

A New, Fast and Sensitive LC/MS/MS Method for the Accurate Quantitation and Confirmation of Melamine and Cyanuric Acid in Pet Food Samples



API 3200™ LC/MS/MS System

Overview

Recent issues with the determination of Melamine and Cyanuric Acid in wheat gluten imported from China and the subsequent animal deaths and recall of millions of pet food products have highlighted the need for both food manufacturers and regulatory agencies to utilize fast and accurate analytical techniques to proactively ensure product safety.¹

A fast and sensitive LC/MS/MS method was developed for the analysis of Melamine and Cyanuric Acid utilizing a simple extraction, with a run time of 10 minutes, and with limits of quantitation of Melamine and Cyanuric Acid below 1 µg/kg. In addition the method provides an extra degree of confirmation through the use of Multiple Reaction Monitoring (MRM) ratios.

Introduction

While GC/MS methods have been developed for the analysis of Melamine and Cyanuric Acid in wheat, rice and other gluten products, these methods require extensive sample clean-up with hazardous solvents and derivatization.² Additionally reported limits of detection are only in the mg/kg range.

In comparison to GC/MS the developed LC/MS/MS method has the following benefits:

- Reduced sample preparation and run time
- Superior quantitative results with less starting material
- Results with a higher degree of confidence
- The ability to analyze a wide range of contaminants

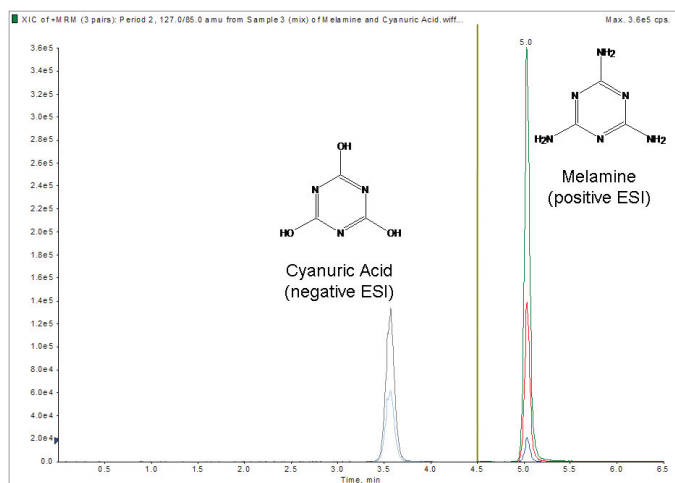


Figure 1. LC separation of Melamine and Cyanuric Acid with MS/MS detection in negative and positive Electrospray Ionization (ESI)

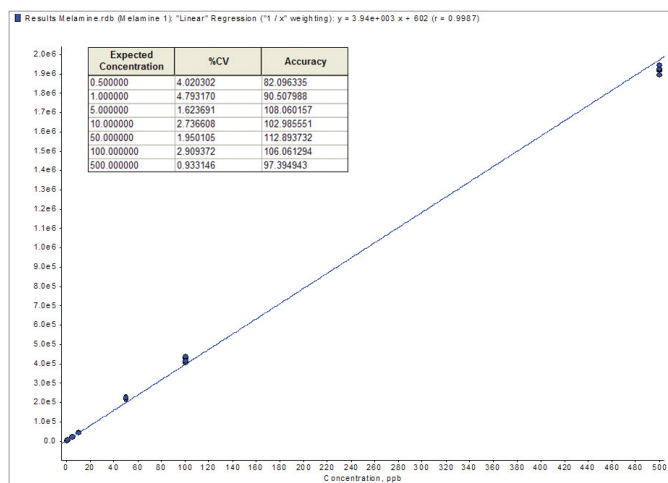


Figure 2. Calibration line of Melamine (MRM: 127/85) over a range of 0.5 to 500ppb with coefficients of variation (%CV) and accuracy for each concentration (5 replicate injections)

Experimental

Chemicals

Melamine (108-78-1), Melamine- $^{15}\text{N}_3$ (98 atom % ^{15}N), and Cyanuric Acid (108-80-5) standards were obtained from Sigma-Aldrich.

