2. MATERIALS AND METHODS

2.1 Apparatus and chemicals

2.1.1 Apparatus

(1) High performance liquid chromatography system, manufactured by Waters Chromatography Division, Milford, U.S.A., consisting of:

a) Autosampler, Waters 717

b) System Controller, Waters 600E and Fluid Handling Unit Connections

c) Spherisorb ODS-25 Micron column (250mm×4.6mm) with Guard-Pak HPLC Precolumn

d) Tunable UV-Visible Absorbance Detector, Waters 486

e) Chromatography Workstation, Maxima 820, NEC.

(2) Filter apparatus, manufactured by Waters, Chromatography Division, Milford, Waters, U.S.A.

(4) Ultrasonicator, model 2200, Branson, U.S.A.

(5) Autoclave SS-240, Tomy Sciko Co.Ltd., Tokyo, Japan

(6) Analytical balance, AC 100 Mettler Instrument A.G., Switzerland

(7) Circulating aspirator, WJ-15 Sibata, Japan

(8) Dispenser Dispet, TM Nichiyo Co., Ltd., Japan

(9) Incubator, B 5050 Heraeus, Germany

(10) Milipore Holder, SX001300, Milipore Corporation, U.S.A.

(11) Refrigerator (-80°C), Sanyo, Thailand

(12) Stereomicroscope, VMZ-4SA-2W, Olympus, Japan

(13) Rotary vacuum evaporator, Buchi, Switzerland

(14) Water bath, Type 1 No. D 7095 Yazama, Japan

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2.1.2 Chemicals

(1) Dichloromethane, organic residue analysis grade, J.T. Baker Inc., USA

(2) Cyclohexane, pesticide grade, J.T. Baker Inc., USA

(3) Acetonitrile, HPLC grade, Riedel-de Haen AG, Germany

(4) Supelpreme-HC PAH Mix (16 EPA-PAHs), 4-8905, methylene chloride : benzene, 50:50, Supelco Inc., USA

(5) Chrysene, Sigma Chemical Co., U.S.A.

(6) Benzo(a)pyrene, Sigma Chemical Co., U.S.A.

(7) Benz(a)anthracene, Sigma Chemical Co., U.S.A.

(8) Dibenz(a,h)anthracene, Sigma Chemical Co., U.S.A.

(9) Anthracene, Sigma Chemical Co., U.S.A.

(10) Anhydrous sodium sulfate, GR grade, Merck, Germany

(11) Silica gel 60-200 mesh, Grade 62, Aldrich Chemical Company, Inc., U.S.A.

(12) Ampicillin (U.S.P), L.B.I.

(13) Bacto agar, Difco laboratories, U.S.A.

(14) d-Biotin, Sigma Chemical Co., U.S.A.

(15) Crystal violet, Sigma Chemical Co., U.S.A.

(16) Dimethylsulfoxide (spectroscopic grade), E. Merck, Germany

(17) Dipotassium hydrogenphosphate, Fluka A.G., Buchs, Switzerland

(18) Disodium hydrogenphosphate, E.Merck, Germany

(19) D-Glucose 6-phosphate, Sigma Chemical Co., U.S.A.

(20) Magnesium chloride, May and Baker Ltd., England

(21) Magnesium sulfate ($MgSO_4.7H_2O$), Fluka A.G., Buchs, Switzerland

(22) Milipore membrane, Nihon Milipore, Kogyo, Yonezawa, Japan

(23) β-Naphoflavone, Aldrich Chemical Company Inc., U.S.A.

