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Survey of maize from south-western Nigeria for zearalenone, α- and β-zearalenols, fumonisin B1 and enniatins produced by *Fusarium* species

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Abstract

A survey for the natural occurrence *of Fusarium* mycotoxins of maize for human consumption in four south western states of Nigeria, using the High Performance Liquid Chromatography coupled with Mass Spectroscopy (HPLC/MS) showed that 93.4% of the total samples were contaminated by zearalenone (ZON), α- and β- zearalenols (α- and β-ZOL), fumonisin B_1 (FB_1) or enniatins (ENN). The fraction of contaminated samples were were 73% for FB_1 (mean: 117 μg kg^{-1}, range: 10-760 μg kg^{-1}); 57% for ZON (mean: 49 μg kg^{-1}, range: 115-779 μg kg^{-1}) and 13% for α-ZOL (mean: 63.6 μgkg^{-1}, range: 32-181 μgkg^{-1}), while ENN A, B and B_1 were present in 3%, 7% and 3% of the samples. There was no β-ZOL present within the detection limits of 50 μg kg^{-1}. Only the FB_1 content was significantly different at 95% confidence level among the four states The *Fusarium* species most frequently isolated from maize seeds was *F. verticillioides* (70%), followed by *F. sporotrichioides* (42%), *F. graminearum* (30%), *F. pallidoroseum* (15%), *F. compactum* (12%), *F. proliferatum* (12%), *F. equiseti* (9%), *F. acuminatum (8%)* and *F. subglutinans* (4%). This is the first report of the occurrence of α- zearalenol and enniatins in Nigerian maize.

Key words: Fumonisin B_1, zearalenone, α-zearalenol, β-zearalenol, enniatins, *Fusarium* species, maize, Nigeria.

Introduction

Maize *(Zea mays* L.) is the most frequently consumed staple crop in all agro ecological zones in Nigeria with 20% of the population consuming it at various numbers of times in a week (IITA, 2004), either as roasted, boiled grain or processed to flour as snacks. Maize is prepared and consumed in a multitude of ways which vary from region to region or$_1$ from one ethnic group to the other. For instance, maize grains are prepared by boiling or roasting as paste ('eko'), 'agbado', and 'elekute' in Nigeria or as popcorn which is eaten all over West Africa (Abdulrahaman and Kolawole, 2006). It also constitutes a major feedstuff for all classes of livestock.

Fusarium species are widespread in nature occurring as saprophytes in many plant materials and as pathogens of various crops especially maize. They have a direct effect on corn yields by causing a variety of diseases and sometimes produce mycotoxins in the infected ears and kernels. The mycotoxins produced by *Fusarium* spp. in cereal grains are second to the aflatoxins and ochratoxin A in attracting the attention of scientists and farmers; these include the zearalenone (ZON), trichothecenes, and fumonisins (Tseng *et al.,* 1985). Fumonisins are produced mainly by *F. verticillioides*, *F. proliferatum*, and *F. nygamai* on cereals (Nelson *et al.,* 1991, 1992, 1994). They are believed to be responsible for a variety of animal diseases, e.g. equine leukoencephalomalacia (ELEM), in horses, pulmonary oedema in swine (Kellerman *et al.,* 1990, Harrison *et al.,* 1990), hepatotoxic and carcinogenic to rats (Gelderblom *et al.,* 1991). Fumonisins have carcinogenic properties; the major target organs believed to be liver and kidney (Engelhaldt *et al*., 2006). Fumonisin B_1 (FB_1) has been found to be a

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potent inhibitor of sphinganine *N*-acyltransferase, causing an elevation of the sphinganine: sphingosine ratio which results in the disruption of cell membrane function (Wang *et al.,* 1991; Riley *et al.,* 1994; Ramasamy *et al.,* 1995). FB_1 has been suggested as a cause of oesophageal and liver cancers in humans (Rheeder *et al.,* 1992, Thiel *et al.,* 1992, Chu and Li, 1994, Ueno, 2000). *F. verticillioides* has been associated with human esophageal cancer risk in the Transkei region of southern Africa (Marasas, 1982, Marasas *et al.,* 1981, 1988) and in China (Li *et al.,* 1980; Yang, 1980). Recent work proved carcinogenicity of FB1 in rodents (CEC, 2005a).

