Residues of cyromazine and its metabolite melamine in eggs of laying hens following consumption of cyromazine contaminated feed

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Abstract

Eggs were recently found to be contaminated with melamine in China. This study was aimed to determine if the cyromazine contaminated feed could metabolite to melamine in eggs. First, a HPLC method was developed to determine cyromazine and melamine residues simultaneously in eggs. The limit of detection and the limit of quantitation were all 0.2 μg/g. Second, a total of 72 laying hens were divided into four groups and were fed diets spiked with 0, 5, 15 or 50 mg of cyromazine per kg of feed for nineteen days, and then non-contaminated feed for next seven days. Eggs collected on days 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 23, 24 and 26 were analyzed for cyromazine and its metabolite melamine in whole eggs and eggs collected on odd days were analyzed in egg whites and egg yolks. This study illustrated that if only treating with cyromazine contaminated feed, there were no melamine residues in eggs. Following exposure to contaminated feed, cyromazine residue in eggs rapidly reached the maximum on the fourth day after drug administration. When administration was discontinued, elimination of cyromazine was quite fast, about less a week. In egg whites and egg yolks, cyromazine but no melamine was detected on the first day after administration. The residues of cyromazine in egg whites were always higher than egg yolks after administering with cyromazine for fifteen days. Since the seventeen day, the residues of cyromazine in egg yolks exceeded egg whites.

Key Words: Cyromazine; Metabolite Melamine; HPLC; Eggs; Egg Whites; Egg Yolks

Introduction

Cyromazine (Trigard or Larvadex; chemical name: N- cyclopropyl- 1, 3, 5- triazine- 2, 4, 6- triamine) (I, Figure 1) is an insect growth regulator used as a chitin synthesis inhibitor for fly control in cattle manure, field crops, vegetables and fruits (Thomson, 1994). The maximum residue levels (MRLs) of cyromazine are not to exceed 5.0 ppm in poultry feed, and the feeding of cyromazine- treated feed must stop at least 72 h before slaughter (EPA, 1999). It can metabolize via dealkylation reactions in both plants and animals and undergo environmental degradation to form melamine (Keiding, 1999; Sancho et al., 2005). The Ministry of Agriculture of China approved cyromazine as a new veterinary drug to be used in animal breeding in 2002. During animal breeding, cyromazine is mainly as cyromazine premix mixed into feed. Into animal body, majorities of cyromazine are excreted into the feces in the form of prototype and its metabolite melamine, thus killing maggots and fly in the stool. Cyromazine is slightly toxic by ingestion, with reported oral LD_{50} values of 3387 mg/kg in rats (Environmental Protection Agency, 2000).

Although cyromazine is slightly toxic, studies have reported that melamine (1, 3, 5- triazine-2, 4, 6-triamine) (II, Figure1), can bind with its analogues, such as cyanuric acid, to form crystals and then may induce significant renal toxicity and carcinogenic effects (Baynes et al., 2008). Due to a nitrogen rich compound, melamine could be illegally added into animal feeds or protein materials to fraudulently increase the apparent protein content. In October 26, 2008, fresh eggs which came from Dalian to Hongkong in China were found to contain excessive melamine, and the problem eggs contain 4.7 ppm for melamine (http://news.ifeng. com/ hongkong/200810/ 1026_19_ 846858.shtml).

Cyromazine and melamine residues have been determined at trace levels by gas chromatography (GC) (Toth and Bardalaye, 1987), by high performance liquid chromatography (HPLC) (Chou et al., 2003; Yokley et al., 2000), and by GC–mass spectrometry (MS) (Yokley et al., 2000). Among them, the HPLC method has already been tested and applied to a great variety of plant and animal substrates, and therefore, would normally be the method of choice.

Usually residues of melamine are in many cases of the same order as those of cyromazine. But, it is not clear whether high residues of cyromazine in eggs are always connected with high residues of melamine. Thus, for food safety and risk assessment purpose, it is essential to investigate the relationship between the cyromazine level in feed and the melamine level in animal-origin foods.

Materials and Methods

Materials and Reagents

Analytical standard of cyromazine and melamine were purchased from Sigma Chemical Co. (St Louis, MO, USA) with 99%+ purities. Ammonia hydroxide, hexane and sodium hydroxide were all analytical reagents. Acetonitrile and methanol (HPLC grade) were from TEDIA (USA).

Standard Solutions

Standard stock solutions were prepared as follows: Each 10.0 mg of cyromazine and melamine was weighted into a 100 ml brown volumetric flask and diluted with methanol to make stock solution (100.0μg/mL). Then, the stock solution was diluted with the mobile phase into a series of standard working solutions (0, 0.10, 0.50, 1.00, 2.00 and 5.00μg/mL). The standard solutions were stored at 4˚C and protected from light.

