CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Bovine serum albumin (Sigma, USA, Lot No. 51K1273)
2. Terephthaloyl chloride (Fluka, Switzerland ,Lot No. 385161/1)
3. Sodium carbonate anhydrous (APS Finechem, Australia, Batch No. FOA249)
4. Sorbitan trioleate, Span85 ® (Sigma, USA, Lot No. 39H0149)
5. Chloroform AR grade (Lab Scan Co.,Ltd. Thailand)
6. Cyclohexane AR grades (Lab Scan Co., Ltd. Thailand)
7. Ethanol (BDH, England)
8. Acetonitrile HPLC (Lab Scan Co., Ltd. Thailand)
9. D-pantothenyl alcohol (Sigma, USA, Lot No. 101K2500)
10. Pantothenyl ethyl ether (Daiichi, Japan)
11. Polysorbate 20, Tween 20® (The East Asiatic Co., Ltd. Thailand)
12. Sodium bicarbonate (APS Finechem , Australia)
13. Ovalbumin (Sigma, USA, Lot No.120K12D1)
14. Gelatin (BDH, England, Lot No. GO1)

EQUIPMENT

1. Analytical balance (BA2105, Sartorius Basic, German)
2. Stirring motor (RW10R, IKA- Laboratechnik , German)
3. UV spectrophotometer (Spectronic 3000 Array ,Milton Roy, USA)
4. Optical light microscope (BH-2, Olympus , Japan)
5. Scanning electron microscope (JSM-5410LV, JEOL Co., Ltd., Japan)
6. pH meter (Beckman , German)
7. High performance liquid chromatography (Shimadzu)

   System controller: SCL-10Avp, Shimadzu, Japan

   UV –VIS detector: SPD-10Avp, Shimadzu, Japan

Liquid chromatography: LC-10Advp.Shimadzu, Japan

8. Column Intersil ODS-3, 4.6*150mm with guard column 4.0*10 mm (GL Science Inc., Japan)

METHODS

1. Preparation of Blank Microcapsules by Interfacial Cross-Linked Proteins with Terephthaloyl Chloride

Preliminary studies of blank microcapsules were studied by interfacial polymerization (IFP) modified from a method described by Levy and Andry (1992) and (Rambourg, Levy and Levy.1982). Various types of protein such as bovine serum albumin (BSA), egg albumin and gelatin were used as polymers in aqueous phase. The microcapsules were prepared by dissolving the proteins in 5 ml of carbonate buffer pH 9.8. Fifteen ml of 5% w/v sorbitan trioleate (Span85®) in solvent mixture of chloroform: cyclohexane (1:4) was added then stirred for 5 min. The w/o emulsion was formed. Twenty ml of terephathaloyl chloride (TC) in solvent mixture was added to the emulsion. The stirring was further continued for 30 min. Microcapsules were obtained. Thirty ml of solvent mixture was added to stop the reaction. The excess solvent was decanted. Microcapsules were washed once with 50 ml of ethanol containing 2 % w/v tween 20® then washed with 50 ml of ethanol and the last three times with deionized water. Microcapsules were separated from the solution by vacuum filtration and dried over night. The microcapsules were kept in a desiccator for further studies. .

The effects of TC concentration on blank microcapsules and proteins concentration were studied. Microcapsules were examined under the optical microscopy and scanning electron microscope to evaluate morphology and the particle size range. Blank microcapsules prepared from various factors were shown in table 3.

Table 3 Blank microcapsules prepared from various factors

Formulation No	Proteins	Variable		Buffer pH 9.8 (ml)	Stirring speed (rpm)
		%w/v protein	%w/v TC		
A1	BSA	20	1.25	5	800
A2	BSA	20	2.5	5	800
A3	BSA	20	5.0	5	800
A4	Ovalbumin	20	1.25	5	800
A5	Ovalbumin	20	2.5	5	800
A6	Ovalbumin	20	5.0	5	800
A7	Gelatin	20	2.5	5	800
A8	Gelatin	20	2.5	5	800
A9	Gelatin	6.0	1.25	5	800

2. Preparation of BSA-TC Microcapsules and BSA-TC walled D-panthenol Microcapsules

From preliminary study in table 3, BSA was selected to be used as polymer wall for preparation of D-panthenol microcapsules. Parameters effecting microcapsule formations were studied in this experiment. The microcapsules were prepared by using interfacial cross-linked of BSA with TC. BSA was weighed and dissolved in 5 ml of carbonate buffer solution pH 9.8. Fifteen ml of 5% w/v Span 85® in solvent mixture of chloroform and cyclohexane (1:4) was added to BSA solution, and then stirred at 800 rpm for 5 minutes to get w/o emulsion. Terephthaloyl chloride in solvent mixture of chloroform and cyclohexane were prepared and added to the emulsion of BSA while stirring. The stirring was further continued for 30 minutes. After 30 minutes, 30 ml of solvent mixture was added. The excess solvent was decanted. The microcapsules were washed once with 50 ml ethanol containing 2% w/v tween 20®, and then washed with 50 ml ethanol and three times with deionized water. The microcapsules were separated

from solution by vacuum filtration and dried over night .The microcapsules were kept in a desiccator for further studies.

