CHAPTER III

MATERIALS AND METHODS

Materials

1. Poly (dl-lactide-co-glycolide) (PLGA) copolymers (Sigma)
   1.1 50% PLA and 50% PGA (PLGA 50:50)
   1.2 75% PLA and 25% PGA (PLGA 75:25)
   1.3 85% PLA and 15% PGA (PLGA 85:15)
2. Curcuminoids (Thai-China Flavours and Fragrances Industry Co., Ltd.) % purity of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were 72.29, 23.56, and 3.63, respectively
3. Polyvinylalcohol, cold water soluble grade (Sigma)
4. Poloxamer 407 (BASF)
5. Vitamin E TPGS (NF grade) (Eastman Chemicals, UK)
6. Acetonitrile HPLC grade (Lab-Scan Co., Ltd., Thailand)
7. Methanol HPLC grade (Lab-Scan Co., Ltd., Thailand)
8. Methanol AR grade (Lab-Scan Co., Ltd., Thailand)
9. Glacial acetic acid AR grade (Lab-Scan Co., Ltd., Thailand)

Equipment

1. Analytical Balance (BA 2105, S/N 21203485, Satorius Basic)
2. Magnetic Stirrer
3. Refrigerated Centrifuge (Himac® CR20B3, Hitachi, Japan)
4. Freeze-dryer (LyoLab w/PC, Lyophilization Systems Inc., USA)

5. High-Performance Liquid Chromatography (HPLC)

   Column: HiQsil C_{18}, 5 μm, 4.6 mm x 150 mm., No. 00W00129 (KYA TECH Corporation, Japan)

   Liquid Chromatography: LC-10ADVP (Shimadzu, Japan)

   UV-VIS Detector: SPD-10AVP (Shimadzu, Japan)

   System Controller: SCL-10AVP (Shimadzu, Japan)

6. Scanning Electron Microscope (SEM) (JSM-5410LV, JEOL Co., Ltd., Japan)

7. Laser Light Scattering Spectroscopy (Mastersizer S long bed version 2.11, Malvern Instruments Ltd., UK)

Methods

1. Preparation of curcuminoids-PLGA nanoparticles

1.1 Formulation ingredients

There were three ingredients used for curcuminoids-PLGA nanoparticles, which were as follows.

a) Polymers: Poly (dl- lactide- co- glycolide) (PLGA) copolymers
   - 50% PLA and 50% PGA (PLGA 50:50)
   - 75% PLA and 25% PGA (PLGA 75:25)
   - 85% PLA and 15% PGA (PLGA 85:15)

b) Active agent: Curcuminoids

a) Stabilizers: Vitamin E TPGS, Polymer 407, or Polyvinyl alcohol

1.2 Preparation method

PLGA nanoparticles containing curcuminoids were prepared according to the modified spontaneous emulsification solvent diffusion (modified-SESD) method, which was developed by Murakami *et al.*, (1999) and Saxena *et al.*, (2004). The preparation procedure was as follows.

a) Organic phase: The accurate weight of 2, 6, or 10 mg of curcuminoids, as explained in Table 3-2, Table 3-3, and Table 3-4, was dissolved in 1.25 ml of methanol and the accurate weight of 100 mg of PLGA was dissolved in 8.75 ml of acetonitrile. Subsequently, the obtained solutions were mixed together under magnetic stirring at speed of 300 rpm to form homogenous mixture.

b) Aqueous phase: The accurate concentration of a stabilizer; vitamin E TPGS, poloxamer 407, or PVA, as explained in Table 3-2, Table 3-3, and Table 3-4, respectively, in 40 ml of deionized water were prepared.

c) The organic phase was then added drop-wise through a 1000 µl pipette tip in to the aqueous phase under magnetic stirring at speed of 700 rpm. The nanoparticles suspension formed was then further stirred for 10 min.

d) The obtained nanoparticles were separated by mean of centrifugation at temperature of 4°C and at force of 16,000 g for 60 min. After centrifugation, the supernatant was then removed and the nanoparticles precipitate was washed for three times with deionized water at the same volume as that of the supernatant at temperature of 4°C and at force of 16,000 g for 30 min.

e) The washed nanoparticles were then freeze-dried from the temperature of -20°C to 4°C for 24 hours to achieve dried powder of nanoparticles. The dried nanoparticles were stored at 0°C in tightly closed-container and protected from light until further use.

