CHAPTER 3

METERIALS AND METHODS

3.1 Instruments apparatus and chemicals

3.1.1. Instruments apparatus and chemicals for lichens study

3.1.1.1 Instruments

1. Compound microscope, Olympus CH-2, Japan
2. Stereo microscope, Olympus SZ-ST, Japan
3. Analytical balance, ADAM AFP-2100L
4. Oven, Scientific model 900, USA
5. Electrochemical analyser for pH meter,Consort C933
6. Global Positioning System; GPS, eTrex, Garmin, Taiwan
7. Untraviolet lamp, vilber Lourmat model VL-6.LC, France

3.1.1.2 Apparatus

1. Grid frame 20 x 50 cm^2
2. Hand lens 20x
3. Measuring tape length 100 cm
4. Compass
5. Pocketknife
6. Envelope for lichens and bark

7. Recording form
8. Writing board
9. Notebook pencil
10. Basket
11. Beaker, Scott Duran, Germany 50 mm
12. Cylinder, Scott Duran, German 10 mm
13. Test tube, Pyrex, USA 30 mm
14. Slide and Cover glass
15. Dropper
16. Small glass bottle
17. Ragging
18. Study survey map
19. Camera

3.1.1.3 Chemicals

1. Deionized water ; DI
2. Potassium hydroxide ; K 10%
3. Lugol's iodine
4. Sodium hypochlorite ; C

3.1.2 Instruments apparatus and chemicals for passive sampling technique

3.1.2.1 Instrument

1. Spectrophotometer, Jasco UV 530, Japan

3.1.2.2 Apparatus

1. Volumetric flask, Duran, Germany 100 and 500 mm
2. Volumetric flask, Vit Lab, Germany 10, 25 and 50 mm
3. Beaker, Scott Duran, Germany 50 and 100 mm
4. Micropipettes 10 – 100, 100 – 1000 µl and 1 - 10 ml
5. Syring 1 ml
6. Syringe filter C.A., 0.45 µm, 13 mm cellulose acetate, Chrom Tech, U.S.A
7. Polypropylene tubes; PP (Syring 10 ml)
8. Whatman (GF/A), Whatman international Ltd., England
9. Protective shelter
10. Paraflim
11. Plastic zip lock bag
12. Foam box
13. Ultrasonic bath, Transsonic Digital S, Elma, USA
14. Ultrasonic bath, model 8891, Cole- Parmar Instrument Co., USA
15. Oven, memmert 100- 800

3.1.2.2 Chemicals

1. Triethanolamine ; TEA; ($C_6H_{15}NO_3$, 149.19), 99%, BDH Chemicals Ltd., England
2. Sodium nitrite ; ($NaNO_2$, 69.00) , 97%, AJAX, Australia
3. N - (1- Napthyl) ethylenediamine dihydrochloride; ($C_{12}H_{16}C_{12}N_2$, 259.18), 90%, Merck, Germany

4. Sulfanilamide; ($C_6H_8N_2O_2S$, 172.21), 99% , Sigma- Aldrich, Germany
5. Phosphoric acid (H_3PO_4, 98.00) 85%, QRec, New Zealand
6. Deionized water ; DI

3.2 Description of investigation area

Chiang Mai province is Thailand's northern capital. It is located at latitude 18°47' North and longitude 98°59' East and about 1027 feet or 310 meters above sea level. Chiang Mai has the boundary with the other provinces and countries as in Figure 3.1. It is located about 720 kilometers north from Bangkok and its land cover 20,107.057 km^2 or 12,566,910 Rai. It can separated the area as the following;

Forest area	69.92 % (8,787,656 Rai)
Agriculture area	12.82 % (1,611,971 Rai)
The area lives and the other	17.26 % (2,167,971 Rai)

(Chiang Mai Province official site, 2009)

The city stand on the Ping River, a major tributary of the Chao Phraya River and among some of the highest mountains in the country. Mountain area; the majority stay in the north and the west of a province. This area was a source of river, forest hills which inappropriate to cultivating. Basin and the foothills plain; general spread between valleys which lie in north - south, for example, Ping River low land where had the plentifully fertile that appropriate for agriculture. Chiang Mai city itself was now Thailand's second largest city and was considered to be the capital of the whole northern region. Similar to other Thai provinces, Chiang Mai is administratively divided into Amphoes or districts, 22 in total as follows; Muang, Chiang Dao, Chom

Thong, Doi Saket, Doi Tao, Fang, Hang Dong, Hod, Mae Ai, Mae Chaem, Mae Rim, Mae Taeng, Omkoi, Phrao, Samoeng, San Kamphaeng, San Pa Tong, San Sai, Saraphi, Wiang Haeng, Chai Prakan and Mae Wang (Figure 3.1) (Chiang Mai Province official site, 2009).

This study was performed in urban and suburban areas in Chiang Mai province, which was a developed area and residential area, respectively. The location of the study area was approximately at 18° 45′ N to 18° 48′ N latitude and 98° 57′ E to 99° 01′ E longitude, with an average elevation about 300 m. The study area was located in Chiang Mai city (Figure 3.2). The land use within the study area was shown in Figure 3.3.

