Hydrophilic Interaction Chromatography (HILIC) for Simultaneous and Fast Analysis of Melamine, Cyanuric Acid, and other Metabolites in Milk and Protein- Rich Powders

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Abstract

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 which 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.

High visibility and the potential public-health threat regarding the concern over melamine adulteration in both animal and human-food sources has prompted many government agencies including the U.S. 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 other metabolites like ammelide and ammeline. Melamine and its metabolites are extremely polar compounds and serve as very good candidates for HILIC chromatography.

The U.S. FDA method for melamine and cyanuric acid was followed with some modifications made to establish a method for all four compounds. This paper provides simultaneous determination and confirmation of melamine, ammelide, ammeline and cyanuric acid in a reliable, efficient, and rapid LC-MS/MS method. A novel amino bonded phase is used in HILIC mode to separate both basic and acidic compounds with base-line resolution within 7 min. Bond Elut Plexa SPE plate is used as a clean-up filter to retain matrix interferences while all the polar analytes pass through.

Introduction

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 and fast determination and confirmation of melamine and cyanuric acid along with two other compounds using a complete solutions approach is presented.

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 other melamine related compounds like ammelide and ammeline (Figure 1).

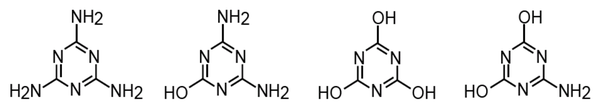

Figure 1. Structures of melamine, ammeline, cyanuric acid, and ammelide, respectively.

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, milk-based formula from local grocery store

Procedure

Sample preparation procedure was based on US FDA's method (1), modified with an additional filtration step with Bond Elut Plexa™ SPE cartridge

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 transferring 100 µL of the extracts into a 1.5 mL micro centrifuge tube and dilute with 900 µL of acetonitrile. For concentrations of ammeline and ammelide above 5µg/g, use 2% ammonium hydroxide in acetonitrile if necessary.
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™ NH2, 5 ⌠, 150 x 3 mm (Varian Part Number A2013150X030)
Mobile Phase: A - Acetonitrile
B - Ammonium acetate 10 mM and 0.1% glacial acetic acid in water
(Both A and B are in separate lines)
LC Program: 78% A isocratic, 0.4 mL/min
Column Temp.: 40ºC
Injection Volume: 20 µL
Instrument: Varian 320-MS Triple Quadrupole Mass Spectrometer
Ionization Mode: ESI Positive/Negative

Compound	Log P	Q1	Q3	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

Table 1. MS/MS details and log P values of melamine and its metabolites.

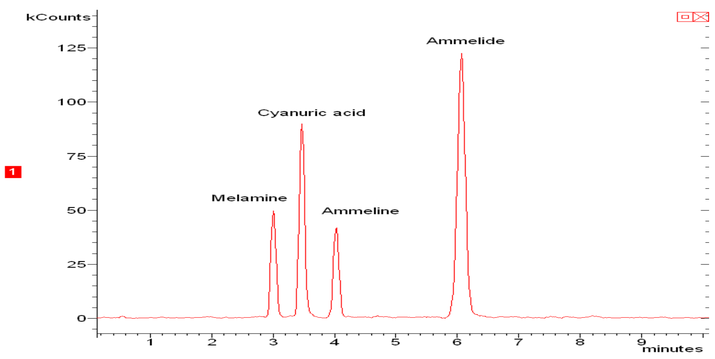

Figure 2. TIC of a standard mix of melamine and related compounds.

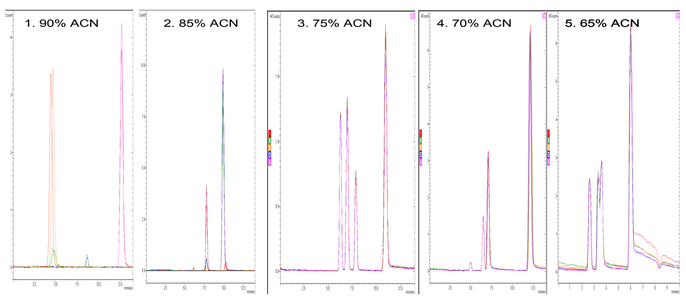

Figure 3. Workable Range of Aqueous Modifier: Reproducible chromatography was attainable between 15% - 30% aqueous modifiers.

