Appendix

Analytical Methods

1. Moisture content (AOAC, 1999)

Method

1. Dry the empty dish and lid in the oven at 105°C 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 105° C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

Calculation

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

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

W_2 = weigh (g) of sample after drying

2. Ash (AOAC, 1999)

Method

1. The crucible and lid is firstly placed in the furnace at 550°C overnight to ensure that impurities on the surface of crucible is burn off. Cool the crucible in the desiccator (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 550°C overnight. During heating, do not cover the lid. Place the lid on after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
5. Weigh the ash with crucible and lid until turning to gray. If not, return the crucible and lid to the furnace for further ashing.

Calculation

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

3. Protein (AOAC, 1999)

Reagents

- Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) anhydrus, nitrogen free with 1 part of copper sulphate ($CuSO_4$)
- Sulfuric acid (H_2SO_4)
- 40% NaOH solution (w/v)
- 0.02N HCl solution
- 4% H_3BO_3 solution (w/v)
- 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_2SO_4.
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently until 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 has distilled.
6. Remove receiver, wash tip of condenser, and titrate excess standard acid in 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 0.02N HCl used sample titration

B = volume (ml) of 0.02N HCl used in blank titration

N = Normality of HCl

W = weigh (g) of sample

14.007 = atomic weigh of nitrogen

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

4. Fat (AOAC, 1999)

Reagents

- Petroleum ether

Method

1. The bottle and lid is firstly placed in the incubator at 105°C overnight to ensure that weight of bottle was 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 was completely evaporate and bottle was 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

$$\% \text{ Fat} = \frac{\text{Weigh of fat} \times 100}{\text{Weigh of sample}}$$

5. Hydroxyproline content (Bergman and Loxley, 1963)

Reagent

- 6 N HCl
- Oxidant solution (the mixture of 7% (w/v) chlororamine T and acetate/citrate buffer, pH 6 at a ratio of 1:4 (v/v))
- Ehrlich's reagent solution (the mixture of solution A (2 g of *p*-dimethylamino-benzaldehyde in 3 ml of 60% (v/v) perchloric acid (w/v))
- Isopropanol
- Hydroxyproline standard solution (400 ppm)

Method

Sample preparation:

1. Weight about 0.1-2.0 g sample (depending on type of sample) into screw cap tube.
2. Add 6 N HCl into the sample at the ratio of 1:10 (solid/acid, w/v).
3. Heat at 110 °C for 24 h in oil bath.
4. Clarify the hydrolysate with activated carbon and filter through Whatman No.4 filter paper.
5. Neutralize the filtrate with 10 M NaOH and 1 M NaOH to obtain the pH 6.0-6.5.

Hydroxyproline determination:

1. Transfer 0.1 ml of the neutralized sample into a test tube and add 0.2 ml of isopropanol then mix well.
2. Add 1 ml of oxidant solution and mix well
3. Add 1.3 ml of Ehrlich's reagent solutuion.

4. Add isopropanol at a ratio of 3:13 (mixture/isopropanol, v/v) and mix well.
5. Heat the mixtures at 60 °C for 25 h in a water bath and then cool for 2-3 min in running water.
6. Dilute the mixture to 5 ml with isopropanol and mix well.
7. Read absorbance at 558 nm.
8. Plot the standard curves and calculate the unknown.

Table: Experimental set up for the hydroxyproline's assay.

Tube number	Water (μL)	400 ppm Hydroxyproline (μL)	Effective Hydroxyproline Concentration (ppm)
1	100	2.5	0
2	97.5	5.0	10
3	95.0	7.5	20
4	92.5	10.0	30
5	90.0	12.5	40
6	87.5	12.5	50
7	85.0	15.0	60
8	0	0	unknown

6. Lowry's procedure for quantitation of proteins (with slight modified Lowry *et al*., 1951)

Reagents

- A: 2% sodium carbonate in 0.1 N NaOH
- B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
- C: 2 N Folin-Ciocalteu's phenol reagent B + distilled water (ratio of 1:1)
- D: 50 ml reagent A + 1 ml reagent B
- Standard reagent: Bovine serum albumin (BSA) at concentration of 1 mg/ml

Method

1. To each of eight disposable cuvette, add the following reagents according to the table.
2. To tubes 8, 200 μL of protein sample were added and mix well by using the vortex mixer.
3. Add 2 ml reagent D to each of the standards and unknown tube and then vortex immediately.
4. Incubate precisely 10 min at room temperature.
5. Add 0.2 ml reagent C (previously dilute 1:1 with distilled water) and vortex immediately.
6. Incubate 30 min at room temperature (sample incubated longer than 60 min should be discarded).
7. Read absorbance at 750 nm.
8. Plot standard curves and calculate the unknown.

Table: Experimental set up for the Lowry's assay.

Tube number	Water (μL)	1 mg/ml BSA (μL)	Effective BSA Concentration (mg/ml)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0
8	0	0	unknown

7. Biuret method quantitation of protein (Robison and Hodgen, 1940)

Reagents

- 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 plastic bottle for storage.
- Distilled water
- Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of seven disposable cuvette, add the following reagents according to the table.
2. To tubes 7, 500 μL of protein sample were added and mix well by using the vortex mixer.
3. Add 2.0 ml of the biuret reagent to each tube, and mix well.

4. Incubate the mixture at room temperature for 30-45 min, and then read the absorbance of each tube at 540 nm.
5. For tube 1-5, plot the absorbance at 540 nm as a function of effective BSA concentration, and calculate the best fit straight line from data. Then, using the average absorbance for the three sample of unknown read the concentration of sample from the plot.

Table: Experimental set up for the Biuret's assay.

Tube number	Water (μL)	10 mg/ml BSA (μL)	Effective 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	0	0	unknown

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

Reagents

- Protein molecular weigh standards
- 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 5-10 mg Bromophenol blue (enough to give dark blue color to the solution). Bring

the volume to 100 ml with distilled water. Divide into 1 ml aliquots, 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, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1 liter to total volume.
- Staining solution: Dissolve 0.05 g of Coomassie blue R-250 in 15 ml of methanol. Add 5 ml of glacial acetic acid and 80 ml of distilled water.
- Destaining solution: 30% methanol-10% glacial acetic acid

Method

Pouring the running gel:

1. Assemble the minigel apparatus according to the manufacture'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 pipettes to the center of sandwich is about 1.5 to 2 cm from the top of the shorter (front) glass plate.

4. Cover the top of the gel with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 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 comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

Table: Experimental set up for running gel and stacking gel

Reagents	5% running gel	4% stacking gel
30% Acrylamide-bis	1.665 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
Distilled water	5.6785 mL	3.00 mL
10% SDS	100 μL	50 μL
2% Ammonium persulfate	50 μL	25 μL
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 hr.
3. Centrifuge at 3,500*xg* for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 4:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 min at 100°C.
2. Remove the comb without tearing the edge of the polyacylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the 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 syring with a flate-tipped needle, load the protein sample into 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 50 V and 150 V.
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 of 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 or 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 about 15 min.
3. Pour off the destaining solution I and replace with fresh solution. Repeat until the gel is clear except for the protein bands.