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Method validation for the detection of formaldehyde in fish using ultra performance liquid chromatography

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Abstract

Formaldehyde, the simplest aldehyde, tops the list of illegal and harmful food contaminants. Recently, formaldehyde has been used indiscriminately to preserve the freshness and quality of fish. Numerous incidents of formalin (a solution of formaldehyde) misuse during the storage and transportation of fish have been reported in several parts of India. In this context, it is pertinent to study formalin contamination in fish and shellfish. Protocol for detection of free and bound formaldehyde by ultra-performance liquid chromatography (UPLC) was attempted in the present study. Formaldehyde peak was observed in the expected retention time and standardisation of the protocol for formaldehyde determination was then carried out. UPLC method was developed and validated for specificity, repeatability, accuracy, and linearity. The average correlation coefficient (R^2) was 0.99. The recovery rates of free and bound formaldehyde were 90.83 to 116.43% and 88.76 to 132.98%, respectively. The limit of detection (LOD) was found to be 0.5 ppm. The study investigated free and bound formaldehyde levels in commonly traded fish species across five major fish markets in Tamil Nadu, India. *Sphyraena barracuda* had the highest recorded concentrations of free and bound formaldehyde at 9.8 and 6.45 mg kg^{-1}, respectively, while *Lethrinus lentjan* exhibited the lowest concentrations at 0.8 mg kg^{-1} for free formaldehyde and 0.5 mg kg^{-1} for bound formaldehyde.

Introduction

Formaldehyde, represented by the chemical formula HCHO (Fig. 1), is an organic substance. It is a colourless gas with a strong odour and is commonly acquired as formalin, which typically contains 37% formaldehyde (IARC, 2012). While it is the most essential member of the aldehyde group, formaldehyde is highly reactive.

Formaldehyde was identified as a naturally occurring substance in food and food products (fruits, vegetables, meats, fish, and crustaceans), and its level differed for each food item. According to the World Health Organisation, fruits and vegetables contain formaldehyde ranging from 3.3 to 60 mg kg^{-1}, milk 1 to 3.3 mg kg^{-1} and fish 1 to 98 mg kg^{-1}. It serves multiple roles in various foods, as preservative, reducing agent, fumigant, and sterilising agent (Norliana *et al*., 2009). It naturally occurs as an intermediate product in the metabolism of fruits and vegetables and is also generated in seafood through the enzymatic breakdown of trimethylamine-oxide (TMAO). Recently, the International Agency for Research on Cancer (IARC) classified formaldehyde as a Group 1 carcinogen to humans, indicating that it poses a significant risk of causing cancer. Specifically, exposure to formaldehyde has been linked to an increased risk of leukaemia and nasopharyngeal cancer (IARC, 2012).

Fish and shellfish are essential for maintaining a balanced and nutritious diet, offering a unique nutritional composition with significant protein content (Ashie *et al*., 1996). As the global population grows, there is a notable increase in individuals' consumption of fish (Immaculate *et al*., 2018).

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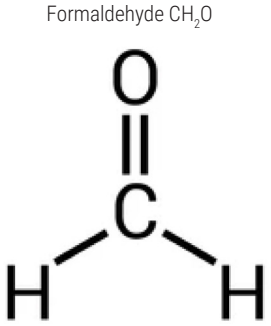

