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Identification & Estimation of Melamine Residue in Powdered Milk by RP-HPLC

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ABSTRACT

Melamine is a toxic triazine used as an adulterant in milk & milk related products to increase the protein content. RP-HPLC method has been developed using water as mobile phase to detect and quantify the melamine residue in powdered milk. Melamine was extracted by precipitation of milk protein at its isoelectric point with dilute acids. Samples were purified by using membrane filter of 0.45 µm pore size, AmChemteq ACI C_{18} (4.6mmx250mm, 5µm) column as the stationary phase. The linearity curve was constructed for the concentration of melamine ranging from 1 mg/kg to 25 mg/kg. The correlation co-efficient was found to be 1.000. Ten marketed powdered milk brands were studied by this method. It was observed that all the ten brands were found to contain melamine residue ranging from 0.00001 mg/kg to 0.00006 mg/kg. The results were below the limits set by Food Safety and Standards Authority of India. This is the first study to confirm the existence of melamine residue in powdered milk marketed in Tamilnadu.

INTRODUCTION

Melamine is a polar organic compound with a 1, 2, 3- triazine. It's commonly used in the production of plastics plates, in finishers for paper, in fertilizer, as a flame retardant, in the manufacture of wrinkle-free textiles Garber [1]. In early 2007, it became a topic of significance as hundreds of pet deaths occurred due to pet food contamination. In September 2008 [2], the World Health Organization (WHO) reported that there have been six infant deaths, and more than 1 lakh of infants and young children in China were hospitalized for urinary problems [3], with possibility of renal tube blockages, the said problems were attributed to the consumption of melamine contaminated infant formula and related dairy products. Researchers confirmed that melamine tableware also releases melamine when used to serve hot foods. In veterinary medicine cyromazine is used as a pesticide/ectoparasiticide which is added to animal feed to chase fly from the manure. Some data have shown that the carry-over occurs from feed to products of animal origin including milk, eggs, meat, and fish [4]. Contamination of melamine into the food chain at baseline level is possible, as it is present in the environment due to widespread use of melamine-containing materials. Several detection methods for melamine have been reported, which includes GC, HPLC, GC-MS, and LC-MS. All the reported methods consume large quantities of organic solvents as mobile phases [5, 8, 9-10]. Risks associated with these solvents extend beyond direct implications as the health of human wildlife and the ecosystem. The present study is eco-friendly and makes use of green chemistry approach. The work presented is first of its kind to be reported in Tamilnadu.

MATERIALS AND METHODS

Chemicals, Reagents & Equipments: Standard Melamine (99 % purity) was purchased from Sigma-Aldrich, Ultra-pure water was obtained from a Millipore system, disposable syringe of pore size 0.45 µm, ultrasonic bath, centrifuge and HPLC (Agilent system).

HPLC system for Analysis: Melamine was determined by HPLC Agilent system equipped with 515 pump from (Milford, MA,USA) stainless steel filter, AmChemteq ACI C_{18} column (5 um, 250 mm x 4.5 mm) was used throughout this study. Agilent column heater module with Agilent Temperature control module, Absorbance Detector, Agilent Automated Gradient Controller associate with Dell computer system using Chem Station software. The temperature of the oven

was set up 25°C. The concentration of the products were determined from the peak areas of the curve using Agilent software for instrument control and data collection. Extraction was performed by precipitating the milk protein. The milk proteins were precipitated by a novel method making use of their isoelectric pH 4.6 by acidification, then centrifuged & filtered.

Chromatographic conditions

Analytical Column: AmChemteq ACI C_{18} (5 µm, 4.6 X 250 mm)
Preparation of Mobile Phase: 100 % HPLC water
Column Temp: 25°C
Flow Rate: One ml per minute
Injection Volume: 20 µL
UV Detection: 210 nm

Sample Collection: A total number of 10 marketed powdered milk samples were collected from various places of Tamilnadu.

Preparation of melamine standards: A 1000 µg/ml melamine stock standard was prepared by accurately weighing 100 mg of melamine into a 100 ml volumetric flask with Millipore water. The stock solution was diluted appropriately to contain variable concentration of 1.00, 5.00, 10.00, 15.00, 20.00, 25.00 µg/mL of melamine.

