CHAPTER 3

MATERIALS AND METHODS

3.1 Raw material

3.1.1 Roselle calyces (Dr. Green, Thailand)

3.1.2 Sucrose (Mitr Phol Sugar Corp., Ltd)

3.1.3 Sugar alcohol

3.1.3.1 Xylitol-crystalline (Danisco, Denmark)

3.1.3.2 Maltitol syrup powder MU90G (Ueno, Japan)

3.1.3.3 Lactitol-monohydratecrystalline (Danisco, Denmark)

3.1.4 Anthocyanin

3.1.4.1 Grape skin (AC 12 WSP, Christian Hansen)

3.1.4.2 Black carrot (ColorFruit Carrot 12 WSP, Christian Hansen)

3.1.5 Chinese-style sausage ingredients (e.g. Chinese herb, seasoning etc)

3.1.6 Thai pork fermented “Nham” ingredients (e.g. salt, sugar, sticky rice etc.)

3.1.7 Vienna sausage ingredients (e.g. seasoning etc)

3.1.8 Collagen casing (Nippi casing, Japan)

3.1.9 Nylon/PE vacuum bag (PA/LLDPE/LDPE), Thickness 80 micron

3.2 Reagents

Rosell anthocyanin extraction

3.2.1 Ethanol 95% (commercial grade)

3.2.2 Hydrochloric acid (Sigma-Aldrich, USA)

Anthocyanins determination

3.2.3 Sucrose (Sigma-Aldrich, USA)

3.2.4 Sodium phosphate, dibasic (J.T. Baker, Austria)

3.2.5 Citric acid, monohydrate (J.T. Baker, Austria)

3.2.6 Acetaldehyde (Sigma-Aldrich, USA)

3.2.7 Sulfur dioxide (Sigma-Aldrich, USA)

3.2.8 Acetronitrile (Merck, Germany)

3.2.9 Acetic acid (Merck, Germany)

3.2.10 Phosphoric acid (Merck, Germany)

Ferrous Ions Chelating Ability (FICA) assay

3.2.11 Ferric chloride (Sigma-Aldrich, USA)

3.2.12 Ferrozine (Sigma-Aldrich, USA)

3.2.13 Methanol (Merck, Germany)

Trolox Equivalent Antioxidant Capacity (TEAC) assay

3.2.14 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Trolox® (Sigma-Aldrich, USA)

3.2.15 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic Acid) Diammonium Salt; ABTS (Sigma-Aldrich, USA)

3.2.16 Potassium persulfate (Merck, Germany)

Ferric thiocyanate (FTC) assay

3.2.17 Ammonium thiocyanate (Sigma-Aldrich, USA)

3.2.18 2,6-Di-tert-butyl-4-methylphenol; BHT (Sigma-Aldrich, USA)

3.2.19 Linoleic acid (Sigma-Aldrich, USA)

3.2.20 Tocopherols (Sigma-Aldrich, USA)

3.2.21 Ferric chloride (Sigma-Aldrich, USA)

3.2.22 Hydrochloric acid (Sigma-Aldrich, USA)

3.2.23 Ethanol 95% (commercial grade)

Thiobarbituric acids reactive substtance (TBARS)

3.2.24 Sulfanilamide (Sigma-Aldrich, USA)

3.2.25 Hydrochloric acid (Sigma-Aldrich, USA)

3.2.26 2-thiobarbituric acid (Sigma-Aldrich, USA)

Protein oxidation (carbonyl) by 2,4-dinitrophenylhydrazones (DNPH)

3.2.27 Potassium chloride (Sigma-Aldrich, USA)

3.2.28 Trichloroacetic acid (Merck, Germany)

3.2.29 Hydrochloric acid (Sigma-Aldrich, USA)

3.2.30 2,4-dinitrpphenylhydrazine (DNPH) (Merck, Germany)

