CHAPTER II
MATERIALS AND METHODS

2.1 Study sites

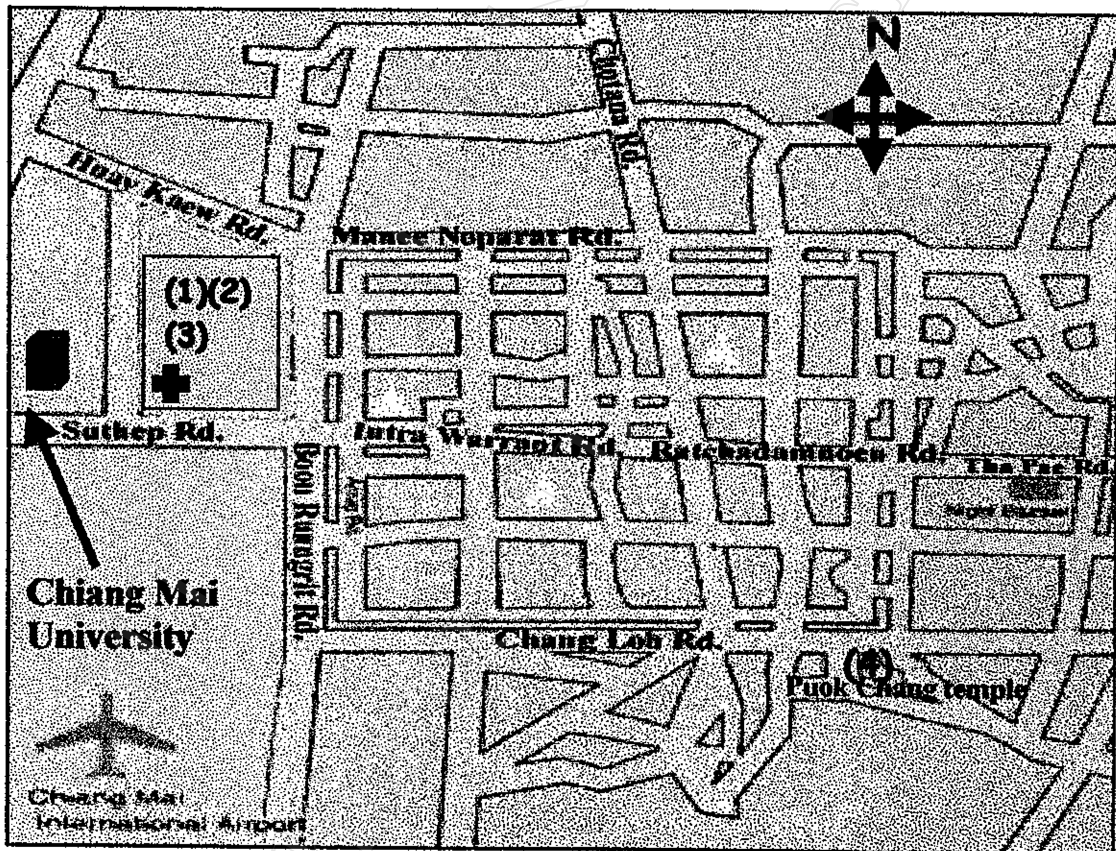


Figure 2.1 Sampling sites for particulate matters measurement in Chiang Mai city

Airborne particulate samples were collected from various sites in Chiang Mai area as describe in the map (Figure. 2.1). As in the map, there were four sites of sampling where;

Site (1) was in Bioassay laboratory in the Department of Biochemistry Faculty of Medicine Chiang Mai University. The building is mostly open to the outside air, but the laboratory room is air-conditioned with 24 hours operating of the air filter machine (Figure 2.2).

Site (2) was the secretary office which was also air-conditioned and located on the 2nd floor in the Department of Biochemistry Faculty of Medicine Chiang Mai University (Figure 2.3).

Site (3) was the terrace of the fifth floor of Multidisciplinary building at the Faculty of Medicine Chiang Mai University. The terrace is approximately 20 meters above the ground. This building is "sandwiched" located between three busy streets the closest of which is ~200 meters. These streets conduct a lot of traffic from 6 am to 9 pm. The sampling location is also approximately 4 km from downtown Chiang Mai (Figure 2.4).

Site (4) was Puok Chang school in down town Chiang Mai and the sampler was located one meter above ground level. This site is just beyond the lower eastern boarder of the old inner walled city of Chiang Mai and also " see much traffic between 6 am to 9 pm" (Figure 2.5).

