

## Appendix 1

### ANALYTICAL METHODS

#### 1. Determination of reducing sugar content (Somogyi, M., 1951)

##### Chemicals

- Reagent A: Dissolve 15 g of sodium potassium tartrate and 30 g of anhydrous  $\text{Na}_2\text{CO}_3$  in about 300 ml water. Add 20 g  $\text{NaHCO}_3$ . Dissolve 180 g of anhydrous  $\text{Na}_2\text{SO}_4$  in 500 ml boiling water and cool. Mix the two solutions and make up to 1 litre with water.

- Reagent B: Dissolve 5 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 45 g anhydrous  $\text{Na}_2\text{SO}_4$  in water and make up to 250 ml.

- Reagent C: Mix reagent A (4 vol.) and B (1 vol.) just before use.

- Reagent D: Dissolve 25 g ammonium molybdate in 450 ml water. Carefully add 21 ml concentrated  $\text{H}_2\text{SO}_4$  with stirring. Dissolve 3 g  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  in 25 ml water and add to the molybdate solution. Incubate for 24-28 h at  $37^\circ\text{C}$  and store in a brown glass-stoppered bottle. Just before use, this reagent should be diluted with 2 vol. of 0.75 M  $\text{H}_2\text{SO}_4$  (4 ml concentrated  $\text{H}_2\text{SO}_4$  in 100 ml solution).

##### Method

MRPs samples with an appropriate dilution (1.0 ml) were mixed with 1.0 ml of reagent C in small stoppered test-tubes. The mixture was heated at  $100^\circ\text{C}$  for 15 min, followed by cooling rapidly to room temperature. Then, reagent D (1.0 ml) was added and mixed well. Water (3.0 ml) was then added. The absorbance was measured at 520.

## **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. 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 and make up volume to 1 liter 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. Saturated  $K_2CO_3$  solution: Take 60 g of potassium carbonate, and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, filter the mixture 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 Tragacanth 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  $\text{MgCO}_3$  to 100 ml of formaline (35 % formaldehyde solution) and shake in order to neutralize the acidity of formaline. Filter and dilute the filtrate with 3 volume of 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  $-20^\circ\text{C}$  freezing if necessary.

### **3.1. 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  $\text{K}_2\text{CO}_3$  solution into outer ring.
6. Close the unit.
7. Mix gently.
8. Stand for 60 min at  $37^\circ\text{C}$  in incubator.
9. Titrate the inner ring solution with 0.02 N HCl using a micro-burette until green color turns pink.

10. Prepare the blank test using 1 ml of 4 % TCA instead of sample extract.

### 3.2. 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 into 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. Titrate the inner ring solution with 0.02 N HCl using a micro-burette until green color turns pink.
11. Prepare the blank using 1 ml of 4 % TCA instead of sample extract.

### Calculation

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

where:  $V_S$  = Titration volume of 0.02 N HCl for sample extract (ml)

$V_B$  = Titration volume

$N_{\text{HCl}}$  = Normality of HCl (0.02 Nxf, factor of HCl)

$A_N$  = Atomic weight of nitrogen (x 14)

$W_S$  = Weight of muscle sample (g)

$V_E$  = Volume of 4 % TCA used in extraction

## Appendix 2

### Melanosis Score



0



2



4



6



8



10