12. BIOCHEMICAL QUALITY ASSESSMENT OF FISH AND FISHERY PRODUCTS

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The seafood is preferred in fresh form and being high in water content needs extra care for preservation. Therefore, fish is simply chilled to extend its shelf life or frozen or converted into different products for consumption. During various stages of post harvest handling fish is exposed to various hazards. The lack of control leads to 'quality deterioration' affecting the quality of the product. The quality of the food in general is a concern from the public health point of view as well.

The term 'quality' means "all those attributes which consciously or unconsciously the fish eater or buyer considers should be present" and which will embrace intrinsic composition, degree of spoilage, damage, deterioration during processing, storage, distribution, sale and presentation to the consumer, hazards to health, satisfaction on buying and eating, aesthetic consideration, yield and profitability to the producer and middle men. The quality of the fish and fishery products is one of the main indices in the activity of any processor. According to ISO, Quality is defined as the totality of features and characteristics of a product or service that bears its ability to satisfy stated or implied needs

Quality of a food material depends on several factors; both intrinsic and extrinsic. The intrinsic factors could be related to the fish species while extrinsic factors are environment related issue which contributes to the contamination with pathogen leading to food borne infections on consumption. Therefore preventing the onset of spoilage and preventing contamination from external sources are to be checked effectively in order to make consumer acceptable fish and shell fish and to avoid the food safety issues.

Seafood differs from other types of food because of its very nature. Fish contains more than 70% water which makes them more prone for spoilage if appropriate measures are not taken. At the time of harvesting fish contains the bacteria and other contaminants naturally present in the ecosystem. Once fish is harvested the new microbes are added from the environment and most of them are pathogenic to consumers. The post mortem changes taking place in the fish provides a suitable environment to the bacteria to multiply, if not controlled by good manufacturing practices. Being perishable, the quality of seafood deteriorates fast resulting in food borne infections on consumption of spoiled fish.

Total Volatile Base Nitrogen (TVBN)

TVBN measures the amount of volatile bases formed from solubilised nitrogen derivatives. It is a measure of decomposition of proteins. TVB-N in fish is mainly composed of ammonia and primary, secondary and tertiary amines. Bacterial catabolism of aminoacids in fish muscle results in the accumulation of ammonia and other volatile bases. Ammonia and primary amines are bound by formalin, therefore this fraction is called the formalin bound nitrogen (FBN). The trimethyl amine (TMA) represents the fraction, which is not bound by formalin. The TVB-N value is used as an index of quality for deciding the state of freshness of fish (along with TMA). A level of 35-40 mg 1VB-N /100g of fish muscle is usually regarded as the limit of acceptability, beyond which the fish can be regarded as spoiled. Generally, there is an increasing trend in TVBN values as the fish gets spoiled.

Principle:-

Volatile basic nitrogen content is mainly constituted by Ammonia. When TCA extract of the sample is treated with saturated Sodium carbonate, Ammonia will liberated which is then trapped in N/100 H2SO4 in the Conway dish. The excess acid in inner chamber is back titrated with N/100 NaOH. The calculated value gives the TVBN of the sample.

Reagents:-

1. N/100 H2SO4 & N/100
2. Saturated Sodium carbonate solution.
3. Mixed indicator.

Procedure:-

Preparation of TCA extract:-

Weigh 10 gms. of fresh muscle sample into a mortar. Add 10 ml. of 20% TCA and ground well. Filter, using Whatman's filter paper No.1 in 50 ml. standard flask. Repeat the extraction with 1% TCA & filter. Collect the washings and make the volume 50ml.

Analysis

1. TVBN is estimated by the micro diffusion Conway method. In the inner chamber of the conway unit place 1 ml. of N/100 H2SO4 and in the outer chamber, 1ml of TCA extract of the sample.
2. Cover the Conway dish with the glass cover smeared with petroleum jelly to give air-tight contact along the outer contact-ring of the unit.
3. Keep just open to draw 1ml of saturated Na_2CO_3 in the outer chamber of the unit, then closed the glass plate to air tight.

