CHAPTER III

MATERIALS AND METHODS

Materials

3.1 Chemicals for Ames Test

Aminopyrene (Aldrich, St Louise, USA) and sodium nitrite (Sigma Chemical, Co. St Louis, MO, USA) were used as precursors of standard mutagen in this assay. Ammonium sulfamate ($NH_2SO_3NH_4$), d-biotin, and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Sigma Chemical, Co. (St. Louis, USA). Magnesium sulphate heptahydrate ($MgSO_4.\ 7H_2O$), histidine monohydrochloride, hydrochloric acid, potassium chloride (KCl), sodium chloride (NaCl), and crystal violet indicator were supplied by E. Merck (Darmstadt, Germany). Sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4.4H_2O$), dipotassium hydrogen phosphate anhydrous (K_2HPO_4), D(+)-Glucose monohydrate and disodium hydrogen phosphate dihydrate ($Na_2HPO_4.\ 2H_2O$) were furnished by Fluka (Biochemica, Switzerland). Citric acid was obtained from BDH Chemicals Ltd.; (Pool, England). Ampicillin sodium salt was purchased from Genereal Drugs House (Bangkok, Thailand). Bacto agar was a product of Difco laboratories (Michigan, USA). Oxoid nutrient broth No. 2 was supplied by Oxoid Ltd. (Basingstoke, Hants, England). Acetonitrile was purchased from J.T. Baker Inc. (Phillipsburg, USA). Other chemicals were of laboratory grade.

3.2 Food Sample

Samples were divided into three groups by their flavours, namely minced pork flavour, pa-lo duck flavour and sour shrimp flavour. They were purchased from a local supermarket in Bangkok. Each brand has several ingredients as shown in the Appendix A.

Methods

3.3 Experimental Design

Determination of the mutagenicity of extracts from instant noodles was carried out according to the experiment protocol shown in figure 5.

Firstly, each sample was divided into three parts (noodles, seasonings and noodles cooked with seasonings). Each part was extracted in the same way with hot water and the volume of the extract was reduced using a vacuum rotary evaporator. Finally, samples of known concentrations were assayed for their mutagenicity with and without nitrite treatment using Ames test.

