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Research Article

Development of spectrophotometric method for quantitative estimation of formaldehyde in milk

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Abstract

Formaldehyde is only a permitted preservative in milk for analytical purposes. However, its presence in milk is unlawful. Many tests are reported for its qualitative detection in milk. The Leach test is one of them. Due to a suitable reaction mechanism, it is viewed as a prospective test for the development of a spectrophotometric method for the quantification of formaldehyde in milk. Formaldehyde was added to pure milk. Leach test was performed, and λ_{max} of colour developed was determined in the visible region. Similarly, a standard curve of formaldehyde-spiked milk serum was prepared using the Leach test. This curve was then used to estimate unknown formaldehyde content in milk. The mean recovery of estimated formaldehyde in milk was calculated to be 93.76%. The recovery factor was calculated by taking the ratio of the amount of spiked formaldehyde to that obtained from the standard curve as 1.06. To validate the method, the formaldehyde content obtained using the developed method was compared with that actually added in milk. The formaldehyde was mixed in the range of 0.008 to 0.0272 g (average 0.0173 g) per 100 mL of milk. The amount of formaldehyde estimated in milk by the developed method ranged from 0.0075 to 0.0277 g (average 0.0170 g) per 100 mL of milk. The performance of the developed method was validated and found to be statistically non-significant, suggesting appropriateness of the method. The developed method is simple, rapid, and suitable for routine analysis of milk compared to sophisticated methods involving costly cumbersome instruments.

Keywords: Formaldehyde, Milk, Preservative, Quantification, Spectroscopy

Highlights

- Leach test, a qualitative test, was used to develop a spectrophotometric method for the quantitative determination of formaldehyde in milk.
- Absorbance maxima (λ_{max}) of the developed colour in Leach test was identified in the visible region using a spectrophotometer.
- A standard curve was prepared by taking known concentrations of formaldehyde in milk and developing colour in milk serum using Leach test procedure.
- Using this standard curve, an unknown concentration of formaldehyde in milk samples was estimated.
- Recovery of the estimated formaldehyde in milk was also determined, and the method was validated for its performance by a true (known) sample approach.

INTRODUCTION

The tropical climate, poor hygienic conditions during milking and the dearth of adequate cold chain facilities are major factors for the spoilage of milk in India. Proliferation of microorganisms in milk causes development of acidity and eventually loses its stability during thermal processing. Formaldehyde (37-40% w/v aqueous solution), a harmful chemical preservative, is added to delay the spoilage of milk by means of ceasing the microbial activity. When added in small amounts, it does not alter the organoleptic behaviour of milk. It is added in the proportion of one part of formaldehyde to 5,000 parts of milk. Being a hazardous chemical, it has serious health implications if ingested. In spite of its preservative effect, the addition of formaldehyde in milk meant for human consumption is unlawful and is prohibited. It also affects nutritive quality of milk products as it binds with proteins.

In order to thwart its addition in milk, several qualitative methods viz. Leach test (Sharma *et al*., 2012), Hehner test (Draaiyer *et al*., 2009), Chromotropic acid test (Sharma *et al*., 2012), etc. have been developed to indicate the presence of formaldehyde in milk. Numerous methods have also been devised for quantitative determination of formaldehyde in milk. Wahed *et al*. (2016) determined formaldehyde in food and feed by an

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in-house validated HPLC method. de Freitas Rezende *et al*. (2017) implemented and validated HPLC with UV detection (HPLC-UV) for formaldehyde determination in bovine milk. Sebaei *et al*. (2018) determined formaldehyde by HPLC with stable precolumn derivatization in Egyptian dairy products. Patyra and Kwiatek (2020) developed an HPLC-DAD method for the determination of formaldehyde in animal feed and silage. Fappiano *et al*. (2022) reviewed several sensor-based methods for the analysis of formaldehyde in agri-food chains and compared them with vis-a-vis conventional analytical approaches. However, these methods involved sophisticated instruments, cumbersome sample preparation procedures, use of expensive reagents, requirement of trained manpower for sample analysis, etc. Instrumental methods may necessitate isolation, purification and concentration of the analyte before subjecting it to analysis. Thus, these methods necessitate highly skilled manpower. Moreover, dairy personnel demand simple quantitative tests for preservatives in milk for routine analysis. Therefore, simple quantitative tests, which can be used for a large number of samples, are needed for the hour. Therefore, using the reaction mechanism in the Leach test, the study was contemplated to develop a spectrophotometric method to estimate the quantity of added formaldehyde in milk.