Apparatus and operating condition

HPLC separation was conducted using an Agilent 1100 Series high-performance liquid chromatograph. The column was a Hypersil NH_2 column (250mm×4.6mm i. d., 5μm particle sizes). The mobile phase containing acetonitrile and water (97:3, V: V) was used. The Solid-Phase Extraction column was an Oasis MCX solid-phase extraction (SPE) cartridge (60 mg, 3 mL, Thermo Scientific from American).

Rotavapor, Model BÜCHI R-200 (Switzerland); High-speed Tissue TearoMMr Homogenizer, Model DS-1 (Shanghai, China); Vortex Machine, Model XW-80A (Shanghai, China); Nitrogen Evaporation Equipment, Model N-EVAPTM112 (America); High-speed centrifuge, Model Biofuge (Germany).

Experiment design and Sampling

This experiment was carried out in the Animal house of Veterinary Institute of the Jiangsu Academy of Agricultural Science (Nanjing, China). Seventy-two HY-LINE VARIETY BROWN laying hens (22 weeks of age and 1.5 kg BW) were obtained from Lukou Laying Hen Farm in Nanjing, China. They were divided into four groups equally with a completely randomized block design experiment. There were six cages for each group, and each cage was composed of three hens. The hens of the four groups were fed a standard corn-soybean meal diet with feed contaminated with 0, 5, 15, or 50 mg/kg cyromazine respectively. The three levels of cyromazine contaminated feeds were obtained by premixing cyromazine with feed followed by further mixing in blender. For each level, the drug concentrations were determined by mixing nine individual eggs each day. During this study, the laying hens were given free access to water and feed. The room temperature was maintained between 20 ^{0}C and 25 ^{0}C with the relative humidity set at 60- 70%. The birds were provided with 16 h of light per day with an intensity of 50 lx.

The experiment lasted 26 days. The hens were given Cyromazine contaminated feeds for nineteen days. Different groups were treated with different Cyromazine dose. And from the twentieth day till the end of the study, the hens were fed non-contaminated feeds for seven days again. Eggs were collected daily during the study. Each egg was labeled indicating date, group and stored at 4 ^{0}C. The eggs collected on days 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 23, 24 and 26 were analyzed for cyromazine and its metabolite melamine concentration in whole eggs. And the eggs collected on days 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25 were analyzed for cyromazine and its metabolite melamine concentration in egg whites and egg yolks.

Analytical Procedures

For the whole eggs, the contents of the eggs from the same replicates were

combined into a 50 mL polypropylene centrifuge tube. For the egg yolks and whites, we separated them and used the same method with whole eggs. After sampling, they were homogenized with a glass-stick. Fifteen milliliters of 1% trichloroacetic acid solution and five milliliters acetonitrile were added to 50 mL polypropylene centrifuge tube containing 2.0 g of homogenate. The samples were capped, vigorously shaken for 30min. First, the sample was ultrasonically extracted for 10 min, and then centrifuged at 1,2000 rpm for 10 min at 4 ^{0}℃. The supernatant was transferred to a clean 50 mL polypropylene centrifuge tube. Five milliliters of saturated hexane was added, and the sample was mixed gently for 2min, and then centrifuged at 1, 2000 rpm for 10 min at 4 □. The hexanes and fat layer of up- layer was then removed. Finally, the extraction solution was subjected to solid phase extraction (SPE) column.

The disposable Oasis MCX SPE column (60 mg, 3 mL, Thermo Scientific, U.S.) was set up. The SPE column was prewashed with 3mL of methanol and 3mL of distilled water. The sample (10mL) was loaded at the speed of 0.5 mL/s and followed by a wash with 3mL distilled water and 3mL methanol. The washings were discarded. Analytes from the column were recovered by eluting with 4.0 mL of 5 % (V: V) ammonia methanol. The eluate was dried in a stream of nitrogen in a 45 ºC water bath and dissolved in 1 mL of methanol and mixed for 1min.

After 0.45 μm nylon syringe filters, an aliquot of 20 μL was injected into HPLC system equipped with a Hypersil NH_2 column (250 mm× 4.6 mm i. d., 5μm particle sizes). The mobile phase consisted of acetonitrile and water (97:3, V: V) pumped at 1.0mL/min. Chromatography was performed with UV-detection at 230 nm. Cyromazine and melamine-free eggs were also treated as described previously.

