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

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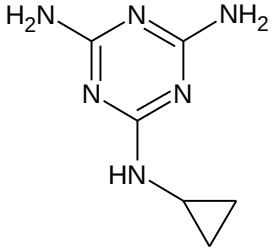
## ABSTRACT

This paper reports the quantities of cyromazine (CYR) and its metabolite melamine (MEL) used as additives in fish and poultry feed. The levels of triazine compounds in fish and poultry feed were determined using 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, were sampled. Samples were extracted using a solid-phase extraction (SPE) consisting of alkaline acetonitrile and phosphate buffer. The extracts were analysed using an Agilent HPLC and a Zorbax Eclipse plus C18 column. The detection and quantification limits (LOD and LOQ) were 1.29 – 1.48 and 3.94 – 4.50 µg/kg for MEL and CYR, respectively. The regression ($r^2$ = 0.989), recovery (99.5 – 102.5%) and precision (RSD < 1) were excellent. Melamine concentration ranged between 85.2±14.3 and 520.0±90.3 µg/kg in fish feed, while CYR was 98.5±9.7 to 345.0±37.7 µg/kg in the samples. The chicken feed had 31.7±6.0 to 54.8±5.7 µg/kg MEL, and 117.6±24.8 to 257.6±16.6 µg/kg CYR. There was no significant difference in MEL concentration of fish feed ($p>0.05$), while there existed a difference a difference between MEL and CYR ($p<0.05$). There existed a significant difference between MEL concentrations in the poultry feed ($p<0.05$) and no difference between MEL and CYR. The two triazine compounds considered in this study were present at a lower concentration than the permissible level.

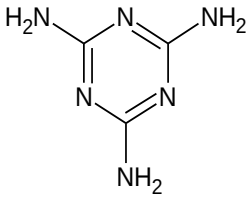
**Keywords:** Cyromazine; Feed additives; HPLC-DAD; Melamine; Quantification.

## INTRODUCTION

The analysis of feeds for cyromazine (CYR) and its metabolite, melamine (MEL) (Figures I and II); a dealkylation product from animal and plant metabolism of CYR as a safety measure becomes necessary because of their health effects that include urolithiasis resulting from renal failure and bladder cancer [1]. Melamine and cyanuric acid have in recent studies been implicated in testicular lesions, ovarian cyst and female reproductive dysfunction in rats [2].




Cyromazine
N-Cyclopropyl-1,3,5-triazine-2,4,6-triazine
CAS No: 66215-27-8




Melamine
1,3,5-triazine-2,4,5-triamine
CAS No:108-78-1

Besides, these nitrogenous compounds could be illegally added to poultry and fish feeds to increase their apparent protein content since the price of feeds depends on the protein content [3-5]. Lately, there have been reports of detection of melamine (up to 150 mg/kg) in fish meal and fish feed in different countries thus raising fears about its consequent transfer to the human food supply system since MEL is deposited as crystals of melamine cyanurate in kidneys [6,7].

Melamine is useful in the manufacture of formaldehyde resins intended for the production of seals, plastics, coatings, commercial filters, adhesives, dishware and kitchenware. The alkaline hydrolysis of MEL yields structurally related compounds that may include ammelide, ammeline and cyanuric acid, and are usually added to foods and feeds to falsely increase their apparent protein contents [8]. Melamine had also been used as a binding agent in the production of fish and shrimp feed [9]. Cyromazine on the other hand 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 and fish feed to control flies, and reduce the environmental menace associated with poultry and fish production. Thus, resulting in the possibility of MEL tainted poultry and fish products [4,10,11]. Melamine, and its metabolic and degradation products have not been permitted as direct additives in feeds, their traces, however, may be detected in feeds due to crops fertilized with MEL related products, or as a breakdown product from CYR that has been included as a veterinary drug [8].

The sources of MEL and its analogues in feed are divided into two viz baseline and adulteration levels. While the latter refers to the intentional addition, unapproved use, or misuse of substances that can degrade to form MEL, the former is the presence of concentration that results from the widespread use of materials that contain MEL and not from adulteration or misuse [12].

