1. INTRODUCTION

1.1 Statement of problems

Chiang Mai Province, with a population of 1.2 million, is located in northern Thailand, and is one of the most fast-growing provinces in the country. The total environment is inevitably polluted along with the development and urbanization if it is not properly protected and managed. Therefore, it is a wise choice to analyze types, sources, and paths of contamination, to evaluate levels of pollution, and to provide environmental information for prevention before serious effects become manifest. Studies on environmental quality of Chiang Mai started only in recent years [1-3]. These studies have revealed that pollution has happened in Chiang Mai to some extent due to traffic, domestic sewage, burning of solid wastes, and others.

Besides environmental monitoring, the epidemiological studies carried out by Martin *et al.*[4,5] showed increased cancer incidence and mortality in recent years. Especially for the lung cancer, Chiang Mai held the highest incidence in comparison to Bangkok, Khon Kaen, and Songkhla. It is recognized that many factors have contributed to this high cancer incidence and mortality. One of the main reasons is likely to be the environmental pollution from air, water, and soil. Polycyclic aromatic hydrocarbons (PAHs), which are widely spread compounds in the human environment, are good indicators for environmental pollution [1,6]. Research on PAHs in airborne particulates showed that the levels of benzo(a)pyrene, benzo(g,h,i)perylene, and benzo(k)fluoranthene in Chiang Mai were higher than those in Tokyo [1]. Most PAHs adsorbed on airborne particulates can be deposited naturally by gravity or are transferred by rain into soil. Therefore, the presence of PAHs in airborne particulates probably influence the occurrence of PAHs in soil. Yang *et al.*[6] reported that the concentrations of PAHs in

soil samples correlated significantly with those in air particulate matter at a significance level of 5%, with a correlation coefficient 0.79. Some other studies also suggested a positive relation between PAHs in air and soil although the clear correlation has not been indicated [7].

Humans may be exposed to the soil pollutants through three pathways, namely, dermal absorption or inhalation of pollutants from dust, plant uptake of pollutants, and leaching of mutagens from the soil to surface of ground water [8]. Evaluation of the mutagenic potential of soil is useful for identification of the source of pollution and provides important information for cancer prevention. Although risk assessment based on the concentration of individual chemicals in a complex mixture holds the advantages in terms of identification and quantification of a variety of chemicals in the mixture, it is limited by several main factors. First, chemical analysis rarely identifies the overall constituent composition of most contaminated mixtures. Secondly, chemical based assessment cannot take into account the potential for synergistic, antagonistic, or additive interactions between component of a complex mixture. Thirdly, biological effects cannot be predicted based on a knowledge of chemical composition alone [8]. Therefore, in addition to chemical analysis, a short-term bioassay, *Salmonella* mutation assay is also employed for detection of mutagens and potential carcinogens in soil because it can often detect the synergistic, antagonistic, or additive interactions of various components. This approach can therefore overcome the limitations of chemical analysis alone [8].

1.2 PAHs as an environmental concern

PAHs are a large group of compounds composed of two or more fused aromatic rings. The simplest members of this group are naphthalene, with two rings, and anthracene and phenanthrene with three rings. Environmental concern has focused on PAHs ranging in molecular size from two- to seven-ring structures. PAHs have received

increased attention in recent years because some of these compounds are highly carcinogenic or mutagenic. The earliest observation of a high frequency of scrotal cancer in chimney sweepers, who exposed to the coal soot which was rich in PAHs, was made more than two centuries ago by Percival Pott [9]. Dibenz(a,h)anthracene was the first such compound to be purified and its carcinogenicity was established in 1930. Benzo(a)pyrene was isolated subsequently from coal tar in 1933 [9]. Because PAHs mostly occur as mixtures, epidemiological studies have not been able to demonstrate the carcinogenicity to humans of individual compounds. However, information from experiments on whole animals and *in vitro* test systems in the last several decades suggests that some compounds are probably or possibly carcinogenic to humans [9,10]. Combining carcinogenicity and occurrence in the environment, sixteen PAHs were recommended by the United States Environmental Protection Agency (US-EPA) as priority pollutants (Table 1.1, Fig. 1.1). The European Community (EC) member states require the determination of six of these PAHs in drinking water.