MATERIALS AND METHODS

Milk samples: Fresh and pure cow and buffalo milk were brought from the Livestock Research Station and Reproductive Biology Research Unit, Anand Agricultural University, Anand, Gujarat, respectively. To prepare mixed milk samples, equal volumes of cow and buffalo milk were mixed. Forty milk samples were analyzed during the research work.

Glassware and chemicals: Cleaned and dried glassware were used during the study. Analytical grade (AR) chemicals (Qualigens) were used in the study.

Reagents used in the test: Formalin (40%, w/v), ferric chloride (Qualigens Fine Chemicals), concentrated hydrochloric acid (S. D. Fine- Chem. Ltd.).

Commercially available formaldehyde (37-40%, w/v) solution was used for the preparation of formaldehyde-added milk samples. The calculation for quantity of formaldehyde to be added to the milk sample is given below (Taking the concentration of formaldehyde as 37%):

100 mL formalin contains 37 g formaldehyde

10 mL formalin contains = 3.7 g

10 mL formalin contains = 3700 mg

1 mL formalin contains = 370 mg

0.1 mL formalin contains = 37 mg

This means to get 37 mg formaldehyde, 0.1 mL formalin is required

$$\text{Then, 10 mg formaldehyde requires} = \frac{0.1}{37} \times 10 = 0.027$$

$$= 0.3 \text{ mL (approx.) formalin}$$

Hydrochloric acid containing ferric chloride: It was prepared by taking 1 mL of 10% ferric chloride solution in a 500 mL volumetric flask, and the volume was made using concentrated hydrochloric acid.

Instrument used in the study: Spectrophotometer (Micro controller based UV-Vis spectrophotometer CL- 1320).

Procedure for Leach test (qualitative test for formaldehyde detection in milk): Ten millilires of milk was mixed with an equal volume of concentrated hydrochloric acid containing 1 mL of ferric chloride solution to 500 mL of acid. The sample is heated directly over a gas flame to 80 to 90°C for about 5 min. Ferric chloride causes the oxidation of protein, which, in turn, reacts with formaldehyde, forming the violet-coloured complex. A violet colour indicates the presence of formaldehyde.

Quantitative determination of formaldehyde in milk: The following procedures were carried out to determine the formaldehyde in milk.

Absorption maxima of colour developed in Leach test for formaldehyde: To get the spectra of colour developed in the Leach test, a known quantity of formaldehyde was added to milk (100 mL) to make a spiked sample. The milk was heated to denature the whey proteins and was coagulated by adding 60 mL of concentrated hydrochloric acid containing ferric chloride. The contents were then filtered through Whatman filter paper No. 1. The filtrate was heated in a boiling water bath for 1 min to develop the colour and scanned for absorption maxima in the visible region using a Micro Controller UV-Vis Spectrophotometer (CL-1320).

Preparation of standard curve for formaldehyde: In a beaker, 100 mL milk was taken, and 0.03 g of formaldehyde was added to it and mixed well. The content was heated in a boiling water bath for 10 min and cooled to room temperature.

Sixty millilitres of concentrated hydrochloric acid

containing ferric chloride was added to the above sample. The content was mixed thoroughly and filtered through the Whatman filter paper grade 1. Simultaneously, the same procedure was followed for pure milk sample (Control).

Filtrates of treated samples (mixed with formaldehyde) and control (without formaldehyde) were collected in clean and dry conical flasks.

For the preparation of the standard curve, different volumes of filtrate (0, 1, 2, 4, 6, 8, 10 mL) of formaldehyde were taken in test tubes so as to get the concentrations of 0, 0.187, 0.375, 0.750, 1.125, 1.500, 1.875 mg formaldehyde per test tube. The total volume was made up to 10 mL in each test tube with control filtrate.

