Application Note 01916

Simultaneous, Fast Analysis of Melamine, Cyanuric Acid, and Related Compounds in Milk and Infant Formula by LC/MS/MS

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Abstract
The current baby milk scandal is a global food safety incident involving milk and infant formula that was allegedly adulterated with melamine. The LC/MS/MS method presented here is based on the US Food and Drug Administration's (FDA) method guidelines to provide reliable and efficient sample preparation and instrument analysis for melamine and related compounds. Simultaneous, fast determination and confirmation of melamine and cyanuric acid along with two other compounds using a complete solutions approach is presented.

Introduction
High visibility and the potential public health threat of melamine adulteration in both animal and human food sources has prompted many government agencies, including the US FDA, to release standard test methods for the analysis of melamine and related compounds, like cyanuric acid, in protein materials (1). Besides these analytes, several food manufacturers require a method for the related compounds, like ammelide and ammeline (Figure 1).

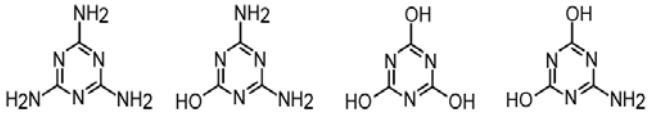

Figure 1. Structures of melamine, ammeline, cyanuric acid, and ammelide, respectively.

These maximum permitted concentrations have been set:

- Approximately 2.5 mg/kg for melamine in food for adults (EU, US, Hong Kong) (1)
- 1 mg/kg in infant foods (Hong Kong)
- 0.5 mg/kg (Japan) (2)
- Limit of detection (LOD) requirement of 2 mg/kg by HPLC and 0.01 mg/kg by LC/MS/MS (China) (3)

This application note provides simultaneous determination and confirmation of melamine, ammelide, ammeline and cyanuric acid in a reliable, efficient, and rapid LC/MS/MS method. Separation is achieved within 7 minutes using a Varian Polaris™ NH_2 column in HILIC mode. Bond Elut Plexa™ is used as a clean-up filter to retain matrix interferences while all the polar analytes pass through. The US FDA method for melamine and cyanuric acid was followed with some modifications made to establish a method for all four compounds.

Instrumentation
- Varian 320-MS Triple Quadrupole Mass Spectrometer with ESI source
- Varian 212-LC binary gradient pumps
- Combi PAL™ autosampler

Materials & Reagents
- SPE cartridge: Bond Elut Plexa, 60 mg, 3 mL cartridge, (Varian Part Number 12109603)
- Melamine (MEL). CAS #: 108-78-1 Ammelide, CAS #: 645-93-2, Ammeline CAS #: 645-92-1 and Cyanuric acid (CYA). CAS # 108-80-5; ChromaDex Inc., California
- Milk, "organic" milk, 100% from local deli store
- Infant formula, a popular brand, concentrated, milk-based formula from local grocery store

Procedure
Standard Preparation

Individual stock solutions, melamine, ammeline, ammelide and cyanuric acid, are all prepared at 100 µg/mL. Melamine and cyanuric acid are dissolved in water, while ammeline and ammelide are dissolved in 2 N ammonium hydroxide.

Standard mixture dilution, 25 µg/mL is used for fortification and matrix calibration standards.

Standard mixture dilutions, 140 ng/mL are used to prepare post-fortified control extracts and solvent standard for calculating matrix effects and percentage recovery.

Suitability Standard, Pre-fortified and Post-fortified Control Samples

Extracted matrix calibration standards are prepared at 0.25, 0.5, 1.0, 2.5, and 5.0 µg/g, by adding 20, 40, 80, 200, and 400 µL of 25 µg/mL standard mix respectively, to 2 g of milk or concentrated infant formula sample.

Pre-fortified control standard is prepared, in addition to the matrix calibration curve standards, at 1.0 µg/mL.

Post-fortified control samples are negative control extracts to which 100 µL of standard mix 140 ng/mL have been added at the final step, to give 1.0 µg/g equivalent extracts. These samples can be used to calculate matrix effects and percent recoveries.

Solvent standards to calculate matrix effects are prepared equivalent to 1.0 µg/g.

