CHAPTER 3

Methodology

The main objective of the present study was to determine the effects of PM_{10} air pollution among the school age children living in 2 separated areas in Chiang Mai province. To achieve the main objective of the present study, this study was consisted of 2 parts as following.

Part 1: To develop a simple and portable EBC collecting device and evaluate the use of developed device with a group of volunteers.

Part 2: To apply the developed EBC collecting device to collect EBC samples of school children who exposed to PM_{10}. After that, to evaluate the effects of PM_{10} exposure to respiratory health as well as pulmonary function, H_2O_2 and MDA concentrations in EBC of children in an urban and a highland school in Chiang Mai province during different seasons.

Part 1: To develop a simple and portable EBC collecting device and evaluate the use of developed device with a group of volunteers.

3.1 Methodology of part 1

3.1.1 Development of EBC collecting device

The device for collection of EBC sample was developed and constructed for the specific objectives of the present study. The device consisted of mouthpiece with one-way valve in which inspiratory and expiratory air were separated. The mouth piece connected to the flexible plastic tubing for letting subjects move freely and having a comfortable position. This flexible plastic tubing was connected to the 50 mL polypropylene collecting tube that was acting as sampling container and placed inside a stainless steel chamber. The polypropylene collecting tube was designed to connect with the second one-way valve to flow the excess air of expired breath towards the top and to prevent the flowing of environmental air into the polypropylene collecting tube and contamination of the sample. A rubber ring was placed between the flexible plastic

tubing and the hole in the stainless steel chamber to achieve an airtight connection. The polypropylene collecting tube was immerged in the stainless steel chamber that contained liquid nitrogen to cool down. The condenser maintains a temperature of less than -20ºC throughout a collection period. The schematic diagram of the device system used to collect EBC sample is shown in Figure 3.1

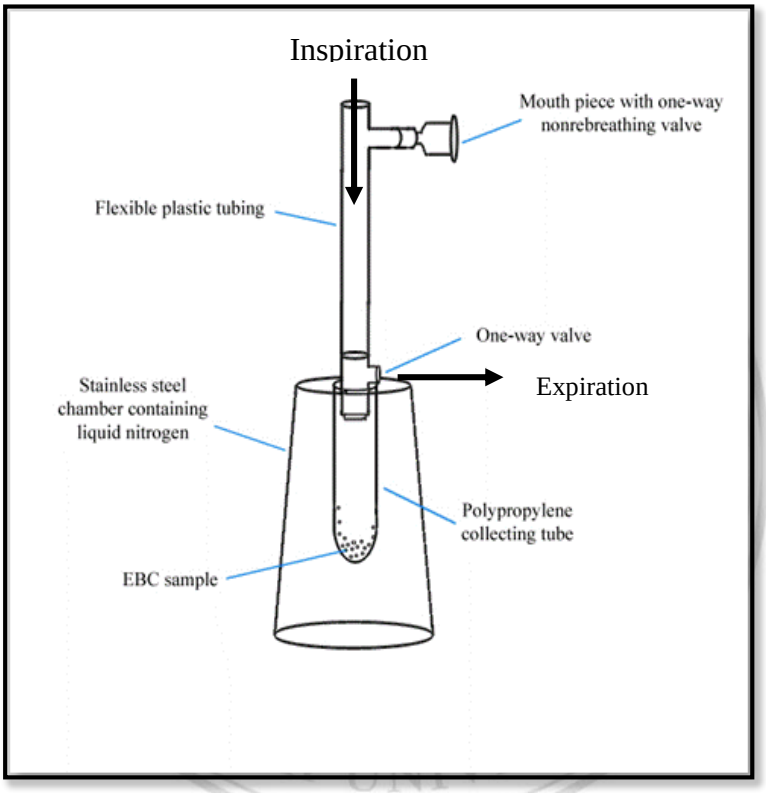

Figure 3.1 Schematic diagram of the system used to collect EBC

The developed EBC collecting device (Figure 3.2) consists of:

- A mouthpiece with one-way valve (Salford non-rebreathing valve 3.5 cm in diameter, Cranlea & Company, Birmingham, UK) (Figure 3.3). The one-way valve allows the device to condense only the exhaled air and avoids contamination with other substances in the environment.

- Flexible plastic tubing that was made from a tube measured 30 cm in length and 2 cm in internal diameter. (Hangzhou Guanghua Oak And Plastics Co, Ltd, Zhejiang, China) (Figure 3.4). The flexible plastic tube allows subjects to move and adapt to a more comfortable position without interruption or increasing resistance to exhalation.

- A polypropylene collecting tube (50 mL) (Figure 3.5) acting as condenser and collector, which is placed into a stainless steel chamber.

- A 0.5 L stainless steel chamber measured 18 cm in height and 4.5 cm in internal diameter (Satien Stainless Steel Public Co, Ltd, Bangkok Thailand) (Figure 3.6). The stainless steel chamber containing liquid nitrogen can be used as a cooling system.

- A second one-way valve that was obtained by modifying a plastic pipe (Figure 3.7). The second one-way valve was used to prevent the flowing of environmental air into the polypropylene collecting tube and the contamination of the sample.

- A rubber ring to achieve an airtight connection (Figure 3.8).

