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Cyromazine Determination in Poultry based Animal Feedstuffs by HPLC/DAD using QuEChERS Methodology

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ABSTRACT

The extensive and uncontrolled use of the larvicide cyromazine used for the control of fly species in poultry operations may lead to residues in poultry based products as well as in higher melamine levels because of the in vivo metabolism of cyromazine. This has already caused the death of cats and dogs and therefore the tolerable daily intake has been re-evaluated by legislation authorities. The aim of this study was to develop a validated HPLC method for the determination of cyromazine in commercial poultry based animal feedstuffs. Solid phase dispersion applying the QuEChERS approach was used. Chromatographic analysis was performed by an Inertsil ODS -3 analytical column (5 µm, 250 × 4 mm) using a mobile phase of MeOH – H_2O (60-40% v/v), delivered isocratically. Cyromazine was detected at 220 nm. Validation was performed in terms of linearity, sensitivity, specificity, accuracy, repeatability and stability. The LOD of the method was found at 0.03 mg / kg, the LOQ was found 0.1 mg / kg, while the linearity extends up to 10 mg / kg. Absolute recovery achieved by the proposed methodology was 78 (±3) %. Several samples from the local market were examined, however no cyromazine residues were identified.

Keywords: cyromazine, melamine, animal feedstuff, HPLC, QuEChERS

INTRODUCTION

The larvicide cyromazine (N-Cyclopropyl-1,3,5-triazine-2,4,6-triamine) is a cyclopropyl derivative of melamine (2,4,6-Triamino-1,3,5-triazine). Their chemical structures can be found in **Figure 1**. Cyromazine is an insecticide approved for use in the EU and in other countries. It is highly soluble in water and relatively volatile. It may be persistent in soil and water systems depending on local conditions. It has a low mammalian toxicity, hence it may cause adverse effects on reproduction. Its use as an insecticide and insect growth regulator is based to its activity by disturbing the nervous system of the immature larval stages of certain insects, mainly *Diptera larvae* and flies on livestock and other insect pests in the field and greenhouse. Therefore, it is used to control fly species, which develop in poultry manure and refuse. Moreover cyromazine may yield melamine as metabolism product. The toxicity of melamine is due to the crystal formation with endogenous uric acid or, cyanuric acid, a structural analogue of melamine, in renal tubules and this leads to acute renal failure [1,2].

Failure to follow directions for use of cyromazine (the active ingredient of Larvadex®) and respective precautions found on the label may result not only in poor fly control, but in illegal residues in the meat or eggs as well. Moreover suggested withdrawal times have to be strictly followed prior to slaughter [3,4].

A residual maximum of 50 ng/g for cyromazine in the edible parts of eggs and poultry meat are allowed as stipulated by the US Code of Federal Regulations (1987); however, residual levels and cyromazine inclusion levels in animal diets differ between countries depending on their application as a veterinary drug [5,6].

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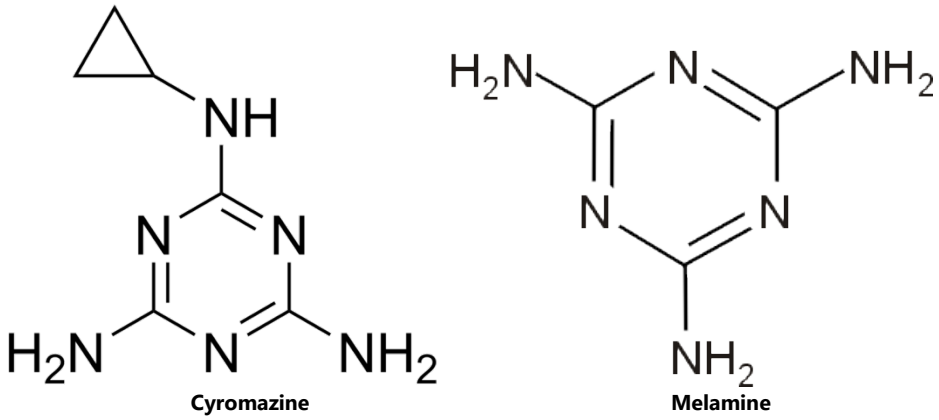

Figure 1. Chemical structures of cyromazine and melamine

Cyromazine is also used on field crops or sprayed onto fruits and vegetables. Patakioutas et al. have reported the detection of melamine residues of less than 1 mg/kg on the edible parts of crops (tomato, lettuce and celery), after applying cyromazine [7].