Table 1.1 Sixteen priority pollutant PAHs by US-EPA [11].

Compound	Abbreviation	Compound	Abbreviation
1. Naphthalene	NAP	9. Chrysene	CHR
2. Acenaphthylene	ACY	10. Benz(a)anthracene	BaA
3. Acenaphthene	ACE	11. Benzo(b)fluoranthene	BbF*
4. Fluorene	FLU	12. Benzo(k)fluoranthene	BkF*
5. Phenanthrene	PHE	13. Benzo(a)pyrene	BaP*
6. Anthracene	ANT	14. Dibenz(a,h)anthracene	DBA
7. Fluoranthene	FLA*	15. Benz(g,h,i)perylene	BPE*
8. Pyrene	PYR	16. Indeno(1,2,3-cd)pyrene	IND*

* PAHs regulated by the EC member states for drinking water.

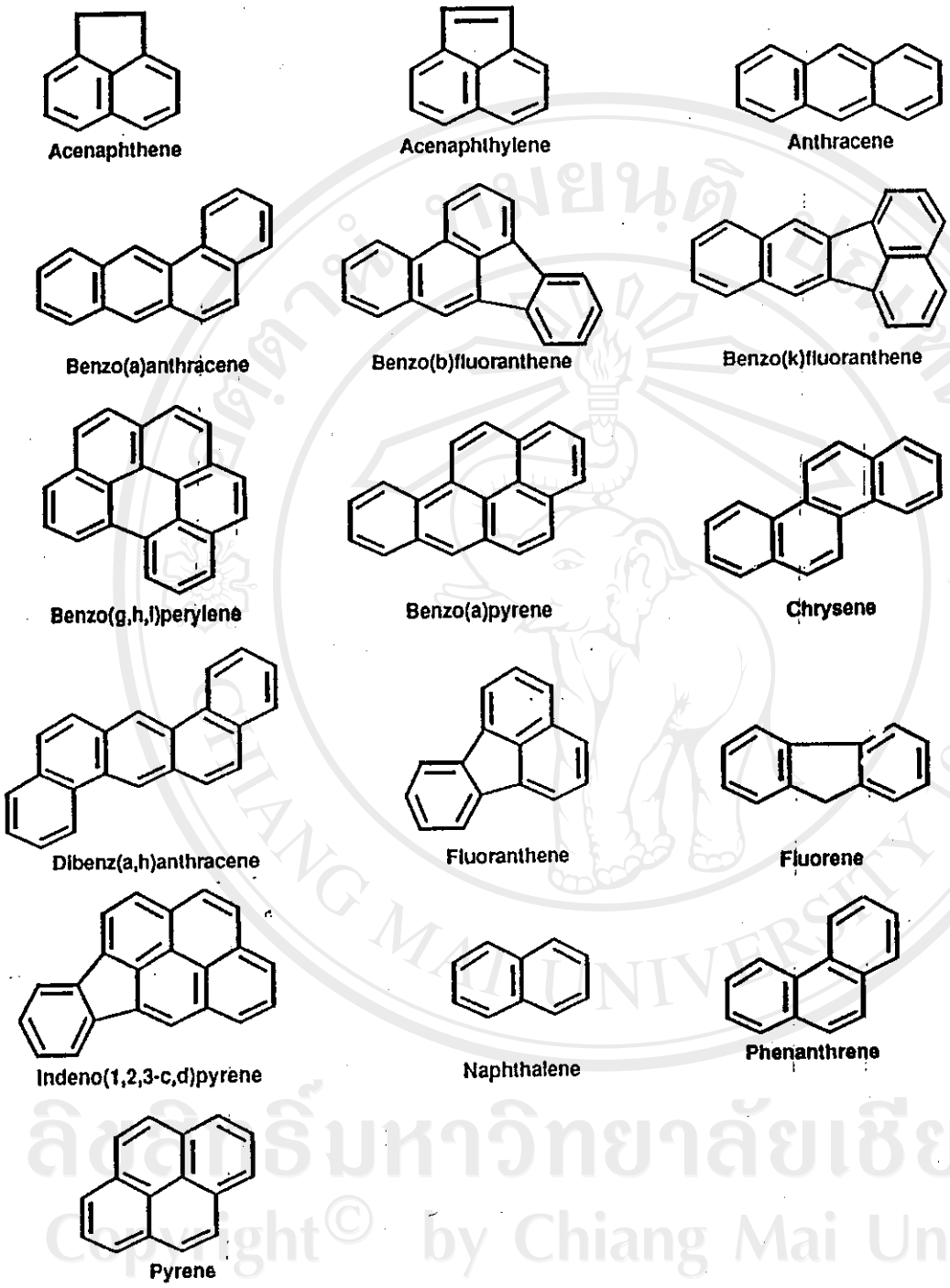


Fig. 1.1 Ring structures of 16 EPA-PAH compounds.

1.3 PAHs in soil and other media

1.3.1 Sources of PAHs in the environment

PAHs occur as a result of any incomplete combustion of organic material which leads to pyrosynthetic reactions. These compounds are typically introduced into the environment by both naturally occurring processes, such as forest fires, and industrial combustion processes, such as the burning of fossil fuels.

PAHs are naturally present at very low concentrations in air, water, soil and smoked food. More recently, because of the increased utilization of fossil fuels, the amounts of PAHs in the environment increase. The sources of environmental contamination are combustion gases from private and industrial heaters, burning of refuse, automobile exhausts, and tobacco smoke (Table 1.2).

Table 1.2 Man made sources of PAHs in the environment[a].

Compound	Cigarette smoke (g/100 cigarettes)	Exhaust condensate (ml/g gasoline consumed)	Coke oven emission (g/g)	Coal tar emission(mg/g)
BaP	2.5	0.05-0.08 340 ppm	4,100	-[b]
BaA	0.3	0.05-0.08	105-2,740	-
DBA	0.4	96 ppm	84-124	0.41
BbF	0.3	0.019-0.048	-	-
IND	0.4	0.032-0.086	101.5	-
ANT	-	0.53-0.64	46.4-942.8	-
CHR	6.0	0.085-0.123	-	-
BPE	-	0.12-0.33	-	-
FLA	-	1.06-1.66	269.7-5,979	144.8
PYR	-	2.15-2.88	206-4,627	105.5

[a] The data of this table are summarized from reference [12].

[b] -, not available.

1.3.2 PAHs in soil

