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Analytical Methods for Estimating Freshness of Fish

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PREFACE

It needs hardly be stressed that freshness is one of the essential factors of fisheries products. The quality of fresh fish as well as that of processed fisheries products depends upon the freshness of fish when it is landed at the port, delivered to the factory or sold to the consumer. Therefore, the problem of fish preservation, in other words "how to keep the freshness of caught fish", is of prime importance in order to increase the added value of marine products.

In seeking to improve the present situation of fish preservation in the region, the technique of "how to measure freshness" or "how to express scientifically the degree of freshness in mathematical terms" is a very important consideration. It is also the first step to be taken before any effective action can be initiated. If we can indicate the degree of freshness in figures instead of such vague terms as "good, medium or bad", based on our own perception of the changing color or smell of fish, we will be able to establish more scientifically and more accurately the existing problems and, at the same time, ascertain ways of improving the present situation.

Dr. Hitoshi Uchiyama, who is an old colleagues of mine, and an eminent expert on fish preservation in Japan, was seconded by JICA to our Training Department, SEAFDEC, as a visiting researcher, from 21 August to 20 September 1978. During his assignment, he was asked by the Department to prepare a text-book on some basic techniques for the measurement of freshness of fish. I hope that this booklet will be of help to readers interested in this subject.

Shigeaki Shindo
Deputy Secretary-General and
Deputy Chief of Training Department

Analytical Methods for Estimating Freshness of Fish
by
Hitoshi Uchiyama

CONTENTS

The term "freshness of fish" should be taken to include two concepts: the lowering of freshness caused by autolytic action of fish tissue; and the so-called deterioration, which is initiated by the onset of spoilage. The former is referred to as "enzymatic freshness" and the latter as "bacterial freshness". These concepts were derived from the facts described below.

1. POST-MORTEM CHANGES IN FISH MUSCLE

Fig. 1 shows the results of changes, during ice storage, in bacterial number, pH value, amounts of free amino acids, salt-soluble proteins, total volatile bases (TVB), and trimethylamine (TMA) in muscle of plaice iced immediately after death.* No significant changes were observed, up to 10 days, in the number of bacteria, nor in the amounts of the bacterial decomposition products such as TVB-N and TMA-N; that is, onset of spoilage took place always after autolysis or lowering of freshness of fish meat had proceeded to some extent. This phenomenon was observed in almost all of the fish species with some differences between the time when autolysis set in and the time when bacteria grew noticeably and their decomposition products increased considerably.

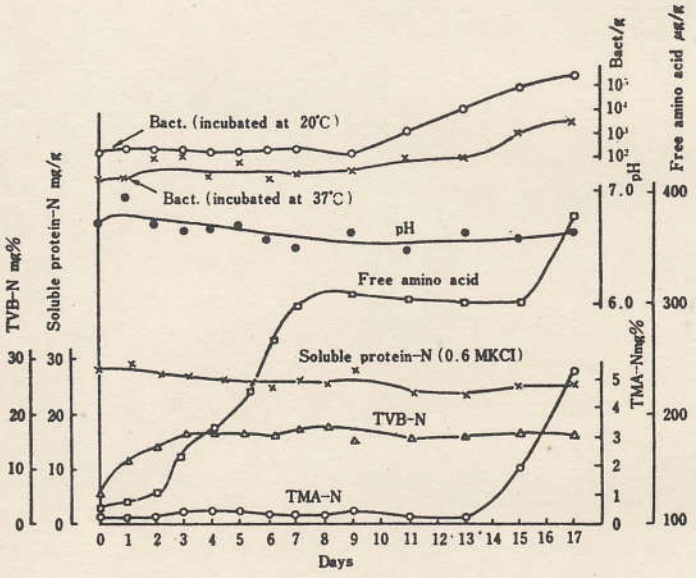

Fig. 1. Changes in bacterial number, amounts of free amino acids, salt-soluble proteins, total volatile bases (TVB) and trimethylamine (TMA) in plaice muscle during ice storage. (H. UCHIYAMA, T. SUZUKI AND S. EHIRA (1966).

