การศึกษาผลของเลซิทินผสมที่เตรียมจากแหล่งที่มีกรดไขมันโอเมก้า 3 ในหนูทดลองต่อไลโปโปรตีนและกรดไขมันในพลาสมา

นางสาวจุลจิตร์ องศ์ปรีชากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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STUDY ON THE EFFECTS OF MIXED N-3 POLYUNSATURATED FATTY ACID-CONTAINING LECITHINS FED IN RATS ON LIPOPROTEINS AND FATTY ACIDS IN PLASMA

Miss Jullajit Ongpreechakul

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	STUDY ON THE EFFECTS OF MIXED N-3 POLYUNSATURATED FATTY ACID-CONTAINING LECITHINS FED IN RATS ON LIPOPROTEINS AND FATTY ACIDS IN PLASMA
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จุลจิตร์ องศ์ปรีชากุล : การศึกษาผลของเลซิทินผสมที่เตรียมจากแหล่งที่มีกรดไขมันโอเมก้า 3 ในหนูทดลองต่อไลโปโปรตีนและกรดไขมันในพลาสมา. (STUDY ON THE EFFECTS OF MIXED N-3 POLYUNSATURATED FATTY ACID-CONTAINING LECITHINS FED IN RATS ON LIPOPROTEINS AND FATTY ACIDS IN PLASMA) อ. ที่ปรึกษา : รองศาสตราจารย์ ดร. วินัย ดะห์ลัน, 153 หน้า.

สารอาหารหลายชนิด เช่น กรดไขมันไม่อิ่มตัวสูงชนิดโอเมก้า 3 มีผลต่อการป้องกันโรคหัวใจและหลอดเลือด แต่ในกรณีของเลซิทินยังให้ผลไม่ชัดเจนนัก งานวิจัยครั้งนี้ทำการศึกษาผลของเลซิทินจากปลาทะเล (LE-FM) ถั่วเหลือง (LE-SB) และเลซิทินผสมที่ได้จากการใช้เลซิทินสองชนิดผสมกันในสัดส่วน 1:1 และ 1:2 (LE-FS 1:1, LE-FS 1:2) ต่อไลโปโปรตีนและกรดไขมันในพลาสมาของหนูทดลอง ซึ่ง LE-FM มีกรดไขมันไม่อิ่มตัวสูงชนิดโอเมก้า 3 ได้แก่ C20:5n-3 และ C22:6n-3 สูงกว่า LE-SB ที่ใช้ทั่วไปทางการค้า และมีกรดไขมันชนิดโอเมก้า 6 ได้แก่ C18:2n-6 และ C20:4n-6 ต่ำกว่า LE-SB จากการศึกษาองค์ประกอบของเลซิทินพบว่า LE-FM และ LE-SB มีปริมาณ phosphatidylcholine (PC) 47.3% และ 36.2%; phosphatidylinositol (PI) 17.9% และ 46.3%; sphingomyelin (SM) 22.9% และ 0% กรดไขมัน C18:2n-6 1.06% และ 54.83%; C18:3n-3 0.37% และ 4.93%; C20:5n-3 5.2% และ 0%; C22:6n-3, 21.11% และ 0% ตามลำดับ ภายหลังการเสริมเลซิทินในสัตว์ทดลองเป็นเวลา 8 สัปดาห์ พบว่าไขมันในเลือดไม่เปลี่ยนแปลง แต่สัดส่วนของ VLDL-TG/HDL-TG ของหนูกลุ่ม LE-FM และ LE-SB ลดลง ($p<0.05$) แสดงถึงอัตราการไหลเวียนและเมแทบอลิซึมของ TG-rich lipoproteins ที่เพิ่มขึ้น นอกจากนี้ particles size ของ VLDL และ LDL ในหนูที่ได้รับเลซิทินทุกกลุ่มมีขนาดเล็กลงเมื่อเทียบกับกลุ่มควบคุม ($p<0.05$) รวมถึง particle size ของ HDL ด้วย ยกเว้น HDL particles ในกลุ่มที่ได้รับ LE-SB มีขนาดใหญ่ขึ้น ($p<0.05$) ความแตกต่างเช่นนี้อาจเป็นผลมาจากชนิดของฟอสโฟลิพิด ได้แก่ SM และ PI ในเลซิทินทั้งสองกลุ่มที่แตกต่างกัน ในกรณีการขนส่งกรดไขมันเพื่อแลกเปลี่ยนกับเซลล์ต่างๆในกระแสเลือด พบว่าสัดส่วนของ C20:5n-3 + C22:6n-3 /C18:2n-6 + C20:2n-6 ซึ่งเป็นกรดไขมันตั้งต้นในการสร้าง eicosanoids serie 2 และ serie 3 ในพลาสมาสูงขึ้นในกลุ่มที่ได้รับ LE-FM เมื่อเทียบกับกลุ่มที่ได้รับ LE-SB บ่งชี้ถึงฤทธิ์ของ LE-FM ในการป้องกันการเกิด thrombogenesis ที่สูงกว่า LE-SB สรุปว่าการเสริมเลซิทินส่งผลดีต่อเมแทบอลิซึมของ TG-rich lipoproteins

ภาควิชาเคมีคลินิก ลายมือชื่อนิสิต.....
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JULLAJIT ONGPREECHAKUL : STUDY ON THE EFFECTS OF MIXED N-3 POLYUNSATURATED FATTY ACID-CONTAINING LECITHINS FED IN RATS ON LIPOPROTEINS AND FATTY ACIDS IN PLASMA. THESIS ADVISOR : ASSOCIATE PROFESSOR WINAI DAHLAN, Ph.D., 153 pp.

The protective effect on cardiovascular disease of many nutrients including omega 3 polyunsaturated fatty acids (n-3 PUFAs) is well established but it has not been investigated this effect in lecithin yet. The objective of the study was to investigate the effects of lecithin which were extracted from fishmeal (LE-FM) containing with high n-3 PUFAs, e.g., C20:5n-3 and C22:6n-3 and low n-6 PUFAs, e.g., C18:2n-6 and C20:4n-6, soybean lecithin (LE-SB) and mixed LE-FM and LE-SB (w/w) in ratios of 1:1 (LE-FS 1:1) and 1:2 (LE-FS 1:2) on lipid profiles in normal rats. For the phospholipids analysis, LE-FM and LE-SB contained phosphatidylcholine (PC) 47.3% and 36.2%, phosphatidylinositol (PI) 17.9% and 46.3%, sphingomyelin (SM) 22.9% and 0% C18:2n-6 1.06% and 54.83%, C18:3n-3 0.37% and 4.93%, C20:5n-3 5.2% and 0% and C22:6n-3 21.11% and 0%, respectively. After 8 weeks administration of each lecithin, no change in plasma lipids was observed in normal rats. However, the group treated with LE-FM and LE-SB significantly decreased VLDL-TG/HDL-TG ratio when compared to control group, indicating that the rate of metabolite of TG-rich lipoprotein increased in the blood circulation. Moreover, VLDL and LDL particles size in normal rats treated with lecithin were decreased when compared to control group. In addition, HDL particles size in normal rats treated with lecithin was also decreased except HDL size of LE-SB group was increased when compared to control group. Such different effects were possibly influenced by dissimilarity of phospholipids subspecies: SM and PI in lecithin. In case of PUFAs, the ratio of serie-3 to serie-2 eicosanoid precursor fatty acids: C20:5n-3 + C22:6n-3 / C18:2n-6 + C20:2n-6 in plasma of normal rats treated with LE-FM was increased when compared to LE-SB group. These findings indicated that LE-FM had the protective effect on thrombogenicity and cardiovascular disease. In conclusion, lecithin supplementation could be the beneficial effects to metabolism of plasma TG-rich lipoproteins.

Department: Clinical Chemistry Student's signature.....

Field of study: Clinical Biochemistry and Molecular Medicine Advisor's signature.....

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CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

ABBREVIATIONS

°C	=	degree Celcius
µl	=	microlitre
AA	=	arachidonic acid
ALA	=	alpha-linolenic acid
C	=	cholesterol
Chol	=	cholesterol
DHA	=	docosahexaenoic acid
DMPE	=	1,2-dimyristoyl-*sn*-glycero-3-[phosphoethanolamine]
DMPS	=	1,2-dimyristoyl-*sn*-glycero-3-[phosphor-l-serine]
EPA	=	eicosapentaenoic acid
FA	=	Fatty acid
FA-C15:0	=	pentadecanoic acid
FAME	=	fatty acid methyl ester
FM	=	fish meal lecithin
FS 1:1	=	fish meal lecithin and soybean lecithin mixed in the ratio of FM : SB 1:1 (w/w)
FS 1:2	=	fish meal lecithin and soybean lecithin mixed in the ratio of FM : SB 1:2 (w/w)
g	=	gram
GC	=	gas chromatography
h	=	hour
HDL	=	high-density lipoprotein
HLPC	=	high performance liquid chromatography
IS	=	internal standard
L	=	litre
LA	=	linoleic acid

LDL	=	low-density lipoprotein
LPC-C15:0	=	1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine
min	=	minute
ml	=	millilitre
mm	=	millimetre
mmole	=	millimole
MS	=	mass spectrometer
MUFA	=	monounsaturated fatty acid
nm	=	nanometre
P	=	phosphorus
PC	=	phosphatidylcholine
PC-C14:1	=	1,2-dimyristelaidoyl-*sn*-glycero-3-[phosphocholine]
PE	=	phosphatidylethanolamine
PI	=	phosphatidylinositol
PL	=	phospholipid
pPE	=	phosphatidylethanolamine plasmalogen
ppm	=	part per million
PS	=	phosphatidylserine
PUFA	=	polyunsaturated fatty acid
rpm	=	round per minute
SB	=	soybean lecithin
SEM	=	standard error of mean
SFA	=	saturated fatty acid
SM	=	spingomyelin
TEM	=	transmission electron microscope
TG	=	triacyglycerol

TG-C15:0	=	tripentadecanoin
TLC	=	thin-layer chromatography
VLDL	=	very low-density lipoprotein

CHAPTER I

INTRODUCTION

Background and rationale

In many countries including Thailand, dietary recommendations aim to reduce lipid consumption so as to reduce the risk of cardiovascular disease (Expert Panel, 1993, Schaefer, 1997). Fat-rich foods are also rich in phosphatidylcholines, the main components of lecithin. One unfavorable consequence of decreasing lipid consumption is a probable decrease in lecithin intake (Canty and Zeisel, 1994). Thus, many studies were carried out the lecithin as a macronutrient in order to evaluate its effects, notably on dyslipidemia and related risk factors of atherosclerosis.

Regarding phospholipids which are called in commercial aspect as lecithin, they are one class of lipids, and a major component of all biological membranes, along with glycolipids, cholesterol and proteins as well as component of monolayer surface of lipoproteins. Due to its polar nature, the head of a phospholipid is hydrophilic (attracted to water); the nonpolar tails are hydrophobic (not attracted to water). When placed in water, phospholipids form a bilayer, where the hydrophobic tails line up against each other, forming a membrane with hydrophilic heads on both sides extending out into the water. This allows it to form liposomes spontaneously, or small lipid vesicles, which can then be used to transport materials into living organisms and study diffusion rates into or out of a cell membrane. In order to study property as lipid and fatty acid transporting vehicle, liposomes as well as fat emulsions which mimicing natural plasma lipoproteins have been used as model.

Generally, fat emulsions are commonly used in intravenously in critically ill and nutritionally depleted patients of all ages as a regimen in both total and partial parenteral nutrition (TPN and PPN). They are utilized mainly to prevent the development of essential fatty acid deficiency and as excellent source of calories (Carpentier, 1989). In general, fat emulsions are made up of three basic components: an aqueous phase of isotonic solution, an emulsifying system of lecithin or phospholipid, and a lipid phase of

oil or triacylglycerol (TG, Dahlan, 1989). As earlier mentioned concerning lipid/fatty acid transport with liposomes or emulsions, Dahlan et al (1992a) demonstrated that during 6-h intravenous infusion of fat emulsion with egg yolk lecithin as emulsifier in healthy men, Red Blood Cell (RBC) membranes altered in their major lipid composition, for instances cholesterol (Chol) decreased while phospholipids (PL) increased.

In addition, Dahlan et al (1992b) showed an evidence that daily infusion of fat emulsions for a long-term period caused a redistribution of the polyunsaturated fatty acids (PUFA) between the n-3 and n-6 families. In their further experiment, Chatnilbandhu (1996) investigated the exchange of fatty acid between red blood cells (RBC) and lecithin-rich fat emulsion *in vitro* by incubating RBC of 40% hematocrit with either fishmeal-, egg yolk- or soya-derived lecithin-rich fat emulsion (FM-LRFE, EY-LRFE or SY-LRFE, respectively) at the lecithin concentrations of 100-300 mg/100 ml incubation mixture for 1 h. They also found that neither emulsion affected RBC membrane cholesterol to phospholipids ratio. However, RBC membrane n-3 PUFA was alternated after the incubations with SY-LRFE and EY-LRFE. Incubating RBC with SY-LRFE, linoleic acid (LA, C18:2 n-6) on RBC membrane was increased whereas DHA (docosahexaenoic acid, C22:6 n-3) was decreased. This led to a marked reduction of n-3/n-6 PUFA ratio ($p<0.05$) corresponding to results obtained from Dahlan et al as earlier referred. In contrast, FM-LRFE provided DHA to RBC and raised membrane n-3 PUFAs significantly ($p<0.05$). They, therefore, concluded that FM-LRFE not only prevents the loss of n-3 PUFA from blood cells but also acts as a good n-3 PUFA supplier.

Charnprasert (1997) demonstrated that platelet membranes' PUFA profile was able to be restructured as needed. FM-LRFE supplied n-3 PUFA whereas SY-LRFE provided n-6 PUFA to the platelet membranes. The higher n-3/n-6 PUFA ratio of platelet membranes was thus concluded that it possibly provided benefit for the production of antithrombogenic prostaglandin (PG).

To the best knowledge, there are no evidences regarding the effect of n-3 PUFA rich lecithin in the *in viv*o model. Thus, the aim of this study was to investigate

the effect of n-3 PUFA rich lecithin on plasma lipid profiles in normal rats. The study also focused on the alterations of lipoprotein subpopulations as well as lipoprotein particle sizes and plasma fatty acid profiles.

Research question

Can the difference sources of lecithin containing various n-3/n-6 PUFAs alter plasma lipoprotein subpopulations and fatty acid profiles in the normal rats?

The objective of the study

To investigate the effects of lecithin which were extracted from various sources on plasma lipoprotein subpopulations and plasma fatty acids in normal rats.

Hypothesis

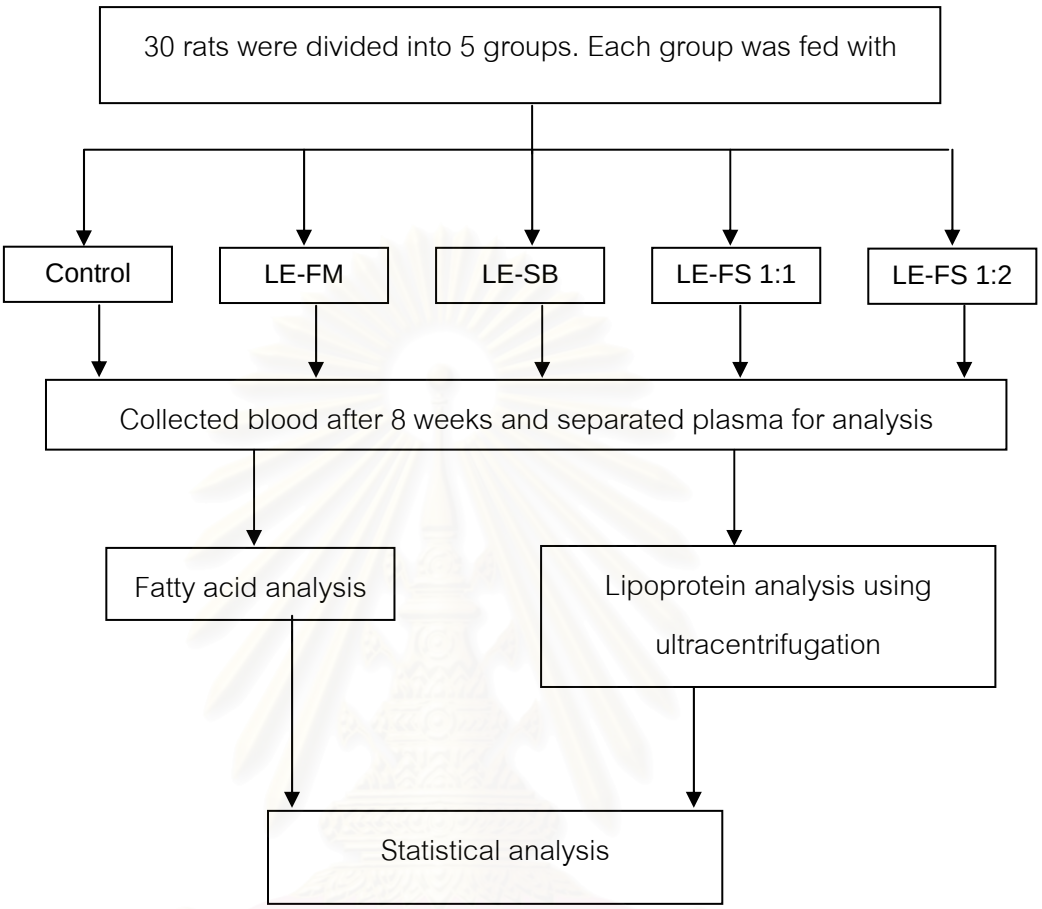
A. Lecithin alters the plasma lipid contents, especially in triacylglycerol content.

B. VLDL particle of normal rat treated with lecithin were smaller than untreated normal rat.

C. The group treated with fishmeal lecithin increased in the component of n-3 PUFA.

D. The group treated with soybean lecithin increased in the component of n-6 PUFA.

Conceptual framework



Control = 1% Tween-20

LE-FM = lecithins derived from fishmeal

LE-SB = lecithins derived from soybean

LE-FS 1:1 = lecithins obtained from blending LE-FM with LE-SB in ratio of 1:1, w/w

LE-FS 1:2 = lecithins obtained from blending LE-FM with LE-SB in ratio of 1:2, w/w

The beneficial of this study

A. This study would be an important fundamental in basic science for further research.

B. This study would indicate the cardioprotective effect of lecithin by alteration on lipid metabolism.

CHAPTER II

LITERATURE REVIEW

The chemistry of fatty acids

Fatty acids are the building blocks from which fats (lipids) are made. Fatty acids found in foods and fats stored in the body are mainly present in the form of triacylglycerols (TG), a glycerol molecule backbone to which three, often different, fatty acids are attached. Fatty acids can be present at any of three positions on the glycerol molecule (termed *sn*-1; *sn*-2 and *sn*-3). The presence of different fatty acids at different positions on the glycerol molecule will also influence its characteristics (i.e. melting point and digestibility).

Fatty acids are made up of a backbone of carbon atoms, with a methyl group (CH_3) at one end [the omega (ω) or n-end] and a carboxyl group (COOH) at the other end [the delta (δ) end]. Hydrogen atoms are joined to the string of carbon atoms, forming a hydrocarbon chain.

Fatty acids vary in length from 2 to 80 carbons, but are typically present in food as 14, 16, 18, 20 and 22 carbon atom chains. Short chain fatty acids, such as butyric acid (4 carbon atoms) or propionic acid (3 carbon atoms) are formed in the gut when polysaccharides are fermented by the anaerobic bacteria present in the large intestine. Certain short chain fatty acids, namely, butyrate, are believed to be important for gut health (Bird et al, 2000). Technically, the long chain fatty acids contain 12 or more carbon atoms. However, this term is often used to describe the longer chain fatty acids that contain more than 20 carbon atoms, which may also be referred to as very long chain fatty acids.

Carbon chain length influences the characteristics of a fatty acid, as does the presence or absence of double bonds between carbon atoms.

a. If all of the carbons in the fatty acid chain are linked by single bonds (i.e. the fatty acid contains all the hydrogen atoms that it can hold), the fatty acid is said to be a saturated fatty acid (SFA).

b. If one or more double bonds are present in the fatty acid chain (i.e. the fatty acid does not hold its potential full complement of hydrogen atoms), the fatty acid is considered to be an unsaturated fatty acid.

- If there is only one double bond present in an unsaturated fatty acid, it is said to be a monounsaturated fatty acid (MUFA).
- If there is more than one double bond present, the fatty acid is said to be a polyunsaturated fatty acid (PUFA).

PUFAs can be further classified as either n-3 (omega 3) or n-6 (omega 6) PUFAs; to which family a PUFA belongs depends on the position of the first double bond in the fatty acid chain. All members of the n-6 family of fatty acids contain their first double bond between the sixth and seventh carbon atoms from the terminal methyl group, while all members of the n-3 family of fatty acids have their first double bond between the third and fourth carbon atoms.

Digestion, absorption and metabolism

Fat enhances the flavour and palatability of foods, and has been shown to slow the rate at which the stomach contents are emptied into the small intestine. As a result, fat leads to general feelings of satiety and satisfaction after a meal that is often difficult to mimic in reduced fat products. In order to be efficiently digested and absorbed, dietary fat must first be emulsified by bile secreted into the small intestine from the gallbladder. The fat droplets form small micelles that are dispersible in water and so can be acted on by pancreatic lipases cleaving the fatty acids at the *sn-1* and *sn-3* positions of the TG. The end-products from the digestion of fat: free fatty acids, a 2-monoacylglycerol, and very limited amounts of glycerol, are absorbed by diffusion across the gut wall into the cells of the intestine. Recently, work by Stremmel et al (2001)

has introduced the idea that fatty acids may be absorbed by carrier-mediated processes, involving transport proteins in the membranes of cells lining the small intestine. However, the importance of this route has been questioned, as the candidate transporter proteins are also found in other cells in the body where they have little to do with fatty acid transport (Tso et al, 2004). Regardless of this, the digestion of fat is very efficient, with typically more than 95% of dietary fat being broken down and absorbed (Sanders and Emery, 2003).

The mode of transport away from the gut is dependent on chain length; the short to medium chain fatty acids (2–12 carbon atoms) are transported bound to the carrier protein albumin *via* the hepatic portal vein to the liver. However, dietary fat mainly consists of the longer chain fatty acids (>12 carbon atoms), which are reassembled in the intestinal cell into TG. These are then packaged into lipoprotein particles known as chylomicrons and are transported *via* the lymph system into the peripheral circulation. The rate of lipolysis (breakdown of fat), absorption of fatty acids and transport might be influenced by the degree of unsaturation (BNF, 1992). However, the nutritional significance of this difference is unclear. The fatty acid at *sn*-2 may influence subsequent metabolism of chylomicrons and ultimately low density lipoprotein (LDL) cholesterol response. It has been suggested that dietary fats containing a predominance of *sn*-2 unsaturated TG might be absorbed more slowly, and cleared more rapidly from the circulation compared with saturated fatty acids, resulting in reduced concentrations of TG in the blood after a meal (Yli-Jokipii et al, 2001). Conversely, SFAs in the *sn*-2 position are preferentially absorbed into the cells lining the intestines over SFAs at positions *sn*-1 and *sn*-2, which also has implications for the balance of TG in the blood after a meal (Berry and Sanders, 2005). It remains controversial as to the relevance of these findings to adults, as most of the work has been conducted in animals, and any work that has been carried out in humans has been conducted in infants.

Once in the bloodstream, chylomicrons acquire apolipoprotein C (apoC) from high density lipoproteins (HDL). At the target cell (e.g. muscle), the apoC subunit

activates the enzyme lipoprotein lipase, which is located within the endothelial cell membrane. This results in cleavage of the fatty acids, which are then transported into tissues for storage or metabolism.

Fatty acids undergo β-oxidation in mitochondria to release energy. The exact energy yield of the different fats is dependent on the chain length and the number of double bonds in the molecule, although 1 g of fat is assumed to release 37 kJ (9 kcal).

Most unsaturated fatty acids can be synthesised in the body following a series of fatty acid chain elongation and desaturation steps. This process is catalysed by the enzyme complex, fatty acid synthase. Plants contain desaturases capable of inserting double bonds at positions 3, 6 and 9 from the terminal methyl group. Animal and human desaturases are only capable of inserting double bonds at specific positions beyond carbon 6. Therefore, there are several fatty acids, termed essential fatty acids (EFA), which cannot be made in the body but are required for normal physiological function in humans. There are two specific EFAs: the cis n-6 PUFA linoleic acid (LA; 18:2 n-6) and cis n-3 PUFA alpha (α)-linolenic acid (ALA; 18:3 n-3). It is from these two 'parent' EFAs that the n-3 and n-6 fatty acid 'families' are derived through a series of enzyme catalysed desaturation and elongation reactions, that generally take place in the cell cytosol or in the mitochondria.

ALA is metabolised to docosahexaenoic acid (DHA; 22:6 n-3) via eicosapentaenoic acid (EPA; 20:5 n-3) and docosapentaenoic acid (DPA; 22:5 n-3), whereas LA is metabolised to arachidonic acid (AA; 20:4 n-6) via gamma (γ)-linolenic acid (GLA; 18:3 n-6) or eicosadienoic acid (20:2 n-6) as two pathways are active. However, conversion is not 100% efficient. Desaturation of DPA at the δ-4 position to produce DHA does not only occur by a single step catalysed by δ-4 desaturase. There is an additional pathway, the 'Sprecher Pathway', which involves elongation and desaturation of DPA and tetracosahexenoic acid in microsomes followed by a chain-shortening step to DHA in peroxisomes (Sprecher, 2000).

It has been estimated that less than 8% of ALA is metabolised to EPA, and the capacity for the body to synthesise DHA appears to be particularly limited. It is estimated that only between 0.02% and 4% of ALA is metabolised to DHA (Vermunt et al, 2000; Pawlosky et al, 2001; Burdge et al, 2002), with women having a greater capacity for DHA synthesis than men (Burdge and Wootton, 2002). Feedback inhibition of enzymes involved in n-3 and n-6 fatty acid synthesis occurs; for example, if the amount of DHA in the diet increases, there is reduced metabolism of ALA to EPA. Furthermore, n-3 and n-6 fatty acids compete for the enzymes involved in fatty acid elongation and desaturation, with certain enzymes having greater affinity for n-3 fatty acids and others having greater affinity for the n-6 series (BNF, 1999).

The metabolites of the EFAs have many different functions. DHA and AA are major constituents of membrane phospholipids, especially in the retina, brain (predominantly as DHA) and platelets (predominantly as AA). As the capacity to synthesise EPA and DHA from the EFA, ALA is very limited, dietary sources make a major contribution to the amount of both these cis n-3 PUFAs in the body (Burdge and Calder, 2005).

The 20 carbon metabolites of EFAs (EPA and AA) function as the basis for important regulatory signals known as eicosanoids. There are several different families of eicosanoids: the prostaglandins (which regulate muscle contraction, immune response and inflammation), prostacyclins (which inhibit platelet aggregation) and thromboxanes (which bring about platelet aggregation) are all formed by the action of cyclo-oxygenase enzymes; the leukotrienes (which affect microvascular and bronchial constriction or dilatation) and hydroxyfatty acids (which regulate cell adhesion) are formed by the action of lipoxygenases. Eicosanoids are produced by cells to act in their immediate environment in response to extracellular stimuli, e.g. blood vessel damage. Eicosanoid formation from the two EFA families follows slightly different pathways. However, they share a common set of enzymes, so the ratio of n-3 to n-6 PUFAs in the diet will affect which pathway is the most active. The activity of the different eicosanoids produced from EFAs varies; eicosanoids derived from the cis n-6 PUFAs typically exert

stronger effects than those derived from n-3. Thus the diet has a role to play in determining the final mix and potency of eicosanoids.

The n-3 PUFAs are also major structural components of membrane phospholipids and influence membrane fluidity and ion transport. The n-3 and n-6 fatty acids compete for receptor sites and therefore incorporation into the phospholipid components of cell membranes. This meta-bolic interaction between the two families suggests that balance in the intake of these fatty acids may be crucial.

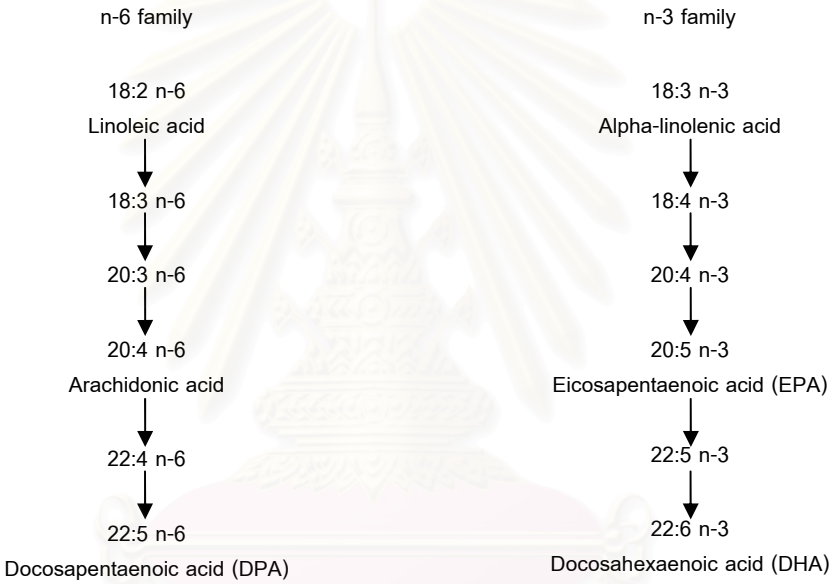


Figure 1 The families of polyunsaturated fatty acid

The richest sources of ALA include some seed oils (e.g., linseed oil, rapeseed oil, soya oil, walnut oil) and nuts (e.g., walnuts, peanuts). Other important sources are meat from grass-fed animals (e.g., beef) and green leafy vegetables (e.g., spinach). The very-long-chain derivatives of ALA, EPA and DHA, are found predominantly in fish oils and are also present in the flesh of oil-rich fish such as mackerel, salmon, kippers, herring, trout and sardines.

The suggested minimum requirement for n-3 fatty acids in the UK is 0.2% of dietary energy (Department of Health, 1991). However in 1994, in response to

the research which showed that eating oil-rich fish could reduce the risk of coronary heart disease mortality, the Government advisory committee recommended an average weekly consumption of very-long-chain n-3 fatty acids of 1.5 g/week (Department of Health, 1994). This is most easily met by the weekly consumption of one to two portions of oil-rich fish.