Sample Preparation

Liquid-Liquid-Extraction of pet food samples was performed using the following procedure:

1. Accurately weigh 5g of homogenized sample into a 250mL conical flask.
2. Add 1250 μL Melamine- $^{15}\text{N}_3$ stock solution (200 $\mu\text{g}/\text{mL}$) to the sample and wait 15 minutes.
3. Add 100mL water and vortex rigorously to mix sample with water.
4. Connect a condenser apparatus to the top of the conical flask and boil the mixture for 10 minutes.
5. Remove the conical flask from the heat and leave on the bench for one minute.
6. Remove 100 μL from the mixture and add to 9900 μL water/acetonitrile (50:50) in a 15mL centrifuge tube and vortex.
7. Centrifuge sample at 5000rpm for 10 minutes at 4°C.
8. Filter the solution through 0.45 μm PVDF syringe filter (Whatman).
9. Transfer to autosampler vial for LC/MS/MS analysis.

TABLE 1. LC CONDITIONS FOR THE ANALYSIS OF MELAMINE AND CYANURIC ACID ON AN INERTSIL HILIC 5 μm (150x3mm) COLUMN

Time (min)	Flow (mL/min)	% (A)	% (B)
0.1	0.5	97	3
5.0	0.5	20	80
5.5	0.5	3	97
5.6	0.5	97	3
10.0	0.5	97	3

TABLE 2. MS PARAMETERS OF THE DETECTION OF MELAMINE AND CYANURIC ACID USING AN API 3200™ LC/MS/MS SYSTEM

Compound	Retention time	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
Cyanuric Acid	3.6min	128	42	-30	-30
		128	85	-30	-13
Melamine	5.0min	127	85	44	26
		127	68	44	48
		127	60	44	27
Melamine- $^{15}\text{N}_3$	5.0min	130	69	44	40
		130	87	44	29

Further dilution of the extract with water/acetonitrile (50/50) might be necessary if the sample is heavily contaminated.

Liquid Chromatography

A Shimadzu Prominence LC system containing a CBM-20A system controller, two LC-20AD pumps, a semi-micro gradient mixer SUS-20A, and a SIL-20AC autosampler was used. Separation

was performed on a GL Science Inertsil HILIC 5 μm (150x3mm) column at a temperature of 40°C with a mobile phase of (A) acetonitrile + 10mM ammonium acetate and (B) water + 10mM ammonium acetate at a flow of 0.5mL/min. The gradient using normal phase conditions is listed in Table 1. An injection volume of 5 μL was used.

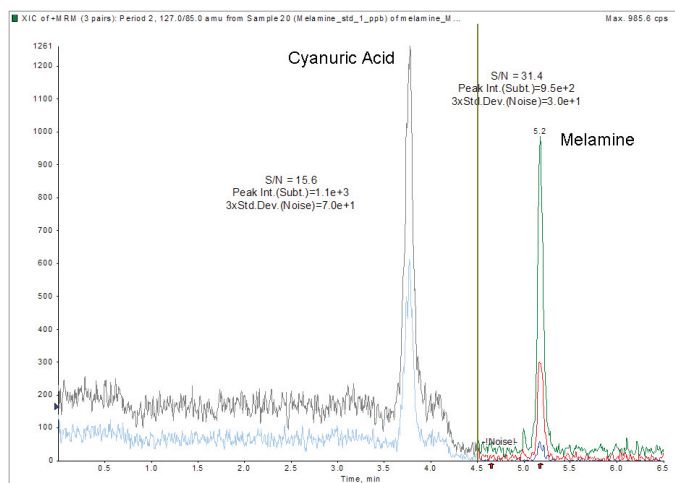


Figure 3. Signal-to-Noise and confirmatory MRM transition at a concentration of 1ppb of Melamine and Cyanuric Acid

