CHAPTER III

MATERIALS AND METHODS

Materials

Chemicals

1. Acetonitrile HPLC grade (Lab-Scan)
2. Cetastearyl alcohol (Sinthai Co., Ltd)
3. Cocamide DEA (Namsiang International Co., Ltd.)
4. Cocamidopropyl betaine (Cognis)
5. Curcuminoid working standard (Curcumin = 71.50% on basis, Desmethoxycurcumin =23.63% on basis, Bisdesmethoxycurcumin = 4.43% on basis)
6. Dimethylsulfoxide (Riedel de Haën®)
7. Ethylcellulose aqueous dispersion, Aquacoat®ECD-30 (Onimex Co.,Ltd.)
8. Evening primrose oil (Namsiang International Co., Ltd.)
9. Glacial acetic acid AR grade (Lab-Scan)
10. Glycerine (Srichand United Dispensary Co., Ltd.)
11. Grape seed oil (Namsiang International Co., Ltd.)
12. Gryceryl triacetate 99% (SIGMA)
13. Methanol AR grade (Lab-Scan)
14. Methanol HPLC grade (Lab-Scan)
15. PEG-100 stearate – glycerol stearate, Simulsol 165 (Adinop Co., Ltd)
16. Phenoxyethanol (Adinop Co., Ltd)
17. Polyethylene glycol average mol.wt.400 (SIGMA)
18. Polymethacrylate aqueous dispersion, Eastacryl™30D (Onimex Co., Ltd.)
19. Polyquaternium-44 (Cognis)
20. Polyvinyl acetate aqueous dispersion, Kollicoat®SR 30D (BASF)
21. Propylene glycol & 5-Bromo-5-Nitro- 1,3 Dioxane, Bronidox L (Cognis)

22. Propylene glycol U.S.P (Srichand United Dispensary Co., Ltd.)
23. Silicone fluid, DC344 (Dow Corning® 344 fluid)
24. Sodium chloride (Namsiang International Co., Ltd.)
25. Sodium laureth sulfate 28%, N 8000 (Cognis)
26. Trilaureth-4-phosphate, KL 340 D (NIKKOL)
27. Turmeric extract powder (Thai-Chaina Flavors & Fragrances Industry Co., Ltd., %purity of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin = 79.24, 20.16, and 2.57, respectively, MW of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin = 368.39, 338.36, and 308.34, respectively.)

Equipment

1. Analytical Balance (Sartorius BA 210S)
2. Desiccator
3. Hot air oven (Model B40, Memmert)
4. Hot air oven (Binder)
5. Incubator (Hotpack)
6. Magnetic stirrer (Mini MR IKA)
7. Mastersizer S long bed version 2.11 (Malvern)
8. Mini spray dryer (Buchi B-290)
9. Moisture analyzer (HR83 Mettler Toledo)
10. Reversed-phase High Performance Liquid Chromatography, HPLC (Shimadzu)

    Liquid Chromatography: LC-10ADvp
    System Controller: SCL-10Avp
    UV-VIS Detector: SPD-10Avp
    Auto Injector: Sil-10ADvp
    Column Oven: CTO-10Avp
    Degasser: DGU-14A
    Column: C18 column, Inersil ODS 5 micron 4.6 mm x 150 mm (GL Science, Japan) with a guard column, Inersil ODS 5 micron 4.6 mm x 150 mm (GL Science, Japan)
11. Scanning electron microscope (JSM-5410LV)

12. Tapping machine
13. Ultrasonic Cleaner (Cavitator, model ME)
14. Vacuum pump (Buchi Vac® V-500)
15. Vortex mixer (Vortex-genic)

Methods

1. Preparation of Curcuminoid Microcapsules by Spray-Drying Technique

1.1. Spray-Dryer and Operating Condition

Curcuminoid microcapsules were prepared using Buchi mini spray-dryer B-290. The operating conditions of spray-drying process were obtained by preliminary study.