The changing dietary patterns which may have distorted the balance of the n-3 and n-6 fatty acids in the diet. The n-6 fatty acid content of the UK diet has risen substantially, as the food industry has replaced traditional sources of fat (for example, lard and butter) with vegetable oils (for example, sunflower oil, corn oil), in response to research concerning heart disease. However, to some extent, very recently, the balance may have been partially redressed with the increased availability of rapeseed oil, which is now widely used in the UK. The consumption of oil-rich fish also seems to have risen slightly.

Most research has focused on a protective role for n-3 fatty acids in coronary heart disease. Although it is now recognised that the n-3 fatty acids have little effect on blood cholesterol levels, postulated cardioprotective actions include a lowering of plasma triacylglycerol (TG) concentration, reduction of blood clotting, inhibition of platelet aggregation, improved endothelial function, antiarrythmic effects and blood pressure lowering. An elevated plasma TG concentration, particularly in the fed state, is now recognised as being associated with the development of atherosclerosis. A substantial number of studies have shown supplementation with fish oils reduces blood TG levels, both in the fasting state and after a meal. Fewer studies have investigated the hypotriaclyglycerolemic effects of ALA, although it does not appear to exert an effect at comparable intakes. While a daily supplement of 2–5 g of EPA/DHA results in a 20–40% reduction in fasting circulating TG levels, little research is available on the effect of more modest intakes (<2 g/day) that might be achieved by more regular consumption of oil-rich fish or foods fortified with fish oils.

The Diet and Reinfarction Trial investigated (2000) men who had recently suffered a heart attack. Subjects were randomly allocated into groups, each given different dietary advice: to reduce total fat intake; to increase fibre intake; and to consume at least two portions of oil-rich fish per week (Burr et al, 1989). After 2 years the group advised to eat more fish had 29% fewer deaths than those not given such advice. This difference was attributable to a reduction in CHD deaths. The recent GISSI trial demonstrated the same protective effect with fish oil supplements; 1 g of EPA and DHA daily reduced coronary heart disease deaths by 20% (GISSI-Prevenzione Investigators, 1999) This level of intervention showed no significant effect on blood TG level.

The n-3 fatty acids appear to reduce the tendency of the blood cells to form a clot. Platelet aggregation (the clumping together of particular types of blood cells) is controlled by the balance between pro-and anti-aggregatory eicosanoids. Arachidonic acid (AA), the n-6 long-chain fatty acid, produces prothrombotic eicosanoids (for example, thromboxane A2), while the n-3 equivalent produces eicosanoids that exert an antithrombotic effect (for example, thromboxane A3). The dietary balance of these PUFAs in the diet will therefore affect the tendency of the blood cells to aggregate and to form a clot. The n-3 fatty acids also appear to protect against cardiac arrythmias. These are irregularities in the electrical conductivity of the heart, which can cause sudden death. This protective effect is likely to be the result of n-3 fatty acids, particularly DHA, playing an important role in the conservation of the normal function of the cells lining the artery walls (the endothelial cells).

Inflammation is a normal protective response to infection, injury or trauma. However an exaggerated immune response directed against the host tissue can cause long-term damage. This occurs in chronic inflammatory diseases such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, psoriasis, type 1 diabetes, multiple sclerosis and atopic disease. There is some evidence to suggest that fish oils may be useful in the treatment of these inflammatory conditions. The dietary intake of n-6 and n-3 fatty acids will determine the types and amounts of eicosanoids in

the body, which are key mediators of inflammation. AA produces eicosanoids (4-series leukotrienes and 2-series prostaglandins) which promote inflammation. The n-3 fatty acids equivalents produced from EPA (5-series leukotrienes and 3-series prostaglandins) are much less potent. The overall effect of fish oil consumption will be partial replacement of the n-6 eicosanoids with the n-3 alternatives. These changes may explain the suggested benefits in inflammatory diseases. Intervention studies have been attempted in a number of chronic inflammatory conditions. Supplementation with large doses of the very-long-chain n-3 fatty acids have been reported to alleviate symptoms such as joint stiffness in rheumatoid arthritis sufferers. However, consistent clinical changes have not been reported for other conditions, and there is no evidence that consumption can prevent the diseases from occurring in the first place.

The role of n-3 fatty acids in infant development. The very-long-chain fatty acids, particularly DHA, are major constituents of the phospholipids of the brain cells and photoreceptor cells of the retina, where they seem to affect membrane fluidity. The proportion of DHA in the membranes of these cells increases steadily during the last trimester of pregnancy. Consequently, an adequate supply of DHA in fetal and early infant life is thought to be essential for normal visual and neurological development. This has led to concern about the adequacy of the diet of pregnant and breast-feeding women. DHA can be synthesised from ALA in the body but the rate of synthesis may be inadequate in preterm babies. Since breast milk contains DHA, the need for supplementation of infant formula has been raised. Children who are breast-fed have been shown to perform better in tests of development or cognition, verbal ability or school performance than those receiving infant formula unsupplemented with long-chain fatty acids. However numerous social and cultural factors are associated with the decision whether or not to breastfeed. Attempts to adjust for such factors have shown the association to persist in preterm babies. However, breast milk differs in a number of other respects, in addition to its PUFA content.

Insulin resistance is an impairment of insulin action that occurs in individuals with noninsulin-dependent diabetes. A high level of n-3 fatty acids in the

phospholipid component of cell membranes may improve their permeability to the hormone insulin, which controls the uptake of glucose into the cells. A high intake of n-6 PUFA could have the opposing effect, and the ratio of intake of the two PUFA families is therefore likely to be of importance. Some studies have suggested that fish oil supplementation may protect against the development of glucose intolerance. However supplementation studies in people who have already developed diabetes have not shown a consistent effect on blood glucose. This suggests that n-3 fatty acids may not be able to reverse insulin resistance once damage to the insulin-producing cells has been done. However these supplementation studies did demonstrate a TG-lowering effect. Raised TG levels are a feature of noninsulin-dependent diabetes.

Intake of n-3 PUFA can most readily be increased by choosing rapeseed oil instead of other vegetable oils for cooking. The consumption of oil-rich fish such as salmon, herring and mackerel once a week makes a significant contribution to the intake of very-long-chain n-3 fatty acids. Small amounts of these are also provided by meat and eggs. For those who do not like fish, supplements containing very-long-chain n-3 fatty acids (either fish oil or algal oil) or foods fortified with them are an option. Foods rich in PUFAs are vulnerable to rancidity through the oxidation of the fatty acids. This calls for particular care in ensuring that the foods themselves are protected by incorporation of appropriate antioxidants and that these products are consumed as part of a diet providing adequate amounts of the nutrients and other substances recognised as being protective (for example, those present in fruit and vegetables).

Unsaturated fatty acids and cardiovascular disease

CVD, encompassing coronary heart disease (CHD), stroke and other diseases of the cardiovascular system, is one of the leading causes of mortality and morbidity worldwide include Thailand. Death rates have been falling in most developed countries. However, in the UK, morbidity associated with CVD appears to be increasing, at least in the oldest age groups (Frayn and Stanner, 2005). More people today survive heart attacks than ever before, but many go on to develop chronic heart failure or

angina, which is associated with a reduced quality of life. For this reason, it is important to reduce the incidence of CVD and the occurrence of heart attacks or stroke and at the same time, reduce morbidity in those who experience CVD.

CVD is a consequence of two interrelated pathological processes: atherosclerosis (hardening and narrowing of blood vessels) and thrombosis (blood clotting). Atherosclerosis is characterised by the accumulation of lipids in the blood vessel walls over a long period of time. This can lead to impaired flow of blood (and therefore oxygen) to the heart, and angina pectoris (chest pain). A heart attack (myocardial infarction: MI) is usually precipitated by a blockage in the coronary artery following the rupture of an atherosclerotic plaque in the arterial lumen. A stroke occurs when a blockage disrupts the blood flow to the brain. (Frayn and Stanner, 2005).

Established risk factors for CVD include age, sex, ethnicity, hypertension, abnormal blood lipids (dyslipidaemia), diabetes, obesity, physical inactivity and smoking. While these established risk factors can explain a high proportion of risk, there still remains some variability unexplained, and a number of emerging risk factors for CVD have been identified, *e.g.* endothelial dysfunction (Sattar and Ferns, 2005), metabolic syndrome (Coppack et al, 2005), and fetal and maternal nutrition (Fall, 2005). Diet is recognised as an important modifiable risk factor for CVD; for example, diets rich in fruit and vegetables are associated with a reduced risk of CVD (Buttriss, 2005). The amount of fat in the diet and type of fatty acids consumed can influence the risk of CVD and its risk factors.

A series of papers published in the 1970s reported that Greenland Inuit, who ate the traditional diet high in seal meat, whale meat and fish, had rates of coronary heart disease ten times lower than their geographical neighbours, the Danes. This low rate of heart disease occurred despite the fact that the diet contained a similar total amount of fat, most of which came from animal sources. The reports on the health and mortality of Inuit focused scientific attention upon the protective and therapeutic potential of fish oils and the n-3 polyunsaturated fatty acids (Stanner, 2000).

Dietary fatty acids and risk of CVD

Evidence for the association between unsaturated fatty acid consumption and CVD comes from a wide-range of sources: cross-cultural comparisons; prospective (cohort) studies; case–control studies and intervention trials.

Studies comparing disease rates in different countries have suggested an inverse association between MUFA intake and death from CHD. Indeed, the mortality rate from CHD is very low in Mediterranean countries where the 'typical' traditional diet is low in SFAs and high in MUFAs (as a result of high intakes of olive oil). Although the diets of Mediterranean countries can vary, MUFAs typically account for 16–29% of energy intake (Kris-Etherton, 1999). Further evidence for an inverse association between MUFA intake and CVD comes from the Nurses' Health Study, a prospective study with a follow-up period of 14 years conducted in more than 80,000 women aged 34–59 years at entry. It reported that a 5% increase in energy derived from MUFA is associated with a significant 19% reduction in risk of developing CHD in women with no history of CHD (Hu et al, 1997). In the Lyon Diet Heart Study, patients randomised to receive a Mediterranean-type diet, including high MUFA, had significant reductions in the risk of death from cardiovascular causes or non-fatal acute myocardial infarction (73%), as well as in cardiac mortality (76%) and total mortality (70%) (de Lorgeril et al, 1994).

While there are a number of other epidemiological studies that confirm a protective effect of MUFAs on CHD (Artaud-Wild et al, 1993, Pietinen et al, 1997), there are also a number of studies that refute an association, including the Framingham Heart Study (Posner et al, 1991).

Little research has been conducted on the influence of n-6 PUFAs and overall risk of CHD in recent years; rather, most research has concentrated on the influence of n-6 PUFAs on risk factors for CHD. The randomised clinical trials testing long-term effects of high-LA diets on CHD incidence were conducted in the 1960s and early 1970s, and are thus often overlooked when conducting literature searches. A statistically significant 20% decrease in the incidence of CHD was observed in the Los

Angeles Veterans' Study. In this instance, men were randomised to a diet containing roughly 40% of energy from fat, of which over a third (38%) was LA in the intervention group compared with only 10% in the control group (Dayton et al, 1969). However, the findings over this period are inconsistent, with four randomised controlled trials showing an independent, protective effect of n-6 PUFA (MRC, 1968, Dayton et al, 1969, Leren 1970, Turpeinen et al, 1979) and nine epidemiological studies showing no significant relation (Hu et al, 2001). It should be noted that no trial to date has shown an adverse effect of a high n-6 PUFA diet on CHD risk. A more recent analysis of the data has suggested that the ratio of PUFA to SFA in the diet might be an important risk factor for CHD, and that it is diets that are rich in n-6 PUFAs, but low in SFAs that are cardio-protective (Garcia-Palmieri et al, 1980, Gordon et al, 1981, McGee et al, 1984).

A number of case–control studies suggest that lower dietary intakes, and lower plasma or adipose concentrations, of LA are associated with a reduced risk of CHD (Simpson et al, 1982, Wood et al, 1987); others do not find this association (Fehily et al, 1987, Thomas et al, 1987). However, case–control studies cannot establish cause or effect as they investigate differences after the onset of disease. Prospective studies reporting the influence of n-6 PUFAs on CHD risk are relatively few as the majority tend to report on overall intake of PUFAs. Miettinen et al (1982) reported an inverse association between the proportion of LA in serum phospholipids at baseline and CHD incidence at 5-year follow-up. Recently, the Kuopio Ischaemic Heart Study reported that LA was strongly inversely associated with CHD mortality (Laaksonen et al, 2005).

A large number of epidemiological studies report an inverse association between intakes of long chain n-3 PUFAs from fish and risk of CHD; several ecological studies have reported a lower incidence of CHD in fish-eating populations, but such studies do not control for other lifestyle factors that may influence the CHD risk (Hirai et al, 1980, Bjerregaard and Dyerberg, 1988). The Zutphen Study, a cohort of the Seven Countries Study, was the first prospective study to report a lower risk of CHD in men who consumed fish (Kromhout et al, 1995), but this relationship was not confirmed in the East Finland cohort of the Seven Countries Study, where intakes were positively

associated with risk (Oomen et al, 2000). The Nurses' Health Study and the Physicians' Health Study have both reported a beneficial effect of fish consumption on sudden cardiac death. In the Nurses' Health Study, consumption of fish was associated with a decreased risk of CHD events and death from CHD (Hu et al, 2002). This protective effect has been attributed to the long chain n-3 PUFAs in fish, i.e. EPA and DHA and confirmed in a nested case–control study where blood concentrations were measured (Albert et al, 2002). When comparing cases with controls, mean blood EPA and DHA concentrations were significantly lower, as were total long chain n-3 PUFA concentrations. Men in the group consuming the highest levels of n-3 PUFAs were 90% less likely to die from a sudden cardiac event (>17 years of follow-up) than the lowest n-3 PUFA consumers. Fish consumption in the same study was also associated with a reduced risk of sudden cardiac death.

Supplementation interventions with long chain n-3 PUFAs appear to have a beneficial effect on cardiac health. A well-respected, large, randomised controlled trial found that a fish oil concentrate providing 0.56 g of DHA and 0.28 g of EPA per day decreased the incidence of sudden cardiac death by 45% and all-cause mortality by 20% (GISSI, 1999). Additionally, in the Diet and Reinfarction Trial (DART), dietary advice to eat two portions of oil-rich fish per week, which provided 1 g of long chain n-PUFA per day, decreased the incidence of fatal CHD by 29% over a period of 2 years (Burr et al, 1989). A recent meta-analysis to establish whether the individual effects found in a number of different studies were consistent, reported that long chain n-3 PUFAs reduced risk of sudden cardiac death by 30%, but not risk of non-fatal MI (Bucher et al, 2002). In the 11 studies analysed, intakes of DHA ranged 0.6–3.7 g/day and EPA 0.3–6.0 g/day. A further randomised controlled trial investigating the influence of long chain n-3 PUFA supplementation and CHD incidence in patients with suspected mycocardial infarction, reported a protective effect of fish oil supplementation on cardiac death (Singh et al, 1997). However, there are misgivings with regards to the way that this study was conducted; there has been considerable debate in the scientific community about the reliability of the findings (White, 2005).

The follow-up of the DART study (DART 2) reported an unexpected 29% increase in CVD mortality in the group advised to eat oil-rich fish (Burr et al, 2003). This is not consistent with other studies, and the authors suggested that other aspects of individuals' lifestyles may have changed when the dietary advice was given. Additionally, the trial was halted for a short period of time because of funding problems, which may have adversely affected the outcome. However, this illustrates the importance of considering all studies when evaluating the effect of a dietary constituent on disease risk. A recent meta-analysis reviewed 48 randomised controlled trials and 41 cohort studies, investigating the effect of n-3 PUFA (EPA, DHA and ALNA) for at least 6 months in adults (both healthy and those with some form of CVD risk factor) on CVD outcomes (Hooper et al, 2006). Pooled estimate showed no strong evidence of a reduced risk of: total mortality; CVD events; or cancer. However, the findings of this meta-analysis appear to have been influenced by the inclusion of the DART 2 study involving more than 3000 men with angina. As mentioned previously, this study reported an unexpected 26% increase in cardiac deaths in the men taking the long chain n-3 PUFA supplements (Burr et al, 2003).

However, as not all individuals consume oil-rich fish, there is also much interest in the n-3 PUFA ALA that is obtained from predominantly plant-based sources. ALA is also associated with a reduced risk of fatal CHD, possibly as it appears to reduce blood LDL cholesterol concentrations in a similar manner to LA or oleic acid (Layne et al, 1996, Freese and Mutanen, 1997). A meta-analysis of five prospective cohort studies assessing ALA intake and CHD risk reported that intakes of 1.2 g/day are associated with a statistically non-significant reduction in risk of fatal CHD of 21% (Brouwer et al, 2004). There have been several clinical trials that have investigated the influence of increased intakes of ALA on CHD risk (Natvig et al, 1968, de Lorgeril et al, 1994, Singh et al, 1997). These trials comprise the Lyon Diet Heart Study, in which individuals who had experienced a first MI were randomised to receive dietary advice to consume a Mediterranean-type diet, including ALA or no dietary advice, and followed up for a period of 5 years. The authors reported a lower incidence of cardiac death and

non-fatal myocardial infarction after consumption of 1.1 g ALA/day, relative to control treatment (de Lorgeril et al, 1994). However, the findings from individual clinical trials have not been consistent, and thus their findings must be interpreted with caution. Some also consider four classic PUFA trials as supportive of the ALA hypothesis, as they included soybean oil, which is rich in ALA as well as the n-6 PUFA LA (MRC, 1968, Dayton et al, 1969, Leren, 1970, Turpeinen et al, 1979).

Lipid components of the cell membrane

At the most fundamental level, the cell membrane is composed of a lipid bilayer with polar hydrophilic head groups that face the cytoplasmic and extracellular spaces and hydrophobic tails that face each other. Integral membrane proteins are embedded in the lipid bilayer or associated with the membrane by posttranslational attachment of a lipid group to the protein.

Phospholipids comprise the most abundant class of membrane lipids. Phospholipids are composed of two fatty acid tails, glycerol, a phosphate group, and a polar head group. Of the phospholipids, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are found primarily in the inner leaflet of the membrane, whereas phosphatidylcholine (PC) is found primarily in the outer leaflet of the membrane. Phospholipids do not flip flop from one leaflet to the other independently. A class of enzymes called phospholipid scramblases catalyzes the movement of phospholipids from one leaflet to the other (Sim and Wiedmer, 2001).

Sphingomyelin (SM) and related molecules such as ceramide and sphingosine make up another class of compound membrane lipids. SM is composed of sphingosine, a fatty acid, a phosphate group, and choline. Ceramide and sphingosine are released by the sequential cleavage of SM. The properties of SM molecules allow them to form hydrogen bonds with each other within the membrane. Cholesterol (Chol) is also an important component of the cell membrane of eukaryotic cells. In eukaryotic cells, Chol fills the gaps among SM molecules. The result is membrane regions with high concentrations of SM and Chol called lipid rafts (Cremesti et al, 2002, Tsui-Pierchala et

al, 2002, Pike, 2005). Some protein components of signal transduction pathways have an affinity for lipid rafts, whereas others are excluded (Cremesti et al, 2002). Extracellular proteins with glycosylphosphatidylinositol (GPI) anchors and myristoylated or palmitoylated intracellular proteins associate with lipid rafts (Pike, 2005). Transmembrane proteins also enter lipid rafts, but the mechanisms that regulate the association of transmembrane proteins with lipid rafts are poorly understood. There does not appear to be a specific protein domain that targets transmembrane proteins to lipid rafts (Pike, 2005). The presence of SM and, therefore, lipid rafts allows SM to serve an important structural role in the cell membrane as well as to participate in signal transduction pathways.

A fatty acid is composed of a long-chain aliphatic carboxylic acid. Fatty acids may be saturated, monounsaturated, or polyunsaturated. The presence of a double bond in a fatty acid adds a kink. Fatty acids that have more double bonds have more kinks in their structure and take up more space. Thus, as the number of double bonds increases, the membrane becomes more fluid because the fatty acids fit less closely together. Similarly, the fewer the number of double bonds (saturated fatty acids), the more tightly the fatty acid tails fit together and the less fluid the membrane. Many membrane lipids contain fatty acid chains. The fatty acid components of membrane lipids vary widely. Two lipids with the same parent structure may have very different fatty acids attached even though they come from the same source or from the same membrane. Physiologically, this means that hydrolysis of a given species of membrane lipid may yield different fatty acids. Also, the identity of a lipid is determined by its parent structure and not by its fatty acids, since the fatty acids may vary.

Phospholipases

The family of phospholipase enzymes cleaves membrane phospholipids (PL); each of the phospholipases acts at a different site on the PL. Both cell membrane and intracellular membrane phospholipids are substrates for phospholipase. The

products that result from these cleavages are involved in a variety of aspects of signal transduction.

Lipids digestion and lipoproteins

Intestinal uptake of lipids

In order for the body to make use of dietary lipids, they must first be absorbed from the small intestine. Since these molecules are oils, they are essentially insoluble in the aqueous environment of the intestine. The solubilization (or emulsification) of dietary lipids is accomplished by means of bile salts, which are synthesized from cholesterol in the liver and then stored in the gallbladder; they are secreted following the ingestion of fat.

The emulsification of dietary fats renders them accessible to pancreatic lipases (primarily lipase and phospholipase A2). These enzymes, secreted into the intestine from the pancreas, generate free fatty acids and mixtures of mono- and diacylglycerols from dietary triacylglycerols. Pancreatic lipase degrades triacylglycerols at the 1 and 3 positions sequentially to generate 1,2-diacylglycerols and 2-acylglycerols. Phospholipids are degraded at the 2 position by pancreatic phospholipase A2 releasing a free fatty acid and the lysophospholipid. The products of pancreatic lipases then diffuse into the intestinal epithelial cells, where the re-synthesis of triacyglycerols occurs.

Dietary triacylglycerols and cholesterol, as well as triacylglycerols and cholesterol synthesized by the liver, are solubilized in lipid-protein complexes. These complexes contain triacylglycerol lipid droplets and cholesteryl esters surrounded by the polar phospholipids and proteins identified as apolipoproteins. These lipid-protein complexes vary in their content of lipid and protein.

Chylomicrons

Chylomicrons are assembled in the intestinal mucosa as a means to transport dietary cholesterol and triacylglycerols to the rest of the body. Chylomicrons are, therefore, the molecules formed to mobilize dietary (exogenous) lipids. The

predominant lipids of chylomicrons are triacylglycerols. The apolipoproteins that predominate before the chylomicrons enter the circulation include apoB-48 and apoA-I, -A-II and IV. ApoB-48 combines only with chylomicrons.

Chylomicrons leave the intestine via the lymphatic system and enter the circulation at the left subclavian vein. In the bloodstream, chylomicrons acquire apoC-II and apoE from plasma HDLs. In the capillaries of adipose tissue and muscle, the fatty acids of chylomicrons are removed from the triacylglycerols by the action of lipoprotein lipase (LPL), which is found on the surface of the endothelial cells of the capillaries. The apoC-II in the chylomicrons activates LPL in the presence of phospholipid. The free fatty acids are then absorbed by the tissues and the glycerol backbone of the triacylglycerols is returned, via the blood, to the liver and kidneys. Glycerol is converted to the glycolytic intermediate dihydroxyacetone phosphate (DHAP). During the removal of fatty acids, a substantial portion of phospholipid, apoA and apoC is transferred to HDLs. The loss of apoC-II prevents LPL from further degrading the chylomicron remnants.

Chylomicron remnants, containing primarily cholesterol, apoE and apoB-48; are then delivered to, and taken up by, the liver through interaction with the chylomicron remnant receptor. The recognition of chylomicron remnants by the hepatic remnant receptor requires apoE. Chylomicrons function to deliver dietary triacylglycerols to adipose tissue and muscle and dietary cholesterol to the liver.

Very low density lipoprotein (VLDL)

The dietary intake of both fat and carbohydrate, in excess of the needs of the body, leads to their conversion into triacylglycerols in the liver. These triacylglycerols are packaged into VLDLs and released into the circulation for delivery to the various tissues (primarily muscle and adipose tissue) for storage or production of energy through oxidation. VLDLs are, therefore, the molecules formed to transport endogenously derived triacylglycerols to extra-hepatic tissues. In addition to triacylglycerols, VLDLs contain some cholesterol and cholesteryl esters and the

apoproteins, apoB-100, apoC-I, apoC-II, apoC-III and apoE. Like nascent chylomicrons, newly released VLDLs acquire apoCs and apoE from circulating HDLs.

The fatty acid portion of VLDLs is released to adipose tissue and muscle in the same way as for chylomicrons, through the action of lipoprotein lipase. The action of lipoprotein lipase coupled to a loss of certain apoproteins (the apoCs) converts VLDLs to intermediate density lipoproteins (IDLs), also termed VLDL remnants. The apoCs are transferred to HDLs. The predominant remaining proteins are apoB-100 and apoE. Further loss of triacylglycerols converts IDLs to LDLs.

Intermediate density lipoprotein (IDL)

IDLs are formed as triacylglycerols are removed from VLDLs. The fate of IDLs is either conversion to LDLs or direct uptake by the liver. Conversion of IDLs to LDLs occurs as more triacylglycerols are removed. The liver takes up IDLs after they have interacted with the LDL receptor to form a complex, which is endocytosed by the cell. For LDL receptors in the liver to recognize IDLs requires the presence of both apoB-100 and apoE (the LDL receptor is also called the apoB-100/apoE receptor). The importance of apoE in cholesterol uptake by LDL receptors has been demonstrated in transgenic mice lacking functional apoE genes. These mice develop severe atherosclerotic lesions at 10 weeks of age.

Low density lipoprotein (LDL)

The cellular requirement for cholesterol as a membrane component is satisfied in one of two ways: either it is synthesized de novo within the cell, or it is supplied from extra-cellular sources, namely, chylomicrons and VLDLs. As indicated above, the dietary cholesterol that goes into chylomicrons is supplied to the liver by the interaction of chylomicron remnants with the remnant receptor. In addition, cholesterol synthesized by the liver can be transported to extra-hepatic tissues if packaged in VLDLs. In the circulation VLDLs are converted to LDLs through the action of lipoprotein lipase. LDLs are the primary plasma carriers of cholesterol for delivery to all tissues.

The exclusive apolipoprotein of LDLs is apoB-100. LDLs are taken up by cells via LDL receptor-mediated endocytosis, as described above for IDL uptake. The uptake of LDLs occurs predominantly in liver (75%), adrenals and adipose tissue. As with IDLs, the interaction of LDLs with LDL receptors requires the presence of apoB-100. The endocytosed membrane vesicles (endosomes) fuse with lysosomes, in which the apoproteins are degraded and the cholesterol esters are hydrolyzed to yield free cholesterol. The cholesterol is then incorporated into the plasma membranes as necessary. Excess intracellular cholesterol is re-esterified by acyl-CoA-cholesterol acyltransferase (ACAT), for intracellular storage. The activity of ACAT is enhanced by the presence of intracellular cholesterol.

Insulin and tri-iodothyronine (T3) increase the binding of LDLs to liver cells, whereas glucocorticoids (e.g., dexamethasone) have the opposite effect. The precise mechanism for these effects is unclear but may be mediated through the regulation of apoB degradation. The effects of insulin and T3 on hepatic LDL binding may explain the hypercholesterolemia and increased risk of atherosclerosis that have been shown to be associated with uncontrolled diabetes or hypothyroidism.

High density lipoprotein (HDL)

HDLs are synthesized de novo in the liver and small intestine, as primarily protein-rich disc-shaped particles. These newly formed HDLs are nearly devoid of any cholesterol and cholesteryl esters. The primary apoproteins of HDLs are apoA-I, apoC-I, apoC-II and apoE. In fact, a major function of HDLs is to act as circulating stores of apoC-I, apoC-II and apoE.

HDLs are converted into spherical lipoprotein particles through the accumulation of cholesteryl esters. This accumulation converts nascent HDLs to HDL2 and HDL3. Any free cholesterol present in chylomicron remnants and VLDL remnants (IDLs) can be esterified through the action of the HDL-associated enzyme, lecithin:cholesterol acyltransferase (LCAT). LCAT is synthesized in the liver and so named because it transfers a fatty acid from the C-2 position of lecithin to the C-3-OH of

cholesterol, generating a cholesteryl ester and lysolecithin. The activity of LCAT requires interaction with apoA-I, which is found on the surface of HDLs.

Cholesterol-rich HDLs return to the liver, where they are endocytosed. Hepatic uptake of HDLs, or reverse cholesterol transport, may be mediated through an HDL-specific apoA-I receptor or through lipid-lipid interactions. Macrophages also take up HDLs through apoA-I receptor interaction. HDLs can then acquire cholesterol and apoE from the macrophages; cholesterol-enriched HDLs are then secreted from the macrophages. The added apoE in these HDLs leads to an increase in their uptake and catabolism by the liver.

HDLs also acquire cholesterol by extracting it from cell surface membranes. This process has the effect of lowering the level of intracellular cholesterol, since the cholesterol stored within cells as cholesteryl esters will be mobilized to replace the cholesterol removed from the plasma membrane.

The cholesterol esters of HDLs can also be transferred to VLDLs and LDLs through the action of the HDL-associated enzyme, cholesterol ester transfer protein (CETP). This has the added effect of allowing the excess cellular cholesterol to be returned to the liver through the LDL-receptor pathway as well as the HDL-receptor pathway.

LDL receptors

LDLs are the principal plasma carriers of cholesterol delivering cholesterol from the liver (via hepatic synthesis of VLDLs) to peripheral tissues, primarily the adrenals and adipose tissue. LDLs also return cholesterol to the liver. The cellular uptake of cholesterol from LDLs occurs following the interaction of LDLs with the LDL receptor (also called the apoB-100/apoE receptor). The sole apoprotein present in LDLs is apoB-100, which is required for interaction with the LDL receptor.

The LDL receptor is a polypeptide of 839 amino acids that spans the plasma membrane. An extracellular domain is responsible for apoB-100/apoE binding.

The intracellular domain is responsible for the clustering of LDL receptors into regions of the plasma membrane termed coated pits. Once LDL binds the receptor, the complexes are rapidly internalized (endocytosed). ATP-dependent proton pumps lower the pH in the endosomes, which results in dissociation of the LDL from the receptor. The portions of the endosomal membranes harboring the receptor are then recycled to the plasma membrane and the LDL-containing endosomes fuse with lysosomes. Acid hydrolases of the lysosomes degrade the apoproteins and release free fatty acids and cholesterol. As indicated above, the free cholesterol is either incorporated into plasma membranes or esterified (by ACAT) and stored within the cell.

