High-Performance Liquid Chromatographic Method for the Determination of Cyromazine and Melamine Residues in Milk and Pork

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Abstract

A high-performance liquid chromatography method is described in this paper. The method uses NH_2 column and 97% acetonitrile eluate to determine the insecticide cyromazine and metabolite melamine residues in milk and pork. Samples were treated with NaOH and extracted with acetonitrile containing 20% NH_4OH. Target analytes of samples were cleaned up and concentrated by C_{18} column solid-phase extraction. A separation for cyromazine and melamine was achieved, and respective retention times were 8 and 12 min. The calibration curves for cyromazine and melamine were linear in a concentration range of 0.01–1.0 µg/mL, with correlation coefficients of 0.9999 and 0.9997, respectively. The limit of detection of both compounds was 0.2 ng, and the limit of quantitation was 0.02 mg/kg. Recoveries of cyromazine and melamine at fortified levels of 0.02, 0.05, and 0.1 mg/kg ranged from 84.5–90.8%, and 83.6–91.3%, respectively, with coefficient of variation of 3.1–7.8%.

Introduction

Cyromazine (*N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine) (Figure 1A), named Larvadex, is an insecticide produced by Syngenta Co., belonging to insect growth regulator of triazine. It is effective in preventing *Diptera* and some kinds of *Coleoptera* larvas from hatching and eclosion. It has been administered as a feed-through larvicide since 1975 in America and was introduced to China in 1994. Nowadays, cyromazine is widely added to animal feed to prevent flies from hatching in the manure, so as to improve the environmental sanitation in domestic animal fields. The widespread use of these agents in food-producing animals raises the possibility of residues remaining in edible tissue after slaughter.

Although cyromazine is slightly toxic by ingestion in rats (1), studies have reported that melamine (1,3,5-triazine-2,4,6-triamine) (Figure 1B), a potential carcinogen (2) which is a degradation product or metabolite of cyromazine and is used as industrial chemical, caused bladder tumors in mice (3).

In 2007, pet feed from China with increased melamine caused American cats' and dogs' illnesses and deaths from liver or kidney failure; this induced people to pay attention to melamine once again. Melamine decomposes cyanuric acid and ammonia in water. Recent experiments have shown that cyanuric acid combined with melamine and cat urine forms crystals that can block the tubes leading out of the kidney. This is probably what is killing the cats. But there is no reason to prove that melamine is not toxic to humans.

The tolerance of cyromazine and melamine is 0.05 mg/kg each in edible livestock products in the USA and EU. In 2001, the Chinese Agriculture Department also established a maximum residue level of 0.05 mg/kg of cyromazine in poultry tissue.

Cyromazine and melamine residues have been determined at trace levels by gas chromatography (GC) (4–6), by high-performance liquid chromatography (HPLC) (7–9), and by GC–mass spectrometry (MS) (6,8). 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. Carbras et al. (7) compared RP-CB_{18} and RP-C_8 columns with acetonitrile–0.5 M sulfuric acid (in different ratios) solvent in analyzing cyromazine and melamine using HPLC. Chou et al. (10) reported that HPLC determined cyromazine and melamine in eggs and poultry meats with NH_2 column and acetonitrile–H_2O (75:25, v/v) solvent, which was a laborious method that consumed a large volume of high purity solvents. It involved duplicate sample preparation, a liquid–liquid partition in separatory funnel, triplicate evaporation under vacuum pressure, and solid-phase extraction

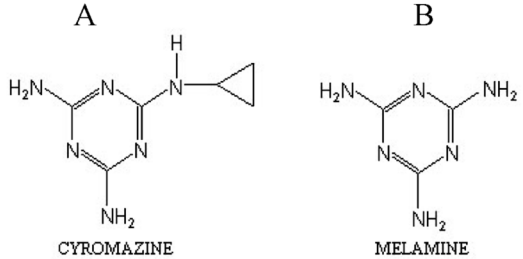

Figure 1. Cyromazine (A) and its major metabolite melamine (B).

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(SPE). Using this technique, it was necessary to perform more clean-up steps to decrease interferences and consume large volumes of high purity solvents, as well as preconcentrating extracts in order to obtain adequate detection levels.

This paper presents a highly sensitive and economical HPLC method for the determination of cyromazine and melamine residues in milk and pork (which can be used in kinetics and metabolism studies) and a routine monitoring program.

