Analysis of Cyromazine in Poultry Feed Using the QuEChERS Method Coupled with LC-MS/MS[†]

Kang Xia,*,§,# Jack Atkins,§ Cindy Foster,§ and Kevin Armbrust§,#

§Mississippi State Chemical Laboratory and #Department of Chemistry, P.O. Box CR,
Mississippi State, Mississippi 39762

Cyromazine is used as an additive in poultry feed to inhibit the development of fly larvae in chicken manure. U.S. Environmental Protection Agency (EPA) method AG-555, modified from method AG-376, has been the standard method for cyromazine analysis in poultry feed. However, these methods are time-consuming (~3 h) and require large volumes (200 mL) of solvent. This study developed an extraction procedure using the QuEChERS method that is faster (~30 min) and uses 20 times less solvent than the AG-555 method. After extraction using the QuEChERS method, the extractant was subjected to cleanup using a C-18 solid phase extraction (SPE) followed by filtering through a 0.45 μm syringe Teflon filter before the liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis. Recovery of 75.0 ± 6.2% was achieved. The method detection limit (MDL) and the limit of quantitation (LOQ) were 0.028 and 0.094 ppm, respectively. Analyses of commercial poultry feed samples using the QuEChERS method yielded results similar to those obtained via EPA method AG-555.

KEYWORDS: QuEChERS method; cyromazine; LC-MS/MS; feed

INTRODUCTION

Cyromazine (trade name Trigard or Larvadex) is an insecticide used as an additive only in the feed for chicken layer hens and chicken breeder hens at < 0.01 pound of cyromazine per ton of poultry feed. It is used for control of flies in manure of treated chickens (*1*). 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 (*1*). Cyromazine is also highly effective as a foliar spray for vegetable crops and ornamentals against leaf miners and various other insects (*2*). The chemical name and structure of cyromazine are shown in **Figure 1**. Although cyromazine is a triazine pesticide, its chemical properties are much different from those of other triazine pesticides. Due to its more ionic and hydrophilic nature, conventional determinative methods for triazines are not applicable for cyromazine.

There have been several methods for the determination of cyromazine in food (*3*), vegetable crops (*4*), and soil (*5*). However, all of those methods require laborious extraction and cleanup and consume large volumes of solvents (**Table 1**). U.S. Environmental Protection Agency (EPA) method AG-376 and the modified method AG-555 for the determination of cyromazine in poultry feed have similar shortcomings (*6*). The EPA methods require refluxing feed samples with large volumes (100−200 mL) of glacial acetic acid for 2 h, followed by cleanup with two SPE cartridges: a strong cation exchange (SCX) cartridge and an Alumina Sep-Pak cartridge. Cyromazine is then analyzed by

high-performance liquid chromatography with UV detection (HPLC-UV). The detection limit for these methods is 1 ppm. Additionally, it is often necessary to perform extensive procedures for cleanup and preconcentration of extracts to remove interferences and to obtain adequate detection levels. In recent years, liquid chromatography−mass spectrometry (LC-MS) has become an accepted technique in pesticide residue analysis, allowing analysis without extensive cleanup and reconcentration steps (*7*, *8*).

Since its introduction, the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method (*9*) has been readily accepted by many pesticide residue analysts because of its low cost and fast and accurate procedures (*10−13*). The original QuEChERS method and its modified versions entail initial single-phase extraction of multiple analytes with a small volume (10 mL) of acetonitrile, followed by liquid−liquid partitioning with the addition of 10 mL of water and 4 g of anhydrous $MgSO_4$ plus 1 g of NaCl. Removal of residual water and cleanup of polar residues are performed simultaneously using a dispersive solid-phase extraction method or SPE cartridges (*9−13*). The QuEChERS method provides the following advantages over traditional techniques: (1) significant reduction of organic solvent; (2) complete removal of the use of any chlorinated solvents; and (3) removal of the use of mechanical homogenizers or blenders. These modifications yield high method recovery of pesticides in a wide range of polarity and volatility and allow high sample throughput at the same time. By coupling this extraction procedure with gas chromatography−mass spectrometry (GC-MS) or LC-MS, a batch of 6−12 extracts can be prepared in < 30 min by a single analyst and up to hundreds of pesticides can be quantitatively analyzed simultaneously in a sample (*12*, *14*). The application of

