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Simultaneous Determination of Melamine and Cyanuric Acid Using LC-MS with the Acclaim Mixed-Mode WAX-1 Column and Mass Spectrometric Detection

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ABSTRACT

Recent investigations of pet animal death and health problems have revealed pet food contaminated by melamine and cyanuric acid.[1] When present together, melamine and cyanuric acid form an insoluble crystal matrix that may cause kidney function failure.[2] In addition, since contaminated wheat gluten, rice protein concentrate, and corn gluten used in animal feed can be also used in human food such as bread, pasta, baby food, etc., it is crucial to monitor for the presence of melamine and cyanuric acid in raw materials as well as in suspicious animal tissue.

Current methods for quantitative determination of melamine and cyanuric acid include gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). GC-MS requires derivatization which is labor intensive and the reported LC-MS methods generally involve a long gradient chromatographic run as well as column clean up.

This presentation introduces a sensitive, simple, and high-throughput method for simultaneous determination of melamine and cyanuric acid by LC-MS and uses stable isotope labeled internal standard (ISTD) for quantification.

Analytes were retained and separated on a recently developed Mixed-Mode WAX-1 column[3] which demonstrates the unique selectivity and retention offered by mixed hydrophobic and ion exchange retention mechanisms. Chromatographic run time was significantly reduced to 8 min. and MS detection was applied to ensure selectivity and sensitivity. Excellent linearities with $R^2 > 0.9995$ through a range of 2–200 ng/mL were achieved for both analytes. Method detection limits (MDL) were estimated to be 3.97 ng/mL for melamine and 3.32 ng/mL for cyanuric acid.

The reliability of this method was evaluated against pet food extracts as well as biological matrices: pork and fish tissue extractions. Pet food samples were prepared by a fast, simple, and automated extraction process (results will be presented elsewhere). And biological samples were obtained from the U.S. FDA and prepared by its current protocol.

INSTRUMENT

A Dionex Summit® HPLC consisting of:

- P680 Pump
- ASI-100 Autosampler
- TCC-100 Column Oven
- UVD-340 UV-Vis Detector

A Dionex MSQ™ Plus single quadruple mass spectrometer with electrospray ionization (ESI) source

Chromeleon® 6.8 Chromatography Management Software

Chromatographic Conditions

Analytical Column:	Acclaim® Mixed-Mode WAX-1 (150 × 2.1 mm, 5 µm)
Flow Rate:	Isocratic 90% acetonitrile (CH_3CN) / 10% 20 mM pH 4 ammonium acetate buffer (v/v) at 0.25 mL/min
Column Temperature:	20 °C
Injection Volume:	5 µL

MSQ Conditions

Analysis Mode:	Selected Ion Monitoring (SIM) with polarity switching
Cone Voltage:	50 V for all scans
Dwell Time:	0.5 s for all SIM channels
Probe Temperature:	500 °C
Scan Events:	Two full scans (100–400 *m/z* in 250 ms) with different polarities were applied throughout the entire analytical run time with 4 SIM scans (500 ms each) for analytes and internal standards (ISTD), shown in Table 1.

Table 1. Scan Functions and Scan Events						
Name	**Start Mass *m/z***	**End Mass *m/z***	**Time Range (min)**	**Scan Time (milliseconds)**	**Polarity**	**Cone Voltage (V)**
Positive Full Scan	100	400	0 ~ 8	250	pos.	50
Negative Full Scan	100	400	0 ~ 8	250	neg.	50
Name	**Mass *m/z***	**Span *m/z***	**Time Range (min)**	**Dwell Time (milliseconds)**	**Polarity**	**Cone Voltage (V)**
SIM GROUP 1						
Melamine	127	0.5	0 ~ 5	500	pos.	50
Melamine-$^{15}N_3$	130	0.5	0 ~ 5	500	pos.	50
SIM GROUP 2						
Cyanuric Acid	128	0.5	5 ~ 8	500	neg.	50
Cyanuric Acid-$^{13}C_3$	131	0.5	5 ~ 8	500	neg.	50

Structures of melamine and cyanuric acid are shown in Figure 1, along with their full scan spectra.

