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Quantitation and Risk Assessment of Chemical Adulterants in Milk Using UHPLC Coupled to Photodiode Array and Differential Refractive Index Detectors

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Abstract Milk can be adulterated illegitimately with formaldehyde, melamine, urea, and sugars for preservation, protein content enhancement, and taste improvement, respectively. In the present study, formaldehyde, melamine, urea, and sugars (fructose, glucose, lactose, sucrose) were analyzed using ultra-fast high performance liquid chromatography in 30 fresh and pasteurized milk samples. Satisfactory chromatographic retention and peak shape investigated and method has been validated by means of recovery experiments in samples spiked at two levels. Average recoveries ranged between 95–105 % with excellent precision; RSD lower than 5 % was achieved. Among the tested samples, formaldehyde was detected in all samples while seven and 20 samples were positive for melamine and sucrose, respectively. Monitoring of these adulterants in milk could help to reduce the human health hazards. Exposure to formaldehyde, melamine, and urea from consumption of milk was estimated, and these were less than 3.3, 1, and 700 μg/mL, respectively, which is an oral reference dose suggested by

the World Health Organization and US Food and Drug Administration.

Introduction

Milk is one of the most important foods in daily life providing rich nutrients which are produced by lacteal secretion from mammal's mammary glands. Verities of milk contains all essential ingredients although differ in odor, taste, and chemical composition. It is an exclusive healthy food which cannot be replaced by any other food required for maintenance of life of both adults and infants. Milk contains good quality carbohydrates, fat, protein, minerals, and vitamins in significant amount especially amino acids which are needed for proper growth of adults and infants (Afzal et al. 2011; Finete Vde et al. 2013; Awan et al. 2014). Sources of fresh milk are the cow, goat, buffalo, sheep, reindeer, and camel. Due to the lack of proper facilities, both the quality and quantity of fresh milk is not satisfactory regarding to the health of consumers. In populous or metro cities where there is a shortage of fresh milk, people prefer to use pasteurized or packed milk. The process of pasteurization was attributed to ultra-high temperature (UHT) which is applied as a safety measure to kill the pathogens (Miller et al. 2000). The pathetic conditions of delivering the fresh or raw milk are one of the causes of a variety of diseases. Fifteen renowned companies (national and multinational) are operative in Pakistan for manufacturing of pasteurized milk, but little is known whether these have any procedure for treatment of raw milk other UHT procedure. Usually, milk is adulterated to maintain the viscosity and

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thicknesses as well as carbohydrates, fat, protein, and the adulterants which are usually starch, rice flour, urea, skimmed milk, vegetable oil, glucose, whey powder, animal fat, melamine, and detergents. The simplest and oldest form of adulterant in milk is water to increase the volume for profitable commodity, but contaminated water has potential risk to human health regarding the waterborne diseases (Campos Motta et al. 2014; Singuluri and Sukumaran 2014). Most of the milk provision in Pakistan is of buffalo (62 % of total milk production) which is more liable to water adulteration than cow's milk because it has more fat content (Imran et al. 2008; Afzal et al. 2011; Mansour et al. 2012). Pond water is also added by some unscrupulous persons to increase the specific gravity of milk because it is a rich source of nitrates. Consumption of such water adulterated milk by humans may cause stomach disorder in elders and serious health hazards in infants and children where milk is their basic diet (Singh and Gandhi 2015). Refrigeration (ice used may be contaminated) of fresh milk is done to avoid spoiling, especially in the summer season during transportation and chemicals such as formalin, sodium carbonate/bicarbonate/hydroxide, and/or calcium hydroxide are added to enhance its shelf life. Cosmetic nature and foamy appearance of milk is maintained by the addition of some detergents; for whiteness and genuine appearance, potassium and calcium salts of thioglycolic acids are also added (Afzal et al. 2011; Soomro et al. 2014). The present work was conducted on fresh and pasteurized or packed milk samples for detection of adulterants in the second largest city (Lahore) of the country. Study of detection of adulteration is of great importance in daily life. With the help of this study, the natural composition of milk will be insured which is necessary for better growth and development of a community. In the present study, physical characteristics (pH, acidity, TDS, EC, and moisture) along with the chemical adulterants (formaldehyde, melamine, urea, and sugars) are investigated by ultra-fast liquid chromatography (UHPLC) coupled to photodiode array and differential refractive index detectors.

Materials and Methods

Instrumentation

HPLC Shimadzu Nexera X2 (Japan) equipped with LC20AD pump, SPD-M20A diode array, RIO10A RI detectors, DGU-20A5 degasser (online), and variable volume loop injection valve were used. The separations were achieved on Acquity BEH-C18 column (100×2.1 mm, 1.7 μm particle size) and Phenomenex

Luna NH_2 column (250×4.6 mm, 5.0 μm particle size). Shimadzu LC program, LabSolutions, analysis data system was used for the integration of peak areas.

Collection of Samples

Fifteen milk samples (pasteurized) were collected from local market shelves in the capital city Lahore (31° 33′ 16.58″ N, 74° 21′ 25.77″ E) Punjab-Pakistan. Each sample was of 250 mL in tetra packing, and none of sample exceeded the storage period of 15 days. Fresh milk samples (15 No's) were collected from dairy shops randomly. Three sites (Fig. 1) were selected for fresh milk sampling, i.e., Kasur-Lahore (Feroz pur road), Sheikhupura-Lahore (Sheikhupura road), and Raiwind-Lahore (Raiwind road) because from these three sides the fresh milk is provided to dairy shops. On these three roads, many industries like pharmaceutical, textile, leather, and chemicals are situated and these industries effluent their wastes along these roads. All the samples were stored at 4 °C and before analysis kept overnight at 25 ± 2 °C.

