A Total Solution for the Analysis of Melamine and Cyanuric Acid in Pet Food by GC/MS and Aqueous Normal-Phase LC/MS/MS

Application

Food

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Abstract

The Agilent 7890A-5975C GC/MS and 1200SL-6410 Triple Quadrupole LC/MS are used to analyze melamine and cyanuric acid in pet food and related raw material. The GC/MS method employs extraction and then trimethylsilyl derivatization and is used here for screening. The detection limit is 10 µg/g. The LC/MS/MS method requires only extraction and then a simple isocratic elution on a normal-phase silica column (Rx-Sil) with water and acetonitrile mobile phase containing 5 mM ammonium acetate. The separation of the two compounds takes 5 min. The linear range is from 50 pg/mL to 50 ng/mL for melamine and from 1 ng/mL to 100 ng/mL for cyanuric acid. The LC/MS/MS method is demonstrated by analysis of the target compounds in complex food matrix.

Introduction

The illicit additives melamine and cyanuric acid in pet food have caused the death of cats and dogs. This resulted in the U.S. FDA recalling millions of packages of pet food in the past year. Consequently, it became a hot issue to determine the content of melamine and cyanuric acid in pet food and related raw materials worldwide.

A screening method using GC/MS for both these compounds was published by the U.S. FDA [1]. According to this procedure, the detection limit for GC/MS screening method is 10 µg/g using a trimethylsilyl (TMS) derivatization process before injection. Although this method is used for screening, it could be used as well for confirmation and quantitation using the SIM/SCAN function of the Agilent 5975. In comparison with the GC/MS method, the LC/MS/MS method presented here simplifies the sample preparation process without derivatization and provides confirmation and quantitation in one step. Additionally, the high sensitivity and selectivity of LC/MS/MS could cover trace-level analysis in animal fluids and tissue to high levels in food and food ingredients.

Experimental

Sample Preparation

Sample preparation for GC/MS was done as per the FDA method [1]. To a 0.5-g sample, a mixed solvent (10:40:50 diethylamine:water:acetonitrile) is added and mixed well to thoroughly wet the entire sample. The mixture is then sonicated for 10 min, centrifuged at 5000 rpm for 10 min, and then filtered into a vial using a 0.45 µm nylon disc. Derivatization is done by transferring 200 µL of the extract to an autosampler vial and evaporating to dryness with a low flow of dry nitrogen at 70 °C. To the dried extract is added 200 µL pyri-

dine and 200 µL N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture is vortexed and allowed to incubate at 70 °C for 45 min.

Sample preparation for LC/MS/MS was done by grinding about 5 g sample by a mill and mixing thoroughly. To a 0.5-g sample, 20 mL of acetonitrile/ water (50:50) is added, capped, and vortexed. The solution is sonicated for 30 minutes and then filtered with a 0.20-µm PTFE syringe filter (Agilent P/N 5185-5843). If the concentration of the target compounds is beyond the linear range of the method, further dilution of the extract with acetonitrile:water should be made. Note that if cyanuric acid and melamine are in the same solution at high concentrations, 10:40:50 diethylamine: water:acetonitrile is needed to disrupt the ion pair.

GC/MS Method Details

Instrument:	7890A GC with 5975C MSD
Software:	MSD ChemStation E.01.00 with NIST 05a MS Library 2.0d

GC Conditions

Column:	DB-5MS 30 m × 0.25 mm × 0.25 µm (P/N: 122-5532)
Inlet temperature:	290 °C
Injection mode:	Splitless or split (1:20)
Injection volume:	1 µL
Carrier gas flow:	He (constant flow) at 1.3 mL/min
Oven program:	75 °C (hold 1 minute) to 300 °C at 30 °C /minute (hold 2 min)
Total run time:	10.5 min.
Transfer line temperature:	280 °C

MS Conditions (SIM/Scan Mode)

Tune:	Autotune
Acquisition mode:	EI; SIM/scan mode
Solvent delay:	3.5 minutes
MS temperature:	230 °C (Source); 150 °C (Quad)

Scan Parameters

Scan range:	40–450 amu
Sampling rate:	2 (scan rate at ~3.6 scans/sec)
Threshold:	100

