CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals for Ames Test

1-Aminopyrene (1-AP) was obtained from Sigma-Aldrich, St. Louis, USA. Merck (Darmstadt, Germany) supplied methanol, Bacto agar, dipotassium hydrogen phosphate anhydrous (K_2HPO_4), L-histidine monohydrochloride, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and potassium chloride (KCl). Ammonium sulfamate ($NH_2SO_3NH_4$), d-biotin, sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4 \cdot 4H_2O$), and sodium dihidrogen phosphate (NaH_2PO_4) were obtained from Sigma Chemical, St. Louis, USA. Oxoid nutrient broth No.2 was purchased from Oxoid Ltd. (Basingstoke, Hants, England). Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), d(+)-glucose anhydrous, di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), hydrochloric acid, and sodium nitrite were purchased from BDH Chemicals Ltd. (Poole, England).

3.1.2 Samples

The chicken extract, the Hom Nil rice and the black glutinous rice were purchased from a supermarket in Bangkok, Thailand.

3.2 Experimental Design

Determination of the antioxidant and the mutagenicity modifying activity of Hom Nil rice and black glutinous rice extracts on nitrite treated 1-aminopyrene and nitrite treated chicken extract were carried out according to the experimental design shown in Figure 10.

First, the antioxidant assay was studied using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay, Ferric Reducing Antioxidant Power (FRAP) assay and determination of total phenolic contents. Second, the mutagenicity of chicken extract and each rice extract were assayed using the standard Ames test in the absence of metabolic activation. Finally, the antimutagenicity and anti-mutagen formation of each rice extract were investigated by adding it to the 1-aminopyrene or chicken extract model

before and after nitration process, then, assayed using Ames test in the absence of metabolic activation.