4. Mix gently by lateral circular movement.
5. Allow the unit undisturbed to stand overnight (or at 37^0C incubation for 2 hrs.).
6. The acid in the inner chamber is titrated against N/100 NaOH using two drops of mixed indicator, the indicator changing from red to green at the end point- (B).
7. Titrate a reagent blank also by taking standard acid at the central compartment- (A)
8. Perform the assay in duplicate for each sample.

Calculation:-

Value(A-B) is the vol. of N/100 acid used up by volatile base.

(1ml of N/100 acid = 0.14 mg of Nitrogen).

$$\text{TVBN mg \%} = \frac{(A-B) \times 0.14 \times 50 \times 100}{\text{Wt. of sample}}$$

Tri-methyl Amine (TMA)

Trimethylamine (TMA) is used to assess the freshness in marine fish. TMA is derived from trimethylamineoxide (TMAO) which is critical for osmo regulation in marine fish. TMAO is a tasteless non-protein nitrogen compound whose content varies with the season, size and age of fish. During spoilage, TMAO is reduced by enzymes to TMA. The concentration of amines in fish tissues is both time and temperature dependent and is related to the deterioration of fish. The determination of TMA as an indicator of freshness (actually of decay) has been a useful criterion for evaluating the quality of fish. TMA-N between 10-15 mg / 100g muscle is considered as the limit of acceptability for round, whole chilled fish. This index is not suitable for freshwater fish and heat treated fish products.

Principle:-

Tri-methyl amine is a non-protein nitrogenous volatile compound. The quantity of TMA formed is depends primarly upon the concentration of its precursor, TMA-O in the fish muscle. TMAO is reduced during spoilage to TMA. The TMA is often determined by the Conway micro-diffusion technique.

Procedure:-

The same procedure is adopted as TVBN, except that 0.5 ml Neutralized Formalin (prepared by shaking formaldehyde with magnessium carbonate and filtering through Whatman 40 no. filter paper) is added to the outer chamber and swirled to mix before adding Sat. Sod. Carbonate. Formaldehyde is added to fix all the bases except TMA.

Calculation:-

$$\text{TMA mg \%} = \frac{(A - B) \times 0.14}{\text{Wt. Of sample}} \times \frac{50}{1} \times 100$$

FREE FATTY ACIDS (FFA)

The deterioration of lipids has always been of primary concern to fishery technologists. Degradation of lipids falls into two categories: oxidation which leads to of odours and flavours and hydrolysis which splits off free fatty acids. FFA gives a measure of hydrolytic rancidity. Fish muscle contains lipase, which is able to catalyse the hydrolysis of short chain triglycerides. Free fatty acids are suspected of deriving primarily from phospholipids, as the latter disappear with time of storage which can be affected by the action of bacteria, enzymes or non-enzymic catalysis. During spoilage, the amount of free fatty acids increases, which can be measured by reacting with alkali and is expressed as %oleic acid.

Principle:-

Fat spoilage can be assessed by estimating the free fatty acids (FFA) and peroxide value (PV) on a common chloroform extract. The FFA in the sample extract is diluted with alcohol and neutralized by titration with sodium hydroxide. The FFA are expressed as % Oleic acid on the extracted fat.

Reagents:-

1. Chloroform.
2. Anhyd. Sod. Sulphate
3. Neutral Ethyl alcohol (Neutralised with NaOH)
4. Phenolphathalein indicator.
5. 0.01N NaOH

Procedure:-

1. Take about 10 gm. of fresh muscle sample in a mortar & grind well with anhyd. Na_2SO_4untill all water is removed.
2. Transfer this into 250 ml. Iodine flask. Add to this 100 ml Chloroform & keep 30min. in dark .
3. Filter the chloroform extract using filter paper and make the vol. 100ml with chloroform.
4. Weigh 2 nos. of 50 ml conical flasks. Add 20 ml of chloroform extract in each conical flask.
5. Evaporate the extract in water bath & then dry them for 3 hrs in Hot air oven at 100^0C.
6. Cool and weigh the conical flask. This will give the fat content (M) in 20 ml of chloroform extract.
7. Add 10 ml. of warm, neutral alcohol & dissolve the fat.
8. Add 1 drop of phenolphthalein indicator & titrate against 0.01 N NaOH.

