ASSESSMENT OF THE LEVELS OF MYCOTOXINS IN VARIETIES OF CEREALS *(Oryza sativa, Zea mays, Pennisetum glaucum and Triticum aestivum)* OBTAINED FROM CALABAR MARKETS, CROSS RIVER STATE, NIGERIA

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ABSTRACT **-** This study assessed the levels of four major mycotoxins (ochratoxin A, zearalenone, fumonisin B_1+B_2 and aflatoxin B_1) in a total of twenty (20) samples consisting of five (5) samples each of *Oryza sativa, Zea mays, Pennisetum glaucum and Triticum aestivum* that were randomly purchased from different markets in Calabar, Cross River State. The four different food items were subjected to the same analytical procedures for extraction with 100ml solvent mixture containing water/ methanol (3:2) and 2g of sodium chloride. The extracts were cleaned-up using a solid phase extraction process to remove interferences while separation and detection of mycotoxins were carried out using high performance liquid chromatography coupled with a fluorescent detector. The results revealed a wide and positive significant ($P<0.05$) variations in the concentrations of the mycotoxins and were mainly dependent on the cereal type. Overall mean concentration of aflatoxin B_1 (1.56 ± 0.14 µg/kg), ochratoxin A (0.49 ± 0.04 µg/kg), fumonisin B_1+B_2 (135.33 ± 3.78 µg/kg) and zearalenone (55.00 ± 0.51 µg/kg) irrespective of cereal type were recorded. An overall order of concentration in the cereals by aflatoxin B_1 was maize > millet > rice > wheat, while by ochratoxin A and fumonisin B_1+B_2 it was maize > rice > millet > wheat. Results indicated that the levels of aflatoxin B_1, ochratoxin A, fumonisin B_1 + B_2 and zearalenone were within Nigerian and European permissible limits of 2µg/kg, 5µg/kg, 800µg/kg and 75µg/kg respectively. This study confirms low levels of mycotoxins contamination of cereals from Calabar markets and suggests that consumption of wheat is relatively safer in terms of mycotoxins concentrations when compared to rice, millet and maize.

Keywords - aflatoxins, ochratoxin A, mycotoxins, cereals, HPLC

1.0 INTRODUCTION

Mycotoxins are toxic, low molecular weight organic compounds which when breathe in, absorbed through the skin or eaten causes immune suppression, sickness or deaths in human beings. Mycotoxins are one group of toxins that are usually found to be present in some cereal grains (e.g. wheat, rice, maize, groundnut, millet etc.) either processed or fresh and these are produced by fungi which have been approximated to contaminate crops to about 25% worldwide.[1] Mycotoxins had attracted worldwide concern owing to the fact that the heavy economic losses connected and attributed with their impact on human beings, farm animals and trade revenues.[2][3] In developing countries like Ghana, Togo, Nigeria, India etc, the combination of humid atmospheric conditions and insufficient drying encourage rapid increase of mold growth which in turn causes intolerable levels of mycotoxins particularly. Presently, the most prominent and economic important ones are: ochratoxin A, aflatoxin B_1, zearalenone, fumonisins B, deoxynivalenol and patulin.[4] Reported studies have shown large-scale mycotoxin poisoning in both developing and developed countries with the presence of these mycotoxins in our cereals which is believed to be carcinogenic, nephrotoxic, teratogenic, immunosuppressive and mutagenic to man.[5][6][7] The aim of this study was to assess the prevailing levels of these four mycotoxins in the cereals obtained from different markets in Calabar, Cross River State and to know which of the cereal grains e.g millet, rice, wheat and maize preference should be given for consumption in situations where diet control is required.

2.0 MATERIALS AND METHODS

2.1 Samples and Sampling

A total of 20 samples were randomly purchased from (Mbukpa Marian, Ikot-Ishie and Bogobiri) markets in Calabar. Samples consisted of local rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*) and millet (*Pennisetum glaucum*) grain. Stratified random sampling was carried out and later quartered to obtain 500g representative samples of laboratory size.[8] Samples were packaged in sealed plastic bags, labelled and taken to the laboratory where they were blended.

2.2 Analysis of mycotoxins

The four different food items were subjected to the same analytical procedures namely: extraction, clean up, separation

and detection for determination of ochratoxin A, zearalenone, fumonisin $B_1 + B_2$ and aflatoxin B_1 mycotoxins.

