# CHAPTER 2

# MATERAILS AND METHODS

## 2.1 Laboratory supplies

**2.1.1** Glass tube with Teflon lined screw caps 16x26 mm. Polypropylene tubes, NIPRO (Thailand) corp., Ltd., Thailand.

**2.1.2** Filter papers, Whatman International Ltd., England**.**

**2.1.3** SKC Filter, Quartz, 1.2 µm pore size, 37 mm, Cat. No.225-1827, SKC Inc, USA.

**2.1.4** 20 mL head space vial, Sigma Aldrich, USA.

## 2.2 Chemicals

### 2.2.1 Chemical for determination of levoglucosan and 2-methoxyphenol

1) Levoglucosan (1,6-anhydro-β-D-glucopyranose,98%), cat. No.498-07-7, Sigma Aldrich, USA.
2) Methyl β-D-xylopyranoside (98%), cat. No.612-05-5, Sigma Aldrich, USA.
3) 2-methoxyphenol (98%), cat. No.90-05-1, Sigma Aldrich, USA.
4) 2-chloro-4-methoxyphenol (98%), cat. No.18113- 03-6, Sigma Aldrich, USA**.**
5) N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 98%)**,** Cat.No.2556-30-2, Sigma Aldrich, USA**.**

6) N-trimethylsilylimidazole (TMSI, 97%), Cat. No.18156-74-6, Sigma Aldrich, USA.

7) Pyridine (99%), cat. No.110-86-1, BDH Laboratory, England.

8) N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, 98%), cat. No.24589-78-4, Sigma Aldrich, USA.

**2.2.2 Organic solvents**

1) Ethyl acetate (100%), J.T. Baker, Germany.

2) Methanol (100%), J.T. Baker, Germany.

3) Acetone (100%), J.T. Baker, Germany.

4) n-Hexane (100%), J.T. Baker, Germany.

**2.2.3 Gases for GC system**

1) Helium gas (HP, 99.99%), TIG, Thailand.

2) Nitrogen gas (HP, 99.99%), TIG, Thailand.

3) Nitrogen gas (UHP, 99.99%), TIG, Thailand.

4) Air gas (HP, 99.99%), TIG, Thailand.

5) Hydrogen gas (HP, 99.99%), TIG, Thailand.

**2.3 Apparatus**

**2.3.1** Gas chromatograph (Model.6890, Agilent) equipped with flame Ionization detector (FID), the separation was accomplished by capillary column (HP-5, 5% phenylmethylpolysiloxane with 30 m×0.25 mm,0.25 µm film thickness), and computerized data handling system (Version.A.10.02 series Chemstation).

**2.3.2** Gas chromatograph (Model.7890, Agilent) equipped with mass spectrometer detector (Model.5975C, Agilent Technology), the separation was accomplished by capillary column (HP-5MS, 5% PhenylmethylsSiloxane with 30 m ×

0.25 mm, 0.25 μm film thickness, Alltech, USA), and computerized data handling system (Version.E.02.02 series Chemstation).

**2.3.3** Rotary evaporator, R-210, Buchi Labortechnik AG, Swizerlan, consisting

a) Water bath, B-480

b) Air pump, KNF Laboport

c) Cooling device, NESLAB, USA.

**2.3.4** Microbalance, 5 decimal places of gram, Mettler Toledo, AB135-S/FACT, Switzerland.

**2.3.5** Ultrasonicator, ELMA Sonic S30H, Elma, Germany.

**2.3.6** Oven, Model.UNE-400, Memmert, Germany.

**2.3.7** Mixer, UZUSIO, VTX-3000L, Tokyo, Japan.

**2.3.8** Personal air sampler, SKC Inc, USA.

## 2.4 $PM_{10}$ sample collection

### 2.4.1 Study site

The study was conducted in Khun Chang Khian (KCK) village, Chang Puak subdistrict, Muang district, Chiang Mai province. KCK village is surrounded by forests and topographic features with altitudes of 1,300 m above mean sea level. The villagers mostly depend on a variety of biomass fuels for their domestic cooking and heating. The common biomass fuel includes wood, dry leaves, plant twigs. In KCK villages the most used biomass fuel is Lychee wood. Typical style of their homes are made of simple wood walls, dirt floors and tile or metal roofs and the residential in this village are generally single roomed, poorly ventilated, and shared for both living and cooking. Characteristics of the house with single layer and wooden board for

prevent cold air at night with a fire in the fire place to cook food and keep warm at night.