2.1 Effect of BSA Concentration

The concentration of BSA was varied from 10,15, and 20% w/v then mixed in 5 ml carbonate buffer pH 9.8. Then microcapsules were prepared as the above procedure with the concentration of terephthaloyl chloride was fixed at 2.5%w/v. The parameters involved are shown in table 4.

Table 4 Formulation of BSA-TC microcapsules prepared by varying concentrations of BSA

Formulation No.	Concentration		Buffer pH 9.8 (ml)	Speed (rpm)	Reaction time (min.)
	% w/v BSA	%w/v TC			
B1	20	2.5	5	800	30
B2	15	2.5	5	800	30
B3	10	2.5	5	800	30

2.2 Effect of Terephtahaloyl Chloride Concentration

The concentration of terephthaloyl chloride was varied from 1.25%, 2.5%, and 5%w/v in solvent mixture of chloroform:cyclohexane (1:4). The microcapsules were prepared as the above procedure by fixed the concentration of BSA at 20% w/v. The parameters involved are shown in table 5.

Table 5 Formulation of BSA-TC microcapsules prepared by varying concentrations of TC

Formulation No.	Concentration		Buffer pH 9.8 (ml)	Speed (rpm)	Reaction time (min.)
	% w/v BSA	%w/v TC			
B4	20	1.25	5	800	30
B1	20	2.5	5	800	30
B5	20	5.0	5	800	30

2.3 Effect of Stirring Rate

The microcapsules were prepared by fixing the BSA concentration at 20% and terephthaloyl chloride at 5% w/v. The stirring rates were varied from 800 rpm and 11000 rpm. The parameters involved are shown in table 6.

Table 6 Formulation of BSA-TC microcapsules prepared by varying stirring rates

Formulation No.	Concentration		Buffer pH 9.8 (ml)	Speed (rpm)	Reaction time (min.)
	% w/v BSA	%w/v TC			
B5	20	5.0	5	800	30
B6	20	5.0	15	11000	30

2.4 Effect of BSA Concentration and D-panthenol Concentration

The concentrations of BSA were varied from 13.3% and 20% w/v while the concentrating D-panthenol in carbonate buffer was loaded from 10-20% w/v. The microcapsules were prepared by the above technique. D-pathenol microcapsules prepared from various concentrations of BSA and D-panthenol are shown in table 7.

Table 7 D-panthenol microcapsules prepared from various concentrations of BSA and D-panthenol

Formulations No	Concentration % w/v		5% TC in solvent (ml)	Buffer pH 9.8 (ml)	Speed (rpm)	Reaction time (min.)
	BSA	D-panthenol				
D1	13.3	10.0	40	15	800	30
D2	13.3	13.3	40	15	800	30
D3	13.3	16.6	40	15	800	30
D4	13.3	20.0	40	15	800	30
D5	20.0	13.3	60	15	800	30
D6	20.0	16.6	60	15	800	30
D7	20.0	20.0	60	15	800	30

3. Characterization of Blank Microcapsules, BSA-TC Microcapsules and BSA-TC walled D-panthenol Microcapsules

3.1 Optical Microscopy

Optical microscopy was used to examine morphology of blank microcapsules, BSA-TC microcapsules and BSA-TC walled D-panthenol microcapsules during preparation.

3.2 Scanning Electron Microscope (SEM)

The morphology of the BSA-TC microcapsules and BSA-TC walled D-panthenol were observed by SEM. The physical appearance of microcapsules was determined such as shape, surface morphology and aggregation.

3.3 Particle Size and Size Distribution

Particle size and size distribution of microcapsules were studied by using Mastersizer, an equipment consisted of optical measurement, provided a collimated

laser that passed through samples to be measured. The scattered laser light from the sample was detected by a receiver of the optical measurement unit. This data from the receiver was transmitted to a computer system where the operating software calculated the size distribution.

