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Occurrence of Ochratoxin A in Nigerian Ready for Sale Cocoa Beans

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Abstract: Ochratoxin A (OTA) is a mycotoxin commonly associated with many food and beverage crops grown in tropical and temperate regions. There is no data available regarding OTA contamination of ready for sale cocoa beans in Nigeria. The objective of this study was to assess the levels of OTA contaminations in ready for sale cocoa bean samples collected from farmers during a survey in three states of Nigeria. An indirect competitive ELISA protocol was used to quantify OTA levels in the samples. Out of 59 samples analyzed, > 90% were positive for OTA, with concentrations ranging from 1.0 to 277.5 μg kg^{-1}. Cocoa bean samples from Cross Rivers State were more contaminated in terms of OTA levels, the higher concentrations observed was 277.5 and 200.8 μg kg^{-1}in 2 samples from Cross Rivers and Edo States, respectively. However, There were no significant differences among the OTA levels of the three States ($p > 0.05$).

Key words: (OTA), ready for sale cocoa beans, Nigeria, States

INTRODUCTION

The increasing occurrence of mycotoxins (secondary metabolites of fungal origin) in agricultural commodities and the subsequent impact on consumer health as well as on national and global trade is of major concern in both the developed and developing countries (Wu, 2005). (OTA), a mycotoxin that was first isolated in 1965 in South Africa from a strain of *Aspergillus ochraceus* (Merue Vandar *et al*., 1965) usually occurs as a trace contaminant in many agricultural products. Since its discovery, OTA had been reported to be produced by a few molds belonging to the *Aspergillus* and *Penicillium* genera (Rizzo *et al*., 2002; Ciegler, 1976; Verga *et al*., 1996; Elbanna *et al*., 1987). These fungi are ubiquitous and can occur in tropical and temperate climates. Growth of the mold and the production of the OTA are dependent upon a number of factors such as the amount of inoculum, substrate, water activity, moisture content, temperature, incubation time and humidity during the growth, harvesting, processing and subsequent drying and storage of the crop. Nigeria has a tropical climate with all year round high ambient temperature and relative humidity that provide optimal condition for the growth of these toxigenic molds and subsequent production of OTA.

The accumulated effects of consuming foods heavily contaminated with OTA is life threatening. This is because OTA is teratogenic, immunosuppressive) immunotoxic and possibly possess neurotoxic, genotoxic and carcinogenic properties (Council and Mycotoxins, 2003). As a result, the International Agency for Research on Cancer has classified it in group 2B as a human carcinogen (IARC, 1993). Its implicated role in an irreversible and fatal kidney disease referred to as Balkan Endemic Nephropathy (BEN) is of concern to humans (Pavlovic *et al*., 1979). The committee on Toxicity of Chemicals in Food, Consumer Products and the Environment concluded that OTA is a genotoxic carcinogen and advised that OTA levels in food should be reduced to the lowest level that is technologically achievable (Cot, 1997).

In 1995, the Joint Expert Committee on Food additives, (JECFA) of the World Health Organization and the Food and Agriculture Organization set a Provisional Tolerable Weekly Intake (PTWI) of 0.1 ng g^{-1} body weight (bw), approximating to 14 μg kg^{-1}bw day^{-1}(WHO, 1996). Because of the toxicological characteristics of OTA, the Scientific Committee on Food of the European Union proposed that the maximum daily OTA admission to the consumer (PTDI value) should be 5 ng g^{-1} bw (Eurpean Union, 2002). In 2002, the European Commission resolved to have maximum levels of OTA in cereals (5 μg kg^{-1}), cereal product (3 μg kg^{-1}) and dried vine fruit (10 μg kg^{-1}), but not yet in cocoa and its related food products (Stefanaki *et al*., 2003). The regulation regarding OTA in cocoa is being considered by the European Union and this may adversely affect cocoa export from Nigeria leading to serious economic implications since cocoa is the second largest foreign exchange earner after petroleum in Nigeria.

