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

2ND EDITION, 1992

MARINE FISHERIES RESEARCH DEPARTMENT
SOUTHEAST ASIAN FISHERIES DEVELOPMENT CENTER
SINGAPORE

In collaboration with

JAPAN INTERNATIONAL COOPERATION AGENCY

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

2ND EDITION, 1992

Edited by

KATSUTOSHI MIWA
LOW SU JI

MARINE FISHERIES RESEARCH DEPARTMENT
SOUTHEAST ASIAN FISHERIES DEVELOPMENT CENTER
SINGAPORE

In collaboration with

JAPAN INTERNATIONAL COOPERATION AGENCY

The Southeast Asian Fisheries Development Center (SEAFDEC) is a technical organisation devoted to the accelerated development of fisheries in the region. The member countries of SEAFDEC are Japan, Malaysia, Philippines, Singapore and Thailand. SEAFDEC has four Departments namely, the Aquaculture Department in the Philippines, the Training Department in Thailand, the Marine Fisheries Research Department in Singapore and the Marine Fishery Resources Development and Management Department in Malaysia.

Southeast Asian Fisheries Development Center
Marine Fisheries Research Department
Postal address: Changi Fisheries Complex
Changi Point, Singapore 1749

Secretariat: Liaison Office
956 Olympia Building
4th floor, Rama IV Road
Bangkok 10500
THAILAND

First Edition: Edited by Hiroshi Hasegawa

Layout:
MOHAMED BIN SALIM

ISBN 9971-88-329-5

PREFACE TO THE SECOND EDITION

Since the release of the first edition of this Laboratory Manual in 1987, the Marine Fisheries Research Department has received favourable comments and suggestions to review and expand the subjects covered in the publication. The Department therefore requested the opinions of SEAFDEC Member Countries, and the task of updating began in 1991 under the direction of Dr Katsutoshi Miwa, Deputy Chief of MFRD and formerly Director of the Tokai Regional Fisheries Laboratory of Japan (now renamed the National Fisheries Research Institute). The items described have been revised under six chapters; as far as possible, the descriptions follow officially recognised methods.

For the convenience of users, this volume incorporates all the entries of the first edition that have remained unchanged, making it unnecessary to refer to the first edition.

The target users of the Manual remain unchanged: they include both experienced laboratory workers and other technical personnel, especially those involved in the analysis of fish and fish products, as well as inspection personnel. The contributors and editors have again endeavoured to present the material as simply as possible, describing each step in the procedure. As before, the use of a ring file allows for easy updating of the material.

Since the target users are mainly from Southeast Asia, the Manual has been designed with special reference to this region. However, we hope that it will be useful as well to other users, and will prove at least as helpful as the earlier edition. We welcome critical comments and suggestions by users in the region and beyond.

I wish to thank all the contributors to this Manual; the quality of the publication is a measure of the effort they have put in so that they can share their experiences.

On behalf of the MFRD, I wish to acknowledge the generous donation of the Japan International Cooperation Agency towards the cost of printing this publication.

Hooi Kok Kuang
Chief
Marine Fisheries Research Department
Southeast Asian fisheries Development Center

ACKNOWLEDGEMENT

There have been many requests for the publication of a revised Laboratory Manual containing new analytical methods from the researchers and analysts in the region. The revision of the Laboratory Manual was started at the end of 1990 at the MFRD Chief's suggestion. Ten items were revised and twenty items were newly added.

The members of the Revised Laboratory Manual Working Group are as follows:

Writers: Katsutoshi MIWA

Lim Pang Yong

Makoto YAMAGATA

Low Lai Kim

Ng Cher Siang

Ng Mui Chng

Layout: Mohamed bin Salim

Finally the editors wish to express their appreciation of efforts of the above persons and the advice of the Food Development Center in Philippines.

CONTENTS

B–6 (Unchanged) : Determination of K-value
NG CHER SIANG

B–7 (Unchanged) : Freshness testing paper
NG CHER SIANG

B–8 (Addition) : Determination of volatile basic nitrogen, trimethylamine oxide nitrogen and trimethylamine nitrogen by Conway's microdiffusion method (barium hydroxide method)
MAKOTO YAMAGATA & LOW LAI KIM

B–9 (Addition) : Determination of ammonia
LOW LAI KIM

B–10 (Addition) : Qualitative test of formaldehyde
LOW LAI KIM

B–11 (Addition) : Determination of formaldehyde by 4-amino-3-hydrazino-5-mercapto-1,2,4-triazo(AHMT) method
MAKOTO YAMAGATA & LOW LAI KIM

B–12 (Addition) : Determination of K-value by Freshness Meter
NG CHER SIANG

B–13 (Addition) : Fractionation and determination of fish proteins
KATSUTOSHI MIWA & LIM PANG YONG

PART C. ANALYSIS OF OILS

C–1 (Unchanged) : Significance of analysis of lipids
LOW LAI KIM

C–2 (Unchanged) : Extraction of lipids (modified Folch's methods)
LOW LAI KIM

C–3 (Unchanged) : Determination of total lipid content
LOW LAI KIM

C–4 (Unchanged) : Determination of phospholipid content
LOW LAI KIM

C–5 (Unchanged) : Determination of acid value
LOW LAI KIM

C–6 (Unchanged) : Determination of saponification value
LOW LAI KIM

C–7 (Unchanged) : Determination of peroxide value
LOW LAI KIM

C–8 (Unchanged) : Determination of thiobarbituric acid (TBA) number
LOW LAI KIM

C–9 (Revised) : Determination of methyl esters of fatty acids by gas chromatographic method
LOW LAI KIM

C–10 (Unchanged) : Determination of the degree of lipid oxidation by gas chromatography
LOW LAI KIM

C–11 (Unchanged) : Preparation of methyl esters by Boron Trifluoride method
LOW LAI KIM

C–12 (Addition) : Extraction and determination method of lean fish lipids
KATSUTOSHI MIWA & LOW LAI KIM

C–13 (Addition) : Lipid composition analysis by thin layer chromatography
KATSUTOSHI MIWA

PART D. ANALYSIS OF ADDITIVES

D–1 (Unchanged) : Detection of polyphosphates
NG MUI CHNG

D–2 (Unchanged) : Determination of monosodium L-glutamate (MSG) content in fish jelly products
NG MUI CHNG

D–3 (Unchanged) : Determination of sugar (sucrose) by Somogyi's method
NG MUI CHNG

D–4 (Unchanged) : Determination of starch
NG MUI CHNG

D–5 (Unchanged) : Determination of salt
NG MUI CHNG

D–6 (Unchanged) : Semi-quantitative analysis of boric acid and borates in meat and meat products
NG MUI CHNG

D–7 (Addition) : Determination of sodium nitrite by colorimetric method
MAKOTO YAMAGATA & LOW LAI KIM

D–8 (Addition) : Determination of sulphur dioxide in frozen shrimps by colorimetric and Conway's microdiffusion method
MAKOTO YAMAGATA & LOW LAI KIM

D–9 (Addition) : Determination of sulphur dioxide by Rankine method (Alkali titration method)
MAKOTO YAMAGATA & LOW LAI KIM

D–10 (Addition) : Determination of sulphur dioxide by Rankine method (Colorimetric method)
MAKOTO YAMAGATA & LOW LAI KIM

D–11 (Addition) : Determination of benzoic acid (AOAC method)
NG MUI CHNG

D–12 (Addition) : Determination of benzoic, sorbic and dehydroacetic acid by steam-distillation and UV spectrophotometric method
MAKOTO YAMAGATA

PART E. MICROBIOLOGICAL PROCEDURE

E–1 (Unchanged) : Handling of food samples
LIM PANG YONG

E–2 (Revised) : Aerobic plate count
LIM PANG YONG

E–3 (Revised) : Coliforms and Escherichia Coli
LIM PANG YONG

E–4 (Revised) : Salmonellae and Shigella
LIM PANG YONG

E–5 (Unchanged) : Staphylococcus aureus
LIM PANG YONG

E–6 (Unchanged) : Faecal streptococci
LIM PANG YONG

E–7 (Unchanged) : Vibrio cholera
LIM PANG YONG

E–8 (Unchanged) : Vibrio parahaemolyticus
LIM PANG YONG

E–9 (Addition) : Anaerobic plate count
MAKOTO YAMAGATA

E–10 (Addition) : Fungi (mould and yeast) plate count
MAKOTO YAMAGATA

E–11 (Addition) : Spore forming bacteria count
LIM PANG YONG

PART F. QUALITY ASSESSMENT AND IDENTIFICATION

F–1 (Addition) : Sensory evaluation of fish and fish products
LOW LAI KIM

F–2 (Addition) : Thin layer isoelectric focusing of fish protein
NG CHER SIANG

APPENDIX

A	(Unchanged)	:	Most probable numbers (MNP) per l g of sample, using 3 tubes with each of 0.1, 0.01 and 0.001 g portions
A1	(Addition)	:	Most probable numbers per 100 ml of sample, planting 5 portions in each of 3 dilutions in geometric series
B	(Revised)	:	Media preparation methods
C	(Unchanged)	:	Biochemical tests in diagnostic microbiology
D	(Unchanged)	:	Preparation methods for reagents
E	(Addition)	:	Examination methods of vibrio cholera in Japan
F	(Addition)	:	Triangle test
G	(Addition)	:	Duo-trio test
H	(Addition)	:	Simple 9 point hedonic scale
I	(Addition)	:	Statistic table for Triangle test
J	(Addition)	:	Statistic table for Duo-trio test

DETERMINATION OF PHYSICAL PROPERTIES OF MEAT

DETERMINATION OF MOISTURE

NG MUI CHNG

INTRODUCTION

There are various methods to determine the moisture content such as drying methods, distillation method and Kahr-Fischer titration method. 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 drying method the amount of moisture in foods is the difference between the weight before and after drying. It is simple and is used (as a standard method) for many kinds of foods. The process of drying is caused by the difference of the relative humidity between a food and the atmosphere, so that the higher the temperature, the faster the drying. Some fermented products are unstable and decompose at high temperature. Such fermented products are dried at 40-70° under vacuum. On the other hand cereals are stable at high temperature and are dried at 135°C under normal atmosphere. Fish and fish products are normally dried at 100-110°C. Simple and rapid drying methods by oven, infra-red balance and microwave moisture checker are used for the drying of fish products.

APPARATUS

Method 1 : Oven of temperature range 100-150°,
Aluminium dish with lid,
Chemical balance (100g),
Desiccator with some moisture absorbent (silica gel, calcium-chloride, concentrated sulfuric acid ~~etc~~).

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

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

SAMPLE PREPARATION

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

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 prepared 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 (about 45 min). 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-weighed 5 g meat prepared 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 as in the oven method i.e.:

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

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

W_2 = weight (g) of sample after drying.

METHOD 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 Table 1).
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 for an additional 30 sec at 300w until the dried weight is constant.

Table 1.

SUITABLE TIME AND HEATING CONDITIONS FOR FISH MEAT SAMPLE

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

CALCULATION

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

REFERENCES

AOAC Official Methods: 13th ed., p. 507-532 (1980)
Japan Food Industrial Technology Society: Analytical methods of food, Kohrin, p. 4-69 (1982).

DETERMINATION OF ASH

NG MUI CHNG

INTRODUCTION

The principle of ashing is to burn off the organic matter and to determine the inorganic matter which remains. This method is applicable to all food materials, and heating temperature is 525-600°C. Fish and fishery products are ashed at 550°C. Heating is carried out in two stages: firstly to remove the water present and to char the sample thoroughly; and finally ashing at high temperature in a muffle furnace.

A phosphorus rich sample which contains acidic ash usually loses some chlorine ions during ashing. On the other hand, alkaline ash from cation rich elements normally absorbs some carbon dioxide during ashing.

APPARATUS

Muffle furnace with temperature display (0-1200°C)
Crucibles and lids (15-20 ml volume)
Tongs
Thick gloves

SAMPLE PREPARATION

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

Place minced meat in a small plastic bag.

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 are burnt off. Cool the crucible in the desiccator (30 mins).

2. Weigh the crucible and lid to 3 decimal places (W_o).

3. Weigh about 5g meat sample prepared into the crucible (W_1). Sometimes high moisture content samples and fatty samples are dried in an oven. Heat over a 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. Ash must be white or light grey. If not, return the crucible and lid to the furnace for further ashing.

6. Weigh the ash with crucible and lid to 3 decimal places (W_2).

CALCULATION

$$\text{Ash Content (\%)} = \frac{W_2 - W_0}{W_1 - W_0} \times 100$$

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 PANG YONG

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 affects 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, thus 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, avoiding 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 a 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 the 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) **CO_2-free distilled water**

Boil the distilled water in a round bottomed 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 PROCEDURE

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 CO_2-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

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

   Add 45 ml of CO_2-free distilled water to the sample and homogenise for 30 seconds. Read pH of sample.

MEASUREMENT OF FREE AND EXPRESSIBLE DRIPS

NG CHER SIANG

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. Thawing procedure affects the drip amount. 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 ø cork borer
Petri dishes
Filter paper (Whatman No. 1, ø 7 cm)
Screw press
Stop watch

II PROCEDURE

1. A 2 cm ø 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-layer filter paper on top and 3-layer filter paper below. Place the whole in the press.

5. Slowly increase the pressure to 1-2 kg/cm^2 within 30 sec.

6. Maintain at 1-2 kg/cm^2 constant pressure for 2 min. then remove the sample, and weigh the pressed sample (Z g).

III CALCULATION

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

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

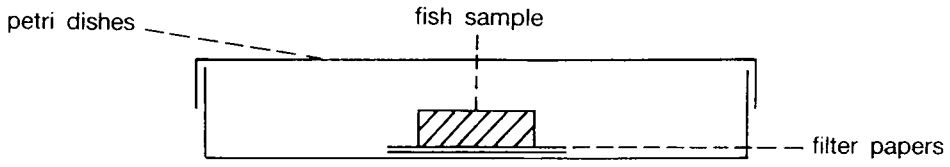
IV PRECAUTIONS

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.



REFERENCE

Shunro KATO (1979) : Theory and Application in Food Freezing, P.398 Korin, Tokyo

FISH PROTEIN EXTRACTIBILITY & ITS DETERMINATION

LIM PANG YONG

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, resulting in the textural changes of fish flesh.

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

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

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 of 22 g or more from the product. Place the sample in a polyethylene bag and store in a 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-103) or equivalent
Refrigerated centrifuge, capable of centrifuging at 12,500 g
Beakers, 100 and 250 ml
Bulb pipettes, 10, 20 & 40 ml
Glass funnels 60 mm ø
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 a 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

1. Total nitrogen. Accurately weigh a duplicate of 1 g of 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 3 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 aliquots used as follows:-

1. For myofibrillar protein nitrogen (MPN)

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

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)

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

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)

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

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 precipitation 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 (I) in protein determination by Kjeldahl method (B-1 Section IV) for calculation of the 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 PANG YONG

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 of 70 g (minimum) from the product. Place the sample in a polyethylene bag and store in a 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 (K_2HPO_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 into a reagent bottle and store in refrigerator.

IV PROCEDURES

1. Weigh ca. 70 g meat sample into the cylinder of the SN type homogeniser.

2. Add 500 g chilled distilled water (ca 10°C) into the cylinder of the homogeniser.

3. Completely remove the air bubbles in the meat sample using the homogeniser at slow speed.

4. Add 100 ml extraction solution and homogenise in 3 mins with speed dial at 3-4.

5. Transfer the meat sol to a 1 litre beaker and keep in ice water (below 5°C) for 20 mins.

6. Measure the 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°C).

7. Read the viscometer when the pointer is 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 MUI CHNG

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 (ø ca 2.5 cm)
Fudoh Rheometer (Model NRM-2002J)
Knife
Cutting board
Trays
Stainless steel moulds — i) ø 2.5 cm, 2.4 cm thick for gel strength measurement.
ii) ø 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 10 min 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, ø 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 ø 2.5 cm for the organoleptic assessment.

For fish jelly products

1. Fish jelly products eg. fish balls, fish cakes, 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:-

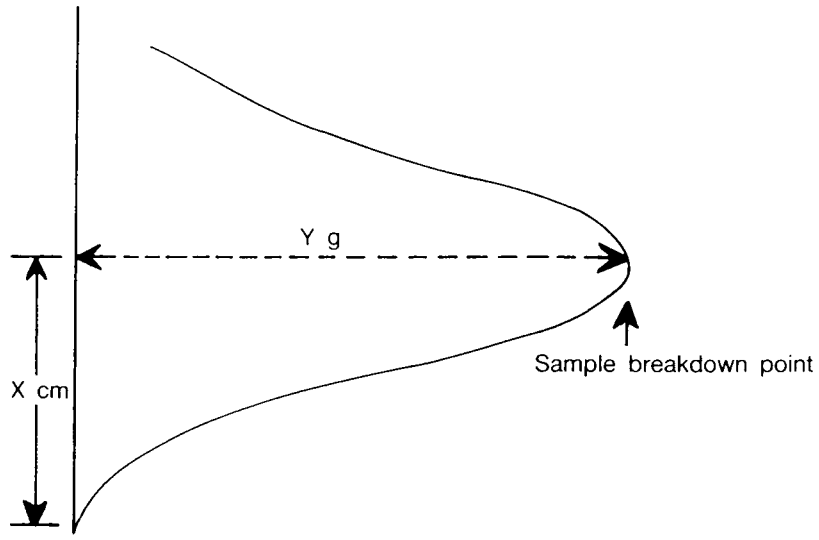
	Rheometer	Chart Recorder	Factor
Speed (cm/min)	6	12	(x ½)
Sensitivity (volts)	1	1/2	(x ½)

2. Place a test piece on the sample holder and switch ON the Fudoh rheometer and chart recorder simultaneously.

3. When test piece is broken as indicated in the recorder chart, switch OFF the chart recorder and rheometer.

4. Repeat with all the test pieces to obtain the average results.

CALCULATION



Recorder Chart

Gel strength = X x Y x F g.cm

where F, factor = ½ x ½ = ¼

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:-

Condition of test samples when folded	Grade
• 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 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.

Score	Grade
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.

WATER ACTIVITY (AW) BY CONWAY'S MICRODIFFUSION METHOD

MAKOTO YAMAGATA

INTRODUCTION

The spoilage of low moisture foods is highly influenced by their water activities. The activity of water in a mixture whether in gas phase or otherwise, is conveniently expressed as the fugacity of water in the mixture divided by the fugacity of pure water at the same temperature and its own pressure. Fugacity on the other hand is defined as the measure of the tendency of a component to escape. It has a unit and dimension of vapour pressure. Fugacity is a "corrected pressure" and water vapour pressures at ambient temperature are still low, i.e. water vapour behaves about perfectly. This justifies for normal cases to define water activity (Aw) as relative humidity shown below

$$Aw = \frac{Pws}{Pws}$$

where Pw = equilibrium water vapour pressure above the food

Pws = water vapour pressure above pure water after saturation

At water activities lower than about 0.90, most pathogenic bacteria cannot grow. For xerophilic moulds, the lowest limit is at Aw of 0.65. In between these, all types of yeasts and moulds may grow.

At very low water activities fat oxidation is promoted. If the water activity is increased, the reaction rate is slowed down at first due to stabilisation of fatty acid hydroperoxides. At higher water contents fat oxidation may be promoted again, which can be partly explained by an increase in the mobility of heavy metal ions catalysing fat oxidation.

Maillard reaction, which causes a decrease in nutritive value and undesirable sensory changes, has a maximum reaction rate in the intermediate range of water activity.

There are 2 methods of deducing water activity using Conway's unit, namely the "Sandwich" Method and the Graph Insert Method. The "Sandwich" method is used for samples whose water activity is previously known or where there is an expected water activity for the sample such as in the case of fish sausage (AW = 0.94). The Graph Insert Method is used when no previous knowledge of expected Aw is available for the sample.

APPARATUS

1. Conway's microdiffusion unit/dish and cover
2. Incubator (25°C)
3. Analytical balance (to 4 decimal places)

REAGENTS

The choice of salts used should be those with water activity in the same range as the expected water activity of the sample.

TABLE 1. Water activity (Aw) of saturated salts at 25°C.

Salts	Aw	Salts	Aw
$K_2Cr_2O_7$	0.980	$CrCl_2.H_2O$	0.708
K_2SO_4	0.968	$NaBr.2H_2O$	0.577
KNO_3	0.924	$Mg(NO_3)_2.6H_2O$	0.528
$BaCl_2.2H_2O$	0.901	$LiNO_3.3H_2O$	0.470
KCl	0.842	$K_2CO_3.2H_2O$	0.427
KBr	0.807	$MgCl_2.6H_2O$	0.330
NaCl	0.752	CH_3COOK	0.224
$NaNO_3$	0.737	$LiCl.H_2O$	0.110

PROCEDURE

Sample preparation

1. Weigh 10 - 20 g of sample and chop into 1 - 2 mm cubes.

Analytical Procedure

1. Pipette 4 ml of the standard salt solutions[*1, *2] (Aw > 0.94 and Aw < 0.94) into the outer ring of each Conway dish.
2. Weigh accurately 1 g of sample in aluminium foil dish and put into the inner ring of each Conway's dish.

3. Fix the cover with fixing reagent (white vaseline), and clip tightly.

4. Place in an incubator at a temperature of 25°± 2°C for 2 ± 0.5 hours.

5. After incubation, weigh the samples using an analytical balance.

CALCULATION

1. "Sandwich" Method

$$Aw = \frac{bx - ay}{x - y}$$

where : Aw = water activity

a = Aw of saturated A salt (Aw > 0.94)

b = Aw of saturated B salt (Aw < 0.94)

x = increase in sample weight when using saturated A salt (mg)

y = decrease in sample weight when using saturated B salt (mg)

Figures used in measurement should be in 2 decimal places e.g. 0.86.

2. Graph Insert Method

To determine the water activity of an unknown sample whose Aw is estimated to be between 0.842 and 0.968, four standards of saturated salts namely K_2SO_4, KNO_3, $BaCl_2.2H_2O$ and KCl should be used. The chopped unknown samples are weighed into different preweighed Conway units each containing one of the four salts. After incubation the percentage change in weight is recorded (Table 2). A graph of percentage weight change (vertical axis) against the water activities of standard saturated salts (horizontal axis) is plotted and the water activity of the unknown sample is the intercept of the graph against the X-axis as shown in Fig. 1 below.

TABLE 2. Graph of percentage weight change of Conway unit against water activities of saturated salts.

Saturated salt	Water activity	Weight change (%)
K_2SO_4	0.968	+4.8%
KNO_3	0.924	+2.8%
$BaCl_2.2H_2O$	0.901	+1.8%
KCl	0.842	−1.8%

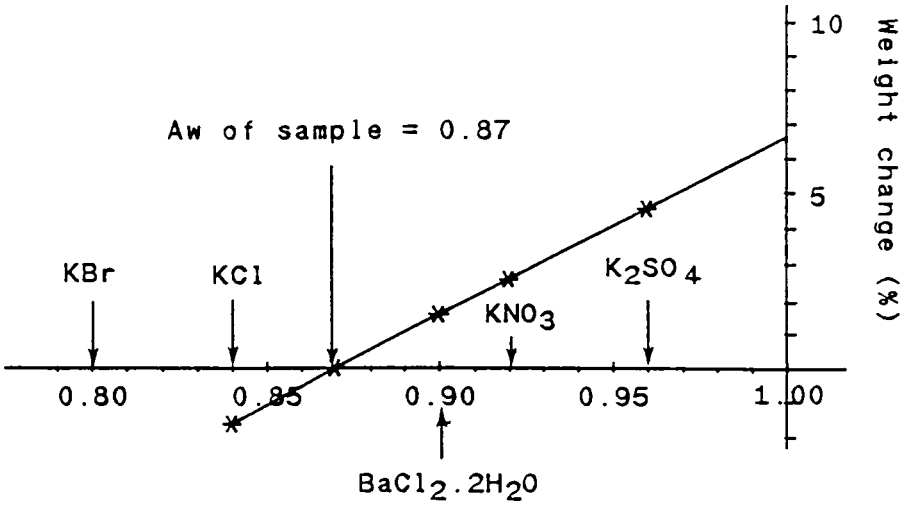


Fig. 1 Graph Insert Method

REMARKS

*1 In the case where the expected Aw is 0.94 (Japanese Agricultural Standard for Aw of fish sausage is less than 0.94), the salts used should be K_2SO_4 (Aw = 0.968) and KNO_3 (Aw = 0.924).

*2 In the case where the expected Aw is not clear, the salts used should be 4 to 5 reagents.

N.B. 1) This method is not applicable to food products containing alcohol.

2) The saturated salt solutions should be kept at 25°C.

3) Instead of using saturated salt solutions, 4 - 5 g of the reference salt (Table 1) is put into the outer ring of the Conway dish and a small amount of distilled water (0.5 - 1.0 ml) is added.

REFERENCES

Environmental Health Bureau, Ministry of Health and Welfare, Japan. Detection of water activity. Kannyu No. 82, 12 December, 1974.

Van den Berg, C. and Bruin, S. (1981). Water Activity and its Estimation in Food Systems: Theoretical aspects. In : Influences on Food Quality. Ed. Louis B. Rockland and George F. Stewart. Academic Press.

Eichner, K. (1986). The influence of Water Content and Water Activity on Chemical Changes in Foods of Low Moisture Content under Packaging Aspects. In : Food Packaging and Preservation Theory and Practice. Ed. M. Mathlouthi. Elsevier Applied Science Publishers.

Gould, G.W. (1989). Drying, Raised Osmotic Pressure and Low Water Activity. In: Mechanisms of Action of Food Preservation Procedures. Ed. G.W. Gould. Elsevier Applied Science Publishers.

Standard Methods of Analysis for Hygienic Chemists - With Commentary - authorized by the Pharmaceutical Society of Japan, Kanehara Shuppan K.K. (1990).

DETERMINATION OF CHEMICAL PROPERTIES OF MEAT

PROTEIN DETERMINATION BY KJELDAHL METHOD

LIM PANG YONG

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:

$$\text{Protein N} \xrightarrow[\text{Catalyst}]{H_2SO_4} (NH_4)_2SO_4 + CO_2\uparrow + H_2O$$

$$(NH_4)_2SO_4 + NaOH \longrightarrow Na_2SO_4 + 2NH_4OH$$

$$NH_4OH \longrightarrow NH_3 + H_2O$$

$$3NH_3 + H_3BO_3 \longrightarrow NH_{4+} + BO_3 \blacksquare$$

$$BO_3 \blacksquare + 3H^+ \longrightarrow H_3BO_3$$

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

a) Sulphuric acid (H_2SO_4), nitrogen free

b) Catalyst

Mix 9 parts of potassium sulphate (K_2SO_4) anhydrous, nitrogen free with 1 part of copper sulphate ($CuSO_4$), anhydrous, nitrogen free.

c) NaOH solution (40% w/v)

Dissolve sodium hydroxide (NaOH), technical grade, mini pearls, in distilled water.

d) Boric acid (4% w/v)

e) Anti-bumping granules

f) Ethanol (95% v/v)

g) 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 is well mixed. Transfer the contents to the automatic burette.

h) 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 (1g) 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 digestor and digest at about 110 - 130°C for 15 mins (ignore this process if non liquid sample is to be digested). Turn the digestor 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 carefully into the 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 a 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 changes from green to pinkish. Read volume of acid used for titration.

IV CALCULATIONS

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

a) solid/semi-solid fish sample

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

where Ws = 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 PANG YONG

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 ($CuSO_4.5H_2O$)

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 $CuSO_4.5H_2O$ 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 these two solutions and dissolve it in 20 ml distilled water. Mix these two solutions and keep in refrigerator. 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 preferable 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)):

   N content (mgN/ml) = (b - a) X 0.1 X 14.00 X 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 O 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.

$Absorbance_{545nm}$ = $(O.D._A - Blank_A) - (O.D._B - Blank_B)$

$O.D._A$ 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 545nm, 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 (VB-N) BY CONWAY'S MICRODIFFUSION METHOD

(1% Boric acid and 0.02N Hydrochloric acid)

NG CHER SIANG AND LOW LAI KIM

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 bacterial activity, and the role of autolysis was negligible. The source of TMA was derived both from bacterial 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 and grouper. TMA is not a good indicator of freshness for lizard fish. Instead, DMA and FA are suitable indices.

The total volatile basic substances (VB-N) in fish meat is mainly composed of ammonia, TMA, and DMA. The level of VB-N 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 VB-N 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 VB-N

The solution in the inner ring of the Conway unit contains 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 under 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.

APPARATUS

1. Conway's unit:

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

2. Micro-burette (to 4 decimal places)

3. Oven (37°C)

4. Volumetric flasks, 10 ml, 100ml, 1000 ml

5. Pipettes, 1 ml, 10 ml

6. Mortar and pestle

7. Centrifuge, 3,000 rpm

8. Centrifuge tubes

9. Weighing balance

REAGENTS

1. Inner ring solution - 1% boric solution containing indicator

   Weigh 10 g of boric acid into 1 litre volumetric flask then add 200 ml of ethanol. After dissolving the boric acid, add 10 ml of mixed indicator solution, then make up to 1 litre with distilled water.

2. Mixed indicator solution

   Dissolve bromocresol green (BCG) 0.01 g and methyl red (MR) 0.02 g in 10 ml of ethanol.

3. 0.02N Hydrochloric acid, HCl

   Dilute 20 ml of 1N HCl standard solution with distilled water and make up to 1000 ml.

4. Saturated potassium carbonate (K_2CO_3) solution

   Weigh 60 g of K_2CO_3 and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, filter through filter paper.

5. 50% potassium carbonate (K_2CO_3) solution

   Dilute saturated K_2CO_3 solution to twice its volume with distilled water.

6. 4% Trichloroacetic acid (TCA, CCl_3COOH) solution:

   Dissolve 40 g of TCA in 960 ml of distilled water.

7. Sealing agent

   Weigh 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.

8. 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 the filtrate 3 times with distilled water.

9. 1% Titanium trichloride ($TiCl_3$) aqueous solution*

   Pipette 6.7 ml of 15% $TiCl_3$ into 100 ml volumetric flask and make up to 100 ml with distilled water.

10. Saturated potassium nitrate (KNO_3) aqueous solution

    Dissolve about 55 g of KNO_3 in 50 ml of distilled water.

* If the stock $TiCl_3$ has been stored for sometime, a recovery test using various concentrations (%) of $TiCl_3$ should be carried out and the appropriate percentage yielding close to 100% recovery should be used.

PROCEDURE
(Refer to Fig. 1)

Sample Preparation

1. Weigh 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. 1) or centrifuge at 3000 rpm for 10 min.
5. Keep the filtrate (sample solution) in -20°C freezer if necessary.

Analytical Procedure

1. Determination of VB-N

   1.1 Apply sealing agent to Conway's unit.

   1.2 Pipette 1 ml of inner ring solution into inner ring.

   1.3 Pipette 1 ml of sample solution into outer ring.

   1.4 Slant the Conway's unit with cover.

   1.5 Pipette 1 ml of saturated K_2CO_3 solution into outer ring.

   1.6 Immediately close the unit and tighten with clip.

   1.7 Mix the outer ring solutions gently.

   1.8 Stand for 60 min at 37°C in incubator.

   1.9 Titrate inner ring solution against 0.02N HCl using a micro-burette until green colour turns pink.

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

2. Determination of TMA-N

2.1 Apply sealing agent to Conway's unit.

2.2 Pipette 1 ml of inner ring solution into inner ring.

2.3 Pipette 1 ml of sample extract into outer ring.

2.4 Pipette 1 ml of neutralized 10% formaldehyde into outer ring and gently mix the outer ring solutions.

2.5 Slant the Conway's unit with cover.

2.6 Pipette 1 ml of saturated K_2CO_3 solution into outer ring.

2.7 Immediately close the unit and tighten with a clip.

2.8 Mix the outer ring solutions gently.

2.9 Stand for 60 min at 37°C in incubator.

2.10 Titrate inner ring solution against 0.02N HCl using a micro-burette until green colour turns pink.

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

3. Determination of TMAO-N

3.1 Take 2 ml of the sample solution into a test tube.

3.2 Add 1 ml of 1% $TiCl_3$ and fully mix. The solution will turn violet.

3.3 Stand in a 80°C water bath for 90 sec. The violet colour should disappear.

3.4 Add saturated KNO_3 dropwise in cases where violet colour persists until it disappears.

3.5 Cool in running water.

3.6 Transfer the solution to a 10 ml volumetric flask.

3.7 Make up to 10 ml with washings and distilled water.

3.8 Proceed as for TMA-N determination

CALCULATION

TMA-N or VB-N (mg/100g)	=	Amt. of HCl used in titration	X	Amt. of ammonium nitrogen equivalent to 1 ml of 0.02N HCl	X	Ratio of the amt. of sample used to 100g muscle

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

TMAO-N (mg/100g) = (TMA-N after $TiCl_3$ reduction X 5) - (TMA-N before $TiCl_3$ reduction)

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

V_B = Titration volume of 0.02N HCl for blank (ml)

N_{HCl} = Normality of HCl (=0.02N x 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

N.B. 1 ml of 0.02N HCl = 0.28 ammonia nitrogen

= $(N_{HCl} \times f \times 14.00)$

Detection limit : 0.2 mg/100g

REFERENCE

Beatty, S.A. and N.E. Gibbons. (1936). The measurement of spoilage of fish. J. Biol. Bd. Can. 3:77-91.

Bysted, J., L. Swenne, H.W. Aas. (1959). Determination of trimethylamine oxide in fish muscle. J. Sci. Food Agric. 10:301-304.

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.

Conway, E.J. (1950). Microdiffusion analysis and volumetric error. Crosby Lockwood and Son Ltd., London.

Yamagata, M., K. Horimoto and C. Nagaoka. (1969). Assessment of green tuna : Determining trimethylamine oxide and its distribution in tuna muscles. J. Food Sci., 34(2):156 - 159.

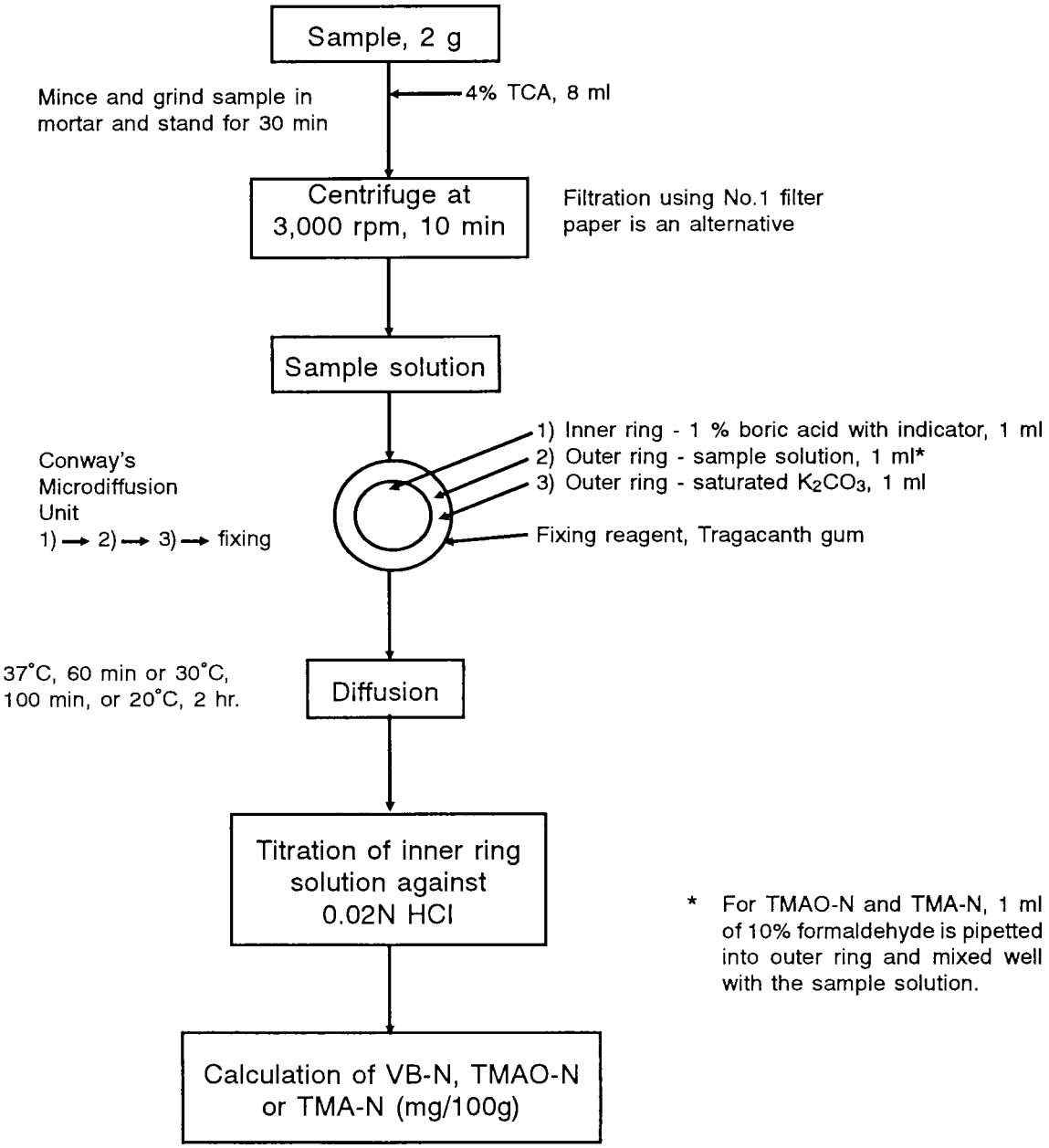


Fig. 1 Analytical procedure for VB-N, TMAO-N, TMA-N analysis

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

NG CHER SIANG

INTRODUCTION

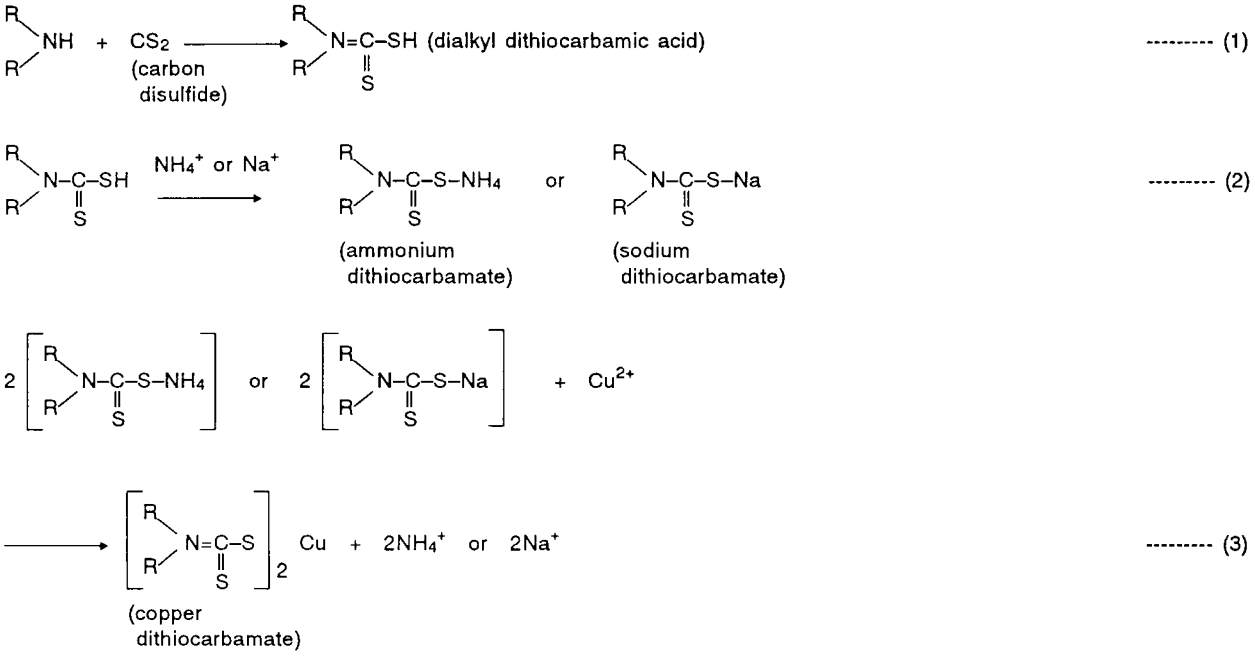
The precursor of dimethylamine (DMA) in fish meat is trimethylamine oxide (TMAO). In the gadoid species, TMAO present in ordinary muscle is decomposed to formadehyde (FA) and DMA simultaneously. This is usually attributed to endogenous enzymes. In the tropics, lizard fish is known to show a similar breakdown 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. react with carbon disulfide to form dialkyl-dithiocarbamic acid (Equation 1). This dialky-dithiocarbamic acid react with NH_4^+ or Na^+ to form 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 (CS_2) 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.

g) 25% TCA, 2% TCA

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% CS_2-toluene solution and then stopper the test tube.
4. Stand for 2 min in 50°C water bath.
5. Shake for 1 min using shaker to bring the colour complex from water layer to solvent layer.
6. Add 1 ml of 30% acetic acid to be added while tube is warm.
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:

$$DMA.HCl \xrightarrow[\Delta,\ \text{steam}]{NaOH} DMA\uparrow + NaCl + H_2O \qquad (1)$$

$$DMA + H_3BO_3 \longrightarrow DMA.H_3BO_3 \qquad (2)$$

$$DMA.H_3BO_3 + H_2SO_4 \longrightarrow DMA.H_2SO_4 + H_3BO_3 \qquad (3)$$

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 $DMA.H_3BO_3$ solution with 0.05N H_2SO_4 using methyl red-bromocresol green mixed indicator.

4. Calculate the factor of DMA.HCl standard solution as:

$$\text{factor, f} = \frac{[(\text{Vol. of sample titration}) - (\text{Vol. of blank titration})] \times (\text{Mol. wt. of N equivalent}) \times (\text{Normality of } H_2SO_4)}{\text{Volume of DMA.HCl used}}$$

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

D. PREPARATION OF CALIBRATION CURVE

1. Take 0.4, 0.8, 1.2, and 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 ug 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 ug) contained in 5 ml of sample solution from the calibration curve.

