CHAPTER IV
MATERIALS AND METHODS

4.1 Chemicals

Urethane, 2, 4, 6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, and ferrous sulfate heptahydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylether and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Propionic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and Folin-Ciocalteu reagent were supplied from Fluka Chemika (Buchs, Switzerland). Trolox was bought from Aldrich Chemical (Milwaukee, WI, USA). All other chemicals and reagents were of analytical grade.

4.2 Preparation of Fermented Rice

Six cultivars of unpolished pigmented rice, namely Sung Yod (red rice), Mon Poo (red rice), Hom Mali Daeng (red rice), Hom Nil (purple rice), Riceberry (purple rice), and black glutinous rice (black rice) were obtained from local markets in Bangkok. Look-Pang was brought from One-hundred Year Samchuck Market in Suphanburi Provice. Rice was fermented in Thai traditional style as suggested by Lotong (1992). It was mixed with distilled water (1:2 w/v) and cooked in an electric cooker. Cooked rice was cool at room temperature and fermented with Look-Pang (0.2% w/w of raw rice) at 30°C for 3 d in a glass container. The fermented rice was dried at 60°C in a hot-air oven for 24 h and stored in a vacuum-sealed polyethylene bag at 4°C before use.

4.3 Antioxidant Activities, Total Phenolic and Anthocyanin Contents

4.3.1 Samples Extraction

Each sample was extracted (both hydrophilic and lipophilic compounds in the same time) with dimethylsulphoxide (DMSO) according to the method of Omata *et al*. (2009) with minor modification. Ground sample (1 g) was extracted with 10 ml DMSO at 30°C for 30 min in a shaking water bath. Then, the mixture was centrifuged at 2500 x g for 10 min and the supernatant was collected. The residue was re-extracted under the same condition. The combined supernatant was again centrifuged at 2500 x g for 10 min and the resultant supernatant was used determined for its antioxidant activities [2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP) assays] and total phenolic content.

4.3.2 Free Radical Scavenging Activity Method (DPPH) Assay

The antioxidant activity from each sample on DPPH was estimated using the procedure described by Fukumoto and Mazza (2000) with some modifications. Each sample was allowed to react with the stable radical (DPPH) in order to evaluate the free radical scavenging activity monitored with the decrease of the absorbance at 520 nm. An aliquot of 22 μl of each sample or standard Trolox (in triplicate) 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 darkness at room temperature. After 30 min, the absorbance of the solution was read in a microplate reader (Sunrise, Tecan Co., Austria) using a 520 mm filter. Standard curve was prepared by using several concentrations of Trolox (0.08 – 0.64 mM in 80% methanol). The antioxidant activity of the extracts was determined using the standard curve expressed as mg of Trolox equivalent per 100 g dry weight.

4.3.3 Ferric Reducing Antioxidant Power Method (FRAP) Assay

The antioxidant activity was measured by its ability to reduce the Fe^{3+}/ferricyanide complex 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 ferrous sulfate heptahydrate was added to each well in a 96-well microtiter plate in triplicate. FRAP reagent (150 µl), freshly prepared and warmed at 37^{o}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 min. The absorbance was read at 600 nm using a microplate reader. The change in absorbance after 8 min 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 and 1000 µM) were used for calibration. The FRAP values of the extracts determined using this standard curve were expressed as mmol of Fe(II) per 100 g dry weight.

4.3.4 Determination of Total Phenolic Content

The total phenolic content of each sample was determined according to method described by Amarowicz *et al*. (2004) and modified the procedures of measurement by using a microplate reader. Briefly, 10 µl of each sample 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 recorded after 30 min at 750 nm with microplate reader (Sunrise, Tecan Co., Austria). The amount of total phenolic content was calculated as a gallic acid equivalent from the calibration curve of gallic acid standard solutions (concentration range between 25 and 800 mg/l), and expressed as mg of gallic acid equivalent per 100 g dry weight.