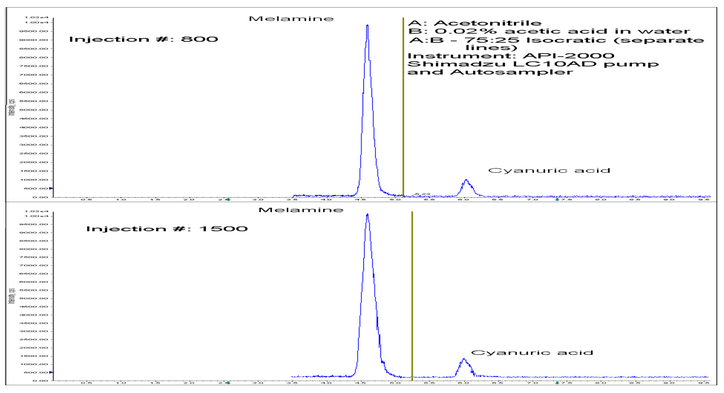

Figure 4. Robustness of Polaris NH2 under HILIC conditions

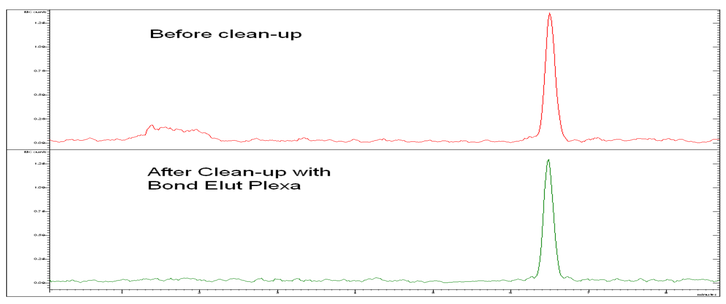

Figure 5. Comparison of the MRM chromatograms of ammelide in fortified milk sample before and after clean-up with Bond Elut Plexa.

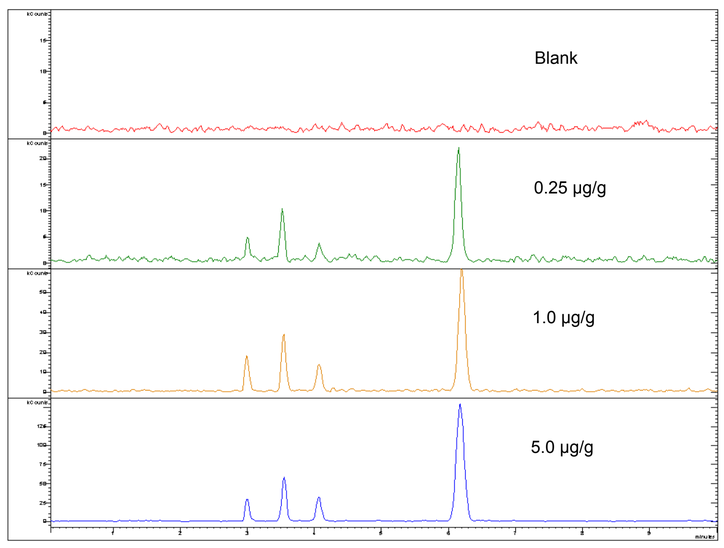

Figure 6. TIC of milk fortified with melamine and related compounds at low, mid, and high concentrations and blank extract

