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# High Performance Liquid Chromatography Assay of Cyclopiazonic Acid in Some Stored Cereals from the Agro-Ecological Zones of Nigeria

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## Abstract
High Performance Liquid Chromatography (HPLC) with a UV detector, was employed to analyse cyclopiazonic acids (CPA) in stored maize, millet, rice, sorghum, wheat and processed cassava (garri) samples from the seven agro-ecological zones of Nigeria namely; Derived Savannah (DS), Mid Altitude (MA), Humid Forest (HF), Northern Guinea Savannah (NGS), Sahel Savannah (SS), Southern Guinea Savannah (SGS) and Sudan Savannah (SuS). CPA a neurotoxin produced by *A. flavus*, with an indole tetramic acid structure, has been reported to be acutely toxic with an $LD_{50}$ value above 2.30 ng/kg, a specific inhibitor of $Ca^{2+}$ -ATPase, and also a potent inhibitor of calcium uptake in the sarcoplasmic reticulum was found in all the samples analysed. The mean concentrations (n = 50) of CPA found were 6.783 µg/kg, 1.173 µg/kg, 1.150 µg/kg, 2.921 µg/kg, 1.481 µg/kg and 1.271 µg/kg for wheat, maize, garri, millet, sorghum and rice respectively. Wheat samples obtained from DS and SG were found to contain the highest amount of CPA with 14.501 µg/kg and 9.601 µg/kg respectively. The recommended acceptable daily intake (ADI) of CPA has been set for 10 µg/kg/day or ≈ 700 and >1000 µg/kg/day for human and animal respectively. All samples (except for wheat samples from DS) collected across the seven agro-ecological zones are less than the recommended ADI by the International Safety and Compliance Agency, USA.

**Keywords:** Cyclopiazonic Acid (CPA), Stored Cereals, High Performance Liquid Chromatography (HPLC), Agro-ecological zones.

## Introduction
Cyclopiazonic acid (CPA) is a mycotoxin produced by several moulds of the *Aspergillus* and the *Penicillium* genus. CPA has been detected in various foods of plant origin, such as peanut, corn, figs, rice, wheat and tomato, and also in food of animal origin, such as cheese, milk and ham (Anshanni & Yu, 2017). Cyclopiazonic acid (α-cyclopiazonic acid; CPA, Figure 1) is an indole-tetramic acid mycotoxin produced by the ubiquitous genera of fungal species such as *Aspergillus* and *Penicillium such as Aspergillus flavus, Aspergillus versicolor, Aspergillus oryzae, Aspergillus fumigatus, Aspergillus tamarii, Penicillium griseofulvum, Penicillium commune* and *Penicillium chrysogenum* (Holzapfel, 1968; Burdock & Flamm, 2000). Chemically, CPA belongs to the family of indole-tetramic acid secondary metabolites. *P. griseofulvum* Dierckx (originally called *P. cyclopium*). This mycotoxin is derived from tryptophan,

mevalonate, and two molecules of acetate (Perng *et al*., 2009. With an empirical formula of $C_{10}H_{20}N_2O_3$, its molecular weight of 336.15.

