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High Performance Liquid Chromatographic Determination of Cyromazine and Its Derivative Melamine in Poultry Meats and Eggs

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ABSTRACT

A high performance liquid chromatography (HPLC) with NH_2 column and 75% acetonitrile eluate to determine the insecticide cyromazine residues in poultry meats and eggs was developed. Samples were treated with NaOH, extracted with acetonitrile containing 20% concentrated NH_4OH, and cleaned up through C18 Sep-Pak cartridge. The peaks of cyromazine and its metabolite melamine were successfully separated within 12 min and cleanly differentiated from interfering peaks of the samples. The detection limit of both compounds was 0.02 ppm. Recoveries of cyromazine and melamine ranged from 92.8-97.3% and 91.0-96.1% at fortified levels of 0.2-0.7 ppm, respectively. Each reaction coefficient of linear standard curve of cyromazine and melamine was better than 0.999. Using this HPLC determination, only one beef sample from 46 tested samples was detected to contain 0.04 ppm cyromazine. This was further confirmed by gas chromatography-mass spectroscopy.

Key words: cyromazine, melamine, insecticide, poultry meats

INTRODUCTION

Cyromazine (Trigard or Larvadex; chemical name: N-cyclopropyl-1,3,5-triazine-2,4,6-triamine) is an insecticide, which acts as an insect growth regulator. It is used for feed-through fly control in caged layers. It is administered as a feed-through larvicide incorporated into feed at 0.50 mg/kg for laying hens to prevent flies from hatching in the manure[(1)]. As a foliar spray it is effective in controlling leaf miners in vegetable crops and ornamentals.

Like other agrochemicals, cyromazine is highly effective in pesticide control but it is also toxic to humans and the environment. In addition, melamine (chemical name: 1,3,5-triazine-2,4,6-triamine), a potential degradation product or metabolite of cyromazine, is a suspected carcinogen[(2,3)]. The tolerance of cyromazine and the melamine metabolite is 0.05 ppm each in edible poultry tissue in USA[(4)].

For safety and environmental impact assessment, analytical methods for determining the chemical residues are indispensable. There have been several methods to determine cyromazine and melamine, including gas chromatography (GC)[(5,6)], high performance liquid chromatography (HPLC)[(3,7-9)] and GC-mass spectroscopy (GC-MS)[(10,11)]. 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. However, the column, solvent system and sample preparation would be very crucial for the HPLC method. Cyromazine has been registered in Taiwan for plant protection use[(12)]. Meanwhile, parts of the poultry meats are imported from foreign countries. The residue of cyromazine and melamine in the domestic and import poultry meats and eggs are of concern. To establish the residue level of cyromazine and melamine in the poultry products, the HPLC approach to determine cyromazine and melamine was examined. The samples of domestic and import poultry meats and eggs were collected and determined using this method.

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MATERIALS AND METHODS

I. *Reagents*

Analytical standard of cyromazine and melamine were purchased from Riedel de Haen AG (Germany) with 99% purities. Sodium hydroxide and ammonia hydroxide (analytical reagents) were from Merck (Darmstadt, Germany). Acetonitrile, methanol and n-hexane (HPLC grade) were also from Merck.

II. *Instrumentation*

A blender (Polytron Pt-3100, Kinematica AG, Littan-Luzern, Switzerland) and Whatman No. 2 filter paper

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(Whatman, Maidstone, UK) were used. The Sep-Pak C18 cartridge (Bakerbond spe Column) was purchased from Baker Company (Philipsberg, NJ, USA). The high performance liquid chromatographic (HPLC) equipment used was Hitachi liquid chromatograph (Hitachi, Tokyo, Japan), consisting of a Model L-6200 pump, a Model L-4000 UV-Vis detector (set at 215 nm), and Model Shimadzu C-R4A Chromatointegrator (Shimadzu, Kyoto, Japan). The gas chromatography-mass spectroscopic(GC-MS) equipment used was HP5890 series II GC-MS (Hewlett-Packard Company, USA), consisting of a Model HP 5970 B quadrupole mass selective detector, a Model HP340C computer system, a Model HP59944 A MS ChemStation, and a capillary column (RTX-5, 30 m × 0.25 mm i.d.)