Figure 3.2 The developed EBC collecting device

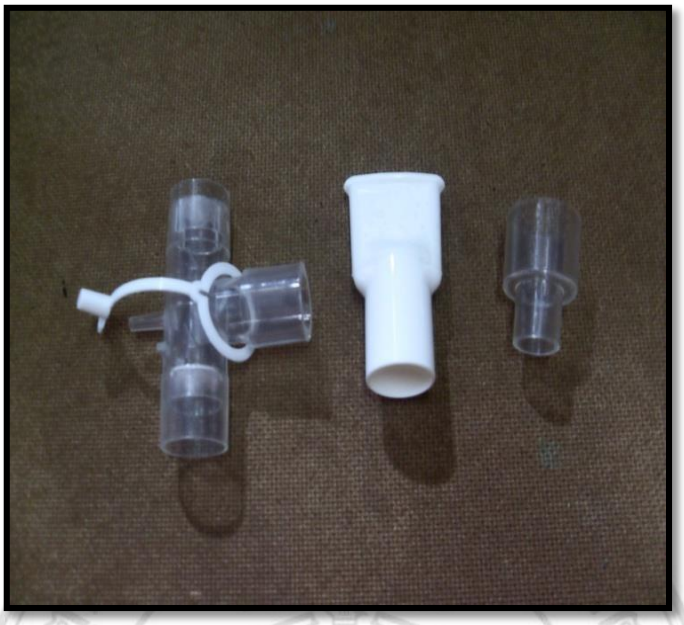

Figure 3.3 The mouthpiece with one-way valve

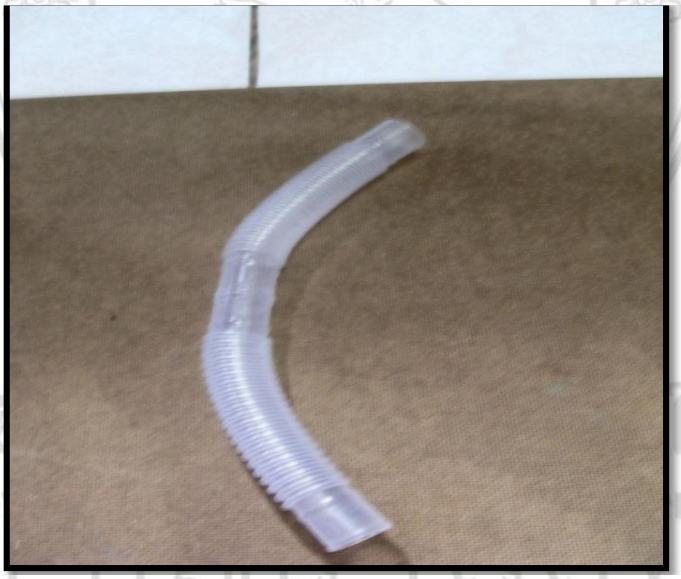

Figure 3.4 The flexible plastic tubing

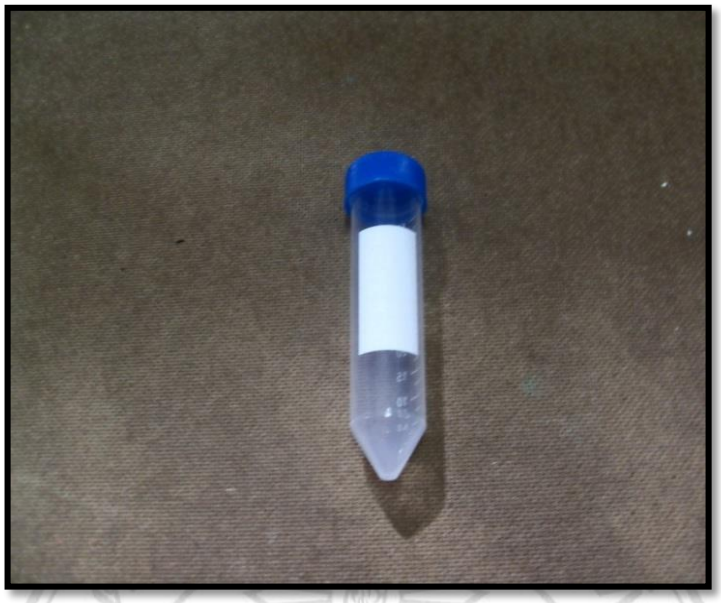

Figure 3.5 The polypropylene collecting tube acting as sampling container.

Figure 3.6 The stainless steel chamber

Figure 3.7 The second one-way valve

Figure 3.8 The rubber ring

3.1.2 Determination of suitable collection periods and breath patterns of EBC sample collection from healthy subjects.

The sampling procedure was setup and used in the study of part 2. The suitable collection periods and breathe patterns was determined. EBC samples were collected by developed EBC collecting device from 5 healthy subjects. EBC samples were collected at 2 consecutive days. On day one, subjects were asked to perform the same EBC collection procedures twice of each subject. They breathed tidally at normal frequency into developed EBC collecting device for 10 minutes to collect EBC samples. After that, they had 30 minutes for technical break and then performed a second EBC collection in the same manner for 20 minutes. On day two, two sampling periods were performed again as day one but the subjects were asked to breathe with increased tidal breathing by breathing with deep inhalations and forcing exhalations. For each of sampling periods, EBC samples were collected separately. Therefore, there were 4 different EBC samples collected from each subject. The collected EBC volume was measured using a calibrated 1000 µL pipette at each collection.

Table 3.1 Timing of EBC collecting in healthy subjects

Day of sampling	1st time collection	Technical break	2nd time collection
Day 1	10 min of normal tidal breathing	30 min	20 min of normal tidal breathing
Day 2	10 min of forced breathing	30 min	20 min of forced breathing

Part 2: To apply the developed EBC collecting device to collect EBC samples of school children who exposed to PM_{10}.

The developed EBC collecting device was used to collect EBC samples from the school children who exposed to PM_{10}. After that, evaluate the effects of PM_{10} exposure to respiratory health as well as selected respiratory symptoms, pulmonary function, exhaled H_2O_2 and exhaled MDA concentration of children in an urban and a highland school in Chiang Mai province.

3.2 Methodology of part 2

3.2.1 Study design

The present study was a follow-up study among school children who lived in two separate areas of Chiang Mai province. The study was designed to determine the effects of ambient PM_{10} exposure to respiratory health as well as pulmonary function, H_2O_2 and MDA concentration in EBC of healthy school-age children living in CM city as an urban school and a highland school of Chiang Mai province.

