CHAPTER II

MATERIALS AND METHODS

Materials

A. Raw Materials

1. Fish Meal

All fish meal samples, grades 1-4, used in the experiment were obtained from Thai fish meal plants. Those two premium grades, 1 and 2, were manufactured by Seri Baanpe plant, Rayong whereas the other two grades, 3 and 4, were from SPP. FEED, Rayong. The characteristic of proximate ingredients of all fish meal samples is shown in **Table 1**. The pictures of all fish meal samples used in the present study is shown in **Figure 1**.

2. Lecithins and Tuna Oils

All samples of crude and refined tuna oils were kindly supplied by the animal feed plant of Mittraparp Products Group of Companies, Lopburi. Four samples of crude soya lecithins were given by T.C. Union Co., Ltd., Bangkok. Egg yolk lecithin was prepared from fresh hen eggs purchased from the nearby market utilizing the extraction procedure as described in the text.

B. Glasswares

TLC chambers used in the experiment were Wheaton model 276860 (Wheaton, Millville, NJ, USA). Micro-reaction vessels (1 ml) with screw cap seals

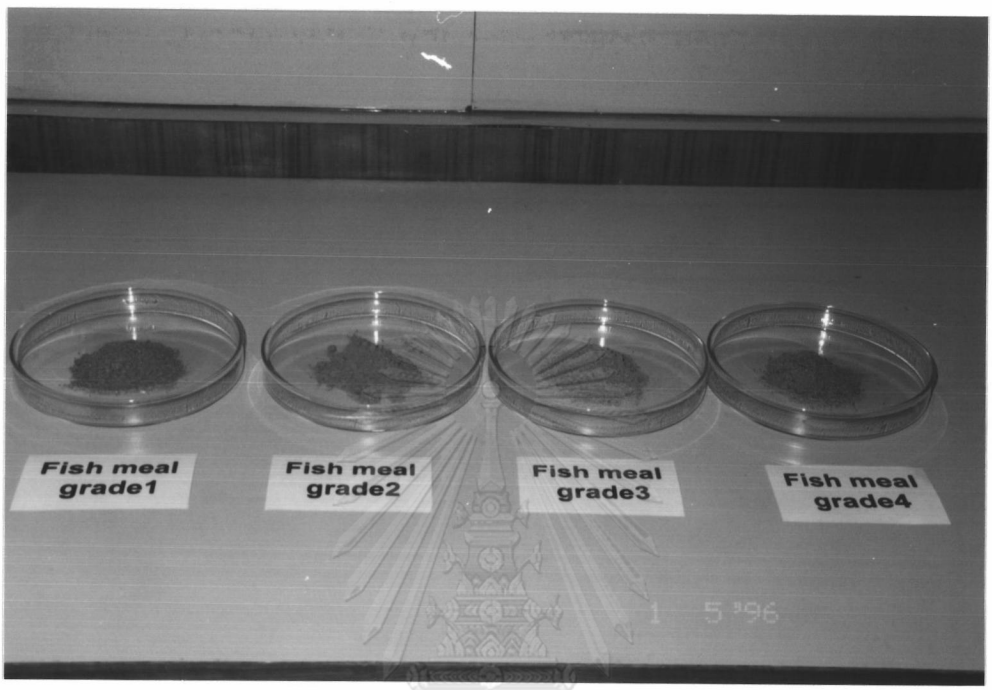

Figure 1 Four samples of fish meals grade 1-4, used in the experiment.

were from Alltech (Alltech Associates, Inc., Deerfield, IL, USA). Borosilicate glass tubes (15x100 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 tubes with reduced weight after boiling were avoided and/or discarded. All glasswares were acid washed and rinsed twice with dichloromethane-methanol (2:1, v/v) and air dried before being used. Glass wool was rinsed with organic solvents and employed under well self protection by wearing rubber gloves and nose mask.