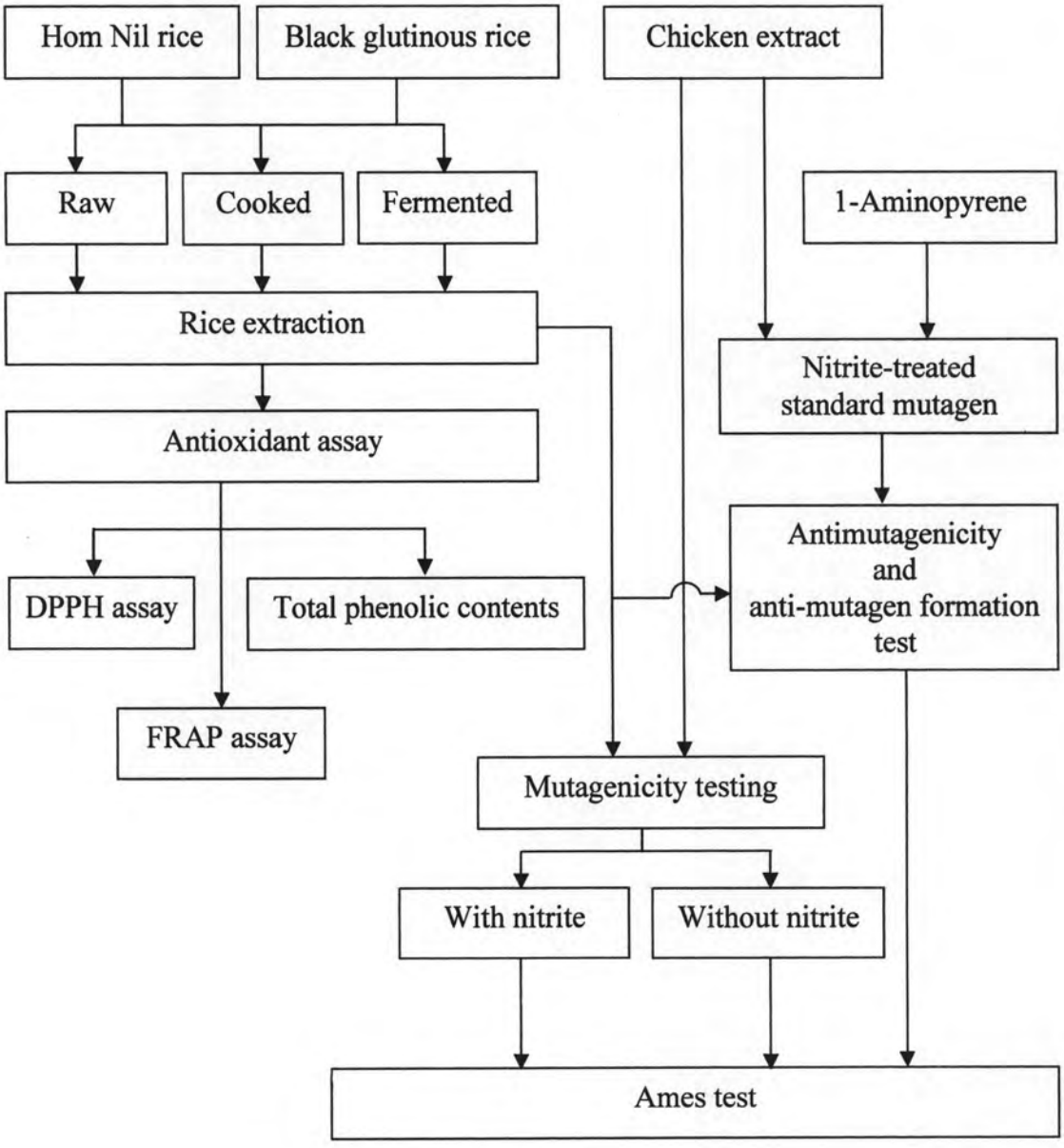

Figure 10 Overall investigations to elucidate the antioxidant activity and the effect of rice extracts using Ames test

3.3 Sample preparations

3.3.1 Preparation of Chicken Extract

Commercial chicken extract was concentrated at 45 °C and dried in a freeze dryer. The freeze-dried chicken extract was pulverized to be find powder. The chicken extract solution (0.486 g/ml) was sterilized by autoclaving before use.

3.3.2 Hom Nil Rice and Black Glutinous Rice Extracts Preparation

Hom Nil rice and black glutinous rice were purchased from a supermarket in Bangkok, Thailand. Each rice was separated into 3 parts for preparation of raw, cooked and fermented rice.

Raw Rice Preparation

The rice (1 kg) was washed with tap water and dried at 40 °C in hot air oven until dried. The dried rice was ground in an electrical blender to be fine powder before the extraction.

Cooked Rice Preparation

The rice (1 kg) was washed with tap water and cooked in an automatic electric rice cooker with water (2 liter). The cooked rice was dried at 40 °C in hot air oven until dried and then it was blended in an electrical blender to be fine powder before the extraction.

Fermented Rice Preparation

The rice (1 kg) was washed with tap water and cooked in an automatic electric rice cooker with water (2 liter). The cooked rice was fermented by adding 1 piece of Thai traditional fermentation starter (Loog-pang) and left in room temperature for 48 hours (อรอนงค์ นัยวิกุล, 2550). The fermented rice was dried at 40 °C in hot air oven until dried and then it was blended in an electrical blender to be fine powder before the extraction.

Rice Extracts Preparation

Dried rice (1 kg) was soaked in 1.5 liter of acid alcohol (0.1 N acetic acid in 70% ethanol) for 1 day and; this process was repeated for 3 times. The filtrates were pooled and evaporated to dryness in a freeze dryer. Each extract was protected from light and stored below 5 °C until used. The rice extract (0.216 g) was dissolved in 1 ml of distilled water and filtered through a sterilized 0.20 μm-membrane (Sartorius, Minisart®) filter set.

3.4 Antioxidant Assay

3.4.1 Sample preparation

Two-hundred milligrams of each rice extract were stirred with 80% methanol (25 ml) at room temperature and assayed for its antioxidant activity. Each rice extract was run simultaneously.

3.4.2 2,2'-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The antioxidant activity of the rice extract on DPPH was estimated using the procedure described by Fukumoto and Mazza (2000) with slight modifications. An aliquot of 22 μl (in triplicate) of each extract or standard Trolox was transferred into a 96-well flat-bottom microplate (Bibby Sterilin Ltd, UK). The solution of 150 μM DPPH in 80% methanol (200 μl) was added to each microplate well. The plate was then covered and left to stand in the dark at room temperature. After 30 minutes, the absorbance of the solution was read in microplate reader (Sunrise, Tecan Co., Austria) using a 520 nm filter. Standard curve was constructed by using several concentrations of Trolox (0.08 – 1.28 mM in 80% methanol) (see Appendix B). The antioxidant activity of the extracts was determined using the standard curve expressed as mg of Trolox Equivalent Antioxidant Capacity (TEAC)/g dry weight (DW) of rice extract. The more value of TEAC, the more the antioxidant activity of the sample is. The radical scavenging activity was also calculated as a percentage of DPPH scavenging activity using the equation: % Scavenging activity = $100 \times (1-A_E/A_D)$; where A_E is the absorbance of the solution when an extract is added, and A_D is the absorbance of the DPPH solution with nothing added. Data were presented as means ± SD of at least triplicate experiments.

