%. The saturated fatty acids detected were C14:0, C16:0 and C18:0.

Seal bubbler oil before and after blending with WGO. Seal blubber oil is an important source of omega-3 fatty acids, including C18:3ω3 (0.42%), C18:4ω3 (0.89%), C20:5ω3 (4.74%), C22:5ω3 (3.5%) and C22:5ω3 (6.08%) (Table 4.1). C14:1ω9 (1.27%), C16:1ω7 (19.03%), C17:1 (0.28%), C18:1ω9 (25.2%), C18:1ω11 (6.68%), C18:2ω6 (2.01%) and

C22:1ω11 (2.14%) were detected as unsaturated forms. In addition, C14:0 (4.32%), C16:0 (5.89%) and C18:0 (0.75%) were saturated forms of fatty acids detected in seal blubber oil. The change in total content of unsaturated fatty acids between the original SBO and its blends was insignificant (P>0.05) (Table 4.1).

Table 4.1. Fatty acid composition of original seal blubber oil, seal blubber oils blended with 5 and 10% wheat germ oil, and original wheat germ oil.

Fatty acids	SBO	SBO (5% WGO)	SBO (10% WGO)	WGO
C14:0	4.32±0.13	4.17±0.02	3.95±0.03	0.14±0.00
C14:1ω9	1.27±0.03	1.19±0.07	1.15±0.01	ND
C16:0	5.89±0.15	6.41±0.05	6.89±0.02	12.25±0.06
C16:1ω7	19.03±0.57	18.35±0.18	17.69±0.18	ND
C17:1	0.28±0.01	0.26±0.01	0.24±0.01	ND
C18:0	0.75±0.02	0.81±0.02	0.92±0.03	2.99±0.08
C18:1ω9	25.2±0.66	25.08±0.05	25.58±0.16	25.47±0.05
C18:1ω11	6.68±0.21	6.31±0.033	5.79±0.05	ND
C18:2ω6	2.01±0.06	5.84±0.07	8.85±0.08	55.53±0.22
C18:3ω3	0.42±0.01	0.63±0.01	0.71±0.01	3.6±0.03
C18:4ω3	0.89±0.02	0.81±0.01	0.74±0.00	ND
C20:1ω9	14.82±0.27	13.29±0.14	12.62±0.07	ND
C20:5ω3	4.74±0.12	4.05±0.06	3.68±0.04	ND
C22:1ω11	2.14±0.13	1.71±0.03	1.63±0.04	ND
C22:5ω3	3.5±0.13	2.71±0.05	2.45±0.02	ND
C22:6ω3	6.08±0.12	5.01±0.11	4.64±0.02	ND

Note: SBO: seal blubber oil; WGO: wheat germ oil; and ND: not detected

Menhaden oil before and after blending with WGO. All the omega-3 fatty acids from seal blubber oil were also detected in the original menhaden oil, such as C18:3ω3 (0.71%), C18:4ω3 (1.8%), C20:5ω3 (16.26%), C22:5ω3 (1.88%) and C22:5ω3 (5.77%) (Table 4.2). The total content of omega-3 fatty acids in menhaden oil was more than 24% of the total fatty acids, which is significantly higher than that of seal blubber oil. On the other hand, unsaturated fatty acids, including C14:1ω9 (0.59%), C16:1ω7 (18.56%), C17:1 (1.47%), C18:1ω9 (9.1%), C18:1ω11 (4.51%), C18:2ω6 (0.88%) and C20:1ω9 (0.81%) were found in menhaden oil. Only C14:0 (12.77%), C16:0 (18.56%) and C18:0 (2.74%) were the saturated fatty acids detected in menhaden oil (Table 4.2). The total content of unsaturated fatty acids of the original MHO and its blends did not change significantly (P>0.05).

Table 4.2. Fatty acid composition of original menhaden oil, menhaden oils blended with 5 and 10% of wheat germ oil, and original wheat germ oil.

Fatty acids	MHO	MHO (5% WGO)	MHO (10% WGO)	WGO
C14:0	12.77±0.03	12.58±0.04	11.62±0.07	0.14±0.00
C14:1ω9	0.59±0.01	0.52±0.01	0.49±0.01	ND
C16:0	18.56±0.17	18.55±0.03	18.08±0.08	12.25±0.06
C16:1ω7	15.73±0.05	15.4±0.05	14.21±0.06	ND
C17:1	1.47±0.01	1.35±0.02	1.25±0.01	ND
C18:0	2.74±0.01	2.61±0.05	2.67±0.02	2.99±0.08
C18:1ω9	9.1±0.01	9.76±0.02	10.68±0.04	25.47±0.05
C18:1ω11	4.51±0.15	4.48±0.07	4.32±0.05	ND
C18:2ω6	0.88±0.03	5.37±0.02	8.67±0.04	55.53±0.44
C18:3ω3	0.71±0.00	0.79±0.01	0.96±0.01	3.62±0.03
C18:4ω3	1.8±0.01	1.58±0.02	1.48±0.01	ND
C20:1ω9	0.81±0.02	0.63±0.00	0.61±0.00	ND
C20:5ω3	16.26±0.04	14.34±0.02	13.53±0.06	ND
C22:5ω3	1.88±0.03	1.27±0.03	1.21±0.02	ND
C22:6ω3	5.77±0.07	4.53±0.05	4.32±0.02	ND

Note: MHO: menhaden oil; WGO: wheat germ oil; and ND: not detected

Tocopherol content. Alpha-tocopherol is the natural antioxidant in marine oils, which has been the major tocopherol homologue detected in both SBO and MHO (Hamam & Shahidi 2006). The results showed that the alpha-tocopherol content was 14.5 mg/100 g in SBO, while it was 8.92 mg/100 g in MHO; these values are higher than those reported previously (Wang & Shahidi *et al.* 2010; Wang & Shahidi 2011; Wanasundara & Shahidi 1999). Tocopherol contents in this work were directly analyzed after dissolving of a small amount of oil samples in the solvent without saponification, and thus the accuracy of determination was improved as loss of tocopherol during alkaline saponification and extraction was prevented. The higher alpha-tocopherol content so obtained may also be due to the different refining process and characteristics of the test oil in this work. All four tocopherol homologues including alpha-, beta, gamma and delta tocopheols were detected in WGO, and its total tocopherol content was 124 mg/100, which is 8 times that in SBO and 13 times that in MHO (Figure 4.1 and Table 4.3). These results demonstrated that WGO is a rich source of tocopherols, and the addition of WGO to SBO and MHO led to an increase of tocopherol content in both oils (Wang & Johnson 2001). Based on the results, a significant ($P<0.05$) increase in the content of α-tocopherol and other tocopherol homologues was noted in both blended SBO and MHO. The total tocopherol contents of SBO (5% WGO) and SBO (10% WGO) increased to 19.98 and 25.18 mg/100 g, respectively; while those in blended MHO reached 14.68 (5%WGO) and 20.42 (10% of WGO) mg/100 g, respectively.

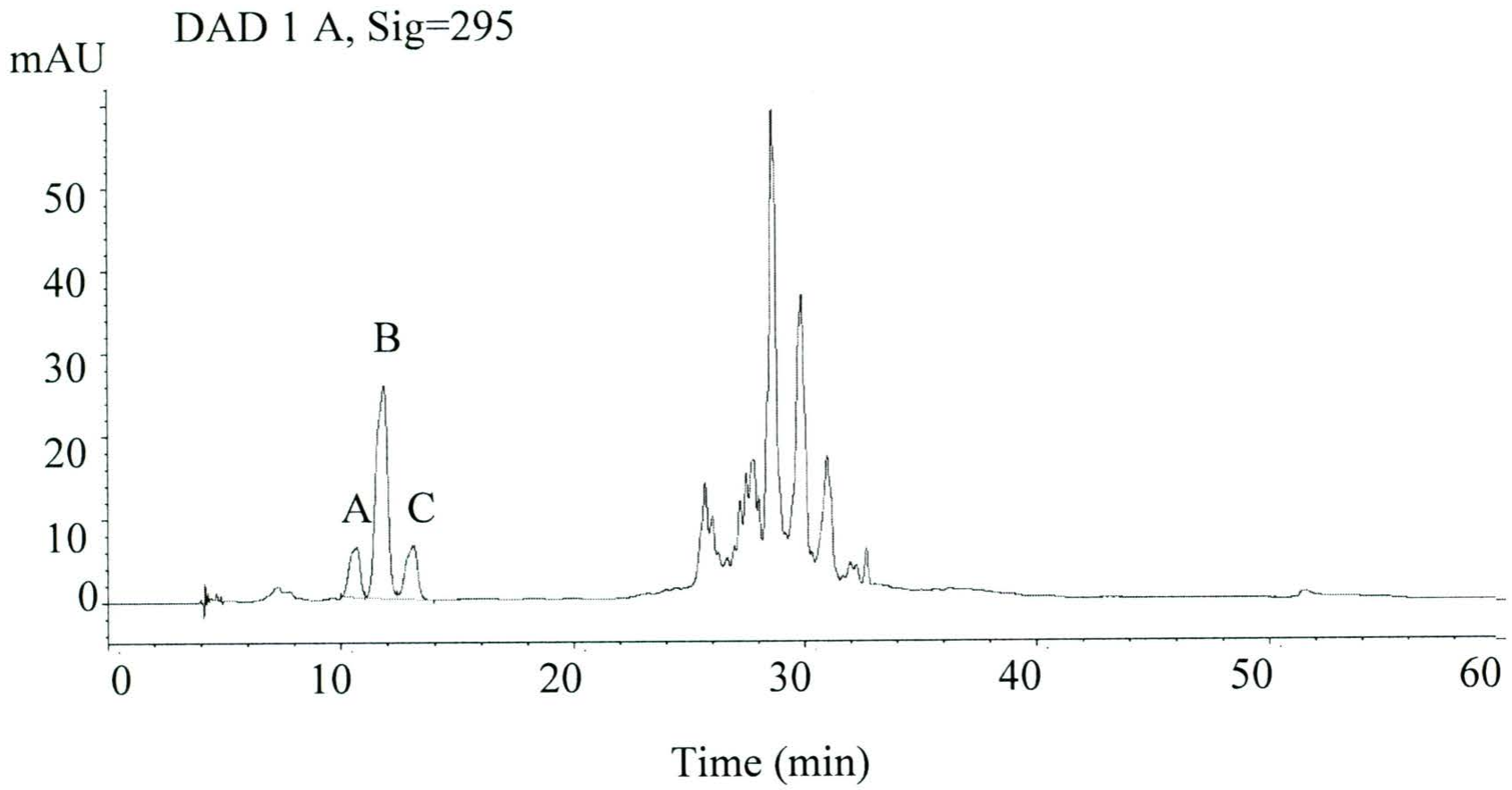

Figure 4.1. High-performance liquid chromatography (HPLC) chromatograms of tocopherol content in wheat germ oil at 295 nm. A: delta-tocopherol; B: beta- and gamma tocopherols; and C: alpha-tocopherol

Table 4.3. Tocopherol contents (mg/100 g of oil) in original seal blubber, menhaden, wheat germ oils and their blends

Oil samples	Alpha-tocopherol	Beta+gamma-tocopherols	Delta-tocopherol
WGO	32.85 ± 0.88	62.71 ± 1.54	28.44 ± 0.70
SBO	14.5 ± 0.52	ND	ND
SBO (10% WGO)	16.07 ± 0.43	6.27 ± 0.15	2.84 ± 0.07
SBO (5% WGO)	15.42 ± 0.45	3.14 ± 0.08	1.42 ± 0.03
MHO	8.92 ± 0.85	ND	ND
MHO (10% WGO)	11.31 ± 0.85	6.27 ± 0.15	2.84 ± 0.07
MHO (5% WGO)	10.12 ± 0.85	3.14 ± 0.08	1.42 ± 0.03

WGO: wheat germ oil; SBO: seal blubber oil; MHO: menhaden oil; and ND: not detected

Oxidative stability

CD test. The CD value of lipids, measured by their absorption at 234 nm, is an indicator of primary oxidation products as conjugated dienes are formed due to a shift in the double bond positions upon oxidation of lipids that contain dienes or polyenes (Logani & Davies 1980). Figure 4.2 shows the CD contents of the original SBO, WGO and their blends under accelerated Schaal oven condition at 60 °C.

The CD values of both original SBO and its blends, SBO (5% WGO) and SBO (10% WGO), increased throughout the entire experimental period due to the formation and accumulation of lipid hydroperoxides, which are the major primary oxidation products (Hamam & Shahidi 2004*)*. Formation of lipid hydroperoxides coincides with that of CD upon oxidation (Hamam & Shahidi 2006; Farmer & Sutton 1946*)*. CD values increased slowly from 0 to 48 h when primary oxidation products, hydroperoxides, were generated and released during lipid oxidation. However, the amount of hydroperoxide present was fairly low at the beginning of oxidation chain reaction. As the storage time was extended to 72 h, large amounts of primary oxidation products were produced due the abundance of free radicals, and this sharply increased CD values which reached the highest values at 72 h (Figure 4.2). Values obtained at 0, 12 and 24 h did not show any significant difference between the original SBO and SBO (5% WGO) blend, while those of the latter were significantly lower when compared to those of the original SBO, indicating better oxidative stability of SBO (5% WGO) blend. The results showed significantly lower CD values at 6, 24, 48 and 72 h for SBO (10% WGO) blend when compared to those of the original SBO, while no significant difference existed at other test points. The CD values did not show any increase for the original WGO, which indicated its excellent stability

under accelerated oxidation conditions. As the most important lipid-soluble antioxidants, tocopherols can effectively decrease lipid oxidation by scavenging free radicals produced from lipid peroxidation chain reactions, and thus may prevenct the oxidation reaction from continuing (Herrera & Barbas 2001; Traber & Atkinson 2007). The mechanism of the ability of α-tocopherol in quenching free radicals is shown in Figure 4.3 (Kamal-Eldin & Appelqvist 1996). The content of α-tocopherol by itself was more than 32.85 mg/100 g of WGO, and the total tocopherol content of WGO was 124 mg/100 g. The large amount of tocopherols, especially gamma and delta tocopheols, helped WGO maintain a good oxidative stability under accelerated oxidation conditions as they possess higher capacity than alpha-tocopherol in preventing lipid oxidation (Liu *et al.* 2002; Wagner *et al.* 2004). The changes of tocopherols content in WGO was monitored throughout the experimental period, and merely 4 % of total tocopherols were lost at the 72 h test point (Figure 4.4). Beta-, gamma and delta tocopherols showed better stability than alpha-tocopherol during the accelerated oxidation as the latter had a significantly ($p<0.05$) higher loss (>4.5%) compared to the loss (<3.1%) of others. The results from fatty acid composition analysis showed no significant changes in the total content of unsaturated fatty acids between the original SBO and its blends (Table 4.1). Thus, the improved oxidative stability of SBO (5% WGO) and SBO (10% WGO) blends was due to the increased tocopherol content contributed by the addition of WGO. The tocopherol content increased 38 and 74 % in SBO (5% WGO) and SBO (10% WGO) blends, respectively, compared to that in the original SBO (Table 4.3).

The CD values of MHO, MHO (5% WGO) and MHO (10% WGO) monitored at each test point are shown in Figure 4.2. The CD values of MHO (5% WGO) blend were

significantly lower than those of MHO at 6, 12 and 48 h, but showed no significant difference at other test points. Significant difference existed in the CD values between MHO (10% WGO) blend and MHO at 0, 6, and 12 h while no significant difference was observed at other test points. MHO (5% WGO) and MHO (10% WGO) blends showed a better oxidative stability when comparing their CD values with those of the original MHO. The results from fatty acid composition analysis did not reveal any significant changes in the total content of unsaturated fatty acids between the original MHO and its blends with WGO (Table 4.2). Therefore, the enhanced stability of MHO blends with WGO is mainly due to the increased content of tocopherols from the addition of WGO as the total tocopherols increased to 14.68 and 20.42 mg/100 g of oil in MHO (5% WGO) and MHO (10% WGO) blends, respectively, while it was 8.92 mg/100 g of oil in the original WGO.

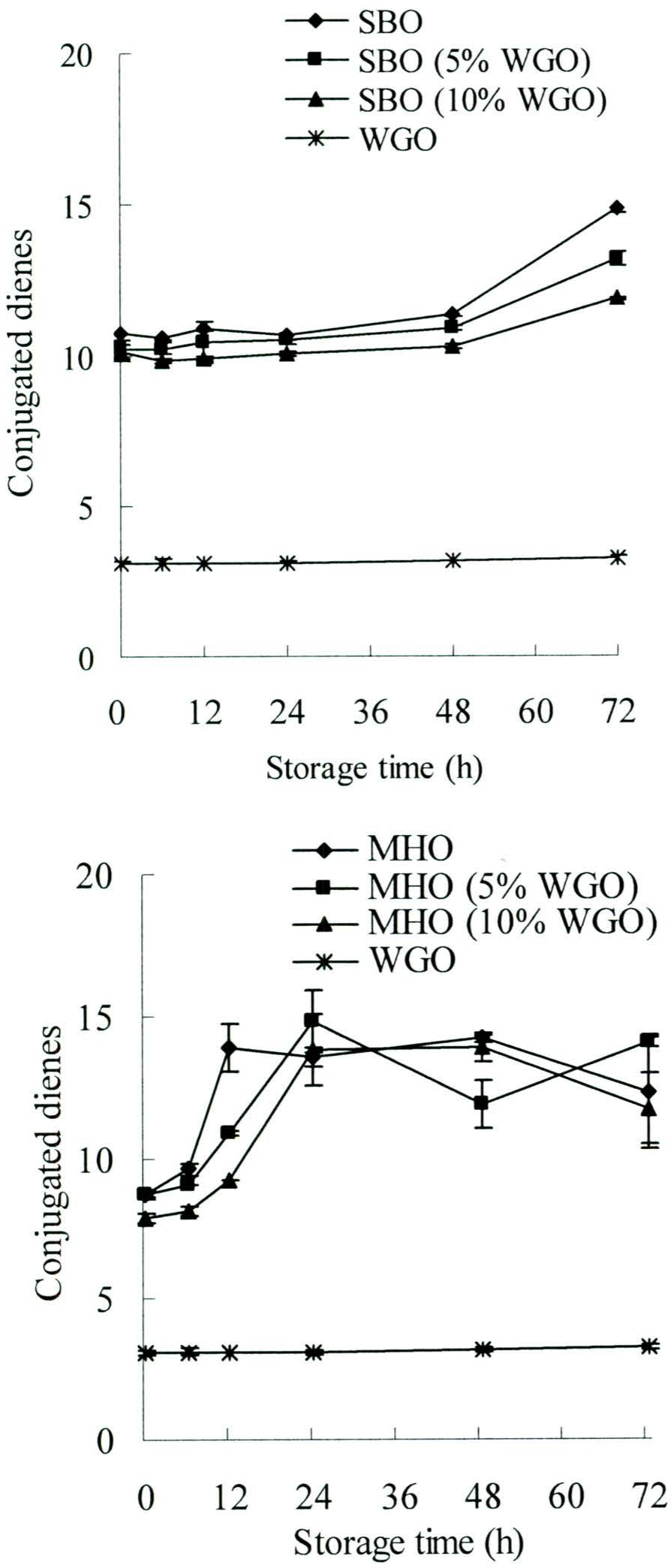

Figure 4.2. Conjugated dienes values of SBO and its blends (top) and MHO and its blends (bottom) stored under Schaal oven condition at 60°C; SBO: original seal blubber oil; MHO: original menhaden oil; and WGO: original wheat germ oil.

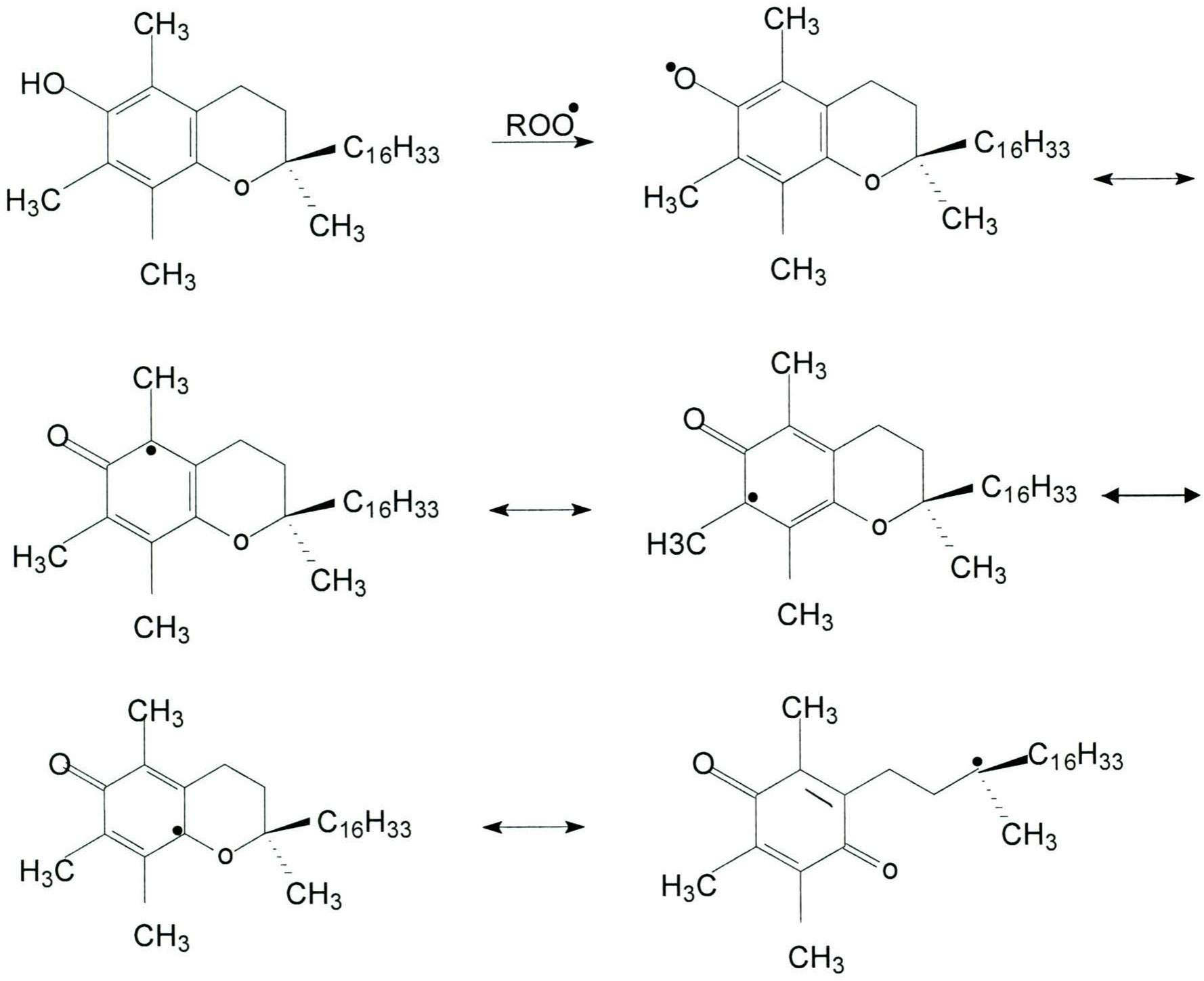

Figure 4.3. Resonance structures of the α-tocopherol radical.

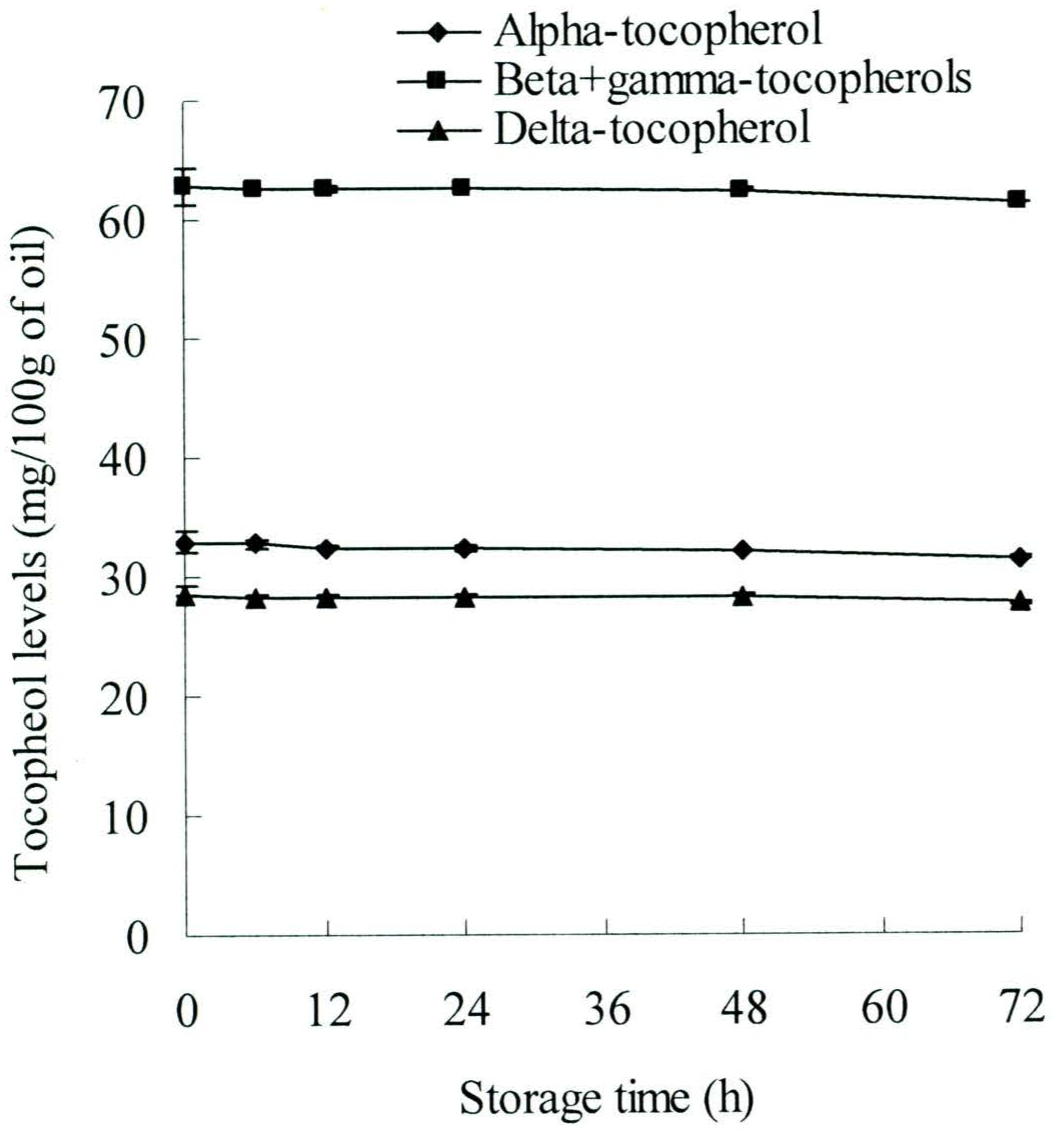

Figure 4.4. Changes of tocopherols contents in the original wheat germ oil stored under Schaal oven condition at 60°C.

TBA test. The TBA test has commonly been employed to assess the level of secondary oxidation products. The rate of formation of secondary oxidation products is mainly affected by three factors, namely the amount of hydroperoxides as primary oxidation products, the level of tocopherols, and the nature of hydroperoxides which may be decomposed to secondary oxidation products at different rates under the same conditions (Nevdakh *et al.* 1984*)*. The type of the hydroperoxide is determined by the nature of fatty acid oxidized.

The results shown in Figure 4.5 summarize TBARS values of SBO and its blends, which increased progressively until reaching their highest values during the storage period in both SBO and its blends, SBO (5% WGO) and SBO (10% WGO). The TBARS values increased in a slow manner from 0 to 48 h, and started having sharp increase after 48 h and reached their highest values at 72 h (Figure 4.5) (Strange *et al.* 1997; Senanyake & Shahidi 2001). Meanwhile, the TBARS values of the original WGO were fairly low and did not show a sharp increase during the entire oxidation period, which coincided with the low CD values of this oil (Figure 4.2). The low TBARS values of WGO indicate its high tocopherol content effectively prevented lipid oxidation. The results did show a significantly lower TBARS value for SBO (5% WGO) blend when compared to that of SBO at 72 h, while showing no significant difference at other test points. On the other hand, the TBARS values of SBO (10% WGO) blend were significantly lower than those the original SBO at both 24 and 72 h, while no significant difference existing at other test points. Based on the results of TBARS, SBO (5% WGO) and SBO (10% WGO) blends were more stable than the original SBO due to increased tocopherols content.

The TBARS results obtained for MHO and its blends showed an increasing trend from 0 to 48 h, but decreased at 72 h (Figure 4.5). The TBARS values of MHO (5% WGO) blend were significantly lower than those of the original MHO at 6, 12 and 48 h, but had no significant difference at other test points. MHO (10% WGO) blend had significantly lower TBARS values than MHO from 0 to 72 h, but showed a higher value at 72 h after reaching the peak value. Compared to the original MHO, a better oxidative stability of both MHO (5% WGO) and MHO (10% WGO) blends was demonstrated as shown by their lower TBARS values.

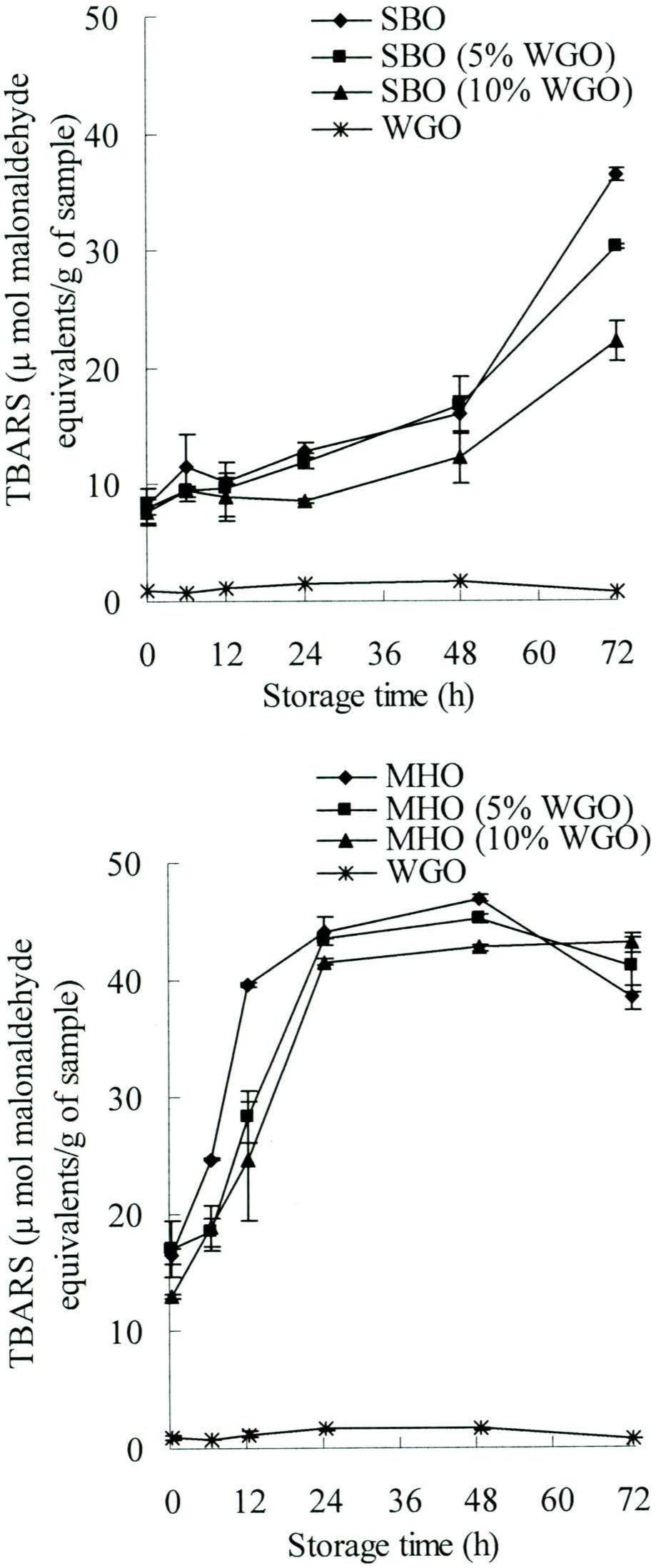

Figure 4.5. TBARS values of SBO and its blends (top) and MHO and its blends (bottom) stored under Schaal oven condition at 60°C; SBO: original seal blubber oil MHO: original menhaden oil; and WGO: original wheat germ oil

Conclusions

Addition of wheat germ oil to seal blubber and menhaden oils modified their fatty acid composition and increased their tocopherol content. The changes of fatty acid composition should not have any major effects on the oxidative stability of the blended oils as the total content of unsaturated fatty acids did not significantly changed. The tocopherol rich wheat germ oil showed an excellent oxidative stability during the oxidation period. The oxidative stability of seal blubber and menhaden oils was improved when blended with different levels of wheat germ oil, mainly due to the increased content of tocopherols, especially gamma- and delta tocopheols. Therefore, wheat germ oil served as a good stabilizer for omega-oils with high contents of polyunsaturated fatty acids.

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CHAPTER 5

Synthesis of mono and dioleyl *p*-coumarates and response surface methodology (RSM) for synthesis optimization

Introduction

Seal blubber and menhaden oils are commonly used as supplements due to their high content of omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Wanasundara & Shahidi. 1996). The beneficial effects of omega-3 fatty acids have been associated with their ability to lower triacylglycerol levels in circulatory system, and in the treatment and/or prevention of hypertension, arthritis, inflammatory and immune disorders (Shahidi & Miraliabari 2005; Wanasundara & Shahidi 1997). In addition, as one of the major membrane components in the brain and retina, DHA is believed to be essential for normal development of central nervous system and visual acuity in humans (Kim & Edsall 1999). However, seal blubber oil and menhaden oil are vulnerable to oxidation due to their high contents of polyunsaturated fatty acids. Traditionally, addition of synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ), as well as natural antioxidants, such as tocopherols, to edible oils and foods has been practiced in order to enhance the oxidative stability and retain the nutritional values of edible oils (Shahidi *et al.* 1992). However, a number of studies have reported that prolonged exposure to high doses of synthetic antioxidants, such as TBHQ, may cause

stomach cancers in an animal model (Hirose et al. 2010). Furthermore, effectiveness of tocopheols as antioxidants can be weakened by the presence of metal ions, including those of iron and copper, which are commonly present in marine oils (Shahidi & Miraliabari 2005; Wanasundara & Shahidi 1997). Therefore, studies on the potential use of other forms of naturally occurring antioxidants to stabilize edible oils are required.

Investigations on the antioxidative efficacy of other natural antioxidants, such as flavonoids and phenolic acids, in foods have been widely reported, and most of them have proven to reduce the extent of lipid oxidation. Antioxidants from these groups are also believed to offer several health benefits to humans. For instance, *p*-coumaric acid occurs in various food sources and beverages, including tomatoes, carrots, garlic as well as wines and vinegars (Ferguson *et al.* 2005). Both its strong antioxidant property and anti-cancer effect have been reported in a number of studies, and its potential to reduce the risk of stomach cancer mainly through reducing the formation of carcinogenic nitrosamines has been demonstrated (Kikugawa *et al*. 1983; Rosskopf *et al*. 1992).

Modifying the structure of triacylglycerols can be used as an effective means to improve their nutritional and health benefits. Acidolysis is a modification process, which incorporates desired fatty acids or other forms of organic acids, such as phenolic acids, into triacylglycerols by using enzyme catalysts (Senanayake & Shahidi 2002). For example, acidolysis of phenolic acids and triacylglycerols has been successfully conducted and reported in several studies (Safari *et al*. 2006; Sabally *et al*. 2006). In this work, a model study using *p*-coumaric acid and triolein was conducted in order to investigate the effects of various experimental conditions on the degree of esterification of *p*-coumaric acid and to determine the proper synthesis conditions to be further

employed in natural oils such as those of highly oxidizable seal blubber and menhaden oils. Response surface methodology (RSM) was used to optimize the degree of esterification of *p*-coumaric acid to triolein. Based on the reported studies and the results from preliminary experiments, enzyme load, reaction time and mole ratio of substrates were selected as variables in the experimental design. The effects of these three experimental conditions on the degree of *p*-coumaric acid esterification as well as the interaction among them were examined in this study. In addition, the predicted conditions to optimize the degree of esterification were also discussed.

Materials and Methods

Materials. Triolein was purchased from Nu-Chek (Elysian, MN, USA). Novozyme 435 and *p*-coumaric acid were obtained from Sigma-Aldrich (Mississauga, ON, Canada). All solvents used were of analytical grade and purchased from Fisher Scientific (Nepean, ON, Canada).

Experimental Design for RSM. Response surface methodology (RSM) is a collection of mathematical and statistical techniques, which has been used for empirical model building to optimize a response influenced by several independent variables. The design of this experiment was generated with a three factors, three level central composite design using RSM. The design was composed of 17 experiments with 3 centre points 8 corner points and 6 facial points. Seventeen runs were performed in a totally random order for minimizing the effects of unexpected variability in the observed responses. The response

is the degree of esterification of *p*-coumaric acid after acidolysis. Enzyme load (5-15 wt%), reaction time (144-288 h) and mole ratio of substrates (triolein to *p*-coumaric acid, 2:1-16:1) were chosen as the three tested variables. Although a higher enzyme load was preferred in previous studies to obtain a higher conversion rate of the substrates, the enzyme load is minimized in industry to reduce the cost. In the preliminary study, the increase of conversion rate continued to 288 h of reaction, which suggested that a longer reaction period was required to reach the peak conversion rate. However, as an unsaturated fatty acid, triolein is vulnerable to oxidation and generates oxidation products during the reaction, and the level of oxidation increases as the reaction time is prolonged. Therefore, 288 h was selected for the longest reaction course in order to minimize the oxidation of this unsaturated oil. Details of the central composite face-centred (CCF) design are shown in Tables 5.1 and 5.2.

Table 5.1. Independent variables in the experimental design.

Variables	Coded levels		
	−1	0	1
Enzyme load (wt%)	5	10	15
Reaction time (h)	144	216	288
Mole ratio of substrates (triolein to *p*-coumaric acid)	2	9	16

Table 5.2. The central composite design matrix employed for the three independent variables.