The routine analysis of poultry and fish feeds based on the Kjeldahl method did not reveal MEL or its analogues because they mimic proteins when tested on the basis of the method [8]. Furthermore, since they are excluded 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 [12,13]. Consequently, international safe limits of 4.5 and 2.5 mg/kg have been set for CYR and MEL, respectively, in feeds [14-16].

However, previous studies have established the presence of CYR and MEL and its analogues in fish and poultry feed stuffs and consequent transfer to their products [17]. Cyromazine was reported in commercial poultry feed in the USA at 2.7 to 6.3 mg/kg with some samples at a concentration level beyond the allowed maximum residue limit (MRL) of 5.0 mg/kg [4]. Melamine had been reported in fish feed and chicken eggs from China, and food items from certain parts of Canada [18-20].

Nigeria depends largely on imports for the supply of its fish and poultry feed ingredients, without comprehensive information on nutritional composition [21,22]. A large number of adulterated versions of imported fish feeds have been reported in Nigeria by fish farmers thus causing losses [23]. Unfortunately, there is no national or established limit for MEL or CYR in feeds in Nigeria, and still no literature on its detection in feeds. Meanwhile, Nigeria imports feed ingredients from countries including China and India where MEL contamination had been reported and punished with death and prison sentences [24]. Consequently, 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, investigates the presence of CYR and its metabolite MEL in fish and poultry feed samples available in Ogun state, Nigeria.

## MATERIALS AND METHODS

### 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).

**Standards**

Standard solutions (500 µg/mL) 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 $^{o}$C. Working solutions were also prepared.

**Fish and poultry feed samples**

One hundred 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 Ogun state, Nigeria. The declared protein content according to the labels on the various fish feed samples ranged between 27 and 45%, and they were all manufactured in 2018. Also, 100 branded poultry feed samples consisting of broilers, growers, finisher and layers mash were sampled from various poultry farms and markets in Ogun state, Nigeria. Samples were collected in Nalgene LDPE sample bags (Thermo Scientific, Massachusetts, USA) to maintain their integrity. The bags were carefully labelled, and on arrival in the laboratory were pulverized by their identity and sizes with a laboratory miller (RETSCH MM 400, Fisher New Hampshire USA) to pass through a 2 mm sieve (Fisherbrand™ , Sigma-Aldrich, St. Louis, MO, USA). They were wrapped in an aluminium foil and kept in the freezer until extracted.

The samples were extracted and cleaned-up following a previously described method with modification [25]. A 3.0 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.

The entire supernatant was loaded onto a C18 SPE column (Supelclean™, Sigma-Aldrich, St. Louis, MO, USA) previously conditioned with 10 mL each of methanol and 0.05 M phosphate buffer, pH 7.0, respectively. After complete effusion, the cartridges were washed with 10 and 5 mL of deionized water and methanol, respectively, and entire effluent discarded. Melamine and CYR 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.

**Chromatographic conditions**

The extracts were determined on an Agilent HPLC (Agilent. Technology 1200 series, Agilent Technologies, Germany) with Zorbax Eclipse plus $C_{18}$ (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). Quantification was performed by using the standard's confirmed retention times and the integrated peak area of the chromatograms, using linear equations. All chromatographic procedures were conducted at room temperature and in triplicate.

**Method validation for the extraction and SPE procedures**

The extraction and SPE procedures were validated as outlined in the EMA, 1995 guidelines [26]**.** The chromatographic conditions and the sensitivity, precision and robustness parameters were the same as those used in the validation method for quantification of MEL and CYR in the extracts. Extraction method efficiency, linearity, selectivity, limit of quantification (LOQ) and detection (LOD), precision, recovery and accuracy were tested. For the SPE procedure, linearity, selectivity, accuracy and precision, recovery, the limit of detection (LOD), and quantification (LOQ), stability and ruggedness were also evaluated. The efficiency of the extraction was computed by com-paring the peak areas of the analytes to those obtained by the analysis of spiked extracts of feed blank samples at six concentration levels (30 - 400