The Hmong uses green bamboo wood to make the walls, splitting and tying it together, while the roof is made from Imperata Cylindrica or Nipa palm. Houses do not have windows because they live, usually, in cold weather areas. There is a main door close to the stove, and seating for the visitor. The stove is situated on the left and used to make food for visitors, and also for boiling food for the pigs. Some houses have a mortar for pounding rice, or a millstone for grinding corn, flour, and soybeans. Further in the house, again on the left hand side, there is normally a bedroom for the members of the family.

**Figure.2.1** Characteristics of the house and cooking stove in KCK village

### 2.4.2 A survey of study household

Almost all of the households in present study relied entirely on the traditional "open fires'' on the floor as cooking kitchen and cooking twice a day (early morning and evening). Inclusion criteria of the households for the present study are the houses which cooking by wood burning inside their houses. Fourteen houses with open wood cooking inside their houses were enrolled as studied houses. Due to very few households using liquid petroleum gas (LPG) for cooking, only one house was

enrolled as a control. Cooking time for both studied and control houses are about 5 - 6 am and 6- 7 pm in each day. The position for studies and control houses as in Figure.2.2

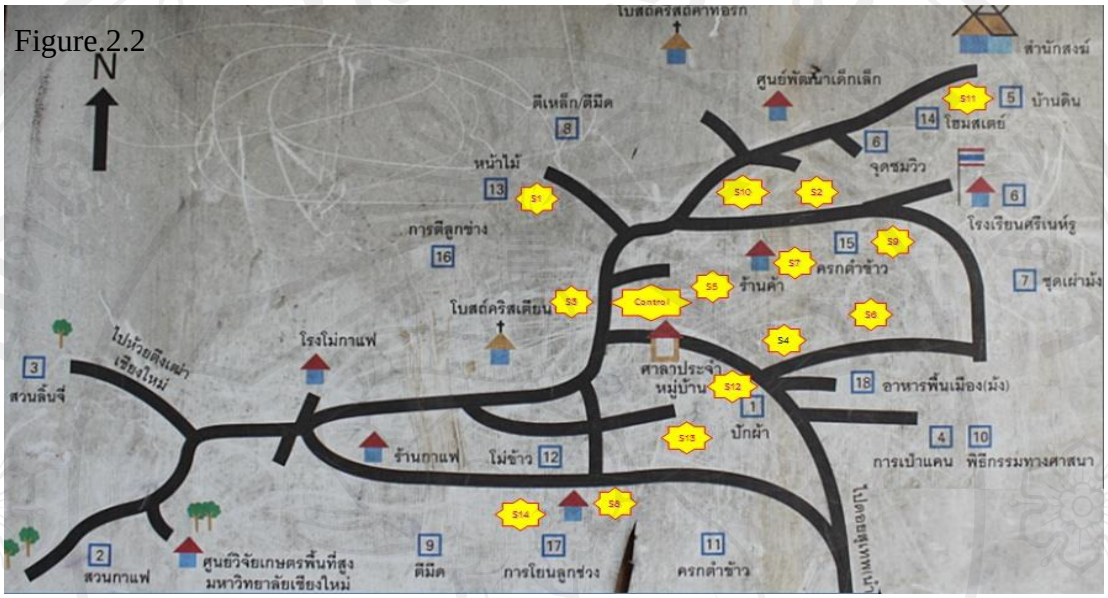


**Figure.2.2** The position of studied houses (S1-S14) and control house

### 2.4.3 Studied subject

The characteristic of studied subjects were the households in KCK village. They must use wood as a fuel for cooking in the household at least 2 times a day. The household owners must be willing to participate to this studied by signing the consent form. Fourteen household from 40 eligible households were selected as studied subjects. The control subject was a household which used LPG fuel for cooking.

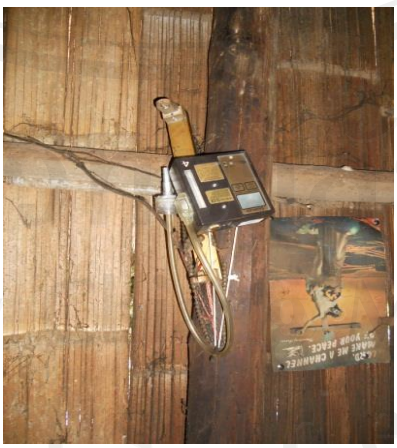

**Figure.2.3** Sample collection in the household

### 2.4.4 Ethical clearance approval

Since the present study involved human interview and engagement in their household areas, the study protocol was submitted and approved by the Human Experimentation Committee of Research Institute for Health Sciences (HEC, RIHES), Chiang Mai University (No.27/2011). Head of the households signed the informed consent before participation in the study.