4.3.5 Determination of Anthocyanin Content

Anthocyanin content of each sample was determined according to the method described by Abdel-Aal and Hucl (1999) with minor modification. A ground sample (3 g) was added in a 50-mL centrifuge tube containing 30 ml of acidified ethanol (95% ethanol and HCl 1.0 N, 85:15 v/v). The mixture was mixed, adjusted to pH 1 with 4 N HCl and shaken at 25°C for 15 min. The mixture was readjusted to pH 1 and shaken for an additional 15 min. Then, the mixture was centrifuged at 2500 x g for 10 min and the supernatant was poured into a 50-ml volumetric flask and made up

to volume with acidified ethanol. Absorbance was measured at 535 nm against a reagent blank. The amount of total anthocyanin content was calculated as a cyanidin 3-glucoside equivalent from the calibration curve of cyanidin 3-glucoside standard solutions (0–27 mg/3 ml) and expressed as mg cyanidin 3-glucoside per 100 g dry weight.

4.3.6 Statistical Methods

All data were reported as means $\pm$ SD for triplicate analyses of the same sample. SPSS (version 13) was used to analyze all data as following. One-Way Analysis of Variance and Schefee's multiple comparison tests were used to determine the effect of fermentation on the antioxidant activities, total phenolic content, and anthocyanin content. Paired-samples t-test was used for evaluation the significant difference between mean values of antioxidant activities, total phenolic content,and anthocyanin contents of unfermented rice and those of fermented rice. Statistical significance was considered for p less than 0.05.

4.4 Antimutagenicity Study using the SMART

4.4.1 *Drosophila* Stock

Two *Drosophila melanogaster* strains were used. Virgin females of *ORR/ORR; flr3/In (3LR)TM3, ri pp sep l(3)89Aa bx34e e Bds* were crossed with males of *mwh/mwh*. Prof. Graf (University of Zurich, Switzerland) kindly provided both strains. Those laboratory stocks were kept on regular medium composed of corn flour 125 g, sugar 100 g, baker's yeast 50 g, agar 14 g, propionic acid 5 ml and water 1000 ml (Robert, 1986). The cultures of the flies as well as the treated larvae were maintained at a constant temperature 16±1 and 25±1ºC, respectively.

4.4.2 Standard Medium

Yeast-glucose-agar *Drosophila* media was the modified formula of Robert (1986). It composed of corn flour (3.75 g), sugar (3.00 g), agar (0.45 g) and yeast (1.50 g). The ingredients were mixed and boiled in 50 ml Erlenmeyer flask containing

30 ml deionized water until it became sticky. Propionic acid was added (0.15 ml) to the medium as a preservative. This medium was used for maintaining the stock of fly culture, mating and collecting larvae.

4.4.3 Experimental Medium

It was prepared by adding each sample to the fly medium (Robert, 1986). Each sample (dried fermented rice) was substituted for 50, 75 and 100 % corn flour (0.25 g) and mix well with other ingredients namely, sugar (0.2 g), agar (0.03 g), yeast (0.10 g) and water (2 ml) to make experimental medium. It was used in mutagenicity evaluation of each sample. Standard medium was used as a negative control while standard medium containing 20 mM urethane was used as a positive control. Toxicity of each sample was determined from data of survival rate of adult files.

4.4.4 Mutagenicity Assay

The mutagenicity assay of each sample was carried out as described by Graf *et al*. (1984). Virgin females of *ORR; flr*^{3} were mated with *mwh* males on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred (with the help of a fine artist's brush) to experimental medium, negative control and positive control medium. They were incubated at 25±1^{o}C until pupation. After metamorphosis, the surviving flies were collected on days 10-12 after egg laying and stored in 70% ethanol as suggested by Graf and van Schaik (1992).

Survival rate of adult files from larvae fed on each experimental medium were collected. Only the highest concentration of each sample providing more than 50% survival was determined for its mutagenicity. Only the insect bearing the marker trans-heterozygous (*mwh*+/+*flr*^{3}) indicated with round wings were mounted on microscope slides (described below) and they were examined for mutant spots. Mutagenicity assay were done twice in order to observe their reproducibility. Toxicity of each sample was determined from data of survival rate of adult files.