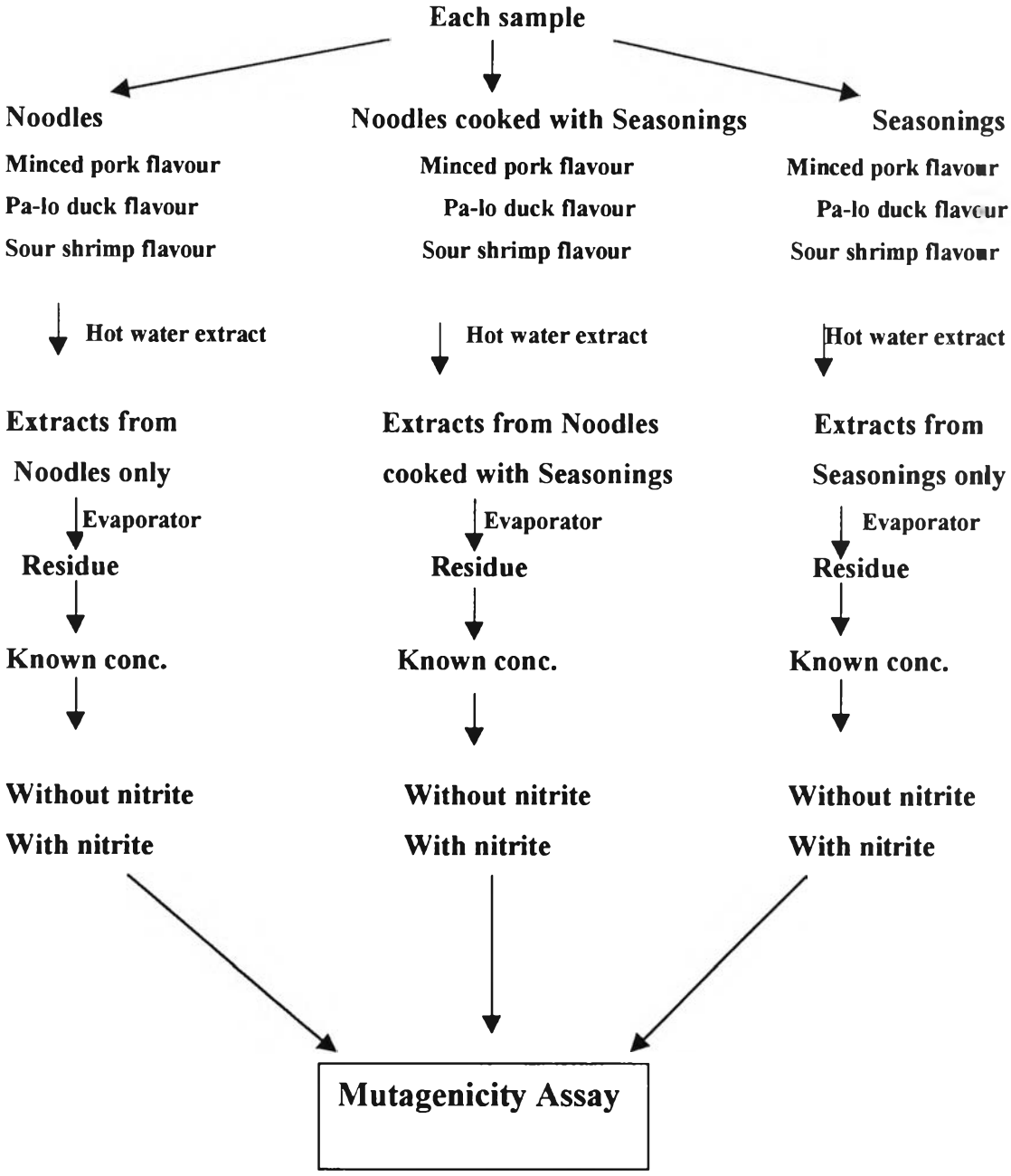

Figure 5 Experimental design of mutagenicity assay

3.4 Sample Preparations

Each sample was composed of noodles, seasonings and noodles cooked with seasonings.

Noodles were cooked according to package instructions for specific temperature and time. Then the noodles were removed by filtration and the filtrate was centrifuged at 1000 rpm for 30 minutes. The supernatant was evaporated down to about 10 % of its original volume under vacuum. Then, the concentrate was autoclaved and subjected to mutagenicity assay.

The extracts of noodles cooked with seasonings were conducted in the same manner as described above.

Each seasoning supplied with each package of instant noodle was cooked according to package instructions for specific temperature and time. Then, the solid matter was removed by filtration and the filtrate was evaporated to about 10 % of its original volume under vacuum. Then, the concentrate was autoclaved and subjected to mutagenicity assay.

3.5 Ames Test

Modified method of Yahagi *et al.* (1975) including pre-incubation of the test sample with *Salmonella typhimurium* was used throughout this study. Two strains of *Salmonella typhimurium* TA 98 and TA 100, which required histidine as a growth factor, were used. They were provided by Assoc. Prof. Dr. Kaew Kangsadalampai of the Institute of Nutrition, Mahidol University. Overnight culture of each strain was prepared in Oxoid nutrient broth No.2 at 37°C with shaking. Manipulation of the culture was done as suggested by Maron and Ames (1983) (Appendix B).

A Minimal agar plate was composed of 30 ml of minimal glucose agar medium consisting of 1.5% Bacto-Difco agar and 2% glucose in Vogel-Boner medium E. A top agar was 0.6% agar and 0.5% sodium chloride. They were prepared as described by Maron and Ames (1983) as shown in Appendix B. Ten ml

of a sterile solution of 0.5 mM L-histidine/biotin was added to each 100 ml of the top agar and mixed thoroughly by swirling before use.