Physical Characteristics

pH was determined by multi-meter (Orion 5 star, Thermo scientific, UK) by direct immersing the electrode in milk sample. Twenty milliliters of milk was weighed and dissolved it in 75 ml of high purity water (0.01 μS/cm prepared by using purification system Millipore system by Milli-Q, USA). 0.05 M sodium hydroxide (Merck, Germany) was used for free acidity by plotting the neutralization curve; and pH was determined at equivalence point and acidity in milliequivalents per kilogram of milk was obtained as ten times the volume of sodium hydroxide. For lactone acidity, excess sodium hydroxide was added to milk solution and back titrated with 0.025 M sulphuric acid (Merck, Germany) for plotting of neutralization curve. Summation of both free and lactone acidity is equivalent to total acidity. TDS in parts per million and EC in milliSiemens per centimeter were measured by Orion 5 star multi-meter (Thermo scientific, UK). Twenty grams of milk was suspended in 75.0 mL of high purity water in a volumetric flask and makes the volume up to 100 mL with the same solvent. Two grams of milk in a china dish was placed in a furnace (Thermolyne™, Thermoscientific-UK) and commenced drying at temperature 105–110 °C. After 1 h, the dish was cooled at room temperature and moisture content was calculated as grams per 100 g of milk (AOAC 2000).

Fig. 1 Sampling sites
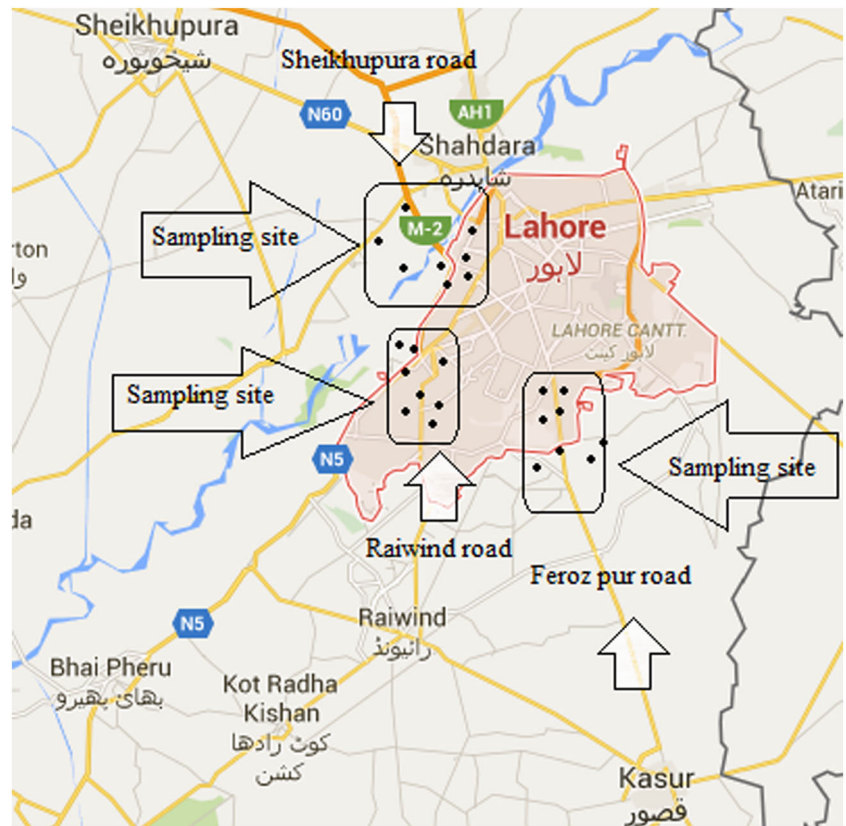
Chemical Adulterants Analysis

Formaldehyde and Melamine

Formaldehyde and melamine were determined by liquid phase extraction followed by HPLC-DAD by slight modification in earlier method reported (Kaminski et al. 1993; Deabes and El-Habib 2012). For formaldehyde, 2.0 mL solution of hexane (Sigma-Aldrich, USA) and 2, 4-dinitrophenyhydrazine (DPPH) from Merck, Germany, was added to 5.0 g of milk sample. Reaction mixture was allowed to stand for 30 min at room temperature. The solution was filtered and washed with hexane, and the solvent was evaporated. The solid matrix was dissolved in acetonitrile (Sigma-Aldrich, USA) for HPLC analysis. The mobile phase comprising of an acetonitrile to water ratio (50:50 v/v) set at a flow rate of 0.45 mL/min and injection volume of 3.0 μL was injected through a column maintained at 40 °C. The wavelength selected was 365 nm. For melamine, 5.0 g of milk sample was mixed with 20 mL of methanol (Sigma-Aldrich, USA) and sonicated for 10 min and then the matrix was centrifuged at 4500 rpm. The supernatant was mixed into a solution of methanol and water (4:1). HPLC conditions remain the same as mentioned in formaldehyde analysis except the λ_{max} is 220 nm and mobile phase is comprised of methanol and water (80:20 v/v). The same procedure was adopted for standard formaldehyde and melamine solutions.

Urea

One milliliter of milk was added to 0.5 mL of trichloroacetic acid (Sigma-Aldrich, USA). and mixture was centrifuge at 5000 rpm for 10 min at 4 °C. 0.75 mL of supernatant was diluted with 0.25 mL distilled water (Milli-Q system, USA). Then, 0.25 mL of acidic p-dimethylaminobenzaldehyde (used 20 % HCl) was added and stirred vigorously for 10 min at room temperature (Czauderna and Kowalczyk 2012). The same procedure was adopted for standard urea solutions. The mobile phase comprising of phosphate buffer of pH 7 (sodium phosphate dibasic and phosphoric acid) and methanol (70:30 v/v) set at flow a rate of 0.45 mL/min and injection volume of 3.0 μL was injected through column maintained at 25 °C. The wavelength was selected at 370 nm.

