CHAPTER II

MATERIALS AND METHODS

Materials

A. Raw Materials

1. Fish Meal

Fish meal used in the experiment was grade A Danish fish meal purchased from P.Chareonpan Ltd.,Bangkok. (**Figure 1**). Danish fish meal contained less fat than grade 1 Thai fish meal employed in our previous experiment (8.08 vs 11.05, g/100 g)(Chatnilbandhu, 1996). However, it was chosen as source of fish lecithins in our present experiment since it contained higher amount of omega-3 polyunsaturated fatty acids (n-3 PUFA's) than that of grade 1 Thai fish meal (38.49 vs 28.1, g/100 g for Danish vs Thai fish meals) . The characteristics of proximate ingredient of this Danish fish meal is shown in **Table 1**.

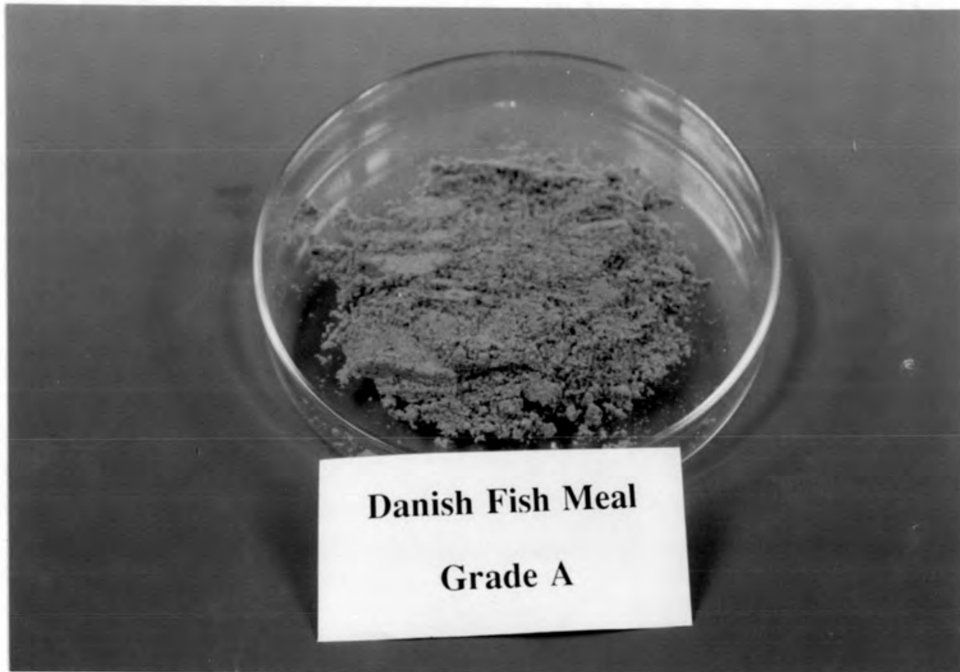


Figure 1 Danish fish meal, used in the experiment.

Table 1 Characteristics of proximate ingradients of Danish fish meal

Fish meal	Moisture	Protein	Fat	Carbohydrate	Fiber	Ash
Danish	7.52	64.55	8.08	3.53	0.72	15.60

The results are average of two determinations and expressed in g/100 g.

Data were obtained from the manufacturers.

2. Lecithins, Fat Emulsions and Fish oils

Crude soya lecithins were given by T.C. Union Co., Ltd., Bangkok. Lipofundin® (MCT/LCT 20%) was commercial fat emulsion and purchased from B.Braun (B.Braun, Melsungen, Germany). Commercial food-grade fish oils were encapsulated fish oil 1000 mg. provided as gift by MaxEPA®, Dr. Ian Brighthope, Hampton, Australia.

B. Platelets

Platelet samples were donated from 122 healthy volunteers and kindly provided to the present experiment in bags of platelet concentrate by the National Blood Centre, Thai Red Cross Society, under the supervision of Mrs. Aroonrat Chantanakajornfung, the Chief of Plasma and Fractionation Section. All samples were various in blood groups (A, AB, B and O) and preliminary investigated as hepatitic viral-free and HIV-free. Platelet concentrate was prepared according to the procedure described later in Methods.

