CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical balance:	- Sartorious LC 6200S, Scientific Promotion Co., Ltd., Germany - Mettler AE 6200S, Mettler-Toledo, Switzerland
Autopipette:	- Pipetman, Gilson, France
Column	- C-18 column Model LUNA 5 μm size 250 mm×4.6 mm i.d., Phenomenex, U.S.A.
Differential scanning calorimetry (DSC)	- NETZSCH-Gerätebay GMbH, DSC 204 phoenix®, Germany
Freeze-dryer	- Flexi-Dry^{TM} μP, FTSsystems, U.S.A.
FTIR	- Perkin Elmer, Model 1760X, U.S.A.
Hot air oven	- Fisher 517G, Scientific worldwide oven, U.S.A.
HPLC	- Hewlett Packard Series 1050, U.S.A.
Laminar	- ISSCO Laminar Flow Model GVF 034, International Scientific Supply Co.,Ltd., U.S.A.
Membrane filter	- Cellulose nitrate, pore size 0.45μm, Whatman, UK
pH meter:	- PHM 83 Autocal pH meter, Radio meter, Denmark
Spectrophotometer:	- Beckman, DU650 spectrophotometer, U.S.A.

UV detector: - SpectroMonitor 3200, Thermo Seperation Products, U.S.A.

Vortex: - Genie Model K-550-GE, Scientific Industries, U.S.A.

Water bath ,shaking - Gyrotory water bath shaker Model G76D, New Brunswick Scientific Co., INC, U.S.A.

2.2 Chemicals

Acetonitrile - HPLC grade from Scharlau Chemie S.A., Spain

Carbaryl: - Analytical grade from Chem Service, U.S.A.
- Commercial grade (Carbaryl 85WP), locally purchased from Mitrpornsombun Co.,Ltd., Thailand

Carbendazim: - Analytical grade from Chem Service, U.S.A.
- Commercial grade (Carbendazim 50WP), locally purchased from Mitrpornsombun Co.,Ltd., Thailand

Cyclodextrin:
- β-cyclodextrin, MW = 1135, Nihon Shokuhin Kako Co.,Ltd., Japan
- Maltosyl-β-cyclodextrin (highly pure G_2-βCD, more than 98% in solid matter, prepared by the action of pullulanase on βCD and maltose substrates, 1 maltosyl unit replaces 1 H atom in C_6-OH of a glucose unit in βCD molecules), Bioresearch Corporation of Yokohama, Japan
- Methyl-β-cyclodextrin (randomly methylated, degree of substitution is 1.8), Bioresearch Corporation of Yokohama, Japan

Dextrin - SANDEX 250, Bioresearch Corporation of Yokohama, Japan

Methidathion: - Analytical grade from Chem Service, U.S.A.

Other common chemicals were at least of reagent grade and obtained from Merck, Fluka or Sigma.

2.3 Methods

2.3.1 Determination of spectrophotometric properties of pesticides

2.3.1.1 The maximum absorption of pesticides and pesticide-CD systems

The UV absorption spectra for free pesticides and their complexes with βCD were compared. Samples were prepared by dissolving excess amount of each pesticide (10 mg) in 10 ml deionized water or 5 mM of βCD solution. These mixtures were shaken at 30 °C and equilibrated for 24 hours and then the samples were filtered through 0.45 μm membrane and scanned for absorbance from 200-400 nm.

2.3.1.2 Calibration curve of pesticides

Pesticides were accurately weighed and dissolved in 100 ml of deionized water at a concentration of 0.1 mM. The stock solution was precisely pipetted into series of volumetric flasks and diluted to the final concentrations of 0.01 to 0.1 mM. The absorbance of each concentration was determined at λmax obtained from 1.1 (284 nm for carbendazim: 276 nm and 210 nm for carbaryl and methidathion, respectively). Standard calibration curve for each pesticide was then plotted.

2.3.2 Solubility studies : Selection of the best types of pesticide and cyclodextrin in soluble complex formation

Solubility measurements were carried out according to the method of Higuchi and Connors (1965). Excess amounts of carbaryl, carbaryl 85 WP, carbendazim, carbendazim 50 WP, and methidathion (10 mg) were added in 10 ml of deionized water containing various concentrations of β-CD, methyl-β-CD or maltosyl-βCD (G_2-β-CD). The concentrations of methyl-β-CD and G_2-β-CD were 0,10,20,40,60 and 100 mM while that of β-CD was 0,5,10,20,30 and 50 mM, due to less soluble property of β-CD. These mixtures were shaken at 30 °C for 24 hours. Then the solution was passed through 0.45 μm membrane filter and diluted with distilled water to make up a suitable concentration for calibration of pesticides. An analysis for solubilized pesticides was performed spectrophotometrically at 284 nm for carbendazim and carbendazim 50 WP, at 276 nm for carbaryl and carbaryl 85 WP and at 210 nm for methidathion against distilled water as the blank; as the effect of cyclodextrins on the wavelength of maximum absorption (λmax) of all pesticides was negligible (see Appendix 1).

