

DATA ARTICLE

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Screening of caffeine, preservatives and antioxidants in dairy products available in Bangladesh using an RP-HPLC method

Md. Shahadat Hossain, Md. Samiul Islam, Subrata Bhadra and Abu Shara Shamsur Rouf*

Abstract

Background: The aim of this current study was to investigate the presence and to determine the contents of caffeine, preservatives and antioxidants in dairy products available in Bangladesh, one of the alarming countries in terms of food security. As high as 41 marketed dairy products, of both locally and internationally manufactured, were collected from local markets of Dhaka, Bangladesh in mid-2013 and then analyzed using an RP-HPLC method to determine the contents of caffeine, benzoic acid, propylparaben, butylparaben, butylated hydroxyanisole and butylated hydroxytoluene. The evaluation was performed using a C₁₈ column (150 mm × 4.6 mm i.d., 5 μm particle size) with a gradient flow rate of acetonitrile and diluted sulfuric acid (0.002 M) as mobile phase from ratio 15:85 to 80:20 (%v/v) at a flow rate of 2.0 ml/min at 265 nm.

Results: The retention times of CF, BA, PP, BP, BHA and BHT were found about 3.6, 11.6, 13.0, 13.3, 13.6 and 16.7 min, respectively. Results revealed that BA was found in 17.1 % of the products within the concentration range of 11 ~ 2067 mg/L; among these, 42.9 % products exceeded the allowable limit of 300 mg/L set by JECFA. Moreover, 14.6 % products showed positive response to CF at a concentration of 10 ~ 18 mg/L, which was well below the tolerance limit (<200 mg/L) set by FDA. However, none of these investigated dairy products were found to contain any detectable amount of PP, BP, BHA or BHT.

Conclusion: Presence of excess amount of BA in dairy products, which are one of the most favorite healthy food items for all generations, can easily jeopardize public health sector. Moreover, CF was found in some CF-free products, which is also an alarming issue to be considered seriously to prevent the future occurrence of it.

Keywords: Dairy products, HPLC, Caffeine, Preservatives, Antioxidants, Food security

Background

People, especially children, consume substantial amount (30 to 150 kg/capita/year) of fresh milk and dairy products every day (FAO 2013a). Milk is a valuable nutritious food with a short shelf-life and requires careful handling as it is highly perishable being an excellent medium for the growth of microorganisms—particularly bacterial pathogens—that can cause spoilage and diseases amongst the consumers. Milk processing like mechanical refrigeration, pasteurization etc. allows the preservation of milk for days, weeks or even months and helps to reduce food-borne illness (FAO 2013b). Processing of dairy products gives

small-scale dairy producers higher cash incomes than selling raw milk, and offers better opportunities to reach regional and urban markets and helps to deal with seasonal fluctuations in milk supply. The transformation of raw milk into processed milk and products can benefit entire communities by generating off-farm jobs in milk collection, transportation, processing and marketing (FAO 2013b).

Bangladesh is nearly self-sufficient in food production, but unfortunately food security remains an elusive goal nowadays. Shortage of storage facilities, lack of academic education, frequent natural disasters, poverty, and lack of strict monitoring systems sometimes lead the crooked producers and businessmen not to apply the standard food processing procedures; rather frequently they add uncontrolled amount of different food preservatives and/or antioxidants in the food products, thus make the

* Correspondence: rouf321@yahoo.com

Institutional address: Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh

product adulterated. It is hard to believe that the percentage of these sorts of debased food products are increasing at an alarming rate and consumed by the common people across the country. Therefore, food-borne illness, intoxication and malnutrition are the common scenario in Bangladesh—more severely in the underdeveloped rural areas (Ali 2013; Rahman and Kabir 2012; Hossain et al. 2008). Numerous reports on adulteration of foods and food products are often published in the mainstream national newspapers as well as broadcasted in the television or radio that also inspired us to do in-depth research in this field. Even the National Food Safety Laboratory (NFSL) of our country found the presence of two harmful and banned pesticides—Aldrin and DDT in their investigated raw milk and milk products in 2014 (NFSL 2014). Beside this, in 2012, Chanda et al. found that all fifty milk samples collected from Barisal district of Bangladesh were adulterated with water and more interestingly, 10 % of those samples were adulterated with toxic formalin and 20 % with sodium bicarbonate. These types of abominable activities are increasing day by day due to lack of government and public awareness, greediness of businessmen, lack of regulations and standards, and finally lack of implementation of existing laws (Rahman and Kabir 2012).

Usually coliforms, lactic acid bacteria, yeasts, and molds are responsible for the spoilage of milk, but psychrotrophic organisms play the most dominant role. In addition, various bacteria of public health concern such as *Salmonella* spp., *Listeria monocytogenes*, *Camphylobacter jejuni*, *Yersinia enterocolitica*, pathogenic strains of *Escherichia coli* and enterotoxigenic strains of *Staphylococcus aureus* may also be found in milk and dairy products (Cempírková and Mikulová 2009; Varga 2007). These microorganisms and their metabolic end products are greatly

harmful for human body. So, suitable preservation techniques are to be applied to prevent microbial and chemical spoilage of dairy products. The most important means of controlling bacteria, yeasts and molds are application of heat, cold, chemicals, radiation etc. Usually food industries incorporate two or more preservation techniques to preserve their products (Potter and Hotchkiss 1999). Mechanical cooling, thermization, pasteurization and ultra-heat treatment are regularly applied to dairy products (Guizani 2007). However, according to WHO/FAO authorized “codex general standard for food additives” guideline published in 1995, our all investigated dairy products have no permission to apply chemical preservation technique using any preservatives and antioxidants, except flavored yogurt in which only benzoic acid (BA) (Fig. 1) can be used in a concentration of less than 300 mg/L (Codex 2015). There is no specific rule on the use of caffeine (CF) in dairy products (Nutri pro 2013), but it may be present in chocolate milk prepared with cocoa powder considering the fact that its concentration should be well below the maximum daily allowable level of it. Excess intake of CF causes headache, insomnia, agitation, tremor, hypertonicity, systemic hypokalemia, tinnitus, tachyarrhythmia, delirium, hyperventilation, hyperthermia, polyuria, dehydration, seizures, and coma (Simmons and Kidner 1998; Davies et al. 2012). On the other hand, BA may cause asthma, urticaria, metabolic acidosis, convulsions etc. if exceeds 5 mg/kg b.w. and it may also form benzene by reacting with ascorbic acid (usually present in yogurt) in the presence of transition-metal catalyst (Qi et al. 2008; Roye et al. 2009). Decline in serum testosterone level in male persons and hypersensitivity reaction may occur if the intake amount of propylparaben (PP) and butylparaben (BP) exceed 10 mg/kg b.w. (WHO 2010; Roye et al. 2009). Moreover, butylated