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Several investigators have reported the natural contamination of OTA in trace quantities (ng to μg) in a variety of foods and feedstuffs from many countries. OTA contaminates cereals (Gollucke *et al*., 2004), dried fruits (Belli *et al*., 2004) coffee (Bonvehi, 2004) wines (Amezqueta *et al*., 2005) cocoa beans and products (Dongo *et al*., 2006; ICCO, 2003) etc. Nigeria has been growing cocoa since the 19th century and is presently the world's fourth largest producer accounting for approximately 6% of the world production (NAFDAC, 2005). Over 80% of cocoa produced in Nigeria is exported to the European trading bloc. The introduction of EU regulation to regulate OTA levels in cocoa beans could threaten exports from Nigeria. However, little is known about the extent of OTA contamination in Nigerian cocoa beans.

This study was initiated to investigate the natural occurrence and levels of OTA in ready for sale cocoa beans in Nigeria with a view of developing intervention strategies necessary to possibly reduce the OTA risks in human and animal health. Ready for sale cocoa beans are cocoa beans that are to be presented for sale by cocoa farmers after immediate drying and bagging.

MATERIALS AND METHODS

Sampling: One ready for sale sample of cocoa beans was collected from each of the 59 cocoa farmers visited during a survey of three cocoa producing states in Nigeria viz: Ondo, Cross Rivers and Edo. Ten cocoa bean samples were from Ondo State, 21 were from Edo and 28 were from Cross River States. The farmers were selected on the basis of the availability and their readiness to supply cocoa beans. The sampled areas within the three states are shown in Fig. 1.

OTA analysis: OTA in cocoa bean samples was extracted following the procedure of ICRISAT (ICRISAT). Five gramme of cocoa beans was placed in a blender, triturated in 25 mL of 70% methanol containing 0.5% potassium chloride until it was thoroughly ground. The suspension in a conical flask was homogenized with an orbital shaker at 300 rpm for 30 min and then filtered through whatman No 1. The filtrates were stored in glass vials at 4°C until required.

Estimation of OTA: Indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA) was used for the estimation of OTA in cacao extracts as per the method described by (Thirumala-davi *et al*., 2000), using OTA-specific rabbit polyclonal antibodies obtained from ICRISAT, Patancheru, India. Microtitre plates (Maxi-sorp-Nunc) were used and at each step the plates were incubated for 1 h at 37 °C. Initially plates were coated with 150 μl of 150 ng mL 1 of OTA-BSA in 0.2 mol/L carbonate coating buffer, pH 9 .6. In the second step plates were

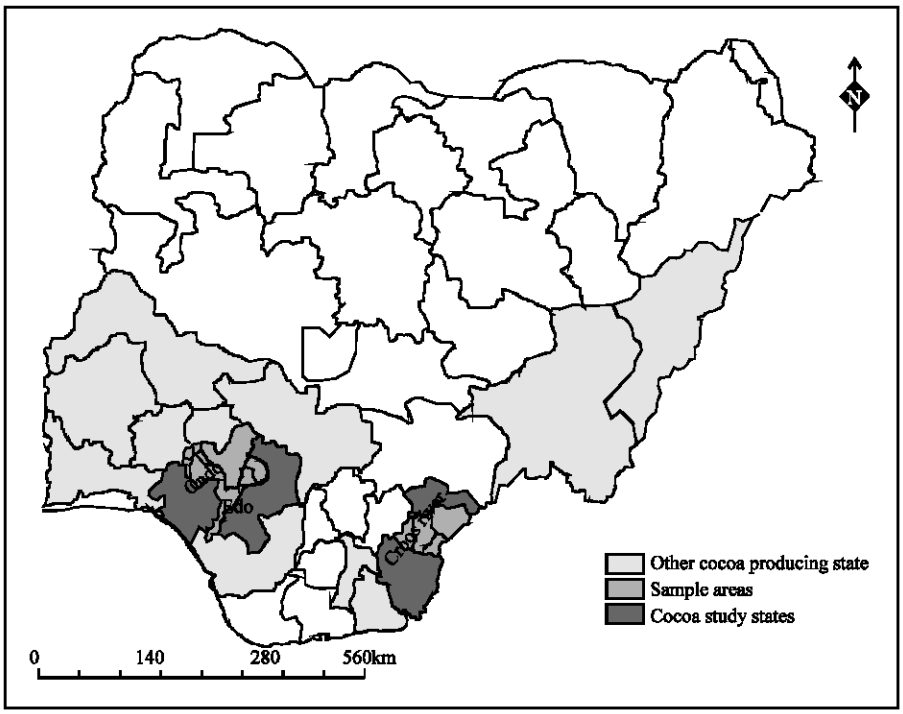

Fig. 1 Map of Nigeria showing sampled areas

Table 1: Recovery of (OTA) A from spiked cocoa bean samples[a]

