

# 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.<sup>1</sup>

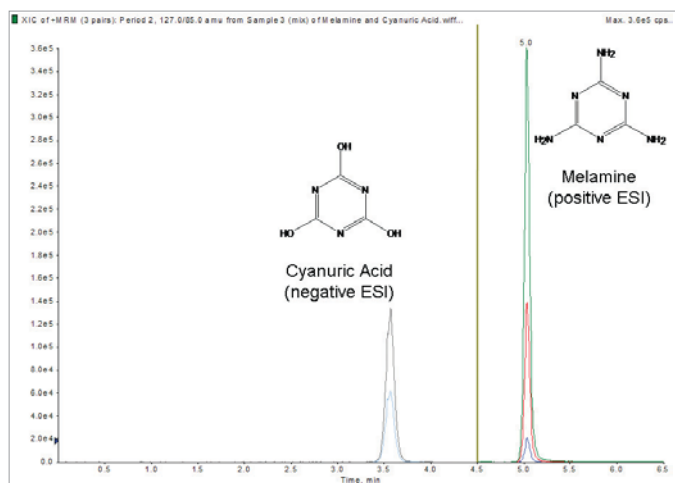
A fast and sensitive LC/MS/MS method was developed for the analysis of Melamine and Cyanuric Acid utilizing a simple extraction, with a run time of 10 minutes, and with limits of quantitation of Melamine and Cyanuric Acid below 1 µg/kg. In addition the method provides an extra degree of confirmation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

## Introduction

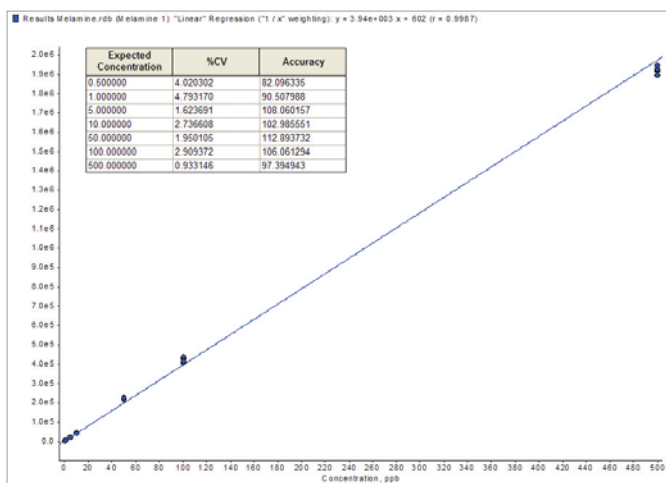
While GC/MS methods have been developed for the analysis of Melamine and Cyanuric Acid in wheat, rice and other gluten products, these methods require extensive sample clean-up with hazardous solvents and derivatization.<sup>2</sup> Additionally reported limits of detection are only in the mg/kg range.

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 500 ppb with coefficients of variation (%CV) and accuracy for each concentration (5 replicate injections)

## Experimental

### Chemicals

Melamine (108-78-1) and Cyanuric Acid (108-80-5) standards were obtained from Sigma-Aldrich.

### Extraction

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

- Add 5mL of acetonitrile to 5g of homogenized sample
- Mix thoroughly and let stand for 15-30 minutes
- Add 5mL of saturated NaCl, mix and let stand for another 15-30 minutes
- Separate by centrifugation and inject the top layer

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 $\mu$ m (150x3mm) column at a

**TABLE 1.** LC CONDITIONS FOR THE ANALYSIS OF MELAMINE AND CYANURIC ACID ON AN INERTSIL HILIC 5 $\mu$ m (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

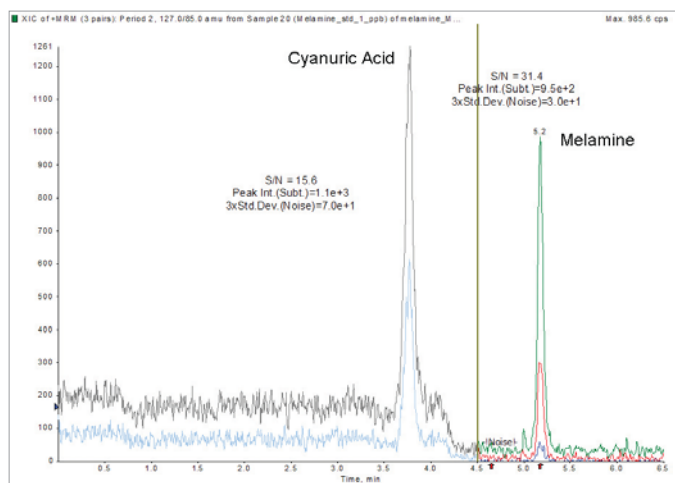
Compound	Retention time	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
Cyanuric Acid	3.6min	128	42	-30	-30
		128	85	-30	-13
Melamine	5.0min	127	85	44	26
		127	68	44	48
		127	60	44	27

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 $\mu$ L was used.

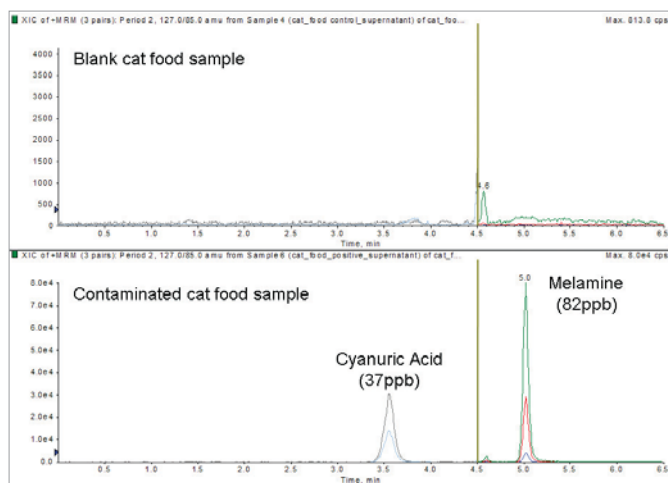
### Mass Spectrometry

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 Multiple Reaction Monitoring (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.