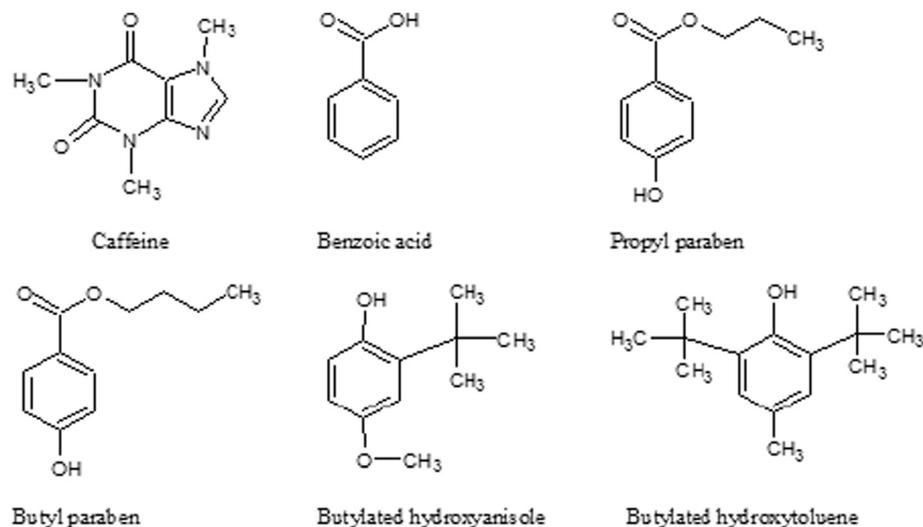


Fig. 1 Structural formula of the six additives

Table 1 The types and sources of collected dairy products

Parameters	Non-heat treated	Heat treated	
		UHT ^a -processed	Pasteurized
Products quantity	16	16	9
Source of origin	Local: 16	Local: 12	Local: 9
	Imported: Nil ^b	Imported: 4	Imported: Nil ^b

^aUHT stands for Ultra Heat Treatment

^bNil means zero product of that type

hydroxyanisole (BHA) may cause carcinogenic effect when the daily ingestion crosses the limit of 500 mcg/kg b.w. (Report on Carcinogens 2014), whereas severe nausea and vomiting may occur following the ingestion of butylated hydroxytoluene (BHT), whose ADI is 125 mcg/kg b.w. (Roye et al. 2009). So, it would be a better approach to collect milk by maintaining proper cleanliness and sanitation, and then applying cooling and/or thermization technique (Guizani 2007).

CF can be quantitatively analyzed using gas chromatography–mass spectrometry (Kerrigan and Lindsey 2005; Ayala et al. 2009), HPLC (Sather and Vernig 2011; Srdjenovic et al. 2008), surfactant–mediated matrix–assisted laser desorption/ionization time–of–flight mass spectrometry (Grant and Helleur 2008), micellar electrokinetic capillary chromatography (Injac 2008), ultraviolet visible absorption spectrometry and thin layer chromatography (Sather and Vernig 2011). The determination of BA and parabens can be carried out by HPLC (Saad et al. 2005; Mroueh et al. 2008; Petronela and Elena 2009; Khosrokhavar et al. 2010; Tawalbeh et al. 2014), capillary electrophoresis (Tang and Wu 2007). The analysis of BHA and BHT can be done with liquid chromatography–mass spectrometry (Lee et al. 2006), non–isothermal pressurized–differential scanning calorimetry (Dunn 2005), gas chromatography (Yang et al. 2002), and square–wave voltammetry (Medeiros et al. 2010).

Studying inquisitively and applying many of these practically, a suitable and precise RP–HPLC method was developed for the simultaneous determination of CF, BA, PP, BP, BHA and BHT. As a part of our continuous interest in the researches of adulteration studies on food, soft drinks, cosmetics and dairy products, we herein report a validated method and the result of our study to explore the presence or absence of CF, BA, PP, BP, BHA and BHT in dairy products, and their compliance with

Table 2 Composition of mobile phase of the RP–HPLC method

Time (min)	Diluted sulfuric acid (0.002 M) (%)	Acetonitrile (%)
0.01–10.00	85	15
10.01–11.00	40	60
11.01–13.00	30	70
13.01–16.00	20	80
16.01–18.00	85	15

Table 3 System suitability of the proposed method

Standards	Peak area (Mean ± %RSD) ^a	Retention time (Mean ± %RSD) ^a	Tailing factor (Mean ± %RSD) ^a
CF	1865839 ± 0.71	3.6 ± 0.04	0.866 ± 0.44
BA	986908 ± 0.50	11.6 ± 0.05	1.075 ± 0.37
PP	994208 ± 0.35	13.0 ± 0.02	1.248 ± 0.33
BP	858343 ± 0.43	13.3 ± 0.04	1.229 ± 0.20
BHA	234037 ± 0.94	13.6 ± 0.03	1.224 ± 0.22
BHT	177374 ± 0.59	16.7 ± 0.03	1.017 ± 0.49

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

^aAll determinants are in sextuplicate

the existing rules and regulations listed by the regulatory bodies. To the best of our knowledge, this is the first study for the assay of such additives in dairy products available in Bangladesh.

