Research Note

Melamine residues in eggs of laying hens exposed to melamine-contaminated feed

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ABSTRACT An experiment was carried out to determine melamine residual levels in eggs by feeding laying hens 200 or 1,000 mg of melamine/kg of diet. Each diet was offered in 3 replicate cages (10 laying hens/cage) from d 1 to 29, followed by a 9-d feeding of a withdrawal diet that contained no melamine. Two eggs were collected from each replicate cage each day for the determination of residual melamine levels after 1 d of feeding. The feeding of melamine resulted in a fast accumulation of melamine in eggs within 3 to 4 d, then maintained 2.00 to 3.88 mg/kg for 200 mg of melamine/kg of diet and 11.09 to 16.46 mg/kg for 1,000 mg of melamine/kg of diet. A withdrawal period of 4.0 d for 1,000 mg of melamine/kg of diet was required based on tolerance values established by the World Health Organization and no withdrawal period was required for 200 mg of melamine/kg of diet.

Key words: melamine, withdrawal period, residue, egg

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INTRODUCTION

Melamine is an important organic chemical raw material (Ruilin, 2006; Ingelfinger, 2008). It is often used in the production of plastics, glues, and flame retardants. Some Asian countries had used melamine as a fertilizer because of its rich nitrogen content (Vail et al., 2007). However, milk powder that was illegally adulterated with melamine led to health problems for thousands of infants in China (Yan et al., 2009; Yang et al., 2009). Moreover, high levels of melamine in eggs imported from northeast China were found in Hong Kong only after 1 mo following the Sanlu brand melamine milk powder incident (Xia et al., 2009).

Previous toxicology studies showed that melamine toxicity in mammals is very low, and this is supported by the large oral half-maximal lethal dose of 3,161 mg/kg in rats (FDA, 2007). The results of a recently conducted experiment on the pharmacokinetics of melamine in pigs following intravenous administration (Baynes et al., 2008) suggested that melamine is readily cleared by the kidney. However, animal studies carried out since the 1980s have demonstrated that the ingestion of melamine by mice can cause bladder stones (Organization for Economic Cooperation and Development, 2002). Renal failure in animals and humans was reported as a result of the illicit use of additives melamine in pet food and in milk powder (Andersen et al., 2008; Yan et al., 2009; Yang et al., 2009). Thus, from a food safety standpoint, a study of melamine residue levels in the eggs of laying hens exposed to melamine-contaminated feed is of interest. At the same time, control and monitoring programs mandated by various government agencies also have interest in melamine residue levels in the eggs of laying hens exposed to melamine-contaminated feed. The purpose of the present study was to determine residue levels in eggs during the dosing and withdrawal period for laying hens fed melamine.

MATERIALS AND METHODS

Melamine

Melamine (purified, ≥99.0%) was purchased from Chemservice (West Chester, PA). Melamine was directly diluted with formula feed for laying hens to a concentration of 10 g/kg and appropriate amounts of this diluted material were then mixed in formula feed for laying hens to prepare treatment diets with 200 and 1,000 mg of melamine/kg of diet.

Animals

In this experiment, 60 laying hens (28 wk old, provided by an animal husbandry and veterinary station of Wu Xiang, Ningbo, China) of the Lohmann Brown strain were randomly allocated into 2 groups with 30 birds each and housed in 6 replicate cages (10 laying hens/cage). All laying hens had free access to water.

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Table 1. Mean recoveries (n = 5) and CV of melamine in eggs collected from control laying hens (exposed to formula feed without melamine) by gas chromatography-mass spectrometry[1]

Fortified concentration (mg/kg)	Mean recovery (%)	Intraassay CV (%)	Interassay CV (%)
0.05	84.7, 86.2, 89.8	10.6, 4.6, 6.0	7.0
0.10	88.1, 87.6, 90.4	7.1, 6.8, 4.6	8.3
0.50	92.6, 84.7, 87.3	6.9, 4.2, 6.0	7.7

[1]Mean recovery values respectively relate to intraassay CV values.

The cages were kept on a 16-h light, 8-h dark cycle. Room temperature was maintained at 20 ± 2°C during the whole experiment period. All laying hens were given 3 times daily for 28 consecutive days formula feed (1,800 g/feeding per cage) that contained either 200 or 1,000 mg/kg of melamine. From d 29 to 38, all laying hens were fed a withdrawal diet that contained no melamine. The formula feeds were formulated based on 62% corn, 18% soybean meal, 3% wheat, 4% cotton seed meal, 3% rapeseed meal, and a vitamin-mineral supplement.

