CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Chemicals	Company	Grade
1. Acetic acid	Merck	Suprapure
2. Adjuvant complete Freund	Sigma	AR
3. Bovine serum albumin	Sigma	AR
4. Citric acid	Merck	AR
5. Conc. Sulfuric acid (H_2SO_4)	Merck	AR
6. Dihydrogen posphase (KH_2PO_4)	Merck	AR
7. Distilled water	-	AR
8. Ethyl acetate	Lab-Scan	HPLC
9. Gelatin	Merck	AR
10. Hexane	Fisher Scientific	HPLC
11. Horseradish peroxidase goat anit-mouse IgA	Zymed Lab.	AR
12. Horseradish peroxidase goat anit-mouse IgG	Zymed Lab.	AR
13. Horseradish peroxidase goat anit-mouse IgM	Zymed Lab.	AR
14. Hydrochloric acid	Merck	AR
15. Isopropanol	Merck	HPLC
16. Nitrogen gas	-	AR
17. O-phenylen-diamine-HCL	Zymed Lab.	AR
18. Peroxide	Sigma	AR
19. Potassium chloride (KCl)	Riedel-de Haen	AR
20. Silica gel	Merck	AR
21. Sodium carbonate	Riedel-de Haen	AR

Chemicals	Company	Grade
22. Sodium chloride (NaCl)	Merck	AR
23. Sodium hydrogen carbonate ($NaHCO_3$)	Riedel-de Haen	AR
24. Sodium hydrogen phosphate-12-hydrate ($Na_2HPO_4.12H_2O$)	Riedel-de Haen	AR
25. Sodium hydroxide (NaOH)	Merck	AR
26. Standard γ-oryzanol	Restek	HPLC
27. Tween 80	Sigma	AR

3.2 Materials and equipments

Materials and equipments	Model	Company	Nation
1. 3-way stopcock	-	Nipro Medical	Japan
2. 96-well microplate	-	Nalge Nunc	Denmark
3. Buchner funnel	-	-	-
4. Centrifuge	Mistral300	MSE co.	England
5. Column Chromatography	25x250 mm	-	Thailand
6. Electronic weight	2842	Sartorius	Germany
7. Filter paper	#4	Whatman	England
8. Guard Column	-	Phenomenex	U.S.A.
9. High Performance Liquid	10A	Shimadzu	Japan
10. Inertsil (5µm, 150x4.6mm I.D.)	CN-3	GL-Science	Japan
11. Membrane filters	0.45µm	Gelman Sciences	U.S.A.
12. Microperpex peristaltic pump	PA-SF	IKA	Germany
13. Micropipette	Pipetman	Gilson	France
14. Microplate reader	2010	Anthos Labtech	Germany
15. Multichannel micropipette 200	-	Eppenddorf	Germany
16. Needle	-	Nippo	Thailand
17. Oven	ULE 400	Memmert	Germany
18. Parafilm	-	American	USA

Materials and equipments	Model	Company	Nation
19. Refrigerated centrifuge	6930	KUBOTA	Japan
20. Refrigerator (- 20 °C)	WCF-95L	Whirlpool	Thailand
21. Rotary evaporator	R-124	BUCHI	Switzerland
22. Sonicate	ME-11	Mettle electronics Corp.	U.S.A.
23. Syringe	-	Nippo	Thailand
24. Vortex	K-500 GE	Labinco	USA
25. Water distiller	MAXIMA	ELGA	England

3.3 Animals

32 Institute of Swiss albino male mice 6 weeks-old from Mahidol University were used in this experiment.

3.4 Methods

There were two experiments in this study. The objective of the first experiment was to find out the effect of pure γ-oryzanol supplemental diets on male mouse humoral immune response in which bovine serum albumin (BSA) antibody titer after immunization was used as an indicator. Experiment II investigated the effect of natural γ-oryzanol in purple glutinous rice bran by feeding purple glutinous rice bran supplemental diets on male mouse humoral immune response in which BSA antibody titer after immunization was used as an indicator. The plan has shown in Figure 18.

3.4.1 Experiment I

The study aimed to investigate the effect of four levels of pure γ-oryzanol supplement to mouse control diet on BSA antibody titer. A complete randomized design was applied to the experiment. 32 Swiss albino 6-week-old male mice, selected randomly, 4 mice per pen. There were 2 pens per treatment, γ-oryzanol were added 4 levels; 0, 280, 800 and 1,340 mg/kg in mice diets, refer by body weight of human level supplement (General Nutrition Centers, 1999). All mice were fed with control diet on preliminary period until 7 weeks old. Feed and water were given *ad libitum*. All mice were given BSA immunization 3 times at day 1, 14 and 28 respectively. Mice

plasma were collected before immunization on day 1 and day 10, 14, 21, 28 and 42 respectively, and then stored at -20 °C. IgA, IgM and IgG titer were evaluated by Enzyme-Linked Immunosorbent Assay (ELISA). The ration and nutrients content has shown in Table 4 and 5.

Treatments:

T1: Control diet + Pure γ-oryzanol 0 mg /kg (Control group)

T2: Control diet + Pure γ-oryzanol 280 mg /kg

T3: Control diet + Pure γ-oryzanol 800 mg /kg

T4: Control diet + Pure γ-oryzanol 1,340 mg /kg

3.4.2 Experiment II

32 Swiss albino male mice 6-week-old were selected randomly and assigned into 4 groups. A complete randomized design was applied to the experiment. There were 4 mice per pen and 2 pens per group. Three groups of mice treated with purple glutinous rice bran at 0, 6 (equal to pure γ-oryzanol 1,340 mg/kg), and 8 % (equal to pure γ-oryzanol 1,800 mg/kg) in diets and the fourth group supplemented with 1,340 mg/kg pure γ-oryzanol. All mice were fed with control diet on preliminary period until 7 weeks old. Feed and water were given *ad libitum*. Mouse humoral immune response in which BSA an antibody titer after immunization were used as an indicator. The BSA antibody titer were evaluated before and after immunizing with BSA for the 42 trial days. All mice were given BSA immunization 3 times at day 1, 14 and 28 respectively. Mice plasma were collected before immunization on day 1 and day 10, 14, 21, 28 and 42 respectively, and then stored at -20 °C. IgA, IgM and IgG titer were evaluated by Enzyme-Linked Immunosorbent Assay (ELISA). The ration and nutrients content has shown in Table 6 and 7.

