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The determination of melamine in muscle tissue by liquid chromatography/tandem mass spectrometry

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In early 2007 it was determined that the compound melamine, suspected of having been involved in the deaths of numerous pets, had been fed to hogs intended for human consumption. This report describes a method for the analysis of melamine in porcine muscle tissue using solid-phase extraction (SPE) and high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). Melamine was extracted in 50% acetonitrile in water. Homogenates were centrifuged and supernatants were acidified and washed with methylene chloride. The aqueous extracts were cleaned up using mixed-mode C8/strong cation exchange SPE and then concentrated, fortified with a stable isotope-labeled analog of melamine, and analyzed by HPLC/MS/MS. Gradient HPLC separation was performed using an ether-linked phenyl column with ammonium acetate/acetic acid and acetonitrile as the mobile phase. Multiple reaction monitoring (MRM) mode of two precursor-product ion transitions for melamine and one for the internal standard was used. A five point calibration curve ranging from 50 to 2000 ng/mL of melamine in solvent was used to establish instrument response. The method was validated by analysis of seven replicate porcine muscle tissue samples fortified with 10 ng/g of melamine. The mean recovery for the seven replicates was 83% with 6.5% relative standard deviation and the calculated method detection limit was 1.7 ng/g. Copyright © 2007 John Wiley & Sons, Ltd.

In March of 2007, pet food ingredients contaminated with melamine and its analogues, ammeline, ammelide, and cyanuric acid, resulted in a major outbreak of renal disease and associated deaths in cats and dogs in the USA.[1] In the course of this outbreak it was discovered that contaminated pet food waste was incorporated into swine and poultry rations which were subsequently consumed by approximately 56 000 swine and 80 000 chickens. Meat from animals fed contaminated feed was consumed by humans, but definitive data on the risk to human health was lacking.

Melamine is an environmental metabolite of the herbicide cyromazine, and its carcinogenic potential was therefore evaluated in order to establish melamine tolerance levels in food. These studies resulted in a tolerance level of 50 ng/g of melamine in meat from hogs[2,3] that was proposed but not adopted. Therefore, it was determined that an analytical method capable of detecting melamine in hog tissue at a level of 50 ng/g or lower was required in order to determine the potential health risk involved with consumption of the affected meat. Because some of this meat had already been consumed by humans, it was essential that this method be developed as rapidly as possible. The goal of this investigation was the rapid development of such a method.

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Several methods have been developed for the analysis of melamine. These include methods utilizing gas chromatography (GC),[4] gas chromatography/mass spectrometry (GC/MS),[5] liquid chromatography (LC),[6–8] capillary electrophoresis,[9] and immunoassay.[10] The only prior report regarding analysis of melamine by LC/MS involved its detection as an environmental metabolite of cyromazine in plant tissue.[11]

This report describes a sensitive and highly specific analytical method for the extraction, purification and quantitative determination of melamine in tissue using a triple stage quadrupole mass spectrometer. The performance of the method was evaluated with regard to the ability to generate accurate and precise qualitative and quantitative data in the relevant concentration range. The method was used for the detection of melamine in tissue from hogs given melamine-adulterated food.

EXPERIMENTAL

Reagents

Water, methanol, formic acid and acetonitrile were of HPLC grade (Fisher Scientific), methylene chloride was of Optima grade (Fisher Scientific), ammonium hydroxide was of

Certified ACS grade (Fisher Scientific), and hydrochloric acid was of Trace Metal grade (Fisher Scientific). All HPLC running solvents were filtered through 0.45 μm nylon filters (Gelman Sciences, Ann Arbor, MI, USA).

Preparation of standard solutions

Melamine (99% purity) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). $^{15}N_3$-Melamine, ring-labeled, was purchased from ICON Services (Summit, NJ, USA) for use as an internal standard. All standard solutions were prepared and diluted in 1:1 acetonitrile/water. Separate stock solutions of 1000 μg/mL of melamine and the labeled melamine were prepared and subsequent dilutions of the standards were made from the stock solution. Six-point calibration curves of melamine were prepared at 50, 100, 250, 500, 1000 and 2000 ng/mL by adding aliquots of melamine standard and the dilution solvent to vials containing 100 μL of 10 μg/mL $^{15}N_3$-melamine. The final volume of each calibration standard was 1 mL.