The round wings of surviving flies stored in 70% ethanol were washed with distilled water. Wings were separated from the body with a fine paintbrush, lined up on a clean slide. A droplet of Faure's solution (30 g gum arabic, 20 ml glycerol, 50

g chloral hydrate, and 50 ml deionized water) as suggested by Graf *et al*. (1984) was dropped on the slide and a cover slip was put on. The round wings of surviving flies (both the dorsal and ventral surface), at least 40 wings, were analyzed under a compound microscope at 400x magnification for the presence of clones of cells showing malformed wing hairs. The position of the spots was noted according to the sector of the wing (Figure 4.1). Different types of spots namely, single spots showing either the multiple wing hairs *(mwh)* or the flare (*flr*^{3}) phenotype and twin spots showing adjacent *mwh* and *flr*^{3} areas were recorded separately. The size of each spot was determined by counting the number of wing cells (hairs) exhibiting the *mwh* or the *flr* phenotype. The spots were counted as two spots if they were separated by three or more wide-type cell rows. Multiple wing hairs (*mwh*) were classified when a wing cell contained three or more hairs instead of one hair per cell as in wide-type. Flare wing hairs exhibited a quite variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon-like extrusions of melanolic chitinous material. All experiments were done twice in order to observe their reproducibility. Toxicity of each sample was determined from data of survival rate of adult files.

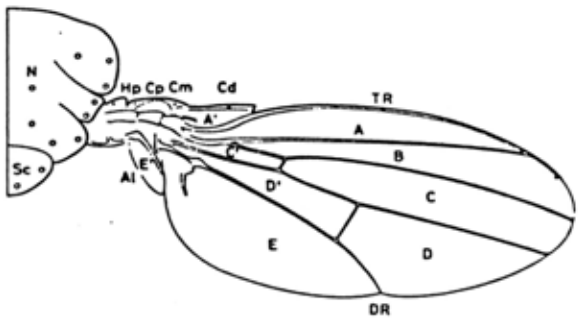

Figure 4.1 Normal half mesothorax showing the regions A-E of the wing surface scored for spots.

The wing spots data were evaluated using the statistical procedure described by Frei and Würgler (1988). Frequencies of induced wing spots of both the treated groups and the negative control were compared. The resulting wing spots were classified accordingly into the following: (1) small single spots of 1 or 2 cells in size, (2) large single spots of 3 or more cells, and (3) twin spots. The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$. A multiple decision procedure was used to decide whether a result was positive, weakly positive, inconclusive or negative according to Frei and Würgler (1988). Statistical consideration and step by step calculation are shown in Appendix A.

4.4.5 Antimutagenicity Assay

The mutagenicity of all samples which provided more than 50% survival of adult flies and did not express their genotoxicity were evaluated in antimutagenicity study (Figure 4.2) of the SMART.

