GC/MS Analysis of Melamine and Cyanuric Acid Below 1 µg/g in Infant Formula

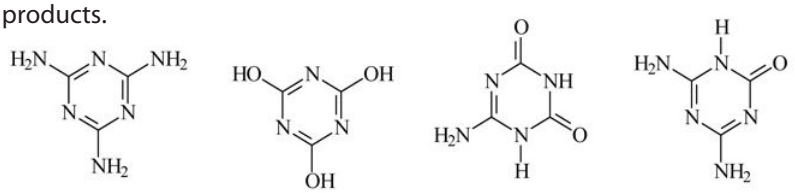
Abstract

The establishment of a 1 µg/g safety threshold for melamine in infant foods has led to a need for sensitive methods. Here, we established GC/MS conditions for highly reproducible analyses and evaluated the effectiveness of both solvent-based and matrix-matched standards. Using this method, melamine and cyanuric acid were reliably detected at and below 1 µg/g in infant formula.

Introduction

Reports linking the presence of melamine in pet food and infant formula to illnesses and deaths have led to the recall of a wide variety of tainted food products and to calls for stricter product testing. Melamine is not considered toxic alone at low doses; however, the observed toxicity has been attributed to melamine exposure in the presence of cyanuric acid. In combination, these compounds form insoluble crystals in the kidneys, causing illness and eventual renal failure. Melamine is not a legal food additive; it is a nitrogen-rich industrial compound used for plastics, flame-resistant products, and some cleaning agents. However, melamine and related byproducts have been added illegally to food products in order to falsely represent the amount of protein present, since protein level in many products is determined using nonspecific assays for nitrogen content (Figure 1).

Figure 1: Melamine and related compounds are rich in nitrogen and have been used to misrepresent protein levels in some food products.



In response to the need for more rigorous melamine and cyanuric acid testing, the U.S. Food and Drug Administration (FDA) has set three different commodity-based minimum reporting levels (MRLs): 10 µg/g for pet food, 2.5 µg/g for human food, and 1 µg/g for infant formula [1,2]. These limits place stringent demands on the analyst to demonstrate adequate instrument sensitivity. The following work was performed to establish conditions for melamine analysis down to 1 µg/g in infant formula and is based on the FDA method, GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide, and Cyanuric Acid [1]. Refer to the FDA method for overall experimental design and semiquantitative calculations.

Procedure

Sample Preparation

All solutions were made using a 1000 µg/mL (each compound) mixed stock solution of melamine, cyanuric acid, ammeline, and ammelide (cat.# 33253). Working standard solutions were then prepared at 1 µg/mL, 10 µg/mL, and 100 µg/mL in extraction solvent (10/40/50 diethylamine/water/acetonitrile). The 10 µg/mL working solution was used to fortify control infant formula at 0.5 µg/g, 1 µg/g, and 5 µg/g (dry formula was prepared according to label instructions prior to fortification). These matrix spike levels were chosen to demonstrate method performance at the MRL for infant formula (1 µg/g) and the MRL for adult human food commodities (2.5 µg/g). High and low standards were also prepared from the working solutions. Standards were prepared in solvent alone and also in extracted matrix at concentrations equivalent to sample fortification levels (0.0125 µg/mL, 0.0249 µg/mL, and 0.123 µg/mL) in order to evaluate possible matrix effects and to determine which technique would yield the most reliable GC/MS data.

The extraction procedure was performed as follows. Multiple 0.5 g matrix samples were prepared in 50 mL centrifuge tubes (cat.# 26227) and fortified if necessary (matrix control blanks were not spiked). After fortification, 20 mL of extraction solvent (10/40/50 diethylamine/water/acetonitrile) was added to each tube, and the samples were then sonicated for 30 minutes. Following sonication, the samples were centrifuged for 10 minutes to pellet particulate matter. The supernatant was then filtered through a 0.45 µm nylon filter (cat.# 26147) to remove any remaining particles and 200 µL aliquots of the resulting extracts were transferred to autosampler vials for derivatization.