The level of intracellular cholesterol is regulated through cholesterol-induced suppression of LDL receptor synthesis and cholesterol-induced inhibition of cholesterol synthesis. The increased level of intracellular cholesterol that results from LDL uptake has the additional effect of activating ACAT, thereby allowing the storage of excess cholesterol within cells. However, the effect of cholesterol-induced suppression of LDL receptor synthesis is a decrease in the rate at which LDLs and IDLs are removed from the serum. This can lead to excess circulating levels of cholesterol and cholesteryl esters when the dietary intake of fat and cholesterol exceeds the needs of the body. The excess cholesterol tends to be deposited in the skin, tendons and (more gravely) within the arteries, leading to atherosclerosis.

Table 1 The major lipoprotein complexes

Complex	Source	Density (g/ml)
Chylomicron	Intestine	<0.95
VLDL	Liver	0.95-1.006
IDL	VLDL	1.006-1.019
LDL	VLDL	1.019-1.063
HDL2	Intestine, liver (chylomicrons and VLDLs)	1.063-1.125
HDL3	Intestine, liver (chylomicrons and VLDLs)	1.125-1.21
Albumin-FFA	Adipose tissue	>1.281

Regulation of triaclyglycerol synthesis

TGs are synthesized in the hepatocyte in response to fluxes of glucose and nonesterified fatty acids (Kersten et al, 1999, Pegorier et al, 2004). De novo TG synthesis is regulated by sterol regulatory element binding protein (SREBP)–1c, a hepatic gene transcription factor that stimulates synthesis of the lipogenic enzymes involved in this pathway (Pegorier et al, 2004). Glucose stimulates SREBP-1c indirectly either by providing TG substrates such as citrate or by increasing the release of insulin. Citrate is derived from glucose through conversion to pyruvate in a reaction mediated by pyruvate kinase, an enzyme that is not regulated by SREBP-1c (Kersten, 2001). Pyruvate is then converted to citrate via the Krebs cycle, ultimately generating acetyl coenzyme A, the primary substrate for fatty acid synthesis. In addition, glucose-stimulated insulin release induces SREBP-1c gene transcription, which elevates levels of SREBP-1c that promote de novo lipogenesis. Glucose also increases lipogenesis by inhibiting the release of glucagon from the pancreas. Together these effects may explain the mechanisms by which a diet rich in simple carbohydrates, which rapidly increase serum glucose levels, can stimulate lipogenesis in both the liver and adipose tissue (Kersten, 2001). Once synthesized, TGs are packaged with apolipoprotein B in a process mediated by microsomal transfer protein and secreted as very-low-density lipoprotein (VLDL, Shelness and Sellers, 2001).

Nuclear receptors and triacylglycerol metabolism

At least 4 nuclear receptors are affected by fatty acids and may regulate TG metabolism (Jump and Clarke, 1999, Price et al, 2000, Clarke, 2001). These include liver X receptor (LXR), hepatocyte nuclear factor–4α (HNF-4α), farnesol X receptor (FXR), and peroxisome proliferator–activated receptors (PPARs). Most nuclear receptors regulate gene expression in predominantly the same fashion. Prior to activation, nuclear receptors often exist in multiprotein complexes that vary depending on the family of receptor in question. When a ligand binds to its cognate receptor, the resulting conformational change (“activation”) will alter the protein-protein interfaces of the molecule. Once this occurs, the activated receptor interacts with a nuclear receptor

response element within the regulatory region of a target gene. Following the recruitment of various transcriptional coactivators and subsequently RNA polymerase II, transcription of the target gene is initiated.

Although SREBP-1c controls lipogenic enzymes such as acetyl coenzyme A carboxylase and fatty acid synthase, it is under the regulation of LXR (Pawar et al, 2003). LXR is activated by binding of ligands such as oxysterol, which prevents excessive cellular cholesterol levels. Nonesterified fatty acids are also LXR ligands and appear to compete with oxysterols for LXR activation (Xu et al, 1999, Coleman and Lee, 2004). Activation of LXR prevents cellular cholesterol toxicity by enhancing the expression of genes that stimulate bile acid synthesis (7-α-hydroxylase, cytochrome P450 [CYP] 7A) and cholesterol excretion into bile and inhibit intestinal cholesterol absorption. LXR also activates adenosine triphosphate–binding cassette transporter (ABC) A1 to promote efflux of cholesterol into HDL as well as ABCG5 and ABCG8 to increase cholesterol efflux from hepatic cells into bile acids and from intestinal cells into the lumen. The net result of these effects is a markedly reduced cellular cholesterol level. For reasons that are uncertain, LXR activation also regulates TG synthesis through enhancing the expression of SREBP-1c.

HNF-4α binds to long-chain fatty acyl coenzyme A with high affinity. Whereas binding of saturated fatty acids stimulates the transcription of HNF-4α, binding of polyunsaturated fatty acids inhibits HNF-4α gene transcription (Clarke, 2001). Fibrates also appear to bind to HNF-4α and inhibit its transcriptional activity (Hertz et al, 2001). HNF-4α affects genes encoding proteins involved in both fat and carbohydrate metabolism, including apolipoproteins CIII, AI, AIV in lipoprotein metabolism, L-pyruvate kinase in carbohydrate metabolism, and CYP7A in bile acid synthesis (Jump and Clarke, 1999).

FXR is activated by bile acids and therefore appears to protect cells from bile acid toxicity. FXR controls bile acid synthesis by inhibiting expression of CYP7A and other bile synthetic enzymes, and it controls bile acid transportation by regulating

expression of the bile salt export pump (Claudel et al, 2005). Bile acids induce FXR expression and also appear to have hypotriacylglycerolemic effects (Sirvent et al, 2004). FXR activation decreases TG levels by increasing hepatic clearance through modulating lipoprotein lipase activity, inducing PPAR-α, and inhibiting SREBP-1c (Claudel et al, 2003). The effects of FXR on SREBP-1c may not be direct: instead, they are mediated by short heterodimer protein, which has a negative feedback effect on LXR. Polyunsaturated fatty acids have also been recently demonstrated to be FXR agonists (Zhao et al, 2004).

Both saturated and unsaturated nonesterified fatty acids are the natural ligands for PPAR. Three subtypes of PPAR (α, β, and γ), varying in expression and biologic function, have been identified. PPAR-α reduces TG levels through decreasing apolipoprotein CIII expression, which inhibits lipoprotein lipase, thereby enhancing TG catabolism, and has a multitude of other effects that modulate TG levels. At the cellular level, PPAR-α enhances microsomal, peroxisomal, and mitochondrial fatty acid oxidation. PPAR-α also induces lipoprotein lipase and fatty acid transport protein, and enhances adipocyte differentiation, all of which result in reduced TG levels. PPAR-α, β, and γ each appear to influence reverse cholesterol transport and increase HDL levels (Clarke, 2001, Pegorier et al, 2004).

The effects of omega 3 polyunsaturated fatty acids on the regulation of genes involved in triacylglycerol metabolism

Polyunsaturated fatty acids, especially those in the class of omega 3 fatty acids, are now known to affect all 4 of the metabolic nuclear receptors that modulate TG levels, LXR, FXR, and HNF-4α, as well as PPAR-α, β, and γ (Jump and Clarke, 1999, Jump, 2002). Initially, the effects of omega 3 fatty acids were believed to be due mostly to their more potent activation of the PPAR subtypes when compared to other nonesterified fatty acids (Pawar and Jumo, 2003). Because both saturated and unsaturated nonesterified fatty acids are natural ligands for binding to PPAR-α, the omega 3 fatty acids were thought to be specific potent agonists of this nuclear receptor. However, PPAR-α knockout mice continue to show an inhibition of lipogenic gene

transcription from PUFA (Clarke et al, 1998). In addition, although omega 3 fatty acids are more potent than omega 6 fatty acids as in vivo activators of PPAR-α, neither of these PUFA is a particularly strong PPAR-α activator (Krey, 1997). However, PUFA metabolites such as eicosanoids or oxidized fatty acids have 1 to 2 orders of magnitude greater affinity for PPAR-α than do their parent fatty acids and are consequently far more potent transcriptional activators of PPAR-α–dependent genes (Krey, 1997). In contrast to the PPAR-α subtype, PPAR-γ shows a clear preference for binding PUFA. As has been observed with PPAR-α, however, polyunsaturated fatty acids are not particularly efficacious activators of PPAR-γ; however, intracellular conversion of fatty acids to eicosanoids, through enhanced expression of 15-lipoxygenase, greatly increased PPAR-γ–mediated gene activation (li et al, 2005).

Because omega 3 fatty acids affect 4 different nuclear receptors, a working model can be developed that explains their hypotriacylglycerolemic effects at a gene transcriptional level. PUFA elicit their hypotriacylglycerolemic effects by coordinately suppressing hepatic lipogenesis through inhibition of SREBP-1c, upregulating fatty acid oxidation in the liver and skeletal muscle through PPAR activation, and enhancing flux of glucose to glycogen through downregulation of HNF-4α. These coordinated effects have the net result of directing fatty acids away from TG storage and toward oxidation. In this repartitioning of fatty acids, by simultaneously downregulating genes encoding proteins that stimulate lipid synthesis and upregulating genes encoding proteins that stimulate fatty acid oxidation, omega 3 fatty acids are more potent hypotriacylglycerolemic agents than are omega 6 fatty acids, on a carbon-for-carbon basis (Jump and Clarke, 1999, Price et al, 2000, Clarke, 2001, Pawar et al, 2003, Pegorier et al, 2004).

Polyunsaturated fatty acids, but not monounsaturated or saturated fatty acids, reduce the precursor content of mature SREBP-1c by 60% to 90% (Worgall et al, 1998, Mater et al, 1999, Clarke, 2001). Polyunsaturated fatty acids may inhibit SREBP1-c gene expression and/or proteolytic release by inhibiting LXR and stimulating FXR. As mentioned previously, polyunsaturated fatty acids may competitively inhibit the natural

ligand binding of oxysterols to LXR, resulting in a reduction in SREBP-1c gene transcription. This suppressive effect can be eliminated by deletion or mutation of LXR-responsive elements located in the promoter region of SREBP-1c (Coleman and Lee, 2004). However, other evidence suggests that PUFA of SREBP1-c is independent of LXR (Pawar et al, 2003). In rats, fish oil feeding suppressed hepatic SREBP-1c–regulated genes but had no effect on other LXR-regulated genes, such as CYP7A1, ABCG5, or ABCG8 (Pawar et al, 2003). In addition, hepatocytes treated with eicosapentaenoic acid suppressed these lipogenic genes both in the absence and the presence of a synthetic LXR agonist (Pawar et al, 2003). The inhibition of LXR may be an indirect effect of PUFA stimulation of both FXR and PPAR subtypes. Stimulation of FXR has been shown to enhance the expression of short heterodimer protein, which has a negative feedback effect on LXR activity (Watanabe et al, 2004). Overexpression of both PPAR-α and PPAR-γ inhibited SREBP-1c promoter activity induced by LXR in a dose-dependent manner. This effect appears to be due to the reduction of LXR/retinoid X receptor, which forms the LXR response elements. PPARs also utilize the retinoid X receptor, suggesting a competitive inhibition because retinoid X receptor supplementation attenuated these inhibitions of LXR by PPAR (Yoshikawa et al, 2003).

The inhibitory effects of polyunsaturated fatty acids on HNF-4α may also explain some of the hypotriglyceridemic effects of these fatty acids. By inhibiting HNF-4α, polyunsaturated fatty acids affect carbohydrate metabolism though decreased activity of both L-pyruvate kinase and glucose-6 phosphatase (Pegorier et al, 2004). As mentioned previously, pyruvate can enter the TG synthetic pathway as citrate formed in the Krebs cycle. HNF-4α may potentially inhibit SREBP-1c formation through competition with the retinoid X receptor heterodimer. Fibrates can also bind to HNF-4α after conversion to coenzyme A thioesters and inhibit its transcriptional activity (Hertz et al, 2001).

Marine omega 3 fatty acids selectively lower TG levels while having a relatively neutral effect on LDL and HDL through targeted effects on specific transcription factors and nuclear receptors. Inhibiting SREBP-1c would selectively lower

TG levels by decreasing synthesis, and PPAR activation would also decrease TG synthesis and increase HDL by enhancing reverse cholesterol transport. However, activation of FXR and downregulation of HNF-4α would tend to decrease HDL levels. LXR inhibition would also selectively lower levels of TGs and may lower HDL. The net effect of stimulating all 4 metabolic nuclear receptors simultaneously with SREBP-1c is likely to achieve the expected effects of marine omega 3 fatty acids of selective TG reduction with minimal effects on LDL and HDL.

Reduction in very-low-density lipoprotein synthesis by marine omega 3 fatty acids

By decreasing hepatic TG synthesis through inhibition of SREBP-1c, marine omega 3 fatty acids will ultimately reduce the secretion of VLDL. However, omega 3 fatty acids may also reduce VLDL secretion through a second mechanism. Recent evidence suggests that the peroxidation of PUFA and subsequent oxidative stress regulates hepatic apolipoprotein B (apoB) degradation and VLDL production (Pan et al, 2004). In the early stages of VLDL synthesis, the cotranslational binding of lipids to apoB in the endoplasmic reticulum by microsomal transfer protein is affected by the various fatty acids available for utilization. The resulting protection of specific apoB domains from proteolysis, which is called endoplasmic reticulum–associated degradation, will promote either secretion of relatively TG-depleted lipoproteins or further lipidation and secretion of larger, more TG-enriched VLDL. Following the early steps of microsomal transfer protein–dependent lipoprotein assembly, a post–endoplasmic reticulum presecretory proteolysis can occur with decreased secretion from the hepatocyte as the result. Saturated fatty acids appear to protect the nascent smaller lipoprotein from post–endoplasmic reticulum presecretory proteolysis, leading to increased secretion. Peroxidation products of omega 3 fatty acids increase post–endoplasmic reticulum presecretory proteolysis, which decreases secretion of larger VLDL and, hence, lowers levels of the catabolic products such as small LDL particles. Evidence for this effect was provided by a study in rat hepatocytes incubated with omega 3 fatty acids either with or without antioxidants (Krauss, 2004). Omega 3 fatty acids, which are prone to peroxidation, increased apoB-100 degradation; however, the

presence of antioxidants suppressed this effect, restoring VLDL secretion. This observation may explain why saturated fatty acid intake is associated with higher levels of larger, more buoyant LDL particles, and why increased intake of omega 3 fatty acids results in a shift from small LDL to large buoyant LDL (Krauss, 2004). It may also explain the mechanism by which antioxidants promote a slight increase in apoB levels (Heart Protection Study Collaborative Group, 2002). In humans, the effects of omega 3, lipid peroxidation on hepatic apoB degradation and VLDL production appear to be modest, because clinical trials have demonstrated only a slight reduction in apoB levels with high intakes of omega 3 fatty acids (Eritsland et al, 1995, Calabresi et al, 2000). Nevertheless, this intriguing hypothesis adds another potential mechanism to explain the lipid-altering effects of omega 3 fatty acids.

LECITHIN AND ITS HEALTH BENEFIT

LECITHIN

Commercial soya lecithin

Lecithin is mostly a mixture of glycolipids, triglycerides, and phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol). However, in biochemistry, lecithin is usually used as a synonym for pure phosphatidylcholine, a phospholipid which is the major component of a phosphatide fraction which may be isolated from either egg yolk (in Greek lekithos) or soy beans from which it is mechanically or chemically extracted using hexane. Lecithin is commercially available in high purity as a food supplement & for medical uses. Phosphatidylcholine, which is a polar lipid, is present in commercial lecithin in concentrations of 20 to 90%. Most of the commercial lecithin products contain about 20% phosphatidylcholine.

Lecithins containing phosphatidylcholine are produced from vegetable, animal and microbial sources, but mainly from vegetable sources. Soybean, sunflower and rapeseed are the major plant sources of commercial lecithin. Plant lecithins are considered to be GRAS (generally regarded as safe). Egg yolk lecithin is not a major

source of lecithin in nutritional supplements. Eggs themselves naturally contain from 68 to 72% phosphatidylcholine, while soya contains from 20 to 22% phosphatidylcholine.

The fatty acids, which make ups of phosphatidylcholine from plant and animal sources, are differing. Saturated fatty acids, such as palmitic and stearic, make up 19 to 24% of soya lecithin; the monounsaturated oleic acid contributes 9 to 11%; linoleic acid provides 56 to 60%; and alpha-linolenic acid makes up 6 to 9%. In egg yolk lecithin, the saturated fatty acids, palmitic and stearic, make up 41 to 46% of egg lecithin, oleic acid 35 to 38%, linoleic acid 15 to 18% and alpha-linolenic 0 to 1%. Soya lecithin is clearly richer in polyunsaturated fatty acids than egg lecithin. Unsaturated fatty acids are mainly bound to the second or middle carbon of glycerol. Choline comprises about 15% of the weight of phosphatidylcholine.

Novel fish lecithins

Of the relatively few investigations that have been made of the phospholipids of fish tissues. These have yielded inexact results, for a variety of reasons. Dahlan et al (1996) have investigated lecithin content and characteristic in fishmeal. It was found that fats and lecithin derived from fishmeal were 9-15 and 2-3 g/100, respectively. Total fatty acids constitute 23-27% monoenes without erucic acid and 24-28% polyenes including 15-19% DHA. Lecithin with 20-50% purity was prepared from grade-1 fish meal by means of consecutive methanol/n-hexane/acetone extraction. The obtained lecithin contains choline up to 66-70 mole% with DHA reaches to 20-23%. Its peroxide value of 57 and acid value of 9 are accepted for food grade lecithin preparation with further refinery process was suggested. The results implied that fish lecithin was probably a promising source of omega-3 fatty acids especially DHA and choline. In such reason, fishmeal lecithin was utilized in the present investigation as source of omega-3 polyunsaturated fatty acids. The detail of fish lecithin characteristics will be described in the following text of this dissertation.

Pharmacological action

Phosphatidylcholine has shown the hepatoprotective activity (Lieber, 1990). Phosphatidylcholine is important for normal cellular membrane composition and membrane repair. Phosphatidylcholine is also the major delivery form of the essential nutrient, choline. Choline itself is a precursor in the synthesis of the neurotransmitter acetylcholine, the methyl donor betaine and phospholipids, including phosphatidylcholine and sphingomyelin among others. Phosphatidylcholine is involved in the hepatic export of very-low-density lipoproteins.

Mechanism of action

The role of phosphatidylcholine is the maintenance of cell-membrane integrity, and is vital to all of the basically biological processes. These are: information flow that occurs within cells from DNA to RNA to proteins; the formation of cellular energy and intracellular communication or signal transduction. Phosphatidylcholine, particularly phosphatidylcholine rich in polyunsaturated fatty acids, has a marked fluidizing effect on cellular membranes. Decreased cell-membrane fluidization and breakdown of cell-membrane integrity, as well as impairment of cell-membrane repair mechanisms, are associated with a number of disorders, including liver disease, neurological diseases, various cancers and cell death.

Pharmacokinetics

Phosphatidylcholine is absorbed into the mucosal cells of the small intestine, mainly in the duodenum and upper jejunum, following some digestion by the pancreatic enzyme phospholipase, producing lysophosphatidylcholine (lysolecithin). Reacylation of lysolecithin takes place in the intestinal mucosal cells, reforming phosphatidylcholine, which is then transported by the lymphatics in the form of chylomicrons to the blood. Phosphatidylcholine is transported in the blood in various lipoprotein particles, including very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL); it is then distributed to the

various tissues of the body. Some phosphatidylcholine is incorporated into cell membranes.

Phosphatidylcholine is also metabolized to choline, fatty acids and glycerol. The fatty acids and glycerol either get oxidized to produce energy or become involved in lipogenesis. Choline is a precursor of acetylcholine. Serum choline levels peak between 2 to 6 hours after oral intake.

Indications and usage

Phosphatidylcholine may be indicated to help restore liver function in a number of disorders, including alcoholic fibrosis, and possibly viral hepatitis. It may also be indicated for the treatment of some manic conditions. There is some evidence that phosphatidylcholine may be useful in the management of Alzheimer's disease and some other cognitive disorders. A possible future role in cancer therapy is also suggested by recent research. It may also be indicated in some with tardive dyskinesia.

Research summary

Clinical studies have demonstrated that choline is essential for normal liver function. Phosphatidylcholine is a better delivery form and is also more tolerable than choline. In addition, phosphatidylcholine, independent of its choline content has shown the hepatoprotective activity. In two studies using baboons fed high alcohol diets, one is supplemented with a soy-derived polyunsaturated lecithin (60% phosphatidylcholine) and the other is unsupplemented, both fibrosis and cirrhosis were largely prevented in the phosphatidylcholine group. Most of the unsupplemented animals in these studies, which continued for up to eight years, developed fibrosis or cirrhosis.

Because these researchers had previously found that choline, equal in amounts contained in the phosphatidylcholine-rich lecithin they subsequently used, had no comparable protective effects on the liver, they concluded that the polyunsaturated phospholipids themselves may have been responsible for the benefits observed.

In vitro studies have shown that these phospholipids increase hepatic collagenase activity and may thus help prevent fibrosis and cirrhosis by encouraging collagen breakdown. Several other mechanisms under investigation may also contribute.

Others have reported similarly encouraging results in animal models. Clearly, human trials are warranted.

In addition, phosphatidylcholine has demonstrated other protective effects in non-alcoholic liver disorders, including protection against various other toxic substances. Its benefits in viral hepatitis were reported some years ago by several different research groups in Europe and elsewhere. In one of these studies, individuals suffering from hepatitis type A and B were given 1.8 grams of phosphatidylcholine daily. Compared with unsupplemented controls, the phosphatidylcholine group enjoyed quicker recoveries, fewer relapses and quicker normalization of liver function tests.

Researchers in Great Britain treated chronic active hepatitis C patients with 3 grams daily of phosphatidylcholine in double-blind fashion. The phosphatidylcholine patients had significantly reduced symptoms, compared with controls. All histologic evidence of the disease disappeared in some cases. These researchers, like others, have hypothesized that phosphatidylcholine's possible antiviral effects are related to the supplement's apparent ability to increase cellular membrane fluidity and repair the membranes of liver cells.

Phosphatidylcholine may help some with tardive dyskinesia, a neurological disorder characterized by defective cholinergic nerve activity. Both supplemental choline and phosphatidylcholine were found to reduce the muscular hyperactivity of this disorder by about 50% in some studies. However, one significant trial did not see a beneficial effect.

There is some very preliminary evidence that phosphatidylcholine may help control manic symptoms in some.

There has been hope, for some time, that phosphatidylcholine would demonstrate clear-cut benefits in cognitive disorders, such as age-related memory loss and Alzheimer's disease. There are a few reports that supplemental choline can improve short-term memory skills and enhance the memories of those who are initial poor learners.

Those with Alzheimer's disease have a diminished ability to synthesize and/or utilize the neurotransmitter acetylcholine, particularly in those areas of the brain related to memory, thus the hope that supplemental choline/phosphatidylcholine might be of benefit. A few studies have suggested some small benefit in memory restoration, but most have not. Research continues.

Recently it has been suggested that phosphatidylcholine might eventually have some therapeutic role in some cancers. There is no evidence of this to date, but animal studies indicate that deficiencies in choline and phosphatidylcholine may disrupt cell membrane signal transduction in ways that could lead to various cancers. There is ample evidence that liver cancer is promoted in various animals by choline-deficient diets, and it has been shown that excess choline can protect against liver cancer in a mouse model.

Phosphatidylcholine has been used to lower serum cholesterol levels, based on the premise that lecithin cholesterol acyltransferase (LCAT) activity has an important role in the removal of cholesterol from tissues. A few studies have shown reduction in serum cholesterol with phosphatidylcholine intake. The results were quite modest, and most studies have not shown any significant cholesterol-lowering activity.

CHAPTER III

MATERIALS AND METHODS

Materials

A. Raw materials

1. Fish meal

The grade A Thai fish meal was manufactured by SKS Group Product of Thailand, Samutsakorn and was donated from Dr. Somkiat Piyathiratitivorakul of the Department of Marine Science, Faculty of Science, Chulalongkorn University. The fish meal sample composed of 65% protein, 12% fat, moisture, ash, sand and salt. The picture of fish meal sample is shown in **figure 2**.

2. Soybean lecithin

Soybean lecithin was purchased from Bangkok Vetlab Company Limited, Samutsakorn, Thailand.

Figure 2 Fish meal sample used in this study

B. Glass wares

TLC chambers (Wheaton model 276860) were purchased from Wheaton, Millville, NJ, USA. Micro-reaction vessels (2 ml) with screw cap seals model C4013-1 and target polyspringe inserts (0.3 ml) model C4010-630 were purchased from National (National Scientific, USA). Borosilicate glass tubes (16×125 mm) with Teflon-lined screw caps were bought from Pyrex and regularly leak proved by re-weighing the tubes containing 2 ml methanol after boiling at 100°C for 1 h in water bath. The reduced weight tubes were avoided and/or discarded. All glasswares were washed by acid solution and rinsed twice with dichloromethane-methanol (2:1, v/v) and air dried before use in the experiment.

C. Chemicals

Nitrogen gas was oxygen-free. Compressed gases were supplied by Chatkorn Lab Center Co., Ltd, Bangkok. All chemicals were analytical grade and purchased from domestic distributors. Chemicals for liquid chromatography-mass spectrometer- mass spectrometer (LC-MS-MS) system were HLPC grade and purchased from Merck KGaA (Darmstadt, Germany). Acetylchloride used for transesterifying lipids, was purchased from Sigma (St Louis, MO, USA). Fatty acid methyl ester (FAME) mixtures code no. 189-19 was obtained from Supelco (Bellefonte, PA, USA). Internal standard (IS) of fatty acid; pentadecanoic acid (FA-C15:0) code no. P6125 and IS of Triacylglycerol; tripentadecanoin (TG-C15:0) code no. T4257 were purchased from Sigma (St Louis, MO, USA). IS of phospholipids; 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC-C15:0) code no. 855676P, 1,2-dimyristoyl-*sn*-glycero-3-[phosphoethanolamine] (DMPE) code no. 850745, 1,2-dimyristelaidoyl-*sn*-glycero-3-[phosphocholine] (PC-C14:1) code no. 850346 and 1,2-dimyristoyl-*sn*-glycero-3-[phosphor-l-serine] (DMPS) code no. 840033 were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The working solutions of above-mentioned ISs were prepared to have the concentration of 1 mg/ml in dichloromethane-methanol (2:1, v/v). Distilled and deionized water were obtained from a Milli-Q synthesis system

(Millipore, Milford, MA, USA) supplied in the Halal Science Center, Chulalongkorn University.

Thin-layer chromatographic plates (20×20 cm TLC) precoated with silica gel 60 without fluorescent indicator, with a layer thickness of 0.25 mm were obtained from Merck (Merck 5721, E.Merck, Darmstadt, Germany). Each plate was prerun twice with dichloromethane-methanol (2:1, v/v) and activated at 120°C in hot air oven for 30 min before being used. Reagent kits for Cholesterol (Chol) and Triacyglycerol (TG) determination purchased from Human, Germany.

D. Instruments

All experiments were carried out at the Lipid and Fat Science Research Center (LiFARC), Faculty of Allied Health Science and the Halal Science Center (HASCI), Chulalongkorn University. The main instruments employed in the experiment are listed below:

1. Gas Chromatograph GC-2010 with Auto injector AOC-20i, Shimadzu, Tokyo, Japan
2. DB-23 Silica column, J&W Scientific, USA
3. Microultracentrifuge CS 150 GXL, Hitachi, Tokyo, Japan
4. Tube sealer STF 2, Hitachi, Tokyo, Japan
5. Tube slicer TSU 2, Hitachi, Tokyo, Japan
6. Refrigerated centrifuge CF 7D2, Hitachi, Tokyo, Japan
7. Photometer Humalyzer 3000, Human, Germany
8. Electronic balance with 4 digits CP2245, Sartorius, germany
9. Rotary evaporator R–114, Buchi, Switzerland

10. Vacuum system, model B–169 Buchi, Switzerland

11. Nitrogen evaporator / heater / stirring module, Pierce, IL, USA

12. Suction pump, model 523-V4-G21DX, MI, USA

13. Deep freezer -80°C, Shin Lab, Japan

14. Hot air oven Thermotec 2300, Contherm, Hutt City, New Zealand

15. Vacuum oven VD 115, Binder, Incheon, Korea

16. Sequential Plasma Spectrometer ICPS-7510 with Autosample Changer, Shimadzu, Tokyo, Japan

17. Microwave Laboratory System Ethos, Milestone, Sorisole, Italy

18. HPLC system HP 1100 series, Agilent Technologies, Palo Alto, CA, USA coupled on-line to Esquire HCT Ion trap mass spectrometer, Bruker Daltonics, GmbH, Germany with an Electrospray ionization source

19. TLC plate scraping system (home-made at LiFARC, Chulalongkorn University)

TLC plate scraping system is shown in **figure 3** which made in house from hard plastic by LiFARC according to the design of Hegstrand (1985). This system was proven to speed up to 2–3 fold of consuming time in the process of scraping TLC plate in comparison to the conventional procedure. The system facilitated the work speed and consequently substantial reduced the oxidation of PUFA possibly occurred during the time delay on dry TLC plate (Dahlan, 1989).

