Determination of Melamine and Its Analogues from Powdered Infant Milk Using Polymeric Solid Phase Extraction

Application Note

Food Safety

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Introduction

Since the infant milk scandal in 2007, several methods have been released for the analysis of melamine from dairy products. However, a comprehensive method for the analysis of the related compounds (ammeline, ammelide, and cyanuric acid) is difficult to find. The US FDA has a comprehensive method, but the lengthy sample preparation steps may not be practical in a production laboratory where low sample preparation time is critical. The hydrophilic nature of the analytes, suggests a HILC LC column would be the most promising and most procedures use a HILIC LC method. HILIC has many pitfalls concerned with reproducibility and high organic solvent usage.

An alternative method was released using Captiva ND plates, which gave quick and reliable results for melamine. Its analogues were only quantifiable at higher concentrations. A two-tiered approach was developed, allowing for the quantification of melamine and its associated analytes at lower concentrations. This method maintains the use of reversed phase chromatography. Good linearity was achieved for each analyte as well as good accuracy and precision.

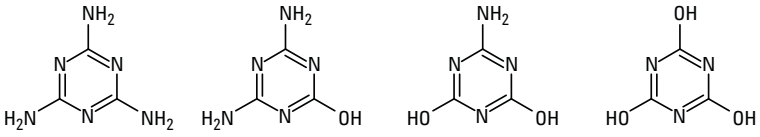

Figure 1. Structures of melamine, ammeline, ammelide, and cyanuric acid respectively.

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Experimental

Standards Preparation

All compounds were ordered from ChromaDex

- Melamine ASB-00013163-100
- Cyanuric acid ASB-00003958-100
- Ammeline ASB-00001657-100
- Ammelide ASB-00001659-100

Melamine and cyanuric acid were prepared in water. Ammeline and ammelide were prepared in a 2% ammonium hydroxide solution. All four compounds were mixed together to a concentration of 100 µg/mL in 2% ammonium hydroxide. Ammonium hydroxide is required to keep all compounds in solution.

Calibration Curve

Powdered infant milk was prefortified to concentrations ranging from 0.1 µg/g to 10 µg/g of melamine and its analogues.

Sample Preparation

1. Weigh out 1 ± 0.01 g of powdered infant milk.
2. Spike the milk with analytes to 1 µg/g.
3. Add 20 mL H_2O.
4. Vortex or shake, there should be no remaining powder.
5. Transfer two, 1 mL aliquots of milk to test tubes.
6. To one sample, add 2 mL 0.1 N HCL (will be applied to Plexa PCX).
7. To the other sample, add 2 mL 0.1 N sodium hydroxide (will be applied to Plexa PAX).
8. Let sit for 30 minutes.

Extraction

Plexa PCX

1. Condition
   a. 3 mL MeOH
   b. 3 mL H_2O
2. Loading – apply sample pretreated with 0.1 N HCL
3. Wash
   a. 3 mL 2% formic acid in H_2O
   b. 3 mL 50:50 MeOH:ACN
4. Elute – 5% ammonium hydroxide in 50:50 MeOH:ACN

Plexa PAX

1. Condition
   a. 3 mL MeOH
   b. 3 mL H_2O
2. Loading – apply sample pretreated with 0.1 N NaOH
3. Wash
   a. 3 mL H_2O
   b. 3 mL MeOH
4. Elute – 5% acetic acid in MeOH

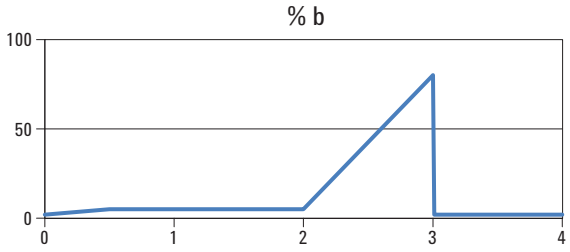
Evaporate both samples to dryness and reconstitute in 500 µL 50:50 MeOH:ACN.

