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Determination of formaldehyde in bovine milk using a high sensitivity HPLC-UV method☆

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ABSTRACT

A method of high performance liquid chromatography with UV detection (HPLC-UV) was implemented and validated for formaldehyde determination in bovine milk. A formaldehyde derivatization reaction with 2,4-dinitrophenylhydrazine, at pH 4.0, enabled its detection in milk at 360 nm. The clean up treatment of milk samples by liquid-liquid extraction with low temperature partitioning (LLE-LTP) was thoroughly optimized. The linearity of the method was shown by analytical curves ranging from 10.0 to 400.0 μg L^{-1} in aqueous solutions and milk samples that were characterized by $R^2 > 0.99$. The limit of quantification of 20.0 μg L^{-1} demonstrated the high sensitivity of the method to determinate formaldehyde residues in milk. Extracts of milk samples fortified with formaldehyde at three concentration levels, led to a mean overall recovery of 102.2 ± 1.3% (n = 9), which satisfies the performance criteria established by the Codex Alimentarius for analytical methods suitable for determination of food residues. The accuracy was evaluated by comparison with a well-established procedure using the Student *t*-test. Comparable results were obtained at a 95% confidence level, demonstrating the usefulness and effectiveness of the proposed method.

1. Introduction

Fraudulent practices in milk occur since antiquity to the present day. They typically involve the addition of the following adulterants: (i) water as diluent; (ii) melamine, urea and milk whey as non-protein nitrogen; (iii) hydrogen peroxide, formaldehyde, salicylic acid, sodium hypochlorite and potassium dichromate to increase product shelf life and mask its poor hygienic conditions; (iv) vegetal oil, animal fat and surfactants to increase fat content [1–4]. Owing to the lack of strong legislation and controls, more sophisticated fraudulent practices frequently emerge endangering population health, and causing chronic diseases or even death, especially of elder people and children, which are the most vulnerable part of the population [5–10].

Among the most common frauds in milk, the addition of formaldehyde (FA) must be highlighted because of its antiseptic and preservative properties, that improve the appearance of the product and keep it odorless [11,12]. Unfortunately, in recent years, many cases of milk contamination with FA have been reported by the Brazilian media, with several contaminated people, including children [13]. However, it is worth adding that FA is an endogenous product of the metabolism of mammals and may be used as a preservative for ruminant feeding [14]. Then, as it can also be produced endogenously, background levels of this compound are found in food products, specifically ranging from 0.1 to 0.3 mg kg^{-1} in milks. This fact prevents the analytical methods and the results obtained to be conclusive and distinguish between endogenous origin or fraudulent addition of FA, when this low range of concentrations is found in milk [10].

Formaldehyde (HCHO) is a colorless gas at room temperature, with a strong characteristic odor and highly flammable, which is produced worldwide in large scale from methanol, and is also a common air pollutant. It is usually marketed in a liquid solution of water and ethanol, called formalin, at concentrations between 37 and 50% w/w of formaldehyde [15]. The electrophilic nature of FA makes it reactive towards a variety of endogenous molecules, including glutathione, proteins, nucleic acids and folic acid [16]. Hence, FA exerts toxic effects on humans and has been classified by the International Agency for Research on Cancer (IARC) as a carcinogen (Group 1), tumorigenic and teratogenic compound and, thus, harmful to human health [17–19].

In Brazil, the determination of FA in milk, which is the objective of this work, is performed by a qualitative official method established by the Ministry of Agriculture, Livestock and Food Supply (MAPA). FA is

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separated from milk by distillation after phosphoric acid addition. An aliquot of the distillate is added to an aqueous solution containing chromotropic acid and sulfuric acid, and the resulting mixture is heated. A violet compound is formed in the presence of FA [20]. However, the poor detection limit (around 1 mg kg^{-1}) of this qualitative method limits its application to determine trace levels of FA in milk [21].

Other techniques, such as GC, HPLC and capillary electrophoresis, have been demonstrated to be more selective and sensitive to the determination of FA not only in milk [10,11,21–26], but also in beer [27], environmental samples [28–30], water [31], fish and other aquatic products [32,33], yogurt and juices [34,35].

This study aimed to contribute to the investigation of fraudulent addition of FA in bovine milk through an improved methodology. With this purpose, a high sensitivity HPLC-UV method was implemented and validated, following the derivatization of FA with 2,4-dinitrophenylhydrazine (DNPH), which was performed directly in milk samples, without the need of distillation or other pre-separation step. A liquid-liquid extraction with low temperature partitioning (LLE-LTP) allowed the appropriate clean-up and the subsequent analysis of the very complex matrix that milk represents by HPLC-UV, without interferences.

2. Materials and methods

2.1. Reagents and solutions

All chemical reagents were of analytical grade or better. Ultrapure water (resistivity of 18.2 MΩ cm) was obtained from a Sartorius system (Arium Confort II, Brazil). Formaldehyde (36.5–38.0%, Vetec, Brazil) was used to simulate milk adulteration, while 2,4-dinitrophenylhydrazine (DNPH 97%, Sigma-Aldrich, USA) and sulfuric acid P.A. (Vetec, Brazil) were employed in the derivatization reactions. Anhydrous ethanol (HPLC grade, Tedia, Brazil) and acetonitrile (ACN - HPLC grade, J.T.Baker, USA) were employed for the recrystallization and purification of the derivatization reagent (DNPH) and in the milk samples treatment, respectively.