Added OTA ($\mu g\ kg^{-1}$)	Cocoa beans ($\mu g\ kg^{-1}$)	Detected (%)[b]
0.02	0.015 ± 0.6	75 ± 0.64
0.06	0.053 ± 1.3	88 ± 2.1
0.1	0.098 ± 5.4	98 ± 1.2
0.14	0.125 ± 3.6	89 ± 0.47
0.18	0.155 ± 0.89	86 ± 7.3

[a] Each sample was replicated thrice. Values are means ± SD, [b] Detected OTA ($\mu g\ kg^{-1}$)/added OTA ($\mu g\ kg^{-1}$) ×100

filled with a suspension of 4% dried milk prepared in phosphate-buffered saline, pH 7.4 containing 0.05% tween-20 (PBS-T). Then 100 µl of OTA standards (10-100 ng mL^{l}) or cacao sample extract diluted 1:10 (v/v) in PBS-T were added to each well and mixed with 50 il of OTA-polyclonal antibody diluted 1:8000 in PBS-T. Goat antirabbit IgG conjugated to alkaline phosphatase (1:4000 dilution in PBS-T; Sigma, USA) was used to detect plate-bound OTA-rabbit polyclonal antibodies. Substrate, 150 µl of *p*-nitrophenyl phosphate at 1 mg mL^{-1}in 0.1% (v/v) diethanolamine buffer, pH 9.8, was allowed to develop for 1 h at room temperature and absorbance at 405 nm was read in an ELISA plate reader. OTA concentration in samples was calculated from the standard curve derived from the OTA-standards and was expressed in $\mu g\ kg^{-1}$.

Recovery experiment: The indirect competitive ELISA technique was validated by performing three replicate analyses of uncontaminated cocoa bean spiked at 5 different levels of OTA (Table 1). The ELISA protocol was as described above.

Preparation of (OTA) a standard stock solution: (OTA) A standard (Sigma, St Louis, MO, USA) was prepared by dissolving l mg OTA in 10 mL of Benzene:Acetic acid 99 (v v^{-1}) (Olsen *et al*., 2003). One milliliter of 10$\mu g\ mL^{-1}$ was calibrated spectrometrically at 333 nm using the value 5550 $mol^{-1}\ cm^{-1}$ for molar absorptivity. After calibration of the OTA solution, an exact volume was evaporated under the fume hood. The residue was redissolved in methanol to get 0.25 $\mu g\ mL^{-1}$ which served as stock solution. Further dilutions were made from the stock solution (stored at 4°C) when needed.

Statistical analysis: Data analysis was carried out by Statistical Package for Social Sciences (SPSS). A non-parametric procedure (median test) was used in order to eliminate difficulties caused by heterogeneous data distribution. For statistical comparison of contamination levels, χ^2 tests were applied within the three states.

RESULTS AND DISCUSSIONS

Recovery experiment: The recovery of OTA averaged 87% in uncontaminated cocoa bean spiked with 0.02, 0.06, 0.1, 0.14 and 0.18 $\mu g\ kg^{-1}$. The detection limit of the assay was 0.01$\mu g\ kg^{-1}$.

Table 2: Occurrence of (OTA) A in ready for sale cocoa beans from three States in Nigeria

States	Number analyzed	OTA positive samples	Mean of positives ($\mu g\ kg^{-1}$)	Range[b] ($\mu g\ kg^{-1}$)
Cross rivers state	28	26	40.4	1.9-277.5
Ondo	10	8	37.2	2.0-118.8
Edo	21	20	34. 5	1.0-200.8
Overall	59	54	37.7	1.0-277.5

b Detection limit 0.01$\mu g\ kg^{-1}$

OTA analysis: Out of the 59 cocoa bean samples from Ondo, Cross Rivers and Edo States tested for OTA contamination, 54 samples (92%) contained detectable amounts of OTA (Table 2).

