2. MATERIALS AND METHODS

2.1 Apparatus

1). Analytical Balance, Satorius Basic, Germany

2). Blender, National MX-110PN, Japan

3). Extraction Glass column for sample preparation, 2 cm i.d. x 30 cm long

4). Evaporator, Rotavapor R-114, manufactured by Buchi, Switzerland

   a. Water bath, B-486

   b. Vacuum system, B-169

5). Helium gas, 99.99% purity, TIG Special Gases, Thailand

6). Nitrogen gas, 99.99% purity, TIG Special Gases, Thailand

7). Air Pressure Regulator, Harris, The Harris Calorific Co., U.S.A.

8). Gas Chromatography manufactured by Hewlett Packard Co., U.S.A. equipped with:

   a). Electron-capture detector, HP 5890 Series II Plus

   b). Capillary column, HP-608, 0.530 mm i.d. x 30 m long x 0.5 μm film thickness

   c). Automatic sampler and injector, HP 7673 GC/SFC

   d). Controller, HP 7673

   e). Data analyzer and printer, HP Chemstation/HP 3365 Series II Chemstation (DOS series) and HP Deskjet 500

2.2 Chemicals

1. Dichloromethane, AR grade, J.T. Baker Inc., U.S.A.
2. Sodium sulfate (anhydrous), AR grade, Fluka, Switzerland
3. Florisil (60-100 mesh), AR grade, Mallinckrodt Chemical Inc., Paris
4. Petroleum Ether, AR grade, J.T. Baker Inc., U.S.A.
5. Iso-octane, GC grade, Fluka, 99.5% purity, Switzerland
6. Silica gel (60-100 mesh), AR grade, Merck, Germany
7. Toluene, AR grade, Merck, Germany
8. Hexane, AR grade, Merck, Germany

9. Pesticide Standards were supplied by Dr. Ehrenstorfer, Germany included:
   - Pesticide Mixture IV (L180004) contains 1 ng/µl each of α-BHC, β-BHC, lindane, heptachlor, cis-heptachlor epoxide, hexachlorbenzene (HCB), α-endosulfan, β-endosulfan in cyclohexane.
   - Pesticide Mixture V (L180005) contains 1 ng/µl each of o,p'-DDD, p,p'-DDD, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT, dieldrin, endrin in cyclohexane
   - Endosulfan sulfate 99% purity
10. Surrogate (Internal Standard Mixture) supplied by "Promochem" company, Germany included 1ng/µl each of tetrachlor-m-xylene, quintozen (pentachloronitrobenzene-PCNB), isodrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydroendo,endo-1,4,5,8-dimethanonaphthalene), and PCBNo.209 (decachlorobiphanyl) in iso-octane. The chemical structures of surrogate compounds are shown in Appendix Figure 3.

2.3 Egg Sampling

The study was divided into two parts. The first part was to optimize and standardize the sampling technique of hens' eggs and the second part was to determine organochlorine residues in hens' eggs in Chiang Mai suburban areas. This approach reduced the labor and chemical costs, but improved the reliability of results for biomonitoring of organochlorine residues by using the hens' eggs.

2.3.1 Standard Operating Procedure for Sampling

In the first part of this research, eggs were collected from one hen, from different hens in one house, and in different houses in one sampling site in order to detect factors which might affect organochlorine residues in eggs. Intentionally, the range of organochlorine residue variation caused by the sampling technique could be determined and the optimum number of eggs which should be sampled representatively for each area for biomonitoring purposes could be estimated.

The experimental site was selected randomly in Group 14, Suthep Subdistrict, Muang District which is located behind Chiang Mai University, at the foot step of Doi Suthep. A total of 17 eggs were collected from: the whole clutch of eggs (10 eggs) of a one-year old hen, the first 3 eggs from a two-year old hen, the first 2 eggs from a three-year old hen from the same house (Figure 2.1), and the other 2 eggs from two different houses in the same sampling site .

Figure 2.1 One egg sampling site where eggs were collected from the 1,2, and 3- year old hens (Suthep sub-district, Muang District, Chiang Mai).

