CHAPTER 3

Material and Method

3.1 Laboratory supplies

3.1.1 Fiber film filter, 47 mm T60A20, Pallflex, Pall Life Science, USA

3.1.2 Aluminum foil

3.1.3 20 mL tube, Borosilicate glass IWAKI TE-32, PYREX, INDONESIA

3.1.4 50 mL tube, Borosilicate glass IWAKI TE-32, PYREX, JAPAN

3.1.5 20, 100 and 200 µL Auto pipette

3.1.6 Beaker

3.1.7 Volumetric flask

3.1.8 Vial tube

3.1.9 Filter holder, 47 mm, ADVANTEC, JAPAN

3.1.10 PTFE membrane filter, 47 mm, ADVANTEC, JAPAN

3.1.11 Centrifugal filter, Ultra free-MC-HV, 0.45 µM, Merck KGaA, Germany

3.2 Chemical reagents (All chemical reagents were analytical grade)

3.2.1Chemical for determination of PAHs

1) Sodium Acetate Trihydrate ($CH_3COOHNa$)
2) Acetic acid (CH_3COOH)
3) 99.5% Ethanol (C_2H_2OH)
4) L-Ascorbic acid ($C_6H_8O_6$)
5) 99% Imidazole Glyoxaline ($C_3H_4N_2$)

6) Acetonitrile (CH_3CN)

7) Perchloric acid ($HClO_4$)

8) Dichloromethane (DCN)

9) Milli-Q water

3.2.2 Standards and Internal standards

1) The EPA 610 PAH mixture including fluoranthene (Flu), pyrene (Pyr), banz[*a*]anthracene (BaA), chrysene (Chr), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*a*]pyrene (BaP), dibenz[*a,h*]anthracene (DBA), benzo[*ghi*]perylene (BghiPe), and indeno[1,2,3-*cd*]pyrene (IDP) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2) Three deuterated PAHs (Pyr-d_{10}, BaA-d_{10} and BaP-d_{12}) were obtained from the Cambridge Isotope Lab. Inc. (Andover, MA, USA).

3.2.3 Mobile phase for HPLC (see appendix A)

1) 95% EtOH/Acetate buffer

2) 30 mM Ascobic acid

3) Imidazole buffer pH 7.6

4) Acetonitrile

3.3 Instruments

3.3.1 Mini Volume Air Sampler, AIRmetric, USA

3.3.2 Desiccator

3.3.3 Microbalance weight, AB135-S/FACT, Mettler Toledo, Switzerland

3.3.4 Microbalance weight, AEX-180, LIBROR

3.3.5 Ultrasonic multi cleaners, W-113, HONDA

3.3.6 Centrifugal evaporator, CVE-3100, EYELA

3.3.7 PH meter, Mettler Toledo, Switzerland

3.3.8 Mini-centrifuge, Benchmark Scientific, USA

3.3.9 HPLC with fluorescence detection, Shimadzu, Kyoto, Japan, consisting

1) Four HPLC pumps (LC-20AD)

2) Fluorescence detector (RF-20Axs)

3) Controller (CBM-10A)

4) Degasser (DGU-20A)

5) Auto sample injector (SIL-20AC)

6) Column oven (CTO-20AC)

3.4 PM_{10} sample collection

3.4.1 Study sites

In this study, ambient PM_{10} samples were collected at five sampling sites located in Mueang Lampang and Mae Moh District, Lampang Province according to their different function. The field descriptions were given as follow and the characteristic of this study sites were show in Table 3.1.

Tha Si Health Promotion Hospital (TS) was chosen as a typical rural area, which surrounded by residential building, grassland and forest. It is situated about 20 meters from a four lanes heavy traffic road.

Sob Pad Temple (SP) was chosen as a residential area. This site is close to the residential community. The major occupation in this village is a chopstick production and where normally they heat chopstick products by burning the waste wood (open burning).

Mae Moh Wittaya School (MW) was chosen as one of the government office center. This site is close to the residential building and some office buildings. No significant sources of industry emission exited at this area.

