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Comparative evaluation of HPLC method and acetyl acetone method in determination of formaldehyde in Fish

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ABSTRACT

The fish and shellfish are the highly perishable food commodity. Formaldehyde is used by fishermen and fish vendors to preserve the freshness and quality of fish and seafood. The present study was conducted to compare the reliability of the quantitative methods for determination of formaldehyde content in fish. The methods selected were colorimetric analysis by using Nash reagent in UV-Vis spectrophotometer and chromatographic detection using Dinitrophenyl hydrazine in High performance liquid chromatography (HPLC). The results showed that both the methods were highly specific without any interference from the reagent blank and fish matrix in formaldehyde determination. The standard curve of formaldehyde by the HPLC method was linear in the range of 0 - 0.1 µg/ml (R^2 =0.9995). The Limit of Detection (LOD) and Limit of quantification (LOQ) for matrix free calibration were 0.0004 µg/ml and 0.0012 µg/ml and for fish were 0.020 µg/g and 0.060 µg/g respectively. Accuracy was 92.63 ±4.60% and repeatability with Relative standard deviation (RSD %) were ranging from 1.77% to 4.29%. The linear range of formaldehyde by the spectrophotometric method was 0-50 µg/ml (R^2 =0.9988), and the LOD and LOQ for matrix free calibration were 0.55µg/ml and 1.6µg/ml whereas for fish were 27.5 µg/g and 80 µ/g respectively Accuracy was 83.67± 5.60% and repeatability with Relative standard deviation(RSD %) were ranging from 2.66% to 6.89%.Both HPLC and UV detection methods can be used for quantitative analysis of formaldehyde in fish, and the major advantages of HPLC are that it is quick, simple and sensitive.

KEYWORDS

Formaldehyde, Nash reagent, DNPH Reagent, Sensitivity

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Fish and seafood are a good source of protein and plays a vital role in a healthy diet (Ashie *et al*., 1996). According to FAO report, “The state of world fisheries and Aquaculture” apparent per capita fish consumption in India is about 20.5 kg. (FAO, 2020)**.** Being a protein rich food, fish is highly perishable. Therefore, different types of preservation methods such as freezing, drying, pickling, marinating have been widely practiced to achieve food safety and quality.

The composition of fish mainly contains fat, free amino acids, and water, which are highly prone to post mortem spoilage (Fernandes and Venkatraman, 1993; Ismail, 2005). Due to an increase in consumer demand, the products have to remain fresh for a long time until they reach the consumers. This prompts the use of many food adulterants which reduces the fish quality and compromises its safety for consumption (Haque and Mohsin, 2009).

Formaldehyde is a potential carcinogen as chronic exposure of formaldehyde can irritate eyes, nose, and throat (Zhang *et al*., 2009). It can also increase breathing problems for people with health conditions like asthma, Chronic Obstructive Pulmonary Disorder. Formalin is occasionally delivered or sprayed to the fishes via the fish traders while transporting to the domestic marketing chain to prevent spoilage and enlarge shelf life (Yeasmin *et al*., 2010). The World Health Organization (WHO) and U.S. Environmental Protection Agency (US EPA) list formaldehyde as a potential danger carcinogen and important environmental pollutant. There are different standard methods used in detection of formaldehyde content of seafood maintained under different conditions like spectrophotometric, HPLC method, calorimetric reaction, GCMS method and rapid assessment using qualitative kits. High performance liquid chromatography (HPLC) method and spectrophotometric method were most commonly used in quantitative detection of formaldehyde.

Nash (1953) developed a calorimetric reaction in detection of formaldehyde. In the Nash method, addition of formaldehyde with a Nash reagent (mixture of ammonium salts like ammonium acetate, acetyl acetone, acetic acid) form a 3, 5-diacetyl-1, 4-dihydrolutidine which develops yellow color on heating along with pH adjustment with maximum absorbance at 412 nm. Formaldehyde can also be detected by High performance liquid chromatography as in this method formaldehyde derivatized with acidic solution of 2, 4–dinitrophenyl hydrazine in methanol water medium to form 2, 4-dinitrophenyl hydrazones followed by analysis with liquid chromatography.

