Analysis and Quantification of melamine in Cow milk collected from different Milk Diaries using cation-exchange based liquid chromatography and tandem mass spectrometry.

Shalini Sharma, Dr.Rajeshwari shivaraj, Dr.A.Radhakrishna

Abstract

Melamine and cyanuric acid have been implicated as adulterants in baby formula in China and pet foods in North America. In China, the effect of melamine or melamine–cyanuric acid adulteration leads to kidney stone development and acute renal failure in thousands of Chinese infants. A selective and sensitive analytical method was developed to measure melamine in Cow milk in order to evaluate the extent of potential health implications resulting from the consumption of these types of adulterated products in the general Indian population. This method involves extraction of melamine from Cow milk using cation-exchange solid-phase extraction, chromatographically separating it from its matrix co-extracts on a silica-based, Inertsil ODS-3 C18 (150mm x 4.6mm x 3µm analytical column using liquid chromatography, and analysis using positive mode electrospray ionization tandem mass spectrometry. Quantification was performed using modified, matrix-based dilution calibration covering the concentration range of 0.50–10 ng/ml. The limit of detection, calculated using replicates of blank by S/N ratio was 0.2 ng/ml and the limit of Quantification was 0.6 ng/ml. The relative recovery of melamine was 113 to 117%. This method was tested for viability by analyzing 80 samples of milk collected from Cows in different Milk dairies located in East, West, North, and south local zone, India. Melamine was detected in traces for 17% of the samples tested, with an Arithmetic mean of 1.31 ng/ml, values which are below the limits set by the US FDA, indicating that this method is suitable for reliably detecting background exposures to melamine or other chemicals from which it can be derived.

Key words: Melamine, Milk, LC-MS/MS, Cow, Zone, Dairy, India.

1 INTRODUCTION

Melamine is a polar organic compound with a 1, 3, 5-triazine structural formula (Fig.1). It is an industrial chemical frequently used as a raw material for the production of multipurpose Melamine –Formaldehyde resins. It is also a common additive in fertilizers because of its Nitrogen rich properties [1]. It is also formed by the degradation of cyromazine, insecticides widely used in crop protection. In September 2008, several children suffered by the toxicity of melamine and several companies have been implicated in the adulteration of milk and infant formula with melamine. Because melamine comprises of 66% Nitrogen, it was used as an additive to fraudulently inflate the detected protein level. Acute toxicity of melamine is found low, it is significantly increased when

administered together with its structural analogue, cyanuric acid (2,4,6-trihydroxy 1-3,5-triazine). Melamine and cyanuric acid formed hydrogen bonding, as a result insoluble melamine-cyanurate crystals which can deposit in the kidney tubules, thus causing damage of renal tissue. Kidney stones and other renal complications due to ingestion of melamine contaminated foodstuffs. [2-3].
As of November 2008, the USFDA has set a 'zero tolerance' level for melamine in infant formula and baby foods; and a maximum tolerance level of 2.5 mg/kg [4, 5] in other foodstuffs. It has been assumed that melamine can yield cyanuric acid by degradation or by the action of microorganisms [6].