Inlet air temperature:	120°C
Aspirator rate:	28 m^3/hr
Pump rate:	5 mL/min
Rotameter:	30 mm (357 Normlitre/hr)

1.2. Variation of Formulation Parameters

Active agent:	Curcuminoid extract
Polymers:	(i) Polymethacrylate, Eastacryl™30D (PM)
	(ii) Polyvinyl acetate, Kollicoat®SR 30D (PVA)
	(iii) Ethylcellulose, Aquacoat®ECD-30 (EC)
Plasticizers/wetting agents:	(i) Propylene glycol (PG)
	(ii) Polyethylene glycol 400 (PEG400)
	(iii) Glyceryl triacetate (GTA)
Drug : polymer ratio:	1:1, 1:2, 1:3
Polymer : plasticizers ratio:	1:1
Solid content:	1% and 2%.

1.3. The Preparation of Curcuminoid Microcapsules

Curcuminoid microcapsules from PM (Eastacryl™30D) were prepared by mixing curcuminoid extract with plasticizers. Then, the resulting mixture was dispersed into polymeric aqueous dispersion of PM. The percents solid content were controlled between 1-2%w/v and the volume of the aqueous dispersion was adjusted to 1000 mL by de-ionized water. Then, the curcuminoid microcapsules were spray-dried by using Buchi mini spray-dryer.

Curcuminoid microcapsules from different polymers and plasticizers with different drug : polymer ratios and percent solid contents were prepared by the same method, in which the different parameters of the feed formulations were shown in table 5.

Table 5. Formulation composition indicating polymers, plasticizers, drug: polymer ratios, and percent solid content.

No.	Polymer	Plasticizer	Drug: Polymer	% Solid Content
1	PM	PG + GTA	1:1	1
2	PM	PG + GTA	1:1	2
3	PM	PG + GTA	1:2	1
4	PM	PG + GTA	1:2	2
5	PM	PG + GTA	1:3	1
6	PM	PG + GTA	1:3	2
7	PM	PG	1:1	1
8	PM	PG	1:1	2
9	PM	PG	1:2	1
10	PM	PG	1:2	2
11	PM	PG	1:3	1
12	PM	PG	1:3	2
13	PM	PG + PEG400	1:1	1
14	PM	PG + PEG400	1:1	2
15	PM	PG + PEG400	1:2	1
16	PM	PG + PEG400	1:2	2
17	PM	PG + PEG400	1:3	1
18	PM	PG + PEG400	1:3	2

Table 5. (Continued)

No.	Polymer	Plasticizer	Drug: Polymer	% Solid Content
19	PVA	PG + GTA	1:1	1
20	PVA	PG + GTA	1:1	2
21	PVA	PG + GTA	1:2	1
22	PVA	PG + GTA	1:2	2
23	PVA	PG + GTA	1:3	1
24	PVA	PG + GTA	1:3	2
25	PVA	PG	1:1	1
26	PVA	PG	1:1	2
27	PVA	PG	1:2	1
28	PVA	PG	1:2	2
29	PVA	PG	1:3	1
30	PVA	PG	1:3	2
31	PVA	PG + PEG400	1:1	1
32	PVA	PG + PEG400	1:1	2
33	PVA	PG + PEG400	1:2	1
34	PVA	PG + PEG400	1:2	2
35	PVA	PG + PEG400	1:3	1
36	PVA	PG + PEG400	1:3	2
37	EC	PG + GTA	1:1	1
38	EC	PG + GTA	1:1	2
39	EC	PG + GTA	1:2	1
40	EC	PG + GTA	1:2	2
41	EC	PG + GTA	1:3	1
42	EC	PG + GTA	1:3	2
43	EC	PG	1:1	1
44	EC	PG	1:1	2
45	EC	PG	1:2	1
46	EC	PG	1:2	2
47	EC	PG	1:3	1
48	EC	PG	1:3	2
49	EC	PG + PEG400	1:1	1
50	EC	PG + PEG400	1:1	2
51	EC	PG + PEG400	1:2	1
52	EC	PG + PEG400	1:2	2
53	EC	PG + PEG400	1:3	1
54	EC	PG + PEG400	1:3	2

