

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

- Raw ostrich-meat trimming and its fat (B.M. Farm, Chiang Mai, Thailand)
- Soy protein isolate (Qingdao Wansheng Chemical Company, Qingdao, China)
- Wheat gluten (Qingdao Wansheng Chemical Company, Qingdao, China)
- Whey protein isolate (Arla Foods Ingredients Amba, Videbaek, Denmark)
- Sodium chloride : Prung Thip (Thai refined salt Co., Ltd., Thailand)
- Sodium tripolyphosphate (Lab P&D, Thailand)
- Pure ground pepper : Raitip (Thai cereals world Co., Ltd., Thailand)
- Garlic (Big C Co., Ltd., Thailand)
- Synthetic sausage casing, 29 mm in diameter (Food EQ Ltd., Thailand)
- DSC Aluminium pans (PerkinElmer Instruments, Shelton, Connecticut, USA)

3.2 Chemical reagents

- 40% (w/v) Acrylamide monomer solution containing 5% (w/v) N, N'-methylenebisacrylamide (Amersham Biosciences AB, Sweden)
- Tris[hydroxymethyl]aminomethane (USB Amersham Life Science, USA)
- Sodium Dodecyl Sulfate (USB Corporation, Japan)
- Ammonium persulfate (USB Corporation, Japan)
- Glycerol (Amersham Biosciences AB, Sweden)
- Glycine (USB Corporation, USA)
- Bromophenol blue (USB Amersham Life Science, Austria)
- 2-Mercaptoethanol (Merck, Austria)
- N, N, N', N'-Tetramethylethylenediamine (TEMED) (USB Corporation, USA)
- CoomassieTM Brilliant Blue G-250 (USB Corporation, UK)

- Full Range Rainbow™ Recombinant Protein Molecular Weight Marker (GE Healthcare UK limited, UK)
- Sodium Thiosulphate 5% (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- Silver Nitrate 2.5% (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- Protein Formaldehyde 37% (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- Glutardialdehyde 25% (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- EDTA-Na₂ (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- Sodium Carbonate (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- Sodium Acetate (Plus One™ Silver Staining kit, Amersham Biosciences AB, Sweden)
- Fluorescein Isothiocyanate (FITC) (SIGMA-ALDRICH, St Louis, MO, USA)
- Nile Red (SIGMA-ALDRICH, St Louis, MO, USA)
- Unless otherwise stated all chemicals were of analytical grade.

3.3 Instruments

- Meat grinder, MK-G20N (Matsushita Electric Industrial Co., Ltd., Osaka, Japan)
- Silent cutter, RS 20 (Meissner Gmb H. & CO. KG. Nahrungsmittelmaschinen Biedenkopf-Wallau, Germany)
- Stuffer (Richmond Brown Inc., Madison, WI, USA)
- High pressure rig (Stansted Fluid Power Ltd, Stansted, UK)
- Texture Analyser, TA-XTPlus (Stable Micro Systems, Co., Surrey, UK)
- Differential Scanning Calorimeter, Diamond DSC (PerKin Elmer Instruments, Shelton, Connecticut, USA)

- Rheometer, Advance Rheometer, AR 2000 (TA Instruments Water, Inc., New Castle, DE, USA)
- Leica Scanning Laser Microscopy (Leica Microsystems, Heidelberg, Germany)
- Electrophoresis and electrotransfer unit, miniVE (Amersham Pharmacia Biotech, Uppsara, Sweden)

3.4 Research designs and methods

3.4.1 Investigation the optimal amount of sodium tripolyphosphate (STPP) and sodium chloride (NaCl) incorporating ostrich-yor formula

3.4.1.1 Preparation of ostrich-meat yor

Fresh ostrich-meat trimming was purchased from B.M. Farm, Chiang Mai which had a chemical composition of $20.92 \pm 0.20\%$ (w/w) protein, $1.78 \pm 0.13\%$ (w/w) fat, $0.01 \pm 0.00\%$ (w/w) carbohydrate, $0.87 \pm 0.02\%$ (w/w) ash and $76.42 \pm 0.04\%$ (w/w) moisture. The ostrich-meat trimming and its fat were ground separately in a laboratory grinder through a 5 mm plate, frozen to -18°C and stored at -18°C until used. Before processing, the meat and fat were thawed at 4°C for 18 h (to approximately 0°C). Yor formulation contained; 1% (w/w) white pepper, 3% (w/w) garlic, 1% (w/w) sugar, 5% (w/w) ice and 5% (w/w) ostrich fat, as well as various levels of STPP (0, 0.25 and 0.50%, w/w) and NaCl (1, 2 and 3%, w/w).

