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**DEGREE**

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**TITLE:** Characterization of Refined Oil from Atlantic Salmon Belly as Affected by Degumming, and Enrichment of Refined Salmon Oil with Capric Acid by Enzymatic Acidolysis

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THESIS

# CHARACTERIZATION OF REFINED OIL FROM ATLANTIC SALMON BELLY AS AFFECTED BY DEGUMMING, AND ENRICHMENT OF REFINED SALMON OIL WITH CAPRIC ACID BY ENZYMATIC ACIDOLYSIS

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The salmon's belly constitutes about 7-9% of the whole body weight and could be used to provide higher valued products such as fish oil. Normally, the main raw material for fish oil production in Thailand comes from by-products of tuna processing. Therefore, salmon belly can serve as an interesting alternate material for fish oil production. Recently, structured lipids (SL) containing fish oil and medium chain fatty acids is considerably important for improving nutritional values of fats and oils. Thus, enhancement of refined salmon oil (RSO) with capric acid (CA) is an interesting avenue for SL production. The objectives of this study were 1) to produce fish oil from salmon belly and to examine the effect of degumming agents (water, phosphoric acid, and citric acid) on the physicochemical properties of the refined oil and to optimize the synthesis of SL from RSO containing CA by using response surface methodology and 2) to examine characteristics of the SL. This study was carried out to produce crude salmon oil from bellies and purified oil by 3 purification steps: degumming with 3 different agents (hot water, 85% phosphoric acid, and 0.3% citric acid), neutralization with alkali, and bleaching with activated carbon. The crude and purified oils were analyzed for yield, color value (CV), free fatty acid (FFA), peroxide value (PV), *p*-anisidine value (*p*-AV), iodine value (IV), saponification value (SV), unsaponifiable matter (USM), heavy metal (HM), and fatty acid composition (FAC). The SL was made by using immobilized lipase, lipozyme IM60 from *Rhiomucor miehei* to incorporate CA at sn-1, 3 positions of salmon oil. Independent variables including substrates molar ratio of RSO/CA (1:2-1:6), enzyme concentration (2-10 wt% of total substrates), incubation temperature (37-60°C) and incubation time (6-48 h) were investigated the optimizing reaction by central composite rotatable design. Responses of experimental data were percentage incorporation of CA, ratio of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA), ratio of monounsaturated fatty acid to polyunsaturated fatty acid (MUFA/PUFA), ratio of omega-3 fatty acid to omega-6 fatty acid (n-3/n-6), and ratio of linoleic acid to docosahexaenoic acid (LA/DHA). The RSO and SL were analyzed for acyl migration, CV, FFA, PV, *p*-AV, IV, and FAC. The result of salmon oil production showed that yield of crude salmon oil was 33.7% of raw material used. After refining process, yield of obtained refined oil was approximately 30% of initial crude salmon oil. The crude oil had red-orange color and changed to be transparent light yellow color after refining process. FFA, PV, *p*-AV, IV, SV, and USM were within acceptable ranges which indicated that the quality of refined oil was acceptable. The degummed oil using citric acid had reduced iron, copper, and phosphorus contents when compared to oil degummed with hot water and phosphoric acid, and the values were within the standard for edible fish oil. Besides, FAC of refined oil showed a higher percentage of MUFA and PUFA. The ratio of n-3/n-6 was about 1.58-2.17. For SL production, the results showed that the optimal conditions for synthesis of SL were at 48.5°C, 48 h with substrates molar ratio at 1:2.66, 1:3.29, and 1:3.51, and enzyme concentration at 8.16, 7.66, and 8.52 wt% of total substrates. The response values were found to have percentage incorporation of CA $\geq$ 10, SFA/PUFA $\geq$ 1.2, MUFA/PUFA$\geq$ 2.7, n -3/n-6 $\leq$ 1.1, and LA/DHA $\geq$ 2.3. The positional distribution of SL at sn-2 position containing long chain fatty acids especially oleic acid, LA, DHA, and eicosapentaenoic acid contents were 42.67-43.74, 13.28-13.93, 7.39-8.59, and 2.78-3.24 mol%, respectively and CA at sn-1, 3 positions was 48.75-56.80 mol%. The SL had transparent light orange yellow color. FFA content, PV and *p*-AV were acceptable and IV was lower than initial oil indicated that the SL was not oxidized and had oxidative stability. The SL produced in this study could be nutritionally useful and have potential uses either for direct consumption or for use as a valuable ingredient oil in various functional food formulations.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| *a** | = | greenness (–*a**)/redness (+*a**) |
| AA | = | arachidonic acid |
| ACAT | = | acyl-coenzyme A: cholesterol acyltransferase |
| AD | = | Alzheimer's disease |
| ADHA | = | Attention deficit hyperactivity disorder |
| ALA | = | alpha-linolenic acid |
| AOAC | = | Association of Official Analytical Chemists |
| AOCS | = | American Oil Chemists' Society |
| *b** | = | blueness (-*b**)/yellowness (+*b**) |
| CA | = | capric acid |
| Cd | = | cadmium |
| cg $I_2$ | = | centigram of iodide |
| CHD | = | Coronary heart disease |
| CLA | = | conjugated linoleic acid |
| cm | = | centimeter |
| Codex | = | Codex Alimentarius Commission |
| CPA | = | caprylic acid |
| Cu | = | copper |
| CV | = | color value |
| CVD | = | Coronary vascular disease |
| d | = | day |
| °C | = | degree Celsius |
| DAG | = | diacylglycerols |
| DHA | = | docosahexaenoic acid |
| DHASCO | = | docosahexaenoic acid single cell oil |
| DPA | = | docosapentaenoic acid |
| EFA | = | essential fatty acids |
| EPA | = | eicosapentaenoic acid |

## LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| ETA | = | eicosatetraenoic acid |
| FAC | = | fatty acid composition |
| FAME | = | fatty acid methylesters |
| FAO | = | Food and Agriculture Organization of the United Nations |
| Fe | = | iron |
| FFA | = | free fatty acid |
| GLA | = | gamma- linolenic acid |
| g | = | gram |
| GC | = | gas chromatography |
| h | = | hour |
| HCl | = | hydrochloric acid |
| HDL | = | High-density lipoprotein |
| Hg | = | mercury |
| HV | = | heavy metal |
| IAFMN | = | International Association of Fish Meal Manufacturers |
| IFOMA | = | International Fish Oil Manufacturing Association |
| ISO | = | International Organization for Standardization |
| IUPAC | = | International Union of Pure and Applied Chemistry |
| IV | = | iodide value |
| kg | = | kilogram |
| KOH | = | potassium hydroxide |
| *L** | = | darkness/whiteness |
| LA | = | linoleic acid |
| LCAT | = | lecithin: cholesterol acyltransferase |
| LCFA | = | long chain fatty acids |
| LCSFA | = | long chain saturated fatty acids |
| LDL | = | Low-density lipoprotein |
| Lipozyme RM-IM | = | lipozyme of immobilized lipase from *Rhizomucor miehei* |

# LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| m | = | meter |
| M | = | molar |
| MAG | = | monoacylglycerols |
| MCFA | = | mediun chain fatty acids |
| MCT | = | mediun chain triacylglycerols |
| meq | = | milliequivalent |
| µl | = | microliter |
| µm | = | micrometer |
| mg | = | milligram |
| ml | = | milliliter |
| mm | = | millimeter |
| min | = | minute |
| MUFA | = | monounsaturated fatty acids |
| N | = | normality |
| NaOH | = | sodium hydroxide |
| $Na_2S_2O_3$ | = | sodium thiosulfate |
| Ni | = | nickel |
| $N_2$ gas | = | nitrogen gas |
| OA | = | oleic acid |
| *n*-3 FA | = | omega-3 fatty acids |
| *n*-6 FA | = | omega-6 fatty acids |
| P | = | phosphorus |
| *p*-AV | = | para-anisidine value |
| % w/w | = | percentage weight by weight |
| % w/v | = | percentage weight by volume |
| PGE3 | = | prostaglandin E3 |
| PKU | = | Phenylketonuria |
| PMA | = | plamitic acid |

## LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| PSL | = | purifined structured lipids |
| PUFA | = | polyunsaturated fatty acids |
| PV | = | peroxide value |
| rpm | = | rotation per minute |
| RSM | = | response surface methodology |
| RSO | = | refined salmon oil |
| S | = | sulphur |
| SCFA | = | short chain fatty acids |
| SDA | = | stearidonic acid |
| Se | = | selenium |
| SFA | = | saturated fatty acids |
| SL | = | structured lipids |
| STA | = | stearic acid |
| STAG | = | structured triacylglycerols |
| SV | = | saponification value |
| TAG | = | triacylglycerols or triglycerides |
| TLC | = | thin layer chromatography |
| USD | = | United States Dollar |
| U.S. FDA | = | United States Food and Drug Administration |
| USM | = | unsaponifiable matter or non-saponifiable material |
| VLDL | = | very low-density lipoprotein |
| v/v | = | volume per volume |
| v/v/v | = | volume per volume per volume |
| wt% | = | weight percent |
| WHO | = | World Health Organization |

# CHARACTERIZATION OF REFINED OIL FROM ATLANTIC SALMON BELLY AS AFFECTED BY DEGUMMING, AND ENRICHMENT OF REFINED SALMON OIL WITH CAPRIC ACID BY ENZYMATIC ACIDOLYSIS

## INTRODUCTION

Commercial process of the frozen salmon generally requires the removal of viscera, heads, tails, frame, and belly, which accounts for approximately 40% of the wet weight of salmon process (Cooper *et al.*, 2014). Belly itself constitutes about 7-9% and could be used to provide higher valued product such as fish oil. Normally, the main raw materials for fish oil production come from by-products of tuna processing but not from salmon processing. Therefore, salmon belly can serve as an interesting alternate material for fish oil production. Recently, fish oil is used in nutraceutical or dietary supplement in the form of capsule for supporting healthiness and preventing some diseases due to its high level of eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3). These fatty acids are long chain n-3 polyunsaturated fatty acids and cannot be synthesized in the human body. They must be obtained through diet or supplementation (Zatsick and Mayket, 2007). These fatty acids have beneficial on the prevention or treatment of several human diseases such as neurological disorders (Freemantle *et al.*, 2006), Alzhemer disease (Eslick *et al.*, 2009), coronary arthritis disease and stroke, cardiovascular heart disease, immunological and inflammatory diseases, asthma, breast and colorectal cancers, hypertension, hyperlipidemia (Zhang *et al.*, 2010), diabetes and obesity (Horrocks and Young, 1999).

For fish oil production, the refining process is considerably important to obtain high quality of the oil. The refined fish oils must have light yellow color, natural-flavor or free from rancid odor and taste, and oxidative stability. Generally, the refining process is composed of degumming, neutralization, and bleaching, which are operated to remove undesirable components of crude oils. Degumming, it is a process

to remove phosphatides that consist of two types; hydratable phosphatides (including phosphatidylcholine and phosphatidylinositol) and non-hydratable phosphatides (including calcium and magnesium salts of phosphatidylethanolamine, and phosphatidic acid) (Johnson, 2002). This process usually uses water and amount of water should be balanced with hydratable phosphatide content of the oil to cause much soluble hydratable phosphatides in sludge or gums. However, using water cannot remove non-hydratable phosphatide, and it is still dissolved in the oil. Therefore, this study aimed to increase efficiency of degumming process by using phosphoric acid and citric acid compared to hot water. There were some reports on using degumming agents for degumming step in the oils, but few were done on salmon oil. Sathivel *et al.* (2003) reported that using 0.3% citric acid solution in degumming process of catfish oil from viscera and menhaden oil could separate the oil and sludge or gums off. This gums included both hydratable and non-hydratable phosphatides and proteinaceous compound. The other study of Crexi *et al.* (2010) indicated that using phosphoric acid (85% v/v) in degumming step of croaker oil resulted in high soluble non-hydratable phosphatide. Degumming can be conducted either as a separate operation or simultaneously with neutralization. Neutralization process, it is a step to remove most free fatty acids by reacting with sodium hydroxide to become sodium soaps. The amount and strength of sodium hydroxide used depend on the amount of free fatty acids present in the oil due to excessive sodium hydroxide can hydrolyze triglycerides and reduce the yield of neutralized oil. Bleaching process, it is mainly used to improve the color quality of the oil by eliminating pigment present in the initial material. It also contributes to the breakdown of peroxide and removal of residual trace of soap.

However, person with fat malabsorption and other metabolism problems particularly elderly people are not taken completely benefits from fish oil consumption due to their efficiency of digestion, absorption, and metabolism are declined especially essential long chain fatty acids. Moreover, chain length of hydrocarbon, degree of unsaturation and the position in triacylglycerols also affect for digestion, absorption, and metabolism fatty acids in the body. Impaired absorption long chain fatty acids could be occur because they are oxidized very slowly and their

melting points are above body temperature, which does not allow adequate incorporation in the liquid phase. This could also be due to the tendency of fatty acids to form hydrated acid calcium soaps, which are insoluble in aqueous media at the pH of the intestine (Carvajal *et al.*, 2000). On the other hand, medium chain fatty acids (MUFA) are more rapidly digested and easily absorbed than long chain fatty acids by pass directly into portal vein and readily oxidize in the liver to serve as an energy source (Akoh, 2006). Thus, MUFA are often used to synthesis structured lipids especially at sn-1 and sn-3 positions and long chain fatty acids at sn-2 position of glycerol backbone from beneficial effects both as an energy source and as source of essential fatty acids.

The aims of this study were 1) to produce fish oil from salmon belly and to examine the effect of degumming agents (water, phosphoric acid, and citric acid) on the physicochemical properties of the refined oils, and 2) to optimize the reaction condition for production of structured lipids (SL) from refined salmon oil containing capric acid (CA) by using response surface methodology involved with the influence of molar ratio substrates, enzyme concentration, incubation time, and incubation temperature of catalyzed enzymatic reaction on the percentage incorporation of CA, ratios of saturated fatty acid to polyunsaturated fatty acid, monounsaturated fatty acid to polyunsaturated fatty acid, omega-3 fatty acids to omega-6 fatty acids, and linoleic acid to docosahexaenoic acid in SL and characteristics of SL. The SL produced in this study may be nutritionally useful and have potential uses either for direct consumption or for use as valuable ingredient oil in various functional food formulations.

# OBJECTIVES

1. To produce fish oil from salmon belly and to examine the effect of degumming agents (water, phosphoric acid, and citric acid) on the physicochemical properties of the refined oil

2. To optimize the synthesis of structured lipids from refined salmon oil containing capric acid by using response surface methodology and to examine characteristics of the structured lipids

3. To study the production of the structured lipids at the optimal condition in gram scale and the properties of the structured oil

# LITERATURE REVIEW

## 1. Salmon

Thailand is the top-three of the world exporter of fish and fishery products during 2007 – 2012 that total Thai export of fish and fishery product grew from USD 5.71 billion in 2007 to USD 8.08 billion in 2012 (FAO, 2014a). Salmon is one of Thai's fishery commodities to export for international trade, especially chilled and frozen salmon fillets. The export value of salmon products also increase 73.36% from USD 95.60 million (17,874 tons) in 2007 to USD 130.31 million (27,512 tons) in 2011 (FAO, 2014b).

Atlantic salmon (*Salmo salar*) is an important raw material to be produced for salmon products. It is an anadromous species in *Salmo* genus of Salmonidae family as well as brown trout (*Salmo trutta*). The natural distribution of the genus is mainly European especially northern Atlantic Ocean around North Atlantic Islands. Only the Atlantic salmon extends to North America. The salmon and trout of the Pacific basin belong to another genus, *Oncorhynchus* (Georgiev, 2011). The Atlantic salmon is fairly large salmonid. Record-sized specimens have been measured to a maximum of 153 cm of long and 45 kg of weight. The color of adult Atlantic salmon has a silver-blue sheen and black spots predominantly above the lateral line though the caudal fin (Figure 1). Juvenile Atlantic salmon spend 1- 8 years in deep-sea feeding grounds. At the onset of maturation, fish cease feeding, and return to their rivers of origin to spawn (October-January). The large numbers of fish die during spawning, although some fish may return to sea (FAO, 2010). The Atlantic salmon included belly approximately 7.2-8.9% of whole body (Figure 1), which consisted of 23.9-28.1% of lipid content, 13.5-15.2% of protein content, 0.32-0.54% of ash, and 50.2-65.7% of moisture content (Worawattanamateekul *et al.*, 2012). The major fatty acid found in wild and farmed Atlantic salmon fillet including plamitic acid (PMA, C16:0), stearic acid (STA, C18:0), oleic acid (OA, C18:1 *n*-9), linoleic acid (LA, C18:2 *n*-6), alpha–linolenic (ALA, C18:3 *n*-3), stearidonic acid (SDA, C18:4 *n*-3), eicosatetraenoic acid

(ETA, C20:4 *n*-3), eicosapentaenoic acid (EPA, C20:5 *n*-3), docosapentaenoic acid (DPA, C22:5 *n*-3), and docosahexaenoic acid (DHA, C22:6 *n*-3) about 62-68% of total fatty acids (Strobel *et al.*, 2010).

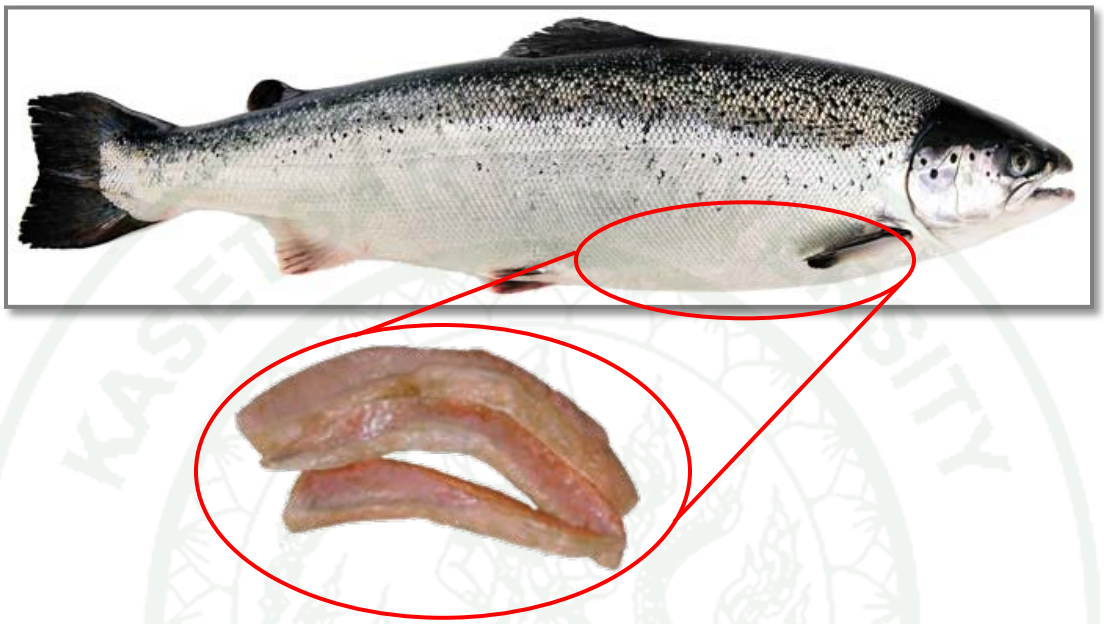


**Figure 1** Characteristics of Atlantic salmon and belly part

**Source:** adapted from Lerøy Seafood Group ASA (2014)

## 2. Production of fish oil

There is a sizable and growing market demand for high quality fish oils, and industrial fish oil production can considerably benefit if appropriate raw materials are available. The fish oil production should be carefully considered to handle by-products from cutting and other fish processing operation due to properties of raw material definitely affect the oil quality.

## 2.1 Production of crude fish oil

### 2.1.1 Raw material

According to Food and Agriculture Organization of the United Nations (FAO, 1986), raw material used for production of fish oil production classified into three categories: 1) fish catches for single purpose of fishmeal and fish oil production such as menhaden, anchovy, capelin and sardine, 2) incident by-catches from another fishery, and 3) fish offal or waste from the edible fisheries such as cutting from fillet operations, fish cannery waste, and frozen processing waste. The Atlantic salmon belly has become a considerable source of material for oil production in this study.

The raw material must be chilled and kept following the legislation of fish oil production unless whole fishery products are used directly for preparing fish oil that is processed within 36 h, where the freshness criteria of total volatile basic nitrogen values are not exceed acceptable ranges (Collins *et al.*, 2010).

### 2.1.2 Cooking

The objective of cooking process is to liberate the oil from fat depots and to coagulate the protein of the fish. The solid and liquid phases can be mechanically separated. Fat cells are also rupture, releasing the oil into the liquid phase. There are two types of cookers in commercial use as indirect and direct steam. Indirect steam cooker rely on surrounding jacket and heated rotary screw conveyer to transfer heat indirectly while direct steam cooker can directly transfer heat from stem pass into raw material, which water is condensed in the mass during the process and has to remove by the pressing and then evaporated from the press liquor (Stansby, 1990). In lab scale, raw material can be cooked by steaming or bake. However, the most common practice cooking, raw material is stream heated to 95-100°C for 15-20 min (FAO, 1986). Most manufacturers

operate cookers to rapid heating of the mass to temperature about 95°C, which similar to report of Sathivel *et al.* (2008a) who indicated that production of unrefined Pollock oil derived from viscera, heads, skins, and frame and other discarded fish from processing plant were ground and cooked at 95°C about 20 min. Also the study of Crexi *et al.* (2010) who denoted that production of crap viscera oil, raw viscera was ground and cooked at 95-100°C for 30 min.

### 2.1.3 Pressing

The purpose of the pressing process is to squeeze out as much liquid as possible from the solid phase, to improve the oil yield of fish oil, and to reduce the moisture content of the press cake. The liquid phase (containing water emulsion, dry matter (both in dissolved and suspended forms, vitamin and minerals), and crude oil about 60-70% of total raw material) are separated from the solid phase (including pressed cake, which contains the residue about 30-40%, is normally further processed to fishmeal) (Collins *et al.*, 2010). The modern presses are very efficient dewatering devices, press cakes with low moisture content. The pressed cake is then dried to produce fish meal rich in protein by dryer while the pressed liquor is further refined to be fish oil rich in polyunsaturated fatty acids especially essential fatty acids such as eicosapentae-noic acid and docosahexaenoic acid. The general pressing appliance for industrial production is hydraulic press and screw press.

### 2.1.4 Separation of press liquor

The separation of three fractions of the pressed liquor containing stickwater, soluble and suspended proteins (sludge), and crude fish oil, is based on their different specific gravity that is settled out in three layers; sludge at the bottom, stickwater in between and crude oil at the top. An important prerequisite for efficient separation is high temperature. The press liquor is reheated to 90-95°C before entering the centrifuge. Then the sludge is removed from the oil by using a decanter and passed through a drying or

dewatering. The separation of stickwater from oil takes place in centrifuge, being the water phase discarded and the oil sent to next step for polishing (FAO, 1986).

### 2.1.5 Oil polishing

Polisher or purifier is used to remove any final traces of moisture and impurities from the oil by stripping steam or hot water and re-centrifuging to extent shelf life of crude fish oil. In practice, the oil phase recovered from the separator is washed and separated into two phases as water and crude fish oil. The water phase including soluble protein, suspended protein, minerals, vitamin, amines/ammonia, and residual oil is mixed with the stickwater and evaporated to concentrate the solid content to about 50%. This concentrate is called fish soluble containing 64-65% of protein content and less than 8% of moisture content. The content of residual oil depends upon the efficiency of separating process, certainly below 1%. The Crude fish oil is tested and can be sold for variety of uses or it can be further refined to be fish oil for human consumption (Stansby, 1990).

## 2.2 Purification of crude fish oil

The crude fish oil is usually further refined in the refining steps. The purpose of refined crude oil is to remove the impurities matter, which include small amount of protein or other solid, phosphatides, undesirable natural flavors and odors, free fatty acids, pigments, waxes, trace solvent residue, and water that cause the original oil to have an unattractive color or taste or that causes harmful metabolic effect (Sikorski, 2002). The refining is an important to refine fish oils, which it contributes to reserve high amount of essential fatty acids especially DHA and EPA.

### 2.2.1 Degumming

Degumming is washing process to remove phosphatides. It may be conducted either as a separate operation or simultaneously with neutralization. There are two types of phosphotides; hydratable and non-hydratable. The hydratable phosphatides are composed of phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidic acid, which are hydrated gum. The first two are always hydratable but the latter two can complex with divalent metal ions (copper, iron, and trace element), rendering them nonhydratable. The non-hydratable phophatides include calcium and magnesium salts of phosphatidylethanolamine and phosphatidic acid, which is caused dark colors and dangerous foaming when the oil is heated. Generally, 1-3% of water is added to cause much soluble hydratable phosphotides and precipitate off, whereas non-hydratable phophotide still remain in the oil. The amount of water should be equivalent to the hydratable phosphatides content of the oil. However, acid can be used as degumming agent. Acid degumming makes more of the phosphatides hydratable. For example, degumming is carried out by heating fish oil to 80°C and adding small amount of phosphoric acid 1.0% (Sathivel*et al*., 2003) or citric acid 0.3% (Crexi *et al*., 2009a), that carboxyl groups of phosphoric and citric acid are thought to be responsible for binding with metals ion and forming complexes (Reische *et al*., 2002) before precipitating (containing phospholipids both hydratable and non-hydratable and any proteinaceous mater) hydrated gum off the oil. This hydrated gum can be dissolved with water during washing and further separated the hot oil by centrifugation.

### 2.2.2 Neutralization

Neutralization is a step to remove most free fatty acid, pigment, residue phospholipids, insoluble matter, water soluble matter, and trace metal by adding sodium hydroxide or aqueous phase to separate oil phase from the difference in specific gravity. In this step, the free fatty acids are combined with alkali to form sodium soap or soapstock and phosphatides and undesirable materials are absorbed alkali to coagulate through hydration. In practice, addition sodium hydroxide leads to

lose the neutral oil and is an important criterion in assessing the efficiency of refining operation (Rossell, 2009). The neutral oil and soapstock are separated by centrifuge and the residual soapstock is washed with water in related with mass of initial oil following further centrifugal separation (Crexi *et al*., 2010).

2.2.3 Bleaching

After neutralization, the oil is usually bleached. Although the main objective of bleaching is to reduce colored matter and natural pigments, partially suspended colloid matter are also removed by absorption with bleaching materials as activated carbon, activated clay, natural clay (Stansby, 1990). For example, bleaching is carried out by heating at 70°C with addition 5% of adsorbents (mixture of activated earth and activated coal at 9:1 ratio) in contact time 20 min, filtration by Bucher funnel with pre-layer of diatomaceous earth, and evaporation by rotary evaporator (Crexi *et al*., 2010).

2.2.4 Winterization

Winterization is a step to remove crystallizes of solid fats which are relatively saturated fatty acids in stearines and solid wax forms. The winterized oil had a higher iodine value and may be made to tolerate various cold tests. Crexi *et al*. (2010) reported that crab viscera oil which was accomplished through winterization 3 steps. First, the nucleation process occurred in a refrigerated bath with a water and alcohol mixture, from 30°C to 5°C with refrigeration rate of 0.62°C/min and agitation. Second and third steps, the crystallization processes were carried without agitation, from 5°C to -4°C with refrigeration rate of 2.7°C/h and from -4°C to -5°C with refrigeration rate of 0.25°C/h, respectively. Crystallization must be driven under slow refrigeration in order to supply large crystals and stable polymorphic forms. Small crystals formed from fast refrigeration cannot be easily filtered.

2.2.5 Deodorization

Deodorization is the last major step in refining of edible oil. It has the responsibility for removing both the undesirable volatile odor and flavor components as free fatty acid monoglyceride, diglycerides, aldehyde, and ketone, which may be produced by previous process such as alkali refining, bleaching or even storage condition (Stansby, 1990). For example, deodorization process, oil is loaded in vessel under vacuum with one opening connected to condenser in order to removing the volatile from the system under steam distillation with vacuum (Crexi *et al.*, 2010).

**3. Measurement of fish oil quality**

Several methods are used to determine the quality of fish oil. These can be classified into chemical and physical methods due to they are rapid, quantitative, and reproducible. Physical methods involve the measurement of melting point, flash point, boiling point, viscosity, caloric value, heat of fusion, and specific heat. While chemical methods rely on the measurement of oxidative rancidity of fish oil as peroxide value during the early stages or *p*-anisidine value during later stages. The measurement of fish oil quality are often divided into two groups, the first being applied after preparation of crude oil to further refine by checking the fundamental parameters following the guideline value for crude fish oil as described in Table 1 and the second examination is to determine the refined oils by checking concentration of the components following guideline for refined fish oil as described in Table 2 and 3.

3.1 Color

Color of fish oil is used to estimate the oil quality. The high quality of fish oil must have light yellow to yellow colors from removing pigments and other impurities in the oil during neutralization and bleaching. In general, crude salmon oil has red-orange color from carotenoid pigment that is a unique

property in Salmonidae family, but it is changed to be light yellow color after refining. Huang and Sathivel (2010) and García-Moreno *et al.* (2013) indicated that crude oil derived from fish offal or by-product from fillet operation, frozen processing, and canned processing had dark brown – bronzed ochre colors and changed to be light yellow color after neutralization and bleaching.

### 3.2 Free fatty acid

Free fatty acid (FFA) is measure the amount of FFA existing in the oil sample, which indicates the susceptible degree to lipid oxidation. Generally, FFA in the oil can be detected and quantified using analysis methods such as ISO 660 (ISO, 1996), AOCS Official method Ca 5a-40 (AOCS, 1992), and IUPAC 2.201 (IUPAC, 1987). The oil with high FFA content tends to increase off-flavors, but it can be reduced or removed during refining step (Wrolstad *et al*., 2005). FFA of crude fish oil should be in the range of 2-5 % as oleic acid (Table 1). After refining, the final oil should be less than 0.1% as oleic acid following the guideline of International Association of Fish Meal Manufacturers (IAFMM) while guideline of Codex standard expressed as acid value (0.6 -3.0mg KOH/g) (Table 2).

### 3.3 Peroxide value

Peroxide value (PV) is the primary measure of rancidity in oil, which indicate amount of hydroperoxide in the oil sample. Oxidation of the oil gives rise to rancid odor and flavor by atmospheric oxygen into fatty acid molecules to form hydroperoxides. PV in the oil can be detected and quantified using analysis methods such as ISO 3961 (ISO, 1996), AOCS Official method Cd 8b-90 (AOCS, 1992), and IUPAC 2.501 (IUPAC, 1987). PV of crude fish oil should be in the range of 3-20 meq/kg (Table 1). After refining, the oil should be less than 0.1 meq/kg according to IAFMM and 5-10 meq/kg following Codex standard (Table 2).

3.4 *p*-Anisidine value

*p*-anisidine value (*p*-AV) is a measure of the level of secondary oxidation products in form of aldehydes (principally 2-alkenals and 2,4-dienals), which contribute to off-flavor formation as a result of oxidation. *p*-AV in oil sample can be detected and quantified using analysis methods including AOCS Official methods Cd 18-90 (AOCS, 1992), ISO 6885 (ISO, 1996), and IUPAC 2.504 (IUPAC, 1987). *p*-AV of crude fish oil should be in the range of 4-60 (Table 1). After refining, the oil should be less than 20 (Table 2).

3.5 Iodide value

Iodide value (IV) is determination of unsaturation degree of fatty acids in oil sample. It is expressed in term of centigram (cg) of absorbed iodide ($I_2$) in 1 gram of oil sample, amount of $I_2$ will react with aliphatic double bonds of unsaturated fatty acids, which is a component in the molecule of the oil (Shahidi and Wanasundara, 2002a; AOCS, 1997). A high IV could indicate less stability to oxidation due to the oil contains high amount of double bond of unsaturated fatty acids. Conversely, a low IV indicates more stability to oxidation due to number of double bond of fatty acids is less (Wrolstad *et al.*, 2005). IV in the oil can be detected and quantified using analysis methods as AOCS Official methods Cd 1d-92 (AOCS, 1992) and ISO 3961 (ISO, 1996). IV of crude fish oil should be in the range of 95-200 (Table 1). Currently, there is no standard of IV of the refined oils.

3.6 Saponification value

Saponification value (SV) is used to determine the average molecular weight of lipid. It is defined as the amount of potassium hydroxide (KOH) in milligrams required to saponify 1 gram of oil sample. A low SV indicates high molecular weight of fatty acid that has low number of TAG molecules per gram sample. Conversely, a high SV indicates low molecular weight of fatty acid, which has high number of TAG molecules per gram sample (Shahidi and Wanasundara,

2002b). SV in the oil can be detected and quantified using analysis methods including AOCS Office method Cd 3b-76 (AOCS, 1992), ISO 3657 (ISO, 1996), and IUPAC 2.202 (IUPAC, 1987). Currently, there is no standard of SV for crude fish oil and refined oils.

3.7 Unsaponifiable matter

Unsaponifiable matter or non-saponifiable material (USM) of fish oil is undesired component including fatty acid alcohols, hydrocarbon, and sterols especially free cholesterol and cholesterol esters. Free cholesterol can be partially removed from the oil by steam stripping whereas cholesterol esters remain in the oil throughout the refining step (Rossell, 2009). USM in the oil can be detected and quantified using analysis methods such as AOCS Official method Ca 6b-53 (AOCS, 1992), ISO 3596-1 (ISO, 1996), and IUPAC 2.401 (IUPAC, 1987). Currently, there is no standard of USM for crude fish oil and refined oils.

3.8 Heavy metal

Crude fish oil in some cases contains amount of heavy metal (HM) such as phosphorus (P), sulphur (S), iron (Fe), and copper (Cu), nickel (Ni), mercury (Hg), selenium (Se), and cadmium (Cd) due to HM accumulation in fish body from effects of feed and habitat. Fe and Cu are considered a pro-oxidant in fish oil and are removed by degumming and neutralization steps. Others also could be removed by refining process (Young, 1986b; Bimbo, 1998). These HM in the oil can be detected and quantified using analysis methods including AOCS Official method Ca 12-55 (AOCS, 1997) for P and S, CAC/RM 9/14 (Codex, 2009) and AOCS Official method Ca 15-75 (AOCS, 1992) for Fe, and AOAC Official method 990.05 (AOAC, 1995), ISO 8294 (ISO, 1996), AOCS Official method both Ca 18-79 and Ca 15-75 (AOCS, 1992), BTM 5.4 (1993), IUPAC 2.631 (IUPAC, 1987) for Cu, EPA method 245.1, 206.3, 270.3 for Hg, Biomedical Test Materials (BTM) 5.4 (Seaborn *et al*., 1993) for Se and Cd. HM of crude fish oil should be in the range of 5-100 mg/kg for P, 0.5-7.0 mg/kg for Fe, less than 0.3 mg/kg for Cu, and less than 30 mg/kg (Table 1). After

refining, the oil should be in the range of 0.12-2.5 mg/kg for Fe, and 0.05-0.10 mg/kg for Cu while there is no standard for P, Hg, Se, and Cd in refined oils (Table 2).

3.9 Fatty acid composition

Fatty acid composition (FAC) is considered to be important for the evaluation of nutritional significance in fish oil as shown in Table 3. However, the fatty acid content of fish oil varies with the species of fish, season, area caught, and age of fish (Bimbo, 1998). For FAC determination, fish oil sample is hydrolyzed by using methanolic sodium hydroxide, followed by methylation with broron trifluoride in methanol, extraction with iso-octane to be fatty acid methylesters (FAME) and determination of the fatty acid composition by gas chromatography according to the AOAC Official method 991.39 or AOCS Official method Ce 1b-89 (AOAC, 1995; AOCS, 1997).

