CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals for Ames Test

- 1-Aminopyrene (1-AP) (Aldrich, St Louis, USA)
- Acetonitrile (J. T. Baker, Phillipsburg, USA)
- Ammonium sulfamate ($NH_2SO_3NH_4$) (Sigma Chemical, St Louis, USA)
- Ampicillin sodium salt (supported by M&H manufacturing, Samutprakarn, Thailand)
- Bacto agar (Merck, Damstadt, Germany)
- Citric acid (BHD Chemical, Pool, England)
- Crystal violet (Merck, Damstadt, Germany)
- Dimethylsulfoxide (DMSO) (Sigma Chemical, St Louis, USA)
- Dipotassium hydrogen phosphate anhydrous (K_2HPO_4) (Fluka, Buchs, Switzerland)
- Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_20$) (Fluka, Buchs, Switzerland)
- D-Biotin (Sigma Chemical, St Louis, USA)
- D (+) – glucose anhydrous (Fluka, Buchs, Switzerland)
- Histidine monohydrochloride (Merck, Damstadt, Germany)
- Hydrochloric acid 34% w/v (Merck, Damstadt, Germany)
- Magnesium sulphate heptahydrate ($MgSO_4.7H_20$) (Merck, Darmstadt, Germany)
- Oxoid nutrient broth No. 2 (Oxiod, Hants, England)
- Potassium chloride (Merck, Damstadt, Germany)
- Sodium ammonium hydrogen phosphate tetrahydrate ($NaNH_4HPO_4.4H_20$) (Fluka, Buchs, Switzerland)

- Sodium chloride (Merck, Damstadt, Germany)
- Sodium dihydrogen phosphate (NaH_2PO_4) (Sigma Chemical, St Louis, USA)
- Sodium nitrite (Sigma Chemical, St Louis, USA)

3.1.2 Samples

The beef (loin) was purchase from local market in Bangkok. And the plant materials, namely Pak Chee Lao [*Anethum graveolens* Linn (ผักชีลาว) Umbelliferae], Pak Ka Yang [*Limnophila aromatica* Merr (ผักแขยง) Scrophulariaceae], Pak Gud [*Diplazium esculentum* (Retz.) (ผักกูด) Athyriaceae], Pak Krad Hua Wan [*Spilanthes acmella* Murr. (ผักคราดหัวแหวน) Compositae], Pak Pai [*Polygonum odoratum* Lour (ผักไผ่) Polygonaceae], were purchased from local market in Samutprakarn during June, 2005. Authentication has been performed by comparison with the herbarium specimen and the Royal Forest Department, Ministry of Natural Resources and Environment, 61 Phaholyathin Ladyao Jatujak Bangkok. The voucher specimen has been deposited in the herbarium of natural museum.

3.1.3 Instruments

- Analytical balance (Sartorius 2200)
- Autoclave (Kenclave, Japan)
- Blender (Imarflex super blender IF-308)
- Colony counter
- Filter paper (Whatman No.1 and No.4)
- High vacuum grease (Dow corning corporation, USA)
- Hot air oven (WTB binder No.960462, Germany)
- Incubator (Memmert, Germany)
- Microprocessor pH tester (Sharp pH, Italy)
- pH paper (Whatman)
- Pipette controller (Brand, Germany)
- Pressure stream sterilizers (All American model No. 1941, USA)
- Rotavapor (Buchi, Switzerland)
- Shaking water bath (Hotech model 905, Taiwan)

- Transfer pipette (Brand, Germany)
- Ultraviolet spectrophotometer (PYE Unicam SP1800)
- Vacuum-system (Buchi B-169, Switzerland)
- Vortex-2 Genie (Scientific Industries, USA)
- Water bath (Memmert, Germany)

3.2 Methods

3.2.1 Experimental Design

Determination of the mutagenicity modifying activity of vegetable extracts on the standard mutagen (1-aminopyrene) and nitrite treated beef extract were carried out according to the experimental design shown in figure 3.1

First, the mutagenicity of beef extract was assayed, nitrite treatment and no treatment, by using the standard Ames test in the absence of metabolic activation.