2.3.2 Egg Collection in Chiang Mai Suburban Areas

According to a report of the Malaria Center Region 2, DDT has been used widely and legally for fighting malaria-disease vectors by the Thai government over large areas in northern Thailand since 1953 [4]. Usually, a 5 % suspension of DDT has been sprayed inside and outside houses at 2 g/m^2 residue spraying. Surrounding Chiang Mai City, Mae Rim and Hang Dong Districts were sprayed more heavily with DDT compared with other Districts.

To biomonitor organochlorine residues in Chiang Mai suburban areas, a total of 64 eggs of domestic hens (*Gallus domesticus*) were collected from various villages in Mae Rim, Hang Dong, Muang, and San Kampaeng Districts, Chiang Mai Province, Thailand between August and December 1995. The locations of the areas surveyed are shown in Figure 2.2. The furthest sampling site is about 40 km from Chiang Mai City.

Eggs were collected randomly from the areas with no DDT spraying (i.e. DDT-unsprayed areas), the areas may have been sprayed with organochlorines some time ago which has now been discontinued (formerly DDT-sprayed areas), and the areas where organochlorines have been applied until now (DDT-sprayed areas). The survey areas also include various land-use types such as high density residential area (Muang District), craft-industrial area (San Kampaeng District), agricultural or mountainous areas (Mae Rim and Hang Dong Districts and Doi Suthep).

In each village eggs were collected randomly from at least two or three houses. At each house two eggs were collected from one hen or sometimes from two different hens depending on the availability. In addition, questionnaires were completed at every egg collection site. The questionnaire was designed to give such information as land and pesticide use, feeding and care of poultry, with the hope of obtaining supplementary information to identify the potential exposure of hens to pesticides (Appendix 1).

