CHAPTER 3

MATERIALS AND METHODS

3.1 INSTRUMENTS

- Critical Point Drier; CPD 020 (Balzers, Germany)
- Diamond Knife (Diatome, U.S.A)
- Freezer (Krungthai, Thailand)
- Gas Chromatography a Hewlett 5892 Series II (Hewlett-packard, U.S.A)
- Gas Chromatography-Mass spectroscopy a Varain Saturn 4D (Varain, U.S.A)
- Incubator BM600(Memmert GambH, Germany)
- Ion Sputter Coater a Union SCD 040 (Balzers, Germany)
- Knife Maker LKB 7800(LKB, Sweeden)
- LKB Ultramicrotome Ultratome V(LKB, Sweeden)
- Light Microscope(Nikon, Japan)
- Magnetic Sterer M21/1 (Franz Morat KG GambH, Germany)
- Microcentrifuge
- Microsyrynx 1μl (Varain, U.S.A)
- Microsyrynx 10μl (Hewlett-packard, U.S.A)
- Scanning Electron Microscope SEM;JSM-5410LV (Tokyo, Japan)
- Stereo Microscope (Nikon, Japan)
- Transmission Electron Microscope TEM; Jeol 200SX (Tokyo, Japan)
- Warmplate (LKB, Sweeden)
- Bioharzard (Bangkok Science, Thailand)

3.2 INVENTORY SUPPLIES

- Beakers 5, 25, 80, 125, 500 and 1000 ml (Pyrex, Germany)
- Capsules; Embedding, Size 00, 8mm I.D (EMS, USA)

- Copper Grid 300mesh (EMS, USA)
- Dissecting Scissors (CV scissors, 4" (EMS, USA)
- Double Edge Coated Blade
- Embedding Capsule Holder (EMS, USA)
- Eppendorf 1 ml
- Filter paper Whatmann No.4 (Whatmann Internation Ltd., England)
- Forcep No.4 INOX (Dumont&Fils, Switzerland)
- Glass Knife Boats (EMS, USA)
- Glass Knife Strips Size 6.4 mm x 25 mm x 400 mm (EMS, USA)
- Glass Stoppered Bottles 125, 250, 500 and 1,000 ml (EMS, USA)
- Micropipette 2, 5, 20, 500 and 1000 p (Axygen-Hayward, USA)
- Mixer Vortex Genies 2 (EMS, USA)
- Parafilm M and Dispenser (EMS, USA)
- Pasteur Pipettes
- Petri Dish (Pyrex, Germany)
- Pipette Tips 2, 5, 20, 500 and 1000 µl (Axygen-Hayward, USA)
- Sectioning set (Chiron Stainless, Germany)
- Single Edge extra long
- Specimen Forceps 4.5" (EMS, USA)
- Tri-Pour Beakers 50, 100 250 400 ml (EMS, USA)
- Tweezers, Style #4, super fine points, short (EMS, USA)

3.3 CHEMICALS

3.3.1 Chemicals for GC and GC-MS

- 2-Heptanone (Sigma Chemical Company, USA)
- n-Hexane (Sigma Chemical Company, USA)
- n-Oxtyl acetate (Sigma Chemical Company, USA)

3.3.2 Chemicals for Electron Microscopy (SEM & TEM)

- Absolute Ethyl Alcohol (Merk, Germany)
- Dodecenyl Succinic Anhydride (Sigma Chemical Company, USA)

- DMP 30 (2,4,6 Tridimethyl Aminomethyl Phenol) (EMS, USA)
- Epon 812 (EMS, USA)
- Glutaraldehyde (Sigma Chemical Company, USA)
- Hydrochloric Acid (Merk, Germany)
- Lead Citrate (Sigma Chemical Company, USA)
- Methyl Nadić Anhydride (EMS, USA)
- Osmium Tetroxide, Crystalline, Highest Purity, 99.95% (EMS, USA)
- Paraformaldehyde,EM grade,Purified (EMS, USA)
- Phosphotungstic acid (Fluka, Switzerland)
- Propylene Oxide, EM grade (EMS, USA)
- Sodiumborate (Fluka, Switzerland)
- Sodium Cacodylate (Sigma Chemical Company, USA)
- Sodium Hydroxide (Fluka, Switzerland)
- Toluidine Blue (Fluka, Switzerland)
- Uranyl Acetate (Fluka, Switzerland)

3.4 SPECIMENS

Five species of honeybees in Thailand namely; *A. andreniformis*, *A. cerana*, *A. dorsata*, *A. florea* and *A. mellifera* are to be investigated. Foragers of each species were collected from the field whist foraging on flowers (*Eupaturium odoratum* and *Mimosa invisa* flowers)

3.5 STUDY SITES

Samples were collected from Bangkok, Chantaburi, Chiang Mai, Saraburi and some other provinces (depending on the foraging activity).

