International Journal of Scientific Engineering and Research (IJSER)
www.ijser.in
ISSN (Online): 2347-3878
Volume 2 Issue 5, May 2014

The Novel Method of Extraction and Analysis of Melamine in Whole Milk Using Sand Material Cleanup Technique and Inertsil C18 Column by LC-MS/MS

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Abstract: ***A simple, precise, accurate and validated Liquid chromatography and Tandem mass spectrometric (LC-MS/MS) method is developed for the analysis of melamine in whole milk. Because of melamine's basic and polar nature, a cation exchange natural sand material is used as cleanup technique. As a result, enhancement in recovery, sensitivity and selectivity has been observed as well made the analysis much cheaper by replacement of use of cartridge. A simple extraction of melamine with phosphate buffer (in acidic condition) and Acetonitrile (precipitation of the protein), matrix cleanup using silica based strong cationic exchangers (sand) and reconstitution with 0.1% Formic acid (v/v) in Acetonitrile. The separation is performed on a Inertsil ODS-3C18 column (150mm x 4.6mm x 3µm. Two selected reaction monitoring (SRM) transitions are monitored for Melamine. After optimization of instrument parameter and settings, the linear response (r^2>0.999) is observed for samples ranging from 0.50 to 10 µg/kg. The method provides recoveries of 70-120% in the concentration range of 0.50 to 10 µg/kg, intra- and inter-day variation in <2.5% RSD. The limit of detection (LOD) and limit of quantification (LOQ) values are 0.2 and 0.6 µg/kg, respectively with an uncertainty of ± 0.03µg/kg at 95 % confidence level.***

Keywords: Melamine, Milk, LC-MS/MS, Sand cleanup, Polar

1. Introduction

Melamine is a polar organic compound with a 1, 3, 5-triazine structural formula (Figure-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 (Carmen and Jyllian, 2013). It is also formed by the degradation of cyromazine, insecticides widely used in crop protection. In September 2008, several childrens suffered by the toxicity of melamine and several companies have been implicated in the adultration 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-cynurate crystals which can deposit in kidney tubules, thus causing damage of renal tissue. Kidney stones and other renal complications due to ingestion of melamine contaminated food stuffs. (McCartney and Jane, 2008; Pickert, Karte, 2008).

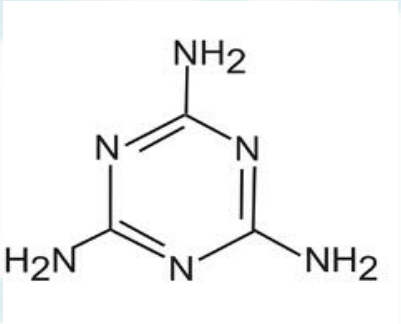

Figure 1: Structure of melamine

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 (FDA, 2008) in other food stuffs. It has been assumed that melamine can yield cyanuric acid by degradation or by the action of microorganisms (Jutzi *et.al.,* 1982). As a result of above scandal 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 levels desired by regulatory authorities (Reimschuessel *et.al.,*2008). 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 (Sheryl Tittlemier, 2009). This requires derivatization,

pretreatment and also shows poor sensitivities, etc. which is a time consuming and reduction of analyte concentration (Gopalkrishnan and John, 2010; Olga and Carmel, 2007). In this study the extraction and analysis of melamine from milk is done by using silica based strong cationic exchanger sand material as cleanup technique with C18 Inertsil ODS column as stationary phase and liquid chromatography & Tandem mass spectrometry LC-MS/MS analysis technique. To validate this method of extraction and analysis some validation studies have been taken up like linearity, precision, recovery, uncertainty etc.