2. Experimental design

2.2 Design structure

A three-factor, three-level Box-Behnken design was used for investigating the effect of formulation ingredients on the obtained nanoparticles and for optimizing the formulation of curcuminoids-PLGA nanoparticles. In this study, the three-experimental factors, defined as independent variables, were as follows.

A = Copolymers (PLA: PGA) ratios of PLGA

B = Percentage amount of curcuminiods loaded in PLGA (%w/w)

C = Percentage amount of stabilizer in the aqueous phase (%w/v)

Three stabilizers; vitamin E TPGS, poloxamer 407, and polyvinyl alcohol, were used for preparing the nanoparticles. Each experimental factor varied at three levels, as listed in Table 3-1. The Box-Behnken design points of nanoparticles formulation prepared using each stabilizer were listed in Table 3-2, 3-3, and 3-4.

Five responses, defined as dependent variables, were investigated for evaluating effect of each experimental factor. They were as follows.

a) %recovery of nanoparticles

b) Particle size (nm)

c) Size distribution, represented as the polydispersity index (PI)

d) %curcuminoids content found in nanoparticles or (% w/w)

e) Entrapment efficiency (%)

Table 3-1. The design structure of experimental factors

Level	Factors					Code Factor*		
	PLA-PGA ratio	Curcuminoids (%)	Stabilizer (%)			A	B	C
			Vit E TPGS	Poloxamer 407	PVA			
High	85:15	10	7	15	7	+1	+1	+1
Middle	75:25	6	5	12	5	0	0	0
Low	50:50	2	3	9	3	-1	-1	-1

*A = Copolymers (PLA-PGA) ratios of PLGA

B = Percentage amount of curcuminiods loaded in PLGA (%w/w)

C = Percentage amount of stabilizer in the aqueous phase (%w/v)

Table 3-2. Design points of curcuminoids-PLGA nanoparticles using vitamin E TPGS as the stabilizer

Formulation Number	**PLA:PGA**	**Curcuminoids (%)**	**Vit E TPGS (%)**
1	50:50	2	5
2	50:50	6	3
3	50:50	6	7
4	50:50	10	5
5	75:25	2	3
6	75:25	2	7
7	75:25	6	5
8	75:25	6	5
9	75:25	6	5
10	75:25	10	3
11	75:25	10	7
12	85:15	2	5
13	85:15	6	3
14	85:15	6	7
15	85:15	10	5

Table 3-3. Design points of curcuminoids-PLGA nanoparticles using poloxamer 407 as the stabilizer.

Formulation Number	PLA:PGA	Curcuminoids (%)	Poloxamer 407 (%)
16	50:50	2	12
17	50:50	6	9
18	50:50	6	15
19	50:50	10	12
20	75:25	2	9
21	75:25	2	15
22	75:25	6	12
23	75:25	6	12
24	75:25	6	12
25	75:25	10	9
26	75:25	10	15
27	85:15	2	12
28	85:15	6	9
29	85:15	6	15
30	85:15	10	12

Table 3-4. Design points of curcuminoids-PLGA nanoparticles using polyvinyl alcohol (PVA) as the stabilizer.