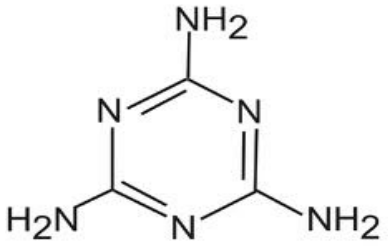

Fig.1 Melamine structure

As a result of the above scandals of melamine adulteration, food/feed safety issues have led to the need for rapid, easy, cheap and reliable analytical methods capable of detecting target analytes at the levels desired by regulatory authorities [7]. Several analytical methods have been developed based on extraction procedures. The reported methods for quantitative determination of melamine include enzyme immunoassay (EIA), IR analysis, gas chromatography-mass spectrometry (GC-MS), Liquid chromatography (HPLC) with UV detection [8]. This requires derivatization, pretreatment and also shows poor sensitivities, etc., which is a time consuming and reduction of analyte concentration [9-10].
In earlier studies Melamine concentration in the breast milk of 77 healthy mothers in Ankara, Turkey, was analyzed by HPLC in accordance with the body mass index (BMI) of the mothers. In 20.78% of the breast milk samples, the concentration of melamine was found to be between 10.09 and 76.43 ng/l, values which are below the limits set by the WHO, CAC and EU authorities. These data provide preliminary evidence suggesting that the presence of trace amounts of melamine in breast milk is unlikely to be a significant health concern and constitutes a basis for meta-analysis in the future [11].

In this study the extraction, analysis, and quantification of melamine in milk was done. These milk samples were collected from different milk dairies located in four different local zones as East, West, North and South. Analysis of melamine was done by using silica based strong cationic exchange sand material as a cleanup technique with C18 Inertsil ODS column as stationary phase and liquid chromatography & Tandem mass spectrometry LC-MS/MS analysis technique.

2 MATERIALS & METHODS

2.1 Standards and chemical

Melamine reference standard material (purity 99.0%), was received from Sigma Aldrich, Formic acid HPLC grade purchased from Merck, Ammonium Formate LCMS grade purchased from Bio solve, Millipore water Purified with the use of Milli-Q-Purification system, Acetonitrile LCMS grade from Labscan, potassium dihydrogen o-phosphate AR grade purchased from seed-fine chemicals, Ammonia (35%) AR –grade purchased from Merck.

2.2 General equipment

Microbalance used from Sartorius M2P, Analytical Balance -Afcoset ER200A, Centrifuge REMI R-24, Micro centrifuge from REMI- RM12C, Sonicator from S. V. Scientific and Vortex Mixer used from Sonca

2.3 Chromatographic equipment

The LC system coupled with tandem mass spectroscope (MS-MS) from Agilent Triple- quad 6460 controlled by Mashunter software.

2.4 Chromatographic conditions

The chromatographic separation was accomplished on an Inertsil ODS C18 analytical column (150mm x 4.6mm I.D. x 3 µm particle size). The LC-MS/MS mobile phase were A: 0.1 % Formic acid in Acetonitrile, B: 20mM Ammonium Formate and Acetonitrile (1:1).
Minimization of sample preparation and automatization of analytical process is a crucial prerequisite to enable high throughput analysis of melamine. Melamine is a polar compound with a pKa of 5.6 and a log p value of -1.37 [12], making a good candidate for aqueous normal phase chromatography. A polar compound may then partitioned on from the moving organic rich mobile phase into the stagnant aqueous solvent.Pump Programming: Gradient, Run 60% A for 4 min to 6 min, 80% A for 6.1min to 10 min, again brings it to equilibrium by running A for 60%, 10 to 12 minute. LC-MS/MS source parameters were well defined (Table 1).

Table-1
Source Parameters optimized

Instrument	LC-MS/MS Agilent 6460 Triple quad

	LC-MS/MS Varian 1200 L Triple quad
Injection volume	5µl
Ionization Mode	ESI Positive
Drying gas temperature	250°C
Gas flow	5 L/min
Nebulizer	45 psi
Sheath gas temperature	350°C
Sheath gas flow	11 L/min
Capillary voltage	4000V
Nozzle voltage	0

2.5 Standard preparation

2.5.1 Melamine stock solution

Precisely weighed melamine (100mg) was dissolved by sonication for 30 minutes in a 100ml volumetric flask with Millipore water. The concentration of melamine standard stock solution was 1000 mg/Kg.

2.5.2 Intermediate Melamine stock solution

This was prepared by diluting 1ml of the *melamine stock solution to* 100ml with Millipore water to give a melamine concentration of 10mg/kg. For further dilutions, the same standard stock solution was applied for the formulation of solvent calibration standards with the mixture of 0.1% Formic acid in Acetonitrile. All the solution was sonicated for 10 min on ultrasonic bath.