ZON is a naturally occurring toxic secondary metabolite produced by several species of *Fusarium* fungi on a variety of cereal grains, with special incidence in corn (Trenholm *et al.,* 1991 and Vinäs *et al.,* 1985). *Fusarium graminearum* has been found to be the major causal agent of ZON contamination of grains (Ichinoe *et al.,* 1983). The clinical signs of exposure to ZON in swine include of vulval tumefaction, vaginal and rectal prolapses, mammary gland enlargement; conception failure, pseudo-pregnancy, decreased pigs per litter, and abortion in mature cycling females (Chang *et al.,* 1979**;** Trenholm *et al.,* 1988). ZON and some of its metabolites have been shown to competitively bind to oestrogen receptors (ER) in a number of in vitro systems and has been demonstrated in uterus, mammary gland, liver and hypothalamus from different species. ZON is fairly rapidly absorbed following oral administration and can be metabolised by intestinal tissue in pigs and possibly in humans during its absorption, with the formation of α- and β-zearalenol (α- and β-ZOL) and α- and β- zearalanol, which are subsequently conjugated with glucuronic acid (Kuiper-Goodman *et al.,* 1987; Eriksen and Alexander, 1998). Some strains of *Fusarium* spp. produce in addition to ZON, *α*-ZOL has also been found in feeds (Mirocha *et al.,* 1979). *α*-ZOL has about 100-fold more estrogenic potency than ZON, and it has been shown to inhibit atherogenesis, lowering plasma LDL-cholesterol and limiting aortic plaque formation in ovariectomized rabbits fed with a high dose of cholesterol (Dai *et al.,* 2004, Murphy *et al.,* 2006). ZON is mainly degraded to the metabolites α- and β- ZOLs; this transformation is not regarded as detoxification because both substances are still oestrogenic (Hagler *et al.* 1979; Kuiper-Goodman et al. 1987; Fitzpatrick *et al*. 1989). Enniatins (ENNs) are cyclic hexadepsipeptides structurally related to beauvericin and are produced by various *Fusarium* species like *F. avenaceum*,, *F. lateritium, F. scirpi, F. oxysporum* (Bottalico and Perrone 2002; Logrieco *et al*., 2002, Nicholson *et al*., 2004). ENNs are synthesised by the multifunctional enzyme, enniatin synthetase (Haese *et al*., 1993), they are virulence factors, increasing the ability of isolates to colonise their host plants (Herrmann *et al*., 1996). ENN have antimicrobial properties, interfere with cholesterol storage in liver cells, and are found to be phytotoxic (Burmeister and Plattner, 1987) and toxic in various bioassays (Strongman *et al*., 1988, Tomoda *et al.*, 1992). It is reported to have antibiotic, insecticidal and ionophoric activity (Grove and Pople, 1980).

Nigeria has a tropical climate with all the year round high ambient temperature and relative humidity; these provide optimal condition for growth of toxigenic molds. Because of the importance of maize as staple food in Nigeria, it is important to monitor contamination of maize. FB1 was the predominant toxin detected in 78.6% of samples with concentration range of 70–1780 $\mu g\ kg^{-1}$ (Bankole and Mabekoje, 2004). The aim of this study therefore was to determine the contaminating *Fusarium* species and examine the occurrence and level of *Fusarium* mycotoxin: FB1, ZON, α- and β-zearalenols and enniatins in maize in four South Western states of Nigeria.

Materials and methods

Study areas

The area chosen for this study is the South western Nigeria comprising of Ondo, Ekiti, Osun and Oyo states, located between latitudes 60 and 90 North of the equator and longitude 30 and 60 East of the Greenwich meridian. The climate is characterized by two seasons; the wet season which lasts from April to October with rainfall distribution ranging between 1500 mm and 2000 mm, while the dry season lasts from November to March. The mean monthly temperatures ranges from 17 to 360 C, mean monthly rainfall of 12.0-314.6 mm, while the mean relative humidity is between 78 and 100% for 2005 (Source: Nigerian Meteorological Agency, Abuja).

Sampling and sample preparation

A total of one hundred and eighty two (182) maize samples were collected from farmers, markets and grain shops in Ekiti, Ondo, Osun and Oyo states with 35, 57, 40 and 50 samples respectively between May and July, 2005. These include a total of 94 white maize and 88 yellow maize samples (21 white and 14 yellow for Ekiti state, 22 white and 35 yellow for Ondo state, 20 white and 20 yellow for Osun state,

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while 31 white and 19 yellow were for Oyo state). Samples were completely dried, prevented from moisture and insects and stored in the cold room prior to analysis. Five hundred gram of seeds per sample was ground with the milling machine (1 mm sieve) and prepared for mycotoxin analysis.