Calculation:-

FFA (as oleic acid on extracted fat), % (m/m) :

$$\text{FFA \%} = \frac{V \times N \times 28.2}{M} \times \frac{100}{20} \times \frac{1}{\text{Wt. of sample}}$$

Where: M = Fat content in 20 ml.of chloroform extract
V = Vol. in ml. of NaOH
N = Normality of NaOH
28.2 = milliequivalent weight of oleic acid (include factor of 100 for %).

Peroxide Value (PV)

The highly unsaturated fatty acids found in fish lipids are very susceptible to oxidation. The primary oxidation products are the lipid hydroperoxides. These compounds can be detected by chemical methods, generally by making use of their oxidation potential to oxidize iodide to iodine or to oxidize iron(II) to iron(III). The concentration of the hydroperoxides may be determined by titrimetric or by spectrophotometric methods, giving the peroxide value (PV) as

milliequivalents (mEq) peroxide per 1 kg of fat extracted from the fish. The most common method is based on iodometric titration which measures the iodine produced from potassium iodide (KI) by the peroxide present in fat. PV is a good guide to assess the quality of fat. Fresh oil should have PV 1 mg.oxygen/kg. On storage it may increase to 10 mg/kg.

Principle:-

During oxidation of fat peroxide is formed. Peroxide value gives measure of oxidative rancidity. The peroxide value is a measure of peroxides contained in the oil.The peroxide value is usually determined volumetrically by method which depends on the reaction of potassium iodide in acid solution with the peroxide oxygen followed by titration of the liberated iodine with Sodium thiosulphate solution.

Reagents:-

1. Glacial acetic acid.
2. 1 % starch solution.
3. N/100 Sod. Thiosulphate solution
4. Pot. Iodide.

Procedure:

1. In a 250 ml. Iodine flask, take 20 ml. of chloroform extract (prepared in FFA)
2. Add about 30 ml of glacial acetic acid and 1 gm of KI &keep in dark for about 30 min. with occasionally swirling.
3. Take out and add 1 cc. 1% starch solution.
4. Titrate liberated iodine with N/100 Sod. Thiosulphate solution.

Calculation:-

$$\textbf{PV}\% = \frac{V \times N}{M} \times \frac{100}{20} \times \frac{100}{\text{Wt. of sample}}$$

V = ml of Sod. Thiosulphate solution used
N = Normality of Sod. Thiosulphate
M = Fat content in 20 ml chloroform extract

Thiobarbituric Acid (TBA)

TBA index is the most used indicator for advanced lipid oxidation. TBA measures the malonaldehyde produced during fat oxidation

Principle:-

Oxidised lipids are formed as fats become rancid. Thiobarbituricacid will react with these fatty lipids to form a red – colored complex which can be determined spectrophotometrically. Malonaldehyde is one of the end products of oxidative rancidity and is believed to be involved in the reaction with TBA. Therefore the TBA value is expressed as mg malonaldehyde per Kg sample. The TBA test is applicable to fatty foods (e.g. meat) as well as fats and oils.

Reagents:-

- TBA reagent:- 0.2883gm in 100ml of 90% glacial acetic acid.

Procedure:-

1. Weigh 10 g of prepared sample in a round bottom flask and add a glass bead and 100ml solution (3ml 2:1 HCl + 97 ml DW = 100ml) & mix.
2. Collect 50 ml distillate by steam distillation.
3. Pipette 5ml of distillate into a glass stoppered tube, add 5mlTBA reagent, stopper, shake and heat in boiling water bath for 40min.
4. Prepare a blank similarly using 5ml DW with 5ml reagent.
5. Then cool the tubes in water for 10min. and measure the absorbance (A) against the blank at 538nm.

Calculation:-

$$\text{TBA no. (as mg malonaldehyde / Kg sample)} = \frac{7.8 \times A}{\text{Wt. of sample}} \times \frac{50}{5}$$

$$= 7.8 \times A$$

7.8 is the TBA standard factor.