C. Plasticwares and Glasswares

The cylindrical polypropylene test tubes (16×100 mm) with screw caps were supplied by LP ITALIANA SPA, Milano, Italy. Plasma transfer sets were supplied by Thai Kawasumi Co., Ltd., Bangkok, Thailand. Borosilicate glass chambers

for thin-layer chromatography (TLC) used in the experiment were from Wheaton® (Wheaton, Millville, NJ, USA). Micro-reaction vessels (1 ml) with screw cap seals were from Alltech (Alltech Associates, Inc., Deerfield, IL, 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 with tightly capped after boiling at 100 °C for 1 hour (h) in water bath. The tubes which maintained constant weight after boiling were chosen whereas those with reduced weight were avoided and/or discarded. Glass bottle (1,000 ml) were from Duran® (Duran, Schott glassware, Mainz, Germany). All glasswares were acid washed and rinsed twice with dichloromethane-methanol (2:1 v/v) and air dried before being used.

D. Chemicals

All compressed gases were supplied by Thonburi-Wattana, Bangkok. Nitrogen used in the experiment was oxygen-free grade. All chemicals were reagent grade. Acetylchloride used for transesterifying lipids was supplied by Sigma (St Louis, MO, USA). Fatty acid methyl esters (FAME's) were purchased from Nu-Chek-Prep (Elysian, MN, USA). They were products code no. GLC-408 and GLC-409 for FAMEs whereas internal standard (IS) of fatty acids were free fatty acids: C 15:0 with code no. N-15-A) and C 19:0 with code no. N-19-A. IS's of lipids: tripentadecanoin (TG-C15:0), cholesteryl pentadecanoin (CE-C15:0), and phosphatidylcholine dipentadecanoyl (PL-C15:0), were from Sigma (St Louis, MO, USA). The working solutions of all above-mentioned IS's were prepared to 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 according to the method of Lepage and Roy, 1984.

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 TLC separation contained butylated hydroxytoluene (BHT; 2-6-di-tert-butyl-4-methylphenol, Fluka Chemica, Switzerland) in the concentrations of respective 5 and 50 mg/dl. The purpose of BHT addition was to protect lipids especially PUFA's from any possible oxidation during their exposures to atmospheric oxygen (Phillips and Dodge, 1967).

TLC plates (20×20 cm) precoated with Silica gel 60 without fluorescent indicator, with a layer thickness of 0.25 mm was 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). Working with all organic solvents and dangerous chemicals were performed under fume hood and hand protected with rubber gloves.

E. Instruments

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

(a) Gas chromatograph 8000 series, Fisons Instruments, Italy

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

(c) Refrigerated centrifuge, Hitachi, himac CF7D2, Japan

(d) Rotary evaporator, model R-114 Buchi, Switzerland

(e) Vacuum system, model B-169 Buchi, Switzerland

(f) Suction pump, model 809 N Kataspir, Model Italiano, Parma, Italy

(g) Suction pump, model 523-U4-G21DX, MI, USA

(h) Nitrogen evaporator/heater/stirring module, Pierce, IL, USA

(i) Sand bath, Gerhardt, Bonn, Germany

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

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

(l) Ultrasonic bath, Decon FS 400b, UK

(m) Spectrophotometer UV-1201, Shimadzu, Tokyo, Japan

(n) Magnetic stirrer, model 815359, IKA Laboratechnik, Germany

(o) Electronic balance with 3 digits, Scaltec SBA41, Germany

(p) Electronic balance with 4 digits, Mettler Toledo, Germany

(q) Hot air oven, Thelco, Precision Scientific Group, IL, USA

(r) TLC plate scraping system

(s) Astecair filtration fume cupboard, model A-5000 E, Astec Environmental Systems Ltd., Avon, UK.

TLC plate scraping system used in the experiments was in house made from hard plastic by FORC according to the design of Hegstrand (1985). This system was proven to speed up to 2-3 times 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's possibly occured during the time delay on dry TLC plate.

