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Determination of Residues of Cyromazine and Its Metabolite, Melamine, in Animal-Derived Food by Gas Chromatography−Mass Spectrometry with Derivatization

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A gas chromatography−mass spectrometric (GC-MS) method was established for the determination of cyromazine and its metabolite, melamine, in animal-derived food. Chicken and tilapia muscle samples were spiked with $^{15}N_3$-melamine, extracted with an acidic acetonitrile/water solution, and defatted with dichloromethane. Egg and milk samples were directly extracted with 3% trichloroacetic acid. The extracts were purified using mixed cation-exchange cartridges, derived with *N,O*-bis(trimethylsilyl)trifluoroacetamide, and detected by GC-MS. Cyromazine and melamine were quantified by external standard methods except for the determination of melamine in animal muscle, which used an internal standard method. Recoveries ranged from 75.0 to 110.0%, and relative standard deviations were <15.0%. In animal muscle the limits of quantification (LOQs) were 20 μg/kg and the limits of detection (LODs) were 10 μg/kg for cyromazine and melamine. In milk and eggs the LOQs were 10 μg/kg and the LODs were 5 μg/kg for both analytes.

KEYWORDS: Gas chromatography−mass spectrometry (GC-MS); animal-derived food; cyromazine; metabolite; melamine; residues

INTRODUCTION

Recently, melamine contamination of human food has become a public event. In the autumn of 2008, infant milk powder contaminated with melamine was found in China and was shortly followed by a report of eggs contaminated with melamine in Hong Kong. Around the same time, the U.S. Food and Drug Administration reported that trace amounts of melamine in infant milk powder had been detected. It is thought from these incidents that melamine was either intentionally added to increase the apparent protein content of the foods or acquired from the packaging. On the other hand, the source of melamine was proposed coming from animals dosed with the insecticide cyromazine, as melamine is a metabolite of it (*1*, *2*).

Cyromazine (*N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine) is an insect growth regulator used to control flies on animals and as a foliar spray to control leafminers on ornamental plants, fruits, and vegetables (*2*, *3*) (**Figure 1**). Cyromazine is also an insecticide approved for use in chickens by the Chinese Ministry of Agriculture administered as a feed-through larvicide incorporated into the feed of laying hens to prevent flies from hatching in the manure. It can be metabolized via dealkylation reactions in both plants and animals to form melamine (*2*−*4*).

Melamine is a triazine-based industrial chemical used in the manufacture of plastics, flame retardants and other products (**Figure 1**). When added to milk or feed, melamine increases the nitrogen concentration, providing a falsely high reading during protein concentration testing. Melamine in combination with cyanuric acid results in the formation of insoluble melamine cyanurate crystal deposits in the kidneys, causing renal failure in those who consume adulterated food (*1*, *2*). Therefore, it is important to be able to identify cyromazine and its metabolite, melamine, in different matrices where the pesticide may be used in the animals' environment. This may help to distinguish agricultural exposure from an intentional adulteration event.

Various analytical approaches of sample preparation and determination of melamine residue in biological samples have been published, including enzyme-linked immunosorbent assay (ELISA) (*5*), nuclear magnetic resonance (NMR) (*6*), high-performance liquid chromatography (HPLC) (*7*), liquid chromatography−tandem mass spectrometry (LC-MS/MS) (*8*−*12*), and gas chromatography−mass spectrometry (GC-MS) (*13*−*19*) methods. ELISA and NMR are often used for screening due to their high throughput. HPLC is not able to confirm the target analyte. To increase the confidence in reported results, mass spectrometry techniques are widely used to identify trace levels of organic residues and contaminants. Andersen et al. (*9*) investigated melamine and triazine in fish and shrimp by LC-MS/MS. Using the same apparatus, Filigenzi et al. (*10*) reported a method to determine melamine, ammeline, ammelide, and cyanuric acid in kidney tissue taken from post-mortem examination of animals suspected of dying from intoxication of the above compounds.