III. *Standard Preparation*

Each 100 mg of cyromazine and melamine was taken and dissolved into 100 mL methanol as stock solution. Then, the stock solution was diluted with acetonitrile into a series of standard solutions (0.02, 0.1, 0.5, 1.0 and 10 ppm).

IV. *Samples*

The numbers of tested samples were as follows: chicken, 10 (domestic); eggs, 10 (domestic); beef, 15 (5 for domestic and 10 for import); mutton, 5 (domestic); and pork, 6 (4 for domestic and 2 for foreign). The domestic samples were collected from different markets in Taipei, and the import commodities were obtained from different codes of imported agencies during June to December 2000.

V. *Analytical Procedures*

To each sample (30 g) of poultry meats and de-shelled eggs was added 1 mL of 1 N NaOH and ground in a blender for 3 min. Ground sample (10 g) were transferred to 100-mL centrifuge tubes and homogenized with 70 mL of ammoniacal acetonitrile containing 20% concentrated NH_4OH for 3 min. The homogenates were centrifuged (10,000 g, 10 min, 4˚C) and filtered through Whatman No.2 filter paper. The above procedure was performed in duplicate. The filtrates were evaporated under vacuum pressure at 45˚C water bath and then placed in volumetric flasks, then acetonitrile was added to a final volume of 50 mL. The solution was transferred to a separatory funnel, added 50 mL of n-hexane, and shaken vigorously; let the phases separate, save the lower acetonitrile phase, and discard the upper n-hexane phase. This procedure was also performed in duplicate. The acetonitrile phase was evaporated into dryness and made up to 1 mL as acetonitrile extract.

The disposable solid phase extraction column, Sep-Pak C18 cartridge with packing 500-mg/6 mL was set up. The extraction column was prewashed with 5 mL of acetonitrile. Acetonitrile extract containing analytes was passed through the column at the rate of ca 1 mL/min. The eluate was discarded. The column was washed with 5 mL of acetonitrile and washings were discarded. Analytes from the column were recovered by eluting with 30 mL of ammoniacal acetonitrile containing 20% concentrated NH_4OH. Eluate was evaporated into dryness, dissolved and made up to 1 mL with acetonitrile as test solution. Use 20-μL for high performance liquid chromatographic (HPLC) analysis.

VI. *Recovery*

The recoveries of cyromazine and melamine were determined by fortifying homogenized poultry meats and eggs with 0.3, 0.5 and 0.7 ppm levels. The standard solution representing known amounts of cyromazine and melamine in 1 mL of acetonitrile was added to 10 g of homogenized sample to obtain desired ppm level fortification in a 100 mL beaker. The beaker was put in a fume hood and sprayed with nitrogen gas for 1 hr before extraction. Three replicates were analyzed at each fortification level for method evaluation.

VII. *Detection Limit*

The detection limits of cyromazine and melamine were determined by extracting homogenized poultry meats and eggs fortified at 0.01, 0.03 and 0.1 ppm levels. The procedure was the same as described above. The detecting limit was evaluated according to the ratio of sample peak area to noise peak area being more than 3 times.

VIII. *Confirmation by GC-MS*

Following HPLC determination, the peak of cyromazine in the detectable sample was collected and reconfirmed by GC-MS. The temperature being raised from 150˚C to 250˚C at a rate of 10˚C/min. The carrier gas helium and flow rate 1.8 mL/min were used. The ionizing voltage was kept at 70 eV at an ion source temperature of 250˚C. Scanning was carried out in a mass range of *m/z* 50-200 at 3 sec intervals.