Run	Variables		
	X_1	X_2	X_3
1[c]	0	0	0
2	1	1	1
3	−1	1	−1
4	0	0	−1
5[c]	0	0	0
6	1	−1	−1
7	−1	1	1
8	1	1	−1
9	0	0	1
10	−1	−1	1
11	1	0	0
12	0	1	0
13	1	−1	1
14	0	−1	0
15[c]	0	0	0
16	−1	−1	−1
17	−1	0	0

Note: X_1: enzyme load; X_2: reaction time; X_3: mole ratio of substrates;
[c]: centre points; actual values of the independent variables seen in Table 5.1

The degree of esterification of *p*-coumaric acid to triolein was modeled from the experimental data using a second-order polynomial regression equation (Equation 5.1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \qquad \text{Equation 5.1}$$

Where Y is the predicted response variable (the degree of esterification), β_0, is the intercept, β_i is the first-order linear effect, β_{ii} is the interaction terms, β_{ij} is the squared effect, and X_i and X_i are the independent variables, including enzyme load, reaction time and mole ratio of substrates.

Equation 5.2 was used to calculate the degree of esterification after acidolysis.

$$\text{Degree of esterification (\%)} = 100 * A_f/A_i \qquad \text{Equation 5.2}$$

Note: A_i is peak area of *p*-coumaric acid in the initial reaction mixture; A_f is total peak area of synthesized phenolic lipid including mono- and dioleyl *p*-coumarates in the final reaction mixture

The statistical software *JMP* version 6.0.0 (SAS Institure Inc. Cary, NC, USA) was used to generate the experimental design and analyze the experimental data.

Acidolysis Reaction. The lipase-catalyzed acidolysis of *p*-coumaric acid with triolein was carried out following the method reported by Safari *et al*. (2006) and Sabally *et al*. (2006). A stock solution of *p*-coumaric acid (10 mM) was freshly prepared in 2-butanone, and three stock solutions of triolein (6.67, 30, 53.36 mM) were prepared in hexane. Subsequently, 1 ml of *p*-coumaric acid stock solution and 3 ml of triolein stock solution were transferred to a 20-mL screwed-capped tube followed by the addition of different amounts of immobilized Novozyme 435 accordingly based on the generated experimental design. The final reaction solutions contained 2.5 mM *p*-coumaric acid and a triolein solution of 5, 22.5 and 40 mM, according to the three different mole ratios of substrates. All tubes were flushed with nitrogen and sealed properly. The acidolysis reaction was performed in an orbital shaking water bath at 50°C and 170 rpm. Reaction mixtures were removed from the water bath at different times according to the experimental design. The reaction mixture (0.1 ml) was transferred to a 4-ml vial, and the solvent was then removed under a stream of nitrogen. The sample was redissolved in 2 ml of solvent mixture consisting of methanol-acetonitrile (41:59, v/v) for HPLC-MS analysis. All reactions were run in duplicate.

HPLC-MS analysis. The composition of the reaction mixture was determined by reversed phase HPLC-MS. An Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV–diode array detector (UV-DAD) was used. Separation of different components in the sample was achieved on a C-18 column (4.6 mm × 250 mm coupled with a guard column, Agilent), using an isocratic elution with a miture of methanol-acetonitrile (41:59, v/v). This was followed by two linear gradients with isopropanol at a

flow rate of 0.8mL/min, and fractions were detected at both 215 and 300 nm. LC flow was further analyzed online by the mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) (LC-MSD-Trap-SL, Agilent) with atmospheric pressure chemical ionization (APCI) at alternative mode for identification of each fraction. The operating conditions used were 121 V for the fragmentor voltage, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, drying gas flow of 7 litre/min.

Results and discussion

Estimated model. The model coefficients were generated with JMP software based on the measured responses given in Table 5.3. Based on the results (Table 5.3) of the experiments, the following second order polynomial equation (Equation 5.3) giving the degree of *p*-coumaric acid esterification as a function of enzyme load, reaction time and mole ratio of substrates of the acidolysis reaction was obtained.

$$Y = 3.17 + 0.79X_1 + 1.21X_2 + 1.18X_3 + 0.39X_1X_2 + 0.48X_1X_3 + 0.34X_2X_3 - 0.35X_1^2 - 0.49X_2^2 - 0.26X_3^2 \quad \text{Equation 5.3}$$

Table 5.3. Central composite design and response for the enzymatic synthesis of mono and dioleyl *p*-coumarates

Run	Pattern	Variables			Response (*Y*) degree of incorporation
		X_1 (wt%)	X_2 (h)	X_3 (ratio)	
1[c]	0 0 0	10	216	9	2.85 ± 0.11
2	+ + +	15	288	16	6.43 ± 0.40
3	− + −	5	288	2	1.25 ± 0.12
4	0 0 a	10	216	2	1.66 ± 0.29
5[c]	0 0 0	10	216	9	3.32 ± 0.08
6	+ − −	15	144	2	0.09 ± 0.04
7	− + +	5	288	16	2.93 ± 0.75
8	+ + −	15	288	2	2.23 ± 0.13
9	0 0 A	10	216	16	4.40 ± 0.67
10	− − +	5	144	16	0.98 ± 0.01
11	A 0 0	15	216	9	4.00 ± 0.34
12	0 A 0	10	288	9	4.13 ± 0.34
13	+ − +	15	144	16	2.31 ± 0.32
14	0 a 0	10	144	9	1.46 ± 0.28
15[c]	0 0 0	10	216	9	2.87 ± 0.07
16	− − −	5	144	2	0.04 ± 0.01
17	a 0 0	5	216	9	1.87 ± 0.45

Note: pattern identifies the coding of the factors. It shows all the codings with "+" for high, "-" for low factor, "a" and "A" for low and high axial values, and "0" for midrange; X_1: enzyme load; X_2: reaction time; X_3: mole ratio of substrates; and [c]: centre points

The coefficient of determination, R^2 was 0.92, which indicates that the sample variation of 92.00% for the degree of esterification is attributed to three selected independent variables, including enzyme load, reaction time and mole ratio of the substrates. The closer the value of R^2 to 1, the better the model fits the experimental data, while the smaller value of R^2, the lesser will be the relevance of the dependent variable for explaining the behaviour of variations in the model (Cao *et al*. 2008; Tan & Shahidi 2012). Therefore, a R^2 of 0.92 indicates a strong fit between the model and the experimental data. The high significance of the model used was also supported by adjusted R^2, which was 0.95. Thus, both R^2 and adjusted R^2 provide a correspondence of the second order polynomial model to the obtained experimental data. Further, statistical testing of the regression model has been done by analysis of variance (ANOVA), which is also used to analyze the significance of the model. In this analysis, the F value indicates the ratio of the mean square regression to the mean square due residuals. The higher the F value is, the greater the variation accounted for the model is compared to the unexplained variation. The ANOVA of the regression model revealed that the model is highly significant, with a high F value of 64.0479 and a small P value (< 0.0001). In addition, each of the observed actual response values were compared with the adequate predicted response values in the parity plot (Figure 5.1), which shows an acceptable level of agreement (Peričin *et al*. 2009).

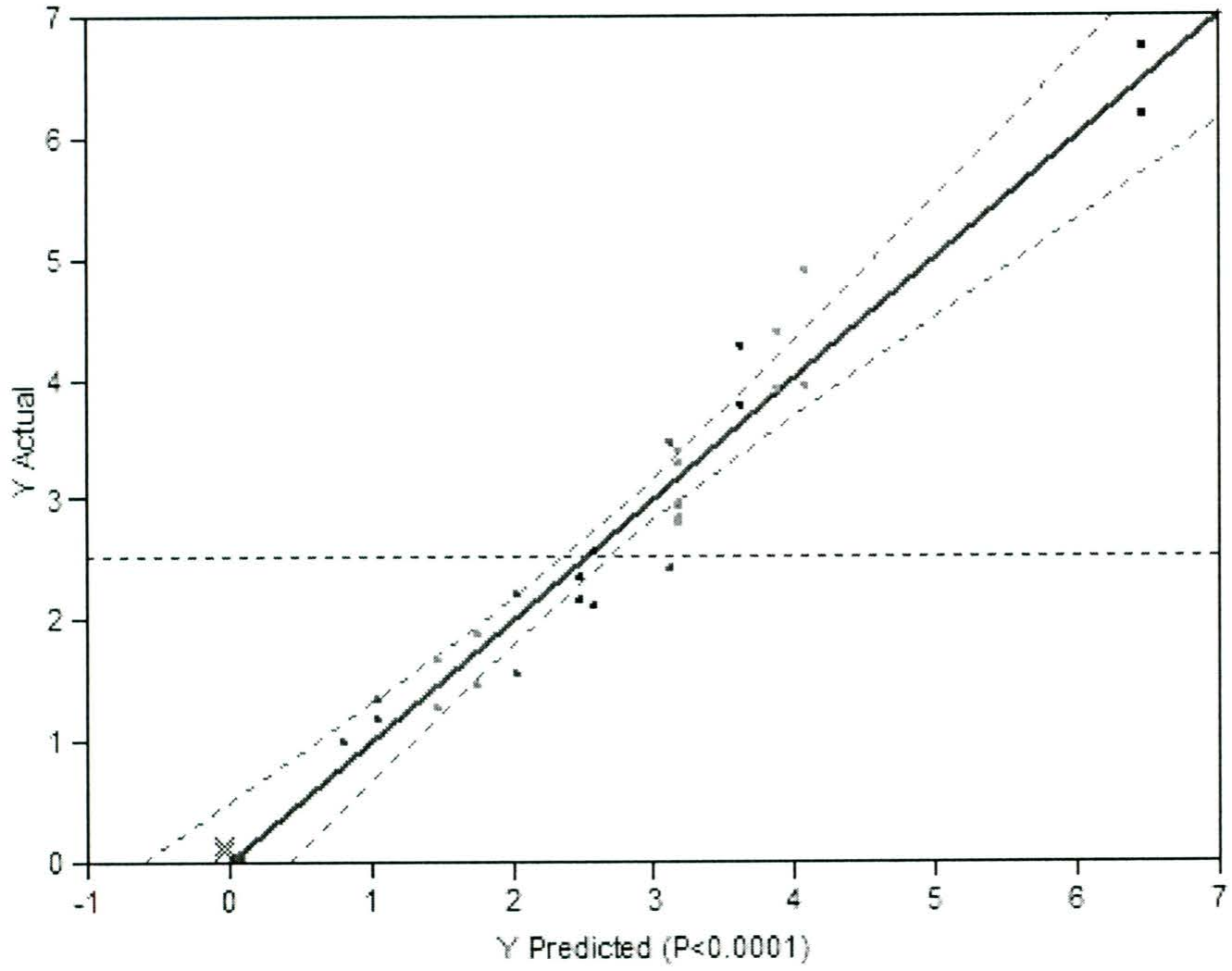

Figure 5.1. Parity plot of actual response values and predicted response values; Y: reponse (the degree of esterification).

The significance of each coefficient was assessed by Student's *t* test and *P* test (Table 5.3). The significance of corresponding coefficient was assessed with their correspondent *t* values and *P* values as they negatively correlated with *P* values and positively correlated with *t* values (Cao *et al.* 2008).

The significance of the coefficient of the first order effects of three independent variables on the response, the degree of esterification of *p*-coumaric acid, can be explained by comparing either their corresponding *P* values or *t* values. Small *P* values (< 0.0001) indicate that significant coefficients of all three variables, enzyme load, reaction time and mole ratio of the substrates, which implies the significant influence of the variables on the investigated response. Furthermore, the magnitudes of the *t* values give the significance of their first order coefficient in a descending order, from reaction time, mole ratio of the substrates to enzyme load, which means the reaction time has the most influence, and enzyme load has the least effect. In addition, the results of the analysis on the quadratic effects of three variables showed that the coefficients of enzyme load and reaction time are significant as small *P* values (< 0.05) occurred. The *P* value of 0.1267 from the third independent variable, i.e., the mole ratio of substrates, indicates an insignificant corresponding coefficient of quadratic effect of this variable.

Based on the analysis results (Table 5.4) of the cross-product, the significance of coefficient of the interactions between each pair of tested variables on the determined response were confirmed with smaller *P* values, all of which were lower than 0.002, and greater *t* value, which were greater than 3.5. For instance, the smallest *P* value, < 0.0001, and the largest *t* value, 4.96, from the cross-product of enzyme load and substrate ratio, indicate the most siginificant coefficient of the interaction between the enzyme load and

mole ratio of the substrates. The less significant coefficient from the other two cross interactions are attested by their larger P values and smaller t values. Such effects are shown graphically in contour plots (Figure 5.2).

Table 5.4. Estimated coefficients and corresponding *t* and *P* values

Term	Coefficient	Std error	*t* Test	*P* value
Intercept	3.17	0.12	27.34	< 0.0001
Linear				
Enzyme load	0.80	0.09	9.35	< 0.0001
Reaction time	1.21	0.09	14.11	< 0.0001
Substrate ratio	1.18	0.09	13.76	< 0.0001
Cross-product				
Enzyme load * reaction time	0.39	0.10	4.06	0.0005
Enzyme load * Substrate ratio	0.48	0.10	4.96	< 0.0001
Reaction time * Substrate ratio	0.34	0.10	3.52	0.0017
Quadratic				
Enzyme load * Enzyme load	-0.35	0.17	-2.13	0.0440
Reaction time * Reaction time	-0.49	0.17	-2.99	0.0064
Substrate ratio * Substrate ratio	-0.26	0.17	-1.58	0.1267

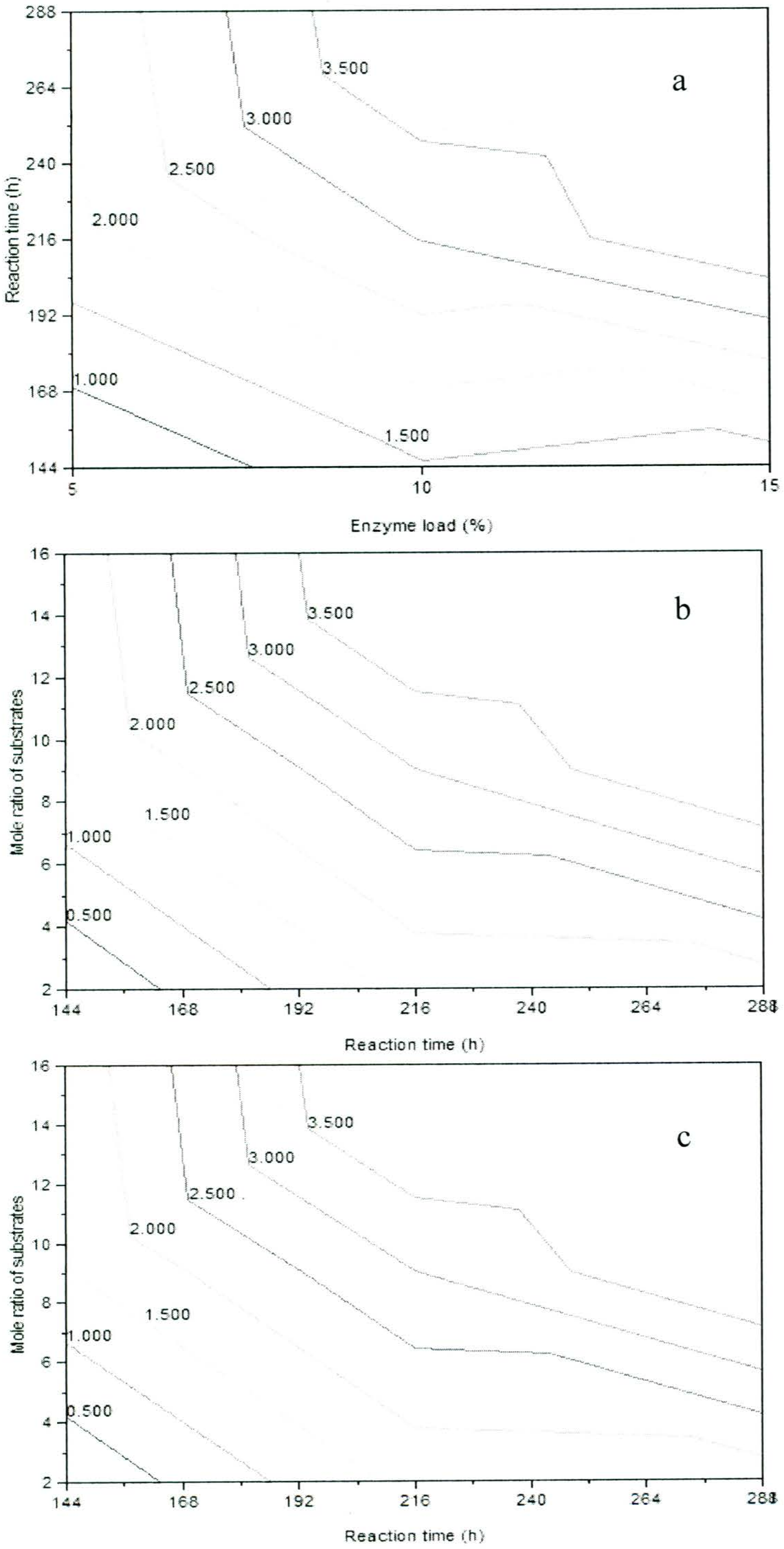

Figure 5.2. Contour plots of two tested variables on the degree of esterification of *p*-coumaric acid; a: reaction time and enzyme load; b: mole ratio of substrates and enzyme load; and c: mole ratio of substrates and reaction time.

Canonical analysis and steepest ascent analysis. The results from canonical analysis showed that the stationary point (a saddle point) predicted was located outside the range of the parameter space employed in the design of optimization, which indicates that the optimization conditions were not in the selected ranges of the three variables. In this situation, the directional search method, steepest ascent analysis was employed to determine the direction toward predictive higher responses with the use of the magnitude and sign of the linear effects in this study (Chen *et al.* 2002). In steepest ascent analysis, the path begins at the centre of the current design space and stretches well outside the design space. A sequence of equally spaced locations along the path is then normally selected and used to generate a new set of optimization experiments. Based on the results of steepest ascent analysis, the path of steepest ascent was to increase all of the three variables, enzyme load, reaction time and the mole ratio of substrates in order to improve the degree of esterification (Figure 5.3). However, there are limitations on options of ranges of selected variables due to the chemical quality and cost controls. For instance, the level of oxidation and concentration of oxidation products are positively correlated with the time course of storage under high temperature (Neff & Byrdwel 1998; Nevdakh *et al.* 1984). Therefore, the reaction time exceeding the used maximum reaction time course, 288 hours, in the experiment should be the avoided. The maximum enzyme dose should not exceed 15% (wt) of the total substrate in order to control the cost in the industry, so the design of experiments regarding the use of enzyme should not be adjusted to the path shown in steepest ascent analysis to achieve a higher degree of esterification by compromising the quality of the lipids and increasing the cost. Therefore, the degree of esterification so yielded in the central composite design should be the closest value to the

possible ideal optimized degree, and the maximum values of the three variables for the synthesis of *p*-coumaric acid with other lipids is recommended.

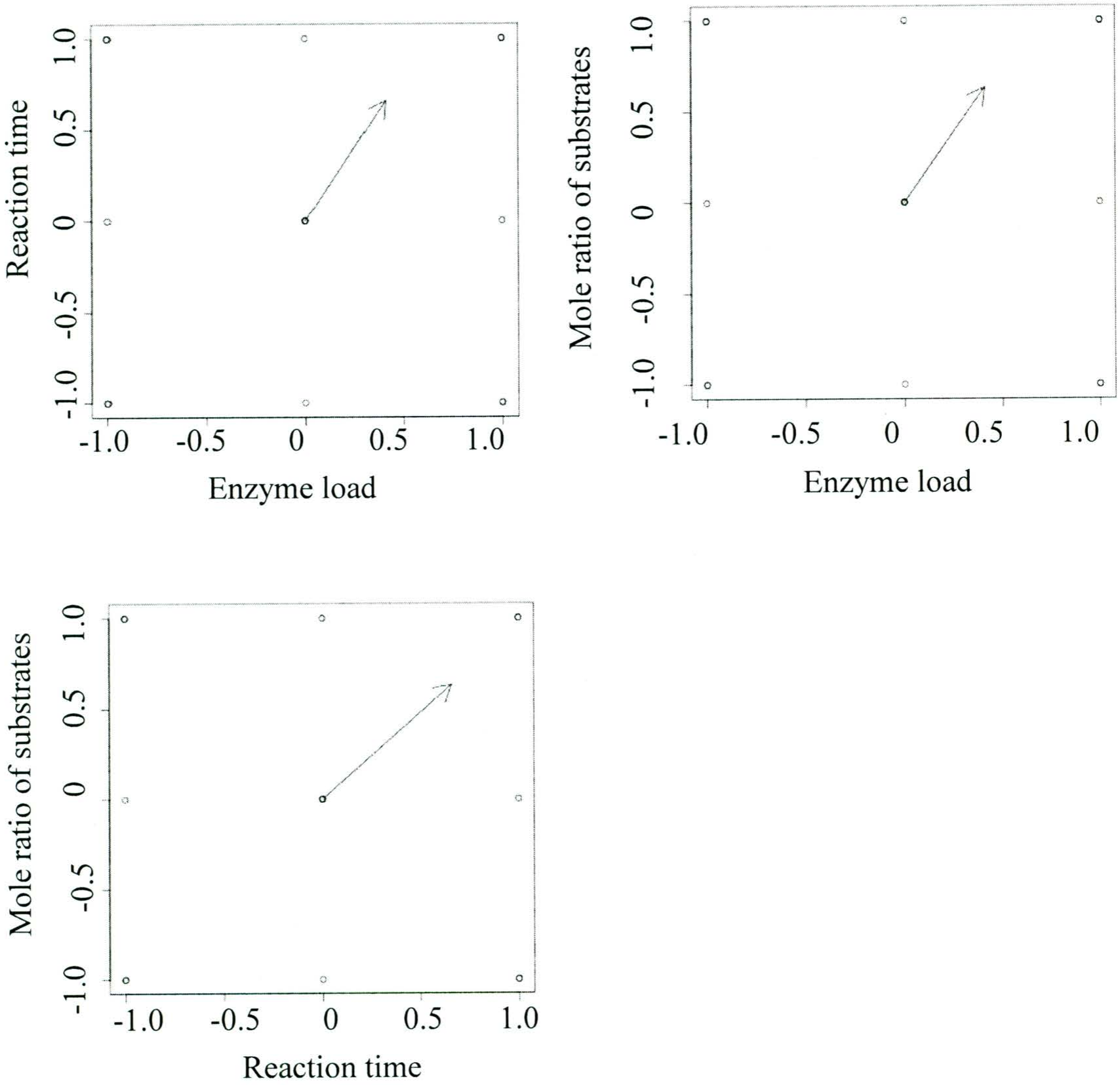

Figure 5.3. Steepest ascent analysis on three dependent variables.

Identification of components from the acidolysis mixtures. The HLPC profiles were monitored by UV at both 215 and 300 nm (Figures 5.4 and 5.5). Different components from the initial and final acidolysis mixtures are shown in Figure 5.4. Each compound was identified by comparing its retention time with that of its corresponding standard and/or the characterization of molecular ion so generated from mass spectrometry.

The compounds in peaks C and F were indentified as *p*-coumaric acid derivatives as only those compounds with a *p*-coumaric acid moiety give an absorbance at 300nm while other compounds from the final acidolysis mixture, including oleic acid, diolein, triolein are not detected at this wavelength. The major compounds in peaks C (t_R, 6.0 min) and peak F (t_R, 23.7 min) were identified as monooleyl *p*-coumarate and dioleyl *p*-coumarate, respectively, based on the fragmentation pattern and molecular ions yielded (Figure 5.6). For monooleyl *p*-coumarate, the major molecular ion generated during fragmentation in peak C showed a m/z at 485.5, representing $[M+H-H_2O]^+$ of monooleyl *p*-coumarate, might have resulted from the loss of a H_2O and gain of a proton. For dioleyl *p*-coumarate, the abundant molecular ion yielded in peak F showed a m/z at 765.7, representing $[M-H]^-$, which resulted from the loss of a proton.

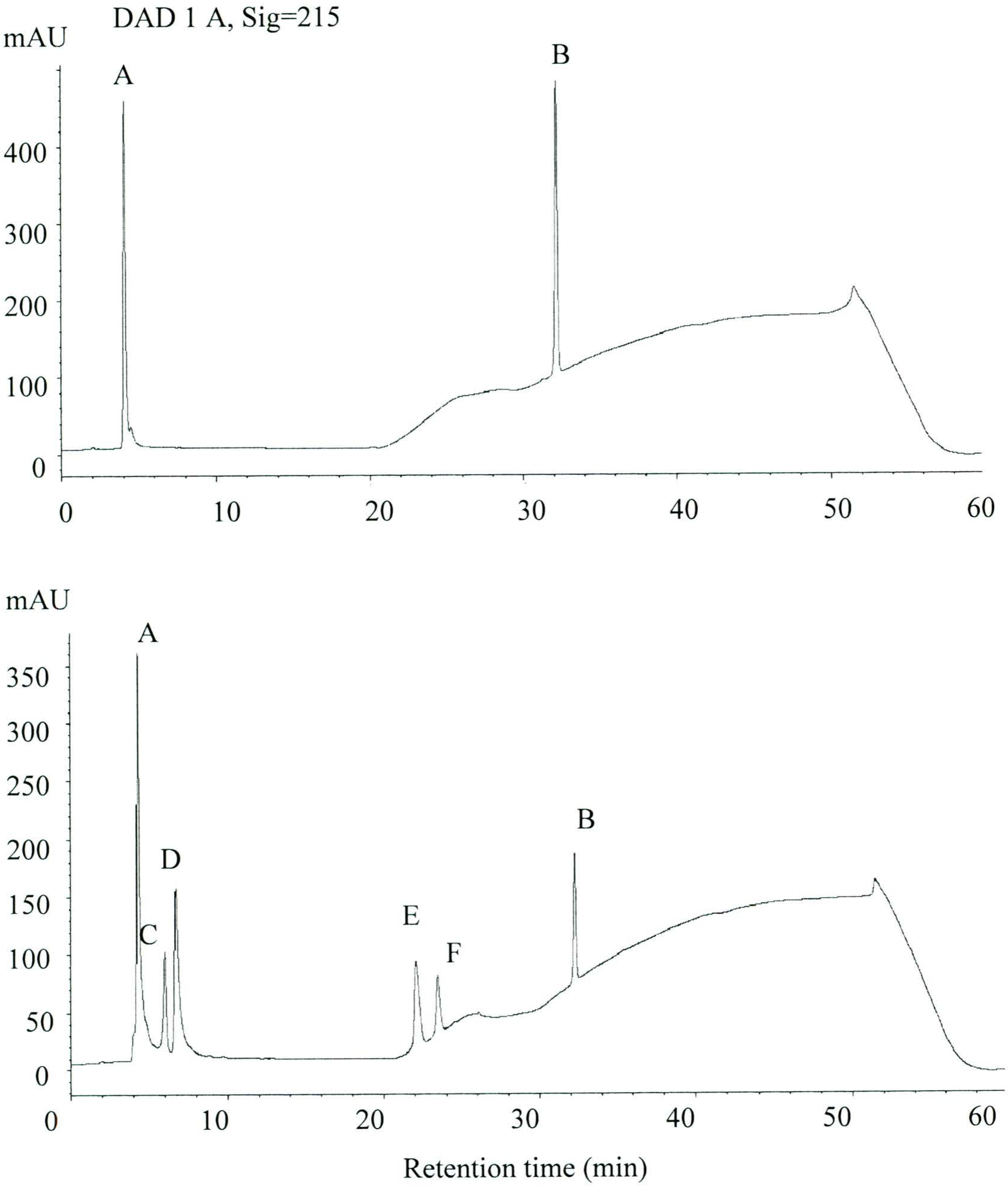

Figure 5.4. High-performance liquid chromatography (HPLC) chromatograms of starting materials (top) and resultants (bottom) of acidolysis of *p*-coumaric acid and triolein at 215 nm. A: *p*-coumaric acid; B: triolein; C: monooleyl *p*-coumarate; D: oleic acid; E: diolein; and F: dioleyl *p*-coumarate.

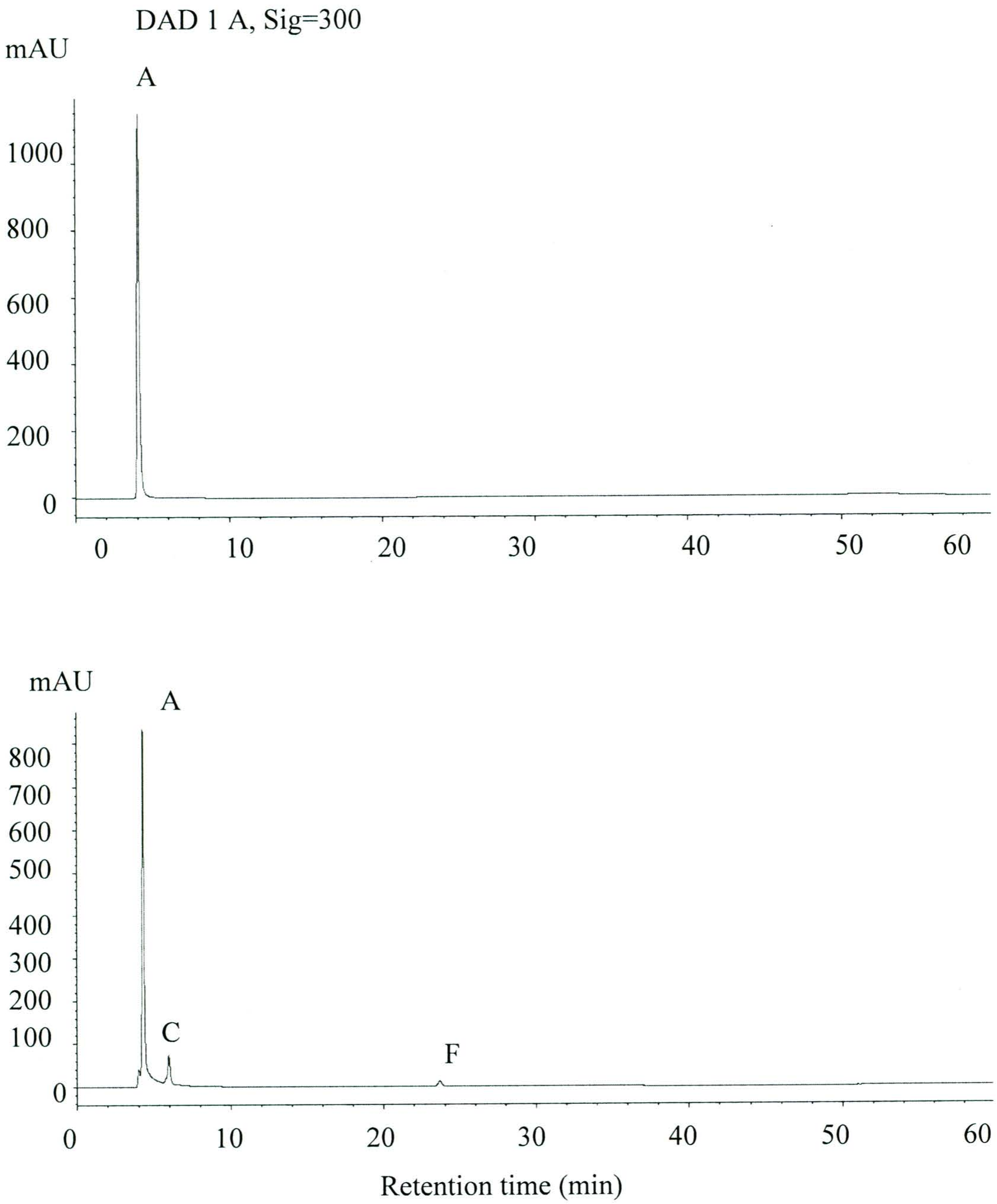

Figure 5.5. High-performance liquid chromatography (HPLC) chromatograms of starting materials (top) and resultants (bottom) of acidolysis *p*-coumaric acid and triolein at 300 nm. A: *p*-coumaric acid; C: monooleyl *p*-coumarate; and F: dioleyl *p*-coumarate.

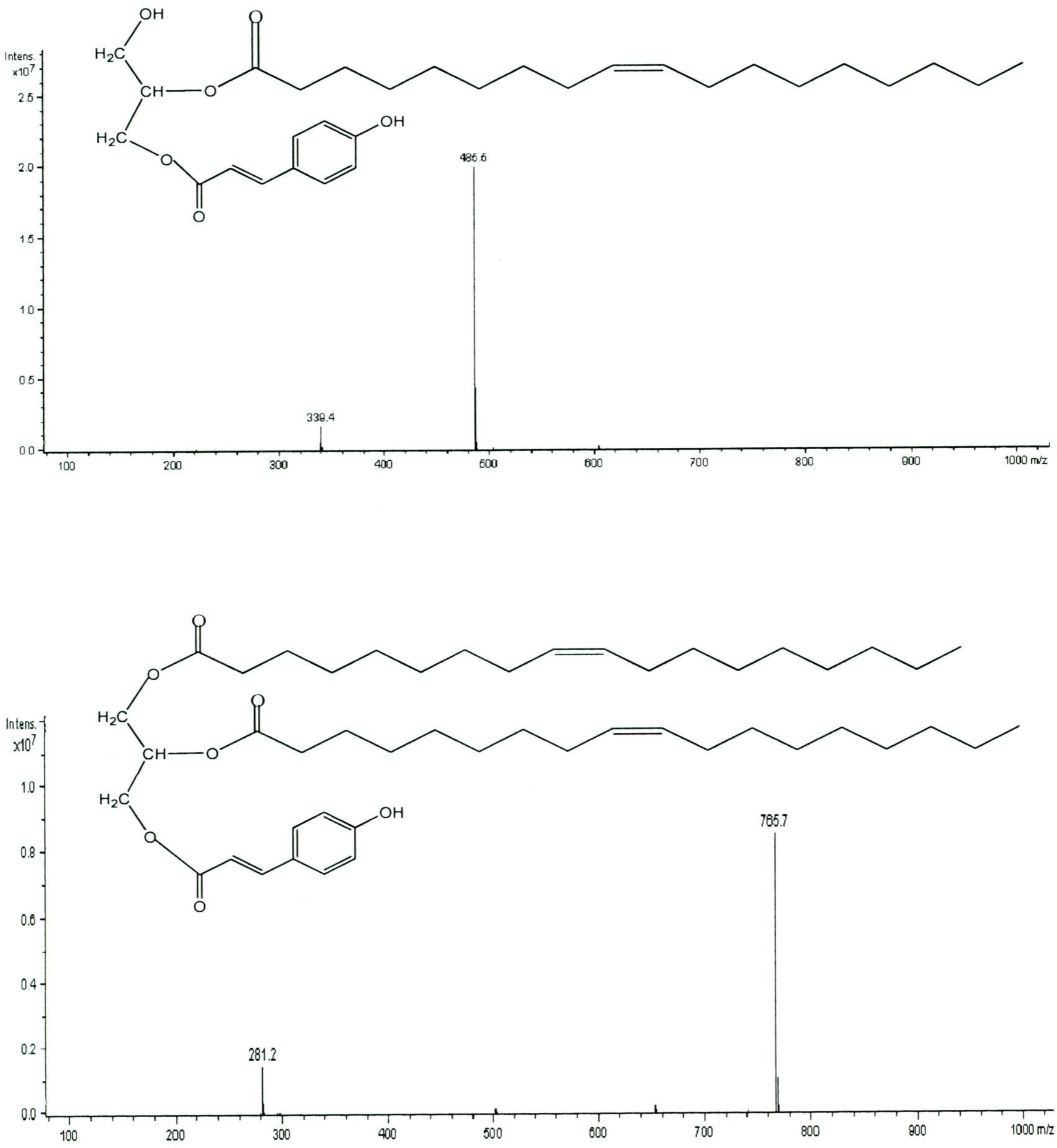

Figure 5.6. Chemical structures and mass spectrometric data of monooleyl *p*-coumarate (top) and dioleyl *p*-coumarate (bottom).

Conlusions

Based on the analysis of experimental data, the model employed was highly sufficient for determining the effectiveness and interaction of three selected variables, enzyme load, reaction time and the mole ratio of substrates, on the dependent variable, the degree of esterification. Although the optimization point was not found in the selected range of the three variables, the steepest ascent analysis suggested that an increase of these three variables might lead to a stationary point. However, based the limitations on increasing the range of tested variables, including possible oxidation of synthesized lipids and increased cost, the degree of esterification so yielded in the designed central composite design should be the one closest to the possible ideal optimized degree. Thus, the maximum values of tested variables were recommended in the use of the acidolysis of *p*-coumaric acid with triolein. Theses conditions may also be employed for acidolysis of seal blubber and fish oils with phenolic acids such as *p*-coumaric acid.

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CHAPTER 6

Antioxidant activity of mono and dioleyl *p*-coumarates in *in vitro* tests, food and biological model systems.

Introduction

Lipid oxidation occurs commonly in food and biological systems. Unsaturated oils, especially those of polyunsaturated type are vulnerable to oxidation caused by free radicals. Lipid oxidation is a major contributor to the deterioration of the edible oils and lipid-based foods. Traditionally, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) together with natural antioxidants such as tocopherols are commonly added to edible oils and lipid rich foods to prevent their oxidation (Shahidi *et al.* 1992). However, a number of studies have reported that prolonged exposure to high doses of synthetic antioxidants, such as TBHQ, may cause stomach cancer in animals (Hirose et al. 2010). Furthermore, effectiveness of tocopheols as antioxidants can be weakened by the presence of metal ions, including those of iron and copper, which are commonly present in oils from different origins, including those from marine sources (Shahidi & Miraliakbari 2005; Wanasundara & Shahidi 1996). Therefore, studies on potential use of other forms of naturally occurring antioxidants to stabilize edible oils and lipid rich foods are required.

Phenolic compounds are a group of natural antioxidants that have been extensively examined for their antioxidant activity and inhibition of lipid oxidation in foods

(Ferguson *et al.* 2005; Kikugawa *et al.* 1983). Antioxidant activity of *p*-coumaric acid, a common phenolic acid occurring in various food sources and beverages, including tomatoes, carrots, garlic as well as wines and vinegars, has been studied in *in vitro* assays as well as in food and biological model systems (Kikugawa *et al.* 1983; Fukumoto & Mazza 2000; Dávalos *et al.* 2004; Medina *et al.* 2006; Gadow *et al.* 1997; Andreasen *et al.* 2001). The great scavenging activity of *p*-coumaric acid towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxyl radicals has been well demonstrated. *p*-Coumaric acid also showed effective metal reduction activity using reducing power assay. Furthermore, it effectively inhibited the bleaching of *β*-carotene in oil-in-water emulsions as well as the oxidation of human low-density lipoprotein (LDL) cholesterol. Like other phenolic compounds, hydrophilic *p*-coumaric acid has poor solubility in media with higher lipophilicity such as fats, oils, lipid rich foods, emulsion formulas in cosmetic as well as biological environments. Lipid derivatives of *p*-coumaric acid, mono and dioleyl *p*-coumarates obtained from the acidolysis of *p*-coumaric acid with triolein, possess improved lipophilicity. Changes of lipophilicity and the steric structure may have great influence on the antioxidant activity of these compounds and their extended use in foods and edible oils.