To date, however, few methods can simultaneously determine cyromazine and melamine. In 1987, Toth et al. (*14*) briefly reported an instrumental method for the analysis of cyromazine and melamine by GC-MS. Likewise, Sancho et al. (*3*) reported the

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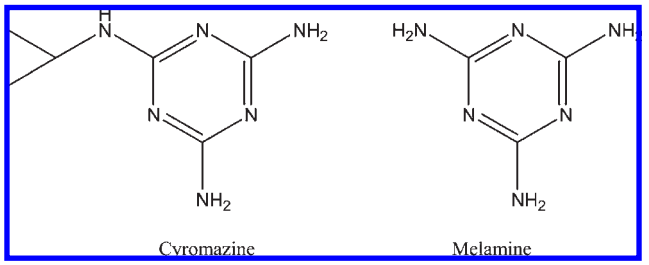

Figure 1. Chemical structures of cyromazine and melamine.

determination of cyromazine residue and its metabolite, melamine, in chard samples by ion-pair liquid chromatography coupled with electrospray tandem mass spectrometry. In 2000, Yokley et al. (*2*) reported an analytical method for the determination of cyromazine and melamine residues in soil using LC-UV and GC-MSD. The simultaneous determination and confirmation of cyromazine and melamine residues in food products such as chicken and tilapia muscle, milk, and eggs using a GC-MS method with derivatization has not yet been reported.

The present study developed a method for the detection of cyromazine and melamine in animal-derived food using GC-MS. First, method development was focused on the optimization of the cleanup procedure to decrease the interference of matrix before injection into the GC. Second, for tissue samples, an internal standard of $^{15}N_3$-melamine was added to account for low recoveries. Finally, lower limits of quantification (LOQs) were achieved by compared with the GC-MS method of the National Standards of the People's Republic of China (*20*). Especially, compared with the method reported by Toth et al. (*15*), this paper reports the use of the most common HP-5MS column. Therefore, the mass spectral properties of derivative cyromazine and melamine, especially the mass spectrum interpretation of cyromazine, are for the first time reported.

MATERIALS AND METHODS

Reagents and Materials. Cyromazine was purchased from Changzhou Zhineng Co. Ltd. (99.92% purity, Changzhou, China). Melamine was obtained from China Institute of Veterinary Drug Control (99.00% purity, Beijing, China). $^{15}N_3$-Melamine was purchased from Toronto Research Chemical Co. (98.00% purity, Toronto, Canada). *N*,*O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco Co. (Bellefonte, PA). HPLC-grade pyridine was purchased from Fisher Scientific Co. (Fair Lawn, NJ). All water used was deionized and purified to 18.2 MΩ-cm (Millipore, Bedford, MA). All other reagents were of analytical purity. Oasis mixed cation-exchange (MCX) solid phase extraction (SPE) cartridges (60 mg, 3 mL) were purchased from Waters Co. (Milford, MA).

Preparation of Standard Solutions. Stock solutions (1000 mg/L) of cyromazine, melamine, and $^{15}N_3$-melamine were prepared by individually dissolving 10 mg of each standard in 10 mL of a methanol/water (1:1 v/v) solution. Each solution was sonicated until crystals were no longer visible. The stock solutions were stored at −20 °C. The mixed working standard solution (1 mg/L) was prepared by combining aliquots of cyromazine and melamine stock solutions and diluting to volume in methanol. The same concentration of $^{15}N_3$-melamine working standard solution was prepared. The working standard solutions were stored at 4 °C.

Sample Preparation. For chicken and tilapia muscle, the homogenized sample (2.0 g) was weighed into a 50 mL round polypropylene centrifuge tube. To this was added 40 μL of $^{15}N_3$-melamine working standard solution and 25 mL of a 50:50 (v/v) solution of acetonitrile/water. The sample was vortex mixed for 30 s, shaken for 10 min, and then centrifuged at 10000 rpm for 8 min. An aliquot (12.5 mL) of the extract was transferred into a 50 mL polypropylene centrifuge tube, and 10 mL of dichloromethane was added and shaken for 5 min. The extract was then centrifuged for 5 min at 5000 rpm, the upper aqueous layer was carefully removed to tube A. Water (5.0 mL) was added to the dichloromethane

