METHODS FOR DETERMINATION OF PRESERVATIVE CHEMICALS (FORMALDEHYDE) IN MARINE / FRESH FISHES

A DISSERTATION REPORT

Submitted in partial fulfillment

Of

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I. INTRODUCTION

1. General Introduction

The most of the people like a fishes to eat and they included in their diet. [1]Also fish is good source of protein[2] [3],also contain the good amount of fat i.e. omega 3,6 and 9, vitamins B and Vitamin D and traces amount of minerals such as Ca , P , Zn , I , mg , K etc. By including fishes in diet can help to reduce the blood pressure and lower the risk of getting a heart attack, and help in developing the brain. [4],[5]

In Bangladesh fisheries are more important as it created a lots of job opportunities, supplying the nutrients, domestic increment and gaining a lot of foreign exchange, which directly or indirectly contributes to the food Security of the Country. Not only fishes but other seafood significantly contributing the nutrients in our daily diet. In Bangladesh reported that the fishes which are imported may contain a more concentration of formaldehyde because it required a more time as it going through a different steps of a supply chain before fishes reaches to the market retailer. Consumer are worried about the presence of the formaldehyde in fishes and other food, so the determination of formaldehyde is needed.

Indian Mackerel are also famous for the good quality of their protein and it is mostly oily. In commercial fish farming mackerel is most important part. Fishery contributes an employment generation so lot of people are earning and poverty getting lower, so there is economic development. As there are many establishment of many aqua culture of fishes so there is shortage of fishes for the growing population. It fulfilling their demand. [6]

There are no proper regulatory control, poor infrastructure for transportation , refrigeration, storage area and day by day highly demand of customer for the fishes, had led to fraudulent practices going on to keep the fishes fresh for a longer time i.e. shelf life is increasing of fishes.[7] So to keep the products fresh these getting contaminated by adding a number of harmful chemicals such as dyes, insecticides, formalin and banned antibiotics.[8]

Moreover 60% of fish are preserved which most of them are consumed are remain fresh. A moreless 40% of the total fish is preserved by adding ice pieces and transported to the inland areas whereas 60% of the total fish is preserved by adding salt cured or dried in the sun or smoked or pickled. [9]

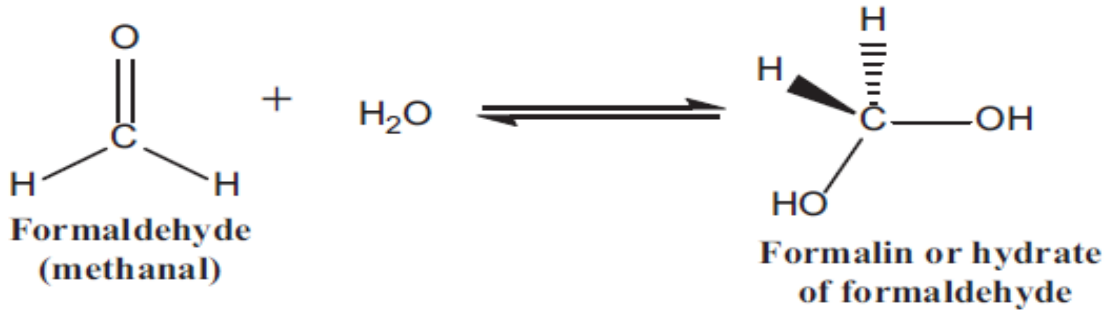
In Bangladesh reported that variety of food items get adulterated by applying the unsafe chemical or preservatives in the market at different steps from farm to seller to consumers. The most of the time formalin is sprayed on the fishes or dipping the fishes in the formalin, so to remain the fishes fresh till it packed, transport, sale, and reaches to the consumers. [1]

The U.S food and Drug Administration (FDA) regulate the economically motivated adulteration (EMA) stated that "the fraudulent, purposely replacing or adding a material in a product for the intention of rising the assumed worth of the product or decreasing the cost for its production "that is in term for the economic gain. In the food supply chain EMA is only example of fraudulent activity types.[10]

To appear the fresh, food, vegetables, fishes and other food stuff can be store for a longer time so they get contaminated by adding a formaldehyde illegally. If the fishes catches area is longer from the market or where the fishes getting sale, formalin is adding as preservative and mixing the ammonia with frozen water.

Formaldehyde (FA) was discovered in 1859 [11] and it has the simple structure. At ambient temperature the formaldehyde is a polymerized gas, colourless and flammable[9] and commercially accessible in form of 30-50% in aqueous solution and it stabilizing with methanol and this is called a ' formalin'.[9] It is a commonly group of a carbonyl compound, so it is usually used in the products to prevent the spoilage by getting contaminated by microbial activities.[9]

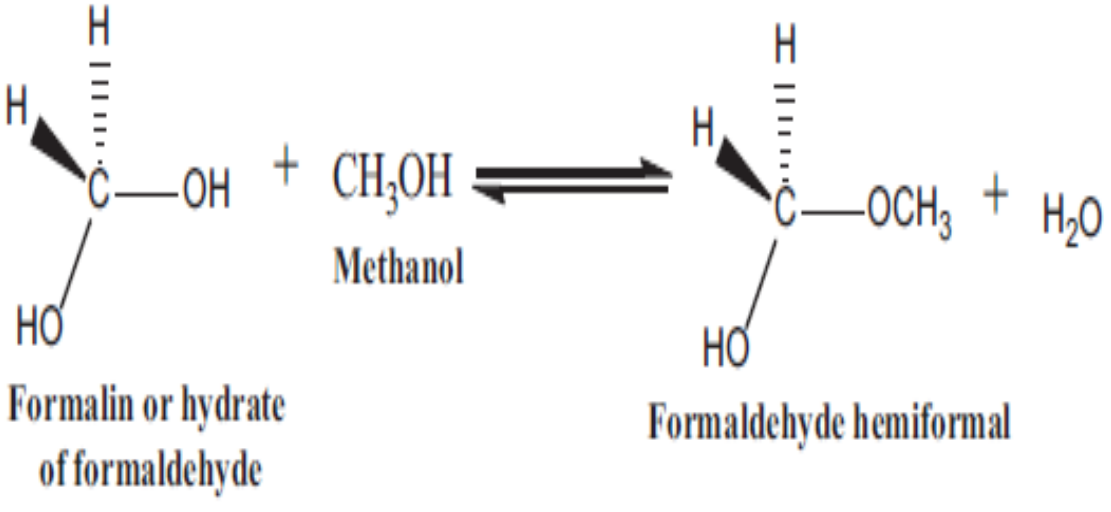
The equilibrium between formaldehyde in water and corresponding hydrates is rapid and complete , giving reaction below : [6]



Scheme 1 : Reaction of formaldehyde with water[6]

Usually formalin contain 37 – 40 % formaldehyde gas weight per weight of water. So there is formation of paraformaldehyde which is white precipitate, that's why it must be kept in the dark. [6]

So formalin is stabilized by adding 10-15% methanol, so there is no formation of paraformaldehyde. So adding the methanol, hemiacetal is forming which is equilibrium with the hydrated formaldehyde because the oxygen bond with hydrogen is substituted by - CH_3 group. In the pH range between 2.8 to 4.0 formalin exist and having a flash point of 60° C. if consider that formalin solution may present 37% formaldehyde and methanol (10%) then its boiling point is 97.8° C and density is 1.089 gmL^{-} . [6]



Scheme 2 : reaction of formalin with methanol to give formaldehyde hemiformal

Formaldehyde / formalin can be converted to a formic acid by the oxidation with the atmospheric oxygen. And this acid get further converted to carbon dioxide and water by the microorganisms. [6]

$$2H_2C=O + O_2 \longrightarrow 2HCOOH$$

$$HCOOH + O_2 \longrightarrow CO_2 + H_2O$$

Scheme 3: Reaction formaldehyde with oxygen to give formic acid
Formic acid reacting with oxygen to give carbon dioxide and water

In some fishes like cod, Pollack, whiting enzymatically formaldehyde is produced by degradation of trimethylamine oxide (TMAO). [12]

Naturally formaldehyde may be formed during the storage in fish flesh, thus giving a flesh deterioration. The amount of dimethylamine and formaldehyde contain in the fish flesh mainly depends on the temperature of the refrigerator and time for the storage.

In the internal organ and red muscle of the fishes there is enzyme present called as Trimethylamine-N-oxide demethylase (TMAOase) which is having an ability of catalysing the conversion of TMAO to DMA and Formaldehyde. [12],[13], [14]

In the muscle of fishes there is presence of trimethylamine oxide (TMAO) which convert to a formaldehyde , so there is naturally presence of formaldehyde in fishes.[15] And as a time pass it get vanishes. Fishes having dark muscle may contain higher amount of natural formaldehyde than the fishes having a white muscle. [16]

There is issues about the formaldehyde residue monitoring program, because formaldehyde produced in the fishes naturally by the deterioration process and it is tuff to

identify the formaldehyde which is produced naturally or added for the preservation purposely.[17]

Formaldehyde used in the construction i.e. furniture, carpeting, textile, wood processing, besides these they may use in the industries. It is by-product of natural activities like forest fires, anthropogenic activities like smoking, tobacco, residential wood and fuel burning. [18].Formaldehyde may be also present in the household products like antiseptics , dish-washing liquids, show care agents, medicines, cosmetics, adhesives etc. [19] In the production of paper plastics and disinfectant and board product formaldehyde is used as the raw material. [19]

The cycle of formaldehyde in biological system has a significantly important part in demethylation and methylation reaction as tis was suggested by some researchers.

In UK diet low level of formaldehyde is present which is causing little harm to the health because formaldehyde get converted to another product through normal metabolic pathway in the body. The World Health Organisation has set a tolerance level of intake daily is 0.15 mg kg^{-1} body weight per day.[5] Recently the announcement was that the formaldehyde is falling in the group 1 carcinogenic in the whole world by the IARC i.e. international organisation. [20], [2], [21],[15] . In Indonesia also using of formaldehyde as a preservative in fishes and other food product is banned by the Minister of Health Regulation No 33/2012. But this was not successfully applied.