3.6 Mutagenicity of Extracts from Noodle, Seasoning and Noodle Cooked with Seasoning

Various amounts (10, 50, 100 and 200 µl) of each extract was introduced into the tube containing appropriate volume of distilled water to obtained the final volume of 200 µl. The content was added with HCl to acidify the reaction mixture to pH 3.0-3.5. Then, distilled water was added to obtain the final volume of 1000 µl. The tube with stopper was shaken in a waterbath at 37 °C for 4 hours and stopped by placing the tube in an ice bath for 1 minute. Distilled water was added to the reaction mixture to obtain the final volume of 1250 µl, and the reaction tube was immersed in an ice bath for another 10 minutes.

Direct mutagenicity of the reaction mixture was assayed according to the pre-incubation (at 37 °C for 20 minutes) method of Yahagi *et al.* (1975), using *Salmonella typhimurium* in the absence of S-9 mix (Figure 6).

Direct Mutagenic assay

To a tube containing 0.1 ml of the reaction mixture, Na_3PO_4-KCl buffer (pH 7.4), was added 0.5 ml, and then 0.1 ml of fresh overnight nutrient broth culture of the tester strain (1-2x10^8 cells). The tube was incubated at 37 °C in a shaking water bath for 20 minutes. After incubation, 2 ml of molten top agar (45 °C) containing 0.5 mM L-histidine/biotin was added. It was mixed well and poured onto a minimal glucose agar plate. The plate was rotated to archived uniform colony distribution and incubated at 37 °C in the dark for 2 days. Induced his^+ revertant colonies were

counted and compared to the established spontaneous mutation. Duplicate plates were carried out for each sample and all experiments were performed at least twice.

For each experiment, positive control and negative control plates were included in the assay. Negative control plate containing the bacteria and distilled water was used to establish the spontaneous mutation of each tester strains. Aminopyrene (0.06, 0.12 μg/plate) was used as positive control with the treatment of excess of nitrite (500 mM) in acid solution as suggested by Kangsadalampai *et al.* (1996).

