VALIDATION OF METHOD AND PHARMACOKINETIC ASSESSMENT OF FORMALDEHYDE IN VARIOUS ANIMAL ORIGIN PRODUCTS BY REVERSE-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

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IN

VETERINARY PHARMACOLOGY AND TOXICOLOGY

BY

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2021

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I hereby declare that the experimental research work and interpretation of the thesis entitled **"VALIDATION OF METHOD AND PHARMACOKINETIC ASSESSMENT OF FORMALDEHYDE IN VARIOUS ANIMAL ORIGIN PRODUCTS BY REVERSE-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)"** or part thereof has not been submitted for any of the other degree or diploma of any university, nor the data has been derived from any thesis or publications of any university or scientific organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

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Dedicated to My Beloved Family

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LIST OF ABBREVIATIONS

Sr. No.	Abbreviations	Name
1.	**>**	Greater Than
2.	**<**	Less Than
3.	**%**	Percentage
4.	**µg**	Microgram
5.	**µl**	Microliter
6.	**µm**	Micro meter
7.	**ALAT**	Alanine Amino Transferase
8.	**ALP**	Alkaline Phosphatase
9.	**APC**	Aerobic Plate Count
10.	**ASAT**	Aspartate Amino Transferase
11.	**BFRI**	Bangladesh Fisheries Research Institute
12.	**CA**	Cornu Ammonis
13.	**CIFT**	Central Institute Of Fisheries Technology
14.	**CORT**	Corticosterone
15.	**CRM**	Certified Reference Material
16.	**DMBA**	Dimethylbenz[A]Anthracene
17.	**DNA**	Deoxyribonucleic Acid
18.	**DNPH**	Dinitro Phenyl Hydrazine
19.	**DPX**	DNA–Protein Cross-Links
20.	**ED50**	Effective Dose 50%
21.	**EPA**	Environmental Protection Agency
22.	**FA (HCHO, CH_2O)**	Formaldehyde
23.	**FSSAI**	Food Safety And Standard Authority Of India
24.	**GC**	Gas Chromatography
25.	**Gm**	Gram
26.	**H_3PO_4**	Phosphoric Acid
27.	**Hr**	Hour

28.	**IARC**	International Agency For Research On Cancer
29.	**ICAR**	The Indian Council Of Agricultural Research
30.	**Kg**	Kilogram
31.	**LC**	Liquid Chromatography
32.	**LCMS**	Liquid Chromatography Mass Spectometery
33.	**LD50**	Lethal Dose 50%
34.	**LOD**	Limit Of Detection
35.	**LOQ**	Limit Of Quantitation
36.	**Mg**	Milligram
37.	**Min**	Minute
38.	**Ml**	Millilitre
39.	**Mm**	Millimeter
40.	MN	Micro Nuclei
41.	**MS**	Mass Spectrometry
42.	**Nm**	Nanometer
43.	NMDA	N-Methyl-D-Aspartate
44.	**^{o}C**	Degree Celsius
45.	**PH**	Potential Of Hydrogen
46.	PND	Post Natal Day
47.	**PPM**	Parts Per Million
48.	**RNA**	Ribonucleic Acid
49.	**RP- HPLC**	Reverse Phase High Pressure Liquid Chromatography
50.	**RPM**	Revolutions Per Minute
51.	**RT**	Retention Time
52.	**SD**	Standard Deviation
53.	**SE**	Standard Error
54.	**SPME**	Solid Phase Micro-Extraction

55.	**TCA**	Trichloro Acetic Acid
56.	**TVB-N**	Total Volatile Basic Nitrogen
57.	**UPLC**	Ultra Performance Liquid Chromatography
58.	**UV**	Ultraviolet
59.	**WHO**	World Health Organization

Introduction

1. INTRODUCTION

Humans are continuously exposed to wide variety of xenobiotics that have shown to be carcinogenic and mutagenic and are of human hazard importance. Xenobiotics are the chemical substances that are found in the animal body which are not naturally produced or are in unusual amount. Cosmetics, food additives, pesticides, drugs, industrial chemicals and environmental pollutants are the various types. Formaldehyde is one of the most commonly used xenobiotics in food industry which is used as a preservative (Patterson *et al.,* 2010) .

Formaldehyde (HCHO) is a reactive agent belonging to the aldehyde family and formalin, a 37% aqueous solution serves as a germicidal and preservative agent (Guidance note of Food Safety and Standard Authority of India, 2018). It is readily soluble in water and present in the gas phase at room temperature, where it evaporates quickly leading to the increase in its harmful effects (Franklin *et al.,* 2000; Ozen *et al.,* 2002; Songur *et al.,*2003). Formalin is commonly used in the industries, as it possesses potent disinfectant activity, and is cheaper, non corrosive and has strong bactericidal and fungicidal action (including their spores) (Braswell *et al.,*1970; Acklund *et al.* 1980; Williams, 1980).

The first report for the use of formaldehyde as a disinfectant was in 1892 (Hugo *et al.,*1992). In 1908, formaldehyde was used as a fumigant agent by Pernot for the fumigation of eggs and incubators as a control measure in poultry diseases.

In medical practice and veterinary sciences, formalin is used as tissue preservative, as a bactericidal agent in mortuaries (Bernstein *et al.,* 1984; Naya and Nakanishi, 2005; Yamato *et al.,* 2005). Occupation exposure is the common means of getting direct contact to formaldehyde by the humans. The most extensive use of formaldehyde is in the production of phenol and melamine, resins along with urea and polyacetal resins which acts as an adhesive agent in plastic, etc in addition to this it is used in different industries like rubber processing plant, lacquering, for finishing of textiles, in cigarettes and other tobacco products, gas cookers, open fireplaces, glue, wood products, shampoos, shaving creams, mascara, drinking

water and food items and as a disinfectant agent. Formaldehyde is released in the environment when used as a fodder preservative in agriculture and as a product of forest fire combustion and engine exhaust. (Morgan, 1997)

The utilization of formaldehyde is widespread in many sectors however, the most common is in the food industry. In food industry, use of formaldehyde in various products is well- known such as meat, milk, seafood's (fish, shellfish and crustaceans) etc. So as to increase its shelf life. In Poultry industry, formaldehyde is used as a disinfectant in hatcheries to control various microbial load such as *Salmonella* and *Escherichia coli* (E.coli). Formaldehyde fumigation is done at the onset of incubation and with the transfer of chicks in hatcheries (Zulkifli *et al.,* 1999). It is also used in some farms as a disinfectant for the buildings and vehicles to clear the pathogen load (Zulkifli *et al.,* 1999; Hayretdag and Kolankaya, 2008). However, the use of formaldehyde for a long time has caused serious adverse effects and reports suggest that it causes destruction of the tracheal ciliary function and decrease the viability of chicks. The use of formaldehyde negatively affects the growing embryos and their further development (The National Toxicology Program, 2011; National Cancer Institute, 2011; Cadirci, 2009; Zulkifli *et al.,* 1999; Hayretdag and Kolankaya, 2008; Banwell, 2013).

Modern incubation practices reduce the ventilation that decreases the microbial contamination of the eggs, but this practice leads to the accumulation of formaldehyde on the egg shells and penetrates the eggs leading to adverse effects on hatchitability (Proudfoot and Stewart, 1970; Sacco *et al.,* 1989; Yildirim *et al.,* 2003; Banwell, 2013). In fumigation, the surrounding premises as well as eggshells comes in contact with the formaldehyde which increases its chances of absorption. In the egg formation, the blastoderm layer from which the embryo develops is placed on the upper layer of the yolk which is held by the vicious albumin and chalazae combination at the center position (Cadirci, 2009; Banwell, 2013).

This positioning leads to the diffusion of carbon dioxide from the porous shell that causes rise in the pH of the egg albumin. A high pH leads to the breakage of interaction between the albumin proteins that is lysozyme and ovamucin, thus further decreasing the albumin viscosity that allows the yolk and the blastoderm to float towards the harmful formaldehyde concentration (Banwell, 2013).

The formaldehyde which is absorbed by the embryos in an early stage leads to the alkylation of purine and pyrimidine nitrogen atoms of RNA and DNA that triggers changes on proteins and nucleic acids which inhibits the function and growth of the birds (Cadirci, 2009). Chicks that are exposed to the formaldehyde develops irritation in the trachea causing blebbing of tracheal cilia and ciliostasis. The adheration of the matted cilia, erupted ciliated area and excessive mucus results in respiratory distress by inadequate mucocilliary action. (Zulkifli *et al.,* 1999; Hayretdag and Kolankaya, 2008).

Formaldehyde gas dissolute with the tracheal mucus that leads to shift in the pH and makes it acidic that further damages the ciliary activities (Sander *et al.,* 1995; Hayretadg and Kolankaya, 2008) which further makes birds more susceptible for disease and deteriorates the overall performance of the bird (Cadirci, 2009).

In fishes, formalin occurs naturally which after the demise undergoes a biological reaction in which formaldehyde and dimethylamine are produced by the breakdown of trimethyamine as a natural product (Jiang *et al.,* 2006). This formaldehyde is also produced by various other biochemical and enzymatic reaction and ageing of the fish. The microbial activity deteriotes the quality of fish further producing some unpleased compounds and chemical amines. Thus, formalin is added by food manufacturers to preserve the texture and quality and tenderness of the meat. (Gram *et al.,* 2002; Arashisar *et al.,* 2004).

Due to the perishability of the fish, vendors dip or spray fishes with formalin water or may inject formalin in the body cavity of fish that to prevent the freshlook or stiffness of the flesh which affects the humans due to its residues showing

carcinogenetic effect (Kibria, 2007). This malpractice of introducing formaldehyde is growing because of inappropriate freezing facilities, inefficient ice storage condition and long travel time. The added formalin can produce a chemical reaction with the fish product and thus affecting the consumer (Jaman *et al.,* 2015) by deteriorating their health due to its carcinogenic, allergic and toxic compound. Naturally present in the human body at a very low concentration as a metabolite in one carbon reaction and leads to symptoms like burning in the throat, headache and breathing difficulty (Herschkovitz *et al.,* 2000).

In fish culture, formalin is also used as an anti fungal agent and anti-parasitic agent for therapeutic management (Schnick, 1988; *Rach et al.,* 1997) while the use of this drug is prohibited by certain countries such as Japan, Australia and Europe in marine fish farming (Guidance note of Food Safety and Standard Authority of India, 2018).

The added formaldehyde usually gets removed with ice-melt water, however the extraction is complete and some residue may remain into it. The Food Safety and Standard Authority of India (FSSAI) has given guidelines regarding the cleaning and cooking of the fishes in where the fishes should be thoroughly washed in running water and formalin is being water soluble and can be removed after more than 75° C.

The general method used for estimation of formaldehyde in foodstuffs are on calorimetric reaction in which sulfuric acid is mixed with simple distillates that yields a purple colour if it contains formaldehyde. The colour intensity obtained is proportional to the concentration of formaldehyde and then is measured by UV-spectrophotometer. These calorimetric methods have certain limitations as they have poor selectivity, specificity, long analysis time and acidic conditions that could lead to false results (Food Preservatives Committee, 1924) while new methods such as liquid chromatography (LC) and Gas chromatography (GC) have shown more accuracy.

A qualitative method for the determination of formaldehyde has been established by ICAR- Central Institute of Fisheries Technology (ICAR-CIFT), Cochin. It has developed an easy and rapid strip test called as CIF-Test which detects the added formaldehyde on the fishes. In this test, a paper strip should be swabbed on the fish surface 3-4 times followed by addition of a chemical reagent which changes the paper strip colour in 2 minutes. this developed colour is then compared with the standard chart (Guidance note of Food Safety and Standard Authority of India, 2018).

In the India, Food Safety and Standard Authority of India (FSSAI) has recommended an ad hoc limit for naturally occurring formaldehyde in fishes and other fishery products. For fresh water fishes, molluscs, crustaceans and echinoderms, the limit is 4 mg/kg while for marine or brackish water fishes, the limit set is 100 mg/kg (FSSAI, 2019). While, the tolerable limit of formaldehyde in fish is 6.5-293 mg/kg and tolerable level for human is 100 mg/kg set by European food safety authority (European food safety authority, 2014). According to the World Health Organization (WHO), for an average adult a range of 1.5-14 mg/d (average 7.75 mg/d) is given and European food safety authority, 2014 has estimated that daily oral exposure of formaldehyde should not exceed 100 mg from the total diet per day.

Ingestion of as little as 30 ml of formalin has been reported to cause death in an adult human being (Wooster *et al.,* 2005). Thus, the formaldehyde has been declared a potential carcinogen and mutagen (Cui *et al.,* 2007) and possibly carcinogenic to humans at LD_{50} 30 gm (WHO, 1989).

Consumption of formalin or exposure to exogenous formaldehyde on a regular basis can be injurious to the nervous system, kidney and liver, and may cause asthma, pulmonary damage and cancer (Abdu *et al.,* 2014, Mamun *et al.*, 2014, Songur *et al.,* 2010). Reports have studied the development of nasal carcinomas in rat exposed to formaldehyde at 6 to 15 ppm. (Albert *et al.,* 1982 , Kerns *et al.,* 1983). Its effects are seen as nasal carcinomas (Hayes *et al.,* 1986, Olsen and Asnaes,

1986), nasopharyngeal (Roush *et al.,* 1987) and buccal carcinomas and even leukemia.

