CHAPTER III

MATERIALS AND METHODS

Materials

1) (±)-α-Tocopherol 95.00% (Sigma,USA) Lot No.055K0674
2) γ-Oryzanol 98.7 % (Tsuno Rice Fine chemicals Co.,Ltd. Japan) Lot No.F03170
3) 1,1- diphenyl-2-picrylhydrazine stable radical (DPPH)(Sigma-Aldrich,Inc.,USA) Lot no. 51K1419
4) Acetonitrile HPLC grade (Labscan Asia Co.,Ltd., Thailand) Batch No.07070231
5) Activated Charcoal (VWR International Ltd, England) Lot No. K334420270
6) Beeswax (supplied by Hong Huat Co.,Ltd., Thailand) Lot No. P12064
7) Cetyl alcohol (supplied by Hong Huat Co.,Ltd., Thailand)
8) Cutina®GMS-SE (Cognis Thai Co., Thailand)
9) DC RM 2051® (SodiumPolyacrylate & Cyclomethicone & Dimethicone & Dimethicone copolyol) (donated from Summit Chemical Co., Ltd., Thailand)
10) Dichloromethane, HPLC grade (Labscan Asia Co.,Ltd., Thailand) Batch No. 06070009
11) Ethanol, analytical grade (Labscan Asia Co.,Ltd., Thailand) Batch No.07020143
12) Glacial acetic acid, analytical grade (Labscan Asia Co.,Ltd., Thailand) Batch No. 04100140
13) Glycerin (S. Tong Chemicals Co.,Ltd., Thailand)
14) Isopropanol, analytical grade (Labscan Asia Co., Ltd., Thailand) Batch No. 01090078
15) Methanol, HPLC grade (Labscan Asia Co., Ltd., Thailand) Batch No. 07020143

16) Methyl paraben (K.H. Co., Ltd., Thailand)
17) Propylene glycol (K.H. Co., Ltd., Thailand)
18) Propyl paraben (K.H. Co., Ltd., Thailand)
19) Rice bran (donated from Thai Edible Oil Co., Ltd., Thailand)
20) Rice bran oil (donated from Thai Edible Oil Co., Ltd., Thailand) Lot No. 040407
21) Stearic acid (K.H. Co., Ltd., Thailand)
22) Tween® 60 (Polysorbate 60) (The East Asiatic., Thailand)

Apparatus

1) 743 Rancimat® (Metrohm Co., Ltd., Switzerland)
2) 7-position Analytical balance (max.2.1 g, d = 0.1 μg) (Mettler MT/UMT) (Mettler Toledo AG, Switzerland)
3) Analytical balance (BA2105, S/N 21203485, Sartorius Basic, Germany)
4) Centrifuge (Model K3 system, Centurion, UK)
5) Cold-pressed Machine (Model 6YL-68 Screw oil press, Shanghai Xuyi Machinery Co., Ltd., China)
6) High Performance Liquid Chromatograph (LC-10 ADvp, Shimadzu, Japan) consisting of:
   - Auto injector (SIL-10A, Shimadzu, Japan)
   - Degasser (DGU-14A, Shimadzu, Japan)
   - Pump (LC-10ADvp, Shimadzu, Japan)
   - System controller (SCL-10Avp, Shimadzu, Japan)
   - UV-VIS detector (SPD-10Avp, Shimadzu, Japan)

   Column: Nova Pak, 3.9 × 150 mm C-18 column, 4 μm (Waters, Ireland)
   Serial No. 1143608013457 Part No. Wat086344
   Nylon syringe filter (Vertical chromatography Co., Ltd.) Lot No. 49001

7) Hot air oven (Memmert model K-550-GE200, Memmert, Germany)
8) Microtiter plate reader (Anthos HTL instrument)
9) pH meter PB 20 (Sartorius, Germany)

10) Ultrasonic bath (Model 4.6.Mettler Electronic Corp., USA)

11) Vortex mixer (Vortex-genie,model G560E,USA)

