

**LABORATORY MANUAL ON ANALYTICAL METHODS AND
PROCEDURES FOR FISH AND FISH PRODUCTS**

Edited by

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PREFACE

This manual represents a continuing effort by the MFRD to provide useful guides for both experienced laboratory workers and other technical personnel. Included in the manual are procedures for determining the physical and chemical properties of fish meat, the analysis of oils and some additives and microbiological procedures.

The methods described in this manual have been compiled from various sources for use in MFRD and may not necessarily be the same methods used by other agencies, although whenever desirable, the officially recognised methods are followed.

In compiling this manual, the contributors have placed emphasis on presenting the material in this manual as simply and as directly as possible by adopting a step by step approach for each method discussed. Constructive criticisms are very much welcomed, for the benefit of future editions. The physical layout of this manual also allows updating of methods easily.

It is hoped that this publication will be a useful reference tool for those involved in laboratory work, especially for those working in the fields of fish and fish products analysis.

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DETERMINATION OF PHYSICAL
PROPERTIES OF MEAT

DETERMINATION OF MOISTURE

NG M. C.

INTRODUCTION

There are various methods to determine the moisture content. The determination depends on the following criteria:-

- a) the form in which water is present
- b) nature of product analysed
- c) rapidity of determination
- d) accuracy desired
- e) availability and cost of equipment required

In the case of fish meat, the methods used are oven method, rapid methods by infra-red balance and microwave moisture checker.

I SAMPLE PREPARATION

Collect meat sample (≤ 100 g) and pass 2-3 times through food mincer, or chop very finely and mix thoroughly.

II INSTRUMENT

Method 1: oven (30-250°C), aluminium dish with lid.

Method 2: infra-red balance (Kett, model F-1A).

Method 3: microwave moisture checker (Anritsu, model K377C).

III ANALYTICAL PROCEDURE AND CALCULATION

METHOD 1: OVEN METHOD

1. Dry the empty dish and lid in the oven at 105°C for 30 min and transfer to the desiccator to cool (30 min). Weigh the empty dish and lid to 3 decimal places.
2. Weigh about 5 g of sample from (I) to the dish. Spread the meat with spatula. Replace the lid and weigh the dish and contents to 3 decimal places.
3. Place the dish with its lid partially covered in the oven. Dry for 16 hrs or overnight at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

CALCULATION

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

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

W_2 = weight (g) of sample after drying.

METHOD 2: INFRA-RED METHOD

1. Balance the infra-red meter at zero level.
2. Evenly spread accurately 5 g meat sample from (I) onto the dish.
3. Place dish with sample on infra-red meter dish holder and level the balance.
4. Set lamp height to mark 7 and switch on the moisture meter. As moisture content in the sample decreases, lower the lamp height gradually until mark 5-4.5.
5. Continue to dry until the readout on the scale is constant (30-45 mins).

CALCULATION

(a) Results can be read directly from the balance scale or

(b) Calculate similarly the oven method i.e.;

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

where W_1 = weight of sample before drying.

W_2 = weight of sample after drying.

METHOD 3: MICROWAVE METHOD

1. Warm up and stabilise the microwave checker for half an hour before use.
2. Tare the sample dish containing glass fiber filter and Teflon ring to zero.
3. Evenly spread about 5 g meat sample on the sample dish and cover with filter paper held in place with Teflon ring.
4. Close the oven door. The weight of sample (g) is displayed on readout.
5. Set the required time at full power, 600w and at variable power, 300w (see below table).
6. Press the start switch to activate the drying.
7. At the end of drying, a buzzer sounds and the moisture content (%) is displayed directly.
8. Press the readout button to obtain the dried weight.
9. Repeat additional 30 sec at 300w until dried weight is constant.

SUITABLE TIME AND HEATING CONDITIONS FOR FISH MEAT SAMPLE

Sample	Power	
	600w	300w
Minced meat	120sec	60sec
Leached meat	300sec	30sec
Surimi	120sec	90sec

CALCULATION

The microwave method is calibrated to give direct readout in % moisture.

DETERMINATION OF ASH

NG M. C.

INTRODUCTION

The principle of ashing is to burn off the organic matter and to determine the inorganic matter remained. Heating is carried out in two stages:- firstly to remove the water present and to char the sample thoroughly; and finally ashing at 550°C in a muffle furnace.

This method is applicable to all food materials.

I SAMPLE PREPARATION

Randomly collect meat sample (≤ 100 g) and pass through a manual mincer twice or chop very finely and mix thoroughly.

Place minced meat in small plastic bag.

II INSTRUMENT AND APPARATUS

Muffle furnace, temperature (0-1200)°C

Crucibles and lids

Thong

Thick gloves

III ANALYTICAL PROCEDURE

1. The crucible and lid is first placed in the furnace at 550°C overnight to ensure that impurities on the surface of crucible is burnt off. Cool the crucible in the desiccator (30 mins).
2. Weigh the crucible and lid to 3 decimal places.
3. Weigh about 5g meat sample from (I) into the crucible. Heat over low bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
4. Heat at 550°C overnight. During heating, do not cover with the lid. Place the lid on after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
5. Weigh the ash with crucible and lid to 3 decimal places.
6. Ash must be white or light grey. If not, return the crucible and lid to the furnace for further ashing.

IV CALCULATION

$$\text{Ash Content (\%)} = \frac{\text{Wt of ash}}{\text{Wt of sample}} \times 100$$

REFERENCE

Official methods of analysis of the Association of Official Analytical Chemists 13th Ed., 1980: 289, 508. See 18.025, 31.012.

MEASUREMENT OF pH

LIM P. Y.

INTRODUCTION

Pre-rigor fish flesh is semi-translucent, glossy and dry in appearance and no moisture can be expressed from it. The flesh is nearly neutral, that is its pH, the degree of acidity or alkalinity is near 7.0. After rigor has resolved, the flesh is wetter in appearance, moisture can be expressed much more easily from it and pH is more acid. The lowering of pH is due to the breakdown of glycogen to lactic acid. Depending upon species, the pH immediately after rigor has resolved is usually 6.4 to 6.8. The pH increases again with increased growth of spoilage bacteria.

The pH of the environment affect bacterial growth. Most bacteria especially spoilage bacteria grow well between pH 6 and pH 8 with progressively less growth at extremes of pH. On the other hand, the pH of other animal meats is between 5.3-6.0, the bacteria grow less readily. This is one reason why fish spoil more quickly than meat. However, the measurement of pH is an indicator of fish freshness.

I SAMPLING AND SAMPLE PREPARATION

Take a representative sample from the experimental material or product lot, avoid taking the red meat portion and store in polyethylene bag prior to preparation for analysis. The sample should be kept in refrigerator or in ice to maintain its integrity.

Homogenise the sample with a mechanical/electrical mincer or chop the sample with knife until homogeneous.

Transfer the homogenised sample into a polyethylene bag and store in refrigerator until required. Ensure that the prepared sample is still homogeneous prior to weighing.

In case of fresh fish meat, the pH of fish homogenate should be determined at once.

II APPARATUS

Round bottom flask
Heating mantle
Mortar & pestle
Tissue homogeniser or grinder with speed control
Beakers, 100 ml
pH meter

III REAGENTS

- a) CO₂ free distilled water

Boil the distilled water with a round bottom flask. Cool the distilled water prior to use. Cap the flask to avoid contact with atmospheric air.

- b) Treated sand

Sieve the sea sand and wash the resulting fine sand 3 times with distilled water.

Boil the washed sand for 15 mins. in a 1N NaOH solution. Allow to cool.

Decant away the NaOH solution and wash the sand 3 times with distilled water or until it is free of the alkali.

Boil the sand for 15 mins. in a 1N HCl solution. Allow to cool.

Decant away the HCl solution, and wash the sand 3 times with distilled water or until it is free of the acid.

Place the treated sand in an oven set at 105°C overnight to dry.

IV PROCEDURES

1. Sample prepared with mortar and pestle

Weigh accurately 2.0 g of sample and place into a mortar.

Add approximately 2.0 g of treated sand to the mortar and grind until the sample is homogenised.

Add 10 ml of CO₂ free distilled water to the homogenate and grind again.

Remove the well ground homogenised sample into beaker and read the pH.

2. Sample prepared with tissue homogeniser/grinder

Weigh accurately 5.0g of sample and place into the beaker.

Add 45 ml of CO₂ free distilled water to the sample and homogenise for 30 seconds.

Read pH of sample.

MEASUREMENT OF FREE AND EXPRESSIBLE DRIPS

NG C. S.

INTRODUCTION

When animal tissues (eg. muscle) are frozen, a certain degree of damage occurs. In muscle tissue, this is reflected in an increased amount of free drips and expressible drips. Free drips is the fluid that exudes from the muscle (or thawed muscle) on standing. Expressible drip is the fluid lost from the meat on application of pressure. No standard method has been established for drips measurements, and the amount of drips measured is a relative value. On reporting drip values, it is therefore important to state the physical parameters involved.

I APPARATUS

2 cm \varnothing cork borer
Petri dishes
Filter paper (Whatman No. 1, \varnothing 7 cm)
Screw press
Stop watch

II PROCEDURE

1. A 2cm \varnothing cylinder of fish muscle is made using the cork borer. Trim the muscle block of the skin and cut the height to 0.5 cm.
2. Weigh the muscle sample (X g) and place it on 2 pieces of filter paper. Place sample in a petri dish with cover. Keep in refrigerator (4°C) for 2 hr.
3. Take the sample out and weigh (Y g).
4. Place the muscle sample between 2-filter paper on top and 3-filter paper below. Place the whole in the press.
5. Slowly increase the pressure to 10 kg/cm² within 30 sec.
6. Maintain at 10 kg/cm² constant pressure for 2 min, then remove the sample, and weigh the pressed sample (Z g).

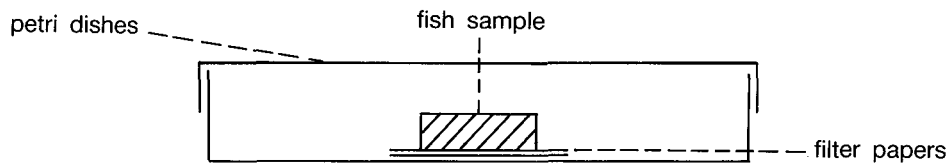
III CALCULATION

$$\text{Free drip, \%} = \frac{(X - Y)}{X} \times 100$$

$$\text{Expressible drip, \%} = \frac{(X - Z)}{X} \times 100$$

IV PRECAUTIONS

- a) The cut sample must be kept frozen until ready for weighing.
- b) Maintain a constant size of sample. Sample size approx. 0.5 cm in thickness and 2.0 cm in diameter.
- c) Take muscle samples from a constant area of the fish.
- d) Weigh the frozen sample quickly to prevent atmospheric moisture from condensing on the sample.



FISH PROTEIN EXTRACTIBILITY & ITS DETERMINATION

LIM P. Y.

INTRODUCTION

Fish proteins gradually become denatured in cold storage. The rate of denaturation depends largely upon storage temperature.

Badly frozen stored fish are easily recognizable. The appearance of the thawed product, instead of being glossy and translucent, is dull and opaque and the texture, no longer firm and elastic, becomes soft and spongy. The cooked flesh loses its succulence and becomes dry, fibrous and tasteless.

The main proteins of fish flesh are called myosin and actin. They are responsible for the mechanism of contraction and relaxation of muscles and are called myofibrillar proteins. Muscle also contains many other proteins, the sarcoplasmic protein which are soluble in tissue fluid and in any salt solution. During freezing and cold storage, the proteins are affected, especially the myofibrillar protein, resulted in the textural changes of flesh.

The myofibrillar protein extractibility, therefore is used as a quality index for the assessment of frozen fish. It is expressed as follows:-

$$\text{Extractibility (\%)} = \frac{\text{MPN} - \text{SPN}}{\text{TN} - \text{NPN}} \times 100 \quad (\text{A})$$

where: MPN = myofibrillar protein-nitrogen (N mg/100 g sample)

SPN = sarcoplasmic protein-nitrogen (N mg/100 g sample)

TN = total nitrogen (N mg/100 g sample)

NPN = non proteinous nitrogen (N mg/100 g sample)

The protein extractibility is applicable to fish and its product in general and can be used as an indicator of the degree of protein denaturation for demersal and pelagic species during cold storage.

I SAMPLING AND SAMPLE PREPARATION

Take a representative sample 22 g or more from the product. Place the sample in polyethylene bag and store in refrigerator or in ice so as to maintain sample integrity before preparation for analysis.

The dark meat, if any, should be removed prior to homogenisation of fish flesh.

Comminute the sample until homogeneous and place the homogenate in a polyethylene bag. Store the sample in a refrigerator or in ice until required. Ensure that the prepared sample is still homogeneous prior to weighing.

II APPARATUS

Chopper or mincer
Analytical balance, decimal to 0.1 mg
Spatula
Bottom-drive homogeniser (Nihon Seiki SN-03) or equivalent
Refrigerated centrifuge, capable of centrifuging at 12,500 g
Beakers, 100 and 250 ml
Bulb pipettes, 10, 20 & 40 ml
Glass funnels \varnothing 60 mm
Whatman filter paper, No. 41

III REAGENTS

a) Phosphate buffer solution

0.03 M potassium di-hydrogen phosphate, 1 litre.

0.03 M di-sodium hydrogen phosphate, 1 litre.

Mix the above solutions into a 2 litre beaker.

Adjust the pH to 6.85 using these solutions.

Store in refrigerator.

b) 0.1 M potassium chloride solution

Weigh KCl required accurately, use distilled water as solvent.

c) 0.5M potassium chloride buffered solution

Accurately weigh KCl required. Dissolve the weighed KCl in the required phosphate buffer solution.

d) Trichloroacetic acid solution (25%, w/v)

Dissolve 25 g TCA in 75 ml distilled water.

IV PROCEDURE OF PROTEIN EXTRACTIBILITY

1. Total nitrogen. Accurately weigh a duplicate of 1 g the homogeneous fish sample for protein digestion (refer to protein determination by Kjeldahl method B-1.)
2. Sarcoplasmic protein nitrogen. Accurately weigh 10 g of the homogeneous fish sample. Blend the sample with 200 ml of 0.1 M KCl solution with the homogeniser for 4 mins (speed set at scale 2). Leave to stand in iced water for 2 hrs. Centrifuge the blended sample at 12,500 g or 9,000 rpm at 5°C for 20 mins. Pipette 20 ml supernatant (sarcoplasmic protein fraction) for digestion (refer to Kjeldahl method).
3. Myofibrillar protein nitrogen. Accurately weigh 10 g of the homogeneous fish sample. Blend sample with 200 ml 0.5 M KCl phosphate buffered solution with the homogeniser for 4 mins (speed set at scale 2). Leave to stand in iced water for 2 hrs. Centrifuge the blended sample at 12,500 g or 9,000 rpm at 5°C for 20 mins. Pipette 20 ml supernatant (myofibrillar protein aliquot) for digestion (refer to Kjeldahl method).
4. Non proteinous nitrogen. Pipette 40 ml of sarcoplasmic protein aliquot (see Section IV 2) into 100 ml beaker. Add 10 ml 25% TCA and leave to stand in iced water for 30 mins with occasional swirling. Filter the content of the beaker with Whatman No. 41 ashless filter paper. Pipette 40 ml of filtrate for digestion (refer to Kjeldahl method).

V CALCULATIONS

Ws in formula (1) in the protein determination by Kjeldahl method (B-1 Section IV) has to be replaced by the meat weight (g) in each of the protein aliquot used as follows:-

- 1) For myofibrillar protein nitrogen (MPN)

$$W_{\text{MPN}} = W_1 \times \frac{20}{W_1 + 200}$$

where W_1 = weight of fish flesh (g) used for myofibrillar protein extraction

20 is the volume (ml) of sarcoplasmic protein aliquot used for digestion

200 is the volume (ml) of 0.5 M KCl — buffered solution used for the extraction of myofibrillar protein

- 2) For sarcoplasmic protein nitrogen (SPN)

$$W_{\text{SPN}} = W_2 \times \frac{20}{W_2 + 200}$$

where W_2 = weight of fish flesh (g) used for sarcoplasmic protein extraction

20 is the volume (ml) of sarcoplasmic protein aliquot used for digestion

200 is the volume (ml) of 0.1 M KCl solution used for the extraction of sarcoplasmic protein

- 3) For non-proteinous nitrogen (NPN)

$$W_{\text{NPN}} = 40^a \times \frac{40^b}{50} \times \frac{w_2}{200 + W_2}$$

where 40^a is the volume (ml) of supernatant of sarcoplasmic protein aliquot used for non-proteinous nitrogen

40^b is the volume (ml) of filtrate used for digestion taken from the sarcoplasmic protein aliquot after precipitated by 25% TCA

50 is the total volume (ml) of sarcoplasmic protein aliquot after addition of 10 ml 25% TCA

W_2 = weight of fish flesh (g) used for sarcoplasmic protein extraction

200 is the volume (ml) of 0.1 M KCl solution used for the extraction of sarcoplasmic protein

Use the above equivalent meat weights and the formula (1) in protein determination by Kjeldahl method (B-1 Section IV) for calculation of respective protein nitrogen aliquot, express in mgN/100 g and calculate the extractibility by formula (A).

N.B. The pH plays an important role in the extraction of fish protein. The optimum pH is between 6.5-7.0. Adjustment of the pH of the buffer solution is important and can be achieved by using di-sodium hydrogen phosphate, potassium di-hydrogen phosphate or sodium carbonate etc.

VISCOSITY OF FISH MEAT SOL

LIM P. Y.

INTRODUCTION

Viscosity is the measure of fluid friction. It may be considered as the internal friction resulting when a layer of fluid is made to move in relationship to another layer. A highly viscous material is one possessing a great deal of internal friction — it will not pour or spread as easily as a material of lesser viscosity.

This procedure can be used as a rapid method to assess the gel forming ability of the fish meat, fish mince, leached meat and surimi etc. Generally, fish flesh with meal sol of a minimum viscosity between 300-400 centipoises can be used to process good quality fish jelly products (e.g. fishballs or fishcakes).

Practically all fluids will become less viscous as their temperature increases, and thicker as they cool. The relationship between viscosity and temperature is exponential in nature; that is, a small temperature change can cause a large viscosity change. The temperature of the material **MUST** be stated along with its viscosity. Not to do so nullifies the meaning of the resulting viscosity value.

The relationship between viscosity and meat concentration is in the form of a power curve. As such, it is important that the meat concentration be constant for comparative studies.

There is a maximum speed at which layers of fluid can move with laminar flow; that is, with no transfer of matter between the layers. Turbulence results beyond this maximum speed, and to maintain this turbulent flow, a larger energy input is necessary. This is reflected by an apparently higher internal friction, and the indicated viscosity will be higher than it should be. The Tokyo Keiki Rotary Viscometer functions at a constant speed of 20 rpm.

I SAMPLING AND SAMPLE PREPARATION

Take a representative sample minimum of 70 g from the product. Place the sample in polyethylene bag and store in refrigerator or in ice so as to maintain sample integrity, in preparation for analysis.

Comminute the sample with a chopper or mechanical mincer until homogeneous and place the homogenate in a polyethylene bag. Store the sample in the refrigerator or in ice until required. Ensure that the prepared sample is still homogeneous prior to weighing.

II APPARATUS

Bottom-drive homogeniser (Nihon Seiki SN-03) or equivalent Rotary Viscometer Type C, CVR-20B, Tokyo Keiki, with 2 spindles (one with a factor of 5 for less viscous fluids and the other with a factor of 20 for viscous fluids)

Beaker, 1000 ml
Chopper or mechanical mincer
Spatula

III REAGENTS

- a) Sodium chloride, extra pure.
- b) Di-potassium hydrogen orthophosphate (KH_2PO_4), cryst. extra pure.
- c) Potassium dihydrogen orthophosphate (KH_2PO_4), cryst. extra pure.
- d) Extraction solution: Dissolve 189 g NaCl, 33.5 g K_2HPO_4 and 8.74 g KH_2PO_4 in 1000 ml distilled water. Transfer the solution in reagent bottle and store in refrigerator.

IV PROCEDURES

1. Weigh ca 70 g meat sample into the cylinder of SN type homogeniser.
2. Add 500 g chilled distilled water (ca 10°C) in the cylinder of homogeniser.
3. Completely remove the air bubble in the meat sample with slow speed of homogeniser.
4. Add 100 ml extraction solution and homogenise for 3 mins with speed dial at 3-4.
5. Transfer the meat sol to 1 litre beaker and keep in ice water (below 5°C) for 20 mins.
6. Measure viscosity of the sol with Type C viscometer with the guard, mesh and selected spindle (temperature of the meat sol should be about $7-10^\circ\text{C}$).
7. Read the viscometer when the pointer stabilised and note the temperature of the meat sol.

V CALCULATION

Multiply the viscometer reading by 5 if the large spindle is used or by 20 if the smaller spindle is used and express the viscosity of the meat sol in centipoises.

QUALITY ASSESSMENT OF FISH JELLY PRODUCTS AND RAW MATERIAL USED FOR PRODUCTION OF FISH JELLY PRODUCTS

NG M. C.

INTRODUCTION

The quality of fish jelly products is assessed by measuring the gel strength objectively and organoleptically by folding and teeth-cutting tests. This quality depends on the following factors:-

1. Fish species
2. Condition of fish
3. Processing method and control
4. Moisture content of final product

The quality assessment would be useful for raw material suppliers and its users (eg fishball processors) to know the quality of the raw material used for the production of fish jelly products; and to assess the quality of the final products.

I INSTRUMENT/APPARATUS AND MATERIAL

Sausage casing (\varnothing ca 2.5 cm)

Fudoh Rheometer (Model NRM-2002J)

Knife

Cutting board

Trays

- Stainless steel moulds —
- i) \varnothing 2.5 cm, 2.4 cm thick for gel strength measurement
 - ii) \varnothing 2.5 cm, 5 mm length for organoleptic assessment

II SAMPLE AND TEST PIECE PREPARATION

For raw material

1. Randomly collect 300 g raw material sample (eg minced meat, leached meat, surimi).
2. Put raw material sample into the mortar grinder.
3. The raw material sample is ground for 25 min as follows:-
 - i) grind the sample for 5 min to break up the muscle fibres.
 - ii) add 1.5% salt based on the weight of fish meat and grind for 5 min.
 - iii) add another 1.5% salt and grind for another 5 min.
 - iv) add water 30% gradually to the ground meat sample and continue to grind for 10min with constant mixing.

4. Fill the ground fish paste into sausage casing taking care not to include air bubbles. This is done by pressing the meat paste onto a board before filling into the casing.
5. Set the sausage-like sample in water bath at 40°C for 20 min followed by heating at 90°C for 20 min.
6. After heating, cool the sample in iced water immediately to prevent further heating.
7. Immerse the sausage-like sample in running water till sample is at room temperature before measurement.
8. Cut the sample for gel strength measurement into 2.4 cm length, \varnothing 2.5 cm with the stainless steel moulds and place on a tray. Five test pieces will be measured for each sample. Slice 5 test pieces of 5 mm thickness. \varnothing 2.5 cm for the organoleptic assessment.

For fish jelly products

1. Fish jelly products eg fishballs, fishcakes, must have a height and thickness of ca 2.0-2.4 cm.
2. These products will be trimmed into standard size of 2.0-2.4 cm by 2.0-2.4 cm.
3. Prepare 5 test pieces for gel strength measurement.
4. Slice 5 test pieces of 5 mm thickness for organoleptic assessment.

III MEASUREMENT AND ASSESSMENT

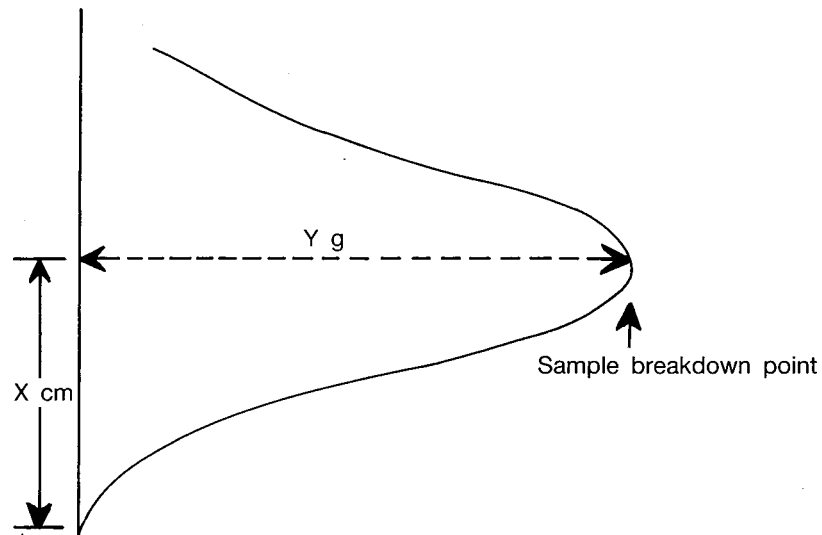
A. GEL STRENGTH MEASUREMENT BY FUDOH RHEOMETER

1. Set the following parameters on the Fudoh rheometer and chart recorder:-

	Rheometer	Chart Recorder	Factor
Speed (cm/min)	6	12	($\times \frac{1}{2}$)
Sensitivity (volts)	1	$\frac{1}{2}$	($\times \frac{1}{2}$)

2. Place a test piece on the sample holder and 'ON' the Fudoh rheometer and chart recorder simultaneously.
3. When test piece is broken as indicated in the recorder chart, 'OFF' the chart recorder and rheometer.
4. Repeat with all the test pieces to obtain the average results.

CALCULATION



Recorder Chart

$$\text{Gel strength} = X \times Y \times F \quad \text{g.cm}$$

$$\text{where } F, \text{ factor} = \frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$$

Fish jelly products of acceptable grade have a gel-strength of 200-300 g.cm.

B. ORGANOLEPTIC ASSESSMENT BY FOLDING AND TEETH-CUTTING TESTS

Organoleptic tests provide a convenient and quick assessment of the “springiness” of final products. Although the tests should be performed by trained personnel, the training is well worth the effort.

(a) Folding Test

Five slices of 5 mm thickness are taken from the prepared samples. Each is then folded in half and if there is no tear or breakage, further folded into quarter. The grading is as follow:-

<u>Condition of test samples when folded</u>	<u>Grade</u>
• No breakage in any of five samples when folded in quarter	AA
• Slight tear in any one of five samples when folded in quarter	A
• Slight tear in any one of five samples when folded in half	B
• Breakage (but 2 pieces still connected) when folded in half	C
• Breaks completely into 2 pieces when folded in half	D

Commercial products of acceptable grade should have a rating of AA.

(b) Teeth-Cutting Test

Samples similar to that for folding test are used to assess the “springiness” using the teeth-cutting test. The grading gives subjective assessment of the resistance experienced by a trained panel when the test piece is bitten between the upper and lower front teeth.

<u>Score</u>	<u>Grade</u>
10	Extremely strong springiness
9	Very strong springiness
8	Strong springiness
7	Quite strong springiness
6	Acceptable springiness
5	Acceptable, slight springiness
4	Weak springiness
3	Quite weak springiness
2	Very weak springiness
1	Mushy texture, no springiness

Local products usually fall in the range of score 5-6.

REFERENCE

Instruction and operational manual, Fudoh rheometer
Fudoh Kogyo Co. Ltd. Available in MFRD laboratory.

DETERMINATION OF CHEMICAL
PROPERTIES OF MEAT

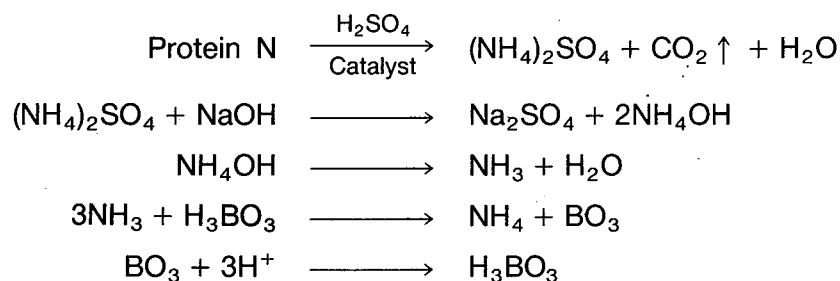
PROTEIN DETERMINATION BY KJELDAHL METHOD

LIM P. Y.

INTRODUCTION

In the presence of sulphuric acid and catalyst, the nitrogen atom in the nitrogenous organic compound is converted to ammonium sulphate. The ammonia is then distilled from an alkaline medium and absorbed in boric acid. The ammonia is then determined by titration with a standard mineral acid.

Taking protein as an example, it is as follows:-



I APPARATUS

Kjeldahl digestion and assembly ("Tecator" brand)
Kjeldahl digestion tube, 250 ml
Kjeldahl distillation apparatus ("Tecator" brand)
Conical flask 250 ml
Automatic burettes 50 ml with 2000 ml reservoir bottle
Magnetic stirrer

II REAGENTS

- Sulphuric acid (H_2SO_4), nitrogen free
- Catalyst
Mix 9 parts of potassium sulphate (K_2SO_4) anhydrous, nitrogen free with 1 part of copper sulphate (CuSO_4), anhydrous, nitrogen free.
- NaOH solution (40% w/v)
Dissolve sodium hydroxide (NaOH), technical grade, mini pearls, in distilled water.
- Boric acid (4% w/v)
- Anti-bumping granules
- Ethanol (95% v/v)
- Standard 0.1N Sulphuric acid
Break ampoule for preparation of standard solution, empty content into 1 L volumetric flask and dilute with nitrogen free distilled water until the mark. Cap and invert the volumetric flask until solution well mix. Transfer the content to automatic burette.
- Indicator
Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol).