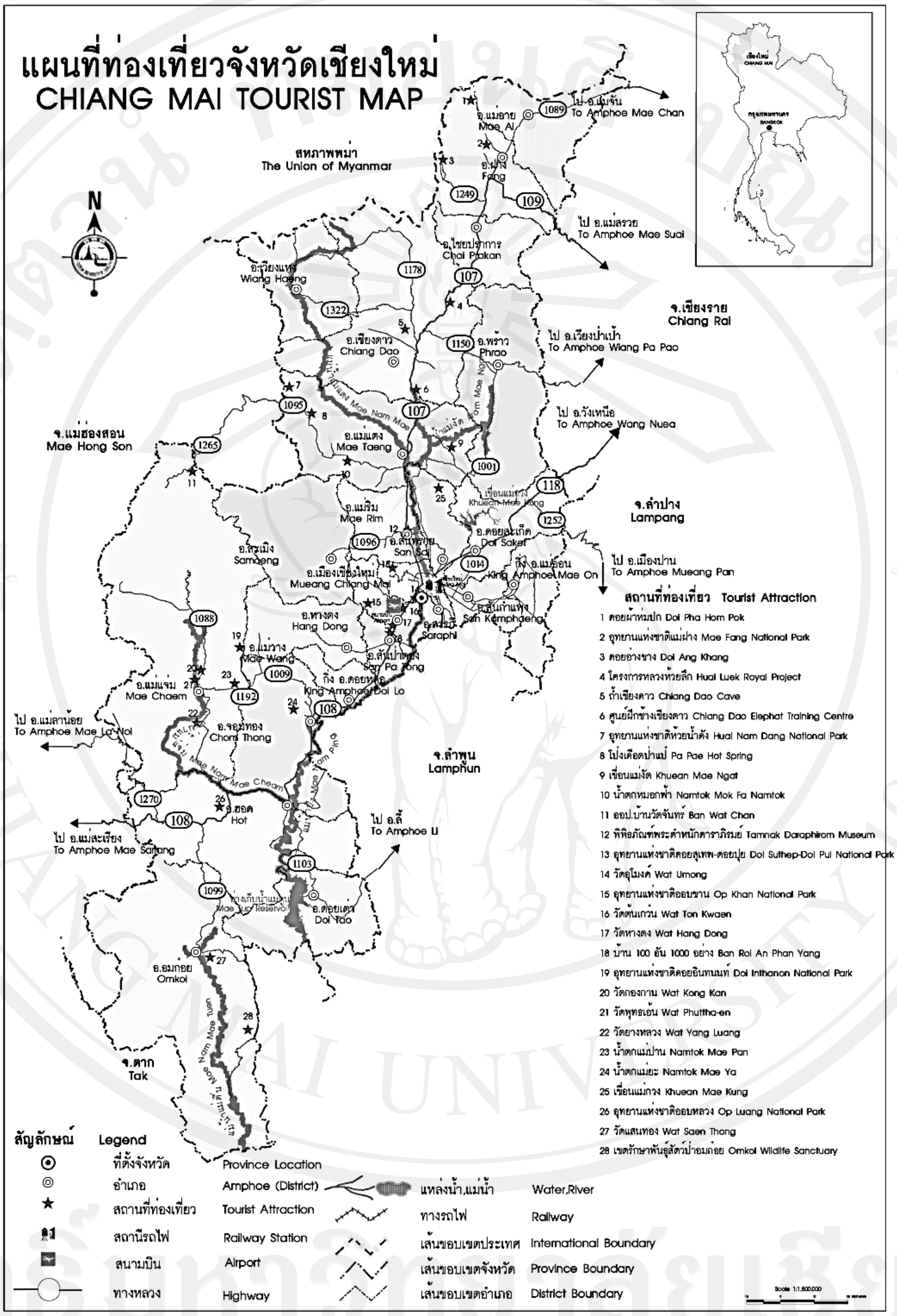

Figure 3.1 Chiang Mai map (Tourismthailand, 2011)

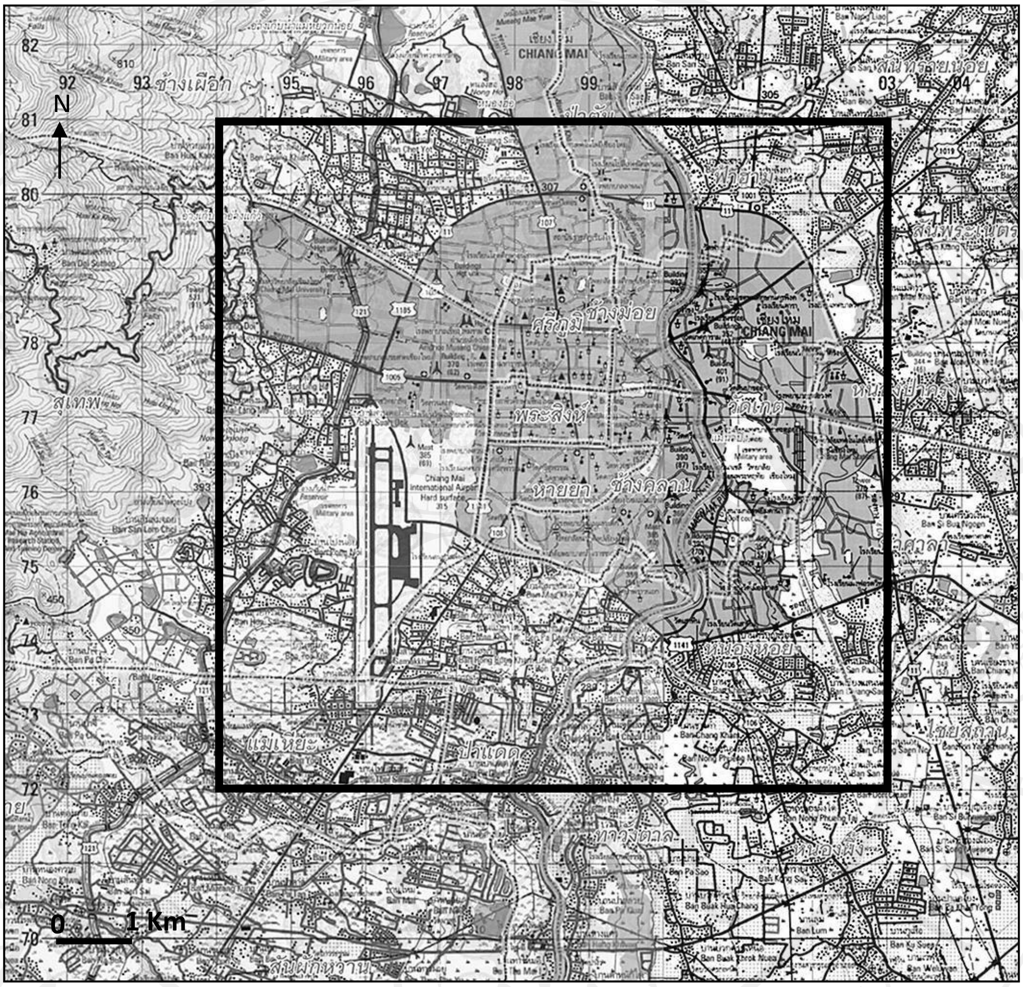

Figure 3.2 The study area (▬) in Chiang Mai (Chiang Mai Province Office,2012)

