DETERMINATION OF NATURAL FORMALIN LEVEL IN SEA FISH COLLECTED FROM SOUTH-EAST COASTAL AREA OF BANGLADESH

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Roll No.: 0115/13

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A thesis submitted in the partial fulfillment of the requirements for the degree of Masters of Science in Applied Human Nutrition and Dietetics

Department of Applied Food Science and Nutrition

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December, 2016

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The author
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List of abbreviations

Words	Abbreviation
%	Percent
2,4-DNPH	2,4-Dinitrophenylhydrazine derivatization
AHMT	4-Amino-3-hydrazino-5-mercapto-1, 2,4-triazol
AOAC	Association of Official Analytical Chemists
BBS	Bangladesh Statistics Bureau
DMA	Dimethylamine
DOF	Department of Fisheries
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GDP	Gross Domestic Product
HCHO-DNPH	Formaldehyde-2,4-DNPH
mg	Milligram
MoFL	Ministry of Fisheries And Livestock
OSHA	Occupational Safety and Health Act
PFBHA	Pentafluorobenzyl-hydroxylamine Hydrochloride
ppm	Parts Per Millions
PRTC	Poultry Research and Training Center
RhB	Rhodamine B
SPME	Solid Phase Microextraction
TMAO	Trimethylamine Oxide
UHPLC	Ultra-High Performance Liquid Chromatography
WHO	World Health Organization
µg	Microgram

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Summary

Formaldehyde is unethically used by some fishermen and fish vendors to preserve the fish and seafood from microbial spoilage. But this compound is produced from the enzymatic reduction of trimethyamine-N-oxide to formaldehyde and dimethylamine during frozen storage and detected by formaldehyde machine by mobile court and impose fine to local seller for that natural formalin content of fish. The present study was conducted to assess the quantitative analysis of natural formalin level in sea fish collected from south-east coastal area of Bangladesh by Ultra-high performance liquid chromatography (UHPLC) method. Most abundant fish species found in South-east Coastal Area of Bangladesh namely Loitta (Harpadon Nehereus), koral (Lates Calcarifur), Rupchanda (Pampus Chinensis), Hilsha (Tenualosa Ilisha), Poa (Pama Pama) and Maittya (Euthynnus Affinis) were collected from Chittagong (Fishery ghat), Sandwip, Hatiya and Cox's Bazar. Freshly caught samples were evaluated for determination of formaldehyde concentration in UHPLC. Peak area from the UHPLC for each sample gives the corresponding formaldehyde concentration compare with the calibration curve. Mean formaldehyde content in different fishes collected from different locations of Bangladesh was estimated from the absorbance and molar concentrations of standard curve. From the present experiment it was evident that fishes viz. Loitta (*Harpadon Nehereus*), koral (*Lates Calcarifur*), Rupchanda (*Pampus Chinensis*), Hilsha (*Tenualosa Ilisha*), Poa (*Pama Pama*) and Maittya (*Euthynnus Affinis*) showed a range of 41.5±0.15811 to 54.28±0.54037 mg/kg formaldehyde at initial. After 2 hours the formaldehyde content were found in the ranges of 53.38±0.75961 to 57.18±0.52154 mg/kg. Based on the result of present study, there were significant differences in the concentration of natural formaldehyde between fish samples at initial and after 2 hours of storage at room temperature. The natural formaldehyde concentration of all fish sample except Koral-1 increases with elapsing of time at room temperature. The present study will differentiate the content of natural formaldehyde and adulterated formaldehyde in fish that is produced in fish muscle by enzymatic conversion of trimethylamine oxide breaks into formaldehyde and dimethylamine in equal parts after the fish dies.

Keywords:

Natural Formaldehyde, Fish, UHPLC

Chapter-I: Introduction

Since the very ancient time of history humans have been eating seafood as for their palatability and nutritional aspects. Fish contributes to the growth and health of living body by providing important nutrients such as protein, lipids, minerals and other essential chemicals. Brain selective nutrients such as omega-3 fatty acids, omega-6 fatty acids, omega-9 fatty acids, iodine and iron are found in sea fish in a higher amount; those reduce the risk of subnormal brain development and also reduce the risk of heart disease, strokes, cancer and some other serious ailments (Cunnane and Stewart, 2010).

Fish and seafood are an important part of a healthy diet and are considered as the biggest source of protein (Ashie et al., 1996). The role of fisheries and livestock sectors in the development of agro-based economy of Bangladesh is very important and promising. According to Food and Agriculture Organization (FAO), Bangladesh has been among the top five fish cultivation countries for many years. In 2006 it jumped to second position, overtaking India. From 2004 till 2014, Bangladesh's fish production increased by 53%. Bangladesh Statistics Bureau (BBS)'s latest economic census says that in the 2013-14 fiscal, the country produced approximately 3.46million tonnes of fish, of which about 2million tonnes were farmed. With the protection of hilsa fries and other initiatives, production of the country's most popular fish hilsa has gone up from 52,000 tonnes to 350,000 tonnes. With prices of fish remaining within the reach of the common people, there has been a 100% increased in per head consumption of fish over the past 10 years. According to a survey of 2010, the annual per head consumption of fish in Bangladesh is 12kg. The people of Chittagong consume the most fish at an annual 17kg per head and the least is in Rangpur at 7.5kb per head. Annual fish consumption globally is 22.4kg per head. They contribute around 8% to national income, which also is 32% of the total agricultural income. About 90% of animal protein in our diet comes from fish and livestock (MoFL, 2011). The fisheries sector contributes 5.10%, of the country's export earnings, 4.91% of its gross domestic product (GDP) and provides 63% of the national animal protein consumption. Fish and fishery products are the country's third largest export commodity contributing 5.10% of its exchange earnings.

According to fisheries department, the country produced about 3.5 million tonnes of fish in the 2013-14. Of this, 2.9 million tonnes came from farms and catches from various Inland water sources, and 0.6 million tonnes were from the sea. It is claimed that the total fish production has increased significantly over the last few decades (DoF, 2009) but it is not sufficient to meet up the growing demand of the country (Yeasmin et al., 2010). As a result imported fishes from neighboring countries enter in the domestic market and it was reported that more than 80 metric ton of fish and fishery products enter into Bangladesh every day through the Teknaf border from Myanmar (Kibria, 2007). Available reports suggest that formalin is sometimes added or sprayed to the fishes by the fish traders while transporting to domestic marketing chain to prevent spoilage and increase shelf life (Yeasmin et al., 2010a). Studies conducted at different markets in Dhaka city (Hossain et al., 2008; Haque and Mohsin, 2009) and Mymensingh Sadar (Yeasmin et al., 2010a) rationalizes the incidence of adding formaldehyde/formalin to fishes especially imported from neighboring countries.

Fish is highly nutritive. It is tasty because of its constituents. The main components of fish are water, protein and fat. The spoilage of fish is a complicated process brought about by actions of enzymes, bacteria and chemical constituents. The spoilage process starts immediately after the death of fish. The process involves three stages. Rigor mortis is a physical effect on the muscle tissue of fish caused by chemical changes following the death. In live fish, its movements are controlled by chemical signals which cause the eythmic contraction (stiffing) and relaxation of the muscles. This produces swimming action. After the death, the normal circulatory system breaks down and chemical signals leak into the muscle causing them to stiffen. This process is known as Rigor Mortis. In other words, in live fish the glycogen present in the muscle is converted to carbon dioxide and water after supply of oxygen to the cells. After the death of fish the blood circulation stops and the supply of oxygen is prevented. The enzymes present in the muscle convert glycogen into lactic acid. The pH of the fish muscle falls. The formation of the lactic acid continues till the supply of glycogen is completely used up. After the completion of rigor mortis, muscle stiffness gradually decreases accompanied by increase in pH, ending up in softening of muscle. This is followed by breakdown of proteins by enzymes. This process is called as autolysis. Freshly caught fish will be almost free from bacteria but the

surface slime, gills and intestine may contain considerable load of bacteria. When the fish is dead, these bacteria start attacking the flesh causing spoilage and produce undesirable compounds. The nature and type of bacteria present in a fish depends upon the water from where it is caught and methods used for handling of the fish after its catch. The activity of organism can be controlled, reduced or even retarded by proper handling and immediate lowering of the temperature. The spoilage is reduced or prevented by applying different processing and preservation method: drying, salting, chilling, canning, curing, freezing, microwave heating, pulsed electric field processing, radio frequency electric field processing and ultrasound. In order to keep the freshness of fish and seafood, fishermen and fish vendors tend to carelessly use formaldehyde as preservation agent.

Formaldehyde is easily flammable, colorless and readily polymerized gas at ambient temperature. The most common commercially available form is a 30-50% in aqueous solution (WHO, 1989). Formaldehyde is the most widespread carbonyl compound and it is widely used in consumer goods to protect the products from spoilage by microbial contamination. Formaldehyde is often added to keep food pleasing to the consumers, but this chemical poses a hazard threat to human health (Cui et al., 2007).

In food industry, it is widely used in food processing as for its bleaching effect and also as preservative in order to prevent the product from spoilage by microbial contamination especially seafood (Wang et al., 2007). Formalin which contains 37% formaldehyde has been used as a therapeutant to control ectoparasites and aquatic fungi disease events occurring at fish culture facilities (Schnick, 1991; Rach et al., 1997). Commonly, the fish are dipped in a formaldehyde bath for this purpose (Greg et al., 2003). Its residues in the food for human consumption are proscribed because of possible carcinogenicity (Jung et al., 2001).

There are 3 commercial formaldehyde products approved by US Food and Drug Administration (FDA), which have similar formulations of about 37% formaldehyde, for use in US aquaculture as parasiticides: Parasite-S (for use on all finfish and penaeid shrimp; Western Chemical), Paracide-F (for use on bluegill, catfish, largemouth bass, salmon and trout; Argent Chemical Laboratories) and Formalin-F (for use on bluegill, catfish, largemouth bass, salmon and trout; Natchez Animal Supply (FDA, 1998). According to the label recommendations, routine treatment

concentrations of formalin range from 15-250 mg L^{-1} for control of protozoan and monogenetic trematodes on fish and shrimp and up to 2000 mg L^{-1} for control of fungi on fish eggs (Jung et al., 2001).