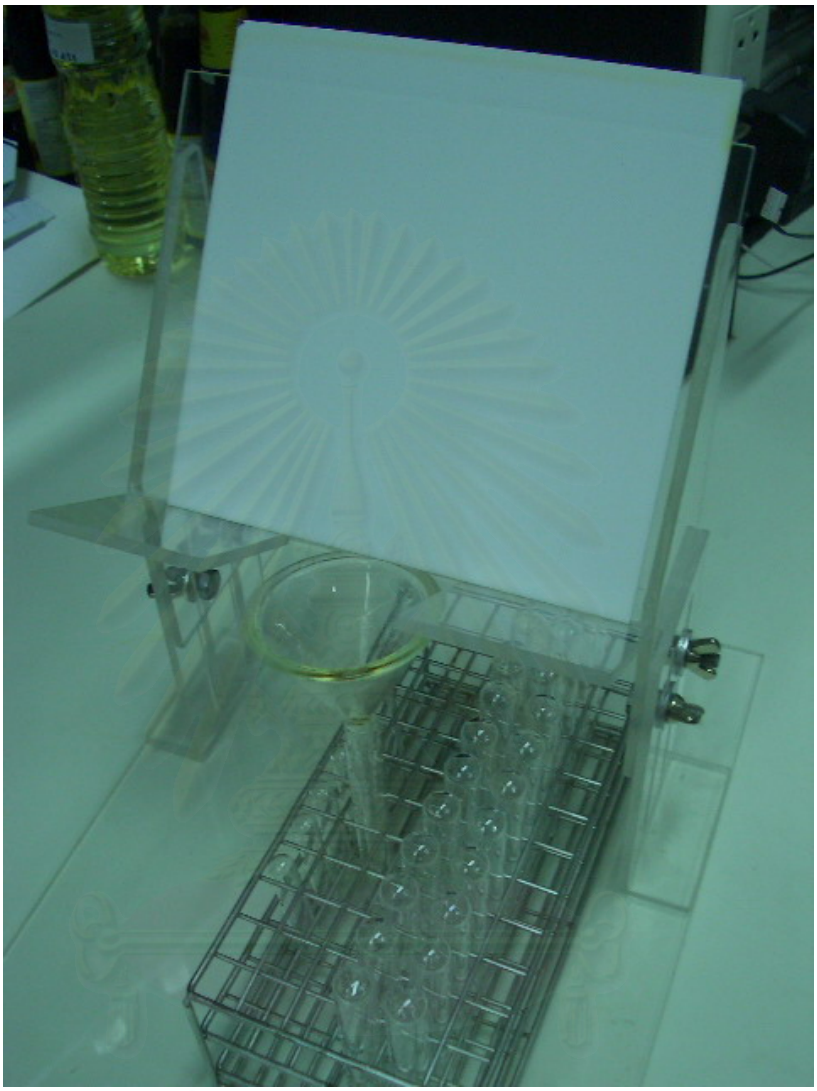


Figure 3 Thin-layer chromatography plate-scraping system made in house by LiFARC according to the design of Hegstrand (1985)

Methodology

A. The extraction of lecithins from raw material

1. Elimination of neutral lipids

Fish meal sample was heated at 80°C for 30 min to evaporate moisture as well as to inactivate any active enzymes before the extraction. Four hundred grams of fish meal were weighed and transferred into a glass beaker (2 L). Fish meal lipids were separated by extracting fish meal twice with 1,200 ml acetone to obtain fish meal-acetone ratio of 1:3 (w/v). The mixture was blended by using magnetic stirrer for 1 h. The miscellae (oil in acetone) were collected by filtering the mixture through a Whatman No. 1 filter paper on Buchner funnel with a slight suction. The residue was compressed with the tip of spatula or glass rod to ensure maximum recovery of filtrate. The remaining residue was carried out for the second extraction following the same procedure. The second filtrate was pooled into the fist fraction and kept in the round bottom flask. The solvent was evaporated from lipids at 40°c under vacuum by rotary evaporator or vacuum oven until thick crude lipids known as crude fish oil was obtained. Any remaining solvent was removed by flushing crude oils with oxygen-free nitrogen gas. The crude oil obtained from acetone extraction constituted neutral lipids exclusively of triacylglycerol (TG). Precipitant of fish meal eliminated partially of neutral lipids was kept for further process.

2. Extraction of polar lipids

Precipitant of fish meal from the previous section was brought for extraction with 1,200 ml of methanol with the ratio of fish meal to methanol 1:3 (w/v). The mixture was blended by using magnetic stirrer for 1 h. The extraction was performed twice. Filtrate was collected in a round bottom flask. Methanol was removed form filtrate under vacuum at 40°C by rotary evaporator or vacuum oven. The remaining residue was kept under nitrogen atmosphere. Later, precipitant of fish meal previously pretreated with methanol was further extracted twice with 3 volumes of n-hexane (w/v)

following the same procedure. The residues of polar lipids majority of lecithins obtained from methanol and n-hexane extractions were combined and weighed.

B. Preparation of lecithin formulas

The extracted lecithin from fish meal as previously described and commercial soybean lecithin were prepared into 4 formulas. Each formula provides lecithin concentration of 0.5 g/ml by homogenized lecithins in 1% Tween-20.

1. Formula 1 used only fish meal lecithin is called LE-FM.
2. Formula 2 used only soybean lecithin is called LE-SB.
3. Formula 3 used fish meal lecithin and soybean lecithin mixed in the ratio of FM : SB 1:1 (w/w) is called LE-FS 1:1
4. Formula 4 used fish meal lecithin and soybean lecithin mixed in the ratio of FM : SB 1:2 (w/w) is called LE-FS 1:2

C. Animal experiment

Animal facilities and protocol were approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Adult male Wistar rats were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. Rats weighing 180-190 g were used in this experiment. They were housed in individual stainless cages at Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand and fed with *ad libitum* commercial pellet diet and free access to water in a room maintained at 24 ± 1°C on approximately 12:12-h light-dark cycle. The commercial pellet diet composed of corn, soybean meal, fullfat soybean, rice, rice by-product, fishmeal, corn gluten meal, vegetable oil, salt, vitamins and minerals.

After 7 days of acclimatization, 30 rats were randomly divided into 5 groups, containing 6 rats (n=6). Each group orally received 600 mg lecithin/kg body weight for 8 weeks as follow

1. Control group received 1% Tween-20 in equal volume of lecithin groups.

2. LE-FM group received fishmeal lecithin

3. LE-SB group received soybean lecithin

4. LE-FS1:1 group received fish meal lecithin and soybean lecithin mixed in the ratio of FM : SB 1:1 (w/w)

5. LE-FS1:2 group received fish meal lecithin and soybean lecithin mixed in the ratio of FM : SB 1:2 (w/w)

The rat body weight, food and water intake were measured everyday. At the end of the experiment, rats were fasted overnight and blood samples were taken by cardiac puncture used heparin as anticoagulant. Plasma was separated by low speed centrifugation for analysis.

D. Chemical analysis

1. Phospholipids analysis

Phosphorus (P) present in all forms of phospholipids (PL) were turned into inorganic phosphorus by acid digestion of the lecithin and assessed quantitatively by Inductively Coupled Plasma Spectrometer (ICPS), precisely, Sequential Plasma Spectrometer ICPS-7510.

Five hundred mg of each formula of lecithin was digested with 7 ml of 65% HNO_3 and 1 ml of 30% H_2O_2 using microwave laboratory system under digestion program setting accordingly to manual from manufacture (Appendix B) and injected into ICPS read P intensity at 178.287 nm. A calibration curve was obtained by

using P standard concentration 6.25, 12.5, 25, 50 and 100 ppm. In calculation, 1 mg of P is equivalent to 25 mg of PL and 1 mmole of P is equivalent to 1 mmole of PL.

2. Phospholipids profiling by using liquid chromatography mass spectrometer (LC-MS)

Phospholipids class of lecithin were separated by LC and identified species by MS according to the method described by Wang et al (2004) and slightly modified by Mr. Sukit Sirikwanpong, Ph.D. student (Biomedical Science, Chulalongkorn University).

i. Sample preparation

Fifty µl of lecithin was taken for lipids extraction according to the method described by Bright and Dyer (1959). Briefly, 950 µl of deionized water (DIW) was added to 50 µl of lecithin then 3.75 ml of dichloromethane-methanol (2:1, v/v) and appropriate amounts of internal standards (IS) were added. The solution was mixed by vortex for 60 seconds(s). Then, added 1.25 ml of dichloromethane and vortex well. Finally, 1.25 ml of DIW was added before the solution was mixed for 60 s and centrifuged at 3000 rpm for 10 min. The lower phase was sampled and dried by evaporation under oxygen-free nitrogen gas using a dry block heater. Prior to analysis, the extracted samples were redissolved in 200 µl of dichloromethane-methanol (2:1, v/v) and further dilued with hexane-isopropanol (3:2, v/v).

ii. Chromatographic conditions

An Agilent HP 1100 series HPLC system was used. The lipids were separated on a diol column, Nucleosil 100-OH (Macherey-Nagel, Germany), (250 mm x 3.0 mm id. x 5 µm particles size). Mobile phase A was hexane-isopropanol (80:20, v/v) and mobile phase B was isopropanol-water-Formic acid-Ammonia (89.4:10:0.2:0.4, v/v). The linear solvent gradient started at 30% B, increased to 60% in 20 min, then increased to 80% in 3 min. After that mobile phase B was maintained at this level for 27 min. Finally, the mobile phase B was decreased to 30% in 3 min and the

column was reequilibrated for about 5 min before the next run. The total flow rate was 0.4 ml/min and the column temperature was kept at 35 °C. The injected volume was 5 µl.

iii. ESI-MS of phospholipids

The HPLC system was coupled on-line to Esquire HCT Ion trap mass spectrometer (Bruker Daltonics) with an ESI source. The analytes eluted from HPLC column were directly entered the MS through a steel ES ionization needle set at 4.5 kV in the negative ion mode. The dry nitrogen gas flow rate was approximately 8.0 l/min at 290°C. All ion source and ion optic parameters were optimized with respect to the negative ion of the phospholipids standards. The MS data were collected under full scan mode (500-1,000 m/z at a rate of four spectra for each time point).

iv. Quantification of phospholipid molecular species

For the single phospholipid class, all acquired MS spectra across each chromatographic peak were averaged to obtain a profile spectrum for a specific class after subtraction from the background. Molecular species of each phospholipids class were quantified by comparisons of individual ion peak intensity with internal standard after correction for ^{13}C isotope effects (Han & Gross, 2001). Identification of ion peaks was achieved by MS^n experiments, which were carried out with AutoMS^n operation function.

3. TLC for separation of TG, free FA and PL

TG, free fatty acids (FFA) and PL were separated from each others by technique of one-dimension TLC. Forty µl of lecithin was diluted with 100 µl TG-C15:0, FA-C15:0 and LPC-C15:0 which is an internal standard (IS). The mixture was applied on the TLC plates by means of a micro syringe with an oblique needle point or a capillary pipette tip with their openings, and then was placed against the adsorbent surface. A one dimension TLC was utilized by n-hexane-diethyl ether-glacial acetic acid (80:20:1, v/v/v) as a developing solvent. Spots of separated PL, FFA and TG were visualized in day light after spraying TLC plate with water. The silica powders contained

PL, FFA and TG were scraped on plate-scraping system and collected in a screw capped tube for fatty acid analysis in the following section.

4. Fatty acid analysis

i. Fatty acid analysis of lecithin

Fatty acid compositions of lecithin were determined after being transesterified according to the technique described by Lepage and Roy (1984, 1986). Forty µl of sample and 100 µl of FA-C15:0 as IS were transferred into a leak-proved Teflon lined screw-capped borosilicate test tube in which 2 ml of methanol-hexane (4:1, v/v) and a small magnetic bar was immediately added. The saponification of lipids and methylation of liberated fatty acids were performed by using acetylchoride. In brief, a 200 µl of acetylchoride was added slowly in the vortexing sample tube. The tube was tightly closed and the fatty acids subjects to methanolysis with slow stirring at 100°C for 1 h in the heating/ stirring dry block. A perfect seal between the cap and the rim of the tube was secured during the heating period. Failure to achieve this would have resulted in a disproportional loss of the more volatile FA esters, making the procedure no longer quantitative.

After cooling the tube in water, 5 ml of 6% of K_2CO_3 solution were slowly added to stop the reaction and to neutralize the mixture. The tube was then shaken and centrifuged (2000 rpm, 10 min) to float hexane which dissolved occurring FAME's inside. At the end of the process, one µl of the hexane upper phase was injected into the gas chromatograph which was conditioned and programmed as described later herewith. A GC-2010 gas chromatograph (GC) with a flame ionization detector was used. The separation of FAME's was performed in a 30 m fused silica capillary column with an internal diameter of 0.25 mm and wall-coated with 0.25 µm, DB-23 P/N 122-2332 (J&W Scientific, USA). Helium was used as carrier gas (1.25 ml/min at 50°C). The split ratio was 50:1. The injection port temperature was 250°C and the detector temperature was set at 300°C. The column temperature was initiated at 50°C. After sample injection the temperature was held for 2 min then the temperature was

programmed to 180°C with an increase rate of 10°C/min and held isothermally for 15 min. The second increment was 4°C/min to 230°C.The latter temperature was maintained constant for 10 min.

ii. Analysis of fatty acid composition in TG, FFA and PL fractions

TG, FFA and PL were separated from each others by a one-dimension TLC using n-hexane-diethyl ether-glacial acetic acid (80:20:1, v/v/v) as a developing solvent as previously described. Spots of TG, FFA and PL were visualized in day light after thin spraying with distilled water; TG fraction migrated nearly to the solvent front whereas PL fraction stayed at the origin and FFA fraction stayed between PL and TG. Equal areas of Silica containing TG, FFA and PL were scraped without delay on a plastic scraping system into a Teflon lined screw-capped borosilicate test tube containing 2 ml of methanol-hexane (4:1, v/v) and the mixture was mixed vigorously. The methylation of fatty acids was performed by using acetylchloride according to the method as previously described.

iii. Analysis of fatty acid composition in plasma

One hundred µl of plasma and 100 µl of FA-C15:0 were transferred into a leak-proved Teflon lined screw-capped borosilicate test tube in which contained 2 ml of methanol-hexane (4:1, v/v) and the tube were vigorously mixed. The direct methylation of plasma fatty acids was performed by using acetylchloride according to the method as previously described.

5. Lipoprotein separations

Lipoproteins were separated from plasma by sequential ultracentrifugation following a series of sample density adjustments, centrifugation, and infranatant / supernatant separation.

i. Preparation of very low-density lipoprotein (Ordovas, 1998 modified by Sittiwichaenwong, 2006)

Sequential ultracentrifugation was used for purifying Very Low-Density Lipoprotein (VLDL) at density < 1.006 g/ml from other lipoproteins. One ml of plasma was transferred into 1.3 ml ultracentrifuge tube (Nalgene, USA) and added 1.006 g/ml KBr solution until it full. The density solution preparations were described in Appendix C. The tube was sealed by the tube sealer and placed into the S 140 AT rotor. Then, centrifuged at 100,000 rpm at 10°C for 3 h using the Hitachi microultracentrifuge CS 150 GXL. The rotor was carefully removed from the ultracentrifuge after it reaches full stop, and getting tubes out trying not to disturb the layers. The centrifuge tubes were cut at the middle of the tube using tube slicing technique. The upper fraction containing concentrated VLDL was removed to the collecting tube by using a transfer pipette trying to recover as much as possible. If some materials appeared to get stuck to the walls of the tube, rinsed carefully using 1.006 g/ml KBr solution. Minimized the foaming during rinse.

ii. Preparation of low-density lipoprotein (LDL)

The infranatant which was collected from the previous isolation was adjusted density to 1.063 g/ml with solid KBr. The weight of KBr was calculated by the equation 1. Centrifuge at 100,000 rpm at 10°C for 4 h. LDL was finally floated and separated. The upper solution which contains LDL was recovered by tube slicing technique, as previously described.

$$KBr\ (g) = \frac{V\ (1.063-1.006)}{0.7057} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where: V = initial volume of the infranatant (ml)

1.063 = required density (g/ml)

1.006 = original density (g/ml)

iii. Preparation of high-density lipoprotein (HDL)

The infranatant which was collected from the previous isolation was adjusted density to 1.21 g/ml with solid KBr. The weight of KBr was calculated by the equation 2. Centrifuge at 100,000 rpm at 10°C for 5 h. HDL was finally floated and separated. The upper solution which contains HDL was recovered by tube slicing technique, as previously described.

$$KBr\ (g) = \frac{V\ (1.21-1.063)}{0.6438} \quad \text{..... (2)}$$

Where: V = initial volume of the infranatant (ml)

1.21 = required density (g/ml)

1.063 = original density (g/ml)

6. Determination of lipoprotein particle diameter

Lipoprotein particle size was determined by electron micrograph of negative stain. Sample from lipoprotein fractions were dialysis with normal saline solution before grid preparation. Dialyzed samples were dropped on the colloidion carbon-coated grids for 3 min. The excess solution was absorbed by Whatman paper. The samples were stained in negative with 1% Phosphotungstic acid for 1 min. The grids were kept in the desiccator at room temperature and analysis using Transmission Electron Microscope (TEM) model JEM-2100 (JEOL, USA) at the Scientific and Technology Research Equipment Center, Chulalongkorn University, Bangkok. The diameters of particles were measured for size determination and calculated as mean ± S.E.M.

7. Determination of triacylglycerol and cholesterol

Concentrations of plasma cholesterol and triacylglycerol and all lipoprotein fractions were measured using commercial kits (Appendix D & E).

E. Statistical analysis

Data are expressed as Mean ± S.E.M. Statistical significance was performed by one-way analysis of variance (ANOVA) by SPSS version 11.5 for windows (SPSS Inc., IL, USA). The Least Significant Difference test (LSD) was used for mean comparisions. *P* value less than 0.05 was considered to be statistically significant.

CHAPTER IV

RESULTS

Characterization of lipid content in lecithin

In the present study, fish meal and soybean were used as sources of lecithin. The grade A Thai fish meal was donated as a gift from Dr. Somkiat Piyathiratitivorakul the Department of Marine Science, Faculty of Science, Chulalongkorn University. Lecithin was obtained from fish meal (LE-FM) which contained high omega 3 polyunsaturated fatty acid (n-3 PUFA) in both eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), whereas soybean lecithin (LE-SB) had high content of linoleic acid (LA; C18:2n-6) and alpha-linolenic acid (ALA; C18:3n-3). Mixed lecithins from fish meal and soybean in the ratio 1:1 (LE-FS 1:1) and 1:2 (LE-FS 1:2) contained these fatty acids between LE-FM and LE-SB.

The picture of lecithin extracted from fish meal and soybean are shown in **figure 4**. Lecithin derived from soybean (LE-SB) was brownish yellow and composed of 50% phospholipids, whereas fish meal lecithin (LE-FM) was dark brown and contained 18% phospholipids. The phospholipids content of LE-FS 1:1 and LE-FS 1:2 were 36% and 39%, respectively. **Figure 5** shows the comparison of phospholipids content in each lecithin.

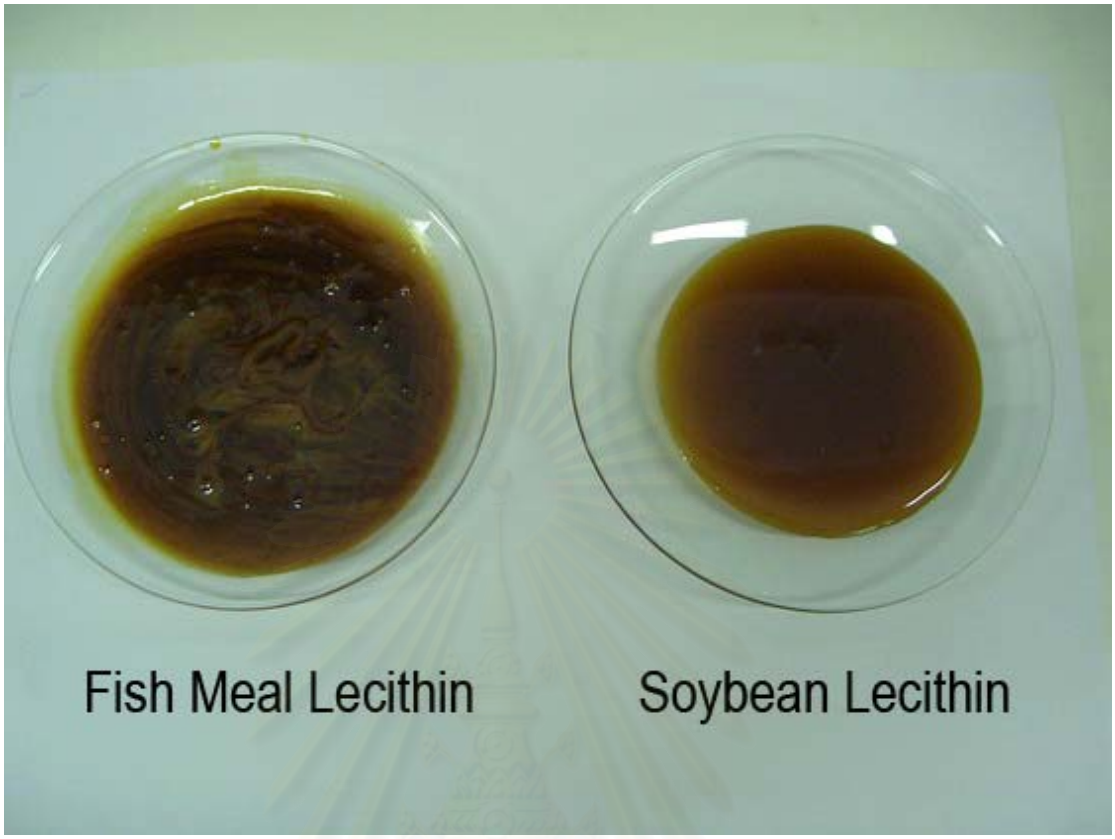


Figure 4 The lecithin extracted from fish meal (left) and soybean (right). Fish meal lecithin was prepared as described in the context whereas soybean lecithin was supplied from the commercial firm.

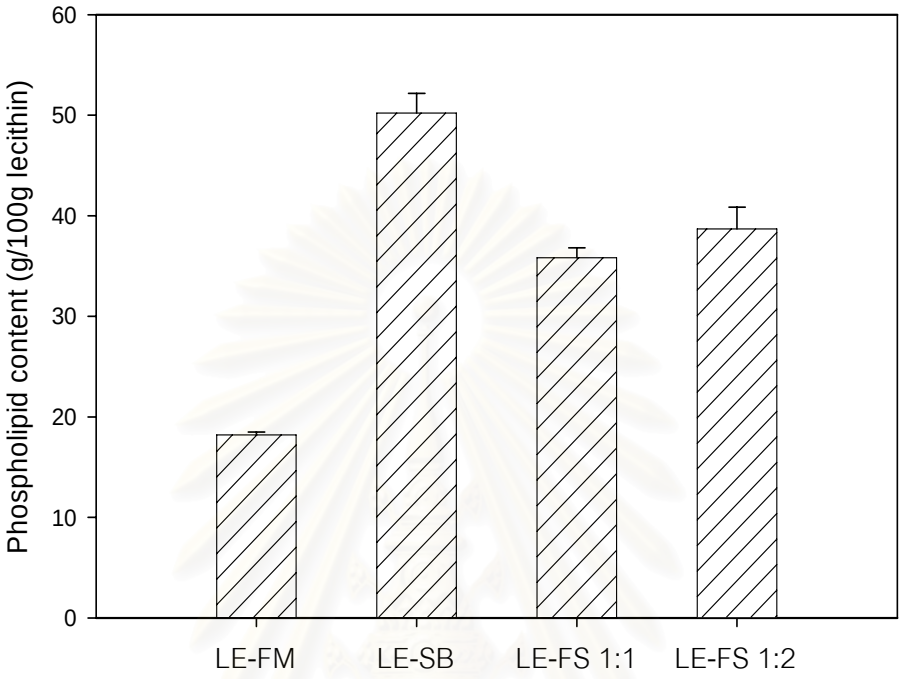


Figure 5 Phospholipids content in each lecithin (g/100g lecithin)

Total fatty acids in lecithin

Figure 6 show fatty acid chromatogram of lecithin. The contents of each fatty acid in lecithin are shown in **table 2**. LE-FM contained the highest content of C20:5n-3 and C22:6n-3 when compared with other lecithin, whereas LE-SB contained the highest content of C18:2n-6. Fatty acids in form of fatty acid methyl ester (FAME) were expressed as means of which per 100 g of fatty acid in respective LE-FM, LE- SB, LE-FS 1:1 and LE-FS 1:2 were as follows: EPA, 5.2 vs 0 vs 0.63 vs 0.39; DHA: 21.11 vs 0 vs 2.42 vs 1.52; LA: 1.06 vs 54.83 vs 47.48 vs 50.02.

Focusing on the n-3 and n-6 PUFAs, LE-FM had the highest content of n-3 PUFAs and the lowest content of n-6 PUFAs. In contrast, LE-SB had the lowest content of n-3 PUFAs and the highest content of n-6 PUFAs. These results showed that the ratio of n-6/n-3 PUFA in LE-SB had higher than LE-FM, whereas the ratio of n-3/n-6 PUFA of LE-SB was lower than LE-FM. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA contents in lecithin are shown in **table 3**.

LE-FS 1:1 was prepared by mixing LE-FM with equal amount of LE-SB (w/w). Fatty acid profile of the obtained lecithin was thus in the range between those of LE-FM and LE-SB. Consequently, LE-FS 1:2 was prepared as aforementioned with ratio of LE-FM to LE-SB 1 to 2 (w/w). Fatty acid profile of the obtained mixed lecithins was also between those of LE-FM and LE-SB. Proportionally, LE-FS 1:1 contained more n-3 PUFAs content and less n-6 PUFAs content than LE-FS 1:2 ($p < 0.05$).

