ANALYTICAL METHODS

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105°C for 3 h and transfer to desiccator to cool. Weigh the empty dish and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample to the uniformity.
3. Place the dish with sample in the oven. Dry for 3 h at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

Calculation

$$\text{Moisture (\%)} = \frac{(W1-W2)}{W1} \times 100$$

where: W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

Reagents

- Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of coppersulphate ($CuSO_4$)
- Sulfuric acid (H_2SO_4)
- 40% NaOH solution
- 0.2 N HCl solution
- 4% H_3BO_3
- Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst and 200 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

$$\text{Protein (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W}$$

Where A = volume (ml) of 0.2 N HCl used sample titration

B = volume (ml) of 0.2 N HCl used in blank titration

N = Normality of HCl

W = weight (g) of sample

14.007 = atomic weight of nitrogen

6.25 = the protein-nitrogen conversation factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.

4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

4. Determination of fat content (AOAC, 2000)

Reagents

- Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105°C overnight to ensure that weight of bottle is stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90°C until solvent is completely evaporate and bottle is completely dry.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\text{Fat (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

5. Determination of lipid content (Bligh and Dyer, 1959)

Reagents

- Chloroform
- Methanol

Method

1. Homogenize the sample (20 g) with 16 ml of distilled water, 40 ml of chloroform and 80 ml of methanol at the speed of 9,500 rpm for 1 min at 4°C.
2. Add 40 ml of chloroform and homogenize for 30 sec.
3. Add 40 ml of distilled water and homogenize again for 30 sec.
4. After centrifugation of the homogenate at 2,000 rpm at 4°C for 20 min, transfer the supernatant into a seperatory funnel and allow to separate.
5. Determine lipid content gravimetrically by measuring triplicate aliquots of the chloroform layer into tared containers, evaporate the solvent and weigh.
6. Calculate the lipid content.

6. Determination of antioxidative activity in a linoleic oxidation system (Sakanaka *et al.,* 2004 modified from Mitsuda *et al.,* 1966; Chen *et al.,* 1995)

Reagents

- 0.1 M phosphate buffer (pH 7.0)
- 50 mM linoleic acid solution in 99.5% ethanol
- 75% ethanol
- 30% ammonium thiocyanate
- 20 mM ferrous chloride solution in 3.5% HCl
- Butylated hydroxytoluene (BHT)

Method

The hydrolysate samples were dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) to obtain the concentrations of 100, 500 and 1,000 ppm. Each solution (0.5 ml) was mixed with 1 ml of 50 mM linoleic acid in 99.5% ethanol and the mixture was kept at 40ºC in dark. At regular intervals, aliquots of the reaction mixtures were taken for measurement of the oxidation using the ferric thiocyanate method. To 50 µl of the reaction mixture, 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was measured at 500 nm. The control was prepared in the same manner except that no protein hydrolysate or antioxidant was added. BHT at level of 25 and 100 ppm was also used and its antioxidative activity in the system was compared.

7. Determination of antioxidative activity in a lecithin liposome system (Frankel *et al*., 1997; Yi *et al*., 1997)

Reagents

- Soybean lecithin
- 0.15 M cupric acetate
- BHT
- TBA solution (15% TCA/ 0.375% TBA/ 0.025N HCl)
- Methanol

Method

Lecithin was suspended in deionized water at a concentration of 8 mg/ml by stirring with a glass rod, followed by sonicating for 30 min using a sonicating bath (Model Transsonic 460/H, Elma, Germany). To test antioxidant activity, each sample with the concentrations of 100, 500 and 1,000 ppm (3 ml) was added to the lecithin liposome system (15 ml). The liposome suspension was then sonicated for 2 min. To initiate the assay, 20 µl of cupric acetate (0.15M) was added. The mixture was shaken at 120 rpm using a shaker (UNIMAX 1010, Heidolph, Germany) at 37^{o}C in the dark. The control and the systems containing 25 or 100 ppm BHT were also prepared. At regular intervals, liposome oxidation was monitored by determining thiobarbituric acid reactive substances (TBARS) and conjugated diene.

1) Measurement of TBARS (Lee and Hendricks, 1997; Duh, 1998)

Liposome sample (1 ml) mixed with 20 µl of butylated hydroxytoluene (0.2%) was added with 2 ml of TBA solution. The mixtures were then heated for 10 min in a boiling water bath (95-100^{o}C) to develop pink color, cooled with tap water, and centrifuged for 20 min at 5,500 × g. The supernatant containing the pink chromogen was quantified at 532 nm.

2) Measurement of conjugated diene (Frankel *et al.*, 1997)

Liposome samples (0.1 ml) were dissolved in methanol (5.0 ml) and conjugated dienes were measured at 234 nm.

8. Determination of nitrogen solubility index (Morr, 1985)

Reagents

- 0.1 M sodium chloride (pH 7.0)

Method

To determine the solubility, 0.5 g of protein hydrolysate sample was dispersed in 50 ml of 0.1 M sodium chloride (pH 7.0). The mixture was stirred at room temperature for 1 h and centrifuged at 2,560 × g for 30 min. The supernatant was filtered through Whatman paper No.1 and the nitrogen content in the total fraction and in the soluble fraction was analyzed by the Kjeldahl method (AOAC, 2000). The nitrogen solubility index (NSI) was calculated as follows:

$$\text{NSI (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

9. Determination of Emulsifying properties (Pearce and Kinsella, 1978)

Reagents

- Soybean oil
- 0.1% sodium dodecyl sulfate (SDS)

Method

Soybean oil (10 ml) and 30 ml of protein hydrolysate solution (0.1, 0.5, 1 and 3% w/v) were mixed and homogenized at a speed of 20,000 rpm for 1 min using an IKA model

T25 basic homogenizer (Selangor, Malaysia). Aliquots of the emulsion (50 µl) were pipetted from the bottom of the container at 0 and 10 min after homogenization and diluted 100-fold into 0.1% SDS solution. The absorbance of the diluted solution was measured at 500 nm. The absorbances measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows:

$$\text{EAI } (m^2/g) = \frac{2 \times 2.303 \times A_{500}}{0.25 \times \text{protein concentration}}$$

$$\text{ESI (min)} = A_0 \times \Delta t/\Delta A$$

where $\Delta A = A_0 - A_{10}$ and Δt = 10 min

10. Determination of foaming properties (Shahidi, 1995)

Method

Twenty ml of protein hydrolysate solution (0.1, 0.5, 1 and 3% w/v) were homogenized in 50-ml cylinder at a speed of 16,000 rpm to incorporate the air for 1 min. The total volume was measured at 0, 0.5, 5, 10, 40 and 60 min after whipping. Foam ability was expressed as foam expansion at 0 min while foam stability was expressed as foam expansion during 60 min. Foam expansion was calculated according to the following equation (Sathe and Salunkhe, 1981):

$$\text{Foam expansion (\%)} = \frac{A - B}{B} \times 100$$

where A = volume after whipping (ml) at different time and B = volume before whipping (ml).