CHAPTER III

MATERIALS AND METHODS

1. Quantitative Analysis of Turmeric Curcuminoids

1.1 Plant Material

The dried rhizomes of various locations of *Curcuma longa* Linn. were kindly provided by Mr. Sermsak Raktham of the Department of Agriculture. The rhizomes were originally obtained from various provinces (Table 17 in Appendix) of Thailand and regrown in the experimental fields in Phichit, Trang and Tak (Doi Musor) provinces. These research stations belong to the Horticultural Research Institute, Department of Agriculture, Thailand. For other zingiberaceous plants, they were purchased from either Chatuchak Market (*Curcuma* species) or Chao Krom Per Traditional Drug Store (*Zingiber* species and *Globba* species).

1.2 Reagents and Standards

1. Chloroform, benzene, methanol (all in AR grade from Merck, Damstadt, Germany)

2. silica gel 60 F 254 plates were purchased from Merck, Damstadt, Germany.

3. Pure natural curcumin, demethoxycurcumin

and bisdemethoxycurcumin were isolated from turmeric samples and purified by preparative thin-layer chromatography followed by crystallization as follows : ten-gram powdered turmeric (passing No.40 sieve) was sonicated with 500 ml. methanol for one hour. The mixture was filtered through Whatman paper No.1 in a Buchner funnel and the filtrate was concentrated *in vacuo* to 50 ml. The concentrated extract was then streaked on a preparative TLC plate (silica gel) and the plate was chromatographed using chloroform : benzene : methanol, 80:15:5. The resulting plate showed three bands of the curcuminoids well separated from one another. Each yellow curcuminoid band was scrapped from the plate and eluted with methanol. The eluate of each curcuminoid was concentrated *in vacuo* and subjected to crystallization by petroleum-ether or chloroform. Each crystalline curcuminoid was identified as curcumin, demethoxycurcumin and bisdemethoxycurcumin by its polarity sequence on TLC plate and confirmed by its ^{1}H NMR spectrum.

1.3 Preparation of Standard Solutions

For the preparation of standard solution, ten milligrams of each pure natural curcuminoid were dissolved in 10 ml methanol to give 1 mg/ml stock solution. From this stock solution, various concentrations of each curcuminoid were prepared. Standard curves in the range 0.25-1.5 μg/5 μl,0.25 - 1.5 μg/5 μl and 0.25 - 1.2 μg/5 μl

were then constructed for curcumin, demethoxycurcumin and bisdemethoxycurcumin, respectively.

1.4 Sample Preparation

For sample preparation,twenty-milligram finely powdered turmeric (passing No. 40 sieve) was accurately weighed and sonicated in 10 ml methanol for one hour. The mixture was filtered through Whatman filter No.1 in a Buchner funnel. Five microliters of the eluate were immediately spotted on a TLC plate. For selected zingiberaceous plants, the extract solutions which were filtered through a Whatman filter No. 1 were concentrated to obtain suitable concentrations before being spotted on a TLC plate.

1.5 Thin Layer Chromatographic Conditions for Curcuminoid Separation

Technique : one way, ascending
Absorbent : silica gel 60F 254 (precoated, Merck)
Plate Size : 10 x 15 cm
Layer thickness : 0.2 mm
Solvent system : chloroform : benzene : methanol (80:15:5)
Sample size : 5 μl
Developing time : 15 min.

Distance : 10 cm.

Temperature : 25-30°C

Rf values : curcumin (0.65),
demethoxycurcumin(0.45) and
bisdemethoxycurcumin (0.33)

1.6 Densitometric Analysis

Each curcuminoid spot obtained after thin layer chromatography was quantitated by densitometric method which were described below.

Model : Shimadsu Dual Wavelength Model CS-930

Lamp : tungsten

Scan mode : linear

Determination mode : absorption

Scan width : x = 6.0 mm.

: y = 0.2 mm.

Sensitivity : medium

Slit width : 1.2 x 1.2 mm^2

Wavelength : 420 nm.

The content of each curcuminoid was calculated based on its standard curve.

1.7 UV-vis Spectrophotometric Analysis

The uv-vis spectrophotometric method was

modified from the method described in the International Organization for Standardization (ISO, 1982) which could determine the total curcuminoid content and compared withTLC-densitometric method. For turmeric samples, the methanolic extract of curcuminoids prepared as described in Material and Methods 1.4, were diluted 1:100 with methanol before reading the absorbance at 420 nm by Perkin-Elmer Lambda 3B UV-vis Spectrophotometer. The standard curve of the total curcuminoids which was obtained from UV-spectrophotometric is shown in Fig.13. This curve showed linearity of the relationship between the content of 0.0025 and 0.02 mg/ml of the standard curcumin which calculated as total curcuminoid contents. Result of the regression analysis and the correlation coefficient (r) was found to be 0.998.