(24) β-Nicotinamide adenine dinucleotide (β-NADH), Orental Yeast Company, Japan

(25) β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH)

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Orental Yeast Company, Japan

(26) Oxoid nutrient broth No. 2, Oxoid Ltd., England

(27) Phenobarbital sodium (U.S.P.), Wako Pure Chemical Industries, Ltd., Japan

(28) Potassium chloride, May and Baker Ltd., England

(29) Sodium ammonium phosphate ($NaNH_4PO_4.4H_2O$), E. Merck, Germany

(30) Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), E.Merck, Germany

(31) Sodium chloride, E. Merck, German

2.2 Soil collection

2.2.1 Study sites

All soil samples were collected from Muang District, Sarapee District, and Doi Suthep area in Chiang Mai Province, Thailand. From previous studies, Muang and Sarapee Districts would be the most polluted Districts in Chiang Mai Province [2-5]. Doi Suthep mountain, which is located about 5 km west of Chiang Mai town, should be relatively clean and subject to less human activities, such as traffic pollution. This area was therefore chosen as the control site in this study. Seven soil samples, collected from Muang District, were distributed randomly in the city, but more samples were collected in the east part of the city because of the higher population density in this area. Most of the 9 samples from Sarapee District were concentrated in the central area in this suburb. The gardens are connected each other in this suburb; roads are narrow and cars seldom pass. Therefore, most samples from Sarapee District were collected from the gardens. Two samples from Doi Suthep were located in the temple area on the mountain. The descriptions of these study sites are given in Table 2.1 and Fig. 2.1.

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Table 2.1 Description of study sites.

Site	District	Location
M1	Muang	Kuang Singh, market area, near a road
M2	Muang	Nawaratt Bridge, garden
M3	Muang	Anusarn, garden
M4	Muang	Wat Prasing, near a road
M5	Muang	Thipanet, near a road
M6	Muang	Srinakrin Health Center, near a park
M7	Muang	Nakorn Ping Bridge, garden, near a road
S1	Sarapee	Kuamung, garden
S2	Sarapee	Yang Nueng (east), garden
S3	Sarapee	Yang Nueng (south), garden
S4	Sarapee	Sarapee (north), school
S5	Sarapee	Yang Nueng (north-west), garden
S6	Sarapee	Nong Pueng, garden
S7	Sarapee	Yang Nueng (center), market area
S8	Sarapee	Sarapee (west), garden
S9	Sarapee	Nong Pueng (north), near a road
D1	Doi Suthep	Wat Pahlaht
D2	Doi Suthep	Wat Prathat, garden

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2.2.2 Sampling method

The upper 0-10 cm soil was collected using a 10 mm i.d. core sampler. At each site, 5 individual portions of soil were taken across a 5×5 m sampling plot. The portions were combined, and stones and roots were removed. All soil samples were sealed in solvent-cleaned aluminum foil containers and immediately transported to the laboratory and stored at -20°C until required for analysis.