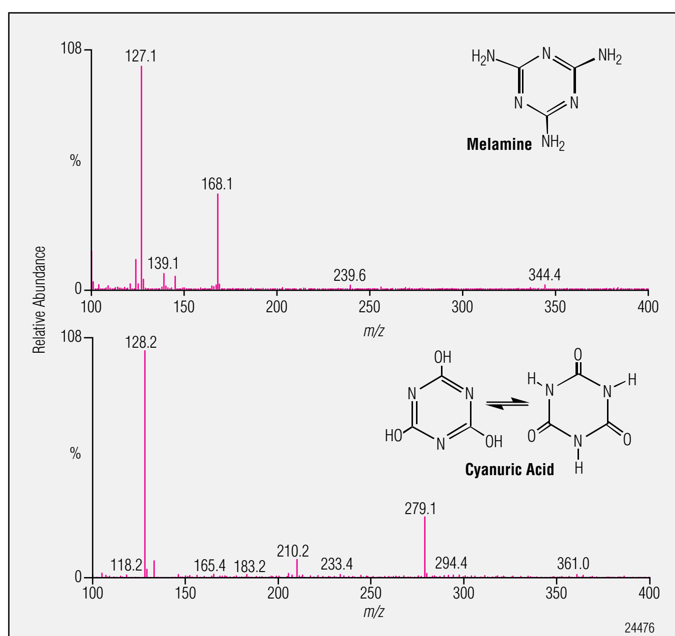

Figure 1. Full scan spectra of melamine and cyanuric acid.

METHOD DEVELOPMENT

Chromatographic Optimization

Mobile phase composition: It is well understood that a mobile phase with higher organic content tends to retain polar analytes longer under HILIC mode, and shows higher MS response under ESI condition. However, separation on a WAX-1 column includes weak anion exchange, which is sensitive to the change of mobile phase in terms of ionic strength and pH.[3] To maintain method reproducibility, proper buffer capacity is required so a mobile phase with 10% pH 4 NH_4OAc buffer in acetonitrile (v/v) was selected.

Buffer pH

When melamine and cyanuric acid are present in different moieties (organic, ionic or a mixture) under different pH conditions, longer retentions were observed when each analyte was switched to a more hydrophilic moiety (ionic form), which can be explained by an ionic exchange mechanism. The effect of pH on retention was investigated and the result is shown in Figure 2.

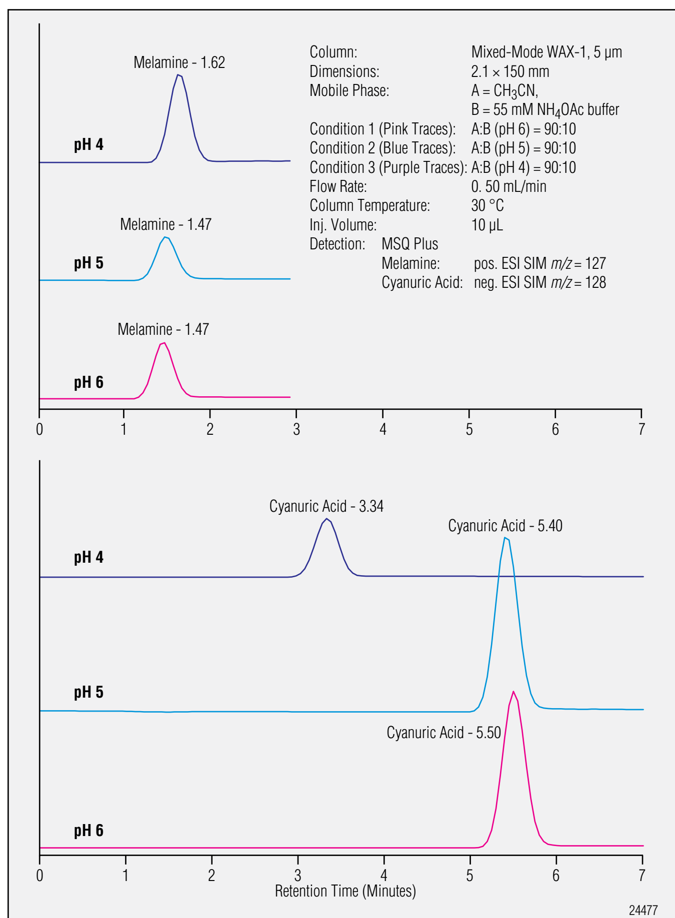

Figure 2. Effect of mobile phase pH on retention time.