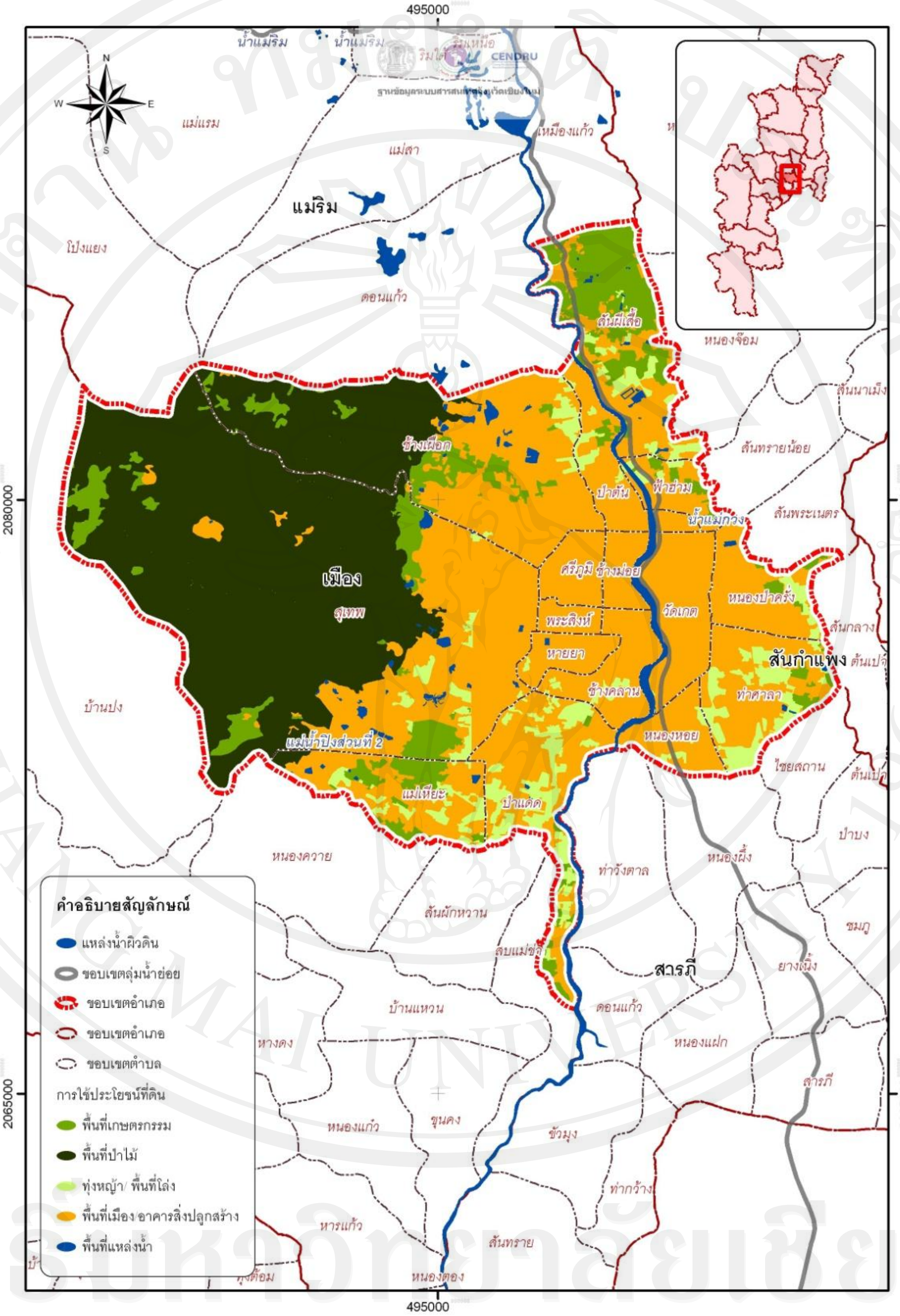

Figure 3.3 Land use types in Chiang Mai (Chiang Mai Province Office, 2012)

3.2.1 General climatic condition

The average of temperature in Chiang Mai was 25.4 °C per year. The average highest temperature was 31.8 °C and average lowest temperature was 20.1 °C. The average of humidity was 72% per year and the average of rain fall was 1,100-1,200 mm. Chiang Mai's climatology was under the effect of two monsoons; south-west's monsoon and north-east's monsoon. The monsoon in Chiang Mai began in May and ends in October, earlier than in Central Thailand. The rain generally fallen sporadically - except during August and September which the streets of Chiang Mai could sometimes flooded. (Chiang Mai Meteorological Station, 2011)

The climatic condition during March – June 2011 from Chiang Mai Meteorological Station, which the lichens sampling was collected, had the annual temperature, annual relative humidity, annual rain fall and wind direction of Chiang Mai were shown in Figure 3.4, Figure 3.5, Figure 3.6 and Figure 3.7, respectively.

The annual temperature during in 2011 was 22.75-27.79 °C (Figure 3.4).

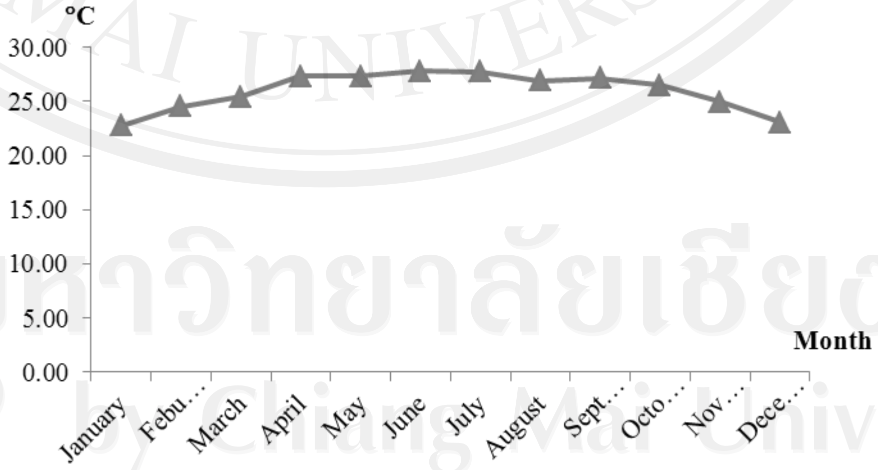

Figure 3.4 annual temperature of Chiang Mai in 2011

(Chiang Mai Meteorological Station, 2011)

The annual relative humidity during in 2011 was 58.6- 82.1%.

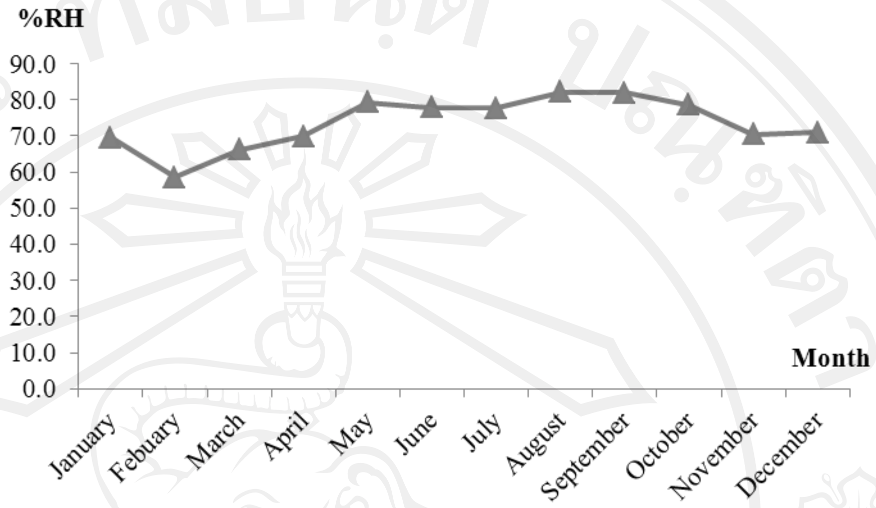

Figure 3.5 annual relative humidity of Chiang Mai in 2011

(Chiang Mai Meteorological Station, 2011)