However, the spectrophotometric methods are most widely used as they are not very sensitive and are subject to numerous interferences by phenols, alcohols and cyclohexane. While HPLC method has been shown to provide adequate sensitivity and have a low limit of detection. In this paper, acetyl acetone spectrophotometry and HPLC methods for the determination of formaldehyde in aqueous solutions were applied, and two methods were explored and compared the reliability of the quantitative determination of formaldehyde in fish.

Materials and Methods

Raw Materials

Freshly caught Indian mackerel (*Rastrelliger kanagurta)* has been collected from nearby Versova landing Centre, Mumbai. Fish was immediately iced in plastic polystyrene insulated container with fish: ice ratio of 1:1 (w/w) and brought to the laboratory of Post-Harvest Technology, CIFE, Mumbai within one hour. The fishes collected had an average weight of 150-200g/ body weight.

Chemicals

Solvents were of analytical grade as in case of Spectrophotometry method formaldehyde stock solution(38%) purity , Ammonium acetate acetyl acetone were purchased from Merck whereas for HPLC the solvents were of analytical grade (SIGMA–Aldrich, Buchs SG, Switzerland) 2,4 dinitrophenylhydrazine (2,4 DNPH) and Formaldehyde in water certified reference material (CRM) (97 mg/l) was from SIGMA– Aldrich (Buchs SG, Switzerland).

Nash Reagent Preparation

The double standard Nash reagent was prepared by mixing 30 g of ammonium acetate, 0.6 ml of acetyl acetone, 0.4 ml of glacial acetic acid, and the volume was made up to 100 ml by adding distilled water. The reagent was stored in a dark glass bottle covered with aluminum foil.

2, 4 Dinitrophenylhydrazine Working Solution

The DNPH solution was prepared by dissolving 300 mg of DNPH crystals in 25 ml of acetonitrile and 1ml of 85% orthophosphoric acid. The solution was made up to 50 ml in a volumetric flask using acetonitrile. The solution was stored in a dark place.

Sample Processing and Derivatization Kinetics

The fish sample (2 g) was taken in a 500 ml conical flask and 100 ml of 1:1 methanol: water was added. The flask was plugged with cotton and allowed to mix in a shaker for 30 minutes at room temperature. After the completion of the time interval, the extract was filtered through Whatman filter paper and kept in separate conical flask.

In Spectrophotometry method the derivatization procedure followed described by (Laly *et al*., 2017) as the five-millilitres (5 ml) of the filtered sample was taken in a test tube and 5 ml of double standard Nash reagent was added. The tubes were placed in a water bath at 55-60°C for 10 minutes for derivatization and the absorbance was measured immediately in an UV-Vis Spectrophotometer at 415 nm. In HPLC method, the derivatization procedure was optimized on trial and error basis as The filtered sample (1 ml) was taken in a 5 ml glass test tube, 50µl of 2, 4 DNPH solution was added and the tube was placed in a hot air oven at 40°C for 60 minutes for derivatization. The solution was allowed to come back to room temperature and sample was proportionally diluted to 3 ml using methanol: water (1:1). The solution was mixed well and filtered through 0.45 µm Nylon syringe filter, followed by HPLC analysis using acetonitrile: water (50:50) mobile phase with a flow rate of 1 ml/min. The sample was injected at 10µl volume and formaldehyde was detected at 355 nm after a run time of 12 minutes.

High Performance Liquid Chromatography Conditions

Chromatographic separation was achieved using Agilent Eclipse (XDB-C18, 4.6mmX150mm with 5.0 µm particle size) under isocratic mode of elution. The mobile phase was a mixture of water and acetonitrile (50:50, v/v). Separation was performed at 35 °C using a 1 mL /min flow-rate and the run time was 12 minutes. The injection volume was 10µL and the detection wavelength was set at 355nm. The chromatographic and the integrated data were recorded in a computer system using Empower data acquiring software (Waters, USA).

Spectrophotometry Method Validation

The method was validated in terms of specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and repeatability. The specificity was determined by scanning the reagent blank(Nash reagent) , sample blank, formaldehyde standard solution and formaldehyde spiked fish sample from 300-500 nm separately as 415 nm was the specific wavelength for measuring formaldehyde if Nash reagent interfered with the measurement of Nash – HCHO complex in reagent blank and fish sample.