3.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity was measured by its ability to reduce the Fe^{3+}/ferricyanidecomplex by forming ferrous products. Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 600 nm. Increased absorbance at 600 nm indicates a stronger reducing power. Each (20 μl) extract or standard or blank reagent was added to each well in a 96-well microtiter plate in triplicate. FRAP reagent (150 μl), freshly prepared (see Appendix B) and warmed at 37 °C according to the procedure described by Griffin and Bhagooli (2004), was added to

each well. The mixture was left at room temperature for 8 minutes. The absorbance was read at 600 nm using a microplate reader. The change in absorbance after 8 minutes from the initial blank reading was compared to that of a standard that was run simultaneously. Aqueous solutions of known standard Fe^{2+} ($FeSO_4.7H_2O$) concentrations (62.5, 125, 250, 500, 1000 μM) were used for calibration (see Appendix B). The FRAP values of the extracts were determined using this standard curve, expressed as mg of ferrous iron (Fe(II)) /g dry weight of rice extract. Data were presented as means ± SD of triplicate wells.

3.4.4 Determination of Total Phenolic Contents

The total phenolic content of methanol extract from each sample was determined according to method described by Swain and Hillis (1959), Naczk and Shahidi (1989) and Amarowicz *et al.* (2004) with slight modification by microplate reader. Briefly, 10 μl of each extract was transferred into a 96-well flat-bottom microplate containing 160 μl of distilled water. After mixing the contents, 10 μl of Folin-Ciocalteu reagent and 20 μl of saturated sodium carbonate solution were added. The microplate was vortexed and absorbance of blue colored mixtures was recorded after 30 minutes at 750 nm with microplate reader (Sunrise, Tecan Co., Austria). The amount of total polyphenols was calculated as a Gallic Acid Equivalent (GAE) from the calibration curve of gallic acid standard solutions (25, 50, 100, 200, 400 and 800 mg/l) (see Appendix B), and expressed as mg gallic acid equivalent/g dry weight of sample. All measurements were done in triplicate. Data were presented as means ± SD.

3.5 Mutagenicity Testing

3.5.1 Preparation of Bacterial Tester Strain

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 Assoc. Prof. Kaew Kangsadalampai, Nutrition Institute, Mahidol University. Overnight cultures of bacteria inoculated from frozen stock culture in Oxoid nutrient broth no. 2 at 37 °C 16 hours were used for mutagenic assay within 24 hours. The cultures were kept in the refrigerator until use.

3.5.2 Nutrient Agar Preparation

Preparation of minimal agar plates

Minimal agar containing 1.5% bacto agar was autoclaved and then mixed with 2% sterile glucose and Vogel-Bonner medium E stock salt solution (VB salt) (see in appendix A). Approximately 30 ml of molten agar was poured into the sterile 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. 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.

3.5.3 Mutagenicity of 1-Aminopyrene

1-Aminopyrene treated with sodium nitrite in acid solution was used as a standard mutagen since it was shown to give direct-acting mutagenicity (Kangsadalampai *et al.*, 1996). In this study, 10 µl and 20 µl of 1-aminopyrene (0.075 mg/ml) was used for testing on *Salmonella typhimurium* strains TA98 and TA100 respectively. 1-AP was added in a tube fitted with a plastic stopper and adjusted the volume into 300 µl with sterile distilled water, then, it was mixed with 550 µl of 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 250 µl of 2 M sodium nitrite to the reaction mixture. The reaction tube was shaken at 37 °C for 4 hours and then was placed in an ice bath for 1 minute to stop the reaction. In order to decompose the residual sodium nitrite, 250 µl of 2M ammonium sulfamate was added to the reaction mixture and then it was allowed to stand for 10 minutes in an ice bath. The reaction mixture was then subjected to be tested as standard mutagen on *Salmonella typhimurium* strains TA98 and TA100 using Ames test with modified preincubation method.

Pre-incubation Method

The pre-incubation method as suggested by Yahagi *et al.* (1975) was used throughout this study. For determination of direct mutagenicity, 100 µl of each tester strain was added to the test tube containing 100 µl of sodium nitrite treated 1-AP and 500 µl of Na_3PO_4-KCl buffer (pH 7.4). The mixture was incubated at 37°C in a shaking water bath for 20 minutes. After incubation, 2 ml of the molten top agar

(45°C) was added. The contents of the tube were well mixed and poured onto the minimal agar plate and the plate was incubated at 37°C for 48 hours. The number of histidine revertants was counted. The procedures are shown in Figure 11.

3.5.4 Mutagenicity of Chicken Extract

With Sodium nitrite Treatment

The procedure was done as described in 3.5.3. The standard mutagen, 1-aminopyrene, was replaced with chicken extract solution from experiment 3.3.1 (100, 150 or 200 µl). Then, the mixture was determined for its mutagenicity by Ames test as described in Figure 12.

Without Sodium nitrite Treatment

The procedure was done as described in 3.5.3. The standard mutagen, 1-aminopyrene, was replaced with chicken extract solution from experiment 3.3.1 (100, 150 or 200 µl). Sodium nitrite and ammonium sulfamate were replaced with sterile distilled water.

3.5.5 Mutagenicity of Rice Extract

The rice extracts were evaluated for mutagenicity with and without sodium nitrite treatment before mutagenic modification test. The procedure was done as described in 3.5.4. The chicken extract was replaced with each rice extract solution from experiment 3.3.2 (50, 75 or 100 µl). Then, the mixture was determined for its mutagenicity by Ames test as described in Figure 13.

