Simultaneous Extraction of Melamine and Cyanuric Acid from Food Products Using Strata® Melamine SPE and Ultra-fast LC/MS/MS Analysis Using Kinetex™ HILIC, Rapid LC/MS/MS Analysis on Luna® HILIC, or Rapid GC/MS Analysis on Zebron™ ZB-XLB HT

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Using Strata Melamine, it is possible to simultaneously extract melamine and cyanuric acid from food samples, achieving very reproducible results at the 20 ng/g LOD level. Post extraction, analysts have the choice of rapid, simultaneous analysis of melamine and cyanuric acid via LC/MS/MS using either a fully porous particle Luna HILIC column for complete analysis in under 3.5 minutes or an ultra-high efficiency Kinetex core-shell technology HILIC column for complete analysis in less than 1 minute, or, via GC/MS using a Zebron ZB-XLB HT column.

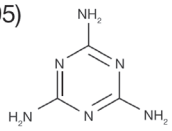
Introduction

Melamine and cyanuric acid are very water soluble compounds that prove to be a challenge for analysts looking to extract both compounds out of different food samples for analysis via liquid chromatography. Over the last two years, rising concern over the presence of both compounds in processed foods has led to increased regulation and required testing by governing and regulatory bodies globally. In concentrations exceeding 2 µg/mL, melamine and cyanuric acid crystallize, in a 1 to 1 ratio, to form melamine cyanurate, a very poorly water-soluble complex. Consumption of melamine and cyanuric acid in concentrations great enough to form melamine cyanurate can result in adverse health problems, including kidney failure and death.

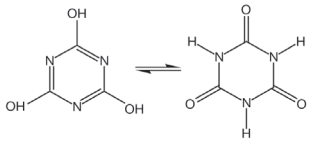
Typically, most analysts look to exploit the functional groups on melamine and cyanuric acid by ionizing and extracting them out of samples by way of ion-exchange solid phase extraction (SPE). Melamine contains three primary amine groups, with a pK_a of around 9, which lend themselves as a proton acceptor under acidic pH, making it possible to retain the compound with a cation-exchange solid phase extraction (SPE) cartridge (**Figure 1**). Cyanuric acid has a pK_a around 6.5, giving it a negative charge under basic pH conditions and making it possible to extract using an anion-exchange SPE cartridge. Extraction of both compounds has thus required two different SPE sorbent cartridges and two separate extraction procedures. In an effort to achieve a greater time savings, we developed a revolutionary SPE cartridge, Strata Melamine, for simultaneously extracting melamine and cyanuric acid out of food products, like baby formula, eliminating the need for two different SPE cartridges and two separate extraction procedures.

Figure 1.
Chemical structures, log P, and pK_a for melamine and cyanuric acid.

Melamine (log P -1.37, pK_a 8.95)



Cyanuric Acid (log P -2.0, pK_a 6.5)



Methods have been developed for the simultaneous analysis of melamine and cyanuric acid using liquid chromatography (LC) and gas chromatography (GC) [1,2], which allows analysts testing for both compounds to combine the two sample extractions into a single sample injection, thus reducing analysis time. Rapid analysis methods were also developed, two for LC/MS/MS and one for GC/MS, giving analysts the option to efficiently analyze the extracted sample using the technique they are most comfortable with. For one of the LC/MS/MS methods we used an ultra-high efficiency Kinetex core-shell technology column and achieved complete separation and analysis of melamine and cyanuric acid in less than 1 minute. Rapid analysis of both compounds is also possible via GC/MS following an optimized method using the Zebron ZB-XLB HT Inferno column, which allows for a complete runtime in less than 9 minutes.

Experimental Conditions

Chemicals

Melamine (99 %) and Cyanuric acid (98 %) solid powder were obtained from Fluka and Acros Organics respectively. For internal standard liquid Melamine (13C3, 99 % Amino-15N3, 98 %) and solid Cyanuric Acid (13C3, 97 % CP) were procured from Cambridge Isotope Laboratories and Isotec respectively. Enfamil®, baby formula, was obtained from a local grocery store. HPLC grade water (Milli-Q, Millipore, Billerica, MA) was used to prepare HPLC mobile phase and for sample preparation. HPLC grade acetonitrile (ACN) was obtained from Honeywell Burdick & Jackson (Muskegon, MI). All other reagents used were obtained from Sigma-Aldrich and used without further purification.

Calibration standards were created by serial dilution. A total of six standards ranging from 4 ng/mL to 2000 ng/mL were created and used to spike baby formula samples.