Instrumentation and Conditions

System	Agilent 1290 Infinity LC Agilent 6460 Triple Quadrupole LC/MS System
Column	Pursuit XRs Ultra 2.8 Diphenyl 100 × 2.0 mm A7521100X020
Mobile phase	A: 0.1% Formic acid in H_2O B: MeOH

Gradient

time	% B
0.00	2
0.50	5
2.00	5
3.00	80
3.01	2



Flow	0.4 mL/min
Injection volume	5 µL
Temperature	ambient
Runtime	3 minutes
EMV	±300
Dwell	300
Cell accelerator voltage	7

Compound	Precursor ion	Product ion	Fragment	Collision energy	Polarity
Melamine	127	85.1	100	18	+
Cyanuric acid	128	42.1	60	14	-
Ammeline	128	69.1	140	34	+
Ammelide	127	84	100	6	-

Source

Gas temperature	300 °C
Gas flow	5 L/min
Nebulizer	20 psi
Sheath gas temperature	275 °C
Sheath gas flow	7 L/min
Capillary	+3,500/–2,000
Nozzle	+0/–500

Results and Discussion

Although this method requires a separate preparation for cyanuric acid, a single reversed phase LC run can be used for all compounds. The samples treated with HCL were applied to Plexa PCX cartridges. From those cartridges melamine, ammeline, and ammelide were extracted. The cyanuric acid samples were pretreated with NaOH and extracted on the Plexa PAX cartridges. Figure 2 demonstrates the baseline separation of the melamine-cyanuric acid pair and the ammeline-ammelide pair. All compounds were reliably detected down to 0.2 µg/g.

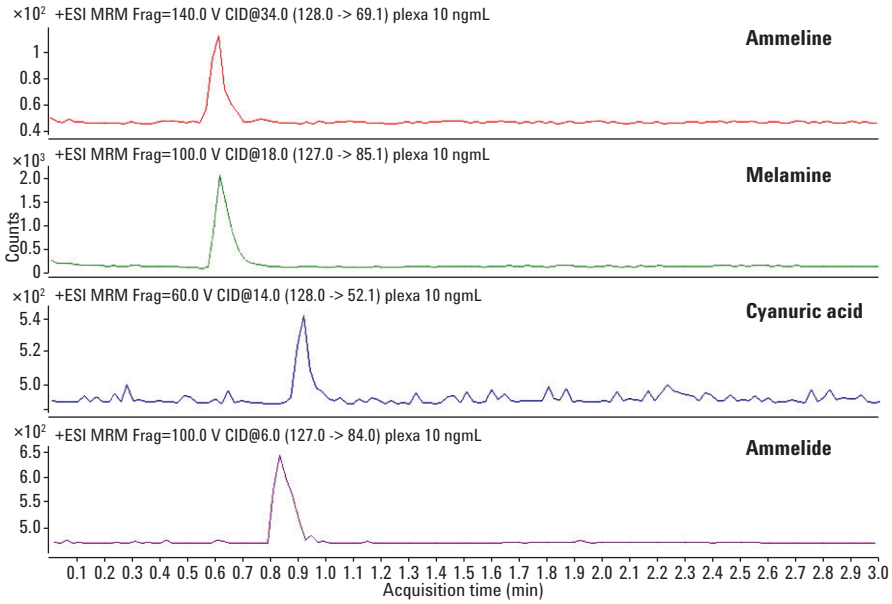


Figure 2. Separation of a 10 ng/mL standard of melamine and its analogues.

All compounds showed linearity from 0.5 µg/g–10 µg/g with a R^2 above 0.993. A minimum of five levels were used for the calibration curves.