Sample preparation

Sample (10 g) was weighed into a 250 mL French, squared homogenization vessel (Fisher Scientific). The sample was homogenized with 50 mL of acetonitrile/water (1:1, v/v) for 1 min at 19 000 rpm using an Ultra-Turrax T-25 tissue homogenizer (IKA-Labortechnik/Tekmar Company, Cincinnati, OH, USA) and centrifuged at 500 rpm (65 *g*) for 5 min using an IEC Centra-7 centrifuge (International Equipment Co., Needham, MA, USA). An aliquot (10 mL) of the clear extract was transferred into a 50 mL, glass, screw-cap centrifuge tube, 0.5 mL of 1 N HCl and 20 mL of methylene chloride were added, and the sample was vigorously mixed by hand for 2 min and centrifuged at 2000 rpm (260 *g*) for 5 min as above. An aliquot (5 mL) of the top, aqueous layer was transferred into a separate tube, 5 mL of water was added to the original (50 mL) tube and the sample was vigorously mixed by hand for 1 min and centrifuged as above. The top layer was removed and combined with the previous aqueous aliquot. After brief vortexing, the combined aqueous aliquots were quantitatively transferred (gravity) onto a 60 mg, 3 cc, Strata X-C solid-phase extraction (SPE) cartridge (Phenomenex Inc., Torrance, CA, USA), conditioned with 3 mL of methanol and 3 mL of water. The column was washed with 2 mL of 0.1 N HCl and 1 mL of methanol; this wash was discarded. Melamine was eluted from the column with 5 mL of 5% ammonium hydroxide in methanol into a glass, disposable test tube. The eluate was evaporated to dryness using a nitrogen evaporator (N-Evap, Analytical Evaporator, Organomation Assoc. Inc., Berlin, MA, USA) set at 60°C, and reconstituted in 100 μL of acetonitrile/water (1:1). Then 100 μL of 50 μg/mL ^{15}N-melamine in acetonitrile/water (1:1) was added to the residue. The mixture was vortexed for 10 s, sonicated for 2 min and filtered through a 0.45 μm HPLC filter (Millipore Corp., Milford, MA, USA) into a small volume autosampler vial. All control and fortified samples were prepared in the same manner.

LC/MS/MS analysis

A high-performance liquid chromatograph (Microm BioResources Inc., Auburn, CA) coupled with a model 4000 Q TRAP hybrid triple stage quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Canada) was used for all analyses. The analytical column was a 150 mm × 4.6 mm × 4 μm Synergi Polar-RP (Phenomenex Inc., Torrance, CA, USA), with a Polar-RP guard column cartridge. The injection volume was 10 μL. The mobile phase consisted of: (A) 25 mM ammonium acetate in 25 mM acetic acid in water; (B) acetonitrile at a flow rate of 500 μL/min under a linear gradient of 75% B to 95% B over 11 min. Mass spectral data were acquired in positive ion electrospray ionization (ESI) mode using the multiple reaction monitoring (MRM) scan function. The TurboIonSpray source was run at a temperature of 450°C with the following settings: curtain gas, 30 (arbitrary units); source gas 1, 60; source gas 2, 60; CAD gas pressure, high; ion spray voltage, 5500. The precursor ion for melamine was the $[M+H]^+$ ion of *m/z* 127. Product ions of *m/z* 85 and 68 were obtained using collision energy (CE) = 35, declustering potential (DP) = 35, collision exit potential (CXP) = 13, and entrance potential (EP) = 10. The precursor ion for $^{15}N_3$-melamine was the $[M+H]^+$ ion of *m/z* 130. The product ion of *m/z* 87 was obtained using CE = 44, DP = 42, CXP = 13, and EP = 10. The scan time for each MRM event was 200 ms.

Method validation

The method was validated by analyzing negative control porcine meat samples (n = 7) fortified with melamine at 10 ng/g. The fortifications were prepared by adding 100 μL of 1 μg/mL melamine standard to 10 g portions of control porcine meat and analyzing them using the method described above. The method was also tested by routine analysis of samples from diagnostic cases.

