

## CHAPTER III

### Materials and Methods

#### 1. Plant material

Sample of various grade of black pepper (A, B, C, D) were obtained from Thai Commodities Co. Ltd. at Amphor Tamai, Chantaburi. These grades were based on their weight using a grading machine. Each grade can be defined as follows :

Grade A : The black pepper with superior characteristic and highest weight of more than 600 grams per litre.

Grade B : The black pepper with nice looking and having the weight approximately 550 grams per litre.

Grade C : The black pepper with inferior appearance and having the weight of less than 500 grams per litre.

Grade D : The black pepper obtained from light berries. It's weight is from 300 to 350 grams per litre. This is not sold in the market. Instead, it is usually ground into powder and mixed with other grades of ground black pepper.

#### 2. Chemicals

Authentic piperine was purchased from Sigma (St. Louis, MO, USA.) Acetone, Hexane and Methylene chloride used for oleoresin extraction were commercial grade (Ward Medic, Bangkok.) Acetonitrile was HPLC grade, Water was distilled in glass.

### **3. Study on the effect of some organic solvents on oleoresin extraction.**

Black pepper grade D was ground to fine powder using Disk Mill (Model FFC-15, Thai-Asako, Ltd.). After passing the sieve no. 20, 330 grams of each powdered sample was weighed and macerated with 1,000 ml of each of the following solvents : acetone, hexane and methylene chloride in a sealed glass container at room temperature. After 48 hours, the oleoresin extract obtained from each organic solvent was filtered through Whatman filter paper No.1 and evaporated until dryness *in vacuo* . The resulted oleoresins were determined for their weight, volatile oil and piperine content.

### **4. Study on the effect of different grades of black pepper on the quality and quantity of oleoresin.**

Black pepper samples of grade A,B,C and D were grounded separately to fine powder using Disk mill (Model FFC-15, Thai - Asako, Ltd.). After passing the sieve no. 20, 330 grams of each powdered sample was macerated with 1,000 ml acetone in a sealed glass container at room temperature. The oleoresin extract obtained from each pepper grade was then filtered through Whatman filter paper No. 1 and evaporated until dryness. The resulted oleoresins were determined for their weight, volatile oil and piperine content.

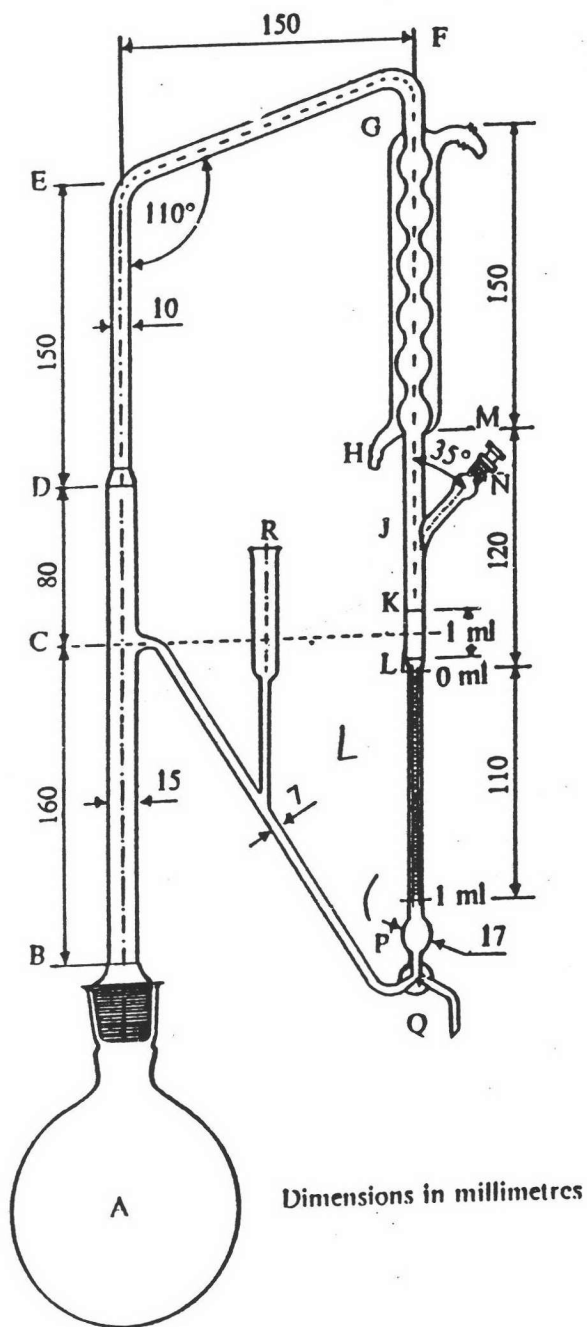


Figure 5: Apparatus for volatile oil content

## 5. Optimization of the maceration time

Finely ground black pepper grade D (330 grams) was macerated with 1,000 ml acetone in a sealed glass container at room temperature. Four containers were prepared and the oleoresin extract was taken from each container at 6,12,24, and 48 hours. Each oleoresin extract was filtered through Whatman filter paper No. 1 and evaporated until dryness before being determined for the weight, volatile oil and piperine content.