In some parts fish sauces is produced by adding salt to fishes in the ratio of fish to salt i.e. 2:1 or 3:1. And after doing this is is stored in concentrated tank where the temperature range exits between the 35 to 40^{o} C. for a longer fermentation method can be used, to ensure colour and flavour development as well as the solubilisation of fishes sauces.[23]

2. HARMFUL EFFECTS

Large amount of free formaldehyde if get into the human body then there is imbalance of normal metabolism like gastrointestinal bleeding and systemic acidosis. When formaldehyde get accumulated in the stomach then it produces gastritis and due to this other processes take place like inflammatory in the upper duodenum and jejunum. [24]Also due to accumulation parenchymatous organ get damage[25] mild swelling in the liver and because of this there is alteration in the vacuolation of protoplast, leukolyte infiltration and cell nucleus.[19] Hence it turn to focal necrosis. Also formalin if inhaled, central nervous system get affected, and due to formation of formic acid, high acidity in the blood results. There has to suffer from the respiratory diseases, blindness and burning in the eyes, nose, throat and irritation in skin, coughing , wheeze, nausea, bronchitis and pneumonia.[24],[9] Formaldehyde exerts embryo toxicity and teratogenicity. Gene mutation also occur and all these symptoms happen when the concentration of formaldehyde is more than 0.1 ppm in air exceeds. Formaldehyde falling in the class of carcinogens.[1] And too much exposure of this may lead to cancer like nasopharyngeal and asthma, and also may lead to death. [24]

II. METHODS FOR THE DETERMINATION OF FORMALDEHYDE:

1. SPECTROPHOTOMETRY

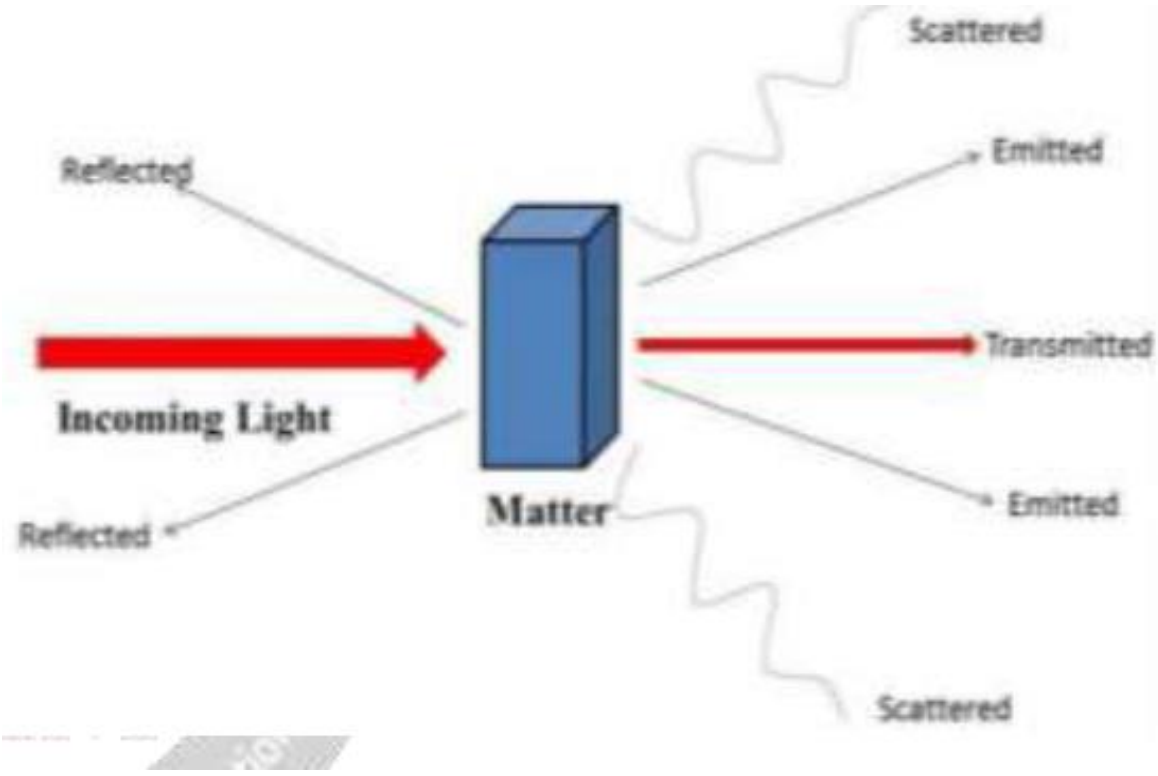
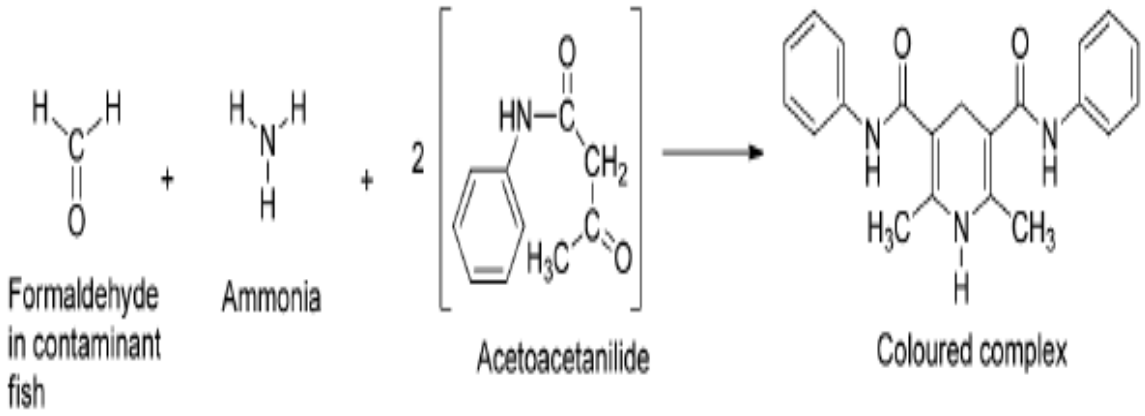


Figure 1: Basic Principle Of Spectrophotometry [24]

(a) Using Nash' Reagent[26](4-amino-3-penten-2-one)

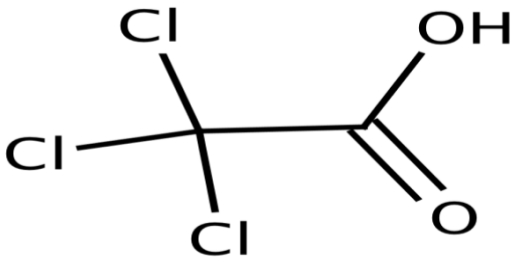
In the Nash' reagent ammonia and acetyl acetone was present which reacted with the formaldehyde present in the fish sample and DDL will formed which is UV active complex. The complex is coloured which use for to measure the intensity which is directly proportional to the concentration of formaldehyde present in the sample. [27]



Scheme 4 : Reaction between formaldehyde and and Nash' reagent containing ammonia and acetoacetanilide to give coloured complex [27]

Collection of fish sample:

Variety of fishes were collected from different market and it used as sample for the detection. The fish sample which are collected were kept in an insulated box containing the piece of ice. This box was carried to check the presence or absence of formaldehyde in the fish sample in the laboratory.



Scheme 5 : Trichloro acetic acid

Chemical Reagent:

For the fish sample extraction method Trichloro acetic acid TCA (60 ml of 6%) was used. To detect the absorbance of formaldehyde Nash' reagent is used as the indicator. 15 g of ammonium acetate was weigh and taken in 100 mL Erlenmeyer flask containing 0.2 ml of acetic acid and 0.3 ml of acetyl acetone and diluted. To store the Nash' reagent, dark glass reagent bottle should be used. This should followed all the time whenever Nash' reagent is stored. The pH range of this solution should be maintain between the ranges of 6.0 to 6.5. If the pH acidic then 0.1 N potassium hydroxide (KOH) is used to bring the pH in the range. And if the pH is basic 0.1 N hydrochloric acid (HCl) is used. The pH is check by the pH – meter.

Fish sample preparation for the detection of formaldehyde:

The collected fish were cut down to a smaller pieces with help of knife. Then these pieces of fish flesh was put in the blender and blended for the 10 minute at least for the homogeneity. After this takes out the extraction of the flesh fish and to these add a 60 ml of 6% trichloro acetic solution. The filter paper whatman number 1 was used to filter the extracted solution of formaldehyde. By using the pH meter the pH of the extracted solution was calculated. Previous addition of trichloro acetic acid solution lower the pH value of the extracted sample and it was maintain in their pH range between 6.0 to 7.0 by adding potassium hydroxide or hydrochloric acid depending the pH values got. In the volumetric flask of 50 mL, 5 ml of sample solution was added by using a measuring cylinder or pipette.

Then this volumetric flask was kept in the refrigerator for 1 hr. The sample was remove from the refrigerator after 1 hr for the analysis. To this solution 2 mL of Nash' reagent which was prepared previous was added. After this the fish sample solution was heated at 600^{o} C in the heating water bath for 30 minutes. Meanwhile check the solution. After cooling the sample solution, poured this solution into the cuvette till the mark and clean the sides of it with the filter or tissue paper and the absorbance was measured at 415 nm in the UV spectrophotometer. Note down the absorbance in triplet of the solution.[1], [28], [15]

Standard Curve Establishment:

First prepared the working solution of known concentration of formaldehyde from the 6.2 % concentration of stock solution. The working solution series are 0.838 ppm , 1.68 ppm, 2,51 ppm, 3.35 ppm and 5.03 ppm. And Nash reagent was added to this working solution. Absorbance of this solutions were calculated one by one. Then the graph was plotted of absorbance v/s concentration of series of working solution. The equation y = mx was used and straight line passing through the origin was obtained.

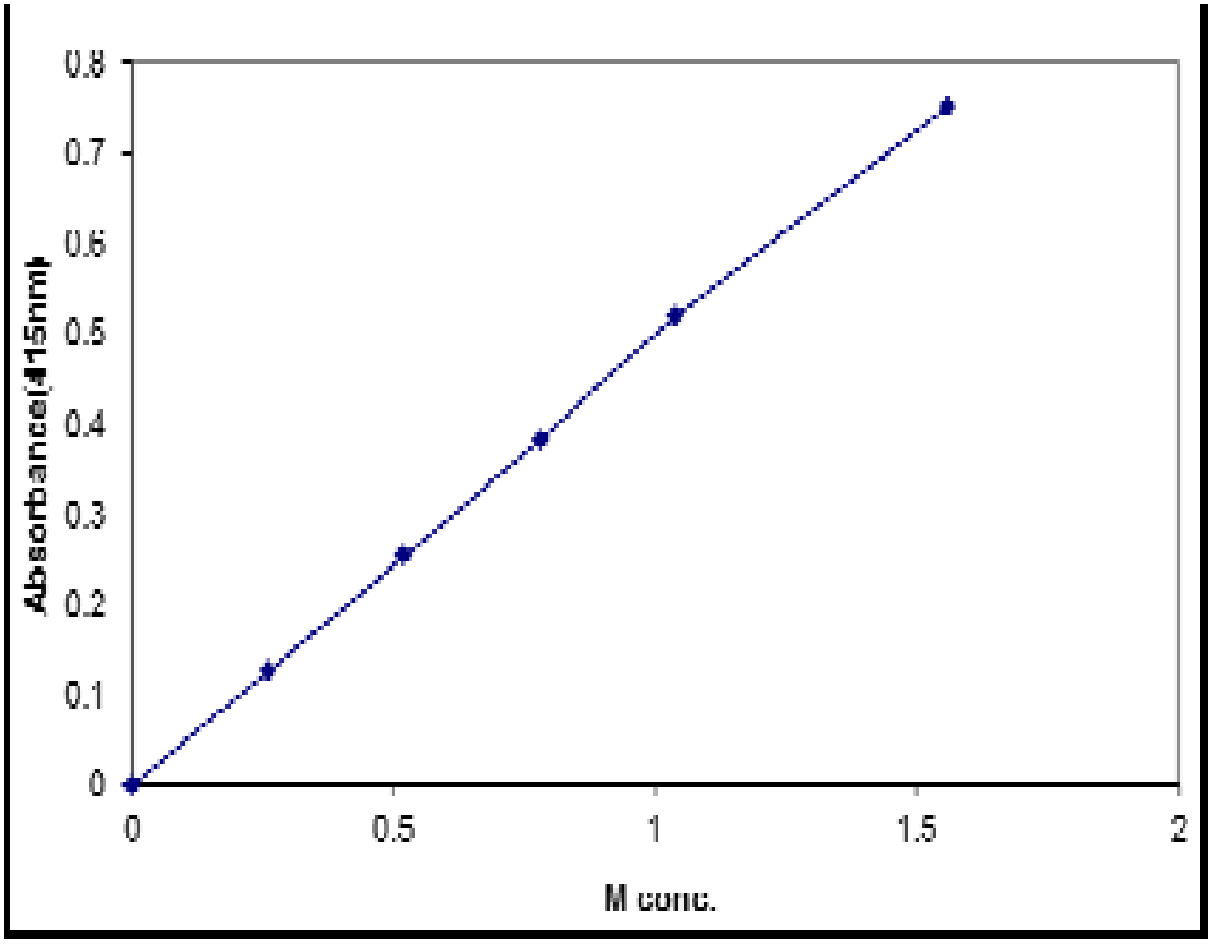
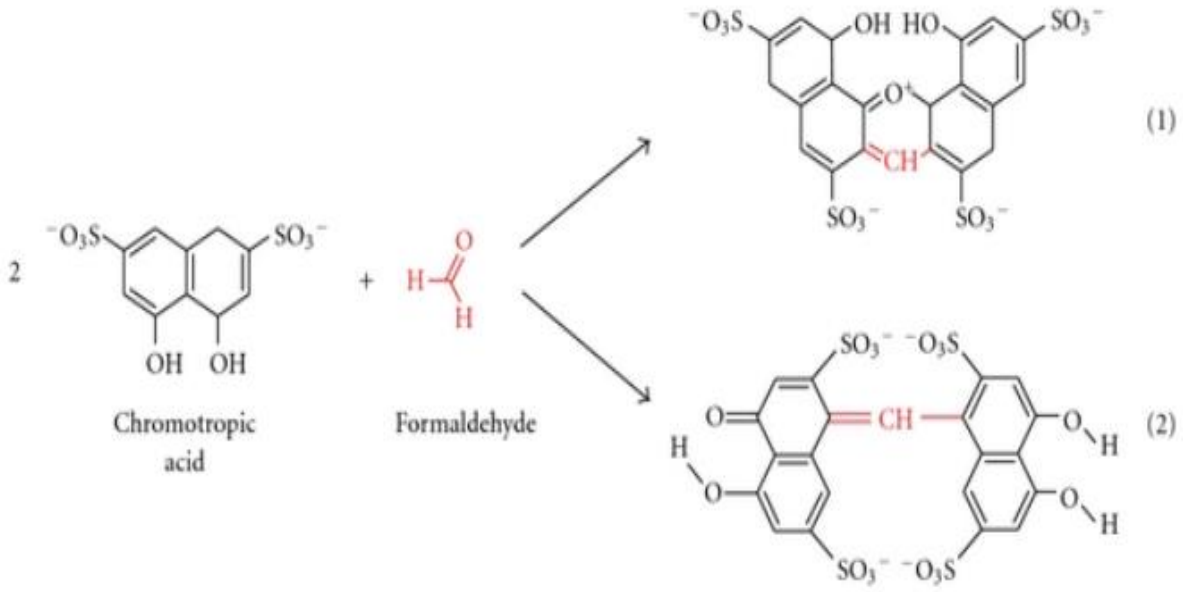


