Appendix

Analytical Methods

1. Moisture content (AOAC, 1999)

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 the dish and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample with spatura.
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

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

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

W_2 = weight (g) of sample after drying

2. pH determination (Benjakul *et al.*, 1997)

Method

1. Weigh 5 g of sample. Add 5 volumes of distilled (w/v).
2. Homogenize for 2 min.
3. Measure pH using pH meter.

3. Solubility (Benjakul *et al.*, 2001)

Reagent

- Tris-HCl buffer (pH 8.0) containing 1 % (w/v) SDS, 8 M urea and 2 % (v/v) β-mercaptoethanol

- 0.5 M NaOH
- 10 % and 50 % trichloroacetic acid (TCA)

Method

To a weighed amount (1 g) of sample (mince or gel), cut into small pieces, in a 100 ml conical flask, 20 ml of solvent was added, then homogenize for 1 min. The mixture was heated in boiling water bath for 2 min and stirred at room temperature for 4 h. All samples were then centrifuged at 10,000xg for 30 min in a Sorvall-RC2 centrifuge. To 10 ml of the supernatant (soluble fraction), cold 50 % (w/v) TCA (2 ml) was added to give a final TCA concentration of 10 %. Samples were kept at 4°C for 18 h, centrifuged at 10,000xg for 20 min and supernatant was removed. The precipitate was washed with 10 % TCA and solubilized in 0.5 M NaOH. Protein content was estimated by biuret method.

Samples were also solubilized in 0.5 NaOH. Protein content in 0.5 M NaOH extract was used as reference value, i.e., 100 %

4. Measurement of TCA-soluble peptide (Morrissey *et al.*, 1993)

Reagents

- 5 % Trichoroacetic acid (TCA) (w/v)
- Tyrosine

Method

1. Weigh 3 g of sample and homogenize in 27 ml of 5 % TCA for 3 min.
2. Keep on ice for 1 h, and centrifuge at 5,000xg for 5 min.
3. Measure tyrosine in the supernatant was measured as an index of autolytic degradation products and express as μmol tyrosine/g sample.

5. Lowry (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: 1 N Folin Phenol reagent
- D: 2 ml reagent B + 100 ml reagent A
- Standard reagent: Tyrosine at concentration 1 mM

Method

1. Add 2 ml reagent D to each of the standards and sample 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 30 min at room temperature
5. Read absorbance at 750 nm.

6. Biuret method (Robinson 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 a 1 liter volumetric flask and bring to 1 liter with distilled water.
- Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To 0.5 ml of sample, 2.0 ml of the biuret reagent were added and mixed well.
2. The mixture was incubated at room temperature for 30 min, then the absorbance at 540 nm was read.

Table: 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

Preparation of actomyosin (Benjakul *et al.*, 1997)

Reagents

- 0.6 M KCl, pH 7
- Distilled water

Method

1. Homogenize 10 g of muscle in 100 ml chilled (4°C) 0.6 M KCl, pH 7.0 for 4 min.
2. Place the beaker containing the sample in ice. Each 20 sec of blending was followed by a 20 sec rest interval to avoid overheating during extraction.
3. Centrifuge the extract at 5,000xg for 30 min at 4°C.
4. Add three volumes of chilled distilled water to precipitate actomyosin.
5. Collect actomyosin by centrifuging at 5,000xg for 20 min at 4°C.
6. Dissolve the pallet by stirring for 30 min at 4°C in an equal volume of chilled 0.6 M KCl, pH 7.

7. Triobarbituric acid-reactive substance (TBARS) (Buege and Aust, 1978)

Reagents

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

Method

Ground sample (0.5 g) was dispersed in 10 ml of TBA solution. The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600xg for 20 min and the absorbance was measured at 532 nm. A standard curve was prepared with malonaldehyde bis (dimethyl acetal) (MDA) at concentration ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malondialdehyde/kg.

8. Determination of trimethylamine (TMA-N) and total volatile basic nitrogen (TVB-N) by Conway's method (Conway and Byrne, 1936)

Reagents

1. Inner ring solution (1 % boric acid solution containing indicator): Take 10 g of boric acid in 1 liter flask, add 200 ml of ethanol. After dissolving with distilled water.
2. Mixed indicator solution: Dissolve bromocresol green (BCG) 0.01 g and methyl red (MR) 0.02 g in 10 ml of ethanol.
3. 0.02 N HCl
4. Satureted K_2CO_3 solution: Take 60 g of potassium carbonate, and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, obtain filtrate through filter paper.
5. 4 % trichloroacetic acid (CCl_3COOH), TCA, solution: Dissolve 40 g of TCA in 960 ml of distilled water.