2. DMA-N (mg/100 g)

$$\text{DMA-N (mg/100 g)} = \frac{(\text{ug DMA-N converted to mg}) \times (\text{Make up volume}) \times (100\,\text{g meat}) \times f \times d}{(\text{Wt. of sample}) \times (\text{Volume of sample})}$$

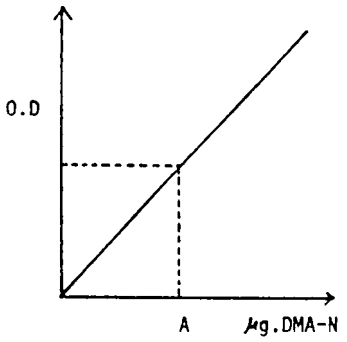
$$= \frac{A}{1000} \times \frac{100 \times 100 \times f \times d}{W \times 5}$$

where W = weight of meat

f = factor of DMA standard solution

d = dilution factor (if any)

A = readout from calibration curve



REFERENCE

Dyer W.J. and Y.A. Mounsey (1945). Amines in fish muscle. II. Development of trimethylamine and other amines. J. Fish. Res. Bd. Can. 6(5):359-367.

DETERMINATION OF FORMALDEHYDE IN FISH MEAT USING NASH'S REAGENT

NG CHER SIANG

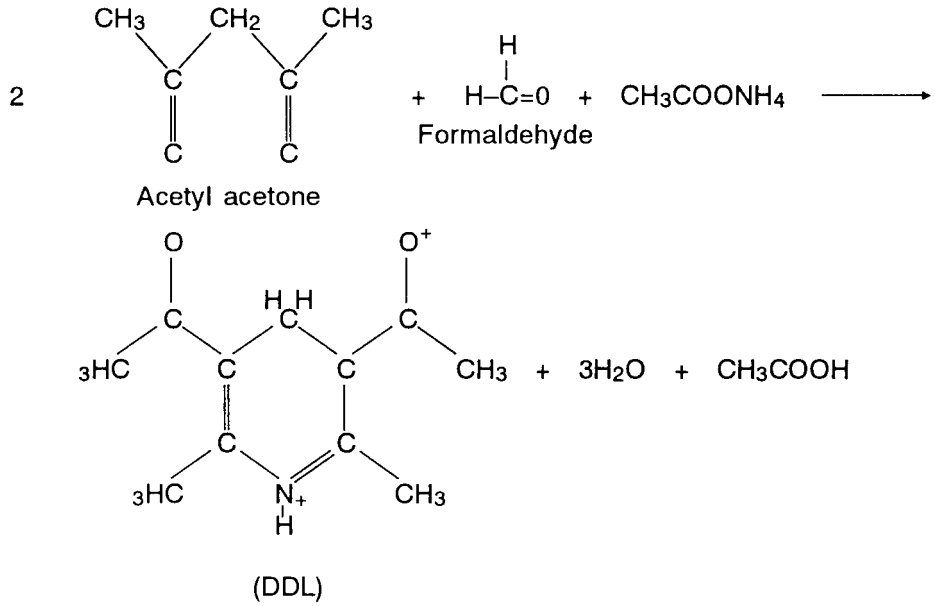
INTRODUCTION

It has been postulated that the enzymatic degradation of trimethylamine oxide (TMAO) results in the simultaneous formation of dimethylamine (DMA) and formaldehyde (FA). This phenomenon had been reported to have a correlation of 0.89. (Amano et al, 1963). FA and DMA formation occurs widely in the gadoid species. In the tropical area, lizard fish (Saurida sp) also exhibits this trend.

Formaldehyde reacts quickly with muscle tissues, causing protein denaturation. Formation of FA is accelerated by freezing.

In this method of FA determination, FA is reacted with an ammonium salt and acetylacetone under neutral conditions to form diacetyldihydrolutidine (DDL). DDL is a yellow compound with maximum absorbance at 412 nm.

MOLECULAR FORMULA AND REACTION



(DDL)

I REAGENTS

a) **Acetylacetone reagent (Nash's reagent)**

Dissolve 150 g of ammonium acetate, 3 ml of acetic acid and 2 ml of acetylacetone in distilled water and make up to 1 litre.

b) **Formaldehyde standard stock solution (1,000 ppm)**

Pipette 0.3 ml of 35% formaldehyde and make up to 100 ml with distilled water to get an approximately 1,000 ppm solution. This aqueous solution is stable for several months.

c) **Formaldehyde standard stock solution, working solution (10 ppm)**

Dilute the stock solution 100 times as follows:

Pipette 10 ml of the stock solution (approximately 1,000 ppm) and make up to 100 ml with 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 thiosulphate standard solution**

Dissolve 25 g of $Na_2S_2O_3.5H_2O$ 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 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 distilled water (blank solution) 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. Titrate the blank with iodine solution until the colour turns brown. Note the volume (A ml) of the iodine solution used.

4. Then titrate against sodium thiosulphate solution using 1 ml of starch as indicator until the solution turns colourless. Note the volume of sodium thiosulphate used.

5. Pipette 5 ml of formaldehyde solution (approximately 1,000 ppm) into a 200-300 ml conical flask, and add 50 ml of distilled water.

6. Add 10 ml of 0.1N sodium bisulfite solution and let it stand for 30 min with occasional shaking.

7. Add the known amount (A ml) of iodine solution, swirl and titrate against sodium thiosulphate solution using 1 ml of starch as indicator until the solution turns colourless. Note the volume of sodium thiosulphate used.

The specific gravity of 35% formaldehyde at 20°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 thiosulphate, using $N_1 V_1 = N_2 V_2$,

N_1, Normality of formaldehyde = factor x Molarity x equivalent

= f x M x e

= f x 0.032375 x 2

V_1 = Volume of formaldehyde = 5 ml

N_2 = Normality of $Na_2S_2O_3$ = 0.1

V_2 = Volume of $Na_2S_2O_3$

= (Vol titrated in sample) – (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 (= 412 nm)
Beckman Model 3560 digital pH meter
Yamato ultra disperser
beakers (50 ml)
long test-tubes (15 ml)
burette (25 ml)
Pipetman micropipette (max vol = 1 ml)
filter paper (Whatman No. 41, ø 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 supernatent 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 ug of formaldehyde, respectively.

2. Add 3, 2.6, 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

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

where A = Reading from calibration curve (ug)

f = factor of formaldehyde of standard solution.

REFERENCES

Amano K., K. Yamada and M. Bito 1963A.
Detection and identification of formaldehyde in gadoid fish. Bull. Jap. Soc. Sci. Fish. 29(7): 695-701.

Amano K., K. Yamada and M. Bito 1963B,
Contents of formaldehyde and volatile amines in different tissues of gadoid fish. Bull. Jap. Soc. Sci. Fish. 29(9): 860-864.

Hebard C.E., J.F. George and R.E. Martin.
Occurence 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
(Ion Exchange Chromatography Method)

NG CHER SIANG

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:-

$$\text{ATP} \longrightarrow \text{ADP} \longrightarrow \text{AMP} \longrightarrow \text{IMP} \longrightarrow \text{HxR} \longrightarrow \text{Hx}$$

ADP	=	adenosine diphosphate
AMP	=	adenosine monophosphate
IMP	=	inosine monophosphate
HxR	=	inosine or hypoxanthine riboside
Hx	=	hypoxanthine

The K value is defined as

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

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 pH 3, 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 1.

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 100-200 mesh Cl (chloride)-form (Bio-Rad Co.).

C. PREPARATION OF ION-EXCHANGE RESIN (SCHEME 2)

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 ø 6 mm) with coarse glass filter at the bottom part. The height of resin is around 50 mm.

IV PROCEDURE

A. PREPARATION OF SAMPLE EXTRACT

See Scheme 1.

B. PREPARATION OF ION-EXCHANGE RESIN

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 NH_4OH.
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 (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 has 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

$$K(\%) = \frac{E250nm\ A}{E250nm\ A + E250nm\ B} \times 100$$

where E250nm A : [Absorbance at 250nm of the solution A-eluate] – [Absorbance at 250nm of the soln A];

E250nm B : [Absorbance at 250nm of the solution B-eluate] – [Absorbance at 250nm of the soln B].

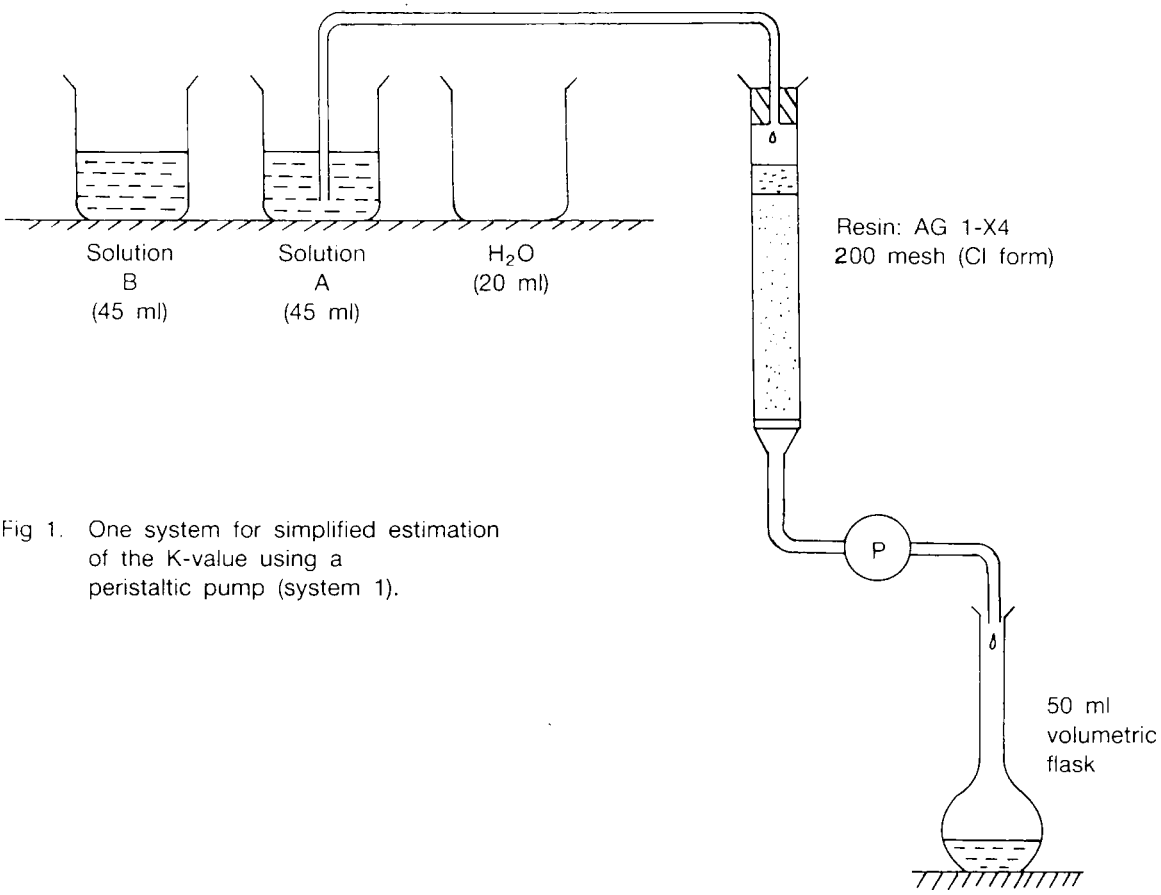


Fig 1. One system for simplified estimation of the K-value using a peristaltic pump (system 1).

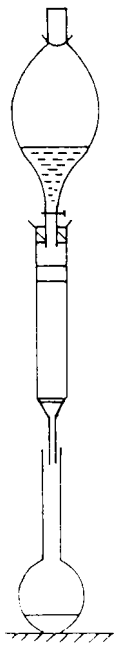


Fig 2. Another system for the estimation of K-value using a separating funnel (system 2).

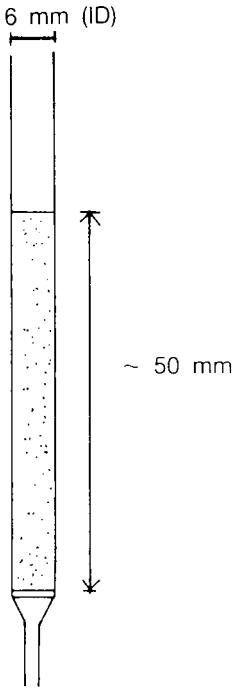
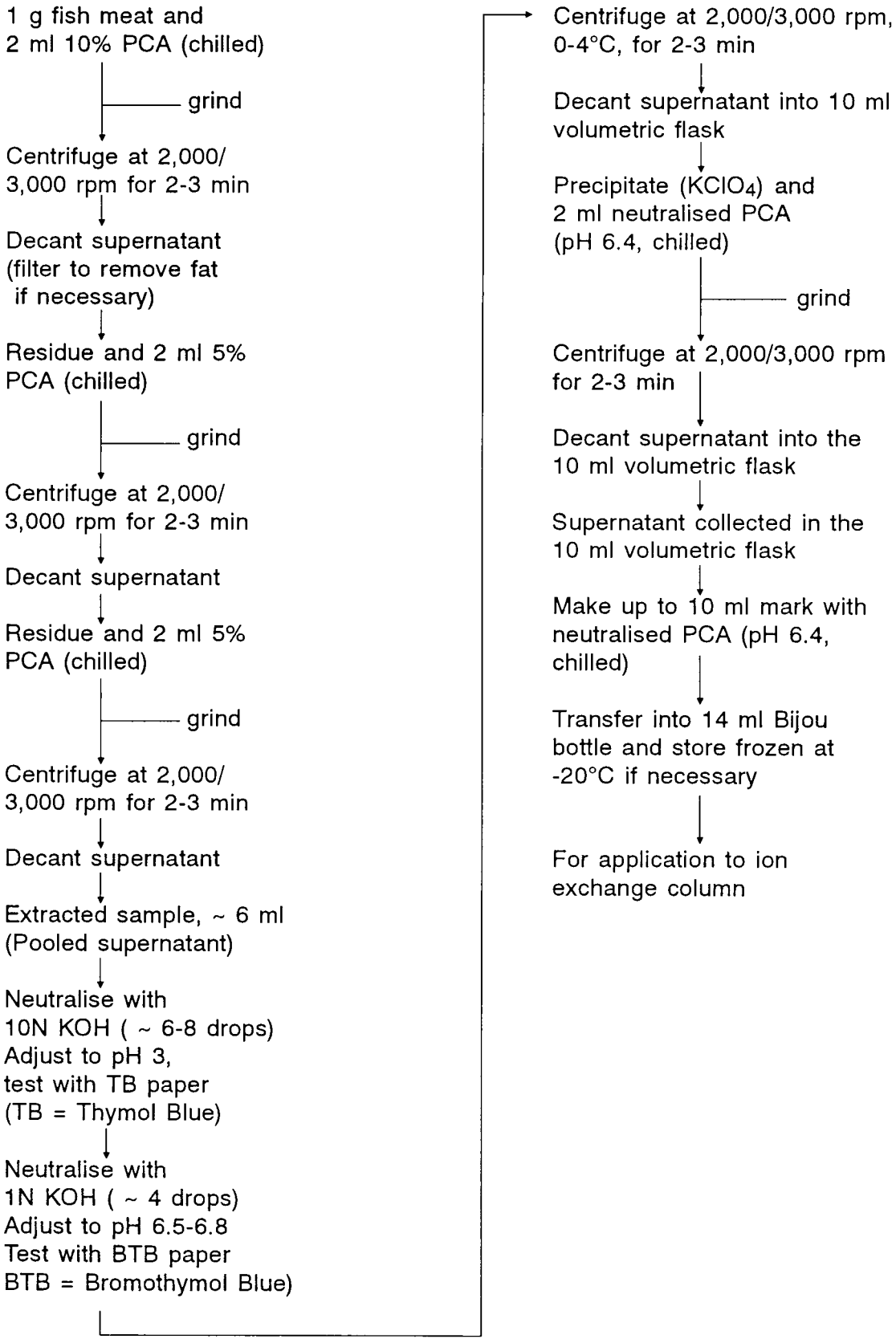
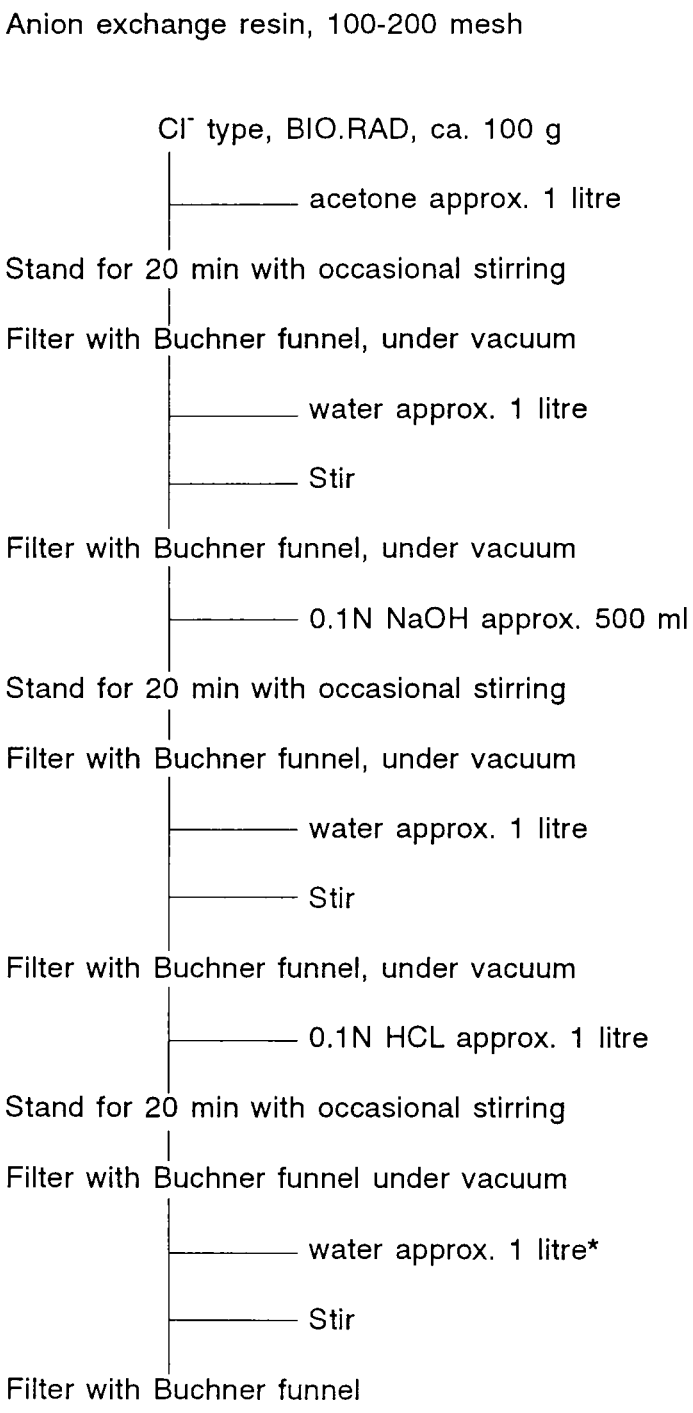


Fig 3. Column

SCHEME 1. PREPARATION OF FISH MUSCLE EXTRACT

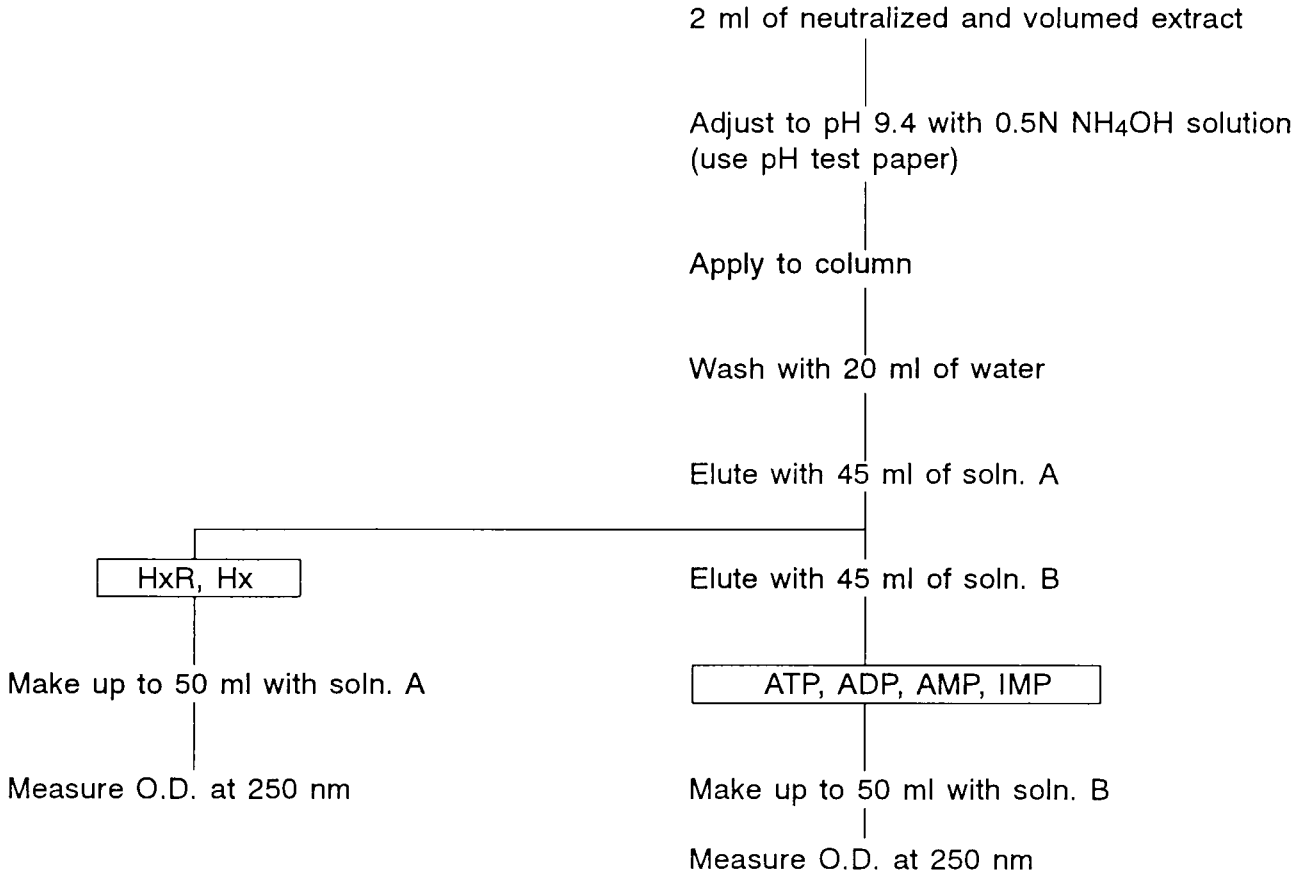


SCHEME 2. PREPARATION OF ION-EXCHANGE RESIN



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

SCHEME 3. SIMPLIFIED FRACTIONATION METHOD FOR K-VALUE



REFERENCES

Ehira S., H. Uchiyama, F. Uda and H. Matsumiya. (1970).
A rapid method for determination of acid soluble nucleotides in fish muscle by concave gradient elution. Bull. Jap. Soc. Sci. Fish. 36:391-496.

Uchiyama H., Ehira S., and Kato N. (1972).
Analytical methods of estimating freshness of fish. In Okada et al (Eds). Utilization of marine products.
Publ: Overseas Technical Cooperation Agency Govt. of Japan.

Uchiyama H. (1978).
Analytical method for estimating freshness of fish. Training Dept. SEAFDEC: 1-9.

Uchiyama H. and Kakuda K. (1984).
A simple and rapid method for measuring K value, a fish freshness index. Bull. Jap. Soc. Sci. Fish. 50(2):263-267.

FRESHNESS TESTING PAPER

NG CHER SIANG

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 has 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 disadvantages 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 $\frac{HxR + Hx}{ATP + ADP + IMP + AMP + HxR + Hx} \times 100$

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.

$$HxR + P_i \underset{\text{nucleoside phosphorylase}}{\rightleftharpoons} 4\ Hx + \text{ribose-1-phosphate}$$

$$Hx + O_2 \xrightarrow[\text{xanthine oxidase}]{} \text{xanthine} + H_2O_2$$

$$\text{xanthine} + O_2 \xrightarrow[\text{xanthine oxidase}]{} \text{uric acid} + H_2O_2$$

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.

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 are 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 has developed.

---

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

DETERMINATION OF VOLATILE BASIC NITROGEN (VB-N), TRIMETHYLAMINE OXIDE NITROGEN (TMAO-N) AND TRIMETHYLAMINE-NITROGEN (TMA-N) BY CONWAY'S MICRO-DIFFUSION METHOD (N/150 Hydrochloric acid and N/70 Barium hydroxide)

MAKOTO YAMAGATA AND LOW LAI KIM

INTRODUCTION

There are two types of titration in the total volatile basic nitrogen (VB-N) and trimethylamine-nitrogen (TMA-N) determinations by the Conway's microdiffusion method. The first involves an acid-alkali reaction using hydrochloric acid and barium hydroxide [HCl-$Ba(OH)_2$] and the second involves a buffering effect using hydrochloric acid and boric acid [HCl-H_3BO_3].

With the acid-alkali reaction method, more sensitive and accurate determinations of VB-N and TMA-N are possible. In addition the colour change at the end-point is very sharp and clear, reducing error caused by human visual judgement.

APPARATUS

1. Scalpels, knives, scissors and pincers
2. Mortars and pestles (ø: 50 - 120 mm)
3. Top loading balance (sensitivity : less than 0.1 g)
4. Filter papers (Whatman No. 1; ø : 55 - 60 mm)
5. Conway's unit
6. Bulb pipettes, 1 ml, 5 ml, 20 ml
7. Komagome pipettes, 1 ml, 2 ml
8. Micro-burette (Capacity 0.15 ml, minimum scale 0.001 ml)

REAGENTS

(Analytical grade)

1. 4% Trichloroacetic acid, TCA

   Dissolve 40 g of TCA (CCl_3COOH, MW = 163.4 g) in distilled water and make up to 1000 ml.

2. N/150 Hydrochloric acid, HCl (containing Tashiro's indicator)

   Pipette 10 ml of Tashiro's indicator solution into a 1000 ml measuring flask. Add 200 ml of ethyl alcohol, followed by about 200 - 300 ml of distilled water. Titrate againstN/10 KOH (potassium hydroxide) till the reddish colour fades away. Then pipette 66.6 ml of N/10 HCl (it is possible to use commercial reagent) into the above measuring flask and make up to 1000 ml with distilled water.

Determine the factor of N/150 HCl and store in brown coloured reagent bottle. 1 ml of N/150 HCl has an equivalent of 0.353 mg Na_2CO_3, therefore 10 ml of N/150 HCl has an equivalent of 3.53 mg Na_2CO_3.

3. N/70 Barium hydroxide, $Ba(OH)_2$

   Dissolve 15.8 g of $Ba(OH)_2$ (MW = 315.50 g) in distilled water[*1] and make up to 1000 ml.

[*1] Distilled water should be boiled and cooled overnight with the mouth of the flask attached to a soda lime trap to remove carbon dioxide (CO_2).

   Pipette 142.9 ml of N/10 $Ba(OH)_2$ into 1000 ml measuring flask and make up to 1000 ml with CO_2-free distilled water. Store with paraffin seals.

4. Tashiro's indicator

   Mix 200 ml of 0.1% methyl red alcohol with 50 ml of 0.1% methylene blue alcohol and store in brown coloured reagent bottle.

5. Saturated potassium carbonate solution, K_2CO_3

   Dissolve 110 g of K_2CO_3 in 100 ml of distilled water and filter through Whatman No. 1 filter paper.

6. Reduced acid formaldehyde, HCHO

   Mix 100 ml of 35 - 37 % formalin well with 10 g of magnesium carbonate ($MgCO_3$) and filter through Whatman No. 1 filter paper. The formaldehyde concentration should be more than 10%.

7. 1% Titanium (III) chloride, $TiCl_3$[*2]

   Pipette 6.7 ml of 15% $TiCl_3$ solution into 100 ml volumetric flask and make up to 100 ml with distilled water.

[*2] If the stock $TiCl_3$ has been stored for sometime, a recovery test using various concentrations (%) of $TiCl_3$ should be carried out and the appropriate percentage yielding close to 100% recovery should be used.

8. Sealing agent

   Mix white vaseline with liquid paraffin in a ratio of 2:1.

PROCEDURE
(Fig. 1)

Sample Preparation

1. Weigh 5 g of well mixed sample into the mortar.

2. Pipette in 20 ml of 4% TCA.

3. Grind well and stand for 30 minutes.

4. Filter through Whatman No. 1 filter paper. Use the filtrate as sample solution.

Analytical Procedure

1. Determination of VB-N

   1.1 Apply sealing agent to the lid of Conway unit.

   1.2 Pipette 1 ml of N/150 HCl containing Tashiro's indicator into the inner ring.

   1.3 Pipette 1 ml of sample solution into the outer ring.

   1.4 Pipette 1 ml of saturated K_2CO_3 into the outer ring, cover the unit immediately and tighten with a clip.

   1.5 Gently mix the solutions in the outer ring well.

   1.6 Stand for 90 mins at 37°C, or 100 mins at 30°C, or for 120 mins at 20°C.

   1.7 Titrate the inner ring solution against N/70 $Ba(OH)_2$ with the aid of a thin glass rod. The end point of titration is reached when the inner ring solution changes from pinkish red to slightly green.

   All titrations should be done in duplicate (i.e. 2 Conway's units per sample).

   1.8 For blank, pipette 1 ml of 4% TCA instead of sample solution into outer ring.

2. Determination of TMA-N

   2.1 Apply sealing agent to the lid of Conway unit.

   2.2 Pipette 1 ml of N/150 HCl containing Tashiro's indicator into the inner ring.

   2.3 Pipette 1 ml of sample solution into the outer ring.

   2.4 Pipette 1 ml of reduced acid formaldehyde into the outer ring.

   2.5 Gently mix the outer ring solutions well and stand for 3 - 4 mins.

   2.6 Pipette 1 ml of saturated K_2CO_3 into the outer ring, cover the unit immediately and tighten with a clip.

2.7 Gently mix the solutions in the outer ring well.

2.8 Stand for 90 mins at 37°C, or 100 mins at 30°C, or for 120 mins at 20°C.

2.9 Titrate the inner ring solution against N/70 $Ba(OH)_2$ with the aid of a thin glass rod. The end point of titration is reached when the inner ring solution changes from pinkish red to slightly green.

All titrations should be done in duplicate (i.e. 2 Conway's units per sample).

2.10 For blank, pipette 1 ml of 4% TCA instead of sample solution into outer ring.

3. Determination of TMAO-N

3.1 Pipette 2 ml of the sample solution from Step 4 of Sample Preparation Part into a small test tube.

3.2 Pipette 1 ml of 1% $TiCl_3$ and mix well. The solution should turn violet.

3.3 Stand in an 80°C water bath for 90 sec. The violet colour should disappear.

3.4 If the violet colour persists, add saturated KNO_3 solution dropwise with shaking, until the violet colour disappears. Saturated KNO_3 is added dropwise only in cases where there is excess $TiCl_3$, if not it can be omitted.

3.5 Cool the tubes under running tap water.

3.6 Pipette 1 ml of the reduced sample solution into the outer ring.

3.7 Proceed as for TMA-N determination.

CALCULATION

$$\text{VB–N or TMA–N (mg/100 g)} = \frac{B - A}{B} \times f \times \left[\frac{14\,(4a + aw)}{150a}\right] \times 100$$

$$\text{TMAO–N (mg/100 g)} = \left\{\frac{B - A}{B} \times f \times \left[\frac{14\,(4a + aw)}{150a}\right] \times 100 \times \frac{3}{2}\right\} - (\text{TMA–N, mg/100 g}) *$$

where, B = titration value (ml) of blank test

A = titration value (ml) of the sample solution

f = factor of N/150 HCl

a = sample weight (g) = 5 g

w = moisture of sample expressed in decimal (e.g. for 80% moisture, use 0.8 for calculations)

* initial content of TMA-N (mg/100g) before reduction of sample solution.

Detection limit : 0.2 mg/100g

REFERENCE

Bysted, J., L. Swenne and H.W. Aas. (1959). Determination of trimethylamine oxide in fish muscle. J. Sci. Food Agric. 10:301-304.

Conway, E.J. (1950). Microdiffusion analysis and volumetric error. Crosby Lockwood and Son Ltd., London.

Yamagata, M., K. Horimoto and C. Nagaoka. (1969). Assessment of green tuna: Determining trimethylamine oxide and its distribution in tuna muscles. J. Food Sci., 34(2):156-159.

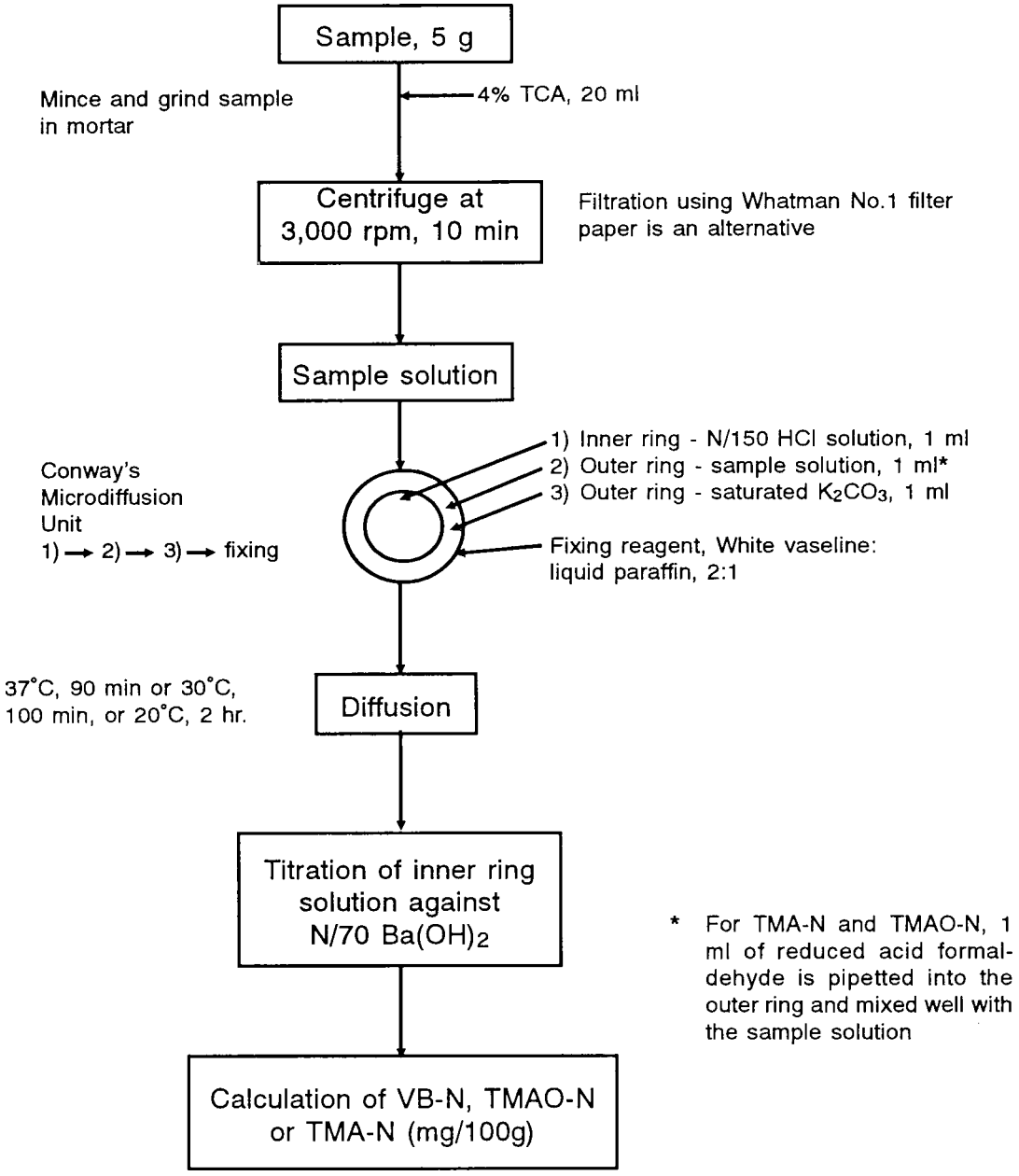


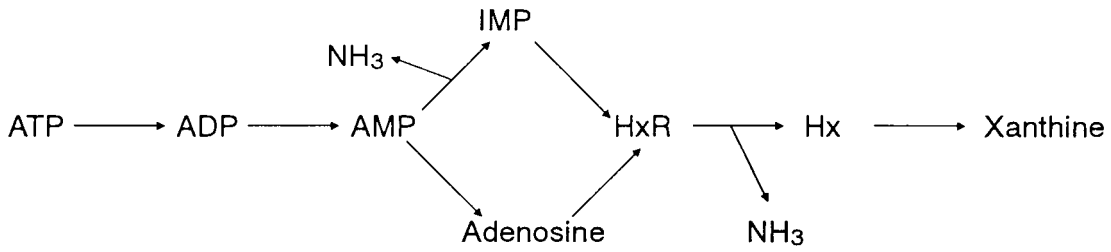
Fig. 1 Analytical procedure for VB-N, TMAO-N, TMA-N analysis

DETERMINATION OF AMMONIA (Colorimetric method)

LOW LAI KIM

INTRODUCTION

Ammonia was assumed to come from the breakdown of nitrogen containing compounds in teleosts and, in the case of elasmobranchs, of urea. Non-protein nitrogen (NPN) constituents and creatine are utilized by Pseudomonads. The primary mode of utilization seems to be oxidative deamination resulting in the accumulation of ammonia and volatile fatty acids. In hake, lactic acid, NPN compounds such as trimethylamine oxide and amino acids are attacked by Pseudomonads to yield trimethylamine, dimethylamine, ammonia and volatile acids. In ice stored shrimps, ammonia can be produced by the action of both micro-organisms and tissue enzymes, adenosine deaminase and adenosine monophosphate deaminase. The degradation pathway is shown as follows :



Where,

ATP	=	adenosine triphosphate,
ADP	=	adenosine diphosphate,
AMP	=	adenosine monophosphate,
IMP	=	inosine monophosphate,
HxR	=	inosine
Hx	=	hypoxanthine
NH_3	=	ammonia

APPARATUS

1. Erlenmeyer flask with stoppers, 100, 250, 500 ml
2. Volumetric flasks, 25, 100, 1000 ml
3. Measuring cylinders, 10, 250 ml
4. Glass filter funnel, ø : 7 cm
5. Filter paper, Whatman No. 1
6. Transfer pipettes, 1, 2, 3, 4, 5, 20 ml
7. Graduated pipettes, 10 ml
8. Separatory flask, 125 ml
9. Timer
10. Glass wool
11. Spectrophotometer (wavelength = 680 nm)

REAGENTS

1. Sodium hydroxide solution, NaOH(1+1)

   Add 1 part distilled water to 1 part NaOH (reagent quality containing <5% Na_2CO_3) in flask. Swirl till solution is completely mixed. Close with rubber stopper. Set aside until Na_2CO_3 has settled, leaving perfectly clear liquid (ca. 10 days).

2. Bromine solution

   Dilute 10 ml NaOH(1+1) from (1) to ca. 100 ml with distilled water. Add 1.0 ml bromine and shake. Dilute to 200 ml with water. Prepare fresh daily.

3. Thymol solution (10% in alcohol)

   Weigh 2.5 g thymol and dissolve in alcohol and make up to 25 ml with alcohol. Prepare fresh daily.

4. Dilute sodium hydroxide solution

   Dilute 25 ml of NaOH(1+1) from (1) to 100 ml with distilled water.

5. Ammonia standard solution

   Dry 5 g of ammonium chloride, NH_4Cl at 100°C for 1 hour. Dissolve 0.314 g NH_4Cl in distilled water and make up to 100 ml. This gives a solution which contains 40 ug/ml. Transfer 4.0 ml to 100 ml volumetric flask, and dilute to volume with distilled water.

6. 2.5% phosphotungstic acid solution

   Dissolve 25 g phosphotungstic acid or tungstophosphoric acid hydrate in distilled water and make up to 1 L. This should not be kept for more than a week. Keep the solution in an amber bottle.

7. n-Butanol (GR)

8. Anhydrous sodium sulphate (Na_2SO_4)

PROCEDURE

Sample Preparation

1. Grind sample 3 times through food chopper, mixing after each grinding.

2. Place 20 g prepared sample in 500 ml Erlenmeyer flask.

3. Add 180 ml 2.5% phosphotungstic acid solution, shake vigorously for 2 min.

4. Filter through Whatman No. 1 or equivalent paper into 250 ml Erlenmeyer flask.

5. Pipette 2 ml filtrate (equivalent to 0.2 g sample) into 125 ml separator. Save remainder of filtrate.

6. To another separator, add 2.0 ml 2.5% phosphotungstic acid solution as blank.

7. To each separator add 8.0 ml distilled water.

8. Then in **immediate** succession add :

   a) 1.0 ml dilute NaOH solution (Reagent 4), swirl to mix.

   b) 2.0 ml thymol solution (Reagent 3), swirl to mix

   c) 5.0 ml bromine solution (Reagent 2) in ca. 30 small additions, swirling vigorously after each addition.

9. Shake vigorously for 1 min. Let stand for 20 min.

10. With series of samples or standards, complete reagent additions in sequence on each separator before proceeding to next.

11. To each separator add 20.0 ml n-butanol and shake vigorously for 1 min. Let stand for 20 min.

12. Drain aqueous layer and pass n-butanol layer through ca. 30 g anhydrous Na_2SO_4 glass funnel plugged with glass wool into 100 ml Erlenmeyer flask.

13. Measure absorbance of solution at wavelength maximum ca. 680 nm in 1 cm cell against blank as reference.

    If absorbance is higher than that of highest ammonia standard, quantitatively dilute reserved filtrate with 2.5% phosphotungstic acid solution so that 2.0 ml diluted solution will produce absorbance below this level.

14. Preparation of standard curve

a) Pipette 0, 1, 2, 3, 4, and 5 ml standard ammonia solution (Reagent 5) into 125 ml separators.

b) Add 2.0 ml 2.5% phosphotungstic acid solution.

c) Dilute up to a total of 10 ml with distilled water.

d) Proceed as in Procedure Step 8.

e) Using 0 solution as reference, measure absorbance of each standard at a wavelength maximum of ca. 680 nm.

f) Prepare standard curve.

