Application Note: Analysis of Melamine in Milk
(updated: 04/17/09)

Product: DPX-CX (1 mL or 5 mL)

INTRODUCTION

There has been great interest recently for detecting melamine in food samples such as milk. High levels of this compound may lead to renal failure in children. Due to being high in nitrogen content, melamine has been suspected to be intentionally added to milk to provide falsely high levels of protein content. Melamine is also used in the manufacturing of various plastics and cleaning supplies, so accidental contamination of food products is also possible.

Regulatory and public concern over melamine contamination in milk has been increasing due to potential health hazards. There is critical need for rapid and reliable analytical methods for determination of melamine in milk.

This application note provides a method for rapid and sensitive detection of melamine from milk. This extraction procedure can be utilized with any chromatographic instrumentation, GC/MS or HPLC/MS/MS. In this study, the analysis was performed using chemical derivatization and GC/MS.

EXPERIMENTAL

Sample Preparation.
1. Pipette 0.25 mL of milk into small test tube.

2. Add internal standard at 0.25 ppm (*melamine-($^{15}N)_3$*).

2. Add 1 mL 50% acetonitrile (ACN)/H_2O, vortex mix 30 sec.

3. Add 0.5 mL 0.1M HCl, vortex mix 10 sec.

4. Aspirate about 0.9 mL of sample solution into DPX-CX 1 mL tip and mix with air.

5. Wait 30 sec, and then dispense to waste.

6. Aspirate the remaining sample solution and mix with air.

7. Wait 30 sec, and then dispense to waste.

8. Add 0.5 mL 10% ACN/H_2O to top of DPX tip and push to waste.

9. Add 0.5 mL ACN to top of DPX tip and push to waste.

10. Add 1 mL of 78/20/2 CH_2Cl_2/isopropanol/ammonium hydroxide to top of DPX tip and elute into GC vial.

11. Take to dryness using N_2 and heat (40°C).

For HPLC/MS/MS analysis:
1. Add 0.5 mL of acetonitrile or mobile phase for HPLC.

2. Inject into instrument.

For GC/MS analysis:
Chemical derivatization:

1. Add 0.1 mL pyridine and 0.1 mL BSTFA (1% TMCS).

2. Heat 30 min at 70-80 $^{\circ}$C.

3. Cool, and transfer contents to GC vial insert.

4. Inject 2 μL using selected ion monitoring (SIM).

GC/MS conditions:

- Agilent Technologies GC/MS: 6890 GC and 5975 Mass Selective Detector.
- DB5-MS Column: 30m, 0.25mm I.D., 0.25μm film thickness.
- GC parameters
  Flow rate: 1 mL/min He @ constant flow.
  Injector: 280 $^{\circ}$C
  Detector: 300 $^{\circ}$C
  Oven: 80 $^{\circ}$C for 1 min, ramp 20 C$^{\circ}$/min to 300 $^{\circ}$C for 5 min for a 17 min
  GC total run time.

SIM Parameters:	Ions	Dwell
	327	20
	342	20
	171	20
	197	20
	330 (I.S.)	20
	345 (I.S.)	20

RESULTS AND DISCUSSION

Previous work has shown that melamine is effectively extracted using cation exchange mechanisms.[1] The cation exchange mechanism permits very high efficient extractions of basic compounds, especially compounds containing amine moieties. Melamine has 3

amine groups, so not surprisingly this compound is efficiently extracted using this mechanism.

The cation exchange sorbent used in the DPX-CX tips are small particle size (10-20 um) with very high efficiency. This material provides unsurpassed capacity to extract basic compounds such as melamine.

Table 1 and Fig. 1 provide statistical data from extractions of melamine from milk using DPX-CX. High recoveries are obtained at concentrations ranging from 10 ppb to 1 ppm with %RSDs less than 5%. The LOD and LOQ values were 6.8 and 20 ppb, respectively. These low levels indicate that the sample solution can be diluted and still provide ample sensitivity to ensure levels are below 1 ppm.

Table 1: Validation results for melamine extracted from milk.

Compound	Recovery	RSD	R^2	LOD (ng/mL)	LOQ (ng/mL)
Melamine	95% (10ppb) (94% (1 ppm))	3.8% (1.6%)	0.9982	6.8	20

LOD=STDEV of the lowest concentration*3.3/slope
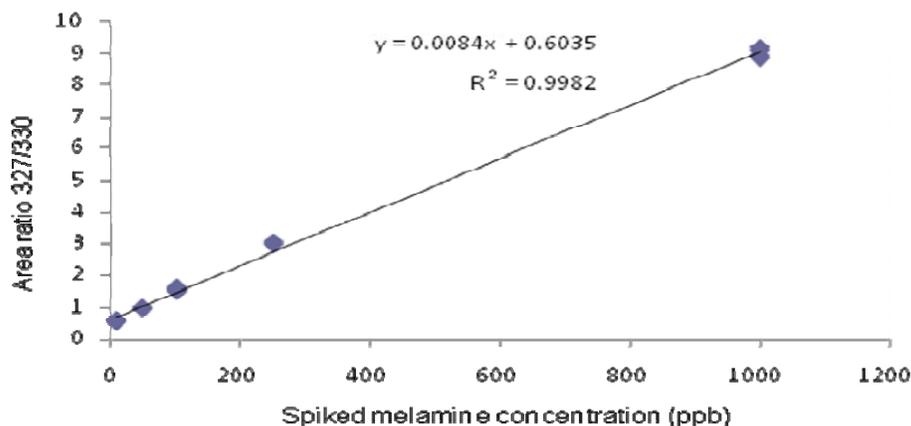LOQ= STDEV of the lowest concentration*10/slope

Figure 1. Calibration plot of melamine extracted from milk using DPX-CX 1 mL tips, showing good linearity from 10 ppb to 1 ppm.