Experimental

Reagents

Analytical standards of cyromazine and melamine were purchased from Sigma Chemical Co. (St. Louis, MO) with 99% purity. Ammonia hydroxide, hexane, and sodium hydroxide were of analytical reagent grade. Acetonitrile and methanol (HPLC-grade) were from Tedia Company Inc. (Fairfield, OH).

Apparatus and operating condition

The HPLC system consisted of an Agilent 1100 Series LC (Palo Alto, CA) equipped with a variable wavelength detector (VWD) and a 20-µL injection loop. The system was controlled through a system controller and a personal computer using an Agilent ChemStation with a data processing system installed on it. The column was a Hypersil NH_2 column (250 mm × 4.6 mm i.d., 5 µm particle size). The mobile phase consisted of acetonitrile and water (97:3). The flow rate was 1.0 mL/min. The column effluent was monitored at 214 nm with VWD. The SPE column was an AccuBONDII ODS-C_{18} Cartridges (500 mg, 6 mL) from Agilent.

Preparation of standard solutions

Standard stock solutions were prepared as follows: 10.0 mg each of cyromazine and melamine was weighed into a 100-mL brown volumetric flask and diluted with methanol to make a stock solution (100.0 µg/mL). Then, the stock solution was diluted with the mobile phase into a series of standard working solutions (0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 µg/mL). All solutions were stored at 4ºC and protected from light. Each level was injected into the HPLC system four times. A certain average peak area was regressed with a certain level to calculate the calibration equation.

Sample preparation and analytical procedures

Milk samples. Milk (2.0 g) was added into 50-mL centrifuge tubes with 0.2 mL 1 M NaOH and 10.0 mL ammoniacal acetonitrile extraction containing 20% concentrated NH_4OH. The whole solution was homogenized for 30 s with the vortex machine, and then vibrated for 10 min with an oscillator at the maximum speed; finally, it was transferred to middle speed for 30 min. The supernatant was moved after centrifugation for 10 min at 10,000 *g* (4ºC). This procedure was performed in duplicate and the organic extracts were combined.

Pork samples. The sample preparation for pork was similar to the procedure previously described. The only difference comes from the homogenizing procedure. The tissues containing buffer and extraction were homogenized by Homogenizer at 18,000 rpm for 2 min.

The combined organic extract was evaporated by rotary evaporator in a 45ºC water bath, and then washed three times with 6 mL (3 × 2 mL) acetonitrile in test tubes. In order to degrease and decolorize the solution, a 5 mL volume of hexane was added to each test tube and stirred with the vortex mixer for 1 min (only tissues). After centrifugation (2,000 *g* for 10 min at 4ºC), the upper hexane phase was discarded. The lower acetonitrile phase was dried in a stream of nitrogen and made up to 1 mL as acetonitrile extract. The disposable SPE column, AccuBONDII ODS-C_{18}, was set up. The SPE column was prewashed with 5 mL of acetonitrile. Acetonitrile extract was passed through the column. The column was washed with 5 mL of acetonitrile and washings were discarded. Analytes from the column were recovered by eluting with 5.0 mL of ammoniacal acetonitrile containing 20% concentrated NH_4OH. The eluate was dried in a stream of nitrogen in a 45ºC water bath and dissolved in 1 mL of the mobile phase. Solutions were filtered through 0.22-µm filter units, and 20 µL was used for HPLC analysis. Cyromazine and melamine-free milk and pork samples were also treated as described previously.

Recovery and precision

Standard working solutions (10.0 µg/mL) of cyromazine and melamine were prepared by diluting 1 mL of stock solution (100.0 µg/mL) with 9 mL of methanol. Recoveries of cyromazine and melamine from milk and pork were determined by preparing three kinds of milk and pork concentrations fortified with standard working solutions (10.0 µg/mL): 0.02 mg/kg, 0.05 mg/kg, and 0.1 0mg/kg. The tubes were put still for 10–15 min and then managed as described in the "Sample preparation and analytical procedures" section. Four replicates of samples at each level were analyzed. The recoveries of two compounds from milk and pork 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 (11).

The limit of quantitation (LOQ) is defined as the lowest concentration of analytes that can be determined with acceptable precision and accuracy (12).