3.6 Mutagenicity Modification Test

3.6.1 Antimutagenic effect of Rice Extracts

3.6.1.1 Effect on Mutagenicity of Sodium Nitrite Treated Chicken Extract

The procedure was done as described in 3.5.3. The standard mutagen, 1-aminopyrene, was replaced with 200 µl of chicken extract solution from experiment 3.3.1. The rice extract from experiment 3.3.2 (50, 75 or 100 µl) was added to a test tube containing the 4-hour incubated mixture of sodium nitrite treated chicken extract (1250 µl) and adjusted the volume into 1350 µl with sterile distilled water before pre-incubation process. Then the mixture was determined for its mutagenicity by Ames test as described in Figure 14.

3.6.1.2 Effect on Mutagenicity of Sodium Nitrite Treated 1-Aminopyrene

The procedure was done as described in 3.6.1.1. The chicken extract was replaced with 10 µl and 20 µl of 1-aminopyrene (0.075 mg/ml) for testing on *S. typhimurium* strains TA98 and TA100 respectively.

3.6.2 Anti-Mutagen Forming Effect of Rice Extracts

3.6.2.1 Effect during Formation of Sodium Nitrite Treated Chicken Extract

The procedure was done as described in 3.5.3. The standard mutagen, 1-aminopyrene, was replaced by 200 µl of chicken extract solution from experiment 3.3.1. The rice extract from experiment 3.3.2 (50, 75 or 100 µl) was added to a test tube containing the chicken extract before adjusting the volume into 300 µl with sterile distilled water. Then, the mixture was determined for its mutagenicity by Ames test as described in Figure 15.

3.6.2.2 Effect during Formation of Sodium nitrite Treated 1-Aminopyrene

The procedure was done as described in 3.6.2.1. The chicken extract was replaced with 10 µl and 20 µl of 1-aminopyrene (0.075 mg/ml) for testing on *S. typhimurium* strains TA98 and TA100 respectively.