There is plenty of capacity to accurately detect melamine at higher concentrations, but the use of a "heavier" melamine internal standard may provide better results (to ensure no contributions of ions due to the analyte).

Most importantly, the chromatograms are free from interferences. There are no sugars or fatty acids present, as shown in Fig. 2. These clean extracts permit the chemical

derivatizations to be efficient and reproducible. As shown in Fig. 3, melamine can be readily detected at concentrations as low as 10 ppb.
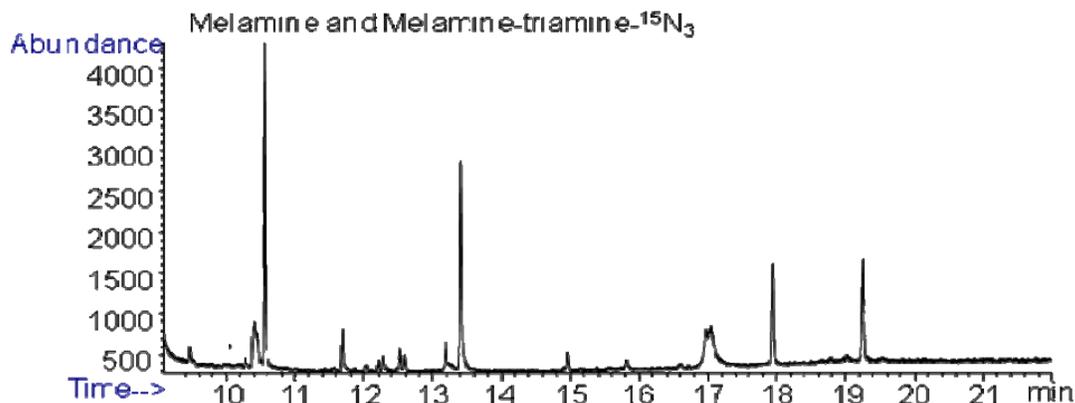

Figure 2. Total ion chromatogram (SIM analysis) of milk spiked at 10 ppb with melamine and 100 ppb with melamine-$(N_{15})_3$ extracted using DPX-CX 1 mL. The derivatized eluent had no interferences with the peak at approximately 10.6 min.
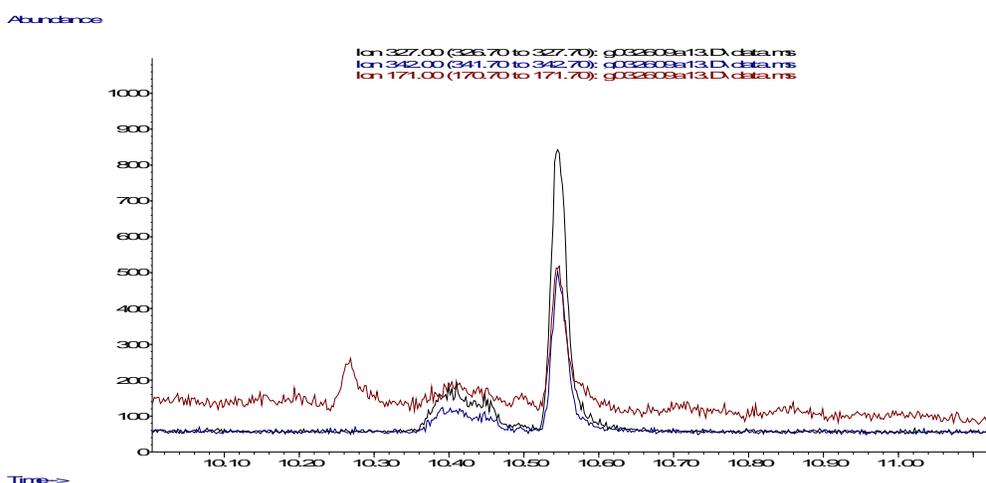

Fig. 3. Extracted ion chromatogram of melamine spiked at 10 ppb in milk.

It should be noted that the DPX-CX tips also work well with the analogues of melamine, ammeline and ammelide. These compounds also contain amine groups, so not surprisingly they are readily extracted using cation exchange mechanisms. Although these analogues were not targeted in this particular study, we have achieved good recoveries of these compounds using DPX-CX previously. Cyanuric acid, however, does not contain a basic moiety and cannot be efficiently extracted using DPX-CX. Moreover, it is desirable to analyze this compound separately from melamine by GC/MS because of

co-precipitation problems associated with the presence of both cyanuric acid and melamine in extracts.

The analysis of cyanuric acid in milk can be efficiently performed with another DPX product, and this procedure is provided in a separate application note.

CONCLUSIONS

This method is very reliable, fast, and easy to perform. By using semi-automation to extract 20 samples simultaneously (using 5 mL DPX-CX tips), this DPX method provides the highest throughput available for extractions of these samples. For GC/MS analysis, the semi-automation can be combined with an automated derivatization station (GERSTEL) that will provide the highest throughput possible for the analysis of melamine and its analogues.

REFERENCES

1. M. Smoker and A. J. Krynitsky, Interim Method for Determination of Melamine and Cyanuric Acid Residues In Foods using LC-MS/MS: Version 1.0, LIB No. 4422 (FDA Laboratory Information Bulletin), Oct. 2008.