Development and validation of an analytical method for determination of bronopol and kathon preservative in milk

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Abstract The bronopol and kathon are chemical preservative which prevent degradation of milk samples and maintain authenticity in analysis. The detection is based on HPLC–UV–Vis spectroscopy, in which C₁₈ column (250 mm × 4.6 mm, 5 µm) was used for chromatographic separations, with a mobile phase comprising 0.1% phosphoric acid in water: Methanol: 0.1% phosphoric acid in acetonitrile (80:10:10) at a flow rate 0.8 ml/min at ambient temperature and with the UV detection at 250 nm for bronopol and 274 nm for kathon. The retention time of bronopol, kathon (MI 2-methyl-4-isothiazolin-3-one) and kathon (CMI 5-chloro-2-methyl-4-isothiazolin-3-one) was 4.52 min, 3.98 min and 6.68 min respectively with a total run time of 10 min. The linearity of the method was satisfactory with regression coefficient (R^2) = 0.99. The limit of quantification was 72, 240, 390 mg L⁻¹ for bronopol, kathon (MI) and kathon (CMI) respectively. Five spiked levels (62.5, 125, 250, 500 and 1000 mg L⁻¹) were used to determine the recovery of bronopol, kathon (MI) and kathon (CMI) which was found to be 95.41 ± 11.84, 95.75 ± 8.21 and 92.22 ± 14.64% respectively, with relative standard deviations in the range 5.9–14.6%. The standardized analytical method was successfully used to rapidly detect bronopol and kathon in milk samples.

Keywords Bronopol · Kathon · HPLC · MI · CMI

Introduction

Authenticity of milk is a major concern for legal authorities as some unscrupulous traders are indulges into malpractice of adulterating milk to combat between great demand and scarce availability of milk. The government appoints food safety officer who randomly collect the samples of milk from market and carry out the chemical examination for determination of its purity. However, milk is a rich source of nutrients, thus allows the growth of microorganisms. Therefore, it is necessary to add chemical preservatives in milk so that milk sample does not spoil until the analysis is complete. These chemical preservatives directly attack microorganisms and acts as either bacteriostatic or bactericidal. Different preservatives have been reported in literature for preservation of milk samples like methyl paraben, propyl paraben (Ranvir et al. 2015), sodium azide, azidiol, bronopol, potassium sorbate (Seskena and Jankevica 2007), kathon (Chaudhary 2013), formalin (FSSAI 2011), dowicil (Durham et al. 1978), mercuric chloride (Grappin and Jeunet 1970) and potassium dichromate (Kroger 1985). Bronopol and kathon are common preservative in cosmetics and hygienic products (Salvador and Chisvert 2011; Chai et al. 2018; Hofmann et al. 2018) and now they are gaining popularity in milk preservation due to their antimicrobial activity against broad range of microorganisms (Bumbadiya et al. 2017).

The bronopol is an antimicrobial compound and its IUPAC name is 2-Bromo-2-Nitro-1,3-Propanediol. It causes oxidation of essential thiol containing amino acids within cell and generates free radical that leads to death of cell (Singh and Gandhi 2015). It is effective against both gram-positive and gram-negative bacteria (Matczuk et al. 2012). At higher temperature bronopol decomposes into formaldehyde that is also known to possess antimicrobial

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activity. Bronopol is found to be unstable in alkaline conditions and degraded into nitromethane, nitrous oxide, hydrogen bromide, formic acid and, methanol (Upadhyay et al. 2014) however stable in acidic medium. Kathon is a combination of two active ingredients 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI). It is a broad-spectrum biocide which is effective against gram positive and gram negative bacteria along with yeast and mold. Kathon attack thiol group of protein and generate intracellular free radicals.