RESULTS AND DISCUSSION

Several types of column including RP-18, RP-8, C18, NH_2 and SCX have been reported for use in HPLC to determine cyromazine and melamine[3,7]. In this study, C18 column with 0.5 μM sulfuric acid/acetonitrile (85:15, v/v) solvent and NH_2 column (200 × 4.6 mm i.d.) with acetonitrile/H_2O (75:25, v/v) solvent were compared in analyzing cyromazine and melamine using HPLC. The retention times of cyromazine and melamine were less than 4 min using a C18 column (200 × 4.6 mm i.d.) with 0.5 μM sulfuric acid/acetonitrile (85:15, v/v) solvent (Figure 1). The acetonitrile ratio could not increase the retention time up to more than 4 min (Figure 2). Therefore, C18 column with 0.5 μM sulfuric acid/acetonitrile solvent was not

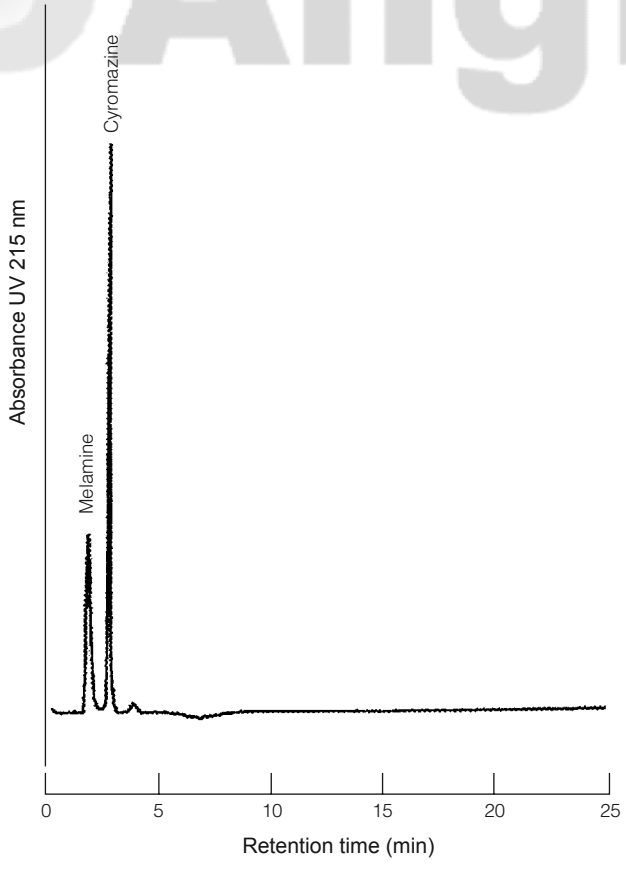

Figure 1. HPLC chromatogram of cyromazine and melamine by C18 column with mobile phase (0.5 μM sulfuric acid/acetonitrile, 85:15).

adequate in analyzing cyromazine and melamine residues in poultry meats because of serious interfere problems. On the other hand, the ionization of both compounds in alkalic solution increased the hydropholic ability so the retention time was extended when alkalic buffer on a reversed-phase column eluted both compounds. However high pH (>7.5) in the eluent extended the separation times of cyromazine and melamine, it might also destroy the stability of resin and shorten the column life[13]. Therefore, we abandoned to study the optimal eluting alkali buffer in using C18 column. The respective retention time of cyromazine and melamine was 6 and 11 min when using NH_2 column with

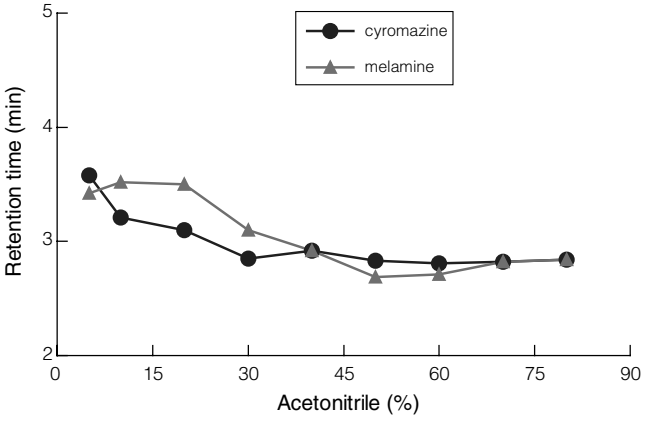

Figure 2. Effect of acetonitrate concentration on the retention time of cyromazine and melamine analyzed with C18 column.