III PROCEDURE

1. Accurately weigh the homogenous fish sample (1 g) or pipette a suitable quantity of protein fraction solution (20 ml myofibrillar or sarcoplasmic protein fraction or 40 ml non-proteinous nitrogen fraction) and place in digestion tube. Add 7 g catalyst, 3 to 5 anti-bumping granules and 20 ml of conc. H_2SO_4 . Also prepare a tube containing the above chemicals except fish sample as blank. Cover tube with exhaust manifold and place tube in the preheated digester and digest at about 110-130°C for 15 mins (ignore this process if non liquid sample is to be digested). Turn the digester to digestion temperature normally around 420°C and digest the sample until the solution is light green (1 hr for fish sample) and then a further 15 mins. Remove tube and leave to stand until sample is cooled. Add cautiously 60 ml distilled water.
2. Switch on distillation apparatus and pre-wash for 10 mins. Dispense 25 ml 4% boric acid into a 250 ml conical flask and place the flask under the condenser, ensuring that the condenser tip is immersed in the boric acid solution. Connect the digestion tube containing the sample digest to the distillation apparatus. Dispense 60 ml 40% NaOH carefully into digested sample. Immediately turn on the steam supply valve to initiate the distillation. Heat for 4 mins until all ammonia has passed over into the boric acid. Lower the conical flask ensuring the condenser tip is not immersed in solution and continue heating for further 1 min. Collect approximately 120 ml distillate. Wash tip of condenser with distilled water.

Place conical flask containing ammonia distillate on magnetic stirrer. Add 1 ml indicator and titrate the sample with standard 0.1N sulphuric acid until the solution change from green to pinkish. Read volume of acid used for titration.

IV CALCULATIONS

Calculate the protein nitrogen (mgN/100 g or 100 ml sample) as follows:-

- a) solid/semi-solid fish sample

$$\text{protein nitrogen} = \frac{(b - a) \times 0.1 \times 14.00}{W_s} \times 100 \quad (1)$$

where W_s = weight (g) or volume (ml) of sample

a = volume (ml) of 0.1N H_2SO_4 used in blank titration

b = volume (ml) of 0.1N H_2SO_4 used in sample titration

14.00 = atomic weight of nitrogen

- b) Calculation of percentage protein

The above protein nitrogen (mgN/100 g or 100 ml sample) can also be presented as percentage protein nitrogen fraction and is expressed as follows:-

$$\% \text{ protein} = \frac{(b - a) \times 0.1 \times 14.00}{W} \times 100 \times \frac{6.25}{1000}$$

where $\frac{(b - a) \times 0.1 \times 14.00}{W_s} \times 100$ is similar to formula (1)

1000 : the conversion of mgN/100 g to gN/100 g sample

6.25 : the protein-nitrogen conversion factor for fish and its by-products.

PROTEIN DETERMINATION BY BIURET METHOD (MODIFIED BY UMEMOTO)

LIM P. Y.

INTRODUCTION

This method is applicable to extracted liquid fish protein aliquots (See A-5 Section IV, 2 and 3) with a protein concentration of between 0.1 to 0.5 mg N/ml.

The method is based on the reaction of Cu^{++} with peptides in alkaline solution to yield a purple Cu^{++} — peptide complex that has a peak of absorption at 545 nm.

Some fish protein fractions contain interfering substances which cause turbidity to the sample solution when the sample is left to stand for attainment of chemical equilibration (for full colour development). These substances include tris — (hydroxymethyl) methylamine used as buffering reagent during the extraction of fish protein and sucrose & sorbitol used as cryoprotective reagent in minced fish flesh during frozen storage. Other interfering chemicals are ammonium sulphate, mercapto-ethanol, Triton X-100 etc. Therefore this method is not suitable for samples containing the above interfering substances.

I APPARATUS

Bulb pipette, 5 ml
Quickfit test tube with stopper, 25 ml
Test tube shaker
Spectrophotometer
Magnetic stirrer
Beaker 250 ml

II REAGENTS

- a) Copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)
- b) Sodium hydroxide (NaOH)
- c) Glycerine
- d) Reagent A.

Dissolve 8 g NaOH in 40 ml distilled water. Add the NaOH solution to 30 ml distilled water containing 0.2 g glycerine. Dissolve 0.4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 30 ml distilled water, add this solution slowly to the above mixture solution with continuous agitation to prevent precipitation. This solution should not be kept in refrigerator for more than 2 months.

- e) Reagent B.

Dissolve 8 g NaOH in 80 ml distilled water. Weigh 0.2 g glycerine and dissolve it in 20 ml distilled water. Mix these two solutions and keep in refrigerator and it should not be kept for more than 2 months.

III PROCEDURE

A. PREPARATION OF CALIBRATION CURVE

1. The Bovin Serum Albumin or myofibrillar protein extract from fish can be used as working solution for the preparation of calibration curve.

- a) Bovin Serum Albumin

Stock solution: Dissolve about 400 mg albumin in distilled water and dilute to 50 ml (about 8 mg/ml)

Working solution: Pipette 5 ml of stock solution into 1000 ml volumetric flask and dilute with distilled water (about 0.4 mg/ml).

- b) Myofibrillar protein extract

It is preferably to use the myofibrillar protein extract obtained from the same group of fish for the preparation of calibration curve.

The protein extract has to be digested following Kjeldahl method and the nitrogen is to be determined accordingly. The following formula is for the calculation of nitrogen concentration in fish protein extract (see B-1 formula (1)):

$$\text{N content (mgN/ml)} = (b - a) \times 0.1 \times 14.00 \times 1/n$$

b: sample titration value (ml)

a: blank titration value (ml)

n: ml of extract used in digestion

Based on the concentration of nitrogen protein in the fish protein extract, appropriate dilution can be made using KCl-phosphate buffered solution for the preparation of calibration curve.

The concentration of the diluted fish protein extract should fall in between 0.1 to 0.5 mgN/ml.

2. Preparation of protein extract for spectrophotometric reading.

Prepare 2 sets of 6 test tubes, each containing 5, 4, 3, 2, 1 and 0 ml (blank) of Bovin serum albumin working solution or fish myofibrillar protein extract and 0, 1, 2, 3, 4 and 5 ml of KCl-phosphate buffered solution respectively. Pipette 5 ml each Reagent A to one set of test tubes and pipette 5 ml each Reagent B to the other set of test tubes. Shake well and leave to stand 2 hrs at room temperature (26°C). Set up the spectrophotometer as specified by the manufacturer, adjust the wavelength to 545 nm and read the absorbance of the solution relative to the reagent blank (contains only KCl-phosphate buffered solution).

3. Calculation & calibration curve

Calculate the solutions' absorbance containing various concentration of protein solutions.

$$\text{Absorbance}_{545\text{nm}} = (\text{O.D.}_A - \text{Blank}_A) - (\text{O.D.}_B - \text{Blank}_B)$$

O.D._A and O.D._B = optical density of sample solutions with Reagent A and B, respectively.

Blank_A and Blank_B = optical density of blank solutions with Reagent A and B, respectively.

Plot the absorbance values of the protein solutions versus the concentrations of the protein solutions to obtain the calibration curve.

B. DETERMINATION OF PROTEIN CONCENTRATION OF UNKNOWN SAMPLE (FISH MYOFIBRILLAR PROTEIN EXTRACT)

Pipette 5 ml protein sample and 5 ml Reagent A into a test tube. To another test tube add 5 ml protein sample and 5 ml Reagent B. Also prepare another 2 test tubes each containing 5 ml KCl-phosphate buffered solution and 5 ml Reagent A and B, respectively. Shake well and leave to stand at room temperature for 2 hrs. Set up the spectrophotometer, adjust the wavelength to 545 nm, and read the absorbance of the solution relative to the reagent blank.

IV CALCULATIONS

Based on the calibration curve, express the result in mgN/ml. Convert the value to equivalent meat wt. and express as mgN/100 g sample, if required.

N.B. Experiments have shown that the results are relatively reliable as compared to results obtained by Kjeldahls' method for fish myofibrillar protein extract in the concentration range of 0.1-0.5 mgN/ml.

REFERENCE

Umemoto, S. (1966)

A modified method for estimation of fish muscle protein by Biuret method. Bull. Jap. Soc. Sci. Fish. 32:427-435.

DETERMINATION OF TRIMETHYLAMINE OXIDE (TMAO-N), TRIMETHYLAMINE (TMA-N), TOTAL VOLATILE BASIC NITROGEN (TVB-N) BY CONWAY'S METHOD

NG C. S.

INTRODUCTION

Trimethylamine oxide (TMAO) is a nitrogenous compound commonly present in marine organisms. It has been suggested that TMAO functions as an osmoregulator in these animals. The degradation of TMAO into simpler compounds such as trimethylamine (TMA), dimethylamine (DMA) and formaldehyde (FA) depends on the enzymes present in the tissue.

Generally, TMAO breaks down to TMA in marine fishes, either by endogenous enzymes, bacteria enzymes or both. However in gadoid fishes, the TMAO is broken down to DMA and FA.

The use of TMA as an index of fish freshness was first proposed by Beatty and Gibbons (1936). This was based on the observation that the production of TMA was dependent on bacteria activity, and the role of autolysis was negligible. The source of TMA in ordinary muscle is due to the bacterial degradation of TMAO to TMA, while in dark muscles, TMA was derived both from bacteria activity as well as from endogenous enzymes.

In recent years, there are opinions that TMA itself may not be a very suitable freshness index. This is because the TMA content in a fish may vary with season, and also, the distribution of TMA within a piece of fillet may not be uniform. Under the local conditions, TMA was found to be a good indicator of freshness for white pomfret, chinese pomfret, grouper and siakap. TMA is not a good indicator of freshness for lizard fish. Instead, DMA and FA are suitable indices.

The total volatile basic substances (TVB) in fish meat is mainly composed of ammonia, TMA, and DMA. The level of TVB increases after spoilage begins (both enzymatic and bacterial). It does not distinguish the origin nor component of these volatile compounds, hence its use is more general.

In this laboratory, the microdiffusion method devised by Conway is adopted. In this method, TMA, TMAO and TVB are determined as their nitrogen. To obtain the actual amount of TMA or TMAO the nitrogen values must be divided by the amount of nitrogen present per molecule of TMA or TMAO.

PRINCIPLE OF THE CONWAY UNIT IN DETERMINATION OF TVB

The solution in the inner ring of the Conway unit contains a 1% solution of boric acid with bromocresol green and methyl red indicator. The sample extract is in the outer ring. On the addition of K_2CO_3 the sample extract becomes alkaline. The TMA and related compounds present in the sample extract are released in alkaline condition as volatile compounds. The volatile compounds diffuse into the boric acid solution to form boric acid salt of these compounds. These salts are reduced to HCl-salts by a strong acid (HCl) during titration.

I REAGENTS

- a) Inner ring solution — 1% boric acid solution containing indicator:
Take 10 g of boric acid in 1 litre flask, add 200 ml of ethanol. After dissolving boric acid, add 10 ml of mixed indicator solution, then make up to 1 litre with distilled water.
- b) Mixed indicator solution:
Dissolve bromocresol green (BCG) 0.01 g and methyl red (MR) 0.02 g in 10 ml of ethanol.
- c) 0.02N HCl:
Dilute 20 ml of 1N HCl standard solution with distilled water and make up to 1000 ml.
- d) Saturated K_2CO_3 solution:
Take 60 g of potassium carbonate (K_2CO_3), and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, obtain filtrate through filter paper.
- e) 50% K_2CO_3 solution:
Dilute saturated K_2CO_3 solution twice with distilled water.
- f) 4% trichloroacetic acid (CCl_3COOH) (TCA) solution:
Dissolve 40 g of TCA in 960 ml of distilled water.
- g) 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.
- h) Neutralized 10% formaldehyde solution:
Add 10 g of $MgCO_3$ to 100 ml of formalin (35% formaldehyde solution) and shake in order to neutralize the acidity of formalin. Filter and dilute filtrate 3 times with distilled water.
- i) 1% $TiCl_3$ aqueous solution:
Take 6.7 ml of 15% $TiCl_3$ solution into 100 ml volumetric flask and make up to 100 ml with distilled water.
- j) Saturated KNO_3 aqueous solution:
Dissolve about 55 g of KNO_3 in 50 ml of distilled water.

II APPARATUS

Conway's unit:

Wash with detergent (use neutral detergent if available), then rinse with running water and leave until dry. Do not wipe with cloth.

Micro-burette

III PROCEDURE

A. 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 temp. with occasional grinding.
4. Filter through filter paper (Whatman No. 41). (or Centrifuge at 3000 rpm, for 10 min.)
5. Keep the filtrate in -20°C freezer if necessary.

B. 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. Titrate inner ring solution with 0.02N HCl using a micro-burette until green colour turns to pink.
10. Do blank test using 1 ml of 4% TCA instead of sample extract.

C. DETERMINATION OF TMA-N

Principle of TMA-N determination is similar to TVB-N determination except addition of formaldehyde to the sample solution. Formaldehyde is added in order to fix any ammonia present in the sample.

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 inner ring solution with 0.02N-HCl using a micro-burette until green colour turns to pink.
11. Do blank test using 1 ml of 4% TCA instead of sample extract.

CALCULATION OF TMA-N OR TVBN

$$\begin{matrix} \text{TMA-N or} \\ \text{TVBN} \\ \text{(mg/100 g)} \end{matrix} = \left(\begin{matrix} \text{Amt. of HCl} \\ \text{used in} \\ \text{titration} \end{matrix} \right) \times \left(\begin{matrix} \text{Amt. of ammonium} \\ \text{nitrogen equivalent to} \\ \text{1 ml of 0.02N HCl} \end{matrix} \right) \times \left(\begin{matrix} \text{Ratio of the amount} \\ \text{of sample used to} \\ \text{100 g muscle} \end{matrix} \right)$$

$$= (V_S - V_B) \times (N_{\text{HCl}} \times A_N) \times \frac{\left[\left(W_S \times \frac{M}{100} \right) + V_E \right] \times 100}{W_S}$$

where

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

V_B = Titration volume for blank (ml)

N_{HCl} = Normality of HCl (=0.02N × f, factor of HCl)

A_N = Atomic weight of Nitrogen (×14.00)

W_S = Weight of muscle sample (g)

M = percentage moisture of muscle sample.

V_E = Volume of 4% TCA used in extraction

NOTE: 1 ml of 0.02N HCl = 0.28 ammonium nitrogen
 = ($N_{\text{HCl}} \times f \times 14.00$)

D. DETERMINATION OF TMAO-N

Reduction of sample extract

1. Take 2 ml of the filtrate (sample extract) or its dilute into a test tube.
2. Add 1 ml of 1% TiCl_3 and fully mix, then confirm that pink colour does not disappear.
3. Stand in an 80°C water bath for 90 sec.
4. Add saturated KNO_3 dropwise until the pink colour disappears.
5. Cool in water.

6. Transfer the solution to 10 ml volumetric flask.
7. Make up to 10 ml with distilled water.
8. Proceed as for TMA-N determination.

CALCULATION OF TMAO-N

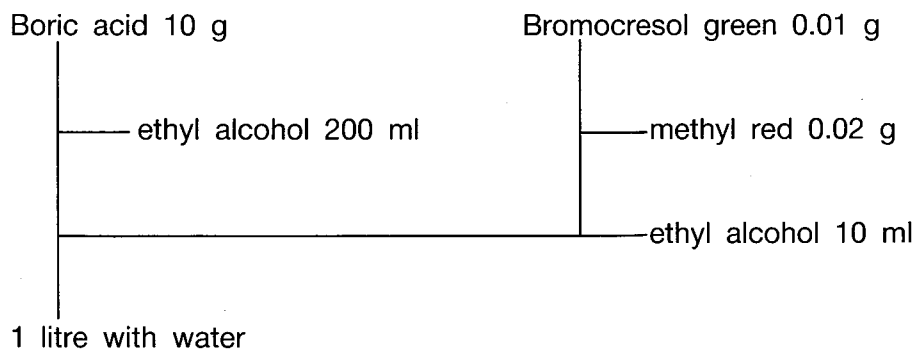
$$\text{TMAO-N} = (\text{TMA-N after TiCl}_3 \text{ reduction})^* - (\text{TMA-N before TiCl}_3 \text{ reduction})$$

* Care must be taken to obtain the correct dilution factor since in TMAO-N determination, the sample was made up to 10 ml before applying into the Conway unit.

ANNEX I. SCHEMATIC FORM FOR PREPARATION OF REAGENTS

REAGENTS

- a) Inner ring solution



- b) 0.02N-HCl (Accurate)
Dilute 20 ml of 1N-HCl with water and make the volume to 1 litre.
- c) Saturated potassium carbonate
Potassium carbonate (K_2CO_3) 60 g
water 50 ml
Boil gently for 10 min.
Cool down
Filtrate
- d) 50% saturated potassium carbonate
Dilute c) solution with water (1:1).

e) 10% TCA

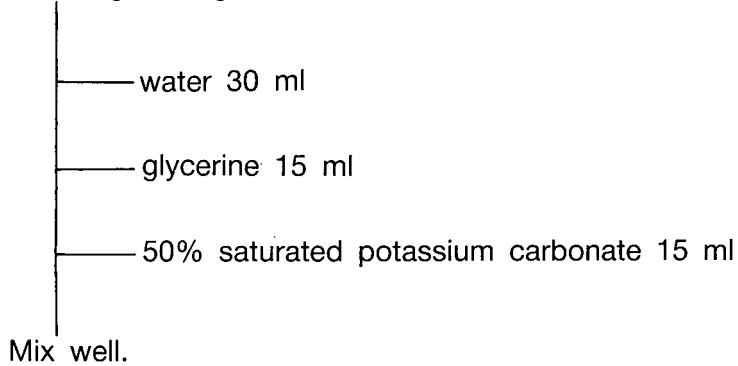
Dissolve 100 g of trichloroacetic acid (CCl_3COOH) in 900 ml of water.

f) 5% TCA

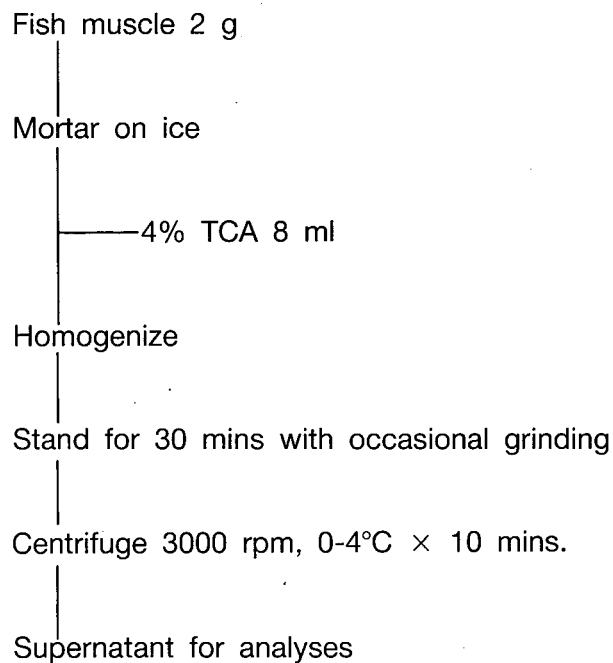
Dilute e) solution with water (1:1).

g) Sealing agent

Tragacanth gum 3 g



ANNEX II. SCHEMATIC FORM FOR PREPARATION OF SAMPLE FOR TVB-N AND TMA



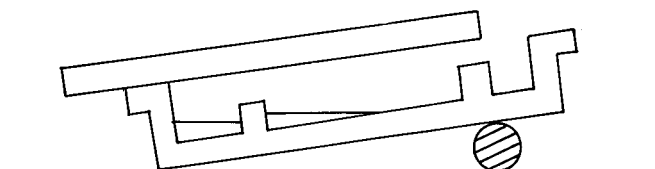
ANNEX III. PROCEDURE FOR TVB-N DETERMINATION

Sealing agent to Conway's unit

Inner solution 1 ml into inner ring

Sample extract 1 ml into outer ring

Slant the Conway's unit with cover



Saturated potassium carbonate
1 ml into outer ring

Close the unit using a band

Mix gently

Stand for 60 min. at 37°C

Titrate inner ring solution with 0.02N-HCl using a micro-burette

Do blank test using 1 ml 4% TCA instead of sample.

ANNEX IV. PROCEDURE FOR TMA-N DETERMINATION

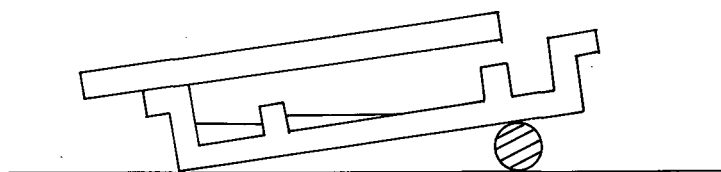
Sealing agent to Conway's unit

Inner solution 1 ml into inner ring

Sample extract 1 ml into outer ring

10% formaldehyde 1 ml into outer ring

Slant the Conway's unit with cover



Saturated potassium carbonate
1 ml into outer ring

Close the unit using a band

Mix gently

Stand for 60 min at 37°C

Titrate inner ring solution with 0.02N-HCl using a micro-burette

Do blank test using 1 ml 4% TCA instead of sample.

REFERENCES

Beatty, S.A. and N.E. Gibbons (1936)

The measurement of spoilage of fish. *J.Biol.Bd. Can.* 3:77-91.

Conway, E.J. and A. Byrne (1936)

An absorption apparatus for the micro-determination of certain volatile substances. I. The micro-determination of ammonia. *Biochem. J.* 27:419-429.

DETERMINATION OF DMA-N BY DYER'S COLORIMETRIC METHOD USING COPPER DITHIOCARBAMATE

NG C. S.

INTRODUCTION

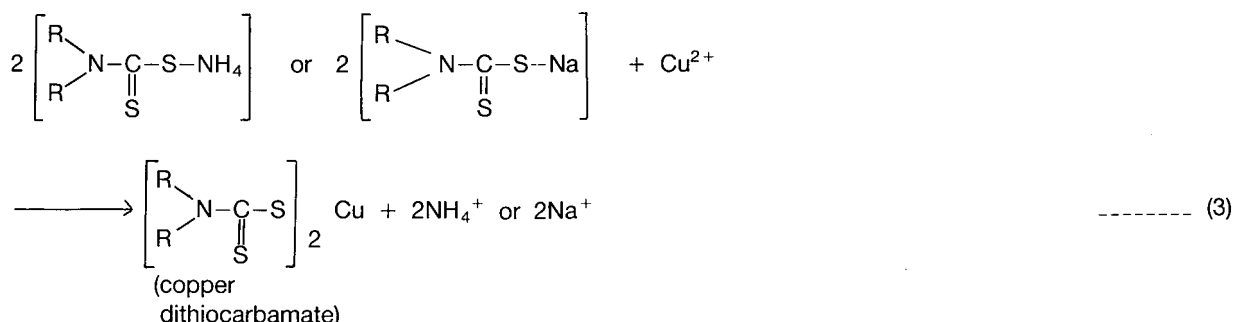
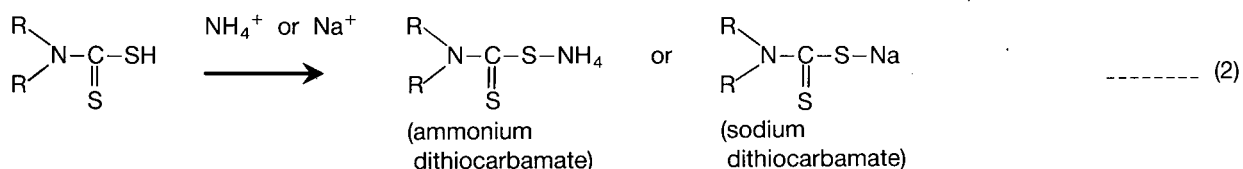
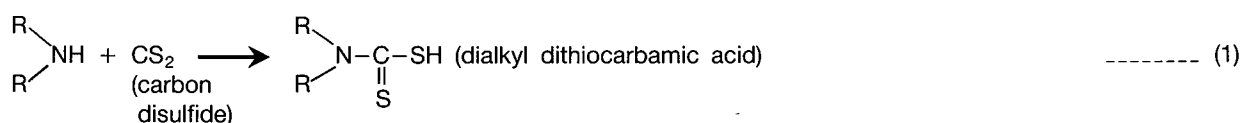
The precursor of dimethylamine (DMA) in fish meat is trimethylamine oxide (TMAO). In the gadoid species, TMAO present in ordinary muscle is decomposed to formadehyde (FA) and DMA simultaneously. This is usually attributed to endogenous enzymes. In the tropics, lizard fish is known to show a similar breakdown sequence. In fresh fish, and fish in the early stages of spoilage, the amounts of primary amines is low. The main secondary amine present is DMA. Hence measurement of DMA can be used as a spoilage indicator. However, at the later stages of spoilage, numerous other secondary amines are formed, and these will interfere with the results of the Dyer's colorimetric method.

In the laboratory determination of TMA-N and TMAO-N, the presence of DMA interferes and yields a higher value for the parameters measured. If the true amounts of TMA-N and TMAO-N are desired, the interference due to DMA must be discounted.

DMA and other secondary amines can react with nitrite salts to form dimethylnitrosoamine, a known carcinogen. Therefore, it is important to determine the amount of DMA present in fish and other food.

PRINCIPLE OF DYER'S COLORIMETRIC METHOD

Volatile secondary amines such as dimethylamine, di-n-propylamine etc. reacts with carbon disulfide to form dialkyl-dithiocarbamic acid (Equation 1). This dialkyl-dithiocarbamic acid reacts with NH_4^+ or Na^+ to form dialkyl-dithiocarbamate (Equation 2). Dialkyl-dithiocarbamate chelates with Cu^{2+} to form a yellow complex, Cu-dialkyl-dithiocarbamate (Equation 3).



I REAGENTS

All reagents should be of GR grade.

- a) 5% (v/v) carbon disulfide-toluene solution

Mix 5 ml of carbon disulfide with 95 ml of toluene.

- b) Copper-ammonium reagent

Dissolve 25 g of ammonium acetate and 0.2 g of cupric sulfate in 30 ml of distilled water, and mix this solution with 25 ml of 40% NaOH. To this add 20 ml of conc. ammonia (s.g. 0.88-0.90) and mix well, then make up to 100 ml with distilled water.

- c) 30% acetic acid.

- d) Anhydrous sodium sulfate.

- e) DMA standard stock solution.

Take 60.0 mg of DMA-HCl salt into 100 ml volumetric flask and make up with distilled water. This solution contains about 0.1 mg DMA-N/ml.

- f) DMA standard working solution

Take 10 ml of DMA stock solution into 100 ml of volumetric flask and make up with 2% TCA solution. This solution contains about 10 ug DMA-N/ml.

II PROCEDURE

A. SAMPLE PREPARATION

1. Take 5 g of sample in a mortar and grind well.
2. Wash the sample into a 100 ml volumetric flask with about 50 ml of distilled water.
3. Stand for 10 min after stirring well.
4. Add 8 ml of 25% TCA and mix well.
5. Make up to 100 ml with distilled water and mix well.
6. Stand for 30 min at ambient temperature.
7. Filter the solution with filter paper (Whatman No. 41).

B. DETERMINATION OF DMA

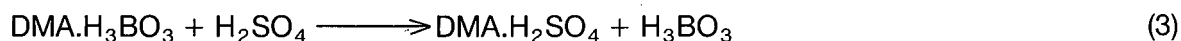
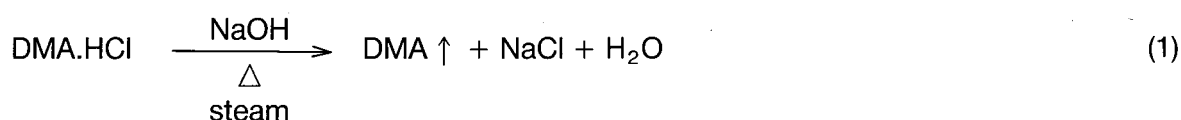
The following procedure should be done in a fume cupboard.

1. Take 5 ml of the filtrate in a test tube with stopper.
2. Add 1 ml of copper-ammonium reagent and mix.
3. Add 10 ml of 5% CS₂-toluene solution and then stopper the test tube.
4. Stand for 2 min in 50°C water bath.

5. Shake for 1 min.
6. Add 1 ml of 30% acetic acid.
7. Shake for 20-30 sec.
8. Stand for 10 min at ambient temperature.
9. Transfer the toluene layer to another tube containing about 0.5 g of anhydrous Na_2SO_4 after the toluene layer becomes clear.
10. Measure the absorbance at 440 nm.
11. Repeat the procedure with 5 ml of 2% TCA as blank.