Soil is a strong matrix for retention of PAHs. The nature of the association of PAHs with soil particles remains unclear. Various mechanisms have been proposed, including partitioning, surface adsorption, intraparticle transport, and trapping with in the interior of aggregates [6,13]. The PAHs occurring in soil are mainly from airborne PAHs by physical clean-up. Most PAHs which are found in airborne particulates are often detected in soil [6].

Soil is a long-term sink for airborne PAHs and the concentrations of PAHs are influenced by a wide variety of environmental factors, including redistribution of PAHs with the soil profiles, surface adsorption factors, photodegradation at soil surface, degradation by microorganisms, and temperature [6,14]. PAHs in soil vary greatly for different sites and different soil profiles. They are mostly distributed in the upper soil layer 0-10 cm, and also can penetrate to 10-20 cm, but rarely reach under 20 cm [7,15]. The concentration in soil can be undetectable in some clean soil, but it may be as high as 450 and 2,690 ppb for benzo(a)pyrene and total PAHs, respectively (Table 1.3).

Table 1.3 PAH concentrations in soil from previous studies (ng/g dry soil).

	Forest soil[a]	Saarland, Germany[b]			Brisbane, Australia[c]
		Urban area	Rural area	Background	Roadside
Compound		(n = 15)	(n = 5)	(n = 8)	
NAP	-	10 (0-50)	3 (0-7)	3 (0-8)	-
ACY	-	1 (0-5)	1 (0-5)	1 (0-2)	-
ACE+FLU	-	30 (0-80)	4 (0-10)	2 (1-3)	174
PHE	-	165 (20-260)	35 (20-70)	15 (6-20)	71.7
ANT	-	40 (10-100)	5 (2-10)	2 (1-7)	27.8
FLA	-	410 (65-1,100)	95 (55-175)	25 (15-50)	-
PYR	-	345 (50-940)	75 (40-150)	20 (10-35)	214
BaA+CHR	-	370 (70-950)	85 (30-185)	25 (15-35)	414
BbF	25-111[d]	170 (45-315)	50 (3-75)	15 (10-25)	-
BkF	-	115 (10-315)	20 (10-30)	7 (4-15)	201
BaP	1.5-4.0	185 (30-450)	30 (10-55)	8 (4-10)	363
DBA	-	75 (10-355)	6 (3-10)	3 (0-9)	42.3
BPE	10-70	145 (25-375)	20 (10-35)	6 (2-10)	598
IND	-	130 (30-290)	25 (10-35)	6 (2-15)	584
Total					2690

[a], data from reference [12].