3.2.2 Study site

Chiang Mai is the second largest province of Thailand which is the largest city in northern Thailand. It is situated down near Ping river valley near the foothill of Suthep Mountain at about 310 m above mean sea level. It is surrounded by mountain range in the northern, eastern and western parts of the province. It is situated approximately 700 kilometers from Bangkok in the upper north region of the country. Chiang Mai has a population about 1,669,590 inhabitants in 20,107 km^2 (http://www.chiangmai.go.th, October 19, 2014).

This study was carried out in two areas of Chiang Mai province, according to the location of the schools. The first primary school is Chiang Mai Rajabhat University Demonstration School to represent the primary school located in an urban area of Chiang Mai city and the other one is Srinaeroo School to represent the primary school located on the highland area of Chiang Mai province. Both school administrators were contacted and agreed to participate.

Chiang Mai Rajabhat University Demonstration School (ST) is located in East of Chiang Mai Rajabhat University, which is at central of Chiang Mai city, and located within 2.5 km from YP air quality monitoring station. This study site is adjacent to a street on one site and surrounded by workplace and commercial areas on the other.

Srinaeroo School (SN) is located 35 km north-west of Chiang Mai city and 20 km from Bhubing Palace. The highland area lies at an altitude of 1,300 m above mean sea level. This area has a cool climate, especially between December and February. In 2011, the population was 1140, of whom 100% were Hmong hilltribe. They set up villages on the hills and their lifestyles are traditional. The majority of the families in the area are subsistence farmers, earning some cash supplement from selling their agricultural products.

Figure 3.9 Map of Chiang Mai province

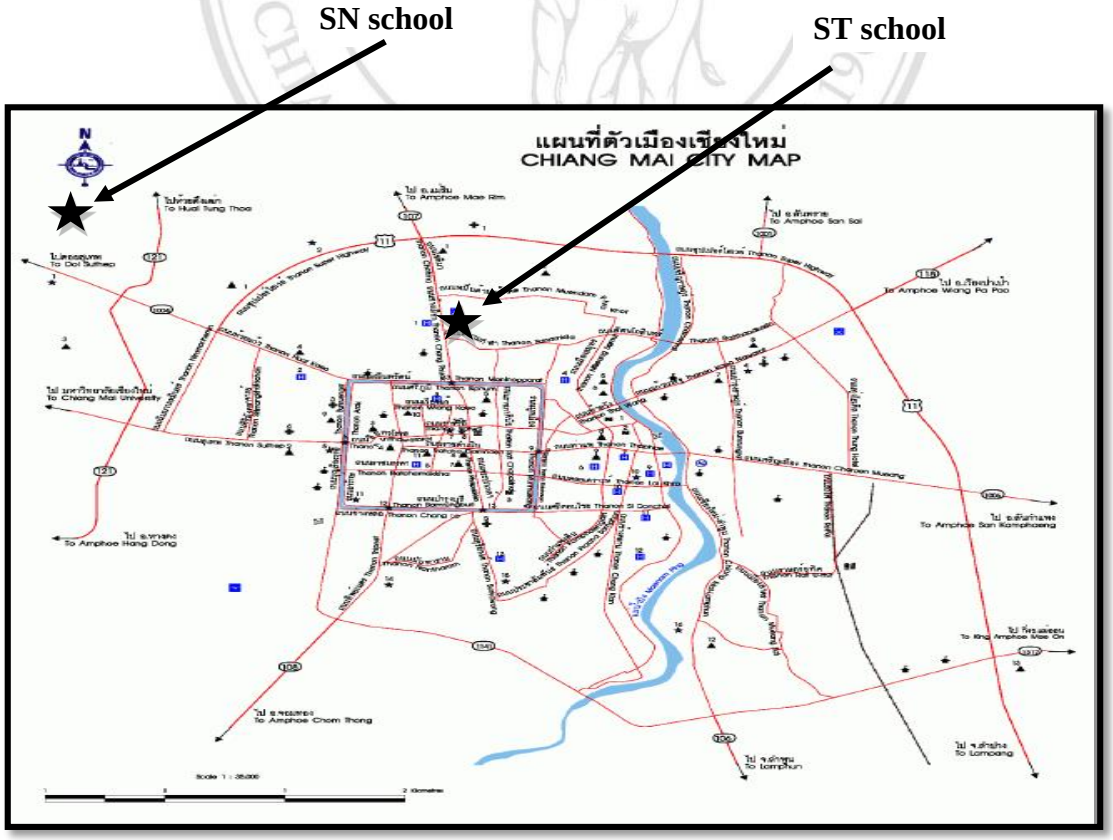

Figure 3.10 Map of Chiang Mai city and study sites

3.2.3 Study subjects

The study subjects were composted of 2 groups:

1) ST students were recruited under the following criteria:

1.1) They had been studying at ST school at least 1 year before the studying start;

1.2) Their homes were locating less than 2.5 kilometers far from the YP station at least 1 year before the studying start;

1.3) Their ages were 10 to 13 years;

1.4) They had not been diagnosed as having asthma or other chronic respiratory disease by the physician and long term medication use and

1.5) They were willing to participate through 8 months of the study.

2) SN students were recruited under the following criteria:

2.1) They had been studying at SN school at least 1 year before the studying start;

2.2) They had been living in Khun Chang Kean (KCK) village at least 1 year before the studying start;

2.3-2.5) They were the same as ST student group.

3.2.4 Study period

The study was conducted during July 2011 and March 2012, excluding October 2011 because of the school's vacation. These periods was chosen based on high variability of PM_{10} levels in Chiang Mai. This study was separated into three study periods following by the season. The first study period was conducted in wet season for 3 months (July to September 2011). The next study period was conducted in November 2011 to represent the transitioned period and dry season for another 4 months (December 2011 to March 2012).