Methods

Chemicals and reagents

All six working standards of CF, BA, PP, BP, BHA and BHT were gifted by Eskayef Bangladesh Limited, Gazipur, Bangladesh. HPLC grade sulfuric acid (Sigma Aldrich, Germany), HPLC grade acetonitrile (RCI Labscan, Thailand) and analytical grade trichloroacetic acid (BDH Chemicals, England) were used for analytical purposes. Milli–Q water was used to prepare the mobile phase.

Sample collection and storage

Forty one marketed dairy products were collected from departmental stores, supermarkets and dairy farms in Dhaka, Bangladesh during mid–2013. Among them, 37 products were local and 4 products were imported. There were 10 full cream milk, 7 locally collected milk (bought from local dairy farms), 6 chocolate milk, 5 mango milk, 8 buttermilk, 2 skimmed milk, 1 banana milk, 1 flavored yogurt and 1 lassi i.e. a sweet or savory Indian drink made from yogurt or buttermilk base with water. The different types of collected dairy products are listed in Table 1. In the product label claim it was observed that only two pasteurized chocolate milk and four UHT–processed

Table 4 Linearity study of the proposed method

Standards	Concentration range (mg/L)	Regression equation (Y = mX + c)	Correlation coefficient (R ²)
CF	2–200	Y = 23019X–42400	0.996
BA	50–500	Y = 14211X–43609	0.992
PP	2–100	Y = 10605X–11766	0.992
BP	2–100	Y = 9294X–84770	0.997
BHA	50–300	Y = 1720X + 10364	0.996
BHT	50–300	Y = 1855X–46598	0.998

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

Table 5 LOD and LOQ of the standards in the proposed method

Standards	LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/L}$)
CF	2.66	8.06
BA	2.70	8.18
PP	0.21	0.64
BP	0.20	0.60
BHA	2.13	6.45
BHT	1.42	4.30

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

Table 6 Results of accuracy determination

Standards	Amount used (mg/L) eq. to (80 %–120 %) respectively	Amount recovered (mg/L)	% Recovery (Mean \pm %RSD) ^a
CF	60	59.66	99.44 \pm 0.025
	67.5	67.36	99.79 \pm 0.04
	75	74.62	99.48 \pm 0.045
	82.5	82.23	99.56 \pm 0.047
	90	89.69	99.64 \pm 0.02
BA	200	198.59	99.30 \pm 0.04
	225	223.65	99.41 \pm 0.03
	250	247.62	99.05 \pm 0.02
	275	273.22	99.33 \pm 0.02
PP	300	297.61	99.20 \pm 0.02
	20	19.23	96.03 \pm 0.4
	22.5	22.03	97.92 \pm 0.25
	25	24.23	96.77 \pm 0.14
	27.5	27.08	98.4 \pm 0.22
BP	30	29.25	97.33 \pm 0.21
	20	19.27	96.18 \pm 0.16
	22.5	21.99	97.78 \pm 0.12
	25	24.28	96.96 \pm 0.19
BHA	27.5	27.03	98.28 \pm 0.2
	30	29.26	97.31 \pm 0.22
	160	158.31	98.9 \pm 0.045
	180	178.33	99.06 \pm 0.036
BHT	200	197.58	98.77 \pm 0.087
	220	218.42	99.25 \pm 0.06
	240	238.42	99.34 \pm 0.015
	160	158.72	99.16 \pm 0.04
BHT	180	178.22	99.0 \pm 0.04
	200	198.75	99.34 \pm 0.09
	220	218.22	99.17 \pm 0.025
	240	238.10	99.2 \pm 0.02

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

^aAll determinants are in triplicate

chocolate milk products declared the presence of cocoa powder, which is a common source of CF but most of the manufacturers did not claim about the presence of BA, PP, BP, BHA and BHT. These samples were stored in a refrigerator below 4 °C till further use.

Equipment

Kubota–2100 centrifuge machine (Kubota, Japan) and Cyberscan 500 pH meter (Eutech, Singapore) were used to prepare the samples. Cole–Parmer Filtration machine (USA) was used to filter the mobile phase. To analyze samples a binary HPLC system (Shimadzu, Japan) equipped with a vacuum degasser (Model: DGU–20A3) and a UV/Vis detector (Model: SPD–20A) was used.

Chromatographic conditions

The separation was achieved using a 5 μm particle sized Capcell Pak octadecylsilyl (ODS) column (150 mm \times 4.6 mm i.d.) (Shiseido, Japan) with the mobile phase consisting of acetonitrile and diluted sulfuric acid (0.002 M) at a binary gradient as shown in Table 2. The mobile phase flow rate was 2.0 mL/min and the injection volume was 20 μL . The column oven temperature was set at 30 °C and the analysis was performed at 265 nm wavelength.

Preparation of solutions

Preparation of standard solution

A standard solution was prepared by adding working standards of CF (75 mg/L), BA (250 mg/L), PP (25 mg/L), BP (25 mg/L), BHA (200 mg/L) and BHT (200 mg/L) and was used to analyze the investigated sample preparations.