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* Abbreviations:

TVB-N:	Total volatile base nitrogen;		TMA-N:	trimethylamine nitrogen;	
ATP	:	adenosinetriphosphate;	ADP	:	adenosinediphosphate;
AMP	:	adenosinemonophosphate;	IMP	:	inosinemonophosphate;
HxR	:	inosine; Hx: hypoxanthine;	PCA	:	perchloric acid;
TCA	:	trichloroacetic acid;	TB	:	thymol blue;
DMA	:	dimethyl amine;	BTB	:	bromthymol blue.

2. METHODS FOR ESTIMATING THE ENZYMATIC FRESHNESS OF FISH USING SIMPLE AND RAPID COLUMN CHROMATOGRAPHY

It is well known that the post-mortem degradation of ATP in fish muscle is effected by a series of enzyme reactions as shown in Fig.2, and proceeds rapidly except for the final stage from HxR to Hx. The enzymes responsible for the reactions have been confirmed, to be identical for all fish species, by many investigators [2),3)]. On the basis of these facts, a K value has been proposed for estimating the enzymatic freshness of fish by SAITO et al [4)]. The K value was given in terms of the ratio of the total amounts of HxR and Hx to the total amounts of ATP and its degradation products in fish muscle. This value can be calculated from the following formula;

$$K = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx} \times 100\ (\%)$$

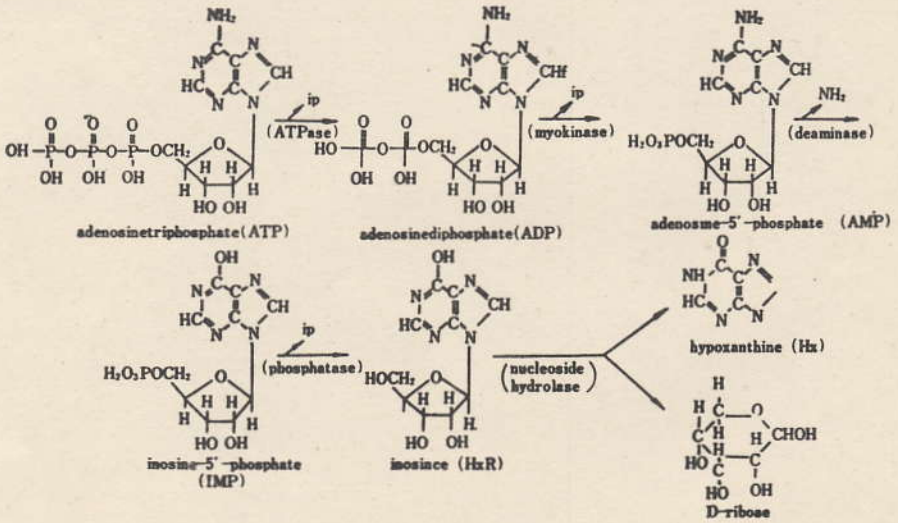

Fig. 2. Degradation of ATP to Hx in fish muscle.

From this formula, it is evident that a K value of fresh fish or fish of good quality is low, whereas that of fish of lower freshness or inferior quality is higher.

Many researchers have carried out investigations to elucidate the relationship between the degradation of ATP in fish muscle and the freshness judged by a sensory test, and a K value has been found to be a useful index for estimating freshness of fish. UCHIYAMA et al [5,6] surveyed the freshness of fish landed at fishing ports in Japan and the freshness of fish in commercial circulation, and obtained the following results; K values of fish muscle immediately after the fish was dead were under 5%, average of K values of fish immediately after being landed at fishing ports, 22.5%, that of "sashimi" or sliced raw fish, a typical Japanese uncooked food, and of material for "sushi", about 20%, and K values of raw material for "kamaboko" (fish cake) and "surimi" (minced fish meat) ranged from 40% to 60%. These conclusions were reached through statistical calculations based on the data obtained from many samples.