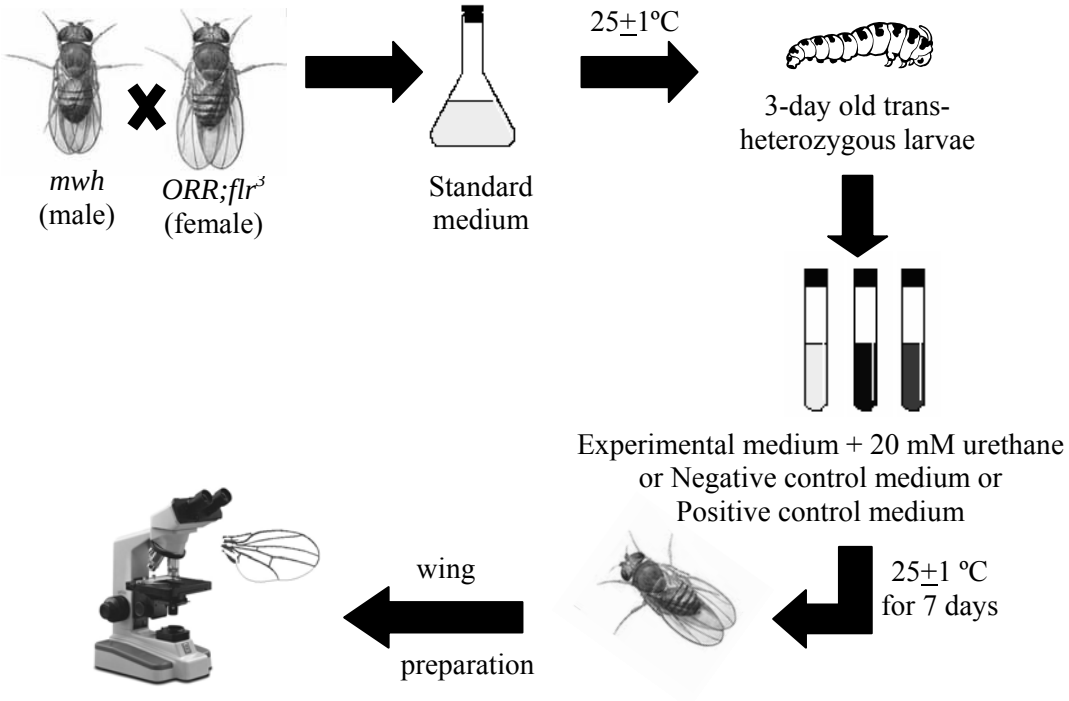

Figure 4.2 Antimutagenicity study of sample against of urethane induced wing spots of *Drosophila melanogaster*.

In this study, virgin *ORR;flr*^{3} females and *mwh* males were mated on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred to experimental medium containing urethane (20 mM), negative control and positive control medium. They were incubated at 25$\pm$1^{o}C until pupation. The surviving adult flies were collected after pupation and were proceeded as of mutagenicity study.

4.4.6 Statistical Methods

The wings of the surviving flies were analyzed for the occurrence of mutant spots and evaluated as described by Frei and Würgler (1988). The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$ (Appendix A). The antimutagenicity of each sample was determined from the percentage of inhibition (Abraham, 1994).

$$\text{Percentage of inhibition} = [(a-b)/a]\times 100$$

When "a" is the number of total spots per wing induced by urethane alone, "b" is the number of total spots per wing induced by urethane in the presence of sample. It is proposed that percentage of inhibition between 0-20 represents a negligible antimutagenicity while percentage of inhibition between 20-40, 40-60 and more than 60 are the evidences of weak, moderate and strong antimutagenicity, respectively. When "b" is higher than "a" the result is classified as enhancement; on the other hand, when "b" is lower than "a" the result is classified as inhibition.

4.5 The Optimal Fermented Rice for Using in the Formulation of a New Functional Food Product

All samples in each test (two antioxidative activities, total phenolic content, anthocyanin content, and antimutagenicity) were ranked from the highest score of 6 to the lowest score of 1. The scores obtained from all five tests of each sample were summed. The fermented rice that has the highest summed score was determined for its nutritive values according to the method of AOAC (2000) namely

moisture, ash, protein, fat, carbohydrate, and energy. It also was used in the formulation of a new functional food product namely, cereal bar.

4.6 Cereal Bar Containing Dried Fermented Rice

4.6.1 Control Cereal Bar

The control cereal bar was prepared as suggested by Ryland *et al*. (2010) with slight modification by substituting corn flakes or crispy rice or the mixture of corn flakes and crispy rice (1:1 w/w) for lentil flake as shown in Table 4.1.

Table 4.1 Formulation of control cereal bars

Ingredient	Amount in g (%ingredient)		
	Formula A	Formula B	Formula C
Granola[a]	220 (36.06)	220 (36.06)	220 (36.06)
Sweetened condensed milk	180 (29.51)	180 (29.51)	180 (29.51)
Corn flakes	180 (29.51)	-	90 (14.75)
Crispy rice	-	180 (29.51)	90 (14.75)
Honey	30 (4.92)	30 (4.92)	30 (4.92)

[a]Granola was prepared by combining 280 g quick cooking oatmeal, 80 g canola oil and 50 g liquid honey and baking at 120ºC for 90 min, stirring every 15 min.