Preparation of sample solution

Accurately measured 40 mL of marketed product was taken into a centrifuge tube with 4 mL of trichloroacetic acid, used to precipitate fat and protein present in milk and milk products and then it was centrifuged for 10 min at 3000 rpm. The supernatant was then filtered using a Whatmann 41 filter paper to obtain the final

Table 7 Precision of the proposed method

Standards	Spike level ^a (%)	Intra-day (Mean \pm %RSD) ^b	Inter-day (Mean \pm %RSD) ^b
CF	100	1865839 \pm 0.71	1855736 \pm 0.63
BA	100	986908 \pm 0.50	982929 \pm 0.56
PP	100	994208 \pm 0.35	985310 \pm 0.46
BP	100	858343 \pm 0.43	852766 \pm 0.76
BHA	100	216226 \pm 0.80	213500 \pm 0.86
BHT	100	234037 \pm 0.94	214671 \pm 0.69

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

^aSpike level indicates the corresponding nominal concentration of each standard

^bAll determinants are in sextuplicate

Table 8 Ruggedness study of the proposed method

Standards	Analyst 1		Analyst 2	
	Amount found (mg/L)	% Recovery (Mean \pm % RSD) ^a	Amount found (mg/L)	% Recovery (Mean \pm % RSD) ^a
CF	74.61	99.48 \pm 0.045	74.67	99.51 \pm 0.05
BA	247.62	99.05 \pm 0.02	247.57	99.03 \pm 0.06
PP	24.19	96.77 \pm 0.41	24.21	96.81 \pm 0.54
BP	24.23	96.96 \pm 0.19	24.16	96.15 \pm 0.63
BHA	197.76	98.77 \pm 0.09	197.56	98.13 \pm 0.07
BHT	198.16	98.88 \pm 0.03	197.99	98.12 \pm 0.09

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

^aAll determinants are in sextuplicate

sample (King 1962). And the blank consisted of acetonitrile, diluted sulfuric acid (0.002 M) and trichloroacetic acid.

Validation of the test procedure

Method validation study was performed based on the current pharmaceutical regulatory guidelines i.e., ICH Q2 (R1). A number of parameters such as precision, accuracy, specificity, linearity, ruggedness and robustness were investigated for this purpose.

System suitability

For the evaluation of system suitability, the repeatability, retention time and tailing factor of six replicates of working standards of CF (75 mg/L), BA (250 mg/L), PP (25 mg/L), BP (25 mg/L), BHA (200 mg/L) and BHT (200 mg/L) were used and percentage relative standard deviation (%RSD) values were calculated in each case.

Linearity

The linearity was checked by analyzing different concentrations of CF (2–200 mg/L), BA (50–500 mg/L), PP (2–100 mg/L), BP (2–100 mg/L), BHA (50–300 mg/L) and BHT (50–300 mg/L). Calibration curves were made using MS Excel 2007 for each standard component. The regression line was calculated as $Y = mX + c$, where X

was the concentration of standard and Y was the response (peak area expressed as AU).

Limits of detection (LOD) and Limits of quantitation (LOQ)

LOD and LOQ were calculated according to ICH Q2 (R1) recommendations in accordance with the 3.3 s/m and 10s/m criteria respectively, where 's' is the standard deviation of the peak area and 'm' is the slope of the calibration curve determined from linearity investigation.

Accuracy (Recovery Test)

Recovery test was done by analyzing a sample of known concentration of standard solutions. Then percent recoveries (mean \pm %RSD of three replicates) were calculated.

Precision

Repeatability (intra-day precision) and intermediate precision (inter-day precision) of the method were determined by using the solution of standards i.e. CF (75 mg/L), BA (250 mg/L), PP (25 mg/L), BP (25 mg/L), BHA (200 mg/L) and BHT (200 mg/L) and the solutions were analyzed in six replicates in the same day (intra-day precision) and daily for six times over a period of three days (inter-day precision).

Table 9 Robustness study of the proposed method

Standards	Standards (mg/L)	Amount detected (mg/L) (mean \pm %RSD) ^a					
		Change in flow rate			Change in pH		
		1.9 mL/min	2.0 mL/min	2.1 mL/min	2.2	2.3	2.4
CF	75	74.69 \pm 0.08	74.62 \pm 0.05	74.59 \pm 0.02	74.63 \pm 0.02	74.62 \pm 0.05	74.63 \pm 0.04
BA	250	247.64 \pm 0.01	247.61 \pm 0.02	247.55 \pm 0.01	247.68 \pm 0.01	247.61 \pm 0.02	247.59 \pm 0.01
PP	25	24.24 \pm 0.04	24.23 \pm 0.04	24.18 \pm 0.04	24.23 \pm 0.04	24.23 \pm 0.04	24.23 \pm 0.04
BP	25	24.25 \pm 0.06	24.28 \pm 0.04	24.23 \pm 0.09	24.27 \pm 0.06	24.28 \pm 0.04	24.25 \pm 0.10
BHA	200	197.61 \pm 0.01	197.58 \pm 0.02	197.52 \pm 0.01	197.58 \pm 0.01	197.58 \pm 0.02	197.58 \pm 0.02
BHT	200	198.7 \pm 0.01	198.75 \pm 0.03	198.67 \pm 0.01	198.71 \pm 0.01	198.75 \pm 0.03	198.75 \pm 0.02