2.2.1 Extraction and clean-up of mycotoxins

Milled samples (50g) were weighed into a 250ml conical flask and 100 ml solvent mixture containing water/ methanol (3:2) and sodium chloride (2g) were added. This was shaken for 30 minutes to homogenise then centrifuged for 15 minutes to separate the liquid from solid. The same method of clean-up via solid phase extraction (SPE) was implemented for all the food products.[9] Elute from the column was allowed to dry and stored in glass vials before HPLC analysis.

2.2.2 Separation and Detection

This was carried out with HPLC Flexar FX-10 System (PerkinElmer, USA), Flexar autosampler tray, FL Flexar detector, KobraCell (100µA), Brownlee Pinnacle DB C_{18} reverse phase analytical column (50mm x 2.1mm, 1.9µm). It involves the use of high pressure (~6000psi) to force the analyte (extract) in solution at a flow rate of 0.7 ml/min with an injection volume of 2µL. The solvents: water, acetonitrile and methanol used as the mobile phase of the HPLC and for re-dissolution of the extracts prior to HPLC were filtered through 0.45µm "Whatman" filter paper on a vacuum pump apparatus prior to attachment to the solvent inlet valves of the solvents before introduction into the pump to abate contamination by particulate matter that could result in column fronting or tailing.[10] The analysis of different mycotoxins involved the use of specific wavelengths. [11,12]

2.2.3 Data Analysis

Data obtained from the determinations were subjected to descriptive statistical analysis using Analyse-it® (version 3.0) statistical software for Microsoft Office. Inferential statistics for correlation were considered significant at 95% confidence interval (i.e. $P<0.05$).

3.0 RESULTS

All four mycotoxins of interest; aflatoxin B_1, ochratoxin A, fumonisin B_1+B_2 and zearalenone were found present and the concentration of each is presented in Table 1-4 and Figure 1.

Table 1: Concentrations of mycotoxins in rice grain samples

Mycotoxins	Concentration range (µg/kg)	Mean	Standard deviation
Aflatoxin B_1	1.40 – 1.49	1.41	0.03
Ochratoxin A	0.51 – 0.53	0.52	0.01
Fumonisin B_1+B_2	138.69 – 138.76	138.72	0.03
Zearalenone	54.24 – 54.27	54.25	0.01

Table 2: Concentrations of mycotoxins in millet grain samples

Mycotoxins	Concentration range (µg/kg)	Mean	Standard deviation
Aflatoxin B_1	1.60 – 1.63	1.60	0.01
Ochratoxin A	0.45 – 0.49	0.47	0.02
Fumonisin B_1+B_2	131.98 – 132.05	132.02	0.02
Zearalenone	55.52 – 55.60	55.55	0.03

Table 3: Concentrations of mycotoxins in maize grain samples

Mycotoxins	Concentration range (µg/kg)	Mean	Standard deviation
Aflatoxin B_1	1.73 -1.78	1.76	0.02
Ochratoxin A	0.50 – 0.54	0.52	0.02
Fumonisin B_1+B_2	139.45 – 139.47	139.46	0.01
Zearalenone	54.79 – 54.84	54.81	0.01

Table 4: Concentrations of mycotoxins in wheat grain samples

Mycotoxins	Concentration range	Mean	Standard deviation

	(µg/kg)		
Aflatoxin B_1	1.40 – 1.42	1.41	0.01
Ochratoxin A	0.42 – 0.44	0.43	0.01
Fumonisin B_1+B_2	131.10 – 131.12	131.12	0.01
Zearalenone	55.39 – 55.42	55.41	0.02

