CHAPTER 2

MATERIALS AND METHODS

2.1. Materials and equipments

2.1.1 Materials

The five varieties of Thai native silkworm pupa and silk cocoons *(Bombyx mori* L.*)* (Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong, and None Ruesee) as shown in **Figure 18** were collected during May-July 2005 and May-July 2006 from the Queen Sirikit Sericulture Center, Nakhon Ratchasima, Udonthani and Sisaket Provinces, Thailand. Specimens were identified by a botanist (Ms.Khobkol Sannamvong). The silkworms were dried before the extraction.

2.1.2 Chemicals

- Cholesterol (Serva Fernbiochemica, New York, USA)
- Petroleum ether (J.T.Baker, USA)
- D.I.Water (Millipore-Quantum Tm EX)
- Hydrochlic acid (Merck, Germany)
- Sodium hydroxide (Merck, Germany)
- Sodium bicarbonate (Merck, Germany)
- Acetic acid (RCI Lab scan, Thailand)
- Ammonium hydroxide (RCI Lab scan, Thailand)
- Sodium acetate (Merck, Germany)
- Iron (III) chloride (Merck, Germany)
- Hydrogen peroxide (Union Chemical, Thailand)
- Linolenic acid ≥ 99% (Sigma-Aldrich, USA)

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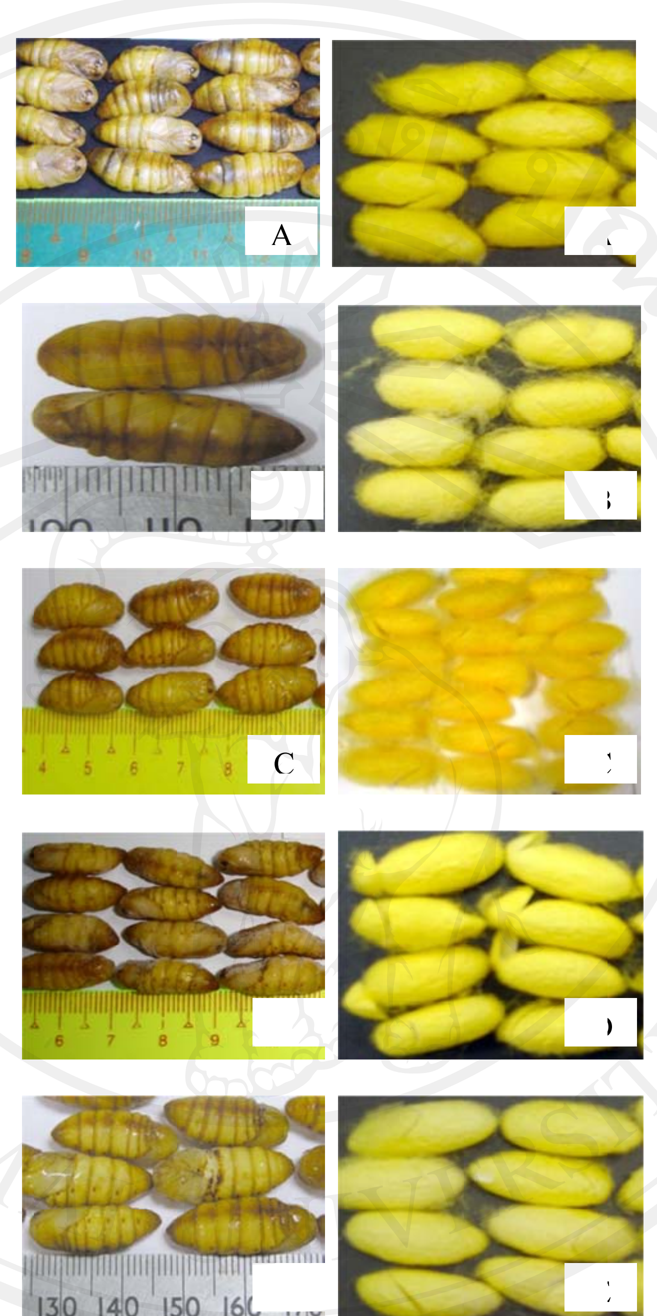

Figure 18 Silkworm pupa (left) and silk cocoon (right) from the five Thai native silkworms *Bombyx mori* (Linn.); (A): Keaw Sakol; (B): Nangnoi Srisaket; (C): Nang Leung; (D): Sam Rong and (E): None Ruesee

- Alpha linolenic acid (Sigma-Aldrich, USA)
- α-Tocopherol (Sigma-Aldrich, USA)
- 2,2-Diphenyl-1-picryhydrazyl (DPPH, Fluka,USA)

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- Ascorbic acid (Sigma-Aldrich,USA).
- Butylated hydroxytoluene (Sigma-Aldrich, USA).
- Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone) (Sigma, USA).
- Tyrosinase enzyme (Sigma-Aldrich, USA).
- Mushroom tyrosinase (Sigma-Aldrich, USA).
- L-tyrosine (Sigma-Aldrich, USA).
- Ethanol (RCI Lab scan, Thailand)
- Methanol (RCI Lab scan, Thailand)
- Culture medium (Merck, Germany)
- Standard amino acid (Sigma-Aldrich, USA)
- Sodium dodecyl sulphate-polyacrylamide gel (Biorad,Laboratories,Canada) Trifluoroacetic acid (Thermo Fisher Scientific,USA)
- Sorbitan, monooctadecanoate, poly (oxy-1,2-ethanediyl) sodium polyacrylate (and)dimethicone (and) cyclopentasiloxane(and)trideceth-6 (and) PEG/PPG -18/18dimethicone (DCRM 2051, Dow Corning,USA
- C_{14}-$_{22}$alkylalcohol and C_{12}-$_{20}$ alkylglucoside (MontanovL, Seppic, France)
- Carbopol®Ultrez21 Polymer (Lubrizol,USA)
- Silsense™ DW-18(Lubrizol,USA)
- Cyclopentasiloxane,dimethiconol,dimethicone crosspolymer (and) phenyltrimethicone blend (DC CB 3031) (Dow Corning,USA)
- Propylene glycol (and) diazolidinyl urea (and) methylparaben (and) propylparaben (ISP Global, NewJersey,USA)
- PEG-100 stearate & glyceryl stearate (Seppic, France)

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- Florasun 90 (FloraTech, Arizona USA)
- Octyldodecanol (Eutanol G, England))
- Shea butter (Namsiang Co.Ltd.,Thailand)
- Ethylene diaminetetraacetic acid (Namsiang Co. Ltd., Thailand)
- Propylene glycol (Namsiang Co. Ltd., Thailand)
- Octyl benzoate (Namsiang Co. Ltd., Thailand)
- Octyl palmitate (EAC Co. Ltd., Thailand)
- Triethanolamine (Namsiang Co. Ltd., Thailand)
- Glycerin (Submit Chemical Co. Ltd., Thailand)
- Floramac® 10(FloraTech, Arizona USA)
- Retinol (Sigma-Aldrich, USA)
- Butylated hydroxytoluene (Sigma-Aldrich, USA)
- Tocopheryl acetate (Namsiang Co. Ltd., Thailand)
- Propyl and methyl paraben (Namsiang Co. Ltd., Thailand)
- Glycerol (Thermo Fisher Scientific, USA)
- 2-mercaptoethanol (Sigma-Aldrich, USA)
- Bromphenol blue (Sigma-Aldrich, USA)
- Glycine (Sigma-Aldrich, USA)
- Coomassie blue R-250(Thermo Fisher Scientific, USA)
- Glacial acetic acid (99%) (LyondellBasell, USA)
- Other solvents (analytical grade)
- Sericin P® (Sinosilk Ltd.,Japan)