Fig. 1. Chemical structure of the formaldehyde

Fish contains water, fat, protein and free amino acids, which render it prone to deterioration through biochemical processes post-mortem and microbial activity (Fernandes and Venkatraman, 1993). Recently, consumers have raised concerns about tainted fish and shellfish with carcinogenic formaldehyde in various fish markets to extend their shelf life (Ali, 2013). The formaldehyde content in finfish and shellfish samples from different markets ranges from 0.33 to 16 mg kg^{-1}. However, the average range of formaldehyde in fish and shellfish is between 2 and 50 mg kg^{-1}. During storage, formaldehyde is gradually produced in frozen marine fish, such as cod, pollock and haddock (Wahed *et al*., 2016; Bhowmik *et al*., 2017). Formaldehyde primarily originates from trimethylamine oxide (TMAO), which is naturally present in marine fish and shellfish muscle tissue. TMAO is broken down into formaldehyde and dimethylamine (DMA) by the enzyme trimethylamine oxide demethylase (TMAO-ase), as observed in studies by Rehbein (1987) and Sotelo *et al*. (1995). During frozen storage, TMAO accumulation can lead to protein interactions and subsequent denaturation, resulting in muscle toughness, as Sotelo *et al*. (1995) documented. It is important to note that formaldehyde production can increase as fish flesh ages and deteriorates. However, fish tissues do not accumulate high levels of formaldehyde due to its conversion into other chemical compounds, as indicated by Tsuda *et al*. (1988).

Various techniques exist for detecting formaldehyde in fish. For example, Nash (1953) developed a colourimetric method using the Hantzsch reaction, which produces a yellow compound called 3,5-diacetyl-1,4-dihydrolutidine through the condensation of ammonium salts, acetylacetone and formaldehyde. Castell and Smith (1973) utilised trichloroacetic acid to extract formaldehyde from fish and employed the NASH test for quantification. Rehbein and Schmidt (1996) used the Reflectoquant test strip and RQFlex to measure free formaldehyde in minced fish after derivatisation with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, resulting in the formation of purple tetrazol derivatives. Their findings showed good agreement with the Nash test. Binachi *et al*. (2007) assessed formaldehyde content in 12 fish species and various fish products using a solid phase microextraction (SPME)-GC-MS method involving fibre derivatisation with pentafluorobenzyl-hydroxylamine hydrochloride. Li *et al*. (2007) developed a high-performance liquid chromatography (HPLC) method for formaldehyde determination, employing steam distillation and 2,4-dinitrophenylhydrazine. The formaldehyde was then analysed by HPLC using an ODS-C18 column and a UV detector at 355 nm. Similarly, Wahed *et al*. (2016) validated a method for detecting formaldehyde by assessing several parameters, including linearity, the limit of detection (LOD), the limit of quantitation (LOQ), recovery, repeatability, intermediate precision and robustness. The results indicated strong linearity (R^2 = 0.99) and a broad detection range (1.08-100 mg l^{-1}). The LOD and LOQ were determined to be 0.32 and 1.08 mgl^{-1}, respectively, with a recovery range of 83.25 to 115.56%. Repeatability and intermediate precision were satisfactory, with relative standard deviations (RSD) below 15%. Yeh *et al*. (2013) also used Gas Chromatography-Mass Spectrometry (GC-MS) to analyse free and bound formaldehyde in squid and squid products. By employing derivatisation with 2,4-dinitrophenylhydrazine (DNPH), they achieved a 2.0 mg kg^{-1} detection limit. Their findings revealed that the total concentrations of free and reversibly bound formaldehyde exceeded the concentration of free formaldehyde alone. Uddin *et al.* (2011) investigated formalin contamination in various fish species, including rohu, mrigal, catla and olive barb, sourced from different markets in Bangladesh. They used the "formalin detection kit" developed by the Bangladesh Council of Scientific and Industrial Research (BCSIR) for their analysis.

Most previous studies on formaldehyde focused on its presence in foods, including fish and shellfish at markets or restaurants. While several studies have separately characterised formaldehyde using techniques such as headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS), GC-MS, GC and UV (Bianchi *et al*., 2007; Paiano *et al*., 2014), only a few have utilised ultra-performance liquid chromatography (UPLC) for this purpose (Jeong *et al*., 2015). The primary objectives of this study were to develop and validate an analytical method for formaldehyde detection in fish using UPLC and assessing the linearity of the standard calibration curve, sensitivity (limit of detection and limit of quantification), repeatability (intra-day and inter-day), and accuracy (recovery).