12) Viscometer (International Rheology Viscometer, Type rotation viscometer,Model RI:H:I2, Shannon Ltd, Ireland)

Methods

1. Extraction of RBO

1.1 Solvent Extraction RBO (SE-RBO) and Refined RBO (RE-RBO)

SE-RBO and RE-RBO were donated from Thai Edible Oil Co., Ltd., Thailand (Figure 12)

1.2 Cold-Pressed RBO (CP-RBO)

Rice bran from Thai Edible Oil Co., Ltd was used to prepare RBO by cold-pressed method (Figure 20). Rice bran was put into the receiving funnel and then the machine was started. After that, the bran was pressed by expeller part. The received oil was kept for further study.

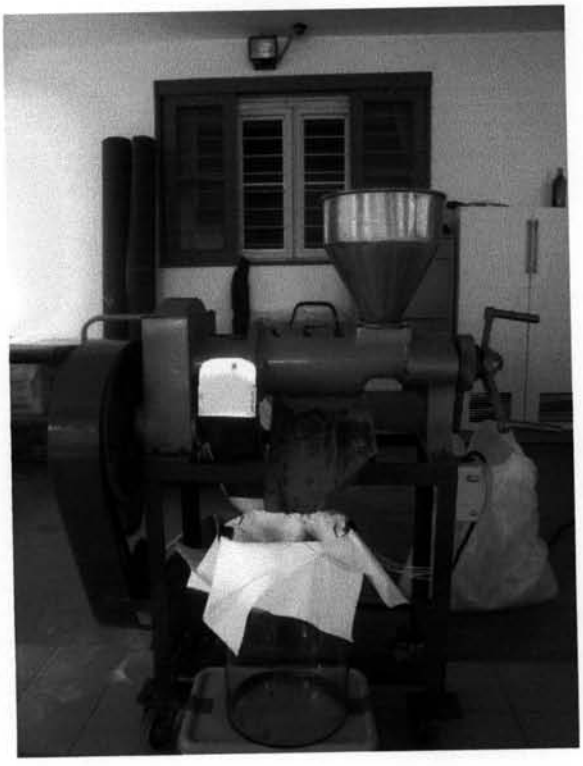

Figure 20 Cold pressed machine: Model 6YL-68 screw oil press

1.3 Bleached RBO

Activated charcoal was immersed in CP-RBO for 15-30 min and then BCP-RBO was separated from activated charcoal by filtering through a filter paper. The oil was stored in a tight container as BCP-RBO for further analysis.

SE-RBO was bleached using the same procedure as CP-RBO. The product was collected as BSE-RBO.

2. Determination of Antioxidant Activity of Various RBO

2.1. Hydrogen-donating Activity (DPPH radical scavenging activity)

2.1.1. Preparation of the DPPH solution

An accurately weight of 1.9715 mg of DPPH (M.W. = 394.32) was dissolved in 50 mL of absolute ethanol.

2.1.2. Preparation of Test Sample Solutions

Each test sample was prepared as an ethanolic solution with initial concentration of 32 mg/mL. For IC_{50} analysis, the serial dilution was performed to give nine concentrations of 32, 24, 20, 16, 10, 12, 8, 4, and 2 mg/mL, respectively

The DPPH solution (1 mL) was added to each of the test sample solutions (1.0 mL) to make the total volume of 2.0 mL. The final concentrations of the sample assay mixtures were 16, 12, 10, 8, 6, 5, 4, 2, and 1 mg/mL, respectively

2.1.3. Measurement of Antioxidant Activity

The assay mixtures contain 1 mL of 0.1 mM DPPH radical solution and 1 mL of test sample solution. The solution was rapidly mixed and after standing for 30 min. The absorbance of the mixture was measured at 517 nm in purple color using UV-VIS spectrophotometer.