RESULTS AND DISCUSSION

The chemical structure of melamine is shown in Fig. 1. ESI of melamine gave the protonated molecular ion of *m/z* 127. Product ions formed by collision-induced dissociation (CID) of melamine agreed with those reported previously,[12] with prominent product ions of *m/z* 85 and 68. $^{15}N_3$-Melamine gave a protonated molecular ion of *m/z* 130 and product ions of *m/z* 87, 69, and 70. Only the $^{15}N_3$-melamine product ion of *m/z* 87 was monitored for routine analysis. Product ion spectra for melamine and $^{15}N_3$-melamine are shown in Figs. 2 and 3, respectively.

Melamine contamination in blank analyses was a recurring problem during the development of this method. This

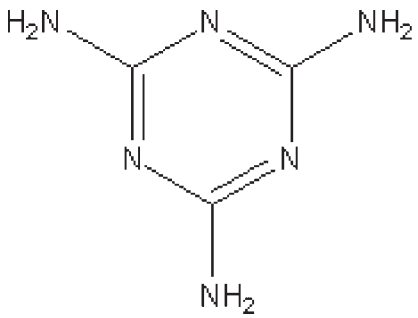

Figure 1. Structure of melamine.

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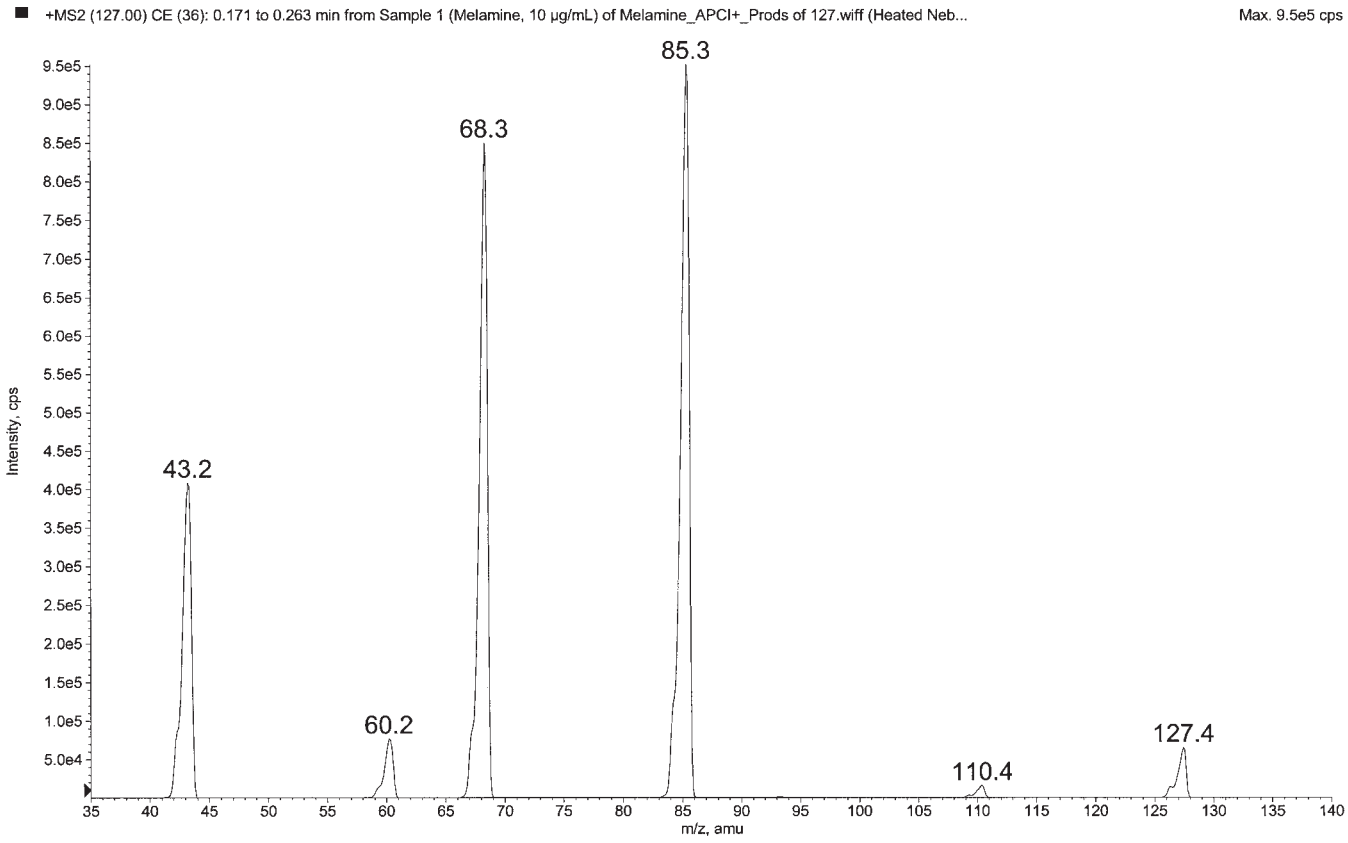

Figure 2. Product ion spectrum of melamine.

