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Detection of free and bound formaldehyde concentration in commercially important fishes and their effect on cooking: A preliminary study

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Abstract
The free and bound formaldehyde concentration in six commercially important fishes from five major fish markets of Tamil Nadu, India were analyzed. The highest concentration of free and bound formaldehyde (9.8 and 6.45 mg/kg) was observed in *Sphyraena barracuda* and the lowest (0.8 and 0.5 mg/kg) concentration was observed in *Lethrinus lentjan*. Storage of S. *barracuda* in ice indicated a significant (p<0.05) increase in formaldehyde concentration after 3 days. *S. barracuda* stored in a deep freezer (-20 °C) for 30 days showed that there was no significant (p>0.05) increase in formaldehyde concentration upto 15 days after which there was a significant increase upto 30 days. *S. barracuda* spiked with various concentration of formaldehyde followed by boiling for 15 min indicated no significant reduction (p>0.05) in formaldehyde concentration. Formaldehyde treated *S. barracuda* washed after different time intervals indicated that washing reduces upto 41 to 60% of treated formaldehyde.

Keywords: Formaldehyde, fish, ice storage, frozen storage, formalin abuse

1. Introduction
Fish is a highly perishable commodity containing water, fat, protein, and free amino acids, which makes them easily susceptible to spoilage by biochemical reaction during the postmortem process and by microorganism (Fernandes and Venkatraman, 1993) [8]. Fish and shellfish can be kept fresh on ice for 8 to 14 days. To keep the freshness of fish and shellfish, fishermen and fish vendors tend to intentionally use formalin (aqueous formaldehyde) as a preservation agent at various concentrations (Noordiana *et al*., 2011) [15].

Trimethylamine oxide (TMAO), a natural constituent in marine fish and shellfish muscle, is considered the most important source of formaldehyde (ref). Soon after the death of fish, TMAO is degraded into formaldehyde and dimethylamine (DMA) by the enzyme TMAO reductase on storage (Rehbein, 1987; Sotelo *et al*., 1995) [17, 20]. Formaldehyde that accumulates during frozen storage, of fish reacts with protein, and subsequently causes protein denaturation and muscle toughness (Sotelo *et al*., 1995) [20]. Formaldehyde may be formed during the aging and deterioration of fish flesh (Tsuda *et al*., 1988) [22].

Formaldehyde, is the simplest aldehyde with the chemical formula, HCHO. It is a colorless gas, with flammable properties and irritating repugnant odor at room temperature. Formalin, an aqueous form of formaldehyde, contains 37% by weight or 40% by volume of formaldehyde gas in water.

According to the International Agency for Research on Cancer (IARC, 2012) [10], formaldehyde is classified as Group 1 carcinogen, primarily based on its association with nasopharyngeal cancer. Chronic exposure to formaldehyde has been reported to have long-term health effects such as pharyngeal congestion, chronic phayrgitis, loss of olfactory functioning, lacrimation and cornea disorder, heartburn, and lethargy (Dai and Bao, 1999) [6]. Intake of formaldehyde through fish consumption causes uncontrolled cell growth or cancer in the stomach, lung, and respiratory system (Li *et al*., 2002) [26].

Formaldehyde occurs in free and bound forms in fish. The level of free and bound formaldehyde varies with fish species. Till date, there is no baseline data on the availability of free and bound formaldehyde in fish and shell fish. Formaldehyde is formed during iced and frozen storage of fish. Postmortem changes influence the formaldehyde content in stored fish. There are also reports that fishes are dipped in formalin before transportation in ice to increase

Their shelf life (Sanyal *et al*., 2017) [19]. Hence, this study was carried out to analyze the free and bound formaldehyde in different commercially important marine fish to get a baseline data and the effect of preservation and processing on formaldehyde concentration in *barracuda*.

2. Materials and Methods

2.1 Raw material collection

Fish samples *viz*., sardine (*Sardinella gibbosa*), *barracuda* (*Sphyraena barracuda*), carangid (*Caranx sexfaciatus*), tuna (*Euthynnus affinis*), emperor (*Lethrinus lentjan*) and mackerel (*Rastrelliger kangurta*), were collected from Thoothukudi, Nagercoil, Tirunelveli, Madurai, and Chennai Fish markets of Tamil Nadu.