Sample Preparation

1. Take 2 mL of milk or concentrated infant formula.
2. Pre-fortify control and matrix calibration standards.
3. After adding standard mix to 2 mL of milk or concentrated infant formula samples, add 12 mL of 2.5% formic acid to each sample. Dissolve by shaking for 15-30 sec, then sonicate in ultrasonic bath and mix on multi-vortex mixer for 30 min each.
4. Centrifuge at 4000 rpm (3750 g) for 10 min at room temperature.
5. Transfer approximately 1.4 mL of the supernatant into a 1.5-mL micro centrifuge tube.
6. Centrifuge at 13200 rpm (16100 g) for 30 min.
7. Dilute sample extracts with acetonitrile by transfer 100 µL of the extracts into a 1.5 mL micro centrifuge tube and dilute with 900 µL of acetonitrile.
8. Vortex mix for 30 sec and centrifuge at 13200 rpm (16100 g) for 30 min.
9. Condition Bond Elut Plexa™ 60 mg, 3 mL SPE cartridge (Varian Part Number 12109603) with 3-mL acetonitrile. Then, transfer the supernatant of the centrifugation step to clean-up each sample, collect the filtrate.
10. Transfer the filtrates to 2-mL sample vials for injections.

LC Conditions

Column:	Varian Polaris™ NH_2, 5 µm, 150 x 3 mm (Varian Part Number A2013150X030)
Mobile Phase A:	Acetonitrile
Mobile Phase B:	Ammonium acetate 10 mM and 0.1% acetic acid in water
LC Program:	78% A isocratic, 0.4 mL/min
Column Temp.:	40 °C
Injection Volume:	20 µL

API Conditions

Ionization Mode:	ESI Positive
Collision Gas:	2.2 mTorr Argon
API Drying Gas:	30 psi at 250 °C
API Nebulizing Gas:	70 psi
SIM Width:	0.7 amu
Needle:	5000/4000 V (± mode)
Capillary:	± 50 V
Shield:	600 V
Detector:	1300 V

Table 1. MS/MS details and log P values of melamine and related compounds. *Ions for quantitation.

Compound	Log P	Parent Ion	Daughter Ion	Capillary Voltage (V)	Collision Energy (V)	Dwell Time (ms)
Melamine	-1.37	(+)127	68*	50	22	100
		(+)127	85	50	14	100
Ammeline	-1.2	(+)128	86*	50	12	100
		(-)126	83	-50	11.5	100
Ammelide	-0.7	(-)127	42	-50	14	100
		(-)127	84*	-50	10.5	100
Cyanuric Acid	-0.2	(-)128	42*	-50	13	100
		(-)128	85	-50	9.5	100

Results & Discussion

LC Separation

Hydrophilic Interaction Chromatography (HILIC) is one of the fastest growing chromatographic techniques employed today for retaining polar analytes. It overcomes the shortcomings of some of the other retention mechanisms for polar compound analysis, like ion-exchange, ion-pair reversed-phase, or making use of polar modified chemistries. These techniques mainly employ buffers that have either high ionic strength and/or are not compatible with MS detection. Moreover, ion pair separation of both an acid and a base in the same analysis is difficult. Melamine and its related compounds are extremely polar compounds as seen by their log P values in Table 1, and serve as very good candidates for HILIC chromatography. All four compounds, having either basic or acidic properties, can be analyzed simultaneously in a single run on an amino bonded phase, like Polaris NH_2, used in a HILIC mode. Separation of this mix is necessary as some of the isotope peaks can interfere with each other (Table 1). Figure 2 shows baseline separation of all four analytes within 7 minutes.

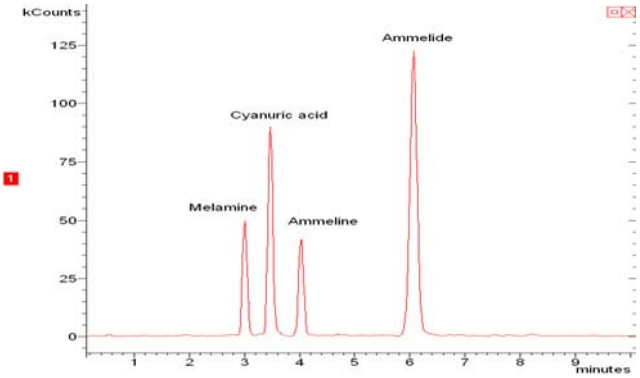

Figure 2. TIC of a standard mix of melamine and related compounds.