Sugars

Two milliliters of milk sample was added to 1.5 mL of distilled water, and the diluted milk was incubated at 60 °C for 10 min. Then, 2.5 mL each of potassium ferricyanide (0.5 M) and zinc acetate (0.5 M) and 1.0 mL of acetonitrile was added. The mixture was allowed to stand for 1 h at room temperature then centrifuged at 10,000 rpm for 10 min at 20 °C. The supernatant was filtered through a membrane filter (0.45 μm). The extract contains the sugars and was injected (20 μL) into HPLC system (Sharma et al. 2009). Mobile phase comprised of acetonitrile

and water (70:30 *v/v*) at a flow rate of 1.0 mL/min, and the temperature of column and RI cell was maintained at 40 °C.

Validation Studies

Linearity

The linearity of proposed method was determined over a concentration range of 0.25–8.0 μg/ml (0.25, 0.50, 1.0, 2.0, 4.0, 8.0 μg/mL) for formaldehyde and for melamine, 2.5–15.0 μg/mL (2.5, 5.0, 7.5, 10.0, 12.5, 15.0 μg/mL) for urea, 100–200 μg/mL (100, 120, 140, 160, 180, 200 μg/mL) for fructose, glucose, sucrose, and 5–10 mg/mL (5, 6, 7, 8, 9, 10 mg/mL) for lactose.

Accuracy and Precision

Accuracy and precision (repeatability and reproducibility) studies were performed by spiking the milk samples at two different levels (L1 and L2) for different days. Repeatability (intra-day precision) and reproducibility (inter-day precision) were determined on same day and three consecutive days, respectively. Spiked concentration for formaldehyde and melamine is 1.5 and 2.4 μg/mL, 7.5 and 12.5 μg/mL concentration spiked for urea, while 10 and 15 μg/mL concentration spiked for each of fructose, glucose, sucrose, and lactose. Five milliliters of milk sample was spiked for accuracy and precision studies, and all measurements were made in triplicate.

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) is the minimum amount of analyte in a sample detectable and larger than uncertainty associated with it, and the limit of quantitation (LOQ) is the amount quantitatively measured with suitable precision and accuracy. LOD and LOQ were determined by standard deviation of the response based on the slope of the calibration by six injections of six working standards each of formaldehyde, melamine, urea, fructose, glucose, sucrose, and lactose under the optimized chromatographic conditions, and calibration curve method (Qadir et al. 2015a, b; Ali et al. 2015; Ahmed et al. 2014) is used for calculation by the following equations:

$$LOD = yB + 3sB \text{ and } LOQ = yB + 10sB$$

Where, yB is intercepts of regression line and sB is standard deviation of intercepts of regression line.

Statistical Analysis

Statistical analysis was performed on SPSS version 15 (SPSS Inc., Chicago, IL, USA) for Windows; analysis

of variance (ANOVA) was performed for significant difference ($p < 0.05$) with post hoc multiple comparisons.

Results and Discussion

Physical Characteristics

pH of milk is correlated with non-fat solids and lactose contents, but it is not influenced by lactation number, month, and season of calving. Non-fat and fatty solids are accounted for total acidity of milk; lactic acid almost shares the 25 % acidity of milk (Ahmad et al. 2013). Total acidity and pH of tested fresh and pasteurized milk samples are presented in Table 1. The mean value of total acidity for fresh milk and pasteurized milk obtained 0.26 and 0.24 while the pH was 6.71 and 6.73, respectively. TDS and EC are interlinked with each other and important parameters to determine the physical characteristics of milk. The TDS and EC (Table 1) obtained was in the range of 10.21–12.63 and 1.3–5.5 for fresh milk and 10.4–12.6 and 1.9–5.8 for pasteurized milk, respectively, which is similar to earlier reported. The moisture content was found in the range of 80.4–86.6 and 75.9–85.4 for fresh and pasteurized milk, respectively.

Formaldehyde and Melamine

Parameter of linear regression equation (linear range, correlation coefficient) and detection limits (LOD and LOQ) for both the adulterants are presented in Table 2. The linear range of 0.25–8.0 μg/mL for both formaldehyde and melamine was selected, and LOD was found to be 0.012 and 0.051 μg/mL, respectively (Table 2). Both the analytes were successfully eluted at the retention time of 3.42 min and 4.21 min (Fig. 2). Accuracy of method was assessed by recovery studies. Known amount of formaldehyde (37 % Sigma-Aldrich, USA) and melamine (Sigma-Aldrich, USA) was spiked into 5.0 mL of milk sample matrix, and measurement was made in triplicate for 3 days. The mean recovery rates (Table 3) for 1.5 and 2.4 μg/mL spiked formaldehyde in 3 days were 99.5 and 100.7 %, respectively, while recovery rates for same spiked melamine were found 98.9 and 99.2 %, respectively. Precision of method was also determined by measuring the spiked concentration as already mentioned. Intra-day precision (Table 3) in terms of % RSD for two spiked concentration was 2.3 and 2.6 %, respectively, for formaldehyde and 1.7 and 1.8 % obtained for melamine while the results of inter-day precision (Table 3) were obtained as 2.9 and 3.2 %, respectively, for formaldehyde while 2.5 % obtained for melamine for both spiked levels. Formaldehyde (Table 4) was found in both fresh and pasteurized milk, in ranges between 0.27–3.12 μg/mL and 0.33–1.76 μg/mL, respectively. Due to the antiseptic nature of formaldehyde, it is added deliberately and illegally in raw milk to increase its shelf life. Formaldehyde content in