REFERENCE

Martin, R.E., Flick, G.J., Hebard, C.E. and Ward, D.R. (1982). 1st Ed. Chemistry & Biochemistry of Marine Food Products. p 28, 31, 323 - 324.

Horwitz, W. (1980). Official Methods of Analysis of the Association of Official Analytical Chemists. 13th Ed. p 289.

QUALITATIVE TEST OF FORMALDEHYDE (Chromotropic Acid Test)

LOW LAI KIM

INTRODUCTION

Formalin (36% formaldehyde), a powerful preservative, is not permitted to be added to foods. Any food however may contain up to 5 mg/kg (ppm) formaldehyde derived from any wet strength wrapping containing formaldehyde based resin, or of plastic food containers or utensils made from any resin of which formaldehyde is a condensing component. The additive dimethylpolysiloxane may contain up to 1000 mg/kg formaldehyde. Traces of formaldehyde may be found in smoked fish derived from the smoke constituents (Shewan, 1949). These however are not considered to be preservatives by definition.

APPARATUS

1. Kjeldahl flask, 800 ml
2. Cone, double plain ends
3. Plain bend, socket to cone
4. Liebig condenser
5. Measuring cylinder, 100 ml
6. Test tube, 15 ml
7. Heating mantle
8. Water bath
9. Pipettes, 1 ml, 5 ml

REAGENTS

1. Phosphoric acid, H_3PO_4
2. 72% Sulphuric acid
   Pour 150 ml H_2SO_4 into 100 ml water and cool.
3. Saturated solution of 1,8-dihydroxynaphthalene 3,6-disulphonic acid in ca. 72% H_2SO_4.

   Weigh about 500 g of the above and make up to 100 ml in 72% H_2SO_4.
   The solution is light straw coloured.

PROCEDURE

1. Macerate 100 g sample (solid or semisolid) with 100 ml distilled water in mortar. If sample is liquid, measure 200 ml of sample.

2. Transfer to 800 ml Kjeldahl flask.

3. Acidify with H_3PO_4 and add 1 ml excess.

4. Connect with condenser through trap.

5. Slowly distil 50 ml at a speed of 3 ml in 12 - 14 min.

6. Place 5 ml of saturated solution of 1,8-dihydroxynaphthalene 3,6-disulphonic acid in ca. 72% H_2SO_4 into a test tube.

7. Add with mixing 1 ml of distillate from Step 5.

8. Place in boiling water bath for 15 min.

9. Observe during heating period for colour development. Presence of formaldehyde is indicated by appearance of light to deep purple (depth of colour depending on amount of formaldehyde present).

REMARKS

Detection limit : 1 ppm.

REFERENCE

Horwitz, W. (1980). 13th Ed. Official Methods of Analysis of the Association of Official Analytical Chemists. p 331, 530-531.

Pearson, D. (1976). 7th Ed. The Chemical Analysis of Foods. p 40-41.

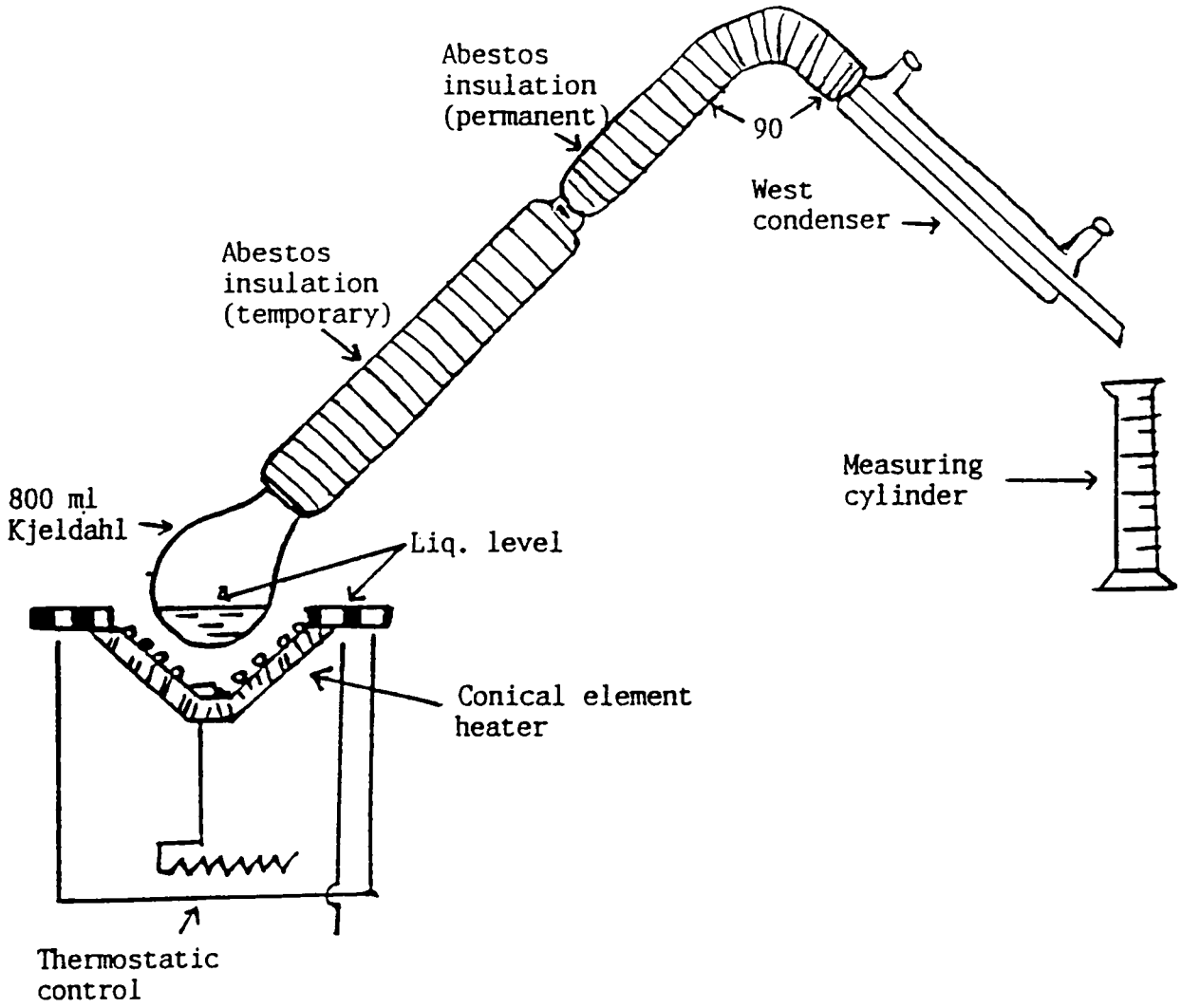


Fig. 1. Distillation unit for formaldehyde determination (chromotropic acid test)

DETERMINATION OF FORMALDEHYDE BY 4-amino-3-hydrazino-5-mercapto-1,2,4-triazol (AHMT) METHOD (Colorimetric method)

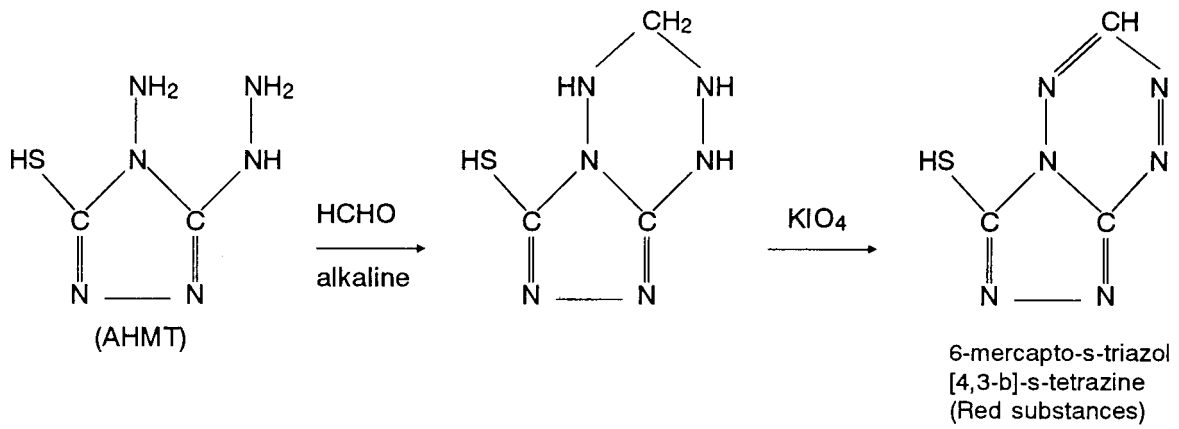
MAKOTO YAMAGATA AND LOW LAI KIM

INTRODUCTION

In this method the formaldehyde undergoes oxidation when reacted with AHMT and KIO_4 to yield violet compounds. The intensity of the compounds is proportional to the concentration of formaldehyde present. This AHMT method is more sensitive than the chromotropic acid method and the acetylacetone method. AHMT is also suitable for determination of residual formaldehyde in bisulfite treated shrimps.

Principle of AHMT Method

$$\text{AHMT plus HCHO} \xrightarrow[KIO_4]{\text{oxidation}} \text{Red substances}$$



APPARATUS

1. Distillation unit (Fig. 1)
2. Conical flasks, 250 ml
3. Volumetric flasks, 200 ml
4. Test tubes with glass stoppers, 20 ml
5. Pipettes, 2 ml, 5 ml, 10 ml
6. Spectrophotometer (550 nm)

REAGENTS

1. AHMT solution

   Weigh 0.5 g of AHMT and dissolve 100 ml of 0.5N HCl solution. Store in a cool, dark place.

2. Potassium periodate (KIO_4) solution

   Weigh 0.75 g of KIO_4 and dissolve in 100 ml of 0.2N KOH solution using a water bath.

3. Formaldehyde(HCHO) standard solution

   Accurately weigh about 1 g of formalin (35%, stock solution) into weighing bottle with 5 ml distilled water and make up to 100 ml with distilled water in a volumetric flask. Pipette 10 ml of this solution into a conical flask and accurately add 50 ml of 0.1N iodine solution. Add 20 ml of 1N KOH solution then stand for 15 min at room temperature. Add 15 ml of 10% H_2SO_4 solution. Titrate against 0.1N sodium thiosulphate ($Na_2S_2O_3$) solution, using 1 ml of starch solution as indicator. Blank should be done using 10 ml distilled water instead of the formaldehyde solution.

   Determination of the actual concentration of stock formaldehyde

   Formaldehyde (HCHO) content, $C(\%) = 1.501 \times (V_B - V)F/W$

   Where
   - V : Titration volume (ml) of 0.1N $Na_2S_2O_3$ solution (Main test)
   - V_B : Titration volume (ml) of 0.1N $Na_2S_2O_3$ solution for blank using distilled water instead of formaldehyde
   - F : Factor of 0.1N $Na_2S_2O_3$ solution
   - W : Sample weight of formalin (stock solution) used

Preparation of standard formaldehyde solutions

Accurately weigh 200/C (g) of formalin (35%, stock solution), dissolve with distilled water and make up to 100 ml. This contains 20,000 mg formaldehyde per litre. Make a 10 times dilution (10 ⟶ 100) of this and repeat 4 times to obtain a formaldehyde solution where 1 ml contains 2 ug HCHO.

PROCEDURE

Sample preparation (Extraction by distillation)

1. Accurately weigh 1 - 10 g of solid sample or pipette 10 ml of liquid sample (100 - 500 ug as HCHO) into flask.

2. Add 5 - 10 ml of distilled water and 1 ml of 20% H_3PO_3 solution.

3. Add 5 - 10 ml of distilled water into receiver and make sure the edge of the condenser is below the distilled water.

4. Start the steam distillation (distillation speed : 6 - 10 ml/ min).

5. When about 200 ml of distillate is collected in the receiver, stop the steam distillation.

6. Make up to 200 ml with distilled water in a volumetric flask.

Analytical Procedure

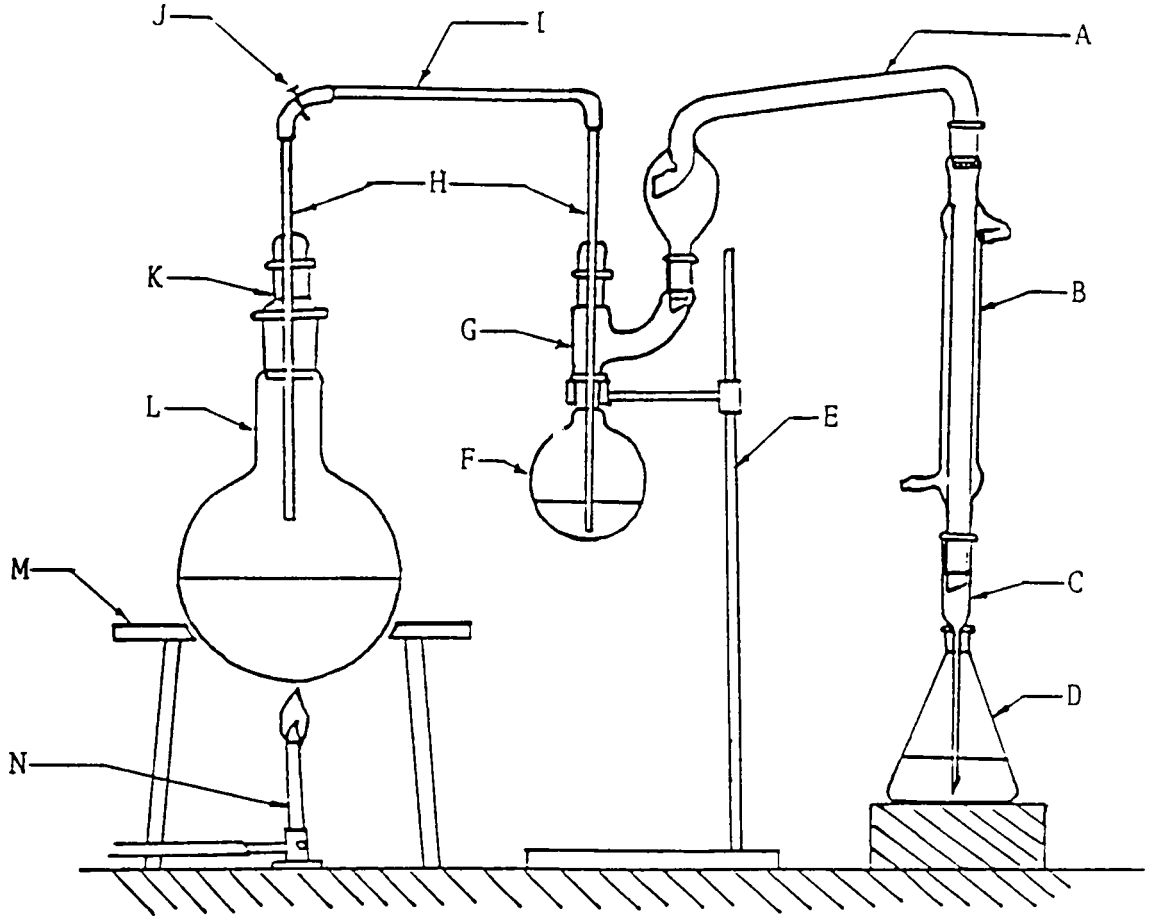
1. Pipette 2.0 ml of sample solution(distillate) into test tube with stopper.

2. Add 2.0 ml of 5N KOH solution followed by 2.0 ml of AHMT.

3. Mix gently and stand for 20 mins at room temperature.

4. Add 2.0 ml of KIO_4 solution and mix gently till the bubbles fade away.

5. Measure its absorption at the wavelength of 550 nm. HCHO concentration (ug/ml) is calculated according to HCHO standard curve (calibration curve).



A	:	Splash head	H	:	Steam inlet tube
B	:	Liebig condenser	I	:	Glass tubing
C	:	Straight delivery adapters	J	:	Clip
D	:	Conical flask	K	:	Reduction adaptors
E	:	Retort stand	L	:	Round bottomed flask, 2L
F	:	Round bottomed flask, 250 ml	M	:	Tripod stand
G	:	Two neck multiple adaptor	N	:	Bunsen burner

Fig. 1. Steam distillation apparatus

Preparation of standard curve

1. Pipette 0.5, 1.0, 1.5 and 2.0 ml of HCHO standard solution each into test tubes with stoppers. Prepare one empty test-tube with stopper.

2. Make up to 2.0 ml with distilled water.

3. Repeat as in Analytical Procedure.

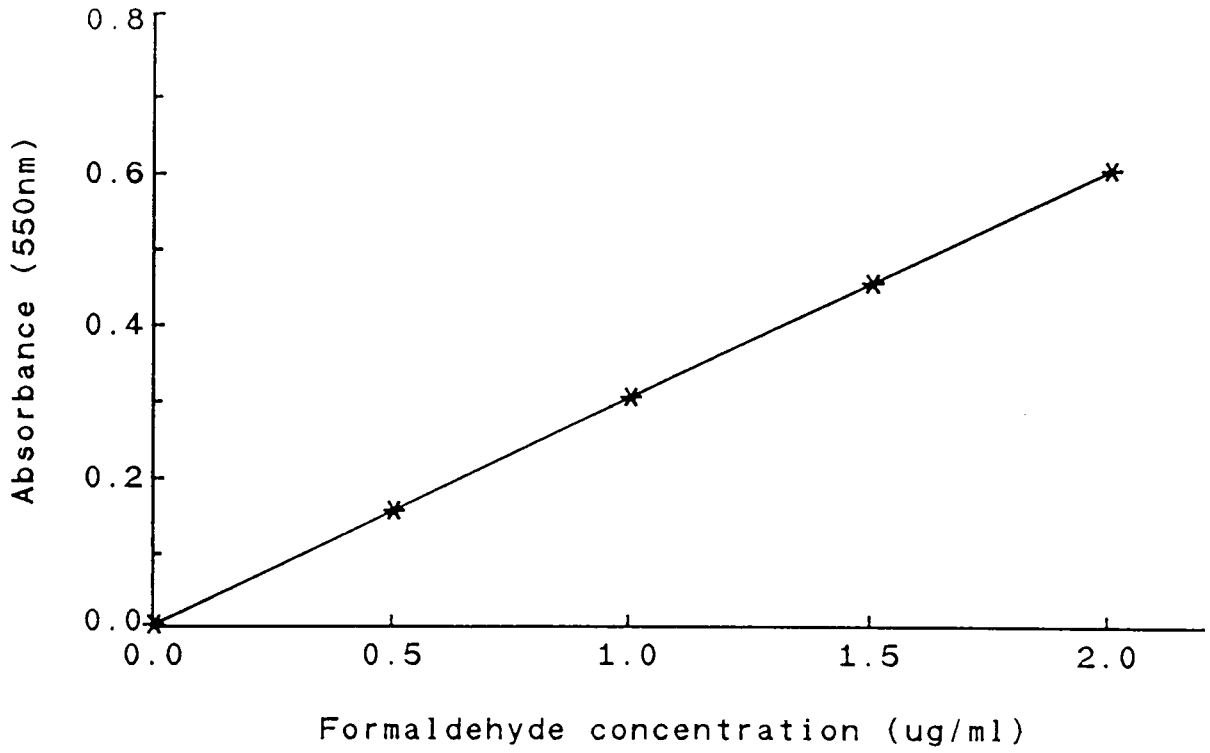


Fig 2. Standard curve for formaldehyde (AHMT Method)

CALCULATION

$$\text{Formaldehye (ug/g)} = \frac{A}{\text{Vol. of filtrate used}} \times \frac{\text{Total vol. of filtrate}}{\text{Wt. of sample}}$$

$$= \frac{A}{2} \times \frac{200}{10}$$

$$= A \times 10$$

where A = Reading from standard curve (ug)

REMARKS

Detection limit of this method is 0.1 ppm.

REFERENCE

Standard Methods of Analysis for Hygiene Chemists - With Commentary - authorized by the Pharmaceutical Society of Japan, Kimbara Publishing Co., Ltd. p. 110. (1990).

Mimura, H., Kaneko, M., Nishiyama, N., Fukui, S., and Kanno, S. (1976). Determination of Formaldehyde by the 4-Amino-3-hydrazino-5-mercapto-1,2,4-triazol Method (AHMT Method). The Journal of Hygiene Chemistry. 22(1), p. 39-41.

DETERMINATION OF K-VALUE BY THE FRESHNESS METER

NG CHER SIANG

INTRODUCTION

The Freshness Meter (Model KV-101, Oriental Co., Tokyo) comprises a water bath incubator chamber fitted with an oxygen electrode, a digital display and a chart recorder. The system uses a series of enzyme reaction steps to break down the ATP and related compounds which are reflected as oxygen consumed during the reactions. The data are presented both in digital read-out and graphically. The ratio of the decrease in oxygen during the various reactions are used to calculate the K-value of the test sample.

The main principles involved in the use of this equipment are presented in Figures 1 and 2.

REAGENTS

A: Sample extraction reagents

a) 10% trichloroacetic acid (TCA)

b) 10 N potassium hydroxide (KOH)

c) Methyl red indicator

B: Reaction reagents

The following reagents are purchased from the equipment supplier or can be mixed in the laboratory.

a) Reagent B
Buffer solution

b) Reagent P is a mixture of alkaline phosphatase and adenosine deaminase.

c) Reagent E_o is a mixture of nucleotide phosphorylase and xanthine oxidase.

PROCEDURE

A Sample Preparation

Take approximately 2 g fish meat. Add 5 ml of 10% TCA and homogenise. Filter, using a coarse filter paper. Add one to two drops of methyl red indicator, and add 10N KOH to neutralise. This neutralised extract is labelled sample S_1.

B Initial Preparation

1) Set the water incubation bath to 37°C and allow the temperature of the reaction chamber to reach 37°C.

2) Place sufficient Reagent B into a bottle and place in water incubation bath. Put in airstone and apply air bubbles to saturate Reagent B with oxygen. Allow Reagent B to equilibrate at 37°C.

3) Zero the digital readout and graph recorder by switching the "FUNC" switch to "ZERO". Use the "ZERO" switch to adjust display to ± 0.1. Similarly, use the "POSITION" switch on the chart recorder to adjust the pen position to zero on the chart paper. Return the "FUNC" switch to "MEAS".

C Preliminary reaction (See Fig. 2)

1) Fill a test tube with 50 ul of Reagent P and 50 ul of extract solution S_1. Place the test tube in the water bath incubator. Allow reaction to occur for 15 minutes. This reactant solution is labelled S_2.

D Main measurement procedure (See Fig. 2)

1) Add approximately 1.2 ml of preheated Reagent B into the reaction cell.

2) Cap the reaction cell tightly. Ensure no air bubbles are trapped inside the reaction cell.

3) Switch on chart recorder and stirrer.

4) Add accurately 20 ul of sample S_1. Wait for one minute.

5) Add accurately 15 ul of Reagent E_0.

6) Take measurement D_1.

7) Add exactly 40 ul of sample S_2.

8) Take measurement D_2.

9) Computation of results. (See Fig. 1)

E Preparing reaction cell for next sample

1) Remove cap and drain out waste reactants.

2) Add distilled water into cell and flush several times.

REFERENCE

Oriental Electric Co. Ltd. (1987) : Manual of Freshness Tester (Model KV-101)

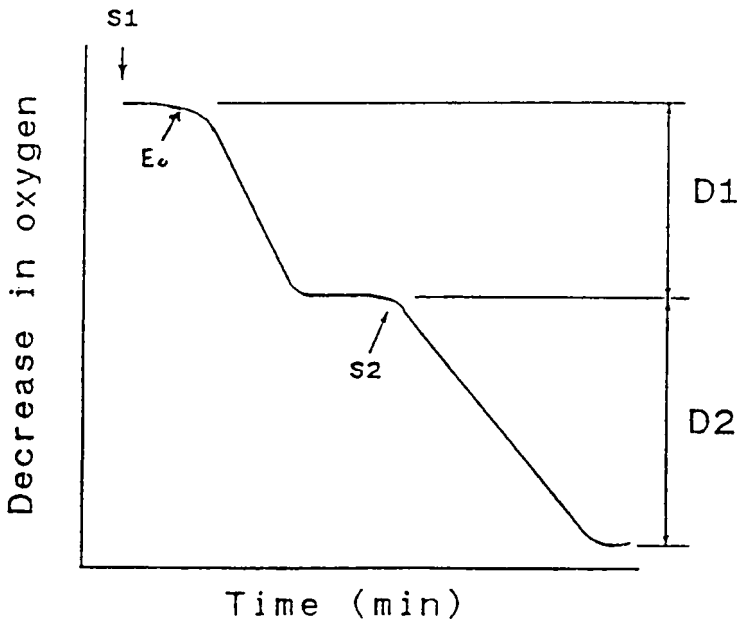


Fig. 1 Calculation of K-Value

$$\text{K-value (\%)} = \frac{D1}{D2} \times 100$$

STEP 1

Original fish extract S_1 is reacted with
Reagent, P (alkaline phosphatase + adenosine deaminase)

ATP + ADP + AMP + IMP + HxR + Hx

Converted to HxR

Resulting solution S_2 contains $\sum$ HxR + Hx

STEP 2

Original fish extract S_1 is reacted with
Reagent Eo (nucleotide phosphorylase + xanthine oxidase)

ATP + ADP + AMP + IMP + HxR + Hx

Converted to uric acid
Oxygen consumed : D_1

STEP 3

Resulting solution from STEP 1, reacted with Eo

$\sum$ HxR + Hx

Converted to uric acid
Oxygen consumed : D_2

STEP 4

$$\text{K value (\%)} = \frac{D_1}{D_2} \times 100$$

Fig. 2 Major Steps in using the Freshness Meter

FRACTIONATION AND DETERMINATION OF FISH PROTEINS

KATSUTOSHI MIWA AND LIM PANG YONG

INTRODUCTION

Myofibrillar proteins (MFP) such as myosin and actin are the major proteins in fish muscle and are soluble in 3-5% salt solution.

MFP solubility in salt solution and ATPase activity have been often used as an index for protein denaturation.

Ironside & Love (1958) reported that the determination of myofibrillar protein solubility, in 5% NaCl or 0.6 M KCl, was very useful for the studies of protein denaturation during freezing storage, and many scientists have since then used this method for the determination of MFP denaturation.

On the other hand the determined values by this method generally showed large deviations, due to the denaturation of MFP during extraction and the insufficient solubility of MFP. The deviation is caused, especially, by the foam formation during homogenization with a normal top driven homogenizer which accelerates the denaturation of MFP. Therefore, a bottom driven homogenizer with a cover immersed in chilled water is recommended. Multiple measurements with the same sample are necessary for the MFP determination by this method.

MFP can be determined not only by measuring the difference between salt soluble protein and water soluble protein (sacroplasmic protein), the precipitated actomyosin in the salt soluble protein solution which has been diluted with cold distilled water can also be measured. Therefore, this method is called myofibrillar protein or actomyosin determination. In this method the precipitated actomyosin is determined together with a small quantity of precipitated sarcoplasmic protein.

Sarcoplasmic proteins consists of regulatory proteins, glycolysis enzymes, myokinase, AMP-deaminase etc. and are more easily soluble in diluted salt solution than in distilled water. But MFP is not soluble in 0.01 - 0.1 M NaCl or KCl solution, therefore, sarcoplasmic protein can be determined by measuring the difference between the diluted salt soluble protein solution and non proteinous compounds.

There are some salt (0.6 M KCl) insoluble proteins in fish muscle, e.g. stroma protein, intercellular protein connected firmly with connective tissues and denatured protein. Stroma protein is not soluble in 0.1 N-NaOH solution, but the other proteins are soluble in it.

Using the differences between the various protein solubilities, the total nitrogen and non proteinous compounds nitrogen, sarcoplasmic protein, myofibrillar protein, residual intercellular protein & denatured protein and stroma protein can be determined.

APPARATUS

1. Chopper or mincer
2. Analytical balance
3. Homogenizer[*1,*2]
4. Refrigerated centrifuge, capable of centrifuging at 9,000 rpm and at 0°-5°C.
5. Beakers, 100 and 250 ml
6. Bulb pipettes, 10, 20 and 40 ml
7. Glass funnels 60 mm ø
8. Whatman filter paper, No. 41
9. Spatula

REAGENTS

1. Phosphate buffer solution.[*3]
   0.03 M potassium di-hydrogen phosphate, 1 litre.
   0.03 M di-sodium hydrogen phosphate, 1 litre.
   Mix the above two solutions and adjust the pH to 6.85 using these solutions.
2. 0.1 M potassium chloride solution.
   Weigh 7.455g KCl accurately, dissolve in 1 litre distilled water.
3. 0.6 M potassium chloride phosphate buffered solution.
   Weigh 44.73g KCl accurately, dissolve in 1 litre phosphate buffer solution.
4. 0.1 N sodium hydroxide solution.
   Weigh 4.0g NaOH accurately, dissolve in 1 litre distilled water.
5. Trichloroacetic acid solution (25%, W/V).
   Dissolve 25g TCA in 100 ml distilled water.

PROCEDURE

1. **Total nitrogen (tN)**

   Accurately weigh a duplicate of 1 g homogeneous fish meat for protein digestion. (refer to Protein determination by Kjeldahl method, Part B, B-1).

2. **Total sarcoplasmic protein nitrogen (tspN)**

   Accurately weigh 10g homogeneous fish meat. Blend the sample with 200 ml of 0.1 M KCl solution with the homogenizer for 4 minutes (speed set at scale 2). Leave to stand in iced-water for 2 hours. Centrifuge the blended sample at 12,500 g or 9,000 rmp at 0°-5°C for 20 minutes. Pipette 20 ml supernatant for digestion with H_2SO_4 (refer to Kjeldahl method).

3. **Non proteinous compounds nitrogen (npN)**

   Pipette 40 ml sarcoplasmic protein aliquot (from No. 2 above) into a 100 ml beaker. Add 10 ml 25% TCA and leave to stand in iced water for 30 minutes with occasional swirling. Filter the content of the beaker with Whatman No. 41 ashless filter paper. Pipette 40 ml of filtrate for digestion with H_2SO_4.

4. **Salt soluble protein nitrogen (sspN)**

   Accurately weigh 10g homogeneous fish meat. Blend sample with 200 ml 0.6 M KCl phosphate buffered solution with the homogenizer for 4 minutes (Speed set at scale 2). Leave to stand in iced-water for 2 hours. Centrifuge the blended sample at 12,500 g or 9,000 rpm at 0-5°C for 20 minutes. Pipette 20 ml supernatant for digestion with H_2SO_4.

5. **Residual intercellular protein and denatured protein nitrogen (rpN)**

   Wash the precipitate of salt soluble protein aliquot with 10 ml 0.6 M KCl phosphate buffered solution and centrifuge at 9,000 rpm for 10 minutes. Repeat this washing procedure twice. Discard the supernatant. Add 15 ml 0.1 N NaOH solution and stir with glass spatula and then centrifuge at 9,000 rpm for 10 minutes. Repeat this procedure three times. Transfer the supernatant into a 50 ml volumetric flask and adjust to 50 ml with 0.1 N NaOH. Pipette 10 ml supernatant for digestion with H_2SO_4.

6. **Stroma protein nitrogen (spN)**

   Suspend the precipitate from No. 5 above in water and transfer into flask for digestion with H_2SO_4.

7. Procedure Nos: 2, 3, 4, 5 and 6 must be repeated at least 3 times.

8. For digestion of the above fractionated protein nitrogen, refer to 'Protein determination by Kjeldahl method' in Part B, B-1.
   (See Fig.1)

CALCULATIONS

The following shows the calculations of equivalent meat weight of fish used in the extraction of various fish proteins:

1) **Meat weight used for total sarcoplasmic protein nitrogen (tspN)**

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

where W_1 is the weight of fish meat (g) used for estimated 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

2) **Meat weight for non proteinous compounds nitrogen (npN)**

$$W_{npN} = 40^a \times \frac{40^b}{50} \times \frac{W_1}{200 + W_1}$$

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 precipitating with TCA

50 is the total volume of sarcoplasmic protein aliquot after addition of TCA solution

W_1 is the weight of fish meat (g) used for sarcoplasmic protein extraction

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

3) **Meat weight for salt soluble protein nitrogen (sspN)**

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

where W_2 is the weight of fish meat (g) used for salt soluble protein extraction

20 is the volume of salt soluble protein aliquot used for digestion

200 is the volume (ml) of 0.6 M KCl phosphate buffered solution used for the sspN extraction

4) **Meat weight for residual intercellular protein & denatured protein nitrogen (rpN)**

$$W_{rpN} = W_2 \times \frac{50}{50}$$

W_2 is the weight of fish meat (g) used for salt soluble protein extraction

20 is the volume of residual intercellular protein and denatured protein aliquot used for digestion

50 is the total volume of residual intercellular protein and denatured protein aliquot

5) **Meat weight for stroma protein nitrogen (spN)**

$$W_{spN} = W_2$$

W_2 is the weight of fish meat (g) used for salt soluble protein extraction

Using the above respective calculated meat weight and the titration value for a particular protein nitrogen obtained by Kjeldahl method, calculate the respective protein nitrogen by the following formula:

$$\text{Protein nitrogen, mgN/100 g} = \frac{(b - a) \times 0.1 \times 14.00}{W} \times 100$$

where W is the weight of equivalent fish meat used for a particular protein extraction

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

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

14.00 is the atomic weight of nitrogen

To determine each protein nitrogen in fish meat, substitute the various calculated protein nitrogen(s) from the above into the following formula

1. **Actual sarcoplasmic protein nitrogen, aspN**

   The difference between total sarcoplasmic protein nitrogen and non proteinous compounds nitrogen:

   aspN = tspN - npN

2. **Myofibrillar protein nitrogen, mfpN**

   The difference between salt soluble protein nitrogen - total sarcoplasmic protein nitrogen:

   mfpN = sspN - tspN

3. **Compositions of various protein nitrogen:**

   i. $\text{aspN, \%} = \frac{\text{aspN}}{\text{tN}} \times 100\%$

   ii. $\text{mfpN, \%} = \frac{\text{mfpN}}{\text{tN}} \times 100\%$

   iii. $\text{rpN, \%} = \frac{\text{rpN}}{\text{tN}} \times 100\,\%$

   iv. $\text{spN, \%} = \frac{\text{spN}}{\text{tN}} \times 100\,\%$

REMARKS

*1 Nihon Seiki SN-03 type homogenizer. The homogenizer has a capacity of 400-500 ml and is bottom driven type, with a cover and a water jacket, so that foam formation can be prevented.

*2 It is very important to prevent the formation of foam during homogenization of fish meat, as denaturation of fish protein take place simultaneously with foam formation. Therefore, the use of the bottom driven homogenizer with a covered cup in water is recommended.

*3 The pH of the salt solution influences the extractibility and stability of fish proteins. The optimum pH is between 6.6 - 7.0. The phosphate buffer solution can be prepared by using di-sodium hydrogen phosphate, potassium di-hydrogen phosphate or sodium carbonate etc.

REFERENCES

J.I.M. Ironside & R.M. Love (1958). Studies on protein denaturation in frozen fish - I., Biological factors influencing the amounts of soluble and insoluble protein present in the muscle of the North Sea Cod. J. Sci. Food Agric., 9, 597-604.

Y. Shimizu & W. Simidu (1960). Studies on muscle of aquatic animals - XXVIII. Protein composition of fish muscle. Nippon Suisan Gakkaishi, Vol. 26, No. 8, p. 806-809.

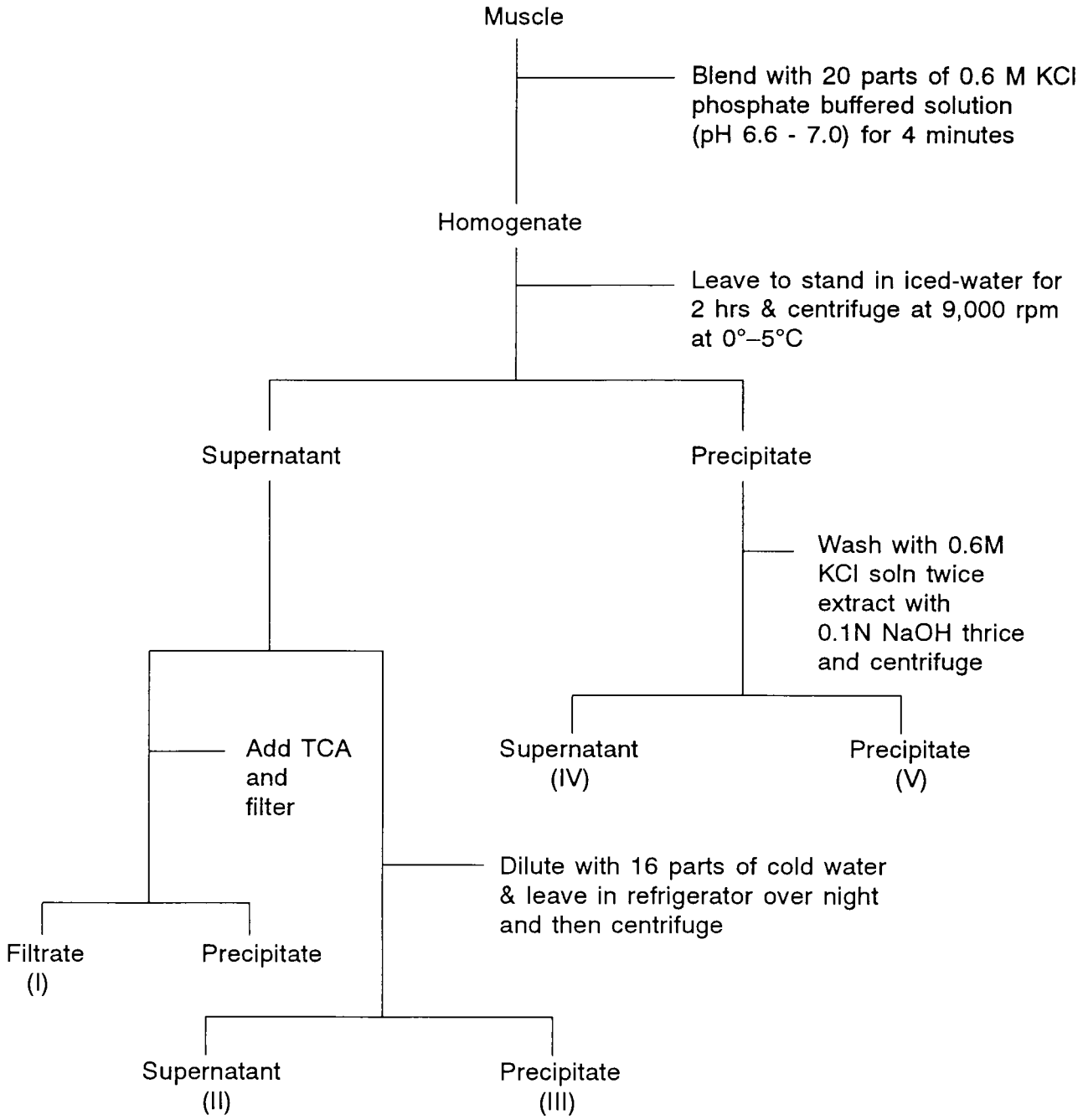


Fig. 1 Extraction and fractionation of protein in fish muscle

(I) Non proteinous compounds.

(II) Sarcoplasmic protein & non proteinous compounds.

(III) Myofibrillar protein or actomyosin protein

(IV) Residual intercellular protein and denatured protein

(V) Stroma protein

ANALYSIS OF OILS

SIGNIFICANCE OF ANALYSIS OF LIPIDS

LOW LAI KIM

INTRODUCTION

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:

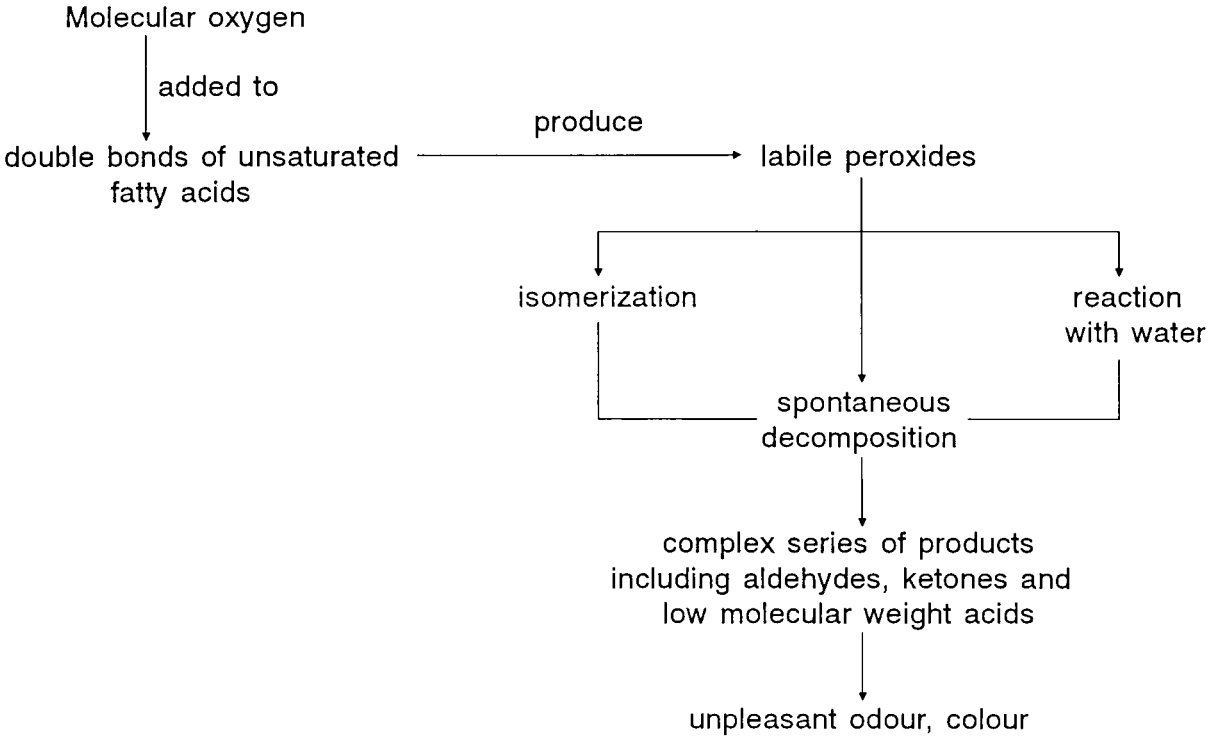
i) the phospholipid content

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 LAI KIM

INTRODUCTION

A mixture of chloroform and methanol in the ratio of 2:1 (v/v) extracts 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.