Formaldehyde exposure also leads to the memory impairment in humans as it is present in minor amount in the nucleus and cytoplasm of all cells (Kalapos, 1999; Kalász, 2003; Trézl *et al.,* 1997; Tyihák *et al.,* 1998). Studies have shown that rats exposed to gaseous formaldehyde leads to its accumulation in brain, decrease the hippocampal neurons (Gurel *et al.,* 2005), and causes memory impairment. While epidemiological studies has confirmed the formaldehyde exposure leads to human cognitive decline and causes demylination of hippocampal neurons and neurofilament protein changes. (Kilburn, 1994; Kilburn *et al.,* 1987; Perna *et al.,* 2001).

Formaldehyde inhalation for a chronic period has shown to cause eye, nose and throat irritation particularly causing nasopharyngeal cancer as its carcinogenic effect (Armstrong *et al.,* 2000, Hauptmann *et al.,* 2004, Vaughan *et al.,* 2000, Hildesheim *et al.,* 2001 and Coggon *et al.,* 2003).

International agency for research on cancer (IARC) has classified formaldehyde as a carcinogenic agent (group 1) to humans since 2006 (Hossain, 2011). The oral reference range set by the US Environmental protection agency (EPA) is 0.2mg/kg. According to a study by the World Health Organization, formaldehyde content ranges from 3.3 mg/kg to 60 mg/kg in fruits and vegetables, 8-20 mg/kg in meats, 1-3.3 mg/kg in milk and 1-98 mg/kg in fish (Yeh *et al.,* 2013)

Thus formaldehyde use in food is prohibited by the FSSAI, 2011, still its adulteration is very rampant. These products therefore when consumed leads to the development of ill effects to the consumers and produces serious health effect. This study aims to study the formaldehyde level in various animal origin samples by using High Pressure Liquid Chromatography. The objective of the study were as follows:

- To validate the method of formaldehyde determination by Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC) technique in various animal origin meat products.
- To determine the concentration of formaldehyde in biological sample / animal origin products.

Review of Literature

2. Review of literature

Formaldehyde (HCHO, formalin, formol) is a reactive agent belonging to the aldehyde family. The first report for the use of formaldehyde as a disinfectant was in 1892 (Hugo *et al.* 1992). Formalin, a 37% aqueous solution serves as a germicidal and preservative agent (Guidance note of Food Safety and Standard Authority of India, 2018). In food industry, use of formaldehyde as a preservative is well - known and is added to various products such as meat, milk, ,seafood's , fish etc. As food items are perishable in nature, these needs to be used as early as possible. The seafood items i.e. fishes, crustaceans and mollusc once caught needs to be stored and transported to places in a cold storage condition. In India, the required facility is not sufficient due to which the vendors dip or spray fishes with formalin water or may inject formalin in the body cavity of fish that to prevent the freshlook or stiffness of the flesh, so as to increase its shelf life that eases its selling. This false practice is followed due to the cheap market price and easy accessibility of formalin to vendors. Formaldehyde is a major threat to humans due to its carcinogenic, allergic and toxic compound and its inhalation for a chronic period has shown to cause eye, nose and throat irritation particularly causing nasopharyngeal cancer as its carcinogenic effect (Armstrong *et al.,* 2000, Hauptmann *et al.*, 2004, Vaughan *et al.,* 2000, Hildesheim *et al.,* 2001 and Coggon *et al.,* 2003).

2.1 Description and properties of formaldehyde

2.1.1 Proprietary Names

Generic names - Formaldehyde, Formalin, Methyl aldehyde , Methylene oxide, Oxomethane, Oxymethylene, Formic aldehyde, Methanal.

2.1.2 Molecular Weight :

30.026 g/mol

2.1.3 Empirical Formula :

C-H2-O

2.1.4 Structural Formula :

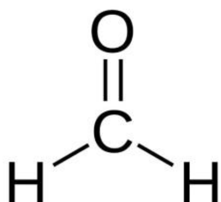

Fig.1. Chemical structure of Formaldehyde

2.1.5 Chemical Name :

Methanal

2.1.6 Melting range :

Formaldehyde melts at -92 ^{o}C (-134 ^{o}F; 181 K)

2.1.7 Appearance, colour and odour :

Formaldehyde is a colourless gas with a characteristic pungent, irritating odour.

2.1.8 Solubility Data :

It is soluble as 40g in 100 ml of water , chloroform and ethanol. It reacts with ammonia, amines, nitromethane, miscible with acetone, benzene, carbon tetrachloride, diethyl ether, ethyl acetate, tetrahydrofuran and toulene.

2.2 Analysis of formaldehyde

2.2.1 High performance liquid chromatography (HPLC)

Junke *et al.,* (2018) carried out a study for detection of formaldehyde in smoked meat products using ultra high performance chromatography. 5 vacuum samples were obtained from different companies. Steam distillation was used as an extraction method in method development on high pressure liquid chromatography and was further derived by 2-4,dinitrophenylhydrazine (DNHP). The optimum conditions maintained for derivatisation of UPLC was : DNHP dose was 0.3 ml with temperature of 600 c and for 60 minutes with twice extraction. The application of this method was done for the estimation of formaldehyde in the smoked meat products. He found that high content of formaldehyde value was on the surface of the meat sample then internal. Which indicates that the formalin has migrated from the surface part to the internal part of the meat. The formaldehyde valve ranged from 25.55 mg/kg to 49.20 mg/kg of meat for the internal part and 34.04 mg/kg to 165.25 mg/kg on the surface part of the meat. With third sample having the highest level of surface formaldehyde while the fifth sample with lowest level of formaldehyde in the meat sample. His study established a fast, simple and reliable method for the formaldehyde level in the smoked meat samples.

Das *et al.,* (2018) conducted a study to verify the presence or absence of formaldehyde from three different retail fish markets in Mumbai. Rohu (Labeo rohita), Catla (Catla catla), Boyal (Wallago attu), Indian Mackerel (Rastrelliger kanagurta) and Bombay duck (Harpodon nehereus) were collected and tested for the presence of formaldehyde. He found that the Catla fish collected from the four

Bunglows fish market contained formaldehyde conc. of 2.76 µg/g and from Andheri fish market, fish contained formaldehyde conc. of 2.88 µg/g. Rohu fish collected from Four Bunglows and Andheri fish market showed the presence of formaldehyde with 3.11 and 2.96 µg/g respectively. Boyal fish collected from Four Bunglows and Andheri fish market has formaldehyde content of 2.38 and 2.22 µg/g correspondingly. Bombay duck fish collected from the same markets contain 1.48, 1.71, 2.08 µg/g whereas Indian Mackerel fish collected were found to have 1.81, 2.27, 2.35 µg/g of formaldehyde content. Both marine and freshwater fishes were noticed to have formaldehyde content in their flesh.

Bhowmik *et al.,* (2017) carried out a study in Bangladesh for determination of formaldehyde in fish sample. 10 samples were taken ,out of which commonly available nine marine and freshwater finfish and one species of shrimp was collected. Samples were taken from fish markets of Dhaka city i.e. the kawran bazaar, raiyer bazaar and the mohakhalli bazaar. Assessment of the samples was done by high performance liquid chromatography (HPLC). He found that a higher level of formaldehyde was in raiyer bazzar fish sample and lower in the kawran bazaar samples. A range of formaldehyde was detected, 5.1 ± 0.71-12.26±1.67 mg kg^{-1} in freshwater finfish sample , 10.8±0.71 -39.68±7.87 mg kg^{-1} in marine finfish samples and 13.57±1.93 mg kg^{-1} in shrimp samples. A complete range of 5.1±0.71 - 39.981±7.87 mg kg^{-1} were observed in all marketed fishes. The level of formaldehyde detected was below the tolerable level for humans. So these samples were declared as safe for human consumption.

Wahed *et al.,* (2016) carried out a study in which a sensitive high performance liquid chromatography method was validated for the quantitative determination of formaldehyde in mango, fish and milk. The validated method was applied to screen samples of fruits, vegetables, fresh fish, milk and fish feed collected from different local markets in Dhaka, Bangladesh. He found that the method was fit-for-purpose and showed good analytical performance in terms of specificity, linearity, precision, recovery and robustness. The expanded uncertainty was < 35%. Levels of formaldehyde in food samples were compared with published data.

Jaman *et al.,* (2015) conducted a study for quantitative analysis of formaldehyde presence by using spectrophotometric method in some important freshwater and marine fish species by using Nash reagent in conjunction with TCA extraction. The freshwater fishes were Rohu (Labeo rohita) , tilapia (Oreochromis nilotica), Thai koi (Anabas testudineus), kachki (Corica soborna) and marine fish loyitta (Harpodon nehereus), chhuri (Lepturacanthus savala) from local markets and from freshly caught samples were evaluated for determination of formaldehyde concentration. He found that the formaldehyde concentration obtained in fishes from three different wet markets of Mymensingh mechhua bazar was ranged between 1.4 and 7.35 µg/g. While formaldehyde concentration in freshly caught fishes Rohu, tilapia and Thai koi collected from ponds of Freshwater Station, Bangladesh Fisheries Research Institute (BFRI), Mymensingh showed natural formaldehyde in their muscle having values of 1.45; 1.85 and 2.60 µg/g, respectively. The marine fish i.e. loyitta and chhuri collected from the landing centre of BFDC at Cox's Bazaar and were investigated in frozen, thawed condition showed to contain naturally occurring formaldehyde as 3.9 and 1.55 µg/g, respectively. In markets of kachki, Spectrophotometrically determination of formaldehyde concentration showed highest value of 7.35 µg/g and in thai koi naturally occurring formaldehyde concentration showed higher value of 2.6 µg/g from freshwater and 3.9 µg/g in loyitta fish from marine source. He further concluded that fish from wet market contained a certain amount of added formaldehyde and fishes from both freshwater and marine sources shows to contain natural occurring formaldehyde in their muscle at different concentration.

Joshi *et al.,* (2015) performed a study on the fishes taken from the local markets of Kathmandu (Nepal) for formaldehyde quantitative assessment. From the three different local markets ,six samples that includes Mrigal carp (Cirrhinus sp.),Spiny eel (Macrognathus pancalus), River catfish (Eutropiichthys vacha), gold fish (Carassius auratus auratus), Rohu (Labeo rohita) and Mayur (Clarias batrachus) were purchased and stored on ice. The sample's formaldehyde estimation was done by using UV- Vis spectrophotometer and pH estimation was also done. He concluded that the pH was in the range of 6 and the formaldehyde concentration was in the range from 0.393 ± 0.004 µg/g to 2.328 ± 0.304 µg/g in

all species, out of which Mayur showed the highest formaldehyde concentration(2.328 ± 0.304 μg/g).

Yeasmin *et al.,* (2010) conducted study for the determination of quality of formalin treated fish (Labeo rohita) during ice storage conditions. Live rohu fishes were purchased from the Bangladesh fish market as samples. Two samples groups were made : one with 20kg fish sample treated with formalin and stored on ice and another 20kg fish sample stored on ice without formalin treatment. These samples were further analyses for bacteriological , organoleptic and biochemical aspects. He found that in organoleptic assessment the formalin treated fishes had a higher shelf life of 28-32 days than the control group than shows a shelf life of 20-23 days. Bacteriological assessment showed a low bacterial load below detection level in Formalin treated fishes even after 16 days of ice storage while fresh rohu stored for 24 days on ice showed a high bacterial load. Biochemical assessment showed an increase in the non-protein nitrogen content of the formalin treated fish gradually with storage time, while non-protein nitrogenous content decreased gradually in non formalin treated fish. Protein content during 24 days of ice cold storage condition in formalin treated fish decreased from 58% TO 28% whereas protein content in non-formalin treated fishes showed a decrease from 86.70% to 40%. Fish treated with formalin showed a reduction in gel forming ability due to the denaturation of muscle protein that resulted in poor quality of fish and it digestibility due to formalin contamination.

Ye *et al.,* (2013) conducted a study for the analysis of free and bound form of formaldehyde in squid and squid products by using gas chromatography-mass spectrometry. Ten squid and squid products were taken for the analysis of free and reversible form of formaldehyde. The formaldehyde derivatisation was done by 2-4 dinitrophenylhydrazine (DNPH) and was analysed by using gas chromatography and mass spectrometry technique. The results were compared with the result of high pressure liquid chromatography. The limit of detection was taken as 2.0 mg /kg. He found that on average ,the total concentration of free and reversible bound formaldehyde was higher than just free form of formaldehyde by 26.6 mg/kg .out of the total concentration of free and reversible bound formaldehyde ,

the free orm comprised of 39% .in the hplc method, the concentration of total free and reversible bound form of formaldehyde was higher than free form of formaldehyde by 19.3mg/kg. the formaldehyde exposure from the consumption of the shredded squid was estimated which was less than 0.2mg/kg, the oral reference dose suggested by the US environmental Protection Agency.