C. STANDARDISATION OF DMA STANDARD SOLUTION.

Principle:



1. Take 10 ml DMA stock solution (DMA.HCl) into distillation tube. Add 20 ml distilled water and 6 ml 10% NaOH.
2. Steam distill to vaporise the DMA, and absorb into 20 ml of 4% H_3BO_3 .
3. Titrate $\text{DMA.H}_3\text{BO}_3$ solution with 0.05N H_2SO_4 using methyl red-bromocresol green mixed indicator.
4. Calculate the factor of DMA. HCl standard solution as:-

$$\text{factor, } f = \frac{\left[\left(\text{Vol. of sample titration} \right) - \left(\text{Vol. of blank titration} \right) \right] \times \left(\text{Mol. wt. of N equivalent} \right) \times \left(\text{Normality of H}_2\text{SO}_4 \right)}{\text{Volume of DMA.HCl used}}$$

$$= \frac{(V_s - V_b) \times 14.00 \times 0.05}{10}$$

D. PREPARATION OF CALIBRATION CURVE

1. Take 0.4, 0.8, 1.2, 1.6 and 2.0 ml of DMA standard working solution into the test tube (volume about 40 ml), and add 4.6, 4.2, 3.8, 3.4 and 3.0 ml of 2% TCA solution, respectively. These solutions contain 4, 8, 12, 16 and 20 μg DMA-N, respectively.
2. Repeat the procedure for determination of DMA.

E. CALCULATION OF DMA-N CONTENT OF SAMPLES

1. Obtain the amount of DMA-N (A μg) contained in 5 ml of sample solution from the calibration curve.
2. DMA-N (mg/100 g)

$$\begin{aligned} \text{DMA-N (mg/100 g)} &= \frac{\left(\frac{\mu\text{g DMA-N}}{\text{converted to mg}} \right) \times \left(\frac{\text{Make up}}{\text{volume}} \right) \times \left(\frac{100 \text{ g}}{\text{meat}} \right) \times f \times d}{(\text{Wt. of sample}) \times (\text{Volume of sample})} \\ &= \frac{A}{1000} \times \frac{100 \times 100 \times f \times d}{W \times 5} \end{aligned}$$

where W = weight of meat

f = factor of DMA standard solution

d = dilution factor (if any)

REFERENCE

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distilled water to get approx. 100 ppm solution. Ten ml of 100 ppm solution is diluted 10 times with distilled water in the volumetric flask. This final dilute gives approx. 10 ppm solution of formaldehyde. This dilute is not stable, so, it is necessary to be renewed in each series of determination.

d) 0.1N Sodium thiosulfate standard solution

Dissolve 25 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in distilled water which is cooled after boiling, and make up to 1 litre. Standardize after standing 1 to 2 days by the procedure described in the determination of peroxide value (C-5).

e) Sodium bisulfite solution (approximately 0.1N)

Dissolve 5.2 g of NaHSO_3 in distilled water and make up to 1 litre.

f) Iodine solution (approximately 0.1N)

Dissolve 12.7 g of I_2 and 40 g of KI in 25 ml of distilled water and make up to 1 litre.

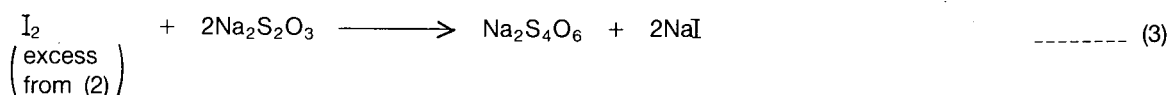
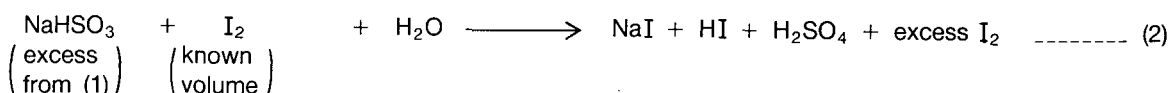
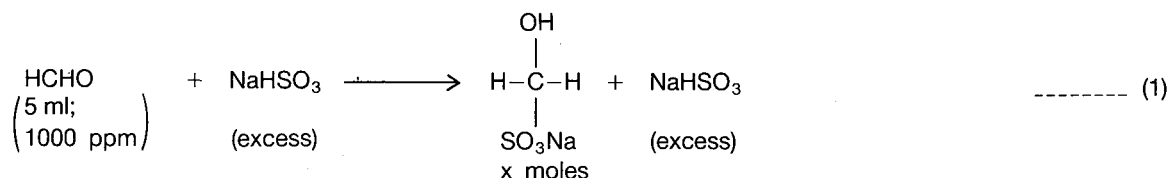
g) 1.5% Starch solution

Weigh 1.5 g of starch and add 100 ml of distilled water, then boil the solution for 30 sec.

STANDARDISATION OF FORMALDEHYDE SOLUTION

1. Pipette 5 ml of formaldehyde solution, approximately 1,000 ppm, into a 200-300 ml conical flask and add 50 ml of distilled water.
2. Add 10 ml of 0.1N sodium bisulfite solution, and let it stand for about 30 min with occasional shaking.
3. Add iodine solution until the colour turns brown. Note the volume of iodine used. Titrate the excess iodine with sodium thiosulfate standard solution until just before the yellow colour disappears. Add 1 ml starch solution as indicator, and continue titration until the dark-blue colour disappears. The volumes of iodine solution and thiosulfate solution are noted.
4. Calculate the factor of formaldehyde standard solution.

The following reactions occur.



$\therefore 2 \text{ moles Na}_2\text{S}_2\text{O}_3 = 1 \text{ mole NaHSO}_3 = 1 \text{ mole HCHO}$

The specific gravity of 35% formaldehyde at 25°C is 1.08. The molarity of the 1000 ppm solution is 0.032375. Since 1 mole of formaldehyde is equivalent to 2 moles of sodium thiosulfate, using $N_1 V_1 = N_2 V_2$,

$$\begin{aligned} N_1, \text{ Normality of formaldehyde} &= \text{factor} \times \text{Molarity} \times \text{equivalent} \\ &= f \times M \times e \\ &= f \times 0.03237 \times 2 \end{aligned}$$

$$V_1 = \text{Volume of formaldehyde} = 5 \text{ ml}$$

$$N_2 = \text{Normality of Na}_2\text{S}_2\text{O}_3 = 0.1$$

$$V_2 = \text{Volume of Na}_2\text{S}_2\text{O}_3 = (\text{Vol titrated in sample}) - (\text{Vol titrated in blank}).$$

$$= V_S - V_B$$

$$(f \times M \times e) \times (5) = (0.1) \times (V_S - V_B)$$

$$\therefore f = \frac{0.1 \times (V_S - V_B)}{5 \times M \times e}$$

$$= \frac{0.1 \times (V_S - V_B)}{5 \times 0.032375 \times 2}$$

II APPARATUS AND INSTRUMENTS

Hitachi Spectrophotometer ($\lambda = 412 \text{ nm}$)
 Beckman Model 3560 digital pH meter
 Yamato ultra disperser
 beakers (50 ml)
 long test-tubes (15ml)
 burette (25 ml)
 Pipetman micropipette (max vol= 1 ml)
 filter paper (Whatman No. 41, \varnothing 15 cm)

III PROCEDURE

A. SAMPLE PREPARATION

1. Weigh 5 g of minced meat accurately in a 30-50 ml beaker.
2. Add 20 ml of 5% TCA solution and homogenize well with homogenizer.
3. Stand in an ambient temperature for 30 min.
4. Filter the supernatant with filter paper, Whatman No. 41.
5. Add 10 ml of 5% TCA solution to the residue, homogenize again, then filter.
6. Neutralize the combined filtrate to pH 6.0-6.5 by using pH meter with 1N or 0.1N KOH dropwise, and make up to 50 ml with distilled water.

B. DETERMINATION OF FORMALDEHYDE

1. Take 3 ml of the neutralized filtrate in a test tube, add 3 ml of the acetylacetone reagent and mix well.
2. Stand in water bath (60°C) for 15 min.
3. Cool the solution in running water.
4. Measure the absorbance of the solution against the blank solution at 412 nm (Blank solution contains distilled water instead of the neutralized filtrate).

C. PREPARATION OF CALIBRATION CURVE

1. Pipette 0, 0.3, 0.6, 1.2 and 2.4 ml of 10 ppm formaldehyde standard working solution into test tube using micro-pipette. These solutions contains 0, 3, 6, and 12 and 24 µg of formaldehyde, respectively.
2. Add 3, 2.7, 2.4, 1.8 and 0.6 ml of distilled water, respectively, then add 3 ml of the acetylacetone reagent.
3. Continue as in the procedure B) 2. to 4. after mixing well.

IV CALCULATION OF FORMALDEHYDE CONTENT

$$\text{Formaldehyde } (\mu\text{g/g}) = \frac{A}{(\text{Vol. of filtrate used})} \times \frac{(\text{Total make up vol. of filtrate}) \times f}{(\text{Weight of sample})}$$

where A = Reading from calibration curve (µg)

f = factor of formaldehyde of standard solution.

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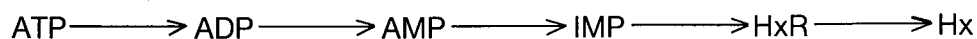
Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. In: Martin R.E. et al (Editors) 1982. Chemistry and biochemistry of marine food products p: 164-165. Publ. AVI., Westport.

DETERMINATION OF K VALUE

NG C. S.

INTRODUCTION

The K value is an index to measure the enzymatic freshness of fish and squids. Immediately after death, ATP (adenosine triphosphate) and related compounds are broken down by endogenous enzymes. A typical schematic breakdown can be represented as:-



ADP = adenosine diphosphate

AMP = adenosine monophosphate

IMP = inosine monophosphate

HxR = inosine or hypoxanthine riboside

Hx = hypoxanthine

The K value is defined as

$$K\% = \frac{[\text{HxR}] + [\text{Hx}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{HxR}] + [\text{Hx}]} \times 100$$

Ideally, the K value should be measured before exogenous enzymatic activities such as bacterial enzymes begin. In applying K value, care should be exercised to ensure that it is reliable. For example, the K value of a processed fillet may be higher as water soluble components such as IMP may have been washed away. Sampling the unexposed meat will prevent such an error. Skins and dark muscles of fish should be excluded during sampling. Guanine found in the skin will be eluted with the hypoxanthine fraction while dark muscles have a high inosine content.

The present method cannot be directly used for measuring the K value of squids. In the squid, the AMP breaks down directly to HxR. Separation of AMP and HxR is more difficult compared to separation of IMP and HxR. A modified method as proposed by Uchiyama (1984) should be adopted.

PRINCIPLE OF ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography distinguishes one component in a mixture from another on the basis of the number of charges of appropriate sign available on each molecule for interaction with the ion exchanger under the conditions imposed. Molecular size is an important factor and the distribution of charges also plays a role.

Ion exchangers can be classified into two categories:-

- (i) those that bear positive charges and are called anion exchangers because they interact with anions.
- (ii) those that bear negative charges and are called cation exchangers because they interact with cations.

In the present method, an anion exchanger (Cl^- form) is used. Uchiyama et. al (1972) had reported that using authentic mixtures of ATP and its related compounds charged with Dowex

1-X4 column, the elution of HxR and Hx takes place in the region of pH 6.0 and 0.1M NaCl, while nucleotides such as ATP, ADP, AMP and IMP are eluted at a acidity of less than pH3, and within the range of up to 0.15M NaCl.

I PREPARATION OF SAMPLE

One gram of ordinary muscle from fish is sufficient. Care should be taken to exclude red muscle, fibrous tissues and skin. Sample treatment procedure is illustrated in Scheme 2.

II REAGENTS

A. FOR SAMPLE PREPARATION

All reagents listed here should be kept at 5°C until used.

- a) 10% perchloric acid (PCA): Dissolve 10 g of PCA (60-70%, HClO_4) in 90 ml of distilled water.
- b) 5% PCA: Dissolve 10 g of PCA in 190 ml of distilled water.
- c) Neutralized PCA: Neutralize 100 ml of 5% PCA to pH 6.4 with 10N-KOH using pH meter, then filter precipitates (KClO_4) through filter paper after cooling the neutralized PCA at 5°C.
- d) 10N-KOH: Dissolve 56 g of potassium hydroxide (KOH) in distilled water and make up to 100 ml.
- e) 1N-KOH: Dissolve 5.6 g of KOH in distilled water and make up to 100 ml.

B. FOR ION-EXCHANGE CHROMATOGRAPHY

- a) 0.5M NH_4OH solution: Dilute 4 ml of 25% NH_4OH with 96 ml of distilled water.
- b) Solution A = 0.001N HCl: Dilute 1 ml of 1N HCl standard solution to 1000 ml with distilled water.
- c) Solution B = 0.01N HCl containing 0.6M NaCl: Dissolve 35.07 g of NaCl in distilled water, then mix this NaCl solution with 10 ml of 1N HCl standard solution and make up to 1000 ml with distilled water finally.
- d) Anion exchange resin: AG (R) 1-X4, 400 mesh Cl (chloride)-form (Bio-Rad Co.).

C. PREPARATION OF ION-EXCHANGE RESIN (SCHEME 1)

- a) Acetone
- b) 0.1N NaOH
- c) 0.1N HCl

III APPARATUS

Chromatography System

Figs. 1 and 2 show two systems for simplified method estimation of K-value.

Column

As shown in Fig 3, use the column (inner \varnothing 6 mm) with coarse glass filter at the bottom part. The height of resin is around 50 mm.

IV PROCEDURE

A. PREPARATION OF ION-EXCHANGE RESIN

See Scheme 1.

B. PREPARATION OF SAMPLE EXTRACT

See Scheme 2.

C. CHROMATOGRAPHY (Also see Scheme 3)

1. Take 2 ml of neutralized muscle extract in a test tube.
2. Adjust pH to 9.4 by using pH test paper, with a few drops of 0.5M NH₄OH.
3. Apply it onto the column.
4. Wash the inside wall of the column with a few ml of distilled water.
5. In system 1 (Fig 1), onto a column attach a siphon tube which is set in a beaker containing 20 ml of distilled water. In system 2 (Fig 2), attach a separating funnel instead of a siphon onto the column and pour 20 ml of distilled water into the separating funnel.
6. Wash out unabsorbed ultraviolet-absorbing-compounds with distilled water from the column.
7. Pour 45 ml of solution A into the beaker or the separating funnel to elute hypoxanthine riboside (HxR) and hypoxanthine (Hx).
8. Collect the eluate in a 50 ml volumetric flask. Maintain the flow rate at 1-1.5 ml/min.
9. After all the solution A had passed into the resin, run 45 ml of solution B into the column to elute ATP, ADP, AMP and IMP.
10. Collect the eluate in another 50 ml volumetric flask.
11. Make up the eluates to 50 ml with solutions A and B, respectively.
12. Measure the absorbance of the two eluates at 250 nm.

V CALCULATION

$$K(\%) = \frac{E_{250\text{nm}} \text{ A}}{E_{250\text{nm}} \text{ A} + E_{250\text{nm}} \text{ B}} \times 100$$

where E_{250nm} A: [OD at 250nm of the solution A-eluate]
– [OD at 250nm of the soln A];

E_{250nm} B: [OD at 250nm of the solution B-eluate]
– [OD at 250nm of the soln B].

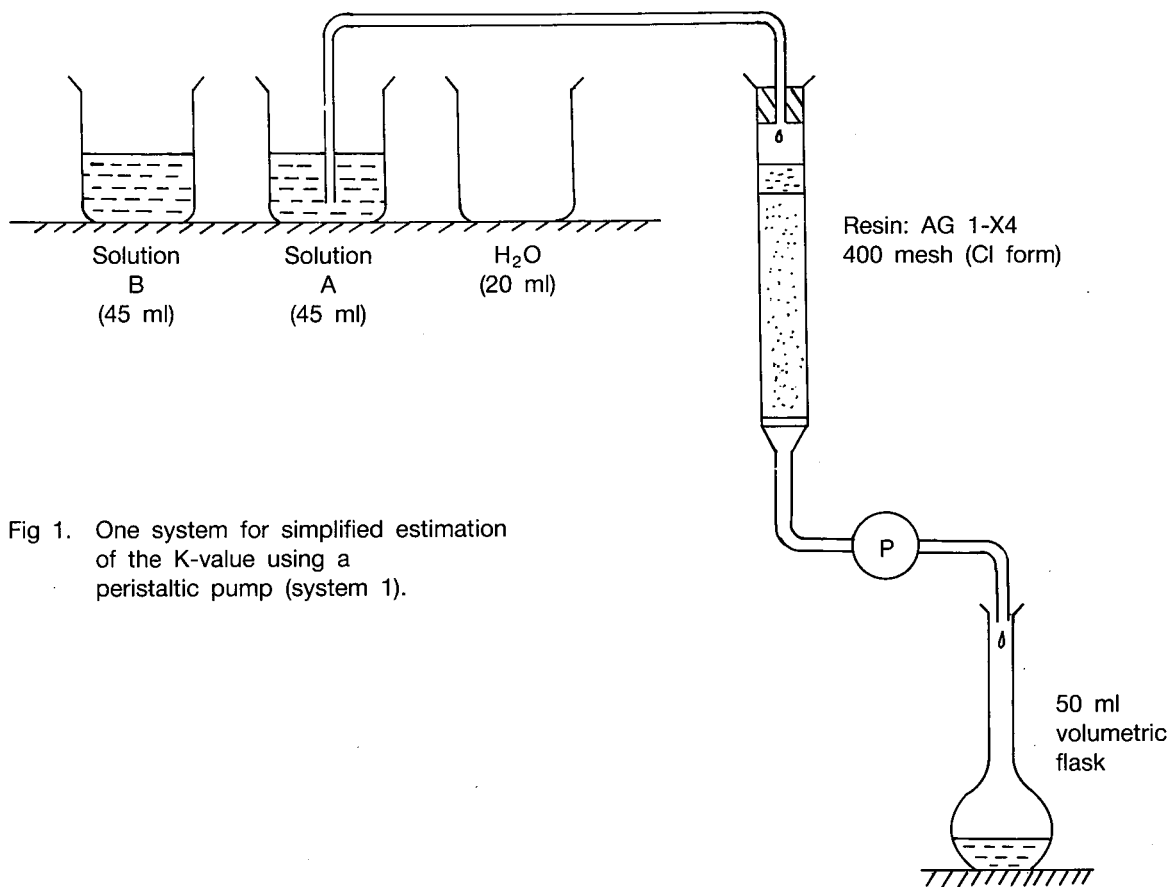


Fig 1. One system for simplified estimation of the K-value using a peristaltic pump (system 1).

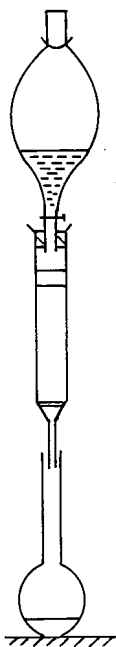


Fig 2. Another system for the estimation of K-value using a separating funnel (system 2).

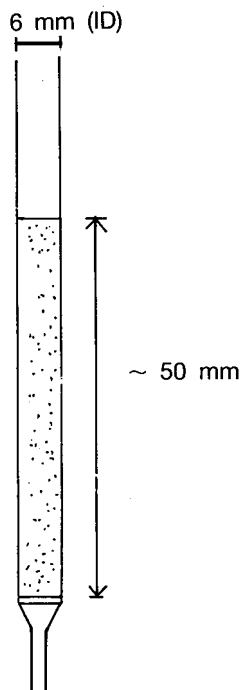
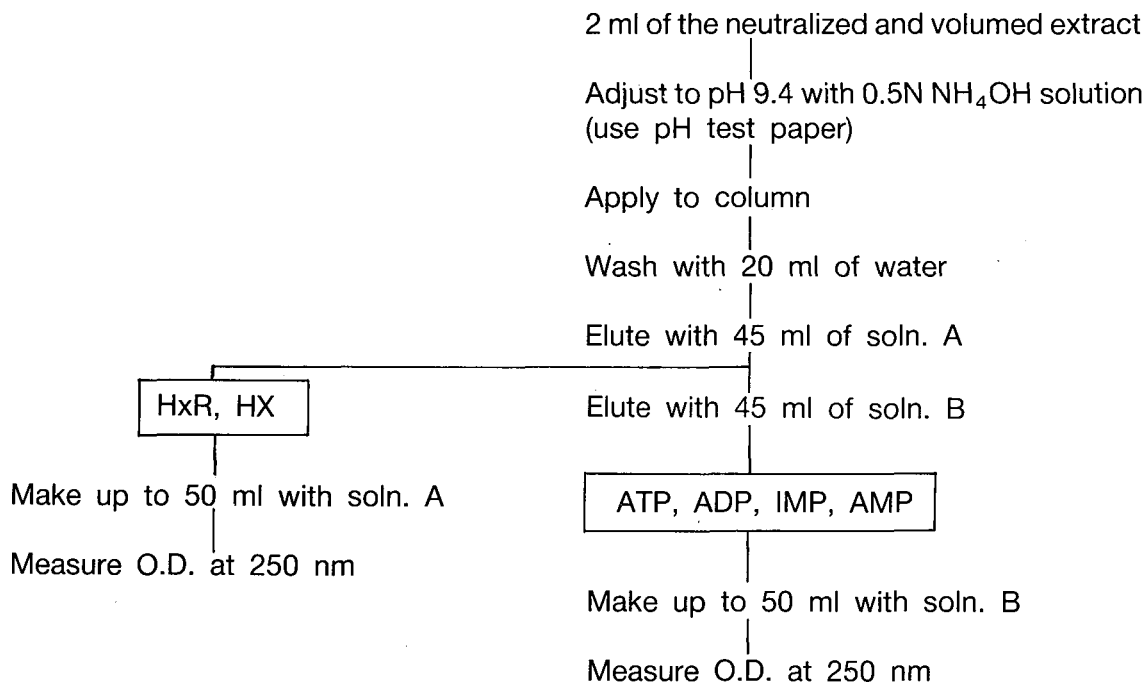


Fig 3. Column

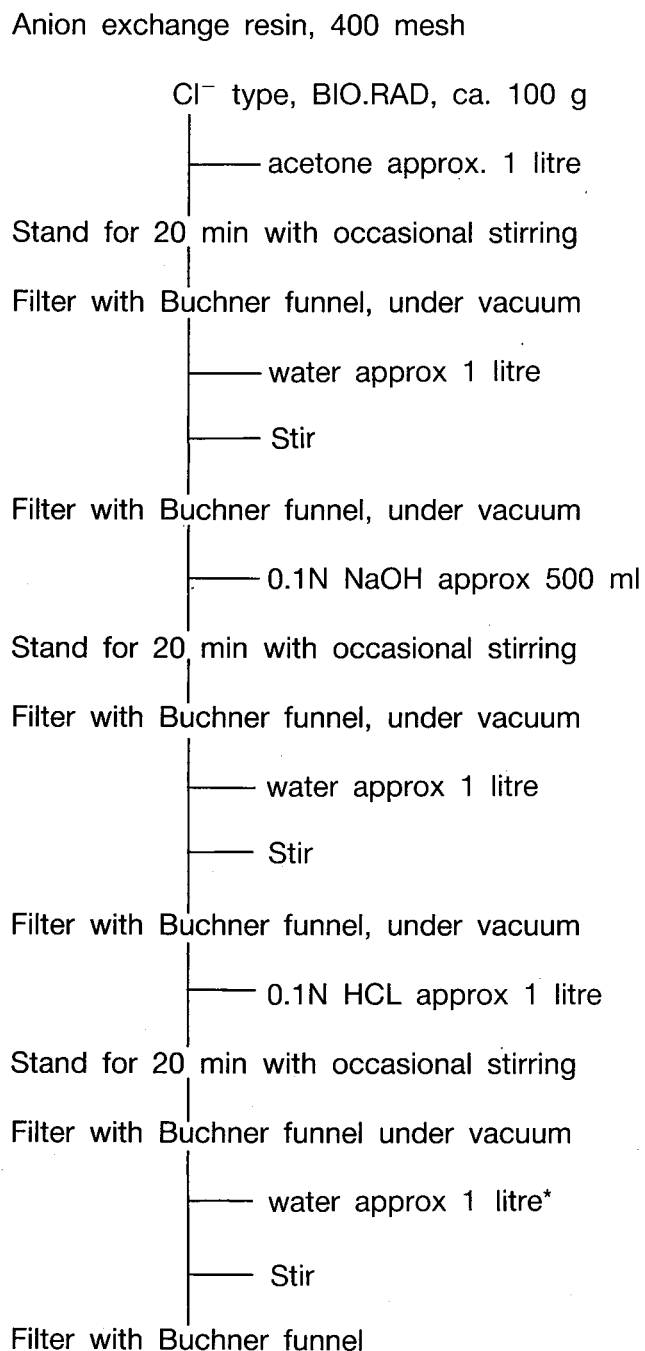
SCHEME 3. SIMPLIFIED FRACTIONATION METHOD FOR K-VALUE



REFERENCES

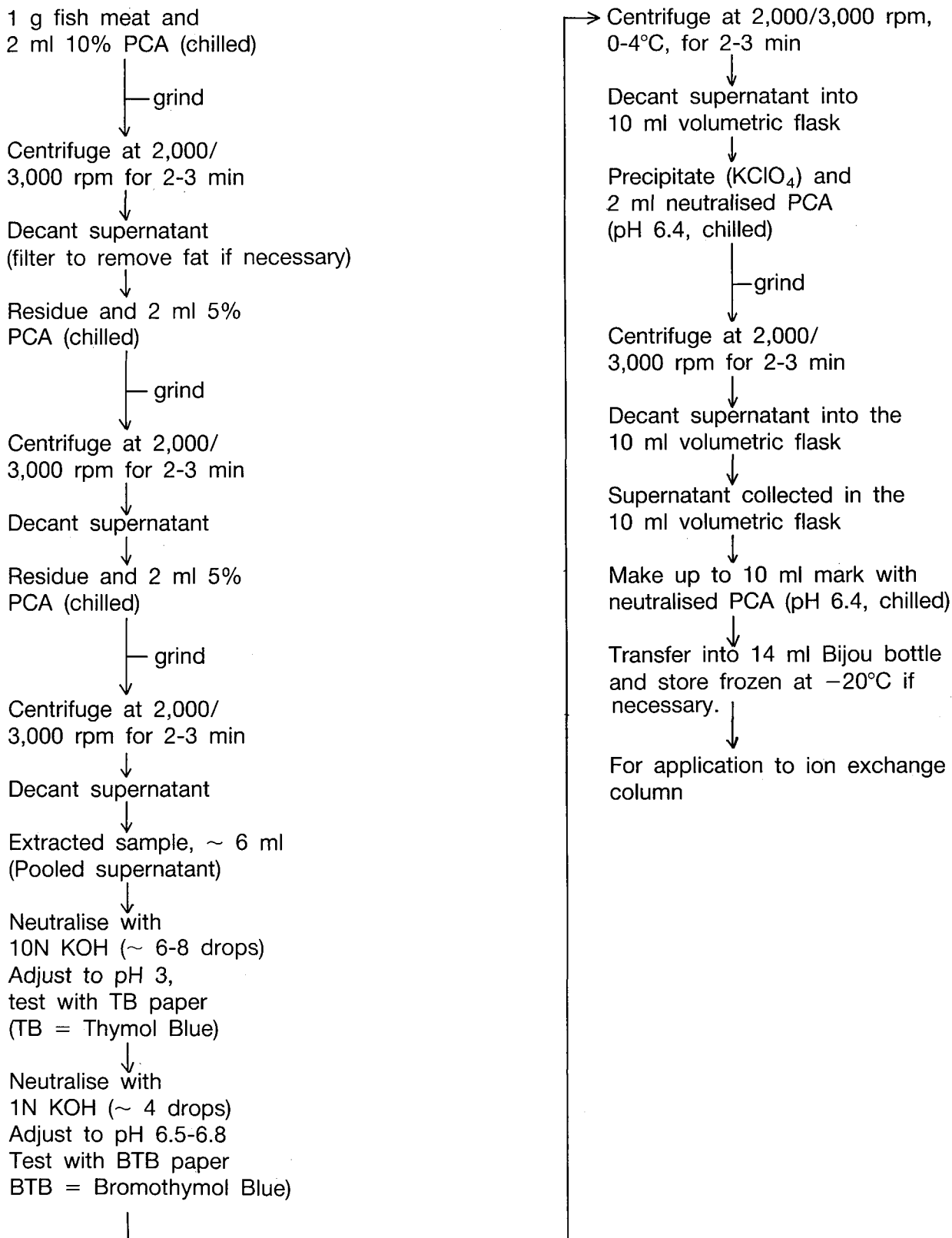
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SCHEME 1. PREPARATION OF ION-EXCHANGE RESIN



* Repeat washing with distilled water until filtrate (water) is neutral. Activated resin is stored at 5°C under water.

SCHEME 2. PREPARATION OF FISH MUSCLE EXTRACT



FRESHNESS TESTING PAPER

NG C. S.

INTRODUCTION

Measuring K value by means of ion exchange chromatography and spectrophotometry is tedious and cumbersome. The Freshness Testing Paper (FTP)* technique, aims at practicality and suitability for use in the field. The principles involved utilise enzyme actions to convert inosine (HxR) and hypoxanthine (Hx) to uric acid, which changes the colour of the dye present in the paper.

This method should preferably be used after it had been calibrated against the ion exchange chromatographic method. The enzymes present in fishes may vary from species to species, and calibration should be conducted for each species. For very crude estimation, no calibration is required.