3.2.31 Ethanol 95% (commercial grade)

3.2.32 Ethyl acetate (Sigma-Aldrich, USA)

3.2.33 Sodium phosphate, dibasic (Sigma-Aldrich, USA)

3.2.34 Guanidine hydrochloride (Sigma-Aldrich, USA)

3.2.35 Bovine serum albumin (BSA) (Merck, Germany)

Nitrite scavenging activity

3.2.36 Sodium nitrite (Sigma-Aldrich, USA)

3.2.37 Sulfanilamide (Sigma-Aldrich, USA)

3.2.38 *N*-(1-naphthyl)-ethyline diamine hydrochloride (Sigma-Aldrich, USA)

3.2.39 Phosphoric acid (Merck, Germany)

3.2.40 Ascorbic acid (Merck, Germany)

3.2.41 Butylated hydroxyanisole (BHA) (Merck, Germany)

Nitric oxide scavenging activity

3.2.42 Sodium nitroprusside (Sigma-Aldrich, USA)

3.2.43 Sulfanilamide (Sigma-Aldrich, USA)

3.2.44 *N*-(1-naphthyl)-ethyline diamine hydrochloride (Sigma-Aldrich, USA)

3.2.45 Naphthylethylenediamine dihydrochloride (Sigma-Aldrich, USA)

Peroxynitrite scavenging activity

3.2.46 Nitric acid (Sigma-Aldrich, USA)

3.2.47 Hydrogenperoxide 30% (Sigma-Aldrich, USA)

3.2.48 Monopotassium phosphate (Sigma-Aldrich, USA)

3.2.49 Dipotassium hydrogen phosphate (Sigma-Aldrich, USA)

3.2.50 Evans blue (Sigma-Aldrich, USA)

3.2.51 Manganese dioxide (Sigma-Aldrich, USA)

3.2.52 Potassium chloride (Sigma-Aldrich, USA)

3.2.53 Sodium chloride (Sigma-Aldrich, USA)

3.2.54 Diethykenetriaminepentaacetic acid (Sigma-Aldrich, USA)

3.2.55 Sodium hydroxide (Sigma-Aldrich, USA)

3.2.56 Sodium nitrite (Sigma-Aldrich, USA)

3.3 Equipments

3.3.1 Nano (grinding and dispersing) machine (JBM-BD35, Taiwan)

3.3.2 Transmission electron microscopy (TEM)

3.3.3 High performance liquid chromatography (Hitachi, Japan)

3.3.4 pH meter couple with Mettler LE438 probe (Mettler Toledo, delta 320, USA)

3.3.5 pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany)

3.3.6 Centrifuge (Harmle Z233 MK-2, Germany)

3.3.7 Centrifuge (Hettich Universal 16, Germany)

3.3.8 UV-Vis spectrophotometer (Hitachi U-2001, Japan)

3.3.9 UV-Vis spectrophotometer (Shimadzu UV-1601, Japan)

3.3.10 Colormeter (ZE 2000, Nippon Denshoku, Japan)

3.3.11 Colormeter (Minolta CR-400, Japan)

3.3.12 Water activity meter (AquaLab™ Series3TE, USA)

3.3.13 Rotary evaporator unit (RII, Büchi Rotavapor, USA)

3.3.14 Homogenizer (Ultra-Turrax® T25Bbasic, Germany)

3.3.13 Texture analyzer, TA-XT2i plus (Stable Micro Systems, U.K.)

3.3.15 Meat grinder (SevenFive, Thailand)

3.3.16 Stuffer (Thailand)

3.3.17 Tray dryer (Patch663, Thailand)

3.3.18 Vacuum packing machine (Alpha-Pack, Thailand)

3.3.19 Convention oven (MR-1302, Mamaru, France)

3.4 methods

3.4.1 Determination of physicochemical and antioxidant properties of roselle extracts prepared by original-grinding extraction and nano-grinding extraction method

Comparative studies on physicochemical and antioxidant properties of roselle extracts prepared by original-grinding and nano-grinding extraction were done. The model solution with different pH, temperature and sucrose concentration was used to evaluate the properties of the roselle extract samples.

