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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 1µg/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

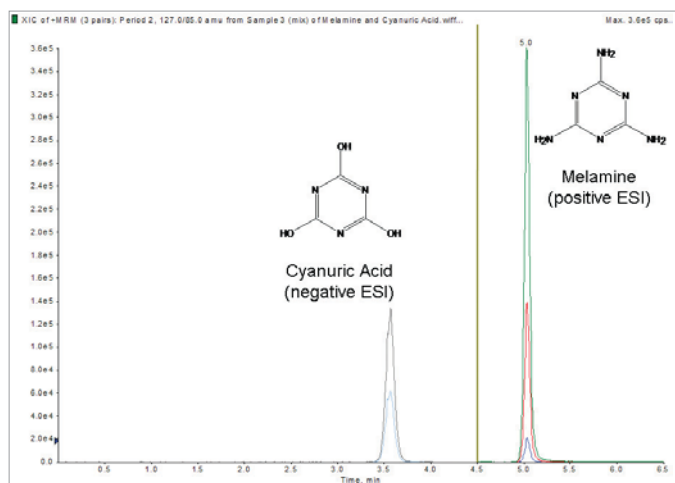- The ability to analyze a wide range of contaminants

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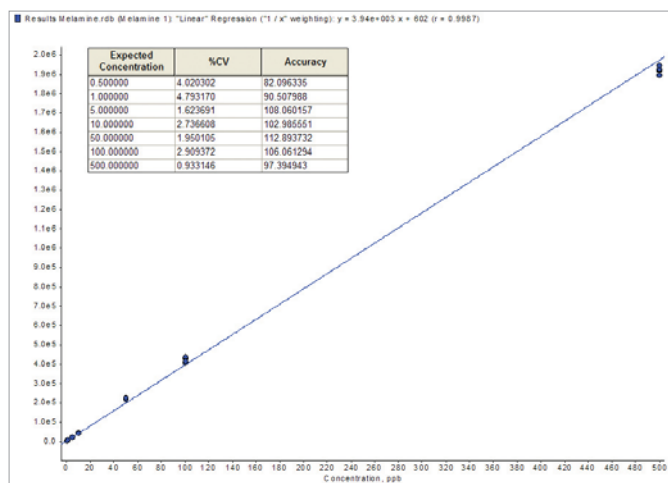

Figure 2. Calibration line of Melamine (MRM: 127/85) over a range of 0.5 to 500 ppb with coefficients of variation (%CV) and accuracy for each concentration (5 replicate injections)

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 5µm (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 5µL 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.

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

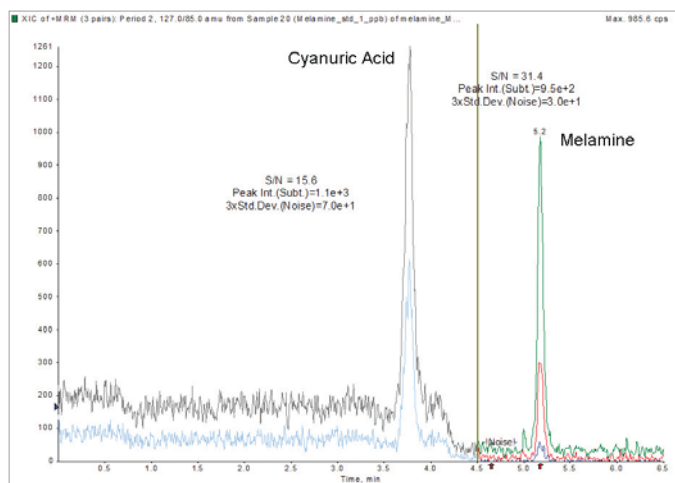| Compound	Retention time	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
Cyanuric Acid	3.6min	128	42	-30	-30
		128	85	-30	-13
Melamine	5.0min	127	85	44	26
		127	68	44	48
		127	60	44	27