Derivatization

Aliquots of standard (50 µL solvent-based, 200 µL matrix-based) and sample extracts (200 µL) were evaporated to dryness at 70 °C with nitrogen gas. Derivatization was performed as follows: 200 µL pyridine, 200 µL BSTFA with 1% TMCS (cat.# 35606), and 100 µL of benzoguanamine (internal standard, available as a custom standard) were added to each vial. Vials were shaken or vortexed for 10 minutes and then incubated at 70 °C for 45 minutes. Derivatization was also tested using 50 µL of BSTFA with 1% TMCS in attempt to minimize instrument contamination from excess derivatization material [3]. Samples processed with the reduced amount of reagent were not completely derivatized; therefore, the FDA method for derivatization using 200 µL of reagent was followed [1].

Analysis

Analyses were performed on a Shimadzu QP 2010 Plus gas chromatograph mass spectrometer (GC/MS) equipped with an AOC 20i+s auto injector and sampler. An Rxi-5Sil MS (30 m x 0.25 mm ID x 0.25 µm) column with a 5 m Integra-Guard integrated guard column (cat.# 13623-124) was used for the analysis. The integrated guard column was chosen since it protects the analytical column from matrix contamination, thus extending column lifetime, and prevents the possibility of leaks at a press-fit connection.

A splitless liner packed with wool (cat.# 22286-200.1) was used to help vaporize the compounds and also to further protect the column by trapping any nonvolatile compounds. GC conditions are shown in Figure 2; mass spectrometer conditions follow and are shown in Table I. The mass spectrometer was operated in SIM acquisition mode with selected ions for each analyte of interest. The transfer line temperature was set at 290 °C and the ion source temperature at 190 °C. The filament delay was set at 8.1 minutes and the dwell time for each ion was 0.15 seconds, giving a total run time of 18.67 minutes.

Calculations were performed using relative response factors as described in the FDA method [1]. Samples were quantified using both solvent-based and matrix-matched quantification standards in order to assess matrix effect and determine which procedure was more effective for infant formula.

Results

This method successfully detected melamine and cyanuric acid to the MRLs required for routine analysis of infant formula and human foods. Highly reproducible chromatographic separation was achieved (Figure 2) and was critical for compound identification, since several quantitation ions were also found in other peaks. Matrix interference was a significant issue for the analysis of ammelide and ammeline at lower concentrations.

Quantitation Ions and Importance of Retention Data

The quantitation ions given in the method were used. However, several of these ions (including m/z 344 and 345) were shared among analytes and were also observed for many of the peaks in samples and derivatizing reagent blanks. This resulted in heavy reliance on chromatographic retention time data for peak identification (in conjunction with confirmation of the ion ratios listed in Table I). The FDA method requires retention times to be within 0.05 minutes for compound identification [1]. This was easily achieved using the Rxi-5Sil MS column, which produced highly reproducible results, even after the approximately 150 injections made during testing (Table II).

Table I: MS conditions (SIM mode).

Compound	t_R (min)	Quant. ion	Qual. ion	Qual. ion	Qual. ion
Cyanuric acid	10.23	345 (100)*	330 (36)	346 (30)	347 (15)
Ammelide	11.07	344 (100)	329 (58)	345 (30)	330 (16)
Ammeline	11.76	328 (100)	343 (79)	329 (29)	344 (24)
Melamine	12.31	327 (100)	342 (53)	328 (30)	343 (17)
Benzoguanamine	14.54	316 (100)	331 (68)	332 (20)	330 (9)
*Expected relative ion ratios from FDA method.					