2. Materials and Methods

2.1 Standards and Chemical

Melamine reference standard material (purity 99.0%), was obtained from Sigma Aldrich. Formic acid HPLC grade purchased from Merck, Ammonium formate LCMS grade purchased from Biosolve,Millipore water Purified with the use of Milli-Q-Purification system, Acetonitrile LCMS grade from Labscan, potassium dihydrogen o-phosphate AR grade purchased from Sd-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 eluents were A: 0.1 % Formic acid in Acetonitrile, B: 20mM Ammonium formate and Acetonitrile (1:1).The effect of Formic acid concentration on melamine retention time and peak shape was evaluated. At 0.1% Formic acid the retention time was identical and consisted and shape of melamine peak were very sharp; however at 0.05% Formic acid the retention time was longer and peak was broad. Minimization of sample preparation and automatization of analytical process are crucial requirement 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(Dobson et.al.,2008) 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 bring it to equilibrium by running a 60%, 10 to 12 min

2.5 Standard Preparation

2.5.1 Melamine Stock Solution

Precisely weighed melamine (100mg) was dissolved by sonication for 30 min 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, same standard stock solution were used for preparation of solvent calibration standards with the mixture of 0.1% Formic acid in Acetonitrile, containing 0.5µg/kg, similarly matrix matched standards were prepared by spiking of blank milk extract obtained by procedures described below in sample preparation. 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, same *Working Melamine solution* was used for preparation 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 upto 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 in Milli-Q water.

2.7 Sample Preparation

Mixed uniformly and homogenized the contents of the sample container. Weighed 5 gm of milk in a centrifuge tube. The sample was spiked with 0.5ml of the above Working Melamine solution (100µg/kg) 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 clean up.
Clean up:

- Take 1 gm of sand material (Fine pore size-Grade-II) in a syringe with cotton plug on the mouth without plunger
- Condition and equilibrate sand containing syringe with 3 ml methanol followed by 3 ml 0.1% Formic acid.
- Load sample (derived from sample pretreatment)

- Wash the sand containing syringe with 3 ml 0.1 % Formic acid followed with 3 ml Methanol.
- Elute melamine from sand material with 5 ml ammonia in methanol.
- Evaporate the eluent to dryness at 5 psi and 50°C, reconstituted in LC Mobile phase A.

2.8 Method Validation

The validation of LCMS/MS method is performed using blank & prefortified samples of milk with melamine (SANCO, 2011) various validation parameters such as Linearity, Precision, Recovery, LOD, LOQ and Reproducibility described below to validate this method:

2.8.1 Linearity

Linearity test solutions were prepared from the melamine working solution at five concentration levels ranging from 0.5 to10 µg/kg. A calibration curve was obtained by plotting the peak area vs. concentration (Figure-2). The %R.S.D. from six repeated injections at each concentration and % y-intercept bias was calculated.

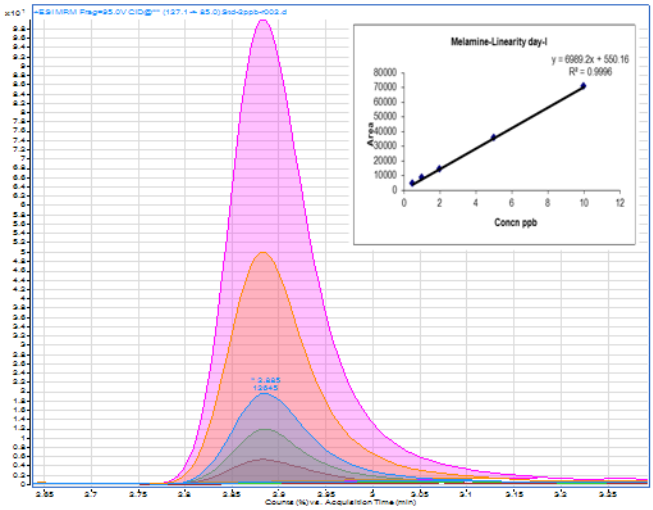

Figure 2: Linearity graph of melamine an overlay mode.

2.8.2 Limit of Detection (LOD) & Limit of Quantification (LOQ)

The LOD and LOQ of the method were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration. Precision study was also carried at the LOQ level by injecting six individual preparations and % R.S.D. of the peak area was calculated. A recovery study was performed in triplicate at 0.5 µg/kg level for liquid samples. Samples spiked at the LOQ level resulted in an average recovery of 101.9 %.

2.8.3 Accuracy

The accuracy of the method was evaluated at three-concentration levels of melamine (0.5, 1, and 10 µg/kg using 5mL of whole milk samples. The % recoveries reflect an average of six analyses at each concentration and are reported in Table 4.