C. Chemicals

Nitrogen used in the experiment was oxygen-free. Compressed gases were supplied by Thonburi-Watana, Bangkok. All chemicals were reagent grade. Acetylchloride used for transesterifying lipids was supplied by Sigma (St Louis, MO, USA). Fatty acid methyl esters (FAMEs), products code no. GLC-408 and GLC-409, and internal standards (IS) of fatty acids, C15:0 code no. N-15-A and C19:0 code no. N-19-A, were obtained from Nu-Chek-Prep (Elysian, MN, USA). IS of lipids: tripentadecanoin (TG-C15:0), cholesteryl pentadecanoin (CE-C15:0), and phosphatidylcholine dipentadecanoyl (PL-C15:0), were purchased from Sigma (St Louis, MO, USA). The working solutions of above-mentioned IS were prepared to have the concentration of 1 mg/ml in dichloromethane-methanol (2:1, v/v). The actual concentrations of C15-FAME as well as C19-FAME derived from transesterification of acid IS's by the reaction of acetylchloride were known by calibrating them with known amount of heptadecanoic acid (C17:0) simultaneously transesterified (Lepage and Roy, 1984).

All standards of phospholipids: phosphatidylcholine (PC; product no. P-7318), phosphatidylethanolamine (PE; P-8193), phosphatidylserine (PS; P-7769), phosphatidylinositol (PI; P-6636), phosphatidic acid (PA; P-9511), Phosphatidylglycerol (PG; P-8318) and lysophosphatidylcholine (LPC; L-0906), were shipped from Sigma (St Louis, MO, USA). All organic solvents were redistilled under vacuum at 40-50 °C in all-glass system using the rotary

evaporator. The solvents used for lipid extraction and for thin layer chromatographic separation were added with butylated hydroxytoluene (BHT: 2,6-di-tert-butyl-4-methylphenol; Fluka Chemika, Switzerland) to have the concentrations of BHT at 5 and 50 mg/dl, respectively. This aimed to protect lipids especially polyunsaturated fatty acids from any possible oxidation during their exposures to atmospheric oxygen (Phillips and Dodge, 1967).

Thin-layer chromatographic plates (20x20 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. The potent carcinogenic chemicals like chloroform and benzene were banned from our experiment for the reason of safe handling. Our alternatives for those two above-mentioned solvents were dichloromethane for chloroform and toluene for benzene (Hamilton and Hamilton, 1992).

D. Instruments

All experiments were carried out at the Fats and Oils Research Unit (FORC), Department of Transfusion Medicine, Faculty of Allied Health Sciences. The main instruments employed in the experiment are listed below:

(a) Gas Chromatograph 8000 series, Fisons Instruments, Italy
(b) Rotary evaporator , model R-114 Buchi, Switzerland
(c) Nitrogen evaporator / heater/ stirring module, Pierce, IL, USA.
(d) Sand bath, Gerhardt, Bonn, Germany
(e) Spectrophotometer UV-1201, Shimadzu, Tokyo, Japan
(f) Vacuum system, model B-169 Buchi , Switzerland
(g) Electronic balance with 3 digits, Scaltec SBA 41, Germany
(h) Electronic balance with 4 digits, Mettler Toledo, Germany

(i) Shaking water bath, model GFL 1083, GFL, Germany

(j) Suction pump, model 809 N Kataspir, Medel Italiano, Parma, Italy

(k) Centrifuge, Kokusan H11 n series, Tokyo Japan

(l) Water bath, model 83, Thelco, Chicago, IL, USA

(m) Hot air oven, Thelco, GCA/Precision Scientific Group, IL USA

(n) Ultrasonic bath, Decon FS 400 b, UK.

TLC Plate scraping system used in the experiment as shown in **Figure 2** was made in house from hard plastic by FORC according to the design of Hegstrand (1985). This system was proven to speed up to 2-3 timed consumed in the process of scraping TLC plate in comparison to the conventional procedure. The system facilitated the work speed and consequently substantial reduce the oxidation of PUFA possibly occurred during the time delay on dry TLC plate.

Methodology

A. Study of Characteristics of Lipids in Fish Meals

1. Extraction of Crude Fats

For quantitative determination of oil content of fish meal the cold extraction with the mixture of dichloromethane and methanol according to the modification of the method of Bligh and Dyer (1959) was employed. Fish meal was heated at 80 °C for 30 min to evaporate moisture as well as to inactivate any active enzymes before the extraction. Fifty grams of fish meal was weighed and homogenized in Waring blender for 5 min with 150 ml of dichloromethane-methanol mixture (1:2, v/v). The homogenate was added with 50 ml of dichloromethane before blending for 2 min followed by 50 ml of distilled water and the mixture was homogenated for another 2 min. The filtrate was then collected by filtering the obtained homogenate through a Whatman No. 1 filter paper on Buchner funnel with a slight suction. The residue was compressed with the tip of a spatula or a