On each day, 2 eggs were randomly selected from each replicate cage after 1 d of feeding. The eggs (only egg contents) from each cage were homogenized using a high-speed blender (Ultra-Turrax T25, IKA, Staufen, Germany) for 2 min and were stored at −20°C until they were analyzed for melamine content.

Sample Preparation

Five grams of homogenate was extracted with 50 mL of 10 g/L trichloroacetic acid water solution and 2 mL of 66 g/L lead acetate water solution. After simple vortexing for 1 min, the sample was extracted for 20 min under ultrasonic conditions and centrifuged at 5,200 × *g* for 5 min. The supernatant was transferred to a clean test tube. Ten milliliters of extract was applied to a PCX cartridge (Beijing Agela Technologies Co. Ltd., Beijing, China) previously conditioned with 3 mL of methanol and 3 mL of water. After the extract had passed through, the cartridge was washed with 3 mL of water followed by 3 mL of methanol. Melamine was finally eluted from the cartridge with 3 mL of ammonium hydroxide/methanol (5/95 vol/vol) solution. The eluate was evaporated to dryness under a nitrogen stream and

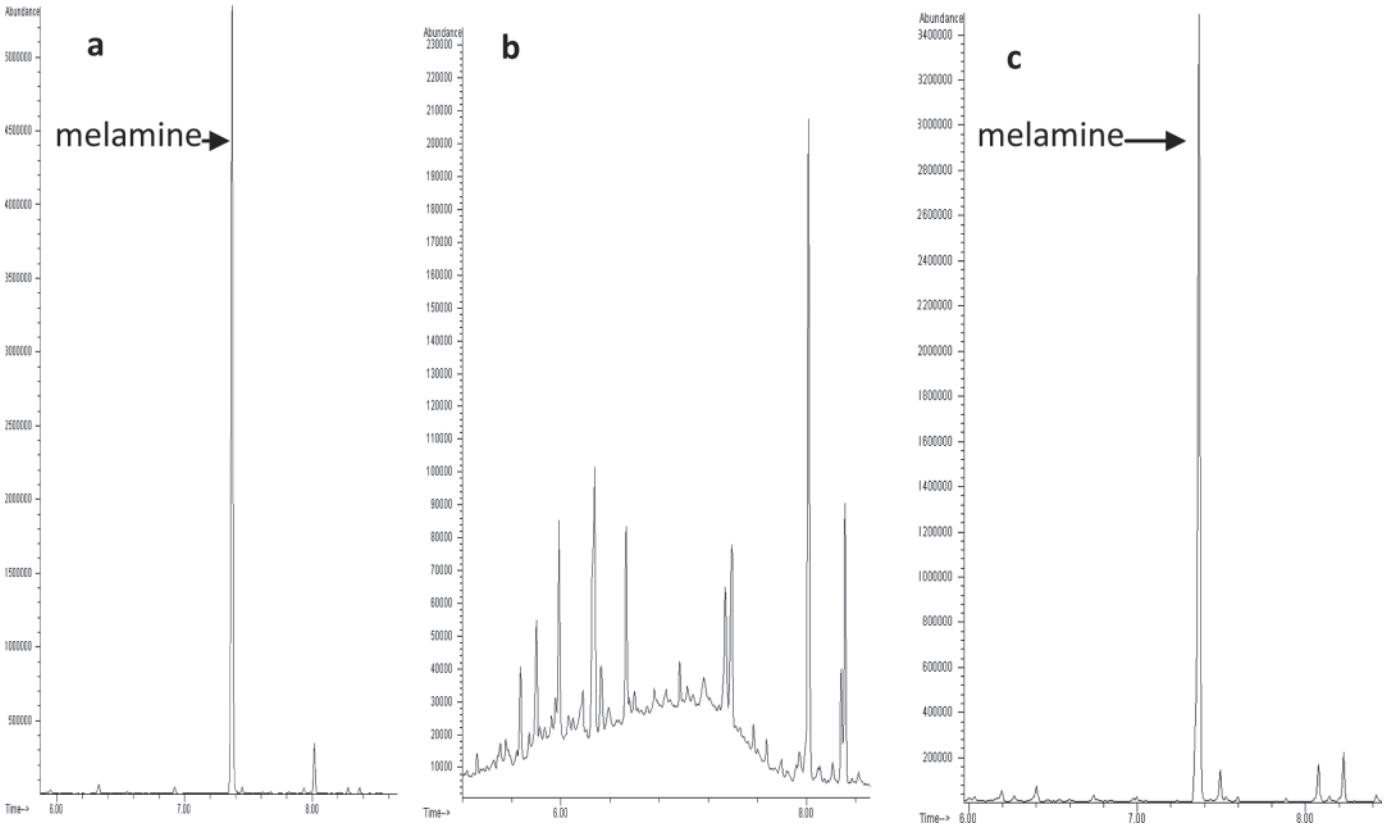

Figure 1. Chromatogram of a) melamine standard (2.5 mg/L), b) blank egg sample, and c) egg sample from elimination experiment.

Table 2. Residues of melamine (mg/kg of wet weight) in eggs of laying hens fed diets containing 200 and 1,000 mg of melamine/kg of diet (n = 6)

	Added melamine (mg/kg of diet)	
Day	200	1,000
1	—	—
2	1.43	3.76
3	1.92	6.67
4	2.00	7.72
5	2.02	11.09
6	2.10	11.40
7	2.19	12.42
8	2.66	12.53
9	2.67	13.44
10	2.86	13.58
11	3.03	13.97
12	3.11	14.32
13	3.12	14.37
14	3.20	14.80
15	3.25	15.03
16	3.30	15.09
17	3.59	16.11
18	3.88	16.28
19	3.42	16.46
20	3.41	15.60
21	2.64	15.03
22	2.60	14.51
23	2.50	14.83
24	2.65	14.11
25	2.47	14.17
26	2.42	13.91
27	2.36	13.64
28	2.43	13.81
29	2.40	13.28

dissolved in a mixture of 200 μL of bis[trimethylsilyl] trifluoroacetamide and 200 μL of pyridine. The glass tube with stopper was kept at 70°C for 30 min to derivatization. After cooling to room temperature, 1 μL of sample was injected for gas chromatography-mass spectrometry (**GC-MS**) analysis.

Instrumentation and Chromatographic Conditions