3.4.1.1 Sample preparation

1) Roselle calyxes

Fresh roselle calyxes were purchased from Peasant Association of Area Taitung, Taiwan. The calyces were dried at 40 °C using hot air oven until a_W ~0.48. Dried calyxes were ground using household blender.

2) Roselle extraction

In this study two methods were used to extraction roselle calyxes;

1) ground roselle calyxes was soaked in ethanolic solution (1.5 M hydrochloric acid in ethanol) for 24 hr, the sample from this extraction refers to “OG”, and

2) ground roselle calyxes was extracted in ethanolic solution coupled with nano machine with set speed at 2000 rpm for 1 hr. Zirconium oxide (ZrO_2) was used as grinder materials. The extract obtained by this procedure refers to “NG”.

The roselle extracts, OG and NG, were evaporated at 30 °C until the acidified ethanol was removed. The extracts were made up to 10 ml with acidified ethanol and stored at -25 °C until further analyzed.

3) Model solutions

Citric acid-Na_2HPO_4 (Mcllvaine) buffer solution was used (see Appendix B1) to prepare a model solution of roselle extracts by dissolving the extracts (OG and NG) in the buffer solution at different pH (pH 3.0 and 4.0) with or without sucrose (0 % and 20 % w/w) and heated at 50, 60, and 70 °C.

3.4.1.2 Analytical methods

1) Color determination

Color of roselle extract solutions were measured using a Colormeter (ZE 2000, Nippon Denshoku, Japan). The absorbance of the roselle extract sample was read at 420, 520, and 700 nm using 1 cm quartz cell by a UV-vis spectrophotometer (U-2001, Hitachi, Japan). Samples were also placed in an optical glass cell and L, a, b and chroma were measured in triplicate. The color density (CD; Equation 1) and degradation index (DI; Equation 2) of samples diluted with pH 3.0 or pH 4.0 buffer were measured.

$$Color\ density\ (CD) = \left(A_{520nm} - A_{700nm}\right) + \left(A_{420nm} - A_{700nm}\right) \quad (1)$$

$$Degradation\ Index\ (DI) = \frac{A_{420nm}}{A_{\lambda_{max}}} \quad (2)$$

2) Co-pigmented, monomeric and polymeric anthocyanins

Co-pigmented (Equation 3), monomeric (Equation 4) and polymeric anthocyanins (Equation 5) were determined according to Tsai and Huang (2004). Briefly, 10 % (v/v) acetaldehyde was added to sample. The mixture was allowed to stand for 45 min at room temperature before measuring A_{520nm} (A^{acet}). To another sample, 5 % (w/v) SO_2 was added, and A_{520nm} was measured (A^{SO_2}). The A_{520nm} was also measured directly from the sample. This reading was multiplied by 10 to give the $A^{extract}$. From these readings, the percent distribution of the various forms of anthocyanins can be calculated as:

$$Copigmented\ anthocyanins\ (\%) = \frac{A^{acet} - A^{extract}}{A^{acet}}\ x\,100 \quad (3)$$

$$Monomeric\ anthocyanins\ (\%) = \frac{A^{extract} - A^{so_2}}{A^{acet}}\ x\,100 \quad (4)$$

$$Polymeric\ anthocyanins\ (\%) = \frac{A^{so_2}}{A^{acet}}\ x\,100 \quad (5)$$

3) Antioxidant capacities

The antioxidant activities of roselle extracts were evaluated by various *in vitro* assays, including the ferrous ion chelating activity (FICA) which was measured according to the method of Chang et al. (2006b) (see Appendix B3), antioxidant activity by trolox equivalent antioxidant capacity (TEAC) was measured according to the method of Arts et al. (2003) (see Appendix B4), and inhibitory activity toward linoleic acid peroxidation by ferric thiocyanate (FTC) was measured according to the method of Manian et al. (2008) (see Appendix B5).