2.2 Preparation of double strength NASH Reagent (DSNR)

Accurately 150 g of Ammonium acetate was dissolved in 2 ml of acetyl acetone and 3 ml of acetic acid in a volumetric flask and made up to 500 ml with distilled water.

2.3 Preparation of Formaldehyde Standard

A stock standard (1000 ppm) was prepared from a formaldehyde (37%) solution. Working standards at a concentration of 0.1,1, 2, 3, 4, 5 and 10 ppm were prepared in test tubes, and 2 ml of DSNR was added to each tube and kept in a water bath at 60 °C for 5 min. Then, all the prepared standards were analyzed in UV-Vis. Spectrophotometer at 415 nm (Jasco, USA) to make a standard linear graph.

2.4 Sample preparation

For the estimation of free formaldehyde, accurately 5.0±0.1 g of fish muscle sample was taken and homogenized with 45 ml of 6% per chloric acid. The homogenate was filtered through Whatman filter paper (No.1), and the pH was adjusted to 5-6 using NaOH and HCl. Then, 2ml of the filtrate was taken in test tubes, and 2 ml of NASH reagent was added and kept in the serological water bath for 5 min at 60 °C. Then, formaldehyde concentration was determined in a UV-Vis spectrophotometer.

For the detection of bound formaldehyde, accurately 10±0.1 g (why variation in weight) fish muscle sample was taken and homogenized with 30 ml of 1% H_2SO_4. The content was steam distilled in kel plus distillation unit (Pelican, Chennai, India). About 100 ml of distillate was collected, and the pH was adjusted to 5-6. From which, 2 ml of distillate was taken in the test tube, and 2 ml of NASH reagent (DSNR) was added and kept in the water bath at 60°C for 5 min. Finally, the formaldehyde concentration was determined in UV-Vis Spectrophotometer.

2.5 Formaldehyde formation in iced and frozen stored barracuda

To examine the effect of iced and forzen storage, *barracuda*, (*Sphyraena barracuda)* was used. For the ice storage study, whole fish was kept in layers of ice in a the rmacole box and kept at 4°C in a chiller. The samples were drawn every day for a period of 5 days and analysed for free and bound formaldehyde concentration. For the frozen storage study, whole fish were packed in poly ethales in a deep freezer at -20°C. Samples were taken for a period of 30 days for the analyses of once in 3 days and free and bound formaldehyde.

2.6 *In vitro* study on effect of cooking on formaldehyde (1kg each)

Whole *barracuda* (1 kg each) was dipped in 50,100,150, and 200 ppm of formaldehyde solution for 15 min. The formalin treated fish were then cut into pieces, placed in a polythene bag, tied and dipped in boiling water (100 °C) for 15 min. Samples were than analyzed for free and bound formaldehyde.

2.7 *In vitro* study on effect of washing on the formaldehyde concentration in fish

Whole *barracuda* were dipped in 200 ppm formaldehyde solution for 15 min. To simulate household preparation, washing was done for different time interval period *viz* 30 sec. 1min. 2 min. 5 min. and 10 min by immersion in water. Samples were analyzed for free and bound formaldehyde.

2.8 UV-Vis. Spectrophotometer analysis of formaldehyde

The prepared sample extract for free and bound formaldehyde was determined in UV-Vis. spectrophotometer. The absorbance value of each sample was recorded at 415 nm, the formaldehyde content (ppm) present in each sample was calculated from the prepared standard curve.

2.9 Statistical analysis

The results were presented as mean ± standard deviation. All experiments were performed in triplicate. One way analysis of variance (ANOVA) was performed to examine, the significant differences between sets by using SPSS version 2.2 Software (IBM, USA).