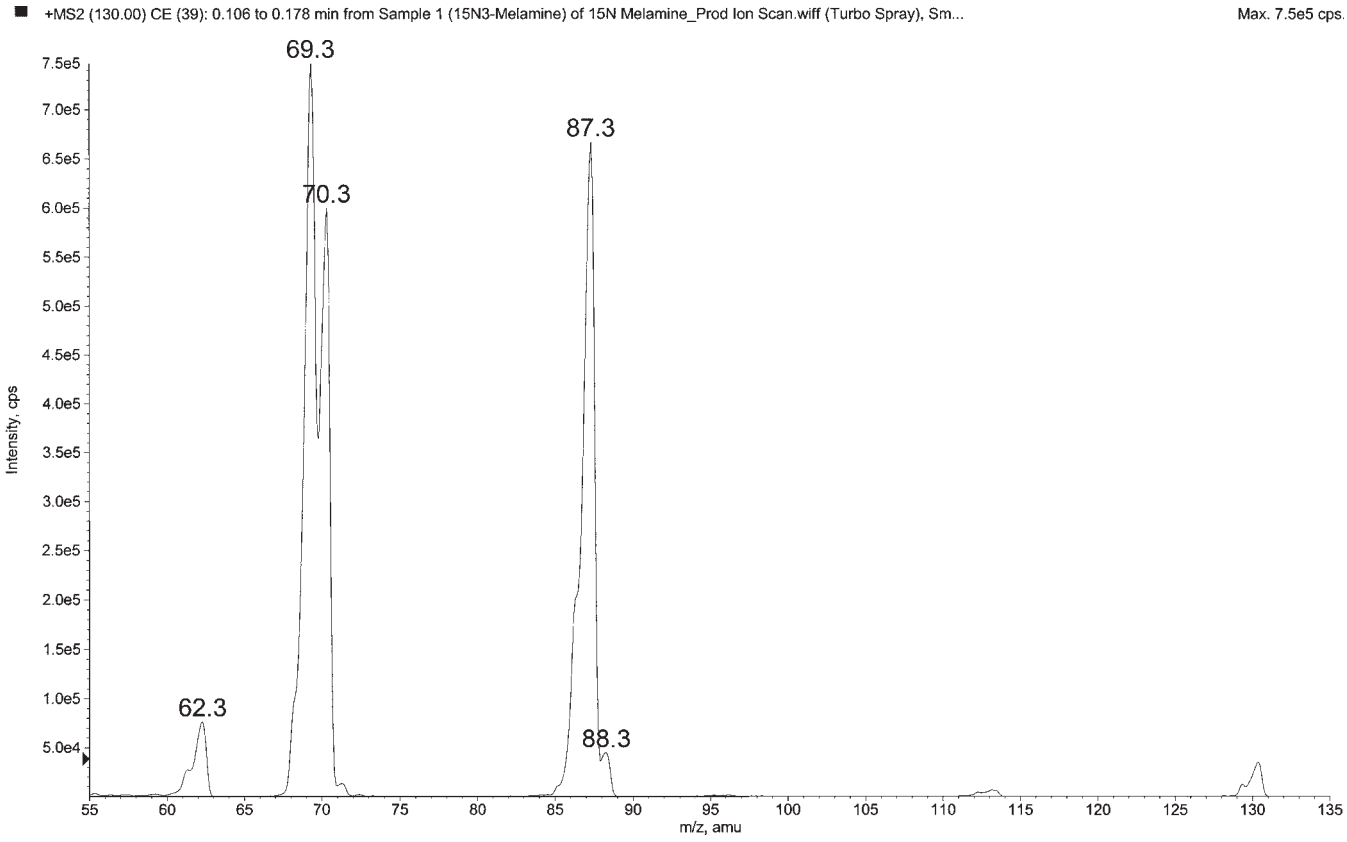

Figure 3. Product ion spectrum of $^{15}N_3$-melamine.