4) HPLC anthocyanin analysis

The roselle anthocyanin solutions were analyzed using HPLC with a UV-vis (Tsai and Huang, 2004). Briefly, the separation of anthocyanins was accomplished on a reverse phase C_{18} column (250 mm × 4.6 mm i.d.; Hitachi High-Technologies Corporation, Japan). In direct analyses, mobile phase were A, 5 % acetic acid and B, acetonitrile. The gradient condition was 0-10 min, 100 % A; 10 to 40 min, 90 to 60 %; and back to 100 % after 45 min. Other chromatographic conditions were as follows: flow fate 1 ml/min, 20 μL injections and the detection wavelength was 280 nm (Hitachi UV-vis detector; Hitachi high-technologies Corporation, Japan).

3.4.1.3 Statistical analysis

Mixed model factorial experiments were used in this study. The statistical analysis of the data was carried out using SPSS software for the analysis of variance (ANOVA) in determining significant differences at a confidence level at 95 % (p<0.05). Variable means were compared by Duncan's multiple rang test.

3.4.2 Evaluation of antioxidant capacity of roselle extracts on lipid and protein oxidation as affected by sucrose and different sugar alcohols in Chinese-style sausage

This objective is intended to evaluate the effect of sucrose and different sugar alcohols on anti-lipid and anti-protein oxidation capacity of roselle extracts in Chinese-style sausage.

3.4.2.1 Effect of sucrose and different sugar alcohols on antioxidant capacity of roselle anthocyanin extracts

1) Materials

Roselle extract: Roselle extract was prepared by soaking ground roselle calyces in 95 % ethanol for 24 hr. The extract is then evaporated at 30 °C until the ethanol is removed.

The extract was kept in brown glass vial with screw cap and stored at -25 °C until further analysis.

Sugar alcohols: Sugar alcohols including Lactitol-monohydratecrystalline (Danisco, Denmark), Xylitol-crystalline (Danisco, Denmark) and Maltitol syrup powder MU90G (Ueno, Japan) were used to replace sucrose in the sausage formulation.

2) Chinese-style sausage preparation

Chinese-style sausage was prepared as described by Pinsirodom (2008). Briefly, fresh lean pork and back fat were purchased from CP Fresh mart (Bangkok, Thailand). The lean pork and pork back fat were trimmed out of connective tissue. They were ground separately through a 4 mm grinder plate (SevenFive, Thailand), and then kept at 4 °C for 2 hr. The mixture of lean pork (65 %) and pork fat (16 %) were formulated to contain 0.3 % roselle extracts. All other ingredients were added: 0.2 % potassium nitrite (prague powder), 0.1 % Chinese five-spice blend, 0.3 % monosodium glutamate, 1.80% salt and 16.6 % sucrose (see Appendix A1). The sausage mixtures were stuffed into collagen casing (Nippi casing, Japan), linked into 10 cm length and then dried at 60 °C for 24 hr. Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (29±1 °C) and analyzed every week for 4 weeks. To compare the effect of sugar alcohols on lipid and protein oxidation in the products, sugar alcohols were used to replace for sucrose at the level of 16.6 % (w/w) based on the control formula weight. Three replicates with batches were conducted in this study.

3) Physical properties

Moisture content: The moisture content of samples were determined using the oven method at 105±1 °C (AOAC, 2000)

Water activity: Water activity (a_W) of the ground samples were measured by a Water activity meter (AquaLab™ Series3TE, USA).

pH: Each 1 g from samples each treatment was blended by homogenizer (Ultra-Turrax® T25Bbasic, Germany) with 10 ml distilled water for 1 min, then pH was determined using pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany).