2.1.4. Calculation of the Percentage of Antioxidant Activity

The percentage of antioxidant activity (% inhibition) was calculated as the following equation (Dasgupta and De, 2004)

$$\%\text{inhibition} = \frac{(\text{Absorbance control} - \text{Absorbance sample}) \times 100}{\text{Absorbance control}}$$

Absorbance control: the absorbance of DPPH solution after incubation and measured at 517 nm

Absorbance sample: the absorbance of reaction mixture after incubation and measured at 517 nm

2.1.5. Calculation of IC_{50}

The concentrations of the test samples versus % inhibition were plotted. The concentration at 50 % inhibition of each sample was obtained from the equation of polynomial regression of the initial portion of graph.

2.1.6. Statistical Analysis

All experiments were carried out in triplicate (n=3) and the data were calculated as mean ± S.D. Statistical comparison of the IC_{50} values among RBOs of different production methods was made using one-way ANOVA and Tukey's test at α-0.05, where appropriate.

3. Determination of γ-Oryzanol and Vitamin E (α-tocopherol) content in Various RBO

The contents of γ-oryzanol and vitamin E in CP-RBO, BCP-RBO, SE-RBO, BSE-RBO, and RE-RBO were determined using the HPLC method.

3.1 HPLC Method Validation of γ-oryzanol

HPLC method was used to quantify γ-oryzanol and vitamin E because of its specificity and high sensitivity. The HPLC analysis method was a modified method of Xu and Godber (1999).

3.1.1 Chromatographic Conditions

The HPLC condition for the analysis of γ-oryzanol was shown as follows

Column	: Nova Pak, 3.9 × 150 mm, C-18 column
Mobile phase	: methanol:acetonitrile:dichloromethane:glacial acetic acid (50:44:3:3 v/v)
Injection volume	: 20 μL
Flow rate	: 1.0 mL/min
UV detector	: the wavelength was set at 325 and 290 nm for measuring γ-oryzanol and vitamin E, respectively
Temperature	: ambient
Run time	: 20 min

The mobile phase was thoroughly mixed, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min prior to use.

3.1.2 HPLC Method Validation

The HPLC method for determination of γ-oryzanol in RBO was developed and validated according to ICH Q2B. The performance parameters were specificity, linearity and range, precision, accuracy and stability of standard and test solutions. Finally, the system suitability was performed

3.1.2.1 Preparation of Standard Curve

Preparation of γ-oryzanol standard solution (Stock I)

The stock standard solution of γ-oryzanol was prepared by dissolving an accurately weight of standard γ-oryzanol 1 g in 100 mL of isopropanol to have a known concentration of 10 mg/mL

Preparation of γ-oryzanol standard solution (Stock II)

Stock I of 25 mL was transferred to 50 mL volumetric flask. The volume was adjusted to 50 mL by mobile phase (conc = 5 mg/mL)

Preparation of γ-oryzanol standard solution (Stock III)

Stock I of 25 mL was transferred to 100 mL volumetric flask. The volume was adjusted to 100 mL by mobile phase (conc = 2.5 mg/mL)

Preparation of γ-oryzanol standard solution (Stock IV)

Stock I of 10 mL was transferred to 100 mL volumetric flask. The volume was adjusted to 100 mL by mobile phase (conc = 1 mg/mL)

The prepared standard solution Stock II, III, and IV of 1 mL were transferred into 10 mL volumetric flasks no. 1, 2, and 3, respectively and prepared standard solution Stock II, III, and IV of 1 mL were also transferred into 100 mL volumetric flasks no.4, no.5 and no.6, respectively. The volumes of six volumetric flasks were adjusted by mobile phase. The preparations were carried out in triplicate at each concentration (conc = 0.010, 0.025, 0.050, 0.100, 0.250, and 0.500 mg/mL). Then, the prepared working standard solutions were analyzed by HPLC using HPLC conditions mention in 3.1.1.

3.1.2.2 Accuracy

Accuracy of an analytical method is degree of closeness between the true value of analytes in the sample and the value determined by the method. Accuracy can be measured by analyzing samples with known concentrations and comparing the measured values with the true values.