Additionally, illegal residues may be found also in crops if cyromazine if applied to manure used in cultivation of small grain crops that will be harvested or grazed [3, 8].

Food and Drug Agency in the USA has established as maximum daily intake 0.63 mg /kg body weight for melamine and 0.02 mg /kg for cyromazine, while the European Food Safety Authority (EFSA) has published a statement on the risks for public health relating to melamine in Chinese infant milk and milk products. This statement included an assessment of dietary exposure for European Union consumers based on imported products and compared these potential dietary exposures with the tolerable daily intake (TDI) of 0.5 mg/kg bw per day established by EFSA for melamine [9,10].

The United States Environmental Protection Agency have reported a dietary exposure estimate for cyromazine of 0.0013 mg/kg bw per day for the population in the USA. They have also stated that approximately 10% of cyromazine is converted to melamine in vivo [11,12].

A great number of studies can be found in literature concerning the determination of melamine and cyromazine in several matrices applying separation techniques, with various detectors such as diode array, or mass spectrometry, after a great variety of sample preparation approaches. These studies include milk, and baby food analysis especially due to the China melamine scandal which lead to the death of babies and infants. With regards to melamine and cyromazine in animal feed there are several studies for their simultaneous determination or single analyte methods. The Quick Easy Cheap Effective Rugged Safe (QuEChERS) approach for cyromazine has been applied only in one paper [13].

He et al used a water compatible molecularly imprinted polymer (MIP) using cyromazine as the template, methacrylic acid as the functional polymer and ethylene glycol dimethacrylate as the cross-linker to extract melamine from feed and milk samples by a MISPE optimized protocol. The cyromazine -MIP demonstrated high cross-reactivity for melamine and low affinity to cyanuric acid [14].

Wei et al reported a High-Performance Liquid Chromatographic method for the determination of insecticide cyromazine and metabolite melamine residues in milk and pork using a NH_2 column and 97% acetonitrile mobile phase. 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. 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 [15].

Xia et al reported on the analysis of cyromazine in poultry feed using the QuEChERS Method Coupled with LC-MS/MS, in 2010. In this method, QuEChERS approach was introduced for the first time. Extracts were further purified by Solid Phase Extraction (SPE) on C18 cartridges and finally filtered by 0.45 μm syringe Teflon microfilters prior to the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Recovery of 75.0 ± 6.2% was achieved. The method detection limit and the limit of quantitation were 0.028 and 0.094 ppm, respectively [13].

A study on the development and validation of a gas chromatography – mass spectrometry for the simultaneous determination of melamine and cyromazine in animal feeds was published in 2011. Sample pretreatment was based on trichloroacetic acid treatment. Recovery rates were within 84.2% and 99.5%. LOD and LOQ values were 0.03 mg / kg and 0.1 mg / kg respectively [16].

A QuEChERS dispersive extraction method was also proposed in 2015 by Tsartsali and Samanidou, for the isolation and clean- up of melamine and cyromazine from chicken egg yolk. Analytes are determined by high performance liquid chromatography (HPLC) using photodiode array detector (PDA). Extraction of isolated compounds was achieved by methanol and acetonitrile mixture (1:1, v/v). Recovery rates ranged between 74.5%-115.8% [17].

Since QuEChERS offers great advantages in the analysis of various food matrices as described in many literature sources [18-22], the aim of the present study was to apply QuEChERS methodology to develop a validated HPLC-DAD method for the determination and quantification of cyromazine in commercial poultry based animal feed.

MATERIALS AND METHODS

Instrumentation

A quaternary HPLC system, consisted of a pump LC – 20AD Shimadzu (Kyoto, Japan), for mobile phase delivery, a diode array detector Shimadzu SPD-M20A, with a Software Shimadzu LC-Solution (Single-PDA) and a degasser Shimadzu DTO-20A. Analytes were separated on an Inertsil ODS -3 (5 μm, 250 × 4 mm) column by MZ Analysentechnik (Mainz, Germany). A Rheodyne 7725i injection valve (Rheodyne, Cotati California, U.S.A), with a 50 μL loop, was used for sample introduction.