glass-bottle top to ensure maximum recovery of filtrate. The remaining residue and filter paper were carried out for the second extraction following the same procedure. The second filtrate was pooled into the first fraction. The combined filtrate was then mixed and allowed to settle for a few minutes in a 500 ml separatory funnel for complete separation and clarification. The clear lower phase of dicholromethane layer which contained total lipids extracted from sample was collected into the round bottom flask. The solvent was evaporated from lipids at 40 °C under vacuum by means of rotary evaporator until a thick crude lipids known as crude fish oils was obtained. Any remaining solvent was removed from crude oils by flushing the residue with nitrogen gas.

2. Chemical Analyses

The lipid extract or fish oil obtained from each grade of fish meal according to the above-mentioned procedure was collected in small portions under nitrogen atmosphere in tightly closed test tubes and kept away from the light at -20 °C. It was divided for subsequent analyses as follows:

(a) crude fat content,

(b) class or profile of lipids,

(c) content and composition of fatty acids present in :-

- total fats,
- triglycerides (TG),
- phospholipid (PL)

(d) lecithin content,

(e) subclasses of PL.

The procedures for all above-mentioned analyses will be explained later in the section of Chemical Analyses.

B. The Extraction of Lecithin

1. Preextraction of Crude Oils

a) Extraction without Alcohol Pretreatment

Fish meal was heated at 80 °C before extraction. Fifty grams of fish meal were transferred into a glass wool-plugged separatory funnel. Lipids were separated from fish meal by extracting twice with 100 ml n-hexane. The mixture was vigorously shaken for 30 min. The miscellae (oil in n-hexane) of both extractions were collected by filtering through glass wool and pooled into a round bottom flask. N-hexane in the mixture was then evaporated from the extract by means of rotary evaporator at 40 °C and collected for reuse. The remaining solvent was removed by flushing the residue with oxygen-free nitrogen gas. The weight of crude oil obtained from the extraction was recorded and its lipid characteristic was analyzed.

b) Extraction with Alcohol Pretreatment

Fifty grams of preheated fish meal were blended twice with 100 ml of alcohol either ethanol or methanol for 30 min in a separatory funnel plugged with clean glass wool. Both filtrates were collected in a round bottom flask. Alcohol was removed from filtrate at 40 °C by means of rotary evaporator. The remaining residue was kept under nitrogen atmosphere for further process. Precipitant of fish meal pretreated with alcohol was extracted twice with n-hexane according to the procedure described earlier. The crude oil obtained from n-hexane extraction as well as from alcohol pretreatment were combined and the pooled crude oils were weighed and their lipid characteristics were determined according to techniques as will be described in the section of Chemical Analyses.

2. Separation of Neutral and Polar Lipids

Crude oil obatined from the previous section constituted polar lipids majorily of lecithins and neutral lipids exclusively of TG. In order to deoil neutral TG from lecithin, crude oils obtained from the extraction with alcohol and n-hexane was blended vigorously for 10 min with a surplus of acetone to have the mixture with crude oil-acetone ratio of 1:3 (w/w). The mixture was then separated under low speed centrifugation yielding lower fraction of acetone insoluble polar lipids and upper fraction of neutral lipid solution in acetone. The lower fractions in each separation of polar lipids were pooled together and the process of deoil using acetone was then repeated. All deoiled lower fractions known as acetone-wet polar lipids was carefully dried under vacuum at 40 °C by means of ratory evaporator. Fish meal lecithin was finally obtained and processed for further experiment and analyses. **Figure 3** shows the pictures of lecithins derived from various sources as described herein. Lecithin derived from fish meal was different in appearance comparing to those obtained from egg yolk and soya. Fish meal lecithin was solid and shorten whereas both latter lecithins were thick liquid. Lecithins of fish meal and soya were dark brown in color but egg yolk lecithin was yellowish.

3. Preparation of Egg Yolk Lecithin

Egg yolk lecithin was prepared at FORC from egg yolk of hen eggs employing the similar procedure as described earlier in the preparation of fish meal lecithin. The obtained lecithin was assayed for the content and subclasses of phospholipids (PL). Fatty acids in both moieties of TG and PL separated by TLC were freed and methylated prior to determine for their composition and concentrations by the technique of GLC.