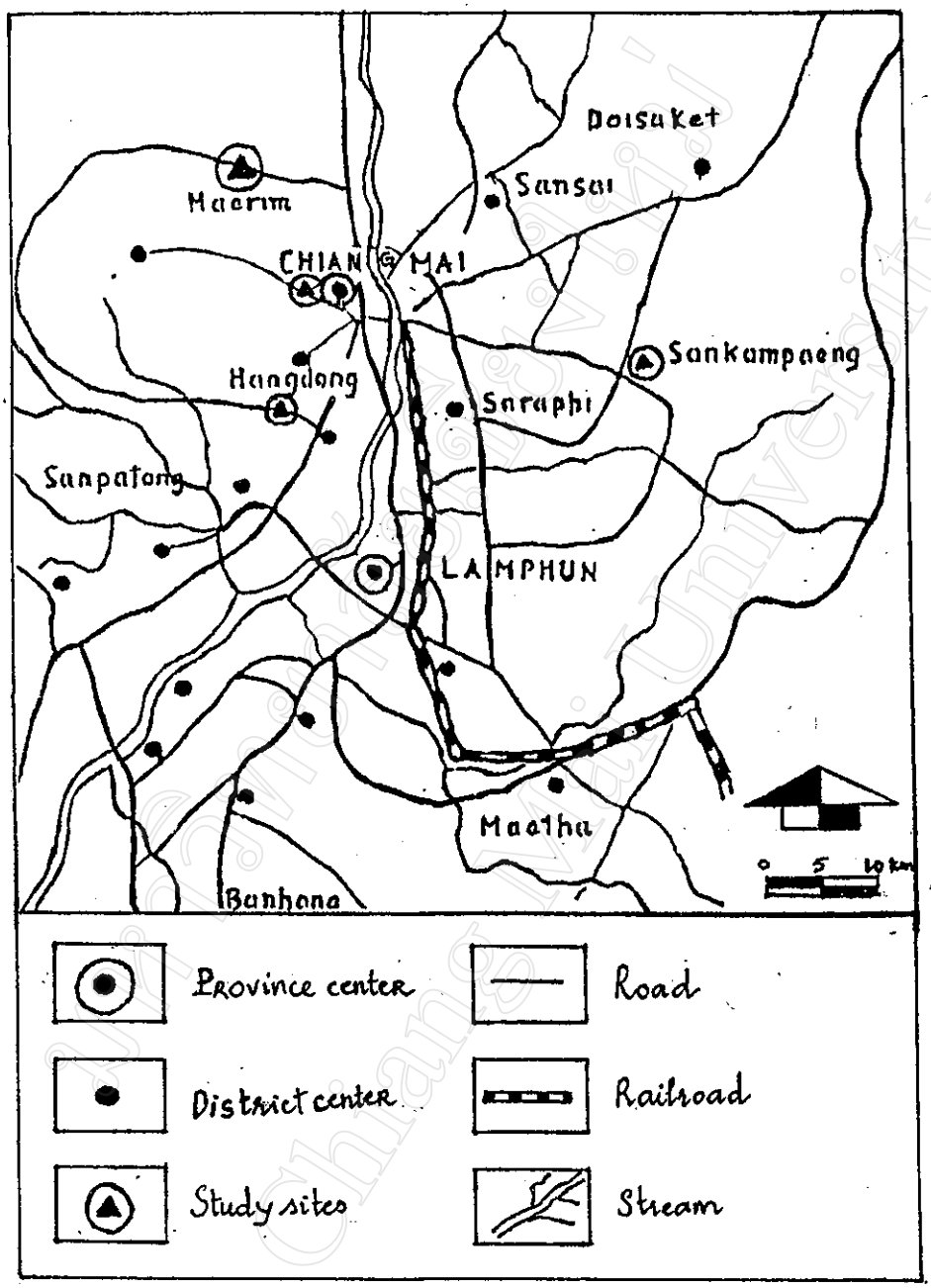

Figure 2.2 Map showing locations of the study sites.

In Mae Rim District, 21 eggs were sampled from eleven villages scattered in three sub-districts Sa Luang, Mae Ram and Don Kaew, including seven DDT-sprayed areas such as Muang Kha, Mae Rim Noi, Pa Kha, Pang Ee Ka, Pang Hai , Huoy Som Suk, and Sa Luong Nua and four areas not having been sprayed with DDT or formerly sprayed areas (Sa Luong Nok, Hua Fai, Don Kaew, and Sa La). The locations of sampling sites are shown in Figure 2.3. The detailed information of sampling sites, hens (e.g. age, feather color), and egg samples (e.g. total number of eggs in the nest) are presented in Appendix Table 3.

Another 19 eggs came from Hang Dong District. They were from eight villages in four sub-districts (Nong Khoai, Ban Pong, Ban Ven, and Mae Kha), including three DDT-sprayed areas (Mae Ha , Huay Sieu, Huay Phak Phay) and five formerly DDT sprayed areas (Ban Pong, Khong Khin, Huay Som Poi, Dong Pai, and Nam Thon) (Figure 2.4 and Appendix Table 4).

Among the four Districts surveyed San Kampaeng is quite renowned for its traditional craft, furniture, handicrafts, lacquerware, and jewelry products. This area is a typical craft-industrial area where DDT spraying has been stopped. Nine eggs were collected from four villages in San Kampaeng, Ton Pao, and Sai Mun Sub-districts (Figure 2.5 and Appendix Table 5).

A total of 15 eggs were sampled from Chan Puag, Su Thep, and Pa Dad Subdistricts, Muang District. These areas are high density residential areas which are no longer sprayed with DDT. Among them, 6 eggs were from Hmong hilltribe and villagers living in Doi Suthep-Pui, National Park (Appendix Table 6).