Two main mechanisms by which antioxidants can play their protective role in food and biological systems have been proposed (Leopoldini *et al.* 2004). In the first one, antioxidants scavenge free radicals by donating a hydrogen atom and themselves become antioxidant radicals, which are then stabilized by electron delocalization. A higher stability of antioxidant radical corresponds to a better capacity of the antioxidant. In the hydrogen donation mechanism, the bond dissociation enthalpy of the O-H bonds in

antioxidants is an important parameter in evaluating the antioxidant action; weaker the O-H bond, the more likely will hydrogen donation occur and deactivate free radicals. The second mechanism involves electron transfer, by which antioxidants donate an electron to free radicals and themselves become radical cations. In this case, the stability of radical cations of antioxidants resulting from the electron transfer is important, which is usually evaluated by the ionization potential of the antioxidant molecules; the lower the ionization potential is, the easier would be the electron transfer, leading to the formation of radical cations of antioxidants. Both the bond dissociation enthalpy and the ionization potential should be considered to assess antioxidant capacity of phenolic antioxidants (Leopoldini *et al.* 2004).

In this work, antioxidant activity of synthesized oleyl *p*-coumarates was investigated in several *in vitro* assays, including DPPH radical scavenging activity, oxygen radical absorbance capacity (ORAC) and reducing power assays. In order to obtain a better understanding of their antioxidant capacity in food and biological systems as well as their potential in food and health applications, antioxidant activity of synthesized phenolic lipids was further examined in a β-carotene-linoleate model system, muscle food model system, human LDL cholesterol oxidation and deoxyribonuclei acid (DNA) scission assays.

Materials and methods

Materials. Triolein was purchased from Nu-Chek (Elysian, MN, USA). Novozyme 435 and *p*-coumaric acid were obtained from Sigma-Aldrich Canada Ltd. (Mississauga, ON,

Canada). Trolox was purchased from Acros Organics (Fair Lawn, NJ, USA). Human LDL cholesterol and supercoiled plasmid pBR322 DNA were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). SYBR safe gel stain was obtained from Invitrogen Corporation (Carlsbad, CA, USA). Fresh ground lean pork was purchased from a local supermarket. All solvents used were of analytical grade and purchased from Fisher Scientific (Nepan, ON, Canada).

Synthesis of mono and dioleyl *p*-coumarates by lipase-catalyzed acidolysis reaction. The Lipase-catalyzed acidolysis of *p*-coumaric acid with triolein was carried out following the method described by Safari *et al.* (2006) and Sabally *et al.* (2006) with minor modification. Reaction conditions were selected based on the study described in Chapter 5. A stock solution of *p*-coumaric acid (10 mM) was freshly prepared in 2-butanone, and a stock solution of triolein (53.36 mM) was prepared in hexane. Then, 30 ml of *p*-coumaric acid stock solution and 90 ml of triolein stock solution were transferred to a 250-ml round-bottom flask followed by addition of 645 mg of immobilized Novozyme 435 (15%, w/w of substrates). The final reaction solutions contained 2.5 mM of *p*-coumaric acid and 40 mM of triolein. The flask was flushed with a stream of nitrogen and sealed. The acidolysis reaction was performed in an orbital shaking water bath at 50°C at 170 rpm. Reaction mixtures were removed from the water bath after 12 days. The reaction mixture (0.1 ml) was transferred to a 4-ml vial, and the solvent was then removed under a stream of nitrogen. The sample was redissolved in 2 ml of a solvent mixture that consisted of methanol-acetonitrile (41:59, v/v) for HPLC-MS analysis.

HPLC-MS analysis synthesized products. The composition of the reaction mixture was determined by reversed phase HPLC-MS. An Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV–diode array detector (UV-DAD) was used. Separation was achieved on a C-18 column (4.6 mm × 250 mm coupled with a guard column; Agilent) by gradient elution with a methanol-acetonitrile-isopropanol mobile phase at a flow of 0.8 ml/min; fractions were detected at both 215 and 300 nm. The mobile phase used initially was methanol-acetonitrile-isopropanol (41:59:0, v/v/v), which was maintained for 15 min. The mobile phase was gradually changed to methanol-acetonitrile-isopropanol (16.5:23.5:60, v/v/v) from 15 to 25 min, and then it reached 100% isopropanol from 25 to 35 min, and was then kept for 10 min with 100% isopropanol. The mobile phase was changed to its initial state, methanol-acetonitrile-isopropanol (41:59:0, v/v/v) in the next 5 min, and it was maintained for 10 min in order to recondition the column. LC flow was further analyzed online by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) with atmospheric pressure chemical ionization (APCI) at alternative mode for identification of each fraction. The operating conditions used were 121 V for the fragmentor voltage, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, and drying gas flow of 7 l/min.

Purification and identification of mono and dioleyl *p*-coumarates. Separation of components, especially mono and dioleyl coumarates in the final acidolysis mixture was achieved using flash column chromatography. The mixture was eluted on a silica column (40 cm × 5 cm i.d.) packed with silica gel (Selecto Scientific, Suwannee, GA, USA) with

a series of solvent mixtures consisting of 350 ml of hexane, 100 ml of hexane-ethyl acetate-formic acid (95:5:2, v/v/v), 400 ml of hexane-ethyl acetate-formic acid (85:15:2, v/v/v), 400 ml of hexane-ethyl acetate-formic acid (75:25:2, v/v/v) and 400 ml of hexane-ethyl acetate-formic acid (65:35:2, v/v/v), in the order stated. Fractions corresponding to each component were collected, and then solvents were removed using a rotary evaporator. Lipid derivatives of *p*-coumaric acid, mono and dioleyl *p*-coumarates, were confirmed by HPLC-MS as described above.

DPPH radicals scavenging activity. The effect of mono and dioleyl *p*-coumarates on the scavenging of DPPH radicals was determined according to the method described by Madhujith and Shahidi (2006) with minor modifications. DPPH radical scavenging capacity of *p*-coumaric acid was used as a reference in this study. *p*-Coumaric acid (2.5 mM), monooleyl *p*-coumarate (2.5 mM) and dioleyl *p*-coumarate (2.5 mM) were dissolved in ethanol, which was followed by an addition of 2 mL of ethanolic solution of DPPH (0.18 mM). Contents of each test solution were thoroughly mixed and injected to the sample cavity of a Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA), and the spectrum was recorded after 10 min. Ethanol was used as the control in place of test compounds. Meanwhile, trolox standards (12.5-200 mM) were prepared for constructing a standard curve. The operating parameters of the Bruker E-scan (EPR spectrometer) were set as follows: 5.02 × 102 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G centre field, 5.12 ms time constant, 9.795 GHz

microwave frequency, and 86.00 kHz modulation frequency. DPPH radical scavenging capacities of test compounds were calculated using Equation 6.1.

DPPH radical scavenging capacity (%) = (1−signal intensity$_{sample}$/signal intensity$_{control}$) × 100 Equation 6.1

Where the control contained no trolox or test compounds. The DPPH radical scavenging capacities of test compounds were expressed as mmol trolox equivalents (TE) per mmol of sample.

ORAC assay. The determination of ORAC was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC, USA) equipped with an incubator and two injector pumps according to Madhujith and Shahidi (2007). Fluorescein and 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) were used as the probe and radical generator, respectively. Solutions of *p*-coumaric acid (5 mM), monooleyl *p*-coumarate (5 mM), dioleyl *p*-coumarate (5 mM) and trolox standards (6.25−100 μM) were prepared in 7% (w/v) randomly methylated *β*-cyclodextrin (RMCD). An aliquot of each solution (20 μl) was injected into a 96-well Costar black plate (Corning Incorporated, Corning, NY, USA), followed by addition of 75 μl of AAPH (17.2 mg/ml in PBS). Phosphate buffer saline (PBS) (75 mM, pH 7.0) was used as a blank. Samples were incubated at 37°C for 10 min prior to the auto-injection of 75 μl of AAPH (17.2 mg/ml in PBS) to each sample. The operating conditions were set as follows: 0.3 s position delay, 8 s orbital shaking before each cycle with 4 mm width, 210 s cycle time, and 48 cycles. Fluorescence was

measured at an excitation wavelength of 485 nm and emission wavelength of 520 nm. A standard curve was constructed from the trolox standards, and results were expressed as mM of trolox equivalents (TE) per mM of sample.

Determination of reducing power. Reducing power of *p*-coumaric acid, monooleyl *p*-coumarate and dioleyl *p*-coumarate was measured according to Duh *et al.* (2001) with slight modifications. One millilitre of test compounds (1mM in 95% ethanol) was transferred to centrifuge tubes containing 2.5 ml of PBS (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide solution. The mixture was incubated at 50°C for 20 min, which was followed by addition of 2.5 ml of trichloroacetic acid (TCA) (10%, w/v), and then the content was centrifuged at 3000 x g for 10 min. After incubation, a portion (2.5 ml) of the supernatant was transferred to the test tube containing 2.5 ml of distilled waster and 0.5 ml of ferric chloride (0.1%). The contents were thoroughly mixed, and the absorbance was read at 234 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent, Palo Alto, CA, USA). Increased absorbance of the reaction mixture indicated increased reducing power. A series of ascorbic acid solutions (1-6 mM in 95% ethanol) were prepared for use in constructing a standard curve.

Antioxidant activity in *β*-carotene-linoleate model system. Antioxidant activity of *p*-coumaric acid, monooleyl *p*-coumarate and dioleyl *p*-coumarate in an oil-in-water emulsion was assessed using the *β*-carotene bleaching assay according to Amarowicz and Shahidi (1995) with minor modifications. *β*-Carotene (10 mg) was dissolved in chloroform (10 ml), and an aliquot (1.0 ml) of this solution was transferred into a 150 ml

round-bottom flask containing linoleic acid (20 mg) and Tween 40 (200 mg). A blank without β-carotene was also prepared (20 mg of linoleic acid + 200 mg of Tween 40). After removal of chloroform under a stream of nitrogen, 50 ml of aerated distilled water were added to each flask, and the contents were then thoroughly mixed using a magnetic stirrer. Test compounds (30 µl, 1mM in ethanol) and the control, ethanol, were injected into the Costar 96-well white plates (Corning Incorporated, Corning, NY, USA). The β-carotene-linoleic acid emulsion and the blank emulsion were injected to the test vials by the built-in injector pump. The absorbance was measured immediately after the addition of emulsion to samples at 450 nm by a Fluostar Optima (BMG LABTECH Inc., Durham, NC, USA) plate reader. The operating parameters of Fluostar Optima were set as follows: 24 cycle No, 420 s cycle time, 4 s shaking time, 5 mm orbital width and 310 µl/s injection speed.

Antioxidant activity of test compounds in protecting β-carotene-linoleic acid oxidation was calculated using the following equation.

$$\% \text{ inhibition} = [1-(A_{s0}-A_{st})/(A_{c0}-A_{ct})] \times 100 \qquad \text{Equation 6.2}$$

where A_{s0}-A_{st} are corrected absorbance values for test samples measured at zero time and after incubation, respectively; while A_{c0}-A_{ct} are corrected absorbance values for control at zero time and after incubation, respectively.

Antioxidant activity in muscle food. The inhibition effect of *p*-coumaric acid, monooleyl *p*-coumarate and dioleyl *p*-coumarate on the production of thiobarbituric acid

reactive substances (TBARS) in cooked pork was determined according to Shahidi and Alexander (1998). Fresh ground pork (40 g) was mixed with 10 ml of distilled water in Mason jars. Test compounds dissolved in ethanol were added separately to the meat samples at a level of 80 μmol/kg (13 ppm of *p*-coumaric equivalent), and then the contents were thoroughly mixed with a glass rod. A control containing no test compounds was also prepared. Samples were cooked in a thermostated water bath at 80°C for 40 min with intermittent stirring. The cooked meat was removed from the water bath and cooled to room temperature, and then samples were homogenized with a Polytron PT 3000 (Brinkmann Instruments, Rexdale, ON, Canada) homogenizer. The homogenized samples were transferred into plastic bags and stored at 4°C for 14 days. The samples were taken on day 0, 3, 5, 7, and 14 for the measurement of secondary oxidation products in terms of TBARS formation according to the method described by Shahidi and Pegg (1994). Two grams of meat sample were weighed into a 50 ml centrifuge tube followed by addition of 5 ml TCA solution (10%, w/v) and 5 ml of 2-thiobarbituric acid (TBA) solution (0.02 M) in water, and then the contents were vortexed for 2 min. The samples were subsequently centrifuged at 3,000 × g for 10 min, and the supernatants were filtered through a Whatman No. 3 filter paper and collected in 20 ml screw-capped tubes. Samples were kept in a boiling water bath for 45 min, and then removed from the water bath and cooled to room temperature under running tap water. The absorbance of the resultant pink-coloured chromogen was read at 532 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent, Palo Alto, CA, USA). A standard curve was prepared using 1,1,3,3-tetramethoxypropane as a precursor of malondialdehyde (MDA). The

TBARS values of samples were calculated using the standard curve and expressed as μmol MDA equivalents per kg of sample.

Inhibition effect on copper-induced LDL cholesterol oxidation. The inhibitory activity of *p*-coumaric acid, monooleyl *p*-coumarate and dioleyl *p*-coumarate against copper-induced LDL cholesterol oxidation was assessed by the method described by Lebeau et al. (2000) with minor modification. Human LDL cholesterol solution was dialyzed in PBS (10 mM, 0.15 M NaCl, pH 7.4) at 4°C under a nitrogen blanket for 12 h. Sample solutions were prepared by dissolving the test compounds in ethanol at 1.0 mM. Sample solutions (10 μl) were transferred into Eppendorf tubes, and the solvent was removed under a stream of nitrogen. To each tube 100 μl of PBS were added, followed by vortexing for 1 min. An aliquot (0.8 ml) of the dialyzed and diluted LDL cholesterol solution (0.125 mg/ml) was added to each tube, and the contents were mixed well and incubated at 37°C for 15 min. The induced oxidation reaction was initiated by the addition of 100 μl of $CuSO_4$ solution (20 μM, previously incubated at 37°C). A blank containing only the sample without LDL or $CuSO_4$ was prepared for each test compound. After incubation of the reaction mixture at 37°C for 22 h, the conjugated dienes formed were measured spectrophotometrically at 234 nm using a Hewlett–Packard model 8452A diode array spectrophotometer (Agilent, Palo Alto, CA, USA). Antioxidant activities of test compounds were expressed as percentage inhibition of conjugated diene formation, as calculated using Equation 7.3.

$$\% \text{ inhibition} = 100 \times (A_{control}-A_{sample})/(A_{control}-A_{native\ LDL}) \qquad \text{Equation 6.3}$$

Where the control contained LDL cholesterol and $CuSO_4$ without antioxidants, and native LDL cholesterol contained LDL cholesterol only. All absorbances for samples were blank-corrected.

Inhibition effect on radical-induced DNA scission. Antioxidant activity of *p*-coumaric acid, monooleyl *p*-coumarate and dioleyl *p*-coumarate against radical-induced DNA scission was determined as described by Zhong and Shahidi (2012) with slight modifications. Oxidation and nicking of DNA strand was induced by hydroxyl and peroxyl radicals and monitored by gel electrophoresis, respectively. For hydroxyl radical-induced DNA oxidation, 2 µl of ethanolic solution (15 µM) of test compounds were added into a 0.5-ml Eppendorf tube, and then the solvent was removed under a stream of nitrogen. To the tube 2 µl of PBS (0.5 M, pH 7.4) were added, followed by thorough vortexing for 1 min. The following reagents were then added to the tube in the order stated: 2 µl of PBS (0.5 M, pH 7.4), 4 µl of supercoiled pBR322 DNA (20µg/ml in PBS), 2 µl of H_2O_2 (0.5 mM) and 2 µl of Fe_2SO_4 (0.5 mM). The mixture was then incubated at 37°C for 1 h. For peroxyl radical-induced DNA oxidation, a higher concentration (50 µM) of samples was used, and H_2O_2 and Fe_2SO_4 were replaced with 4 µl of AAPH (9mM). A blank (contained only DNA) and a control (containing DNA and one of the radicals) were also prepared for both assays.

Upon the completion of incubation, l µl of loading dye (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was added, and the whole mixture was centrifuged and loaded onto an agarose gel, which was prepared by dissolving 0.7% (w/v) agarose in Tris-acetic acid–ethylenediaminetetraacetic acid (EDTA) buffer (TAE, pH 8.5) and stained

with SYBR safe gel stain (1:10000, v/v). Gel electrophoresis was performed at 80 V for 80 min in TAE buffer using a horizontal submarine gel electrophoresis apparatus (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada). The DNA bands were visualized under ultraviolet light. Images were photographed by a GelDoc apparatus equipped with a Sony digital camera and analyzed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA, USA). The intensity of the bands (obtained from densitometer) was used as an indicator of the concentration of the supercoiled and nicked DNA fractions. Antioxidant activity was calculated as % retention of DNA according to equation 6.4.

$$\% \text{ retention} = 100 \times (\text{Intensity of supercoiled } DNA_{sample}/\text{Intensity of supercoiled } DNA_{blank}) \quad \text{Equation 6.4}$$

Results and discussion

Identification of lipid derivatives of *p*-coumaric acid. The identity of purified mono and dioleyl *p*-coumarates was confirmed by their fragmentation patterns and molecular ions yielded. For monooleyl *p*-coumarate, the major molecular ion generated during the fragmentation had a m/z at 485.5, representing $[M+H-H_2O]^+$ of monooleyl *p*-coumarate; this might have resulted from the loss of a molecule of H_2O and addition of a proton. For dioleyl *p*-coumarate, the abundant molecular ion had a m/z at 765.7, representing $[M-H]^-$, resulting from the loss of a proton.

DPPH radical scavenging activity. The DPPH radical scavenging assay is commonly used in evaluating antioxidant effectiveness because of the relatively short time required for analysis (Chen *et al.* 1999). Phenolic compounds may act as free radical scavengers by virtue of their hydrogen donating ability (Castelluccio *et al.* 1996). As one of the hydroxycinnamic acid, *p*-coumaric acid possesses higher antioxidant activity than other isomers as the hydrogen-donating ability of the hydroxyl group at the *para*-position is stronger than that at other positions (Nabi & Liu 2012). The strong DPPH radical scavenging capacity of *p*-coumaric acid has been reported by Fukumoto and Mazza (2000). The antioxidant potency of the synthesized phenolic lipids, mono and dioleyl *p*-coumarates may be contributed by the *p*-coumaric acid moiety. However, the antioxidant efficacy of mono and dioleyl *p*-coumarates may differ from that of *p*-couamric acid due to possible steric and electronic effects as well as lipophilicity change rendered by the mono and diolein moieties. The antioxidant potential of *p*-coumaric acid and its phenolic derivatives, mono and dioleyl *p*-coumarates was evaluated using DPPH radical, which is a stable hydrophobic radical frequently used in antioxidant assessment (Siriwardhana & Shahidi 2002).

The results of the DPPH radical scavenging assay on test compounds are summarized in Figure 6.1. The signal intensity in EPR spectra of DPPH radical as affected by the test compounds decreased significantly, which revealed the scavenging effect of *p*-coumaric acid and its lipid derivatives on DPPH radical. All test compounds exhibited fairly effective DPPH radical scavenging activity. When comparing the scavenging capacity of *p*-couamric acid and its lipid derivatives, *p*-coumaric acid demonstrated a significantly greater capacity as trolox equivalent than dioleyl *p*-coumarate, but showed similar

capacity to monooleyl *p*-coumarate. Meanwhile, the monooleyl *p*-coumarate showed superior DPPH radical scavenging activity compared to dioleyl *p*-couamrate. Lipophilicity of phenolic compounds plays an important role in their antioxidant activity, higher lipophilicity of phenolic compounds may lead to greater antioxidant activity (Zhong & Shahidi 2011). In this case, esterification of *p*-couamric acid improved its lipophilicity, and thus its lipid derivatives should have showed higher DPPH radical scavenging capacity. However, the great antioxidant activity of the lipid derivatives was not observed, and that may be due to the influence of changes of the bond dissociation enthalpy, which could lead to electron redistribution and alteration of electron density on the aromatic ring of the *p*-couamric acid moiety (Zhong & Shahidi 2011). The bond dissociation enthalpy is strongly associated with the hydrogen donation capacity of the hydroxyl group. Therefore, the difference of DPPH radical scavenging activity between *p*-couamric acid and its lipid derivatives may due to a combination influence from changed lipophilicity and bond dissociation enthalpy.

Although the improved lipophilicity of mono and dioleyl *p*-coumarates may extend their application in more lipophilic environments, further investigation is required to obtain a better understanding of their antioxidant efficacy in different model systems.

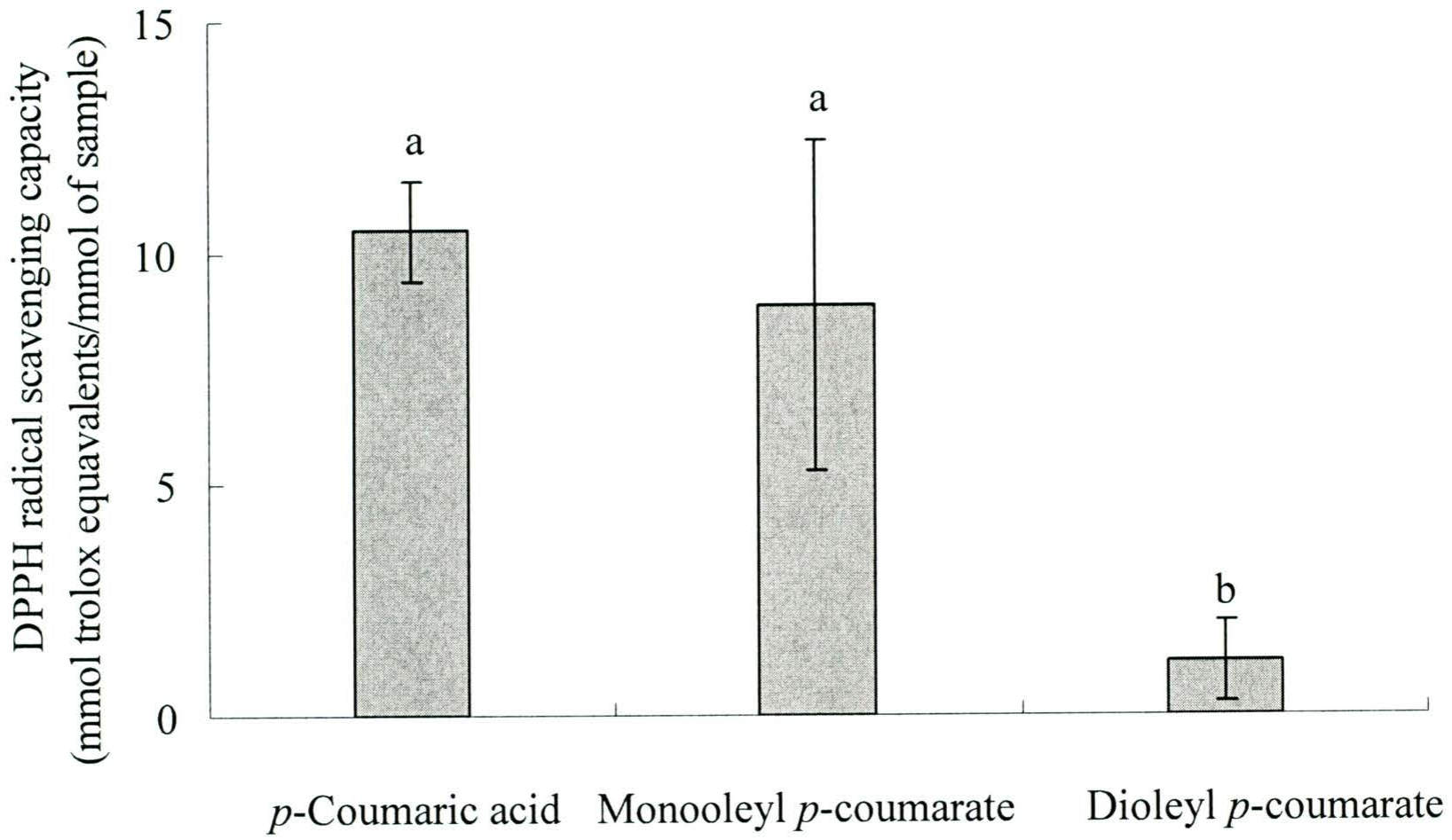


Figure 6.1. DPPH radical scavenging capacity of *p*-coumaric acid, mono and dioleyl *p*-coumarates as trolox equivalents (TE); bars with different letters are significantly different ($P< 0.05$).

Oxygen radical absorbance capacity (ORAC). *p*-Coumaric acid and its lipid derivatives, mono and dioleyl *p*-coumarates demonstrated various radical scavenging activities in the DPPH radical scavenging assay. However, the results obtained from the DPPH radical scavenging assay may not well reflect the antioxidant capacity of these compounds in the real food and biological systems, where life time of their radicals are much shorter than the stable and artificial radical, DPPH radical (Tan & Shahidi 2011). Therefore, radical scavenging efficacy of *p*-coumaric acid and its lipid derivatives were further evaluated by oxygen radical (peroxyl radical) absorbance capacity (ORAC) assay, which uses a biologically relevant source, in order to gain a better understanding of their antioxidant activity in biological systems (Zhong & Shahidi 2012). ORAC was established as a standard method for examining the antioxidant activity of hydrophilic antioxidants, which introduce acetone-water as a solvent medium and randomly methylated-β-cyclodextrin (RMCD) as solubility enhancer to improve lipophilicity of the solvent to test compounds (Huang *et al.* 2002).

Dávalos et al. (2004) reported that ORAC value of *p*-coumaric acid was 4.5 fold that of trolox, which was 2 times higher than that of BHA. The ORAC of *p*-coumaric acid and its lipid derivatives are summarized in Figure 6.2. All test compounds exhibited strong peroxyl radical scavenging activity. *p*-Coumaric acid showed the highest ORAC value, which was 12 times that of trolox, followed by monooleyl *p*-coumarate and dioleyl *p*-coumarate. The trend of the results was correlated with the result of the DPPH radical scavenging assay. The antioxidant activity of *p*-coumaric acid was negatively affected by esterification as monooleyl *p*-coumarate and dioleyl *p*-coumarate showed much lower ORAC values than *p*-coumaric acid. Such a phenomenon may be caused by changes of

chemical and physical characteristics such as lipophilicity, electron distribution and steric effect. For instance, the improved lipophilicity may have a positive effect on radical scavenging activity as higher lipophilicity of phenolic compounds may lead to greater antioxidant activity (Zhong & Shahidi 2011). The 3-dimensional conformation and molecular environment of *p*-coumaric acid might also be altered after esterification, and the fatty acid chain or chains may hinder the hydroxyl group from hydrogen donation to free radicals, thus weakening its antioxidant activity. Moreover, the weak electron donating ester group may also compromise the hydrogen donation activity of *p*-coumaric acid (Wright *et al.* 2001).

It appears that the improved lipophilicity was overwhelmed by the effect of steric hindrance as well as weaker hydrogen donation ability, thus leading to negative effect of esterification on peoroxyl radical scavenging capacity.

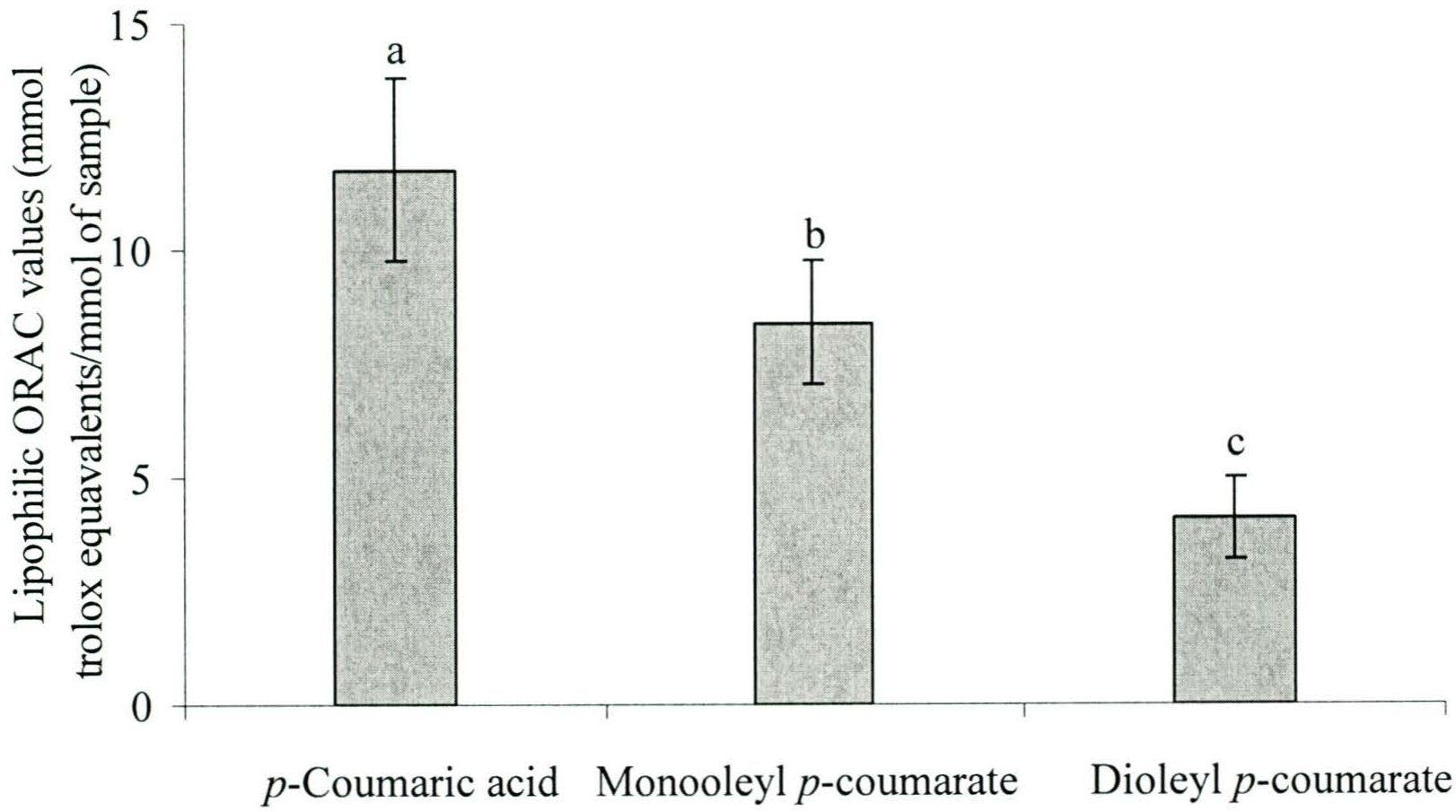


Figure 6.2. Oxygen radical absorbance capacity of *p*-coumaric acid, mono and dioleyl *p*-coumarates as trolox equivalents (TE); bars with different letters are significantly different ($P< 0.05$).

Reducing power. A number of methods have been developed to measure the antioxidant activity of phenolic compounds. For instance, the hydrogen donation capacity of test compounds is examined using the DPPH and ORAC assays. Antioxidants cannot only achieve their goal of deactivating free radicals and preventing oxidation through hydrogen donation, but can also reach that by electron transfer (Leopoldini *et al.* 2004; Lin *et al.* 1996). The efficacy of an antioxidant to donate an electron, so called reducing power, is dependent on both intrinsic factors such as the ionization potential and extrinsic factor such as medium environment. The ferric reducing antioxidant power assay is frequently employed to assess the efficiency of antioxidants for their electron transfer capacity. The reducing power of antioxidants and its measurement is based on the reduction of the Fe^{3+} to the Fe^{2+} by antioxidants in an acidic environment (Wright *et al.* 2001; Wojdylo *et al*. 2007).

The strong reducing power of *p*-coumaric acid was reported by Medina *et al.* (2006). In this study, the results (Figure 6.3) showed effective reducing power of all test compounds, and *p*-coumaric acid showed similar reducing power to its lipid derivatives, mono and dioleyl *p*-coumarates. Compared to the scavenging ability on the DPPH radical, the antioxidant difference between *p*-coumaric acid and its lipid derivatives showed a decrease. This phenomenon may be caused by the decrease of the ionization potential of *p*-coumaric acid when it was esterified to bulky substituents (Sato *et al.* 1983) as decreased ionization potential facilitates the electron transfer. The results of the reducing power of mono- and dioleyl *p*-coumarates correlated well with those of the DPPH radical scavenging assay as shown in Figure 6.1. The bulkier compound, dioleyl *p*-coumarate, exhibited a lower reducing power than monooleyl *p*-coumarate, possibly due

to the existing difference in their hydrophilicity. In reducing power assays, the capacity of antioxidants is strongly solvent-dependent as the solvent facilitates the stability of charged ions (Wright *et al.* 2001). Dioleyl *p*-coumarate, consisting of two oleic acid chains, has a lower solubility than monooleyl *p*-coumarate that only has one oleic acid chain in aqueous testing medium, and thus dioleyl *p*-coumarate showed a compromised efficacy under the hydrophilic test environment.

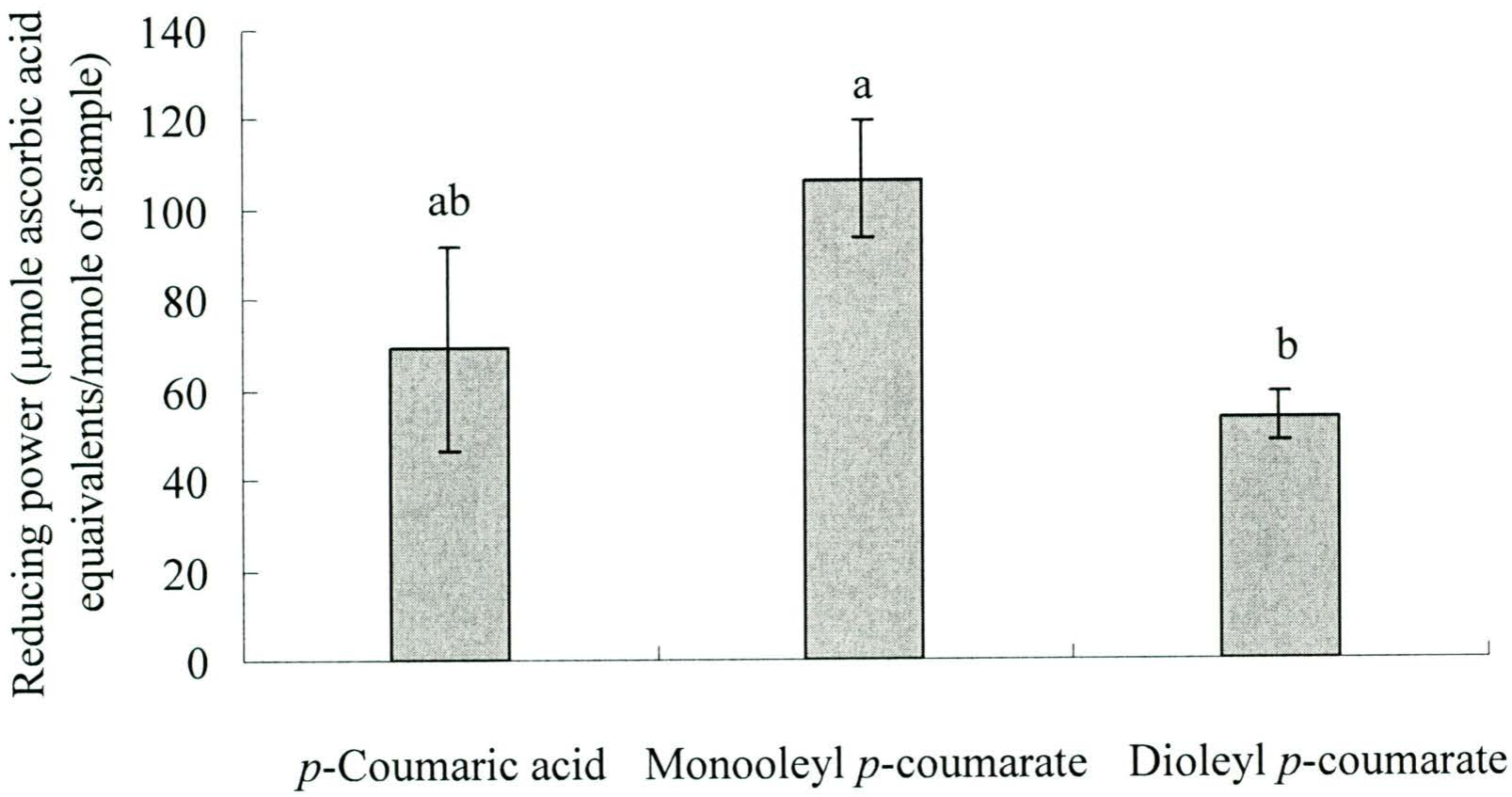


Figure 6.3. Reducing power of *p*-coumaric acid, mono and dioleyl *p*-coumarates as ascorbic acid equivalents; bars share a common letter are not significantly different ($P > 0.05$).

Antioxidant activity in *β*-carotene-linoleate model system. High surface-to-volume ratio of oil-in-water emulsions normally occur in food systems. Therefore, it is essential to investigate the performance of antioxidants in oil-in-water emulsions to achieve a comprehensive assessment of their antioxidant activity prior to the application of these compounds in food systems (Zhong & Shahidi 2011). In this work, the antioxidant capacity of *p*-coumaric acid and its lipid derivatives, mono and dioleyl *p*-coumarates, in oil-in-water emulsion was examined using a *β*-carotene/linoleic acid emulsion model system. The decolouration of *β*-carotene is a free radical-mediated phenomenon resulting from oxidation of linoleic acid in the emulsion. Addition of antioxidants to the emulsion can minimize the loss of *β*-carotene during the coupled oxidation of linoleic acid and *β*-carotene in the aqueous test medium through the elimination of free radicals generated from linoleic acid oxidation. Their antioxidant activity was detected by monitoring the bleaching of *β*-carotene as affected by test compounds.

