APPENDIX

ANALYTICAL METHODS

1. Moisture content (AOAC, 1999)

Method

1. Dry the empty dish and lid in the oven at 105ºC for 30 min and transfer to desiccator to cool (30 min). Weigh the empty dish and lid.
2. Weigh about 5 g of sample to the dish. Spread the sample with spatula.
3. Place the dish with sample in the oven. Dry for 16 h or overnight at 105ºC
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

Calculation

$$\% \text{ Moisture} = \frac{(W_1 - W_2)}{W_1} \times 100$$

Where: W_1 = weight (g) of sample before drying

W_2 = weight (g) of sample after drying

2. Ash (AOAC, 1999)

Method

1. The crucible and lid is firstly placed in the furnace at 550ºC overnight to ensure that impurities on the surface of crucible is burned off. The crucible is then cool in the desicator (30 min).
2. Weigh the crucible and lid to 3 decimal places.
3. 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.
4. Heat at 550ºC overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desicator.
5. 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

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

3. Protein (AOAC, 1999)

Reagents

- Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) anhydrous, nitrogen free with 1 part of copper sulphate (Cu SO_4)
- Sulfuric acid (H_2 SO_4)
- 40% NaOH solution (w/v)
- 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 Kjeldahl catalyst, and 200 ml of conc. H_2 SO_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 conversion factor for fish and its by-products

4. Measurement of TCA-soluble peptide (Morrissey et al., 1997)

Reagents

1. 5% Trichloroacetic acid (TCA) (w/v)
2. Tyrosine

Method

1. Weigh 3 g of sample and homogenize in 27 ml of 5% TCA.
2. Keep in ice for 1 h, and centrifuge at 7,500xg for 5 min.
3. Measure the peptides in the supernatant and express as mmole tyrosine/ g sample.

5. Lowry (Lowry et al., 1951)

Reagents

1. A: 2% sodium carbonate in 0.1 N NaOH
2. B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
3. C: 1 N Folin Phenol reagent
4. D: 2 ml reagent B + 100 ml reagent A
5. Standard reagent:

   BSA: 1 mg/ ml (used to determine protein concentration)

   Tyrosine: 1 mM (used to determine protein concentration)

Method

1. Add 2 ml reagent D to each of the standards and samples (200 µl)
2. Incubate precisely 10 min at room temperature.
3. Add 0.2 ml reagent C (previously diluted 1:1 with distilled water) and vortex immediately.
4. Incubate for 30 min at room temperature.
5. Read absorbance at 750 nm.

6. Biuret method (Robinso and Hodgen, 1940)

Reagents

1. Biuret reagent: Combine 1.50 g $CuSO_4.5H_2O$; 6.00 g sodium potassium tartrate and 500 ml distilled water in a beaker and stir;

add while stirring 300 ml of 10% NaOH (w/v); transfer to 1 liter volumetric flask and bring to 1 liter with distilled water.

2. Standard reagent : 10 mg/ml bovine serum albumin (BSA)

Method

1. To 0.5 ml of sample, add 2.0 ml of biuret reagent and mixed well.
2. Incubate the mixture at room temperature for 30 min and then measure the absorbance at 540 nm.

Experimental set up for the biuret's assay.

Tube number	Water (µl)	10 mg/ml BSA (µl)	BSA concentration (mg/ml)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	0	500	10

7. Determination of reducing sugar content (slightly modified method of Somogyi, 1951)

Reagents

- A: Dissolve 15 g of sodium potassium tartrate anhydrous Na_2CO_3 in about 300 ml; add 20 g $NaHCO_3$; dissolve 180 g of anhydrous

Na_2SO_4 in 500 ml boiling water and cool; mix the two solutions and make up to 1 litre with water.

- B: Dissolve 5 g $CuSO_4.5H_2O$ and 45 g anhydrous Na_2SO_4 in water and make up to 250 ml.
- C: Mix reagent A (4 vol.) and B (1 vol.) just before use.
- D: Dissolve 25 g ammonium molybdate in 450 ml water.