The annual rain fall during January – June 2011 were 0.02- 9.44 mm

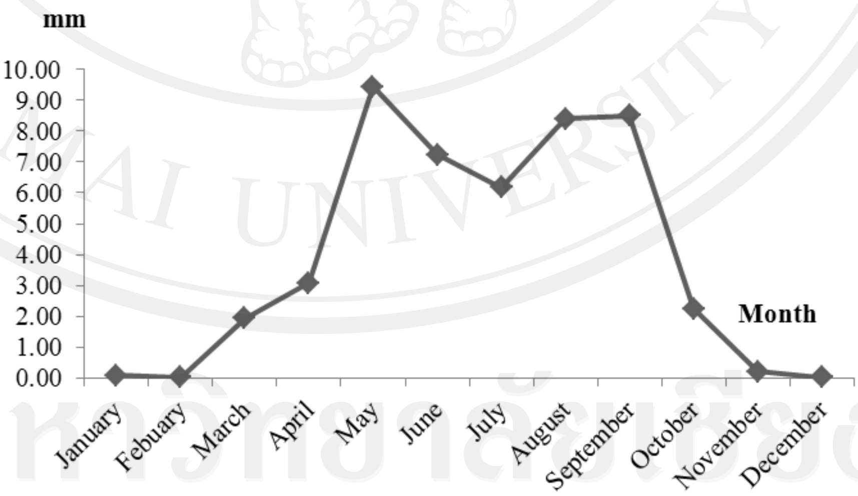

Figure 3.6 annual rain fall of Chiang Mai in 2011

(Chiang Mai Meteorological Station, 2011)

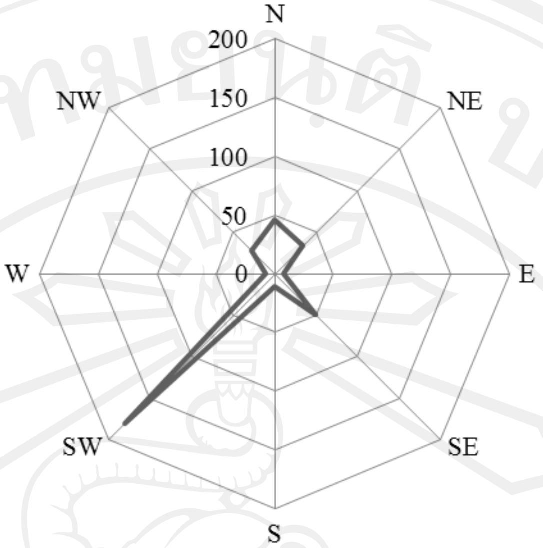

Figure 3.7 wind direction of Chiang Mai in 2011

(Chiang Mai Meteorological Station, 2011)

3.3 Lichen mapping for assessment of air quality in Chiang Mai City

3.3.1 Sampling procedure

The method for mapping lichen diversity was slightly modified from the VDI method (VDI, 1995). The study sites in urban and suburban areas of Chiang Mai city was divided into grid-squares size of 0.5 x 0.5 km^2 and 1.0 x 1.0 km^2 , the center of the city and its surrounding, respectively. The small squares will produce better results therefore a grid square of 0.5 km side-length was selected in the center of the city (urban area) where was supposed to high pollution. On the contrary, a grid square of 1 km side-length was selected outside of the center of the city (suburban area) where was supposed to has less pollution. The squares were then numbered. The squares coded with “A” were located in urban areas, while “B” were located in suburban areas (Figure 3.8). The total number of investigated grid square was 73 squares.

Six mango trees (*Mangifera indica* L.) or bullet wood trees, Pikul in Thai named, (*Mimusops elengi* L.**)** was chosen as a suitable tree in each grid square for lichen frequency investigation. Mango trees were the main investigation trees in this study but in study area that had no mango trees, bullet wood trees were substituted for mango trees. Because bark pH testing found that had no significant between mango bark pH and bullet wood bark pH (Appendix A). The blue point represented of mango tree and the red one is represented of bullet wood tree (Figure 3.9).

Species and frequency of lichens on the tree trunk were registered by using a grid-frame size of 20 x 50 cm^2 which was divided to 10 subsquares, size of 10 x 10 cm^2. The part of the tree trunk where was most densely covered with lichens was selected. A rope was used for fixing the grid frame onto the tree trunk at 1m above the ground (Figure 3.10). The inclination should not exceed 10° degree from the vertical. Tree that were injured by fungicides, liming, grazing animals and human activities were not selected (VDI, 1995). Other environmental factors, for examples; trees diameter, type of bark, pH bark, the orientations where the grid frames place on the trunk were recorded in recording form (Appendix G).

All lichens inside the grid frame, expect specimens whose diameters were smaller than 3 mm, were registered. Specimens which were smaller than 3 mm were not registered in order to avoid misidentification. Frequency of each lichen species were recorded, frequency being the number of small squares wherein each species occurred. Frequency of species which were not found inside the grid frame but occurred in the immediate vicinity were recorded as one.

Only well-known and easily recognized lichens species were determined in the field but for those that were not identified, chip of bark containing the lichens were removed from the trunk by a pocketknife. They were identified later in the laboratory using lichen identification keys such as Wolseley and Aguirre-Hudson (1997) and Sipman, (2003).