2.1.3 Human volunteers

- 82 females and 8 males, age 25-65 years

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2.1.4 Equipments

- Analytical balance (Sartorius, BP 2100, Sentorius Co.,Ltd.,Germany)
- High performance liquid chromatography (HPLC, AS 1000, Thermo Finigan, USA)
- Freeze dryer / shell freeze system (Labcon Co.Ltd., USA)
- Probe sonicator (Vibra-Cell Model VC 505, Sonics & Materials Inc., Newtown, CT, USA)
- pH meter (Cyberscan pH 500, Euteoh Instruments, Singapore)
- Rotary vacuum evaporator (Eyela,N-N Series, New York USA)
- Transmission electron microscope (TEM 1200SJEOL,Ltd.,Tokyo, Japan)
- High speed refrigerated centrifuge (Tomy Seko Co., Tokyo, Japan)
- Zeta potential analyzer ZS (Brookhaven Instruments Corporation, New York USA)
- Light microscope equipped with digital camera(Model BH-2 Olympus, Japan)
- Water bath with thermostat (Memmert WB22,Gmbh+, Germany)
- Soxhlet extraction (Capacity 5 kg)
- Gas chromatography mass spectrophotogramphy (Shimadzu,GC-MS17Aver.3 Series, Japan)
- Mixer (IKAR Labortechnik RW20 digtial,RW20DZM,Germany)
- Brookfield, RVDV-II+ (Brookfield engineering laboratories,USA)
- Microplate reader (Zenyth 340 Reader, England)
- UV-VIS spectrophotometer V-530 (Jasco,USA)
- Ultra low temperature freezer -80 C ,MDF,Sonyo,Japan

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- Glass microfiber filter (Scientific Promotion, Thailand)
- Cutometer MPA 580 (Courage + Khazaka electronic GmbH, Cologne Germany)
- Skin diaganostic SD 27 (Courage+ Khazaka electronic GmbH, Cologne Germany)
- Tray dryer (Kluay Nam Thai Tow Op Co.,Ltd, Thailand)
- Shaken (Charn intertech Co.,Ltd, Thailand)
- Rancimat apparatus Model 743 (Metrohm Siam, Switzerland)
- Homogenizer (Pro Scientific Inc,USA)

2.2 Method

2.2.1 Extraction of the native Thai silkworm pupa oil

2.2.1.1. Soxhlet extraction: The five varieties of Thai native silkworms were boiled at 100°C until floating above the water level **(Figure 19)** then, the water was drained, and the worms were roasted until reaching a level of about 8% moisture. After that, the dried pupas were ground to powder. Oils from the powder were hot extracted using a Soxhlet apparatus (Doneanu et al., 1997; Kobkol et al., 2002), for the hot process, the dried pupa powder was boiled with petroleum ether at the ratio of 100 g of powder to 300 mL of solvent for 18 hours in a Soxhlet apparatus. The extracted oils were filtered, and the solvent was evaporated by a rotary vacuum evaporator at 40 °C The silkworm oil was collected and further used for characteristic determination.

2.2.1.2. Maceration extraction: The five varieties of Thai native silkworms were kept fresh at -20 °C until use the maceration extraction petroleum ether as solvent **(Figure 20)**. The ratio of silkworm pupa to the solvent was 100 g to 750 mL.

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Figure 19 Extraction processes of the native Thai silkworm pupa oil by Soxhlet extraction

The sample was blended for 3 minutes until homogeneous and then shaken 200 rpm at 10 $^{\circ}$C for 7 days. After that, the sample was removed and the solvent was evaporated by a rotary vacuum evaporator at 40°C (adapted from Nipha and Arunyakorn, 1997; Doneanu et al., 1997). The silkworm oil was collected and further used for characteristic determination.

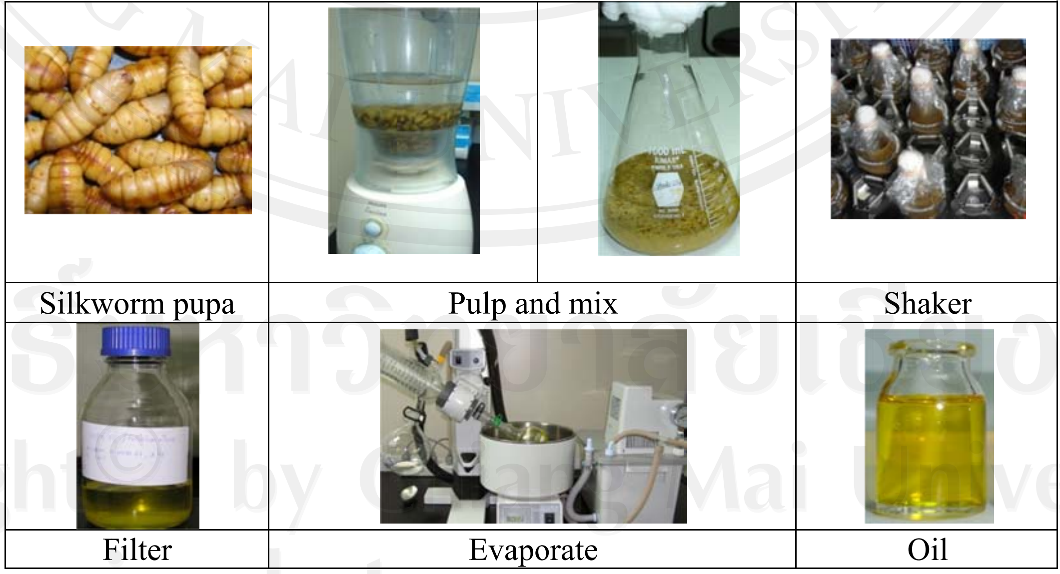

Figure 20 Extraction process of the native Thai silkworm pupa oil by the maceration extraction

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2.2.1.3. Characteristics of the native Thai silkworm oil

2.2.1.3.1 Physicochemical stability of oils extracted from the native Thai silk. The oil and sericin samples (0.1 g each) were dissolved in 99% ethanol and distilled water, respectively. The pH of the solution was measured by a pH meter (Cyberscan 510; In Experiment Instruments, Singapore). The physicochemical stability of the resulting solution was observed in 10% HCl, 10% CH_3COOH, 10% NaOH, 10% NH_4OH, 10% CH_3COONa, 10% $FeCl_2$, and 10% H_2O_2 solutions. Then, 0.1% oil solvent was used to measure pH. Next, the test oil solutions were dropped into the oil sample solution until changes were observed. It no changes, at 50 drops were stop. The changes including color, turbidity and sedimentation test were observed (Helena and Veronika, 2005).

2.2.1.3.2 Determination of linoleic acid contents in the oil sample by HPLC

The linoleic acid contents were determined by high performance liquid chromatography (HPLC). The spectra system instrument equipped with Luna C 18, 5 μm, 250 x 4.0 nm Phenomenex, USA columns and the LC1200 UV/VIS detector, and LC1100 HPLC pump were used. The mobile phase of 90% acetonitrile and 10% trifluoroacetic acid (0.1% (v/v) and the injection volume of 10 μL with the flow rate of 1 mL/min and with the ultraviolet (UV) detector set at 210 nm were used. The linoleic acid contents were calculated by comparing with the standard linoleic acid (Sigma Co., St. Louis, USA) (David et al.,1999).

2.2.1.3.3 Determination of the oxidation induction time of the oil sample

The Rancimat method may also provide some other useful information regarding oxidative stability of edible fats and oils and fat-containing foods. Oils sample size, airflow rate, and temperature are the operational parameters that can be

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adjusted easily in the Rancimat method and may affect the determination of the oxidative stability index (OSI), temperature coefficient and Q10 number (oxidative stability measures), and shelf-life prediction of edible fats and oils and fat-containing foods. The oxidation stability was measured using a Rancimat apparatus Model 743 (Metrohm Siam). A flow of air (10l/h) was bubbled through the oil heated at 110 °C, and the volatile compounds were collected in cold water, increasing the water conductivity. The time to reach a fixed level was recorded (Aparicio, et al., 1999; Farhoosh, 2007).

