MONITORING OF MELAMINE IN MILK AND FEED USING ELISA AND LCMS/MS SCREENING METHODS

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ABSTRACT. A monitoring program for melamine in milk and feed was conducted in response to global melamine alertness in the year 2008. Two screening methods were adopted i.e., a liquid chromatography triple quadrupole tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assay (ELISA). The liquid chromatography method developed by several international research centers was adapted. This method consisted of an initial extraction with 10% trichloroacetic acid (TCA) for milk samples or 60% methanol/water for feed samples, followed by a series of centrifugation, dilution and filtration steps. Melamine was analysed in the chromatographic program using a zwitterionic HILIC LC column. Electrospray ionisation in positive ion mode was used. The quantity of melamine present was determined with a calibration curve consisting of sample extracts from milk or feed fortified from 25 to 50 ppb that were taken through the extraction procedure. The ranges of recovery from fortified raw milk samples (n=20) and feed samples (n=21) was 70–80% and 68%, respectively. The limit of detection was estimated at 10 ppb for both matrixes. Milk samples were found negative for melamine, however 4.5% of feed samples were found to contain the compound at concentrations between 1 to 5 ppm.

Keywords: Melamine, Milk, Feed, Screening, LCMS/MS, ELISA

INTRODUCTION

Residue monitoring and surveillance of food of animal origin by the Department of Veterinary Services Malaysia (DVSM) is an important part of an overall national food safety program. This is to minimise unwanted chemical residues in food for public consumption.

The incidences of melamine contamination which led to human or animal illness and fatality in 2007 to 2009, especially in China (WHO, 2008; WHO, 2009), was brought to the attention of DVSM to tackle the problem at the farm level. The major tasks were concurrently taken by the Ministry of Health Malaysia. Other countries have also reported detection of melamine in eggs (probably arising from contaminated feed) and milk-containing products manufactured in China, such as liquid milk, biscuits, candies, frozen yogurt dessert and beverages. It appears that contamination with melamine happened

during milk production, where it had been intentionally added to raw milk at milk collection centres for at least 9 months (WHO, 2008).

Chemical and histological analyses of tissues from the 2004 and 2007 incidents by Brown (2007) and Thompson (2008) have shown that the acute renal failures in animals from both pet food incidents were associated with the presence of melamine and cyanuric acid.

Consequently, the screening for melamine residues in fresh milk, milk products and feed were conducted in a 6-month food safety program established by the Veterinary Public Health Laboratory, DVSM as a quick response to the global melamine alertness. A recommended enzyme-linked immunosorbent assay (ELISA) or liquid chromatography triple quadrupole tandem mass spectrometry (LCMS/MS) screening method (VARIAN, 2008), without solid phase extraction, was adapted and applied to accomplish this urgent task.

MATERIALS AND METHOD

Screening Procedure. Samples of raw cattle milk were received from the milk collection centers of DVSM, which are mostly situated in the western states of Peninsular Malaysia. Milk product samples were received from the Quarantine Unit, DVSM, while feeding stuff were collected randomly from samples submitted by private farm owners for routine quality testing at this laboratory. The samples were screened using a competitive ELISA (Abraxis Melamine Plate Kit Cat. #50005B). When the samples showed a positive reading beyond cut-off point of 20 ppb for both matrixes by ELISA, they would be further analyzed using LCMS/MS method.

Only milk and feed samples were targeted for testing during the period of October 2008 to March 2009. Raw milk samples were also screened for their quality to observe any significant difference in their protein content using Milkoscan 4000 (Foss) prior to the testing for melamine.

Crude Protein Determination. Crude protein was determined on feed and meat and bone meal (MBM) samples according to Kjeldahl method (AOAC, 1995) using Protein Digestion System (VELP) and Micro Kjeldahl Distillation System (Foss). One gram of the samples were digested for 4 hours in 15 mL H_2SO_4 using 1 g $CuSO_4/TiO_2$ as catalyst, until a clear solution without black particles was obtained. The sample was cooled for 1 hour, then dissolved in a 100 mL distilled water and transferred to a semi micro kjeldhal distillation apparatus which has been previously conditioned by passing steam for several minutes. 50 mL of 40% NaOH solution was added to the Kjeldhal apparatus. 25 mL of 4% boric acid solution and 3 drops of methyl red-bromocresol green mixed indicator solution were added into a titration flask and kept at the end of the apparatus to trap the ammonia liberated. Steam was passed through the flask until about 15 mL of distillate was

received. This solution was collected at the titration flask and titrated with 0.2N standard H_2SO_4 solution until pink colour was seen as end point.