Ground ostrich-meat trimming was emulsified with these ingredients to form a homogeneous mass (with a final temperature of 10°C), and subsequently stuffed into synthetic sausage casing, 2.9 cm in diameter. The raw meat yors were cooked by steaming for 60 min then immersed in cold water until cooled to room temperature and stored at 5°C for analysis (a modified method from Fernández-López *et al.*, 2003); the flow chart for the production of heat-treated ostrich-meat yor is shown in Fig. 3.1.

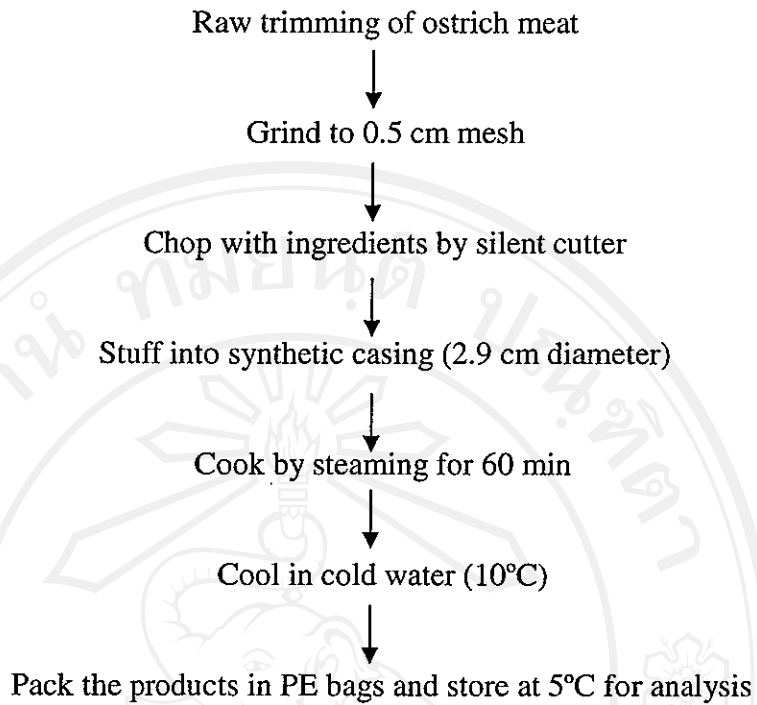


Figure 3.1 Processing scheme for heat-treated ostrich-meat yor.

3.4.1.2 Water holding capacity (WHC)

The amount of released plus expressible water from a meat gel is an indicator of its WHC; a lower amount reflects a greater WHC (Funami *et al.*, 1998).

3.4.1.2.1 Released water

Percentage of water released from the products was measured immediately upon sample collection according to the method of Funami *et al.* (1998). A sample with casing was weighed (A) and after removing the casing, the surface water of both product (B) and casing (C) was wiped with filter paper (Whatman No.2) then reweighed (B, C). The percentage of released water was calculated according to the equation:

$$\text{Released water (\%)} = 100 \times \{(A-B)-C\} / (A-C)$$

3.4.1.2.2 Expressible water

Percentage of expressible water was measured as described by Funami *et al.* (1998). Samples with 29 mm diameter 15 mm thickness were placed between double

layers of filter paper (Whatman No. 2) and subsequently subjected to compression using a Texture Analyser with cylindrical aluminium probe (50 mm in diameter). The measurement was performed by loading 50 kg cell at a crosshead speed of 5 mm/sec to 70% strain for 60 sec.