Free Alpha – amino Acids

In Crustaceans, the free alpha – amino acid is upto 40% of the NPN and in teleosts is only 6%. The attractive flavour invariably present in prawns and other crustaceans is attributable to their comparatively higher contents of free amino acids. The comparatively quicker rates of spoilage occurring in invertebrates than in teleosts may be attributed to the presence of large quantities of free amino – acids in their muscles.

Principle:-

The method depends on the formation of soluble copper compounds through the complex reaction between the amino acids and excess copper in the form of $CuSO_4$. The amount of copper taken into solution by amino acids or similar material is determined iodometrically.(Pope and Stevens method).

Reagents:-

1) Cupric Chloride - $CuCl_2.2H_2O$ - 27.3gms/lit.
2) Tri Sodium Phosphate - Na_3PO_4. 12 H_2O - 64.5gm.
3) Borate Buffer - $Na_2B_4O_7$.10 H_2O - 57.2gms in 1.5 lit. of water, add 100ml 1N HCl. Dilute to 2 liters with water.
4) Cupric – Phosphate Suspension:- 1 vol. of $CuCl_2$ + 2 vol. of Na_3PO_4+ 2 vol. of Borate buffer and mix.
5) Thymolphthalein indicator
6) Std. N/100 Sod. Thiosulphate solution
7) Starch solution:- 0.5% (preapare fresh)
8) 1% NaOH soln.
9) Potassium Iodide (KI)
10) Glacial acetic acid

Procedure :-

1) Pipette out 25ml of TCA extract of the sample (as prepared for TVBN) in 100ml std. Flask)
2) Add 2 drops of Thymolphthalein indicator. Neutralise this TCA acid with 1% NaOH soln. till light blue color appears.
3) Then add 35ml Cupric – Phosphate Suspention. Make the vol. upto 100ml with DW. Mix it properly and filter.
4) Pipette out 20ml of filtrate in 150ml conical flask. Add about 1gm of KI and 15ml of glacial acetic acid.

5) Titrate this rapidly with N/100 $Na_2S_2O_3$ till light yellow color.
6) Then add 1ml of starch soln. again titrate with N/100 $Na_2S_2O_3$ till blue color gets disappear. Note down the reading.

Calculation :- 1ml of 0.01 N $Na_2S_2O_3$ = 0.28 mgm of alpha amino nitrogen.

Alpha amino nitrogen (mg%)= $\frac{0.28 \times V}{\text{Wt of sample}} \times \frac{50}{25} \times \frac{100}{20} \times 100$

V = Titre value.

Non – Protein Nitrogenous Compounds (NPN)

NPN compounds generally encountered in fish muscle comprise Ammonia, TMA-bases, Guanidine and Imidazole derivatives and miscellaneous substances like Urea, Amino-acids, Purines and Pyrimidines.

NPN can be determined by the micro kjeldahl distillation method.

Procedure :-

1) Take 10ml of TCA extract in the Kjeldahldigetion flask.
2) Add a pinch of digestive mixture and 10ml of conc. H_2SO_4.
3) Digest the mixture until the contents are clear.
4) Cool and dilute to 50ml ammonia free distilled water.
5) Take 10ml for distillation.
6) Titrate the distillate with N/100 H_2SO_4.

Calculation :-

(1ml of N/100 acid = 0.14 mg of Nitrogen)

$$\% \text{ of NPN} = \frac{0.14 \times V}{\text{Wt of sample}} \times \frac{50}{10} \times \frac{50}{10} \times 100$$

Determination of Indole in Shrimp

Indole is used as an index of decomposition. Indole formation in shrimp is supposed to be due the action of bacteria such as Proteus morganii, *E. coli*. on shrimp protein. The amount of indole produced is proportional to the extent of decomposition. Shrimp can decompose in

the absence of indole-producing organisms. Therefore the presence of indole in shrimp definitely decomposition but the absence cannot ensure that the product is free from spoilage.

Indole is extracted with light petroleum from trichloroacetic acid – precipitated shrimp muscle. The extracted indole, soluble in light petroleum, is reacted and re-extracted with Ehrlich's reagent. Indole in the form of a rose indole complex can be determined spectrophotometrically.