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appeared to be due to carryover of melamine in the HPLC/MS system. The current mobile phase was chosen after receiving information that its use would minimize carryover (P. Martos, University of Guelph, Ontario, personal communication). This mobile phase gave adequate chromatographic performance and subsequent blank analyses showed no detectible melamine.

Figure 4 shows typical ion chromatograms for 50 ng/mL of melamine and 1000 ng/mL of the internal standard in 80:20 water/acetonitrile. The relative response of melamine vs. the internal standard was consistently linear over the calibration range of 50–2000 ng/mL, with r^2 values >0.995. The signal-to-noise (S/N) ratio of melamine in the 50 ng/mL standard was consistently greater than 10.

The extraction procedure reported here gave adequate recovery of melamine from the porcine tissue matrix. The initial analysis of tissue samples from hogs given melamine-contaminated feed indicated the presence of melamine at or near levels of 10 ng/g. Such low levels in tissue are consistent with the limited information available on the toxicokinetics of melamine. There is no known metabolism of melamine in mammals and the compound does not appear to bind to plasma proteins. In rats, 90% of an orally administered ^{14}C-labeled melamine dose was excreted unchanged in urine within 24 h.[13] In dogs, 50 to 60% of orally administered melamine was excreted in urine within 6 h and up to 85% were recovered within 24 h.[14] A small amount of melamine appeared to be recovered in urine of dosed dogs from 25 to 27 h after exposure. The rapid clearance from the body shows that melamine is unlikely to accumulate in tissues. The method was therefore evaluated for melamine concentrations expected in these samples.

Method evaluation was performed by analysis of seven replicates of negative control muscle tissue, each fortified with melamine at a level of 10 ng/g. (equivalent to 100 ng/mL in the concentrated extract). The average recovery from this set was 83% with 6% relative standard deviation (RSD). The method detection limit, as calculated using Student's t test at the 99% confidence level for 6 degrees of freedom, was 1.7 ng/g of tissue. Because of the requirement for rapid method development and the expectation that melamine levels would be low or not detectible in the samples, the dynamic range of the method and its performance at higher analyte concentrations were not evaluated. Further investigation would be required to determine the method's performance at higher concentrations. For all tissue samples analyzed, the concentration range of melamine in positive samples was 2.5–12 ng/g.

The ratio of response for *m*/*z* 85 vs. *m*/*z* 68 in the seven fortified control samples averaged 1.40 with 7.21% RSD. Ion ratios in ten associated calibration standards averaged 1.41 with 1.22% RSD. Figure 5 shows a representative chromatogram of a control sample fortified with melamine at the 10 ng/g level from the MDL study. Figure 6 shows a representative chromatogram of a negative control sample, indicating no carryover of melamine.

For quality control purposes, each batch of 12 or fewer diagnostic samples included a negative control porcine meat