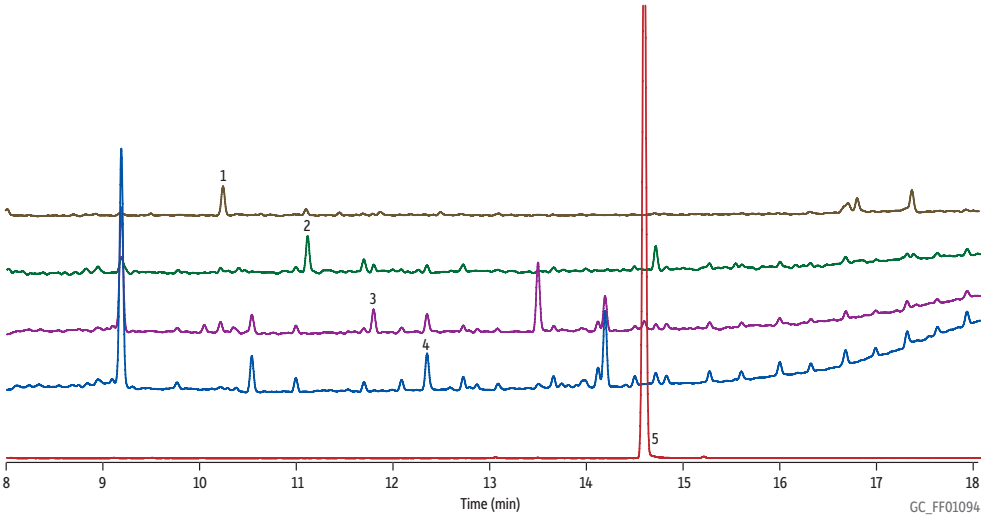
Table II: Retention time is critical to accurate peak identification. Highly reproducible results were achieved using an Rxi-5Sil MS column (n=3).

Compound	Retention time (min)		
	0.5 µg/g	1 µg/g	5 µg/g
Cyanuric acid	10.26 ± 0.05	10.23 ± 0.0006	10.23 ± 0.001
Ammelide	11.08 ± 0.003	11.07 ± 0.002	11.08 ± 0.003
Ammeline	11.76 ± 0.001	11.76 ± 0.003	11.76 ± 0.002
Melamine	12.31 ± 0.002	12.31 ± 0.000	12.31 ± 0.004
Benzoguanamine	14.54 ± 0.002	14.54 ± 0.001	14.54 ± 0.002

Figure 2: Analysis of melamine and related compounds in infant formula (1 µg/g MRL spike level).

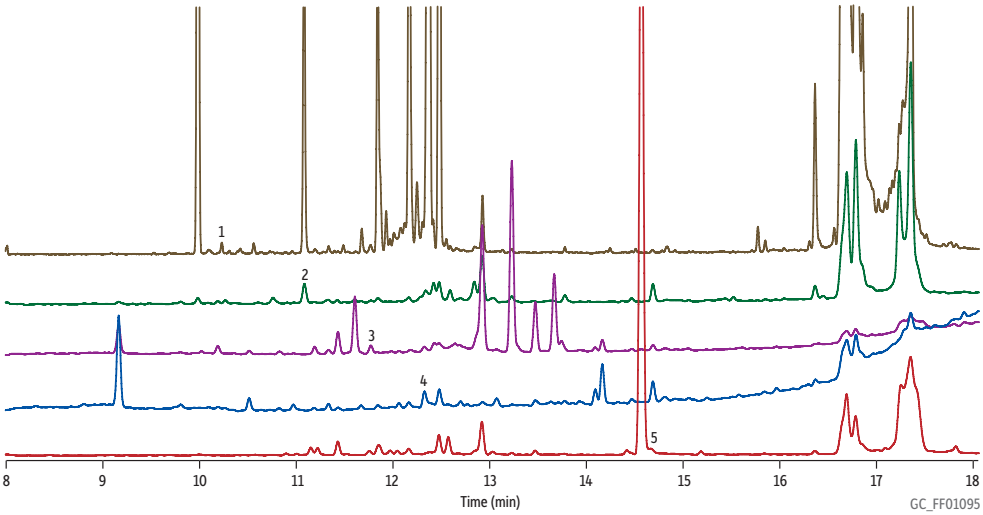
A. Solvent-only standard (0.01 µg/mL injection concentration)

Peaks	t_R (min)	Quant. Ion	Qual. Ion	Qual.Ion	Qual. Ion
1. Cyanuric acid	10.238	345	330	346	347
2. Ammelide	11.090	344	329	345	330
3. Ammeline	11.770	328	343	329	344
4. Melamine	12.318	327	342	328	343
5. Benzoguanamine	14.553	316	331	332	330