Feed with added melamine. A standard solution of melamine (1 µg/mL) was prepared by diluting 1 g of melamine (99% purity, Acros, USA) in 1 L of acetonitrile. Feed sample (1 g) was added with 1 mL of standard solution. Crude protein was determined according to the procedure as stated above.

Sample Preparation for Melamine in Milk. Liquid milk sample (2 mL) was extracted with 8 mL of 10% TCA in a 50 mL polypropylene centrifuge tube. The extract was sonicated and subjected to centrifugation at 4000 rpm for 10 min. Then, 0.2 mL supernatant was transferred to 5 mL glass test tube followed by dilution with 1.8 mL buffer for ELISA.

For LCMS/MS analysis, the supernatant was diluted with 1.8 mL acetonitrile and filtered with 0.4 micron nylon filter prior to analysis.

Sample Preparation for Melamine in Feed. Granular feed sample (1 g) was extracted with 10 mL of 60% methanol/ water in a 50 mL polypropylene centrifuge tube. The extract was vortexed, sonicated for 1 min then vortexed again and allow the sample to settle for 5 min. The upper layer was pipetted into 25 mL glass test tube followed by centrifugation at 10,000 rpm for 5 min. Then, 0.1 mL supernatant was transferred into 5 mL glass tube followed by dilution with 1.9 mL 10% methanol/20 mM phosphate buffer saline (PBS) for ELISA.

For LCMS/MS analysis, the supernatant was diluted with 1.9 mL acetonitrile and filtered with 0.4 micron nylon filter prior to analysis. Sample preparations for meat and bone meal (MBM) with unknown composition, were similar to that of granular feed samples.

Experimental Conditions

Liquid chromatography mass spectrometry (LCMS/MS). Analysis was performed on a Waters liquid chromatography equipped with binary pump 2695 Alliance Separation Module and Micromass triple quadrupole Quattro Ultima detector using MassLynx 4.0 software for instrument control and data acquisition. The chromatographic separation was accomplished with gradient elution on an Atlantis HILIC Silica (3.0 mm × 50 mm i.d., 3 µm particles) at 30°C in a column oven. The flow rate was 0.2 ml/min. The injection volume was set to 10 µL. The mobile phase A consisted of 10 mM ammonium acetate in acetonitrile and mobile phase B consisted of 10 mM ammonium acetate in water. The gradient elution performed was 0-4.2 min: 97% A; 4.2-5.5 min: 3% A; 5.6-15 min: 97% A.

Ionspray source (Electrospray Ionisation) was operated in positive ionization mode with source temperature of 100°C. Pure nitrogen was used. Desolvation temperature was set at 350°C and desolvation gas flow was 600 L/hr.

Cone gas flow was 55 L/hr. Cone voltage was set at 70 V and the dwell time 0.1 sec. Ion transitions of m/z 127/68 (collision energy 49 eV) and m/z 127/85 (collision energy 17 eV) were used for quantitative and qualitative detection of melamine.

Calculation. For the LCMS/MS method, the analyte concentration was calculated automatically using external standard calibration with a standard curve prepared in a pre-fortified control matrix which had been carried out through the extraction procedure.

RESULTS AND DISCUSSION

A total of 322 raw milk samples received in four batches from different sources were tested for their quality profile using Milkoscan 4000 (Foss). Figure 1 shows the ranges of milk protein (2.0–4.4%; mean = 3.1%); total solid (9.7–22.3%; mean = 13.5%); solid non-fat (2.8–9.5%; mean = 8.6%) and pH (6.7–7.1; mean = 6.9). It was observed that the ranges for protein content and pH were narrow and relatively consistent as compared to ranges for total solid and solid non-fat. Melamine compound was not detected in all raw milk samples when screened with ELISA. A few samples that had reading above the cut-off point of 20 ppb were further subjected to analysis by LCMS/MS method.

Melamine has a high nitrogen content (66.6% by weight), which makes it attractive for economic adulteration as a fraudulent substitute for protein, especially when indirect protein assays using Kjeldahl method is used to determine total nitrogen. The purpose of crude protein determination by this method in this study was to observe the effect of addition of at least 1 ppm of melamine standard solution into the feed samples.