Table 1 Physical characteristics of fresh and pasteurized milk ($n = 15$)

pH		Acidity (%)		TDS (%)		EC (ms/cm)		Moisture (g/100 g)	
Fre. milk	Pas. milk	Fre. milk	Pas. milk	Fre. milk	Pas. milk	Fre. milk	Pas. milk	Fre. milk	Pas. milk
6.56 ± 0.02[a]	6.66 ± 0.01[i]	0.21 ± 0.01[a, f, l]	0.10 ± 0.01	10.46 ± 0.21[a]	10.46 ± 0.11[a]	3.90 ± 0.31[a]	3.76 ± 0.22	82.60 ± 0.23[a, k]	82.60 ± 0.43[a]
6.64 ± 0.04[b]	6.63 ± 0.03[b, e, g, j, o]	0.20 ± 0.02[b, a, f, l]	0.20 ± 0.01[a, b, f, l]	10.40 ± 0.12[b]	10.40 ± 0.22[b]	3.50 ± 0.32[b]	3.78 ± 0.11	80.40 ± 0.22[b, l]	81.54 ± 0.52
6.84 ± 0.03[c]	6.76 ± 0.01[d, k, l]	0.24 ± 0.04[m]	0.24 ± 0.03[c, m]	11.20 ± 0.11[c]	11.20 ± 0.13[c, m]	2.10 ± 0.13[c]	3.13 ± 0.21	85.50 ± 0.34[c, m]	75.89 ± 0.14
6.75 ± 0.02[d]	6.74 ± 0.02[d, l]	0.27 ± 0.01[d, n]	0.27 ± 0.02[d, n]	10.69 ± 0.09[d]	10.60 ± 0.09	5.50 ± 0.23[d]	5.67 ± 0.11	80.88 ± 0.43[d]	81.11 ± 0.21
6.64 ± 0.01[e, b]	6.84 ± 0.05[c, m]	0.29 ± 0.02[e, o]	0.29 ± 0.04[e, o]	11.62 ± 0.21[e]	11.60 ± 0.11[n]	1.28 ± 0.09[e]	2.13 ± 0.13	83.00 ± 0.56[e, o]	83.00 ± 0.21[e, o]
6.89 ± 0.05[f]	6.79 ± 0.01[h, n]	0.21 ± 0.03[f, a, b, l]	0.10 ± 0.01	12.63 ± 0.23[f]	12.60 ± 0.21	3.70 ± 0.14[f]	3.65 ± 0.23	81.96 ± 0.45[f]	81.43 ± 0.23[p]
6.64 ± 0.02[g, b, e]	6.64 ± 0.03[b, e, g, j, o]	0.17 ± 0.05[g]	0.10 ± 0.01	11.67 ± 0.11[g]	10.80 ± 0.11	1.40 ± 0.09[g]	2.33 ± 0.22	85.60 ± 0.67[g]	85.44 ± 0.43
6.78 ± 0.03[h]	6.56 ± 0.02[a]	0.40 ± 0.02[h]	0.40 ± 0.03[h, j]	12.23 ± 0.12[h]	12.40 ± 0.23	3.20 ± 0.11[h]	3.32 ± 0.11[p]	86.60 ± 0.42[h]	82.60 ± 0.53[k, a]
6.66 ± 0.05[i, b, e, g]	6.74 ± 0.04[d, k, l]	0.35 ± 0.02[i]	0.35 ± 0.02[i]	11.00 ± 0.11[i]	11.00 ± 0.14[i]	1.63 ± 0.11[i]	1.98 ± 0.11	84.78 ± 0.44[i]	84.21 ± 0.42[q]
6.63 ± 0.04[j, b, e, g]	6.84 ± 0.06[c, m]	0.40 ± 0.04[j, h]	0.40 ± 0.04[h, j]	10.21 ± 0.21[j]	10.00 ± 0.16	3.10 ± 0.23[j]	3.34 ± 0.32[p]	80.50 ± 0.54[j]	81.33 ± 0.42
6.76 ± 0.06[k, d]	6.75 ± 0.07[d, k, l]	0.18 ± 0.01[k, g]	0.10 ± 0.01	10.72 ± 0.11[k]	10.46 ± 0.18[a]	3.90 ± 0.24[k, a]	3.43 ± 0.22	82.60 ± 0.56[k, a]	82.34 ± 0.41[r]
6.74 ± 0.05[l, d]	6.74 ± 0.04[d, k, l]	0.20 ± 0.01[l, a, b, f]	0.20 ± 0.02[a, b, f, l]	10.43 ± 0.11[l]	10.40 ± 0.09[b]	3.50 ± 0.31[l, b]	3.55 ± 0.21	80.40 ± 0.43[l, b]	81.43 ± 0.21[p]
6.84 ± 0.06[m, c]	6.89 ± 0.06[f]	0.24 ± 0.03[m, c]	0.24 ± 0.01[c, m]	11.20 ± 0.09[m, c]	11.20 ± 0.21[c, m]	2.10 ± 0.11[m, c]	2.45 ± 0.21	85.50 ± 0.27[m, c]	79.76 ± 0.32
6.79 ± 0.07[n, h, j]	6.64 ± 0.05[b, e, g, i, j, o]	0.27 ± 0.02[n, d]	0.27 ± 0.02[d, n]	11.60 ± 0.11[n]	10.60 ± 0.11	5.50 ± 0.34[n, d]	5.78 ± 0.34	84.88 ± 0.76[n]	84.22 ± 0.22[q]
6.64 ± 0.06[o, b, g, e, i, j]	6.78 ± 0.04[h, n]	0.29 ± 0.01[o, e]	0.29 ± 0.03[o]	11.45 ± 0.09[o]	11.60 ± 0.13[n]	1.28 ± 0.05[o, e]	1.97 ± 0.11	83.00 ± 0.88[o, e]	82.34 ± 0.31[r]

All measurements in triplicate; mean ± SD; significantly different values are represented by different letters (a–r) using one way ANOVA