Mobile Phase Preparation

100 mM ammonium formate buffer (pH 3.2) was prepared fresh daily by dissolving approximately 6.31 g of ammonium formate in 1 liter of water and adding 10.5 mL of concentrated formic acid. 100 mL of buffer was then added to 900 mL of ACN and mixed completely.

100 mM ammonium acetate buffer (pH 5.8) was prepared fresh daily by dissolving approximately 7.71 g of ammonium acetate in 1 liter of water and adding 400 µL of concentrated acetic acid. 100 mL of buffer was then added to 900 mL of ACN and mixed completely.

Sample Preparation

50 µL (4.0 µg/mL) of melamine and cyanuric acid were added to a homogeneous mix of 200 mg of baby formula in 1 mL of water. 100 µL of HCl was also added to the mix, along with the melamine and cyanuric acid internal standards (IS) at a volume of 50 µL (15.0 µg/mL). A protein precipitation was performed by adding 3 mL of ACN to the 1 mL of baby formula sample. After vortexing and centrifuging the sample, the supernatant was collected for the SPE step.

Solid Phase Extraction Procedure

200 mg/3 mL Strata Melamine SPE cartridges from Phenomenex (Torrance, CA) were used for the simultaneous extraction of melamine and cyanuric acid. The SPE cartridges were conditioned

with 3 mL of methanol and equilibrated with 3 mL of 50/50 ACN and water. Approximately 4 mL of the supernatant from the initial protein precipitation step was loaded onto the SPE cartridge and vacuumed at a rate of 1 to 2 drops per second. Three different wash aliquots consisting of two 500 µL 50/50 ACN/water and one 500 µL 50/50 methanol/water were passed through at a rate of 1 to 2 drops per second. The cartridge was dried for 2 minutes under 10 mm Hg of vacuum. Analytes were eluted with one 500 µL aliquot of methanol and two 500 µL aliquots of 5 % ammonium hydroxide in methanol at a rate of 1 drop per second. All three elution aliquots were combined, dried down under heated nitrogen (45-55 °C), and reconstituted in 1 mL of mobile phase.

Chromatographic Conditions

LC/MS/MS was performed for the analysis of the extracted baby formula samples. An Agilent 1100 series binary pump equipped with on-line solvent degasser, autosampler, and column temperature module (Palo Alto, CA) interfaced with an Applied Biosystems API3000™ tandem mass spectrometer with TurboIonSpray® electrospray ionization (ESI) interface was for the analysis. The mass spectrometer was set for MRM; the heater gas flow was 7000 cc/minute with the heater temperature set at 450 °C. The ionization mode was switched from negative to positive at 1.7 minutes in order to detect cyanuric acid and melamine respectively. See **Table 1** for other MS/MS conditions and compound ionization data. The LC system was controlled using Analyst 1.41 software.

LC/MS/MS Analysis

Table 1.
MS/MS settings and compound mass to charge ratios for the analysis of melamine and cyanuric acid on Luna HILIC.

Analyte	Ionization	Q1	Q3	Dwell Time (sec)	Nebulizer Gas Flow (NEB)	Curtain Gas (CUR)	Ion Spray Voltage (IS)	Collision Gas (CAD)
Cyanuric Acid	-Ve	128	42	150	8.00	12.00	-4500.00	12.00
Cyanuric Acid-13C3	-Ve	131	43	150				
Melamine	+Ve	127	85	150	4.00	7.00	5000.00	12.00
Melamine - 13C3, 15N3	+Ve	133.2	89.1	150				

Results and Discussion

The addition of 100 µL of 0.2 N HCl during the protein precipitation step of the extraction gives the sample loaded onto the Strata Melamine SPE cartridge a pH reading of ca. 5.5. At this pH we can assume that melamine has a positive charge, allowing for cationic retention, and cyanuric acid has a neutral charge, allowing for retention via polar interactions. The sample load onto the Strata Melamine SPE cartridge consisted of 75 % ACN, necessary to crash out proteins, which we found to be essential in achieving acceptable recoveries of cyanuric acid. ACN levels less than 75 % during the load step of the SPE procedure lead to a significant decrease in cyanuric acid recovery, however, concentrations greater than 75 % during the load and wash steps had no adverse affect on recoveries and were seen to improve absolute recoveries.

The combined protein precipitation and solid phase extraction of melamine and cyanuric acid out of baby formula yielded relative recoveries of 115 % and 94.5 % respectively (**Table 2**). Acceptable coefficients of variation (CVs) for both compounds, 4.10 for cyanuric acid and 3.05 for melamine, were also achieved.