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

^aAll determinants are in triplicate

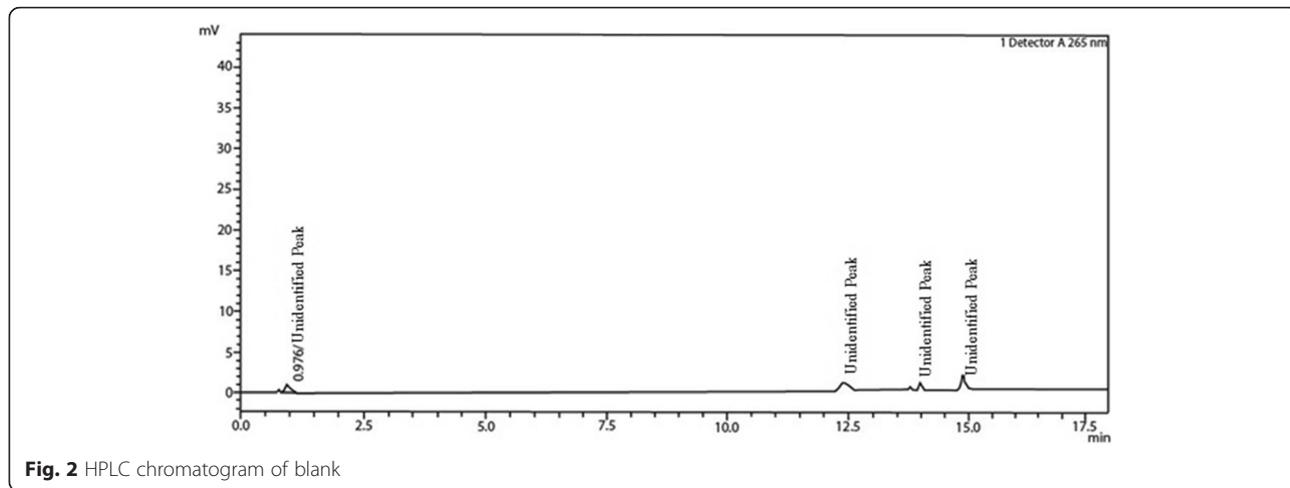


Fig. 2 HPLC chromatogram of blank

Ruggedness

Ruggedness of the method was determined by analyzing six assay sample solutions of standard CF (75 mg/L), BA (250 mg/L), PP (25 mg/L), BP (25 mg/L), BHA (200 mg/L) and BHT (200 mg/L) by two analysts in the same laboratory to check the reproducibility of the result. The percentage recovery and %RSD were calculated in both cases.

Robustness

To determine the robustness of the method, flow rate of the mobile phase and the pH of the diluted sulfuric acid were slightly changed. The %RSD of robustness testing under these conditions was calculated in all cases.

Results and discussion

Method validation

System suitability

The results (Mean ± %RSD of six replicates of the standards) of the chromatographic parameters are shown in Table 3, which indicate the good performance of the system.

Linearity

The relevant information is shown on Table 4. The correlation coefficient values of all six investigated ingredients are shown well within (0.99–1.00), which indicate the linearity of the proposed method.

Limits of detection (LOD) and Limits of quantitation (LOQ)

The LOD and LOQ values of CF, BA, PP, BP, BHA and BHT are shown in Table 5, indicating that the proposed method is well sensitive.

Accuracy (Recovery Test)

The overall recoveries of the standards are summarized in Table 6. Almost all results showed very good recoveries at five different concentration levels.

Precision

The results obtained are presented in Table 7. Intra- and inter-day results were found very close to those of the