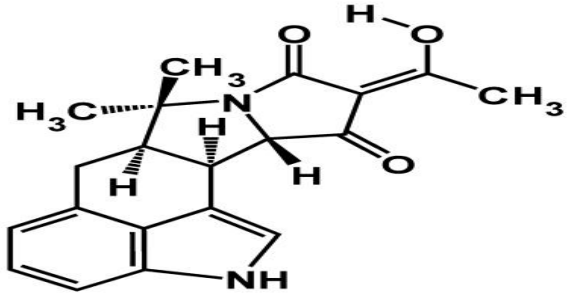


**Figure 1:** Chemical Structure of Cyclopiazonic Acid.

Cyclopiazonic acid (CPA) is one of the mycotoxins rarely studied despite its demonstrated cytotoxicity and immunotoxicity on human cells (Hymery, Masson, Barbier & Coton, 2014). In addition, this mycotoxin displays immunosuppressive properties and haematological disorders in humans (Hymery *et al*., 2014). These adverse effects in human beings together with those observed in animals (Burdock & Flamm, 2000; Hymery, Masson, Barbier, & Coton, 2014) support the optimisation of a reliable analytical method to detect and quantify this toxic compound for minimising the risk associated with its presence in foods. Recently, high-performance liquid chromatography (HPLC) (Finoli *et al*., 1999) have been widely used for detection and quantitative determination of CPA in fungal cultures and agricultural commodities. This method have been optimised to evaluate CPA amounts in various food matrices (Ansari & Häubl, 2016)

There are many reports on other mycotoxins such as aflatoxins contamination in cereals including wheat, maize and rice cultivated in Nigeria (Makun *et al*., 2013; Makinde *et al*., 2020). However, there is a paucity of data on CPA contamination of mainly cultivated cereals (maize, millet, rice, sorghum, and wheat) and processed cassava flour (garri) in Nigeria. Therefore, the main aim of this study is to determine CPA contamination in cereals and garri collected across the seven agro-ecological zones of Nigeria, using High Performance Liquid Chromatography (HPLC).

## Materials and Method

### Sample Collection

Twenty one (21) samples each of maize, millet, rice, sorghum, wheat and garri were collected randomly across the seven agro-ecological zones of Nigeria from storage facilities in 2019. The samples were properly labelled and preserved prior to analysis.

### Reagents and Chemicals

Acetonitrile and methanol were purchased from Fisher Scientific (Fisher Scientific UK Ltd., UK); Sodium chloride and anhydrous magnesium sulphate were supplied by Scharlab S.L. and Fisher Scientific, respectively. All solvents were HPLC grade. Distilled water was obtained from a Central Research Laboratory (Tanke, Ilorin). Vials, inserts, closures and 0.22µm filters used for mycotoxins were provided by Cosela S.L. (Spain).

### Standards preparation

CPA standard (Sigma-Aldrich, Germany) was dissolved in acetonitrile at a concentration of 1 mg/mL and stored at -20 °C in a sealed vial until use. Different amounts of the CPA stock standard were placed in amber 1.5mL vials and were evaporated to dryness under a gentle stream of $N_2$. Working standards (10–1000µg/mL of CPA in methanol) were prepared by appropriate dilution of known volumes of the stock solution with methanol.

### Extraction of Cyclopiazonic Acid

Milled samples (10 g each) of the cereals (maize, millet, rice, sorghum, and wheat)/processed cassava flour (garri) were weighed into labelled conical flasks and treated with 20 $cm^3$ methanol: water (60:40 v/v) in a mechanical shaker for 2 hours. The content was filtered through a Whatmann No. 2 filter paper and the filtrate (≈5 $cm^3$) cleaned by passing it through a micro-filter (5µm pore size) preconditioned with methanol: water (3:1 v/v). The filtrate was stored for further analyses at 4°C.

**Validation of High Performance Liquid Chromatography Method for Cyclopiazonic Acid Quantification**

The parameters of linearity (quantification), accuracy (% recovery) and sensitivity (limit of detection (LOD) and limit of quantification (LOQ)) were estimated for all the mycotoxins analysed according to the method described by Abia *et al*. (2013). For quantification purposes, external calibration curves were established based on serial dilutions of the mycotoxin standard solutions as indicated above

Linear calibration curves generated for the mycotoxin standards were considered satisfactory when correlation coefficients ($r^2$) were greater than 0.99.

