CHAPTER III

MATERIALS AND METHODS

Materials

A. Raw Materials

1. Fish Meal

The high quality Danish and local fish meals, used in the experiment were purchased from Charoenpokhapant Co., Ltd. Bangkok **(Figure 3).** The characteristics of proximate nutrient ingredient of fish meal are shown in **Table 1.**

2. Soybean lecithins

Soybean lecithins were donated by Dakuza Co., Ltd., Bangkok.

3. Fish oils

Fish oils were kindly supplied by T.C.Union Co., Ltd., Bangkok.

4. Vitamin C, Astraxantin, Vitamin mix and Mineral mix.

All of these vitamins and minerals were kindly delivered by Rovithai Co., Ltd., Bangkok.

5. Carrageenan

Carrageenan was gift from Dr. Charoen Nitithamyong.

6. Casien, Dextrin, Corn starch,Cellulose and Cholesterol

All of these materials were purchased from Sigma (St Louis, MO, USA).

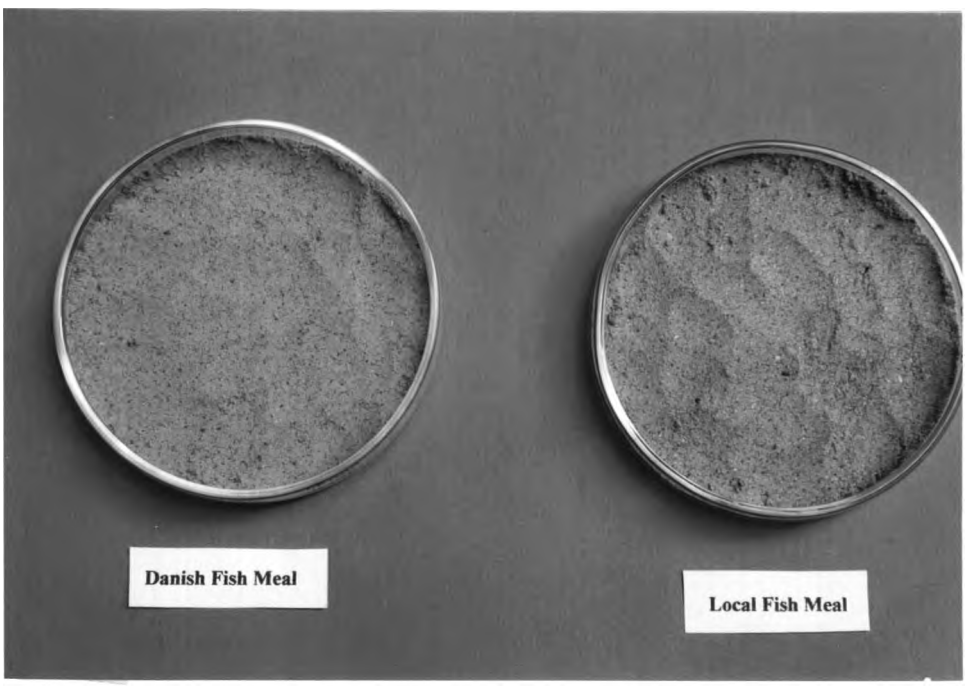

Figure 3 Danish and local fish meal, used in the experiment.

Table 1 Characteristics of proximate ingredients of fish meals used in present experiment

Ingredients	Danish fish meal	local fish meal
Protein	64.55	63.90
Carbohydrate	3.53	4.00
Fat	8.08	6.80
Moisture	7.52	7.10
Fiber	0.72	0.60
Ash	15.60	17.60

Data were obtained from the Fish Nutrition Laboratory, CU.

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

B. Plastic wares and Glasswares

Chambers for thin layer chromatography (TLC), Wheaton model 276860 (Wheaton, Millville, NJ, USA), were used in this experiment. Micro-reaction vessels (1 ml) with screw cap seal were obtained from Alltech (Alltech Associate Inc., Deerfield, IL, USA). Borosilicate glass tube (16 × 125 mm) with Teflon-lined screw caps were Pyrex® and regularly leak proved by re-weighing the tubes containing 2 ml methanol with tighly capped after boiling at 100 ° C for 1 h in water bath. The tubes retaining constant weight after boiling were chosen whereas those with weight lost were discarded. Glass bottle (1000 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.