## 6. Determination of oleoresin weight

The filtered oleoresin extract was evaporated at 40°C *in vacuo* using preweighed round-bottom flask (500 ml) until dryness. The dried substance called oleoresin was weighed with the flask. The oleoresin weight was simply the difference of the final weight and empty-flask weight..

## 7. Volatile oil content and composition determination

### 7.1 Volatile oil content determination

(Volatile oil content was determined by the method described in the British Pharmacopoeia, 1988 (A-137))

Two to three grams of the obtained oleoresin was weighed and put in a 500 ml round bottom flask. The flask was then added with 250 ml distilled water followed by a few pieces of boiling chips.

The flask was set with the apparatus for determination of volatile oil (Fig. 5). and distilled until 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 percent dry weight of oleoresin. The volatile oil obtained was then collected and stored at 4°C until being analysed for its chemical composition by Gas chromatography.

## **7.2 Determination of volatile oil composition by Gas chromatographic analysis.**

The chemical constituent of the volatile oil in black pepper oleoresin were examined by gas chromatography (GC). The conditions of GC are as follows :

### **GC conditions :**

Instrument	:	Varian 3400 equipped with 8100 Autosampler (Sugar Land, Texas, USA)
Injector	:	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 µm (SGE, Victoria, Australia)
Column programming	:	60-200°C rate 4°C per 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 µl

## 8. Piperine content determination.

### 8.1 Sample preparation for UV-spectrometric analysis.

Five milligrams of the dried oleoresin was dissolved with 250 ml acetone. After filtering through Whatman No. 1, the solution was determined for piperine content by UV-spectrophotometer using the following conditions :

Instrument model	: Lambda 3B double-beam UV-visible spectrophotometer (Perkin-Elmer, Connecticut, USA)
UV detector	: 336 nm
Spectrometer cuvet	: silica (Quartz)
Light path	10 mm
Minimum volume	1 ml

The obtained absorbance value was used for calculation of piperine content based on a calibration curve of the compound.

### 8.2 Calibration curve of piperine

Authentic piperine (12.80 milligrams) were dissolved and adjusted with acetone in a 50 ml volumetric flask to give 256  $\mu\text{g/ml}$  stock solution. From this stock solution, various concentrations (16-256  $\mu\text{g/ml}$ ) of piperine were prepared and detected by a UV-spectrophotometer for their absorbance. The resulted values were used for constructing a calibration graph by plotting between the absorbances and concentrations of the authentic piperine. At least five analyses were performed for each determination.

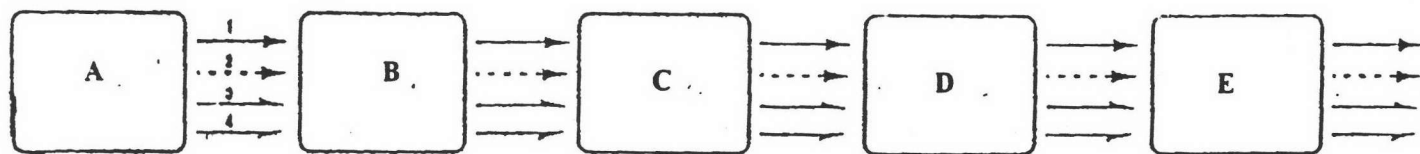
### 8.3 HPLC analysis for piperine detection in black pepper oleoresin

The oleoresin solution prepared as described in 8.1 was filtered through a membrane (0.45  $\mu\text{m}$ ) and the filtrate was injected into a HPLC using the following conditions :

Pump	:	varian 9010 equipped with varian 9095 autosampler (Walnut Creek, CA, USA.)
Detector	:	varian 9050
Integrator	:	varian 4400
Column	:	125 mm. x 4 mm, particle size 5 $\mu\text{m}$ . (Damstadt, Germany)
Mobile phase	:	55% acetonitrile in water
Flow rate	:	1 ml/min
UV detector	:	336 nm
Injection volume	:	10 $\mu\text{l}$
Chart speed	:	0.25 cm/min

### 9. Study on the step-wise maceration of black pepper for oleoresin extraction.

The system consisted of 5 containers (2.50 litre capacity each). Each container contained 330 grams of grade D black pepper and extracted with 1,000 ml acetone according to either Method 1 or Method 2 as described below. The weight of oleoresin, volatile oil and piperine content were evaluate from the oleoresin filtrate as described of black pepper in sections 6, 7 and 8 respectively.