Murthy ***et al.,*** **(2019)** conducted the study to analyze food safety parameters for different outlets by collecting equal numbers of fish samples from all three markets of Navi Mumbai region. Proximate composition, biochemical and microbiological parameters were evaluated. Antibiogram for the isolated Staphylococcus aureusa and Escherichia colifrom these fishes were studied. He found that in dry fish market, most of the fish had higher total volatile basic nitrogen (TVB-N) and formaldehyde than permissible level. His evaluation showed that the formaldehyde content of fishes from different markets exhibited a higher percentage (1.03-1.93 mg %) than the recommended value. Local retail fresh fish market samples had higher levels of Aerobic Plate Count (APC), E. coli and S. aureus. One sample from dried fish market exceeded the permissible limit of APC (2,36,000 cfu/g). Supermarket samples contained all the microbial and biochemical levels within the limit but exhibited higher formaldehyde content. Multiple drug resistant bacteria also existed in both local and supermarket samples.

Nageswari ***et al.,*** **(2012)** conducted a study to quantify formaldehyde in active pharmaceutical ingredient by using HPLC-UV method. As formaldehyde does not possess chromophore, the developed HPLC method involved derivatization with 2,4-dinitrophenylhydrazine.She found that by using this method, the detection and quantitation limits achieved are 0.5 and 1.5 ppm, respectively. The calibration curve of formaldehyde was linear over the concentration range of 1.5–20 ppm. The method was found to be sensitive, precise and accurate and the proposed method has been successfully applied to estimate formaldehyde content in scale-up batches of bulk drug.

Soman *et al.,* (2008) carried out a study to develop and validate the reverse phase high pressure liquid chromatography (RP-HPLC) for the quantitative determination of formaldehyde in a drug substance. Formaldehyde (HCHO) is reacted with 2,4- dinitrophenylhydrazine (DNPH) to form a Schiff base (HCHODNPH derivatization product),having absorbing maximum (λmax) at 360 nm. The HPLC method employs a C8, 3-μm particle size analytical column (150 mm × 4.6 mm), 15-μL injection volume, column temperature controlled at 30°C, detection at 360 nm, and a water–acetonitrile (55:45, v/v) mobile phase at a flow rate of 1 mL/min. These conditions resolve the HCHO–DNPH product from unreacted DNPH, the drug substance and related impurities, as well as diluent peaks within 20 min. he found that the retention time of the HCHO–DNPH product was approximately 6.4 min. The method was linear, accurate in the specified range (0.33–333 ppm), and robust based on analyte (HCHO–DNPH derivatization product) stability in standard and sample. Detection limit was 0.03 ng (0.1 ppm).

Gani *et al.,* (2013) developed a simple tool known as optical chemical sensor based on immobilised pararosaniline in tetraethyl orthosilicate as sol-gel matrix for the detection of the formalin (formaldihide) in various food samples. This tool generates an optical signal that turns the colour from purple to yellow when contacted with food that contains formaldihide. His results shows the chemo sensor optic have characteristic maximum wave length 576.42 nm, with linier range 0 - 100 ppm, linearity coefficient R2 = 0.999, limit detection (LOD) 0.504 ppm, limit of quantification (LOQ) 1.680 ppm, sensitivity 0.087, disturbed matrix selectivity 1.716 %. The optimum is operational at pH 4, and response time at 150 seconds of 2 ppm. This sensor can be used to detect formalin in food sample in a simple mode and reusable for 4 times application. He further concludes that this tool is feasible and can be used for the determination of formaldehyde in food samples.

Sibirny *et al.,* (2011) studied alcohol oxidase and formaldehyde dehydogenase based enzymatic methods for formaldehyde assay in fish food

products. fish meat product such as muscle tissues of frozen hake and cod, as well as of freshly-killed carp were taken and two different deproteinising procedures were used. AOX-based enzyme method has the ability to oxidise a hydrated form of formaldehyde to formic acid. Hydrogen peroxide monitored in peroxidase-catalysed colorimetric reaction. While in FdDH-based method, in formaldehyde-dependent reaction, from nitrotertrazolium by the reductive action of NADH, a coloured formazane is formed .From the results, he demonstrated some products of fishes(cod and hake) had high concentration of formaldehyde (up to 100mg/kg wet weight) .Both of these methods were useful for the analysis of formaldehyde in fish products.

Bianchi *et al.,* (2007) demonstrated the formaldehyde content in the fish products having variable formaldehyde content by using a method - solid phase micro-extraction (SPME)-GC-MS based on derivatisation of fibre with pentaflurobenzyl-hydroxylamine hydrochloride. Various types of fish products were analysed for the quality assessment.12 species of fishes (fresh water, sea-fish and crustaceans) were taken . And Fresh fishes, deep frozen, boiled fish, canned and roasted fish products were analysed. He found that the highest concentration of formaldehyde was in fish samples belonging to the gadidae family fish (6.4 $\pm$1.2 mg/kg to 293 $\pm$ 26 mg/kg).out of the 14 samples four samples showed valves exceeding 60 mg/kg, while samples stored on ice showed a moderate formaldehyde concentration at $0^{o}C$. Rest of the samples showed valve of less than 22 mg/kg.

Radford and Dalsis (1982) carried out a study to analyse the formaldehyde level by using high pressure liquid in shrimps. Samples tested were brought from the local markets and also shrimps which are reared in laboratories. 2-4 dinitrophenylhydrazone is formed by the conversion of formalin is estimated by HPLC. The total time given for this method was 2hrs, detection limit of 0.05mg of formaldehyde per kg of shrimp, and at 10g/kg level, he found that the average recovery of 72.3% and the method used was valid.

Claeys *et al.,* (2009) carried an experiment on detection of formaldehyde in cultivated mushrooms, in which an evaluation was done to assess whether its

presence in food poses a risk to public health. He observed levels of formaldehyde in mushrooms are lower than the levels reported for vegetables, fruit, meat, fish and dairy products. On the basis of available data, a rough estimate of the dietary exposure to formaldehyde was performed. The exposure through the consumption of cultivated mushrooms (approximately 0.19 mg kg^{-1} body weight day^{-1} on average, consumers only) appeared to be small compared with the total dietary intake of formaldehyde (approximately 99.0 mg kg^{-1} body weight day^{-1} , total population). Based on comparison with toxicological safety limits for chronic exposure and given that formaldehyde is carcinogeniconly through inhalation and not by ingestion, he concluded that the dietary exposure to formaldehyde was not a cause for concern.

Chang *et al.*, (1983) carried out a study on inhalation exposure of formaldehyde. F-344 rats B6C3F1 mice were selected and were exposed to formaldehyde. Control group and pre-treated rats and mice were exposed to formaldehyde at 6hrs per day for mice and 4 days for rats at the dos of 6 or 15 ppm for 6 hours and their respiratory parameters were checked. Tidal volume and respiratory rate were recorded in these groups on nasal epithelium. Further assessment of formaldehyde deposition was one by autoradiography ,cell turnover and histopathological. He found that mice were better able to reduce minute ventilation upon repeated exposures, they had less HCHO available for deposition than rats, resulting in less tissue damage and a lower rate of cell turnover in the nasal epithelium.

Lee *et al.*, (1984) carried out an experiment to study the potency of formaldehyde as a sensitizing chemical in animals. Three routes of exposure were utilized: inhalation, dermal, and injection (with Freund's complete adjuvant). For inhalation exposure, animals divided in three groups, they were exposed to 6 ppm (Group I) or 10 ppm HCHO (Group II) for 6 hr/day or 8 hr/day (Group III) on 5 consecutive days. Animals were evaluated for three parameters i.e skin sensitivity, production of anti-formaldehyde antibody, and respiratory sensitivity (both immediate- and delayed-onset) to HCHO. He found that the animals who received inhalation exposure, two of four animals in Group III displayed dermal

sensitivity. No antibodies or pulmonary sensitivity were observed in any animals of these groups. Animals exposed to HCHO by injection who skin tested, displayed extensive dermal reactions. While ,two of four animals in this group developed antibodies to HCHO in low titre and no pulmonary sensitivity was detected. Animals exposed to HCHO by dermal contact developed neither pulmonary sensitivity nor antibodies to HCHO. However, all of these animals developed skin sensitivity. He further concludes that HCHO acts as skin sensitizer in the guinea pig without any respiratory hypersensitivity.

Sorg *et al.,* (2011) carried out a study to determine whether repeated formaldehyde (Form) exposure would alter corticosterone (CORT) levels in a rat model of MCS. Male Sprague–Dawley rats were given acute chamber exposures to Air or Form (0.7 or 2.4 ppm), and trunk blood was collected 20 or 60 min later. He found that all groups showed increased CORT levels above naïve basal levels at 20 min and a return to baseline by 60 min, with no differences between treatment groups. The second experiment examined the effect of repeated Form exposure (1 h/day×5 days/week×2 or 4 weeks) on basal CORT levels and after a final challenge. Basal CORT was increased above naïve values after 2 week exposure to Air or 0.7 ppm Form. By 4 week, CORT levels in the Air group returned to naïve values, but remained elevated in the 0.7 ppm Form group. There were no differences in basal CORT levels among either 2.4 ppm exposed groups. After a final Air or Form challenge, the 2 and 4 week Air and 0.7 ppm Form groups had elevated CORT levels similar to their acute response, while the 2 and 4 week 2.4 ppm Form groups had elevated CORT levels compared to their acute response, indicating enhanced reactivity of the HPA axis to subsequent Form. These findings suggest that altered HPA axis functioning occurs after repeated low-level Form exposure, and may have implications for mechanisms mediating MCS in humans.

Kerns *et al.,* (1983) carried out an experiment on groups of approximately 120 male and 120 female Fischer 344 rats and C57BL/6 x C3H F, mice were exposed by inhalation to the concentration of 0, 2.0, 5.6, and 14.3 ppm of formaldehyde gas 6 hr/day, 5 days/week, for 24 months. This was followed by

non exposure for 6 months. Interim sacrifices of the animals were conducted at 6, 12, 18, 24, 27, and 30 months. Significant formaldehyde-induced lesions were seen on the nasal cavity and proximal trachea. He found that the distribution and severity of these lesions were concentration dependent. Rhinitis, epithelial dysplasia, and squamous metaplasia were seen on all exposed groups of rats and in the intermediate and high exposure groups of mice. At 27 months ,there was regression of rhinitis, dysplasia, and metaplasia in the 14.3- and 5.6-ppm groups of mice and in the 2.0- and 5.6-ppm groups of rats. Squamous cell carcinomas were observed in the nasal cavities of 103 rats (52 females and 51 males) and 2 male mice exposed to 14.3 ppm and in 2 rats (one male and one female) that were exposed to 5.6 ppm of formaldehyde gas. Formaldehyde inhalation was weakly associated with an increase in the frequency of polypoid adenomas in the nasal cavity of male rats

Sarsilmaz ***et al.,*** **(2007)** conducted a study to determine whether exposure of neonatal rats to formaldehyde had either early or delayed effects on the numbers of pyramidal cells in the cornu ammonis (CA) of the hippocampus. Neonatal Wistar rats divided int0 three groups and were exposed to 0 ppm (control group), 6 ppm and 12 ppm (high concentration group) of FA concentrations throughout the 30-day period after the birth and were placed into a glass chamber for 6 h/day containing FA vapour., Some of the animals from each FA-treated group were anesthetized and decapitated at the day 30 while the remaining ones were killed at the day 90. The brains were removed immediately and fixed in 10% neutral-buffered FA solution. He found that the appearance of pyramidal cells was normal under light microscope both postnatal day (PND) 30 and PND 90 in all groups. There were concentration-related volume changes of CA at PND 30 and PND 90; low concentration of FA significantly increased, whereas high concentration decreased the volume of CA in comparison of the control at PND 30.Importantly, high concentration of FA at PND 90 increased the volume of CA in comparison of the low concentration but not with the control. Furthermore, low and high concentrations of FA decreased the volume of hemisphere at PND 30, whereas a reverse effect of these concentrations was observed at the hemisphere of PND 90 in comparison of the control. In both CA

and cerebral hemisphere, he found an age-related volume decrease in both control and low/high concentration groups. On the other hand, there were significant age-related reductions in the total number of pyramidal cells at 90 days of age irrespective of the groups examined. Rats treated with high concentration FA were seen to have significantly fewer pyramidal cell neurons than either the animals treated with low concentration FA or control groups ($p<0.01$). He concluded that these observations indicate that pyramidal cells in the hippocampus may be vulnerable to FA exposure during the early period of life.

Tong *et al.,* (2013) conducted an experiment Gaseous formaldehyde exposure is known to induce animal memory loss and human cognitive decline; carried out a study, where he found that hippocampal formaldehyde gets accumulated in memory deteriorating disease such as age-related dementia .normal Sprague–Dawley rats were persistently given intraperitoneal injection with formaldehyde. Furthermore, this excess formaldehyde treatment suppressed the hippocampal LTP formation by blocking N-methyl-D-aspartate (NMDA) receptor. He found that the chronic excess formaldehyde treatment over a period of 30 days markedly decreased the viability of the hippocampus and down-regulated the expression of the NR1 and NR2B subunits of the NMDA receptor. He found that excess endogenous formaldehyde is a critical factor in memory loss in age-related memory-deteriorating diseases.