This compound is also produced from the enzymatic reduction of trimethyamine-N-oxide to formaldehyde and dimethylamine during frozen storage and it causes protein denaturation and muscle toughness (Bianchi et al., 2007; Sotelo et al., 1995). Moreover, it result in the loss of food quality because of unacceptable texture, undesirable flavour, odour, colour and its harm for consumers. Formaldehyde accumulates during the frozen storage of some fish species, including cod, pollack and haddock (Sotelo et al., 1995). Deterioration in quality due to micro-organisms and various biochemical processes is nearly eliminated, but some enzymatic activities cause changes in the products of frozen fish. These changes are of great commercial importance, because they are limiting factor for the shelf-life of frozen seafood (Benchman, 1996).

From previous studies, formaldehyde has been detected as a result of postmortem change in the tissues of cod (Gadus macrocephalus), Alaska pollock (Theragra chalcogramma), blue shimp (Penaeus stylirostris) and pacific shrimp (Pandalus jordani) (Amano and Yamada, 1964; Flores and Crawford, 1973; Hose and Lightner, 1980). Endogenous formaldehyde residues ranging from 0.1-31.8 μg g^{-1} were detected in several species including eel (Anguilla japonica) (Ueno et al., 1984), striped bass (Morone saxatilis) (Xu and Rogers, 1995), banana shimp (Penaeus merguiensis) (Yamagata and Low, 1995) and Nile tilapia (Tilapia niloticus) (Xu and Rogers, 1995). The highest level of formaldehyde (e.g. 10-20 mg kg^{-1}) in fish may not be considered as palatable as human food source (Yasuhara and Shibamoto, 1995). An acceptable daily intake of 0.2 mg kg^{-1} body weight has been set by the United States Environment Protection Agency, whereas, values of 60 mg kg^{-1} for Gadidae and crustaceans, respectively were proposed in 1985 by the Italian Ministry of Health (Bianchi et al., 2007).

In recent time formalin is a panic word in Bangladesh. Fishes are the one of the major source of food and protein to human being. In Bangladesh it is called as a national food. Almost every person is taking fishes more or less every day. But now a days, this consumable fishes are being contaminated by formalin by some evil traders. But

some fishes contain formalin in their fleshes naturally which are detected by formaldehyde machine by mobile court and impose fine to local seller for that natural formalin content of fish.

1.1 Aim of the Study:

1. To determine the level of natural formalin in sea fish.

1.2 Objectives of the Study:

1. To determine the level of formalin in sea fish fluctuate with time.
2. To determine the corresponding change of natural formalin with time among different species of sea fish

Chapter-2: Review of Literature

2.1 Fish and Sea Fish

Fish cold-blooded, limbless, completely aquatic vertebrates, having gills, commonly fins, and typically elongated torpedo-shaped body mostly covered with scales. Seafish is considered as a main source of high biological value protein, polyunsaturated oil and minerals such as calcium, potassium, fish form an important target for bio-magnifications of heavy metals as they are at the top of food pyramid and act as a possible transfer media to human beings

Bangladesh is a country with hundreds of rivers and ponds and is notable for being a fish-loving nation, acquiring the name "Machh-e Bhat-e Bangali" which means, "Bengali by fish and rice". According to fisheries department, the country produced about 3.5 million tonnes of fish in the 2013-14. Of this, 2.9 million tonnes came from farms and catches from various Inland water sources, and 0.6 million tonnes were from the sea (DoF, 2016). Among 32,000 species of fishes worldwide and almost 40% of the species live in fresh water. 401 species of marine fishes and 251 species of inland fishes (in freshwaters and brackish waters) is found in Bangladesh.

Fish play a crucial role in the Bangladeshi diet, providing more than 60% of animal source food, representing a crucial source of micro-nutrients, and possessing an extremely strong cultural attachment. Fish (including shrimp and prawn) is the second most valuable agricultural crop, and its production contributes to the livelihoods and employment of millions. The culture and consumption of fish therefore has important implications for national food and nutrition security, poverty and growth.

2.2 Methods used to determine formaldehyde in sea fish

Fish and fish products play an important role in human nutrition as a source of biologically-valuable proteins, fats and fat-soluble vitamins and frozen and fresh fish are the most commercialized products. There are different methods used to determine formaldehyde content of seafood maintained under different conditions.

Spectrophotometer methods used were Nash mehod, 4-Amino-3-hydrazino-5-mercapto-1, 2,4-triazol (AHMT) method and Kinetic spectrophotometric method. In Nash method the occurrence of Hantzsch reaction between acetylacetone, ammonia

and formaldehyde to form 3,5-diacetyl-1,4-dihydrolutidine (λmax = 412 nm). To complete the reaction, the procedure requires pH adjustment with ammonium acetate and heating.

In 4-Amino-3-hydrazino-5-mercapto-1, 2,4-triazol (AHMT) method procedure determines formaldehyde in fish, olive flounder (Paralichthys olivaceus) and black rockfish (Sebastes schlegeli). Two milliliters of deionized water as control and 2 mL of the extract are pipetted into separate tubes and 2 mL of 5 N KOH are added to each tube. Then 2 mL of 0.5% AHMT dissolved in 0.5 N HCl and the solutions are gently mixed. A stopper is put on each test tube, which is allowed to stand for 20 min at room temperature. Afterwards, 2 mL of 0.75% KIO solution prepared in 0.2 N KOH 4 are added. The mixture is shaken gently and by spectrophotometry (DMS 80 UV-visible, Varian, England). The absorbance of the violet color is read at 550 nm. The amount of formaldehyde in fish (μg g^{-1} or μg mL^{-1}) is calculated from standard curves (Jung et al., 2001).

Spectrophotometric catalytic kinetic methods are based on the catalytic effect of the element upon the reactions whether in colored (Vis) or colorless (Dirksen et al., 2001) solutions (Rancic et al., 2005). Rhodamine B (RhB) compounds are a group of xanthene dyes that have been widely used in analytical chemistry and the photophysical properties of rhodamines in solution have been extensively studied. Formaldehyde could act as an effective catalyst for the catalysis of formaldehyde in the developed system. The reaction rate was spectrophotometrically monitored by measuring the decrease colour from purple to colorless in the absorbance at 515 nm (Lazrus et al., 1988). The catalytic effect of formaldehyde on the indicator reaction between RhB and potassium bromate is very sensitive. The method has a detection limit as low as 2.90 μg L-1 and an analytical working range of 10-100 μg L-1.

High performance liquid chromatography method is based on steam distillation and 2,4-Dinitrophenylhydrazine derivatization (2,4-DNPH). Formaldehyde is analyzed by HPLC using ODS-C18 column at UV detector (355 nm) and have been applied to squid (Li et al., 2007a). By this method, the formaldehyde content of squid muscle and viscera, dried squid thread and boiled squid were determined.

The formaldehyde content in different fish products is evaluated using a Solid Phase Microextraction (SPME)-GC-MS method based on fiber derivatisation with

pentafluorobenzyl-hydroxyl-amine hydrochloride (Bianchi et al., 2007). As formaldehyde is a very volatile compound, solvent-free techniques like Solid Phase Microextraction (SPME) can be easily applied for the analysis of organic compounds especially formaldehyde, thus combining sampling and preconcentration in a single step (Pawliszyn, 1997). An innovative method based on SPME with in situ derivatisation with Pentafluorobenzyl-hydroxylamine Hydrochloride (PFBHA) have been developed and validated for the determination of formaldehyde at ultratrace levels in frozen fish samples (Bianchi et al., 2005).

Electronic noses have been applied in lots of applications (Ampuero and Bosset, 2003; Zhang et al., 2005, 2006), especially in the quality control of food industry (Haugen et al., 2006; Marilley and Casey, 2004; Marti et al., 2005) and food safety detection (Magan and Evans, 2000; Needham et al., 2005; Rajamäki et al., 2006).

2.3 Research conducted on various aspects of formaldehyde in fishes

As a product of normal metabolism, formaldehyde has been documented to be naturally present in many common food items, including fruits and vegetables, meats, fish, crustacea and dried mushrooms etc., at a wide range of levels. In some seafood species, formaldehyde is a natural breakdown product of a chemical known as trimethylamine oxide (TMAO) that exists in their bodies. Trimethylamine oxide breaks down into formaldehyde and dimethylamine in equal parts after the animal dies. The level of formaldehyde can accumulate in certain marine fish during frozen storage and crustacea after death. A search of literature revealed that little work has been done in the past on the determination of formaldehyde in fish The literature reviewed here is based on the study conducted elsewhere various aspects of formaldehyde in fishes.

Formalin is widely used compounds in fish culture for therapeutic and prophylactic treatment of fungal infection and external parasites of fish and fish egg. Schick (1974) reviewed uses of formalin in fish culture. Phelps (1975) and Bill et al. (1977) studied toxicities of formalin to fishes in water.

Parkin and Hultin, (1982) reported that Nash's reagent method can cause the interferences during the extraction of formaldehyde. The trichloroacetic acid used in

Nash's reagent method could interfere the formaldehyde determination due to the presence of ferrous or ferric ion.

Rehbein et al. (1995) reported that high amounts of FA result in tough texture and low water binding capacity.

Floyd (1996) reported that formalin removed the oxygen present in fish. If 5 mg/l of formalin is used, 1 mg/l of dissolved oxygen will be excluded. In addition it also reduced the ability of algae to produce oxygen in water treated with formalin. Lately, public concerns towards the usage of formalin in seafood and its toxicity increased. Formalin is approved by FDA and can used onto fish eggs at a concentration range of 1000 mg/l or 2000 mg/l. Occupational Safety and Health Act (OSHA) (1999) reported on AOAC 931.08 method used concentrated sulphuric acid and bromine while Nash's method used trichloroacetic acid. The used of these tree compounds has been shortlisted as hazardous chemicals where the threshold limit values for bromine is 0.1 while for both phosphoric and sulphuric acid is 1.

Yildiz et al., (1999) studies on the secondary stress in healthy Nile tilapia after treatment with mixture of formalin, malachite green and methyl blue, plasma phosphorus levels dropped after FMC treatment and calcium level in general were lower than those of the controls. Magnesium levels were not influenced by FMC treatment.

Kozinska and Antychowicz (2000) studies on immunization of carp (Cyprinuscarpio) against Aeromonas. They reported that the effectiveness of vaccination with the antigens inactivated with formalin or heat, and given i.p. or by immersion (imm), were compared. Fish developed a significantly higher level of immunity after the administration of formalin antigen than heatinactivated antigen.

Jung et al. (2000) studies on effect of formalin on hematological and blood chemistry in olive flounder, Paralichthysolivaceus (Temminck et Schlegel). They reported that formalin exposure also caused significant increases in alkaline phosphate, lactate dehydrogenates, potassium, chloride, magnesium and inorganic phosphorus. However total protein decreased significantly in the formalin exposure group ($p<0.05$).