**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

## Results and Discussion

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  $1 \mu\text{g}/\text{kg}$  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 can be used stand-alone or integrated into Cliquid™ Software for Routine Food Testing. Cliquid™ Food and Beverage Software provides a simple four step work flow, 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 presence of Melamine and Cyanuric Acid in contaminated cat food samples.

## 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 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  $1 \mu\text{g}/\text{kg}$ . The method provides an extra degree of confirmation through the use of Multiple Reaction Monitoring (MRM) ratios.

Future investigation will include the optimization of recovery by adjusting pH during sample preparation and the use of isotopically labeled internal standards to correct for possible matrix effects during ionization.

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## Reference

- [http://en.wikipedia.org/wiki/2007\\_pet\\_food\\_crisis](http://en.wikipedia.org/wiki/2007_pet_food_crisis)
- <http://www.fda.gov/cvm/MelaminePresence.htm>

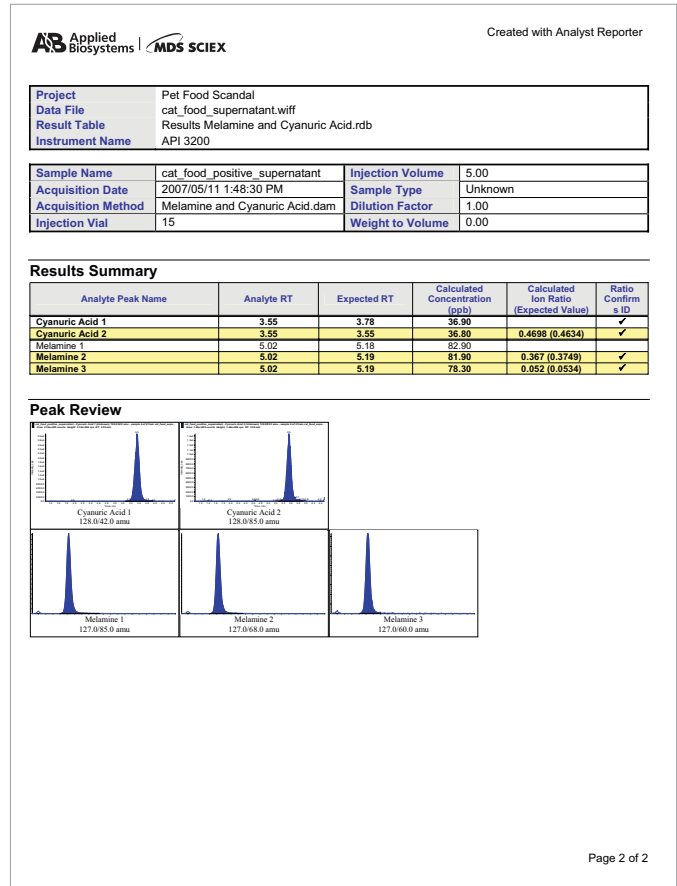
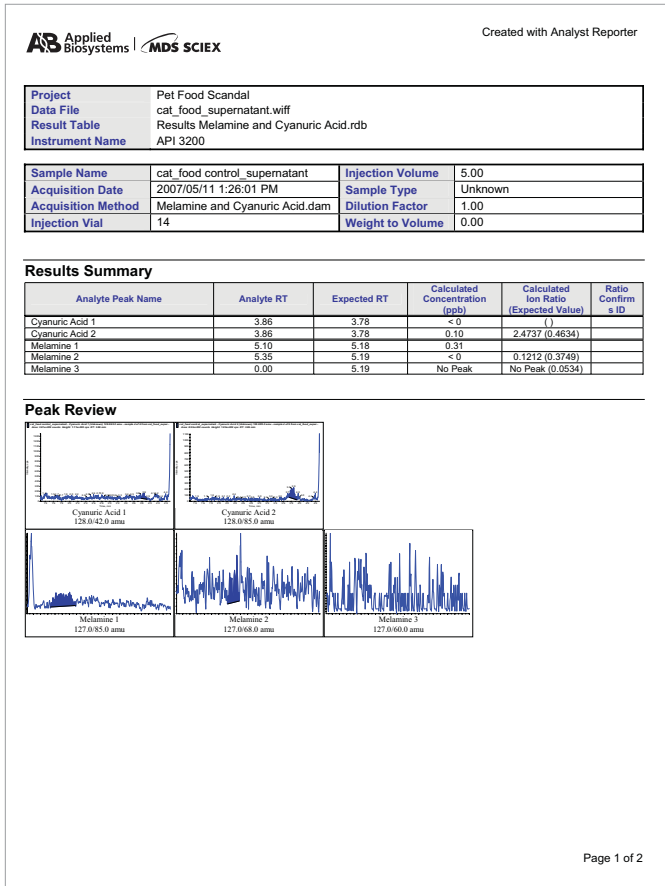


Figure 5. A report generated automatically by Cliquid™ Food and Beverage Software with confirmatory results of Melamine and Cyanuric Acid findings in cat food samples

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