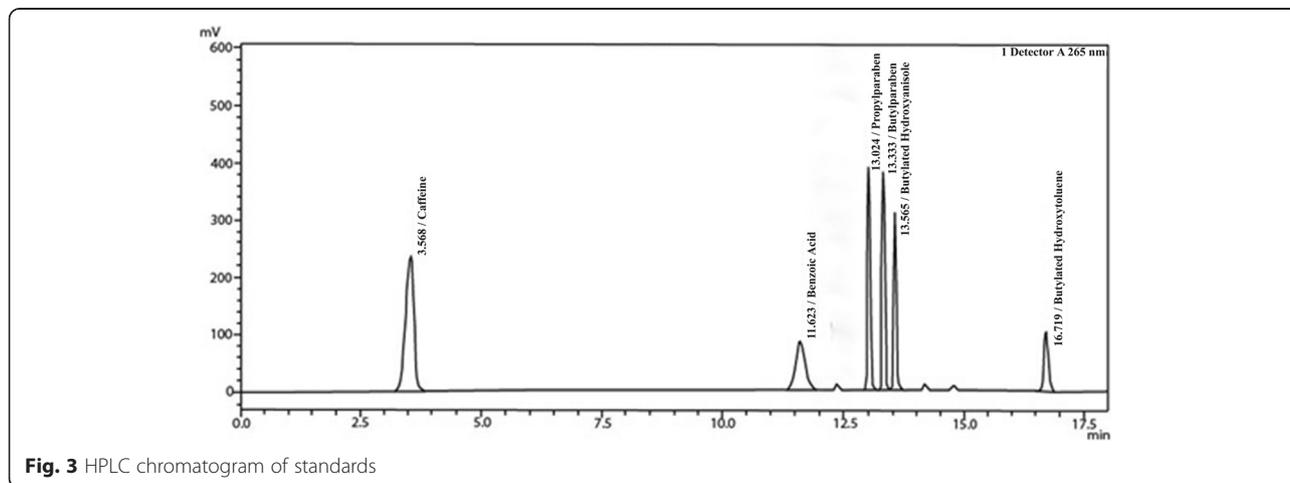


Fig. 3 HPLC chromatogram of standards

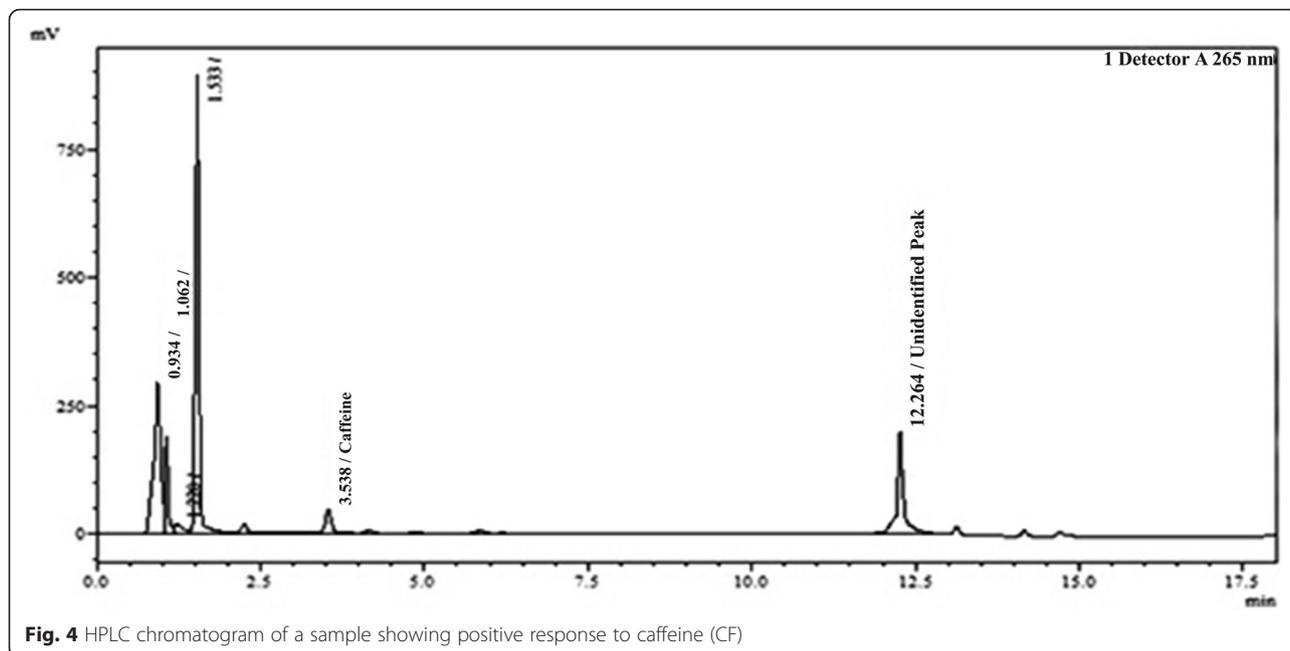


Fig. 4 HPLC chromatogram of a sample showing positive response to caffeine (CF)

corresponding freshly prepared nominal concentration, but inter-day results are slightly more deviated from the norms.

Ruggedness

The results (mean of %recovery ± %RSD of six assay samples) of the ruggedness test are presented in Table 8.

Robustness

The pH of the 0.002 M diluted sulfuric acid was reproducibly found about 2.3. The robustness of the method

has been checked changing the pH of diluted sulfuric acid as well as flow rate of mobile phase. The results (mean ± %RSD of three assay samples) of the robustness study are given in Table 9.

Quantitation of CF, BA, PP, BP, BHA and BHT in the dairy products

A precise, robust and rugged RP-HPLC method for simultaneous analysis of CF, BA, PP, BP, BHA and BHT was developed to investigate 41 marketed dairy products within a shortest possible time. Our designed analytical

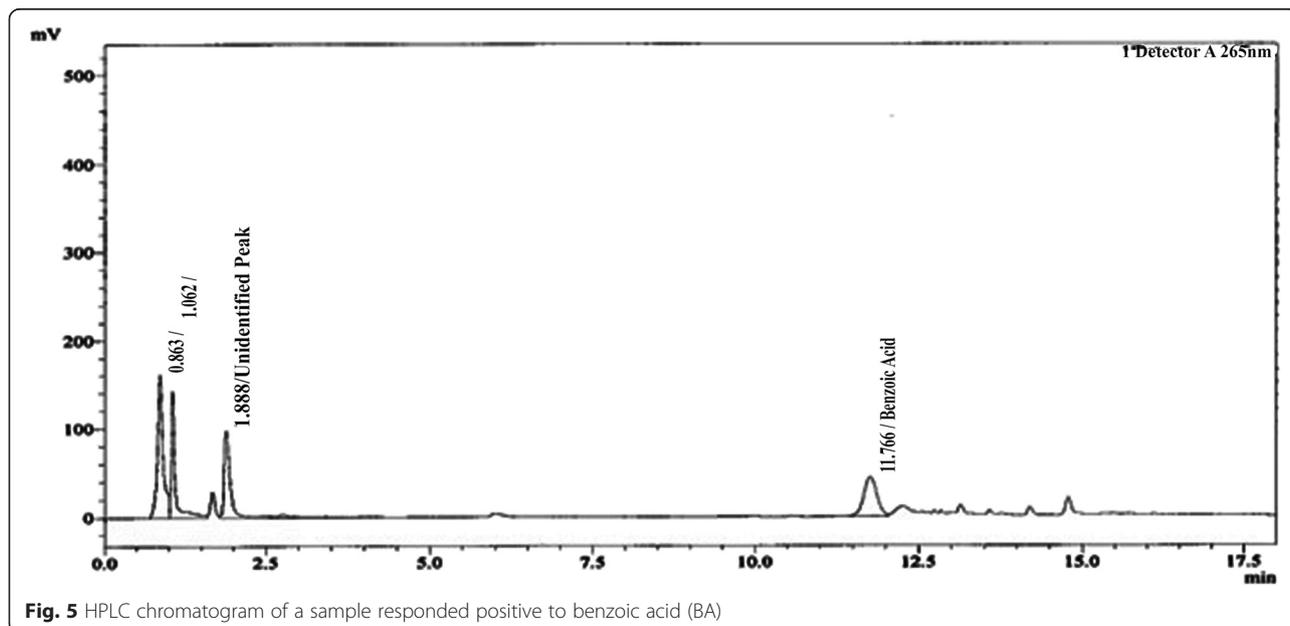


Fig. 5 HPLC chromatogram of a sample responded positive to benzoic acid (BA)

Table 10 The concentrations of CF and BA in dairy products

Estimation of CF		Estimation of BA	
CF positive dairy products	Concentration of caffeine present (mg/L) (mean ± %RSD) ^a	BA positive dairy products	Concentration of BA present (mg/L) (mean ± %RSD) ^a
C1	12.28 ± 0.66	M1	65.22 ± 0.09
C2	14.4 ± 0.40	M5	42.28 ± 0.16
C3	17.76 ± 0.34	B1	55.30 ± 0.12
C4	10.93 ± 0.33	Y1	11.68 ± 0.39
C5	13.57 ± 0.28	G1	1875.65 ± 0.15
C6	14.79 ± 0.26	G2	1074.85 ± 0.16
-	-	P1	2066.39 ± 0.15

CF caffeine, BA benzoic acid, PP propylparaben, BP butylparaben, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene
^aAll determinants are in triplicate

method revealed their retention times of about 3.56, 11.62, 13.02, 13.33, 13.56 and 16.72 min, respectively, which is so far the best approach to analyze such a large number of dairy products. Figs. 2, 3, 4 and 5 represent the chromatograms of blank, six standards, a CF-positive product and a BA-positive product sequentially. Among the 41 dairy products, none was labeled to contain CF; rather 6 chocolate milks (composed of 14.6 % of milk products) were labeled to contain cocoa powder instead. Only these 6 products showed positive response towards CF in the concentration range of 10 ~ 18 mg/L. This range is well below the maximum allowable limit of CF, which is 200 mg/L. The resultant concentrations of CF in these 6 milk products are individually presented in Table 10.