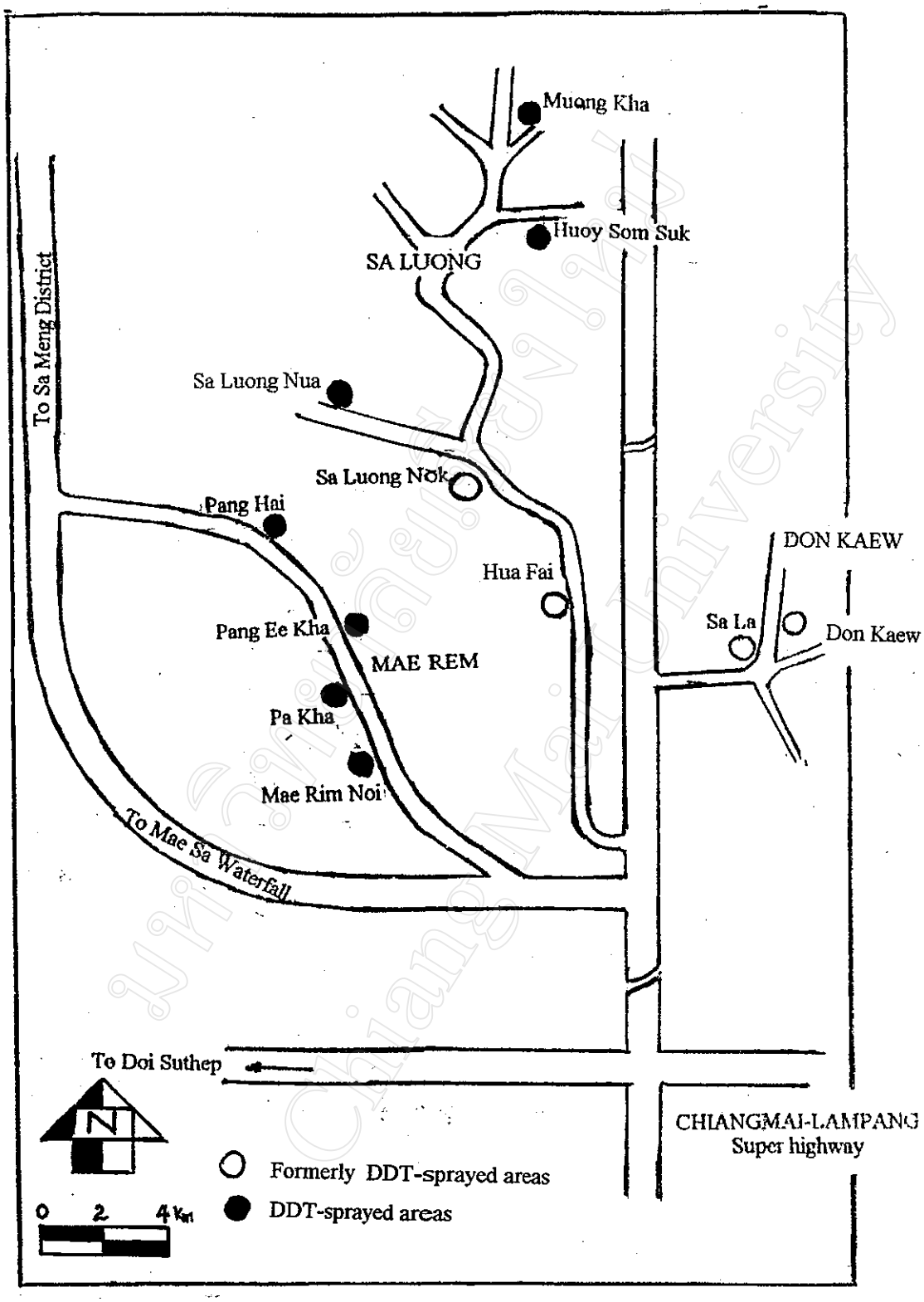

Figure 2.3 Map showing locations of egg sampling sites in Mae Rim District.