Fre. fresh, *Pas.* pasteurized

raw milk ranges between 1–3.3 μg/mL according to WHO (Sandhu et al. 1984; WHO 1989). One sample of fresh milk was quantified as borderline concentration of formaldehyde (3.12 μg/mL). Formaldehyde should not be added to milk although it preserves the milk for a long time at room temperature and also increase its heat stability. Even in small amount, formaldehyde is highly toxic and classified as carcinogenic. A maximum 0.2 mg/kg oral dose is suggested by Environmental Protection Agency (EPA) of the USA. Various illnesses such as vomiting, abdominal pain, diarrhea, shallow respiration, decrease in body temperature, memory loss, weak irregular pulse, insomnia, mood and balance alteration, unconsciousness, and blindness due to damage of the optical nerve can be caused by ingestion of formaldehyde in humans (USEPA 1999; Afzal et al. 2011). Data in Table 4 presented that only four samples of fresh milk (0.16, 0.21, 0.09, and 0.18 μg/mL) and three samples of pasteurized milk (0.19, 0.22, and 0.23 μg/mL) were positive for melamine contents. In recent

years, melamine became topic of discussion because hundreds of thousands of pets died due to melamine-contaminated feeds. There was a urinary stone outbreak in Chinese children who consumed melamine tainted milk. Melamine is illegally added to milk to increase protein contents. Due to its high nitrogen contents (66.6 %), the addition of 1 g melamine in 1 kg of milk increased up to 0.4 % protein contents. Melamine also contains reaction by products, viz, ammeline, ammelide, and cyanuric acid which are also toxic to humans (Tyan et al. 2009; Hau et al. 2009). Maximum residue limit (MRL) for melamine suggested by the European Union (EU) and Food and Drug Administration (FDA-USA) is 2.5 and 0.25 mg/kg, respectively, whereas the former stressed that infant formula should be completely free from it. Experts from WHO/FAO stated a safety margin for infant formula is 1 mg/kg. Melamine adulterated milk ingestion in humans may cause renal failure, urolithiasis, bladder cancer, and even

Table 2 Linear regression equation parameters for tested components

Components	Concentration (μg/mL)	r^2	Regression equation	LOD (μg/mL)	LOQ (μg/mL)
Formaldehyde	0.25, 0.50, 1.0, 2.0, 4.0, 8.0	0.9989	$y = 34594x - 523.31$	0.012	0.04
Melamine	0.25, 0.50, 1.0, 2.0, 4.0, 8.0	0.9956	$y = 8641.7x + 3258$	0.05	0.17
Urea	2.5, 5.0, 7.5, 10.0, 12.5, 15.0	0.9989	$y = 22501x - 39,779$	0.027	0.09
Fructose	100, 120, 140, 160, 180, 200	0.9935	$y = 756.46x + 4575.3$	0.026	0.09
Glucose	100, 120, 140, 160, 180, 200	0.9967	$y = 16938x + 5372.9$	0.031	0.10
Sucrose	100, 120, 140, 160, 180, 200	0.9935	$y = 741.33x + 4483.8$	0.026	0.90
[a]Lactose	5, 6, 7, 8, 9, 10	0.9927	$y = 67752x + 21,492$	0.36	1.2

[a] Concentration in mg/mL

Fig. 2 Chromatograms of formaldehyde and melamine
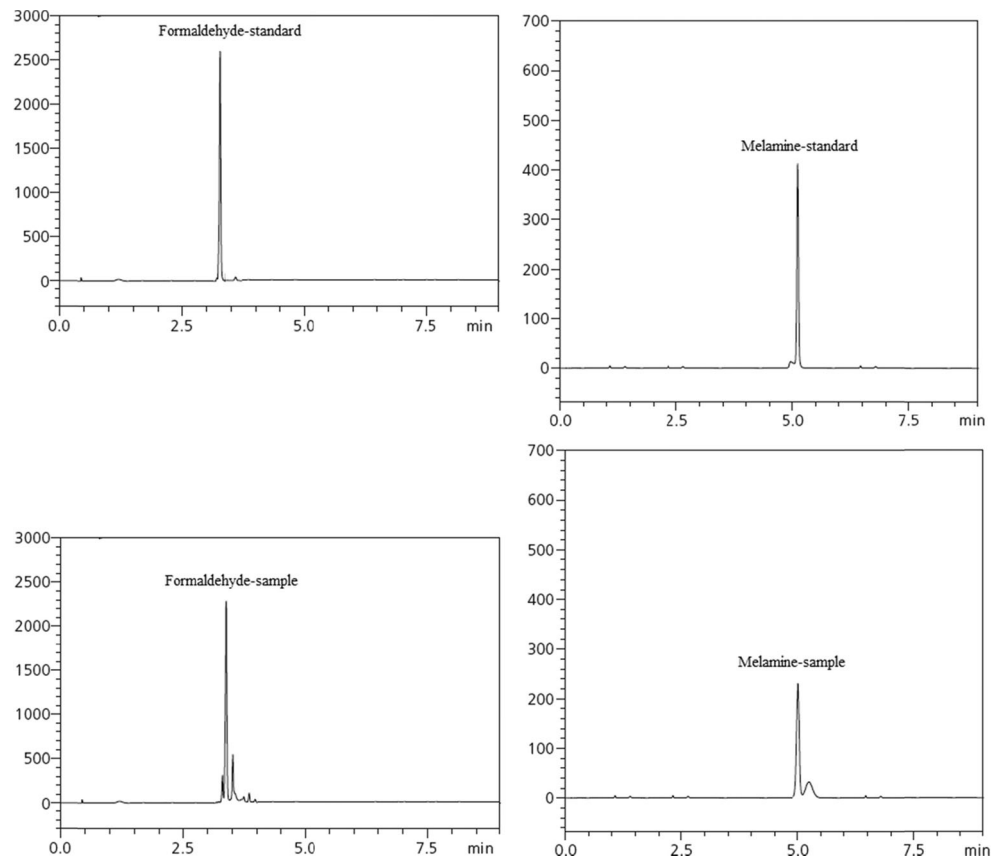
Table 3 Accuracy and precision studies