Gadow et al. (1997) studied the antioxidant activity of *p*-coumaric acid in the *β*-carotene/linoleic acid emulsion model system and reported its strong inhibitory effect on *β*-carotene bleaching. In this work, the results (Figure 6.4) indicated that *p*-coumaric acid inhibited the bleaching of *β*-carotene by 8.3% over the 161-min incubation period, followed by dioleyl *p*-coumarate (8.2%) and monooleyl *p*-coumarate (1.6%). Based on the antioxidant "polar paradox" theory, nonpolar antioxidants are more effective than polar ones in emulsion systems (Zhong & Shahidi 2012), which implies that the lipid derivatives should have exhibited higher inhibitory effect on *β*-carotene bleaching than *p*-coumaric acid; however, such an effect was not observed in the study due to two possible reasons. First of all, esterification of *p*-coumaric acid may have changed the surrounding

molecular structure of its hydroxyl group on the aromatic ring as well as the bond dissociation enthalpy of the O−H bond. Secondly, esterification also improved the lipophilicity of the lipid derivatives. The combination effect of changed bond dissociation enthalpy of the O-H bond and the change in lipophilicity may contribute to different inhibitory effects of test compounds. The inhibitory activity of *p*-coumaric acid and its lipid derivatives, especially dioleyl *p*-coumarate on *β*-carotene bleaching indicates excellent antioxidant activity of these compounds through hydrogen donation and suggests their greater potential in emulsions of foods, cosmetics and biological systems.

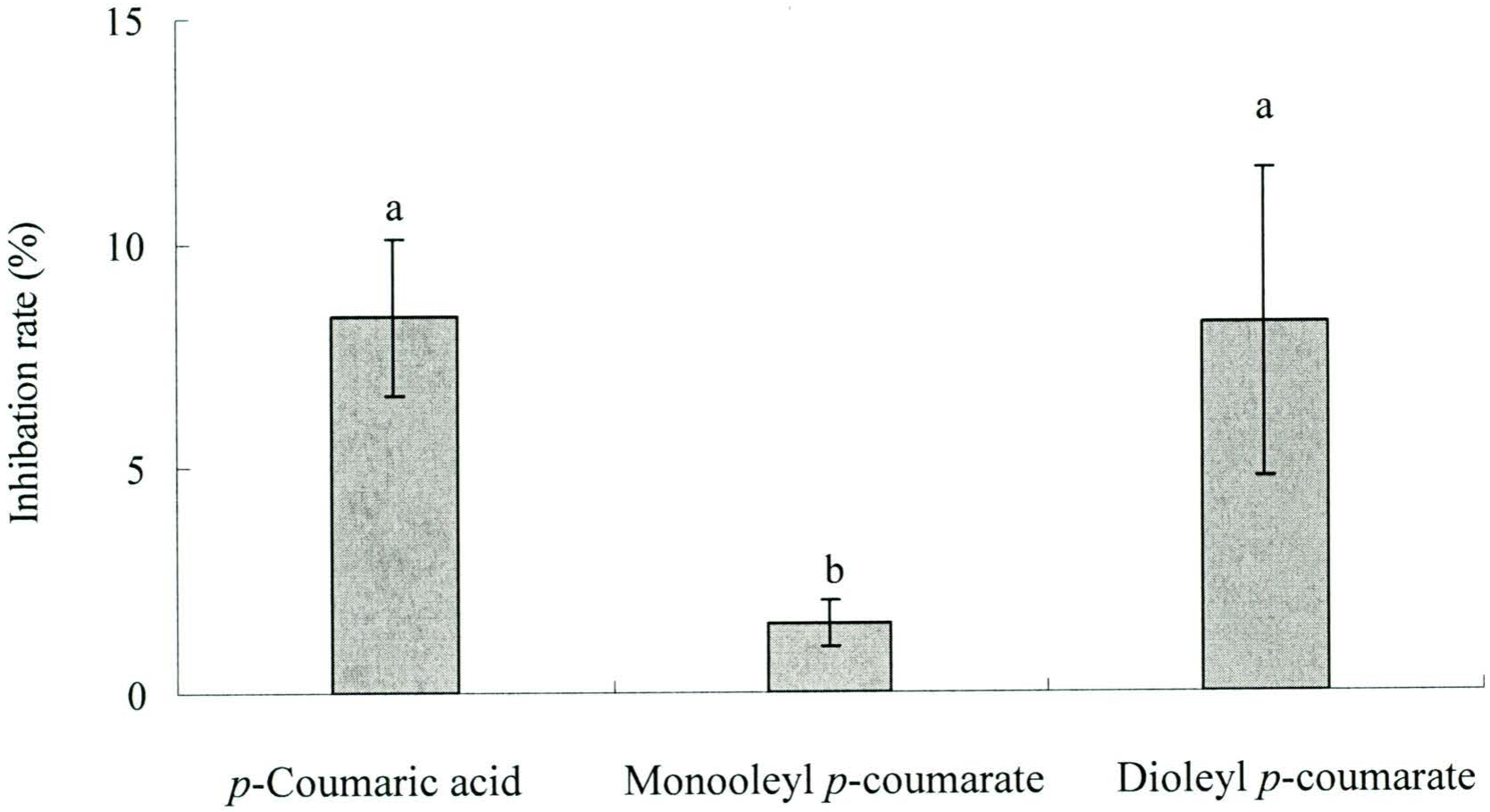


Figure 6.4 Inhibitory effects of *p*-coumaric acid, mono and dioleyl *p*-coumarates against β-carotene bleaching; bars with different letters are significantly different ($P< 0.05$).

Antioxidant activity in muscle foods. In addition to oil-in-water emulsion model systems, the antioxidant capacity of phenolic compounds can also be assessed in other high surface-to-volume ratio systems such as the lipids in processed whole tissue foods (Porter 1993). Other than being one of the major components of oil-in-water emulsions such as salad dressing and spreads, lipids are also incorporated into processed whole tissue foods such as sausages and hams. Lipids, especially those containing high levels of unsaturated fatty acids, undergo oxidation when exposed to oxygen and generate oxidation products; therefore, lipid components are responsible for deterioration of processed whole tissue foods. Thus, lipid components require effective antioxidants to prevent their oxidation in lipid rich muscle foods as they are more vulnerable to oxidation during precooking and long storage time under high surface-to-volume conditions (Porter 1993). Thus, the inhibitory capacity of antioxidants in thermally processed whole or modified muscle foods, which are frequently associated with rapid lipid oxidation and development of "warmed-over flavour", is better evaluated (Jayathilakan *et al.* 2007; Shahidi *et al.* 1987). The antioxidant potential of various synthetic and natural antioxidants in three common domestic meat species including sheep, beef and pork have been studied by Jayathilakan *et al.* (2007), and the antioxidants, ascorbic acid, TBHQ and BHA showed great potential to control lipid oxidation as well as warmed-over flavour development in all the test meat species. The strong inhibitory effects of epigallocatechin gallate (EGCG), the major phenolic compound from green tea, and its lipid derivatives synthesized from corresponding polyunsaturated fatty acids and phenolic extracts of millet on lipid oxidation in meat model systems have been demonstrated by Zhong and Shahidi (2012), Chandrasekara and Shahidi (2012) and He and Shahidi (1997).

In this work, the inhibitory effect of *p*-coumaric acid and its lipid derivatives, mono and dioleyl *p*-coumarates, on the production of thiobarbituric acid reactive substances (TBARS), the secondary products of lipid oxidation, in cooked ground pork was measured and expressed as μmol MDA equivalents per kg of sample. The results summarized in Figure 6.5 shows that TBARS values of all test samples increased throughout the test period. The TBARS values of the sample group with added *p*-coumaric acid were significantly lower ($P < 0.05$) than the control group at all test points, which indicated effective inhibitory effects of *p*-coumaric acid on lipid oxidation during the entire storage period. Monooleyl *p*-coumarate showed a slight prooxidative effect on its sample group as its TBARS value was significantly higher than that of the control group at day 3 test point while the TBARS values were similar to that of the control group at other test points. Dioleyl *p*-coumarate performed as a weak antioxidant against lipid oxidation as the TBARS values of its group were significantly lower than those of the control group only on day 0 and 7 test points and did not show significant difference at other test points. The test compounds added to fresh ground meat exerted effects at different levels on lipid oxidation during cooking of the meat prior to storage, which explains the presence of different TBARS values from test groups on day 0. The oxidation of oleic acid may compromised the effectiveness of the lipid derivatives in preventing lipid oxidation in meat and rendered higher TBARS values in the groups added with monooleyl *p*-coumarate or dioleyl *p*-coumarate. Therefore, mono and dioleyl *p*-coumarates showed no or slight antioxidant activity in meat, and that may be explained by the weak resistance of oleic acid chain or chains in mono and dioleyl *p*-coumarates to oxidation under high thermal conditions and longer storage period. In addition, higher

lipophilicity of antioxidants may contribute to their better performance as it appears to be positively associated with their activity in cooked muscle food (Zhong & Shahidi 2012). Thus, difference of antioxidant activity between monooleyl *p*-coumarate or dioleyl *p*-coumarate in this test may be caused by better lipophilicity of dioleyl *p*-coumarate compared to monooleyl *p*-coumarate. Hence, mono and dioleyl *p*-coumarates with their weaker antioxidant activity may not be the best choice for use in cooked muscle foods for improving their oxidative stability and extending their shelf life.

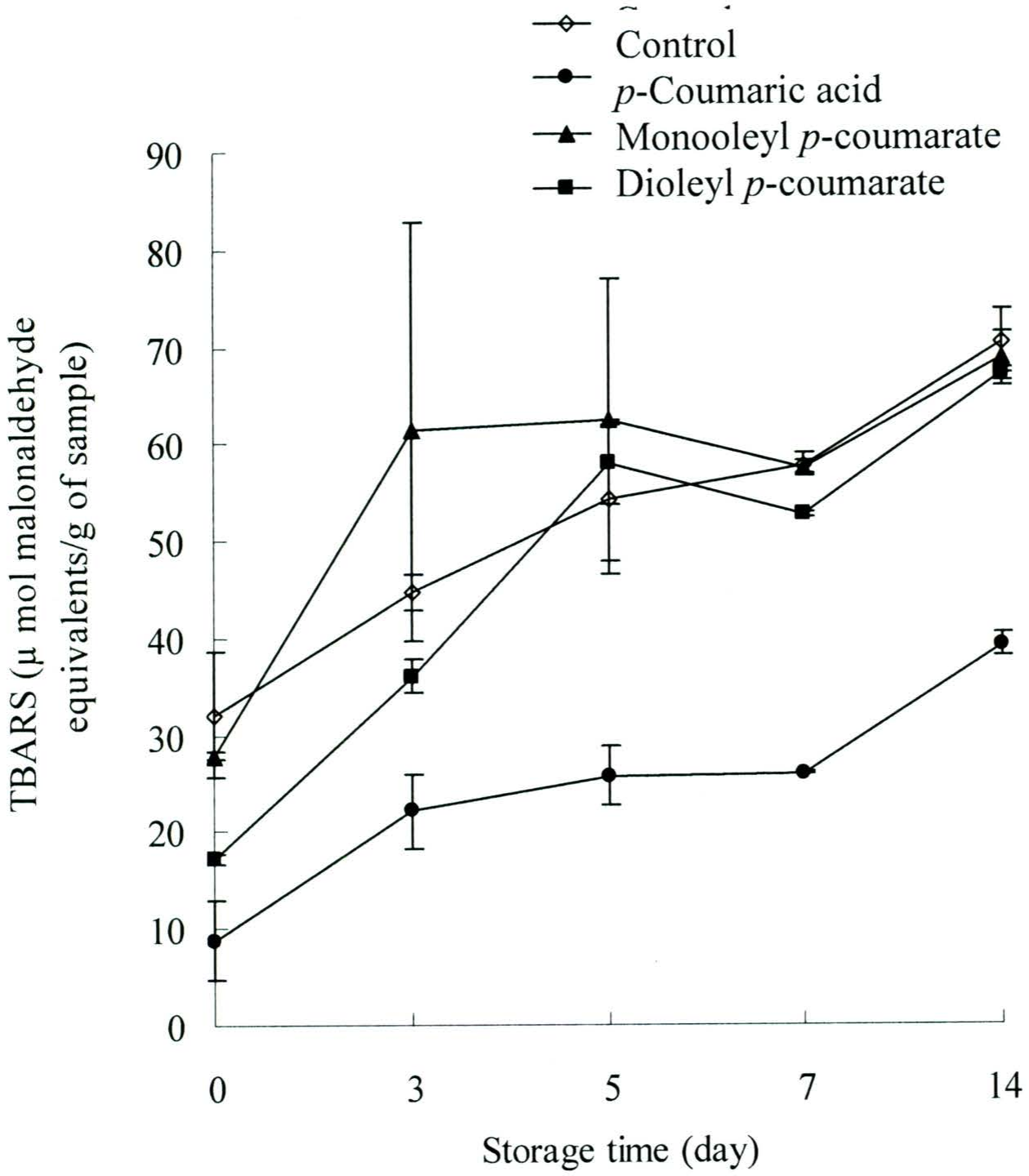


Figure 6.5 TBARS values in cooked ground pork as affected by *p*-coumaric acid, mono and dioleyl *p*-coumarates.

Inhibitory effect on human LDL cholesterol oxidation. LDL cholesterol is one of the five major cholesterol carriers in the blood. It has been proven that the risk of atherosclerosis is positively associated with the increased plasma level of LDL (Ross 1993). Recently, the oxidized forms of LDL have been recognized as the contributor to the formation of atherosclerotic plaques in arteries other than the native form (Esterbauer *et al.* 1992), hence heightening interest in preventing LDL oxidation. Dietary antioxidants such as phenolic acids are believed to be effective inhibitors of LDL oxidation caused by free radical attack (Slavin *et al.* 1997; Nardini *et al.* 1995; Andreasen *et al.* 2001). The LDL cholesterol oxidation assay is one of the tests that can evaluate the effectiveness of antioxidants in biological systems. The inhibitory effect of test antioxidants on LDL cholesterol oxidation was monitored by subjecting LDL cholesterol to induced oxidation in the presence of a known concentration of test antioxidants and monitoring the progression of the oxidation (Lebeau *et al.* 2000), in which conjugated dienes (CD) were used as an indicator of the level of LDL cholesterol peroxidation.

In this work, the inhibitory effects of *p*-coumaric acid, mono and dioleyl *p*-coumarates on Cu^{2+}-induced LDL cholesterol oxidation were evaluated and the results are summarized in Figure 6.6. LDL cholesterol was oxidized to different levels at the end of a 22-h incubation period, which depended on the type of antioxidants added to the samples. All test compounds showed high inhibitory capacity on LDL cholesterol oxidation. Inhibition rate (%) of LDL cholesterol oxidation with added *p*-coumaric acid was 37.3%, which is much higher than the value reported by Andreasen *et al.* (2001) due to the higher *p*-coumaric acid concentration employed in this work. Dioleyl *p*-coumarate had a significantly ($P<0.05$) greater inhibitory effect than *p*-coumaric acid, while the inhibition

efficacy of monooleyl *p*-coumarate was slightly, but not statistically significantly ($P>0.05$) higher than that of *p*-coumaric acid. The improved inhibitory capacity of dioleyl *p*-coumarate against LDL cholesterol oxidation may be due to the improved lipophilicity of the molecule as the higher lipophilicity allowed dioleyl *p*-coumarate to have greater affinity to the major components on the surface of LDL cholesterol, i.e., the phospholipids. Moreover, hydrophilic *p*-coumaric acid and lipophilic oleic acid chains may lead to better amphiphilicity of dioleyl *p*-coumarate, which further allows these lipid derivatives to exert their antioxidant activities within the LDL particle and/or on the surface or extra-particle environment of LDL cholesterol (Zhong & Shahidi 2012). Thus, the potential use of mono and dioleyl *p*-coumarates as anti-atherosclerosis agents is supported by their high inhibitory activity on LDL cholesterol oxidation.

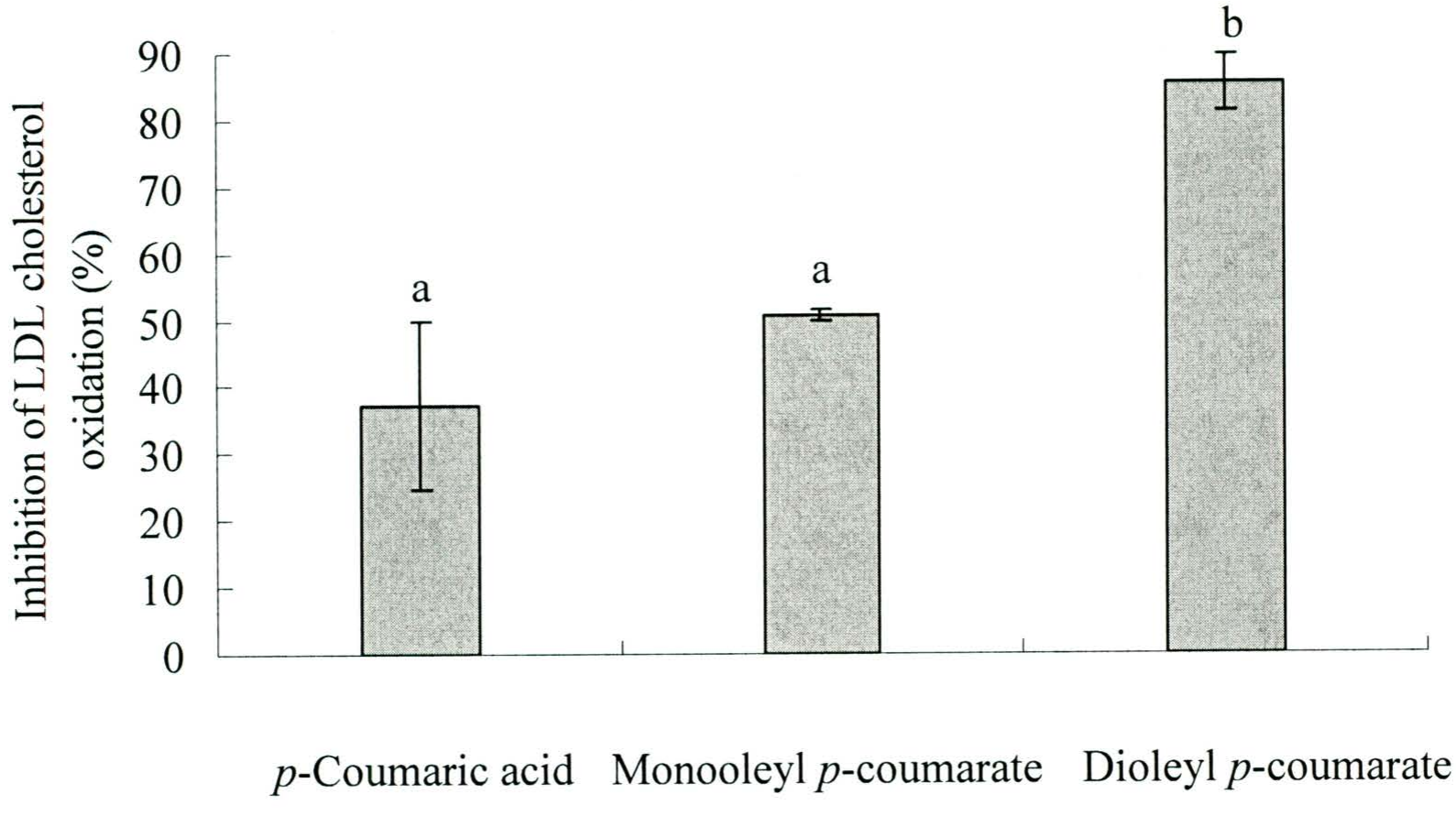


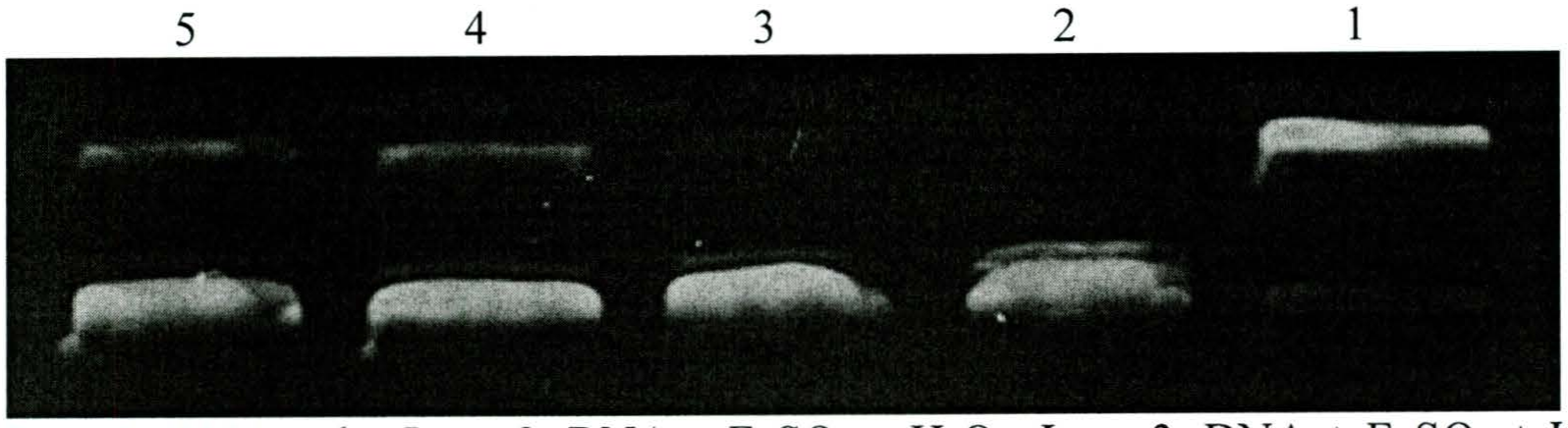
Figure 6.6. Inhibitory effect of *p*-coumaric acid, mono and dioleyl *p*-coumarates on LDL cholesterol oxidation; bars with different letters are significantly different ($P< 0.05$).

DNA scission assay. Oxidative stress in cells caused by reactive oxygen species (ROS) such as hydroxyl and peroxyl radicals leads to DNA damage. Hydroxyl and peroxyl radicals react with DNA by possible addition to double bonds of DNA bases or abstraction of a hydrogen atom from the methyl group of thymine, among others (Cooke *et al.* 2003). Therefore, oxidative damage of DNA may occur at both the nucleotide bases and the phosphate backbone resulting in strand breakage and sister chromatid exchange, DNA-DNA and DNA-protein cross linking, and base modification (Dizdaroglu *et al.* 1993). Background level of oxidative DNA damage is an inevitable consequence of cellular metabolism and will be repaired in normal tissues. However, the oxidative stress caused by excess free radicals may lead to severe DNA damage, which is implicated in mutagenesis, carcinogesis and cytostasis. Thus, the effect of various antioxidants, including phenolic acids, on DNA oxidation inhibitory activity has been widely studied (Lodovici *et al.* 2001; Nabi & Liu 2012).

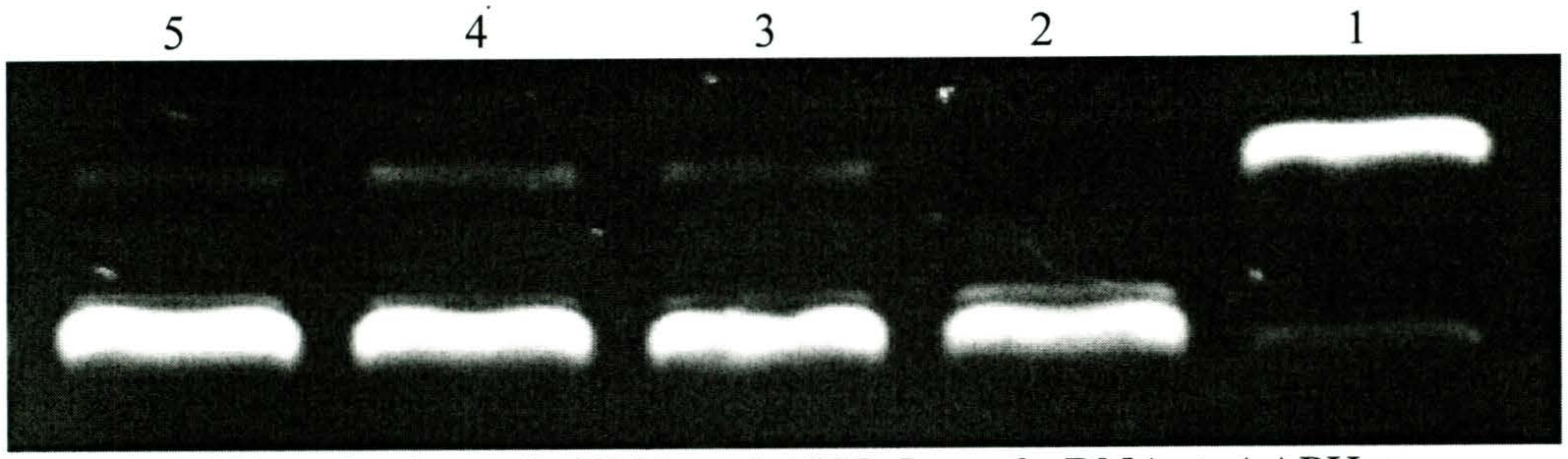
In this study, the inhibitory activity of *p*-coumaric acid, mono and dioleyl *p*-coumarates on hydroxyl and peroxyl radical-induced supercoiled DNA strand oxidation was evaluated, and the results are shown in Figures 6.7 and 6.8. The oxidative damage caused by hydroxyl and peroxyl radicals led to the supercoiled plasmid DNA strand nicking and formation of nicked open circular forms as a result of single strand cleavage (Figure 6.7). At higher concentrations, the free radicals can even cause double bond breakage and convert the supercoiled plasmid DNA strand to linear form DNA. The results from Figure 6.8 showed effective DNA retention in all test compounds in hydroxyl radical-induced DNA tests due to their high radical scavenging capacity and for eliminating hydroxyl radicals generated from H_2O_2 through Fenton reation (Perron *et al.* 2008). Of these, the

lipid derivative, monooleyl *p*-coumarate, showed slightly better, but not significant ($P>0.05$) higher retention percentage than *p*-coumaric acid; meanwhile, dioleyl *p*-coumarate exhibited a significantly ($P<0.05$) higher retention than *p*-coumaric acid. The trend of retention percentage of *p*-coumaric acid, mono and dioleyl *p*-coumarates in hydroxyl radical-induced DNA nicking is positively correlated to that of the inhibitory effect of these compounds in LDL cholesterol oxidation assay, which may result from the amphiphilic nature of mono and dioleyl *p*-coumarates.

The oxidation of supercoiled DNA was successfully induced by peroxyl radical and resulted in the occurrence of nicked open circular DNA at the end of incubation, but the linear DNA was absent (Figure 6.7). All the test compounds showed high retention of DNA, varying from 8.4 to 10.5% (Figure 6.8). Monooleyl *p*-coumarate showed a similar inhibitory capacity to *p*-coumaric acid and significantly ($P<0.05$) higher capacity than dioleyl *p*-coumarate. The peroxyl radical has a better stability than hydroxyl radical, which helps it reach targets before being degraded or eliminated, and this may partialy explain the different trend of retention percentage exerted by test compounds between hydroxyl radical and peroxyl radical-induced DNA oxidation.



Lane 1: DNA only; Lane 2: DNA + $FeSO_4$ + H_2O_2; Lane 3: DNA + $FeSO_4$ + H_2O_2 + *p*-coumaric acid; Lane 4: DNA + $FeSO_4$ + H_2O_2 + monooleyl *p*-coumarate; Lane 5: DNA + $FeSO_4$ + H_2O_2 + dioleyl *p*-coumarate.



Lane 1: DNA only; Lane 2: DNA + AAPH; Lane 3: DNA + AAPH + *p*-coumaric acid; Lane 4: DNA + AAPH + monooleyl *p*-coumarate; Lane 5: DNA + AAPH + dioleyl *p*-coumarate.

Figure 6.7. The inhibitory effect of *p*-coumaric acid, mono and dioleyl *p*-coumarates on radical-induced DNA scission (top: hydroxyl-induced DNA scission; bottom: peroxyl-induced DNA scission).

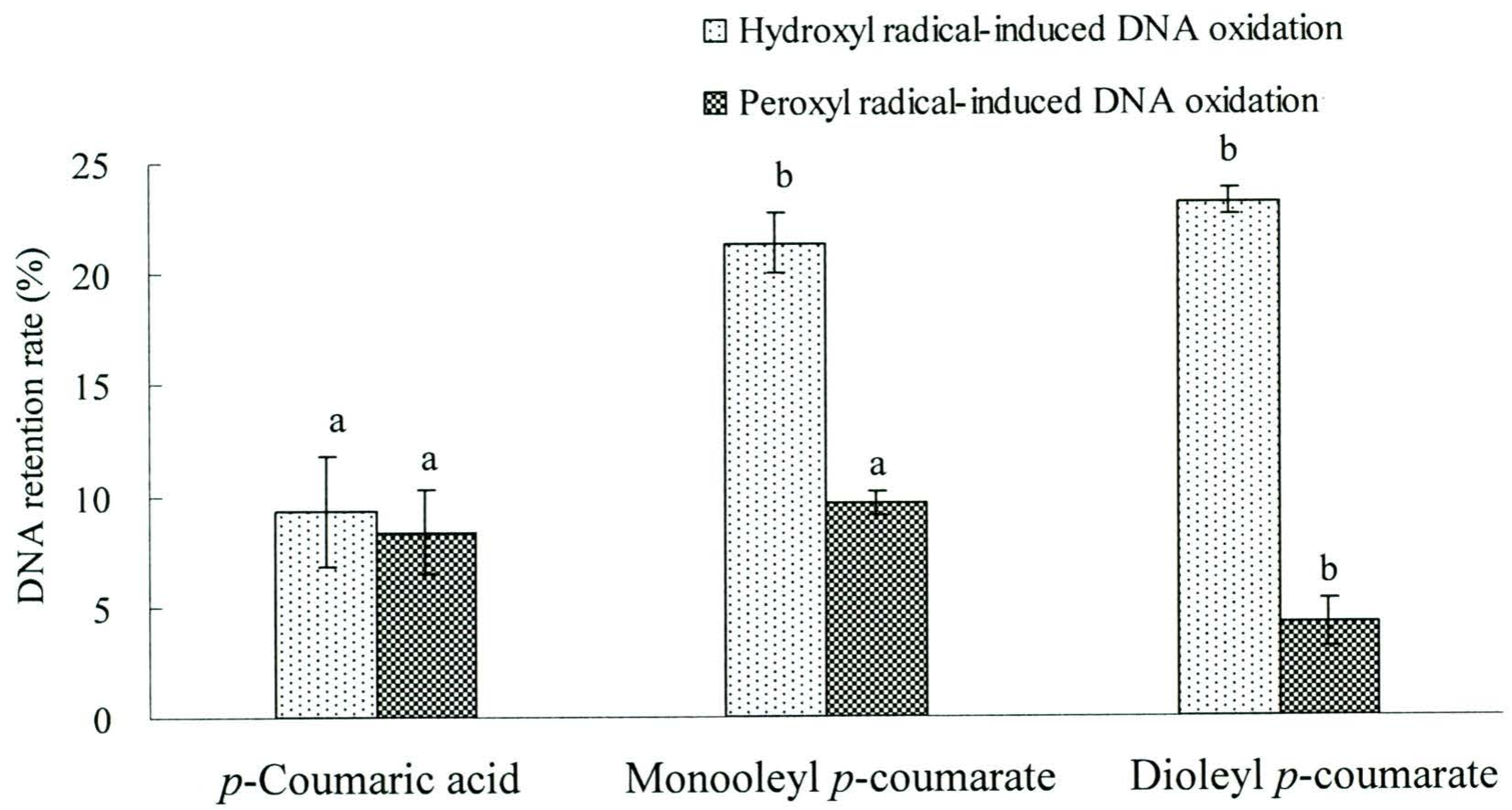


Figure 6.8. DNA retention rate (%) in radicals-induced oxidative scission; bars within the same group with different letters are significantly different ($P< 0.05$).

Conclusions

Phenolic lipids, mono and dioleyl *p*-coumarates, derived from *p*-coumaric acid, exhibited different antioxidant capacity due to various antioxidant mechanisms and different testing systems employed. Mono and dioleyl *p*-coumarates showed lower DPPH and oxygen radicals scavenging capacity. These might be due to steric hindrance exerted by esterified bulky moieties and weakened hydrogen donation capacity caused by the formation of ester bonds. Mono and dioleyl *p*-coumarates exhibited better reducing power compared to their radical scavenging capacities, possibly due to the decreased ionization potential brought about by bulky moieties. Mono and dioleyl *p*-coumarates exhibited excellent antioxidant potential against *β*-carotene bleaching, which suggests their potential use in more lipophilic environment; however, such activity was not confirmed in a muscle food model as only dioleyl *p*-coumarate showed limited inhibitory effect on lipid oxidation in cooked muscle food due the oxidation of oleic acid at high thermal processing temperatures and long storage periods. Both mono and dioleyl *p*-coumarates demonstrated high antioxidant capacity in human LDL cholesterol oxidation assay, in which dioleyl *p*-coumarate showed significantly higher activity than that of *p*-coumaric acid, which could be explained by its improved lipophicility. These compounds performed in a similar manner in the hydroxyl radical-induced DNA oxidation assay, but a similar activity was not observed in the peroxyl radical-induced DNA oxidation assay due to the different nature of radicals employed.

The great antioxidant activity of mono and dioleyl *p*-coumarates shown in antioxidant testing assays employed suggests that these synthesized phenolic lipids may be used as

potential ingredients in functional foods, drugs or cosmetics for health promotion and disease risk reduction. Synthesis and antioxidant activity assessment of phenolic lipids from the acidolysis between *p*-coumaric acid and omega-3 oils such as seal blubber and menhaden oils should further be conducted in order to improve their stability and health benefits.

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CHAPTER 7

Acidolysis of *p*-coumaric acid with seal blubber oil and menhaden oil and antioxidant activity of its lipid derivatives in *in vitro* tests, food and biological model systems.

Introduction

The omega-3 oils from marine origin, especially those of fish such as menhaden and those of marine mammals such as seal blubber oils, are commonly used as supplements due to their high content of omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Wanasundara & Shahidi 1996). The beneficial effects of omega-3 fatty acids have been associated with their ability to lower triacylglycerol levels in circulatory system, and in the treatment and/or prevention of hypertension, arrhythmia, arthritis, inflammatory and immune as well as mental disorders (Shahidi & Miraliabrai 2005; Wanasundara & Shahidi 1997; Reiffel & McDonald 2006). For instance, DHA is believed to be essential for normal development of the central nervous system and visual acuity in humans (Kim & Edsall 1999). However, the high content of long chain polyunsaturated fatty acids (PUFA) in seal blubber oil and menhaden oil decreases their oxidative stability. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ), as well as natural antioxidants such as tocopherols are conventionally added to edible oils and foods in order to enhance their stability (Shahidi *et al.* 1992). Of these, tocopherols have been

commonly used to improve the stability and retain the nutritional value of seal blubber oil. However, a number of studies have reported that prolonged exposure to high doses of synthetic antioxidants, such as TBHQ, may cause cancer in an animal model (Lindenschmidt *et al.* 1986; Bauer *et al.* 2001; Hirose et al. 2010). Furthermore, effectiveness of tocopheols as antioxidants can be compromized by the presence of metal ions, including those of iron and copper, which are commonly present in marine oils (Shahidi & Miraliabari 2005; Wanasundara & Shahidi 1997). Therefore, studies on the potential use of other forms of naturally occurring antioxidants to stabilize edible oils are required.

Investigations of the antioxidative efficacy of other natural antioxidants, such as phenolic acids, in foods have been widely reported, and most of them have proven to reduce the extent of lipid oxidation. Antioxidants from these groups are also believed to offer several health benefits to humans. For instance, *p*-coumaric acid occurs in various food sources and beverages, including tomatoes, carrots, garlic as well as wines and vinegars (Ferguson *et al.* 2005). Both its strong antioxidant property and anti-cancer effect have been reported in a number of studies, and its potential to reduce the risk of stomach cancer mainly through reducing the formation of carcinogenic *N*-nitrosamines has been demonstrated (Kikugawa *et al.* 1983; Rosskopf *et al.* 1992).

Modifying the structure of triacylglycerols can be used as an effective means to improve nutritional and health benefits of the original oils. Acidolysis is a modification process, which incorporates desired fatty acids or other forms of organic acids, such as phenolic acids, into triacylglycerols using enzyme catalysts (Senanayake & Shahidi 2002). For example, acidolysis of phenolic acids and triacylglycerols has been successfully

conducted and reported in several studies (Safari *et al.* 2006; Sabally *et al.* 2006). In Chapters 5 and 6, synthesis of phenolic lipids using *p*-coumaric acid and triolein was successfully conducted. The purified lipid derivatives of *p*-coumaric acid, mono and dioleyl *p*-coumarates, demonstrated great antioxidant activity in *in vitro* assays, both in food and biological model systems, which may be explained by changes of lipophilicity and the steric conformation of modified lipids. In this study, acidolysis of *p*-coumaric acid with seal blubber oil and menhaden oil was conducted. Antioxidant activity of synthesized phenolic lipids was investigated in several *in vitro* assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, oxygen radical absorbance capacity (ORAC) and reducing power assays. In addition, antioxidant activity of synthesized phenolic lipids was further examined in a β-carotene-linoleate model system and muscle food model system. The efficacy of the phenolic lipids so obtained on the extent of oxidation of human low-density lipoprotein (LDL) cholesterol and prevention of DNA scission was also examined in order to obtain a better understanding of their antioxidant capacity and their potential use in food as well as health applications.

Materials and methods

Materials. Triolein was purchased from Nu-Chek (Elysian, MN, USA). Novozyme 435 and *p*-coumaric acid were obtained from Sigma-Aldrich Canada Ltd. (Mississauga, ON, Canada). Trolox was purchased from Acros Organics (Fair Lawn, NJ, USA). Human LDL cholesterol and supercoiled plasmid pBR322 DNA were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). SYBR safe gel stain was obtained from

Invitrogen Corporation (Carlsbad, CA, USA). Fresh ground lean pork was purchased from a local supermarket. All solvents used were of analytical grade and purchased from Fisher Scientific (Nepean, ON, Canada).