Figure 2.1 Particulate matter measurement in the Bioassay laboratory in the Department of Biochemistry (site 1)

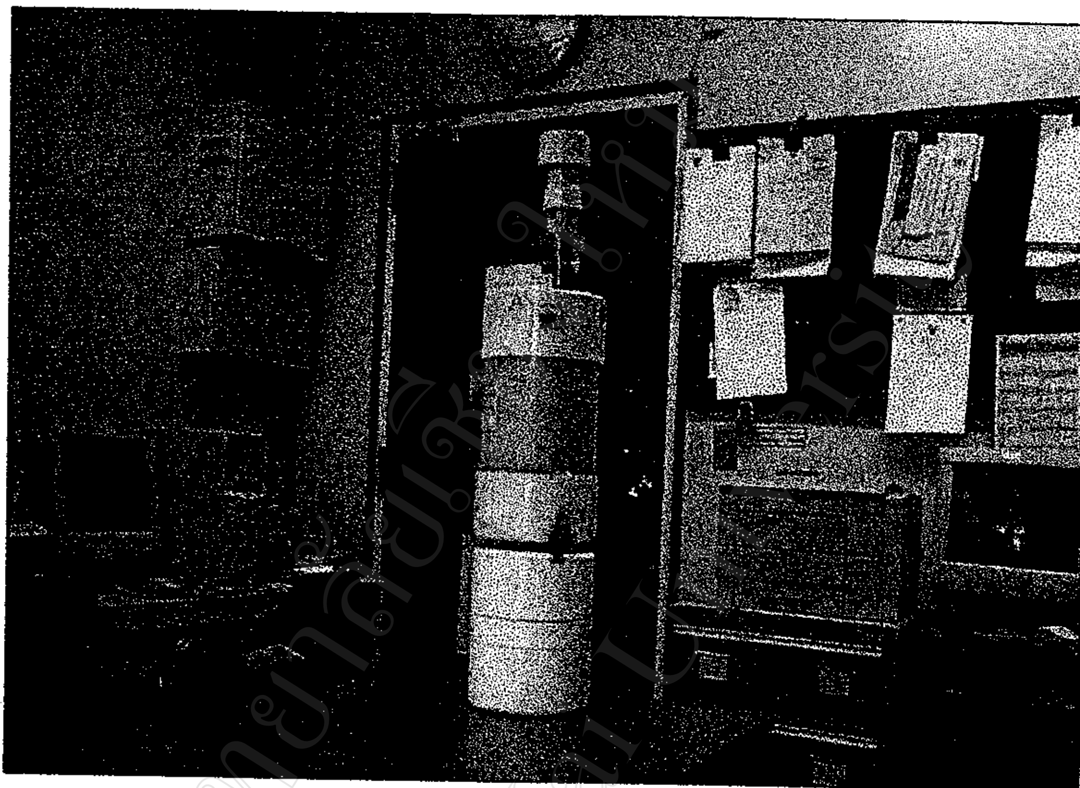


Figure 2.2 Particulate matter measurement in the secretary office of Biochemistry department (site 2)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่ Chiang Mai University