On the other hand, JONES et al [7] has proposed Hx in fish muscle as a useful index of freshness of fish. However, the conversion of degradation products of ATP in fish muscle from HxR to Hx is predominantly influenced by the enzymes, nucleosidehydrolase or nucleosidephosphorylase (Fig. 2). The activity of these enzymes in fish muscle differs in different species. For example, it was found that muscle of horse mackerel either lacks both these enxymes or their activity is extremely weak, whereas these enzymes in plaice muscle are extremely active [8]. Accordingly, in muscle of horse mackerel, HxR accumulates to a considerable extent (HxR accumulating type), whereas in that of plaice Hx accumulates predominently during ice storage of the fish. In some species both HxR and Hx are accumulated (intermediate type). Moreover, of some 110 species of fish investigated, [9] 40 species were classified into the HxR accumulating type and about 50 species were classified into the intermediate type. This fact shows that it is impossible or difficult to estimate the enzymatic freshness of fish by determining Hx in its muscle. As for the K value, however, as described above, it is expressed as the ratio of the amounts of HxR and Hx to the total amount of ATP and its degradation products in fish muscle. Therefore, a K value can be applied to all of the fish species, without regard to accumulation of HxR or Hx, as an index of the enzymatic freshness of fish.

It is necessary, in the analitycal procedures described below, to use standard solutions prior to measuring K values of fish samples, until good recoveries are obtained [10].

2.1 Reagents

ATP, ADP, AMP, IMP, HxR, and Hx purchased from Sigma Chemical Co., or Boehringer & Soehne Co., are preferably used for preparing the standard solutions. These solutions are prepared as 10 mM aqueous solutions except for the Hx solution. A 10 mM Hx solution is prepared by dissolving the reagent in 0.05 N sodium hydroxide because of its low solubility in water. All the standard solutions are stored in a refrigerator at -20°C.

2.2 Preparation of muscle extract

ATP and its post-mortem degradation compounds in fish muscle are soluble in PCA or TCA which is widely used for preparing a deproteinized extract for determining TVB or TMA-N in fish tissue. Nucleotides and their related compounds have ultraviolet spectra, and are determined quantitatively from optical density values at 250 μ or 260 μ. Therefore, muscle extract for measuring the K value must be prepared with PCV which, differing from TCV, has no ultraviolet spectra. The procedure for the extraction of ATP and its related compounds from fish muscle is shown in the flowsheet in Fig.3. In a 10 ml centrifugal tube 1 g of muscle is homogenized at ice temperature with 2 ml of chilled 10% PCA by using a glass rod. The homogenate is centrifuged at 3,000 rpm for 3 min. The residue is washed with 2 ml of chilled 5% PCA, and recentrifuged. After this process has been repeated twice, the supernatants are combined and immediately neutralized at ice temperature with a small quantity of 10 N