Figure 2 : Standard curve: : Absorbance of formaldehyde V/S concentration of formaldehyde [1]

(b) By Derivatization with chromotropic acid



Scheme 6 : Reaction between formaldehyde and chromotropic acid

Extraction of formaldehyde:

Take the fishes flesh in the flask and add 120 mL water and 1.32 N sulphuric acid. To this flask attach the distillation set up and place the flask in heating mantle. The collection rate should be approximate 2 to 3 drops per second of the distillate and as per that adjust the thermostat. The distillation was collected in a bottle of polypropylene screw tapped. This is sealed and stored in the refrigerator. And whenever analysis is performed that time removed it.[20]

Preparation of Chromotropic Acid Derivatization Reagent

Weigh 0.5 g of chromotropic acid and put in a beaker containing 100 mL of water. Mix well and see that it should be dissolved completely, and to this add slowly 150 mL concentrated sulphuric acid while stirring. After cooling used this as a chromotropic acid derivatization reagent. [8] [18]

Derivatization with Chromotropic acid:

Take 1 mL of distillate and add 9 mL of water i.e. the dilution with water 1: 10 ratio. Take 1 mL aliquot of the diluted solution and poured to test tube containing screw-capped. To that 5 mL of chromotropic acid derivatizatiion reagent was added. Again the test tube was sealed and mixed the content using Vortex mixer. Slightly loosed the caps and for the next 15 min heat this test tube in a boiling water bath, violet colour will formed. After this cooled this mixture at room temperature.[8]

Standard (Calibration) Curve:

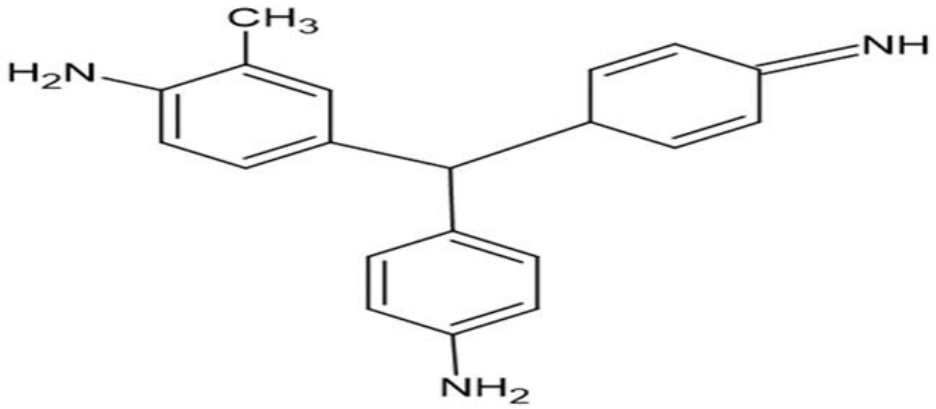
Working solution of known concentration of formaldehyde were prepared from the stock solution of formaldehyde as similar way done for the Nash' reagent.

Analysis:

The above extracted solution was taken in the quartz and measured the absorbance at 570 nm which is (lamda) max. Also the absorbance of the standard solution was measured. Water is used as the reference. Then plot the graph absorbance V/S concentration of formaldehyde chromotropic acid derivative, then find out the concentration of a sample solution.

(c) Using Schiff reagent

This method is more sensitive when the concentration of formaldehyde is low.



Scheme 7: structure of Schiff reagent

Schiff reagent Preparation:

Take the approximately 900 mL of boiling water, to its dissolve the 5 g basic fuchsin dye. Cool this solution. Then add slowly 100 mL of 1 mL HCl to the above fuchsin solution. Again cool this solution to 25° C. Add 10 g of $K_2 S_2O_5$ to the cooled solution. Then above solution was shaken for 3-4 min and then filter this. Clear crystal solution should be obtained otherwise retreatment and re - filtration has to do. Store the above prepared solution at 4° C in reagent bottle which has to be sealed with the foil. Schiff reagent gave a pink colour when it react with the formaldehyde. And this coloured solution used to detect the concentration of formaldehyde by using a spectrophotometer. Beside aldehyde, Acrolein, acetaldehyde, sulphur dioxide and oxides of nitrogen also reacting with the Schiff reagent. This is the interference. This method does not follow the Beer's lambert law whereas other method followed. [18]

RESULTS:

TABLE 1 : Formaldehyde content was detected by using different fishes and listed below. [29]

	Hake	Cod	Carp
Nash' reagent	121.6 ±9	96.8±3.1	0

TABLE 2 : Fresh water Fishes collected from BRFI ponds and determine the formaldehyde content by spectrophotometry using a Nash' reagent and results tabulated below.[1]				
Fishes sample	Sources	Absorbance	Molar concentration	Formaldehyde content (ug/g)
1. Rohu	BFRI	0.143 ± 0.002	0.29	1.45
2. Tilapia		0.184 ± 0.002	0.37	1.85
3. Thai Koi		0.250 ± 0.001	0.52	2.6

TABLE 3 : Formaldehyde content from frozen marine fishes collected from BFDC landing centre in Cow's Bazar were calculated by the Nash's reagent method [1]				
Fish species	Sources	Absorbance	Molar conc. Lo	Formaldehyde (ug/ g)
1. Loyitta	BEDC landing centre	0.919 ±0.002	0.78 X 10^{-4}	3.9
2. Chhuri		0.149 ±0.002	0.31 X 10^{-4}	1.55

Different concentration of standard formaldehyde was taken and find the absorbance to calculate the recovery test and reliability of the method. To find out the precision each sample takes in duplicate form.

In the imported fishes the concentration of formaldehyde are found in the range of 0.701 and to 3.710 ug g^{-1} .[5]

2. HPLC METHOD

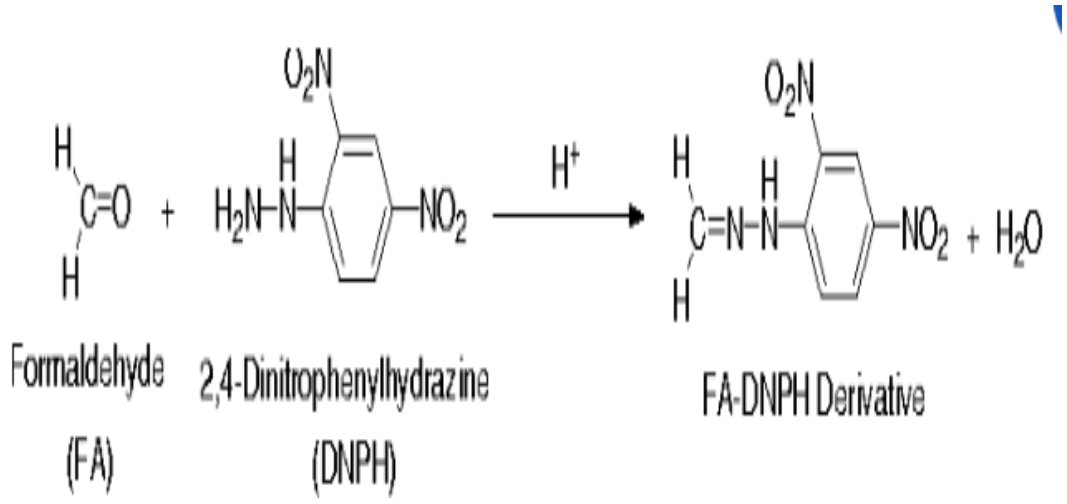
A total 10 number of fishes were selected and bring this to laboratory and stored in the refrigerator for the determination purpose. In the analysis muscle part of the fish was used.

Requirement:

Chromatographic grade acetonitrile, guaranteed grade formaldehyde (100ug mL^{-1}) , pure water , 30% methanol for the recovery of formaldehyde.[7], [30]

Preparation of 2, 4- dinitrophenyl hydrazine:

Before utilizing these should be recrystallized. Take 10 mL of anhydrous acetonitrile acetate and in it dissolved the 2, 4 DNPH to give a saturated solution. After complete dissolving the solution should be cooled at a room temperature and fill this solution into a brown bottle and kept overnight at 40° C for the crystallisation. Filter and collected by vacuum filtration. Accurately weigh 150 mg of DNPH recrystallized crystal and should be 49.5 mL of acetonitrile and add a 0.5 mL of H_3 PO_4 and mixed well. [3], [7] , [31], [32],[33]



Scheme 8 : reaction of formaldehyde with 2,4-Dinitrophenylhydrazine giving FA-DNPH Derivatives

Standard curve establishment:

First the stock solution of formaldehyde from the standard formaldehyde material was prepared. From this the working solution was prepared in series. This was injected in the HPLC instrument and note down the retention time. The spectra will obtained, from this peak area is noted and plot the graph of peak area V/S concentration of formaldehyde. [7]

Procedure:

Take the fish sample approximately 5 g and add 5 mL of acetonitrile to it. Then mixed it well. After mixing this was kept 30 min for the sonication which is important. It remove if any air bubble or dissolved gases is present in the mixture. It speed up the reaction. After these mixture put in the centrifugal tube and run these at 5000 rpm for 5 to 10 min. any suspended particles or solid particles if present in the sample mixture get separated. After centrifugation the supernatant was decay into another clean container and filter this by using a whatman filter paper of 90 nm diameter. [7],[32]

Some amount of 2, 4 DNPH (two and half mili liter) was taken and added to the extracted solution and mixed this well. The sample was kept for shaking in the water bath at 40^{o}C for 1 hr for the incubation purposed. In this 1 hr formaldehyde were converted quantitatively to its base. After 1 hr of incubation, the layer of acetonitrile was collected by using the membrane filtered of 0.45 um, and injected into the injection port of the HPLC instrument.