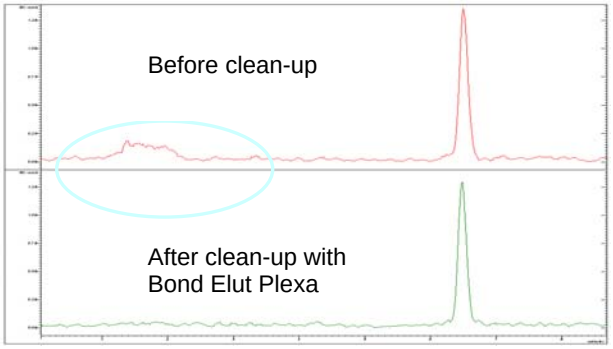

Figure 3. Comparison of the chromatograms of ammelide in fortified milk sample before and after clean-up with Bond Elut Plexa.

Matrix Impurities Removal

One modification made from the published US FDA method (1) is the inclusion of a clean-up step after dilution of sample extracts with acetonitrile in the sample preparation protocol. Bond Elut Plexa is used as a cleanup filter to retain matrix interferences while all the polar analytes pass through. Figure 3 demonstrates a comparison of chromatograms of ammelide in fortified milk sample before and after clean-up. Impurities at the front end are completely removed after the extracts are passed through a clean-up filter.

Calibration Range and Sensitivity

Both matrices are fortified with concentrations of 0.25, 0.5, 1.0, 2.5 and 5.0 µg/g of melamine and related compounds. Figures 4 and 5 illustrate target ion chromatogram (TIC) comparisons at three different concentrations, including blank extracts.

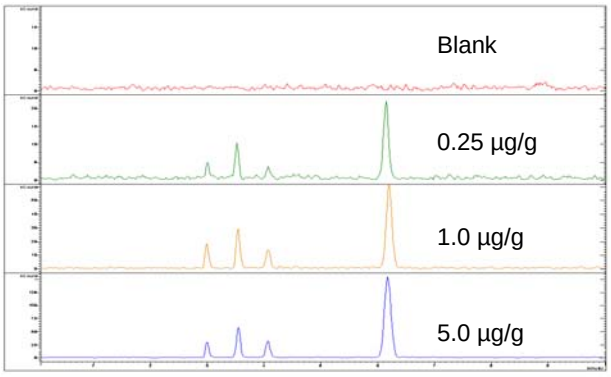

Figure 4. TIC of milk fortified with melamine and related compounds at low, mid, and high concentrations and blank extract.

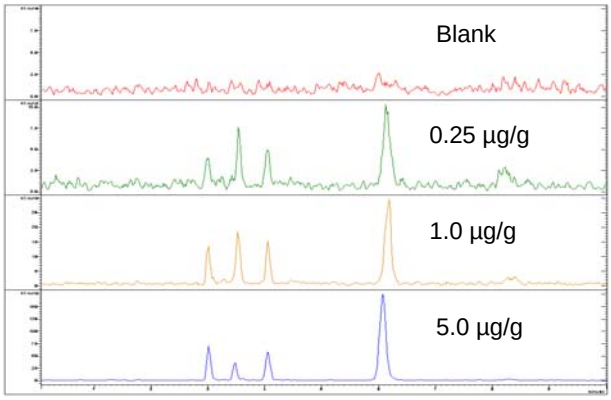

Figure 5. TIC of infant formula fortified with melamine and related compounds at low, mid, and high concentrations and blank extract.