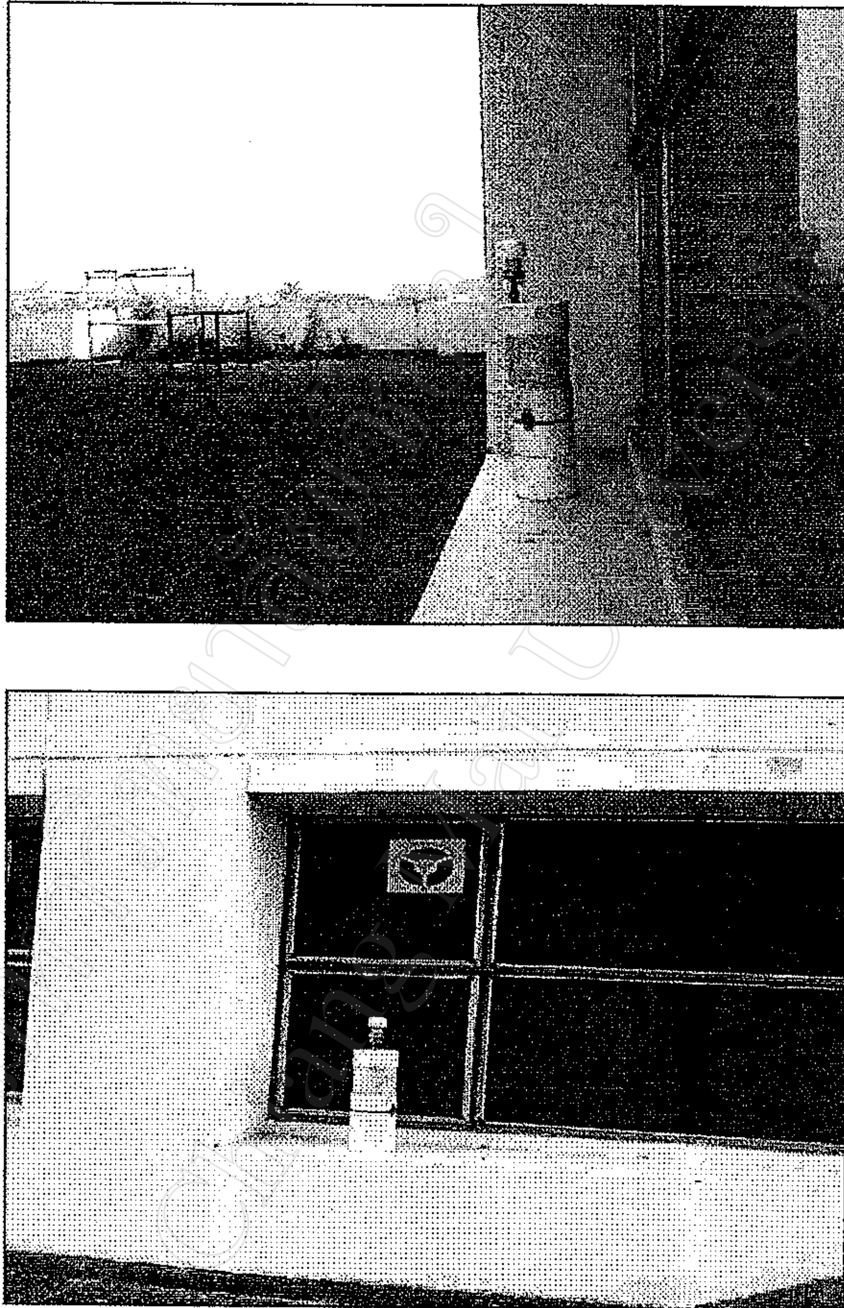


Figure 2.3 Particulate matter measurement at the terrace of the fifth floor of Multidiciplinary building, Faculty of Medicine (site 3)

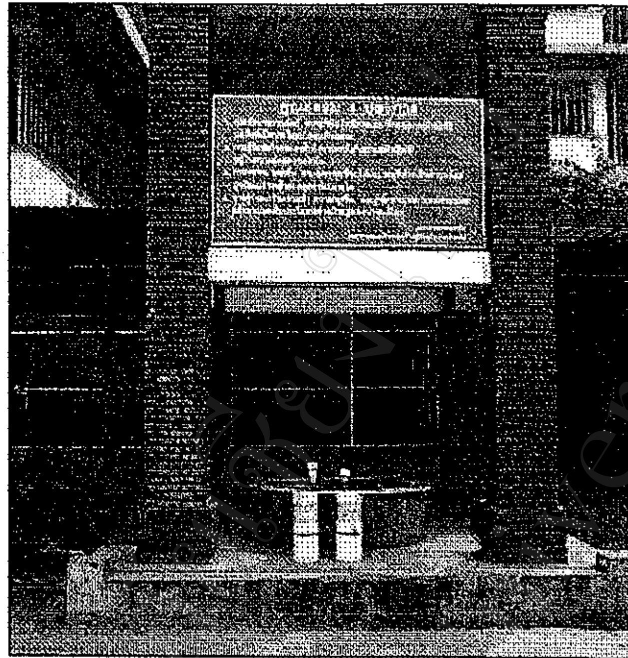


Figure 2.4 Particulate matter measurement at Puok Chang school (site 4)

Airborne particulate 24 hour samples were collected from May to October 1999 at various sites as described in Figure 2.1;

- PM 2.5 was collected from site 1
- PM 10 (50% aerodynamic cut size with diameter equal to or less than 10 µm) was collected from site 2 and from site 3.
- PM 10 and PM 2.5 were collected one meter above ground level at site 4.

In order to get both the day and night-time series distribution of PM 10 and PM 2.5, airborne particulate matter was sampled two times each day from November 2000 to March 2001; once over the period of 9.00-17.00 and at night (from 18.00 to 02.00). This was done at the following stations:

- PM 2.5 was collected from site 1, site 2, and site 4.
- PM 2.5 and PM 10 were collected from site 3.