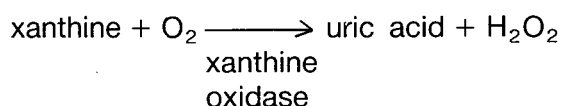
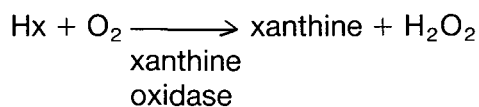
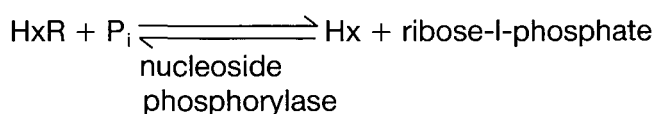
Since the FTP uses enzymes, storage of the test paper at low temperature is essential (preferably at -60°C) to ensure the functionality of the enzymes. This is one of the major disadvantage of this technique.

PRINCIPLE OF FRESHNESS TESTING PAPER

This technique uses enzymatic degradation and the subsequent colour conversion of a redox dye to indicate "freshness"

The K value (%) is defined as
$$\frac{\text{HxR} + \text{Hx}}{\text{ATP} + \text{ADP} + \text{IMP} + \text{AMP} + \text{HxR} + \text{Hx}} \times 100$$

In the FTP, the enzymes nucleoside phosphorylase and xanthine oxidase are embedded in the test paper. On application of the sample extract, the following reactions occur.



The uric acid formed changes the colour of the redox dye present in the FTP. The colour intensity is proportional to the content of HxR + Hx.

* The FTP Test Kit is patented and sold by Kankyo Bunseki Centre K.K. Tokyo.

I PROCEDURE

1. Take 0.5 g minced fish meat and add 0.5 g treated sand. Grind well in a small mortar.
2. Add 1.5 ml of FB* solution. Grind well. Add another 3.5 ml FB solution and grind well.
3. Dip FTP into homogenised solution. Remove and blot off excess solutions on filter paper. Keep the FTP in a plastic bag (transparent; and keep at room temperature for 10-15 min. (Standardise the time for each species).
4. Compare the colour (red to purple) of FTP with the colour chart provided. Read the corresponding K value.

STANDARDISE FTP TO K VALUE BY ION-EXCHANGE CHROMATOGRAPHY

1. Samples of the species under study of varying freshness is required.
2. Meat samples were individually prepared. A portion of the meat is used in the FTP, while the corresponding portions are subjected to conventional K value analysis.
3. The colour intensity and the corresponding K value are correlated. Care should be taken to standardise the time of reading of the coloured strips of FTP.

PRECAUTIONS

1. All the materials supplied with the FTP kit are easily degraded, and should be stored frozen (-60°C preferable) and in the dark. Anaerobic conditions will prolong the shelf life.
2. Check the expiry date before use. Expired products will give unreliable results.
3. The resulting colour is unstable in light, and will eventually fade. Readings should be conducted immediately after the full colour had developed.

* The procedure for FB solution preparation as stated in the test kit.

ANALYSIS OF OILS

SIGNIFICANCE OF ANALYSIS OF LIPIDS

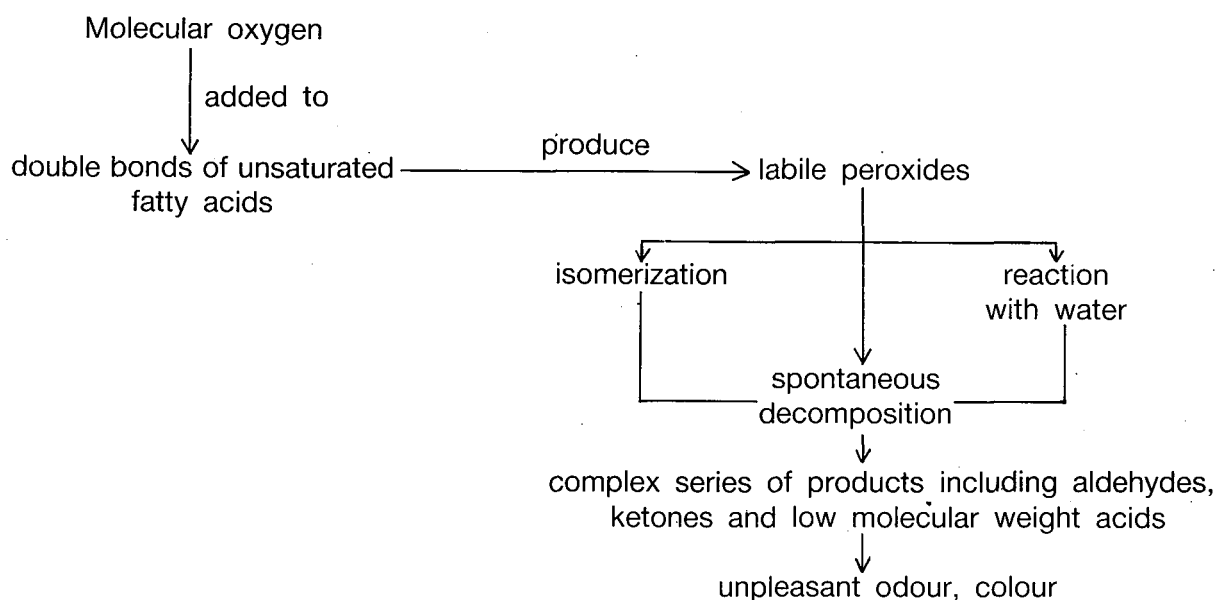
LOW L. K. & NG C. S.

Fish lipids exist as phospholipids (tissue fat) and triglycerides (depot fats or neutral lipids). During storage, fish lipids deteriorate by hydrolysis and oxidation.

Both phospholipids and triglycerides are hydrolyzed by enzymes into free fatty acids. The indices used for measuring the degree of hydrolysis are:

- i) the phospholipid content
- ii) the acid value (AV)
- iii) the free fatty acid value (FFA)
- iv) the saponification value (SV)

During oxidation, the highly unsaturated fatty acids of fish fats react with atmospheric oxygen to yield a complex series of compounds including aldehydes, ketones, and acids of lower molecular weight. These by-products contribute to the unpleasant taste and rancid odour of spoilt fish. Exposure of the fish to heat and light, to moisture, and to the presence of traces of certain metals (eg. copper, nickel and iron) accelerates this oxidation reaction. The reaction involved may be summarized as follows:-



Chemical parameters which are used for determining the extent of spoilage due to oxidation of fish lipids are:-

- i) the peroxide value (POV)
- ii) the thiobarbituric acid number (TBA)
- iii) the oxidation index

EXTRACTION OF LIPIDS (MODIFIED FOLCH'S METHOD)

LOW L. K. & NG C. S.

INTRODUCTION

A mixture of chloroform and methanol in the ratio of 2:1 (v/v) extract lipid more exhaustively from animal tissues than most other simple solvents systems. With most tissues, the lipids are removed almost completely after two or three treatments with the mixture. Most of the contaminating compounds in the extract can be removed from the chloroform-methanol (2:1 v/v) mixtures simply by shaking the combined solvents with a quarter of the total volume of water. The lower phase which comprises 60% of the total volume contains the purified lipid. This extraction yields approximately a 95-99% recovery of lipids.

I SAMPLE PREPARATION

The fish sample is chopped into a mince. Depending on the tissue, the following approximate sample sizes are used.

- i) ordinary muscle (20 – 50 g)
- ii) dark muscle (15 g)
- iii) skin (10 g)

II APPARATUS

Homogenizer with ice jacket.
Buchner flask and funnel
Vacuum pump
Nitrogen gas
Separating flasks (1000 ml)
Volumetric flasks (50 ml)
Measuring cylinders (100 and 250 ml)
Whatman No. 1 filter paper (qualitative, 7 cm Ø)

III REAGENTS

- a) Purified and distilled chloroform

Wash chloroform once with concentrated sulphuric acid (10 ml H_2SO_4 for 1 litre of chloroform). Then wash 2 to 3 times with distilled water using a separating funnel. Collect washed chloroform and add anhydrous calcium chloride. Stand overnight, then transfer to distillation flask. Distil and collect fraction which distills over at 60.5°C. Add purified and distilled methyl alcohol (1% by volume) as stabilizer. Keep in dark. Should be used within one month.

- b) Purified and distilled methyl alcohol

Add granular potassium hydroxide to methyl alcohol to remove acids, aldehydes and moisture. Distill and collect fraction which distills over at 64.5°C. Keep in the dark. Should be used within one month.

- c) Chloroform-methyl alcohol (C-M mixture)

Mix reagent from a) with b) in the proportion of 2:1 (v/v).

d) 1% BHA-BHT antioxidant solution

Dissolve 1 g of butylated hydroxyanisole (BHA) and 1 g of butylated hydroxytoluene (BHT) in 100ml C-M mixture.

e) Anhydrous sodium sulphate

IV PROCEDURE

1. Weigh the chopped sample into the homogenizer cup.
2. Add C-M mixture volume of about 3.5 times the weight of sample, and 2-3 drops of antioxidant solution.
3. Homogenize for 1 min and filter with Whatman No. 1 filter paper using a Buchner funnel and vacuum pump.
4. Transfer the residue into the cup and repeat homogenization twice.*
5. Transfer the combined filtrate into a separating flask.
6. Pour distilled water, volume approximately a quarter of that of the extract, into the separating flask.
7. Shake very gently 2-3 times, and stand overnight.**
8. Drain off the chloroform phase through a Whatman No. 1 filter paper into an Erlenmeyer flask containing about 2-5 g anhydrous sodium sulphate. Shake well and leave for about 5 min. Decant into an evaporating flask.
9. Wash the filter paper 2-3 times with C-M Mixture.
10. Concentrate the extract with a rotary evaporator under reduced pressure at 40°C (water-bath temperature).***
11. Dissolve the concentrated extract with C-M Mixture and transfer to 50 ml volumetric flask using a pipette.
12. Make up to the mark with C-M Mixture.
13. Flush with nitrogen gas and store at -20°C. This sample is used for other tests unless otherwise specified.

* Not necessary to add antioxidant solution.

** When mixture does not separate well, centrifuge at 8,000 rpm for 10 min.

*** Do not allow to evaporate to dryness.

N.B. Residual water vapour cannot be removed completely.

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DETERMINATION OF TOTAL LIPID CONTENT

LOW L. K. & NG C. S.

INTRODUCTION

This method enables the total lipid of the fresh fish sample to be determined without the destruction of the lipid extract.

I APPARATUS

Analytical balance (at least 1 mg sensitivity)
Rotary vacuum pump (max vacuum = 3×10^{-2} mbar)
Water bath with temperature control system (40°C)
Desiccator
Test-tubes
Pipette (5 ml)

II PROCEDURE

1. Dry test-tube in desiccator for half an hour and weigh accurately.
2. Pipette accurately 5 ml of the extract into the dry preweighed test-tube.
3. Remove solvent completely using the rotary evaporator under reduced pressure at 40°C (Water-bath temperature)
4. *Attach the test-tube to a rotary vacuum pump and dry the sample for about 5 min.
5. Leave the test-tube in a desiccator for 30 min and weigh the test-tube and contents accurately.

* Drying can also be done in an electric air oven at 105°C for 30 min. However, the lipid may oxidise and hence increase the weight of the dry sample by about 4 to 10%.

III CALCULATIONS

$$\text{Total lipid content (\%)} = \frac{W_1}{V_e} \times \frac{V_t}{W_s} \times 100$$

where W_1 = weight of dried lipid

W_s = weight of skin or meat used

V_e = volume of extract used

V_t = total volume of extract prepared

DETERMINATION OF PHOSPHOLIPID CONTENT

LOW L. K. & NG C. S.

INTRODUCTION

Phospholipids are hydrolyzed by the action of phospholipase. The action of phospholipase is usually stronger than that of lipase. Therefore the extent of hydrolysis of phospholipids is adopted as an index of lipid deterioration.

Phospholipid content is obtained by using column chromatography to separate the triglycerides (neutral lipids) from the phospholipids. The polar phospholipids are absorbed by the silicic acid and is eluted by the methanol. The neutral lipids which are not absorbed by the silicic acid are first eluted out by the chloroform.

I APPARATUS

Glass chromatograph column (\varnothing :1-2 cm; length: 30 cm) with Teflon tap.
Cotton wool
Filter paper (Whatman No. 1)
Preweighed, dry evaporating flask (50 ml capacity)
Analytical balance
Desiccator
Rotary evaporator with water bath (28°C)

II REAGENTS

- a) Silicic acid (Mallinckrodt, 100 mesh)
- b) Celite 545
- c) Methanol (analytical grade)
- d) Chloroform (analytical grade)

A. PREPARATION OF THE PACKING MATERIAL

1. Wash the silicic acid and celite 545 separately with warm methanol for 5 to 10 mins.
2. Allow the material to settle and decant the washing solution.
3. Repeat steps 1. and 2. twice.
4. Wash with warm acetone twice.
5. Air dry at room temperature overnight.
6. Then oven dry at 120°C for 2 hours.
7. Cool and mix silicic acid and Celite 545 in a ratio 2:1.

B. PREPARATION OF COLUMN

1. Weigh packing material 10-15 times that of the lipid sample weight.
2. Soak cotton wool in chloroform and pack into bottom of column. Exclude as much air as possible.

3. Place 2 layers of Whatman No. 1 filter paper cut into size of columns.
4. Mix the packing material in chloroform and pour gently into column with the aid of a glass rod.
5. Allow the packing material to settle.
6. Place 2 layers of Whatman No. 1 filter paper on the packing material.
7. Drain column of excess chloroform leaving a 1 cm high column of chloroform.

III PROCEDURE

1. Dissolve 1 g sample lipid in pure chloroform, making a 5 to 10% solution.
2. Introduce thin sample onto the column.
3. Drain off excess chloroform till solvent level is about 1 cm above the packing material.
4. Drain off with 250 ml chloroform and collect the neutral lipids in preweighed evaporating flask (elution speed: 3 drops per second).
5. Evaporate off the solvent using the rotary evaporator, dry up using a rotary vacuum pump for 5 mins, cool in desiccator for another 30 min and weigh the neutral lipids.
6. Drain off with 100 ml methanol and collect the phospholipids in preweighed evaporating flask.
7. Evaporate off the solvent using the rotary evaporator, dry up using a rotary vacuum pump for 5 mins, cool in desiccator for 30 min and weigh the phospholipids.

IV CALCULATION

$$\text{Neutral lipid (\%)} = \frac{(\text{Weight of Neutral Lipid})}{(\text{Weight of PL} + \text{Wt. of NL})} \times 100$$

$$\text{Phospholipid (\%)} = \frac{(\text{Weight of Phospholipid})}{(\text{Weight of PL} + \text{Wt. of NL})} \times 100$$

NL = Neutral lipid

PL = Phospholipid

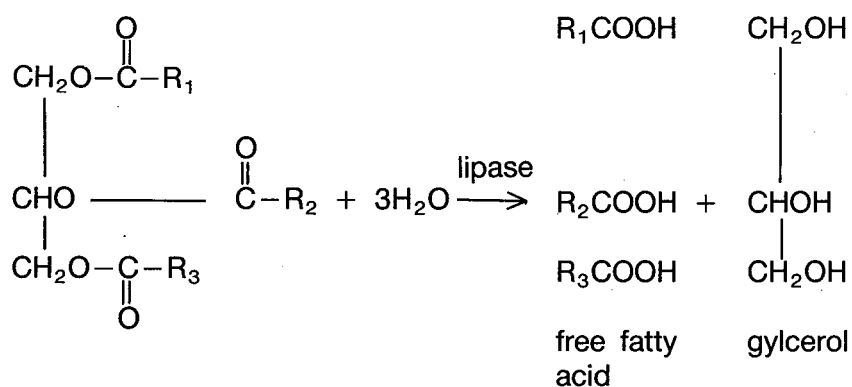
The phospholipid content is expressed as the percentage of phospholipid over the total lipid present per gram of sample lipid.

DETERMINATION OF ACID VALUE

LOW L. K. & NG C. S.

INTRODUCTION

The acid value is a measure of the extent to which the glycerides in the oil have been hydrolysed by lipase action. The glycerides are also hydrolysed with water in the presence of air and possibly bacteria. The decomposition is accelerated by heat and light.



As rancidity is usually accompanied by free fatty acid formation, determination of acid value is often used as a general indication of the condition and edibility of oils.

The acid value is the number of milligrams of potassium hydroxide required to neutralize the free fatty acids in 1.0 g of fat or oil.

I APPARATUS

Microburette (2 ml with 0.01 ml intervals)
Conical flasks (100 ml)
5 ml pipettes

II REAGENTS

a) 0.02N KOH in ethyl alcohol

Weigh 5.6 g of potassium hydroxide, dissolve in distilled water and make up to 100 ml with distilled water (1N solution). Dilute 50 times with ethyl alcohol when required.

b) n-Hexane

c) 1% phenolphthalein or thymolphthalein in ethyl alcohol

Dissolve 1 g of either indicator in 100 ml of ethyl alcohol.

III PROCEDURE

1. Take 0.1-0.3 g of fat sample or A ml of the extract containing 0.1-0.3 g of fat in a 100 ml Erlenmeyer flask.
2. Add [10 — A] ml of n-Hexane and 1-2 drops of indicator.
3. Titrate the solution against 0.02N KOH solution. The end point is reached when pink (phenolphthalein) or blue (thymolphthalein) colour persists for 30 seconds.
4. Carry out a blank test using A ml of C-M Mixture instead of the extract.

IV CALCULATION

$$\text{Acid value (mg/g)} = \frac{56.11 \times 0.02 \times (V_s - V_b) \times F}{W}$$

where V_s = titration volume of sample (ml);

V_b = titration volume of blank (ml);

W = weight of fat in the volume of extract used (g);

F = factor of 0.02 KOH solution, where

$F = \frac{5}{V_f}$: V_f is the volume of 0.02N KOH required to neutralize 5 ml of the 0.02N H_2SO_4 solution.

56.11 = Molecular weight of KOH

0.02 = Concentration of KOH

DETERMINATION OF FREE FATTY ACID (FFA)

LOW L. K. & NG C. S

INTRODUCTION

The FFA figure is usually calculated as oleic acid by dividing the acid value by 2. With most oils the acidity begins to be noticeable to the palate when the FFA calculated as oleic acid is about 0.5-1.5%.

When the FFA cannot be estimated in terms of oleic acid, it can be calculated from the saponification value.

CALCULATION

1. Determination of Free Fatty Acid from Acid Value

$$\begin{aligned}\text{FFA (\%)} &= \text{acid value} \times \frac{\text{mol. wt. of oleic acid}}{\text{mol. wt. of KOH}} \times \frac{100}{1000} \\ &= \text{acid value} \times \frac{282.27}{56.11} \times \frac{1}{10} \\ &= \text{acid value} \times \frac{1}{2}\end{aligned}$$

2. Determination of Free Fatty Acid from Acid Value And Saponification Value Expressed as mg Number per 100 g Meat

$$\text{FFA (mg/100 g)} = \frac{\text{acid value} \times \text{total lipid}}{\text{saponification value}} \times 100$$

* N.B. 2. Personal communication from Dr. Tsukuda.

REFERENCE

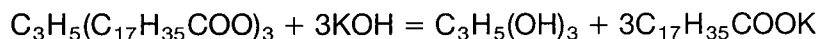
Pearson, D. (1976) In: The chemical analysis of foods (7th Ed.) Churchill Livingstone:493.

DETERMINATION OF SAPONIFICATION VALUE

LOW L. K. & NG C. S.

INTRODUCTION

Saponification is the hydrolysis of esters. Oils and fats are the fatty acid esters of the trihydroxy alcohol, glycerol. The saponification value of an oil is defined as the number of milligrams of potassium hydroxide required to neutralise the fatty acids resulting from the complete hydrolysis of 1 g of the sample. A soap is formed during saponification, for example:



Stearin

Glycerol

Potassium stearate

The esters of the fatty acids of lower molecular weight require more alkali for saponification, so the saponification value is inversely proportional to the mean of the molecular weights of the fatty acids in the glycerides present.

As many oils have somewhat similar values, the saponification value is not, in general, so useful for identification purposes. It is useful for detecting the presence of oil and fats which contain a high proportion of lower fatty acids.

I SAMPLE PREPARATION

The fish lipid is extracted with C-M mixture and the solvent evaporated using the rotary evaporator. About 0.2-0.5 g of lipid is used. The approximate sample sizes to be used for each type of lipid is as follows:-

Type of lipid	Sample size (g)	N/2 KOH solution (ml)
fish lipid	0.2 – 0.5	10
animal fat	0.5 – 1.0	20
plant oil	0.5 – 1.0	20
wax	1.0 – 2.0	20

II APPARATUS

Bulb condensers

Erlenmeyers flasks (50-300 ml depending on sample size)

Water bath

Pipettes

Burette

III REAGENTS

a) 0.5N HCl standard solution

Use 1N HCl standard solution and dilute exactly two times.

b) 0.5N Ethanolic potassium hydroxide standard solution

Weigh 35 g of KOH, dissolve in 20 ml of water, then make up to 1000 ml with 95% (v/v) ethanol or absolute alcohol.

c) Indicator

Phenolphthalein

Take 1 g of phenolphthalein and make up to 100 ml with 95% ethanol.

Methylene blue

Take 0.1 g of methylene blue and make up to 100 ml with water.

IV PROCEDURE

1. Take 0.2 to 0.5 g of lipid in a 50-100 ml Erlenmeyer flask.
2. Add 10 ml of 0.5N ethanolic potassium hydroxide solution and mix.
3. Heat at 80-85°C in a water bath for 30 min.
4. Cool to between 30-40°C liquid state, then titrate with 0.5N HCl standard solution (Add 2-3 drops of indicator).
5. Carry out a blank test (without lipid).

V CALCULATION

$$\text{Saponification value (mg/g)} = \frac{28.05^* \times (A - B) \times F}{S}$$

where S = sample weight

A = titration volume of blank (ml)

B = titration volume of sample (ml)

F = Factor of 0.5N HCl standard solution

* Half of molecular weight of KOH

REFERENCES

Jacobs, M.B. (1973). The chemical analysis of foods and food products (Reprint of 3rd Ed): 380-381.

Pearson, D. (1976). The chemical analysis of foods (7th Ed):491-492.

DETERMINATION OF PEROXIDE VALUE

LOW L. K. & NG C. S.

INTRODUCTION

Unsaturated fish oils are particularly susceptible to oxidation, developing peroxides under poor cold-storage or frozen storage conditions. Peroxides are the precursors of breakdown products that cause rancid flavours in fat. The concentration of peroxides is indicative of oxidation during the early stages of lipid deterioration. This index becomes less reliable during the later stage of deterioration, because peroxide degradation increases.

The peroxide value (POV) is defined as the reactive oxygen contents expressed in terms of milliequivalents (meq) of free iodine per kilogramme of fat. It is determined by titrating iodine liberated from potassium iodide with sodium thiosulphate solution.

Oils with POV well below 10 meq/kg are considered fresh. A rancid taste begins to be noticeable when the POV is between 20 and 40 meq/kg. In interpreting such figures, however, it is necessary to take into account the particular oil or fat involved.

I APPARATUS

Evaporating flasks with stoppers (250 ml capacity)
Rotary evaporator with vacuum pump
Pipettes (1 ml, 5 ml, 10 ml, 20 ml)
Measuring cylinders (25 ml, 100 ml)
Stop watches
Microburette (2 ml)
Burette (50 ml)
Erlenmeyer flasks (100 ml, 200 ml) with stoppers
Balance with at least 0.1 g sensitivity

II REAGENTS

a) 0.01N $\text{Na}_2\text{S}_2\text{O}_3$ solution

Dissolve 25 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in freshly boiled distilled water and make up to 1000 ml. Stand for 2-3 days. Add 10 ml of iso-amylalcohol as stabilizer. When required, dilute 10 times with freshly boiled distilled water. Keep in a dark brown bottle.

Standardization of the $\text{Na}_2\text{S}_2\text{O}_3$ Solution

1. Take 20 ml of 0.01N $\text{K}_2\text{Cr}_2\text{O}_7$ solution in a 250 ml flask with stopper.
2. Add 10 ml of 10% KI solution and 5 ml of 25% H_2SO_4 .
3. Immediately stopper the flask and stand for 5 min in the dark.
4. Add 100 ml of distilled water and shake.
5. Titrate with 0.01N $\text{Na}_2\text{S}_2\text{O}_3$ solution until yellow colour almost disappears.
6. Add 1 ml of 1.5% starch solution as indicator, and continue the titration until dark blue colour disappears.
7. Carry out blank test by using 20 ml of distilled water instead of $\text{K}_2\text{Cr}_2\text{O}_7$ solution.

8. Calculation:

$$F = \frac{20 \times F'}{V_s - V_b}$$

where F = factor of 0.01N $\text{Na}_2\text{S}_3\text{O}_3$ solution

F' = factor of 0.01N $\text{K}_2\text{Cr}_2\text{O}_7$ solution

V_s = titration volume of sample (ml)

V_b = titration volume of blank (ml)

b) Chloroform-acetic acid mixture (2:3).

Mix CHCl_3 and CH_3COOH , 2:3 by volume. Flush with pure, dry nitrogen gas.

c) Saturated KI solution

Dissolve 100 g KI in 70 ml freshly boiled distilled water. Keep the solution with precipitated crystals in a dark brown bottle.

d) 1.5% starch solution

Weigh 1.5 g of soluble starch in a beaker. Add 100 ml of distilled water. Heat and boil for 30 sec.

e) 0.01N $\text{K}_2\text{Cr}_2\text{O}_7$ standard solution

Weigh 4.9035 g of $\text{K}_2\text{Cr}_2\text{O}_7$ which had been dried at 100-110°C for 3-4 hr. Dissolve it in distilled water and make up to 1000 ml. When required, dilute 10 times with distilled water.

Factor $F' = \frac{4.9035}{W}$; where W is the actual weight of $\text{K}_2\text{Cr}_2\text{O}_7$ used.

f) 10% (w/v) KI solution

Dissolve 10 g of KI in distilled water and make up to 100 ml

g) 25% H_2SO_4 solution

Mix 25 g (13.5 ml) of concentrated H_2SO_4 and 75 ml of distilled water.

III PROCEDURE

1. Take about 0.3 g of fat sample or A ml of the extract containing about 0.3 g of fat into a 250 ml flask with stopper.
2. Remove solvent using rotary evaporator under reduced pressure at 40°C (water-bath temperature).
3. Add 10 ml of CHCl_3 - CH_3COOH mixture and dissolve the fats by shaking.
4. Add 1 ml of saturated KI solution.
5. Immediately stopper and stand in the dark for 5 min.
6. Add 20 ml of distilled water, then shake.

7. Titrate the liberated iodine with 0.01N $\text{Na}_2\text{S}_2\text{O}_3$ solution until light yellow colour. Add 1 ml of 1.5% starch solution as indicator and titrate till colourless.
8. Carry out blank test in the same manner without fats.

IV CALCULATION

$$\text{POV (meq per 1000 g)} = \frac{(V_s - V_b) \times F \times N \times 1000}{W} = \frac{(V_s - V_b) \times F \times 1 \times 1000}{W \times 100}$$

$$= \frac{(V_s - V_b) \times F \times 10}{W}$$

where V_s = titration volume of sample (ml);

V_b = titration volume of blank (ml);

F = factor of 0.01N $\text{Na}_2\text{S}_2\text{O}_3$ solution;

W = weight of fat in volume of extract used (g);

N = normality of $\text{Na}_2\text{S}_2\text{O}_3$ solution (in this case N/100)

$$\text{POV (millimoles per 1000 g)} = \frac{0.5 \times (V_s - V_b) \times N \times 1000}{W}$$

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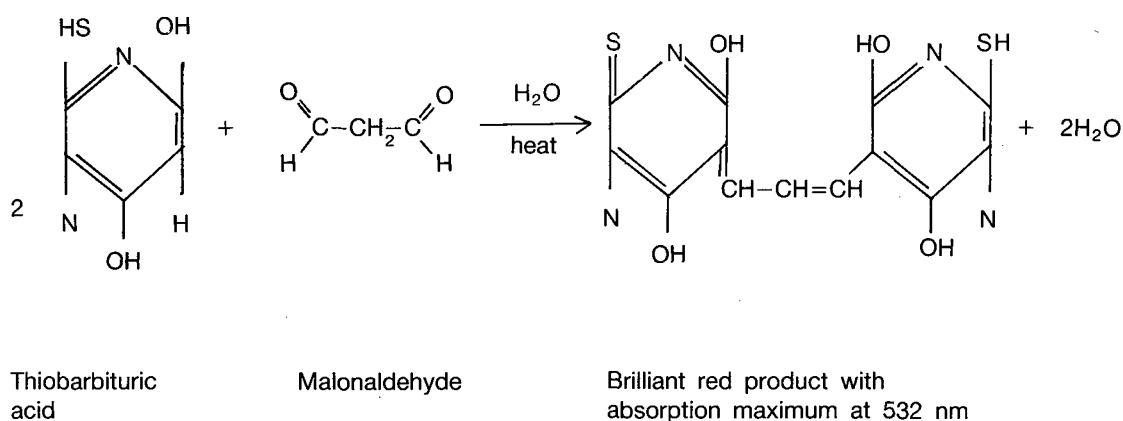
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DETERMINATION OF THIOBARBITURIC ACID (TBA) NUMBER

LOW L. K. & NG C. S.

INTRODUCTION

In autoxidised lipids, most malonaldehyde does not appear in the free state but seems to exist mainly in a weakly-bound state and is released when the system is heated with a mild acid. The TBA test measures malonaldehyde in autoxidising systems. The basic reaction can be represented as follows:-



It is a sensitive test and can be correlated with the development of off-odours and flavours. It is especially well-suited for the detection of oxidative rancidity in lipids which are unsaturated and contain 3 or more double bonds. The TBA number is defined as the number of milligrammes of malonaldehyde per kilogramme of sample.