---

the QuEChERS method to cyromazine analysis has not been previously reported. Therefore, the goal of this investigation was to develop an efficient method for cyromazine analysis in poultry feed by coupling the QuEChERS method with liquid chromatography—tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Chemicals, Materials, and Solvents. The cyromazine standard (neat material, 99% pure, 100 μg mL^{-1} dissolved in methanol) was purchased from AccuStandard Inc. (New Haven, CT). Optima grade acetonitrile, water, methanol, and acetone, glacial acetic acid (99.9% purity, 17.4 N), and ammonium hydroxide were all purchased from Fisher Scientific (Waltham, MA). Prepackaged test tubes each containing 6 g of anhydrous magnesium sulfate and 1.5 g of anhydrous sodium acetate were purchased from United Chemical Technologies, Inc. (Bristol, PA).

Sample Preparation. *Sample Extraction and Cleanup Using the QuEChERS Method.* The blank (control) poultry feed sample used for spiking and the commercial poultry feed samples were provided by
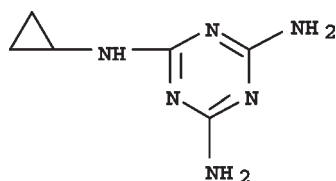

Figure 1. Chemical name and structure for cyromazine (SciFinder database).

Novartis Animal Health US, Inc. (Greensboro, NC). The samples were homogenized by grinding each sample to a fine powder (0.5 mm average particle size) in a Thomas-Wiley laboratory mill (model 4, Swedesboro, NJ). To prepare cyromazine-spiked samples, an appropriate amount of 100 μg mL^{-1} cyromazine standard was transferred into 2 g of the blank feed sample in a 50 mL polypropylene centrifuge tube and thoroughly mixed by hand under the fume hood until the methanol in which the standard was dissolved was evaporated.

Two grams of homogenized blank or spiked poultry feed was weighed into a 50 mL polypropylene centrifuge tube. Ten milliliters of acetonitrile/acetic acid (75:25, v/v) was added to the sample and sonicated at 50/60 Hz for 15 min. After sonication, 10 mL of water, 6 g of magnesium sulfate, and 1.5 g of sodium acetate were added to the sample and shaken by hand vigorously for 1 min. After shaking, the sample was centrifuged at 3400 rpm for 10 min at ambient temperature, and 1 mL of supernatant was pipetted into a 10 mL calibrated test tube and diluted to 10 mL with a mixture of water/acetonitrile (95:5, v/v) containing 0.1% acetic acid. For the cleanup, the 10 mL diluted sample extract was eluted through a Bond Elut C18 SPE cartridge (500 mg/3 cm^3, Varian, Palo Alto, CA) at 1 mL min^{-1} under vacuum using a vacuum manifold, followed by filtration through a 0.45 μm syringe Teflon filter (Millipore, Billerica, MA). Two milliliters of the filtrate was transferred to a HPLC vial before analysis by LC-MS/MS.

Sample Extraction and Cleanup Using Method AG-555. EPA method AG-555 was modified on the basis of method AG-376 (6). Briefly, 10 g of homogenized finely ground poultry feed was weighed into a 500 mL round-bottom flask containing several boiling chips. Two hundred milliliters of glacial acetic acid was added to the flask, and the mixture was subjected to heating under reflux for 2 h. After cooling to room