2.5.3 Working Melamine solution

This was prepared by diluting 1ml of the *Intermediate melamine stock solution to* 100ml with the mixture of 0.1% Formic acid in Acetonitrile to give a melamine concentration of 100µg/kg. For further dilutions, the same Working Melamine solution was applied for the formulation of solvent calibration standards with the mixture of 0.1% Formic acid in Acetonitrile.

2.6 Reagent Preparation

a) 100 mM Phosphate Buffer: prepared by dissolving 1.36 gm of KH_2PO_4 in 100 ml Milli-Q water and adjusted the pH to 2.5

b) 20 mM Ammonium Formate: dissolved 0.63 gm Ammonium Formate in 500 ml of Milli-Q water

c) 5% Ammonia in methanol: 20 ml of 25% Ammonia solution and made up to 100 ml in methanol.

d) 0.1% Formic acid: 0.1 ml of Formic acid in 100 ml volumetric flask and made up the volume to 100 ml with Milli-Q water.

2.7 Sample Collection

India is the largest producer of milk. Each and every part of India is contributors for this white revolution. There are so many local cow milk dairies available in the city market. For our experiment we used four different zones as East, West, North, and South for collection of milk. Twenty samples of milk were collected from every zone, individually five each, weekly for four weeks. So, total eighty samples of milk were collected (fig. 2).

Fig.2 Milk samples collected from local milk dairies (Set-I)

2.8 Sample Preparation

Mixed uniformly and homogenized the contents of the sample container. Weighed 5 gm of milk in a centrifuge tube and diluted with 5ml 100 mM phosphate buffer, pH 2.5 and 1 ml Acetonitrile. The sample was sonicated for 5 min using an ultrasonic water bath followed by centrifugation at 3500 RPM for 10 minutes. The supernatant layer was isolated for further cleanup.

2.8.1 Cleanup procedure

- Took 1 gm of sand material (Fine pore size-Grade-II) in a syringe with cotton plug in the mouth without plunger
- Conditioned and equilibrated sand containing syringe with 3 ml methanol followed by 3 ml 0.1% Formic acid.
- Loaded sample (derived from sample pretreatment)
- Washed the sand containing syringe with 3 ml 0.1 % Formic acid followed with 3 ml Methanol.
- Eluted melamine from sand material with 5 ml ammonia in methanol.
- Evaporated the eluent to dryness at 5 psi and 50°C, reconstituted in LC Mobile phase A.

2.9 Melamine peak identification

The mass spectrum of melamine (fig. 3) exhibit the molecular ion at m/z 127 (parent ion) while 85, 68 and 43 expressed as daughter ions (Table 2). The standard of

melamine was analyzed along with samples on the same day to confirm the identification. Melamine recovery studies also performed to show method's accuracy. The standard was resolved and eluted at 6.6 min with respect to melamine

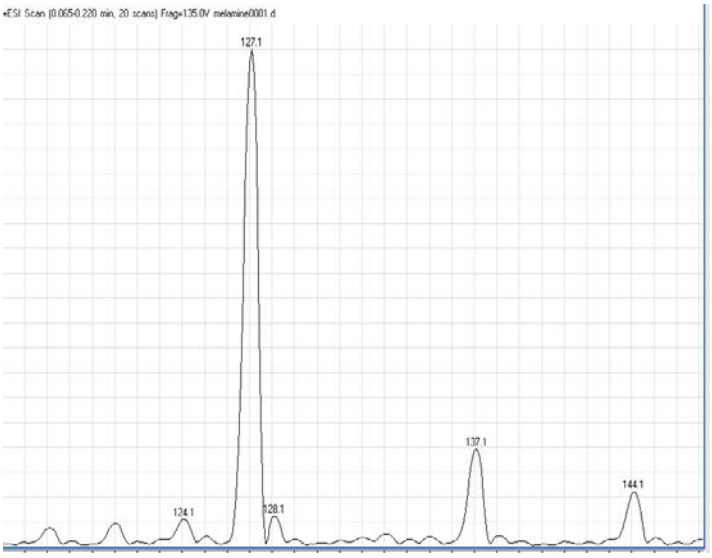

Fig.3 Melamine Mass breakdown

Table-2 Mass fragments monitored:

Reference Standard	Precursor Ion (m/z)	Product Ion (m/z)	Dwell	Fragmentor Voltage (V)	C.E(V)	Polarity
Melamine	127.1	85	100	95	16	Positive
	127.1	68.1	100	95	32	Positive
	127.1	43.1	100	95	36	Positive

3 RESULTS AND DISCUSSION

India has wide diversities in the field of agriculture. Day today farmers use lots of pesticides, insecticides and other chemicals for their crop protections. These chemicals were, however beneficial to farmers in their crop protection whereas certain chemicals on as is basis and their degradation products are harmful to living beings. Melamine is also a degradation product of urea and cyromazine. When some of the animals during their grazing, or taking in animal feeds, chances of melamine introduction in their body may take place as a consequence of this nephrotoxicity may occur. Also, when animals like Cow, Buffalo, and Goat etc. faces the same situation, chances of melamine release in milk might take place. And as a result, while consumptions of this contaminated milk with melamine, causes nephrotoxicity in human too, if the level of melamine is more than the tolerance level.

So this paper describes the analysis of milk samples collected from cow to study the melamine contamination. Many Cow milk dairies are available in India. Cow milk is usually fed to infants who, as has been shown in China, are a vulnerable group. Testing methods, thus need to be developed to determine if such additives are present and at what levels. In our present experiment, all the tested samples (80) were collected from the cow from different milk dairies. These milk samples were collected in sterile glass bottles stored in refrigerated conditions.

3.1 Linearity

A linear calibration plot (Fig. 4) is obtained over the calibration range of 0.5 to 10µg/kg with a correlation coefficient (r2) of 0.999. Both solvent based standards and fortified samples are used for calibration for day I, day II, and day III analysis. The % R.S.D. values of the repeated injections are <2% within each concentration and the y-intercept bias is less than 2%.

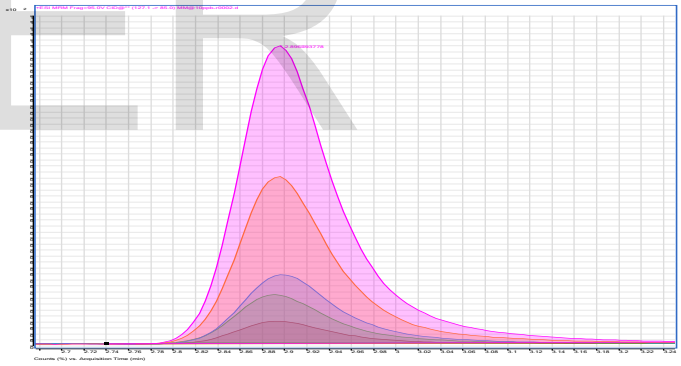

Fig.4 Melamine Linearity graph

3.2 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was determined to be 0.2µg/kg and the LOQ was determined to be 0.6µg/kg. The % R.S.D. of the precision study carried at the LOQ level was within 5%.

3.3 Accuracy

The percentage recovery of melamine in milk samples ranged from 113 to 117% (Table 3). The method shows re

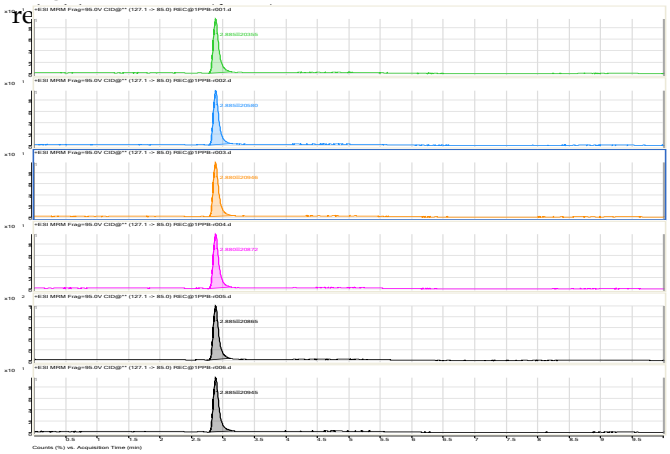

Fig.5 Melamine Recovery graph

Table-3

Recovery results of melamine spiked at three different concentration 0.5µg/kg, 1 µg/kg and 10µg/kg