C. Preparation of Lecithin-Rich Fat Emulsions (LRFE) (Figure 4)

1. Fish Meal-Derived Lecithin-Rich Fat Emulsion (FM-LRFE)

Lecithin extracted from fish meal grade 1 was found to comprise of both PL and TG in the ratio of PL-TG 2.7:10 (w/w) or 0.27. FM-LRFE with PL-TG ratio ranging between 0.25-0.28 was prepared from the obtained fish meal lecithin by physical dispersion method according to New (1994). In brief, the stock FM-LRFE was prepared to provide PL concentration of 1.2 g/dl by blending fish meal lecithins in normal saline solution (NSS, 0.85% NaCl) with waring blender for 10 min, or until its has given a homogeneous milky suspension free of visible particles. The mixture was transfer into a polypropylene screw cap bottle and flush the mixture with oxygen-free nitrogen gas and seal the cap. The mixture was sonicated in sonic bath for 1 h with occasional shaking. The solution was kept at 4 °C under nitrogen atmosphere until being used within several days. Freshly prepared working solutions of FM-LRFE were achieved by diluting stock emulsion with NSS to have PL concentrations of 100, 150, 200, and 300 mg/dl.

2. Egg Yolk-Derived Lecithin-Rich Fat Emulsion (EY-LRFE)

The prepared egg yolk lecithins was found to contain PL higher than that of fishmeal lecithins (58 vs 27 g/100g, see also the results). To prepare stock EY-LRFE with similar PL-TG ratio of FM-LRFE, egg yolk TG present in crude lecithin was thus diluted by soya oil to adjust PL-TG ratio from original 0.58 to 0.27 mimicing PL-TG ratio of FM-LRFE. The resulted egg yolk lecithin was then employed for the preparations of stock EY-LRFE with PL concentration of 1.2 mg/dl and of working solutions of EY-LRFE at PL concentrations of 100, 150, 200 and 300 mg/dl following the similar procedures as described for FM-LRFE above.

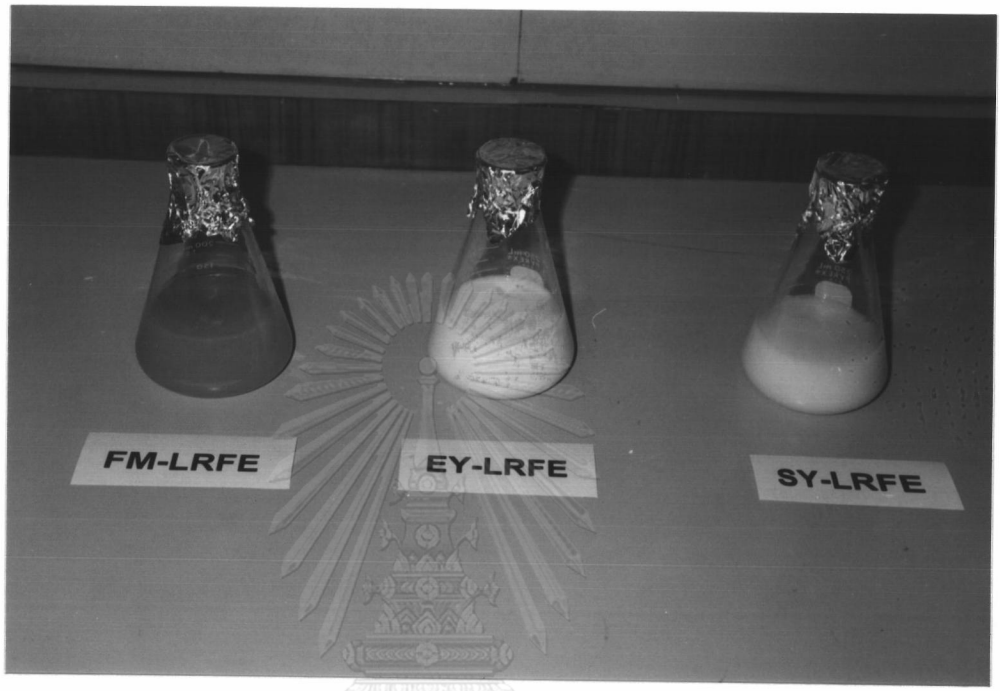

Figure 4 Three fat emulsions with lecithin rich prepared according to the dispersion procedure method as described in the text. Abbreviations : FM-LRFE, fish meal-derived lecithin-rich fat emulsion ; EY-LRFE, egg yolk-derived lecithin-rich fat emulsion ; SY-LRFE, soya-derived lecithin-rich fat emulsion.