SIM Parameters

GROUP 1

Group identification:	Auto_1							
Resolution:	Low							
Plot 1 Ion:	345.10							
Ions/dwell in group:	Mass	Dwell	Mass	Dwell	Mass	Dwell		
	188.00	25	330.10	25	345.10	25		

GROUP 2

Group identification:	Auto_2							
Resolution:	Low							
Group start time:	6.76							
Plot 1 ion:	327.20							
Ions/dwell in group:	Mass	Dwell	Mass	Dwell	Mass	Dwell	Mass	Dwell
	197.00	25	285.10	25	327.20	25	342.20	25

LC/MS Method Details

Instrument:	1200 SL RRLC with 6410 Triple Quad MS
Software:	MassHunter B.01.03

LC Conditions

Column:	ZORBAX Rx-Sil, 2.1 mm × 150 mm, 5 µm (P/N 883700-901)
Column temperature:	40 °C
Mobile phase:	A = 5 mM ammonium acetate in water B = 5 mM ammonium acetate in acetonitrile
Flow rate:	0.4 mL/min
Injection volume:	10 µL
Isocratic:	95% B
Stop time:	5.5 minutes
Needle wash:	50:50 acetonitrile/water; flush port 10 seconds

MS Conditions

Ion source:	ESI
Polarity:	Positive and negative
Nebulizer gas:	Nitrogen
Ion spray voltage:	4000 V
Source temperature:	350 °C
Resolution:	Q1 (unit) Q3 (unit)
Scan mode:	Multiple reaction monitoring (MRM)
Segment:	Segment 1 = 0~2 min negative mode for cyanuric acid Segment 2 = 2~5.5 min positive mode for melamine
Delta EMV:	600 V

Parameters of multiple reaction monitoring (MRM) are shown in Table 1.

Table 1. MRM Parameters for Cyanuric Acid and Melamine

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
1.45	Cyanuric acid	128	42	200	100	30
		128	85	200	100	5
4.92	Melamine	127	85	200	100	20
		127	68	200	100	35

Results and Discussion

The Selection of Monitoring Ions for SIM Mode in GC/MS

Selective ion monitoring (SIM) is often used to improve the detection limit and quantitative reproducibility. With SIM mode, the MS monitors only a few ions for each target compound within the retention time (RT) range that the target elutes from the column. By monitoring only a few specific ions, the signal-to-noise ratio (S/N) improves significantly. Therefore, the selection of the ions monitored for SIM mode is very important for good analytical result. Usually, a few ions of highest abundance are chosen for SIM to maximize the response of the target compounds. However, if the interference from the matrix is significant, the most characteristic ions should be chosen to minimize signal disturbance even if their abundance is relatively lower. In this application, the influence of ion selection was demonstrated in Figure 1. With the use of more characteristic ions, the chromatogram has much less interference than the chromatogram with the highest abundance ions. The use of SIM/SCAN allows both the highest sensitivity and reproducibility with the additional benefit of full identification contained in the complete spectra of the analytes.