[b], data from reference [16], expressed as mean (min-max).

[c], data from reference [6].

[d], including benzo(j)fluoranthene.

1.3.3 PAHs in airborne particulates and water

More studies on PAHs in the environment are focused on airborne particulates because PAHs in the air are more directly in contact with humans from inhalation [1,17,18]. The fate of PAHs in the atmosphere depends on the size of the specific compound. Many lower molecular weight PAHs are volatile. Adsorption onto airborne particles is the likely fate of many large molecular weight PAHs. These airborne particles may be inhaled by organisms resulting in exposure to PAHs. These adsorbed PAHs photodecompose readily in the atmosphere with ozone and various oxidants. Degradation times range from less than one day to several weeks [19]. Airborne PAHs that do not photodecompose are eventually returned to aquatic and terrestrial ecosystems by precipitation.

Airborne PAHs vary with different seasons, with relatively low concentration in summer time because of washing by rain and stronger photodecomposition, and higher in winter along with the increased use of fuels for heating. Generally, the amount of PAHs in air is lower when traffic volume is lower, the mean temperature higher, and the mean wind speed higher [6].

The possible sources of water PAHs include atmospheric deposition, surface runoff, municipal waste effluents, industrial effluents, spills and leakage during transport and production of fossil fuels, natural seepage, and erosion of exposed shales and coal seams [19,20]. PAHs are also formed through direct biosynthesis and from biogenic precursors [19]. Due to their hydrophobic nature, most PAHs in aquatic ecosystems rapidly become associated with particles and are deposited in sediments. They return from sediment due primarily to resuspension. A variety of processes, including volatilization, sedimentation, chemical oxidation, photodecomposition, and microbial degradation, are important mechanisms of environmental loss of PAHs [20,21].

1.4 General toxicity and carcinogenicity of PAHs

1.4.1 General toxicity of PAHs

PAHs have little inherent biological activity; their toxicity results from the formation of reactive metabolites. Metabolism of PAHs is via the cytochrome P450 mediated mix function oxidase system with oxidation or hydroxylation as the first step. The resultant epoxides or phenols may then go through a detoxification reaction to produce glucuronides, sulfate, or glutathione conjugates [22]. Some of the epoxides, however, may be metabolized to dihydrodiols, which in turn may undergo conjugation to form soluble detoxification products or oxidation to diol-epoxides. These latter compounds are thought to be the ultimate carcinogens in cases where carcinogenicity has been demonstrated. The metabolites of PAHs are primarily eliminated by urine and faeces as water-soluble compounds.

The toxicity of individual PAHs differs with molecular weight. Unsubstituted lower molecular weight compounds containing two or three rings (e.g., naphthalene, fluorene, phenanthrene, and anthracene) exhibit acute toxicity and other adverse effects to some organisms and humans, but are not carcinogenic. In contrast, higher molecular weight compounds (four-seven rings) are significantly less acutely toxic, but many are demonstrably carcinogenic, mutagenic, or teratogenic to a variety of organisms, including fish, amphibians, birds, and mammals.

Significant ways of exposure to PAHs are through inhalation, ingestion, and dermal contact [23,24]. The most important source of lung exposure is through inhalation of fuel-combustion products or cigarette smoke. Contact of PAHs with lung tissue can cause severe pulmonary edema, pneumonitis, and hemorrhage [25]. PAHs are highly lipid-soluble and readily absorbed from the gastrointestinal tracts of mammals. They are rapidly distributed to a wide variety of tissues, with a particular tendency for localization in

body fat. PAHs are primary irritants when in contact with skin, thus causing dermatitis. Information on the acute, subacute, and chronic toxicity of PAHs is limited. Previous researches [22,25] demonstrated that exposure to PAHs could depress the activity of immune systems, causing significant decrease in levels of IgA, IgM, IgG, and IgE as well as T-lymphocytes. Exposure to PAHs could also decrease the body weight, inhibit mitochondrial respiration and increase liver weight.