Monticello *et al.,* (1996) conducted a formaldehyde carcinogenicity study in which a major end point was checked correlation of cell proliferation indices with sites of formaldehyde-induced SCC. For up to 24 months, rats were exposed (6 h/day, 5 days/week) to formaldehyde (0, 0.7, 2, 6, 10, or 15 ppm) with sacrifice time points at 3, 6, 12, and 18 mo. A unit length labelling index (ULLI; S-phase nuclei/mm basement membrane) was determined for specific nasal regions in addition to a population-weighted ULLI (PWULLI). The PWULLI was defined as the product of regional ULLI and total number of nasal epithelial cells in the respective site. Nasal SCC sites of origin were mapped. He found that formaldehyde induced SCC in a highly nonlinear fashion, with no observed effect at the level of 2 ppm, a minimal response at 6 ppm, and a steep increase at 10 and

15 ppm. The tumor incidence was 1, 22, and 47% at 6, 10, and 15 ppm, respectively. Further, ULLI was significantly ($P < 0.05$) increased at 10and 15ppm but not at the lower concentrations .He saw that there was a good correlation between PWULLI and regional tumor incidence (R2 0.88), while the correlation of regional SCC with ULLI was relatively poor (R2 0.46). He concluded that target cell population size and sustained increases of cell proliferation in these populations, determined by differences in regional airflow-driven formaldehyde dose to these sites, coupled with the known nonlinear kinetics of formaldehyde binding to DNA, can together account for the nonlinearity and site specificity of formaldehyde-induced nasal SCC in rats.

Swenberg *et al.,* (1980) carried out a study on groups of 120 male and 120 female rats who were exposed to formaldehyde vapour by inhalation to 0, 2, 6, or 15 ppm 6 hr/day, 5 days/week, for 18 months of a complete 24-month study. Data available shows the results after 18 months of exposure. He found that squamous cell carcinomas occurred in the nasal cavities of 36 rats exposed to 15 ppm formaldehyde. The tumors ranged from small early carcinomas of the nasal turbinate to large invasive osteolytic neoplasm's which further extended into the sub cutis of the premaxilla. But similar tumors were not detected in rats exposed for 18 months to 2 or 6 ppm or in mice which were exposed to 2, 6, or 15 ppm formaldehyde. He also found that rhinitis, epithelial dysplasia, and squamous metaplasia occurred in rats from all exposure levels of formaldehyde; however, the severity and extent of the lesions were dose related. In contrast, papillary hyperplasia and squamous atypia was seen only in animals exposed to 15 ppm formaldehyde.

Pitten *et al.,* (2000) carried out a study for determination of neurotoxicological impact from formaldehyde inhalation. Forty Wistar rats (Lew.1/K) were trained to find food in a maze within a particular time. When all animals were at an equal level, 13 rats were exposed to inhaled 2.6 ppm and 13 others inhaled 4.6 ppm formaldehyde 10 min/d, 7 d/week for 90 d. The control group 14 animals were inhaling water steam according to the same exposure pattern. During the exposure period and the post-trial observation stage (30 d), the

time required to find the food and the number of mistakes made on the way were recorded. He recorded that between the animals exposed to formaldehyde and the control group a statistically significant difference for both parameters was observed ($p<0.05$). The animals exposed to formaldehyde needed more time and showed more mistakes than the animals of the control group while going through the maze. His results underline the necessity for a systematic observance of precautions in classify formaldehyde as “probably neurotoxic”. Further investigations are required to assess the neurotoxicologic impact of sub chronic formaldehyde exposure.

Morgan *et al.,* (1986) conducted a study to determine the nature and distribution of acute effects of inhaled formaldehyde on the nasal mucociliary apparatus of male F-344 rats by using whole body exposures. Formaldehyde inhalation exposures were given in a range from a single 6-hr period up to multiple 6-hr exposures daily for 3 weeks, at a concentration of 15, 6, 2, 0.5, and 0 ppm. Further, Within 1 hr of the last exposure, the rats were killed and the nasal passages examined for effects on nasal mucociliary function. He found that the rats Exposed to 15 ppm formaldehyde induced inhibition of mucociliary function in specific regions of the nose, and mucostasis was generally more extensive than cilia stasis. With 2 weeks of exposure, The effects, initially confined to the anterior regions of the nose, became progressively more extensive with only very slight progression during the third week. Inhibition of mucociliary function was much less severe with 6 ppm, minimal at 2 ppm, and not detected in rats following exposure to 0.5 ppm. The distribution of epithelial lesions, identified by histopathology, correlated well with the distribution of defective mucociliary function, but mucociliary function was a more sensitive indicator of toxicity. Localized defects in mucociliary function represent a potentially important consequence of exposure to formaldehyde.

Wouterson *et al.,* (1987) carried out a sub chronic study, in which Male and female albino Wistar rats were exposed to concentrations of 0, 1, 10 or 20 ppm formaldehyde vapour during 6 h/day, 5 days/week for 13 weeks. Treatment-related changes observed at 20 ppm included in both sexes: stared

coats, uncoordinated locomotion and excitation during the first 30 minutes of each exposure, yellowing of the fur, growth retardation, a decreased level of plasma protein, severe and extensive keratinized stratified squamous metaplasia of the nasal respiratory epithelium, and focal degeneration and squamous metaplasia occasionally accompanied by keratinisation of the olfactory epithelium; in males only; increased activities of plasma aspartate amino transferase (ASAT), alanine amino transferase (ALAT) and alkaline phosphatase (ALP) and squamous metaplasia of the laryngeal epithelium. Lesions seen at 10 ppm included yellowing of the fur and moderate squamous metaplasia of the nasal respiratory epithelium. He found that the only change observed in three out of twenty 1 ppm exposed animals that might or might not be treatment-related was minimal focal epithelial hyperplasia and squamous metaplasia of the respiratory epithelium lining the nasal septum and maxillary turbinate's. It was concluded that under the conditions of the present 13-week inhalation study, formaldehyde at concentrations up to 10 ppm was not hepatotoxic to rats. At the 20 ppm formaldehyde level, a slight effect on the fiver of mate rats cannot be completely excluded. The study was inconclusive with respect to 1 ppm formaldehyde being a cytotoxic or a no-cytotoxic effect level for the nasal epithelium.

Till *et al.,* (1989) carried out an experiment in which **f**ormaldehyde was administered in the drinking-water to groups of 70 male and 70 female Wistar rats for up to 24 months. Survivors of subgroups of ten rats/sex/group each were killed after 12 or 18 months. The mean formaldehyde doses administered were 0, 1.2, 15 or 82 mg/kg body weight/day for males, and 0, 1.8, 21 or 109 mg/kg/day for females. Body weight and food intake were decreased in the high-dose group. Liquid intake was decreased by 40% in the high-dose group in both sexes in comparison with the controls. There was a slight temporary increase in the density of urine, whereas there was a tendency towards lower urine production in the high-dose group. The relative kidney weights were increased in the high-dose females. Gross examination at autopsy revealed a raised and thickened limiting ridge of the fore stomach in most high-dose rats. In addition, several rats in the high-dose group showed irregular mucosal thickenings in the fore- and/or glandular stomach. Treatment-related histopathological gastric changes seen in

most of the animals of the high-dose group included papillary epithelial hyperplasia frequently accompanied by hyperkeratosis and focal ulceration in the fore stomach and focal chronic atrophic gastritis, occasionally accompanied by ulceration and/or glandular hyperplasia, in the glandular stomach. A higher incidence and/or degree of renal papillary necrosis occurred in the high-dose rats. From this study, he found out that the ‘no-observed-adverse-effect level’ of formaldehyde was 15 and 21 mg/kg body weight/day for male and female rats, respectively. Oral administration of formaldehyde at doses of 82 and 109 mg/kg/day to male and female rats, respectively, caused severe damage to the gastric mucosa but did not result in gastric tumours or tumours at other sites. The study did not provide any evidence of carcinogenicity of formaldehyde after oral administration.

Wouterson *et al.,* (1989) carried out study the significance of damage to the nasal mucosa for the induction of nasal tumours by formaldehyde in rats, a long-term inhalation study was conducted in which male rats with severely damaged or undamaged nose were exposed 6 h/day for 5 days/week to 0, 0.1, 1.0 or 10 ppm formaldehyde vapour for 28 months, or for 3 months followed by a 25-month observation period. The damage to the nasal mucosa was induced by bilateral intranasal electro coagulation. The total number of rats used was 720, 480 with damaged and 240 with intact nose.Compound-related degenerative, inflammatory and hyper plastic changes of the nasal respiratory and olfactory mucosa were invariably observed when rats with intact nose were exposed to 10 ppm but not when exposed to 1.0 or 0.1 ppm formaldehyde. Nasal electro-coagulation increased the incidences of formaldehyde induced rhinitis, hyper- and metaplasia of the respiratory epithelium, and degeneration and hyper- and metaplasia of the olfactory epithelium. In addition, exposure to 10 ppm formaldehyde for 28 months produced nasal squamous cell carcinomas in rats with damaged nose (15/58) but not in rats with intact nose. He found that three months of exposure to 10 ppm formaldehyde or exposure to 0.1 or 1.0 ppm formaldehyde for 28 months had no such effect. It was concluded that severe damage to the nasal mucosa may contribute to the induction of nasal tumours by formaldehyde.

Teng *et al.,* (2001) conducted out a study in which groups of 32 animals (16 males/16 females) were given topical application of 200 µg 1% formaldehyde or 10% formaldehyde in water on the back skin twice a week. Two other groups were painted with 51.2 µg DMBA in 100 µL reagent grade acetone: one was followed 9 days later with applications of 200 µl 10% formaldehyde in water twice a week, and the other with 17 nmol TPA in 100 µL acetone twice a week. A fifth group of 176 mice was treated once with 51.2 µg DMBA in acetone and given no further treatment. The animals were painted and/or observed for 60 weeks. The animals painted once with DMBA alone were observed for 80 weeks. Animals receiving 1 and 10% formaldehyde alone developed no tumors. The 176 mice painted once with DMBA developed 225 skin tumors in 85 animals. Six of them were squamous cell carcinomas and 2 animals had lymph sarcomas. In the group painted with DMBA and then followed by formaldehyde, three animals had lung adenomas, and 11 had neoplastic growths of the skin (three squamous cell carcinomas and 22 papillomas). The final tumor rate after DMBA initiation followed by formaldehyde was not significantly different from the final tumor rate after DMBA alone, but the time of appearance of the first tumor and the mean latency time was significantly or very significantly, reduced. He concluded that under the experimental conditions used formaldehyde had no carcinogenic potency of its own but did shorten the latency time in DMBA-induced carcinogenesis. Formaldehyde has also acute toxic and irritating effects. It is therefore important to induce practical methods for reducing exposure to formaldehyde in pathology laboratories.

Speit and Schmid (2006) conducted a study in which he found that formaldehyde (FA) is genotoxic in vitro in cultured mammalian cells . When FA reaches the nuclear DNA, it forms DNA–protein cross-links (DPX). Incomplete repair of DPX can lead to the formation of mutations, in particular chromosome mutation and micronuclei (MN) in proliferating cells. Due to its high reactivity, FA leads primarily to local genotoxic effects at the site of contact. In humans, local genotoxic effects of FA have been studied with the micronucleus test (MNT) in exfoliated nasal and buccal mucosa cells. This approach is considered to be highly relevant because these tissues are the actual targets of FA, and MN

are a sensitive indicator for the mutagenic action of FA. The published studies suggest that inhalation of FA leads to increased MN frequencies in nasal and/ or buccal mucosa cells. However, a critical review of the data reveals that the effects are not consistent, and the studies should be interpreted with caution.

Heck *et al.,* (1985) carried out a study to check the effect of exposure to formaldehyde (CH_2O) on the CH_2O concentration of the blood. Eight male F-344 rats were exposed to 14.4 ± 2.4 ppm of CH_2O for 2 hours and the blood was collected immediately after exposure. Formaldehyde concentrations in the blood were determined by gas chromatography/ mass spectrometry. The blood of eight rats unexposed to CH_2O was collected and analyzed in the same manner. Measured CH_2O concentrations (µg/g of blood) were: controls, 2.24 ± 0.07; exposed, 2.25 ± 0.07 (mean ± S.E.). Formaldehyde concentrations in human blood were determined by analyzing samples of venous blood collected before and after exposure of six human volunteers (4 M, 2 F) to 1.9 ± 0.1 ppm of CH_2O for 40 min. Average CH_2O concentrations (µg/g of blood) were: before exposure, 2.61 ± 0.14; after exposure, 2.77 ± 0.28. He found that in neither experiment was there a statistically significant effect of exposure on the average CH_2O concentration of the blood. However, human subjects differed significantly with respect to their blood CH_2O concentrations, and significant differences (either an increase or a decrease) were found between the CH_2O concentrations of the blood taken before and after exposure from some of the subjects, suggesting that blood CH_2O concentrations may vary with time.