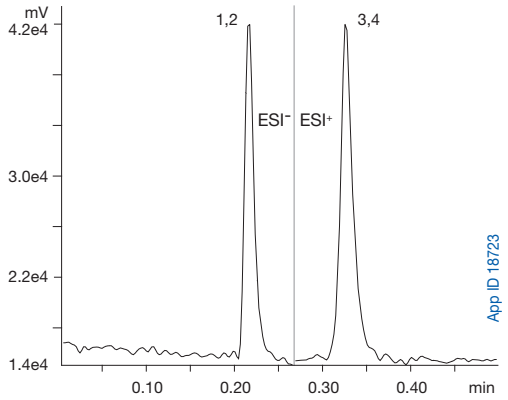
Table 2.
Absolute recoveries, relative recoveries, and coefficients of variation (CV) data for the extraction of melamine and cyanuric acid using Strata Melamine SPE cartridges.

	Absolute Recoveries	Relative Recoveries
Melamine	67 % (CV=3.10)	67 % (CV=3.10)
Cyanuric Acid	68 % (CV=2.02)	94.5 % (CV=4.10)

Complete separation and detection of melamine and cyanuric acid was achieved by LC/MS/MS in less than 0.4 minutes using a Kinetex HILIC column and in less than three minutes using a Luna HILIC column (**Figures 2 and 3**). The MS ionization mode was initially set for negative ionization to facilitate the detection of cyanuric acid, but following elution of the cyanuric acid peak, the source must be switched to positive ionization mode in order to allow for detection of melamine.

Calibration curves created for melamine and cyanuric acid were based on actual baby formula extracts (**Figures 4a and 4b**). Both calibration curves took into account concentrations ranging from 20 ng/g to 2,000 ng/g, resulting in R^2 values equal to 0.9998 and 0.9991 for melamine and cyanuric acid respectively.

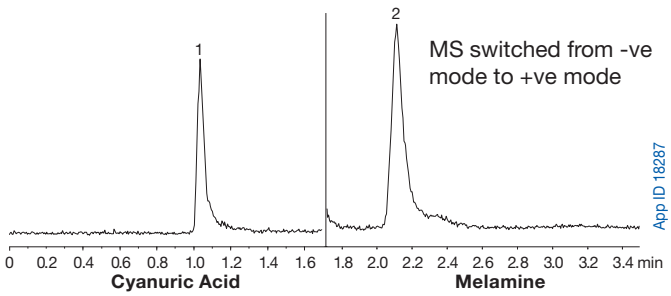
Figure 2.
LC/MS/MS chromatogram of melamine and cyanuric acid extracts from baby formula separated on a Kinetex HILIC column



Column: Kinetex 2.6 µm HILIC
Dimension: 50 x 2.1 mm
Mobile Phase: Acetonitrile/100 mM Ammonium acetate, pH 5.8 (90:10)
Flow Rate: 1.0 ml/min
Temperature: 25 °C
Detection: Mass Spectrometer (MS)
Switch from Negative Ion Mode (Cyanuric acid) to Positive Ion (for Melamine) at 0.26 minutes until 1 minute; MRM
Sample: 1. Cyanuric acid 128-85.0 (quant ion), 128.0-42.0 (qualifier ion)
2. Cyanuric acid 13C3 ISTD 131.1-87.0
3. Melamine 127.1-85 (quant ion), 127.1-68 (qualifier ion)
4. Melamine-13C3.15N3 ISTD 133.2-89.1

Note:
If using an HPLC system with needle wash capability, the needle wash solution should be 95/5 ACN/water for best results

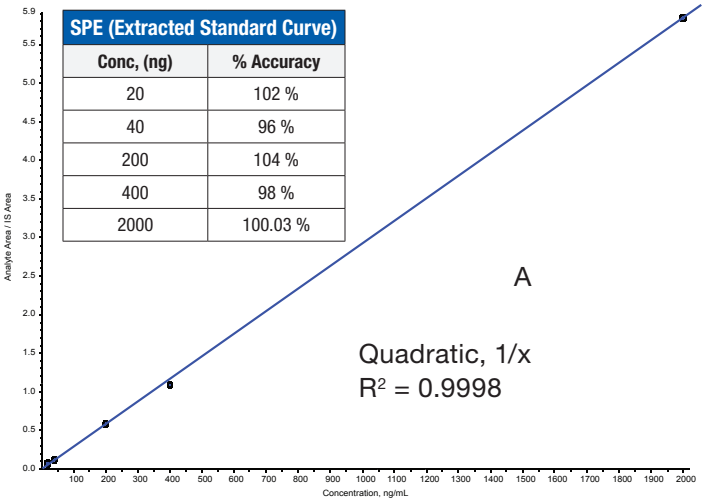
Figure 3.
LC/MS/MS chromatogram of melamine and cyanuric acid extracts from baby formula separated on a Luna HILIC column