The content was heated in boiling water bath for 1 min to develop violet colour. The content was cooled immediately in a chilled water bath.

Absorbance was measured at 545 nm using 10 mL blank as filtrate obtained from pure milk with concentrated hydrochloric acid containing ferric chloride with the help of a spectrophotometer.

A standard curve was plotted using absorbance and formaldehyde concentration (mg).

Estimation of spiked formaldehyde in milk samples: Milk samples were spiked with formaldehyde by adding 0.008, 0.0128, 0.016, 0.0224 and 0.0272 g formalin in 100 mL milk respectively. The content was heated in a boiling water bath for 10 min and cooled to room temperature. Sixty millilitres of concentrated hydrochloric acid containing ferric chloride was added to the samples. The content was mixed thoroughly and filtered through Whatman filter paper grade 1. Aliquots of 10 mL filtrate were collected in test tubes. The test tubes were heated in a boiling water bath for 1 min to develop a violet colour. Cooled immediately in the chilled water bath. The absorbance was measured at 545 nm against a blank using a spectrophotometer.

Calculations for formaldehyde content in milk samples: The concentration of formaldehyde (mg) in the sample was obtained corresponding to absorbance from the graph (standard curve).

Suppose, absorbance for the sample is X and the corresponding concentration from the standard curve for formaldehyde is Y mg. Then, 10 mL filtrate from the sample contains Y mg formaldehyde

160 mL (100 mL milk + 60 mL reagent) filtrate has

$\frac{Y}{10}$ x 160 mg formaldehyde

i.e. 100 mL milk contains $\frac{Y}{10}$ x 160 mg formaldehyde

= Y x 16 mg formaldehyde

= Y x 0.016 g formaldehyde

Statistical analysis of the data: Appropriate statistical tools/descriptive statistical tests were used to analyze the data obtained in this study. A completely randomized design (CRD) and t-test were used for the analysis of the data.

RESULTS

Qualitative tests known as the Hehner test (Vishweshwar and Krishnaiah, 2005; Draaiyer *et al*., 2009; Kamthania *et al*., 2014) and the Leach test (Vishweshwar and Krishnaiah, 2005; Farrington and Woll, 1910; Sharma *et al*., 2012) have been reported for qualitative determination of added formaldehyde in milk.

In the Leach test, 10 mL of milk is mixed with an equal volume of concentrated hydrochloric acid containing 1 mL of ferric chloride solution to 500 mL of acid. The sample is heated directly over a gas flame to 80 to 90°C for about 5 min. Ferric chloride causes the oxidation of protein, which, in turn, reacts with formaldehyde, forming the violet-coloured complex. A violet colour indicates the presence of formaldehyde. The formaldehyde reaction is a general one for proteins, and it depends on the presence of the tryptophan group in the protein molecule. The intensity of the reaction with different proteins varies in direct proportion to the amount of tryptophan present in the protein molecule (Rosenheim, 1907).

Reaction mechanism in the above test is made use of to develop a spectrophotometric method to estimate amount of formaldehyde in milk. The study was carried out in phases: a) finding the maxima of the coloured complex formed during the qualitative test, b) preparing the standard curve, c) estimating recovery using the method, and d) validating the developed method.

Absorption maxima of colour developed in Leach test for formaldehyde: To get the spectra of colour developed in the Leach test, the test procedure mentioned in the 'quantitative determination of formaldehyde in milk' part in the Materials and Methods section was followed. The spectrum obtained for the same is presented in (Fig. 1). The highest peak of absorbance was observed at 545 nm. Hence, this wavelength was used to take absorbance during further course of study.