Briefly, sweetened condensed milk and honey were blended in an electric mixture at low speed for 1 min. Granola and the other ingredients were added and mixed well for 2 min. The mixture was spread in a baking tray lined with aluminum foil, baked at 150^{o}C for 20 min in a household electric oven and cooled on a stainless steel rack at ambient temperature (approximately 28ºC) for 10 min. An aluminum foil was removed and the cereal bar was cooled for another 15 min. The product was cut into an appropriate size (approximately 3.5×10 cm.), kept in polyethylene bags at ambient temperature (approximately 28ºC) for sensory screening test.

4.6.2 Sensory Screening Test for a Selected Control Cereal Bar

A randomized complete block design (RCBD) was conducted for sensory test in order to determine the acceptability of the control cereal bars. The samples were prepared on the day before the sensory screening test. Each formula was labeled with a three-digit random number and was randomly served to each panelist, one at a time. The sensory screening test was performed at the Sensory Science Laboratory of the Institute of Nutrition, Mahidol University. Thirty panelists including staffs and graduate students of the Institute of Nutrition, Mahidol University were participated in the test. Subjects were asked to rinse their mouth with water between samples.

A nine-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely) was used to rate the 3 samples for general appearance, color, overall acceptability, odor, flavor, and texture. The questionnaire is shown in Appendix B. The control cereal bar with the highest score and higher than 6 of overall acceptability would be selected as a control formula.

4.6.3 Formulation of Cereal Bar Containing Dried Fermented Rice

Dried fermented rice was substituted for the mixture of corn flakes and crispy rice in a control cereal bar (formula C) described earlier (Table 4.1). The formulas of cereal bar containing dried fermented rice were shown in Table 4.2. The optimum level of dried fermented rice for the cereal bar was obtained after the second sensory screening test.

Table 4.2 Formulation of cereal bars containing dried fermented rice.

Ingredient	Amount in g (%ingredient)		
	Formula D	Formula E	Formula F
Granola[a]	220 (36.06)	220 (36.06)	220 (36.06)
Sweetened condensed milk	180 (29.51)	180 (29.51)	180 (29.51)
Mixture of corn flakes and crispy rice	90 (14.75)	45 (7.38)	-
Dried fermented rice	90 (14.75)	135 (22.13)	180 (29.51)
Honey	30 (4.92)	30 (4.92)	30 (4.92)

[a]Granola was prepared by combining 280 g quick cooking oatmeal, 80 g canola oil and 50 g liquid honey and baking at 120ºC for 90 min, stirring every 15 min.

4.6.4 Sensory Screening Test for Selecting the Optimum Level of Dried Fermented Rice in Cereal Bar

The cereal bar containing dried fermented rice (mentioned in 4.6.3) was prepared on the day before the sensory screening test. It was stored in a polyethylene bag at ambient temperature (approximately 28ºC). A nine-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely) was used to rate the samples for general appearance, overall acceptability, and odor. A five-point just-about right (1 = much too little, 3 = just about right and 5 = much too much) was used for evaluating color, taste (sweetness), and texture. The questionnaire is shown in Appendix C. The product with the highest score and the mean acceptability rating of at least 6 (like slightly) for overall acceptability and the score of five-point just-about right, which was not significantly different from the score of 3, would be selected for further study in the in-house consumer test; it was called new cereal bar.

4.6.5 In-House Consumer Test

The new cereal bar obtained from sensory screening test (see 4.6.4) and control cereal bar were evaluated by fifty panelists who were recruited from the staffs and graduate students of the Institute of Nutrition, Mahidol University. A nine-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely) was used to rate the samples for general appearance, color, overall acceptability, odor, flavor, and texture liking. The questionnaire is shown in Appendix B.