Recovery and precision Study

Blank eggs (2.00g) were used. Recoveries of cyromazine and melamine were determined by preparing three kinds of concentrations fortified with standard working solutions (10.0 μg/mL): 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg. Four replicates of samples at each level were analyzed. The recoveries at each concentration were calculated by means of the standard calibration curves with the peak area. The limit of detection (LOD) for this method, defined as the concentration at which the signal-to-noise ratio (measured from the injection of standard solutions containing cyromazine and melamine) was 3:1 (Titato and Lanças, 2006) The limit of quantitation (LOQ) is defined as the lowest concentration of analytes that can be determined with acceptable precision and accuracy (Boon, et al., 2006).

Results and Discussion

Method optimization

Under the selected chromatographic conditions, peak area and concentration of cyromazine and melamine standard solution in range of 0.1- 5.0 μg/mL were made linear. The regression equation and correlation coefficient respectively: y = 83.208x+ 3.6296, R^2= 0.9995 (cyromazine); y = 81.284x- 0.1681, R^2 = 0.9998 (melamine); x is the concentration of drug representatives (μg/mL); y represents the peak area. Results of recoveries were shown in Table 1. Recoveries of cyromazine and melamine at fortified levels of 0.5 μg/g、1.0 μg/g and 2.0 μg/g ranged from 71.86- 83.65% and

78.15- 81.53% respectively, with relative standard deviation (RSD) of 3.75%- 7.23%. The limit of detection was 0.2μg/g for cyromazine and melamine. And the limit of quantitation was 0.2μg/g for cyromazine and melamine.

The simultaneously extraction of cyromazine and its metabolite melamine was a modification method of our lab developed to extract them from eggs and chicken excretion (Wei Ruicheng, et al., 2008; Bao Hongduo, et al., 2010). Cyromazine and melamine are both highly polar basic compounds, they are normally difficult to obtain enough retention in a C_{18} column, so we choose a Hypersil NH_2 column. And the mobile phase we used consisted of acetonitrile and water (97:3, V: V) pumped at 1.0 mL/min. Cromatography was performed with UV- detection at 230 nm. These conditions were all agreed with the HPLC recommended by USDA for determining cyromazine and melamine (FSIS, 1991).

Deposition and depletion of cyromazine and its metabolite melamine in whole eggs

Deposition (for nineteen days) and depletion (for seven days) of cyromazine in whole eggs after oral administration of cyromazine in a dose of 5 mg/kg (group one), 15mg/kg (group two), and 50 mg/kg (group three) contaminated feeds were shown in Figure 2. No melamine was detected in eggs originating from the three treatment groups. Cyromazine residues in eggs was detectable on the first day after the beginning of administration, at the concentration of 0.05 ug/g (group two) and 1.30 ug/g (group three) respectively, but was not detected in group one, which indicated that there was rapid deposition of cyromazine in eggs. And then cyromazine residue in eggs increased rapidly to 0.33 ug/g (group one), 0.62 ug/g(group two) and 3.06 ug/g (group three) on the fourth day after drug administration, which demonstrated an apparent dose-response relationship. This was the same with the study of Yiqiang et al, 2010, the average melamine concentrations in eggs were 0.00 (below limit of detection), 0.16, 0.47, 0.84, and 1.48 mg/kg for the 0, 5, 25, 50, and 100 mg/kg treatment groups, respectively. Thereafter, cyromazine residues were relatively steady until the nineteen day administration.

Nearly all the residues data submitted to the (WHO/FAO) Joint Meeting on Pesticide Residues (JMPR) in 1990 were on both cyromazine and its main metabolite melamine. Residues of melamine were in many cases of the same order as those of cyromazine. But this study illustrated that if only treating with cyromazine, there were no melamine residues in eggs. This result was accord with the reference (China Inspection report, 2008).

When administration was discontinued on the nineteenth day, cyromazine residues in egg dropped rapidly from 2.25 ug/g on the twentieth day to 0.03 ug/g on the twenty-fifth day in group three, and the group one and group two were not detectable respectively on the twenty-second and twenty-third day post administration in any eggs (showed in Table 2). It is evident, therefore, elimination of cyromazine was quite fast in eggs.

It was worth noting that excess of cyromazine of l5mg/kg and 50mg/kg feeding for 19 days, eggs would not be detected with melamine. Therefore, adding normal amount of cyromazine was safe.