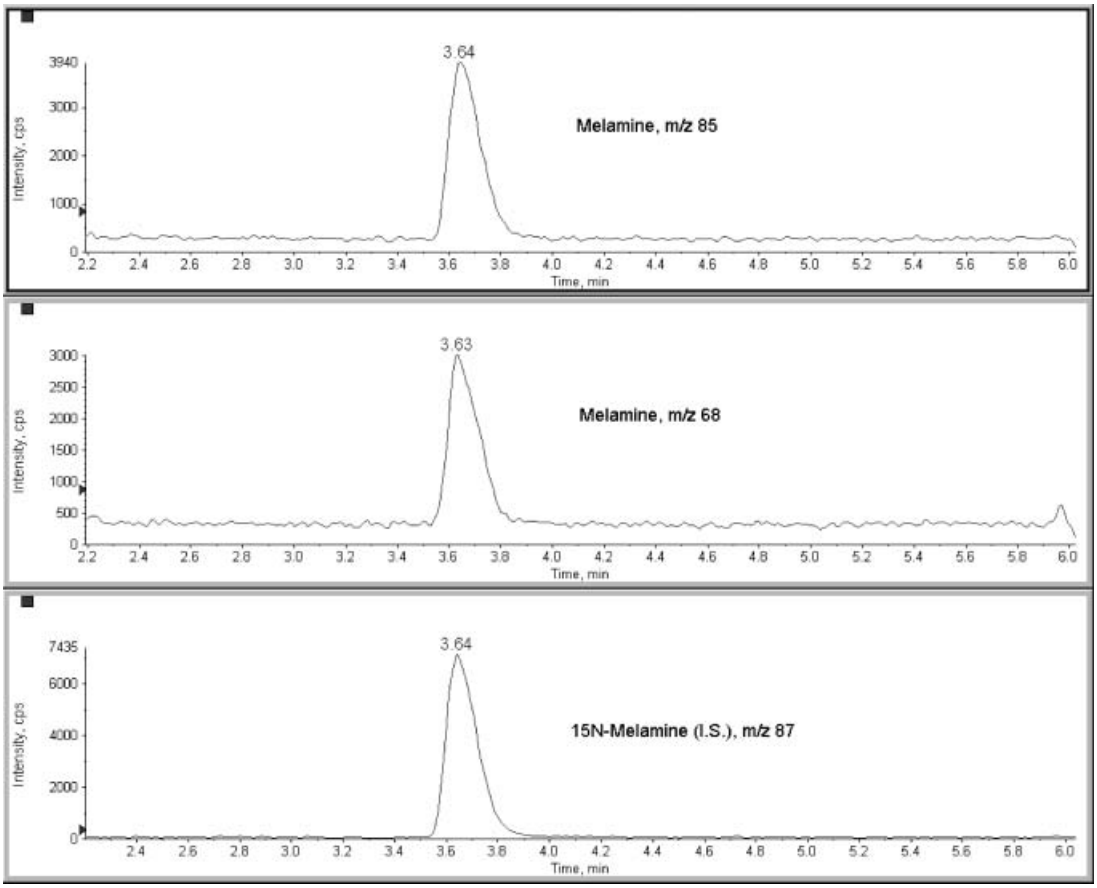

Figure 4. Ion chromatogram of 50 ng/mL melamine standard (upper chromatogram) with 1000 ng/mL $^{15}N_3$-melamine internal standard (lower chromatogram).

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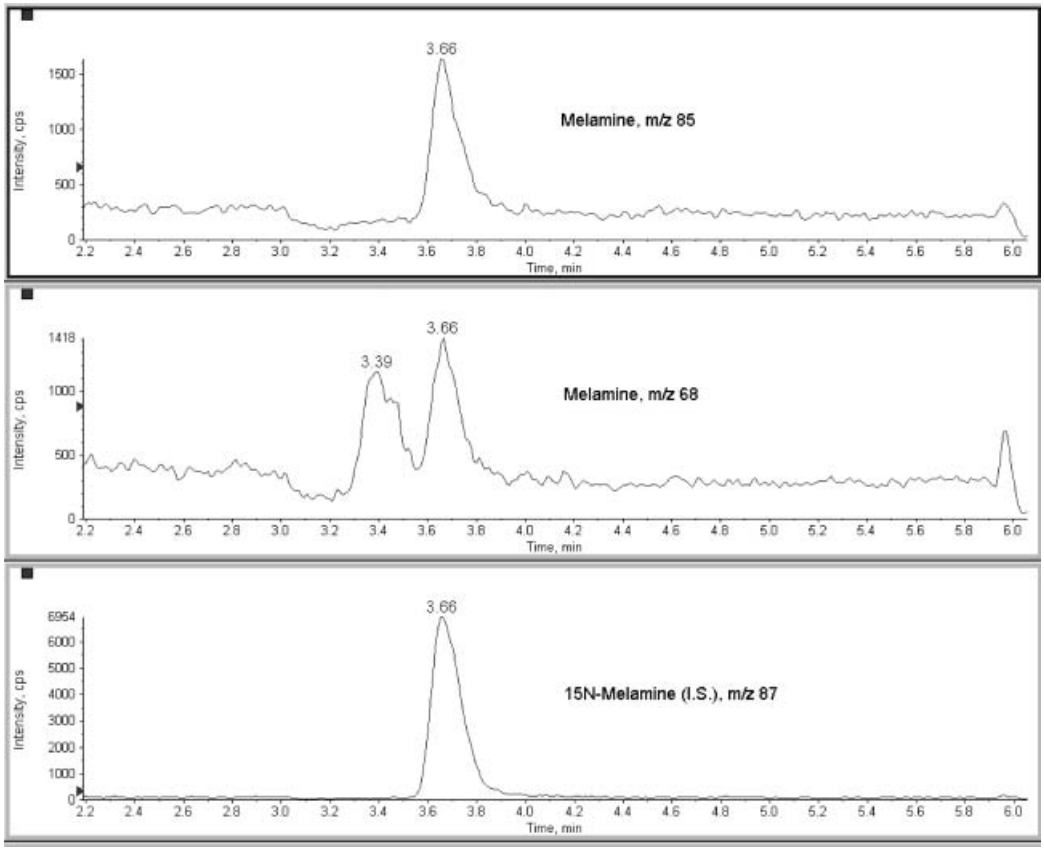