APPARATUS

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

REAGENTS

1) 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.

2) 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.

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

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

4) 1% BHA-BHT antioxidant solution

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

5) Anhydrous sodium sulphate

PROCEDURE

Sample Preparation

1. 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)

2. Weigh the chopped sample into the homogenizer cup.

3. Add C-M mixture volume of about 3.5 times the weight of sample, and 2-3 drops of antioxidant solution.

4. Homogenize for 1 min and filter with Whatman No. 1 filter paper using a Buchner funnel and vacuum pump.

5. Transfer the residue into the cup and repeat homogenization twice.*

6. Transfer the combined filtrate into a separating flask.

7. Pour distilled water, volume approximately a quarter of that of the extract, into the separating flask.

8. Shake very gently 2-3 times, and stand overnight.**

9. 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.

10. Wash the filter paper 2 - 3 times with C-M Mixture.

11. Concentrate the extract with a rotary evaporator under reduced pressure at 40°C (water-bath temperature).***

12. Dissolve the concentrated extract with C-M Mixture and transfer to 50 ml volumetric flask using a pipette.

13. Make up to the mark with C-M Mixture.

14. 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.

REFERENCE

Christie, W.W. (1982). In: Lipid analysis (2nd Ed.) Pergamon Press:22

Folch et al. (1951). Journal of Biological Chemistry, 191:833.

DETERMINATION OF TOTAL LIPID CONTENT

LOW LAI KIM AND NG CHER SIANG

INTRODUCTION

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

APPARATUS

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

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%.

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 LAI KIM

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 eluted by the methanol. The neutral lipids which are not absorbed by the silicic acid are first eluted out by the chloroform.

APPARATUS

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

REAGENTS

1. Silicic acid (Mallinchrodt, 100 mesh)
2. Celite 545
3. Methanol (analytical grade)
4. Chloroform (analytical grade)

PROCEDURE

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

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.

ANALYTICAL 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.

CALCULATION

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

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

NL = Neutral lipid

PL = Phospholipid

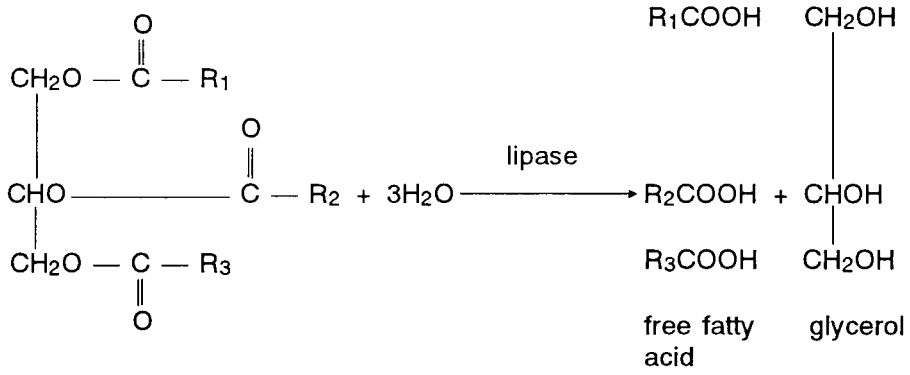
The phospholipid content is expressed as the percentage of phospholipid over the total lipid present per gram of sample lipid.

DETERMINATION OF ACID VALUE

LOW LAI KIM

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.

APPARATUS

1. Microburette (2 ml with 0.01 ml intervals)
2. Conical flasks (100 ml)
3. 5 ml pipettes

REAGENTS

1. 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.

2. n-Hexane

3. 1% phenolphthalein or thymolphthalein in ethyl alcohol

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

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.

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 LAI KIM

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

$$\text{FFA (\%)} = \text{acid value} \times \frac{\text{mol. wt. of oleic acid}}{\text{mol. wt. of KOH}} \times \frac{100}{1000}$$

$$= \text{acid value} \times \frac{282.27}{56.11} \times \frac{1}{10}$$

$$= \text{acid value} \times \frac{1}{2}$$

2. Determination of Free Fatty Acid from Acid Value And Saponification Value expressed as mg 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 LAI KIM

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:

$$C_3H_5(C_{17}H_{35}COO)_3 + 3KOH = C_3H_5(OH)_3 + 3C_{17}H_{35}COOK$$

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.

APPARATUS

1. Bulb condensers
2. Erlenmeyer flasks (50 - 300 ml depending on sample size)
3. Water bath
4. Pipettes
5. Burette

REAGENTS

1. 0.5N HCl standard solution

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

2. 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.

3. 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.

PROCEDURE

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

Analytical 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, then titrate with 0.5N HCl standard solution (Add 2-3 drops of either indicator).
5. Carry out a blank test (without lipid).

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 LAI KIM

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.

APPARATUS

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

REAGENTS

1. 0.01N $Na_2S_2O_3$ solution

   Dissolve 25 g of $Na_2S_2O_3.5H_2O$ 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 $Na_2S_2O_3$ Solution

1. Take 20 ml of 0.01N $K_2Cr_2O_7$ 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 $Na_2S_2O_3$ 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 $K_2Cr_2O_7$ solution.

8. Calculation:

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

where F = factor of 0.01N $Na_2S_3O_3$ solution

F' = factor of 0.01N $K_2Cr_2O_7$ solution

V_s = titration volume of sample (ml)

V_b = titration volume of blank (ml)

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

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

3. 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.

4. 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.

5. 0.01N $K_2Cr_2O_7$ standard solution

   Weigh 4.9035 g of $K_2Cr_2O_7$ 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.

   $$\text{Factor } F' = \frac{4.9035}{W}$$

   where W is the actual weight of $K_2Cr_2O_7$ used.

6. 10% (w/v) KI solution

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

7. 25% H_2SO_4 solution

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

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 $Na_2S_2O_3$ 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.

CALCULATION

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

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

where V_s = titration volume of sample (ml);

V_b = titration volume of blank (ml);

F = factor of 0.01N $Na_2S_2O_3$ solution;

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

N = normality of $Na_2S_2O_3$ solution (in this case N/100)

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

REFERENCES

Japanese Association of Oil Chemists: Standard methods of oil analysis in Japan, (1972) 2(4): 12-71.

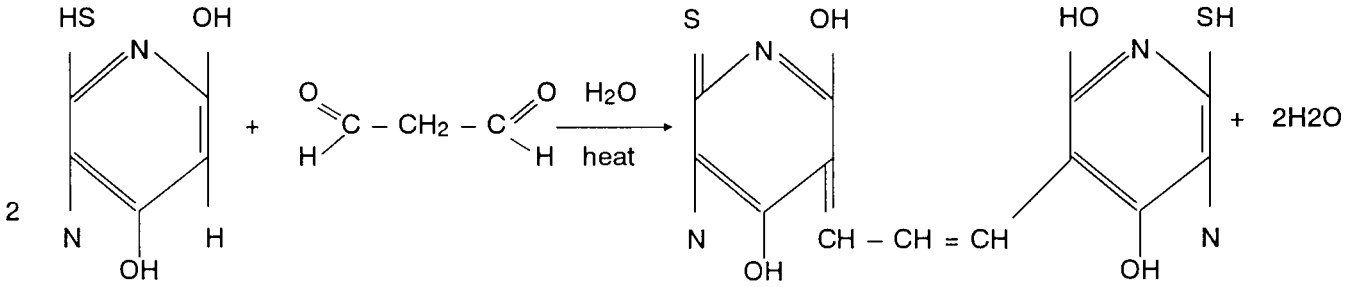
Pearson, D. (1976). The chemical analysis of foods (7th Ed.): 494-495.

DETERMINATION OF THIOBARBITURIC ACID (TBA) NUMBER

LOW LAI KIM

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:



Thiobarbituric acid

Malonaldehyde

Brilliant red product with absorption maximum at 532 nm

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 hydroloysis.

APPARATUS

1. Spectrophotometer (λ = 532 nm)
2. Test tubes with screw caps
3. Hot water bath (boiling water)
4. Pipettes (3, 5, 10, 25 ml)
5. Rotary evaporator with vacuum pump and water bath
6. Vortex mixer
7. Test tube basket
8. Glass centrifuge tubes
9. Centrifuge
10. Source of N_2 gas

REAGENTS

1. 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).

2. Trichloroacetic acid (TCA) solution

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

3. 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.

4. Chloroform (Analytical grade)

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 stoppers.
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 N_2 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 centrifuge 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 into another test tube and read absorbance at 532 nm.
13. Blank test should be carried out in the same manner without fats.

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.

REFERENCES

Pearson, D. (1976). The chemical analysis of foods (7th Ed.):496-497.

Sinnhuber, R.O. and Yu, T.C. (1958). The 2-thio-barbituric acid method for the measurement of rancidity in fishery product II. The quantitative determination of malonaldehyde. Food Tech., 12:9.

Sinnhuber, R.O., and Yu, T.C. (1977). The 2-thio-barbituric acid reaction. An objective measure of the oxidative deterioration occuring in fats and oils. Abura Kagaku, 26:259-267.

DETERMINATION OF METHYL ESTERS OF FATTY ACIDS BY GAS CHROMATOGRAPHIC METHOD

LOW LAI KIM

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 nonvolatile substances such as epoxy, oxidized, or polymerized fatty acids.

Esters appear in order of increasing number of C atoms and of increasing unsaturation for same number of C atoms. C_{16} ester is ahead of the C_{18} Me-esters which appear in order : stearate (18:0), oleate (18:1), linoleate (18:2), and linolenate (18:3). C_{20} 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.

APPARATUS

1. Gas chromatograph (Shimadzu GC-9A)

2. Glass Spiral Columns

   2.1 For DEGS : length = 1.6 m, i.d. = 3 mm

   2.2 For Unisole 3000 : length = 2 m, i.d. = 3 mm

3. Packing material

   3.1 Diethylene glycol succinate (DEGS), 5 - 20% on support Shimalite W or Chromosorb W,(acid washed and silanized diatomaceous earth), mesh 60 - 80. Maximum aging temperature = 210°C.

   3.2 Unisole 3000 on support Uniport C, mesh 80/100. Maximum aging temperature = 250°C.

4. Microliter syringes

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

   4.2 Maximum volume 1 ul, graduated to 0.01 ul (Hamilton 7001 SN)

5. Recorder (Chromatopac C-R1B, Shimadzu)

REAGENTS

1. Carrier gas

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

2. Other gas

   Purified grade air with oxygen = 21 ± 1%, moisture < 3.0 ppm, hydrocarbons < 5.0 ppm.

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

3. 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.

OPERATING CONDITIONS

1. Column aging condition

   1.1 DEGS column

Aging temperature	=	200°C
Carrier (N_2) gas flow	=	60 ml/min
Duration	=	16 - 18 hours

   1.2 Unisole 3000 column

Aging temperature	=	240°C
Carrier (N_2) gas flow	=	70 ml/min
Duration	=	16 - 18 hours

   1.3 Connect the long arm of column into the inlet or injection port of the gas chromatograph.

   1.4 Do not connect the short arm to the detector. Leave it hanging in the oven.

1.5 Apply carrier gas flow at normal flow rate.

1.6 Then programme the oven temperature at 1°C/min rise to a temperature which is at least 10°C above that at which the column is operated.

1.7 Hold the final temperature for at least 2 hours and preferably for 16 hours.

1.8 Reduce temperature to the normal operating temperature and connect column to detector. The column is ready for use.

2. Isothermal programme for DEGS column

Column initial temperature	=	180°C
Column intial time	=	0.0 min
Column programme rate	=	0°C/min
Column final temperature	=	180°C
Column final time	=	100 min
Injection port temperature	=	200°C
FID detector temperature	=	200°C
Range	=	10^2

3. Isothermal programme for Unisole 3000 column

Column initial temperature	=	220° or 230°C
Column initial time	=	0.0 min
Column programme rate	=	0°C/min
Column final time	=	100 min
Injection port temperature	=	270°C
FID detector temperature	=	260°C
Range	=	10^2

4. Gas Flow rate and pressure

Carrier (N_2) gas	=	30 - 40 ml/min
Hydrogen gas	=	0.6 kg/cm^2
Purified air	=	0.5 kg/cm^2

5. Recorder condition

Width	=	5 sec
Slope	=	100 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 (DEGS column)
	=	3 mV/full scale (Unisole 3000 column)
Speed	=	10 mm/min
Method	=	41
Sample weight	=	100 (default value)
Internal standard weight	=	1 (default value)

PROCEDURE

Sample Preparation

The fish oils used are first esterified by the boron trifluoride method. (See C-11)

Analytical Procedure

1. Open the carrier gas tank, set regulator outlet to 400 pKa, open the carrier valve on GC-9A and adjust carrier flow rate on GC-9A to that required.

2. Switch on GC-9A main switch, power and heater.

3. Select the File with the appropriate programme required and press ENTER. Then press START to start the programme. Press MONI COL to display the column initial temperature (CITP) on the display panel.

4. Wait for the temperature to reach that of the programmed temperature.

5. Open the purified air tank and set regulator outlet to 400 pKa.

6. Open the hydrogen gas tank and set regulator outlet to 400 pKa. Open the Hydrogen gas valve on GC-9A.

7. To ignite the FID detector, set the hydrogen gas to 0.9 kg/cm^2 and the purified air to 0.2 kg/cm^2. Turn the ignitor to the detector till there is a "pop" sound. The detector is ignited and can be checked for vapour condensation on the flat surface of a metal spatula. Then set the hydrogen gas to 0.6 kg/cm^2 and the purified air to 0.5 kg/cm^2. The GC-9A is ready for use.

8. Switch on the recorder and set the black key at open.

9. Press the PLOT key and adjust the baseline. Check SLOPE to be less than 300.

10. When the baseline is stable, inject 0.1 - 0.3 ul of 5 - 10% esterified sample in redistilled n-Hexane or chloroform by piercing the septum of the injection port and quickly discharging the sample.

11. Immediately press the START keys on GC-9A and the recorder.

12. Withdraw needle and note on chart the air or solvent peak as the start reference point.

13. If trace components are desired, the sample may be increased by < 10 times.

14. Adjust sample size so that major peaks is not attenuated > 8 times, preferably less (2-4).

15. Change setting of attenuator as necessary to keep peaks on chart paper. Mark attenuator setting on chart.

16. At end of run press the STOP key on recorder. The results will be printed out.

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 of sample from these graphs, interpolating if necessary.

5. Avoid conditions which permit "masked peaks" which are not sufficiently resolved.

CALCULATION

Method 41 of Chromatopac C-RIB 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 Chromatopack 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 < %.

REFERENCES

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

Mirio SAITO et.al.(1985): Fatty acid composition of Fish Lipids, Jpn. J.Nutr;, 43(6) 301-318.

Through personal communication with Mr Kinumaki, Dr Tsukuda and Dr Miwa.

DETERMINATION OF THE DEGREE OF LIPID OXIDATION BY GAS CHROMATOGRAPHY

LOW LAI KIM

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 decosahexaenoic 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 occured during frozen storage.

PROCEDURE

Sample Preparation

The sample is prepared by the boron trifluoride method.

Analytical Procedure

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

CALCULATION

The index of oxidation, I, is defined as:

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

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 LAI KIM

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.

APPARATUS

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

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

REAGENTS

1. 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 BF3 vapours with effective fume removal device. Avoid contact with skin, eyes, and respiratory tract).

2. Methanolic sodium hydroxide solution (0.5N)

   Dissolve 2 g NaOH in 100 ml MeOH containing < 0.5% H_2O. White precipitate of Na_2CO_3 forming on long standing may be ignored.

3. n-Hexane pure, as determined by GLC.

4. Nitrogen gas containing < 5 mg oxygen/kg.

5. Methyl red solution - 0.1% in 60% ethyl alcohol.

PROCEDURE

Sample Preparation

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

Analytical 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 BF_3 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 Na_2SO_4 to remove H_2O. If necessary, dilute solution to concentration of 5 - 10% for GLC.

N.B. BF_3 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 N_2 in refrigerator.

REFERENCE

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

EXTRACTION AND DETERMINATION METHOD OF LEAN FISH LIPIDS

KATSUTOSHI MIWA AND LOW LAI KIM

INTRODUCTION

Ethyl ether extraction method has been used generally and widely as extraction and determination method of lipids in foods. Neutral lipids such as triglyceride and wax are mostly extracted by ethyl ether, but most polar lipids such as phospholipids and galactolipids are not extracted by ethyl ether without acidic digestion.

Polar lipids contents of raw fish and shellfish are usually 0.5 - 1.0%, with the seasonal variation and the difference from fish species being less than that of neutral lipids.

Warm water fish normally has very low level fat content of several percent, so that the ratio of polar lipids to total lipids is larger than that of cold water fish. The difference between ethyl ether extracted lipid content and chloroform-methanol mixture extracted lipid content is larger than that of cold water fish.

Otherwise in the case of extraction from lean fish lipids, chloroform-methanol 1:1 mixture is more effective than chloroform-methanol 2:1 mixture and the former uses less volume of solvents than the latter.

APPARATUS

1. Homogeniser
2. Buchner flask and funnel
3. Vacuum pump or aspirator
4. Vacuum distillator or vacuum rotary evaporator
5. Nitrogen gas
6. Separating flask (1L)
7. Measuring cylinders (100 ml & 250 ml)
8. Filter paper, Whatman No. 1.

REAGENTS

1. Purified and distilled chloroform ($CHCl_3$).

2. Purified and distilled methanol (CH_3OH).

3. Anhydrous sodium sulfate ($NaSO_4$), analytical grade.

SAMPLE PREPARATION

(Refer to Fig. 1)

The raw fish sample (moisture content about 80%, fat content a few %) is chopped into a mince. 100g of the minced sample is homogenised with chloroform-methanol mixture using a homogeniser or a blender.

PROCEDURE

(Refer to Fig. 1)

1. Weigh 100 g of the chopped sample into the homogeniser cup.
2. Add 100 ml of chloroform and 200 ml of methanol and then homogenise for 2 mins with a homogeniser.
3. Add 100 ml more of chloroform to the homogenate and homogenise for 30 secs.
4. Add 100 ml of distilled water to the homogenate and homogenise it again for 30 secs.
5. Filter the homogenate using a Buchner funnel and vacuum pump.
6. Transfer the residue into the cup and repeat homogenisation once more using 50-100 ml of chloroform. Then filter the homogenate with Buchner funnel.
7. Transfer the combined filtrate into a separating flask and stand about an hour.
8. Drain off the chloroform phase into a flask containing about 5g of anhydrous sodium sulphate.
9. Filter the chloroform solution into a vacuum evaporating flask.
10. Wash the flask and the filter paper 2-3 times with chloroform.
11. Evaporate the extract with a rotary evaporator under reduced pressure.
12. Dry in a vacuum desiccator and weigh the crude lipids.
13. Flush with nitrogen gas and store at -20°C. This sample is used for other tests such as determination of lipids composition, fatty acid composition, fatty indexes and deterioration of fat.

REFERENCES

Folch, J., Lees, M. and Stanley, G.H.S. (1957) : J. Biol. Chem., 226, 497.

Bligh, E.G. and Dyer, W.J. (1959) : Can. J.Biochem. Physiol., 37, 911.

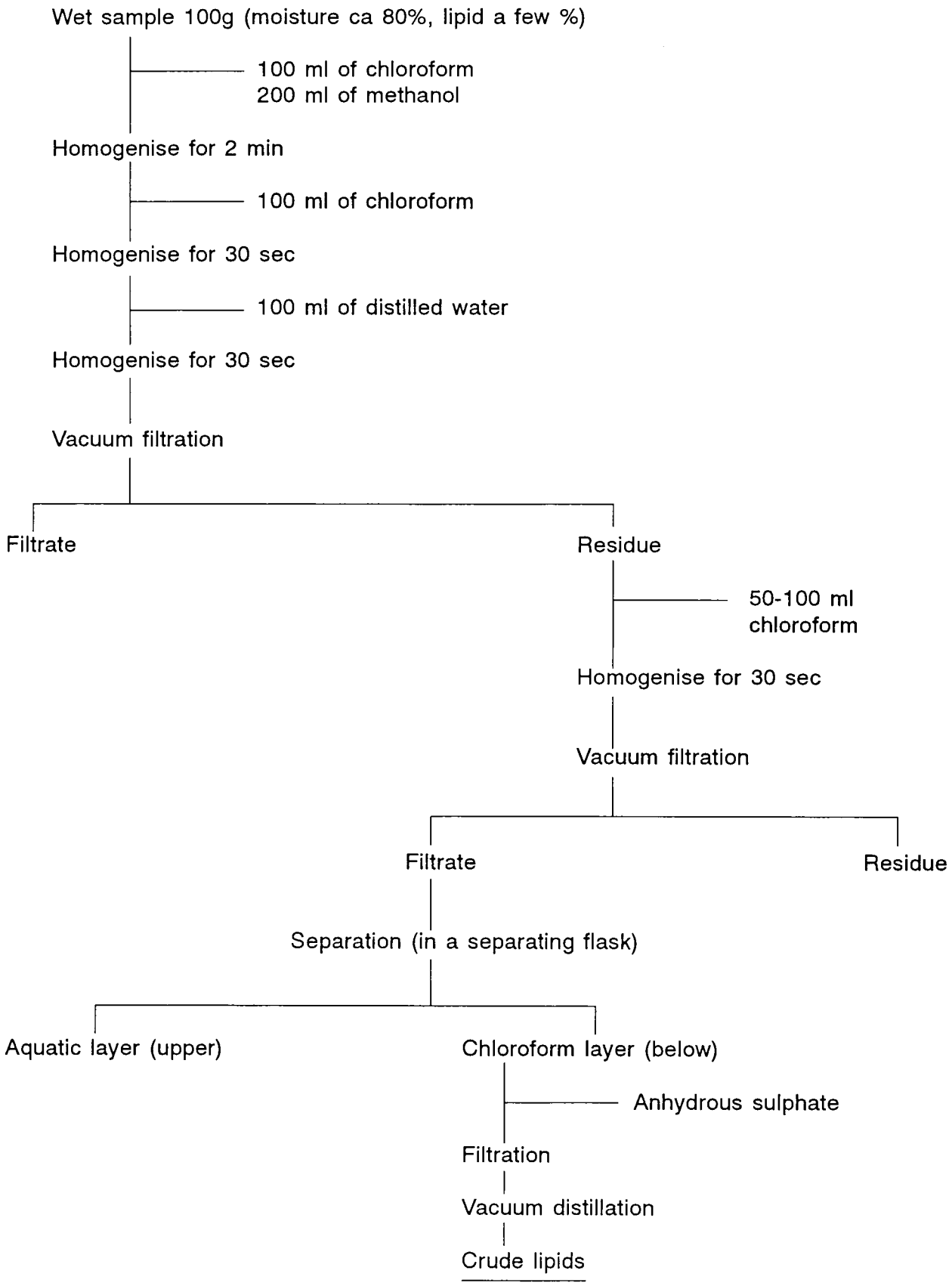


Fig. 1. Extraction Method of Lean Fish Lipids

(Taken from Bligh et. al (1959))

LIPID COMPOSITION ANALYSIS BY THIN LAYER CHROMATOGRAPHY

KATSUTOSHI MIWA

INTRODUCTION

Fish lipids are generally composed of neutral fat (triglyceride, wax, cholesterol ester, glyceryl ether etc.) and polar lipids (phospholipids and galactolipids). Furthermore some pigments, fat-soluble vitamin and hydrocarbon are dissolved in neutral fat which is contained in hypodermal tissue, dark muscle and fatty tissue around viscera.

Lipid composition varies with fish species, and fish tissues and organs, therefore lipid composition analysis using chromatographic methods has an important role in the study of fish lipid metabolism. Silica gel column chromatography and thin layer chromatography (TLC) are available for the isolation, identification and determination of each lipid component.

TLC is able to analyze a small amount of lipids faster than column chromatography.

TLC was first reported by Izmailov & Shriber, who were scientists of USSR, in 1938. After that E. Stahl who was a pharmacologist in Germany, applied TLC to fields of medicine, pharmacy and food technology and built up the foundation of TLC method.

THIN LAYER PLATE PREPARATION[*1]

1. Apply the absorbent, which is usually a mixture of 1 g of silica gel, 0.05 g of calcium sulphate and 2 ml of water, on the glass plates (20 x 20 or 10 x 20 cm) in the form of an aqueous slurry by means of a suitably designed spreader to obtain even layers of 0.25 mm thickness.

2. Air dry and activate the plates by heating in oven at 110 - 120°C for one or two hours.

3. Store in an airtight box or in a desiccator.

SAMPLE PREPARATION

Crude lipids are extracted by modified Folch method or Bligh method (see C2 or C-12 this book). A preparation of 5-10% of this crude lipid in chloroform and methanol mixture (2:1 v/v) is put into a glass test tube with glass stopper [*2], flushed with nitrogen gas and stored frozen.

REAGENTS

1. Developing Solvent Systems

   The following two kinds of developing solvent systems are generally used for separation of neutral fat components.

   a) petroleum ether : diethyl ether : acetic acid

      (90 : 10 : 1, V/V/V)

   b) n-hexane : diethyl ether : acetic acid

      (80 : 20 : 1, V/V/V)

   The following two developing solvent systems are usually used for separation of phospholipids.

   c) Chloroform : methanol : water

      (65 : 25 : 4 V/V/V)

   d) Chloroform : methanol : acetic acid : water

      (25 : 15 : 4 : 2, V/V/V/V)

2. Detecting reagents (spray)

   The following two reagents are common detecting reagents of all lipids:

   a) 50% sulphuric acid (w/v)

   b) 3% copper (II) acetate monohydrate (w/v), $(CH_3COO)_2 Cu.H_2O$, in 8% phosphoric acid (w/v)

   These detecting reagents break down and carbonize the lipids, so that the lipid spots blacken after heating the plates.

   By means of looking at the feature and thickness of black spots on TLC, we are able to find out the lipid composition and the estimated amount of lipids. Densitometric determination method[*3] is one of the measurement methods for estimating lipid content.

If the lipid components separated on TLC are needed to be determined accurately, mild detecting reagents should be used and the developed spots recovered from the plate. The common mild detecting reagents are:

c) Iodine vapour : TLC plate is put into an iodine vapour filled tank for 2-3 min, after which the lipid spots turn brown. If an iodine tank is not available, a 2% iodine-ethanol solution can be used instead. TLC spots coloured with iodine vapour are discolored in air.

d) Ultraviolet irradiation : If the TLC plate is viewed under ultraviolet light, the lipid spots show fluorescence or appear as dark spots. When fluorescence reagents such as 0.2% 2', 7' - dichlorofluorescence ethanol solution or 0.05% rodamine B ethanol solution are sprayed on TLC plates, the lipid spots will appear as splendid fluorescence spots under ultra-violet light. Iodine vapor and the fluorescence materials do not break down the lipids, so the lipid spots can be recovered and the composition or quantity be determined.

Some specific detecting reagents of phospholipids, such as Dittmer-Lester reagent, ninhydrin reagent, Dragendorff reagent et. al., are quite well known. Dittmer-Lester reagent reacts with inorganic phosphorus and becomes blue spots. Ninhydrin reagent reacts with amino compounds, phosphatidyl ethanolamine & phosphatidyl serine, and becomes violet spots. Dragendorff reagent[*4] reacts with choline, phosphatidyl choline, and becomes reddish-brown spots. Dittmer-Lester reagent is suitable for detecting phospholipids.

e) Molybdenic ammonium - perchloric acid reagent (Dittmer-Lester reagent).

3g of molybdenic ammonium is dissolved in 50 ml of water followed by 5 ml of 6M hydrochloric acid and 13 ml of 70% perchloric acid.

Spray this reagent on TLC and heat the TLC plate at 80°C for 10 min. The spots of phospholipids will develop as blue spots.

APPARATUS

1. Spreader
   Fig. 1 is a kind of spreader called "applicator" devised by Kirchner. It is very popular and convenient.

2. Developing tank
   Normally a box type tank made of glass is used (Fig. 2). The size of the tank is usually 25 x 15 x 25 cm with an air-tight glass cover.

3. Spray
   A spray as shown in Fig. 3 can be used.

4. Oven drier
   Oven drier capable of temperature control up to 200°C.

5. Micro-syringe (50 μl) and spotting capillary tube.

PROCEDURE

1. Apply sample in a solvent as discrete spots 2.0 cm from the bottom of the plate by means of a syringe or a capillary tube.

2. Place plate in developing tank containing developing solvent system.

3. Remove plates when the solvent front reaches 10 cm from the spotted bottom of the plate.

4. Dry in air or vacuum for about 30 min to remove solvent.

5. For lipid composition analysis, spray with detecting reagents.

6. When using 50% sulphuric acid or 3% copper acetate-phosphoric acid as detecting reagent, heat the plate at 150-180°C for 15-20 min after spraying. The plate will turn white and the spots black. (See Fig. 4[*5] & Fig. 5[*6]).

7. Identification of spots is done by means of comparing with the retention time (R_f) of standard samples and analysis of qualitative reactions. Each lipid has its own R_f if the same developing solvent system is used, but the R_f may vary slightly depending on the activity of TLC plates and room temperature.

8. If it is necessary to accurately determine the lipid content, a definite amount of sample has to be spotted using a syringe and then developed with the solvent system. After the spots are detected by mild detecting methods such as iodine vapor or fluorescence under ultra-violet light, the spots are raked out with a small spatula, then the lipids are extracted from the spot powder with chloroform-methanol (2:1) and determined.

9. In keeping a record of TLC condition, the following should be included: date of experiment, room temperature, sample type, absorbent type, thickness of absorbent, developing solvent system, developing time, detecting reagent, and R_f of spots and standards.

REMARKS

*1 Commercially prepared plates are available:
TLC plates silica gel G, F_{254} pre-coated, 10 x 20 cm, layer thickness 0.25 mm.

*2 Gummy stopper cannot be used as a stopper for the sample container, because gummy stopper is soluble in chloroform-methanol which is the solvent of samples.

*3 The estimated lipid content of the spot can be determined by means of measuring the concentration of spot colour and the size of spot using a densitometer.

*4 Dragendorff reagent is prepared as follows:

Basic bismuth nitrate 1.7 g is dissolved in 100 ml of 20% acetic acid solution (A solution). 40g of potassium iodide is dissolved in 100 ml of water (B solution). 20 ml of A solution, 5 ml of B solution and 70 ml of water are mixed prior to spraying.

*5 Fig. 4. shows the developing pattern of whole shark liver oil on TLC using n-hexane - diethyl ether - acetic acid developing solvent systems.

*6 Fig. 5. shows the developing pattern of shark liver phospholipids on TLC using chloroform-methanol - water developing solvent systems.

REFERENCES

N.A. Izmailov & M.S. Shriber (1938) : Farmatsiya, 3, 1.

E. Stahl (1969) : Dünnschicht-chromatographic, Springer-verlag, Berlin.

Chemical society of Japan (1978) : New handbook of chemical experiments (Biochemistry I), Maruzen.

T. Saito et.al. (1974) : Biochemistry of fisheries & food technology experimental handbook, Koseisha-Koseikaku.

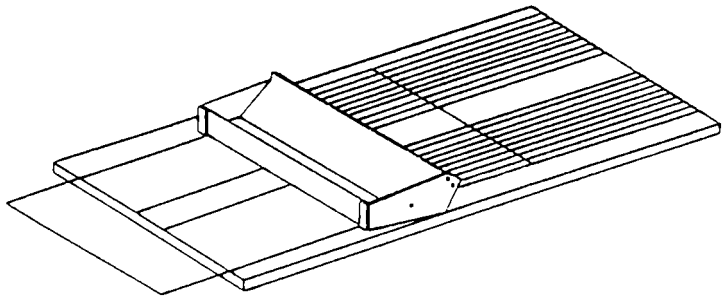


Fig. 1. Kirchner's applicator

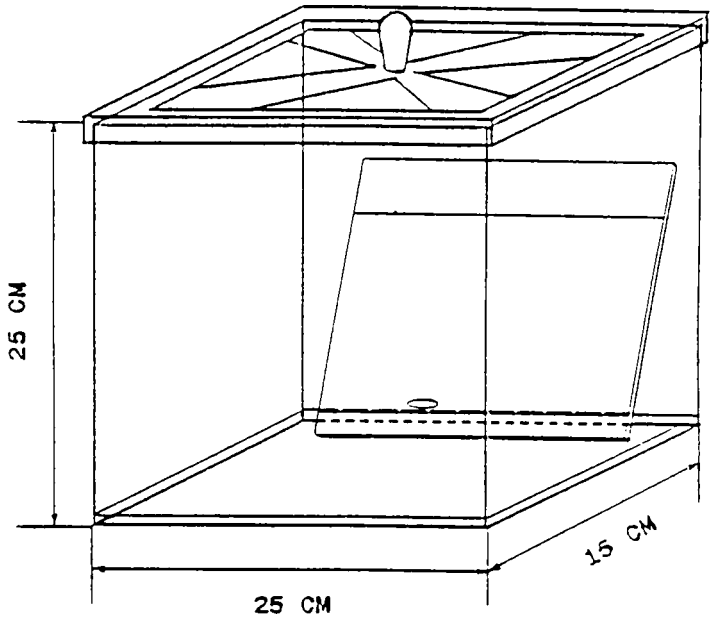


Fig. 2. Developing tank made of glass

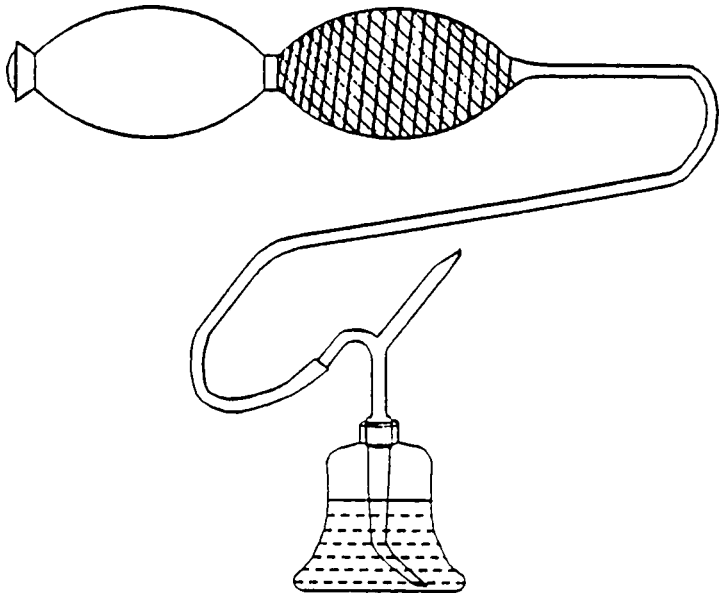


Fig. 3. Spray for detecting reagents

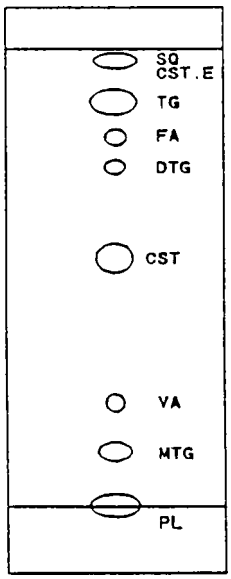


Fig. 4. TLC of whole shark liver oil (developing solvent system; n-hexane : diethyl ether : acetic acid, 80 : 20 : 1)

SQ : squalene
CST.E : cholesterol ester
TG : triglyceride (triacylglyceride)
FA : fatty acid
DTG : diglyceride
CST : cholesterol (free)
VA : vitamin A
MTG : monoglyceride
PL : phospholipid

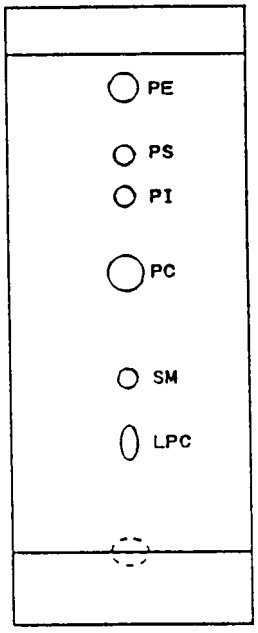


Fig. 5. TLC of phospholipid of shark liver oil (developing solvent system, chloroform:methyl alcohol: water, 65 : 25 : 4)

PE : phosphatidyl ethanolamine
PS : phosphatidyl serine
PI : phosphatidyl inositol
PC : phosphatidyl choline
SM : sphingomyelin
LPC : Lyso-phosphatidyl choline

ANALYSIS OF ADDITIVES

DETECTION OF POLYPHOSPHATES

NG MUI CHNG

INTRODUCTION

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

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.

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.

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 x 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, $NaH_2PO_4.H_2O$
Tetrasodium diphosphate decahydrate, (sodium pyrophosphate) $Na_4P_2O_7.10H_2O$
Pentasodium triphosphate, (sodium tripolyphosphate) $Na_5P_3O_{10}$
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.

APPARATUS

1. Oven (Temp 30 - 200°C)
2. Glass chamber tank with cover
3. TLC plates (20 x 20 cm)
4. Glass tips

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 µl of the standard solutions on the plate at an interval of 1.5 - 2 cm, 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).

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 MUI CHNG

INTRODUCTION

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

The quantity 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 iodonitro tetrazolium chloride (INT) to a formazan which is measured in the visible range at 492 nm (see reaction 2).

$$\text{(1)}\quad \text{L-Glutamate} + NAD^+ + H_2O \xrightarrow{\text{GIDH}} \alpha\text{-ketoglutarate} + NADH + NH_{4+}$$

$$\text{(2)}\quad NADH + INT + H^+ \xrightarrow{\text{diaphorase,}} NAD^+ + \text{formazan}$$

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.

SAMPLE PREPARATION

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

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 is pipetted into 1.8 ml of distilled water. This contains 8.0 ug 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

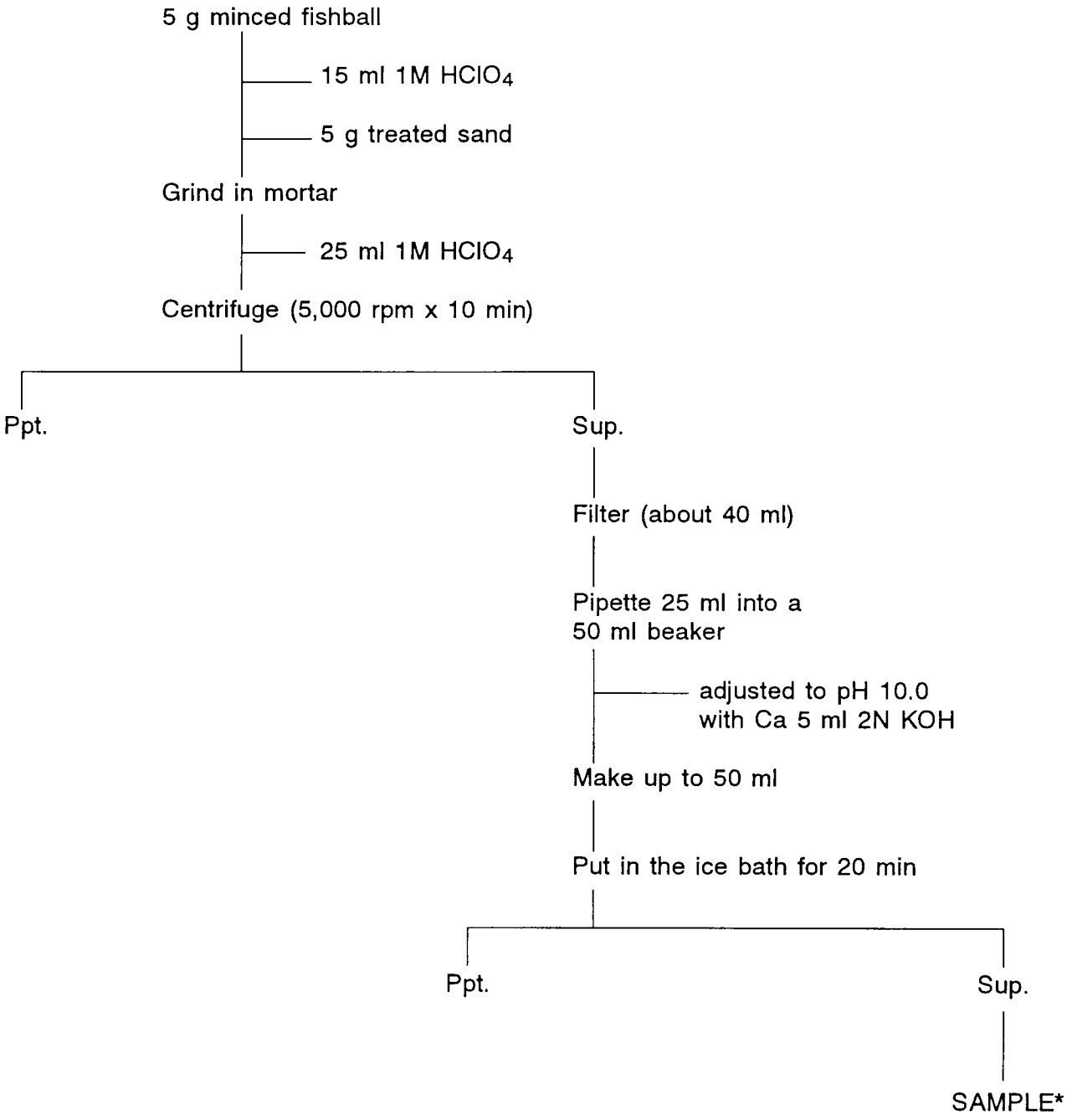
f) Enzyme solution (1 set contains 4 enzyme solutions named solution 1, 2, 3 and 4)

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

Food Analysis; Boehringer
Mannheim, GMBH, WEST GERMANY

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

CALCULATION

To calculate the L-glutamic acid in fishball

$$\text{Corrected O.D.}_{\text{sample}} = \text{O.D.}_{\text{sample}} - \text{O.D.}_{\text{blank}}$$

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

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

where $\text{O.D.}_{\text{standard}}$ = 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 MUI CHNG

INTRODUCTION

Sugar is widely used in the manufacture of food as a 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.