2.2.1.3.4 Determination of tocopherol and cholesterol contents by HPLC

Tocopherols and cholesterols were measured following the IUPAC Standard Method (IUPAC, 1992). A solution of 100 mg of oils were vigorously mixed in 1 mL of chloroform, centrifuged and injected into the HPLC. Tocopherols and cholesterol were separated using a normal phase HPLC system (LC1200 UV/VIS Detector, LC1100 HPLC Pump). Luna C18 10 µm 250 x 4.0 mm. silica column, (Phenomenex U.S.A) and a 10-min isocratic method using 98% (v/v) methanol in water at 42 °C at 1 mL/min. Tocopherols and cholesterol were quantified through their absorbance at 280 nm (Diode array detector, HP1100 Series, Agilent Technologies, Santa Clara, CA).

2.2.1.3.5 Determination of fatty acid contents in the oil sample

The fatty acid determinations in oil by GC-MS were measured following the analytical methods described in Regulations EEC/2568/91 and C/1429/92 of European Union Commission (EC, 1992). Briefly, 1 μl of the analytical sample was injected into the chromatograph for the GC-MS analysis. The injector temperature was fixed at 250 °C, and the injection was in the split-splitless mode. The splitter was

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opened (50:1) for 0.5 min, closed for 3.5 min, and then opened at 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature, 50 °C (held for 2 min), increased at 8 °C /min to 250 °C, followed by a second gradient at 3 °C /min to 260 °C (held for 20 min), and finally, increased at 3 °C /min to 300 °C (held for 10 min). The total analysis time was 70 min, and 5 min extra time was necessary for re-establishing and equilibrating the initial conditions. The ion-trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current, 80 μ. a transfer line, ion trap, and manifold temperatures, 220, 200, and 50°C. Fatty acids were reported as relative percentage of the total peak area.

2.2.1.3.6 Physical and chemical properties of the oil

The oil obtained from different extraction methods of difference native Thai silkworms were characterized using the following physical and chemical parameters (AOCS, 1983): density, peroxide value, refractive index, acid value, saponification value, unsaponification value and iodine value.

A: Determination of saponification value; Indicator method was used as specified by ISO 3657 (1988). An amount of 2g of the oils was weighed into a conical flask; 25ml of 0.1N ethanolic potassium hydroxide was then added. The content which was constantly stirred was allowed to boil gently for 60 min. A reflux condenser was placed on the flask containing the mixture. Few drops of phenolphthalein indicator was added to the warm solution and then titrated with 0.5M HCl to the end point until the pink colour of the indicator just disappeared. The same procedure was used for other samples and blank. The expression for saponification value (S.V.) is given by: S.V = 56.1N $(V_0-V_1)/M$, where V_0 = the volume of the

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solution used for blank test; V_I = the volume of the solution used for determination; N = actual normality of the HCl used; M = mass of the sample.

B: Determination of unsaponification value; the unsaponifiable matter was extracted five times with 30 mL of n-hexane. The combined extract was washed repeatedly with 100 mL of distilled water/ethanol (90:10, vol/vol) to neutralize the excess hydroxide. The solution was dried with anhydrous sodium sulfate. The solvent was removed by evaporation under reduced pressure.

C: Determination of Iodine Value (ISO. 3961,1989); The method specified by ISO 3961 (1989) was used. The 0.4g of the sample was weighed into a conical flask and 20ml of carbon tetrachloride was added to dissolve the oil. Then 25ml of Dam's reagent was added to the flask using a safety pipette in fume chamber. Stopper was then inserted and the content of the flask was vigorously swirled. The flask was then placed in the dark for 2 hours and 30 minutes. At the end of this period, 20ml of 10% aqueous potassium iodide and 125ml of water were added using a measuring cylinder. The content was titrated with 0.1M sodium-thiosulphate solutions until the yellow colour almost disappeared. Few drops of 1% starch indicator were added and the titration continued by adding thiosulphate drop wise until blue coloration disappeared after vigorous shaking. The same procedure was used for blank test and other samples. The iodine value (I.V) is given by the expression: I.V = 12.69C $(V_1-V_2)/M$, where C=Concentration of sodium thiosulphate used; V_1 = volume of sodium thiosulphate used for blank; V_2 = Volume of sodium thiosulphate used for the determination, M = Mass of the sample.

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D: Determination of Density; Density bottle was used to determine the density of the oil. A clean and dry bottle of 25ml capacity was weighed (W_0) and then filled with the oil, stopper inserted and reweighed to give (W_1). The oil was substituted with water after washing and drying the bottle and weighed to give (W_2). The expression for specific gravity (Sp.gr) is: Sp.gr = (W_1-W_0) / (W_2-W_0) = mass of the substance / Mass of an equal volume of water.

E: Peroxide value; Weigh 5.00±0.05 g sample into a 250 ml erlenmeyer flask and then add 30 ml acetic acid: chloroform (3:2) solution (under the hood). Swirl the flask until the sample is dissolved and add 0.5 ml saturated potassium iodide (KI) solution. Allow the solution to stand with occasional swirling for one minute and then add 30 ml of distilled water. Slowly titrate with 0.01 N sodium thiosulfate ($Na_2S_2O_3$) by adding it with constant and vigorous shaking. Continue titrating until the color changes to light yellow. Add 0.5 ml of 1% soluble starch indicator which will give a blue color. Continue titrating, shaking the flask vigorously near the endpoint which is a faint blue color to liberate all of the iodine from the chloroform ($CHCl_3$) layer. Add the sodium thiosulfate ($Na_2S_2O_3$) drop-wise until the blue color just disappears. Calculate the peroxide value as meq of peroxide /kg of oil, S x M x 1000/ weight of sample in grams, where S = ml of $Na_2S_2O_3$ and M = 0.01, the concentration of the $Na_2S_2O_3$, solution.

F: Determination of Refractive Index; Refractometer was used in this determination. Few drops of the sample were transferred into the glass slide of the refractometer. Water at 30°C was circulated around the glass slide to keep its temperature uniform. Through the eyepiece of the refractometer, the dark portion viewed was adjusted to be in line with the intersection of the cross. At no parallax

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error, the pointer on the scale pointed to the refractive index. This was repeated and the mean value noted and recorded as the refractive index.

G: Determination of Acid Value; 25ml of diethyl ether and 25ml of ethanol was mixed in a 250ml beaker. The resulting mixture was added to 10g of oil in a 250ml conical flask and few drops of phenolphthalein were added to the mixture. The mixture was titrated with 0.1M NaOH to the end point with consistent shaking for which a dark pink colour was observed and the volume of 0.1M NaOH (V_0) was noted. Free fatty acid (FFA) was calculated as: $V_0/W_0 \cdot 2.82 \cdot 100$, where 100ml of 0.lM NaOH = 2.83g of oleic acid, W_0 = sample weight; then the acid value = FFA·2.

2.2.1.4 Biological activities of the oil samples

2.2.1.4.1 Free radical scavenging assay

Oil samples at 200, 100, 50, 25 and 12.5 mg/ml and the standard antioxidants (vitamin C , vitamin E ,BHT and linoleic acid) in the mixture of 95% ethanol and 10%(v/v) DMSO (1:1) were assayed for free radical scavenging activity by the DPPH method (Jung, B. K. et al., 2006). Briefly,100 μl of the samples or standards, 25 µl of 2mg/ml DPPH in 95% ethanol and 25 µl of 95% ethanol were mixed in 96-well microplates and incubated at room temperature (25°C) for 30 min. The absorbance was measured at 515 nm. (Purple) The percentages of DPPH radical scavenging activity were calculated according to the following equation:

$$\% \text{ DPPH radical scavenging activity} = \frac{A - B}{A} \times 100$$

Where A is the absorbance of the control reaction and B is the absorbance of the test samples. Sample concentrations providing 50% scavenging (SC_{50}) were

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calculated from the graph plotted between free radical scavenging inhibition percentages and the sample concentrations.