The column i.e. the stationary phase used is c_{18} column (ODS-C_{18}) which is reverse stationary phase and wavelength set at 355 nm and the temperature of the oven set in between the 30 to 40 ^{o} C. The separation is carried out by high pressure and the elution is isocratic. The solvent used is mixture of water/methanol (35:65 V/V) and the flow rate is set to 1 mL/ min. paper 18 20uL of sample volume was injected, run for 12 to 15 min. And note down the retention time. From the peak, calculate the peak area and this value plotted in the standard calibration graph and from this will get the concentration of formaldehyde present in sample solution.[7], [34]

Result:

The concentration of formaldehyde in the squid were find out by using the HPLC technique with the detection limit of 0.20 mg/kg and R^2 in the standard curve were found out 0.9983. And the 91.8% got the recovery rate by adding known concentration of formaldehyde to a known sample.[20]

Formaldehyde level was ranged from 0.38 ± 0.01– 15.75 ± 0.08 mg kg^{-1} in fresh fish. [3]

The chromatogram were obtained without the interference peak in the HPLC technique. As the analysis and preparation of sample were taking place in the closed and automated system, the volatilization of formaldehyde is avoided and the method was improved with good reliability and repeatability. [31], [32]

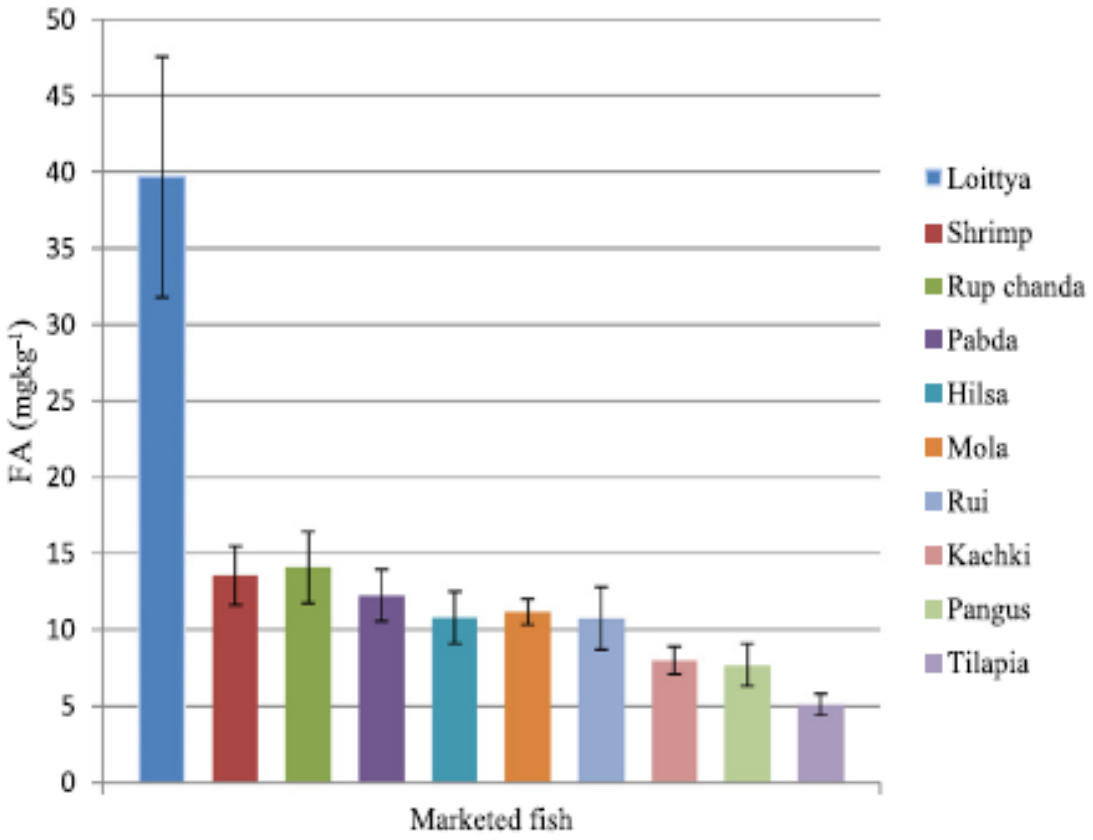


Figure 3 : Formaldehyde content in the market fishes[3]

In the HPLC sample derivatives were analysed and compared the retention time of sample to the standard formaldehyde for the qualification. In the calibration equation peak area of the sample solution was substituted and from that concentration of formaldehyde was calculated. [3]

TABLE 4 : Fish sample was testes for the determination of formaldehyde by the HPLC technique paper[31]					
Analyte	Matrix	Limit of detection LOD (mg Kg^{-1})	Limit of quantification LOQ (mg Kg^{-1})	Recovery range	Relative standard deviation (RSD_r) (%)
Formaldehyde	Fish	1	5	81 – 100	5 % (n=28)

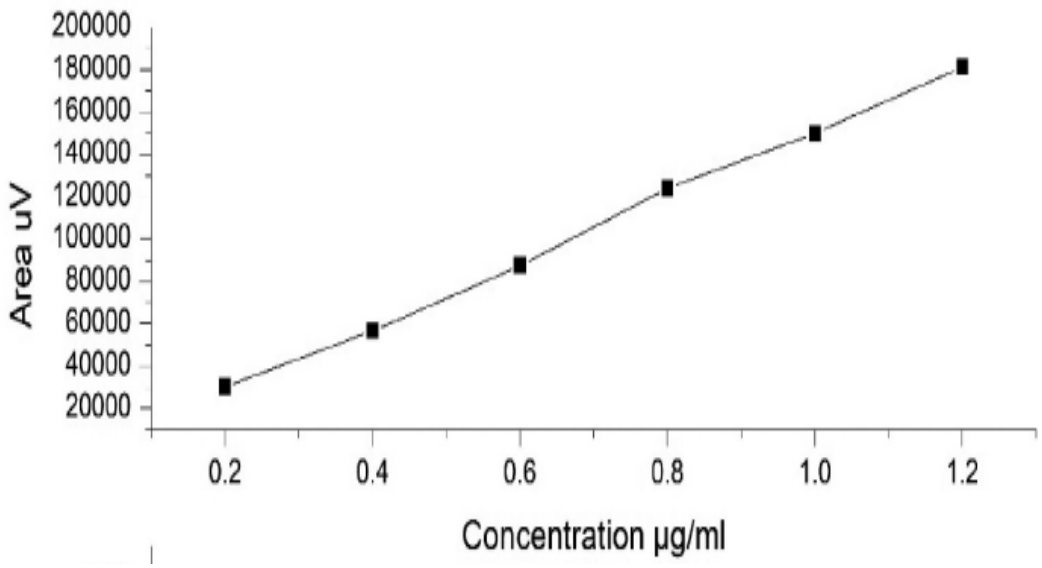


Figure 4 : Calibration curve from HPLC analysis showing concentration versus area of peak height [27]

- # METHOD VALIDATION FOR HPLC

Method validation of HPLC is important. By doing the proper validation of a method gives the evidence in the documented form of a method performance and providing in term of linearity, specificity, limit of detection, repeatability etc. [7]

Materials:

Analytical grade solvents, 2,4 DNPH , certified reference material of formaldehyde in the deionised water.[7]

Procedure:

Prepare the stock solution of formaldehyde of a CRM in the deionised water. To obtain the standard calibration curved free of matrix was prepared to a 6 different concentration i.e. 1,2,5,25,50 and 100 mg/L from the stock solution of CRM of formaldehyde. This known concentration of formaldehyde is spiked to the sample before the extraction. Then this was injected one by one in the HPLC instrument and the retention time was noted of the peak which is useful in the identification, quality checking purposed. It helps to identifying if any impurities or other interference is present.

Result:

By injecting a blank reagent (2,4 DNPH and phosphoric acid) , blank sample and solution of formaldehyde individually to check the specificity of the method[7].

Check the response of 0.1, 1, 2, 5, 25, 50 and 100 mg/L of standard to find out the linearity of the method. [7]

Calculate the 3.3 $S_{y/x}$ /slope for the checking the limit of detection and also determine the 10 $S_{y/x}$ /slope for the quantification limit. [7]

For the recovery and repeatability determination 5 sample from each matrices was spiked at nominal concentration i.e. LOQ, 2XLOQ and 5XLOQ level. In the % form recoveries was expressed and standard deviation and the relative standard deviation express the

repeatability. The repeatability limit (r) were determined to coverage factor of 99.9% (by applying the expression , r =2.8 X S_r)[7]

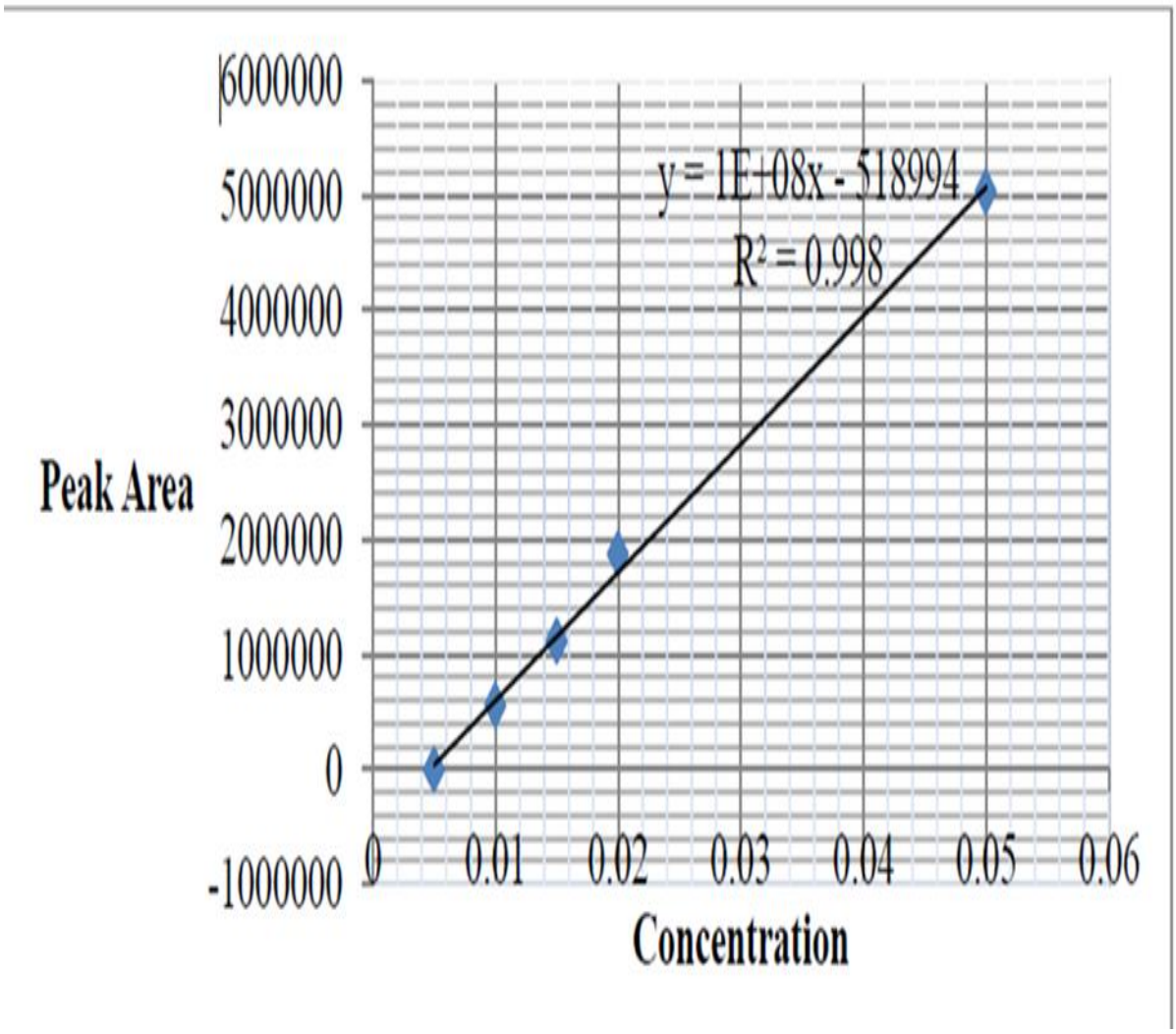


Figure 5 : standard calibration curve for the formaldehyde , peak area v/s concentration