Jaafer et al. (2000) studies on formaldehyde formed in marine fish caused by several factors such as, the amount of dark muscle tissue, the quantity of substrate present,

temperature, cofactors, storage time and the degree of combination of the flesh. Moreover, he reported that most of people are not able to tolerate air containing formaldehyde which is more than 10 ppm. Thus OSHA's promulgated that the safety level of formaldehyde in air at working area must not more than 3 ppm. However, the formaldehyde content added onto fish is still a concern as consumers are more aware of food safety.

Jung et. al., (2001) reported that a scientific relevant database suitable for determination of an appropriate withdrawal time and environmental risk assessment for therapeutic use of formalin in the aquaculture industry of marine finfish. Keck and Blane (2002) studies on effect of formalin chemotherapeutic treatments on biofilter efficiency in marine recirculation fish farming system. They reported that formalin (33-38%) aqueous formaldehyde solution is currently used for bath treatments to control ecto-parasitic infections of fish. Its effects on nitrification wereevaluation in a semi-closed pilot scale saltwater re-circulating culture system. Repeated treatments may be hazardous for nitrifying bacteria, which could induce an increase in nitric concentration.

Onusiriuka (2002) studies on effect of sub lethal concentration of formalin on weight gain in the African Catfish, Clariasgariepinus. He reported that decreasing in weight gain, directly proportional to the toxicant concentration, was observed in fish exposed to concentrations above 3.12 mg/l. There was significant difference in weight gain of fish exposed to the various toxicants and the fish in the control tank ($p<0.05$). Though formalin is recommended for ectoparasitic infection of farmed fishes, its used in fish farms must therefore be under stipulated controlled procedures. Threshold values should be determined for the fish cultured.

According to Chu et. al., (2003), RSM is a method use which has the ability to improve the results of experiment by changing the value of test variables that control the process in order to obtain the optimum condition. Fajer-Avila etal. (2003) reported that safety of formalin to bullseye puffer fish and determine the efficiency of formalin to control parasitic infection. The difference in toxicity between the bulls' eye puffer fish and parasites indicated that formalin was efficacious in the control of parasitic epizootics in bulls eye puffer fish culture.

Shahoo (2003) studies on the immunomodulatory effect of dietary administration of 3, 3, 5- triiodo thyronine in rohu (Labeorohita). He reported that a significantly higher specific antibody titre against formalin killed Aeromonas hydrophilaand lowering the mortality percentage against A. hydrophilachallenge. He also reported that oral administration of T3 at all dose levels resulted in significantly ($p<0.05$) higher serum levels, total serum protein and globulin levels and reduced albumin-globulin ratio (A: G) compared with the control group.

Kodama et al. (2004) reported that changes of serum c-reactive protein (CRP) levels in rainbow trout (Oncorhynchusmykiss) after exposure to formalin, metriphonate or potassium permanganate, which are used in aquaculture as anti-ectoparasitic chemicals. At 18 days after treatment, the CRP level had decreased too significantly below the normal level. Measurement of CRP levels in trout serum can be used as a bio-indicator of the health condition of the fish.

Araujo et al, (2004) studies on effect of formalin therapeutic bath on stress of indicator in tambaqui. They reported that formalin has been used to control fish diseases; however there is little on the secondary effects of this chemical on the fish. Fish did not show signs of stress in100mg/l formalin concentration at all exposure times. In 200 and 250 mg/l formalin concentrations significantly increased blood glucose level after 30 min exposure.

Buchmann et al. (2004) studies on effect of formalin treatment on epithelial structure and mucous cell densities in rainbow trout. They reported that exposure of rainbow trout fry 2 months post-hatching to various concentrations of formalin affected the mucous cell dynamics of fish. Limited exposure (50 ppm, 1 h) stimulated mucous cell proliferation as indicated by the slight increase in densities of Alcian blue positive cells in tail fin epidermis. In contrast, high concentrations (200-300 ppm, 1 h) or lengthy exposure (24) at lower concentrations caused a decrease in mucous cell density. The implications of formalin bating for susceptibility or resistance of fish to subsequent pathogen exposure are discussed.

In year 2004, International Agency for Research on Cancer (IARC) classified formalin as toxicity value of group 1 where it is known as to be carcinogenic to humans. Bianchi et al. (2005) reported that an innovative method based on SPME with in situ derivation with Pentafluorobenzyl-hydroxylamine Hydrochloride

(PFBHA) have been developed and validated for the determination of formaldehyde at ultratrace levels in frozen fish samples.

Bianchi et al. (2007) reported that the eyes are the most sensitive part when exposed to formalin. Biachan et al. (2007) studies on determination of formaldehyde (FA) content in different fish products was evaluated using a solid phase microextraction (SPME)-GC-MS method based on fiber derivatisation with pentafluorobenzyl-hydroxyl-amine hydrochloride. Hhigher formaldehyde levels were founds in species belonging to the Gadidae family, whereas, freshwater fish as well as crustaceans were generally characterized by lower values. Using this method, LOD and LOQ values of 17 and 28 μg/kg, respectively were calculated.

Yeasmin et al. (2010) studies on many commercially important fishes such as catla and rohu were imported from neighboring countries and sold in the retail markets. Study showed that formalin was not detected in any fish produced locally, but was detected in the importedones of catla and rohu ranging from 0.5% to 1% which was sold in different markets of Mymensingh with comparatively lower price than those produced locally. The survey also indicated that the overall hygienic condition of the retailers and sanitary conditions of surveyed markets were poor except KR and SP fish markets, where both sanitary and hygiene conditions were found to be in acceptable condition.

Uddin et al. (2011) studies that fish items in Bangladesh contain formalin which is a highly hazardous and carcinogenic chemical. An attempt was taken to detect the extent of formalin use in fish available in Dhaka city. From five different local markets five species of fishes were collected and presence of formalin was detected using the “formalin detection kit in fish” developed by Bangladesh Council of Scientific and Industrial Research (BCSIR). The study indicates that 70% rohu fish was formalin contaminated and almost 50% of fish samples contain formalin.

Rahman et al. (2012) studied were conducted on the detection of formalin on fish obtained from different markets by formalin detection kit. They reported that 26 formalin treated fish out of 150 samples in which 16% in Modina Market, 26% in Ambar Khana, 13% in Lal Bazar, 23% in Kazir Bazar and 6% in Tucker Bazar.

Joshi et al. (2014) determine the Formaldehyde content of selected fish from the wet markets of Kathmandu valley. From three different local markets, six species of fishes were collected and quantitative determination of formaldehyde was performed using UV-Vis spectrophotometer. Formaldehyde content was found from 0.393±0.004 μg g^{-1} to 2.328±0.304 μg g^{-1}. Of the species analyzed Magur contained the highest concentration of formaldehyde (2.328±0.304 μg g^{-1}).

Jaman et al. (2016) determine formaldehyde concentration of freshly caught samples: freshwater fish viz. Indian major carp, rohu (Labeo rohita), tilapia (Oreochromis nilotica), SIS, kachki (Corica soborna), climbing perch, Thai koi (Anabus testidineus), and marine fish viz. Bombay duck, loyitta (Harpodon nehereus), ribbon fish, chhuri (Lepturacanthus savala) from market. Formaldehyde conc. obtained in fishes from three different wet markets of Mymensingh Sadar ranged between 1.4 t 7.35 μg/g. On the other hand, formaldehyde conc. in fresh fishes, rohu, tilapia and Thai koi collected from ponds of Freshwater Station, BFRI, Mymensingh showed natural formaldehyde in their muscle having values of 1.45; 1.85 and 2.60 μg/g respectively. The marine fish viz. loyitta and chhur collected from the landing center of BFDC at Cox's Bazar and investigation in frozen condition showed to contain naturally occurring formaldehyde as 3.9 and 1.55 μg/g respectively.

Studies were also conducted on the quantitative changes of bacterial load in fish. It was observed that in formalin treated fish, there was a chance of washing out of formalin from the surface of the fish body along with melting of ice.

Chapter-3: Materials and Methods

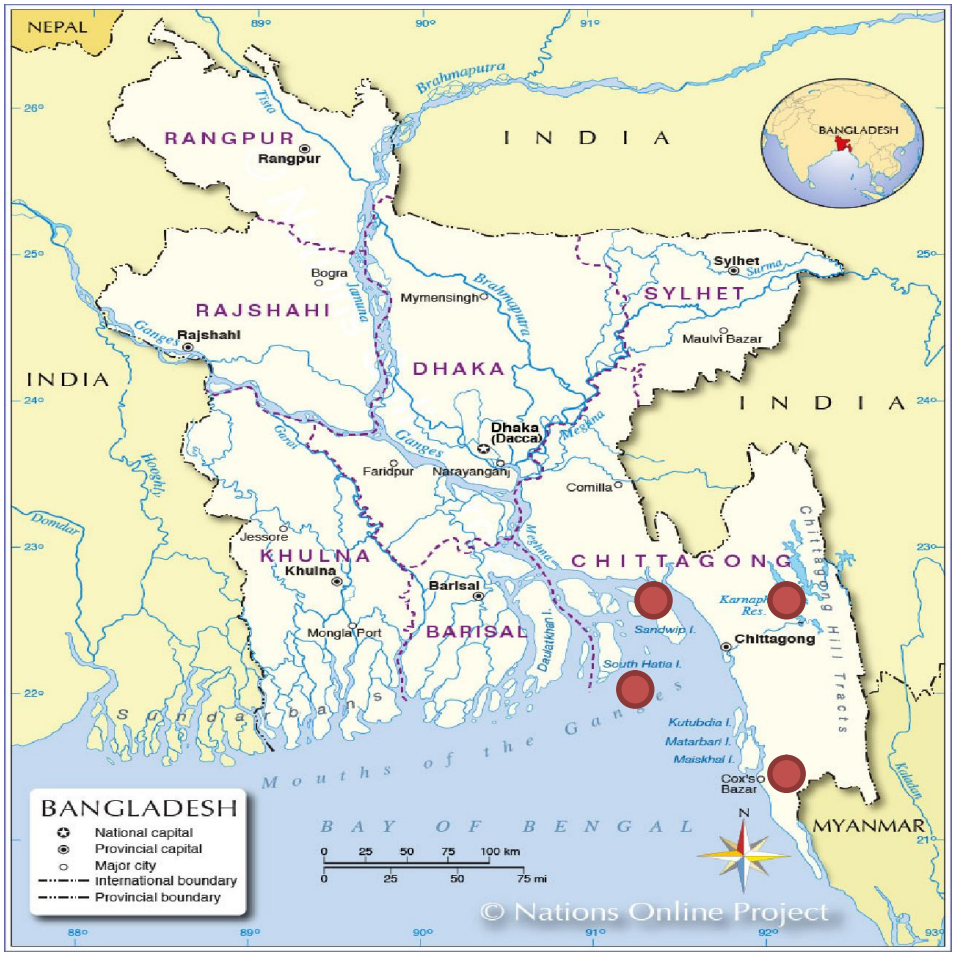
3.1 Location and study period

The experiment was conducted in the laboratory of Poultry Research and Training Center (PRTC), Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh. The experiment was conducted for a period of six months from 1st January, 2016 to 30th June, 2016.