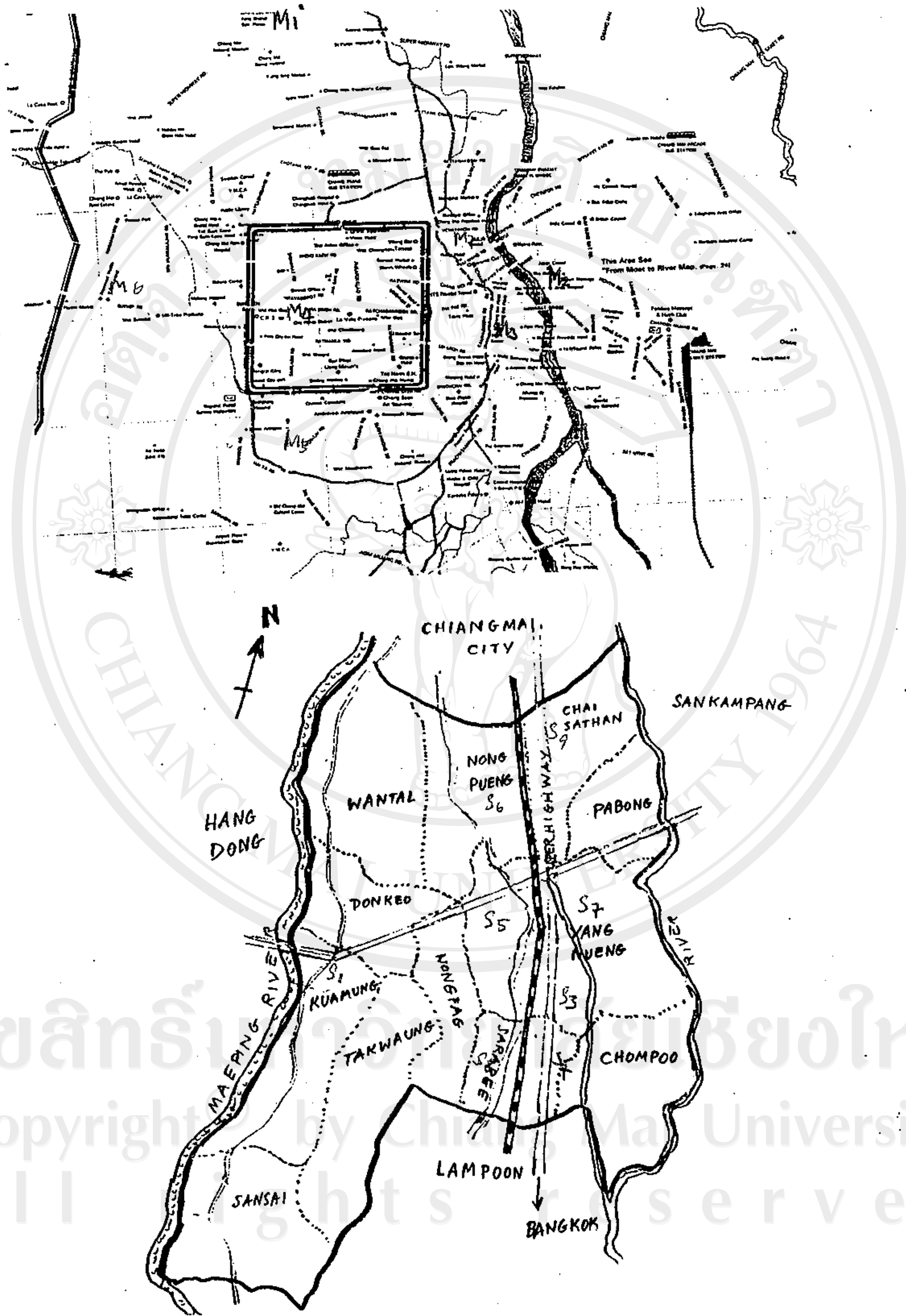

Fig. 2.1 Map of Muang and Sarapee Districts showing sampling sites.

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2.3 Soil sample preparation

2.3.1 Procedure for HPLC analysis of EPA-PAHs in soil

The following procedure was modified from the Standard Operating Procedure for analysis of EPA-PAHs in soil [16].

2.3.1.1 Sample handling

Each air-dried soil sample was homogenized and let to pass through a 2 mm sieve. About 20 g of soil was put into a conical flask, with the weight recorded to the nearest 0.01 gram.

2.3.1.2 Extraction

Approximately 60 ml of dichloromethane was added to the soil sample and the mixture was sonicated in an ultrasonic bath for 10 minutes. The extract was filtered through Whatman No. 41 filter paper into a round bottom flask. Sodium sulfate was put into the funnel before filtering in order to remove water from the sample. Ultrasonic extraction was then repeated for a second time. The flask and the funnel were rinsed with dichloromethane after subsequent filtration. The solvent was evaporated by means of a vacuum rotary evaporator at about 35°C to approximately 2 ml. Then 2 ml of cyclohexane was added and treatment with the vacuum evaporator was resumed until the volume of the solution was reduced to 1 ml.

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2.3.1.3 Sample clean-up

A 10 mm i.d. chromatographic glass column was filled with a slurry of 5 g silica (10% water) in cyclohexane and air bubbles were avoided. The column was then washed with 10 ml cyclohexane. The extract was pipetted onto the column, and no action was taken until the extract was in the sorbent layer. The flask was rinsed 3 times with

cyclohexane, using about 1 ml each time. The impurities and interferences were eluted with 10 ml cyclohexane. These were collected in a 10 ml volumetric cylinder and discarded. PAHs were eluted in the second fraction of 10 to 60 ml cyclohexane, and collected in a round bottom flask. The eluate (10 to 60 ml) was evaporated to about 5 ml using a rotary evaporator. Then it was transferred to a small V-shaped flask. The round bottom flask was rinsed. The eluate was carefully evaporated to about 2 ml with the aid of a rotary evaporator and further to dryness under a stream of nitrogen. The residue was then redissolved in acetonitrile and stored in a refrigerator before any injection into the HPLC commenced (Fig. 2.2).