3.4 Yield of Microcapsules

An amount of the microcapsules was weighed and % yields was calculated according to the equation.

$$\% \text{ Yield} = \frac{\text{actual yield (g) X 100}}{\text{theoretical yield (g)}} \quad (1)$$

$$\% \text{ Yield} = \frac{\text{Wt. of product microcapsules (g) X 100}}{\text{Wt of BSA +D-panthenol +TC}} \quad (2)$$

3.5 Percent Entrapment and Encapsulation Efficiency

The percentage of D-panthenol entrapment was calculated from the following equation.

$$\% \text{ Observed content} = \frac{\text{Assay amount of D-panthenol X 100}}{\text{Amount of produced microcapsules}} \quad (3)$$

$$\% \text{ Entrapment} = \frac{\% \text{ observed content X 100}}{\% \text{ theoretical content}} \quad (4)$$

Where,

$$\% \text{ Theoretical content} = \frac{\text{Wt. of D-panthenol X 100}}{\text{Wt. of D-panthenol + wt. of BSA + TC}} \quad (5)$$

4. Method of Quantitative Analysis of D-panthenol in Microcapsules

4.1 UV Spectrophotometer

In order to determine the maximum absorption wavelength of both standard D-panthenol and internal standard pantothenyl ethyl ether (PEE), each compound was dissolved in water and then transferred to cell for UV detection. The blank solution was water .The concentration of standard D-panthenol and internal standard PEE, were both 1 mg/ml.

4.2 HPLC Assay for D-panthenol Standard Solution

4.2.1 HPLC Condition

The high performance liquid chromatography was used for the analysis of D-panthenol. The system was composed of two pumps, adjustable wavelength UV detector, system controller, 20 microliters loop, and degasser. The condition used for analyzing D-panthenol was based on the application of reversed- phase HPLC of pantothenic acid derivatives. The condition was as follows :

Column	:	Intersil ODS. C18 (4.6 x 150 mm) with guard column.
Detector wavelength	:	210 nm.
Flow system	:	Binary gradient
Mobile phase	:	Acetonitrile and water (20:80)
Total flow rate	:	1 ml/min
Injection volume	:	40 µl

4.2.2 Preparation of Standard Solution

A stock solution of D-panthenol was prepared by dissolving 250 mg of D-panthenol in water, and then the solution was adjusted to 25 ml in a volumetric flask.

A stock solution of pantothenyl ethyl ether (PEE) was prepared by dissolving 250 mg of pantothenyl ethyl ether in water, and then the solution was adjusted to 25 ml in a volumetric flask.

Six appropriate dilutions of standard D-panthenol were prepared by firstly, individual pipetting 0.25, 0.5, 0.75 1, 1.25 and 1.5 ml of D-panthenol stock solution into six 10 ml volumetric flasks. Secondly, 0.5 ml of PEE was pipetted into each of these volumetric flasks. The solution was adjusted to volume with water. So the concentrations of each standard solutions contained were 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mg/ml of D-panthenol and 0.5mg/ml of PEE, respectively. Forty microliters of these solutions were injected for analysis.

4.2.3 Validation for the Quantitative Determination of D-panthenol by HPLC

The analytical method was validated to ensure its suitability. According to USPXXIII, the parameters for validation methods included specificity, linearity, accuracy and precision.

4.2.3.1 Specificity

The specificity of the HPLC method used to determine D-panthenol content in the microcapsules was evaluated by comparing the chromatograms of vehicles used to prepare microcapsules, standard solution and samples containing PEE in each samples. The peak area of D-panthenol and PEE must not be interfered by the other components.

4.2.3.2 Linearity

The linearity was determined by plotting the standard curve between peak area ratios of D-panthenol to PEE and the concentration of D- panthenol (mg/ml). The standard curve was fitted using the linear regression analysis; the coefficient of correlation (r^2) and the equation were calculated.

4.2.3.3 Accuracy

Accuracy of an analytical method is the closeness of the test result obtains with this method to the true value. The accuracy of D-panthenol analysis by HPLC was done by comparing percent recoveries of three different concentration of D-panthenol in water with the known concentrations. Percent recovery of each injection was calculated by dividing the concentration fitted from the calibration curve by the known concentrations. The mean, standard deviation (SD), and percent coefficient of variation (%CV) of each concentration were determined.

4.2.3.4 Precision

Precision describes the reproducibility of measurements; in other word, the closeness of results that have been obtained in exactly the same way. The precision of a measurement is readily determined by simply repeating the measurement

on replicate samples. Three terms are widely used to describe the precision: standard deviation, and coefficient of variation.

4.2.3.4.1 Within Run Precision

The within run precision was evaluated by analyzing peak area ratio of D-panthenol to PEE. Each concentration of standard solution was triplicately analyzed within the same day.

4.2.3.4.2 Between Run Precision

The between run precision was evaluated by analyzing peak area ratio of D-panthenol to PEE of three sets of calibration curves injected in the different days for the other three days.

4.2.3.5 Analysis of D-Panthenol in Microcapsules

An accurate weight of 500mg of D-panthenol microcapsules powder were crushed in mortar and extracted 2 times with 25 ml of water. The filtered water was transferred to 100 ml volumetric flask, 5.0 ml of PEE stock solution was added and then the solution was adjusted to volume with water. The sample solution was analyzed by same HPLC method that was used to analyze D-panthenol standard solution. The percentage of entrapment was calculated as equation (4).