Dairy products are available in Bangladesh as pasteurized form, UHT form and some are not heat treated. Two products out of 9 pasteurized dairy products and 4 products out of 16 UHT treated products contained CF and all of 16 not heat treated products did not contain CF. Independent of this type of treatment, every chocolate milk products whether they are heat treated or not as well as pasteurized or UHT treated were found CF positive. So, our study could not establish any relationship between heat treatment of the milk and the presence of CF. The presence of CF in pasteurized, UHT treated and not heat treated products are shown in Fig. 6.

There were 37 domestic milk and milk products and 4 products were imported from Australia, New Zealand, Ukraine and South Africa. Six out of 37 domestic dairy products were found to contain CF. The imported dairy products did not contain CF. Moreover, none of the imported products was chocolate milk products. There is no imported chocolate milk product available in Bangladesh. So, caffeine content could not be correlated with the products' origin i.e. whether they are imported or domestic. Interestingly, the presence of CF in the chocolate milks observed in this study is similar to the average content of 11 mg/Kg collected by Craig and Nguyen (1984). However, though the CF content found is low, the CF present in milk products may contribute to the exceeding of tolerance limit along with other CF rich products like tea, coffee, energy drinks etc.

Again, 7 out of 41 analyzed dairy products were found BA positive. The concentration of BA in these products was between 11 ~ 2067 mg/L. Among these products, 2 were mango milk, 1 each from banana milk, flavored yogurt, lassi, and 2 from buttermilk. The comparison is shown in Fig. 7. Beside this, all the pasteurized milk and milk products were BA negative, 5 among 16 UHT milk and milk products were found to contain BA whereas 2 out of 16 not heat-treated products showed positive response to BA which is shown in Fig. 8. Seven dairy

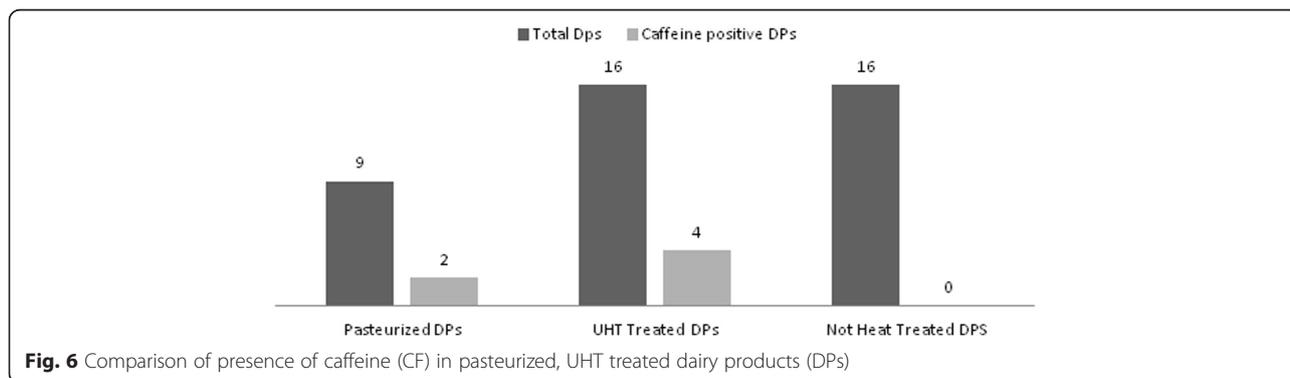
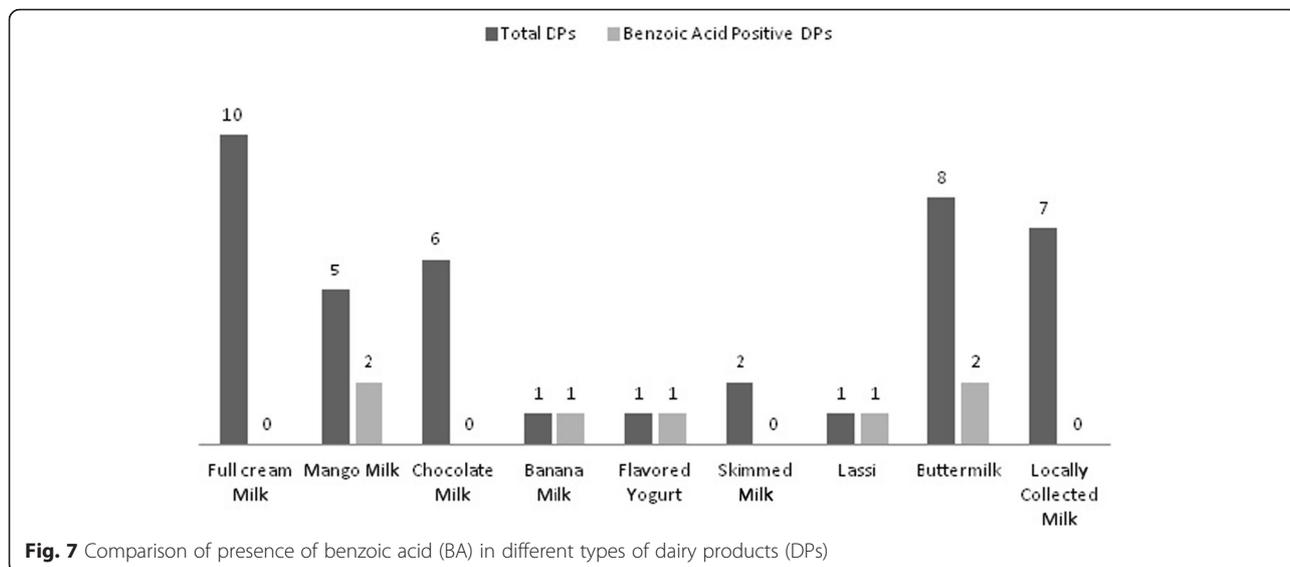