Accuracy was performed by spiked sample method. The prepared standard solution Stock I, II, and III of 1 mL were transferred into 10 mL volumetric flasks no. 1, 2, and 3, respectively. Then, the γ-oryzanol was extracted from RBO sample by the method shown in Figure 21.

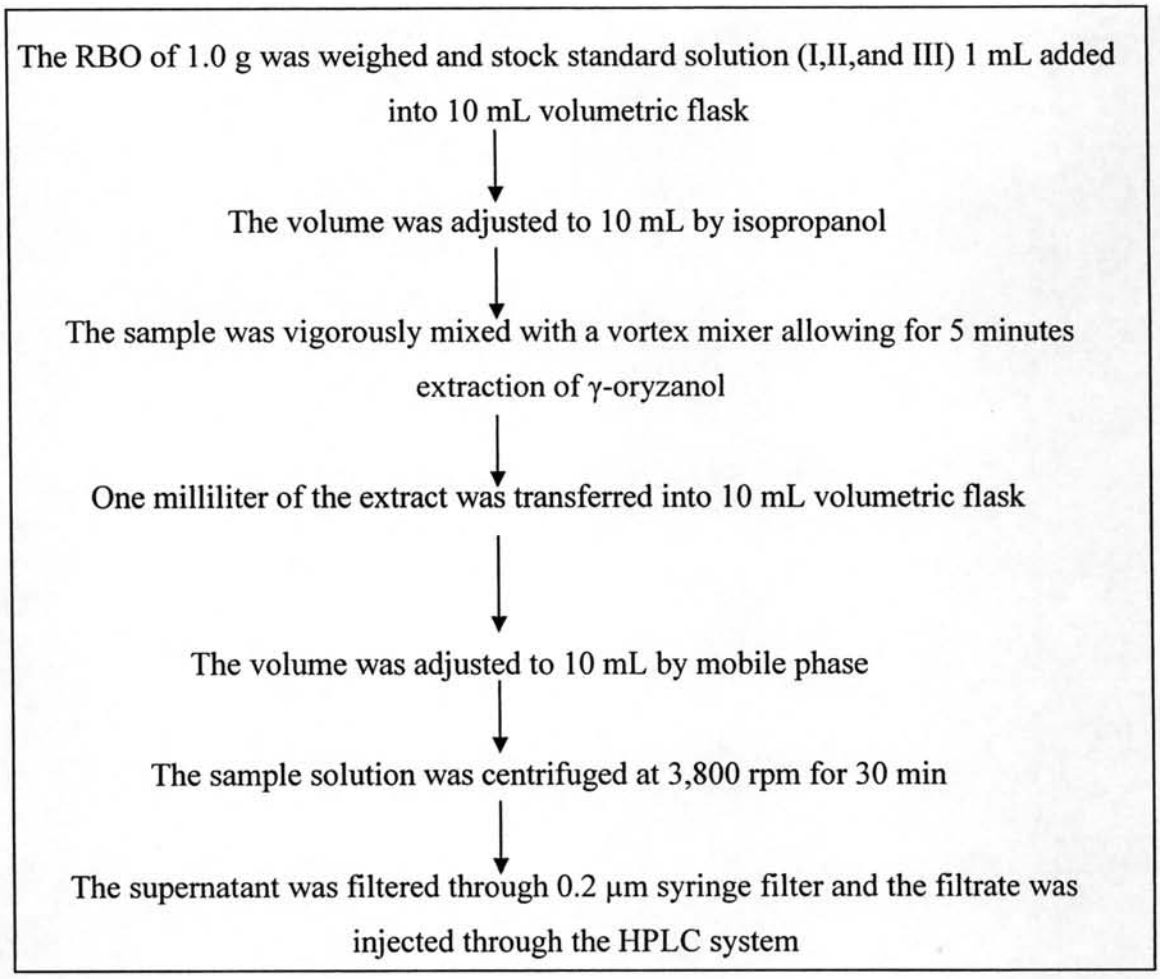

Figure 21 The schematic diagram of the extraction of γ-oryzanol in RBO.