2. Analysis of Curcuminoid microcapsules

2.1. Yield of Microcapsules

The percent yield of microcapsules was calculated by using the formula below.

$$\%\text{Yield} = \frac{\text{Wt. of microcapsules obtained at the end of the process (g)}}{\text{Wt. of initial solid substances added (g)}} \times 100$$

Where:

Initial solid substances added = curcuminoids extract (g) + dried wt. of polymer (g)

2.2 HPLC Analysis

2.2.1. Analytical Method Development

The high performance liquid chromatography (HPLC) was used to determine the amount of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in microcapsules. The HPLC conditions employed in this study were validated and proved to be stability-indicating assay (SIA) for the determination of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin (Sunipon et al., 2003) and were shown as following:

HPLC column:	C18 column, Inersil ODS 5 micron 4.6 mm x 150 mm
Mobile phase:	2% acetic acid: acetonitrile (60: 40)
Flow rate:	2.0 mL/min
Injection volumn:	20 μL
Detector wavelength:	425 nm
Temperature:	ambient temperature

2.2.2. Analytical Method Validation of Curcumin, Desmethoxycurcumin, and Bisdesmethoxycurcumin in Cleansing Gel Preparation

2.2.2.1. Preparation of Standard Curve

Preparation of curcuminoid working standard solution (Stock I)

Curcuminoids working standard of 20 mg was weighed into 200-mL volumetric flask. The volume was adjusted to 200 mL by methanol. (conc = 100 μg/mL)

Preparation of curcuminoid working standard solution (Stock II)

Stock I of 5 mL was transferred to 100-mL volumetric flask by 5-mL transferring pipette. The volume was adjusted to 100 mL by methanol (conc = 5 μg/mL).

Preparation of curcuminoid working standard solution (Stock III)

Stock I of 25 mL was transferred to 100-mL volumetric flask by 25-mL transferring pipette. The volume was adjusted to 100 mL by methanol (conc = 25 μg/mL).

Preparation of standard curve

The prepared working standard solution Stock II of 1, 2, and 5 mL were transferred into 25-mL volumetric flasks no.1, 2, and 3, respectively, by transferring pipettes and prepared working standard solution Stock III of 2, and 5 mL were also transferred into 25-mL volumetric flasks no.4, and 5, respectively, by transferring pipettes. The volumes of five volumetric flasks were adjusted to 25 mL by methanol. The preparations were carried out in triplicate at each concentration (conc = 0.2, 0.4, 1.0, 2.0, and 5.0 μg/mL). Then, the prepared working standard solutions were analyzed by HPLC using HPLC conditions mentioned in 2.2.1 and the amount of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were calculated from the chromatograms.

2.2.2.2. Accuracy Assay

Accuracy was performed by spiked placebo method. The prepared working standard solution Stock II of 1, 2, and 5 mL were transferred into 25-mL volumetric flasks no.1, 2, and 3, respectively, by transferring pipettes and prepared working standard solution Stock III of 2, and 5 mL were also transferred into 25-mL volumetric flasks no.4, and 5, respectively, by transferring pipettes. Then, the curcuminoids were extracted from cleansing gel by the method shown in figure 7.