Bender *et al.,* (1983) conducted a study in which human panellists sensitive to formaldehyde eye irritation were exposed to low concentrations of formaldehyde vapour (0.35 to 1.0 ppm) for 6 minutes. Eye irritation was evaluated by time to detection of the first trace of irritation and by subjective ranking of severity. He found that both time to response and severity appeared to be functions of formaldehyde concentration. Also severity of response was above “slight” only with highest test concentration, 1.0 ppm.

Suruda *et al.,* (1993) conducted a study to check the effect of low-level exposure to formaldehyde on oral, nasal, and lymphocyte biological markers in a

group of 29 mortician students who were about to take a course in embalming. During the 85-day study period, the subjects performed an average of 6.9 embalming and had average cumulative formaldehyde exposures of 14.8 ppm-h, with an average air concentration of 1 .4 ppm during embalming. Since the average time spent embalming was 1 25 mm, he found that the formaldehyde exposures calculated as an 8-h time-weighted average were 0.33 ppm on days when embalming were done, which was less than the Occupational Safety and Health Administration permissible exposure limit of 0.75 ppm. Epithelial cells from the buccal area of the mouth showed a 1 2-fold increase in micronucleus frequency during the study period, from 0.046 ± 0.1 7/1 000 cells pre-exposure to 0.60 ± 1.27/1000 cells at the end of the course ($P < 0.05$). Nasal epithelial micronuclei increased 22%, from 0.41 ± 0.52/1000 cells to 0.50 ± 0.67/1000 cells ($P = 0.26$). In blood cells, the frequency of micro nucleated lymphocytes was increased from 28%, from 4.95 ± 1.72/1000 cells to 6.36 ± 2.03/1000 cells ($P < 0.05$), while sister chromatid exchanges decreased 7.5% ($P < 0.05$). A dose-response relationship was observed between cumulative exposure to formaldehyde and that increases in buccal micronuclei in the 22 male subjects but not in the 7 female subjects. He concluded that low-level exposure to formaldehyde is associated with cytogenetic changes in epithelial cells of the mouth and in blood lymphocytes. These cytogenetic effects may be useful as markers of biologically effective dose.

Yilmaz *et al.,* (2016) developed a simple high-performance liquid chromatography method for the determination of formaldehyde in human tissue. Formaldehyde was derivatized with 2,4-dinitrophenylhydrazine. It was extracted from human tissue with ethyl acetate by liquid–liquid extraction and analyzed by high-performance liquid chromatography. He found that the calibration curve was linear in the concentration range of 5.0–200 μg/mL. Intra-and inter-day precision values for formaldehyde in tissue were less than 6.9%, and accuracy (relative error) was better than 6.5%. The extraction recoveries of formaldehyde from human tissue were between 88and 98%. The limits of detection and quantification of formaldehyde were 1.5 and 5.0 μg/mL, respectively. Also, this assay was applied to liver samples taken from a biopsy material.

Materials&Methods

3. MATERIAL METHOD

3.1. Study Area

The fresh fish samples and ready-to-eat were collected from local fish vendors of Mumbai Suburban region. The collection was done in an insulated ice-box filled with ice- packs and processing of these samples was done on the same day. The processing procedure for the ready-to-eat products was same as that of raw fish samples.

3.2 Sample Size

A total of 100 samples of meat (fish and ready-to-eat products) was taken for further extraction procedure. Out of the total samples, 80 samples of fishes (fresh water and marine water) and 20 samples of ready-to-eat products were analysed.

3.3 Analysis of samples

3.3.1 High pressure Liquid Chromatography

3.3.1.1 Apparatus

The High Pressure Liquid Chromatography (Shimadzu, Japan) system consisted of Quaternary pump (Model DGU-20A5R), Auto sampler (Model SIL-20AHT) and UV Detector (Model CTO-10ASVP) was used.

3.3.1.2 Principle of HPLC

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation

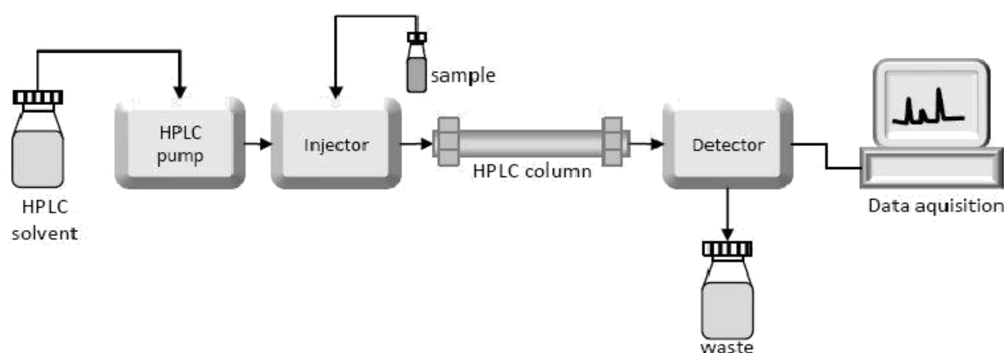

Fig.2. - Basic principles of HPLC

3.3.1.3 Columns and packings of the HPLC

The column details are -

ACE C18 column, (150 cm x 4.6 mm, 5 µm);

Pore Size: 300 A˚

3.3.1.4 Chromatographic conditions :

Instrument used: Shimadzu HPLC; Isocratic

Mobile Phase: Methanol (60) : Water (40)

Flow rate: 1.0 ml/min

Column: ACE C18 column, (150 cm x 4.6 mm, 5 µm);

Pore Size: 300 A˚

Detector: UV at 365 nm

Injection volume: 10 µL

Column Temp: ambient

Run Time: 11 minutes

Diluent :Mobile phase

The method was validated on research findings of Bhowmik *et al.,* (2017). In brief, the methodology used in current experiment is as follows -

3.3.1.5 Reagent preparation:

DNPH was recrystallized before use. Recrystallization was carried out by dissolving and excising DNPH 10 mL of anhydrous acetonitrile acetate to form a saturated solution. After complete dissolution, the solution was cooled to room temperature, capped in a brown bottle and stored overnight at 40 °C for further crystallization. The crystals was collected by vacuum filtration. About 150 mg of DNPH crystals was accurately weighted and was dissolved in 49.5 mL of acetonitrile and mixed with 0.5 mL of H_3PO_4 (85%).

3.3.1.6 Standard preparation from CRM (certified reference materials)

Standard was prepared from CRM first stock solution whose concentration was 1000 µg/mL in water. The dilution was performed according to the concentration required for analysis.

3.3.1.7 Sample preparation:

About 5 g samples was taken; blank and spiked formalin was added into the samples. Then added 5 mL acetonitrile, analytical grade and vortex. The samples were sonicated for 30 min at room temperature (25–30 °C), shaken for 30 min in a shaking water bath at room temperature at 150 rpm, centrifuged for 5 min in 6000 rpm at 22 °C, filtered through a Whatman filter paper (90 mm). The upper layer of the extract of approximately 5 mL was carefully taken. 2.5 mL working DNPH solution and vortex was added well. The samples was derivatised by shaking at 150 rpm, at 40 °C for 1 hr in a shaking water bath and after incubation the supernatant got filtered by a syringe micro filter (0.45 µm).

3.3.1.8 Qualification and quantification of formaldehyde:

The sample derivatives were analyzed in HPLC and compared to the standard formaldehyde retention time for qualification. The peak area of the sample solution was substituted in the calibration equation of the standard curve to calculate the formaldehyde concentration.

3.3.1.9 Recovery test:

To determine recovery, known concentration of formaldehyde CRM (1 mg /L, 2 mg/L, and 3 mg /L) were spiked in meat sample. The spiking levels were within the calibration range. Samples were extracted following sample preparation procedure outlined in material and methods section and recovery was calculated as follows:

Recovery (%): Concentration of formaldehyde quantified in the sample * 100/ Spiked concentration.

Results&Discussions

4. RESULT AND DISCUSSION

Formaldehyde an active disinfectant, has a potent preservative use in food industry. The food items commonly the perishable ones are preserved by the addition of formaldehyde in an acceptable range to increase its shelf life. Addition of this agent leads to the decrease in microbial activity that causes deteriorating action.

As per WHO, formaldehyde has been declared to be a potent carcinogen and mutagen to humans. In India, FSSAI (Food safety and standard authority of India) has set an ad hoc limit for formaldehyde in fish and fishery products only.

In the present study, the samples were analysed and its pharmacokinetic assessment was done for detecting the formaldehyde level that might be an indicative of adulteration. The samples were mainly fishes of various species and ready to eat products. Formaldehyde is rampantly used as an adulterant and no data is available in India where formaldehyde level has been analysed by HPLC machine.

4.1 ASSAY OF SAMPLES

4.1.1 High Pressure Liquid Chromatography (HPLC)

The samples were analysed on the HPLC. The HPLC method was validated for various parameters.

(A) Method Validation.

(a) Specificity

(a.i) Placebo Study

Specificity is the ability to access unequivocally the analyte of interest in other components that can be expected to be present those might be typically matrix, degradants, impurities, etc. (Q2 (R1) , 2005). Fig. 3 shows the standard chromatogram of 3 ppm formaldehyde-2,4- DNPH. The placebo mixture showed

only 2,4-DNPH peak in the whole chromatogram, indicating the specificity of the method.

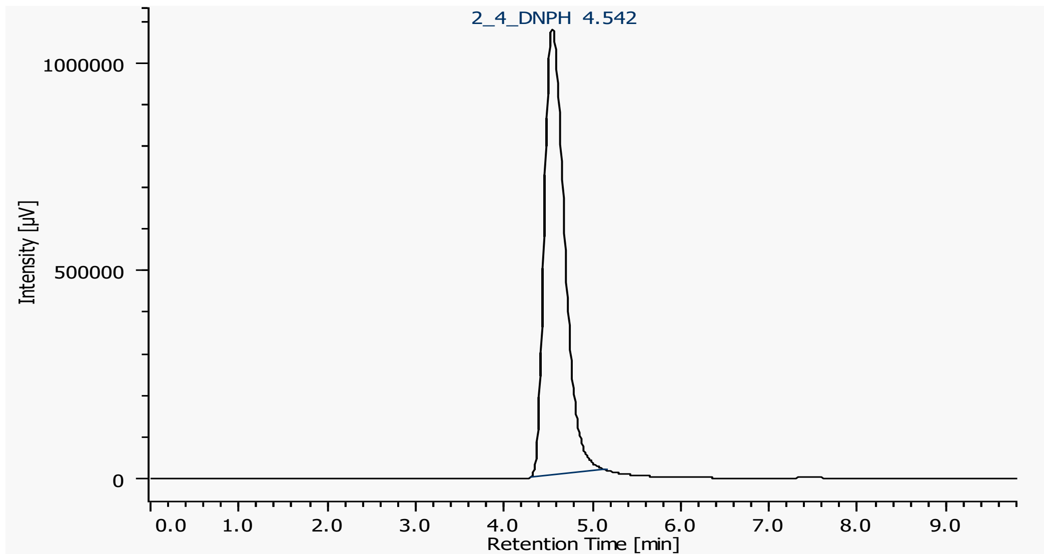

Fig. 3 Chromatogram of Placebo mixture

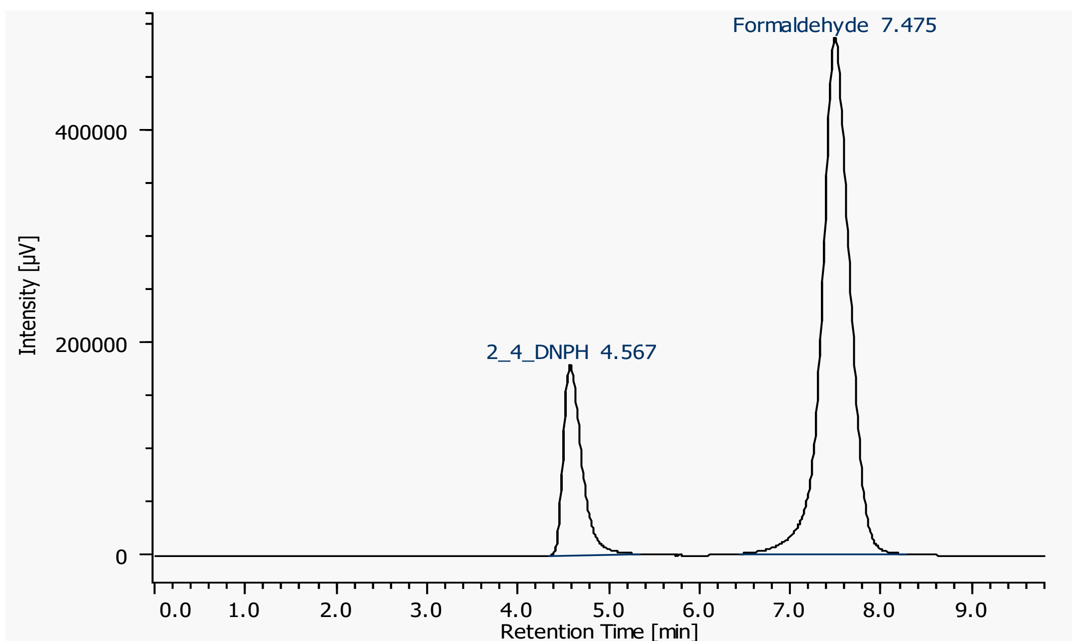

Fig.4 Standard chromatogram of Formaldehyde-2,4-DNPH at 100 ppm concentration

(b) LINEARITY

(b.i) Linearity of formaldehyde in mobile phase

The calibration curve of formaldehyde standard in the mobile phase was plotted by taking range of 0.5 -3 ppm. The method was found to be linear with the selected concentration range with the regression coefficient of 0.99 and slope of 10443 and intercept of 2249. The standard plot was depicted in Fig. 5.