Second, the mutagenicity of each vegetable extract was assayed, nitrite treatment and no treatment, by using the standard Ames test in the absence of metabolic activation.

Finally, the antimutagenicity of each vegetable extract was investigated by adding it to the 1-AP or beef extract after nitrosation process, then, assayed by using Ames test in the absence of metabolic activation.

3.2.2 The Preparation of Bacterial Tester Strains

Salmonella typhimurium tester strains used in this study were histidine-dependent strains (His^+) TA98 and TA100, which were capable of detecting frameshift mutation and base-pair substitution respectively. The both strains were kindly provided by associate professor Dr. Kaew Kangsadalampai, Nutrition Institute, Mahidol University. The tester strains were manipulated as shown in appendix B. Overnight cultures of bacteria inoculated from frozen stock culture in Oxoid nutrient broth no. 2 at 37°C was used for mutagenic assay within 24 hours.

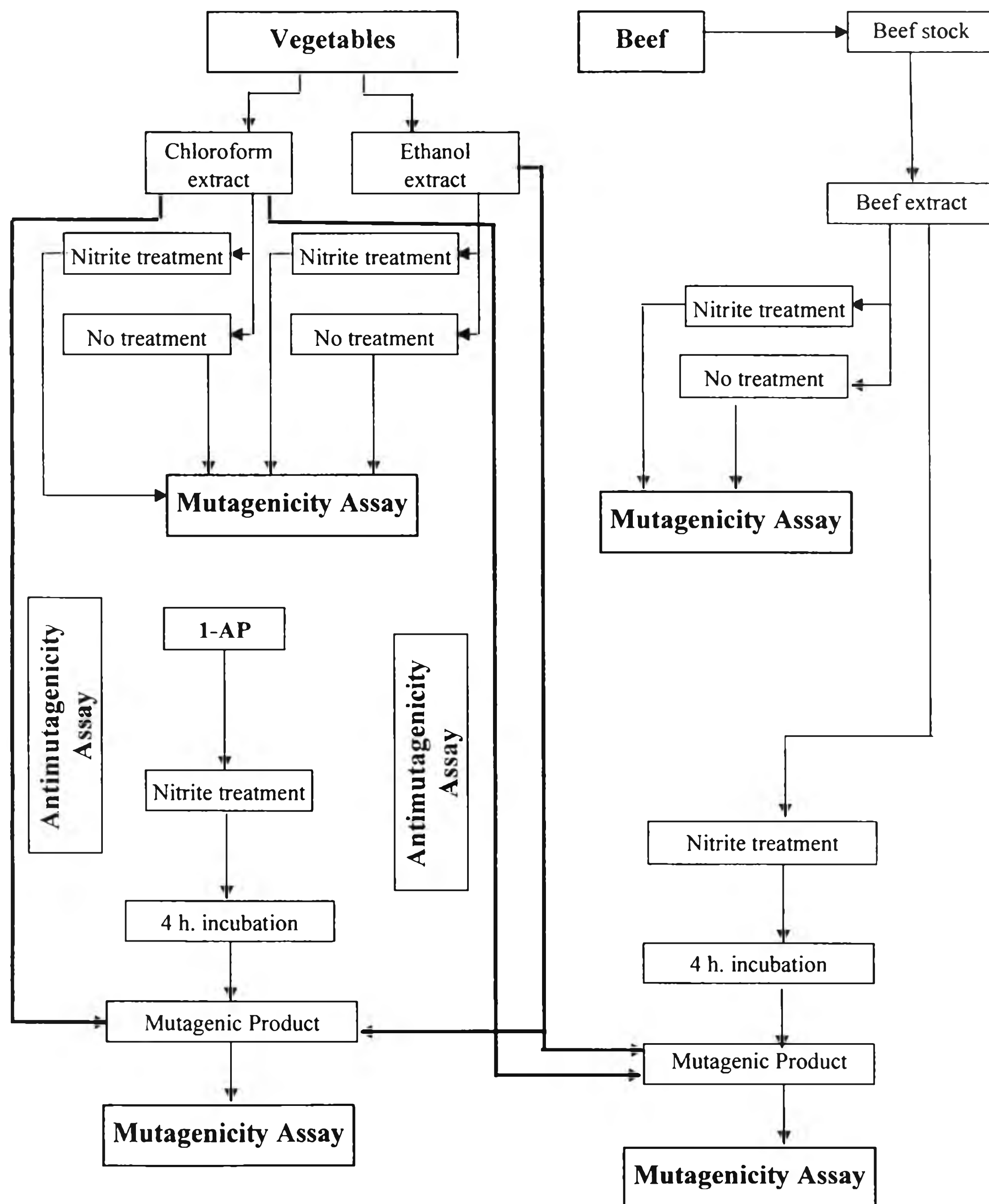

Figure 3.1 Experimental designs for determining the mutagenicity modification of some vegetable extracts on the mutagenicity of nitrite treated 1-Aminopyrene and beef extract.

3.2.3 Nutrient Agar Preparation

Preparation of minimal agar plates

Minimal agar containing 1.5% Bacto agar was autoclaved at 121°C 20 minutes and then mixed with 2% sterile glucose and Vogel-Bonner medium E stock salt solution (VB salt) (see in appendix B). Approximately 30 ml of molten agar was poured into the steriie Petri dish. It was left until solidify and stored at 37°C in the incubator for 48 hours before using.

Preparation of top agar

Top agar containing 0.6% Bacto agar and 0.5% sodium chloride was autoclaved at 121°C 20 minutes. 10% (v/v) of a sterile solution of 0.5 mM histidine and biotin were added to the molten top agar, then, was maintained at 45°C in the water bath.

3.3 Preparation of Beef Extract