6. Sealing agent: Take 3 g of Trangacanth gum, add 30 ml of distilled water, 15 ml of glycerine and 15 ml of 50 % saturated K_2CO_3 solution and mix well.
7. Neutralized 10 % formaldehyde solution: Add 10 g of $MgCO_3$ to 100 ml of formaline (35 % formaldehyde solution) and shake in order to neutralize the acidity of formaline. Filter and dilute filtrate 3 volume with distilled water.

Method

Sample extraction:

1. Take 2 g of fish meat in a mortar and grind well.
2. Add 8 ml of 4 % TCA solution and grind well.
3. Stand for 30 min at ambient temperature with occasional grinding.
4. Filter through filter paper (Whatman No. 41) or centrifuge at 3,000 rpm, for 10 min.
5. Keep the filtrate in -20°C freezing if necessary.

8.1 Determination of TVB-N

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Slant the Conway's unit with cover.
5. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
6. Close the unit.
7. Mix gently.
8. Stand for 60 min at 37°C in incubator.
9. Titrated inner ring solution with 0.02 N HCl using a micro-burette until green color turns pink.
10. Do blank test using 1 ml of 4 % TCA instead of sample extract.

8.2 Determination of TMA-N

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Pipette 1 ml of neutralized 10 % formaldehyde unto outer ring.
5. Slant the Conway's unit with cover.
6. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
7. Close the unit.
8. Mix gently.
9. Stand for 60 min at 37°C in incubator.
10. Titrated inner ring solution with 0.02 N HCl using a micro-burette until green color turns pink.
11. Do blank test using 1 ml of 4 % TCA instead of sample extract.

Calculation

$$\text{TMA-N or TVB-N (mg N/100g)} = \frac{(V_S - V_B) \times (N_{HCl} \times A_N) \times V_E \times 100}{W_S}$$

where:
- V_S = Titration volume of 0.02 N HCl for sample extract (ml)
- V_B = Titration volume
- N_{HCl} = Normality of HCl (0.02 Nxf, facter of HCl)
- A_N = Atomic weigh of nitrogen (x 14)
- W_S = Weigh of muscle sample (g)
- V_E = Volume of 4 % TCA used in extraction

9. Determination of total sulfhydryl content (Benjakul *et al.*, 1997)

Reagents

1. 0.1 % 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)
2. 0.2 M Tris-HCl buffer, pH 6.8 (containing 8 M urea, 2 % SDS, and 10 mM EDTA)

Method

1. Mix actomiosin (1 ml, 4 mg/ml) with 9 ml of 0.2 M Tris-HCl.
2. Take 4 ml-aliquot of the mixture and add with 0.4 ml of 0.1 % DTNB solution. Incubate the mixture at 40°C for 25 min.
3. Measure the absorbance at 412 nm with spectrophotometer.
4. Prepare a blank by replacing the sample with 0.6 M KCl, pH 7.0.
5. Calculate SH content from the absorbance using the molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ and express as mole/10^5 g protein.

Calculation

$$C \text{ (mole/}10^5 \text{ g protein)} = \frac{A}{\varepsilon b}$$

where: C = concentration

A = absorbance at 412 nm

ε = extinction coefficient of 13,600 $M^{-1}cm^{-1}$

b = path length 1 cm

10. Determination of disulfide bond (Thannhauser *et al.*, 1987)

Reagents

1. 5,5′-dithiobis-2-nitrobenzoic acid (Ellman' s reagent)
2. Na_2SO_3
3. glycine
4. sodium sulfite
5. EDTA

- NTSB assay solution (Thannhauser *et al.*, 1987)

- NTSB assay solution is prepared from the stock solution by diluting it 1:100 with a freshly prepared solution that is 2 M in guanidine thiocyanate, 50 mmole in glycine, 100 mmole in sodium sulfite, and 3 mmole EDTA. The pH should be adjusted to 9.5. The assay solution is used directly to measure disulfide bond concentrations and is stable for up to 2 weeks when stored at room temperature.

Method

1. Add 10-200 µl of protein solution into 3 ml of the NTSB assay solution.
2. Incubate the reaction mixture in the dark for 25 min.
3. Measure the absorbance at 412 nm against a blank.
4. Calculate disulfide bond concentration from the absorbance using the molar extinction coefficient of 13,900 $M^{-1}cm^{-1}$ and express as mole/10^5 g protein.

Calculation

$$C \text{ (mole/}10^5 \text{ g protein)} = \frac{A}{\varepsilon b}$$

where: C = concentration

A = absorbance at 412 nm

ε = extinction coefficient of 13,900 $M^{-1}cm^{-1}$

b = path length 1 cm

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

Reagents

- 30 % Acrylamide-0.8 % bis Acrylamide
- 2 % (w/v) Ammonium persulfate
- 1 % (w/v) SDS
- TEMED (N,N,N'N'-tetramethylenediamine)
- 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.
- 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. 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 I: 50 % methanol-7.5 % glacial acetic acid
- Destaining solution II: 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 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 h.
3. Centrifuge at 3,500xg 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.