3. Results and Discussion

3.1 Presence of the free and bound formaldehyde in fish available in markets

The free and bound formaldehyde present in commercially important fish purchased from various fish markets ranged from 0.5 to 9.8 mg/kg and 0.2 to 6.45 mg/kg respectively (Table 1). There was a significant difference (p<0.05) in the free and bound formaldehyde concentration in fishes obtained from the same market as well as same fish species from different markets. The formaldehyde was high in the fishes purchased from Madurai fish market. In contrast, minimum concentration of formaldehyde was found in fishes obtained from Thoothukudi fish market. Thoothukudi, Nagercoil, and Chennai fish markets are located in the coastal region, whereas Tirunelveli and Madurai fish markets are in interior part of the state. Fishes collected from the interior markets have a high formaldehyde concentration when transporting fish to distant interior location, fish traders do not use sufficient ice, which can result in shorter shelf life. Loss in quality is attributable to improper management, when trucks, carriers, and railways move fish to near and distant areas (Reza *et al*., 2009) [18]. Because of this, a significant part of the whole fish loses its quality before it reaches the consumers. To overcome this problem, fish traders use formalin to maintain fish quality when it reaches the consumers. To overcome this problem, fish traders use formalin as a fish preservative to increase the shelf life of fish. The high concentration of formaldehyde in the fishes of Madurai fish market might be due to the intentional addition of formalin to preserve the fish in ice for a longer period.

The free and bound formaldehyde content in different fishes

varied significantly. The free and bound formaldehyde were high in *barracuda*, According to Jaafar *et al*. (2013) [12], the presence of formaldehyde in marine fish is influenced by various factors such as the amount of dark muscle, substrate, cofactors, temperature, storage time and degree of contamination. There are few reports on the various amounts of formaldehyde in the dark muscle of fish which states that the variation is due to the TMAO content in the fish species. Similar to our results of the present study, Aminah *et al*. (2013) [2] also reported significant difference in the formaldehyde contents in among fishes collected from three wholesale markets *viz*. Borong Selayang, *Harian* Selyang, Borong Klang in Malaysia. An earlier study carried out by Haque and Mohsin (2009) [9] in fishes obtained from four fish markets of Dhaka city (Kawaran Bazar fish market, Savar Bazar fish market, Mirpur Bazar fish Market and Jatrabari fish markets) stated that formaldehyde was highest in fishes sold in Kawaran Bazar fish market (48%) and low in Savar Bazar fish market (14%). In another study carried out in Bangladesh, it was found that 82% of imported fish and 18% of local fish were formalin contaminated in Tongi town (Bari *et al*., 2013) [3]. Lipi *et al*. (2014) reported that 4.2% of 500 fish samples collected from Myanmar and India were formalin contaminated. In the present study, the formaldehyde concentration in the fish samples were found to be higher than the residual limit set by the US Environmental Protection Agency (0.2 mg/kg EFSA 2006), and Malaysian Food Regulation Act (1985) (5mg/kg but lower than the 100 ppm set by Food Safety and Standards Authority of India (FSSAI, 2019) formalin contents in the sampled fishes from different fish markets of Tamil Nadu were lower than the limit set by the Food Safety and Standards Authority of India (FSSAI, 2019). In this study, the fishes obtained from Madurai and Tirunelveli fish markets had much higher concentration of formaldehyde than the limit set by the Malaysian Food Regulation Act, 1985.

Table 1: Free and bound formaldehyde concentration in fishes collected from different fish markets of Tamil Nadu

Name of the fish sample	Thoothukudi		Nagercoil		Tirunelveli		Chennai		Madurai	
	Free FA (mg/kg)	Bound FA (mg/kg)	Free FA (mg/kg)	Bound FA (mg/kg)	Free FA (mg/kg)	Bound FA (mg/kg)	Free FA (mg/kg)	Bound FA (mg/kg)	Free FA (mg/kg)	Bound FA (mg/kg)
Sardinella gibbosa	0.8±0.05^{a}	0.3±0.2	1.2±0.05^{a}	2.8±0.09	6.8±0.35^{a}	2.95±0.15	5.5±0.05^{a}	2.25±0.07	7.1±0.1^{a}	3.4±0.15
Lethrinus lentjan	0.5±0.1^{ab}	0.3±0.05	0.8±0.1^{b}	1.45±0.64	3.8±0.05^{b}	2.45±0.32	1.2±0.05^{b}	2.3±0.07	4.1±0.1^{bf}	3.19±0.20
Rastrelliger kanagurta	0.9±0.20^{c}	0.2±0.15	1.5±0.05^{c}	0.5±0.21	4.3±0.1^{c}	2.45±0.27	2.4±0.05^{c}	1.85±0.07	4.3±0.19^{cf}	1.95±0.35
Sphyraena barracuda	3±0.25^{d}	2±0.20	4.4±0.1^{d}	3.85±0.15	9.8±0.1^{d}	6.45±0.02	8.7±0.1^{d}	2.8±0.27	9.1±0.10^{d}	6.15±0.35
caranx sexfaciatus	1±0.1^{ce}	0.2±0.1	0.9±0.07^{be}	1.35±0.45	0.9±0.02^{e}	0.8±0.17	1.8±0.1^{e}	1.95±0.22	1.2±0.15^{e}	1.05±0.02
Euthynnus affinis	1.3±0.15^{cf}	0.3±0.20	1.6±0.05^{cf}	1.4±0.07	0.6±0.05^{f}	0.75±0.08	2.2±0.05^{f}	3.05±0.17	2.2±0.09^{fc}	3.05±0.35

n = 10, values – Mean ± SD. Different alphabets in rows indicates significant difference