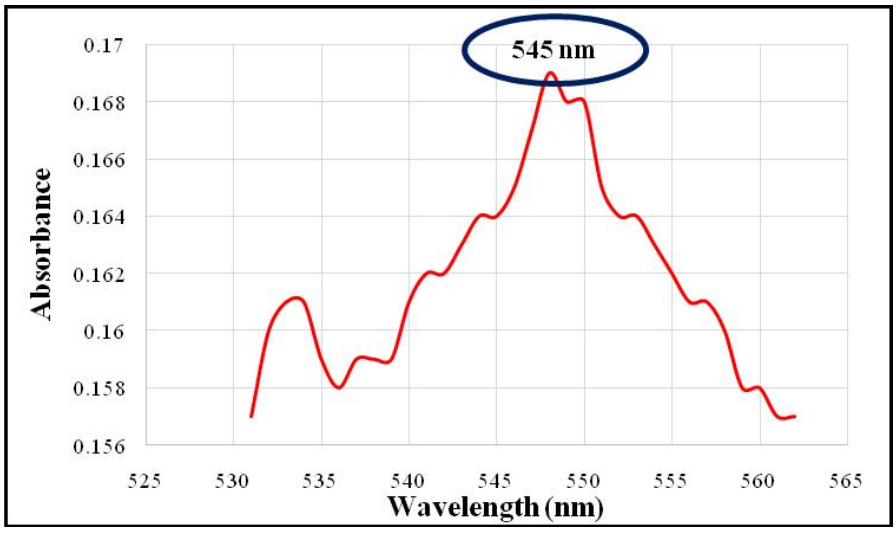

Fig. 1. Absorption maxima (λ_{max}) of coloured complex formed in Leach test

Preparation of standard curve for spiked formaldehyde in milk: During the preparation of the standard curve, 0.03 g formalin was mixed in 100 mL milk, and the filtrate was prepared according to the procedure described in 'quantitative determination of formaldehyde in milk' part in Materials and Methods section. Aliquots (0, 1, 2, 4, 6, 8, 10 mL) of the filtrate were taken in different test tubes so as to get corresponding formaldehyde concentrations of 0, 0.187, 0.375, 0.750, 1.125, 1.500, 1.875 mg. The total volume in the test tubes was made to 10 mL with the filtrate obtained from the control milk sample (no formaldehyde was mixed). Colour was developed using the test procedure. The intensity of the developed violet colour gradually increased with the increase in concentration of the formaldehyde in milk (Fig. 2). The absorbance was measured at 545 nm wavelength. The graph (standard curve) of absorbance versus concentration of formaldehyde was plotted (Fig. 3). Three replications were conducted, and average values of the same were calculated (Table 1) as shown in the Materials and Methods section.

The absorbance of the developed colour in the test reaction was linearly increasing with the increase in the concentration of the formaldehyde mixed in the milk, which concurred with the corresponding increase in colour intensity (Table 1 and Fig. 3). The slope ($R^2 = 0.984$) indicated that the regression line fits very well in the data.

Table 1. Preparation of standard curve for spiked formaldehyde in milk

Sl. No.	Formaldehyde (mg/10 mL filtrate) *	Absorbance (545 nm)
1	0.000	0.000
2	0.187	0.060
3	0.375	0.105
4	0.750	0.190
5	1.125	0.265
6	1.500	0.353
7	1.875	0.389

*The value is the average of three replications

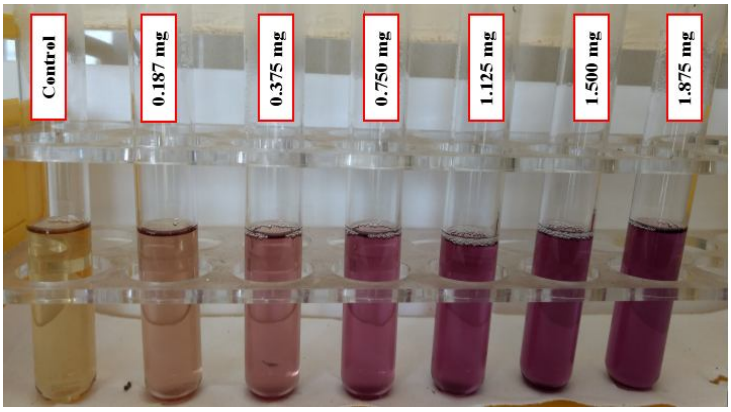

Fig. 2. Intensity of violet colour with varying amounts of formaldehyde in Leach test