Formulation Number	PLA:PGA	Curcuminoids (%)	PVA (%)
31	50:50	2	5
32	50:50	6	3
33	50:50	6	7
34	50:50	10	5
35	75:25	2	3
36	75:25	2	7
37	75:25	6	5
38	75:25	6	5
39	75:25	6	5
40	75:25	10	3
41	75:25	10	7
42	85:15	2	5
43	85:15	6	3
44	85:15	6	7
45	85:15	10	5

2.2 Statistic analysis

A computer software package, Design Expert® version 6, was used to provide convenience for fitting of the response surface and to construct the contour and three-dimensional (3-D) plots of the data. Each response was analyzed individually, along the following steps. The definition and details of statistical terms are explained in Appendix A.

a) Consider the data and, if required, select an appropriate data transformation, e.g. base 10 log, inverse, square root, etc., for improving the fit of the model to the data.

b) Choose the appropriate regression model, by generating "Fit Summary" from Design Expert® software for fitting the regression models, which were linear, 2-factor interaction (2FI) and quadratic models, to each response of the obtained nanoparticles.

c) Calculate an analysis of variance (ANOVA) and post-ANOVA analysis of individual model coefficients to generate the final equation of each response. In some case, the model reduction might be needed to eliminate the term of factor, which was not statistically significant in the regression model.

d) Construct model plots, contour and three-dimensional (3-D) plots, for interpreting the effect of factors on each response.

e) Finally, the optimal formulations of PLGA nanoparticles containing curcuminoids were achieved by performing the multiple response optimization.

2.3 Optimization of the formulation ingredients

After generating the correlation equations of the independent variables (factors) and dependent variables (responses), the optimal formulation of PLGA nanoparticles containing curcuminoids using each stabilizer were designed. The

desirability function approach, which is a simultaneous optimization technique, was used as the criteria for making decision in the multiple response optimization. The desirability function approach was accomplished by the followings.

a) Obtaining the individual desirability (*d*) for each response.

The individual desirability (*d*) were obtained by specifying the goals and boundaries required for each response. Upper and lower boundaries for goal also needed to be specified. Three goals could be chosen, which were "minimize", "target" and "maximize" the response. The scale of the desirability function range was between 0 to 1, where $d = 0$ for a completely undesirable response and $d = 1$ for a fully desired response.

b) Combining the individual desirabilities to provide a measurement of the composite desirability (*D*) of the multiple response system.

c) Maximizing the composite desirability and identifying the optimal factor settings at constrained conditions of all responses.

3. Characterization of curcuminoids-PLGA nanoparticles

3.1 Nanoparticles recovery

The dried powder of PLGA nanoparticles containing curcuminoids, obtained after freeze-drying were weighed and the nanoparticles recovery of each formulation could be archieved by calculating the percentage of weight of the obtained dried nanoparticles compared with the weight of PLGA and curcuminoids added in each formulation, as equation 3-1.

$$\text{Recovery (\%)} = \frac{\text{Amount of obtained dried powder nannoparticles}}{\text{Amount of PLGA and curcuminoids used in formulation}} \times 100 \quad \text{(Equation 3-1)}$$

3.2 Particle size and size distribution

The mean diameter (particle size) and the size distribution, defined as polydisperse index (PI), of the obtained nanoparticles were measured by mean of laser light scattering spectroscopy. The instrument used was Mastersizer S long bed version 2.11 (Malvern Instruments Ltd., UK).

The samples of nanoparticle suspension were prepared and analyzed within three days after preparation. For particle size measuring, the nanoparticles suspension samples were diluted with appropriate volume of deionized water and then analyzed in triplicate. By this technique, the data was reported in the format of the plot between the cumulative percentage of the particle number and the particle size ranges. The size distribution was respresented in term of polydispersity index, which can be calculated from Equation 3-2. The data of the mean particle size and the polydispersity index were shown in Appendix B.

$$\text{Polydispersity index} = \frac{D(v, 0.9) - D(v, 0.1)}{D(v, 0.5)} \qquad \text{(Equation 3-2)}$$

Where: D(v, 0.1), D(v, 0.5), and D(v, 0.9) equal to mean particle size at 10%, 50%, and 90 % cumulative number of particles, respectively.