Standard stock solutions of formaldehyde-2,4-dinitrophenylhydrazone (FA-DNPH, Sigma-Aldrich, USA), were prepared by dissolving appropriate masses previously weighed in an analytical balance (GR-202, AND, Japan) in ultrapure water. Standard solutions of FA-DNPH (from 10 to 400 μg L^{-1}) employed to obtain the analytical curves were prepared by dilution of appropriate aliquots of the stock solutions.

Aiming to have the appropriate conditions for the derivatization reactions of FA, a pH-meter (Digimed, model DM-22, SP, Brazil) was employed to adjust the pH of the solutions. The standards and samples solutions were all filtered using PTFE filters (0.45 μm, Agela Technologies, USA) before injection in the chromatographic system.

2.2. HPLC–UV system and operating conditions

Sample analysis was carried out by HPLC-UV, under the conditions previously studied and optimized by Ochs et al. [36], except for a cleaning step that was included in the gradient to allow the complete elution of the matrix components. The chromatographic system was controlled by an Agilent ChemStation, and consisted of a vacuum degasser, a binary pump, an automated injector, a column oven and an UV-DAD detector. A Supelcosil C-18 (250 × 4.6 mm; 5 μm; Supelco) column connected to a pre-column with the same characteristics was used for chromatographic analysis. The mobile phase consisted of acetonitrile (A) and water (B), and was previously filtered through a Millipore filtration system, using 0.45 mm membranes (Agilent, USA) and degassed in an ultrasonic bath prior to use. The gradient was as follows: 65% v/v of A was kept for 6 min with a subsequent linear increase to 90% v/v of A until 8 min. A flow-rate of 1.0 mL min^{-1}, a column temperature of 30 °C and an injection volume of 10 μL were used for standards and samples solutions. The detection wavelength was 360 nm and the retention time for FA, in the derivatized form (FA-DNPH), was 4.6 min.

2.3. Formaldehyde derivatization reaction and stability of the derivative

The FA molecule has no chromophore group capable of allowing its quantification at low concentrations and, therefore, its derivatization is always necessary. The derivatization reaction of FA used in this work can be seen at Fig. SM.1 of the Supplementary material.

In order to evaluate the effect of pH in the derivatization reaction, a kinetic study of the FA-DNPH formation was carried out at pH 3.0, 4.5 and 6.0. With this purpose, aqueous solutions of FA (300 μg L^{-1}) were acidified using sulfuric acid solution (0.01 mol L^{-1}) and an excess of 0.1 g L^{-1} of DNPH, previously recrystallized in ethanol, was added. The resulting solutions were analyzed under the HPLC conditions presented above (Section 2.2).

After assessing the optimal pH for the derivatization reaction, a study of FA-DNPH stability was carried out in aqueous solutions and milk samples submitted to the freezing process at different time intervals (12, 36, 60, 84 and 108 h). The solutions were treated and analyzed as described in Sections 2.2 and 2.4.

2.4. Samples description and treatment

The studied samples were obtained from thirteen different brands and types (whole, semi-skimmed and skimmed milk) of powdered and liquid bovine milk, that were purchased in supermarkets of the cities of Rio de Janeiro and Niterói, in Rio de Janeiro State, Brazil.

A simple sample treatment (Fig. 1) by LLE-LTP, together with the FA derivatization reaction, was accomplished through the following procedure:

A diluted solution (400 mg L^{-1}) from FA 36.5–38.0% (Vetec, Brazil) was prepared, and an aliquot of it was added to 2.5 mL of milk sample. The pH of the resulting solution was adjusted to 4.0 ± 0.2 by the addition of 1.0 mol L^{-1} sulfuric acid, and 5.0 mL of 0.1 g L^{-1} DNPH solution in ACN were added to the mixture, which was vortex-mixed for 1 min and centrifuged at 6000 rpm (2.012*g*) for 20 min. Subsequently, this solution was cooled to −4 °C overnight aiming freezing the water, whereas the organic phase remained liquid [37–39]. The organic phase was quickly removed from the tube using an automatic micropipette, transferred into a 5.0 mL volumetric flask and the volume reconstituted with ACN. This resulting solution was filtered through a syringe filter (0.45 μm, 25 mm, Millipore, USA) and transferred into glass vials for HPLC-UV analysis.

Although the freezing step may be considered time-consuming, no supervising is required and it is possible to carry out the extraction of several samples simultaneously. Moreover, the process of FA derivatization reaction and sample treatment as a whole is simple and fairly reproducible considering the complexity of the matrix. However, it is noteworthy that this freezing time can be reduced if the available freezer reaches lower temperatures, as reported in the work of E.C.P. Rego et al. [39], in which the tube was cooled down to −20 °C for only 4 h.

3. Results and discussion

3.1. Evaluation and optimization of the conditions of formaldehyde derivatization reaction

A study of the effect of pH on the derivatization reaction of FA using DNPH as derivatizing reagent was performed in aqueous solutions. Previous studies have been indicated that the formation of the desired hydrazone (FA-DNPH) is slow at very low or very high pH values, being generally faster in the pH range between 4.0 and 5.0 [29,40].

The obtained results evaluated as described in Section 2.3, showed that the optimum reaction occurs in a pH range between 3.0 and 4.5,