2.2.1.4.2 Tyrosinase inhibition assay

Oil samples at 200, 100, 50, 25 and 12.5 mg/ml and the standard antioxidants (vitamin C and kojic acid) were mixed with 5% (v/v) DMSO and assayed by the modified dopachrome method using tyrosine as a substrate as previously described (Piao et al., 2002). Briefly, 40 µl of samples or standards, 40 µl of 0.1 mg/mlL-tyrosine, 50 µl of 0.1 mg/ml mushroom tyrosinase and 80 µl of 0.1M phosphate buffer were added in 96-well microplates. The 5% (v/v) DMSO solution was used as a negative control. The mixture was incubated at 37 °C for 60 min. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 450 nm. The experiment was done in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation:

$$\% \text{ inhibition activity} = \frac{(A-B) - (C-D)}{(A-B)} \times 100$$

Where A is the absorbance of the blank after incubation, B is the absorbance of the blank before incubation, C is the absorbance of the sample after incubation, and D is the absorbance of the sample before incubation. Sample concentrations providing 50% inhibition (IC_{50}) were calculated from the graph plotted between tyrosinase inhibition activity percentages and the concentrations.

2.2.2 Preparation of sericin from the Thai native silk cocoon

2.2.2.1 Basic hydrolysis: the sericin proteins from the five Thai native silk cocoons were prepared by extraction using basic hydrolysis (Terada et al., 2007; Kato et al., 1998). 100 g of cocoon was cut into small pieces before the addition of 5 L of

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0.05% (w/v) sodium carbonate solution (pH 10–11) and boiled at 95 °C for 2 hour. Then, the extracts were filtered to remove fibroin using a glass microfiber. The filtrates were dialyzed with deionized water for 24 hours and the dialysate was centrifuged for 8000 rpm at 4 °C for 20 minute and then, lyophilized by a freeze-dryer as shown as in **Figure 21.**

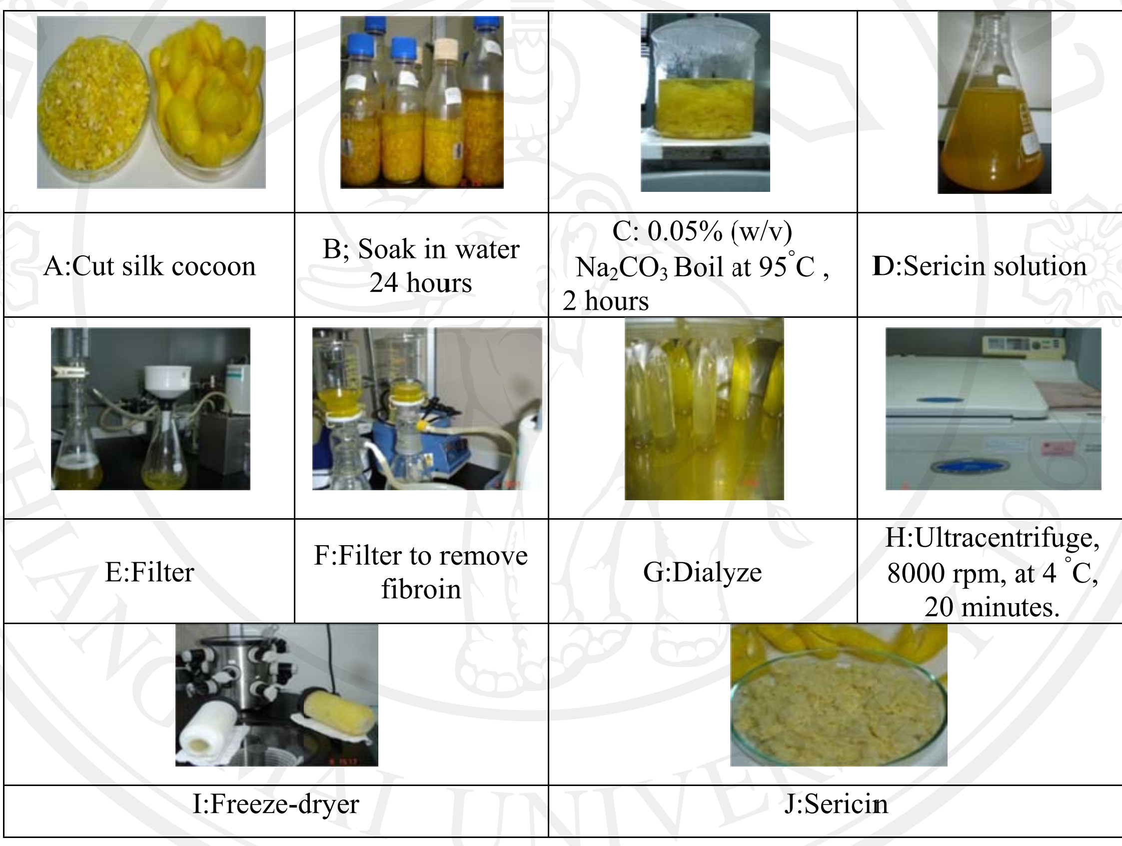

Figure 21 Extraction of process sericin from the Thai native silk cocoon prepared by extraction using basic hydrolysis

2.2.2.2 Autoclave hydrolysis: the sericin proteins from the five Thai native silk cocoons were prepared by hydrolysis according to the method previously described (Zhang et al., 2004; Akiyama et al., 1993). For the autoclaving, 100 g of cocoon was washed with hot water three times and cut into small pieces before the addition of

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1.5 L of distilled water and left overnight. Then, the mixture was put into a high pressure boiler at 121°C for 2 hours and filtered to remove fibroin using a glass microfiber filter. The filtrates were dialyzed with deionized water for 24 hours and the dialysate was centrifuged for 8000 rpm at 4 °C for 20 minute and then, lyophilized by a freeze-dryer as shown in **Figure 22**.

