Application Note: Analysis of Cyanuric Acid in Milk
(updated: 04/23/09)

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

INTRODUCTION

There has been great interest recently for detecting melamine and its analogues such as cyanuric acid in food samples such as milk. High levels of these compounds 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 and cyanuric acid are also found in various plastics and cleaning supplies, so accidental contamination of food products is possible.

Regulatory and public concern over melamine and cyanuric acid 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 and cyanuric acid in milk.

Cyanuric acid is a difficult analyte to analyze. It is a small compound that is very hydrophilic, and it is not soluble in polar organic solvents such as acetonitrile. To analyze this substance by GC/MS, it must be chemically derivatized, so it is imperative that the analyte be removed from "water". Most methods for cyanuric acid have focused on essentially no cleanup. The samples are extracted (or diluted for liquids) with acetonitrile and water (or 4% formic acid in acetonitrile), centrifuged (multiple times), and filtered.[1] A small aliquot of the solution is then dried using gas flow (or vacuum) and heat. Due to the dilutions and small volumes, the methods are not very sensitive and LC/MS/MS analysis has been a primary focus.

This application note provides a much improved method for rapid and sensitive detection of cyanuric acid in milk. The sample preparation involves the use of a "cleanup" procedure for the removal and precipitation of sample matrix interferences using a "Cleanup Tip" (DPX-WAX). 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 0.10 mL of milk into small test tube.

2. Add internal standard at 1 ppm.

3. Add 0.5 mL of 50% acetonitrile and water.

4. Vortex mix for a couple of seconds.

5. Add to "top" of DPX-WAX "Cleanup Tip", 5 mL.

6. Aspirate and mix solution with sorbent (preferably using Lever Extractor).

7. Dispense "clear solution" into vial.

8. Take to dryness using N_2 and heat (70°C).

For HPLC/MS/MS analysis:
1. Add 0.5 mL of DI water (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 MTBSTFA.

2. Heat 30 min at 70-80 ^{o}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 $^{°}$C
    Detector: 300 $^{°}$C
    Oven: 80 $^{°}$C for 1 min, ramp 20 C$^{°}$/min to 300 $^{°}$C for 5 min for a 17 min
        GC total run time.

SIM Parameters:

Ions	Dwell
414	20
415	20
456	20
242	20
417 (I.S.)	20
459 (I.S.)	20

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RESULTS AND DISCUSSION

Many procedures for cyanuric acid focus on little to no sample preparation. In these procedures, small aliquots of the sample is dried after centrifugation steps and filtration.[1] For GC/MS analysis, the extracts are derivatized with BSTFA.[2]

We have used these procedures for analyzing milk, and a representative chromatogram is shown in Fig. 1. We have found the TMS derivatives of cyanuric acid in these extracts to be unstable. Even the spiked standards of blank extracts of these samples are not stable. We believe this is probably due to the presence of sugars (intense peaks from 14-16 minutes) and fatty acid components (app 9.5 and 10 min). This sample preparation may be good for many sample matrices, but it does not seem to be ideal for milk.
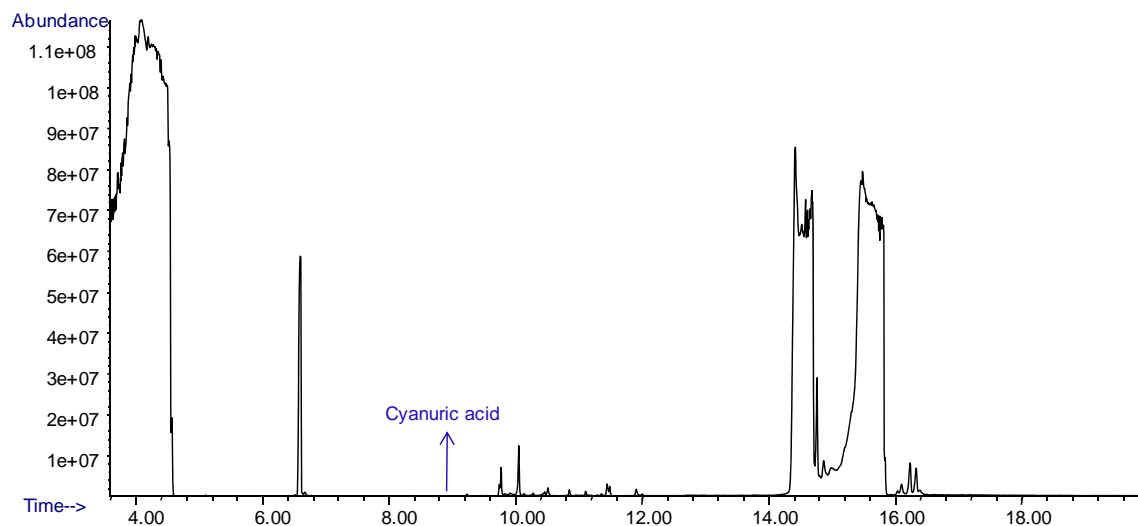

