APPENDIX

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 copper sulphate ($CuSO_4$)
- 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 until frothing ceases. Boil briskly until the solution becomes clear.
4. Cool and add 60 ml of distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 drops of 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 250 ml of petroleum ether into the bottle and place 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 evaporated 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^{o}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^{o}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 salt content (AOAC, 2000)

Reagents

- 0.1 N $AgNO_3$
- 0.1 N KSCN
- Conc. HNO_3
- 5% Ferric alum indicator

Methods

1. Add the sample (1g) with 10 ml of 0.1 N $AgNO_3$ and 10 ml of conc. NHO_3.
2. Boil the mixture gently on a hot plate until all solids except $AgCl_2$ is dissolved (usually 10 min).
3. Cool the mixture using running water.
4. Add 5 ml of ferric alum indicator.
5. Titrate the mixture with standardized 0.1 N KSCN until solution becomes permanent light brown.
6. Calculate the percentage of salt as follows:

$$\text{Salt (\%)} = \frac{5.8 \times (\text{volume of } AgNO_3 \times N) - (\text{volume of KSCN} \times N)}{\text{Weight of sample}}$$

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

Method

1. Weigh 5 g of sample and add 10 volumes of distilled water (w/v).
2. Homogenize for 2 min.
3. Measure pH using pH meter.

8. 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

1. Suspend the lecithin 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).

2. Add 3 ml of sample with the concentrations of 200, 500 and 1,000 ppm to the lecithin liposome system (15 ml) and then sonicate for 2 min.

3. Add 20 µl of cupric acetate (0.15M).

4. Shake the mixture at 120 rpm using a shaker (UNIMAX 1010, Heidolph, Germany) at 37^{o}C in the dark.

5. Prepare the control and the systems containing 50 or 200 ppm BHT.

6. At regular intervals, monitor liposome oxidation by determining thiobarbituric acid reactive substances (TBARS) and conjugated diene.

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

1.1 Mix liposome sample (1 ml) with 20 µl of butylated hydroxytoluene (0.2%) and 2 ml of TBA solution.

1.2 Heat the mixtures for 10 min in a boiling water bath (95-100^{o}C) to develop pink color.

1.3 Cool with tap water, and centrifuge for 20 min at 5,500 × g.

1.4 Measure the absorbance of the supernatant containing the pink chromogen at 532 nm.

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

Dissolve liposome samples (0.1 ml) in methanol (5.0 ml) and measure conjugated dienes at 234 nm.

9. Determination of antioxidant activity in β-carotene-linoleic acid (Taga *et at*., 1984)

Reagents

- β-carotene
- Chloroform
- Linoleic acid
- Tween 4

Method

1. Dissolve β-carotene (1mg) in 10 ml of chloroform.
2. Add 3 ml aliquot of the solution to 20 mg linoleic acid and 200 mg Tween 40.
3. Remove chloroform by purging with nitrogen.
4. Add oxygenated distilled water (50 ml) to the β-carotene emulsion and mixed well.

5. Mix 200 µl of Mungoong soluble fraction (200, 500 and 1,000 ppm) with oxygenated β-carotene emulsion (3 ml) and incubated at 50°C.

6. Monitor oxidation of β-carotene emulsion spectrophotometrically at 470 nm after 0, 10, 20, 30 and 40 min incubation at 50°C.

7. Prepare the control by using the distilled water and the systems containing 50 or 200 ppm BHT instead of antioxidant in the assay system.

10. Determination of antioxidative activity in comminuted fish model system (Benjakul *et al*., 2005)

Material

- Round scads (*Decapterus maruadsi*)

Reagents

- BHT

Method

1. Wash round scads, fillet and mince.

2. Mix the mince (250 g) with 20% distilled water (w/w) and use as the control.

3. Mix 20 % (w/w) of Mungoong soluble fraction (200, 500 and 1,000 ppm) and BHT (200 ppm) with the mince (250g).

4. Place the mixtures (50g) in the polyethylene bags and keep at 4 °C.

5. Monitor oxidation of fish mince by determining thiobarbituric acid reactive substances (TBARS) after 0, 2, 4, 6 and 8 day of storage.

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

Reagents

- 0.1 M sodium chloride (pH 7.0)

Method

1. Disperse 0.5 g of sample in 50 ml of 0.1 M sodium chloride (pH 7.0).

2. Stir the mixture at room temperature for 1 h and centrifuge at 2,560 × g for 30 min.

3. Filter the supernatant through Whatman paper No.1

4. Determine the nitrogen content in the total fraction and in the soluble fraction by the Kjeldahl method (AOAC, 2000).

5. Calculate the nitrogen solubility index (NSI) as follows:

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