Sonication was performed using an Elmasonic S40 H (40 Hz, 340 W, Elma, Singen, Germany). Mobile phase water was filtered by Whatman (Buckinghamshire, England) using membrane cellulose nitrate filters 0.2 μm Whatman GmbH (Dassel, Germany). A mixing device MIX by FALC (Treviglio, Italy) was used for sample homogenisation. An analytical balance ±0.0001 g KERN & Sohn Gmbh, ALS-220-4 (Balinden, Germany) was used for the preparation of samples. Samples were centrifuged in an EBA 20 Hettich centrifuge (Balingen, Germany). A 20 fold (SPE) Vacuum Manifold Agilent Technologies, (Santa Clara, USA), a 6 port Mini-Vap Supelco (St Luis, USA) were used for SPE and evaporation.

SPE cartridges SEP-PAK Vac (6 cc, 500 mg) from Waters (Wilmslow, England), LiChrolut RP-18 (500 mg, 3 mL) from Merck (Darmstadt, Germany), AccuBond II ODS-C18 Cartridges (500 mg, 3 mL) from Agilent Technologies (Santa Clara, California, USA), ABS ELUT-NEXUS (60 mg, 3 mL) from Agilent Technologies (USA). QuEChERS Dispersive SPE 2 mL (Bondelut from Agilent), PSA 50 mg, C18ec 50 mg, $MgSO_4$ 150 mg were used for sample preparation.

Syringe microfilters Q-Max® (25 mm, membrane-0.22 μm) Frisenette Aps (Knebel, Denmark) were used for sample filtration prior to HPLC analysis.

Materials and Methods

Reagents used in the study were: HPLC Lichrosolv® methanol by Merck (Darmstadt, Germany), HPLC acetonitrile by Fisher Scientific (Loughborouh, Leicestershine, UK), ammonium acetate and NaCl, acetone), n-hexane, cyclohexane by Merck (Darmstadt, Germany) and triethylamine p.a. by Merck (Darmstadt, Germany), melamine > 99% Sigma – Aldrich (St. Luis, USA), cyromazine > 99,8% Sigma – Aldrich (St. Luis, USA), caffeine > 99% from Sigma – Aldrich (St. Luis, USA). Water Membrapure (Henigsdorf, Germany) was used throughout study.

Chromatographic Conditions

An Inertsil ODS - 3 (5 μm, 250 × 4 mm) column was found to be suitable for the separation under isocratic elution, using Methanol-water 60-40 % v/v, at a flow rate of 1 mL/min, at ambient temperature, within 5 min. Retention time of melamine was 2.651 min, of cyromazine 3.425 min and of caffeine 4.251 min, which was evaluated as internal standard at the concentration of 1 ng/μL. Backpressure observed was 190-200 bar and injected volume was 50 μL. Analytes were detected by DAD at 220 nm.

Standard Solutions

Stock aqueous solutions of melamine, cyromazine and caffeine were prepared at 100 ng/μL and stored at 4°C. These were found to be stable for at least one month. Working standards were prepared by serial dilution at 0.1 ng/μL, 0.2 ng/μL, 0.5 ng/μL, 2 ng/μL, 5 ng/μL and 10 ng/μL.

Sample Preparation-SPE Evaluation

Various SPE sorbents were initially investigated for the isolation of cyromazine and melamine extraction from standard solutions (10 ng/μL), by applying different protocols (data not shown), which were not further used as

they were not able to sufficiently purify the matrix. The following cartridges: SEP-PAK Vac (6 cc, 500 mg) from Waters (Ireland), LiChrolut RP-18 (500 mg, 3 mL) from Merck (Germany), AccuBond II ODS-C18 (500 mg, 3 mL) Agilent Technologies (UK), ABS ELUT-NEXUS (60 mg, 3 mL) Agilent Technologies (USA) were investigated. The generic protocol used was:

1. Cartridge conditioning with 2 mL CH_3OH and 2 mL H_2O.
2. 200 µL Sample loading.
3. Washing with 200 µL H_2O of high purity Membrapure water.
4. Analytes' elution using various solvent systems.
5. Evaporation under gentle nitrogen stream at 30-40°C.
6. Reconstituted with 200 µL with Membrapure H_2O.