Percentage of expressible water could be calculated by the ratio of the apparent expressible water to the total moisture content. The moisture contents of ostrich-meat yor were determined using the method of AOAC (2000) No. 930.15. The percentages of expressible water were calculated according to the following equation:

$$\text{Expressible water (\%)} = 100 \times (A/B)$$

$$A (\%) = 100 \times \{(C-D)/C\}$$

Where A = Apparent expressible water (%)

B = Total moisture content of ostrich-meat yor (%)

C = Weight before compression

D = Weight after compression

3.4.1.3 Sensory evaluation

Sensory evaluation was carried out using 36 panelists. These included students and staff from the Department of Food Science and Technology, Chiang Mai University. The evaluation method applied 9-point hedonic scale (9 = like extremely, 5 = neither like nor dislike, 1 = dislike extremely), testing scores of above 5.0 were considered to be acceptable. Sensory attributes considered were, appearance, colour, firmness, saltiness, juiciness and overall acceptability. Samples consisted of 1 cm thick slices of the yor and were identified using a 3-digit random number. Samples were presented to the panelists on a plastic plate and served at room temperature. Panelists were advised to rinse their mouth with plain water between each test (Kuo and Chu, 2003).

3.4.1.4 Statistical analysis

The data of water holding capacity were analysed by an analysis of variance (ANOVA) using a Factorial Experiment in Completely Randomized Design with two

independent variables. The first variable was the added STPP concentration (0, 0.25 and 0.50%, w/w) and the second variable was the added NaCl levels (1, 2 and 3%, W/W). Duncan's New Multiple Range Test was applied to determine differences within treatments. All data were analysed using SPSS 10.0.1 software (SPSS Inc., Chicago, USA).

Data from the sensory evaluation studies were analysed using analysis of variance (ANOVA) by a Balanced Incomplete Block (BIB) Design. Duncan's New Multiple Range Test was applied to determine differences within treatment means (Montgomery, 2001).

3.4.2 Investigation the optimal amount of pepper and garlic added in ostrich-yor formula

3.4.2.1 Ostrich-meat yor preparation

The optimal percentage of STPP and NaCl from section 3.4.1 were assigned in this experiment (0.25 and 2% (w/w) for STPP and NaCl, respectively) and incorporating 1, 2 and 3% (w/w) pepper; 3, 5 and 7% (w/w) garlic in the yor formulas and mixed following the method in section 3.4.1.1.

3.4.2.2 Sensory evaluation

Sensory evaluation was carried out the same method as described in section 3.4.1.3. However, in this case the only sensory attributes assessed were appearance, pepper flavor, garlic flavor and overall acceptability.

3.4.2.3 Statistical analysis

All data were analysed using analysis of variance (ANOVA) by a Balance Incomplete Block (BIB) Design. Duncan's New Multiple Range Test was applied to determine differences within treatment means (Montgomery, 2001).

3.4.3 Determination of the optimal condition of pressure, temperature and holding time for processing the yor

An optimal raw yor batter from 3.4.2 was assigned for this experiment subsequently cased and processed according to the condition set for 3×2×2 factorial experiment in completely randomized design which had pressure treatments of 300, 500 and 700 MPa, at temperatures of 40 and 60°C with holding times of 40 and 60 min. The processed samples were then subjected to the various physicochemical studies.

3.4.3.1 Measurement of colour

Colour measurements on the pressurised samples (29 mm diameter × 10 mm thickness) were performed in a Minolta Chroma Meter CR-300 at ambient temperature and expressed as CIE Lab L^* (lightness), a^* (redness) and b^* (yellowness). The instrument was calibrated to standard white tiles before analysis (Minolta, 1994). An average value was determined by taking observations from three different cut surfaces of the samples.

3.4.3.2 Water holding capacity

The same method was used as described in section 3.4.1.2

3.4.3.3 Determination of gel strength

Analysis of gel strength was carried out at ambient temperature by a texture analyser, using 5 mm diameter ball-end probe to puncture through 90% of the sample height whose diameter of 29 mm and 30 mm thickness with a penetrated speed of 5 mm/sec. The first recorded peak corresponded to penetration force (kg). Gel strength was calculated by multiplying the penetration force (kg) with distance of the penetration (mm) (Tabilo-Munizaga and Barbosa-Cánovas, 2004b).

3.4.3.4 Stress relaxation test

Stress relaxation test was performed using a texture analyser. A cylindrical aluminium probe (35 mm in diameter) was used to compress sample with diameter, 10 mm thickness. Edges of samples were coated with “light” low viscosity silicone oil

to prevent the sample from drying out. A 50 kg load cell was used to compress the samples with a test speed of 5 mm/sec. A constant compressive strain of three percent (which was determined initially from stress versus strain curve, shown in Fig. 3.2) was imposed throughout the experiment and maintained for 1,800 sec. (Supavititpatana and Apichartsrangkoon, 2007). Mathematical models of these curves were also determined.