Deposition and depletion of cyromazine and its metabolite melamine in egg whites and egg yolks

The present study of our lab showed that melamine had different deposition profiles in egg whites and yolks (haven't been published). To obtain information on the distribution of cyromazine between the white and yolk, the group three (50 mg/kg) was selected. Egg white and yolk were separated and the residue content was determined. The results were shown in Figure 3. Cyromazine was detected out in the egg whites and egg yolks on the first day after administration, but its metabolite melamine was not detected out in either egg whites or egg yolks until the end of experiment. The residues of cyromazine in egg white were always higher than egg yolk after administering cyromazine for fifteen days. However, since seventeen day, the residues of cyromazine in egg yolk exceeded egg white. Cyromazine accumulated to a maximum of 3.08 ug/g at day 15 in white. And in yolk, the maximum concentration of cyromazine 3.05 ug/g was observed at day 19. During depletion of cyromazine, the dispelling rates of cyromazine in egg yolks were little slowly than egg whites. The first peak of cyromazine concentration was observed at the fifth day after administration. The peak gradually increased reaching its maximal height at the seventh day with 1.08 μg/g (egg yolks) and 1.24 μg/g (egg whites). Residues of cyromazine were detected until three days post treatment in yolks and two days in whites.

The distribution of veterinary drugs between egg whites and yolks has been reviewed (Cornelis and Michae, 2000; Furusawa, 2001). Residues of drugs appear first in egg white, at least when the drug is distributed towards that compartment. Although it depends strongly on the compound, yolk in general contains a higher residue concentration than egg white. Being relatively polar compounds, it is no surprise that cyromazine was predominantly accumulated more in the egg white initially. At the beginning of the treatment, a more equal distribution was observed in white, but rapidly the relative content of the egg yolk increased. Physicochemical properties of the drugs and the physiology of the hen and physiology of egg formation will determine how much drug will be deposited and at what place.

Conclusion

A HPLC method was developed for simultaneously determination of cyromazine and its metabolite melamine in eggs. This method was then employed to investigate the residues in eggs. The results clearly indicated there were no melamine residues in eggs, egg whites and egg yolks from the hens treated only with cyromazine contaminated feed.

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Table 1 Recoveries of cyromazine and melamine from spiked eggs (n=4)

Drug	Added concentration (μg/g)	Recoveries (%)	RSD(%)
Cyromazine	0	0	0
	0.5	71.86±5.20	7.23
	1.0	79.09±2.96	3.75
	2.0	83.65±3.61	4.31
Melamine	0	0	0
	0.5	78.15±4.52	5.79
	1.0	80.09±4.36	5.44
	2.0	81.53±3.65	4.48

Table 2 Concentrations of cyromazine in whole egg after discontinues giving cyromazine

Cyromazine level (mg/kg)	Concentrations of cyromazine (mg/kg)				
	Day 20	Day 22	Day 23	Day 24	Day 26
0	ND	ND	ND	ND	ND
5	0.12±0.04	ND	ND	ND	ND
15	0.39±0.05	0.21±0.06	0.03±0.06	ND	ND
50	2.25±0.31	0.23±0.33	0.12±0.10	0.03±0.01	ND

ND, not determined. Expressed as cyromazine concentration ± SD

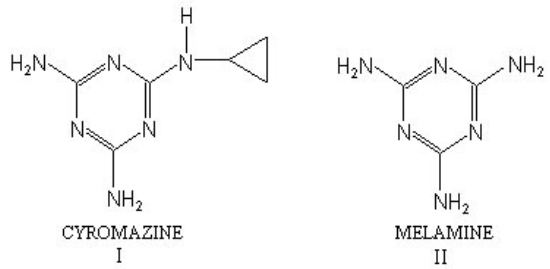

Figure 1 Cyromazine (I) and its major metabolite melamine (II).

