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TRIMETHYLAMINE FORMATION IN FILLETS OF YELLOWFIN TUNA AS AN INDEX OF FRESHNESS: VARIATION WITH STORAGE TIME AND TEMPERATURE

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ABSTRACT

Trimethylamine in fillets of yellowfin tuna was determined qualitatively by paper chromatography and quantitatively by monitoring the absorbance of the trimethylamine picrate salt at 410 mm. The results show that the concentration of trimethylamine increase with storage time and temperature.

INTRODUCTION

Of the 2,000 species of aquatic flora and fauna listed by the Proposed Fishery Industry Development Act of 1972, only a few species are harvested for commerce, trade and home consumption (Rillo, 1973). One of these is the Yellowfin tuna, which is number one in the list of fish and fishery products being exported by the Philippines (BFAR, 1975). In 1975, the quantity of yellowfin tuna caught by commercial vessels was 9.7 million kilograms, 8.1 million kilograms of which were exported in the frozen state.

The taxonomy of tuna is quite conflicting.. It is classified under the family Thunnidae by some ichthyologists (Herre, 1953), and under the family Scombridae by others (Perlmutter, 1961). The yellowfin tuna, scientifically known as *Thunnus albacares*, is locally known as *tuna*, *tambakol*, *tulingan*, *bareles*, *buyo*,

badlawan, *balarito*, and *panit*. It has a robust, compact, rounded but streamlined, bullet-shaped body completely covered with scales. It has a dark back and silvery sides without the stripes, bars and dark spots which other finlet fishes like the bonitos possess.

A mature yellowfin tuna can be identified without trouble because no other tuna has unusually long second dorsal and anal fins of a brilliant cadmium yellow color, characteristics which the young fish does not possess. The first and pelvic fins are tinged with yellow and the finlets are bright yellow with narrow, dusky edges. Irregular, spotted white markings which fade quickly can be seen on the lower body of the live fish. The pectoral fins are as long or slightly longer than the head. The first dorsal fins have 13 spines, the second dorsal fins have 14 rays followed by 8-11 separate dorsal finlets and 8-10 anal finlets. The yellowfin has a gill raker count of 36-22. At sexual maturity, a yellowfin tuna may range in size from 2-4 ft.

Although the tuna is a temperate fish, its spawning area includes the entire equatorial Pacific. The season extends throughout the year, the low months being November, December and January and the high months being April, May and June. In the Pacific, commercial catches range in size from 6-200 lbs. with most of them being under 40 lbs. (Migdalski, 1953).

Because the onset of deteriorative changes occurs rapidly, fish is probably the most perishable of flesh foods, its marketability affected to a great extent by the degree of freshness. The assessment of fish freshness thus assumes some importance in the industry.

Despite numerous attempts, the search for chemical methods of determining fish freshness that are universally applicable and correlate well with organoleptic freshness continues. The methods that have been developed invariably consists in the measurement of

either a specific chemical component such as skatole, indole and trimethylamine which form at the onset of spoilage, or a group of related substances such as total volatile bases of acids (Stansby, 1976).

Of the many single chemical tests for fish freshness, that which involves the determination of trimethylamine (TMA) is most widely used. So far, three techniques for the quantitation of trimethylamine formation have been developed and modified: microdiffusion, gas-liquid chromatography (GLC) and spectrophotometry. The first method is reported to give reproducible results if properly carried out but is believed to be rather inconvenient and inaccurate. The second, GLC, is quite accurate and sensitive, capable of measuring even minute concentrations of trimethylamine. The third method uses readily available reagents and is quite conveniently performed. Basically, it involves the volatilization of trimethylamine by an alkali from the acid fish extract into a toluene phase to which picric acid has been added. This results in the formation of the picrate salt of trimethylamine, and the ionization of which is then measured spectrophotometrically. First formulated by Dyer (1939), the method has been modified successively (Castell, et al. 1968, 1970; Murray and Gibson, 1972), the accuracy of which equals GLC and the results of which agree excellently with the modified picrate method.