Results and Discussion

Optimization of sample treatment

In choosing extracts, this experiment compared the extraction effects achieved by acetonitrile, ethyl acetate, and ammoniacal acetonitrile containing 20% concentrated NH_4OH. Extractions with acetonitrile and ethyl acetate were found to be of lower recovery or to have interference with other peaks, even after further purification. Because cyromazine and melamine contain three amines in their chemical structures, which has a larger solubility in alkaline solution, ammoniacal acetonitrile extraction containing 20% concentrated NH_4OH

provided optimum extraction efficiency for high recoveries and easily separated them from interference substances in these experiments.

After high-speed centrifugation, the milk sample extract was divided into three levels: the lower level was the precipitation, the middle level was the fat, and the upper level was the supernatant. The fat level was extremely thin. Low temperature centrifugation created mist on the tube's outer layer, which caused difficulty in observing the middle level. So the quota shift supernatant may avoid this level to disturb next step works.

Ammoniacal acetonitrile is a kind of medium polarity solvent, which could extract lipoid and pigments along with valid organic extract. So after the evaporation procedure, lipoid and pigments have to be removed, especially for pork. Therefore, hexane was applied in this experiment to degrease lots of fat and decolorize to make the solution clear, which is in favor of purification. This degreasing method saved reagent supply and avoided transfer loss using separatory funnels.

Optimization of chromatographic conditions

Several types of columns (including RP-18, RP-8, C_{18}, NH_2, and SCX) and several mobile phases have been reported for use in HPLC to determine cyromazine and melamine. The retention times of cyromazine and melamine were less than 4 min using RP-C_{18} and RP-C_8 columns with acetonitrile–0.5 M sulfuric acid solvent, and increasing the acetonitrile ratio could not raise the retention time up to more than 4 min. Therefore, Chou et al. suggested that RP-C_{18} and RP-C_8 columns with acetonitrile–0.5 M sulfuric acid solvent were not suitable in analyzing cyromazine and melamine residues in eggs and meats because of serious interference problems. But an ion-pairing LC mechanism using a C_{18} stationary phase could improve the retention of this very hydrophilic compound (13).

On the other hand, the ionization of both compounds in alkaline solution increased the hydrophilic ability, so the retention times of cyromazine and melamine were extended when eluted with alkaline buffer on a reversed-phase column. But Chou et al. (10) thought that the high pH (> 7.5) in the eluent might destroy the stability of the resin and shorten the column life.

Therefore, the NH_2 column (200 × 4.6 mm i.d.) with acetonitrile–H_2O (75:25, v/v) solvent in analyzing cyromazine and melamine was selected in the experiment. The separation effects were much better with few interfering peaks. But the peak shape was not good enough because the retention time was 4.6 min, much closer to interfering peaks. After changing the acetonitrile ratio in the mobile phase, a better separation for cyromazine and melamine was achieved with acetonitrile–H_2O (97:3, v/v) solvent. Their respective retention times were 8 and 12 min.

The SPE C_{18} column was effectively used to separate and concentrate cyromazine and melamine from the samples. This was consistent with the reports (6–8,10).

Most screening chromatographic assays have employed UV detection (214 nm) for cyromazine and melamine (7,8,10). Before acetonitrile extract was passed through the SPE column, the interferences were observed with endogenous compounds from the milk and pork samples at 214 nm. But fewer background residues of target analytes were observed in matrix samples treated by the SPE column.

Response linearity

The calibration curves for the determination of cyromazine and melamine in milk and pork were linear with concentrations of 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 µg/mL. The linear regression equation and correlation coefficients were $Y = 236.29X + 0.0381$ ($r = 0.9999$) for cyromazine and $Y = 200.03X - 0.7654$ ($r = 0.9997$) for melamine. The correlation coefficients were satisfactory and indicated that these methods were dependable for quantitative detection.

Liquid chromatograms and retention times

Because the column thermostat was not used, the retention times of cyromazine and melamine were approximately 8 min and 12 min under the chromatographic conditions in the 20ºC to 25ºC range with relative standard deviations (RSD) of 1.64 and 3.55, respectively. The chromatograms are appreciable because both peaks could clearly separate from the interfering peaks (Figure 2).