μgkg-1 for MEL and CYR). The linearity, linear range and sensitivity were established from the analytical curve obtained by six replicates of analysis for the two analytes at the concentrations described above in the feed matrix. Analytical curves were obtained by plotting the peak area versus the concentration of each analyte and evaluated by least squares regression analysis. The sensitivity is the slope of the calibration graph. The selectivity was determined by analysis of chromatograms of sample extractant solution without analytes to verify the absence of interferents. The accuracy was assessed through recovery tests conducted by adding known amounts (25, 50 and 75 μgkg-1 of MEL and CYR standards) to the sample at three different levels, three solutions each in triplicate. The per cent bias was determined by comparing the results of the analyses of the fortified samples. The limit of detection (LOD) and limit of quantita-tion (LOQ) were determined at the signal-to-noise ratios of 3 and 10, respectively, measured at the approximate retention time of the corresponding analyte peak.

**Statistical analysis**

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

## RESULTS AND DISCUSSION

The linearity, selectivity, precision, accuracy, and detection and quantification limits of the method were determined from calibration curves obtained by least-squares linear regression analysis of the peak area versus analyte concentration at six levels. The validation parameters, regression coefficients and range of linearity for MEL and CYR and external calibration are as shown in Table 1. The excellent linearity obtained for the range studied were higher than 0.98. Table 1 also gives the detection and quantification limits (LOD and LOQ) calculated. Recovery ranged between 99 and 102% for the two analytes at different spiking levels (Table 2). Precision was satisfactory because the RSD was < 1 (Table 2) in all instances. No interfering peaks were observed at the retention times corresponding to MEL and CYR matrix blank and extracts of spiked samples.

Table 1: Quantification of MEL and CYR

| | MEL | CYR |
|---|---|---|
| Linear range (μk/kg) | 30-400 | 30-400 |
| Regression equation | y = 16.5x + 1.81 | y = 75.76x + 4.45 |
| $R^2$ | 0.989 | 0.989 |
| Detection limits (μk/kg) | 1.48 | 1.29 |
| Quantification limits (μk/kg) | 4.52 | 3.94 |

Table 2: Accuracy of the recovery of the standard solution of MEL and CYR added to the samples

| Standard | Added conc. ($\mu gkg^{-1}$) | [a]Recovery (%) | RSD |
|---|---|---|---|
| MEL | 25 | 102.5 | 0.97 |
| | 50 | 99.5 | 0.48 |
| | 75 | 102.0 | 0.39 |
| CYR | 25 | 101.7 | 0.88 |
| | 50 | 102.0 | 0.65 |
| | 75 | 101.9 | 0.89 |

[a]Mean of triplicate determinations

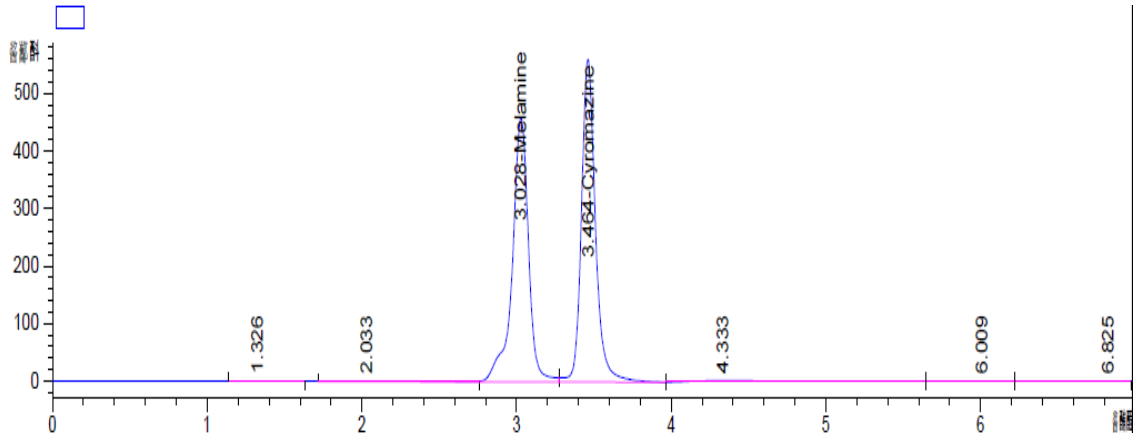



**Fig. 1: Chromatogram for MEL and CYR standards**

The 150 samples collected for the five fish feed types were analysed for MEL and CYR as revealed in Table 3.