3.2 Effect of iced storage on the formaldehyde concentration

The results of free and bound formaldehyde present in *barracuda* stored in ice for a period of 5 days are given in the Table 2. *Barracuda* had an initial free formaldehyde concentration of 2.55 mg/kg, which increased up to 5.6 mg/kg, on day 6. Similarly, the bound formaldehyde increased from 1.79 to 10.77 mg/kg. Statistical analysis showed that there was no significant increase in formaladehyde concentration from day1 to day 3(p>0.05), whereas increase was significant (p<0.05) from day 3 to day 5 (Fig. 1). Immaculate *et al*. (2018) [11] reported that the formaldehyde was significantly higher in un-iced fish (10.64 - 18.75 mg/kg) than in iced fish (0.001-0.32 mg/kg), and attributed the increase to the natural production by postmortem enzymatic reaction on storage.

In vitro study undertaken with *barracuda* treated with 200 mg/L for 10 days showed that the formaldehyde increased every day, but the amount of increase was lower compared to the fish held in ice without treatment. However, Sanyal *et al*. (2017) [19] have reported higher concentration of formalin (11.52 –13.10mg/kg) in mrigal treated with 5mg/kg formalin immediately after treatment, after which it remained constant until 14 days of iced storage.

Yeasmin *et al*. (2010) [25] reported that fish treated with 5% formalin and stored in ice can increase the shelf life more than the fish held in ice without any formalin treatment.

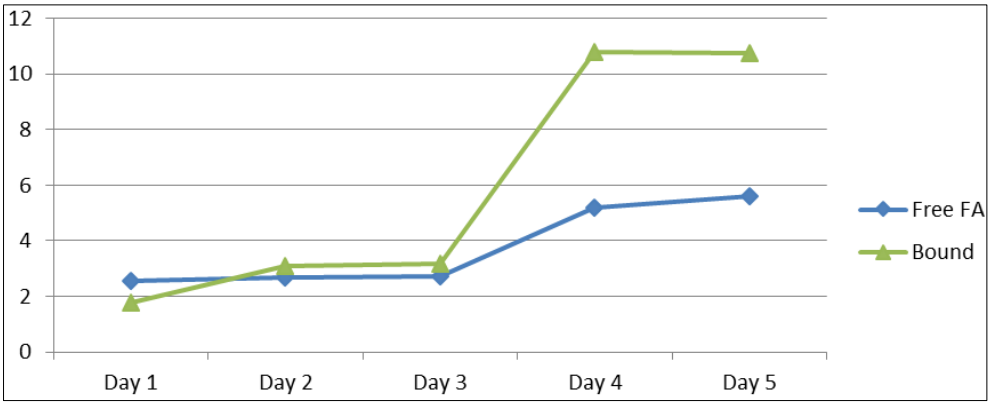

Fig 1: Free and bound formaldehyde concentration (mg/kg) in barracuda during iced storage

3.3 Effect of frozen storage on the formaldehyde barracuda

Table 2: Free and bound formaldehyde concentration (mg/kg) in barracuda during iced storage

Period of storage (Days)	Free FA	Bound FA
Day 1	2.56±0.01[a]	1.79±0.02 [a]
Day 2	2.67±0.01[ab]	3.08±0.05 [ab]
Day 3	2.7±0.01[b]	3.17±0.03 [b]
Day 4	5.2±0.05[c]	10.8±0.09 [c]
Day 5	5.6±0.02[d]	10.77±0.04 [d]