Table 1. Current Methods for Determination of Cyromazine in Different Matrices

matrix	extraction	cleanup	analysis	MDL[a] (ppm)	recovery (%)	ref
chicken, beef, mutton, pork meats, eggs	grind 30 g of sample with 1 mL of x1 N NaOH; homogenize with 70 mL of acetonitrile containing 20% concentrated NH$_4$OH; centrifuge at 10000g for 10 min at 4 °C and filter through Whatman no. 2 filter paper; evaporate the filtrates at 45 °C and bring to volume with acetonitrile to 50 mL; exchange with 50 mL of *n*-hexane and collect the acetonitrile phase; evaporate the acetonitrile phase to dryness and redissolve in 1 mL of acetonitrile	prewash Sep-Pak C18 cartridge with 5 mL of acetonitrile; pass the 1 mL acetonitrile extract through the column at 1 mL min^{-1}; wash the column with 5 mL of acetonitrile; elute analyte with 30 mL of acetonitrile containing 20% concentrated NH$_4$OH; evaporate the elute to dryness, redissolve in 1 mL of acetonitrile	HPLC-UV; NH$_2$ column; mobile phase: acetonitrile/ water (75:25, v/v)	0.02	91—96	3
chard	homogenize 20 g sample with 120 mL phosphate buffer at pH 2 for 8 min; add 30 mL of methanol and mix for 5 min; centrifuge, filter supernatant, adjust to a final volume of 200 mL with methanol, and dilute 5 to 25 mL with water; filter through a 0.45 μm syringe filter; mix 1 mL of filtered solution with 50 μL of 250 mM TFHA in a 2 mL vial	not required	LC-MS/MS; Discovery C18 column; mobile phases (A) water containing 0.5 mM TFHA and (B) methanol; ionization: electrospray positive ion mode with *m/z* 167.1 > 84.8	0.05	93—103	4
soil	shake 20 g of sample with 100 mL of acetonitrile containing 30 0.05 M (NH$_4$)$_2$CO$_3$ for 30 min; centrifuge and collect supernatant; shake the sediment with 100 mL of acetonitrile containing 30 mL of 0.05 M (NH$_4$)$_2$CO$_3$ for 30 min; centrifuge, collect the supernatant, and combine with the previous one; adjust 100 mL of pooled supernatant to pH ≤2 with 4 N HCl	pass pH adjusted 100 mL of supernatant through strong cation exchange cartridge at 5 mL min^{-1}; wash column at 5 mL min^{-1} first with 50 mL of acetonitrile/water (90:10, v/v), then with 50 mL of methanol/water (90:10, v/v), and finally with 10 mL of methanol; elute analyte with 20 mL of NH$_4$OH/ methanol (5:95, v/v) at 5 mL min^{-1}; evaporate the eluent to dryness, redissolved in 1 mL of water for HPLC-UV and in 1 mL of acetone for GC-MS analysis	HPLC-UV; Zorbax SCX column; mobile phase: methanol/ K$_3$PO$_4$ buffer (25:25, v/v); GC-MS; DB-Wax capillary column; electron impact ionization with *m/z* 151	0.01	73—142 (HPLC-UV); 93—127 (GC-MS)	5

[a] MDL, method detection limit.

Article

J. Agric. Food Chem., Vol. 58, No. 10, 2010 **5947**

Table 2. Mass Spectrometry Conditions for Cyromazine Quantitation

|	
capillary voltage	3.1 kV
cone voltage	35 V
collision energy	21–24 V
source temperature	120 °C
desolvation temperature	350 °C
cone gas flow	135 L/h
desolvation gas flow rate	750 L/h
collision gas	argon
parent ion (*m/z*)	167
product ion (*m/z*)	85

temperature, 2 mL of the supernatant was removed and passed through a preconditioned Bond Elut SCX SPE cartridge (500 mg/3 cm^3, Varian) at 1–2 drops min^{-1}, and the eluent was discarded. The SCX SPE cartridge was first washed at gravity flow with 5 mL of methanol/water (90:10, v/v) and then with 5 mL of methanol. The eluent from each wash was discarded. Cyromazine was eluted at gravity flow from the SCX cartridge with 5 mL of NH$_4$OH/methanol (5:95, v/v). The eluent containing cyromazine was evaporated to dryness using a rotary evaporator at 30–35 °C, and the residue was redissolved in 5 mL of acetone/methanol (95:5, v/v) in an ultrasonic bath to ensure the residue was completely dissolved. The redissolved sample was passed through a Sep-Pak Classic Alumina A cartridge (1850 mg sorbent weight, 120 Å pore size, 175 μm particle size, Waters, Milford, MA) at gravity flow. The cartridge was first washed at gravity flow with 5 mL of acetone/methanol (90:10, v/v) and then with 5 mL of acetone/methanol (75:25, v/v). The eluent from each wash was discarded. Cyromazine was eluted at gravity flow from the cartridge with 10 mL of acetone/methanol (50:50, v/v) and was collected in a 50 mL flask. The eluent containing cyromazine was evaporated to dryness using a rotary evaporator at 30–35 °C. The residue was redissolved in 5 mL of methanol before analysis on LC-MS/MS.