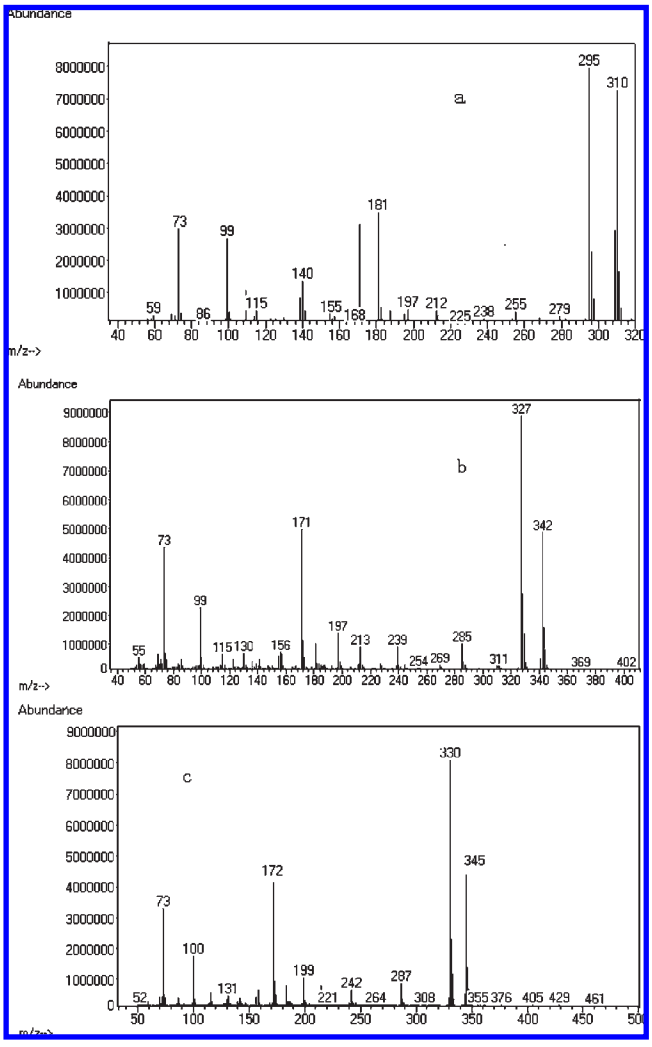

Figure 2. Full-scan spectra of the derivatives of (**a**) cyromazine, (**b**) melamine, and (**c**) $^{15}N_3$-melamine internal standard.

layer, and the same extraction procedure was repeated twice. The upper aqueous layer was removed and combined with the first aqueous extract in tube A. Finally, the extract in tube A was subjected to SPE.

For milk and egg samples, 2.0 g of homogenized sample was weighed into a 50 mL round polypropylene centrifuge tube. Ten milliliters of 3% (v/v) trichloroacetic acid was added, and the tube was vortex mixed for 30 s and shaken for 10 min. After centrifugation (10 min, 10000 rpm), the supernatant was subjected to SPE.

An Oasis MCX SPE cartridge was used to clean up the sample extract. The SPE cartridge was conditioned with 5 mL of methanol followed by 5 mL of water. The extracted sample solution was applied to the conditioned cartridge at 1−2 mL/min. The cartridge was then washed with 5 mL of 0.1 N HCl, followed by 5 mL of methanol, and dried by applying vacuum for 1 min. The sample was eluted into a glass tube using 3 mL of 5% (v/v) ammonium hydroxide in methanol to the column. The eluate was evaporated to dryness in a water bath at 50 °C.

To the dried extract were added 300 μL of pyridine and 100 μL of BSTFA, followed by mixing for 1 min with a vortex mixer and heating at 80 °C for 1 h. The sample was cooled to room temperature and injected into the GC column.