This paper examines the applicability of the modified picrate method in the determination of trimethylamine in fillets of yellowfin tuna, the Philippines' primary fish export. Trimethylamine formation is correlated with storage time and temperature and the results compared with standards of freshness for fillets from another fish species.

MATERIALS AND METHODS

Sample preparation

Yellowfin tuna of the same catch were obtained from

one and the same fish vendor in Quiapo Market Manila. Their past history is unknown but they can be presumed to have been previously stored in ice for 1-2 days, granting that standard storage procedures were followed. The fishes ranged in weight from 3-5 kg and measured 1.5 - 2 ft. long. They exhibited all the characteristics described for fresh fish and were taken to be at the rigor mortis stage. After scaling, gutting, and washing with tap water, they were cut into 1/2 - 3/4 inch, thick fillets. Fillets from fishes of the same catch were randomly mixed, individually wrapped in polyethylene bags and and grouped into three batches. One batch was stored at room temperature (28 to 32°C): the second was refrigerated (8 to 12°C). Fillet samples from each batch were then withdrawn from storage at varying time intervals from TMA analysis.

Extraction of TMA

Several fish fillets from each batch were withdrawn from storage. Frozen fillets were allowed to thaw but not completely defrosted to prevent any bacterial deterioration of the defrosted muscle. The skin and bones were separated from the flesh and discarded. The muscles were then minced and mixed. Around 100 g of minced fish muscle was weighed and homogenized with 300 ml 5% trichloroacetic acid (TCA) solution for 2-3 min. in a Virtis "23" homogenizer. The resulting slurry was filtered through Whatman #4 filter paper until a clear extract was obtained.

Qualitative determination of TMA by paper chromatography

To the extract obtained above was added 75 ml of 50% formalin followed by 150 ml toluene and 200 ml 45% KOH solution. The mixture was shaken vigorously and the toluene layer was collected and washed with 100 ml 0.6 N HCL to remove the amines. The aqueous layer was collected, over-dried under vacuum to around 5.0 ml and analyzed by paper chromatography against a TMA-HCL standard with n-butanol: acetic acid: water (4:1:5) as irrigant. The chromatograms were dried and developed with I_2 vapor for the detection of tertiary amines and

with 1% muhydrin solution for the detection of primary and secondary amines.

Quantitative determination of TMA

To 20 ml of acid fish extract was added 5 ml of 50% formalin. Depending on the expected TMA-Nitrogen (TMA-N) content of the extract, a 0.2-4. ml sample of the extract was taken and made up to 4.0 ml with distilled H_2O in a test tube. Formalin (1.0 ml of a 50% solution), toluene (10 ml) and KOH (3.0 ml of a 45% solution) were then added in succession. The test tube was stoppered and shaken vigorously about 50 times and the phases were allowed to separate for 10 minutes. The toluene layer was collected, dried over about 0.1 g anhydrous Na_2SO_4, and 5.0 ml transfered into another test tube containing 5.0 ml picric acid. The test tube was swirled gently. The absorbance of the resulting solution was read on a Bausch and Lomb Spectronic 20 at 410 nm against a reference blank consisting of 4.0 ml distilled H_2O treated in the manner described above.

Preparation of standard curve

Several aliquot portions of freshly standardized TMA-HCL solution were taken, each of which was made up to 4.0 ml with distilled H_2O and treated according to the procedure just described. The absorbance of each was measured and the total weight of nitrogen in each aliquot (TMA-N) was calculated as mg TMA-N/4 ml solution and plotted against absorbance. The slope of the line was calculated using the method of least squares.