Recovery assay was carried out in triplicates on 3 least contaminated samples by spiking 5 g of each with 100 µl known standard concentration. Eventually, spiked samples were mixed and kept for 16 hours in a fume cupboard at a room temperature to establish the equilibrium between the sample matrix and the toxins. From each spiked sample, 20 µl of the extract was injected into the HPLC. The analyte detected was quantified by comparing its peak area on the calibration plot to that of equivalent mycotoxin standard. The percentage recovery are presented in Table 1 while the recovery obtained are in line with the allowable limits of the recovery recommended by Codex or Association of Official Analytical Chemists (Scott, 1995). The Codex recommends 60–120 % of recovery rates of mycotoxins and the guideline for the recoveries by AOAC is 70–125%.

$$\%\ Recovery = \frac{Concentration\ Eqv.to\ the\ peak\ area\ measured\ from\ the\ spike\ sample}{Each\ toxin\ concentration\ used\ for\ spiking\ the\ sample} \times 100$$

LOD and LOQ for the CPA in cereals and processed cassava flour were estimated using the lowest concentrations in the spiked samples estimated as signal-to-noise ratio (S/N) of 3:1 and 10:1 respectively.

**Table 1**: **Calibration Parameters for Quantification of CPA by HPLC method**

| Analytes | Calibration level (µg/kg) | Percentage recovery (%) | $r^2$ | Equation of straight line |
|---|---|---|---|---|
| **CPA** | 0.001, 0.01, 1.0 | 96 | 0.9998 | y = 4E+07x - 27468 |

The calibration curve used for validation experiments was plotted in the range from 0.1 to 10 ng/mL and showed good linearity with the regression coefficient of $R^2$=0.999. Additional linearity testing was done in the 0.5 to 20 ng/mL range ($R^2$=0.996) due to the fact that CPA concentrations found in the samples were outside the original calibration range. Given that calibration standards were prepared from standard solutions, but not in the matrix, such regression coefficients were to be expected. These values show a slight improvement in sensitivity of the HPLC method of CPA detection in the cereals analysed as compared to the studies of Peromingo *et al*. (2018) and Delgado *et al*. (2019). The method recovery was evaluated at two levels (3µg/kg and 10µg/kg), and the average recoveries were 96.0 %. Validation results showed that the method is fit for purpose and can be employed in the analysis of CPA in the cereal samples.

**Result and Discussion**

From the results presented in Table 2, CPA was detected in the samples of collected from DS agro-ecological zone, including garri, maize, millet, rice, sorghum and wheat while in SGS agro-ecological zone CPA was not detected. For SS, all samples except garri had CPA in the range 1.283 – 9.601 µg/kg.

**Table 2: Average Concentration of Cyclopiazonic acid across the Agro-Ecological Zones of Nigeria (µg/Kg)**

| Sample | DS | SGS | SS | NG | SuS | MA | HF |
|---|---|---|---|---|---|---|---|
| **Garri** | 2.014±0.006a | ND | ND | ND | ND | ND | ND |
| **Maize** | 7.251±1.233g | ND | 2.014±0.01 | 1.124±0.120c,d | 3.059±0.012a | 2.252±0.172a,b | ND |
| **Millet** | 2.013±0.006a | ND | 2.032±0.00 | ND | 2.214±0.127b | 2.840±0.152c | 2.507±0.198a,b |
| **Rice** | 2.014±0.006a | ND | 1.283±0.119c | ND | 4.253±0.116b | 3.376±0.180d | ND |
| **Sorghum** | 1.027±0.015a | ND | 3.215±0079a | 2.997±0.094c,d | 4.806±0.098c | ND | ND |
| **Wheat** | 14.918±0.130e | ND | 9.601±0.007a | 2.063±0.0118a | ND | 2.013±00.006a | 2.211±0.135b |