PREPARATION OF SAMPLE

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

REAGENTS

a) Somogyi solution A

Anhydrous Na_2CO_3 25g

- KNa – tartarate.$4H_2O$ 25g
- 500 ml H_2O
- $CuSO_45H_2O$ 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

$K_2C_2O_4.H_2O$ (potassium oxalate) 28g

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 in a volumetric flask.

e) 2N H_2SO_4

Conc. H_2SO_4 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 KIO_3

Weigh about 2 g of KIO_3, dry 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 $Na_2S_2O_3$ solution

Sodium thiosulphate $Na_2S_2O_3.5H_2O$, 13 g.

Na_2CO_3 0.2 g

Make up to 1 litre with decarbonated H_2O

j) 0.005N $Na_2S_2O_3$

Dilute 100 ml of 0.05N $Na_2S_2O_3$ to 1 litre.

DETERMINATION OF FACTOR (F) OF 0.05N $Na_2S_2O_3$

Weigh about 1.5 g dried KIO_3 accurately

|

Make up to 500 ml with H_2O in volumetric flask

|

Pipette 10 solution — Blank: Pipette H_2O, 10 ml

|

Add 2.5% KI, 20 ml

Add 2N H_2SO_4, 20 ml

|

Titrate with 0.05N $Na_2S_2O_3$ with starch indicator

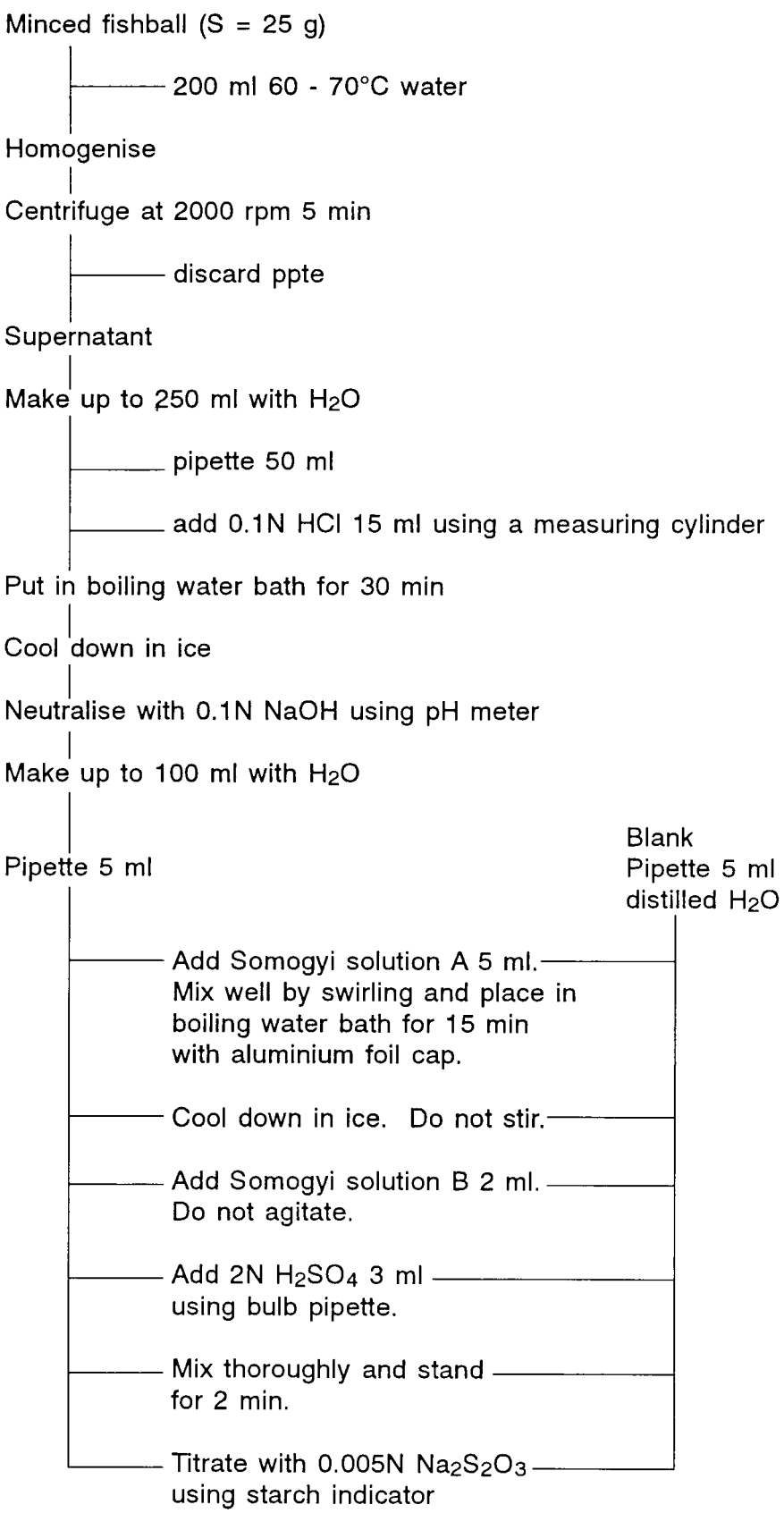
$$\text{Factor, } F = \text{wt. of } KIO_3 \times \frac{10}{500} \times \frac{1}{0.0017835} \times \frac{1}{(B - A)}$$

0.0017835 : conversion factor of 1 ml 0.05N $Na_2S_2O_3$ to KIO3 (g)

A : titration volume of KIO_3 solution (ml)

B : titration volume of blank (ml)

PROCEDURE


Minced fishball (S = 25 g)
200 ml 60 - 70°C water
Homogenise
Centrifuge at 2000 rpm 5 min
discard ppte
Supernatant
Make up to 250 ml with H_2O
pipette 50 ml
add 0.1N HCl 15 ml using a measuring cylinder
Put in boiling water bath for 30 min
Cool down in ice
Neutralise with 0.1N NaOH using pH meter
Make up to 100 ml with H_2O
Pipette 5 ml
Blank
Pipette 5 ml
distilled H_2O
Add Somogyi solution A 5 ml.
Mix well by swirling and place in
boiling water bath for 15 min
with aluminium foil cap.
Cool down in ice. Do not stir.
Add Somogyi solution B 2 ml.
Do not agitate.
Add 2N H_2SO_4 3 ml
using bulb pipette.
Mix thoroughly and stand
for 2 min.
Titrate with 0.005N $Na_2S_2O_3$
using starch indicator

CALCULATION

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

$$= 13.7655\ (B - A)\ F \times \frac{1}{S}$$

where 0.0001449 : 1 ml 0.005N $Na_2S_2O_3$ = 0.0001449 g glucose

A : Sample titration volume (ml)

B : Blank titration volume (ml)

F : Correction factor of $Na_2S_2O_3$

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. 1980:226, 14.114(d)
877, 50.037, 50.038
515, 31.052, 31.053

DETERMINATION OF STARCH

NG MUI CHNG

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 (see D-3). The content of starch in the sample is then back calculated from the content of the glucose.

For confirmation of the presence of starch in a 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.

SAMPLE PREPARATION

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

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) dissolved in 85 ml distilled water.

e) Somogyi solution A

Sodium potassium tartrate 90 g
(K Na $C_4H_4O_6.4H_2O$)

$CuSO_4.5H_2O$ 30 g

H_2O 500 ml

Trisodium phosphate
$Na_3PO_4.12H_2O$ 225 g

H_2O 700 ml

KIO_3 3.5 g

H_2O 100 ml

Make up to 2 litres with distilled water

Keep at room temperature.

f) Somogyi solution B

Potassium oxalate 90 g
($K_2C_2O_4.H_2O$)

KI 40 g

Make up to 1 litre with distilled water.

Keep at room temperature.

g) 2N H_2SO_4

Conc. H_2SO_4 (60 ml) dilute to 1 litre with distilled water. (Conc. H_2SO_4, 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 KIO_3

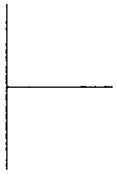
Weigh about 2 g KIO_3 and dry in oven at 120°C for 1 hour.

j) 2.5% KI

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

k) 0.05N $Na_2S_2O_3$ solution

Sodium thiosulphate $Na_2S_2O_3.5H_2O$ 13g



Na_2CO_3 0.3 g

Make up to 1 litre with decarbonated distilled water.

Determination of factor, F, of 0.05N $Na_2S_2O_3$

Weigh 1.5 g dried KIO_3 accurately and dissolve in 500 ml distilled water in volumetric flask.

To 10 ml of KIO_3 solution and 10 ml distilled water (BLANK) each add 2.5% KI (20 ml) and 2N H_2SO_4 (20 ml).

Titrate with 0.05N $Na_2S_2O_3$ using starch indicator.

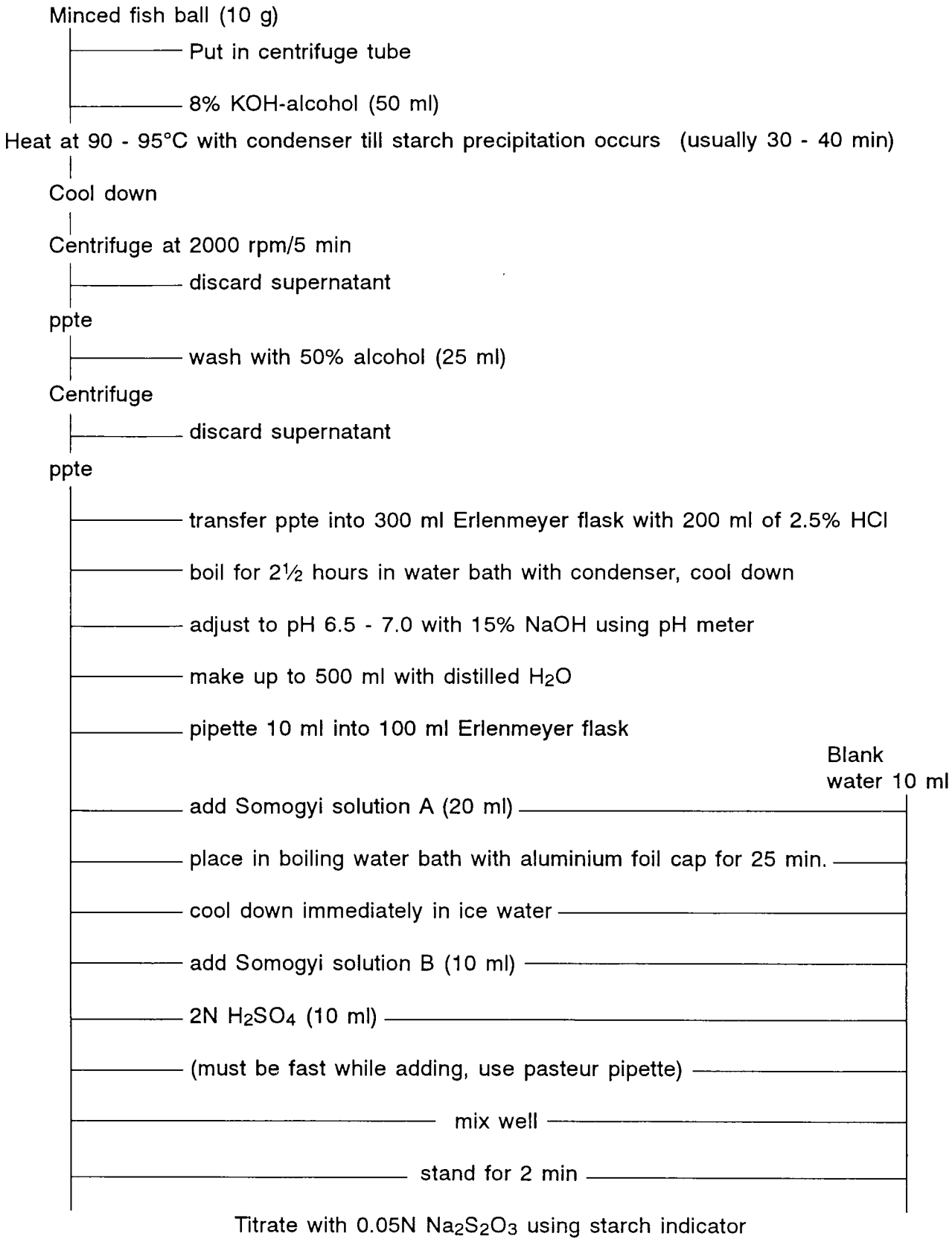
$$\text{Factor, } F = \text{Wt. of } KIO_3 \times \frac{10}{500} \times \frac{1}{0.0017835} \times \frac{1}{(B - A)}$$

where 0.0017835 : conversion factor of 1 ml 0.05N $Na_2S_2O_3$ to KIO_3

A : titration volume of KIO_3 solution (ml)

B : titration volume of blank (ml)

PROCEDURE



(Note: colour changes to light blue).

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

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 $Na_2S_2O_3$
0.001449	=	Conversion factor of 0.05N $Na_2S_2O_3$ (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 MUI CHNG

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.

PREPARATION

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

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 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.

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 glass wool. Collect the filtrate in a 250 ml volumetric flask. Make up to the volume and shake well.

* Centrifuging is only necessary if the particles in suspension are very fine.

4. Transfer 10 ml filtrate with bulb pipette into a 100 ml conical flask. Add 50 ml distilled water using the measuring cylinder and add 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).

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 $AgNO_3$ to 0.005844 g NaCl

REFERENCES

Pearson D. (1976) : The chemical analysis of food. 7th Ed: 519.

Official methods 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 MUI CHNG

INTRODUCTION

Boric acid and borates are commonly used as preservatives. They act as anti-microbiological agents. 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 the test paper to dark blue-green, but it is 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.

PREPARATION OF SAMPLE

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

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 a 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. petri dish). Hang paper to dry. After 1 hour, cut into 6 x 1 cm strips and store in a 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 reference 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.

APPARATUS

1. Erlenmeyer flask (125 ml)
2. Glass rod for stirring
3. Watch glass
4. Bunsen burner
5. Test tubes
6. Petri dish
7. Forceps
8. Scissors and string

PROCEDURE

1. Disperse 25 g of ground meat in 50 ml distilled water in a 125 ml Erlenmeyer flask, using a 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 is solidified (30 min).

4. Filter through pledget of glass wool.

5. Transfer 10 ml filtrate to a 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 ½ 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.

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.

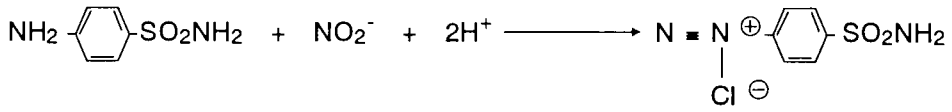
DETERMINATION OF SODIUM NITRITE (residual NO_2) BY COLORIMETRIC METHOD

MAKOTO YAMAGATA AND LOW LAI KIM

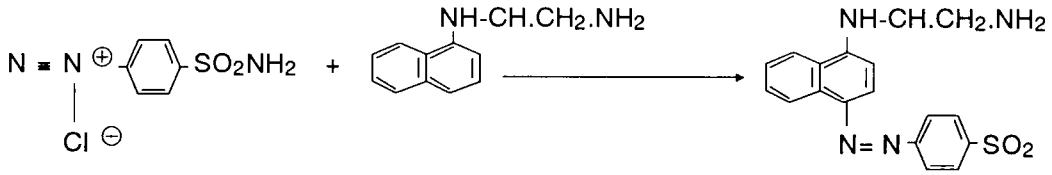
INTRODUCTION

In this method the sulfanilamide combines with the nitrite under acid conditions to become a diazotized salt. The diazotized salt then combines with naphthyl ethylene diamine to form the reddish-violet colour of the azo dye. The intensity of the colour is proportional to the concentration of the nitrite present. The nitrite concentration is determined by the absorbance of the azo dye at a wavelength of 540 nm.

The types of fish products analysed for nitrite are fish sausages, fish hams, salmon roe (or "ikura") and alaska pollack roe.



Sulfanilamide — Diazotized salt



Diazotized salt — Naphthy ethylene diamine — Azo dye

APPARATUS

1. Homogenizer and cup (capacity : 100 ml)
2. Volumetric flasks (25 ml and 200 ml)
3. Water bath (80°C)

REAGENTS

1. 0.5N Sodium hydroxide (NaOH)

2. 12% Zinc sulphate ($ZnSO_4.7H_2O$)

3. 10% Ammonium acetate (CH_3COONH_4) buffer solution :
   Weigh 100 g of ammonium acetate and dissolve in 900 ml of distilled water. Adjust the pH to pH 9.0 using 10% ammonia water (NH_4OH).

4. 1% Ammonium acetate (CH_3COONH_4) buffer solution :
   Dilute the 10% ammonium acetate (pH 9.0) 10 times.

5. 0.5% sulfanilamide (p-aminobenzene sulfonamide, $C_6H_8N_2O_2S$, or sulfanilamidum) : Weigh 0.5 g of sulfanilamide and dissolve in 100 ml of hydrochloric acid with warming (Concentrated HCl : distilled water is 1:1, v/v). Keep in amber glass reagent bottle with glass stopper. This solution can be kept for 4 weeks at 5 - 10°C.

6. 0.12% N-(1-naphthyl)ethylene diamine dichloride ($C_{12}H_{16}Cl_2N_2$).
   Weigh 0.12 g and dissolve in distilled water. Make up to 100 ml. Keep in amber reagent bottle. This solution can be kept for 4 weeks at 5 - 10°C. The solution must be colourless. If there is colour development, the solution should be discarded.

7. HCl (1:1, v/v)
   Dilute concentrated HCl (min. 37%) with an equal volume of distilled water.

8. NO_2 standard solution:

   Preparation of the standard original solution

   Dry sodium nitrite ($NaNO_2$) well in a concentrated sulphuric acid (H_2SO_4) desiccator for 24 hours. Weigh 0.493 g of the dried $NaNO_2$ and dissolve in sterilized, distilled water. Make up to 1000 ml. This is the standard original solution (0.493g $NaNO_2$/1000 ml). Store in amber reagent bottle.

   Preparation of the standard solution.

   Take 10 ml of the standard original solution and make it up to 100 ml with distilled water. Take 2 ml from this and add 10 ml of 1% ammonium acetate. Then make up to 100 ml with distilled water. This is the standard solution. Store in amber reagent bottle. Unstable reagent, must not be stored for more than 2 weeks.

   1 ml of standard sodium nitrite solution contains 0.2 ug NO_2-N.

Preparation of standard curve

Pipette 2.5, 5, 10, 15 and 20 ml of the standard sodium nitrite solution into 25 ml volumetric flasks. Make up to 20 ml with 1% ammonium acetate buffer solution.

These 20 ml solutions contain 0.5, 1, 2, 3 and 4 ug NO_2 respectively.

Spectrophotometric readings of standard curve

Add 1 ml 0.5% sulphanilamide solution into each of the volumetric flasks containing the standard solutions. Shake well. Then add 1 ml 0.12% naphthylethylene diamine solution and shake. Make up to 25 ml with distilled water and shake thoroughly. Let stand for 20 min and read at 540 nm.

PROCEDURE

Sample Preparation (Fig. 1)

1. Weigh 10.0g sample into homogeniser cup.
2. Add about 20 ml to 30 ml of distilled water (ca. 80°C).
3. Homogenize for 30 sec.
4. Pour into 250 ml conical flask.
5. Wash homogenizer cup with distilled water, and make up to 150 ml with hot distilled water (ca. 80°C).
6. Pipette 10 ml of 0.5N NaOH, followed by 10 ml of 12% $ZnSO_4.7H_2O$.
7. Cover the mouth of the flask with aluminium foil and heat for 20 min. at 80°C in a water bath.
8. Cool to room temperature with cool running water.
9. Add 20 ml of 10% NH_4CH_3COOH buffer solution.
10. Make up to 200 ml with distilled water in a volumetric flask.
11. Mix well and stand for 10 min.
12. Filter through Whatman No. 1 filter paper, discarding about 10 ml of first filtrate.
13. Use filtrate as test solution.
14. For a blank test use 10 ml of distilled water instead of sample and repeat steps 1 to 13 to obtain a blank test solution.

Analytical Procedure (Fig. 2)

1. Pipette 20 ml of sample test solution into a 25 ml volumetric flask.
2. Pipette 1 ml of 0.12% naphthylethylene diamine solution into the volumetric flask.
3. Follow up with 1 ml 0.5% sulfanilamide.
4. Make up to 25 ml with distilled water, mix well and stand for 20 min.
5. Measure the absorbance for the colour developed using a spectrophotometer at a wavelength of 540 nm.
6. For a blank test, pipette 20 ml of blank test solution into a 25 ml volumetric flask, and repeat steps 2 to 4.
7. Into a third 25 ml volumetric flask, pipette 20 ml of sample test solution, followed by 1 ml of HCl(1:1, v/v). Then repeat steps 4 and 5.

CALCULATION

NO_2 (ppm) = 3.28 A

A = Aa – (Ab + Ac)

where Aa = Absorbance of sample test solution

Ab = Absorbance of blank test solution

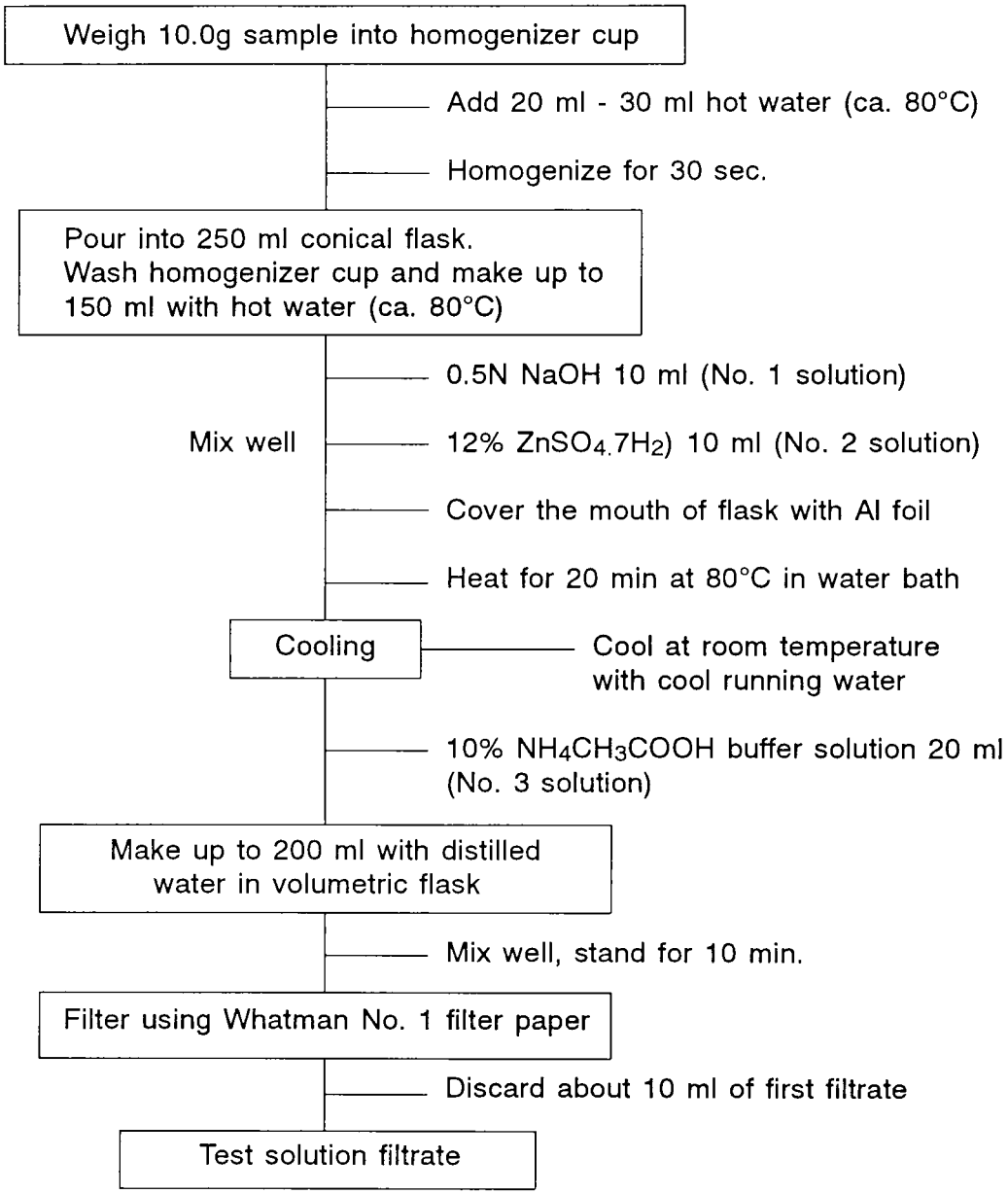
Ac = Absorbance of sample test solution and HCl (1:1)

REMARKS

1. In cases where the fat content is high such as salmon roe and cheese, the filtrate may be turbid. Thus 10 ml of 1N NaOH should be added with 24% $ZnSO_4.7H_2O$ or 20 ml of 0.5N NaOH and 12% $ZnSO_4.7H_2O$. The filtrate will become clear or transparent after this treatment.
2. Detection limit : 0.1 ppm (0.0001 g/kg)

REFERENCE

Shokuhin Eisei Kensa Shishin (I), Guidelines for Food Hygiene Inspection (I), Japan Food Hygiene Association (1973).



N.B. Blank test should be done using 10 ml of distilled water instead of sample. Repeat the above procedure with the blank.

Fig. 1 Sample Preparation for NO_2 analysis

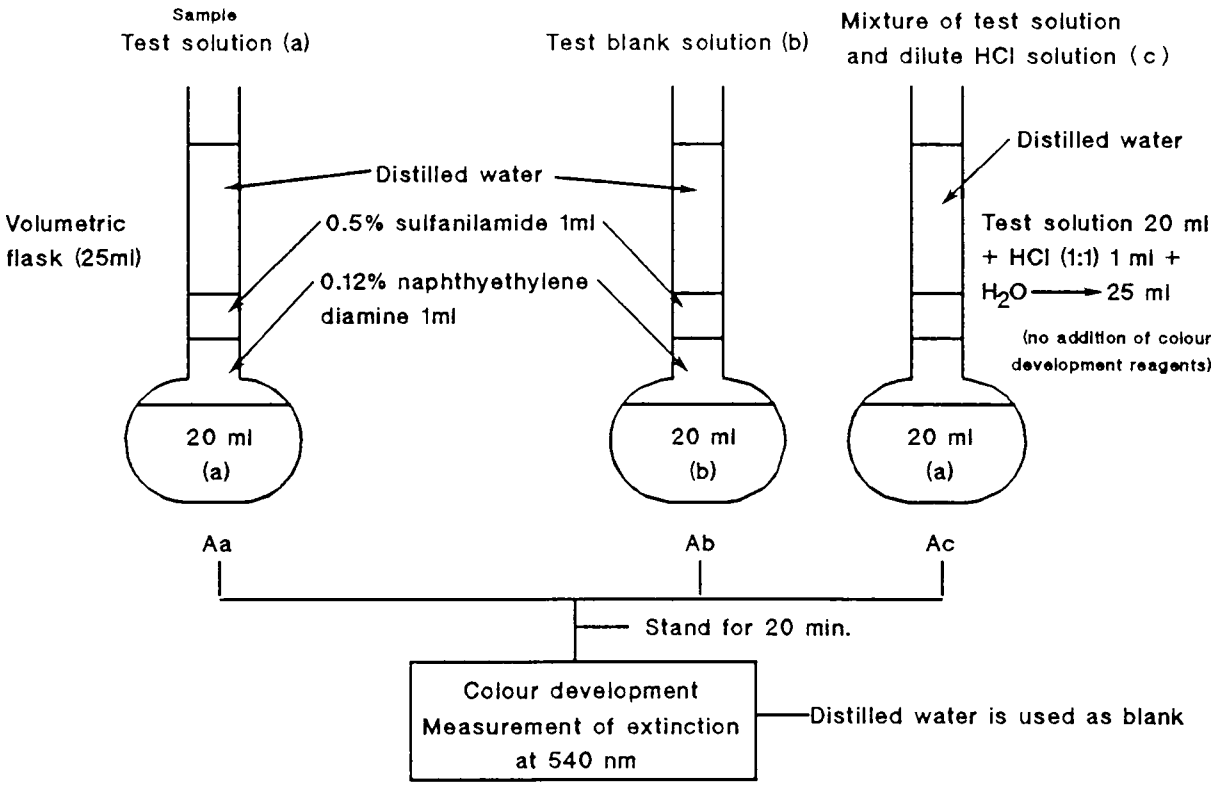


Fig. 2 Analytical Procedure for NO_2 analysis

DETERMINATION OF SULPHUR DIOXIDE (residual SO_2) IN FROZEN SHRIMPS BY COLORIMETRIC AND CONWAY'S MICRODIFFUSION METHOD

MAKOTO YAMAGATA AND LOW LAI KIM

INTRODUCTION

This method uses a combination of colorimetric method and Conway's microdiffusion method. By regulation (under the Food Sanitation Law, Japan), peeled shrimps should have a residual SO_2 of less than 100 ppm (0.1g/kg).

APPARATUS

1. Volumetric flasks (100 ml, 250 ml, 1000 ml)
2. Conway Microdiffusion Units with covers.
3. Pipettes (1 ml, 5 ml, 10 ml, 20 ml)
4. Mortar and pestle
5. Centrifuge (4,000 rpm)
6. Centrifuge tubes
7. Oven (37°C)

REAGENTS

1. Absorbent solution (Mercuric chloride* solution of SO_2)

   Weigh 27.2 g of mercuric chloride ($HgCl_2$) and 11.7 g of sodium chloride (NaCl) and dissolve in an adequate volume of 5% glycerine solution in a volumetric flask. Make up to 1000 ml with 5% glycerine. Then add 0.03 g of sodium azide (NaN_3) to the solution.

2. Diluting solution

   Take one volume of the absorbent solution and mix thoroughly with three volumes of distilled water (1:3, v/v).

   *Caution : Mercuric chloride should be handled with care because it is highly toxic. Proper disposal method for mercuric chloride should be used.

3. ρ-rosaniline formalin solution

   Dissolve 0.2 g of ρ-rosaniline hydrochloride into 100 ml of distilled water and filter if necessary. Measure 20 ml of this solution into a volumetric flask (100 ml) and add 6 ml of HCl solution (hydrochloric acid :12N, 36%). Make up to 100 ml with distilled water. Shake the diluted solution well. Transfer to a 250 ml flask and add 100 ml of 0.2% of formalin (HCHO).

N.B. ρ-rosaniline solution and 0.2% of formalin should be stored separately and mixed on the day of use. It is recommended that 0.2% formalin should be prepared fresh on the day of use.

4. Sulphurous standard solution

Weigh 0.5 g of sodium bisulphite ($NaHSO_3$) and dissolve in distilled water in a 100 ml volumetric flask. Make up to 100 ml with distilled water. This solution contains 5 mg $NaHSO_3$ / ml.

Take 10 ml of the sulphurous standard solution and add 15 ml of 0.1N iodine solution. Then add 2 ml conc. HCl (12N, 36%). Titrate against 0.1N sodium thiosulphate solution. Note the volume of titrant as **a** ml.

For blank use 10 ml of water and add 15 ml of 0.1N iodine solution. Then add 1 ml of conc. HCl (12N, 36%) and titrate against 0.1N sodium thiosulphate solution. Record as **b** ml.

$$A = \frac{93.75}{b - a} \times \frac{1}{f}$$

where :

a = volume of 0.1N sodium thiosulphate (ml) used in titration of sulphurous standard solution

b = volume of 0.1N sodium thiosulphate (ml) used in titration of blank

f = factor of 0.1N sodium thiosulphate solution used

Take A ml of the sodium bisulphite solution and make up to 300 ml with the diluting solution. This is the original sodium bisulphite solution.

5. Standard solution

Pipette 1 ml of the original sodium bisulphite solution into a 100 ml volumetric flask. Make up to 100 ml with diluting solution. Shake thoroughly. This gives the standard sodium bisulphite solution and contains 0.001 mg SO_2 in 1 ml.

PROCEDURE

Sample Preparation

1. Semi-thaw the frozen shrimps.
2. Wash slightly in running water.
3. Peel the shrimps, removing the carapace, shell and tail.
   (**N.B.** Do not wash shelled shrimps).
4. Weigh 10g of minced sample into mortar.
5. Pipette 70 ml of diluting solution followed by 20 ml of saturated (8%) mercuric chloride into mortar.
6. Mince and grind peeled shrimp in the mortar.
7. Centrifuge at 4,000 rpm for 10 min. or filter through Whatman No. 1 filter paper.
8. Collect the supernatant or filtrate as the sample solution.

Analytical Procedure (Fig. 1.)

1. Pipette 1 ml of absorbent solution into inner ring of Conway unit.
2. Pipette 1 ml of sample solution into outer ring of Conway unit.
3. Pipette 0.2 ml of 25% phosphoric acid solution into the outer ring.
4. Place fixing reagent (85% phosphoric acid or white vaseline) on cover of Conway unit.
5. Cover the dish and tighten with clip.
6. Stand at 37°C for 90 min or 30°C for 100 min or 20°C for 2 hours.
7. Pipette sample solution from inner ring into absolute test tube.
8. Wash inner ring with absorbent solution and make up to 5 ml with the absorbent solution.
9. Pipette 1 ml of ρ-rosaniline formalin solution into absolute test tube, mix well and stand at 20° - 25°C for 35 min.
10. Measure the absorbance of the colour developed at a wavelength of 560 nm.

CALCULATION

SO_2 in shrimp muscle(ppm)

$$= \frac{5 \times A \times \text{Total sample solution (100 ml)}}{As \times \text{Sample weight (10 g)}}$$

$$= \frac{5 \times A \times 100}{As \times 10}$$

$$= \frac{A}{As} \times 50$$

or SO_2 in shrimp muscles (g/kg)

$$= \frac{A}{As} \times 0.05$$

Where A = – log T (5 ml sample solution)
As = – log T (5 ml of standard solution)
Blank solution is 5 ml of absorbent solution.

REMARKS

The detection limit : 5 ppm (0.005 g/kg)

REFERENCE

Kanshoku No. 110. Ministry of Health and Welfare, Japan. 24th May 1973.

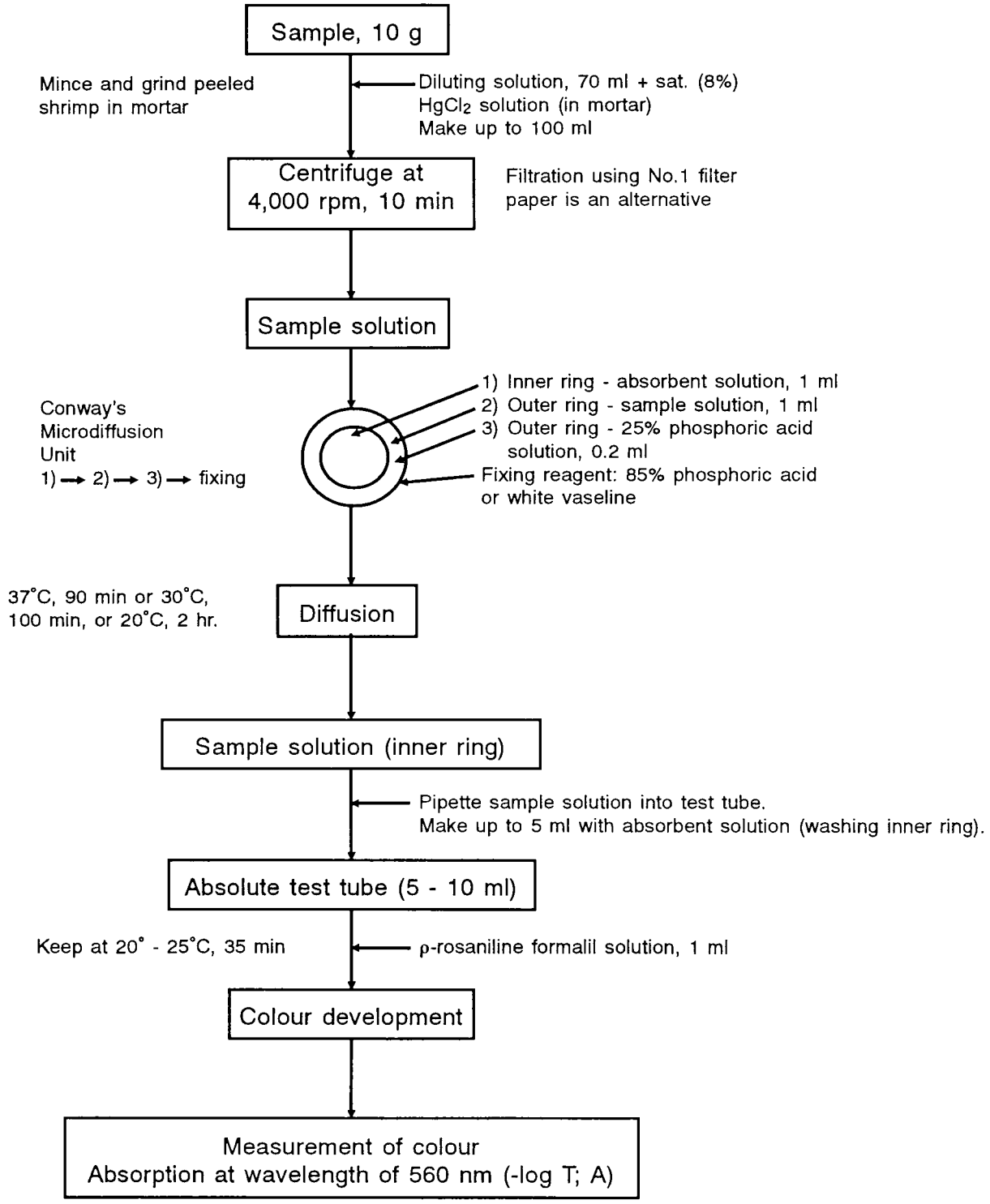


Fig. 1 Analytical procedure for SO_2 analysis

DETERMINATION OF SULPHUR DIOXIDE (residual SO_2) BY RANKINE METHOD A : ALKALI TITRATION METHOD

MAKOTO YAMAGATA AND LOW LAI KIM

INTRODUCTION

Sulphur dioxide in foods is extracted by heating with phosphoric acid (H_3PO_4). The sulphur dioxide is changed to sulphuric acid (H_2SO_4) in the presence of hydrogen peroxide (H_2O_2). The sulphuric acid is then titrated against sodium hydroxide (NaOH).

This method is applicable to all foods. However, the colorimetric method is better than this alkali method for foods containing a large amount of sulphide and salt.

APPARATUS

1. Round bottomed flasks, 100 ml
2. Oval shaped flasks, 50 ml
3. Burette, 50 ml
4. Apparatus for distillation as shown in Fig. 1.

REAGENTS

1. Carbon dioxide (CO_2) free distilled water

2. Indicator solution

   Dissolve 0.2 g methyl red and 0.1 g methylene blue in ethanol (95%, v/v) and make up to 100 ml.

3. 0.3% hydrogen peroxide (H_2O_2) solution

   Pipette 1 ml of 30% H_2O_2 (analytical grade) into distilled water in a 100 ml volumetric flask, and make up to 100 ml with distilled water.

4. 25% phosphoric acid (H_3PO_4) solution

   Add 240 ml of distilled water to 100 ml of 85% H_3PO_4. Mix thoroughly.

5. 0.01N (N/100) sodium hydroxide (NaOH) solution

6. Silicon oil as antifoaming agent (food additive).

PROCEDURE

Sample Preparation

The weight required for various samples are as follows :

20 g ———	Wine, natural fruit juice, soybean sauce.
10 g ———	Green mustard paste, molasses, millet jelly, candied cherry, dried fruit.
1 g ———	Paste made from the arum root powder (Konniaku), boiled bean, cut and dried radish, miso (soybean paste), chocolate, sugared beans, garlic, frozen shrimp, gelatin.
0.1 - 0.2 g ———	Dried gourd shavings (Kanpyo).

Analytical Procedure (Figs. 1 and 2)

1. Into Flask A pipette 10 ml of 0.3% H_2O_2 followed by 3 drops of indicator solution, 1 - 2 drops of 0.01N NaOH[*1]. The colour should change to olive green.
2. Fix Flask A to double cooling tube C.
3. Weigh sample into Flask B[*2] and add 40 ml of distilled water, followed by 1 - 2 drops of silicon oil[*3] and 10 ml of 25% H_3PO_4.
4. Quickly fix Flask B to glass capillary E.
5. Flow in air or nitrogen gas at a speed of 0.6 l/min.
6. Set micro-burner flame height at 4 - 5 cm and heat Flask B for 10 min.
7. Pull out Flask A, and wash the tip of the glass delivery tube with a small amount of distilled water into Flask A.
8. Titrate the solution in Flask A against 0.01N NaOH till solution in Flask A turns olive green.

CALCULATION

SO_2 (g/kg) = 0.3205 X (a – b) X f X 1/W

Where a = titration volume of sample solution (ml)

b = titration volume of blank test solution (ml)

f = factor of 0.01N NaOH

W = sample weight (g)

0.3205 = 1 ml of 0.01N NaOH contains 0.3205 mg of SO_2

Detection limit : 5 ppm (0.005 g/kg)

REMARKS

*1 = For the purpose of neutralising solution in SO_2 receiver i.e. Flask A.

*2 = Sulphur dioxide is unstable. Thus, after weighing, the sample must be analysed immediately.

*3 = For use as antifoaming agent.

REFERENCE

Shokuhin Eisei Kensa Shishin, Guidelines for Food Hygiene Inspection. (Analytical Methods for Food Additives in Food), Japan Food Hygiene Association (1989).