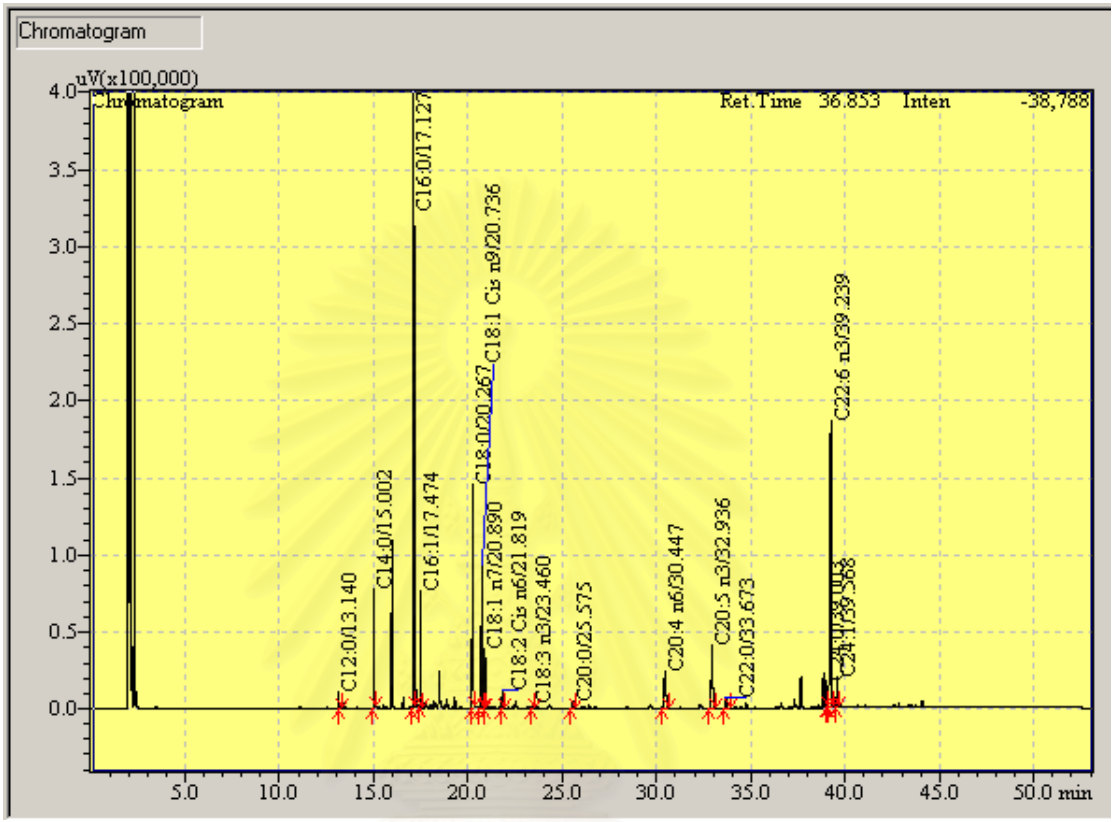


Figure 6a Fatty acid chromatogram of LE-FM

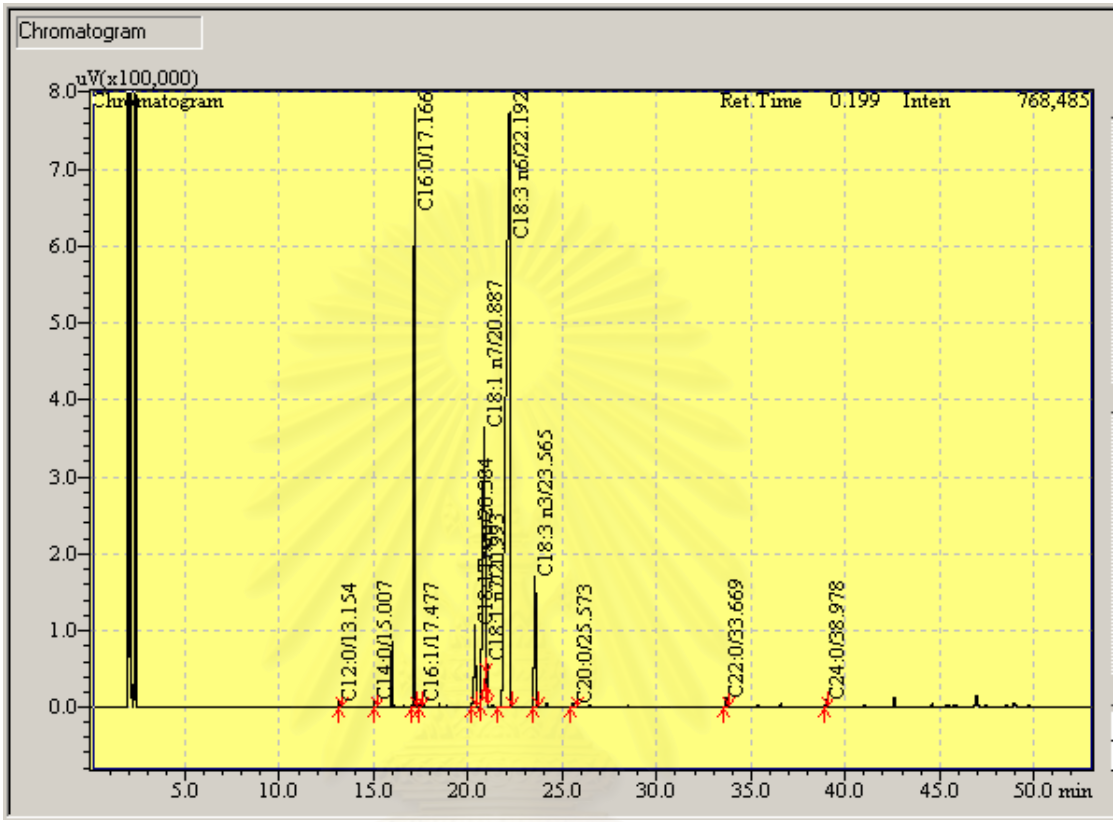


Figure 6b Fatty acid chromatogram of LE-SB

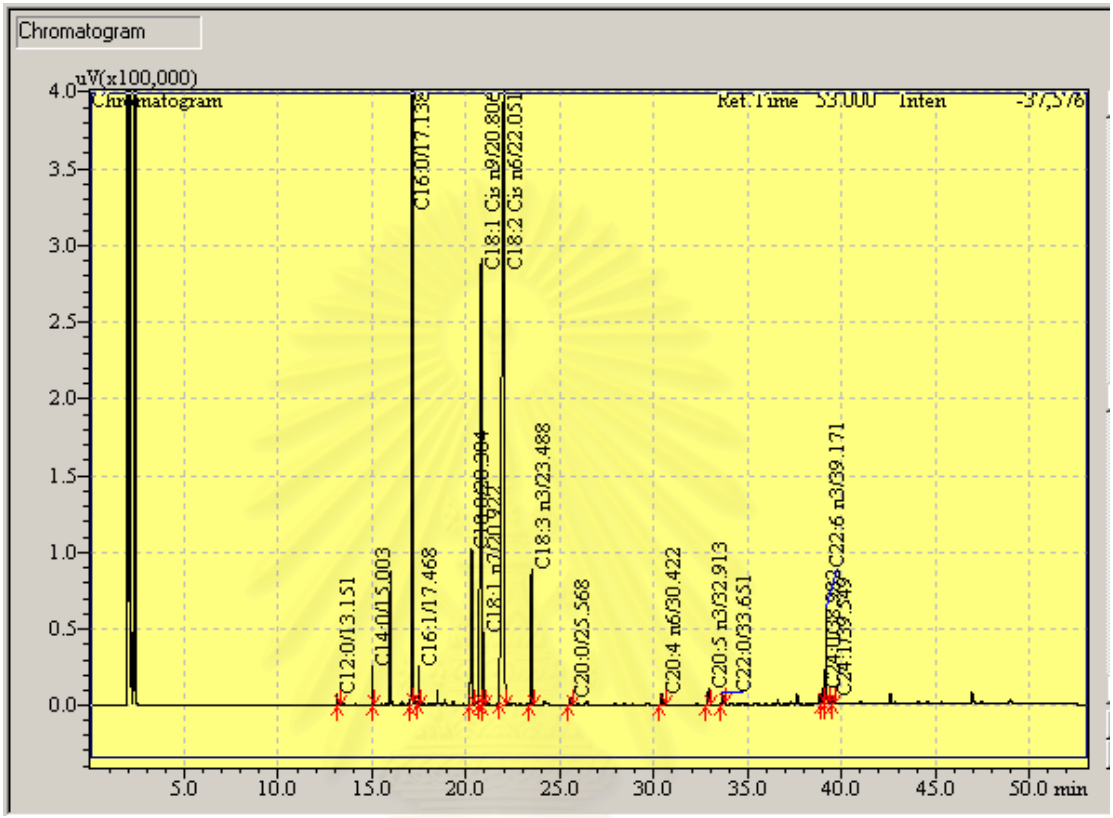


Figure 6c Fatty acid chromatogram of LE-FS 1:1

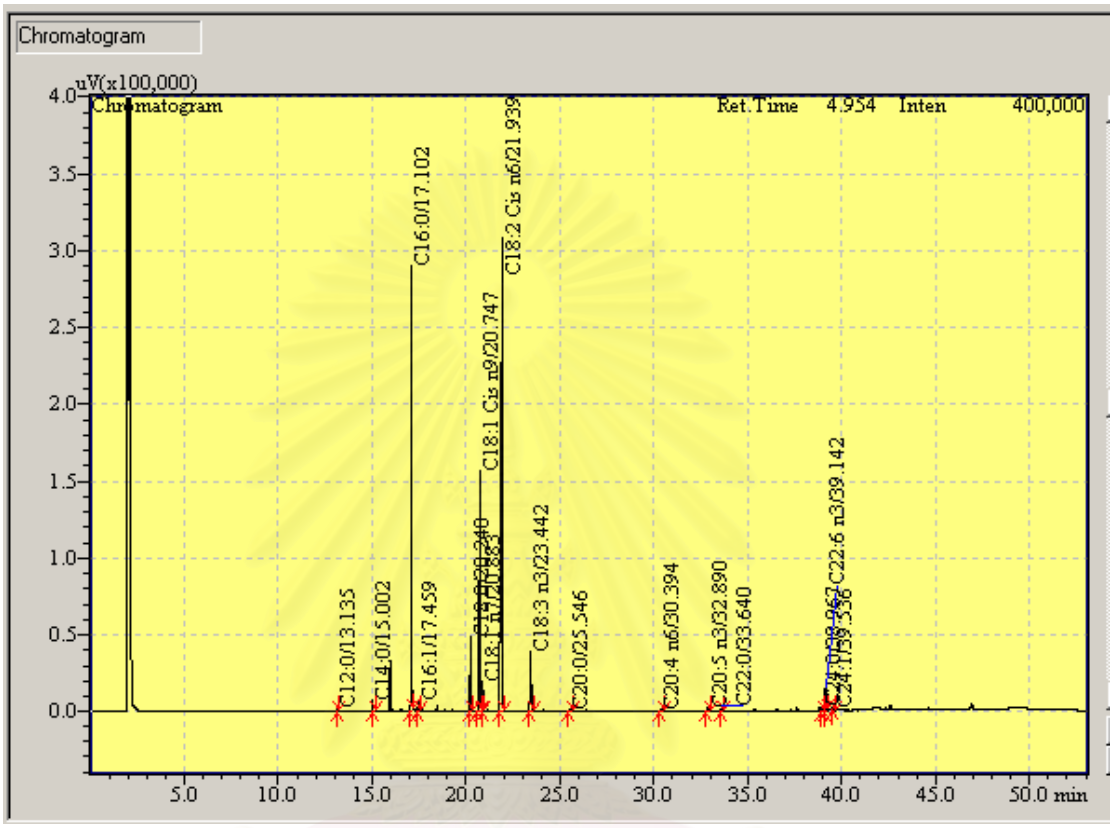


Figure 6d Fatty acid chromatogram of LE-FS 1:2

Table 2 Fatty acid profile of each lecithin in form of fatty acid methyl esters (FAMEs) (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
C12:0	0.67 ± 0.00	0.11 ± 0.01	0.19 ± 0.17	0.16 ± 0.01
C14:0	3.64 ± 0.19	0.14 ± 0.00	0.58 ± 0.02	0.39 ± 0.00
C16:0	30.73 ±0.01	18.22 ±0.18	19.70 ± 6.01	18.88 ± 0.04
C16:1 n-7	4.11 ± 0.01	0.11 ± 0.01	0.57 ± 0.01	0.39 ± 0.00
C18:0	13.58 ± 0.01	3.43 ± 0.09	4.76 ± 0.26	4.13 ± 0.03
C18:1 n-9	8.10 ± 0.01	15.30 ± 1.45	16.06 ± 0.06	16.68 ± 0.04
C18:1 n-7	3.07 ± 0.00	2.18 ±1.06	1.79 ± 0.04	1.63 ±0.03
C18:2 n-6	1.06 ± 0.02	54.83 ± 0.48	47.48 ± 0.15	50.02 ± 0.14
C18:3 n-3	0.37 ± 0.00	4.93 ± 0.08	4.20 ± 0.02	4.45 ± 0.04
C20:0	0.54 ± 0.01	0.20 ± 0.00	0.23 ± 0.02	0.24 ± 0.00
C20:4 n-6	3.98 ± 0.01	-	0.44 ± 0.02	0.29 ± 0.00
C20:5 n-3	5.20 ± 0.00	-	0.63 ± 0.02	0.39 ± 0.00
C22:0	0.85 ± 0.01	0.33 ± 0.01	0.38 ± 0.16	0.38 ± 0.01
C22:6 n-3	21.11 ± 0.02	-	2.42 ± 0.06	1.52 ± 0.01
C24:0	1.33 ± 0.01	0.23 ± 0.01	0.32 ± 0.04	0.31 ± 0.01
C24:1 n-9	1.67 ± 0.01	-	0.26 ± 0.05	0.14 ± 0.01

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

Table 3 Saturated, monoenic and polyenic fatty acids of each lecithin in form of fatty acid methyl esters (FAMEs) (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
SFA	51.34 ± 0.02	22.66 ± 0.30	26.16 ± 6.01	24.50 ±0.07
MUFA	16.95 ± 0.02	17.58 ± 0.77	18.67 ± 0.02	18.83 ± 0.04
PUFA	31.71 ± 0.03	59.76 ± 0.56	55.17 ± 0.07	56.67 ±0.11
n-6 PUFA	5.04 ± 0.02	54.83 ± 0.48	47.93 ± 0.13	50.31 ± 0.14
n-3 PUFA	26.68 ± 0.01	4.93 ± 0.08	7.24 ± 0.07	6.36 ± 0.05
n-6/n-3 ratio	0.19 ± 0.00	11.13 ± 0.09	6.62 ± 0.08	7.91 ± 0.08
n-3/n-6 ratio	5.30 ± 0.02	0.09 ± 0.00	0.15 ± 0.00	0.12 ± 0.00

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

SFA = saturated fatty acids

MUFA = monounsaturated fatty acids

PUFA = polyunsaturated fatty acids

Fatty acids in triacylglycerols, free fatty acids and phospholipids fractions of lecithin

LE-FM, LE-SB, LE-FS 1:1 and LE-FS 1:2 were assessed for lipid subclasses by pre-extraction and purification of triacylglycerol (TG), free fatty acids (FFA) and phospholipid (PL) fractions using chromatographic technique of one-dimensional thin layer chromatography prior to transesterification of all fatty acids by digesting with acetylchloride as described earlier. The actual concentrations of individual fatty acids in TG, FFA and PL fractions separated from all lecithins are shown in **tables 4**, **6** and **8**, respectively. SFA, MUFA and PUFA of TG, FFA and PL fractions of lecithin are shown in **tables 5**, **7** and **9**, respectively.

In TG fractions of each lecithin (**table 4**), C22:6n-3 was a major n-3 PUFAs content of LE-FM whereas C18:2n-6 was a minor substitute in this lecithin. Regarding fatty acid in omega 6 family, C18:2n-6 was a major n-6 PUFAs content found in LE-SB as well as mixed LE-FS 1:1 and LE-FS 1:2 about 50 g/100 g fatty acids and they were significantly higher than that of LE-FM ($p < 0.05$). **Table 5** shows the groups of fatty acids, it was obvious that n-6 PUFAs were found exceed to nearly 60 g/100g for LE-SB and for LE-SB containing mixed lecithins leading n-6/n-3 ratios of such lecithins: LE-SB, LE-FS 1:1 and LE-FS 1:2, had the ratios of n-6 PUFAs/n-3 PUFAs much higher than that of LE-FM ($p < 0.05$). In contrast, ratio of n-3 PUFAs to n-6 PUFAs of LE-FM was thus the highest content when compared to other kinds of lecithins ($p < 0.05$). Focusing on PUFAs, it was found that n-3 PUFAs were the highest content in LE-FM, whereas n-6 PUFAs were found the highest content in LE-SB.

Table 4 Fatty acid profile in TG fraction in form of fatty acid methyl esters (FAMEs) of each lecithin (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
C14:0	9.04 ± 0.14	-	0.36 ± 0.05	0.23 ± 0.04
C16:0	38.58 ± 0.62	11.09 ± 0.31	11.66 ± 0.12	12.16 ± 0.63
C16:1	9.14 ± 0.05	0.10 ± 0.01	0.28 ± 0.01	0.24 ± 0.00
C18:0	13.49 ± 0.69	3.40 ± 0.06	3.45 ± 0.09	3.64 ± 0.23
C18:1 n-9	12.28 ± 0.59	24.52 ± 1.25	23.06 ± 0.51	25.31 ± 1.70
C18:1 n-7	5.33 ± 0.12	1.75 ± 0.07	1.84 ± 0.05	1.86 ± 0.04
C18:2 n-6	2.22 ± 0.06	53.92 ± 1.17	53.32 ± 0.36	51.42 ± 1.68
C18:3 n-3	-	5.21 ± 0.53	5.71 ± 0.19	4.92 ± 0.76
C22:6 n-3	9.93 ± 0.55	-	0.33 ± 0.03	0.22 ± 0.04

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

Table 5 Saturated, monoenic and polyenic fatty acids in TG fraction in form of fatty acid methyl esters (FAMEs) of each lecithin (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
SFA	61.10 ± 0.07	14.49 ± 0.37	15.47 ± 0.15	16.03 ± 0.82
MUFA	26.75 ± 0.42	26.37 ± 1.33	25.18 ± 0.45	27.41 ± 1.66
PUFA	12.15 ± 0.49	59.14 ± 1.70	59.35 ± 0.55	56.56 ± 2.47
n-6 PUFA	2.22 ± 0.06	53.92 ± 1.17	53.32 ± 0.36	51.42 ± 1.68
n-3 PUFA	9.93 ± 0.55	5.21 ± 0.53	6.03 ± 0.22	5.14 ± 0.80
n-6/n-3 ratio	0.23 ± 0.02	10.43 ± 0.84	8.86 ± 0.29	10.20 ± 1.26
n-3/n-6 ratio	4.48 ± 0.37	0.10 ± 0.01	0.11 ± 0.00	0.10 ± 0.01

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

See abbreviations in Table 3.

Table 6 shows fatty acid profile in free fatty acid (FFA) fraction. The results correspond to those found in TG. The content of C22:6n-3 in FFA fraction was noticeably higher than that in TG fraction ie, 23.67 in FFA *vs* 9.93 g/100 g in TG. This was possibly explained by the fact that ester bond of highly PUFA as C22:6 were fragile, easily broken and released from TG which produced FFA with high C22:6n-3 proportion as seen. Furthermore, high proportion of C22:6n-3 in FFA was also found in LE-FS 1:1 and LE-FS 1:2 (8.95 and 6.06 g/100 g, respectively). C18:2n-6 was still the major FFA in LE-SB (47.88 g/100 g) corresponding to that found in TG moiety (53.92 g/100 g).

It was also shown in **table 7** that mostly fatty acid presented in FFA fraction of all lecithin was PUFAs. The content of n-3 PUFA was found predominantly in LE-FM whereas n-6 PUFAs content were found predominantly in LE-SB, LE-FS 1:1 and LE-FS 1:2. The ratio of n-3/n-6 PUFA of LE-FM was markedly higher than those found in three lecithins containing LE-SB in their mixtures ($p < 0.05$).

Table 8 shows fatty acid profile in phospholipids fraction that separated from lecithin. Again, omega 3 fatty acids exclusively C22:6n-3 and C20:5n-3 in LE-FM were markedly higher than those found in LE-SB, LE-FS 1:1 and LE-FS 1:2. In contrast, LE-FM contained trace amount of C18:2n-6. It content was fewer than those of soybean containing lecithins (1.25 *vs* 53.26 *vs* 47.11 *vs* 50.32 g/100 g in LE-FM, LE-SB, LE-FS 1:1, LE-FS 1:2, respectively).

In **table 9**, n-3 fatty acids of both C20:5n-3 and C22:6n-3 in phospholipids fraction of LE-FM were prominent confirming that such lecithin was rich source of n-3 PUFAs.

Table 6 Fatty acid profile in free fatty acid (FFA) fraction in form of fatty acid methyl esters (FAMEs) of each lecithin (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
C14:0	3.53 ± 0.10	0.42 ± 0.18	1.68 ± 0.04	1.22 ± 0.04
C16:0	32.27 ± 0.11	21.39 ± 0.35	27.19 ± 0.09	24.71 ± 0.49
C16:1 n-7	4.74 ± 0.18	-	1.86 ± 0.01	1.45 ± 0.01
C18:0	14.17 ± 0.69	5.20 ± 0.59	9.15 ± 0.40	7.72 ± 0.01
C18:1 n-9	6.45 ± 0.06	18.76 ± 0.43	13.28 ± 0.19	15.34 ± 0.50
C18:1 n-7	2.89 ± 0.28	2.73 ± 1.06	2.54 ± 0.21	2.25 ± 0.10
C18:2 n-6	1.30 ± 0.10	47.88 ± 2.47	28.78 ± 0.58	35.50 ± 0.17
C18:3 n-3	-	3.00 ± 0.43	1.98 ± 0.19	2.44 ± 0.12
C20:0	-	0.63 ± 0.28	0.71 ± 0.31	0.38 ± 0.01
C20:4 n-6	4.75 ± 0.25	-	1.88 ± 0.08	1.20 ± 0.24
C20:5 n-3	6.23 ± 0.04	-	2.35 ± 0.04	1.72 ± 0.15
C22:6 n-3	23.67 ± 0.21	-	8.95 ± 0.24	6.06 ± 0.83

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

Table 7 Saturated, monoenic and polyenic fatty acids in FFA fraction in form of fatty acid methyl esters (FAMEs) of each lecithin (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
SFA	49.97 ± 0.68	27.64 ± 1.40	38.73 ± 0.23	34.04 ± 0.55
MUFA	14.08 ± 0.15	21.49 ± 1.50	17.68 ± 0.41	19.04 ± 0.62
PUFA	35.94 ± 0.53	50.88 ± 2.90	43.93 ± 0.65	46.93 ± 1.17
n-6 PUFA	6.05 ± 0.36	47.88 ±2.47	30.66 ± 0.51	36.71 ± 0.07
n-3 PUFA	29.90 ± 0.17	3.00 ± 0.43	13.27 ± 0.37	10.22 ± 1.10
n-6/n-3 ratio	0.20 ± 0.01	16.17 ± 1.49	2.31 ± 0.07	3.63 ± 0.38
n-3/n-6 ratio	4.96 ± 0.26	0.06 ± 0.01	0.43 ± 0.01	0.28 ± 0.03

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

See abbreviations in Table 3.

Table 8 Fatty acid profile in PL fraction in form of fatty acid methyl esters (FAMEs) of each lecithin (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
C12:0	1.00 ± 0.07	0.24 ± 0.01	0.37 ± 0.05	0.29 ± 0.02
C14:0	2.72 ± 0.16	0.19 ± 0.00	0.60 ± 0.07	0.38 ±0.00
C16:0	30.74 ± 0.25	22.76 ± 0.27	24.28 ± 0.13	23.93 ± 0.05
C16:1 n-7	3.38 ± 0.60	0.11 ± 0.00	0.47 ± 0.01	0.31 ± 0.00
C18:0	14.28 ± 0.20	3.69 ± 0.25	5.09 ± 0.07	4.41 ± 0.01
C18:1 n-9	11.17 ± 1.54	13.98 ± 1.69	11.98 ± 0.18	11.96 ± 0.01
C18:1 n-7	3.01 ± 0.13	1.54 ± 0.02	1.76 ± 0.01	1.67 ± 0.00
C18:2 n-6	1.25 ± 0.09	53.26 ± 1.48	47.11 ± 0.28	50.32 ± 0.06
C18:3 n-3	0.29 ± 0.01	3.41 ± 0.19	3.07 ± 0.07	3.32 ± 0.00
C20:0	0.73 ± 0.14	0.17 ± 0.03	0.21 ± 0.00	0.17 ± 0.01
C20:4 n-6	3.53 ± 0.19	-	0.51 ± 0.02	0.27 ± 0.00
C20:5 n-3	3.80 ± 0.19	-	0.57 ± 0.03	0.32 ± 0.01
C22:0	1.30 ± 0.04	0.35 ± 0.02	0.50 ± 0.02	0.48 ± 0.00
C22:6 n-3	17.89 ± 0.92	-	2.51 ± 0.06	1.44 ± 0.01
C24:0	2.14 ± 0.02	0.30 ± 0.01	0.56 ± 0.03	0.48 ± 0.01
C24:1	2.77 ± 0.04	-	0.41 ± 0.01	0.25 ± 0.00

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

Table 9 Saturated, monoenic and polyenic fatty acids in PL fraction in form of fatty acid methyl esters (FAMEs) of each lecithin (g/100g fatty acid)

Fatty Acid	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
SFA	52.92 ± 0.65	27.71 ± 0.03	31.61 ± 0.09	30.14 ± 0.05
MUFA	20.34 ± 1.42	15.63 ± 1.67	14.62 ± 0.20	14.19 ± 0.02
PUFA	26.75 ± 1.20	56.66 ± 1.66	53.77 ± 0.24	55.68 ± 0.05
n-6 PUFA	4.77 ± 0.11	53.26 ± 1.48	47.62 ± 0.26	50.58 ± 0.06
n-3 PUFA	21.97 ± 1.09	3.41 ± 0.19	6.16 ± 0.03	5.08 ± 0.01
n-6/n-3 ratio	0.22 ± 0.01	15.69 ± 0.45	7.74 ± 0.07	9.96 ± 0.02
n-3/n-6 ratio	4.60 ± 0.12	0.06 ± 0.00	0.13 ± 0.00	0.10 ± 0.00

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

See abbreviations in Table 3.

Phospholipids profiling in lecithin

Phospholipids subclasses of lecithin were separated by liquid chromatography and identified the subspecies especially their fatty acid moieties by mass spectrometer according to the method described by Wang et al (2004) with slightly modified by Mr. Sukit Sirikwanpong, Ph.D. student (Program of Biomedical Science, Chulalongkorn University) as previously described. **Figure 7** show LC-chromatogram and mass spectrum of lecithin. Molecular species as well as in m/z ratio of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine fused with plasmalogen (PE & pPE) and spingomyelin (SM) in mixed lecithins are shown in **tables 10**, **11**, **12 13** and **14**, respectively. The distribution of phospholipids (PL) in each lecithin is shown in **table 15**. The results of phospholipids subspecies would confirm those of fatty acid profile that LE-FM was rich source of n-3 PUFA as well as LE-SB was accepted as rich source of n-6 PUFAs. However, the results obtained from LC-ESI-MS provide information with more specific description of not only phospholipids subspecies but fatty acid moieties present in individual phospholipids subspecies as well.

Phosphatidlycholine in lecithin

The results showed that LE-SB was significantly rich source of PC when compared to LE-FM (10.83 *vs* 2.68 μmol/100 mg lecithin, respectively). Focusing on molecular species, the mainly PC species in LE-FM were 16:0/22:6 (m/z 850) and 16:0/18:1 (m/z 804) while the main PC species in LE-SB, LE-FS 1:1 and LE-FS 1:2 were 18:2/18:2 (m/z 826) and 16:0/18:2 (m/z 802). The total PC contents in LE-FM, LE-SB, LE-FS 1:1 and LE-FS 1:2 were 2.68, 10.83, 10.32 and 10.09 μmole/100 mg lecithin, respectively.

Phosphatidylserine in lecithin

In LE-FM, the major PS molecular specie was 16:0/22:6 (m/z 806) while in LE-SB, LE-FS 1:1 and LE-FS 1:2 were 16:0/20:4 + 18:1/18:3 (m/z 782). The total PS contents in LE-FM, LE-SB, LE-FS 1:1 and LE-FS 1:2 were 120, 154, 91 and 141 nmole/100 mg lecithin, respectively.

Phosphatidylinositol in lecithin

The major PI molecular species in FM were 18:0/22:6 + 18:1/22:5 (m/z 909) while in SB, FS 1:1 and FS 1:2 were 16:0/18:2 (m/z 833). The total PI contents in LE-FM, LE-SB, LE-FS 1:1 and LE-FS 1:2 were 1.02, 13.98, 10.52 and 11.92 μmole/100 mg lecithin, respectively.

Phosphatidylethanolamine and PE plasmalogen in lecithin

PE and pPE were fused and inseparated by liquid chromatography thus analyzed together as fused PE/pPE. The major molecular species found in this fused phospholipid subspecies of LE-FM were 18:0/22:6 + 18:1/22:5 + 18:2/22:4 (m/z 790) while in LE-SB, LE-FS 1:1 and LE-FS 1:2 were 16:0/20:4 + 18:2/18:2 (m/z 738). The total PE & pPE contents in LE-FM, LE-SB, LE-FS 1:1 and LE-FS 1:2 were 0.55, 5.11, 3.76 and 4.23 μmole/100 mg lecithin, respectively.

Sphingomyelin in Mixed Lecithins

There was no SM content in LE-SB. The major SM molecular species in LE-FM, LE-FS 1:1 and LE-FS 1:2 were 16:0 (m/z 747). The total SM contents in LE-FM, LE-FS 1:1 and LE-FS 1:2 were 1.29, 1.22 and 0.69 μmole/100 mg lecithin, respectively.

Most abundant of PL in LE-FM was thus PC and SM while in LE-SB, LE-FS 1:1 and LE-FS 1:2 were PC and PI.

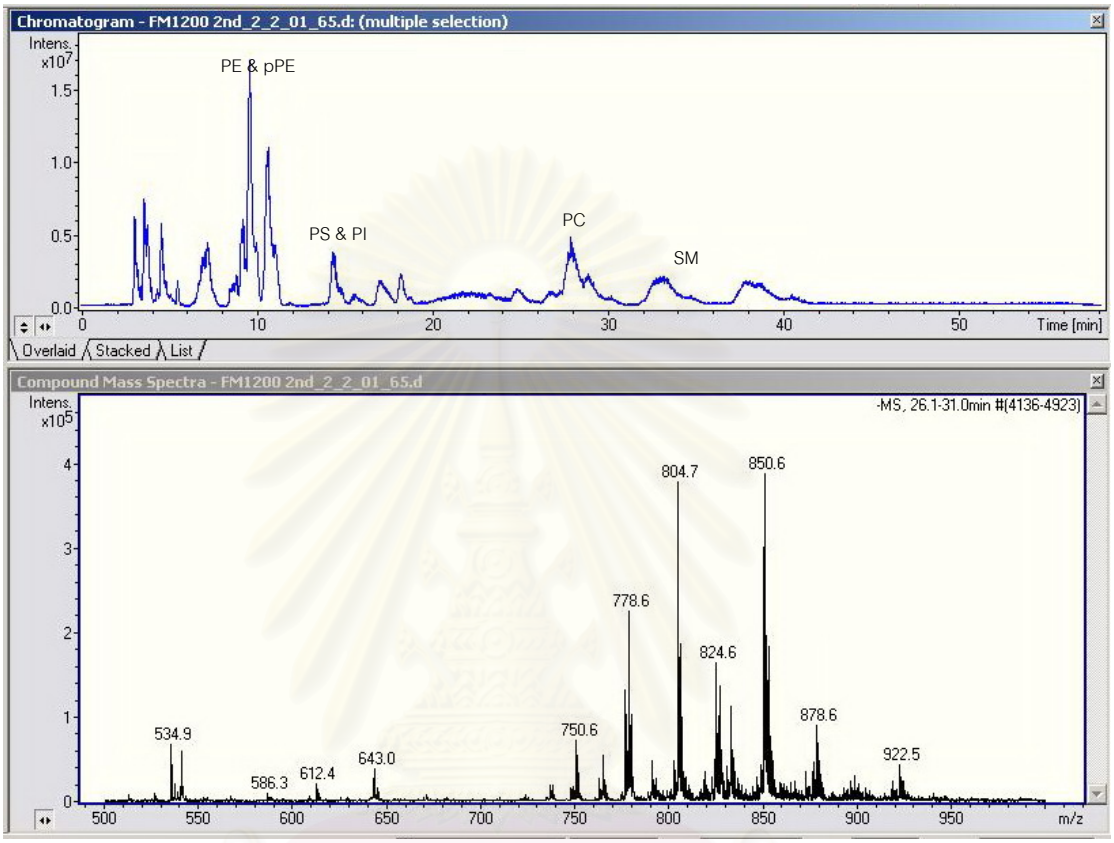


Figure 7a LC-chromatogram (upper part) and mass spectrum (lower part) of LE-FM

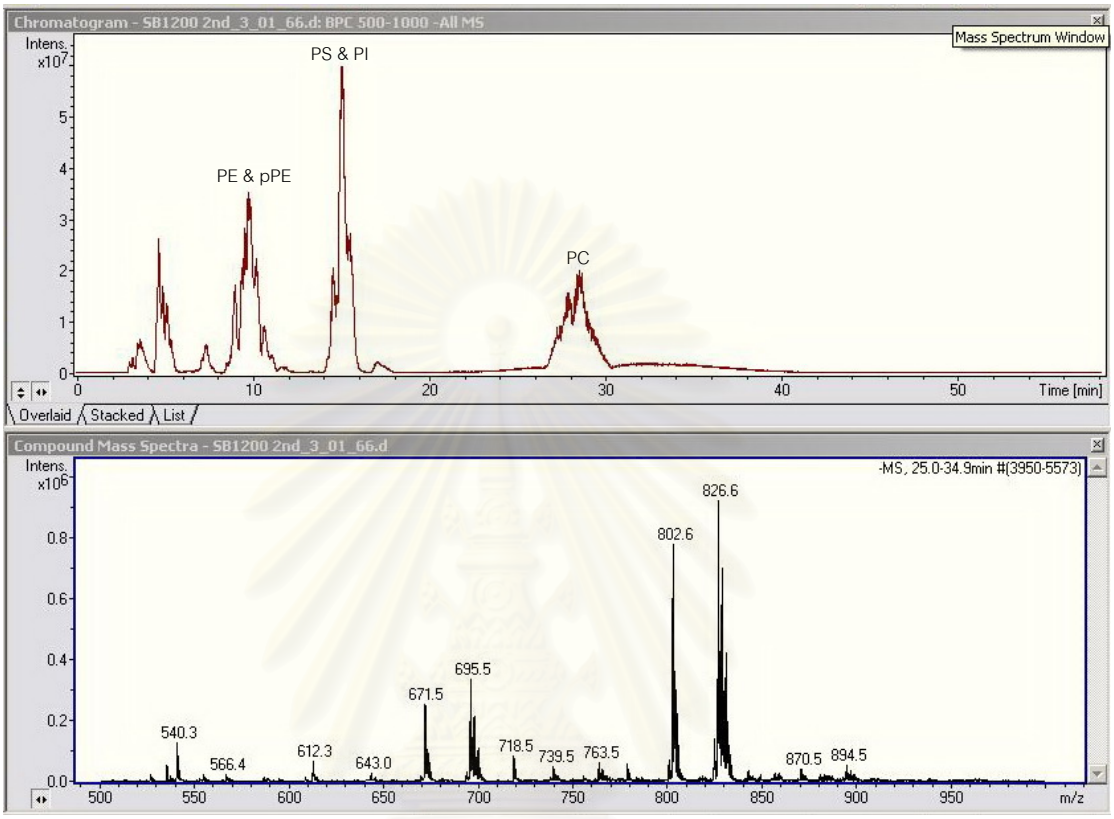


Figure 7b LC-chromatogram (upper part) and mass spectrum (lower part) of LE-SB

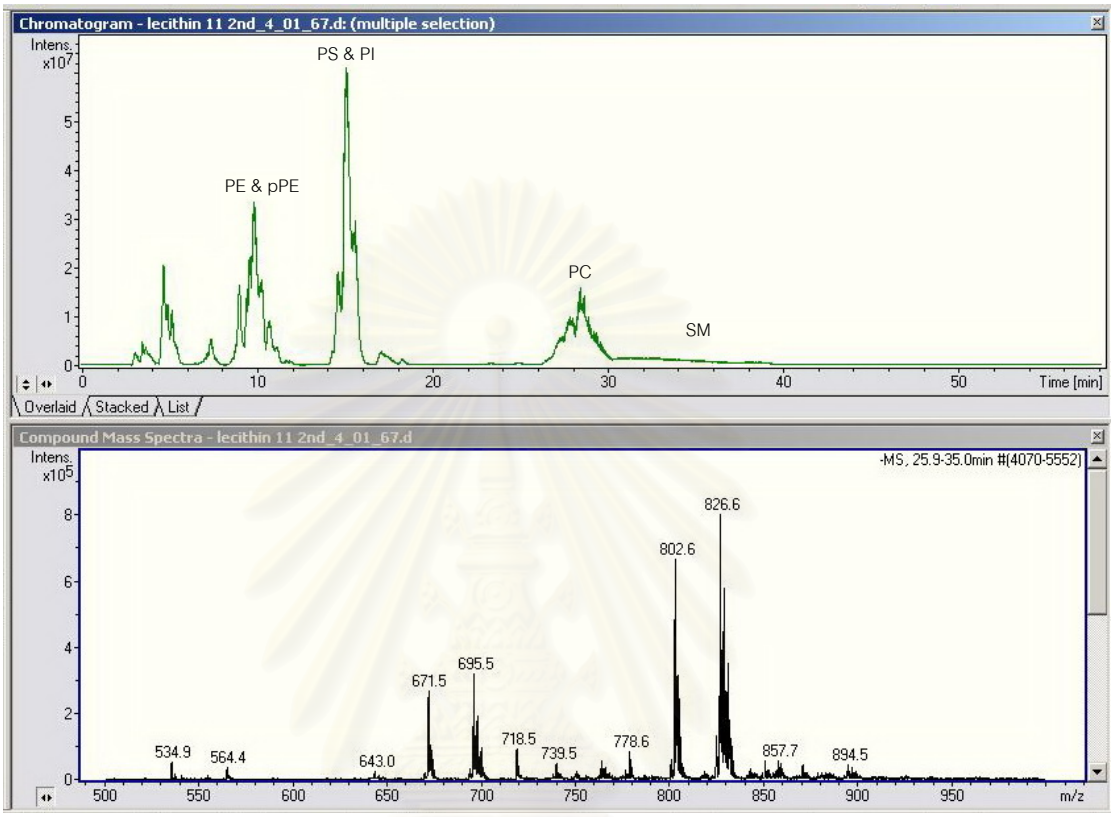


Figure 7c LC-chromatogram (upper part) and mass spectrum (lower part) of LE-FS 1:1