Values - Mean ±S. D. Different alphabets in rows indicate significant difference

The results of free and bound formaldehyde in *barracuda* held in freezer for a period of 30 days are given in Table 3. The initial free formaldehyde concentration was 1.06 mg/kg, on day 0 which increased each day upto 3.3 mg/kg on day 30. The bound formaldehyde concentration also increased from 2.69 mg/kg on day 1 to 10.45 mg/kg on day 30. There was no significant increase in formaldehyde concentration from day 1 to day 15(p>0.05), whereas the increase was significant from day 20 to 25 (p<0.05) (Fig. 2). Tunhun *et al.* (1993) [23] in rake-gilled mackerel and lizard fish, it was reported that formaldehyde increased from 0.50 to 1.4 mg/kg in rake-gilled mackerel, and in lizard fish during frozen storage (-20 °C) of six months. The quantity of formaldehyde formed differed with species due to the presence of inherent TMAO in each fish (Sotelo *et al*., 1995) [20] the accumulation of formaldehyde in fish tissue takes place during the frozen storage. Formaldehyde and dimentylamine (DMA) are the products of enzyme-catalysed reaction, which has trimethyl lamine oxide (TMAO) as its substrate (Amano *et al*., 1963; Tomioka *et al*., 1974) [1, 21]. TMAO demethylase (TMA Oase) is the enzyme which catalayses the breakdown of TMAO to formaldehyde and DMA, which is located mainly in the viscera (Amano *et al*., 1963, Rehbein, 1987) [1, 17] and in the microsomal fraction of muscle especially in the dark muscle (Castell, 1971; Parkin *et al*., 1982) [4, 16].

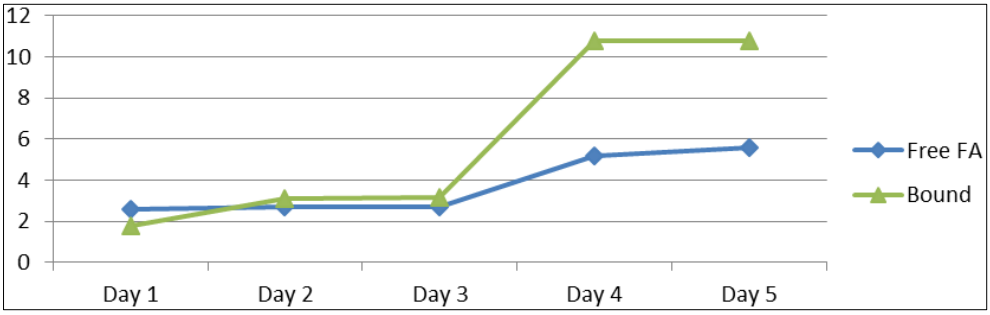

Fig 2: Free and bound formaldehyde concentration (mg/kg) in barracuda during iced storage

Table 3: Free and bound formaldehyde concentration in fish sample during frozen storage

Period of storage (Days)	Free FA (mg/kg)	Bound FA (mg/kg)
Day 1	1.06±0.01[a]	2.69±0.02 [a]
Day 2	1.11±0.01[ab]	2.76±0.03 [ab]
Day 3	1.2±0.05[ab]	2.72±0.05 [ab]
Day 7	1.21±0.01[ab]	2.76±0.02 [ab]
Day 10	1.22±0.02[ab]	2.75±0.02 [ab]
Day 15	1.35±0.05[ab]	3.15±0.01 [ab]
Day 20	2.3±0.1[b]	6.95±0.15 [b]
Day 25	2.9±0.05[c]	8.12±0.48 [c]
Day 30	3.3±0.05[d]	10.45±0.02 [d]

Values – Mean ± S.D. Different alphabets in rows indicates significant difference

3.4 *In vitro* of cooking on the residual formaldehyde in barracuda

In vitro study, undertaken with *barracuda* pre-treated with different concentration of foramaldehyde was examined for free and bound formaldehyde concentration and the results are given in the Table 4. The absorption (%) of formaldehyde was calculated in fish tissue based on the detected free formaldehyde. The mean absorption of 8.5% was found in fish treated with 50, 100 and 150mg/l formaldehyde, and it was slightly higher (10%) in fish treated with for 200 mg/l formaldehyde. The mean absorption of bound formaldehyde almost remained similar at all concentrations.