Materials and methods

Fish sample

This study investigated the free and bound formaldehyde levels in commonly traded fish species across five major fish markets in Tamil Nadu, India. Six types of commercially important fish, namely sardine (*Sardinella gibbosa*), barracuda (*Sphyraena barracuda*), carangid (*Caranx sexfaciatus*), tuna (*Euthynnus affinis*), emperor fish (*Lethrinus lentjan*) and mackerel (*Rastrelliger kanagurta*) were identified for the analysis. Fish samples intended for method validation were collected from the Thoothukudi fishing harbour, transported to the laboratory under iced conditions (4-7°C), and the length and weight of each fish were documented. Subsequently, the fish were carefully dissected on a sanitised plastic board and the edible muscle portion was separated and used for further extraction.

Sample preparation for formaldehyde detection by UPLC

The Ultra Performance Liquid Chromatography (UPLC), specifically the Waters H-Class system equipped with a quaternary pump and

tunable UV detector (M/s. Water Pvt. Ltd, USA) was utilised for formaldehyde detection. Steam distillation of fish samples was carried out using the Kjeldahl system (Pelican, Chennai). Other essential equipment and chemicals employed in the sample extraction process included a water bath (Technico, Chennai), N2 evaporator, ultrasonic bath (Labman, Chennai), refrigerated centrifuge (5804R Eppendorf, Germany) and electronic balance (Sartorius India Pvt Ltd, Chennai). Additionally, for the analysis, Formaldehyde DNPH mix (RESTEK, USA), dichloromethane (Rankem, India), 2,4-dinitrophenylhydrazine (Merck, India) and acetylacetone (Himedia, India) were used.

The sample preparation and analysis of formaldehyde were carried out with slight modifications of Yeh *et al*. (2013) and Wahed *et al*. (2016) methods. For free formaldehyde analysis by UPLC, 5±0.5 g of homogenised sample was taken in a 50 ml centrifuge tube, 10 ml of milliQ water was added and vortexed well for 3 min. The tubes were kept in a sonicator for 30 min and centrifuged at 7000 rpm at 4°C for 10 min. From this, 1 ml of supernatant was taken in a 15 ml centrifuge tube and 0.25 ml of 2,4 DNPH solution (3 mg ml^{-1}) was added and incubated in a water bath at 60ºC for 10 min. The tubes were then cooled at room temperature; volume was made up to 5 ml using acetonitrile and vortexed for 2 min. Finally, centrifuged at 7000 rpm at 4°C for 5 min and filtered using a 0.2 PVDF syringe filter. The filtered extract was transferred to sample vials and kept for injection in UPLC.

A 5±0.5 g homogenised sample was taken in a distillation tube for bound formaldehyde analysis. In addition, 1 ml of 20% phosphoric acid and 40 ml of water were added. Steam was then distilled in a kelplus distillation unit until 100 ml of distillate was collected. From that, 20 ml of distillate was taken in a 50 ml centrifuge tube and 1 ml of DNPH solution was added and vortexed. The tubes were kept in the water bath at 60ºC for 10 min. The tubes were then cooled to room temperature and 3 ml of dichloromethane was added and vortexed for 10 min. The tubes were centrifuged at 7000 rpm at 4°C for 5 min. Finally, 2.5 ml of the bottom dichloromethane layer was taken and evaporated to dryness in an N_2 evaporator. The N_2 flow was set as 9 psi at 40ºC. Then, 1 ml of acetonitrile was added, and the dried extract was reconstituted. The tubes were centrifuged at 7000 rpm at 4°C for 5 min. The extract was filtered, transferred to sample vials and kept for injection in UPLC.

Method validation for free and bound formaldehyde in UPLC

Method validation is done by analytical characteristics of the method such as accuracy, precision, specificity, detection limit, quantitation limit, linearity, range and robustness. Method validation is crucial to confirm the reliability and accuracy of the analytical technique used to determine formaldehyde levels in different samples. This process involves evaluating parameters like linearity, accuracy, precision and robustness to ensure that the method meets the necessary standards.