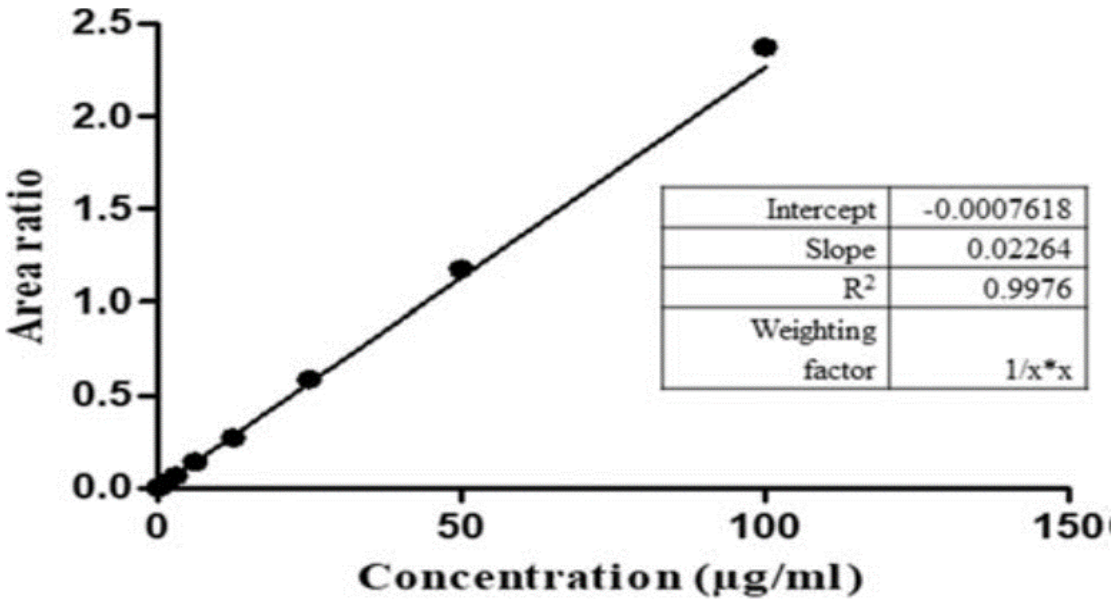


Figure 6 : Graph of calibration curve plotted area ratio V/S concentration of formaldehyde [35]

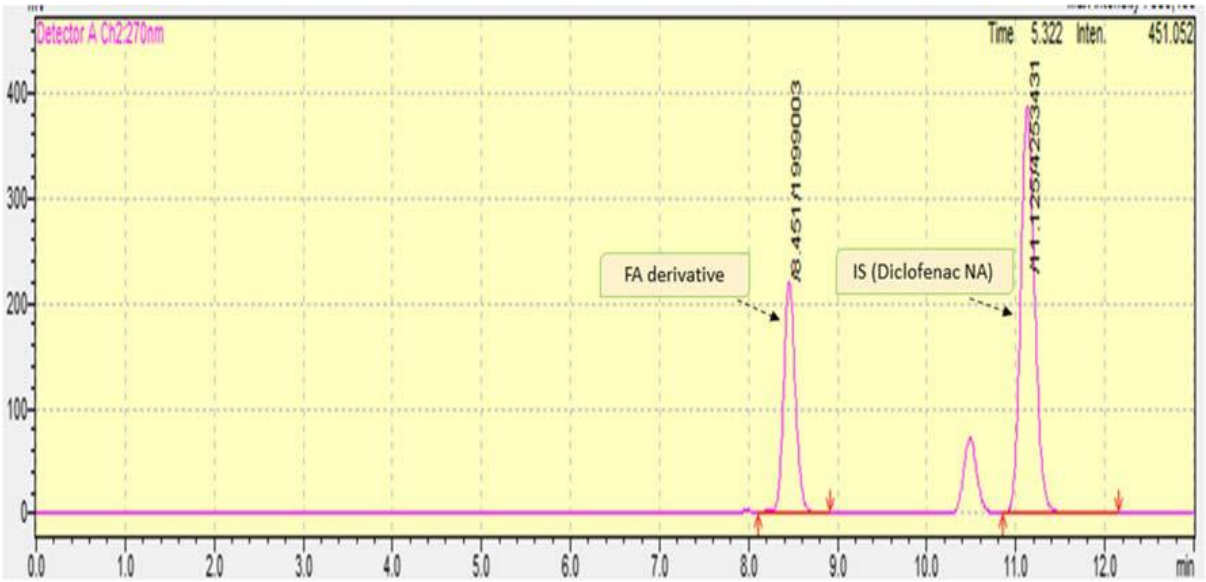


Figure 7: formaldehyde derivative showing retention time of 8.45 min and internal standard showing 11.12 min. [35]

3. CAPALLIARY ELECTROPHORESIS

Now a days capillary electrophoresis recognises an important technique in the analytical separation due to its good reproducibility, speed, small volume of sample required, consumptions of solvents is few and no contaminants. There are better separation of aldehydes of low molecular weights by the CE. Miniaturised devices has been used for capillary electrophoresis performance and it achieving a growth in the last decades. It is having advantages for e.g. high speed, low reagent consumption, efficient separation and small dimensions. Capillary electrophoresis is coupled with the electrochemical detection because it having tuneable selectivity, high sensitivity and compatibility with technology of advance micro machining.[36]

Basically formaldehyde exits as a molecules having no charge so electrophoretic mobility is zero, and it does not contain a chromophore for the sensitive detection by UV. The electro active compound i.e. 2-thiobarbituric acid (TBA) was chosen as a derivatization reagent to determine the formaldehyde content.

Reagents:

Analytical grade solvents and reagents, standard compound of FA. formaldehyde stock solution was prepared i.e. 1.0×10^{-2} g/m with distilled water, TBA (7.25×10^{-3} g/mL) prepared using ethanol solution (1:1), and these kept for one week for stabilisation and store in refrigerator at around 4 ^{o} C .[36]

Sample preparation and derivatisation

The extract of sample prepared, and it is filter using 0.22 um nylon filters before derivatisation. Take an appropriate quantity of filtrate and 165 uL TBA was added and 250 uL HCl (2.90 mol/L) and some distilled water was added to make the volume to 1 mL and these was put in the 5 mL flask and capped it and kept for shaking under the magnetic stirrer for 1 hour at room temperature.[36]

Set up

For the mini-CE-ED system ± 5 kV direct current is used and giving voltage for the separation between ends of the column. Capillary having 2 end, one end is held to a positive potential which is inlet and other is held near the negative potential which is outlet. The capillary column packed with a fused-silica having the dimension of 19.5 cm X 25 um and inner diameter 360 um. The 3 cell are in the combination i.e. saturated calomel electrode, platinum electrode and reference electrode which making the electrochemical cell and used with an amperometric detector. And this is connected at opposite side of capillary column outlet end and in between place a guiding tube which was fabricated. [36]

Process:

Before using 0.1 $molL^{-1}$ NaOH, the capillary column was washed and rinsed with deionised water for at least 5 min each time and then the capillary column was flushed with the buffer (pH 9.0) and applying 2500 V for 15 min. the same buffer was filled in the detection cell. In the sample vial sample solution was introduced by applying 2500 V of voltage between the ground detection cell and the sample vial. At the same time high voltage leading wire and capillary column inlet was immersed into the buffer which is a running and voltage required for the separation which is applied between grounding and high voltage wire for the separation. To maintain the same viscosity of running buffer the assembly placed in the room having the temperature having 20^{o} C. These is useful for producing high reproducibility and good experimental results.

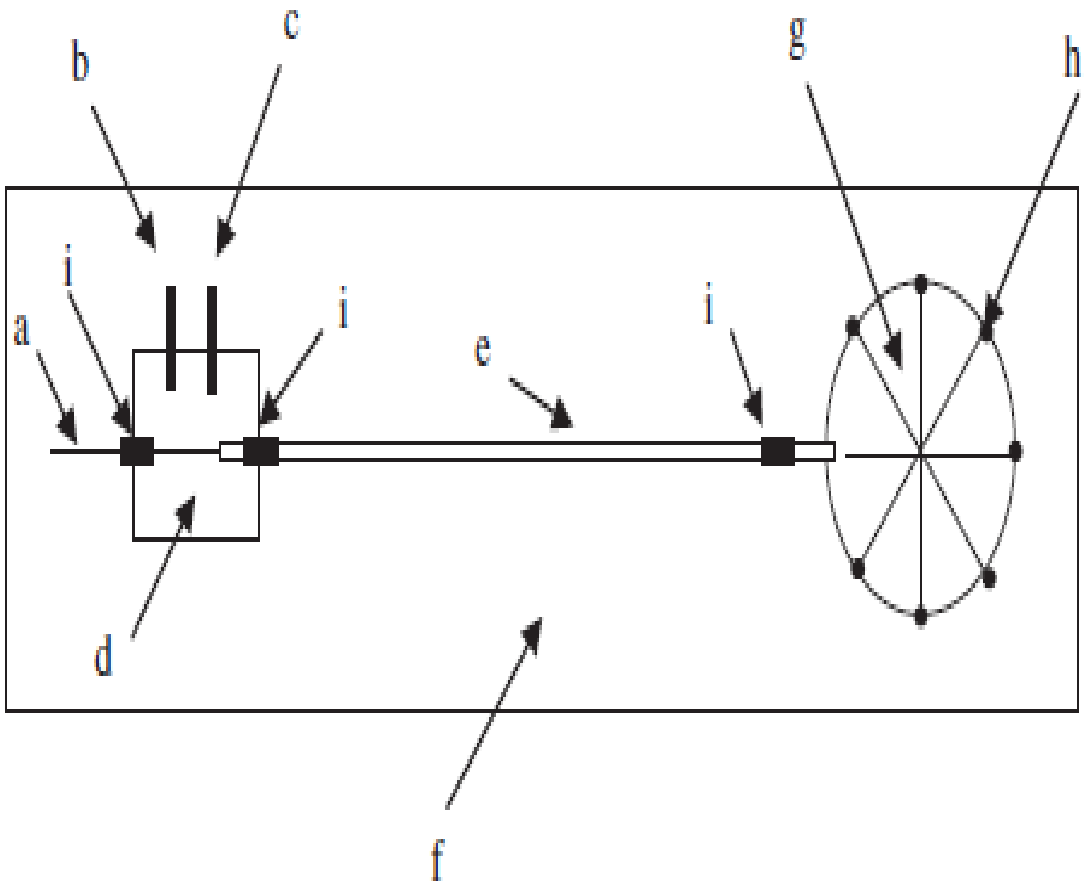


Figure 8 : This showing mini-CE-ED system: (a) Working electrode; (b) Pt. auxiliary electrode; (c) saturated calomel electrode (SCE) reference electrode; (d) detection cell; (e) 25 lm i.e. fused silica capillary column; (f) plexiglass plate; (g) turning plexiglass disc; (h) plastic vial for running buffer or sample solution and (i) metal tube.