GC-MS Conditions. GC-MS confirmation analyses were performed using an Agilent model 6890 series gas chromatograph interfaced (capillary direct) to a 5973i mass detector (MSD), operated in the selected ion monitoring (SIM) mode. The ions of interest for each analyte were obtained after inspection of their full-scan mass spectra via electron ionization (EI) at 70 eV. The MSD transfer line was maintained at 280 °C. An HP-5MS column (0.25 mm i.d., 30 m, 0.25 μm film thickness) was employed for the separation. The temperature of the column was

programmed from 75 to 300 °C at 30 °C/min. Injections of 1 μL were made in the splitless mode, with an injection port temperature of 270 °C. The carrier gas was helium with a linear velocity of 1.3 mL/min.

Method Validation. The method was evaluated by spiking blank chicken and tilapia muscle samples (2 g, $n = 6$) at concentrations of 20, 40, and 80 μg/kg with mixed working standard solution. Egg and milk blank samples were each spiked at concentrations of 10, 20, and 40 μg/kg. These samples were analyzed using the method described above.

RESULTS AND DISCUSSION

GC-MS Analyses. Figure 2 shows the full-scan EI mass spectra of derivative molecules of cyromazine, melamine, and $^{15}N_3$-melamine. The spectrum of derivative cyromazine is given in **Figure 2a** and the MS interpretation in **Figure 3a**. The molecular ion was observed at m/z 310, where a base fragment peak was observed at m/z 295 ($M^+ - CH_3$). In addition, fragments at m/z 181 ($M^+ - CH_3 - C_3H_5N - Si(CH_3)_2$) and 99 ($M^+ - CN - Si(CH_3)_3 - C_3H_5N - Si(CH_3)_2$) were present. Monitoring ions were determined at m/z 310, 295, 181, and 99, and m/z 295 was the quantifying ion.

The mass spectrum and derivative molecule of melamine are shown in **Figures 2b** and **3b**, respectively. As previously reported (*20*), m/z 342, 327, 171, and 99 were selected as monitoring ions, and m/z 327 was selected as the quantifying ion.

The mass spectrum of the derivative molecule of $^{15}N_3$-melamine and its MS interpretation are shown in **Figures 2c** and **3c**, respectively. As **Figure 2c** shows, m/z 345 is the molecular ion, and the abundant fragment ion at m/z 330 is ascribed to the molecular ion losing a CH_3 group. For the derivative of $^{15}N_3$-melamine, three nitrogen atoms on the heterocyclic ring amidogen are substituted by ^{15}N, and therefore its base peak is m/z 330, and the m/z 327 fragment ion is no longer visible. From the structural formula and the mass spectrum, fragment ions of m/z 342, 181, and 99 are not apparent and, therefore, do not interfere with the determination of melamine. It was on this basis that m/z 330 was selected as the quantifying ion of the internal standard.

Total ion chromatograms of spiked samples of cyromazine, melamine, and $^{15}N_3$-melamine are shown in **Figure 4**, and retention times t_R were 7.48, 7.07, and 7.07 min, respectively.

Optimization of the Sample Treatment Procedure. For chicken and tilapia muscle samples, the extraction method for the determination of melamine in catfish muscle reported by Andersen et al. (*9*) was improved. The $^{15}N_3$-melamine internal standard was added to chicken and tilapia muscle. The samples were extracted and then subjected to purification using MCX cartridges. The isotope-labeled analogue of melamine was used to compensate for recovery loss of melamine because the recovery was typically below 50% for animal muscle. For egg and milk samples, the extract method for the determination of melamine in raw milk (*20*) was improved. Samples were extracted using 3% trichloroacetic acid and then purified by MCX cartridges. This extraction procedure gave adequate recoveries of melamine and