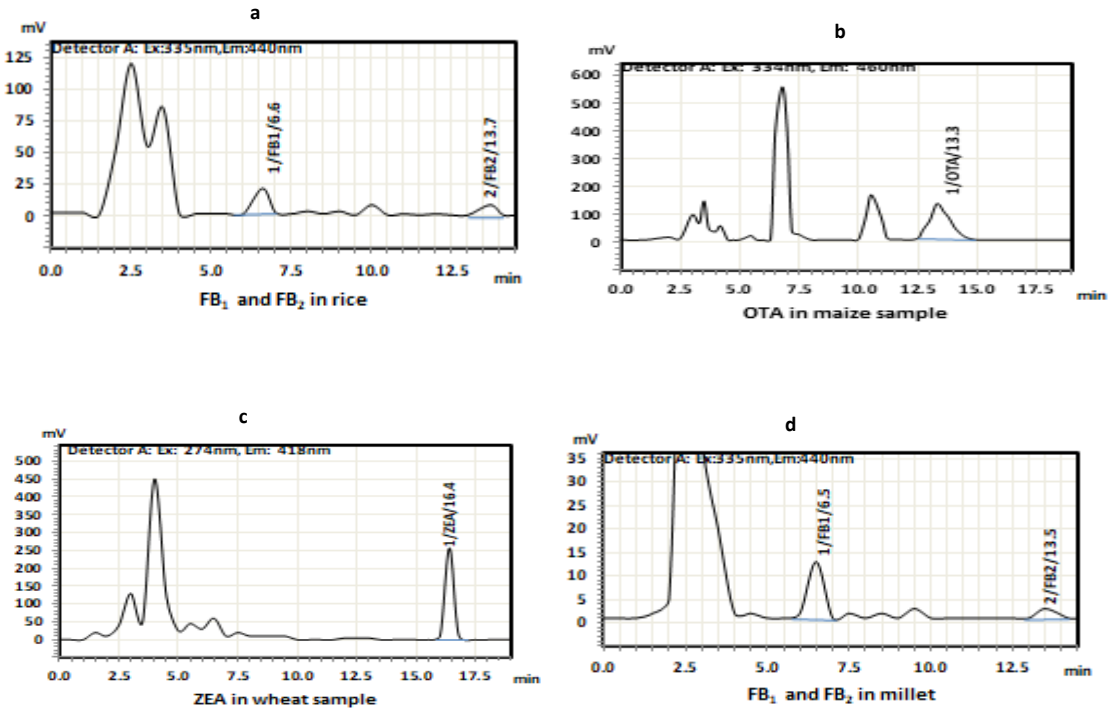

FIGURE 1: Chromatograms of some of the samples.

4.0 DISCUSSION

Ezekiel *et al.* (2012) [13] reported aflatoxin B_1 concentration range of 0.08 – 1.4µg/kg in millet samples analysed which is lower than the concentration range of 1.60 – 1.63µg/kg in millet in this study but was below the Nigerian and European Union permissible limit of 2µg/kg for aflatoxin B_1 in this staple cereal; it is however relatively safe. Roasted groundnuts, raw groundnuts obtained from farm lands and raw groundnuts in storage locations were analysed for aflatoxin B_1 by (Ifeji *et al.* 2014) [14] and was reported to contain 64.78 ± 5.11µg/kg, 54.32 ± 4.36µg/kg and 42.78 ± 4.27µg/kg respectively. This very high concentration in the groundnut samples required an urgent need for constant monitoring because it is one of the major staple foods in the country. Dimitrokallis *et al.* (2008) [15] reported that control cultures that were positive with only aflatoxinB_1 had a concentration of 197.20ng/kg reduced to 82.52ng/kg after incubating with 60ng/kg of ochratoxin A for 14 days. This was attributed to the inhibitory effect of ochratoxin A to the production ofaflatoxins and also divergent uptake behaviours of mycotoxins which indicates the transport of aflatoxins being different from that of ochratoxin A in samples as a result of physico-chemical effects. Data obtained from this study for aflatoxin B_1 (1.40 – 1.78µg/kg) and ochratoxin A (0.42 – 0.54µg/kg) are in agreement with this reportof having a lower concentration of aflatoxin B_1 because of the presence of ochratoxin A.

The mean concentration of total aflatoxins (B_1+B_2) and ochratoxin A in rice samples obtained from the farm lands, store and markets in Niger State as reported by (Makun *et al.*, 2007)[16] was 372µg/kg and 200 - 1000µg/kg respectively while the respective concentration values of zearalenone, deoxynivalenol and fumonisin B_1+B_2 in the study was not stated, it was reported to be relatively low. However, the rice samples were unsafe for human consumption and a cause for concern because of the high level of ochratoxin A and aflatoxins concentrations which exceeded the Nigerian and European Union permissible limit of 5µg/kg and 4µg/kg respectively. High level of aflatoxins could interact with other carcinogens like the hepatitis B virus and have a synergistic adverse effect thereby causing the prevalent liver cancer noticeable in Nigeria.[17] Also, the high concentration of ochratoxin A in the study was far above the Nigerian and European Union permissible limit of 5µg/kg in maize and rice grown in Niger state can also promote the occurrence of renal diseases when people suffering from diabetes, hypertension and malaria consume these contaminated cereals as the case is in Nigeria.[17]

Furthermore, persistent intake of aflatoxins can increase the likelihood of premature delivery, childhood mortality, immunosuppression with vulnerability to other

abnormality such as: stunted growth, infectious diseases like pneumonia.[18] For example, in the year 2004 the consumption of aflatoxin infected food with concentration between 1,600 - 12,000µg/kg was implicated in the poisoning that lead to the death of hundreds in Kenya.[19] In this study maize had aflatoxin B_1 and ochratoxin A concentration of 1.73 – 1.78µg/kg and 0.50 - 0.54µg/kg respectively while rice samples had aflatoxin B_1 concentration of 1.40 – 1.49µg/kg and ochratoxin A concentration of 0.51 - 0.53µg/kg which were below the Nigerian and European union permissible limit of 5µg/kg hence, the cereal analysed do not pose any health danger with respect to aflatoxin B_1 and ochratoxin A.