Repeatability

About 5 g of each homogenised sample was taken in six centrifuge tubes. A 50 ppm formaldehyde solution was added to this. The free and total formaldehyde concentration was determined as per the above procedure. The mean, standard deviation and coefficient of variation (CV) values were determined to validate repeatability of the method. The CV was calculated using the formula:

$$CV(\%) = \frac{SD}{Mean} \times 100$$

Linearity

About 5 g of homogenised sample was taken in 50 ml centrifuge tubes. Standard formaldehyde solution was spiked at different concentrations (1, 2, 5, 10, 25, 50 and 100 ppm). The free and total formaldehyde concentration was determined as per the above procedure. A graph was plotted with concentration on the X-axis and area on the Y-axis. Linearity of standard formaldehyde DNPH solution at the above concentration was also determined.

Accuracy

Accuracy refers to the closeness of test results obtained from an analytical method to the actual value. The accuracy of a technique can be measured by calculating the recovery. The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response received for the actual concentration of the analyte in the solvent.

About 5 g of each homogenised sample was taken into three centrifuge tubes. The samples were spiked with 10, 25 and 50 ppm standard formaldehyde solution. The formaldehyde concentration was determined as described earlier. The recovery was calculated using the formula:

$$\text{Recovery (\%)} = \frac{\text{Conc. of FA obtained}}{\text{Spiked conc.}} \times 100$$

Limit of detection (LOD)

Formaldehyde DNPH at various concentrations of 1, 2, 5, 10, 25, 50 and 100 ppm and that of 5 g sample spiked with the same formaldehyde concentrations was determined using UPLC. The lowest concentration that was detected was recorded as LOD for formaldehyde and that of matrix, respectively.

UPLC analysis of formaldehyde

Formaldehyde content was analysed using UPLC (Waters ACQUITY H-Class, USA) following the method described by Yeh *et al.* (2013). Methanol and water (60:40) were used as mobile phases at a flow rate of 0.6 ml min^{-1} with a run time of 3 min. The Phenomex Synergy 2.5 µ fusion RP, 50 x 2.0 mm column, was used for separation and the column temperature was 40ºC. The injection volume was set at 5 µl and the wavelength of the tunable UV detector was set at 355 nm. Concentration of the sample was calculated using the formula:

$$\text{Sample concentration} = \frac{\text{Peak area (sample)}}{\text{Peak area (standard)}} \times \text{Dilution factor}$$

Results and discussion

Method validation for free and bound formaldehyde in UPLC

The method for determining free and bound formaldehyde by UPLC was developed and optimised for the steps namely recovery, time and reagents, and matrix interference. The UPLC working wavelength of the tunable UV detector was selected for maximum absorbance at 355 nm (Seibei *et al*., 2018). Various mixtures of mobile phases were tried for good separation and methanol: Milli-Q (60: 40 v/v) was found to perform well. Pre-column derivatisation with 2,4-dinitrophenyl hydrazine reagent is a powerful technique for detecting formaldehyde using HPLC (Bhowmik *et al*., 2017). The reagent 2,4-dinitrophenyl hydrazine selectively condenses with formaldehyde to produce a stable hydrazine derivative. This method was one of the most reliable methods to determine formaldehyde in cosmetic products (Maneli *et al*., 2014). Several workers have studied detecting formaldehyde in fish using HPLC (Wahed *et al*., 2016; Bhowmik *et al*., 2017).