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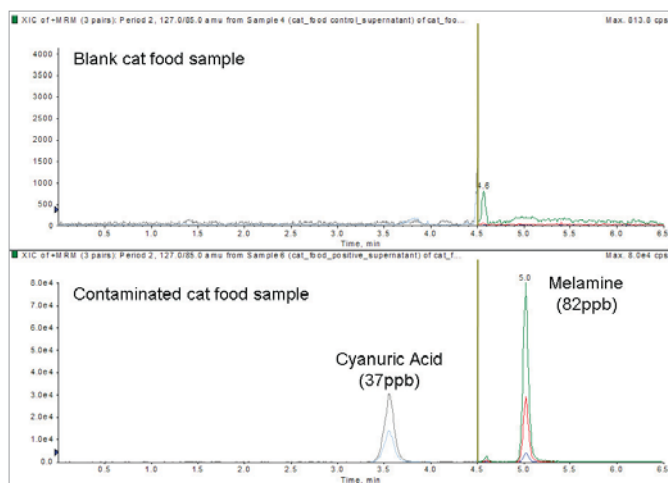

Figure 4. LC/MS/MS chromatograms of a blank and contaminated cat food sample

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 1µg/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 1µg/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.

Authors

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Reference

[1] http://en.wikipedia.org/wiki/2007_pet_food_crisis

[2] http://www.fda.gov/cvm/MelaminePresence.htm

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Project	Pet Food Scandal
Data File	cat_food_supernatant.wiff
Result Table	Results Melamine and Cyanuric Acid.rdb
Instrument Name	API 3200

Sample Name	cat_food control_supernatant	Injection Volume	5.00
Acquisition Date	2007/05/11 1:26:01 PM	Sample Type	Unknown
Acquisition Method	Melamine and Cyanuric Acid.dam	Dilution Factor	1.00
Injection Vial	14	Weight to Volume	0.00

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ppb)	Calculated Ion Ratio (Expected Value)	Ratio Confirms ID
Cyanuric Acid 1	3.86	3.78	< 0	( )	
Cyanuric Acid 2	3.86	3.78	0.10	2.4737 (0.4634)	
Melamine 1	5.10	5.18	0.31		
Melamine 2	5.35	5.19	< 0	0.1212 (0.3749)	
Melamine 3	0.00	5.19	No Peak	No Peak (0.0534)	

Peak Review

Cyanuric Acid 1 128.0/42.0 amu / Cyanuric Acid 2 128.0/85.0 amu / Melamine 1 127.0/85.0 amu / Melamine 2 127.0/68.0 amu / Melamine 3 127.0/60.0 amu

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Project	Pet Food Scandal
Data File	cat_food_supernatant.wiff
Result Table	Results Melamine and Cyanuric Acid.rdb
Instrument Name	API 3200

Sample Name	cat_food_positive_supernatant	Injection Volume	5.00
Acquisition Date	2007/05/11 1:48:30 PM	Sample Type	Unknown
Acquisition Method	Melamine and Cyanuric Acid.dam	Dilution Factor	1.00
Injection Vial	15	Weight to Volume	0.00

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ppb)	Calculated Ion Ratio (Expected Value)	Ratio Confirms ID
Cyanuric Acid 1	3.55	3.78	36.90		✔
Cyanuric Acid 2	3.55	3.55	36.80	0.4698 (0.4634)	✔
Melamine 1	5.02	5.18	82.90		
Melamine 2	5.02	5.19	81.90	0.367 (0.3749)	✔
Melamine 3	5.02	5.19	78.30	0.052 (0.0534)	✔