**Table 1** Guideline of crude fish oil quality

| Parameter | Quantities |
|---|---|
| Color (gardner scale) | 12-14 |
| Free fatty acid(% as oleic acid) | 2-5 |
| Moisture and impurities (%) | 0.5-1.0 |
| Peroxide value (meq/kg) | 3-20 |
| Anisidine value | 4-60 |
| Iodine value (cg $I_2$/g) | |
| Capeline | 95-160 |
| Herring | 115-160 |
| Menhaden | 150-200 |
| Sardine | 160-200 |
| Anchovy | 180-200 |
| Jack mackerel | 160-190 |
| Sand eel | 150-190 |
| Iron (mg/kg) | 0.5-7.0 |

**Table 1** (Continued)

| Parameter | Quantities |
|---|---|
| Copper (mg/kg) | < 0.3 |
| Phosphorus (mg/kg) | 5-100 |
| Sulphur (mg/kg) | < 30 |

**Source:** adapted from Young (1986a); Bimbo (1998)

**Table 2** Guideline of refined fish oil quality

| Parameter | Quantities | |
|---|---|---|
| | International Association of FishmealManufacturers | CODEX STAN 19-1981 |
| Color | < 3.0 Red, 30 Yellow (Livibond, 5¼"cell) | Depending on designed product |
| Flavor and odor | bland | bland |
| Matter volatile at 105°C (%) | < 0.2 | < 0.2 |
| Free fatty acid (% as oleic acid) | < 0.1 | - |
| Acid value (mg KOH/g) | - | < 0.6 |
| Insoluble impurities (%) | < 0.05 | < 0.05 |
| Soap content (%) | < 0.005 | < 0.005 |
| Peroxide value (meq/kg) | < 0.1 | < 10 |
| Iron(mg/kg) | < 0.12 | < 2.5 |
| Copper (mg/kg) | < 0.05 | < 0.1 |
| Nickel (mg/kg) | < 0.2 | - |

**Source:** adapted from Young, (1986b); Codex (2009)

**Table 3** Fatty acid composition of refined fish oil from four fish species

| Fatty acid | Atlantic Salmon | | Herring | Tuna | Pollock |
|---|---|---|---|---|---|
| | Wild | Farmed | | | |
| Myristic acid (C14:0) | 2.0-4.5 | 2.5-5.5 | 3.0-10.0 | 2.0-5.0 | 4.0-5.5 |
| Pentadecanoic acid (C15:0) | ND-1.0 | ND-0.5 | ND-0.5 | ND-2.0 | ND-0.5 |
| Palmitic acid (C16:0) | 12.0-13.5 | 7.0-16.5 | 8.0-25.0 | 14.0-24.0 | 8.0-11.0 |
| Palmitoleic acid (C16:1 n-7) | 4.5-5.0 | 3.0-8.0 | 3.5-12.0 | 1.0-12.5 | 9.0-12.0 |
| Heptadecanoic acid (C17:0) | ND-1.0 | ND-0.5 | ND-0.5 | 1.0-3.0 | ND-1.0 |
| Stearic acid (C18:0) | 2.5-5.0 | 2.0-5.0 | 0.5-4.0 | 1.0-7.5 | 1.0-2.0 |
| Vaccenic acid (C18:1 n-7) | na | na | na | 2.0-7.0 | Na |
| oleic acid (C18:1 n-9) | 16.0-17.5 | 16.0-40.0 | 4.0-22.0 | 10.0-25.0 | 7.0-13.5 |
| Linoleic acid (C18:2 n-6) | 1.5-2.0 | 2.5-11.0 | ND-2.0 | ND-3.0 | 0.5-1.5 |
| Linolenic acid (C18:3 n-3) | 11.5-14.0 | 0.5-6.0 | ND-2.0 | ND-2.0 | ND-0.5 |
| γ-Linolenic acid (C18:3 n-6) | 5.0-5.5 | ND-0.5 | ND-2.0 | ND-4.0 | ND-0.5 |
| Stearidonic acid (C18:4 n-3) | 2.0-2.5 | 0.5-1.5 | 0.5-5.0 | ND-2.0 | 1.0-2.0 |
| Arachidic acid (C20:0) | na | na | na | ND-2.5 | na |
| Eicosenoic acid (C20:1 n-9) | 4.5-6.0 | 1.5-7.0 | 8.5-14.0 | ND-2.5 | 4.0-5.5 |
| Eicosenoic acid (C20:1 n-11) | 4.5-6.0 | 0.5-2.0 | na | ND-3.0 | 10.0-16.0 |
| Arachidonic acid (C20:4 n-6) | 5.0-5.5 | ND-1.0 | ND-0.5 | ND-3.0 | na |
| Eicosatetraenoic acid (C20:4 n-3) | 14.0-16.5 | 0.5-2.0 | ND-1.5 | ND-1.0 | 0.3-0.5 |
| Eicosapentaenoic acid (C20:5 n-3) | 8.5-9.5 | 6.0-9.0 | 4.0-15.0 | 2.5-9.0 | 9.5-11.0 |
| Heneicosapentaenoic acid (C21:5 n-3) | ND-1.0 | ND-1.0 | ND-1.0 | ND-0.5 | na |
| Docosanoic acid (C22:0) | na | na | na | na | na |
| Erucic acid (C22:1 n-9) | 4.0-6.0 | ND-4.0 | na | ND-1.0 | 0.5-1.5 |
| Cetoleic acid (C22:1 n-11) | 4.0-6.0 | 0.5-7.0 | 11.0-27.0 | ND-1.0 | 11.5-15.5 |
| Docosapentaenoic acid (C22:5 n-3) | 2.5-3.0 | 1.5-5.0 | ND-1.5 | ND-3.0 | 0.5-1.0 |
| Docosahexaenoic acid (C22:6 n-3) | 10.5-11.0 | 3.0-14.5 | 2.0-12.0 | 21.0-42.5 | 4.5-5.5 |

**Note** na = not available, ND = non-detect as defined at 0.05%

**Source:** Join FAO/WHO Food Programme Codex committee on Fats and Oil (2013)

**5. Benefit of fish oil**

Fish oils is a good source of the polyunsaturated fatty acids (PUFA) especially omega-3 (*n*-3) and omega-6 (*n*-6) families. PUFA cannot be synthesized in the human body but must be obtained through diet or supplementation due to human cells lack the converting enzyme, *n*-3 desaturase, which cannot convert *n*-6 PUFA to *n*-3 PUFA. On the other hand, saturated and monounsaturated fatty acids can be synthesized in the body. *n*-3 PUFA families especially eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3) is synthesized from α-linolenic acid (ALA, 18:3 *n*-3) while n-6 PUFA families as arachidonic acid (AA, 20:4 *n*-6) is synthesized from linoleic acid (LA, 18:2 *n*-6) by enzymes δ-5 and δ-6 desaturases as shown in Figure 2 (Simopoulos, 2008). These PUFA compete to react with the same enzymes for desaturation and elongation and each class of PUFA has a different effect on human health. AA and EPA are the parent compounds for eicosanoids production. The eicosanoids made from AA can act in a pro-inflammatory manner by enhancing platelet aggregation to generate thrombus and atheromas that are cause of coronary heart disease (CHD) and stimulate tumor cell proliferation. Conversely, eicosanoids made from EPA can increase immune system and their anti-inflammatory effects play a role in reducing both colon carcinogenesis and risk of CHD (Hibbeln *et al*., 2006; Roynette *et al*., 2004). Action mode of fish oil especially *n*-3 PUFA on functions mediated by *n*-6 PUFA is summarized as follows;

- Impair uptake of *n*-6 PUFA
- Inhibit desaturases especially δ-6-desaturase
- Compete with *n*-6 PUFA acyltransferases
- Displace AA from specific phospholipid pools
- Dilute pools of free AA
- Competitively inhibit cylooxygenase and lipoxygenase,
- Form eicosanoid analogs with less activity or competitively bind to eicosanoid site
- Alter membrane properties and associated enzyme and receptor functions

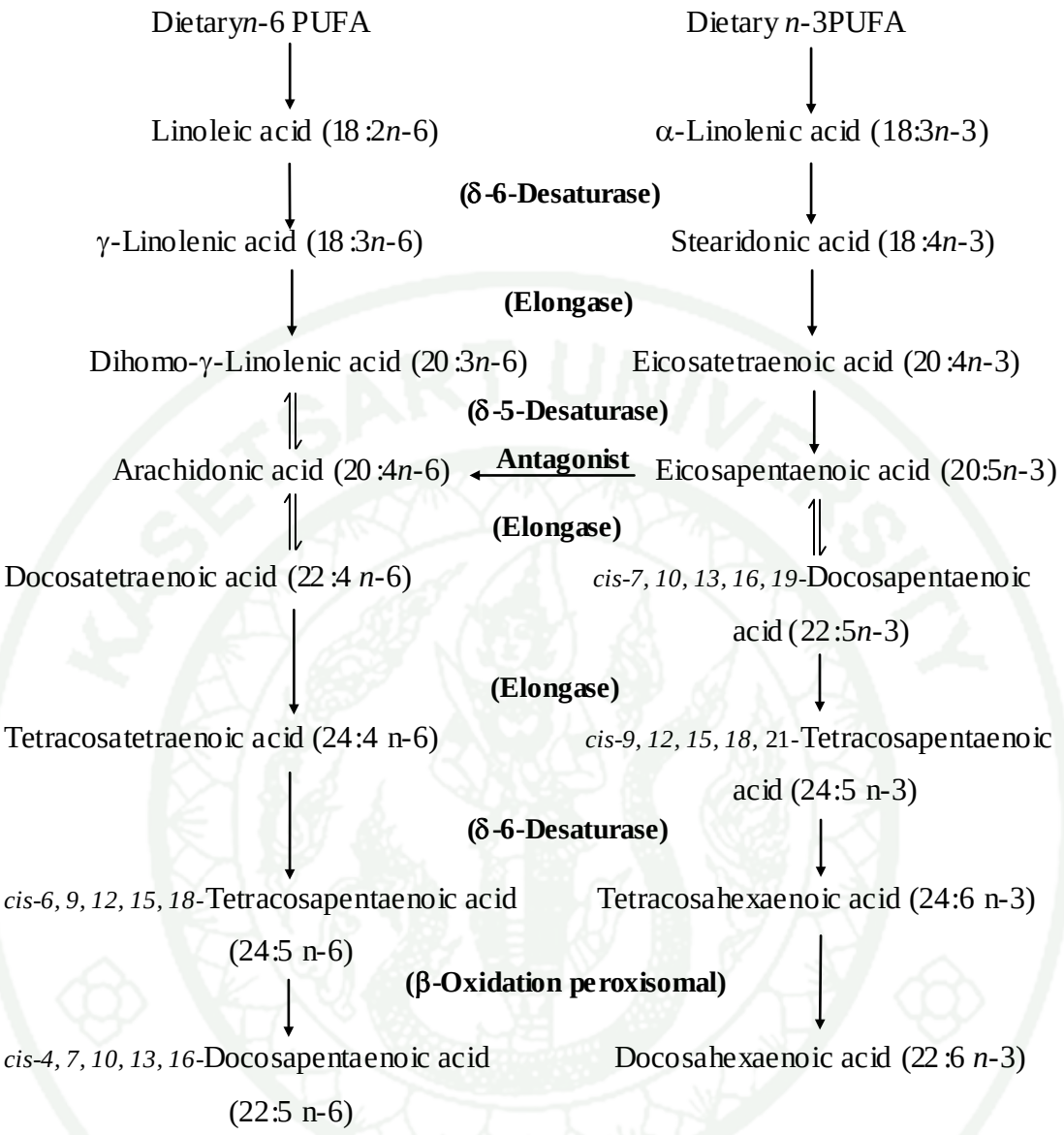



**Figure 2** Pathways leading to the metabolism of dietary *n*-6 and *n*-3 polyunsaturated fatty acids

**Source:** Simopoulos (2008)

Fish oil also has a high content of *n*-3 PUFA in form of DHA and EPA. A large number of studies have been done on the potential benefits in each stage of age.

5.1 Infants, pregnancy, and lactation

Adequate supplies of AA and DHA are essential for growth, functional brain development, and central nervous system development in infant especially visual acuity. Both neural integrity and function can be permanently disturbed by deficits of *n*-6 and *n*-3 essential fatty acids during fetal and neonatal development (Horrocks and Young, 1999). The suggested dose of WHO (2003) and FAO (2010) for adequately DHA intake in infant during 0-6 months should be in the range of 0.20-0.36% total fatty acids based on breast milk, 10-12 mg/kg of body weight for 6-24 months based on combination between breast milk and non breast milk diet, while EPA is a natural constitute of breast milk, but in amounts more than 0.1% in infant formula may antagonize AA and interfere with infant growth. The pregnancy and lactation women are recommended to take DHA 300 mg/d. Currently, a large number of infants have been fed formula including DHA and other *n*-3 fatty acids due to DHA deficiencies are associated with fetal alcohol syndrome and attention deficit hyperactivity disorder. Lapillonne and Craig (2009) reported that increased DHA content above 1% of total fatty acids of human milk either by providing them others with a DHA supplement or by adding DHA directly to the milks appears to be safe and may enhance neurological development particularly that of infants with a birth weight below 1,250 g.

5.2 Children

Children without sufficient *n*-3 PUFA in their diet may suffer from neurological and visual disturbances, dermatitis, and growth retardation especially DHA is involved in cell signaling that lead to learning ability and memory. DHA can be associated and treated diseases in children as attention deficit hyperactivity disorder (ADHD), which is a developmental problem resulting in poor concentration

and control of impulses. Children with ADHA have low levels of DHA in their blood than children without ADHD can also affect children's learning, social skills, and family functioning. There is some evidence that increasing DHA levels reduces the severity of ADHD-type behaviour, such as inattention in some children (Horrocks and Young, 1999) and Phenylketonuria (PKU) caused by deficient activity of hepatic phenylalanine hydroxylase in newborn. Children (aged 1-11 years) patients with PKU, who obtained fish oil supplement containing DHA 15 mg/d and EPA 22.5 mg/d during 90 days had higher DHA level more than 3 fold and higher EPA level more than 8 fold in plasma phospholipids compared with the normal plasma phospholipids both 2 types in the same age (Beblo *et al.*, 2007). Moreover, the suggested dose of adequately DHA and EPA intake in children should be in the range of 100-150 mg/d for 2-4 years, 150-200 mg/d for 4-6 years, 200-250 mg/d for 6-18 years (WHO, 2003; FAO, 2010).

5.3 Adults

DHA is important for maintenance of the brain and learning during adults by formation of new cell in hippocampus continues to proliferate. DHA and EPA have a positive effect on increasing the ratio of high-density lipoprotein (HDL) to low-density lipoprotein (LDL) cholesterol, decreased risk for coronary artery disease, prevent and treat the several diseases such as arthritis, atherosclerosis, diabetes mellitus, myocardial infarction, thrombosis, hypertension, depression, inflammatory, and some cancers (Horrocks and Young, 1999). The comparison of serum fatty acid composition between Japanese students (33 males and 29 females) and Dutch students (20 males and 19 females) in university were surveyed the risk factor of coronary vascular disease (CVD). There was found that Japanese students had low EPA while Dutch students had high AA, which indicated the ratios of EPA/AA and n-3/n-6 PUFA of Dutch students were lower than those the Japanese students and influenced greater incidence of CVD (Hirai *et al.*, 2000). Furthermore, Harris (1997) found that consuming 3-4 g/d of DHA and EPA resulted in a plasma TAG decrease to 24% in normolipemic subjects and 34% in hypertriacylglycerolemic patients. Also the study of Kris-Etherton *et al.* (2003) reported that supplementation 2-4 g/d of EPA and DHA

can reduce plasma triglyceride levels about 25-30% in patients with hypertriacylglycerollemia. The other study reported that consumption refined fish oil 3 g/d for 6 weeks can reduce inducible ventricular tachycardia and risk of sudden cardiac death in patients with coronary artery disease (Metcalf *et al.*, 2008). For patients with colorectal adenomas, consumption 2 g/d of EPA led to formation of series-3 prostaglandins as PGE3, the suppression of crypt cell proliferation, and increased apoptosis in colonic mucosa (Courtney *et al.*, 2007). However, WHO (2003) and FAO (2010) recommend DHA and EPA intake in adult should be in the range of 0.25-2.00 g/d following acceptable macronutrient distribution range, that is an intake range for an energy source associated with reduces risk of chronic disease.

5.4 Elder

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss, intellectual decline, and eventual global cognitive impairment. Most patients with AD are elderly person during 55-89 years and aged 75-89 years was found approximately 35.6% for women and 30.4% for men (Ott *et al.*, 1995). Consumption high amount of fish (chinook salmon 20 g and cod 100 g amount 2-3 meals per week) or fish oil supplement (EPA and DHA intake about 380 mg/d) can prevent cognitive impairment, cognitive decline, and development of dementia or AD for elderly person aged 70-89 years (Gelder *et al.*, 2007). Similarly, the study was done with a group of men aged 69-89 years. Cognitive impairment was evaluated with mini-mental state examination. This group was received the optimal intake of *n*-3/*n*-6 ratio through fish consumption, which influenced to maintain the cognitive function of the brain and delayed onset of sporadic Alzheimer's disease (Horrocks and Young, 1999). However, WHO (2003) and FAO (2010) indicated that the strength of the evidence is regarded as insufficient to date for DHA and EPA concentration in relation to function of central nervous system.

Suggested dose for fish oil consumption [WHO (2003), FAO (2010), and U.S. FDA (2003)]

| | | | |
|---|---|---|---|
| Infant | 0-6 months | DHA | 0.20-0.36% of total fatty acids based on breast milk |
| | 6-24 months | DHA | 10-12 mg/kg of body weight based on combination between breast milk and non breast milk diet |
| Children | 2-4 years | DHA& EPA | 100-150 mg/d |
| | 4-6 years | DHA & EPA | 150-200 mg/d |
| | 6-18 years | DHA & EPA | 200-250 mg/d |
| Adult | 18-60 years | DHA & EPA | 200-250 mg/d |
| | | Fish oil | 1,000 mg/d |

**6. Structured lipids**

Structured lipids (SL) or structured triacylglycerols (STAG) are edible oil, which have been restructured by incorporation with desired fatty acids or modified to change triacylglycerols (TAG) with altered fatty acid composition or location in the glycerol backbone for specific function and nutrition properties of TAG via chemical or enzymatic reactions (Iwasaki *et al.*, 2001; Zhou *et al.*, 2000). SL are also defined as TAG containing mixture of short- and/or medium- and/or long-chain fatty acids composition residues at specific positions in the glycerol backbone (Figure 3), which influence to change or improve physicochemical characteristics of TAG of oils or fats in food and medicine industries. The distributed fatty acid on the glycerol backbone of TAG influences their digestion, absorption, and tissue uptake (Irimescu *et al.*, 2001).

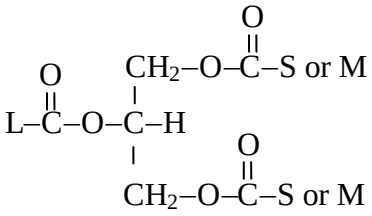


**Figure 3** General structure of structured lipids containing short chain fatty acids (S), medium chain fatty acids(M), and long chain fatty acids (L) in distributed position of glycerols backbone

**Source:** adapted from Akoh and Min (2002)

6.1 Sources of fatty acid for structured lipids synthesis

SL have been developed to optimize the benefit of fat substrate mixture. A variety fatty acids are used in the synthesis of SL, taking advantage of specific functional and medical purposes of each to obtain maximum benefit from a given SL as *n*-3 PUFA, which are often referred to as nutraceuticals and functional foods including neurological disorders in the adult or elder (Freemantle *et al.*, 2006), reducing or preventing the symptom of metabolic pathologies such as cardiovascular, hypertension, hyperlipidemia (Zhang *et al.*, 2010), immunologic, and inflammatory diseases (Eslick *et al.*, 2009), diabetes and obesity (Horrocks and Young, 1999). The component fatty acids and their position in TAG molecule determine the functional and physical properties, metabolic fate, and health benefits of the SL.

6.1.1 Short chain fatty acids

The short chain fatty acids (SCFA) contain 2-6 carbon atoms in chain length. They are found in gastrointestinal tract of mammals, where are the end products of microbial digestion to being carbohydrates. SCFA are usually present in bovine milk and other milk fat-based product as butter. Cow's milk is composed of 5-10% of butyric acid (C4:0) and 3-5% of caproic acid (C6:0) (Shahidi and Senanayake, 2006). SCFA are volatile fatty acids and more rapidly absorbed in the stomach than

medium chain fatty acids (MCFA) and long chain fatty acid (LCFA) due to their higher water solubility, smaller molecular size, and shorter chain length. SCFA are mainly esterified to the sn-3 position of TAG and completely hydrolyzed in lumen of the stomach and small intestine (Osborn and Akoh, 2002). SCFA are useful ingredients in the synthesis of low calories SL and are lower in caloric value than MCFA and LCFA. This is accordance with the study of Kanda *et al.,* (2010), who indicated that structured oils containing SCFA was produced by trilaurin and SCFA (butyric acid; C4:0, valeric acid; C5:0, and caproic acid; C6:0) combined with lipase from *Alcaligenes* sp.

### 6.1.2 Medium chain fatty acids

Medium chain fatty acids (MCFA) range from 8-12 carbons in chain length including caprylic acid (C8:0), capric acid (C10:0), and lauric acid (C12:0). Like all triglycerides, medium chain triacylglycerols (MCT) are composed of a glycerol backbone and three fatty acids, thus the name triglyceride; in the case of MCT, 2 or 3 of the fatty acid chains attached to glycerol are medium chain in length. MCFA are used for making lipid emulsion or blending with LCFA (long chain fatty acids) for parenteral and enteral nutrition (Figure 3). MCT are liquid or solid at room, their melting points depend on the fatty acid composition. MCT are used as carrier for colors, flavors, vitamins, and pharmaceuticals. MCFA are commonly found in palm kernel oil and coconut oil contains caprylic acid or capric acid approximately 7-17% and is a raw material of synthesis MCT (O'Brien, 2004). The produced MCT are odorless, tasteless, and high oxidative stability that their calorific value is merely high 8.3 cal/g. These characteristic of MCT attract to produce SL in low calorie diets. Metabolically, MCT do not require carnitine for transport or chylomicron formation, and are transported to the liver directly through the portal system (Traul *et al.*, 2000). In many cases, MCFA are not readily re-esterifies into TAG. They have more than twice the calorific density of proteins and carbohydrates, MCFA are absorbed and metabolized as rapidly as glucose, whereas LCT are metabolized more slowly.MCT have clinical applications in the treatment for fat malabsorption disorders, gall bladder disease, hyperlipidemia, obesity and deficiency of the carnitine system (Calabrese *et*

*al.*, 1999). However, these lack essential fatty acids (EFA), and animal fed 100% MCT emulsion show side effect such as vomiting, defecation and in some cases coma (Lingren *et al.*, 2001). Therefore, MCFA are the most useful in a structured lipid that combines their inherent mobility, solubility, and ease of metabolism with more healthful polyunsaturated fatty acids (PUFA) (Akoh, 2006).

a) Capric acid

Capric acid (CA, C10:0) is saturated fatty acid of 10 carbon atoms and occurs naturally in coconut oil and palm kernel oil, as well as in the milk of various mammals and to a lesser extent in other animal fats (Beare-Rogers *et al.*, 2001). Many researchers have successfully incorporated CA into fish oil TAG to synthesized SL for medical, nutritional, and food applications. The study of Hamam and Shahidi (2005) denoted that CA incorporation into docosahexaenic acid single cell oil (DHASCO) was synthesized by lipase enzyme from *Pseudomona*s sp., which resulted in highest CA incorporation up to 10.17±0.11%, DHA content was 37.1±0.29%. Furthermore, Jennings and Akoh (2001) indicated that CA incorporation into TAG of menhaden oil was synthesized by lipase enzyme from *Rhizomucor miehei*, which resulted in highest CA incorporation up to 31.1±4.6 mol%, EPA and DHA contents was 12.6±3.1 and 13.7±4.4 mol%, respectively. The synthesis of MLM-type SL comprises MCFA in sn-1, 3 positions and LCFA in sn-2 position of TAG. The effect of MCFA in sn-1, 3 positions of TAG lead to fast hydrolyzed through intestinal cell, while residual LCFA in sn-2 position is micellarized and absorbed through lymphatic.

6.1.3 Long chain fatty acids

Long chain fatty acids (LCFA) are fatty acids with aliphatic tails 14-24 carbons as saturated and polyunsaturated fatty acids. Essential fatty acids (EFA) are an important LCFA including *n*-3 and *n*-6 PUFA, which are required by the human body but cannot be made in sufficient quantity from synthesis in body. Therefore, they must be obtained from food.

a) Long chain saturated fatty acids

Long chain saturated fatty acids (LCSFA) containing high amounts of behenic acid (C22:0) has been reported to raise plasma cholesterol levels due to it poorly absorbed in human to exhibit poor emulsion formation and poor micellar solubilization from melting point higher than body temperature (Shahidi and Senanayake, 2006). However, the poor adsorption of LCSFA influences low calorie SL synthesis.

b) Polyunsaturated fatty acids

The polyunsaturated fatty acids (PUFA) are fatty acid with more than one double bond in carbon chain. The PUFA are considerably referred to *n*-3 and *n*-6 families, which are differed from one another based on the location of the double bond from the terminal methyl group of fatty acid molecule.

(1) Omega-6 polyunsaturated fatty acids

Omega-6 polyunsaturated fatty acid (*n*-6 PUFA) present the basis of *n*-6 family that compose of linoleic acid (LA, C18:2 *n*-6) and arachidonic acid (AA, C20:4 *n*-6). LA is the most fatty acid of this type and is found in all vegetable oils in the seeds of most plants except coconut, palm nuts, and cocoa. LA serves as a precursor compound of n-6 PUFA family, which is formed by desaturation and elongation chains. Therefore, LA can be metabolized into γ-linolenic acid (GLA, 18:3 *n*-6) and AA as shown in Figure 3 (Simopoulos, 2008). Of these, AA is greatly important as an essential component of membrane phospholipids and as a precursor of eicosanoid formation while GLA is an important intermediate in the biosynthesis of AA from LA (Shahidi and Senanayake, 2006). *n*-6 PUFA are recommend to supply through the diet about 1-2% dietary sufficient calories to cure the symptoms of essential fatty acid deficiency in infants (Zatsick and Mayket, 2007). Nutritionists have suggested *n*-6 PUFA content in SL about 3-4% to full fill EFA requirements of the SL (Akoh and Min, 2002). The study was done with structured lipids containing

fatty acid mixture from Brazilian sardine oil into soybean oil by using lipozyme RM-IM (lipase obtained from *Rhizomucor miehei*) for improving nutrient delivery to critically ill patients (De Araújo *et al.*, 2011).

(2) Omega-3 polyunsaturatedfatty acids

Omega-3 polyunsaturated fatty acids (*n*-3 PUFA) represent the basis of *n*-3 family, which contain α-linolenic acid (ALA, C18:3 *n*-3), eicosapenta-enoic acid (EPA, C20:5 *n*-3), and docosahexaenoic acid (DHA, C22:6 *n*-3). DHA and EPA are referred to benefit health and prevent many diseases, which is described previously in section 5. DHA and EPA are mainly found in fatty fish such as mackerel, herring, salmon, and tuna or small fish as anchovy and capelin, blubber of marine mammals such as seals and whales, liver of white lean fish, algal and fungal oils (Barrow and Shahidi, 2008). The enrichment of structured lipids with DHA and EPA is nutritionally useful and has potential use as valuable ingredient oil in various functional food formations. This is accordance with the report of Şahín et *al.*, (2006), who demonstrated that structured lipids resembling human milk enriched with EPA and DHA was synthesized by enzymatic acidolysis reactions (Liposyme RM-IM) between tripalmitic, hazelnut oil fatty acids, and EPA and DHA concentrates.

6.2 Influence of triglycerides structure on digestion and absorption of lipid

The physical properties and physiological effects of dietary fatty acids depend not only on their structural parameters such as chain length and degree of unsaturation but also on their position in the triglyceride molecule. The glycerol backbone of triglycerides (TAG) is esterifies by fatty acids in a specific manner, and large differences are seen in the positional distribution of TAG molecular species in dietary oils and fats which are unique to each fat (Christophe, 2006). Although most dietary fatty acids are esterifies in the form of TAG, relatively little is known about the importance of the steriospecific distribution of fatty acids in a TAG molecule and their importance to biological activities. The fatty acids distribution in the naturally occurring TAG is not random. The taxonomic patterns of vegetable oils consist of

TAG following the sn-1, 3 random distributions, with SFA being located almost exclusively at sn-1, 3 positions of TAG and unsaturated fatty acids present at sn-2 position. Conversely, animal fats from tallow, lard, bovine milk contain SFA at the sn-2 position (Akoh and Min, 2002). Such differences in the positional distribution of fatty acids among natural fats have been implicated to have specific effects on the profile, structure and composition of lipoprotein. The TAG structure plays an important role in the digestion, absorption and metabolism of fats. The major phases of digestion and absorption of TAG occur when the food reaches the small intestine where they are exposed to bile and pancreatic juice. As intact TAG cannot be absorbed by the intestinal epithelial cells, they undergo hydrolysis in the gut. In the presence of co-lipase, pancreatic lipase acts on emulsified glycerides and catalyzed the hydrolysis of primary ester bond. The pancreatic lipase complex preferentially hydrolysis fatty acids at the sn-1 and sn-3 positions of TAG, leaving initially fatty acid at sn-2 position that are further hydrolyzed to 2-monoacylglycerols (2-MAG) due to their polar (glycerol) and non polar moieties they function as excellent emulsifying agent (Jeukendrup and Aldred, 2004). The lipolysis products which include free fatty acids, MAG and diacylglycerols (DAG) together with phospholipids and cholesterol form micelles by bile salts and diffuse through both the stagnant water layer and the brush border membrane into enterocytes (Haman and Shahidi, 2005). As the rate of hydrolysis of fatty acids at the sn-2 position is low, the fatty acids at sn-2 position remain as ester in 2-MAG during digestion and absorption. A variety of fatty acids, MAG and DAG intermediates are formed during digestion of fat whose nature is determined by the positional distribution of the fatty acids in the original TAG molecules (Haman and Shahidi, 2005). Many of these molecules have specific melting points, which may influence subsequent digestion, absorption and metabolism. MAG readily form mixed micelles have variable incorporation into mixed micelles (Christophe, 2006). Digestion and absorption of long chain fatty acids (LCFA) occur less readily than short chain fatty acids (SCFA), as the latter and 2-MAG require lower concentrations of the bile salts to achieve emulsification into micelle form (Carriere *et al.*, 1997). Impaired absorption of LCFA in the free state could also occur because of their melting points, some of which are above body temperature, which does not allow adequate incorporation in the liquid phase (Shahidi

and Senanayake, 2006). This could also be due to the tendency of free fatty acids to form hydrated acid calcium soaps, which are insoluble in aqueous media at the pH of the intestine (Carvajal *et al.*, 2000). Many studies have indicated that the content and arrangement of LCSFA are the major factors that determine their digestibility (Kanjilal *et al.*, 2013). Fatty acids have an effect on apoprotein secretion by intestinal cell, and the magnitude of the effect depends on the nature of the fatty acids (Murthy *et al.*, 1992) and consequently on the structure of TAG. Medium chain triglycerides (MCT) are more rapidly and easily digested than long chain triglycerides (LCT) and they can be taken up as such and hydrolyzed in the intestinal cell. LCT are hydrolyzed to 2-MAG and fatty acids. 2-MAG is micellarized and absorbed more readily than free fatty acids. The hydrolysis product are reutilized to from TAG in the mucosal cells and are transported to the lymph in chylomicrons, approximate 75% of the fatty acids located at the sn-2 position of the TAG are conserved in the original position, whereas fatty acids released from sn-1, 3 positions bind to glycerol at random (Jeukendrup and Aldred, 2004). MCT are hydrolyzed readily and completely free fatty acids and glycerol via 1-MAG. MCFA being more hydrophilic, solubilization as micelles is not a prerequisite for absorption. MCT can also be incorporated into mucosal cells without hydrolysis and can readily be hydrolyzed in the cell (Che Man and Manaf, 2006).

### 6.2.1 Cylomicron metabolism

The LCFA are re-esterified into TAG and incorporated into chylomicrons in the enterocytes and then enter the lymphatics to reach the general circulation through the thoracic duct. MCT undergo near complete intra luminal hydrolysis and are absorbed mainly as free fatty acids (Traul *et al.*, 2000). As MAG provides the basic structure for the re-synthesis of chylomicron TAG in the enterocytes, sn-2 located saturated fatty acids (SFA) present in ingested TAG are conserved and incorporated into circulating chylomicron TAG (Jeukendrup and Aldred, 2004). Studies on human subjects have shown that the feeding of lard which is high in palmitic acids at the sn-2 position results in the increased proportions of plamitate in the sn-2 position of very low-density lipoprotein (VLDL) and

chylomicron TAG (Forsythe *et al.*, 2007). Nestel *et al.* (1995) showed that positional distribution was maintained in TAG chylomicrons after feeding an interesterified palm oil blend to hypercholesterolemic men. The intramolecular structure of dietary TAG have been suggested to influence the metabolism of chylomicrons to chylomicron remnants because the lipoprotein lipase like pancreatic lipase has positional specificity for the sn-1, 3 position of TAG (Carriere *et al.*, 1997). Several kinetic studies have suggested that the presence of SFA at the sn-2 position in dietary TAG administered orally or intravenously, slows down the metabolism of chylomicrons via both lipoprotein lipase hydrolysis of TAG and chybmicron remnant removal by the liver (Mortimer *et al.*, 1994).

6.2.2 Fatty acid and cholesterol metabolism

The liver is the site where biosynthesis of very low-density lipoprotein (VLDL) occurs. VLDL circulating in plasma is degraded by lipoprotein lipase. The VLDL remnants appear first in circulation as intermediate density lipoproteins (IDL) and then as low density lipoprotein (LDL). In the transformation of VLDL to LDL, all its apoproteins except apo B-100 are removed and much of their cholesterol is esterified by high density lipoprotein (HDL) associated enzyme lecithin: cholesterol acyltransferase (LCAT). The enzyme transfers a fatty acid molecule from sn-2 of lecithin to cholesterol with concomitant formation of lysolecithin (Kasim-Karakas, 2007). LDL formed from VLDL is then taken up by the liver via LDL receptors, which are cell surface trans-membrane glycoprotein that specifically binds both apo B-100 and apo-E. Thus, LDL uptake is receptor mediated endocytosis. In the liver cells, the LDL's apo B-100 is rapidly degraded to its component amino acids. The cholesterol esters are hydrolyzed by a lysosomal lipase to yield cholesterol, which is subsequently incorporated into the cell membrane. Any excess intra cellular cholesterol is re-esterified for storage within the cell through the action of acyl-CoA: cholesterol acyltransferase (ACAT), mainly with oleic acid. Dietary cholesterol suppresses LDL receptor activity and increases the amount of VLDL in circulation, thus increasing the amount of LDL circulating in the plasma. Although fatty acids modify this regulatory process, they have little effect in the absence of dietary

cholesterol. Since LDL is involved in distributing cholesterol to various tissues, it is sometimes called as "bad cholesterol" (Fisher *et al.*, 1998). HDL has essentially the opposite function of LDL. It removes cholesterol from various tissues and converts it into cholesterol esters through the action of LCAT in the liver, which is the only organ capable of disposing significant quantities of cholesterol as bile acids. HDL cholesterol is therefore called as "good cholesterol" (Barter and Rye, 1996). SFA cause modification in the composition, structure, and relative density of LDL, culminating in decreased binding, internalization and degradation of LDL. In addition, feeding with SFA like C12:0, C14:0 and C16:0 have shown to enrich hepatic lipid pools with SFA and suppress cholesterol ester formation (Woolett and Dietschy, 1994) as these saturates are poor substrates for ACAT. As a consequence of the decrease in the cholesterol ester formation, more sterol is diverted into the free cholesterol pool, which is believed to regulate LDL receptor transcription. Hence, as hepatic tissue concentrations of free cholesterol increase, a greater inhibition of LDL receptor endocytosis is observed resulting in higher concentrations of plasma LDL cholesterol (Woollett and Dietschy, 1994). On the other hand, with an enrichment of hepatic tissue with unsaturated fatty acid such as oleic acid, the preferred substrate of ACAT, cholesterol is removed out of the circulatory free sterol pool into the biologically inert pool of cholesterol ester. As a result, LDL-receptor transcription is increased, leading to increased uptake of LDL-cholesterol from the circulation (Dietschy *et al.*, 1993). In comparison, caproic, caprylic, capric, stearic and trans fatty acids do not alter relative receptor activity or LDL-cholesterol production, and thus have a neutral effect on circulating LDL-cholesterol.

### 6.3 Synthesis of structured lipids

Chemical and enzymatic interesterifications are two of the major reactions used by the industry for modification of natural fats and oils. It is a process that is used to modify the physical and functional properties of TAG mixture in fats and oils. The reaction involves the exchange of acyl residues between an ester an acid (acidolysis), an ester and an alcohol (alcoholysis), and an ester with another ester (transesterification) that define in term "interesterification" to synthesize SL.

6.3.1 Chemical interestrification

In general, chemical interesterification involves the random removal of fatty acids from the original TAG, shuffling these fatty acids, and random replacement of them on the new TAG. Chemical interesterification results in a complete randomization of acyl groups in the TAG. Interesterification, alone or in combination with other processes, extends the utility of edible oils are extensively employed in a wide of food application. Commercially, chemical interesterification is used for processing of edible fats and oils to produce margarines, spreads, cooking oils, shortenings, confectionary fats, and reduced calories lipids. Two mechanisms have been proposed for alkaline-catalyzed chemical interesterification including enolate anion formation and carbonyl addition.

a) Enolate anion formation

This mechanism suggests that the reaction begins when the sodium methoxide (catalyst) attacks the acidic hydrogen from the carbon to the carbonyl carbon to produce an enolate structured. This reaction produces a carbanion, which is a strong nucleophile. The enolate anion the reacts with another ester group in the TAG molecule to produce a β-keto esters and a glycerylate, which reacts further with other carbonyl carbons to afford other β-keto esters. In this way, all ester groups in the TAG may react and move around their initial position. The same mode of action applies to ester interchange between two or more TAG molecules. The intra-ester interchange is believed to predominate in the initial stages of the reaction (Senanayake and Shahidi, 2005).

b) Carbonyl addition

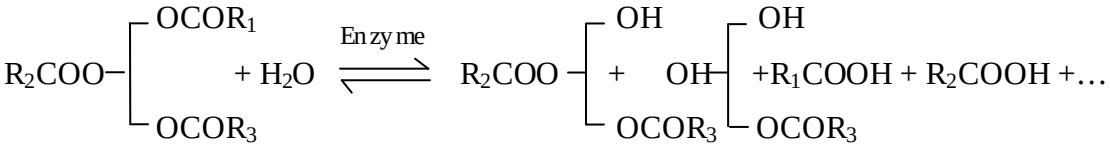
In this reaction, the alkylate ion (nucleophile) adds on to the slightly positively-charged carbonyl carbon at one of the three fatty acylglycerol ester bonds and forms a tetrahedral intermediate. The fatty acid methyl ester is then released, regenerating a glycerylate anion for further reaction. The glycerylate anion

functions as the real catalyst and transfers acyl group around the glycerol backbone. It should be noted that with the carbonyl addition mechanism, both intra- and inter-esterification reactions are possible. The similarity in the two mechanisms is that glycerylate anions are formed via both mechanisms. The basic difference is that, in the first instance, both β-keto esters and glycerylate ions are the acyl donors for interesterification, and in the second, only glycerylate ions are the acyl donor. (Marangoni and Rousseau, 1995).

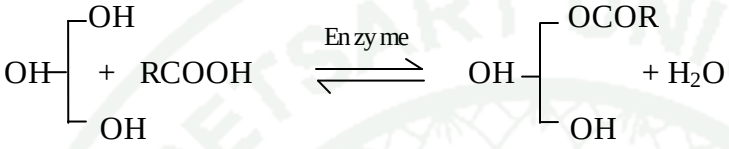
### 6.3.2 Enzymatic interesterification

Enzymatic interesterification has several advantages over the chemical assisted reactions, such as mild reaction condition lead to reduced energy consumption and less thermal damage to reactants and products, the possibility of lipase specificity toward their natural substrates, as well as high catalytic efficiency. The obtained products depend on the specific of the lipase used. Enzymatic interesterification is accomplished by using lipase. Such systems are composed of the continuous water immiscible phase (containing the lipid substrate) and an aqueous phase (containing the lipase). In the presence of water, lipase catalyzes the hydrolysis of TAG. Transesterification is the exchange of acyl group between two esters, either two TAG or a TAG and a fatty acid. It is used to alter the physical properties of individual fats and oils or fat-oil blends by altering the positional distribution of fatty acids in the TAG. In acidolysis, the transfer of an acyl group between an acid and an ester is an effective means of incorporating novel fatty acids into TAG. Alcoholysis occurs between TAG and glycerol (Figure 4).