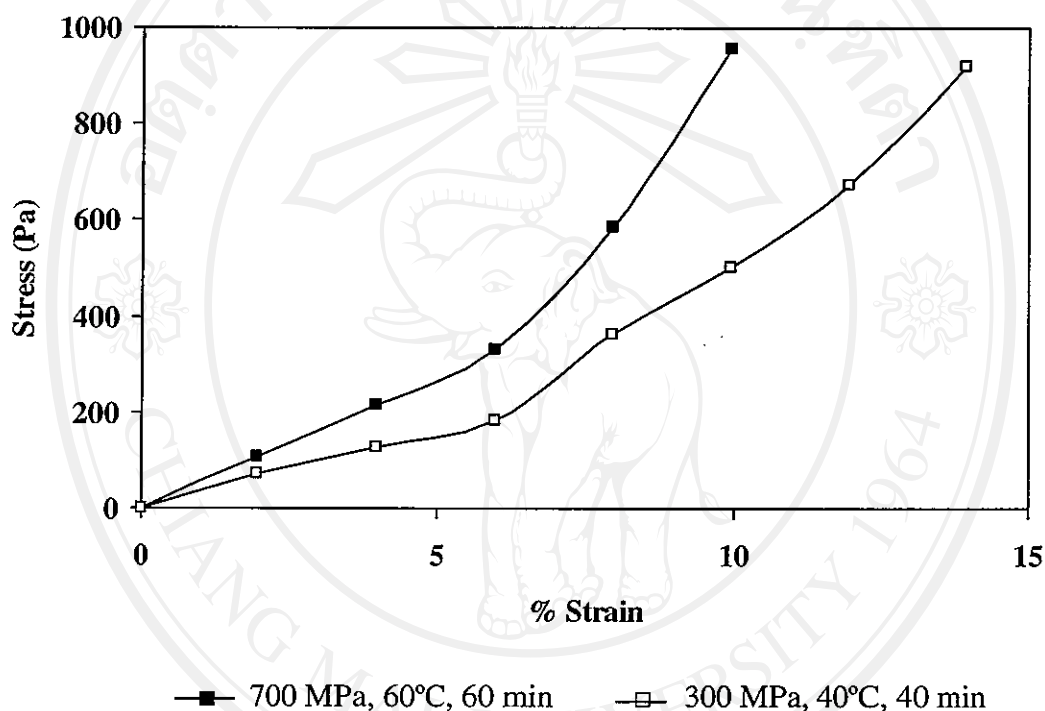


Figure 3.2 Stress versus strain curve of pressurised ostrich-meat yor, showing the initial linear viscoelastic region (LVR).

3.4.3.5 Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry was used in this study to follow the thermal denaturation of the proteins present in the pressurised and unpressurised samples. The instrument was calibrated using Indium over a range 40-200°C. Thermal analysis was carried out using sample 20±3 mg with scanning rate of 10°C/min over the temperature range of 40-100°C, an empty aluminum pan used as a reference. All samples were rescanned to assess reversibility (Molina *et al.*, 2002). All experiments were done in triplicate.

3.4.3.6 Electrophoretic analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out the proteins from samples. The samples (0.2 g) were dissolved in 1 ml of 0.5 M Tris-HCl (pH 6.8) buffer containing 10% (v/v) SDS, 0.02% (w/v) bromophenol blue and 20% (v/v) glycerol. Some 7 μ L of each of the extracted samples were applied to each well. A broad Range standard (Full Range Rainbow Protein Molecular Weight Marker; GE Healthcare UK limited, UK) was used for assessment of molecular weight. The bands of protein were identified using 7.5% polyacrylamide “running” gels. The gels were subsequently stained for 4 h in a solution containing 0.25% (w/v) Coomassie Brilliant Blue G-250, 40% (v/v) methanol and 7% (v/v) acetic acid. Excessive stain was removed by immersion in destain solution I contained 40% (v/v) methanol and 7% (v/v) acetic acid, and destain solution II consisted of 5% (v/v) methanol and 7% (v/v) acetic acid, respectively. For reduced samples, the samples were reduced in warmed 5% 2-mercaptoethanol for 15 min prior to loading (Apichartsrangkoon and Ledward, 2002). To visualize the protein bands two methods were applied, either silver staining or Coomassie Brilliant Blue staining (Apichartsrangkoon, 2003).