Peak Review

Cyanuric Acid 1 128.0/42.0 amu / Cyanuric Acid 2 128.0/85.0 amu / Melamine 1 127.0/85.0 amu / Melamine 2 127.0/68.0 amu / Melamine 3 127.0/60.0 amu
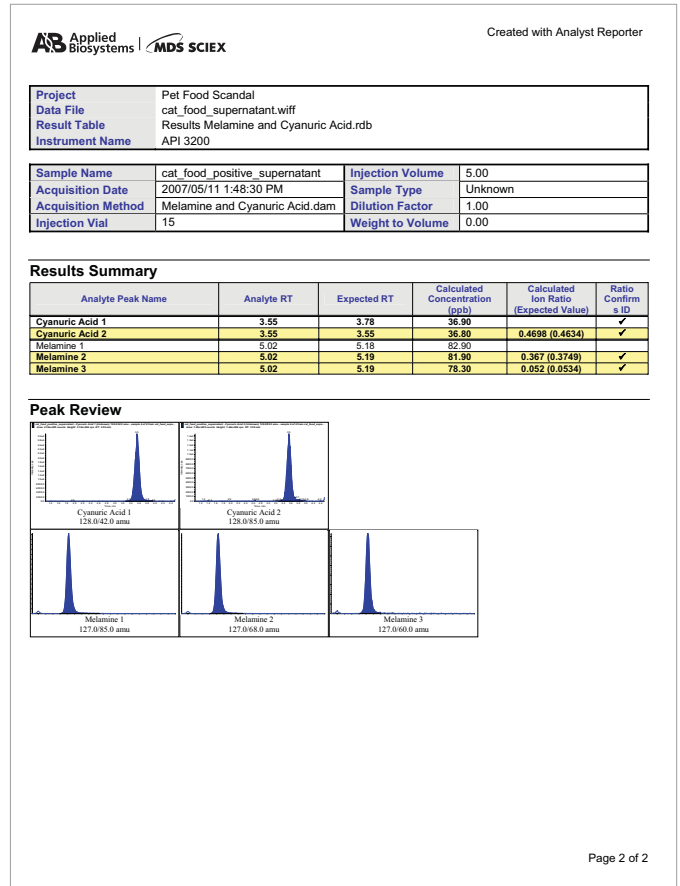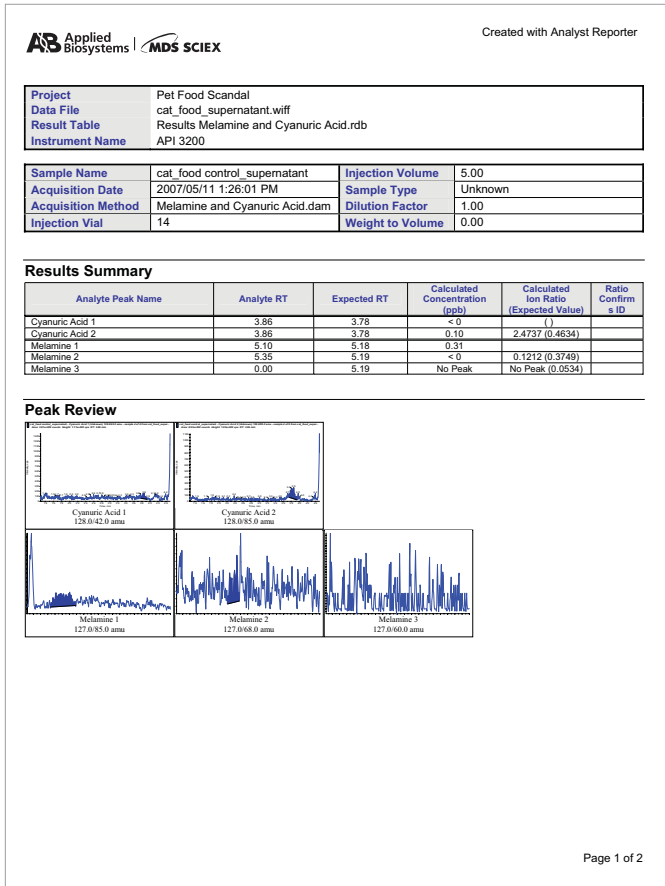
Figure 5. A report generated automatically by Cliquid™ Food and Beverage Software with confirmatory results of Melamine and Cyanuric Acid findings in cat food samples

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