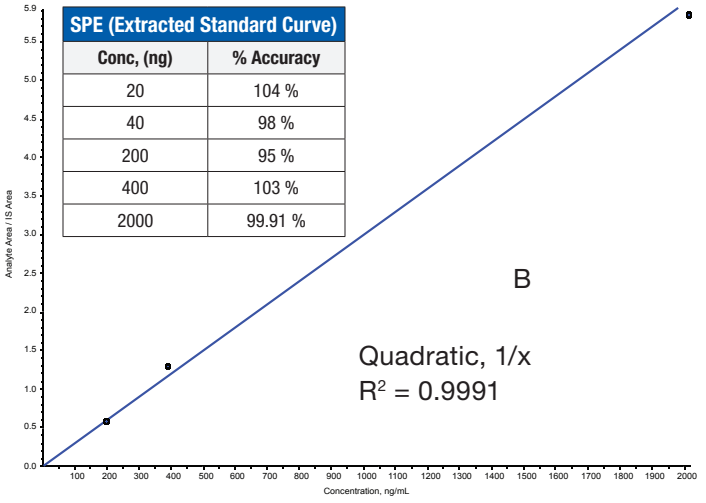
Column: Luna 3 µm HILIC
Dimension: 100 x 2.0 mm
Part No.: 00D-4449-B0
Mobile Phase: A: Acetonitrile
B: 100 mM Ammonium Formate pH 3.2 A/B (90:10)
Flow Rate: 0.4 mL/ min
Detection: Mass Spectrometer (MS) (ambient)
Sample: 1. Cyanuric acid (-ve)
2. Melamine (+ve)

Figure 4.
Calibration curves based on baby formula extracts of melamine at varying concentrations, and, % accuracy data

SPE (Extracted Standard Curve)	
Conc, (ng)	**% Accuracy**
20	102 %
40	96 %
200	104 %
400	98 %
2000	100.03 %



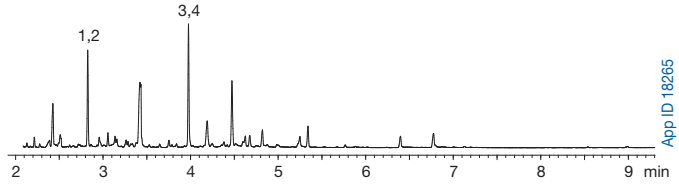
SPE (Extracted Standard Curve)	
Conc, (ng)	**% Accuracy**
20	104 %
40	98 %
200	95 %
400	103 %
2000	99.91 %



The lower limit of quantitation (LLOQ) was determined to be 200 ng/g for melamine and cyanuric acid in accordance with FDA guidelines (CV <20 % and S/N > 5:1).[3] The lower limit of detection (LLOD) for melamine and cyanuric acid was found to be 20 ng/g. For both the LLOD and LLOQ, lower limits were achieved for the detection of melamine; however the limits were set where both compounds met FDA guideline requirements.

Complete separation and detection of melamine and cyanuric acid was achieved by GC/MS in less than 9 minutes using the Zebron ZB-HT Inferno column (**Figure 5**). Following the Strata Melamine extraction and cleanup procedure, compounds were derivatized, eliminating the need for an ionization mode switch during MS analysis.

Figure 5.
GC/MS/MS chromatogram of melamine and cyanuric acid extracts from baby formula separated on a Zebron ZB-XLB-HT Inferno column



Column: Zebron ZB-XLB-HT Inferno
Dimensions: 15 meter x 0.25 mm x 0.25 µm
Part No.: 7EG-G024-11
Injection: On-Column @ 103 °C, 1 µL
Carrier Gas: Helium @ 1.4 mL/min (constant flow)
Oven Program: 100 °C for 0.5 min to 320 °C @ 25 °C/min
Detector: Mass Selective (MSD) @ 325 °C
Sample: Analytes are 200 ng / 100 µL in BSTFA / Pyridine (1:1)
1. Cyanuric Acid 13C3 (IS)
2. Cyanuric Acid
3. Melamine 13C3 15N3 (IS)
4. Melamine

Extraction from Tissue
For the extraction of melamine and cyanuric acid out of tissue, like pork or fish, the procedure stated in the US FDA Laboratory Information Bulletin No. 4422 was proven effective in combination with the Strata Melamine SPE cleanup method.