The beef was cleaned with tap water and left to dry, then was chopped. The 100 g of the chopped meat was heated in 200 ml of ultrapure water at 80°C for 8 hours in a condenser boiling flask. The beef stock was filtered through filter paper No. 1. The filtrate was centrifuged to obtain clear solution and then evaporated. The beef extract was adjusted to be 10 ml with distilled water in a volumetric flask and autoclaved for 20 min. The sterile beef extract was stored below -8°C and used for mutagenicity evaluation.

3.4 Preparation of Vegetable Extracts

The extract from each vegetable, namely Pak Chee Lao [*Anethum graveolens* Linn (ผักชีลาว) Umbelliferae], Pak Ka Yang [*Limnophila aromatica* Merr (ผักแขยง) Scrophulariaceae], Pak Gud [*Diplazium esculentum* (Retz.) (ผักกูด) Athyriaceae], Pak Krad Hua Wan [*Spilanthes acmella* Murr. (ผักคราดหัวแหวน) Compositae], and Pak Pai [*Polygonumodoratum* Lour (ผักไผ่) Polygonaceae] (Appendix A) were carried out according to the chart shown in figure 3.2. The edible parts of them were used in

sample preparation. All of the selected vegetables, except Pak Gud, were prepared by following method. First, the vegetables were cleaned with tap water and left to dry. Then, the edible parts were chopped, and then resized by a home-use blender. After that, 100 g of the resized plants were soaked in 200 ml of chloroform (for extracting non-polar substances from vegetables) for 3 days. Next, the chloroform extracts were filtered through filter paper No. 4 and the filtrates were evaporated in a rotary evaporator. The concentrated samples were diluted with DMSO to obtain the appropriate concentration and stored below 5°C. Then the marcs were soaked in 200 ml of 95% ethyl alcohol for 3 days. The alcohol extracts were filtered through filter paper No. 4 and the filtrates were evaporated with rotary evaporator. The marcs were then discarded. The extracted samples were diluted with DMSO to obtain the appropriate concentration and stored below 5°C.

For Pak Gud, its edible parts were chopped then dried in hot air oven at 60°C for 8 hours because fresh plant adsorbed chloroform solvent. After that, 10 g of the dried plant were soaked in 20 ml of chloroform for 3 days. It was then following the aforementioned method.