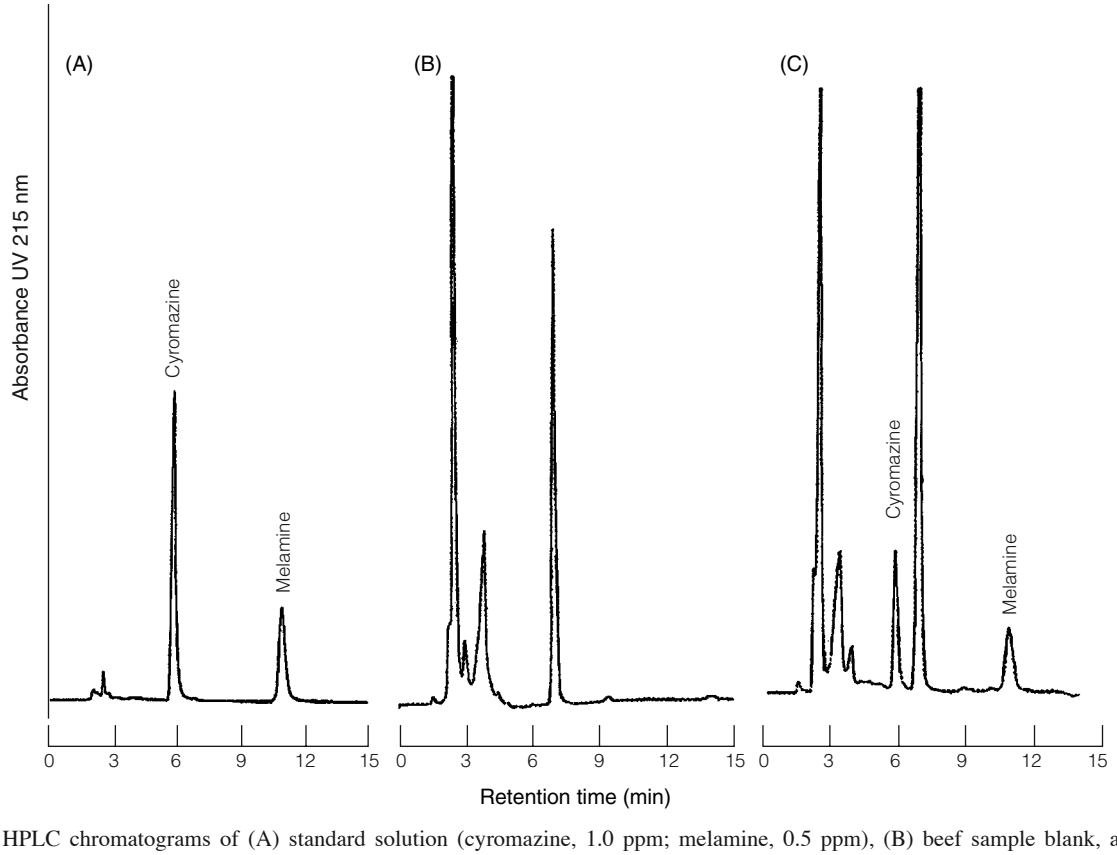

Figure 3. HPLC chromatograms of (A) standard solution (cyromazine, 1.0 ppm; melamine, 0.5 ppm), (B) beef sample blank, and (c) beef sample spiked with standard (cyromazine, 0.5 ppm; melamine, 0.5 ppm).

acetonitrile/H_2O (75:25, v/v) solvent (Figure 3A). Both peaks could clearly separate from the interfering peaks of poultry meat sample (Figure 3B,C). The effect of the concentration of acetonitrile on the retention time was shown in Figure 4. Since the retention time increased when the concentration of acetonitrile was increased in the eluting solvent, NH_2 column with acetonitrile/H_2O (75:25, v/v) solvent was suggested to be appreprate in analyzing cyromazine and melamine residues in the poultry meats.

