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Rapid extraction of melamine in powdered milk for direct electrospray ionization tandem mass spectrometry analysis

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ABSTRACT

A combination of a simple pretreatment for melamine extraction and direct analysis in electrospray ionization tandem mass spectrometry (ESI–MS/MS) is proposed. Three pretreatments were evaluated. The first was based on suppressing interference using acetonitrile. The second used sulphuric acid and trichloroacetic acid to suppress interference and for melamine extraction, respectively. The third used sulphuric acid to suppress milk interference, trichloroacetic acid for melamine precipitation, and ethyl acetate for melamine extraction. However, only the last pretreatment suppressed milk interference in melamine detection and a good linearity (R^2=0.99) was obtained. The presence of MS/MS 85 on melamine fragmentation spectrum showed the selectivity of this method. The limit of detection and limit of quantification were 0.269 μg L^{-1} and 0.897 μg L^{-1}, respectively. The recoveries and relative standard deviation (RDS) of method were lower than 114% and 7.86%, respectively. Further, the research was extended to elucidate the nature of the melamine in the extract through infrared spectroscopy and microscopy analyses. The precipitate was characterized as melaminium bis(trichloroacetate) dihydrate, which is generated through hydrogen bound formation in an interaction between melamine and trichloroacetic acid. Therefore, a simple, fast, and easy method for melamine extraction and direct ESI–MS/MS analysis was developed.

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1. Introduction

Milk is a complete food and source of essential nutrients, including carbohydrate, protein, fatty acids, calcium, phosphorus and magnesium. Moreover, the milk constituents can affect dairy products performance and quality; therefore, the industries usually define milk quality based on the nutrient levels, mainly protein and fat. These parameters have been used to calculate the payout to the supplier [1]. Unfortunately, adding nonauthentic nutrients has been used to adulterate milk and, consequently, increase economic gain [2].

This adulteration is defined as the removal or replacement of milk components and addition of substances without a purchaser's knowledge, including water, whey, sucrose, starch, salt, sodium hydroxide, and formaldehyde [2,3]. Melamine is a nitrogen-rich compound commonly used to manufacture plastics, laminates, glues and adhesives [4]. Although it is not an ingredient in food, it was reported as present in Chinese powdered milk and infant formula in 2008. It was used to falsely increase the apparent milk protein and was not discovered because the routinely used methods could not distinguish between nitrogen from protein and non-protein sources, which resulted in incorrectly high protein measurements [5]. Consuming tainted products by the Chinese population affected 294,000 infants and children with more than 50,000 hospitalizations and at least 6 deaths [6]. This scandal scared the world and created the need for sensitive, specific, rapid, and reliable screening methods.

The Food and Drug Administration (FDA) from the United States of America (USA) established a liquid chromatography–mass spectrometry (LC–MS/MS) method to detect melamine in infant formula. This technique is accurate, but it is time consuming and requires hazardous solvent [7,8]. Many researchers have reported analytical methods for melamine detection and quantification, such as gas chromatography-single quadrupole mass spectrometry [9], a spectrophometric method [10], liquid chromatography tandem mass spectrometry [1,4,11–14], and an enzyme-linked immunosorbent assay [7]. However, these most available methods are time-consuming,

List of abbreviations: ESI–MS/MS, electrospray ionization tandem mass spectrometry; FDA, Food and Drug Administration; HB, hydrogen bond; HMF, 5-hydroxymethylfurfural; HTST, high temperature short time; LOQ, limit of quantification; LOD, limit of detection; LC–MS/MS, liquid chromatography tandem mass spectrometry; R^2, linearity; USA, United States of America

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labor-intensive and expensive. Therefore, to simplify the sample preparation and improve the methods for detecting melamine, it is important to monitor contamination and prevent damage to consumer health.

Recent advances in ambient ionization mass spectrometry facilitate direct, rapid, real-time, and high-throughput analyses with little or no sample pretreatment [19,20]; it has been used to characterize food and identify adulterants, and authors report it as a reliable method [21,22]. In this paper, we proposed a simple and rapid sample preparation to detect melamine in powdered milk using electrospray ionization tandem mass spectrometry (ESI–MS/MS). Further, the research was extended to infrared spectroscopy and microscopy analyses to identify the nature of the precipitate formed during extraction.

2. Material and methods

2.1. Sample and reagents

Powdered milk was prepared from pasteurized milk (Grain Lait, Perdões, Brazil; high temperature short time [HTST]). Melamine was obtained from Sigma (St. Louis, USA) with chemical purity of greater than 98% and dissolved in Milli-Q purified water.

2.2. Powdered milk preparation

Triplicates of powdered milk were produced from pasteurized milk concentrated in a rotary evaporator (70 °C, TE-0581, TE-210, Tecnal, Piracicaba, Brasil) until the concentration of 52% of total solids; thereafter, the concentrate was dried in mini spray dryer (MSD 1.0, Labmaq, Ribeirão Preto, Brasil). The temperature for the inward and outward drying air was 160 and 100 °C, respectively, and the feed flow rate was 0.79 L h^{-1} in system with dual fluid nozzle atomization.