3.2.5 Ethical clearance approval

The present study protocol was approved by the Human Experimentation Committee of Research Institute for Health Sciences (RIHES), Chiang Mai University. Children and their parents were informed about the study protocol. After they agreed to participate in the study, children signed informed assent and parents signed informed consent.

3.2.6 Exposure assessment

The level of PM_{10} was used to represent the subjects' exposure from PM_{10}. The present study was determined level of PM_{10} from outdoor and classroom air.

1) Outdoor PM_{10} level

The PM_{10} levels from the nearest air quality monitoring station were used to represent the subject's exposure to PM_{10}. The exposure from PM_{10} pollution of ST children was obtained daily ambient concentration from the YP station which locating within 2.5 kilometers radius of the ST school. However at the highland area was lacked of the air quality monitoring station of PCD; therefore the portable air sampler (E-sampler, Met One Instruments Inc., USA) was used to operate at SN school to represent the exposure from PM_{10} of SN children during the study period. Prior to operate the portable air sampler at SN school, the portable air sampler was used to collect PM_{10} samples at YP School. The samples concentrations were compared with levels obtained from the PCD air quality monitoring station at YP School at the same sampling period. Daily outdoor concentration of PM_{10} in SN school was measured for 24 hours/day on Monday to Friday at the concurrent week of the health outcome assessment. The sampling position was a courtyard within the school ground about 1.5 meters above the ground level.

Figure 3.11 Portable air sampler used to monitored PM_{10} level at the courtyard of SN school

2) Classroom PM_{10} level

In addition to measure personal exposure to PM_{10} of the subjects during the study period, the personal air sampler was assigned to monitor the PM_{10} level in each classroom. Each samples was collected for 7-8 hours using single-stage impactor (Personal Environmental Monitor-PEM; SKC Inc., USA), connected with flow-controlled battery-operated pumps (Air-Check Sampler; SKC Inc.). Air flow was maintained constant 2 liters per minute (simulating almost human respiratory rate). The sampler was charged for 8-10 hours every night prior to sampling. Each sample was collected on a 37-mm filter on Monday to Friday in a concurrent week of the health outcomes assessment. Pre- and post-weights were gravimetric measurement, 3 time, in a controlled clean room with 5 place-decimal micro-balance (Add name, company, city, country made) at RIHES' laboratory. Filters were handled with gloves and forceps in the field and laboratory. The support pads were changed daily. Loaded filters were placed in the petri dishes and kept in desiccators' cabinet for at least 48 hours before pre-and post-weighing. Filter mass from pre- and post- sampling was obtained as the average of three weighing. Researcher visited schools at about the same time of each day, every day, to change the filters.

The sampling position in each classrooms were opposite to the black board, about 1 meter above the floor level, the level at which the subjects would normally inhale, and away from the door. A total of 4 classrooms at ST school and 3 classrooms at SN school were selected for classroom PM_{10} monitoring. The personal air sampler used to monitor PM_{10} level in classroom as shown in Figure 3.12 and 3.13.

Figure 3.12 Personal air sampler was placed to monitor PM_{10} level in classroom

Figure 3.13 Personal air sampler, close-up, was used to monitor PM_{10} level in the classroom

3.2.7 Health outcome assessment

All study subjects were visited at the school including collected EBC samples and performed their pulmonary function every month during the follow-up period. The average time of each visit was between 20-30 days. School chlidren's height, weight, pulmonary function testing, EBC samples collection and the presence or absence of respiratory symptom were recorded during each visit.

1) Pulmonary function testing

Pulmonary function was measured by a portable electric mini-spirometer (Datospir, Sibel SA, Barcelona, Spain) using Spiro Thai 2.0 Program, according to the predicted pulmonary function parameters from reference values in the Thai population (Dejsomritrutai *et al.*, 2000). A trained technician performed pulmonary function testing according to the recommendation of the American Thoracic Society (1995) in each school after regular calibration of spirometers. Each subject underwent a forced spirometry to obtain the following parameters: forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1), and relation of FEV_1/FVC. Pulmonary function indices used in the analyses are expressed as the percentage of the predicted values based on the subject-specific sex, age, height, and weight. All subjects were trained by technician for their suitable blowing as fast, hard, and long as possible, which the best of three consecutive spirometer's recordings was chosen for further analyses. Height and weight were measured for each subject before performed pulmonary function. Ambient temperature and pressure monitored throughout the procedure. Weight was determined using a digital scale. Height was measured standing with feet together and arms relaxed at the sides.