2.3.2 Procedure of preparation for *Salmonella* mutation assay

Fifty grams soil sample was extracted by 100 ml dichloromethane with sonication for 10 minutes. This extraction was repeated twice. After filtering through No. 41 Whatman filter paper, the filtrate was evaporated to dryness in a vacuum rotary evaporator at 35°C. The dry residue was weighed, redissolved in dimethylsulfoxide (DMSO) and passed through Millipore filter membrane to obtain a sterile solution for using in *Salmonella* assay. Samples were stored at 0°C in the dark prior to mutation assay.

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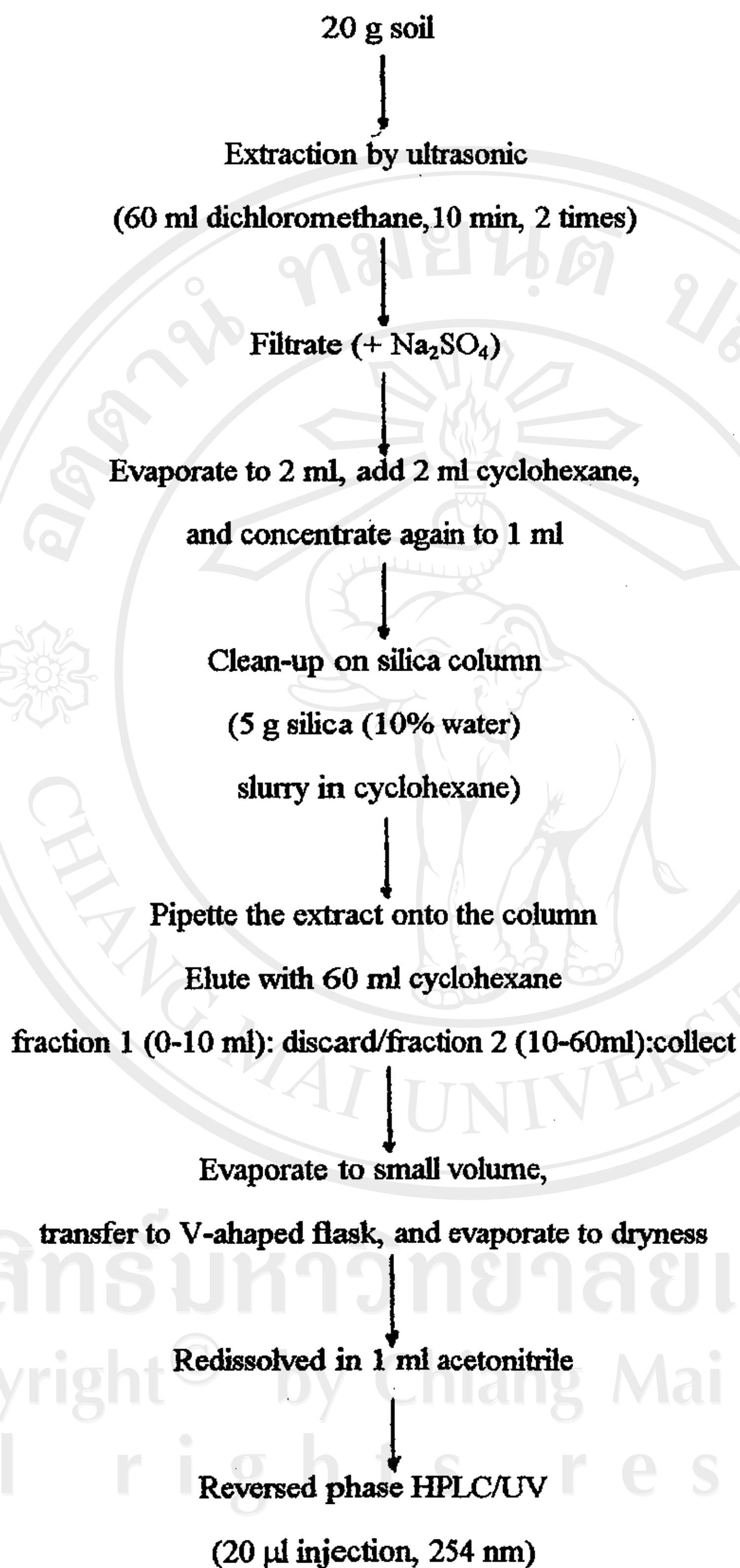

Fig. 2.2 Scheme of sample preparation for HPLC analysis of EPA-PAHs in soil.