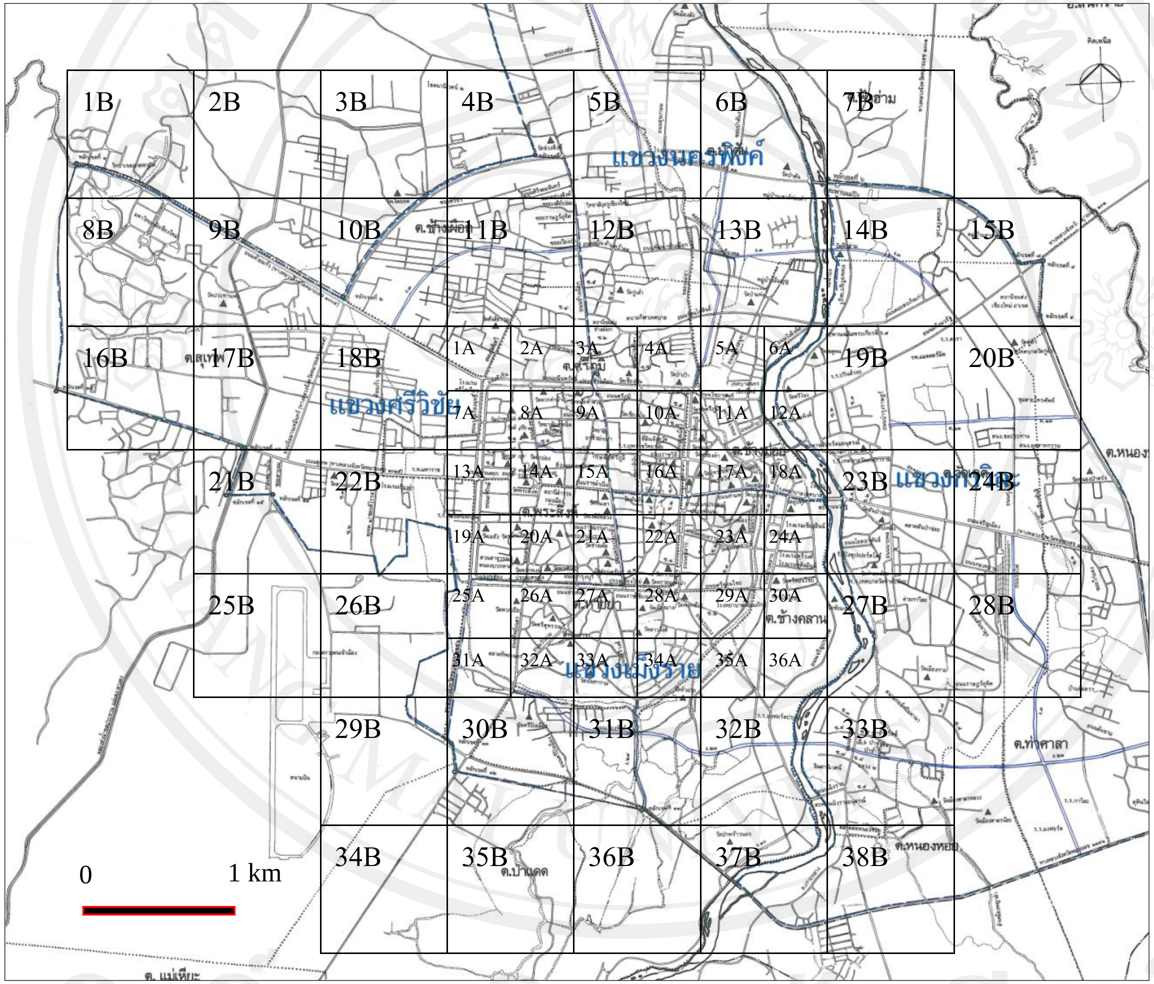

Figure 3.8 Investigated grid square in Chiang Mai (Map from Chiang Mai Municipal)

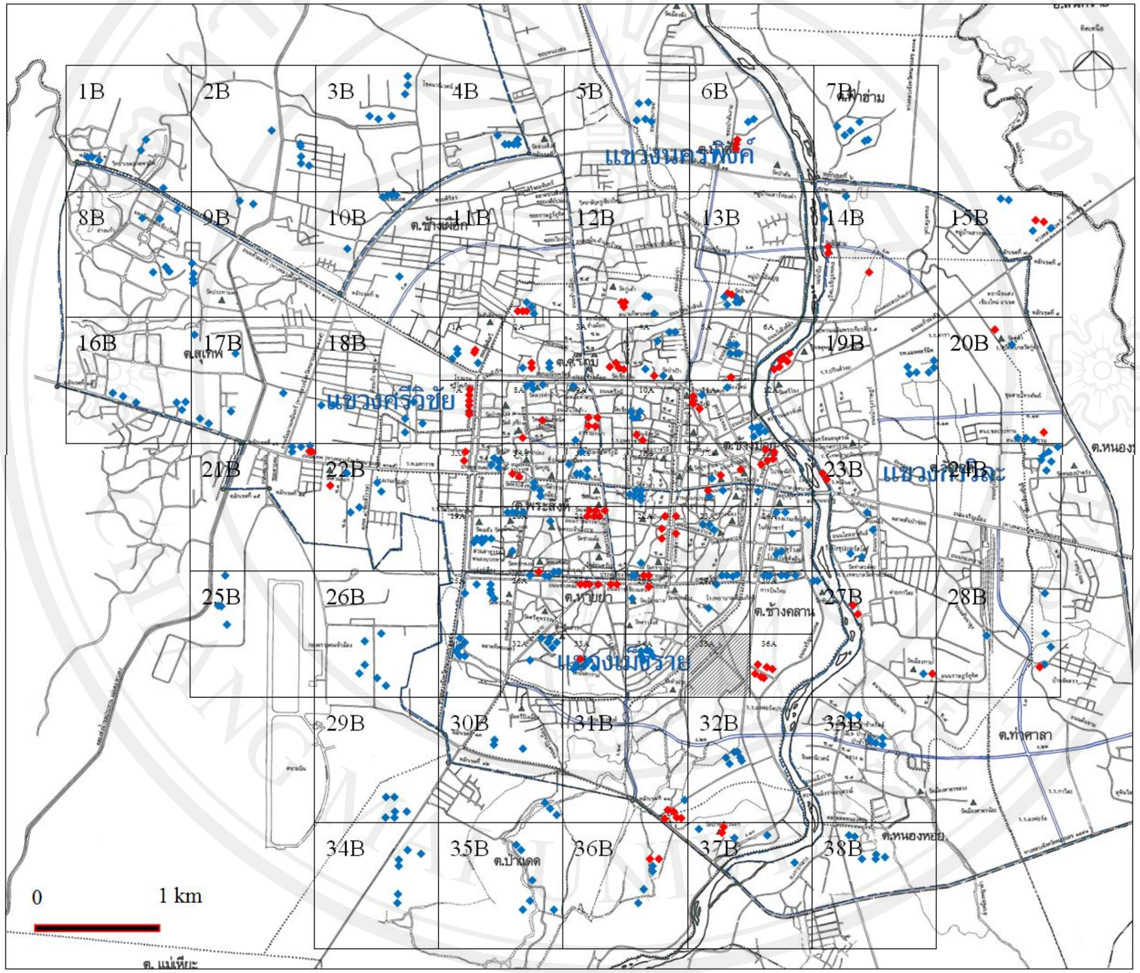

Figure 3.9 The point of selected trees in each grid square in Chiang Mai; blue point = mango tree, red point = bullet wood tree (Map from Chiang Mai Municiple)