Figure 1. Cyanuric acid spiked at 10 ppm, extracted using 4% formic acid-acetonitrile w/o cleanup (BSTFA derivative), 10 µL injection

We investigated other possible derivatization procedures, and found that MTBSTFA seemed to provide a more stable derivative. Figures 2 and 3 show the mass spectra of cyanuric acid and cyanuric acid-$^{13}C_3$, respectively. These derivatives appeared to be more stable in the milk extracts, and we were able to more readily detect the analyte. Figure 4 shows a chromatogram of cyanuric acid with internal standard spiked in milk.
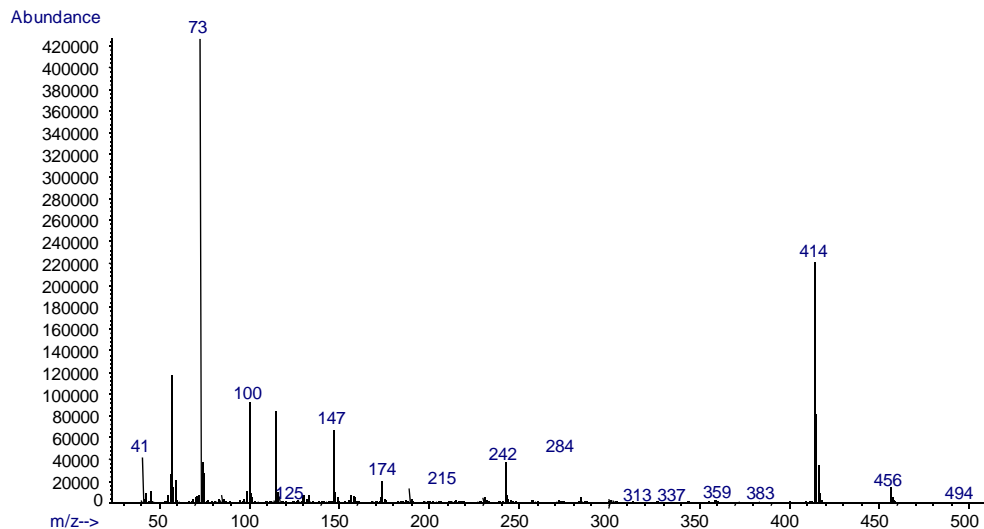
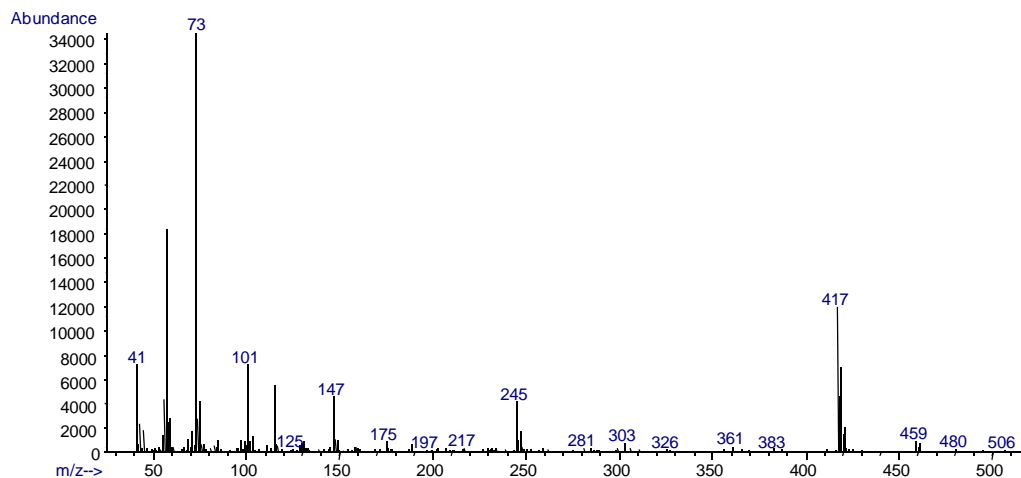**Figure 2. Mass spectra of cyanuric acid/MTBSTFA derivative**