The free and bound formaldehyde concentration were determined in formaldehyde pre-treated fish cooked for a period of 15 min the results are given in the Table 4.The results indicated that the effect of cooking on the formaldehyde in fish is almost negligible because 87 to 92% of the added formaldehyde were retained in the cooked fish. Therefore, it can be inferred that cooking does not reduce or remove the free and bound formaldehyde from fish. Aminah *et al.* (2013) [2] studied the effect of boiling and frying on formaldehyde content in seven commercial fish species and reported that the concentration varied from 2.38 to 2.95 mg/kg in fresh, 2.08 to 2.35 mg/kg in boiled and 2.28 to 2.49 mg/kg in fried fish. This emphasis that formaldehyde added or present in fish gets highly bound to the fish tissue due to strong molecular interaction. Chandralekha *et al.* (1992) [5] reported that in formaldehyde treated fish, 75% of it was retained in fish after cooking, and 80% of it was retained after deep frying.

Table 4: Free and bound formaldehyde concentration in formalin treated uncooked and cooked fish

FA Conc. (mg/l)	Free FA (mg/kg) in uncooked sample	Free FA (mg/kg) in cooked sample	Bound FA (mg/kg) in uncooked sample	Bound FA (mg/kg) in uncooked sample	Removal (%) of formaldehyde conce. after cooking
50	4.1±0.05[a]	3.8±0.2[a]	2.15±0.07 [a]	1.95±0.15 [a]	8.4
100	8.7±0.15[b]	7.8±0.55[b]	2.3±0.02 [b]	1.45±0.05 [b]	10.4
150	13.3±0.2[c]	11.2±1[c]	2.45±0.16 [c]	3.8±0.5 [c]	13.3
200	20±0.1[d]	19.2±0.05[d]	2.25±0.02 [d]	2.55±0.07 [d]	4

Values – Mean±S. D. Different alphabets in rows indicates significant difference

Table 5: Effect of washing on formaldehyde concentration in formalin treated fish

Washing time	Free FA (mg/kg)	Removal (%)	Bound FA (mg/kg)
Initial	21.8 ppm	-	2.95
30 sec.	16.5±1.2	24.3	3.0±0.7
1 min.	13.9±0.15	36.2	2.35±0.62
2 min.	12.8±0.16	41.0	2.5±0.19
5 min.	8.9±0.1	59.1	4.15±0.15
10 min.	8.1±0.1	62.8	4.15±0.19

Values – Mean ± S.D.; ND

3.5 *in vitro* effect of washing on the residual formaldehyde in barracuda

In vitro study undertaken to examine the effect of washing in the formaldehyde pre-treated *barracuda* showed that in the fish treated with 200 ppm formaldehyde the fish observed only 10% to 12% of formaldehyde. The free formaldehyde was 21.8 mg/kg and bound formaldehyde was 2.95mg/kg prior to washing step, which was equivalent to an absorption rate of 10 to 12%. The free formaldehyde decreased significantly with increase in washing duration. When the fish was dipped for 10 min in water the free formaldehyde concentration got reduced upto 60%. There was not much difference in the conc. of bound formaldehyde and washing duration which indicate that added formaldehyde mostly remained in free form and free formaldehyde can be removed by washing.

Although washing can remove free or added formaldehyde from treated fish, there will be loss in the quality of fish due to textural changes. Chandralekha *et al*. (1992) [5] reported that washing of fish with water prior to cooking removed up to 43% of formaldehyde and the concentration of the treated fish depends on the size of the fish, the strength of formalin solution, the absorption coefficient, duration of dipping and presence or absence of the skin in the fish

4. Conclusion

In present study free and bound formaldehyde concentration in the commercially important fish collected from different fish markets of Tamil Nadu were analysed. The concentration of formaldehyde in *barracuda* exceeded the limit set by USEPA but below the limit of FSSAI. When *barracuda* was held in iced storage and frozen storage, an increase in bound formaldehyde was noticed than that of free formaldehyde due to TMAO breakdown. The increase in bound formaldehyde was significant beyond day 3 of iced storage and beyond day 15 of frozen storage. Cooking of formaldehyde treated fish showed negligible reduction (8-13%) in free and bound formaldehyde concentration. Washing of formaldehyde treated fish indicated a significant reduction in free formaldehyde by 41 to 60%. The study provided very important findings that prolonged storage of fish in ice and in cold store increased only the bound formaldehyde. Washing reduced free or added formaldehyde concentration. Therefore, consumer are advised to wash the fish for 15 min in water, to get rid of added formaldehyde (if any) used to treat the fish, as further cooking will retain the added formaldehyde and cause health hazard.

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