LC-MS/MS Conditions. Cyromazine analyses of the sample extracts by LC-MS/MS were performed using a Waters Alliance 2695 HPLC (Waters) coupled with a micromass Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.). The HPLC conditions include a guard column (Alltima, C18, 5 μm, 2.1 × 7.5 mm, Deerfield, IL), a reversed phase analytical column (Alltima, C18, 5 μm, 2.1 × 250 mm, Waters), and two mobile phases, (A) acetonitrile containing 0.1% formic acid and (B) water containing 0.1% formic acid. The following gradient condition was used for the analyses: 0–2 min, 5% A; 2–5 min, from 5 to 10% A; 5–5.5 min, from 10 to 90% A; 5.5–8 min, 90% A; 8–10 min, from 90 to 5% A; and 10–12 min, 5% A. The mobile phase flow rate was 0.2 mL min^{-1}. The injection volume was 25 μL. The mass spectrometer was operated in the positive ion mode electrospray ionization and set up to monitor the ion transition of the precursor ion (*m/z* 167) to the product ion (*m/z* 85) in multiple reaction monitoring (MRM). The detailed mass spectral conditions are listed in **Table 2**.

Preparation of Calibration Solutions and Quantification Calculation. Concentrations (ng mL^{-1}) of cyromazine in the cleaned up extractants from both the QuEChERS method and method AG-555 were quantified using external standards consisting of cyromazine dissolved in water/acetonitrile (95:5, v/v) at 2, 10, 40, and 200 ng mL^{-1}. The external standards were prepared by mixing appropriate amounts of 1 or 10 μg mL^{-1} cyromazine standard with 1 mL of solvent containing 5% HPLC mobile phase A and 95% HPLC mobile phase B. Because no matrix effect was observed, matrix calibration standards were not used for the determinative assay.

When using the QuEChERS method, the final cyromazine concentration (μg g^{-1}, ppm) in a poultry feed sample was calculated by multiplying cyromazine concentration in the cleaned up extractant by a factor of 0.1 or 0.05 when using the QuEChERS method and method AG-555, respectively. Eight commercially available poultry feed samples were tested using both methods.

Performance Evaluation. The performance of the developed method was assessed using blank poultry feed samples spiked with cyromazine. The coefficient of linearity was determined using poultry feed spiked with cyromazine at levels of 0.2, 0.4, 1, 2, 4, and 8 ppm. The experiments for testing recovery and intraday precision were conducted using poultry feed samples spiked with cyromazine at levels of 0.4, 2, and 4 ppm, making seven replicate measurements on the same day. The interday precision was
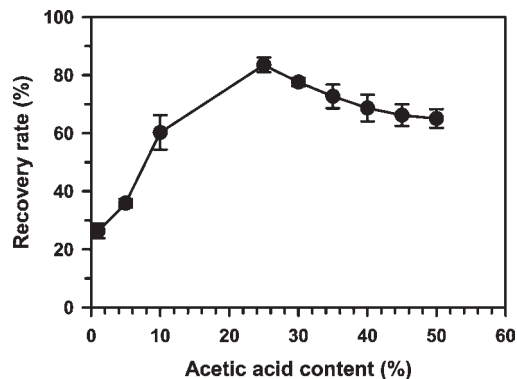
Figure 2. Percent method recovery of cyromazine from poultry samples extracted using the modified QuEChERS method with different volume ratios of acetonitrile and glacial acetic acid.

determined using spiked samples with 2 ppm cyromazine on seven different days. The intraday precision and the interday precision are reflected by percent relative standard deviation (%RSD) calculated using equation

$$\%RSD = 100 \times S/mean$$

where S and mean are standard deviation and the mean, respectively, of the detected concentrations of the seven replicates.