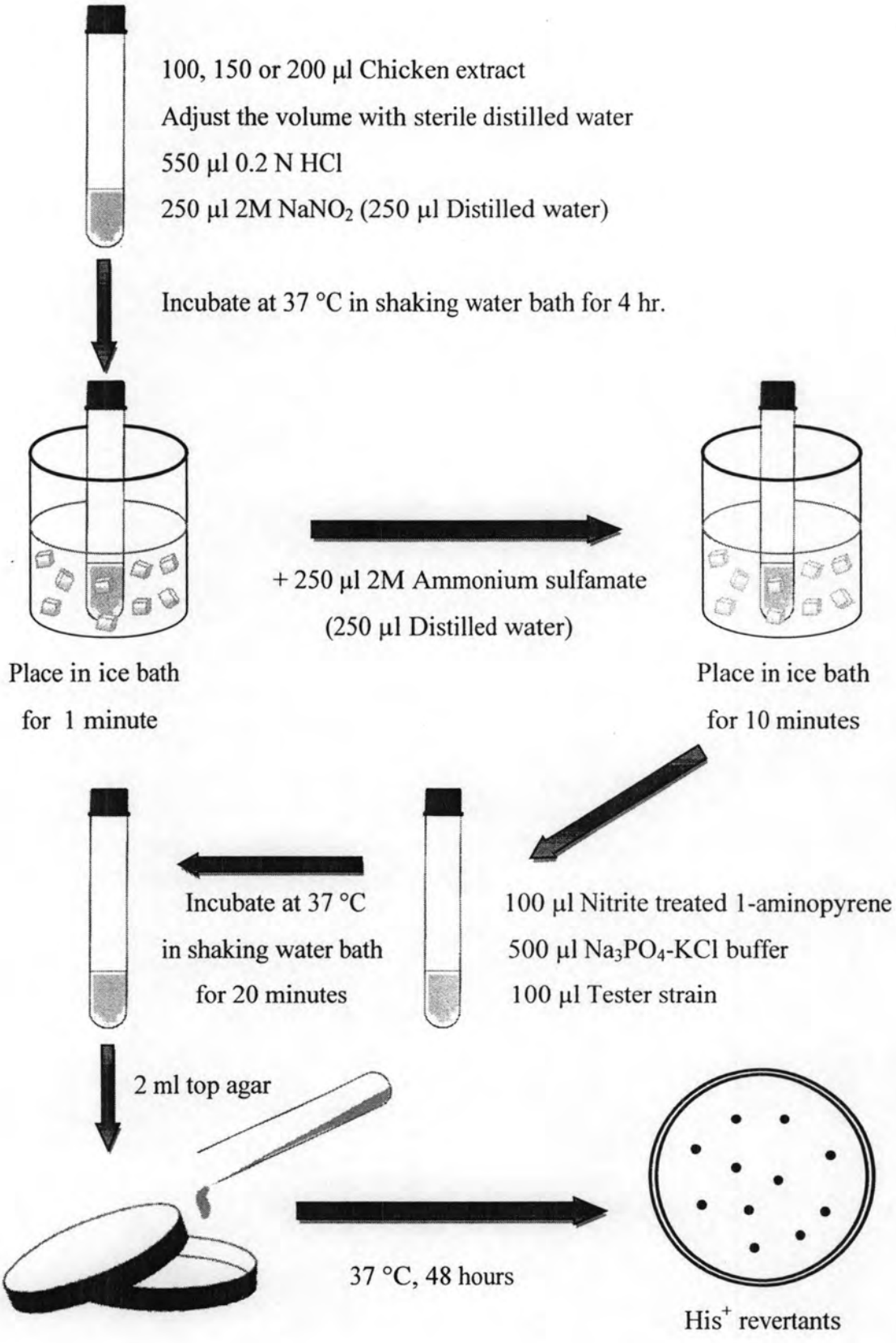

Figure 11 Steps to determine the mutagenicity of the chicken extract, with and without sodium nitrite, using Ames mutagenicity test (preincubation modification)

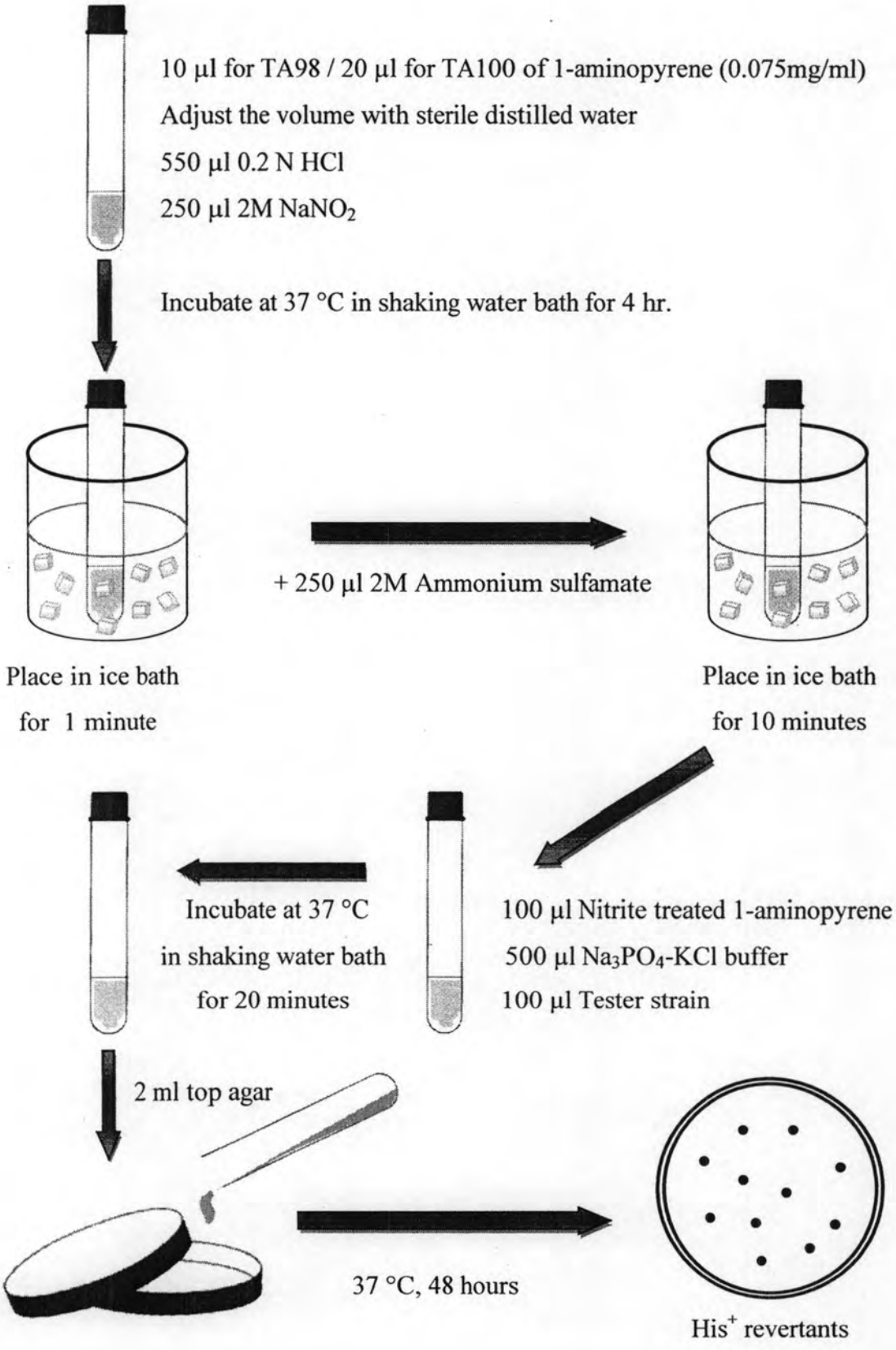

Figure 12 Steps to determine the mutagenicity of the sodium nitrite treated 1-aminopyrene using Ames mutagenicity test (preincubation modification)