Fig. 6 Comparison of presence of caffeine (CF) in pasteurized, UHT treated dairy products (DPS)



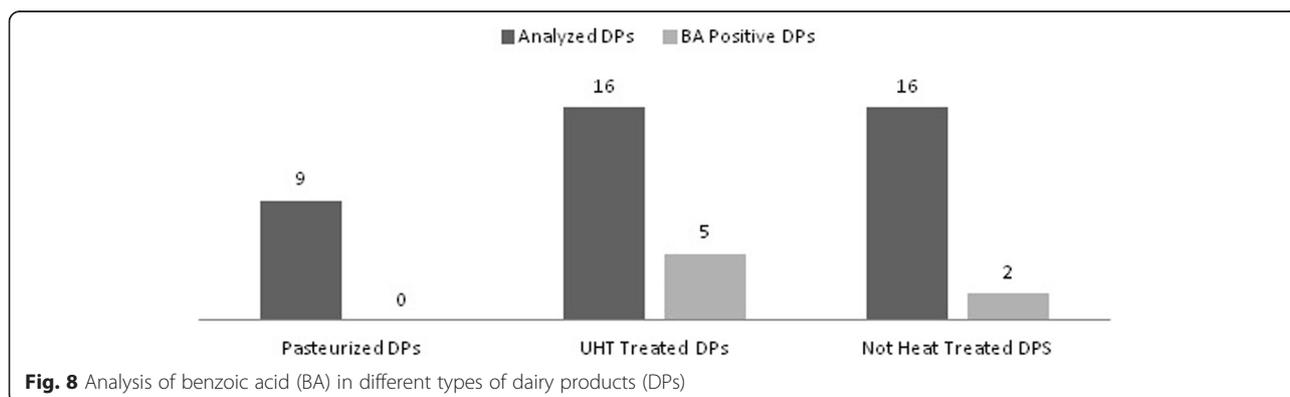
products among 37 Bangladeshi milk products were found to contain BA. None of the imported milk products was found to contain BA.

According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), BA is only permitted to use in flavored yogurt up to the maximum use level of 300 mg/L. Among 7 BA positive dairy products, only 1 product was flavored yogurt. Therefore, BA was approved to be used in only 1 product and the concentration of this product was about 11.68 mg/L, which was well below the maximum use level set by JECFA. Six other BA positive products are not permitted to use BA. Moreover, 3 of them crossed the maximum permitted level of 300 mg/L, even though BA is not permitted to be used in these products. Apart from these 3 products, 4 other BA positive products contained relatively very low amount of the additive which may be originated due to feeding practices, contamination, storage condition, and veterinary drugs. It is also well known that hippuric acid, a natural component of milk, may be converted to benzoic acid by the fermentation of lactic acid bacteria.

Further study needs to be carried out to confirm these sources.

However, the mean concentrations of benzoic acid observed by Qi et al. (2008) in pasteurized and UHT milk products in China were 3.6 ± 3.3 and 2.4 ± 2.2 mg/kg, ranging between 0.51–8.8 and 0.62–13 mg/Kg, respectively, where, the UHT milk products was plain milk. In our study, none of the analyzed pasteurized and non-flavored UHT milk products was found BA positive. Beside this, in Jordan, Mihyar et al. (1998) found BA in labaneh (concentrated yogurt) ranging from not detected to 2000 mg/Kg, whereas, Tfouni and Toledo (2002) did not find BA in yogurt in Brazil while our study detected BA in the flavored yogurt. The concentration of BA found in the product was well below the maximum use level.

But, every analyzed milk products showed negative response to PP, BP, BHA and BHT. However, Pattono et al. (2009) revealed the presence of BHT in conventional milk and organic milk ranging from not detected to 1.45 mg/L and not detected to 1.67 mg/L, respectively, in Italy,



though, they did not find BHA in their samples. Though these 4 additives were not found within detectable range in our samples, continuous monitoring is necessary to maintain the situation.

Finally, since this is the first report from Bangladesh about using these six additives in dairy products, more studies should be carried out on a regular basis by researchers and respective regulatory bodies to protect common people from the uncontrolled consumption of these potentially harmful additives with highly nutritious dairy products.

Conclusion

The aim of this study was to explore the six additives in dairy products available in Bangladesh by developing a simple RP–HPLC method. The proposed time–programmed method can do it within 20 min, including reequilibration. Our screening revealed the presence of CF and BA in several dairy products. The non–permitted use of BA in dairy products and the presence of CF in CF–free products can be injurious to human health. So, we expect that the respective policy makers of Bangladesh will consider this outcome seriously and devise rules on them as quickly as possible.

Abbreviations

ADI, Acceptable Daily Intake; b.w., Body–weight; BA, Benzoic Acid; BHA, Butylated hydroxyanisole; BHT, Butylated hydroxytoluene; BP, Butylparaben; CF, Caffeine; FDA, Food and Drug Administration; JECFA, Joint FAO/WHO Expert Committee on Food Additives; PP, Propylparaben; RP–HPLC, Reversed Phase High Performance Liquid Chromatography; WHO, World Health Organization.

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Authors' contributions

SB and ASSR did the experimental design of this study. MSH and MSI performed the laboratory analyses. MSH, MSI, and SB interpreted the data. MSH wrote the manuscript. MSH, SB, and ASSR edited and proofread the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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