Sample Preparation of Poultry based Animal Feed (Dog and Cat)

A quantity of 0.3 g of homogenized commercial dog or cat food was spiked with 300 µL cyromazine standard at 10 ng/µL. A volume of 300 µL water was added for blank samples.

A volume of 1.5 mL of MeOH – H_2O (50 – 50 % v/v), was added and the sample was sonicated for 10 min and centrifuged at 3500 rpm for 10 min.

The supernatant was transferred by a Pasteur pipette to a glass beaker and filtered by 0.22 µm membrane syringe filters. The filtrate was added to a vial containing QuEChERS sorbent and was vortexed and centrifuged for 10 min. The supernantant was removed and transferred to clean tubes and subsequently evaporated to dryness under gentle nitrogen stream at 30°C and reconstituted to 300 µL MeOH prior to HPLC analysis.

Defatting of animal feed was also examined. A quantity of 0.3 g animal feed was extracted twice by n-hexane, cyclohexane, their mixture (1:1v/v) and triethylamine. After addition of 1.5 mL H_2O – MeOH (50 – 50% v/v), the sample was sonicated for 10 min, centrifuged for 10 min filtered and applied to SPE for further clean up and elution by methanol or acetonitrile prior to injection to the HPLC-DAD. Washing step included H_2O – MeOH (90 – 10 % v/v), as well as H_2O – acetone (90 – 10 % v/v), but with no satisfactory results.

The same procedure was applied with no defatting step by adding 5 mL of H_2O – MeOH (50 – 50 % v/v) to 0.3 g.

Method Validation

Method validation was performed in terms of linearity, sensitivity, selectivity, accuracy, precision, repeatability and stability.

Linearity was checked using seven standard solutions 0.1 and 10 ng/µL. Linearity was also checked for spiked dog feed in the range 0.1 mg/kg and 10 mg/kg. LOD and LOQ values were calculated using the 3.3×S/N formula, and 10×S/N respectively, where S is the signal and N is the noise in the blank.

Selectivity of the method was investigated by the analysis of various dog and cat feed samples. The accuracy and precision of the method was examined at three concentration levels of spiked animal feed samples, of final concentrations 0.3 mg/kg, 5 mg/kg, and 10 mg/kg. Within-day repeatability of the method was examined by six replicates within day. Intermediate (between-day) precision) was evaluated by duplicates in a period of seven days at two concentration levels: 0.3 mg/kg and 10 mg/kg.

Stability was evaluated by the analysis of spiked samples at 4°C, after one day, one week and one month. Samples were found to be stable for one month according to degradation criterion of 10%.

RESULTS AND DISCUSSION

A typical chromatogram of standard solution at 5 ng/µL, using the chromatographic conditions, as described in experimental part, is illustrated in **Figure 2**.