This was determined by measuring absorbance of Nash reagent (reagent blank) and formaldehyde in the fish sample from 300 -500 nm. Linearity of formaldehyde was calculated from the regression equations and correlation coefficient (R^2) for the range from 0 to 50 µg/ml. Limit of detection and Limit of quantification was determined by using the slope (S) and the standard deviation of y-intercept of regression line (σ) using the formula for LOD ($3.3\sigma / S$) and for LOQ ($10\sigma/S$) (ICH, 2005). The accuracy is evaluated by means of recovery assays carried out by adding known amounts of standard spiked to the fish sample, at LOQ level and also calculated % of Relative standard deviation. Repeatability was expressed as relative standard deviation (%) as the spiked fish sample was evaluated over three different days at the same concentration levels as per the method.

HPLC Method Validation

The specificity of the method was evaluated by injecting the reagent blank (2-4 DNPH), sample blank, formaldehyde standard solution, and formaldehyde spiked fish sample separately. Linearity of formaldehyde was calculated from correlation coefficient (R^2) for responses of 0 ,0.005, 0.010, 0.025, 0.05 and 0 .1 µg/ml. Limit of detection and Limit of quantification was determined by using the slope (S) and the standard deviation of y-intercept of regression line (σ) using the formula for LOD ($3.3\sigma / S$) and for LOQ ($10\sigma/S$)(ICH,2005). The accuracy is evaluated by means of recovery assays carried out by adding known amounts of standard spiked to the fish sample, at LOQ level and also calculated % of Relative standard deviation. Repeatability was expressed as relative standard deviation (%) as the spiked fish sample was evaluated over three different days at the same concentration levels as per the method.

Statistical Analysis

The results were performed for at least three repetitions and results were tested for statistical significance using Microsoft Excel. The SPSS 16.0 (SPSS, 2000) statistical package was used for analysis of the experimental results. The results were expressed as mean ± standard deviation and Duncan's multiple range tests was used for Post hoc comparison to asses statistical significance ($P < 0.05$) between the triplicates.

Results and Discussion

Spectrophotometry Method Validation

Formaldehyde reacts with Nash reagent to form 3, 5-diacetyl-dihydrolutidine compound (Nash, 1953). It had maximum absorbance at 415 nm (Castell *et al*., 1973).The specificity of the method was evaluated and the matrix did not interfere in the analysis. The formaldehyde peak developed had high resolution and the reagent blank impurities did not interfere in the analysis because the reagent blank does not develop any peak at 415nm whereas the formaldehyde sample and standard develops peak at 415 nm due to presence of formaldehyde (fig 1 and fig 2). The formaldehyde determination by spectrophotometry method is highly specific with high resolution. The study was similarly conducted by (Landim *et al.*, 2013) analyzed the specificity of spectrophotometric method for the detection of flavonoids in a crude extract of *Dimorphandra gardneriana* and the formaldehyde peak developed had high resolution and the reagent blank impurities did not interfere in the analysis. Linearity calibration curve was plotted in the range from 0 to 50 µg/ml and the correlation coefficient (R^2) was found as 0.9988.The LOD and LOQ were 0.55 µg/ml and 1.6µg/ml, respectively lower than 1.3681 µg/ml and 4.5603 µg/ml reported by (Rohyami *et al*., 2017).

Table 1 represents the LOD and LOQ in spectrophotometer. The LOD and LOQ values for fish samples in this study were 27.5µg/g and 80µg/g respectively. The LOD value of fish were higher than 10 mg /kg in squid sample reported by (Lee *et al*., 1984).Compared to LOD and LOQ of matrix-free samples, the LOD and LOQ of fish matrix were higher due to the other compounds present in the matrix that might have got co-extracted during the extraction process. This study showed that the detection of formaldehyde by spectrophotometer was reliable, but less sensitive. The recovery rate of the formaldehyde was determined by spiking fish with known concentrations of formaldehyde at LOQ level of 100µg/g and showed a recovery rate of 83.67± 5.60% similar to 78.97–89.23% accuracy of formaldehyde detection in smoked meat products reported by (Li *et al*., 2018). The repeatability was determined as % of RSD (Relative standard deviation) and formaldehyde was spiked into fish samples at two fortification levels of 100 µg/g and 250 µg/g and the % RSD was found in the range from 2.66 to 6.89% was higher than 1.39%-2.25% reported by (Sun *et al*., 2013).