OTA was detected in all, except one sample from Edo State at levels ranging from 1-201 $\mu g\ kg^{-1}$ and the mean value of all the samples was over 34 $\mu g\ kg^{-1}$. The most contaminated samples were from Owan East Local Government Area (LGA) of the State. Among the cocoa bean samples from Ondo State, 80% were positive with an average OTA content of 37 $\mu g\ kg^{-1}$. Twenty six out of 28 cocoa bean samples from Cross Rivers State were positive for OTA with an average value of 40.4 $\mu g\ kg^{-1}$. Only four samples had OTA levels greater than 50 $\mu g\ kg^{-1}$and the most OTA level (277) was detected in a sample from Etung LGA of Cross Rivers State. OTA has been detected in several food crops. 57% of 6476 food samples marketed in Italy were reported to be OTA contaminated (Wolff *et al*., 2000; MAFF, 1997) while 22% of the cocoa products marketed in Italy, were contaminated above detection limits of 0.01 $\mu g\ kg^{-1}$(Tafuri *et al*., 2004). (OTA) A was detected in cocoa bean at levels from 0.1-3.5 $\mu g\ kg^{-1}$, the mean concentration being 0.45 $\mu g\ kg^{-1}$; only one sample exceeded 2 $\mu g\ kg^{-1}$ (Bonveh, 2004).

The frequency distribution patterns of different levels of OTA contamination are given in Fig. (2) for the cocoa beans from Cross Rivers, Edo and Ondo States. This shows that cocoa bean samples from Cross Rivers State with very high levels (260-280 $\mu g\ kg^{-1}$) were just one. Over 80% of the samples had OTA levels within the range 0.1-50 $\mu g\ kg^{-1}$. For cocoa bean samples from Edo State, one cocoa bean sample had very high OTA level (200-220 $\mu g\ kg^{-1}$) while 80% had OTA levels within 0.1-50 $\mu g\ kg^{-1}$. None of the samples from Ondo State had OTA levels beyond 100-120 $\mu g\ kg^{-1}$. Figure 3 shows the comparison of the incidences of OTA contamination for the three States. Over 40% of the samples from Cross Rivers State had OTA levels lower than 50 $\mu g\ kg^{-1}$and samples with high levels (>250$\mu g\ kg^{-1}$) were very exceptional. Only about 30% of the cocoa bean samples

