CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical balance : Sartorious A200S, Scientific Promotion Co., Ltd.,Germany
Mettler AG 204 , Mettler Toledo, Switzerland

Centrifuge : Centrifuge 5810 , Eppendorft, Germany

Column : OV-5 capillary column size 30m x 0.32 mm id., 0.25 μm film thickness, Ohio Valley Specialty Chemical.

DSC : DSC 822e , Mettler Toledo, Switzerland

FTIR : Model 1760X, Perkin Elmer, USA

Freeze dryer : LYO-LAB , Lyophilization Systems, Inc.USA

GC : Agilent 6890 N , Agilent Technologies,Inc.USA

Hot air oven : Memmert model 100, GmbH^{+} Co.,KG., Germany

Shaker water bath : PolyScience® , Model 28L/B/SH/C, supplier by Harikul Group Co., Ltd,

UV spectrophotometer : Jasco V-530 , supplier by Analytical Lab Science Co., LTD.

2.2 Materials

AHTN (Fixolide) : A gift from the Thai-China Flavors and Fragrances Industry Co., Thailand

Cyclodextrin : β-cyclodextrin , MW = 1135 , Ensuiko Sugar Refining Co. (Yokohama , Japan)
Methyl-β-cyclodextrin (randomly methylated with a degree of substitution of 1.7), Ensuiko Sugar Refining Co. (Yokohama , Japan)
Hydroxypropyl-β-cyclodextrin (degree of substitution = 0.91), Ensuiko Sugar Refining Co. (Yokohama , Japan).

Dipropylene glycol : A gift from the Thai-China Flavors and Fragrances Industry Co., Thailand

Ditallow dimethyl ammonium chloride : Commercial grade from Hong Huat Co.,Ltd.

Ethanol : Analytical grade from MERCK , Germany

n-Hexane : Pesticide residue grade from Fisher Scientific

Naphthalene : GC grade from Fluka , Germany

2.3 Methods

2.3.1 Analysis of Fixolide

2.3.1.1 Spectrophotometric Method

2.3.1.1.1 The maximum absorption of fixolide

The UV absorption spectra for free fixolide and its soluble complex with CD were compared. Samples were prepared by dissolving 5 mg of fixolide in 10 ml of 50 % (v/v) aqueous ethanolic solution or of 5 mM βCD in the same solvent. These mixtures were sonicated at 30 °C for 20 minutes and then the samples were filtered through 0.45 μm membrane and scanned for absorbance from 200-400 nm.

2.3.1.1.2 Calibration curve of fixolide

50 mg of fixolide was accurately weighed and dissolved in 100 ml of 50 % (v/v) aqueous ethanolic solution. Ten ml of this solution was pipetted in 100-ml volumetric flask and adjusted to volume. This solution was a stock solution of fixolide.

Standard solutions of fixolide were prepared by pipetting 2, 3, 4, 5, 6, 7 and 8 ml of fixolide stock solution into 25-ml volumetric flasks and diluted to the final concentrations of 4, 6, 8,10, 12, 14 and 16 μg/ml, respectively.

All standard solutions were analyzed spectrophotometrically at λ_{max} obtained from 2.3.1.1.1. Standard calibration curve was then plotted.

2.3.1.2 GC Method

2.3.1.2.1 Chromatographic condition

The chromatographic condition used was modified from Osemwengie (2001). The condition was as follows:

Column	: OV-5 capillary column (30m x 0.32 mm I.D., 0.25 μm film thickness)
Carrier gas	: Helium , velocity 36 cm/sec
Detector	: FID , 250 °C
Injection	: 1 μl, split ratio 40:1, injection temperature 250 °C
Oven temperature	: 90 – 230 °C, flow rate 10 °C/min
Program	230 °C, hold for 2 min.

2.3.1.2.2 Standard solution

A stock solution of internal standard was prepared by accurately weighing 250 mg of naphthalene. n-Hexane was used to dissolve and adjust to final volume in a 25-ml volumetric flask.

A stock solution of fixolide was prepared by accurately weighing 100 mg of fixolide. n-Hexane was used to dissolve and adjust to final volume in a 25-ml volumetric flask.

A stock solution of fixolide was prepared by pipetting 0.5, 1, 2, 3, 4 and 5 ml of the fixolide stock solution in 10-ml volumetric flasks. Then 1 ml of the naphthalene stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with n-hexane so that the concentrations of the standard solutions were 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ml, respectively, and that of naphthalene was 1.0 mg/ml.

2.3.1.2.3 Standard curve of fixolide

Fixolide standard solutions ranging from 0.2-2.0 mg/ml were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations were preformed.