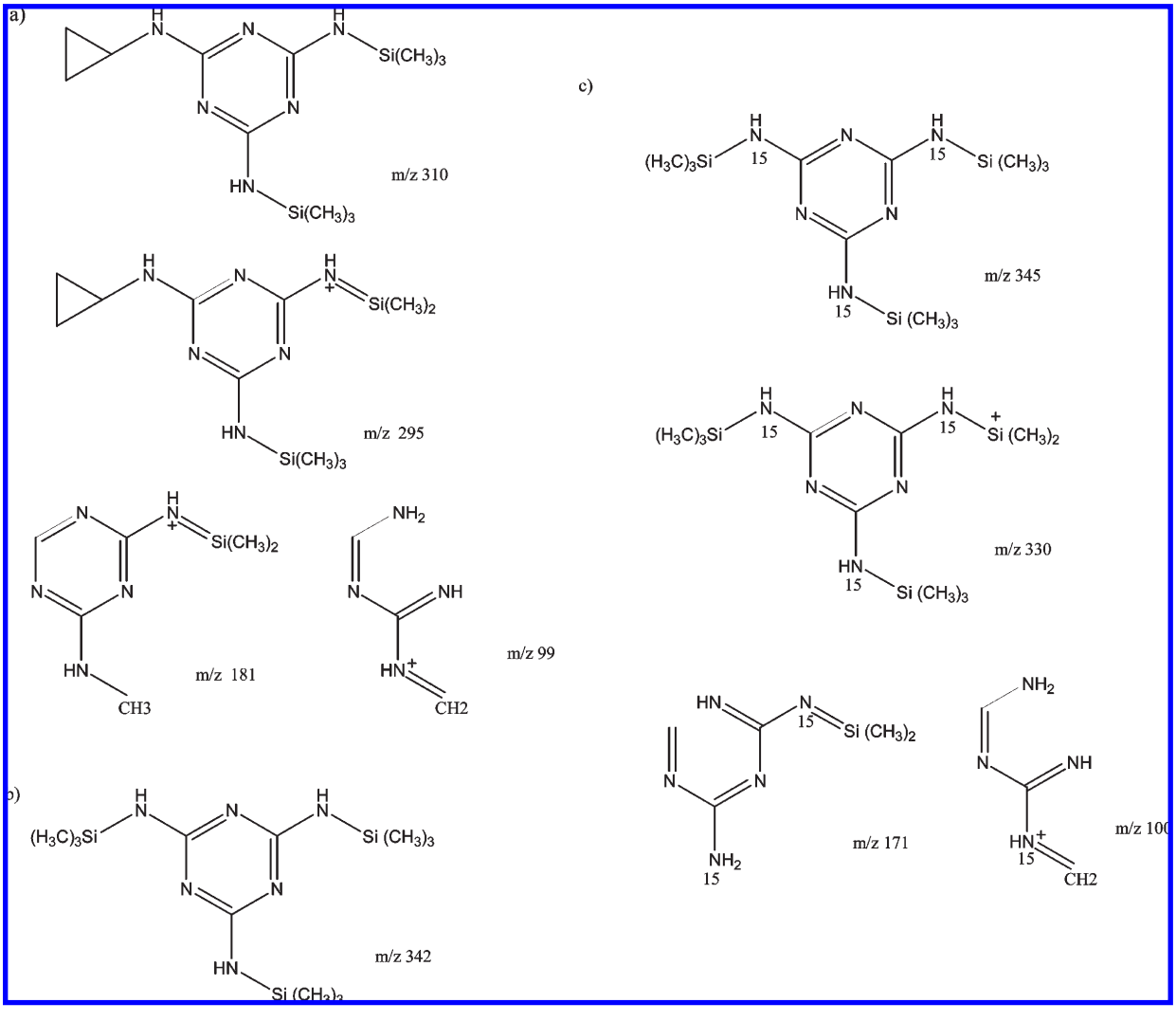

Figure 3. Structures of derivatives and/or MS interpretation of (**a**) cyromazine, (**b**) melamine, and (**c**) $^{15}N_3$-melamine internal standard.