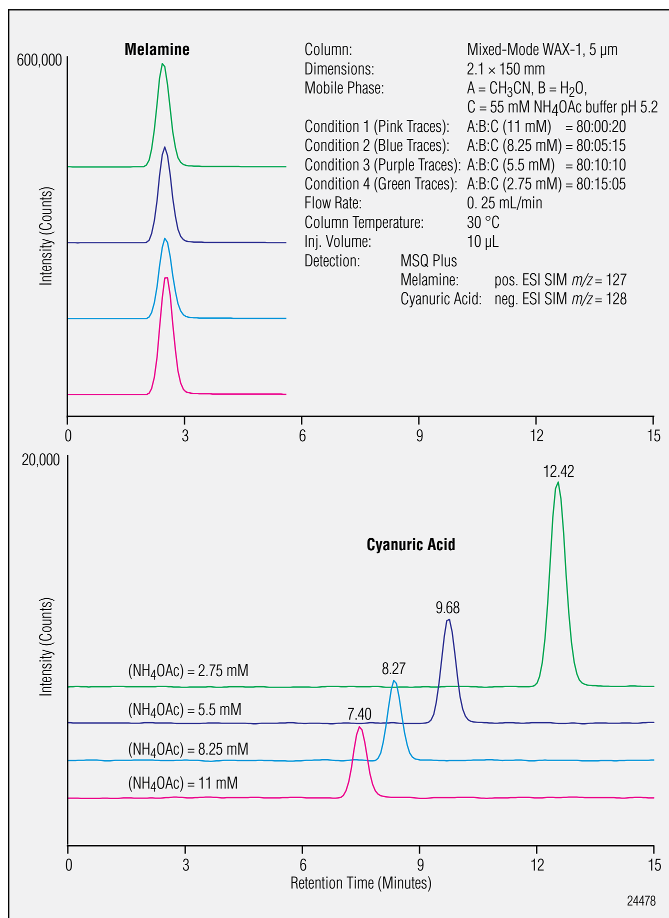

Figure 3. Effect of buffer ionic strength on retention time.

Ionic Strength

A WAX-1 mixed mode column offers a unique selectivity for polar analytes due to the weak anionic exchange mechanism. An analyte with greater polarity is expected to demonstrate greater dependence on mobile phase ionic strength. As shown in Figure 3, the retention time of cyanuric acid changed from 12.42 min to 7.40 min by increasing the mobile phase total ionic strength from 2.75 mM to 11.0 mM.

The combination of the three chromatographic factors offers greater flexibility and control of the range of retention time for cyanuric acid, shown in this study to be from approximately 6 to over 15 min.

Mass Spectrometric Optimization

Mass spectrometric and chromatographic conditions were optimized in combination. The chromatograms of a calibration standard are shown in Figure 4 with the obtained optimization of resolution, sensitivity, and throughput.

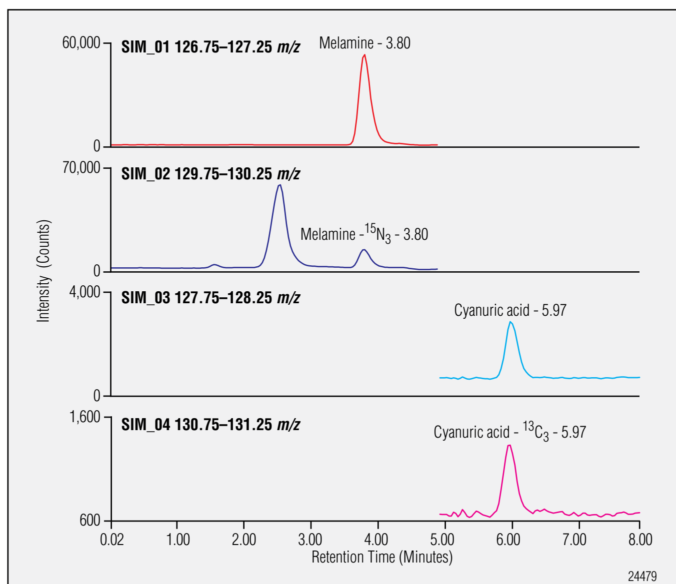

Figure 4. SIM chromatograms of a calibration standard under optimized conditions.