Color: Color parameters were measurement by a colormeter (as CIE L*, a*, b*). The color measurements of Chinese-style sausage were performed at room temperature. Total color difference (TCD; Equation 6), Hue angle (Equation 7), chroma (saturation index; Equation

8), and browning index (BI; Equation 9) was calculated using Hunter L*, a*, and b* values (Bozkurt and Bayram, 2006) as:

$$TCD = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad (6)$$

$$Hue\ angle = \arctan(b/a) \quad (7)$$

$$Chroma = \sqrt{a^2 + b^2} \quad (8)$$

$$BI = \frac{[100\ x\ (X - 0.31)]}{0.17} \quad (9)$$

$$\text{Where: } X = \frac{(a + 1.75\ x\ L)}{(5.645\ x\ L + a - 3.012\ x\ b)}$$

4) Antioxidant capacities

Lipid oxidation measurement: Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBARS) (Min et al., 2009). Results were expressed as mg MDA per kg of sausage (see Appendix B6).

5) Statistical analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

3.4.2.2 Effect of sugar alcohol concentration on antioxidant capacity of roselle extracts

In this study, a sugar alcohol was selected (from 3.4.2.1) according to the physical properties and oxidative stability of the sausage samples. The Chinese-style sausages were then prepared with varied concentrations of the sugar alcohol. All samples were compared with Chinese-style sausage containing 16.6 % (w/w) sucrose as a control sample.

1) Materials

Roselle extract: Preparation was done as described in 3.2.1.1

Sugar alcohol: Type of sugar alcohol was selected from the results in experiment 3.4.2.1

2) Sausage preparation

Chinese-style sausage was prepared as described earlier (3.4.2.1.2). To compare the effect of sugar alcohol concentration on lipid and protein oxidation in the products, the sugar alcohol was used to replace for sucrose at the level of 11.6, 16.6 and 21.6% (w/w) based on the control formula weight (see Appendix A2). Three replicates with batches were conducted in this study. Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (30±1 °C) and analyzed every week for 4 weeks.

3) Physical properties: Physical properties of the samples products were analyzed as mentioned in experiment 3.4.2.1 number 3)**.**

4) Antioxidant capacities

Lipid oxidation measurement: Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBARS) method (Min et al., 2009). Results were expressed as mg MDA per kg of sausage (see Appendix B6).

Protein Carbonyl Content: Protein oxidation in the sausage was determined by measuring the formation of protein carbonyls by converting them to 2,4-dinitrophenylhydrazones (DNPH) and the derivatives were measured spectrophotometrically according to method described by Viljanen et al. (2005) (see Appendix B7).

5) Statistical analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

3.4.2.3 Effect of sugar alcohol on texture profile and sensory qualities of Chinese-style sausage during storage

In this study, the Chinese-style sausage sample (from 3.4.2.2) was selected by the consideration of physical properties and as lipid oxidation (TBARS) and protein carbonyl by DNPH method.

1) Materials

Roselle anthocyanins extract: Preparation was done as described in 3.2.1.1

Sugar alcohol: The sugar alcohol was selected from the results in experimental 3.4.2.2

2) Sausage preparation

Chinese-style sausage was prepared as described in experiment 3.4.2.1 number 2). To compare the effect of sucrose and sugar alcohol on texture profile analysis and sensory quality of the products, sucrose was added at 16.6 % (w/w) in the control sample and the level of sugar alcohol added was selected from the result in experiment 3.4.2.2 (see Appendix A3). Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (30±1 °C) and 4 °C and measured every week for 4 weeks.