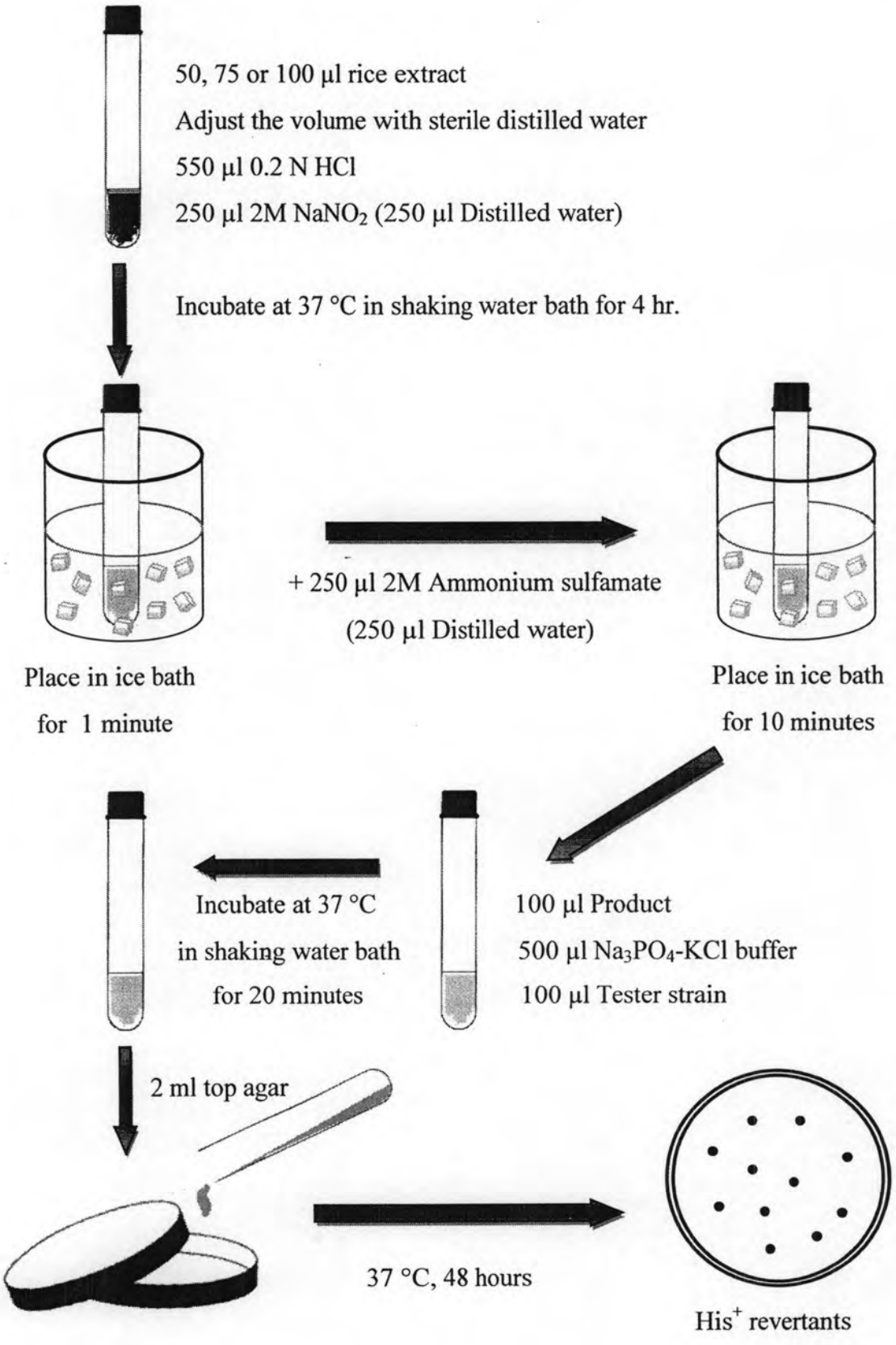

Figure 13 Steps to determine the mutagenicity of the rice extract, with and without sodium nitrite, using Ames mutagenicity test (preincubation modification)