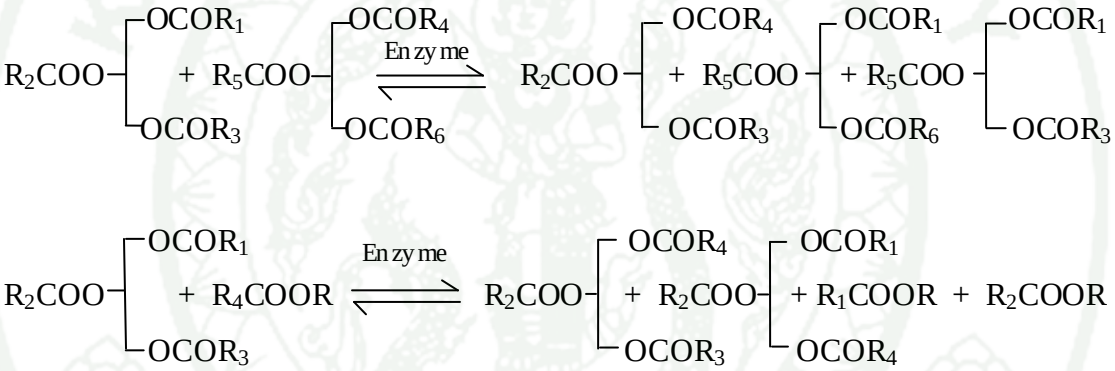
Hydrolysis



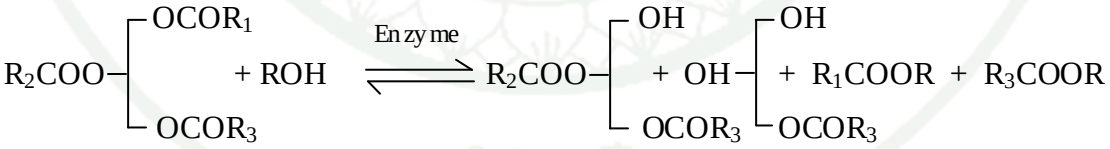

Esterification




Tranesterification




Alcoholysis




Acidolysis

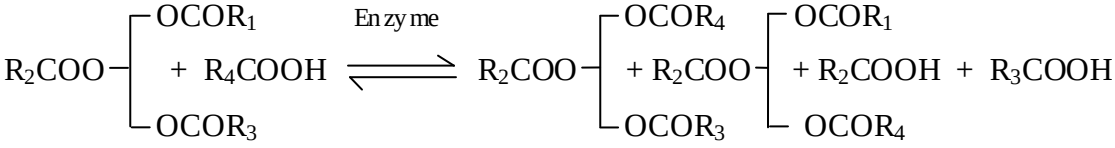



**Figure 4** Principal lipase catalyzed reaction in oil and fat modifications

**Source:** adapted from Villeneuve and Foglia (1997)

a) Lipase

Lipase is enzyme that catalyzes the hydrolysis of TAG forming free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. When reaction conditions are strictly controlled, lipase may also catalyze the formation of acylglycerol from free fatty acids and glycerol. Extracellular microbial lipase are produced by microorganisms and released into their growth environment to digest lipid materials (Senanayake and shahidi, 2005). The naturally occurring LCFA are water insoluble, and lipases are characterized by their ability to catalyze the hydrolysis of ester bonds at the inter phase between the insoluble substrate phase and the aqueous phase in which the enzyme is soluble. Specificity of lipase, one of major advantages of enzymes is their ability to catalyze reaction with high selectivity and specificity. Lipases show positional and substrate specificity.

Lipases can be sub-divided into five different classes. The first group comprises lipase, which hydrolyzed fatty acids from the TAG independent of their type or position on the TAG. They have no regiospecificity. Use of this lipase for interesterification reaction results in the random distribution of fatty acids in TAG. Examples for such enzyme include lipases produced by *Candida cylindracea*, *Corynebacterium acnes*, *Staphylococcus aureus*. The second group includes 1, 3-specific lipases, which catalyze reaction at sn-1, 3 positions of the TAGs molecule. Lipase from *Aspergillus niger*, *Rhizopus delemar*, *Rhizopus arrhizus*, *Mucor miehei* exhibit 1, 3 specificity (Senanayake and shahidi, 2005). The third group covers lipase with different rates of hydrolysis or MAG, DAG, and TAG. Some of these enzymes have been found in tissues of rats and humans. The fourth group of lipases catalyzes the exchange of specific types of fatty acids. An example for this is the extracellular lipase from the fungus *Geotrichum candidum*, which preferentially releases unsaturated *cis n*-9 fatty acid groups (Senanayake and shahidi, 2005). The fifth group contains special enzymes that show a faster hydrolysis rate for the fatty acids placed on the sn-1 position than the sn-3 position. This is commonly referred to as stereospecificity. Examples include lipoprotein lipases from milk, adipose tissue and post heparin plasma, which preferentially cleave the ester bond in the position

sn-1; and human and rat lingual lipase, which react preferentially with the fatty acids at position sn-3 (Hanel and Gelb, 1995; Lehner, 1993). The selectivity and specificity of these lipases are highly dependent on the reaction conditions used (such as water activity, temperature, pH, and immobilization). TAG, the preferred substrates for lipases are insoluble in water. They self-associate to from monomolecular films, micelles or emulsions in aqueous medium. Due to this self-association lipolysis takes place at the lipid water interface (Villeneuve and Foglia, 1997). Enzyme catalyzed reaction can occur at low temperatures and in non-solvent systems. Lipase specificity towards different fatty acids or different acylglycerol moieties has also been studies and used for the enrichment of different fatty acids. Several investigators have focused on the enrichment of EPA, DHA, ALA, AA, LA and conjugated linoleic acid (Haas *et al*., 1999). Some lipases were found to have specificity towards *trans* fatty acids. Different fatty acids exhibit different reactivity in interesterification reactions due to the specificities and inhibitory effects on lipases. Under similar conditions, different fatty acids show different rates and extent of incorporation during interesterification reactions (Pinsirodom and Parkin, 1999).

b) Lipase in lipid modification

There are a variety of specific lipases with regiospecificity and stereospecificity, which could be used for the production of specific structured lipids. Although widespread in nature, lipases have become available only recently in large quantities for industrial purposes. Some of the possible applications of lipases have been established in the last 35-50 years with the realization that lipases could be used in microaqueous organic systems at high temperatures of up to 70°C (Gupta and Roy, 2004). The interesterification reactions with sn-1, 3 specific lipase offers high catalytic efficiency, specificity and selectivity. It provides a useful way to improve the nutritional properties of lipids by incorporation of a required acyl group into a specific position of the TAG. The chemical methods do not provide this specificity (Akoh, 2006). Microbial lipases have attracted great attention, as they are thermostable, functions without co-lipase requirement and possess different specificities. Using genetic engineering, recombination or mutation, lipases may be produced with desired

properties and efficient expression. Lipases from different microbial sources like *Rhizomucor miehei, Cadida antartica, Geotrichum candidum, Rhizopus delemar, Chromabacterium viscosum, Pseodomonas,* and *Candida* species have been used for interesterification reactions. Lipase from Carica papaya latex is an example of the lipase from plant source used for interesterification.

Microbial lipases, Lipozyme IM 60 from *Rhizomucor miehei* and SP435 from *Cadida antartica* have been most commonly used. Lipozyme IM 60 is immobilized on to an anion exchange resin with well worked out specificity (Chandler *et al.*, 1998). It has been used to the maximum extent in various systems studied so far. It is evident from different studies that lipase has varying substrate specificities. Lipase from *R. miehei* has been reported to show preference for free fatty acids over methylesters (Akoh and Yee, 1997). Lipases have also been shown to have specificity towards individual fatty acids. Sonnet *et al.* (1993) reported the high selectivity of *G. candidum* lipase for oleic acid compared to together longer chain MUFA. Similar observation have been made with *R. miehei* lipase specificity for saturated, mono and PUFA (Pedersen and Holmer, 1995).

c) Lipase structure and interesterification mechanism

One of the major advances that took place in understanding lipase activity is the elucidation of structural aspects of lipase based on the knowledge gained from protein engineering. Lipases operate at oil-water interfaces and undergo a conformational change before a substrate can diffuse into the active site (Derewenda, 1994). All lipases tend to have similar three-dimensional structures and consist of the catalytic triad of His-Ser-Asp (Glu). The serine residue is the nucleophile and the other two amino acids are involved in a charge-relay system designed to improve catalysis (Figure 5). The catalytic triad of lipase is buried under a lid of a surface loop, which undergoes a conformational change, exposing the substrate to the active site. At the same time, the hydrophobic environment around the active site becomes exposed and forms a strong binding site for the interface (Derewenda *et al.*, 1992). The interestification reaction consists of four steps (Figure 5). In the first step, the active

site serine attacks the carbonyl carbon of TAG, an undissociated fatty acid or, a fatty acid alkyl ester, forming a tetrahedral intermediate (step 2). The role of the histidine and the aspartic acid residues is to make the serine hydroxyl group a stronger nucleophile without requiring strong basic conditions. This tetrahedral intermediate is stabilized by backbone amide groups, which form a structure referred to as the oxynion hole. The carbon-oxygen bond of the ester is subsequently broken (step 3), releasing either an alcohol or water depending on whether the substrate is a TAG, a fatty acid methyl ester or fatty acid. In step 4, an alcohol reacts with the acyl-enzyme intermediate, again forming a tetrahedral intermediate, which will rearrange, releasing a new TAG and regenerating the active site (Marangoni and Rousseaue, 1995).

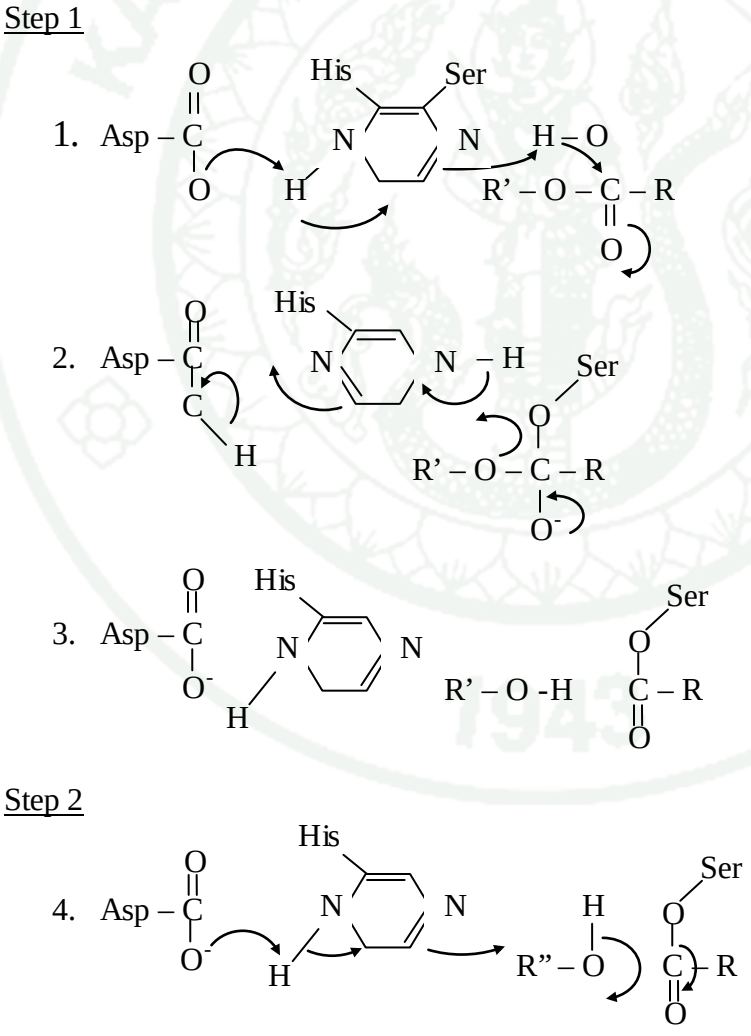
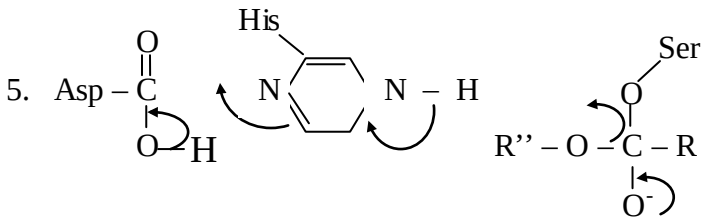



**Figure 5** Catalytic mechanism for lipase-mediated enzymatic interesterification

Step 3




Step 4

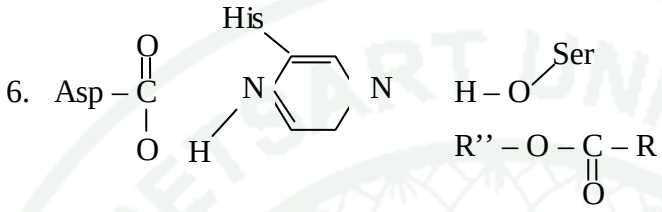



**Figure 5** (Continued)

**Source:** adapted from Maranggoni and Rousseau (1995)

d) Stability and Reusability of lipases

The stability of lipases is one of the important attributes required for their industrial applications. The stability of a lipase depends on its characteristic nature and the conditions under which it is used. Immobilization of lipases can alter enzyme stability. Generally, the activity of enzyme is reduced due to immobilization. However, the stability is often enhanced because the ternary structure and/or conformation of the enzyme are held in active forms by the support linkage. Immobilized lipases have been studies for hydrolytic (Vitro *et al.*, 1994), synthetic (Sonnet *et al.*, 1994), and inter/tranesterification (Kosugi and Azuma, 1994) reaction in both aqueous and organic media. The carrier is chosen on the basis of its mechanical strength, loading capacity, cost, chemical durability, functionality and hydrophobic or hydrophilic character. Materials tested as solid supports for immobilization of lipases include ion exchange resins (Deaesephadex A-50 or Amberlite IRA94) (Yang and Rhee, 1992), silica gel (Sonnet et al., 1994) and microporous polypropylene (Vitro *et al.*, 1994). In other cases, lipases have been immobilized by entrapping them in gels of photo-cross-linkable resins (Yang and Chen, 1994). Immobilization of lipases helps in the physical removal of the enzyme

after reaction and the enzyme can be used. This also allows one to terminate the reaction at a desired degree of esterification by physically removing the immobilized enzyme from the medium. Furthermore, immobilization usually increases the thermostability of the enzyme and they can be used even in microaqueous systems. The stability of lipases is also affected by lipid quality. It was found that minor compounds in oils and fats such as lipid hydroperoxides, phospholipids, emulsifiers, chlorophylle, carotenoids, lipid polymers, heavy metal ions and even some antioxidant affect the stability of lipases. Inhibition of enzyme activity occurs probably because all these minor compounds cover the surface of the enzyme and eventually prevent the contract between the enzyme and the substrates, except lipid hydroperoxides, or possibly, heavy metal ions which react with the enzymes and cause the conformational changes (Xu *et al.*, 1998b).

### 6.4 Applications of structured lipids

#### 6.4.1 Food

The consumer demands reduced-fat food products that has generated considerable interest in the development of fat replacers or fat substitutes. Reduced calorie SL is designed by taking advantage of either limited absorption of LCSFA or lower caloric value of SCFA. For example, SL containing behenic acid (C22:0) at sn-1, 3 positions of TAG from kokum, sal and mango fat are suitable for use in bakery and confectionery due to it has long melting ranges (Bebarta *et al.*, 2013). SL containing butyric acid (C4:0), valeric acid (C5:0) and caproic acid (C6:0) in sn-1, 3 positions of TAG of trilaurin are used in cacao butter substitutes in chocolate product due to they are easy digestible, light calorie, metabolism-stimulating and oxidation-stabilized fats especially the fatty acid composition closely resembles that of cocoa butter (Kanda *et al.*, 2010). Moreover, the synthesis of TAG with MCFA is another method used to obtain low calorie fats. Such examples include TAG containing behenic, caproic, and caprylic acids. The absorption of behenic acid in the intestine was approximately 28% in the bodyand MCFA is metabolized as rapidly as glucose that is a source of energy (Yang *et al.*, 2001). Incorporation of

MCFA and behenic acids in sn-1,3 position of TAG frompalm olein and palm stearin are suitability for use in plastic fats industries such as margarine, shortening, and cocoa butter (Chnadhapuram and Sunkireddy, 2012). Furthermore, SL resembling human milk enriched with *n*-3 fatty acids (EPA and DHA) was synthesized by enzymatic acidolysis reaction between tripalmitin, hazelnut oil fatty acids, and *n*-3 fatty acids (Şahín *et al.*, 2006).

### 6.4.2 Medicine

SL can be synthesized to obtain specific metabolic effects or to improve physical characteristics of fats. The effects of a structured lipid made from MCT and fish oil was compared with conventional LCT on the tumor growth in experimental animals. The SL was found to decrease tumor protein synthesis, reduce tumor growth in Yoshida Sarcoma-bearing rats, decrease body weight and improve nitrogen balance (Ling *et al.*, 1991). In addition, the effect of fish oil/MCT in reducing tumor growth was synergistic with tumor necrosis factor. A similar study by Mendez *et al.* (1992) compared the effects of SL made from fish oil and MCFA with a physical mix of the same components and found that the structured lipids improved the nitrogen balance in animals. Park *et al.* (1997) have demonstrated that a structured lipid containing conjugated linoleic acid (CLA) at the sn-1, 3 positions and LCFA in the sn-2 position is more rapidly hydrolyzed and efficiently absorbed that a typical LCT. Similarly, metabolic infusion of SL emulsion in healthy humans showed that the capacity of these subjects to hydrolyze SL is at least as high as to hydrolyze LCT (Nordenstrom *et al.*, 1995). This finding is significant because of earlier concern about the interference of MCT in the metabolism of LCT when both are present in a physical mix. An investigation into the in *vivo* fate of the fat emulsions based on structured lipids showed potential use of SL as core material in fat emulsion-based drug delivery systems (Hedeman *et al.*, 1996). Rats, with burn injuries, when fed on SL made from safflower oil and MCFA were found to gain higher body weight, show greater positive nitrogen balance and higher serum albumin concentration when compared to controls receiving a physical mix of these lipids (Rao and Lokesh, 2003). Enhanced absorption of LA was observed in cystic fibrosis patients fed SL containing

LCFA and MCFA (Hubbard and McKenna, 1987). A mixed acid type of TAG composed of LA and MCFA have been reported to improve immune functions (Shahidi and Haman, 2006; Kishi *et al.*, 2002). SL appeared to preserve reticuloendothelial function while improving nitrogen balance (Hyltander *et al.*, 1995). Long term feeding studies with SL containing MCFA and fish oil fatty acids showed that SL modified plasma fatty acid composition, reflecting dietary intake and induced systemic metabolic changes that persisted after the diet was discontinued (Matsuo, 2001). SL made by reacting triplamitin with unsaturated fatty acids using sn-1, 3 specific lipase closely mimicked the fatty acids distribution of human milk and was commercially released under the trade name of Betapol$^{TM}$ (Quinlan and Moore, 1993). These studies have indicated that SL have more positive beneficial effects on physiological functions and on disease conditions as compared to that could be observed with LCT or physical mixture of TAG.

**7. Response surface methodology**

Response surface methodology (RSM) uses mathematical and statistical designs for optimizing reaction conditions. It is used for the modeling and analysis of a response variable, which is influenced by multiple independent factors. The method evaluates the effects of multiple factors (reaction conditions), the interactions between them, and optimizes them in terms of the response variables (product yield or percentage incorporation of fatty acid). Based on the effect of the factors on the response variables in the range designed, a response surface that contains the optimal points for the reaction can be obtained. Several investigations have used RSM to optimize reaction condition for enzymatic reaction in organic solvents. Huang and Akoh (1996) optimized enzymatic interesterification reactions employing RSM for the incorporation of pure ethyl caprylate into soybean oil and sunflower oil. In this model, incubation time, molar ratio of ethyl caprylate to total TAG and LCT were assumed to be the most important factors affecting the reaction. Similarly, RSM was employed to elucidate acyl migration occurring during enzymatic interesterification between rapeseed oil and CA in a solvent-free media (Xu *et al.*, 1998a). Five factors, via water content, reaction temperature, enzyme load, reaction time and substrate ratio

were varied at three levels. RSM was also used to modify menhaden oil to incorporate CLA, while conserving the PUFA in the TAG. In this case, parameters such as residence time, substrate molar ratio and reaction temperature were optimized employing RSM. All parameters had a positive influence on the incorporation of carprylic acid but residence time and substrate molar ratio had a negative effect on the retention of PUFA (Xu *et al.*, 2000).

# MATERIALS AND METHODS

## Materials

### 1. Material

1.1 Atlantic salmon was raised in Lerøy Seafood Group ASA's farm around 3 years and was caught during September-November. The salmon belly derived from fillet processing of Lerøy factories from Norway.

### 2. Reagents

2.1 Lipozyme IM 60 (a commercially immobilized 1, 3-specific lipase obtained from *Rhizomucor miehei* with particle size 300-1,000 μm) on ion exchange resins was received from Novo Nordisk Bioindustrial Inc., (Danbury, CT, USA)

2.2 Capric acid (purity minimum 98%) (Fluka Chem. Co., Thailand)

2.3 Boron trifluoride in methanol (S.M. Chemical Supplies Co., Ltd., Thailand)

2.4 Chloroform (RCl Labscan Ltd., Thailand)

2.5 Cholic acid or sodium salt (Hi Media laboratories Pvt. Ltd., India)

2.6 Diethyl ether (S. Science Equipment Chemical Ltd. Part., Thailand)

2.7 Ethanol absolute (S. Science Equipment Chemical Ltd. Part., Thailand)

2.8 Glacial acetic acid (S. Science Equipment Chemical Ltd. Part., Thailand)

2.9 Hydrochloricacid (S. Science Equipment Chemical Ltd. Part., Thailand)

2.10 Lipid standard mono-, di- and triglycerides (S.M. Chemical Supplies Co., Ltd., Thailand)

2.11 Pancreatic lipase (S.M. Chemical Supplies Co., Ltd., Thailand)

2.12 Methanol (S. Science Equipment Chemical Ltd. Part., Thailand)

2.13 n-hexane (RCl Labscan Ltd., Thailand)

2.14 Petroleum ether (S. Science Equipment Chemical Ltd. Part., Thailand)

2.15 Potassium iodide (S. Science Equipment Chemical Ltd. Part., Thailand)

2.16 Polyunsaturated Fatty Acid No.3 from Menhaden Oil Standard (S.M. Chemical Supplies Co., Ltd., Thailand)

2.17 Standard C23:0 Methyl ester (S.M. Chemical Supplies Co., Ltd., Thailand)

2.18 2, 2, 4-Trimethylpentane or iso-octane (S. Science Equipment Chemical Ltd.Part., Thailand)

2.19 Tris-HCl 1 M (S.M. Chemical Supplies Co., Ltd., Thailand)

2.20 Wijs' reagent (S.T. Inter Ltd. Part., Thailand)

All chemicals used in this study were analytical grade.

**3. General Equipment**

3.1 Hydraulic press model

3.2 Steam cooker, Sure Chef Combi-steamer MCS (Henny Penny®, USA)

3.3 Stirrer withpropellers (IKA®, Fisher model, UK)

3.4 Centrifuge, Suprema 21 (Tomy, Japan)

3.5 Orbital – shaking incubator, WB 14 (Memmert, Germany)

3.6 Hot air oven, UNB 400 (Memmert, Germany)

3.7 Homogenizer, R 104 basic (IKA®, Fisher, Japan)

3.8 Suction filter and vacuum pump (GE motor, USA)

3.9 Rotary evaporator, R-124 (BUCHI, Japan)

3.10 Vortex mixer (Torika, Japan)

3.11 Freezer, temperature -18 °C (Sanyo, Thailand)

3.12 Refrigerator, temperature 4°C (Hitachi, Thailand)

3.13 Autopipette, 200 and 1,000 µl (Eppendorf, USA)

**4. Equipment for Analysis**

4.1 Colorimeter, CM 3500 D (Minolta, Japan)

4.2 Gas chromatography, GC-17A (Shimadzu, Japan) and DB-WAX capillary column (30 m ×0.25 mm ×0.25 µm) (J&W Scientific Folsom, USA)

4.3 Gas chromatography, 7890A GC (Agilent Technologies, USA) and DB-wax capillary column (30 m × 0.25 mm× 0.25 μm) (J&W Scientific Folsom, USA)

4.4 Spectrophotometer, UV-1700 (Shimadzu, Japan)

4.5 TLC-Silica gel 60 (Merck, Germany)

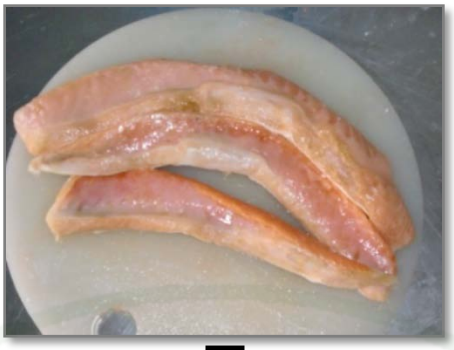
**Methods**

**1. Production of crude oil from salmon bellies and properties of the crude salmon oil**

1.1 Production ofcrude salmon oil

Atlantic salmon (*Salmo salar*) bellies were obtained from Food Project (Siam) Company Limited, Bangkok (Figure 6-a). They were transported in frozen condition to laboratory of Department of Fishery Products, Faculty of Fisheries, Kasetsart University. Upon arrival, they were kept in freezer (-18°C) for further study.

Frozen salmon bellies were thawed at room temperature (30-32°C), trimmed to remove bones and fins portions, cut at small pieces (Figure 6-b), heated with steam cooker at 95-100°C for 15 min (Figure 6-c), and crushed with silent cutter for 5-10 min (Figure 6-d). Solid fraction was separated from the liquor fraction by primary filtering through the cheesecloth. The remaining solid fraction was pressed by hydraulic press to separate the pressed cake (Figure 6-e) from the pressed liquor (Figure 6-f). The pressed liquor was composed of salmon oil, soluble protein solution, and water. The upper phase (salmon oil) was taken to be heated at 80°C for 15 min to separate the remaining soluble protein by centrifugation at 6,000 rpm (5,640 ×g), 4°C for 20 min. The obtained crude oil (Figure 6-g) was kept in amber bottles, flushed with nitrogen gas, and stored at -18°C in a freezer until the crude oil was further analyzed for physicochemical properties.



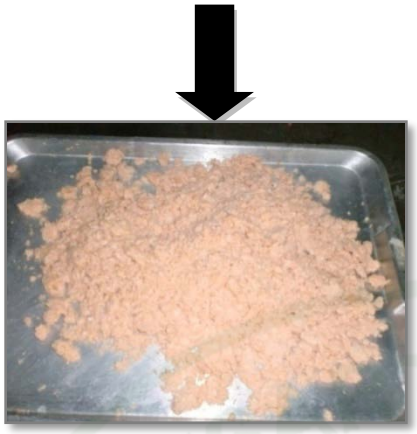
a. Atlantic salmon bellies

b. Salmon bellies after trimming

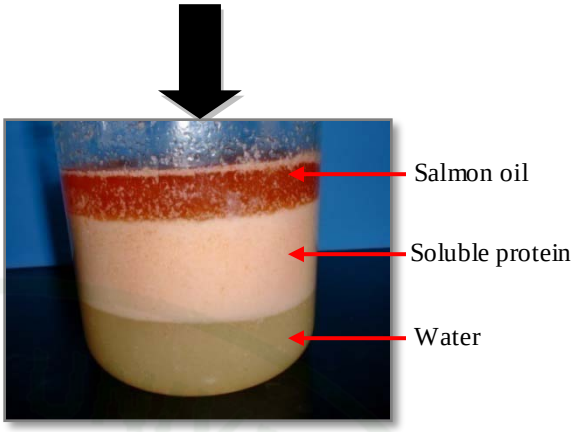
c. Heat with steam cooker

d. Crush with silent cutter

**Figure 6** Production of crude salmon oil from salmon belly



e. Solid fraction or pressed cake after pressing




f. Liquor fraction or pressed liquor after pressing

g. Crude salmon oil

**Figure 6** (Continued)

1.2 Determination of physicochemical properties of crude salmon oil

1.2.1 Yield

The yield of products obtained from crude salmon oil production including crude salmon oil, pressed cake, soluble protein solution, and water were expressed as percentage of raw material used.

1.2.2 Color value

The color analysis was carried out using a colorimeter (Minolta, Japan) and color value (CV) expressed as $L^*$ (darkness/whiteness), $a^*$ indicated in form of $-a^*$ (greenness) and $+a^*$ (redness), and $b^*$ indicated in form of $-b^*$ (blueness)

and +$b^*$ (yellowness) in CIE ($L^*$, $a^*$, $b^*$) color space (or CIELAB) system. The sample oils (20 ml) were filled into a sample holder then put it in the slot of instrument for analysis. Measurement was done in triplicate and reported an average values.

1.2.3 Peroxide value

Determination of peroxide value (PV) was followed AOCS Official method Cd 8-53 (AOCS, 1997). The oil sample (5.00±0.05 g) was dissolved with 30 ml acetic acid: chloroform solutions (ratio 3:2 (v/v)) and 0.5 ml of saturated potassium iodide (KI) solution in erlenmeyer flask. The sample mixer was occasionally shaken for 1 min, and then immediately added 30 ml of distilled water. The sample mixer and blank were titrated with 0.1 N solution of sodium thiosulfate ($Na_2S_2O_3$) by vigorous shaking. Continuous titration until the yellow color of iodine was almost disappeared and added with 2.0 ml of starch indicator solution. Continuous titration with constant agitation until blue color of starch was completely disappeared. Each result of oil sample was calculated to express as the miliequivalent (meq) peroxide to 1 kg sample following Equation 1.

PV (meq/kg) = [volume of $Na_2S_2O_3$ used to titrate sample (ml) – volume of $Na_2S_2O_3$ used to titrate blank (ml)] × concentration of $Na_2S_2O_3$ solution (N) × 1,000 / weight of sample (g) (1)

1.2.4 *p*-Anisidine value

Analysis of anisidine value (*p*-AV) was according to AOCS Official method Cd 18-20 (AOCS, 1997). The oil sample (1.00±0.01 g) was dissolved with 25 ml of iso-octane solution in volumetric flask. Sample solvent (5 ml) was pipetted into one test tube and 5 ml of iso-octane solution was also pipetted into second test tube, and then each tube was added with 1 ml of *p*-anisidine reagent and shaken. After exactly 10 min, the solvent in the first test tube in a cuvette was measured the absorbance at 350 nm of spectrophotometer by using the solution from the second test

tube as a blank in the reference cuvette. Each result of oil sample was calculated to express as *p*-AV following Equation 2.

*p*-AV= 25 × [(1.2 × absorbance of sample solution after reaction with the *p*-anisidine reagent) – absorbance of blank] / weight of sample (g) (2)

1.2.5 Iodine value

Iodine value (IV) was determined following AOCS Official method Cd 1-25 (AOCS, 1997). The oil sample (0.50±0.01 g) was dissolved with 15 ml of chloroform solution and 25 ml of Wijs solution in erlenmeyer flask then it was covered with aluminium foil and was stored in a dark place for exactly 30 min. The sample mixer and blank were immediately added 20 ml of 15% potassium iodide solution and 100 ml of distilled water. The titrand both of the sample solvent and the blank were titrated with 0.1 N solution of $Na_2S_2O_3$ combined with vigorous shaking. Continue the titration until the yellow color of iodine was almost disappeared, starch indicator solution (0.5 ml) was added and continue the titration until blue color of starch was completely disappeared. The results of unsaturation of the fatty acids were calculated to express in term of centigram (cg) of iodine absorbed to 1 grams of oil sample shown in Equation 3.

IV (cg $I_2$/ g) = (volume of $Na_2S_2O_3$ used to titrate blank (ml) – volume of $Na_2S_2O_3$ used to titrate sample (ml)) × concentration of $Na_2S_2O_3$ solution (N) ×12.69 / weight of sample (g) (3)

1.2.6 Free fatty acid

Analysis of free fatty acid (FFA) was according to AOCS Official method Ca 5a-40 (AOCS, 1997). The oil sample (7.00±0.05 g) was dissolved with 75 ml of hot neutralized alcohol in erlenmeyer flask and phenolphthalein indicator solution (2 ml) was added. The titrand mixer was titrated with 0.25 N solution of sodium hydroxide (NaOH) and vigorously shaken until the appearance of the first

permanent pink colors. Each result of oil sample was calculated for FFA expressed as % oleic acid following Equation 4.

FFA (% oleic acid) = volume of NaOH used to titrate sample (ml) × concentration of NaOH solution (N) × 28.2 / weight of sample (g) (4)

1.2.7 Sponification value

Analysis of saponification value (SV) was according to AOCS Official method Cd 3-25 (AOCS, 1997). The oil sample (5.00±0.01 g) was dissolved with 50 ml of alcoholic potassium hydroxide (containing 5-10 g of potassium hydroxide (KOH) mixed with 1.0-1.5 liters of ethyl alcohol 95%) in round bottom flask, while blank was only 50 ml of alcoholic KOH. The samples solvent and blank were completely saponified about 1 h by reflux and washed condenser with ethyl alcohol 95%. After the flask and condenser were cooled, the sample in flask was added with 1 ml of phenolphthalein indicator and titrated with 0.5 N solution of hydrochloric acid (HCl) until pink color of titrand disappeared comparing with blank. The volume of 0.5 N HCl solution required to titrate blank and sample were recorded and calculated following the Equation 5 to express as number of milligram of KOH required to sponify 1 gram of oil sample.

SV (mg KOH/g) = 56.1× (volume of HCl used to titrate blank (ml) – volume of HCl used to titrate sample (ml)) × concentration of HCl solution (N) / weight of sample(g) (5)

1.2.8 Unsponifiable matter

Analysis of unsponifiable matter (USM) was according to the AOCS Official method Ca 6b-53 (AOCS, 1997). The oil sample (2.0±0.1 mg) was dissolved in 25 ml of 95% ethyl alcohol with 1.5 ml of KOH solution in round bottom flask. The samples solvent and blank were completely saponified under reflux for 30 min

and immediately transferred to separatory funnel. The mixed solvent (from rinsing flask with 50 ml of diethyl ether) in the separatory funnel was shaken gently and was drained off the lower layer (alcoholic soap). The solvent in separatory funnel was extracted with 50 ml of diethyl ether two times by keeping only the upper layer of diethyl ether that added with 20 ml of distillated water, shook vigorously, and drained off the lower aqueous layer. The diethyl ether layer was washed with the distillated water two times then added 20 ml of 0.5 N KOH solution, shook vigorously, and drained off the lower layer. These processes were repeated two times for extracting with KOH solution. The diethyl ether was evaporated to remain the unsaponified residue on the bottom flask and was weighted to calculate mass of residue. After that the residue in flask was dissolved with 2 ml of diethyl ether and 10 ml of 95% ethyl alcohol and then added with phenolphthalein indicator. The titrand was titrated with 0.02 N NaOH solution until pink color of the end point appeared to determine mass of fatty acid in grams following weight of residue of free fatty acid content under relationship; 1 ml of 0.02 N NaOH is equivalent to 0.0056 g of oleic acid to calculation. Any reagent blank by conducting the unsaponifiable matter procedure without any fat and oil present was determined by this procedure to calculate mass of blank. Each result of oil sample was calculated to express as percentage of unsponifiable matter (USV) following the Equation 6.

$$\text{USV (\%)} = \text{mass of residue (g)} - [\text{mass of fatty acid (g)} + \text{mass of blank (g)}] \times 100 / \text{weight of sample (g)} \quad (6)$$

1.2.9 Heavy metal

Heavy metal (HM) including copper (Cu), iron (Fe), and phosphorus (P) ions of crude salmon oil and degummed oils were determined according to AOAC Official method TE-CH-134 (AOAC, 2005). The oil sample was digested with nitric acid and hydroperoxide under pressure in closed Teflon vessels and heated by microwaves oven (Ethos Sel, Milestone, Italy). The temperatures was set to digest sample in three steps starting from room temperature (25°C) to 80°C for 4 min, from 80°C to 130°C for 7 min, and from 130°C to 170°C for 5 min, and held at 170°C for

10 min. Then the sample solution was diluted with Mili-Q water and analyzed by a inductively coupled plasma optical-emission spectrometer (ICP-OES, Optima 4,300 DV, Perkin-Elmer, USA), which was expressed as mg/kg by comparison with calibration standards of Cu, Fe, and P. The limits of detection were 0.10 mg/kg of Cu and 0.65 mg/kg of Fe.

1.2.10 Fatty acid composition

Fatty acid composition (FAC) was determined by preparation of fatty acid methyl esters (FAME) as described by AOCS Official method Ce 1b-89 (AOCS, 1997). Oil sample was saponified with methanolic NaOH and free fatty acids were methylated with boron trifluoride in methanol. The FAME was extracted with iso-octane, dried over anhydrous sodium sulphate, and identified by gas chromatography (GC 17A, Shimadzu, Japan) equipped with flame ionization detector. The capillary column (30 m × 0.25 mm, 0.25 μm film thickness) was used for analysis. The FAME analysis was carried out in triplicate by injecting 1.0 μl; split ratio 1:80. The injection and detector temperatures were held at 250°C and 260°C, respectively. The flow rate of nitrogen carrier gas at 1.0 ml/min, linear speed 30 cm/s, oven temperature held at 170°C for 5 min and increased to 240°C at 5°C/min and then held at 240°C for 30 min. FAME sample (1 μl) was injected into GC in splitless mode. The Peak of FAME sample was identified and compared to the retention times of each peak of fatty acid standard PUFA No.3 from Menhaden oil (Sigma-Aldrich, USA), which was expressed as the percentage area of each FAME mixture. Saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), omega-3 fatty acid (*n*-3), omega-6 fatty acid (*n*-6), and ratio of omega-3 to omega-6 (*n*-3/*n*-6) were calculated.

**2. Production of refined salmon oil and determination of physicochemical properties of the oil**

The refining step of crude salmon oil was carried out in three stages: degumming, neutralizing, and bleaching.

2.1 Degumming

The crude salmon oil sample was heated in water bath at 80-85°C for 15 min and agitated by stirrer with two-bladed propellers at 250 rpm for 15 min. The hot crude oil was equally divided into 3 portions. There different agents were used for the degumming process. There included 1) hot water at 100°C (1% w/w of oil), 2) 85% of phosphoric acid (1% w/w of oil), and 3) 0.3% of citric acid (1% w/w of oil). Each degumming agent was added into hot crude oil with agitation at 250 rpm for 15 min. After cooling down at room temperature shown in Figure 7 (a1-3), the mixture was centrifuged with a speed of 6,000 rpm for 20 min at 4°C to separate the hydrated gum of phosphatides from degummed oil. The degummed oils were weighed, kept in amber bottles, flushed with nitrogen gas, and stored at -40°C in a freezer until further used for analysis of physicochemical properties (CV, PV, *p*-AV, IV, FFA, SV, USM, HM, and FAC), which methods used as previous described in 1.2.2-1.2.10, respectively. The degummed oils were further subjected to neutralization.