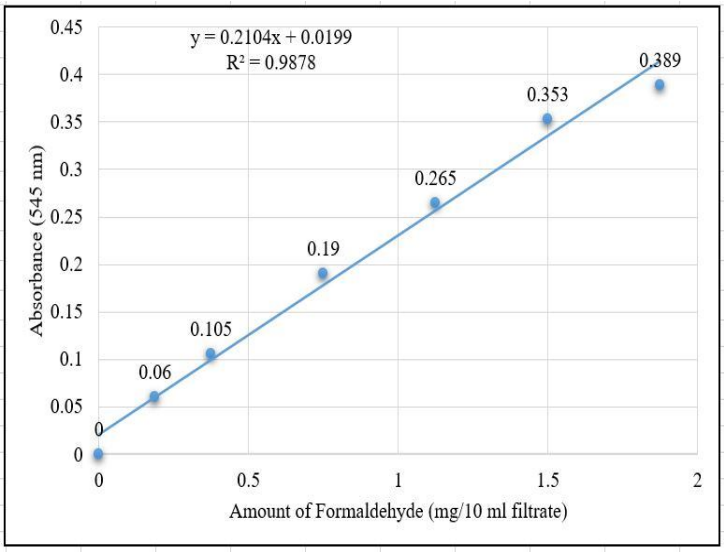

Fig. 3. Standard curve for formaldehyde in milk

R^2 value of 0.984 is suggestive of goodness of fit of a standard curve.

Recovery of estimated formaldehyde in milk: Experiments were conducted to calculate the amount of formaldehyde obtained from the standard curve after mixing a known amount of formaldehyde in the milk. The formaldehyde was mixed in the range of 0.008 to 0.0272 g per 100 mL of milk. Different formaldehyde concentrations were tried to cover the entire range of the standard curve. The amount of formaldehyde in each case was estimated from the standard curve. The per cent recovery of formaldehyde obtained in the experiment was derived from the amount of formaldehyde expected in the 10 mL of filtrate and the amount of formaldehyde estimated in the 10 mL of filtrate from the standard curve. Three replications were considered. The recovery of formaldehyde in the experiments varied from 89.20 to 99.30 per cent, with an average of 93.76 per cent (Table 2). The recovery factor was calculated taking 100 as a numerator with recovery as a denominator (i.e.100/93.76). The recovery factor for the formaldehyde in this study was found to be 1.06. Henceforth, to estimate the actual amount of formaldehyde mixed in the milk sample, the results obtained through the standard curve from the experiment would be multiplied by the recovery factor.

Table 2. Estimation of recovery of formaldehyde in the experiment

Replication	Amount of formaldehyde expected (mg/10 mL filtrate)	Amount of formaldehyde estimated (mg/10 mL filtrate)	Recovery (per cent)
R1	0.5	0.475	95.00
	0.8	0.765	95.62
	1.0	0.941	94.10
	1.4	1.340	95.71
	1.7	1.640	96.47
R2	0.5	0.485	97.00
	0.8	0.746	93.25
	1.0	0.993	99.30
	1.4	1.298	92.71
	1.7	1.531	90.05
R3	0.5	0.446	89.20
	0.8	0.722	90.25
	1.0	0.932	93.20
	1.4	1.274	91.00
	1.7	1.592	93.64
Average		93.76	

Modi *et al*. (2018) calculated the recovery factor for added sodium hydroxide estimation in milk to be 1.049 using the Rosolic acid test during the development of a spectrophotometric method for quantification of sodium hydroxide in milk. Similarly, Desai *et al*. (2018) calculated the recovery factor for added starch estimation in milk using the Iodine test to be 1.046 during the development of a spectrophotometric method for quantification of starch in milk.

Validation of developed method: After obtaining the recovery factor, the amount of formaldehyde (g) derived from the standard curve was multiplied by the recovery factor to get the amount of formaldehyde mixed in 100 mL of milk in this experiment. The data obtained for the amount of formaldehyde actually mixed in the milk and the corresponding amount of formaldehyde practically estimated in the experiment, along with their statistical analysis, are presented in Table 3.