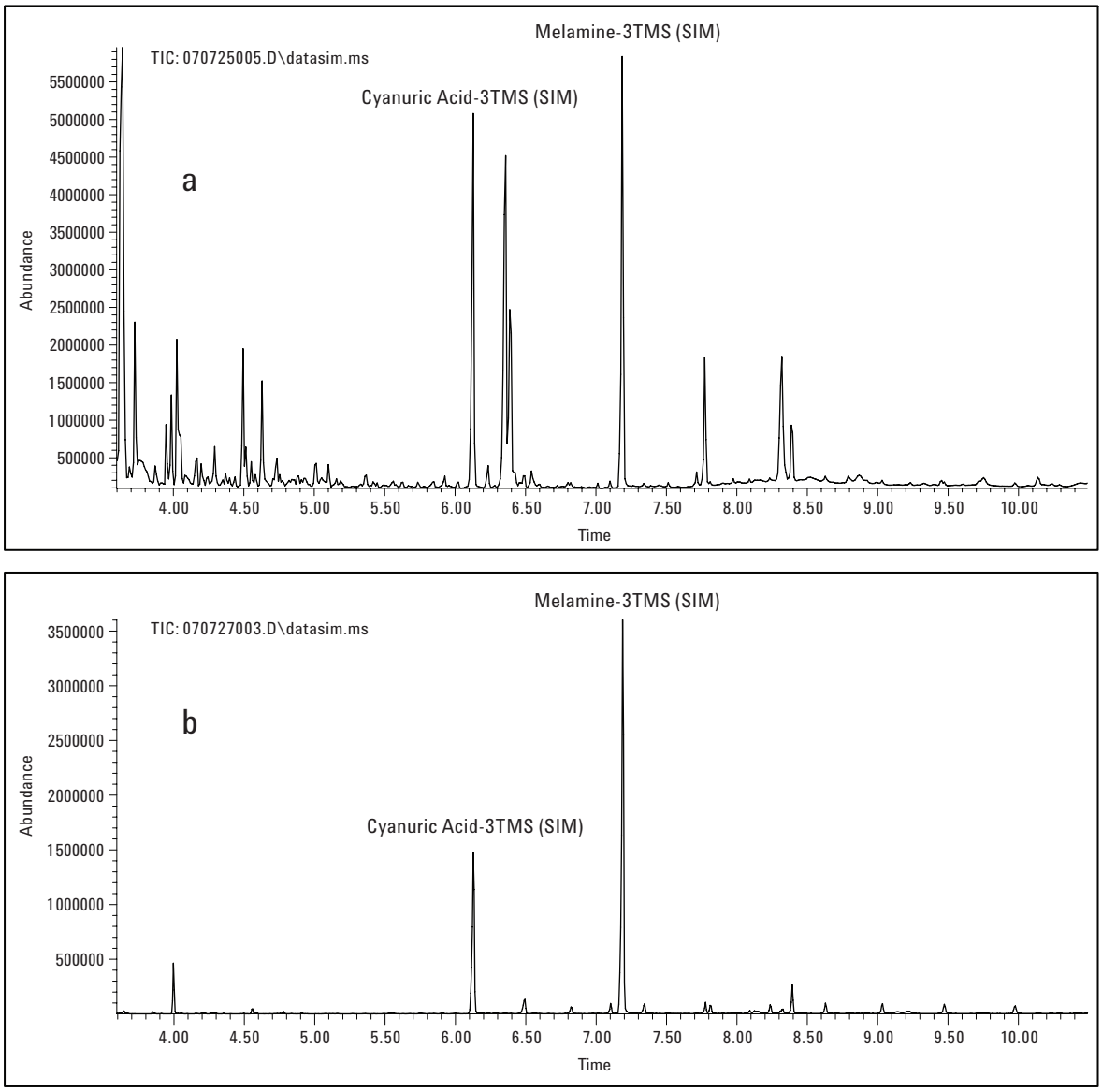

Figure 1. The influence of the selection of the ions monitored in SIM mode shown with spiked dry cat food; a) the ions of highest abundance were used (cyanuric acid: *m/z* 73, 147, 171; melamine: *m/z* 327, 330, 342, 345) and b) characteristic ions were used (cyanuric acid: *m/z* 188, 330, 345; melamine: *m/z* 197, 285, 327, 342).

The Retention of Melamine and Cyanuric Acid on a Normal-Phase Column

Liquid chromatographic separation of small highly polar compounds like melamine and cyanuric acid can be achieved by retention mechanisms such as ion-exchange and ion-pair reversed-phase liquid chromatography (IP-RPLC). In general, the ion-exchange approach is not very suited to the electrospray interface (ESI) due to the use of buffers with high ionic strength. Likewise, volatile ion-pair reagents can provide satisfactory results, but again are not well suited for routine use with LC/MS. In addition, ion pair separation of both an acid and a base in the same analysis is difficult.

A much more suitable approach for these compounds is known as aqueous normal-phase chromatography or hydrophilic interaction chromatography (HILIC) [2]. This mode of separation is defined as a partition of a polar analyte between a relatively nonpolar mobile phase and a polar semi-immobilized liquid phase associated with the stationary phase. Normal phase chromatography is usually associated with adsorption of the analyte on the stationary phase. It is believed that in this mode water is trapped on the surface of the stationary phase and evokes partitioning instead of adsorption. Both cyanuric acid and melamine can be successfully retained by the Rx-Sil column with 1.45 minutes and 4.92 minutes of retention time, respectively, under these conditions.