3.2 Collection of fish sample

Totally 36 fish samples were collected from Chittagong (Fishery ghat), Sandwip, Hatiya and Cox's Bazar. Immediate after collection the fishes were frozen and brought to the laboratory in insulated box which took about 12 hours journey. Then the fishes

were kept frozen for about 2-3 days until the detection of formaldehyde.



Sampling Location

Figure 1. Fish Sampling Location in Bangladesh

3.3 Selection of fish sample

The most abundant fish species found in South-east Coastal Area of Bangladesh namely Loitta (*Harpadon Nehereus*), koral (*Lates Calcarifur*), Rupchanda (*Pampus Chinensis*), Hilsha (*Tenualosa Ilisha*), Poa (*Pama Pama*) and Maittya (*Euthynnus Affinis*) were collected.

3.4 Chemical Reagents used:

Acetonitrile and ethanol (HPLC grade)

Acetonitrile and ethanol (HPLC grade) were obtained from Aldrich (Milwaukee, WI) and Merck (Darmstadt, Germany). Purified water was obtained by distillation and filtration through an E-pure Alltech (Deerfield, IL) system. The other reagents were of analytical grade.

2,4- dinitrophenylhydrazine (DNPH)

The 2,4-DNPHi solution (pH = 1.85) was prepared at 0.05% (w/v) in acetonitrile–H_2O–H_3PO_4 (20:79:1, v/v/v) and then purified by liquid–liquid discontinued extraction with CCl_4. It was stored at 4°C in total darkness. The purity of the solution was verified by HPLC–UV analysis. A more detailed account of reagent preparation can be found elsewhere.

Perchloric acid, Dichloromethane, phosphoric acid and methanol

Perchloric acid, dichloromethane and phosphoric acid were analytical grade, and methanol was HPLC grade purchased from TEDIA.

Formaldehyde standard

Formaldehyde 37% wt. % solution in water, A.C.S. reagent, was purchased from Sigma Aldrich (St. Louis, MO).

3.5 Sample preparation:

The frozen fish were thaw at room temperature and then filleted, minced and homogenized. For determining the formaldehyde content, 10g mince sample, 200mL

water and 10mL 10 percent weight phosphoric acid were mixed and distilled, then the distilling liquid was collected about 100mL. For extraction of the formaldehyde, 4 mL of distilling liquid was added into the mixer of 1 mL of 0.4% 2,4 DNPH solution and 50 l perchloric acid solution. Then stirred for 45 minutes with dichloromethane. After vigorous mixed and dehydrated, dichloromethane was volatilized and was re-dissolved with 1mL methanol. The mixture was filtered through a 0.45um HV filter before injection. For each sample three replicates were analyzed. Results were expressed as mg of formaldehyde /kg.

3.6 Quantitative analysis of formaldehyde

3.6.1 Standard curve establishment

The standard formaldehyde solution containing 0.005, 0.01, 0.015, 0.02, 0.05 mg/L formaldehyde concentration was prepared. Three injections of each standard solution were made and the corresponding peak area 558908, 1117800, 1676735, 2235600, 5589500 were obtained to establish the calibration curve.

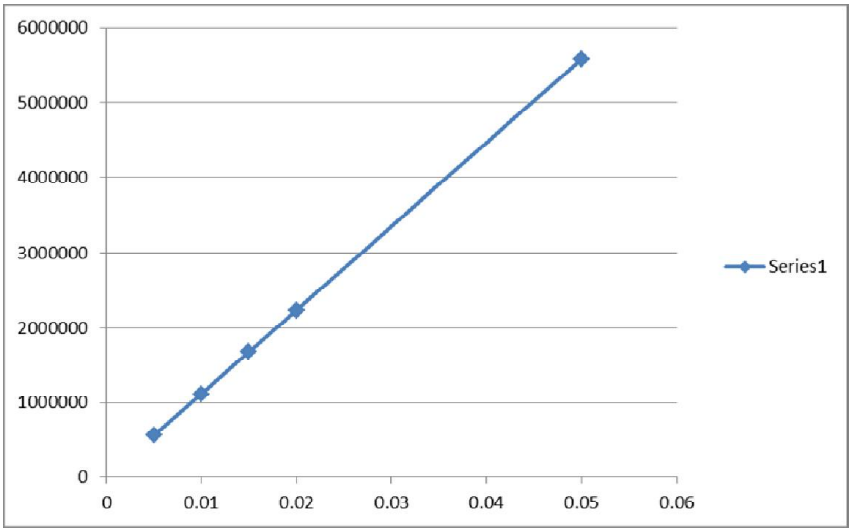


Figure 2. Standard curve of formaldehyde concentration calculated as on the basis of absorbance vs molar conc.

3.6.2 High Performance Liquid Chromatography (HPLC) analysis method

The high-performance liquid chromatography (HPLC) system was Agilent 1100 HPLC instrument (Agilent Technologies, USA), which consisted of a pump, a column chamber, a VU detect, and an Agilent ChemStation for LC system.

The HPLC column was a Hypersil ODS2-C18, 5µm, 150mm ×4.6mm, DAD Lamp mode: D2. Syringe volume of autosampler is 100µl and Autosampler Temperature and Column Oven Temperature are 20°C and 30°C respectively. The sample volume was set at 15µl and the absorb wavelength of detector was set at 365nm. The Mobile phase is the mixture of A: Acetonitrile, B: Deionized water And mobile phase ratio is (A:B=70:30, v/v) with a flow rate of 0.5 ml/min. The peak area was used for quantitative calculation of formaldehyde.

3.7 Statistical Analysis

The obtained data were stored in Microsoft Excel 2007 and then exported into SPSS Version 17.0 software (SPSS Inc., USA) for statistical analysis. Descriptive analysis was performed by using percentages, mean and standard deviation for different variables. Finally one -way ANOVA was used to compare the level of Natural Formalin Level in Sea Fish Collected from South-east Coastal Area of Bangladesh. The level of significance was set ≤ 0.05.

Chapter- 4: Results

Figure 3 showed chromatogram of formaldehyde standard solution by HPLC. Two main chromatographic peaks were found in the 2.829 min and 5.377min respectively. Only one peak in 2.829 min was appeared in blank solution, the other peak in 5.377min was considered to be a derivatized product of Formaldehyde-2,4-DNPH (HCHO-DNPH) in the formaldehyde standard solution.

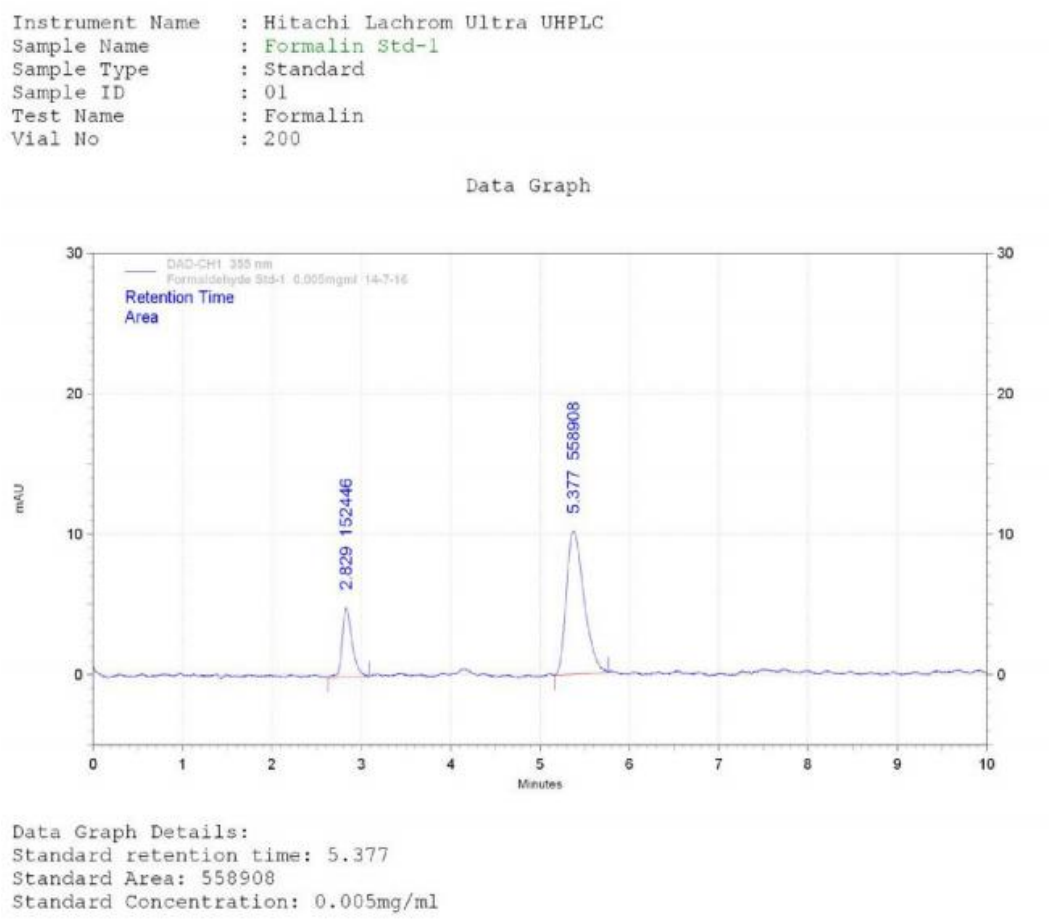


Figure 3. Typical chromatogram of formaldehyde standard solution

Mean formaldehyde content in different fishes collected from different locations of Bangladesh was estimated from the absorbance and molar concentrations of standard curve. The formaldehyde content different fishes from different locations were presented in Table 1.