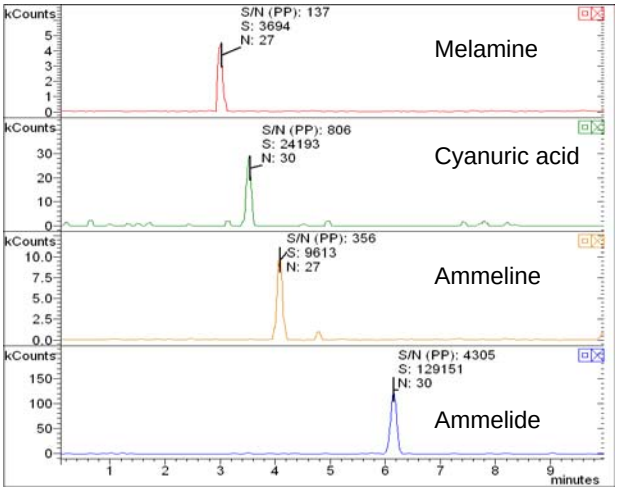

Figure 6. MRM chromatogram of milk fortified with melamine and related compounds at the lowest concentration, 0.25 µg/g.

One of the acceptance criteria for confirmation as specified by the US FDA requires the presence of critical ions and a S/N ratio > 5:1. Figures 6 and 7 show multiple reaction monitoring (MRM) chromatograms of melamine and related compounds at the lowest concentration, 0.25 µg/g. The S/N ratio for each analyte is higher than 16:1, which exceeds the FDA specifications.

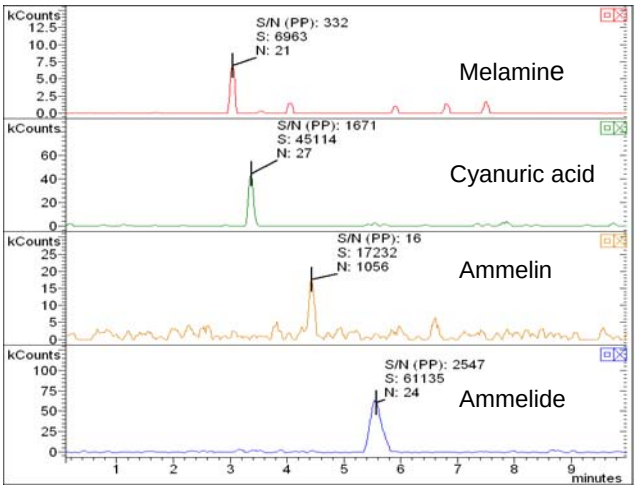

Figure 7. MRM chromatogram of infant formula fortified with melamine and related compounds at the lowest concentration, 0.25 µg/g.

Linearity

Figure 8 demonstrates five point calibration curves with good linearity in the range of 0.25 to 5 µg/g, with linear regression coefficient r^2 higher than 0.99.

Recoveries

Table 2 shows the recoveries of melamine and cyanuric acid at 1.0 µg/g (n = 3) to be in the range of 84% - 95%. At the same concentration, the recoveries of ammeline and ammelide are in the range of 68% - 89%. RSDs are below 20% for all sample sets (n=3).

Table 2. Recoveries of melamine and related compounds from fortified infant formula and milk at 1.0 µg/g.

Sample	1.0 µg/g Fortified Infant Formula	1.0 µg/g Fortified Milk
Melamine Average % Recovery ± % RSD (n)	88.3 (n = 3) ±14.7%	83.8 (n = 3) ±19.6%
Cyanuric Acid Average % Recovery ± % RSD (n)	92.5 (n = 3) ±6.2%	94.9 (n = 3) ±14.2%
Ammeline Average % Recovery ± % RSD (n)	77.5 (n = 3) ±16.4%	67.8 (n = 3) ±6.5%
Ammelide Average % Recovery ± % RSD (n)	89.4 (n = 3) ±14.7%	87.3 (n = 3) ±18.0%