Lampang Meteorological Station (MS) was chosen as a typical commercial area. It is close to the airport and downtown business center with many shops, markets and restaurants. It is situated about 500 meters from a four lanes heavy traffic road.

Sa Det Subdistrict Administrative Organization (SD) was chosen as a typical rural area, which surrounded by government officers, residential communities, grassland and some forest area where forest fires often occur during in dry season. It is situated about 20 meters from a four lanes heavy traffic road.

Table 3.1 Characteristic of the study sites

Study sites	Latitude, Longitude	Elevation (MASL)
Tha Si Health Promotion Hospital (TS)	18°42’69.96”N 99°75’78.89”E	368
Sob Pad Temple (SP)	18°24’96.60”N 99°.76’22.45”E	313
Mae Moh Wittaya School (MW)	18°27’87.38”N 99°65’45.71”E	384
Lampang Meteorological Station (MS)	18°27’83.17”N 99°50’65.78”E	244
Sa Det Subdistrict Administrative Organization (SD)	18°39’01.76”N 99°61’89.53”E	264

In addition, ambient PM_{10} concentrations have been monitored at the Air Quality Monitoring (AQM) stations set up by the Pollution Control Department (PCD) of Thailand. There are four stations in Lampang province which located at Lampang Meteorological Station (37T), Sob Pad Health Promotion Hospital (38T), Tha Si Health Promotion Hospital (39T) and Mae Moh Government Center (40T). PM_{10} were measured with the tapered element oscillating microbalance (TEOM) detector and Beta Ray method. The locations of this study and four AQM sites by PCD were show in Figure 3.1.

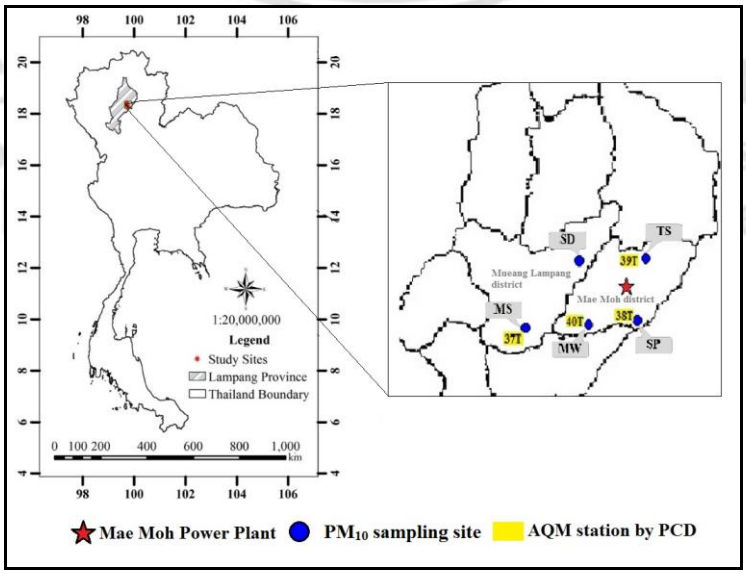

Figure 3.1 Location of this study sites and AQM stations by PCD

3.4.2 PM_{10} collection

The thirty of PM_{10} sampling was continuously conducted for 24 hours periods during the dry season (February – April) and wet season (May – July) 2015. The PM_{10} samples were collected on 47-mm Fiberfilm filters (T60A20, Pallflex, Pall Life Science, USA) using a Mini Volume air sampler (AIRmetric, USA) at a flow rate of 5.0 L/min above the ground level of 1.5 m as show in Figure 3.2.

The filter papers were equilibrated in a desiccator under controlled relative humidity (30-40%) and temperature (15-30°C) for 24 hours and then weighed on a five-digit electronic microbalance before and after sampling. The exposed filters were stored individually in sealed containers wrapped with aluminum foil and kept in plastic bags. After weighing, the sample filters ware stored in the same containers and kept in a refrigerator (at -20°C) until analysis.