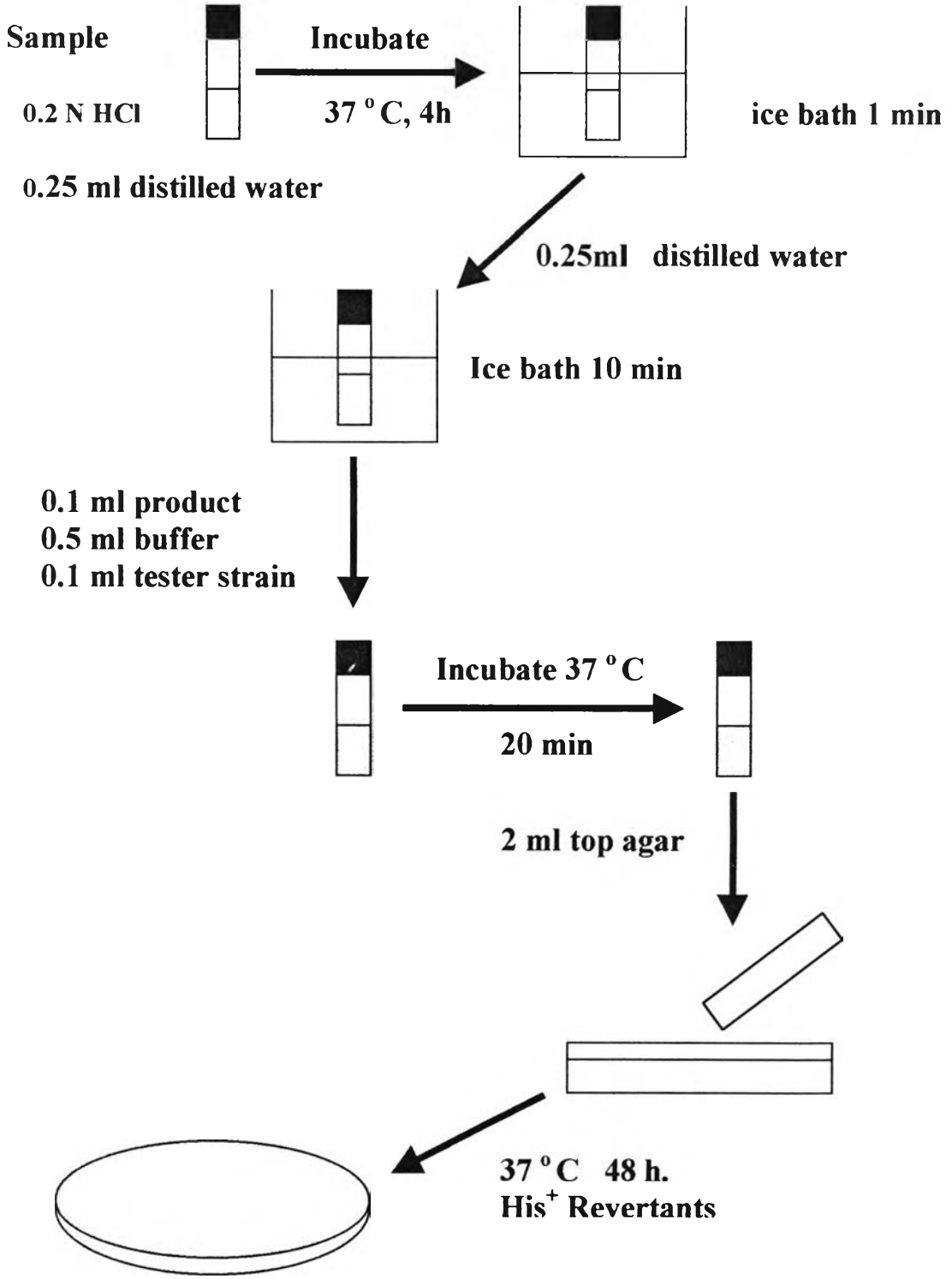

Figure 6 Direct mutagenicity evaluation of sample using the Ames test (pre-incubation modification)

3.7 Mutagenicity of Nitrite Treated Extracts from Noodle, Seasoning and Noodle Cooked with Seasoning

To determine the mutagenicity of extracts from sample treated with nitrite, the extract of 10, 50, 100, 200 µl into the tube. Diluted HCl, which containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added. The adding of 0.25 ml of 2 M sodium nitrite to the tube started the reaction. The final volume was 1000 µl. Then the tube was incubated at 37 °C with shaking for 4 hours. The reaction was stopped by allowing the mixture to stand for 1 minute in an ice bath. Ammonium sulfamate (2M, 0.25 ml) was added to the reaction mixture to decompose the residue nitrite and the reaction tube was immersed in an ice bath for another 10 minutes. Finally, the mixture was determined for its mutagenicity by Ames test as described in Figure 7.

3.8 Data Manipulation

The mutagenicity of each sample was presented as number of histidine revertants per plate. The data were reported as means with standard deviation of four plates from two different experiments. And for easier to compare the degree of mutagenicity, the results are also expressed as mutagenicity index (MI) which was calculated from the average value of number of histidine revertants per plate of sample divided by average value of spontaneous revertants. Sample expressed its mutagenicity higher than two times of spontaneous revertants with a dose-response relationship was evaluated mutagenic. In addition, the data were reported as specific mutagenic activity and total mutagenic activity. Specific mutagenic activity of samples expressed as number of revertants per gram and total mutagenic activity expressed as number of revertants per package. Expected values of noodles cooked

with seasonings were obtained by summing the mutagenicity of individual components from nitrite treated extracts from noodles and nitrite treated extracts from seasonings while actual values were obtained from actual experiment.