Homogenize 5 g of tissue with 20 mL of 50/50 acetonitrile and water. Spike the homogenized sample with internal standards and vortex thoroughly. Centrifuge sample at 7000 rpm for 15 minutes. Take 1 mL of the supernatant and add 2 mL of acetonitrile and 100 µL of 0.2 N HCl. Vortex the sample thoroughly and then centrifuge for 15 minutes at 7000 rpm. Load the supernatant onto the Strata Melamine SPE tube and proceed with SPE cleanup method.

Conclusions

Simultaneous extraction of melamine and cyanuric acid from baby formula can be achieved using 200 mg/3 mL Strata Melamine SPE cartridges. The lower limit of quantitation set by the World Health Organization (WHO) of 1 mg/kg is easily achieved with the experimental LLOQ of 200 ng/g for both compounds.[4] Good linearity was also established, with R2 values greater than 0.994 over the concentration range of 20 ng/g to 2,000 ng/g for both melamine and cyanuric acid.

Ultra-fast, simultaneous analysis in less than 30 seconds of melamine and cyanuric acid is possible using the new Kinetex HILIC column. For a longer run time, a longer Kinetex HILIC column can be used or the Luna HILIC LC/MS/MS method can be followed to yield a complete analysis in under 3 minutes.

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The optimized Zebron ZB-XLB HT Inferno GC/MS method yields fully resolved peaks in less than 9 minutes, which is more than 50 % faster than the GC method suggest by the FDA. With temperature stability up to 400 °C, the Zebron ZB-XLB HT Inferno column allows for the removal of residual on-column contaminants, resulting in a longer column lifetime when compared to traditional phases.

References

1. Litzau J, Mercer G, Mulligan K. http://www.fda.gov/cvm/GCMSMelamine htm. Accessed February 25, 2007.
2. Tosoh Bioscience, TSK-GEL Amide-80 HILIC Columns for the Analysis of Melamine and Cyanuric Acid in Milk by LC-MS/MS, Application Note AN191108.
3. *Guidance for Industry Bioanalytical Method Validation*; U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, 2001.
4. *Toxicological and Health Aspects of Melamine and Cyanuric Acid: Report of a WHO Expert Meeting, In Collaboration with FAO, Supported by Health Canada;* World Health Organization, WHO Document Production Services: Geneva, Switzerland, 2009.

Ordering Information

SPE

Strata® Melamine

Part No.	Description	Sorbent Mass	Unit
8B-S049-EBJ	Strata Melamine	100 mg	3 mL (50/box)
8B-S049-FBJ	Strata Melamine	200 mg	3 mL (50/box)

LC

Kinetex™

Part No.	Description	Dimensions (mm)	Unit
00B-4474-AN	Kinetex™ 1.7 µm HILIC	50 x 2.1	ea
00B-4461-AN	Kinetex™ 2.6 µm HILIC	50 x 2.1	ea
00F-4461-AN	Kinetex™ 2.6 µm HILIC	150 x 2.1	ea
00B-4461-E0	Kinetex™ 2.6 µm HILIC	50 x 4.6	ea
00F-4461-E0	Kinetex™ 2.6 µm HILIC	150 x 4.6	ea

Other phases available, contact your Phenomenex technical consultant.

Luna® HILIC Columns

Part No.	Description	Dimensions (mm)	Unit
00A-4449-B0	Luna 3 µm HILIC	30 x 2.0	ea
00B-4449-B0	Luna 3 µm HILIC	50 x 2.0	ea
00D-4449-B0	Luna 3 µm HILIC	100 x 2.0	ea
00F-4449-B0	Luna 3 µm HILIC	150 x 2.0	ea
00B-4449-Y0	Luna 3 µm HILIC	50 x 3.0	ea
00D-4449-Y0	Luna 3 µm HILIC	100 x 3.0	ea
00F-4449-Y0	Luna 3 µm HILIC	150 x 3.0	ea
00D-4449-E0	Luna 3 µm HILIC	100 x 4.6	ea
00F-4449-E0	Luna 3 µm HILIC	150 x 4.6	ea

GC

Zebron™ GC Columns

Part No.	Description	Dimensions	Unit
7EG-G024-11	Zebron ZB-XLB-HT Inferno	15 m x 0.25 mm x 0.25 µm	ea
7HG-G024-11	Zebron ZB-XLB-HT Inferno	30 m x 0.25 mm x 0.25 µm	ea

guarantee

If Kinetex, Luna and Zebron columns do not provide at least an equivalent separation as compared to a competing column of the same particle size, similar phase and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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