2.3.2 Phase solubility studies

The phase solubility studies for investigation of soluble complex formation were carried out according to the method of Higuchi and Connors (1965). Excess amounts of fixolide (100 mg) were added in 10 ml of 50 % (v/v) aqueous ethanolic solution containing various concentrations of βCD, methyl-βCD and HP-βCD. The concentrations of methyl-βCD and HP-βCD were 0, 10, 20, 40, 60, and 100 mM while that of βCD was 0, 2, 4, 8, 12 and 16 mM, due to less soluble property of βCD. These mixtures were shaken at 30 °C for 24 hours. Then the solution was filtered through 0.45 μm membrane filter. A portion of the filtrate was adequately diluted and analyzed spectrophotometrically at 258 nm. Phase solubility diagram, the molar concentration of fixolide versus molar concentration of each CD, was plotted. The apparent stability constant or formation constant (K_c) was calculated from the slope of the phase solubility diagram by following equation:

$$K_c = \frac{\text{slope}}{\text{intercept (1 - slope)}}$$

2.3.3 Preparation of Fixolide : Cyclodextrin (βCD, Methyl-βCD, HP-βCD) Solid Complexes

Three methods were used for preparation of the solid complexes. These included co-precipitation, kneading and freeze-drying. Physical mixing was prepared as control and for comparison. Three mole ratios of fixolide : CD (1:1, 1:2 and 1:3) were used in this study.

2.3.3.1 Preparation of solid complexes by co-precipitation method

Fixolide and cyclodextrin were accurately weighed according to certain ratios shown in Table 3. The cyclodextrin was dissolved in 30 ml of ethanol : water (1:3) at 55 °C, then 2 ml of fixolide in ethanol was added drop-wise to the solution. The heater was switched off, and then the mixture was magnetic stirred for 24 hours. The complex, which was precipitated out of solution, was vacuum filtered, washed with 24 ml of ethanol:water (1:2), and dried at 40 °C for 24 hours. The powder was kept in a desiccator for further study.

2.3.3.2 Preparation of solid complexes by kneading method

Fixolide and cyclodextrin were accurately weighed according to certain ratios shown in Table 3. The knead mixture was prepared by gently triturating the fixolide and CD in a glass mortar for 15 minutes. Then 0.5 ml of ethanol : water (1:3) was added drop-wise and the mixture was kneaded for 15 minutes to obtain homogeneous paste. The paste was dried in a hot air oven at 40 °C for 24 hours. The powder was kept in a desiccator for further study.

2.3.3.3 Preparation of solid complexes by freeze-drying method

Fixolide and cyclodextrin were accurately weighed according to certain ratios shown in Table 3. The cyclodextrin was dissolved in 27.5 ml of distilled water to obtain a clear solution, then 27.5 ml of fixolde in ethanol was added to the solution. The mixture was magnetic stirred for 2 hours, and then the solution was operated in the freeze dryer (LYO-LAB, Lyophilization Systems). The freeze dried-product was prepared by freezing at -40 °C, 500 millitor. During the primary drying step, the temperature started at -30 °C and then gradually increased by 10 °C until it reached 20 °C. The secondary drying step was performed at 25 °C, 100 millitorr for 5 hours. The powder products were kept in a desiccator for further study.

2.3.3.4 Preparation of solid complexes by physical mixing method

Fixolide and cyclodextrin were accurately weighed according to certain ratios shown in Table 3. The two compounds were physically mixed in a small plastic bag for 5 minutes at room temperature. Then the powder was kept in a desiccator for further study.

Table 3. Composition of fixolide : CD (βCD, Methyl-βCD, HP-βCD) inclusion complex

Method for preparation	Ratio* (guest:host)	Fixolide (g)	βCD (g)	Methyl-βCD (g)	HP-βCD (g)
Co-precipitation	1:1	0.2273	1.0000	1.1559	1.3215
	1:2	0.2273	2.0000	2.3118	2.6432
	1:3	0.2273	3.0000	3.4678	3.9647
Kneading	1:1	0.1136	0.5000	0.5777	0.6605
	1:2	0.0568	0.5000	0.5777	0.6605
	1:3	0.0379	0.5000	0.5777	0.6605
Freeze-drying	1:2	0.1136	1.0000	1.1560	1.3216
	1:3	0.0758	1.0000	1.1560	1.3216
Physical mixing	1:2	0.1136	1.0000	1.1560	1.3216

*** Approximate mole ratio**

2.3.4 Detection of Fixolide : Cyclodextrin (βCD, Methyl-βCD, HP-βCD) Solid Complexes

2.3.4.1 Differential Scanning Calorimetry (DSC)

DSC thermograms were obtained from DSC 822e apparatus (Mettler Toledo, USA). All samples were examined using 3-4 mg of samples in aluminium pans and heated at 10 °C/min in the temperature range from 30 to 250 °C. The measurements were carried out under dry nitrogen at the flow rate of 60 ml/min.