METHOD VALIDATION

Linearity and Calibration Curve

Calibration standards were obtained from the U. S. FDA with stable isotope labeled analytes (Melamine-$^{15}N_3$ and Cyanuric Acid-$^{13}C_3$) as internal standards (ITSD). These standards were used to generate calibration curves and demonstrate linearity. Excellent linearity was achieved throughout the range from 2 ng/mL to 200 ng/mL, as shown in Figures 5 and 6.

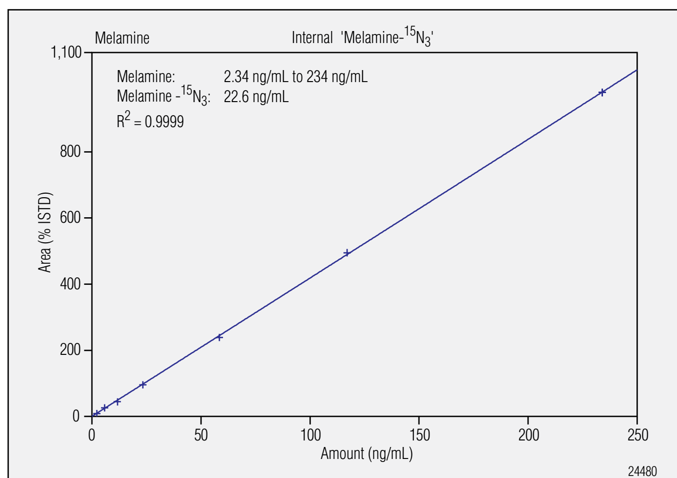

Figure 5. Calibration curve, Melamine.

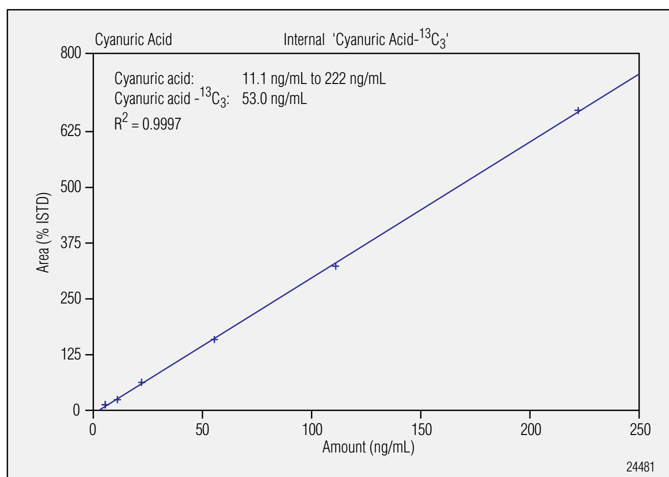

Figure 6. Calibration curve, Cyanuric acid.

Method Detection Limit (MDL)

Method detection limits were calculated by seven replicate injections of calibration standards and calculated by the following equation:

$$MDL = t_{99\%} \times S_{(n = 7)}$$

Where t is student's t at 99% confidence intervals ($t_{99\%,\ n = 7} = 3.143$) and S is the standard deviation. The MDLs of melamine and cyanuric acid were calculated to be 3.97 ng/mL and 3.92 ng/mL, respectively.