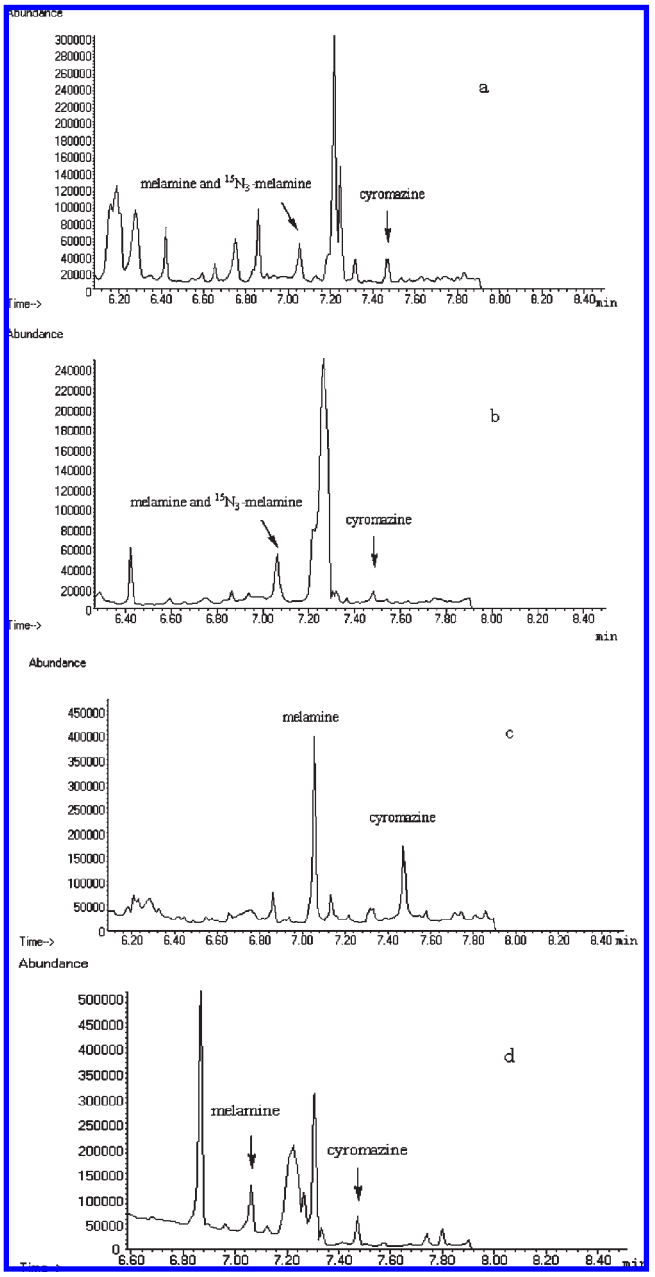

Figure 4. Total ion current chromatograms of cyromazine, melamine, and $^{15}N_3$-melamine internal standard spiked at 40 μg/kg in (**a**) chicken, (**b**) tilapia, (**c**) milk, and (**d**) egg samples.

cyromazine and, consequently, an internal standard was not added. Compared with the procedure of the National Standards of the People's Republic of China for the determination of melamine in raw milk, the current study avoided the use of poisonous lead acetate solution and/or acetonitrile for sample preparation and, therefore, was better for the environment. Furthermore, the use of SPE for purification provided much cleaner extraction samples than those obtained by direct derivatization (*16*) and resulted in lower limits of quantification (LOQs).

Calibration Graphs and Method Validation. To compensate for compound loss during sample preparation, matrix-matched standard curves ranging from 50 to 1000 μg/L were prepared. The calibration curves for detection of melamine in chicken and tilapia muscle were derived by using the ratio of peak areas of melamine to isotope-labeled melamine against melamine concentrations. The calibration curves for detection of melamine and cyromazine in milk and eggs and for the detection of cyromazine in animal muscle were obtained using the peak areas of target compounds against concentrations. These analyses showed that good linearity was obtained for the two analytes with correlation coefficients of $r^2 > 0.995$ (**Table 1**).

Table 1. Matrix-Spiked Standard Curves of Cyromazine and Melamine

matrix	compound	calibration graph[a]	r^2
chicken	cyromazine	$y = 729x - 102$	0.995
	melamine	$y = 0.0201x + 0.00659$	1.000
tilapia	cyromazine	$y = 2129x + 487.5$	0.998
	melamine	$y = 0.0154x + 0.00488$	0.999
milk	cyromazine	$y = 1995x + 487.5$	0.999
	melamine	$y = 3556x + 909$	0.997
eggs	cyromazine	$y = 8334x + 4291.5$	0.999
	melamine	$y = 14246x + 420.5\ 5$	0.998

[a] $y = ax + b$, where y is the area, a is the slope, x is the concentration, and b is the y-intercept.