**Method 1 :**

**Step 1 :** Soaked 330 g. black pepper powder in container A with 1,000 ml pure acetone for 48 hours.

**Step 2 :** Filtered the oleoresin extract from container A and squeezed the residue to get maximum volume of the extract.

**Step 3 :** Took 50 ml. of the filtrate to evaluate oleoresin weight, volatile oil and piperine content.

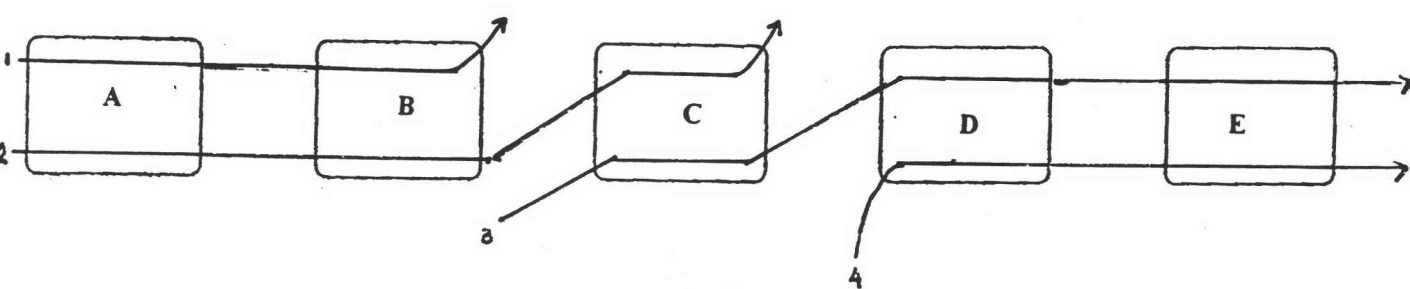
**Step 4 :** Adjusted the filtrate volume to 1,000 ml with acetone and poured into the next container (container B) containing another 330 g. of black pepper powder.

**Step 5 :** After the black pepper in contained B was macerated for 48 hours, then repeated step 2 to step 5 with the same acetone extract until finishing to container E.

\* These 5 steps were repeated 4 times, each time with pure acetone (1,000 ml)

**Method 2 :**

This system also consisted of 5 containers but the sequence of maceration was different from the method 1 as shown in the following diagram.







- Step 1 :** Soaked 330 g black pepper powder in container A with 1,000 ml pure acetone for 48 hours.
- Step 2 :** After filtration and 50 ml of the filtrate was taken for analysis, the filtrate (added acetone to 1,000 ml) was poured to container B for further 48 hours maceration. The filtrate obtained from container B was again taken for the analysis.
- Step 3 :** Pure acetone solvent (1,000 ml) was added to the residue in container A for complete extraction of oleoresin. The same filtrate was again poured to container B followed by container C in a similar way of step 1 and 2.
- Step 4 :** Another 1,000 ml of pure acetone was added to the residue in container C followed by containers D and E.
- Step 5 :** The last portion of 1,000 ml pure acetone was added to the residue of containers C and D for completing the process.

## **10. Study on residual solvent present in the prepared oleoresin**

### **10.1 Sample preparation**

One gram of oleoresin was dissolved with 10 ml methanol. The solution was filtered through a membrane (0.45  $\mu\text{m}$ ) before injected into a GC system which was set using the following conditions :

Instrument	:	Varian 3400 equipped with 8100 Autosampler (Sugar Land, Texas, USA).
Injector	:	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 $\mu\text{m}$ (SGE, Victoria, Australia)
Column programming	:	60-90°C rate 4°C per min hold 10 min
Injector temperature	:	250°C
Detector temperature	:	250°C
Split ratio	:	100 : 1
Chart speed	:	0.5 cm./min
Sample size	:	0.2 $\mu\text{l}$

## 10.2 Calibration

One millilitre of acetone was diluted with 100 ml methanol in a volumetric flask to give 1% concentration (stock solution). From this stock solution, various concentrations of acetone from 0.5% to 0.25% were prepared and analyzed by GC. The results were used for constructing a calibration graph by plotting between area unit and concentrations of acetone. At least three analyzes were performed each determined.