2.3.4.2 Fourier Transform Infrared Spectrometry (FTIR)

The infrared spectra of pure materials, all the solid complexes and the physical mixture were obtained using potassium bromide (KBr) disc in the range of 400-4000 cm^{-1} on Perkin Elmer Model 1760X at The Technological Research Equipment Center of Chulalongkorn University.

2.3.5 Determination of suitable mixing time for complex formation by co-precipitation method

There are many factors that could affect the complex formation of guest and CDs, such as solvents and temperature. The amount of time and good mixing are also the main factor for co-precipitation method, especially high molecular weight molecules have a tendency to associate with themselves rather than interacting with CDs. Therefore, in order to know the suitable mixing time for inclusion complex formation by co-precipitation method, the samples were mixed at various times (3, 6, 12, 18 and 24 hours) and the products were analyzed as described in 2.3.6.

2.3.6 Determination of Fixolide in the Solid Complexes

The amount of fixolide in the complex was determined by solvent extraction method, and followed by GC analysis using the internal standard method. One hundred mg of complex powder were dissolved in 30 ml water in 50 ml thick-wall screw cap tubes. n-Hexane (4 ml) was added together with 1 ml of naphthalene as internal standard (10 mg/ml). The tubes were shaken vigorously for 1 min, then centrifuged at 2500 rpm for 5 minutes. The hexane phase was separated and the aqueous phase was re-extracted again with hexane. The hexane extracted portions were pooled, and hexane was added to make final volume of 10 ml, each extracted sample was analyzed by GC according to the method described in section 2.3.1.2. Triplicate samples were extracted.

Recovery of the method

Recovery of the method was determined for each mole ratio by using fixolide and βCD (Table 4) in place of fixolide-CD complex. These samples were spiked with naphthalene (internal standard) and extracted similar to the complex powder under the same conditions. The extracted samples were then subjected to GC analysis. The recovery was calculated using equation as follows:

$$\text{Recovery (\%)} = \frac{\text{Amount of fixolide determined } (g)}{\text{Amount of fixolide added } (g)} \times 100$$

Table 4. Composition of fixolide and βCD used in the determination of recovery of the method

Mole ratio	Fixolide (g)	βCD (g)
1:1	0.0185	0.0815
1:2	0.0102	0.0898
1:3	0.0070	0.0930

2.3.7 Determination of Properties of the Solid Complexes

2.3.7.1 Thermal stability test

Thermal stability experiments were performed in the solid state. Fixolide or selected fixolide : CDs inclusion complex was placed in hot air oven at 35, 50 and 80°C. At appropriate time intervals, three samples from each group were taken for analysis of fixolide remaining by hexane extraction and GC analysis according to the method described in section 2.3.1.2.

2.3.7.2 UV stability test

UV stability experiments were performed in the solid state. Fixolide or selected fixolide : CDs inclusion complex was placed under the UV lamp, with wavelength ranged from 100-280 nm (Philip lamp, 30 watt, 45 cm) at a distance of 12.5 inches. At various time intervals, three samples from each group were analyzed for the amount of fixolide remaining using GC method.

2.3.7.3 Dissolution study

Dissolution studies of all samples were carried out in 100 ml of 50 % (v/v) ethanolic aqueous solution (as a medium solution). Fixolide (20 mg) or its equivalent in solid complexes were added in the medium and shaken at 30°C, 5 ml was withdrawn at different time intervals (0, 5, 10, 15, 20, 30, 40, 50 and 60 minutes) for analysis of fixolide content. The medium was replaced with 5 ml of fresh medium solution. The sampling was diluted for appropriate concentration, and analyzed by UV spectrophotometer at 258 nm. The dissolution studies were performed in triplicate.

In this Methods section, the most suitable method of complex formation and the best type of cyclodextrin were selected for the properties study.

2.3.8 Application of the Inclusion Complex in Fabric Softener

Formulation of fabric softener was prepared using ditallow dimethylammonium chloride (DTDMAC), which was main ingredient in the formula. The formulation was similar to that used in commercial preparation. (The "Comfort" formula was shown in Appendix E). DTDMAC (1.25 g) was dissolved in 40 ml of warm deionized water with stirring, then 400 mg of free fixolide in 2 ml dipropylene glycol solution or its equivalent in solid complex was added. The formula was mixed until the mixture was smooth and homogeneous. The mixtures possessed the final concentration of fixolide of 10 mg/ml. One ml of the mixture was pipetted and transferred to 4 ml glass vials. These samples were studied for thermal and UV stabilities of fixolide in fabric softener according to the method described in section 2.3.7.1 and 2.3.7.2.