

## Technical Report

# The Extraction and Analysis of Melamine in Milk-Based Products using Discovery DSC-SCX SPE and Ascentis Express HILIC LC-MS/MS

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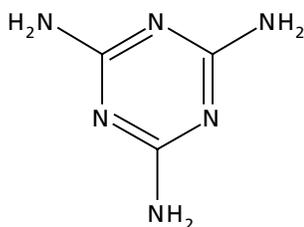
### Introduction

Melamine is a polar organic compound with a 1,3,5-triazine skeleton (Figure 1). It is commonly used for its fire retardant properties and is often combined with formaldehyde in the molding of plastics. It is also a common additive in fertilizers because of its nitrogen rich properties. In September 2008, several Chinese companies have been implicated in the adulteration of milk and infant formula with melamine. Because melamine comprises 66% nitrogen, it was used as an additive to fraudulently inflate the detected protein level above the true level. By September 22, 2008, up to 13,000 children were inflicted with kidney stones and/or other renal complications due to ingestion of melamine contaminated foodstuffs (1,2).

As of November 2008, the US FDA has set a “zero-tolerance” level for melamine in infant formula and baby foods; and a maximum tolerance level of 2.5 ppm in other foods. However, according to the US FDA, the presence of melamine in infant formula below 1 ppm does not present public health concerns (3,4).

In this report, we discuss the extraction and analysis of melamine from milk, milk powder, and dry infant formula using a silica-based strong cation exchanger (SCX) SPE phase and HILIC (hydrophilic interaction liquid chromatography) coupled with tandem mass-spectrometry.

**Figure 1. Structure of Melamine**



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### Experimental

Milk, dry milk powder, and dry infant formula were spiked with melamine and subjected to sample pre-treatment via extraction with a low pH buffer or dilute acid. Note that the low pH environment was important to facilitate protein removal/precipitation and to adequately ionize melamine for SCX SPE. Each blank sample matrix was confirmed to be melamine free prior to analyte spiking. After sample pre-treatment, the extracts were processed using Discovery®

DSC-SCX SPE via the procedure described in Table 1. Discovery DSC-SCX SPE is a silica-based SPE phased functionally bonded with benzene sulfonic acid. The phase offers high capacity (0.8 meq/g); and capacity is a critical attribute for recovering highly polar basic compounds such as melamine.

#### Whole Milk Sample Pre-Treatment

5 mL of store purchased milk was spiked with melamine (Cat. No. M2659) and diluted with 5 mL 100 mM phosphate buffer, pH 2.5 and 1 mL acetonitrile. The sample was sonicated for 5 minutes using an ultrasonic water bath followed by centrifugation at 3500 rpm for 10 minutes. The middle supernatant layer was isolated for further SPE processing. Note that 2.2 mL of the middle supernatant layer (equivalent to 1 mL milk sample) was further processed using SPE.

#### Dry Milk Sample Pre-Treatment

1 g of store purchased dry milk was spiked with melamine (Cat. No. M2659). The spiked milk powder was thoroughly dissolved in 4 mL DI water and 6 mL 2.5% formic acid was added. The acidified milk powder was sonicated for 10 minutes using an ultrasonic water bath. The sample was centrifuged at 3500 rpm for 10 minutes; and the middle supernatant layer was isolated for further SPE processing. Note that the entire middle supernatant layer (equivalent to 1 g dry milk) was further processed using SPE.

#### Dry Infant Formula Sample Pre-Treatment

1 g of store purchased dry infant formula was spiked with melamine (Cat. No. M2659). The spiked infant formula was thoroughly dissolved in 4 mL DI water, and 6 mL 100 mM phosphate buffer, pH 2.5 was added. The buffered infant formula was sonicated for 10 minutes using an ultrasonic water bath. The sample was centrifuged at 3500 rpm for 10 minutes; and the middle supernatant layer was isolated for further SPE processing. Note that 5 mL of the middle supernatant layer (equivalent to 0.5 g) was further processed using SPE.

**Table 1. Discovery DSC-SCX SPE Cleanup Procedure**

SPE Cartridge: Discovery DSC-SCX SPE, 500 mg/6 mL (Cat. No. 52688-U)

1. Condition and equilibrate SPE cartridge with 3 mL methanol followed by 3 mL 0.1% formic acid.
2. Load sample (derived from sample pre-treatment).
3. Wash SPE cartridge with 3 mL 0.1% formic acid followed by 3 mL methanol.
4. Elute melamine from SPE cartridge with 4 mL 5% ammonia diluted in methanol.
5. \*\*Evaporate SPE eluent to dryness with nitrogen at 5 psi and 50 °C. Reconstitute in LC mobile phase A.

**\*\*Note:** For a quick evaluation, the SPE eluent can be directly injected as is. However, for accurate quantitation, evaporation and reconstitution is recommended.