1.4.2 Mechanisms of carcinogenesis of PAHs

The International Agency for Research on Cancer (IARC) has evaluated a number of chemicals for carcinogenicity to humans. The chemicals are classified into four groups according to experimental data (Appendix 2). Among the EPA-PAHs, benzo(a)pyrene, benz(a)anthracene, and dibenz(a)anthracene are considered to be probably carcinogenic to humans on the basis of experimental data obtained from both whole animals and *in vitro* test systems (Group 2A). Experimental data also indicate that benzo(b)fluoranthene, benzo(k)fluoranthene and indeno(1,2,3-cd)pyrene are possibly carcinogenic to humans (Group 2B) [9.10]. The carcinogenicity of other PAHs is given in Table 1.4.

PAHs have been primarily tested for skin carcinogens; they are very active in mice, much less active in rats and hamsters and inactive in guinea pigs [26]. It was long believed that metabolites of PAH compounds were the proximate carcinogenic agents, initially epoxides at the so-called "K-region". The later proposal [27] that dihydrodiol-epoxides, formed in the Bay-region of benzo(a)pyrene and similar hydrocarbons, were the proximate carcinogens was more consistent with the data. Many PAHs are metabolized to Bay-region diol-expoxides (Fig. 1.2).

Benzo(a)pyrene is the most commonly studied PAHs of the environment. It is useful as a model PAH for studying interactions with DNA. As shown in Fig 1.3, benzo (a)pyrene is metabolized by P450 to benzo(a)pyrene-7,8 epoxide which is hydrated by epoxide hydrolase to form the proximate carcinogen, benzo(a)pyrene-7,8-diol, and then

further metabolized by P450 to form the ultimate carcinogen, benzo(a)pyrene-7,8-diol-9,10-epoxide [22,28,29]. This reactive intermediate binds covalently to DNA forming DNA adducts. Benzo(a)pyrene-7,8-diol-9,10-epoxide binds to several bases in DNA but binds preferentially to deoxyguanine residues. Benzo(a)pyrene-7,8-diol-9,10-epoxide is highly mutagenic in eukaryotic and prokaryotic cells and carcinogenic in rodents. DNA adduct formation is thought to be involved in mutation and neoplastic transformation of target organs. The major DNA adducts have been well characterized in animals. PAH-DNA adducts could also be found in human peripheral blood lymphocytes, human lung tissues and human placenta [30-32].

Table 1.4 Degree of evidence for carcinogenicity and overall evaluations of cacinogenicity to humans for selective PAHs [9,10].

Compound	Degree of evidence for carcinogenicity		Overall evaluation
	Human	Animal	
BaP	ND	S	2A
BaA	ND	S	2A
DBA	ND	S	2A
BbF	ND	S	2B
BkF	ND	S	2B
IND	ND	S	2B
ANT	ND	I	3
BPE	ND	I	3
CHR	ND	L	3
FLA	ND	I	3
FLU	ND	I	3
PHE	ND	I	3
PYR	ND	I	3

ND = not adequate data, I = inadequate evidence, L = limited evidence, S = sufficient evidence.