Calculations

TMA-N/100 g fish was calculated in the following manner:

$$\text{Total mg TMA-N standard} = \frac{\text{Absorbance}}{\text{slope of std. curve}} - \frac{\text{absorbance}}{21.40}$$

$$\text{Total mg TMA-N aliquot} = \frac{\text{Total mg TMA-N standard}}{\text{ml aliquot}} \times 25 \text{ ml}$$

$$\text{Total mg TMA-N TCA extract} = \frac{\text{Total mg TMA-N aliquot}}{20 \text{ ml}} \times 300 \text{ ml}$$

$$\text{mg TMA-N/100 g fish} = \frac{\text{Total mg TMA-N TCA extract}}{\text{weight of fish}} \times 100$$

RESULTS AND DISCUSSION

The paper chromatograms of the fish extracts when developed with iodine vapor invariably showed only one spot with an R_f value that closely matches that of the TMA-HCL standard. No spots developed when the chromatograms were sprayed with a 1% ninhydrin solution, a reagent specific for primary and secondary amines. These results indicate that only the tertiary amine, trimethylamine, was liberated from the trichloroacetic acid extract into the toluene layer after the addition of KOH, the alkalising agent; and that it is the colored trimethylamine picrate salt that is formed on the subsequent addition of picric acid to the toluene layer.

A standard curve of absorbance as standard TMA-N concentration expressed as mg TMA-N/4 ml was prepared. Altogether, 11 aliquot portions were each made up to the volume of 4 ml. The concentrations used and their corresponding absorbances are given in Table 1. Application of the method of least squares gave a straight line with a slope of 21.40. this slope together with the observed absorbance was used in the computation of the TMA-N concentration in each of the fish extracts as detailed in the preceding section.

The fish fillets were stored under three sets of conditions: at room temperature, under refrigeration and under frozen storage. The formation of trimethylamine under each condition was monitored at different time intervals. The results are summarized in Tables 2,3 and 4. In each case, day 0 denotes the day sample batches were prepared prior to storage. It will be noted that the starting TMA-N concentrations are not uniform

Table 1. Concentration and absorbance values used in the preparation of the standard curve.

Standard Solution	Concentration in mg TMA-N/4 ml	Absorbance at 410 mm
1	0.0047	0.065
2	0.0095	0.152
3	0.0142	0.234
4	0.0189	0.375
5	0.0190	0.390
6	0.0236	0.451
7	0.0284	0.560
8	0.0331	0.600
9	0.0378	0.760
10	0.0380	0.755
11	0.0570	1.200

for all cases. This suggests variations in the initial conditions of freshness of the fish samples used in the experiments. This situation arises because the fish used for each set of experiments were purchased on different days.

The results clearly show that on day 0, even before

sample work-up, TMA formation had already occurred. This indicates that deteriorative changes in fish take place quite readily, possibly setting in as soon as the fish is taken out of the water. Deterioration progresses with time until fish spoilage occurs, the rate being strongly dependent on the conditions of storage. It is evident from the results obtained that trimethylamine accumulates as spoilage proceeds, its rate of formation being a function of storage temperature. The data in Table 2 show that the concentration of TMA-N in the fillets almost quadruples (from 0.678 to 2.48 mg/100 g fish), after only a quarter of a day's storage at room temperature increasing, but not as abruptly to 17.2 mg after 2 days. A similar rate of increase in TMA-N concentration is not observed in fillets kept under refrigeration, the initial amount just nearly doubling, but only after 5 days' storage (Table 3). Freezing effectively deters trimethylamine formation, the concentration being nearly constant for 12 days, doubling on or a few days before day 25 (Table 4). These observations are graphically summarized in Figure 1 which shows a plot of TMA-N concentration vs storage time. Any apparently anomalous decrease in TMA concentration with storage time may be attributable to experimental error possibly arising from the loss of small amounts of the extremely volatile amine during work-up.

The progressive accumulation of trimethylamine parallels the sensory observations made of the fillets for the entire duration of the storage. This test has proved to be useful particularly with certain low-oil content species such as cod and haddock. For haddock fillets, for example, Castell and Triggs (1955) have set the following standards of freshness:

0-1 mg TMA-N/100 g fish = fresh

1-5 mg TMA-N/100 g fish = doubtful or spoiling

5 mg TMA-N/100 g fish = spoiled

The same authors take note of the alternating standards

Table 2. Quantitative determination of TMA in fillets of yellowfin tuna stored at room temperature (28-32°C).