3.6 METHOD FOR PHEROMNE ANALYSES

3.6.1 Pheromone Extraction

(i) Mandibular glands were removed from the head capsules and cleaned from adhering tissues under stereomicroscope.

(ii) Glands were transferred into n-haxane. Thirty foragers for each species were used.

(iii) Samples were homogenized in glass Teflon homogenizer in batches of **sixty** glands in 500 µl solvent, centrifuged at 10,000 g for 15 min and stored at –18°C until used.

(iv) N-octyl acetate was used as the internal standard.

(v) Samples were analyzed by gas chromatography on a Howlett 5892 series II (GC) for quantity and a Varian Saturn 4D constructed with Incos Ionization (GC-MS) for quality.

3.6.2 Protocol for Pheromonal Quantitative (GC Hewlett 5892 series II)

(i) Injected 1 µl of samples (extracted from mandibular glands) in the injection port (in splitless injection mode on a fused silica capillary column (30m. x 0.25 mm. i.d.) with bonded 0.25 µm DB-5 stationary phase and a flame ionization detector which was used at a detector temperature of 260°C.

(ii) Set the oven temperature at 40°C, it was programmed at 40°C for 2 minute and increased at a rate of 7°C/min until to 250°C is reached.

(iii) Adjust the helium carrier gas flow rate of 1ml/min.

(iv) Data were analyzed.

3.6.3 Standard Calibration Curve of 2-Heptanone (GC Hewlett 5892)

(i) Injected 1 µl of variety concentration of 2-Heptanone in N-hexane with constant concentration (10.0E-03 ml/ml) of N-octyl acetate in the injection port; The concentrations of 2-Heptanone are as follows,

- 1.0E-03 ml/ml
- 1.5E-03 ml/ml
- 2.0E-03 ml/ml
- 3.0E-03 ml/ml
- 4.0E-03 ml/ml

(ii) Set the oven temperature at 40°C, it was programmed at 40°C for 2 minute and and it was increased at a rate of 7°C/min until to 250°C is reached.

(iii) Adjust the helium carrier gas flow rate of 1ml/min.

(iv) The calibration curve were made from relative peak areas of 2-Heptanone and N-octyl acetate.

(v). Quantifications of compounds were calculated from flame ionization detector responses to know amounts of each of the compounds.

3.6.4 Protocol for Pheromonal Qualitative by GC-MS

(i) Injected 0.30 µl of samples (extracted from mandibular glands) in the injection port.

(ii) Set the oven temperature at 40°C and it were increased at a rate of 7°C/min until to 260°C is reached.

(iii) Adjust the carrier gas flow rate of 1ml/min.

(iv) Data were analyzed.

3.6.5 Confirmation of Pheromones by Using Standards

(i) Injected 1 µl of standard which were dissolved in N-hexane and adding of N-octyl acetate with the same concentrations in the injection port.

(ii) Set the oven temperature at 40°C and it were increased at a rate of 7°C/min until to 260°C is reached.

(iii) Adjust the carrier gas flow rate of 1ml/min.

(iii) The relative retention time of standard and internal standard were compared to the results from GC-MS.

3.7 Protocol for Scanning Electron Microscope

(i) The mandibular glands were removed from head capsules of each species under stereomicroscope. Ten foragers were used for each species.

(ii) Fix specimens in Karnowsky fixative for 3 h. at 4°C.

(iii) Wash by washing buffer at pH 7.4 for 3 times-10min each.Post -fix in 2% osmium tetroxide in cacodylate buffer pH 7.4 for 1 h.

(iv) Dehydrate in alcohol series; 30%, 50%, 70%, 90% and 100% for ten min. each.

(v) Dry samples by critical point drying temperature. Sputter-coating by gold and examined by Sem; JSM-5410 LV.

3.7 Protocol for Transmission Electron Microscope

(i) Mandibular glands were removed from the head capsules and cleaned from adhering tissues. **Ten foragers** for each species will be used.

(ii) They were fixed immediately in Karnowsky fixative at 4°C for 3 h.

(iii) The tissues were next rinsed twice at 25°C in 0.27 M fixing buffer pH 7.4 for ten minutes each.

(iv) The specimens were post fixed 1 h. in 2% osmium tetroxide in the same buffer.

(v) Dehydrated by gradation ethanol; 30%, 50%, 70%, 90% and 100% for ten minutes each.

(vi) Tissues were agitated for 12 h. in mixture of propyline oxide and embedding media.

(vii) Embedded tissues in Epon 812-aradite mixture in capsules.

(viii) Put tissues capsules into the incubator for polymerization at 35°C for 24h., 45° C for 48h. and 65°C for 72h.

(ix) Semi-thin sections (1μ m) by LKB ultramicrotome and stained with 1 % toluidine blue in sodium borate solution to select target areas.

(x) Ultra-thin sections (90nm) and floated in distilled water.

(xi) Tissues were collected on 200 mesh grids, stained by uranyl acetate and contrast enhanced by lead citrate.

(xii) They were examined by Jeol 200SX (Tokyo, Japan).