Sample preparation: 2g of powdered milk sample was homogenized with 100 ml of water, acidified with dilute hydrochloric acid to a pH - 4.6 & centrifuged (setting rpm to 6000 for 30 minutes). The resulting supernatant solution was filtered with syringe filter and injected.

Method: The standard solution of melamine of varied concentration was injected by using Rheodyne injector into the column. Detection of the eluate was carried out at 210 nm. Similarly the sample solution of suitable concentration was also analyzed using the system. The Retention time was found to be 5.8 for both sample and standard.

Recovery study: To study the accuracy and precision of the proposed method, recovery studies were performed on spiked samples at two levels. The percentage recovery range of samples were found to be 95% - 103%.

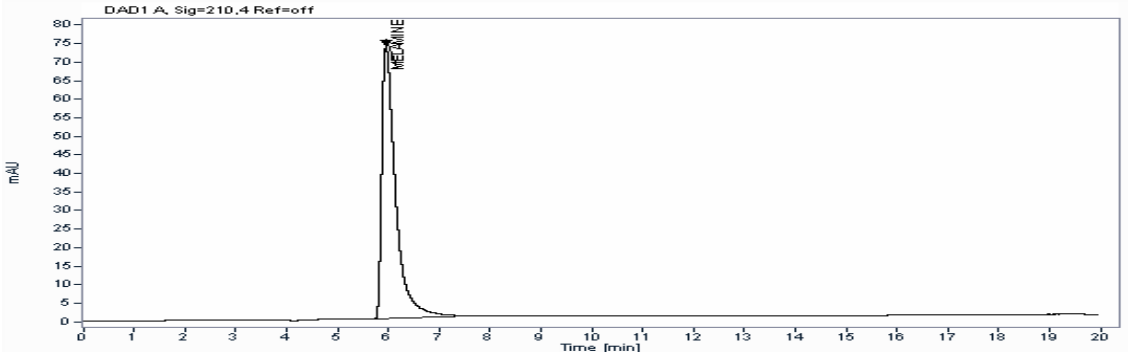

Figure 1: Chromatogram of melamine standard

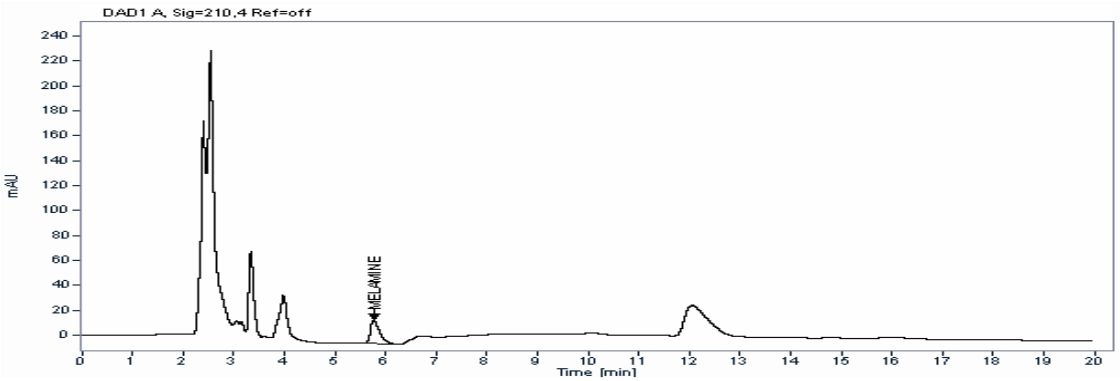

Figure 2: Chromatogram of powdered milk sample

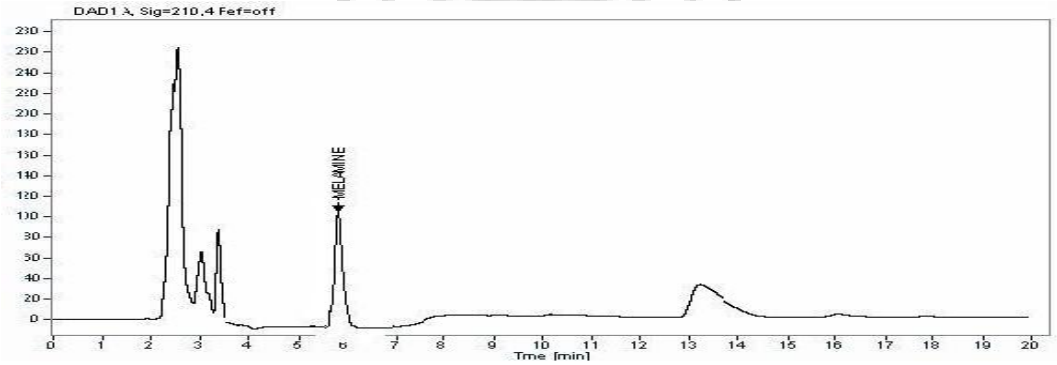

Figure 3: Chromatogram of Spiked powdered milk

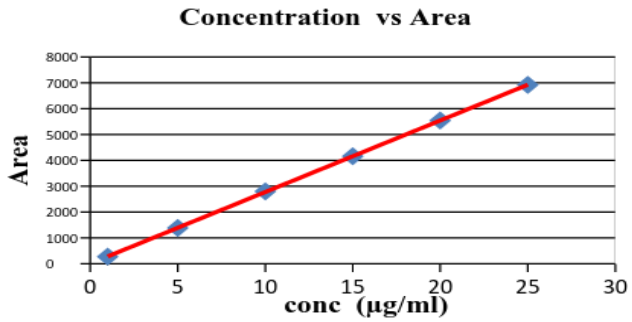

Figure 4: Calibration curve for melamine standard

Table 1: System suitability parameters

Sr. No.	Parameter	Values
1	Theoretical plates	4109.6
2	Tailing factor	1.19
3	Linearity (µg/ml)	1-25
4	Correlation coefficient (r^2)	1.000
5	Limit of Detection (LOD)	100 µg/kg
6	Limit of Quantification (LOQ)	300 µg/kg

Table 2: Detection of melamine in powdered milk Samples

Name of sample	No. of sample	% of positive sample	Melamine residue in (mg/kg) #	Limit by FSSAI (mg/kg)
Powdered milk sample	Brand 1	100 % (all the 10 samples contain melamine residue)	0.00006	Not More Than 2.5 mg/kg
	Brand 2		0.00004	
	Brand 3		0.00001	
	Brand 4		0.00002	
	Brand 5		0.00002	
	Brand 6		0.00001	
	Brand 7		0.00002	
	Brand 8		0.00003	
	Brand 9		0.00003	
	Brand 10		0.00002	

Mean of three determinations.

Table 3: Recovery study of spiked milk sample