Table 1. Formaldehyde content in fish sample

SL No	Sample Name	Formalin Concentration (mg/ml)		P value
		Initial Value ±SD	After 2hours Value ±SD	
1	Loitta	41.5±0.15811	54.40±0.29155	<0.0001
3	Koral	52.58±0.67602	53.38±0.75961	<0.11
5	Poya	51.58±73959	55.06±0.64265	<0.0001
7	Hilsa	42.5±0.62048	55.38±0.70498	<0.0001
9	Maitta	52.30±0.38079	57.18±0.52154	<0.0001
11	Rupchanda	54.28±0.54037	56.42±0.57619	<0.0001

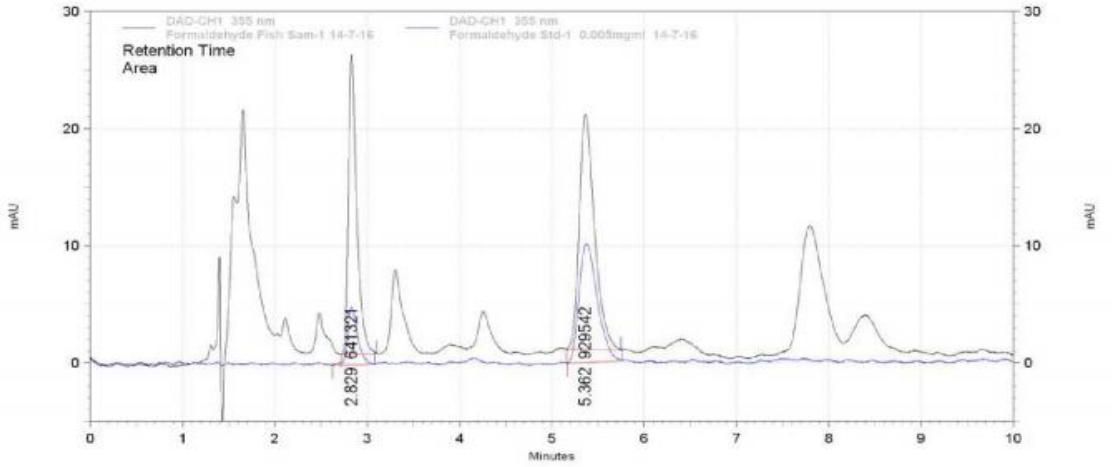
The Chromatogram obtained by HPLC for formaldehyde content of different fishes were presented in Figure 4-10.

From the present experiment it was evident that fishes *viz.* Loitta (*Harpadon Nehereus*), koral (*Lates Calcarifur*), Rupchanda (*Pampus Chinensis*), Hilsha (*Tenualosa Ilisha*), Poa (*Pama Pama*) and Maittya (*Euthynnus Affinis*) showed a range of 41.5±0.15811 to 54.28±0.54037 mg/kg formaldehyde at initial. After 2 hours the formaldehyde content were found in the ranges of 53.38±0.75961 to 57.18±0.52154 mg/kg

From the standard curve, the result obtained that the Loitta fish collected from the mentioned locations were found mean formaldehyde conc. of 41.5±0.15811mg/kg at initial and 54.40±0.29155 mg/kg after 2 hour interval (Table1). There were no significant difference of formaldehyde conc. in Loitta fishes from different location of Bangladesh.

Sample Name : **Loitta-1**
Sample Type : Marine fish
Sample ID : 02
Vial No : 199

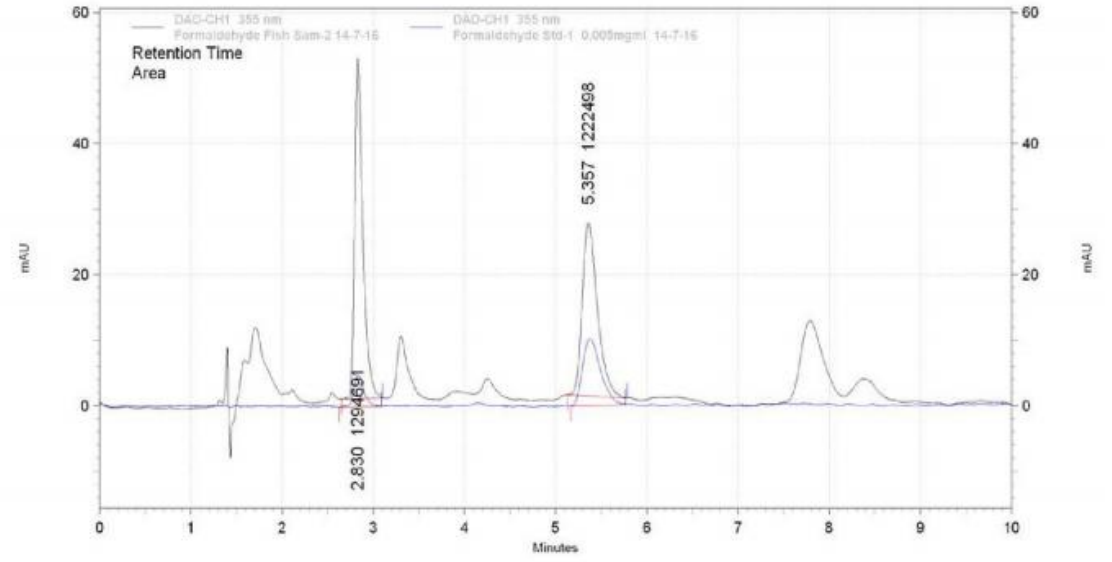
Data Graph



Data Graph Details:
Sample retention time: 5.362
Sample Area: 929542
Loitta-1 Sample Concentration: 0.0083mg/ml

Sample Name : **Loitta-2**
Sample Type : Marine fish
Sample ID : 03
Vial No : 198

Data Graph



Data Graph Details:
Sample retention time: 5.357
Sample Area: 1222498
Loitta-2 Sample Concentration: 0.0109mg/ml

Figure 4. Typical chromatogram of Loitta fish.

The estimated mean formaldehyde conc. of Maitta fish were 52.30±0.38079 mg/kg at initial and 57.18±0.52154 mg/kg after 2 hour interval (Table1). There were no significant differences of formaldehyde conc. in Maitta fish from different location of Bangladesh.

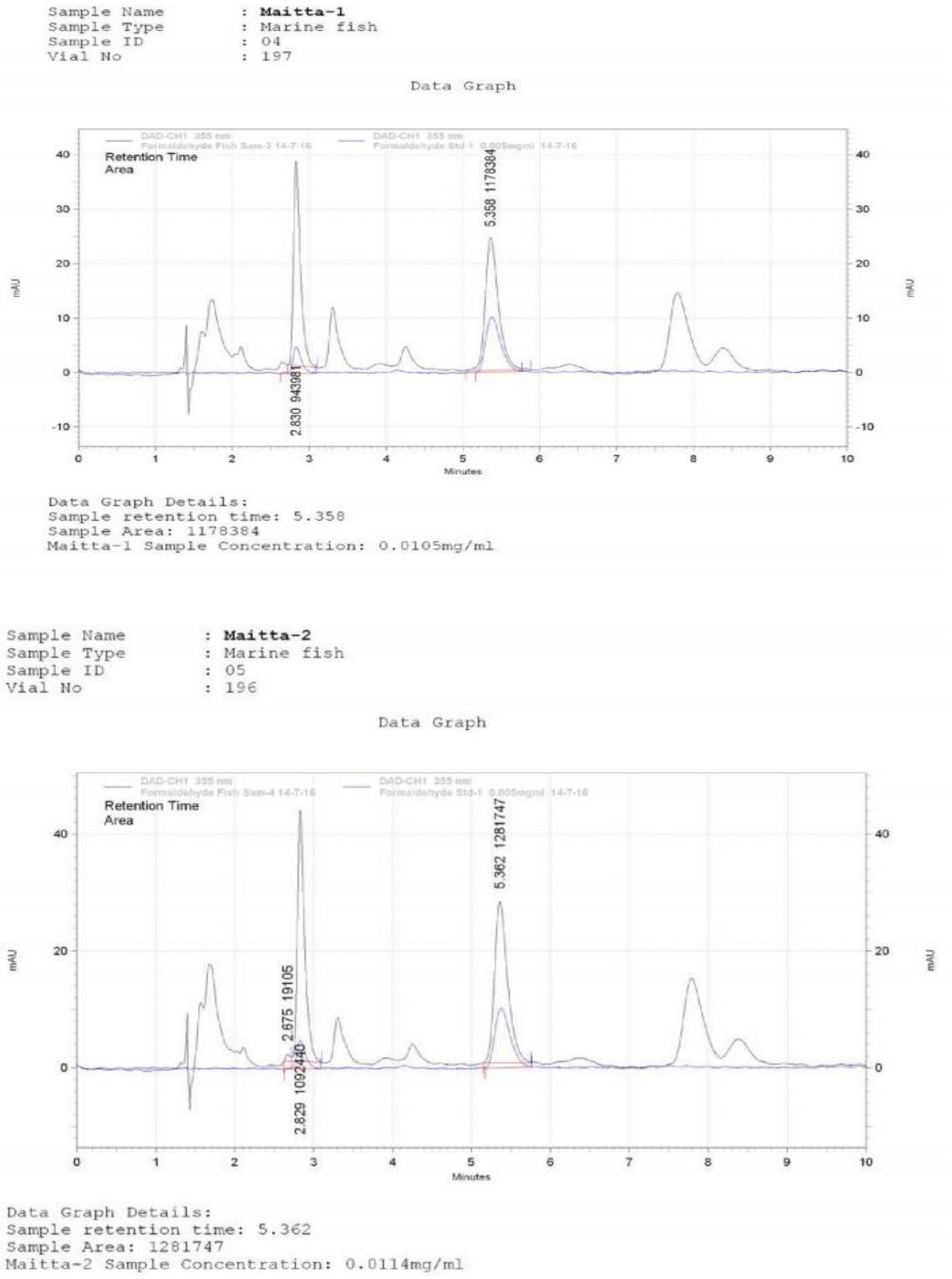


Figure 5. Typical chromatogram of Maitta fish.

The mean formaldehyde conc. of Hilsha fish were 42.5±0.62048 mg/kg at initial and 55.38±0.70498 mg/kg after 2 hour interval (Table1). There were no significant differences of formaldehyde conc. in Hilsha fishes from different location of Bangladesh.