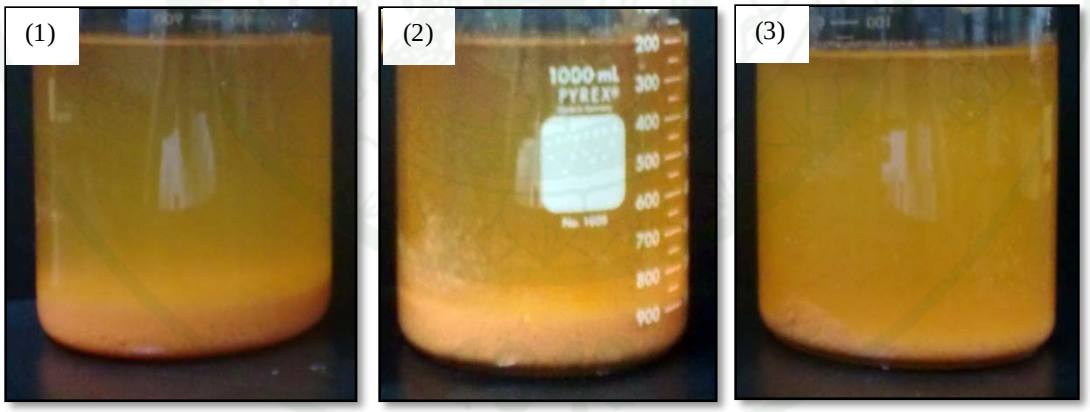
2.2 Neutralization

Degummed oils were heated at 85°C and agitated at 250 rpm for 10 min. Then addition of 20° Baumé of sodium hydroxide (14.07-14.65% w/w of actual NaOH) was added following AOCS Official method Ca 9b-52 (1998). The oils were heated again at 85°C for 60 min and centrifuged at 6,000 rpm, 20 min at 4°C to separate neutralized oil from soapstock shown in Figure 7 (b-d). The neutralized oils were washed with hot water (10% of oil), followed by agitation at 250 rpm for 10 min. This step was repeated three times until the oils were clear. The subsequent neutralized oils were maintained at 50°C and centrifuged at 6,000 rpm to separate the

residue. The neutralized oils were weighed, kept in amber bottles, flushed with nitrogen gas, and stored at -40°C in a freezer for analysis of physicochemical properties (CV, PV, *p*-AV, IV, FFA, SV, USM, and FAC), which methods used as previous described in 1.2.2-1.2.8 and 1.2.10, respectively. The neutralized oils were further processed for bleaching.

2.3 Bleaching

The neutralized oils were added with 2% (w/w) of activated carbon were agitated at 500 rpm for 15 min (room temperature). Filtration was done with Buchner funnel and filter paper No.5 containing diatomaceous earth and then the water was removed by using a rotary evaporator. The bleached oils (Figure 7 (e1-3)) were weighed, kept in amber bottles, flushed with nitrogen gas, and stored at -40°C in a freezer until further analysis for physicochemical properties (CV, PV, *p*-AV, IV, FFA, SV, USM, and FAC), which methods used as previous described in 1.2.2-1.2.8 and 1.2.10, respectively.




a. Degummed salmon oils and gums after degumming with hot water (1), phosphoric acid (2), and citric acid (3)

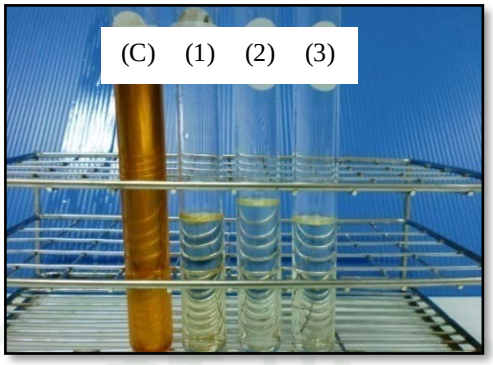
**Figure 7** Characterization of crude salmon oil and refined oils in each purification step using different degumming agents

b. Neutralized salmon oil and soapstock after degumming with hot water

c. Neutralized salmon oil and soapstock after degumming with phosphoric acid

d. Neutralized salmon oil and soapstock after degumming with citric acid

**Figure 7** (Continued)




e. Crude salmon oil (c) and bleached salmon oils after neutralization and degumming with hot water (1), phosphoric acid (2), and citric acid (3)

**Figure 7** (Continued)

**3. Production of structured lipid from refined salmon oil incorporation with capric acid by response surface methodology**

3.1 Optimal condition for structured lipids production with enzymatic acidolysis reactions by Response Surface Methodology

Four-variable five-level central composite rotatable design according to the principle of response surface methodology (RSM) was used to study the response patterns to determine the optimum combination of variables. Four variables were considered for the reaction including substrate molar ratio of refined salmon oil (RSO) to capric acid (CA) at 1:2, 1:3, 1:4, 1:5, and 1:6, incubation temperature at 37, 42.75, 48.5, 54.25, and 60°C, enzyme concentration at 2, 4, 6, 8, and 10% of total substrates weight, and incubation time at 6, 16.5, 27, 37.5, and 48 h, which represented at five levels in each factor including -2.0, -1.0, 0.0 , 1.0, and 2.0., respectively. The independent variables and experimental design are shown in Table 4 and 5.

Enzymatic acidolysis reaction mixture was carried out in 25 ml conical flask with stopper under inert atmosphere ($N_2$ gas), which consisted of substrate mixture (Figure 8-a) between RSO (100 mg) and CA (344.54-1,033.62 mg) taken at molar ratios ranging from 1:2 to 1:6 in 5 ml of hexane and then immobilized lipase

IM 60 (Figure 8-b) was added at a concentration range of 2 – 10% (w/w) as shown in Table 4 and 5. The acidolysis reaction mixtures were incubated in an orbital-shaking incubator (Figure 8-c) at 150 rpm under the specified times (16.5-48 h) and temperatures (37 - 60°C) following the experimental design both independent variables in each levels (Table 4) and reaction condition in each treatment (Table 5). After enzymatic reaction, the acidolysis product was passed through a filter paper to separate the immobilized lipase off and poured through an anhydrous sodium sulfate. Excessive amount of hexane was further evaporated by rotary evaporator. The acidolysis product was kept in amber bottle, flushed with nitrogen gas, closed tightly with stopper, and stored at -18°C in freezer until further used for triacylglycerols selection by thin layer chromatography (TLC) and analysis of FAC by gas chromatography (GC). The FAC was used to determine the efficiency of incorporation CA into triglycerides or triacylglycerols (TAG) of refined salmon oil.

**Table 4** Variables and their levels for four-variable five-level central composition rotatable design

| Variables | Symbols | | Levels | | | | |
|---|---|---|---|---|---|---|---|
| | Coded | Uncoded | -2 | -1 | 0 | 1 | 2 |
| Substrate molar ratio | $X_1$ | $x_1$ | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 |
| Temperature of reaction (°C) | $X_2$ | $x_2$ | 37.0 | 42.8 | 48.5 | 54.3 | 60.0 |
| Enzyme concentration (% w/w) | $X_3$ | $x_3$ | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 |
| Time of reaction (h) | $X_4$ | $x_4$ | 6.0 | 16.5 | 27.0 | 37.5 | 48.0 |

**Table 5** Treatment schedule for central composition rotatable design

| Runs | Substrate ratio | | Temperature of reaction (°C) | | Enzyme Concentration (% w/w) | | Time of reaction (h) | |
|---|---|---|---|---|---|---|---|---|
| | Actual ($x_1$) | Coded ($X_1$) | Actual ($x_2$) | Coded ($X_2$) | Actual ($x_3$) | Coded ($X_3$) | Actual ($x_4$) | Coded ($X_4$) |
| 1 | 3.00 | -1 | 42.75 | -1 | 4.00 | -1 | 16.50 | -1 |
| 2 | 5.00 | 1 | 42.75 | -1 | 4.00 | -1 | 16.50 | -1 |
| 3 | 3.00 | -1 | 54.25 | 1 | 4.00 | -1 | 16.50 | -1 |
| 4 | 5.00 | 1 | 54.25 | 1 | 4.00 | -1 | 16.50 | -1 |
| 5 | 3.00 | -1 | 42.75 | -1 | 8.00 | 1 | 16.50 | -1 |
| 6 | 5.00 | 1 | 42.75 | -1 | 8.00 | 1 | 16.50 | -1 |
| 7 | 3.00 | -1 | 54.25 | 1 | 8.00 | 1 | 16.50 | -1 |
| 8 | 5.00 | 1 | 54.25 | 1 | 8.00 | 1 | 16.50 | -1 |
| 9 | 3.00 | -1 | 42.75 | -1 | 4.00 | -1 | 37.50 | 1 |
| 10 | 5.00 | 1 | 42.75 | -1 | 4.00 | -1 | 37.50 | 1 |
| 11 | 3.00 | -1 | 54.25 | 1 | 4.00 | -1 | 37.50 | 1 |
| 12 | 5.00 | 1 | 54.25 | 1 | 4.00 | -1 | 37.50 | 1 |
| 13 | 3.00 | -1 | 42.75 | -1 | 8.00 | 1 | 37.50 | 1 |
| 14 | 5.00 | 1 | 42.75 | -1 | 8.00 | 1 | 37.50 | 1 |
| 15 | 3.00 | -1 | 54.25 | 1 | 8.00 | 1 | 37.50 | 1 |
| 16 | 5.00 | 1 | 54.25 | 1 | 8.00 | 1 | 37.50 | 1 |
| 17 | 2.00 | -2 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 18 | 6.00 | 2 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 19 | 4.00 | 0 | 37.00 | -2 | 6.00 | 0 | 27.00 | 0 |
| 20 | 4.00 | 0 | 60.00 | 2 | 6.00 | 0 | 27.00 | 0 |
| 21 | 4.00 | 0 | 48.50 | 0 | 2.00 | -2 | 27.00 | 0 |
| 22 | 4.00 | 0 | 48.50 | 0 | 10.00 | 2 | 27.00 | 0 |
| 23 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 6.00 | -2 |
| 24 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 48.00 | 2 |
| 25 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |

**Table 6** (Continued)

| Runs | Substrate ratio | | Temperature of reaction (°C) | | Enzyme concentration (% w/w) | | Time of reaction (h) | |
|---|---|---|---|---|---|---|---|---|
| | Actual ($x_1$) | Coded ($X_1$) | Actual ($x_2$) | Coded ($X_2$) | Actual ($x_3$) | Coded ($X_3$) | Actual ($x_4$) | Coded ($X_4$) |
| 26 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 27 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 28 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 29 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 30 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |
| 31 | 4.00 | 0 | 48.50 | 0 | 6.00 | 0 | 27.00 | 0 |

Where $X_1 = (x_1 - 4.0)/1.0$; $X_2 = (x_2 - 48.5)/5.75$; $X_3 = (x_3 - 6.0)/2.0$; $X_4 = (x_4 - 27)/10.5$


a. Substrates of reaction (1) capric acid and (2) refined salmon oil

b. Lipozyme IM 60 (immobilized lipase)

**Figure 8** Materials and production of structured lipids by enzymatic acidolysis reaction

c. Incubation of acidolysis reaction mixtures in an orbital-shaking water bath

**Figure 8 (**Continued)

3.2 Determination of properties of acidolysis products

3.2.1 Triacylglycerol

Acidolysis product (40 µl) was dissolved in 200 µl of hexane to separate TAG fraction by TLC. The aliquot of the product was spotted on TLC-silica gel plates and developed with 80:20:1 (v/v/v) of petroleum ether/diethyl ether/glacial acetic acid (Jennings and Akoh, 2001) in chamber until the developing solvent reached the top of the plate. The plate was removed from the developing chamber and then dried. The bands were visualized under iodine vapor in iodine chamber. The TAG bands were identified and compared to distance of TAG bands of triolein standard (Sigma-Aldrich, USA), which were extracted with 2:1 (v/v) of chloroform: methanol. Excessive amount of solvent was further evaporated by rotary evaporator and further analyzed for fatty acid composition by GC.

3.2.2 Fatty acid composition

The TAG fraction in each acidolysis product was saponified by methanolic KOH and FFA of product was methylated with boron trifluoride in

methanol according to method of Morrison and Smith (1963). The fatty acid methyl esters (FAME) were extracted with hexane and dried over anhydrous sodium sulphate. Fatty acid composition of product was analyzed to identify by GC (Agilent 7890A GC) (Agilent Technologies, USA) equipped with flame ionization detector. The DB-wax capillary column (30 m × 0.25 mm × 0.25 μm film thickness) (J&W Scientific Folsom, USA) was used for analysis. The injection and detector temperatures were held at 250°C and 260°C, respectively. The flow rate of nitrogen carrier gas held at 1.0 ml/min, linear speed at 30 cm/s, and oven temperature at 170°C for 5 min and then increased to 240°C at 5°C/min for 30 min. The FAME samples were run with internal standard as tricosanoic acid (C23:0) and was done in triplicate by injecting 1.0 μl; splitless mode at 1:80. The peak of FAME sample was identified and compared to the retention times of each peak of FAME standards from menhaden oil (Sigma-Aldrich, USA) that was used as an external standard. Average results were expressed the percentage area of each FAME mixture and then calculated in term of mol percentage (mol%).

The results of fatty acid composition of TAG fraction for each acidolysis product from 3.2.2 was used to determine the response values including percentage incorporation of CA, ratios of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA), monounsaturated fatty acid to polyunsaturated fatty acid (MUFA/PUFA), *n*-3 fatty acids to *n*-6 fatty acids (*n*-3/*n*-6), and linoleic acid to docosahexaenoic acid (LA/DHA) for selection of second-order polynomial model at the optimal condition to produce structured lipids and verification of the model compared with the predicted value.

**4. Production of structured lipid at the optimal condition and determination of properties of the structured lipid**

4.1 Production of structured lipids at the optimal condition

The optimal condition for structured lipid (SL) production obtained from previous section at small scale (mg-scale) was up in gram scale production that was performed by increasing concentration of refined salmon oil (RSO) from 100 mg to 20 g and also concentration of CA raised from 344.54-1,033.62 mg to 41.34-120.93 g. The substrates mixture was dissolved with 60 ml of hexane and then immobilized lipase enzyme was added following increased proportion of substrates mixture. Reaction temperature and time were fixed at 48.5°C for 48 h. After the enzymatic reaction, the reaction product was passed through a filter paper to separate the immobilized lipase, poured through an anhydrous sodium sulfate, kept in amber bottle, flushed with nitrogen gas, closed tightly with stopper, and stored at -18°C in freezer until further used for purification. In purification step, excessive amount of FFA in purified structured lipids (PSL) was removed from the reaction product following the method described by Hita *et al.* (2007). The reaction product (30 g) was dissolved in 45 ml of n-hexane and 300 ml of KOH 0.8 N in ethyl alcohol 30% solution. The product was intensively agitated to separate phases including hexane phase as upper layer and hydroalcoholic phase as lower layer in separated funnel. The hexane phase was transferred to new separated funnel while hydroalcoholic phase was extracted once more with 100 ml of n-hexane to increase the purified TAG recovery yield. Twice hexane solvents were collected together and then evaporated by rotary evaporator to remove hexane off. The PSL was flushed with nitrogen gas and kept at -18°C in freezer until further used for determination of the positional distribution of fatty acids by pancreatic lipase hydrolysis.

4.2 Determination of purified structured lipids

4.2.1 Hydrolysis of purified structured lipids

The PSL were hydrolyzed with pancreatic lipase specific in sn-1 and sn-3 positions of TAG according to Official method of European community (1991) to determine the positional distribution of fatty acids in TAG. The PSL (0.1 g) was dissolved with 2 ml of Tris-HCl buffer (1.0 M, pH 8.0), 0.5 ml of sodium cholate (0.1% w/v), and 0.2 ml of calcium chloride (22% w/v) in test tube with vigorous shaking. The mixture tube was incubated in water bath at 40°C for 1 min for preparation of hydrolysis condition, 20 mg of pancreatic lipase was added, shaken carefully, and placed in water bath at the same temperature (40°C) for 2 min. The tube was vigorously shaken for 1 min and left to cool down at the room temperature after that it was added with 1 ml of ethyl alcohol 95% to stop the reaction. The hydrolytic products in this tube was dissolved with 1 ml of HCl (6.0 M) and 1 ml of diethyl ether, vigorously shook, and centrifuged with speed 3,000 rpm for 10 min. The diethyl ether layer as upper phase was transferred to another tube and then the solvent was removed under a flow of nitrogen. The hydrolyzed PSL was further analyzed for acyl migration and fatty acid composition of fatty acids at sn-2 position and sn-1, 3 positions.

4.2.2 Acyl migration

The hydrolyzed PSL was dissolved in 200 µl n-hexane to separate acyl migration. The aliquot of hydrlyzed PSL was spotted on TLC-siliga gel plates and developed with 50:50:1 (v/v/v) of n-hexane: diethyl ether: glacial acetic acid (Jennings and Akoh, 2001) in developing chamber until the developing solvent reached the top of the plate. The plate was removed from the developing chamber and then dried. The band was visualized under iodine vapor in iodine chamber. The bands of mono-, di-, and triacylglycerols of PSL related with the bands of mono-, di-, and triacylglycerols of olein standard (Figure 9) were scraped off and extracted with 2 ml of diethyl ether. The excessive solvent was remove under a flow of nitrogen for

preparation of FAME and analyzed the fatty acid composition of fatty acids at sn-2 and sn-1, 3 positions by GC.

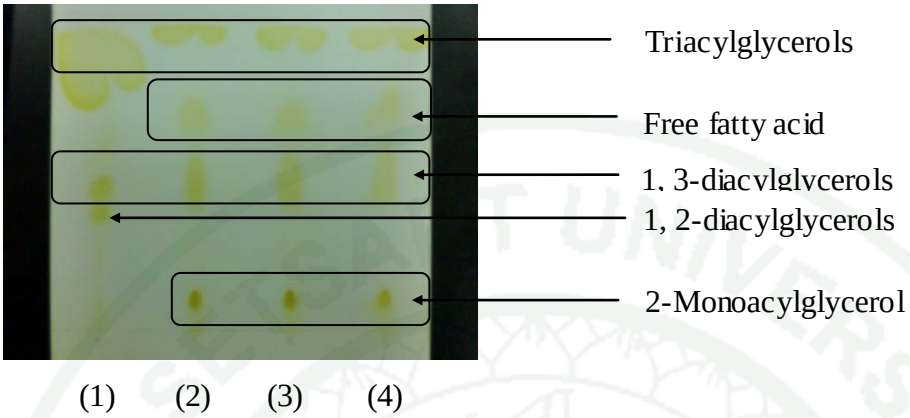



**Figure 9** Chromatograms of olein standard (1) and purified structured lipids (2-4) by thin layer chromatography method

4.2.3 Fatty acid composition of fatty acids at sn-2 and sn-1, 3 positions

The mono-, di-, and triacylglycerols of PSL were saponified by methanolic KOH and these FFA was methylated by boron trifluoride in methanol following the method described earlier in section 3.2.2.

4.2.4 Determination of physicochemical properties of purified structured lipids

PSL was analyzed the physicochemical properties including CV, PV, *p*-AV, IV, FFA, and FFC following the methods used as previous described in 1.2.2-1.2.6, and 1.2.10, respectively.

**5. Experimental design and statistical analysis**

5.1 Production of crude salmon oil and refined salmon oil

5.1.1 Experiment was run in triplicate with completely randomized design. Means and deviations of the data collected were reported. Analysis of variance (ANOVA) was performed (SPSS statistics 22.1, IBM Corporation, Armonk, NC., USA.) to detect differences of data among samples from three replications followed by Duncan's Multiple Range Test with a $P<0.05$ levels of significant treatment mean.

5.2 Production of structured lipid from refined salmon oil incorporation with capric acid

5.2.1 A five-level and four-variable central composite rotatable design was adopted (Table 4). Variables considered important for the reaction were substrate molar ratio, enzyme concentration, reaction time, and reaction temperature. The design consisted of 16 factorial points, 8 axial points and 7 center points in hypercube, which are given by 2*n* factorial design (Table 5). For creating response surface, the experimental data obtained based on the above design was fitted to a second order polynomial model of the form given as following Equation 7.

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + \varepsilon \quad (7)$$

Where $Y_i$ (i = 1-5) is the predicted response

$Y_1$ = the predicted response of percentage incorporation of CA,

$Y_2$ = the predicted response of ratio of SFA/PUFA,

$Y_3$ = the predicted response of ratio of MUFA/PUFA,

$Y_4$ = the predicted response of ratio of n-3/n-6 PUFA,

$Y_5$ = the predicted response of ratio LA/DHA

$b_0$ = the value of the fitted response at the center point of design,

$b_i$ = linear term coefficients of $X_i$ when i=1,…, n,

$b_{ii}$ = quadratic term coefficients of $X_i$ when i=1,…, n,

$b_{ij}$ = cross-product term coefficients of $X_i$ and $X_j$ when i<j and i=1,…, n-1 while j=1,…, n,

ε = the random error

The coefficients of equation 7 were obtained using STATISTICA 21.0 software package based on the data provided in Table 5. The insignificant terms were deleted based on Student's t-test. Four responses were analyzed using analysis of variance, which indicated significant sum of squares due to regression (first and second order terms) and insignificant lack of fit. The high values of coefficient of determination ($R^2$) also suggested that the model is a good fit. In order to predict workable optimum conditions, optimization of fitted polynomial for the responses was performed by a non-linear mathematical optimization method. Responses were monitored and results compared with model predictions. The fitted polynomial equation was expressed as surface and contour plots to visualize the relationship between the response and experimental levels of each factor and predict the optimum conditions.

## Place and Duration of Experiments

### 1. Places

The experiments were conducted at Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand and Central Food Technological Research Institute, Mysore, India.

### 2. Duration

The experiments were carried out from November 2009 to September 2013

# RESULTS AND DISCUSSIONS

## 1. Production of crude oil from bellies and properties of the crude salmon oil

### 1.1 Yield of crude salmon oil

Percentage of severed salmon products derived from crude salmon oil process is shown in Table 6. The products included crude salmon oil, pressed cake, soluble protein, and water, resulting in 33.7±6.1%, 36.4±1.2%, 10.3±0.6%, and 17.6±4.9% by wet basis, respectively. The crude salmon oil was found to have one third of initial salmon belly flesh that was higher than the study of Strobel *et al.* (2010), who reported total fat content of salmon fillets (wild Atlantic salmon, farmed Atlantic salmon, rainbow trout, and herring) were found to have 2.1±1.1%, 12.3±4.7%, 7.9±6.3%, and 12.7±3.1%, respectively. The color characteristics of salmon oil had red-orange color due to its carotenoid pigment contains in salmon fish. The products derived from crude salmon oil production, such as pressed cake and soluble protein could be further used for protein hydrolysate production (Zhong *et al.*, 2002). The results indicated that salmon belly is suitable to be used as raw material for salmon oil production due to high amount of lipid accumulated abundantly within bellies flesh.

**Table 6** Percentage of salmon products derived from salmon oil production

| Products | Percentage (% wet basis) |
|---|---|
| Crude salmon oil | 33.7±6.1 |
| Pressed cake | 36.4±1.2 |
| Soluble protein | 10.3±0.6 |
| Water | 17.6±4.9 |

**Note** Values are given as mean ±standard deviation from triplicate determination.

1.2 Yield of refined salmon oil

The crude salmon oil was transferred into purification process including degumming, neutralization, and bleaching. For degumming process, it is aimed to remove hydrated gum or phosphatides, which consisted of complex lipids, resin, sugar, and proteinaceous compound (Sikorski, 2002). The percentage of weight loss compared to initial crude oil is shown in Table 7. The results showed that effect of using hot water, phosphoric acid, and citric acid on percentage of weight loss in degumming process were not significantly different ($p \geq 0.05$), but there were significant difference ($p<0.05$) compared to the oil after neutralization and bleaching steps. The neutralization step gave higher weight loss than degumming process because this step can eliminate free fatty acid, carotenoid pigment, phospholipids, sulfur compound, insoluble substances in oil, and soluble matter in water to become soapstock (Sikorski, 2002). During bleaching processing, activated carbon can trap undesirable odor from oxidation product and absorb pigment, residual trace of soap, and trace metals (Rossi *et al*., 2003). Their resulted in higher in weight loss and pale color. After purification process, weight loss of purified salmon oil was almost 67-70% or the obtained purified oil was approximately 30% of initial crude salmon oil. The results indicated that high amount of undesirable compounds could be eliminated by purification process.

**Table 7** Percentage of weight loss in each purification step of salmon oil

| Steps | Degumming agent | Weight loss (%) |
|---|---|---|
| Degumming | Hot water | 6.70±0.15[a] |
| | Phosphoric acid | 4.51±0.17[a] |
| | Citric acid | 5.95±0.08[a] |
| Degumming and neutralization | Hot water | 38.99±0.33[b] |
| | Phosphoric acid | 38.59±0.67[b] |
| | Citric acid | 37.58±0.26[b] |
| Degumming, neutralization, and bleaching | Hot water | 67.02±1.25[c] |
| | Phosphoric acid | 69.80±2.32[c] |
| | Citric acid | 68.32±2.23[c] |

**Note** Values are means of triplicate determinations±standard deviation.
Different superscript letters in column under the same physical analysis were significantly different ($p<0.05$).

**2. Physicochemical properties of crude salmon oil and purified oil**

2.1 Color value

The color of salmon oil is used as one of index to indicate the purity of the oil. The results showed that crude salmon oil had red-orange color (Figure 10 (a1), (b1), and (c1)) as shown by high brightness ($L^*$), redness ($a^*$) and yellowness ($b^*$) values (81.59±0.16, 30.53±.13 and 60.13±0.14, respectively) in Table 8. The results were quite different from the report by Huang and Sathivel (2008), who denoted that crude salmon oil derived from viscera, heads, skin, and frame had brown color with $L^*$, $a^*$, and $b^*$ values of 26.95, 4.24, and 8.65, respectively. Sathivel (2005) also indicated that crude oils from heads of red salmon and pink salmon had caramel and ochre colors, respectively. $L^*$, $a^*$, and $b^*$ values of red salmon oil was 32.1, 4.9, and 14.6, respectively and for pink salmon oil was 40.3, 2.7, and 16.6, respectively. For

degumming, *L** values of degummed oil using phosphoric acid was significantly increased when compared to degummed oils with hot water and citric acid, and crude oil whereas crude oil and degummed oil with citric acid were not significantly different ($p \geq 0.05$) and they were slightly higher than degummed oil with hot water. However, *a** and *b** values of crude salmon oil was significantly decreased when compared to degummed oils with 3 degumming agents. Especially, *b** value of degummed oils using hot water and citric acid were not significantly different ($p \geq 0.05$). Due to hydrogen ions can dissociate from phosphoric acid to bind with hydroxyl groups of phospholipids, carotenoid, and proteinaceus compound higher than hot water and citric acid. While *L** value of oil was significantly increased after neutralization and bleaching steps (Figure 10 (a3-4), (b3-4), and (c3-4)). These could be explained that carotenoid pigments could be eliminated during neutralization by alkali, which was able to precipitate pigments in the form of soapstocks. The bleaching by addition activated carbon directly affected the color of salmon oil by increasing lightness and transparency. Porous structure of carbon can absolutely trap residue pigment (Rossi *et al.*, 2003). The obtained salmon oil after neutralization and bleaching processes was changed to be light yellow with increasing *L**, and reducing both *a** and *b** values. The results were different from the study of García-Moreno *et al.* (2013) who reported that the degummed and neutralized sardine oil had a dark brown color. After bleaching, the oil was changed to lighter and slightly more transparent. Huang and Sathivel (2010) also indicated that neutralized salmon oil had bronzed ochre color. After bleaching, the oil was in golden ochre color.

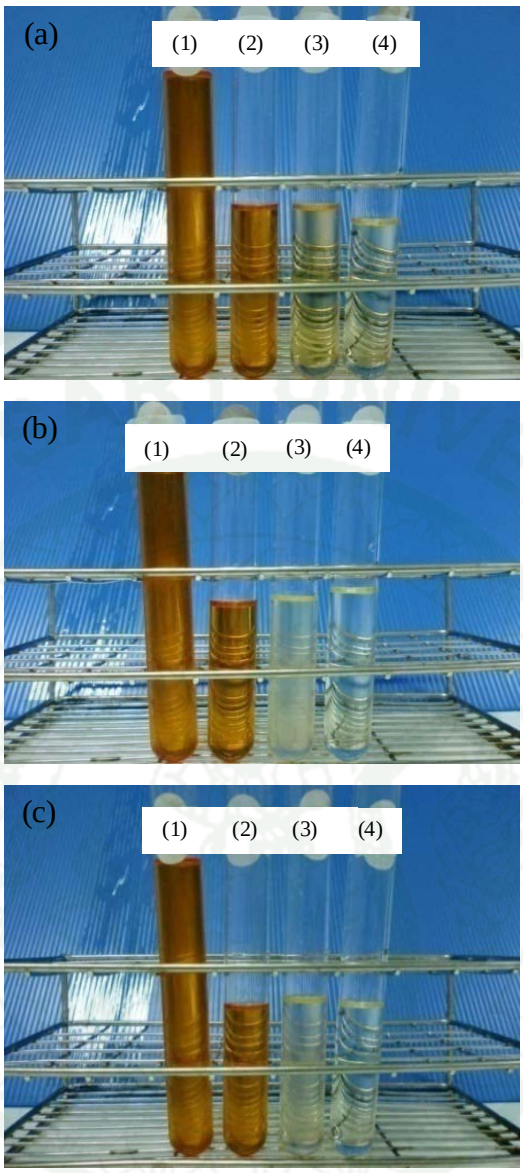



**Figure 10** Color characteristics of crude salmon oil and refined oils in each purification step; (a) refined oil through addition hot water during degumming process, (b) refined oil through addition phosphoric acid during degumming process, (c) refined oil through addition citric acid during degumming process, and (1.2.3.4) crude salmon oils, degummed oils, neutralized oils, and bleached oils, respectively

**Table 8** Color values of crude salmon oil and refined oils in each purification step using different degumming agents

| Steps | Degumming agent | Color value | | |
|---|---|---|---|---|
| | | *L** | *a** | *b** |
| Crude oil | | 81.59±0.16$^{g}$ | 30.53±0.13$^{a}$ | 60.13±0.14$^{a}$ |
| Degumming | Hot water | 80.10±0.07$^{h}$ | 29.23±0.05$^{c}$ | 58.85±0.01$^{b}$ |
| | Phosphoric acid | 84.98±0.39$^{f}$ | 11.95±0.06$^{d}$ | 41.30±0.16$^{c}$ |
| | Citric acid | 81.53±0.06$^{g}$ | 29.67±0.02$^{b}$ | 58.88±0.07$^{b}$ |
| Neutralization | Hot water | 93.23±0.07$^{c}$ | 2.09±0.03$^{f}$ | 15.56±0.10$^{f}$ |
| | Phosphoric acid | 91.53±0.27$^{d}$ | -1.05±0.05$^{h}$ | 18.65±0.02$^{d}$ |
| | Citric acid | 90.76±0.23$^{e}$ | 2.35±0.03$^{e}$ | 16.51±0.02$^{e}$ |
| Bleaching | Hot water | 97.98±0.13$^{b}$ | -0.42±0.02$^{g}$ | 8.68±0.08$^{h}$ |
| | Phosphoric acid | 98.07±0.46$^{b}$ | -2.43±0.06$^{j}$ | 10.69±0.14$^{g}$ |
| | Citric acid | 99.22±0.08$^{a}$ | -1.31±0.01$^{i}$ | 7.41±0.11$^{i}$ |

**Note** Values are means of triplicate determinations±standard deviation. Values in each column with different superscript (a-j) indicate significant differences ($p<0.05$).

2.2 Free fatty acids

The presence of free fatty acid (FFA) in the oil is an indication of insufficient process, lipase activity, or hydrolyzed contents that the oil with high quality should be low in FFA content. The FFA content of crude oil and purified oil are shown in Table 9. The crude oil gave initial FFA content of 1.39% as oleic acid. The content was not significantly different ($p>0.05$) after degumming step by using hot water and citric acid (1.42±0.06 and 1.45±0.06% as oleic acid, respectively), but was slightly higher ($p<0.05$) after degumming with phosphoric acid (1.85±0.06% as oleic acid). These could be explained that phosphoric acid is decomposed to give hydrogen ions for three times (as $7.25\times10^{-3}$, $6.31\times10^{-8}$, and $4.89\times10^{-13}$ at 25°C, respectively) that is similar to dissociation of citric acid (as $7.10\times10^{-4}$, $1.70\times10^{-5}$, and

$6.4\times10^{-6}$ at 25°C, respectively) (Zumdahl and Zumdahl, 2007), but phosphoric acid gives hydrogen ions in a wide period more than citric acid and can hydrolyze ester bond of triglycerides more than citric acid and water. FFA content of crude salmon in this study was lower than those derived from viscera, head, and skin (3.48±0.04%) reported by Huang and Sathivel (2010), but was higher than crude sardine oil (0.21±0.009%) from the study of Noriega-Rodriiguez *et al.* (2010).The results of FFA content of crude salmon oil of this study was in accordance with guideline of required quality for producing oil to be ranged from 1 to 7 (Young, 1986a). However, the FFA content in crude oil was significantly decreased after neutralization and bleaching processes. Most of FFA in degummed oils were eliminated by reacting with NaOH and formed soapstock to precipitate off and the residue FFA in neutralized oils were absorbed with activated carbon. The results of FFA in neutralized oils after degumming with phosphoric acid and citric acid were similar to neutralized oil derived from viscera, head, and skin reported by Huang and Sathivel (2010). The FFA of bleached oils were slightly increased. There was similar to bleached sardine oil from the study of Noriega-Rodriiguez *et al.* (2010). However, the FFA content of salmon oil after neutralization and bleaching processes did not exceed 0.5% as oleic acid. The acceptable level of FFA in refined fish oil should be in the range of 0.1-1.3% as oleic acid ( Rubio-Rodríguez *et al.*, 2012).

2.3 Peroxide value

Peroxide value (PV) is an indication of primary changes of oil, which is oxidized to generate hydroperoxide. Incident peroxide compound is not odoriferous substance so it cannot identify by sensory evaluation. However, peroxide compounds can be decomposed and changed to be small volatile compounds, such as carbonyl, aldehyde, ketone, alcohol, and hydrocarbons, which are the products of the secondary changes to affect the rancid off-flavors of oxidized oil (Boran *et al.*, 2006). Table 9 shows PV of crude oil compared to purified oil. Degummed oils with 3 degumming agents were significantly increased when compared to crude oil. Particularly, using hot water during degumming gave the highest PV in the oil due to broken molecule of water and repeated heat in this steps are catalysts to formation hydroperoxide from

reacting between double bond of released free fatty acid and oxygen by autoxidation. PV was decreased during neutralization because NaOH reacted with the most of FFA and oxidation products and slightly increased during bleaching due to oxygen restarted to attach double bond of long chain unsaturated fatty acid again. PV of crude salmon oil was 6.16±0.02 meq/kg, which was in agreement with guideline of required quality for producing oil (3-20 meq/kg for crude oil) (Young, 1986a). PV of degummed oils was slightly higher than crude oil, but it was decreased after neutralization and bleaching by reacting with NaOH to form soapstock and absorption with activated carbon. The results indicated that PV of the refined salmon oil after purification process did not to exceed 10 meq/kg according to the standards of edible oils (Codex, 2009).

2.4 ρ-Anisidine value

ρ-Anisidine value (ρ-AV) is a measure of the secondary products of fats and oils oxidation especially aldehydes form (principally 2-alkenals and 2, 4-dienals) and can be evaluated by the sensory with given the strong rancid smell of fish oil (Wrolstad *et al.*, 2005). Table 9 shows ρ-AV of crude salmon oil and purified oil that were significantly different ($p<0.05$). The ρ-AV of crude oil was 1.85±0.04, which is in agreement with guideline of required quality for producing oil with a desirable and acceptable shelf life (4-60 for crude oil) (Young, 1986a). The ρ-AV of all fish oil samples in every purification process did not exceed 3 that is in accordance with Noriega-Rodriiguez *et al.*, (2010) who suggested that the refined oil after purification process should be lower than 10. The results of ρ-AV of refined salmon oil after purification process was acceptable, which indicated that the oil was oxidized to a low extent.

2.5 Iodide value

Iodide value (IV) is used to determine the unsaturated degree of fatty acids in oils. It is expressed in term of centigram (cg) of absorbed iodide ($I_2$) in 1 gram of oil sample, amount of $I_2$ will react with aliphatic double bonds of unsaturated fatty

acids, which is a component in the molecule of the oil (Shahidi and Wanasundara, 2002a; AOCS, 1997). A high IV could indicate less stability to oxidation due to the oil contains high amount of double bond of unsaturated fatty acids. Conversely, a low IV indicates more stability to oxidation due to number of double bond of fatty acids is less (Wrolstad *et al*., 2005). Table 9 shows IV of crude salmon oil compared to refined oils. The crude oil had the highest IV at 144.37±0.02 cg $I_2$/g compared to the refined oils. These could be explained that crude salmon oil had the highest amount of unsaturated fatty acids up to 83.38% (sums of MUFA and PUFA) compared to refined oils as shown in Table 11. Similarly, the study of Chantachum *et al.* (2000) demonstrated that high IV of crude tuna heads oil (154 cg $I_2$/g) was in agreement with high amount of unsaturated fatty acids (51.2%). Crexi *et al.* (2009b) also indicated that high IV of crude carp viscera oil (115 cg $I_2$/g) was in accordance with high amount of unsaturated fatty acids (73.16%). IV of crude salmon oil was also similar to various crude marine fish such as capelin (95-160 cg $I_2$/g), herring (115-160 cg $I_2$/g), and menhaden (120-200 cg $I_2$/g) (Bimbo, 1998). IV of degummed oils using hot water and phosphoric acid (128.33±0.07 and 131.96±0.05 cg $I_2$/g, respectively) were significantly decreased ($p<0.05$) when compared to crude oil and degummed oil using phosphoric acid. While IV of neutralized oil after degumming with phosphoric acid and citric acid and all bleached oils tended to decrease during neutralization and bleaching, but they were not significant different ($p\geq0.05$). This decrease in IV for these oils during the refining process may be due to the reduction of unsaturated fatty acids, which is confirmed by the results showed in Table 10 especially for MUFA. These result was similar to the study of Menegazzo *et al.* (2014) demonstrated that IV of nile tilapia and hybrid sorubim refined oils were decreased from 84.54 to 82.42 cg $I_2$/g and 82.47 to 80.04 cg $I_2$/g, respectively after neutralization and bleaching when compared to crude oil.