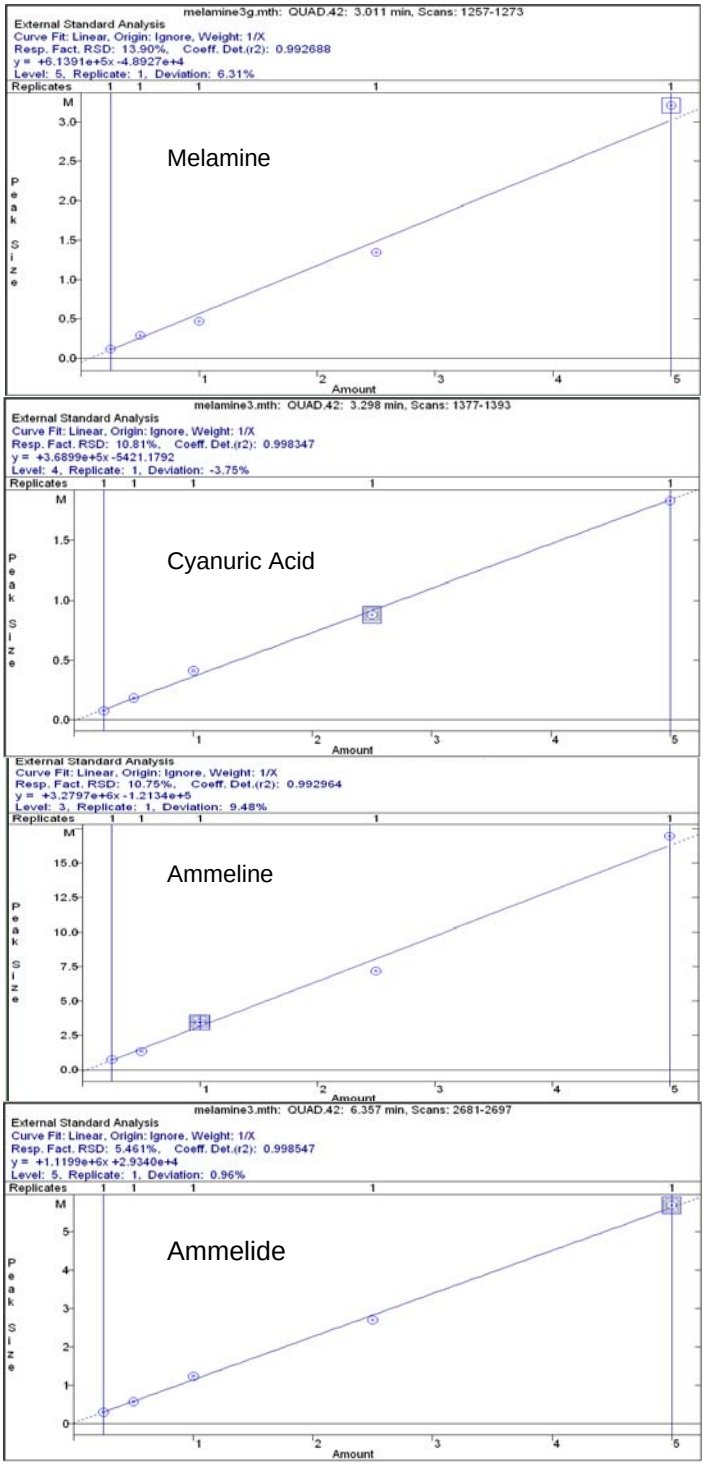

Figure 8. Calibration curves of melamine, cyanuric acid, ammeline and ammelide.

Conclusion

- Simultaneous, fast determination and confirmation of melamine, ammeline, ammelide and cyanuric acid using a complete solutions package comprising Varian Bond Elut Plexa™, Polaris™ NH_2, and 320-MS was achieved.
- The Varian Polaris NH_2 column can be used in HILIC mode. It offers baseline separation within 7 minutes for both basic and acidic compounds, like melamine and related compounds.
- Clean-up using Bond Elut Plexa reduces matrix interferences in both milk and infant formula.
- The linearity of LC/MS/MS analysis with the Varian 320-MS triple quadrupole mass spectrometer for melamine and related compounds is very good in the range of 0.25 to 5 µg/g.
- The signal-noise ratio at the lowest concentration 0.25 µg/g is 16 or higher, which exceeds the US FDA specification.
- Recoveries of melamine and cyanuric acid observed are in the range of 84% - 95%. For ammeline and ammelide, the recoveries are in the range of 68% - 89%. RSDs observed were below 20% for all sample sets.

References

1. Determination of Melamine and Cyanuric Acid Residues in Infant Formula using LC-MS/MS, Sherri Turnipseed, Christine Casey, Cristina Nochetto, David N. Heller *FDA Laboratory Information Bulletin*, LIB No. 4421, Vol 24, Oct 2008 http://www.cfsan.fda.gov/~frf/lib4421.html
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