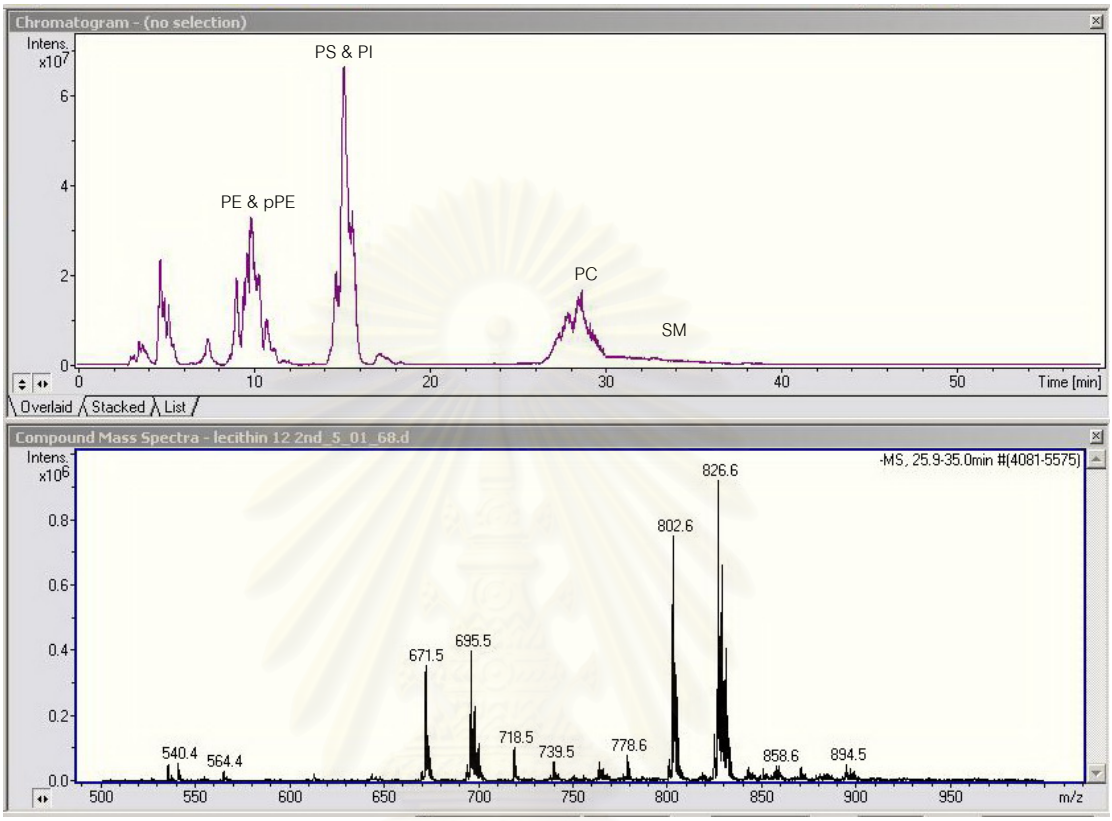


Figure 7d LC-chromatogram (upper part) and mass spectrum (lower part) of LE-FS 1:2

Table 10 Molecular species of phosphatidylcholine (PC) in each lecithin (nmol/100 mg lecithin) by negative-ion LC-ESI-MS analysis

m/z	Molecular species	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
750	14:0/16:0	83 ± 18	-	70 ± 5	47 ± 3
776	16:0/16:1	168 ± 16	23 ± 4	86 ± 2	58 ± 0
778	16:0/16:0	276 ± 20	190 ± 8	270 ± 16	230 ± 5
792	16:0/17:0	33 ± 4	-	28 ± 1	-
800	16:0/18:3	19 ± 2	235 ± 4	200 ± 8	204 ± 10
802	16:0/18:2	60 ± 6	2,586 ± 136	2,201 ± 100	2,251 ± 43
804	16:0/18:1	484 ± 34	715 ± 48	810 ± 40	735 ± 7
806	16:0/18:0	73 ± 8	22 ± 2	44 ± 4	29 ± 2
824	16:0/20:5 + 16:1/20:4	213 ± 15	459 ± 24	435 ± 18	433 ± 7
826	16:0/20:4 + 18:2/18:2	152 ± 13	3,016 ± 177	2,582 ± 86	2,676 ± 4
828	16:0/20:3	22 ± 3	1,905 ± 181	1,574 ± 34	1,631 ± 3
830	18:0/18:2 + 18:1/18:1	46 ± 7	1,082 ± 114	923 ± 22	973 ± 1
832	18:0/18:1	116 ± 34	221 ± 26	258 ± 0	240 ± 4
834	18:0/18:0	23 ± 7	11 ± 4	64 ± 45	14 ± 5
850	16:0/22:6	497 ± 43	-	168 ± 4	100 ± 4
852	16:0/22:5 + 18:0/20:5 + 18:1/20:4	165 ± 21	-	70 ± 0	47 ± 1
854	18:0/20:4 + 18:1/20:3 + 16:0/22:4	37 ± 14	35 ± 1	60 ± 2	45 ± 2
856	18:0/20:3 + 18:1/20:2	9 ± 2	83 ± 4	76 ± 2	75 ± 1
858	18:0/20:2	23 ± 5	77 ± 7	142 ± 1	117 ± 6
860	18:0/20:1	21 ± 3	22 ± 4	38 ± 1	30 ± 2
876	18:1/22:6	55 ± 10	-	32 ± 0	-
878	18:0/22:6 + 18:1/22:5	87 ± 31	20 ± 1	66 ± 1	45 ± 2
880	18:0/22:5 + 18:1/22:4	20 ± 7	66 ± 0	67 ± 8	61 ± 5
882	18:0/22:4 + 20:0/20:4	-	62 ± 3	56 ± 7	54 ± 5
	Total	2,682 ± 324	10,829 ± 745	10,320 ± 376	10,094 ± 59

Data were expressed as Mean ± S.E.M.; n=2 independent experiments.

Table 11 Molecular species of phosphatidylserine (PS) in each lecithin (nmol/100 mg lecithin) by negative-ion LC-ESI-MS analysis

m/z	Molecular species	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
762	16:0/18:0	19 ± 7	-	-	-
782	16:0/20:4 + 18:1/18:3	-	62 ± 6	38 ± 2	47 ± 3
784	16:0/20:3 + 18:1/18:2 + 18:0/18:3	-	27 ± 1	-	20 ± 1
786	18:0/18:2 + 18:1/18:1	-	32 ± 1	-	23 ± 1
788	18:0/18:1 + 16:0/20:1	25 ± 7	-	25 ± 1	20 ± 1
806	16:0/22:6	35 ± 8	-	-	-
808	18:0/20:5 + 18:1/20:4 + 16:0/22:5	21 ± 5	-	-	-
810	18:0/20:4 + 16:0/22:4 + 18:1/20:3	21 ± 6	33 ± 5	28 ± 1	30 ± 5
	Total	120 ± 19	154 ± 10	91 ± 3	141 ± 11

Data were expressed as Mean ± S.E.M.; n=2 independent experiments.

Table 12 Molecular species of phosphatidyinositol (PI) in each lecithin (nmol/100 mg lecithin) by negative-ion LC-ESI-MS analysis

m/z	Molecular species	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
809	16:0/16:0 + 14:0/18:0	-	56 ± 11	47 ± 1	52 ± 3
831	16:0/18:3	-	801 ± 108	589 ± 45	701 ± 51
833	16:0/18:2	-	7,098 ± 919	5,361 ± 177	5,987 ± 403
835	16:0/18:1 + 16:1/18:0	-	1,376 ± 244	1,028 ± 38	1,231 ± 15
855	16:0/20:5	-	253 ± 66	178 ± 10	213 ± 3
857	16:0/20:4 + 18:2/18:2	46 ± 10	1,420 ± 282	1,003 ± 6	1,187 ± 5
859	16:0/20:3 + 18:1/18:2 + 18:0/18:3	-	958 ± 112	696 ± 45	770 ± 55
861	18:0/18:2 + 18:1/18:1	-	1,671 ± 189	1,160 ± 57	1,382 ± 93
863	18:0/18:1	16 ± 7	343 ± 49	222 ± 9	278 ± 24
881	16:0/22:6	83 ± 16	-	-	-
883	18:1/20:4 + 16:0/22:5 + 18:0/20:5	111 ± 18	-	27 ± 1	-
885	18:0/20:4 + 16:0/22:4 + 18:1/20:3	243 ± 30	-	67 ± 2	48 ± 1
887	18:0/20:3	9 ± 5	-	-	-
907	18:1/22:6	39 ± 9	-	25 ± 0	-
909	18:0/22:6 + 18:1/22:5	395 ± 42	-	96 ± 4	70 ± 5
911	18:0/22:5; 18:1/22:4; 20:1/20:4	72 ± 8	-	20 ± 1	-
	Total	1,015 ± 145	13,976 ± 1981	10,520 ± 373	11,919 ± 653

Data were expressed as Mean ± S.E.M.; n=2 independent experiments.

Table 13 Molecular species of phosphatidylethanolamine (PE) and plasmalogen PE (pPE) in each lecithin (nmol/100 mg lecithin) by negative-ion LC-ESI-MS analysis

m/z	Molecular species	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
	PE				
714	16:0/18:2	11 ± 1	1,526 ± 90	1,077 ± 111	1,200 ± 50
716	16:0/18:1	13 ± 1	378 ± 17	271 ± 21	303 ± 4
736	16:0/20:5	-	243 ± 22	172 ± 3	204 ± 5
738	16:0/20:4 + 18:2/18:2	17 ± 0	1,398 ± 140	1,008 ± 62	1,118 ± 1
740	16:0/20:3 + 18:1/18:2 + 18:0/18:3	-	742 ± 47	494 ± 29	578 ± 21
742	18:0/18:2 + 18:1/18:1 + 16:0/20:2	-	422 ± 5	295 ± 31	358 ± 17
744	18:0/18:1	17 ± 1	83 ± 2	61 ± 2	76 ± 1
762	16:0/22:6	83 ± 6	-	27 ± 3	-
764	18:1/20:4 + 18:0/20:5 + 16:0/22:5	31 ± 0	-	-	-
766	18:0/20:4 + 16:0/22:4	21 ± 0	25 ± 5	44 ± 8	39 ± 1
772	18:0/20:1 + p18:1/22:6	-	35 ± 13	-	-
790	18:0/22:6 + 18:1/22:5 + 18:2/22:4	110 ± 6	-	37 ± 6	26 ± 1
792	18:0/22:5 + 18:1/22:4	26 ± 2	-	-	-
794	18:0/22:4 + 20:0/20:4	9 ± 0	-	-	-
	pPE				
722	p16:0/20:4	8 ± 0	-	-	18 ± 2
728	p18:0/18:1	24 ± 2	18 ± 4	-	17 ± 0
746	p16:0/18:2	22 ± 0	92 ± 55	121 ± 8	152 ± 3
748	p18:1/20:4 + p18:0/20:5 + p16:0/22:5	30 ± 1	37 ± 7	34 ± 4	34 ± 3
750	p18:0/20:4 + p16:0/22:4	16 ± 0	-	-	-
774	p18:0/22:6 + p18:1/22:5	31 ± 1	31 ± 1	32 ± 3	33 ± 1
776	p18:0/22:5 + p18:1/22:4	32 ± 0	-	-	-
778	p18:0/22:4	34 ± 3	-	-	-
806	p22:0/20:4	16 ± 2	81 ± 2	82 ± 0	80 ± 7
	Total	550 ± 3	5,111 ± 246	3,756 ± 269	4,234 ± 81

Data were expressed as Mean ± S.E.M.; n=2 independent experiments.

Table 14 Molecular species of sphingomyelin (SM) in each lecithin (nmol/100 mg lecithin) by negative-ion LC-ESI-MS analysis

m/z	Molecular species	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
719	14:0	132 ± 11	-	-	60 ± 23
747	16:0	385 ± 22	-	380 ± 171	203 ± 12
761	17:0	25 ± 2	-	37 ± 3	-
771	18:2	30 ± 3	-	31 ± 6	19 ± 3
773	18:1	9 ± 1	-	-	11 ± 1
775	18:0	39 ± 10	-	42 ± 10	26 ± 4
799	20:2	26 ± 4	-	28 ± 8	19 ± 1
801	20:1	25 ± 4	-	15 ± 7	13 ± 7
803	20:0	15 ± 6	-	40 ± 1	14 ± 1
829	22:1	95 ± 14	-	123 ± 2	68 ± 9
831	22:0	92 ± 8	-	116 ± 16	55 ± 6
857	24:1	328 ± 23	-	318 ± 24	152 ± 44
859	24:0	90 ± 2	-	91 ± 3	45 ± 10
	Total	1,292 ± 109	-	1,222 ± 199	686 ± 16

Data were expressed as Mean ± S.E.M.; n=2 independent experiments.

Table 15 Distribution of phospholipids in lecithin (%)

Phospholipids Class	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
PC	47.3	36.2	39.8	37.3
PS	2.1	0.5	0.4	0.5
PI	17.9	46.3	40.6	44.0
PE & pPE	9.8	17.0	14.5	15.6
SM	22.9	0	4.7	2.6

N=2 independent experiments

PC = phosphatidylcholine

PS = phosphatidylserine

PI = phosphatidylinositol

PE = phosphatidylethanolamine

pPE = phosphatidylethanolamine plasmalogen

SM = sphingomyelin

Characteristic of rat diet

Rat diet was commercial pellet diet manufactured by C.P. Pet Food (Thailand). Raw material and nutritional composition of rat diet are shown in **table 16** and **17. Figure 8** show fatty acid chromatogram of rat diet and **table 18** shows fatty acid profiles in rat diet. It contained high n-6 PUFA with little proportion of n-3 PUFA that it resulted in an increase of n-6/n-3 ratio (**table 19**).

Effect of lecithin on animal

Adult male Wistar rats were used in this experiment. After 7 days of acclimatization, 30 rats were randomly divided into 5 groups (n=6), each group orally received different lecithin as previously described. The control group received 1% Tween-20 in equal volume of lecithin mixtures which was given to normal rats in lecithin groups. Food, water and rats were weighed a daily. At the end of the experiment, there was no significant change in food and water consumption between each group of rats. Weight gain from initial weight of LE-SB and LE-FS 1:1 group was significantly increased when compared to control group despite of indifferent intake of food. All data are shown in **table 20**. This was possibly explained by high fat intake as well as metabolizable energy consumption of LE-SB group rats ($p < 0.05$) as shown in **table 21**.

Table 22 and 23 also show average mineral and vitamin intakes that all groups of normal rats received the same amount of all those indispensable nutrients.

In all groups treated with lecithin, there were no significant change in blood chemical parameters, except HDL-cholesterol and creatinine. HDL-cholesterol of group treated with LE-SB and LE-FS 1:1 showed a significant increase when compared to control group ($p< 0.05$). Creatinine of group treated with LE-FS 1:1 and LE-FS 1:2 were higher than that of control, LE-FM and LE-SB groups ($p< 0.05$). In addition, blood chemical parameters of liver and kidney functions were no significant difference from control (**table 24**).

Table 16 Raw material and nutritional composition in rat diet

Composition	Amount	
Raw Material		
Corn, Corn Gluten Meal, Rice and Rice by-product	53	%
Soybean Meal and Steamed Soybean	34	%
Fishmeal	8	%
Soybean Oil	2	%
Vitamins and Minerals	3	%
Total	100	%
Nutritional Composition		
Moisture	12	% max
Crude Protein	24	%min
Fat	4.5	%min
Fiber	5	%max
Carbohydrate	48.5	%
Metabolizable Energy	3,040	kcal/kg

Data was obtained from the manufacturer.

Table 17 Minerals and vitamins in rat diet

Composition	Amount	
Minerals		
Ca	1.0	%
P	0.90	%
Na	0.20	%
K	1.17	%
Mg	0.23	%
Mn	171	ppm
Cu	22	ppm
Zn	100	ppm
Fe	180	ppm
Co	1.82	ppm
KI	1	ppm
Se	0.1	ppm
Vitamins		
A	20,000	IU/kg
D	4,000	IU/kg
E	100	
B1	5	mg/kg
B2	20	mg/kg
B6	20	mg/kg
B12	0.036	mg/kg
Niacin	100	mg/kg
Folic acid	6	mg/kg
Biotin	0.4	mg/kg
Pantothenic acid	60	mg/kg
Choline Chloride	1,500	mg/kg

Data was obtained from the manufacturer.

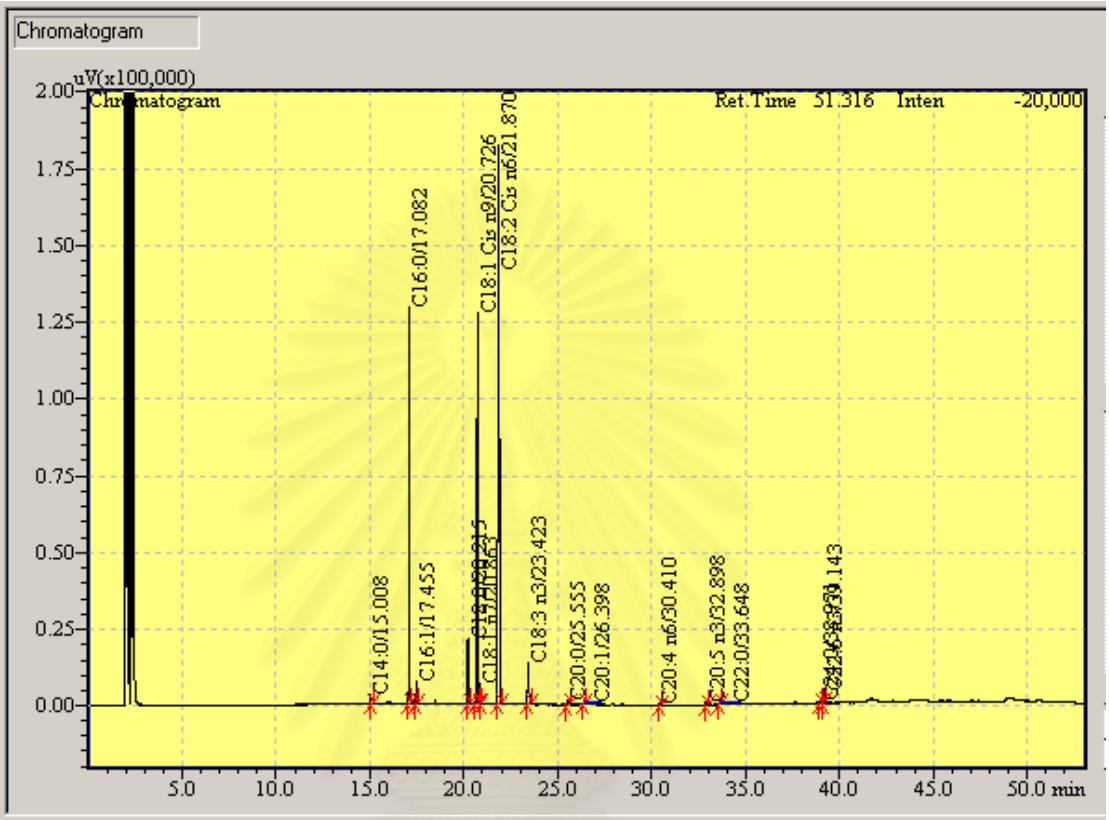


Figure 8 Fatty acid chromatogram of rat diet

Table 18 Fatty acid profile in rat diet in form of fatty acid methyl esters (FAMEs) (g/100g fatty acid)

Fatty acid	Amount
C14:0	0.37 ± 0.01
C16:0	15.83 ± 0.03
C16:1 n-7	0.95 ± 0.00
C18:0	3.98 ± 0.02
C18:1 n-9	25.88 ± 0.02
C18:1 n-7	1.16 ± 0.02
C18:2 n-6	45.96 ± 0.03
C18:3 n-6	3.38 ± 0.01
C20:0	0.49 ± 0.01
C20:1 n-9	0.21 ± 0.00
C20:4 n-6	0.20 ± 0.01
C20:5 n-3	0.28 ± 0.02
C22:0	0.29 ± 0.00
C22:6 n-3	0.77 ± 0.01
C24:0	0.24 ± 0.00

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

Table 19 Saturated, monoenic and polyenic fatty acids in rat diet in form of fatty acid methyl esters (FAMEs) (g/100g fatty acid)

Fatty acid	Amount
SFA	21.20 ± 0.05
MUFA	28.20 ± 0.03
PUFA	50.59 ± 0.01
n-6 PUFA	49.54 ± 0.02
n-3 PUFA	1.05 ± 0.02
n-6/n-3 ratio	47.33 ± 0.85
n-3/n-6 ratio	0.02 ± 0.00

Data were expressed as Mean ± S.E.M.; n=3 independent experiments.

See abbreviations in Table 3.

Table 20 The body weight, food and water intake per day of rat

Parameter	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
Initial body weight (g)	203 ± 3	199 ± 3	193 ± 1	196 ± 5	199 ± 3
Body weight after 8 weeks (g)	353 ± 9	365 ± 16	380 ± 3	375 ± 13	370 ± 8
Weight gain (g)	150 ± 8	166 ± 13	187 ± 4 [a]	179 ± 10 [a]	171 ± 8
Food intake/day (g)	16.25 ± 0.19	16.09 ± 0.44	16.24 ± 0.26	16.60 ± 0.37	16.3 ± 0.32
Water intake/day (g)	59.24 ± 1.09	57.98 ± 2.82	56.41 ± 5.38	60.98 ± 1.71	59.59 ± 3.87

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to control.

Table 21 Average nutrient daily intake of rat

Composition	*Control (1% Tween-20; n=6)*	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
Moisture (g/day)	1.95 ± 0.02	1.93 ± 0.05	1.95 ± 0.03	1.99 ± 0.05	1.96 ± 0.04
Crude Protein (g/day)	3.90 ± 0.45	3.86 ± 0.10	3.90 ± 0.06	3.99 ± 0.09	3.91 ± 0.08
Fat (g/day)	0.73 ± 0.01	1.06 ± 0.02 [a]	1.07 ± 0.01 [a]	1.09 ± 0.02 [a]	1.07 ± 0.01 [a]
Fiber (g/day)	0.81 ± 0.01	0.81 ± 0.02	0.81 ± 0.01	0.83 ± 0.02	0.82 ± 0.02
Carbohydrate (g/day)	7.88 ± 0.09	7.81 ± 0.21	7.88 ± 0.13	8.05 ± 0.18	7.90 ± 0.15
Metabolizable Energy (kcal/day)	49.40 ± 0.57	52.67 ± 1.39 [a]	52.42 ± 0.79 [a]	53.54 ± 1.14 [a]	52.60 ± 0.97 [a]

Data were calculated from food consumption per day and are expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to control.

Table 22 Average minerals daily intake of rat

Composition	*Control (1% Tween-20; n=6)*	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
Ca (mg/day)	162.5 ± 1.88	160.92 ± 4.36	162.37 ± 2.59	166.05 ± 3.75	162.95 ± 3.18
P (mg/day)	146.25 ± 1.7	144.83 ± 3.93	146.13 ± 2.33	149.45 ± 3.37	146.66 ± 2.87
Na (mg/day)	32.5 ± 0.38	32.18 ± 0.87	32.47 ± 0.52	33.21 ± 0.75	32.59 ± 0.64
K (mg/day)	190.13 ± 2.2	188.27 ± 5.1	189.97 ± 3.03	194.28 ± 4.38	190.65 ± 3.73
Mg (mg/day)	37.38 ± 0.43	37.01 ± 1	37.34 ± 0.6	38.19 ± 0.86	37.48 ± 0.73
Mn (mg/day)	2.78 ± 0.03	2.75 ±0.08	2.78 ± 0.04	2.84 ± 0.06	2.79 ± 0.06
Cu (μg/day)	357.5 ± 4.14	354.02 ± 9.59	357.21 ± 5.7	365.31 ± 8.24	358.49 ± 7.01
Zn (mg/day)	1,625 ± 18.84	1,609.17 ± 43.61	1,623.67 ± 25.88	1,660.5 ± 37.46	1,629.5 ± 31.84
Fe (mg/day)	2,925 ± 33.91	2,896.5 ± 78.5	2,922.6 ± 46.59	2,988.9 ± 67.42	2,933.1 ± 57.32
Co (μg/day)	29.58 ± 0.34	29.29 ± 0.79	29.55 ± 0.47	30.22 ± 0.68	29.66 ± 0.58
KI (μg/day)	16.25 ± 0.19	16.09 ± 0.44	16.24 ± 0.26	16.6 ± 0.38	16.3 ± 0.32
Se (μg/day)	1.63 ± 0.02	1.61 ± 0.04	1.62 ± 0.03	1.66 ± 0.04	1.63 ± 0.03

Data were calculated from food consumption per day and were expressed as Mean ± S.E.M.

Table 23 Average vitamins daily intake of rat

Composition	*Control (1% Tween-20; n=6)*	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
A (IU/day)	325 ± 3.77	321.83 ± 8.72	324.73 ± 5.18	332.1 ± 7.49	325.9 ± 6.37
D (IU/day)	65 ± 0.75	64.37 ± 1.74	64.95 ± 1.04	66.42 ± 1.5	65.18 ± 1.27
E (mg/day)	1.63 ± 0.02	1.61 ± 0.04	1.62 ± 0.03	1.66 ± 0.04	1.63 ± 0.03
B1 (μg/day)	81.25 ± 0.94	80.46 ± 2.18	81.18 ± 1.29	83.03 ± 1.87	81.48 ± 1.59
B2 (μg/day)	325 ± 3.77	321.83 ± 8.72	324.73 ± 5.18	332.1 ± 7.49	325.9 ± 6.37
B6 (μg/day)	325 ± 3.77	321.83 ± 8.72	324.73 ± 5.18	332.1 ± 7.49	325.9 ± 6.37
B12 (ng/day)	585 ± 6.78	579.3 ± 15.7	584.52 ± 9.32	597.78 ± 13.48	586.62 ± 11.46
Niacin (mg/day)	1.63 ± 0.02	1.61 ± 0.04	1.62 ± 0.03	1.66 ± 0.04	1.63 ± 0.03
Folic acid (μg/day)	97.5 ± 1.13	96.55 ± 2.62	97.42 ± 1.55	99.63 ± 2.25	97.77 ± 1.91
Biotin (μg/day)	6.5 ± 0.08	6.44 ± 0.17	6.5 ± 0.1	6.64 ± 0.15	6.52 ± 0.13
Pantothenic acid (μg/day)	975 ± 11.3	965.50 ± 26.17	974.20 ± 15.53	996.30 ± 22.47	977.7 ± 19.12
Choline Chloride (mg/day)	24.38 ± 0.28	24.14 ± 0.65	24.36 ± 0.39	24.91 ± 0.56	24.44 ± 0.48

Data were calculated from food consumption per day and were expressed as Mean ± S.E.M.

Table 24 Blood chemical parameters of rats after 8 weeks administration

Parameters	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
Triacylglycerol (mg/dl)	138.5 ± 20.02	117 ± 20.27	106.5 ± 14.5	97.7 ± 7.21	108.3 ± 8.4
Cholesterol (mg/dl)	67 ± 2.89	65.2 ± 3.89	68.5 ± 1.78	59 ± 3.67	67.5 ± 4.93
HDL (mg/dl)	34.68 ± 1.77	31.9 ± 1.84	35.98 ± 1.15	30.5 ± 2.03 [a]	34.48 ± 2.1
Creatinine (mg/dl)	0.8 ± 0.03	0.83 ± 0.02	0.77 ± 0.04	0.88 ± 0.02 [a, b]	0.93 ± 0.03 [a, b]
BUN (mg/dl)	25.78 ± 0.1	24.47 ± 1.13	25.93 ± 1.04	24.82 ± 1.5	25.98 ± 0.99
Uric (mg/dl)	1.38 ± 0.2	1.37 ± 0.14	1.42 ± 0.27	1.23 ± 0.24	1.03 ± 0.18
SGOT (u/l)	88.5 ± 5.7	97 ± 17.6	91.67 ± 11.58	99.83 ± 7.68	98.67 ± 8.69
SGPT (u/l)	35 ± 2.37	33.17 ± 3.68	30.83 ± 1.74	33.33 ± 1.89	29.83 ± 3.23
Alkaline Phosphatase (u/l)	95.5 ± 8.16	85.5 ± 6.87	99.83 ± 12.29	87 ± 3.62	79.5 ± 2.81

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to LE-SB.