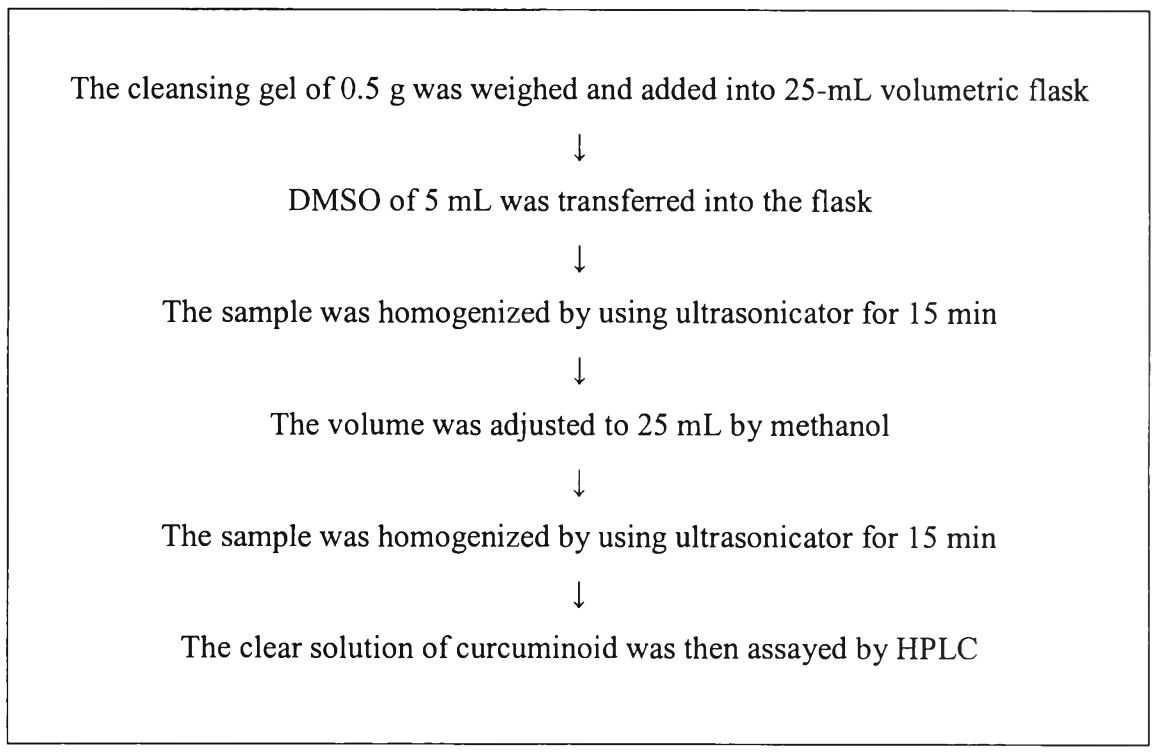

Figure 7. The schematic diagram of the extraction of curcuminoids in cleansing gel.

The preparations were carried out in triplicate at each concentration (conc = 0.2, 0.4, 1.0, 2.0, and 5.0 μg/mL). The actual concentrations, the observed concentrations, and %recovery of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were determined.

2.2.2.3. Precision Assay

Precision was performed by spiked placebo method. The prepared working standard solution Stock I of 5 mL was transferred into six 25-mL volumetric flasks. Then, the sample was undergone the curcuminoids extraction steps described in 2.2.2.2. The preparations were carried out in six replications. The actual concentrations, the observed concentrations, and %recovery of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were determined.

2.2.2.4. Linearity Assay

The actual concentrations and the observed concentrations of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin determined in 2.2.2.2 were averaged and the linearity graphs of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were constructed.

2.2.2.5. Specificity Assay

Specificity was performed by spiked placebo method. The prepared working standard solution Stock II of 5 mL was transferred into 25-mL volumetric flasks. Then, the sample was undergone the curcuminoids extraction steps described in 2.2.2.2. The resulting clear solutions were analyzed by HPLC using HPLC conditions mentioned in 2.2.1 and the chromatograms were recorded.

2.3. Percent Content of Curcuminoids and Entrapment Efficiency of Curcuminoid Microcapsules

The percent content of curcuminoids and the entrapment efficiency of curcuminoid microcapsules were determined using HPLC method. Triplicate samples of microencapsulated curcuminoids of 100 mg were accurately weighed and dissolved with 10 mL of methanol. The resulting solution of 100 μL was taken and diluted with 10 mL of mobile phase. This solution was then assayed by HPLC and the amount of

curcuminoids was determined from standard curves. The percent content of curcuminoids and the percent entapment of curcuminoid microcapsules were calculated as followed:

$$\%\text{Curcuminoid content} = \frac{\text{Observed amount of curcuminoids } (\mu g/mL)}{\text{Actual amount of microcapsules } (\mu g/mL)} \times 100$$

$$\%\text{Curcuminoid entrapment} = \frac{\%\text{Observed curcuminoid content}}{\%\text{Theoretical curcuminoid content}} \times 100$$