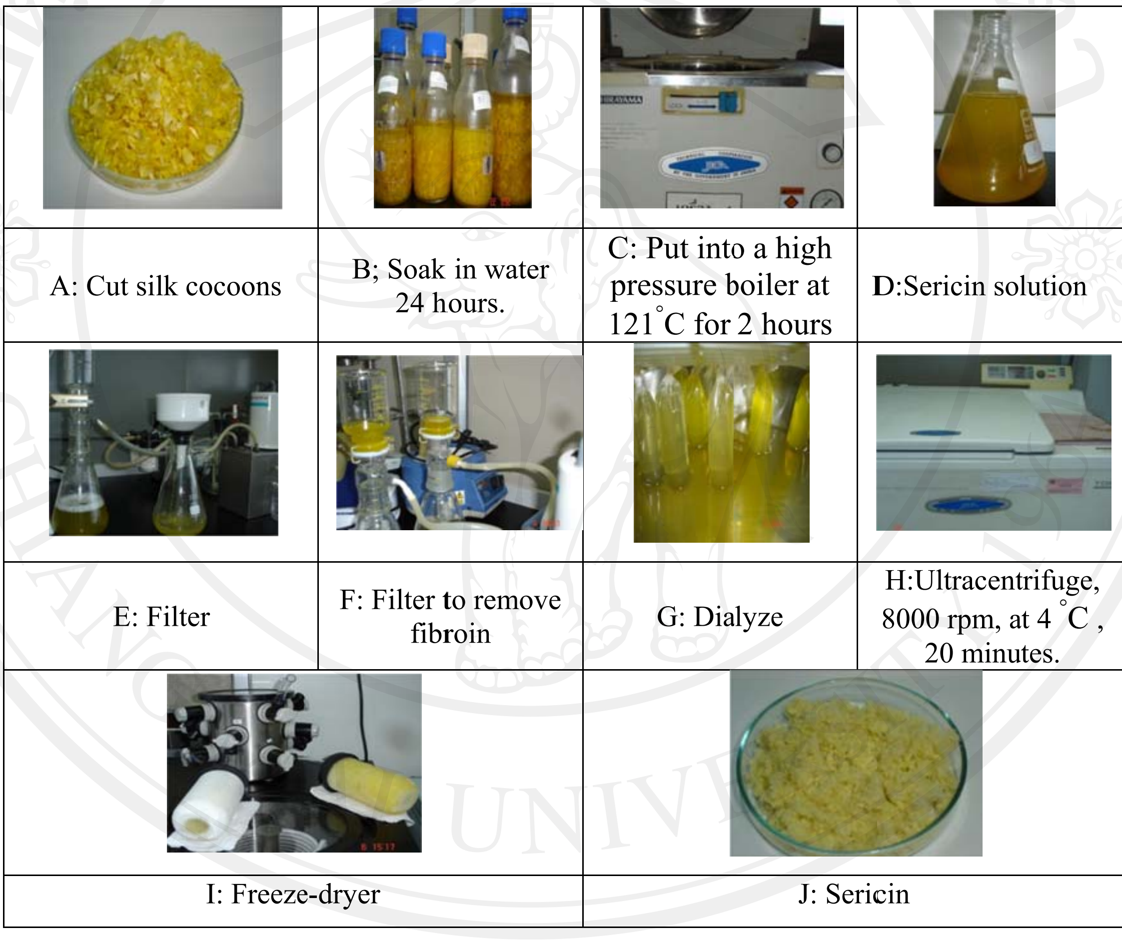

Figure 22 Extraction of process sericin from the Thai native silk cocoon prepared by extraction using autoclave hydrolysis

2.2.2.3 Characteristics of sericin from the native Thai silk cocoon

2.2.2.3.1 Characterization of sericin from the native Thai silk cocoon

The silk protein product obtained from the experiment was characterized for

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total nitrogen, loss on drying, ash, pH and water solubility (AOAC, 1995).

A: Total nitrogen by Kjeldahl Method; this method involves the conversion of nitrogen in biological materials into $(NH_4)_2SO_4$ by digestion with H_2SO_4 followed by distillation of NH_3 in an alkaline medium. The ammonia is collected in (0.05 N) sulphuric acid of known strength which is back titrated with standard sodium hydroxide solution.

B: Loss on drying; Weigh an empty crucible to 3 decimal places. Add between 1 to 2 grams of wet sediment being careful to keep sediment away from the rim of the crucible where it may become dislodged and fall out during handling. Re-weigh the crucible and place in the oven for at least 12 hours or overnight. Using tongs, remove the crucibles from the oven and allow to cool in a desiccator (to prevent re-absorption of the moisture) before re-weighing. The percentage weight remaining after drying can then be calculated. The same sample can also be used for loss on ignition analysis.

C: Ash content or A_c (% m/m) was measured by using a Heraeus muffelofen oven according to the method described in DIN EN ISO 6245 (2003).

D: pH; the pH measurements were performed with a digital pH meter (Cyberscan pH 500, Euteoh Instruments, Singapore)

E: Water solubility; the sericin from the native Thai silk cocoon were placed in 100 mL of water, stirred and dissolved at 90-100 °C for setting up the times. The remained sericin was filtered and then dried at 70 °C to obtain the dry weight. The solubility of the sericin could be calculated by Tao et al. (2007) as follow:

$$\text{Solubility (\%weight)} = \frac{\text{Original weight (g) - Remained weight (g)}}{\text{Original weight (g)}} \text{ x100}$$

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F: Total plate count (*E.coil*);Sericin is free of specified pathogens. The amount of non-pathogenic microorganisms with less than 100 CFU per /mL of sericin should meet the CTFA microbiology guidelines.

2.2.2.3.2 Gel Electrophoresis of sericin from the native Thai silk cocoon

To determine the molecular mass of sericin, SDS-PAGE was performed as previously described with some modifications (Takasu et al., 2002). Pigments from silk cocoon were removed prior to sericin extraction to confirm that carotenoids or flavonoids would not interfere with the molecular mass determination. Briefly, sample solutions for SDS/PAGE were prepared by adding an equal volume of the buffer (0.25 M Tris/HCl, pH 7.0, containing 4% (w/v) SDS, 10% sucrose,10% (v/v) 2-mercaptoethanol and 0.025% bromophenol blue to each protein solution (**APPENDIX B**). Each sample solution was then incubated at 98 $^{\circ}$C for 2–3 minutes and loaded on to a 5-20% gradient gel (Bio-rad, Japan). Electrophoresis was performed in 125 mM Tris base with 0.96 M glycine and 0.5% SDS; polypeptide bands were detected by silver staining.

2.2.2.3.3 Fourier Transform Infrared (FT-IR)

Sericin powders were prepared from silk cocoon solution by freeze drying method (Khan et al., 2010). The investigated functional group properties and absorbency of the three species of sericin powder were determined using by Fourier Transform Infrared (FT-IR) spectroscopy measured with a Nicolet IR-200 infrared spectrometer by the ATR method in the region of 4000-400 cm^{1} spectral resolution and 128 scans.

2.2.2.4 Biological activities of the sericin samples

2.2.2.4.1 Free radical scavenging assay of sericin extracted from the native Thai silk cocoon

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Sericin samples at 200, 100, 50, 25 and 12.5 mg/ml and the standard antioxidants (vitamin C , vitamin E ,BHT and linoleic acid) in the mixture of 95% ethanol and 10%(v/v) DMSO (1:1) were assayed for free radical scavenging activity by the DPPH method (Jung et al., 2006). Briefly, 100 µl of the samples or the standards, 25 µl of 2mg/ml DPPH in 95% ethanol and 25 µl of 95% ethanol were mixed in 96-well microplates and incubated at room temperature (25°C) for 30 minutes. The absorbance was measured at 515 nm. The percentages of DPPH radical scavenging activity were calculated according to the following equation:

$$\text{\% DPPH radical scavenging activity} = \frac{A - B}{A} \times 100$$

where a was the absorbance of the control reaction and B was the absorbance of the test samples. Sample concentrations providing 50% scavenging (SC_{50}) were calculated from the graph plotted between free radical scavenging inhibition percentages and the sample concentrations.

2.2.2.4.2 Tyrosinase inhibition assay of sericin extracted from the native Thai silk cocoon

Sericin samples at 200, 100, 50, 25 and 12.5 mg/ml and the standard antioxidants(vitamin C and kojic acid) were mixed with 5% (v/v) DMSO and assayed by the modified dopachrome method using tyrosine as a substrate as previously described (Long et al., 2000). Briefly, 40 µl of the samples or standards, 40 µl of 0.1 mg/ml L-tyrosine, 50 µl of 0.1 mg/ml mushroom tyrosinase and 80 µl of 0.1M phosphate buffer were added in 96-well microplates. The 5% (v/v) DMSO solution was used as a negative control. The mixture was incubated at 37 °C for 60 minutes. Before and after incubation, the amount of dopachrome produced in the reaction

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mixture was measured at 450 nm. The experiment was done in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation:

$$\% \text{ inhibition activity} = \frac{(A-B) - (C-D)}{(A-B)} \times 100$$

where a was the absorbance of the blank after incubation, B was the absorbance of the blank before incubation, C was the absorbance of the sample after incubation, and D was the absorbance of the sample before incubation. Sample concentrations providing 50% inhibition (IC_{50}) were calculated from the graph plotted between tyrosinase inhibition activity percentages and the concentrations.

2.2.2.4.3 Total amino acid profile of sericin extracted from the native Thai silk cocoon

Amino acid profile of sericin silk from *Bombyx mori* Linn (Native Thai silk) was analyzed using high performance liquid chromatography (HPLC, AS 1000, Thermo Finigan, USA) connecting with AccQ Tag column (1993). Samples for the analysis were hydrolysed in 4 M methane sulfonicacid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Tokyo, Japan) at 100 °C for 24 h under vacuum. All experiments were performed in triplicate.