Table 3. Amount of formaldehyde mixed and estimated in 100 mL of milk

Sl. No.	Formaldehyde from std. curve (A) (g/100 mL milk)	Formaldehyde estimated (A x 1.06*) (g/100 mL milk)	Formaldehyde mixed (g/100 mL milk)
1	0.0076	0.0080	0.0080
2	0.0122	0.0129	0.0128
3	0.0150	0.0159	0.0160
4	0.0210	0.0222	0.0224
5	0.0262	0.0277	0.0272
6	0.0077	0.0080	0.0080
7	0.0120	0.0127	0.0128
8	0.0160	0.0160	0.0160
9	0.0210	0.0223	0.0224

Cont. Table 3.

Table 3., Cont. ...

Sl. No.	Formaldehyde from std. curve (A) (g/100 mL milk)	Formaldehyde estimated (A x 1.06*) (g/100 mL milk)	Formaldehyde mixed (g/100 mL milk)
10	0.0245	0.0259	0.0272
11	0.0071	0.0075	0.0080
12	0.0115	0.0122	0.0128
13	0.0150	0.0159	0.0160
14	0.0203	0.0215	0.0224
15	0.0254	0.0269	0.0272
Average	0.0161	0.0170	0.0173
t Stat		0.1124	
P(T<=t) two-tail		0.9112	
Test		NS	

* Recovery factor, NS = Non-significant

The formaldehyde was mixed in milk in the range of 0.008 to 0.0272 g per 100 mL of milk, with an average of 0.0173 g per 100 mL of milk. The amount of formaldehyde estimated in the milk by the method developed in this study ranged from 0.0075 to 0.0277 g per 100 mL of milk, with an average of 0.0170 g per 100 mL of milk. The comparison between the actual amount of formaldehyde mixed per 100 mL milk and the amount of formaldehyde estimated per 100 mL milk by the method of the study revealed that the difference between the two was statistically non-significant.

DISCUSSION

For quantitative estimation of added formaldehyde in milk, the Leach test was used. In this study, the Leach test was used to find out the absorption maxima (λ_{max}) of the violet colour developed in the test. Using this λ_{max}, the standard curve is prepared using a known quantity of formaldehyde in milk. The standard curve is used to quantify the spiked formaldehyde in the milk and its recovery during estimation. Finally, the method was validated.

Spectral characteristics developed colour due to formaldehyde in milk: Formaldehyde was added to 100 mL milk, so as to get spectra of developed colour due to formaldehyde in milk. The milk was heated to denature the whey proteins and was coagulated by adding 60 mL of concentrated hydrochloric acid containing ferric chloride. Then filtered through Whatman filter paper No 1. The filtrate was heated in a boiling water bath for 1 min to develop the colour, and the same was scanned for absorption maxima in the visible region using a Micro Controller UV-Vis Spectrophotometer (CL-1320). The spectrum is presented in (Fig. 1). The highest peak observed was at 545 nm wavelength and henceforth selected for further study. Modi *et al*. (2018), in their study of quantification of sodium hydroxide in milk, identified absorbance maxima as 554 nm for the developed colour by Rosolic acid test. Similarly, Desai *et al*. (2018), in their study of the quantification of starch in milk, identified absorbance maxima as 549 nm of the developed colour by the Iodine test.

Preparation of standard curve for spiked formaldehyde in milk: In the second phase, standard curve was prepared. For the standard curve, 0.03 g formalin was mixed in 100 mL milk, and the filtrate was prepared according to the procedure described in the Materials and Methods section. Different aliquots (0, 1, 2, 4, 6, 8, 10 mL of filtrate from the sample mixed with formaldehyde) were taken in test tubes so as to get corresponding concentrations of 0, 0.187, 0.375, 0.750, 1.125, 1.500, 1.875 mg of formaldehyde in a test tube. The volume in a test tube was made to 10 mL with the filtrate of the control milk sample, wherein no formaldehyde was mixed. Colour was developed. The intensity of the violet colour formed gradually increased with the increase in concentration of the formaldehyde in milk (Fig. 2).