Where,

$$\%\text{Theoretical curcuminoid content} = \frac{\text{Wt. of curcuminoids extract (g)}}{\text{Wt. of curcuminoids extract (g) + Wt. of dried polymer (g)}} \times 100$$

The suitable preparations of microencapsulated curcuminoids were selected for further studies on physical properties, chemical stability at different storage temperatures, and chemical stability in skin-care preparations.

2.4. Determination of Physical Properties of Curcuminoid Microcapsules

2.4.1. Morphology and Particle Size

A small amount of spray-dried curcuminoid microcapsules were placed on one surface of a double-faced adhesive tape that stick to the sample support, coated with gold under vacuum condition and then observed on a scanning electron microscope JSM-5410LV for evaluation of the outer topographies of the microencapsulated products.

The particle size of microencapsulated curcuminoid was measured by laser light scattering using a model Mastersizer S long bed version 2.11 (Malvern).

2.4.2. Bulk Density, Tapped Density, and Hausner Ratio

The bulk density of curcuminoid microcapsules was determined by measuring the volume of a certain mass of microcapsules sample that had been passed through a screen into a graduated cylinder.

The tapped density of curcuminoid microcapsules was determined by mechanically tapping a measuring cylinder containing microcapsules sample. The initial volume was recorded, the cylinder was mechanically tapped, and volume readings were taken until little further volume change was observed.

Hausner ratio is the measure of the relative importance of interparticular interactions. In a free-flowing powder, such interactions are generally less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticular interactions, and a greater different between the bulk and tapped densities will be observed. The Hausner ratio was calculated as the ratio of the initial volume of a certain mass of microcapsules and the final volume of the same mass of microcapsules after mechanical tapping.

2.4.3. Moisture Content

The moisture content of the microencapsulated curcuminoids was determined by using Moisture analyzer (HR83 Mettler Toledo) with heating temperature of 105 °C. The sample of microcapsules of approximately 1 g was weighed into the aluminum pan and the percent moisture content was subsequently assessed. The measurements were performed in triplicate for each sample.

2.5. Determination of the Chemical Stability of Spray-Dried Curcuminoid Microcapsules Stored at Different Storage Temperatures

The preparations of microencapsulated curcuminoids were firstly put in a brown-colored glass bottle and sealed with the film. The preparations were then stored at 4°C, room temperature, 40°C and 50°C. The content of curcuminoids in microcapsules was assayed at the 0, 1, 2, and 3 month by HPLC method mentioned in

2.2.1. The percent retention, which was defined as the ratio between the content of curcuminoids that retained in the formulation after some time and the initial content of curcuminoids in the formulation, was used to evaluate the storage stability of curcuminoid microcapsules at different storage temperatures.

The curcuminoids solution to be assayed by HPLC was prepared using the method which was mentioned in 2.3.

2.6. Determination of the Chemical Stability of Curcuminoid Microcapsules in Skin-Care Preparations

2.6.1 Preparation of Cleansing Gel Containing Curcuminoid Microcapsules

The chosen cleansing gel formula, of which the stability had been Tested by 3-round of freeze-thaw cycles, was shown below:

Cleansing gel formula:

Glycerine	2.0
Polyquaternium-44	1.0
Sodium chloride	0.5
Sodium laureth sulfate 28%	25.0
Cocamide DEA	4.0
Cocamidopropyl betaine	10.0
Preservative	0.2
Water q.s. to	100.0

The pH of prepared cleansing gel was subsequently adjusted by citric acid solution to obtained final pH of 5, 7, and 8.