The results are expressed as malonaldehyde or 1,1,3,3,-tetra-ethoxypropane, which yields malonaldehyde by acid hydrolysis.

I APPARATUS

- Spectrophotometer ($\lambda = 532 \text{ nm}$)
- Test tubes with screw caps
- Hot water bath (boiling water)
- Pipettes (3,5,10,25 ml)
- Rotary evaporator with vacuum pump and water bath.
- Vortex mixer
- Test tube basket
- Glass centrifuge tubes
- Centrifuge
- Source of N₂ gas

II REAGENTS

a) TBA solution

Dissolve 1 g of TBA in 75 ml of 0.1N NaOH. Dilute to 100 ml with distilled water (can be kept for more than 1 month in refrigerator).

b) Tri-chloroacetic acid (TCA) solution

Mix 50 ml of 25% TCA solution, 30 ml of 0.6 N HCl and 420 ml of distilled water.

c) Antioxidant solution

Dissolve 0.3 g BHA (butylated hydroxyanisole) in 5.4 g propylene glycol. Dissolve 0.3 g of BHT (butylated hydroxytoluene) in 4.0 g of warm Tween 20. Mix the two solutions.

d) Chloroform (Analytical grade)

III PROCEDURE

1. Take 0.2-0.4 g of fat sample or A ml of the extract containing 0.2-0.4 g of fat in a test tube with screw caps.
2. Add 3 drops of antioxidant solution.
3. Remove the solvent using the rotary evaporator under reduced pressure at 35-40°C (water-bath temperature).
4. Add 3 ml of TBA solution and 17 ml of TCA solution.
5. Flush N₂ gas into the test tube and immediately stopper.
6. Heat at 100°C in a boiling water-bath for 30 min till the colour appears.
7. Cool to room temperature in tap water.
8. Add about 5 ml of chloroform and mix for a few seconds with a Vortex mixer.
9. Transfer about 15 ml of the colour solution to a GLASS centrifuging tube.
10. Centrifuge for 10 min at 3,000 rpm.
11. If the aqueous solution is not clear, centrifuge again at 10,000 rpm for 10 min.
12. Transfer a part of the clear aqueous solution and read absorbance at 532 nm.
13. Blank test should be carried out in the same manner without fats.

IV CALCULATION

$$\text{TBA No. (mg malonaldehyde/kg fat)} = \frac{\text{Abs.} \times F \times 0.2}{W}$$

where Abs = absorbance at 532 nm

W = weight of fat in volume of extract (g)

F = factor = 46.

N.B. The absorbance of a 1 g sample (in 100 ml reagent) multiplied by the factor 46 is the TBA number, or the milligram of malonaldehyde per 1000 g (1 kg) of sample (Sinnhuber et. al., 1958). As the amount of reagent used is only 20 ml, the results must be multiplied by 0.2 to give the absorbance of the sample in 100 ml reagent as specified by the definition.

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DETERMINATION OF METHYL ESTERS OF FATTY ACIDS BY GAS CHROMATOGRAPHIC METHOD

LOW L. K. & NG C. S.

INTRODUCTION

Methyl esters of fatty acids from fish and animal fats having 8-24 carbon atoms are separated and determined by gas chromatography. This method is not applicable for epoxy, oxidized, or polymerized fatty acids.

I SAMPLE PREPARATION

The fish oils used are first esterified by the boron trifluoride method.

II APPARATUS

The following conditions are for use with flame ionization detector (FID).

a) Gas chromatograph (Shimadzu GC-9A).

With minimum dead space in injection system, which is maintained at 20-50°C higher than column temperature. The column temperature should be maintained within $\pm 1^\circ\text{C}$ to at least 220°C. If programmed heated, dual columns are used.

b) Columns

1.600 mm \times 3 mm (i.d.) glass spiral columns.
Maximum aging temperature = 210°C.

c) Packing

Chromosorb W, (Acid-washed and silanized diatomaceous earth) mesh 60-80.
Coated with 5-20% diethylene glycol succinate (DEGS).

Condition column while disconnected from detector at 200°C with current of nitrogen gas at 60 ml/min for 16-18 hours.

d) Microliter syringes

Maximum volume 10 ul, graduated to 0.1 ul (Hamilton 701-N).

e) Recorder (Chromatopac C-RIB, Shimadzu)

Method of recording : Thermal printer-plotter

Chart width : 21 cm

Chart speed : 0-50 mm/min

Pen speed : 0.89 sec/full scale;
57 characters/sec.

Span : 1 mv (automatic attenuation by time programming). With attenuation switch to change range.

Integration sensitivity : 1 μV . sec (= 1 digit)

Linearity : $\pm 0.1\%$ or better.

III REAGENTS

a) Carrier gas

Purified grade nitrogen gas with oxygen <4.0 ppm, moisture <2.5 upm, hydrocarbons <1.0 ppm.

b) Other gas

Purified grade air with oxygen $21 \pm 1\%$, moisture <3.0 ppm, hydrocarbons <5.0 ppm.

Purified grade hydrogen with oxygen <3 ppm, moisture hydrocarbons <1 ppm.

c) Reference standards

Known mixtures of methyl esters of fatty acids or methyl esters of oil of known composition, preferably similar to that of material to be analyzed.

IV OPERATING CONDITIONS

a) Isothermal program

Column initial temperature = 180°C.

Column initial time = 0.0 min.

Column final temperature = 180°C.

Column final time = 500 mins.

Injection port temp. = 200°C.

Range = 10^2 .

b) Gas flow rate and pressure

Hydrogen gas : 0.6 kg/cm²

Purified air : 0.5 kg/cm²

Nitrogen gas : 60 ml/min.

c) Recorder conditions

Width : 5 sec

Slope : 300 uV/min

Drift : 0 uV/min

Min Area : 10 count

T-DBL : 0 min

Lock : 1.3 min

Stop time : 1000 min

Attenuation : 4 mV/full scale

Speed : 5 mm/min

Method : 41

Sample weight : 100 (default value)

Internal standard weight : 1 (default value)

V PROCEDURE

1. With recorder showing stable baseline, inject 0.1-0.3 ul 5-10% n-hexane solution of methyl esters.
2. If trace components are desired, the sample may be increased by <10 times.
3. Pierce septum of inlet port and quickly discharge sample.
4. Withdraw needle and note on chart small peak due to air or solvent, marking start reference point.
5. Press 'Start' on both GC-9A and recorder.
6. Adjust sample size so that major peak is not attenuated >8 times, preferably less.
7. Change setting of attenuator as necessary to keep peaks on chart paper. Mark attenuator setting on chart.

VI IDENTIFICATION

1. Analyze reference standard mixtures under same operating conditions as for sample.
2. Measure retention time (S) for known esters by measuring the distances from start point.
3. Plot log S as a function of number of C atoms of acids. Under isothermal conditions, graphs of straight chain esters of same degree of unsaturation should be straight lines, approximately parallel.
4. Identify peaks from sample from these graphs, interpolating if necessary.
5. Avoid conditions which permit "masked peaks" which are not sufficiently resolved.

N.B. Esters appear in order of increasing number of C atoms and of increasing unsaturation for same number of C atoms. C₁₆ ester is ahead of the C₁₈ ester and C₁₈ Me esters appear in order: stearate (18:0), oleate (18:1), linoleate (18:2), and linolenate (18:3). C₂₀ saturated ester (arachidic, 20:0) usually appears before 18:3 ester, but may be reversed on some columns, or positions may change with column used.

VII CALCULATIONS

Method 41 of Chromatopac C-R1B is a normalization method. Use method of normalization, which assumes all components of sample are represented on chromatogram, so that sum of areas under peaks represent 100% of constituents (total elution). As the Chromatopac C-R1B is equipped with integrator, the figures shown can be used directly for calculation. Report results to following significant figures, with 1 figure beyond decimal point in all cases: 3 for >10%, 2 for 1-10% and 1 for <1%.

REFERENCES

Official methods of analysis of the Association of Official Analytical Chemists (13th Ed.), 1980 :447-449.

Through personal communication with Mr Kinumaki and Dr Tsukuda.

DETERMINATION OF THE DEGREE OF LIPID OXIDATION BY GAS CHROMATOGRAPHY

LOW L. K. & NG C. S.

INTRODUCTION

Fish oils, in general, consist predominantly of triglycerides and phospholipids, and minor proportions of free fatty acids, vitamins, etc.

Fish oils contain approximately 15-40% (on the weight of total fatty acids) of saturated fatty acids. The main saturated fatty acid is palmitic acid $C_{16}H_{32}O_2$.

Polyenoic acids of the C_{16-24} series occurs in fish oils. The acids of the C_{20} and C_{22} series are the most abundant. An eicosapentaenoic acid, $C_{20:5}$, and a docosahexaenoic acid, $C_{22:6}$ occurs as a major component in most marine oils. It has been suggested that in the docosahexaenoic acid the double bonds are either in the 4-5, 8-9, 12-13, 15-16, 18-19 and 21-22 or the 4-5, 8-9, 11-12, 14-15, 17-18 and 20-21 position.

Since both palmitic acid and docosahexaenoic acid are abundant in fish oils, we can use them to measure the degree of lipid oxidation that has occurred during frozen storage.

I SAMPLE PREPARATION

The sample is prepared by the boron trifluoride method.

II PROCEDURE

The procedure is the same as that for the determination of Methyl esters of fatty acids by gas chromatography.

III CALCULATION

The index of oxidation, I, is defined as:-

$$I = 1 - \frac{x'/y'}{x/y}$$

where x' = % of $C_{22:6}$ of stored sample

y' = % of $C_{16:0}$ of stored sample

x = % of $C_{22:6}$ of fresh sample

y = % of $C_{16:0}$ of fresh sample.

Hence, by measuring the ratio of $C_{22:6}$ and $C_{16:0}$ at the initial and subsequent stages, we can use the index of oxidation as a measure of the degree of docosahexaenoic acid.

REFERENCE

By personal communication with Mr Kinumaki (1983).

PREPARATION OF METHYL ESTERS BY BORON TRIFLUORIDE METHOD

LOW L. K. & NG C. S.

INTRODUCTION

Glycerides and phospholipids are saponified, and fatty acids are liberated and esterified in presence of BF_3 catalyst for further analysis by gas liquid chromatography (GLC).

This method is applicable to common animal and vegetable oils and fats, and fatty acids. Unsaponifiables are not removed, and if present in large amounts, may interfere with subsequent analyses.

This method is not suitable for preparation of methyl esters of fatty acids containing major amounts of epoxy, hydroperoxy, formyl, oxo, cyclopropyl, and cyclo-propenyl groups, and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.

I SAMPLE PREPARATION

Precise weighing is not required. Sample size need be known only to determine size of flask and amounts of reagents, according to following table:

Sample (mg)	Flask (ml)	0.5N NaOH (ml)	BF_3 Reagent (ml)
<50	10	1-2	2
50-75	10	2	3
75-100	10	3	4
100-250	50	4	5
250-500	50	6	7
500-750	100	8	9
750-1000	100	10	12

N.B. Though a 350 mg sample is preferred, it may be difficult to obtain this amount of oil from low fat sample. Hence, 50-100 mg sample could be used.

II APPARATUS

Reaction flasks: 50 and 125 ml flasks with outer joints.

Condenser: Water-cooled, reflux, with 20-30 cm jacket and inner joint.

III REAGENTS

a) Boron trifluoride reagent — 125 g BF_3 /1 MeOH

Available commercially as boron trifluoride methanol complex with about 14% BF_3 . This reagent is stable for 2 years.

(Caution: Remove BF_3 vapours with effective fume removal device. Avoid contact with skin, eyes, and respiratory tract).

- b) Methanolic sodium hydroxide solution (0.5N)
Dissolve 2 g NaOH in 100 ml MeOH containing <0.5% H₂O. White precipitate of Na₂CO₃ forming on long standing may be ignored.
- c) n-Hexane pure, as determined by GLC.
- d) Nitrogen gas containing <5 mg oxygen/kg.
- e) Methyl red solution — 0.1% in 60% ethyl alcohol.

IV PROCEDURE

1. Add sample (ca. 350 mg preferred for GLC) to flask and then add 0.5N methanolic NaOH solution and anti bubbling stone.
2. Attach condenser, and reflux until fat globules disappear (usually 5-10 mins at 85°C ± 5°C).
3. Add BF₃ solution from bulb or automatic pipette through condenser and continue boiling for 2 min (at 90-100°C).
4. Add 2 ml n-hexane through condenser and boil for 1 min.
5. Remove heat, then condenser, and add several ml saturated NaCl solution.
6. Rotate flask gently several times.
7. Add additional saturated NaCl solution to float the n-hexane solution into neck of flask.
8. Transfer about 1 ml upper n-hexane solution into test tube and add small amount anhydrous Na₂SO₄ to remove H₂O. If necessary, dilute solution to concentration of 5-10% for GLC.

N.B. BF₃ is very toxic. Work in hood. Wash all glassware immediately after use. If fatty acids containing >2 double bonds are present, remove air from MeOH and flask by passing in stream of nitrogen gas for a few min. Methyl esters should be analysed as soon as possible. If necessary, n-hexane solution may be kept under N₂ in refrigerator.

REFERENCE

Official methods of analysis of the Association of Official Analytical Chemists (13th Ed.), 1980: 447.

ANALYSIS OF ADDITIVES

DETECTION OF POLYPHOSPHATES

NG M. C.

INTRODUCTION

Polyphosphates (food grade) are commonly used in the production of fish jelly products. The addition of polyphosphates help to smoothen the ground fish paste and increase the gel strength of the final fish jelly products. The commercial polyphosphate is a mixture of sodium pyrophosphate and sodium tripolyphosphate (sodium triphosphate) of 1:1.

The principle of detection involves extracting the polyphosphates present in the sample with trichloroacetic acid, separating the phosphates by thin layer chromatography (TLC) and finally detecting the phosphates by spraying with colour reagent.

This method is also applicable to meat and meat products.

I PREPARATION OF SAMPLE SOLUTION

1. Mix well 50 g minced sample with 15 ml warm water using a spatula.
2. Add 10 g trichloroacetic acid and mix.
3. Store in the refrigerator for one hour to allow separation.
4. Filter the separated solution.
5. Collect the clear solution for chromatographic separation.

- N.B.
1. If the filtrate is turbid, add an equal volume of diethylether and shake. Remove the ether layer with small pipette and add an equal volume of 95% ethanol to the water phase. Shake for a minute. Allow the mixture to stand for a few minutes before filter.
 2. Use the sample solution on the day of preparation. Store it chilled if the chromatography analysis cannot be done immediately.

II REAGENTS

a) Preparation of TLC plates

1. Weigh 15 g of cellulose powder, Whatman thin layer chromedia CC41, into a beaker.
2. Add 30 ml distilled water and mix well with glass rod.
3. Apply this slurry onto glass plates (20 × 20 cm) with the spreading device to obtain a layer of 0.25 mm in thickness.
4. Air-dry the plates undisturbed for 60 min at room temperature.
5. Heat them finally for 10 min at 100°C.
6. Store the plates in a desiccator.

b) Preparation of developing solvent of TLC

Isopropyl alcohol, 140 ml.

Trichloroacetic acid (TCA), 40 ml of 13.5% solution.

Ammonia, 0.6 ml (SG 0.91).

Mix these solutions. If the solvent is not to be used on the same day of preparation, keep it in a tightly closed bottle.

c) Preparation of spray reagents

Spray reagent I

7.5% ammonium molybdate solution

Concentrated nitric acid (analytical grade)

Mix equal volumes (1:1). Prepare the reagent on the day of use.

Spray reagent II

195 ml of 15% sodium metabisulphite solution

5 ml of 20% sodium sulphite

0.5 g 1-amino-2-naphthol-4-sulphonic acid

Mix and store the reagent in a closed brown bottle in the refrigerator.

Spraying with reagent II is not an absolute necessity. However the intense blue spots produced by these reagents improve the detection considerably.

d) Preparation of polyphosphate standards

Sodium dihydrogen orthophosphate monohydrate, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

Tetrasodium diphosphate decahydrate, (sodium pyrophosphate) $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$

Pentasodium triphosphate, (sodium tripolyphosphate) $\text{Na}_5\text{P}_3\text{O}_{10}$

Sodium hexametaphosphate (NaPO_3)₆, Gramham's salt

Dissolve 200 to 300 mg of each of the standards in 100 ml of distilled water. These standard solutions can be kept at 40°C for 4 weeks.

III INSTRUMENTS/APPARATUS

Oven (Temp 30-200°C)

Glass chamber tank with cover

TLC plates (20 × 20 cm)

Glass tips

IV CHROMATOGRAPHIC SEPARATION OF POLYPHOSPHATES

1. Fill a paper-lined developing chamber with the developing solvent up to a layer of 0.5 to 1 cm over the bottom. Close the chamber immediately with a tightly fitting lid.
2. Allow to stand for at least 30 min at ambient temperature in order to saturate the chamber atmosphere with the vapour of the developing solvent. This system should be protected from sunlight and draught.
3. Apply 5 µl of the sample solution on to the TLC plate at about 2 cm from the bottom end of the plate. Keep the spots small by applying 1 µl at a time. Use a cold air stream for drying.

N.B. Hot air should be avoided because of danger of hydrolysis of polyphosphates.

4. In the same way, apply 5 μ l of the standard solutions on the plate at an interval of 1.5-2cm, but at exactly the same distance from the bottom end of the plate.
5. Remove the lid from the chamber and quickly but carefully place and dip the spotted plate in the developing solvent in the chamber. Replace the lid immediately.
6. Develop the plate until the solvent front has ascended about 10 cm.
7. Remove the developed plate from the chamber, mark the position of the solvent front with pencil, and allow to dry at ambient temperature for 30 min or, alternatively, in a stream of air.
8. Place the plate under a fume hood and spray the plate lightly but uniformly with spray reagent I.
9. Air-dry the plate under a fume hood. Subsequently heat for 30 min at 100°C in the oven in order to remove the last traces of nitric acid and decompose polyphosphate.
10. Remove the plate from the oven and verify the absence of the pungent smell of nitric acid. Yellow spots will slightly appear in the presence of phosphate.
11. Allow the plate to cool to room temperature and then replace it under the fume hood. Spray the plate lightly but uniformly with spray reagent II. Blue spots will appear immediately on phosphate areas.
12. Measure the migrating distance from the spotting position to the center of the phosphate spot (A) and also to the solvent front (B).
13. Calculate the ratio A/B (Rf value).

V INTERPRETATION OF RESULTS

Compare the migrating distance of the phosphate spots from the sample with those of the standard solution. The Rf values of some phosphates are:-

Orthophosphate	0.80 – 0.90
Diphosphate (Pyrophosphate)	0.40 – 0.45
Triphosphate	0.20 – 0.23
Hexametapolyphosphate	0.00

These values are changeable according to developing conditions. It is advisable to analyse sample solution together with the standard solution.

REFERENCE

International Standard Orgn. ISO/TC 34/Sc 6/WG 2. The Netherlands.

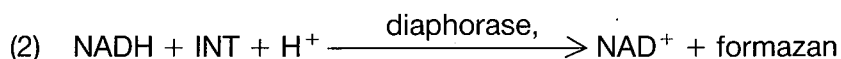
DETERMINATION OF MONOSODIUM L-GLUTAMATE (MSG) CONTENT IN FISH JELLY PRODUCTS

NG M. C.

INTRODUCTION

Monosodium L-glutamate is usually used as a taste enhancer in the production of fish jelly products.

The presence of MSG present in fish jelly products can be determined by enzymatic reaction. In the presence of the enzyme, glutamate dehydrogenase (GIDH), the L-glutamic acid present is deaminated oxidatively by nicotinamide-adenine dinucleotide (NAD^+) to α -ketoglutarate (see reaction 1). In the reaction catalyzed by diaphorase, the NADH formed converts idonitro tetrazolium chloride (INT) to a formazan which is measured in the visible range at 492 nm (see reaction 2).



The equilibrium of reaction (1) lies far on the side of glutamate. By trapping the NADH formed with INT (2), the equilibrium is displaced in favour of α -ketoglutarate.

I SAMPLE PREPARATION

Collect fish jelly products sample (≤ 100 g) and pass 2-3 times through food mincer, or chop very finely and mix thoroughly.

II REAGENTS

a) Preparation of standard L-glutamic acid solution

1. Dissolve 100 mg L-glutamic acid with 25 ml distilled water.
2. Adjust to pH 7.0 with 2N KOH.
3. Make up to 100 ml with distilled water.
4. Pipette 10 ml solution into a volumetric flask.
5. Make up to 250 ml with distilled water.

This is the standard solution which contains 40 mg L-glutamic acid/litre.

b) Dilution of standard L-glutamic acid (40 mg/litre)

0.2 ml of the above solution was pipetted into 1.8 ml of distilled water. This contains 8.0 μg L-glutamic acid.

c) 1 M perchloric acid, HClO_4

Dissolve 143.51 g perchloric acid in 1 litre distilled water.

d) 2N KOH

Dissolve 11.2 g KOH in distilled water and make up to 100 ml in a volumetric flask.

e) Treated sand

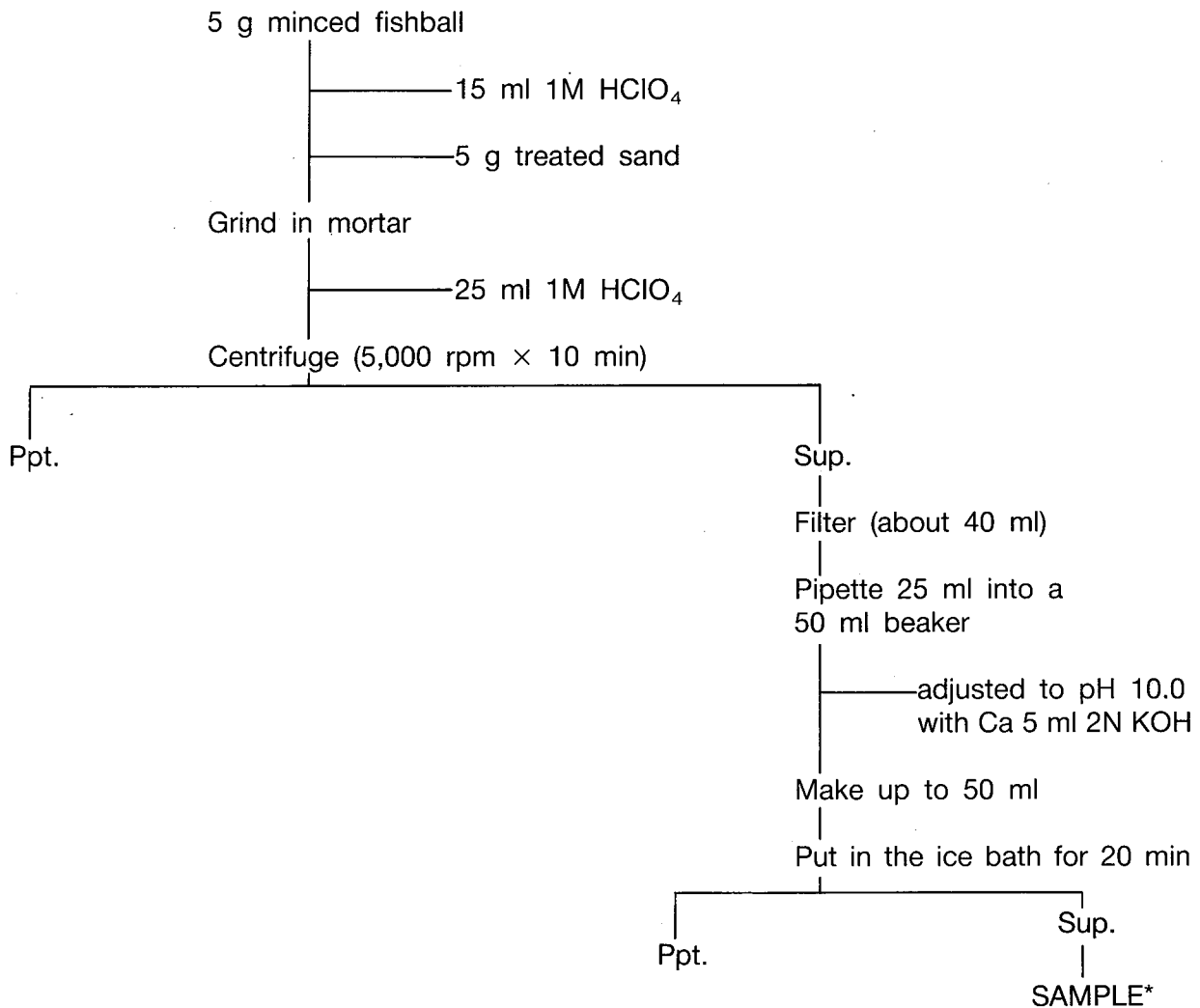
f) Enzyme solution (1 set contains 4 enzyme solutions named solution 1, 2, 3 and 4)

N.B. This enzyme solution can be purchased from:

Food Analysis
Boehringer
Mannheim GmbH
Mannheim, WEST GERMANY

III PROCEDURE

A. PREPARATION OF L-GLUTAMIC ACID SAMPLE SOLUTION



* Supernatant sample is to be diluted if too concentrated.

B. PREPARATION OF SUPERNATANT SAMPLE IN ENZYME SOLUTION

Pipette the enzyme soln and sample soln into test-tubes according to the following table (duplicate) and mix. Add soln 4 and mix again. Stand for 30 min at 25°C water bath. Read the optical densities of the soln at 492 nm.

	Blank (ml)	Standard (ml)	Sample (ml)
Enzyme Soln. 1	0.60	0.60	0.60
Enzyme Soln. 2	0.20	0.20	0.20
Enzyme Soln. 3	0.20	0.20	0.20
Distilled Water	2.00	—	—
Std. Soln.	—	2.00	—
Sample Soln.	—	—	2.00
Soln. 4	0.03	0.03	0.03

IV CALCULATION

To calculate the L-glutamic acid in fishball

$$O.D._{sample} = O.D._{sample} - O.D._{blank}$$

$$\text{L-glutamic acid (mg/100 g)} = \frac{O.D._{sample}}{O.D._{standard}} \times 8 \times 10^{-3} \times D \times \frac{50}{2} \times \frac{[40 + 5M]}{25} \times \frac{100}{W}$$

where $O.D._{sample}$ = optical density of sample

where $O.D._{standard}$ = optical density of standard

8×10^{-3} = standard solution used in mg L-glutamic acid

M = moisture of sample

D = Dilution ratio

W = Wt. of sample (5 g)

$\frac{50}{2}$ = $\frac{\text{Make-up volume}}{\text{Sample of volume of reaction}}$

[40 + 5 m] = Volume of perchloric acid solution used.

REFERENCE

Colorimetric method for the determination of L-glutamic acid in foodstuffs. Cat. No. 139092.
Available from Boehringer Mannheim, GMBH. West Germany.

DETERMINATION OF SUGAR (SUCROSE) BY SOMOGYI'S METHOD

NG M. C.

INTRODUCTION

Sugar is widely used in the manufacturing of food as taste and flavour enhancer. It is also capable of inhibiting, retarding or arresting the process of fermentation, acidification or any other decomposition of food. Thus sugar is also used as a preservative.

The sugar extracted from the sample is converted into glucose with diluted HCl. The glucose content is determined by Somogyi's method. The content of sugar is then back calculated from glucose content. The recovery of sugar was found to be 91% and the reproducibility was satisfactory.

I PREPARATION OF SAMPLE

Take a representative sample of the product, pass it through the mincer, transfer into a labelled polyethylene bag and keep it chill.

II REAGENTS

a) Somogyi solution A

Anhydrous Na_2CO_3	25 g
— KNa - tartarate.4 H_2O	25 g
— 500 ml H_2O	
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 g
	— 200 ml H_2O
— add drop by drop while stirring	
— NaHCO_3	20 g
— KI	5 g
— KIO_3 (1M = 6N)	0.892 g

Make up to 1 litre with distilled H_2O .

b) Somogyi solution B

KI	25g
— $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ (potassium oxalate)	28 g

Make up to 1 litre with distilled H_2O .

- c) 0.1N HCl
Dilute 10 ml 1N HCl in 100 ml volumetric flask.
- d) 0.1N NaOH
Weigh 1 g NaOH, dissolve in distilled water and make up to 250 ml volumetric flask.
- e) 2N H₂SO₄
Conc. H₂SO₄ 60 ml dilute to 1 litre.
- f) Starch indicator
Weigh 1 g soluble starch and 0.1 g salicylic acid, dissolve both in 99 ml distilled water. Boil to dissolve the starch.
- g) Dried KIO₃
Weigh about 2 g of KIO₃, dried in the oven at 120°C for 1 hr.
- h) 2.5% KI
Weigh 2.5 g KI, dissolve in 97.5 ml of distilled water.
- i) 0.05N Na₂S₂O₃ solution
Sodium thiosulphate Na₂S₂O₃.5H₂O, 13 g.
Na₂CO₃ 0.2 g
Make up to 1 litre with decarbonated H₂O
- j) 0.005N Na₂S₂O₃
Dilute 100 ml of 0.05N Na₂S₂O₃ to 1 litre.