ND **=Not detected**

The percentage of positives in the samples collected by agro-ecological zones was 100%, 0%, 83.3%, 67.4%, 67.4% and 32.4% for DS, SGS, SS, NG, SuS, MA and HF respectively while the total average across the zones was 57.1 %, which can be considered as a significant CPA occurrence when it comes to staple commodities. The CPA concentration found in the samples ranged from 1.027 µg/kg up to 14.918 µg/kg (Table 3). As compared to the study by Chilaka *et al*., (2014) conducted on cereals, our results revealed a lower CPA concentrations. These differences can be attributed to difference in location, effect of temperature and time of sample collection. Although the concentrations of CPA in most of the grains were below the acceptable daily intake (ADI) for CPA in humans of 10 µg/kg/day or 700 µg/day (Burdock and Flamm, 2000), its 100 % occurrence in the grains is a source of concern as regular consumption of foods containing low levels of the toxin can lead to acute toxicity (Adetunji *et al*., 2014). This finding also corroborates the work of Lombaert (2002) that though CPA is among the least toxic of mycotoxins, it is the most frequently detected and its occurrence is considered to be an indicator of possible presence of other more toxic mycotoxins.

**Table 3: Cyclopiazonic acid Positive Samples.**

| Type of Sample | Number of Sample | Number of Positive | CPA Con range (µg/kg) |
|---|---|---|---|
| **Garri** | 21 | 3 | 2.008 – 2.024 |
| **Maize** | 21 | 12 | 1.124 - 2.840 |
| **Millet** | 21 | 12 | 2.013 – 2.840 |
| **Rice** | 21 | 9 | 1.283 – 4.253 |
| **Sorghum** | 21 | 9 | 1.027 – 4.806 |
| **Wheat** | 21 | 12 | 2.063 – 14.918 |

The low amount of rainfall (650–1,000 mm) and long period of dry season (6–9 months) in the SS and NGS zones may be responsible for the low concentration of aflatoxins found in maize grains in these regions (Adetunji *et al*., 2014). Atehnkeng *et al*. (2008) earlier recorded the least level of other mycotoxins contamination in the NGS zone. Furthermore, Udo *et al*. (2000) also did not detect aflatoxins in farmers store in the NGS zone and attributed this to the fact that farmers usually store their grains in "rhumbu" (local granary). Higher temperatures and drier conditions favour infection by *Aspergillus* fungi, and the development of mycotoxins such as CPA in cereals prior to harvest and these contamination frequently accompany heat and water stress that may be associated with drought (Atehnkeng *et al*., 2008). Thus, CPA may be expected to be higher in maize grains collected in the DS and NGS zone with a relatively warm and dry climate as compared with the NG and the SGS where crops are grown under more moderate conditions with less water stress. However, our results show more CPA contamination in the SGS zone compared to NGS zones, a trend common across West Africa (Atehnkeng *et al*., 2008). The low concentration of CPA in the HF zone as compared to the SGS zones may be due to the fact that farmers in the zone do not usually store their grains for long periods as they usually sell their maize in the fresh state because the zone is highly urbanized. The grains are usually consumed by the populace as snacks and by local industries. Wild and Hall (2000) also reported that urban dwellers generally have lower levels of mycotoxins exposure than rural population in developing countries because urban populations typically consume more diversified diets

than do rural dwellers and may have food that is better controlled for contaminants. The high concentration of CPA observed in the DS zone is probably due to the high amount of rainfall (1,300–1,500 mm) usually recorded in the zone in collaboration with the various storage practices employed by the farmers in the zone (Adetunji *et al*., 2014).

The high incidence of CPA contamination also observed in SS could be due to the mixed cropping systems practiced by farmers in the zone. Farmers in this zone are in the habit of planting crops like yam, maize, cowpea, peanut and soybeans on the same piece of land. This practice encourages cross-transfer of toxigenic strains from one infected crop to another (Atukwase *et al*., 2009).

## Conclusion

The cereals collected across the agro-ecological zones were contaminated with low doses of CPA but their consistency are associated with negative public health consequences. Furthermore, there is a high risk of contamination by Nigerian cereals consumers to the CPA especially in the Derived and Southern Guinea Savannas. Intervention strategies are therefore needed across the Agro-Ecological Zones to ensure that safe and wholesome foods are made available to the populace. Local farmers also need to be trained on how to cultivate good agricultural and storage practices in order to reduce the prevalence of CPA.

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