Parameters	Components						
	Formaldehyde	Melamine	Urea	Fructose	Glucose	Sucrose	Lactose
Amount added (µg/mL) L1 (L2)	1.5(2.4)	1.5(2.4)	7.5(12.5)	10(15)	10(15)	10(15)	10(15)
Accuracy (% recovery)							
Day 1 L1 (L2)	99.9(101.3)	98.5(99.3)	97.9(98.3)	100.2(98.7)	98.7(99.1)	99.4(99.6)	100.1(102.3)
Day 2 L1 (L2)	98.3(100.9)	99.2(100.1)	99.1(99.4)	99.1(99.7)	98.5(99.8)	98.7(100.8)	104.3(101.8)
Day 3 L1 (L2)	100.2(99.8)	99.1(98.2)	100.3(97.8)	100.6(99.8)	98.9(98.4)	99.5(100.1)	101.4(100.7)
Mean L1 (L2)	99.5(100.7)	98.9(99.2)	99.1(98.5)	99.9(99.4)	98.7(99.1)	99.2(100.2)	101.9(101.6)
Repeatability (% RSD): intra-day precision							
[a]Session 1 L1 (L2)	2.1(2.4)	1.7(1.6)	1.8(2.2)	2.6(3.2)	1.2(0.9)	2.4(2.1)	1.1(1.3)
Session 2 L1 (L2)	2.6(2.5)	1.9(2.1)	1.2(2.6)	3.2(3.3)	2.3(2.7)	1.8(1.9)	1.2(1.6)
Session 3 L1 (L2)	2.3(2.9)	1.6(1.6)	2.5(3.5)	3.5(4.1)	3.2(4.3)	2.1(2.1)	1.3(1.3)
Mean L1 (L2)	2.3(2.6)	1.7(1.8)	1.8(2.8)	3.1(3.5)	2.2(2.6)	2.4(2.1)	1.2(1.6)
Reproducibility (% RSD): inter-day precision							
Day 1 L1 (L2)	2.4(2.1)	1.9(1.7)	2.8(1.9)	3.1(3.3)	2.1(1.9)	2.8(2.4)	3.2(2.7)
Day 2 L1 (L2)	3.1(3.5)	2.9(2.2)	2.8(2.9)	3.1(3.6)	2.1(2.2)	3.1(2.9)	2.5(2.4)
Day 3 L1 (L2)	3.3(3.9)	2.6(3.6)	3.5(3.4)	3.8(4.3)	2.9(3.8)	2.8(3.3)	2.8(2.8)
Mean L1 (L2)	2.9(3.2)	2.5(2.5)	3.0(2.7)	3.3(3.7)	2.4(2.6)	2.9(2.9)	2.8(2.6)

[a] Interval of 4 h

L level

Table 4 Concentration of formaldehyde, melamine, and urea in fresh and pasteurized milk ($n = 15$)

Formaldehyde (μg/mL)		Melamine (μg/mL)		Urea (μg/mL)	
Fre. milk	Pas. milk	Fre. milk	Pas. milk	Fre. milk	Pas. milk
1.12 ± 0.11[a]	0.82 ± 0.09[f]	–	–	595.1 ± 1.12[a]	550.2 ± 1.11
1.64 ± 0.14[b]	1.04 ± 0.11	–	–	549.6 ± 1.23[b]	570.6 ± 1.52
0.96 ± 0.03[c]	1.06 ± 0.11	0.16 ± 0.05[a]	–	590.5 ± 0.97[c]	545.4 ± 1.53
0.45 ± 0.10[d]	0.33 ± 0.12	–	–	625.8 ± 1.26[d]	589.1 ± 1.82
0.94 ± 0.12[e]	0.72 ± 0.09	0.21 ± 0.03[b]	–	616.2 ± 1.65[e]	677.3 ± 1.72
0.81 ± 0.05[f]	0.76 ± 0.08	–	0.19 ± 0.05[d]	514.4 ± 2.1[f]	654.3 ± 1.54
0.88 ± 0.09[g]	1.12 ± 0.11[a]	–	–	505.3 ± 1.56[g]	632.2 ± 1.75
0.92 ± 0.06[h, k]	1.02 ± 0.09	–	0.22 ± 0.06[b]	540.8 ± 1.34[h]	625.3 ± 1.72
0.29 ± 0.03[i]	1.29 ± 0.07	0.09 ± 0.01[c]	–	631.4 ± 1.5[i]	678.1 ± 1.72
0.27 ± 0.04[j]	1.08 ± 0.12	–	0.23 ± 0.07[e]	577.5 ± 1.87[j]	571.7 ± 1.32
0.93 ± 0.08[k, h]	0.86 ± 0.06	–	–	557.1 ± 1.21[k]	520.7 ± 1.43
0.71 ± 0.02[l]	0.91 ± 0.08	–	–	573.3 ± 1.43[l]	528.8 ± 1.25
1.54 ± 0.15[m]	1.76 ± 0.13	0.18 ± 0.04	–	612.5 ± 1.54[m]	590.4 ± 1.32
2.29 ± 0.18[n]	1.55 ± 0.12[m]	–	–	598.8 ± 1.91[n]	610.7 ± 1.29
3.12 ± 0.21[o]	1.09 ± 0.11	–	–	595.1 ± 1.65[o]	550.4 ± 1.73

All measurements in triplicate; mean ± SD; significantly different values are represented by different letters (a–o) using one way ANOVA

Fre. fresh, *Pas.* pasteurized

death particularly for vulnerable individuals such as young children and infants (Jawaid et al. 2013). The concentrations found in both the tested samples are within the permissible level suggested by regulatory authorities (WHO and EU).