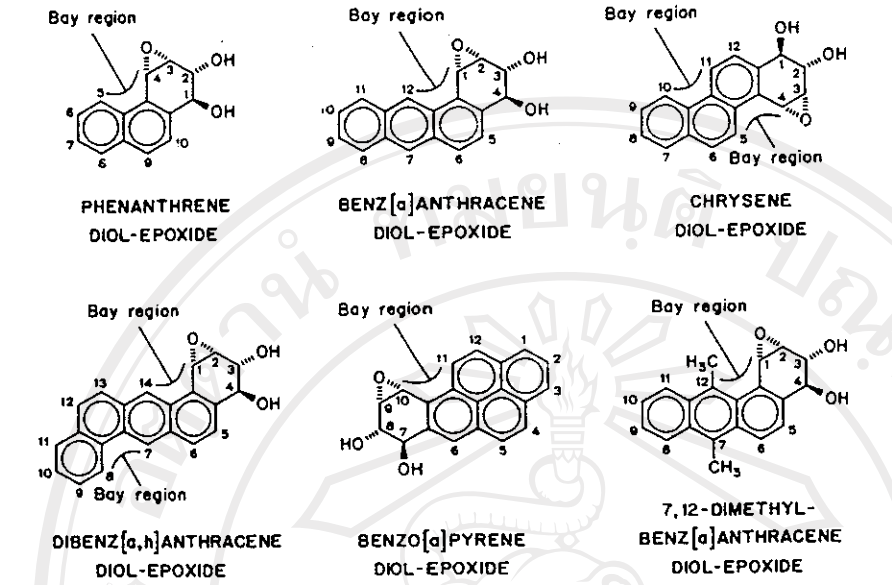
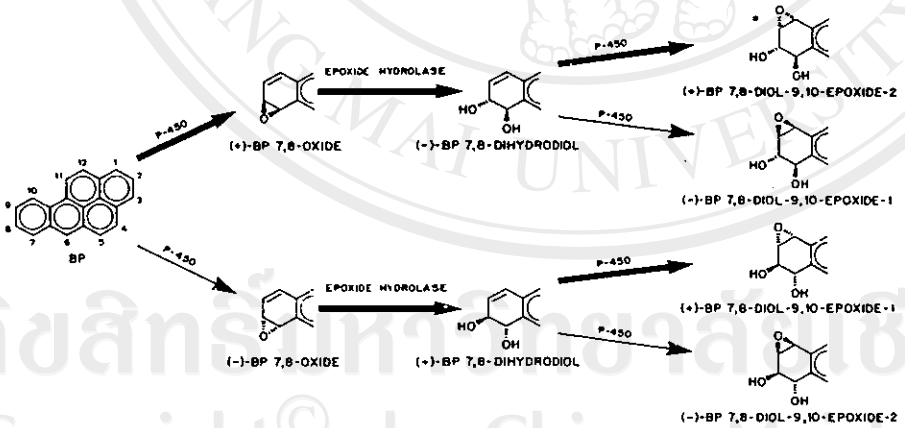


Fig. 1.2 Structures of bay-region diol-epoxides of some PAHs [28].



* Ultimate carcinogen — note stereochemistry.

Fig. 1.3 Formation and absolute stereochemistry of BaP metabolites responsible for the carcinogenicity of parent hydrocarbon. Heavy arrows indicate the major metabolic pathway [28].

1.5 Analytical techniques for determination of PAHs in the environment

The carcinogenicity of PAHs is dependent on their structure. While benzo(e)pyre is a weak carcinogen, isomeric benzo(a)pyrene is strongly carcinogenic. Therefore, it is important to analyze individual PAHs in each environmental sample.

Several analytical techniques have been established. These techniques are based on gas chromatography (GC) [14,33,34], GC with mass spectrometry (MS) [35], or high performance liquid chromatography (HPLC) [36-38]. EPA method 610 describes the analysis of PAHs in municipal and industrial waste water while EPA method 8100 and 8310 provide for the analysis of liquid and solid hazardous wastes. These methods specify GC with flame ionization detector (FID) or HPLC with ultraviolet (UV) and fluorescence detectors. Although FID is a commonly used detector, compound identification is difficult since this detector responds to all carbon containing materials.

HPLC offers significant advantages: 1) PAHs are high molecular weight compounds with low volatility. With HPLC, these compounds can be separated at ambient temperatures. 2) Liquid chromatography can be connected to UV/Visible diode-array detectors which are valuable for positive identification and for quantification of co-eluting components. 3) Liquid chromatography can be connected to a fluorescence detector which is highly selective and sensitive.

A common method for PAHs determination uses HPLC with UV detection at 254 nm because these compounds can be determined with good sensitivity under these conditions. This is easily done with a UV/Visible detector. Another popular option is the use of fluorescence detection for some of the PAHs, as many of these compounds have a high natural fluorescence. Fluorescent methods are capable of measuring subnanogram quantities of PAHs. However, this method tends to be fairly non-selective. The normal spectra obtained tended to be intense and lacked resolution. Efforts to overcome this

difficulty leads to the use of UV absorption spectroscopy as the detection method complete with pre-speciated techniques involving liquid chromatography (LC) and thin layer chromatography (TLC) to isolate specific PAHs, partially BaP [39].