3) Physical properties: Physical properties of the samples products were analyzed as mentioned in experiment 3.4.2.1 number 3)**.**

4) Texture profile analysis: Texture profile analysis (TPA) was applied as described by Bourne (2002) using a TAXT2i texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe P/25 to determine hardness, cohesiveness, adhesiveness, gumminess, chewiness and springiness and a reversible probe to determine the maximum cutting force and the cutting work. The cutting samples are approximately 1.5 cm long and 2.5 cm wide which will be compressed twice to 50 % of their thickness. The following parameters are defined: hardness (H) = maximum strength required to achieve compression; area of the first compression (A_1) = total energy required for the first compression; area of the second compression (A_2) = total energy required for the second compression, adhesiveness = area under the abscissa after the first compression; springiness (S) = height the sample recovers between the first and second compression; cohesiveness $\left[(C)=\frac{A_2}{A_1}\right]$; gumminess $[(G)=HxC]$; chewiness $[(ch)=SxG]$.

5) Sensory analysis: Sensory evaluations were following the procedure of Chambers and Wolf (1996) and conducted at days 0, 7, 14, 21 and 28 of storage times. The Chinese-style sausages were evaluated by an experienced 25-members panel consisting of master and PhD students, aged from 25 to 35, were selected from the Agro-Industry, KMITL. Panelists were chosen on the basis of previous experience in consuming traditional Chinese-style sausage. Sausages were first cooked in a convention oven (Mamaru, MR-1302, France) at 150 °C for 20 min. Cooled at room temperature (30 °C), then sliced into 3 mm thick pieces and served on plastic dishes covered with plastic films and coded with three-digit random number. The method performed to describe the organoleptic of the Chinese-style sausage was inspired by a

Quantitative descriptive analysis (QDA) was carried out as described by García et al (2010) and Pérez-Cacho et al. (2005). The attributes of this evaluated including appearance, odor, taste, mouth feel and overall acceptability (see Appendix C1). The panelists evaluated sausage over structure scales of 15 cm delimited at both ends. The sensory attributes use in this study including color, odor, taste, texture and overall acceptance were determined using 1-7 point hedonic scale, with 1, 4 and 7 representing extremely dislike, neither like nor dislike and extremely like, respectively (see Appendix C2).

6) Statistical analysis

A completely randomized experimental design (CRD) was used for texture profile analysis and randomized completely block design (RCBD) was used for sensory analysis. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was be determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.

3.4.3 Investigation of the ability of anthocyanins in scavenging of reactive nitrogen species in model system and evaluation of their effect on nitrite reduction in meat products.

These studies were intended to evaluate the ability of roselle extracts in scavenging of reactive nitrogen species (RNS) comparing to the anthocyanins from different sources, and their effect on the nitrite reduction in Vienna sausage and traditional Thai fermented sausage (Nham).

3.4.3.1 Materials

Natural anthocyanins: Roselle anthocyanin extracts was prepared following the method in 3.2.1.1. Anthocyanins from various sources; grape skin (AC 12 WSP, Christian Hansen) and black carrot (ColorFruit Carrot 12 WSP, Christian Hansen) were obtained from The Each Asiatic (Thailand) Public Company Limited and used for the comparative study.

3.4.3.2 Reactive nitrogen species (RNS) scavenging capacities

Nitrite scavenging activity: This assay was carried out as described by Kang et al. (2006) and Saha et al. (2004). The reaction mixtures were prepared in pH 3.0, 6.0 and 9.0 (see Appendix B1 and B2) measured using a UV-vis spectrophotometer at 540 nm (see Appendix B8).

Nitric oxide scavenging activity: Nitric oxide scavenging activity was measured spectrophotometrically as described by Govindarajan et al. (2003). The sample was reacted with Griess solution, the absorbance of reaction mixtures were measured using a UV-vis spectrophotometer at 546 nm (see Appendix B9).

Peroxynitrite ($ONOO^-$) scavenging activity: The peroxynitrite scavenging activity was measured according to the method of Küçük et al. (2007). The absorbance of the color mixtures after incubated at 25 °C were measured using UV-vis spectrophotometer at 611 nm (see Appendix B10).

All RNS scavenging tests were performed three times. Ascorbic acid, Butylated Hydroxyanisole (BHA), and/or Gallic acid were used as a positive control.