Method Evaluation by Biological Samples

Chromatogram

Pork and fish tissue extracts (control and spiked samples) were obtained from the U.S. FDA and prepared using current FDA protocol. All biological samples were evaporated to dryness and reconstituted in mobile phase. Figure 7 shows the SIM chromatograms of a control sample (extracted blank pork sample, ISTD spiked before extraction; and spiked with melamine (melamine portion) or cyanuric acid (cyanuric acid portion); and then evaporated to dryness and reconstituted in mobile phase).

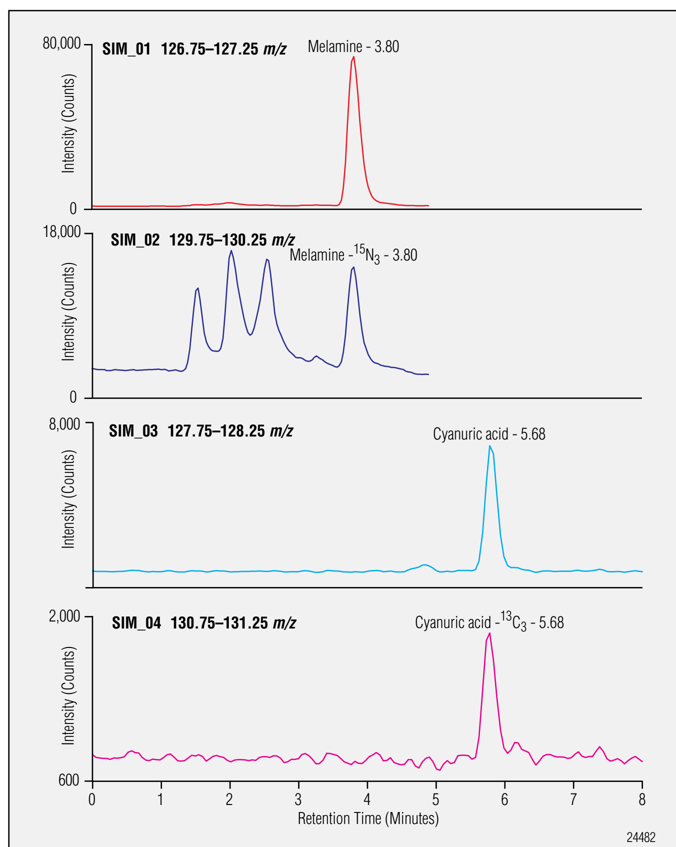

Figure 7. SIM chromatograms of FDA control samples after evaporation and reconstitution.

Reproducibility

Chromatography may become distorted after repeated injections and interferences may arise from late eluting unknown compounds from previous injections. Since these interferences may require extra measures, such as gradient long runs or column clean up, it is important to evaluate method reproducibility. In this study, method reproducibility was evaluated by repeated injections of melamine and cyanuric acid control samples spiked respectively with the relevant analyte. The result is shown in Table 2. Excellent reproducibility was observed for melamine, both for retention time and observed amount. A slight variation was observed for cyanuric acid retention time, which may be explained by the presence of formic acid residue from the sample preparation process.

Table 2. Method Reproducibility

Analyte	Retention Time		Observed Amount	
	Mean (minutes)	% RSD	Mean (ng/mL)	%RSD
Melamine	3.80	0.00	61.2	1.38
Cyanuric Acid	5.64	0.74	141	1.02

CONCLUSION

- A simple and fast LC-MS method has been developed for simultaneous determination of melamine and cyanuric acid.
- Melamine and cyanuric acid can be retained and resolved with satisfactory retention time and total analytical cycle time.
- Reproducibility has been demonstrated.
- MDLs of <5 ng/mL were achieved by mass spectrometric detection.
- Excellent linearity was achieved with R^2>0.9995 within 2 to 200 ng/mL.
- This method has been applied to the analyses of biological samples prepared by current U.S. FDA protocols.

ACKNOWLEDGEMENTS

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REFERENCES

1. *Pet Food Recall/Tainted Animal Feed,* U.S. FDA, May 31, 2007.
2. *Melamine and Cyanuric Acid Interaction May Play Part in Illness and Death from Recalled Pet Food,* AVMA, May 1, 2007.
3. Dionex Corporation, Acclaim Mixed-Mode WAX-1 Column, LPN 1899, February 2007.

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