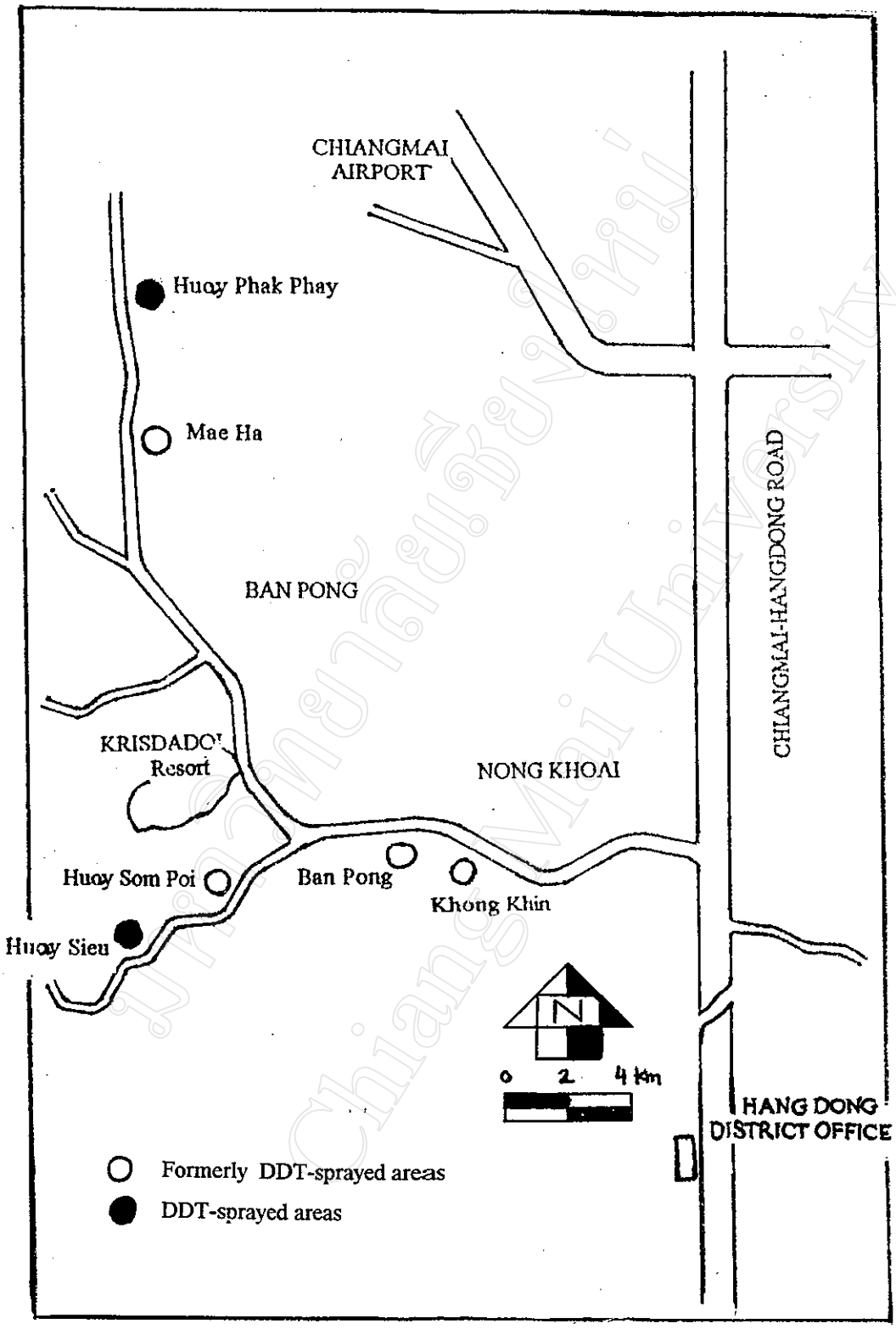

Figure 2.4 Map showing locations of egg sampling sites in Hang Dong District.