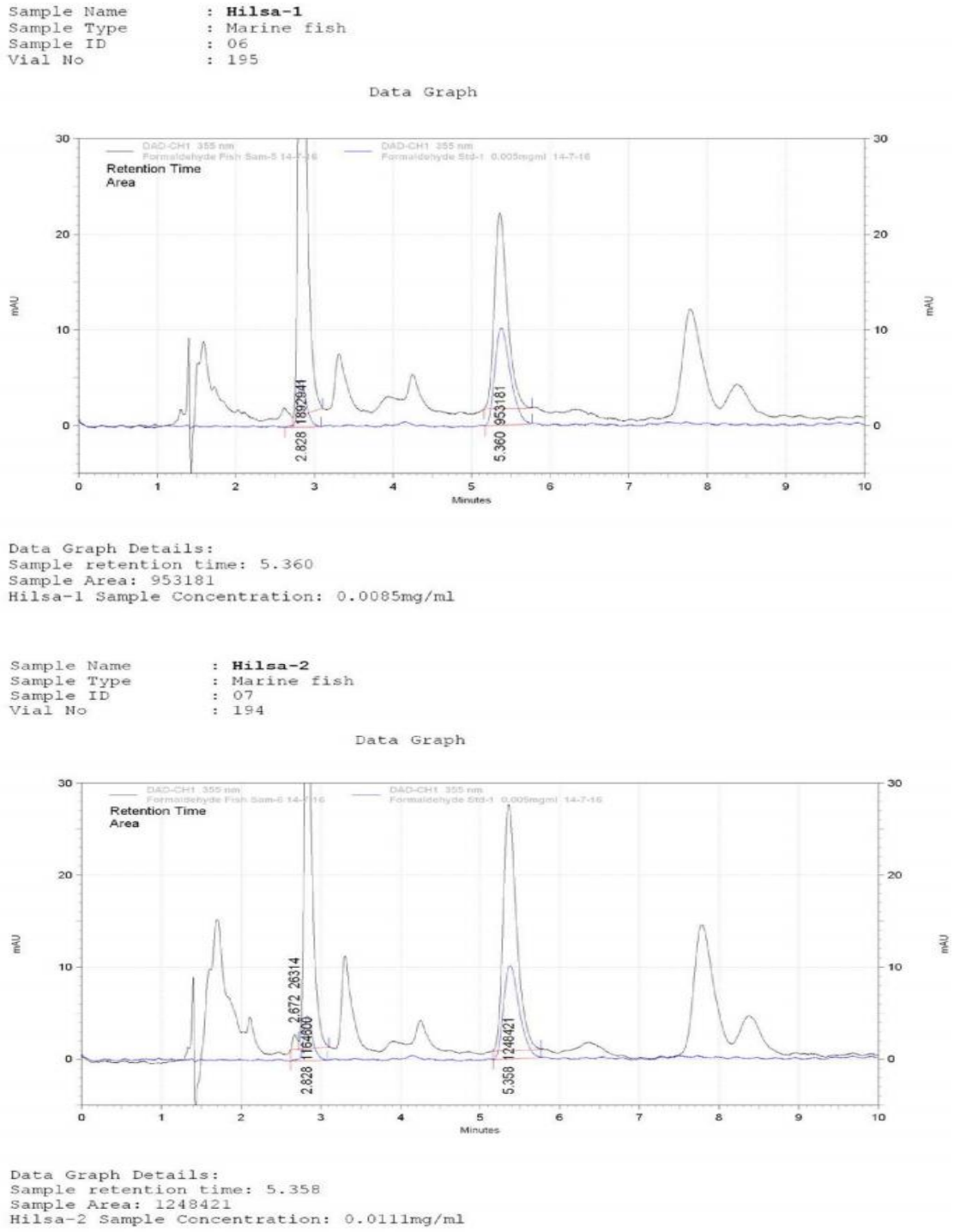


Figure 6.Typical chromatogram of Hilsha fish.

Among the different source of Poya fish, it was observed that mean formaldehyde conc. of 51.58±73959 mg/kg at initial and 55.06±0.64265 mg/kg after 2 hour interval (Table1). There were no significant differences of formaldehyde conc. in Poya fishes from different location of Bangladesh.

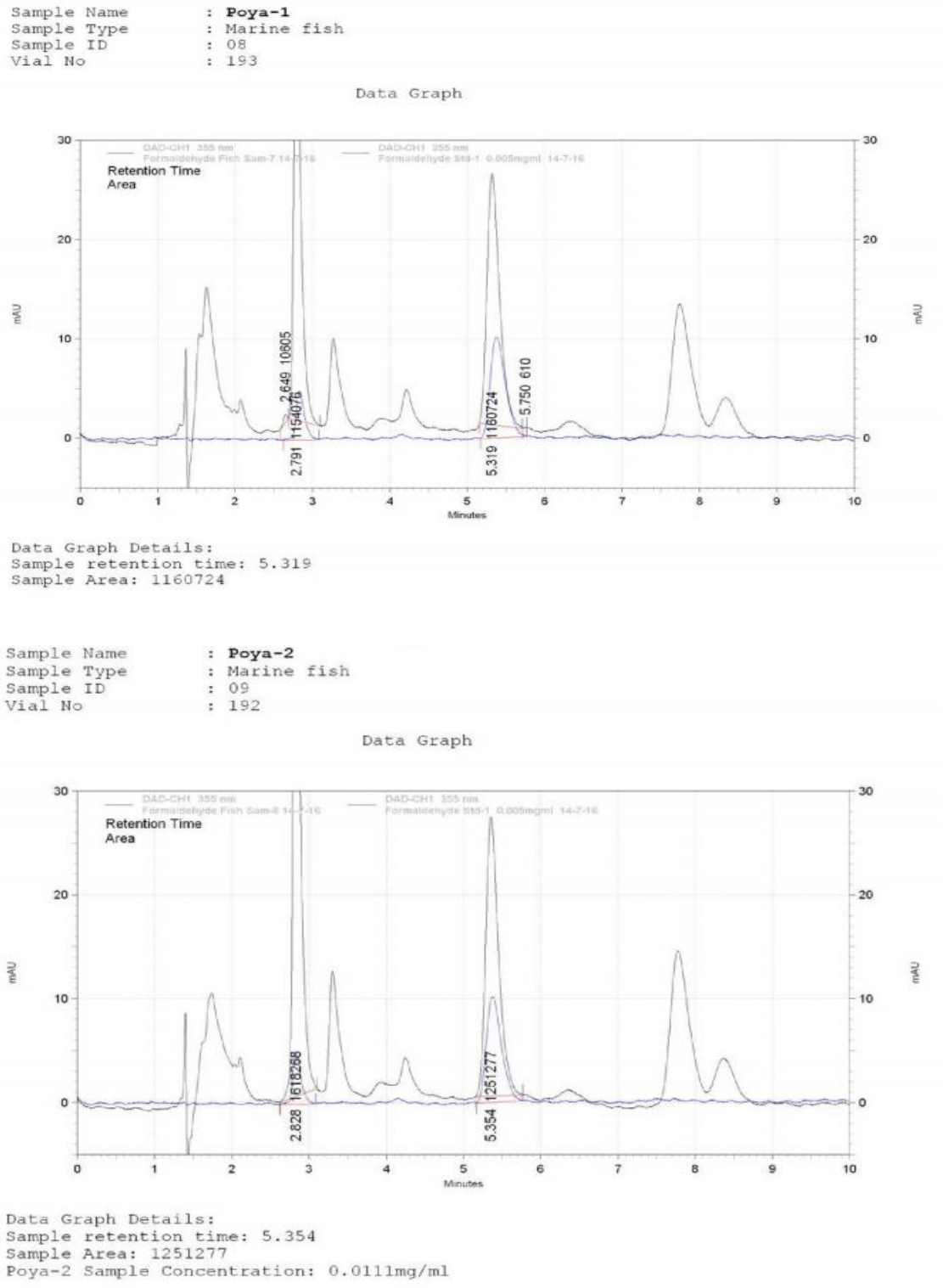


Figure 7. Typical chromatogram of Poya fish.

In case of Koral fish collected from the mentioned locations were found mean formaldehyde conc. of 52.58±0.67602 mg/kg at initial and 53.38±0.75961 mg/kg after 2 hour interval (Table1). There were significant differences of formaldehyde conc. in Koral fish from different location of Bangladesh.

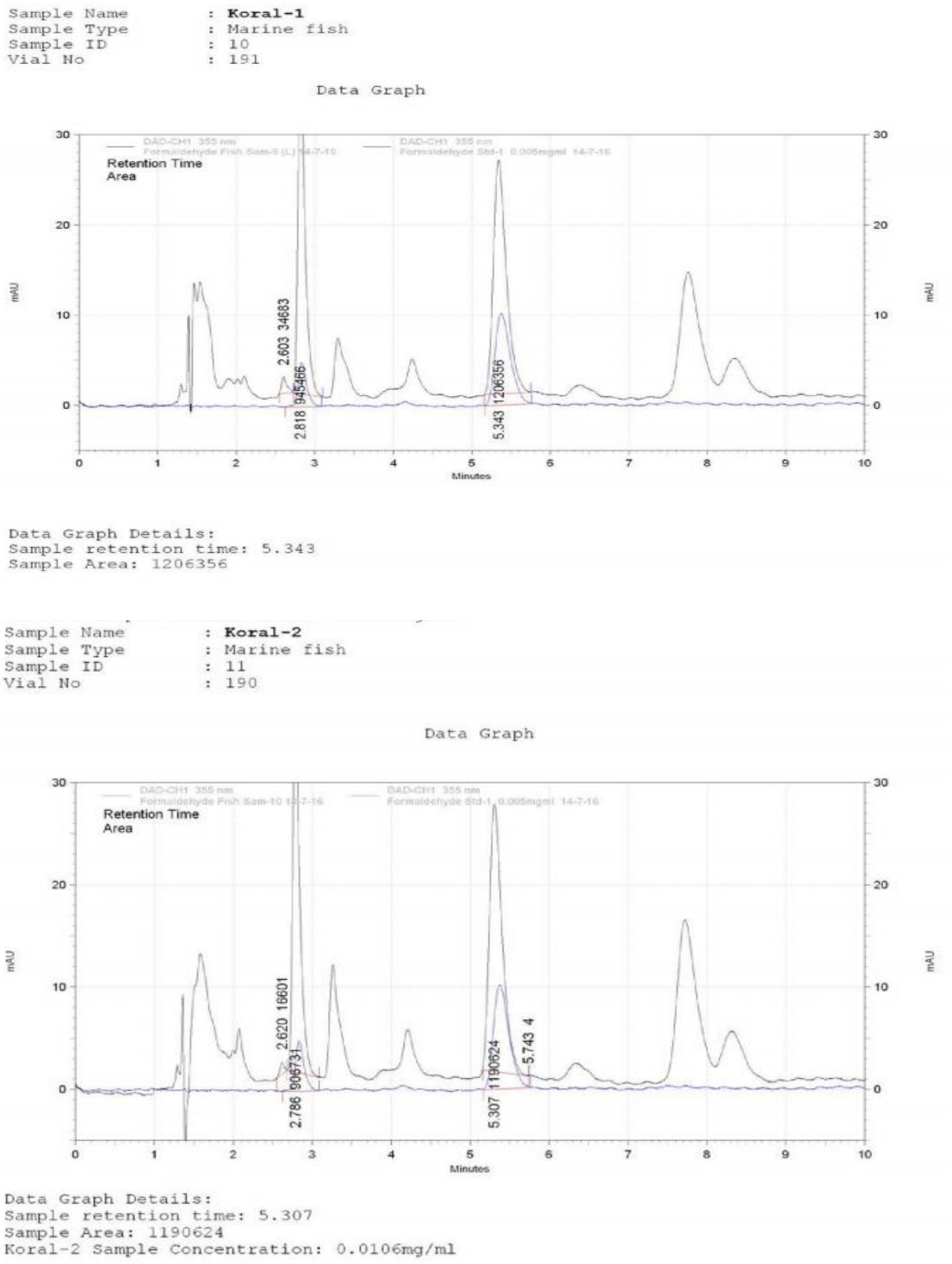


Figure 8. Typical chromatogram of Koral fish.