Treatment

T1: Control diet without rice bran (0 mg /kg γ-oryzanol approximately), (Negative control)

T2: Control diet with 6 % purple glutinous rice bran (1,340 mg /kg γ-oryzanol approximately)

T3: Control diet with 8 % purple glutinous rice bran (1,800 mg /kg γ-oryzanol approximately)

T4: Control diet + Pure γ-oryzanol 1,340 mg /kg (Positive control)

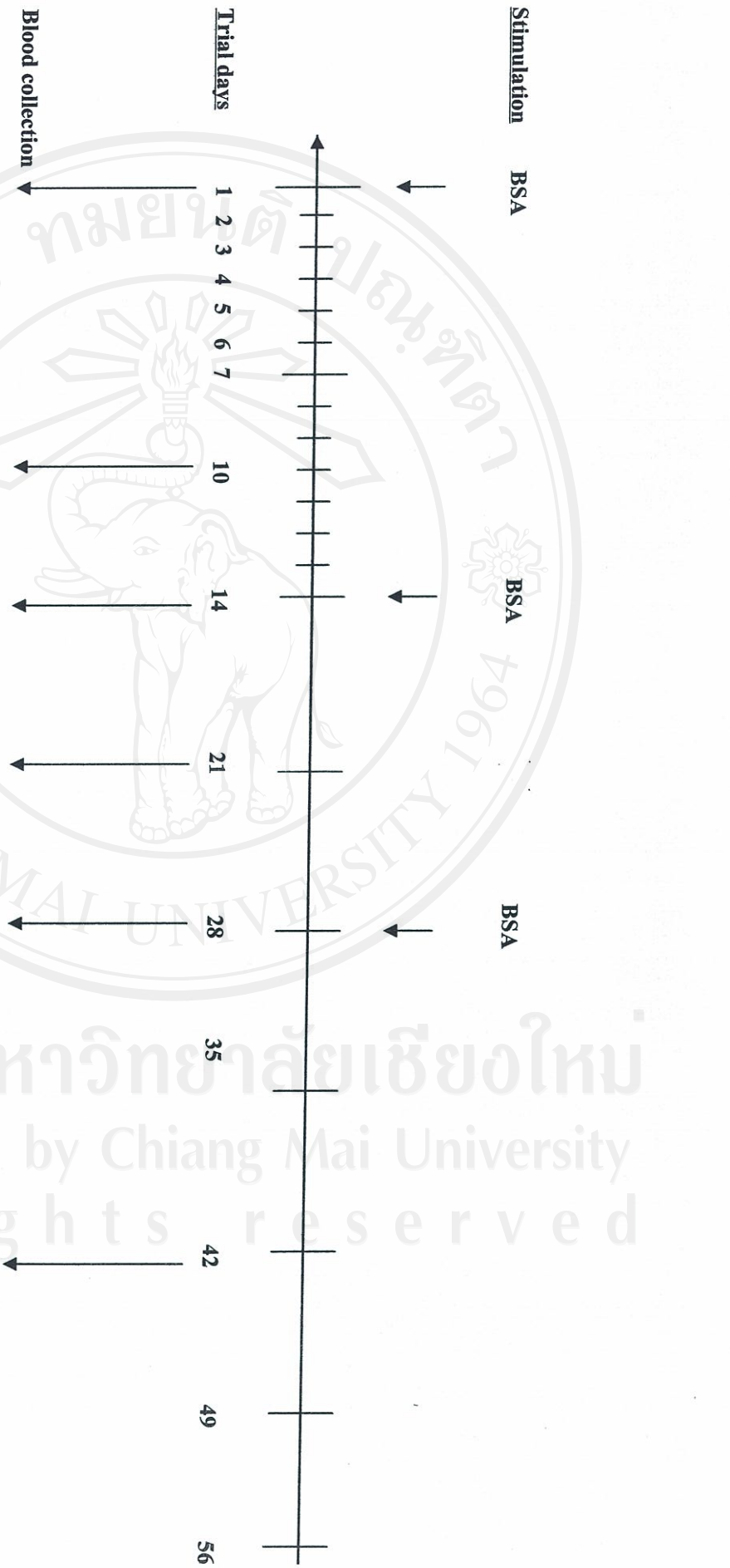

Figure 18. Program of blood collection and immunization in the experiment.

3.5 Determination of γ-oryzanol in purple glutinous rice bran

The method was modified from Xu and Godber (1999) to detect γ-oryzanol in purple glutinous rice bran. γ-oryzanol was concentrated using reverse phase HPLC by three steps as follows.

3.5.1 Step I extraction of crude oil.

Twenty five grams of rice bran were placed in a 500-ml round-bottom flask with 1 gm of ascorbic acid, 35 ml of hexane, and 15 ml of ethyl acetate. The flask was attached to a rotary evaporator, with vacuum and placed in a 60 °C water bath for 40 minutes with rotation at 180 rpm. Then 25 ml of distilled water was added to the flask. The flask was placed on the rotary evaporator at the same temperature and rotation speed for 10 minutes. Solvent was separated from rice bran residue by filtration. Rice bran residue was reextracted 2 more times. The extracted were pooled and centrifuged at 4,100 g for 10 minutes. The organic solvent layer was evaporated in a rotary evaporator under vacuum at 60 °C to obtain crude oil. The diagram of method was shown in Figure 19.