Figure 5. Ion chromatogram of from analysis of negative control tissue fortified with 10 ng/g melamine (upper chromatogram). The lower chromatogram shows the $^{15}N_3$-melamine internal standard.

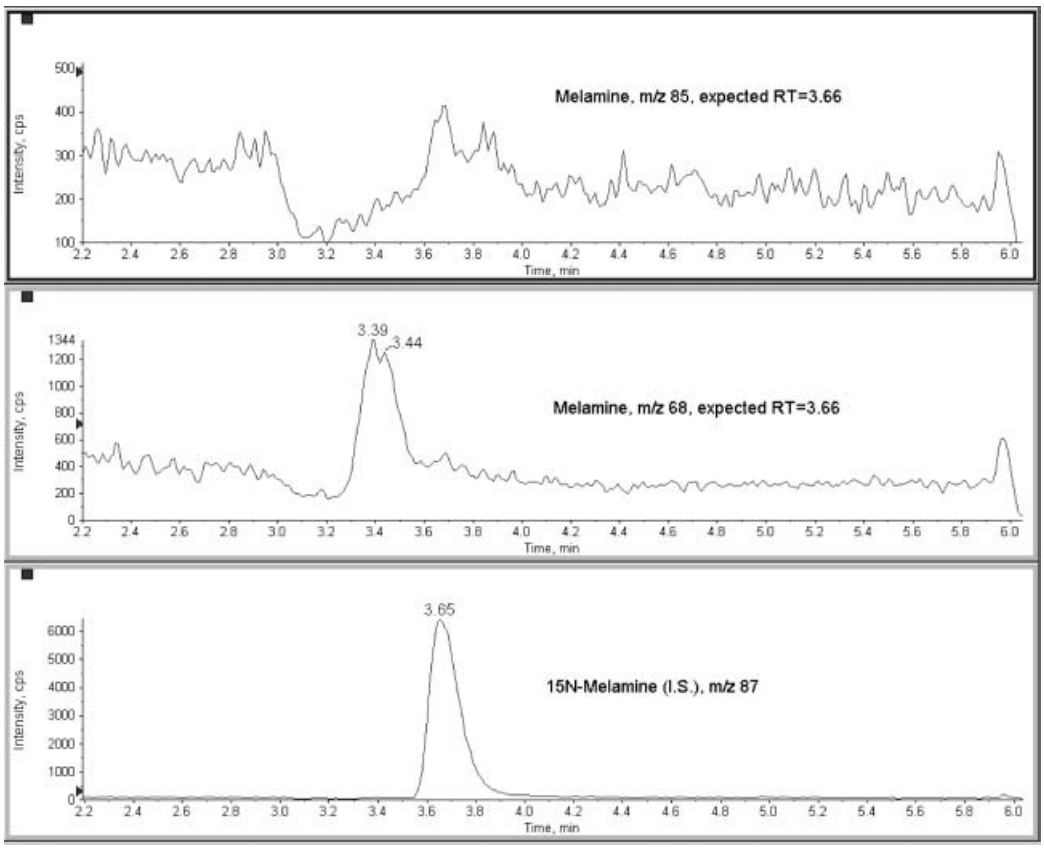

Figure 6. Ion chromatogram of unfortified negative control tissue showing no detectible melamine. The lower chromatogram shows the $^{15}N_3$-melamine internal standard.