Melamine spiked concentration (µg/kg)	% Recovery
0.5	113.17
1.0	108.34
10.0	117.39

3.4 Sample analysis

Hundred milk samples were analyzed for identification of melamine contamination. Every twenty samples per week, five from each diary were classified as set I, Set II, Set III and Set IV. Fourteen milk samples were tested for the presence of melamine every week collected from different milk dairies. Two milk samples contain melamine in North zone, six samples contain melamine in East zone, three milk samples contain melamine in West zone, and three milk samples contain melamine in South zone. Total 14 samples were found positive for the presence of melamine whereas 61 milk samples shows negative for melamine contamination (Table 4)

Table 4
Analysis of Melamine in Cow Milk by LC-MS/MS

Local Zone	Melamine Detection,(ppb)			
	I SET	II SET	III SET	IV SET
Sample North-N1	0.95	Nil	Nil	Nil
Sample North-N2	0.82	Nil	Nil	Nil
Sample North-N3	Nil	Nil	Nil	Nil
Sample North-N4	Nil	Nil	Nil	Nil
Sample North-N5	Nil	Nil	Nil	Nil
Sample East-E1	Nil	Nil	Nil	Nil
Sample East-E2	Nil	Nil	Nil	Nil
Sample East-E3	Nil	Nil	Nil	Nil
Sample East-E4	1.09	Nil	1.79	0.92
Sample East-E5	1.15	Nil	0.79	1.01
Sample West-W1	1.12	Nil	2.82	Nil
Sample West-W2	0.90	Nil	Nil	Nil
Sample West-W3	Nil	Nil	Nil	Nil
Sample West-W4	Nil	Nil	Nil	Nil
Sample West-W5	Nil	Nil	Nil	Nil
Sample South-S1	1.15	Nil	Nil	Nil
Sample South-S2	Nil	Nil	Nil	Nil
Sample South-S3	Nil	Nil	Nil	Nil
Sample South-S4	Nil	Nil	Nil	Nil
Sample South-S5	Nil	1.07	3.67	Nil

4 CONCLUSION

A selective and sensitive analytical method was developed to measure melamine in Cow milk in order to evaluate the extent of potential health implications resulting from the consumption of these types of adulterated products in the general population. For analysis four different local zones as East, West, North, and South were selected for collection of milk samples. Twenty samples of milk were collected from every zone; individually five each, weekly for four weeks. Eighty milk samples were analyzed for identification of melamine contamination. Total 14 samples were found positive for the presence of melamine whereas 66 milk samples shows negative for melamine contamination. The method was found linear over a concentration range of 0.5 to 10µg/kg. The percentage recovery of melamine in milk samples ranged from 113 to 117%. This LC-MS/MS method for analyzing melamine in milk samples is cost effective, fast, and sensitive.

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Shalini sharma
Karpagam University, Polachi road, Coimbatore, Tamilnadu, India Email: shalinisharma1984@gmail.com

Dr.Rajeshwari shivaraj
Head of the department of Biotechnology, FCS, Karpagam University, Polachi road, Coimbatore, Tamilnadu, India
Email: rajeshwarishivaraj@gmail.com

Dr. A.Radhakrishna
Joint Director, Shriram Institute for Industrial Research (A unit of Shriram Scientific) Plot No. 14& 15 Sadarmangala Industrial area, Bangalore-560048, India Email: ark@shriraminstitute-bangalore.org