The preparations were carried out in triplicate at each concentration (conc = 0.025, 0.050, and 0.100 mg/mL). The actual concentrations, the observed concentrations, and % recovery of γ-oryzanol were determined.

The recovery was obtained from the mean of observed concentrations calculated from the calibration curve divided by the mean of actual concentrations and multiplied by 100. The results were calculated in percentage terms. According to guidance, the recovery should be coverage in the range of 98-102 %RSD.

3.1.2.3 Precision

3.1.2.3.1 Intra-day precision

Precision was obtained in terms of repeatability (intra-day precision) when the analysis was performed in one laboratory by one analyst using the same equipment at the same day. Repeatability was measured by analysis through six determinations without serial dilution.

3.1.2.3.2 Inter-day precision

Inter-day precision was determined by comparing the estimated concentration of six determinations without serial dilution for three different days.

Precision was performed by spiked sample method. The prepared standard solution stock II of 1 mL was transferred into six 10 mL volumetric flasks. Then, the samples were undergone the γ-oryzanol extraction step described in 3.1.2.2. The preparations were carried out in six replications. The actual concentrations, the observed concentrations, and % recovery of γ-oryzanol were determined.

The precision of an analytical method was calculated as coefficient of variation (C.V.), i.e., relative standard deviation (RSD) as percentage terms (the percentage of RSD (%RSD)). Precision should be < 2 % of RSD value.

3.1.2.4 Linearity

The actual concentrations and the observed concentrations of γ-oryzanol in 3.1.2.2 were averaged and the linearity graphs of γ-oryzanol were constructed.

The linearity, the R^2 value, was calculated by plotting the observed concentration against the actual concentration. The linear correlation coefficient (R^2) should be greater than 0.9995 accordingly to guidance.

3.1.2.5 Selectivity

Selectivity refers to a method that gives responses for a number of substances and can distinguish the analyte response from all other responses. Selectivity of the method should be evaluated by comparison of chromatograms among standard solution, sample solution with standard solution and blank samples.

3.2 HPLC Analysis of γ-oryzanol in Various Production Method of RBO (modified from Roger et al., 1993)

The RBO of 1.0 g was weighed and added into 10 mL volumetric flask.The volume was adjusted to 10 mL by isopropanol. Then, the sample was vigorously mixed with a vortex mixer allowing for 5 minutes for extraction of γ-oryzanol. After that, one milliliter of the extract was transferred into 10 mL volumetric flask. The volume was adjusted to 10 mL by mobile phase. Then, the sample solution was centrifuged at 3,800 rpm for 30 min. Finally, the supernatant was filtered through 0.2 μm syringe filter and the filtrate was injected through the HPLC system.

3.3 HPLC Analysis of Vitanin E (α-tocopherol) in Various Production Method of RBO

To quantify vitamin E, a standard solution containing 400 μg/mL of tocopherol (Sigma) in the mobile phase was prepared. Predetermined volumes of the standard solution were diluted with the mobile phase to have 0, 4, 8, 12, 16, 20, and 40 μg/mL of tocopherol. The samples were then analyzed by HPLC. The wavelength was set at 290 nm following the procedures described for the RBO samples (3.2). The peak areas were used to make a standard curve, and a regression equation was generated to calculate the amount of vitamin E in the samples, expressed as micrograms per gram of RBO.

4. Oxidative Stability in RBO from Various Production Methods

RBO Oxidation were compared among five production methods using 743 Rancimat® at accelerated temperature 120 °C and exposed to a steam of air.

The apparatus has worked following the standard of oil stability index (OSI). In the Rancimat method, 3.0 g of each sample was transferred to the measuring vessel and exposed to the air steam and absorbed there in the measuring solution (distilled water). The conductivity of this measuring solution was continuously recorded. An oxidation curve was obtained as a plot of conductivity versus time, where point of inflection was known as "the induction time". The long induction time means the long oxidative stability. The induction times of RBO samples were compared using one-way ANOVA and Tukey's test at α-0.05.