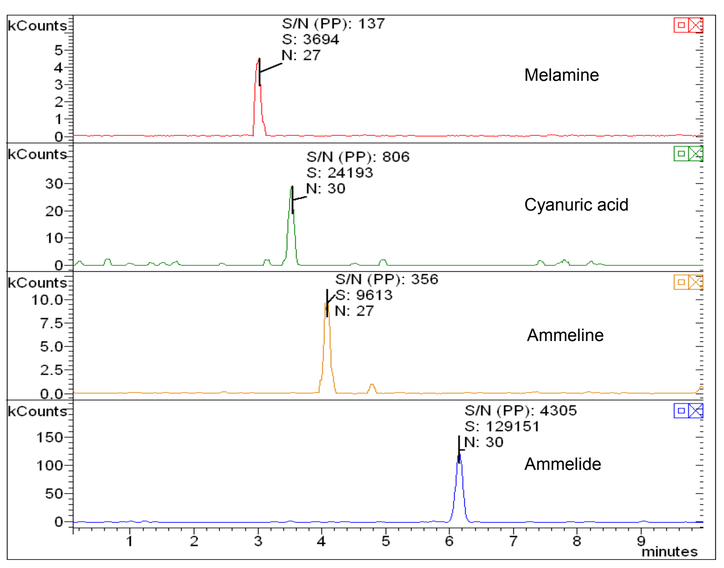

Figure 7. MRM chromatogram of milk fortified with melamine and related compounds at the lowest concentration, 0.25 µg/g.

Results & Discussion

LC Separation

Melamine and its metabolites 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 NH2, 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.

Solvent Strength, Reproducibility, and Robustness

The data in Figure 2 was generated using both mobile phase counterparts, A and B in separate lines. If pre-mixed mobile phase is used, the same ionic strength needs to be maintained as that used in separate lines in order to get similar results, i.e. pre-mixed mobile phase containing 78% acetonitrile and 22% water needs to be modified with 2.2 mM ammonium acetate and 0.02% glacial acetic acid. If the analytes screened are melamine and cyanuric acid only, less acid in mobile phase (0.01% or less) provides greater separation. HILIC typically involves usage of high organic (>70%) mobile phases with the aqueous component being volatile salts. Concentration of aqueous modifier in HILIC plays an important role as it directly affects solvent strength. If there is too much water, cross-over into other modes of chromatography can take place, depending on the analyte and phase, i.e. separations begin to look like IEX or RP (IEX most likely with NH2 phase). Experiments were conducted to investigate this effect on Polaris NH2 using a mix of melamine and related compounds. Reproducible chromatography was attainable between 15%-30% aqueous modifiers (70% - 85% organic concentrations) as seen in Figure 3 (2). Figure 4 demonstrates robustness of Polaris NH2 for the analysis of melamine and cyanuric acid standards after 1500 injections, the column being used for routine analysis of these compounds in milk powder, soybean powder and snack food using the US FDA method (1). As seen, the retention times, reproducibility, and resolution between melamine and cyanuric acid are consistent after over a thousand injections.

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 5 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 6 illustrates target ion chromatogram (TIC) comparisons at of milk fortified at three different concentrations, including blank extracts (3). 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 7 shows multiple reaction monitoring (MRM) chromatograms of milk fortified with 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 (3).