3.5.2 Step II semipurification of γ-oryzanol using a low-pressure silica column.

A glass column (2.5 cm x 25 cm) packed with 20 gm of silica was used to remove the triglycerides and other lipids. Initially, the crude oil was solubilized in 50 ml of the solvent (hexane/ethyl acetate 9:1) for flushing through the column. Then 50 ml of solvent (hexane/ethyl acetate 7:3) was allowed to flow through the column, and the eluant were collected. The column was then washed with 50 ml of hexane/ethyl acetate (1:1), and the semipurified γ-oryzanol was obtained after the solvent was evaporated. The diagram of the method was shown in Figure 20.

3.5.3 Step III determination of γ-oryzanol using a reverse-phase HPLC.

The reverse-phase HPLC system consisted of an inertsil CN-3 (5μm , 150 x 4.6mm I.D.) HPLC column, a guard-pak, a 10A auto-injector, a 10A pump, a 10A LC spectrophotometer detector and 10A chromatography workstation (Shimadzu) were used to record the chromatogram and calculate the γ-oryzanol concentrations. The diagram of method was shown in Figure 21.

Extraction of crude oil

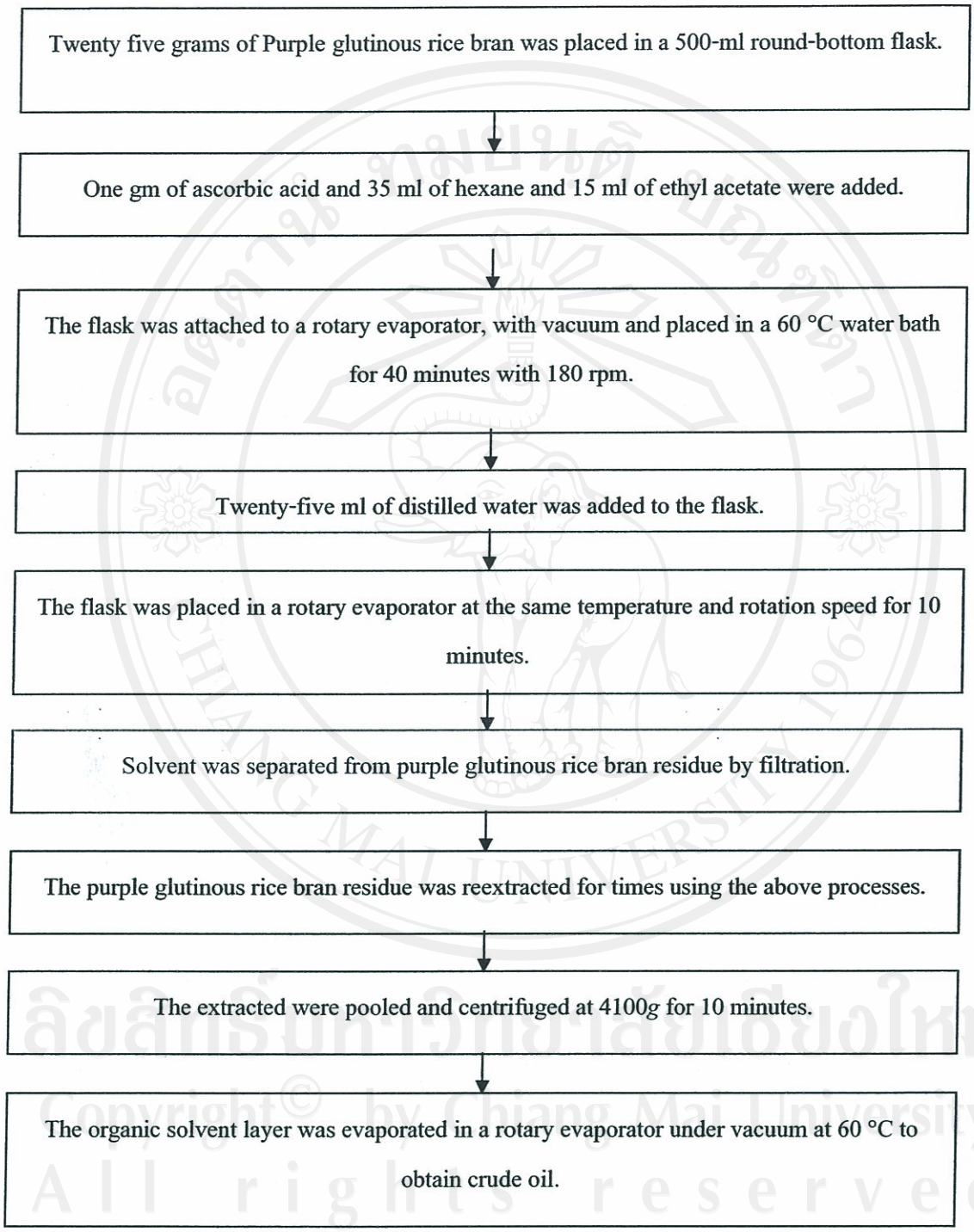

Figure 19. Diagram of extraction of crude rice bran oil by solvent extracted method (Adapted from Xu and Godber 1999).