### 2.6 Saponification value

Saponification value (SV) is determination of molecular weight of fatty acid in the oil via amount of alkali required to saponify a defined amount of oil sample. It is expressed in mg potassium hydroxide per gram oil sample that indicates

the mean molecular weight of the oil's triglycerides (TAG) and when divided by 3 give the mean molecular weight of the constituent fatty acids (Wrolstad *et al.*, 2005). Therefore, a low SV indicates high molecular weight of fatty acid that has low number of TAG molecules per gram sample. Conversely, a high SV indicates low molecular weight of fatty acid, which has high number of TAG molecules per gram sample (Shahidi and Wanasundara, 2002b). Table 9 shows SV of crude oil compared to refined oils. Crude salmon oil had the highest SV at 215.24±0.10 mg KOH/g, which was similar to the report of SV in tuna head oil (217.3-235.7 mg KOH/g) (Chantachum *et al.* 2000). SV of degummed oil using hot water, citric acid and phosphoric acid (212.42±0.23, 202.23±0.17, and 197.96±0.37 mg KOH/g, respectively) were significantly decreased when compared to crude oil. After neutralization and bleaching, SV of neutralized oils after degumming with hot water and phosphoric acid and all bleached oils tended to decrease, but they were not significantly different ($p \geq 0.05$). The results indicated that crude salmon oil composed of the lowest molecular weight of fatty acids that affected the highest number of TAG molecules per gram sample, resulting in the highest SV. After refining, molecular weight of fatty acids in the oil was increased while number of TAG molecules per gram sample was reduced due to most FFA was hydrolyzed by alkali during neutralization step.

2.7 Unsaponifiable matter

Unsaponifiable fraction or non-saponifiable material (USM) is contaminated compound in oils that will remain after saponification as sterols, fatty alcohol, glyceryl ethers, hydrocarbons and minor quantities of pigments, vitamins, and various oxidation products (Rossell, 2009; Boran *et al.*,2006). Table 9 shows USM of crude salmon oil compared to refined oils. USM of crude salmon oil was 0.16±0.05%, which was lower than the reports of Chantachum *et al.* (2000) and Tengku-Rozaina and Birch (2013) who indicated that crude tuna heads oil and hoki oil had USM at 3.6-4.1% and 4.10%, respectively. The amount of USM in fish oil varies according to fish species (Bimbo, 1998) and is usually less than 2%, but it can increase to 8% under certain seasonal and feeding conditions (Young,1986a). USM of

degummed oils with phosphoric acid and citric acid were significantly increased when compared to crude oil and degummed oil with hot water. These could be explained that hydrogen ions can dissociate from phosphoric acid and citric acid to bind with hydroxyl groups of phospholipids, carotenoid, and proteinaceus compound higher than hot water, resulting in increasing non-triglyceride substances (including hydrocarbon, alcohols and sterols) that released during degumming. After neutralization and bleaching, USM of neutralized oil after degumming with citric acid and all bleached oils were decreased compared to degummed oils with phosphoric acid and citric acid and neutralized oils after degumming with hot water and phosphoric acid, but they were not significantly different ($p \geq 0.05$) compared to crude oil and degummed oil with hot water. These results were different from the study of Tengku-Rozaina and Birch (2013) indicated that USM of refined hoki oil was increased from 4.10 to 7.24% after bleaching when compared to crude oil. However, there is no standard of USM for crude and refined fish oil according to Codex standard (Codex, 2009).

2.8 Heavy metals

The remaining residue of heavy metals (HM), such as copper (Cu), Iron (Fe), and phosphorus (P) in fish could come from environmental condition to cause the HM accumulation in fish body and can lead to remain in fish oil products. Consumption of fish oil contaminated high HM residual might cause harmful health for consumers from their accumulation in the body (Rossell, 2009). The results in Table 9 shows that Cu, Fe, and P ions in crude salmon oil derived from belly were less than 1.00, 2.30, and 69.86 mg/kg, respectively, which was in agreement with suggestion of required crude oil after extraction should be less than 2.0 mg/kg of Cu (Rubio-Rodríguez *et al.*, 2012), 0.5-7.0 mg/kg of Fe and 5.0-100.0 mg/kg of P (Young, 1986b). After degumming, the residual of Cu in degummed oil with hot water was less than 1.00 mg/kg, but they were not detected in degummed oils using phosphoric and citric acids. The residual of Fe in degummed oil with hot water was up to 6.70 mg/kg due to hydration caused phosphatides insoluble in the oil especially phosphatidylethanolamine and phosphatidic acid that can form complex with Fe ions

to become increasing non-hydratable phosphatides (Johnson, 2002). However, it was lower for degummed oils with phosphoric acid and citric acid (1.40 and 1.38 mg/kg, respectively). The residue of P in degummed oil with phosphoric acid was up to 129.28 mg/kg due to phosphoric acid was added during degumming to remove phosphatides, which affected higher P content. However, P content could be reduced or removed during neutralization according to the report of Sathivel *et al.* (2003). Also the study of Crexi *et al.* (2010) demonstrated that P content in crude and degummed carp viscera oil were found up to 201 and 94.2 mg/kg, respectively and were not detected after neutralization. Noriega-Rodríguez*et al.* (2010) reported that P content in crude sardine oil was 1.86±0.20 mg/kg and was not detected after neutralization. These results were in agreement with guideline of refined oils following Codex standard (Codex, 2009) that Cu and Fe should be less than 0.1 and 2.5 mg/kg, respectively. However, there is no standard for P in refined oils. Furthermore, Cu and Fe ions in degummed oils with phosphoric acid and citric acid were lower than degummed oil with hot water. These could be explained that citric acid and phosphoric acid could chelate metals by reducing their redox potentials and stabilizing the oxidized form of the metal. Moreover, carboxyl groups of citric acids and phosphate groups of phosphoric acids could bind with metals and form complexes to precipitate off during process (Akon and Min, 2002). The results demonstrated that degummed oil using citric acid could lower Cu, Fe, and P contaminated in the oils better than using hot water and phosphoric acid.

### 2.9 Fatty acid composition

The example of fatty acid composition (FAC) in methylesters form of refined oils in this study is shown in Figure 11. The results of FAC of crude salmon oil and refined oils showed in Table 10. There were not significantly different ($p>0.05$) in fatty acid composition of purified oil compared to the crude oil. There were 3 groups of fatty acids found in salmon oil including 1) saturated fatty acids (SFA) consisted of myristic acid (MRA, C14:0), plamitic acid (PMA, C16:0), and stearic acid (STA, C18:0); 2) monounsaturated fatty acids (MUFA) including plamitoleic acid (PTA, C16:1 *n*-7), veccenic acid (VCA, C18:1 *n*-7), oleic acid (OA,

C18:1 *n*-9), gadoleic acid (GDA, C20:1 *n*-9), and eucic acid (EUA, C22:1 *n*-9); and 3) polyunsaturated fatty acids (PUFA) consisted of linoleic acid (LA, C18:2 *n*-6), alpha–linolenic (ALA, C18:3 *n*-3), stearidonic acid (SDA, C18:4 *n*-3), eicosatetraenoic acid (ETA, C20:4 *n*-3), arachidonic acid (AA, C20:4 *n*-6), eicosapentaenoic acid (EPA, C20:5 *n*-3), docosapentaenoic acid (DPA, C22:5 *n*-3), and docosahexaenoic acid (DHA, C22:6 *n*-3). There were 16 fatty acids found in salmon oil for this study that differed from the report by Ackman (2000) who found only 14 fatty acids excluding VCA and ETA in salmon oil obtained from fillet. Strobel *et al.,* (2010) also showed that salmon oil derived from fillets of wild Atlantic salmon, farmed Atlantic salmon, rainbow, and herring had only 13 fatty acids excluding MRA, VCA, and ETA. The major fatty acid found in salmon oil derived from belly included OA, PMA, LA, DHA, EUA, EPA, and GDA accounting for 74.70 to 77.08% of the total fatty acids. Table 10 shows percentage of SFA, MUFA, and PUFA of crude salmon oil and purified oil in the range of 16.80-20.41%, 47.67-49.45%, and 39.41-34.27%, respectively. The results were similar to report of Blanchet *et al.* (2005), who suggested that fatty acid composition of wild and farmed Atlantic salmons were 19.0±1.0% and 25.6±2.9% of SFA, 53.7±3.9% and 33.4±7.9% of MUFA, and 27.3±3.0% and 41.0±5.8% of PUFA, respectively.

Dietary ratios of *n*-3/*n*-6 PUFA have been considered to implicate in controlling markers of metabolic syndrome that the long chain *n*-3 PUFA has been shown to decrease insulin resistance, triglyceride levels, heart rate, and blood pressure, and increase high density lipoprotein cholesterol (Carpentien *et al*., 2006), Conversely, *n*-6 PUFA can increase inflammatory signals and have been associated with cardiovascular heart disease (Hibbeln *et al.,* 2006). The ratio of *n*-3/*n*-6 of salmon oil in this study was in the range of 1.58-2.17 that was in agreement with the study of Strobel *et al.,* (2010) who denoted that ratio of *n*-3/*n*-6 of salmon oil from fillet was 2.89±1.96. In addition, AA, EPA, and DHA are considered to be the important fatty acids as precursors for eicosanoids in the human body. Normally, AA, DHA and EPA can be synthesized from LA and ALA, respectively by enzymes (δ-5 and δ-6 desaturases). However, LA and ALA are essential fatty acids and cannot be synthesized in human body, but they must be obtained from plants and animals in the

diet. Although humans have the capacity to convert ALA to EPA and DHA, the efficiency of conversion is low, in particular to DHA. Generally, ALA intake increases EPA and DPA, but there is very little increase in DHA in plasma fractions (platelets, white cells and red blood cells) or breast milk (Francois *et al*., 2003; Burdge and Calder, 2005). The results showed that AA in salmon oils of this study were 1.06-1.22%, which were higher than that reported by Ackman (2000) in which AA of salmon oil derived from fillet was 0.5%. Strobel *et al.,* (2010) also indicated that salmon oils from both wild and farmed Atlantic salmon were found to be AA at 0.40±0.11% and 0.40±0.13%, respectively. EPA of refined salmon oils were found to have in range of 5.11-5.26%, which also were higher than the study of Ackman (2000) who found that EPA was 4.6%. DHA of refined salmon oils were found to be in range of 7.03-7.85%, which were lower than Ackman (2000) who reported that DHA was up to 11.9%. The results were little different from the report of Strobel *et al.*, (2010) showed that salmon oils from both wild and farmed Atlantic salmons had DHA at 16.90±6.18% and 8.04±2.67%, respectively. Therefore, the difference between fatty acid composition and fatty acid content in refined salmon oil depends on types of initial raw material, extraction process, and refining process, which keep the essential fatty acid and the specific chemical structures.

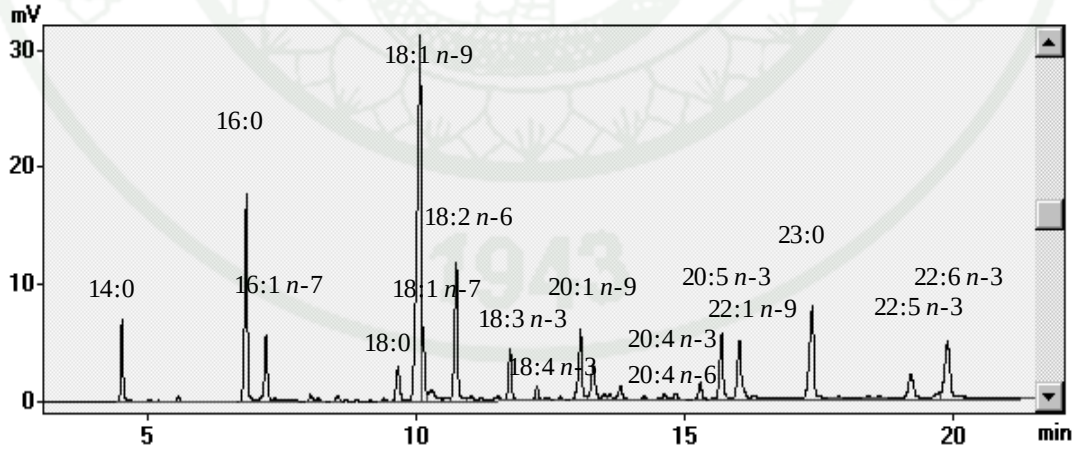



**Figure 11** Fatty acid composition in methyl esters form of bleached oil after neutralization and degumming with citric acid by gas chromatography

**Table 9** Chemical properties of crude salmon oil and refined oils in each purification step using different degumming agents

| Steps | Degumming agent | Free fatty acid (% as oleic acid) | Peroxide value (meq/kg) | Anisidine value | Iodine value (cg $I_2$/g) | Sponification (mg KOH/g) | Unsaponi-fiable matter (%) | Heavy metals (mg/kg) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Copper | Iron | Phos phorus |
| Crude oil | | 1.39±0.00$^{d}$ | 6.16±0.02$^{bc}$ | 1.85±0.04$^{c}$ | 144.37±0.02$^{d}$ | 215.24±0.10$^{d}$ | 0.16±0.05$^{ab}$ | <1.00 | 2.30 | 69.86 |
| Degumming | Hot water | 1.42±0.06$^{d}$ | 10.95±0.20$^{d}$ | 1.55±0.06$^{b}$ | 128.33±0.07$^{ab}$ | 212.42±0.23$^{bc}$ | 0.14±0.03$^{a}$ | <1.00 | 6.70 | 63.24 |
| | Phosphoric acid | 1.85±0.06$^{e}$ | 7.53±0.08$^{c}$ | 0.93±0.02$^{a}$ | 140.90±0.06$^{cd}$ | 202.23±0.17$^{ab}$ | 0.42±0.07$^{e}$ | ND | 1.40 | 129.28 |
| | Citric acid | 1.45±0.06$^{d}$ | 10.96±0.05$^{d}$ | 1.47±0.04$^{b}$ | 131.96±0.05$^{abc}$ | 197.96±0.37$^{a}$ | 0.33±0.01$^{de}$ | ND | 1.38 | 62.23 |
| Neutralization | Hot water | 0.27±0.06$^{b}$ | 7.51±0.07$^{c}$ | 2.01±0.03$^{c}$ | 122.38±0.05$^{a}$ | 193.76±0.22$^{a}$ | 0.31±0.13$^{cde}$ | - | - | - |
| | Phosphoric acid | 0.20±0.00$^{a}$ | 4.11±0.01$^{a}$ | 2.96±0.01$^{e}$ | 134.14±0.07$^{bcd}$ | 194.53±0.33$^{a}$ | 0.30±0.05$^{cde}$ | - | - | - |
| | Citric acid | 0.20±0.00$^{a}$ | 4.10±0.01$^{a}$ | 2.87±0.05$^{e}$ | 137.52±0.03$^{bcd}$ | 201.60±0.13$^{b}$ | 0.19±0.07$^{abc}$ | - | - | - |
| Bleaching | Hot water | 0.40±0.02$^{c}$ | 9.59±0.01$^{d}$ | 1.39±0.02$^{b}$ | 137.94±0.05$^{bcd}$ | 196.55±0.27$^{a}$ | 0.09±0.05$^{a}$ | - | - | - |
| | Phosphoric acid | 0.39±0.02$^{c}$ | 4.10±0.01$^{a}$ | 2.44±0.01$^{d}$ | 134.57±0.04$^{bcd}$ | 197.84±0.17$^{a}$ | 0.18±0.04$^{abc}$ | - | - | - |
| | Citric acid | 0.37±0.01$^{c}$ | 4.47±0.02$^{a}$ | 2.12±0.02$^{c}$ | 130.33±0.02$^{ab}$ | 191.43±0.39$^{a}$ | 0.12±0.02$^{a}$ | - | - | - |

**Note** Values are means of triplicate data±standard deviation, ND = Not detected, values in each column with different superscript (a-e) indicate significant differences ($p<0.05$).

**Table 10** Fatty acid composition (%) and sum of fatty acid content (%) of crude salmon oil and refined oils in each purification step using different degumming agents

| Fatty acids | Crude oil | Degumming | | | Neutralization | | | Bleaching | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Hot water | Phosphoric acid | Citric acid | Degummed with hot water | Degummed with phosphoric acid | Degummed with citric acid | Degummed with hot water | Degummed with phosphoric acid | Degummed with citric acid |
| C14:0 | 3.81±0.51[a] | 4.26±0.28[a] | 4.09±0.21[a] | 3.81±0.72[a] | 3.74±0.19[a] | 4.12±0.31[a] | 4.02±0.10[a] | 3.97±0.23[a] | 3.99±0.08[a] | 3.95±0.15[a] |
| C16:0 | 10.70±0.23[a] | 13.30±1.15[a] | 12.71±1.00[a] | 12.09±1.10[a] | 11.72±1.75[a] | 11.96±0.86[a] | 12.02±0.72[a] | 12.28±0.81[a] | 11.91±0.82[a] | 11.99±1.12[a] |
| C18:0 | 2.29±0.05[a] | 2.85±0.29[a] | 2.68±0.27[a] | 2.53±0.27[a] | 2.59±0.46[a] | 2.29±0.49[a] | 2.23±0.79[a] | 2.66±0.37[a] | 2.54±0.22[a] | 2.50±0.21[a] |
| C16:1 *n*-7 | 4.44±0.15[a] | 4.62±0.04[a] | 4.57±0.14[a] | 4.70±0.16[a] | 4.58±0.13[a] | 4.66±0.07[a] | 4.53±0.43[a] | 4.65±0.16[a] | 4.40±0.18[a] | 4.51±0.09[a] |
| C18:1 *n*-7 | 3.58±0.28[a] | 3.74±0.63[a] | 4.00±0.18[a] | 3.59±0.47[a] | 4.11±0.74[a] | 3.81±0.96[a] | 3.30±0.42[a] | 3.84±0.06[a] | 4.03±0.13[a] | 3.66±0.17[a] |
| C18:1 *n*-9 | 29.87±0.93[a] | 29.51±1.19[a] | 29.56±0.76[a] | 29.09±1.17[a] | 28.50±0.84[a] | 27.79±1.18[a] | 29.51±1.08[a] | 30.04±0.80[a] | 29.29±1.08[a] | 30.69±1.21[a] |
| C20:1 *n*-9 | 5.34±0.20[ab] | 5.52±0.12[ab] | 5.49±0.08[ab] | 5.59±0.42[ab] | 5.14±0.34[ab] | 5.62±0.27[ab] | 5.83±0.08[b] | 4.91±0.31[ab] | 5.29±0.25[ab] | 4.47±0.09[a] |
| C22:1 *n*-9 | 6.22±0.69[a] | 5.47±0.14[a] | 5.56±0.20[a] | 5.46±0.40[a] | 5.34±0.61[a] | 5.89±0.29[a] | 5.71±0.25[a] | 5.17±0.14[a] | 5.31±0.19[a] | 5.53±0.29[a] |
| C18:2 *n*-6 | 9.78±0.75[a] | 8.84±0.53[a] | 10.05±1.03[a] | 10.61±1.22[a] | 11.70±1.27[a] | 10.53±0.37[a] | 10.42±0.90[a] | 10.07±0.56[a] | 9.92±0.48[a] | 10.43±0.33[a] |
| C18:3 *n*-3 | 3.60±0.37[a] | 3.07±0.13[a] | 3.50±0.67[a] | 3.68±0.55[a] | 3.61±0.80[a] | 3.94±0.34[a] | 3.66±0.31[a] | 3.77±0.24[a] | 3.84±0.35[a] | 3.88±0.35[a] |
| C18:4 *n*-3 | 1.32±0.42[a] | 0.80±0.08[a] | 0.95±0.23[a] | 0.85±0.23[a] | 1.01±0.30[a] | 1.04±0.08[a] | 0.97±0.10[a] | 1.09±0.07[a] | 1.09±0.02[a] | 1.10±0.03[a] |
| C20:4 *n*-3 | 1.41±0.13[a] | 1.14±0.09[a] | 1.22±0.08[a] | 1.28±0.04[a] | 1.16±0.17[a] | 1.34±0.04[a] | 1.30±0.15[a] | 1.29±0.05[a] | 1.29±0.11[a] | 1.27±0.06[a] |

**Table 10** (Continued)

| Fatty acids | Crude oil | Degumming | | | Neutralization | | | Bleaching | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Hot water | Phosphoric acid | Citric acid | Degummed with hot water | Degummed with phosphoric acid | Degummed with citric acid | Degummed with hot water | Degummed with phosphoric acid | Degummed with citric acid |
| C20:4 *n*-6 | 0.94±0.18$^{a}$ | 1.41±0.21$^{a}$ | 1.26±0.47$^{a}$ | 1.56±0.67$^{a}$ | 1.55±0.54$^{a}$ | 1.72±0.79$^{a}$ | 2.03±0.22$^{a}$ | 1.06±0.24$^{a}$ | 1.22±0.19$^{a}$ | 1.10±0.32$^{a}$ |
| C20:5 *n* -3 | 5.49±0.44$^{c}$ | 4.30±0.29$^{ab}$ | 4.79±0.45$^{abc}$ | 4.44±0.67$^{abc}$ | 4.22±0.61$^{ab}$ | 4.58±0.56$^{abc}$ | 4.09±0.05$^{a}$ | 5.26±0.13$^{bc}$ | 5.13±0.12$^{abc}$ | 5.11±0.18$^{abc}$ |
| C22:5 *n*-3 | 2.84±0.59$^{a}$ | 2.55±0.16$^{a}$ | 2.49±0.06$^{a}$ | 2.23±0.23$^{a}$ | 2.27±0.13$^{a}$ | 2.49±0.11$^{a}$ | 2.41±0.06$^{a}$ | 2.66±0.47$^{a}$ | 2.77±0.14$^{a}$ | 2.61±0.12$^{a}$ |
| C22:6 *n*-3 | 8.55±1.00$^{a}$ | 8.30±0.26$^{a}$ | 8.92±0.46$^{a}$ | 8.58±0.33$^{a}$ | 8.75±0.09$^{a}$ | 8.33±1.16$^{a}$ | 7.97±1.01$^{a}$ | 7.25±0.34$^{a}$ | 7.85±0.77$^{a}$ | 7.03±0.47$^{a}$ |
| ΣSFA | 16.80±0.76$^{a}$ | 20.41±1.67$^{a}$ | 19.48±1.47$^{a}$ | 18.43±1.95$^{a}$ | 18.05±1.29$^{a}$ | 18.37±1.06$^{a}$ | 18.27±1.36$^{a}$ | 18.91±1.06$^{a}$ | 18.44±1.11$^{a}$ | 18.44±1.42$^{a}$ |
| ΣMUFA | 49.45±0.93$^{a}$ | 48.86±1.98$^{a}$ | 49.18±0.56$^{a}$ | 48.43±1.21$^{a}$ | 47.67±1.72$^{a}$ | 47.77±1.18$^{a}$ | 48.88±0.55$^{a}$ | 48.61±0.66$^{a}$ | 48.32±0.93$^{a}$ | 48.86±0.78$^{a}$ |
| ΣPUFA | 33.93±1.18$^{a}$ | 30.41±1.24$^{a}$ | 33.18±1.05$^{a}$ | 33.23±1.02$^{a}$ | 34.27±1.29$^{a}$ | 33.97±1.53$^{a}$ | 32.85±1.04$^{a}$ | 32.45±0.70$^{a}$ | 33.11±1.47$^{a}$ | 32.53±0.79$^{a}$ |
| Σ*n*-3 | 23.21±1.20$^{a}$ | 20.16±1.40$^{a}$ | 21.87±1.29$^{a}$ | 21.06±0.87$^{a}$ | 21.02±0.78$^{a}$ | 21.73±1.30$^{a}$ | 20.40±1.38$^{a}$ | 21.32±0.81$^{a}$ | 21.97±1.42$^{a}$ | 21.00±0.84$^{a}$ |
| Σ*n*-6 | 10.72±0.57$^{ab}$ | 10.25±0.08$^{a}$ | 11.31±0.66$^{abc}$ | 12.17±0.57$^{abc}$ | 13.25±0.74$^{c}$ | 12.25±0.57$^{abc}$ | 12.45±0.84$^{bc}$ | 11.13±0.41$^{ab}$ | 11.14±0.3$^{ab}$ | 11.53±0.13$^{abc}$ |
| *n*-3/*n*-6 | 2.17±0.00$^{a}$ | 1.97±0.34$^{a}$ | 1.93±0.12$^{a}$ | 1.73±0.28$^{a}$ | 1.58±0.34$^{a}$ | 1.77±0.12$^{a}$ | 1.64±0.21$^{a}$ | 1.92±0.12$^{a}$ | 1.97±0.09$^{a}$ | 1.82±0.08$^{a}$ |

**Note** Values are given as mean±standard deviation from triplicate determination. Values in each row with different superscript (a-c) indicate significant differences ($p<0.05$). ∑ SFA: sum of saturated fatty acid; ∑ MUFA: sum of monounsaturated fatty acid; ∑ PUFA: sum of polyunsaturated fatty acid; ∑*n*-3: sum of omega-3 fatty acid; ∑*n*-6: sum of omega-6 fatty acid; *n*-3/*n*-6: ratio of omega-3 fatty acid to omega-6 fatty acid.

**3. Structured lipids made from refined salmon oil incorporation with capric acid by response surface methodology design**

3.1 Fatty acid composition

The new triacylglycerols (TAG) of structured lipids (SL) (after acidolysis reaction) could be separated from free fatty acids and other intermediary acylglycerols (monoacylglycerols (MAG) and diacylglycerols (DAG)) by TLC and was further determined for fatty acid composition by GC. The main fatty acid composition of RSO (refined salmon oil) and SL (Table 11) under the different experimental conditions was used to select the optimal conditions (temperature and time) for SL production. The major fatty acids found for RSO were OA, which counted for 37.14 mol%, followed by PMA (11.95 mol%), LA (10.54 mol%), EPA (8.20 mol%), EUA (6.12 mol%), and DHA (5.82 mol%). The ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, LA/DHA were 0.63, 1.81, 1.52, and 1.29, respectively (Table 12). After enzymatic acidolysis reaction, SL tended to increase in SFA and MUFA and decrease in *n*-3 fatty acids and LA from incorporation of CA. The highest CA incorporation into TAG of salmon oil was achieved under the experimental conditions of run number 15 (substrate molar ratio at 1:3, enzyme concentration at 8% (w/w), reaction temperature at 54.25°C, reaction time at 37.50 h) and 22 (substrate molar ratio at 1:4, enzyme concentration at 10% (w/w), reaction temperature at 48.50°C, and reaction time at 48 h) at 13.69 and 10.84 mol%, respectively while under the other experimental conditions, the incorporation of CA was lower than 9 mol% (Table 12). Under the conditions of run number 15 and 22, the SL were lower in EPA and DHA contents when compared to their contents in the initial oil (8.20 mol% of EPA and 5.82 mol% of DHA in initial oil versus 5.10 mol% of EPA and 3.76 mol% of DHA in run 15 and 4.56 mol% of EPA and 4.52 mol% of DHA in run number 22), leading to decrease in the ratio of *n*-3/*n*-6 to approximately 1.20 and 1.21, respectively. In contrast, the ratios of SFA/PUFA, MUFA/PUFA, and LA/DHA were increased to 1.30, 2.46, and 1.65, respectively in run number 15 and 1.28, 2.42, 1.93, respectively in run number 22 (Table 12). The SFA especially MRA and PMA contents were decreased in the SL. Although reaction temperature at 54.25°C (condition of run

number 15) gave the highest incorporation of CA, but the optimal temperature was chosen at 48.5°C (condition of run number 22) due to the temperature at 54.5°C provided a positive effect only increasing incorporation of CA, but it gave a negative effect on decreasing LA/DHA ratio and other responses (ratios of SFA/PUFA, MUFA/PUFA, n-3/n-6), which were not influenced the reaction rate (Table 14). However, the lower reaction temperature at 48.50°C was similar to report of Senanayake and Shahidi (2005) who suggested that the optimum temperature for the incorporation of CA into seal blubber oil was 45°C. Haman and Shahidi (2005), also indicated that the optimal incorporation of CA into docosahexaenoic acid single cell oil (DHASCO) was obtained at 45°C. As previous explanation, the proper reaction temperature of CA incorporation into RSO was selected at 48.5°C.

The reaction time showed the greatest effect on the responses, which means that the high value (48 h) of reaction time had a positive effect on increasing percentage incorporation of CA and ratios of SFA/PUFA, MUFA/PUFA, and LA/DHA (3.81, 0.94, 1.97, and 1.63, respectively in condition of run number 24) except ratio of *n*-3/*n*-6 (1.49) had a negative effect on decreasing when compared these responses to RSO (including percentage incorporation of CA and ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA counted for 0, 0.63, 1.81, 1.52, and 1.29, respectively) (Table 12).

In conclusion, the optimal reaction temperature and time were chosen at 48.5°C and 48 h based on the responses including percentage incorporation of CA and ratios of SFA/PUFA, MUFA/PUFA, LA/DHA, and *n*-3/*n*-6. The reaction temperature and time were two variable fixed for SL production under the optimal condition, while the other variables including substrate molar ratio and enzyme concentration were further determined by response surface plotting.

**Table 11** Fatty acid composition (mol%) of refined salmon oil and structured lipids derived from acidolysis of salmon oil with capric acid under different conditions

| Runs | Saturated fatty acids | | | | Monounsaturated fatty acids | | | | Polyunsaturated fatty acids | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CA | C14:0 | C16:0 | C18:0 | C16:1 n-7 | C18:1 n-9 | C20:1 n-9 | C22:1 n-9 | C18:2 n-6 | C20:4 n-6 | C18:3 n-3 | C20:4 n-3 | C20:5 n-3 | C22:5 n-3 | C22:6 n-3 |
| Control | 0 | 3.13 | 11.95 | 3.19 | 4.69 | 37.14 | 4.69 | 6.12 | 10.54 | 0.99 | 1.01 | 0.88 | 8.20 | 1.67 | 5.82 |
| 1 | 4.46 | 1.86 | 9.08 | 3.09 | 3.29 | 41.36 | 2.85 | 6.31 | 8.27 | 0.95 | 0.72 | 1.26 | 7.98 | 1.37 | 7.14 |
| 2 | 1.38 | 1.52 | 11.10 | 4.36 | 3.39 | 41.76 | 3.46 | 6.76 | 8.59 | 1.42 | 0.74 | 1.01 | 7.05 | 1.76 | 5.69 |
| 3 | 2.04 | 1.97 | 9.96 | 2.92 | 3.71 | 39.31 | 4.03 | 7.85 | 9.53 | 1.31 | 1.05 | 0.99 | 7.34 | 2.32 | 5.67 |
| 4 | 2.50 | 2.07 | 12.23 | 4.81 | 3.99 | 37.69 | 3.72 | 7.16 | 9.65 | 1.10 | 0.84 | 0.74 | 6.94 | 1.60 | 4.96 |
| 5 | 1.91 | 1.95 | 11.22 | 4.58 | 3.58 | 38.49 | 2.94 | 4.94 | 9.17 | 1.07 | 0.70 | 0.92 | 7.45 | 2.83 | 8.26 |
| 6 | 2.01 | 1.60 | 13.14 | 6.41 | 3.56 | 39.41 | 2.52 | 6.57 | 8.28 | 0.95 | 0.87 | 0.71 | 7.05 | 0.77 | 5.87 |
| 7 | 4.42 | 1.84 | 11.48 | 4.15 | 3.45 | 40.77 | 2.61 | 5.11 | 8.75 | 0.77 | 0.70 | 0.77 | 7.91 | 1.33 | 5.95 |
| 8 | 0.15 | 1.79 | 14.49 | 6.92 | 4.42 | 37.81 | 2.54 | 7.36 | 8.53 | 1.32 | 0.97 | 0.79 | 6.31 | 1.44 | 5.14 |
| 9 | 0.56 | 2.43 | 12.16 | 5.12 | 3.74 | 43.88 | 2.12 | 6.09 | 8.48 | 0 | 0.68 | 0 | 6.93 | 1.89 | 5.91 |
| 10 | 0.17 | 2.01 | 14.79 | 6.40 | 4.48 | 41.97 | 3.17 | 7.61 | 10.14 | 1.19 | 0.82 | 0.60 | 4.73 | 0 | 1.93 |
| 11 | 2.24 | 1.98 | 10.65 | 3.53 | 3.94 | 41.85 | 2.41 | 7.56 | 9.36 | 0.94 | 1.04 | 0.94 | 6.19 | 2.46 | 4.88 |
| 12 | 1.46 | 1.93 | 12.23 | 4.81 | 3.72 | 42.36 | 2.75 | 7.29 | 8.84 | 0.70 | 0.86 | 0.70 | 6.28 | 1.27 | 4.80 |
| 13 | 5.97 | 1.73 | 10.10 | 3.11 | 1.73 | 48.26 | 1.23 | 3.21 | 14.64 | 0.43 | 0.53 | 0.43 | 3.88 | 0.78 | 3.74 |

**Table 11** (Continued)

| Runs | Saturated fatty acids | | | | Monounsaturated fatty acids | | | | Polyunsaturated fatty acids | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CA | C14:0 | C16:0 | C18:0 | C16:1 n-7 | C18:1 n-9 | C20:1 n-9 | C22:1 n-9 | C18:2 n-6 | C20:4 n-6 | C18:3 n-3 | C20:4 n-3 | C20:5 n-3 | C22:5 n-3 | C22:6 n-3 |
| 14 | 0.78 | 2.41 | 13.96 | 6.43 | 1.72 | 44.69 | 1.22 | 3.57 | 17.54 | 1.61 | 0.63 | 0.69 | 1.59 | 0 | 3.22 |
| 15 | 13.69 | 1.79 | 8.65 | 3.08 | 3.37 | 37.75 | 3.32 | 7.34 | 8.44 | 1.10 | 0.67 | 0.55 | 5.10 | 1.39 | 3.76 |
| 16 | 0.75 | 1.73 | 15.58 | 6.79 | 3.04 | 39.22 | 2.82 | 7.71 | 8.52 | 0 | 0.91 | 0 | 6.89 | 0 | 6.06 |
| 17 | 8.67 | 2.07 | 10.09 | 3.12 | 3.49 | 40.05 | 2.82 | 5.26 | 9.11 | 0.97 | 0.76 | 0.70 | 5.95 | 1.36 | 5.56 |
| 18 | 3.11 | 1.72 | 13.52 | 6.22 | 3.07 | 39.58 | 2.20 | 5.36 | 8.45 | 0.92 | 0.63 | 0.69 | 6.61 | 1.00 | 6.93 |
| 19 | 0.40 | 1.62 | 12.09 | 4.78 | 3.61 | 43.57 | 2.51 | 5.90 | 9.87 | 0.59 | 0.90 | 0.79 | 6.25 | 0.85 | 6.36 |
| 20 | 5.31 | 2.84 | 10.39 | 3.54 | 3.57 | 38.92 | 3.51 | 5.49 | 8.64 | 1.32 | 1.05 | 0.99 | 7.68 | 1.25 | 5.50 |
| 21 | 0.20 | 2.09 | 11.29 | 3.90 | 3.93 | 42.14 | 3.71 | 6.58 | 9.78 | 1.04 | 0.95 | 0.70 | 6.74 | 1.51 | 5.45 |
| 22 | 10.84 | 1.95 | 10.59 | 3.91 | 3.50 | 39.61 | 1.91 | 6.43 | 8.82 | 0.80 | 1.09 | 0.60 | 4.56 | 0.87 | 4.52 |
| 23 | 1.69 | 2.37 | 12.33 | 3.73 | 3.75 | 40.43 | 2.84 | 5.73 | 8.89 | 0.83 | 0.76 | 0.83 | 8.26 | 1.81 | 5.74 |
| 24 | 3.81 | 1.95 | 12.99 | 5.28 | 2.00 | 41.30 | 2.31 | 4.83 | 8.60 | 0.87 | 0.60 | 0.43 | 7.77 | 0.94 | 6.33 |
| 25 | 2.00 | 1.50 | 11.63 | 4.87 | 2.31 | 43.40 | 1.63 | 3.07 | 13.28 | 0.46 | 0.42 | 0.77 | 6.55 | 1.00 | 7.12 |
| 26 | 2.39 | 1.22 | 10.82 | 4.86 | 1.63 | 46.94 | 1.21 | 1.99 | 13.79 | 0.65 | 0.45 | 0.65 | 7.60 | 0.88 | 4.92 |
| 27 | 2.11 | 1.63 | 12.02 | 5.07 | 2.72 | 40.59 | 2.15 | 3.62 | 10.27 | 0.78 | 0.71 | 0.93 | 7.40 | 0.84 | 8.71 |
| 28 | 2.01 | 1.81 | 12.76 | 4.82 | 2.56 | 42.16 | 3.61 | 2.87 | 11.58 | 0.81 | 0.44 | 0.81 | 5.60 | 1.22 | 6.96 |

**Table 11** (Continued)

| Runs | Saturated fatty acids | | | | Monounsaturated fatty acids | | | | Polyunsaturated fatty acids | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CA | C14:0 | C16:0 | C18:0 | C16:1 n-7 | C18:1 n-9 | C20:1 n-9 | C22:1 n-9 | C18:2 n-6 | C20:4 n-6 | C18:3 n-3 | C20:4 n-3 | C20:5 n-3 | C22:5 n-3 | C22:6 n-3 |
| 29 | 2.21 | 1.36 | 12.17 | 4.93 | 2.57 | 42.15 | 2.47 | 3.20 | 11.74 | 0.68 | 0.52 | 0.84 | 6.54 | 1.02 | 7.62 |
| 30 | 2.06 | 1.72 | 12.41 | 4.96 | 2.64 | 41.45 | 2.89 | 3.26 | 10.95 | 0.80 | 0.58 | 0.87 | 6.51 | 1.03 | 7.85 |
| 31 | 2.13 | 1.54 | 11.82 | 4.90 | 2.30 | 43.31 | 2.15 | 2.89 | 12.24 | 0.68 | 0.50 | 0.79 | 6.80 | 0.99 | 6.94 |

**Note** Control = refined salmon oil, CA = capric acid, C14:0 = myristic acid, C16:0 = plamitic acid, C18:0 = stearic acid, C16:1 *n*-7 = plamitoleic acid, C18:1 *n*-7 = veccenic acid, C18:1 *n*-9 = oleic acid, C20:1 *n*-9 = gadoleic acid, C22:1 *n*-9 = eucic acid, C18:2 *n*-6 = linoleic acid, C18:3 *n*-3 = alpha–linolenic, C18:4 *n*-3 = stearidonic acid, C20:4 *n*-3 = eicosatetraenoic acid, C20:4 *n*-6 = arachidonic acid, C20:5 *n*-3 = eicosapentaenoic acid, C22:5 *n*-3 = DPA, and C22:6 *n*-3 = docosahexaenoic acid

**Table 12** Response parameters of refined salmon oil and structured lipid obtained from salmon oil with capric acid under the different conditions

| Runs | % Incorporation of CA | SFA/PUFA ratio | MUFA/PUFA ratio | *n*-3/*n*-6 ratio | LA/DHA ratio |
|---|---|---|---|---|---|
| Refined salmon oil (control) | 0.00 | 0.63 | 1.81 | 1.52 | 1.29 |
| 1 | 3.83 | 0.67 | 1.94 | 2.82 | 0.69 |
| 2 | 4.46 | 0.70 | 2.11 | 1.57 | 1.19 |
| 3 | 2.04 | 0.60 | 1.95 | 1.60 | 1.30 |
| 4 | 2.50 | 0.84 | 2.03 | 1.40 | 1.74 |
| 5 | 3.91 | 0.65 | 1.64 | 1.97 | 1.23 |
| 6 | 2.01 | 0.95 | 2.12 | 1.65 | 1.17 |
| 7 | 4.42 | 0.84 | 1.98 | 1.62 | 1.12 |
| 8 | 1.50 | 0.95 | 2.13 | 1.49 | 1.35 |
| 9 | 0.56 | 0.85 | 2.34 | 1.82 | 1.59 |
| 10 | 1.50 | 1.20 | 2.95 | 0.71 | 2.14 |
| 11 | 3.24 | 0.71 | 2.16 | 1.41 | 1.56 |
| 12 | 1.46 | 0.87 | 2.39 | 1.46 | 1.41 |
| 13 | 5.97 | 0.86 | 2.23 | 1.02 | 3.00 |
| 14 | 1.78 | 0.93 | 2.03 | 0.77 | 2.06 |
| 15 | 13.69 | 1.30 | 2.46 | 1.20 | 1.65 |
| 16 | 4.75 | 1.11 | 2.36 | 1.63 | 1.24 |
| 17 | 8.67 | 0.98 | 2.11 | 1.37 | 1.50 |
| 18 | 3.11 | 0.97 | 1.99 | 1.69 | 1.28 |
| 19 | 0.40 | 0.75 | 2.31 | 1.45 | 1.58 |
| 20 | 5.31 | 0.84 | 1.95 | 1.65 | 1.13 |
| 21 | 1.96 | 0.67 | 2.15 | 1.36 | 1.53 |
| 22 | 10.84 | 1.28 | 2.42 | 1.21 | 1.93 |
| 23 | 1.69 | 0.74 | 1.95 | 1.79 | 1.08 |
| 24 | 3.81 | 0.94 | 1.97 | 1.49 | 1.63 |

**Table 12** (Continued)

| Runs | % Incorporation of CA | SFA/PUFA ratio | MUFA/PUFA ratio | *n*-3/*n*-6 ratio | LA/DHA ratio |
|---|---|---|---|---|---|
| 25 | 2.00 | 0.68 | 1.70 | 1.15 | 2.03 |
| 26 | 2.39 | 0.67 | 1.79 | 1.00 | 1.81 |
| 27 | 2.11 | 0.70 | 1.66 | 1.18 | 1.79 |
| 28 | 2.01 | 0.78 | 1.87 | 1.21 | 2.07 |
| 29 | 2.00 | 0.68 | 1.70 | 1.15 | 2.03 |
| 30 | 2.39 | 0.67 | 1.79 | 1.00 | 1.81 |
| 31 | 2.11 | 0.70 | 1.66 | 1.18 | 1.79 |

**Note** CA = capric acid, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, *n*-3 = omega-3 fatty acids, *n*-6 = omega-6 fatty acids, LA = linoleic acid, and DHA = docosahexaenoic acid

3.2 Response surface plotting

The fatty acid composition of achieved SL (Table 11) was used to predict responses including percentage incorporation of CA ($Y_1$), and the ratios of SFA/PUFA ($Y_2$), MUFA/PUFA ($Y_3$), *n*-3/*n*-6 ($Y_4$), and LA/DHA ($Y_5$) to produce appropriated SL were shown in Table 12. The coefficient for the actual functional relations for predicting response was shown in Table 14. Multiple regression analysis was applied to achieve the fitting model by analysis of variance (ANOVA). For first and second order models were significant for all responses (Table 13). The lack of fit was not significant ($p \geq 0.05$) for the ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA (Table 13) that mean these second order models described adequately the data except percentage incorporation of CA. The regression coefficients of multiple determinations ($R^2$) were high for percentage incorporation of CA, SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA ratios approximately 0.92, 0.92, 0.91, 0.91, and 0.90, respectively (Table 13), which mean these models were good fit.