Figure 3.2 PM_{10} sampling sites

3.5 PM_{10}-bound PAHs analysis

3.5.1 Preparation of solution

1) Preparation of Stock standard PAHs solution. Stock PAHs solution concentration of 2,000 µL was prepared in acetonitrile.

2) Preparation of Spiked PAHs standard solution. Spiked PAHs solution concentration of 1,000 µL was prepared in acetonitrile.

3) Preparation of Internal PAHs standard solution

$5x10^{-6}$ mM of Pyr-d_{10}	125 µL
$5x10^{-6}$ mM of BaA-d_{10}	125 µL
$1x10^{-5}$ mM of BaP-d_{12}	125 µL
EtOH	625 µL
Total volume	1,000 µL

The solution was added 15 µL into extracted samples to get final concentration of 450 µL.

3.5.2 Method validation

Method validation is an important regulatory requirement in HPLC analysis. Method validation provides a high degree of assurance, that an analytical method employed for a specific test, is suitable for its intended use.

The most widely applied validation characteristic parameters was presented in this study in terms of accuracy, recovery, precision, repeatability, reproducibility, limit of detection (LOD) and limit of quantitation (LOQ).

1) Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration (reference materials), and comparing the measured value to the true value. The second approach is to compare test results from the new method with results from an existing alternate well-characterized procedure that is known to be accurate. The third approach is based on the recovery of known amounts of analyze. This is performed by spiking analyze in

blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50-150% of the target concentration. The percent recovery should then be calculated. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyze. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyze.

In this study, the accuracy of the proposed method was determined by evaluating the analyze recovery from the sample. The samples ware spiked with a known amount of analyze and the percentage recovery is calculated by comparing the difference of the spiked and non-spiked samples. Recoveries range between 73-107% for particulate PAHs with relative standard deviation (RSD) of less than 10%. Overall, the recovery of PAHs in PM_{10} was considered to be good.

Table 3.2 Acceptable recovery of the concentration of method validation

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%

2) Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation, and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. Precision may be performed at three different levels: repeatability, intermediate precision, and reproducibility. In the present work, 5 replicates of a certain concentration were performed.

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

$$\%RSD = SD/X$$

Where

SD = standard deviation

X = mean of observed values

Repeatability (intra-day assay precision) is the results of the method operating over a short time interval under the same conditions (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each), or from a minimum of six determinations at 100% of the test or target concentration. A precision criterion for an assay method is that the instrument precision (RSD) will be ≤1%, and for the impurity assay, at the limit of quantitation, the instrument precision (repeatability) will be ≤ 5%. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and confidence interval.

The repeatability and reproducibility standard deviation varies with concentration and acceptable values in the following Table 3.3.

Table 3.3 Acceptable repeatability and reproducibility of method validation

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%

In this study, the precision of method was evaluated by finding the intra-day RSD of sample concentrations (n=5). Sample was spiked with a known amount of analyzed and the RSD of the absolute values of concentrations calculated. Precision was less than 10% for particulate PAHs, with the values ranging between 1.9 to 8.7%.

3) LOD and LOQ

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyze. LOD is defined as the lowest amount of analyze that can be detected above baseline noise ratio (S/N). The instrumental LOD and LOQ were calculated as a signal to noise ratio of 3 and 10 respectively. The value of this study ranged from 0.0063-0.1004 ng/ml for 10 PAHs tested.

3.5.3 Determination of PM_{10}-bound PAHs samples

A circle of the filter samples was thoroughly cut into small pieces and placed in a test tube. After adding the internal standard (Pyr-d_{10}, BaA-d_{10} and BaP-d_{12}) of 15 µL to correct analytical variability, the cut filters were ultrasonically extracted with 5 mL of dichloromethane (DCM) for 15 min in three times. The extracted solution was in final volume of 15 mL, centrifugal evaporation was carried out until the DCM was completely evaporated. The residue was added with 225 µL of ethanol, and the solution was then filtered through a centrifugal filter (Ultra free MC-HV, PVDF 0.45 µM, Merck KGaA, Darmstadt, Germany). The resulting solution was in final volume of 450 µL. A 100 µL aliquot of the crude extracted solution was subjected to HPLC-FL for detecting PAHs.