5. Kinetic Study on the Stability of γ-Oryzanol in RBO from Various Production Methods

RBO from various production methods were filled in each container. The containers were then stored in ovens at 40, 60, and 70°C. Samples were taken after predetermined time intervals and γ-oryzanol was extracted from RBO using the method follow (3.2). The amount of γ-oryzanol remaining was assayed using the HPLC (n =3). Percentage of γ-oryzanol remaining was calculated as a relative percentage of initial amount of γ-oryzanol at time zero to amount of γ-oryzanol at time t. These data were used to predict the stability of γ-oryzanol using Arrhenius relation.

6. Formulation of O/W Emulsions Using RBO from Various Production Methods (CP-RBO, BCP-RBO, SE-RBO, BSE-RBO, and RE-RBO)

The oil phase composes of 5 % RBO from various production methods for comparison o/w emulsions properties.

6.1 Preparation of Various RBO Emulsions

Each RBO emulsion was prepared by separately mixing the ingredients of water phase and oil phase (Table 5). The ingredients in water phase and oil phase were heated to 75 °C and 70 °C, respectively. The water phase was then slowly added to the oil phase and continued stirring by magnetic stirrer at 1000 rpm for 30 mins or until the emulsion was congealed and homogeneous.

Table 5 Percent ingredient of oil-in-water emulsions

Ingredient	Formulation (%w/w)				
	RE-RBO	SE-RBO	BSE-RBO	CP-RBO	BCP-RBO
	OIL PHASE				
RBO	5	5	5	5	5
Glyceryl monostearate self- emulsifier	2.7	2.7	2.7	2.7	2.7
Tween 60	2.3	2.3	2.3	2.3	2.3
Steric acid	0.5	0.5	0.5	0.5	0.5
Cetyl alcohol	2	2	2	2	2
Bee wax	0.5	0.5	0.5	0.5	0.5
	WATER PHASE				
Sodium Polyacrylate &Cyclomethicone &Dimethicone &Dimethicone copolyol	0.3	0.3	0.3	0.3	0.3
Glycerin	1	1	1	1	1
Propylene Glycol	4	4	4	4	4
Paraben concentration (mL)	1	1	1	1	1
Water qs to	100	100	100	100	100

Note: *Paraben concentrate consists of 20%w/v methyl paraben and 2%w/v propyl paraben in propylene glycol.

6.2 Determination of Physical Properties

6.2.1 Physical Appearance

The physical appearance of emulsions was visually observed.

6.2.2 Determination of pH

pH of emulsion was determined using pH meter.

6.2.3 Determination of Viscosity

An apparent viscosity of each formulation was measured by viscometer. An approximately 250 g of each samples was measured in triplicate. Spindle ASTM 3 was used for all samples. A speed was set at 70 rpm and kept spindle rotating for about 60 s or until constantly mean viscosity was obtained. The mean viscosity (mPas) was recorded.

6.3 Skin-feeling Evaluation

The evaluation consisted of volunteers from among the faculties, students at Faculty of Pharmaceutical Sciences, Chulalongkorn University. Each formulation was evaluated by 15 volunteers.

The prepared oil-in-water emulsions were evaluated for their texture, spreadability, tackiness, color, and odor. Recorded scores were 1, 2, 3, 4 and 5 according to the degree of satisfaction from "least satisfied" (1) to "most satisfied (5).

The graded scores were averaged and the 'mean score' was used in the comparison to find any differences among five various RBO emulsions.

6.4 Determination of Emulsion Stability by Accelerated Temperature Test

6.4.1 Temperature Cycling (Heating -Cooling Cycle)

The products should pass six cycles of temperature testing from 4 °C and 45 °C. The products were kept at 4 °C for 24 h and subsequently kept at 45 °C for another 24 h. This completed one cycle, six cycles were repeated (Grimm, 1985). After 6 completed heating-cooling cycle, emulsions were visually observed for any changes in physical appearance such as flocculation, creaming, coalescence and phase separation. The emulsions were also determined for any changes in physicochemical properties as described in 6.2.2 and 6.2.3.