Based on the observation of the data relation with the criterion of individual responses optimization, the reaction temperature and time were kept constant at 48.5°C and 48 h, which were two variables at the pre-selected level. The substrate molar ratio and enzyme concentration were further varied within the experimental domain at point and their effect on percentage incorporation of CA, and ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA were studied. The response surfaces indicated a complex interaction between the variables.

**Table 13** Analysis of variance for the fitted second order polynomial model and lack of fit for percentage incorporation of CA and ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA

| Source of variation | df | Sum of squares | | | | |
|---|---|---|---|---|---|---|
| | | % incorporation of CA | SFA/PUFA ratio | MUFA/PUFA ratio | *n*-3/*n*-6 ratio | LA/DHA ratio |
| Regression | | | | | | |
| First order terms | 4 | 111.42[a] | 0.47[a] | 0.57[a] | 1.28[a] | 1.94[a] |
| Second order terms | 10 | 124.11[a] | 0.51[a] | 2.79[a] | 2.74[a] | 3.33[a] |
| Total | 14 | 235.53 | 0.98 | 3.36 | 4.02 | 5.27 |
| Residual | | | | | | |
| Lack of fit | 10 | 19.45[a] | 0.08[ns] | 0.28[ns] | 0.39[ns] | 0.48[ns] |
| Pure error | 6 | 0.18 | 0.01 | 0.06 | 0.01 | 0.10 |
| Total error | 16 | 19.63 | 0.09 | 0.34 | 0.40 | 0.58 |
| Grand Total | 30 | 255.16 | 1.06 | 3.70 | 4.42 | 5.85 |
| Coefficient of determination ($R^2$) | | 0.92 | 0.92 | 0.91 | 0.91 | 0.90 |

**Note** [a] significant ($p<0.05$), [ns] not significant ($p\geq0.05$)

3.2.1 Effect of substrate ratio and enzyme concentration on percentage incorporation of capric acid

Table 14 shows the effect of substrate ratio and enzyme concentration on percentage incorporation of capric acid (CA) in structured lipids (SL). The percentage incorporation of CA was found to be a function of linear, quadratic, and interaction terms of substrate ratio and enzyme concentration. The linear coefficient of substrate ratio was significantly negative effect on percentage incorporation of CA ($P$<0.001), which resulted in decrease in percentage incorporation of CA with an increase in the level of substrate ratio, whereas linear coefficient of enzyme concentration was significantly positive effect on percentage incorporation of CA ($P$<0.001), which resulted in increase in percentage incorporation of CA with an increase in enzyme concentration. The quadratic coefficient both of substrate ratio and enzyme concentration were significantly positive effect ($P$<0.001) and interaction of substrate ratio and enzyme concentration had significantly negative effect on percentage incorporation of CA ($P$<0.001). At the lowest level of substrate ratio (2), percentage incorporation of CA was found to continuously increase up to 28% combining with increasing enzyme concentration from 2 to 10%. At the highest level of substrate ratio (6), incorporation of CA was reduced to 1% in the range 2-5% of enzyme concentration and then slowly increased up to 9% in the range 6-10% of enzyme concentration. A decrease in substrate ratio resulted in increase in the incorporation of CA.

At the lowest level of enzyme concentration (2%), percentage incorporation of CA decreased from 3 to 0 % at 2-3 of substrate ratio then thoroughly remained constant at 0% in the range of 3-5 of substrate ratio and little increased to 1% at the substrate ratio 6. At the highest level of enzyme concentration (10), percentage incorporation of CA continuously decreased from 27 to 9% with an increase substrate ratio from 2 to 6. An increase in enzyme concentration resulted in increase in the incorporation of CA (Figures 12-a and 13-a). The model for percentage incorporation of CA can therefore be written in Equation 8 as below, but this model was not described adequately the data due to its lack of fit was significantly different.

$$Y_1 = 2.14 - 1.20Sr + 0.81T + 1.51Ec + 0.52t + 0.79Sr^2 + 0.92Ec^2 - 0.54Sr \times T \quad (8)$$
$$- 1.14Sr \times Ec - 0.64Sr \times t + 0.74T \times Ec + 1.07T \times t + 1.28Ec \times t$$

Where $Y_1$ = the predicted response of percentage incorporation of capric acid (CA),

$S_r$ = substrate molar ratio,

T = reaction temperature,

t = reaction time,

$E_c$ = enzyme concentration

3.2.2 Effect of substrate ratio and enzyme concentration on ratio of saturated fatty acid to polyunsaturated fatty acid

Effect of substrate ratio and enzyme concentration on ratio of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA) for synthesis SL shows in Table 14. The linear and quadratic coefficients of substrate ratio was significantly positive effect on ratio of SFA/PUFA ($P$<0.001), which resulted in increase ratio of SFA/PUFA with an increase in the level of substrate ratio. The linear and quadratic coefficient of enzyme concentration was also significantly positive effect on ratio of SFA/PUFA ($P$<0.001), which resulted in increase ratio of SFA/PUFA with an increase in the level of enzyme concentration. The interaction of substrate ratio and enzyme concentration had significantly negative effect on ratio of SFA/PUFA ($P$<0.05). At the lowest level of substrate ratio (2), ratio of SFA/PUFA remained constant at 1.2 in the range 2-6% of enzyme concentration and then slowly increased up to 1.9 combining with increasing enzyme concentration from 7 to 10%. At the highest level of substrate ratio (6), ratio of SFA/PUFA decreased from 1.6 to 1.4 at 4-8% of enzyme concentration and then returned to increase up to 1.6 at the enzyme concentration 10%. An increase in substrate ratio resulted in increase in ratio of SFA/PUFA.

At the lowest level of enzyme concentration (2%), ratio of SFA/PUFA thoroughly remained constant at 1.2 in the range 2-4 of substrate ratio and then continuously increased up to 1.6 in the range of substrate ratio from 5 to 6. At the highest level of enzyme concentration (10%), ratio of SFA/PUFA decreased from 1.9

to 1.6 in the range of 2-3 of substrate ratio then remained constant at 1.6 in the range of 3-5 and little increased to 1.7 at the substrate ratio 6 (Figures 12-b and 13-b). An increase in enzyme concentration resulted in increase in ratio of SFA/PUFA. The model for ratio of SFA/PUFA can therefore be written in Equation 9 as follows;

$$Y_2 = 0.70 + 0.06Sr + 0.09Ec + 0.08t + 0.05Sr^2 + 0.09Ec^2 + 0.05t^2 - 0.05Sr \times T \quad (9)$$
$$- 0.0Sr \times Ec + 0.09T \times Ec$$

Where $Y_2$ = the predicted response of ratio of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA),

$S_r$ = substrate molar ratio,

T = reaction temperature,

t = reaction time,

$E_c$ = enzyme concentration

3.2.3 Effect of substrate ratio and enzyme concentration on ratio of monounsaturated fatty acids to polyunsaturated fatty acids

Table 14 shows effect of substrate ratio and enzyme concentration on ratio of monounsaturated fatty acids to polyunsaturated fatty acids (MUFA/PUFA). The linear coefficient of substrate ratio was significantly positive effect on ratio of MUFA/PUFA ($P$<0.01), which resulted in increase ratio of MUFA/PUFA with an increase in the level of substrate ratio, whereas the linear coefficient of enzyme concentration was not significant ($P$≥0.05). However, the quadratic coefficient of enzyme concentration was significantly positive ($P$<001). Conversely, the quadratic coefficient of substrate ratio was not significant ($P$≥0.05) and interaction of substrate ratio and enzyme concentration had significantly negative effect on ratio of MUFA/PUFA ($P$<0.05). At the lowest level of substrate ratio (2), ratio of MUFA/PUFA decreased from 2.8 to 2.3 in the certain extent at 2-6% of enzyme concentration and then continuously increased up to 3.6 in the rage of 7-10% of enzyme concentration. At the highest level of substrate ratio (6), ratio of MUFA/PUFA continuously decreased from 4.1 to 2.7 in the range of 2-6% of enzyme

concentration and slightly increased up to 3.2 through increasing of enzyme concentration at 8-10%. An increase in substrate ratio resulted in increase in ratio of MUFA/PUFA.

At the lowest level of enzyme concentration (2%), ratio of MUFA/PUFA was found to continuously increase from 2.8 to 4.1 combining with increasing substrate ratio from 2 to 6. At the highest level of enzyme concentration (10%), ratio of MUFA/PUFA was found to slowly decrease from 3.5 to 3.1 through increasing of substrate ratio from 2 to 10. The results could be suggested that increase in enzyme concentration led to no change in ratio of MUFA/PUFA (Figures 12-c and 13-c). The model for ratio of MUFA/PUFA can therefore be written in Equation 10 as follows;

$$Y_3 = 1.72 + 0.11Sr + 0.09t + 0.11T^2 + 0.21E_c^2 + 0.12t^2 - 0.11S_r \times T - 0.12S_r \times E_c + 0.19T \times E_c \quad (10)$$

Where $Y_3$ = the predicted response of ratio of monounsaturated fatty acids to polyunsaturated fatty acids (MUFA/PUFA)

$S_r$ = substrate molar ratio,

T = reaction temperature,

t = reaction time,

$E_c$ = enzyme concentration

3.2.4 Effect of substrate ratio and enzyme concentration on ratio of omega-3 fatty acids to omega-6 fatty acids

Table 14 shows effect of substrate ratio and enzyme concentration on ratio of omega-3 fatty acids to omega-6 fatty acids (*n*-3/*n*-6). The linear coefficient of substrate ratio was significantly negative effect on ratio of *n*-3/*n*-6 ($P<0.01$), which resulted in decrease ratio of *n*-3/*n*-6 with an increase in the level of substrate ratio. Similarly, the linear coefficient of enzyme concentration was also significantly negative effect on ratio of *n*-3/*n*-6 ($P<0.05$), which resulted in decrease ratio of *n*-3/*n*-6

with an increase in the level of enzyme concentration. The quadratic coefficient of substrate ratio was significantly positive effect ($P<0.001$) and interaction of substrate ratio and enzyme concentration had also significantly positive effect on ratio of *n*-3/*n*-6 ($P<0.01$). At the lowest level of substrate ratio (2), ratio of *n*-3/*n*-6 was found to continuously decrease from 2.6 to 1.0 through increasing of enzyme concentration from 2 to 6%. At the highest level of substrate ratio (6), ratio of *n*-3/*n*-6 was found to increase from 1.8 to 2.2 with an increase in enzyme concentration from 2 to 6%. An increase in substrate ratio resulted in decrease in ratio of *n*-3/*n*-6.

At the lowest level of enzyme concentration (2%), ratio of *n*-3/*n*-6 was found to continuously decrease from 2.6 to 1.6 at 2-4 of substrate ratio and then slightly increased up to 1.8 at substrate ratio 6. At the highest level of enzyme concentration (10%), ratio of *n*-3/*n*-6 was found to continuously increase from 1.0 to 2.2 with an increase in substrate ratio from 2 to 10 (Figures 12-d and 13-d). An increase in enzyme concentration resulted in decrease in ratio of *n*-3/*n*-6. The model for ratio of *n*-3/*n*-6 can therefore be written in Equation 11 as follows;

$$Y_4 = 1.16 - 0.10S_r - 0.08E_c - 0.20t + 0.14S_r^2 + 0.11T^2 + 0.12t^2 + 0.19S_r \times T + 0.14S_r \times E_c + 0.13T \times E_c + 0.18T \times t \quad (11)$$

Where $Y_4$ = the predicted response of ratio of omega-3 fatty acids to omega-6 fatty acids (*n*-3/*n*-6),

$S_r$ = substrate molar ratio,

T = reaction temperature,

t = reaction time,

$E_c$ = enzyme concentration

3.2.5 Effect of substrate ratio and enzyme concentration on ratio of linoleic acid to docosahexaenoic acid

Table 14 shows effect of substrate ratio and enzyme concentration on ratio of linoleic acid to docosahexaenoic acid (LA/DHA) for synthesis SL. The

linear coefficient of enzyme concentration was significantly positive effect on ratio of LA/DHA ($P$<0.05), which resulted in increase ratio of LA/DHA with an increase in the level of enzyme concentration, whereas linear coefficient of substrate ratio was not significant ($P$≥0.05). The quadratic coefficient of substrate ratio was significantly positive effect on ratio of LA/DHA ($P$<0.001), whereas quadratic coefficient of enzyme concentration was not significant ($P$≥0.05). The interaction of substrate ratio and enzyme concentration had significantly negative effect on ratio of LA/DHA ($P$<0.01). At the lowest level of substrate ratio (2), ratio of LA/DHA was found to continuously increase from 0.9 to 2.9 with an increase in enzyme concentration from 2 to 6%. At the highest level of substrate ratio (6), ratio of LA/DHA was found to slowly decrease from 0.9 to 0.7 with an increase in enzyme concentration from 2 to 6%. An increase in substrate ratio led to no change in ratio of LA/DHA.

At the lowest level of enzyme concentration (2%), ratio of LA/DHA was found to increase from 0.8 to 1.3 at 2-4 of substrate ratio and then continuously decreased to 0.9 at substrate ratio 6. At the highest level of enzyme concentration (6%), ratio of LA/DHA was found to continuously decrease with an increase in substrate ratio from 2 to 10 shown in Figures 12-e and 13-e. An increase in enzyme concentration resulted in decrease in ratio of LA/DHA. The model for ratio of LA/DHA can therefore be written in Equation 12 as follows;

$$Y_5 = 1.91 - 0.11T + 0.08E_c + 0.25t - 0.12\,S_r^2 - 0.13T^2 - 0.13t^2 - 0.16S_r \times E_c - 0.13Sr \times t - 0.16T \times E_c - 0.26\,T \times t \quad (12)$$

Where $Y_5$ = the predicted response of ratio of linoleic acid to docosahexaenoic acid (LA/DHA),

$S_r$ = substrate molar ratio,

T = reaction temperature,

t = reaction time,

$E_c$ = enzyme concentration

**Table 14** Estimated coefficient for the fitted second order polynomial representing the relationship between the response (percentage incorporation of CA and ratios of SFA/PUFA, MUFA/PUFA, n-3/n-6, and LA/DHA) and process variables of structured lipids

| Term of regression coefficient | % incorporation of CA | SFA/PUFA ratio | MUFA/PUFA ratio | n-3/n-6 ratio | LA/DHA ratio |
|---|---|---|---|---|---|
| Intercept ($b_0$) | 2.14 $^{a}$ | 0.70 $^{a}$ | 1.72 $^{a}$ | 1.16 $^{a}$ | 1.91 $^{a}$ |
| $S_r$ ($b_1$) | -1.20 $^{a}$ | 0.06 $^{a}$ | 0.11 $^{b}$ | -0.10 $^{b}$ | -0.01 $^{ns}$ |
| T ($b_2$) | 0.81 $^{b}$ | 0.02 $^{ns}$ | -0.04 $^{ns}$ | -0.01 $^{ns}$ | -0.11 $^{b}$ |
| $E_c$ ($b_3$) | 1.51 $^{a}$ | 0.09 $^{a}$ | -0.04 $^{ns}$ | -0.08 $^{c}$ | 0.08 $^{c}$ |
| t ($b_4$) | 0.52 $^{c}$ | 0.08 $^{a}$ | 0.09 $^{b}$ | -0.20 $^{a}$ | 0.25 $^{a}$ |
| $S_r \times S_r$($b_{11}$) | 0.79 $^{a}$ | 0.05 $^{b}$ | 0.04 $^{ns}$ | 0.14 $^{a}$ | -0.12 $^{b}$ |
| T × T ($b_{22}$) | 0.03 $^{ns}$ | 0.02 $^{ns}$ | 0.11 $^{a}$ | 0.10 $^{b}$ | -0.13 $^{b}$ |
| $E_c \times E_c$($b_{33}$) | 0.92 $^{a}$ | 0.09 $^{a}$ | 0.21 $^{a}$ | 0.03 $^{ns}$ | -0.03 $^{ns}$ |
| t × t ($b_{44}$) | 0.01 $^{ns}$ | 0.05 $^{b}$ | 0.12 $^{a}$ | 0.12 $^{a}$ | -0.13 $^{b}$ |
| $S_r \times T$ ($b_{12}$) | -0.54 $^{ns}$ | -0.05 $^{b}$ | -0.11 $^{b}$ | 0.19 $^{a}$ | 0.00 $^{ns}$ |
| $S_r \times E_c$($b_{13}$) | -1.14 $^{a}$ | -0.05 $^{c}$ | -0.12 $^{b}$ | 0.14 $^{b}$ | -0.16 $^{b}$ |
| $S_r \times t$($b_{14}$) | -0.64 $^{c}$ | -0.01 $^{ns}$ | 0.00 $^{ns}$ | 0.07 $^{ns}$ | -0.13 $^{b}$ |
| T× $E_c$($b_{23}$) | 0.74 $^{b}$ | 0.09 $^{a}$ | 0.19 $^{a}$ | 0.13 $^{b}$ | -0.16 $^{b}$ |
| T× t($b_{24}$) | 1.07 $^{a}$ | -0.01 $^{ns}$ | -0.03 $^{ns}$ | 0.18 $^{a}$ | -0.26 $^{a}$ |
| $E_c \times t$($b_{34}$) | 1.28 $^{a}$ | 0.00 $^{ns}$ | 0.01 $^{ns}$ | -0.04 $^{ns}$ | 0.08 $^{ns}$ |

**Note** $S_r$ =Substrate molar ratio, T =Reaction temperature, t =Reaction time, and $E_c$ =Enzyme concentration

Values in each column with different superscript (a, b, and c) indicated significant differences ($p<0.001$, 0.01, and 0.05, respectively) and (ns) was not significant ($p>0.05$).

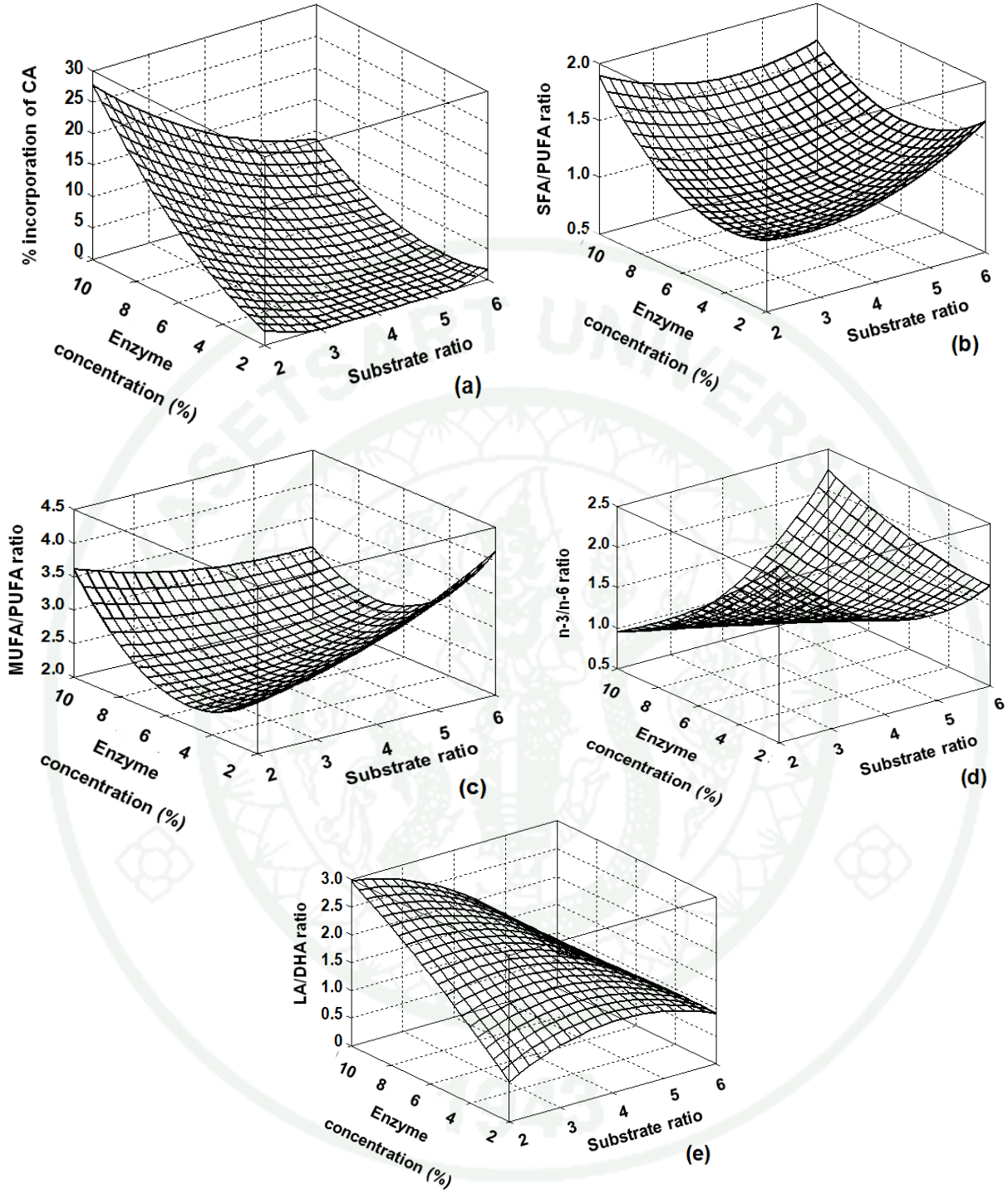



**Figure 12** Surface plots showing the effect of enzyme concentration and substrate ratio on (a) % incorporation of CA; (b) ratio of SFA/PUFA; (c) ratio of MUFA/PUFA; (d) ratio of *n*-3/*n*-6; (e) ratio of LA/DHA. Temperature and time of the reaction were kept constant at 48.5 °C and 48 h, respectively.

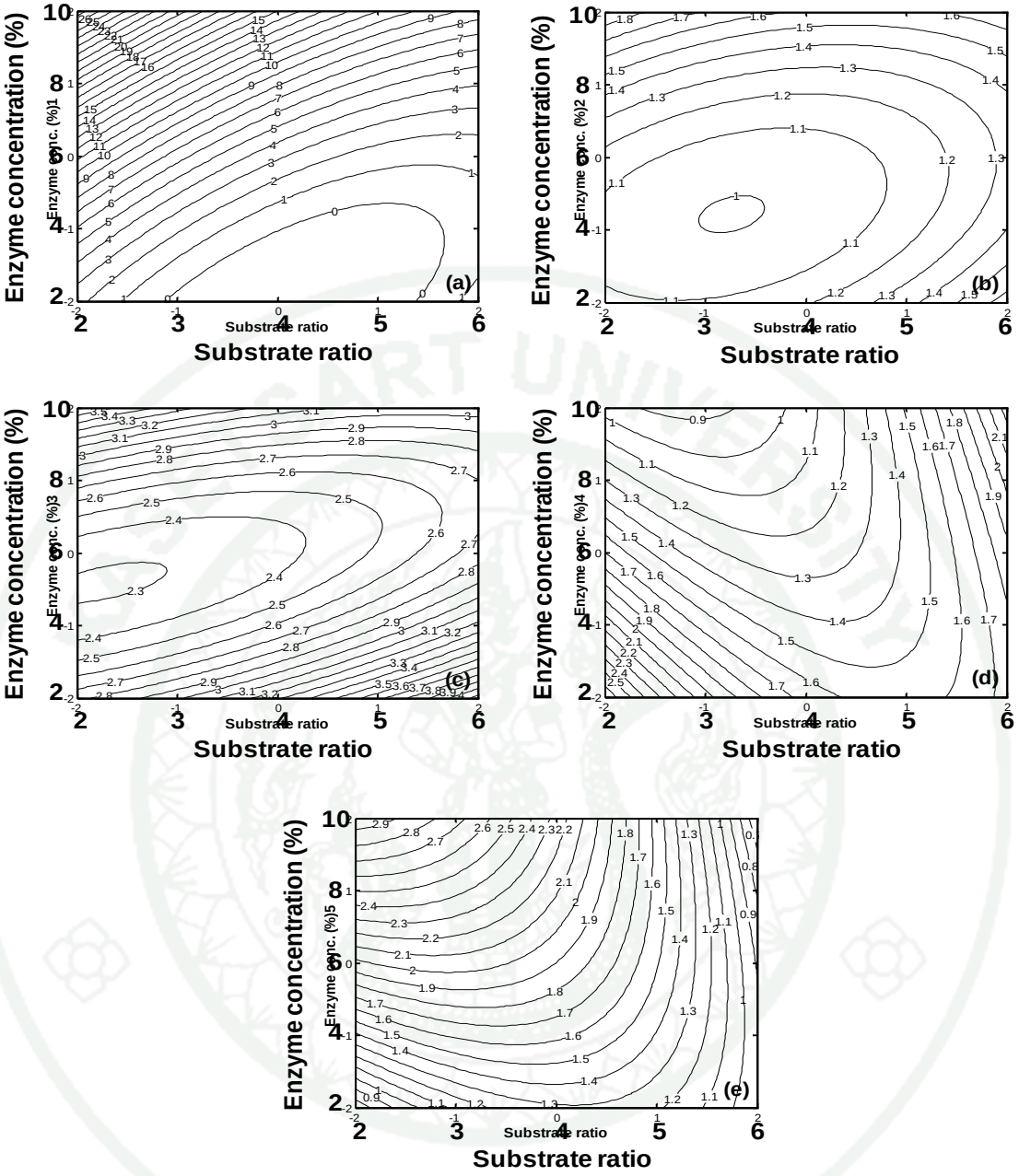



**Figure 13** Contour plots showing the effect of enzyme concentration and substrate ratio on (a) % incorporation of CA; (b) ratio of SFA/PUFA; (c) ratio of MUFA/PUFA; (d) ratio of *n*-3/*n*-6; (e) ratio of LA/DHA. Temperature and time of the reaction were kept constant at 48.5 °C and 48 h, respectively.

The relationship between responses and process parameters were examined by three dimensional surface plots as shown in Figure 12 (a-e) with two variables fixed at optimum level based on these coefficients keeping at 48.5°C (coded value 0) and 48 h (coded value 2) for reaction temperature and time and changing the other two variables (substrate ratio and enzyme concentration) within the experimental range. In the surface plots (Figure 12 (a-e)), only the significant effects of fitting second order polynomial models were Equation 9-12 for each response that were further assessed appropriate boundary for SL production especially substrate ratio and enzyme concentration at the optimal level.

3.3 Optimization of the reaction

All response parameters (ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA) were optimized the following the recommended adequate intake in diet to assess efficiency for production of SL. The ratio of SFA/PUFA should be taken about 0.5-1.98 to avoid the accumulation of cholesterol in the body (Jenning and Akoh, 2001). The ratio of MUFA/PUFA should get in the range of 0.39-2.70 that can reduce the excessive triglycerides to be adipose storage in the body (Kris-Etherton and Yu, 1997). The ratio of *n*-3/*n*-6 should be ranged from 0.5 to 1.0 for reducing risk of coronary artery disease and stroke, inflammatory disease, asthma, breast cancer and colorectal cancer (Simopoulos, 2002).The ratio of LA/DHA should be ranged from 1.62 to 2.52 for reducing plasma total cholesterol and low-density lipoprotein cholesterol and risk of sudden cardiac death, cardiovascular disease, hyper-triglyceridemia, and hypertension. (Horrocks and Young, 1999; Simopoulos, 2002; Strobel *et al.*, 2010). The optimum combination, which satisfied the above requirement, based on individual the optimum conditions for the ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA shows in Table 15 and Figure 14. An acceptable compromise was made following the criteria for the percentage incorporation of CA $\geq$ 10, ratio of SFA/PUFA $\geq$ 1.2, ratio of MUFA/PUFA $\leq$ 2.7, ratio of *n*-3/*n*-6 $\leq$ 1.1, and ratio of LA/DHA $\geq$ 2.3 (Figure 14). The contour plots for the responses were superimposed and the regions that best satisfy all constraints were selected as the optimum conditions. Superimposed contour plots for each response is

shown in Figure 14. A combination of optimum working conditions (A, B, and C) can be selected from the shaded area as present in Table 15. The overlapped area could be recommended as practical optimum zone for enzyme concentration (7.66 to 8.52%, coded level 0.83 to 1.26) and substrate ratio (2.66 to 3.51, coded level -1.34 to -0.49). Consequently, the production of SL contained CA incorporation into TAG from RSO under the optimal working conditions as follows;

Condition A; molar ratio of substrates between RSO/CA at 1:2.66, enzyme concentration at 8.16% (w/w), incubation temperature and time at 48.5°C for 48 h.

Condition B; molar ratio of substrates between RSO/CA at 1:3.29, enzyme concentration at 7.66% (w/w), incubation temperature and time at 48.5°C for 48 h.

Condition C; molar ratio of substrates between RSO/CA at 1:3.51, enzyme concentration at 8.52% (w/w), incubation temperature and time at 48.5°C for 48 h.

**Table 15** Feasible and optimal conditions and predicted and experimental values of response at optimal condition

| Optimum condition | Condition A | | Condition B | | Condition C | |
|---|---|---|---|---|---|---|
| | Coded levels | Actual levels | Coded levels | Actual levels | Coded levels | Actual levels |
| Variables | | | | | | |
| Substrate molar ratio ($X_1$) | -1.34 | 2.66 | -0.71 | 3.29 | -0.49 | 3.51 |
| Temperature of reaction (°C) ($X_2$) | 0 | 48.50 | 0 | 48.50 | 0 | 48.50 |
| Enzyme concentration (% w/w) ($X_3$) | 1.08 | 8.16 | 0.83 | 7.66 | 1.26 | 8.52 |
| Time of reaction (h) ($X_4$) | 2.00 | 48.00 | 2.00 | 48.00 | 2.00 | 48.00 |
| Responses | Pred. value | Exp. value[a] | Pred. value | Exp. value[a] | Pred. value | Exp. value[a] |
| % incorporation of CA | 15.04 | 14.94 | 10.07 | 10.00 | 11.87 | 10.96 |
| SFA/PUFA ratio | 1.36 | 1.21 | 1.21 | 1.12 | 1.33 | 1.23 |
| MUFA/PUFA ratio | 2.70 | 2.60 | 2.51 | 2.31 | 2.70 | 2.62 |
| n-3/n-6 PUFA ratio | 1.10 | 1.01 | 1.09 | 1.02 | 1.03 | 1.00 |
| LA/DHA ratio | 2.51 | 2.42 | 2.31 | 2.15 | 2.36 | 2.31 |

**Note** [a] Mean value of five determinations, Pred. value = predicted value, Exp. value = Experimental value,
Condition A; molar ratio of substrates between RSO/CA at 1:2.66, enzyme concentration at 8.16% (w/w), reaction temperature and time at 48.5°C for 48 h.
Condition B; molar ratio of substrates between RSO/CA at 1:3.29, enzyme concentration at 7.66% (w/w), reaction temperature and time at 48.5°C for 48 h.
Condition C; molar ratio of substrates between RSO/CA at 1:3.51, enzyme concentration at 8.52% (w/w), reaction temperature and time at 48.5°C for 48 h.

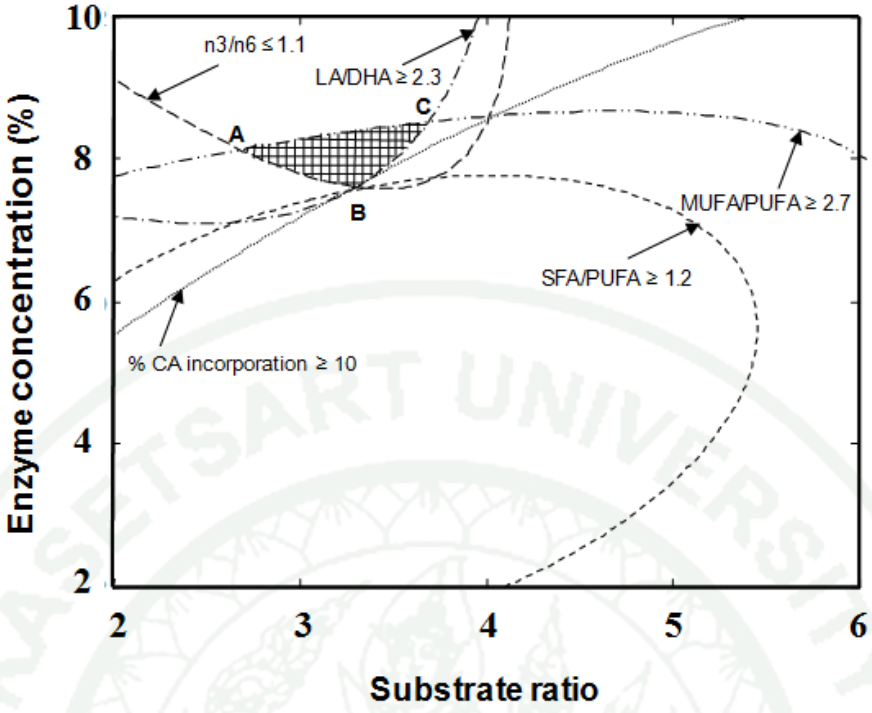



**Figure 14** Superimposed contour plots showing the shaded overlapping area for percentage incorporation of CA ≥ 10, ratio of SFA/PUFA ≥ 1.2, ratio of MUFA/PUFA ≤ 2.7, ratio of *n*-6/*n*-3 ≤ 1.1 and LA/DHA ≥ 2.3

**4. Structured lipid derived from refined salmon oil incorporation with capric acid at the optimal condition in gram scale**

4.1 Verification of the model and optimization

The results obtained from a batch containing 100 mg of refined salmon oil (RSO) was scaled up by using 20 g of RSO as a starting material to validate the results obtained at gram scale following the optimal working conditions (A, B, and C) as previous described in session 3. The fatty acid composition of RSO and SL are provided in Table 15. The results of experimental value for production SL under

the optimal working conditions were found to be in agreement with predicted response on percentage incorporation of CA, ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA in the range of 10.00-14.94, 1.12-1.23, 2.31-2.60, 1.00-1.02, and 2.15-2.42, respectively. Moreover, DHA content of SL was ranged from 3.98 to 4.83 mol%, which were lower than RSO (5.88±0.09 mol%), whereas EPA content were in the range of 4.02-4.29 mol% that were higher than RSO (3.64±0.30 mol%) as shown in Table 16.