Table 1. Calibration curve of formaldehyde-2,4-DNPH in mobile phase

Sr. No.	**Concentration (µg/mL)**	**Mean Peak Areas**	**Standard deviation**	**%RSD**
1.	0.5	51653	182.9125	0.35
2.	1	102598	269.9018	0.26
3.	1.5	153817.7	358.4025	0.23
4.	2	203931	1628.014	0.79
5.	2.5	255885.3	499.0955	0.19
6.	3	315178.3	5246.096	1.66

* Mean of triplicate readings

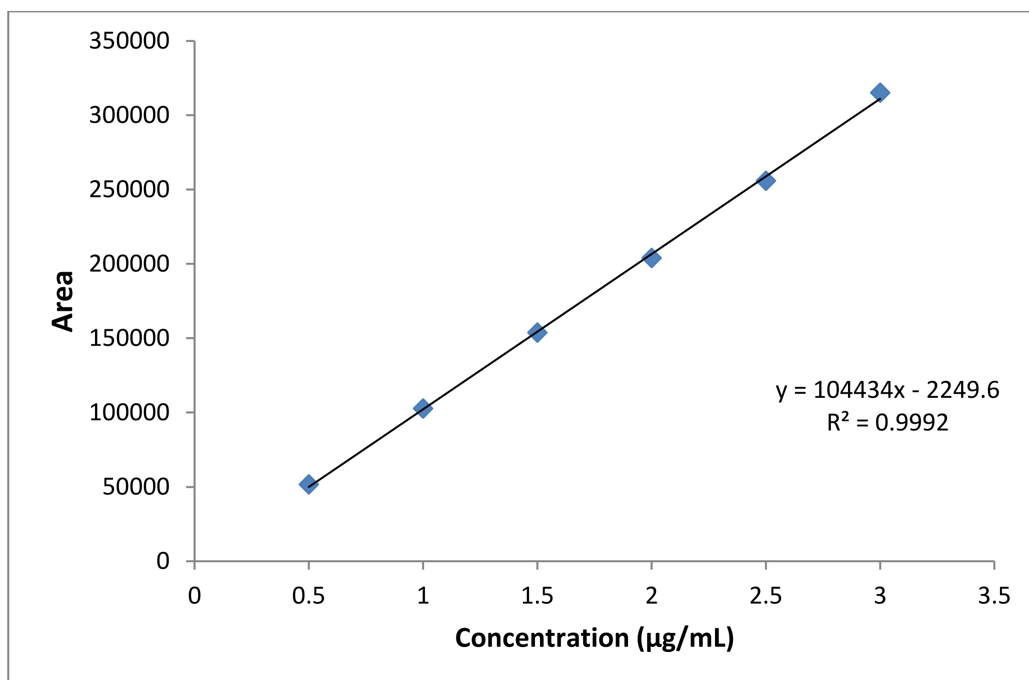

Fig.5 Standard calibration curve of formaldehyde-2,4-DNPH

(c) Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ values were found to be 0.14 µg/mL and 0.44 µg/mL, respectively by ANOVA analysis.

(d) Precision

The precision of an analytical procedure is the closeness of agreement (degree of scatter) between series of measurement obtained from multiple sampling of same homogenous sample under prescribed conditions (Q2 (R1) , 2005).

(d.i) System precision

The % RSD value of area of all six replicate injections of standard solution (3 µg/mL) was 0.56 % which meets the system suitability criteria of % RSD NMT 2%.

Table 2. System Precision

Injection Number	Retention Time (min)	Area
1	7.45	315698
2	7.4	317855
3	7.46	314520
4	7.41	316589
5	7.43	313855
6	7.5	318320
Mean	7.44	316139.5
standard deviation	0.03	1785.285
%RSD	0.49	0.56

(d.ii) Repeatability/Intra-assay precision

Also known as Inter-assay precision, repeatability expresses the precision under same condition over a short period of time that is on the same equipment at normal operating condition of analytical method (Q2 (R1) , 2005). Data represented in Table. 3 shows the variation obtained after injecting six samples of 3 µg/mL. The % RSD of analyte obtained was 0.78 % which shows that the method is precise.

Table 3. Repeatability / Intra-assay precision

Sample	Area
S1	316326
S2	318230
S3	317624
S4	312526
S5	319553
S6	315221
Mean	316580
Standard deviation	2489.879
%RSD	0.78

(d.iii) Intermediate Precision (Ruggedness)

Intermediate precision expresses the within laboratory variations that is different days.

Table 4. Intermediate precision

Sample	Day 1	Day 2
	Area	Area
S1	312224	316526
S2	311229	319501
S3	316239	326231
S4	318228	319825
S5	312102	319229
S6	317228	319527
Mean	314541.7	320139.8
Standard Deviation	3032.613	3221.01
%RSD	0.96	1.00
Pooled data for 12 samples		
Mean	317340.8	
Standard Deviation	4176.521	
%RSD	1.31	

(e) Accuracy

(e.i) Determination of Accuracy

Accuracy expresses the closeness agreement between the value that is accepted as conventional true value or an accepted reference value and the value found. This is also called trueness.

Table 5. Accuracy- Determination by spiking placebo with analyte

Level (ppm)	Sample	Recovery (%)	Mean Recovery (%)
1	S1	89.45	90.34
	S2	91.23	
2	S1	88.96	92.655
	S2	96.35	
3	S1	90.36	93.29
	S2	94.22	
Mean		92.095	------
Standard deviation		1.552683	------
% RSD		1.68	------

(f) Robustness

Robustness is the measure of analytical procedure capacity to remain unaffected by small, but deliberate variations in method parameters which provides an indication of reliability in normal range.

The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the area, it was changed by 0.3 units from 0.7 to 1.5 mL/min while the other mobile phase components were held constant.

The effect of percent organic strength on resolution was studied by varying acetonitrile from -5 to +5 (20 to 30%) while the other mobile phase components were held constant.

The standard solution and sample solution were injected at each changed condition.

Formaldehyde estimation in different animal origin samples

In the present study, a total of 100 fish samples of various species were collected and processed in the department for detection of formaldehyde or its residue content into the aliquot, the analysis was done by using RP-HPLC. The samples were divided into fresh water fishes, marine water fishes and ready-to-eat products. The chromatogram of these samples is presented in the Appendix xi and tables containing respective values have been shown in Table no. 6. The statistical analysis was done by using one-way ANOVA.

Catla (*Catla catla*), Rohu (*Labeo rohita*), Catfish (*Siluriformes*), and Nila Tilapia (*Oreochromis niloticus*) species of freshwater fishes were evaluated for the formaldehyde residue in which it was found that Catla (*Catla catla*) and Nila Tilapia (*Oreochromis niloticus*) had formaldehyde concentration of 13.90±0.89 mg kg^{-1} and 11.85±1.02 mg kg^{-1} respectively while no formaldehyde content was found in Rohu (*Labeo rohita*) and Catfish (*Siluriformes*) species. The values found in the freshwater fishes were above the permissible limit given by the FSSAI, 2019. Therefore, it can be speculated that the fishes might have been adulterated with formaldehyde for preservation.

To estimate formaldehyde in marine water fishes, the fish species selected were Indian Mackerel (*Rastrelliger kanagurta*), Bombay duck (*Harpadon nehereus*), Sardine (*Sardina pilachardus*), and Pomfret (*Bramidae*). Out of the fish samples analysed, Indian Mackerel (*Rastrelliger kanagurta*), Sardine (*Sardina pilachardus*), and Pomfret (*Bramidae*) had formaldehyde content of value 23.01±1.29 mg kg^{-1}, 22.19±1.03 mg kg^{-1} and 20.37±1.69 mg kg^{-1} respectively. Whereas, no formaldehyde content was observed in Bombay duck (*Harpadon nehereus*) species. The detected values in these samples were below the standard limit of the FSSAI, 2019.

The analysed samples values were compared with the given standard and significant difference ($p < 0.001$) were observed between the groups i.e. in both fresh water and marine water fishes.

Along with these fish samples, some of the ready-to-eat products were also analysed in which no formaldehyde concentration was detected.

Table 6. Data Of different Animal Origin Samples

SAMPLE NAME	SAMPLES NO.										
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**MEAN ± SE (mg/kg)**	**SD**
1. LOBEO ROHITA	0	0	0	0	0	0	0	0	0	0	0	0
2. CATLA CATLA	13.78	10	14.58	16.00	13.91	18.00	16.80	11.65	9.36	15.00	*13.90± 0.89	2.83
3. SILURIFORMIS	0	0	0	0	0	0	0	0	0	0	0	0
4.OREOCHROMIS NILOTICUS	14.25	18.00	7.90	13.56	14.65	9.23	10.23	8.65	9.69	12.35	* 11.85 ± 1.02	3.24
5.RASTRELLIGER KANAGURTA	24	25	26.6	23.78	25.3	15	19	26.82	18.36	26.25	* 23.01 ± 1.29	4.08
6. HARPADON NEHEREUS	0	0	0	0	0	0	0	0	0	0	0	0
7. SARDINA PILACHARDUS	22.65	18.36	23.53	15.32	24.65	22.00	24.65	20.56	25.36	24.93	* 22.20 ± 1.03	3.26
8. BRAMIDAE	14.85	19.22	24.23	26.50	16.56	10.50	20.36	26.98	24.65	19.85	* 20.37 ± 1.69	5.35
9. READY TO EAT SAMPLES	0	0	0	0	0	0	0	0	0	0	0	0

SD = Standard deviation; SE = Standard error; the samples were analysed by RP-HPLC method. * $P < 0.001$ Vs Standard Group.

Table 7. FSSAI, 2019 limit for naturally occurring formaldehyde in food products

Food category	Permissible limit (max.) Standard
Fresh water (Molluscs, Crustaceans and Echinoderms)	4 mg/kg
Marine / Brackish water	100 mg/kg

Bhowmik *et al.,* (2017) estimated formaldehyde levels in fresh water, marine water and shrimps from different market places of Bangladesh by HPLC method. He observed that the formaldehyde range was 5.1 ±0.71 - 12.26 ± 1.67 mg/kg , 10.8 ± 1.72- 39.68 ± 7.87 mg/kg and 13.57± 1.93 mg/kg for shrimps which were below the tolerable levels for humans.

Wahed *et al.,* (2016) performed formaldehyde level estimation by HPLC method in various food products. He observed that the formaldehyde level in Kachki fish was 11.39 ± 0.62 mg/kg, in mixed dead fish 8.39 ± 1.58 mg/kg and in milk 1.76 ± 0.04 mg/kg.

Das *et al.,* (2018) by using UV-spectrophotometer carried out a study in different fishes i.e. Rohu (Labeo rohita), Catla (catla catla), Bombay duck (Harpadon nehereus) and Boyal (Wallago attu) were collected from different retail markets of Mumbai. He found that the formaldehyde level in Catla fish was 2.76 µg/g , 2.88 µg/g from Four bungalow and Andheri market respectively. For Rohu fish, it was 3.11 µg/g/g and 2.96 µg/g, for boyal fish had 2.38 µg/g. and 2.22 µg/g. For Bombay duck it was 1.48 µg/g,1.71 µg/g, 2.22 µg/g and Indian mackerel content 1.81 µg/g, 2.27 µg/g,2.35 µg/g of formaldehyde.

Jaman *et al.,* (2015) quantitively estimated the formaldehyde content in different fishes by spectrophotometer. The formaldehyde content from different markets ranged between 1.4µg/g and 7.35 µg/g. The highest concentration was

found in Kachki i.e. 7.35 μg/g , in marine fish it was 3.9 μg/g in loyitta fish and 2.6 μg/g in Thai freshwater fish.

Joshi *et al.,* (2014) estimated formaldehyde and pH in fishes from different fish markets by Uv-Vis spectrophotometer. The mean range of formaldehyde found was 0.393 ± 0.004 μg/g to 2.238 ± 0.304 μg/g. The highest concentration was found in Magur fish (2.238 ± 0.304 μg/g).

Yeh *et al*., (2012) analysed the free and bound form of formaldehyde in squid and squid products by gas chromatography-mass spectrophotometer. The total concentration of formaldehyde level in free and reversibly bound was more than free formaldehyde alone and for shredded squid it was less than 0.2mg/kg.