Ayejuyo *et al.* (2008) [20] reported a mean concentration of 0.34µg/kg of ochratoxin A in twenty different brands of imported rice sold in Lagos market while (Gwary *et al.* 2008)[21] reported an overall mean concentration of 0.12 ± 0.07µg/kg in maize, millet and guinea corn obtained from Niger State. However, the mean concentration of ochratoxin A in this study: 0.43 ± 0.01µg/kg in wheat, 0.47 ± 0.02µg/kg in millet, 0.52 ± 0.01µg/kg in rice and 0.52 ± 0.02µg/kg in maize respectively were higher than the reports by (Ayejuyo *et al.* 2008; Gwary *et al.* 2008)[20][21] but they were all below the Nigerian and European union permissible limit of 5µg/kg hence do not pose any health challenge. The results of this study revealed wide variation in the concentrations of aflatoxin, ochratoxin A, fumonisin B_1+B_2 and zearalenone mycotoxin in the cereal samples and they were mostly dependent on the cereal type with an overall order of concentration in the cereals by aflatoxin B_1: maize > millet > rice > wheat, while for ochratoxin A and fumonisin B_1+B_2 it is maize > rice > millet > wheat. The trend in concentration of maize and millet in this study is the same as reported by (Gwary *et al.* 2008)[21] with maize having a higher concentration than millet. There are several reported studies of simultaneous relationship linking aflatoxin and ochratoxin A[22], penicillic acid and ochratoxin A[23] and other pairs which are known to co-exist in cereal grains. Aflatoxins, zearalenone, fumonisins, ochratoxin A and the trichothecenes (e.g.: nivalenol, deoxynivalenol, HT-2 toxin, diacetoxyscirpenol, T-2 toxin, 3-acetyl deoxynivalenol, etc.) mycotoxins have been simultaneously isolated from a single rice sample obtained in South East Asia (Reddy *et al.*, 2008)[24] and rice samples grown in Niger state, Nigeria (Makun, *et al.*, 2007)[16] which confirms co-contamination with more than 5 different mycotoxins. The 4 major mycotoxins: aflatoxin B_1, zearalenone, fumonisin and ochratoxin A assessed and found to be present in all rice, wheat, maize and millet samples in this study agrees with reports of (Makun, *et al.*, 2007; Reddy *et al.*, 2008)[16][24] on the co-contamination of food products by more than one mycotoxin.

In this study, an overall correlation analysis was carried out to determine the co-contamination of mycotoxins in all four cereal types sampled. Table 5 contains varying r-values in the cereals analysed at a significant level of $P < 0.05$.

Table 5: Correlation matrices of mycotoxins in the four cereal samples

	Aflatoxin B_1	**Ochratoxin A**	**Zearalenone**	**Fumonisin B_1+B_2**
Aflatoxin B_1	-	0.329	-0.084	0.189
Ochratoxin A	0.329	-	0.628	0.808
Zearalenone	-0.084	0.628	-	0.940
Fumonisin B_1+B_2	0.189	0.808	0.940	-

Number of samples = 20

People living with diabetes are advised to abstain from consuming food such as rice, garri, yam etc which are high in carbohydrate and replace with unripe plantain, wheat flour, etc which are rich in fiber, iron and with less composition of carbohydrate. With that in mind, Pearson's correlation co-efficient was used to determine the relationship of individual mycotoxin between two different cereal grains analysed so the results can be used as a guide to advise affected individuals suffering from oesophageal cancer, breast cancer, liver cancer, kidney disease and hyper estrogenic effects. Also, these suggestions will act as preventive measures for healthy persons. Table 6 is the summary of correlation analysis between cereal pair for the four mycotoxins analysed from which aflatoxin B_1 had a positive significant value of r = 0.75 between maize and wheat which implies that the more these cereals are consumed, the more one is prone to this hepatotoxin which causes liver diseases. It is however safe to suggest that wheat is a better alternative to maize for people suffering from liver diseases like cancer because it has a lower concentration of the aflatoxin when compared to maize. Table 6 showed a positive significant value of r=0.8 between wheat and rice, maize and millet respectively for fumonisin mycotoxin which implies that the more these cereals are consumed, the more one is prone to this mycotoxin which causes oesophageal cancer.