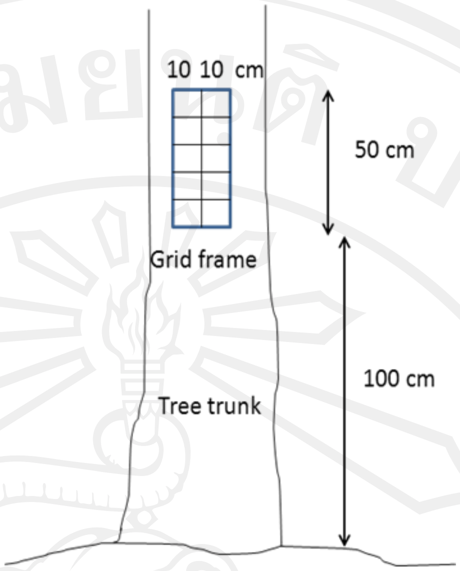

Figure 3.10 Grid frame on the tree trunk

3.3.2 Calculation of the air quality index

After all lichens species were determined, the sum of frequencies of lichen species on the investigated trees within the grid squares was calculated to air quality index. For the calculation, the following formulas were used;

Mean sum of frequencies in the grid square; (Air quality Index: AQI)

$$AQI_j = \frac{\sum \mathrm{F_{ij}}}{\mathrm{n_j}}$$

Standard deviation

$$S_j = \sqrt{\frac{\Sigma(F_{ij} - AQI_j)^2}{n_j - 1}}$$

The accuracy of the estimation depended on the standard deviation of the results and was best described by the confidence limits (in this case 95% were recommended). These were calculated by the following formulas;

Lower, upper confidence limits (L_{1j}, L_{2j})

$$L_{1j} + L_{2j} = AQI_j \pm t_j \cdot \frac{S_j}{\sqrt{n_j}}$$

Abbreviations in the formulas;

i =	The number of the individual trees on grid squares of the investigation area
j =	The number of the grid squares of the study area
F_{ij} =	The sum of the frequencies of lichens on trees no. I in the examined unit j
n_j =	The number of surveyed trees within the examined unit j
S_j =	The standard deviation of the examined unit j
L_{1j}, L_{2j} =	The lower and upper confidence limits of the AQI_j, the confidence interval is $L_{2j} – L_{1j}$
t_j =	Critical value of the Student Distribution for n_{j-1} degree of freedom

3.3.3 Registration of air quality classes (AQC)

The air quality values (AQI) were classified into air quality classes. The width of classes was calculated from the mean standard deviation of all grid squares of the whole investigation area. The following formulas were used to calculate;

Mean standard deviation of the investigation

$$S_p = \sqrt{\frac{\sum_j \sum_i (F_{ij} - AQI_j)^2}{m(n_p - 1)}}$$

Width of the air quality classes

$$t_p = \frac{S_p}{\sqrt{n_p}}$$

Abbreviations in the formulas;

S_p	mean standard deviation of the grid squares of the whole investigation area
n_p	mean number of sample trees per grid square of the whole investigation area
m	number of the investigated grid squares in the whole investigation area
t_p	Critical value of the Student Distribution for n_p – 1 degrees of

freedom

The air quality values of the individual grid squares j were classify to the air quality classes according to following convention was applied;

Table 3.1 The convention for classification of the air quality classes.

Lower class limit		Upper class limit
0	< AQI ≤	1 x class width
1 x class width	< AQI ≤	2 x class width

3.3.4 Evaluation and representation of the air quality classes

An exposure scale was used for evaluation whose threshold limits were defined by air quality values of 0.0 – 12.5 – 25.0 – 37.5 – 50.0 (VDI, 1995) (Figure 3.11). A suitable evaluation and a color scale which were used for the mapping represent of the result were combined with the impact classes defined before. The air quality classes were put parallel to the exposure scale and accordingly receive the best-fitting color and suitable evaluation.

If the air quality classes and the impact classes overlapped, the evaluations were chosen and the colors in overlapped classes were mixed. One air quality class could distributed over two impact classes. In the case that air quality classes valued as one single impact class, the different air quality classes had to be separated by black hatching.

The result of this study was presented by colored grid squares in accordance with air quality classes. Then, isolines were drawn in order to show more details of the situation of the air quality in the investigation area.

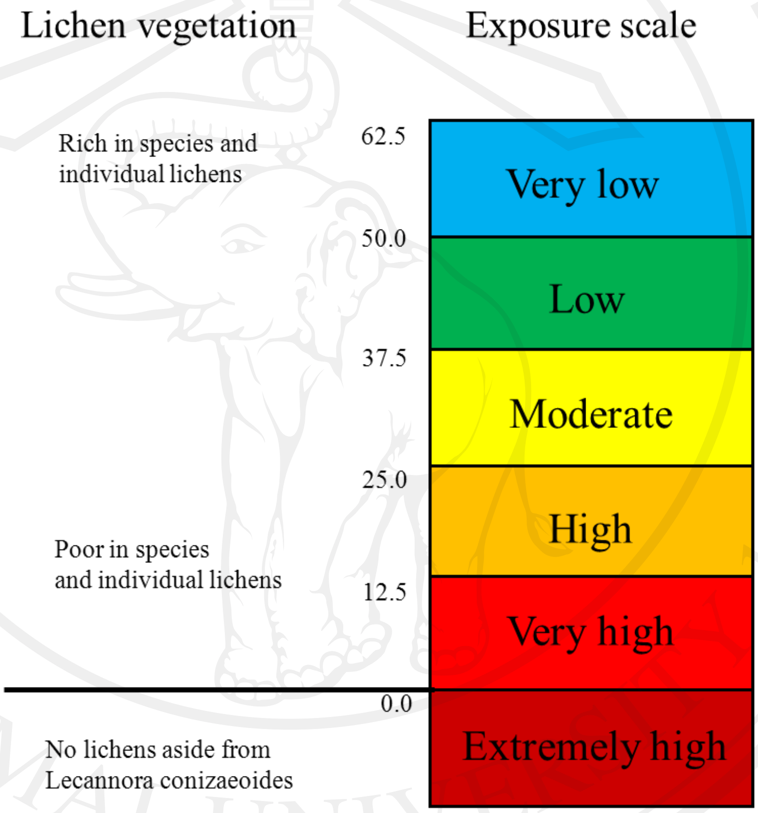

Figure 3.11 The standard exposure scale (VDI, 1995)

To draw the isolines in mapping, first the air quality values were entered to the center of gravity of each grid square. All directly neighboring air quality values were connected to each other by connecting lines. Then between the centers of each pair of squares, the crossing points between the isolines and the connecting lines were calculated and marked on the connecting lines accordingly. The positions of the crossing points were calculated from the following formulas;

$$x = \frac{A}{B_1 - B_2}$$

and

$$D = (C_1 - C_2) \cdot X$$

Abbreviations in the formulas;

A = Distance between the center of gravity of both squares in cm

B_1 = Air quality value of the square No. i

B_2 = Air quality value of the neighboring squares of the square No. i

X = 1 unit of air quality value in cm on the connecting line between the center of gravity of both squares

C_1 = Value of an isoline

C_2 = Air quality value of the square No. i

D = Position of the connecting point measured from the center of gravity of square No. I (distance in cm)

The crossing point was always calculated from the air quality values of the neighboring closest squares. The points which belong to the same border of one class were connected, forming a line. The number of isolines corresponded with the number of the air quality classes of investigation area.