Isolation and identification of Fusarium *species*

Fungi were isolated by surface-sterilizing 15 seeds per sample in 1% sodium hypochlorite solution in a 50 ml beaker for 1 min and washed with three changes of sterile distilled water. Five seeds each were placed on Potato-Dextrose Agar (PDA) (Roth, Karlsruhe, Germany) and incubated for 4 to 7 days at 25°C. The fungi were isolated and sub-cultured to obtain pure cultures and the *Fusarium* species were then inoculated to both Potassium Chloride Agar (KClA) (Merck, Darmstadt, Germany) and Spezieller Närstoffarmer Agar (containing 1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4.7H2SO_4$, 0.5 g KCl, 0.2 g Glucose, 0.2 g Saccharose, 20.0 g Agar, 1 L distilled water, (Gerlach and Nirenberg, 1982)), incubated for 7 days in the dark and placed under long-wavelength UV at 22°C for two to four weeks. Fungi were identified by the descriptions of Gerlach and Nirenberg (1982) and Burgess *et al*. (1994). Contamination level of *Fusarium* species was determined by the presence or absence of fungal species in the samples and calculated as percentage of samples infected.

Reagents and reference standards

ZON, FB1, and α- and β-ZOLs standards were obtained from Sigma (Taufkirchen, Germany) in highest purity available. ENN mixture was extracted from Locabiosol (Servier, München, Germany) and used only as a reference for mass transitions and retention time. In addition to ENNs, Locabiosol contains isopropylmyristat, ethanol, saccharin and flavours. The pharmaceutical was extracted with hexan to remove oils and the defatted solution was used as a qualitative standard for HPLC-MS. The ratios of ENNs in the extract determined by HPLC with UV detection was as follows: ENN B1 38%, ENN B1 44%, ENN A1 18%. Acetonitrile and methanol of gradient quality grade were obtained from VWR (Darmstadt, Germany), ammonium acetate and formiate were from Merck (Darmstadt, Germany). HPLC water was prepared by twofold distillation in a distillation apparatus consisting of only glass and Teflon components.

Mycotoxin analysis

Methods of Royer *et al.*, 2004 was used for analysis of ZON, α- and β-ZOLs and FB1, while those of Monti *et al.*, 2000 were used for enniatin (ENN) A, B and B_1. The toxins were extracted from 4 g of ground maize in 40 ml acetonitrile-water (84:16) in 50 ml Falcon tubes as follows: samples were incubated and shaken vigorously overnight in a wrist-action shaker, centrifuged at 4,500 rpm for 10 min and decanted. One milliliter of the supernatant was pipetted unto 1.5 ml Eppendorf tubes, evaporated to dryness at 45°C in a Speed vacuum chamber and stored in the freezer at -20°C. Samples were dissolved in a mixture of 375 µl methanol and 375 µl buffer (10 mM ammonium acetate, pH set to 4.5 with formic acid, containing 1% acetonitrile) and defatted with 500 µl of hexane. After phase separation (20°C, 14,000 rpm for 1 min) the lower phase was filtered (OPTI-FLOW, 13mm, 0.2 µm; PTFE; WICOM, Heppenheim, Germany) into HPLC vials.

LC analysis was performed using a Varian system consisting of two pumps, a degasser, an autosampler and a column oven. The analytes were separated on a polar modified RP-18 column (Synergi Fusion RP 80A column; 4 µm, 100 x 2 mm i.d.; Phenomenex, Aschaffenburg, Germany). The column was maintained at a temperature of 40°C. The flow rate was set to 0.2 ml/min, the injection volume was 10 µl. Solvent A was water, solvent B was methanol. ZON and the ZOLs were separated as follows: 0-7 min 20-40% B, 7-10 min 40-70% B, followed by washing and re-equilibration steps. FB1 and ENNs were separated as follows: 0-1 min 20% B, 1-10 min 20-60% B, followed by washing (98% B) and equilibration steps. Since it is possible to ionize FB1 in positive and negative ESI, the FB1 samples was scanned twice. Peak integration was performed from the positive mode scan.