Results:

There is a good separation and temperature is playing important role in the separation. The concentration and acidity of the buffer which is flowing affects the electro osmotic flow and zeta potential which determine the separation of analytes. When the value of flowing buffer increases there is better resolution with rise in migration time. Greater separation voltage gives lesser migration time. [36]

TABLE 5 : This showing the assay results of two aldehydes in real samples (n = 3) [36]		
Samples		Formaldehyde (ug/mL) (RSD %)
Waterishlogged products	1.Sea cucumber	3.15 (4.6)
	2. Jelly fish	17.1 (3.0)
	3. Squid	0.601 (2.7)
	4. Tripe	0.812 (5.5)
	5. Pork skin	0.278 (5.2)

4. FIBER BUNDLE SENSOR

It is a simple experiment and does not required any pre-treatment for the sample. Contaminated food is placed and irradiated with a laser, some radiation will absorbed and some will be reflected by the sample and the reflected light is collected and used to determine the concentration of the formaldehyde present in the sample. [37],[24]

Helium – Neon laser is used and emits a radiation and at 633 nm it is visible that the fish sample will illuminate which is connected to fibre bundle probe with 2 parts. from the one part light is coming from the laser and focus on the fish sample and through another part he light intensity which get reflected is collected and which is connected to the detector. The detector used are digital voltmeter. Also the 2 probe is having a support of micrometric movement.[24],[37]

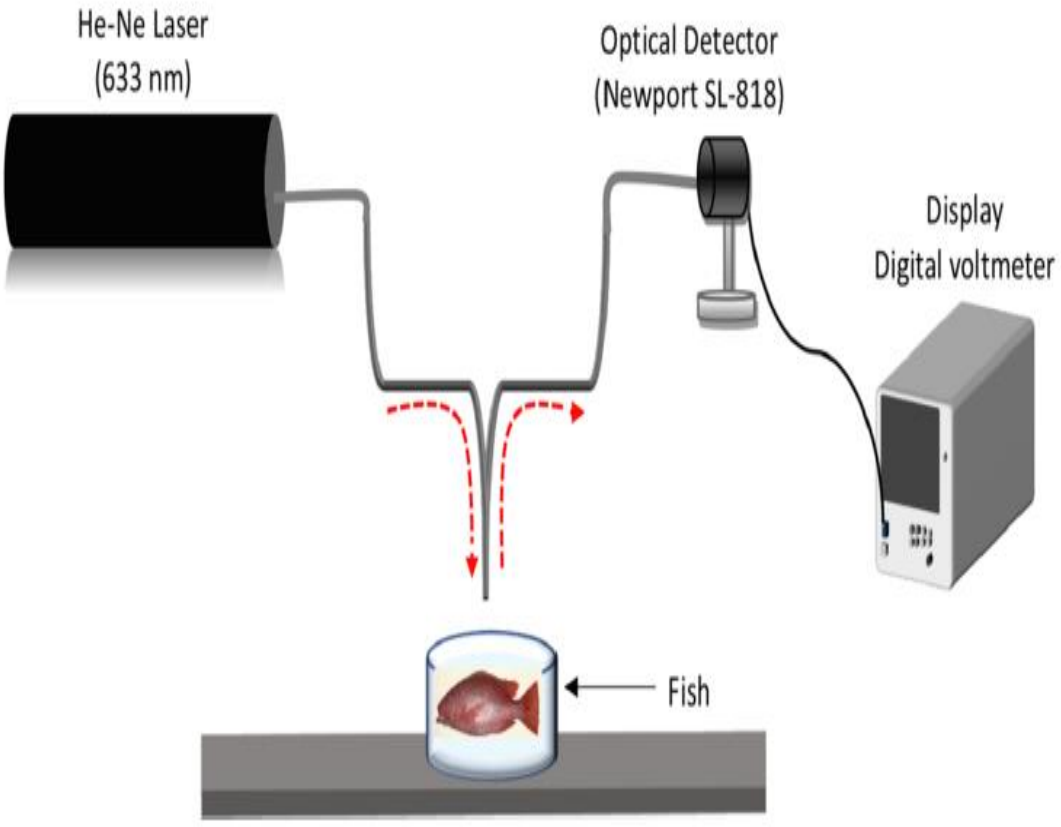


Figure 9 : Experimental set-Up [37]

Results:

Different types of fishes were tested by using a sensor, and measured the output voltage given by the detector and also the fishes dipped in the known concentration of formaldehyde and calculate the voltage and plotted the graph of voltage V/S concentration and it proves that the sensor is stable. [37]

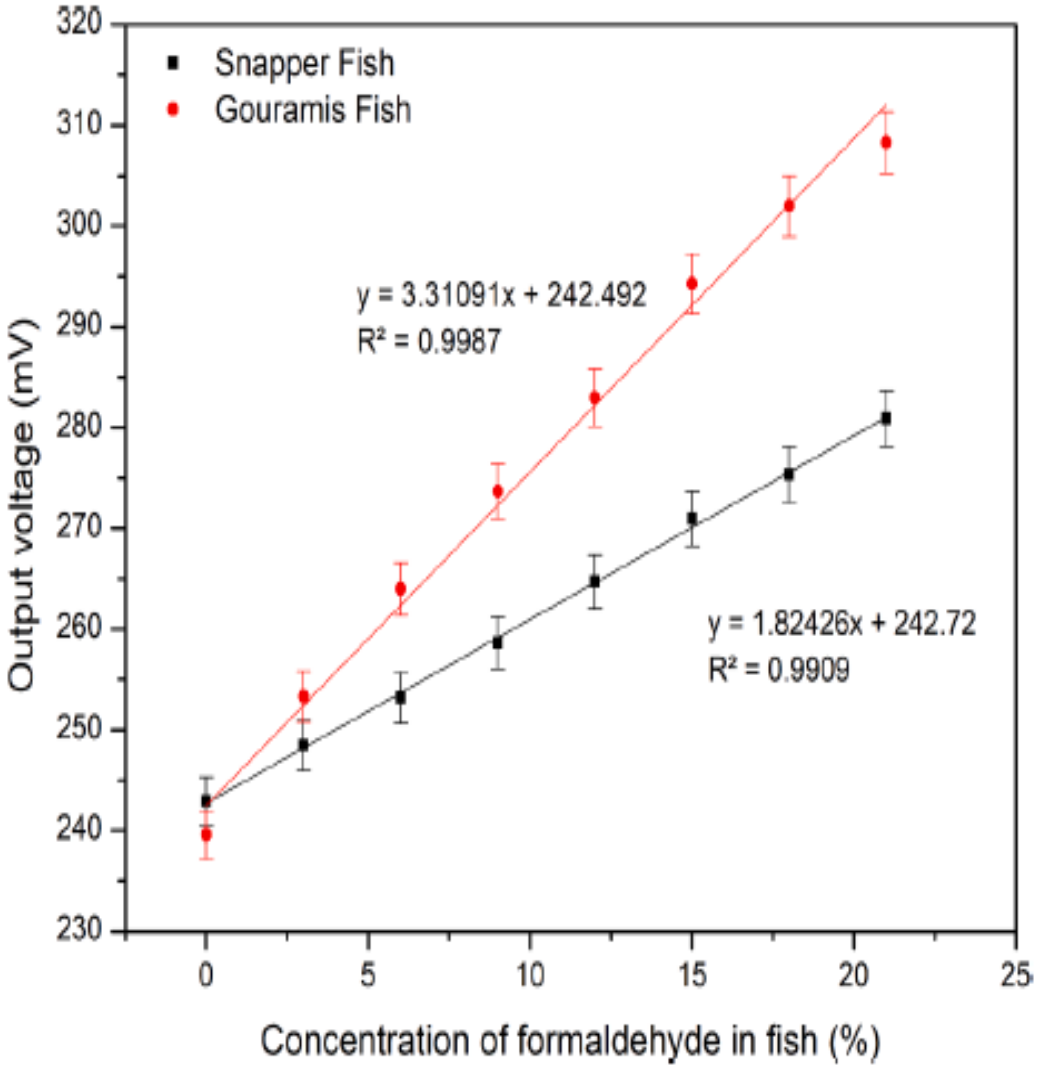


Figure 10 : Graph of output voltage (mV) V/S concentration of formaldehyde in % [37]

This below figure tells that, different concentration was taken and increases the distance between sample and probe, and result obtained, suggesting that no change in the concentration occur while increasing or decreasing the distances, hence it is proved that the sensor is linear and giving a good results. [37]

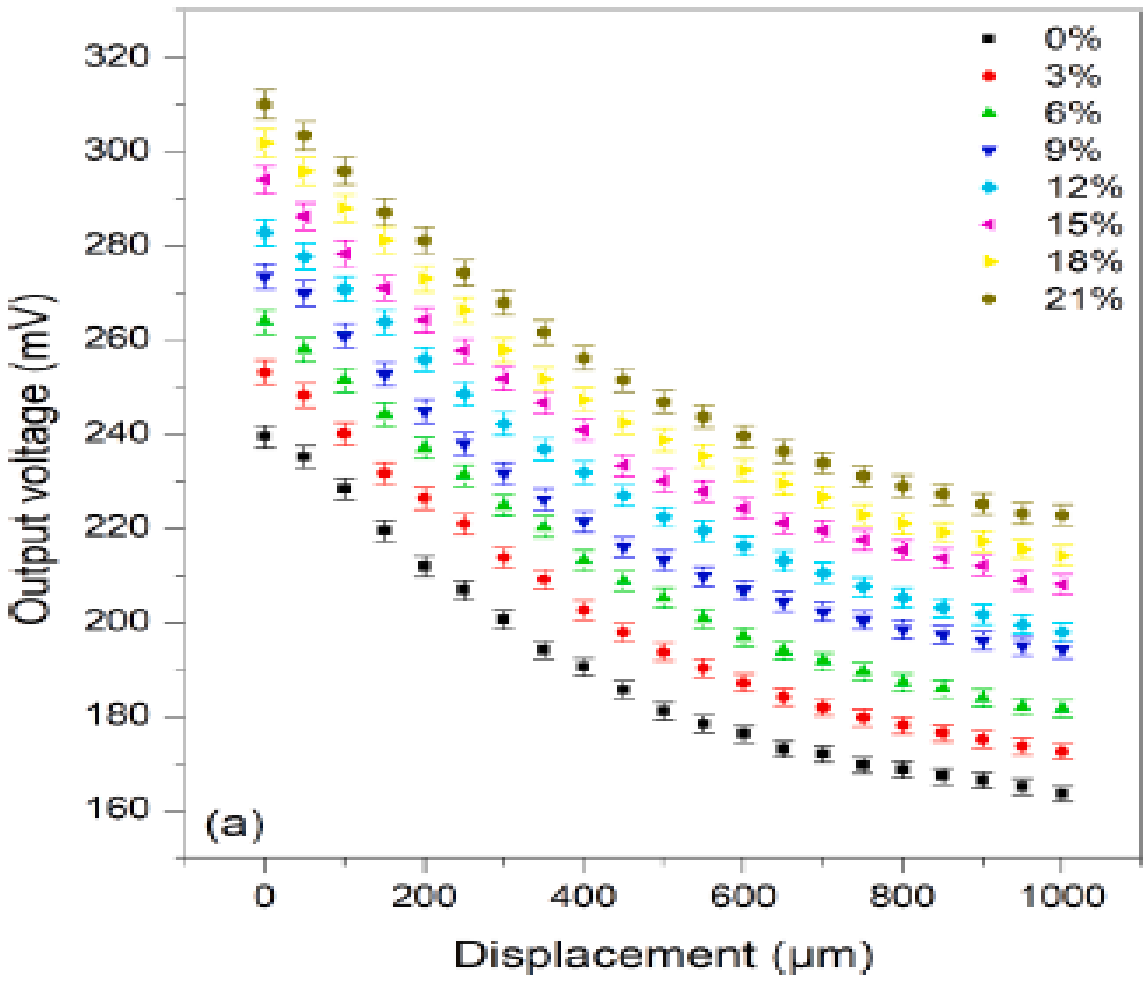


Figure 11 : The graph of output voltage (mV) V/S Displacement (um) [37]