3.3 Particle morphology

The shape and surface morphology of PLGA nanoparticles containing curcuminoids were studied by means of Scanning Electron Microscopy (SEM) technique. The nanoparticles samples were dried and then sputter coated with gold prior to visualization.

3.4 Curcuminoids content and entrapment efficiency

The content of curcuminoids entrapped in nanoparticles were analyzed in triplicate, followed the method described by Gevender *et al.*, (1999).

a) The accurate amount of 25 mg of freeze-dry nanoparticles was weighed in a 10-ml volumetric flasks.
b) Five milliliters of methanol were added into volumetric flasks to dissolve curcuminoids from nanoparticles, and sonicated for 30 min.
c) The volume was adjusted to 10 ml with 50% methanol.
d) The amount of curcuminoids were analyzed by HPLC and calculated against the standard curve as described below.
e) The curcumonoids contents and entrapment efficiency were calculated by Equation 3-2 and 3-3, respectively.

$$\text{Content (\% w/w)} = \frac{\text{Amount of drug in nanoparticles}}{\text{Amount of nanoparticle}} \times 100 \qquad \text{(Equation 3-3)}$$

Entrapment efficiency (%)

$$= \frac{\text{Amount of drug in nanoparticles}}{\text{Amount of drug used in formulation}} \times 100 \qquad \text{(Equation 3-4)}$$

The reversed-phase HPLC with UV detector was used to determine the content of curcuminoids in the obtained nanoparticles. The method used in this research was modified from the high resolution HPLC condition for curcumonoids analysis which was developed by Jayaprakasha *et al.*, (2002). The procedure is as follows.

Column:	HiQsil® C_{18} (150 x 4.6 mm., 5 μm.)
Detector wavelength:	425 nm.
Mobile phase:	2% Acetic acid (in water):Acetonitrile (50:50)
Flow system:	Binary gredient
Flow rate:	0.75 ml/min
Injection volume:	20 μl
Run time:	15 min
Temperature:	Ambient

Standard curves for determination of curcuminoids amount were constructed between peak areas and concentrations of standard curcuminoids solutions. Various concentrations of standard curcuminoids solutions in methanol at 0.02, 0.04, 0.06, 0.08, and 0.10 mg/ml were prepared as follows.

a) The accurate amount of 25 mg, of curcuminoids was weighed in a 25-ml volumetric flask and dissolved with 5 ml of methanol. The volume was adjusted with 50% methanol. The final concentration of stock standard solution was 1 mg/ml.

b) The accurate volume of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of 1 mg/ml stock standard curcuminoids solution was put into 10-ml volumetric flasks, and adjusted to volume with 50 % methanol. The final concentrations of working standard solutions were 0.02, 0.04, 0.06, 0.08 and 0.10 mg/ml, respectively.

c) These solutions were analyzed by HPLC, in triplicate, and standard curve were constructed by plotting the peak areas against curcuminoids concentrations.

3.5 *In vitro* release study

The *in vitro* release profile of curcuminoids-PLGA nanoparticles obtained from the three optimal formulations, which were prepared from different emulsifier, were determined by the technique as follows.

a) An appropriate amount of nanoparticles samples, 100 mg, were suspended in 200 ml of phosphate buffer saline, PBS (pH 7.4), with 2% sodium lauryl sulphate under sink condition. The study was performed at constant temperature 37 ± 0.5 °C with constant stirring rate of 100 rpm, in triplicate.

b) At specific time intervals of 15 min, 30 min, 45 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 8 hr, 10 hr, 12 hr, 24 hr, 36 hr and 48 hr, 5 ml of buffer solutions were discharged. Then the equivalent volume of fresh buffer solution was added immediately.

c) The sampling buffer solutions were then analyzed for the amount of curcuminoids release using HPLC.

d) The graphs of the cumulative release over time of each formulation were plotted to show the release profile of each formulation.