Lipase-catalyzed acidolysis of *p*-coumaric acid with seal blubber oil (SBO) and menhaden oil (MHO). Lipase-catalyzed acidolysis of *p*-coumaric acid with SBO and MHO was carried out following the method described in Chapter 6. In brief, a stock solution of *p*-coumaric acid (10 mM) was freshly prepared in 2-butanone, and stock solution of oils (53.36 mM) were prepared in hexane. Then, 30 ml of *p*-coumaric acid stock solution and 90 ml of each oil stock solution were transferred to a 250-ml round-bottom flask followed by addition of 645 mg of immobilized Novozyme 435 (15%, w/w of substrates). The final reaction solutions contained 2.5 mM of *p*-coumaric acid and 40 mM (calculated based on the average molecular weight of SBO and MHO) of SBO or MHO. Flasks were flushed with a stream of nitrogen and sealed properly. The acidolysis reaction was performed in an orbital shaking water bath at 50°C at 170 rpm. Reaction mixtures were removed from the water bath after 12 days. The reaction mixture (0.1 ml) was transferred to a 4-ml vial, and the solvent was then removed under a stream of nitrogen. The sample was redissolved in 2 ml of methanol-acetonitrile (41:59, v/v) for HPLC-MS analysis.

HPLC-MS analysis synthesized products. The composition of the reaction mixtures was determined by reversed phase HPLC-MS. An Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV–diode array detector (UV-DAD) was

used. Separation was achieved on a C-18 column (4.6 mm × 250 mm coupled with a guard column, Agilent) by gradient elution with a methanol-acetonitrile-isopropanol mobile phase at a flow of 0.8 ml/min; fractions were detected at both 215 and 300 nm. The mobile phase used initially was methanol-acetonitrile-isopropanol (41:59:0, v/v/v), and it was maintained for 15 min. The mobile phase was gradually changed to methanol-acetonitrile-isopropanol (16.5:23.5:60, v/v/v) from 15 to 25 min, and then it reached 100% isopropanol from 25 to 35 min, and kept there for 10 min. The mobile phase was changed to its initial state, methanol-acetonitrile-isopropanol (41:59:0, v/v/v) in the next 5 min, and it was maintained for 10 min in order to recondition the column. LC flow was further analyzed online by the mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) with atmospheric pressure chemical ionization (APCI) at alternative mode for identification of each fraction. The operating conditions used were 121 V for the fragmentor voltage, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, and drying gas flow of 7 l/min.

Purification and identification of lipid derivatives of *p*-coumaric acid. Separation of phenolic lipid components from the final acidolysis mixture was achieved using flash column chromatography. The mixture was eluted on a silica column (40 cm × 5 cm i.d.) packed with silica gel (Selecto Scientific, Suwannee, GA, USA) with a series of solvent mixtures consisting of 350 ml of hexane, 100 ml of hexane-ethyl acetate-formic acid (95:5:2, v/v/v), 400 ml of hexane-ethyl acetate-formic acid (85:15:2, v/v/v), 400 ml of hexane-ethyl acetate-formic acid (75:25:2, v/v/v) and 400 ml of hexane-ethyl acetate-formic acid (65:35:2, v/v/v), in the order stated. Fractions corresponding to each

component were collected, and then solvents were removed using a rotary evaporator. Lipid derivatives of *p*-coumaric acid, including phenolic lipids mixture obtained from SBO (PSBO) and MHO (PMHO) were confirmed by HPLC-MS as described above.

DPPH radical scavenging activity. The effect of phenolic lipids on of the scavenging of DPPH radicals was determined according to the method described by Madhujith and Shahidi (2006) with minor modifications. DPPH radical scavenging capacity of *p*-coumaric acid was used as a reference in this study. *p*-Coumaric acid (2.5 mM), PSBO (2.5 mM) and PMHO (2.5 mM) were dissolved in ethanol, followed by the addition of 2 ml of ethanolic solution of DPPH (0.18 mM). Contents of each test solution were thoroughly mixed and injected to the sample cavity of a Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA), and the spectrum was recorded after 10 min. Ethanol was used as the control in place of test compounds. Meanwhile, trolox standards (12.5-200 mM) were prepared for constructing a standard curve. The operating parameters of the Bruker E-scan (EPR spectrometer) were set as follows: 5.02 × 102 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, and 86.00 kHz modulation frequency. DPPH radical scavenging capacities of test compounds were calculated using Equation 7.1.

$$\text{DPPH radical scavenging capacity (\%)} = (1 - \text{signal intensity}_{\text{sample}}/\text{signal intensity}_{\text{control}}) \times 100 \qquad \text{Equation 7.1}$$

Where the control contained no trolox or test compounds. The DPPH radical scavenging capacities of test compounds were expressed as mmol trolox equivalents (TE) per mmol of sample.

ORAC assay. The determination of ORAC was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC, USA) equipped with an incubator and two injector pumps according to Madhujith and Shahidi (2007). Fluorescein and 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) were used as the probe and radical generator, respectively. Solutions of *p*-coumaric acid (5 mM), PSBO (5 mM), PMHO (5 mM) and trolox standards (6.25−100 μM) were prepared in 7% (w/v) randomly methylated *β*-cyclodextrin (RMCD). An aliquot of each solution (20 μl) was injected into a 96-well Costar black plate (Corning Incorporated, Corning, NY, USA), followed by addition of 75 μl of AAPH (17.2 mg/ml in PBS). Phosphate buffer saline (PBS) (75 mM, pH 7.0) was used as a blank. Samples were incubated at 37 °C for 10 min prior to the auto-injection of 75 μl of AAPH (17.2 mg/ml in PBS) to each sample. The operating conditions were set as follows: 0.3 s position delay, 8 s orbital shaking before each cycle with 4 mm width, 210 s cycle time, and 48 cycles. Fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 520 nm. A standard curve was constructed from the trolox standards, and results were expressed as mM of trolox equivalents (TE) per mM of sample.

Determination of reducing power. Reducing power of *p*-coumaric acid, PSBO and PMHO was measured according to Duh *et al.* (2001) with slight modifications. One

millilitre of test compounds (1 mM in 95% ethanol) was transferred to centrifuge tubes containing 2.5 ml of PBS (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide solution. The mixture was incubated at 50°C for 20 min, which was followed by addition of 2.5 ml of trichloroacetic acid (TCA) (10%, w/v), and then the content was centrifuged at 3000 x *g* for 10 min. After incubation, a portion (2.5 ml) of the supernatant was transferred to the test tube containing 2.5 ml of distilled waster and 0.5 ml of ferric chloride (0.1%). The contents were thoroughly mixed, and the absorbance was read at 234 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent, Palo Alto, CA, USA). Increased absorbance of the reaction mixture indicated increased reducing power. A series of ascorbic acid solutions (1-6 mM in 95% ethanol) were prepared for their use in constructing a standard curve.

Antioxidant activity in *β*-carotene-linoleate model system. Antioxidant activity of *p*-coumaric acid, PSBO and PMHO was assessed using the *β*-carotene bleaching assay according to Amarowicz and Shahidi (1995) with minor modifications. *β*-Carotene (10 mg) was dissolved in chloroform (10 ml), and an aliquot (1.0 ml) of this solution was transferred into a 150 ml round-bottom flask containing linoleic acid (20 mg) and Tween 40 (200 mg). A blank without *β*-carotene was also prepared (20 mg of linoleic acid + 200 mg of Tween 40). After removal of chloroform under a stream of nitrogen, 50 ml of aerated distilled water were added to each flask, and the contents were then thoroughly mixed using a magnetic stirrer. Test compounds (30 µl, 1 mM in ethanol) and the control, ethanol, were injected into a Costar 96-well white plate (Corning Incorporated, Corning, NY, USA). The *β*-carotene-linoleic acid emulsion and the blank emulsion were injected

into the test vials by the built-in injector pump. The absorbance was measured immediately after the addition of emulsion to samples at 450 nm by a Fluostar Optima (BMG LABTECH Inc., Durham, NC, USA) plate reader. The operating parameters of Fluostar Optima were set as follows: 24 cycle No, 420 s cycle time, 4 s shaking time, 5 mm orbital width and 310 μl/s injection speed.

Antioxidant activity of test compounds in protecting β-carotene-linoleic acid oxidation was calculated using the following equation.

$$\% \text{ inhibition} = [1-(A_{s0}-A_{st})/(A_{c0}-A_{ct})] \times 100 \qquad \text{Equation 7.2}$$

where A_{s0}-A_{st} are corrected absorbance values for test samples measured at zero time and after incubation, respectively; while A_{c0}-A_{ct} are corrected absorbance values for control at zero time and after incubation, respectively.

Antioxidant activity in muscle food. The inhibition effect of *p*-coumaric acid, PSBO and PMHO on the production of thiobarbituric acid reactive substances (TBARS) in cooked pork was determined according to Shahidi and Alexander (1998). Fresh ground pork (40 g) was mixed with 10 ml of distilled water in Mason jars. Test compounds dissolved in ethanol were added separately to the meat samples at a level of 80 μmol/kg (13 ppm of *p*-coumaric equivalents), and then the contents were thoroughly mixed with a glass rod. A control containing no test compounds was also prepared. Samples were cooked in a thermostated water bath at 80°C for 40 min with intermittent stirring. The cooked meat was removed from the water bath and cooled to room temperature, and then

samples were homogenized with a Polytron PT 3000 (Brinkmann Instruments, Rexdale, ON, Canada) homogenizer. The homogenized samples were transferred into plastic bags and stored at 4°C for 14 days. The samples were taken on day 0, 3, 5, 7, and 14 for the measurement of secondary oxidation products in terms of TBARS formation according to the method described by Shahidi and Pegg (1994). Two grams of meat sample were weighed in a 50 ml centrifuge tube followed by addition of 5 ml trichloroacetic acid (TCA) solution (10%, w/v) and 5 ml of 2-thiobarbituric acid (TBA) solution (0.02 M) in water, and then the contents were vortexed for 2 min. The samples were subsequently centrifuged at 3,000 × *g* for 10 min, and the supernatants were filtered through a Whatman No. 3 filter paper and collected in 20 ml screw-capped tubes. Samples were kept in a boiling water bath for 45 min, and then removed from the water bath and cooled to room temperature under running tap water. The absorbance of the resultant pink-coloured chromogen was read at 532 nm using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent, Palo Alto, CA, USA). A standard curve was prepared using 1,1,3,3-tetramethoxypropane as a precursor of malondialdehyde (MDA). The TBARS values of samples were calculated using the standard curve and expressed as μmol MDA equivalents per kg of sample.

Inhibition effect on copper-induced LDL cholesterol oxidation. The inhibitory activity of *p*-coumaric acid, PSBO and PMHO against copper-induced LDL cholesterol oxidation was assessed by the method described by Lebeau et al. (2000) with minor modification. Human LDL cholesterol solution was dialyzed in PBS (10 mM, 0.15 M NaCl, pH 7.4) at 4°C under a nitrogen blanket for 12 h. Sample solutions were prepared by dissolving the

test compounds in ethanol at 1.0 mM. Sample solutions (10 μl) were transferred into Eppendorf tubes, and the solvent was removed under a stream of nitrogen. To each tube 100 μl of PBS were added, followed by vortexing for 1 min. An aliquot (0.8 mL) of the dialyzed and diluted LDL cholesterol solution (0.125 mg/ml) was added to each tube, and the contents were mixed well and incubated at 37°C for 15 min. The induced oxidation reaction was initiated by the addition of 100 μl of $CuSO_4$ solution (20 μM, previously incubated at 37°C). A blank containing only the sample without LDL or $CuSO_4$ was prepared for each test compound. After incubation of the reaction mixture at 37°C for 22 h, the conjugated dienes formed were measured spectrophotometrically at 234 nm using a Hewlett–Packard model 8452A diode array spectrophotometer (Agilent, Palo Alto, CA, USA). Antioxidant activities of test compounds were expressed as percentage inhibition of conjugated diene formation, as calculated using Equation 7.3.

$$\% \text{ inhibition} = 100 \times (A_{control}-A_{sample})/(A_{control}-A_{native\ LDL}) \qquad \text{Equation 7.3}$$

Where the control contained LDL cholesterol and $CuSO_4$ without antioxidants, and native LDL cholesterol contained LDL cholesterol only. All absorbances for samples were blank-corrected.

Inhibition effect on radical-induced DNA scission. Antioxidant activity of *p*-coumaric acid, PSBO and PMHO against radical-induced DNA scission was determined as described by Zhong and Shahidi (2012) with slight changes. Oxidation and nicking of DNA strands was induced by hydroxyl and peroxyl radicals and monitored by gel

electrophoresis, respectively. For hydroxyl radical-induced DNA oxidation, 2 μl of ethanolic solution (15 μM) of test compounds were added into a 0.5-ml Eppendorf tube, and then the solvent was removed under a stream of nitrogen. To the tube 2 μl of PBS (0.5 M, pH 7.4) were added, followed by thorough vortexing for 1 min. The following reagents were then added to the tube in the order stated: 2 μl of PBS (0.5 M, pH 7.4), 4 μl of supercoiled pBR322 DNA (20 μg/ml in PBS), 2 μl of H_2O_2 (0.5 mM) and 2 μl of Fe_2SO_4 (0.5 mM). The mixture was then incubated at 37 °C for 1 h. For peroxyl radical-induced DNA oxidation, a higher concentration (50 μM) of samples was used, and H_2O_2 and Fe_2SO_4 were replaced with 4 μl of AAPH (9 mM). A blank (contained only DNA) and a control (containing DNA and one of the radicals) were also prepared for both assays.

Upon the completion of incubation, l μl of loading dye (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was added, and the whole mixture was centrifuged and loaded onto an agarose gel, which was prepared by dissolving 0.7% (w/v) agarose in Tris-acetic acid–ethylenediaminetetraacetic acid (EDTA) buffer (TAE, pH 8.5) and stained with SYBR safe gel stain (1:10000, v/v). Gel electrophoresis was performed at 80 V for 80 min in TAE buffer using a horizontal submarine gel electrophoresis apparatus (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada). The DNA bands were visualized under ultraviolet light. Images were photographed by a GelDoc apparatus equipped with a Sony digital camera and analyzed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA, USA). The intensity of the bands (obtained from densitometer) was used as an indicator of the concentration of the supercoiled and nicked DNA fractions. Antioxidant activity was calculated as % retention of DNA according to equation 7.4.

$$\% \text{ retention} = 100 \times (\text{Intensity of supercoiled } DNA_{sample}/\text{Intensity of supercoiled } DNA_{blank}) \quad \text{Equation 7.4}$$

Results and discussion

Identification of components from the acidolysis mixtures. The HLPC profiles were monitored by UV detector at both 215 and 300 nm. Different components from the initial and final acidolysis mixtures are shown in Figures 7.1-7.4. Each compound was identified by comparing its retention time with that of its corresponding standard and/or the characterization of molecular ion so generated from mass spectrometry. The compounds in peaks C were indentified as lipid derivatives of *p*-coumaric acid as only those compounds with a *p*-coumaric acid moiety give an absorbance at 300 nm while other compounds from the final acidolysis mixture, including free fatty acids and acylglycerols are not detected at this wavelength. The major phenolic lipid compounds identified in peaks C from both acidolysis mixtures with SBO and MHO are listed in Table 7.1, which includes 8 of phenolic monoacylglycerols and 8 of phenolic diacylglycerols. The mass spectrometry of the representative identified ions and their corresponding chemical structures are shown in Figures 7.5-7.9. Other identified derivatives are shown in Appendixes 7.1-7.14, in which compounds in Appendixes 7.1-7.6 are lipid derivatives of SBO while these in Appendixes 7.7-7.14 are lipid derivatives of MHO; however, several of these phenolic lipids can be derived from both SBO and MHO.

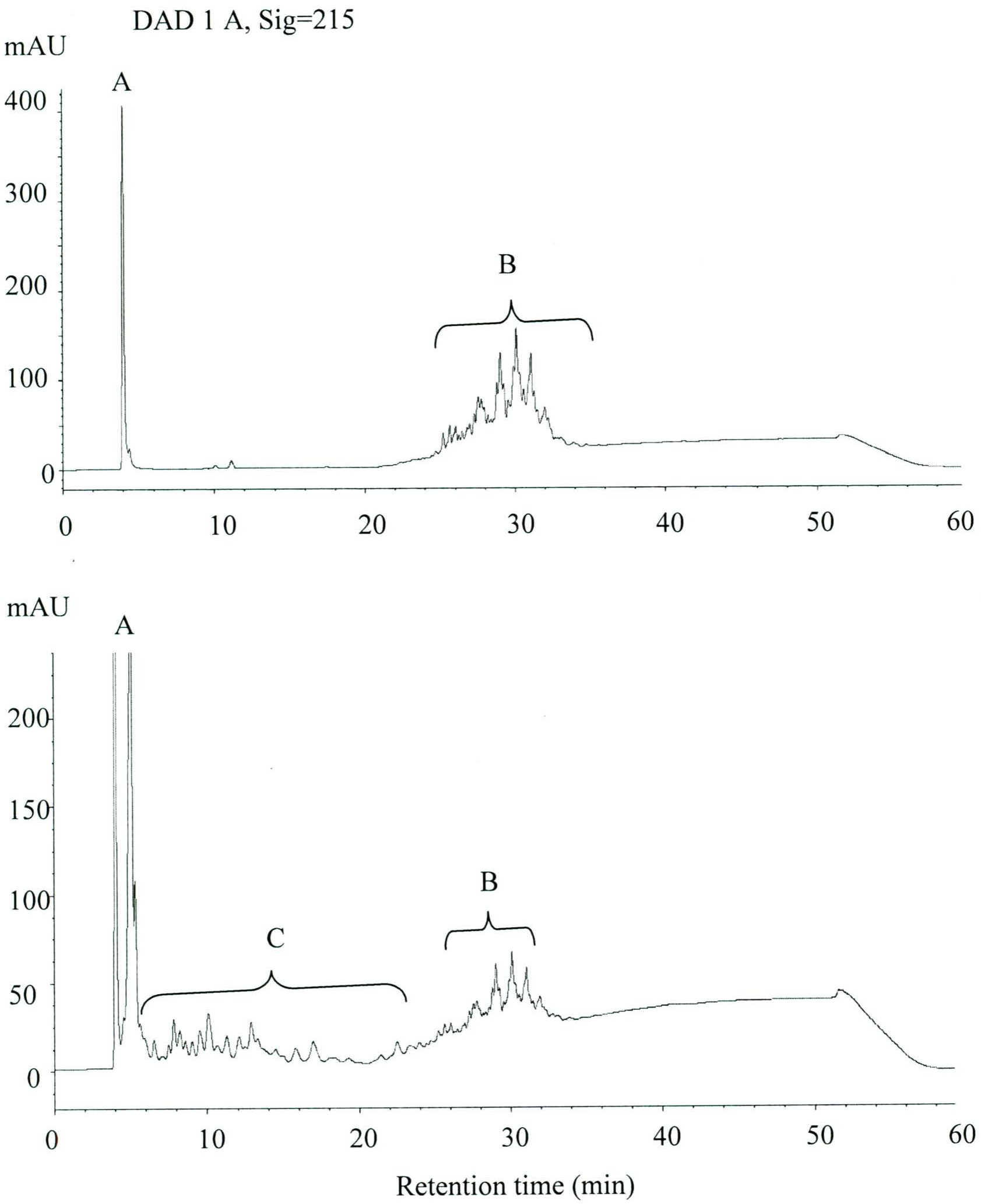


Figure 7.1. High-performance liquid chromatography (HPLC) chromatograms of starting materials (top) and resultants (bottom) of acidolysis of *p*-coumaric acid and SBO at 215 nm. A: *p*-coumaric acid; B: SBO; and C: PSBO (phenolic lipids mixture obtained from SBO).

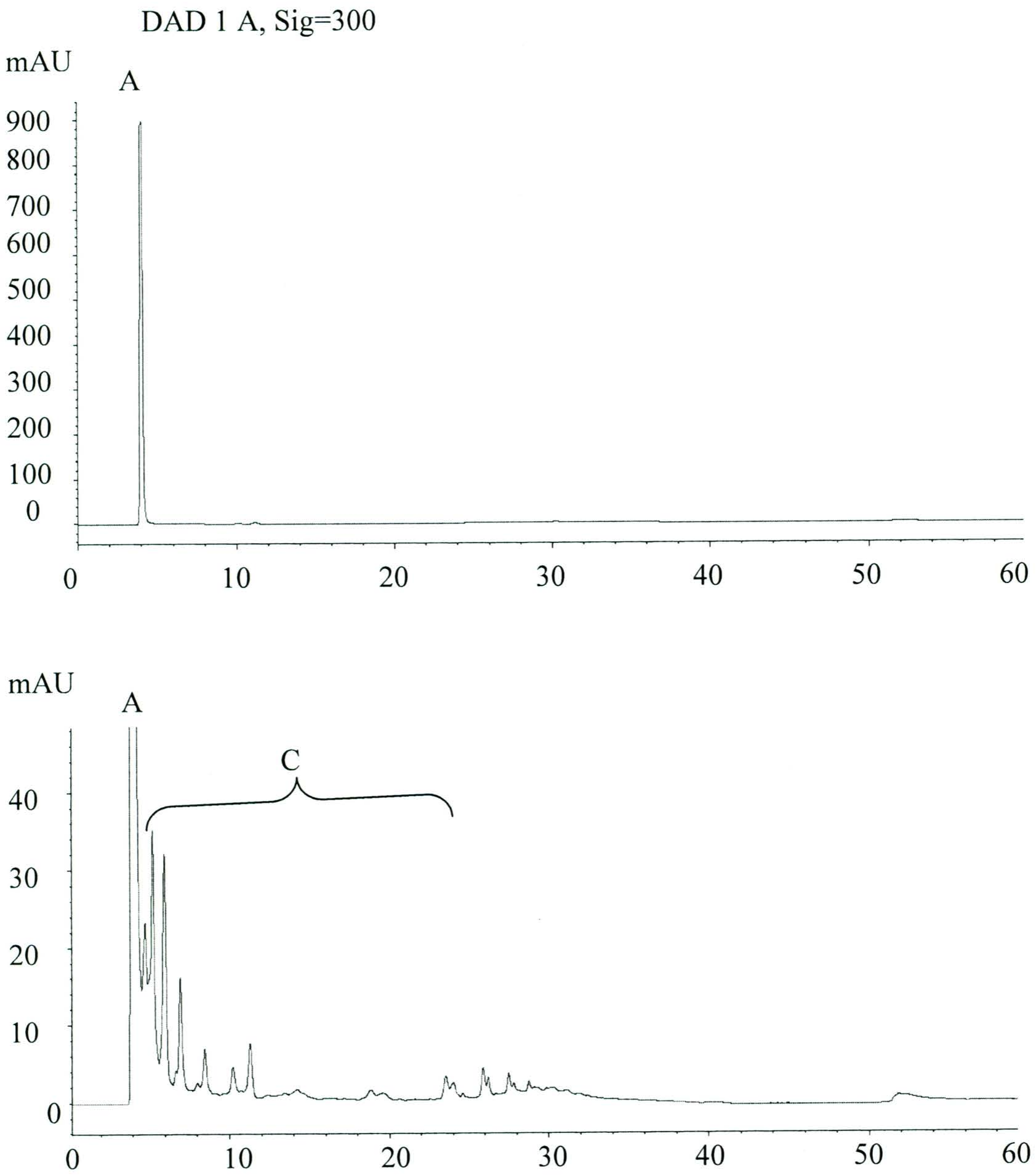


Figure 7.2. HPLC chromatograms of starting materials (top) and resultants (bottom) of acidolysis of *p*-coumaric acid and SBO at 300 nm. A: *p*-coumaric acid; and C: PSBO (phenolic lipids mixture obtained from SBO).

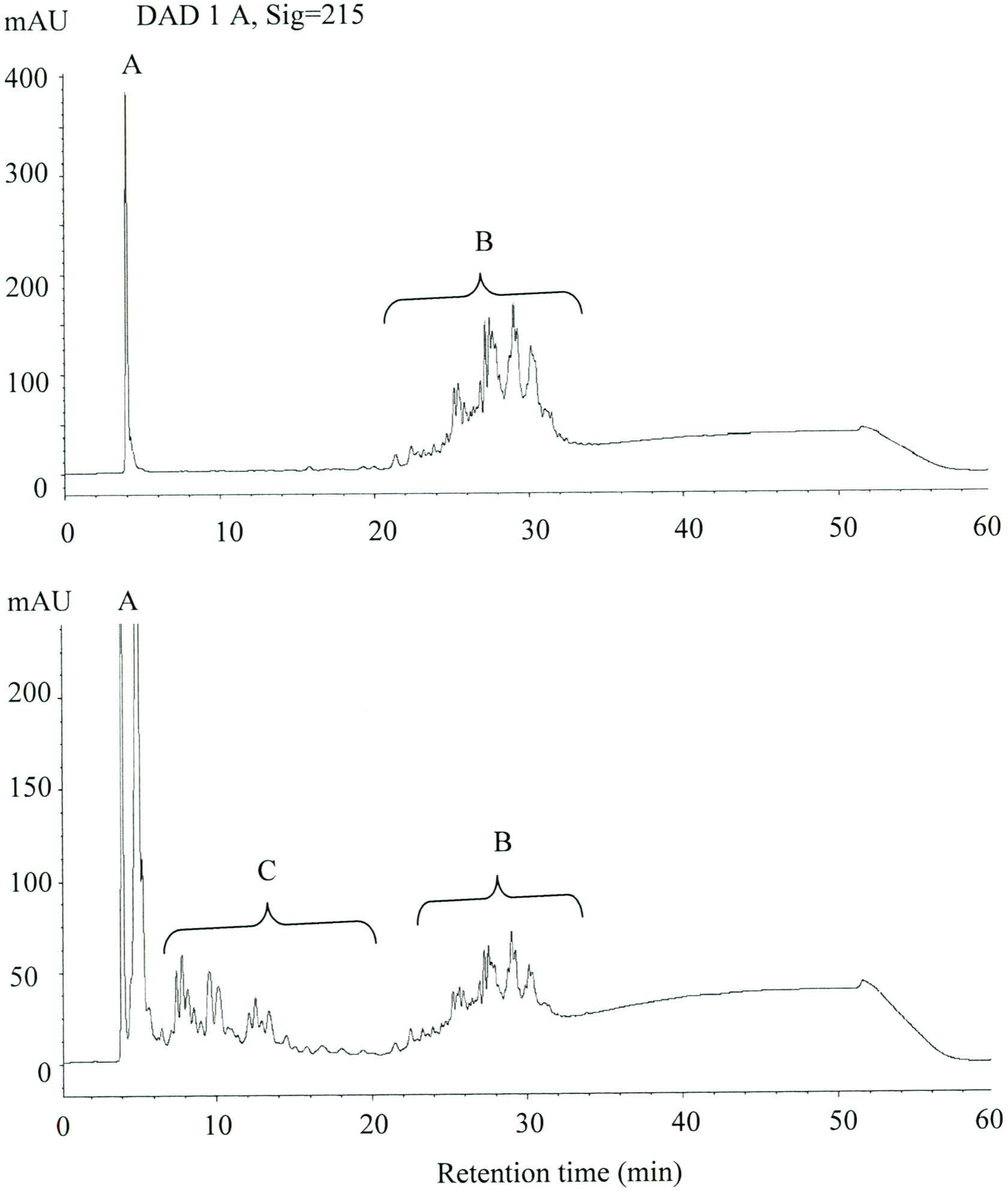


Figure 7.3. HPLC chromatograms of starting materials (top) and resultants (bottom) of acidolysis of p-coumaric acid and MHO at 215 nm. A: *p*-coumaric acid; B: MHO; and C: PMHO (phenolic lipids mixture obtained from MHO).

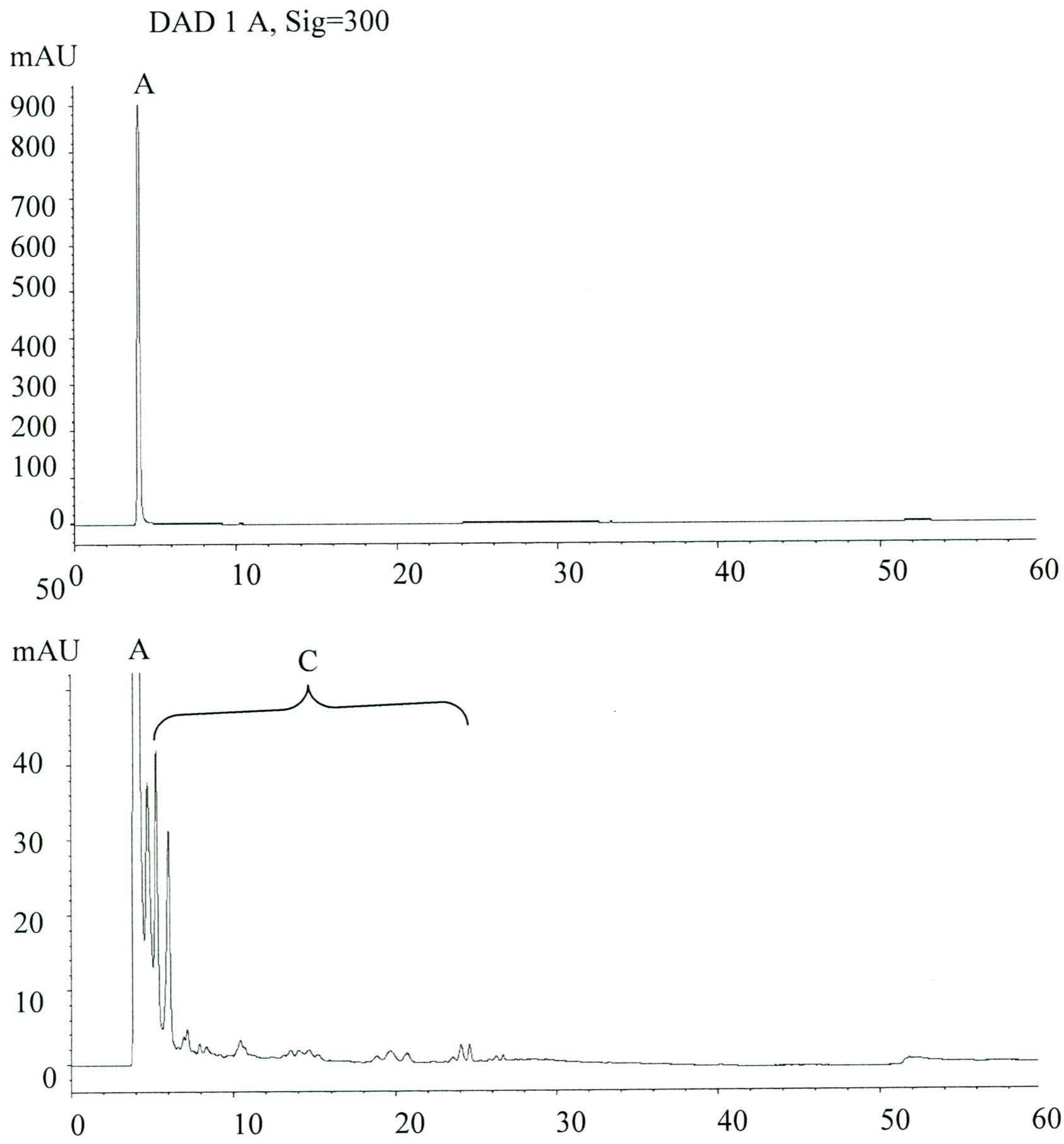


Figure 7.4. HPLC chromatograms of starting materials (top) and resultants (bottom) of acidolysis *p*-coumaric acid and MHO at 300 nm. A: *p*-coumaric acid; and C: PMHO (phenolic lipids mixture obtained from MHO).

Table 7.1. Lipid derivatives of *p*-coumaric acid identified in both acidolysis mixtures of *p*-coumaric acid with SBO and MHO.

Phenolic lipids	Fragmentation pattern	m/z
Phenolic monoacylglycerols		
Monomyristyl *p*-coumarate[2]	$[M+H-H_20]^+$	431.3
Monocetyl *p*-coumarate[1&2]	$[M+H-H_20]^+$	459.3
Monopalmitoleyl *p*-coumarate[1&2]	$[M+H-H_20]^+$	457.3
Monostearyl *p*-coumarate[1&2]	$[M+H-H_20]^+$	487.3
Monooleyl *p*-coumarate[1&2]	$[M+H-H_20]^+$	485.3
Monolinoleyl *p*-coumarate[2]	$[M+H-H_20]^+$	483.3
Monoeicosenyl *p*-coumarate[1&2]	$[M+H-H_20]^+$	513.3
Monoeicosapentaenyl *p*-coumarate[1&2]	$[M]^+$	523.5
Phenolic diacylglycerols		
Dimyristyl *p*-coumarate[1&2]	$[M]^+$	661.5
Myristyl myristeyl *p*-coumarate[2]	$[M+H-H_20]^+$	639.5
Myristeyl cetyl *p*-coumarate[1]	$[M+H-H_20]^+$	667.5
Myristyl cetyl *p*-coumarate[2]	$[M+H-H_20]^+$	669.5
Cetyl palmitoleyl *p*-coumarate[1]	$[M+H-H_20]^+$	695.5
Myristeyl elaidolinolenyl *p*-coumarate[2]	$[M+H-H_20]^+$	689.5
Myristeyl stearidonyl *p*-coumarate[1]	$[M+H-H_20]^+$	687.5
Myristeyl eicosapentaenyl *p*-coumarate[2]	$[M+H-H_20]^+$	713.5

Note: [1] indicates phenolic lipid compound derived only from SBO; [2] indicates phenolic lipid compound derived only from MHO; [1&2] indicates phenolic lipid compounds derived from both SBO and MHO; $[M+H-H_2O]^+$ resulting from the loss of a molecule of H_2O and addition of a proton; and $[M]^+$ resulting from the loss of an electron.

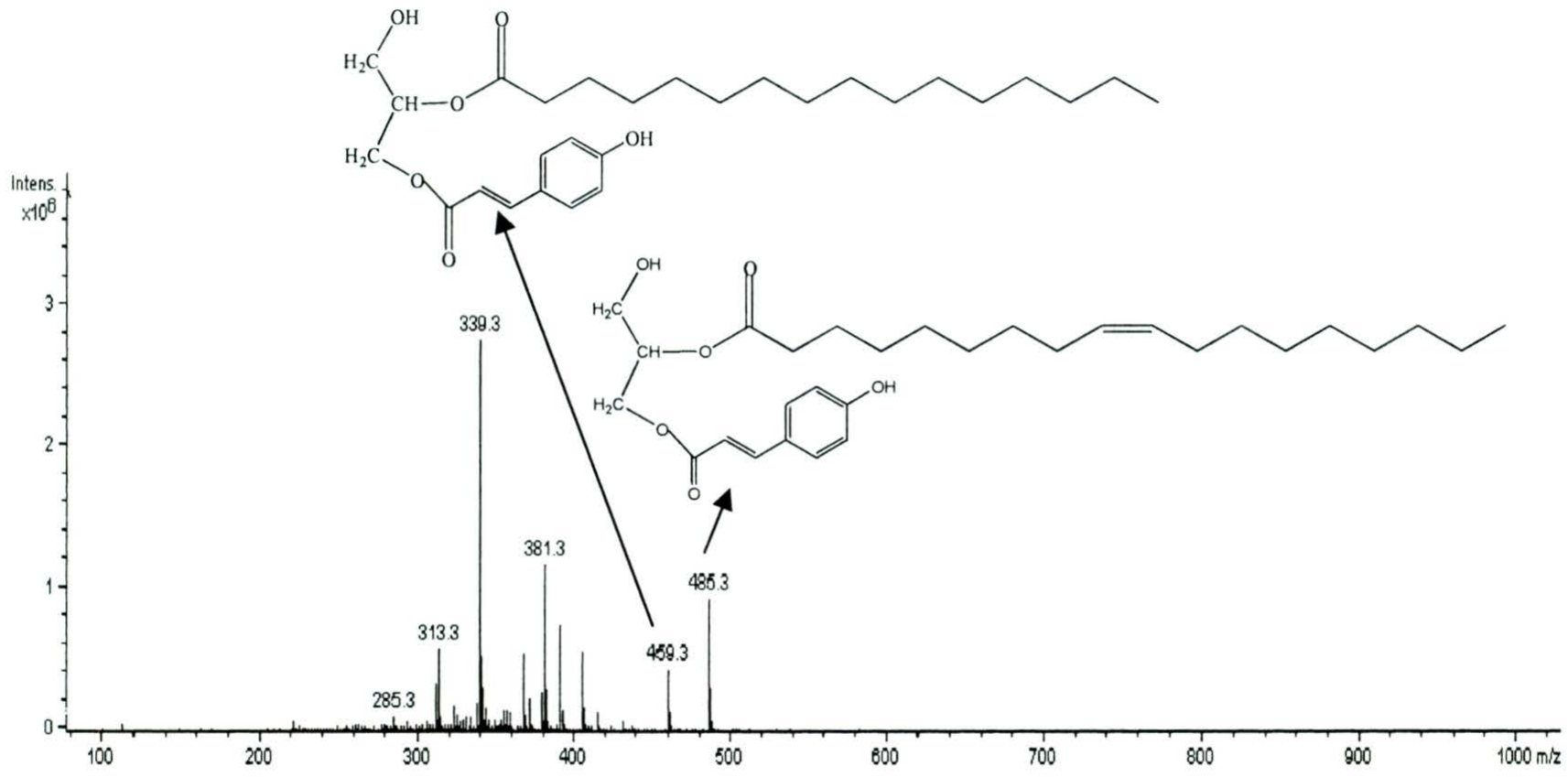


Figure 7.5. Chemical structures and mass spectrometric data of monocetyl *p*-coumarate and monooleyl *p*-coumarate.