2.2.3 Preparation of niosomes entrapped with sericin and oil extracted from the None Ruesee silkworm

2.2.3.1 Preparation of blank niosomes

Niosomal formulations were prepared by chloroform film method with

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sonication. Briefly, the total amount of 20 mM of Tween® 61 together with cholesterol at 1:1 molar ratio was dissolved in chloroform in a flask. Then, the solvent was removed by a rotary evaporator (Eyela,N-N Series, and USA). The thin lipid or surfactant films on of the flask were hydrated by adding distilled water. The resulting dispersion was shaken vigorously at 60 ± 1 °C until the film was completely dispersed. The dispersion was further sonicated by a probe sonicated (Vibra-Cell Model VC 505, Sonics & Materials Inc., Newtown, CT, USA) at 25 amplitude for 20 minutes. Then, the dispersion was centrifuged at 8000 rpm for 20 minutes by ultracentrifuge (Tomy Seko Co.Ltd., Tokyo, Japan) to eliminate the traces of titanium from sonication step and then, lyophilized by a freeze-dryer **(Figure 23).**

Figure 23 Preparation processes of niosomes

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2.2.3.2 Characteristics of blank niosomes

A. Particle size and zeta potential determination of the blank niosomes

The particle size and zeta potential were measured by Zeta-sizer nanoseries. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 70 °C This measurement is based on a dynamic light scattering method. The vesicular dispersions were diluted about 20 times with distilled water before the measurement.

B. Physical stability of the blank niosomes

The niosomes were kept in transparent cover vials protected from light and stored at room temperature (25±2 °C), 4±2 and 45±2 °C for 3 months. At 0, 1, 2 and 3 months, the physical stability of the dispersions was observed visually (color, odor, clarity ,pH and precipitation). The best niosomal formulations which showed the proper physical stability were selected.

C. Selection of the best blank niosomal formulation

The blank niosomal formulations which gave the best physical characteristics were selected to entrapped the sericin and oil extracts

2.2.3.3 Entrapment of silkworm sericin and oil in the selected niosomal formulation

The preparation process of niosomes entrapped with sericin and silkworm oil was described as follows. The appropriate amount of Tween 61 and cholesterol at 1:1 molar ratios and 1% (w/v) silkworm oil were added together with the Tween 61 and cholesterol, and prepared by the chloroform film and sonication (CFS) (Kikuchi et al., 1999). Briefly, the surfactants together with cholesterol and oil were dissolved in chloroform, and the solvent was removed by a rotary evaporator (Eyela, N-N Series,

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New York USA) to get a thin film. The film was evacuated in a desiccator under reduced pressure for more than 24 hours, and resupended in 1% (w/v) sericin water solution. The dispersion was obtained and swelled by swirling in a water bath at 60 ±1 °C for 20 minutes. The dispersion was further sonicated by a probe sonicator (Vibra-Cell Model VC 505, Sonics & Materials Inc., Newtown, CT, USA) at 25 amplitude for 20 minutes at pulse on 3.0 and pulse off 1.0. Then, the dispersion was centrifuged at 8000 rpm for 1 minute by ultracentrifuge (Tomy Seko Co.Ltd., Tokyo, Japan) to eliminate the traces of titanium from the sonication step. Then, the dispersion was lyophilized by a freeze dryer (Freeze dry / shell freeze system, (Labcon Co. Ltd., USA). The resulting dried powder was kept at 4 °C until use.

2.2.3.4 Physicochemical properties and stability observation

A. Appearance and morphology

Sedimentation, flocculation and turbidity were visually observed and reported as degrees of sedimentation using the criteria as shown as in **Figure 24**

No sedimentation reported as –

Partial sedimentation (1-25%) reported as +

Nearly complete sedimentation (26-75%) reported as ++

Complete sedimentation reported as +++

Figure 24 The bottom-view of bottle showed different degrees of sedimentation

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B. pH measurement

The pH of niosomal dispersion was measured by a pH meter (Cyberscan pH 500, Euteoh Instruments, Singapore).

C. Appearance of niosomal dispersion

The appearance of niosomal dispersion was investigated by means of an Olypus-BH-2 microscopy (magnification 400X) equipped with digital camera. A small droplet of the suspension was placed on a glass microscopy slides, diluted with a few drops of distilled water and covered with a glass cover slip. The samples were examined for vesicle formation, crystal formation and vesicular appearences.

D. Determination of entrapment efficiency

The niosomal dispersion was centrifuged at 50,000 rpm 4 °C for 30 minutes. The vesicle was then precipitated and the supernatant was clear with yellow color. The supernatant was further completely removed by pipetting. The amount of sericin and alpha-linolenic acid in the supernatant and in the vesicles (precipitate) were determined by HPLC respectively, and the amounts found were considered as the un-entrapped and entrapped sericin and oils, respectively.

The percentage of entrapment efficiencies were calculated from the ratio of the amount of sericin or oil entrapped in the niosmes (I_{in}) and the total initial amount of sericin or oil (in the form of linoleic acid) (I_{total}) and multiplied by 100, according to the following equation:

$$\text{Entrapment efficacy (\%)} = \frac{I_{in}}{I_{total}} \times 100$$

E. Particle size and zeta potential measurement

The particle size and zeta potential values were measured using Zeta-

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sizer nanoseries (Brookhaven Instruments Corporation, New York, USA). The temperature was set at 25 °C Charges on the vesicles and their mean zeta potential values with the standard deviation were obtained directly from the measurement. This measurement is based on a dynamic light scattering method. The vesicular dispersions were diluted to about 30 times with deionized-distilled water before the measurement. The measurement was taken about 120 second each and repeated 3 times for each sample. The refractive index was set at 1.33. The particle size and zeta potential of niosomes were internally determined in terms of mean diameter, 95% limits and the standard deviation.

F. Stability study of the niosomes entrapped with sericin and oil

The niosomes entrapped with sericin and oil were kept in transparent vials and stored at room temperature (25±2 °C), 4±2 and 45±2 °C for 3 months. At 0, 1, 2, and 3 months, the physical stability of the dispersions were observed visually (color, odor, clarity, precipitation and pH). At 3 months, the vesicular size and the polydispersion including was determined using by Zetasizer (Uchegbu et al.,1996).

G. Transmission electron microscopy (TEM)

The vesicle morphology was examined by transmission electron microscopy (TEM) in order to determine size, shape, and lamellarity. A 200 mesh formvar copper grid was floated on a droplet of niosomal dispersion on parafilm or dental wax for 10 minutes to allow some vesicles to adheres on the formvar. The remaining dispersion was removed by absorbing with the corner of the filter paper. The grid was then transferred onto a nearly drop of the negative stain solution (1% (w/v) of uranyl acetate solution) and left for 10 minutes, blotted excess solution with a

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filter paper, and air-dried for 30 minutes. Finally, the sample was observed under a transmission electron microscope.

2.2.4. Development of anti-wrinkle serum containing niosomes entrapped with oil and sericin extracted from silk

2.2.4.1 Five serum base formulations were developed and one formulation was selected

Serum were prepared from 5 basic formulations as shown in **Table 22 and Appendix F**

2.2.4.2 The quality of the serum base was compared with the typical facial moisturizing products sold on the market. Their compositions were listed in **Table 22** and the preparation process was indicated in **Figures 25-29**. Three different brands of the facial cream formulations including Brand A, Brand B, and Brand C were selected with the type and compositions that were similar to the serum base formulation. The quality characteristics including, sensory quality, and clinical quality were used as guidelines for the development of the serum base in order to get a new formula that was in the same standard as the items sold in the market. Details of the quality study include the followings:

2.2.4.2.1 Characteristic determination

A) Color was measured with a handy colorimeter (Nippon, model no. NR-3000, Tokok, JAPAN).