Reagents:-

1) Trichloroacetic acid (TCA) :- 6gm of TCA dissolve in 100ml DW .
2) Petroleum ether, Boiling point 40 – 60^0C.
3) **Ehrlich's reagent**:- Dissolve 9gm para-dimethylaminobenzaldehyde in 45ml conc. HCl acid in 250ml volumetric flask and dilute to volume with ethanol.
4) Std. Indolesolutions :- Accurately prepare stock solution of 10mg indole in 100ml light petroleum. Use 1:10 dilution (with petroleum) working solution. Refrigerate indole solutions.

Procedure:-

1) Homogenise 40gm shrimp with 80ml ice-cold TCA solution in a warring blender one min. Add 80ml ice-cold light petroleum and blend for one min.
2) Transfer homogenate to 250ml centrifuge bottle and cenrifuge 10min. at 10,000 rpm. Filter supernate through whatman no. 1 paper under suction.
3) Transfer filtrate to 250ml separatory funnel. After the two layers have separated, transfer acid layer (lower) to second 250ml separatory funnel.
4) Wash TCA- denatured protein precipitate separated by centrifugation with 40ml light petroleum and filter as described above.
5) Transfer filtrate to second 250ml separatory funnel already containing TCA layer from first extraction.
6) Shake 1 min. and let 2 layers separate. Transfer lower acid layer to third separatory funnel and extract for third time with 40ml light petroleum .
7) Combine all light petroleum extracts into 1 separatory funnel.
8) Extract indole with exactly 5ml freshly prepared Ehrlich's reagent by vigorously shaking 1 min.
9) The rose indole complex formed is quantitatively transferred to Ehrlich's reagent layer.
10) When layers have separated, transfer lower layer to 1 cm path cell and read at 570nm against reagent blank solution.
11) Prepare standard curve as follows.
12) Accurately measured volumes from 0.5 to 4ml (5 to 40 microgm) stock indole solution (working solution) into 80ml TCA in separatory funnel.

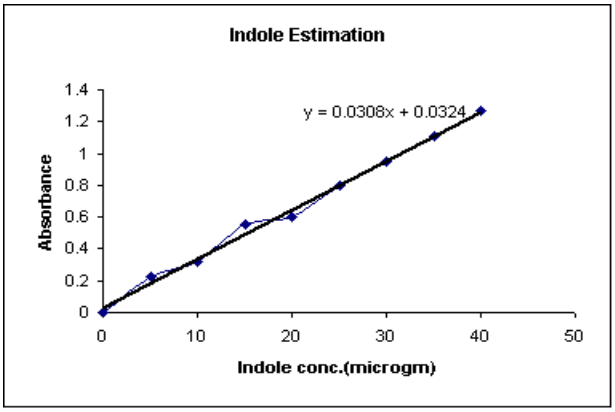
13) Extract indole by procedures described above and construct standard curve.
14) Rose indole complex from indole standard and from TCA- extracted shrimp is stable up to 4 hour.

Calculation:-

With the help of the standard curve the amount of indole present in 40gm shrimp can be determined. Indole content is usually expressed as the amount of indole in microgram per 100gm shrimp muscle. 250 microgram per kilogram is the limit.

Standard Graph

Conc.	Absorbance
0	0
5	0.23
10	0.32
15	0.55
20	0.6
25	0.8
30	0.95
35	1.11
40	1.27



Sulphur dioxide Estimation by Modified Monier – Williams Apparatus

Reagents Required:-

1) 3% Hydrogen peroxide
2) 0.25% Methyl red indicator
3) 0.1N Pot. Permangnate
4) 1:2 Hydrochloric acid
5) 0.1N Sod. Hyroxide soln.

Procedure :-

1) Assemble as shown in the figure.
2) Take 40ml of neutralized Hydrogen peroxide soln. in each U- tubes.
3) Place 100gm of sample (homogenized) in 3 necked flasks.
4) Add 75ml of 1:2 HCl
5) Add 325ml DW.

6) Start water flow and gas flow.
7) Then switch on heater. Reflux the sample for 30 min.
8) Sulphur dioxide in the sample gets entrapped in hydrogen peroxide in U-tubes
9) Remove and transfer into a flask and titrate against 0.1N NaOH.

Calculation:- 1 ml of 0.1N NaOH = 3.203 mg of SO_2