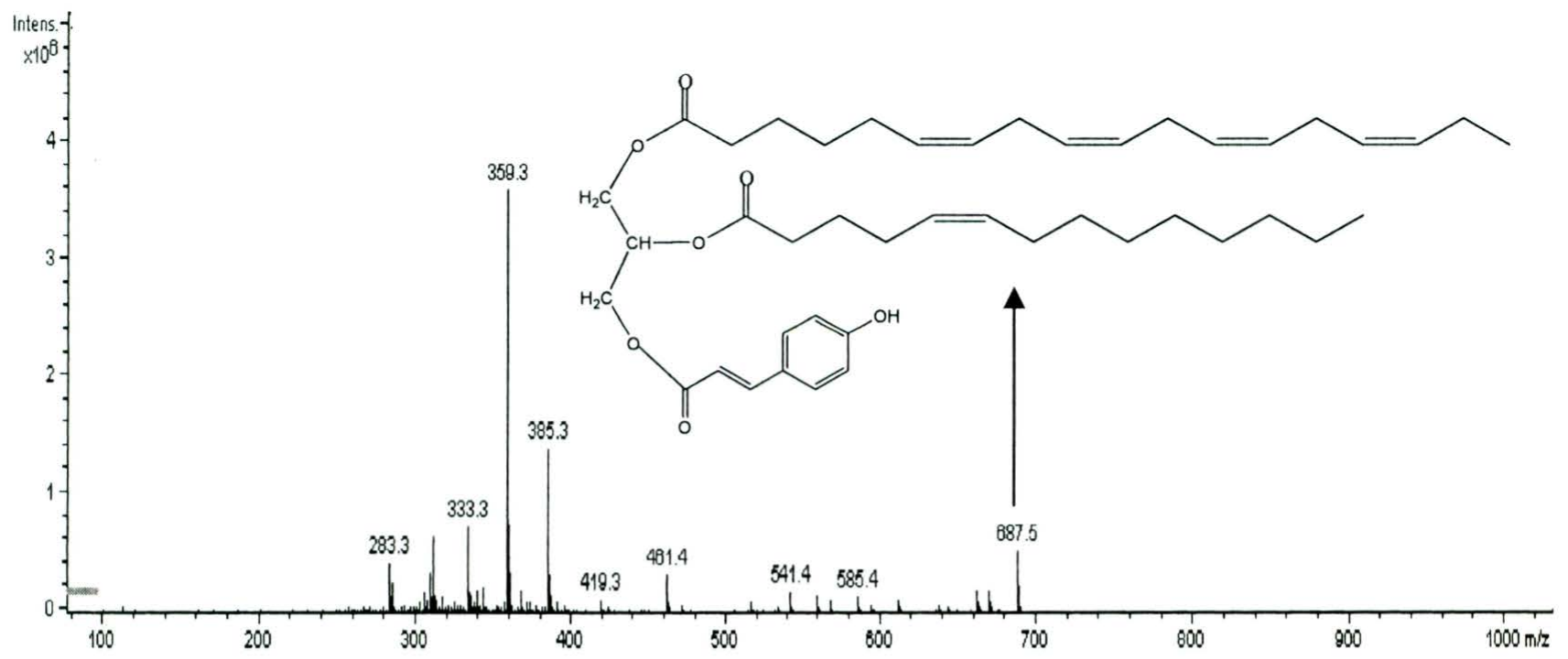


Figure 7.6. Chemical structure and mass spectrometric data of myristeyl stearidonyl *p*-coumarate.

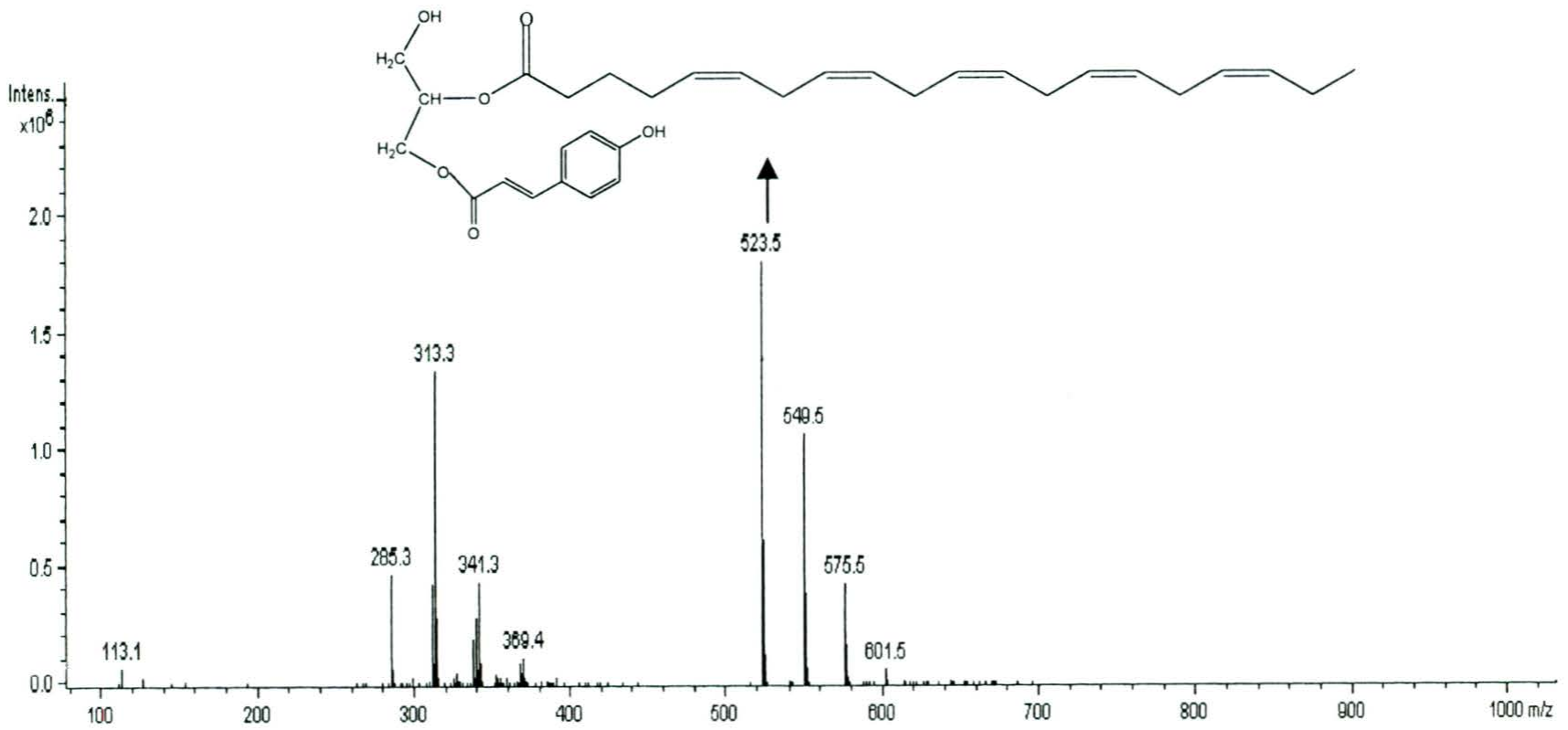


Figure 7.7. Chemical structure and mass spectrometric data of monoeicosapentaenyl *p*-coumarate.

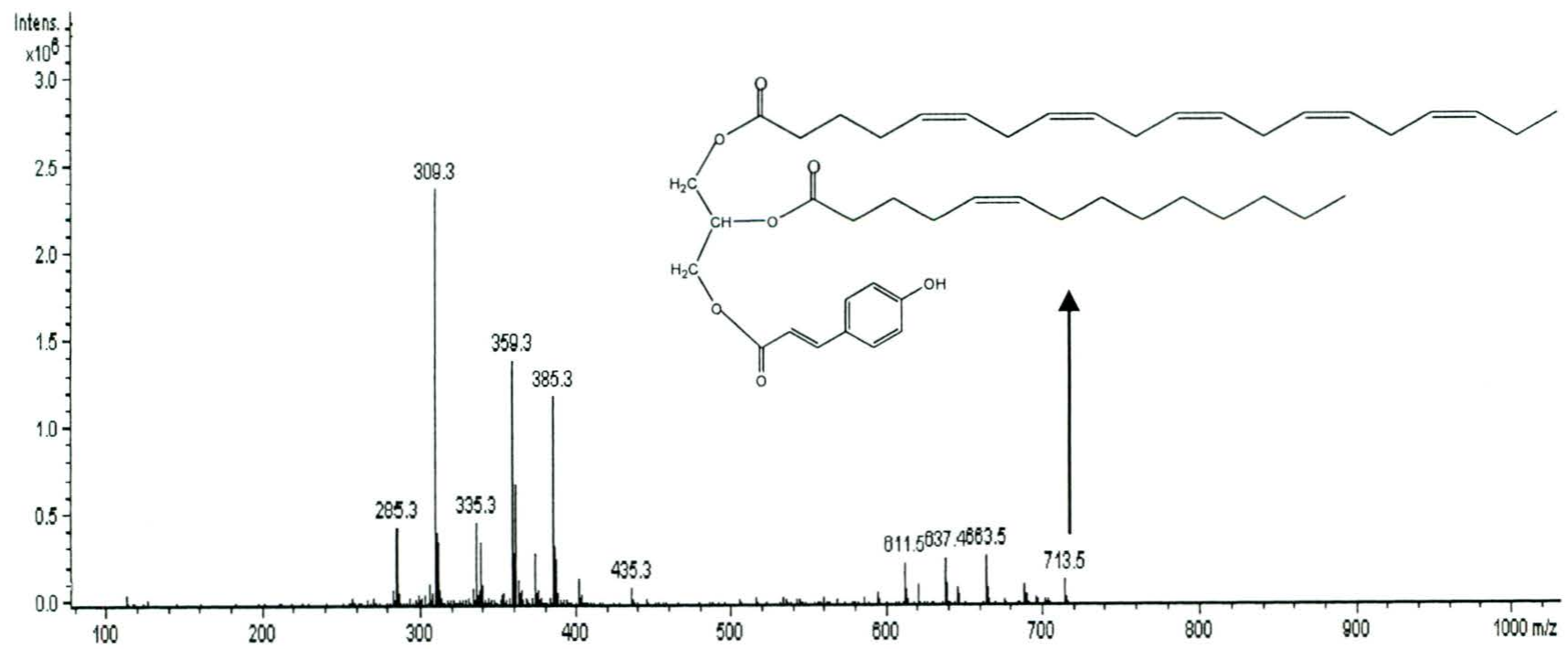


Figure 7.8. Chemical structure and mass spectrometric data of myristeyl eicosapentaenyl *p*-coumarate.

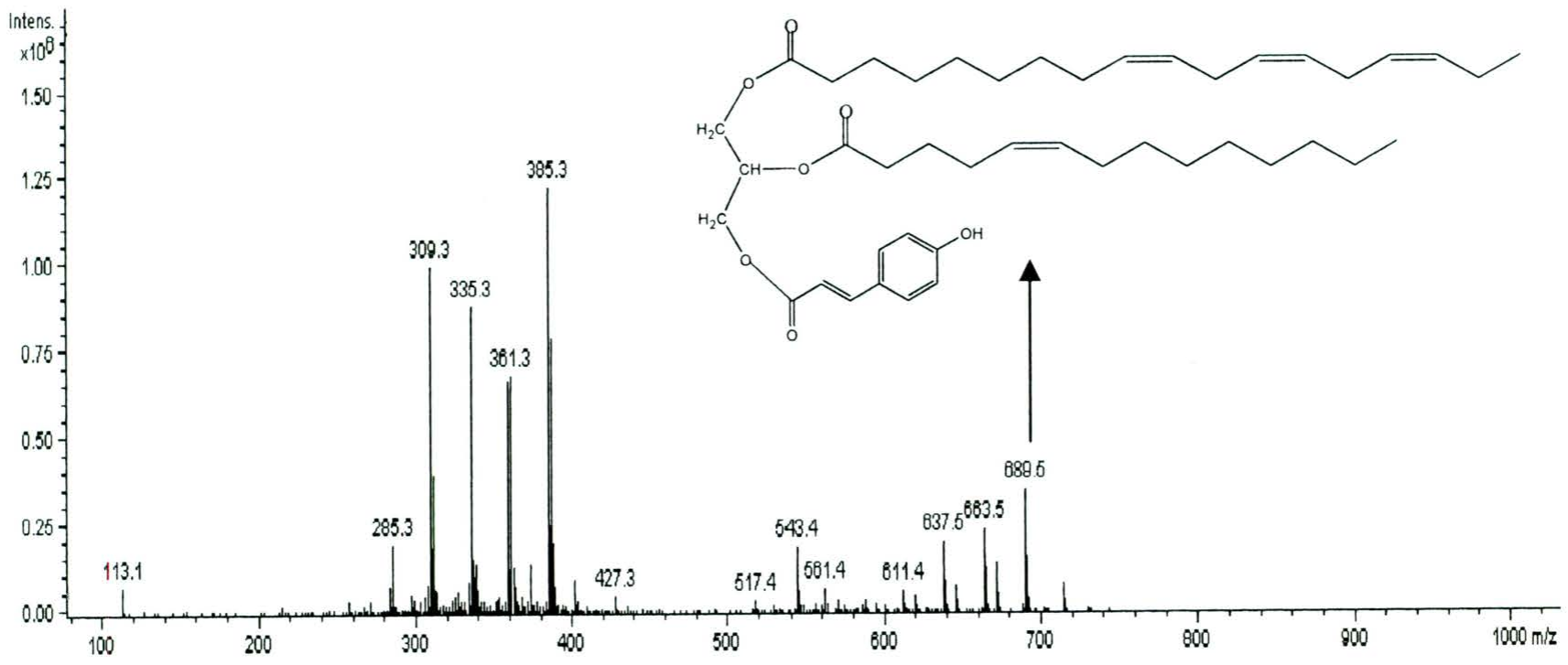


Figure 7.9. Chemical structure and mass spectrometric data of myristeyl elaidolinolenyl *p*-coumarate.

DPPH radical scavenging activity. The DPPH radical scavenging assay is frequently used in examining antioxidant activity because of the relatively short time required for analysis (Chen *et al.* 1999). Phenolic compounds may act as free radical scavengers by virtue of their hydrogen donating ability (Castelluccio *et al.* 1996). As a hydroxycinnamic acid, *p*-coumaric acid possesses higher antioxidant activity than other isomers as the hydroxyl group at the *para*-position exhibits stronger hydrogen donation ability than that at other positions (Nabi & Liu 2012). The strong DPPH radical scavenging capacity of *p*-coumaric acid has been reported by Fukumoto and Mazza (2000). The antioxidant potency of the synthesized phenolic lipids, PSBO and PMHO may be contributed by the *p*-coumaric acid moiety. However, the antioxidant efficacy of PSBO and PMHO may differ from that of *p*-couamric acid due to possible steric and electronic effects as well as lipophilicity change rendered by the mono and diolein moieties. The antioxidant potential of *p*-coumaric acid and its phenolic derivatives, PSBO and PMHO was evaluated using DPPH radical, a stable hydrophobic radical frequently used in antioxidant assessment (Siriwardhana & Shahidi 2002).

The results of the DPPH radical scavenging assay on test compounds are summarized in Figure 7.10. The signal intensity in EPR spectra of DPPH radical as affected by the test compounds decreased significantly, which revealed the scavenging capacity of *p*-coumaric acid and its lipid derivatives on DPPH radical. All test compounds exhibited fairly effective DPPH radical scavenging activity. When comparing the scavenging capacity of *p*-coumaric acid and its lipid derivatives, *p*-coumaric acid demonstrated a significantly lower ($P< 0.05$) capacity as trolox equivalent than both PSBO and PMHO. Meanwhile, PMHO showed superior DPPH radical scavenging activity compared to

PSBO. Lipophilicity of phenolic compounds plays an important role in their antioxidant activity; higher lipophilicity of phenolic compounds may lead to greater antioxidant activity (Zhong & Shahidi 2011). In this case, esterification of *p*-couamric acid improved its lipophilicity, and thus its lipid derivatives showed higher DPPH radical scavenging capacity. Although the improved lipophilicity of PSBO and PMHO may extend their application in more lipophilic environments, further investigation is required to obtain a better understanding of their antioxidant efficacy in different model systems with different test media.

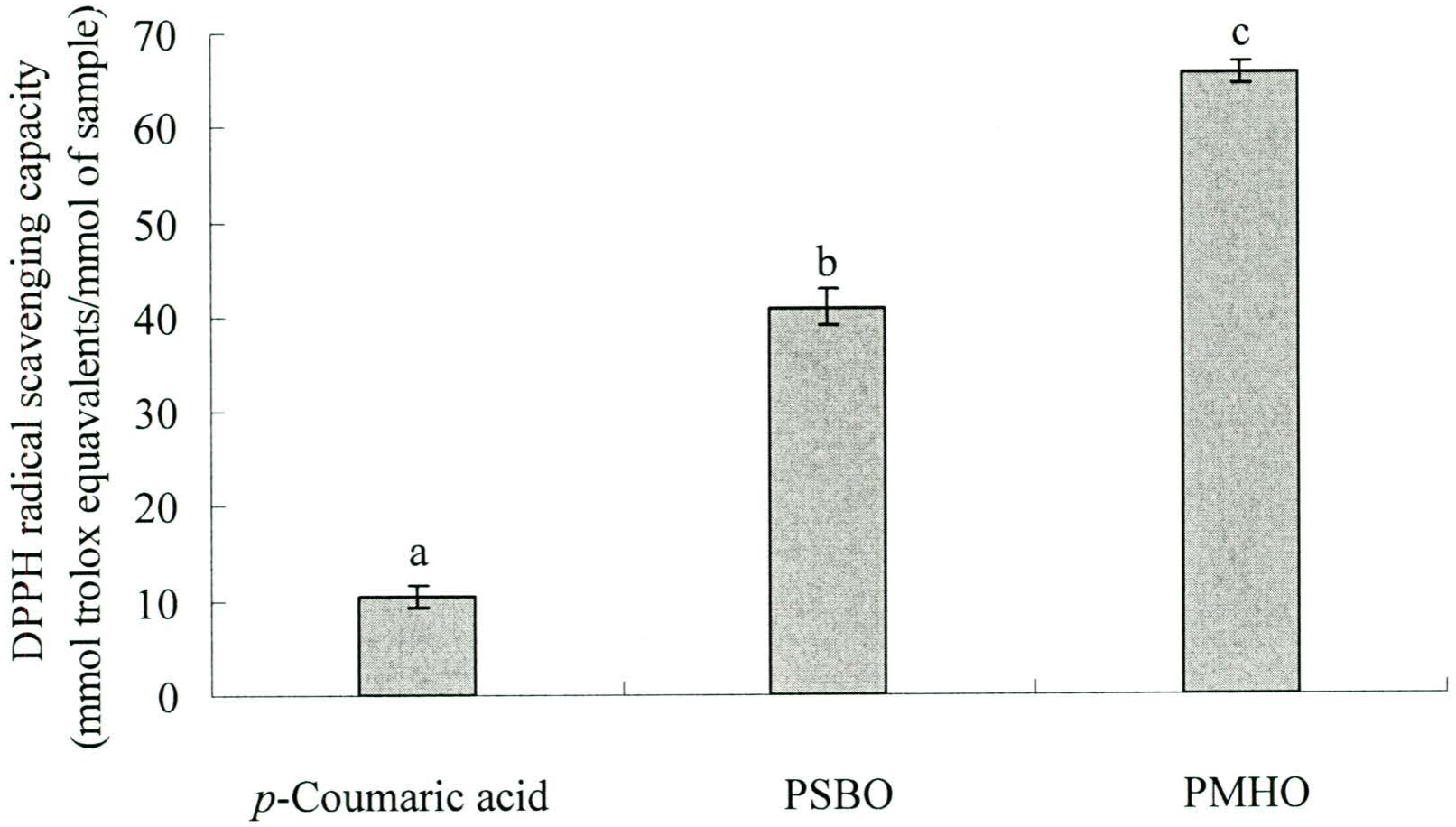


Figure 7.10. DPPH radical scavenging capacity of *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO) as trolox equivalents (TE); bars with different letters are significantly different ($P< 0.05$).

Oxygen radical absorbance capacity (ORAC). *p*-Coumaric acid and its lipid derivatives, PSBO and PMHO demonstrated strong activity in the DPPH radical scavenging assay. However, the results obtained from the DPPH assay may not well reflect the antioxidant capacity of these compounds in the real food and biological systems, where life time of their radicals are much shorter than the stable and artificial radical, DPPH radical (Tan & Shahidi 2011). Therefore, radical scavenging efficacy of *p*-coumaric acid and its lipid derivatives were further evaluated by oxygen radical (peroxyl radical) absorbance capacity assay, which uses a biologically relevant source, in order to gain a better understanding of their antioxidant activity in biological systems (Zhong & Shahidi 2012). ORAC was established as a standard method for evaluating the antioxidant activity of hydrophilic antioxidants, in which acetone-water is used as a solvent medium and randomly methylated-β-cyclodextrin (RMCD) employed as a solubility enhancing agent in order to improve lipophilicity of the solvent to test compounds (Huang *et al.* 2002).

Dávalos et al. (2004) reported that ORAC value of *p*-coumaric acid was 4.5 fold that of trolox, which was 2 times higher than that of BHA. The ORAC of *p*-coumaric acid and its lipid derivatives are summarized in Figure 7.11. All test compounds exhibited strong peroxyl radical scavenging activity. Both PSBO and PMHO showed significantly higher ($P< 0.05$) ORAC values than *p*-coumaric acid, and the trend of the results was correlated with the result of the DPPH radical scavenging assay. The improved antioxidant activity of PSBO and PMHO may be caused by changes of chemical and physical characteristics of the lipid derivatives, such as lipophilicity, electron distribution and steric effect. For instance, the improved lipophilicity may have a positive effect on radical scavenging

activity as higher lipophilicity of phenolic compounds may lead to a greater antioxidant activity (Zhong & Shahidi 2011). However, the weak electron donating ester group may compromise the hydrogen donation activity of *p*-coumaric acid, which leads to lower antioxidant activity (Wright *et al.* 2001). It appears that the weaker hydrogen donation ability caused by the ester group was overwhelmed by the effect of improved lipophilicity, and thus postitive effect of esterification on peroxyl radical scavenging capacity became evident.

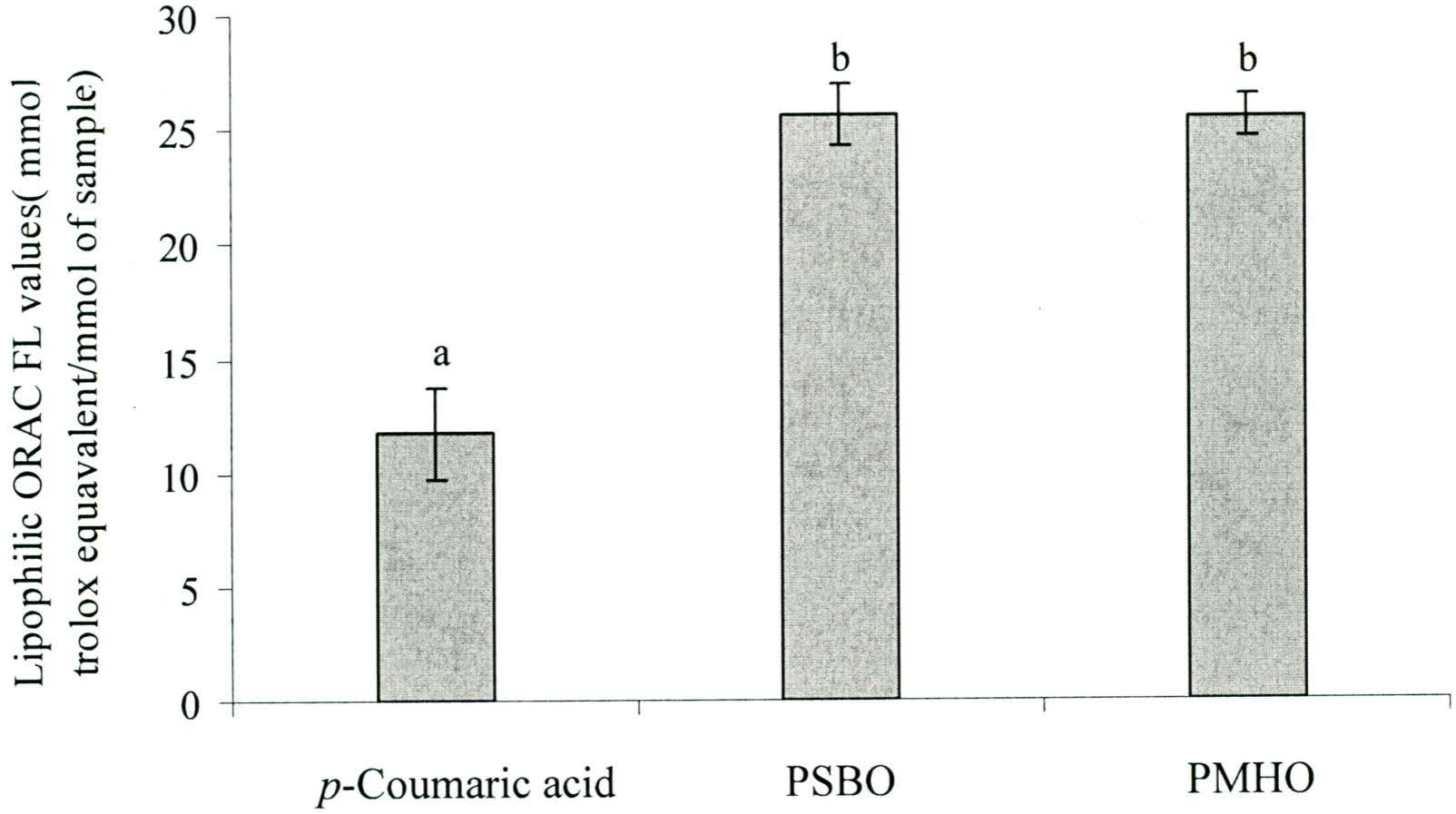


Figure 7.11. Oxygen radical absorbance capacity of *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO) as trolox equivalents (TE); bars with different letters are significantly different ($P< 0.05$).

Reducing power. Antioxidant activity of phenolic compounds can be assessed with a number of methods. For instance, the hydrogen donation capacity of test compounds was examined using the DPPH and ORAC assays. Antioxidants cannot only achieve their goal of eliminating free radicals and preventing oxidation through hydrogen donation, but can also reach that by an electron transfer mechanism (Leopoldini *et al.* 2004; Lin *et al.* 1996). Electron donation efficacy of an antioxidant, so called reducing power, is dependent on both intrinsic factors such as the ionization potential and extrinsic factor such as medium environment. The ferric reducing antioxidant power assay is frequently employed to assess the efficiency of antioxidants for their electron transfer capacity. The reducing power of antioxidants and its measurement is based on the reduction of the Fe^{3+} to the Fe^{2+} by antioxidants in an acidic environment (Wright *et al.* 2001; Wojdylo *et al.* 2007).

p-Coumaric acid demonstrated strong reducing power in the study conducted by Medina *et al.* (2006). In this work, the results summarized in Figure 7.12 showed effective reducing power of *p*-coumaric acid and its lipid derivatives. Compared to *p*-coumaric acid, PSBO did not show significant difference ($P>0.05$) and PMHO showed significantly higher ($P<0.05$) reducing power. This phenomenon may be caused by the decrease of the ionization potential of *p*-coumaric acid when it was esterified to bulky acylglycerol substituents (Sato *et al.* 1983) as decreased ionization potential facilitates the electron transfer.

The results of the reducing power of PSBO and PMHO are correlated with those of both DPPH radical scavenging and ORAC assays as shown in Figures 7.24 and 7.25. However, the reducing power of PSBO is not significantly different to that of *p*-coumaric

acid, which may be partially due to the decreased hydrophilicity of *p*-coumaric acid after esterification in aqueous test system. In reducing power assay, the capacity of antioxidant is strongly solvent-dependent as the solvent facilitates the stability of charged ions (Wright *et al.* 2001). The reducing power of lipid derivatives might be compromised under the hydrophilic test environment; however, such an effect was overwhelmed by the effect of decreased ionization potential.

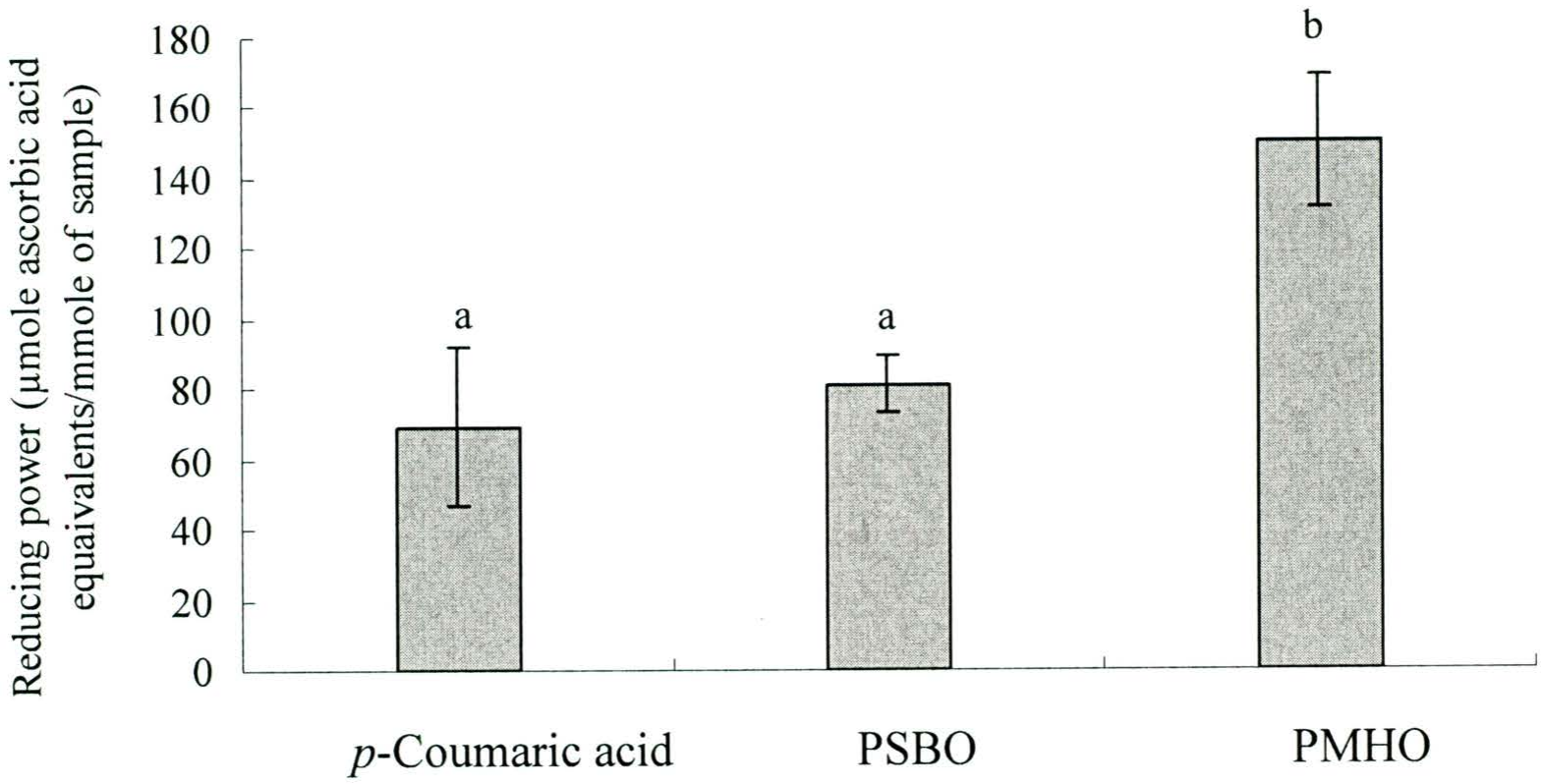


Figure 7.12. Reducing power of *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO) as ascorbic acid equivalents; bars with different letters are significantly different ($P< 0.05$).

Antioxidant activity in a *β*-carotene-linoleate model system. High surface-to-volume ratio of oil-in-water emulsions normally occurs in food systems. Therefore, it is essential to investigate the effectiveness of antioxidants in oil-in-water emulsions to achieve a comprehensive assessment of their antioxidant activity prior to their application in food systems (Zhong & Shahidi 2011). In this work, the antioxidant capacity of *p*-coumaric acid and its lipid derivatives, PSBO and PMHO, in oil-in-water emulsion was examined using a *β*-carotene/linoleic acid emulsion model system. The decolouration of *β*-carotene is a free radical-mediated phenomenon resulting from oxidation of linoleic acid in the emulsion. Addition of antioxidants to the emulsion can minimize the loss of *β*-carotene during the coupled oxidation of linoleic acid and *β*-carotene in the aqueous test medium through the elimination of free radicals generated from linoleic acid oxidation. Their antioxidant activity was detected by monitoring the bleaching of *β*-carotene as affected by test compounds.

Gadow et al. (1997) studied the antioxidant activity of *p*-coumaric acid in the *β*-carotene/linoleic acid emulsion model system and reported its strong inhibitory effect on *β*-carotene bleaching. In this work, the results (Figure 7.13) indicated that PSBO inhibited the bleaching of *β*-carotene by 50.1% over the 161-min incubation period, followed by PMHO (12.2%) and *p*-coumaric acid (8.35%). Based on the antioxidant "polar paradox" theory, nonpolar antioxidants exhibit higher efficacy than that of polar ones in emulsion systems (Zhong & Shahidi 2012), which implies that the lipid derivatives with improved lipophilicity should exhibit higher inhibitory effect on *β*-carotene bleaching than that of *p*-coumaric acid. However, the efficacy of an antioxidant is also determined by its hydrogen donation capacity. In this case, esterification of *p*-coumaric acid may have changed the

surrounding molecular structure of its hydroxyl group on the aromatic ring as well as the bond dissociation enthalpy of the O−H bond. The steric hindrance to hydroxyl group and the changed bond dissociation enthalpy of O−H bond may decrease the hydrogen donation capacity and lead to compromised antioxidant activity of the test lipid derivatives. This may partially explain that the inhibitory activity of PMHO on β-carotene bleaching did not show not significant difference ($P > 0.05$) to that of *p*-coumaric acid.

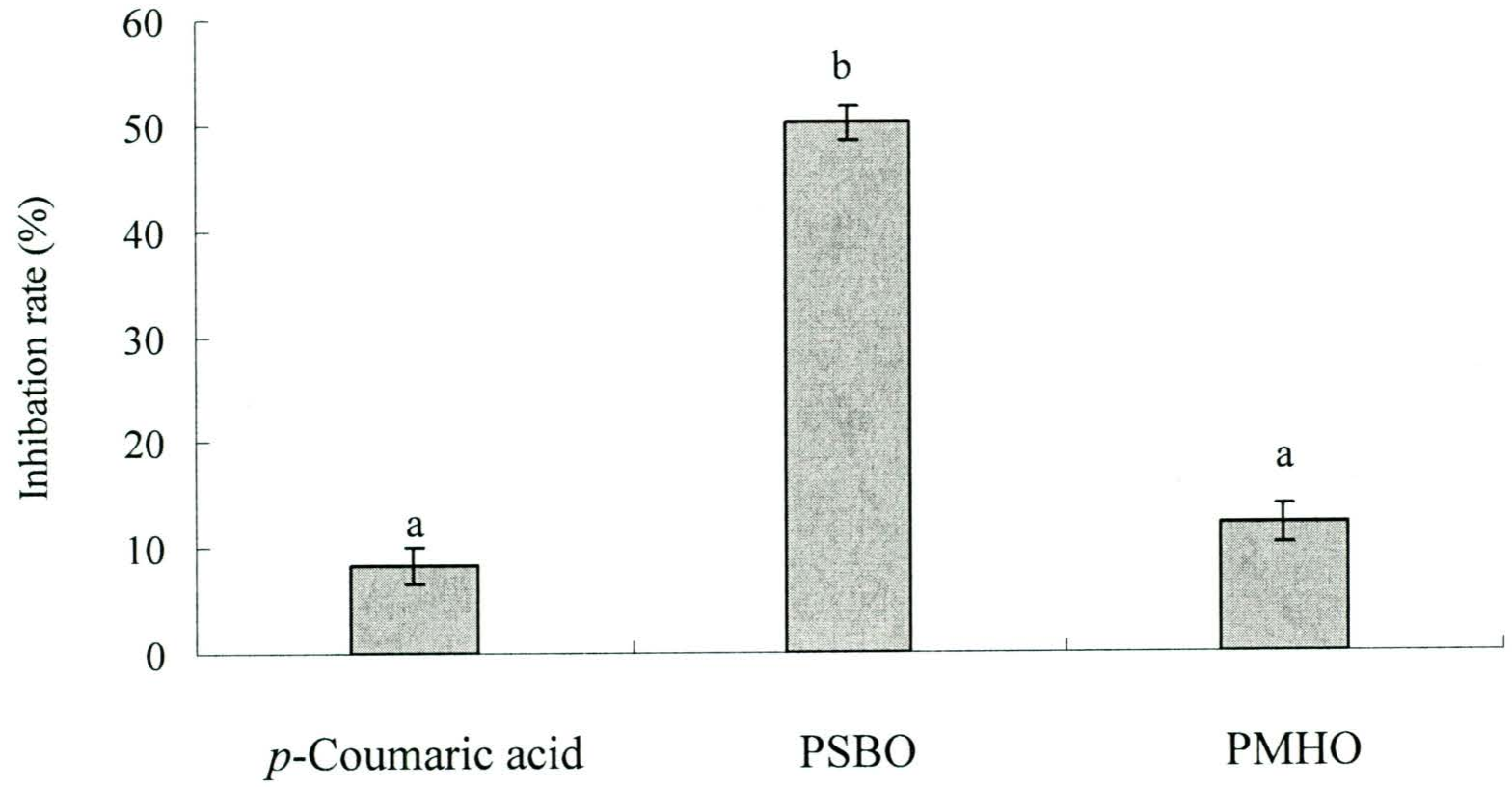


Figure 7.13. Inhibitory effects of *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO) against β-carotene bleaching; bars with different letters are significantly different ($P< 0.05$).

Antioxidant activity in muscle foods. In addition to the oil-in-water emulsion model systems, the antioxidant capacity of phenolic compounds can also be evaluated in other high surface-to-volume ratio systems such as the lipids in processed whole tissue foods (Porter 1993). Other than being one of the major components of oil-in-water emulsions such as salad dressing and spreads, lipids are also incorporated into processed whole tissue foods such as sausages and hams. Lipids, especially those containing high levels of unsaturated fatty acids, undergo oxidation when exposed to oxygen and generate oxidation products; therefore, lipid components are responsible for deterioration of processed whole tissue foods. Thus, effective antioxidants are required in order to prevent oxidation of lipid rich muscle foods as they are more vulnerable to oxidation during precooking and long storage time under high surface-to-volume conditions (Porter 1993). Thus, the inhibitory activity of antioxidants in thermally processed whole or modified muscle foods, which are frequently associated with rapid lipid oxidation and development of "warmed-over flavour", is better assessed (Jayathilakan *et al.* 2007; Shahidi *et al.* 1987). The antioxidant potential of various synthetic and natural antioxidants in three common domestic meat species including sheep, beef and pork have been studied by Jayathilakan *et al.* (2007), and the antioxidants, ascorbic acid, TBHQ and BHA showed great potential to control lipid oxidation as well as warmed-over flavour development in all the test meat species. The strong inhibitory effects of epigallocatechin gallate (EGCG), the major phenolic compound from green tea, and its lipid derivatives synthesized from corresponding polyunsaturated fatty acids and phenolic extracts of millet on lipid oxidation in muscle model systems have been demonstrated by Zhong and Shahidi (2012), Chandrasekara and Shahidi (2012) and He and Shahidi (1997).

