Chapter 3

Materials and Methods

1. Chemicals

Standard alpha-tocopherol, gamma-oryzanol, α,α-diphenyl-β-picrylhydrazyl (DPPH), ferric cyanine, bythylatedhydroxyanisole (BHA), ferulic acid and cathechin were purchased from Sigma-Aldrich Chemical Co., (St. Louis, Mo, USA). Hexane, heptane, dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific. All chemicals and reagents were analytical grade. Food grade glyceryl momostearate (Flakes) were purchased from Foreverest Resources Development Co., Ltd. (China). The remaining reagents and solvents were procured from Fluka or Merck unless stated otherwise. Double-distilled and deionized water was used for the preparation of all solutions.

2. Methods

2.1 Effect of stabilization of rice bran by domestic heating on mechanical extraction yield, quality, and antioxidant properties of cold-pressed rice bran oil.

Preparation of rice bran and its stabilization by domestic heating

Rice bran samples (Sangyod Phatthalung rice) (*Oryza sativa* L) were obtained by milling rice grain in a local grinding mill, Kaow Klang Village, Pantae, Khunkanun, Phatthalung, Thailand, during February to April in 2010. Freshly milled bran samples were directly collected from the milling system in polyethylene bags. The grains were immediately separated from

rice bran by sieving with 177–297 µm sieves. Different domestic heating methods (as describe in Table 1) of rice bran were applied within 1 hour after milling. Unstabilized rice bran was used as the control in this study.

Five hundred grams of rice bran was placed in the container for domestic heating with heating condition as describe in Table 3.1. Temperature during domestic heating was controlled and measured with a thermocouple (Union, UN 305A, Japan). However, temperature of stabilized bran with microwave was immediately measured after heating. Then, rice bran samples were cooled down in the cooling tray about 30 min to reach room temperature. The sample was placed into the polyethylene bag and kept in a cooler (6 ± 2ºC). Each sample was collected until the rice bran reached 20 kg. Rice bran samples were separated into two portions. The first portion (1 kg) was transported to the Department of Food Science and Technology, Thaksin University. Stabilized rice brans were kept at -20 ºC for analysis of moisture content (AOAC, 1999). The second portion (19 kg) was transported to Ban Thai Herbs Co. Ltd, Tanhodduan, Khunkanun, Phatthalung, Thailand, for RBO extraction by pressing.

Table 3.1 Rice bran stabilization methods.

Domestic heating methods	Heating conditions	Instruments
Hot air heating	150 ± 2 ºC, 10 min	Domestic hot air oven (TRIMOND, BO-300D-HT, China)
Roasting	150 ± 2 ºC, 10 min	Domestic cooking pan with diameter 106.68 cm
Steaming	130 ± 2 ºC, 60 min	Domestic cooking steamer with diameter 106.68 cm
Microwave heating	150 ± 2 ºC, 3 min Power 800 w, frequency 2450 MHz	Domestic microwave oven (R-218H, Sharp, Japan)

Oil extraction by pressing

RBO were obtained by pressing 5 kilograms of stabilized and unstablilzed rice bran with a screw type expeller (475.70 w motor, Oriental Motor, Gear Head DY9 97575, Japan). This operation was carried out three times and the extracted oil was quantified. Later, all the extracted oil was collected for its analysis. Fine particles in the expressed oil were separated by vacuum filtration (Vacuumbrand, ME 2C, Germany) with a double layer of Whatman filter paper no.1. RBO samples were kept at -20 ºC for the further analysis. Oil extraction yield is defined as gram per hundred gram rice bran (g/100g bran).

Color measurement

Color of RBO was measured, using a colorimeter (HunterLab, Model ColorFlex, Virginia, USA), and recorded by using the CIE color system profile of L*, a* and b*. Fifteen milliliters of each oil was pipetted into a sample cup, and color value was obtained using a D65/10° setting (daylight 65 illuminant/10° observer).

Analysis of chemical properties

Peroxide value (PV), Acid value (AV), free fatty acid (FFA) content were determined following AOCS official methods (AOCS, 2004). FFA was calculated as oleic acid and expressed as percentage of the total lipids.

Analysis of phytochemical contents and antioxidant activity

Gamma oryzanol content in RBO was determined spectrophotometrically (UV-VIS 1700, Shimadzu, Japan) following the method of Mezouari and Eichner (2007), slightly modified. Briefly, RBO samples were diluted in heptane. The content of gamma oryzanol was determined by UV absorption at 315 nm gamma oryzanol, contents in RBO were quantified against the standard curve. The calibration curve was obtained with pure oryzanol in a concentration range of 0–200 mg/L. The concentration of gamma oryzanol was calculated and expressed as g/100 g oil.