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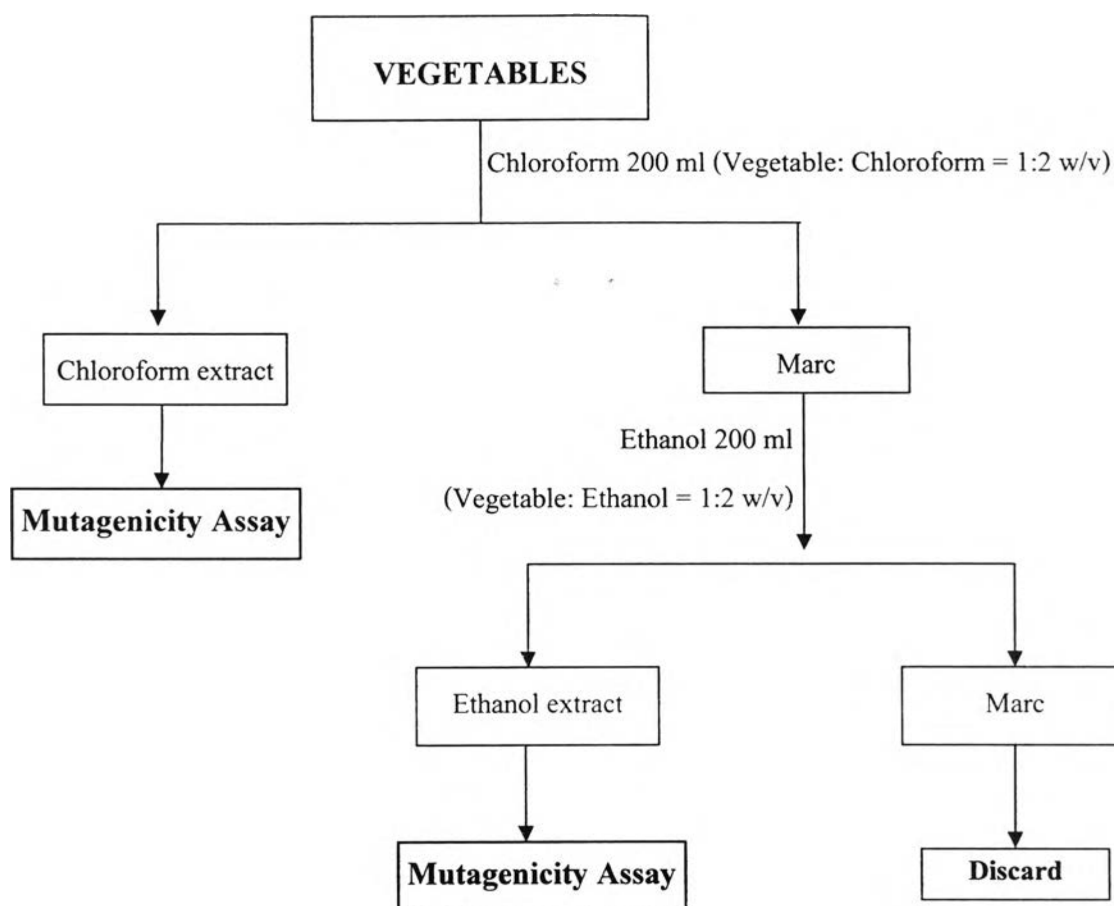

Figure 3.2 Solvent extractions of vegetable samples

3.5 Mutagenicity of 1-Aminopyrene-Nitrite and Beef Extract-Nitrite

1-AP treated with nitrite in acid solution was used as a standard mutagen since it was shown to give direct-action mutagenicity in the condition similar to that occurred during stomach digestion (Kangsadalampai and Butryee, 1995; Kangsadalampai, Butryee, and Manoonphol, 1996). Briefly explain, 20 μl (tested on TA98) or 40 μl (tested on TA100) of 1-AP (0.0375 mg/ml) was added into a tube fitted with a plastic stopper and then mixed with 0.73 or 0.71 ml 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and added 0.25 ml of 2 M sodium nitrite to the reaction mixture. The final concentration of nitrite was 0.5 M. The reaction tube was shaken at 80 rpm, 37°C in a water bath for 4 hours and was stopped by placing the tube in an ice bath for 1 minute. In order to decompose the residual nitrite, 0.25 ml of 2 M ammonium sulfamate was added to the reaction mixture and allowed to stand for 10 minutes in an

ice bath. The reaction mixture was mixed with 0.5 ml phosphate buffer (pH 7.4), and 100 μl of fresh overnight cultures of the tester strain and incubated at 37°C in a shaking water bath for 20 minutes. After incubation, 2 ml of the molten soft agar (45°C) was added. It was mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37°C in the dark for 48 hours. After incubaton, His^+ revertant colonies were counted.