HPLC Method Validation

HPLC method is one of the most widely used methods for determination of formaldehyde. The reagent 2, 4-dinitrophenyl hydrazine selectively condenses with formaldehyde to produce a stable hydrazine derivative. This method is one of the most reliable methods to determine formaldehyde in cosmetic products (Rivero, and Topiwala, 2004; Maneli *et al*., 2014). The specificity was demonstrated by running the reagent blank containing 2-4 Dinitrophenyl hydrazine, formaldehyde reference standard and formaldehyde spiked fish sample. The reagent blank had only 2-4 DNPH. It developed the peak and it was eluted at 3.3 minutes of retention time. The reagent blank did not develop a peak after 3.3 minutes as it had no formaldehyde. The formaldehyde spiked fish sample was analysed. 2-4 DNPH was first eluted at 3.3 minutes, followed by formaldehyde at 5.5 minutes suggesting that 2-4 DNPH did not interfere with formaldehyde detection (fig 3, fig 4). There is no interference from fish matrix was observed and formaldehyde from spiked fish could be efficiently detected and quantified using HPLC.

The formaldehyde determination by HPLC method is highly specific with high resolution which were similar to (Soman *et al*., 2008), who detected specificity formaldehyde in drug substance by running the HCHO, DNPH, un-derivatized sample and derivatization standard, and derivatized sample. These authors reported that there was no interference between HCHO-DNPH derivatization product of the drug substance and reagent blank Linearity curve was plotted in the range from 0 to 0.1µg/ml and the correlation coefficient (R^2) was found as 0.9995. Table 2 represents the LOD and LOQ in HPLC method. The LOD and LOQ were analysed using calibration curve and were found to be 0.0004 µg/ml and 0.0012µg/ml respectively and for the fish matrix was found as 0.020µg/g and 0.060 µg/g respectively which were lower than 0.39 µg/ml and 1.30 µg/ml in case of matrix free calibration and whereas for fish sample was 1.75 µg/g and 5.83 µg/g reported by (Wahed *et al*., 2016). Compared to matrix free samples, the LOD and LOQ for fish matrix were higher due to the other compounds present in the matrix that interfere in the analysis. Nevertheless, HPLC is a highly reliable method for the detection of formaldehyde in fish matrix.

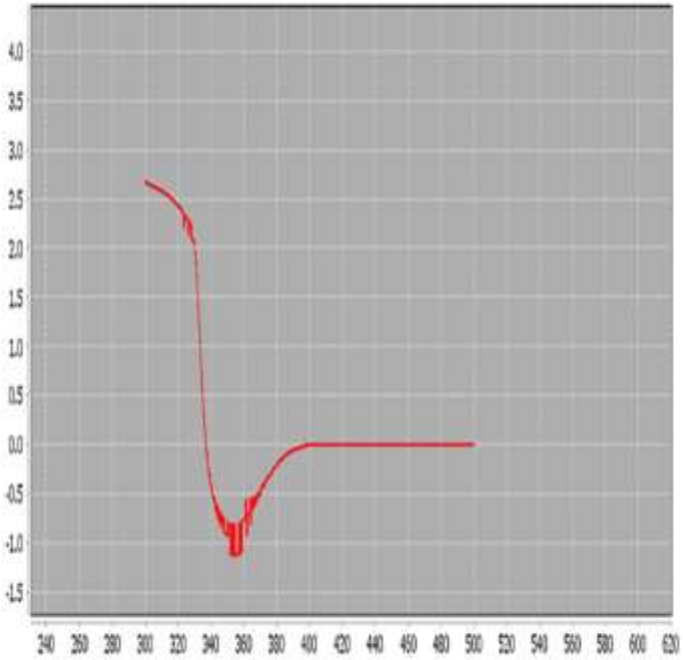

Fig 1. Scanning performance of reagent blank (Nash reagent) in UV Spectrophotometer