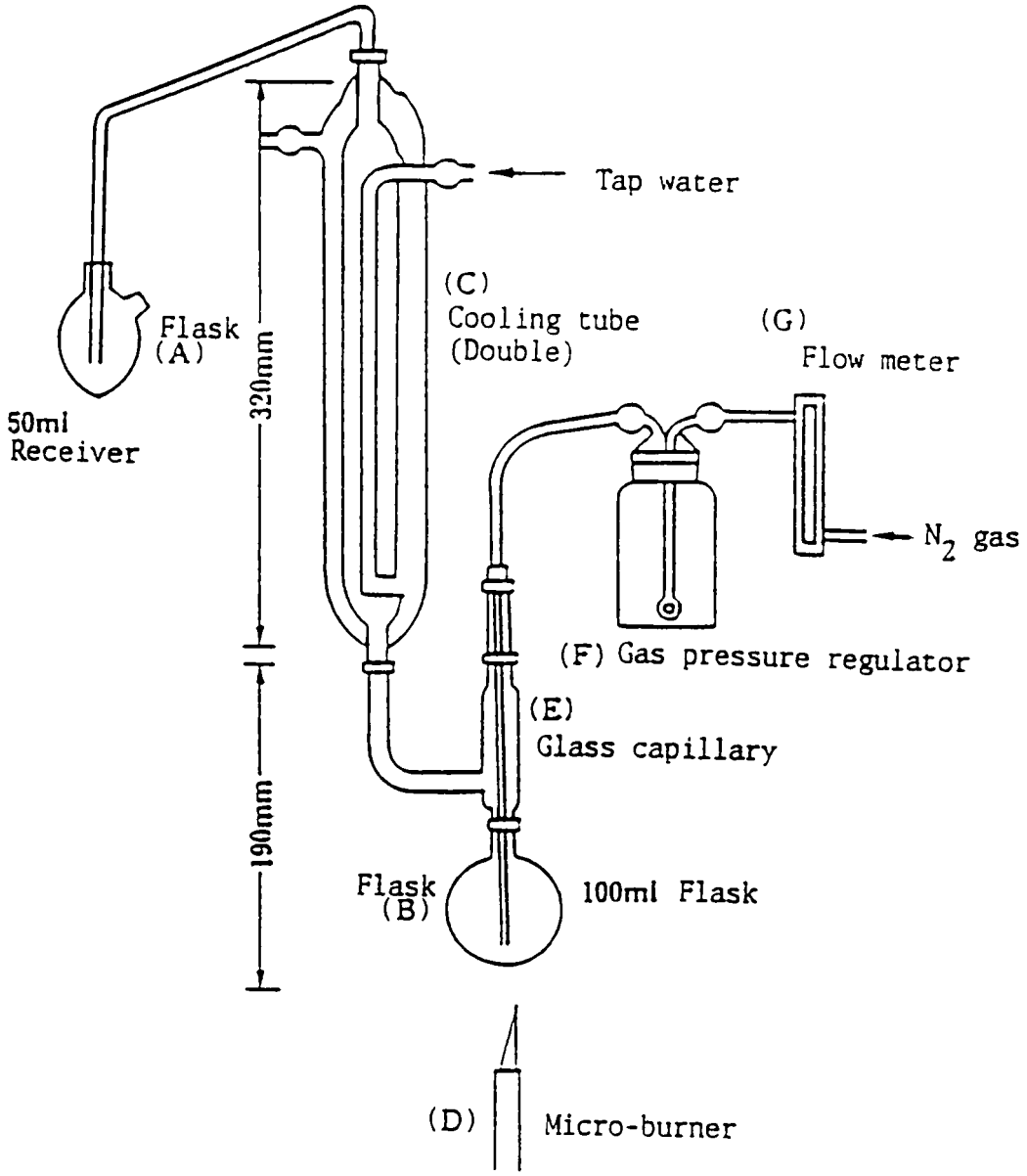


Fig. 1. APPARATUS FOR DISTILLATION
(RANKINE METHOD)

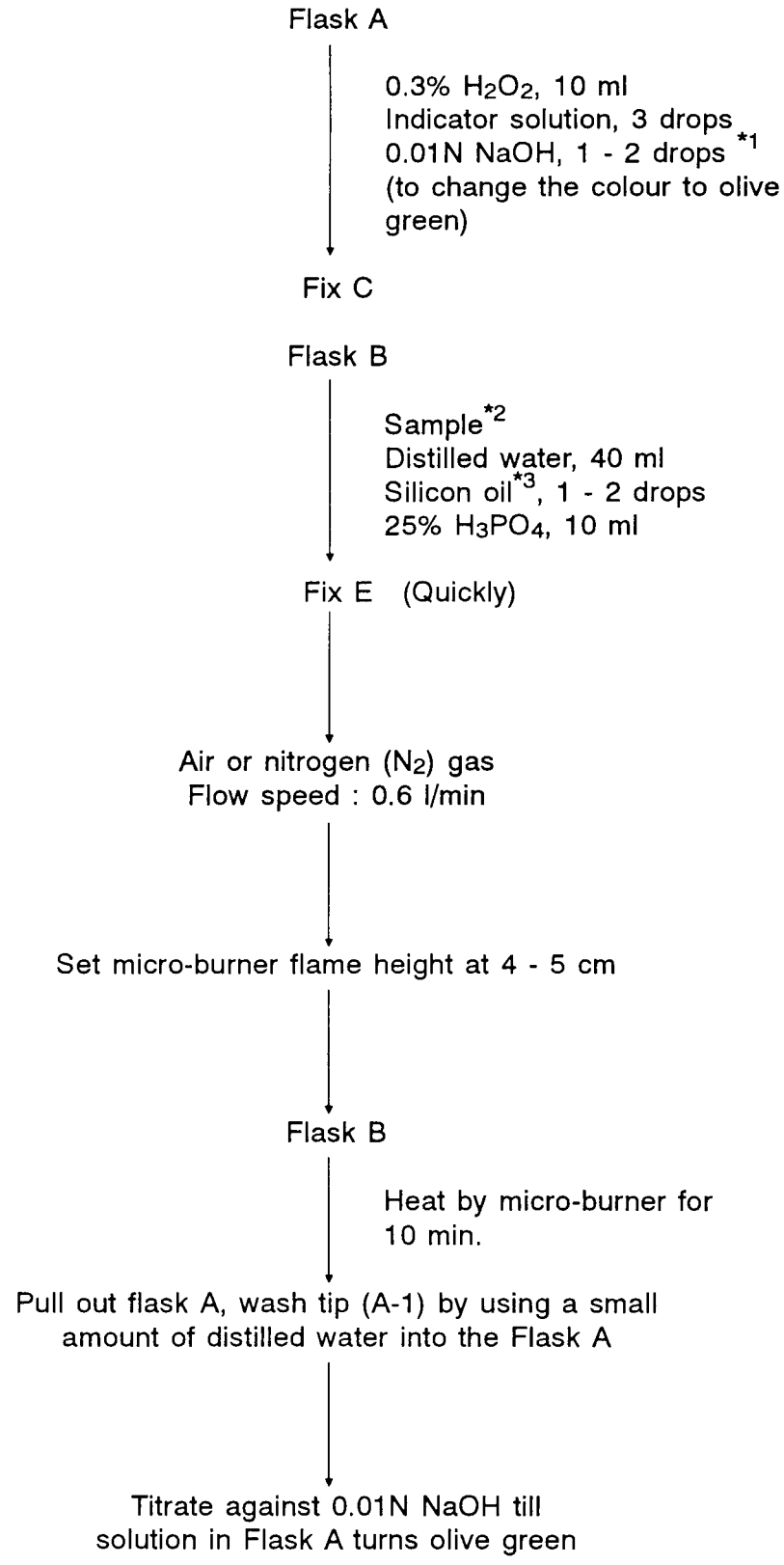


Fig. 2. Analytical procedure for the analysis of SO_2 by Rankine method.

DETERMINATION OF SULPHUR DIOXIDE (residual SO_2) BY RANKINE METHOD B : COLORIMETRIC METHOD

MAKOTO YAMAGATA AND LOW LAI KIM

INTRODUCTION

The sulphur dioxide in foods is distilled under acidic condition (phosphoric acid, H_3PO_4) in the presence of nitrogen (N_2) gas.

The receiver contains alkali (sodium hydroxide). By heating, the sulphur dioxide from foods are immediately stabilized in the form of a salt-alkali complex in the receiver.

An aliquot of the solution from the receiver is used and acidified using phosphate buffer solution. Then the addition of ρ-rosaniline-formaldehyde solution results in the development of a reddish colour. The intensity of the colour developed is measured by the colorimetric method.

This method is applicable to all foods.

APPARATUS

1. Round bottomed flasks, 100 ml
2. Oval shaped flasks, 50 ml
3. Graduated test tubes with stoppers, 10 ml
4. Distillation apparatus (Fig. 1)

REAGENTS

1. 1% sodium azide (NaN_3)

   Weigh 1 g of NaN_3 and dissolve in distilled water. Make up to 100 ml.

2. 95% (v/v) ethanol

3. Hydrochloric acid - sodium acetate solution

   Measure 600 ml hydrochloric acid (HCl, 1N). Add 500 ml of sodium acetate ($CH_3COONa.3H_2O$, 1 M) and mix well.

4. ρ-rosaniline-HCl solution ($[NH_2C_6H_4]_3COH$-HCl)

   Weigh 0.2 g ρ-rosaniline-HCl (analytical grade) and add 100 ml distilled water. Mix well. Stand overnight, then filter using filter paper (No. 1). Take 20 ml of the filtrate and add 6 ml HCl (12N or 36%). Make up to 100 ml with distilled water.

5. ρ-rosaniline-formaldehyde solution

   Add 1 volume of 0.2% ρ-rosaniline-HCl solution with 1 volume of 0.2% formaldehyde solution (1:1). Mix well.

6. 0.2% formaldehyde solution

   Take 1 ml of 37% formalin and add 184 ml of distilled water or 1 ml of 35% formalin and add 174 ml distilled water. To be prepared fresh for each use.

7. 5% dimedone ($C_8H_{12}O_2$)-ethanol

   Weigh 5 g dimedone and dissolve in ethanol. Make up to 100 ml.

8. Silicon oil as antifoaming agent (food additive)

9. 25% phosphoric acid (H_3PO_3) solution

   Take 100 ml of 85% H_3PO_3 and add 240 ml of distilled water. Mix well.

10. 0.1N sodium hydroxide (NaOH) solution.

11. Sulfurous standard solution

    Weigh 0.5 g sodium bisulphite ($NaHSO_3$) and dissolve in distilled water. Make up to 100 ml in a volumetric flask.

    Take 10 ml of this solution and add 15 ml of 0.1N iodine (I_2) solution. Also add 2 ml of HCl (12N or 36%). Titrate against 0.1 N sodium thiosulphate solution. From the titration, calculate A ml using formula below :

$$A\ (ml) = \frac{93.75}{(b - a)} \times \frac{1}{f}$$

Where a = titration volume of sulfurous standard solution (ml)

b = titration volume (ml) of blank (using 10 ml of distilled water instead of $NaHSO_3$)

f = factor of 0.1N sodium thiosulphate

Take A ml of $NaHSO_3$ solution and make up to 300 ml with 0.1N NaOH solution to give the **original sodium bisulphite** solution. Finally take 2 ml of the original bisulphite solution and make up to 100 ml with 0.1N NaOH solution, in a volumetric flask. This gives the **sulphurous standard solution** ($NaHSO_3$ solution). 1 ml of this solution contains 0.002 mg (2 ug) SO_2. This solution must not be used after more than 2 - 3 days in the refrigerator.

PROCEDURE
(Refer to Figs. 1 and 2)

1. Pipette 8 ml of 0.1N NaOH solution into Flask A, then fix Flask A to C.

2. Pipette 1 ml 5% dimedone-ethanol into Flask B, followed by 1 ml 1% NaN_3, 2 drops of silicone oil and 10 ml of 25% H_3PO_4.

3. Flow in nitrogen gas at a speed of 0.5 - 0.6 l/min for 5 min.

4. Remove Flask B from E and quickly put the sample into Flask B.

5. Connect Flask B to glass capillary E.

6. Flow nitrogen gas at 0.5 - 0.6 l/min.

7. Heat Flask B with micro-burner for 10 min using a flame height of 4 - 5 cm.

8. Remove Flask A and wash the tip of the delivery tube with small amounts of 0.1N NaOH solution into Flask A.

9. Make up the solution in Flask A to 10 ml in a graduated test tube (stoppered) with washings from Flask A.

10. Take 5 ml of the above sample solution and add 5 ml of ρ-rosaniline-formaldehyde solution. Stand for 35 min at room temperature (20° - 25°C).

11. Determine by colorimetry at wavelength of 560 nm.

12. For testing standard solution, 5 ml of standard solution is used instead of sample solution.

CALCULATION

$$SO_2\ (g/kg) = 10 \times \frac{\text{absorption value of sample solution (A)}}{\text{absorption value of standard solution (B)}} \times \frac{10}{5} \times W \times \frac{1}{1000}$$

$$= \frac{W}{50} \times \frac{A}{B}$$

Where, A = absorption value of sample solution

B = absorption value of standard solution

W = sample weight(g)

Detection limit = 0.2 ppm (0.0002 g/kg)

REMARKS

1. This colorimetric method is suitable for samples with titration values of less than 0.1 ml of 0.01N NaOH solution (alkali titration method).

2. Nitrogen gas is used to prevent the oxidation of SO_2 to H_2SO_4.

3. If a high level of SO_2 is obtained, dilute 5 ml of the sample solution (in a graduated test tube with stopper) with 0.1N NaOH solution and make up to 10 ml i.e. two times dilution.

4. This method is suitable for low levels of SO_2 (0.2 ppm) as well as high levels of SO_2.

5. To prevent carbonization in the case of low moisture foods an adequate amount of distilled water must be used in the Flask B.

REFERENCE

Shokuhin Eisei Kensa Shishin, Guidelines for Food Hygiene Inspection. (Analytical Methods for Food Additives), Japan Food Hygiene Association (1989).

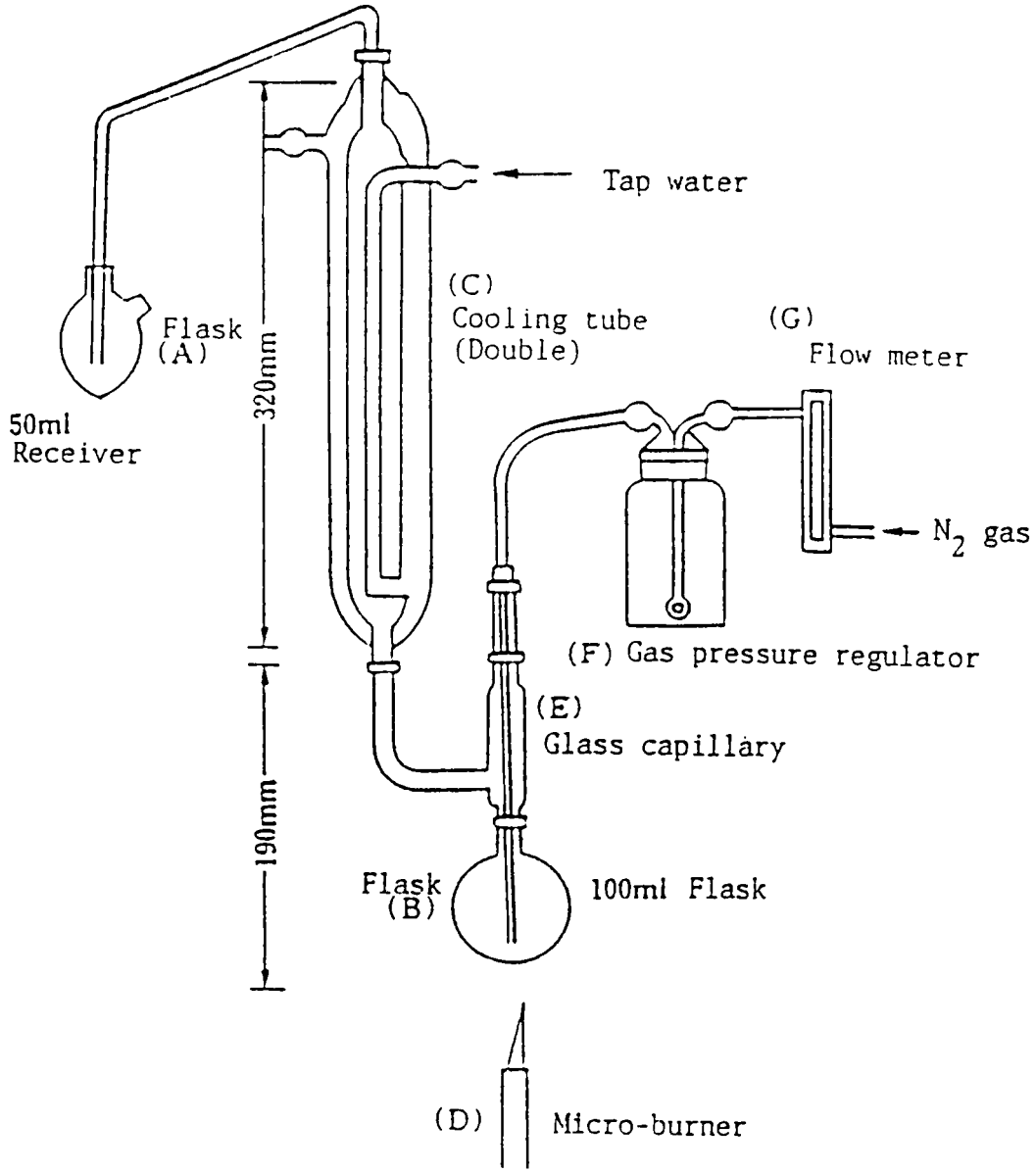


Fig. 1. APPARATUS FOR DISTILLATION
(RANKINE METHOD)

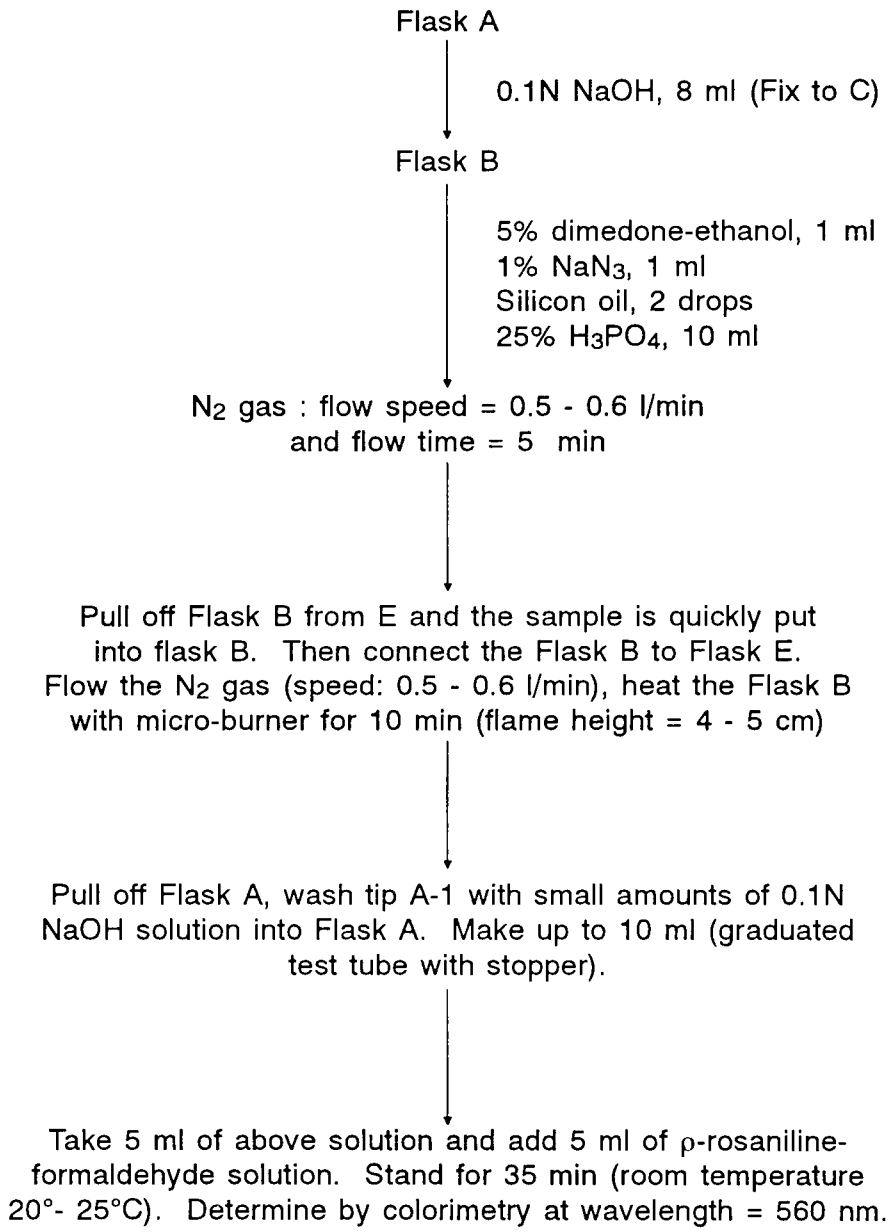


Fig. 2. Analytical procedure for the analysis of SO_2 by Rankine Method

DETERMINATION OF BENZOIC ACID

NG MUI CHNG

INTRODUCTION

Benzoic acid is used as a preservative in the form of the sodium or potassium salt. The amount present is calculated as the acid itself. The undissociated acid is effective in retarding the growth of yeasts and moulds. It is therefore used as an anti-fungal agent in some food.

In Singapore, benzoic acid is not permitted in fish products. Its content should therefore be absent in the products.

PREPARATION OF SAMPLE

I GENERAL METHOD

Transfer 150 g minced sample into 500 ml volumetric flask. Add saturated NaCl solution. Use 10% NaOH solution to adjust the pH in flask to alkali (> pH 7).

Shake well manually and then with magnetic stirrer for about 2 hours. Filter. Retain filtrate for further test.

II SALTED DRIED FISH

Transfer 50 g minced sample into 500 ml volumetric flask with water. Make slightly alkaline with 10% NaOH solution using litmus paper. Stand for 2 hours shaking frequently or using magnetic stand and stirrer. Filter.

Pipette as large a measured portion of filtrate as possible (> 300 ml) into a second 500 ml volumetric flask. Add 30 g pulverised NaCl for each 100 ml solution. Shake until NaCl dissolves and make-up to the mark with saturated NaCl solution. Mix well and filter off precipitated protein and extraneous matter.

REAGENTS

a) Saturated NaCl solution

b) 10% NaOH

c) Chloroform (GR grade)

d) Conc. H_2SO_4

e) Ethyl alcohol

f) 0.05N NaOH

g) Indicator phenolphthalein

h) Diluted HCl (1part conc. HCL + 3 parts distilled water)

PROCEDURE

1. Pipette 100-200 ml filtrate from SAMPLE PREPARATION into a 500 ml separator. Neutralise with diluted HCl using litmus paper and add excess 5 ml HCl.

   NOTE : With salted fish, protein usually precipitates on acidifying, but the precipitate does not interfere with extraction.

2. Extract carefully with $CHCl_3$, using successive portions of 70, 50, 40 and twice 30 ml.

3. Shake slowly using rotary motion to avoid formation of emulsion. The $CHCl_3$ layer will separate easily after a few minutes.

   NOTE : If emulsion forms, break it by stirring $CHCl_3$ layer with glass rod or by drawing $CHCl_3$ layer into a second separator and giving 1-2 sharp shakes.

4. Carefully drain out as much **clear** $CHCl_3$ solution as possible after each extraction into a 400 ml beaker.

5. Leave the beaker of $CHCl_3$ to evaporate overnight in fume cupboard till dry.

6. Put beaker of dry residue in desiccator containing a small beaker of concentrated H_2SO_4 to remove the moisture in residue. Leave it for half day or overnight.

7. Dissolve residue of benzoic acid in 40 ml ethyl alcohol. Add few drops of indicator and 10 ml distilled water.

8. Titrate with 0.05N NaOH till end point. Carry out a blank titration without benzoic residue.

CALCULATION

1 ml 0.05N NaOH = 0.0072 g anhyd. Na benzoate

Therefore, **Sample = (A-B) 0.0072 g anhyd. Na benzoate**

where A = Sample – titrated volume (ml) of 0.05N NaOH

B = Blank – titrated volume (ml) of 0.05N NaOH

REFERENCES

David Pearson. The Chemical Analysis of food. 7th Edition, p 33.
Official Methods of analysis of the Association of Official Analytical Chemists (1984). p 376.

DETERMINATION OF SORBIC ACID, BENZOIC ACID AND DEHYDROACETIC ACID BY STEAM DISTILLATION AND UV SPECTROPHOTOMETRIC METHOD

MAKOTO YAMAGATA

INTRODUCTION

The ultraviolet spectrophotometric method can be used for both qualitative and quantitative tests. The quality and quantity of the contents can be determined by measuring the specific preservatives such as sorbic acid, benzoic acid and dehydroacetic acid at specific maximum UV absorption wavelength. This method is very useful for the effective determination of many samples. However, in using this method, sometimes off odours are emitted.

In principle, preservatives from foods are extracted by steam distillation. The fraction is then purified by solvent extraction. Each acidified preservative from the extract solution is then measured at the specific absorption wavelength using UV spectrophotometer.

APPARATUS

1. Analytical balance
2. Steam distillation apparatus (Fig. 1)
3. UV absorption spectrophotometer
4. Shaker

REAGENTS

1. Buffer solution

   Add 50 ml of 2M KCl to 10.6 ml of 2N HCl and make up to 200 ml with distilled water.

2. Standard solution of sorbic acid

   Dissolve 50 mg sorbic acid in 4.5 ml of 0.1N NaOH and make up to 100 ml with distilled water. From this, make a 100 times dilution with distilled water (take 5 ml to make up to 500 ml with distilled water). 1 ml of this sorbic acid standard solution contains 5 ug of $C_2H_8O_2$.

3. Standard solution of benzoic acid

   Dissolve 100 mg benzoic acid in 8.5 ml of 0.1N NaOH and make up to 100 ml with distilled water. 1 ml of this benzoic acid standard solution contains 10 ug of $C_7H_6O_2$.

4. Standard solution of dehydroacetic acid

   Dissolve 100 mg dehydroacetic acid in 6 ml of 0.1N NaOH and make up to 100ml with distilled water. From this, make a 50 times dilution with distilled water (take 10 ml to make up to 500 ml with distilled water). 1 ml of this dehydroacetic acid standard solution contains 20 ug $C_8H_8O_4$.

PROCEDURE

Sample Preparation
(Fig. 2)

1. Weigh 50.0g sample in beaker. If the sample is a solid, add 100 ml of distilled water and mix well.

2. Adjust to pH 7 using 10% NaOH or 10% HCl.

3. Put sample into round bottomed flask (500 ml). Add 15 ml 15% tartaric acid (pH 2 - 3), 60 g NaCl, 1 drop of silicon oil and make up to 200 ml with distilled water.

4. Steam distil at a rate of 10 ml/ min. Collect 500 ml of distillate.

5. Pipette 50 ml of distillate into separatory funnel. Add 4 ml 10% HCl, 10 g NaCl, and extract 3 times with 40 ml, 30 ml and 30 ml ether respectively.

6. Drain the ether extract and pool the 3 extracts.

7. Wash ether extract with 15 ml distilled water.

8. Drain ether layer and discard water layer.

9. Add 20 ml of 1% $NaHCO_3$ and shake thoroughly.

10. Drain the $NaHCO_3$ extract and repeat Step 9. Pool the 2 $NaHCO_3$ water based extracts. The ether extract can be used for paraoxy-benzoic acid determination.

11. Neutralize the water based $NaHCO_3$ extract with 10% HCl and make up to 50 ml with distilled water. This is sample solution I and is used for benzoic acid, sorbic acid and dehydroacetic acid tests.

Analytical Procedure

1. Qualitative analysis

   1.1 Pipette 10 ml of each sample solution I, except standard solution into a test tube.

   1.2 Add 2 ml of buffer solution and 2 ml of distilled water to each sample solution.

   1.3 Measure absorption using UV Spectrophotometer at wavelength spectrum of 220 - 320 nm with liquid layer height of 10 mm in cuvette.

   1.4 Compare readings of sample solutions with those of standard solutions.

   1.5 A control solution containing a mixture of distilled water with buffer solution should be used. However, if the concentration is too high, use buffer solution to dilute 10 times and test again.

2. Quantitative analysis

Procedure same as that for Qualitative analysis.

CALCULATION

$$\text{Sorbic acid (g/kg)} = 5 \times \frac{A\,(265\ nm)}{As\,(265\ nm)} \times \frac{1}{2} \times \frac{1}{\text{sample (g)}}$$

$$\text{Benzoic acid (g/kg)} = 10 \times \frac{A\,(230\ nm)}{As\,(230\ nm)} \times \frac{1}{2} \times \frac{1}{\text{sample (g)}}$$

$$\text{Dehydroaceticacid (g/kg)} = 20 \times \frac{A\,(308\ nm)}{As\,(308\ nm)} \times \frac{1}{2} \times \frac{1}{\text{sample (g)}}$$

where,

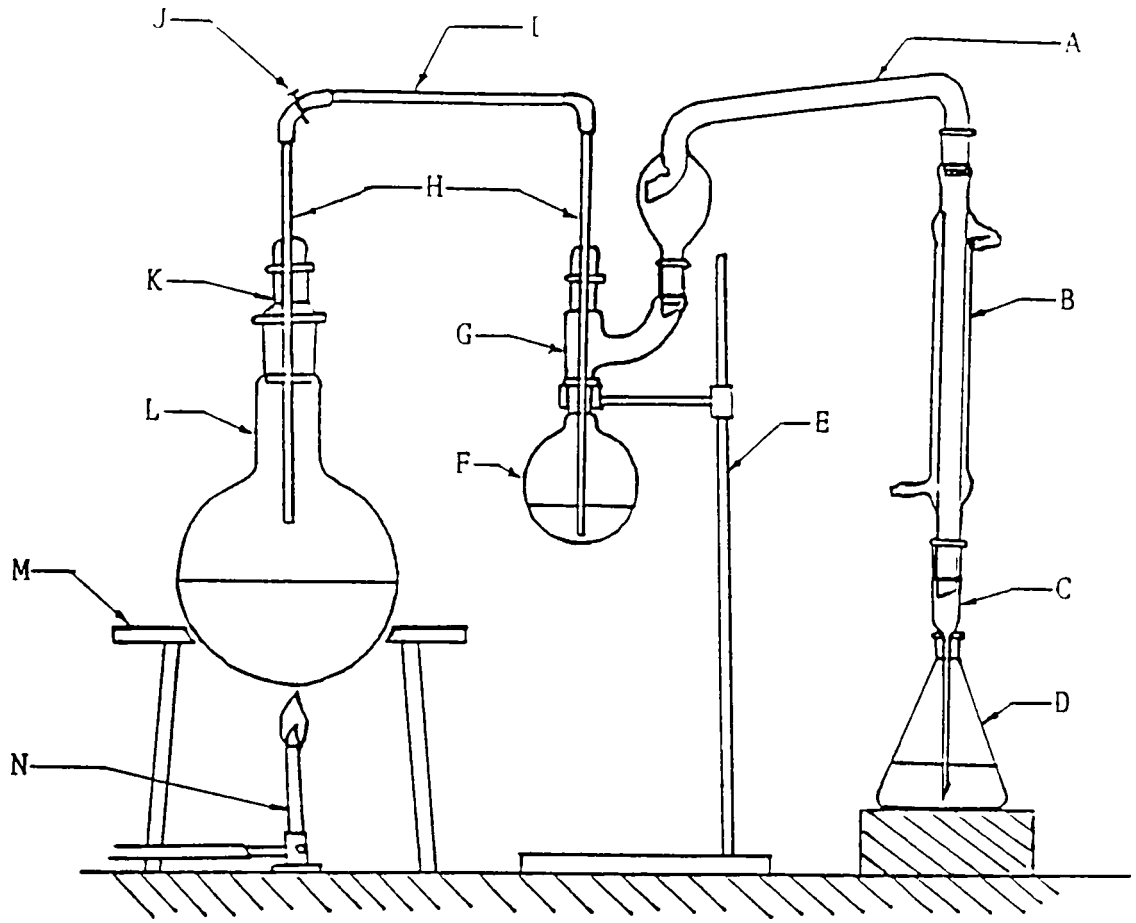
A = UV absorption of sample solution at wavelengths 230, 265 and 308 nm (Fig. 3).

As = UV absorption of standard solution at wavelengths 230, 265 and 308 nm.

Detection limit : 5 ppm (0.005 g/kg)

REFERENCE

Standard Methods of Analysis for Hygiene Chemist - With Commentary - authorized by the Pharmaceutical Society of Japan, Kanehara Shippan K.K. (1990).



A	:	Splash head	H	:	Steam inlet tube
B	:	Liebig condenser	I	:	Glass tubing
C	:	Straight delivery adapters	J	:	Clip
D	:	Conical flask	K	:	Reduction adaptors
E	:	Retort stand	L	:	Round bottomed flask, 2L
F	:	Round bottomed flask, 250 ml	M	:	Tripod stand
G	:	Two neck multiple adaptor	N	:	Bunsen burner

Fig. 1. Steam distillation apparatus.

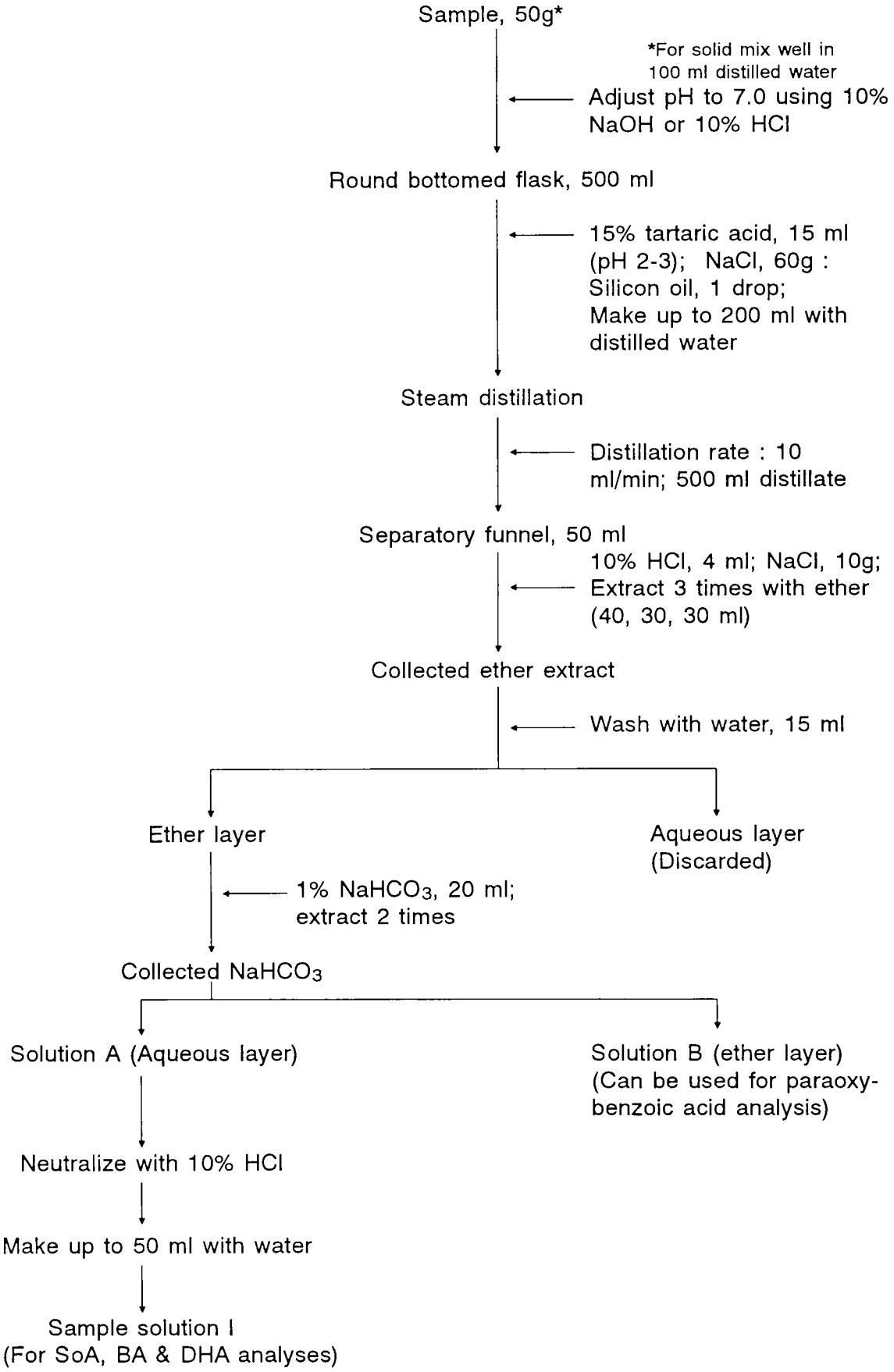


Fig. 2. Sample preparation for sorbic acid, benzoic acid and dehydroacetic acid analyses.

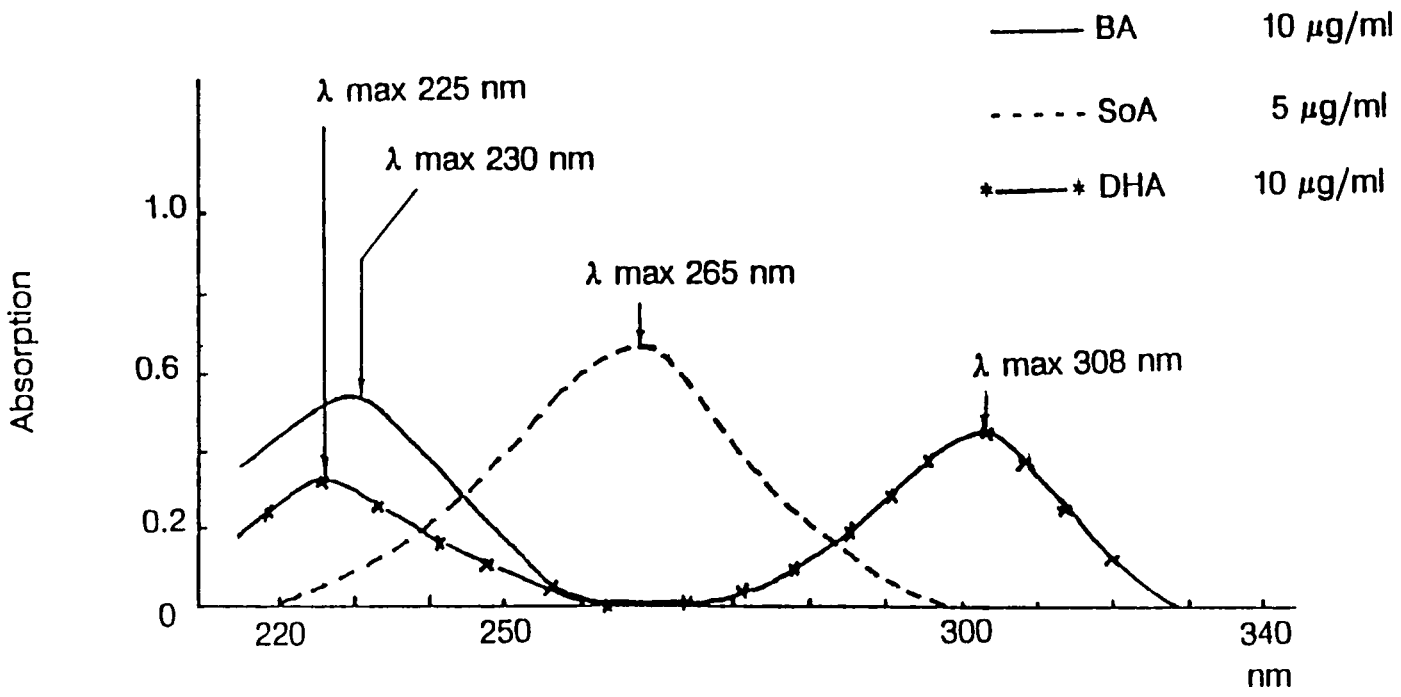


Fig. 3. UV absorption spectrum for standard solutions of each preservative at pH 2.

MICROBIOLOGICAL PROCEDURE

HANDLING OF FOOD SAMPLES

LIM PANG YONG

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 (eg. in laminar flow chamber) throughout the handling of the product. If the sample is frozen, thaw it in the original container or in a 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, eg. 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 PANG YONG

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 and pour 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 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 steps in the series provides 30 to 300 colonies when plated on a nutrient medium. (It is this range that will give the most accurate colony count.)

I. APPARATUS

'Waring' blender & flasks or
Stomacher ('Lab-blender' 400)

Pipettes, 1 & 5 ml

Scissors, scalpel & forceps

Alcohol (70% v/v) swabs

Bent glass spreader

Incubator

Colony Counter

Autoclave

Weighing balance

Laminar flow chamber

Bunsen burner

Sterile petri dish
(90 mm ø x 15 mm H)

II. CULTURE MEDIA

1. Plate count agar (PCA) or Standard Method agar

   Refer to respective manufacturer's instruction for the preparation of the medium.

2. Butterfield's buffered phosphate diluent

a. Stock solution:

Monopotassium hydrogen phosphate	34.0 g
Distilled water	500.0 ml

Adjust to pH 7.2 with about 175 ml 1N sodium hydroxide solution; dilute to one litre. Sterilise at 121°C for 15 minutes and store in refrigerator.

b. Working solution:

Dilute 1.25 ml stock solution to 1.0 litre with distilled water and dispense 45 ml each in glass bottles. Sterilise at 121°C for 15 minutes.

III. PROCEDURE

1. SAMPLING PROCEDURE

Randomly pick 150-200 g of sample. Aseptically cut each piece of the sample in half and keep the half-cut portions in a sterile polyethylene bag or sterile container. Store the bag/container in refrigerator (2° to 5° ± 1°C) to maintain sample's integrity and examine the sample within 18 hours. If test cannot be performed shortly, sample should be well frozen until further examination.

2. SAMPLE PREPARATION

i. Weigh aseptically 25 g of the above sample into a sterile 'Waring' blender jar or stomacher bag. Add 225 ml sterile Butterfield's buffered phosphate diluent and blend for 1 minute.

ii. Transfer 10 ml of the above suspension into 90 ml buffered phosphate diluent to give a dilution of 10^{-2}. Prepare next dilution (10^{-3}) by mixing 1 ml of the well mixed diluted sample solution (10^{-2}) with 9 ml buffered phosphate diluent. Prepare further dilutions: 10^{-4}, 10^{-5}, ... if required.