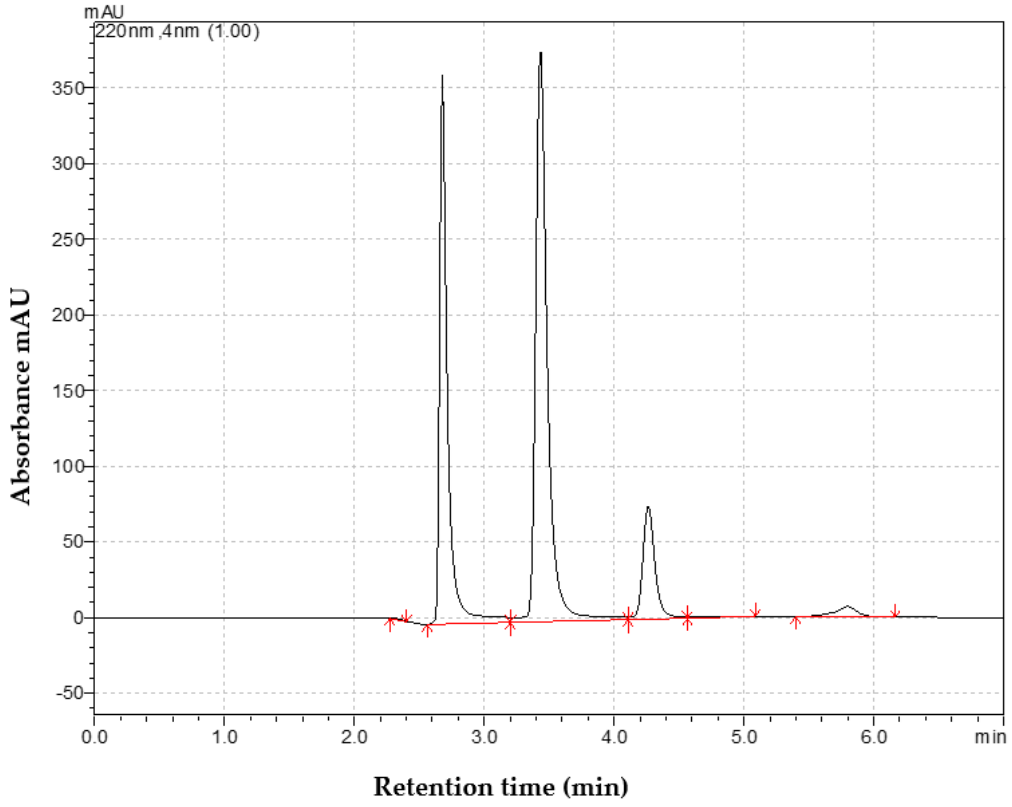

Figure 2. Typical chromatogram of standard solution at *5* ng/μL. *Mel: 2.651 min, Cyr: 3.425 min, Caf: 4.251 min.*

Table 1. Melamine and cyromazine recovery rates from standard solutions after SPE

SPE cartridge– eluent solvent	Recovery %	
	Melamine	Cyromazine
Oasis – 2 mL MeOH	105.4	100.5
Nexus – 2 mL MeOH	89.5	87.8
Lichrolut – 2 mL MeOH	95.3	88.4
SEP – PAK - 2 mL MeOH	96.5	95.4

Resolution factor is 2.3 for melamine and cyromazine and 1.9 for cyromazine and caffeine.

Sample Preparation

Best SPE results were obtained by SEP- PAK and OASIS with 2 mL MeOH as eluent solvent. Recovery rates using standard solutions were 96.5% for melamine and 95.4% for cyromazine. The other two examined sorbents gave also considerable amounts. Results are shown in **Table 1**. However they were not eventually applied, since they were not sufficiently purifying sample matrix.

No satisfactory results were obtained even after trying various combinations of SPE clean up, protein denaturation, and defatting. The most promising approach was dispersive SPE with QuEChERS, which offered good results for cyromazine, however not for melamine.

Further purification by SPE with above described protocols and elution by methanol no satisfactory clean-up was achieved.

The addition of acetonitrile for protein denaturation did not improve the efficiency of sample purification method.

Due to the fact that clean-up was not effective for melamine, the method was validated for cyromazine. External calibration was performed since caffeine could not be used as internal standard due to co-elution of endogenous interferences. Recovery rates of cyromazine after QuEChERS approach was 78.0 (±3.0) % from dog food and 88.0 (±2.5) % from standard solutions.

Figure 3 illustrates a blank chromatogram of dog feed and **Figure 4** a spiked one at 10 mg/kg.