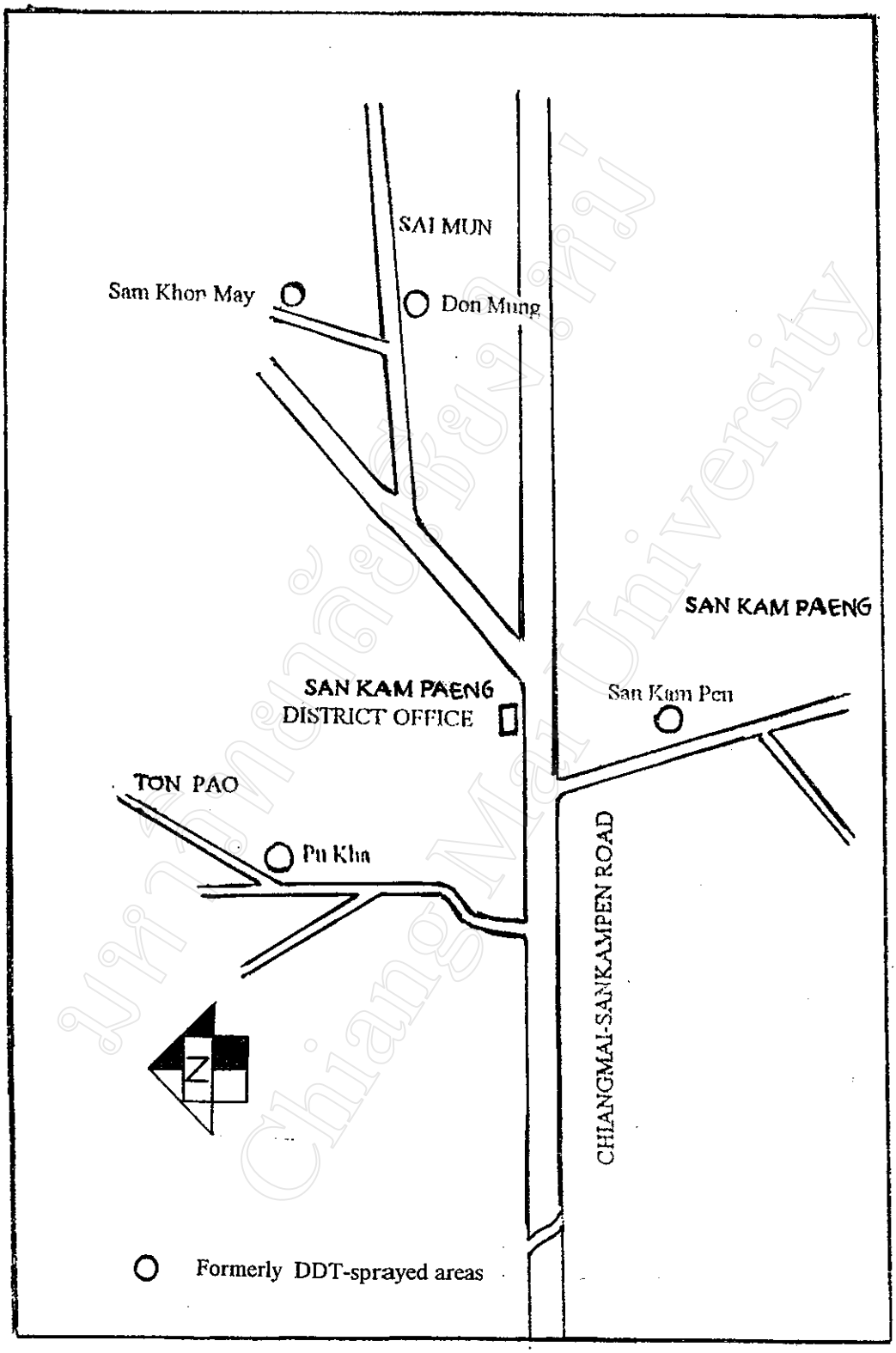

Figure 2.5 Map showing locations of egg sampling sites in San Kampaeng District.

2.4 Organochlorine Analysis

Organochlorine residues in hens' eggs were analyzed qualitatively and quantitatively in accordance with the US-EPA Method 608 and the Standard Operating Procedure of the University of Saarland Institute of Biogeography (USIB-SOP) for eggs by capillary gas chromatography with an electron-capture detector (ECD). The main steps are sampling, extraction, clean up, qualitative and quantitative determination, and confirmation analysis.

2.4.1 Egg Sample Storage

In the laboratory, the weight of the whole egg was recorded intentionally to find out the relationship between organochlorine residues in eggs and the egg weight . Each egg was broken and the whole shell contents were homogenized by a blender, then transferred into a glass tube and stored at -20°C in a freezer until being analyzed.

2.4.2 Homogenization

Approximately 3 g of mixed egg sample was placed in a porcelain mortar and ground well with sodium sulfate to yield dry free-flowing powder. A known amount of surrogate mixture (100 ng of each) was added to the homogenized sample.