The recovery rate was calculated using the equation

$$recovery\ rate = 100\% \times (detected\ concentration/spiked\ concentration)$$

The method detection limit (MDL) and limit of quantitation (LOQ) were determined using the method described in the *Code of Federal Regulations,* Part 136, Appendix B (*15*). Briefly, eight blank poultry feed samples were spiked with cyromazine at 0.2 ppm, extracted, and analyzed. The values of MDL and LOQ were calculated using the equations

$$MDL = S \times (t\ value)$$

$$LOQ = 10 \times S$$

where S is the standard deviation of the detected concentrations of the eight samples. The t value = 2.998 when n = 8.

RESULTS AND DISCUSSION

QuEChERS Method Optimization. The QuEChERS method was modified by mixing glacial acetic acid (pK_a = 4.75) with acetonitrile as an extraction solvent. Acetic acid was added to increase the extraction efficiency. **Figure 2** shows that when using acetonitrile as an extraction solvent, approximately 25% of the cyromazine spiked to the blank poultry feed sample was recovered. The cyromazine recovery rate increased with increasing glacial acetic acid content in the acetonitrile extractant and reached a maximum of 80% when the acetonitrile/acetic acid volume ratio was 75:25 (v/v). Higher glacial acetic acid contents resulted in lower recoveries.

Method Performance. The linearity of the spiked samples (0.2–8 ppm) was >0.999 (**Figure 3**). The MDL and LOQ calculated on the basis of the data shown in **Table 3** were 0.028 and 0.094 ppm, respectively. The current MDL value by the QuEChERS method was comparable with those for tissue and soil samples (*3−5*) but was lower than the 1 ppm MDL achieved by EPA method AG-555 for poultry feeds (*6*). The intraday precision (%RSD) ranged from 4.68 to 11.5% at three levels of cyromazine, and the interday precision (%RSD) at 2 ppm was 5.21% (**Table 4**). The method recovery ranged from 71.0 ± 6.2 to 78.3 ± 9.0% for the four groups of cyromazine-spiked samples, with an overall average method recovery of 75 ± 6.2%. Signal
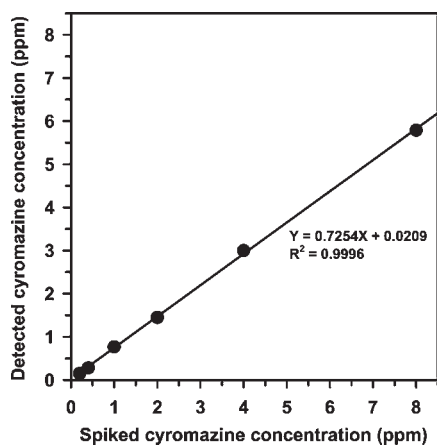
5948 *J. Agric. Food Chem.,* Vol. 58, No. 10, 2010

Xia et al.

Figure 3. Regression curve showing linearity range of cyromazine determination in spiked poultry feed samples using the modified QuEChERS method.

Table 3. Determination of MDL, LOQ, and S/N (Blank Samples Spiked with Cyromazine at 0.2 ppm)

replicate	concentration (ppm)	recovery (%)
1	0.146	73.0
2	0.152	76.0
3	0.161	80.5
4	0.157	78.5
5	0.151	75.5
6	0.132	66.0
7	0.153	76.5
8	0.161	80.5
mean (X)	0.152	75.8
standard deviation (S)	0.00944	4.72

MDL = $S \times (t$ value$)$ =
  $0.00944 \times 2.998 = 0.028$ ppm
LOQ = $10 \times S = 10 \times 0.00944 =$
  0.0944 ppm
S/N = $(X/S) = 0.152/0.00944 = 16.1$

Table 4. Recovery, Intraday Precision, and Interday Precision

	spiked level (ppm)	recovery (%)	%RSD
intraday precision test ($n = 7$)	0.4	78.3 ± 9.0	11.5
	2	71.0 ± 6.2	8.61
	4	74.9 ± 3.5	4.68
interday precision test ($n = 7$)	2	74.8 ± 3.9	5.21

reduction (e.g., matrix suppression) was not observed during LC-MS/MS detection of blank poultry feed extract spiked with cyromazine. Therefore, the loss of recovery could not be due to ion suppression during LC-MS/MS detection.