At last for Rupchanda fish mean formaldehyde conc. were found 54.28±0.54037 mg/kg at initial and 56.42±0.57620 mg/kg after 2 hour interval (Table1). There were no significant differences of formaldehyde conc. in Rupchanda fish from different location of Bangladesh.

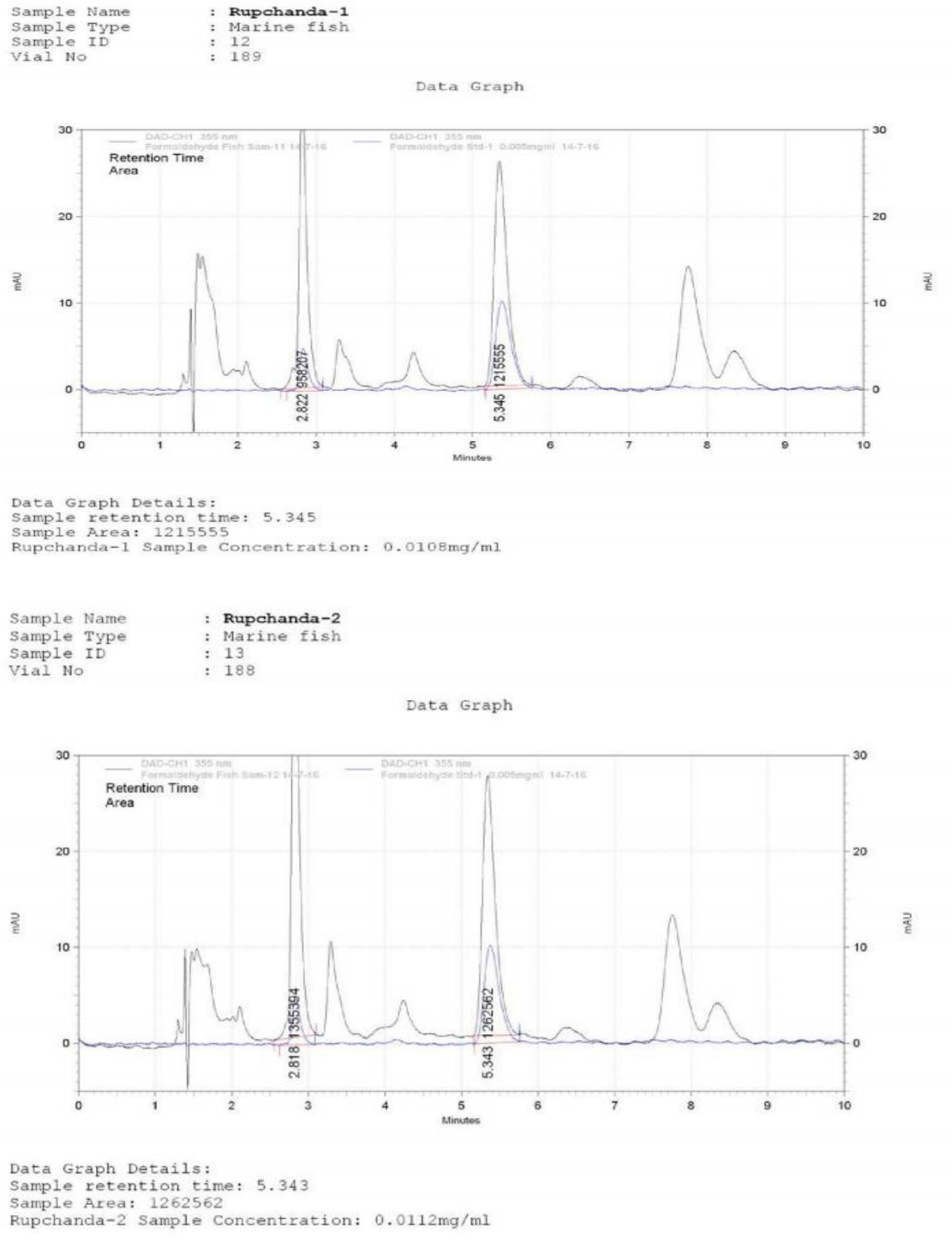


Figure 9. Typical chromatogram of Rupchanda fish.

However, the estimated formaldehyde conc. among different species, at initial Loitta fish have the lowest 41.5±0.15811 mg/kg and Rupchanda fish have the highest 54.28±0.54037 mg/kg. After 2 hours, Koral fish formaldehyde concentration was lowest 53.38±0.75961 mg/kg and Maitta fish was highest 57.18±0.52154 m/kg among the fish species.

Chapter-5: Discussion

The widespread use of formalin, in preservation of fish, fruit and other food items is posing a threat to public health. The chemical used as a solution in water keeps fish fresh and makes fruits like mangoes attractive. This chemical, usually used to stop dead bodies from rotting, is now being used to preserve edible items. Exposure from its gas or vapor can cause irritation to the eyes, nose and respiratory tract, causing sneezing, sore throat, larynx constriction, bronchitis and pneumonia. Multiple exposures can lead to asthma. It can also affect the skin, causing dermatitis or allergic reaction. Serious inhalation or ingestion can cause severe pain with inflammation ulceration and necrosis of the mucous membranes, which line almost every internal organ. This may show as symptoms of nausea, vomiting blood, diarrhea with bloody stool, blood from the urine, acidosis, vertigo, and circulation failure, then death. 30mL is suggested the lethal dose of formalin. The limit allowed in air that is still safe for human is less than 2 ppm.

Formaldehyde can be found naturally in food including fruits and vegetables, meats, fish and other dried and preserved foods. Based on the result of present study, there were significant differences in the concentration of natural formaldehyde between fish samples at initial and after 2 hours of storage at room temperature. The natural formaldehyde concentration of all fish sample except Koral-1 increases with elapsing of time at room temperature. The concentration of naturally occurring formaldehyde increased slowly in fresh fish sample with respect to time that is described by Uddin et al. (2015). According to Sotelo et al., 1995, the amount of formaldehyde formed depends mainly on the time and temperature of the storage which causes muscle toughening and water loss in fish, leading to lower acceptability as well as functionality. The reduction of TMAO process also caused the bacteria activity to increase.

In case of Loitta fish the increase of natural formaldehyde after 2 hours was highest (13.2 mg/kg) in Loitta-1 and Loitta-4 and was lowest in Loitta-3 (12.3 mg/kg). In Koral fish the increase of natural formaldehyde after 2 hours was highest (2.1 mg/kg) in Koral-2. But for Koral-1 the natural formaldehyde concentration tends to decrease 1 mg/kg after 2 hours of storage. The increase in natural formaldehyde of Poya fish after 2 hours was highest (4.1 mg/kg) in Poya-2 and Poya-5 and was lowest in Loitta-

3 (3 mg/kg). For Hilsha fish the increase of natural formaldehyde after 2 hours was highest (13.7 mg/kg) in Hilsha -4 and was lowest in Hilsha -1 (3 mg/kg). Considering the increase of natural formaldehyde in Maitta fish it was found an increase of 5.7 mg/kg for Maitta-2 sample (Highest) and 4.2 mg/kg in case of Maitta-5 sample (Lowest). For Rupchanda fish the increase of natural formaldehyde after 2 hours was highest (3.5 mg/kg) in Rupchanda-3 and was lowest in Rupchanda-4 (1.2 mg/kg).

In case of overall increase of natural formaldehyde after 2 hours was highest 12.9 mg/kg in Loitta fish and was lowest in Koral fish 0.8 mg/kg.

As a product of normal metabolism, formaldehyde has been documented to be naturally present in many common food items, including fruits and vegetables, meats, fish, crustacea and dried mushrooms etc., at a wide range of levels. In some seafood species, formaldehyde is a natural breakdown product of a chemical known as trimethylamine oxide (TMAO) that exists in their bodies. Trimethylamine oxide breaks down into formaldehyde and dimethylamine in equal parts after the animal dies. The level of formaldehyde can accumulate in certain marine fish during frozen storage and crustacea after death. Its levels were reported to be up to 400 mg/kg in Bombay-duck after cold storage. TMAO constitutes a characteristic and important part of the NPN-fraction in marine species. This component is found in all marine fish species in quantities from 1 to 5 % of the muscle tissue (dry weight) but is virtually absent from freshwater species and from terrestrial organisms (Anderson and Fellers, 1952; Hebard *et al.,* 1982). Stroem *et al.* (1979) have shown that TMAO is formed by biosynthesis in certain zooplankton species. These organisms possess an enzyme (TMA mono-oxygenase) which oxidizes TMA to TMAO. TMA is commonly found in marine plants as are many other methylated amines (monomethylamine and dimethylamine). Plankton-eating fish may obtain their TMAO from feeding on these zooplankton (exogenous origin). Belinski (1964) and Agustsson and Stroem (1981) have shown that certain fish species are able to synthesize TMAO from TMA, but this synthesis is regarded as being of minor importance. The TMA-oxidase system is found in the microsomes of the cells and is dependent on the presence of Nicotinamide ademine denucleotide phosphate (NADPH): $(CH_3)_3N + NADPH + H^+ + O_2 \rightarrow (CH_3)_3NO + NADP^+ + H_2O$. The amount of TMAO in the muscle tissue depends on the species, season, fishing ground, etc. In general, the highest amount is found in elasmobranchs and squid (75-250 mg N/100 g); cod have somewhat less (60-

120 mg N/100 g) while flatfish and pelagic fish have the least. An extensive compilation of data is given by Hebard *et* al., (1982). According to Tokunaga (1970), pelagic fish (sardines, tuna, mackerel) have their highest concentration of TMAO in the dark muscle while demersal, white-fleshed fish have a much higher content in the white muscle.