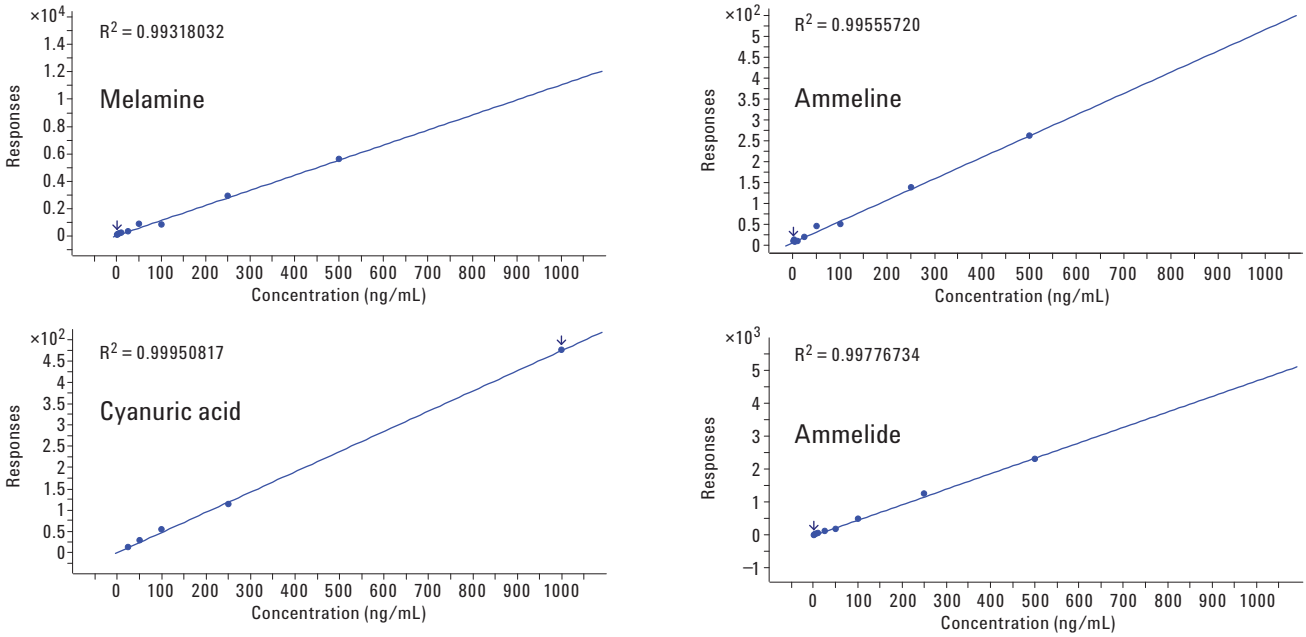


Figure 3. Calibration curves of melamine, cyanuric acid, ammeline, and ammelide.

Table 2 lists the relative recoveries of melamine and its analogues following sample preparation with Plexa PCX and Plexa PAX. All analytes extracted with Plexa PCX showed relative recoveries within 14% of true value with RSDs below 8%. Cyanuric acid, extracted from Plexa PAX, showed relative recovery within 17% of true value with a RSD of less than 6%.

Table 1. Recoveries of Melamine and its Analogues from Fortified Powdered Infant Formula Compared to Captiva Method, 1.0 µg/g (n = 6)

	Average % Recovery ± RSD	
	SPE	**Captiva**
Melamine	100 ± 7.9	94 ± 12.4
Cyanuric acid	117 ± 5.9	n/a
Ammeline	114 ± 6.8	n/a
Ammelide	112 ± 5.8	n/a