Methods

A. The Extraction of Lecithins from Raw Materials

1. Elimination of Neutral Lipids

Fish meal sample was heated at 80°C for 30 min to evaporate the moisture as well as to inactivate any active enzymes before the extraction. Three hundred grams of fish meal were weighed and transferred into a glass bottle (1,000 ml). Fish meal lipids were separated by extracting fish meal twice with 900 ml acetone to obtain fish meal-acetone ratio of 1:3 (w/v). The mixture was vigorously shaken for 30 min. 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 and filter paper were carried out for the second extraction following the same procedure. The second filtrate was pooled into the first fraction and kept in 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 by flushing crude oils with oxygen-free nitrogen gas. The crude oil obtained from acetone extraction constituted neutral lipids exclusively of triacylglycerols (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 900 ml of methanol with the ratio of fish meal to methanol 1:3 (w/v). The mixture was vigorously shaken for 30 min in a glass bottle. Filtrate was collected in a round bottom flask. Methanol was removed from filtrate under vacuum at 40°C by means of rotary evaporator. The remaining residue was kept under nitrogen atmosphere. The extraction with methanol was performed twice. Later, precipitant of fish meal previously pretreated with methanol was furtherly extracted twice with 3 volumes of n-hexane (w/v) following the same procedure. The residues of polar lipids majorily of lecithins obtained from methanol and n-hexane extractions were combined and weighed. Lecithin's lipid characteristics were investigated according to techniques

as will be described in the section of Chemical Analysis. **Figure 2** shows the pictures of lecithins derived from fish meal in comparison to commercial soya lecithins. Both types of lecithins were viscous liquid. Fish meal lecithin was dark brown whereas soya lecithin was brownish yellow.

B. Preparation of Lecithin-Rich Fat Emulsions (LRFE)

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

Lecithin extracted from fish meal was found to comprise of both PL and TG in the ratio of PL-TG 1:3 (w/w) or 0.33. FM-LRFE with PL-TG ratio ranging between 0.3-0.4 was prepared from the obtained fish meal lecithin by physical dispersion method as described by 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) by means of magnetic stirrer for 30 min, or until it provided an opaque-homogeneous suspension without any visible particles. The mixture was transferred into a polypropylene screw cap bottle and flushed with oxygen-free nitrogen gas. The bottle was capped and sealed with parafilm. The mixture was sonicated in ultrasonic bath (Decon FS 400b, UK) for 1 h with occasional shaking. The homogeneity of the prepared stock emulsion was maintained for several days under nitrogen atmosphere at 4°C. Working emulsions of FM-LRFE were freshly prepared by diluting stock emulsion with NSS to have PL concentrations of 100, 300 and 600 mg/dl.

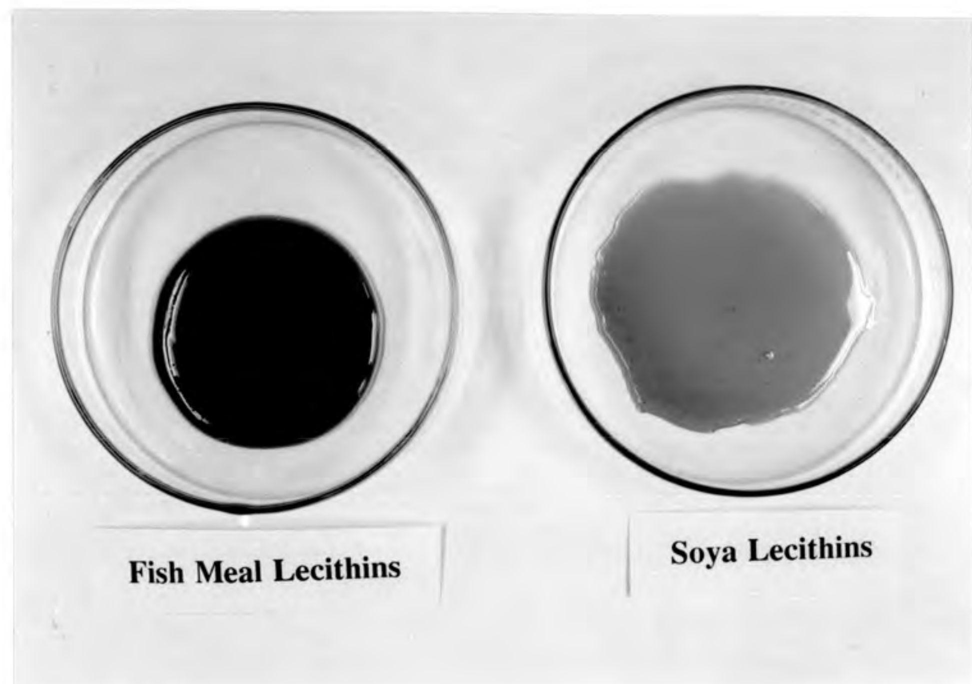


Figure 2 Crude lecithins derived from fish meal and soya. Fish meal lecithin was prepared as described in the text whereas soya lecithin was supplied from the commercial firm.