Total phenolic content of RBO was measured according to the method reported by Lai *et al*., (2009) by using Folin–Ciocalteu reagent with some modification. A 0.1 mL RBO solution (1.0 mg/mL DMSO) was sampled into 2 mL of 0.02 mg/mL Na_2CO_3 and mixed for 3 min. After adding 0.1 mL of Folin–Ciocalteau reagent, the final mixture was left for 30 min before reading the absorbance at 750 nm (UV-VIS 1700, Shimadzu, Japan). All measurements were conducted in triplicate and the data were expressed as mg ferulic acid equivalent (FAE) per g of the oil (mg FAE/g oil), based on the calibration curve of ferulic acid.

Total flavonoid content of RBO was determined according to the method reported by Jia *et al*. (1998), slightly modified. Briefly, RBO was dissolved in DMSO (1.0 mg/mL DMSO) and an appropriate dilution of RBO (250 µL) was diluted with distilled water 1.25 mL and 75 µL of 0.05 mg/mL $NaNO_2$ solution were added. The mixture was allowed to stand at room temperature for 6 min before 150 µL of 0.1 mg/mL $AlCl_3$ were added. This mixture was allowed to stand for a further 5 min before 0.5 mL of 1 mol/L NaOH was added. The solution was shaken vigorously before absorbance at 510 nm was measured with a UV–vis spectrophotometer (UV-VIS 1700, Shimadzu, Japan). The results were expressed as mg catechin equivalents per g of the oil (mg CE/g oil).

DPPH radical scavenging activity was determined using the method originally developed by Blois (1958) with slight modifications by Lai et al. (2009). A portion (0.1 mL) of the extract solution (1.0 mg/mL DMSO) in a test tube was well mixed with 3.9 ml of methanol and 1.0 mL of α,α-diphenyl-β-picrylhydrazyl (DPPH) solution (1.0 mmol/L in methanol). The mixture was kept at ambient temperature for 30 min prior to measurement of the absorbance at 517 nm. BHA was used as the reference. All measurements were done in triplicate. The scavenging effect was derived by the following equation:

$$\text{DPPH scavenging effect (\%)} = [1- (A_{517\ nm,\ sample} / A_{517\ nm,\ control})] \times 100$$

Inhibition on linoleic acid peroxidation was measured following the method of Lai et al. (2009), rice bran extract (0.5 mg) dissolved in 0.5 mL of DMSO was mixed with linoleic acid (LA) emulsion (2.5 mL, 0.02 mol/L, pH 7.0) and phosphate buffer (2.0 mL, 0.2 mol/L, pH 7.0) in a test tube. The mixed solution was then incubated at 37 ºC for 72 h for completing color development rising from $FeCl_2$–thiocyanate interaction. The absorbance at 500 nm, an index of the peroxide value, of the resultant solution was then checked (UV-VIS 1700, Shimadzu, Japan). Butylated hydroxyl anisole (BHA) was examined for reference. All data

were means of triplicate analyses. The percentage of LA peroxidation inhibition was calculated by the following equation:

$$\text{Inhibition on LA peroxidation (\%)} = [1- (A_{500\ nm,\ sample} / A_{500\ nm,\ control})] \times 100$$

2.2 Comparative Study on Yield and Chemical Properties of Solvent Extracted and Cold-Pressed Rice Bran Oil (*Oryza saltiva* L.)

Stabilization of Sangyod Rice Bran

SRB samples were obtained by milling rice grain in a local grinding mill; Kao Klang Village, Pantae, Khuankanoon, Phatthalung, Thailand during February to April in 2010. Freshly milled bran samples were directly collected from the milling system in polyethylene bags. The grains were suddenly separated from rice bran by sieving with 177–297 μm sieves. Within 2 hours after milling, SRB samples were heated at 150 ºC for 10 min to inactivate endogenous lipase, lipoxygenase and microbes (Juliano, 1985). Thereafter, stabilized SRB was stored at 4 ºC in a cooler immediately and transported to Department of Food Science and Technology, Thaksin University within 1 hour. Stabilized SRB were kept at -20 ºC for the further study.

Proximate Analysis of Sangyod Rice Bran

Proximate composition of SRB was determined in triplicate using AOAC methods (AOAC, 1999) for protein, fat, moisture, and ash. The percentage of carbohydrate was determined by difference: [100 - (% protein + % fat + % moisture + % ash)]. Energy levels were calculated by multiplying protein and carbohydrate contents by a factor of 4 and fat by 9.