In this work, the inhibitory effect of *p*-coumaric acid and its lipid derivatives, PSBO and PMHO, on the production of thiobarbituric acid reactive substances (TBARS), the secondary products of lipid oxidation, in cooked ground pork was measured and expressed as μmol MDA equivalents per kg of sample. The results summarized in Figure 7.14 show that TBARS values of all test samples increased throughout the entire test period. The TBARS values of the sample group with added *p*-coumaric acid were significantly lower ($P<0.05$) than the control group at all test points, which indicated strong inhibitory effects of *p*-coumaric acid on lipid oxidation during the storage period. PSBO showed a prooxidative effect on its sample group as its TBARS value were significantly higher than those of the control group at day 7 and 14 test points while the TBARS values were similar to those of the control group at other test points. On the other hand, PMHO exhibited an even higher prooxidative effect as the TBARS values of its group were significantly higher than those of the control group during the entire storage period except day 0. The test compounds added to fresh ground meat exerted effects at different levels on lipid oxidation during cooking of the meat prior to storage, which explains the presence of different TBARS values from test groups on day 0.

The lipid derivatives of *p*-coumaric acid contain a number of unsaturated fatty acid. For instance, C14:1, C16:1, C18:1, C18:4 and C20:1 were identified in PSBO, while C14:1, C16:1, C18:1, C18:1, C18:3, C18:4, C20:1 and C20:5 were identified in PMHO. The oxidation of unsaturated fatty acids, expecially PUFAs may have compromised the effectiveness of the phenolic moiety of the lipid derivatives in preventing lipid oxidation in meat and rendered higher TBARS values in the groups added with PSBO or PMHO. Therefore, PSBO and PMHO showed prooxidant activity in meat due to the weak

resistance of unsaturated fatty acids in PSBO and PMHO to oxidation under high thermal conditions and longer storage period. The TBARS values of the group with added PMHO are significantly higher than that of the group added with PSBO, which may be explained by the higher PUFA content of PMHO to PSBO. Hence, PSBO and PMHO will not be effective for use in cooked muscle foods for improving their oxidative stability and extending their shelf life due to their prooxidant activity in such system.

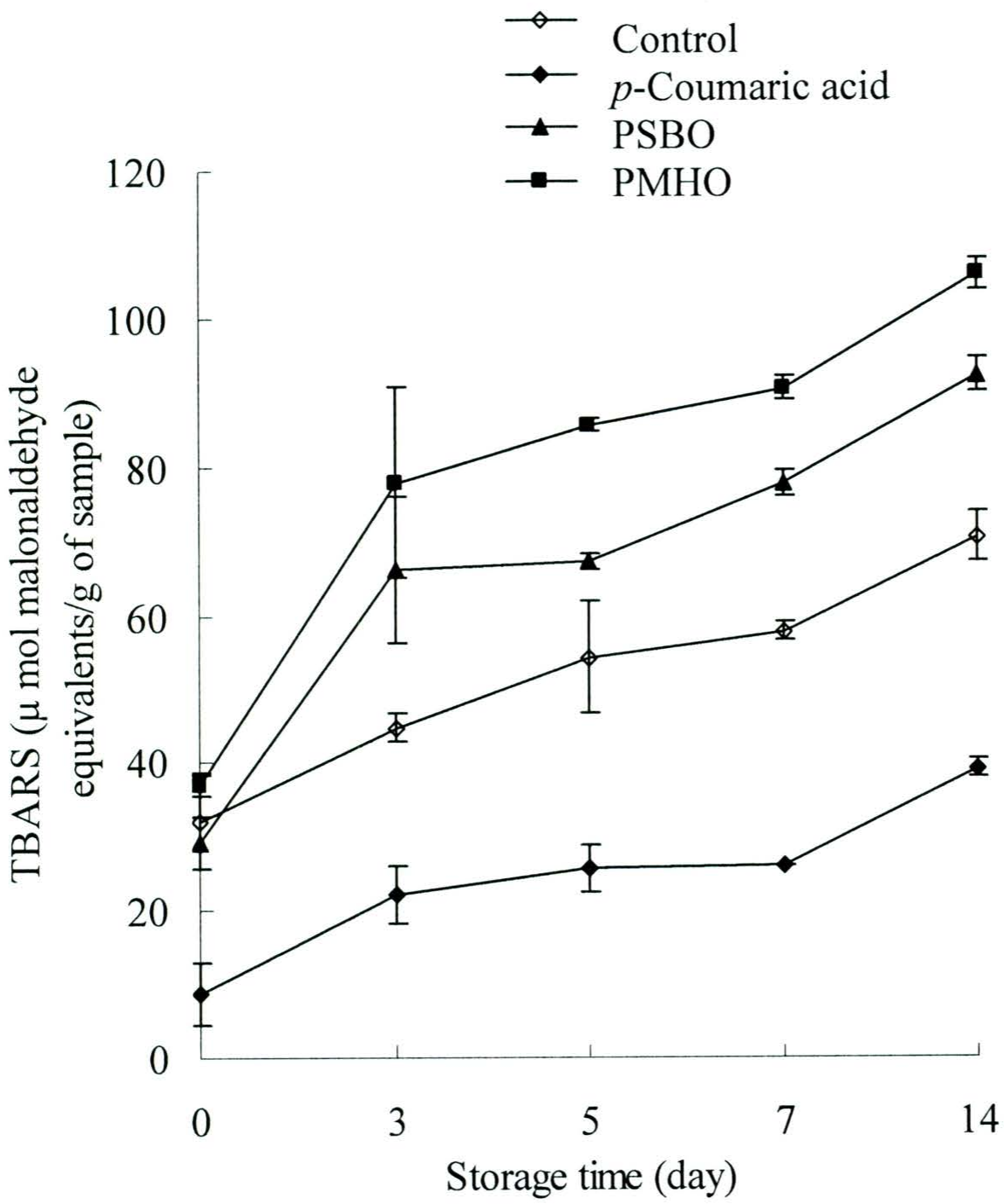


Figure 7.14. TBARS values in cooked ground pork as affected by *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO).

Inhibitory effect on human LDL cholesterol oxidation. LDL cholesterol is one of the five major cholesterol carriers in the blood. It has been proven that the risk of atherosclerosis is positively associated with the increased plasma level of LDL (Ross 1993). Recently, the oxidized products of LDL have been recognized as the contributor to the formation of atherosclerotic plaques in arteries other than the native form (Esterbauer *et al.* 1992), hence providing incentive for preventing LDL oxidation. Dietary antioxidants such as phenolic acids are believed to be effective inhibitors of LDL oxidation caused by free radical attack (Slavin *et al.* 1997; Nardini *et al.* 1995; Andreasen *et al.* 2001). The LDL cholesterol oxidation assay is frequently employed to examine the effectiveness of antioxidants in biological systems. The inhibitory effect of test antioxidants on LDL cholesterol oxidation was monitored by subjecting LDL cholesterol to induced oxidation in the presence of a known concentration of test antioxidants and monitoring the progression of the oxidation (Lebeau *et al.* 2000), in which conjugated dienes (CD) formed were used as an indicator of the level of LDL cholesterol peroxidation.

In this work, the inhibitory effects of *p*-coumaric acid, PSBO and PMHO on Cu^{2+}-induced LDL cholesterol oxidation were evaluated and the results are summarized in Figure 7.15. LDL cholesterol was oxidized to different levels at the end of a 22-h incubation period, which depended on the type of antioxidant added to the samples. All test compounds showed high inhibitory capacity on LDL cholesterol oxidation. Inhibition percentage of LDL cholesterol upon addition of *p*-coumaric acid was 37.3%, which is much higher than the value reported by Andreasen *et al.* (2001) due to the higher *p*-coumaric acid concentration employed in this work. Meanwhile, the inhibition percentage

shown by lipid derivatives of *p*-coumaric acid, PSBO and PMHO, was 42.8 and 48.4%, respectively, which are not significantly higher than that of *p*-coumaric acid ($P>0.05$). The slightly improved inhibitory capacity of PSBO and PMHO against LDL cholesterol oxidation may be due to their improved lipophilicity as the higher lipophilicity allowed these lipid derivatives to have greater affinity to the major components on the surface of LDL cholesterol, i.e., the phospholipids. Moreover, hydrophilic *p*-coumaric acid and lipophilic fatty acid chain may lead to better amphiphilicity of the lipid derivatives, which further allowed them to exert their antioxidant activities within the LDL particle and/or on the surface of LDL cholesterol (Zhong & Shahidi 2012). Thus, the potential use of PSBO and PMHO as anti-atherosclerosis agents is supported by their high inhibitory activity on LDL cholesterol oxidation.

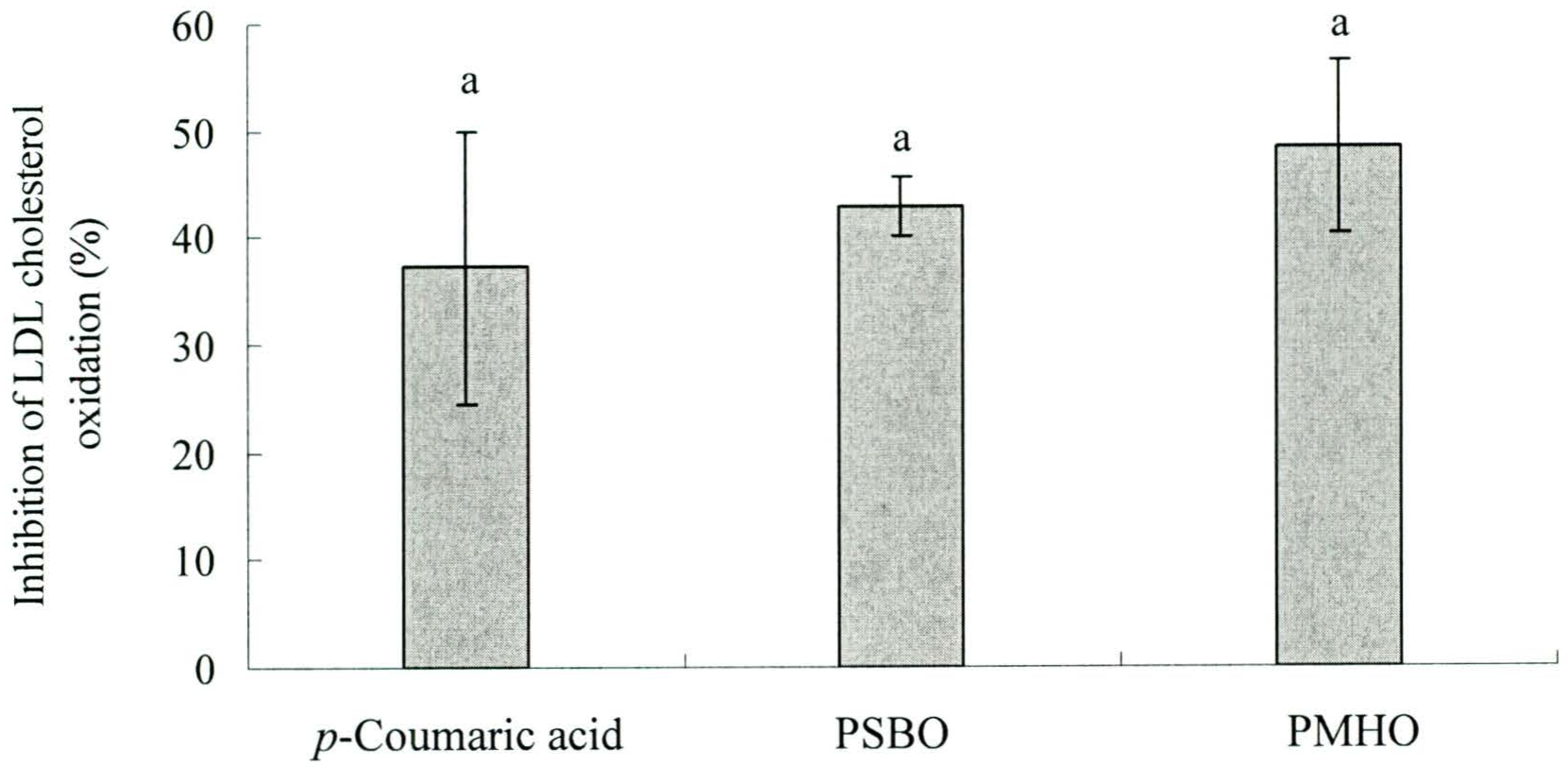


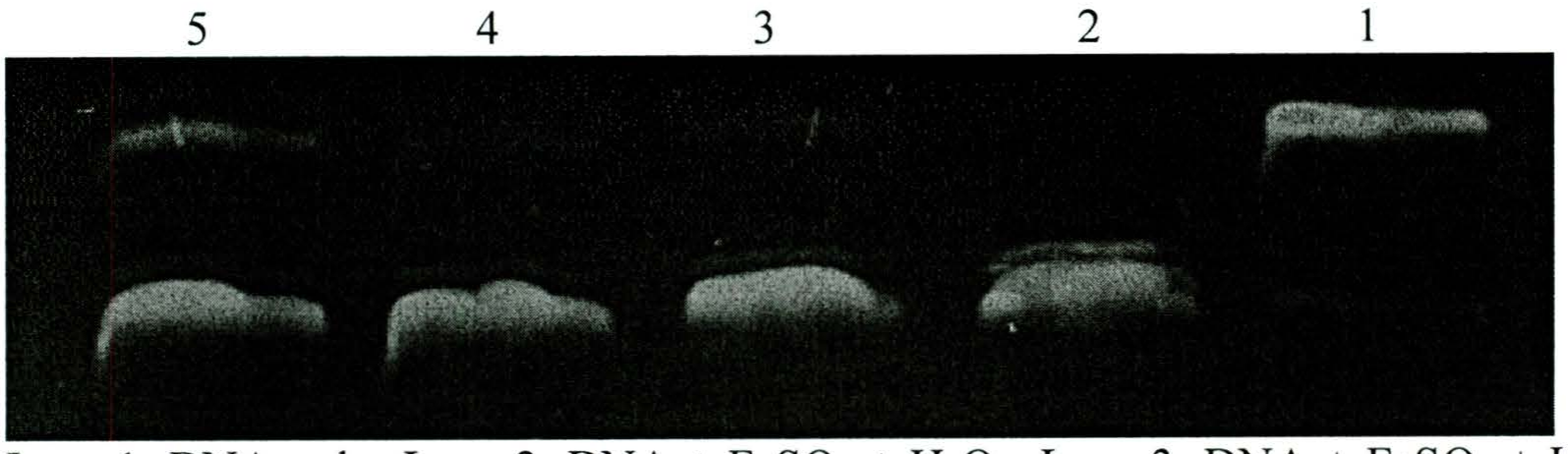
Figure 7.15. Inhibitory effect of *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO) on LDL cholesterol oxidation; bars with different letters are significantly different ($P< 0.05$).

DNA scission assay. Oxidative stress in cells caused by reactive oxygen species (ROS) such as hydroxyl and peroxyl radicals leads to DNA damage. Hydroxyl and peroxyl radicals react with DNA by possible addition to double bonds of DNA bases or abstraction of a hydrogen atom from the methyl group of thymine, among others (Cooke *et al.* 2003). Therefore, oxidative damage of DNA may occur at both the nucleotide bases and the phosphate backbone resulting in strand breakage and sister chromatid exchange, DNA-DNA and DNA-protein cross linking, and base modification (Dizdaroglu *et al.* 1993). Background level of oxidative DNA damage is an inevitable consequence of cellular metabolism and can be repaired in normal tissues. However, the oxidative stress caused by excess free radicals may lead to severe DNA damage, which is implicated in mutagenesis, carcinogenesis and cytostasis. Thus, the effect of various antioxidants such as phenolic acids, on DNA oxidation inhibitory activity has been widely studied (Lodovici *et al.* 2001; Nabi & Liu 2012).

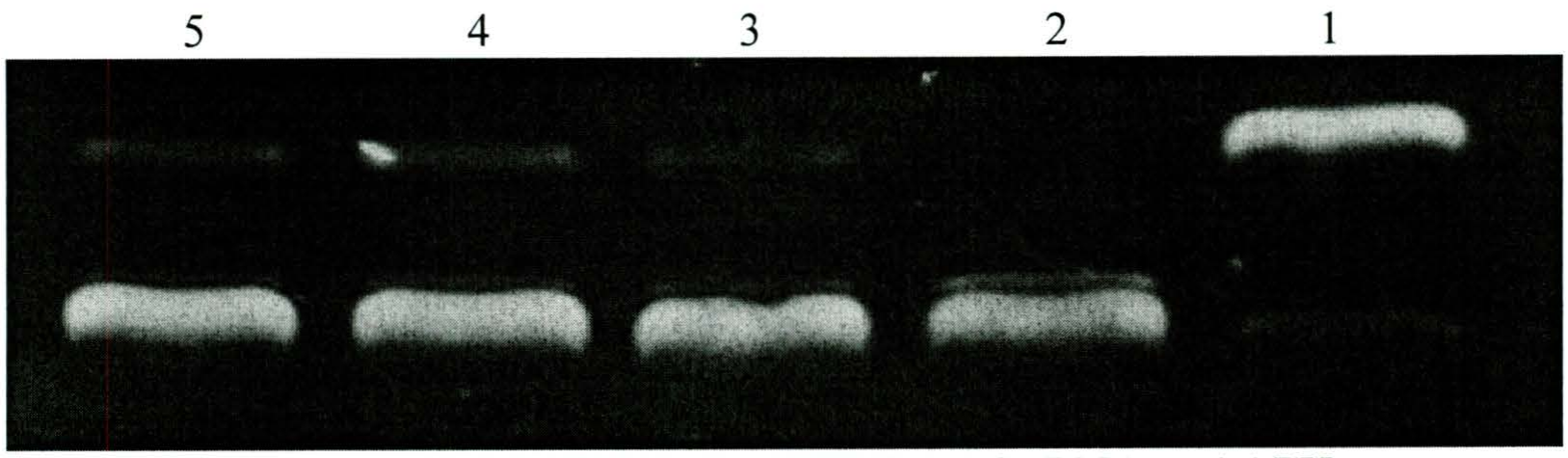
In this study, the inhibitory activity of *p*-coumaric acid, PSBO and PMHO on hydroxyl and peroxyl radical-induced supercoiled DNA strand oxidation was evaluated, and the results are shown in Figures 7.16 and 7.17. The oxidative damage caused by hydroxyl and peroxyl radicals led to supercoiled plasmid DNA strand nicking and formation of nicked open circular form as a result of single strand cleavage (Figure 7.30). At higher concentrations, the free radicals can even cause double bond breakage and convert the supercoiled plasmid DNA strand to linear form of DNA. However, no linear form of DNA was observed due to the modest concentration of radicals employed in the test. The results from Figure 7.31 showed effective DNA retention in all test compounds in hydroxyl radical-induced DNA tests due to their high radical scavenging capacity and for

eliminating hydroxyl radicals generated from H_2O_2 through Fenton reation (Perron *et al.* 2008). Of these, the lipid derivatives, PSBO showed slightly higher retention percentage than *p*-coumaric acid at an insignificant level ($P>0.05$); meanwhile, PMHO exhibited a significantly ($P<0.05$) higher retention percentage than *p*-coumaric acid. The trend of retention percentage of *p*-coumaric acid and its lipid derivatives in hydroxyl radical induced DNA nicking is positively correlated to that of the inhibitory effect of these compounds in LDL cholesterol oxidation assay, which may result from the greater amphiphilic nature of these lipid derivatives.

The oxidation of supercoiled DNA was also successfully induced by peroxyl radical and resulted in the occurrence of nicked open circular DNA at the end of incubation, but no linear DNA was observed (Figure 7.30). All test compounds showed high retention of DNA (Figure 7.31). The lipid derivatives, PSBO and PMHO, exhibited a slightly higher inhibitory capacity compared to *p*-coumaric acid although this was statistically insignificant ($P>0.05$). The different performance of test compounds on DNA retention between hydroxyl radical- and peroxyl radical-induced DNA oxidation may be due to the better stability of peroxyl radical compared to hydroxyl radical which helps it reach specific targets before being degraded or eliminated.



Lane 1: DNA only; Lane 2: DNA + $FeSO_4$ + H_2O_2; Lane 3: DNA + $FeSO_4$ + H_2O_2 + *p*-coumaric acid; Lane 4: DNA + $FeSO_4$ + H_2O_2 + PSBO; Lane 5: DNA + $FeSO_4$ + H_2O_2 + PMHO.



Lane 1: DNA only; Lane 2: DNA + AAPH; Lane 3: DNA + AAPH + *p*-coumaric acid; Lane 4: DNA + AAPH + PSBO; Lane 5: DNA + AAPH + PMHO.

Figure 7.16. The inhibitory effect of *p*-coumaric acid, PSBO (phenolic lipids mixture obtained from SBO) and PMHO (phenolic lipids mixture obtained from MHO) on radical-induced DNA scission (top: hydroxyl-induced DNA scission; bottom: peroxyl-induced DNA scission).

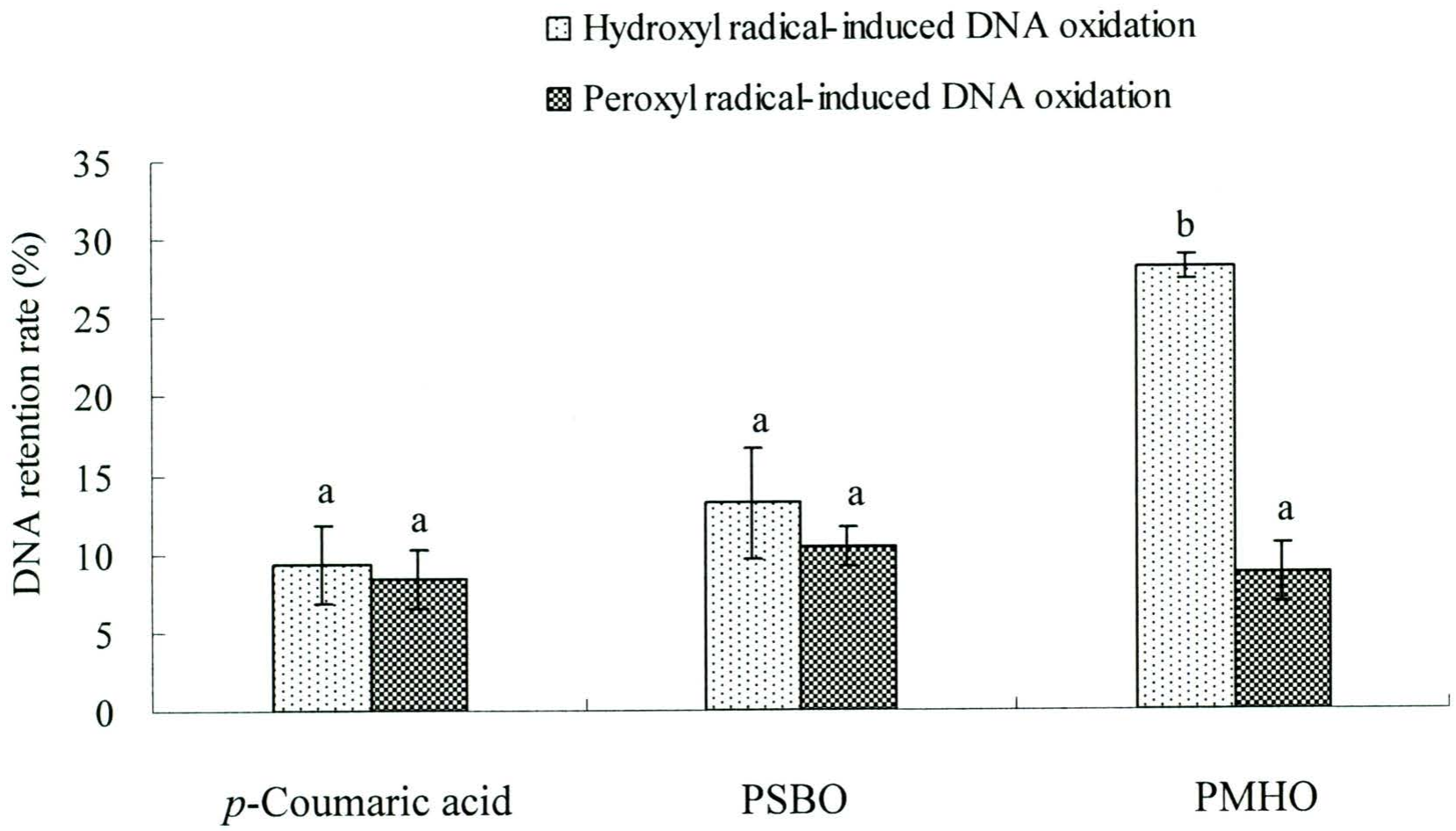


Figure 7.17. DNA retention rate (%) in radical-induced oxidative scission; bars within the same group with different letters are significantly different ($P<0.05$); PSBO: phenolic lipids mixture obtained from SBO; and PMHO: phenolic lipids mixture obtained from MHO.

Conclusions

Lipid derivative of *p*-coumaric acid, PSBO and PMHO, showed great antioxidant capacity at different levels due to various antioxidant mechanisms and different testing systems employed. PSBO and PMHO exhibited greater DPPH and oxygen radicals scavenging capacity. These might mainly be due to the improved lipophilicity of these lipid derivatives. PSBO and PMHO showed higher reducing power compared to *p*-coumaric acid, possibly due to the decreased ionization potential brought about by bulky moieties as lower ionization potential facilitates the electron transfer. Lipid derivatives, especially PSBO, demonstrated excellent inhibitory efficacy against β-carotene bleaching, which suggests their potential use in lipophilic environments; however, such activity was not observed in muscle food models as both PSBO and PMHO showed prooxidant activity on lipid oxidation in cooked muscle food due to the oxidation of their unsaturated fatty acids at high thermal processing temperatures and long storage periods.

Lipid derivatives of *p*-coumaric acid demonstrated slightly higher antioxidant activity in human LDL cholesterol oxidation assays than *p*-coumaric acid due to their improved lipophilicity and amphiphilicity. These lipid derivatives performed strong antioxidant activity in both hydroxyl radical and peroxyl radical-induced DNA oxidation assay. The great antioxidant activity of the phenolic lipid compounds obtained from the acidolysis of *p*-coumaric acid with SBO and MHO, shown in antioxidant testing assays employed suggests that these synthesized phenolic lipids may be used as potential ingredients in functional foods, supplements or cosmetics for health promotion as well as disease risk reduction.

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CHAPTER 8

Summary and recommendation for future research

Chemical and enzymatic randomizations lead to the redistribution of fatty acids among the stereoisomeric *sn*-1,3 and *sn*-2 positions of the glycerol moiety of TAGs of seal blubber and menhaden oils. However, the effect of randomizations on positional distribution of each fatty acid occurred at different levels in the oils tested. In the case of chemical randomization, results obtained from GC and ^{13}C NMR spectral analysis were different when comparing positional distributions of C18:4, C20:5, C22:5 and C22:6 in seal blubber and menhaden oils before and after randomization, but they followed a similar trend. The results of chemical randomization revealed that redistribution of fatty acids, especially unsaturated fatty acids together with changes of alpha-tocopherol upon randomization altered the chemical and physical properties of the oils and hence their oxidative stability. The sole effect of positional distribution of fatty acids on the oxidative stability of the oils were further confirmed in the study of enzymatic randomization when the α-tocopherol content was replenished to its original level in the randomized oils. The results showed that redistribution of fatty acids, in particular, unsaturated fatty acids, was the major factor affecting the oxidative stability of omega-3 oils; higher distribution of PUFA at the terminal positions led to lower oxidative stability of the resultant oils.

Blending of seal blubber and menhaden oils with wheat germ oil modified fatty acid compositions and increased tocopherol content of these omega-3 oils. The oxidative stability of seal blubber and menhaden oils was improved to different extents when

blended with different percentages of wheat germ oil, and such effects were mainly due to the increased content of tocopherols, especially gamma- and delta tocopherols as the total content of unsaturated fatty acids did not changed significantly. Therefore, wheat germ oil served as a good stabilizer for the marine oils tested.

Optimization conditions of acidolysis of *p*-coumaric acid with triolein was determined by using response surface methodology and the steepest ascent analysis. The degree of esterification of *p*-coumaric acid yielded in the central composite design should be the one closest to the possible ideal optimized degree. Maximum values of tested variables, reaction time: 288 h, enzyme load: 15% (w/w of starting materials) and ratio of substrates: 1/16 (mole of *p*-coumaric acid/mole of lipid), were recommended in the use of the acidolysis of *p*-coumaric acid with triolein as well as acidolysis of *p*-coumaric acid with seal blubber and fish oils. The synthesized phenolic lipids, mono and dioleyl coumarates were identified by HPLC-MS. The results obtained from *in vitro* assays, food and biological model systems showed that phenolic lipids, mono and dioleyl *p*-coumarates, derived from *p*-coumaric acid, exhibited different antioxidant capacities due to various mechanisms involved and different testing systems employed. Mono and dioleyl *p*-coumarates showed the lower DPPH and peroxyl radical scavenging capacity compared to that of *p*-coumaric acid, possibly due to steric hindrance exerted by esterified bulky moieties and weakened hydrogen donation capacity caused by the formation of ester bonds. Mono and dioleyl *p*-coumarates exhibited better reducing power compared to their radical scavenging capacities, possibly caused by the decreased ionization potential brought about by bulky moieties. Mono and dioleyl *p*-coumarates exhibited excellent antioxidant potential against *β*-carotene bleaching, which suggests their potential use in

more lipophilic environments; however, such activity was not confirmed in muscle food models as only dioleyl *p*-coumarate showed limited inhibitory effect on lipid oxidation in cooked muscle food may due to the oxidation of oleic acid at high thermal processing temperatures and long storage periods. Both mono and dioleyl *p*-coumarates demonstrated high antioxidant capacity in human LDL cholesterol oxidation assays than *p*-coumaric acid, which could be explained by their improved lipophilicity. These compounds performed in a similar manner in the hydroxyl radical-induced DNA oxidation assay, but a similar activity was not observed in the peroxyl radical-induced DNA oxidation assay due to the different nature of radicals employed.

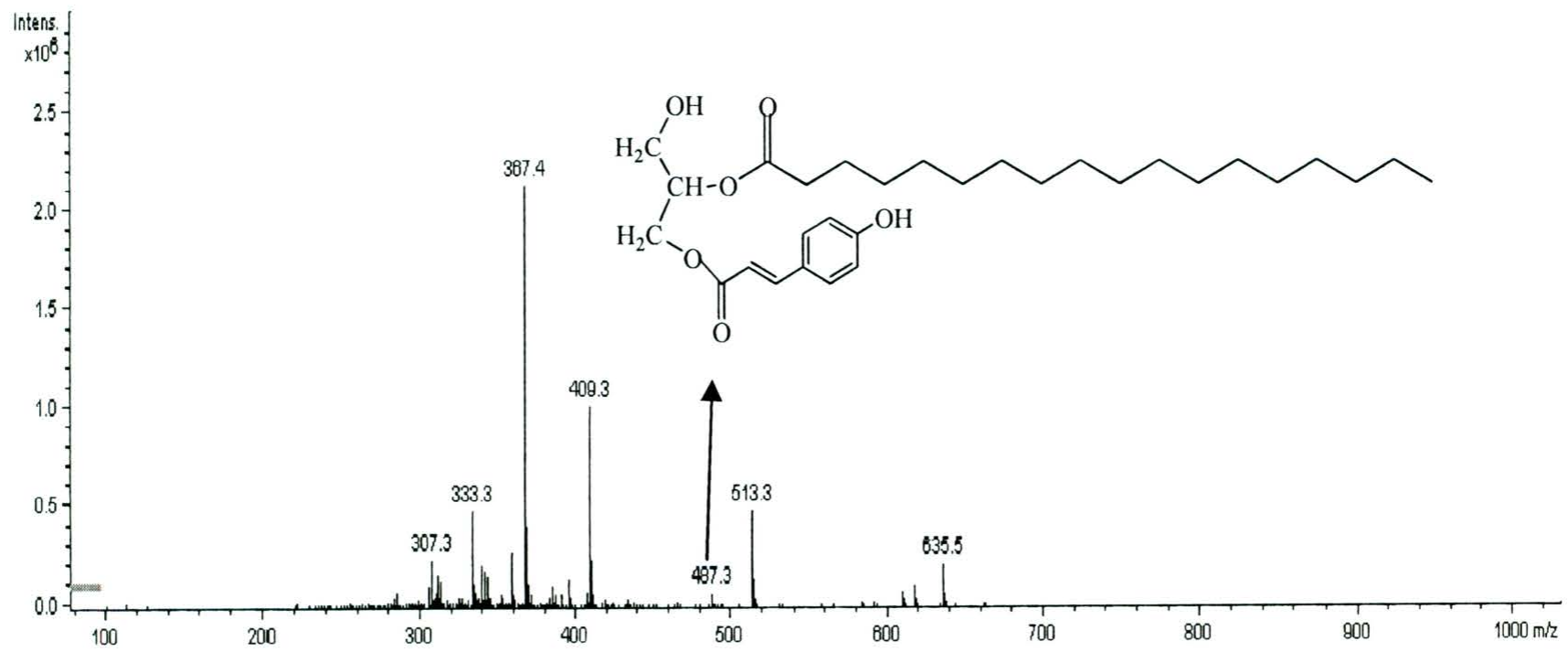
Phenolic lipids obtained from acidolysis of *p*-coumaric acid with seal blubber and menhaden oils were identified by HPLC-MS, in which 8 of them are phenolic monoacylglycerols and 8 of them are phenolic diacylglycerols. The results obtained from various antioxidant activity assays indicated strong antioxidant capacity of these phenolic lipids. Both groups of phenolic lipids derived from SBO and MHO exhibited greater DPPH and peroxyl radicals scavenging capacities compared to *p*-coumaric acid, which might mainly be due to the improved lipophilicity of the phenolic lipids. They also showed higher reducing power compared to *p*-coumaric acid, possibly due to the decreased ionization potential brought about by bulky moieties as lower ionization potential facilitates electron transfer. Phenolic lipids, especially those originating from seal blubber oil, demonstrated excellent inhibitory effect against *β*-carotene bleaching, which suggests their potential use in lipophilic environment; however, such activity was not observed in muscle food model systems employed. Both groups of phenolic lipids showed prooxidant activity on lipid oxidation in cooked muscle food due to the oxidation

of their unsaturated fatty acids at high thermal processing temperatures and long storage periods. However, these phenolic lipids demonstrated insignificantly higher antioxidant activity in human LDL cholesterol oxidation assays than *p*-coumaric acid due to their improved lipophicility. In addition, they performed strong antioxidant activity in both hydroxyl radical and peroxyl radical-induced DNA oxidation assays. The great antioxidant activity of the phenolic lipids obtained from the acidolysis of *p*-coumaric acid with triolein as well as seal blubber and menhaden oils shown in antioxidant testing assays suggests that these synthesized phenolic lipids may be used as potential ingredients in functional foods, supplements or cosmetics for health promotion as well as disease risk reduction.

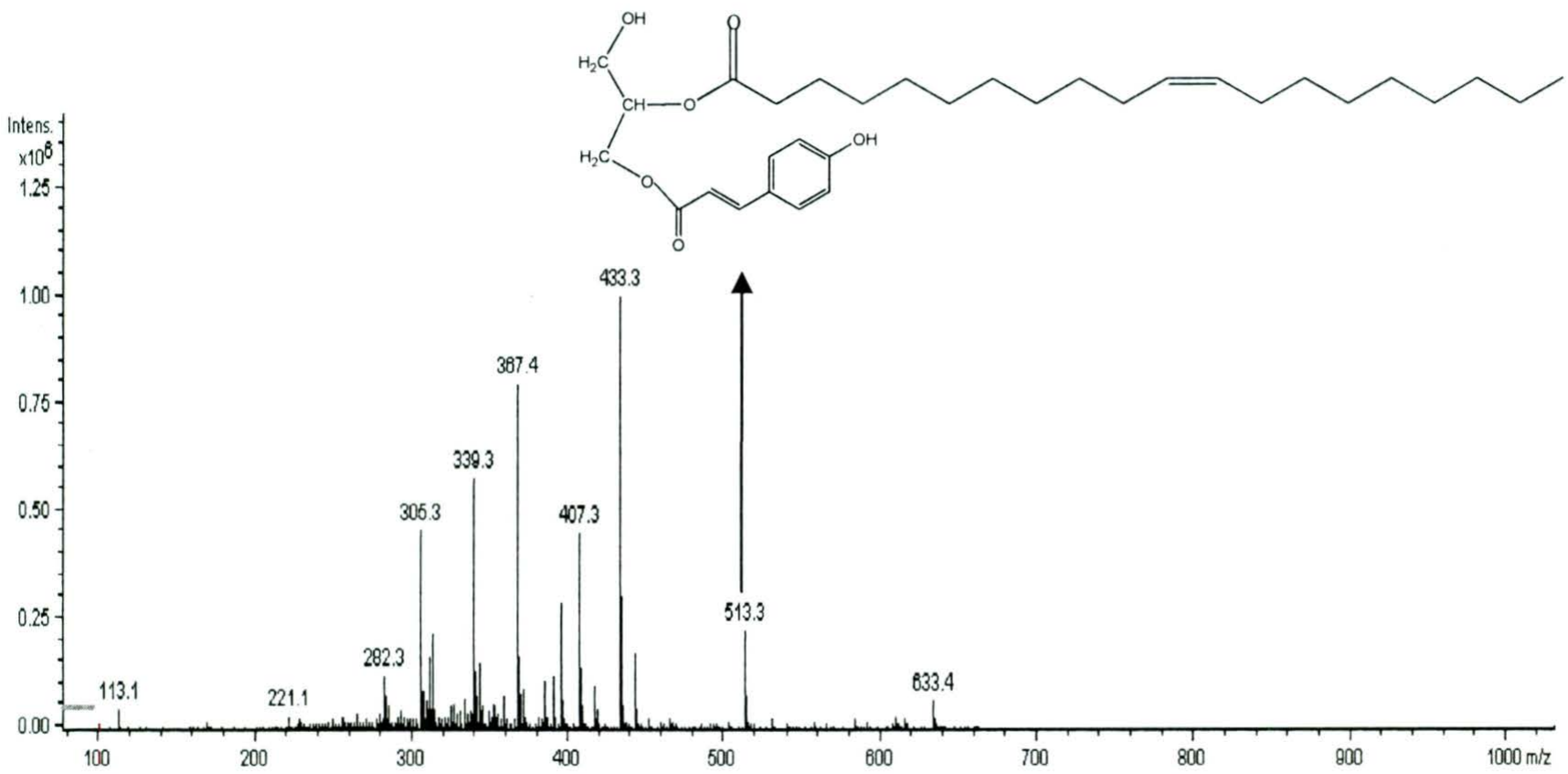
Further studies on synthesized phenolic lipids are needed in order to examine their antimicrobial potential and anti-inflammatory activity, among others. Meanwhile, bioactivities of these synthesized phenolic lipids *ex vivo* and *in vivo* using cell lines, animal models and eventually clinical studies need to be investigated. Furthermore, the activity of other enzymes on catalyzing the esterification of *p*-coumaric to phenolic lipids should be assessed in order to obtain alternatives to Novozyme 435, which may help achieve higher esterification rates of *p*-coumaric and to reduce the cost of enyzme in larger scale production of phenolic lipids. The effect of using higher mole ratio of *p*-coumaric to triacylglycerols on the variety of synthesized phenolic lipids may also need to be investigated as it may lead to the formation of variety of increased phenolic lipid species as well as higher conversion rate of triacylglycerols to their phenolic counterparts. In addition, the scale-up production of phenolic lipids derived from seal blubber and menhaden oils should also be studied.