Figure 2 shows the percentages of crude protein content of the feed samples tested that ranged between 14.0 and 61.9% (mean = 21.1%). The addition of melamine

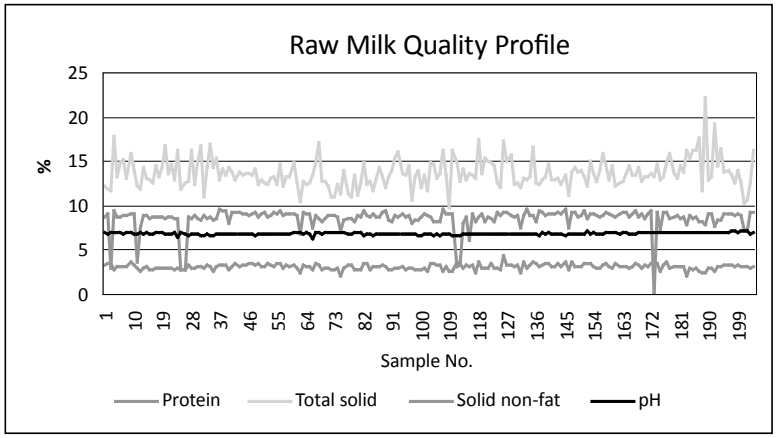

FIGURE 1. Quality profile of tested raw milk using Milkoscan4 000 (Foss)

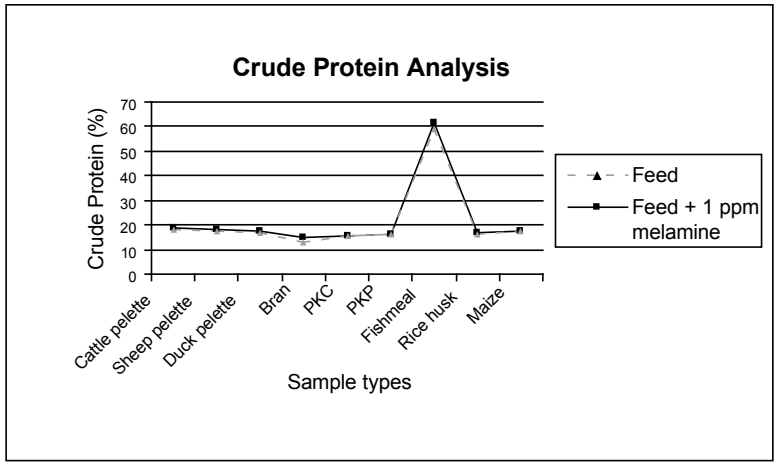

FIGURE 2. Percentage of crude protein of different types of feed and feed with added melamine using Kjeldahl method

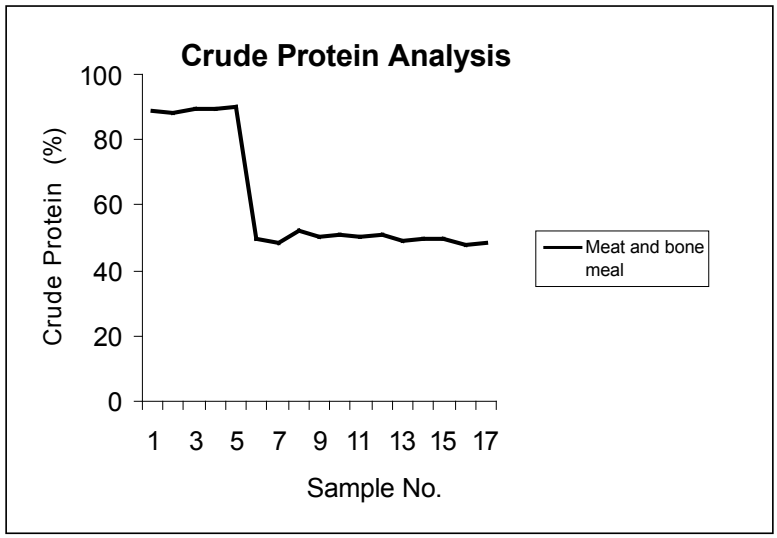

FIGURE 3. Percentage of crude protein of meat and bone meal (MBM) using Kjeldahl method

into these feed samples showed an overall increase of 4.5% crude protein content (mean = 23.1%). However, this increase was not significant in certain types of these feed samples. The crude protein determination of MBM samples similarly using Kjeldahl method ranged between 48 and 89% (Figure 3). Melamine compound was not detected in all MBM samples as determined by ELISA method.

Chromatograms of LCMS/MS analysis

The chromatograms (Figures 1 to 8) show the presence of melamine compound in bird and pig feeds detected by LCMS/MS

screening method with the concentrations ranging between 1 to 5 ppm (Figures 4, 5 and 6). Unknown peaks at 7.70 min and 7.73 min were noticed in both pig samples after the melamine peak. It could be speculated that this peak may represent that of a melamine related compound with a molecular weight closer to that of melamine. However it is suggested that a further clean up step in sample preparation should be conducted, for the purpose of this study to enhance the melamine peak more effectively. Figure 7 shows the chromatogram of a blank feed sample and Figure 8 show the calibration curve as part of quality control and in this analysis.