The optimal conditions A, B, and C in this study for synthesis purified structured lipids (PSL) was substrate molar ratio at 1:2.66, 1:3.29, and 1:3.51 of RSO/CA, respectively, loaded enzyme concentration at 8.16, 7.66, and 8.52% (w/w), respectively, and the same both reaction temperature and time at 48.5 °C for 48 h. There were found to have the high incorporation of CA up to 14.94±0.23, 10.00±0.29, and 10.96±0.22 mol%, respectively, EPA content increased to 4.02±0.11, 4.29±0.14, and 4.03±0.09 mol%, respectively (from 3.63±0.30 mol% of RSO) whereas DHA content of RSO decreased from 5.88±0.90 mol% to 3.98±0.03, 4.83±0.03, and 4.13±0.02 mol% of conditions A, B, and C, respectively. The ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA of PSL were 1.21, 2.60, 1.01, and 2.42, respectively for condition A, 1.12, 2.31, 1.02, and 2.15, respectively for condition B, and 1.23, 2.62, 1.00, and 2.31, respectively for condition C. The fatty acid compositions of PSL were found to increase amount of CA, OA, and EPA for all three conditions, except EUA increased only PSL in the condition A and C.

The results expressed that PSL obtained from condition A (including substrate molar ratio at 1:2.66 of RSO/CA, loaded enzyme concentration at 8.16% (w/w), and incubation temperature and time at 48.5 °C for 48 h) gave the highest amount of CA at 14.94±0.23 mol%, EPA and DHA contents remained 4.02±0.11and 3.98±0.03 mol%, respectively, and the ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and LA/DHA were in the acceptable ranges. The results were different from report by Jennings and Akoh (2001), who indicated that the optimal condition for SL to obtain the highest CA incorporation at 31.1±4.6 mol% including mole ratio of 1:2 (menhaden oil/CA), lipase enzyme from *Rhizomucor miehei* of 10% (w/w), incubation

temperature 55°C, and time 24 h. The fatty acid composition of SL was found to increase amount of CA, MRA, PMA, and PTA, whereas STA, OA, EPA, and DHA were decreased. Hamam and Shahidi (2005) also denoted that the optimal conditions for synthesis SL containing CA incorporation into docosahexaenoic acid single cell oil (DHASCO) was substrate ratio of 1:3 (DHASCO/CA), lipase enzyme from *Pseudomona***s** sp. of 4% (w/w), incubation temperature and time at 45°C for 24 h, which resulted in the highest CA incorporation up to 10.17±0.11% and DHA content was 37.1±0.29%. The fatty acid composition of the SL was found to increase only amount of CA, while DHA content was similar to original DHASCO, and other fatty acids content were lower than the initial oil. These could be explained that the effect of different sources of raw materials as fish oil and lipase enzyme and working condition as reaction temperature and time influenced to produce the various SL.

**Table 16** Fatty acid composition of refined salmon oil and purified structured lipid at the optimal condition in gram scale (mol%)

| Fatty acids | Salmon oil | Purified structured lipids (condition) | | |
|---|---|---|---|---|
| | | A | B | C |
| 10:0 | 0 | 14.94±0.23 | 10.00±0.29 | 10.96±0.22 |
| 14:0 | 3.48±0.32 | 1.10±0.01 | 2.11±0.05 | 2.07±0.11 |
| 16:0 | 12.24±0.18 | 6.16±0.18 | 10.09±0.29 | 10.20±0.06 |
| 18:0 | 3.13±0.12 | 2.96±0.05 | 3.07±0.04 | 2.10±0.03 |
| 16:1 *n*-7 | 4.90±0.08 | 3.67±0.12 | 3.43±0.07 | 3.72±0.07 |
| 18:1 *n*-9 | 37.71±0.51 | 39.98±0.10 | 41.39±0.34 | 40.73±0.22 |
| 20:1 *n*-9 | 5.62±0.88 | 3.75±0.04 | 3.18±0.09 | 3.43±0.12 |
| 22:1 *n*-9 | 5.88±0.49 | 6.65±0.34 | 4.16±0.09 | 6.14±0.07 |
| 18:2 *n*-6 | 10.52±0.57 | 9.64±0.13 | 10.39±0.08 | 9.56±0.06 |
| 20:4 *n*-6 | 0.97±0.06 | 0.70±0.01 | 0.78±0.01 | 0.74±0.02 |
| 18:3 *n*-3 | 3.32±0.24 | 0.97±0.05 | 0.90±0.04 | 0.88±0.12 |
| 20:4 *n*-3 | 0.91±0.02 | 0.53±0.12 | 0.41±0.10 | 0.46±0.02 |
| 20:5 *n*-3 | 3.64±0.30 | 4.02±0.11 | 4.29±0.14 | 4.03±0.09 |

**Table 16** (Continued)

| Fatty acids | Salmon oil | Purified structured lipids (condition) | | |
|---|---|---|---|---|
| | | A | B | C |
| 22:5 *n*-3 | 1.80±0.11 | 0.95±0.03 | 0.99±0.15 | 0.85±0.06 |
| 22:6 *n*-3 | 5.88±0.09 | 3.98±0.03 | 4.83±0.03 | 4.13±0.02 |
| SFA/PUFA ratio | 0.70 | 1.21 | 1.12 | 1.23 |
| MUFA/PUFA ratio | 2.00 | 2.60 | 2.31 | 2.62 |
| n-3/n-6 ratio | 1.35 | 1.01 | 1.02 | 1.00 |
| LA/DHA ratio | 1.79 | 2.42 | 2.15 | 2.31 |

**Note** All data are mean values±standard deviation.
Condition A; molar ratio of substrates between RSO/CA at 1:2.66, enzyme concentration at 8.16% (w/w), reaction temperature and time at 48.5°C for 48 h.
Condition B; molar ratio of substrates between RSO/CA at 1:3.29, enzyme concentration at 7.66% (w/w), reaction temperature and time at 48.5°C for 48 h.
Condition C; molar ratio of substrates between RSO/CA at 1:3.51, enzyme concentration at 8.52% (w/w), reaction temperature and time at 48.5°C for 48 h.

4.2 Positional distribution of fatty acids

The purified structured lipids (PSL) was performed to determine acyl migration in sn-2 and sn-1, 3 positions by using pancreatic lipase and analyzed by TLC to separate acylglycerols in each type off. Their fatty acid compositions at sn-2 and sn-1, 3 positions were determined again by GC as shown in Table 17. The results of TLC showed that the relative content of TAG (89.29-92.43%) was much higher than that of diacylglycerols (DAG; 6.18-8.75%) and monoacylglycerols (MAG; 1.36-1.96%). The distribution of fatty acids at sn-2 position of PSL compared to RSO (Table 19) showed that total amount of MUFA (including PTA, OA, GDA, and EUA) and PUFA (including LA, AA, ALA, ETA, EPA, DPA, and DHA) were reduced from 55.74 to 51.42 mol% of MUFA and from 36.07 to 29.84 mol% of PUFA, whereas SFA (including CA, MRA, PMA, and STA) was increased from 8.20 to 18.74 mol%.

There were found that OA at sn-2 position of RSO had the highest concentration (46.34±0.91 mol%), followed by LA (14.04±0.44 mol%), DHA (8.71±0.54 mol%), and ALA (5.90±0.17 mol%). Similarly, there were also found OA with the highest concentration at sn-2 position of PSL (42.67±0.36-43.74±0.79 mol%), followed by LA (13.28±0.22-13.93±0.74 mol%),CA (8.24±0.20-10.93±0.12), and DHA (7.39±0.25-8.59±0.58 mol%).

The distribution of fatty acids at sn-1, 3 positions of PSL compared to RSO were found that the amount of SFA was increased from 22.98 to 65.13 mol%, whereas MUFA and PUFA were decreased from 54.32 to 18.25 mol% of MUFA and from 22.70 to 14.56 mol% of PUFA. There were found that CA at sn-1, 3 positions of PSL had the highest concentration (48.75±0.22-56.80±0.31 mol%), followed by OA (11.94±0.32-21.00±0.19 mol%), LA (4.91±0.06-5.79±0.06 mol%), and PMA (4.68±0.10-5.26±0.02 mol%). The distributed EPA and DHA contents of PSL were located to a higher extent at sn-2 position (2.78±0.07-3.24±0.39 mol% of EPA and 7.39±0.25-8.59±0.58 mol% of DHA) when compared to EPA and DHA contents at sn-1, 3 positions (1.52±0.16-1.98±0.05 mol% of EPA and 4.22±0.19-4.75±0.45 mol% of DHA). The results showed that the effect of pancreatic lipase hydrolyzed MCFA over LCFA and agreed with those of Jennings and Akoh (2001) who reported that CA was mainly located at sn-1, 3 positions (31.1 mol%) of SL produced via acidolysis of menhaden oil with CA. Similarly, Xu *et al.* (2000) reported that caprylic acid (CPA) at sn-1, 3 positions of menhaden oil after acidolysis were high incorporation (38.8 mol%). Shimada *et al.* (1996) also indicated that after modification high amount of CPA was incorporated at sn-1, 3 positions of tuna oil (41.9 mol%).Finally, the achieved PSL was produced in MLM type (sn-MLM type; M = medium chain fatty acids, L = long chain fatty acids), which would have the potential to deliver many health benefit associated with PUFA for the prevention of some cardiovascular diseases, inflammations, and rheumatoid arthritis, diabetes, skin problems. At the same time, the effect of MUFA contributed to associate reducing calorie for controlling obesity by increasing serum total cholesterol concentration and also for persons with fat malabsorption and other metabolism problems.

In conclusion, PSL obtained from condition A containing SFA at sn-1, 3 positions of RSO especially CA was increased to 56.80 mol% and LCFA at sn-2 position particularly OA, LA, DHA and EPA remained as 42.67, 13.92, 7.39, and 2.78 mol%, respectively. These results can confirm successful synthesis SL in MLM type.

**Table 17** Positional distribution of refined salmon oil and purified structured lipids at the optimal condition (%)

| Fatty acids | Refined salmon oil | | | Purified structured lipids | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Condition A | | | Condition B | | | Condition C | | |
| | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 |
| 10:0 | 0 | 0 | 0 | 14.94± 0.23 | 10.93±0.12 (24.39) [a] | 56.80±0.31 (75.61) [b] | 10.00± 0.29 | 8.24±0.20 (27.47) [a] | 50.65±1.95 (72.53) [b] | 10.96± 0.22 | 8.67±0.24 (26.37) | 48.75±0.22 (73.63) |
| 14:0 | 3.48± 0.32 | 2.31±0.07 (22.13) [a] | 4.09±0.27 (77.87) [b] | 1.10± 0.01 | 2.09±0.06 (63.33) [a] | 1.65±0.02 (36.67) [b] | 2.11± 0.05 | 2.20±0.02 (34.76) [a] | 1.46±0.06 (65.24) [b] | 2.07± 0.11 | 2.29±0.18 (36.88) | 1.57±0.03 (63.12) |
| 16:0 | 12.24± 0.18 | 5.34±0.09 (14.54) [a] | 14.79±0.20 (85.46) [b] | 6.16± 0.18 | 5.19±0.08 (28.08) [a] | 5.26±0.02 (71.92) [b] | 10.09± 0.29 | 5.14±0.14 (16.98) [a] | 4.68±0.10 (83.02) [b] | 10.20± 0.06 | 5.26±0.37 (17.19) | 5.24±0.03 (82.81) |
| 18:0 | 3.13± 0.12 | 0.55±0.10 (5.86) [a] | 4.10±0.02 (94.14) [b] | 2.96± 0.05 | 0.53±0.01 (5.97) [a] | 1.42±0.02 (94.03) [b] | 3.07± 0.04 | 0.52±0.04 (5.65) [a] | 1.31±0.05 (94.35) [b] | 2.10± 0.03 | 0.52±0.05 (8.25) | 1.47±0.06 (91/75) |
| 16:1 n-7 | 4.90± 0.08 | 5.34±0.12 (36.33) [a] | 4.19±0.04 (63.67) [b] | 3.67± 0.12 | 4.76±0.08 (43.23) [a] | 2.18±0.03 (56.77) [b] | 3.43± 0.07 | 4.94±0.08 (48.01) [a] | 2.33±0.01 (51.99) [b] | 3.72± 0.07 | 4.91±0.35 (44.00) | 2.52±0.18 (56.00) |
| 18:1 n-9 | 37.71± 0.51 | 46.34±0.91 (40.96) [a] | 37.99±0.24 (59.04) [b] | 39.98± 0.10 | 42.67±0.36 (35.58) [a] | 11.94±0.32 (64.42) [b] | 41.39± 0.34 | 43.74±0.79 (35.23) [a] | 18.59±0.92 (64.77) [b] | 40.73± 0.22 | 42.86±0.56 (35.08) | 21.00±0.19 (64.92) |
| 20:1 n-9 | 5.62± 0.88 | 3.15±0.23 (18.68) [a] | 7.89±0.03 (81.32) [b] | 3.75± 0.04 | 3.13±0.04 (27.82) [a] | 1.83±0.19 (72.18) [b] | 3.18± 0.09 | 3.13±0.05 (32.81) [a] | 2.64±0.13 (67.19) [b] | 3.43± 0.12 | 3.11±0.20 (30.22) | 2.78±0.08 (69.78) |

**Table 17** (Continued)

| Fatty acids | Refined salmon oil | | | Purified structured lipids | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Condition A | | | Condition B | | | Condition C | | |
| | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 |
| 22:1 n-9 | 5.88±0.49 | 0.90±0.13 (5.10)[a] | 7.76±0.13 (94.90)[b] | 6.65±0.34 | 0.86±0.05 (4.31)[a] | 2.30±0.15 (95.69)[b] | 4.16±0.09 | 0.85±0.07 (6.81)[a] | 1.95±0.14 (93.19)[b] | 6.14±0.07 | 0.84±0.13 (4.56)[a] | 2.11±0.24 (95.44)[b] |
| 18:2 n-6 | 10.52±0.57 | 14.04±0.44 (44.49)[a] | 9.76±0.01 (55.51)[b] | 9.64±0.13 | 13.92±0.13 (48.13)[a] | 5.79±0.06 (51.87)[b] | 10.39±0.08 | 13.28±0.22 (42.61)[a] | 5.24±0.24 (57.39)[b] | 9.56±0.06 | 13.93±0.74 (48.57)[a] | 4.91±0.06 (51.43)[b] |
| 20:4 n-6 | 0.97±0.06 | 0.35±0.04 (12.03)[a] | 0.34±0.02 (87.97)[b] | 0.70±0.01 | 0.28±0.01 (13.33)[a] | 0.19±0.01 (86.67)[b] | 0.78±0.01 | 0.33±0.03 (14.10)[a] | 0.19±0.01 (85.91)[b] | 0.74±0.02 | 0.31±0.04 (13.96)[a] | 0.17±0.03 (86.04)[b] |
| 18:3 n-3 | 3.32±0.24 | 5.90±0.17 (59.24)[a] | 3.09±0.01 (40.76)[b] | 0.97±0.05 | 2.12±0.11 (70.67)[a] | 2.21±0.04 (29.33)[b] | 0.90±0.04 | 2.26±0.20 (83.70)[a] | 2.05±0.08 (16.30)[b] | 0.88±0.12 | 2.50±0.50 (94.70)[a] | 1.81±0.05 (5.30)[b] |
| 20:4 n-3 | 0.91±0.02 | 1.06±0.06 (38.83)[a] | 1.36±0.01 (61.17)[b] | 0.53±0.12 | 1.02±0.04 (64.15)[a] | 0.97±0.02 (35.85)[b] | 0.41±0.10 | 1.03±0.06 (83.74)[a] | 1.01±0.04 (16.26)[b] | 0.46±0.02 | 0.96±0.18 (69.57)[a] | 0.85±0.05 (30.43)[b] |
| 20:5 n-3 | 3.64±0.30 | 3.36±0.25 (30.77)[a] | 3.96±0.23 (69.23)[b] | 4.02±0.11 | 2.78±0.07 (23.05)[a] | 1.98±0.05 (76.95)[b] | 4.29±0.14 | 3.24±0.39 (25.17)[a] | 1.97±0.07 (74.83)[b] | 4.03±0.09 | 2.99±0.47 (24.73)[a] | 1.52±0.16 (75.27)[b] |
| 22:5 n-3 | 1.80±0.11 | 2.65±0.11 (49.07)[a] | 2.10±0.06 (50.93)[b] | 0.95±0.03 | 2.33±0.09 (81.75)[a] | 1.25±0.04 (18.25)[b] | 0.99±0.15 | 2.59±0.26 (87.21)[a] | 1.19±0.10 (12.79)[b] | 0.85±0.06 | 2.26±0.45 (88.63)[a] | 0.98±0.06 (11.37)[b] |

**Table 17** (Continued)

| Fatty acids | Refined salmon oil | | | Purified structured lipids | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Condition A | | | Condition B | | | Condition C | | |
| | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 | Sn-1, 2, 3 | Sn-2 | Sn-1, 3 |
| 22:6 n-3 | 5.88± 0.09 | 8.71±0.54 (49.38) [a] | 2.09±0.23 (50.62) [b] | 3.98± 0.03 | 7.39±0.25 (61.89) [a] | 4.22±0.19 (38.11) [b] | 4.83± 0.03 | 8.51±0.18 (58.73) [a] | 4.75±0.45 (41.27) [b] | 4.13± 0.02 | 8.59±0.58 (69.33) [a] | 4.32±0.03 (30.67) [b] |
| Σ SFA | 18.85 | 8.20 | 22.98 | 25.15 | 18.74 | 65.13 | 25.27 | 16.10 | 58.10 | 25.33 | 16.74 | 57.03 |
| Σ MUFA | 54.11 | 55.74 | 54.32 | 54.03 | 51.42 | 18.25 | 52.16 | 52.66 | 25.51 | 54.02 | 51.72 | 28.41 |
| Σ PUFA | 27.04 | 36.07 | 22.70 | 20.82 | 29.84 | 16.62 | 22.59 | 31.24 | 16.39 | 20.65 | 31.54 | 14.56 |
| Σ*n*-3 | 15.55 | 21.68 | 12.60 | 10.28 | 15.64 | 10.64 | 11.42 | 17.63 | 10.96 | 10.35 | 17.3 | 9.48 |
| Σ*n*-6 | 11.49 | 14.39 | 10.10 | 10.34 | 14.20 | 5.98 | 11.17 | 13.61 | 5.43 | 10.30 | 14.24 | 5.08 |

**Note** All data are mean values±standard deviation of triplicate measurement. [a] (fatty acids at sn-2 position (mol%) / (fatty acids at sn-1, 2, 3 positions (mol%) × 3) × 100, and [b] (100 - sn-2)

Condition A; molar ratio of substrates between RSO/CA at 1:2.66, enzyme concentration at 8.16% (w/w), reaction temperature and time at 48.5°C for 48 h.

Condition B; molar ratio of substrates between RSO/CA at 1:3.29, enzyme concentration at 7.66% (w/w), reaction temperature and time at 48.5°C for 48 h.

Condition C; molar ratio of substrates between RSO/CA at 1:3.51, enzyme concentration at 8.52% (w/w), reaction temperature and time at 48.5°C for 48 h.

## 4.3 Characteristics of refined salmon oil and purified structured lipid

The physicochemical properties of RSO and PSL are shown in Table 18. The results showed that RSO had a brilliant and transparent light yellow color (Figure 10 (c4)), that *L**, *a**, and *b** values of the oil were 98.59±0.02, -0.97±0.01, and 8.26±0.06 respectively. After acidolysis reaction and purification steps, PSL in conditions A and B were changed from light yellow color to be light orange yellow color, while PSL in condition C was slightly clouded than conditions A and B (Figure 15). Due to condition of enzymatic acidolysis cause partial semi-solid fat of SL from increasing SFA content (Shukla, 1995). *L**, *a**, and *b** values of PSL were ranged from 92.56 to 95.38, -1.40 to -1.45, and 32.86 to 36.28, respectively. FFA contents of PSL were found in the range of 0.14-0.20% as oleic acid that they were lower than initial oil (0.29±0.02 % as oleic acid) due to most of FFA (excessive CA and liberated LCFA) were eliminated again by reacting with potassium hydroxide in purification step to form soft soap and precipitate off. However, these results were not exceeded 0.2% as oleic acid following an acceptable level of refined fish oil (Rubio-Rodríguez*et al.,* 2012). PV of PSL were in the range of 0.74-0.86 meq/kg and lower than RSO (5.96±0.01 meq/kg) due to their lower unsaturated fatty acids content compared to RSO, which consisted of high PUFA content and led to faster progression of oxidation. However, these were not exceed 10 meq/kg according to the standards of edible oils (Codex, 2009). *p*-AV of PSL were in the range of 0.40-0.46 and lower than initial oil (1.48±0.01), which agreed with suggestion of Noriega-Rodriiguez *et al.,* (2010). IV of PSL were in the ranged of 20.67-24.48 cg $I_2$/g indicated that PSL composed of low amount of unsaturated fatty acid and to enhance the stability and to delay an oxidative change.

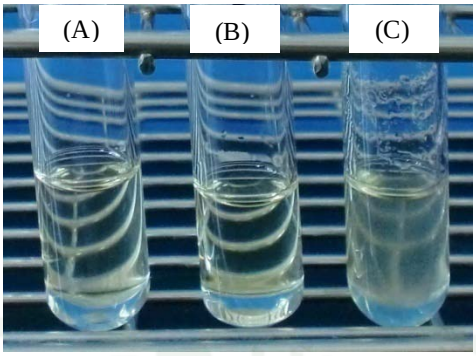



**Figure 15** Structured lipids derived from refined salmon oil incorporation with capric acid at the optimal conditions.
Condition A; mole ratio of substrates between RSO/CA at 1:2.66, enzyme concentration at 8.16% (w/w), incubation temperature and time at 48.5 °C for 48 h,
Condition B; mole ratio of substrates between RSO/CA at 1:3.29, enzyme concentration at 7.66% (w/w), incubation temperature and time at 48.5 °C for 48 h,
Condition C; mole ratio of substrates between RSO/CA at 1:3.51, enzyme concentration at 8.52% (w/w), incubation temperature and time at 48.5 °C for 48 h

**Table 18** Physicochemical properties of refined salmon oil and purified structured lipid

| Parameters | | Refined salmon oil | Purified structured lipids | | |
|---|---|---|---|---|---|
| | | | Condition A | Condition B | Condition C |
| Color | *L** | 98.59±0.02 | 95.38±0.16 | 93.07±0.04 | 92.56±0.20 |
| | *a** | -0.97±0.01 | -1.40±0.01 | -1.43±0.02 | -1.54±0.04 |
| | *b** | 8.26±0.06 | 32.86±0.06 | 34.14±0.08 | 38.28±0.02 |
| Free fatty acid (% as oleic acid) | | 0.29±0.02 | 0.14±0.01 | 0.20±0.01 | 0.16±0.01 |
| Peroxide value (meq/kg) | | 5.96±0.01 | 0.86±0.01 | 0.99±0.00 | 0.74±0.02 |
| *p*-Anisidine value | | 1.48±0.01 | 0.40±0.05 | 0.46±0.01 | 0.42±0.04 |
| Iodine value (cg $I_2$/g) | | 101.77±0.02 | 20.67±0.04 | 22.61±0.02 | 24.48±0.01 |

**Note** Values are given as mean±standard deviation from triplicate determination.

# CONCLUSION AND RECOMMENDATION

## Conclusion

Salmon belly is a good source to be used as raw material for crude oil production with high percentage of yield (33.7±6.1%). Most of physicochemical properties of refined oils after degummed with three different degumming agents (water, phosphoric acid, and citric acid) were not significantly different ($p>0.05$) except that degummed oils using citric acid had higher capacity in lowering Cu, Fe, and P in the oils than water and phosphoric acid. The crude oil had red-orange color from carotenoids component and changed to be transparent light yellow color after purification process. Free fatty acid contents, peroxide values, and ρ-anisidine values for all oil samples were in an acceptable range indicated the oils were not rancid due to oxidation reaction. Fatty acid composition showed that percentage of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were higher than saturated fatty acids (SFA). The ratio of omega-3 fatty acids to omega-6 fatty acids was found to be 1.58-2.17.

The optimal working condition A including substrate molar ratio between refined salmon oil (RSO)/capric acid (CA) at 1:2.66 mol%, incubation temperature and time at 48.5 °C for 48 h, and loaded enzyme concentration at 8.16% (w/w) used to synthesize structured lipids (SL) derived from RSO and CA gave the highest CA incorporation in triglycerides of salmon oil up to 14.94±0.23 mol%. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contents remained at 4.02±0.11 and 3.98±0.03 mol%, respectively, and the ratios of SFA/PUFA, MUFA/PUFA, *n*-3/*n*-6, and linoleic acid to docosahexaenoic acid (LA/DHA) were in acceptable ranges indicated SL containing fatty acid proportions at the appropriated levels relative to a good second order model. The positional distribution of SL at sn-2 position containing long chain fatty acids especially oleic acid (OA), LA, DHA, and EPA contents were 42.67±0.36, 13.92±0.13, 2.78±0.07, 7.39±0.25, and 2.78±0.07 mol%, respectively and CA at sn-1, 3 positions was 56.80±0.31 mol %. The SL had transparent light orange yellow color by increasing its yellowness and decreasing both

brightness and redness. Free fatty acid content, peroxide values, and *p*-anisidine values were acceptable indicated the oil was not oxidized and iodine value was lower than initial oil showed that SL had oxidative stability. The structured lipids produced in this study may be nutritionally useful and have potential uses either for direct consumption or for use as valuable ingredient oil in various functional food formulations.

## Recommendation

The structured lipid (SL) obtained from this study including capric acid (CA) incorporation into triacylglycerols (TAG) of refined salmon oil increased medium chain saturated fatty acid especially CA at sn-1, 3 position and remained long chain fatty acids at sn-2 position of TAG especially oleic, linoleic, docosahexaenoic, and eicosapentaenoic acids. The obtained SL expected to be an energy source due to rapid digestion and easy absorption by direct pass into portal vein. The quality of SL depends on temperature and time of incubation, substrate molar ratio, and enzyme concentration, which the latter two variables impart dominating effect in the lipase-catalyzed reaction of SL production. However, the response functions (incorporation of CA, ratios of saturated fatty acids to polyunsaturated fatty acids, monounsaturated fatty acids to polyunsaturated fatty acids, omega-3 fatty acids to omega-6 fatty acids, and linoleic acid to docosahexaenoic acid) can be related to those four independent variables by a second order polynomial. The absorption capacity, toxicity, and novel functional foods produced from SL are suggested to be further studied.

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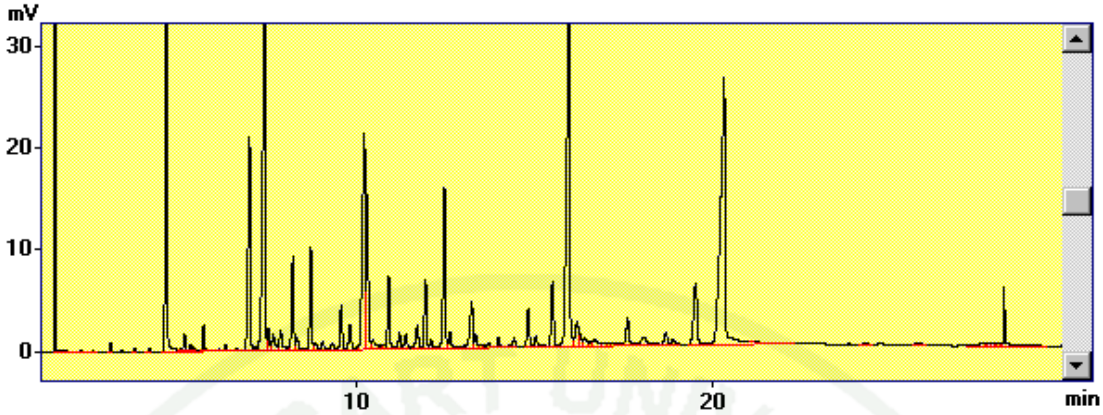
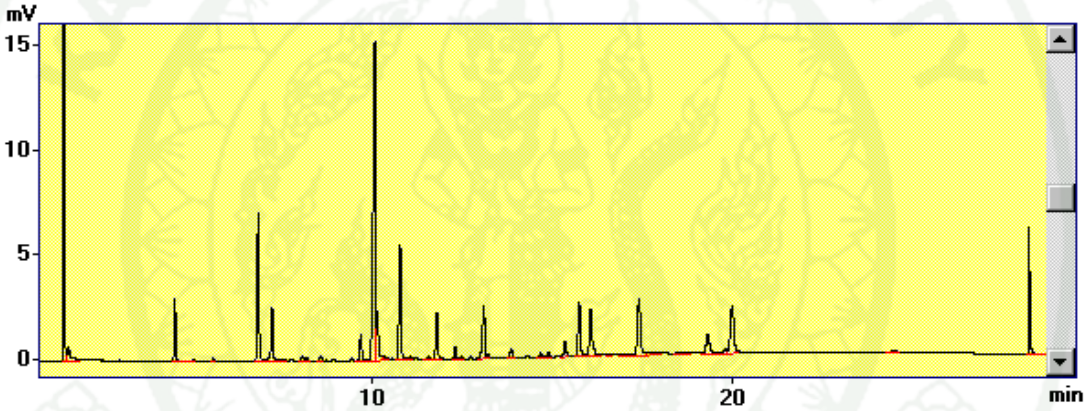
# APPENDICES

**Appendix A**

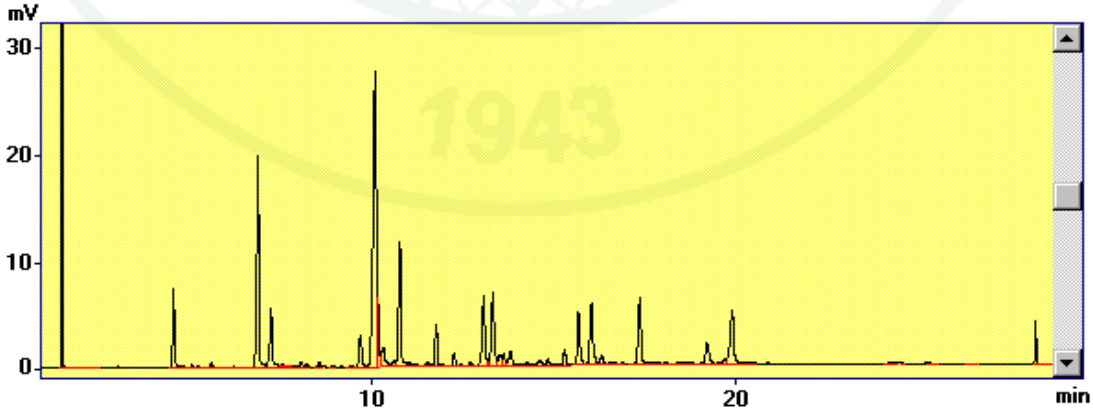
Fatty acid composition of standard oil, crude salmon oil and refined oils from determination by gas chromatography