The recovery of analytes using this procedure was evaluated by spiking mixed working standard solution to blank samples at three levels in replicates of six. **Figure 4** shows the chromatograms of different matrices spiked at 40 μg/kg. The results are listed in **Table 2**, which shows that the average recovery of cyromazine and melamine ranged from 75.0 to 110.0%. The reproducibility of this method was represented by the relative standard deviation (RSD) at the spiked level for each compound, and these values are also summarized in **Table 2**. For each analyte, the within- and between-day reproducibilities were determined by testing six replicates independently, with samples extracted at levels of 20, 40, and 80 μg/kg for animal muscle and at 10, 20, and 40 μg/kg for milk and eggs. The within-day reproducibilities ranged from 1.5 to 13.0%, and the between-day reproducibilities ranged from 6.4 to 14.0%. In milk and eggs, the limits of detection (LODs), defined as the concentration that yields an S/N equal to 3, were 5 μg/kg for cyromazine and melamine; the LOQs, defined as the concentration that yields an S/N equal to 10, were 10 μg/kg for both analytes. In animal muscle, the LODs were 10 μg/kg and the LOQs were 20 μg/kg for cyromazine and melamine, which are lower than the LOQs of the GC-MS method of the National Standards of the People's Republic of China (*20*).

Sample Analysis. The newly developed method was applied to the analysis of market-ready samples. Samples of 10 kinds of chicken and tilapia muscle, eggs, and milk that were commercially available from the market in Beijing in September 2008 were assayed. **Figure 5** shows the total ion chromatograms for these samples. Melamine in the egg and milk samples were detected at 14.8 and 171.2 μg/kg, respectively, below the maximum residue limit (MRL) of 2.5 mg/kg set by the Chinese Administration of Quality Supervision, Inspection and Quarantine (AQSIQ) (*20*). The MRL is the maximum amount of a residue of a veterinary drug that can be in a specific animal tissue (muscle, fat, liver, etc.). The reasons for the detection of melamine are as follows: First, milk products were most probably manufactured using ingredients made from melamine-contaminated milk. Second, milk and eggs are likely contaminated through animal feed tainted with melamine. Third, cyromazine is an insecticide approved for use in chickens and for use in the growth of various vegetables in China. Melamine is a metabolite of cyromazine. Epstein et al. (*21*) reported that cyromazine and melamine metabolite residues can be detected in cyromazine-dosed cattle and are related to the amount of cyromazine administered to the cattle. It appears to be plausible that milk and eggs from animals exposed to cyromazine may contain melamine. Further studies are needed to confirm

Table 2. Recoveries and RSDs of Spiked Blank Samples (n = 5)