Figure 3. Mass spectra of ^{13}C- cyanuric acid/MTBSTFA derivative

Although this change in derivatization improved the stability and detection of cyanuric acid, the chromatograms are still characterized with intense sugar (14 to 17 min) and fatty acid components (app. 9, 10.5 and 12 min). In fact, the heavier derivative had shifted the retention time to longer times that overlap with fatty acid components. Although the use of selected ion monitoring keeps these interferences to a minimum, we noted two major problems. The inlet liner would get very dirty and would need replacement almost after every 2 or 3 injections. Secondly, the mass spectrometer source would also need to be cleaned more often. (This was readily noticed by observing increasing EM voltages in the MS tune files.)
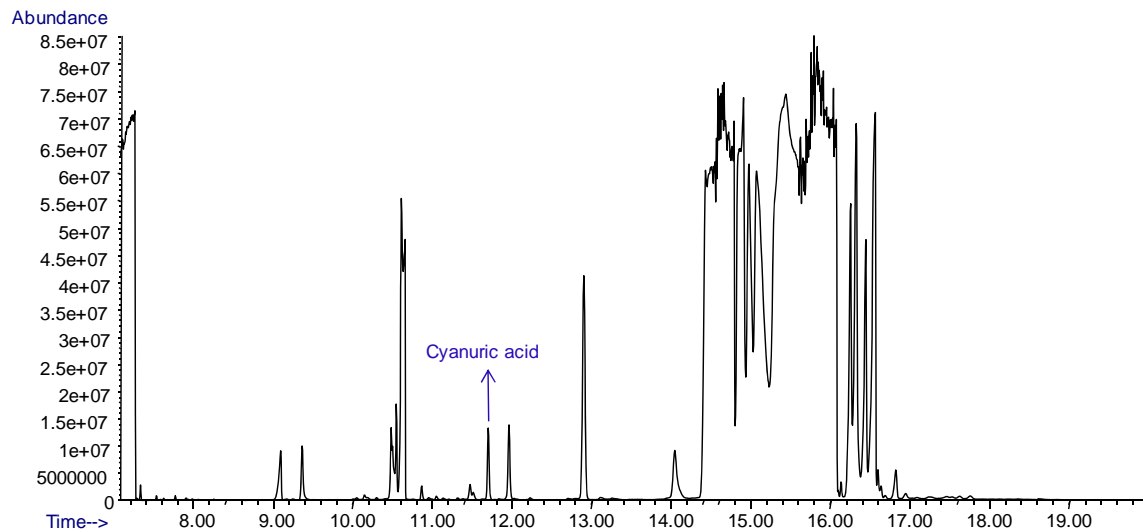
Figure 4. Cyanuric acid spiked at 10 ppm, extracted using 4% formic acid-acetonitrile w/o cleanup (MTBSTFA derivative), 10 µL injection
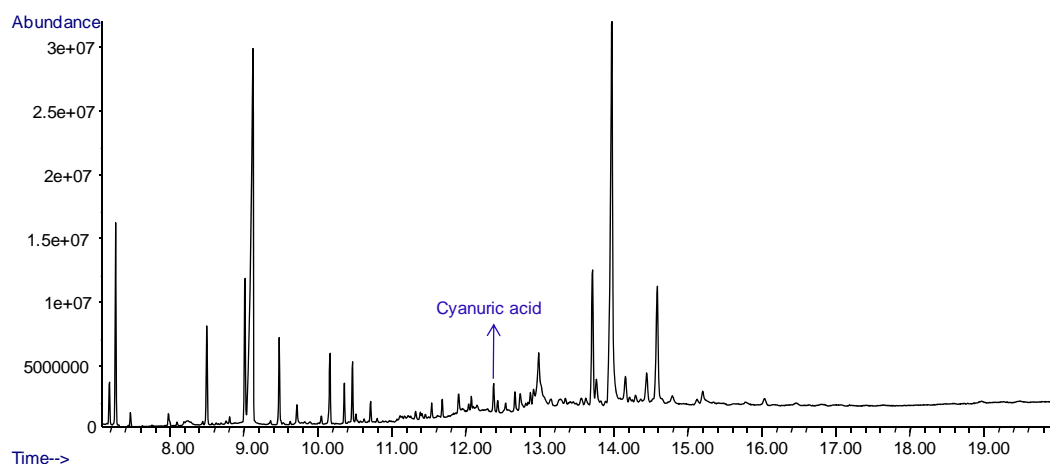