DETERMINATION OF FACTOR (F) OF 0.05N Na₂S₂O₃

Weigh about 1.5 g dried KIO₃ accurately

Make up to 500 ml with H₂O in volumetric flask

Pipette 10 solution

Blank

Pipette H₂O, 10 ml

Add 2.5% KI, 20 ml

Add 2N H₂SO₄, 20 ml

Titrate with 0.05N Na₂S₂O₃ with starch indicator

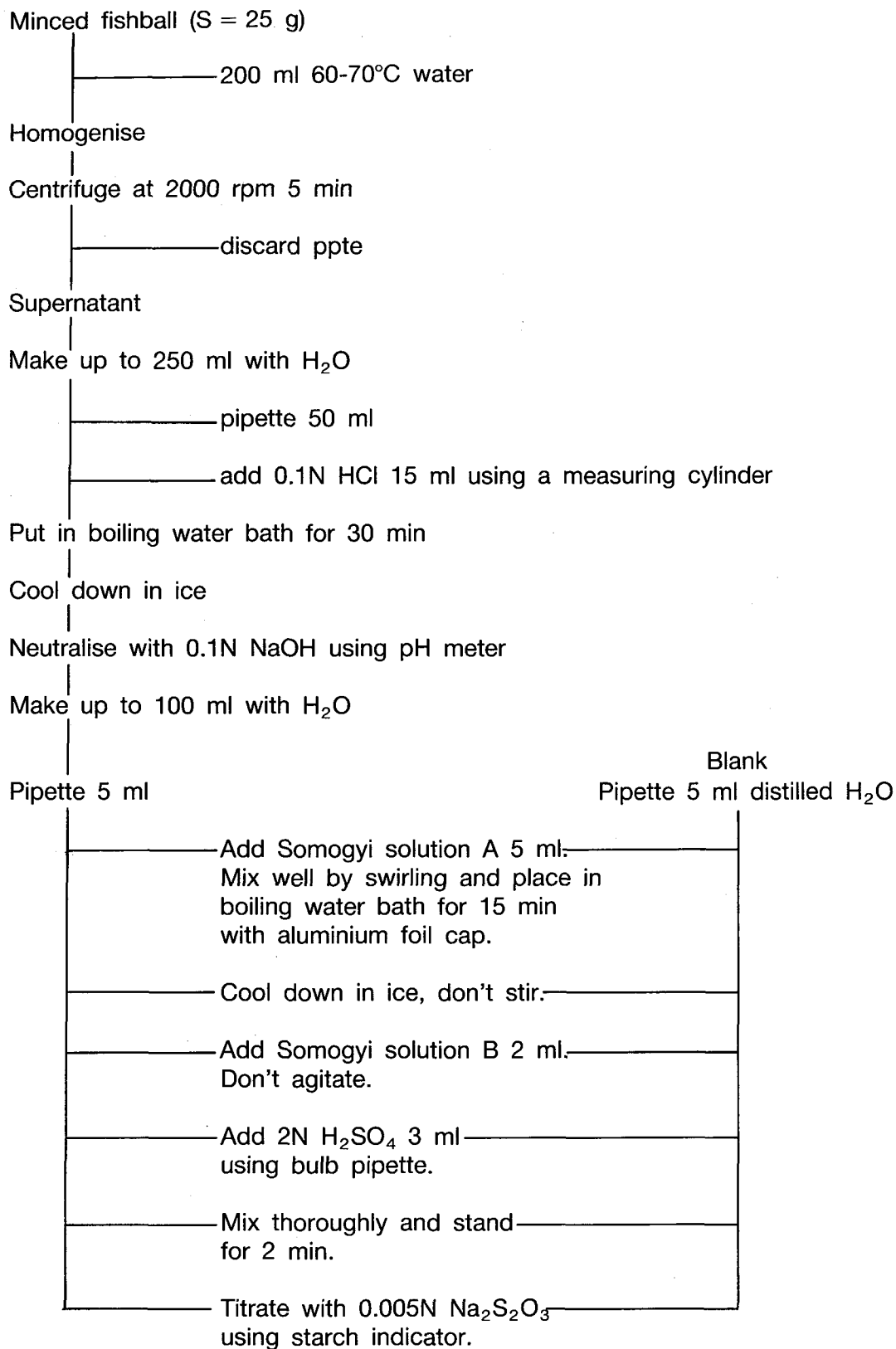
$$\text{Factor, } F = \text{wt. of KIO}_3 \times \frac{10}{500} \times \frac{1}{0.0017835} \times \frac{1}{(B - A)}$$

0.0017835: conversion factor of 1 ml 0.05N Na₂S₂O₃ to KIO₃ (g)

A: titration volume of KIO₃ solution (ml)

B: titration volume of blank (ml)

III PROCEDURE



IV CALCULATION

$$\begin{aligned}\text{Sucrose (\%)} &= 0.0001449 (B - A) F \times \frac{100}{5} \times \frac{250}{50} \times 0.95 \times \frac{1}{S} \times 100 \\ &= 13.7655 (B - A) F \times \frac{1}{S}\end{aligned}$$

where 0.0001449 : 1 ml 0.005N $\text{Na}_2\text{S}_2\text{O}_3$ = 0.0001449 g glucose

- A : Sample titration volume (ml)
B : Blank titration volume (ml)
F : Correction factor of $\text{Na}_2\text{S}_2\text{O}_3$
S : Sample weight
0.95 : Conversion factor of glucose to sucrose

REFERENCES

David Pearson. The chemical analysis of food. 7th Ed: 128.

Official methods of analysis of the Association of Official Analytical Chemists. 13th Ed. 1980.
1980: 226, 14.114(d)
877, 50.037, 50.038
515, 31.052, 31.053

DETERMINATION OF STARCH

NG M. C.

INTRODUCTION

Starch is commonly used in the production of fish jelly products. Its main functions are:-

- (1) as an extender to increase the bulk of production
- (2) as a binding agent

Starch is readily convertible into glucose by hydrolysis either by an enzyme such as diastase or by heating with an acid and its estimation usually depends upon this reason.

The hydrolysed glucose is determined by Somogyi method (D-3). The content of starch in the sample is then back calculated from the content of the glucose.

For the confirmation of presence of starch in fish jelly product, the sample is first heated with water. The starch granules will swell up and burst at about 70°C, resulting in a sticky feel. When iodine solution is added, a characteristic blue colour is developed, due to starch iodide, which is decomposed on heating, but is reformed on cooling.

I SAMPLE PREPARATION

Collect fish jelly product sample (≤ 100 g) and pass 2-3 times through food mincer, or chop very finely and mix thoroughly.

II REAGENTS

- a) 8% potassium hydroxide (KOH) in alcohol.
Dissolve 8 g KOH in 4 ml of distilled water completely and mix with 96 ml absolute alcohol. (Potassium hydroxide must be dissolved in water first as it is insoluble in ethanol).
- b) 50% ethanol.
- c) 2.5% hydrochloric acid (HCl)
14 ml conc. HCl in 186 ml distilled water (Conc. HCl is 35%).
- d) 15% Sodium hydroxide (NaOH)
15 g NaOH (Technical grade) dissolve in 85 ml distilled water.

e) Somogyi solution A

Sodium potassium tartrate (K Na C ₄ H ₄ O ₆ ·4H ₂ O)	90 g	CuSO ₄ ·5H ₂ O	30 g
Trisodium phosphate Na ₃ PO ₄ ·12H ₂ O	225 g	H ₂ O	500 ml
H ₂ O	700 ml		
		KIO ₃	3.5 g
		H ₂ O	100 ml

Make up to 2 litres with distilled water

Keep at room temperature.

f) Somogyi solution B

Potassium oxalate (K ₂ C ₂ O ₄ ·H ₂ O)	90 g
KI	40 g

Make up to 1 litre with distilled water.

Keep at room temperature.

g) 2N H₂SO₄

Conc. H₂SO₄ (60 ml) dilute to 1 litre with distilled water. (Conc. H₂SO₄, 36N, is 95-97 wt %).

h) Starch indicator

Dissolve 1 g soluble starch and 0.1 g salicylic acid in 99 ml distilled water. Boil to dissolve the suspension.

i) Dried KIO₃

Weigh about 2 g KIO₃ and dry in oven at 120°C for 1 hour.

j) 2.5% KI

Dissolve 2.5 g KI in distilled water and make up to 100 ml.

k) 0.05N Na₂S₂O₃ solution

Sodium thiosulphate Na₂S₂O₃.5H₂O 13g

Na₂CO₃ 0.3 g

Make up to 1 litre with decarbonated distilled water.

Determination of factor, F, of 0.05N Na₂S₂O₃

Weigh 1.5 g dried KIO₃ accurately and dissolve in 500 ml distilled water in volumetric flask.

To 10 ml of KIO₃ solution and 10 ml distilled water (BLANK) each add 2.5% KI (20 ml) and 2N H₂SO₄ (20 ml).

Titrate with 0.05N Na₂S₂O₃ using starch indicator.

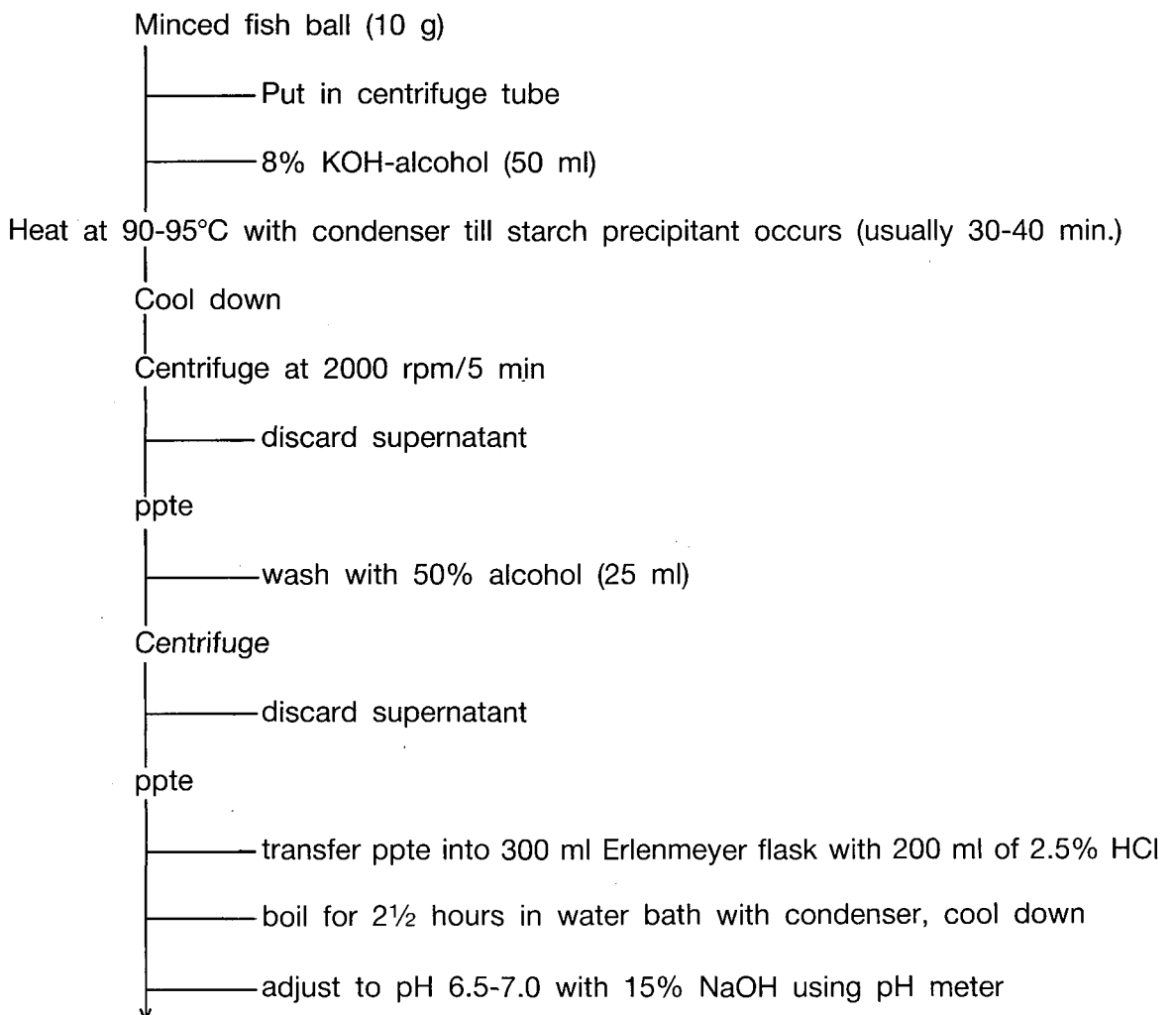
$$\text{Factor, } F = \text{Wt. of KIO}_3 \times \frac{10}{500} \times \frac{1}{0.0017835} \times \frac{1}{(B - A)}$$

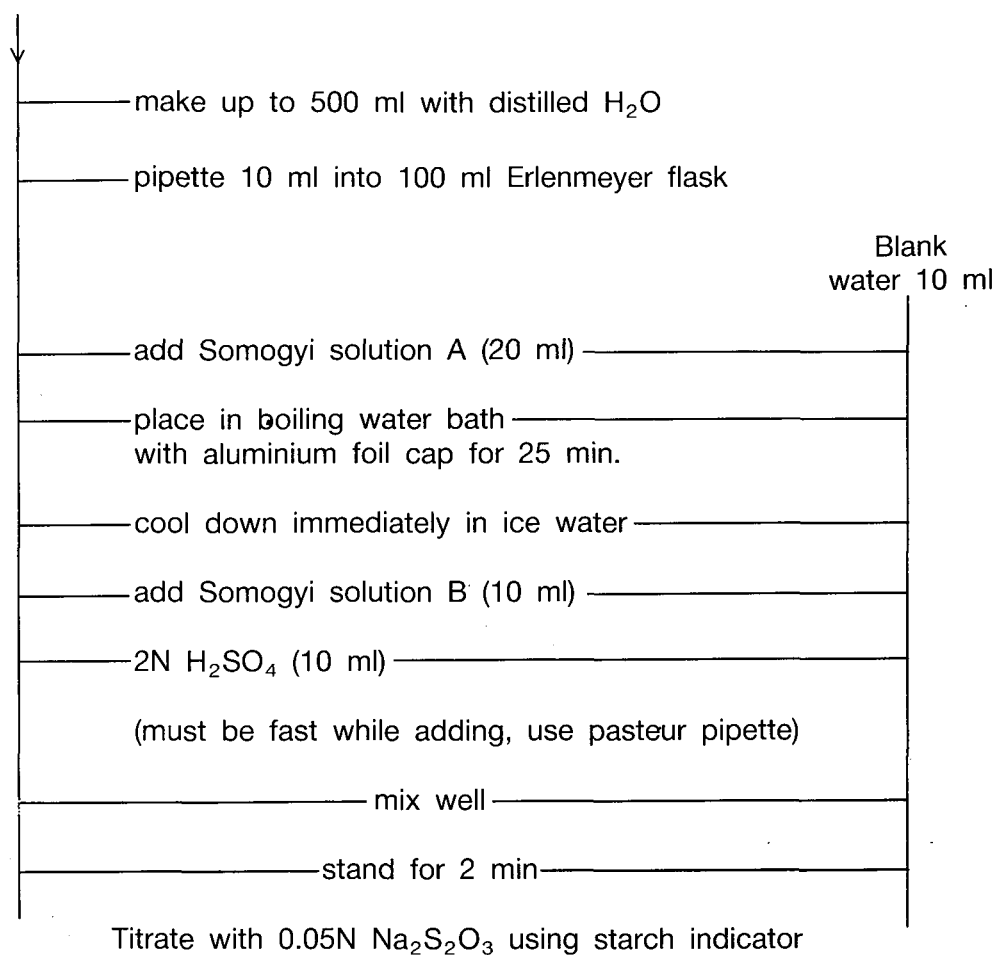
where 0.0017835: conversion factor of 1 ml 0.05N Na₂S₂O₃ to KIO₃

A: titration volume of KIO₃ solution (ml)

B: titration volume of blank (ml)

III PROCEDURE





(Note: colour changes to light blue).

The blank test with distilled water (10 ml) should be carried out simultaneously with the supernatant sample.

IV CALCULATION

$$\text{Starch (\%)} = 0.001499 (B - A) F \times \frac{500}{10} \times 0.9 \times \frac{1}{S} \times 100$$

A = titration volume of sample (ml)

B = titration volume of blank (ml)

F = Factor of 0.05N Na₂S₂O₃

0.001499 = Conversion factor of 0.05N Na₂S₂O₃ (ml) to glucose (g)

0.9 = Conversion factor of glucose to starch.

S = Weight of sample (g).

REFERENCE

Official methods of analysis of the Association of Official Analytical Chemists. 13th Ed. 1980. 24.057:383.

DETERMINATION OF SALT

NG M. C.

INTRODUCTION

Sodium chloride (Food grade) is an important additive for the production of fish jelly products. Its main function is to extract the salt soluble protein to give the gel strength of the final product.

The amount of sodium chloride present in such products can be determined by titrating the extract containing the chloride ion with silver nitrate, AgNO_3 . Potassium chromate (K_2CrO_4) is used as the indicator and the end point is indicated by the change in colour from yellow to reddish brown.

I PREPARATION

Collect fish jelly products sample (≤ 100 g) and pass 2-3 times through food mincer, or chop very finely and mix thoroughly.

II REAGENTS

All reagents should be of GR grade or AR grade:-

- a) 0.1N silver nitrate (AgNO_3) solution

Dissolve 17 g of AgNO_3 in distilled water and make up to 1 litre in volumetric flask. Keep it in a brown colour glass bottle in the dark.

- b) Potassium chromate indicator, K_2CrO_4

Dissolve 5 g K_2CrO_4 in distilled water and dilute to 100 ml.

III PROCEDURE

1. Weigh accurately 25 g sample into a 400 ml beaker.
2. Add 200 ml hot boiled water and stir for 60 mins.
3. Filter through the glass wool. Collect the filtrate in a 250 ml volumetric flask. Make up to the volume and shake well.
4. Transfer 10 ml filtrate with bulb pipette into 100 ml conical flask. Add 50 ml distilled water using the measuring cylinder and 1 ml K_2CrO_4 indicator.
5. Titrate with 0.1N AgNO_3 (S ml). At the end point, the colour changes from yellow to brownish red.
6. Carry out a blank determination using 60 ml distilled water and 1 ml K_2CrO_4 indicator (B ml).

IV CALCULATION

$$\text{Salt (\%)} = \frac{250 \text{ ml}}{10 \text{ ml} \times 25 \text{ g}} \times (S - B) \times F \times 100$$

where S = Titration volume of sample (ml)

B = Titration volume of blank (ml)

F = Conversion factor of 1 ml 0.1N AgNO₃ to 0.005844 g NaCl

REFERENCES

David Pearson. The chemical analysis of food. 7th Ed: 519.

Official method of analysis of the Association of Official Analytical Chemists 13th Ed. 1980:289, 18.034.

SEMI-QUANTITATIVE ANALYSIS OF BORIC ACID AND BORATES IN MEAT AND MEAT PRODUCTS

NG M. C.

INTRODUCTION

Boric acid and borates were commonly used as a preservatives. It acts as an anti-microbiological agent. However these preservatives are not permitted in the fishery products.

In the presence of boric acid (H_3BO_3) or sodium borate ($Na_2B_4O_7$) the turmeric test paper turns methyl red. This can be further confirmed by addition of NH_4OH which changes test paper to dark blue-green, but restored to red by acid.

In the semi-quantitative analysis, the amount of boric acid or borates detected is compared with the degree of redness on the turmeric test paper prepared from a range of standard boric acid (0-1%).

This method is applicable to meat and meat products.

I PREPARATION OF SAMPLE

Collect meat sample (≤ 100 g) and pass 2-3 times through food mincer, or chop very finely and mix thoroughly.

II REAGENTS

a) Hydrochloric acid, conc.

b) 80% Ethanol

c) Preparation of turmeric paper

Add 100 ml 80% ethanol to 1.5-2.0 g turmeric powder in 250 ml Erlenmeyer flask. Shake 5 min and filter. Dip sheets of Whatman No. 1 paper into the clean filtrate in flat-bottom dish (eg petric dish). Hang paper to dry. After 1 hour, cut into 6×1 cm strips and store in tightly stoppered container protected from light.

d) Preparation of reference standards

1. Dissolve 1.000 g H_3BO_3 in distilled water and dilute to 100 ml with distilled water.
2. Transfer 0.00, 0.10, 0.20, 0.50, 0.75, 1.00, 2.50 and 5.00 ml of the above H_3BO_3 solution to 15 ml test tubes.
3. Dilute to 10 ml with distilled water and add 0.7 ml HCl to prepare the reference standard.
4. Keep tubes tightly stoppered to prevent evaporation.

These references standard solutions represent 0.00, 0.02, 0.04, 0.10, 0.15, 0.20, 0.50 and 1.00 % H_3BO_3 in meat (based on 25 g sample extracted with 50 ml distilled water and 10 ml aliquot used for test). The standard solutions may be stored in pyrex test tubes for more than 6 months.

III APPARATUS

Erlenmeyer flask (125 ml)
Glass rod for stirring
Watch glass
Bunsen burner
Test tubes
Petri dish
Forceps
Scissor and string

IV PROCEDURE

1. Disperse 25 g of ground meat in 50 ml distilled water in 125 ml Erlenmeyer flask, using flat-end stirring rod. Cover with watch glass.
2. Bring to boil over medium flame with agitation. Do not over-heat.
3. Cool in ice bath until fat solidified (30 min).
4. Filter through pledget of glass wool.
5. Transfer 10 ml filtrate to 15 ml test tube, add 0.7 ml HCl, stopper, and mix.
6. Mark identification on end of piece of turmeric paper and dip unmarked end into unknown solution to $\frac{1}{2}$ the length of paper.
7. Quickly remove moistened paper and place on sheet of white filter paper. Flat-tipped forceps are useful in handling paper.
8. Place freshly prepared standard strips of test paper (made by dipping turmeric papers in similar manner into series of standard solutions) alongside sample turmeric strips.
9. After more than 1 hour (but < 2 hour) at room temperature, strips are dry enough for comparison. Good natural light is preferred.

V INTERPRETATION OF RESULTS

Place standard strips ca 1 cm apart on white filter paper background and bring "unknown" sample strips between adjacent standard strips for close colour matching.

If colour intensity is beyond range of standards, repeat test with dilution of meat filtrate (eg 5 ml filtrate, 5 ml distilled water, 0.7 ml HCl, and multiply final reading by 2). Use freshly prepared set of standards with each series of samples tested.

REFERENCE

Official methods of analysis of the Association of Official Analytical Chemists. 13th Ed. 1980. 20.33:328.

MICROBIOLOGICAL PROCEDURE

HANDLING OF FOOD SAMPLES

LIM P. Y.

I COLLECTION, TRANSPORT AND STORAGE OF SAMPLES

- a) Samples shall be transported to the laboratory as soon as possible after sampling, and shall reach the laboratory within 24 hours of sampling.
- b) Samples shipped frozen should be frozen when received by the laboratory. Fresh perishable samples should register a temperature from 0°C to 4°C.
- c) Ideally, samples should be examined immediately upon receipt by the laboratory. Practically however, initiation of analysis may have to be postponed. Store frozen samples at -20°C until they are to be examined. Fresh or refrigerated products are stored between 0° and 4°C for not longer than 24 hours. Store non-perishable, canned, or low-moisture food at room temperature until ready for analysis.

II CONDITION OF SAMPLES CONTAINER

Checking sampling containers for gross physical defects. Carefully inspect plastic bags and bottles for tears, pinholes and puncture marks. Any cross-contamination resulting from one or more of the above defects would invalidate the sample. Samples should be adequately sealed and labelled.

III THAWING

When necessary to thaw the sample, use aseptic technique (e.g. in laminar flow chamber) throughout the handling of the product. If the sample is frozen, thaw it in the original container or in the container in which it was received in the laboratory. Whenever possible, avoid transferring the sample to a second container for thawing. If the sample can be easily handled without thawing, e.g. ice cream, proceed directly to the next step. If the frozen sample must be thawed, do it in a manner that minimizes destruction or proliferation of the sample microflora. Normally, the sample can be thawed at 2-5°C within 18 hours. If rapid thawing is desired, thaw the sample at less than 45°C for not more than 15 mins. When thawing a sample at elevated temperatures, agitate the sample frequently, or preferably, continuously. Such rapid thawing is best carried out in a controlled temperature water-bath.

AEROBIC PLATE COUNT

LIM P. Y.

INTRODUCTION

The aerobic plate count provides an estimate of the number of viable micro-organisms in food according to the medium used and the time and temperature of incubation. The spread plate method described below is based on the assumption that each viable cell will form a colony, thus it is important that:-

- the sample is adequately dispersed
- the cells do not lose their viability
- the cells do not multiply during the preparation of the dilutions.

The material under investigation is diluted in known volumes of sterile diluent to provide a set of serial dilutions of the microbial population so that an aliquot at some step in the series provides 30 to 300 colonies when plated on a nutrient medium. (It is this count that will give the most accurate colony count.)

I CULTURE MEDIA*

Plate count agar (PCA) or Standard Methods agar
Butterfield's buffered phosphate diluent.

* Refer to Appendix B for methods of media preparation.

II APPARATUS

'Waring' blender & flasks	Autoclave
Pipettes	Incubator
Scissors & forceps	Water-bath
Alcohol lamps	Weighing balance
Alcohol (70% v/v) swabs	Laminar flow chamber
Bent glass spreader	

III SAMPLING PROCEDURE

Randomly pick 150-200 g of sample. Aseptically slice each piece of the sample in half and keep the half-cut portions in a sterile polyethylene bag or container. Store in refrigerator (5°C) to maintain its integrity.

IV SAMPLE PREPARATION

1. Weigh about 50 g of the above sample and put them into a 'Waring' blender flask. Add in 450 ml of sterile Butterfield's buffered phosphate diluent. Blend for 1 min at low speed.
2. Transfer 5 ml of this suspension into 45 ml of phosphate diluent to give a dilution of 10^{-1} . Prepare further dilutions by mixing 1 ml of the well mixed diluted sample solution with 9 ml of phosphate diluent.

V PROCEDURES

1. Select the appropriate dilutions and for every dilution, inoculate 0.1 ml aliquots to each of two PCA plates.
2. Spread the inoculum gently and evenly over the surfaces of the agar plates with a sterile bent glass spreader.
3. Allow the plates to stand until the inoculum has been absorbed completely, which should be within 15 mins after the spreading.
4. Invert the plates and incubate at 35°C for 48 hrs or at any suitable temperature and period.
5. Count those plates which have between 30-300 colonies.
6. The Aerobic plate count for the sample is calculated as below:-

Average number of colonies on the plate \times Dilution factor \times 10

Calculate to per gram of the sample. (See below).

VI CALCULATION OF AEROBIC PLATE COUNT (Spread Plate Method)

$$APC = \frac{450 + W}{W} \times \frac{1}{d} \times 10 \times \frac{En + En'}{p + (0.1)q} \text{ organisms/g}$$

where d : lowest dilution

En : total count of 2 plates at lowest dilution

En' : total count of 2 plates at highest dilution

p : number of plates at lowest dilution

q : number of plates at highest dilution

W : weight of sample

VII BACTERIOLOGICAL LIMITS OF APC FOR FISH/FISHERY PRODUCTS (COOKED & RAW)

Total plate count at 35°C for 48 hrs for

cooked products : 1×10^6 orgs/g

raw products : 2.5×10^6 orgs/g

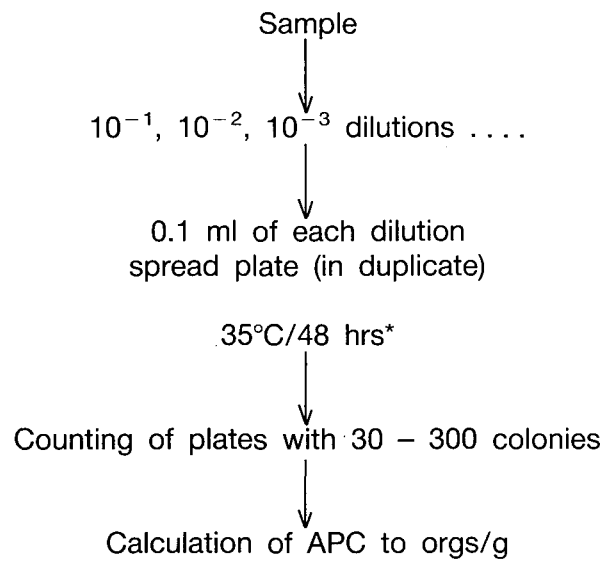
REFERENCES

A. Hazzard. (1985) ASEAN Training Course in Fish Quality Control. Training Course organised by HAWKAID, Hawkesbury Agricultural College Research and Development Co. Ltd. Chapter: Fish quality control microbiology. Section 4:63-65

Veterinary Public Health Laboratory, Primary Production Department unpublished manual.

A flow diagram of the procedure for Aerobic Plate Count (APC) is included as the following figure.

FLOW DIAGRAM OF THE PROCEDURE FOR AEROBIC PLATE COUNT (APC)



* or any suitable temperature and period of incubation.

COLIFORMS AND ESCHERICHIA COLI

LIM P. Y.

INTRODUCTION

Coliforms are Gram-negative, non-sporing, facultatively anaerobic rods which ferment lactose, producing acid and gas within 48 hrs and they belong to the family Enterobacteriaceae. The coliform group includes several genera, some of which are of intestinal origin (Escherichia) while others are associated with plant and soil material (Enterobacter). Thus it is actually a misconception to consider the coliform group as simply an indicator of faecal pollution.