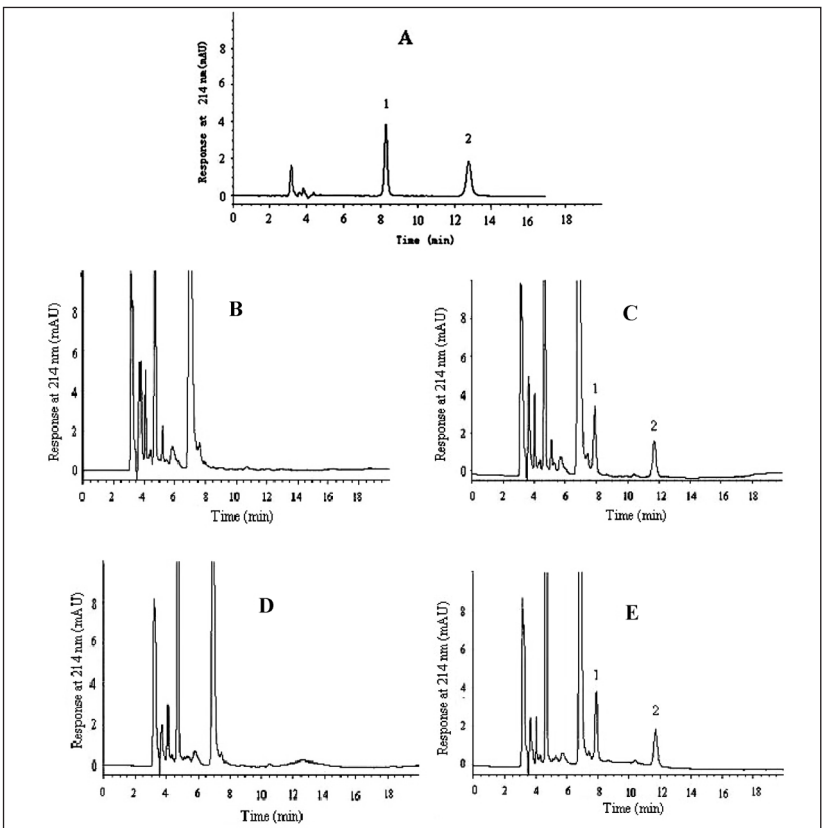

Figure 2. Chromatogram obtained from HPLC–VWD analysis of standard solutions of cyromazine and melamine (A); blank pork sample (B); pork sample spiked at 0.05 mg/kg with cyromazine and melamine (C); blank milk sample (D); milk sample spiked at 0.05 mg/kg with cyromazine and melamine (E). Peaks: cyromazine, 1; melamine, 2.

Recovery and sensitivity

Equation 1 can be used to calculate the content of target analytes in samples. Where "*X*" represents the content of cyromazine or melamine in the samples (µg/kg), "*c*" is the content of cyromazine or melamine calculated through regression equation (µg/mL), "*V*" is the final volume of sample (mL), "*m*" is the sample's weight (g), and "1000" is the unit converter coefficient.

$$X = \frac{c \times V \times 1000}{m \times 1000} \qquad \text{Eq. 1}$$

Tables I and II summarize the recoveries and coefficients of variation of the control samples and incurred milk and pork for cyromazine and melamine. At the ranges of 0.02 mg/kg ~0.1 mg/kg, the recoveries of cyromazine in milk ranged from 84.5% to 90.8% with coefficients of variation ranging from 3.1% to 4.4%, while the recoveries of cyromazine in pork ranged from 85.2% to 89.6% with a coefficients of variation ranging from 4.6% to 5.2%. As shown in Table II, samples of milk and pork fortified with melamine in the range of 0.02 mg/kg ~0.1 mg/kg have recoveries of more than 83.6% and coefficients of variation ranging from 5.6% to 7.8%. The LOD of the method for two kinds of samples was 0.2 ng, while the LOQ was 0.02 mg/kg.

Table I. Recoveries of Cyromazine from Spiked Milk and Pork*

Sample	Fortified concentration (mg/kg)	Determined concentration (mg/kg)	Recoveries (%)	Coefficients of variation (%)
Milk	0	–	–	–
	0.02	0.0169 ± 0.0005	84.5 ± 2.62	3.1
	0.05	0.0431 ± 0.0019	86.2 ± 3.79	4.4
	0.10	0.0908 ± 0.0029	90.8 ± 2.91	3.2
Pork	0	–	–	–
	0.02	0.0172 ± 0.0009	86.0 ± 4.47	5.2
	0.05	0.0426 ± 0.0020	85.2 ± 4.09	4.8
	0.10	0.0896 ± 0.0041	89.6 ± 4.12	4.6

* Mean ± SD; $n = 4$.