2.3. Melamine-added milk standards

Melamine stock solutions (500 mg L^{-1}) were prepared by dissolving melamine in water. The powdered milk was diluted in water and contaminated with melamine at different concentrations (0, 2.5, 5, 10, 15 and 20 mg L^{-1}).

2.4. Melamine extraction

Three extraction procedures were evaluated. The first pretreatment was performed in accordance with the methodology described by Desmarchelier et al. [12]. The powdered milk (1 g) and melamine aliquots were placed into a falcon polypropylene tube that was mixed and allowed to stand for 10 min. The water (5 mL) and acetonitrile (5 mL) were added successively, and the resulting slurry was thoroughly mixed after each solvent was added. The slurry was further diluted with acetonitrile (30 mL) and water (10 mL) and placed onto an automated shaker for 5 min. The tube was then centrifuged at 4000*g* at room temperature for 10 min (Centrifuge, Beckman, GS-15R), and the liquid supernatant was used for direct injection into ESI–MS/MS.

The second pretreatment was performed by adjust to pH 4.6 with 2 mol L^{-1} sulphuric acid (pHmeter, Quimis, Q400AS); thereafter, the samples were filtered. The filtered aliquot (5 mL) was added to 24% trichloroacetic acid (5 mL) and left to stand for 15 min. The mixture was filtered and the permeate was divided in two parts. The first aliquot of extract was used for injection into ESI–MS/MS. The remaining portion (4 mL) was added to ethyl acetate (4 mL) followed by stirring (Vortex, Phoenix-AP56); thereafter, the supernatant was used for direct injection into ESI–MS/MS (the third pretreatment sample). The extraction steps are described in Fig. 1.

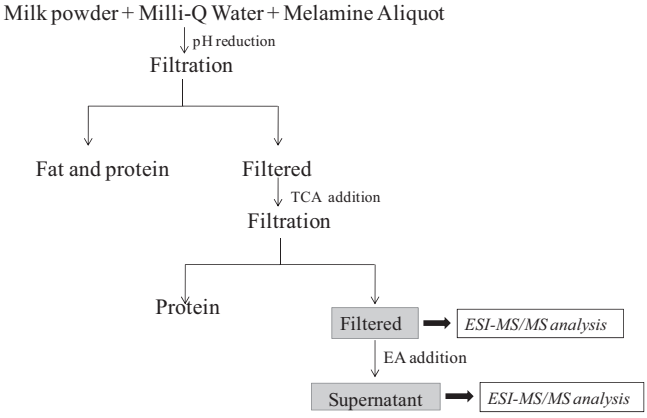

Fig. 1. The second and third pretreatment steps for powdered milk. TCA—trichloroacetic acid; EA—ethyl acetate; ESI–MS/MS—electrospray ionization tandem mass spectrometry.

2.5. ESI–MS/MS analysis

An aliquot of formic acid was added to yield 0.1% as the final concentration in the extract. Sample introduction was performed by using a micro syringe at a flow rate of 10 μL min^{-1}. To avoid cross-interference between samples, HPLC-grade methanol (Merck, Brasil) was injected between each infusion.

ESI–MS/MS analyses were performed using Agilent 1100 Series LC/MSD trap equipment in positive ion mode to identify the melamine. The spectra were obtained through 50 scans at 0.2 s intervals. The MS parameters were as follows: heat capillary temperature 350 °C, dry gas flow 6 L min^{-1}, nebulizer pressure 15 psi, and capillary voltage −3.5 kV. Mass spectra were acquired and accumulated over 60 s.

2.6. Method performance

The linearity (R^2) was evaluated from three calibration curves with six levels of melamine concentrations in powdered milk [23].

The limit of detection (LOD) and limit of quantitation (LOQ) were detected on blank samples ($n=10$) and calculated by Eqs. (1) and (2), respectively:

$$\mathrm{LOD} = 3\frac{sb}{b} \quad (1)$$

$$\mathrm{LOQ} = \frac{10sb}{b} \quad (2)$$

where *sb* is the standard deviation of the areas of the blank samples in mass spectrum, and *b* is the slope of the calibration curve [23]. The recovery and accuracy (%) were calculated according to Eq. (3), using blank matrices spiked with melamine at two fortification levels (8 mg L^{-1}; 12 mg L^{-1}).

$$R(\%) = \frac{(C_1 - C_2)}{C_3} \times 100 \quad (3)$$

where C_1 is the melamine concentration determined in adulterated milk, C_2 is the melamine concentration determined in blank sample, and C_3 is the concentration added [24]. The precision was calculated by the relative standard deviation (Eq. (4)).

$$\mathrm{RSD} = \frac{s}{x} \times 100 \quad (4)$$

where *s* is the standard deviation of replicate, and *x* is the mean of replicate. The selectivity was evaluated by the presence of melamine fragment MS/MS 85 [24].

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