Appendix 1

Analytical Methods

1. Total nitrogen (AOAC, 2000)

Sample preparation

Samples (20 ml) were diluted with 180 ml of distilled water

Reagents

1. Kjedahl catalyst: Mix 10 part of potassium sulphate (K_2SO_4) anhydrous, nitrogen free with 1 part of copper sulphate ($CuSO_4$)
2. Sulfuric acid (H_2SO_4)
3. 40% Sodium hydroxide (NaOH) solution (w/v)
4. 0.02 N Hydrochloric (HCl) solution
5. 4% Boric acid (H_3BO_3) solution (w/v)
6. indicator solution: Mix 100 ml of 0.125g methyl red and 0.082g methylene blue (in 95% ethanol) with 20 ml of 0.1g bromocresol green (in 95% ethanol)

Method

1. Pipette sample 1 ml in digestion flask (use 1 ml of distilled water as blank).
2. Add 5 g Kjeldahl catalyst, and 20 ml of conc. H_2SO_4.
3. Place flasks in inclined position and heat gently until frothing ceases.
4. Boil briskly until solution clears.

5. Cool and add 50 ml distilled water cautiously.
6. Immediately connect flask to digestion bulb on condenser, and with tip of condenser immersed in standard acid and 3-5 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 has distilled.
7. Remove receiver, wash tip of condenser, and titrate excess standard acid in distilled with standard HCl solution.

Calculation

$$\text{Total nitrogen} = \frac{(A - B) \times 14.007 \times N}{W}$$

Where:

	A	=	volume (ml) of 0.02 N HCl used sample titration
	B	=	volume (ml) of 0.02 N HCl used blank titration
	N	=	Normality of HCl
	W	=	weight (g) of sample
	14.007	=	atomic weight of nitrogen

2. Formol nitrogen content (Thai Industrial Standard, 1983)

Sample preparation

Samples (20 ml) were diluted with 180 ml of distilled water

Reagents

1. Formaline solution (38% v/v; pH9)
2. 0.1 N NaoH
3. Phenolphthalein

Method

1. Pipette 10 ml of sample with an appropriate dilution
2. Titrate to pH 7.0 with 0.1 N NaOH
3. Add 10 ml of formalin solution (38% v/v, pH 9)
4. Titrate to obtain pH of 9 with 0.1 NaOH
5. Calculate formal nitrogen content

Calculation

Formol nitrogen content (mg N/ml) = ml of A (pH 7-pH 9) ×N×14

Where: A = volume (ml) of 0.1 N NaOH used sample titration

N = Normality of NaOH

14 = atomic weight of nitrogen

3. Ammonia nitrogen content (Thai Industrial Standard, 1983)

Sample preparation

Samples (20 ml) were diluted with 180 ml of distilled water

Reagents

1. Magnesium oxide (MgO)
2. 0.05 N Sulfuric acid (H_2SO_4) solution
3. 4% Boric acid (H_3BO_3) solution (w/v)
4. Indicator solution: Mix 100 ml of 0.125g methyl red and 0.082g methylene blue (in 95% ethanol) with 20 ml of 0.1g bromocresol green (in 95% ethanol)

Method

1. Transfer sample with an appropriate dilution (50 ml) in 400 ml Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO.
2. Distill the sample and collect the distillate in 50 ml of 4% boric acid consisting of the mixed indicator (methyl red: bromocresol green: methylene blue).
3. Titrate with 0.05 N H_2SO_4 to reach the end-point.
4. Calculate ammonia nitrogen content

Calculation

Ammonia nitrogen content (mg N/ml) = $5.6 \times N \times$ (ml of A)

Where: A = volume (ml) of 0.05 N H_2SO_4 used sample titration

N = Normality of H_2SO_4

4. Amino nitrogen content (Thai Industrial Standard, 1983)

Amino nitrogen was calculated based on the formol and ammonia nitrogen contents.

Calculation

Amino nitrogen content (mg N/ml) = Formal nitrogen content – Ammonia nitrogen content.

