**QUANTIFICATION OF CYROMAZINE AND MELAMINE IN FISH AND POULTRY FEEDS BY HIGH PERFOMANCE LIQUID CHROMATOGRAPHY – DIODE ARRAY DETECTION**

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**Abstract**

This paper reports the quantities of cyromazine and its metabolite melamine used as additives in fish and poultry feeds. The two triazine compounds were assayed in fish and poultry feeds obtained from Ilaro, Ogun State in Nigeria by high-performance liquid chromatography coupled with diode-array detection (HPLC-DAD). Fish and poultry feed samples meant for various sizes of fishes and different types of chickens, respectively produced locally and imported were after sampling pulverized, treated with acetonitrile and adjusted to pH 7 with phosphate buffer prior to extraction with alkaline acetonitrile (acetonitrile: 25% ammonia solution (95/5; v/v)) from solid-phase extraction (SPE) cartridges previously conditioned with methanol and phosphate buffer. The extracts were concentrated to 2 mL in a water bath at 20oC, filtered through a 0.45 µm syringe and separated on a Zorbax eclipse plus C18 column (150 x 4.6 mm, 5 µm) with mobile phases consisting of acidified ultrapure water and acetonitrile in gradient elution mode within a run time of four minutes. The two triazine compounds considered in this study were present at a lower concentration in the feeds than the permissible level, but their combined effects could be a potential health risk. It is concluded that their presence resulted from the widespread use of materials that contain melamine and not from adulteration or misuse.

**Keywords:** Baseline levels, feeds, feed additives, HPLC-DAD, quantification, triazine compounds.

**1. Introduction**

The analysis of feeds for cyromazine and its metabolite, melamine; a dealkylation product from animal and plant metabolism of cyromazine [1] as a safety measure becomes necessary because of their health effects that include urolithiasis resulting from renal failure and bladder cancer [1] Besides, these nitrogenous compounds could be fraudulently added to poultry and fish feeds to increase their apparent protein content since the price of feeds depends on the protein content. Lately, there have been reports of detection of melamine (up to 150 mg/kg) in fish meal and fish feed in different countries [2], thus raising fears about its consequent transfer to the human food supply system.

 

Cyromazine Melamine

N-Cyclopropyl-1,3,5-triazine-2,4,6-triazine 1,3,5-triazine-2,4,5-triamine

CAS No: 66215-27-8 CAS No: 108-78-1

Melamine is useful in the manufacture of formaldehyde resins intended for the production of seals, plastics, coatings, commercial filters, adhesives, dishware and kitchenware while its alkaline hydrolysis yields structurally related compounds that may include ammelide, ammeline and cyanuric acid, and are usually added to foods feeds to falsely increase their apparent protein contents [3]. Melamine had also been reportedly used as a pelletizing agent in the production of fish feed. Cyromazine is additionally used as a pesticide to control insects by inhibiting their metamorphosis in crops and animal feed production; it is equally included in poultry feeds to control flies thereby reducing the environmental menace associated with poultry production, thus resulting in the possibility of melamine being detected in the tissue or eggs [4]. Melamine, and its metabolic and degradation products have not been permitted as direct additives in feeds [3], their traces, however, may be detected in feeds due to crops fertilized with melamine related products, or as a breakdown product from cyromazine that has been included as a veterinary drug. The sources of melamine and its analogues in feed are divided into baseline and adulteration levels. While the latter refers to the intentional addition, unapproved use, or misuse of substances that can degrade to form melamine, the former is the presence of concentration that results from the widespread use of materials that contain melamine and not from adulteration or misuse [5].

The routine analysis of poultry and fish feeds for safety did not reveal the presence of melamine or its analogues because they mimic proteins when tested based on the Kjeldahl method [3]; and since they are not included on the target list of compounds for control, this has resulted in several illnesses and deaths across countries including Italy, China, USA, Netherlands, and France [5, 6]. Consequently, an international safe limit of 4.5 and 2.5 mg/kg have been set for cyromazine and melamine, respectively in feeds [7-9].

Nigeria depends largely on imports for the supply of its fish and poultry feed ingredients [10], without comprehensive information on nutritional composition. Unfortunately, there is no national or established limit for melamine or cyromazine in feeds in Nigeria, and still no literature on its detection in feeds. Meanwhile, Nigeria imports foods and feed ingredients from countries where melamine contamination had been reported. Consequently, an all-inclusive analyses of these ingredients and finished feeds are necessary to forestall the occurrence of prohibited additives and their attendant health issues. This study, therefore, aims to investigate the presence of cyromazine and its metabolite melamine in fish and poultry feed samples available in Ilaro, Ogun state, Nigeria.