Semipurification of γ-oryzanol

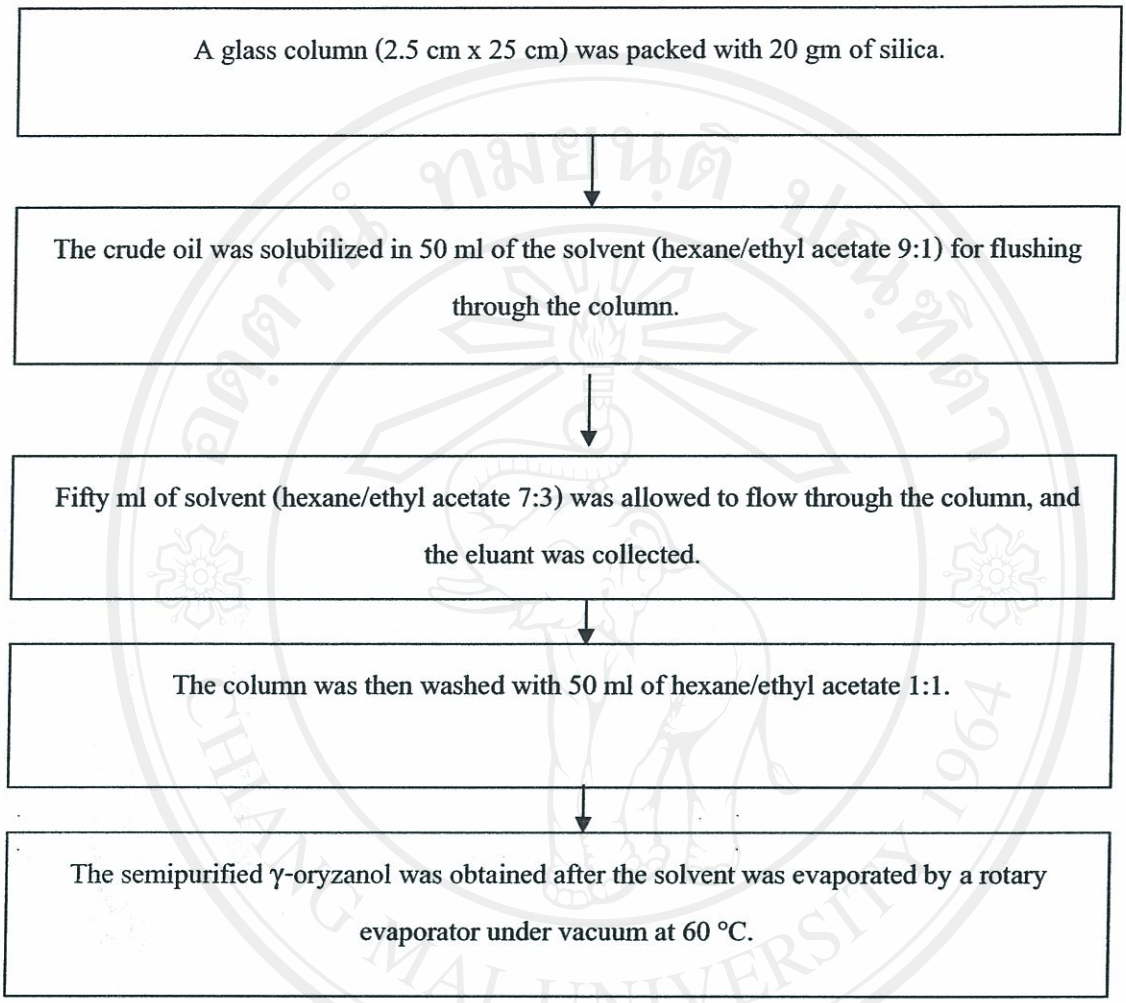

Figure 20. Diagram of semipurification of γ-oryzanol by low-pressure silica column method (Adapted from Xu and Godber 1999).

Determination of γ-oryzanol

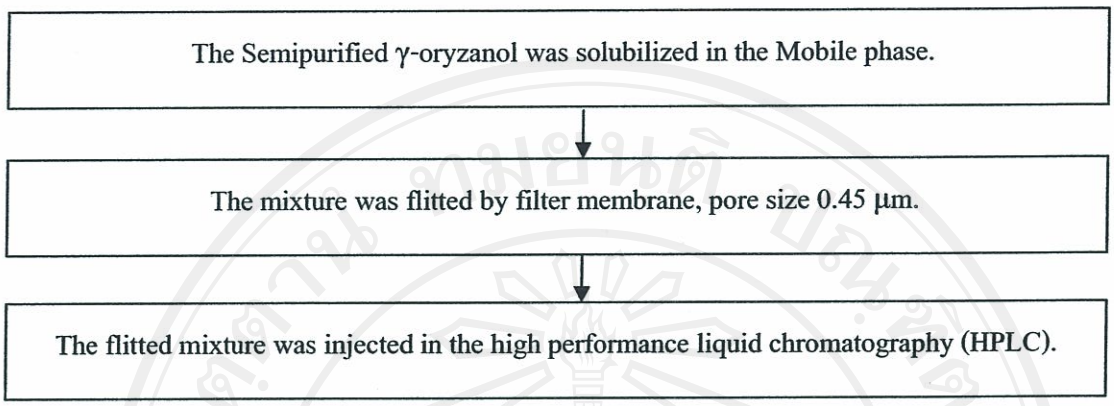

Figure 21. Diagram of determination of γ-oryzanol by reverse phase HPLC method (Adapted from Xu and Godber 1999).

HPLC condition for γ-oryzanol determination are as followed:

- Column : Inertsil CN-3 (5μm , 150 x 4.6mm I.D.)
- Flow rate : 1.0 ml/min
- Eluent : Hexane : Isopropanol : Acetic acid (94:5:1)
- Oven Temp. : 30 °C
- Detector : UV 320 nm
- Standard : γ-oryzanol 10 μl
- Sample volume : 2 μl

3.6 Feed preparation

There were two experimental rations in this study. Experimental rations were formulated according NRC (1978) standard. In the first experiment, rations were supplemented at four levels (0, 280, 800 and 1,340 mg per kg) of pure γ-oryzanol to mouse control diet. In the second experiment ration was assigned into 4 groups. Three groups were supplemented at three levels (0, 6 and 8 percent in diets) of purple glutinous rice bran and the fourth group was supplemented with pureγ-oryzanol 1,340 mg per kg to mouse control diet. The ration was packed tightly and placed in a dry, cool, dark room. Purple glutinous rice bran was tasted to determine total γ-oryzanol, from its natural state before feed formulation.