Analysis of Commercially Available Samples. No significant background interference was observed in the LC-MS/MS chromatograms of the samples prepared using a modified QuEChERS method (**Figure 4**). The modified QuEChERS method yielded results for the eight commercially available poultry feed samples similar to those obtained with EPA method AG-555 (**Figure 5**). The cyromazine concentrations in the eight samples ranged from 2.7 to 6.3 ppm using the modified QuEChERS method, whereas 3.0–5.9 ppm cyromazine was detected using EPA method AG-555. In conclusion, compared to method AG-555 (6), the modified QuEChERS method provides comparable results but requires less solvent and fewer cleanup steps and is
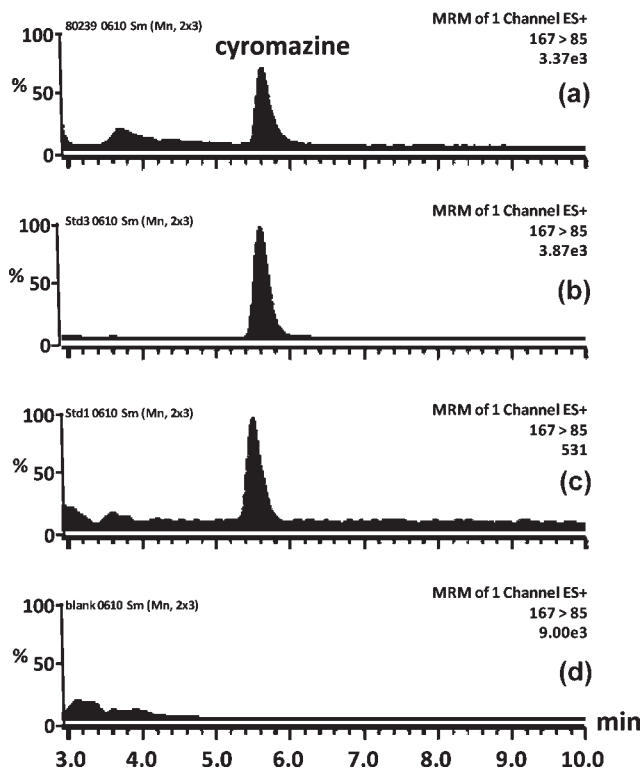

Figure 4. LC-MS/MS MRM chromatograms for a commercial poultry feed sample containing cyromazine at 3.53 ppm (**a**, top), cyromazine external standards at 40 ng mL^{-1} (**b**) and 2 ng mL^{-1} (**c**), and a blank poultry feed sample (**d**, bottom).
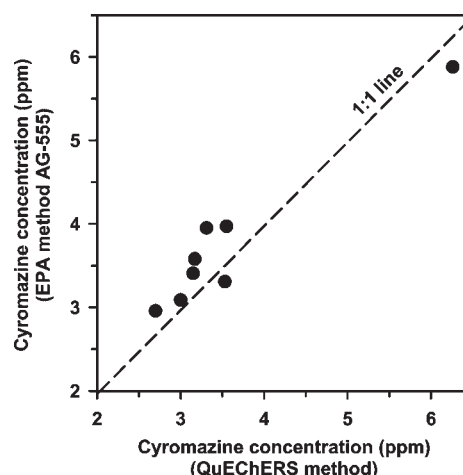

Figure 5. Comparison of cyromazine concentrations in eight poultry feed samples extracted using the modified QuEChERS method and EPA method AG-555 followed by LC-MS/MS analysis.

faster. Although the effectiveness and efficiency of the QuEChERS method have been illustrated in many previous works (7–14), to our best knowledge this method has not been previously applied to the analysis of cyromazine in poultry feed.