3.5.4 HPLC-FL analyzes

The PM_{10} samples for PAHs determination was analyzed by HPLC-FL with the systems as shown in Table 3.4.

Table 3.4 PAHs analysis system

Equipment	System
Four pumps	LC-20AD, Shimadzu, Kyoto, Japan
a. Imidazole	
b. Acetonitrile	
c. Ascorbic acid	
d. EtOH/Acetate acid	
Fluorescence detector	RF-20Axs, Shimadzu, Kyoto, Japan
System controller	CBM-10A, Shimadzu, Kyoto, Japan
Degasser	DGU-20A, Shimadzu, Kyoto, Japan
Integrator	CBM-20A, Shimadzu, Kyoto, Japan
Auto sample injector	SIL-20AC, Shimadzu, Kyoto, Japan
Column oven	CTO-20AC, Shimadzu, Kyoto, Japan
Guard column	5NPE, 4.6ID × 150 mm (COSMOSIL)
Separator column	πNAP, 4.6ID × 50 mm (COSMOSIL)

The extracted samples were injected to HPLC-FL. Retention time and peak areas of PAHs were investigated by Shimadzu LCsolution software. The quantitation was based on peak area between PAHs standard and internal standard (Pyr-d_{10}, BaA-d_{10} and BaP-d_{12}). The Structures of 10 PAHs analyzed and Schematic diagrams of PAHs analysis system were showed in Figure 3.3 and 3.4 respectively.