2.8.4 Precision

Precision was measured as repeatability and reproducibility of whole milk samples. Using a sample processed according to Section 2.7, the repeatability (intra-day) and reproducibility (inter-day) of the method was demonstrated by injections (in triplicate) of the sample on the initial day and two consecutive days. The recoveries ranged from 72 to 117% (2.5% R.S.D.).

2.8.5 Uncertainty of Measurement

The maximum possible error that is measurement of uncertainty is calculated at LOQ level at 95 % confidence level is 0.6 ± 0.03 µg/kg.

3. Results and Discussion

Liquid chromatography & Tandem Mass spectroscopy (LC-MS/MS) is an excellent separation technique for ionizable molecules. Since melamine is a basic analyte (pKa=5.6), under acidic conditions (pH <3), the analyte is fully protonated in the mobile phase and residual silanol groups on the silica support of the column packing are also protonated. We have developed a simple analysis of whole milk spiked with melamine. The method uses mobile phase A: 0.1 % Formic acid in Acetonitrile, B: 20mM Ammonium formate and Acetonitrile (1:1). The method is advantageous as it avoids the use of expensive cartridges in sample cleanup procedure as the cleanup was done by a natural sand grade-II material.

The breakdown pattern of melamine was done (figure- 3) and SRM (Table 1) in order to check its abundance in response by optimization in LC-MS/MS source parameters (Table 2). The chromatograms (Figure-4) indicate that there is no interference from the milk and the melamine signal is clearly distinguished at 2.9 min. A linear calibration curve is demonstrated in (Figure-2), in addition samples spiked with 0.5 to 10 µg/kg of melamine shows recoveries in the range of 72.4 to 117% with % RSD values ranging from 0.2 to 2.5% indicating that the method is accurate over this concentration range (Table 5).

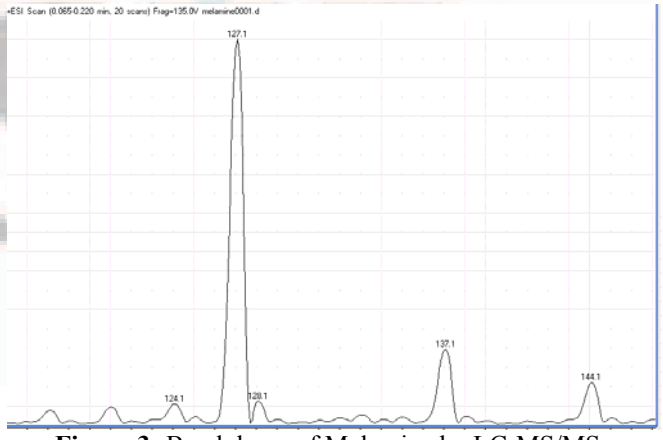

Figure 3: Breakdown of Melamine by LC-MS/MS

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Table 1: Mass fragments monitored

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

Table 2: Source Parameters Optimized

Instrument	LC-MS/MS Agilent 6460 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

3.1 Linearity

A linear calibration plot (Figure-2) is obtained over the calibration range of 0.5 to 10µg/kg with a correlation coefficient (r^2) of 0.999. Both solvent based standards and fortified samples are used for calibration for day I, day II, and day III analysis (Table 3). The % R.S.D. values of the repeated injections are <2% within each concentration and the y-intercept bias is less than 2%.

Table 3: Linearity performed for three different days over a linear range of 0.5 to 10µg/kg

Compound Name		Day-I	Day-II	Day-III
Melamine	Standard Linearity(R^2)	0.9996	0.9986	0.9992
	Fortified Linearity(R^2)	0.9968	0.9964	0.9954

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 72.4 to 117% (Table 4).The method shows reliable recovery (Figure 4).