No. of Days	Trial No.	Weight fish (g)	Absorbance at 410 nm	mg TMA-N 100 g fish	Average	Sensory Evaluation
0	1	134.6507	0.215	0.699	0.678	Firm flesh, no slime but seaweedy fishy odor
	2	118.3960	0.178	0.658		
1/4	1	103.2689	0.560	2.38	2.48	Firm flesh slightly ammoniacal odor
	2	122.1500	0.720	2.58		
1	1	100.5528	0.840	7.32	6.18	Soft flesh, stale and very ammoniacal odor
	2	97.6110	0.560	5.03		
1 1/3	1	107.5440	0.600	14.00	15.3	Putrid
	2	108.8478	0.718	16.50		
2	1	98.2557	0.330	19.60	17.2	Putrid, flesh retains finger indentations, slime layer formed
	1	104.8883	0.178	14.90		

Table 3. Quantitative determination of TMA in fillets of yellowfin tuna stored under refrigeration (8-12°C).

No. of Days	Trial No.	Weight Fish (g)	Absorbance at 410 nm	mg TMA-N 100 g fish	Average	Sensory Evaluation
0	1	99.4543	0.93	6.55	7.14	Ammoniacal
	2	106.9105	1.18	7.73		
1	1	111.7960	0.598	6.25	5.72	Ammoniacal
	2	110.7380	0.492	5.19		
2	1	118.8513	0.710	6.98	6.88	Very Ammoniacal
	2	123.8646	0.720	6.79		
5	1	89.8969	0.412	11.5	11.8	Soft flesh, slightly putrid, slime layer form
	2	102.5247	0.500	12.2		

Table 4. Quantitative determination of TMA in fillets of yellowfin tuna under frozen storage (-5 to -10°C).

No. of Days	Trial No.	Weight Fish (g)	Absorbance at 410 nm	mg TMA-N 100 g fish	Average	Sensory Evaluation
0	1	131.3754	0.232	0.884	0.865	
	2	117.1091	0.198	0.846		
2	1	106.2388	0.202	0.952	0.972	Seaweedy fish odor, no slime for entire duration of storage
	2	113.3815	0.225	0.993		
12	1	101.6300	0.178	0.877	0.822	
	2	98.9960	0.152	0.768		
25	1	100.5718	0.305	1.520	1.72	
	2	90.0718	0.295	1.910		