Figure 5. Full scan chromatogram of 1.0 ppm cyanuric acid spiked in 0.1 mL milk, 10 µL injection. The extract was "cleaned up" using DPX-WAX.

We also explored the use of strong anion exchange to extract cyanuric acid directly, which has been reported.[2] We still had problems associated with interferences using GC/MS analysis, and we did not achieve very high extraction efficiencies. During these development studies, however, we found that the use of a weak anion exchange resin simultaneously solved 3 problems for our analysis. First, as we expected, the weak anion exchange sorbent removed the fatty acid components without binding cyanuric acid (at approximately neutral pH). The second and very unique feature was that a precipitation occurred when this sorbent was mixed with the milk solutions. The sorbent apparently

causes precipitation of the proteins, and therefore time-consuming centrifugation steps are not required. The third solution, and most surprising, was that the sugar interferences were not detected (Figures 5 and 6).
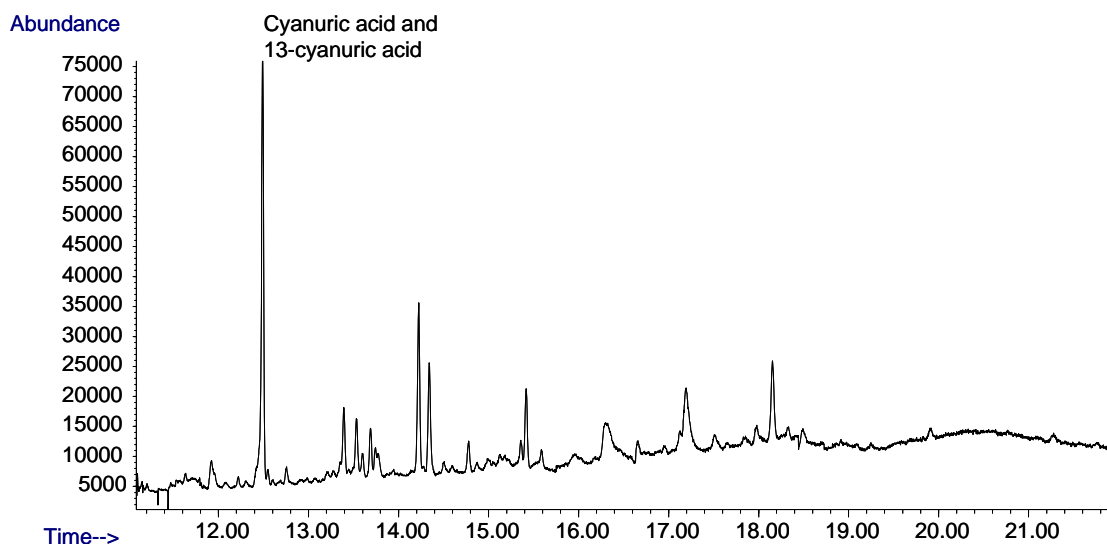