Table 4. Feed ingredients of experimental diets in experiment I

Ingredients	T1 (%)	T2 (%)	T3 (%)	T4 (%)
Cassava	40.5	40.5	40.5	40.5
SBM 45%	20.3	20.3	20.3	20.3
FM 60%	15.2	15.2	15.2	15.2
Sugar	10.0	10.0	10.0	10.0
Skim milk	5.1	5.1	5.1	5.1
Tallow	2.0	2.0	2.0	2.0
Whey 13%	2.0	2.0	2.0	2.0
Molasses	2.0	2.0	2.0	2.0
Lysine	1.2	1.2	1.2	1.2
DL-Met	0.6	0.6	0.6	0.6
Salt	0.2	0.2	0.2	0.2
Pure γ-oryzanol (mg/kg)	0	240	800	1,340

Soy been meal (SBM), fish meal (FM)

Table 5. Chemical composition of experimental diets of in experiment I by proximate analysis

Composition (%DM)	T1	T2	T3	T4
Dry matter	91.0	91.3	91.4	91.4
Crude protein	24.5	24.3	24.4	24.5
Crude fat	5.0	5.0	4.9	5.0
Crude fiber	3.7	36.7	36.0	36.5

Table 6. Feed ingredients of experimental diets in experiment II

Ingredients	T1 (%)	T2 (%)	T3 (%)	T4 (%)
Cassava	38.8	40.6	39.8	38.8
FM 60%	-	15.2	14.9	-
SBM 45%	29.1	10.2	10.0	29.1
Sugar	9.7	10.2	10.0	9.7
PGRB	0	6.0	8.0	0
Skim milk	4.8	5.1	5.0	4.8
Vegetable oil	4.8	5.1	5.0	4.8
Molasses	4.8	-	-	4.8
MCP	2.9	3.0	3.0	2.9
Whey 13%	2.4	2.5	2.5	2.4
DL-Met	0.78	0.15	0.15	0.78
Salt	0.48	0.50	0.50	0.48
γ-oryzanol (mg/kg)	0	0	0	1,340

Soy been meal (SBM), fish meal (FM), purple glutinous rice bran (PGRB), mono-calcium-phosphate (MCP)

Table 7. Chemical composition of experimental diets of in experiment II by proximate analysis

Composition (%DM)	T1	T2	T3	T4
Dry matter	90.3	91.2	91.4	90.4
Crude protein	17.9	17.0	16.5	17.7
Crude fat	6.3	7.4	7.5	6.2
Crude fiber	3.8	4.0	4.3	3.8

3.7 Humoral immunity experiment

3.7.1 Immunization the mice with BSA

The antigen was prepared by mixing BSA with complete Freund adjuvant in equal volume and homogenized by 3-way stopcock. The mice were immunized by subcutaneous injection at days 1, 14 and 28.

3.7.2 Blood samples collection

The blood samples were collected from the tail vein by hematocrit capillary tube into non-anticoaggulant tube at days 1, 10, 14, 21, 28 and 42. Then let it stand to clot. Serum samples were obtained by centrifugation; the supernatants were transferred and diluted to 1:10. Samples were kept at -20 $^{\circ}$C and thawed for measuring BSA antibody titer.

3.7.3 Determination of BSA antibody titer

This method was used to detect antibody against BSA in the plasma of the mice. BSA was diluted at 10 μg/ml by coating buffer and coated in the 96-well microplate 100 μl/well and incubated at 4 $^{\circ}$C overnight. After incubation, the plate was washed by washing buffer 200 μl/well 3 times. 1 % gelatin 200 μl/well was added and incubated at 37 $^{\circ}$C for 1 hour. After incubation, the plate was washed by washing buffer 200 μl/well 3 times. Added mice plasma 100 μl/well and incubated at 37 $^{\circ}$C for 1 hour. After incubation, the plate was washed by washing buffer 200 μl/well 3 times. Horseradish peroxidase conjugated goat anti-mouse IgA or IgM or IgG 20 μl/well was added and incubated at 37 $^{\circ}$C for 1 hour. After incubation, the plate was

washed by washing buffer 200 µl/well 5 times. Substrate solution 100 µl/well and incubated at 37 °C for 30 minutes. Stopping solution 50 µl/well was added and the optical density at 492 nm was read by ELISA reader. The methods are shown in Figure 22.