Poikilotherm nature of fresh fish allows a wide variety of bacteria to grow, including the Gram-negative, rod-shaped bacteria which belong to the genera Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Aero monadaceae, and Vibrionaceae, and Gram-positive bacteria such as Bacillus, Micrococcus, Clostridium, Lactobacillus, and Corynebacterium. Psychrotrophs are bacteria that can tolerate cold temperature and grow at 0 degree Celsius but grow optimally around 25 degrees Celsius. Another possible cause is anaerobic bacteria that are able to utilize TMAO as the terminal electron acceptor in an anaerobic respiration process with trimethylamine (TMA) as the primary product and also increases the natural formaldehyde content.

In Bangladesh, fishes are adulterated by hazardous chemicals at different steps from farm to consumers. Formalin (FA) is reported to be frequently added as preservative either by dipping or spraying to the fresh fishes by the fish traders while transporting to domestic marketing chain to prevent spoilage and extend shelf life (Hoque et al., 2016). It was observed in the study conducted in Dhaka city (Islam et al., 2015) that almost 5% shops of total consumable fishes contain formalin treated fishes in the fish markets. They found this intensity varies market to market and species to species. During the study of Paul et al. (2014) a total of 21 formalin treated fishes which accounts 4.2% of the total examined fishes (500 samples) were found in Jessore district. Yeasmin et al., (2010) found that formalin was detected in the imported Catla and Rohu ranging from 0.5% to 1% which were sold in different markets of Mymensingh with comparatively lower price than those produced locally. So formalin is a major concern in the southeast coastal region in Bangladesh. The natural formaldehyde content of fish must take into account before estimating the total formaldehyde content of fish.

Chapter-6: Conclusions

The present study revealed the presence of natural formaldehyde in sea fish samples of Loitta (*Harpadon Nehereus*), koral (*Lates Calcarifur*), Rupchanda (*Pampus Chinensis*), Hilsha (*Tenualosa Ilisha*), Poa (*Pama Pama*) and Maittya (*Euthynnus Affinis*) from different location. The estimation of formaldehyde was calculated in line with standard curve obtained from concentration of formaldehyde solution used for this study in HPLC. Major finding of present of natural formaldehyde in sea fish are: the presence of dark muscle tissue, the quantity of substrate present, temperature, cofactors, storage time and the degree of comminution of the flesh. There are higher content of nitrogenous compounds in dark muscle of marine fish than the white muscle of marine fish. 95% of nitrogenous content in dark muscle tissue contains higher amount of free amino acids, imidazole dipeptides, trimethylamine oxide (TMAO) and its degradation products such as formaldehyde. The dark muscle also contains more lipid and TMAO where enzyme breaks down the component into trimethylamine (TMA), dimethylamine (DMA) and Formaldehyde. At present consumable fishes are being contaminated by formalin by some evil traders. But some fishes contain formalin in their fleshes naturally which are detected by formaldehyde machine by mobile court and impose fine to local seller for that natural formalin content of fish. So poor fish sellers are economically loses from this type of fine although they do not adding formalin in their fishes. To rescue the local fish seller from such fine, the present study will differentiate the content of natural formaldehyde and adulterated formaldehyde.

Chapter-7: Recommendations and Future perspectives

As formalin is one of the threats to the modern world, few suggestions that can be considered to overcome the formaldehyde content in fish. Several methods have been taken and proposed in order to control or reduces formaldehyde content in fish. In Bangladesh, the government advised to the public to choose the fish that are fresh and avoid those with unusual smell and also avoid buying noodle fish that are stiff (formaldehyde could stiffen flesh of fish). Freshness is a property of fish that has a considerable influence on its quality.

Besides, public also advised to wash and cook the fish thoroughly as formaldehyde is water soluble and it could be dissipated upon heating. When formaldehyde is released into water, it does not move into other media but it is broken down because formaldehyde is readily soluble in water, alcohols and other polar solvent. The USEPA's Exposure Factors Handbook has reported cooking the fish will result in weight (moisture and fat) loss which subsequently decreases the formaldehyde concentration in cooked fish. The formaldehyde concentration was decrease after roasting and boiling. The decrease of formaldehyde content was due to the evaporation of the sample during the cooking process.

As a product of normal metabolism, formaldehyde has been documented to be naturally present in many common food items, including fruits and vegetables, meats, fish, crustacea and dried mushrooms etc., at a wide range of levels. The present study only determines the level of natural formalin in sea fish in southeast coastal region of Bangladesh. Food items such as fruits and vegetables, meat and meat products can be of great concern.

However, there were some limitations in this study such as the temperature change, time of storage and handling could possibly influenced the concentrations of formaldehyde since it is a volatile compound. Additionally, only edible parts of fish were analysed and no results were shown in the bones and fins. The fish consumption was an approximate since no diet recall interview was carried out. Epidemiological studies of potential carcinogenic hazards associated with the ingestion of formaldehyde were not identified.

Consequently, the development of simple and sensitive methods for monitoring formaldehyde is of great interest from the analytically and toxicological viewpoints that can differentiate the natural and added formaldehyde content. In order to control or reduces formaldehyde content in fish and other food, several measures has been taken and proposed.

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Appendix A: HPLC reading for natural formalin determination in fish

Sample SL No	Sample Name	Vial Number	Area	tR	Formalin Concentration (mg/ml)
	Standard-1	200	558908	5.377	0.005
1	Loitta-1	199	929542	5.362	0.0083
2	Loitta-2	198	1222498	5.357	0.0109
3	Maitta-1	197	1178384	5.358	0.0105
4	Maitta-2	196	1281747	5.362	0.0114
5	Hilsa-1	195	953181	5.360	0.0085
6	Hilsa-2	194	1248421	5.358	0.0111
7	Poya-1	193	1160724	5.319	0.0103
8	Poya-2	192	1251277	5.354	0.0111
9	Koral-1	191	1206356	5.343	0.0107
10	Koral-2	190	1190624	5.307	0.0106
11	Rupchanda-1	189	1215555	5.345	0.0108
12	Rupchanda-2	188	1262562	5.343	0.0112

Appendix B: Fish Sample Analyzed

B. Rupchanda (*Pampus Chinensis*)

A. Hilsha (*Tenualosa Ilisha*)

C. Maittya (*Euthynnus Affinis*)

D. Loitta (*Harpadon Nehereus*)

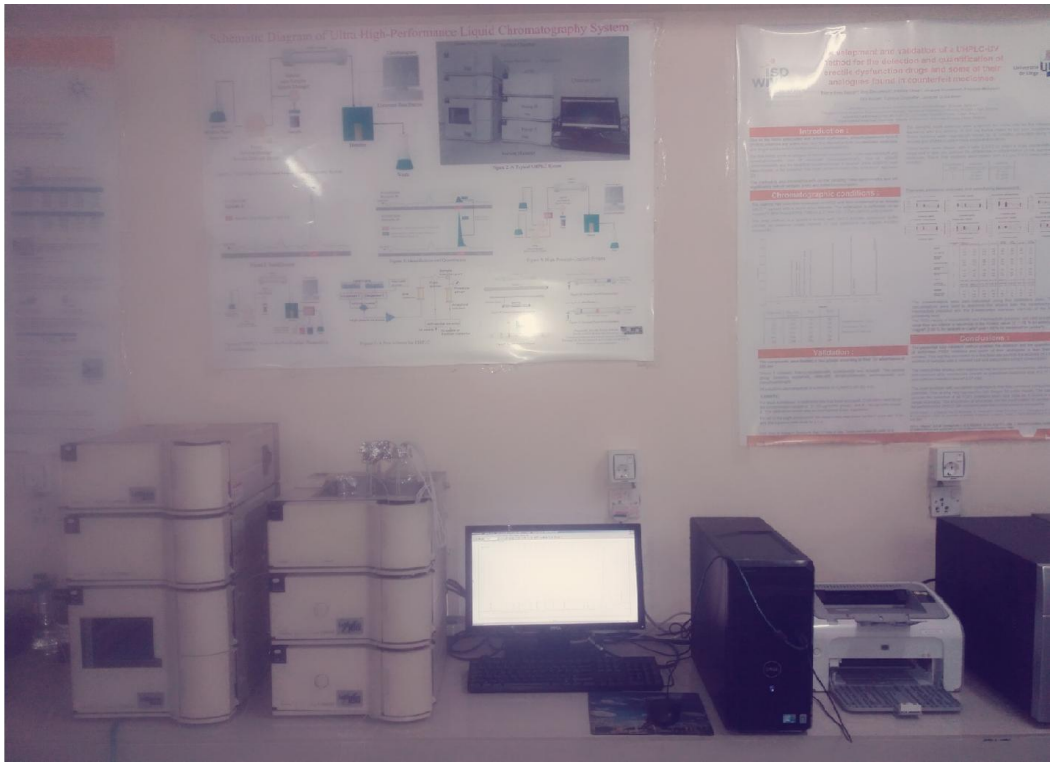
E. koral (*Lates Calcarifur*)

F. Poa (*Pama Pama*)

Appendix C: Analytical works carried out during research

A. Sample Preparation

B. Reagent Preparation



C. Analysis in HPLC

Brief Biography

Md. Altaf Hossain received the B.Sc. (Hon's) in Food Science and Technology from the Faculty of Food Science and Technology of Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh. Now, he is a candidate for the degree of M.S. in Applied Human Nutrition and Dietetics under the Department of Applied Food Science and Nutrition, Faculty of Food Science and Technology, Chittagong Veterinary and Animal Sciences University (CVASU). He joined as a Lecturer in the Dept. of Applied Food Science and Nutrition under the Faculty of Food Science and Technology, CVASU in 2015 and still now holds the same post. His research interests are in Food safety, Malnutrition, Dietetics, Reduction of nutritional changes in food processing, Nutraceuticals products development and improve the food pattern in human being by awareness building. Md. Altaf Hossain has great eagerness of research in his area to improve the nutritional status of malnourished people in undeveloped and developing countries.