5. BIOSENSOR

Either used of a harmful chemicals it is more convenient to use the method which is using a less toxic chemical. Now a days advance techniques coming and more direct methods are coming for the detection of formaldehyde in fishes or other food items with a good sensitivity.[38]

Basically polymer membrane are using as to support a development of biosensor due to easy and convenient procedure and less expensive methods. These uses a different trappers like enzymes and antibodies. Having the capacity of polymeric membrane to trap the ions and giving a specific site immobilization of a probe for sensing are of a significant important to develop a ability of high achievement of biosensor. [9]

For the ion- selective membrane fabrication poly vinyl chloride (PVC) is used, but due to its rigid character it is not applicable for the supporting biological molecule in the membrane. So to suit the PVC for the supporting material it is converted to another flexible soft compound by adding an additive like plasticizer. To give the biocompatibility and mechanical properties hydrogel based polymer like poly (2- hydroxyethyl methacrylate) (pHEMA) and poly (methyl methacrylate-co-hydroxylethyl methacrylate) are used. Other membrane like os(bpy)2-poly (vinylpyridine) (POs-EA) is used which entrapped the formaldehyde dehydrogenase enzyme. The membrane which is based on poly (n-butylacrylate-co-N-acryloxysuccinimide) is used for the finding out the formaldehyde concentration quantitatively. This biosensor are potentiometric enzyme type and this involved the transfer of H^+ ion at the interface between the electrode and electrolyte. This can be used without addition of plasticizer. This membrane is doped in a Sodium tetrakis(3,5-bis(trisfluromethyl)phenyl) borate which is a anionic lipophilic salt and inophore of hydrogen to make a membrane selective for H^+ ion. Also the modification of membrane done i.e. the succinimide group is replace by AOX enzyme via the peptide covalent bond. At the surface of the acrylic membrane immobilised AOX catalysed the transformation of formaldehyde to formic acid and hydrogen peroxide (H_2O_2). The formic acid which is formed get dissociates into the H^+ and $HCOO^-$ ions in the solution.

Basically there is involvement of changes in the H^+ ion due to conversion of formaldehyde. So there will be variation in the pH of the solution. So in between the reference electrode and enzyme electrode there is generation of electromotive force (emf) which is used to determine the concentration of formaldehyde present in the sample which is exactly proportional to the amount of H^+ ion formed. [38]

Chemical required:

N-acryloxy-succinimide, acryl film base biosensor , Ag/AgCl screen printed electrode (SPE)[38]

Preparation of sample:

Collect the fishes and put in the ice containing container and bring this to a laboratory. Take out the muscle part of the fishes. And cut down the muscle into pieces. Weigh the 2.5 g of muscle and put in a ceramic pot, and pestle for some time. Then add 10 mL water and mixed this and again pestle this. Then add a 2 mL of both solution i.e. Carrez reagent I (15%, m/v, aqueous solution $k_3Fe\ (CN)_6.3H_2O$) and Carrez reagent II (30%, m/v, aqueous solution $ZnSO_4.7H_2O$). After this add a 34 mL of water so the total volume will be 50mL and stirred well this mixture. Then filter this solution using a filter paper (folded filtrak -88). And kept this filtrate at 4^o C before the analysis. [38]

Preparation of the Biosensor Electrode:

Take the Ag/AgCl SPE electrode and immersed in the solution containing the mixture of 1.6 wt % DMPPE polymerised initiator and 0.5 uL HEMA monomer. Under the continues flow of the nitrogen gas, the light of UV focus on the surface of Ag/AgCl SPE for 18 s. Upto here poly2 (hydroxyethylmethacrylate) pHEMA membrane was ready. Then for 15 min it is immersed in the solution of Tris-HCl buffer and by doing this it is functioned as the solid state inner solution. These will increase the detection limit of electrode for ion selection.

Then make the homogeneous mixture containing 1 wt% HDDA, 0.1 wt % nBA monomer, 1.6 %DMPP as a photo initiator, 0.8 wt% NaTFPB lipophilic anion salt, 1.9 wt% hydrogen ionophore and appropriate amount of NAs monomer was coated on the top of the inner

solution of PHEMA layer, then the photo-cured under the nitrogen environment take place for next 180 s and then poly (n-butylacrylate -co – N - acryloxy - succinimide) for H^+ selection membrane was ready. And to form a bond between succinimide and amine functional group via nucleophilic ring opening reaction, the membrane was dispensed on the H^+ ion or in 5uL of alcohol oxidase (AOX) enzyme. [38]

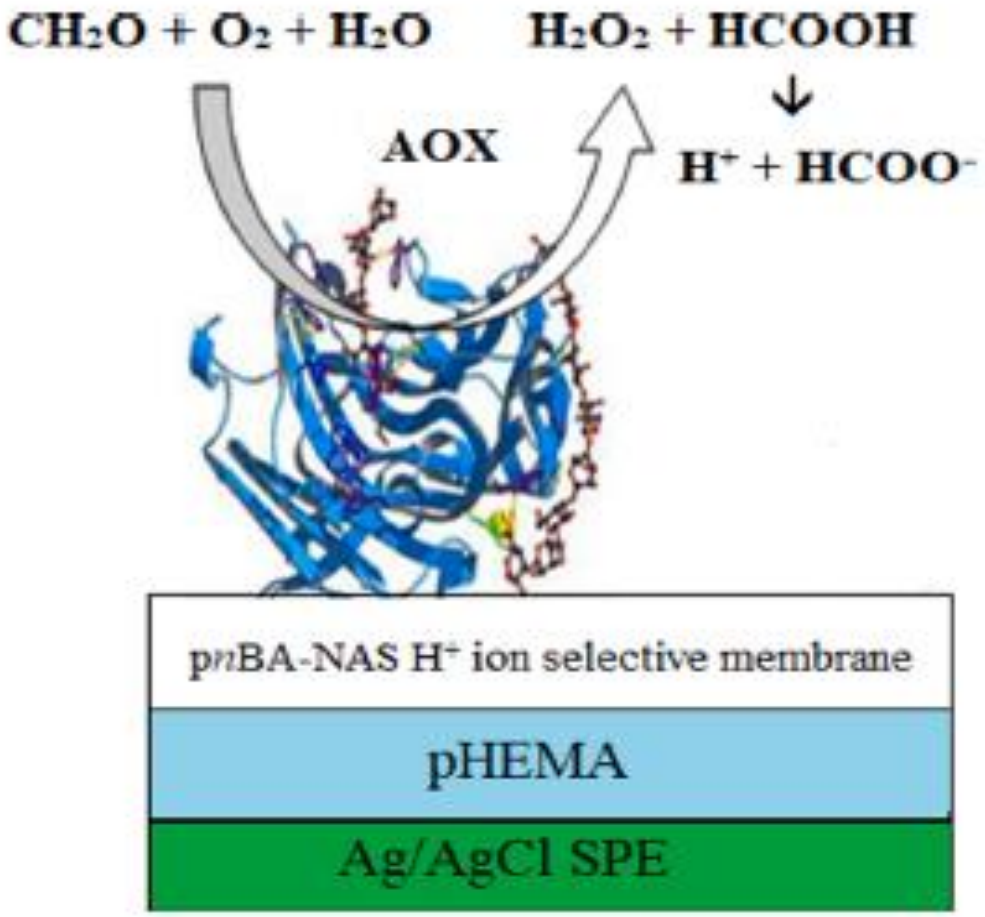


Figure 12 : Potentiometric formaldehyde biosensor with AOX enzyme on the surface of the pnBA-NAS copolymer membrane. [38]

Experiment:

The response of biosensor was measured in the electro chemical cell which consists of working electrode i.e enzyme-modified H^+ ion selective Ag/AgCl SPE and containing 0.1 M Tris- HCl as internal solution in the Ag/AgCl reference electrode and bridge electrolyte of 1 M lithium acetate gel. Both this electrode connected to the Orion ion meter to record the potential difference between this two electrodes in the form of emf. [38]

Results:

The response of the formaldehyde biosensor remain stable by having sensitivity more than 95%. Longer lifetime of biosensor attributing the chemical immobilization of enzymes by the strong covalent bond formation on the surface of electrode. The different concentration of formaldehyde prepared and check by the biosensor and it gives a good results having average relative standard deviation calculated at 1.4% (n=7) and also it showing the satisfactory repeatability RSD at 7.8 % (n=7) . This suggested that biosensor electrode can be reused 3 times. [38]

TABLE 6 :Concentration of formaldehyde in different fish sample (n=3) [38]

Samples	Formaldehyde concentration by potentiometric biosensor (mM)
1. Snapper fish (Lutjanus johnii)	5.100 ± 0.610

2. Pomfret fish (Pampus argenteus)	2.470 ± 0.090
3. Threadfins fish (Leptomelanosoma indicum)	5.010 ±0.150
4. Snapper fish (Lutjanus johnii)	3.910 ± 0.090
6. Pomfret fish (Pampus argenteus)	3.980 ± 0.090
7. Threadfins (Leptomelanosoma indicum)	2.380 ± 0.080

TABLE 7 : Comparison of the analytical performances of formaldehyde biosensors fabricated based on different materials and modifications of electrode with the developed formaldehyde biosensor based on AOX-modified H+ ion-selective Ag/ AgCl SPE. Parameters [38]

Parameters	Siti et al.	korpan	Yew & Lee	
1. Enzymes	AOX	AOX	AOX	AOX
2. Response times (s)	_	10 – 60	1 – 8	1- 8
3. Sensitivity (mV/decade)	43.90 ±2.10	26.00	59.41 ± 0.66	59.23 0.85
4.Linear response (mM)	-	5.0 – 200.0	0.3 – 316.2	0.5 – 220.0
5.Limit of detection (mM)	-	-	0.3	0.1
6.Reproducibility (% RSD)	15.0	2.0	3.0	1.4
7.Stability (day)	-	60	48	80

III . CONCLUSION:

There are lots of steps in chemical methods, and to perform these well trained person is required.

The chromotropic acid derivatives giving good reasonable specificity for formaldehyde compare with other aldehydes.

As our study/ survey suggested that comparing the two methods spectrophotometry and HPLC, HPLC was more sensitive, effective provided good accuracy, therefore HPLC is preferred over the Spectrophotometry for the quantitification / qualification of formalin. [15]

Spectrophotometry consider a reliable, safer procedure, fast, convenient for the detection of formaldehyde in fishes.

HPLC is more expensive technique and they require well specialised operator to operate the equipment, which is not available mostly in the every places.

HPLC was giving good result in terms of analytical section i.e. robustness, specificity, linearity, precision.

Spectrophotometry method are giving a low colour stability and less interference of many substances.

Spectrophotometry required a complex pre-treatment processes, have a long detection time and low sensitivity. In place of UV spectrophotometry IR-Spectrophotometry may be used, which is non-destructive and rapid method.

Schiff reagent has a less sensitivity, less accurate results as it interference with the other co-pollutants. Our study/survey suggested that this method is need to be modified to give a satisfactory results.

The proton NMR experiment was used for the detection purposed, in the future there will be more scoped if proper procedure and instrument if available.[39]

Also paper based titration can be used to detect the formaldehyde content, which is easy technique, but little expensive and not portable. So to overcome this paper based analytical devices had been discovered which is easy to used and cheaper.[40]

Electrophoresis is fast techniques, easy to used, the derivatisation used is easily available. No complex procedure involved.