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2.4 HPLC analysis of soil samples

2.4.1 HPLC conditions

Gradient HPLC system consisting of acetonitrile and water phase reservoirs was used. The operating conditions, which were modified from EPA method 8310, are as follows:

Column: Spherisorb ODS-25 Micron

Gradient elution:

Min	%Acetonitrile	%Water
0	50	50
30	100	0
35	100	0
36	50	50
46	50	50

Flow rate: 1.0 ml/min.

Helium sparge: 20 ml/min.

Injection volume: 20 μl.

Detection: UV 254 nm.

2.4.2 Qualitative analysis

The identification of sample components was undertaken in this study by means of comparison of the retention times with those of PAH standards. When any peak of a sample chromatogram matched well with one of the standard PAHs in the standard chromatogram, further confirmation would be needed.

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2.4.3 Quantitative analysis

The individual PAHs in the samples was determined by the external standard method. Quantitative data were taken from chromatographic peaks by means of peak height. The amounts of PAHs in the samples were calculated from the calibration curves employing the Maxima data system.

2.4.4 Confirmation analysis

Confirmation analysis was carried out by comparing the ratio of the peak heights of a component at two different wavelengths, 254 nm and 297 nm, to that of the standard.

Attempts to confirm identifies of PAHs by GC/MS in the Department of Chemistry at Chiang Mai University yield no significant peaks when 10 ppm PAH standard mixture was injected onto the capillary column of the GC/MS. Some samples were then sent to the University of Saarland, Germany and further confirmed by GC/MS conducted by Dr. Joachim Krueger.

2.4.5 Construction of calibration curves

Stock standard solutions with PAH concentrations of 1.00 μg/ml each were prepared by diluting Supelpreme-HC PAH Mix (16 EPA-PAHs, 2,000 ppm each) in acetonitrile in a 10 ml volumetric flask. Calibration standards at a concentration levels of 0.1, 0.2, 0.5, 1.0 μg/ml were then prepared by appropriate dilution. Data of the peak height response vs. concentration injected were obtained from these standards under the HPLC conditions employed. All calibration runs were performed as described for sample analysis.

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2.4.6 Detection limit and determination limit

The detection limit was achieved as two times greater than the noise level of base line. The determination limit includes the whole sample preparation and HPLC conditions,

for instance, sample weight, final volume of the cleaned up extracts and injection volume. This limit is called by the USA-EPA the "method detection limit (MDL)". This is calculated after determination of detection limit.

2.4.7 Surrogate standard recovery

The PAH standards were surrogated into the soil sample for measuring the recovery rate. The surrogated soil samples were subject to the same preparation and analysis procedure as the other soil samples. The percent recovery was calculated by the following equation:

$$\text{recovery\%} = \frac{A_{ssp} - A_{sp}}{A_{std}} \times 100\%$$

where A_{ssp} = the amounts of PAHs detected in surrogated soil sample

A_{sp} = the amounts of PAHs detected in soil sample

A_{std} = the amounts of standard PAHs surrogated into the soil sample

2.5 *Salmonella* mutation assay

2.5.1 The bacteria tester strains

Salmonella typhimurium strains TA98 and TA100, supplied by Prof. Dr. Taijiro Matsushima, Japan Bioassay Laboratory, Japan, were used. They were kept at -80 °C frozen and were cultured in Oxoid nutrient broth No. 2 at 37 °C for 14 hours. The culture contains about 10^9 cells per ml. The genotype of the tester strains was confirmed before use, including histidine requirement, *rfa* mutation, R-factor, *uvr*B mutation, and positive mutagen response and spontaneous reversion (Appendix 3).