Extraction of Sangyod Rice Bran Oil

Organic solvent (hexane) extraction and cold pressing were used for producing SRBO. Twenty grams of stabilized SRB was mixed with hexane 80 ml, 120 ml and 160 ml (ratio 1: 4, 1:6 and 1:8 respectively). The samples were placed in the shaking water bath at room temperature (30 ºC) for 15, 30, 45 and 60 min. Hexane was removed by vacuum evaporation with a rotary evaporator (Rotavapor® R-210/R-215, Buchi, Switzerland) at 40 ºC. Crude SRBO samples were kept at -20 ºC for the further analysis. The SRBO yield (%) was calculated on the basis of following formula:

SRBO yield (%) = (Weight of oil / Weight of sample) x 100

SRBO were obtained by pressing 5 kilograms of stabilized rice bran with a screw type expeller (475.70 w motor, Oriental Motor, Gear Head DY9 97575, Japan). This operation was carried out three times and the extracted oil was quantified. Later, all the extracted oil was collected for its analysis. Fine particles in the expressed oil were separated by vacuum filtration (Vacuumbrand, ME 2C, Germany) with a double layer of Whatman filter paper no.1. SRBO samples were kept at -20 ºC for the further analysis. The SRBO yield (%) was calculated on the basis of the formula as describe above.

Analysis of Chemical Characteristics and Fatty Acid Profile

Peroxide value, acid value, free fatty acid content and iodine value were determined following AOCS official methods (AOCS, 2004). Free fatty acid content was calculated as oleic acid and expressed as percentage of the total lipids.

The fatty acid compositions were determined as fatty acid methyl esters (FAME) using gas chromatography, GC-14A (Shimadzu, Japan) equipped with fused silica capillary column Carbowax-30 M (30 m, 0.25mm ID) and flame ionization detector (FID). Helium was used as

the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170 ºC and increased to 225 ºC with a rate of 1 ºC /min and then held at 220 ºC for an additional 20 min. The detector temperature was set at 270 ºC, while the temperature at the injection port was maintained at 250 ºC. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid (AOAC, 1999).

Analysis of Vitamin and Phytochemical Contents

Vitamin A, Vitamin B1, Vitamin B2, Vitamin B3, Vitamin B6, Vitamin E, biotin and cholesterol of SRBO were analyzed by AOAC standard method (AOAC, 2005)

Gamma oryzanol content in SRBO was determined spectrophotometrically (UV-VIS 1700, Shimadzu, Japan) following the method of Mezouari and Eichner (2007), slightly modified. Briefly, SRBO samples were diluted in heptane. The content of gamma oryzanol was determined at the UV absorption of 315 nm. Gamma oryzanol contents in SRBO were quantified against the standard curve. The calibration curve was obtained with pure oryzanol in a concentration range of 0–200 mg/L. The concentration of gamma oryzanol was calculated and expressed as mg/g oil.

Total phenolic content of SRBO was measured according to the method reported by Lai *et al*., (2009) by using Folin–Ciocalteu reagent with some modifications. A 0.1 mL of the SRBO solution (1.0 mg/mL DMSO) was sampled into 2 mL of 2% Na_2CO_3 and mixed for 3 min. After adding 0.1 mL of Folin–Ciocalteau reagent, the final mixture was left for 30 min before reading the absorbance at 750 nm (UV-VIS 1700, Shimadzu, Japan). All measurements were conducted in triplicate and the data were expressed as mg ferulic acid equivalent (FAE) per g of the oil (mg FAE/g oil), based on the calibration curve of ferulic acid.

Total flavonoid content of SRBO was determined according to the method reported by Jia *et al*.(1998), slightly modified. Briefly, SRBO was dissolved in DMSO (1.0 mg/mL DMSO) and an appropriate dilution of SRBO (250 µL) was diluted with distilled water 1.25 mL and

75 µL of 5% $NaNO_2$ solution were added. The mixture was incubated at room temperature for 6 min before 150 µL of 10% $AlCl_3$ were added. This mixture was stand for a further 5 min before 0.5 mL of 1 M NaOH was added. The solution was shaken vigorously before absorbance at 510 nm was measured with a UV–vis spectrophotometer (UV-VIS 1700, Shimadzu, Japan). The results were expressed as mg catechin equivalents per g of the oil (mg CE/g oil).