Any preservative used for analytical milk samples should not interfere during chemical analysis of milk. Different research reports are available that bronopol and kathon does not interfere in the chemical analysis of milk. Bronopol does not interfere in the fatty acid analysis of milk using gas chromatography or by near-infrared reflectance spectroscopy (Coppa et al. 2010). Bronopol (0.05–0.10%) and kathon (0.20–0.40%) both have good antimicrobial activity against all groups of microorganisms like total bacterial count, coliforms, lactic acid bacteria and yeast and moulds (Bumbadiya et al. 2017). Because of very low effective concentration, these preservatives have a great potential to be used in preservation of analytical milk samples without affecting original composition and outcome of the test procedure. Different methods based on HPLC are available for detection of kathon (Baranowska and Wojciechowska 2013; Wittenberg et al. 2015; Alvarez-Rivera et al. 2012) and bronopol (Matczuk et al. 2012; Miralles et al. 2016). However no literature is available for identification and determination of kathon and bronopol in milk. Sample preparation before analysis is crucial to obtain accurate results. As milk contains fat, protein, lactose and other water soluble components and due to their chemical structure, bronopol and kathon interacts with constituents of milk especially milk proteins. Reports from literature are available that bronopol reacts with cysteine, glutathione and proteins via thiol/disulphide reaction (Stretton and Manson 1973). Kathon reacts with protein thiol via multiple pathways (Supelco Bulletin 912 1997). Therefore, the extraction of bronopol and kathon from milk will require sample preparation step for good recovery and reproducible results. For the present study, a method was standardized for the extraction of bronopol and kathon from milk and their subsequent determination using high performance liquid chromatography-UV spectrometry (HPLC–UV).

Materials and methods

Chemicals and reagents

Standard of bronopol (purity, 99.81%) and kathon CG (MI:CMI ratio 1:3) with brand name Proclin® was purchased from Sigma Aldrich Ltd. HPLC-grade methanol, acetonitrile (ACN), tetrahydrofuran (THF), orthophosphoric acid, ethanol and Water were obtained from Fisher Scientific, zinc sulphate and copper sulphate of analytical grade was purchased from Himedia.

Instrumentation

The chromatographic system used for HPLC analysis was an Agilent Technologies (Model No. 1260 Infinity) equipped with a C₁₈ column (250 mm × 4.6 mm, 5 µm) and UV–Vis detector Data acquisition was performed with Agilent Open LAB CDS (Version A.04.05) software.

Extraction of bronopol and kathon from milk

Cow milk was collected from Livestock Research Centre (LRC) of National Dairy Research Institute (NDRI), Karnal Haryana and spiked with bronopol and kathon at known concentration of 500 mg L⁻¹. In the first screening, the bronopol and kathon were extracted from milk using 50% ammonium sulphate, 50% sodium chloride, 4% acetic acid, absolute ethyl alcohol (Fox et al. 1998) and Zinc Copper precipitant (IS 8479-Part II -1977) with a 1:1 solvent-milk ratio. The mixture was kept in water bath maintained at 90 ± 3 °C for 10 min and filtered through Whatman No. 40 filter paper. The obtained supernatant was subjected to HPLC analysis. Zinc-copper precipitant was prepared by dissolving 3.0 g of zinc sulphate and 0.6 g of copper sulphate in water and volume was made to 100 mL with water.

Analytical method assessment

Cow milk was spiked with bronopol and kathon at various known concentrations (50–2000 mg L⁻¹). The standardized analytical method was validated according to ICH Steering Committee (1996) and evaluated for linear response of the calibration curve, precision (intra-day and reproducibility), level of detection (LOD) and level of quantitation (LOQ).

The linearity of the method was determined by plotting the detector response (peak area) versus the concentration of bronopol and kathon. Accuracy was calculated as the percentage of recovery by assay of the known added amount of analyte (bronopol and kathon) in the sample.

Precision/reproducibility were determined using method of Kayesh et al. (2013) with five replications. The precision was checked by inter day repeatability response after replicate sample injections and expressed as RSD%.

Level of detection (LOD) and Level of quantitation (LOQ) for bronopol and kathon was calculated theoretically by using following formulas (ICH Steering Committee 1996).

$$\text{LOD} = (\sigma/\text{slope}) \times 3.3$$

$$\text{LOQ} = (\sigma/\text{slope}) \times 10$$

Results and discussion

Standardization of analytical conditions for quantification of kathon and bronopol over HPLC