2.6.2. Preparation of Moisturizing Cream Containing Curcuminoid Microcapsules

The chosen moisturizing cream formula, of which the stability had been tested by 3-round of freeze-thaw cycles, was shown below:

Moisturizing cream formula:

Glycerine	2.0
Propylene glycol	3.0
PEG-100 stearate-glycerol stearate	2.0
Cetastearyl alcohol	5.0
Trilaureth-4-phosphate	1.0
Grapeseed oil	2.0
Evening primrose oil	0.5
Silicon oil	5.0
Phenoxyethanol	0.6
Water q.s. to	100.0

2.6.3. Chemical Stability of Curcuminoid Microcapsules in Skin-Care Preparations

The microcapsules samples were incorporated into cleansing gel and moisturizing cream in 0.01%w/w of the formulations, kept in glass containers and in the dark. The content of curcuminoids was tested every two weeks. The percent retention of curcuminoids in microcapsules, which was defined as the ratio between the content of curcuminoids that retained in the microcapsules after some time and the initial content of curcuminoids in the microcapsules, was used to evaluate the chemical stability of curcuminoid microcapsules in different skin-care preparations.

Curcuminoids were extracted from skin-care preparations by the mothod shown in figure 8.

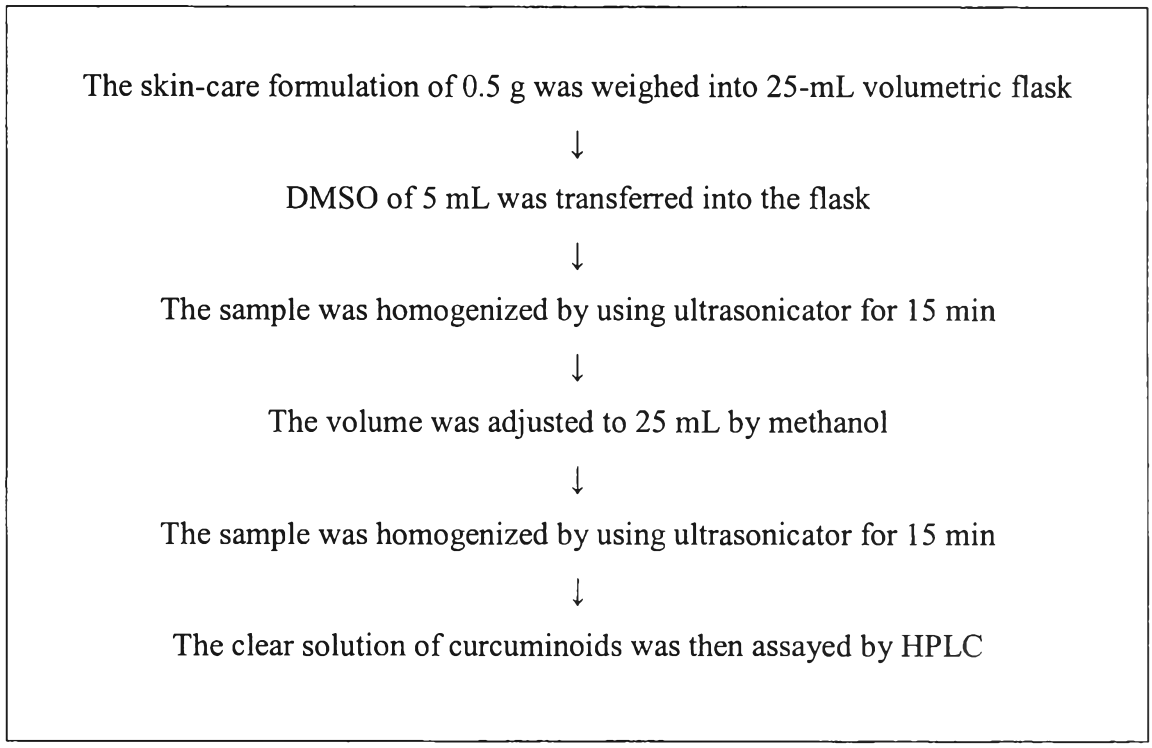

Figure 8. The schematic diagram of the extraction of curcuminoids in skin-care preparations