C. Chemicals

Nitrogen gas used in the experiment was oxygen-free. Compressed gases were supplied by Thonburi – Watana Ltd., Part., Bangkok. All chemicals were reagent grade. Acetylchloride used for transesterifying lipids was purchased from Sigma (St Louis, MO, USA). Fatty acid methyl ester (FAME) products code no. GLC-408 and GLC-409 , and internal standards (IS) of fatty acids, C 15:0 code no. N-15-A and C 19:0 code no. N-19-A, were delivered by Nu-Chek-Prep (Elysian, MN, USA). IS of lipids: tripentadecanoin (TG – C 15:0), cholesteryl pentadecanoin (CE – C 15:0), and phosphatidylcholine dipentadecanoyl (PL – C 15:0), were purchased from Sigma (St Louis, MO, USA). The working solutions of 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 after the transesterification of IS's by the reaction of acetylchloride were known by calibrating them with known amount of heptadecanoic acid (C 17:0) 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 (Buchi R 114, Buchi Labortecnik AG, Flawil, Switzerland). The solvents used for lipid extraction and for thin layer chromatographic separation were dissolved with butylated hydroxytoluene (BHT: 2,6 – di–tert– butyl –4-methylphenol; Fluka Chemika, Switzerland) to 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 exposure to atmospheric oxygen (Phillips and Dodge, 1967).

TLC plates (20 × 20 cm) precoated with silica gel 60 without fluorescence 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 both 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.

D. Instruments

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) Sandbath , 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 H 11 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) Suction pump, model 523-U4-G21DX, MI, USA

(o) Ultra sonic bath, Decon FS 400 b, UK

(p) Magnetic stirrer, model 815359, INK Laboratechnik, Germany

(q) TLC plate scraping system (home-made at FORC, Chulalongkorn University)

TLC plate scraping system used in the experiment 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 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 reduce the oxidation of PUFA possibly occurred during the time delay on dry TLC plate (Dahlan 1989).

METHODOLOGY

All biochemical analyses were carried out at the Fats and Oil Research Center (FORC), Department of Transfusion Medicine, Faculty of Allied Health Sciences, Chulalongkorn University.

A. Extraction of Lecithins from Danish and local fish meals

1. Elimination of Neutral Lipids

Danish fish meal and local fish meal samples were heated at 80 °C for 30 min to lessen the moisture content as well as to inactivate any active enzymes before the extraction. Three hundred grams of each fish meal were weighed and transferred into a glass bottle (100 ml). 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 processed for the second extraction following the same procedure. 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 triglycerides (TG). Precipitant of fish meal partially eliminated neutral lipids was kept for further extraction.

2. Extraction of Polar Lipids

Precipitant of fish meal from the previous section was brought for extraction of polar lipids 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 majority of lecithins obtained from methanol and n-hexane extractions were combined and weighed. Lecithin's lipids were characterized according to techniques as will be described in the section of Chemical Analysis.

B. Chemical Analyses

1. Phospholipid Analysis

a) Preparation of reagents

Phosphorus presenting 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 on 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 percent anhydrous sodium disulphite ($Na_2S_2O_5$), followed by the addition of 1 g anhydrous sodium sulphite (Na_2SO_3). The solution was filtrated by passing through the Whatman paper No.1 into a dark bottle. The solution was freshly prepared each month and stored at 4°C.

b) Lecithin preparation and analysis of lecithin

Lecithin was dissolved in to 10 volumes of dichloromethane - methanol mixture (2:1, v/v) and 20 µl of the solution was transferred into a 16× 150 mm acid – washed glass tube.

c) Procedure

The tube containing diluted lecithin sample as described above was heated in sandbath at 190°C until all solvent were evaporated. The lecithin residue was

redissolved in 0.2 ml of concentrated sulfuric acid. The mixture was re-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 the sand bath for another 45 min for completing the combustion and to decomposing 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) was added and the contents was mixed thoroughly. The contents was boiled for 10 min. in water bath. The blue color of ended reaction solution was assessed at optical density of 830 nm with the UV-1201 split-beam spectrophotometer. Di–sodiumhydrogen phosphatedihydrate (Na_2HPO_4. $2H_2O$, mw = 178 with P = 31) at the concentration of 8 mg phosphorus/dl was used as standard. A calibration curve was lined between 3 points of phosphorus standard solutions of 2, 4 and 8 μg phosphorus per tube . For the 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. Small volume of concentrated extracts of lipid was 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 under spray of water and scraped on plate-scaping system. The scraped powder was 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 composition of oils were determined by the preparation of methylester according to the technique described by Lepage and Roy (1985). Twenty μl of either diluted lecithin solution or diluted oil or all scraped powder was 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 magnatic bar were immediately added. The saponification of lipids and methylation of liberated fatty acids were achieved by the reaction of acetylchloride in methanol. In brief, a 200 μl of acetylchloride was added slowly in the vortexing sample tube, the tube was tightly closed and the fatty acid was 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. Any leak if occurred 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% K_2CO_3 solution were slowly added to stop the reaction and to neutralize the mixture. The tube was then shaken and centrifuged in order to float hexane with dissolved FAME. At the end of the process, a 1-2 μl of the hexane upper phase was injected into the gas chromatograph (GC) which was conditioned and programmed as described later herewith. A 8000 series GC with a flame ionization detector (FID) was used. The separation of individual FAME was performed in a 30 m fused silica capillaly 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 1:10. The temperature of injection port 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