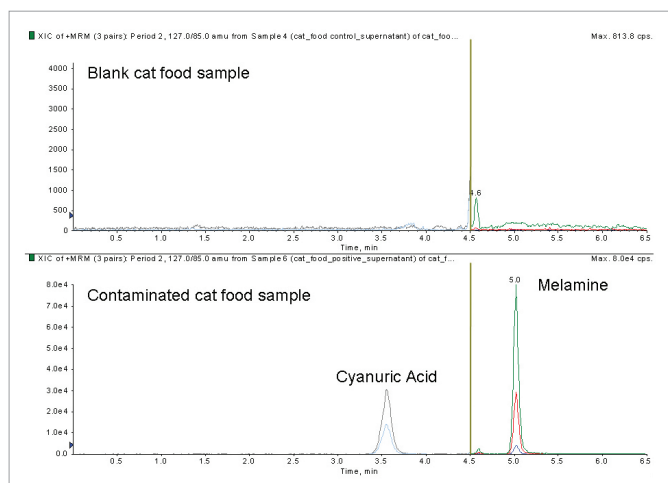


Figure 4. LC/MS/MS chromatograms of a blank and contaminated cat food sample

Mass Spectrometry

An API 3200™ LC/MS/MS System equipped with Turbo V™ Source and Electrospray Ionization (ESI) probe was used to detect both targeted analytes in MRM mode. Precursor and product ions with corresponding Declustering Potentials (DP) and Collision Energies (CE) of the detection of Cyanuric Acid in negative polarity and Melamine in positive polarity are given in Table 2.

Results and Discussion

It is now known that pet foods were contaminated with high concentrations of Melamine. Melamine in combination with Cyanuric Acid caused formation of melamine-cyanurate crystals in the kidneys of exposed animals, which can cause kidney failure and death.

A number of solubility tests were performed in water to determine that at room temperature these crystals are soluble below 2µg/mL. The extraction procedure was optimized to make sure that the melamine-cyanurate crystals will dissolve completely at concentrations that are likely to be in the pet food. Dilution steps and temperature was adjusted carefully to avoid precipitation at a concentration

level up to 100µg/mL. While still warm, the extract is diluted finally to a concentration at which the complex is known to be soluble at room temperature. Further dilution of the extract with water/acetonitrile (50/50) might be necessary if the sample is heavily contaminated.

The developed extraction procedure was validated by spiking 2 types of pet food, namely biscuit and two different wet foods from different manufacturers at high and low concentration levels. These spiked samples were analyzed in triplicates to evaluate recovery and reproducibility.

The analytical recoveries were 116% (±8.7%) from spiked quality control samples containing Melamine concentrations of 20 and 2000 mg/kg and 68% (±4.3%) for Cyanuric Acid.

Melamine and Cyanuric Acid were separated using a normal phase gradient on a Hydrophilic Interaction Chromatography (HILIC) column. Periods were adjusted to allow detection in negative and positive polarity with highest sensitivity. An example chromatogram is given in

Figure 1. Linearity and reproducibility of the developed method was studied by 5 replicate injections at each concentration level. The example calibration line of Melamine given in Figure 2 highlights the linear range of 3 orders of magnitude and the reproducibility and accuracy of this method. Limits of detection were found below 1ppb for Melamine and Cyanuric Acid. Figure 3 shows a chromatogram with Signal-to-Noise ratios and detected MRM transitions used for confirmation.

Several pet food samples were extracted and analyzed for both targeted analytes. Examples of a blank and a contaminated cat food sample are given in Figure 4.

Cliquid™ Software for Routine Food Testing

The presented method is available as an iMethod™ test to download into Cliquid® Software for Routine Food Testing. Cliquid® Food and Beverage Software provides a simple four step workflow, to perform the analysis and to automatically generate both quantitative and confirmatory reports according to regulatory guidelines. Figure 5 shows a report with MRM ratios to confirm the