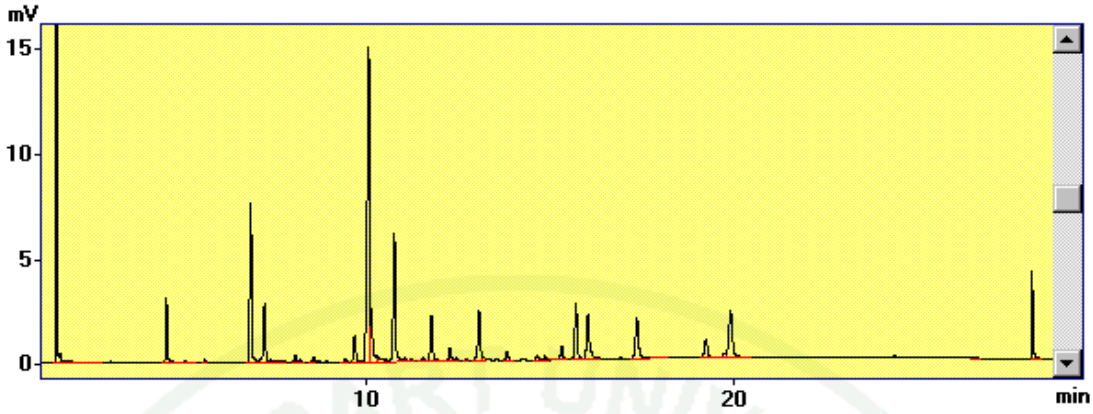

**Appendix Figure A1** Fatty acid composition of standard menhaden oil



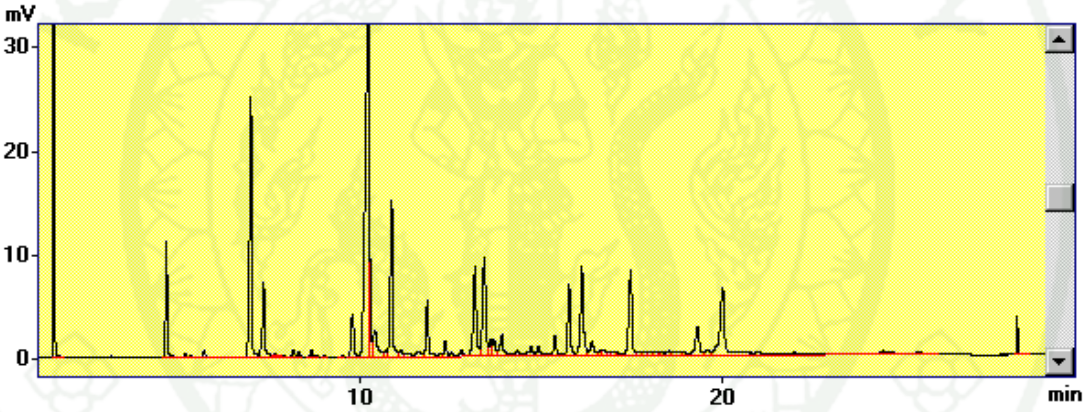

**Appendix Figure A2** Fatty acid composition of crude salmon oil



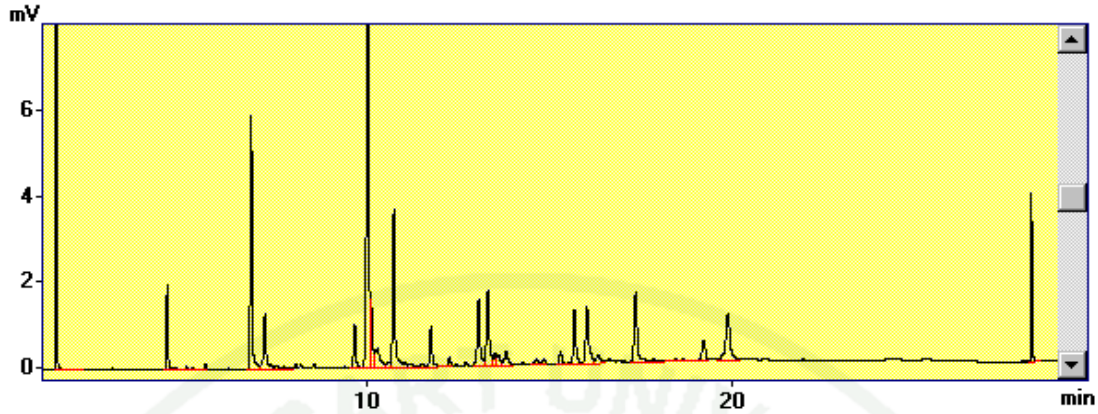

**Appendix Figure A3** Fatty acid composition of degummed salmon oil with hot water



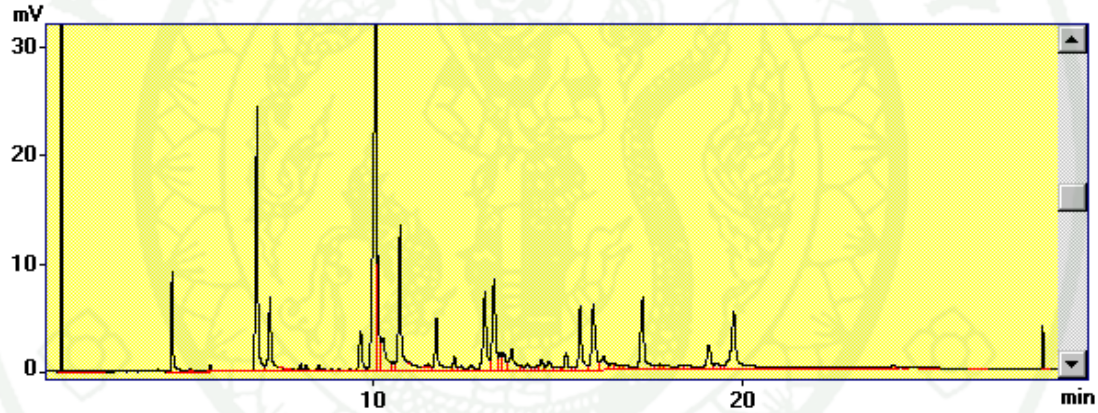

**Appendix Figure A4** Fatty acid composition of degummed salmon oil with phosphoric acid



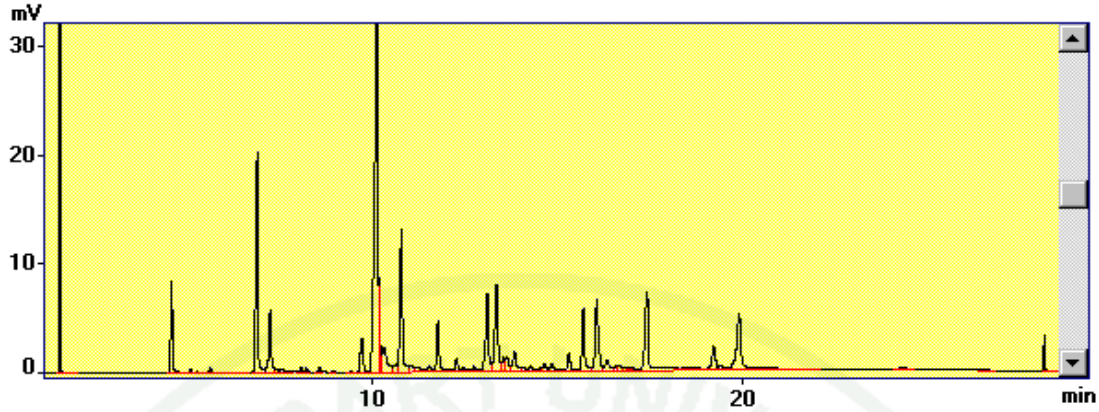

**Appendix Figure A5** Fatty acid composition of degummed salmon oil with citric acid



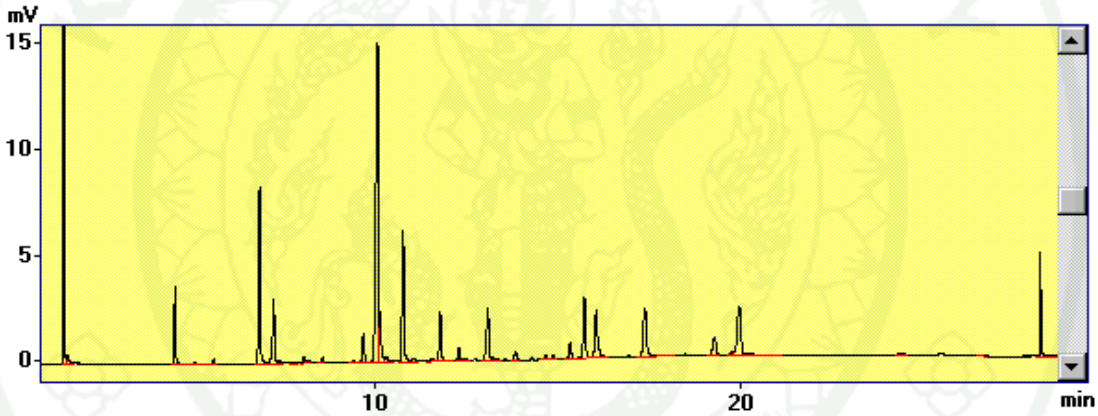

**Appendix Figure A6** Fatty acid composition of neutralized salmon oil after degumming with hot water



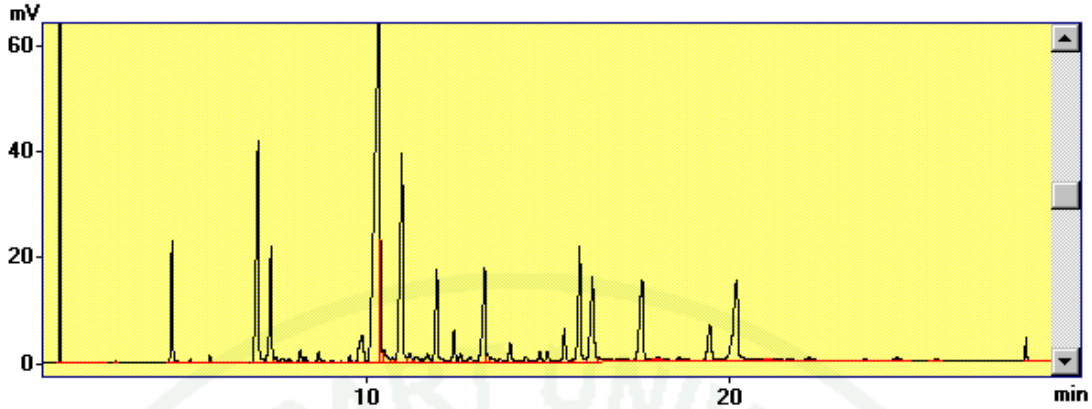

**Appendix Figure A7** Fatty acid composition of neutralized salmon oil after degumming with phosphoric acid



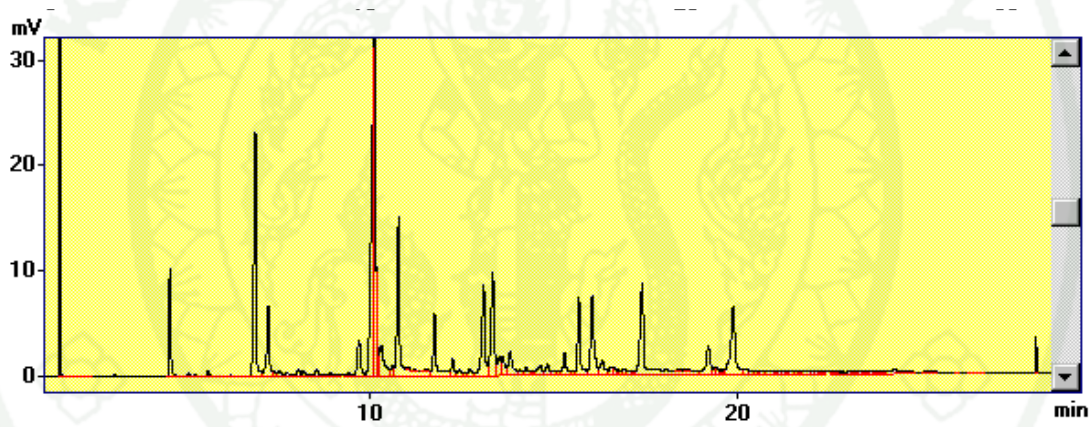

**Appendix Figure A8** Fatty acid composition of neutralized salmon oil after degumming with citric acid




**Appendix Figure A9** Fatty acid composition of bleached salmon oil after neutralization and degumming with hot water



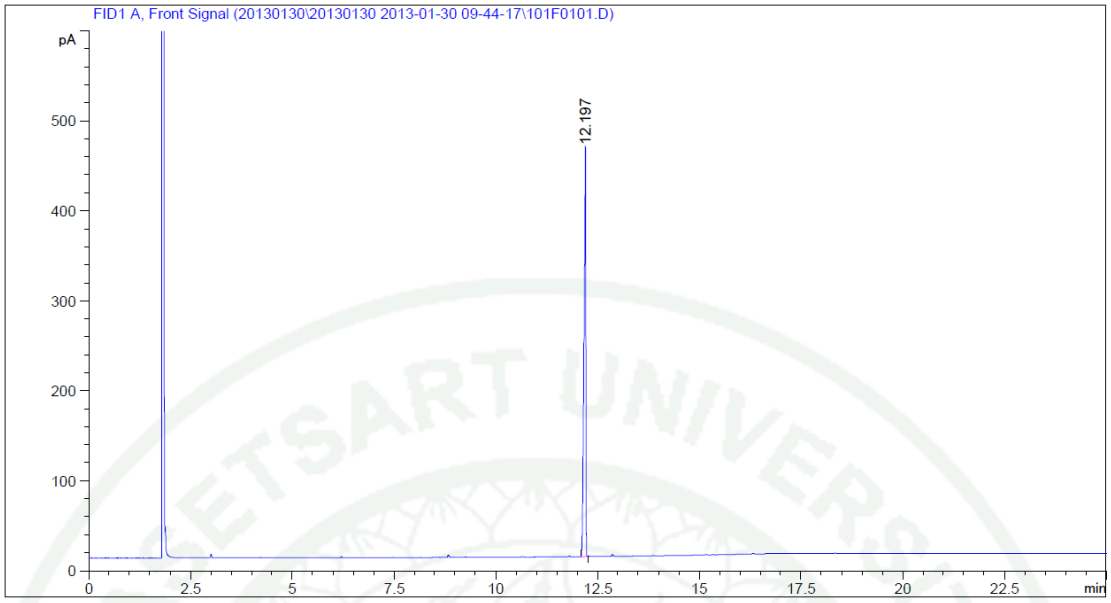

**Appendix Figure A10** Fatty acid composition of bleached salmon oil after neutralization and degumming with phosphoric acid



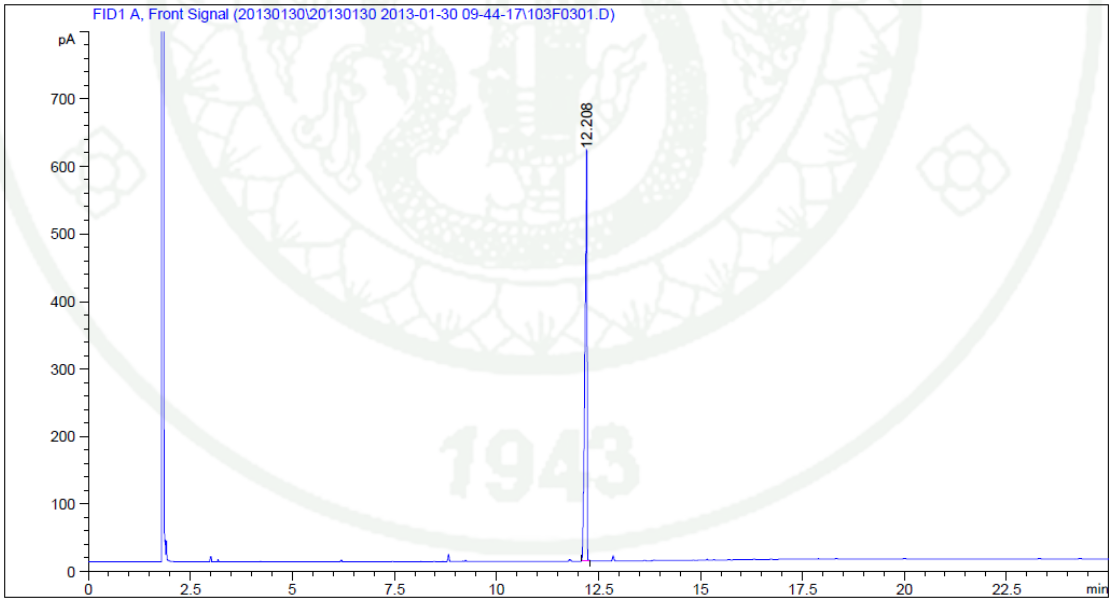

**Appendix Figure A11** Fatty acid composition of bleached salmon oil after neutralization and degumming with citric acid

**Appendix B**

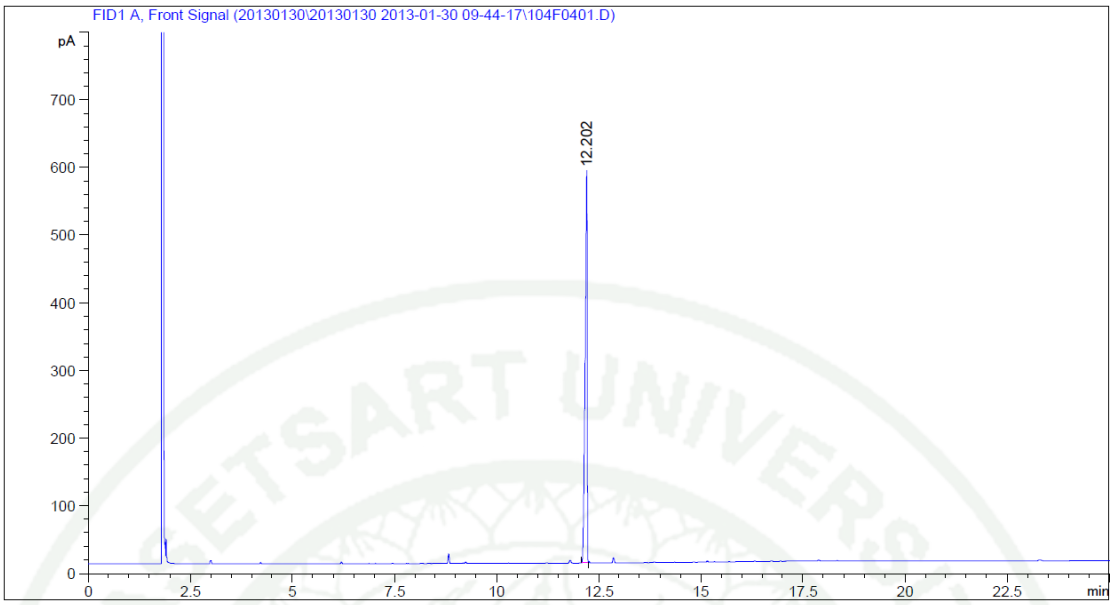
Fatty acid composition of fatty acids at sn-2, sn-1, 3, and sn-1,2,3 positions of mono-, di-, and triolein standard, refined salmon oil and structured lipids from determination by gas chromatography



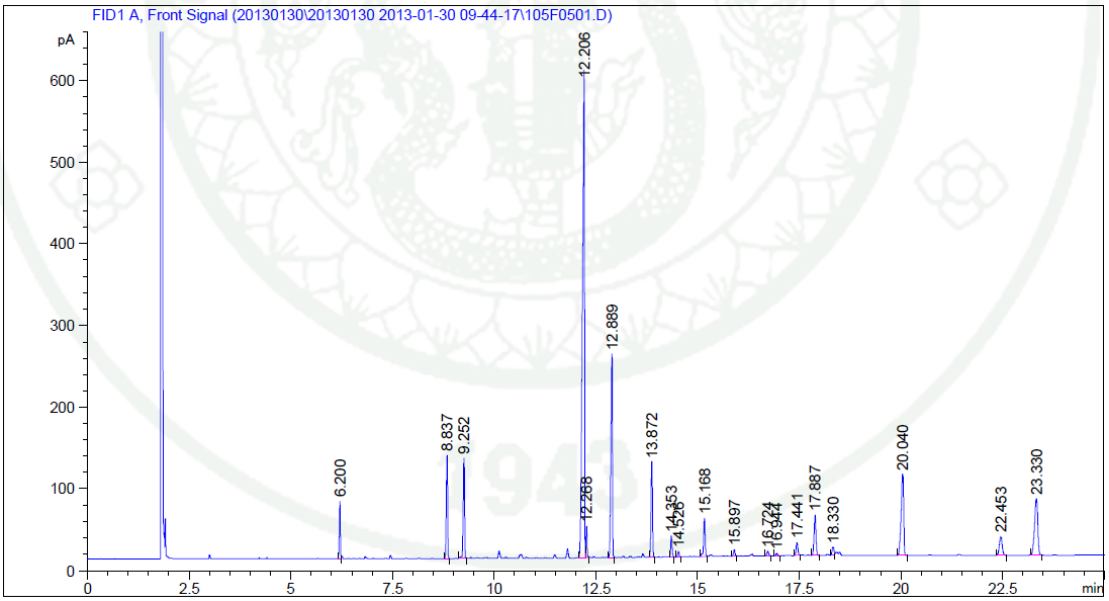

**Appendix Figure B1** Fatty acid composition of fatty acids at sn-2, of monoolein standard



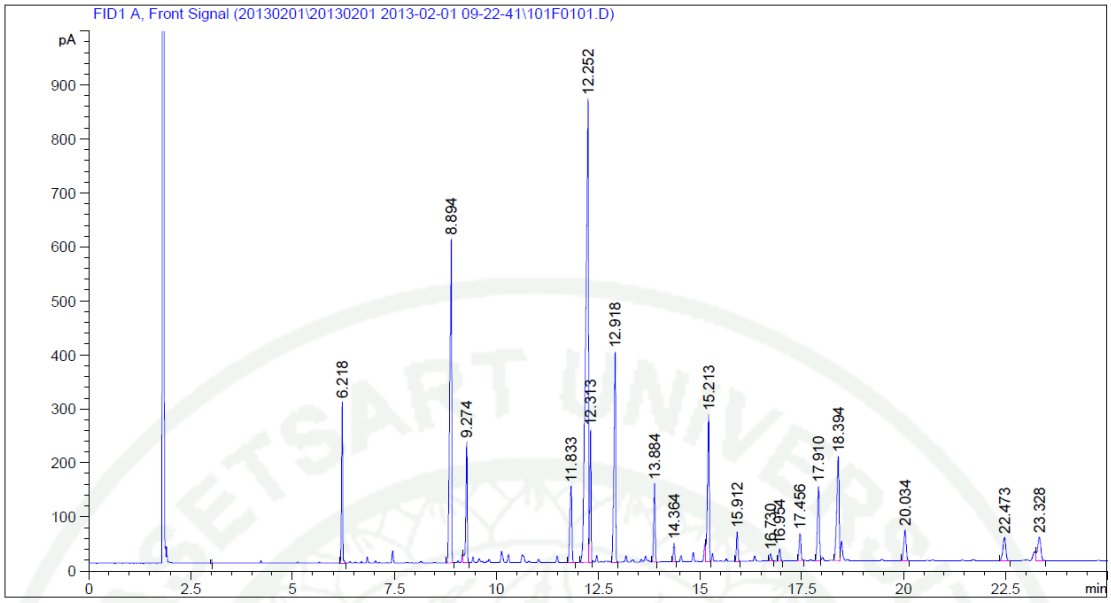

**Appendix Figure B2** Fatty acid composition of fatty acids at sn-1, 3 of diolein standard



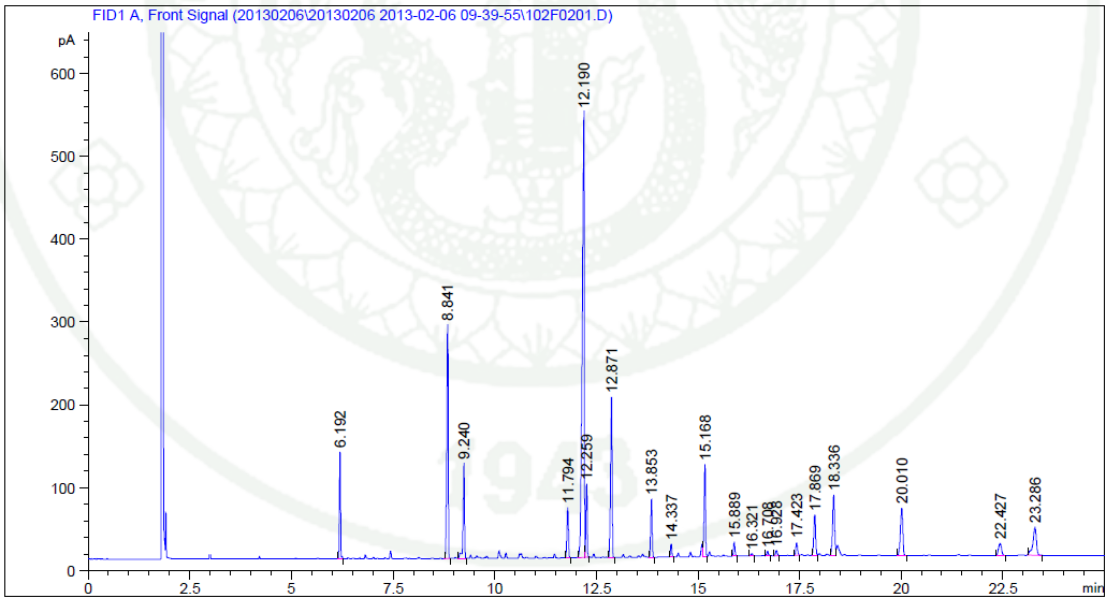

**Appendix Figure B3** Fatty acid composition of fatty acids at sn-1, 2, 3 of triolein standard



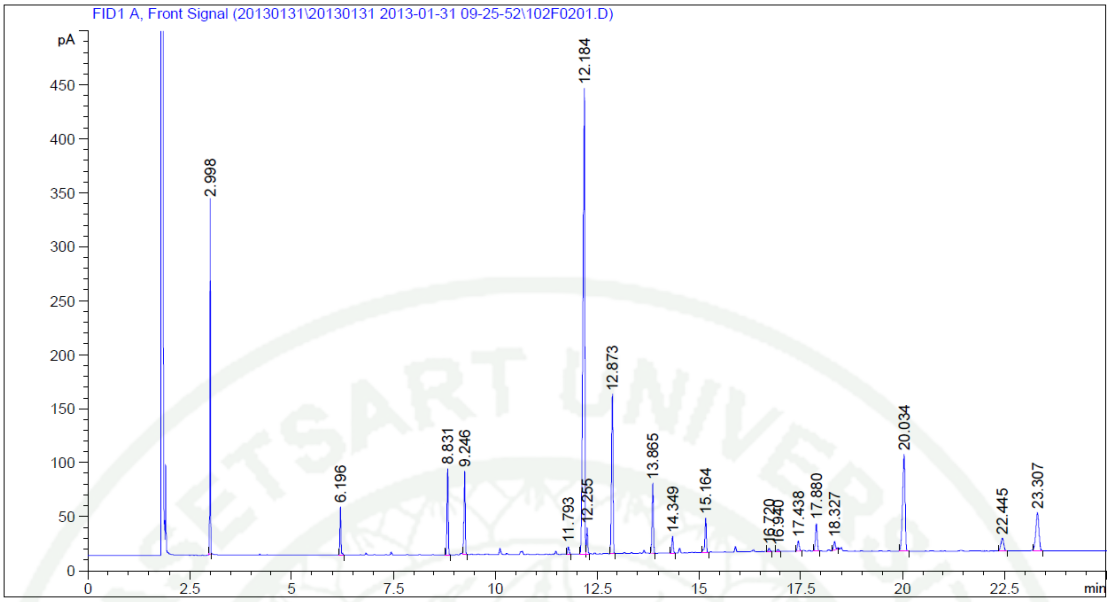

**Appendix Figure B4** Fatty acid composition of fatty acids at sn-2 of refined salmon oil



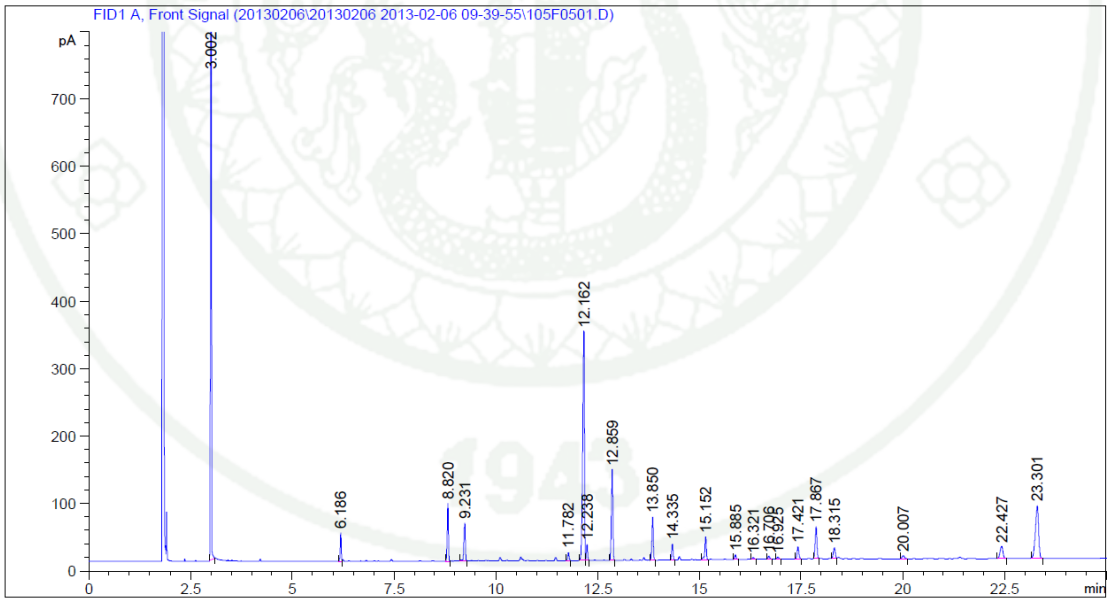

**Appendix Figure B5** Fatty acid composition of fatty acids at sn-1, 3 of refined salmon oil



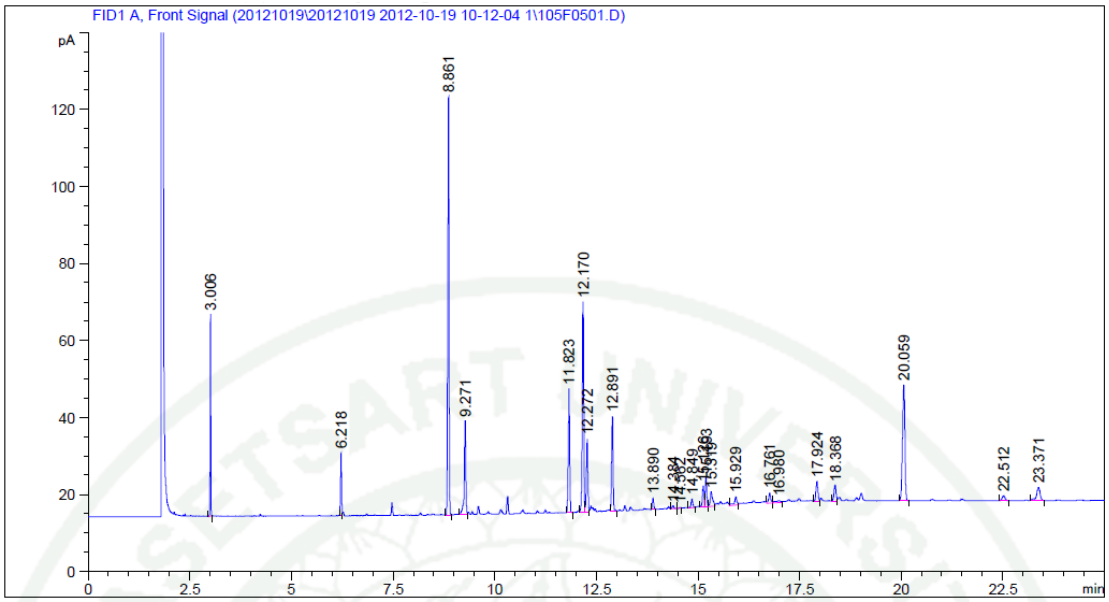

**Appendix Figure B6** Fatty acid composition of fatty acids at sn-1, 2, 3 of refined salmon oil



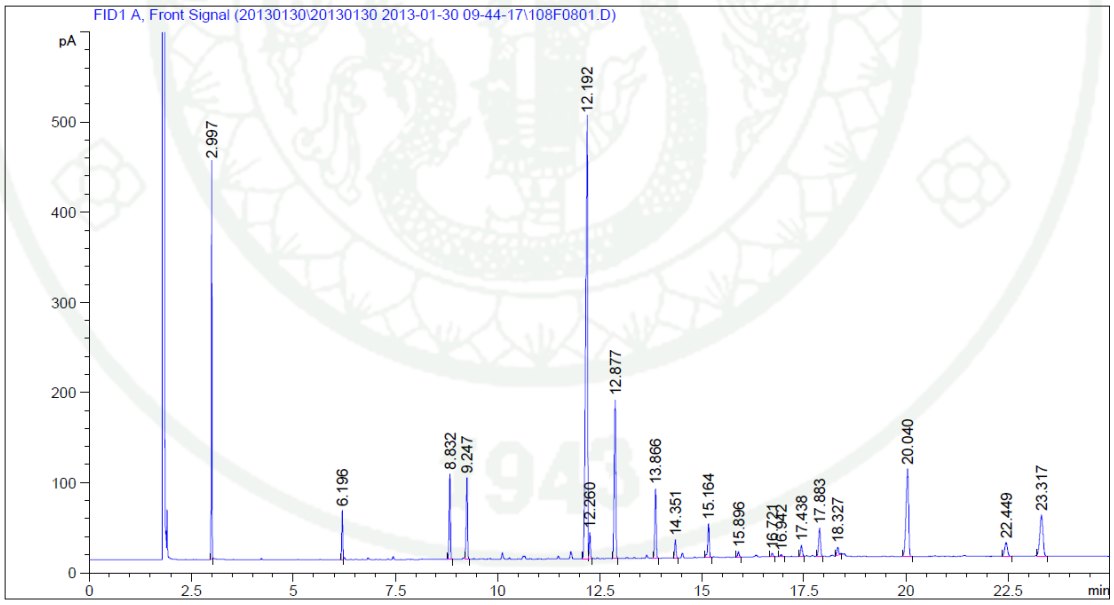

**Appendix Figure B7** Fatty acid composition of fatty acids at sn-2 of structured lipids of condition A



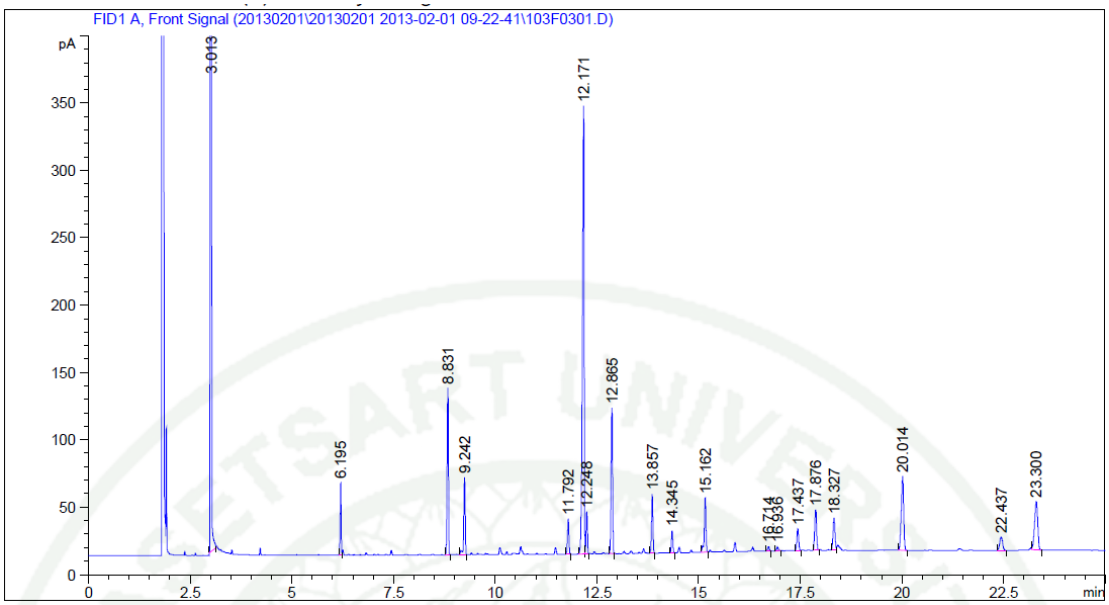

**Appendix Figure B8** Fatty acid composition of fatty acids at sn-1, 3 of structured lipids of condition A



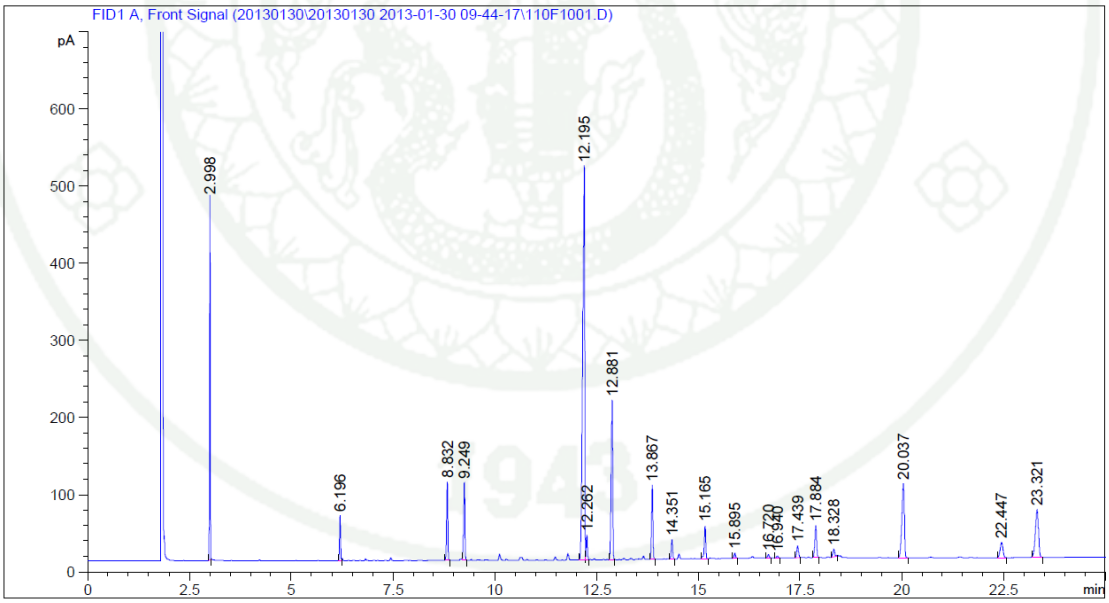

**Appendix Figure B9** Fatty acid composition of fatty acids at sn-1, 2, 3 of structured lipids of condition A



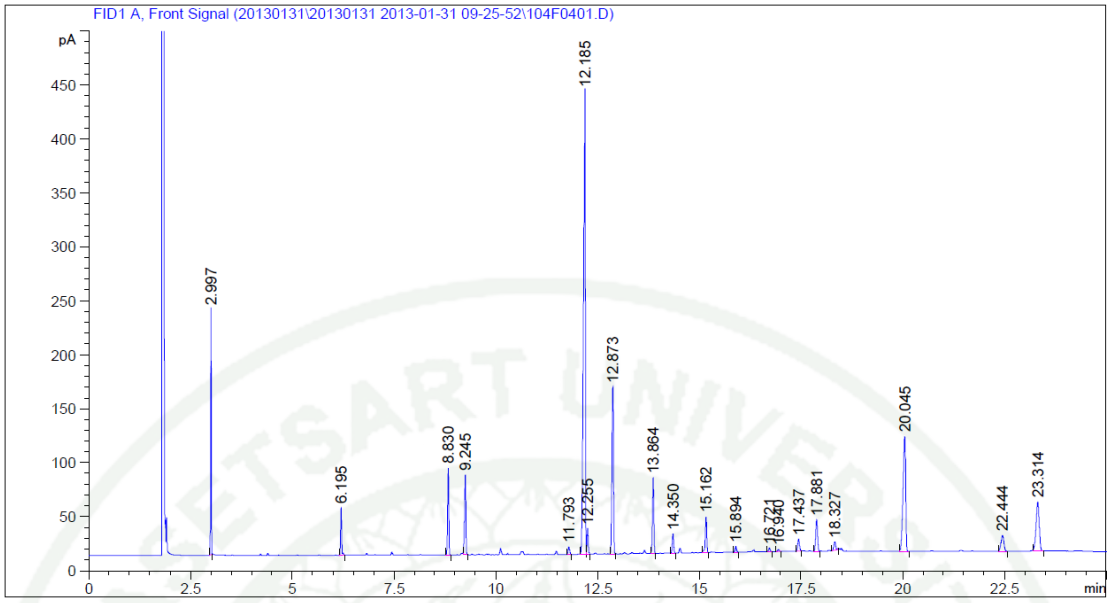

**Appendix Figure B10** Fatty acid composition of fatty acids at sn-2 of structured lipids of condition B



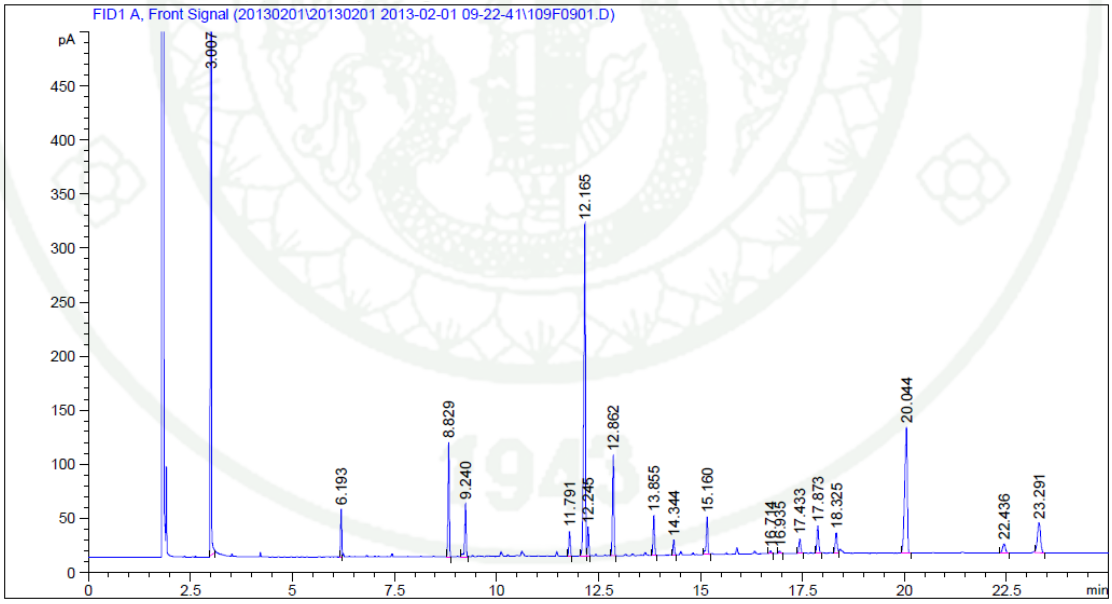

**Appendix Figure B11** Fatty acid composition of fatty acids at sn-1, 3 of structured lipids of condition B



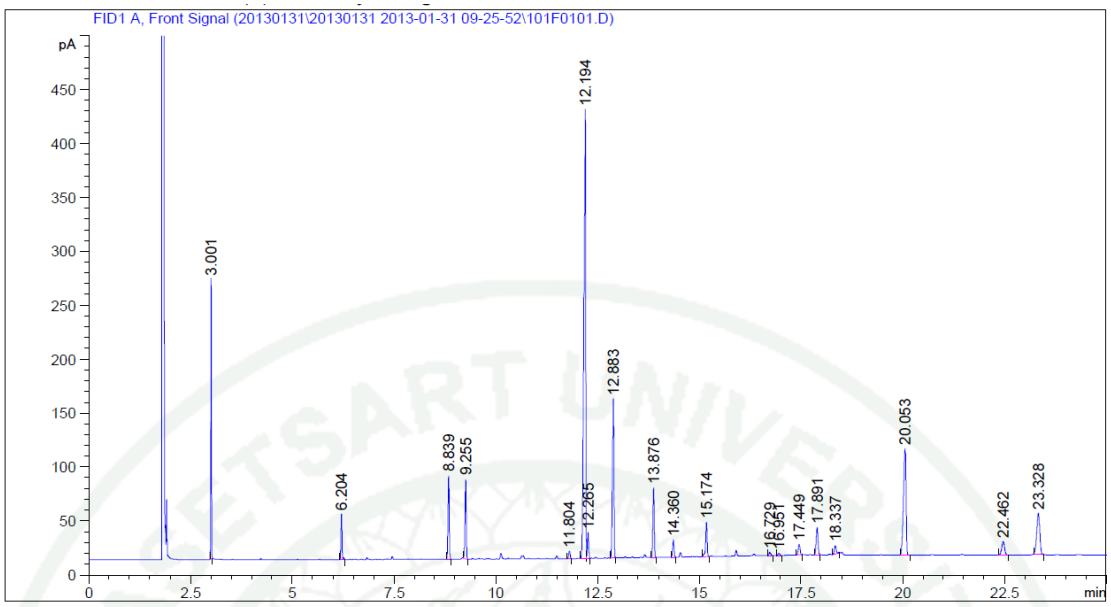

**Appendix Figure B12** Fatty acid composition of fatty acids at sn-1, 2, 3 of structured lipids of condition B




**Appendix Figure B13** Fatty acid composition of fatty acids at sn-2 of structured lipids of condition C




**Appendix Figure B14** Fatty acid composition of fatty acids at sn-1, 3 of structured lipids of condition C




**Appendix Figure B15** Fatty acid composition of fatty acids at sn-1, 2, 3 of structured lipids of condition C

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