Specificity

The peak development was mainly based on the compound and its retention time. The elution peak for blank (2,4 DNPH) and standard are shown in Fig. 2 (a, b) The reagent blank had a peak for 2,4 DNPH only and this compound was eluted at 0.355 min. The reagent blank did not develop a peak after 0.355 min., as it had no formaldehyde.The specificity of formaldehyde was analysed using the UPLC method. In the current study, two separate peaks for 2-4 DNPH and formaldehyde were observed at 0.355 and 0.561 min, suggesting that DNPH did not interfere with formaldehyde detection (Fig. 2). Soman *et al*. (2008) studied the specificity of formaldehyde in drug substances by running the HCHO, DNPH and derivatised standard and sample. They reported no interference between the HCHO-DNPH derivatisation product of the drug substance and the reagent blank. The retention times of DNPH and the DNPH HCHO derivatisation products were 3.8 and 6.4 min, respectively. Moreover, there was no interference between the sample matrix and the HCHO DNPH derivatised product. In the present study, the elution time was much less than that of other studies. Wahed *et al*. (2016) reported elution/retention times of 2,4 DNPH and HCHO-2,4 DNPH as 5 and 10.5 min, respectively.

Repeatability

Formaldehyde was spiked at 50 ppm in five fish samples and analysed for free and bound formaldehyde concentration and the results are given in Table 1. The free formaldehyde had a mean of 45.82, SD of 2.72 and CV (%) of 5.95, while total formaldehyde had a mean of 48.07, SD of 2.84 and CV (%) of 5.93. Wahed *et al*. (2016) reported CV (%) of fish sample matrix spiked with 5, 10 and 25 ppm

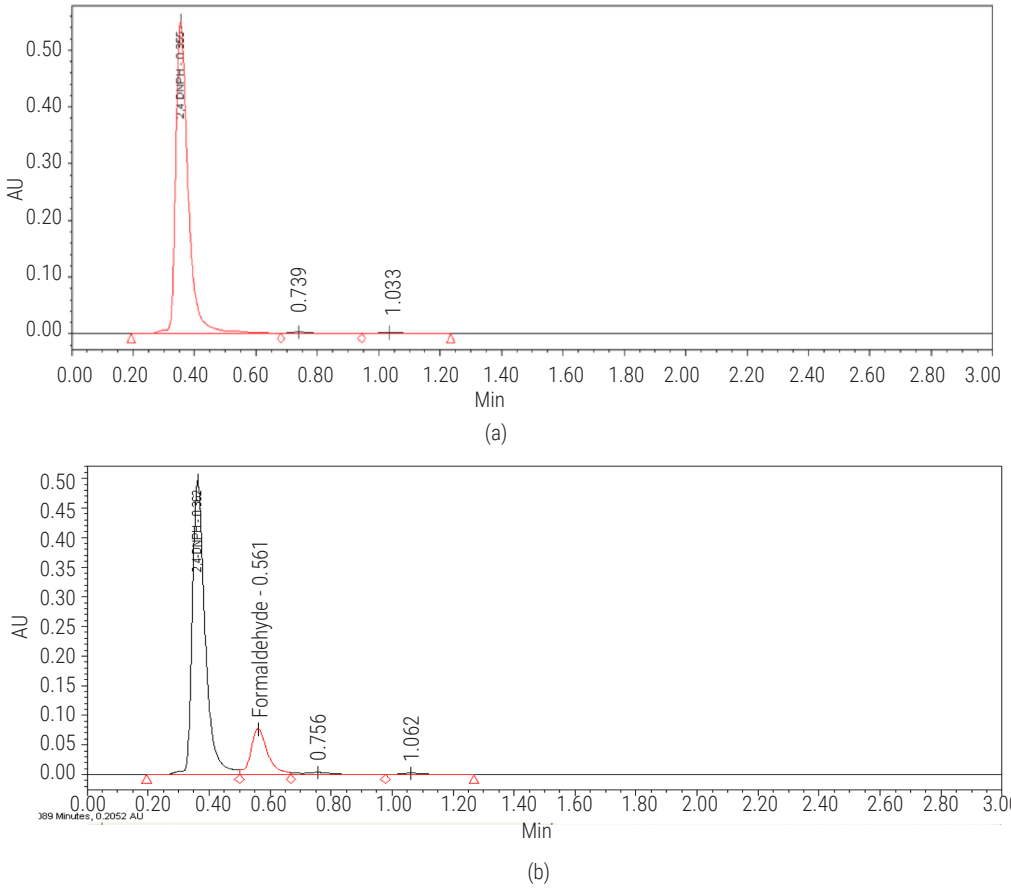

Fig. 2. Chromatograph of (a) 2,4 DNPH reagent blank and (b) Reference formaldehyde standard at 10 ppm