The other positive direct-acting mutagen also used in this study was beef extract treated with nitrite (Münzner, 1986). The beef extract was treated with and without nitrite in acidic condition (figure 3.3 and figure 3.4 respectively) as follows. Briefly explain, the beef extract was diluted with ultrapure water to obtain the concentration of 0.4, 0.8, 1.6, and 3.2 mg/plate which were calculated from residue weight before treated with nitrite and mixed with 650 μl of 0.5 N HCl (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 250 μl of 2 M $NaNO_2$ or ultra pure water. The final concentration of nitrite was 0.5 M. The reaction tube was shaken at 80 rpm, 37°C for 4 hours and was stopped by placing the tube in an ice bath for 1 minute. The nitrosated product (100 μl) was mixed with 500 μl phosphate butter (pH 7.4), and 100 μl of overnight cultures of the tester strain and incubated at 37°C in a shaking water bath for 20 minutes. After incubation, 2 ml of the molten agar (45°C) was added. The contents of the tube were well mixed and poured onto the minimal agar plate. The plate was evenly distributed by rotating the dish. After solidification, the plate was inversely placed in an incubator at 37°C for 48 hours. After incubation, the amount of His+ revertant colonies was determined.

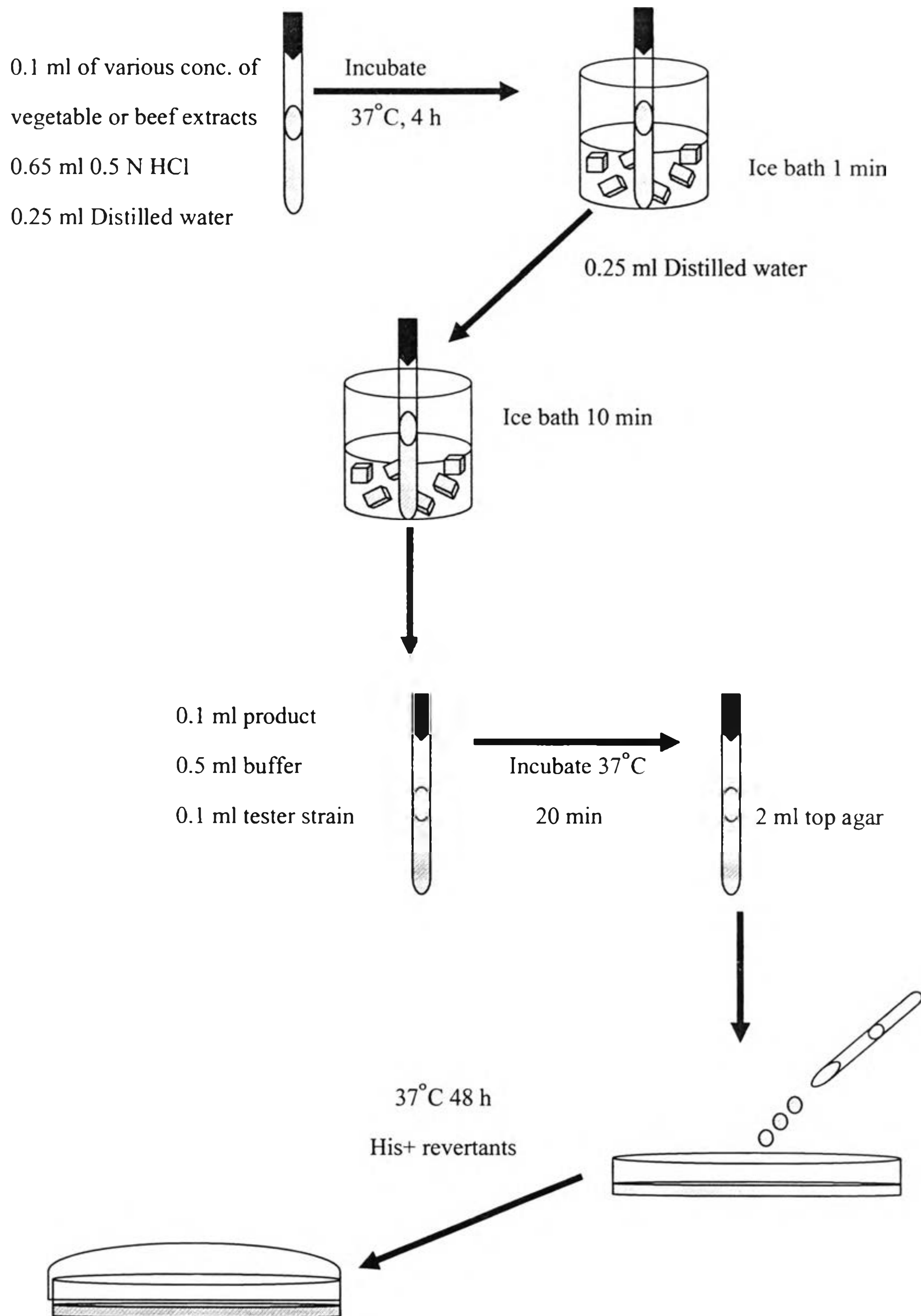

Figure 3.3 Steps to determine the mutagenicity of the sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