B) pH was measured by a pH Meter (Cyberscan pH 500, Euteoh Instruments, Singapore)

C) Viscosity was measured with a Brookfield Viscometer (Model

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RVDV -II+, Brookfield engineering laboratories, USA) controlling temperature of the sample at 25±0.2 °C

D) The physical stability of the formula was done by Freeze-Thaw cycle (-4 °C/24 hours and 45 °C/24 hours) for 5 rounds. pH value and viscosity were measured in each round to evaluate the stability and to investigate the layering of the cream. (Viscosity was measured by the viscometer with the needle size 5 at speed 50 rpm).

2.2.4.2.2 Sensory quality

Sensory quality was evaluated by 30 volunteers (21 females 9 males, aged between 20-65 years old) using hedonic scaling test and just about right to evaluate quality of the products for 3 stages; a) before used, i.e. appearance, b) during used, spreadability, absorbency and stickiness on the skin, c) After used, moisturization and overall liking to observe rash on the skin (Moskowitz et al., 1993; Epler, S. et al., 1998).

Table 22 Ingredients of the 5 selected serum base formulations

Phase	Raw Material	Formulation Nos. (%)				
		1	2	3	4	5
A	Montanov 202	3	1.5			
	Montanov 68	1				
	Lanoll 99	8	4			
	Finsolv TN	2				
	Lipacide DPHP	1				
	Olivem 1000		2			
	Oliwax LC		2			
	Capric triglyceride		6			
	Floraester 60		6			
	Laurex CS		1			

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Table 22 Ingredients of the 5 selected serum base formulations (continued)

Phase	Raw Material	Formulation Nos. (%)				
		1	2	3	4	5
	SFE 839		1			
	Simusol 165		1			
	Dimethicone		1			
	Glycerin			3	3	
	Carbopol ® ultrez 21 polymer			0.3		
	Sodium chloride				1	
	Peptamide 6				3	
	DMDM hydration				1	
	Carbomer 940					0.6
	Triethanolamine					0.5
	Distilled water			qs.to 100	65.5	26.7
B	Demineralized water	82.3				
	Distilled water		qs.to 100			
	Magnesium aluminum silicate	0.5				
	Xanthan gum		0.15			
	Glycerin		3			
	Propylene glycol			2		
	Florasun 90			3		
	Silsense™ DW-18			3		
	Finsolv TN			2		
	Octyldodecanol			2		
	Octyl palmitate			2		
	Floramac® 10			1		
	SF 1328				10	
	SF 1284				8.5	
	Gel base 2				1.5	
	DCRM 2051				1.5	
	Crodafos CS-20 acid					2

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Table 22 Ingredients of the 5 selected serum base formulations (continued)

Phase	Raw Material	Formulation Nos. (%)				
		1	2	3	4	5
	Stearyl alcohol					1
	Crodamol STS					2
	Shea butter					0.3
	Avocado oil					0.3
	Dimethicone					1
	Methyl paraben					0.2
	Propyl paraben					0.2
	BHT					0.1
C	Sepic gel 305	1.5				
	DMDM hydration		0.3			
	Perfume		0.3			
	Shea butter			0.5		
	Peptamide 6			2		
	Phytelene chamomile			0.3		
	Distilled water					
	Triethanolamine					0.3
D	Sepicide HB	0.5				
	Perfume	0.2				
	Germaben® II E			0.5		
	Simugel EG				3	
	Distilled water					14
E	Triethanolamine	qs.		0.25		
	Perfume				0.3	
F	Sepic gel 305					1.2
	Perfume			0.3		

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Heat the water and oil phase (A and B) at 75-80 °C

⇩

Add water into oil and mix them with homogenizer at the speed of 25,000-30,000 rpm for 3 minutes

⇩

Add C into the mixture and mix them with homogenizer at speed 25,000-30,000 rpm for 5 minutes and Let it cool down until its temperature was 35-40 °C

⇩

Add D mix them with homogenizer at the speed of 35,000-40,000 rpm for 5 minutes

Figure 25 Preparation process of the serum base formulation 1

(Modified from firming and moisturizing care (www.seppic.com, 2006)

Dissolve xantham gum by glycerin and B. gradually add water.

⇩

Heat parts A and B to 75-80 °C

⇩

Gradually pour parts A into B slowly and stir constantly

⇩

Mix it by the homogenizer at 35,000 rpm for 2 minutes.

Reduce the temperature to 40 °C add C in A and B and stir constantly

⇩

Add simugel EG and perfume homogenizer at 35,000 rpm for 5 minutes

Figure 26 Preparation process of the serum base formulation 2

(Modified from the O/W formula moisturizing cream PC49031/5

(www.eac.com, 2008)

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Weigh carbomer 21 and glycerin into water and spin by a mixer

⇩

Add part B into A and mix together by a mixer for 5 minutes

⇩

Add C and D and mix with a mixer for 10 minutes

⇩

Add F and Adjust pH by triethanolamine

Figure 27 Preparation process of the serum base formulation 3

(Modified from anti-cellulite cream gel 0-001 4 (www.noveon.com)

eigh part A and mix together and dissolve into homogenization

⇩

Weigh part B and mixed by a mixer into homogenization

⇩

Pour A into B and mix well with a mixer for 1 hour into homogenization

⇩

Adjust pH by triethanolamine

Figure28 Preparation process of the serum base formulation 4

(Modified from the formula firming cream gel (www.arch.com, 2006)

Weigh Carbomer 940 in a beaker and spun by a mixer at 70 °C until all dissolved and adjusted by triethanolamine

⇩

Weigh part B and heat to 70-75 °C until all dissolved

⇩

Pour B into A and bring to be homogenized at 30,000 rpm for 5 minutes

⇩

Cool it to 40 °C and add C and TEA to be homogenized at 35,000 rpm for 5 minutes

⇩

Add D homogenized at 35,000 rpm for 3 minutes

Figure 29 Preparation process of the serum base formulation 5

(Modify from firm up firming cream ST-50(www.arch.com, 2005)

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2.2.4.3 Optimizations of the best anti-wrinkle serum formulation

The selected best anti-wrinkle serum formulation from the topic 2.2.4.2 was developed to get a better formula that has similar quality to the formula sold in the market. Levels of the 3 studied factors of the 3 formulae of serum were measured for chemical, physical and sensory quality as in the topics of 2.2.4.2.1 and 2.2.4.2.2. **Table 23.**

Table 23 Factors and levels used in the study

Serum formula	Levels of the studied factor		
	CDRM 2051	Simugel EG	Seppic gel 305
1	0.5	-	-
2	-	0.5	-
3	-	-	0.5

Note: CDRM 2051, Simugel EG and Seppic gel 305 are thickening agent used to develop the serum.