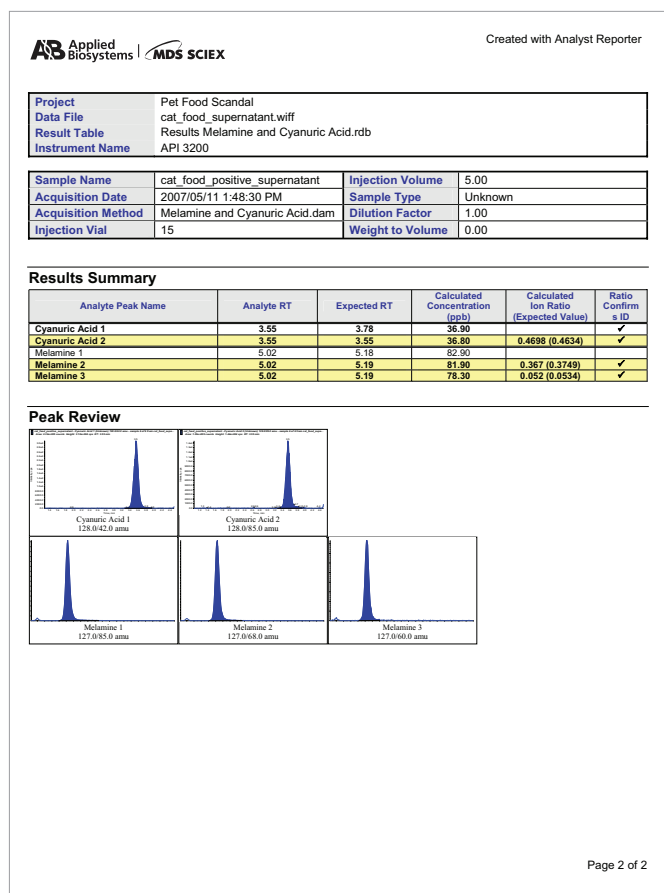
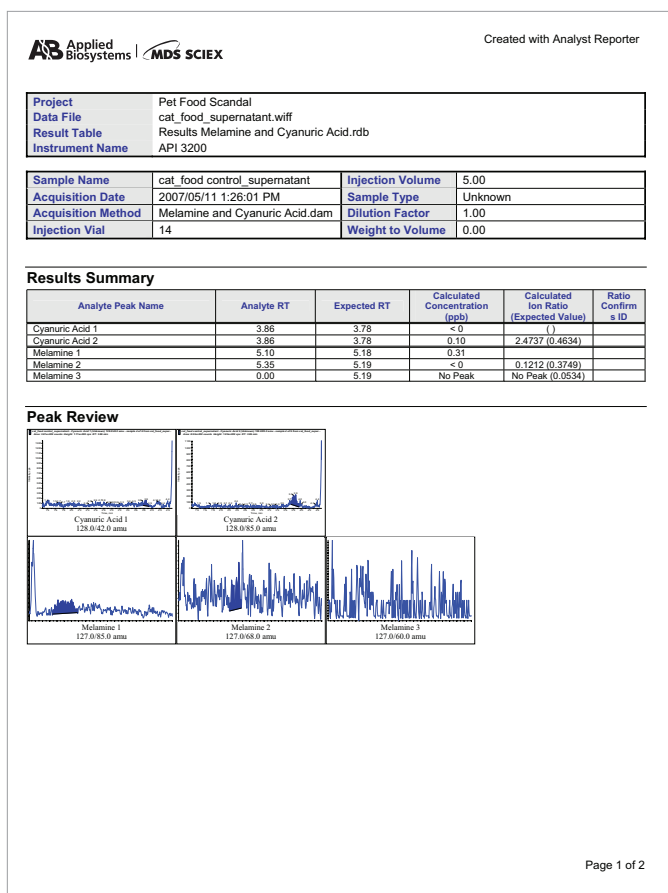


Figure 5. A report generated automatically by Cliquid® Food and Beverage Software with confirmatory results of Melamine and Cyanuric Acid findings in cat food samples

presence of Melamine and Cyanuric Acid in contaminated cat food samples. Visit info.appliedbiosystems.com/iMethods

Conclusion

A fast and sensitive LC/MS/MS method was developed for the quantitation and confirmation of Melamine and Cyanuric Acid in pet food samples. A simple extraction with high recovery followed by LC separation on a HILIC column and MS/MS detection in negative and positive Electrospray Ionization using an

API 3200™ LC/MS/MS System enables the quantitation of Melamine and Cyanuric Acid below 1ppb. The method provides an extra degree of confirmation through the use of MRM ratios.

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Reference

- http://en.wikipedia.org/wiki/2007_pet_food_crisis
- <http://www.fda.gov/cvm/MelaminePresence.htm>

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