Table 3: Description of MEL and CYR detected in fish feed samples

| Feed type (mm) | Usage | MEL | | | | | CYR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | n (%)[a] | Content (µg/kg) | | | | n(%)[a] | Content (µg/kg) | | | |
| | | | Percentiles | | Max | x̄ ±SD | | Percentiles | | Max | x̄ ±SD |
| | | | 75th | 95th | | | | 75th | 95th | | |
| 1.5 | Fingerlings | 27(48.2) | 97.5 | 99.6 | 101.4 | 85.2±14.3 | 27(40.7) | 112.7 | 116.7 | 118.1 | 107.2±8.s7 |
| 2.0 | Post-fingerlings and Juveniles | 26(61.5) | 547.5 | 693.9 | 694.8 | 520.0±90.3 | 26(50.0) | 328.8 | 356.7 | 359.8 | 287.7±64.2 |
| 3.0 | 50 – 150 g fishes | 36(36.1) | 279.8 | 258.8 | 301.8 | 258.2±37.5 | 36(44.4) | 258.8 | 270.2 | 279.8 | 244.5±24.8 |
| 4.0 | 150 – 400 g; 300 – 600 g fishes | 27(40.7) | 258.2 | 693.9 | 189.8 | 119.1±27.6 | 27(59.3) | 104.5 | 110.7 | 117.0 | 98.5±9.7 |
| 6.0 | 600 – 1 kg fishes | 34(52.9) | 440.4 | 453.7 | 470.6 | 411.5±38.5 | 34(38.2) | 359.7 | 400.9 | 401.6 | 345.0±37.7 |

x̄±SD (Mean±Standard Deviation); [a]Positive detection (detection frequency, %)

The distribution of MEL and CYR in fish feed samples are as indicated in Table 3. The 2.0 mm feed had the highest concentration of MEL, per cent frequency of occurrence, and the maximum MEL load. The use of 2.0 mm feed cuts across several stages of fish production since it is well suited for different fish sizes as a floating and sinking feed. Fish feed meant for the biggest (market-ready) size of fish (6.0 mm) had MEL next to the 2.0 mm. The per cent frequency of occurrence was higher than those of 1.5, 3.0, and 4.0 mm, respectively. The high level of MEL in the 6.0 mm sample is attributable to the efficient use of dietary protein by big size fishes for energy. Cyromazine was highest in the 6.0 mm fish feed with a 38.2% occurrence frequency. The concentration of CYR in the 6.0 mm feed is attributable to its sinking ability. And also, the need to increase the apparent protein content of feed becomes necessary since the feeding requirement of fish reduces with age and size [27]. Uneaten feed degrades water quality, and thus the addition of CYR to 6.0 mm prevents fouling resulting from an uneaten feed.

Both MEL and CYR were determined in the different poultry feeds, as shown in Table 4. The layers' mash had the highest concentration of the two analytes with respect to frequency of occurrence and mean concentration. The occurrence of MEL and CYR in broiler starter presented a scenario similar to that of the layers' mash. Using F-statistics as indicated in Table 5, there was no significant difference in the concentration of the analytes in the fish feeds. However, there was a difference in the fish feed types. A reversal of these observations was made for the poultry feeds