4.4.6 Statistical Methods

One-Way ANOVA and Schefee's multiple comparison tests were used to detect significant differences in sensory screening test which was test by a nine-point hedonic scale. One sample t-test was used for evaluation the significant difference in sensory screening test which was test by a five-point just-about-right scale. Data from in-house consumer test was analyzed by independent-samples t-test. Statistical significance was considered for p less than 0.05.

4.7 Physical Properties of the Control Cereal Bar and Cereal Bar Containing Dried Fermented Rice

All samples were prepared and kept in polyethylene bags at ambient temperature (approximately 28ºC) for 24 h before determination. Color values (L*, a*, b*) of each sample was measured using a spectro-colorimeter model JS555 (Color Techno System Corporation, Tokyo, Japan). A tungsten halogen lamp was used as a light source. The color values by a colorimeter were expressed as L*, a*and b* values. The maximum for L* value is 100 which represents a perfect reflecting diffuser. The zero is minimum value for L* which represents black color. Positive a* value is red while a negative one is green. Positive and negative b* value are yellow and blue, respectively. The a* and b* values have no specific numerical limits. In case of cereal bars, they were ground before measuring.

Water activity was measured using a water activity meter (Novasina IC-500 A_w-Lab, Axair Ltd., Pfäffikon, Switzerland) at a controlled temperature (25$\pm$1°C). Each ground sample was placed in the equilibrium chamber until the constant water activity was gained.

The texture of each sample was measured using a texture analyzer model TA.XT plus with Texture Exponent 32 software (Stable Micro Systems Ltd., YL, UK). The texture analyzer was calibrated with a 1000 gram weight and programmed to measure hardness and fracturability using the small three-point bend rig (HDP/3PB) probe. The probe was located on a heavy duty platform. The sample was placed on the stage. The probe descended into the geometric center of the piece to a distance of 30 mm at the rate of 1 mm/sec and withdrew after the piece was broken. Graphs of texture profile analysis are shown in Appendices D and E.

Data of physical properties were evaluated by One-Way ANOVA and Schefee's multiple comparison tests using SPSS (version 13). All data were reported as means $\pm$ SD. Statistical significance was considered for p less than 0.05.

4.8 Antioxidant Activities, Total Phenolic content, Anthocyanin Content, and Antimutagenicity of the Control Cereal Bar and New Cereal Bar

The sample was ground and stored in a vacuum-sealed laminated aluminum foil bag at 4°C before the determination of its antioxidant activities, total phenolic content, anthocyanin content, and antimutagenicity. Each sample was extracted with DMSO as mentioned in section 4.3.1 and was determined for its antioxidant activities and total phenolic content followed the methods shown in section 4.3.2, 4.3.3, and 4.3.4, respectively. Anthocyanin content and antimutagenicity of each sample were analyzed according to the methods in sections 4.3.5 and 4.4, respectively.

All data were analyzed by independent-samples t-test using SPSS (version 13) and reported as means $\pm$ SD for triplicate analyses of the same sample. Statistical significance was considered for p less than 0.05.

4.9 Nutritive Values of the Control Cereal Bar and New Cereal Bar

Each sample was ground and determined for its nutritive values according to the method of AOAC (2000) namely moisture, ash, protein, fat, carbohydrate (by subtracting the percentage of moisture, ash, protein and fat from 100), energy (by calculation), soluble and insoluble dietary fiber. All data were reported as averages for duplicate analyses.

4.10 Effect of Storage on Various Properties of the New Cereal Bar

The sample was prepared and stored in a vacuum-sealedlaminated aluminum foil bag at ambient temperature (approximately 28ºC) for 0, 45 or 90 days. The sample was subjected to acceptability test and evaluated for physical properties (color, water activity, and texture), antioxidant activities, total phenolic content, anthocyanin content, and antimutagenicity as mentioned above.

One-Way ANOVA and Schefee's multiple comparison tests were used for evaluation the significant difference between mean values of physical properties, antioxidant activities, total phenolic content, and anthocyanin content of each product. The statistical method described by Frei and Würgler (1988) and Abraham (1994) was used to determine the mutagenicity and antimutagenicity, respectively.