Junke *et al.,* (2018) used UPLC method for determination of formaldehyde in smoked meat products from five companies. The formaldehyde content was in the range of 25.55 mg/kg - 49.20 mg/kg for internal part while for the surface formaldehyde it was in the range of 34.04mg/kg - 165.25 mg/kg.

Bianchi *et al.,* (2013) demonstrated formaldehyde in fish samples by solid phase micro-extraction (SPME)- GC-MS based on derivatisation of fibre with pentaflurobenzyl-hydroxylamine hydrochloride. 12 fish samples (fresh water, crustaceans and sea-fish), also some fresh fishes, deep frozen, boiled fish, canned and roasted fish products were analysed He found that the highest concentration of formaldehyde was in fish samples belonging to the Gadidae family fish (6.4 ±1.2 mg/kg to 293 ± 26 mg/kg). Out of the 14 samples four samples showed valves exceeding 60 mg/kg, while samples stored on ice showed a moderate formaldehyde concentration at $0^{o}C$. Rest of the samples showed value of less than 22mg/kg.

Radford and Dalsis, (1982) studied the formaldehyde level in shrimps by using HPLC. The method was validated and 10 mg/kg level was found in the shrimps.

Sibirny *et al.,* (2011) carried out a study for estimation of formaldehyde in fish products y using alcohol oxidase and formaldehyde dehydrogenase based

enzymatic method. Muscle tissues of frozen hake and cod, as well as of freshly-killed carp were taken in which fish (cod and hake) had highest amount of formaldehyde that is up to 100 mg/kg by wet weight.

Lee *et al.,* (1983) studied the effect of formaldehyde in rats by inhalation , injection and dermal route.

The above method scientific findings given by different researchers indicate the importance of estimation of formaldehyde content in fishes. The international scenario and awareness about the formaldehyde estimation can be drawn by looking at the reports where most of the samples in these investigations have been reported positively.

To control this adulteration practice, the Food Safety Standard Authority Of India (FSSAI, 2019) has set a permissible limit for naturally occurring formaldehyde in freshwater (crustaceans, molluscs, and echinoderms) and marine water fishes of 4 mg/kg and 100 mg/kg respectively.

In the investigated region of Maharashtra, Konkan, and Sea coastal areas, we have got the positive samples for formaldehyde content below the standard limits of FSSAI, 2019. These findings can be corroborated with the normal physiological pathway of marine fish concerning formaldehyde content. However, the surprising level of formaldehyde content in freshwater fishes signifies that the continuous observation of such samples is of utmost importance. Though the number of samples and area covered is comparatively less, the detailed sampling plan and analysis can be done for further investigation into the area where formaldehyde content was noted.

The results in the present study gives the importance of the need for continuous observation of xenobiotic residue in biological samples as adulteration is nowadays happening at the basic level of the supply chain which can be detrimental to human health in the short as well as long term.

Summary & Conclusions

5. SUMMARY AND CONCLUSION

Formaldehyde or formalin (37 % in water) has been declared as a carcinogen and possess a major threat to public health. the consumption of this leads to deleterious effects in the human body. The formalin enters the human chain indirectly by the uptake of various products adulterated by this chemical. People are exposed to this chemical by various routes such as inhalation (workers working in industries), and oral route (by consuming formalin laced products). People exposed suffers from eye, nose and throat irritation causing further damage to nervous system, kidney and liver. Continuous exposure by inhalation route damages the mucosa, tracheal tract and pulmonary tissue that leads to its carcinogenic effect.

In food industry, formaldehyde is used as a preservative to increase the shelf life of products. Local vendors and agents misuse this chemical, by using it in a rampant way without noticing its harmful effects. Commonly this practice is followed for fishes and fishery products as they are highly perishable items.

Trimethylamine oxide acts as an agent for the fluctuating amount of formaldehyde content in different fishes. After the post-mortem, trimethylamine oxide is broken down to dimethylamine and formaldehyde as its main products. Beside this natural formation of formaldehyde, microbial activities leading to biochemical reactions can also produce formaldehyde, which leads to the physical damage of the fish and its quality (Jiang *et al.,* 2006).

Formalin estimation in tissue samples by the use of RP- HPLC has not been studies in India. Therefore, this study was carried out to estimate the formaldehyde level in biological products as the practice of adulteration is widespread.

In the present study, formaldehyde was detected in two species of fresh water and in three species of marine water fishes. The fresh water fishes *Catla (Catla catla)* and Nila Tilapia (*Oreochromis niloticus*) had formaldehyde with the value of 13.90±0.89 mg kg $^{-1}$ and 11.85±1.02 mg kg $^{-1}$ respectively. In the case of marine water fishes, the values detected were 23.01±1.29 mg kg $^{-1}$, 22.19±1.03

mg kg $^{-1}$ and 20.37±1.69 mg kg $^{-1}$ for Indian Mackerel (*Rastrelliger kanagurta*), Sardine (*Sardina plachardus*) *and* Pomfret (*Bramidae*) respectively. The analysed samples values were compared with the given standard and significant difference were observed between the groups i.e.in both fresh water and marine water fishes.

Formaldehyde concentration was not found in any of the ready to eat products.

From the current study following conclusions are made :-

- It is concluded that the RP-HPLC validated and standardized in the present study is an accurate method of detection of formaldehyde at picogram level.
- In the present study, the RP-HPLC analysis detected the formaldehyde content in fresh and marine water fishes. The utilization of formaldehyde as a preservative in the food industry has therefore importance and needs to be monitored continuously.
- The higher levels of formaldehyde reported in fishes might be due to the physiological metabolism or might be due to adulteration at the ground level.
- Further speciation can be done by increasing the number of samples from the same region where positive values are reported.

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Appendices

Appendix I

The representative chromatograph of Formaldehyde -2,4-DNPH in various animal origin products.

< Sample information >

Sample Name : Labeo Rohita
Sample ID : 1
Data file name : Labeo Rohita.lcd
Method Filename : HCHO- 2 4 DNPH.lcm
Batch Filename : Labeo Rohita.lcd
Vial # : 1- 82 Sample Type : Unknown
Injection volume : 10 µL level : 1
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Data processed : 1/18/2021 11:46:25 AM Processed by : System Administrator

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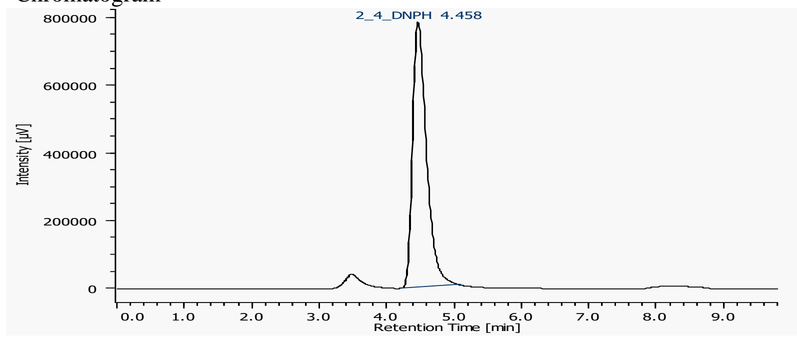

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	11251900	4.458	780929	100.0%	----	M	
2.	Formaldehyde	----	----	----	----	----	M	

< Sample information >

Sample Name : Catla catla
Sample ID : 1
Data file name : Catla catla.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Catla catla.lcd
Vial # : 1-134 Sample Type : Unknown
Injection volume : 10 µL level : 1
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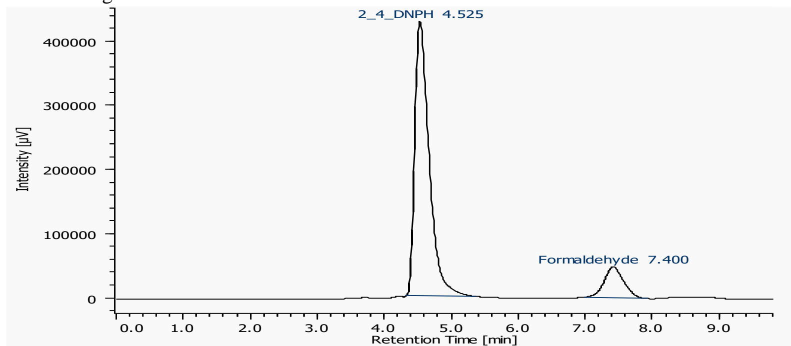

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	6375085	4.525	426649	89.887%	----	M	
2.	Formaldehyde	961141	7.400	48001	13.101%	----	M	

< Sample information >

Sample Name : Oreochromis niloticus
Sample ID : 1
Data file name : Oreochromis niloticus.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Oreochromis niloticus.lcd
Vial # : 1-103
Injection volume : 10 µL
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Data processed : 1/18/2021 11:59:25 AM

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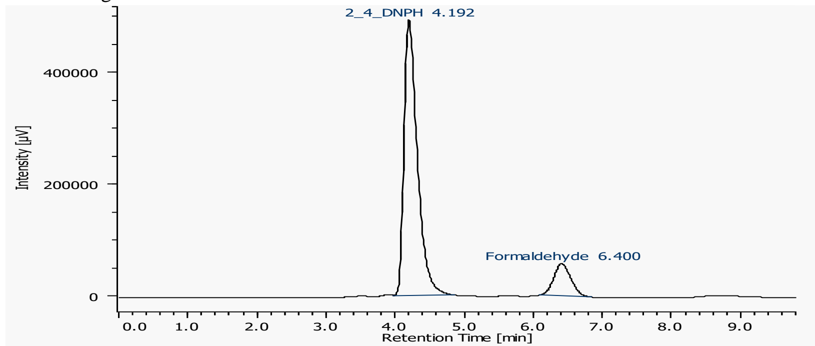

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	6588797	4.192	490726	86.890%	----	M	
2.	Formaldehyde	994145	6.400	57371	13.125%	----	M	

< Sample information >

Sample Name : Siluriformes
Sample ID : 1
Data file name : Siluriformes.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Siluriformes.lcd
Vial # : 1-144
Injection volume : 10 µL
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Data processed : 1/18/2021 12:52:25 PM

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Processed by : System Administrator

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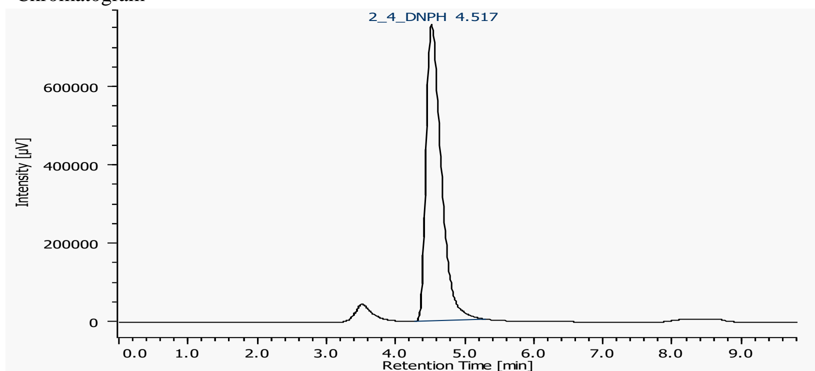

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	11526538	4.517	755884	100.0%	----	M	
2.	Formaldehyde	-----	-----	-----	-----	----	M	

< Sample information >

Sample Name : Rastrelliger kanagurta
Sample ID : 1
Data file name : Rastrelliger kanagurta.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Rastrelliger kanagurta.lcd
Vial # : 1-93
Injection volume : 10 µL
Data Acquired : 1/18/2021 11:22:55 AM
Data processed : 1/18/2021 11:38:25 AM

Sample Type : Unknown
level : 1
Acquired by : System Administrator
Processed by : System Administrator

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	5903037	4.367	422946	77.886	----	M	
2.	Formaldehyde	1676002	7.000	85757	22.114	----	M	

< Sample information >

Sample Name : Harpadon nehereus
Sample ID : 1
Data file name : Harpadon nehereus.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Harpadon nehereus.lcd
Vial # : 1-103
Injection volume : 10 µL
Data Acquired : 1/18/2021 11:38:55 AM
Data processed : 1/18/2021 11:59:25 AM

Sample Type : Unknown
level : 1
Acquired by : System Administrator
Processed by : System Administrator

<Chromatogram>

< Peak Area >

Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	9336533	4.383	665059	100.0%	----	M	
2.	Formaldehyde	----	----	----	-----	----	M	

< Sample information >

Sample Name : Sardina pilchardus
Sample ID : 1
Data file name : Sardina pilchardus.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Sardina pilchardus.lcd
Vial # : 1-114 Sample Type : Unknown
Injection volume : 10 µL level : 1
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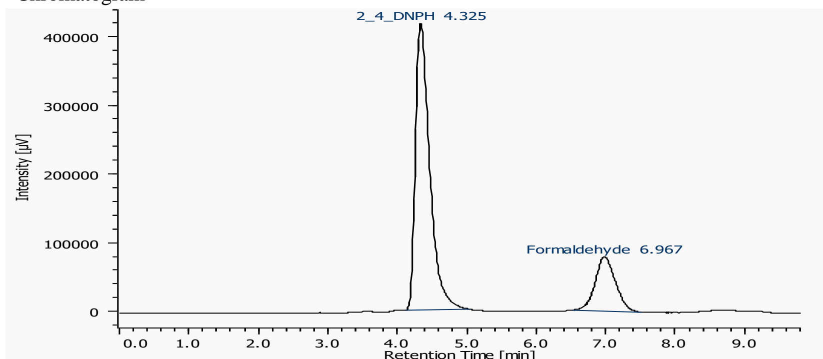