Table 4: Description of MEL and CYR detected in poultry feed samples

| Feed type | MEL | | | | | CYR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n (%)[a] | Content (µg/kg) | | | | n(%)[a] | Content (µg/kg) | | | |
| | | Percentiles | | Max | x̄ ±SD | | Percentiles | | Max | x̄ ±SD |
| | | 75th | 95th | | | | 75th | 95th | | |
| Broiler starter | 34(23.5) | 58.6 | 61.5 | 61.8 | 54.3±5.8 | 34(26.5) | 150.5 | 167.9 | 167.9 | 142.0±17.5 |
| Broiler finisher | 36(27.8) | 52.7 | 59.0 | 61.9 | 51.4±5.4 | 36(44.4) | 139.0 | 150.9 | 157.0 | 117.6±24.8 |
| Growers | 34(38.2) | 33.8 | 41.9 | 46.0 | 31.7±6.0 | 34(47.1) | 160.0 | 174.6 | 178.3 | 157.5±10.3 |
| Layers' mash | 46(34.8) | 58.3 | 62.4 | 63.7 | 54.8±5.7 | 46(37.0) | 270.7 | 273.6 | 274.4 | 257.6±16.6 |

x̄±SD (Mean±Standard Deviation); [a]Positive detection (detection frequency, %)

Table 5: ANOVA test of MEL and CYR detected in fish feed poultry feed samples

| **Feeds** | **Tests** | **F-statistics** | **P-value** | **Remarks** |
|---|---|---|---|---|
| Fish feeds | Concentrations[a] | 1.93 | 0.24 | Not significant |
| | Feed type[b] | 8.33 | 0.03 | Significant |
| Poultry feeds | Concentrations[a] | 16.13 | 0.03 | Significant |
| | Feed type[c] | 1.16 | 0.45 | Not significant |

[a]MEL and CYR;
[b]Fish feeds: 1.5mm, 2.0mm,3.0mm,4.0mm and 6.0mm
[c]Poultry feeds: Broiler Mash, Broiler Finisher, Growers and Layers mash.

Animal feeds have been reported to contain 3.3 to 21 000 mg/kg of MEL, while whole eggs, dried eggs, dried egg powder and liquid eggs also contained 0.1–5 mg/kg MEL, further demonstrating that carry-over from feed to fish and eggs does occur [11]. The levels of MEL and

CYR in the layers' mash confirm the assertion of Gossner et al [11]. Previous reports have shown the presence of MEL at 3.5 mg/kg of MEL in fish feeds from China, and 2.7 to 6.3 mg/kg of CYR in eight commercially available poultry feed samples from the USA [4, 11]. The levels of MEL and CYR in feeds from previous studies are much higher compared to what obtained in this study. The difference in levels of the analytes compared to previous results can be attributed to the scale of production on the Nigerian poultry and aquaculture sub-sectors. The Nigerian poultry and aquaculture sub-sectors are ravaged with high importation of poultry meat and fish; and are thus not under production pressure to warrant the use of additives. It however becomes worrisome when the MEL is considered alongside CYR for co-exposure since they are similar. Meanwhile, MEL accumulates in tissues of fish when fed with feed with lower concentration of the additive and therefore can be transferred to man. To confirm the transfer of MEL from feeds to fish, a survey of market-ready cat fish and other fishes reported MEL at a concentration of 50–237 µg/kg [1]. The consumption of MEL in fish and other foods at above 50 µg/kg presents a greater health risk in the presence of CYR [28]. The level of MEL in this study is in agreement with the 13.9–294 µg/kg MEL found in foodstuff collected from Albamy, New York United States and France [29,30]. The presence of MEL with its analogues elevates its toxic potentials, thus the presence of CYR as found though little in the feed also elicits negative implications of MEL. Both MEL and CYR were detected in chicken feed samples and eggs from China by capillary electrophoresis at a concentration of above 0.42 mg/kg, that is similar with some of the results from the present study [31]. However, Christogiorgos et al. did not detect MEL and CYR in poultry and animal feedstuffs obtained in Greece [32].

## CONCLUSION

The concentrations of MEL and CYR` found in this study were generally below the acceptable lower limit for the two compounds. Food and agricultural agencies are however encouraged to step-up their surveillance activities to forestall the inclusion of the additives.

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