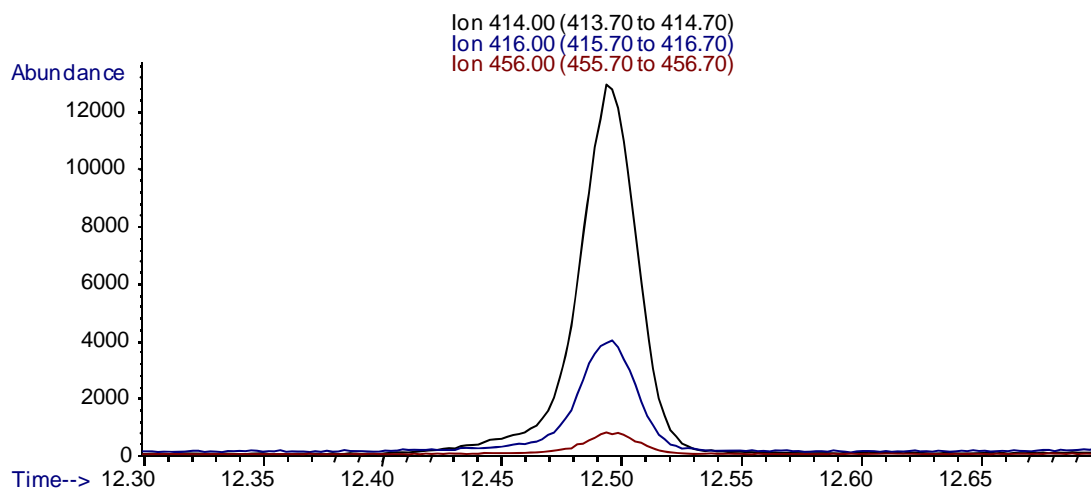**Figure 6. SIM chromatogram of 1.0 ppm cyanuric acid spiked in 0.1 mL milk, 2μL injection.**

Figure 7. Extracted ion of cyanuric acid/MTBSTFA derivative (spiked at 1.0 ppm)

Most importantly, this rapid "Cleanup Tip" provides a very rapid and efficient procedure to readily detect cyanuric acid at the threshold level of 1 ppm in milk (Figure 7). The sensitivity is very good even though just 0.10 mL of milk is used for this analysis.

Table 1 and Fig. 8 provide statistical data from extractions of cyanuric acid in milk using DPX-WAX for "cleanup". High recoveries are obtained at concentrations ranging from 0.25 ppm to 10 ppm with %RSD of 5.3% at 1 ppm. The LOD and LOQ values of 70 and 220 ppb, respectively.

Table 1. summary of R2, LOD, LOQ, %Recovery and %RSD.