Triglyceride (TG) and phospholipid (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 on a plastic scraping system and the sraped powder was collected directly without delay in lined screw-capped borosilicate test tube containing 2 ml of methanol- hexane (4:1,v/v) and the content was mixed vigorously. The methylation of fatty acids was acheived under the reaction of acetylchloride according to the method as previously described.

c) Analysis of Fatty Acid in Shrimp's Diets

Two grams of each diets was transferred into screw capped borocilicate test tube containing 6 ml of dichloromethane - methanol (2:1,v/v). The mixture was vortexed occasionally during 30 min. period and the filtrate was then collected in the centrifuge tube, measured the volume and filtered through a Whatman No.1 filter paper . The filtrate was transferred into new screw capped borosilicate test

tubes. Approximately 0.2 volume of 0.88% potassium chloride solution was added to the filtrate then mixed . The mixture was then left for 15 min. to allow the apparent separation of 2 phases. Two hundred μl of the lower phase was transferred to a new screw capped borosilicate test tube. The methylation of fatty acids was perfromed under acetylchloride reaction according to the method as previously described.

d) Analysis of Fatty Acid in Shrimps

Approximately 70 mg of Shrimp tissue was weighed and transferred for homogenizing 1 ml in 10 ml of methanol, followed by the addition of 20 ml of dichloromethane then the mixture was continuously homogenized for further 2 min. The homogenate was filtered through a Whatman No. 1 filter paper. The filtrate was kept in a glass stoppered bottle. The solid residue left on filter paper was then mixed with 30 ml of chloromethane:methanol (2:1, v/v) and furtherly homogenized for 3 min. The residual was rinsed one more time with 20 ml of dichloromethane folowed by 10 ml of methanol and the filtrates were combined in a measuring cylinder. One – quarter volume of 0.88 % potassium chloride was added into the combined filtrates then the mixture was swirled before allowing to settle for 20 min. The upper phase, which is the aqueous layer, was removed by aspiration. The lower phase was mixed with one – quarter of water :methanol (1:1, v/v). The mixture was allowed to settle for 20 min and the upper layer of polar solvent was removed by aspiration. The lower layer of nonpolar was evaporated by rotary evaporator at 27 ^{o}C in order to concentrate the dissolved lipid. Fatty acid composition of the obtained lipid was investigated according to the methylation method as previously described.

C. Effect of Lecithin on Growth, Survival and Stress Resistance of *P. monodon* Larvae at Salinity 25 and 30 ppt

The study was carried out at the Marine Biotechnology Research Unit, Department of Marine Science, Chulalongkorn University. A complete randomized design involving factorial 4 diets containing different sources of lecithin and two salinity conditions with three replications were done in each treatment combination. The sources of lecithin were soybean lecithin (SL), Danish lecithin (DL) and local lecithin (LL). The amount of lecithin in each diet was controlled at 1.5 g/100 g diets. Salinity used in this experiment was 25 and 30 ppt. Four diets employed in this experiment were soybean lecithin - added diet (SAD), Danish fish meal lecithin - added diet (DAD), local fish meal lecithin - added diet (LAD) and lecithin -free diet or control diet (CD).

Shrimp nauplii obtained from Banchong's Farm, Chachoensao province were used in this experiment. The feeding tests were separated into three stages of larvae as follows: zoea I to zoea III, mysis I to mysis III and postlarva 1 to postlarva 15. The shrimp larva were assigned into each plastic aquarium containing 5 ml of sea water as shown in **Figure 4**. The density was 300 larvae/aquarium in zoea stage, 100 larvae/aquarium in mysis stage and 50 larvae/ aquarium in postlarva stage.