### 2.4.5 $PM_{10}$ collection

The $PM_{10}$ samples were collected using personal air sampler (SKC. U.S.A.) in accordance with the reference method defined in the NIOSH Manual of Analytical Methods (NMAN, 1994) and quartz filters (QM-A,Whatman,37 mm diameter). The position of personal air sampler in the houses was located about 1.5 meters above the floor level on houses wall with flow rate was 2.0 L/min.

The sampling duration in each house was performed for 12 hr (06.00 pm to 06.00 am of next morning) for 3 days a week on Monday, Wednesday, and Friday. The $PM_{10}$ concentration of each household was obtained by the mean concentration of 3-day collection. The sampling period was in October 2011 for wet season and January 2012 for dry season.

After collection, the collected filters were covered with aluminum foil to protect the sample from sunlight. Then, they were placed in the desiccators for 24 hours prior weighing by a microbalance, 5 places in a clean room at 25 ˚C and less than 50% relative humidity. All filters were then kept in the freezer (-20 ˚C) until analysis.

## 2.5 Analysis of levoglucosan and 2-methoxyphenol in $PM_{10}$ sample

### 2.5.1 Preparation of solution

**1) Preparation of levoglucosan spiking standard solution**

Levoglucosan spiking solution concentration of 1,000 mg/L was prepared in methanol.

**2) Preparation of methyl β-D-xylopyranoside spiking solution**

Methyl β-D-xylopyranoside, an internal standard, spiking solution concentration of 1,000 mg/L was prepared in methanol. The solution was spiked into extracted samples to get final concentrations of 20 mg/L.

**3) Preparation of 2-methoxyphenol stock standard solution**

The 2-methoxyphenol standard stock solution concentration of 1,000 mg/L was prepared in ethyl acetate. The stock concentration was diluted to 10 mg/L (an intermediate solution) and then 1 mg/L (spiking solution) in ethyl acetate.

**4) Preparation of 2-chloro-4-methoxyphenol spiking solution**

The 2-chloro-4-methoxyphenol spiking solution concentration of 1 mg/L (an internal standard) was prepared in ethyl acetate. The solution was spiked into extracted samples to get final concentrations of 0.05 mg/L.

### 2.5.2 Method validation

Method validation was the process using to confirmation that the analytical procedure employed for a specific test is suitable for its intended. Result from method validation can be used to judge the quality of analytical results, it is an integral part of any good analytical practice (Horwitz, 2000).

In the present work, the method validation was presented in terms of precision, repeatability, and reproducibility, limit of detection (LOD), limit of quantitation (LOQ), recovery, and linearity of calibration curve**.**

**1) Precision**

The general term “precision” is used to describe the magnitude of random (indeterminate) errors associated with the use of an analytical method. The sources of random error evaluated depend upon the range of conditions over which the data are collected. Precision and trueness evaluations, known as accuracy studies, are often performed concurrently (Horwitz, 2000). In the present work, 5 replicates of a certain concentration were performed.

Relative standard deviation in percentage (%RSD) is calculated as follows:

$$\%\text{RSD} = \frac{SD}{\overline{X}}$$

where:

- SD = standard deviation with n-1 degrees of freedom (n = total number of observed values)
- $\overline{X}$= mean of observed values.

Precision was indicated closeness by individual result from the same analyst on the same instrument with identical reagent within a short period of time (<1 day) was indicates by closeness of individual results obtained by the different days after. Precision is usually defined in terms of repeatability and reproducibility.

Repeatability refers to the degree of agreement of results when conditions are maintained as constant as possible. It usually refers to the standard deviation of simultaneous duplicates or replicates. Theoretically the individual determinations

should be independent but this condition is practically impossible to maintain when determinations are conducted simultaneously and therefore this requirement is generally ignored. Calculate the standard deviation of repeatability from at least 5 pairs of values obtained from at least one pair of replicates analyzed with each batch of analyses. The objective is to obtain a representative value, not the "best value", for how closely replicates will check each other in routine performance of the method.

Reproducibility precision refers to the degree of agreement of results when operating conditions are as different as possible. It usually refers to the standard deviation (SD) or the relative standard deviation (RSD) of results on the same test samples by different instruments, different analysts, different days, and different laboratory environments and therefore it should reflect the maximum expected precision exhibited by a method.

