

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 spatula.
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

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

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

W2 = weight (g) of sample after drying

2. 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

Weighed 1 g of sample (mince or gel). Cut into small pieces and place in a 100 ml conical flask. Add 20 ml of solvent and homogenize for 1 min. Heat the mixture in boiling water bath for 2 min. Stir at room temperature for 4 h. Centrifuge at 10,000xg for 30 min in a Sorvall-RC2 centrifuge. To 10 ml of the supernatant (soluble fraction), add cold 50 % (w/v) TCA (2 ml). Kept the mixture at 4°C for 18 h and centrifuge at 10,000xg for 20 min. Wash the precipitate with 10 % TCA and solubilize in 0.5 M NaOH. Determine the protein content by biuret method.

To determine the total protein content, solubilize sample in 0.5 NaOH. Use protein content in 0.5 M NaOH extract as reference value, i.e., 100 %.

3. Biuret method (Robinson and Hodgen, 1940)

Reagents

- Biuret reagent: Combine 1.50 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 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, add 2.0 ml of the biuret reagent and mixed well.
2. Incubate the mixture at room temperature for 30 min.
3. Read the absorbance at 540 nm.

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

Tube number	water (μ l)	10 mg/ml BSA (μ l)	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

4. 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 pellet by stirring for 30 min at 4°C in an equal volume of chilled 0.6 M KCl, pH 7.

5. Electrophoresis (SDS-PAGE) (Leampli, 1970)

Reagents

- 30 % Acrylamide-0.8 % bis Acrylamide
- 2 % (w/v) Ammonium persulfate
- 1 % (w/v) SDS
- TEMED (N,N,N',N'-tetramethylethylenediamine)
- 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.

- 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:

- Pour off completely the layer of isobutyl alcohol.
- Prepare a 4 % stacking gel solution by adding as defined in table.
- Transfer stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers.
- 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 μ l	50 μ l
Distilled water	4.012 ml	3 ml
2 % Ammonium persulfate	50 μ l	25 μ l
TEMED	5 μ l	3 μ l

Sample preparation:

- Weigh 3 g of sample and homogenize with 5 % (w/v) SDS in a final volume of 30 ml.
- Incubate the mixture at 85°C for 1 h.
- 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 μ l 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.