However, generally speaking, it is the count of E. coli that is a more reliable indicator of faecal contamination. Its presence indicates recent faecal contamination as it generally does not survive for long in environments other than the intestine.

Faecal coliforms are a group of coliforms capable of fermenting lactose to produce acid and gas at both 37°C and 44.5 ± 0.5°C in 48 hrs and generally contain a high proportion of E. coli. As a significant number of non-faecal coliforms can give a positive faecal coliform test, the test can be made more specific for E. coli by testing for the production of indole at 44.5 ± 0.5°C.

I CULTURE MEDIA*

Brilliant green bile broth (BGB)
Butterfield's buffered phosphate diluent
Eosin methylene blue agar (EMB)
Koser citrate medium
Lauryl sulphate tryptose broth (LST)
MRVP medium
SIM medium
Simmons citrate agar
Nutrient broth

* Refer to Appendix B for methods of media preparation.

II CHEMICAL REAGENTS#

- a) Kovac's reagent
- b) Methyl red solution
- c) α-naphthol solution (5% w/v)
- d) KOH solution (40% w/v)

Refer to Appendix D for methods of reagent preparation.

III APPARATUS

'Waring' blender & flasks	Autoclave
Pipettes	Incubator
Scissors & forceps	Water-bath
Alcohol lamps	Weighing balance
Alcohol (70% v/v) swabs	Laminar flow chamber

IV SAMPLING PROCEDURE

Refer to 'AEROBIC PLATE COUNT' (E-2) SECTION III

V SAMPLE PREPARATIONS

Refer to "AEROBIC PLATE COUNT" (E-2) SECTION IV

VI PROCEDURE

A. EXAMINATION FOR PRESUMPTIVE COLIFORMS

1. Select appropriate dilutions and for every dilution, transfer 1 ml aliquots into each of 3 LST tubes.
2. Invert tubes to ensure Durham tubes do not contain gas bubbles.
3. Incubate the tubes at 35°C for 48 hrs.
4. Any tube producing gas is considered positive for the presence of coliforms.

B. CONFIRMATION TESTS FOR COLIFORMS

1. Transfer a loopful of suspension from a positive LST tube into a tube of BGB broth.
2. Invert tubes to ensure Durham tubes do not contain gas bubbles.
3. Incubate the BGB tubes at 35°C for 48 hrs.
4. Examine for gas production.
5. Using the MPN Tables (Appendix A), calculate the MPN of coliforms based on the proportion of confirmed LST tubes (with gas production) for 3 consecutive dilutions.

C. EXAMINATION FOR PRESUMPTIVE E. COLI

1. Transfer a loopful from each LST tube (with gas production) into a tube of BGB broth, prewarmed to 44.5°C.
2. Incubate the BGB tubes at 44.5°C for 48 hrs.
3. Examine for gas production at 24 hrs and, if negative, again at 48 hrs.
4. Any tube showing gas production is considered positive for the presence of presumptive E. coli.

D. CONFIRMATION TESTS FOR E. COLI

1. Subculture all positive BGB tubes by streaking onto plates of EMB agar.
 2. Incubate at 35°C for 18-24 hrs.
 3. Examine the plates for suspicious E. coli colonies, ie black or dark centred with or without the greenish metallic sheen.
 4. Subculture the suspected E. coli colonies in nutrient broth and incubate at 35°C for 18-24 hrs.
 5. Perform the following biochemical tests#:
Indole production
Methyl-Red & Voges-Proskauer tests
Citrate utilization
- # Refer to Appendix C for biochemical tests procedures.
6. Interpret results as follow:-

	<u>Indole</u>	<u>MR</u>	<u>VP</u>	<u>Citrate</u>
typical <u>E. coli</u>	+	+	-	-
atypical <u>E. coli</u>	-	+	-	-

7. Using the MPN Tables (Appendix A), calculate the MPN of E. coli based on the proportion of BGB tubes in 3 successive dilutions which were shown to contain E. coli.

VII CALCULATION OF MPN

$$\text{Most Probable Number (MPN)} = \frac{\text{Index}}{10} \times (450 + W) \times \frac{1}{W}$$

where W : weight of sample in g

Index : from MPN Tables

VIII BACTERIOLOGICAL LIMITS OF E. COLI FOR FISH/FISHERY PRODUCTS (COOKED & RAW)

Cooked products : 100 MPN/g

Raw products : 100 MPN/g

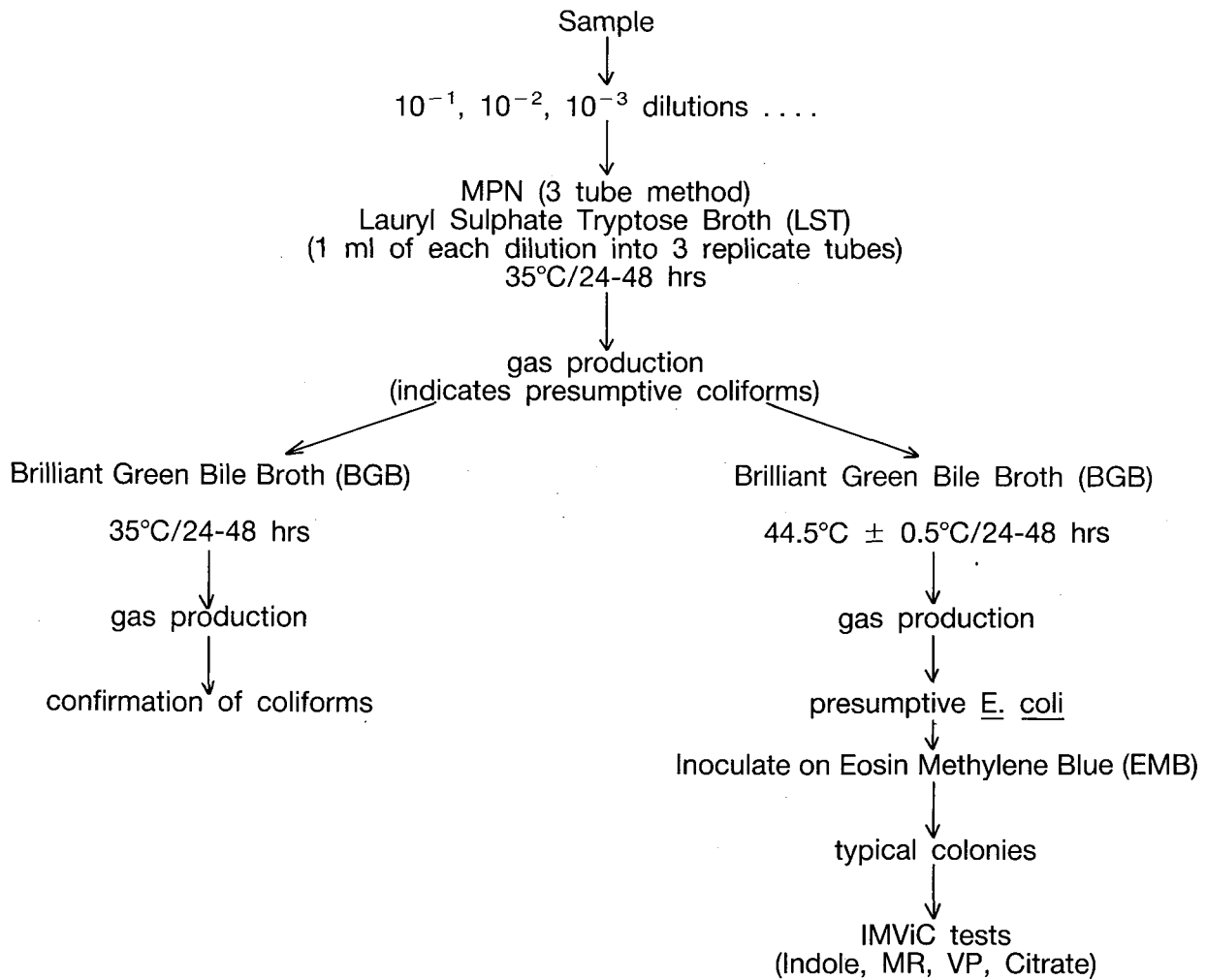
REFERENCES

- A. Hazzard. (1985). ASEAN Training Course in Fish Quality Control. Training course organised by HAWKAID, Hawkesbury Agricultural College Research and Development Co. Ltd. Chapter: Microbiology in Seafood Quality Control. Section 2:16.
Chapter: Fish quality control microbiology Section 6:88.

See Reference 2 in E-2.

A flow diagram of the examination procedures for coliforms and E. coli is included as the following figure.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR COLIFORMS AND E. COLI



SALMONELLAE & SHIGELLA

LIM P. Y.

INTRODUCTION

The presence in foods of any serotype of Salmonella is potentially dangerous as a source of human disease, either directly upon consumption of food, or indirectly through secondary contamination of utensils, processing equipments or processed foods. A further risk arises through induction of the carrier state in food-handlers.

I CULTURE MEDIA*

Nutrient broth
Selenite broth
Tetrathionate broth
Desoxycholate citrate agar (DCA)
Xylose lysine deoxycholate (XLD)
Triple sugar iron agar (TSI)
MacConkey agar (MCA)
GN broth
Salmonella anti-sera: Polyvalent "O" (somatic)
Polyvalent "H" specific and non-specific (flagellar)

* Refer to Appendix B for methods of media preparation.

II APPARATUS

'Waring' blender & flasks	Autoclave
Pipettes	Incubator
Scissors & forceps	Agitated water bath
Alcohol (70% v/v) swabs	Weighing balance
Plating loops	Laminar flow chamber
Inoculating needle	Glass slides
Conical flasks or screw-cap jars, 250 ml	Petri dish (90 × 15 mm)

III SAMPLING PROCEDURE

Refer to "AEROBIC PLATE COUNT" (E-2) Section III

IV PROCEDURE

A. RESUSCITATION (PRE-ENRICHMENT)

1. Weigh 50 g of the above sample and put them into a 'Waring' blender flask and add approximately 200 ml of sterile nutrient broth. Homogenise for 1 min at low speed.

Also blend 50 g of above sample with 200 ml of GN broth for Shigella.

2. Incubate at 35°C for 24 hrs; for Shigella incubate at 35°C for 18 hrs.

B. SELECTIVE ENRICHMENT

1. Mix the resuscitated culture gently and add 1 ml each to 10 ml of tetrathionate broth and 10 ml of selenite broth.
2. Incubate the selective enrichment broths at 35°C for 24 hrs.

C. PLATING ON SELECTIVE AGAR MEDIA

1. Each culture of enrichment medium is inoculated onto DCA and XLD agar plates. Inoculate the same for Shigella from GN broth culture (from Step A-2) on MCA, DCA and XLD agar plates.
2. Transfer a loopful of culture and streak to obtain isolated colonies.
3. Incubate at 35°C for 24 hrs.
4. Examine the plates for the presence of Salmonella & Shigella colonies.

For Salmonella:

- a) On XLD agar: appear as pink colonies with black centres of H₂S.
- b) On DCA agar: appear as colourless colonies.

For Shigella:

- a) On XLD agar: appear as red or pink colour colonies, about 1 mm Ø.
- b) On DCA & MCA: appear as opaque or transparent colonies.

D. SCREENING AND BIOCHEMICAL TESTS

1. Pick a suspected colony with inoculating wire and inoculate the TSI agar slant by streaking the slant and stabbing the butt. Incubate at 35°C for 24 hrs.
2. Salmonella cultures typically produce an alkaline (red) slant and acid (yellow) butt, with or without production of H₂S (blackening of butt) in TSI agar. Shigella cultures typically produce red slant and yellow butt, with no H₂S or gas.
3. Purify TSI cultures by streaking onto MCA and incubate for 24 hrs at 35°C. Typical colonies appear transparent and colourless, sometimes with a dark centre.
4. Subculture Salmonella colony in nutrient broth and incubate at 35°C for 24 hrs. Screen typical Shigella cultures in urea agar and motility medium. Shigella is urease negative and non-motile.

5. Using the nutrient broth culture as inoculum perform the following biochemical tests.

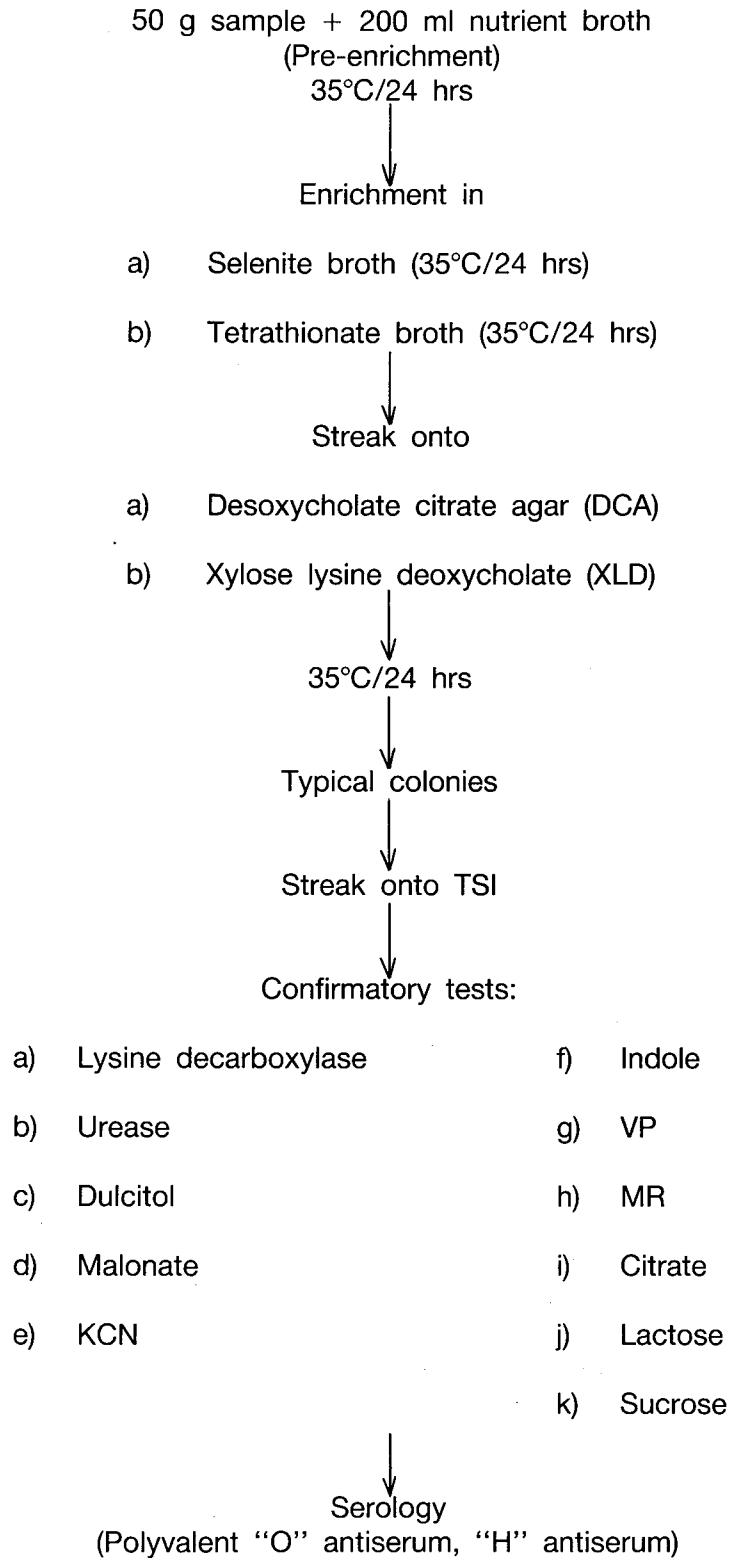
<u>Tests (Salmonella)</u>	<u>Results</u>	<u>Tests (Shigella)</u>	<u>Results</u>
Lysine decarboxylase	+	Glucose (gas)	-
Urease	-	VP	-
Dulcitol	+	MR	+
KCN	-	Indole	+/-
Malonate	-	Lysine	-
Indole	-	Arginine	+/-
VP	-	Ornithine	+/-
MR	+	Citrate	-
Citrate	+	Mannitol	+/-
Lactose	-	Lactose	-
Sucrose	-		

6. Incubate the tests for 24-28 hrs at 35°C.
7. Note that a large percentage of Salmonella arizonae strains are negative for dulcitol utilization; positive for malonate and lactose utilization.
8. Perform serological tests for cultures giving reactions typical of Salmonellae & Shigella.

E. SEROLOGICAL CONFIRMATION

1. Emulsify the culture in 2 drops of saline on a clean glass slide.
2. Add one some loopful of polyvalent "O" antiserum to the first drop only. Use the second drop as a saline control.
3. Tilt the slide back and forth for 1 minute and examine for agglutination. A positive reaction is when there is agglutination in the test mixture but not in the saline control.
4. Repeat similarly with polyvalent "H" antiserum.
5. Salmonella isolates causes agglutination for both antisera.
6. Conduct the serology for Shigella from Step 1 to Step 3.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR SALMONELLA



FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR SHIGELLA

50 g sample + 200 ml GN broth

35°C/18 hrs



Streak onto

1) MCA

2) DCA

3) XLD

35°C/24 hrs



TSI

35°C/24 hrs



if alkaline/acid; no H₂S, no gas

urease: -

motility: -



Confirmatory tests:

Glucose (gas)

Arginine

VP

Ornithine

MR

Citrate

Indole

Mannitol

Lysine

Lactose



Serology

STAPHYLOCOCCUS AUREUS

LIM P. Y.

INTRODUCTION

Staphylococcus aureus is a common organism on the skin and in the nasal passages of approximately 50% of the population. Heat treated seafood may become contaminated with this organism by poor handling, then storage at improper temperatures allows the organism to multiply and produce its toxin.

This type of food poisoning may be avoided by practising strict personal hygiene, thorough cleaning and disinfection of equipment, and storage of susceptible food at temperatures below 10°C or above 60°C.

Examination of a product for S. aureus does not guarantee protection against staphylococcal food poisoning because the organism may be killed, without destruction of the heat stable enterotoxin produced during growth of the organism. A direct microscopic smear of the food may be helpful, as direct detection of toxin in food requires methods which are too involved for routine use. A smear reveals viable and killed cells of staphylococci.

I CULTURE MEDIA*

Baird Parker medium
Brain heart infusion broth (BHI)
Citrated human plasma
Trypticase soy broth + 10% NaCl (TSB)
Butterfield's buffered phosphate diluent

* Refer to Appendix B for methods of media preparation.

II APPARATUS

'Waring' blender & flasks	Autoclave	Test-tubes
Pipettes	Incubator	Plating loops
Scissors & forceps	Water-bath	
Alcohol lamps	Weighing balance	
Alcohol (70% v/v) swabs	Laminar flow chamber	

III SAMPLING PROCEDURE

Refer to "AEROBIC PLATE COUNT" (E-2) Section III

IV SAMPLE PREPARATION

Refer to "AEROBIC PLATE COUNT" (E-2) Section IV

V PROCEDURE

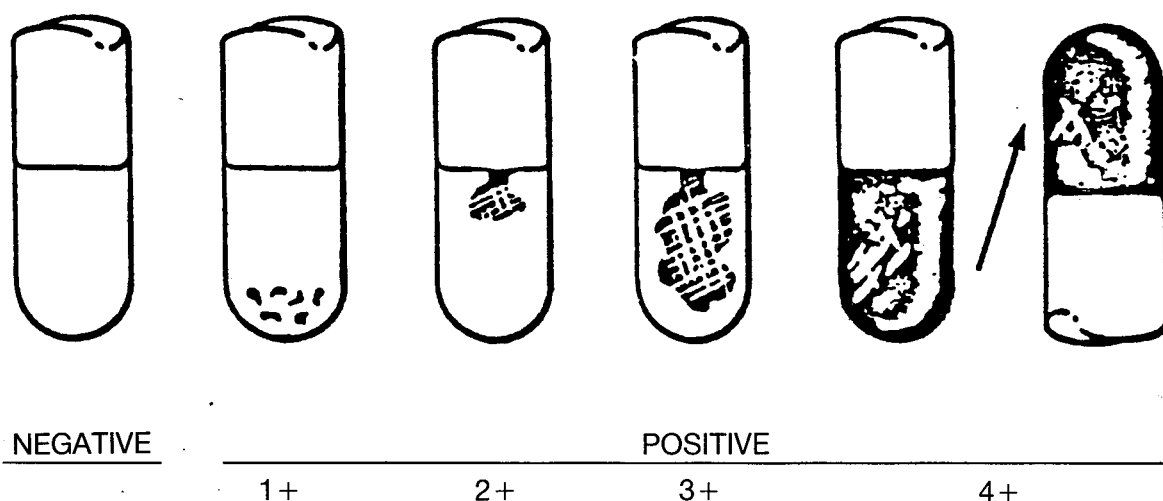
1. Select appropriate dilutions and for every dilution, transfer 1 ml aliquots into each of 3 TSB tubes.
2. Incubate the tubes at 35°C for 48 hrs.
3. The presence of turbidity indicates presumptive S. aureus.
4. Streak a loopful of the culture from a positive tube onto Baird Parker agar plate.
5. Incubate the plates at 35°C for 48 hrs.
6. Typical colonies of S. aureus on Baird Parker agar appear as smooth, black, convex and shiny with narrow white entire margins and are surrounded by clear zones extending into the opaque medium.
7. Subculture all suspected colonies in BHI broth and incubate at 35°C for 24 hrs.
8. Transfer 0.5 ml of the broth culture into a test-tube and add 1 ml of citrated human plasma. Mix by gentle rotation of the tube.
9. Incubate at 35°C for about 6 hrs, and if negative, examine again after 24 hrs.
10. A 3+ or 4+ clot formation is considered a positive reaction for S. aureus. A 3+ reaction refers to formation of a large organized clot and a 4+ reaction is when the entire contents of the tube coagulate and is not displaced when the tube is inverted. (See illustration overleaf).
11. Using the MPN Tables (Appendix A), calculate the MPN of S. aureus based on the proportion of confirmed turbid TSB tubes for 3 consecutive dilutions.

VI CALCULATION OF MPN

$$\text{Most Probable Number (MPN)} = \frac{\text{Index}}{10} \times (450 + W) \times \frac{1}{W}$$

where W : weight of sample in g.
Index : from MPN tables.

TYPES OF COAGULASE TEST REACTIONS



	NEGATIVE	NO EVIDENCE OF FIBRIN FORMATION
1+	POSITIVE	SMALL UNORGANIZED CLOTS
2+	POSITIVE	SMALL ORGANIZED CLOT
3+	POSITIVE	LARGE ORGANIZED CLOT
4+	POSITIVE	ENTIRE CONTENT OF TUBE COAGULATES AND IS NOT DISPLACED WHEN TUBE IS INVERTED

VII BACTERIOLOGICAL LIMITS OF S. AUREUS FOR FISH/FISH PRODUCTS (COOKED & RAW)

Cooked products : 100 MPN/g

Raw products : 250 MPN/g

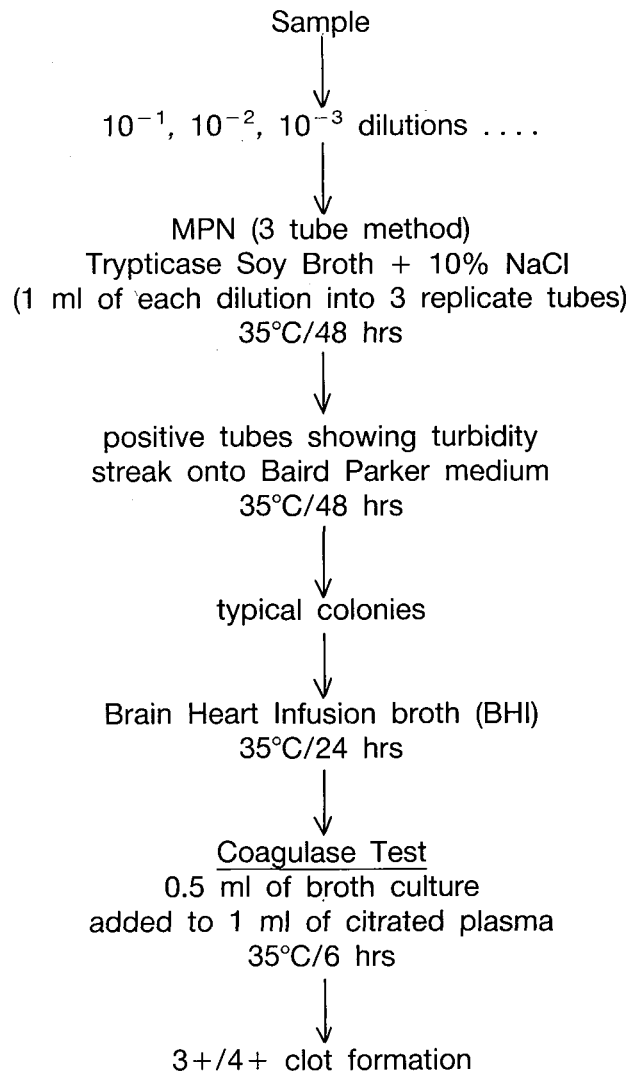
REFERENCES

A. Hazzard. (1985). ASEAN Training Course in Fish Quality Control. Training course organised by HAWKAID, Hawkesbury Agricultural College Research Development Co. Ltd. Chapter: Microbiology in seafood quality control. Section: 68, 114 & 115.

See Reference 2 in E-2.

A flow diagram of the examination procedures for Staphylococcus aureus is included as the following figure.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR STAPHYLOCOCCUS AUREUS



FAECAL STREPTOCOCCI

LIM P. Y.

INTRODUCTION

Streptococci are gram positive cocci, sometimes coccobacilli, arranged in chains. This group of streptococci resides in the intestine of warm-blooded animals. They are bile resistant and capable of growth at 45°C.

Faecal streptococci form part of the microflora of many food without necessarily indicating poor hygiene. They are found in many fermented food, such as cheese and raw sausage, and often take part in the fermentation process. However, in meat products which have received a severe heat process, the presence of excess numbers of faecal streptococci indicates unhygienic handling and/or faulty storage.

I CULTURE MEDIA*

Azide dextrose broth (ADB)
Bromocresol purple azide broth
Butterfield's buffered phosphate diluent

* Refer to Appendix B for methods of media preparation.

II APPARTUS

'Waring' blender & flasks	Autoclave
Pipettes	Incubator
Scissors & forceps	Water-bath
Alcohol lamps	Weighing balance
Alcohol (70% v/v) swabs	Laminar flow chamber

III SAMPLING PROCEDURE

Refer to 'AEROBIC PLATE COUNT' (E-2) SECTION III

IV SAMPLE PREPARATION

Refer to "AEROBIC PLATE COUNT" (E-2) SECTION IV

V PROCEDURE

1. Select appropriate dilutions and for every dilution, transfer 1 ml aliquots into each of 3 ADB tubes.
2. Incubate the tubes at 35°C for 24 hrs.
3. The presence of turbidity indicates presumptive faecal streptococci.

4. Transfer a loopful of suspension from a positive ADB tube into a tube of bromocresol purple azide broth.
5. Incubate the tubes at 35°C for 24 hrs.
6. The bromocresol purple azide broth turning purple red confirms the presence of faecal streptococci.
7. Using the MPN tables (Appendix A), calculate the MPN of faecal streptococci based on the proportion of confirmed positive bromocresol purple azide broth tubes for 3 consecutive dilutions.

VI CALCULATION OF MPN

$$\text{Most Probable Number (MPN)} = \frac{\text{Index}}{10} \times (450 + W) \times \frac{1}{W}$$

where W : weight of sample in g.
 Index : from MPN tables.

VII BACTERIOLOGICAL LIMITS OF FAECAL STREPTOCOCCI FOR FISH/FISHERY PRODUCTS (COOKED & RAW)

Cooked products : —
 Raw products : 1,000 MPN/g

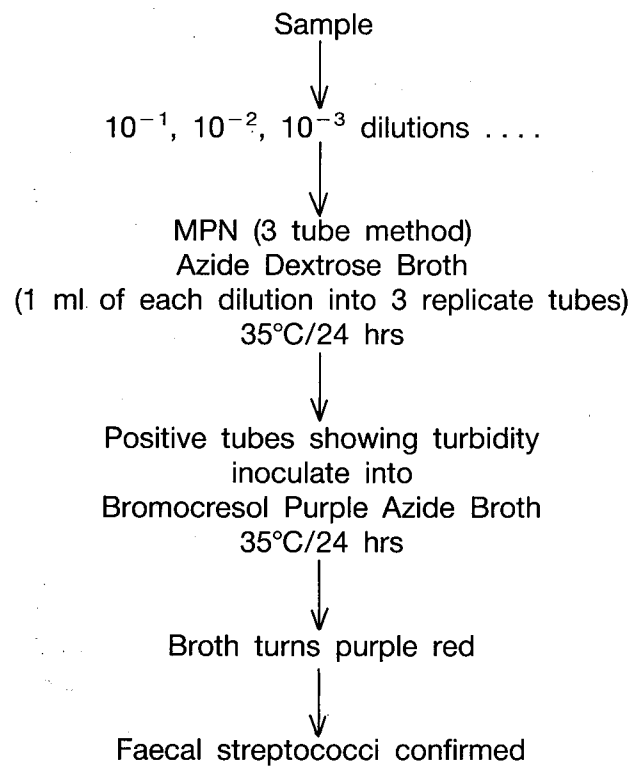
REFERENCES

- A. Hazard. 1985. ASEAN Training Course in Fish Quality Control. Training course organised by HAWKAID, Hawkesbury Agricultural College Research and Development Co. Ltd.
 Chapter: Microbiology in seafood quality control. Section 2: 17 & 28.
 Chapter: Fish quality control microbiology. Section 6. Page 88.