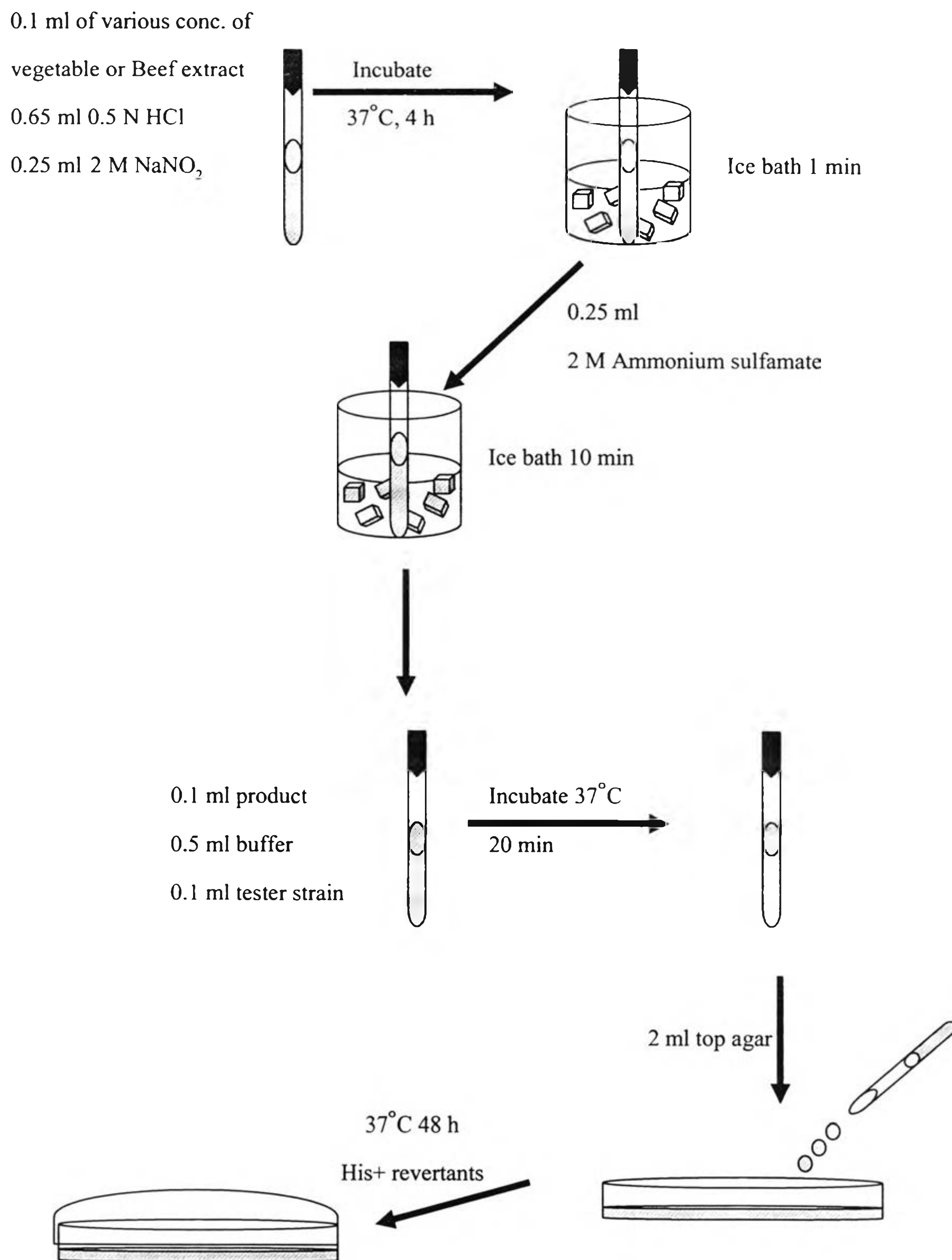

Figure 3.4 Steps to determine the mutagenicity of the nitrite treated sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

3.6 Mutagenicity of Vegetable Extracts

Each extract from vegetable was also treated with or without nitrite and determined for its mutagenicity. Briefly, different volume of each sterile vegetable extracts was measured into a sterile test tube fitted with a plastic stopper and mixed with hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 250 μl of distilled water (figure 3.3) or 2 M sodium nitrite (figure 3.4). The final volume was 1250 μl. The reaction tube was shaken at 37°C for 4 h. Then it was placed in an ice bath to stop the reaction. Added 250 μl of 2 M ammonium sulfamate to the reaction mixture, for decomposing nitrite, or 250 μl of distilled water and allowed to stand for 10 min in an ice bath.

3.7 Antimutagenicity of Vegetable Extracts

The pre-incubation method as suggested by Yahagi (1975) was used to investigate antimutagenicity property of the vegetable extracts. An aliquot of 100 μl of nitrite treated 1-AP or nitrite treated beef extract (3.2 mg of beef residue weight/plate) was used as a standard mutagen. The procedure was performed as follows (figure 3.5). 0.1 ml of nitrosated product, 0.5 ml of phosphate buffer, 0.1 ml of the tester strain and an aliquot of 0.1 ml of each vegetable extracts were mixed together in a test tube and incubated at 37°C for 20 minutes. After incubation, 2 ml of the molten agar (45°C) was added. The contents of the tube were well mixed and poured onto the minimal agar plate. The plate was evenly distributed by rotating the dish. After solidification, the plate was inversely placed in an incubator at 37°C for 48 hours. After incubation, the number of His+ revertant colonies was determined.