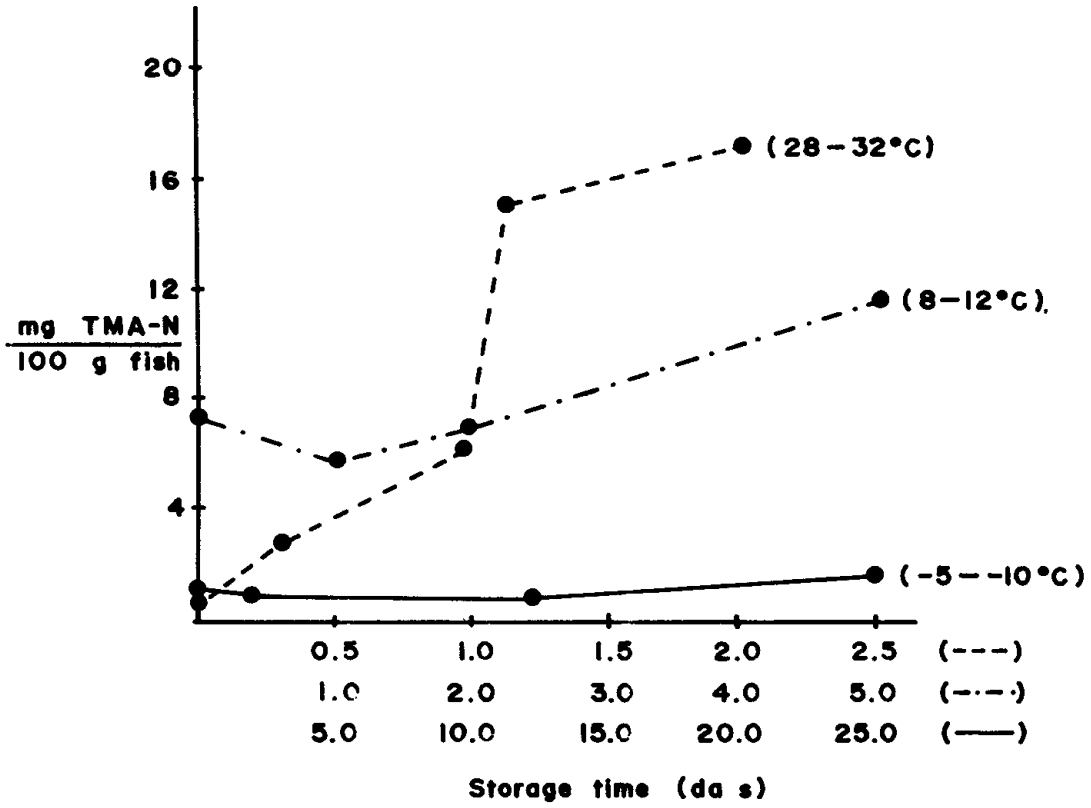

Figure 1. Concentration of TMA in fillets of yellowfin tuna with respect to storage time and temperature.

set by other workers, e.g., fish containing 0.6-5 mg TMA-N/100 g fish is considered fresh by some while 4-6 mg TMA-N/100 g is considered by others to be the range where initial spoilage occurs. What these differences point to is that freshness standards might differ for each species, possibly because of inherent variations in spoilage patterns. This notwithstanding, the standards set by Castell and Triggs appear to be at least roughly applicable to the fillets of yellowfin tuna used in this study, particularly if the sensory observations are correlated with TMA-N concentration. Thus, samples in which TMA-N concentration was found to exceed 5 mg/100 g fish invariably gave off highly ammoniacal to putrid odors and showed other indications of near spoilage. Conversely, those with TMA-N concentrations below 5 mg. emitted only normal fish odors. It becomes quite obvious from this correlation that all the fish samples used in obtaining the data given in Table 3 have suffered a significant loss in freshness. The process of spoilage is somehow retarded by refrigerating the fillets.

Spoilage of fish has been attributed to either bacterial activity or autolysis which is the spontaneous disintegration of cells or tissues by the action of their own autogenous enzymes (Frazier, 1967). Spoilage gives rise to numerous, mostly volatile chemical products like amines, sulfides and many others which altogether are thought to be responsible for the foul smell of stale or rotting fish.

It is thought that trimethylamine is a product of the bacterial reduction of trimethylamine oxide (TMAO), a compound known to be present in large amounts in marine fishes (Rillo, 1973; Vaisey, 1956). Two equally plausible mechanisms for the reduction have been proposed, one being enzyme-mediated and the other being a non-enzymic, catalytic process. This study shows that bacterial activity, and consequently TMA formation is most effectively inhibited by freezing.

Trimethylamine is a foul-smelling volatile compound and thus is expected to contribute to the spoilage

odors of fish. Its contribution is however dependent on pH. On the acidic side of neutrality--pH 6.7 or below, its odor is not perceptible. As pH increases, however, the characteristic ammoniacal odor becomes increasingly strong, a phenomenon which is chemically explainable: at low pH, the amine is protonated and thus tied down as a non-volatile non-smelling salt. This condition obtains before or during the early stages of spoilage where pH is low; an accumulation of TMA in the fish muscles at this point contributes little to the fish odor. However as pH approaches neutrality, becoming increasingly basic as spoilage progesses, the accumulated TMA is freed (deprotonated, on the molecular level) and becomes an important contributor to spoilage odors--an observation borne out, although to a limited extent, by the results of this investigation.

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