Table II. Recoveries of Melamine from Spiked Milk and Pork*

Sample	Fortified concentration (mg/kg)	Determined concentration (mg/kg)	Recoveries (%)	Coefficients of variation (%)
Milk	0	–	–	–
	0.02	0.0173 ± 0.0010	86.5 ± 4.84	5.6
	0.05	0.0418 ± 0.0030	83.6 ± 6.02	7.2
	0.10	0.0873 ± 0.0055	87.3 ± 5.50	6.3
Pork	0	–	–	–
	0.02	0.0178 ± 0.0011	89.0 ± 5.52	6.2
	0.05	0.0433 ± 0.0031	86.6 ± 6.24	7.2
	0.10	0.0913 ± 0.0071	91.3 ± 7.12	7.8

* Mean ± SD; $n = 4$.

Conclusion

The described HPLC method was specific, reliable, and sufficiently sensitive for the analysis of cyromazine and melamine residues in milk and pork. The sample preparation, extraction procedure, and the established chromatographic conditions were valid and precise for determination of cyromazine and melamine residues in a short time. Therefore, the described methods are appreciable in monitoring cyromazine and melamine residues in livestock products.

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References

1. A. Sergio, Q. Guillermo, G. Salvador, et al. Determination of cyromazine in pesticide commercial formulations by vibrational spectrometric procedures. *Anal. Chim. Acta* **524:** 257–64 (2004).
2. R.L. Melnick, G.A. Boorman, J.K. Haseman, et al. Urolithiasis and bladder carcinogenicity of melamine in rodents. *Toxicol. Appl. Pharmacol.* **72:** 292–303 (1984).
3. Z.Y. Ning. A new veterinary drug—cyromazine. *Veterinary Pharmaceuticals & Feed Additives* **4:** 18–19 (2001).
4. P.C. Bardalaye, W.B. Wheeler, and C.W. Meister. Gas chromatographic determination of cyromazine and its degradation product, melanine, in Chinese cabbage. *J. Assoc. Off. Anal. Chem.* **70:** 455–57 (1987).
5. H. Ishimwata, T. Inoue, and A. Tanimura. Migration of melamine and formaldehyde from tableware made of melamine resin. *Food Addit. Contam.* **3:** 63–70 (1986).
6. J.P. Toth and P.C. Bardalaye. Capillary gas chromatographic separation and mass spectrometric detection of cyromazine and its metabolite melamine. *J. Chromatogr.* **408:** 335–40 (1987).
7. P. Cabras, M. Meloni, and L. Spanedda. High-performance liquid chromatographic separation of cyromazine and its metabolite melamine. *J Chromatogr. A* **505:** 413–16 (1990).
8. R.A. Yokley, L.C. Mayer, R. Rezaaiyan, et al. Analytical method for the determination of cyromazine and melamine residues in soil using LC-UV and GC-MSD. *J. Agric. Food Chem.* **48:** 3352–58 (2000).
9. A. Kaune, R. Bruggemann, M. Sharma, et al. Soil adsorption coefficients of s-triazines estimated with a new gradient HPLC method. *J. Agric. Food Chem.* **46:** 335–43 (1998).
10. S.S. Chou, D.F. Hwang, and H.F. Lee. High performance liquid chromatographic determination of cyromazine and its derivative melamine in poultry meats and eggs. *Journal of Food and Drug Analysis* **11:** 290–95 (2003).
11. G.M. Titato and F.M. Lanças. Optimization and validation of HPLC–UV–DAD and HPLC–APCI–MS methodologies for the determination of selected PAHs in water samples. *J. Chromatogr. Sci.* **44(1):** 35–40 (2006).
12. V. Boon, B. Glass, and A. Nimmo. High-performance liquid chromatographic assay of indomethacin in porcine plasma with applicability to human levels. *J. Chromatogr. Sci.* **44(1):** 41–4 (2006).
13. V. David, A. Medvedovici, and F. Albu. Retention behavior of metformin and related impurities in ion-pairing liquid chromatography. *J. Liq. Chromatogr. Rel. Technol.* **28(1):** 81–95 (2005).

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