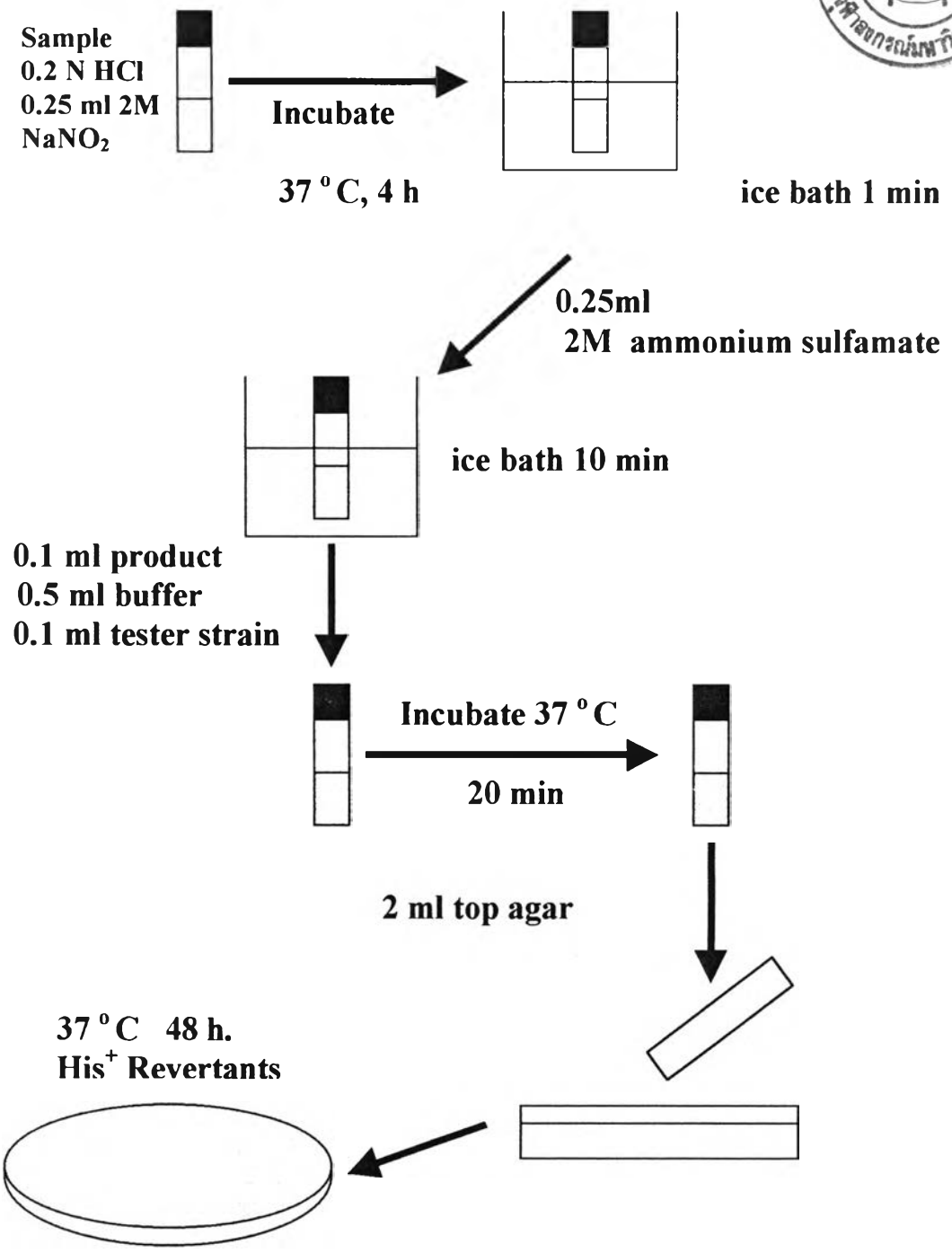

Figure 7. Steps to determine the mutagenicity of nitrite treated food sample using the Ames *Salmonella* mutagenicity test (pre-incubation modification) in the absence of S-9 mix.