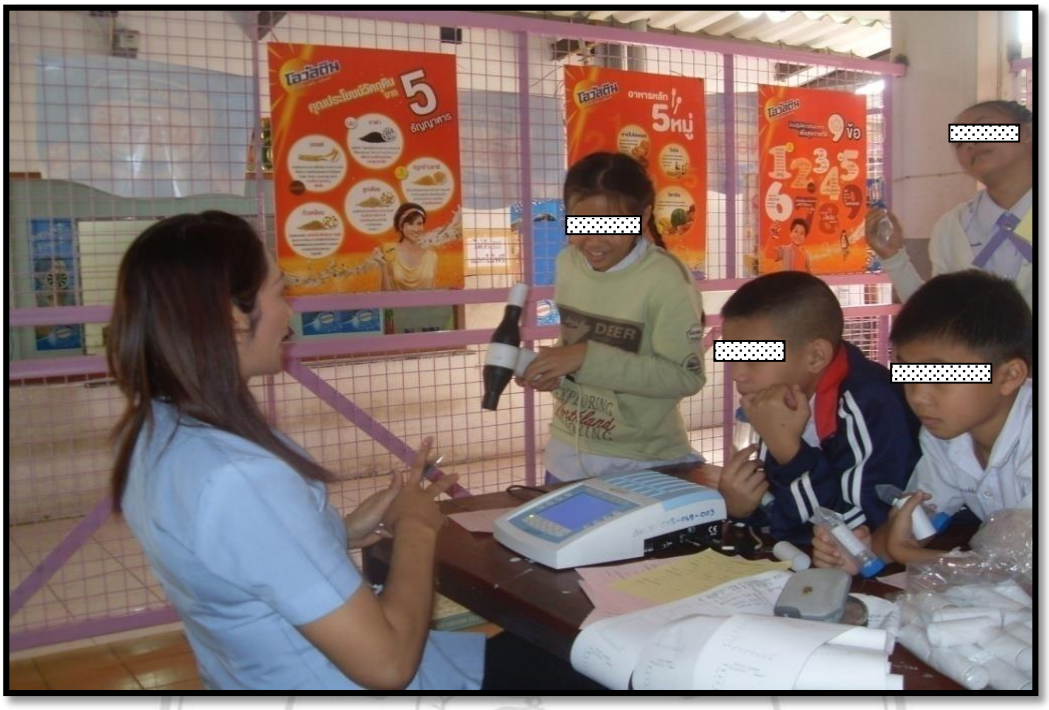

Figure 3.14 School children were advised on and tested for pulmonary function by a trained technician

2) Respiratory symptoms

The school children will be defined as having respiratory symptom if they had at least one symptom of respiratory symptom such as cough, sputum induction, shortness of breath, wheezing and chest discomfort in the concurrent week of PM_{10} monitoring. The respiratory symptoms were considered for odd ratio analysis. The risk was calculated between ST and SN children having different exposure to risk factor. The odd ratio above 1.0 suggests a relationship between exposure and risk (Ingle *et al.*, 2005).

3) EBC samples collection

EBC samples were collected from the subjects using the developed EBC collecting device. After rinsing their mouths, subjects were instructed to form a complete seal around the mouthpiece with their mouths and maintain dry mouths during collection by periodically swallowing excess saliva. The mouthpiece was used as a saliva trap. The subjects sat comfortably and wore nose clip. They were instructed to breathe normal tidally through the mouthpiece for 10 minutes to collect the EBC samples. The collected EBC samples were kept on dry ice and frozen immediately at –70°C until analysis. All EBC samples were analyzed at Toxicology Laboratory, Environmental and Health Research Unit, Research Institute for Health Sciences, Chiang Mai University.

Figure 3.15 Photograph of school children during EBC collection.

4) Method validation of biomarker determination in EBC

In the present study, method validation of H_2O_2 and MDA were presented in terms of calibration curve, repeatability and reproducibility, limit of detection and limit of quantification, and percent recovery, describe as following.

4.1) Calibration curve

4.1.1) Calibration curve of H_2O_2 standard

The calibration curve of H_2O_2 standard was constructed by triplicate spectrophotometric (at 450 nm) measurement of 8 concentrations ranging from 0, 0.08, 0.15, 0.31, 0.62, 1.25, 2.50, 5.0 µM of standard H_2O_2 solution in urified water. H_2O_2 standard concentrations were plotted against their mean absorbance.

4.1.2) Calibration curve of MDA standard

The calibration curve of MDA standard was constructed in a range of MDA concentrations of 0.08, 0.15, 0.30, 0.60, 1.20, 2.40 and 4.80 µM for HPLC analysis.

4.2) Repeatability and reproducibility

4.2.1) Repeatability and Reproducibility of H_2O_2

The repeatability in the present study was obtained with 8 repeated measurements continuous of 1.0 µM H_2O_2 standard solution on a spectrophotometer at 450 nm. Reproducibility of the system was obtained by preparing 5 solutions of 1.0 µM H_2O_2 standard solution and followed by analysis in the same manner for 5 continuous days. The results were estimated by SD and the relative standard deviation (% RSD).

4.2.2) Repeatability and Reproducibility of MDA

The repeatability was done by 5 injections of 0.15 µM MDA standard solution on HPLC under the optimum conditions. The reproducibility was checked by injections of 0.15 µM MDA standard solution on HPLC analysis under the optimum conditions for 5 continuous days.

4.3) Limit of detection (LOD) and limit of quantification (LOQ)

4.3.1) LOD and LOQ of H_2O_2 standard

The present study was obtained with 7 measurements of pooled EBC samples on a spectrophotometer at 450 nm. LOD and LOQ were calculated from 10 sample pooled measurement using equation as described below:

$$LOD = 3SD$$

$$LOQ = 10SD$$

Where SD = the standard deviation of sample pooled concentration

4.3.2) LOD and LOQ of MDA standard

The limit of detection and limit of quantification were assessed by 7 injections of pooled EBC samples into the HPLC analysis. LOD and LOQ were calculated from 10 sample pooled measurement using equation as described below (Currie, 1999):

$$LOD = 3SD$$

$$LOQ = 10SD$$

Where SD = the standard deviation of sample pooled concentration

4.4) Recovery

Recovery was calculated as described below:

$$\text{Recovery (\%)} = ((C_s - C_u) \times 100) / C_a$$

Where C_s= the individual standard levels in spiked sample (µM)

C_u = the individual standard levels in unspiked sample (µM)

C_a = the known spiked levels of individual

4.4.1) Percent recovery of H_2O_2 standard

The analytical EBC recovery was calculated by 10 measurements of pooled EBC samples were unspiked and spiked with 0.1, 1.0, and 2.0 µM of H_2O_2.

4.4.2) Percent recovery of MDA standard

Percent recovery was conducted by comparing peak areas of replicates from EBC unspiked and spiked with 0.15, 0.6, and 2.4 µM of MDA.