Appendices

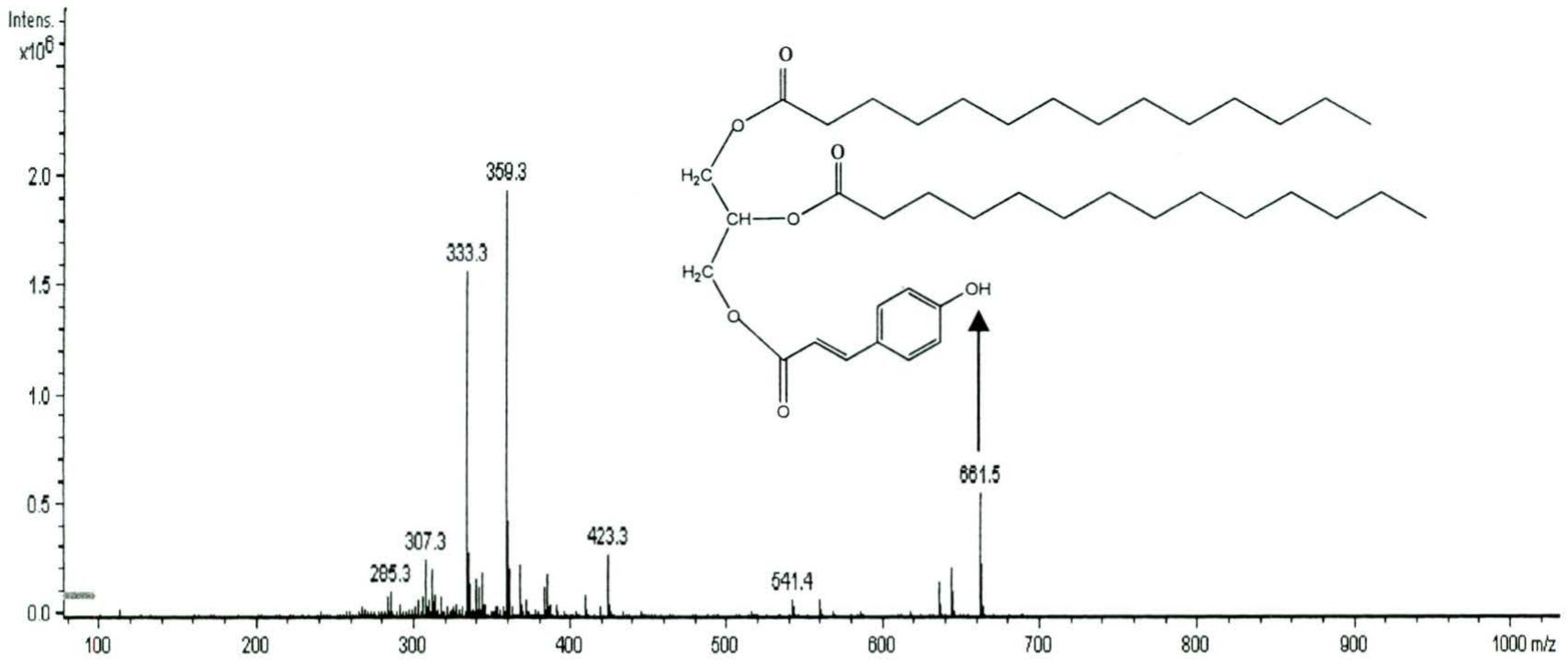
Appendix 7.1. Chemical structure and mass spectrometric specmetric data of monopalmitoleyl *p*-coumarate.



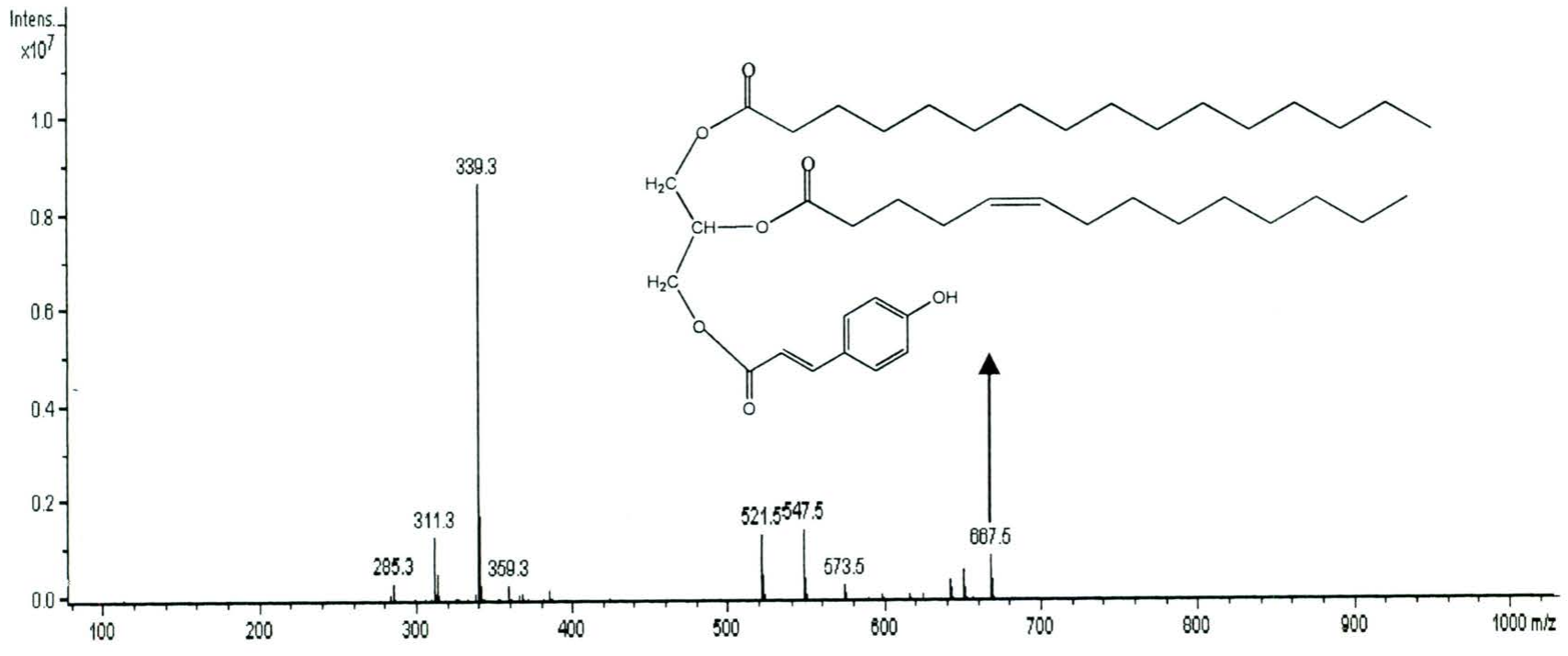
Appendix 7.2. Chemical structure and mass spectrometric data of monostearyl *p*-coumarate.



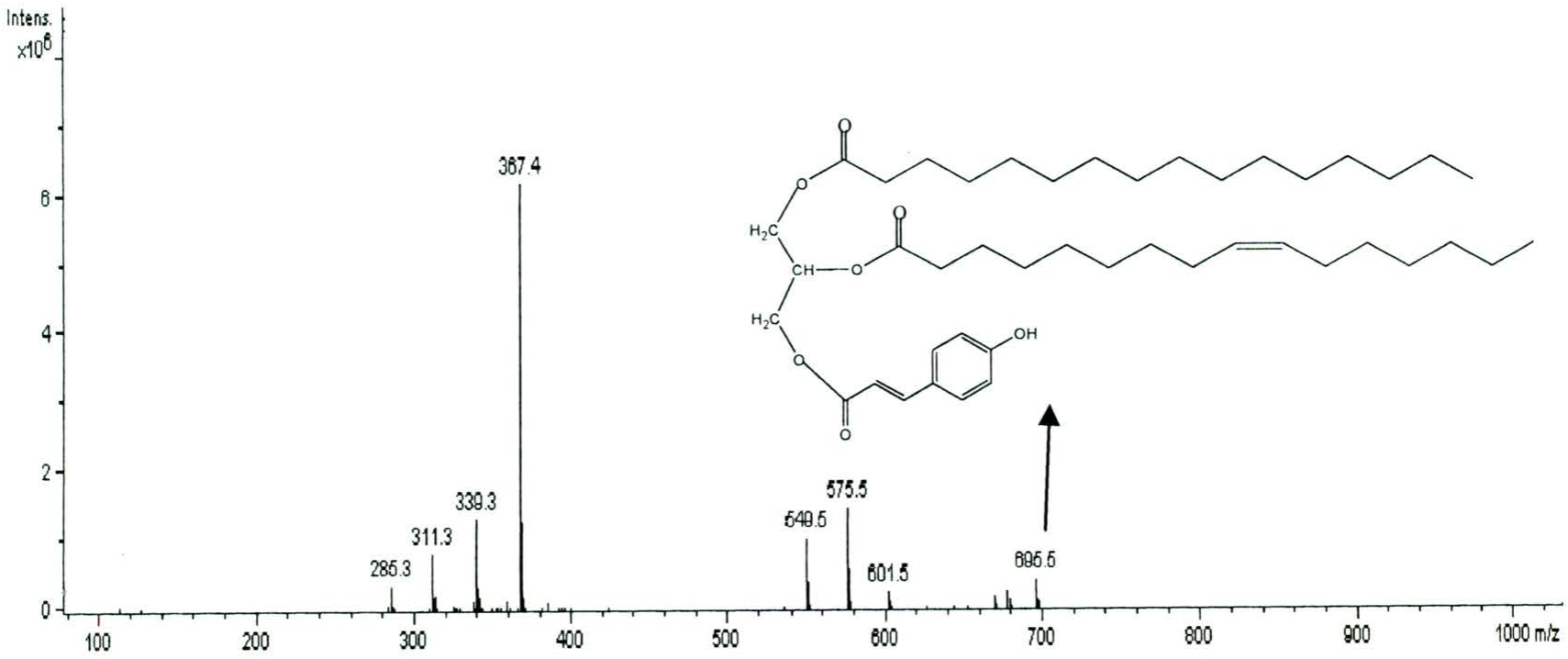
Appendix 7.3. Chemical structure and mass spectrometric data of monoeicosenyl *p*-coumarate.



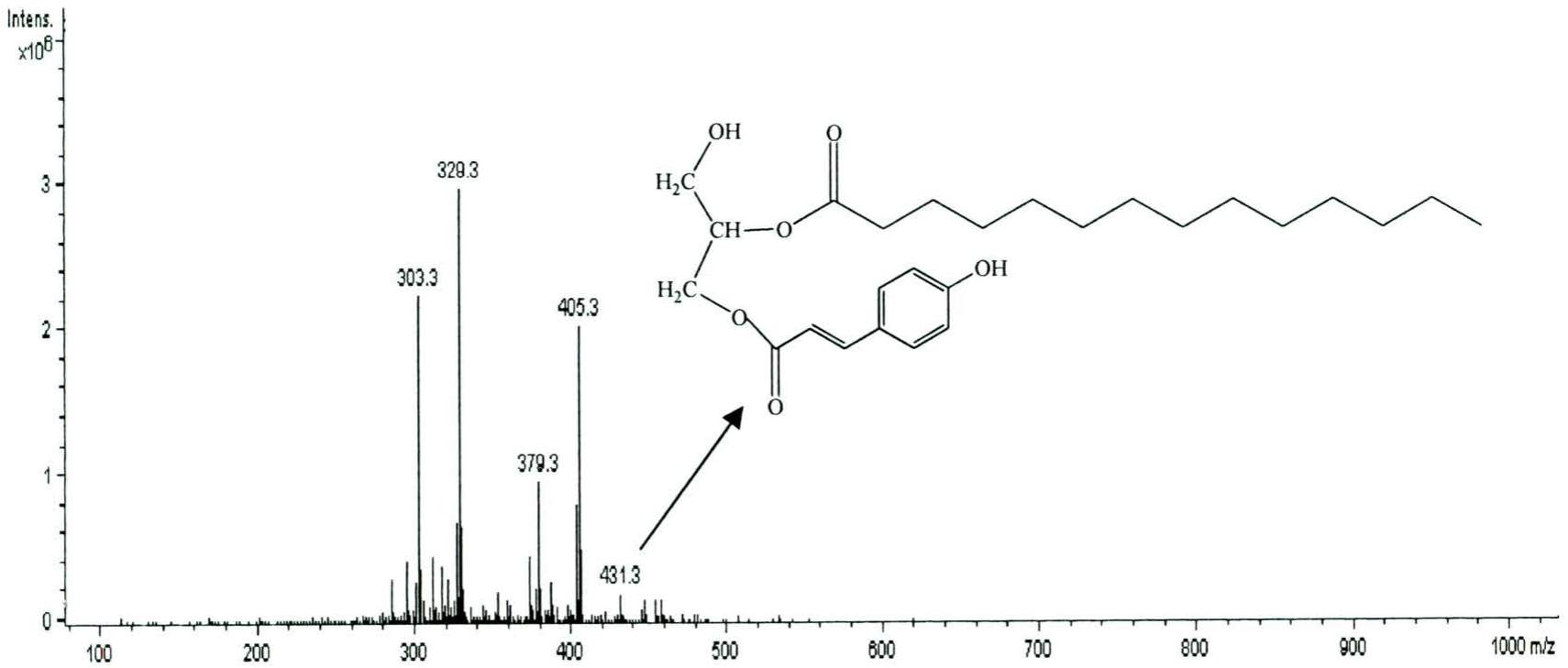
Appendix 7.4. Chemical structure and mass spectrometric data of dimyristyl *p*-coumarate.



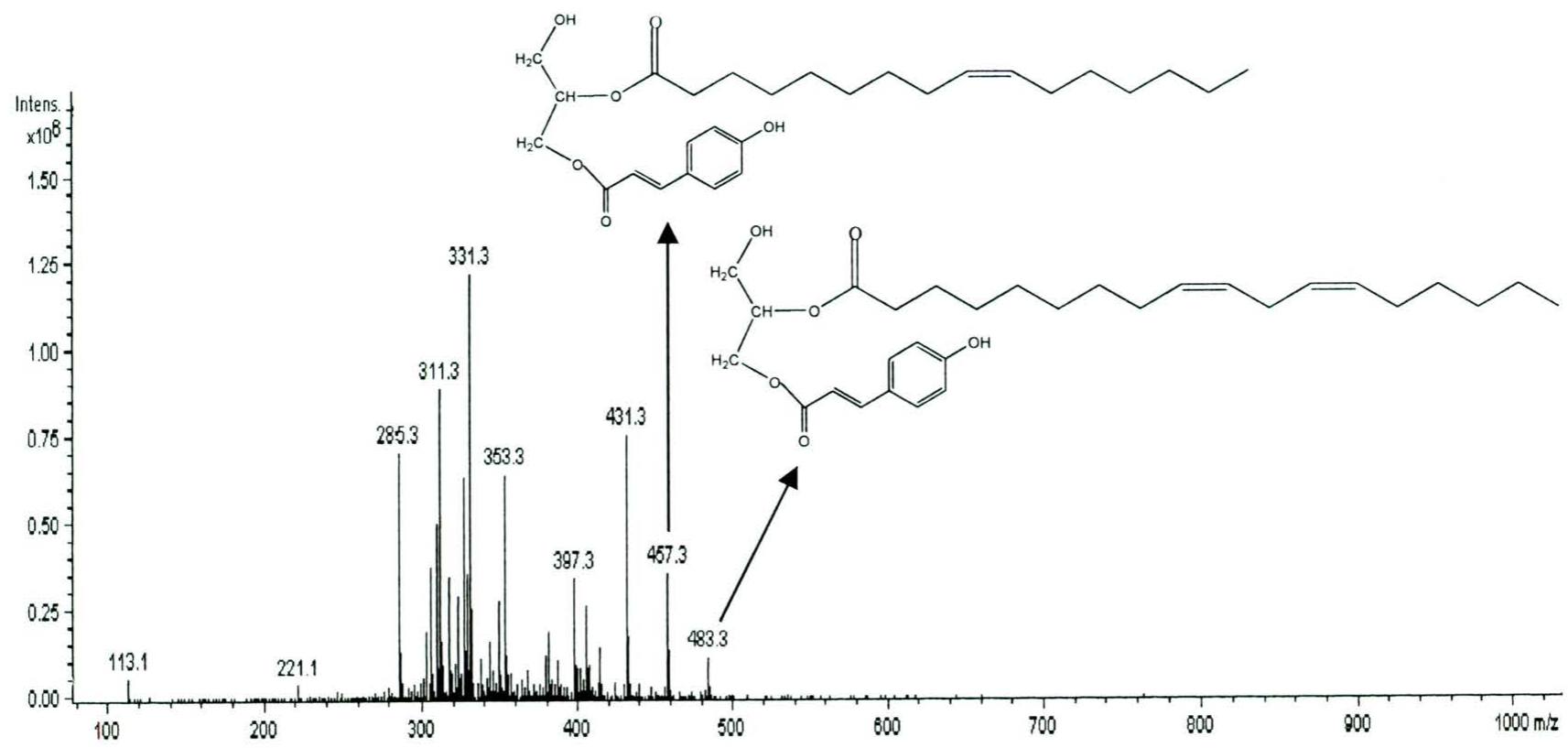
Appendix 7.5. Chemical structure and mass spectrometric data of myristeyl cetyl *p*-coumarate.



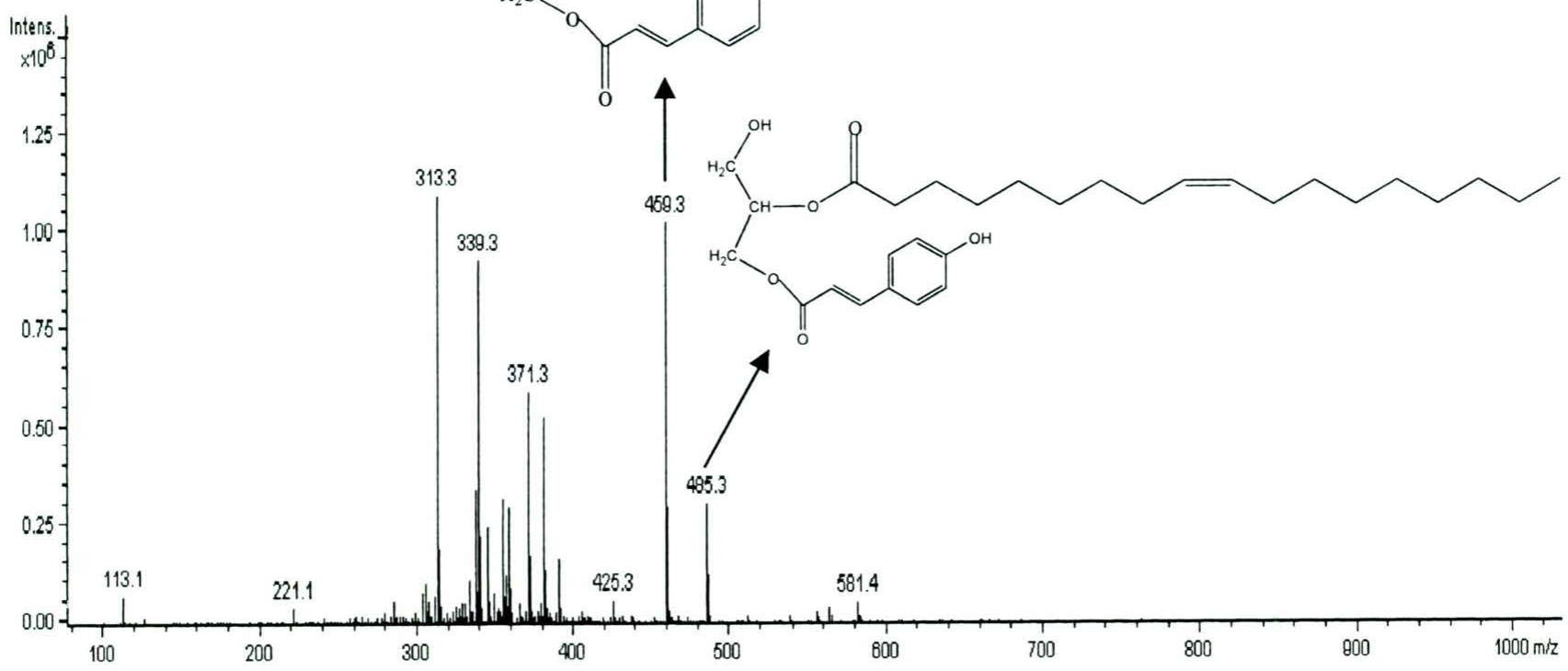
Appendix 7.6. Chemical structure and mass spectrometric data of cetyl palmitoleyl *p*-coumarate.



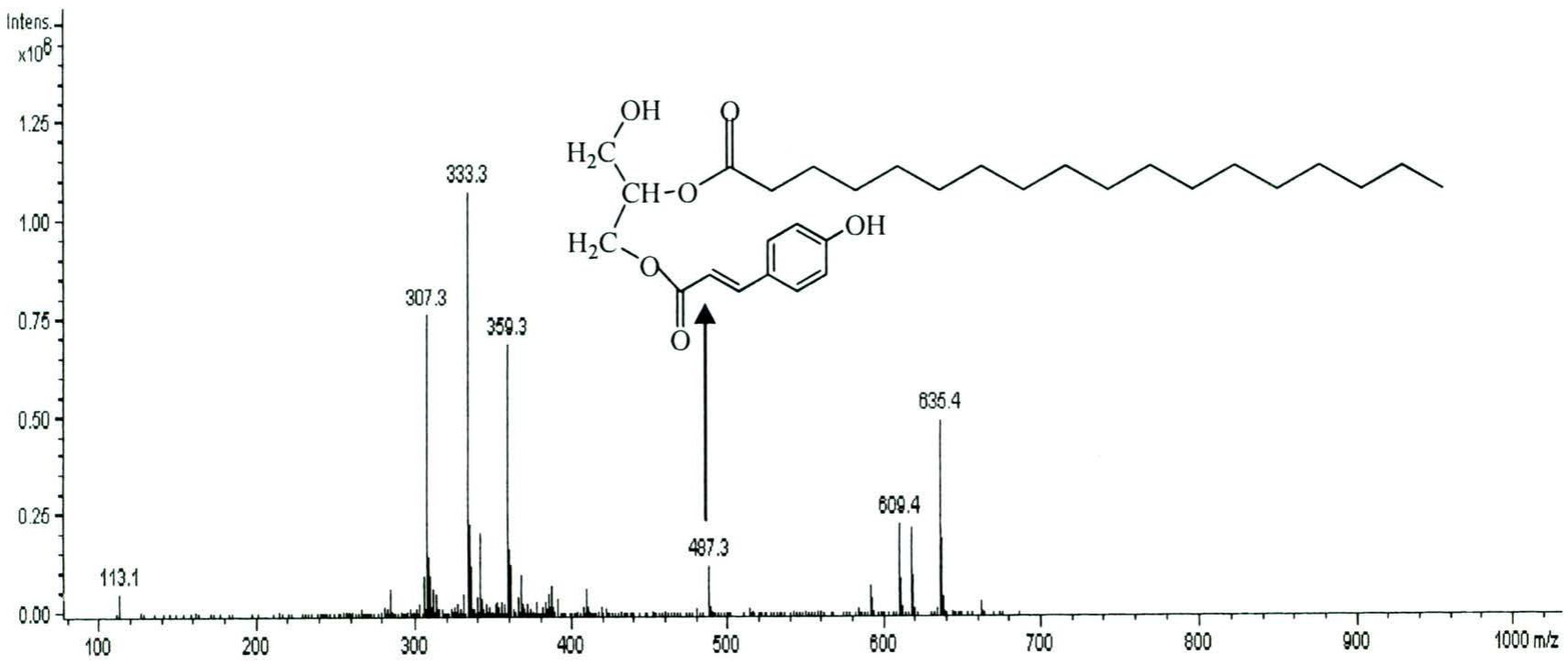
Appendix 7.7. Chemical structure and mass spectrometric data of monomyristyl *p*-coumarate.



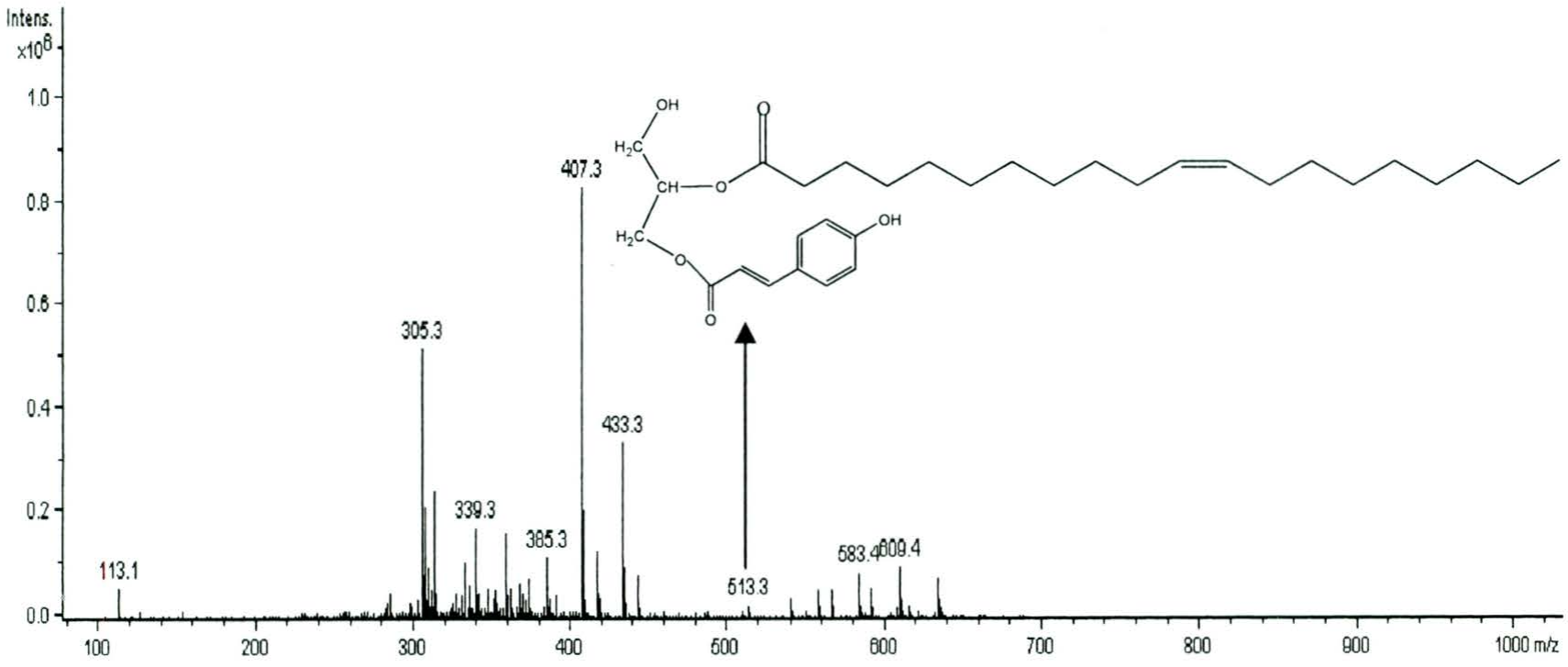
Appendix 7.8. Chemical structures and mass spectrometric data of monopalmitoleyl *p*-coumarate and monolinoleyl p-coumarate.



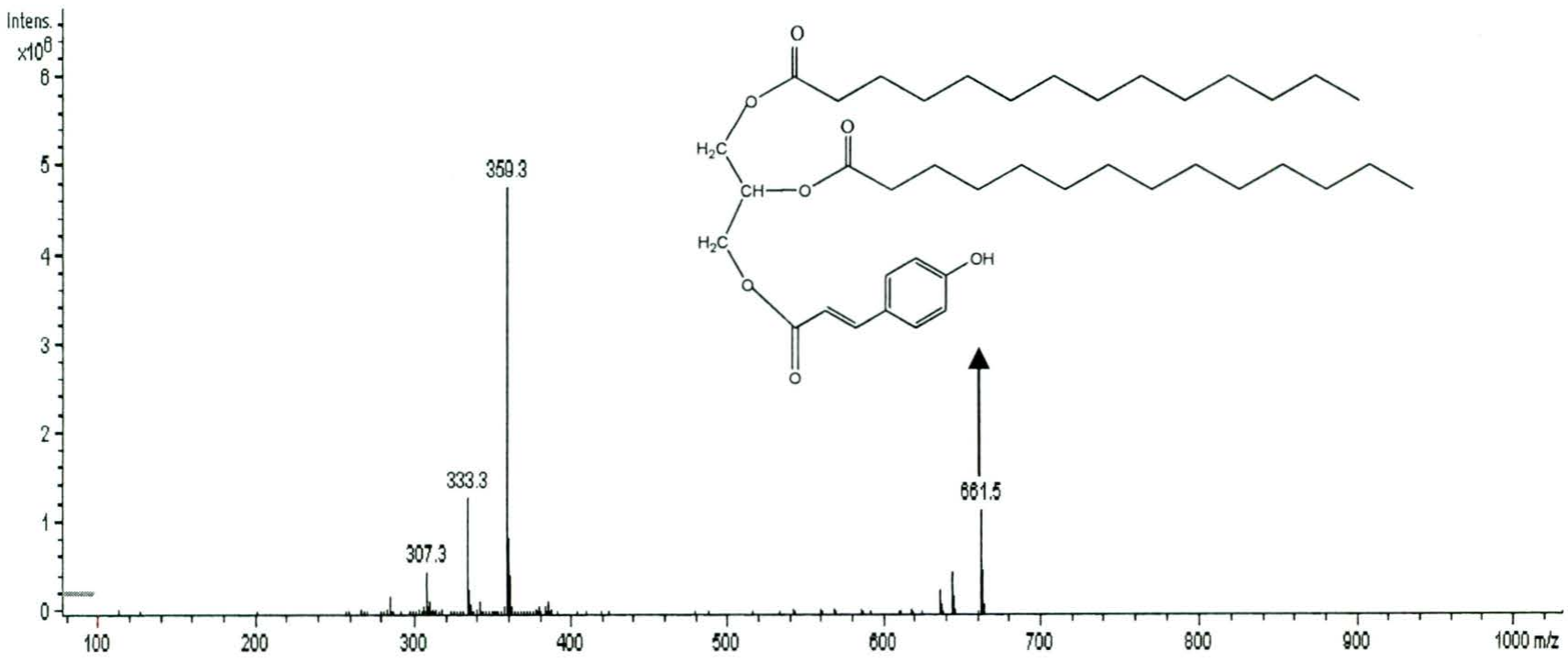
Appendix 7.9. Chemical structures and mass spectrometric data of monocetyl *p*-coumarate and monooleyl p-coumarate.



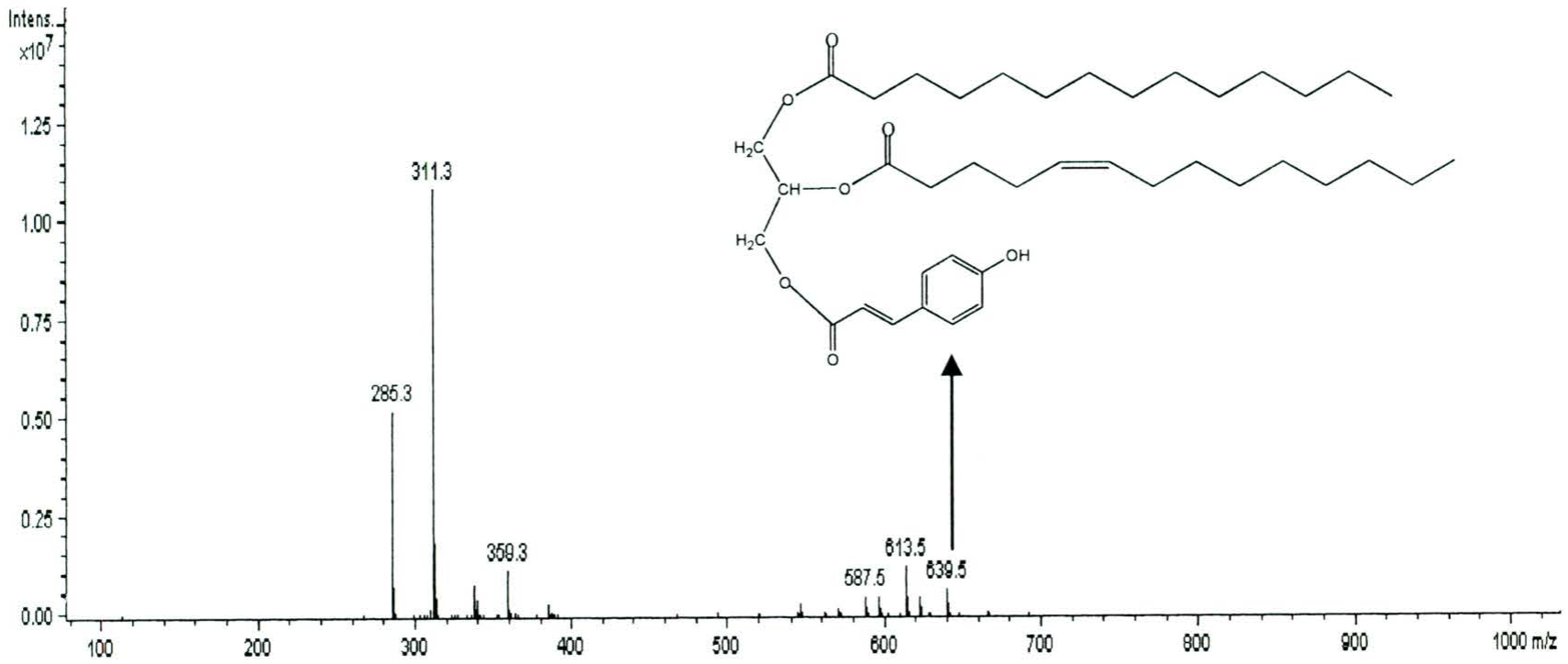
Appendix 7.10. Chemical structure and mass spectrometric data of monostearyl *p*-coumarate.



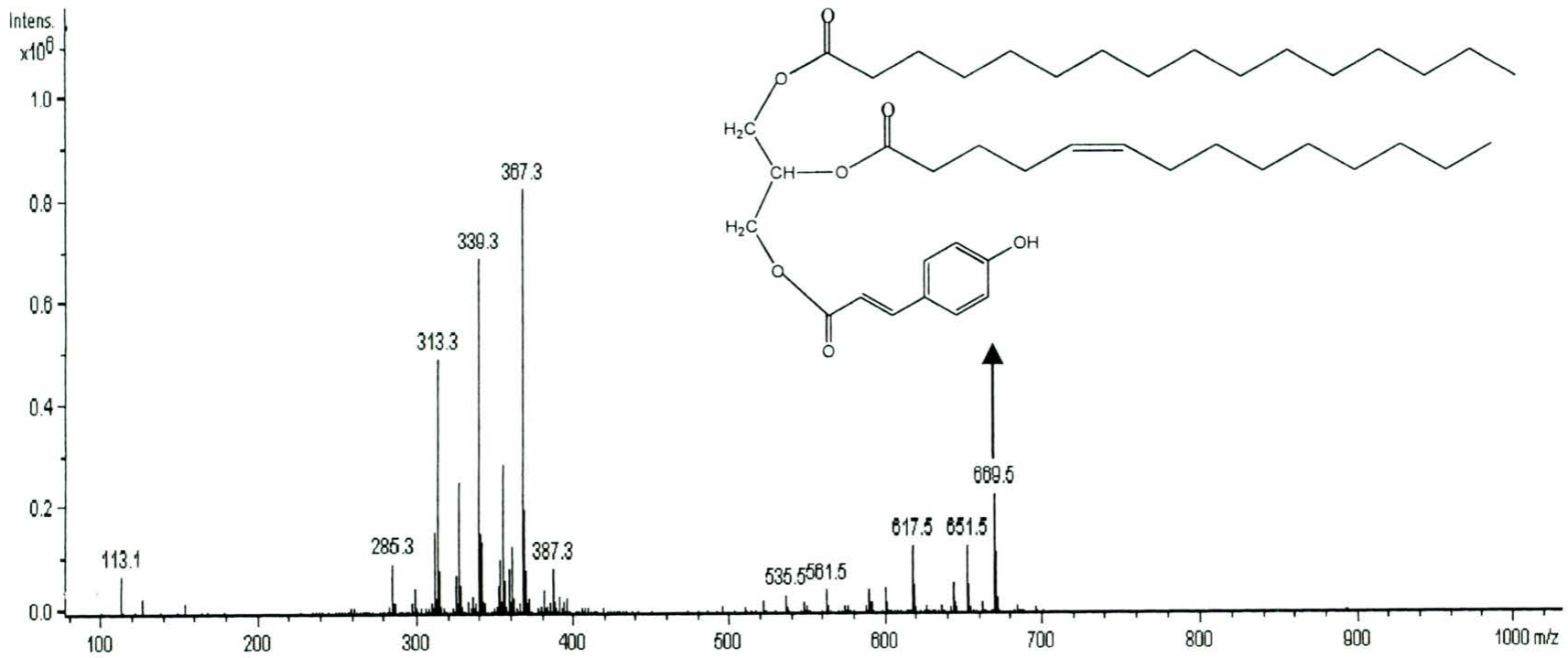
Appendix 7.11. Chemical structure and mass spectrometric data of monoeicosenyl *p*-coumarate.



Appendix 7.12. Chemical structure and mass spectrometric data of dimyristyl *p*-coumarate.



Appendix 7.13. Chemical structure and mass spectrometric data of myristyl myristeyl *p*-coumarate.



Appendix 7.14. Chemical structure and mass spectrometric data of myristyl cetyl *p*-coumarate