2.4.3 Extraction

The powder was transferred into an extraction glass column. The mortar and pestle were cleaned with small portions of dichloromethane which was also poured into the column. Cold extraction was done with 60 ml dichloromethane in two steps. The first 30 ml of dichloromethane was allowed to stay in the extraction column, keeping contact with the powder for 20 minutes before eluting into pre-weighed (± 1 mg) round bottom flask. Then the extraction was repeated with another 30 ml dichloromethane and eluted right away into the same flask.

2.4.4 Gravimetric Determination of Fat

The solvent (dichloromethane) was evaporated to dryness by using a vacuum evaporator at 35°C. The flask was weighed and this procedure was repeated until the constant weight was obtained. This part of the sample, the raw extract, is defined as the "fat content" of the egg sample throughout this study.

2.4.5 Clean-up

The florisil column chromatography method is usually employed for clean-up of chlorinated pesticides. Prior to use, florisil was activated at 600°C for 2 hours, adjusted with 3% (w/w) water, and allowed to stand for 24 hours. Glass wool was plugged into the conical end of a chromatographic column which was filled with about 10 cm of petroleum ether. Ten grams of standardized florisil was added slowly into the column. The adsorbent was covered with about 1 cm of anhydrous sodium sulfate and the supernatant petroleum ether was drained to the top of sodium surface layer. Formation of bubbles in the column packing should be avoided.

The raw extract (fat content) in the round-bottom flask was dissolved with 1-2 ml petroleum ether. The redissolved fat extract was transferred into 5 ml volumetric flask and the round-bottom flask was washed 3 times with small portions of petroleum ether. The rinses were collected into the volumetric flask and filled up to 5 ml. A known volume of this solvent corresponding to less than 200 mg fat was pipetted to the top of column packing. The solution was allowed to percolate and was eluted with 120 ml petroleum ether : dichloromethane (4:1 v/v) at a rate of 5 ml/min. The eluate was collected into a 250 ml round bottom flask and was concentrated to about 3 ml. This solution was transferred to a pre-weighed V-shaped flask and concentrated again to about 1 ml. The last traces of solvent were removed with the aid of a gentle stream of air to dryness and the rest was redissolved with 1 ml iso-octane for final examination by GC. The V-shaped flask containing the concentrate was

weighed to determine the final volume through calculation using the density of the iso-octane of 0.70 g/ml. The flow diagram of the whole analytical procedure for organochlorine residue analysis is displayed in Figure 2.6.

2.4.6 Gas Chromatographic Determination

An aliquot of the final volume was transferred into a vial and injected onto the GC column with the following operating conditions:

Injection volume :	1μl
Injection port temperature:	250°C
Column temperature :	250°C
Detector temperature :	300°C
Carrier gas flow rate :	Helium gas at 2.4 ml/min
Analysis time :	26.7 min
Oven temperature program :	80°C (1 min) to 190°C at 30°C/min,
	to 280°C (1 min) at 6°C/min,
	to 300°C (5 min) at 20°C/min.

Egg-sample

Weigh about 3 g of mixed egg

↓

Homogenization

Mix with Na_2SO_4 anhydrous and surrogate (100ng each)

↓

Extraction

Cold extract with 60 ml CH_2Cl_2

*1st: stand for 20 min then elute with 30 ml CH_2Cl_2

*2nd: without waiting, elute with 30 ml CH_2Cl_2

Collect eluate into pre-weighed round bottom flask

↓

Fat determination

Rotary- evaporate the eluate at 35°C until dryness

re-weight round bottom flask => "fat content"

redissolve fat content in 5 ml petroleum ether

↓

Clean-up

Column chromatography: stationary phase : 10 g of florisil 3% H_2O

Mobile phase : 120 ml of petroleum ether : dichloromethane (4:1,v/v)

Take an aliquot of raw extract content less than 200 mg fat

↓

Enrichment

Rotary-evaporate the eluate at 65°C until dryness

fill up with 1 ml iso-octane- "final volume"

↓

GC/ECD

Figure 2.6 Flow diagram of the analytical procedure for organochlorine residue analysis.