5) Analysis of exhaled H_2O_2 (Gallati and Pracht, 1985)

5.1) Principle

The concentration of exhaled H_2O_2 was measured using a spectrophotometric assay by means of horseradish peroxidase-catalyzed oxidation of tetramethylbenzidine. The reaction product was measured spectrophotometrically at an absorbance 450 nm using an automate microplate reader.

5.2) Equipments and materials

-10, 25, 100, 500 mL volumetric flasks, DURAN Glastechnik GmbH & Co.KG, Wetheim/Main, Germany

-250 mL Erlenmeyer flask, DURAN Glastechnik GmbH & Co.KG, Wetheim/Main, Germany

- 100, 250 mL beaker, DURAN Glastechnik GmbH & Co.KG, Wetheim/Main, Germany

- Automatic micro pipette, Pipetman model P20, P100, and P1000; Gilson Compant Inc., Wisconsin, USA

- UV Plate, Corning Incorporate, New York, USA

- Magnetic stirrer, Fisher, New Hampsire, USA

-5 place decimal Micro balance, Mettler Toledo, AB135-S/FACT, Ohio, USA

- Microplate spectrophotometer, Sunrise model, TECAN, Mannidorf, Austria

- Vortex, LMS Co. Ltd., Tokyo, Japan

- pH meter, Beckman Coulter, Atlanta, USA

5.3) Chemical reagents

- Sulfuric acid (H_2SO_4), Merck, Darmstadt, Germany
- Potassium iodide (KI), Ajax Finechem Pty Ltd, Victoria, New Zealand
- Starch, Ajax Finechem Pty Ltd, Victoria, New Zealand
- Sodium thiosulphate ($Na_2S_2O_3$), Fisher Scientific, Loughborough, UK
- Citric acid monohydrate, Fisher Scientific, Loughborough, UK
- Sodium citrate dehydrate, Fisher Scientific, Loughborough, UK
- 30% Hydrogen peroxide (H_2O_2), Merck, Darmstadt, Germany
- 3´,3, 5, 5´ tetramethylbenzidine, Sigma-Aldrich, Steinheim, Germany
- Dimethyl sulfoxide (DMSO), Fisher Scientific, Loughborough, UK
- Horseradish peroxidase (HRP), Sigma Chemicals, Missouri, USA

5.4) Evaluation of the assay

5.4.1) Chemical preparation

- 1 M Sulfuric acid (H_2SO_4): Slowly add 5.6 mL concentrated H_2SO_4 to 90 mL water, make to 100 mL with water.
- 10% w/v Potassium iodide (KI): Dissolved 10 g of KI in 100 mL water. Stored in a cool, dark place.
- 1% w/v Starch: Weight 1 g soluble starch into 90 mL water, heat to dissolve and make to 100 mL with water. Store in a cool, dark place.
- 0.10 M Sodium thiosulphate ($Na_2S_2O_3$): Dissolve 2.4818 g of $Na_2S_2O_3$ in 100 mL water.
- 0.42 M citrate buffer, pH 3.8: Mix 0.42 M citric acid 15 mL with 0.42 M sodium citrate 9.3-9.9 mL and adjust with pH meter.
- 420 µM 3´,3,5,5´tetramethylbenzidine (TMB) in 0.42 M citrate buffer, pH 3.8: dissolved 0.0010 g in 1 mL DMSO and dilute to 10 mL with citrate buffer.
- HRP 26.5 U/mL: dilute stock HRP 10 µL with 990 µL water.

- Sulfuric acid (H_2SO_4) 9 N: pipette 12.5 mL concentrated H_2SO_4 into water

5.4.2) Titration method

H_2O_2 approximately 30% must be titrated to obtain the actual concentration by Iodometric titration.

- Pipette 50 mL water into Erlenmeyer flask 250 mL.
- Add 0.10 mL stock H_2O_2.
- Add 1.00 mL 1M of H_2SO_4.
- Add 5.0 mL 10% KI, wrap with parafilm and keep in the dark for 10 minutes.
- Titrate with 0.10 M $Na_2S_2O_3$ from the burette until the solution turns pale yellow.
- Add 2 mL of 1% starch, the solution turns dark purple. Continue titrating until the solution becomes colorless.
- Record the volume of thiolsulphate used and calculate the concentration of stock H_2O_2 from the equation:

$$\text{Concentration of stock } H_2O_2 = \frac{\text{vol. of thiosulphate used (mL) x Na2S2O3 conc. (M)}}{\text{2 x vol. of stock hydrogen peroxide (mL)}}$$

5.4.3) H_2O_2 standard preparation

- Prepare intermediate I of H_2O_2 standard 1000 µM in 500 mL water by diluting stock H_2O_2
- Prepare intermediate II of H_2O_2 standard 100 µM in 10 mL water by diluting 1.00 mL of intermediate I of H_2O_2 standard to 10 mL with water.
- Prepare working H_2O_2 standard 0.16, 0.31, 0.63, 1.25, 2.5, 5.0 µM in 10 mL water by serial diluting (1:2) of the intermediate II of H_2O_2 standard.

5.5) Procedure

The exhaled H_2O_2 concentration was measured using a spectrophotometric assay by means of horseradish peroxidase-catalyzed oxidation of tetramethylbenzidine according to the method previously described (Gallati and Pracht, 1985). Briefly, 250 µL of EBC sample was mixed with 250 µL of TMB in 0.42 M citrate buffer, pH 3.8 and 20 µl of HRP (26.25 U/mL). The samples were incubated at room temperature for 20 minutes, and the reaction was stopped by addition of 20 µL of 9 N sulfuric acid. The product of this reaction was then immediately analyzed with an automated microplate reader at the wavelength for absorbance of 450 nm. The concentration of H_2O_2 was calculated from a standard curve of know concentration of H_2O_2 prepared for each assay.