Table 1. Repeatability of formaldehyde detection at 50 ppm

Concentration injected (ppm)	Area	Recovered (ppm)	CV (%)
50	137396	50	5.95
	119576	43.5151	
	124799	45.4158	
	126998	46.2160	
	118884	43.2633	
	Mean	45.6820	
	Std Dev	2.7179	

at 6.72, 6.65 and 0.56, respectively. Sebaei *et al*. (2018) reported the coefficient of variation in milk, cheese and yoghurt spiked with 10 ppm formaldehyde as 9.8, 10.9 and 10.5 ppm, respectively.

Linearity

The linearity was measured using different concentrations (1, 2, 5, 10, 25, 50 and 100 ppm) of standard formaldehyde derivatised in 2,4 DNPH. The average correlation coefficient (R^2) was 0.99 (Table 2, 3; Fig. 3 a, b). Pina *et al*. (1995) validated a method for detecting formaldehyde in the enteric coating of hard gelatin capsules by HPLC and reported a correlation coefficient (R^2) of 0.99. Wahed *et al*. (2016) also measured linearity using different concentrations (1, 2, 5, 25, 50 and 100 ppm) of derivatised standard formaldehyde and reported an R^2 value of 0.99. In this study, the linearity was also measured by spiking standard formaldehyde derivatised with 2,4 DNPH at 1 - 100 ppm concentrations. The correlation coefficient (R^2) obtained was 0.98 and 0.99 for free and total formaldehyde, respectively. Wahed *et al*. (2016) reported an R^2 value of 0.99 for a matrix-matched sample, *i.e.,* fish sample spiked at above said concentrations of derivatised standard formaldehyde.

Accuracy

For testing the accuracy of formaldehyde detected by UPLC in the fish samples, the recovery rate of the formaldehyde from the samples was determined by measuring the free and bound formaldehyde spiked at the concentration of 10, 25 and 50 ppm and the results are given in Tables 4 and 5. The recovery rate of the free and total formaldehyde determined by spiking with various

Table 2. Linearity of different concentrations of free formaldehyde detected using UPLC

Concentration (ppm)	Area
0	0
2	22863
5	24608
10	26123
25	30659
50	69402
100	137396
R^2	0.989

Table 3. Linearity of different concentrations of total formaldehyde detected using UPLC

Concentration (ppm)	Area
0	921290
2	947589
5	895679
10	1140112
25	1620265
50	3045874
100	5435421
R^2	0.997

concentrations ranged from 90 to 100% (Tables 4 and 5). The results are similar to earlier studies, which reported the accuracy of formaldehyde detection in fish by HPLC method as 91.2 -105.3% (Wahed *et al*., 2016); in squid as 83.1- 103 (Li *et al*., 2007) and in milk as 90-94% (Sebaei *et al*., 2018).

Limit of detection (LOD)

The standard linear graph was plotted using 0.5, 1, 2, 5, 10, 25, 50 and 100 ppm formaldehyde DNPH solution. The lowest concentration detected was 0.5 ppm against the blank; hence, it becomes the LOD. The homogenised fish sample was spiked with standard formaldehyde solutions (1, 2, 5, 10, 25, 50 and 100 ppm), and the formaldehyde concentration was determined using