B. Matrix spike (0.01 µg/mL injection concentration)

Peaks	t_R (min)	Quant. Ion	Qual. Ion	Qual.Ion	Qual. Ion
1. Cyanuric acid	10.258	345	330	346	347
2. Ammelide	11.073	344	329	345	330
3. Ammeline	11.760	328	343	329	344
4. Melamine	12.328	327	342	328	343
5. Benzoguanamine	14.538	316	331	332	330



Column Rxi-5Sil MS w/5 m Integra-Guard column, 30 m, 0.25 mm ID, 0.25 µm (cat.# 13623-124)
Sample Infant formula fortified at 1 µg/g with melamine and related analogs stock standard (cat.# 33253) Benzoguanamine (cat.# 33251)
Conc.: 0.01 µg/mL as tri-TMS derivatives
Injection
Inj. Vol.: 1.0 µL splitless (hold 1 min)
Liner: 3.5 mm Gooseneck Splitless w/Wool (cat.# 22286-200.1)
Inj. Temp.: 280 °C

Oven
Oven Temp.: 75 °C to 320 °C at 15 °C/min (hold 4 min)
Carrier Gas He, constant flow
Flow Rate: 1 mL/min
Detector MS
Mode: SIM
Transfer
Line Temp.: 290 °C
Ionization Mode: EI
Instrument Shimadzu 2010 GC & QP2010+ MS
Notes all method ions in table, only quantification ions were plotted

Recovery of Target Compounds

Recovery data can be determined using solvent-based standards (no matrix) or matrix-matched standards (prepared in matrix extract and then derivatized). Since some matrices provide an analyte-protecting effect that results in an increased response compared to an identical analysis with solvent-based standards, we evaluated both methods here [4,5,6]. Recovery values using solvent-based standards varied from 50 to 250%, compared to the 81 to 143% recoveries shown in Table III, which were determined using matrix-matched standards. Though this method is not intended for true quantitation, recoveries based on matrix-matched standards indicate that determining melamine and cyanuric acid at different minimum reporting levels is possible, even below the 1 µg/g MRL required for infant formula.

Table III: Recovery of melamine and related compounds from spiked infant formula using matrix-matched standards (n = 3).

µg/g Detected	% Recovery (mean±std.dev.)	
0.5 µg/g fortification		
Cyanuric acid	0.4	81±29
Ammelide	MI	MI
Ammeline	MI	MI
Melamine	0.42	85±35
1 µg/g fortification		
Cyanuric acid	1.3	131±17
Ammelide	1.3	132±20
Ammeline	MI	MI
Melamine	1.3	132±57
5 µg/g fortification		
Cyanuric acid	7.2	143±46
Ammelide	4.9	97±18
Ammeline	5.3	106±20
Melamine	4.3	86±34
MI= matrix interference prevented accurate integration.		

Matrix Interference

As shown in Table III, ammelide and ammeline could not be integrated reliably below the MRL due to partial coelution with a matrix compound that had isobaric interferences. The same effect was observed for ammeline at 1 µg/g. Accurate integration was not possible since the ion ratios of the target analyte did not align with those expected based on known spectra or spectra of standards determined on the same system. While this method was optimized for melamine and cyanuric acid, the matrix interference seen for the other compounds illustrates the importance of evaluating and minimizing matrix carryover, especially when analyzing a new matrix. In this work, derivatizing reagent blanks were analyzed between each sample to ensure there was no carryover to subsequent injections.

Conclusion

Analysis of melamine and related compounds in infant formula is challenging since it has the lowest MRL of all commodities, and also because it is rich in sugars, which derivatize and chromatograph easily, thus increasing the potential for significant interferences. While this method successfully determined melamine and cyanuric acid to the MRLs required for routine analysis, coelutions complicated the analysis of ammelide and ammeline. Reliable retention time identification was critical for compound identification and the Rxi-5Sil MS column used here detected target analytes reproducibly and thus is recommended for GC/MS analysis of melamine. In addition, this column includes an Integra-Guard integrated guard column, which extends analytical column lifetime without the risk of leaks that can occur with manually connected guards.

References

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Compound	CAS #	cat.#
BSTFA w/1% TMCS (N,O-bis[trimethylsilyltrifluoroacetamide] w/1% trimethylchlorosilane)		
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25 g vial	25561-30-2	35607

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