3.4.3.7 Statistical analysis

All data were analysed by analysis of variance (ANOVA) using SPSS 10.0.1 software (SPSS Inc., Chicago, USA). A confidence level of 5% was used to compare sample population means ($P \leq 0.05$). When a significant difference was detected between treatments, the mean values were compared using Duncan's New Multiple Range Test (DMRT) procedure (Montgomery, 2001).

3.4.4 Investigation of the interactions between soy protein isolate (SPI), whey protein isolate (WPI) and wheat gluten (WG) in ostrich-meat yor

3.4.4.1 Ostrich-meat yor preparation

The yor was prepared following the method outlined in section 3.4.3. This gave a basic yor to which was added various concentrations of SPI, WPI and WG (0-4%, w/w).

The protein materials had the following compositions; SPI, $91.26 \pm 0.01\%$ (w/w) protein, $0.75 \pm 0.01\%$ (w/w) fat, $0.72 \pm 0.03\%$ (w/w) carbohydrate, $3.66 \pm 0.02\%$ (w/w) ash and $3.61 \pm 0.02\%$ (w/w) moisture, WPI, $92.46 \pm 0.15\%$ (w/w) protein, $0.18 \pm 0.01\%$ (w/w) fat, $1.88 \pm 0.13\%$ (w/w) carbohydrate, $2.96 \pm 0.04\%$ (w/w) ash and $2.52 \pm 0.03\%$ (w/w) moisture and wheat gluten, $90.40 \pm 0.45\%$ (w/w) protein, $1.11 \pm 0.06\%$ (w/w) fat, $4.94 \pm 0.50\%$ (w/w) carbohydrate, $0.78 \pm 0.06\%$ (w/w) ash and $2.77 \pm 0.04\%$ (w/w) moisture.

3.4.4.2 Small deformation experiment

Samples were assessed rheologically by oscillatory testing for both storage (G') and loss (G'') moduli as well as transient measurement for creep compliance using a controlled stress rheometer. All samples were measured at 25°C with a gap setting of 2 mm using 25 mm parallel plate geometry with a coarse surface to avoid the specimen slippage. Edges of samples were coated with a low viscosity silicone oil to prevent water loss. In order to ensure that all measurements were carried out within the linear viscoelastic region (LVR) of the samples under test, a stress sweep was carried out at a frequency of 1 Hz (Fig. 3.3). All rheological measurements were the mean values determined from assessment of three replications.