3.4 Analysis of bark pH

Pieces of bark 2 – 3 mm thick without lichens were removed selected tree trunk at 150 cm above the ground by using a pocketknife. Chips of bark were collected in a bark's envelopes. The bark samples were dried at 80 °C for 48 hours and then pounded down. Samples of 2 g of bark were soaked with 10 ml distilled water. After 24 hours, pH was determined directly in the solution by pH meter. (van Herk, 2001)

3.5 Passive sampling

This method was developed by Khaodee (2006) and Bootdee *et al.* (2012).

3.5.1 Description of sampling sites

To determine the NO_2 concentration of each air pollution zone in the investigation area, the sampling sites for installing the diffusion tubes were decided after the air quality map was created. The locations of sampling sites were presented in Chapter 4.

3.5.2 Climatic condition during the exposure period

The daily temperature, relative humidity, rain fall and wind direction during the exposure period, January 21- 28, February 17- 24, March 13- 20 and April were shown in Figure 3.12, Figure 3.13, Figure 3.14 and Figure 3.15 respectively (Chiang Mai Meteorological Station, 2012)

The temperature during January to April in 2012 was 23.09-29.42 °C.

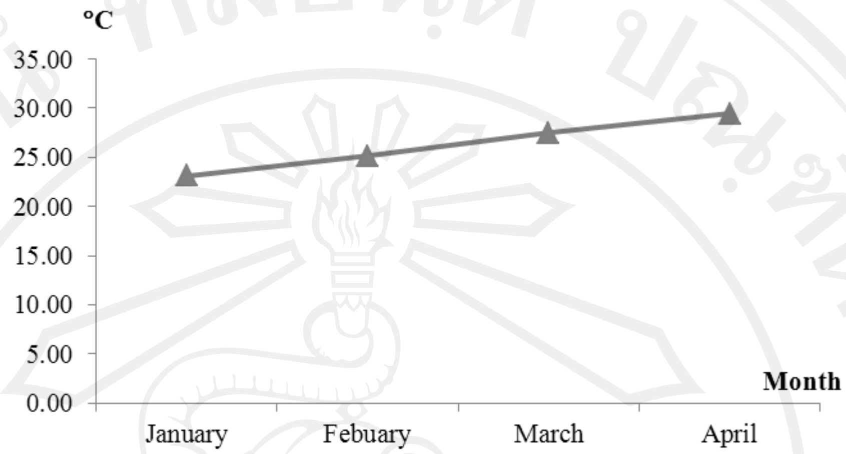

Figure 3.12 Daily temperatures during the exposure period (Chiang Mai Meteorological Station, 2012)

The relative humidity during January to April in 2012 was 55.0- 66.3%.

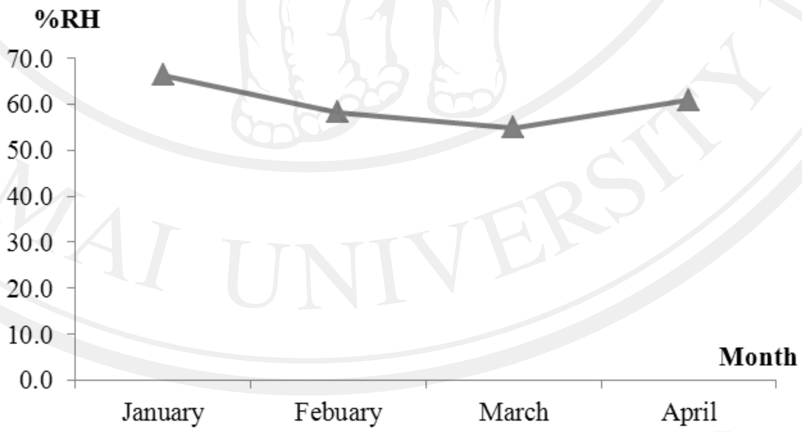

Figure 3.13 Relative humidity during the exposure period

(Chiang Mai Meteorological Station, 2012)

The rain fall during January to April in 2012 was 0.00- 6.98 mm.

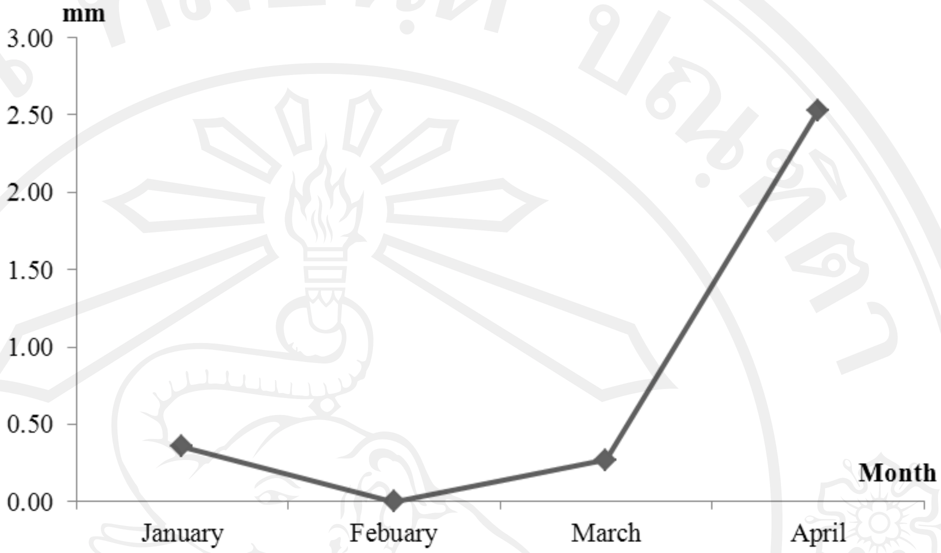

Figure 3.14 Rain fall during the exposure period

(Chiang Mai Meteorological Station, 2012)

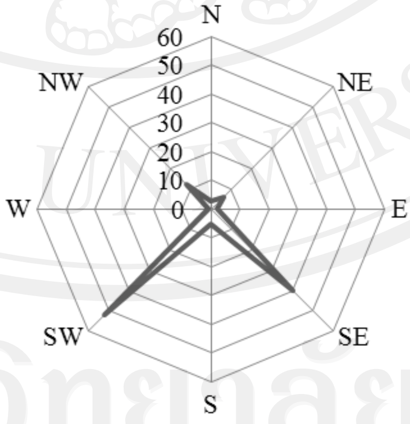

Figure 3.15 Wind directions during the exposure period

(Chiang Mai Meteorological Station, 2012)