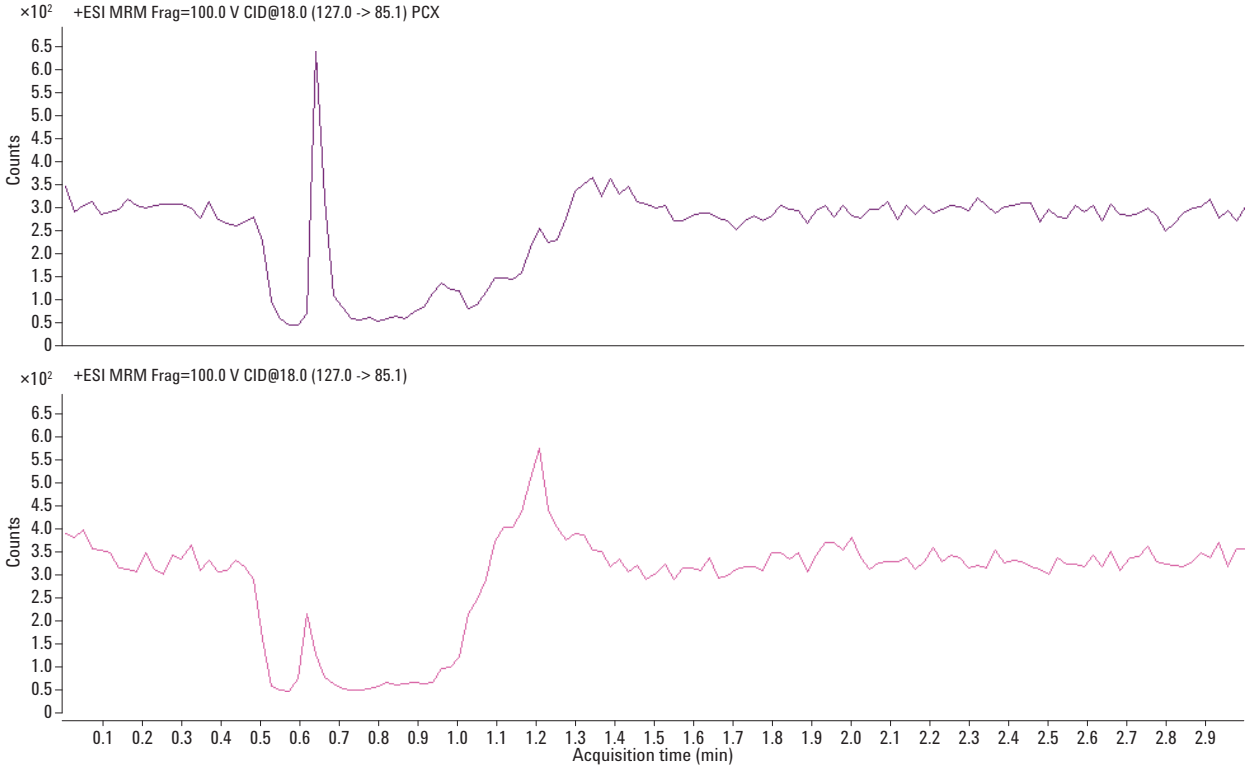


Figure 4. Melamine extracted through Plexa PCX (top), Captiva ND (bottom) at 1.0 µg/g.

Analyte sensitivity increased across the board for all compounds. Figure 4 demonstrates a 3 fold sensitivity increase for melamine compared to a similar sample prepared through Captiva ND, both were prefortified to 1.0 µg/g.

Conclusions

The method employed in this application note provides another quick method for the analysis of melamine and its analogues from powdered dairy products using generic sample preparation procedures for Plexa PCX and Plexa PAX and reversed phase chromatography.

Using Plexa PCX and Plexa PAX quick analysis of melamine and its analogues were accomplished down to the regulatory levels of 1 µg/g [2] and below. Linearity was demonstrated for all compounds with a linear regression coefficient greater than 0.993. The previous Captiva ND method was sensitivity enough only for melamine, maintaining a signal-to-noise ratio > 5:1 required by the US FDA at the 1 µg/g level [1]. This method is sensitive for melamine and all of its analogues below the regulatory level of 1 µg/g. Ammeline, ammelide, and cyanuric acid maintained an SNR above 5:1 all the way down to the 0.1 µg/g level. Melamine was capable of maintaining that SNR down to 0.5 µg/g. At regulatory levels, melamine, ammeline, and ammelide showed relative recoveries within 14% of true value with RSDs below 8%. Cyanuric acid showed a relative recovery within 17% of true value with a RSD of less than 6%.

Employing a two-tiered approach using Plexa PCX and Plexa PAX to the analysis of melamine and its analogues allows for increased sensitivity without sacrificing ease of use and time.

References

1. Turnipseed SB, Casey C, Nochetto C, and Heller DN. U.S. FDA Laboratory Information Bulletin, 2008: 24 : 4421. Lib. No. 4421[http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/DrugChemicalResiduesMethodology/ucm071637.htm] accessed September 1, 2011.
2. Chinese Ministry of Health (2011). China sets limits of melamine levels tolerable in food products, 21 April, 2011 (http://english.gov.cn/2011-04/21/content_1849392.htm)

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