3.4.3.3 Nitrite reduction in meat products

Meat products preparation: Preparation of meat products including Vienna sausage (see Appendix A4) and Nham (traditional Thai fermented pork) (see Appendix A5) were followed the method described by Heinz and Hautzinger (2007) and by Swetwiwathana *et al*. (2007), respectively.

The Vienna sausage and Nham were produced under typical processing conditions, with 0.3 % of roselle extracts and two levels of sodium nitrite, 0 and 125 mg/kg (125 ppm), the highest permitted level for use in meat products by Thai Industrial Standards Institute (TISI 3540-2006; TSI 1219-2004).

The Vienna sausage mixtures were stuffed into collagen casing (Nippi casing, Japan), linked into 10 cm length and dried at 60 °C for 1 hr, then cook in hot water (65±3 °C) for 45 min. After cooled, Vienna sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at refrigerated temperature (4±1 °C) and analyzed every 3 days for 24 days. For the traditional Thai fermented pork sausage "Nham", the 100 g of mixtures were stuffed into 2.5 cm diameter polyethylene (PE) bag and tied with thread. After that, the samples were kept at room temperature (30±1 °C) and analyzed every day for 7 days.

1) Physical properties:

Physical properties of the samples products were analyzed as mentioned in experiment 3.4.2.1 number 3)**.**

2) Antioxidant capacities

Lipid oxidation determination: Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBARS) method (Min et al., 2009). Results were expressed as mg MDA per kg of sample (see Appendix B6).

Nitrite determination: Sodium nitrite in the samples was measured according to the method of Liu et al. (2010). The residual nitrite of all samples was reacted with the Griess

solution. Absorbance values of each sample were measured using a UV-vis spectrophotometer at 540 nm (see Appendix B11).

3.4.3.4 Kinetic degradation of nitrite in traditional Thai fermented pork

The traditional Thai fermented pork "Nham" was produced, with or without 0.3 % of roselle extracts and positive control (BHA 200 ppm) and three levels of sodium nitrite; 125, 250 and 500 ppm (see Appendix A6). Briefly, minced pork was mixed thoroughly with roselle extracts following salt and sodium nitrite for 1 min. Then ground garlic and sugar were added and mixed for 1 min. Cooked sticky rice was mixed additionally for 2 min, and then minced pork lard was added and mixed for 2 min. The 100 g mixture was stuffed into 2.5 cm diameter polyethylene (PE) bag and tied with thread. After that, the samples were kept at room temperature (30±1 °C) and analyzed every day for 4 days.

3.4.3.4.1 Chemical properties:

pH: The pH was measured after homogenization (Ultra-Turrax® T25Bbasic, Germany) with distilled water at a ratio of 1:10 using pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany)**.**

Total acidity (as lactic acid): Ten gram samples were homogenized with 90 ml distilled water. Titratable acidity was determined as % lactic acid by titrating with 0.1 M NaOH using phenolphthalein as an indicator (AOAC. 2000).

Nitrite determination: Sodium nitrite in products was measured according to the method of Liu et al. (2010). The nitrite residual of samples were reacted with the Griess solution. Absorbance values of each sample were measured using a UV-vis spectrophotometer at 540 nm (see Appendix B11).

Kinetics of nitrite degradation: The degradation kinetics of most biological materials of food system follows the first-order (Equation 10) reaction (Villota and Hawkes, 2007). The first-order reaction rate constant (k) (Equation 10) and half-life time ($t_{1/2}$) values (Equation 11) were expressed by the following equations:

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \tag{10}$$

$$t_{1/2} = -\ln 0.5k^{-1} \tag{11}$$

Where C_o is the concentration of sodium nitrite at zero time, C_t is the concentration of sodium nitrite at time t (hour) of fermentation time, k is the first-order kinetic constant.

3.4.3.5 Statistical analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant ($P<0.05$), mean values was determined by Duncan's New Multiple Range Test. The statistical analysis was conducted with SPSS software.