There is no that much used of optical Biosensor nowadays ,and not fully developed but in the future with better applications will arises, as it is does not need of any pre-treatment and it is non-destructive.

Since the formaldehyde is volatile and to find out the accurate concentration of formaldehyde in fishes solid phase micro-extraction technique can be used , which is based on the derivatisation with pentaflurobenzyl-hydroxylamine.[21]

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V. BIBLIOGRAPHY

1. Jaman N, Sazedul Hoque M, Chandra Chakraborty Professor S, et al. International Journal of Fisheries and Aquatic Studies 2015; 2(6): 94-98 Determination of formaldehyde content by spectrophotometric method in some fresh water and marine fishes of Bangladesh. Published online 2015. www.fisheriesjournal.com

2. Rahman M, Sheikh B, Rahman M, Hosen M. *DETECTION OF FORMALIN AND QUALITY CHARACTERISTICS OF SELECTED FISH FROM WET MARKETS AT SYLHET CITY IN BANGLADESH*.; 1998. http://www.bdresearchpublications.com/admin/journal/upload/09326/09326.pdf

3. Bhowmik S, Begum M, Hossain MA, Rahman M, Alam AKMN. Determination of formaldehyde in wet marketed fish by HPLC analysis: A negligible concern for fish and food safety in Bangladesh. *Egypt J Aquat Res*. 2017;43(3):245-248. doi:10.1016/j.ejar.2017.08.001

4. Jinadasa BKKK, Elliott C, Jayasinghe GDTM. A review of the presence of formaldehyde in fish and seafood. *Food Control*. 2022;136(January):108882. doi:10.1016/j.foodcont.2022.108882

5. Asare-Donkor NK, Adaagoam RA, Voegborlo RB, Adimado AA. Risk assessment of Kumasi metropolis population in Ghana through consumption of fish contaminated with formaldehyde. *J Toxicol*. 2018;2018. doi:10.1155/2018/4785031

6. Leal JF, Neves MGPMS, Santos EBH, Esteves VI. Use of formalin in intensive aquaculture: properties, application and effects on fish and water quality. *Rev Aquac*. 2018;10(2):281-295. doi:10.1111/raq.12160

7. Wahed P, Razzaq MA, Dharmapuri S, Corrales M. Determination of formaldehyde in food and feed by an in-house validated HPLC method. *Food Chem*. 2016;202:476-483. doi:10.1016/j.foodchem.2016.01.136

8. Chandralekha APL, Baranage C. *FORMALDEHYDE LEVELS IN FISH FROM THE KANDY*

MARKET. Vol 199220.; 1992.

9. Marzuki N, Abu Bakar F, Salleh AB, Heng LY, Yusof A, Siddiquee S. *Electrochemical Biosensor Immobilization of Formaldehyde Dehydro-Genase with Nafion for Determination of Formaldehyde from Indian Mackerel (Rastrelliger Kanagurta) Fish*. Vol 8.; 2012.

10. Manning L, Soon JM. Food Safety, Food Fraud, and Food Defense: A Fast Evolving Literature. *J Food Sci*. 2016;81(4):R823-R834. doi:10.1111/1750-3841.13256

11. Aksornneam L, Kanatharana P, Thavarungkul P, Thammakhet C. 5-Aminofluorescein doped polyvinyl alcohol film for the detection of formaldehyde in vegetables and seafood. *Anal Methods*. 2016;8(6):1249-1256. doi:10.1039/c5ay02719e

12. Benjakul S, Visessanguan W, Tanaka M. Partial purification and characterization of trimethylamine-N-oxide demethylase from lizardfish kidney. *Comp Biochem Physiol - B Biochem Mol Biol*. 2003;135(2):359-371. doi:10.1016/S1096-4959(03)00082-4

13. Nielsen MK, Jørgensen BM. Quantitative: Relationship between trimethylamine oxide aldolase activity and formaldehyde accumulation in white muscle from gadiform fish during frozen storage. *J Agric Food Chem*. 2004;52(12):3814-3822. doi:10.1021/jf035169l

14. ESTHER WEI YIN CHANG1, AI MAY LOONG1, WAI PENG WONG1 SHIT FUN CHEW2JONATHAN M. WILSON3, AND YUEN KWONG IP1Volsi ECFR, Mendes MA, Marques MR, et al. Change in tissue free amino acid contents branchial Na+/K+-ATPase activity and bimodial perch, Anabas tetudias (Bloch), during Sea water acclimation. *J Exp Biol*. 2011;1(4):397. doi:10.1002/jez

15. Hoque M d. S, Jacxsens L, Rahman M d. B, et al. Evaluation of artificially contaminated fish with formaldehyde under laboratory conditions and exposure assessment in freshwater fish in Southern Bangladesh. *Chemosphere*. 2018;195:702-712. doi:10.1016/j.chemosphere.2017.12.111

16. M.A. H, M.M H, A.F.M.I.U Z, M.A A, K.N. S. Association of Natural Formaldehyde Level With Quality Attributes of Selected Sea Fish, Collected From Southeast Coast of

Bangladesh. *Sci Res J*. 2020;08(04):1-7. doi:10.31364/scirj/v8.i4.2020.p0420757

17. Devaraj P, Babu V, Cengiz EI. Qualitative detection of formaldehyde and ammonia in fish and other seafoods obtained from Chennai's (India) fish markets. *Environ Monit Assess*. 2021;193(2). doi:10.1007/s10661-021-08871-z

18. Agrawal A, Pandey LP. Review of spectrophotometric methods for the determination of magnesium. *Rev Anal Chem*. 1994;13(4):253-295. doi:10.1515/REVAC.1994.13.4.253

19. Kim KH, Jahan SA, Lee JT. Exposure to formaldehyde and its potential human health Hazards. *J Environ Sci Heal - Part C Environ Carcinog Ecotoxicol Rev*. 2011;29(4):277-299. doi:10.1080/10590501.2011.629972

20. Mason DJ, Sykes MD, Panton SW, Rippon EH. Determination of naturally-occurring formaldehyde in raw and cooked Shiitake mushrooms by spectrophotometry and liquid chromatography-mass spectrometry. *Food Addit Contam*. 2004;21(11):1071-1082. doi:10.1080/02652030400013326

21. Bianchi F, Careri M, Musci M, Mangia A. Fish and food safety: Determination of formaldehyde in 12 fish species by SPME extraction and GC-MS analysis. *Food Chem*. 2007;100(3):1049-1053. doi:10.1016/j.foodchem.2005.09.089

22. Jawahar LS, John C, Shafeekh M, Anupama TK, Sankar T V. Retention of residual formaldehyde in treated Indian mackerel (Rastrelliger kanagurta) under iced storage and related food safety concern. *Indian J Fish*. 2017;64(4):87-93. doi:10.21077/ijf.2017.64.4.61228-12

23. Klomklao S, Benjakul S, Visessanguan W, Kishimura H, Simpson BK. Effects of the addition of spleen of skipjack tuna (Katsuwonus pelamis) on the liquefaction and characteristics of fish sauce made from sardine (Sardinella gibbosa). *Food Chem*. 2006;98(3):440-452. doi:10.1016/j.foodchem.2005.06.013

24. Deepika S, David R, Salim S, Swathi J, Varghese DM, Azad A. A Review on the Traditional Methods and Novel Techniques for Detection of Formaldehyde Concentration in Fish. *Int J Res Eng Appl Manag*. 2020;(April 2020):286-290.

doi:10.35291/2454-9150.2020.0299

25. Teerasong S, Amornthammarong N, Grudpan K, et al. A Multiple processing hybrid flow system for analysis of formaldehyde contamination in food. *Anal Sci*. 2010;26(5):629-633. doi:10.2116/analsci.26.629

26. Eckert RK, Elliott HW, Elliott KAC, et al. *Science, 110, 279. Happold, F. C. (1950)*. Vol 12.; 1947.

27. Valadares J, Majik MS, Tilve SG. Comparison of the Formaldehyde Content in Indian Mackerel (Rastrelliger kanagurta) Fish Using High Performance Liquid Chromatography and UV-Vis Spectrophotometry. *J Aquat Food Prod Technol*. 2021;30(8):980-987. doi:10.1080/10498850.2021.1961961

28. Rovina K, Vonnie JM, Shaeera SN, Yi SX, Halid NFA. Development of biodegradable hybrid polymer film for detection of formaldehyde in seafood products. *Sens Bio-Sensing Res*. 2020;27:100310. doi:10.1016/j.sbsr.2019.100310

29. Sibirny V, Demkiv O, Klepach H, Honchar T, Gonchar M. Alcohol oxidase- and formaldehyde dehydrogenase-based enzymatic methods for formaldehyde assay in fish food products. *Food Chem*. 2011;127(2):774-779. doi:10.1016/j.foodchem.2010.12.146

30. Delikhoon M, Fazlzadeh M, Sorooshian A, et al. Characteristics and health effects of formaldehyde and acetaldehyde in an urban area in Iran. *Environ Pollut*. 2018;242:938-951. doi:10.1016/j.envpol.2018.07.037

31. Chung SWC, Chan BTP. Trimethylamine oxide, dimethylamine, trimethylamine and formaldehyde levels in main traded fish species in Hong Kong. *Food Addit Contam Part B Surveill*. 2009;2(1):44-51. doi:10.1080/02652030902858921

32. Chen L, Jin H, Xu H, et al. Microwave-assisted extraction coupled online with derivatization, restricted access material cleanup, and high-performance liquid chromatography for determination of formaldehyde in aquatic products. *J Agric Food Chem*. 2009;57(10):3989-3994. doi:10.1021/jf900136x

33. Rovina K, Vonnie JM, Shaeera SN, Yi SX, Halid NFA. Development of biodegradable

hybrid polymer film for detection of formaldehyde in seafood products. *Sens Bio-Sensing Res*. 2020;27. doi:10.1016/j.sbsr.2019.100310

34. Gu DC, Zou MJ, Guo XX, et al. A rapid analytical and quantitative evaluation of formaldehyde in squid based on Tri-step IR and partial least squares (PLS). *Food Chem*. 2017;229:458-463. doi:10.1016/j.foodchem.2017.02.082

35. Kundu A, Dey P, Bera R, et al. Adverse health risk from prolonged consumption of formaldehyde-preserved carps in eastern region of Indian population. *Environ Sci Pollut Res*. 2020;27(14):16415-16425. doi:10.1007/s11356-020-07993-0

36. Zhang D, Zhang J, Li M, et al. A novel miniaturised electrophoretic method for determining formaldehyde and acetaldehyde in food using 2-thiobarbituric acid derivatisation. *Food Chem*. 2011;129(1):206-212. doi:10.1016/j.foodchem.2011.04.025

37. Yasin M, Irawati N, Zaidan AH, et al. Fiber bundle sensor for detection of formaldehyde concentration in fish. *Opt Fiber Technol*. 2019;52(July):101984. doi:10.1016/j.yofte.2019.101984

38. Nurlely, Ahmad M, Yook Heng L, Ling Tan L. Potentiometric enzyme biosensor for rapid determination of formaldehyde based on succinimide-functionalized polyacrylate ion-selective membrane. *Meas J Int Meas Confed*. 2021;175(February):109112. doi:10.1016/j.measurement.2021.109112

39. Anissah U, Ariyani F, Barokah G, Januar HI. NMR Metabolomics of Saurida tumbil Fish Treated with Formaldehyde Solution as Misconduct Food Preservation Method. *J Aquat Food Prod Technol*. 2021;30(3):263-270. doi:10.1080/10498850.2021.1880510

40. Taprab N, Sameenoi Y. Rapid screening of formaldehyde in food using paper-based titration. *Anal Chim Acta*. 2019;1069:66-72. doi:10.1016/j.aca.2019.03.063