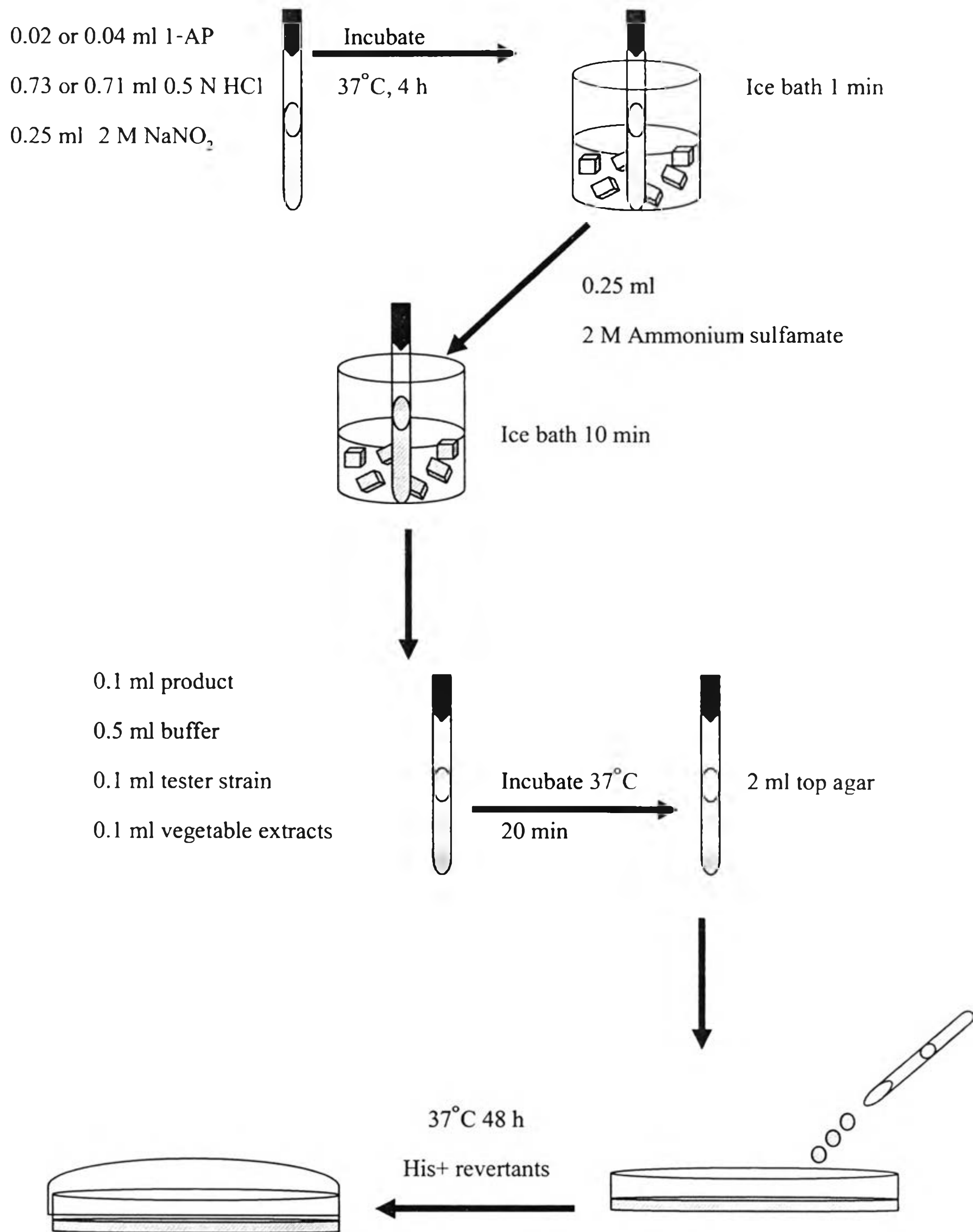

Figure 3.5 Steps to determine the antimutagenicity of varies vegetable extracts on nitrite treated 1-aminopyrene using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

3.8 Data Manipulation

The mutagenicity of each sample was presented as a number of histidine revertants per plate. The data were reported as means with standard deviation of six plates from two experiments. To simply compare the degree of mutagenicity, the results were also expressed as a mutagenicity index (MI) (equation 3.1):

$$MI = \frac{Average(N)}{Average(S)} \qquad \text{(Equation 3.1)}$$

N = a number of histidine revertants per plate of the sample

S = a number of spontaneous revertants per plate of the negative control

The mutagenicity of samples was determined by number of histidine revertants at least one concentration which should be higher than 2 times of spontaneous revertants with a dose-response relationship. A percentage of modification (either increase or decrease on mutagenicity of sample) was calculated as following (equation 3.2):

$$\%\ modification = \left(\frac{A - B}{A - C}\right) x\ 100 \qquad \text{(Equation 3.2)}$$

A = a number of histidine revertants induced by nitrite treated standard mutagen (1-AP) or beef extract

B = a number of histidine revertants induced by standard mutagen (1-AP) or beef extract in the present of vegetable extract

C = a number of spontaneous revertants (negative control)

From the equation 3.2, the inhibition (or enhancement) of mutagenicity may be divided into four classes (Calomme *et al.*, 1996) as shown in table 3.1.

Table 3.1 Criteria of evaluation as the inhibition or enhancement of mutagenicity

% modification	inhibition or enhancement
± 0 – 20 %	no effect
± 20 – 40 %	weak activity
± 40 – 60 %	effective or moderate activity
> 60 %	strong or potent activity

+ = inhibitory effect

- = enhancing effect