HPTLC-MS analysis of melamine in milk: standardization and validation

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Abstract Melamine containing large amount of nitrogen may be deliberately added to milk in order to increase the protein content for economic adulteration in food stuffs. High performance thin layer chromatography-mass spectrometry (HPTLC-MS)-based method of quantification of melamine has been developed using Silica gel 60 F_{254s} HPTLC plates, with optimized mobile phase iso-propanol/dichloromethane/water, 5:2.5:3, v/v/v in a twin trough chamber saturated (5 min) at pH 6.8. Validation was carried out by testing its specificity, linearity (1–50 µg), accuracy (95.908–98.369%), inter- and intraday precision (1.578 and 1.670%), respectively, limits of detection (2.688 ng) and quantification (8.146 ng). HPTLC in combination with MS has been found suitable for routine analysis of melamine added as adulterant to market-purchased milk samples.

Keywords Melamine · Adulteration · Validation · HPTLC-MS

1 Introduction

Melamine (2, 4, 6-triamino-1, 3, 5-triazine, C₃H₆N₆) is used in the manufacturing of plastics, fertilizers, melamine-formaldehyde resins for surface coatings, laminates, fire retardant, and adhesives (Wen et al. 2010). Being enriched with nitrogen (66.7%), melamine is sometimes added to food or animal feed for increasing the apparent protein content (European Food Safety 2007) which is not measurable by Kjeldahl or Dumas method (Chan et al., 2008). Melamine content if added to the food stuff is associated with several health disorders like urolithiasis, tissue injury, and even bladder cancer (Newton and Utley 1978). Fatality of several infants in China due to the presence of high concentrations of melamine (0.1–2500 µg.mL⁻¹) in infant milk products has attracted worldwide attention (Sun et al. 2010). The USFDA has set tolerance level of less than 1 mg.L⁻¹ melamine in infant formula and maximum tolerance level of 2.5 mg.L⁻¹ in other foods (Smith et al. 2007).

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Analytical methods such as gas chromatography (Toth and Bardalaye 1987), liquid chromatography/mass spectrometry (Fligenzi et al. 2007), diode array thin layer chromatography (Broszat et al. 2008), capillary electrophoresis (Garber 2008), and enzyme-linked immunosorbent assay (Shan-Shan et al. 1999) are officially recognized reference methods for the detection of melamine contamination in food stuffs. However, in order to develop a faster, low-cost, and routine method for economic adulteration of melamine in milk particularly for the laboratories not having expensive LC-MS infrastructure, high performance thin layer chromatography (HPTLC) is proved to be a boon. It possesses the necessary flexibility for combining various complementary detection modes such as mass spectrometry (MS), which has proven to be useful to identify such adulterant in food stuffs. The present short communication reports a HPTLC-MS method for the detection of melamine in milk.

2 Materials and method

2.1 Chemicals and reagents

Aluminum-baked Silica gel 60 F_{254s} HPTLC plates obtained from E. Merck, Darmstadt, Germany; standard melamine (Sigma-Aldrich Chemicals); and isopropanol, dichloromethane, and HPLC grade water (Sigma Aldrich Chemicals) were used. All the organic solvents used in the study were of analytical grade.

2.2 Instrumentation

High performance thin layer chromatograph (Camag, Muttanz, Switzerland) consisted of an auto sample applicator Linomat V connected to a nitrogen cylinder, TLC scanner attached to a PC running win-CATS software (version 1.4.4), TLC Visualizer, and Camag twin-trough chambers were used in analysis.

HPTLC-MS analysis was carried out by TLC-MS interface using acetonitrile as eluting agent at a flow rate of 1 mL.min⁻¹. It extracts circular zones in the form of bands from the developed HPTLC plate. The eluted material was automatically transferred to single-quadrupole mass spectrometer, and mass spectra was recorded with capillary voltage +6 kV to -6 kV, nebulizer gas pressure 20 psi, flow rate 10 L.min⁻¹ at temperature 300 °C, fragmentator voltage 100 V, gain 1, threshold 10, and step size 0.25.

2.3 Standard solution preparation

Melamine was dissolved in MeOH/H₂O (1:1, v/v) to prepare standard stock solution (1.0 mg.mL⁻¹). Stock solution was diluted twice its volume to prepare working solution (0.5 mg.mL⁻¹) and stored at 4 °C.

2.4 Sample preparation

Milk samples, collected from two different dairies in Agra, India, were spiked with known concentration of melamine (2–12 µg) to prepare seven aliquots. The spiked sample solutions were sonicated (Ultrasonic bath, Systronics) for 15 min using

ultrasonic bath at 20 kHz. Samples were filtered (Whatman filter paper, 0.50 μm) and were used for analysis.

