

CHAPTER III

MATERIALS AND METHODS

1. Plant material

Samples of green and black pepper of Sri Lankan and Sarawak cultivars were harvested fully ripened from six different gardens at Amphor Tamai, Chantaburi which is the major site of pepper plantation in Thailand. The plants of each cultivar are five years old. Some black pepper samples were obtained from the warehouse station of Thai Commodities Co. Ltd. at Amphor Tamai, Chantaburi and were purchased from various markets in Bangkok. Black pepper samples from Brasil, India and Malaysia were obtained from the Dr. E. Rendlen Gmb & Co. OHG, Stuttgart, Germany.

Samples of green pepper were harvested at 1, 2, 3, 4, 5, 6 months old of pepper berries from Sarawak and Sri Lankan cultivars which are grown locally in Thailand. These samples were obtained from six different gardens at Amphor Tamai, Chantaburi. The green pepper berries were used for preparing black pepper in our laboratory by hot air oven at 50°C for 12 hours. The pepper samples of each cultivars of six replication were used for analysis. These samples were used for studying the changes in volatile oil and piperine contents during the development of pepper berries.

2. Chemicals

Authentic samples of α -pinene, β -caryophyllene, tridecane and piperine were purchased from Sigma (St. Louis, MO, USA.), β -pinene was from Chem Service (West Chester, PA, USA.), Δ^3 -carene was from Fluka (Buchs, Switzerland), limonene and sabinene were from Extrasynthese (Z.I. Lyon Nord, Genay, France). Acetone, toluene and methylene dichloride were analytical reagent grade. Acetonitrile and methanol were HPLC grade. Water was distilled in glass.

3. Sample preparation

Black pepper samples were ground to a fine powder in grinder connected with a cool water circulator. After passing the sieve no. 20, the powder samples were weighed and immediately determined for their moisture, volatile oil and piperine contents. Samples of green pepper were prepared by grinding the berries using mortar and pestle. The resulted paste was then weighted and subjected to the analyses.

4. Moisture content determination

Moisture was determined by the method of British Pharmacopoeia 1988. Two hundred milliliters of toluene and 2.0 ml of water were added into a 500-ml round-bottom flask connected with an apparatus for determination of moisture content (Fig. 12). The solvent was distilled

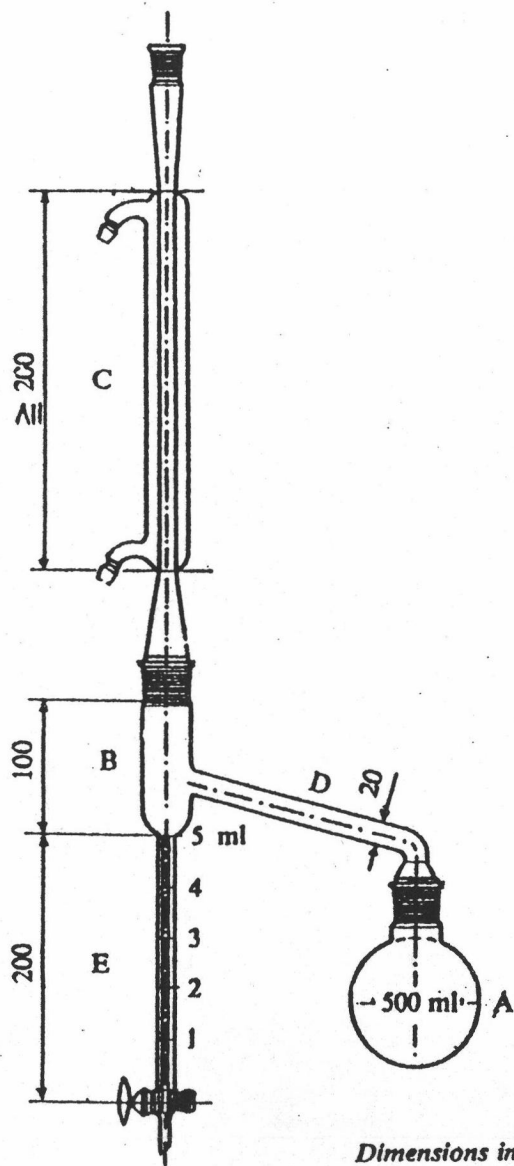


Figure 12 Apparatus for moisture content determination.

for two hours and cooled for 30 min. before reading the water volume. Fifteen grams of each ground pepper sample were then added to the flask with a few pieces of boiling chips. The flask was heated until the water and toluene were completely separated. The volume of water was recorded and calculated as percentage of water in sample.