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

Commercial soya lecithins with PL content of 50 g/100 g was added with soya oil to have PL-TG ratio similarly to that of fish meal lecithins (1:3 w/w). The diluted lecithins were then blending in NSS to prepare SY-LRFE. Working emulsions of SY-LRFE at PL concentrations of 100, 300 and 600 mg/dl was prepared according to the procedure employed for the preparation of FM-LRFE as described above.

3. Soya Lecithin - Fish Oil Mixed Fat Emulsion (SL-FOFE)

Stock SL-FOFE was prepared to have PL-TG ratio similar to FM-LRFE as follows: crude lecithin was added with MaxEPA® fish oil until the origin PL-TG ratio of 1:1, w/w, was diluted to 1:3, w/w, mimicing PL-TG ratio of FM-LRFE. Working emulsions of SL-FOFE at PL concentrations of 100, 300 and 600 mg/dl were prepared according to the procedure employed for the preparation of FM-LRFE as described above.

4. 20% Lipofundin

Commercial fat emulsion, 20% Lipofundin contained egg lecithin at PL concentration of 1.2 g/dl together with medium-chain triacylglycerols (MCT) and long-chain triacylglycerols (LCT) at TG concentrations of each 10 g/dl. Working emulsions

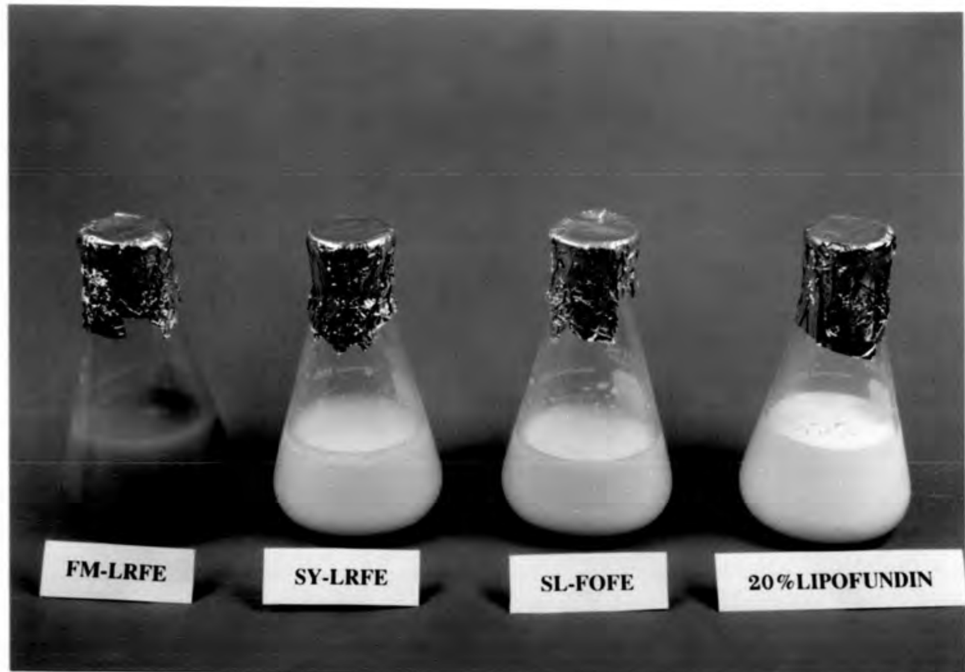


Figure 3 Four lecithin-rich fat emulsions (LRFE) prepared by the dispersion procedure as described in the text. From left to right: FM-LRFE, fish meal-derived lecithin-rich fat emulsion; SY-LRFE, soya-derived lecithin-rich fat emulsion; SL-FOFE, soya lecithin-fish oil mixed fat emulsion; 20% Lipofundin, commercial egg yolk-derived lecithin-rich fat emulsion.

were prepared by diluting 20% Lipofundin with NSS to have PL concentrations of 100, 300 and 600 mg/dl.