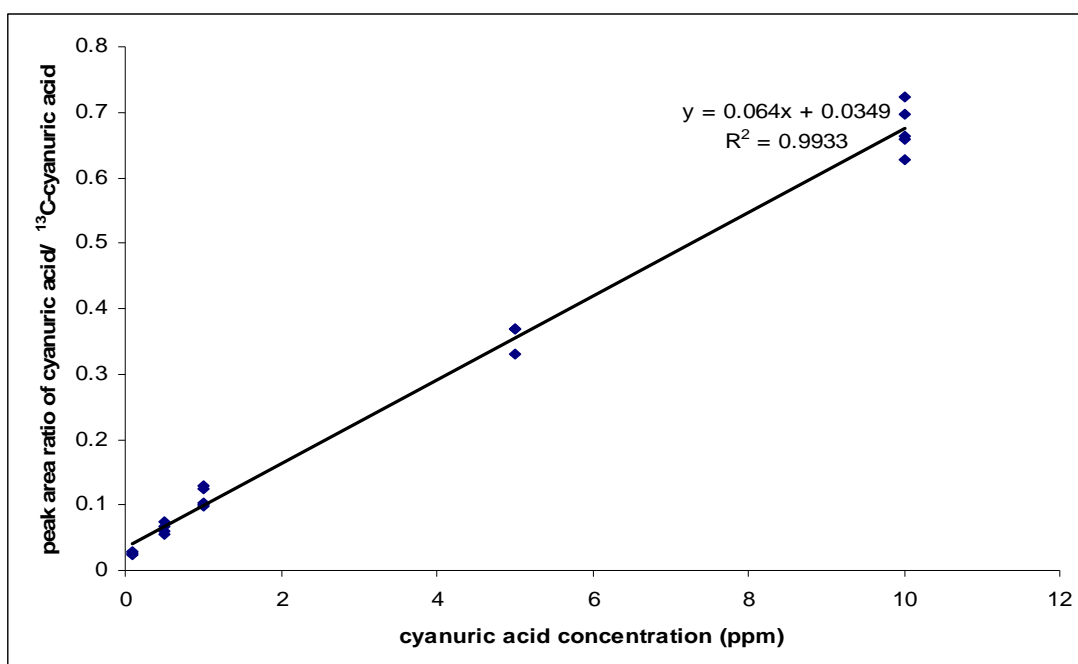| R^2	LOD (ppm)	LOQ (ppm)	Recovery% (1.0 ppm)	RSD% (1.0 ppm)
0.9933	0.07	0.22	80.21	5.33

Figure 8. Calibration of cyanuric acid 0.1 ppm to 10 ppm

It should be noted that the dynamic range can be easily increased above 10 ppm without requiring dilution. The calibration plot indicates that there is variance at the high calibrator at 10 ppm, but this is due to the fact that high concentrations of cyanuric acid contribute peak intensities to the "internal standard"; ie, the mass spectrum of cyanuric acid has a small intensity at 417 amu, which is the ion monitored for the internal standard. This can be addressed by using a "heavier" internal standard which is commercially available. We chose the present internal standard because it was less expensive, and the solution to this problem can be addressed by simply diluting the sample if it tests greater than 10 ppm.

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CONCLUSIONS

This method is very reliable, fast, and easy to perform, and provides the most sensitive detection reported for cyanuric acid. By using semi-automation to extract 20 samples simultaneously (using 5 mL DPX-WAX tips), this DPX method provides the highest throughput available for extractions of these samples. The overall analysis and throughput can be improved for GC/MS analysis by combining semi-automation with an automated derivatization station (GERSTEL). For LC/MS/MS, the extraction can be completely automated using DPX-WAX-1 mL TA (1 mL with a transport adaptor) and the GERSTEL MPS-3, with the "cleanup" taking less than 2 minutes to perform for each sample. This latter procedure provides the highest throughput which allows for "ready for analysis" sample preparation, with a sample being extracted while the previous sample is being chromatographically analyzed.

REFERENCES

1. S. Turnipseed, C. Casey, C. Nochetto, and D. N. Heller, Determination of Melamine and Cyanuric Acid Residues In Infant Formula using LC-MS/MS, LIB No. 4421 (FDA Laboratory Information Bulletin), Vol. 24, Oct. 2008.

2. 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.