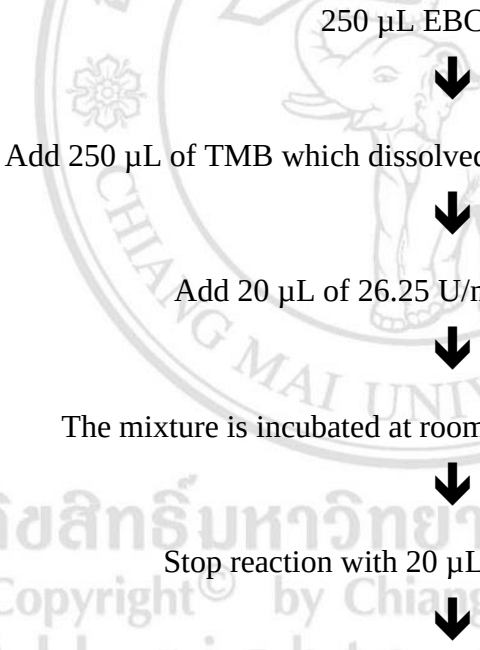

Figure 3.16 Flow chart of H_2O_2 determination

6) Analysis of exhaled MDA.(Wong *et al*., 1987)

6.1) Principle

EBC lipoperoxides are hydrolyzed by boiling in dilute phosphoric acid. MDA, one of the hydrolysis products, is reacted with TBA to form $MDA(TBA)_2$ adduct. EBC proteins are precipitated with methanol and removed from the reaction mixture by centrifugation. The protein-free extract is fractionated by HPLC on a column of octadecy silica gel, to separate the MDA-TBA adduct from the column with methanol/phosphate buffer and quantified spectrophotometrically at 532 nm. EBC lipoperoxide concentrations are computed by reference to a calibration curve prepared by assays of tetraethoxypropane (TEP), which undergoes hydrolysis to liberate stoichiometric amount of MDA.

6.2) Equipment and materials

- 10, 25, 50 mL volumetric flasks. Duran, Munich, Germany
- 100, 250, 500, 1500 mL beaker, Duran, Munich, Germany
- Micropipette, Pipetman P20, P100, and P1000, Gilson, Hoenbergstr, Germany
-Micro balance weight, Mettler Toledo, AB135-S/FACT, Ohio, USA
- Polycarbonate filter (0.45 μm pore diameter), Waters-Millipore, Massachusetts, USA
- Water bath, BECThai Co. Ltd, Bangkok, Thailand
- Refrigerated centrifuge, Thermo Scientific Sorvall, Porton Down, UK

6.3) Chemical reagents

-1, 1, 3, 3-tetraetoxypropane (TEP), Sigma-Aldrich, Steinheim, Germany
- Orthophosphoric acid (H_3PO_4), JT Baker, Detventer, Netherland
- Potassium dihydrogen phosphate (KH_2PO_4), Merck, Damstadt, Germany
- Potassium hydroxide (KOH), Merck, Damstadt, Germany
- Sodium hydroxide (NaOH), Merck, Damstadt, Germany
- Thiobarbituric acid (TBA), Sigma Chemicals, Missouri, USA

- Methanol, JT Baker, Deventer, Netherland
- Ethanol, JT Baker, Deventer, Netherland

6.4) HPLC condition

Column:	SUPELCO C18, 150 X 4.6 mm
Column temperature	40º c
Mobile phase	50 mmol/L phosphate buffer, pH 6.8: methanol(60:40, v/v)
Flow rate:	1.0 mL/min
Detector:	UV detection at 532 nm (Shimadzu SPD 20AV)
Injection volume:	50 µL
Retention time	5.8 minute
Method length	10 min

6.5) Evaluation the assay

6.5.1) Chemical preparation

- 0.44 mol/L Phosphoric acid solution: Dilute 10 mL of H_3PO_4 reagent to 1 l with water
- 42 mmol/L TBA solution: Dissolve 0.6 g of thiobarbituric acid in proximately 80 mL of water, with stirring on a hot-plate (55ºc). Cool the solution to 25º c and dilute to 100 mL with water.
- 400 mL/L Ethanol solution: Dilute 420 mL of 95% ethanol to 1 l with water

6.5.2) TEP standard preparation

- TEP standard solution: In a 25-mL volumetric flask, dilute 50 µL of 1,1,3,3-tetraetoxypropane reagent to the mark with 400 ml/L ethanol solution.
- For intermediate standard, pipette 0.5 mL of this TEP stock standard solution into a 100 mL volumetric flask and dilute to the mark with 400 ml/L ethanol solution.

- To prepare TEP working standard solutions (0.08, 0.15, 0.31, 0.61, 1.22, 2.43, 4.86 µmol/L), pipette into seven 25-mL volumetric flasks 0.05, 0.10, 0.19, 0.38, 0.75, 1.5, and 3.0 mL, respectively, of TEP intermediate standard solution and dilute the contents to the mark with water.
- Methanol-NaOH solution: Pipette 4.5 mL of 1 mol/L NaOH solution into a 50 mL volumetric flask and dilute to the mark with methanol.
- 50 mmol/L, pH 6.8 Potassium phosphate buffer: Dissolve 13.6 g of anhydrous KH_2PO_4 in approximately 1.6 l of distilled water and titrate to pH 6.8 with 1 mol/L KOH solution, monitoring constantly with a pH meter. Dilute to 2 l with water and filter through a polycarbonate filter (0.45 µm pore diameter).
- Mobile phase: Mix 400 mL of methanol and 600 mL of potassium phosphate buffer solution.

6.6) Procedure

MDA was determined by measuring MDA–thiobarbituric acid (TBA) adducts according to the method previously described by Wong *et al*. (1987) with slight modifications.