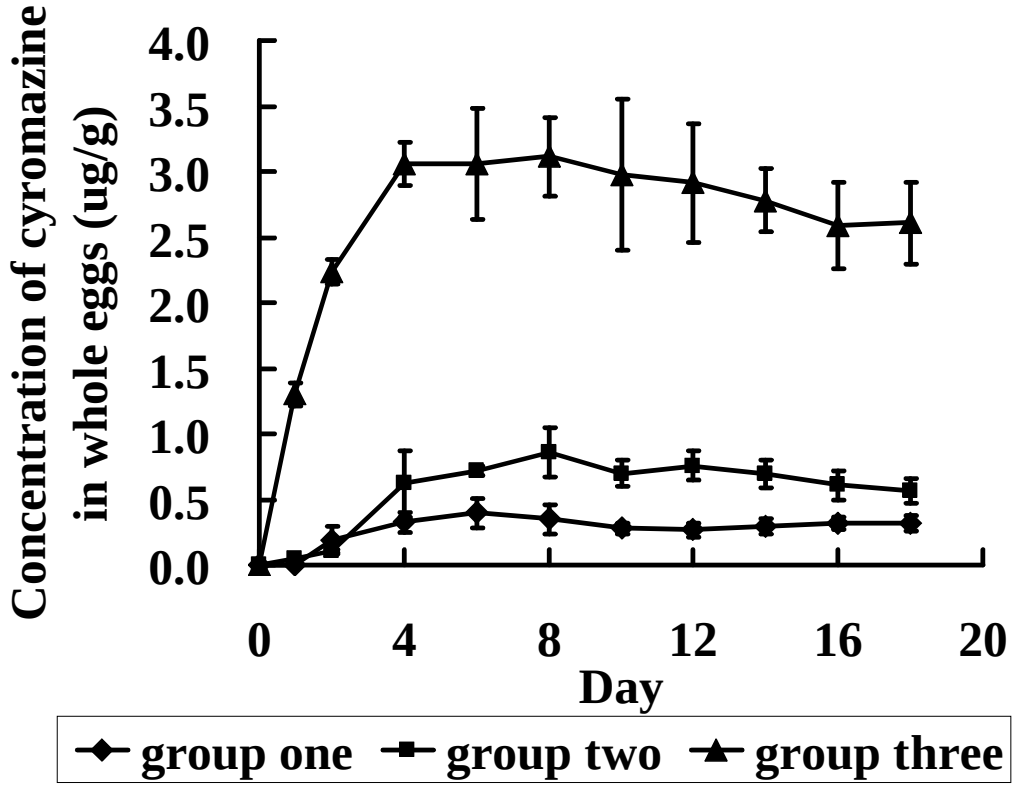

Figure 2 Deposition of cyromazine in whole eggs after oral administration of cyromazine in a dose of 5mg/kg (group one), 15mg/kg (group two), and 50 mg/kg (group three) contaminated feeds at days 0,1,2,4, 6, 8, 10, 12, 14, 16, 18.

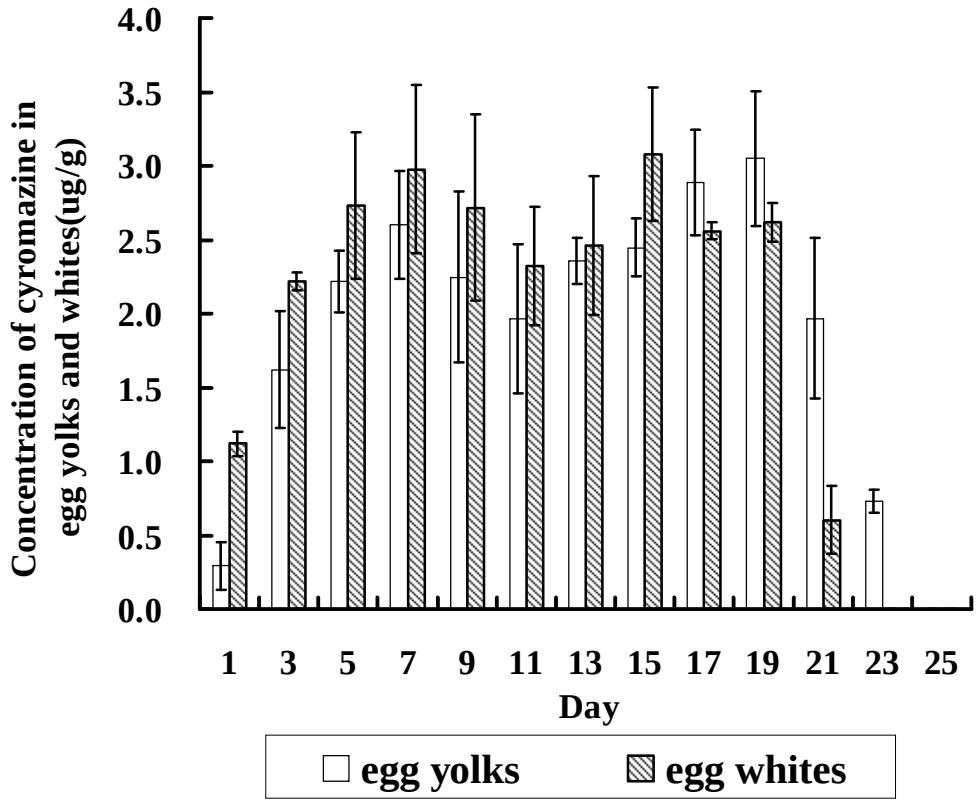

Figure 3 Deposition and depletion of cyromazine in egg whites and egg yolks after oral administration of cyromazine in a dose of 50 mg/kg contaminated feeds at days 0,1,3,5, 7, 9, 11, 13, 15, 17, 19 (deposition) and 21, 23, 25 (depletion).