C. Handling of Platelets

1. Qualification of Blood Donor

(a) Healthy volunteer with age in the ranges of 17-60 years old .

(b) More than 45 kg in body weight.

(c) Non - menstrual or non - pregnant woman.

(d) Never been jaundice or infected with Aids and/or malaria during the period of 3 years.

(e) Not taking any type of anticoagulants, antiplatelet agents, non steroidal antiinflammatory drugs or Aspirin.

2. Blood Used for Platelet Preparation

(a) Blood must be drawn and kept in a multiple bag containing citrate phosphate dextrose (CPD) as anticoagulant.

(b) Blood is kept at 22±2 °C and processed for platelet separation within 6 hours .

3. Preparation of Platelet Concentrates (PC)

(a) Centrifuge bags of whole blood (WB) with Heraeus 6000i for first spin at 2,490 rpm, 4 min, 22°C, accel = 9, decel = 4. **Figure 4** shows a bag of WB with labels of identification number and blood group. Steps of cupping and balancing the bags before the centrifugation are shown in **Figures 5** and **6**.

(b) Transfer centrifuged blood with care to the plasma separators. Beware of remixing red blood cell (RBC) with plasma. Press bag gently in order to transfer platelet-rich plasma (PRP) to another bag (**Figure** 7). Packed red cell (PRC) and PRP are fully separated at this step. **Figure 8** shows PRC and PRP after separation.

(c) Centrifuge PRP for second spin at 3,420 rpm, 6 min, 22 °C, accel = 9, decel = 5.

(d) Press out platelet-poor plasma (PPP) from centrifuged PRP bag into another bag. At this step PPP and PC containing with approximately 50-60 ml of plasma are fully separated. PPP is kept at -20°C as fresh frozen plasma (FFP). **Figure 9** shows the plasma separators with 3 PC bags at the top and 3 PPP bags at the bottom. The pictures of PC bags (three lower bags) and PPP bags (three upper bags) are shown in **Figure 10**.

(e) Hang the PC bags in a Melco linear platelet reciprocator at 22 ± 2°C and regularly shake the bags for 1-2 h in order to suspend platelets in

plasma and prevent them from aggregation. Shelf life of PC in this condition is 72 hours. The picture of PC bags in shelf of platelet reciprocator is shown in **Figure 11**.

4. Blood Cell Count in PC

PC is diluted with 10 volumes of Isoton T® balanced electrolyte solution (Gee Chang Hong Centre, Hong Kong). RBC, white blood cell (WBC) and platelets in diluted PC were then counted with Coulter, Model T-540.

D. Study the Effect of LRFE on Human Platelets

1. Effect of Plasma

a) Incubation without Plasma

Five ml of PC at the concentration of 1.86×10^9 cells/cm^3 were transferred into graduated polypropylene tube by plasma transfer set and the platelets were washed 3 times with phosphate buffer pH 7.4. The platelets were sedimented after each wash by centrifuging at 1,500 rpm for 3 min at 22 °C. The plasma was removed by aspirating with suction pump. After the last centrifugation at 3,000 rpm for 5 min, the freshly prepared platelets without plasma were immediately processed for incubation with fat emulsions. Five ml of the working LRFE solution of either 100,