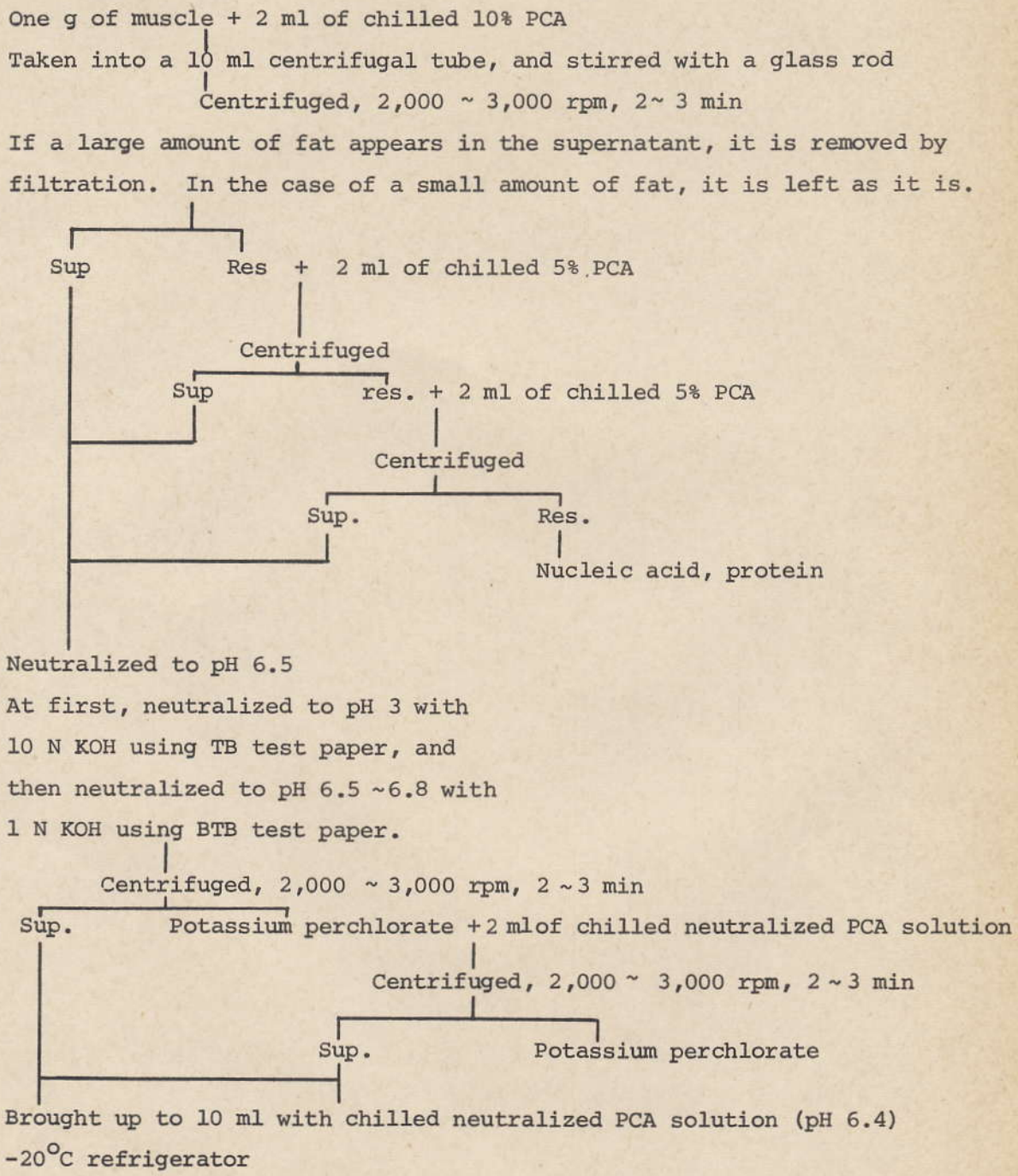

Fig. 3. Preparation of fish muscle extract.

potassium hydroxide to about pH 3.0 using TB test paper, and finally to pH 6.5-6.8 with a few drops of 1 N potassium hydroxide using BTB test paper. The neutralized extract is centrifuged and the precipitate of potassium perchlorate formed is washed twice at ice temperature with 2 ml portions of chilled neutralized PCA (5%) solution (pH 6.4). The supernatant and washings are combined and the total volumes are brought up to 10 ml with the chilled, neutralized solution pH 6.4. Extracts thus prepared are stored at -20°C in a refrigerator to await analysis.

2.3 Preparation of ion exchange resin

Dowex 1 x 4 (Cl type, 200-400 mesh) resin is washed with acetone, followed by 1 N sodium hydroxide, deionized water, and 1 N hydrochloric acid in this order. After being washed finally with deionized water, it is stored in refrigerator at 5° - 10°C.