[b] $p < 0.05$ compared to control and LE-FM.

Effect of lecithin on lipoprotein subpopulations

Lipoproteins were separated from plasma by sequential ultracentrifugation following a series of sample density adjustments, centrifugation, and infranatant / supernatant separation as previously described.

The contents of triacylglycerol (TG), cholesterol (Chol) and TG/Chol ratio in plasma and lipoprotein fractions of rats are shown in **table 25** and **26**. TG in HDL (HDL-TG) of LE-FM and LE-SB groups was higher than that of control and LE-FS 1:2 group ($p< 0.05$). HDL-Chol in LE-SB and LE-FS 1:1 groups was lower than that of LE-FS 1:2 ($p< 0.05$). TG in plasma, VLDL and LDL tended to decrease in normal rats fed with lecithin when compared with control group. The ratio of TG/Chol in VLDL fractions tended to decrease in all groups of normal rats fed with lecithin when compared with control ($p< 0.05$). TG/Chol ratio in HDL fraction was significantly increased in rat treated with LE-SB when compared with control group. In addition, TG/Chol ratio in HDL fraction of rat treated with LE-FM and LE-SB also increased when compared with rat treated with LE-FS 1:2 ($p< 0.05$) (**figures 9** and **10**).

As shown in **table 27**, particle sizes of lipoprotein were studied and assessed. Electron micrograph of lipoprotein particle was shown in **figure 11**. Sizes of VLDL and LDL in normal rats fed with lecithin were smaller than that of control group ($p< 0.05$). It was interesting to find that average sizes of HDL in LE-FS 1:1 and LE-FS 1:2 were smaller in comparison to control group whereas those in LE-SB group were larger than that of control ($p< 0.05$).

Table 25 Triacylglycerol and cholesterol in lipoprotein fractions of rats fed with lecithin after 8 weeks administration (mg/dl)

Parameters	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
Triacylglycerol					
Plasma	133.26 ± 16.14	111.78 ± 18.42	103.46 ± 11.94	96.1 ± 6.99	100.66 ± 7.36
VLDL	88.62 ± 10.42	70.72 ± 9.22	67.08 ± 12.05	62.35 ± 9.86	72.11 ± 9.32
LDL	7.71 ± 1.49	6.9 ± 0.67	7.31 ± 0.68	6.3 ± 0.23	6.85 ± 0.9
HDL	3.54 ± 0.68	5.49 ± 0.53 [a]	5.75 ± 0.66 [a]	3.91 ± 0.89	3.37 ± 0.43 [b]
Cholesterol					
Plasma	63.04 ± 2.37	62.51 ± 3.94	65.95 ± 1.79	57.65 ± 4.01	64.69 ± 4.76
VLDL	6.12 ± 0.72	5.77 ± 0.32	6.23 ± 1.1	5.02 ± 0.48	5.65 ± 0.81
LDL	4.23 ± 0.66	4.34 ± 0.71	4.77 ± 0.65	3.41 ± 0.92	4.13 ± 1.84
HDL	37.13 ± 2.19	34.13 ± 2.64	32.35 ± 1.08	32.07 ± 2.87	39.86 ± 2.55 [c]

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM and LE-SB.

[c] $p < 0.05$ compared to LE-FS 1:1.

Table 26 Triacylglycerol/cholesterol ratio in lipoprotein fractions of rats fed with lecithin after 8 weeks administration

Parameters	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
Plasma	2.10 ± 0.21	1.83 ± 0.33	1.62 ± 0.21	1.75 ± 0.27	1.58 ± 0.14
VLDL	15.12 ± 1.82	12.33 ± 1.69	10.78 ± 0.71 [a]	12.21 ± 1.18	13.39 ± 1.45
LDL	2.10 ± 0.56	1.75 ± 0.25	1.74 ± 0.36	4.1 ± 1.73	2.9 ± 0.69
HDL	0.10 ± 0.02	0.16 ± 0.01	0.18 ± 0.02 [a]	0.14 ± 0.04	0.08 ± 0.01 [b]

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to control.

[b] $p < 0.05$ compared to LE-FM and LE-SB.

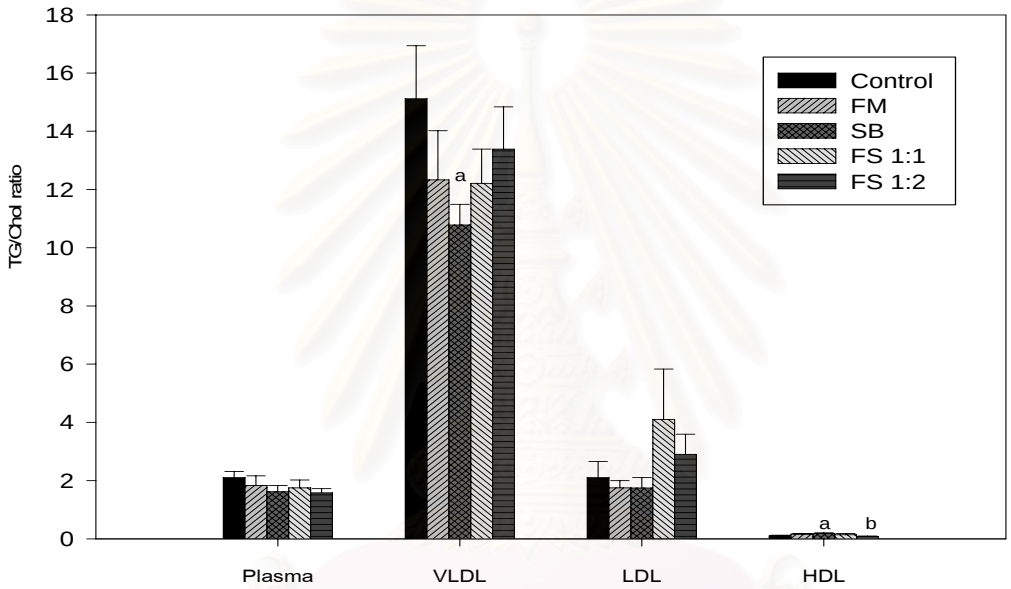


Figure 9 Triacylglycerol/cholesterol ratio in lipoprotein fractions of rats fed with lecithin after 8 weeks administration

[a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM and LE-SB.

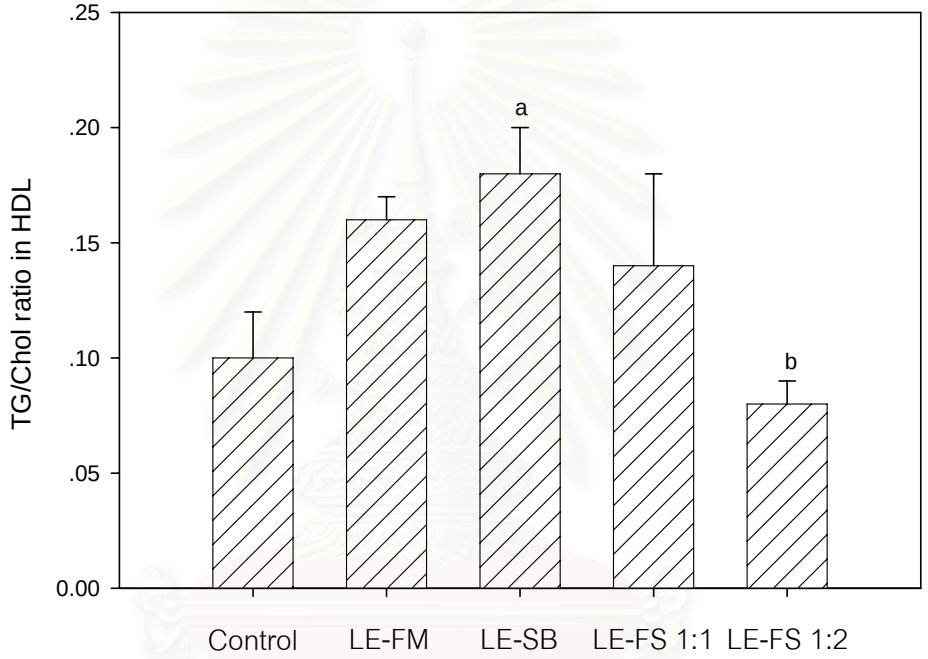


Figure 10 Triacylglycerol/cholesterol ratio in HDL fractions of rats fed with lecithin after 8 weeks administration

[a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM and LE-SB.

a) b) c)

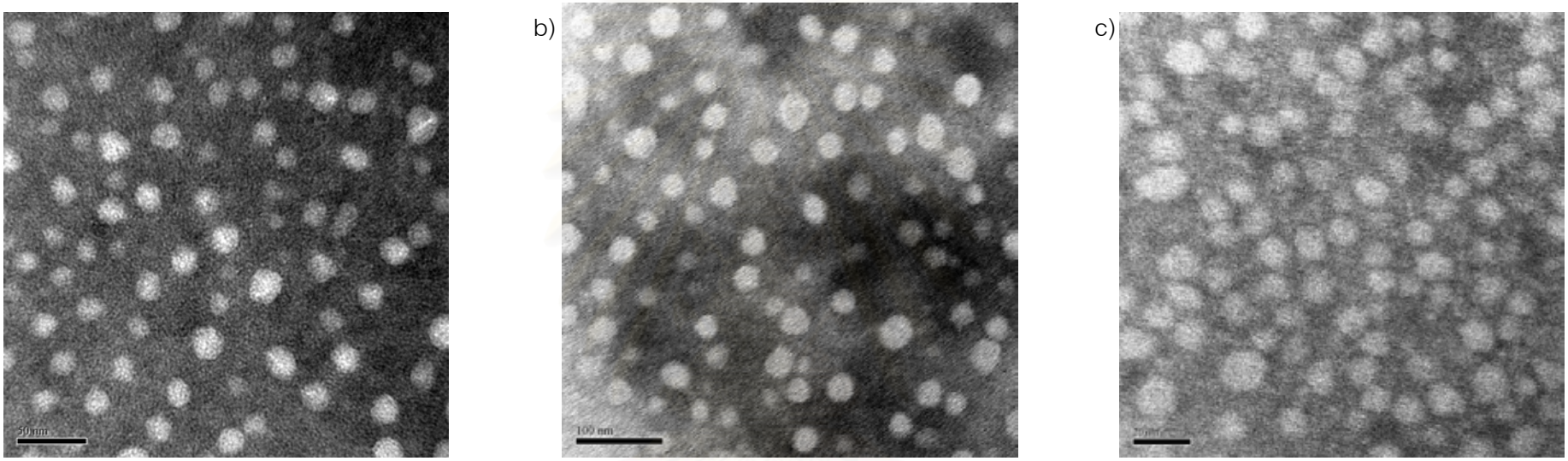


Figure 11 Electron micrograph of lipoprotein particle of rats fed with lecithin after 8 weeks administration

a) very low density lipoprotein b) low density lipoprotein c) high density lipoprotein

Table 27 Lipoprotein particle size in nm of rats fed with lecithin after 8 weeks administration

Parameter	Control (1% Tween-20)	LE-FM	LE-SB	LE-FS 1:1	LE-FS 1:2
VLDL Size	37.50 ± 0.85 (n=433)	26.15 ± 0.42 [a] (n=428)	22.93 ± 0.27 [a, b] (n= 379)	31.37 ± 0.61 [a, b, c] (n=404)	22.09 ± 0.34 [a, b] (n=366)
LDL Size	19.83 ± 0.23 (n=439)	17.74 ± 0.19 [a] (n=261)	17.93 ± 0.23 [a] (n=308)	17.5 ± 0.54 [a] (n=442)	16.60 ± 0.18 [a, b, c, d] (n=437)
HDL Size	12.94 ± 0.25 (n=207)	12.31 ± 0.2 (n=240)	15.63 ± 0.31 [a] (n=151)	11.56 ± 0.15 [a, c] (n=284)	10.48 ± 0.37 [a, d] (n=334)

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM.

[c] $p < 0.05$ compared to LE-SB. [d] $p < 0.05$ compared to LE-FS 1:1.

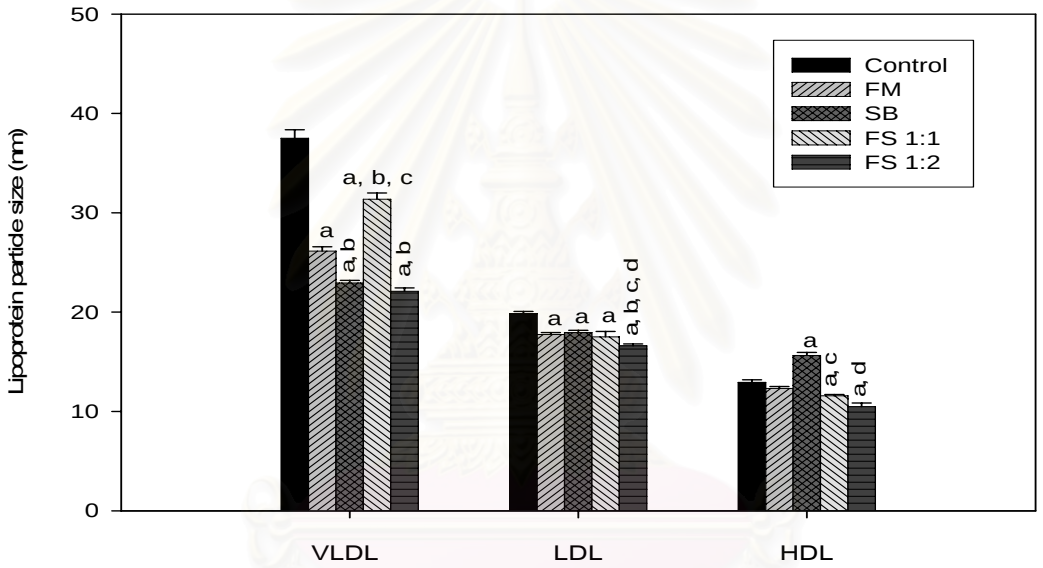


Figure 12 Lipoprotein particle size of rats fed with lecithin after 8 weeks administration (nm)

[a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM.

[c] $p < 0.05$ compared to LE-SB. [d] $p < 0.05$ compared to LE-FS 1:1.

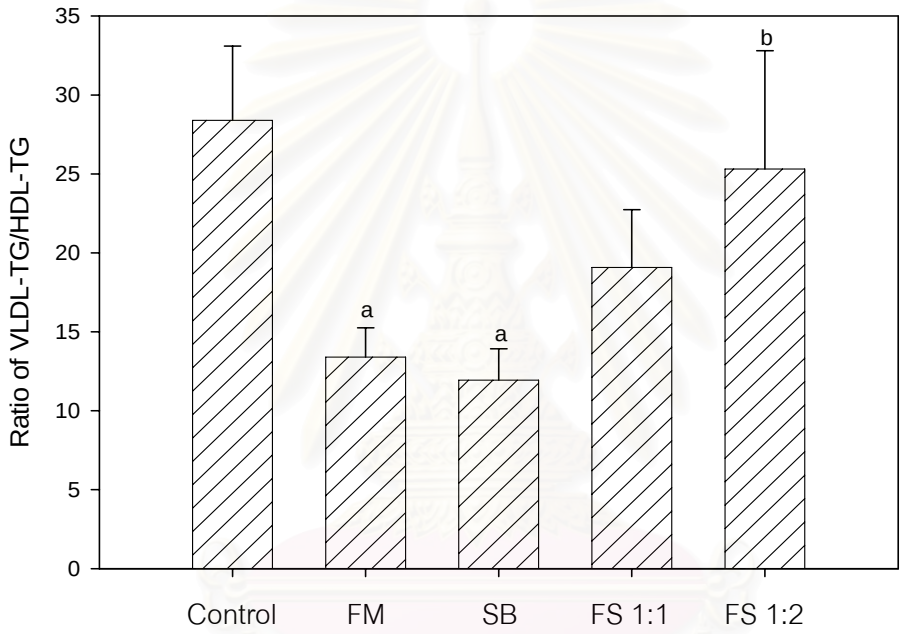


Figure 13 Ratio of VLDL-TG to HDL-TG of rats fed with lecithin after 8 weeks administration

[a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-SB.

Effect of lecithin on plasma fatty acid

Individual plasma fatty acid in normal rat was separated and determined by gas-liquid chromatographic technique as described earlier.

Figure 14 show chromatogram of plasma fatty acid. The contents of plasma fatty acids are shown in **table 28**. Lecithin altered plasma fatty acids content. C18:2n-6 (LA) and C20:5n-3 (EPA) significantly decreased, while C20:4n-6 (AA) markedly increased when compared with control. Moreover, C18:2n-6 of group treated with LE-FS 1:1 and LE-FS 1:2 were significantly different change from control ($p< 0.05$). C18:3n-3 and C20:4n-6 content in LE-SB group was significant difference from control ($p< 0.05$).

Table 30 shows plasma fatty acid profile in form of FAME of normal rats fed with mixed lecithins. LE-SB had proportion of C22:6n-3 lower than LE-FM, LE-FS 1:1 and LE-FS 1:2 ($p< 0.05$). In group fed with LE-FS 1:1 and LE-FS 1:2, C20:4n-6 was higher than that of control group ($p< 0.05$). C18:3n-3 of group treated with LE-FM was lower than that of control and LE-FS 1:1 group ($p< 0.05$). In group fed with LE-SB, C18:2n-6 was lower than that of control group ($p< 0.05$). Total n-3 PUFA of group treated with LE-SB was lower than that of LE-FS 1:1 and LE-FS 1:2 group ($p< 0.05$).

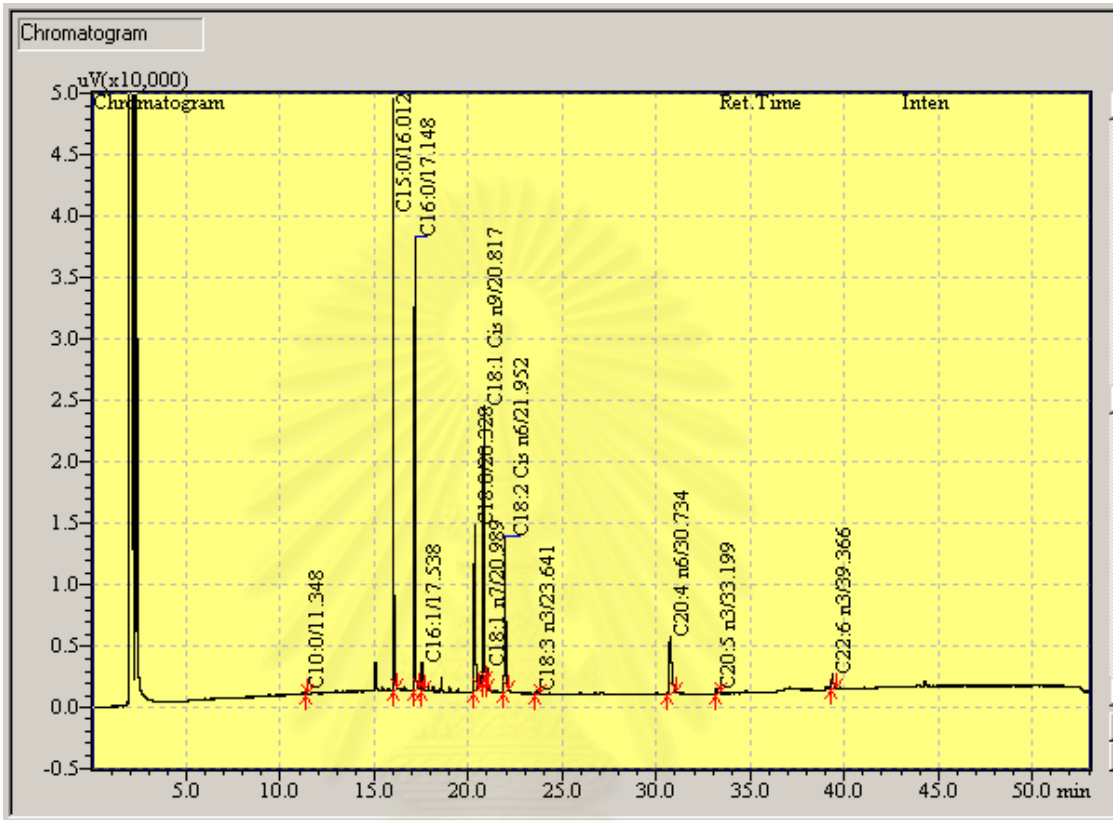


Figure 14 Plasma fatty acid chromatogram of rats fed with lecithin after 8 weeks administration

Table 28 Plasma fatty acid concentration in form of fatty acid methyl esters (FAMEs) of rats fed with lecithin after 8 weeks administration (mg/l)

Parameter	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
C10:0	37.78 ± 8.99	10.93 ± 1.36 [a]	9.54 ± 0.82 [a]	22.88 ± 2.44 [a]	16.88 ± 2.83 [a]
C16:0	494.11 ± 43.93	492.81 ± 71.97	594.45 ± 58.94	408.82 ± 25.07 [c]	433.82 ± 29.04 [c]
C16:1 n-7	47.81 ± 11.97	38.46 ± 8.02	39.39 ± 7.94	27.05 ± 2.68	28.49 ± 6.06
C18:0	163.77 ± 11.5	223.44 ± 56.58	219.63 ± 22.59	168.40 ± 11.21	182.02 ± 15.36
C18:1 n-9	308.50 ± 44.37	293.46 ± 73.87	571.24 ± 293.79	212.61 ± 22.02	207.52 ± 20.24
C18:1 n-7	74.92 ± 10.43	41.11 ± 6.15 [a]	41.55 ± 8.23 [a]	42.09 ± 3.35 [a]	46.3 ± 4.63 [a]
C18:2 n-6	751.74 ± 76.39	588.93 ± 48.51	689.62 ± 75.82	540.11 ± 52.48 [a]	550.69 ± 41.05 [a]
C18:3 n-3	28.98 ± 4.57	19.07 ± 2.51	29.52 ± 4.35 [b]	23.76 ± 3.76	19.29 ± 1.74
C20:4 n-6	388.37 ± 32.12	405.35 ± 40.26	478.73 ± 19.18 [a]	421.61 ± 26.64	474.73 ± 32.25
C20:5 n-3	36.31 ± 4.06	32.61 ± 4.83	34.83 ± 5.70	26.67 ± 3.02	30.28 ± 3.58
C22:6 n-3	121.3 ± 13.21	125.14 ± 14.39	113.39 ± 11.36	114.74 ± 10.11	135.18 ± 18.12
Total	2,266 ± 218	2,819 ± 421	2,454 ± 232	2,009 ± 131 [c]	2,125 ± 129

Data were expressed as Mean ± S.E.M. [a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM. [c] $p < 0.05$ compared to LE-SB.

Table 29 Saturated, monoenic and polyenic fatty acid in form of fatty acid methyl esters (FAMEs) of rats fed with lecithin after 8 weeks administration (mg/l)

Parameter	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
SFA	695.65 ± 57.94	721.71 ± 125.17	825.56 ± 78.01	600.1 ± 32.33[a]	632.72 ± 36.45
MUFA	431.23 ± 63.05	373.02 ± 80.71	669.04 ± 296.98	297.08 ± 18.87	302.26 ± 25.71
PUFA	1,326.7 ± 122.59	1,171.1 ± 73.57	1,346.09 ± 102.22	1,126.88 ± 73.1	1,210.18 ± 73.72
n-6 PUFA	1,140.12 ± 106.16	994.28 ± 55.61	1,168.36 ± 83.03	961.71 ± 60.63	1,025.42 ± 65.74
n-3 PUFA	186.59 ± 19.58	176.82 ± 20.38	177.73 ± 19.82	165.16 ± 14.67	184.76 ± 20.97
n-6/n-3 ratio	6.24 ± 0.42	5.85 ± 0.43	6.76 ± 0.37	5.96 ± 0.38	5.84 ± 0.68
n-3/n-6 ratio	0.17 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.18 ± 0.02

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to LE-SB.

See abbreviations in Table 3.

Table 30 Plasma fatty acid profile in form of fatty acid methyl esters (FAMEs) of rats fed with lecithin after 8 weeks administration (g/100g fatty acid)

Parameter	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
C10:0	1.22 ± 0.24	0.41 ± 0.13 [a]	0.29 ± 0.06 [a]	0.91 ± 0.1 [c]	0.64 ± 0.08 [a]
C16:0	22.08 ± 0.76	23.22 ± 1.01	23.38 ± 1.68	22.18 ± 0.65	22.56 ± 0.76
C16:1 n-7	1.93 ± 0.37	1.65 ± 0.25	1.41 ± 0.26	1.36 ± 0.12	1.36 ± 0.24
C18:0	7.5 ± 0.39	10.4 ± 1.43 [a]	8.88 ± 0.85	9.32 ± 0.49	9.64 ± 0.6
C18:1 n-9	13.92 ± 0.65	13.72 ± 1.81	18.2 ± 5.6	11.81 ± 0.6	11.09 ± 0.5
C18:1 n-7	3.01 ± 0.18	1.87 ± 0.31 [a]	1.46 ± 0.25 [a]	2.07 ± 0.07 [a, c]	2.18 ± 0.12 [a, c]
C18:2 n-6	30.88 ± 0.91	27.08 ± 2.47	25.11 ± 2.17 [a]	26.87 ± 1.39	26.4 ± 0.53
C18:3 n-3	1.05 ± 0.07	0.77 ± 0.08 [a]	0.95 ± 0.12	1.04 ± 0.11	0.83 ± 0.04
C20:4 n-6	14.04 ± 0.7	16.22 ± 2.01	15.92 ± 1.93	18.59 ± 1.26 [a]	19.99 ± 1.1 [a]
C20:5 n-3	1.2 ± 0.12	1.16 ± 0.14	0.99 ± 0.14	1.05 ± 0.09	1.16 ± 0.12
C22:6 n-3	3.17 ± 0.17	3.71 ± 0.46	2.35 ± 0.47 [b]	3.68 ± 0.27 [c]	4.16 ± 0.46 [c]

Data were expressed as Mean ± S.E.M. [a] $p < 0.05$ compared to control. [b] $p < 0.05$ compared to LE-FM. [c] $p < 0.05$ compared to LE-SB.

Table 31 Saturated, monoenic and polyenic fatty acid in form of fatty acid methyl esters (FAMEs) of rats fed with lecithin after 8 weeks administration (g/100g fatty acid)

Parameter	Control (1% Tween-20; n=6)	LE-FM (n=6)	LE-SB (n=6)	LE-FS 1:1 (n=6)	LE-FS 1:2 (n=6)
SFA	30.8 ± 0.92	33.83 ± 2.21	32.62 ± 2.34	32.4 ± 0.5	32.84 ± 0.66
MUFA	18.86 ± 0.99	17.24 ± 1.82	21.74 ± 5.35	16.37 ± 0.6	14.63 ± 0.83
PUFA	50.34 ± 1.3	48.94 ± 3.87	45.31 ± 3.69	51.23 ± 0.51	52.53 ± 1.14
n-6 PUFA	44.92 ± 1.42	43.29 ± 3.33	41.03 ± 3.33	45.46 ± 0.29	46.39 ± 1.23
n-3 PUFA	5.42 ± 0.24	5.64 ± 0.64	4.28 ± 0.55	5.77 ± 0.33[a]	6.14 ± 0.52[a]
n-6/n-3 ratio	8.41 ± 0.57	7.96 ± 0.56	10.15 ± 1.24	8.02 ± 0.49	7.9 ± 0.87
n-3/n-6 ratio	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.01

Data were expressed as Mean ± S.E.M.

[a] $p < 0.05$ compared to LE-SB.

See abbreviations in Table 3.

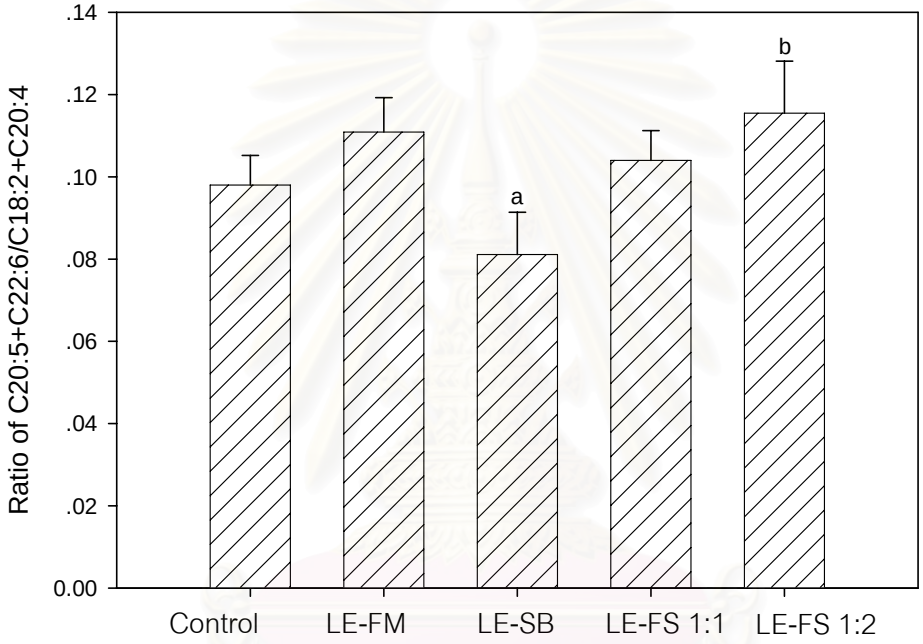


Figure 15 The ratio of C20:5+C22:6/C18:2+C20:4 of rats fed with lecithin after 8 weeks administration

[a] $p < 0.05$ compared to LE-FM. [b] $p < 0.05$ compared to LE-SB.

CHAPTER V

DISCUSSION

Long chain n-3 PUFAs (polyunsaturated fatty acids) are found in fatty fish and fish oils. Substantial evidence from epidemiological and case-control studies indicates that consumption of fish, fatty fish and long-chain n-3 PUFAs reduces the risk of cardiovascular mortality. Secondary prevention studies using long-chain n-3 PUFAs in patients' post-myocardial infarction have shown a reduction in total and cardiovascular mortality, with an especially potent effect on sudden death. Long-chain n-3 PUFAs have been shown to decrease blood triacylglycerol concentrations, decrease production of chemoattractants, growth factors, adhesion molecules, inflammatory eicosanoids and inflammatory cytokines. It also shows to decrease blood pressure, increase nitric oxide production, endothelial relaxation and vascular compliance, decrease thrombosis and cardiac arrhythmias and increase heart rate variability. These mechanisms most likely explain the primary and secondary cardiovascular protection afforded by long-chain n-3 PUFA consumption. A recent study suggested that long-chain n-3 PUFAs might also act to stabilize advanced atherosclerotic plaques, perhaps through their anti-inflammatory effects. As a result of the robust evidence in their favour, a number of recommendations to increase intake of long-chain n-3 PUFAs have been made (Calder, 2004).