2.4.7 Qualitative and Quantitative Analysis

Identification of organochlorine compounds in egg samples was done by comparison of the retention time of the suspected peak in the sample chromatogram to the retention time of the related standard in the standard chromatogram. When the correspondence was found within limits the identification was considered "possible". The limits could be checked by comparing the retention times of surrogate compounds in the sample and standard chromatograms. In cases of doubt the confirmation analysis would be done.

For organochlorine quantification, the external standard method was applied. The injection of appropriate concentration of standard solutions was repeated after every third injections of sample solution. Organochlorine residue contents in samples were calculated by the following equation:

$$\text{Residue Concentration based on fresh weight (mg/kg)} = (A_{fin} \times V_{fin} \times K) \div W_{ini} \quad (2.1)$$

Where

A_{fin} : concentration of residue found in the final solution (μg/ml)

V_{fin} : final volume (ml)

W_{ini} : initial sample weight (g)

K : dilution factor

K= (volume of petroleum ether used for dissolve fat) ÷ (volume of petroleum ether used for clean-up) .....(2.2)

2.4.8 Detection Limit and Percentage Recovery

The low concentration of organochlorine standard mixture was prepared and injected onto the GC column to determine the noise signal level and the peak of the lowest concentration.

The height of that noise signal level was measured and in this study the detection limit was estimated as two times of this value using the following equation.

Detection Limit = (2 × noise level × amount of component injected) ÷ (peak response) ..... (2.3)

To determine the percentage recovery, a known amount of four surrogate compounds (100 ng each) was spiked into each egg sample. The percentage recovery was reported as the average number of four percentage recoveries from four surrogate components spiked. The percentage recovery was calculated by the following equation :

$$\text{Recovery (\%)} = (A_{istd} \times V_{fin} \times K \times 100) \div A_{spi} \quad \text{.....(2.4)}$$

where

A_{istd}: concentration of surrogate component found in the final solution (μg/ml)

A_{spi} : amount of surrogate spiked in egg sample (μg)

V_{fin} : final volume (ml)

K : dilution factor (see equation 2.2)

2.5 Confirmation Technique

In case of suspense, the confirmation would be carried out by applying the following techniques: fractionation analysis on a silica column, sample treatment with concentrated sulfuric acid, and standard spiking.

For the organochlorines such as endrin, dieldrin , α-endosulfan and endosulfan sulfate which are destroyed easily by concentrated sulfuric acid, the "quick" confirmation found to be of practical use was to treat samples with concentrated H_2SO_4 and then re-injecting sample without sulfuric acid onto the GC system. The peaks represent for these pesticides would not appear in the subsequent

chromatograms while the peaks of other pesticides as HCB, HCHs, DDT and its derivatives were still stable.

Another confirmation was done by doing fraction analysis for both standard and sample solution on a silica column. Before using, silica gel was activated at 550°C for 2 hours and adjusted with 1.5%(w/w) water. One gram of silica was packed in a small glass column (10 ml pipette) and covered with 5 mm sodium sulfate. The silica column was conditioned by washing with 5 ml hexane. One ml solvent of standard or sample extract was transferred to the top of sodium sulfate and eluted with 8 ml hexane: toluene 65:35 (v/v) for the first fraction. The column was eluted with another 8 ml toluene which was collected in second V-shaped flask and this would be regarded as the second fraction. Both fractions were evaporated to a small volume , added with 5 ml iso-octane and were re-evaporated again to about 1 ml. These solvent were re-weighed for calculation of the final volume of iso-octane and then were injected onto the GC system. Among target organochlorine compounds in this study DDT and its derivatives, heptachlor, cis-heptachlor epoxide, BHC (all), HCB would be eluted in the first fraction while α-endosulfan, β-endosulfan, endosulfan sulfate, endrin, dieldrin would be eluted in the second fraction.

Besides, 15 egg samples were sent to Dr. Joachim Krueger of the University of Saarland, Germany for additional confirmation analysis.

2.6 Data Analysis

All the gathered data were analyzed using the statistical package SPSS version 6.0 for windows.