Although sample preparations with different cartridges (C18 and SCX) and resins have been reported in several papers[3,5,7,10], we found Sep-Pak C18 cartridge with acetonitrile containing 20% concentrated NH_4OH could effectively purify the poultry meats and eggs. Recoveries of cyromazine and melamine in the poultry meats and eggs were shown in Table 1, more than 92.8% for cyromazine and between 91.0 and 96.1% for melamine. Standard curves of cyromazine and melamine were prepared separately in the range of 0.02-12 ppm and peak area (Y) vs. amount (X) of insecticide regression coefficients for standard curves were subjected to linear regression analysis. The correlation coefficients and linear regression coefficients were Y = 581553 X – 6330 (r = 0.9997) for cyromazine and Y = 742458 X + 600 (r = 0.9998) for melamine. This indicated a definite linear relationship between insecticide concentration and detector response. The detection limit of both cyromazine and melamine was 0.02 ppm.

Using this HPLC determination, the level of cyromazine and melamine in the poultry meats and eggs is

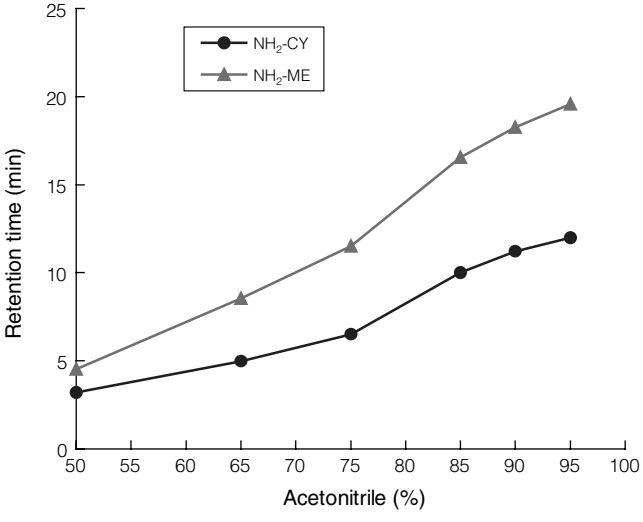

Figure 4. Effect of acetonitrile concentration on the retention time of cyromazine (CY) and melamine (ME) analyzed with NH_2 column.

Table 1. Recoveries of cyromazine and melamine spiked into chicken, beef, mutton, pork and eggs

Sample	Recovery (%)			
	Spiked level (ppm)[a]			
	0.7	0.5	0.3	Mean
	Cyromazine			
Chicken	97.3(4.5)[b]	95.8(4.3)	95.4(3.9)	96.1(2.8)
Eggs	95.7(4.3)	94.5(4.3)	92.8(3.7)	94.3(2.2)
Beef	94.4(5.1)	93.8(4.6)	93.3(4.8)	93.8(3.9)
Mutton	95.6(4.5)	94.3(5.6)	93.9(4.1)	94.6(2.9)
Pork	96.5(4.2)	95.3(4.7)	95.9(3.9)	95.9(3.4)
	Melamine			
Chicken	94.3(3.7)	94.1(3.7)	92.7(5.6)	93.7(3.6)
Eggs	95.2(4.1)	96.1(4.6)	92.8(7.7)	94.7(4.8)
Beef	94.1(5.3)	93.8(5.2)	94.0(4.8)	94.0(3.9)
Mutton	95.7(5.1)	95.2(5.6)	93.3(6.4)	94.7(3.6)
Pork	95.0(5.5)	94.1(5.8)	91.0(7.4)	93.4(3.1)

[a]Average of triplicate.
[b]Number in parentheses represents coefficient of variation.

Table 2. Concentration of cyromazine and melamine in the sample

Sample name	Sample No.	Origin of sample	Concentration (ppm)	
			Cyromazine	Melamine
Chicken	10	R.O.C	<0.02	<0.02
Eggs	10	R.O.C	<0.02	<0.02
Beef	4	New Zealand	<0.02	<0.02
Beef	1	U.S.A.	<0.02	<0.02
Beef	5	Australia	<0.02	<0.02
Beef	5	R.O.C	<0.02~0.04[a]	<0.02
Mutton	4	Australia	<0.02	<0.02
Mutton	1	New Zealand	<0.02	<0.02
Pork	2	U.S.A	<0.02	<0.02
Pork	4	R.O.C	<0.02	<0.02

[a]Only one sample was detected to contain 0.04 ppm.