2.2 Sample collection and PM levels measurement

Particle samples were collected on filters using portable battery operated samplers (MiniVol Portable Air Samplers, AIRmetrics 225, 5th street, Suite 501, Springfield Oregon, 97477,USA) for individual periods of 12 to approximately 23 hours. The samplers located in an undisturbed area in each sampling station, at least 30 cm from any obstacle to air flow. The samplers are cylindrical in shape with a diameter of 15 cm and a height of 74 cm. They weigh about 7 kg and use a flow rate of 5 l/min, are battery operated and can run unattended for 24 consecutive hours. Filters and batteries were changed in the field. Air first enters the sampler though an

impactor that was designed to pass particles either 10 µm and less (PM 10) or particles that are 2.5 µm or less with a 50% cut size as described by Reist (1993) . Immediately down stream of the impactor is filter holder.

Critical to the collection of the correct particle size is the correct flow rate through the impactor. For the MiniVol, the actual volumetric flow rate must be 5 liters per minute (5 lpm) at ambient conditions. To assure a constant 5 lpm flow rate through the size separator at differing air temperature and atmospheric pressure, the sampler must be adjusted for each sampling period and the pumps must be calibrated before sampling. A soap bubble flow meter was used to calibrate the flow and the temperature and pressure corrections applied as per the Airmetrics Minivol manual.

Filters were pre-weighted on a 6 place balance at ~30 °C (Sartorius AG, Goettingen, Germany). They were then re-weighed after being transported back to the lab each day in their filter holders. Particulate matter was collected on 47mm diameter, 0.45 µm pore size filters (Gelman Sciences, Ann Arbor, Michigan). The mass of particulate matter associated with these filters was determined on a 6 place gram balance and permitted the measurement of particle concentrations down to 5 g/µm^3. The precision of 8% (2 relative standard deviations) for six different samplers sampling the same out door air (avg. conc = 121 µg/m^3) for a 24 hour period. In another test, two side by side samplers agreed to within 3% when the outdoor PM 2.5 concentration was 160 µg/m^3 for a 24 hour period. Recent comparisons with the MiniVols as reported by AIRmetrics suggest a relative accuracy and pooled precision of 2 percent and 7 percent when compared to a sampler which complies with at the new USEPA Federal Register Method (FRM) for PM 2.5 particle samplers.

2.3 Sample extraction

In order to obtain enough extractable material from filter, one-month's samples were mixed together according to the sampling time, i.e. day-time, night-time, and 24-hours. This tends to compensate for the fluctuations in airbome particulate matter over the course of one-month's sampling. The filter was cut into small pieces and placed in 250 ml beaker.

The extraction method was modified from the standard USEPA method 3550 (USEPA,1996). Approximately 200 ml of dichloromethane was added to a beaker. The filters were cut into small pieces (~ 0.5 cm) and sonicated in an ultrasonic bath for 15 minutes. The extract was filtered through a Whatman No. 41 filter paper into a round bottom flask. Anhydrous sodium sulfate was used to remove water form the sample before filtering. The sample was sonicated another two times but with only ~100 ml of dichloromethane. All of the beakers and funnels were rinsed with dichloromethane after each subsequent filtration. The extract solvent was evaporated with a vacuum rotary evaporator at about 35 °C to dryness and weighed. The residue was resolved in DMSO to make the highest concentration for *Salmonella* mutation test. An approximate 0.1ml of the aliquot was used for the comet assay.

2.4 Preparation for *Salmonella* mutation test

The extract aliquot was passed through the Millipore filter membrane to get sterile solution used in *Salmonella* mutation test (Matshushima *et al.*, 1980). Extract was stored at 4 °C in refrigerator in dark prior to *Salmonella* mutation assay.

2.5 *Salmonella* mutation test

The assay was performed in the Bioassay laboratory. The bacteria tester strains *Salmonella typhimurium* TA100 was provided by Prof. Dr. Taijiro Matsushima, Director of Japan Bioassay Reaearch Center, Japan. The bacteria was stored at -80 °C and was cultured in Oxiod nutrient broth No.2 at 37 °C for 14 hours before using. The overnight culture contains about $2x10^9$ cell per ml.

The 0.05 ml of sample extract, 0.5 ml of S9 mix for direct acting or 0.5 ml of mixture of 0.2 M phosphate buffer (pH7.4) and water (50:50,v/v) for direct acting, and a overnight culture of tester stain were added into a sterilized capped culture tube. They were mixed and preincubated at 30 °C for 30 min in a shaking water bath. Then molten top agar at 45 °C, supplemented with 0.5 mM Histidine-Biotin, was added mixed by rotating the tube between the palms and poured onto a 30 ml Vogel-Bonner plate. The overlay agar was allowed to solidify in room temperature. The plates were kept in 37 °C incubator at dark for 48 hours within one hour. The revertant colonies per plate were counted and the toxic effect were examined under a stereomicroscope (Matsushima *et al.*,1980).