Analysis of Element Contents

Calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), iron (Fe), copper (Cu), cadmium (Cd), arsenic (As) and lead (Pb) contents were determined by the inductively coupled plasma optical emission spectrophotometer, ICP-OES (Perkin Elmer, Model 4300 DV, USA) according to the method of AOAC (1999). Rice bran oil samples (4 g) were homogenized with 4 mL of concentrated nitric acid. The homogenate was heated using a hot plate until digestion was completed. The digested samples were transferred to a volumetric flask and the volume was made up to 10 mL with deionized water. The solution was subjected to ICP-OES analysis. Flow rates of argon to plasma, auxiliary and nebulizer were kept at 15, 0.2, and 0.8 L/min, respectively. Sample flow rate was set at 1.5 mL/min. The concentration of mineral was calculated and expressed as mg/kg oil.

2.3 Emulsion properties of cold-pressed rice bran oil nano-emulsion stabilized by glyceryl monostearate (GMS)

Preparation of cold-pressed RBO emulsion

The effect of cold-pressed RBO and emulsifier (GMS) concentration on the formation of oil-in-water emulsions emulsion was studied. GMS was used as an emulsifier in this study because it is widely used in cosmetics, pharmaceutical and the food industry. Oil-in-water emulsions were prepared using different concentrations of cold-pressed RBO (10 to 40 wt%) and GMS (1 to 5 wt%). The ratio of GMS and cold-pressed RBO of emulsion was controlled at 1: 10.

Cold-pressed RBO samples and aqueous buffer solutions (10.0 mM phosphate buffer, pH 7.0) were heated in a water bath up to 70 °C. GMS was added in to the heated cold-pressed RBO and mixed till it completely dissolve in cold-pressed RBO (the clear oil solution). Heated buffer solution was added into the oil phase solution. The emulsification was carried out by a two-step homogenization process. First, the oil and aqueous phases were blended using a high-shear mixer (Tissue-Tearor; Biospec Products, Bartlesville, OK) operated at 20000 rpm for 2 min to produce a coarse oil-in-water emulsion. Second, the coarse emulsion was immediately passed through a high-pressure homogenizer (Microfluidizer M-110L processor, Microfluidics Inc., Newton, MA) three times at 12000 psi to produce a fine oil-in-water emulsion. During the homogenization process, the temperature was controlled with the hot water at 70-80 °C. The cold-pressed RBO emulsion samples were kept at room temperature for 24 h for further analysis.

Particle size measurement

The particle size distribution (PSD) of the emulsions was measured using a laser light scattering instrument (Master Sizer 2000, Malvern Instruments Ltd.,Worcestershire,U.K.). This instrument measures the intensity of laser light scattered from a dilute emulsion, and then reports the PSD that gives the closest fit between theoretical calculations (Mie theory) and

experimental measurements of intensity versus scattering angle. To avoid multiple scattering effects emulsions were diluted with the same buffer as the continuous phase. Particle size measurements are reported as volume-surface mean diameters d32 or volume-weighted mean diameters d43. The refractive indices of the dispersed and continuous phases used in the calculations of the PSD were 1.464 and 1.330, respectively. We assumed that the imaginary part of the refractive index of the RBO was zero, although it did have a slight yellowish color, which may therefore have had some effect on the reported PSDs. It should be noted that dilution and stirring are likely to disrupt any weakly flocculated droplets, and so the particle size data on highly aggregated emulsions should be interpreted with caution.

ζ -potential measurement

The electrical charge (ζ-potential) of lipid droplets in the emulsions was determined using a particle electrophoresis instrument (ZEN3600, Nano-series, Zetasizer, Malvern Instruments). Emulsions were diluted until they gave an instrument attenuation factor of approximately 6 using buffer solution at the same pH as the initial sample. The emulsions were agitated prior to analysis to ensure that they were homogeneous. The ζ-potential of each individual sample was calculated from the average of 2 freshly prepared samples with at least 2 replications per sample. The instrument used the Smoluchowski approximation to calculate the ζ-potential from the measured electrophoretic mobility of the particles.

Color measurement

Color of RBO was measured, using a colorimeter (X-rite, Color Munki Design, China), and recorded by using the CIE color system profile of L*, a* and b*.

Creaming index measurement

Emulsion samples (10 mL) were placed in glass test tubes (16 × 150 mm) and then stored at ambient temperature for 30 days before analysis. The susceptibility of the emulsions to creaming was ascertained by measuring the height of the boundary layer between the opaque droplet-rich layer at the top and the transparent or turbid droplet-depleted layer at the bottom of the test tubes. Creaming results are reported as the Creaming Index (CI) = 100 × (height of interface) /(height of total emulsion) (Demetriades and McClements 2000). The pictures of emulsion were taken by the digital camera (Sony, Cyber shot, Exmor R 10.2 mega pixels, Japan).