Different mobile phase Water: ACN: Phosphate Buffer (94.5:5:0.5), Methane: Water: Orthophosphoric acid, Ethanol: 1% acetic acid (13:87), Methanol: THF: Water (5:5:100), 0.1% phosphoric acid in Water: Methanol: 0.1% phosphoric acid in ACN were tried for detection of bronopol and kathon as per reported literature. The reported wavelength range for detection of bronopol was 210–260 nm and for kathon was 250–280 nm (Paul et al. 2011; Lasmar et al. 2011; Jie and Jinhui 2000). In HPLC analysis isocratic elution using 0.1% phosphoric acid in water: Methanol: 0.1% phosphoric acid in acetonitrile (80:10:10) as mobile phase and wavelength of 250 nm for bronopol and 274 nm for kathon was standardized on the basis of maximum height and peak area. Bronopol is single compound therefore one peak was observed and Kathon is a combination of two active components 2-methyl-4-isothiazolin-3-one (MI) and 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) in 1:3 ratio, therefore, two peaks were obtained. In literature (Medley et al. 2014; Kawakami et al. 2015; Baranowska and Wojciechowska 2013) peaks of MI and CMI were also reported in HPLC chromatogram of kathon. In standardized protocol, the retention time of bronopol, kathon (MI) and kathon (CMI) was 4.52 min, 3.98 min and 6.68 min respectively. Total run time of standardized method was 10 min.

Extraction of bronopol and kathon from milk

Out of different treatments given to milk samples, it was found that treatment of milk with ammonium sulfate and zinc copper precipitant was most satisfactory. However, in case of ammonium sulfate the protein and fat was separated as a very hard layer on the top and collection of clear

filtrate was difficult. During HPLC analysis of the filtrate, recovery was too less and some protein peaks (unknown) were also detected. During HPLC analysis of filtrate obtained from the zinc copper precipitate a number of unknown peaks were detected with peaks of bronopol and kathon. Therefore, the extraction protocol was again modified. The standardized extraction protocol for bronopol and kathon from milk is presented in Fig. 1. The addition of NaOH was critical step in extraction of preservatives from milk system. The addition of NaOH is required to precipitate remaining whey proteins and non-protein nitrogen content. However, reports are available that at higher pH bronopol is unstable and hydrolyzed at very fast rate (Moore and Stretton 1981) as well as kathon undergoes degradation in alkaline solution (Barman and Preston 1992). Out of different concentration tried for extraction of preservatives (0.05 N, 0.1 N, 0.2 N, 0.4 N, 0.6 N, 0.8 N, 1.0 N and 2.0 N), it was observed that 0.1 N NaOH was sufficient to precipitate whey proteins without change in final pH of the filtrate. On increasing concentration of NaOH, whey protein precipitated very efficiently but due to rise in pH the stability of preservatives in filtrate was decreased that resulted in lower recovery. Therefore, 0.1 N NaOH was selected for extraction of preservatives.

Quantification of bronopol and kathon in milk

After using the standardized extraction protocol, in control milk (without preservative) at 250 nm and 274 nm, no

Fig. 1 Standardized protocol for extraction of bronopol and kathon in milk sample

Fig. 2 **a** HPLC chromatogram of Control Milk at 250 nm. **b** HPLC chromatogram of Milk with bronopol and kathon at 250 nm. **c** HPLC chromatogram of Control Milk at 274 nm. **d** HPLC chromatogram of Milk with bronopol and kathon at 274 nm

peak was observed for bronopol and kathon (Fig. 2a, b). In extracted filtrate from milk samples (individually added with bronopol and kathon at 500 mg L⁻¹ concentration) at 250 nm one peak (bronopol) was observed at 4.52 min and at 274 nm two peaks (MI and CMI) were observed at 3.98 min and 6.68 min as presented in Fig. 2c and d. On comparison of HPLC chromatograms of control milk and milk sample added with bronopol and kathon, the method standardized for extraction found to be sensitive and specific, as only peaks of bronopol and kathon were obtained with minimum interference of other components of milk especially amino acid.