			% mean recovery within-day (RSD%)			
matrix	compound	spiked (μg/kg)	first day	second day	third day	% mean recovery between-days (RSD%)
chicken	cyromazine	20	80.8 (6.6)	87.2 (5.9)	105.0 (3.5)	90.9 (14.0)
		40	80.9 (5.2)	95.8 (8.3)	105.0 (4.5)	93.8 (13.0)
		80	75.0 (2.7)	78.9 (8.6)	95.6 (2.8)	83.2 (11.0)
	melamine	20	106.0 (3.8)	95.0 (5.2)	107.0 (3.5)	103.0 (6.5)
		40	84.8 (6.5)	105.0 (1.5)	108.0 (5.7)	99.4 (12.0)
		80	75.2 (5.5)	83.1 (5.5)	82.3 (1.7)	80.2 (6.4)
tilapia	cyromazine	20	92.0 (6.4)	96.6 (8.3)	78.0 (8.5)	88.8 (11.0)
		40	102.0 (3.7)	85.0 (4.2)	84.3 (11.0)	90.4 (11.0)
		80	78.1 (6.2)	87.3 (5.2)	89.3 (8.0)	71.6 (8.0)
	melamine	20	106.0 (3.8)	95.0 (5.2)	107.0 (3.5)	103.0 (6.5)
		40	84.8 (6.5)	105.0 (1.5)	108.0 (5.7)	99.4 (12.0)
		80	75.2 (5.5)	83.1 (5.5)	82.3 (1.7)	80.2 (6.6)
milk	cyromazine	10	86.1 (13)	105.0 (3.3)	109.0 (3.8)	100.0 (13.0)
		20	88.2 (5.6)	95.9 (6.8)	101.1 (5.3)	95.7 (8.4)
		40	91.5 (3.6)	96.9 (4.1)	103.2 (7.3)	96.8 (6.4)
	melamine	10	92.7 (4.2)	98.1 (4.6)	94.6 (4.9)	92.1 (7.5)
		20	85.6 (6.8)	92.3 (4.5)	90.0 (5.5)	97.1 (7.2)
		40	88.1 (8.0)	95.2 (4.6)	83.6 (3.8)	96.1 (9.1)
eggs	cyromazine	10	88.5 (7.5)	101.2 (5.3)	90.9 (4.8)	101.1 (10.1)
		20	98.1 (7.6)	85.9 (6.6)	99.8 (5.2)	92.5 (7.6)
		40	91.5 (6.1)	90.2 (5.8)	81.1 (4.6)	89.8 (8.0)
	melamine	10	96.2 (8.2)	102.0 (5.3)	100.1 (6.8)	95.0 (8.2)
		20	88.2 (4.6)	97.3 (5.0)	93.8 (8.2)	92.1 (7.4)
		40	81.6 (6.4)	85.9 (5.9)	81.5 (6.3)	86.8 (9.0)

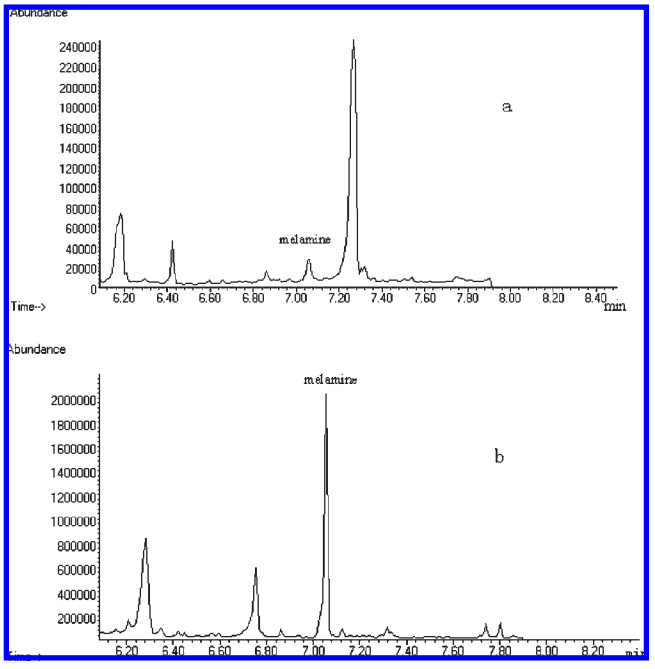

Figure 5. Total ion current chromatograms of melamine in (**a**) egg and (**b**) milk samples.

these results. Finally, melamine has been shown to migrate into food samples from melamine−formaldehyde plastic ware. Epstein et al. (*21*) proposed that melamine found in canned beef muscle tissue from both control animals and those dosed with the insecticide cyromazine was due to the melamine−formaldehyde resin linings in the cans. However, our studies, in which cans from the milk containing melamine were extracted with various solvents, did not provide any indication that melamine was present in the packaging. Therefore, it does not appear that the melamine detected in the products analyzed in this study originated from the packaging.

Therefore, the method described in this paper provides an important tool to monitor violative residues present in animal-derived foods and to prevent them from entering the human food supply. These studies provide crucial information to the Chinese Ministry of Agriculture for the scrutiny of food products that could contain cyromazine and melamine residues.

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