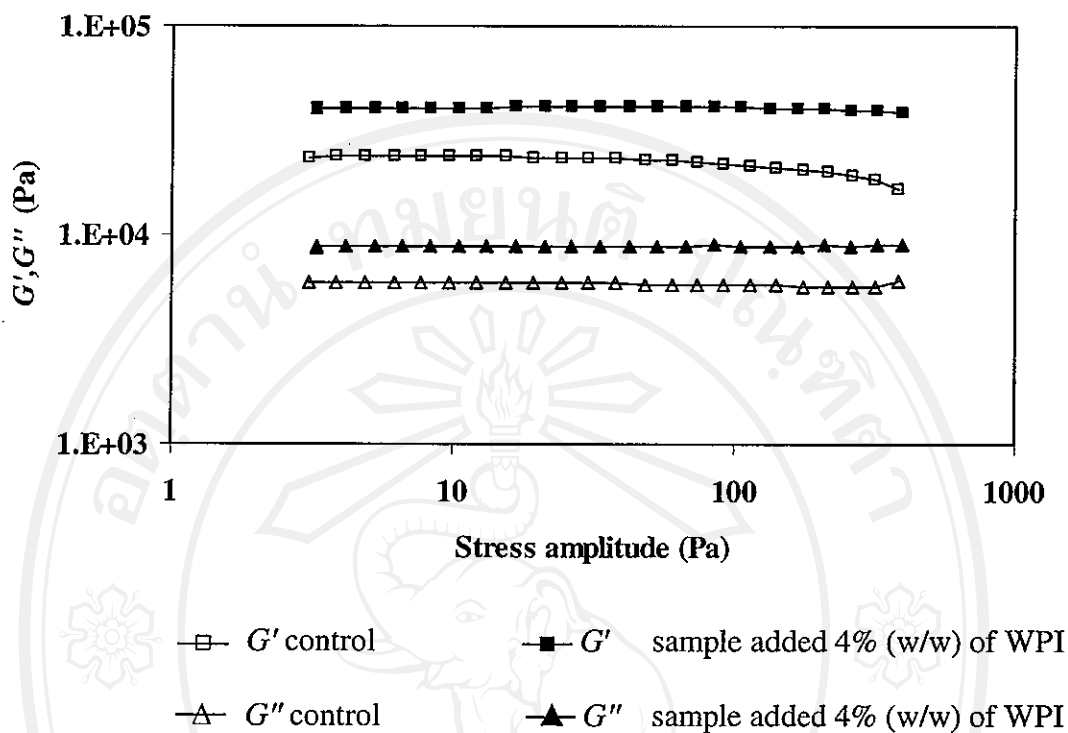


Figure 3.3 Typical stress amplitude sweep (frequency 1 Hz) of a pressure treated ostrich-meat yor.

3.4.4.2.1 Transient creep testing

The treated samples were cut into 2 mm slices prior to analysis. Both creep and recovery of the samples was determined (Initial load 50 Pa for 300 s and unloaded recovery for 900 s). The stress used (50 Pa) was found to be well within the linear viscoelastic region for all of the samples tested (Fig. 3.3). Maxwell and Kelvin-Voigt models were used for data analysis (“curve fitting”). Initial compliance (J_0), retarded compliance (J_i), retardation time (λ_i) and asymptotic viscosity (μ_0) were determined according to the model (Ferry, 1980; Steffe, 1996).

3.4.4.2.2 Dynamic oscillatory measurements

Samples were measured over a frequency range of 0.01–10 Hz at a control stress of 50 Pa (linear viscoelastic region, Fig. 3.3). Both their storage (G') and loss (G'') modulus assessments were taken using parallel plate geometry (20 mm diameter

with a gap of 2 mm). Edges of samples were covered by silicone oil to prevent water loss (a modified method of Apichartsrangkoon and Ledward, 2002).

3.4.4.3 Determination of gel strength

The same method was used as described in section 3.4.3.3

3.4.4.4 Water holding capacity

The same method was used as described in section 3.4.1.2

3.4.4.5 Electrophoretic analysis

The same method was used as described in section 3.4.3.6

3.4.4.6 Differential scanning calorimetry (DSC)

The same method was used as described in section 3.4.3.5

3.4.4.7 Confocal scanning laser microscopy

The samples for microscopic examination (1-2 mm thickness) were stained with a mixture of Fluorescein Isothiocyanate (FITC, 0.02% (w/v) in water and ethanol at a ratio of 1:1) and Nile Red (0.02% (w/v) in water and ethanol at a ratio of 1:1) for protein and fat, respectively about 30 min. The stained samples were dehydrated at room temperature for 10 min. A Leica Scanning Laser Microscopy equipped with a Helium/Neon laser was used for the excitation (500-530 nm for FITC and 505-586 nm for Nile Red). Pictures of the selected areas from each sample were taken using a 10× magnification objective lens (a modified method of Michalski *et al.*, 2002).

ImageJ software was used for image processing and analysis. The digital image files (*.TIF) were converted to 8-bit greyscale images to assess particle size distribution of the two major components.

3.4.4.8 Statistical analysis

The data were analysed by an analysis of variance (ANOVA) using a Mixture Experiment in Simplex Centroid Design. The mixture design was a special cubic model (Montgomery, 2001). All data were analysed by SPSS 10.0.1 software

(SPSS Inc., Chicago, USA). The equation used to measure the dependent variables was as follows:

$$Y = \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ijk} X_i X_j X_k$$

The three variables SPI, WPI and WG were added to the ostrich-meat yor at three levels. These variables were X_i (SPI), X_j (WPI), X_k (WG) at the same levels of 0, 1.33, 2 and 4% (w/w).

3.4.5 Comparison of physicochemical and sensory qualities with the rheological measurements of pressurised and heat-treated ostrich yor

An optimal raw yor batter as described in section 3.4.4 was subjected to both pressure (700 MPa, 40°C, 40 min) and heat-treatments (cooked in steam water for 60 min). The processed samples were subjected to various physicochemical studies.