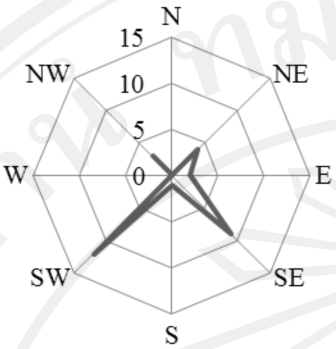

Figure 3.15a- January

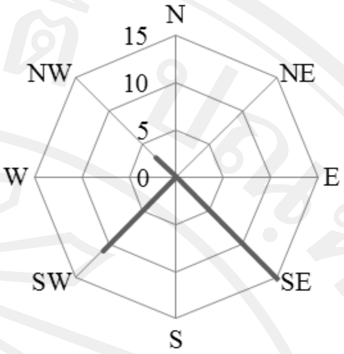

Figure 3.15b- February

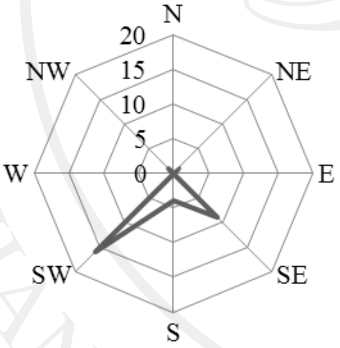

Figure 3.15c- March

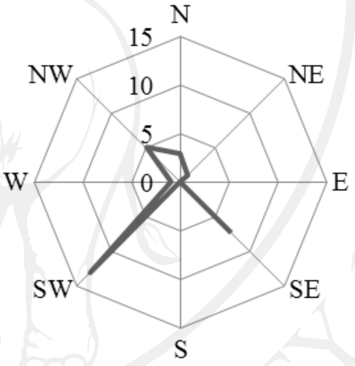

Figure 3.15d- April

Figure 3.15a- d Wind directions in each month during the exposure period (Chiang Mai Meteorological Station, 2012)

3.5.3 Preparation of solutions for NO_2 sampling and analysis (Bootdee, 2009)

Preparation of absorbing solution (20% v/v of Triethanolamine, TEA), sulfanilamide solution, N- (1Napthyl) ethylenediamine dihydrochloride (NEDA) solution, saltzmann reagent and nitrite strand stock solution (1,000 mg/L) see Appendix D

3.5.4 Analytical characteristics (Bootdee, 2009)

Analytical characteristics of linear dynamic range, calibration curve, limit of detection (LOD), limit of quantitation (LOQ), repeatability and reproducibility see Appendix D

3.5.5 Preparation of diffusion tube

Polypropylene (PP) tubes with a length of 7.7 cm and an internal diameter of 1.6 cm were cleaned and sonicated for one hour, then rinsed with deionized water. Filter paper, Whatman GF/A, was cut in a circle with the diameter being equal to inner diameter of the tube. The filter paper was sonicated for 30 minutes for clean and rinsed twice with distilled water before drying at 60 °C for 24 hour. After that, the filter paper was placed inside the bottom of the tubes (Khaodee, 2006; Pomphueak, 2005; Bootdee, 2009).

3.5.6 Exposure of diffusion tube

On the exposure day, 50 µl of 20% TEA in deionized water was directly added onto the Whatman GF/A filter paper (Pomphueak, 2005; Bootdee, 2009). The diffusion tubes were vertically fixed, with the open end facing upward, inside the shelter to protect them from meteorological effect i.e. wind, sunlight and rain. Configuration of the passive samplers is illustrated in Figure 3.16. The protective shelter containing five replications of samplers and three blank of tube was hung at 1.5 – 2.0 m above ground level in Figure 3.17. The samplers were exposure for 7 days.

After 7 days of exposure, the tubes were collected, closed with caps immediately, then sealed with parafilm. They were placed in plastic zip lock bags, transferred to the laboratory and stored in a refrigerator until the time of analysis. The exact times of installation and collection were noted for calculation of the NO_2 amount.

In the field, the diffusion tubes were prepared using the same procedure. Three replications of sampler and one blank tube were fixed inside the protective shelter at the field and the caps of the sampling tube were removed.

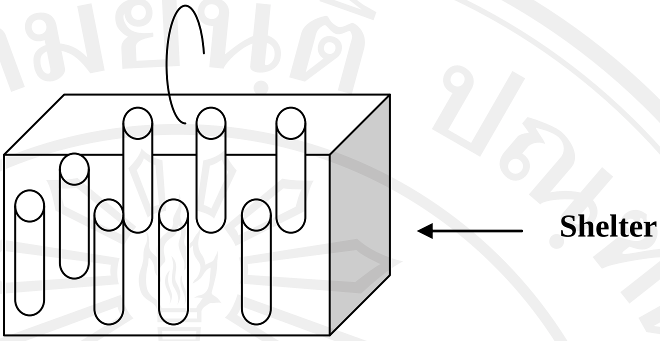

Figure 3.16 The configuration of passive samplers

Figure 3.17 The protective shelter was hung at 1.5 – 2.0 m above ground level

3.5.7 Extraction of sample

After the sampling was completed, 2 ml of deionized water were added directly into the sampling tube. The tube was capped and shook for 15 minutes. The sample solution was then filtered through 0.45 µm cellulose acetate membrane by the help of syringe.

3.5.8 Analysis of sample

One ml of the extracted sample solution was mixed with 2 ml of Saltzman reagent in a test tube and stood for 10 minutes until color development was completed. After that, the solution was measured for its absorbance by spectrophotometry at 540 nm by using reagent as blank.

The absorbance values were calculated by the equation called Fick's law. According to Fick's law, the concentration of gases in passive sampler in µg/m^3 unit is calculated as follow:

$$C = [\frac{Q \times L}{A \times t \times D}] \qquad \text{………3.5.8-1}$$

Where:

C = concentration measured by passive sampling tube (µg/m^3)

Q = quantity of absorption products present in the sampler (µg)

L = diffusion length (m)

A = cross- sectional area (m^2) = ¶r^2

t = sampling times (s)

D = diffusion coefficient (m^2/s)

The diffusion coefficient of nitrogen dioxide in air must be corrected to average ambient temperature (k) during sampling period, according to equation:

$$D_T = D_{298}\ (T)^{1.75} \times (298.15)^{-1.75}$$

Where:

D_T = diffusion coefficient (cm^2s^{-1}) of NO_2 through air at T (K)

D_{298} = the diffusion coefficient of NO_2 through air at 298.15 K (0.154 cm^2s^{-1})

T = temperature (k)

Quantity of absorption products in the sampler (Q)

Q value of NO_2 determination was calculated by multiplication NO_2^- concentration obtained from calibration curve (ppm) with 2 (2 ml of extraction volume). The NO_2^- in ppm unit was converted to the total amount of NO_2 in passive sampler in μg unit.

$$Q\ (\mu g) = NO_2^-\ \text{concentration (ppm)} \times 2\ \text{ml}$$