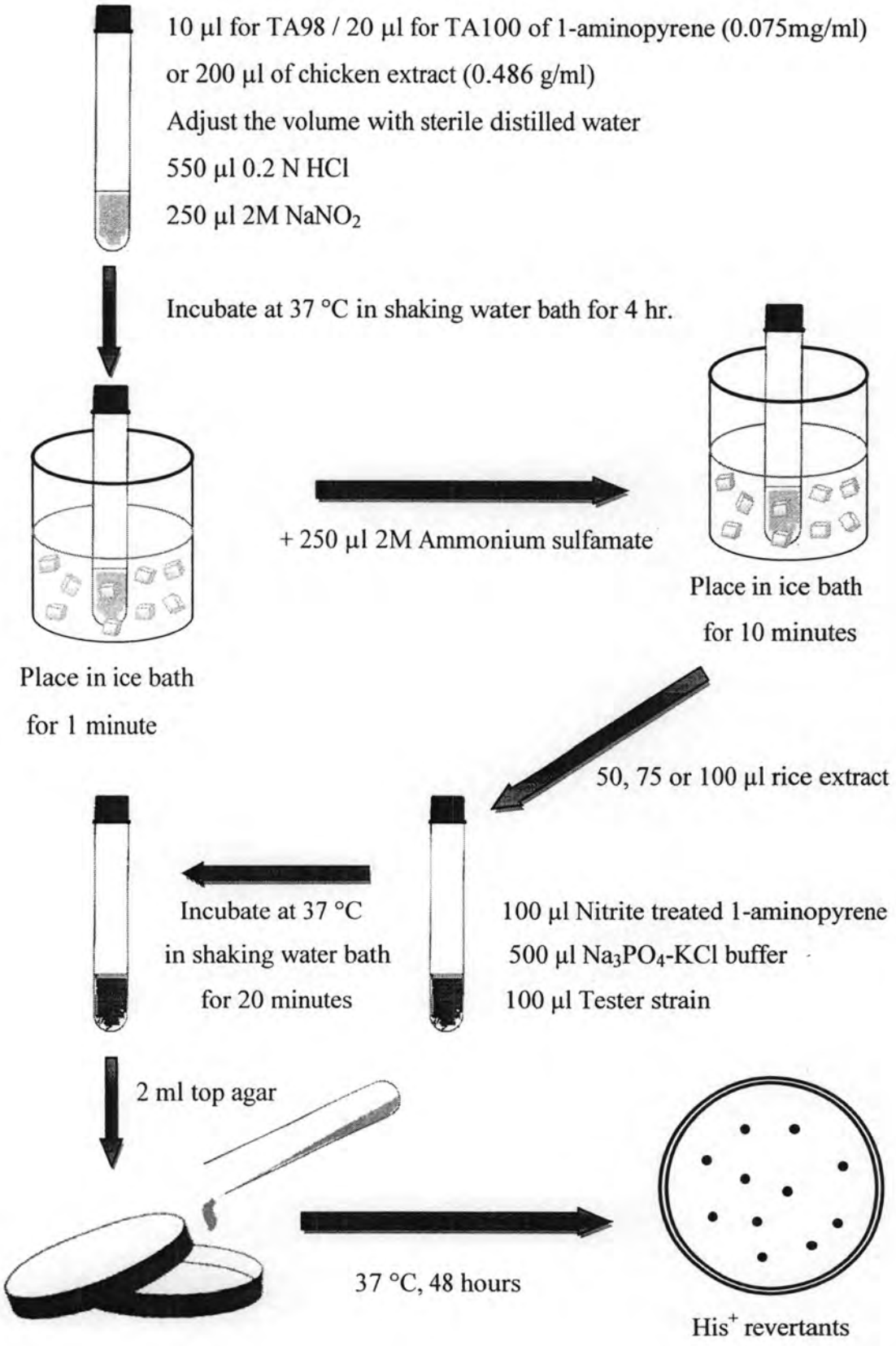

Figure 14 Steps to determine the antimutagenic effect of the rice extract using Ames mutagenicity test (preincubation modification)