Urea

The linear range selected for determination of urea in milk was 2.5–15 μg/mL, and detection limit was found to be 0.027 μg/mL (Table 2). The urea was eluted at the retention time of 5.12 min (Fig. 3) and parameters of linear regression are presented in Table 2. For accuracy and precision studies, 7.5 and 12.5 μg of standard urea were spiked to milk samples separately; recovery rates (Table 3) and % RSD was determined. The mean recovery rates for 7.5 and 12.5 μg spiked urea in 3 days were obtained 99.1 and 98.5 %, respectively. Intra-day precision (Table 3) in terms of % RSD for two spiked concentration was 1.8 and 2.8 %, respectively, while the results of inter-day precision (Table 3) were obtained as 3.0 and 2.7 %, respectively. The urea contents in fresh milk obtained in ranges between 505–625 μg/mL while pasteurized milk contained in ranges between 520–678 μg/mL. Non-protein nitrogen source (55 %) of milk is urea. Milk is adulterated with urea because it is cheaper, easily available, and a rich source of nitrogen. It is added to milk for non-fat solid content standardization, increase whiteness, consistency, and shelf life. Typically, the contents of urea in milk are 180–400 mg/L; excess supply of crude protein in animal feeds contribute to milk urea concentration. The maximum acceptable limit for urea in milk is 700 mg/L (Kohn 2000; Trivedi et al. 2009). Higher level of urea in feeds influences the milk production as well as fertility. Urea exceeding the upper limit is harmful especially for pregnant women, children, and young girls where it hastens the puberty process; it also causes severe illnesses such as acidity, malfunctioning of the kidney, ulcer, osteoporosis, and cancer. At high temperature, boiling of milk containing urea decompose to acetic acid, ammonia, and carbonic acid and may be converted into biuret which causes strong irritation in the urinary tract and decrease in blood pressure of humans (Singh and Gandhi 2015). In the present study, none of the sample exceeded the upper limit of 700 mg/L.

Sugars

The amino column was used to elute the sugars because sugars can interact with amines; four sugars, viz. fructose, glucose, sucrose, and lactose were eluted in isocratic mode using RI detector at retention times of 4.91, 5.5, 8.1, and 9.2 min, respectively (Fig. 3). The linear ranges used for the four sugars were 100–200 μg/mL (fructose, glucose, sucrose) and 5–10 mg/mL (lactose). The detection limit for the four sugars 0.21 μg/mL (fructose), 0.31 μg/mL (glucose), 0.26 μg/mL (sucrose), and 3.6 μg/mL (lactose) was investigated (Table 3). For accuracy and precision studies, 10 and 15 μg of each sugar was spiked to milk samples separately and recovery rates and % RSD were determined. The mean recovery rates for 10 and 15 μg spiked standards in 3 days were obtained 99.9 and 99.4 % (fructose), 98.7 and 99.1 % (glucose), 101.9 and 101.6 % (lactose), and 99.2, 100.2 % (sucrose). Repeatability (Table 3) in terms of % RSD for two spiked concentration was 3.1 and 3.5 % for fructose, 2.2 and 2.6 % for glucose, 1.2 and 1.4 % for lactose, and 2.4 and 2.1 % for sucrose, respectively, while the results of reproducibility (Table 3) were obtained as 3.3 and 3.7 % for fructose, 2.4

Fig. 3 Chromatograms of urea and sugars
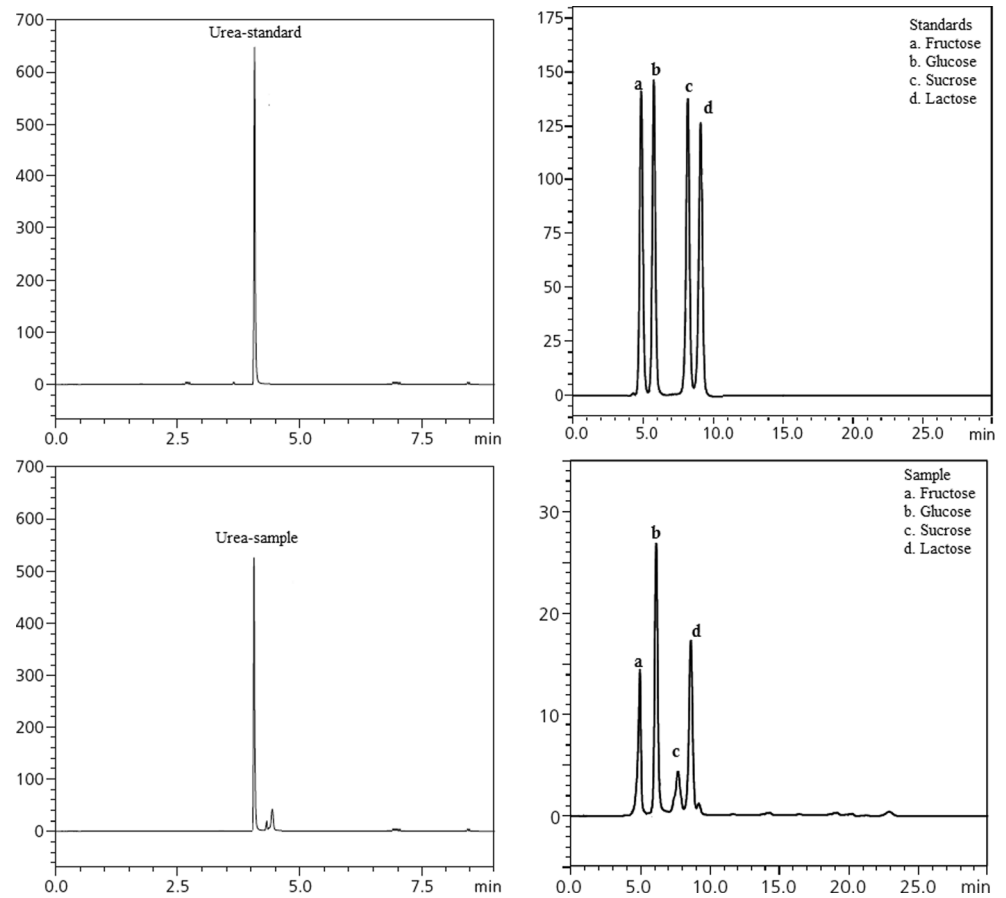
Table 5 Concentration of fructose, glucose, lactose, and sucrose in fresh and pasteurized milk ($n = 15$)