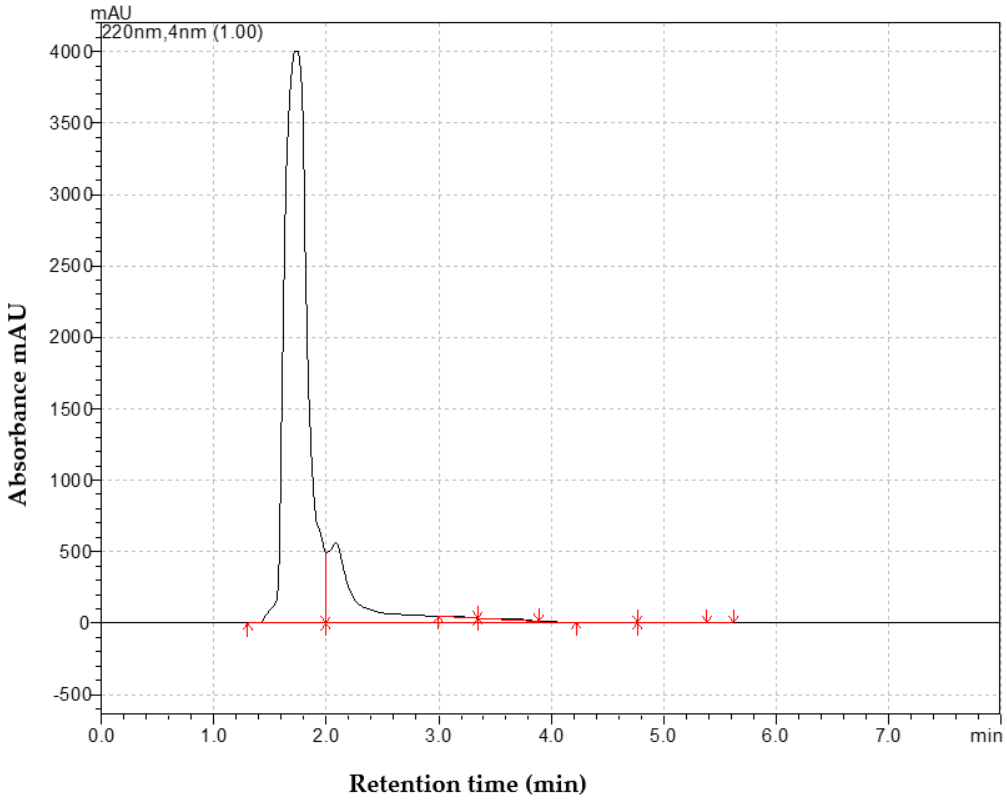

Figure 3. HPLC chromatogram of blank sample of dog feed

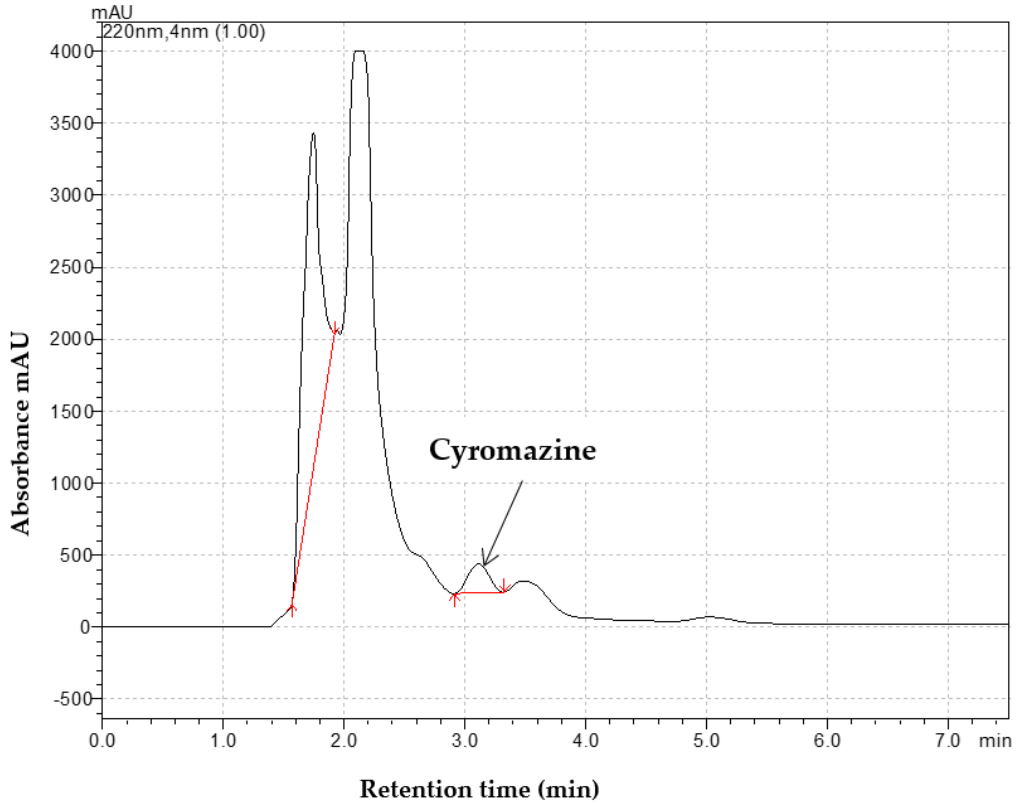

Figure 4. HPLC chromatogram of dog feed spiked at 10 mg/kg

Table 2. Linear regression analysis data, where: y= peak area and *x concentration of standard solution* ng/μL

Analyte	Regression equation	Coefficient of determination R^2
Melamine	y = 372760x + 37604	R^2 = 0.9994
Cyromazine	y = 420386x + 104535	R^2 = 0.9963

Table 3. Repeatability of cyromazine determination (n=6 replicates)

Concentration level (mg/kg)	Found ± SD (mg/kg)	Recovery (%)	RSD
0.3	0.296±0.02	98.7	6.7
5	4.7±0.28	94.0	5.9
10	9.58±0.55	95.8	5.5

Table 4. Between day precision and accuracy of cyromazine determination (n=2×7 days)

Concentration level (mg/kg)	Found ± SD (mg/kg)	Recovery (%)	RSD
0.3	0.282±0.025	94.0	8.9
10	9.88±0.59	98.8	5.9

Table 5. Stability study of cyromazine in spiked feed samples

	Recovery (%)
Stability at *4*°C	
1 day	99.9
1 week	98.0
1 month	97.5

Method Validation

Method validation was performed in terms of linearity, sensitivity, selectivity, accuracy, precision, repeatability and stability.

Linearity

Satisfactory linearity was observed with regards to standard solutions as shown in **Table 2**.

Respective regression equation using spiked dog feed was y = 291206x + 65805 with coefficient of determination, R^2 = 0.9972, where *x*= cyromazine concentration (mg/kg). Negative matrix effect is observed for cyromazine due to the complexity of animal feed sample.

Method's LOD was found to be 0.03 mg/kg, while LOQ was 0.1 mg/kg. Linearity was observed up to 10 mg/kg.

Selectivity

Selectivity of the method was investigated by the analysis of various dog and cat feed samples.

As shown by the chromatograms in **Figures 3** and **4**, melamine could not be resolved from endogenous matrix components. Therefore the method can be characterized as selective only for cyromazine, since no endogenous analytes were eluted at the retention time of the latter.