Name of the Sample	Number of Sample	Percentage of recovery	Percentage Recovery range
Powdered Milk Sample	Brand 1	96 %	95 % - 103 %
	Brand 2	98 %	
	Brand 3	95 %	
	Brand 4	96 %	
	Brand 5	98 %	
	Brand 6	102 %	
	Brand 7	96 %	
	Brand 8	95 %	
	Brand 9	103 %	
	Brand 10	102 %	

RESULTS AND DISCUSSION

Preparation of powdered milk sample for melamine detection and quantification consists of 2 steps which involves sample extraction and purification on activated SPE column. But the optimized procedure used in this study is simple and efficient, and does not require purification by SPE, The reconstituted powdered milk was precipitated with dilute hydrochloric acid, subsequently centrifuged and filtered. The infant formula should not contain melamine more than 1 ppm and other foods more than 2.5 ppm as per Food Safety and Standard Authority of India [6]. WHO in 2008 has also specified that the tolerable daily intake of melamine is 0.2 mg/kg [7]. The recovery studies were performed at two levels, the percentage recovery of the spiked milk sample is shown in table 3. The limit of detection is 100 µg/kg and limit of quantification is 300 µg/kg. The marketed brands of powdered milk analyzed by the proposed method were found to contain less than 2.5 mg/kg.

CONCLUSION

The present study showed that the milk samples had an acceptable level of melamine and complied with FSSAI. Recent product recalls and food safety incidents due to melamine adulteration or contamination have caused a worldwide food security concern. This has led to many methods being developed to detect melamine in foods, but few methods have been reported reliable measurement of melamine in environmental samples. The suggested method is simple, sensitive and robust and allows for analysis of large number of samples, without

degradation in column performance. The proposed method could be used for routine analysis of melamine.

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