The extracts of samples were tested at one level for the one-month pooled samples which was 350 µg/plate for *Salmonella typhimurium* TA100 after preliminary test on the samples. In preliminary test, the TA100 showed high sensitivity. The 5 µg/plate of B(a)P used as positive control in the presence of S9 was done in every batch of test. The three plates were given for each dose in each experiment. All of the results were repeated to confirm and the average revertants in each different samples were calculated as the mean of six plates with standard deviation of mean.

2.6 The Comet assay

2.6.1 Peripheral blood Mononuclear Cell (PBMC) preparation from blood samples by Ficoll Hypaque gradient centrifugation technique.

PBMCs were prepared by mixing heparin blood sample in centrifuge tube with phosphate buffer saline (PBS) in a proportion 1:1 by volume, underlaying with Ficoll-Hypaque in propotin 1:2, then centrifuge at 1200 g for 30 minutes. The PBMC layer was separated and washed 3 times by centrifuging at 1200 g for 5 minutes with PBS. The last step was to adjust cell to the desired concentration in RPMI 1640.

2.6.2 Lymphocytes treatment

Lymphocytes (40,000 cells/cm^3) suspended in 500 µl medium (RPMI 1640) were treated *in vitro* in a CO_2 incubator for 3 hours at 37 ^{o}C with 10 µl of the extract aliquot after both in the presence and absence of metabolic activation system (50 µl S9mix). After incubation, cells were wash 3 time with PBS each for 5 minutes by centrifuging at 1200 g, then added 30 µl RPMI 1640.

2.6.3 Cell embedding and electrophoresis

Determination of DNA damage by single-cell microgel-electrophoresis was conducted according to the original method of McCarthy *et al.* (1997), with minor modifications. 10 µl cell suspensions were embedded in 90 µl of 0.75 % low-melting agarose and topped on to the 90 µl of 0.1% normal-

melting agarose which was layer on to the frosted part of microscope slide. To lyse cellular and nuclear membranes of the embedded cells and to permit DNA unfolding in alkaline conditions, the slides were immersed in ice-cold freshly prepared lysis solution for 1 hour, at 4 ^{o}C in the dark. The slides were then placed in electrophoresis alkaline buffer and the embedded cells were exposed to alkaline for 20 minutes to allow DNA unwinding.

Electrophoresis was performed in the same buffer (pH>13) for 20 minutes by applying an electric field of 17 Volt and adjusting the current to 300 mA. After electrophoresis, the slides were first washed gently three times, each for 5 minutes, with neutralization buffer (pH 7.5) to neutralize the alkaline, and the DNA was then stained by adding 25 µl of ethidium bromide (100 µg /ml). The slides were kept in a humidified sealed box at 4 ^{o}C to prevent drying of the gel and analysed within 48 hours (Villarini *et al.*,1998). All of the results were repeated another two times in each experiment to confirm and the average of tail length and head diameter was calculated as the mean of three slides with standard deviation of mean.

2.6.4 Image analysis

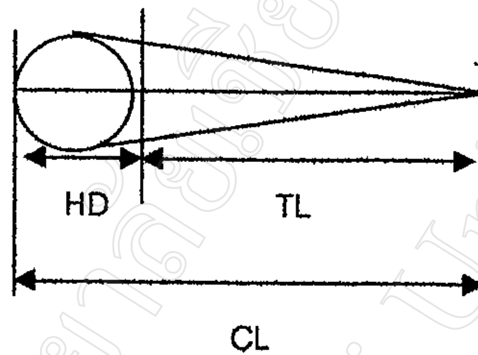
In each experiment, to evaluate the extent of DNA damage, images of 100 randomly selected cells were analysed from each sample using an fluorescent microscope equipped with an excitation filter of 515-560 nm and barrier filter of 590 nm. A 100x immersion objective was used with an ocular to project the comet cell image onto a high sensitivity camera. Imaging was performed using a special analysis system (McCarthy *et al.*,1997; Puaninta *et al.*, 2001). The DNA damage was considered by the tail length which was calculated by ;

$$TL = HD - CL$$

Where, TL = Tail length (μm)

HD = Head diameter (μm)

CL = Comet length (μm)



2.7 Data analysis

The data analysis from COMET assay was performed by using ANOVA and PM levels were calculated by spread sheet of Quattro – Pro for Window Version 6 (Kamens,1994).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University