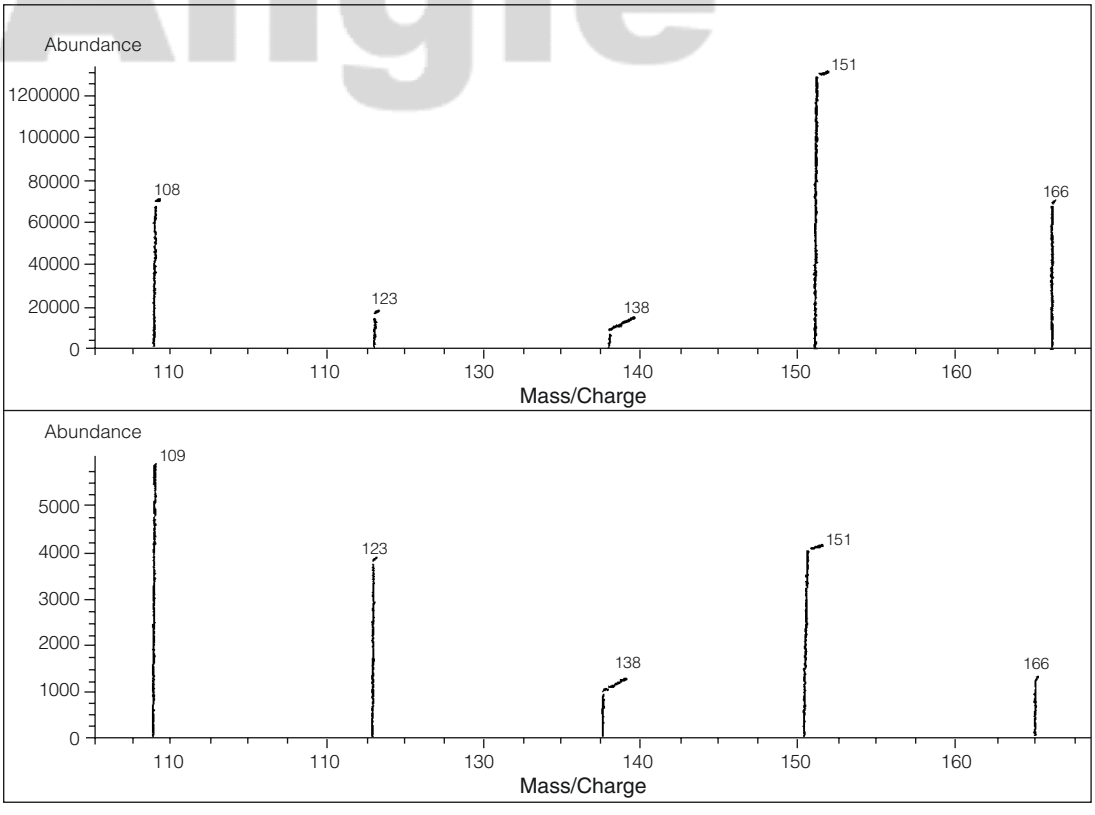

Figure 5. GC-MS chromatograms of standard cyromazine (1 μg) and beef sample.

shown in Table 2. It was found that only one domestic beef was detected to contain 0.04 ppm cyromazine-like compound. For confirmation, the cyromazine-like compound in the detectable beef sample was examined by GC-MS, the retention time of standard cyromazine and cyromazine-like compound was the same (9.2 min). Both cyromazine and cyromazine-like compound displayed a parent peak at *m/z* 166 and four fragment peaks at *m/z* 151, 138, 123 and 109 (Figure 5). These electron impact mass spectra were the same as those reported by Toth and Bardalaye[10]. Hence, the detectable beef sample was identified to contain 0.04 ppm cyromazine.

Judging from the above data, the HPLC method with NH_2 column and acetonitrile/H_2O (75:25, v/v) solvent was valid, accurate, and precise for the determination of cyromarine and melamine. The sample preparation using C18 cartridge with acetonitrile containing 20% concentrated NH_4OH was available for purifying the cyromazine and melamine in the poultry meats and eggs. In this study, the residue of cyromazine was detected on domestic beef samples. Therefore, the illegal use of cyromazine in Taiwan poultry farms should be carefully monitored.

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