2.4 Column used

A 18 x 0.6 cm (diam.) column fitted at its lower end with a glass filter is used (Fig. 4). Instead of the filter, glass wool can be used. The lower end of the column is narrowed to minimize head space, thereby

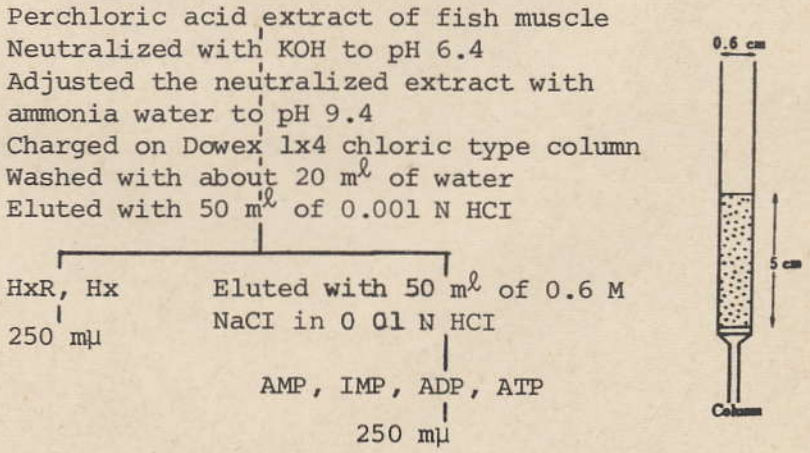

Fig. 4. Rapid fractionation method for the mixtures of HxR + Hx and AMP + IMP + ADP + ATP.

ensuring sharp separation of the compounds. As Plate 1 shows, a separate funnel is connected to the top of the column for convenient and rapid separation of the compounds. The ion exchange resin washed is packed 5 cm high using a pipette. Suspended resin is poured carefully along the inside wall of the column so as to avoid air entering the resin bed. It is convenient to use a small pump in order to speed up packing the resin in the column. When the column is used later, it is stored in a refrigerator at 5°C after its top and lower ends have been sealed with parafilm or an ordinary rubber stopper. It is convenient to prepare a number of columns and store them in a refrigerator so that they can be used immediately at need.

2.5 Preparation of eluting solution

The following solutions are prepared with sodium chloride (guaranteed reagent) and 1 N hydrochloric acid of factor 1.00 which is commercially available.

Solution A: 0.001 N hydrochloric acid

Solution B: 0.6 M sodium chloride containing 0.01 N hydrochloric acid.

2.6 Procedure for separating HxR and Hx from nucleotides ATP, ADP, AMP and IMP

The flow sheet in Fig. 4 shows a procedure for estimating a K value. At first, 2 ml of neutralized muscle extract is placed in a beaker of about 20 ml, adjusted to pH 9.4, using TB test paper, with a few drops of 0.5 M liquid ammonia, and then charged in the column. The inside wall of the beaker in which the sample solution has been placed is washed with a small quantity (about 2 ml) of deionized water which has been adjusted to pH 9.4 with the liquid ammonia, and the washings are also charged in the column. A small

pump is conveniently used here, too. When the meniscus of the sample solution has been lowered to the top of the resin bed in the column, a separate funnel is attached to the column, 20 ml of deionized water is poured into it, and amino acids and other ultraviolet absorbing compounds are eluted with water from the column. In order to elute HxR and Hx, 45 ml of solution A is poured into the separate funnel, and continually dropped into the column at a flow rate of about 1 - 2 ml per min. The eluate is received in a 50 ml volumetric flask. When the meniscus of solution A used nears the top of the resin bed, 45 ml of solution B is poured into the separate funnel and elution is performed as described above. By this process, ATP, ADP, AMP and IMP can be eluted under the influence of pH and the concentration of sodium chloride in solution B. The elutes of solutions A and B received in the volumetric flasks are brought up to 50 ml with solutions A and B.