5. Volatile oil content and composition

5.1 Volatile oil content determination

Volatile oil was determined by the method described in the Association of Official Analytical Chemists (method 962.17, AOAC, 1990). Fifteen grams of each ground pepper sample were put into 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 set with the apparatus for determination of volatile oil (Fig. 13). The flask was distilled until two consecutive readings 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 samples. The volatile oil obtained was then collected and stored at 4°C until being analyzed for its chemical composition by gas chromatography and GC-MS.

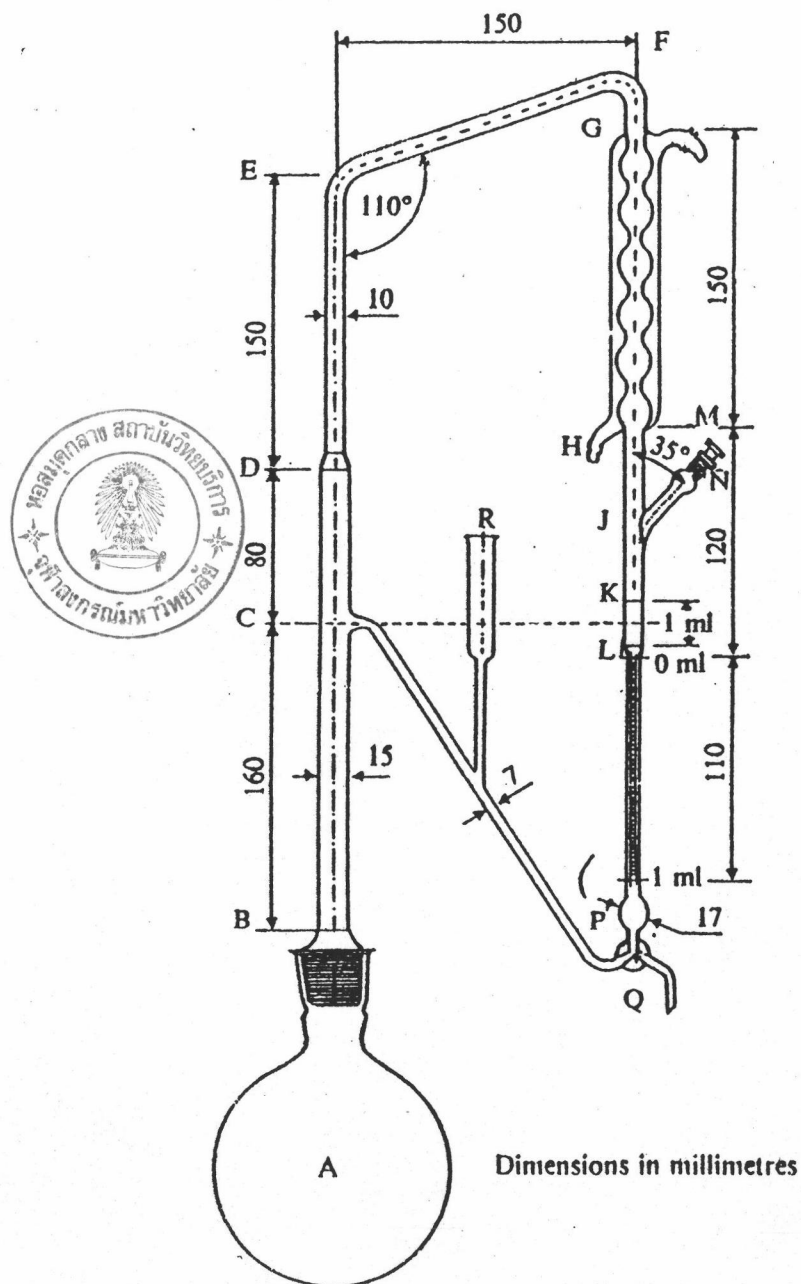


Figure 13 Apparatus for volatile oil content determination.

5.2 Gas chromatographic analysis

5.2.1 Sample preparation for GC analysis

Each pepper oil sample 0.1 ml was added with 20 μ l tridecane as internal standard and diluted with 0.1 ml acetone before injected (0.5 μ l) into the GC system. At least three preparations were performed for each sample.