The repeatability and reproducibility standard deviation varies with concentration and acceptable values approximate the values in the following Table.2.1

**Table.2.1** The acceptable repeatability and reproducibility was a function of the concentration and the purpose of the analysis (Horwitz, 2000).

| Concentration | Repeatability (%RSD) |
|---|---|
| 100% | 1% |
| 10% | 1.5% |
| 1% | 2% |
| 0.10% | 3% |
| 0.01% | 4% |
| 10 µg/g (ppm) | 6% |
| 1 µg/g (ppm) | 8% |
| 10 µg/kg (ppb) | 15% |

**2) Accuracy**

The term “accuracy” has been given so many meanings that it is better to use a more specific term. Ordinarily it means closeness of the test result to the “true” or accepted value. But the test result can be an individual value, the average of a set of values, or the average of many sets of values. Therefore, whenever the term is used, the number of values it represents and their relationship must always be stated, e.g., as an individual result, as the average of duplicates or *n* replicates, or as the average of a set of a number of trials. The difference of the reported value from the accepted value, whether it is an individual value, an average of a set of values, or the average of a number of averages, is the bias under the reported conditions. The frequently used term when the average of a set of values is reported is “trueness”.

**Table 2.2** The acceptable recovery is a function of the concentration and the purpose of the analysis (Horwitz, 2000).

| Concentration | Recovery limits |
|---|---|
| 100% | 98-101% |
| 10% | 95-102% |
| 1% | 92-105% |
| 0.10% | 90-108% |
| 0.01% | 85-110% |
| 10 µg/g (ppm) | 85-115% |
| 1 µg/g (ppm) | 75-120% |
| 10 µg/kg (ppb) | 70-125% |

Accuracy means closeness of the test result to the "true" or accepted value. But the test result can be an individual value, the average of a set of values, or the average of many sets of values (Horwitz, 2000).

In the present work, the clean quartz filters were spiked with known levels of two compounds (levoglucosan and 2-methoxyphenol) and two internal standards (methyl β-D-xylopyranoside and 2-chloro-4-methoxyphenol)**.** The extraction was followed the same procedure daily in parallel with the samples'analysis. Recovery was calculated from these spiked filters to monitor method efficiency and instrument performance. The spiking solutions were all prepared in ethyl acetate, Calibration standards were also made at least once a week to monitor solutions and instrument calibration.

It was calculated as described below:

Recovery (%) = $((C_f-C_u)/C_k) \times 100$

Where: $C_f$ = the detected levels in spiked clean quartz filters (mg/L)

$C_u$ = the detected levels in unspiked clean quartz filters (mg/L)

$C_k$ = the known spiked levels in clean quartz filters (mg/L)

**3) Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

The concentration of detected levels (X axis) and S.D. (Y axis) of individual series were plotted as a linear curve for determining Y-intercept (Taylor, 1990). Limit of detection was calculated as described below:

Limit of detection (LOD) = 3 x Y-intercept

Limit of quantification (LOQ) = 10 x Y-intercept

### 2.5.3 Determination of levoglucosan and 2-methoxyphenol in $PM_{10}$ samples

The quartz filters which contained $PM_{10}$ sample were analyzed for levoglucosan and 2-methoxyphenol as following. The filter sample was placed in a 20-mL glass vial and spiked with 20 µL of 1,000 mg/L of methyl β-D-xylopyrano side (IS for levoglucosan) and 50 µL of 1 mg/L of 2-chloro-4-methoxyphenol (IS for 2-methoxyphenol). The vials were capped and left at room temperature to allow the internal standards to be adsorbed onto the filter for ten minutes, the vial was de-capped and added 15 mL of ethyl acetate sonicated (Elma, Germany) for 30 minutes at controlled temperature (~10ºC) by using ice to extract the target compounds, the filter was removed and the extract was filtered through a Whatman 0.45 µm glass filter to remove particulates. Then, the extract was evaporated until nearly dried through evaporation under a stream of air in the water bath at 35 °C. The residue was added with 1 mL of ethyl acetate. The sample was then divided into two 200 µL portions. The portions for levoglucosan analysis was dried under a stream of $N_2$ at room temperature and reacted with 200 µL of derivatizing reagent N-methyl-N-bis (trimethylsilyl) trifluoroacetamide with 2% N-trimethylsilylimidazole at 70 ºC for 1 hr. The derivatized sample for levoglucosan determination was analyzed by GC-FID. The another portion for 2-methoxyphenol determiantion was dried under a stream of $N_2$ at room temperature and reacted with 50 µL of derivatizing reagent N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylsilyl chloride and 10 µL of pyridine at 70 ºC for 2 hr to derive –COOH to TMS ester and –OH to TMS ether. After the reaction completed, derivatives were added with 140 µL of n-hexane. The derivatized sample for 2-methoxyphenol determination was analyzed by GC-MS.