ABBREVIATIONS USED

HPLC-UV, high-performance liquid chromatography with UV detector; LC-MS, liquid chromatography—mass spectrometry; QuEChERS, quick easy cheap effective rugged and safe method; LC-MS/MS, liquid chromatography—tandem mass spectrometry; SPE, solid phase extraction; SCX, strong cation

exchange; MRM, multiple-reaction monitoring; MDL, method detection limit; LOQ, limit of quantitation.

LITERATURE CITED

(1) U.S. EPA. Cyromazine; Pesticide Tolerance. In *Federal Register* **1999**, *64*, 50043−50050 (http://www.epa.gov/EPA-PEST/1999/September/Day-15/p24047.htm).

(2) Ware, G. W. *The Pesticide Book*, 4th ed.; Thomson Publications: Fresno, CA, 1993.

(3) Chou, S. S.; Hwang, D. F.; Lee, H. F. High performance liquid chromatographic determination of cyromazine and its derivative melamine in poultry meats and eggs. *J. Food Drug Anal.* **2003**, *2*, 290–295.

(4) Sancho, J. V.; Ibáñez, M.; Grimalt, S.; Pozo, O. J.; Hernández, F. Residue determination of cyromazine and its metabolite melamine in chard samples by ion-pair liquid chromatography coupled to electrospray tandem mass spectrometry. *Anal. Chim. Acta* **2005**, *530* (2), 237–243.

(5) Yokley, R. A.; Mayer, L. C.; Rezaaiyan, R.; Manuli, M. E.; Cheung, M. W. Analytical method for the determination of cyromazine and melamine residues in soil using LC-UV and GC-MSD. *J. Agric. Food Chem.* **2000**, *48*, 3352–3358.

(6) U.S. EPA. Pesticides: Analytical Methods & Procedures-Residue Analytical Methods (RAM); http://www.epa.gov/oppbead1/methods/ramcl.htm#Cl.

(7) Jansson, C.; Pihlström, T.; Österdahl, B.-G.; Markides, K. E. A new multi-residue method for analysis of pesticide residues in fruit and vegetables using liquid chromatography with tandem mass spectrometric detection. *J. Chromatogr., A* **2004**, *1023* (1), 93–104.

(8) Picó, Y.; Font, G.; Moltó, J. C.; Mañes, J. Pesticide residue determination in fruit and vegetables by liquid chromatography−mass spectrometry. *J. Chromatogr., A* **2000**, *882* (1−2), 153–173.

(9) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC. Int.* **2003**, *86*, 412–431.

(10) Schenck, F. J.; Hobbs, J. E. Evaluation of the quick, easy, cheap, effective, rugged, and safe (QuEChERS) approach to pesticide residue analysis. *Bull. Environ. Contam. Toxicol.* **2004**, *73* (1), 24–30.

(11) Payá, P.; Anastassiades, M.; Mack, D.; Sigalova, I.; Tasdelen, B.; Oliva, J.; Barba, A. Analysis of pesticide residues using the quick easy cheap effective rugged and safe (QuEChERS) pesticide multi-residue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Anal. Bioanal. Chem.* **2007**, *389* (6), 1697–1714.

(12) Nguyen, T. D.; Lee, B. S.; Lee, B. R.; Lee, D. M.; Lee, G.-H. A multiresidue method for the determination of 109 pesticides in rice using the quick easy cheap effective rugged and safe (QuEChERS) sample preparation method and gas chromatography/mass spectrometry with temperature control and vacuum concentration. *Rapid Commun. Mass Spectrom.* **2007**, *21* (18), 3115–3122.

(13) Anastassiades, M.; Mastovská, K.; Lehotay, S. J. Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. *J. Chromatogr., A* **2003**, *1015* (1−2), 163–184.

(14) Walorczyk, S. Development of a multi-residue screening method for the determination of pesticides in cereals and dry animal feed using gas chromatography-triple quadrupole tandem mass spectrometry. *J. Chromatogr., A* **2007**, *1165* (1−2), 200–212.

(15) Guidelines establishing test procedures for the analysis of pollutants. *Code of Federal Regulations*, Part 136, Appendix B; U.S. Government Printing Office: Washington, DC, 2002.