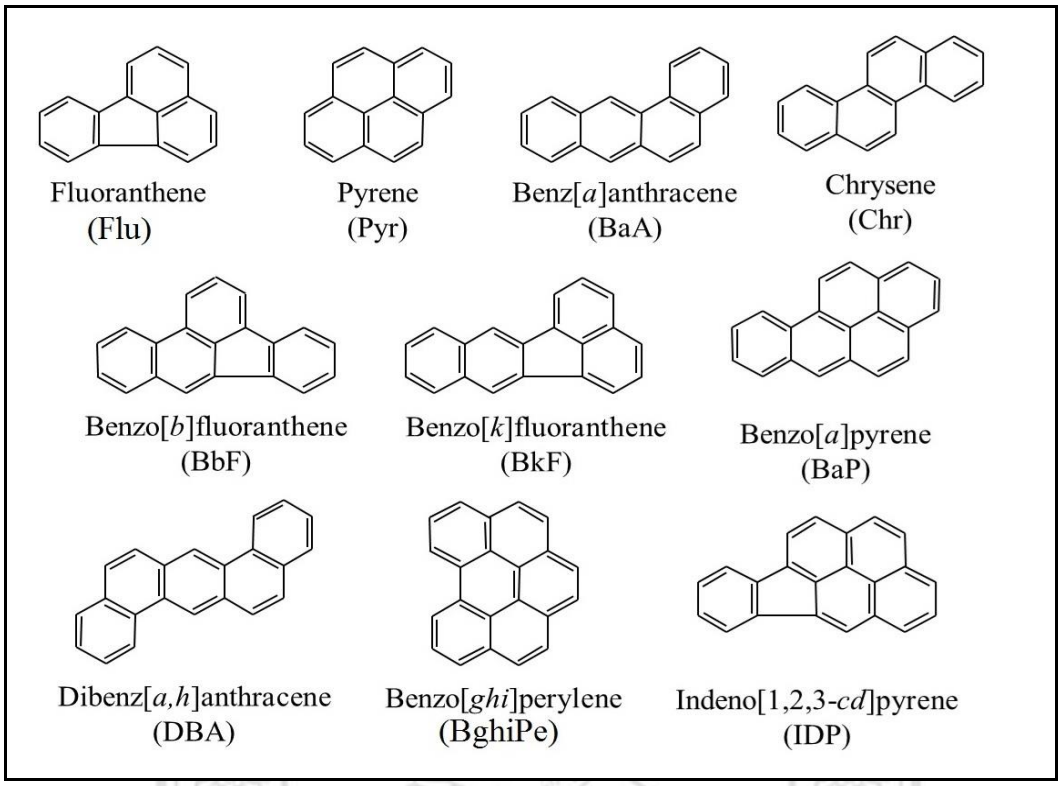

Figure 3.3 Structures of 10 analyzed PAHs

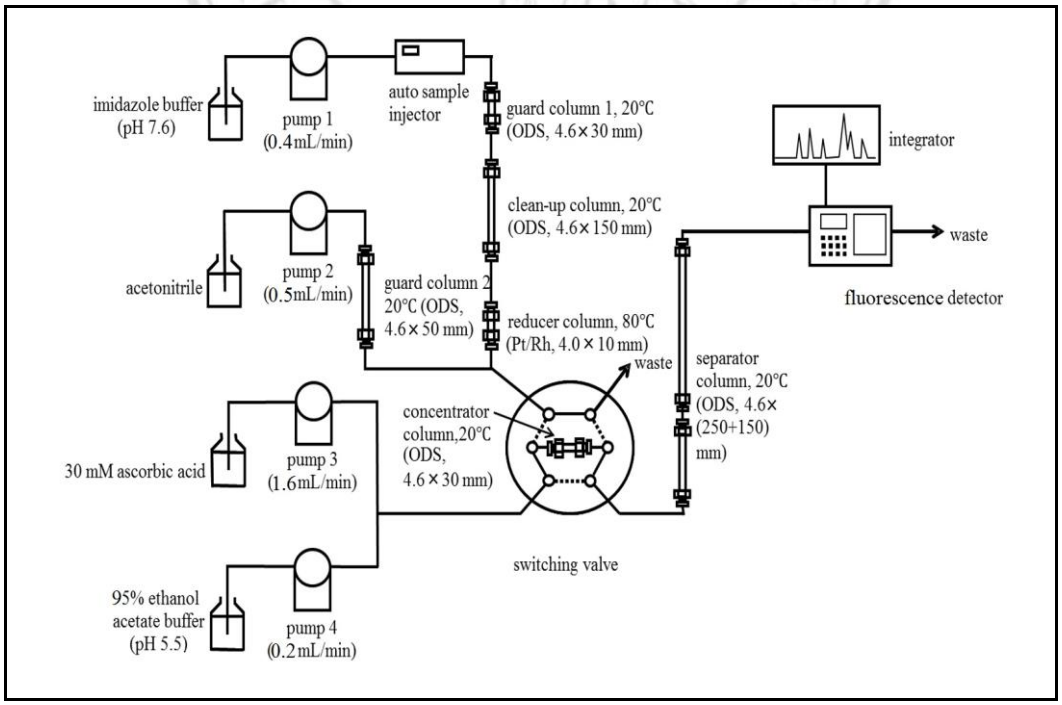

Figure 3.4 Schematic diagram of PAHs analysis system

Table 3.5 Analytical conditions for PAHs analysis

Parameters	Conditions
HPLC-FL	
Mobile phase pumps	Total flow of 0.5 mL/min
	Maximum Pressure of 44.0 MPa
	Minimum Pressure of 0.0 MPa
a. Imidazole	Flow rate of 0.4 mL/min
b. Acetonitrile	Flow rate of 0.5 mL/min
c. Ascorbic acid	Flow rate of 1.6 mL/min
d. EtOH/Acetate acid	Flow rate of 0.2 mL/min
Injection mode	Split mod, Split flow 15-35 µL/sec
Guard column temperature	20ºC
Separator column temperature	85ºC
Binary gradient	
00.00 – 08.20 min	Acetonitrile 100%
08.20 – 08.21 min	Acetonitrile 20%
08.21 – 27.02 min	Acetonitrile 70%
27.03 – 47.01 min	Acetonitrile 80%
47.02 – 90.02 min	Acetonitrile 100%
90.03 – 103.00 min	Acetonitrile 20%
Fluorescence wavelength	
53.25 – 55.55 min	Ch1 : Ex/Em = 283/513 nm Flu
	Ch2 : Ex/Em = 264/407 nm Pyr-d_{10}, Pyr, BaA-d_{10}, BaA, Chr, BbF, BkF, BaP-d_{12}, BaP, DBA, BghiPe
79.50 – 103.00 min	Ch1 : Ex/Em = 294/482 nm IDP