Quantitative Analysis of Melamine and Cyanuric Acid in Different Matrices

A time segment program was used to switch between the negative mode for cyanuric acid and the positive mode for melamine. Linearity of the developed method was studied by three replicate injections at each concentration level. The calibration curves of melamine and cyanuric acid are displayed in Figure 2. The linear range of the method is 50 pg/mL to 50 ng/mL for melamine and 1 ng/mL to 100 ng/mL for cyanuric acid. The detection limits for melamine and cyanuric acid are shown in Figure 3. The S/N ratio (peak-to-peak) is 12.7 for 50 pg/mL melamine and 12.8 for 1 ng/mL cyanuric acid.

Pet food, wheat gluten, corn meal, and rice protein samples were extracted and analyzed for both target compounds. Examples of a spiked wheat gluten sample are given in Figure 4. A mixture of acetonitrile and water (50:50) is most efficient for the extraction of melamine and cyanuric acid from the different matrices. When injecting 10 µL of sample, the extract should be diluted to 5% or less water or the peaks will be distorted. Otherwise, reducing the injection volume to 1 µL is the easiest way to eliminate the distortion.

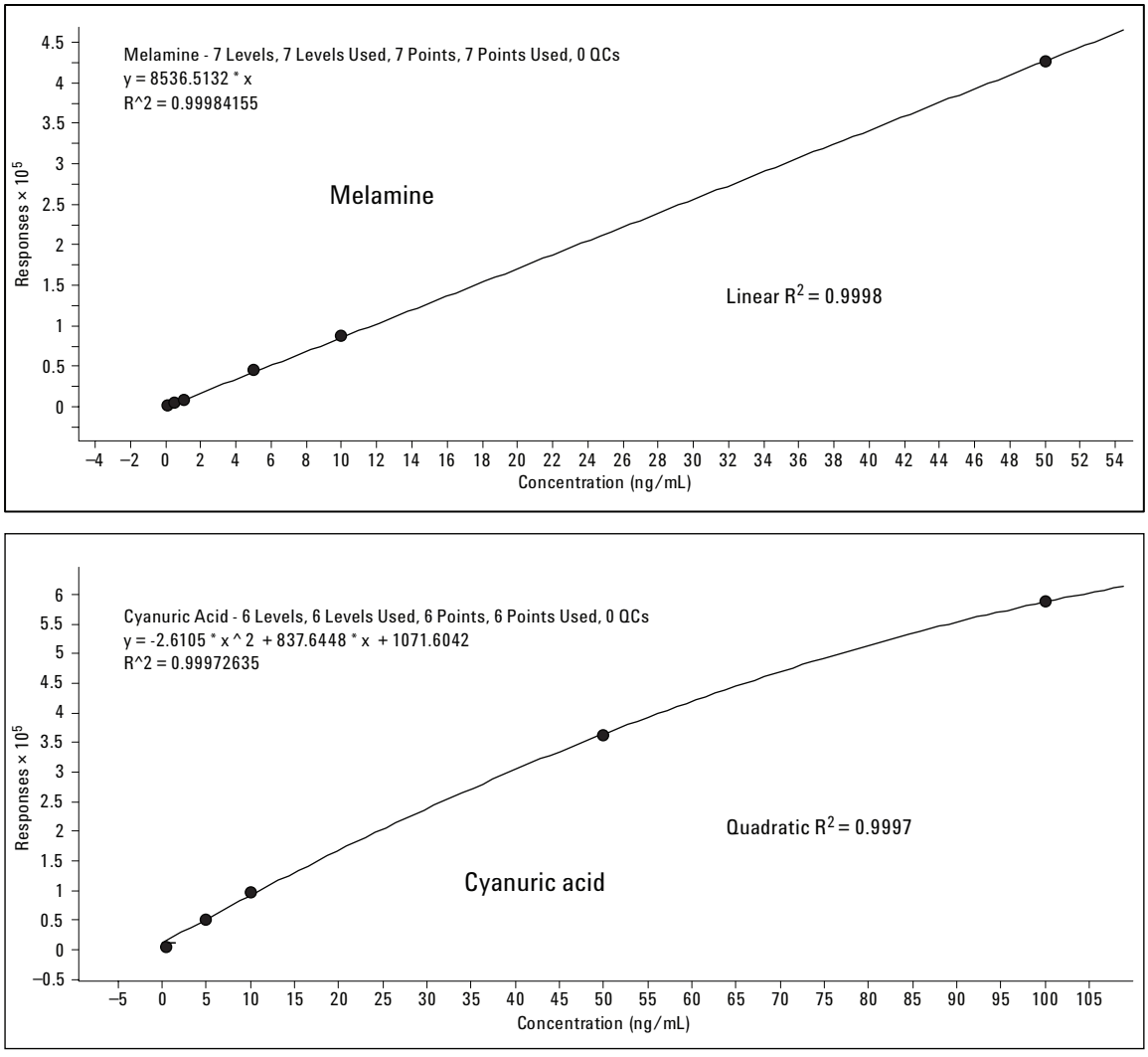

Figure 2. Linearity of melamine and cyanuric acid.