3.4.5.1 Small deformation experiment

Same method as described in section 3.4.4.2

3.4.5.2 Determination of gel strength

Same method as described in section 3.4.3.3

3.4.5.3 Water holding capacity

Same method as described in section 3.4.1.2

3.4.5.4 Electrophoretic analysis

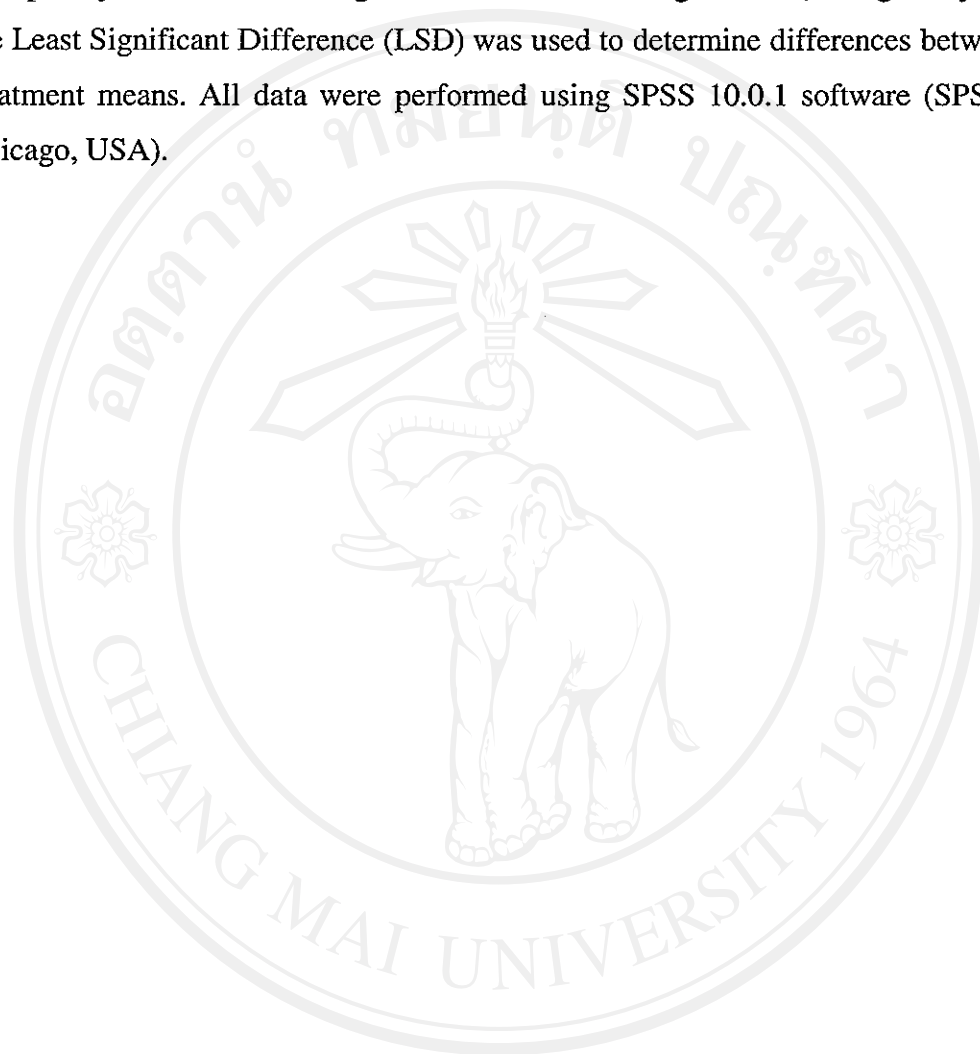
Same method as described in section 3.4.3.6

3.4.5.5 Sensory evaluation

Sensory evaluation was carried out the same method as described in section 3.4.1.3. Only the sensory attributes were assessed for colour, flavour, juiciness, texture and overall acceptability.

3.4.5.6 Statistical analysis

All data were analyzed by an analysis of variance (ANOVA) using a Completely Randomized Design. If the F value was significant (Montgomery, 2001), the Least Significant Difference (LSD) was used to determine differences between the treatment means. All data were performed using SPSS 10.0.1 software (SPSS Inc., Chicago, USA).



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