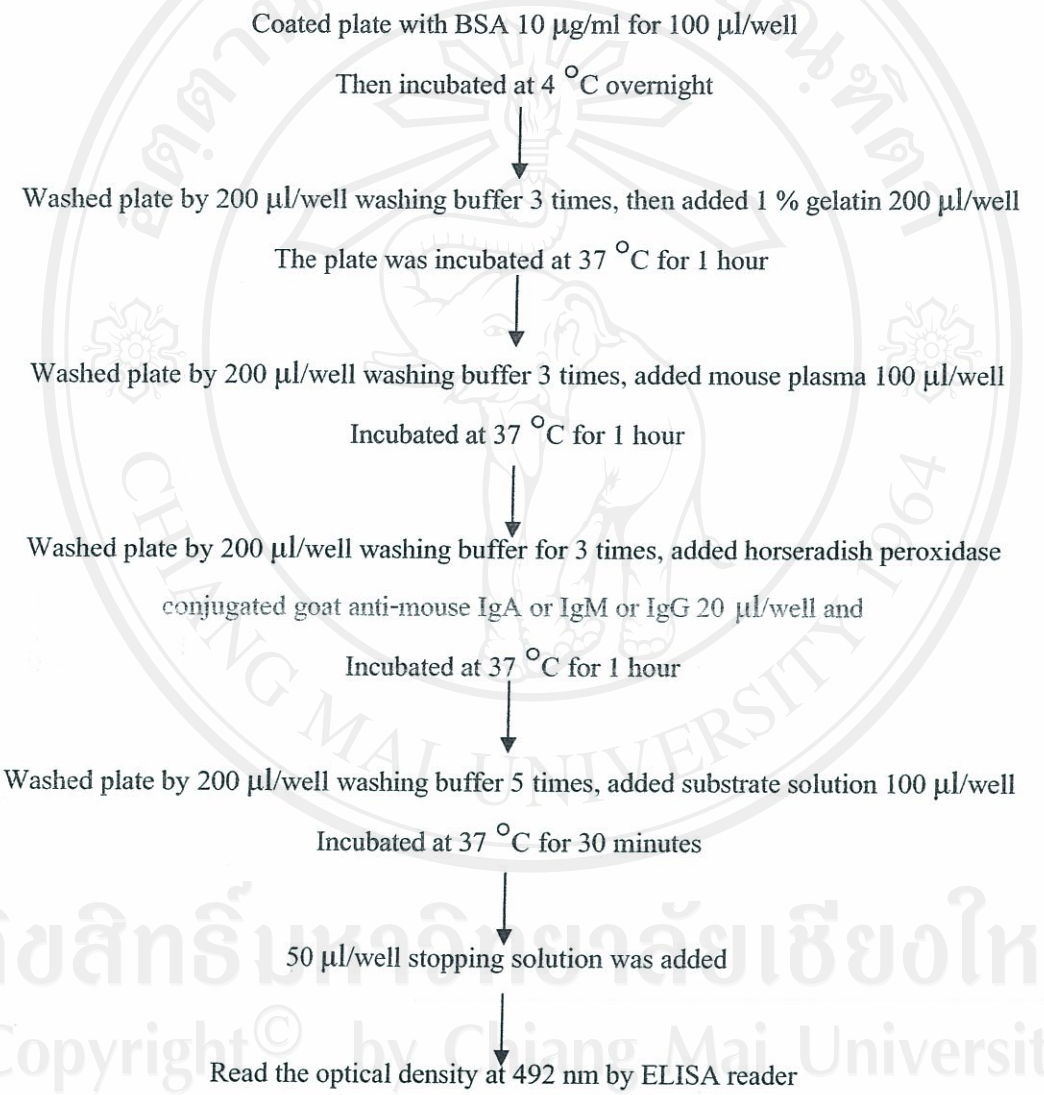

Figure 22. Diagram of indirect ELISA method for screening antibody titer in mouse serum (adapted from Crowther 1995).

3.8 Inter and intra coefficient of variance (CV) assay

The method was adapted from Butter *et al.,* (2001). Three plates were coated with BSA 10 μg/ml for 100 μl/well and incubated at 4 °C overnight. After incubation, the plate was washed by washing buffer 200 μl/well 3 times. 1 % gelatin 200 μl/well was added and incubated at 37 °C for 1 hour. After incubation, the plate was washed by washing buffer 200 μl/well 3 times. Mouse plasma from only one 100 μl/well was added and incubated at 37 °C for 1 hour. After incubation, the plate was washed by washing buffer 200 μl/well 3 times. Horseradish peroxidase conjugated goat anti-mouse IgA or IgM or IgG 20 μl/well was added and incubated at 37 °C for 1 hour. the plate was washed by washing buffer 200 μl/well 5 times. Substrate solution 100 μl/well was added and incubated at 37 °C for 30 minutes. Stopping solution 50 μl/well was added and the optical density at 492 nm was read by ELISA reader. The coefficient variance was calculated from the equation below:

$$CV = (\sqrt{MS\ error} / Grand\ mean) \times 100$$

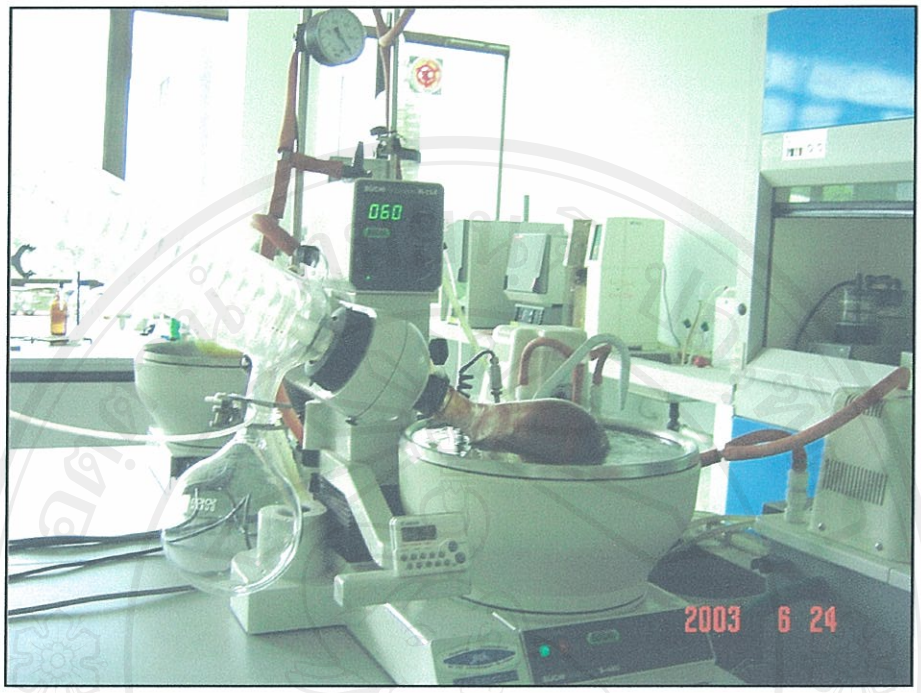

Figure 23. Crude oil extraction by using rotary evaporator.