Table 6: Summary of correlation analysis between cereal pair for the mycotoxins analysed

Cereal pair	**Aflatoxin B_1 (r-value)**	**Ochratoxin (r-value)**	**Zearalenone (r-value)**	**Fumonisin B_1+B_2 (r-value)**
Millet and Rice	0.22	0.17	0.27	0.40
Rice and Maize	0.00	0.00	0.29	0.35

Millet and Wheat	0.11	0.46	0.41	0.03
Wheat and Rice	0.47	0.00	0.26	0.80
Maize and Millet	0.16	0.00	0.22	0.80
Maize and Wheat	0.75	0.00	0.68	0.03

Also, from the result in this study, the maximum fumonisin concentration in rice, millet, maize and wheat are 138.76 µg/kg, 132.05 µg/kg, 139.48 µg/kg and 131.13 µg/kg respectively so an order of decreasing concentration can be suggested for preferential consumption (i.e wheat > millet > rice > maize) to reduce the potential risk of oesophageal cancer caused by fumonisin contamination since the richer the cereal is in carbohydrate, the higher the potential for mycotoxin production and contamination. However, wheat has less carbohydrate level than maize which suggests consumption preference for wheat with a lower fumonisin concentration value of 131.13µg/kg.

There is limited information on contamination and effect of production processes on fumonisin although in the Republic of Benin, Fandohan *et al*. (2005)[25] reported a mean concentration of 1.99 ± 0.06µg/g in raw maize and a 80% reduction in the fumonisin concentration of the sample to 0.37 ± 0.06µg/g after separation by handpicking and up to 75% reduction in concentration to 0.54 ± 0.03µg/g after washing and fermentation of the raw maize sample. Martins *et al*. (2001) reported high level of fumonisin B_1 in orange leaves (350 – 700µg/kg), black tea (80 - 280µg/kg), leaves and flowers of linden tree (20 - 200µg/kg) with lower levels in corn silk (50 – 150µg/kg) and chamomile (20 – 70µg/kg). The fumonisin B_1 + B_2 concentration in this study (131.10 – 139.48µg/kg) falls within the range of 0.01 – 3644µg/kg in other studies[26][27][28][29][30] on fumonisin contamination although less pronounced than levels detected in some of the cited studies. The cereals analysed for in this study do not pose any health challenge because high level of fumonisins in other reported studies is neurotoxic, hepatotoxic, and nephrotoxic in animals and so it has been classified as a possible carcinogen to humans. Data from the analysis of zearalenone in this study indicated its presence in all the cereal samples analysed with mean concentration of 54.25 ± 0.01µg/kg in rice, 55.55 ± 0.03µg/kg in millet and 54.81 ± 0.01µg/kg in maize which are within the concentration range of 24 - 1169µg/kg in rice samples from Niger State as reported by (Makun, 2007).[16] The concentrations of zearalenone in the cereals analysed in this study were below the set Nigerian and European union regulatory limit of 75µg/kg and do not pose any health challenge.

Mycotoxigenic fungi are notable to inhabit several cereals and form mycotoxins when environmental conditions are highly favourable for their development at the respective storage locations[31]. Factors such as climatic conditions (e.g. temperature and moisture level), good farm practice (early harvest) and post-harvest handling (transport, drying and storage) are major factors responsible for mycotoxin production and contamination in food as discussed in the literature review. Rice, maize, wheat, millet, cocoa, groundnut and guinea corn are some of the major substrates for mycotoxin production. It was observed that cereals from the open markets undergo another round of sun drying, blowing of air through the grains in order to remove the unwanted debris, handpicking of some damaged grains in order to make the cereals look attractive on display may significantly reduce mycotoxin formation and contamination. These processes combined with transport and storage practices could be contributory factors to the low incidence of mycotoxins in the grains analysed as compared with those from farm lands and storage in reported studies.[32]

5.0 CONCLUSION

The low levels of mycotoxins contamination in the four cereals analysed suggest that consumption of wheat is relatively safer than maize, millet and rice. More research should be conducted on other food products as well as the health implications with relation to dietary exposure of people in Nigeria with organisations and governmental bodies playing key roles in this regard, collaboration in research involving multidisciplinary teams is needed for effective research, documentation, monitoring and evaluation of mycotoxins in Nigeria.

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