Table 3.6 Characteristic of PAHs standard and internal standard

Compounds	Channel (Ch)	Rt (min)
Flu	1	52.74
Pyr-d_{10} (IS)	2	53.50
Pyr	2	54.04
BaA-d_{10} (IS)	2	56.56
BaA	2	57.30
Chr	2	58.71
BbF	2	63.14
BkF	2	66.01
BaP-d_{12} (IS)	2	67.34
BaP	2	69.21
DBA	2	73.87
BghiPe	2	79.57
IDP	1	84.25

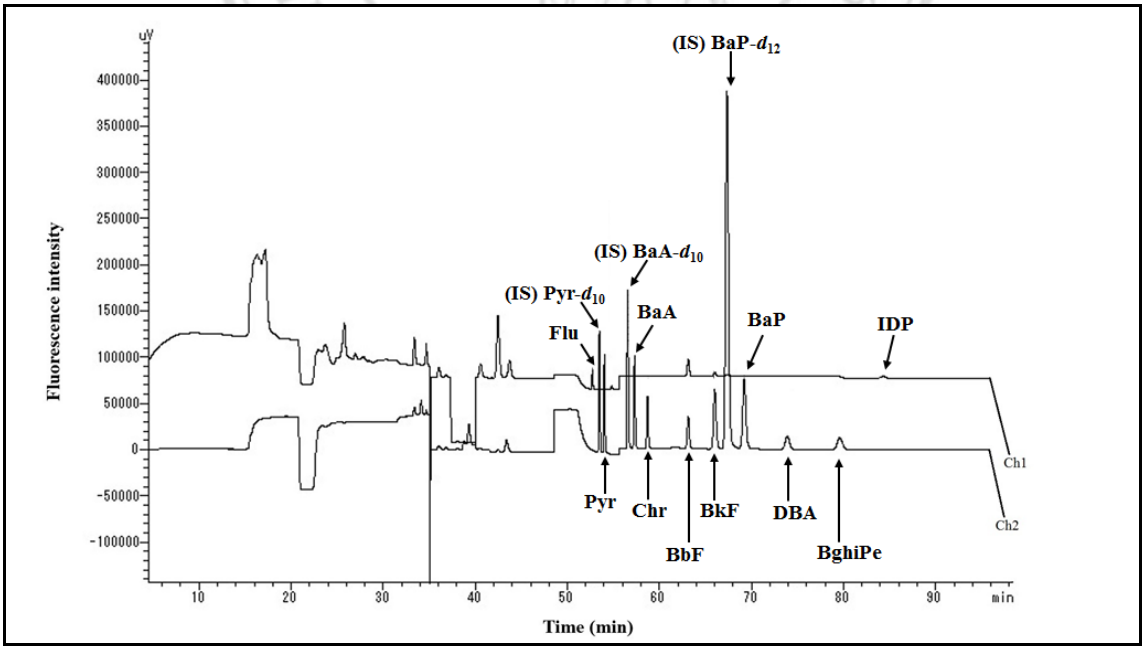

Figure 3.5 Chromatograms of 10 PAHs standard and 3 internal standards

3.6 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 expected as mean ± SD data and the t-test is used to compare the values of means from two related samples (wet and dry season). In order to identify the relationships between the concentration of PAHs and PM_{10}, Pearson correlation was implemented in forms of correlation coefficient (r).