Carefully add 21 ml concentrated H_2SO_4 with stirring. Dissolve 3 g $Na_2HASO_4.7H_2O$ in 25 ml water and add to the molybdate solution. Incubate for 24-28 h at 37ºC and store in a brown glass-stoppered bottle. Just before use, this reagent should be diluted with 2 vol. of 0.75 M H_2SO_4 (4 ml concentrated H_2SO_4 in 100 ml solution).

Method

1. Mix samples with an appropriate dilution (1.0 ml) with 1.0 ml of reagent C in small stoppered test-tubes.
2. Heat the mixture at 100ºC for 15 min, followed by cooling rapidly to room temperature.
3. Add reagent D (1.0 ml) and mix well.
4. Add water (3.0 ml)
5. Read the absorbance at 520 nm.

8. Determination of thiobarbituric acid substance (TBARS) (Buege and Aust, 1978).

Reagents

- TBA solution: 0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric acid were mixed thoroughly in 100 ml of distilled water.

Method

1. Mix sample (0.5 g) with 2.5 ml of TBA solution.
2. Heat the mixture for 10 min in a boiling water bath (95-100˚C) to develop pink color and cool with tap water and centrifuge at 5500 rpm for 25 min.
3. Read the absorbance of the supernatant at 532 nm.
4. Prepare a standard curve with malonaldehyde bis(dimethyl acetal) (MDA) at concentration ranging from 0-10 ppm.
5. Calculate the quantity of TBARS in each sample using standard curve as mg MDA/kg sample.

9. Electrophoresis (SDS-PAGE) (Leammli, 1970)

Reagents

- 30 % Acrylamide-0.8 % bis Acrylamide
- Sample buffer: Mix 30 ml of 10 % of SDS, 10 ml of glycerol, 5 ml of β-Mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 10

mg Bromophenol blue. Bring the volume to 100 ml with distilled water and stored at -20°C.

- 2 % (w/v) Ammonium persulfate
- 1 % (w/v) SDS
- TEMED (N,N,N'N'-tetramethylenediamine)
- 0.5 M Tris-HCl, pH 6.8
- 1.5 M Tris-HCl, pH 8.8
- Electrode buffer: Dissolve 3 g of Tris-HCl, 14.4 g of glycine and 1 g of SDS in distilled water; adjust to pH 8.3 and add distilled water to 1 liter total volume.
- Staining solution: Dissolve 0.04 g of Coomassie blue R-250 in 100 ml of methanol; add 15 ml of glacial acetic acid and 85 ml of distilled water.
- Destaining solution l: 50 % methanol-7.5 % glacial acetic acid
- Destaining solution ll: 5 % methanol-7.5 % glacial acetic acid

Method

Pouring the separating gel:

1. Assemble the minigel apparatus according to the manufacturer's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution by adding, as defined in following Table.
3. Transfer the separating gel solution using a pasture pipette to the center of sandwich is 2 cm from the top of the shorter glass plate.

4. Cover the top of the gel with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 45 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4 % stacking gel solution by adding as defined in table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of one of the spacers.
4. Insert a comb into the layer of stacking gel solution by placing one corner of the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 45 min at room temperature.

Reagents	10 % running gel	4 % stacking gel
30% Acrylamide-bis	3.333 ml	0.665 ml
1.5 M Tris-HCl buffer, pH 8.8	2.5 ml	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.25 ml
10% SDS	100 µl	50 µl
Distilled water	4.012 ml	3 ml
2% Ammonium persulfate	50 ml	25 ml
TEMED	5 µl	3 µl

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5 % (w/v) SDS in a final volume of 30 ml.
2. Incubate the mixture at 85°C for 1 h.
3. Centrifuge at 3,500 xg for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 1:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 h.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.
5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 µl syringe with a flate-tipped needle, load the protein sample in to the wells by carefully applying the sample as a thin layer at the bottom of the well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Running the gel:

1. Connect the power supply to the anode and cathode of the gel apparatus and run at 15 mA per gel.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge the sandwich along its entire length.
3. Insert a spatula between the glass plates at one corner where the spacer was, and gently pry the two plates apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent of dye and swishing the plate.

Staining the gel:

1. Place the gel in a small plastic box and cover with the staining solution. Agitate slowly for 3 h or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for 15 min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat until the gel is clear except for the protein bands.