3. Soya-Derived Lecithin-Rich Fat Emulsion (SY-LRFE)

Commercial soya lecithin with PL content of 54 g/100g was adjusted to have PL-TG ratio similarly to that of fish meal lecithin (27 g/100g) by the addition of soya oil. The diluted soya lecithin was then employed for the preparation of SY-LRFE by blending in NSS. Stock and working solutions of SY-LRFE were prepared as described earlier.

D. Study on the Effect of LRFE on Human Red Blood Cells (RBC)

1. Preparation of Plasma-Free RBC

Hepatitic viral-free and HIV-free packed erythrocytes with group O were donated by healthy volunteers and kindly provided to the present study by the National Blood Center, Thai Red Cross. The erythrocytes were washed 3 times with cold isotonic NSS. The buffy coat layered on top of packed cell was removed after each wash by aspiration using a suction pump. The prepared plasma-free erythrocytes were collected in small portion and immediately processed for incubation with liposomes as described below.

2. Incubation with LRFE

Four ml of packed erythrocytes were transferred into 15 ml graduated glass centrifuge tube and 6 ml of the working LRFE solution of either 100, 150, 200 or 300 mg PL/dl was added slowly into the tube. The hematocrit value in each incubation mixture was actually 40%. Each incubation with either concentrations of LRFE was performed in 5 replications. The mixtures of erythrocytes and liposomes were mixed gently by converting the tube and incubation was carried out at 37 °C for 1 h in shaking water bath. Later, the LRFE solutions were separated from RBC by centrifuging at 3000 g for 15 min. Packed RBC was then washed 3 times with cold NSS in order to remove the

remaining LRFE. Washed RBC was employed for furthur analyses. RBC simultaneously incubated with NSS was considered as 0 mg PL/dl incubation and used as control .

E. Chemical Analyses

1. Erythrocyte Membrane Lipid Extraction

Two 0.5 ml aliquots of the packed erythrocytes were taken for lipid extraction. The erythrocyte membrane lipids were extracted into a mixture of 11 volumes of isopropanol and 7 volumes of dichloromethane according to the method described by Rose and Oklander (1965). All organic solvents were reagent grade and used without further purification. In brief, half a ml of distilled water was added to the packed erythrocytes and the contents of the tube were mixed with a Vortex mixer and allowed to stand for 15 min. Isopropanol (5.5 ml) was added slowly with mixing. The cells gradually turned dark and clumpy. After one hour and occasional mixing, 3.5 ml of dichloromethane were added and mixed. At the end of another hour with occasional mixing, the tube was centrifuged at 500 x g for 30 min. The total fluid volume was 9.8 ml. The clear extract was directly sampled for further analysis

2. Phospholipid Analysis

a) Preparation of Reagents

Phosphorus present in all forms of PL was turned into inorganic phosphorus and assessed quantitatively by its reaction with Fiske-Subbarow reagent according to the modified technique of Bartlett (1959). The Fiske-Subbarow reagent was prepared at 60 °C heating-stirring plate by adding 0.5 g 1-amino-2-naphthol-4-sulfonic acid under mechanical stirring to 200 ml of freshly prepared

15 per cent anhydrous sodium disulphite ($Na_2S_2O_5$), followed by addition of 1 g anhydrous sodium sulphite (Na_2SO_3) . The solution was filtered on Whatman paper No. 1 into a dark bottle. The solution was freshly prepared each month and stored at 4 °C.

b) Sample Preparations

1) Fish oil or Lecithin

Oil or lecithin was diluted to 1:10 (w/v) with dichloromethane-methanol mixture (2:1 v/v) and 20 µl of these solution was transferred into a 16 x 150 mm acid-washed glass tube for further analysis.

2) LRFE

A 20 µl of LRFE solution was transferred into the tube for further analysis.