5. pH determination (Benjakul et al., 1997)

Method

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

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

Reagents

1. 5% Trichloroacetic acid (TCA) (w/v)
2. Tyrosine

Method

1. Weight 3 g of sample and homogenize in 27 ml of 5% TCA.
2. Keep in ice for 1 hr, and centrifuge at 7,500xg for 5 min.
3. Measure the peptides in the supernatant and express as μmole tyrosine / g sample.

7. Lowry (Lowry *et al*., 1951)

Reagents

1. A: 2 % sodium carbonate in 0.1 N NaOH
2. B: 0.5 % $CuSO_4.5H_2O$ in 1 % sodium citrate
3. C: 1 N Folin Phenol reagent
4. D: 2 ml reagent B + 100 ml reagent A
5. 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.

8. Biuret method (Robinson and Hodgen, 1940)

Reagents

1. 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.
2. 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

9. 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. 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, 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 4 ml of sample in a beaker and stir.
2. Add 16 ml of 4 % TCA solution and stir.
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.

9.1 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.

9.2 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 weight of nitrogen (x 14)
- W_S = Weight of muscle sample (g)
- V_E = Volume of 4 % TCA used in extraction

10. Total aerobic bacteria counts (Tanasupawat *et al.*, 1992)

Chemicals / Media

1. Sodium chloride (NaCl)
2. Standard plate count agar
3. Peptone

Method

1. Transfer sample (15 g or ml) aseptically to a stomacher bag.
2. Add 135 ml of 0.1% (w/v) peptone solution having 10% (w/v) NaCl (0.1% peptone solution) and blend for 1 min by stomacher.
3. Dilute mixture to 1:10, 1:100, 1:1,000 and 1:10,000 in 0.1 % peptone solution.
4. Pipette 1 ml of diluted mixture into sterilized plate. Add 20 ml of plate count agar (PCA) containing 10% NaCl.
5. Incubate at 37ºC for 48h.
6. Express the microbial counts as log colony-forming unit (CFU)/g.

Calculation

CFU/g sample = Average number of colonies X dilution factor

11. Proteolytic bacteria (Tanasupawat *et al.*, 1992)

Chemicals / Media

1. Sodium chloride (NaCl)
2. Standard plate count agar
3. Peptone
4. Casein

Method

1. Transfer sample (15 g or ml) aseptically to a stomacher bag.
2. Add 135 ml of 0.1% (w/v) peptone solution having 10% (w/v) (0.1% peptone solution) and blend for 1 min by stomacher.
3. Dilute mixture to 1:10, 1:100, 1:1,000 and 1:10,000 in 0.1 % peptone solution.
4. Pipette 0.1 ml of diluted mixture into sterilized plate (spread on the surface of media).
5. Incubate at 37ºC for 72h.
6. Express the microbial as log colony-forming unit (CFU)/g.

Calculation

CFU/g sample = Average number of colonies X dilution factor

12. Halophilic bacteria (Namwong *et al.*, 2005)

Chemicals / Media

1. Sodium chloride (NaCl)
2. Standard plate count agar
3. Peptone
4. casamino acid
5. yeast extract
6. sodium glutamate
7. Trisodium citrate
8. $MgSO_4 \cdot 7\ H_2O$
9. KCl
10. $FeCl_2 \cdot 4H_2O$
11. $MnCl_2 \cdot 4\ H_2O$
12. agar

Method

1. Transfer sample (15 g or ml) aseptically to a stomacher bag.
2. Add 135 ml of 0.1% (w/v) peptone solution having 10% (w/v) (0.1% peptone solution) and blend for 1 min by stomacher.
3. Dilute mixture to 1:10, 1:100, 1:1,000 and 1:10,000 in 0.1 % peptone solution.
4. Pipette 0.1 ml of diluted mixture into sterilized plate (spread on the surface of media).
5. Incubate at 37ºC for 96h.

6. Express the microbial counts as log colony-forming unit (CFU)/g.

Calculation

CFU/g sample = Average number of colonies X dilution factor

Appendix 2

% Weight Organ

Organs	%
Liver	14.4047 ± 5.5565
Pancreas	3.9140 ± 1.2839
Intestine	12.8680 ± 3.7108
Stomach	17.6508 ± 0.6713
Bile sac	2.8476 ± 0.6713
Spleen	42.8886 ± 3.6189
Etc.	5.4263 ± 7.5820