The intensity of the colour was measured by taking absorbance at 545 nm. Three replications were conducted, and average values of the same were calculated as mentioned in the Materials and Methods section (Table 1). The graph (standard curve) of absorbance versus concentration of formaldehyde was plotted (Fig. 3).

The absorbance values of the reaction mixture were linearly increasing with the increase in concentration of the formaldehyde mixed in the milk and coinciding very well with the increasing intensity of the colour (Table 1 and Fig. 3). The slope ($R^2 = 0.984$) indicated that the regression line was very well fit in the data.

Recovery of estimated formaldehyde in milk: The average recovery of 93.76 per cent of formaldehyde was obtained in the experiments (Table 2). The recovery factor of 1.06 was calculated by taking 100 as a numerator with recovery as a denominator (i.e.100/93.76). The amount of formaldehyde recovered in the filtrate was lower than the amount of formaldehyde mixed in the milk. This might be attributed to some losses of formaldehyde during preparation of the filtrate. Some formaldehyde might be retained in the coagulum owing to its interaction with casein. Some formaldehyde might be retained in the serum portion of the coagulum as moisture. Similar observations with lower recoveries in regard to sodium hydroxide and starch in milk were reported by Modi *et al*. (2018) and Desai *et al*. (2018), respectively.

Validation of method: After developing the recovery factor, the amount of formaldehyde (g) derived from the standard curve was multiplied by the recovery factor to get the amount of formaldehyde mixed in 100 mL of milk in this experiment. The data obtained for the amount of formaldehyde actually mixed in the milk and the corresponding amount of formaldehyde practically estimated in the experiment, along with their statistical analysis, are presented in Table 3. The formaldehyde was mixed in milk in range of 0.008 to 0.0272 g per 100 mL of milk, with an average of 0.0173 g per 100 mL of milk. The amount of formaldehyde estimated in the milk by the method developed in this study ranged from 0.0075 to 0.0277 g per 100 mL of milk, with an average of 0.0170 g per 100 mL of milk. The statistical comparison between the actual amount of formaldehyde mixed and the estimated amount of formaldehyde per 100 mL milk by the developed method in this study revealed a non-significant difference between the two estimations. The results suggested that the method worked out in the study was suitable for practical purposes. Similar kind of work had been also carried out by other researchers. Modi *et al*. (2018) and Desai *et al*. (2018) developed a spectrophotometric method for quantification of sodium hydroxide and starch in milk, respectively.

The Leach test was used for the quantification of formaldehyde in milk using a spectrophotometer. Absorption maxima of the violet colour developed in the Leach test was identified in the visible region as 545 nm. Then standard curve was constructed by developing the colour by adding formaldehyde at the rate of 0. 0.187, 0.375, 0.750, 1.125, 1.500 and 1.875 mg per 10 mL milk serum. Milk samples containing known amount of formaldehyde were subjected to the Leach test and colour was developed. Using the standard curve, content of formaldehyde in the milk samples were calculated. The recovery of added formaldehyde in milk worked out to be 93.76%. The recovery factor was calculated taking 100 as a numerator with recovery as a denominator (i.e.100/93.76) and was found to be 1.06. The amount of formaldehyde obtained from the standard curve was converted into the amount of formaldehyde in 100 mL of milk by back calculation. The amount of formaldehyde thus obtained per 100 mL of milk was multiplied by the recovery factor to compensate for the losses of formaldehyde occurring during the procedure followed in the experiment. The method was validated by comparison of amount of formaldehyde obtained in milk by the developed method with actual amount of formaldehyde added in milk. The difference between these values was statistically non-significant. The developed method was found to be simple, relatively faster and suitable for routine analysis of milk.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contributions: ZSM: Involved in investigation, data generation, and preparing original draft; SCP: Engaged in conceptualization, data curation, and supervision; AKJ, AIS: Engaged in supervision and final editing of the manuscript.

Data availability statement: Data will be provided on the request to corresponding author.

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