5.2.2 Gas chromatographic conditions

The chemical constituents of the pepper volatile oils were examined by gas chromatography. The conditions of GC were described below.

GC conditions

Instrument model	Varian 3400 equipped with 8100 Autosampler (Sugar Land, Texas, USA.)
Injector model	1077 Split/Splitless
Detector	FID
Column	Fused silica capillary column (50 m. x 0.22 mm. i.d.) coated with BP 20 film thickness 0.25 μ m (SGE, Victoria, Australia)
Column programming	60-200°C rate 4°C/min hold 10 min.
Injector temperature	250°C
Detector temperature	250°C
Nitrogen carrier gas	0.85 ml/min
Hydrogen supply	30 ml/min
Air supply	300 ml/min

Split ratio	100:1
Chart speed	0.5 cm/min
Sample size	0.5 μ l

5.2.3 Calibration

Various working standard solutions (15-350 μ l/ml) were prepared. One hundred microliters of each solution were added with 20 μ l tridecane as internal standard and diluted with 0.1 ml acetone. After mixing, 0.5 μ l of the solution was injected into the GC column. The resulted chromatogram was used for constructing calibration graphs by plotting between the peak area ratio (peak area of authentic sample/peak area of internal standard) and concentrations of the authentic samples (μ l/ml). At least three analyses were performed for each determination.

5.3 Gas chromatography-mass spectrometry.

For indentification of composition in pepper oil, a gas chromatography-mass spectrometry (GC-MS) was used. The pepper oil was diluted 1:1000 in ethanol before injected into GC-MS system. The conditions of GC-MS were described below. The spectra were recorded and compared in the Terpene library (RP Adams "The Analysis of Essential Oil by GC-MS").

GC-MS conditions

Instrument model	Varian Saturn II GC/MS System
Column	fused silica capillary column (30 m. x 0.25 mm. i.d.) coated with DB-5 (J & W) film thickness 0.25 μm
Column programming	60-180°C rate 3°C/min
Injector temperature	250°C
Helium carrier gas	1 ml/min
Split ratio	100:1
Accelerating voltage	1,700 volts
Emission current	20 micro amps
Sample size	1 μl

6. Piperine content determination**6.1 Sample preparation for HPLC analysis**

Fifty milligrams of each ground pepper samples and 10 ml of methylene dichloride were put into a 50-ml tube and extracted under reflux at 50°C for 1 hour. The extract of each pepper sample was filtered by membrane (0.45 μm .). After filtering, the filtrate was adjusted with methylene dichloride in a 10-ml volumetric flask and injected into HPLC system.

6.2 HPLC conditions

The piperine in extract of each pepper sample was examined by HPLC. The conditions of HPLC were described below.

HPLC condition

Instrument model	Varian 9010 equipped with Varian 9095 Autosampler (Sugar Land, Texas, USA.)
Detector model	Varian 9050
Integrator model	Varian 4400
Column	Merck LiChroCart RP-18, 125 mm x 4 mm , Particle size 5 μ m (Darmstadt, Germany)
Mobile phase	55% Acetonitrile in water
Flow rate	1 ml/min
UV detector	336 nm
Injection volume	10 μ l
Chart speed	0.25 cm /min

6.3 Calibration

Thirty milligrams of authentic piperine were dissolved and adjusted with methylene dichloride in 100-ml volumetric flask to give 300 μ g/ml stock solution. From this stock solution, various concentrations of piperine were prepared. Ten microliters of piperine solutions in the range 50-300 μ g/ml were injected into the HPLC system. The resulted chromatogram was used for constructing calibration graphs by plotting between peak areas and concentrations of the authentic piperine. At least three analyses were performed for each determination.

7. Studies on volatile oil content and composition in pepper during storage

Black pepper samples from the warehouse station of Thai Commodities Co. Ltd., Chantaburi were kept in tight container at room temperature for 8 months. Black pepper samples from this condition at 0, 1, 2, 3, 4, 5, 6, 7, 8 months were studied volatile oil content and composition as the methods described above.

8. Study on volatile oil composition in pepper oil during storage

The pepper oil from hydrodistillation of black pepper was kept in a tight container at both room temperature in dessicator and at 4°C in a refrigerator for 8 months. The samples from each condition at 0, 1, 2, 3, 4, 5, 6, 7, 8 months were studied volatile oil composition by GC as described above.