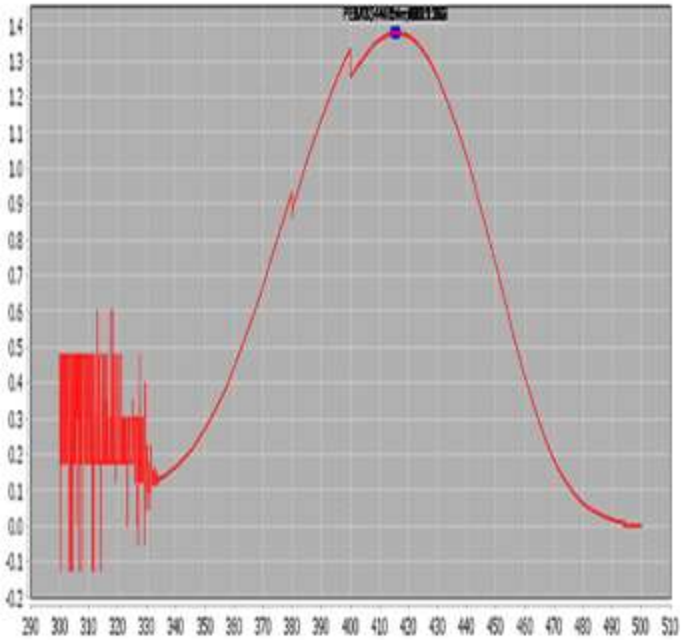

Fig 2. Scanning performance of formaldehyde (0.5%) spiked fish sample

Table 1. LOD AND LOQ for formaldehyde detection using UV-Spectrophotometer

Set	Standard Concentration (µg/ml)	Absorbance	Equation	R^2	LOD and LOQ (µg/ml)	LOD and LOQ in Fish Sample (µg/g)
1	0	0.003	y = 0.0158x + 0.0064	0.9977	LOD = 0.55 µg/ml	LOD =27.5 µg/g
	2.5	0.053				
	5	0.085				
	10	0.176				
	25	0.375				
	50	0.808				
2	0	0.004	y = 0.0156x + 0.0105	0.9990	LOQ = 1.6 µg/ml	LOQ = 80 µg/g
	2.5	0.055				
	5	0.086				
	10	0.179				
	25	0.387				
	50	0.797				
3	0	0.002	y = 0.0159x + 0.0053	0.9993		
	2.5	0.052				
	5	0.084				
	10	0.169				
	25	0.388				
	50	0.807				
Slope (S) = 0.0157						
SD of intercept = 0.0026						