See Reference 2 in E-2.

A flow diagram of the examination procedures for Faecal Streptococci is included as the following figure.

FLOW DIAGRAM OF EXAMINATION PROCEDURES OF FAECAL STREPTOCOCCI



VIBRIO CHOLERA

LIM P. Y.

INTRODUCTION

Cholera is an acute specific infection caused by the organism, Vibrio cholera. Diagnosis may be confirmed by the presence of large numbers of the comma-shaped bacilli on direct microscopic examination of a faecal or vomitus smear, and by the isolation of the organism on culture.

Fish and shellfish have been identified as vehicles of cholera. Large numbers of V. cholera must usually be ingested to cause cholera, thus problems often occur when poor handling and inadequate refrigeration have allowed the organism to multiply.

I CULTURE MEDIA*

Alkaline peptone water (pH 8.6-9.0)	Phenylalanine agar (PPA)
Andrade peptone water	SIM medium
Aesculin broth	Simmons citrate agar
Decarboxylase medium base	Thiosulphate citrate bile
Koser citrate medium	salts sucrose agar (TCBS)
MRVP medium	Triple sugar iron agar (TSI)
Nutrient agar (+ 3% NaCl)	Sodium chloride (NaCl)
Nutrient gelatin	

* Refer to Appendix B for methods of media preparation.

a) 1% solution (w/v) of each of the following amino acids:-

L-arginine HCl
L-lysine HCl
L-ornithine HCl

b) 1% solution (w/v) of each of the following sugars:-

Arabinose	Lactose	Melibiose
Glucose	Mannitol	Salicin
Inositol	Mannose	Sucrose

II CHEMICAL REAGENTS#

- Tetramethyl-p-phenylenediamine di-HCl aq. soln. (1% w/v)
- Kovac's reagent
- 0.1N HCl
- Methyl red solution
- KOH solution (40% w/v)
- α -naphthol solution (5% w/v)
- FeCl₃ aq. soln. (10% w/v)

Refer to Appendix D for methods of reagent preparation.

III APPARATUS

'Waring' blender & flasks	Autoclave
Pipettes	Incubator
Scissors & forceps	Water-bath
Alcohol lamps	Weighing balance
Alcohol (70% v/v) swabs	Laminar flow chamber
Plating loops	

IV SAMPLING PROCEDURE

Refer to "AEROBIC PLATE COUNT" (E-2) Section III

V PROCEDURE

1. Weigh about 50 g of the sample and add approximately 200 ml of alkaline peptone water in a 'Waring' blender flask. Blend for 1 min at low speed.
2. Incubate at 35°C for 6-8 hrs.
3. At the end of the incubation period, transfer a loopful obtained from the pellicle (surface growth) onto TCBS agar and streak to obtain isolated colonies.
4. Incubate the plates at 35°C for 18-24 hrs.
5. V. cholera colonies on TCBS agar appear as large, smooth and yellow.
6. Screen isolates with the following tests*:-

<u>Tests</u>	<u>Results</u>
TSI	acid slant/acid butt; no gas; no H ₂ S
Indole (SIM)	+
Motility (SIM)	+
Lysine decarboxylase	+
Peptone water (+3% NaCl)	growth

* Refer to Appendix C for biochemical tests procedures.

7. From the TSI slant, inoculate a nutrient agar (+3% NaCl) slant and incubate at 35°C for 24 hrs.

8. Perform the oxidase test from the nutrient agar slant and use the peptone water culture as inoculum for the following biochemical tests*.

<u>Tests</u>	<u>Results</u>
Oxidase	+
Lysine	+
Ornithine	+
Arginine	-
Sucrose	+
Mannitol	+
Inositol	-
MR	+ ^w (Reaction delayed & weak)
VP	+/- (Indefinite)
PW + 0% NaCl	+
PW + 3% NaCl	+
PW + 7% NaCl	d (16-84% strains positive)
PW + 9% NaCl	-
PW + 11% NaCl	-

* Refer to Appendix C for biochemical tests procedures.

9. Carry out the following confirmatory biochemical tests*:-

<u>Tests</u>	<u>Results</u>
Citrate	+ ^w (Reaction delayed & weak)
Phenylalanine	-
Gelatin (5°C)	+
Gas from glucose	-
Lactose	-
Arabinose	-
Mannose	+
Salicin	-
Melibiose	-
Aesculin	-

* Refer to Appendix C for biochemical tests procedures.

10. Serological agglutination tests are performed on confirmed isolates using polyvalent O anti-serum and Ogawa and Inaba anti-sera.

VI BACTERIOLOGICAL LIMITS OF VIBRIO CHOLERA FOR FISH/FISHERY PRODUCTS (COOKED & RAW)

This organism should not be detected in 50 g sample.

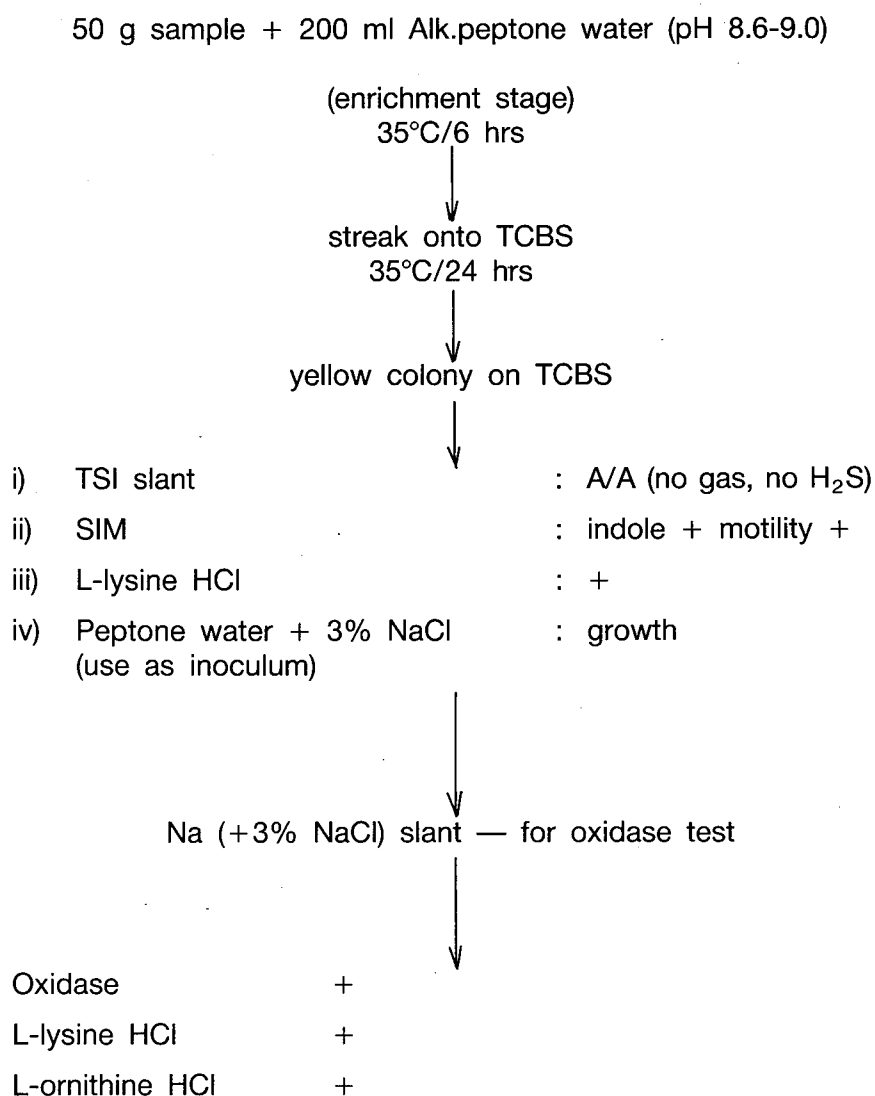
REFERENCES

A. Hazzard. (1985). ASEAN Training Course in Fish Quality Control. Training course organised by HAWKAID, Hawkesbury Agricultural College Research and Development Co. Ltd. Chapter: Microbiology In Seafood Quality Control. Section 6: 68 & 77.

See Reference 2 in E-2.

A flow diagram of the examination procedures for V. cholera is included as the following figure.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR VIBRIO CHOLERA



L-arginine HCl	-	
Sucrose	+	
Mannitol	+	
Inositol	-	
MR	+ ^w	(Reaction delayed & weak)
VP	+/-	(Indefinite)
PW + 0% NaCl	+	
PW + 3% NaCl	+	
PW + 7% NaCl	d	(16-84% strains positive)
PW + 9% NaCl	-	
PW + 11% NaCl	-	



Confirmatory biochemical tests

Citrate	+ ^w	(Reaction delayed & weak)
Phenylalanine	-	
Gelatin (5°C)	+	
Gas from glucose	-	
Lactose	-	
Arabinose	-	
Mannose	+	
Salicin	-	
Aesculin	-	
Melibiose	-	



serology for V. cholera

VIBRIO PARAHAEMOLYTICUS

LIM P. Y.

INTRODUCTION

Food poisoning due to V. parahaemolyticus is a food-borne infection resulting from the ingestion of a large number of this organism (about 10^6 - 10^9 viable cells). The major symptoms are diarrhoea and abdominal pain with headache, fever and vomiting also occurring. The organisms are excreted during the acute stage of the illness after which they decrease rapidly.

The differentiation of V. parahaemolyticus from other pathogenic species of *Vibrio* is based mainly on salt tolerance, Voges-Proskauer reaction, fermentation of sucrose and growth at 43°C.

I CULTURE MEDIA*

Glucose salt teepol broth (GSTB)
Modified Wagatsuma agar
Thiosulphate citrate bile salts sucrose agar (TCBS)
MRVP medium
Triple sugar iron agar (TSI)
Andrade peptone water
Koser citrate medium
Simmons citrate agar
Phenylalanine agar (PPA)
Bacto-peptone (PW)
Decarboxylase medium base
Nutrient gelatin
Aesculin broth
SIM medium
Butterfield's buffered phosphate diluent
Nutrient agar (+3% NaCl)
Sodium chloride (NaCl)

* Refer to Appendix B for methods of media preparation.

a) 1% solution (w/v) of each of the following amino acids:-

L-arginine HCl
L-lysine HCl
L-ornithine HCl

b) 1% solution (w/v) of the following sugars:-

Arabinose	Mannitol	Salicin
Glucose	Mannose	Sucrose
Lactose	Melibiose	

II CHEMICAL REAGENTS**

Tetramethyl-p-phenylenediamine di-HCl aq. soln. (1% w/v)

- | | |
|---|--|
| a) Kovac's reagent | d) 01.N HCl |
| b) Methyl red solution | e) KOH solution (40% w/v) |
| c) α -naphthol solution (5% w/v) | f) FeCl ₃ aq. soln. (10% w/v) |

** Refer to Appendix D for methods of reagent preparation.

III APPARATUS

'Waring' blender & flasks	Autoclave
Pipettes	Incubator
Scissors & forceps	Water-bath
Alcohol lamps	Weighing balance
Alcohol (70% v/v) swabs	Laminar flow chamber
Plating loops	

IV SAMPLING PROCEDURE

Refer to "AEROBIC PLATE COUNT" (E-2) Section III.

V SAMPLE PREPARATION

Refer to "AEROBIC PLATE COUNT" (E-2) Section IV.

VI PROCEDURE

1. Select appropriate dilutions and for first dilution, transfer 10 ml aliquots into each of 3 tubes of double strength GSTB.
2. For each of the next 2 further dilutions, transfer 1 ml aliquots into each of 3 tubes of single strength GSTB.
3. Incubate the tubes at 35°C for not more than 18 hrs.
4. Transfer a loopful of suspension from the top 1 cm of a positive GSTB tube onto a TCBS plate and streak to obtain isolated colonies.
5. Incubate the plates at 35°C for 18 hrs.
6. Examine the plates for typical V. parahaemolyticus colonies which are large and blue-green with a dark centre.
7. Screen suspected isolates by inoculating the following media* and incubate at 35°C for 24 hrs.

TSI agar	K/Acid (no gas; no H ₂ S)
Indole (SIM)	+
Motility (SIM)	+
L-lysine HCl	+

* Refer to Appendix C for biochemical tests procedures.

8. Inoculate the TSI culture into peptone water (+3% NaCl) and nutrient agar (+3% NaCl) slant and incubate at 35°C for 24 hrs.
9. Perform the oxidase test from the nutrient agar slant and use the peptone water culture as inoculum for the following biochemical tests.*

Oxidase	+
Voges-Proskauer	-
Sucrose	-
Mannitol	+
Peptone water (PW) + 0% NaCl	-
Peptone water (PW) + 3% NaCl	+
Peptone water (PW) + 7% NaCl	+
Peptone water (PW) + 9% NaCl	+
Peptone water (PW) + 11% NaCl	-

* Refer to Appendix C for biochemical tests procedures.

10. Carry out the following confirmatory biochemical tests*

Methyl Red (MR)	+
Citrate	+
L-arginine HCl	-
L-ornithine HCl	+
Phenylalanine (PPA)	-
Nutrient gelatin (5°C)	+
Gas from glucose	-
Lactose	-
Arabinose	+
Mannose	+
Mannitol	+
Salicin	-
Aesculin	+
Melibiose	-

* Refer to Appendix C for biochemical tests procedures.

11. Calculate the MPN of V. parahemolyticus based on the proportion of positive GSTB tubes which are confirmed for the presence of V. parahaemolyticus. (See below)

VII CALCULATION OF MPN

$$\text{Most Probable Number (MPN)} = \frac{\text{Index}}{10} \times (450 + W) \times \frac{1}{W}$$

where W : weight of sample in g

Index : from MPN Tables (Appendix A)

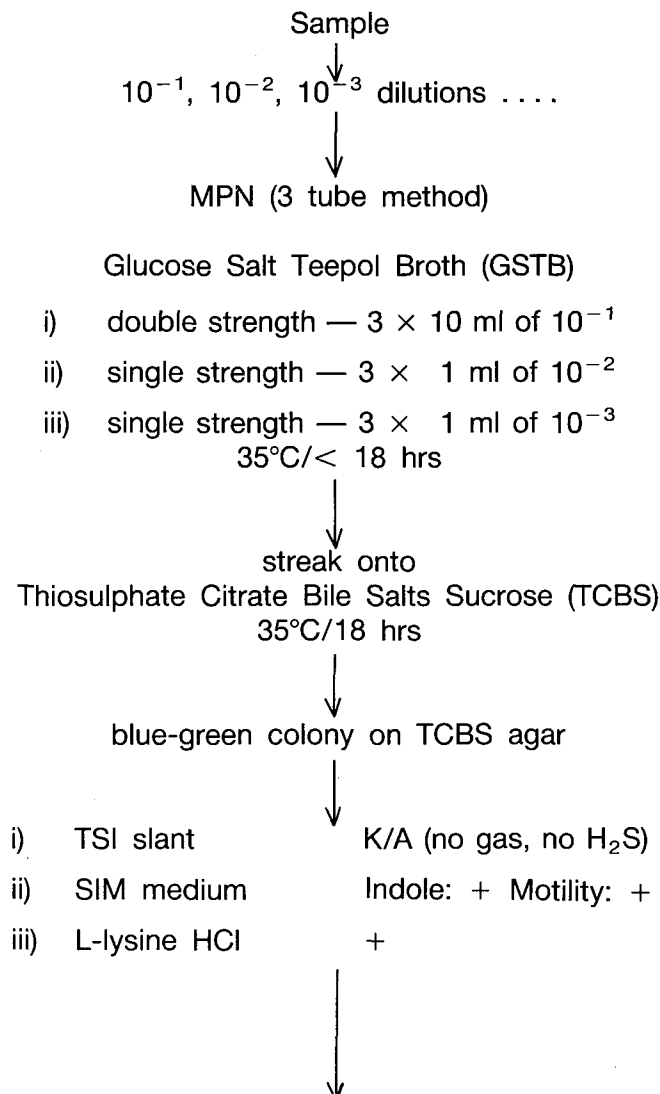
REFERENCES

A. Hazzard. (1985). ASEAN Training Course in Fish Quality Control. Training course organised by HAWKAID, Hawkesbury Agricultural College Research and Development Co. Ltd. Chapter: microbiology in seafood quality control. Section 6: P. 69-70.

Isolation and identification of Vibrio parahaemolyticus. Bacteriological analytical manual. Jan. 1969.

A flow diagram of the examination procedures for V. parahaemolyticus is included as the following figure.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR V. PARAHAEMOLYTICUS





- Inoculate
- i) Peptone water (+3% NaCl) (as inoculum)
 - ii) Nutrient agar (+3% NaCl) slant (for oxidase test)

- i) Oxidase +
- ii) Voges-Proskauer -
- iii) Sucrose -
- iv) Mannitol +
- v) PW + 0% NaCl -
- PW + 3% NaCl +
- PW + 7% NaCl +
- PW + 9% NaCl +
- PW + 11% NaCl -



confirmatory biochemical tests

- Methyl Red (MR) +
- Citrate +
- L-arginine HCl -
- L-ornithine HCl +
- Phenylalanine (PPA) -
- Nutrient gelatin (5°C) +
- Melibiose -
- Gas from glucose -
- Lactose -
- Arabinose +
- Mannose +
- Mannitol +
- Salicin -
- Aesculin +



Kanagawa reaction test (if required) (see next page)

KANAGAWA REACTION OF *V. PARAHAEMOLYTICUS*

APPLICATION

The Kanagawa reaction tests for the presence of specific haemolysis on Wagatsuma agar.

A positive reaction has been found to correlate closely with the pathogenicity of *V. parahaemolyticus* isolates. The isolates that have caused illness in humans are almost always Kanagawa-positive, although isolates from seafood are almost always Kanagawa-negative.

PROCEDURE

1. Subculture the isolate into 3% NaCl peptone water and incubate at 35°C for 18 hrs.
2. Spot a loopful of this culture onto a freshly prepared, dried modified Wagatsuma agar plate. Several spottings may be made on the same plate.
3. Incubate at 35°C for 18 ± 2 hrs.
4. A positive test consists of β-haemolysis: a zone of transparent clearing of the blood cells around the colony.
5. It is very important to remember that only observations within 24 hrs is valid in this test.

APPENDIX

APPENDIX A

MOST PROBABLE NUMBERS (MPN) PER 1 G OF SAMPLE, USING 3 TUBES WITH EACH OF 0.1, 0.01 AND 0.001 G PORTIONS

MPN INDEX TABLE			
No. of Tubes Giving Positive Reaction out of			MPN Index per 10 ml
3 of 1 ml Each	3 of 0.1 ml Each	3 of 0.01 ml Each	
0	0	0	<3
0	0	1	3
0	0	2	6
0	0	3	9
0	1	0	3
0	1	1	6.1
0	1	2	9.2
0	1	3	12
0	2	0	6.2
0	2	1	9.3
0	2	2	12
0	2	3	16
0	3	0	9.4
0	3	1	13
0	3	2	16
0	3	3	19
1	0	0	4
1	0	1	7
1	0	2	11
1	0	3	15
1	1	0	7
1	1	1	11
1	1	2	15
1	1	3	19
1	2	0	11
1	2	1	15
1	2	2	20
1	2	3	24
1	3	0	16
1	3	1	20
1	3	2	24
1	3	3	29
2	0	0	9
2	0	1	14
2	0	2	20
2	0	3	26
2	1	0	15
2	1	1	20
2	1	2	27
2	1	3	34
2	2	0	21
2	2	1	28
2	2	2	35
2	2	3	42
2	3	0	29
2	3	1	36
2	3	2	44
2	3	3	53
3	0	0	23
3	0	1	39
3	0	2	64
3	0	3	95
3	1	0	43
3	1	1	75
3	1	2	120
3	1	3	160
3	2	0	93
3	2	1	150
3	2	2	210
3	2	3	290
3	3	0	240
3	3	1	460
3	3	2	1,100
3	3	3	≥2,400

MEDIA PREPARATION METHODS

B1 BUTTERFIELD'S BUFFERED PHOSPHATE DILUENT

1. Stock solution

Dissolve 6.8 g KH_2PO_4 in 100 ml H_2O , adjust pH 7.2 with ca 35 ml 1N NaOH and dilute to 200 ml. Store in refrigerator.

2. Diluent

Dilute 1.25 ml stock solution to 1 litre with distilled H_2O . Prepare diluent with this solution, dispensing enough to allow for losses during autoclaving. Autoclave for 15 mins at 121°C.

B2 MEDIA PREPARATION

For the method of preparing the following media, refer to the respective manufacturer's manual. Available manuals: BBL, Difco, Merck & Oxoid.

- Aesculin broth
- Andrade peptone water
- Azide dextrose broth (ADB)
- Bacto-peptone (PW)
- Baird Parker medium
- Brain heart infusion broth (BHI)
- Brilliant green bile (2%) broth (BGB)
- Bromocresol purple azide broth
- Decarboxylase medium base
- Desoxycholate citrate agar (DCA)
- Eosin methylene blue agar (EMB)
- GN broth
- Koser citrate medium
- Lauryl sulphate tryptose broth (LSB)
- MacConkey agar (MCA)
- Modified Wagatsuma agar
- MRVP medium
- Nutrient agar
- Nutrient broth
- Nutrient gelatin
- Phenylalanine agar (PPA)
- Plate count agar (PCA)
- Selenite broth
- SIM medium
- Simmons citrate agar
- Tetrathionate broth
- Thiosulphate citrate bile salts sucrose agar (TCBS)
- Triple sugar iron agar (TSI)
- Trypticase soy broth (TSB)
- Xylose lysine deoxycholate (XLD)

APPENDIX C

BIOCHEMICAL TESTS IN DIAGNOSTIC MICROBIOLOGY

C1 AESCULIN HYDROLYSIS

Inoculate Aesculin broth and examine daily up to 7 days for blackening, this indicates hydrolysis of the aesculin. Alternatively, inoculate Aesculin agar and look for blackening in and around the bacterial growth.

C2 CARBOHYDRATE BREAKDOWN

Inoculate the Andrade peptone water sugar and examine after 24 hrs of incubation. Acid production is indicated by a change in the colour from colourless to pinkish or reddish. Formation of gas is indicated by a bubble in the inverted Durham tube.

C3 CITRATE UTILIZATION

Method 1.

Inoculate Koser citrate broth and incubate at 35°C for at least 24 hrs. Examine for turbidity.

Turbidity	—	citrate utilized
No turbidity	—	citrate not utilized

Method 2.

Inoculate by making a single streak over the surface of a slope of Simmons citrate. Examine for growth and colour change.

Blue colour and streak of growth	—	citrate utilized
Original green colour	—	citrate not utilized

C4 COAGULASE TEST

Method 1. Slide test

Emulsify a colony from a culture plate in a drop of saline on a microscope slide.

Emulsify the culture well to make a milky suspension of the organisms. Mix a loopful of human plasma into the drop of bacterial suspension.

Tilt the slide back and forth and observe for formation of granular precipitate of white clumps. Clumping of bacteria indicates presence of coagulase (coagulase-positive), and usually occurs within 15 to 20 seconds. The test is considered negative if clumping is not observed within 2 to 3 minutes.

Method 2. Tube test

To 0.5 ml of a 24 hrs broth culture of the organism in a test-tube, add 1 ml of human plasma. Mix by gentle rotation of the tube, avoiding stirring or shaking of the mixture. Incubate at 35°C for 1 to 4 hrs.

Observe for formation of a visible clot.

C5 DECARBOXYLASE REACTIONS

Inoculate tubes of the Decarboxylase medium containing 1% (w/v) solution of amino acid (L-arginine HCl or L-lysine HCl or L-ornithine HCl) and incubate at 35°C. Examine daily for up to 4 days. Decarboxylation is indicated by a purple colour, whereas the control and negative tubes are yellow.

C6 GELATIN HYDROLYSIS

Inoculate Nutrient gelatin and incubate at 35°C for up to 14 days. For every 2 to 3 days, cool in a refrigerator for half an hour and then examine for liquefaction. Set up a control tube of uninoculated medium in parallel.

C7 HYDROGEN SULPHIDE PRODUCTION

Method 1.

Inoculate a tube of Triple sugar iron (TSI) agar by stabbing the butt and streaking the slope. Observe for blackening due to H₂S production.

Method 2.

Inoculate a tube of SIM medium by stabbing into the butt. Observe for blackening due to H₂S production.

C8 INDOLE PRODUCTION

Inoculate a tube of SIM medium by stabbing into the butt. Incubate for 48 hrs at 35°C. Add Kovac's reagent down the side of the tube. A red colour in the reagent layer indicates indole.

C9 MOTILITY

Method 1.

Transfer a loopful of a young broth culture of the organism to a clean microscope slide. Cover with a cover-slip. Examine for motility using a high-power dry objective and reduced illumination.

Method 2.

Inoculate a tube of SIM medium by stabbing to a depth of 1 cm from the bottom. After incubation at 35°C for 24 hrs, examine the growth pattern of the organism.

A motile organism migrates from the stab line and diffuses into the medium, causing a turbidity; or it may exhibit fuzzy streaks of growth. Growth of a non-motile organism is concentrated along the stab line, with the surrounding medium remaining clear.

C10 METHYL-RED REACTION

Inoculate MRVP broth and incubate at 35°C for 24 to 48 hrs. Add 2 drops of methyl red solution, shake and examine.

red colour	—	+
orange	—	±
yellow	—	

C11 OXIDASE ACTIVITY

On a piece of filter paper in a petri-dish, place 2 to 3 drops of the oxidase reagent (1% w/v tetramethyl-p-phenylenediamine di-HCl aq. solution); do not allow the drops to dry on the plate. The test organism is removed with a platinum wire and smeared across the surface of the impregnated paper. A positive reaction is shown by the development of a dark purple colour within 10 seconds.

C12 PHENYLALANINE DEAMINATION

Method 1.

Inoculate Malonate-phenylalanine medium and incubate for 24 hrs at 35°C. Acidify with 0.1-0.2 ml of 0.1N HCl; add 0.2 ml 10% FeCl₃ aq. solution; shake and observe immediately any colour change. A positive reaction is indicated by a green colour which quickly fades.

Method 2.

Inoculate heavily a Phenylalanine agar slope. Incubate overnight and run 0.2 ml 10% FeCl₃ aq. solution over the growth. A positive result gives a green colour on the slope and in the free liquid at the base.

C13 VOGES-PROSKAUER (VP) REACTION

Inoculate MRVP broth and incubate at 35°C for 24 hrs. After completion of the methyl red test, add 0.6 ml of 5% α-naphthol solution followed by 0.2 ml 40% KOH solution. Shake, slope the tube (to increase the size of the air/liquid interface) and examine after 15 minutes and 1 hr. A positive reaction is indicated by a strong red colour.

C14 TRIPLE SUGAR IRON (TSI) TEST

With the inoculating needle, touch a well-isolated colony on a culture plate and stab the needle into the deep of the tube to a depth of 1 cm from the bottom. Remove the needle from the deep and streak the slant surface. Incubate at 35°C for 24 hrs.

Record the TSI results with the slant reaction first followed by the deep reaction, separated by a slash mark (slant reaction/deep reaction). The slant reaction involves the presence or absence of acidity (carbohydrate fermentation). When interpreting the deep reaction observe for:-

- a) absence or presence of acidity

- b) presence of CO_2 and H_2 gases, as evident by a splitting of the medium, a single gas bubble, complete displacement of the medium from the bottom of the tube leaving a clear area, or a slight indentation of the medium from the side of the tube.
- c) presence of a black precipitate indicating that H_2S gas was produced.

Use the following standard abbreviations to record the TSI results:-

Acidity (yellow)	—	A
Alkalinity (purplish/red)	—	K
CO_2 and H_2 gases	—	gas
Hydrogen sulphide	—	H_2S
No change	—	NC

PREPARATION METHODS FOR REAGENTS

D1 KOVAC'S (1928) REAGENT FOR INDOLE

p-dimethylaminobenzaldehyde	5 g
Iso-Amyl alcohol	75 ml
Conc. HCl	25 ml

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50-55°C). Cool and add the acid. Protect from light and store at 4°C.

Note: The reagent should be light yellow to light brown in colour; some samples of amyl alcohol are unsatisfactory, and give a dark colour with the aldehyde.

D2 METHYL RED SOLUTION

Methyl red	0.04 g
Ethanol	40 ml
Distilled water to	100 ml

Dissolve the methyl red in the ethanol and dilute to volume with distilled water.

D3 α -NAPHTHOL SOLUTION

5%(w/v) α -naphthol in ethanol

The solution should not be darker than straw colour; if necessary the α -naphthol should be redistilled (Fulton, Halkias & Yarashus, 1960).

D4 OXIDASE TEST REAGENT

1%(w/v) tetramethyl-p-phenylenediamine dihydrochloride aq. solution

The reagent should be colourless and be stored in a glass-stoppered bottle, protected from light, at 4°C. The solution should not be used if it becomes deep blue. The autoxidation of the reagent may be retarded by the addition of 1% ascorbic acid (Steel, 1962b). If ascorbic acid is not used to delay the process of autoxidation, the solution should be freshly prepared each week.

REFERENCE

COWAN, S.T. (1974) 'Manual for the identification of medical bacteria' 2nd Ed Cambridge University Press.