**2. Materials and Methods**

**2.1 Chemicals and reagents**

Melamine 99% (Sigma-Aldrich Missouri. USA.), cyromazine, HPLC grade methanol, acetonitrile, sodium dihydrogen phosphate, disodium hydrogen phosphate, and ammonia solution were obtained from Merck life Scientific Industries (Darmstadt, Germany), formic acid 90% was purchased from M&B (May and Baker) England, Water was purified using Milli-Q system and solid-phase extraction (SPE) cartridges were obtained from Agilent Technology (California, USA).

**2.2 Standards**

500 µg/mL standard solutions of the two triazine compounds were prepared by accurately weighing and dissolving 5 mg standard in 5 mL formic acid:water (50:50 v/v) and preserved at 4 oC. Working solutions were prepared.

**2.3 Fish and poultry feed samples**

Fifty domestic and imported fish feed samples with various brand names and sizes of between 1.5 and 6.0 mm meant for fingerlings, juvenile, post-fingerlings and table size fishes whose weight ranged from 3 - 1000 g were collected from fish farms and stores in Ilaro, Nigeria. The declared protein content as per the labels on the various fish feed samples ranged between 27 and 45 %, and they were all manufactured in 2018. Also, fifty (50) poultry feed samples (branded and locally formulated) consisting of broilers, growers, finisher and layers mash were sampled from various poultry farms and markets in Ilaro, Nigeria. Samples upon collection were carefully labelled, and on arrival in the laboratory were pulverized with a laboratory miller (RETSCH MM 400, Fisher New Hampshire USA) to pass through a 2 mm sieve (FisherbrandTM , Sigma-Aldrich, St. Louis, MO, USA) wrapped in an aluminium foil and kept in the freezer until extracted.

**2.4 Sample extraction and clean-up**

The samples were extracted and cleaned-up following a previously described method with modification [11]. A 3.0 ± 0.1 g of each pulverized sample was weighed into a 50 mL beaker and extracted with 15 mL of acetonitrile and 30 mL of 0.05 M phosphate buffer, pH 7.0, it was sonicated (Sonicator - 300VT, BioLogics Instruments, Manassas, USA) for 10 mins, and thereafter vortex mixed for 10 mins using a vortex mixer (VM18, Schiltern Scientific, Beds, UK). It was centrifuged (Centrifuge-34b187, Thermo Scientific, Swedesboro, USA) at 3,500 rpm for 20 mins and the supernatant was collected. C18 SPE columns (SupelcleanTM, Sigma-Aldrich, St. Louis, MO, USA) were conditioned with 10 mL each of methanol and 0.05 M phosphate buffer, pH 7.0, respectively, and loaded with the supernatants. After complete effusion, the cartridges were washed with 10 and 5 mL of deionized water and methanol, respectively, and entire effluent discarded.Melamine and cyromazine were thereafter eluted with 4 mL of alkaline acetonitrile (acetonitrile: 25% ammonia solution (95/5; v/v)). The eluent was evaporated to 2 mL at 40°C in a water bath, and filtered through a 0.45µm syringe (Acrodisc syringe filters, GHP membrane, diam. 25 mm, pore size 0.45 μm, Sigma-Aldrich, St. Louis, MO, USA) into a vial for analysis.

The extracts were determined on an Agilent HPLC (Agilent. Technology 1200 series, Agilent Technologies, Germany) with Zorbax Eclipse plus C18 (Dimensions: 150 x 4.6 mm, 5 µm particle) column also from Agilent Technology. The mobile phases consisted of acidified purified water and acetonitrile (30:70) in gradient at a flow rate of 0.5 mL /min with an injection volume of 5 µL. Analytes were measured at 214 nm with a diode array detector (DAD).

**2.5 Statistical analysis**

Microsoft excel was used for data entry and descriptive statistics, while SigmaPlot version 14 (Systat Software, USA) was used for statisical analysis. Single factor ANOVA was used to test for significant differences among different pairs.

**3. Results and discussion**

The two triazine compounds were eluted singly from the column upon the optimisation of chromatographic conditions, and their different retention times obtained. A 10 point calibration curve was prepared with the mixed standards of the compounds at a concentration range of 0 – 1000 µg/kg. Also, using the standard’s retention time and the integrated peak area of the chromatograms, linear equations with r2 ≥ 0.9889 were obtained. Samples analytes were quantified from the linear equations using their various peak areas as obtained in the different chromatograms, thus giving the concentration. No interfering peaks were observed in the blank chromatograms at the quantification wavelength (214 nm, Fig. 1). The analytes were eluted with clear peak separation in less than 4 minutes. Melamine was first eluted followed by cyromazine with retention times of 3.028 and 3.464 minutes, respectively having good resolution and symmetric peaks.

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**Fig. 1: Chromatogram for melamine and cyromazine standards**

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**Fig. 2: A representative chromatogram for melamine and cyromazine in feed samples**

Ten samples were assayed for each of the five different fish feed types of sizes one and half, two, three, four, and six millimetres for fishes whose weight ranged between 3 and 1000 g. Table 1 shows the levels of melamine and cyromazine in each feed type. Nine of the ten one and half millimetre sample contained melamine and cyromazine while a sample had neither of the two triazine compounds. The maximum levels of melamine and cyromzine in the sample were 198 and 190 µg/kg, respectively.