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2.5.2 Preparation of S9 mix

The procedure for induction of rat liver microsomal enzymes was modified from the method described by Matsushima *et al.*[55]. The male Sprague-Dawley rats (6 weeks old) were injected intraperitoneal with sodium phenobarbital at a dosage 30 mg/kg body weight on the first day of treatment, and 60 mg/kg body weight daily on the second, third and last day. The rats were also inoculated intraperitoneal with 5,6-benzoflavone at a dosage of 80 mg/kg body weight on the third day.

On the fifth day, the rats were scarified by cervical dislocation, and the livers were removed and prepared for S9 fraction following the modified method described by Garner *et al.*[56]. The freshly removed livers were placed in preweighed beakers containing 1 ml of chilled 0.15 M KCl and weighed. The livers were washed in chilled 0.15 M KCl for several times and transferred to a beaker containing 3 volume of chilled 0.15 M KCl solution (3 ml/g wet liver). The livers were thus minced, homogenized, and centrifuged at 9,000 x g for 10 minutes. The supernatant (S9 fraction) was divided into small aliquots and stored at -80°C until used. For each mutation assay, an aliquot of S9 fraction was thawed at room temperature and kept in ice.

The components of S9 mix were 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4 mM NADH, 100 mM sodium phosphate pH 7.4, and S9 fraction in a concentration of 0.1 ml per ml of mixture. The S9 mix was freshly prepared for each mutation assay.

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2.5.3 Procedure for mutagenicity assay

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The assay was performed essentially as "preincubation technique" [57]. A fresh overnight culture of bacterial tester strain 0.1 ml, the test soil extracts 0.1 ml, and 0.5 ml of S9 mix (or 0.5 ml of 0.2 M phosphate buffer, pH 7.4) were added into a capped culture tube. The contents were mixed and preincubated at 30°C for 30 minutes. Then the molten top agar (at 45°C) supplemented with 0.5 mM of Histidine-Biotin was added and

mixed by rotating the tube between the palms and poured on a 30 ml Vogel-Bonner agar plate. The top agar was uniformly distributed on the surface of the agar and overlaid agar was allowed to solidify. Within an hour the plates were kept in a dark, 37°C incubator. The revertant colonies per plate were counted and examined for toxic effect under a steromicroscope after 48 hours.

The extracts of soil were tested at four dose levels, 0.5, 1.0, 2.0, 4.0 mg/plate for TA98 in addition to solvent control and positive control. Two or three plates were used for each dose in each experiment. The experiments were repeated and the results were given as the mean of 4-6 plates with standard deviation of mean. Two dose levels, 0.5 and 4.0 mg/plate, were used for TA100 at first. Some samples that showed possible mutagenic activity were tested again in four dose levels same with the doses for TA98.

2.6 Physical and chemical parameters of soil

2.6.1 Soil moisture content

About 5-6 g amount of soil was accurately weighed in a tared crucible immediately after weighing the sample for extraction, and put in an oven at 105-110°C for 14 hours. After the sample was cooled in a desiccator and weighed, percent weight loss can be reported as moisture as follows.

$$\% \text{ moisture} = \frac{\text{g of sample} - \text{g of dry sample}}{\text{g of sample}} \times 100\%$$

2.6.2 Organic matter content

Ignition at moderate temperature was chosen as a method for determination of total organic matter in soil in this study. First, the water was driven off at 110°C, the organic matter was oxidized by heating at 350°C to 400°C for 8 hours and the mineral matter could

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be assumed to be unchanged at these temperatures. The weight of organic matter is calculated as follows:

$$\% \text{ organic matter} = \frac{(\text{oven-dried soil weight}) - (\text{soil weight after heating})}{\text{oven-dried soil weight}} \times 100\%$$

2.6.3 Soil texture

Soil texture is concerned with the size of mineral particles. The "feel" method, by rubbing soil between the thumb and fingers, was used to determine each soil studied.

2.7 Data analysis

The data analysis was conducted by SPSS statistical package for student *t*-test and correlation analysis.

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