### LC-MS/MS Analysis using HILIC

Melamine (Figure 1) is a polar molecule with a pKa of 5.6 and a Log P value of -1.37 (5), making it a good candidate for Aqueous Normal Phase (ANP) chromatography. In ANP chromatography, a polar hydrophilic analyte partitions between a relatively polar stationary phase and a relatively non-polar mobile phase. ANP is commonly referred to as HILIC, but the term HILIC implies a mechanism that is one of several mechanisms that may be operative under ANP conditions.

This HILIC mechanism describes the process of preferential solvation of the polar stationary phase with the aqueous component of the mobile phase and subsequent depletion of the mobile phase of water. This sets up a biphasic system where there is a semi-immobilized layer of water near the surface and an organic-rich mobile phase layer. A polar compound may then partition from the moving organic-rich mobile phase into the stagnant aqueous solvent near the surface. Water then becomes the "strong" elution solvent, and analytes generally elute (assuming HILIC is the dominant mechanism) in order of "decreasing" hydrophobicity (a lower log P, indicating a more polar molecule, and consequently more retention).

Detailed HILIC LC-MS/MS conditions are described in Table 2.

**Table 2. HILIC LC-MS/MS Conditions**

|                 |   |             |     |     |
|-----------------|---|-------------|-----|-----|
| column:         | Ascentis® Express HILIC, 5 cm x 2.1 mm I.D., 2.7 µm particles (Cat. No. 53934-U); or Ascentis Express HILIC, 10 cm x 2.1 mm I.D., 2.7 µm particles (Cat. No. 53939-U) |             |     |     |
| instrument:     | Agilent 1100 HPLC system with ABI/MDS Sciex 3200 Q Trap MS-MS   |             |     |     |
| mobile phase A: | 10 mM ammonium formate in 90:10 acetonitrile:water  |             |     |     |
| mobile phase B: | 10 mM ammonium formate in 70:30 acetonitrile:water  |             |     |     |
| gradient:       | Min   | Flow Rate   | %A  | %B  |
|                 | 0   | 0.2 mL/min. | 100 | 0   |
|                 | 5   | 0.4 mL/min. | 0   | 100 |
|                 | 10  | 0.4 mL/min. | 100 | 0   |
|                 | 15  | 0.2 mL/min. | 100 | 0   |
| det.:           | MS/MS; MRMs (127/85 & 127/68 m/z) – MRM 127/85 was used for quantitation  |             |     |     |

| Q1  | Q3   | Declustering Potential (DP) | Entrance Potential (EP) | Collision Energy (CE) | Collision Cell Exit Potential (CXP) |
|-----|------|-----------------------------|-------------------------|-----------------------|-------------------------------------|
| 127 | 85.0 | 46                          | 4                       | 31                    | 4                                   |
| 127 | 68.0 | 41                          | 4                       | 31                    | 4                                   |

|                    |        |
|--------------------|--------|
| curtain gas:       | 20     |
| gas 1:             | 20     |
| gas 2:             | 40     |
| ion spray voltage: | 5000   |
| temp.:             | 350 °C |
| LC temp.:          | 30 °C  |
| inj.:              | 2 µL   |

## Results and Discussion

### Calibration Curve

Whole milk samples were quantified against external calibration standards prepared in buffer. Dry milk powder and dry infant formula were quantified against matrix match calibration standards. To prepare matrix match standards, blank dry milk and infant formula were pre-treated and subjected to SPE processing as described in Table 1. The resulting blank SPE eluent was spiked with melamine.

Note that no signs of ion-suppression were evident for the extraction/analysis of whole milk and dry milk powder allowing for accurate quantitation against external standards diluted in buffer. However, up to 45-50% ion-suppression occurred for infant formula. Therefore, it is important to quantitate against matrix-match standards when analyzing melamine in infant formula.

### High Recoveries & Low Background

Both milk and infant formula were spiked with melamine in triplicate at the levels of 10-2500 ng/mL and ng/g, respectively. Absolute recovery was determined for each spike concentration (Table 3). For dry milk powder, only one spike level was tested (1000 ng/g) and is not included in Table 3. Recovery and reproducibility was high across all the sample matrices and spike levels tested. The average recovery for whole milk and infant formula were 89% and 82%, respectively. RSDs were less than 11% for all the spike levels tested. Recovery for dry milk powder was 81% at the 1000 ng/g spike level tested.