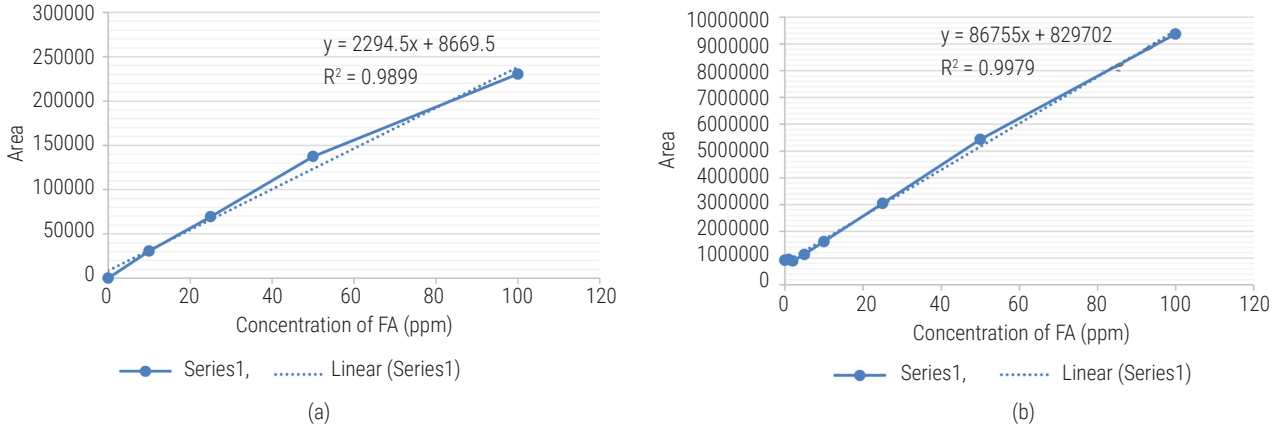

Fig. 3. Linearity of (a) free formaldehyde and (b) total formaldehyde detected using UPLC

Table 4. Determination of method accuracy for free formaldehyde by UPLC

Spiked concentration (ppm)	Area	Recovered concentration (ppm)	Recovery (%)
10	31915	11.61	116.14
25	69874	25.42	101.71
50	124799	45.41	90.83

Table 5. Determination of method accuracy for total formaldehyde by UPLC

Spiked concentration (ppm)	Area	Recovered concentration (ppm)	Recovery (%)
10	1620265	13.29	132.98
25	2703620	22.19	88.76
50	5843484	47.96	95.92

UPLC. The lowest concentration detected was 2 ppm, which is the LOD of the method for the sample matrix. The LOD obtained was 0.5 ppm for formaldehyde solution, slightly higher than 0.39 ppm as reported by Wahed *et al*. (2016) and lower than 2 ppm reported by Tai-Sheng *et al*. (2013). The LOD was slightly higher (2 ppm) for the spiked sample due to matrix interference. Patyra and Kwiatek (2020) reported the limits of detection and quantification for the formaldehyde in feed and silage as 1.6 to 2.7 and 2.6 to 5.2 mg kg^{-1}, respectively. Similar to our results, Wahed *et al*. (2016) reported an LOD of 1.7 ppm in matrix matched sample.

In the present investigtion, a chromatographic method using UPLC was developed. A modified rapid extraction procedure involving derivatisation with 2-4 dinitrophenyl hydrazine at 60^0C was also developed and standardised. The UPLC method developed has a run time of only 3 min. A separate DNPH peak was observed at 0.355 min followed by formaldehyde peak at 0.561 min. The method was validated, and the recovery of free and bound formaldehyde was 90.83 to 116.14% and 87.76 to 132.96%, respectively. The correlation coefficient (R^2) of the linear curve obtained for free and bound FA was 0.98 and 0.99 respectively. The method has good repeatability with a CV (%) of 5.95 andis very sensitive, as the limit of detection (LOD) was 0.5 ppm, which was less than the MRL prescribed by FSSAI. The UPLC method offers several advantages being faster, simpler and more sensitive compared to the spectrophotometric method.

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