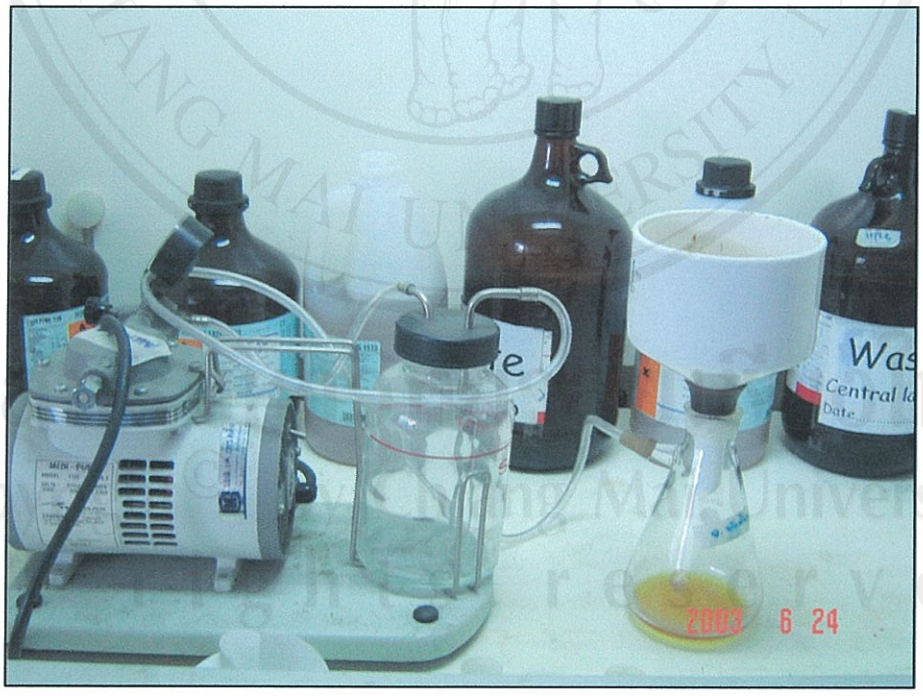

Figure 24. Crude oil filtrations through Buchner funnel.

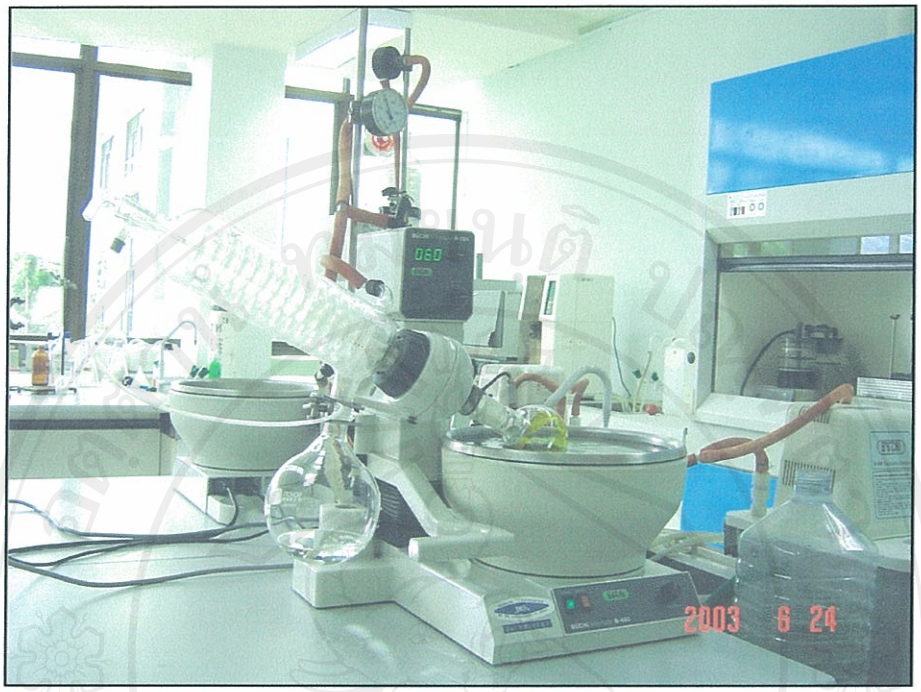

Figure 25. Solvent was evaporated from crude oil using rotary evaporator.

Figure 26. Crude oil drying using nitrogen gas flow.