Identification has been performed in terms of spectra provided by PDA detector for respective standards and peak purity wa-s taken into account (data not included).

Accuracy and precision

Accuracy and precision results are shown in **Table 3**, while intermediate (between-day) precision) results are summarised in **Table 4**.

Stability

Samples, as shown in **Table 5**, were found to be stable for one month according to degradation criterion of 10% [23].

Real sample analysis

Ten samples, namely five dog and five cat poultry based feedstuffs from local market were analysed by the method as described at the experimental part. However no cyromazine residues were detected.

CONCLUSIONS

Cyromazine, the active ingredient of Larvadex®, which is included in laying-hen diets for fly control, in and around chicken layer and breeder operations may result in the meat or eggs, which can be further used for house pet animal feedstuff production. Cyromazine is in vivo metabolized to melamine, which leads to acute renal failure.

Herein a simple and fast method using QuEChERS approach is developed and applied to commercial animal feed. Method validation was performed in terms of linearity, sensitivity, precision, accuracy, selectivity, repeatability and stability.

At the specific chromatographic condition retention time of cyromazine was 3.410 min. LOD was 0.03 mg/kg, and linearity holds up to 10 mg/kg LOQ was 0.1 mg/kg with recovery rates at 78 (±3)%. Spiked samples were found to be stable at least for one month where stored refrigerated.

The described method is quick, easy and friendly to the environment. . Since it requires no highly sophisticated mass spectrometry hyphenated to liquid chromatography instrumentation, it can be readily applied in any laboratory. Melamine though could not be isolated from the background interference and this is the main drawback of the determination. The analysis of real samples from local market revealed no cyromazine levels.

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