Sample analysis was performed on an Agilent 6890 GC coupled with a 5973 mass selective detector (Agilent Co., Santa Clara, CA) operated in the electron impact mode. The GC was fitted with an HP-5MS capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA). The temperature program employed was 75°C for 1 min, 30°C/min to 300°C, and hold for 2 min. The injector temperature was 250°C and all injections were made in splitless mode. Helium was used as carrier gas at flow rate of 1.3 mL/min. The temperature of interface, quadruple, and ion source was 280, 150, and 230°C, respectively. The full scan acquisition was performed in the mass range mass:charge ratio 70 to 550 amu; all data for quantification were collected in the selected ions monitoring mode. Solvent delay was 5 min; the intensity of the characteristic ions at mass:charge ratio 99, 171, 327, and 342 were monitored.

GC-MS Method Validation

The linearity of the analytical method was constructed using working standard solutions by plotting the peak area of standard at concentrations of 50, 100, 200, 500, 1,000, and 2,500 ng/mL. The limit of detection (**LOD**) and the limit of quantitation (**LOQ**) were determined using the lowest signal:noise ratio of fortified sample. The LOD and LOQ were defined as the concentration of melamine that produced a chromatographic peak with signal:noise ratio greater than 3 and 10, respectively. The accuracy and precision (interday and intraday) of the analytical method were determined using blank samples spiked at 3 levels (0.05, 0.10, and 0.50 mg/kg). The intraday precision was assessed by performing 5 repetitions of each level during a single day and the interday precision was assessed by 5 repetitions of each level per day over 3 different days.

Statistical Analyses for Withdrawal Time

Withdrawal time was calculated with the program WT1.4. This program, suggested by the European Medicines Agency, calculates withdrawal period using specific experimental data and is a computer translation of the method described in Committee for Veterinary Medicinal Products (1996).

RESULTS AND DISCUSSION

The GC-MS method was validated. A standard curve was built in the range of 50 to 2,500 ng/mL for melamine with correlation coefficients >0.999. The mean recoveries for melamine ranged from 84.7 to 92.6%, with CV ranging from 4.2 to 10.6% (Table 1). The LOD, defined as a signal:noise ratio of 3:1, was 3 ng/g; the LOQ, defined as a signal:noise ratio of 10:1, was 10 ng/g. The chromatograms obtained from the analysis of the standard, the blank, and the egg sample from the elimination experiment are shown in Figure 1. The determined melamine levels in the experimental diets were 171 ± 14 mg/kg (n = 3) and 879 ± 52 mg/kg (n = 3) for 200 and 1,000 mg of melamine/kg of diet, respectively.