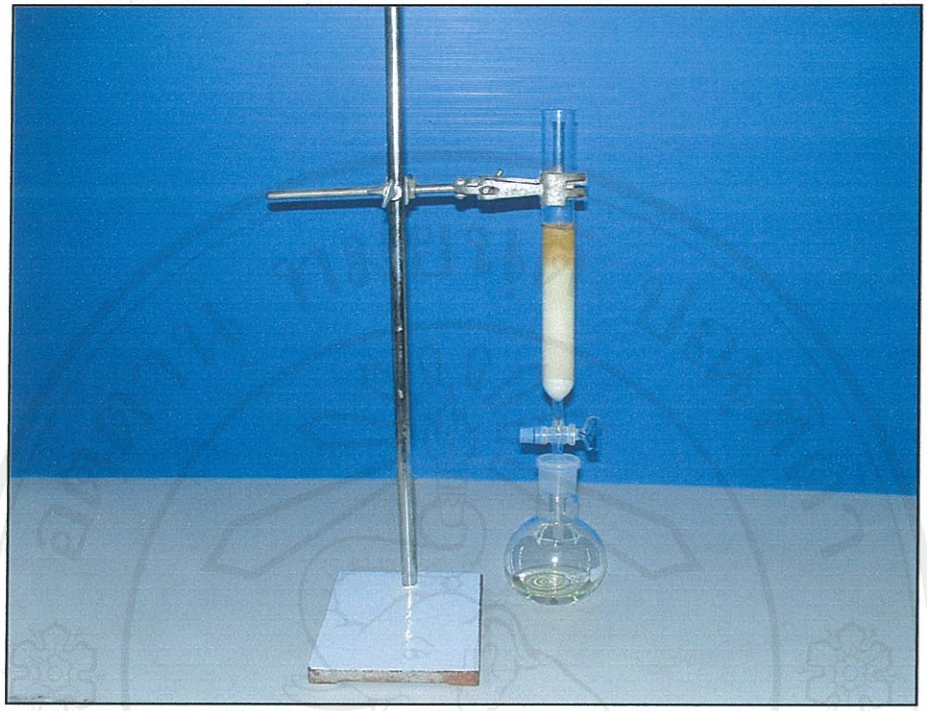

Figure 27. Semi-γ-oryzanol extraction from crude oil using column chromatography.

Figure 28. Determination of γ-oryzanol using high performance liquid chromatography (HPLC).

Figure 29. Bovine serum albumin homogenization with adjuvant complete Freund.

Figure 30. Immunization the mice with BSA at subcutaneous (Tip cap of needle was cut 10 mm approximately before injection to protect muscle injured).

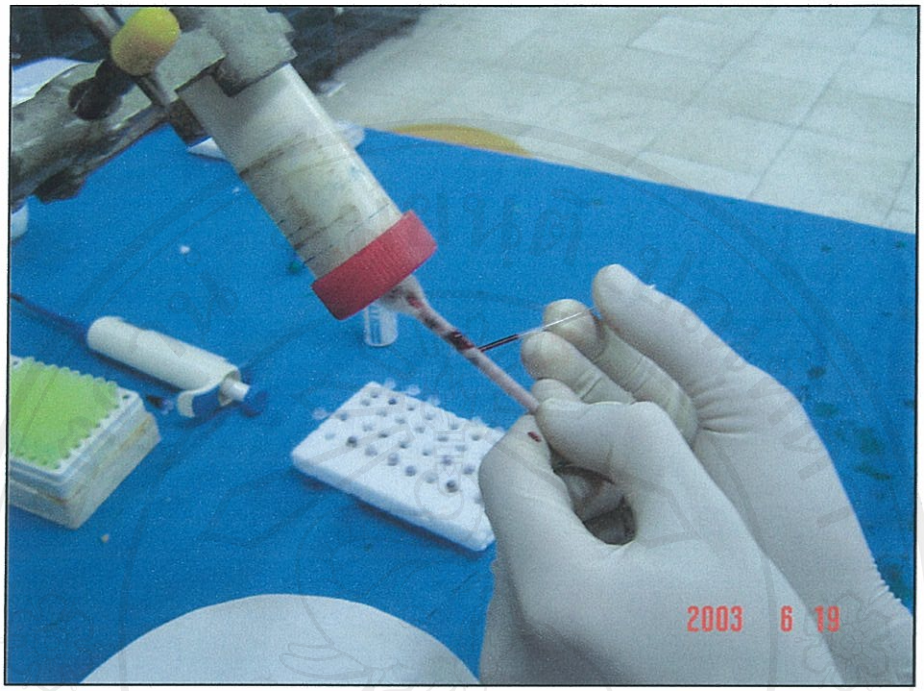

Figure 31. Blood collection at the tail vein by hematocrit capillary tube.

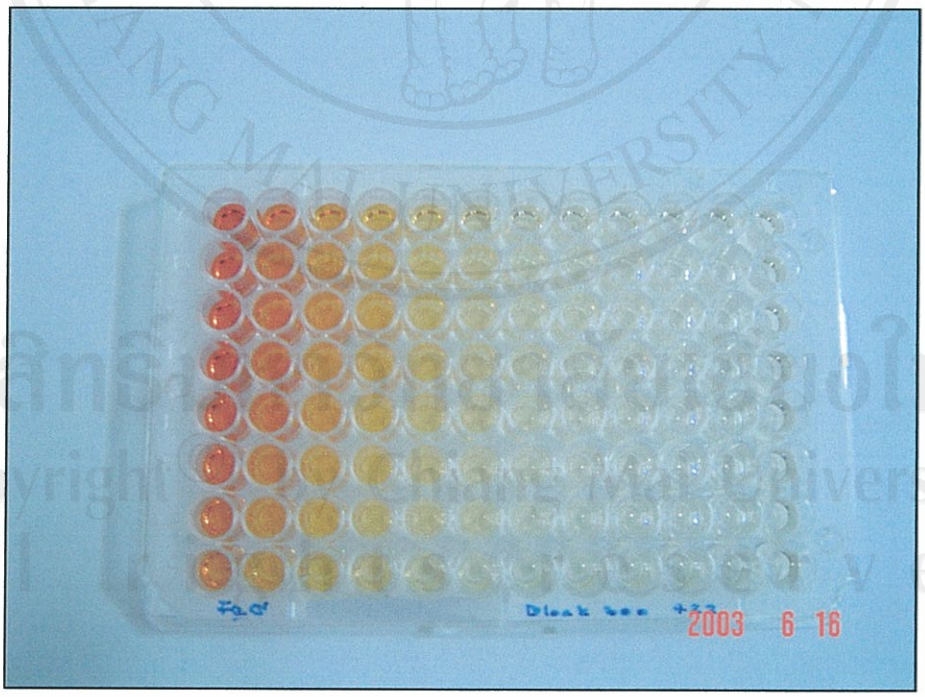

Figure 32. Determination of antibody titer using indirect ELISA method.