For MS/MS detection a Varian 1200 triple quadrupole was used with positive and negative electrospray ionisation (ESI). Gas flow and temperature were 250°C and 21 psi. Needle, shield and capillary voltage were -4400/ -600 /-40 volts for negative ESI, and 5000/250/80 volts for positive ESI. Fragmentation was performed by collision induced fragmentation. System control was done by Varian MS Workstation 6.42. Quantitative determination was per-formed in single reaction monitoring (SRM) for FB1 and multiple reaction monitoring (MRM) for ZON and ZOLs, respectively. Transitions used for quantification were as follows: FB1 in positive ESI: 722>352; ZON: 317>175; ZOLs: 319>275. The presence of ENN was determined as follows: ENN A1: 685.5>455, ENN B: 657>445, ENN B1: 671.5>427. Calibration curves were prepared by spiking maize extract prepared from mycotoxin-free grain with a standard solution in the range 6.25-2000 µg kg^{-1}. Limits of detection and quantification were estimated as follows.

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Blank matrix was prepared in ten repetitions, Blank maize flour was spiked to 5, 10 , 20, 50 and 200 µg kg^{-1} ZON and ZOL, and 20, 50, 200 and 500 µg kg^{-1} FB1 in three repetitions each. The noise range was estimated in the peak-to peak-mode. Toxin concentrations generating signals which exceeded the noise level threefold were set as limits of detection, concentrations corresponding to signals exceeding the background noise tenfold we set as the limits of quantification. Detection limits were 20 µg kg^{-1} for α-ZOL, 50 µg kg^{-1} for β- ZOL and 10 µg kg^{-1} for FB1. %RSD for both ZOLs were 30%, for ZON 20%, and for FB1 8%.

Statistical analysis

Statistical analyses were performed using Statistix 8.1 Analytical Software. Data were arcsined transformed and Analyses of variance (ANOVA) were performed and Tukey HSD All-Pairwise Comparisons Test at 5% significance level was used to compare the means of mycotoxin levels. Spaearman Rank Correlation coefficient was used to evaluate the strength of the relationships between the incidence of Fusarium and the mycotoxins as well as co- contamination of mycotoxins.

Results

Occurrence of fungi

Nine *Fusarium* species were isolated from maize seeds: *F. acuminatum, F. compactum*, *F. equiseti*, *F. graminearum, F. oxysporum, F. pallidoroseum, F. sporotrichioides*, *F. subglutinans* and *F. verticillioides*. Four *Aspergillus* species (*A. flavus* and *A. niger, A. terreus and A. ochraceus*), 2 *Penicillium* species, *Cladosporium* sp., *Curvularia* sp., *Mucor* sp. and 7 unidentified fungal species. The highest frequency among the *Fusarium* species was 70% for *F. verticillioides*, followed by *F. sporotrichioides* (42%), *F. graminearum* (30%), *F. pallidoroseum* (15%), *F. compactum* (12%), *F. proliferatum* (12%), *F. equiseti* (9%), *F. acuminatum* (8%) and *F. subglutinans* (4%).

Incidence and levels of Fusarium *mycotoxins*

The occurrence of *Fusarium* mycotoxins (FB_1, ZON, α- and β-ZOLs, ENN A, B and B_1) in Nigerian maize is summarized in Table 1. At least one mycotoxin was detected in 170 samples (93.4%), while 12 samples were toxin free. FB1 was detected in 133 (73%) of the samples, 9 of them below the quantification limit of 10 µgkg^{-1}. ZON was detected in 103 (57%) of the samples, 79 of them below the quantification limit. The mean and maximum levels of ZON were 48.8 µg kg^{-1} and 778.9 µg kg^{-1}, respectively. α- ZOL was detected in 23 (13%) of the samples, 12 of them below the quantification limit. The mean and maximum levels for α- ZOL were 63.6 µg kg^{-1} and 181.3 µg kg^{-1}, respectively. ENN A, B and B_1 occurred in 5 samples, while further 4 samples contained only ENN B_1. None of the maize samples contained β-ZOL above the detection limits of 50 µg kg^{-1}.

The distribution of *Fusarium* mycotoxins in maize seeds in four south western states of Nigeria is shown in Table 2. The highest mean contents of 149 µg kg^{-1} FB1 was detected in Oyo state in 46 (25.2%) of the samples, followed by Ekiti state with 130 µg kg^{-1} in 19 (10.4%) samples, while the least mean FB1 contents of 66 µg kg^{-1} was obtained in Osun state in 31 (17.0%) samples. The means of FB1 contents across the four states were significantly different at P=0.05. The content of of the other toxins did not differ significantly among the states. Out of the 133 samples that showed presence of FB1, 52 and 59 samples were in the concentration range of <50 and 51-200 µg kg^{-1} respectively (Figure 1). Two samples from Ondo state and 3 samples from Oyo state showed the highest FB1 levels of 500-800 µg kg^{-1}.