3. SPREAD PLATE METHOD

i. Select the appropriate dilutions from 2.ii above and for every dilution, inoculate 0.1 ml aliquot to each of two pre-dried PCA plates.

ii. Spread the inoculum gently and evenly over the surface of the agar plates with a sterile bent glass spreader.

iii. Allow the plates to stand until the inoculum has been absorbed completely, which should be within 15 minutes after the spreading.

iv. Invert the plates and incubate at 35° ± 1°C for 48 ± 2 hours or at selected temperature and period.

v. Count those plates which have 30-300 colonies.

vi. The aerobic plate count (Spread Plate Method) for the sample is calculated as follows:

APC = x.d/s colony forming unit(cfu)/g

where d : dilution at which colonies are counted.

x : average count of colonies.

s : volume of aliquot.

vii. Method of calculation:

Example:

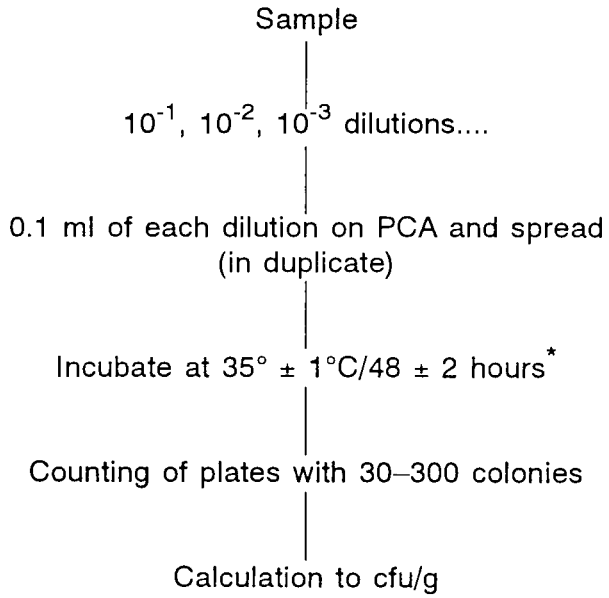
1. Colony count less than 30 per petri dish:

At dilution	Wt of sample/ml	Record result as
10^{-1}	0.1 g	< 300/g
10^{-2}	0.01 g	< 3,000/g
10^{-3}	0.001g	< 30,000/g

2. Colony count less than 30 and more than 300 per petri dish:

At dilution	Presumed average colony count from duplicate petri dish	Calculation
10^{-2}	2,854*	*Disregarded
10^{-3}	291	(291000+360000)/2 = 3.3 x 10^5/g
10^{-4}	36	
10^{-5}	4*	*Disregarded

viii. The procedure for Aerobic Plate Count (Spread Plate Method) is summarised as follows:



* or at selected temperature and period of incubation.

4. POUR PLATE METHOD

i. Select the appropriate dilutions from 2.ii above and for every dilution inoculate 1.0 ml aliquot each to a sterile petri dish.

ii. A 10-15 ml portion of molten PCA is poured into each of the above sterile petri dishes. The plates are then rotated 5 times clockwise, 5 times anti-clockwise 5 times back and forward. Care should be taken not to splash agar on the lid of the dish. Plates are left to set.

iii. Invert the plates and incubate at 35° ± 1°C for 48 ± 2 hours or at selected temperature and period.

iv. Count those plates which have 30-300 colonies on surface or subsurface of the agar.

v. The aerobic plate count (Pour Plate Method) for the sample is calculated as follows:

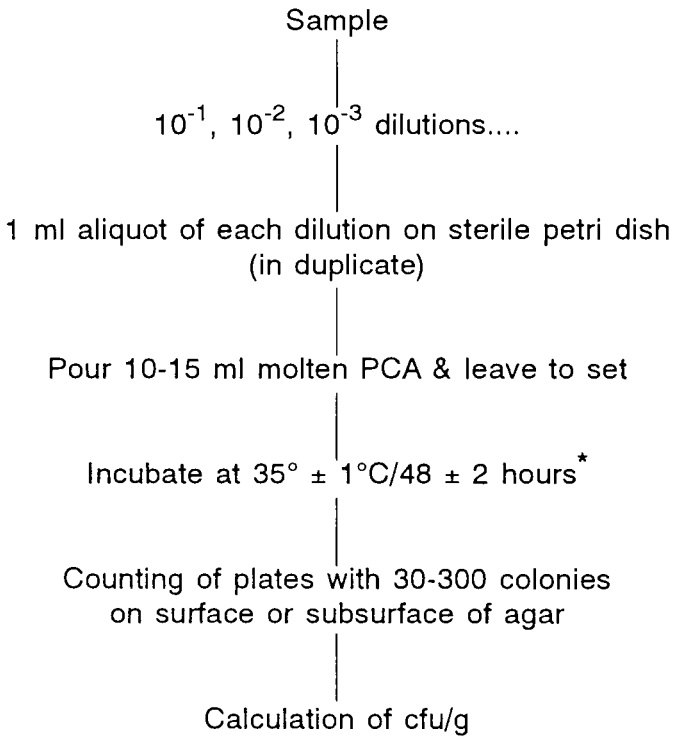
APC = x.d/s colony forming unit(cfu)/g

where d : dilution at which colonies are counted.

x : average count of colonies.

s : volume of aliquot.

vi. The procedure for Aerobic Plate Count (Pour Plate Method) is summarised as follows:



* or at selected temperature and period of incubation.

IV. REFERENCES

1. Official Methods of Analysis of the Association of Official Analytical Chemists. Edited by Sidney Williams. 14th edition.

2. Bacteriological Analytical Manual. Food and Drug Administration, Bureau of Foods, Division of Microbiology. 5th edition, August 1978.

3. Compendium of Methods for the Microbiological Examination of Foods. Compiled by the APHA Technical Committee on Microbiological Methods for Foods. Edited by Marvin L Speck. 2nd edition. American Public Health Association, 1984.

COLIFORMS AND ESCHERICHIA COLI

LIM PANG YONG

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) or EC medium
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 & flask	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 EC medium 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 EC 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:

Indole	MR	VP	Citrate	Type
+	+	–	–	Typical *E. coli*
–	+	–	–	Atypical *E. coli*
+	+	–	+	Typical Intermediate
–	+	–	+	Atypical Intermediate
–	–	+	+	Typical *Enterobacter aerogenes*
+	–	+	+	Atypical *Enterobacter aerogenes*

Other groupings may appear : in such cases cultures are usually mixed. Restreak to det. their purity.

Compute MPN of *E. coli*/g, considering Gram neg., nonspore-forming rods producing gas in lactose and producing ++-- or -+-- IMViC patterns as *E. coli*.

Refs. : JAOAC 49 : 270, 276 (1966); 51 : 865, 867 (1968); 58 : 1154 (1975).

VII CALCULATION OF MPN

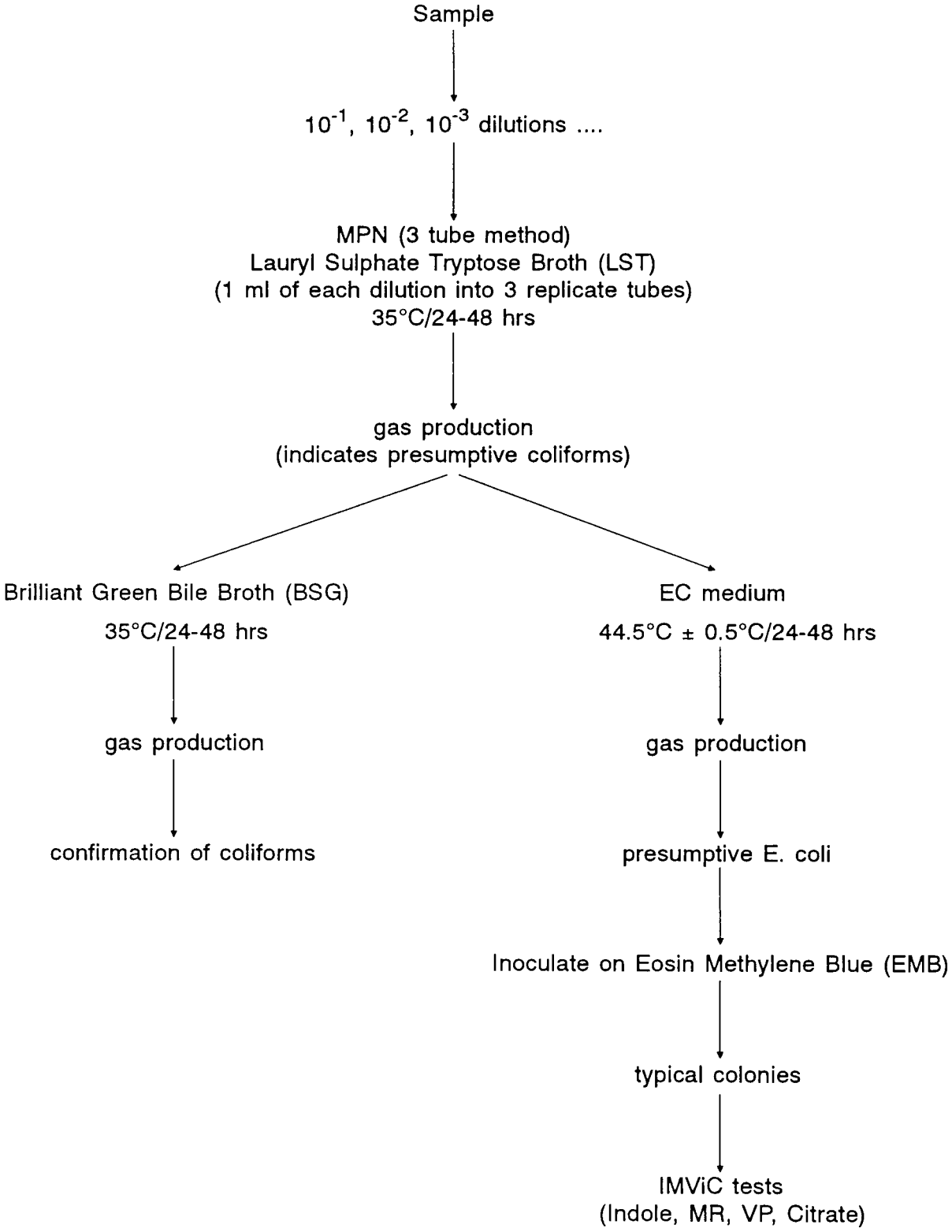
Most Probable Number = Index/g
See Appendix A and A1

REFERENCES

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.

Official Methods of Analysis, Association of Official Analytical Chemists, 14th Edition, 1984.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR COLIFORMS AND E. COLI



SALMONELLAE & SHIGELLA

LIM PANG YONG

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

III SAMPLING PROCEDURE

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

IV PROCEDURE

A RESUSCITATION (PRE-ENRICHMENT)

1. Weigh 25 g of the above sample and put them into a 'Waring' blender flask and add approximately 225 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 H_2S.

   b) on DCA agar: appear as colourless colonies.

   For Shigella

   a) On XLD agar: appear as red or pink colour colonies, about 1 mm ∅.

   b) On DCA & MCA: appear as opague or transparent colonies.

D SCREENING AND BIOCHEMICAL TESTS

1. Pick up 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 H_2S (blackening of butt) in TSI agar. Shigella cultures typically produce red slant and yellow butt, with no H_2S 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:

Tests (Salmonella)	Results	Tests (Shigella)	Results
Lysine decarbonxylase	+	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 Salmonella & Shigella.

E SEROLOGICAL CONFIRMATION

1. Emulsify the culture in 2 drops of saline on a clean glass slide.

2. Add one 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.

REFERENCE

Official Methods of Analysis, Association of Official Analytical Chemists, 14th Edition, 1984.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR SALMONELLA

25 g sample + 225 ml nutrient broth
(Pre-enrichment)
35°C/24 hrs

↓

Enrichment in

a) Selenite broth (35°C/24 hrs)

b) Tetrathionate broth (35°C/24 hrs)

↓

Steak onto

a) Desoxycholate citrate agar (DCA)

b) Xylose lysine deoxycholate (XLD)

↓

35°C/24 hrs

↓

Typical colonies

↓

Streak onto TSI

↓

Confirmatory tests:

a) Lysine decarboxylase
b) Urease
c) Dulcitol
d) Malonate
e) KCN
f) Indole
g) VP
h) MR
i) Citrate
j) Lactose
k) Sucrose

↓

Serology
(Polyvalent "O" antiserum, "H" antiserum)

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 H_2S, no gas

urease: -

motility: -

↓

Confirmatory tests:

Glucose (gas)	Arginine
VP	Ornithine
MR	Citrate
Indole	Mannitol
Lysine	Lactose

↓

Serology

STAPHYLOCOCCUS AUREUS

LIM PANG YONG

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

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 tubes 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 content of the tube coagulates 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.

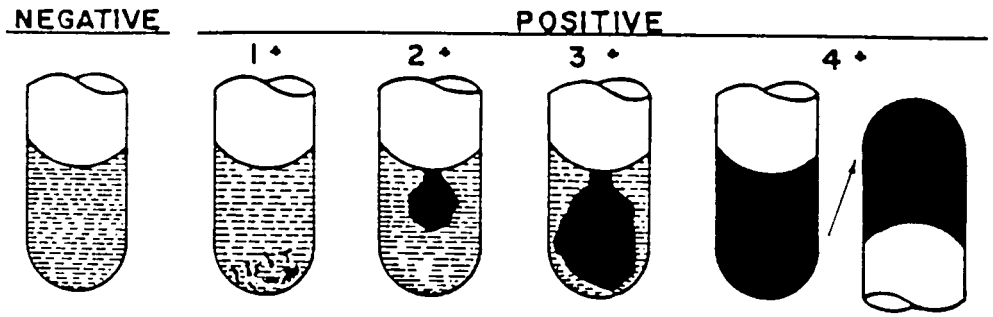
VI CALCULATION OF MPN

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

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/FISHERY PRODUCTS (COOKED & RAW)

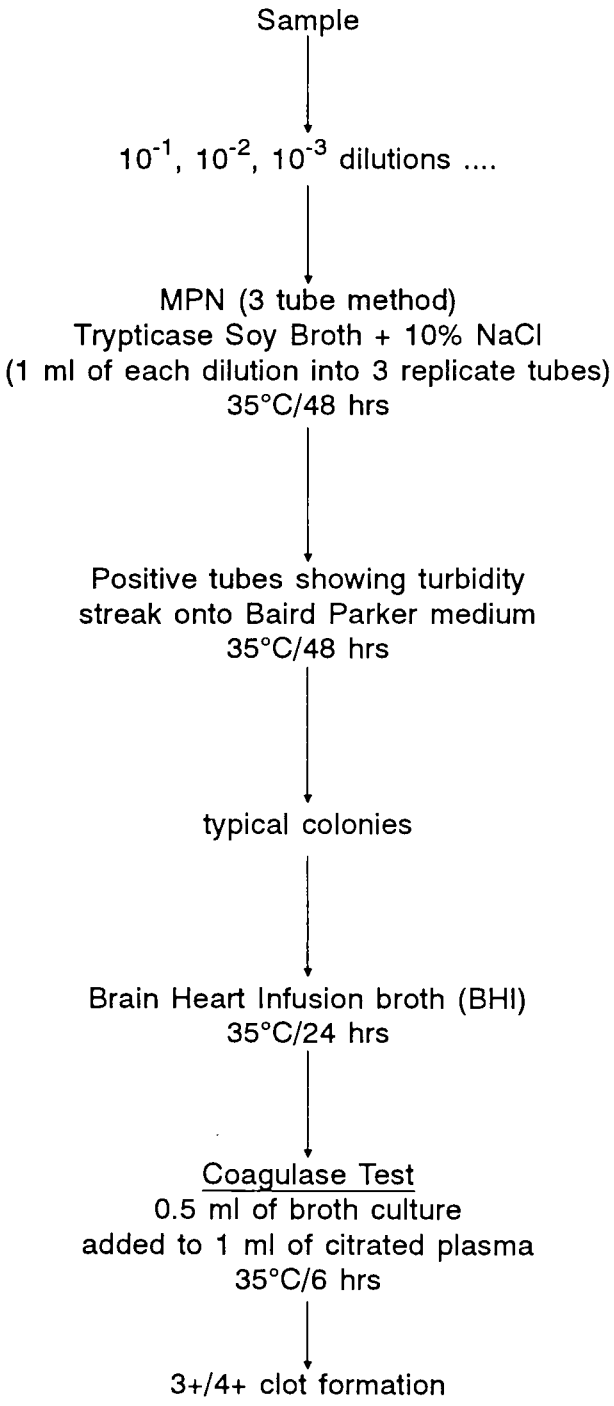
Cooked products : 100 MPN/g

Raw products : 250 MPN/g

REFERENCE

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: 68, 114 & 115

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR STAPHYLOCOCCUS AUREUS



FAECAL STREPTOCOCCI

LIM PANG YONG

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 foods without necessarily indicating poor hygiene. They are found in many fermented foods, 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 APPARATUS

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

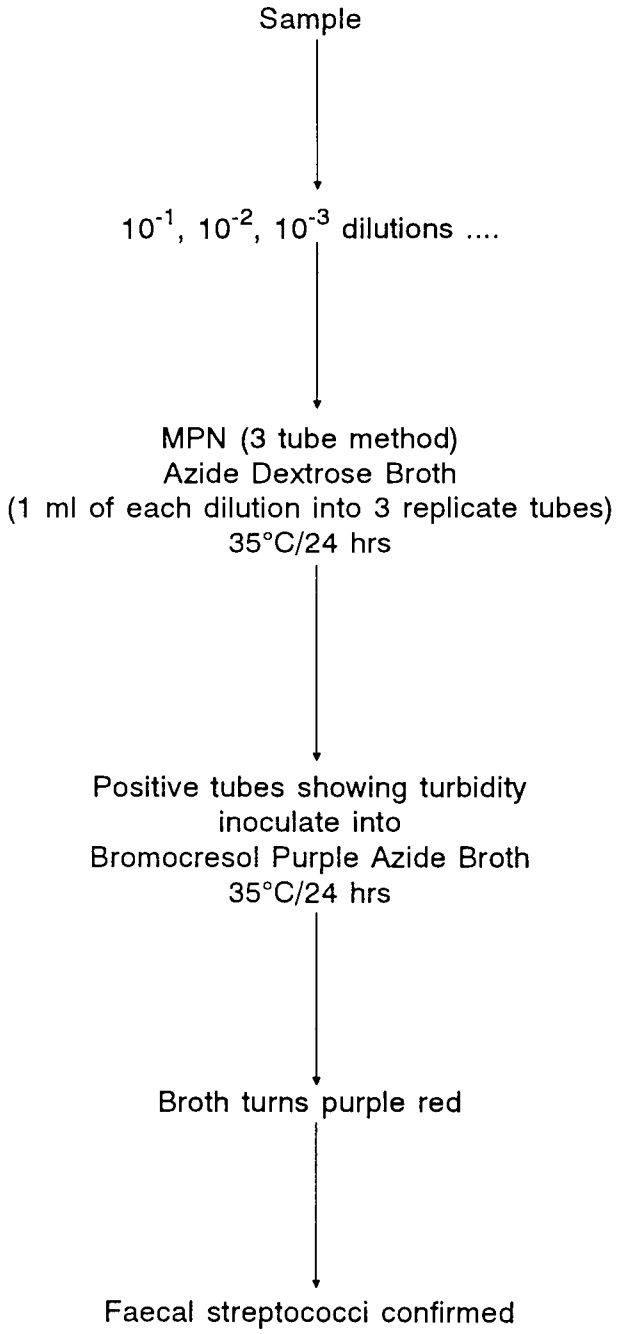
Cooked products : -

Raw products : 1,000 MPN/g

REFERENCE

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: 17 & 28.
Chapter: Fish quality control microbiology. Section 6: 88.

FLOW DIAGRAM OF EXAMINATION PROCEDURES FOR FAECAL STREPTOCOCCI



VIBRIO CHOLERA

LIM PANG YONG

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)
Andrade peptone water
Aesculin broth
Decarboxylase medium base
Koser citrate medium
MRVP medium
Nutrient agar (+3% NaCl)
Nutrient gelatin
Phenylalanine agar (PPA)
SIM medium
Simmons citrate agar
Thiosulphate citrate bile salts sucrose agar (TCBS)
Triple sugar iron agar (TSI)
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 each of the following sugars:

Arabinose	Lactose	Melibiose
Glucose	Mannitol	Salicin
Inositol	Mannose	Sucrose

II CHEMICAL REAGENTS*

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

b) Kovac's reagent

c) 0.1N HCl

d) Methyl red solution

e) KOH solution (40% w/v)

f) α-naphthol solution (5% w/v)

g) $FeCl_3$ aq. soln. (10% w/v)

* Refer to Appendix D for methods of reagent preparation.

III APPARATUS

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

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**:

Tests	Results
TSI	acid slant acid butt; no gas; no H_2S
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*.

Tests	Results
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*:

Tests	Results
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.

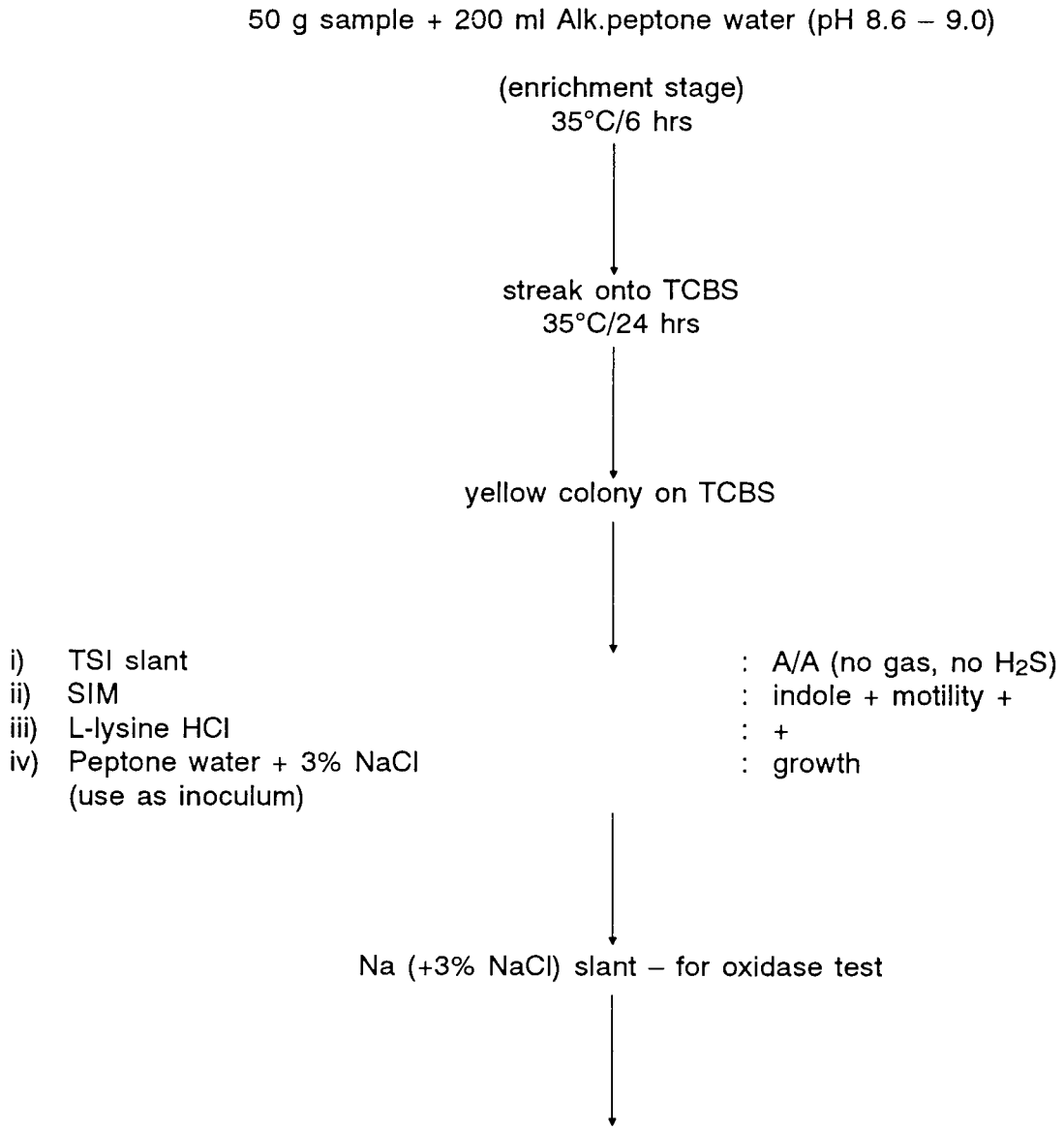
VI BACTERIOLOGICAL LIMITS OF VIBRIO CHOLERA FOR FISH/FISHERY PRODUCTS (COOKED & RAW)

This organism should not be detected in 50 g sample.

REFERENCE

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.

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 PANG YONG

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

b) Methyl red solution

c) α-naphthol solution (5% w/v)

d) 0.1N HCl

e) KOH solution (40% w/v)

f) $FeCl_3$ aq. soln. (10% w/v)

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

III APPARATUS

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

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_2S)
Indole (SIM)	+
Motility (SIM)	+
L-lysine HCl	+

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. parahaemolyticus 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

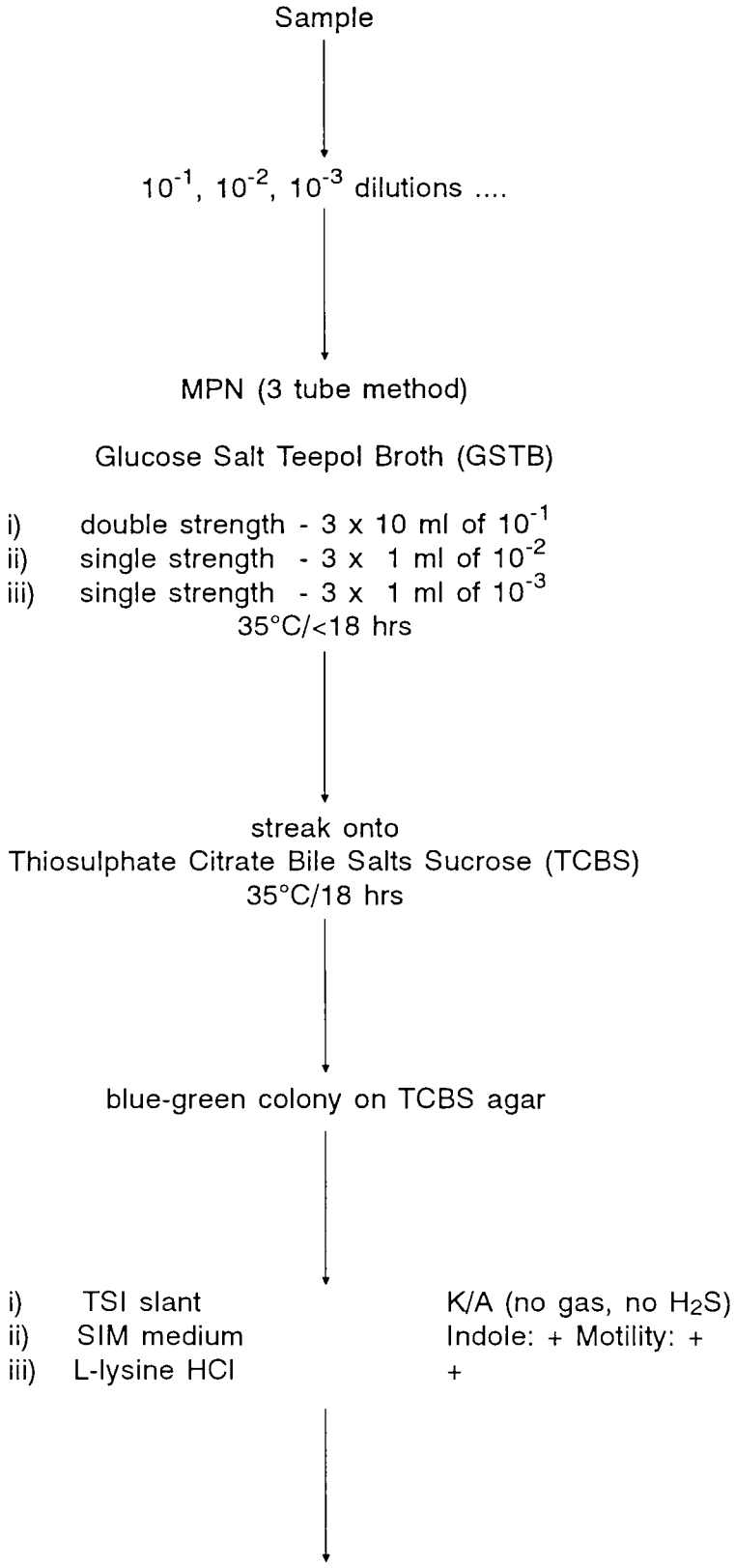
Index : from MPN Tables (Appendix A)

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 : 69-70

Isolation and identification of Vibrio parahaemolyticus. Bacteriological Analytical Manual. Jan. 1969.

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 Watgatsuma 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.

ANAEROBIC PLATE COUNT
(Spread and Pour Plate Method)

MAKOTO YAMAGATA

INTRODUCTION

The anaerobic plate count provides an estimate of the number of viable anaerobic microorganisms in food and is affected by the type of medium used and the length and temperature of incubation.

The spread and pour plate methods described below are the same as those used in determining the aerobic plate count. The only difference is in the anaerobic incubation of the media.

APPARATUS

1. Anaerobic jar
2. Vacuum pump and nitrogen gas bomb, if necessary
3. BBL Gas pack plus anaerobic jar system and gas pack pouch (see instruction manual of BBL) is a convenient and easier method for anaerobic condition.
4. "Waring" blender & flasks or Stomacher ("Lab-blender" 400)
5. Pipettes, 1 ml, 5 ml
6. Scissors, scalpels & forceps
7. Alcohol (70%, v/v) swabs
8. Bent glass spreader
9. Incubator (35° ± 1°C)
10. Autoclave
11. Weighing balance
12. Laminar flow cabinet
13. Bunsen burner
14. Sterile petri dish (ø : 90 mm, H : 15 mm)

CULTURE MEDIA
(Refer to Appendix B for methods of media preparation)

1. Anaerobic agar

Trypticase peptone	17.5 g
Phytone peptone	2.5 g
Sodium chloride	2.5 g
L-cysteine	0.4 g
Dextrose	10.0 g
Agar	15.0 g
Sodium thioglycollate	2.0 g
Sodium formaldehyde sulfoxylate	1.0 g
Methylene blue	0.002 g
Distilled water	1 litre
Final pH	7.2 ± 0.1

Autoclave for 15 min at 121°C.

2. SPS agar

Sodium sulfite	0.50 g
Polymyxin sulfate	0.01 g
Sulfadiazine	0.12 g
Trypticase peptone	15.00 g
Yeast extract	10.00 g
Agar	13.90 g
Iron citrate	0.50 g
Distilled water	1 litre
Final pH	7.0 ± 0.1

Autoclave for 15 min at 118°C.

Suitable media for detection of *Clostridium perfringens* (welchii) in food. Black colonies are sulfite reducers.

3. TSN agar

Trypticase peptone	15.00 g
Sodium sulfite	1.00 g
Neomycin sulfite	0.02 g
Polymycin sulfite	0.05 g
Yeast extract	10.00 g
Ferric citrate	0.50 g
Agar	13.50 g
Final pH	7.2 ± 0.1

Autoclave for 15 min at 118°C.

Suitable media for detection of *Clostridium perfringens* (welchii) in food. Black colonies are sulfite reducers. 5 ml of buffered thioglycollate solution may be added to 200 ml of TSN agar at 77°C, if desired.

PROCEDURE

1. Sampling Procedure

   Same as in aerobic plate count method.

2. Sample Preparation

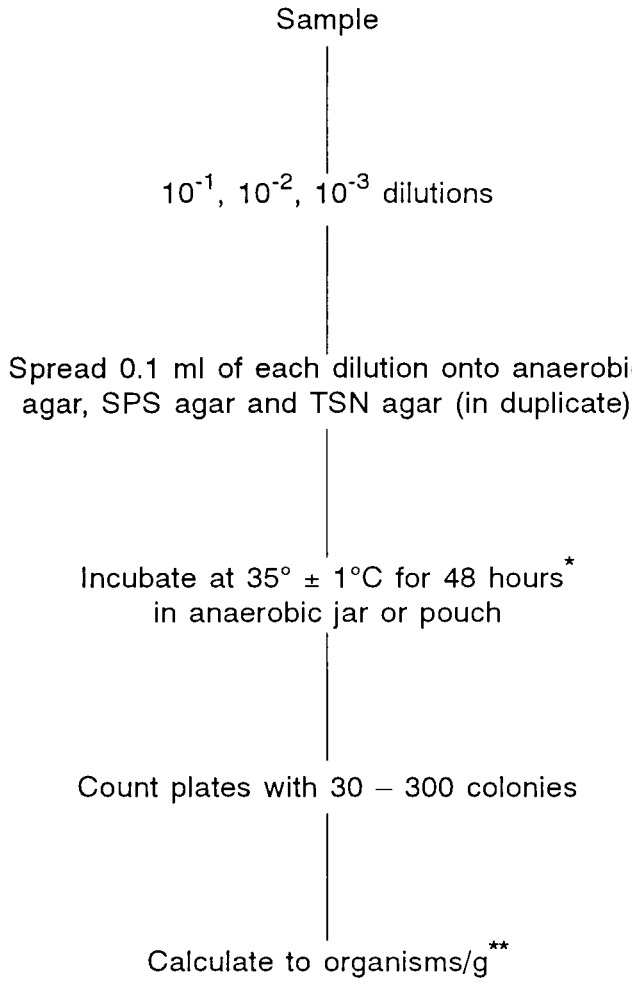
   Same as in aerobic plate count method.

3. Spread plate method (Fig. 1)

   Same as in aerobic plate count method, however culture media used are anaerobic agar (SPS agar and TSN agar).

4. Pour plate method (Fig. 2)

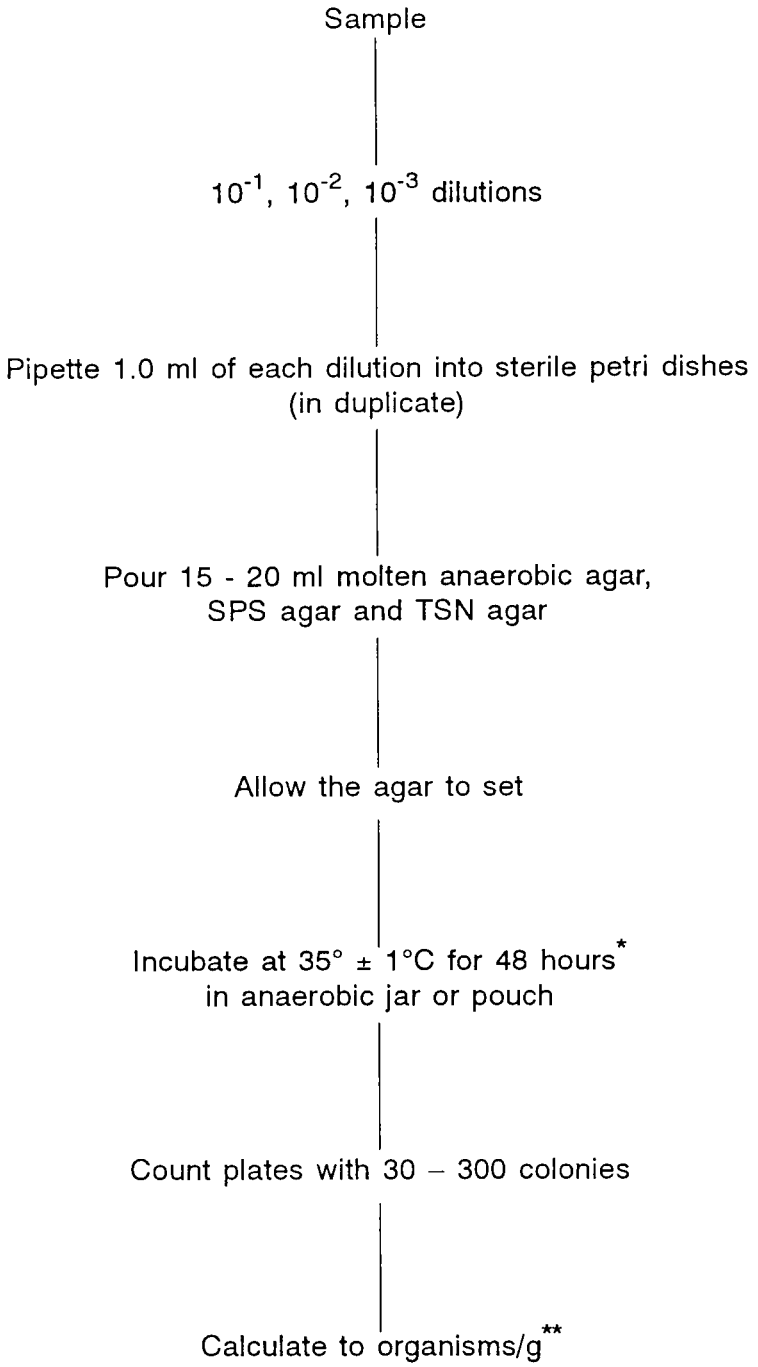
   Same as in aerobic plate count method, however culture media used are anaerobic agar (SPS agar and TSN agar).



* or at any selected temperature and specific period of incubation

** if only black colonies are counted, describe as anaerobic sulphite reducers per gram

Fig. 1. Flow diagram of the procedure for anaerobic plate count (Spread Plate Method)



* or at any selected temperature and specific period of incubation

** if only black colonies are counted, describe as anaerobic sulphite reducers per gram

Fig. 2. Flow diagram of the procedure for anaerobic plate count (Pour Plate Method)

REFERENCE

Shokuhim Eisei Kensa Shishin, Guide to Food Hygiene Examination (Authorized by the Ministry of Health and Welfare), Japan Hygiene Association (1990).

FUNGI (MOULD AND YEAST) PLATE COUNT

MAKOTO YAMAGATA

INTRODUCTION

Mould growth on foods with its fuzzy, cotton-like and sometimes coloured appearance is familiar to everyone. Usually a mouldy or "mildewed" food is considered unfit to eat. While it is true that moulds are concerned with the spoilage of many kinds of foods, special moulds are useful in the manufacture of certain kinds of foods or ingredients of foods.

Yeasts are as difficult to define as moulds. In Henrici's "Molds, Yeasts, and Actinomycetes", yeasts are defined as true fungi whose usual and dominant growth form is unicellular. Some of the moulds in their conidial stage are like budding yeasts, and some yeasts have a mycelial stage. An example of a genus which is sometimes listed with the moulds and sometimes with the yeasts is *Geotrichum*.

Yeasts may be useful or harmful in foods. The manufacture of foods like bread, beer, wines, vinegar, and surface-ripened cheese involves yeast fermentation. Yeasts are grown for enzymes and for food. They are undesirable when they cause spoilage of sauerkraut, fruit juices, syrups, molasses, honey, jellies, meats, wines, beer and other food.

Partial classification of Eumycetes, or true fungi, and the classification of yeasts to include genera found in foods are shown in Figs. 1 and 2 respectively.(Source : W.C. Frazier, Food Microbiology, TATA McGraw-Hill Publishing Co. Ltd., New Delhi, 1977).

APPARATUS

1. Petri dishes (ø = 90 mm, depth = 20 mm)
2. Waring blender and flasks
3. Pipettes
4. Scissors and forceps
5. Alcohol lamps
6. Alcohol (70% v/v) swabs
7. Bent glass spreader
8. Autoclave
9. Incubator
10. Weighing balance

CULTURE MEDIA* AND REAGENTS

1. Potato dextrose agar

2. Sabouraud dextrose agar

3. 0.05% agar in 0.85% saline (NaCl) solution

4. Chloramphenicol (antibiotics)

   Before autoclaving the media, weigh 50 – 100 mg of chloramphenicol and dissolve in 10 ml of 95% ethanol (medium : 1000 ml).

   * Refer to Appendix B for methods of media preparation.

PROCEDURE

Sampling procedure

Refer to Aerobic Plate Count.

Sample preparation

1. Weigh 25 g of the sample and put them into a Waring blender flask.

2. Add in 225 ml of 0.05% agar in sterile saline (0.85% NaCl).

3. Blend for about 3 min at low speed (sample 0.1 g / 1 ml : One ml of this suspension contains 0.1 g of sample i.e. a 10^{-1} dilution.

4. Pipette 10 ml of the suspension from the 10^{-1} dilution into 90 ml of 0.85% saline diluent to give a dilution of 10^{-2}.

5. Prepare further dilutions by mixing 1 ml of the well mixed diluted sample solution with 9 ml of 0.85% saline diluent.

Analytical procedure

1. Using 10^{-1} stage dilutions, pipette 0.1 ml aliquots to each of the 2 potato dextrose agar plates or the Sabouraud dextrose agar plates, for every dilution.

2. Spread gently and evenly over the surfaces of the agar plates with a sterile bent glass spreader.

3. Allow the plates to stand until the aliquot has been absorbed completely, which should be within 15 min after the spreading.

4. Invert the plates and incubate at 23° ± 2°C for 5 days (maximum for 7 days).

5. Count the plates which have 10 – 100 colonies.

6. The fungi plate count for the sample is calculated as shown below :

Table 1. Calculation of fungi plate count (Spread plate method) using 0.1 ml of 10^{-1} dilution.

Dilution	The average colony count obtained on duplicate petri dishes	Record (fungi/g)
10^{-2}	19	1.9×10^3
10^{-3}	2*	
10^{-4}	0	

* Not recorded because number of colonies is <10. As this method uses 0.1 ml of 10^{-1} dilution (0.1 g/ml) sample solution, care should be taken when the results are obtained from using 10^{-2} dilution (0.01 g/ml) as the beginning stage.

If the number of colonies obtained is <10 in 10^{-2} stage, it implies that the count is <1000/g.

If necessary the number of colonies of 1 ml of 10^{-1} (1 g/ml) are determined using 3 agar plates with 0.3 ml, 0.3 ml and 0.4 ml (total of 1 ml) of the 10^{-1} dilution or 2 agar plates with 0.5 ml each (total of 1 ml). In this case if the number of colonies obtained is <10 in the 10^{-1} dilution, it implies that the count is <100/g.

Table 2. Fungi plate count (Pour plate method) using 1 ml of 10^{-1} dilution.