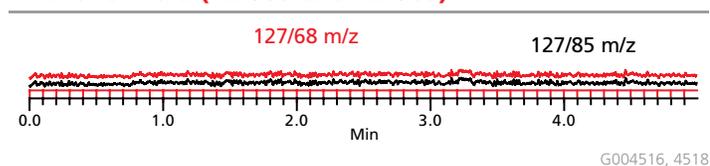
**Table 3. Average Absolute Recovery for Melamine in Whole Milk and Dry Infant Formula**

| Sample Matrix & Spike Level (n=3) | 10 ng/g  | 100 ng/g | 500 ng/g | 1000 ng/g | 2500 ng/g |
|-----------------------------------|----------|----------|----------|-----------|-----------|
| Whole milk**                      | 71 ± 11% | 85 ± 7%  | 83 ± 2%  | 112 ± 8%  | 95 ± 10%  |
| Dry infant formula                | 83 ± 11% | 76 ± 7%  | 90 ± 7%  | 82 ± 11%  | 79 ± 10%  |

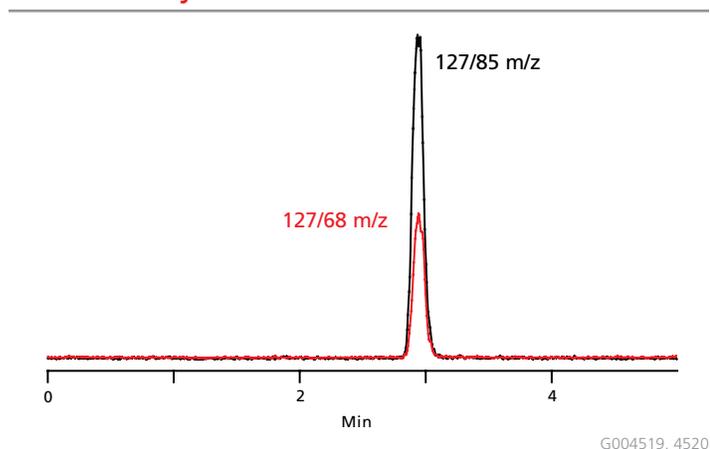
\*\*Whole milk spiked at the equivalent 10-2500 ng/mL level.

Figure 2 depicts chromatograms of SPE extracts of blank infant milk formula. The blank chromatograms were free of melamine and other interferences for the mass transitions monitored. Figure 3 is a representative chromatogram of a whole milk sample spiked with 100 ng/mL melamine and extracted using Discovery DSC-SCX SPE. HILIC chromatography of melamine provided good peak shape and retention coupled with a short analytical run time of less than 4 minutes.

**Figure 2. Chromatograms for SPE Extracts of Blank Infant Formula (127/85 and 127/68)**



**Figure 3. Chromatogram of Whole Milk Spiked with Melamine (100 ng/mL) and Extracted with Discovery DSC-SCX SPE**



### Low Limits of Quantitation

Based on a signal-to-noise ratio of 10:1, the estimated lower limit of quantitation is 4 ng/g (ppb) for dry milk powder and 4 ng/mL for whole milk. Note that background levels of melamine can be found in plasticware, solvents, and reagents; and should be monitored carefully. For example, we have found up to 2-3 ng/g melamine when processing 1 g of blank sample. In addition, the FDA has reported up to 40 ng/g of background melamine when analyzing infant formula using a polymeric SPE phase (6)

### Melamine Analysis Coupled with Cyanuric Acid

When melamine is present with cyanuric acid, supramolecular aggregates are formed that are not soluble in water and other solvents. Previous reports provided by the FDA have suggested that exposing the samples to very high or low pH in conjunction with dilution is an effective means for disrupting this complex, thereby aiding solubility (4,7,8).

Whole milk samples were spiked with a mixture of melamine and cyanuric acid at the levels of 100 ng/mL and 1000 ng/mL prior to sample pre-treatment (described previously) and SPE cleanup (Table 1). Because sample pre-treatment required dilution with a low pH buffer, melamine was adequately dissolved prior to SPE processing; and melamine recovery was in the range of 99-102%.

### Conclusion

In this report, we described an assay for the extraction and analysis of melamine in whole milk, dry milk powder, and infant formula. Because of melamine's basic and polar properties, a strong cation exchange SPE phase was required for adequate recovery and selectivity during sample preparation. When coupled with HILIC chromatography, excellent analyte retention was observed and MS response was enhanced due to the high organic mobile phase inherent with this mode of chromatography. Using Discovery DSC-SCX SPE and Ascentis Express HILIC coupled with tandem mass-spectrometry, we were able to achieve average recoveries in the range 80-90% and lower limits of quantitation of 4 ng/g (ppb) for dry milk powder and 4 ng/mL for whole milk.

## Ordering Information

| Description   | Cat. No. |
|---|----------|
| Discovery DSC-SCX SPE, 500 mg/6 mL, pk. 30                    | 52688-U  |
| Ascentis Express HILIC, 5 cm x 2.1 mm I.D., 2.7 µm particles  | 53934-U  |
| Ascentis Express HILIC, 10 cm x 2.1 mm I.D., 2.7 µm particles | 53939-U  |
| Melamine standard, 99% purity                                 | 52549    |
| Cyanuric acid standard, 98% purity                            | 16614    |
| Melamine solution, 100 µg/mL in acetonitrile/water            | 44642-U  |

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