2.7 Calculation

The value of K can be calculated from the following formula:

$$K = \frac{E\ 250\ m\mu\ A}{E\ 250\ m\mu\ A + E\ 250\ m\mu\ B} \times 100\ (\%)$$

Where E 250 mμ A and E 250 mμ B are the optical densities at 250 mμ of the fraction of solutions A and B, respectively.

This process can be conveniently carried out using a simple apparatus as shown in Plate 1, whereby it is possible to determine the K values of approximately 30 samples per day.

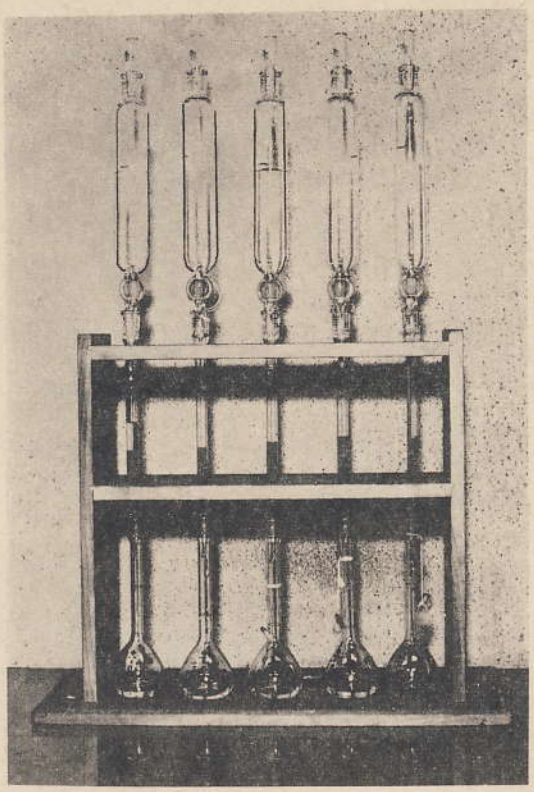

Plate 1. Simple apparatus for rapid estimation of the K value

3. METHOD FOR ESTIMATING THE BACTERIAL FRESHNESS USING VALUE OF TVB-N

While passing through the stages of rigor mortis, resolution of rigor mortis and autolysis caught fish spoils. The bacterial number at the initial stage of spoilage has been said to be 10^5 celles per g (of muscle). However, this value is not a precise one, but can be used as a rough standard. During the time when fish or shell fish are alive, their gills and viscera are being contaminated by bacteria. The bacteria multiply at the later stage of autolysis. They are mostly sycrotrophilic bacteria, and mainly of the genus Pseudomonas, which are Gram negative bacteria [11]. As a means for the detection of fish spoilage, therefore, methods to measure bacterial metabolites have been widely employed. The typical substances of the metabolites are TVB-N and TMA-N.

Measurement of TVB-N

Volatile bases include compounds such as ammonium, one of the decomposition products of amino acids in fish muscle, and TMA-N described below. Measurement of TVB-N is one of the methods which has been most widely used for estimating bacterial freshness of fish, and can be divided into the two processes, distillation and the micro-diffusion method. Because of its simple procedure, the latter method is described here.

3.1 Preparation of muscle extract

Two g of fish muscle are ground manually in a mortar with 10 ml of 5% TCA. While being occasionally homogenized, the brei is allowed to stand at room temperature for 30 min. If the temperature is higher than 30°C, the extract is prepared at a lower temperature by using a mortar chilled with ice, for at higher temperatures amide bonds in fish muscle are decomposed by the TCA used to give bases. The muscle homogenate is filtrated with filter paper, and the filtrate is stored in a refrigerator to await analysis.

3.2 Apparatus

Conway's cell made of hard glass as shown in Fig. 5. is used. The dish, 75 mm in diameter and 15-20 mm in depth, with an inner ring 10 mm in height, is commercially available. The cell consists of a dish, a cover and a band made of steel which is used for keeping the inside of the unit airtight. A unit in which complete airtightness is not attained cannot be used.