Dilution	The average colony count obtained on duplicate petri dishes	Record (fungi/g)
10^{-1}	19	1.9×10^2
10^{-2}	2	
10^{-3}	0	

If the number of colonies obtained is <10 in 10^{-1} dilution, it implies that the count is <100/g.

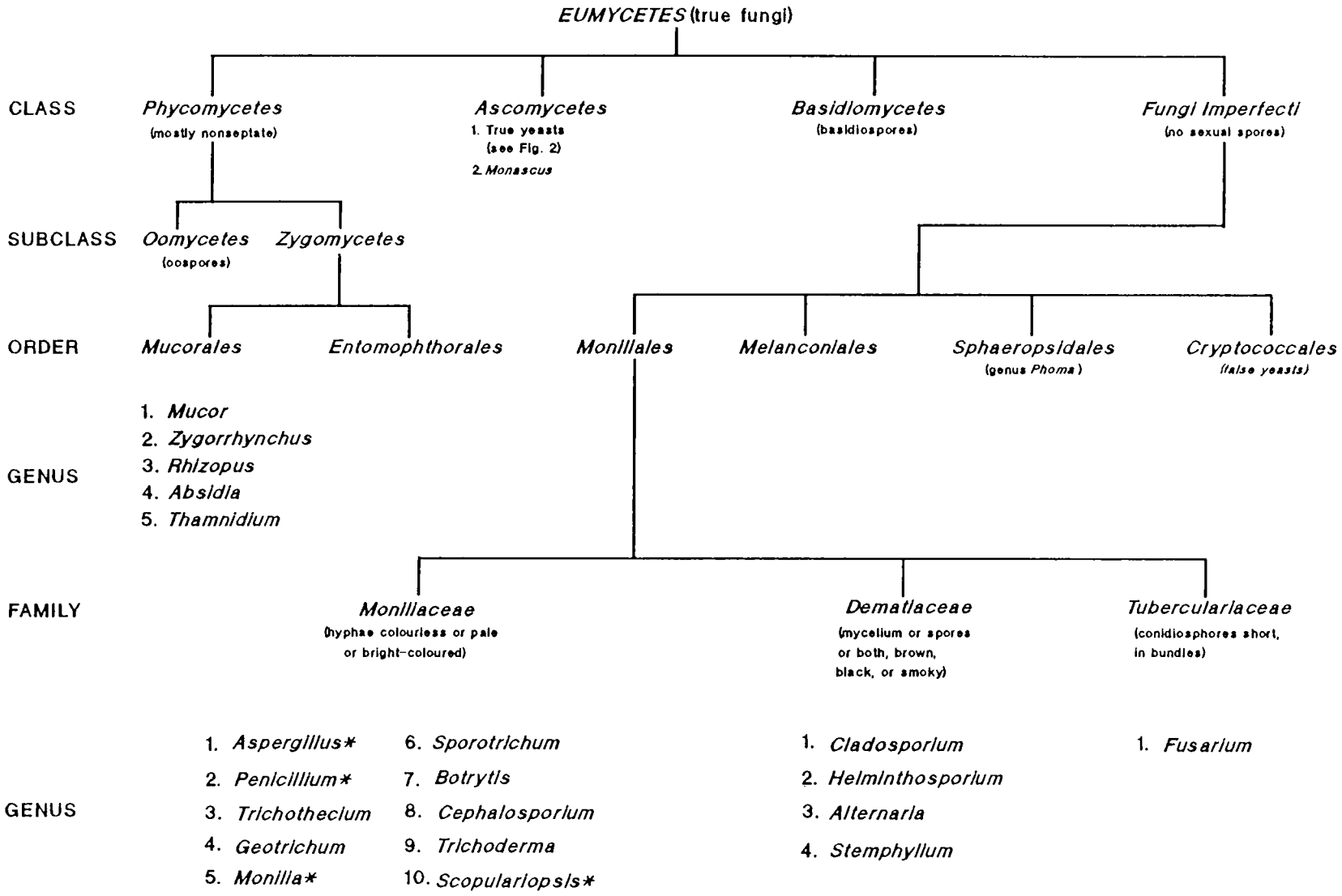
7. Mould and yeast plate count.

Judgement is based on the shape of colonies and the counts of mould and yeast respectively.

REFERENCES

Shokuhin Eisei Kensa Shishin, Guide to Food Hygiene Examination (authorized by the Ministry of Health and Welfare), Japan Food Hygiene Association. (1990).

Frazier, W. C. (1977). Food Microbiology, TATA McGraw-Hill Publishing Co. Ltd., New Delhi.



* Perfect species in Ascomycetes

Fig. 1. Partial classification of *Eumycetes*, or true fungi

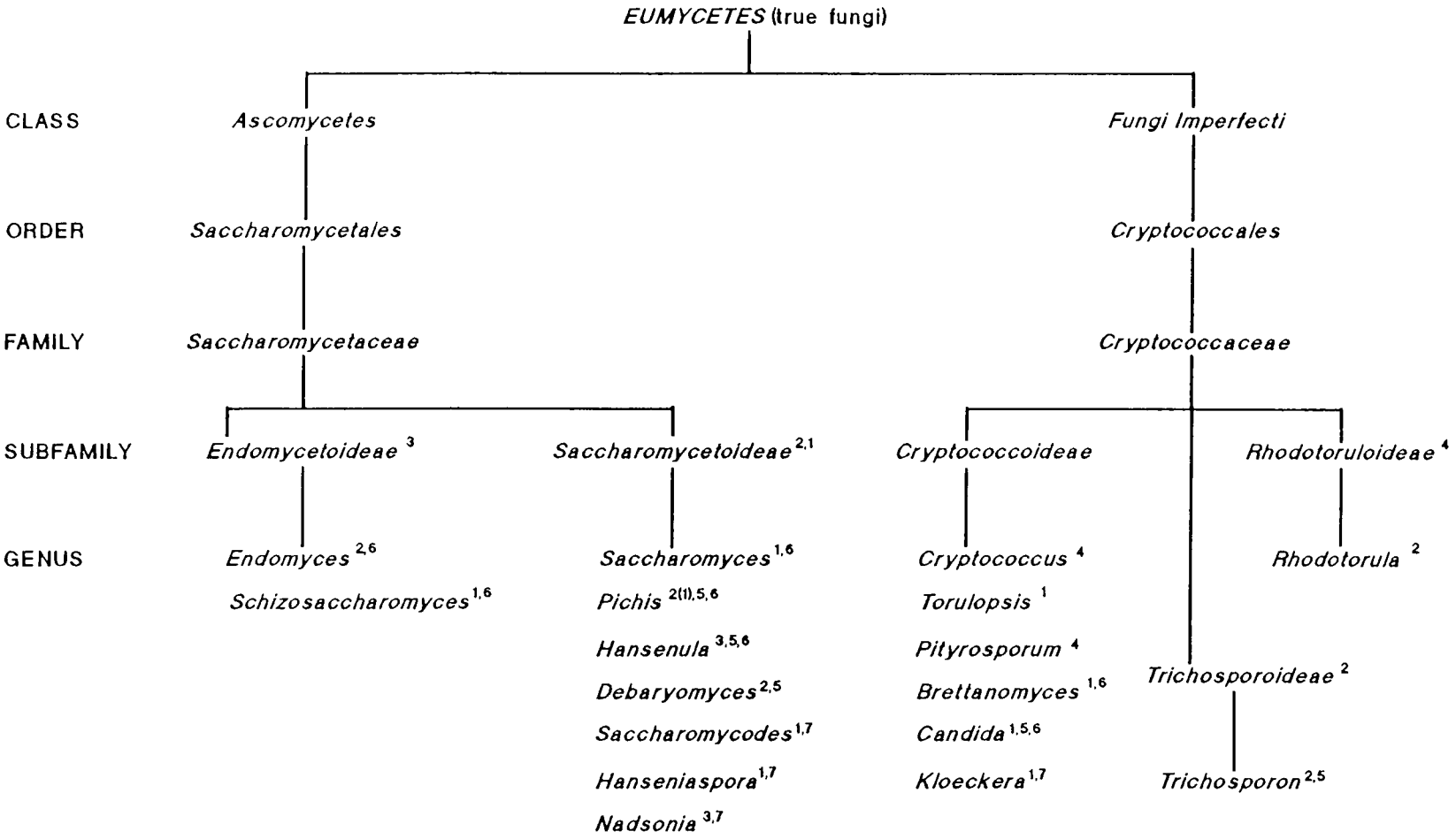


Fig. 2. Classification of yeasts to include genera found in foods.

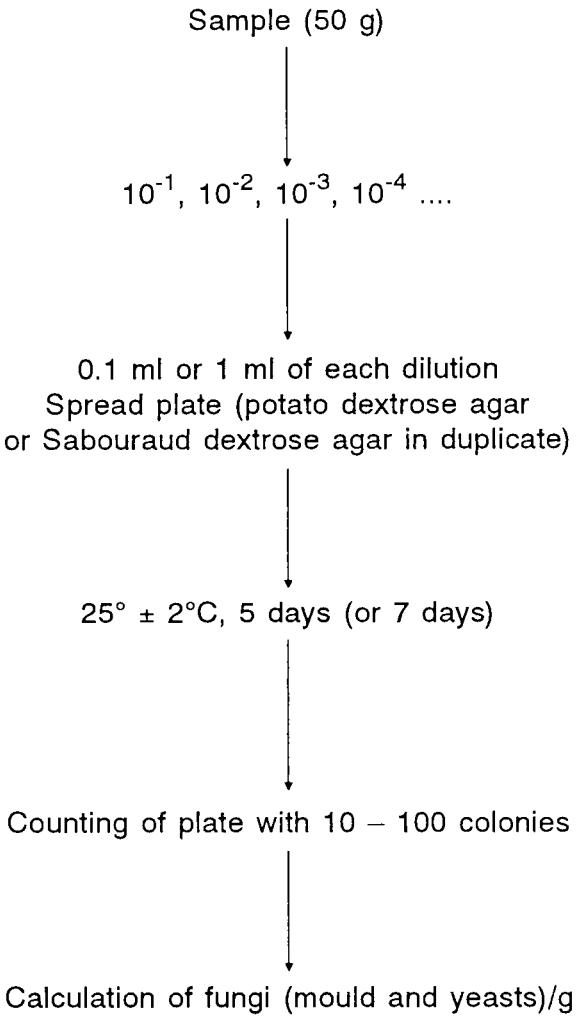


Fig. 3. Flow diagram of the procedure for Fungi Plate Count

SPORE FORMING BACTERIA COUNT

LIM PANG YONG

INTRODUCTION

Spores of bacilli could survive in mixed population of micro-organisms and could be found in foods commonly. These spores usually have greater resistance to temperature, germicidal chemicals and lethal radiations etc than the vegetative cells. Therefore, it is not surprising that spores are detected readily in many foods and ingredients such as starches, dried food stuff etc.

The following method uses heat shock to kill the vegetative cells and specific incubation temperature is selected to let the surviving spores to germinate for enumeration. However, the method described is not suitable for the enumeration of facultative and stenothermophilic organisms.

I. APPARATUS

Autoclave	Incubator
Weighing balance	Pipettes, 1 ml & 10 ml
'Waring' blender & flasks or Stomacher ('Lab-blender' 400)	Bunsen burner
Thermostatic controlled water bath with stirrer	Laminar flow chamber
Scissors, scalpel & forceps	500 ml Erlenmeyer flask
Alcohol (70% v/v) swabs	Sterile petri dish (90 mm ø x 15 mm H)
Colony Counter	

II. CULTURE MEDIA

1. Diluent: Select one of the following as diluent

   a. 0.1% Peptone Water

Peptone	1.0 g
Distilled water	1.0 liter

   Dissolve peptone in distilled water. Adjust to pH 7.0 ± 0.1. Dispense 45 ml each in glass bottle and sterilise at 121°C for 15 minutes.

   b. Butterfield's buffered phosphate diluent*

   *Refer to Appendix B.

c. 0.85% Saline Solution

Sodium chloride	8.5 g
Distilled water	1.0 liter

Dispense 45 ml each into flasks, bottles, or tubes and sterilise at 121°C for 15 minutes.

2. Tryptone glucose extract (TGE) agar (Difco) or Trypticase glucose agar (BBL)

Prepare 100 ml of Tryptone glucose extract (TGE) agar or trypticase glucose agar in a 500 ml Erlenmeyer flask and one additional flask of medium is used as a sterility control. Sterilise at 121°C moist heat for 15 mins and transfer to a 45°C water bath for cooling and let it remain in the water bath.

III. PROCEDURE

1. SAMPLING PROCEDURE

Randomly take 150-200 g of sample. Aseptically cut the sample into smaller portions and keep them in a sterile polyethylene bag or container. Store the cut sample in refrigerator (5°C) to maintain sample's integrity.

2. EXAMINATION PROCEDURE

i. Weigh 25 g of sample in a sterile blender jar and add 225 ml of sterile 0.1% peptone water into it. Blend the sample at high speed for 2 mins. If a stomacher is used, it requires 60 seconds.

ii. Pipette the blended sample into a set of 3 flasks of TGE agar which are held in the bath in the following sequence: 10 ml into the first, 1 ml into the second, and 0.1 ml into the third flask. Swirl the flasks gently to disperse the blended sample throughout the medium.

iii. Transfer the flasks without delay to a stirred water bath pre-adjusted to 80°C and heat for 30 minutes. (For fish paste product, temperature would be set at 100°C for 10 minutes.) The water level must be above the liquid level in the flasks. Agitate the flasks occasionally and gently to ensure the heat is evenly distributed.

iv. Transfer the flasks to a 45°C water bath after rapid cooling (cooling is done in cold tap water taking care that the temperature does not fall to the point where agar solidifies) and keep in the water bath for a period not more than 10 mins.

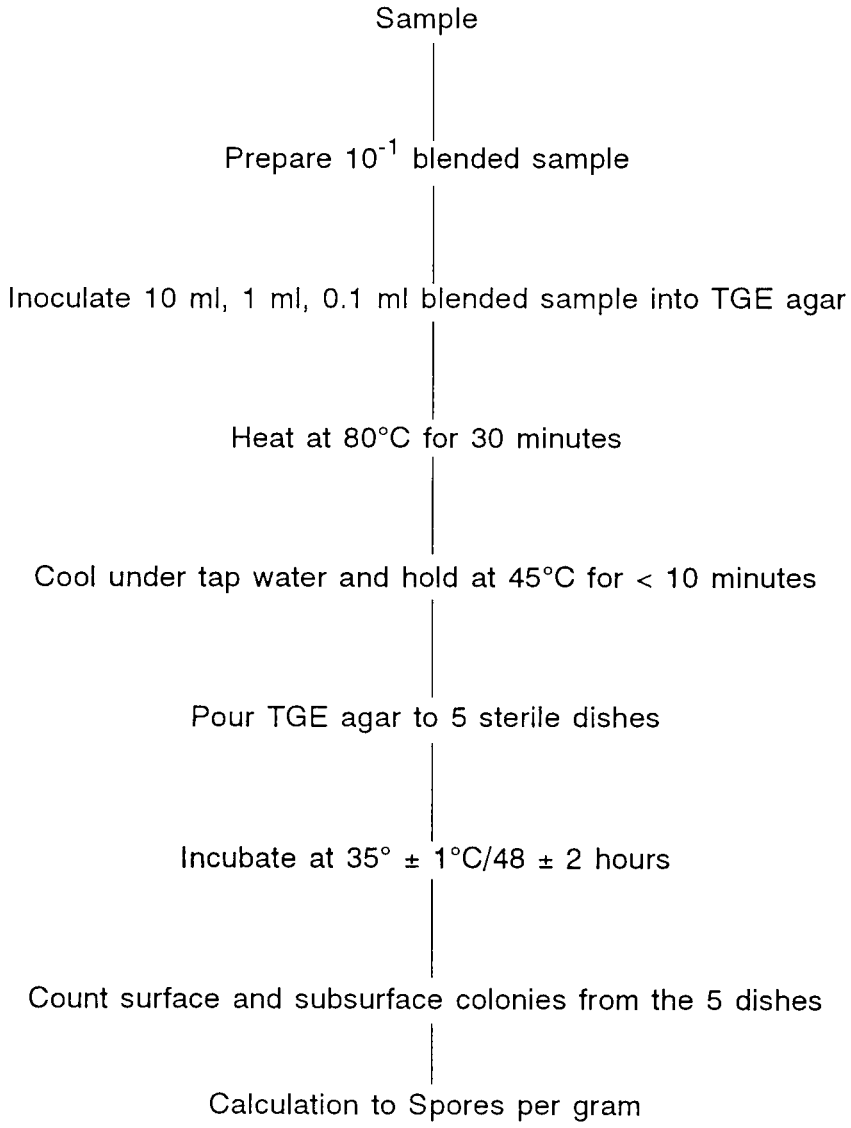
v. Pour the content in each flask, representing test sample and sterility control, respectively, into a set of 5 sterile petri dishes in equal volumes, i.e. about 20 ml per dish.

vi. When agar has solidified, invert and incubate the petri dishes at 35° ± 1°C for 48 ± 2 hours.

vii. Count the colonies on the surface and subsurface of the agar. The sum of colonies on the set of 5 plates poured from TGE agar containing 10 ml of blended sample, represents the number of aerobic, mesophilic spores per gram. Similarly, the number of colonies in sets of plates receiving 1.0 and 0.1 ml of blended sample is equal to 0.1 and 0.01 of the number of spores per gram and must be multiplied by 10 and by 100 respectively to get the count per gram.

viii. The number of spores which can be enumerated by this method ranges from 1 to 150,000 spores per gram.

ix. Flow diagram for aerobic spore-forming count:



REFERENCE

Compendium of Methods for the Microbiological Examination of Foods. Compiled by the APHA Technical Committee on Microbiological Methods for Foods. Edited by Marvin L Speck. 2nd edition. American Public Health Association, 1984.

QUALITY ASSESSMENT AND IDENTIFICATION

SENSORY EVALUATION OF FISH AND FISH PRODUCTS

LOW LAI KIM

INTRODUCTION

Sensory evaluation or organoleptic assessment of fish and fish products involve the use of the five human senses (namely sight, smell, taste, touch and hearing) to gauge the quality of food. Sensory evaluation is necessary to complement the chemical analysis of food to give an overall quality assessment of the food product.

Sensory tests involve a panel of taste panelists either trained or otherwise. Trained panel members are usually used in difference tests. Untrained panel members are usually made up of a consumer panel for a preference test. The food product is evaluated for its appearance, odour, taste and texture.

There are two types of tests, the difference test and the preference test. The difference tests come in many forms, the two more commonly used ones are the triangle test and the duo-trio test. The difference test is used for experiments where it is required to see if a treatment results in a difference in the food product as compared to a control. The preference test is used for market surveys of consumer preference for a new food product, etc.

FACILITIES

The basic sensory testing facilities include a sample preparation area, a panel booth area and a panel discussion area.

1. **Sample preparation area**

It should include the following facilities :

a) Cooking facilities (stoves, microwave oven, steamer, warmer or ovens, fryer, kettle)

b) Exhaust hood over cooking area

c) Storage facilities

d) Cutlery, crockery, trays, casseroles, etc.

e) Refrigerator

f) Stainless steel table for food preparation

g) Serving bench connected to the panel booths

h) Sinks with hot and cold water if necessary.

2. **Panel booth area**

The panel booth area should include the following :

a) Booths with neutral grey (Munsel value N/7) walls which reflect 40-45% light, to separate panel members

b) A sink in each booth

c) Water for rinsing (a tap in each booth)

d) Space for samples and questionaires

e) Provision of drinking water

f) Communication system between panel members and test organiser

g) Comfortable chairs which can be adjusted in height to suit panel members.

h) Each booth to be fitted with daylight light. Coloured light may be necessary in masking the colour of food product.

3. **Panel discussion area**

This area can be in the same room as the panel booths. The area is provided for round table discussions tests and discussion of results among test organizer and panel members. The area should have the following :

a) Round neutral grey table to comfortably sit 8 to 10 panel members

b) Whiteboard

c) Notice board

d) Comfortable chairs with adjustable height to suit the panel members

e) Daylight light fittings (adequate and uniform, with comfortable level of illumination)

The panel booth and panel discussion areas should be air-conditioned (constant at 20°C, with controlled temperature and humidity) with a slightly positive pressure to reduce inflow of air from the sample preparation area. The air-conditioning should be installed with activated carbon filters to remove all odours. Use odourless materials, paints and equipment in these two areas.

PANELIST

1. Difference tests

A discrimination panel is used for difference tests. The panel members should undergo prior screening. For triangle tests the panel members should be selected on the basis that their correct responses should not exceed 80%. Each member should have two trials at the same session, and selection should be based on no fewer than 20 judgements per member made on 10 different tests in 10 different sessions.

a) Number of panel members = usually 10

b) Minimum number for a given test = 5

c) Maintain a pool of qualified persons if possible.

2. Preference tests

Panel members must be representative of some consumer population and randomly selected. Persons who have expert knowledge of the product type and all who have specific knowledge of the samples and variables being tested should not be included in the panel. No prior training is required.

a) For small laboratories = usually 30

b) 16 to 20 panelists are sometimes employed

c) about 50 to 100 people are usually considered adequate

Panel members should be kept highly motivated by giving them due recognition in terms of small tokens after each test. Test organizer must create interest in the test activity, maintain a high degree of status for the programme and the panelists, and make panelists aware of the importance of their contribution.

3. Physiological sensitivity of panelists

To eliminate physiological effects in panelists,

a) conduct tests 1 hour after meals

b) wait at least 20 minutes after smoking, chewing gum, or eating or drinking between meals

c) encourage panelists to avoid eating highly spiced foods for lunch on days tests are to be run in the afternoon

d) when running odour tests, panelists must not use perfumes, lipsticks or perfumed face lotions

e) in taste testing, have subjects rinse their mouths with room temperature water just prior to starting tests and between samples

f) with odour stimuli, normal breathing would usually suffice if one waits 20 to 30 seconds between samples.

4. **Physiological control**

a) Maintain a pleasant and relaxed environment

b) Use coding (2 or 3 digit) for samples, refer to Table of random numbers

c) Use random presentation of samples e.g. for a 3 sample test :

Sample code	840	257	503
Order of presentation	A	B	C
	B	C	A
	C	A	B
	A	C	B
	B	A	C
	C	B	A

SAMPLES

1. Random selection of samples.

2. Uniformity in sample preparation, using standard procedures and preparation methods. Usually a panel member is presented with a tray containing a fork and a knife, serviettes, a glass of warm drinking water, a tumbler for waste disposal, samples, questionnaire, pencil and eraser.

3. Presentation of samples should be uniform. The sizes of samples should be sufficient for at least 3 tastes (normal sips or bites). About 1/2 oz of liquid or 1 oz of solid should suffice.

4. Temperature of samples should not be above 77°C for hot food or drinks, or at normal temperature in which product is usually consumed.

TEST DESIGN AND METHODS

1. Triangle test

Samples are presented either simultaneously or successively. In this test two samples are the same and one is not. The panelist must select the odd one out. A sample of the questionaire for triangle test is in Appendix F. When frequency of correct solutions is above the chance level, a difference is inferred (obtained by consulting the Statistical table for triangle test, Appendix I).

If the products are A and B, the samples should be presented as follows:

Sample set	Sample code		
	486	927	184
1	A	B	A
2	B	A	B
3	A	A	B
4	B	B	A
5	B	A	A
6	A	B	B

2. Duo-trio test

In this test two samples are the same and one is different. One of the similar pair is identified as R and the panelist must select that one from the two unknown that is similar to the identified sample, R. A sample questionaire for the duo-trio test is in Appendix G. The criteria for statistic analysis is based on the number of responses correctly identified as the similar sample. The statistical table for duo-trio test is in Appendix J.

If the two samples are A and B, the presentation of sets of samples are as follows:

Sample set	Reference sample(R)	Sample code		
		498	276	956
1	Product A	R	A	B
2	Product B	R	B	A
3	Product A	A	R	B
4	Product B	B	R	A
5	Product A	A	B	R
6	Product B	B	A	R

3. Hedonic preference test

The term "Hedonic" implies psycological expression to "pleasure" and "displeasure". The test is carried out using terms "like" and "dislike" to indicate psychological responses when testing food products. Numerical scores are assigned to the hedonic scale which can be 5-point or 9-point, and the data can be statistically analysed using One-way Analysis of Variance. If the calculated F-value is greater than that from the F-value statistical table, a significant difference is detected in the sample. A sample of the 9-point hedonic scale questionaire is shown in Appendix H. The samples should be randomly presented to the panelist and an example for a 3-sample (A, B, C) test and a 10 member panel is shown below.

Panel member	Sample code		
	579	286	408
1	A	B	C
2	B	C	A
3	C	A	B
4	A	C	B
5	B	A	C
6	C	B	A
7	A	B	C
8	B	C	A
9	C	A	B
10	A	C	B

REFERENCES

Manual on Sensory Testing Methods. Sponsored by ASTM Committee E-18 on Sensory Evaluation of Materials and Products. ASTM Special Technical Publication 434. Published by American Society for Testing and Materials. (1968).

Piggott, J.R. (1984).Sensory Analysis of Foods. Elsevier Applied Science Publishers, London and New York.

THIN LAYER ISOELECTRIC FOCUSING OF FISH PROTEIN

NG CHER SIANG

INTRODUCTION

Proteins are made up of many units of amino acids. All the amino acids have both positive and negative charges in their dissociated form. The net charge of a protein molecule in a given media is either positive, neutral or negative. These charged states are determined by the dissociation characteristics of the component amino acids in that protein. Under the influence of an electric field, the differently charged proteins will move towards the point of neutrality within the media. This point of neutrality is called the isoelectric point of that protein.

APPARATUS

The apparatus described in this procedure include the electrophoretic chamber (LKB 2117 Multiphor II), power supply (LKB 2197 Power Supply) and the water bath (LKB 2219 Multitemp II).

REAGENTS

1. Fixing solution

Methanol (MeOH) ..... 150 ml
Distilled water ..... 350 ml
Sulfosalicylic acid, dihydrate ($C_7H_6O_6S.2H_2O$) ..... 17.25 g
Trichloroacetic acid (CCl_3COOH) ..... 57.50 g

2. Destaining solution

Ethanol (C_2H_5OH) ..... 500 ml
Acetic acid (CH_3COOH) ..... 150 ml
and dilute to 2,000 ml with distilled water.

3. Staining solution

Coomasie Brilliant Blue R-250 ..... 0.345 ml
Destaining solution ..... 300 ml
Stand for 48 hours and filter before use.

4. Preserving solution

Destaining solution ..... 500 ml
Glycerol ($C_3H_8O_3$) ..... 50 ml

5. Phosphate buffer (pH 7.0), 0.01 M

1.79 g Sodium phosphate, dibasic $Na_2HPO_4.12H_2O$ in 300 ml distilled water. Add 1M H_3PO_4 dropwise to adjust to pH 7.0 and dilute to 500 ml with distilled water.

6. Anode buffer

1M H_3PO_4

7. Cathode buffer

1M NaOH

8. Kerosene

9. Thin layer polyacrylamide gel

Polyacrylamide gel concentration (T)	= 5%
Degree of cross linking (C)	= 3%
Ampholine concentration	= 2.4% (w/v)
Gel dimension 245 X 110 X 1 mm	

Commercially prepared gels are used as a matter of convenience. For laboratory preparation of gel, refer to LKB Handbook.

SAMPLE PREPARATION

1) Homogenise approximately 1 g fish muscle with 10 ml distilled water or phosphate buffer (pH 7.0).

2) Centrifuge homogenised sample at 10,000 rpm for 10 mins (0°-10°C).

3) Collect supernatant for use. Supernatant can be frozen until required.

PROCEDURE

Setting the electrophoresis apparatus

1) LKB 2219 Multitemp II

Set the cooling water bath between 5°C and 10°C.

2) LKB 2197 Power supply

Set Power at constant of 25W
Set Current at maximum (50mA)
Set Voltage at maximum (1500V)

Set the apparatus to run at constant power for maximum electrofocusing efficiency.

Isoelectric focusing

1) Switch on the cooling unit.

2) Place the paper template provided on the cooling plate.

3) Adjust the position of the electrodes (attached to the electrode holder) to the appropriate width.

4) Apply a layer of kerosene on the template for good contact.

5) Place the polyacrylamide gel on the template, with the plastic base in contact with the paper template.

CAUTION

If the gel is wrongly placed, and has been in contact with kerosene, **DO NOT USE**. The kerosene may catch fire under the high voltage during the run.

6) Wet a paper wick with the anode buffer, and separately wet another wick with the cathode buffer. Wet the wicks in excess buffer and blot away the excess buffer.

7) Place the wet wicks on the anode and cathode ends of the gel.

CAUTION

Wicks placed wrongly will cause short circuit and is very dangerous. Always check before placing the wicks on to the gel.

8) Wet the applicator strips with the protein solution and place on the gel at about two thirds distance from the anode end of the gel.

9) Connect the electrodes to the power pack (for anode, use the outer port). **See Precaution 1.**

10) Run for 30 mins., recording the voltage and current every 5 mins. **See Precaution 2.**

11) Stop the run by switching off the power pack. Remove the connection between the power pack and the gel chamber before opening the chamber cover.

12) Using forceps remove the applicator strips carefully.

13) Resume the run for 60 mins. **See Precaution 3.** Stop the run as before.

14) Remove the two strips of wicks and gels. Cut one corner of the anode end to mark the direction of the anode.

15) To proceed with the pH measurement, cut off a strip of the gel before fixing the main gel (see later section).

16) Transfer the gel to fixing solution. Keep for 45 mins.

17) Drain off the fixing solution. Rinse twice with destaining solution. Soak gel in destaining solution for 45 to 60 mins.

18) Drain off destaining solution. Introduce preheated stain (50°C). With gentle agitation, stain for 5 mins. **See Precaution 4**.

19) Discard stain. Rinse twice with destaining solution. Soak gel in destaining solution at room temperature, with occasional agitation. Destain overnight. **See Precaution 5.**

20) The end point is when the background of the gel is clear. Remove destaining solution, and replace with preserving solution. Keep for 1-2 hours.

21) When the gel is satisfactorily destained, tape the gel and plastic support onto a preheated glass surface. Dry at 50°C for 45 mins. The gel should not be too dry.
See Precaution 6.

22) Carefully place the protective cover over the gel, ensuring no trapped air bubbles. The gel is very sticky, and once the mylar sheet comes into contact with the gel, it cannot be removed without destroying the gel.

DETERMINATION OF THE pH GRADIENT

After the focusing and removal of the wicks, cut off a strip of the gel, approximately 1.5 cm broad breadthwise (i.e. from + to – ends). The small strip of the gel is overlaid onto a piece of graph paper. It is cut into 19 or 20 portions, each close to 0.5 cm. Each of these strips is placed in 10 ml of CO_2 free distilled water. It is covered and left to stand for an hour or so. Before measuring the pH, it is given a vigorous shaking, using a vibrator. The pH is then measured.

The distribution of the pH gradient by the ampholine is such that the anode end is acidic and the cathode end is basic. The pH gradient can be plotted on the graph provided.

PRECAUTIONS

1) Care should be taken when handling the unit. Always remove the connection between the power pack and the gel chamber before opening the chamber.

2) After setting the unit to run, keep an eye on the gel to ensure that the high voltage does not cause the gel to burn. If it happens, stop the run immediately.

3) Observe the cover placed directly over the gel for presence of condensation during the run. If much condensation occurs, it is better to stop the run temporarily, and wipe away the excess moisture before continuing. The usual procedure of disconnecting the power pack applies.

4) The stains should preferably be filtered before use. The temperature set at 50°C ensures rapid staining.

5) During destaining, it is necessary to agitate occasionally to ensure good de-staining. The destaining time is not fixed, but depends on each case.

6) During drying of the gel, the plastic support should be firmly taped to a good support to prevent it from buckling.

REFERENCE

Anders Winter (1977) : Analytical Electrofocusing in Thin Layers of Polyacrylamide Gels, LKB Application Note 250

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

APPENDIX A1

Most Probable Numbers per 100 mL of Sample, Planting 5 Portions in Each of 3 Dilutions in Geometric Series

Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes				Number of Posivite Tubes				Number of Positive Tubes			
10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN
0	0	0		1	0	0	2.0	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4.0	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6.0	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8.0	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9.0	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4.0	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1,600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81				

APPENDIX B

B1 BUTTERFIELD'S BUFFERED PHOSPHATE DILUENT

1. Stock solution

Dissolve 6.8 g KH_2PO_4 in 100 ml H_20, 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 water. Prepare diluent with this solution, dispensing enough to allow for losses during autoclaving. Autoclave for 15 mins for 121°C.

B2 MEDIA PREPARATION

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

Merck & Oxoid.

Aesculin broth

Andrade peptone broth (ADB)

Azide dextrose broth (ADB)

Anaerobic agar

Bacto-peptone (PW)

Baird Parker medium

Brain heart infusion broth (BHI)

Brilliant green bile (2%) broth (BGB)

Bromcresol 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)

Potato dextrose agar*

Sabouraud agar*

Selenite broth

Sodium sulfite-polymyxin sulfadiazine agar (SPS)

SIM medium

Simmons citrate agar

Tetrathionate broth

Thiosulphate citrate bile salts sucrose agar (TCBS)

Triple sugar iron agar (TSI)

Triplecase soy broth (TSB)

TSN agar

Xylose lysine deoxycholate (XLD)

* Before sterilizing by autoclaving at 121°C for 15 mins, add 50-100 mg of chloramphenicol (antibiotics) dissolved in 10 ml of 95% ethyl-alcohol into 1,000 ml of medium to inhibit of the growth of bacteria. If chloramphemicol is not available, adjust to pH 3.5 ± 0.1 by adding sterilized 10% tartaric acid to the sterilized medium.

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 H_2S production.

Method 2.

Inoculate a tube of SIM medium by stabbing into the butt. Observe for blackening due to H_2S 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% $FeCl_3$ 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% $FeCl_3$ 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 evidenced 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

APPENDIX D

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.

APPENDIX E

EXAMINATION METHOD OF *Vibrio cholerae* IN JAPAN

MAKOTO YAMAGATA

INTRODUCTION

Vibrio cholerae infection is a fatal infectious disease. There are two types of *Vibrio cholerae*. One is a classical type and the other is the eltor type. Recently, most of the *V. cholerae* are of the eltor type, such as those identified by Ogawa, Inaba and Hikoshima.

Symptoms of cholera are heavy watery diarrhoea and vomitting. It is caused by oral ingestion of *Vibrio cholerae* contaminated foods. Cholera epidemic is commonly found in developing countries through waterborne infection. Thus fish and shellfish has a high *Vibrio cholerae* contamination rate even though the bacteria counts are very low ($< 10^3$ cells/100g). In the distribution chain of fish and shellfish, the temperature should be kept below 10°C. This will help prevent the onset of cholera infectious disease. Furthermore, the contamination of *Vibrio cholerae* could be eliminated by subjecting the food to proper cooking operations. It is vitally important to prevent secondary or cross-contamination after cooking.

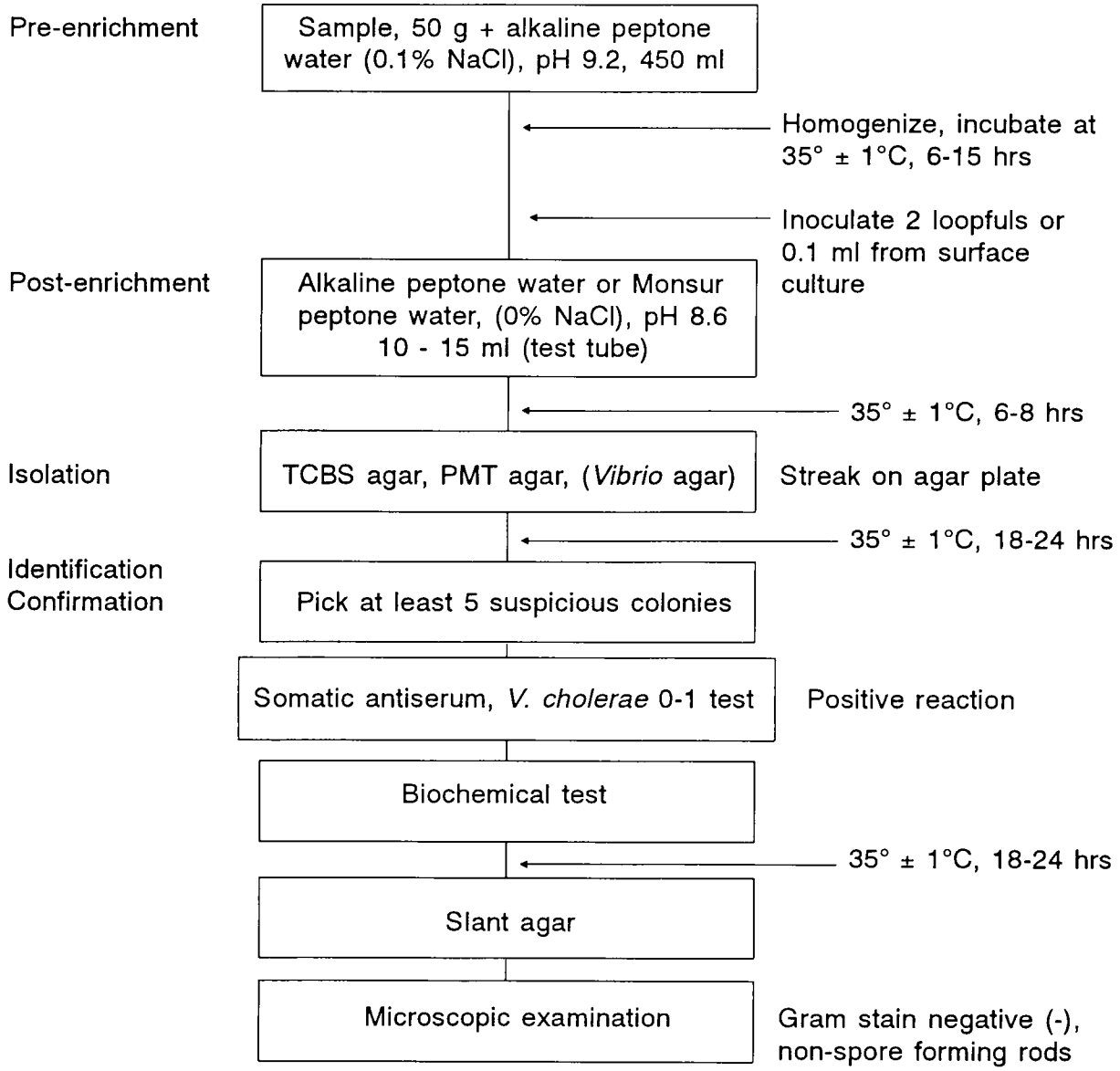


Fig. 1. Flow diagram of procedure for the examination of *Vibrio cholerae*

Source : Method of Japanese Ministry of Health and Welfare, Guidelines for *Vibrio cholerae* detection. (1988).

Table 1. Biochemical tests for *Vibrio cholerae*.

Tests	Results
TSI (stab)	acid + (Y^*/Y) gas – H_2S –
Oxidase test	+
Indole	+
Lysine decarboxylase test	+
Ornithine decarboxylase test	+
Arginine hydrolysis test	–
Mannitol	+
Inosite (inositol)	–
Growth in 0% NaCl peptone water	+
Growth in 7% NaCl peptone water	–
Growth in 10% NaCl peptone water	–

* Sometimes negative reaction (red colour on surface)

Source : Method of Japanese Ministry of Health and Welfare, Guidelines for *Vibrio cholerae* detection. (1988).

Table 2. Serological types of *Vibrio cholerae*.

Serological types	Somatic antiserum	Polyvalent serum	Serum factor	
			Anti Ogawa type serum (Anti B serum)	Anti Inaba type serum (Anti C serum)
Ogawa	AB	+	+	–
Inaba	AC	+	–	+
Hikoshima	ABC	+	+	+

Vibrio cholerae only produces cholera enterotoxin in fish and shellfish (Eiken No. 232, 28 September, 1988).

APPENDIX F

TRIANGLE TEST

DIFFERENCE ANALYSIS

DATE: _____ NAME: _____

PRODUCT: _____

Instructions: Here are three samples for evaluation. Two of these samples are duplicates. Separate the odd sample for difference in taste only.

Odd Sample is Number _____

Comments:

APPENDIX G

DUO-TRIO TEST

DIFFERENCE ANALYSIS

DATE: _____ NAME: _____

PRODUCT: _____

Instructions: Here are three samples for evaluation. One sample R is the reference sample. The other two samples contain one that is identical to R. Taste the two unknown samples and select the one that is identical to R and write down the code in the space below.

The sample that is identical to R is Number _____

Comments:

APPENDIX H

PREFERENCE TEST : SIMPLE 9-POINT HEDONIC SCALE

NAME:_____ AGE: _____ DATE: _____

APPEARANCE	Sample Code		
	351	554	750
Excellent (9)			
Very Good			
Good (7)			
Fair			
BORDERLINE (6)			
Slightly Poor			
Poor (4)			
Very Poor			
Inedible (2)			

Comments on appearance : _____

_____

ODOUR	Sample Code		
	351	554	750
Excellent (9)			
Very Good			
Good (7)			
Fair			
BORDERLINE (6)			
Slightly Poor			
Poor (4)			
Very Poor			
Inedible (2)			

Comments on appearance : _____

_____

APPENDIX I

Statistical Table for Triangle Test

NUMBER OF PANELISTS	NUMBER OF CORRECT CHOICES TO ESTABLISH SIGNIFICANCE AT	
	5% LEVEL	1% LEVEL
1	–	–
2	–	–
3	3	–
4	4	–
5	5	5
6	5	6
7	5	6
8	6	7
9	6	7
10	7	8
11	7	8
12	8	9
13	8	9
14	9	10
15	9	10
16	9	11
17	10	11
18	10	12
19	11	13
20	11	13

APPENDIX J

Statistical Table for Duo-Trio Test

NUMBER OF PANELISTS	NUMBER OF CORRECT CHOICES TO ESTABLISH SIGNIFICANCE AT	
	5% LEVEL	1% LEVEL
1	–	–
2	–	–
3	–	–
4	–	–
5	–	–
6	6	–
7	7	–
8	8	8
9	8	9
10	9	10
11	10	11
12	10	11
13	11	12
14	12	13
15	12	13
16	13	14
17	13	15
18	14	15
19	15	16
20	15	17