Briefly, 50 µL of EBC sample was mixed to 0.44 mol/L of H_3PO_4 (750 µL) and 42 mmol/L of TBA solution (250 µL) and the mixture was heated at 95°c for 60 minutes. Then, the mixture was cooled in ice water for 5 minutes and then kept at room temperature for 20 minutes. Three hundred µL of the mixture were transferred into another centrifuge tube and added with 300 µL of Methanol-NaOH solution. And then, the mixture was centrifuged at 13000 rpm for 10 minutes. The absorbance was measured at 532 nm after separation with an HPLC system equipped with a mobile phase of 50 mmol/L of KH_2PO_4 (pH 6.8): methanol (60:40, v/v). MDA concentration was calculated from the standard calibration curve and express as µmol/L of EBC.

The determination of MDA in EBC samples was performed with a eight-point calibration curve based on water blank measurement and seven concentration (0.08, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 µM). Since the calibration curve may change with the influences of daily prepared TEP standard and running buffer, calibration points are obtained daily to calculated MDA concentrations of the samples analyzed in that day. The process of MDA measurement is described by Figure 3.17.

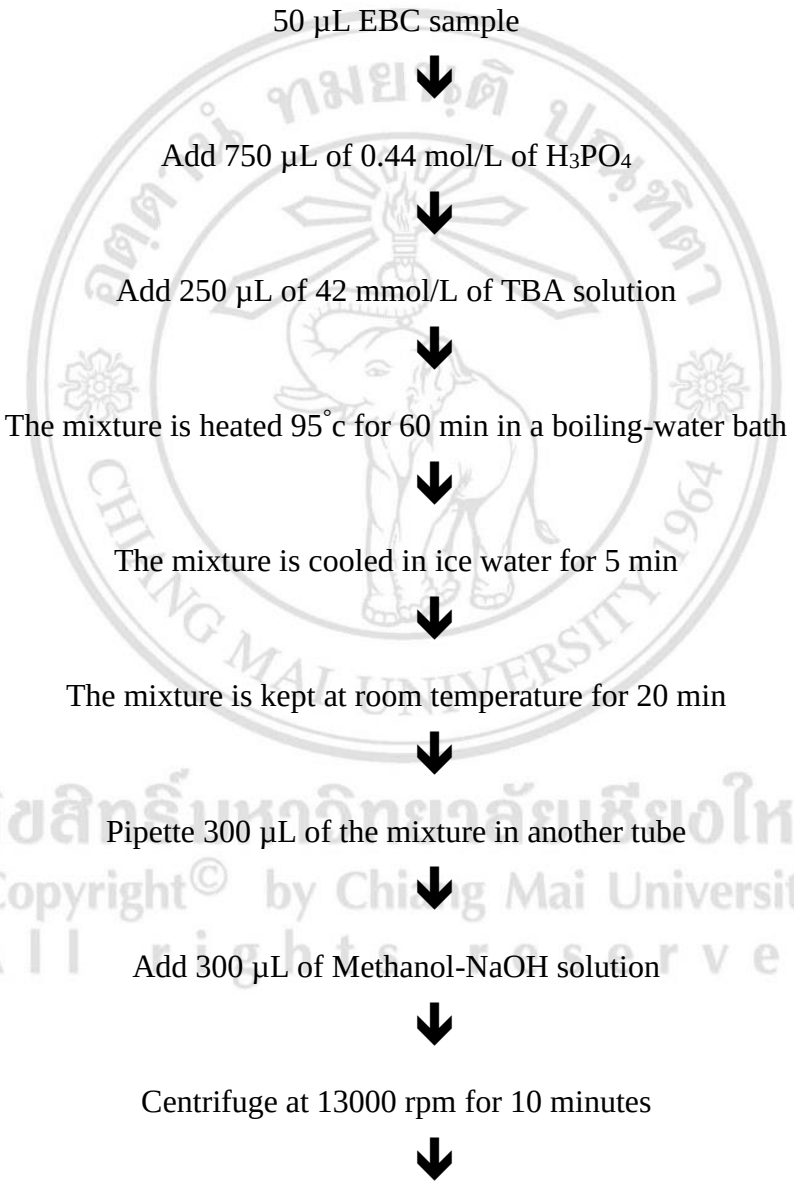

Figure 3.17 Flow chart of MDA determination

3.2.8 Statistical analysis

The Kolmogorov-Smirnov test was used for the fitness of the variables to the normal distribution. Mean or median values were reported accordingly to describe central tendencies. The results were expressed as mean ± sd for normally distributed data and median and range for non-normally distributed data.

The percentage of samples above the limits of detection (LOD) for each analyze was calculated. The imputed value was used for concentrations less than the LOD. Geometric mean (GM) and geometric standard deviation (GSD) was calculated instead of mean and SD when data was highly skew. For those data with high skew, a value of LOD/√2 was used where a concentration less than LOD; similarly, those data with low skew, a value of LOD/2 was used (Finkelstein and Verma, 2001).

Baseline characteristics between the two study groups were compared by a Student t-test for independent groups (continuous variables) and by Chi-square test (categorical variables). The independent t-test was used to compare outdoor PM_{10} exposures, classroom PM_{10} exposures, and health outcomes variables between the subjects from two study areas. Outdoor PM_{10} exposures, classroom PM_{10} exposures, and health outcomes variables between seasons were compared by ANOVA test. The concentrations of exhaled H_2O_2 and MDA were log-transformed to approach a normal distribution. The odd ratios and 95% confidence interval were calculated to evaluate the risk of respiratory symptom between ST and SN school children. The association between PM_{10} exposure and health outcomes was examined using the non-parametric by Pearson's correlation coefficient. A statistical software package (SPSS Version 17; SPSS Thailand) was used. Statistically significance was defined as $p < 0.05$.