Appearance viscosity

A controlled stress rheometer (Kinexus, Malvern Instruments Ltd., Worcestershire, U.K.) with a coaxial cylinder attachment, having a ratio of 0.922 between the external diameter of the rotating bob and the internal diameter of stationary cylinder, was employed to determine the rheological behaviour of emulsions. The gap between the cup and rotor was 850 mm. A constant temperature of 20 ± 0.1 ºC was maintained during the measurement using a circulatory water bath. The controlled rate measurement technique was employed by progressively increasing the shear-rate up to 600 s^{-1} to obtain 50 shear-rate/ shear-stress data points. All rheological measurements were conducted on triplicate samples.

2.4 The influence of environmental stresses on emulsion stability.

Stability to pH

Emulsion samples were prepared in aqueous buffer solutions and then the pH was adjusted to the desired final value (pH 2 to 9) using either NaOH and/or HCl solution. Emulsion samples (10 mL) were then transferred into glass test tubes (160 × 15 mm) and stored at ambient temperature overnight prior to analysis.

Stability to ionic strength

Emulsions (pH 7) were diluted with different amounts of NaCl and buffer solution to form a series of samples with the same droplet concentration but different saltconcentrations (0 to 400 mM NaCl). The emulsions were stirred for 30 min and then transferred into glass test tubes (160 × 15 mm) and stored at ambient temperature overnight prior to analysis.

Stability to heating

Emulsions (pH 7) samples were transferred into glass test tubes (160 × 15 mm), which were stored in a water bath for 30 min at a fixed temperature ranging from 30 to 90 ◦C. The emulsion samples were then immediately placed at room temperature and stored overnight prior to analysis.

Measurements of emulsion stability

Cold pressed-RBO emulsion samples (50 mL) were placed in 100 mL Duran glass bottle, and then incubated at room temperature (about 25°C) in the dark. The samples were kept for 90 days. The samples were taken at 0, 30, 60 and 90 days for study the stability of emulsion.

Analysis of oxidative stability

Lipid hydroperoxides of cold-pressed RBO emulsion were measured with a modified ferric thiocyanate method (Alamed *et al*., 2009) by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/ 2-propanol (3:1) by mixing (10 s, 3 times) and isolation of the organic solvent phase by centrifugation at 1000g for 2 min (Beckman Centrifuge model J2-21, Beckman Instruments Inc., Fullerton, CA). The organic solvent phase (200 μL) was added to 2.8 mL of methanol/n-butanol (2:1), followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of a ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$ $7H_2O$). After 20 min of incubation at

room temperature, absorbance was measured at 510 nm (Genesys 20; Thermo Scientific, Waltham, MA) Hydroperoxide concentrations were determined using a standard curve from cumene hydroperoxide.

Thiobarbituric acid reactive substances (TBARS) (McDonald and Hultin, 1987) were determined by mixing 1.0 mL of emulsion with 2.0 mL of TBA reagent in 10 mL test tubes. TBA reagent was prepared by combining 100 mL of TCA-TBA-HCl solution [15% w/v trichloroacetic acid (TCA) and 0.375% w/v thiobarbituric acid (TBA) in 0.25 M HCl] with 3.0 mL of ethanol (containing 2% BHT). Samples were heated in a boiling water bath for 15 min, cooled to room temperature (10 min), and then centrifuged for 15 min at 3400g (Beckman Centrifuge model J2-21, Beckman Instruments Inc., Fullerton, CA). Samples were held at room temperature for 10 min before the absorbance of each sample was measured at 532 nm (Genesys 20; Thermo Scientific, Waltham, MA). TBARS concentrations were determined using a standard curve from 1,1,3,3-tetraethoxypropane.

Analysis of phytochemical contents and antioxidant activity in cold press rice bran oil emulsion

Phytochemicals in cold–pressed RBO emulsion were extracted by mixing 1.5 mL of emulsion with 7.5 mL of isooctane/ 2-propanol (3:1) by mixing (10 s, 3 times). The organic solvent phase was isolated by centrifugation at 1000g for 2 min (Beckman Centrifuge model J2-21, Beckman Instruments Inc., Fullerton, CA). The supernatant was collected for the measurement of gamma oryzanol and DPPH radical scavenging activity (method as describe above). The methanol was used instead of the isooctane/ 2-propanol for extraction of phenolic compounds from the emulsion. Methanol extracted was measured the phenolic compound content (method as describe above).

2.4 Statistical Analysis

All analyses were run in triplicate. The mean values and standard deviations were calculated. Statistical analysis of SRBO extraction was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1980). The data obtained from the study of chemical properties was subjected to an independent sample T test. The statistical analysis was performed by SPSS 11.0.