After that, the factors affecting the formula from the topic 2.4.2 including, viscosity, spreadability, smoothness and gloss characteristics from the study by experimental design 3x3 factorial. C_{14-22} alkylalcohol and C_{12-20} alkylglucoside (MONTANOV L) were added in the serum formula at 0.5, 1 and 1.5 % respectively and C_{14-22} alkylalcohol and C_{12-20} alkylglucoside and cyclospentasiloxame, dimethicomol, dimethicone crosspolymer (and) phenyltrimethicone blend (DCCB3031) were at 1, 1.5 and 2% respectively. After that, gave the measured preference scores with the 9-point scale. The reactions between factors and the average difference from the measurement of the quality values. Then, the selected optimized product was based on the factors in the formula, i.e viscosity, absorbency,

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gloss, moisturization, firming and overall liking. Then, the data from each of the treatment were used to create a mathematical model or regression equation by all variables using combined equation (enter) and the selected equation with $p \leq 0.05$ and the coefficients of Parasites decision (R^2) more than 0.75 to the analysis graph contour plot. The analysis of the determinate was done using Response Surface Methodology (RSM) with Quadratic Model quality of consumer products compared with the commercial quality for the selection of the optimized formulation. After that, the experiment chemical, physical, and sensory qualities of the treatments were measured according to the direction in 2.2.4.2.1 and 2.2.4.2.2 in order to examine the effectiveness of each formula and the clinical quality as follow:

A. Skin test by the clinical method was performed in 10 volunteers before and after the application of the serum. The selected volunteers must have healthy complexion and no record of skin allergy. The volunteers applied the serum to their face skin, around the eyes, and neck. The testing area size of 2 x 2 cm was cleaned with clean water and dried with towel. The volunteers applied 2 g of the serum on their face puff in the same direction. Then, the skin quality was measured. The process of application was done at the controlled temperature (20±2 $^{\circ}$C) and relative humidity (40-60%) RH (Hashmi et al., 2007; Dobrev, 2005).The following skin quality was investigated:

B. Skin elasticity was measured with a cutometer MPA 580 (Courage & Khazaka, Colong, Germany) before and after the application of the serum, using a 2 mm-diameter probe and vacuuming pressure at 450 mbar. Vacuuming condition was on-time 5 seconds and off-time (relaxation time) 3 seconds with 10 repetitions for one measurement. Afterwards, the pressure was cut off and the skin returned to its original

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shape. The following parameters were calculated automatically by a built-in database file where; R5 was the net elasticity of the skin (Ur/Ue), R8 was the viscoelastic character of skin (Uv). Each measurement was performed in triplicate. The measuring process was done every other week for 1 month.

C. Skin moisture was measured before and after the serum application with the skin diaganostic SD (Courage & Khazaka, electronic GmbH, Germany). The measurement was based on the capacitance method that used the relatively high dielectric constant of water compared to the substances of the skin. Three measurements were performed in each testing area on the human volunteers volar face.

2.2.4.4 Development of serum containing niosomes entrapped with the silk oil and sericin

The selected serum base formulation from 2.2.4.3 was used to develop the product containing niosomes. Niosomes were added into the serum base formulation at 3, 6 and 9 % w/w. Then, the chemical quality, including pH value, physical quality including color value, and viscosity were measured. Sensory quality including were measured with 9 point Hedonics scale satisfactory level by 30 untrained volunteers. The formulation base was selected on the criteria of having the highest satisfactory level and the highest sensory quality evaluated by the just-about- right method (Epler et al., 1998).

2.2.4.5 Development of color and fragrance of the selected serum formulation

2.2.4.5.1 Color development of the selected serum formulation

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Preference ranking was used to evaluate appearance i.e. white, natural white, and off-white, respectively, by 50 untrained respondents. The result was analyzed by the Friedman's Test method. The formulae and calculation were shown in **Appendix C**. The color that gave the highest preference was chosen

2.2.4.5.2 Fragrance development of the selected serum formulation

The selected serum from topic 2.2.4.5.1 was examined to develop the right fragrance. The top 3 preferable fragrances including floral fruit, spring time, and fruit were used by adding 0.5% of each fragrance into the final process of the serum preparation. Evaluation was done by 30 volunteers using 9-point Hedonic scale on each stage, i.e. smell before use, smell during use (during rubbing on the skin), and smell after use (smell after applying for 15 minutes). The formula that gave the highest satisfactory level and has the highest percentage of just-about-right evaluation was chosen (Lawless and Heymann, 1998).

2.2.4.6 Quality assessment on the developed serum containing niosomes entrapped with the silk oil and sericin

The developed serum was evaluated on the following characteristics:

A.: Measure the pH value with a pH Meter (TISI 152-1996).

B. Physical quality

B1.Measure the viscosity by Brookfield Viscometer DV+II

B2.Measure the color value with a handy colorimeter

C. Microbiological quality (TISI 152-1996)

Ten fold dilutions of 10 g of each sample (randomly selected) were performed to achieve dilution factor of 10^{-2} and 10^{-5}. An exactly amount (0.1 ml) of the diluents were poured in three nutrient agar plates for total bacteria count

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determinaton, with the MacConkey agar (Oxoid) for the coliform count and Sabourad dextrose agar with chloramphenicol (250 mg/100 ml) for the fungal counts. All plates were incubated for 48 hours at 30 °C except the Sabouraud dextrose agar plates which were incubated at 25 °C for 6 days. The identification of the microbial counts was based on the classification scheme proposed by Adebayo-Tayo. et al (2009).

D. Sensory quality

Sensory quality was evaluated by examining the characteristics during use. These included greasy residue and the absorption feel on the skin, and the characteristics after use including, skin softness and smoothness, skin moisture, dryness of the skin, and irritation

E. Clinical quality of the developed serum was measured by comparing with the products sold in the market (brand A and brand B) on skin elasticity with Cutometer MPA 580 and skin moisture with Skin Diaganostic SD 27 before and after serum application according to the direction in (Kikuchi, K. and Tagami H., 2008; Ezure. et al., 2009). Eight subjects participated in the entire study. The subjects washed their face and were then acclimated for 20 minutes under the standard environmental conditions, in which the temperature and relative humidity were adjusted to 20 ± 2 °C and 40-60 %, respectively. Briefly, using a 2-mm probe, a negative pressure of 400 mbar was applied on the skin for a period of 2 minutes, followed by a 2 minutes relaxation time, and the ratio of the immediate retraction (Ur) to the final distension (Uf) was analyzed (Ur/Uf). Measurement points were the intersections of the two lines of the upper cheek and lateral cheek elasticity. Measurements were carried out three times, and the average values were used for analysis.

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2.2.5 Stability of the developed serum at difference storage temperatures

The developed serum containing niosomes entrapped with the silk oil and sericin were stored in polyethylene bottles, and kept at room temperature (30 °C) and in retorting conditions at 35 and 45 °C. Then, at different time intervals of 0,1,2,3,4 and 8 weeks, the serum were determined for physical qualities (viscosity and color) and chemical quality (pH comparing with at initial).

2.2.6. Consumer acceptance study

The consumer acceptance study on the developed serum by Home Use test method (Gacula et al., 1986) with 82 target females consumers aged over 35 years in Bangkok during December 2009 to January 2010 was performed. Non-probability sampling was used by distributing the survey sheets and product samples to the respondents to use for 8 weeks. The product preference levels (1 = dislike extremely and 9 = like extremely) were calculated for the difference of each preference level as follow (**Appendix D)** (Cooper, 2003; Rosas et al., 2005).

Average preference = (the highest – the lowest) / number of levels

Substitution: Average preference = (9 – 1) / 9 = 0.88

Each preference level was interpreted as follow:

1.0 – 1.88 = dislike extremely

1.89 – 2.77 = dislike very much

2.78 – 3.66 = dislike moderately

6.67 – 4.55 = dislike slightly

4.56 – 5.44 = neither like nor dislike

5.45 – 6.33 = like slightly

6.34 – 7.22 = like moderately

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7.23 – 8.11 = like very much

8.12 – 9.00 = like extremely

For the satisfaction level on the characteristics of the developed serum, the volunteers graded the 5 satisfaction as 5 levels (1 = much too weak and 5 = much too strong). The difference of each satisfaction level was evaluated by the method of Cooper, (2003). Each satisfaction level was interpreted as follow:

1.00 – 1.80 = much too weak

1.81 – 2.60 = slightly to weak

2.61 – 3.40 = just-right

3.41 – 4.20 = slightly to strong

4.21 – 5.00 = much too strong

2.2.7 Cost calculation of the developed serum containing niosomes entrapped with oil and sericin silk protein

The production cost calculation of the sericin from silk cocoon, oil from silkworm pupa and the anti-wrinkle serum were estimated shown in **Appendix E.**

2.2.8 Statistical analysis

The data in this study were analyzed statistically with the suitable operation programs including SPSS12, STATISTICA, and SIGMA PLOT-STAT

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