**Table 1: Levels of melamine and cyromazine in fish feed samples (µg/kg)**

|  |  |  |
| --- | --- | --- |
| Sample (mm) | Melamine  (Mean ± SD) | Cyromazine  (Mean ± SD) |
| 1.5 | 93.13±49.76aA | 109.00±62.47dA |
| 2.0 | 561.5±395.12bA | 359.9±277.96dA |
| 3.0 | 279.17±236.08cA | 254.25±199.97dA |
| 4.0 | 123.43±74.32dA | 100.29±88.63dA |
| 6.0 | 447.00±294.04dA | 348.29±217.03dA |

Means in rows and columns with different alphabets are significantly different (p<0.05). Upper and lowercase alphabets are for rows and columns, respectively.

All of the ten two millimetre fish feeds contained both melamine and cyromazine at 987 and 752 µg/kg, respectively. Meanwhile, only six of the ten three millimetre sample had melamine with the highest level been 650 µg/kg; eight samples had cyromazine with 630 µg/kg as highest. The four and six millimetre fish feeds had seven and five samples containing melamine, respectively, while seven of the ten samples examined in each case also contained cyromazine. Maximum melamine levels of 210 and 699 µg/kg were obtained in the four and six millimetre samples, respectively, whereas they were 264 and 700 µg/kg for cyromazine. There was significant difference (p < 0.05) between the mean levels of melamine in the 1.5, 2.0, and 3.0 mm fish feed samples, while no such difference was observed for the 4.0 and 6.0 mm samples. There was no difference between the mean levels of cyromazine in the poultry feeds, and also between melamine and cyromazine in the feeds.

**Table 2: Levels of melamine and cyromazine in poultry feed samples (µg/kg)**

|  |  |  |
| --- | --- | --- |
| Sample | Melamine  (Mean ± SD) | Cyromazine  (Mean ± SD) |
| Broiler starter | 56.11±43.86 dA | 149.20±153.57 Da |
| Broiler finisher | 52.22±51.49 dA | 157.00±121.54 dA |
| Grower | 31.5±22.89 dA | 169.00±113.66 dA |
| Layers mash | 57.00±29.09 dA | 274.4±249.14 dA |

Means in rows and columns with different alphabets are significantly different (p<0.05). Upper and lowercase alphabets are for rows and columns, respectively.

All the poultry feed samples contained cyromazine at 149.20±153.17 and 274.4±249.14 µg/kg, (Table 2). The maximum concentration of cyromazine obtained in all the poultry feed samples were below the highest limit of 2.5 mg/kg set for the compound. Melamine was also found in some of the poultry feed samples, but at concentrations far below the set limit (4.5 mg/kg).

Animal feeds have been reported to contain 3.3 to 21 000 mg/kg of melamine, while whole eggs, dried eggs, dried egg powder and liquid eggs also contained 0.1-5 mg/kg melamine [4], further demonstrating that carry-over from feed to fishes and eggs does occur. Yan et al. [13] had reported 3.5 mg/kg of melamine in fish feeds from China, while Xia et al. [14] reported 2.7 to 6.3 mg/kg of cyromazine in eight commercially available poultry feed samples from the USA.

The levels of melamine in feeds from these previous studies are much higher compared to what obtains in the present study, but becomes worrisome when the melamine is considered alongside cyromazine for co-exposure since they are similar. Meanwhile, melamine accumulates in tissues of fish when fed with feeds with lower concentration of the additive. Andersen et al. [15] in a survey of market-ready cat fish and other fishes reported melamine at a concentration of 50-237 µg/kg. The consumption of melamine in fish and other foods at above 50 µg/kg is likely to present a health risk [15] that might be greater in the presence of cyromazine. Melamine found at 13.9-294 µg/kg in animal feed was reported as an important source of melamine in foodstuffs collected from Albamy, New York United States [16], corroborating an earlier report from France by Valat et al. [17]. The level of melamine in the reports is in the range of melamine found in the present study. The authors also asserted that the presence of melamine with its analogues elevates its toxic potentials, thus the presence of cyromazine as found though little in the feeds could also elicit the negative implications of melamine. However, Christogiorgos et al. [18] did not detect melamine and cyromazine in poultry and animal feedstuffs obtained in Greece.

**4. Conclusions**

The concentrations of melamine and cyromazine` found in this study could be considered as baseline, meaning they did not result from adulteration or misuse; but from concentrations of melamine present in the environment and in the food chain as a result of the wide-spread use of materials that contain melamine. The use of environmentally friendly additives and probiotics is encouraged to forestall the negative consequences of unauthorised additives while our regulatory agencies should be proactive to check the activities of feed manufacturers.

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