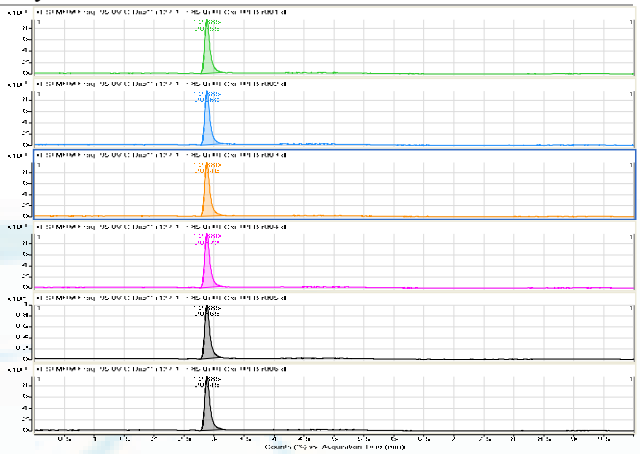

Figure 4: Recovery chromatogram by LC-MS/MS

Table 4: Recovery results of melamine spiked at three different concentration 0.5µg/kg, 1 µg/kg and 10µg/kg

Melamine spiked concentration (µg/kg)n=6	*% Recovery*		
	Day-I	*Day-II*	*Day-III*
0.5	113.17	84.10	108.48
1.0	108.34	79.97	100.98
10.0	117.39	72.40	97.66

3.4 Precision

The method shows good repeatability (intra-day) and reproducibility (inter-day) for processed samples (figure- 5) as indicated by recoveries of 72.4 to 117% (2.5% R.S.D.). In addition, the reproducibility of a sample separately processed (in triplicate) on day 0, day-2 and day 3 (Table-5) resulted in recoveries of 72.4–97.7% (2.3% R.S.D.)

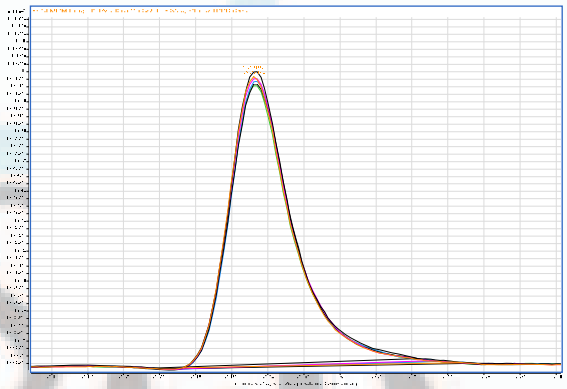

Figure 5: Precision graph overlay mode of six injections

Table 5: Repeatability (intra-day) and reproducibility (inter-day) for processed samples

Melamine	RSD (%) at 0.5 µg/kg (n=6)	RSD (%) at 1 µg/kg (n=6)	RSD (%) at 10 µg/kg (n=6)
Day-I	1.6	0.8	1.0
Day-II	2.5	1.5	0.9
Day-III	2.3	0.2	1.1

The relative standard deviation is within the limits i.e., % RSD is less than 5.

3.5 Uncertainty of Measurement

The maximum possible error that is measurement of uncertainty is calculated at LOQ level at 95 % confidence level is 0.6 ± 0.03 µg/kg.

4. Conclusion

This paper describes a simple method to determine the melamine contamination in milk samples using LC-MS/MS. The validation study carried out for the determination of Melamine in Milk demonstrates that the parameters studied have shown satisfactory results meeting the acceptance criteria. Because of Melamine's basic & polar nature a cation exchange natural sand material is used for cleanup, which enhances the recovery and selectivity during sample preparation. Usage of sand material replaces the SPE Cartridge and makes the analysis much cheaper. Starting with an analyte concentration of 0.5 to 10 µg/kg, a linear response (r^2>0.999) was observed for samples.LC coupled with Tandem mass-spectrometry and usage of high organic mobile phase, enhance the recoveries and we are able to achieve average recoveries in the range of 72-117 % in the concentration range of 0.5 to 10 µg/kg .Intra- and inter-day reproducibilities are less than 5% R.S.D. The LOD & LOQ are 0.2 and 0.6 µg/kg, respectively. The uncertainty found in the range of ± 0.03 µg/kg at 95% confidence level. This analytical method provides excellent sensitivity, reproducibility, accuracy, and precision for the detection of melamine in milk.

Acknowledgement

The support by Shriram institute for industrial research, Bangalore is gratefully acknowledged.

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