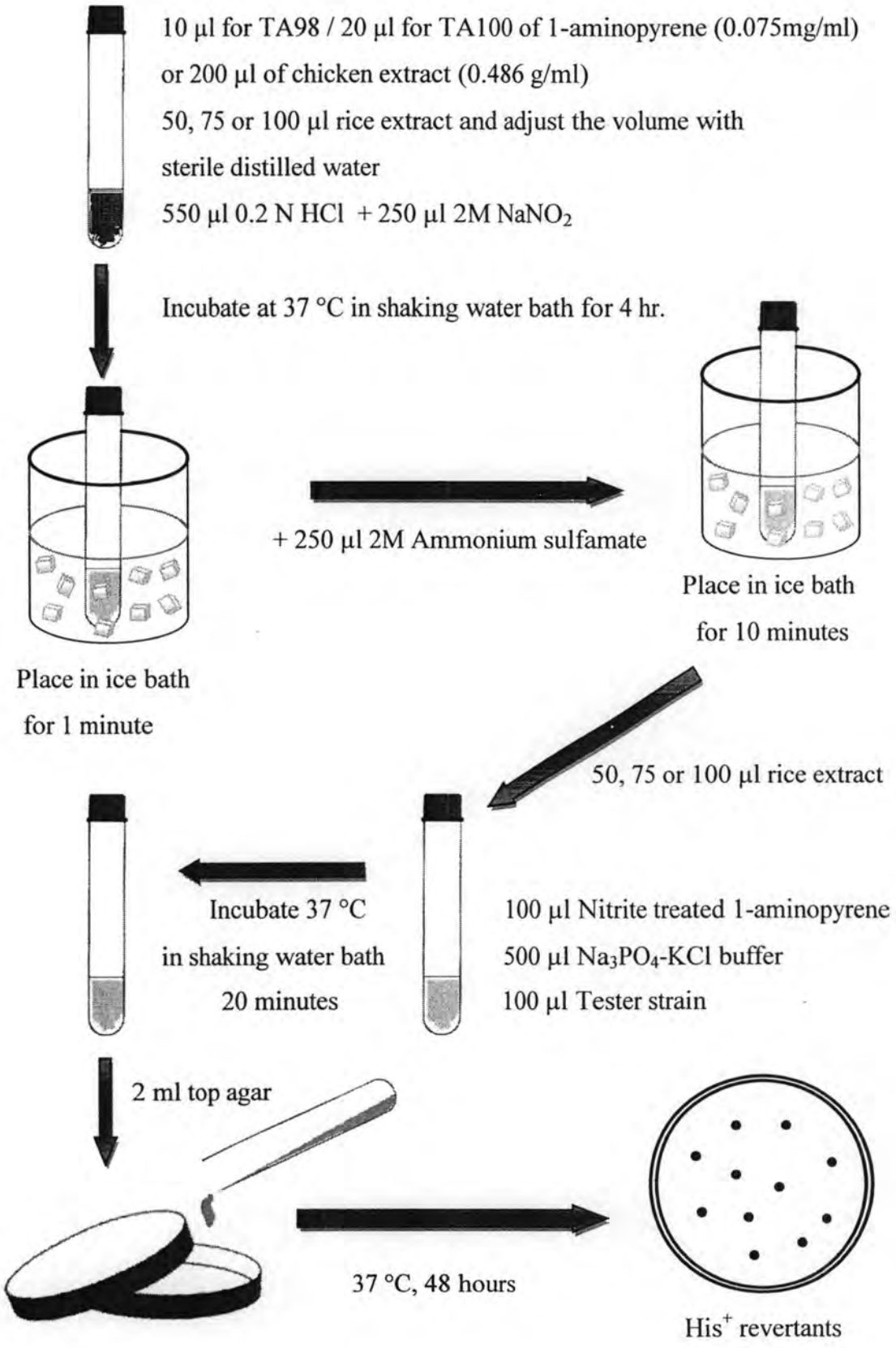

Figure 15 Steps to determine the anti-mutagen forming effect of the rice extract using Ames mutagenicity test (preincubation modification)

3.7 Data Evaluation

The results were reported as mean with standard deviation of histidine revertants per plate. The mutagenicity of each sample was pronounced when number of histidine revertants per plate was higher than twice of spontaneous revertants with a concentration-response relationship (Maron and Ames, 1983). The results were also expressed as a mutagenicity index (MI), which was calculated as following:

$$MI = \text{Average (N)} / \text{Average (S)}$$

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

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

The percentage inhibition for antimutagenicity was calculated as following:

$$\% \text{ inhibition} = \left(\frac{A - B}{A - C} \right) \times 100$$

A = a number of histidine revertants induced by sodium nitrite treated standard mutagen (1-Aminopyrene) or chicken extract

B = a number of histidine revertants induced by mutagen in the presence of rice extract before pre-incubation process

C = a number of spontaneous revertants (negative control)

The inhibition (or enhancement) of mutagenicity may be divided into four classes as shown in Table 8.

Table 8 Criteria for evaluation as the inhibition or enhancement of mutagenicity

% inhibition	Inhibition or enhancement
± 0 - 20	Negligible effect
± 20 - 40	Weak activity
± 40 - 60	Effective or moderate activity
± > 60	Strong or potent activity

Modified from Calomme *et al.*, 1996
+ = inhibitory effect , - = enhancing effect

The percentage of modification for anti-mutagen formation (either increase or decrease on mutagenicity of standard direct mutagens) of each rice extract was calculated as following:

$$\% \text{ modification} = \frac{\text{Expected MI} - \text{Actual MI}}{\text{Expected MI}} \times 100$$

Expected MI = MI calculated from the summation of MI of sodium nitrite treated mutagen, either chicken extract or 1-aminopyrene, and sodium nitrite treated each rice extract.

Actual MI = MI of sodium nitrite treated mutagen, either chicken extract or 1-aminopyrene, in the presence of rice extract before nitration process.