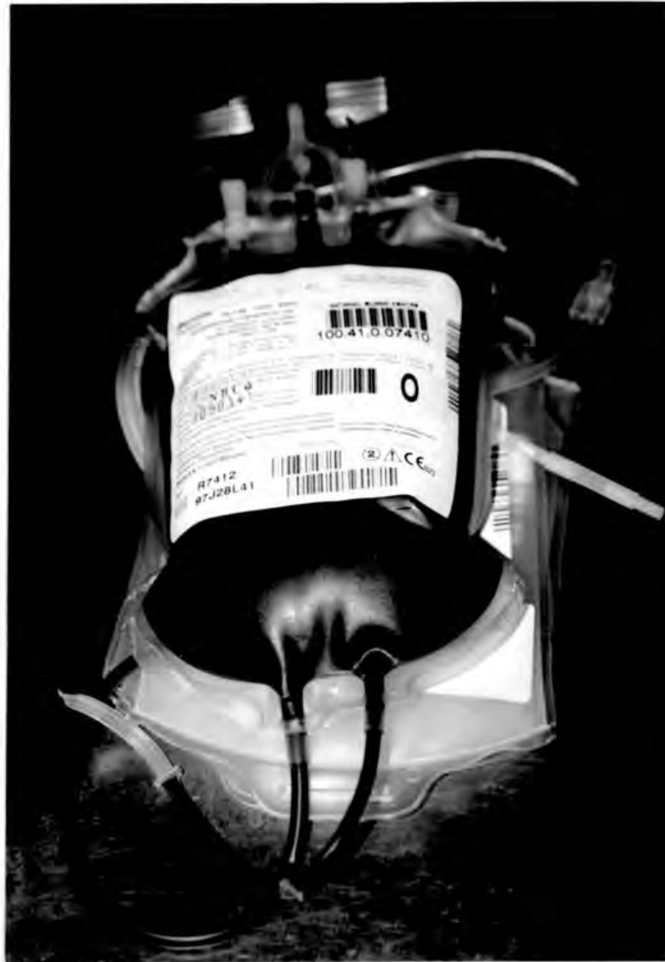
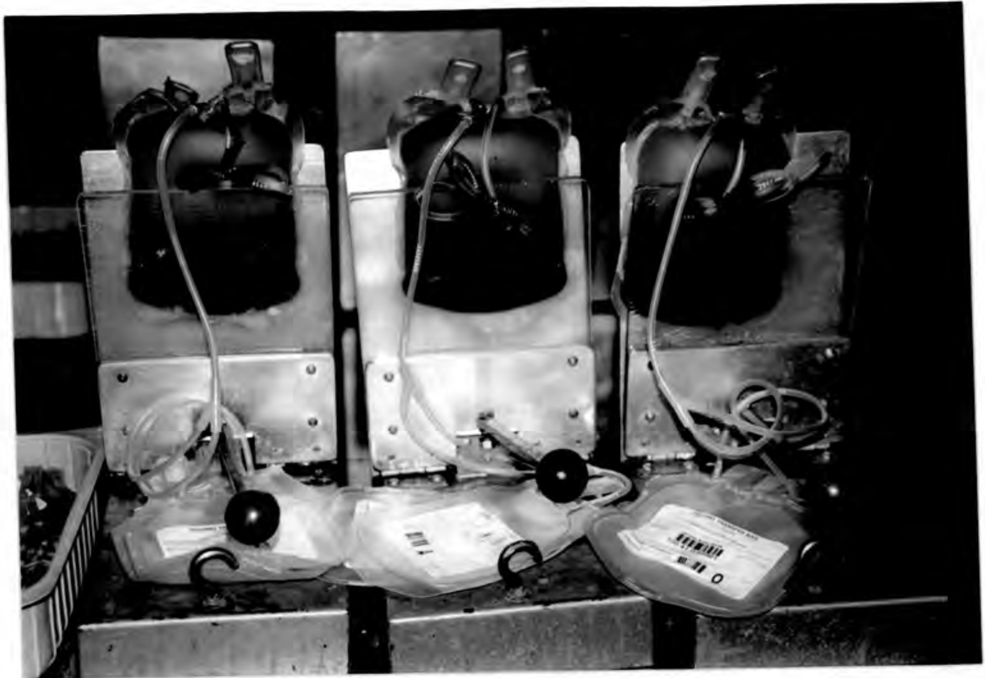


Figure 4 Whole blood donated by volunteer packed in Terumo® triple bags. The label shows identification number and blood group.

Figure 5 The triple bags with whole blood are prepared for centrifugation.

Figure 6 Balancing 2 centriguge cups on the 2 digit balance before centrifugation.