Fishmeal lecithin had high content of n-3 PUFAs especially C20:5n-3 or eicosapentaenoic acid (EPA) and C22:6n-3 or docosahexaenoic acid (DHA). The high contents of C20:5n-3 and C22:6n-3 were found in all fractions of lipid in fishmeal: triacylglycerol, free fatty acids and phospholipids fractions. However, there was not much C20:5n-3 in triacylglycerol fractions. n-3 PUFAs in soybean lecithin was alpha-linolenic acid (ALA) whereas it had no C20:5n-3 and C22:6n-3 in soybean lecithin. Most PUFAs in soybean lecithin were C18:2n-6 or linoleic acid (LA) which was the one of the n-6 PUFA family. High contents of C20:5n-3 and C22:6n-3 in fishmeal lecithin was also found in phospholipids subspecies especially phosphatidylcholine (PC) and phosphatidylinositol (PI). There was high content of C18:2n-6 in PC, PI and PE

(phosphatidylethanolamine). Focusing on phospholipids classes, most abundant of phospholipids in fishmeal lecithin was PC and SM (sphingomyelin) and soybean lecithin was PI and PC. There was no SM content in soybean lecithin but it was found that soybean lecithin had high PI content. This is probably explained by alteration of phospholipid subclasses during storage after extraction from their sources as reported by Chu and Lin (1993).

Dahlan and colleagues have demonstrated in several *in vitro* studies that PUFA profile on blood cell membranes was able to be reconstructed as need. Fishmeal lecithin supplied n-3 PUFA whereas soybean lecithin provided n-6 PUFA to the blood cell membranes (Chatnilbandhu, 1996, Charnprasert, 1997). From previous experiments, lecithins of both fishmeal and soybean were prepared as lecithin liposomes and used them for incubation with blood cells of both erythrocytes and platelets. Modification of blood cells in accumulation of n-3 polyunsaturated fatty acids (n-3 PUFA) replacing n-6 PUFA was found after incubation of blood cells with n-3 PUFA rich lecithin, to inducing anti-atherogenicity and anti-thrombogenicity to those blood cells. The raised question was whether such phenomenon could result in animal model by oral administration of lecithin or not. The present experiment was thus aimed to answer that raised question by studying the effect of lecithin in normal rat.

In group treated with lecithin, there was no significant difference found in food and water intake when comparing to control group of rats. This evidence showed that lecithin had no direct effect to the appetite. After supplementation, no change in plasma lipids was observed. However, Triacylglycerol (TG) contents in plasma, VLDL and LDL tended to decrease. In addition, VLDL-TG/HDL-TG ratio was significant decreased in rats fed with LE-FM and LE-SB when compared with control group.

In the present study, normal rat treated with LE-FM had markedly increased plasma n-3 PUFA exclusively C22:6n-3 compared to control and LE-SB group. It was implying that rat plasma lipoproteins of LE-FM group were ready to carrying n-3 PUFA for exchange with blood cells. The results indicated that increasing

n-3 PUFA could stimulated the plasma lipoprotein activity on exchange n-3 PUFA of blood cell membrane. n-3 PUFA rich lecithin affected to modification of fatty acid component on blood cell. This phenomenon reduced the risk factors of cardiovascular disease.

Electron micrographs of VLDL, LDL and HDL were also studied. Diameters of these particles decreased when compared to control. These effects occurred in group treated with lecithins. In contrast, HDL particle size in LE-SB group is significantly increased ($p< 0.05$). Hedrick et al (2001) showed that the increased size of HDL results in part from the inhibition of the ability of hepatic lipase (HL) to hydrolyze phospholipids and triglycerides in HDL. Vasandani et al (2002) investigated the effects of dietary n-6 and n-3 PUFAs on parameters of plasma lipoprotein and hepatic lipid metabolism in LDL receptor (LDLr) knockout mice. Dietary n-3 PUFA decreased the rate of appearance and increased the hepatic clearance of IDL/LDL resulting in a marked decrease in the plasma concentration of these particles. Moreover, dietary n-3 PUFA increased the hepatic clearance of IDL/LDL through a mechanism that appears to involve apolipoprotein (apo) E but it is independent of the LDLr, the LDLr related protein (LRP), the scavenger receptor B1, and the VLDLr. The decreased rate of appearance of IDL/VLDL in the plasma of animals fed n-3 PUFA could be attributed to a marked decrease in the plasma concentration of precursor VLDL. Decreased plasma VLDL concentrations were due in part to decreased hepatic secretion of VLDL triglyceride and cholesteryl esters, which in turn was associated with decreased concentrations of these lipids in liver. Decreased hepatic TG concentrations in animals fed n-3 PUFA were due in part to suppression of fatty acid synthesis as a result of a decrease in sterol regulatory element binding protein-1 (SREBP-1) expression and processing. These studies indicate that n-3 PUFA can markedly decrease the plasma concentration of apoB-containing lipoproteins and enhance hepatic LDL clearance through a mechanism that does not involve the LDLr pathway or LRP (Vasandani et al, 2002).

The relationship between increased plasma cholesterol levels and atherogenesis has been established in a large number of studies (Martin et al, 1986,

Castelli et al, 1996), but it is now generally recognized that hypertriacylglycerolemia is also a risk factor for the disease (Brewer, 1999, Miller, 2000). Dietary intake of n-6 polyunsaturated fatty acids (PUFA), found in vegetable oils, and n-3 PUFA, found in oily fish, is known to decrease blood cholesterol and triacylglycerol levels, and consequently to reduce the risk atherosclerosis development (Shepherd et al, 1980, Thornberg and Rudel, 1992). However, dietary n-6 PUFA exert their effects mainly by lowering plasma low-density lipoprotein (LDL) cholesterol levels and cause only a modest reduction in blood TG, whereas consumption of n-3 PUFA leads to a substantial decrease in plasma TG concentrations, but has little effect on blood cholesterol in most circumstances (Von Lossonczy et al, 1978, Philipson et al, 1985).

Dietary n-3 PUFA lower plasma TG by decreasing plasma levels of VLDL (Von Lossonczy et al, 1978, Philipson et al, 1985) and this is believed to be caused by suppression of VLDL secretion by the liver. Feeding studies have shown that hepatic secretion of TG, cholesterol, and apolipoprotein B (apoB) in VLDL secretion is reduced by n-3 PUFA (Harris et al, 1990, Ribiero et al, 1991, Brown et al, 1999). The assembly of VLDL particles in the liver is believed to occur in two steps; first, lipid is transferred by the microsomal triacylglycerol transfer protein (MTP) to apoB during translation, and second, the apoB-containing precursor particles fuse with TG droplets to form mature VLDL (Shelness and Sellers, 2001). In addition, hepatic levels of component lipids such as cholesteryl ester and TG also play a role in modulating VLDL formation (Maosn, 1998). Cholesteryl ester synthesis is regulated by acyl coenzyme A:cholesterol acyltransferase (ACAT), an enzyme that exists in two forms, ACAT1, which is widely expressed in tissues, and ACAT2, which is found mainly in the liver and intestine. However, current evidence suggests that ACAT2 is responsible for supplying cholesteryl ester for VLDL (Shelness and Sellers, 2001, Joyce et al, 1999, Buhman et al, 2000, Lee et al, 2000). The only committed step in TG synthesis is the last in the pathway, which is catalyzed by acyl coenzyme A:diacylglycerol acyltransferase (DGAT) (Cases et al, 1998).

Ramsamy et al (2003) showed that HDL is able to displace hepatic lipase (HL) directly from the surface of the hepatoma cell line, HepG2, and Chinese hamster ovary cells stably overexpressing human HL. ApoA-I is more efficient at displacing cell surface HL than HDL, and different HDL classes vary in their ability to displace HL from the cell surface. HDL[2]s have a greater capacity to remove HL from the cell surface and intracellular compartments, as compared with the smaller HDL particles. The different HDL subclasses also uniquely affect the activity of the enzyme. HDL[2] stimulates HL-mediated hydrolysis of VLDL-TG, while HDL[3] is inhibitory. Inhibition of VLDL hydrolysis appears to result from a decreased interlipoprotein shuttling of HL between VLDL and the smaller, denser HDL particles. Their study suggested that high HDL[2] levels are positively related to efficient TG hydrolysis by their ability to enhance the liberation of HL into the plasma compartment and by a direct stimulation of VLDL-TG hydrolysis.

In the present study, it was found that TG contents in VLDL tended to decrease. In addition, VLDL-TG to HDL-TG ratio in normal rat treated with LE-FM and LE-SB were significantly decreased when compared to control group. These results were reflected to the increment of circulation and metabolism of TG-rich lipoproteins. Moreover, these effects also resulted in reduction of VLDL particle sizes whereas HDL particle size in rat treated with LE-SB was increased. Such differences were possibly influenced by dissimilarity of phospholipids subspecies of both lecithins: SM and PI. It was found that high content of SM found on HDL surface might retard transformation of nascent HDL to mature HDL by inhibiting activity of lecithin-cholesterol acyltransferase (LCAT). Consequently, regeneration of cholesteryl ester from free cholesterol on HDL surface possibly was original in nascent HDL released from shrunken surface of hydrolyzed VLDL or extracting from pheripheral tissues. This indicated the slow production of mature HDL. Small particle sizes of HDL in normal rat treated with LE-FM was possibly from the effect of high SM present in LE-FM. It was imply by corresponding proportion of SM on generated HDL.

LCAT catalyzes the formation of cholesterol esters on high density lipoproteins (HDL) and plays a critical role in reverse cholesterol transport. Sphingomyelin (SM), an important constituent of HDL, may regulate the activity of LCAT at any of the key steps of the enzymatic reaction: binding of LCAT to the interface, activation by apo A-I, or inhibition at the catalytic site. In order to clarify the role of SM in the regulation of the LCAT reaction and its effects on the structure of apolipoprotein A-I, Bolin and Jonas (1996) prepared reconstituted HDL (rHDL) containing egg phosphatidylcholine, cholesterol, apolipoprotein A-I, and up to 22 mol % SM. Because the interfacial properties of substrate particles can dramatically affect LCAT binding and kinetics, they also prepared and analyzed proteoliposome substrates having the same components as the rHDL, except for a 4-fold higher ratio of phospholipid to apolipoprotein A-I. The reaction kinetics of LCAT with the rHDL particles revealed no significant change in the apparent V_{max} but showed a concentration-dependent increase in slope of the reciprocal plots and in the apparent K_m values with SM content. The dissociation constant (K_d) for LCAT with these particles increased linearly with SM content up to 22 mol %, changing in parallel with the apparent K_m values. No structural changes of apolipoprotein A-I were detected in the particles with increasing content of SM, but fluorescence results with lipophilic probes revealed that significant changes in the acyl chain, backbone, and head group regions of the lipid bilayer of the particles are introduced by the addition of SM. On the other hand, the proteoliposome substrates also had increased K_d values for LCAT at high SM contents but compared with the rHDL particles had a 6-10-fold lower affinity for LCAT binding and exhibited kinetics consistent with competitive inhibition by SM at the active site. These results show conclusively that the dominant mechanism for the inhibition of LCAT activity with rHDL particles by SM is the impaired binding of the enzyme to the interface. The results also underscore the significant differences in the enzyme reaction kinetics with different substrate particles (Bolin and Jonas, 1996).

Dietary SM is hydrolyzed by intestinal alkaline sphingomyelinase and neutral ceramidase to sphingosine, which is absorbed and converted to palmitic acid

and acylated into chylomicron triglycerides (TGs). SM digestion is slow and is affected by luminal factors such as bile salt, cholesterol, and other lipids. In the gut, SM and its metabolites may influence TG hydrolysis, cholesterol absorption, lipoprotein formation, and mucosal growth. SM accounts for 20% of the phospholipids in human plasma lipoproteins, of which two-thirds are in LDL and VLDL. It is secreted in chylomicrons and VLDL and transferred into HDL via the ABCA1 transporter. Plasma SM increases after periods of large lipid loads, during suckling, and in type II hypercholesterolemia, cholesterol-fed animals, and apolipoprotein E-deficient mice. SM is thus an important amphiphilic component when plasma lipoprotein pools expand in response to large lipid loads or metabolic abnormalities. It inhibits lipoprotein lipase and LCAT as well as the interaction of lipoproteins with receptors and counteracts LDL oxidation. The turnover of plasma SM is greater than can be accounted for by the turnover of LDL and HDL particles. Some SM must be degraded via receptor-mediated catabolism of chylomicron and VLDL remnants and by scavenger receptor class B type I receptor-mediated transfer into cells (Nilsson and Duan, 2006).

Regarding the role of phosphatidylinositol (PI) on HDL metabolism, LE-SB contained high proportion of PI. This possibly leaded to increase reverse cholesterol transport and clearance of HDL from plasma. In this experiment, HDL-TG in rat treated with LE-FM and LE-SB were significantly increased when compared to control group whereas HDL-Chol tended to decrease. These results leading to markedly decreased of HDL-TG/HDL-Chol ratio. It reflects high HDL hydrolysis by lipase before rapidly removing out of the plasma. Burgess et al (2003) reported that administration of PI to New Zealand white rabbit increases HDL negative charge and stimulates reverse cholesterol transport. Intravenous administration of PI (10 mg/kg) associated almost exclusively with the HDL fraction in rabbits. PI promoted an increase in the hepatic uptake of plasma free cholesterol (FC) and a 21-fold increase in the biliary secretion of plasma-derived cholesterol. PI also increased cholesterol excretion into the feces by 2.5-fold. PI directly affects cellular cholesterol metabolism. In cholesterol-loaded macrophages, PI stimulated cholesterol mass efflux to lipid-poor reconstituted HDL. PI

was about half as effective as cAMP at stimulating efflux, and the effects of cAMP and PI were additive. In cultured HepG2 cells, PI-enriched HDL also enhanced FC uptake from HDL by 3-fold and decreased cellular cholesterol synthesis and esterification. PI enrichment had no effect on the selective uptake of cholesterol esters or on the internalization of HDL particles. PI-dependent metabolic events were efficiently blocked by inhibitors of protein kinase C and the inositol signaling cascade. The data suggest that HDL-PI acts via cell surface ATP binding cassette transporters and signaling pathways to regulate both cellular and intravascular cholesterol homeostasis.

Studies have shown that PI can stimulate reverse cholesterol transport by enhancing the flux of cholesterol into HDL and by promoting the transport of high density lipoprotein-cholesterol (HDL-C) to the liver and bile. Burgess et al (2005) determined the safety and therapeutic value of PI after oral administration to normolipidemic human subjects. They performed a randomized 2 week study in 16 normolipidemic subjects. Subjects received either 2.8 or 5.6 g of PI, with or without food. PI was well tolerated by all subjects. PI significantly affected the levels of HDL-C and triglyceride in the plasma of subjects receiving PI with food. The lower dose showed a 13% increase in HDL-C, whereas the high dose showed an increase of 18% over the 2 week period. Both low- and high-dose groups showed significant increases in plasma apolipoprotein A-I. The high dose of PI also decreased plasma triglycerides by 36% in the fed subjects. These data suggest that after only 2 weeks, PI may have a comparable therapeutic value to niacin, with negligible side effects.

In case of PUFAs transported by lipoproteins for exchanges with blood cells, it was found that ratio of serie-3 to serie-2 eicosanoid precursor fatty acids: C20:5n-3 + C22:6n-3 /C18:2n-6 + C20:2n-6 increased in plasma of rats fed with LE-FM compared to LE-SB reflecting anti-thrombogenicity of LE-FM.

The 20- and 22-carbon *n*-3 PUFAs are unique lipids that when added to the diet or to cells can alter membrane phospholipid composition, impact eicosanoid synthesis and action, and regulate transcription factor activity and abundance. N-3

PUFAs affect diverse physiological processes including cognitive functions and visual acuity, immunosuppressive and anti-inflammatory actions, and anti-thrombotic and anti-arrhythmia activities along with having major effects on whole body glucose and lipid metabolism. It is important to distinguish those effects that are specific for n-3 PUFA from those effects that are seen with unsaturated fatty acids in general. Whereas n-6 PUFAs stimulate, n-3 PUFAs inhibit eicosanoid synthesis and signaling and NFκB activation. This feature accounts for the anti-inflammatory and anti-thrombotic action of n-3 PUFA. There is also a strict requirement for C22:6n-3 over C22:5n-6 for normal CNS development and function. In contrast, PUFA effects on membrane raft composition as well as the regulation of transcription factors like PPARs, LXRs, or SREBP-1c are determined more by changes in cellular levels of unsaturated fatty acids rather than specific effects of n-3 PUFA. The modest resistance of n-3 PUFA to β-oxidation or assimilation into neutral lipids might be sufficient to elevate intracellular n-3 NEFA or PUFA-CoA levels allowing these factors to serve as regulatory ligands for transcription factors or substrates for protein acylation. Unfortunately, no direct evidence has been reported to support this concept (Jump, 2002).

Some effects of n-3 PUFA on physiological processes remain poorly defined. The rapid attenuation of arrhythmias in cardiomyocytes treated with n-3 PUFA involves changes in the activity of several membrane channels (Leaf, 2001, Pound et al, 2001). Whether this effect involves changes in membrane phospholipid composition or targeting of membrane channel proteins to specific microdomains is unknown. Nevertheless, considerable progress has been made in understanding how n-3 PUFAs affect cell function. Many mechanisms have been described, and new mechanisms are likely to be discovered that will better define how these unique lipids impact human health and disease.

CHAPTER VI

SUMMARY AND CONCLUSION

The present study are summarized herewith

1. Phospholipid content of LE-FM, LE-SB, LE-FS 1:1 and LE-FS 1:2 assessed in form of phosphorus containing lipids were 18, 50, 36 and 39 g/100g lecithin, respectively.

2. LE-FM had high n-3 PUFA and low n-6 PUFA content whereas LE-SB had low n-3 PUFA and high n-6 PUFA content.

3. The most abundant n-3 PUFA content in LE-FM was C22:6n-3. In contrast, the most abundant n-3 PUFA in LE-SB was C18:3n-3. In addition, all lecithins contained n-6 PUFA which was C18:2n-6.

4. For phospholipids subclasses, LE-FM contained high PC and SM, whereas LE-SB contained high PC and PI. There was no SM content in LE-SB.

5. Lecithin had effect on body weight whereas it had no effect on the appetite.

6. Plasma TG of normal rats fed with lecithin tended to decrease. These findings were found not only in rat plasma but also in VLDL and LDL fractions. HDL-TGs in normal rats fed with LE-FM, LE-SB and LE-FS 1:1 increased. Lecithin had no effect on cholesterol (Chol) contents. The ratio of plasma, VLDL and LDL TG/Chol of rats fed with lecithin tended to decrease except for LDL of group treated with LE-FS 1:1 and LE-FS 1:2. For HDL fractions, TG/Chol ratio in rat treated with LE-FM, LE-SB and LE-FS 1:1 was increased whereas LE-FS 1:2 group was decreased.

7. VLDL and LDL particle diameters of rats fed with lecithin was reduced. HDL particle was also decreased except that of HDL in LE-SB group was increased. These data including reduction of TG/Chol and VLDL-TG/HDL-TG ratios reflect to more rapid clearance of VLDL of rats fed with lecithin. Noticeably, ratio of TG/Chol of HDL increased significantly corresponding to marked increment of HDL particle size in

rat treated with LE-SB. This result implied the benefit of high proportion of PI phospholipids in LE-SB which it was reported to stimulate reverse cholesterol transport leading to expansion of HDL size whereas LE-FM had disadvantage of high content of SM phospholipids which possibly retard activity of LCAT enzyme as previously reported by other investigators leading to insignificant change of HDL particle.

8. Focusing on plasma fatty acids, the content of C18:2n-6 and C20:5n-3 was decreased in all groups of rat treated with lecithin whereas C20:4n-6 was increased. C18:3n-3 content in rat treated with LE-FM, LE-FS 1:1 and LE-FS 1:2 were decreased whereas there was increased in rat treated with LE-SB. The content of C22:6n-3 in rat treated with LE-FM and LE-FS 1:2 was increased whereas there was decreased in rat treated with LE-SB and LE-FS 1:1.

In conclusion, supplementation of lecithin provides the beneficial effects to metabolism of plasma TG-rich lipoproteins. Thus, quality improvement of phospholipid subspecies and PUFAs by using mixed lecithins still requires further investigation especially in cellular and tissue levels.

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APPENDICES

APPENDIX A

Ethic approval for animal experiment

No.360/2005

Study Protocol and Consent Form Approval

The Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol and informed consent dated and/or amended as follows in compliance with the ICH/GCP.

Study Title	: Study on the Alterations of Phospholipids Subspecies and Polyunsaturated Fatty Acids in Experimental Aninals' Tissues Consuming Diet Mixed with Lecithins Derived from Fishery and Agricultural Products
Study Code	: -
Centre	: Chulalongkorn University
Principle Investigator	: Miss Sathaporn Ngamukote
Protocol Date	: May 16, 2005
Document Reviewed	:

: .....
(Professor Anek Aribarg, M.D.)
Chairman of Ethics Committee

: .....
(Associate Professor. Vilai Chentanez, M.D.)
Associate Dean for Research Affairs

Date of Approval	: September 23, 2005
Approval Expire	: September 23, 2008

* A list of the Ethics Committee members (names and positions) present at the Ethics Committee meeting on the date of approval of this study has been attached. This Study Protocol Approval Form will be forwarded to the Principal Investigator.

APPENDIX B

Sample preparation for phospholipids analysis

LECITHIN

Application field: Food / Feed — Digestion Application Note **DG-FO-25**

SUMMARY

This method provides for the acid digestion of the Lecithin sample in a closed vessel device using temperature control microwave heating for the metal determination by spectroscopic methods.

MICROWAVE EQUIPMENT

Milestone ETHOS labstation with easyWAVE or easyCONTROL software HPR1000/10S high pressure segmented rotor.

SAMPLE AMOUNT

0.4 g

REAGENTS

7 ml of HNO_3 65%, 1 ml of H_2O_2 30%

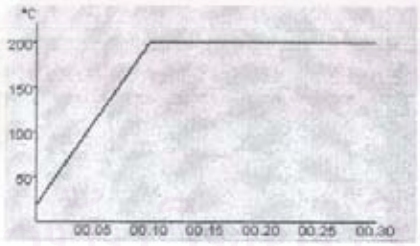
PROCEDURE

1. Place a TFM vessel on the balance plate, tare it and weigh of the sample.
2. Introduce the TFM vessel into the HTC safety shield.
3. Add the acids; if part of the sample stays on the inner wall of the TFM vessel, wet it by adding acids drop by drop, then gently swirl the solution to homogenize the sample with the acids.
4. Close the vessel and introduce it into the rotor segment, then tighten by using the torque wrench.
5. Insert the segment into the microwave cavity and connect the temperature sensor
6. Run the microwave program to completion.
7. Cool the rotor by air or by water until the solution reaches room temperature.
8. Open the vessel and transfer the solution to a marked flask.

MICROWAVE PROGRAM

Step	Time	Temperature	Microwave power
1	10 minutes	200°C	Up to 1000 Watt*
2	20 minutes	200°C	Up to 1000 Watt*

TEMPERATURE PROFILE



NOTES

*Use up to 500 Watt for operations with 3 or less vessels simultaneously.
This procedure is only a guideline and it may need to be modified or changed to obtain the required results on your sample.
Always use hand, eye and body protection when operating with the microwave system.

APPENDIX C

Density solution preparation

KBr is hygroscopic salt, then, KBr used in the preparation of the density solutions should be thoroughly dried overnight in an oven at 115°C. To avoid repeating this procedure every time new solutions are made, the dried KBr solution should be maintained in a desiccator. The several KBr solutions were use (Ordovas, 1998).

Density (g/ml)	KBr (g)
1.006	10.1
1.019	30.6
1.063	90.3
1.21	316.6

The KBr salt was dissolved in 950 ml of double-distilled (dd)H_2O. Add 1 ml of 10% Na_2 EDTA and 10% azide, adjust the pH to 7.0 and finish the volume adjustment to 1liter.

APPENDIX D

Total cholesterol determination

Principle: The cholesteryl esters are cleaved by the action of cholesteryl esterase to yield free cholesterol and further hydrolyzed by cholesterol oxidase. H_2O_2 created from the reaction reacts with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity is directly proportional to the concentration of cholesterol and can be determined photometrically. The reactions are following;

$$\text{Cholesteryl esters} + H_2O \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{cholest-4-en-3-one} + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4\ H_2O$$

Sample collections:

Collect serum, heparinized or EDTA plasma using standard sampling tubes. Do not use citrate, oxalate or fluoride. Stability of the plasma: 5-7 days at 4°C or 3 months at -20°C. Fasting and non fasting samples can be used.

Standard curve preparation:

The concentrations of the cholesterol standard are following:

50 mg/dl = 1.29 mmol/l 100 mg/dl = 2.59 mmol/l

200 mg/dl = 5.17 mmol/l 400 mg/dl = 10.34 mmol/l

Standard conc^{n} (mg/dl)	volume (μl)	H_2O (μl)
Blank	-	100
0	-	100
50	10	90
100	10	90
200	10	90
400	10	90

Sample preparation:

Samples	Volume (μl)	H_2O (μl)
Plasma	20	80
VLDL fractions	100	-
LDL fractions	100	-
HDL fractions	50	50

Testing:

1. Pipette standards, samples and H_2O following the table in to the tubes.
2. Add 1000 µL of colour reagent in each cuvette, mix well.
3. Allow the color complex developed at room temperature for 30 min. Read the OD at 500 nm. The color complex will be stable for 1 hour.

Remarks:

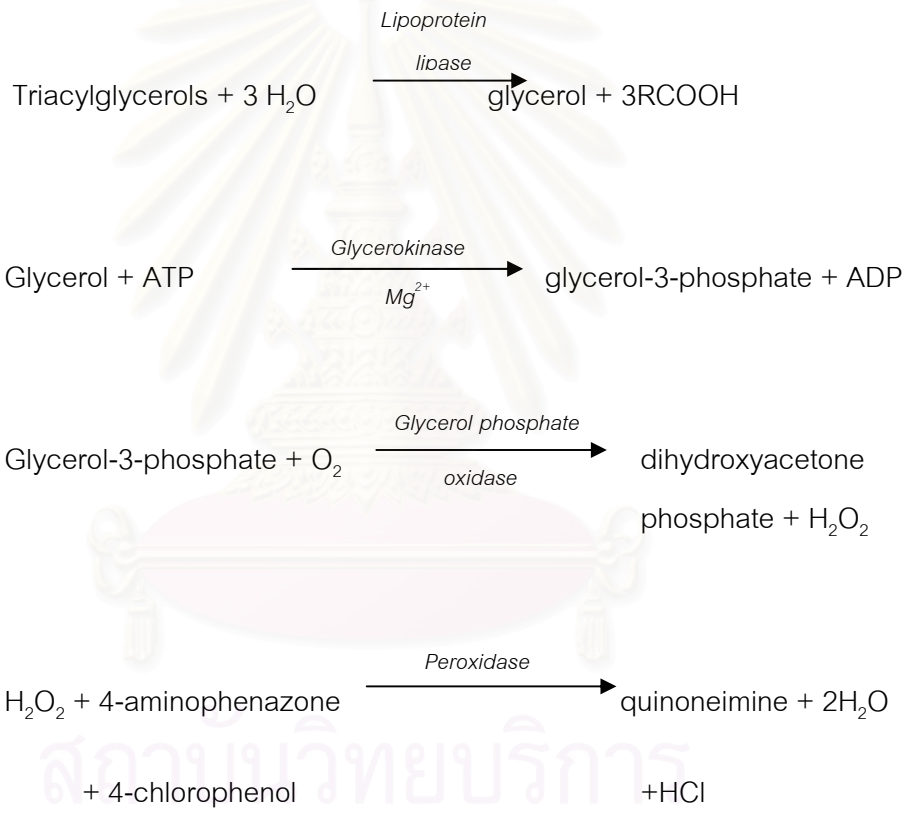
-The test is not influenced by haemoglobin values up to 200 mg/dl or bilirubin values up to 5 mg/dl.

-If the cholesterol concentration of the sample more than the linearity range, dilute sample with 0.9% NaCl or distillated / deionised water. Multiply the result by the dilution factor.

APPENDIX E

Triacylglycerol determination

Principle: Lipoprotein lipase hydrolyzes triacylglycerols to glycerol followed by oxidation to dihydroxy-acetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff. The reactions are following:



Sample collections:

Collect serum, heparinized or EDTA plasma using standard sampling tubes. Plasma stable for 5-7 days at 4°C or 3 months at –20°C.

Standard curve preparation:

Standard conc^{n} (mg/dl)	volume (μl)	H_2O (μl)
Blank	-	100
0	-	100
50	10	90
100	10	90
200	10	90
400	10	90

Remarks:

-It should be avoiding the carry over while pipette the triacylglycerol standard. Its may affect to the triacylglycerol concentration in the samples.

-If the triacylglycerol concentration of the sample more than the linearity range, dilute sample with 0.9% NaCl or distillated / deionized water. Multiply the result by the dilution factor.

Sample preparations:

Samples	Volume (μl)	H_2O (μl)
Plasma	20	80
VLDL fractions	20	80
LDL fractions	100	-
HDL fractions	50	50

Methods:

1. Pipette standards, samples and H_2O following the table in to the microcuvettes.
2. Add 500 µl of colour reagent in each cuvette, mix well.
3. Allow the color complex developed at room temperature for 30 min. Read the OD at 500 nm. The color complex will be stable for 1 hour.

Remark:

- Determination of triacylglycerols in normal and pathogenic plasma sedentary during the sample determinations.

Interference:

- Hemolysis: No significant interference up to an H index of 600. (hemoglobin concentration approximate: 600 mg/dl).
- Lipemia: The L index correlates with the turbidity but not triacylglycerol level.

BIOGRAPHY

Miss Jullajit Ongpreechakul was born on November 10, 1979 in Bangkok, Thailand. She graduated with Bachelor degree of Sciences in Medical Technology from Faculty of Allied Health Sciences, Chulalongkorn University in 2001. She has studied for Master degree in Clinical Biochemistry and Molecular Medicine Program at Faculty of Allied Health Sciences, Chulalongkorn University since 2005.