Note: SD –Standard deviation, R^2 - Coefficient of determination

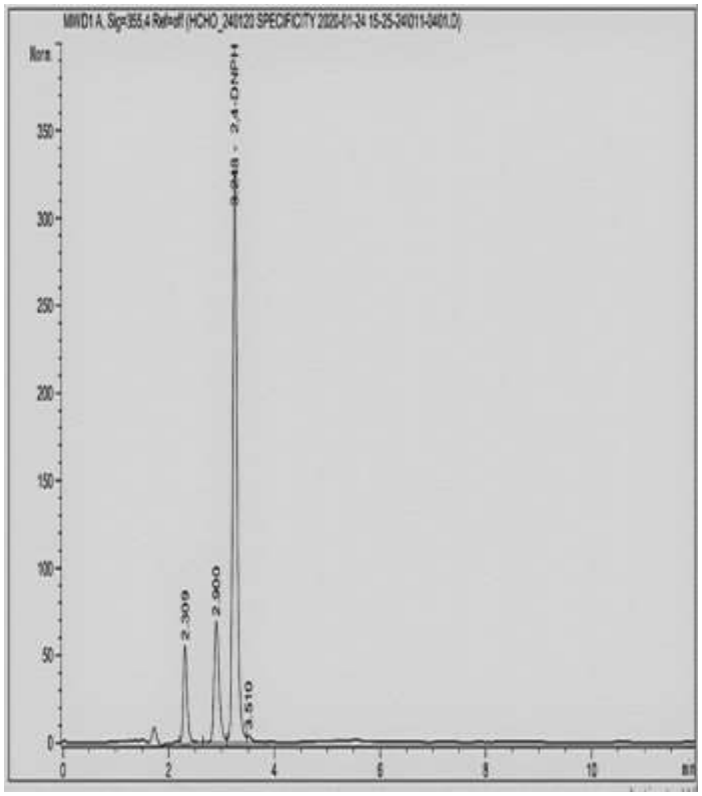

Fig 3. Chromatogram of 2-4 DNPH reagent blank

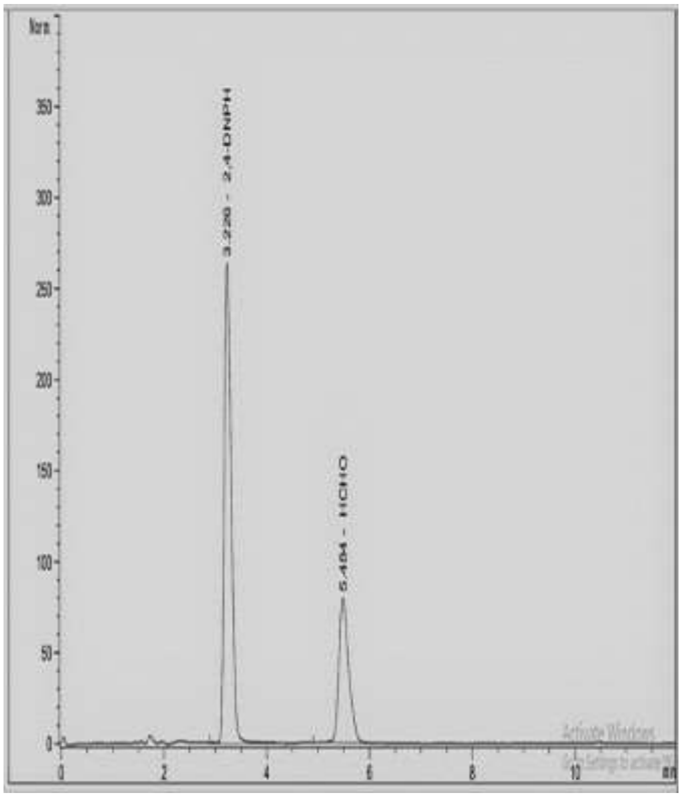

Fig 4. Chromatogram of formaldehyde spiked in the fish sample at 5 μg/g

Table 2. LOD AND LOQ for formaldehyde detection using HPLC

Set	Standard concentration (μg/ml)	Area	Equation	R^2	LOD and LOQ(μg/ml)	LOD and LOQ in fish sample (μg/g)
1	0	4.17				
	0.005	79.67	y = 12866x + 9.654	0.9988		
	0.010	139.87				
	0.025	329.54			LOD = 0.0004 μg/ml	LOD = 0.020μg/g
2	0	5.53	y =12263x + 12.401			
	0.005	78.92		0.9981	LOQ = 0.0012 μg/ml	LOQ = 0.060μg/g
	0.010	139.55				
	0.025	316.14				
	0	5.95				
	0.005	77.64				
3	0.010	137.21	y =12134 x + 12.102	0.9984		
	0.025	312.98				

Slope (S) = 12.42
SD of intercept = 1.507

Note: SD – Standard deviation, R^2 - Coefficient of determination

Table 3 Comparative analysis of HPLC and spectrophotometric methods

Detection Method	Specificity	Linearity (R^2)	Sensitivity (µg/ml)	Theoretical Sensitivity of Fish Sample (µg/g)	Recovery % at LOQ Level	Repeatability (RSD %)
			LOD LOQ	LOD LOQ		
HPLC	No interference was found close to the retention time of formaldehyde	0.9995	0.0004µg/ml 0.0012µg/ml	0.020µg/g 0.060µg/g	88.20 ± 3.02 to 105.41 ± 1.87	1.77 to 4.29
Spectrophotometry	No interference was found at specific nm used for detecting formaldehyde	0.9988	0.55µg/ml 1.6µg/ml	27.5µg/g 80µg/g	83.67 ± 5.60 to 100.26 ± 2.66	2.66 to 6.94