All samples tested and their results are summarized as in Table 1.

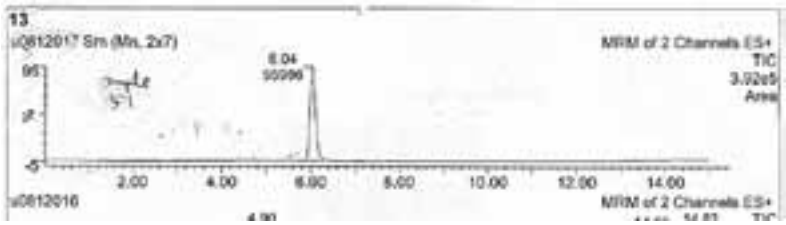

FIGURE 4. Chromatogram (TIC) of positive bird feed sample at 6.04 min

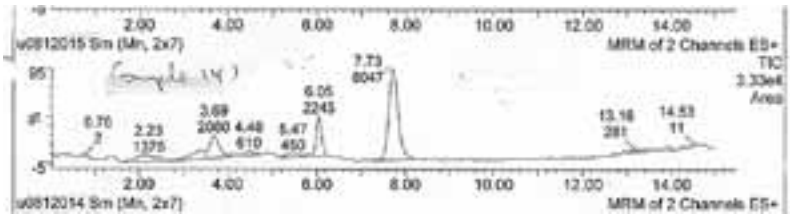

FIGURE 5. Chromatogram (TIC) of positive pig feed (yeast) sample at 6.05 min

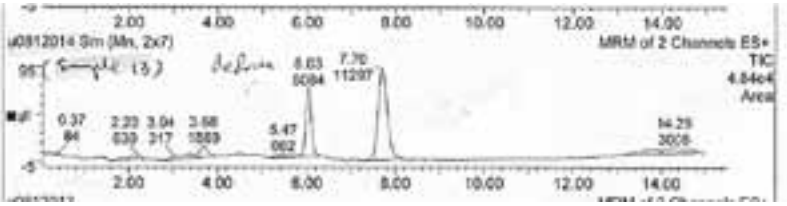

FIGURE 6. Chromatogram (TIC) of positive pig feed sample at 6.03 min

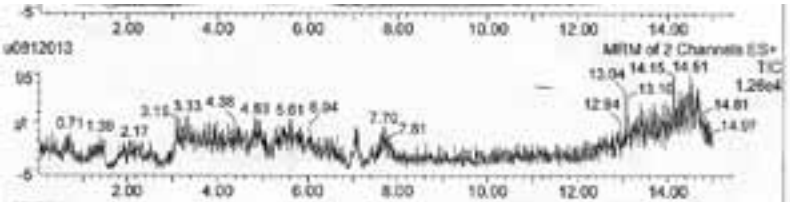

FIGURE 7. Chromatogram of blank feed sample

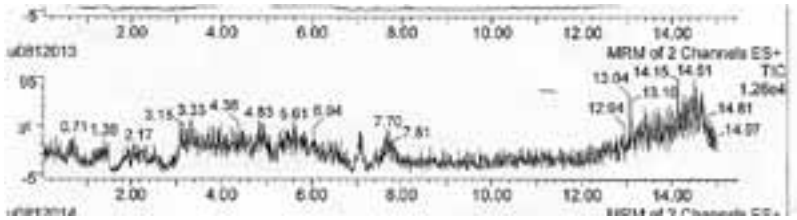

FIGURE 8. Calibration curve of fortified Melamine in feed samples (x 20)

TABLE 1. Types and number of samples tested during the monitoring programme

Sample type	No. of samples Tested	Number of samples that detected positive for melamine	Source
Raw milk	322	0	Local
Milk products	41	0	Imported
Feeds	55	4	Imported
Others (MBM)	33	0	Imported

CONCLUSION

Due to the incidences of melamine contamination, there is a need for a rapid and sensitive method for the analysis of melamine in food, especially of milk products and feed. The application of the above LCMS/MS screening method with linearity (R2) of the calibration standard approaching 0.98 for a range of concentration from 5 to 100 ppb and recovery of 68% (feed), without going through solid phase extraction, has helped this laboratory to conduct the melamine monitoring program quite timely. At least 4.5% of feed samples had been detected to contain melamine compound ranging between 1 to 5 ppm.

It is recommended that the DVSM to continue with this monitoring program from time to time and to conduct a confirmatory test, especially on the imported animal feed samples and animal tissues of interest using other confirmatory methods.

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