A diode array detector can simultaneously provide qualitative and quantitative information. The diode-array detector is used to identify peaks by UV/Visible spectra. With the diode-array detector, amounts as low as around 0.5 ng per component could be detected. If lower detection limits were required, a fluorescence detector should be used. For PAHs, this detector is about 1,000 times more sensitive than a UV detector. In order to get the highest sensitivity for the whole analysis, the fluorescence detector is the detector of choice. Optimal values from such a detector can be found by using the scan function incorporating in this instrument. The individual compounds pass the detector flow cell and the fluorescence light is measured as a function of the excitation and emission wavelengths. After the optimization of parameters, the minimum detectable level of a particular PAH is generally lower to less than 0.5 pg per compound. Therefore, high performance liquid chromatography in combination with UV/Visible diode-array and fluorescence detection is a selective and sensitive method for the analysis of PAHs in environmental samples.

The choice of GC and HPLC as the analysis techniques for PAHs is influenced by their sensitivity, selectivity, their ability to analyze complex samples as well as availability. The appropriate choice of detectors depends upon the actual method being used and the detection limits required for the samples being studied. Generally, methods for the analysis of samples with few matrix components, other than drinking water, may be analyzed by UV absorbance at 254 nm. Drinking water samples require the added sensitivity of fluorescence detection. More complex samples such as soil, may require fluorescence detection for added selectivity.

1.6 Short-term tests (STT) for screening environmental mutagens and carcinogens

The carcinogenic potential of a chemical can be measured with a life time animal bioassay, which usually takes two or three years. Mutagenicity experiments are often used to evaluate the potential for inducing tumors because of basic similarities in the postulated molecular mechanisms of chemical carcinogenesis and mutagenesis [40]. Short-term tests have become predominant biological assays for detecting and assessing genotoxic and mutagenic efforts. STT are in wide-spread use and many different systems have been developed [41]. More than a hundred procedures for short-term assay have been developed and more than thirty kinds of short-term bioassay are used for screening and detecting genotoxins [42-44]. In short-term test systems for detecting the mutagenicity of compounds, microorganisms, fungi, plants, insects, and cultured cells of humans, hamsters and mice, are extensively used. Various damages produced in chromosomes and DNA are detected by these test systems. The most widely used STT assay is the *Salmonella*/ microsome test due to its simplicity, less cost, and good correlation to carcinogenicity. It is estimated to be used worldwide in more than 2,000 laboratories [41]. More than 5,000 compounds have been published by using this assay [45]. The tests are increasingly being used to determine the mutagenicity of complex environmental mixture[8,17,46-49]. The *Salmonella*/microsome assay is based on the detection of mutated histidine-dependent strains (his^-) of *Salmonella typhimurium* which can mutate back (reversion) to the wild-type (his^+) if they are exposed to mutagenic compounds.

Most mutagens are not direct acting compounds, but need metabolic activation to make them reactive. Bacteria do not contain the spectrum of monooxygenase found in higher animals, thus, Ames [50] incorporated his assay a crude fraction of rat liver obtained by centrifugation at 9,000 g, consisting mainly of microsomes, as an activating system.

One of the objectives for detecting mutagen is to detect predictive carcinogens by screening the chemicals which induce gene mutations and chromosome aberrations. Early studies from three laboratories [51-53] suggested that *Salmonella* mutation assay could identify nearly 90% of possible carcinogens. The test failed to detect some classes of carcinogens such as polychlorinated pesticides (DDT, DDE) [45,52]. Nevertheless, the *Salmonella* assay is an instant success in the screening for the potential carcinogenicity of chemical compounds. The simplicity of this assay made it more useful when complex and expensive animal carcinogenicity tests were impractical [51-54].

1.7 Purposes of the study

The aims of this thesis work are:

1) to determine 16 EPA-PAH compounds in soil samples from Chiang Mai Province by HPLC with UV detection,
2) to measure the mutagenicity of extracts from soil samples by *Salmonella* mutation bioassay, and
3) to evaluate the correlation between mutagenicity and PAH levels.