### 2.5.4 GC analyses

#### 1) Analysis of levoglucosan

The derivatized sample for levoglucosan determination was analyzed by GC-FID with the conditions as shown in Table 2.3.

**Table 2.3** GC-FID condition

| GC Parameter | Condition |
|---|---|
| GC-FID | Model.6890, Agilent, USA. |
| Column | HP-5, 5% Phenyl methylsiloxane, 30 mm I.D., 0.25 x 0.25 µm film thickness, Alltech, USA. |
| Carrier gas | Helium, flow-rate 2.1 mL/min |
| Injection mode | Split mode, split ratio 0.1:1.0 ,Split flow 0.4 mL/min |
| Injector temperature | 250 ºC |
| Temperature Program | |
| Initial column temperature | 250 ºC |
| Temperature program | 25 ºC/min to 160 ºC increased by 5 ºC/min to 200 ºC and increased by 30 ºC/min to 290 ºC |
| Post run temperature | 290 ºC |
| Post run hold time | 0 ºC |
| FID | |
| Detector temperature | 290 ºC |
| Hydrogen carrier gas flow rate | 40 mL/min |
| Nitrogen make-up gas flow-rate | 30 mL/min |
| Air flow-rate | 400 mL/min |

The samples were injected to GC-FID. Retention time and peak area of levoglucosan were integrated by GC ChemStation software. Quantitation was based on ratio of peak area between levoglucosan standard and methyl β-D-xylopyranoside. The identification of levoglucosan compound in $PM_{10}$ sample was carried out by comparing the retention time between levoglucosan sample and standard. Levoglucosan concentration in terms of mg/L was calculated from the calibration curve of extracted levoglucosan standards. Finally, it was transferred to a unit of $\mu g/m^3$. Levoglucosan standard concentrations are in the range of 1-100 mg/L with 20 mg/L internal standards.

**2) Analysis of 2-methoxyphenol**

The derivatized sample for 2-methoxyphenol determination was analyzed by GC-MS with the conditions as shown in Table 2.4

**Table 2.4** GC-MS condition

| GC Parameter | Condition |
|---|---|
| GC | Model.7890, Agilent, USA. |
| Column | HP-5 MS, 5% PhenylMethylSiloxane, 30 mm I.D., 0.25 x 0.25 µm film thickness, Alltech, USA. |
| Carrier gas | Helium, flow-rate 1 mL/min |
| Injection mode | Split Vent 50 mL/min at 1 min |
| Injector temperature | 250 ºC |

**Table 2.4** GC-MS condition (Continued)

| | |
|---|---|
| Temperature Program | |
| Initial column temperature | 80 ºC |
| Temperature program | 15 ºC/min to 190 ºC increase by 20 ºC/min to 210 ºC and increase by 40 ºC/min to 280 ºC and hold time 1 min |
| Post run temperature | 290 ºC |
| Post run hold time | 3 ºC |
| MS | Model.5975, Agilent, USA. |
| Detection mode | SIM mode |
| Transfer line temperature | 280 ºC |
| Source temperature | 230 ºC |
| Electron energy | 70 eV |
| Ionization mode | EI |

The sample were injected to GC-MS. Retention time and peak area of 2-methoxyphenol were integrated by using MSD Chemstation software. The identification of 2-methoxyphenol compound in $PM_{10}$ sample was carried out using retention time and constant selected ion (shown in Table 2.5). Concentration of 2-methoxyphenol in terms of mg/L was calculated from the calibration curve of extracted 2-methoxyphenol standards. Finally, it was transferred to a unit of $ng/m^3$. Concentrations of 2-methoxyphenol standards were in the range 0.02-0.2 mg/L with 0.05 mg/L internal standards.

**Table 2.5** Characteristic ions of 2-methoxyphenol

| Compound | $t_R$ | Primary Ion | Secondary Ion |
|---|---|---|---|
| 2-methoxyphenol | 6.05 | 166 | 151,181,196 |
| 2-chloro-4-methoxyphenol | 7.96 | 179 | 180,181 |

**2.6 Data analysis**

The statistical analysis was performed using the Statistical Package for the Social Sciences for Windows (SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± SD data and the paired t-test is used to compare the values of means from two related samples (wet season and dry season). In order to identify the relationships between the concentration of levoglucosan and $PM_{10}$, the concentration of 2-methoxyphenol and $PM_{10}$, Pearson correlation was implemented in forms of correlation coefficient (r).