A



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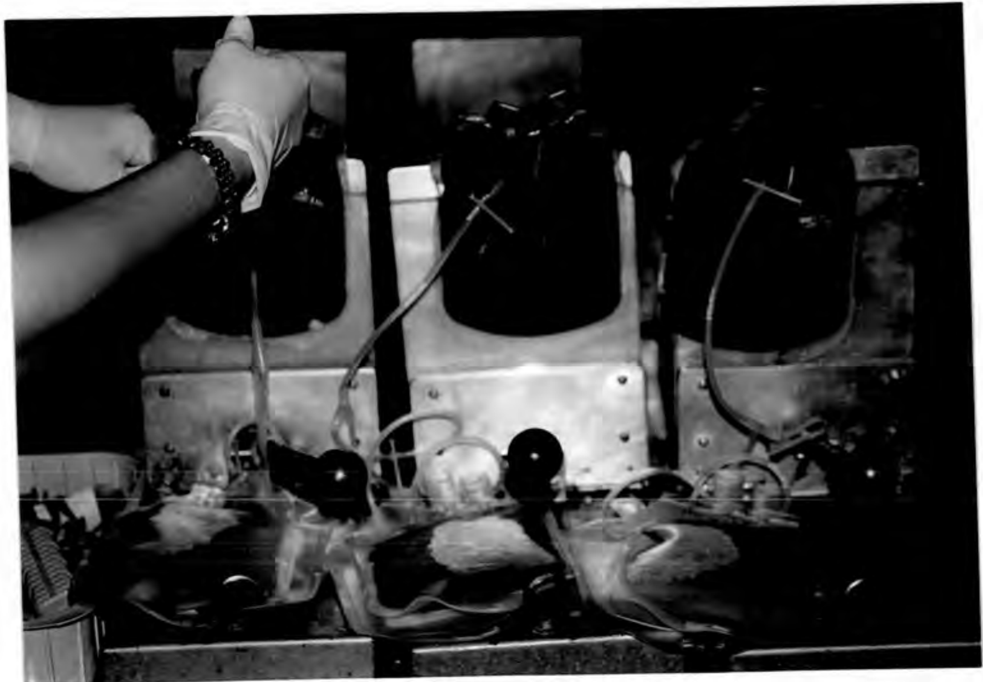

Figure 7 Three manual plasma separators with the triple bags of bloods after first spin centrifugation. Platelet-rich plasma (PRP) (on the top) is pressed out of the first bag to the the second one of the triple bags with the facility of the hand lever (**A**). PRP and packed red cell (PRC) are finally separated (**B**).

Figure 8 Triple bags with packed red cell (PRC) in the first bag and platelet-rich plasma (PRP) in the second one. The bag of PRC is then remove before subjecting two remaining bags for the preparation of platele concentrate (PC) under centrifugation with second spin at 3,420 rpm, 6 min, 22°C, Accel=9, Decel=5.

Figure 9 Plasma as platelet-poor plasma (PPP) is pressed out of the second bag of triple (on the top) to the third one (at the bottom) and left 50-60 ml of plasma inside the second bag as platelet concentrates (PC).

Figure 10 Platelet concentrates (PC) (three lower bags) after removed out of platelet poor plasma (PPP) (three upper bags).

Figure 11 The bags of platelet concentrates after removed out of PPP bags are being shaked on shelf of Melco linear platelet reciprocator in order to prevent platelets from clumping.

300 and 600 mg/dl were slowly added onto the pellet of platelets and the mixture was vortexed gently. Each incubation was performed in 5 replications. During incubation, the mixtures of platelets and fat emulsions were gently and regularly mixed by converting the tube and the incubation was carried out at 22°C for 1 h in shaking water bath. Later, the LRFE solutions were separated from platelets by centrifuging at 3,000 rpm, 10 min, 22°C, Accel = 3, Decel = 3. Platelets incubated with FM-LRFE and 20% Lipofundin were then washed 3 times with cold NSS whereas platelets incubated with SY-LRFE and SY-FOFE were washed 3 times with 1.055 potassium bromide (KBr) density solution and 1 time with cold NSS in order to remove the remaining LRFE. Washed platelets were employed for further analyses. Platelets incubated with liposome-free NSS were used as control and considered as concentration of PL at 0 mg/dl.

b) Incubation with Plasma

FM-LRFE working emulsions with PL concentrations of 100, 300 and 600 mg/dl were prepared by using plasma (not NSS) as diluent. Platelets were then incubated with FM-LRFE in autologous plasma following the procedure of incubation without plasma as described above. Thus, all conditions of incubation were corresponding to those performed with FM-LRFE in NSS diluent.

2. The Stability of Transferred PUFA on Platelets

Platelets were incubated with FM-LRFE at PL concentration of 600 mg/dl for 1 h at 22°C. They were then separated from the incubation mixture and stayed in NSS for 1, 3 and 5 h before washing 3 times with cold NSS. Washed platelets were processed for further analyses.