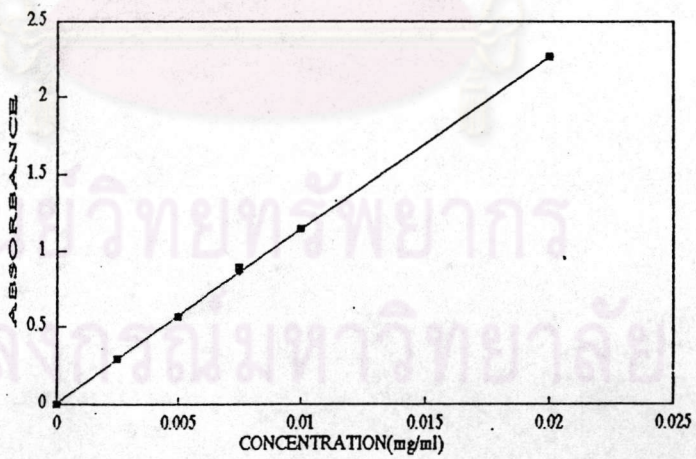

Fig 13. Standard curve of the curcumin on uv-vis spectrophotometric method calculated as total curcuminoid content.

2. Determination of Turmeric Oil Content and Composition

2.1 Plant Material

Plant material is as described in Meterial and Methods 1.1.

2.2 Reagents and Apparatus

Water triple distilled in glass, volatile oil traps-elevenger type with joints for oils with densities near or less than water and round bottom flask 500 ml.

2.3 Sample Preparation

Turmeric and selected zingiberaceous rhizomes were ground to fine powder. After passing the sieve No.40, the powder of each sample was weighed and immediately determined for volatile oil content.

2.4 Volatile Oil Content Determination

Volatile oil was determined by the method described in the Association of Official Analytical Chemists (method 962.17, AOAC, 1990). Ten grams of the ground turmeric and selected zingiberaceous plants were put in a 500 ml round-bottom flask. Water was added into the flask to about half full and also a few pieces of boiling chips. The flask was then set with the apparatus (Fig 14.) for determination of volatile oil (lighter

than water apparatus) (Kenneth, 1990). The flask was distilled until the two consecutive reading taken at one hour intervals showed no change in oil content (about four hours). After cooling, the oil volume was read, calculated and expressed as milliliter of the oil per one hundred grams of sample (%v/w). The volatile oil obtained was then collected and stored at 4°C until being analyzed for its chemical compositions by gas chromatography.

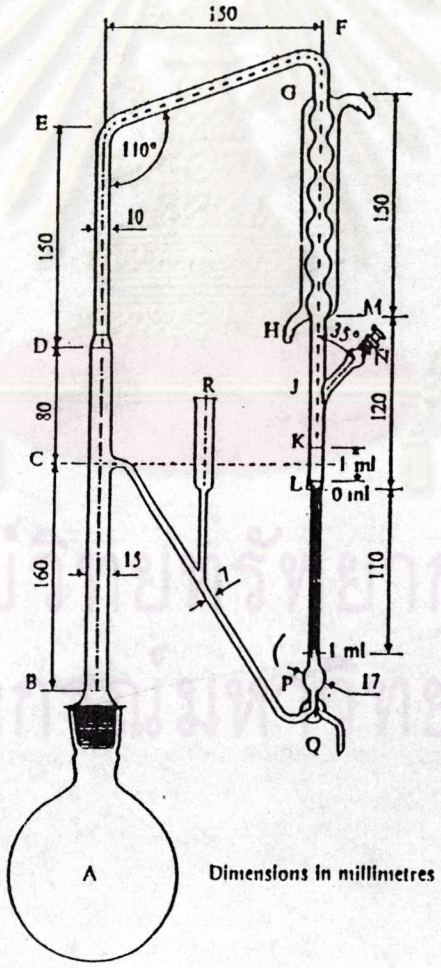

Fig.14 Apparatus for volatile oil content determination

2.5 Gas Chromatographic Conditions

The chemical constituents of the turmeric oil and selected zingiberaceous oil were examined by a Varian 3400 gas chromatography (Sugar Land, Texas, USA) equipped with 8100 Autosampler, a 1077 Split/Splitless Capillary Injector, FID detector and a fused silica capillary column (50 x 0.22 mm I.D) coated with BP 20 (film thickness 0.25 μm, SGE, Victoria Australia). The operating parameters were as follows :-

Nitrogen gas flow rate	: 0.85 ml/min
Hydrogen flow rate	: 30 ml/min
Air flow rate	: 300 ml/min
Injector temperature	: 250°C
Detector temperature	: 250°C
Initial column temperature	: 60°C
Final column temperature	: 200°C, rate 4°C/min, hold 20 min at 200°C
Split ratio	: 100:1
Chart speed	: 0.5 cm/min
Sample size	: 0.3 μl

2.6 GC-MS Conditions

For identification of composition in turmeric oil, a gas chromatography mass spectrometry (GC-MS) Varian Saturn II GC/MS System was used. The system was connected with a 30 m x 0.25 mm (I.D.) capillary column coated with

DB-5, J & W (film thickness 0.25 μm). The operating parameters were as follows :-

Helium carrier gas flow rate	: 1 ml/min
Injector temperature	: 250°C
Initial column temperature	: 60°C
Final column temperature	: 180° C, rate 3°C/min
Split ratio	: 100:1
Accelerating voltage	: 1700 volts
Emission current	: 20 microamps

The spectra were recorded and compared in the Terpene library (RP Adams "The Analysis of Essential Oil by GC-MS").