The concentrations in molarity of soluble carbaryl, carbaryl 85 WP, carbendazim, carbendazim 50 WP and methidathion were determined from standard curve of each compound. Phase solubility diagram was constructed by plotting the pesticide molar concentration in the solution versus molar concentration of each CD. Apparent formation constant (Kc) was calculated from the slope of the phase solubility diagram by the following equation:

$$Kc = \frac{[\text{pesticide-CD}]}{[\text{pesticide}]\,[\text{CD}]} = \frac{\text{slope}}{S_0\,(1\text{-slope})}$$

where S_0 is the pesticide solubility (intercept).

2.3.3 Determination of the amount of active ingredient of Carbaryl in commercial formular

Commercial grade carbaryl and carbendazim were used in this work in addition to the analytical grade. The check for purity and the amount of active ingredient should be carried out. Only the carbaryl 85WP was analyzed since carbendazim 50WP was proved in Result Table 6 to be not as good in forming complex with CDs so it was not used in further experiments.

2.3.3.1 Determination of carbaryl by HPLC

The carbaryl content in carbaryl 85WP was determined by reverse-phase HPLC method using analytical C-18 column (Octadecyl silane chemically bonded to < 10 μm porous microsilica packing, 250 mm × 4.6 mm i.d.). HPLC analysis was performed with HP series 1050, 4 pumps and UV detector which was set at 276 nm. Acetonitrile in aqueous (55% by volume) was used as mobile phase and the run was operated at the flow rate of 1.0 ml/min.

2.3.3.1.1 Standard curve of carbaryl

The carbaryl standard curve was made. A stock standard solution was prepared by adding acetonitrile (10 ml) to 0.0028 g of carbaryl. Working range standard solutions at 0-280 μg/ml were prepared by diluting the stock solution with acetonitrile. Ten μl of each concentration was injected into C-18 column and run with above condition described in 2.3.3.1. The carbaryl standard curve was plotted between standard carbaryl concentration and peak area.

2.3.3.1.2 Determination of percent carbaryl in carbaryl 85WP

Carbaryl 85WP (0.0023 g) was dissolved in 10 ml acetonitrile. Ten μl was injected into C-18 column and run with above condition described in 2.3.3.1. Carbaryl content was determined from the standard curve prepared in 2.3.3.1.1.

2.3.4 Preparation of carbaryl-methyl-βCD solid complexes

From solubility studies, carbaryl 85WP and methyl-βCD were chosen as the best pair in complex formation. Solid complexes were then prepared by various methods using the 1:1, 1:2 and 2:1 molar ratios of carbaryl 85WP to methyl-βCD. All samples were kept desiccated after preparation.

2.3.4.1 Preparation of carbaryl-methyl-βCD by physical mixing

For the preparation of the physical mixtures, carbaryl 85 WP and methyl-β CD powders were dried at 40°C overnight. Only the 2:1 guest: host was prepared by this technique. Carbaryl 85 WP 0.402 g (40 % active ingredient) and methyl-βCD 0.5665 g were mixed together for 10 minutes at room temperature. The mixture was then kept in a desiccator.

2.3.4.2 Preparation of carbaryl-methyl-βCD by co-precipitation

The solid complex of carbaryl with methyl-βCD was prepared by magnetic stirring the aqueous solution of methyl-βCD to which carbaryl 85 WP was added, at 30°C for 3 hours. Carbaryl 85 WP and methyl-βCD were accurately weighed in the ratios as shown in Table 4. The complex, which was precipitated out of solution, was filtered and dried at room temperature for 1 day. In the case of 2:1 ratio, the freeze-drying of the supernatant solution was further performed, this was called " co-precipitation freeze-dried mixture".

2.3.4.3 Preparation of carbaryl-methyl-βCD by freeze-drying

Methyl-βCD was dissolved in distilled water, then carbaryl 85WP was added and magnetic stirred at 30°C for 3 hours. Carbaryl 85 WP and methyl-βCD were

accurately weighed in the ratios as shown in Table 4. The solution was frozen at - 80 °C and freeze-dried in a Flexidry μP lyophilized apparatus.

2.3.4.4 Preparation of carbaryl-methyl-βCD by kneading

Carbaryl 85 WP and methyl-βCD were accurately weighed in the ratios as shown in Table 4. The kneaded mixture was prepared by mixing these ratios together. Water (15% of total weight) was gradually added and kneaded was performed on the concave glass for another 10 minutes to obtain homogeneous paste. The paste was dried at room temperature (approximate 30°C) overnight. Then, the kneaded mixture was grounded and finely screened through a 40 mesh sieve.

Table 4. The ratio of carbaryl 85 WP: methyl-βCD used in the solid complex preparations.