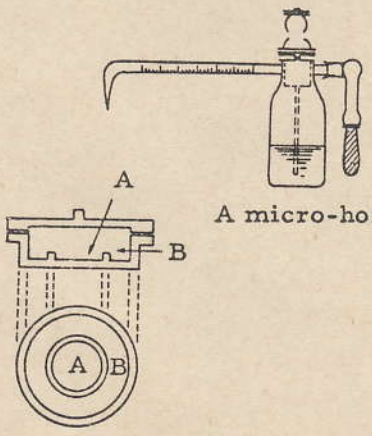

Fig. 5. Apparatus for micro-diffusion analysis.

Three g of tragacanth gum is dissolved in 30 ml of deionized water, mixed with 15 ml of glycerine and 15 ml of 50% saturated potassium bicarbonate, and the mixture is shaken.

Reagent: The following reagents are used:

a) 0.1 g of bromcresol green and 0.2 g of methyl red are dissolved in ethyl alcohol and the volume is brought up to 100 ml with ethyl alcohol.

b) Indicator containing boric acid

Ten g of special grade boric acid, 200 ml of ethyl alcohol and 10 ml of the indicator described above are mixed, and the final volume is brought up to 1 L with deionized water.

c) Saturated Solution of potassium bicarbonate.

3.3 Procedure

A small amount of glue is applied uniformly to the inside of the cover with the finger. One ml portion of the indicator containing boric acid, and the sample solution are pipetted into the inner ring (A) and the outer ring (B), respectively, and a cover is set on the dish. The cover is slid back a little, 1 ml of the saturated potassium bicarbonate is immediately pipetted into the outer ring (B) of the dish, and the cover is promptly placed back on the dish. The dish is fixed with a steel band described above. Being volatile under alkaline conditions, the base in the sample solution is absorbed in the boric acid in the inner ring. Due care must be taken in dropping the alkaline solution. The sample solution and the potassium bicarbonate in the outer ring are mixed by rotating the unit. Similarly, the blank test unit, which contains 1 ml of 5% TCA solution, instead of muscle extract, and other reagents, is rotated 3-4 times,

as mentioned above. The sample solution and the blank test solution thus treated are kept at 37°C for 90 min., in a thermostat, and titrated as follows:

A micro-horizontal burette as shown in Fig. 5 is used for titration. The boric acid in the inner ring is titrated with $\frac{1}{50}$ N hydrochloric acid in order to determine the basic substances which were absorbed in it. The boric acid is titrated by dropping hydrochloric acid, and mixing the solution using a glass rod. This procedure is repeated until the green colour of the indicator disappears. The use of 1 N hydrochloric acid of factor 1,000, which is commercially available, is convenient.

3.4 Calculation

The amount of TVB-N is calculated from the following formula:

TVB-N = (X-b) 0.28 a ratio of dilution of sample

Where X is the amount (ml) of hydrochloric acid used for the titration; b, blank value (ml); 0.28, the amount of ammonium-nitrogen equivalent to 1 ml of $\frac{1}{50}$ N hydrochloric acid, and the ratio of dilution is a ratio of the amount (g) of the sample used to 100 g of muscle.

Two determinations are made for each sample. After it has been confirmed that the two values obtained are similar, their mean value is calculated.

4. METHODS FOR ESTIMATING THE BACTERIAL FRESHNESS USING VALUE OF TMA-N

Measurement of TMA-N

It has long been known that TMA is a substance [12] peculiar to fish and shell fish and distributed in their muscle, viscera etc. After the fish or shell fish has died, the substance is converted to TMA by the action of a reductase in bacteria as follows:

$$(CH_3)NO \quad \text{-----} \quad (CH_3)N$$

It has been reported that, in some fish species, the reductase occurs in their red muscle. Even in these species, however, the enzyme does not occur in their ordinary muscle. Therefore, the measurement of TMA-N has been widely employed as a useful method for detecting spoilage of fish. Three methods, distillation, micro-diffusion and Dyer's colorimetric method using picric acid, are commonly used. The micro-diffusion method is the simplest to manipulate.