3) Erythrocyte Membrane Lipid Extract

A 200 µl erythrocyte membrane lipid extract was transferred by means of micropipette into 13 x 100 mm acid-washed glass tube. The extract was dried under a gentle stream of nitrogen in a water bath at 50 °C. The dried residue was redissolved in 1 ml dichloromethane-methanol (2:1, v/v). Methanol and inorganic phosphate remaining in the membrane lipid extract were eliminated by washing the dichloromethane-methanol phase with 0.2 ml of 0.05 N KCl (Folch et al., 1957; Rose and Oklander, 1965; Terpstra, 1984). The inorganic phosphate-free erythrocyte membrane lipid dichloromethane extract was then completely dried under a gentle stream of nitrogen at 50 °C. The dried residue was analyzed for PL analysis as described below.

c) Procedure

The tube which containing sample as described above was redissolved in 0.2 ml of concentrated sulfuric acid and heated in a 190 °C sand bath for at least 30 min. Two drops of 30 percent hydrogen peroxide were added and the solution was returned to the sand bath for another 45 min to complete the combustion and to decompose all the peroxide. Five ml of freshly prepared chromogen solution containing Fiske-Subbarow reagent-5% ammonium molybdate-distilled water, 1:1:23 (v/v/v) were added and the contents were mixed thoroughly. The contents were heated for 10 min in a boiling water bath. The optical density was recorded at 830 nm with the UV-1201 split-beam spectrophotometer. Di-sodiumhydrogenphosphate dihydrate ($HNa_2O_4P.2H_2O$) (phosphorus content, 31 g in a total of 178 g) at the concentration of 8 mg phosphorus/dl was used as standard. A calibration curve was obtained by using 2, 4, and 8 μg phosphorus per tube. In the calculations, 1 mg of phosphorus is equivalent to 25 mg of PL and 1 mmole of phosphorus is eqivalent to 1 mmole of PL.

3. Separation of Phospholipid Subclasses

Phospholipid subclasses in the biological samples were separated by the two-dimensional TLC procedure described by Broekhuyse (1969) and slightly modified by van Meer et al. (1980).

a) One-dimension TLC for Separation of TG and PL

The concentrated lipid extracts were redissolved in 20-40 μl of dichloromethane-methanol (2:1, v/v) and were proceeded directly for the separation of PL subclasses as described in the following section. Due to their high TG content, the concentrated extracts of lipid were proceeded through a one-dimension TLC using n-hexane-diethyl ether-glacial acetic acid (80:20:1,

v/v/v) as solvent in order to remove TG. The silica powder of the origin area which contains PL was scraped directly into a tube using a TLC plate scraping system as previously shown in **Figure 2**. PL was eluted from the scraped silica powder with three solvent rinses, centrifuged and the supernatant filtered through a capillary pipette plugged with glass wool. The rinsing solvents were 2 ml dichloromethane-methanol-acetic acid-water (25:14:4:2, v/v/v/v) followed by 2 ml methanol and 2 ml methanol-acetic acid-water (95:15:5, v/v/v) (Field et al., 1985). The pooled solvents were removed at 40 °C under nitrogen and PL residual was redissolved with 20-40 µl of dichloromethane-methanol (2:1, v/v).

b) Two-Dimensional TLC

The concentrated lipid extracts (20-40 µl) were applied to the TLC plates by means of a microsyringe with an oblique needle point or a capillary pipette tip, the opening of which was placed against the adsorbent surface. The chromatogram was developed in the first direction with dichloromethane-methanol-25% ammonia-distilled water (90:54:5.7:5.3, v/v/v/v) and in the second direction with dichloromethane-methanol-glacial acitic acid-distilled water (90:30:8:2.85, v/v/v/v). Between these two runs, the plates were dried for 15 min in an air stream. Spots were coloured with iodine vapour after chromatography. After iodine sublimation, the areas of silica powder containing the different PL subcomponents were scraped into the tubes; PL's were eluted as described in the previous section. Silica powder taken from an area which contains no lipid was used as a blank. PL-phosphorus was determined according to Bartlett (1959) as described previously.