Table I. Occurrence of *Fusarium* mycotoxins in Nigerian maize

Mycotoxin[1]	Positive samples[2]	Mean (µg/kg)	Range[3] (µg/kg)	Median (µg/kg)	Quantification limit (µg/kg)
Fumonisin B1 (FB1)	133 (73%)	117	10–760	75	20
Zearalenone (ZON)	103 (57%)	49	115–779	4	20
α-Zearalenol (α-ZOL)	23 (14%)	64	32–181	11	30
Enniatin B	5 (3%)	–	–	–	–
Enniatin B1	12 (7%)	–	–	–	–
Enniatin A1	5 (3%)	–	–	_	–

[1]Number of samples contaminated with mycotoxins: 170 (93%)
[2]Total number of samples: 182
[3]Range for positive samples

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Table II. Distribution of fumonisin B_1, zearalenone, α- and β-zearalenols, and enniatins in four south-western states of Nigeria.

	South-western states of Nigeria (μg kg^{-1})			
Mycotoxins[1]	Ondo	Ekiti	Osun	Oyo
Fumonisin B_1 (FB_1)				
Number (% of contaminated samples)	37 (20.3%)	19 (10.4%)	31 (17.0%)	46 (25.2%)
Mean*	115	130	66	149
Zearalenone (ZON)				
Number (% of contaminated samples)	32 (17.6%)	23 (12.6%)	21 (11.5%)	27 (14.8%)
Mean	21	63	41	75
α-Zearalenol (α-ZOL)				
Number (% of contaminated samples)	4 (2.2%)	7 (3.8%)	5 (2.7%)	7 (3.8%)
Mean	67	84	60	44
Enniatin B				
Number (% of contaminated samples)	3 (1.6%)	0	0	2 (1.1%)
Enniatin B_1				
Number (% of contaminated samples)	5 (2.7%)	3 (1.6%)	1 (0.5%)	3 (1.6%)
Enniatin A1				
Number (% of contaminated samples)	3 (1.6%)	0	0	2 (1.1%)

[1]Total number of samples = 182.
*Means across the four states are significantly different at $p = 0.05$ (Turkey HSD all-pairwise comparisons test).

Contamination frequency and levels for ZON were comparatively low with most of the samples below 50 μg kg^{-1} (Figure 2). Maximum levels of 351-800 μg kg^{-1} was found in one sample from Oyo state. Results in Figure 2 shows that most samples contaminated with α-ZOL were within 11 to 50 μg kg^{-1}, while 2 samples from Ondo state, 4 samples from Ekiti state, 2 samples from Osun state and 3 samples from Oyo sate showed the highest level of 51-200 μg kg^{-1}. The Spearman rank correlation analysis showed that ties were found between the incidence of *F. verticillioides* and FB1 levels (r=0.35). There was coexistence and multiple contaminations of 6, 4, 3 and 2 mycotoxins in 2, 1, 18 and 48 samples respectively. The study revealed a high frequency but low level of contamination of FB1, ZON, α-ZOL in maize samples obtained from four states in Nigeria. In contrast, the incidence of ENN was low. Table 3 shows the distribution of mycotoxins in white and yellow maize. Yellow maize generally showed higher concentrations of mycotoxins than white maize. The mean concentration level for white and yellow maize are FB1 (115 and 119 μg kg^{-1}), ZON (39 and 61 μg kg^{-1} and α-ZOL (55 and 75 μg kg^{-1}) respectively.

Discussion

In this study, we found that that *F. verticillioides* was the most prevalent among the 9 *Fusarium* species in Nigerian maize, contaminating 71% of samples.

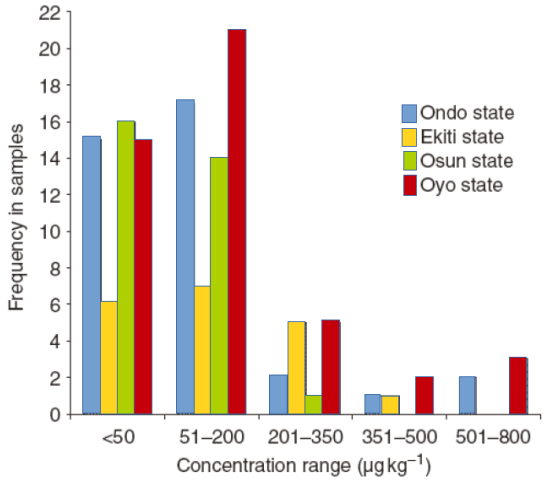

Figure 1. Concentration levels of fumonisin B1 in maize samples from four states of Nigeria.