E. Chemical Analyses

1. Phospholipid Analysis

a) Preparation of Reagents

Phosphorus present in all forms of PL was turned into inorganic phosphorus and quantitatively assessed by its reaction with Fiske-Subbarow reagent according to the modified technique of Bartlett (1959). The Fiske-Subbarow reagent was prepared at 60 °C on heating-stirring plate. Half g of 1-amino-2-naphthol-4-sulfonic acid was added onto 200 ml of freshly prepared 15 percent anhydrous sodium disulphite ($Na_2S_2O_5$) under mechanical stirring followed by the addition of 1 g anhydrous sodium sulphite (Na_2SO_3). The finished solution was stored at 4°C and monthly prepared.

b) Sample Preparations

1) Fish Oil or Lecithins

One volume of oil or lecithins were dissolved in 10 volumes of dichroromethane-methanol mixture (2:1 v/v) and 20 μl of these solution was transferred into a 16 × 150 mm acid-washed glass tube for analysis.

2) LRFE

A 20 μl of lecithin-rich fat emulsion in each concentration was transferred into the tube for analysis.

c) Procedure

The tube containing samples as described above was heated to dry before being reconstituted in 0.2 ml of concentrated sulfuric acid. The mixture was heated in a 190°C sand bath for 30 min. Two drops of 30 percent hydrogen peroxide were then added and the mixture was returned to sand bath for another 45 min for completing the combustion and decomposing all the peroxide. Five ml of freshly prepared chromogen solution containing Fiske-Subbarow reagent-5% ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$-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 of blue color ended solution was recorded at 830 nm with the UV-1201 split-beam spectrophotometer. Di-sodiumhydrogenphosphate dihydrate ($HNa_2O_4P.2H_2O$, mw 178 with P = 31) at the concentration of 8 mg phosphorus/dl was used as standard. A calibration curve was performed with 3 phosphorus standard solutions of 2, 4 and 8 μg phosphorus per tube. For calculation, 1 mg of phosphorus is equivalent to 25 mg of PL and 1 mmole of phosphorus is equivalent to 1 mmole of PL.

2. TLC for Separation of TG and PL

TG and PL in the concentrated lipid extracts were separated from each other by technique of one-dimension TLC. The concentrated extracts of lipid were applied to the TLC plates by means of a microsyringe with an oblique needle point or a capillary pipette tip with their openings were placed against the adsorbent surface. A one dimension TLC utilized n-hexane-diethyl ether-glacial acetic acid (80:20:1, v/v/v) as a developing solvent. Spots of separated PL and TG were visualized in day light after spraying TLC plate with water. The silica powders contained PL and TG were scraped on plate-scaping system and collected in a screw capped tube for fatty acid analysis in the following section.

3. Fatty Acid Analysis

a) Fatty Acid Analysis of Oils

Fatty acid compositions of oils were determined after being transesterified 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 magnetic bar was immediately added. The saponification of lipids and methylation of liberated fatty acids were performed by using acetylchloride. In brief, a 200 µl of acetylchloride was added slowly in the vortexing sample tube, the tube was tightly closed and the fatty acids subjected to methanolysis with slow stirring at 100°C for 1 hour 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 to float hexane which dissolved occurring FAME's inside. 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 later herewith. A 8000 series gas chromatograph (GC) with a flame ionization detector was used. The separation of fatty acids methylesters (FAME's) 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-23 P/N 123-2332 (J&W Scientific, USA). Helium was used as carrier gas (1.5 ml/min at 80°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 80°C and after sample injection 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 220°C. The latter temperature was maintained constant for 15 min.

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

TG and 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 as 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 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.

c) Analysis of Fatty Acid Composition in Platelets

Platelets were treated for LRFE removal and the washed platelets 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 were vigorously mixed. The direct methylation of platelets fatty acids was performed by using acetylchloride according to the method of Guy and Lepage (1984) 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 SAS (Statistical Analysis System). The letter: 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 the similar letters were non-significant differrent. The changes of individual platelet fatty acid were calculated as relative membrane fatty acid changed as follow :

$$\text{Relative membrane FA changed} = (FA_x - FA_o) / FA_o \times 100$$

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

FA_o= platelets fatty acid (%) without incubation

The values of coefficient of determination (r^2), slope and Y-intercept in equation were calculated according to the regression analysis using programme of Microsoft Excel version 5.0 for Windows. The correlation (r), t and p values were calculated.