Table 3. Residues of melamine (mg/kg of wet weight) in eggs of laying hens fed diets containing 200 and 1,000 mg of melamine/kg of diet for 28 d followed by 0, 1, 2, 3, 5, 7, and 9 d of withdrawal (n = 6)

	Added melamine (mg/kg of diet)	
Withdrawal day	200	1,000
0	2.40	13.28
1	0.32	6.80
2	0.27	5.95
3	0.18	2.08
5	0.09	0.65
7	0.008	0.25
9	0.006	0.03

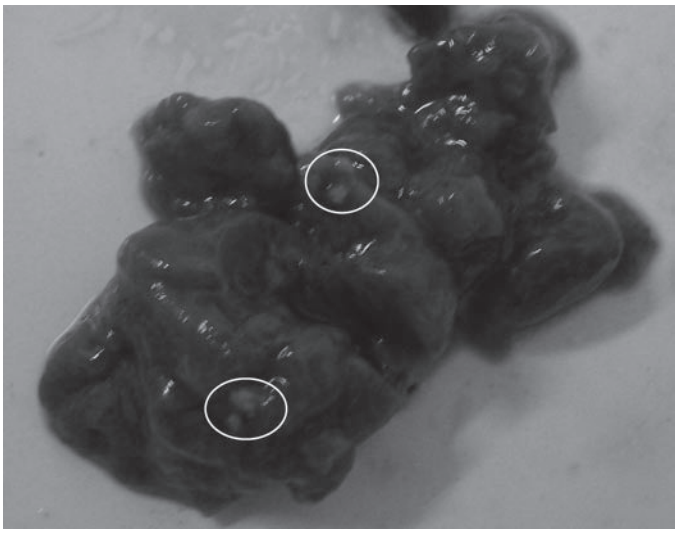

Figure 2. Chicken kidney, with stones shown in ovals.

The residue data are presented in Tables 2 and 3. First, the residue levels of melamine increased with the feeding days from d 1 to 18 and from d 1 to 19 for 200 and 1,000 mg of melamine/kg of diet, respectively. Then, the concentration of melamine decreased slightly after feeding d 18 and 19 for 200 and 1,000 mg of melamine/kg of diet, respectively. Similarly, Lü et al. (2009) found that the melamine residue levels in the tissues were lower on d 42 compared with d 28 when graded levels of melamine in diets were fed to broiler chicken. They thought that broiler chickens may have developed more capacity to clear melamine from body tissues with advancing age. However, the decrease of feed intake was the main reason based on our investigation of feed intake because the remains of formula feed after d 18 increased. Moreover, the reason for the decrease of feed intake may be that the function of the kidney of laying hens was harmed by melamine and cyanuric acid; the stone appeared in all kidneys of 10 slaughtered laying hens (Figure 2). Second, melamine concentrations decreased rapidly in eggs after withdrawal. Nine days after withdrawal, the eggs contained only 0.25 and 0.23% of melamine of the concentration of 0 d withdrawal for 200 and 1,000 mg of melamine/kg of diet, respectively. Similar findings have been reported by Baynes et al. (2008) and Lü et al. (2009). Baynes et al. (2008) observed that melamine appears to be cleared rapidly (half-life = 4.07 ± 0.39 h) in the pig. Lü et al. (2009) observed that melamine residues in chicken tissues were depleted after a 7-d withdrawal period. Third, the limit of 2.5 mg/kg for melamine in food was proposed by the World Health Organization and accepted by many countries including the United States, the European Union, and China. The concentrations of melamine in eggs of laying hens at the withdrawal period of 0 d were lower than the limit for 200 mg of melamine/kg of diet. A withdrawal period of 4.0 d was required by calculation of the data (Table 3) with WT1.4 for 1,000 mg of melamine/kg of diet based on the limit.

The GC-MS method used in this work was effective in detecting the presence of melamine in eggs. The results presented in this study demonstrate an accumulation of melamine in eggs of laying hens. A 4.0-d withdrawal period was sufficient to obtain acceptably low residue levels (<2.5 mg/kg) for 1,000 mg of melamine/kg of diet; no withdrawal period was required for 200 mg of melamine/kg of diet.

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