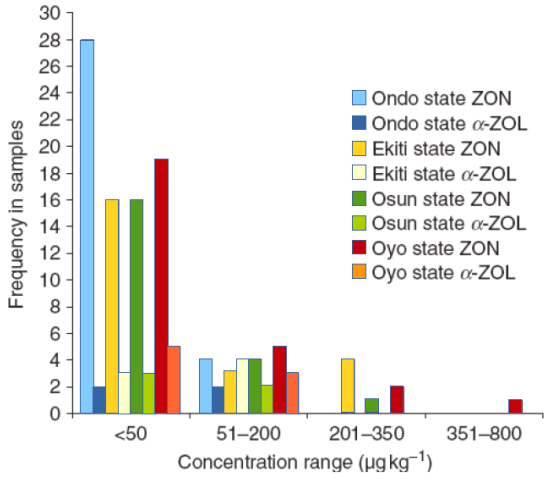

Figure 2. Concentration levels of zearalenone (ZEN) and α-zearalenol (α-ZOL) in maize samples from four states of Nigeria.

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This result is in agreement with the reports of Oyeniran, 1977; Bankole, 1994; Bankole and Mabekoje, 2004 that *F. verticillioides* was the most prevalent fungus in freshly harvested maize. The same observation was reported by Owolade et al, 2001 and Bankole and Adebanjo, 2003 for Nigerian stored maize. We confirmed the observation of these authors that *F. verticillioides* was often isolated from symptomless kernels.

Maximum limits for ZON and FB1 have not been established in Nigeria yet. Eight samples (3 from Oyo State, 1 from Osun State and 4 from Ekiti state (Figure 1) were not fit for human consumption by European Union (EU) standards, because they contained ZON

Table III. Concentration levels of *Fusarium* mycotoxins in white and yellow maize seeds in Nigeria.

	White		Yellow	
Mycotoxins[1]	($\mu g\,kg^{-1}$)	SE[2]	($\mu g\,kg^{-1}$)	SE[2]
Fumonisin B_1	115	18.32	119	17.38
Zearalenone	39	13.61	61	15.45
α-Zearalenol	55	15.59	75	17.78

[1]Total number of samples = 182.
[2]SE, Standard Error of means.

above the permissible level of 200 µg kg^{-1} (CEC, 2005a). No sample exceeded the EU maximum limit of 2,000 µg kg^{-1} for FB1. α-ZOL was detected in 14% of the maize samples with mean levels of 64 µg kg^{-1} and maximum of 181 µg kg^{-1}, though recommended legal limits for α-ZOL have not yet been established.

The percentage of maize samples contaminated and mean levels of FB1 in this study agrees with previous reports from pre-harvest maize in Nigeria (Bankole and Adebanjo, 2003, Bankole and Mabekoje, 2004). However, this work reports a lower FB1 concentration range of 10-760 µg kg^{-1} probably due to unsuitable climate for fungal contamination in the four states where the samples were obtained, though, a high level of contamination may occur in other years. The Spearman Rank correlation analysis showed that ties were found between ZON and α-ZOL (r=0.52), meaning that as the grain was being contaminated by the production of ZON, the chances of production of more the potent α-ZOL increases, thereby having greater impact on health of animals and livestock.

The co-occurrence of FB1, ZON, α-ZOL, ENN A, B and B1 in 69 samples calls for urgent concern and monitoring of mycotoxins in maize, owing to its importance as a major staple food in Nigeria. The simultaneous contamination is significant from the standpoint of potential risks to human and animal health (Yazdanpanah et al., 2001). A more comprehensive survey in all other agroecological zones is desirable, in order to assess the extent of which this Fusarium toxins is a problem in Nigeria. The maintenance of fumonisins at undetectable levels from post-harvest to the drying interval is a challenge (Marín et al., 1999). Therefore, efforts to reduce the harvest/drying interval, as well as the constant monitoring of toxigenic fungi and fumonisin contamination in corn and corn-based foods are essential in order to assure the quality and safety of products and to minimize the potential hazards to human and animal health.

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