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	5940332	4.325	415849	79.029%	----	M	
2.	Formaldehyde	1576317	6.967	78970	15.959%	----	M	

< Sample information >

Sample Name : Bramidae
Sample ID : 1
Data file name : Bramidae.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Bramidae.lcd
Vial # : 1-124 Sample Type : Unknown
Injection volume : 10 µL level : 1
Data Acquired : 1/18/2021 11:39:55 AM Acquired by : System Administrator
Data processed : 1/18/2021 12:21:25 AM Processed by : System Administrator

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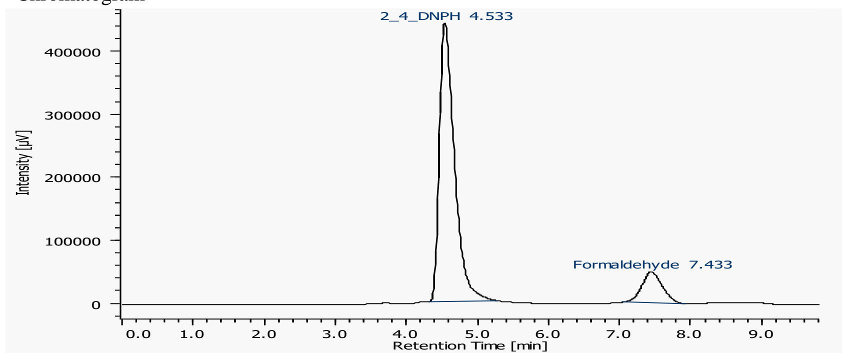

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	6514776	4.533	440391	89.971%	----	M	
2.	Formaldehyde	977597	7.433	49088	10.029%	----	M	

< Sample information >

Sample Name : Ready to eat products
Sample ID : 1
Data file name : Ready to eat products.lcd
Method Filename : HCHO - 2 4 DNPH.lcm
Batch Filename : Ready to eat products.lcd
Vial # : 1-154
Injection volume : 10 µL
Data Acquired : 1/18/2021 11:39:55 AM
Data processed : 1/18/2021 01:20:25 PM

Sample Type : Unknown
level : 1
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Processed by : System Administrator

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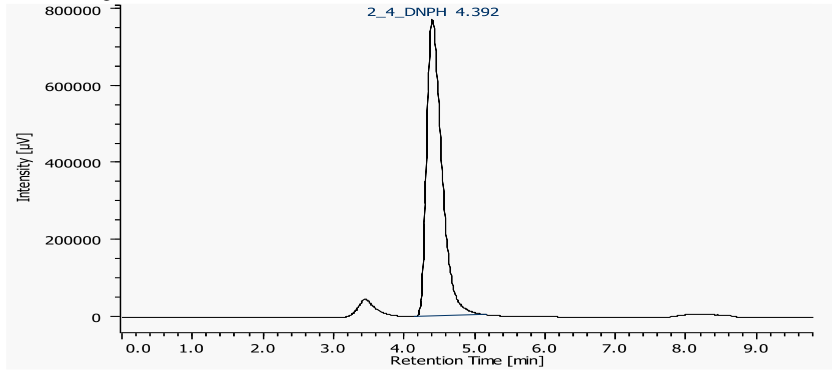

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Sr.No.	Peak Name	Area	Ret. Time	Height	Area %	Unit	Mark	Name
1.	2-4-DNPH	11497391	4.392	767835	100.0%	----	M	
2.	Formaldehyde	-----	-----	-----	-----	----	M	

Abstract

THESIS ABSTRACT

1.	Title of the thesis (in Capital letters)	:	VALIDATION OF METHOD AND PHARMACOKINETIC ASSESSMENT OF FORMALDEHYDE IN VARIOUS ANIMAL ORIGIN PRODUCTS BY REVERSE-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)
2.	Full name of student	:	Miss. Krutika Pramod Khiratkar
3.	Name and address of Major Advisor	:	Dr. S. S. Sole, Assistant Professor, Department of Veterinary Pharmacology and Toxicology, Mumbai Veterinary College, Parel, Mumbai-400012.
4.	Degree to be awarded	:	M. V. Sc.
5.	Year of award of Degree	:	2021
6.	Major subject	:	Veterinary Pharmacology & Toxicology
7.	Total number of pages in the thesis	:	
8.	Number of words in the abstract	:	
9.	Signature of Student	:	
10.	Signature, Name and address of forwarding authority (HOD/SH)	:	Dr. R. V. Gaikwad Professor and Head, Dept. of Vet. Clinical Medicine, Preventive Medicine, Pharmacology & Toxicology, Mumbai VeterinaryCollege, Parel, Mumbai-400 012
11.	Signature of the Associate Dean		

ABSTRACT

Formaldehyde adulteration in biological samples is an emerging issue concerning human health. Thus, this study was carried out to estimate the formaldehyde concentration in various products by using the RP-HPLC method. The validated method has shown the limit of detection and limit of quantification of 0.14 µg/mL and 0.44 µg/mL, respectively. The samples (freshwater fishes, marine water fishes, and ready to eat products) were collected from the local markets and analyzed. The level of formaldehyde found in Catla (*Catla catla)* and Nila Tilapia *(Oreochromis niloticus)* were 13.90 ± 0.89 mg kg $^{-1}$ and 11.85 ± 1.02 mg kg $^{-1}$ respectively, while no formaldehyde was detected in Rohu *(Labeo rohita)* and Catfish *(Siluriformes).* In marine water fishes, out of the four species analyzed, Indian Mackerel *(Rastrelliger kanagurta),* Sardine *(Sardina pilachardus),* and Pomfret (*Bramidae)* were found positive for formaldehyde with a value of 23.01 ± 1.29 mg kg $^{-1}$, 22.19 ± 1.03 mg kg $^{-1}$ and 20.37 ± 1.69 mg kg $^{-1}$ respectively and it was absent in Bombay duck *(Harpadon nehereus)* species. Formaldehyde was not detected in any of the ready to eat products. The analysed samples values were compared with the given standard and significant difference were observed between the groups i.e.in both fresh water and marine water fishes.

Level of formaldehyde detected in marine water fishes was under the permissible limit of 100 mg kg $^{-1}$ and can be corroborated with the natural physiological pathway whereas, the level in fresh water fishes was above the given permissible level of 4 mg kg $^{-1}$. The current validated and standardized method of RP-HPLC in the present study is an accurate method of detection and samples which were found positive in analysis should be further investigated by increasing the sample size and performing speciation study.

प्रबंधसारांश

१	प्रबंधाचेनाव	:	प्राणीउत्पत्तीच्याउत्पादनांमध्ये रिर्वस फेझ हायप्रेशर लिकुइड क्रोमटोग्राफीद्वारे फोर्मल्डहयडेचे फर्माकोकिनेटिक मूल्यांकन आणि पद्धतीचे प्रमाणीकरण
२	विद्यार्थाचेनाव	:	कृतिका प्रमोद खिरटकर
३	मार्गदर्शकचेनावआणिपत्ता	:	डॉ. एस.एस.सोले सहाय्यकप्राध्यापक,पशुऔषधशास्त्रवविषशास्त्रविभाग,मुंबईपाशुवैद्यकमहाविद्यालय,परेल,मुंबई 400012
४	पदवी	:	एम. व्ही. एस. सी.
५	पदवीप्रदानकरण्याचेवर्ष	:	२०२१
६	मुख्यविषय	:	पशुऔषधशास्त्रवविषशास्त्र
७	प्रबंधाचीएकूणपाने	:	
८	सारांशचेएकूणशाब्द	:	
९	विद्यार्थाचीसही	:	
१०	प्रबंधपाठविणाऱ्याअधिकाऱ्याचे संपूर्णनाव,पत्ताआणिसही	:	डॉ. आर. व्ही. गायकवाड प्राध्यापकआणिविभागप्रमुख, पशुऔषधशास्त्रवविषशास्त्रविभाग, मुंबईपाशुवैद्यकमहाविद्यालय,परेल,मुंबई 400012
११	सहयोगीअधिष्ठातामुंबईपाशुवैद्यकमहाविद्यालय,परेल,मुंबई-400012	:	

प्रबंध सारांश

जैविक नमुन्यांमध्ये फॉर्मल्डिहाइड भेसळ ही मानवी आरोग्याशी संबंधित एक उदयोन्मुख समस्या आहे.अशा प्रकारे, आरपी-एचपीएलसी पद्धतीने विविध उत्पादनांमध्ये फॉर्मल्डिहाइड मात्रेचा अंदाज घेण्यासाठी हा अभ्यास केला गेला.प्रमाणित पद्धतीने अनुक्रमे 0.14 μg / mL आणि 0.44 μg / mL चे प्रमाणीकरण शोधण्याची मर्यादा दर्शविली आहे. स्थानिक बाजारपेठेतून (ताजे पाण्यातील मासे, सागरी पाण्यातील मासे आणि उत्पादने खाण्यासाठी तयार) नमुने घेण्यात आले व त्यांचे विश्लेषण करण्यात आले.कॅटला (कॅटला कॅटला) आणि निला टिलापिया (ओरीओक्रोमिस नीलोटिकस) मधील फॉर्मल्डिहाइडची पातळी अनुक्रमे 13.90 ± 0.89 मिलीग्राम किलोग्राम -1 आणि 11.85 ± 1.02 मिग्रॅ किलो -1 होती, तर रोहू (लाबेओ रोहिता), कॅटफिशमध्ये फॉर्मलडीहाइड आढळला नाही. सिल्युरीफॉर्म्स) समुद्री पाण्याच्या माशांमध्ये विश्लेषित केलेल्या चार प्रजातींपैकी इंडियन मॅकेरेल (रास्ट्रेलिगर कानगुर्ता), सार्डिन (सारडीना पायलाकार्डस) आणि पोम्फ्रेट (ब्रॅमिडे) 23.01 ± 1.29 मिलीग्राम किलो -१, 22.19 ± 1.03 मूल्य असलेल्या फॉर्मल्डिहाइडसाठी सकारात्मक आढळले. मिलीग्राम किलो -१ आणि 20.37 ± 1.69 मिलीग्राम किलोग्राम -१ अनुक्रमे बॉम्बे डक (हरपाडॉन नेहेरियस) प्रजातीत अनुपस्थित होता.कोणत्याही पदार्थ खाण्यासाठी तयार असलेल्या ठिकाणी फॉर्मलडीहाइड आढळला नाही. विश्लेषण केलेल्या नमुन्यांची मूल्ये दिलेल्या प्रमाणांशी तुलना केली गेली आणि गट म्हणजेच ताजे पाणी आणि सागरी पाण्याच्या मासे या दोन्ही गटांमध्ये महत्त्वपूर्ण फरक आढळला. सागरी पाण्याच्या माशांमध्ये आढळलेल्या फॉर्मल्डिहाइडची पातळी १०० मिलीग्राम/किलोग्राम च्या

परवानगी मर्यादेअंतर्गत होती आणि नैसर्गिक शारिरीक मार्गाने त्याचे प्रमाणन केले जाऊ शकते, तर ताज्या पाण्यातील माशांची पातळी दिलेल्या ४ मिलीग्राम/किलो च्या अनुज्ञेय पातळीपेक्षा जास्त आहे.सध्याच्या अभ्यासामध्ये आरपी-एचपीएलसीची सध्याची मान्यताप्राप्त आणि प्रमाणित पद्धत शोधण्याची अचूक पद्धत आहे आणि विश्लेषणात सकारात्मक आढळलेल्या नमुन्यांची नमुने आकार वाढवूनस्पष्टीकरण अभ्यास करून अधिक तपासले जावे.

Vita

VITA

Miss. Krutika Pramod Khiratkar was born on 4th October 1995, in Chandrapur District, Maharashtra State. She has completed her S.S.C from Narayana Vidyalayam, Chandrapur in the year 2011. She completed her H.S.C. Shri Major Hemant Jakate High School, Board of Secondary & Higher Secondary Education in 2013. She was a bright student who actively participated and won in various extra-curricular activities like elocution, Badminton in school and college.

She has successfully completed her graduation as Bachelor of Veterinary Science & Animal Husbandry (B.V.Sc. and A.H.) from Mumbai Veterinary College, Mumbai during 2013 to 2018 securing 77.8% in first class.

Being interested, she joined the Department of Veterinary Pharmacology and Toxicology, in 2018 for pursuing her M.V.Sc. and was enthusiastically involved in various research projects carried out there. She has successfully completed her post-graduation.

Apart from academics, She is also interested in travelling and reading.