4.1 Micro-diffusion method

The procedure is the same as that for TVB-N described above, except that 1 ml of formaldehyde is added to the sample solution in the outer ring of the unit. The formaldehyde solution is added in order to fix any ammonia present in the sample solution; thus only TMA is measured. The amount of TMA-N is calculated in the same way as that of TVB-N.

4.2 Dyer's method

Recently Dyer's method was modified by SHEWAN et al.[13] the modified method is described here.

Formaldehyde is added to a TCA extract of fish muscle in order to fix any ammonia present, and TMA in the

sample solution is extracted with toluene. Colour is developed by TMA reacting with picric acid, TMA being colorimetrically determined. DYER et al. employed potassium bicarbonate as the alkaline agent, which was used in order to liberate TMA from the extract. SHEWAN et al, however, advocate the use of 45% potassium hydroxide as the alkaline agent. The purpose of this modification is to eliminate the effect of DMA, which occurs in fish muscle. According to them, potassium hydroxide gave results corresponding more closely to the Conway method, and 45% potassium hydroxide was superior to the 25% potassium hydroxide used by HASHIMOTO et al.

4.3 Reagents

The following reagents are used: 10% formaldehyde, toluene, 45% potassium hydroxide, 0.02% picric acid-toluene solution, and sodium sulfide anhydrous.

4.4 Procedure

Five ml of TCA extract of fish muscle is taken into a separate funnel, and 1 ml of 10% formaldehyde, 10 ml of toluene, and 3 ml of 45% potassium hydroxide are added to the extract in this order. The mixture is vigorously shaken 60 times by hand and the phases of toluene and water are allowed to stand for a time to ensure complete separation. About 7 ml of toluene phase, i.e. the upper layer, is taken into a test tube, and anhydrous sodium sulfide is added to it little by little (ca. 1 g) until the sodium sulfide added is no longer adhesive even by shaking. Five ml of the toluene thus treated is taken into a test tube and 5 ml of 0.02% picric acid toluene solution are added to it; the color that develops is estimated colorimetrically. Optical density at 410 mμ is measured relative to a blank in which 5% TCA was treated in place of muscle extract. Prior to sample analysis a standard calibration curve must be constructed by treating TMA-hydrochloride solutions of different concentrations as

described above. Fig. 6 shows an example of the calibration curve. However, a calibration curve must be constructed by each investigator.

The content of TMA in sample tissue is calculated from the following formula using the standard curve;

$$TMA = (X-b) \times K \ (mg\%)$$

where X is optical density at 410 mμ of sample solution; b, optical density at 410 mμ of blank test; K, a constant, the amount of TMA per unit of optical density which was obtained from the standard curve.

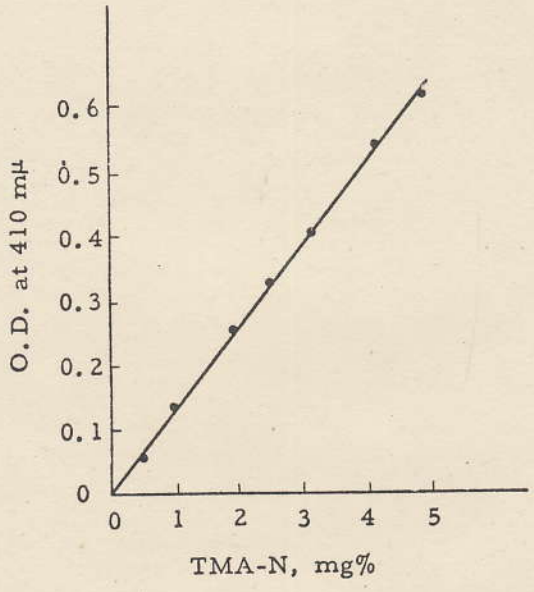

Fig. 6. Standard curve for TMA-N

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