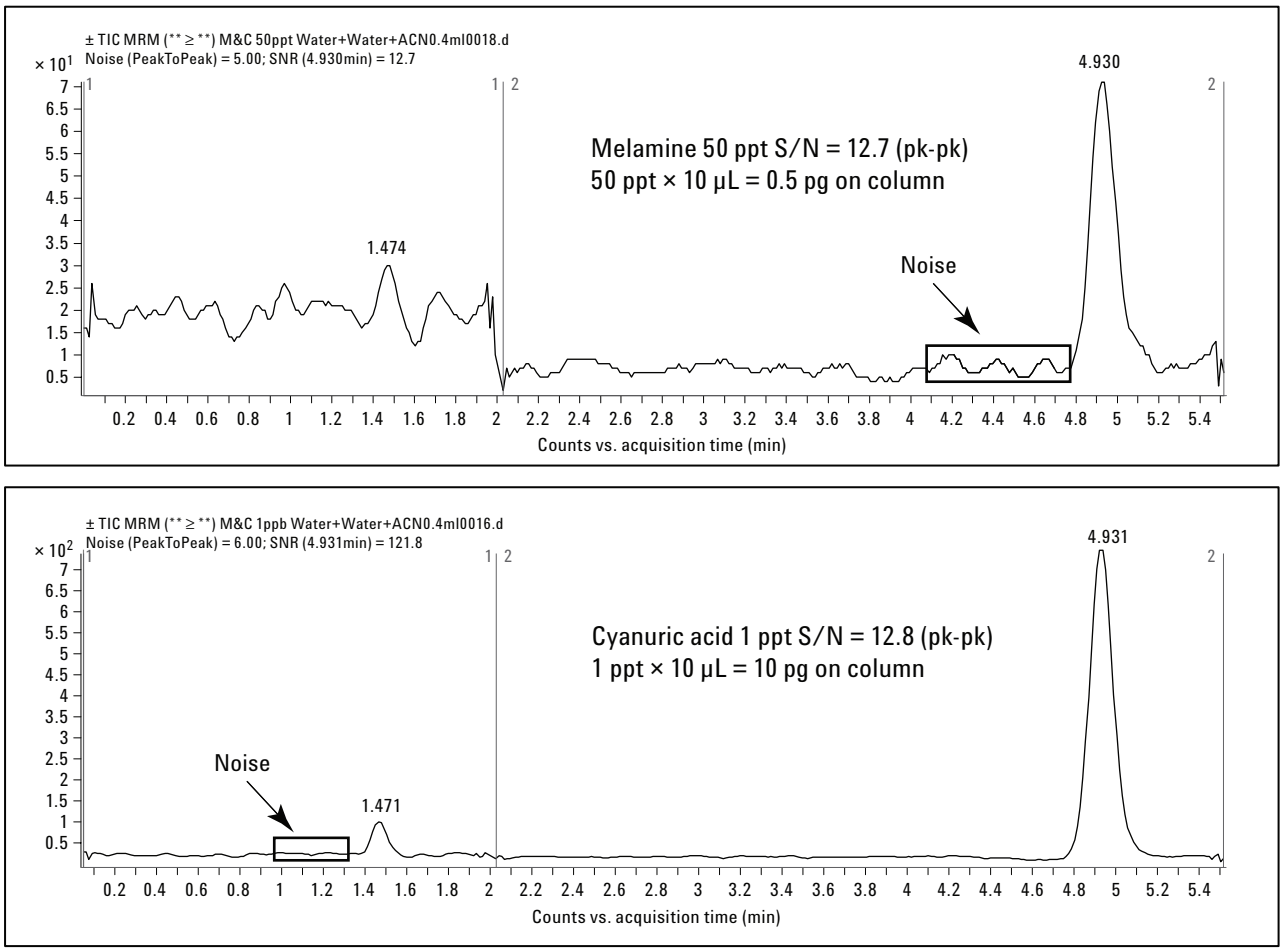

Figure 3. Detection limits for melamine and cyanuric acid.

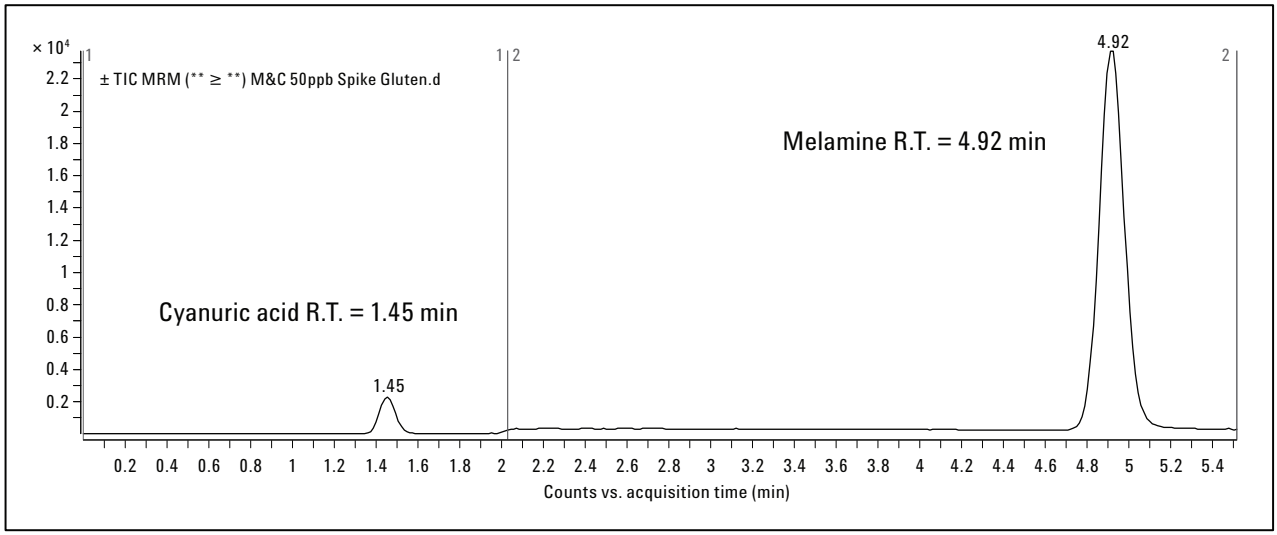

Figure 4. Total reaction monitoring chromatogram of spiked wheat gluten sample.

Conclusions

This application demonstrates that GC/MS or LC/MS/MS methods can readily be used for the analysis of melamine and cyanuric acid in pet food and its ingredients. In the example shown, the GC/MS method was used to screen the presence of melamine and cyanuric acid in pet food using SIM mode. However, the method can be used for quantitation and confirmation using SIM/SCAN so long as very low detection levels are not required. In addition, an LC/MS/MS method has been shown with both a simple sample preparation procedure and an aqueous normal-phase separation. The method is highly sensitive and selective and is used both for the confirmation and quantitation of melamine and cyanuric acid in pet food and related raw materials. The detection limits of 50 pg/mL for melamine and 1 ng/mL for cyanuric acid would readily allow for the analysis of these compounds at trace levels in animal fluids and tissues.

References

1. GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide and Cyanuric Acid, Version 2, May 7, 2007. http://www.fda.gov/cvm/MelaminePresence.htm
2. K. I. Petrus Hemström, "Hydrophilic interaction chromatography." *Journal of Separation Science* **2006**, 29, (12), 1784-1821.

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