Fructose µg/mL		Glucose µg/mL		Lactose g/100 mL		Sucrose µg/mL	
Fre. milk	Pas. milk	Fre. milk	Pas. milk	Fre. milk	Pas. milk	Fre. milk	Pas. milk
216.11 ± 1.27[a]	186.12 ± 1.11	124.34 ± 1.22[a]	118.22 ± 1.43	3.8 ± 0.8[a]	3.3 ± 0.3	3.90 ± 0.12[a]	–
198.43 ± 1.43[b]	208.76 ± 1.43	102.78 ± 1.64[b]	124.43 ± 1.22[h]	3.5 ± 0.3[b, h]	3.2 ± 0.6[f, i, k]	3.50 ± 0.21[b]	–
186.34 ± 1.32[c]	175.11 ± 1.54	132.92 ± 1.23[c]	119.22 ± 1.34	2.9 ± 0.2[c, d]	2.9 ± 0.4[c, d]	–	2.10 ± 0.2[i]
178.54 ± 1.65[d]	183.43 ± 1.62	115.91 ± 1.54[d]	123.11 ± 1.34	2.9 ± 0.3[d, c]	2.7 ± 0.5	–	5.50 ± 0.34[k]
189.65 ± 1.76[e]	182.54 ± 1.82	109.23 ± 1.33[e]	131.34 ± 1.72	3.2 ± 0.5[e, f]	3.1 ± 0.4[e, f, i, k]	1.28 ± 0.91[c]	–
202.44 ± 1.65[f]	211.43 ± 1.92	111.43 ± 1.32[f]	124.43 ± 1.26[h]	3.2 ± 0.3[f, e]	3.2 ± 0.6[e, f, i, k]	3.70 ± 0.98[d]	3.70 ± 0.89[d]
206.43 ± 2.13[g]	209.76 ± 2.42	124.45 ± 1.22[g]	119.33 ± 1.65	4.6 ± 0.7[g]	3.7 ± 0.8[a]	–	1.40 ± 0.92
213.76 ± 1.87[h]	211.54 ± 1.82	124.43 ± 1.32[h]	117.32 ± 1.37	3.6 ± 0.2[h, b]	3.4 ± 0.9[b]	3.20 ± 0.21[e]	–
216.54 ± 1.54[i]	215.34 ± 1.92	109.76 ± 1.54[i]	109.21 ± 1.72	3.1 ± 0.2[i, e, f]	3.6 ± 0.3[h]	–	1.60 ± 0.96
201.93 ± 1.72[j]	194.65 ± 1.57[k]	119.54 ± 1.29[j]	121.44 ± 1.32	4.1 ± 0.5[j, l]	3.6 ± 0.3[h]	3.10 ± 0.91[f, e]	3.10 ± 0.22[e]
194.66 ± 1.32[k]	182.76 ± 1.34	111.44 ± 2.11[k, f]	119.65 ± 2.34	3.1 ± 0.5[k, e, f]	3.6 ± 0.5[h]	3.90 ± 0.21[h, a]	–
176.44 ± 1.43[l]	198.54 ± 1.82	104.98 ± 2.21[l]	114.76 ± 2.34	4.1 ± 0.7[l]	3.4 ± 0.6[b]	–	3.50 ± 0.43[b]
203.33 ± 1.72[m]	194.32 ± 1.83	114.67 ± 1.43[m]	116.87 ± 1.65	3.6 ± 0.3[m, h]	3.1 ± 0.3[e, f, i, k]	2.10 ± 0.92[i]	2.10 ± 0.92[i]
187.76 ± 2.13[n]	182.72 ± 2.38	124.98 ± 1.32[n]	123.23 ± 1.54	4.3 ± 0.2[n]	3.8 ± 0.9[a]	–	5.50 ± 1.01[k]
193.22 ± 2.11 [o]	190.23 ± 2.33	119.87 ± 1.22 [o]	120.54 ± 1.43	3.8 ± 0.4[o, a]	3.3 ± 0.7[b]	1.28 ± 0.82[j, c]	1.20 ± 0.43

All measurements in triplicate; mean ± SD; significantly different values are represented by different letters (a–o) using one way ANOVA

Fre. fresh, *Pas.* pasteurized

and 2.6 % for glucose, 2.8 and 2.6 % for lactose, and 2.9 % (both spiked levels) for sucrose, respectively. Data in Table 5 presents the sugar contents present in milk samples under investigation. The concentration of fructose ranged between 176–216 μg/mL for fresh milk and 175–215 μg/mL for pasteurized milk; glucose ranged between 102–132 μg/mL for fresh milk while 109–131 μg/mL for pasteurized milk samples. Sixty and 67 % of fresh and pasteurized milk samples were identified positive for sucrose contents; the maximum amount of sucrose was found to be 3.9 and 5.5 μg/mL, respectively. The lactose content in the range between 2.9–4.6 g/100 mL and 2.7–3.8 g/100 mL was found in fresh and pasteurized milk samples, respectively. These values of sugars obtained in tested samples are comparable with values reported earlier in literature (Afzal et al. 2011; Czauderna and Kowalczyk 2012). Still no regulatory body like WHO, EU, USFDA suggested the permissible level of these sugars reported in this study. But the milk is adulterated with glucose and sucrose to mask the addition of extraneous water in milk and improve the taste. Lactometer reading is also managed by the addition of these sugars by masking the addition of water. High contents of sucrose or glucose may contribute to diabetics in the elevation of blood sugar. It can be hazardous for peoples who already are diabetic or have a cardiac problem (Malik et al. 2006; Singh and Gandhi 2015).

Conclusion

In present study, chemical adulterants, viz, formaldehyde, melamine, urea, and sugars especially sucrose added to milk illegally were successfully analyzed by UHPLC coupled to diode array and differential refractive index detectors. Out of 30 samples, one sample has borderline content of formaldehyde of 3.12 μg/mL while remaining samples are in compliance with contents suggested by WHO and USFDA. Melamine and urea were also in the safe range given by EU and WHO/FAO. Natural occurring of urea in milk should be investigated. With knowledge that there is endogenous urea present in milk, a proper regulation should be put in place against use of urea for protein content enhancement. The monitoring of deliberately added formaldehyde for preservation, melamine and urea for protein contents enhancement, and sucrose for taste improvement by using proper analytical technique is very important.

Compliance with Ethical Standards

Informed Consent Not applicable.

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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