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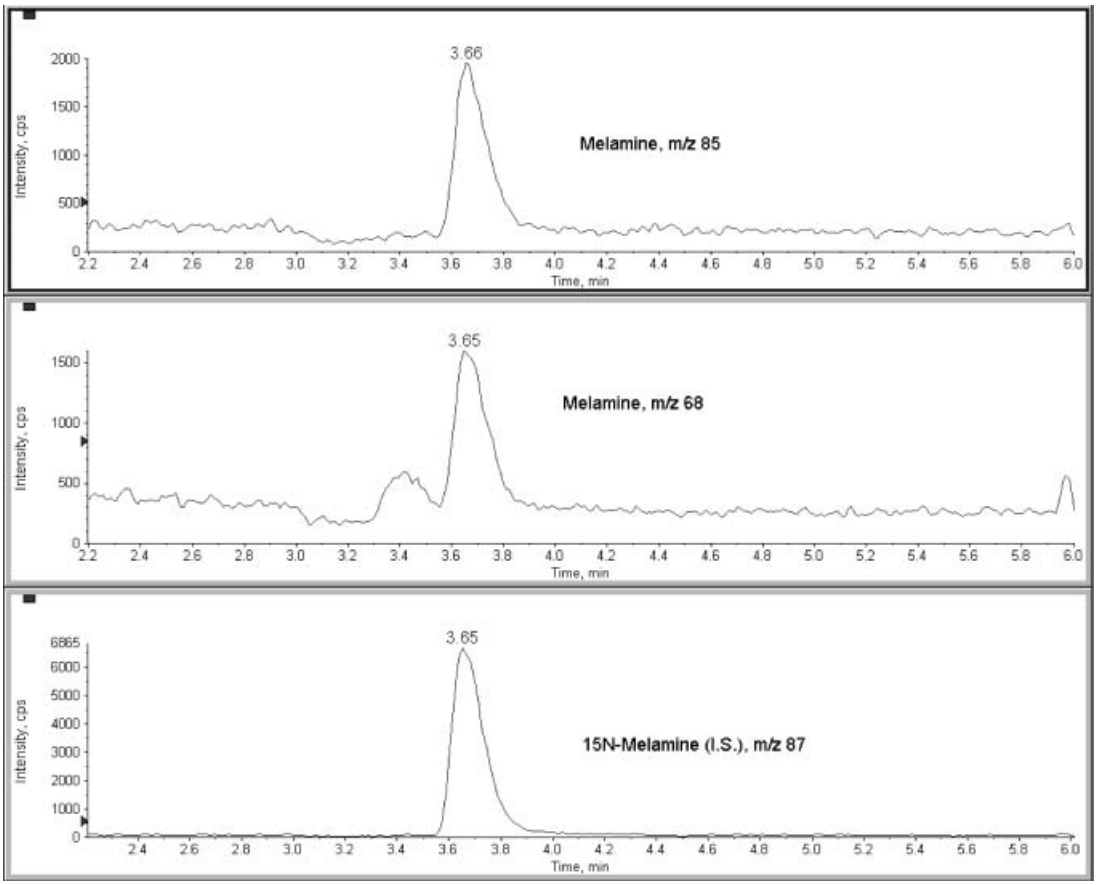

Figure 7. Ion chromatogram from analysis of a tissue sample from an affected hog. Melamine is shown in the upper chromatogram and the 15N3-melamine is shown in the lower chromatogram. The melamine level from this sample was calculated to be 11 ng/g.

sample (method blank) and one or more negative control samples fortified with melamine at the 10 ng/g level. One analyst was able to extract and analyze a complete batch of samples within an 8-h day. Figure 7 shows a chromatogram from a diagnostic sample determined to be positive for melamine at 11 ng/g.

CONCLUSIONS

A quantitative HPLC/MS/MS method was rapidly developed for the analysis of melamine in edible hog tissue. Data generated by this method provided crucial information to the USDA and FDA[15] in conducting a safety and risk assessment of melamine. This interim risk assessment indicates that melamine residues in edible tissues of animals inadvertently exposed to melamine are unlikely to pose a human health risk. Further research is needed, however, to evaluate the health risk of a combination of melamine and cyanuric acid, because in the course of the pet food recall investigation, cyanuric acid was also identified in pet food as a co-contaminant.[16]

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