4. <u>Cholesterol Analysis</u>

a) In Erythrocyte Membrane Lipid Extracts

Cholesterol (exclusively in form of free cholesterol) content in erythrocyte membrane lipid extracts was analyzed by enzymatic kits (cat. no. 10028 Human, Germany). Two hundred µl of erythrocyte membrane lipid extract were transferred into a 13 x 100 mm acid-washed glass tube. The extract was dried under a gentle stream of nitrogen at 50 °C. One ml reagent solution containing 0.5% (v/v; final concentration) Triton X-100 was added into the tube. After careful mixing, the incubation was carried out at 37 °C for 10 min and the absorbance was read at 500 nm against a reagent blank. Cholesterol aqueous solution , 200 mg/dl, was used as standard.

b) LRFE Solution or Highly-Turbid Fluid Specimen

Cholesterol content in LRFE or highly-turbid fluid specimen was analysed by enzymatic-spectrophotometric technique which developed by Dahlan et al. (1992). Two hundred µl of aqueous specimen were transferred into a 13 x 100 mm acid-washed glass tube. Two ml reagent solution containing 0.5% (v/v; final concentration) Triton X-100 was added into the tube and mixed carefully. The incubation was carried out at 37 °C for 15 min after that added 2 ml of n-butylacetate-n-butanol (4:1, v/v) into the tube and the contents of the tube were mixed vigorously with a Vortex mixer for 45 sec. The reaction tube was centrifuged at 3000 rpm for 10 min and the yellow color of the upper phase was measured the absorbance at 440 nm by using the Quart cuvette. The standard and reagent blank was run simultaneously according to the same method.

5. <u>Fatty Acid Analysis</u>

a) Fatty Acid Analysis of Oils

Fatty acid compositions of oils were determination by preparation of metylesters according to the technique described by Lepage and Roy (1986).

Twenty µl of sample 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 megnetic bar was immediately added. The saponification of lipids and methylation of liberated fatty acids was performed by using acetylchoride. Briefly a 200 µl of acetylchloride was added slowly while the tube was mixing, the tube was tightly closed and the fatty acids subjected to methanolysis with slow stirring at 100 °C for 1 hour in the heat/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. At the end of the process, a 1-2 µl aliquot of the hexane upper phase was injected into the gas chromatograph which was conditioned and programmed as described: A 8000 series gas chromatograph (GC) with a flame ionization detector was used. The separation of fatty acids methylesters (FAMEs) was performed in a 30 m fused silica capillary column with an internal diameter of 0.32 mm and wall-coated with 0.25 µm, DB-WAX P/N 123-7032 (J&W Scientific, USA). Helium was used as carrier gas (1.5 ml/min at 100 °C). The split ratio was 10:1. The injection port temperature was 250 °C and the detector was set at 300 °C. The column temperature was initiated at 100 °C and after sample injection the temperature was programmed to 200 °C with an increase rate of 25 °C/min and held isothermally for 15 min. The second increment was 5 °C/min to 220 °C this temperature was maintained constant for 5 min.

b) Analyses of Fatty Acid Composition in TG and PL Fractions

Triglycerides (TG) and phosphilipid (PL) were separated from each other by a one dimension TLC using n-hexane-diethyl ether-glacial acetic acid (80:20:1), v/v/v) as a developing solvent which previously described . Spots

of TG 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. Equal areas of silica containing TG and PL were scraped without delay on a plastic scraping system into a Teflon lined screw-capped borosilicate test tube in which 2 ml of methanol-hexane (4:1, v/v) and the tube was mixed vigorously. The methylation of fatty acids was performed by using acetylchoride according to the method as previously described.

c) Analysis of Fatty Acid Composition in Erythrocytes

Five hundreds µl of packed RBC which washed 3 times with 3 volumes of cold normal saline (NSS) were transferred into a Teflon lined screw-capped borosilicate test tube in which 2 ml of methanol-hexane (4:1, v/v) and the tube was mixed vigorously. The direct methylation of RBC fatty acids was performed by using acetylchoride according to the method as previously described.

F. Statistical Analysis

The results were expressed as Means ± S.D. All statistical significances were calculated by one-way analysis of variance (ANOVA) with Duncan' s new multiple range test by programme of SPSS/PC+ for Windows. The letters: a, b, c and d, labelled over figures or pictures were used for indicating statistical differences among those comparison. Any different letters if indicated were statistically different at $p<0.05$ whereas any similar letters were non-significant different. The values of slope and coefficient of determination (r^2) were calculated according to the regression analysis using programme of Microsoft Excel 5.0 for Windows. The changes of individual RBC fatty acid was calculated as relative membrane fatty acid changed as follow:

relative membrane FA changed = $(FA_x-FA_0)/FA_0 \times 100$

FA_x = RBC fatty acid (%) after incubation with certain PL concentration

FA_0 = RBC fatty acid (%) without incubation