Method for preparation	ratio* (guest : host)	Carbaryl 85 WP (g)	Methyl-βCD (g)	Water (ml)
Co-precipitation	1:1	0.201	0.5665	200
	1:2	0.201	1.1330	200
	2:1	0.402	0.5665	200
Freeze-drying	1:1	0.201	0.5665	100
	1:2	0.201	1.1330	100
	2:1	0.402	0.5665	100
Kneading	1:1	0.201	0.5665	0.1
	1:2	0.201	1.1330	0.2
	2:1	0.402	0.5665	0.1
Physical mixture	2:1	0.402	0.5665	-

* **Approximate mole ratio,** molecular weight of methyl-βCD used in calculation was that of βCD with substitution of 2 H atoms by 2 CH_3 groups

Investigation of the carbaryl 85 WP -methyl-βCD solid complexes

2.3.5.1 Differential scanning calorimetry (DSC)

Solid complexes prepared from carbaryl: methyl-βCD in the molar ratios of 2:1 by different methods were subjected to investigation by DSC. Samples ranging from 3-7 mg were placed in pierced aluminum pans and scanned at a rate of 10 °C/min. DSC measurements were carried out under dry nitrogen (10 ml/min) on a NETZS CH GERATEBAU GMbH Thermal analysis apparatus at The Technological Research Equipment Center of Chulalongkorn University.

2.3.5.2 Fourier Transform Infrared spectrometry (FTIR)

Solid samples were prepared by the potassium bromide (KBr) disc method and scanned from 400-4000 cm^{-1}. Only the samples of carbaryl 85 WP-methyl-βCD complex in 2:1 ratio prepared by physical mixing, co-precipitation, freeze-drying and kneading were investigated. Fourier Transform Infrared spectra were performed on a Perkin Elmer Model 1760X at The Technological Research Equipment Center of Chulalongkorn University.

2.3.6 Detection of suitable conditions for complex formation by freeze-dried method

There are many factors that could affect the encapsulation of guest into CD's cavity. One of the main factor is the temperature. Therefore, in order to know the suitable temperature for preparing the inclusion complex formation, carbaryl 85WP and methyl-βCD were mixed at various temperatures before freeze-drying and the procedure was the same as in protocol 2.3.4.3.

In this experiment, different temperatures (20,30,40,50 and 60°C) were used for complex formation. The result was analyzed by dissolution method which

measures the amount of carbaryl dissolved from the complex into solution which reflects dispersion property of the complex. Another important condition is the ratio of guest: host. So in the preparation of the solid complexes by various methods (see section 2.3.4), three main ratios, 1:1, 1:2 and 2:1 were prepared. These samples prepared by kneading and freeze-drying were further characterized in section 2.3.7.

2.3.7 Characterization of solid state inclusion complexes

2.3.7.1 Dissolution study of inclusion complex

To measure the dissolution in the solid state, excess amount of samples (200 mg) were added in 100 ml deionized water in 250 ml flask. The sample was shaken at 30°C and 1 ml was withdrawn at time intervals (0,5,10,15,30,60,120 and 180 minutes) for analysis of carbaryl content. Dilution with deionized water to appropriate concentration, filtration and analysis at 276 nm were performed. From this experiment, the most suitable method of solid complex preparation and condition were selected. The criteria of chosen were highest amount of carbaryl solubilized and low quantity of methyl-βCD used. Those conditions were used for next study.

2.3.7.2 Thermal stability test

To measure the thermal stability in the solid state, each solid complex was placed in a hot air oven at 80°C. At appropriate time intervals, the samples were taken out for analysis of remaining carbaryl spectrophotometrically at 276 nm. The curves of carbaryl concentration versus time of heat exposure at 80°C (0,5,10,15,30,60,120 and 180 minutes) were plotted.

2.3.7.3 UV stability test

To measure the UV stability in the solid state, each solid complex was exposed in laminar, the UV lamp of 30 W at a distant of 22.5 inches. The residual carbaryl in the solid complex at appropriate time (0,5,10,15,30,60,120 and 180 minutes) intervals were analyzed at 276 nm.

Thermal and UV stability of carbaryl: methyl-βCD mixtures in the molar ratio of 1:1, 1:2 and 2:1 prepared from freeze-dried and kneading method were studied. In addition to comparing with thermal and UV stability of free carbaryl, comparison was also made with carbaryl: dextrin mixture. Carbaryl 85WP: dextrin at a molar ratio of 3:1 was prepared by freeze-drying and kneading method.

2.3.7.4 Brine Shrimp (Artemia salina* Linnaeus*) cytotoxicity Lethality Test (Solis et al.,1993)

Brine shrimp eggs (*Artemia Salina*) were hatched in artificial seawater prepared from NaCl 38 g/l. After 24 hrs. incubation at room temperature (approximate 22°C) , nauplii were collected with a micropipette after attracting the organisms to one side of the box with a light source. Carbaryl 85WP and Carbaryl 85WP-methyl-β CD at 2:1 molar ratio were tested, 20 mg/l stock solution prepared by 5mg of sample were dissolved in 250 ml artificial seawater. Serial solutions were made in artificial sea water in microwell. The concentrations of sample were 1, 5 and 10 mg/l. Control wells with only artificial sea water were included in each experiment. A suspension of nauplii containing 8-12 organisms (100 µl) were transferred to each well (thereby 60 nauplii for each concentration of each test sample) and the covered plate was incubated for 6 hours. The number of dead (non-motile) nauplii in each well were counted. LC_{50} values were then calculated using Probit analysis (Finney, 1971).