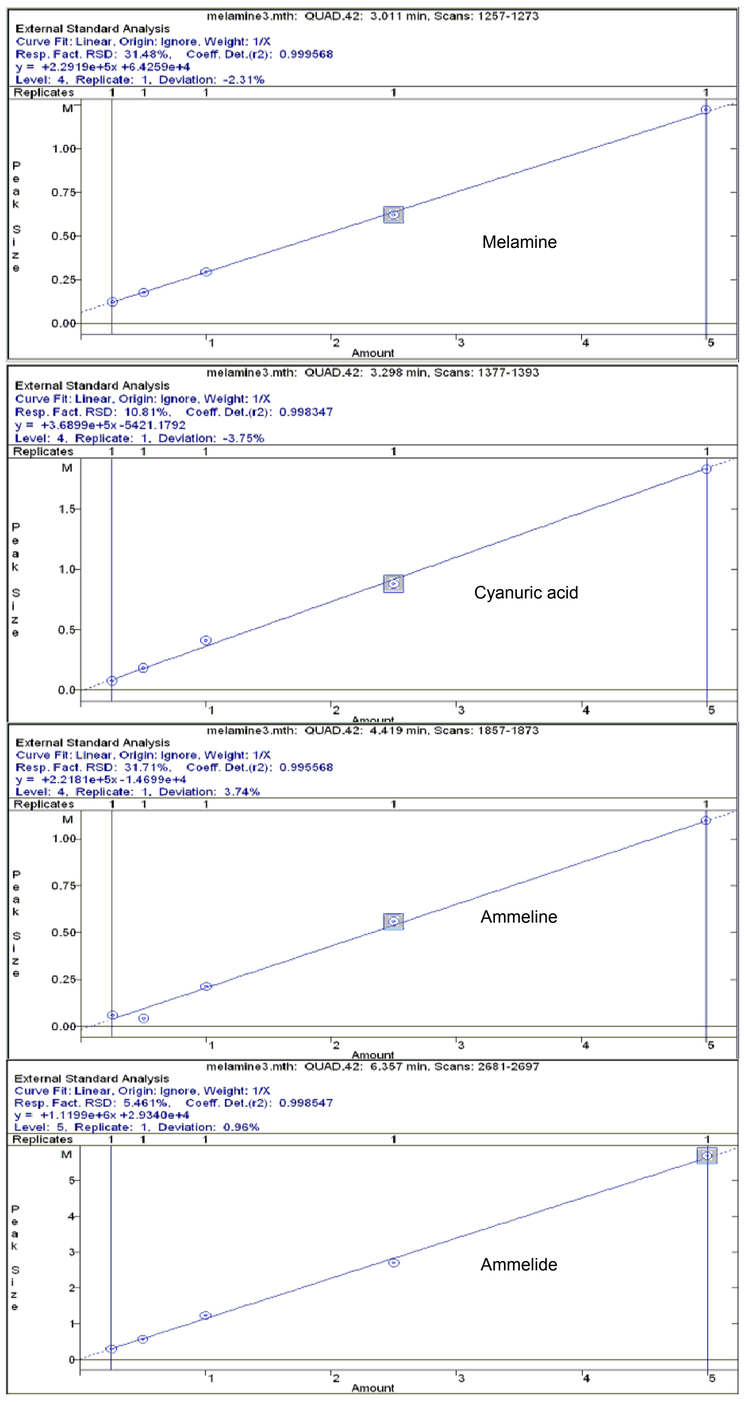

Figure 8. Calibration curves of melamine, cyanuric acid, ammeline and ammelide.

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%

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

Results & Discussion (cont)

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).

Conclusions

- Polaris NH2 column can be used in HILIC mode for simultaneous and fast determination and confirmation of melamine, ammeline, ammelide and cyanuric acid in milk and infant formula. A complete solutions package using Varian Bond Elut Plexa™, Polaris™ NH2, and 320-MS is presented.
- It offers baseline separation within 7 minutes for both basic and acidic compounds, like melamine and related analogs. Column robustness and longevity is proven for melamine and cyanuric acid standards in milk powder, soybean powder, and snack food for over 1,000 injections.
- 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, Vol24, Oct 2008 http://www.cfsan.fda.gov/~frf/lib4421.html

2. Effect of Stationary Phase Chemistry on Selectivity of Pharmaceuticals, Pesticides, and Oligosaccharides in HILIC Separations, Ritu Arora, Richard Robinson, Mike Chang, Eugene Chang, Poster #: 600-5P, Pittcon 2009.

3. Simultaneous and Fast Analysis of Melamine, Cyanuric Acid, and Related Compounds in Milk and Infant Formula by LC/MS/MS, Eugene Chang, Ritu Arora, Varian Application Note SI-01916.

Acknowledgements

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