2.5 Chromatographic analysis

HPTLC plate (20 $\times$ 10 cm^2 ; 0.2 mm thick coated with silica gel 60 F_{254s}) containing 15 tracks of samples and standards were used under following conditions: band width 8 mm, 11.3 mm apart from each other, 10 mm from lower edge, and 15 mm from left and right edge of the plate sprayed using Linomat V auto sample applicator fitted with 100 μl syringe at temperature 22 ± 2 $^{\circ}\text{C}$ and relative humidity $55 \pm 5\%$. Loaded plates were developed to a distance of 80 mm using mobile phase (iso-propanol/dichloromethane/water, 5:2.5:3, v/v/v) in Camag twin-trough plate development chamber (Broszat et al. 2008). It was lined with a filter paper and pre-saturated for 5 min with mobile phase (10 mL) before use. The developed plate was dried using warm air and scanned at 200 nm. Densitometric measurements, spectra recording, and data processing were carried out using planar chromatography manager. UV scanner was optimized as: slit dimension, 6.00 mm $\times$ 0.30 mm (micro); scanning speed, 100 nm.s⁻¹; and data resolution, 1 nm per step. Regression analysis and statistical data were automatically generated by the win-CATS software. The plate images were recorded using HPTLC Visualizer at 254 nm. Melamine was chromatographed and quantified on HPTLC plates and further confirmed by mass spectrometer.

2.6 Method validation

Various parameters (specificity, linearity, accuracy, precision, LOD, LOQ, and robustness) for validation of the method were considered in accordance with ICH Guidelines (1999).

Fig. 1 UV Spectra for standard melamine and S1–S7 were spiked milk samples

Fig. 2 HPTLC-MS of spiked milk sample having characteristic peak at $m+1$ for melamine

3 Results and discussion

3.1 Method development

The proposed HPTLC method was optimized for separation and resolution based on the simulation for chemical composition of mobile phase, nature of stationary phase, time of saturation, and pH. Standardized conditions for HPTLC analysis were as follows: optimized mobile phase, iso-propanol/dichloromethane/water; 5:2.5:3; v/v/v; stationary phase, Silica gel 60 F₂₅₄; time of saturation, 5 min at pH 6.8. Densitometric scanning at 200 nm resulted into a single peak of standard melamine at R_f value 0.57 ± 0.02 . Visualization was carried out at 254 nm and image was recorded. After optimization, adulterated test samples 15 μ L were loaded on the same plate and quantified for accuracy. The matching of UV spectra of standard melamine among the observed peaks in the adulterated test samples was considered a basis for the presence of melamine adulteration (Fig. 1). The confirmation of the peak at R_f 0.57

Fig. 3 **a** Figure showing no peak in blank solution; **b** peak of melamine in spiked milk sample at 50 ng concentration

Fig. 4 Calibration curve of standard melamine and quantification of spiked samples

for melamine present in spiked test samples was done by recording its characteristic molecular ion peak ($m+1$) at 127.23 amu using HPTLC-MS study (Fig. 2).

3.2 Method validation

A sharp peak of standard melamine was observed exhibiting no interference of impurities indicating the specificity of developed method (Fig. 3). The calibration curve of the standard melamine working solution was obtained by plotting the peak area versus concentration with the help of win-CATS software (Fig. 4) and linearity was obtained in the range 1–10 μL or 1–10 μg , with the linearity parameters as slope, 3461.734; intercept, 1926.009; standard deviation, 2.82%; regression coefficient (r^2), 0.99929. Milk samples were spiked with standard melamine working solution, and percentage recovery was measured at three loadings (10, 15, and 20 μg) on the plate (Table 1). The accuracy of the method was found to be in the range (95.908–98.369%). For intraday precision, the protocol was carried out repeatedly three times under similar experimental conditions, while for interday precision, the protocol was repeated for five consecutive days to get the changes in the peak area and results were reported in terms of coefficient of variability which was found in the range 1.69–1.72% and 0.420–0.569% respectively. The LOD and LOQ were calculated as 2.688 and 8.146 ng, respectively. Robustness of the method was determined by performing the same analysis using $\pm 2\%$ variations in mobile phase composition, absorption wavelength, and pH (Table 2). The statistical data obtained indicate that these minor modifications

Table 1 Measurement of accuracy of the proposed HPTLC-MS method

Concentration of melamine in 25 mL of milk samples (ng per band)	Mean peak area $\pm$ SD	Concentration recovered (ng per band)	Percent recovery
10	2546 $\pm$ 0.54	33.56784	95.908
20	3405 $\pm$ 0.24	44.98763	97.804
30	4264 $\pm$ 0.76	54.10298	98.369

Table 2 Measurement of robustness of the proposed HPTLC-MS method

No.	Parameters	Rf	Peak area
1	Iso-propanol/dichloro methane/water (5.5:2.5:3, v/v/v)	0.57	12,815
2	Iso-propanol/dichloro methane/water (5:2.5:3, v/v/v)	0.55	12,475
3	Iso-propanol/dichloro methane/water (4.5:2.5:3, v/v/v)	0.54	11,987
4	Absorption wavelength 198 nm	0.56	13,465
5	Absorption wavelength 200 nm	0.57	14,120
6	Absorption wavelength 202 nm	0.57	14,998
7	pH 7.0	0.57	10,298
8	pH 6.8	0.53	11,910
9	pH 7.2	0.58	10,926

with the experimental parameters did not affect the analysis and accurately and precisely quantify the melamine content in samples.

4 Conclusion

The present short communication reports a simple HPTLC-MS method for the routine analysis of melamine in milk. The methodology involved in the present paper avoids chemical steps and wet chemistry like chlorination and derivatization under 12 principles of green chemistry and provides a comparatively simpler and rapid method for routine analysis of milk samples.

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Conflict of Interest Roopa Rani, Sharad Medhe, and M M Srivastava declare that they have no conflict of interest and are grateful to the Ministry of Human Resource and Development, Govt. of India for rendering financial assistance. This article does not contain any studies with human or animal subject performed by any of the authors.

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