The shrimp larva were daily fed diets for five periods at 8.00, 11.00, 14.00 , 17.00 and 20.00 h. The rearing condition was performed in a static system with partial water change (about two-third of total volume everyday) and gently aerated. Water temperature during experiment was 27-30 $^{\circ}$ C .

1. Processing of Larval Diets

The diet was a purified microparticulated diet which was modified from Kanazawa (1985). The diets were prepared by blending all dry ingredients with particle size ≤ 20 micron (**Table 2**), mixed well, then fish oil and lecithin were added and mixed for 20 min. Thirty ml of water was then added to the mixture. Carrageenan was dissolved in 20 ml of water and heated at 80° C for 10 min, then added into the mixture. Vitamin C dissolved in 20 ml distilled water was also added to the mixture. After well mixing, the mixture was poured into the tray, left at cool temperature . The diet was cut into small pieces (about 2×2 cm) and dried under freeze-dryer. After completely drying, the diet was blended and sieved for the separation into three parts; size≤55 micron (for zoea) , > 55 –150 micron (for mysis) and > 150 –300 micron (for postlarva). The microparticulated diets were kept in a dark, sealed container, flushed with nitrogen gas and stored at 4° C until used. Proximate analysis of diets was carried out according to AOAC (1980).

2. Salinity stress testing

The stress resistance of the larva was studied at the stage of postlarva 15 as follows. Ten larvae from each replication of diets were immediately transferred from 25 ppt and 30 ppt (normal condition) to 0 ppt (stress condition). Number of shrimp mortaltity was recorded every 10 min. The total period of low salt tolerance was conducted for two hours. The details of the techniques was described by Ree et al. (1994)

Figure 4 Experimental units for rearing *P. monodon* larvae

Table 2 The Composition of the diet

Ingredients	Content (g/ 100 g feed)			
	SAD	DAD	LAD	CD
Casein	55.0	55.0	55.0	55.0
Dextrin	15.5	15.5	15.5	15.5
Fish oil	8.0	8.0	8.0	8.0
Mineral mix[a]	8.0	8.0	8.0	8.0
Vitamin mix[b]	4.0	4.0	4.0	4.0
Lecithin	1.5 (SL)	1.5 (DL)	1.5 (LL)	0.0
Cholesterol	1.0	1.0	1.0	1.0
Carrageenan	5.0	5.0	5.0	5.0
Cellulose	1.8	1.8	1.8	3.3
Vitamin C	0.2	0.2	0.2	0.2
Astraxanthin	0.0025	0.0025	0.0025	0.0025

[a] Mineral mix 100 g contains: K_2HPO_4 , 2.0g; $Ca_3(PO_4)_2$, 2.720 g; $MgSO_4.7H_2O$, 3.041 g; $NaH_2PO_4.2H_2O$, 0.790 g.

[b] Vitamin mix 100 g contains: ρ-Aminobenzoic acid, 10.0 mg.; Biotin, 0.40 mg.; Inositol, 400.0 mg.: Ca - Pantothenate, 60.0 mg.; Pyridoxine- HCl, 12.0 mg.; Riboflavin, 8.0.; Thiamin-HCl, 4.0 mg.; Menadione, 4.0.; α -Tocopherol, 20.0 mg.; Cyanocobalamine, 0.08 mg.; Calciferol , 1.20 mg.; Folic acid , 0.80 mg.; Choline chloride, 120.0 mg.

SL = soybean lecithin

DL = Danish fish meal lecithin

LL = local fish meal lecithin

3. Data collection

Survival of the larvae was recorded at the end of each larval stage. Survival rate was calculated at the final number in proportion to the initial number of the larvae. Growth rate was monitored by measuring the length of postlarva 15. Ten larvae from each replication were randomly sampled for growth measurement. For salinity stress test, number of shrimp mortality was recorded every 10 min during 2 h. period.

For water quality, two – thirds volume of the total water volume was withdrawn and replaced by the filtered 25 and 30 ppt sea water daily. Salinity was determined by a reflectometer. Levels of ammonium, nitrate and pH of water were monitored by Merck′ s test kits once for zoea stage and mysis stage and twice for postlarva stage.

D. Statistical Analysis

All statistical significances were calculated by one –way analysis of variance (ANOVA) with Duncan′ s new multiple range test by program of SPSS / PC + for windows and Statistic Analysis System (SAS) program (SAS, 1985). Means between treatments were compared with Duncan′s new multiple-range test.