The recovery rate of the formaldehyde was determined by spiking formaldehyde with known concentration in the range of 0.1µg/g at LOQ level in the fish sample. The recovery rate of formaldehyde was 92.63 ± 4.60% similar to 96-100.6% reported by (Pina *et al*., 1995) and 91.25 - 105.36% by (Wahed *et al*., 2016). The repeatability was determined as % of RSD (Relative standard deviation) and formaldehyde was spiked into fish samples at two fortification levels of 0.25 µg/g and 2.5 µg/g and the % RSD was found in the range from 1.77 to 4.29% was lower than 2.28% reported by (Sun *et al*., 2013).

Comparative Evaluation of Spectrophotometry and HPLC Method

Table 3 represents the comparative results of HPLC and spectrophotometry method. The validation parameters like specificity, Linearity, Sensitivity, Accuracy and Repeatability were calculated for both the methods. The method was found to be specific as there were no interferences from impurities, either from reagent blank or from the fish matrix. In linearity assay, the correlation coefficient (R^2) for HPLC method was found to be 0.9995 and for spectrophotometry method, the value was 0.9988. The sensitivity was calculated as Limit of Detection (LOD) and Limit of quantification (LOQ). LOD and LOQ for HPLC method were 0.0004µg/ml and 0.0012µg/ml, whereas for spectrophotometry method the corresponding values were 0.55µg/ml and 1.6µg/ml, respectively.

The sensitivity (LOD and LOQ) for fish samples were calculated after multiplying with dilution factor. The LOD and LOQ of fish sample for HPLC method were 0.020µg/g and 0.060µg/g, respectively whereas for Spectrophotometry method, the values were 27.5µg/g and 80µg/g, respectively. The sensitivity of HPLC method was higher than spectrophotometry method. The accuracy for HPLC method was 92.63 ± 4 .60 % and 83.67± 5.60% for spectrophotometry method. Based on this, HPLC method can be considered as more accurate than spectrophotometry method. The Repeatability (RSD %) for HPLC method was in the range from 1.77 – 4.29% and for spectrophotometry method, 2.66 – 6.89 %. Comparatively, the HPLC method was more precise than spectrophotometry method. Sun *et al*. (2013) compared the HPLC and spectrophotometry methods on quantitative determination of formaldehyde in water-based coatings and measured linearity, sensitivity, accuracy and repeatability. The linearity (R^2) of HPLC and spectrophotometry method were 0.9998 and 0.9995. LOD and LOQ of HPLC were 0.96 mg/ kg and 32.3mg/ kg, whereas for spectrophotometry method the values were 5.93 mg/kg and 41.7 mg/kg. The accuracy rate of formaldehyde in HPLC and spectrophotometry method was 96.2–101.3 and 95.5–105.3, respectively. The repeatability (% RSD) of HPLC and spectrophotometry methods was 0.85% and 1.21%, respectively. It was concluded that the HPLC was an ideal method for detection of formaldehyde in water based coatings.

Therefore, from the results that the two methods are quick, simple, sensitive and suitable for the determination of formaldehyde. However, for sample containing a lower level of formaldehyde, the HPLC method was an ideal method for detection of formaldehyde in fish sample. All validation parameters were within the acceptance performance criteria recommended by (AOAC Official Methods of Analysis, 2012).

Conclusion

Formaldehyde, the simplest of the aldehydes used in chemical industry, is a prominent harmful food additive which is the highly hazardous and carcinogenic. It is therefore important to adopt and optimize methods for sensitive detection of formaldehyde in fresh fish. Many methods are used for determination of formaldehyde. The most widely used methods for detection of formaldehyde content in fish include the UV-Vis spectrophotometer and the High Performance Liquid Chromatography (HPLC).The present study showed that both spectrophotometric and HPLC methods can be used for quantitative detection of formaldehyde detection in fish. The HPLC method is particularly advantageous as it is quick, simple and highly sensitive compared to spectrophotometric method.

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