Method validation

Linearity, LOD, and LOQ

Linearity was studied using five solutions in the concentration range from 62.5 to 1000 mg L⁻¹. The regression equation was found by plotting the peak area (y) versus the preservative concentration (x) expressed in mg L⁻¹. The correlation coefficients obtained in each case was above 0.99, it demonstrates that there is a strong linear relationship between the peak area and the concentration of the analyte. Analytical characteristics Calibration Equation,

Correlation coefficient, Accuracy/Recovery (Mean ± SD), Slope, Intercept, Linearity range, LOD, LOQ and RSD% of bronopol, kathon (MI) and kathon (CMI) using HPLC methods are presented in Table 1. The limit of detection was found to be 24, 0.1 and 0.1 mg L⁻¹ for bronopol, kathon (MI) and kathon (CMI) respectively. This was the minimum concentration that can be detected in HPLC with standardized protocol. However, the limit of quantification was calculated theoretically by $10 \cdot \sigma / \text{slope}$ and was found to be 72, 240 and 390 mg L⁻¹ for bronopol, kathon (MI) and kathon (CMI) respectively. This was the minimum concentration that can be quantified in HPLC with standardized protocol. The reported effective concentration of bronopol and kathon ranged from 500 to 1000 mg L⁻¹ for bronopol and 2000 to 4000 mg L⁻¹ for kathon in preservation of milk (Bumbadiya et al. 2017). In standardized method the limit of detection and quantification for bronopol and kathon in milk was found to be far lower than that of their level of addition. Therefore the standardized method is effective and sensitive to determine the added bronopol and kathon in milk.

Accuracy and precision

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. The accuracy was determined by recovery percent assays and the percent recovery for individual spiked milk sample is tabulated in Table 1. The average

Table 1 Analytical characteristic of bronopol and kathon

Parameter	Bronopol	Kathon	
		MI	CMI
Calibration equation	$y = 1106.4x + 10,024$	$y = 195.38x + 8549.4$	$y = 392.76x + 5527.5$
Correlation coefficient	$R^2 = 0.9994$	$R^2 = 0.9971$	$R^2 = 0.9921$
Accuracy/recovery (Mean ± SD)	95.41 ± 11.84	95.75 ± 8.21	92.22 ± 14.64
Slope	1106.4	195.38	392.76
Intercept	10,024	8549.4	5527.5
Linearity range (µg mL ⁻¹)	62.5–1000	62.5–1000	62.5–1000
LOD (µg mL ⁻¹)	24	0.1	0.1
LOQ (µg mL ⁻¹)	72	240	390
RSD%	5.9	8.6	14.6
% Recovery			
Concentration of spiked standard (µg mL ⁻¹)			
1000	99.56 ± 7.72	94.79 ± 7.25	93.89 ± 10.55
500	101.74 ± 6.13	102.01 ± 8.02	106.41 ± 9.92
250	100.47 ± 6.10	102.94 ± 9.46	102.61 ± 9.08
125	101.67 ± 8.97	82.44 ± 8.52	73.08 ± 7.71
62.5	87.96 ± 8.82	96.62 ± 9.72	85.10 ± 10.89

recovery obtained for bronopol, kathon (MI) and kathon (CMI) was $95.41 \pm 11.84\%$, $95.75 \pm 8.21\%$ and $92.22 \pm 14.64\%$ respectively. At lower concentration of spiking, the recovery was found to be lower compared to higher concentration of spiking. In the quantitative determination of kathon and bronopol in cosmetic products (Jie and Jinhui 2000) with methanol: THF: water (5:5:100), the recoveries ranged from 91.3 to 106.8% and RSD were 0.5% and 3.7% respectively. The recoveries of bronopol in lactulose solution using isocratic elution with water: acetonitrile: phosphate buffer (94.5:5:0.5) at 214 nm ranged from 97.07 to 99.02% and RSD were 1.91% (Paul et al. 2011). The recovery of kathon from shampoo, conditioner and moisturizer sample was reported to be 93–111% when analyzed by HPLC–MS/MS (Wittenberg et al. 2015). The limit of quantification was $0.1 \mu\text{g g}^{-1}$ that was comparable with limit of kathon detected by our standardized protocol. By using HPLC–MS/MS (Alvarez-Rivera et al. 2012) reported recovery of 60% for kathon (MI), which was far lower than the recovery obtained in standardized method. Therefore on the basis of average recoveries for bronopol and kathon added in milk the method is quite sensitive and specific with the studies reported in literature.

Conclusion

A simple, rapid and sensitive HPLC method with UV–Vis detector was standardized and validated for the estimation of bronopol and kathon in milk. The standardized method includes a simple sample preparation procedure and offers a combination of sensitivity, simplicity, and a relatively short time of analysis. The method was validated and demonstrated a good linear range, as well as a good precision, accuracy, and specificity. Thus, the method can be used for quantification of bronopol and kathon in milk.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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