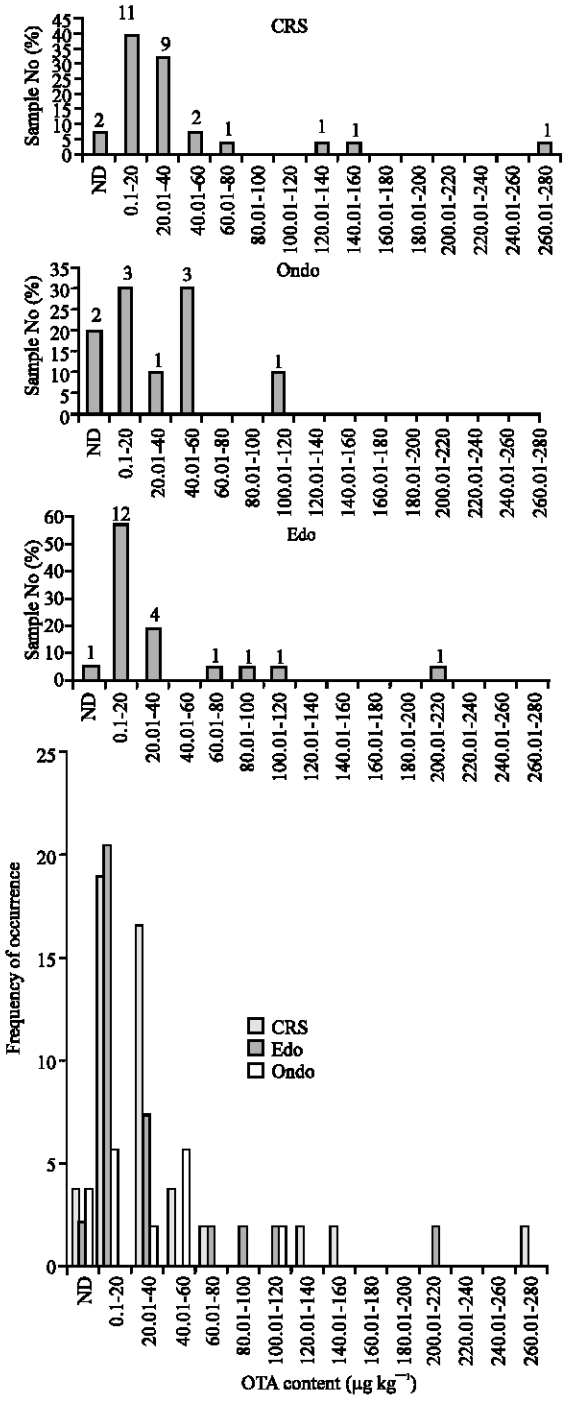

Fig. 2: OTA frequency distribution in cocoa beans from Cross Rivers, Edo and Ondo States. Numbers indicated on histograms represent total numbers in each range

from Edo State showed values lower than 50 µg $kg^{-1}$and about 2% of the samples showed OTA levels > 200 µg kg^{-1}. For cocoa bean samples from Ondo State,

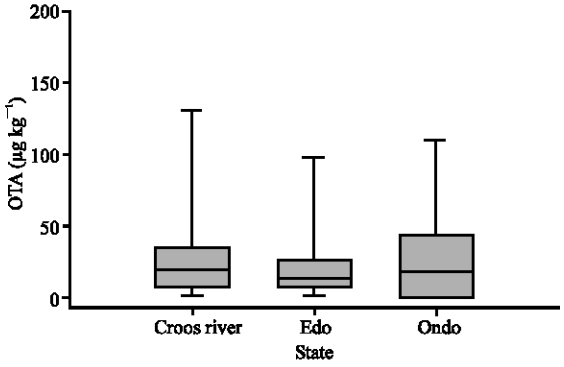

Fig. 3: Box and whisker plots of positive cocoa bean samples OTA distribution as a function of their states

Table 3: Median and x^2 Tests of OTA levels in the cocoa bean samples from three different States

States	Mean	χ^2	df	Significance
CRS	28.42			
Edo	25.25	1.454	2	> 0.05
Ondo	30.13			

OTA Content (µg kg^{-1})

only about 13% of the samples showed values lower than 50 µg kg^{-1}, whereas only 2% samples showed OTA levels over 100 µg kg^{-1}.

The box plots showing the distribution in terms of median, maximum and minimum values of the positive samples in each State are shown in Fig. 4. The OTA level of cocoa bean samples from Cross Rivers State show a much higher dispersion than the other two States. It further shows that OTA levels of cocoa bean samples from Edo State fall within the median. The results of the median test and x^2 test performed on the cocoa bean samples (Table 3) show that there were no significant differences among the OTA values of cocoa bean samples from the three different states.

This study documents that the extent of OTA contamination is high in ready for sale cocoa beans from Edo, Cross River and Ondo States of Nigeria were the mean levels of OTA were similar across the three States. This could be attributed to the prevailing climatic conditions in those States with respect to harvesting and drying. The three States fall within the humid forest zone of Nigeria that experiences an average 269 mm rainfall, 61-95% relative humidity and 22.4-30.3°C of temperature during the peak harvesting season (September to December). These conditions favor mold development and subsequent production of OTA (Naresh *et al*., 2003; Mitchell *et al*., 2004). Interestingly, it has been reported that OTA concentration in cocoa beans is concentrated in the shell (Amezqueta *et al*., 2005). Fourteen of the twenty-two cocoa bean samples (64%) suffered a loss of OTA of more than 95% due to shelling, 6 samples had a

loss of OTA in the range 65-95% and only one sample presented a reduction of less than 50%.

In Nigeria, the maximum tolerable limits of OTA in cocoa based products are yet to be established; however the high values recorded, indicate that cocoa bean products may contribute to daily intake of OTA in the diet. This poses a public health problem as OTA is currently being linked to many cancers and even kidney and liver malfunctions (BCERF). The result therefore calls for research towards developing intervention strategies to reducing the levels of OTA in human and animal feeds to safe limits.

This result calls for future research on the OTA levels of cocoa beans from different ecologies in order to validate the dependence of OTA to climatic conditions. This will form the basis for developing intervention strategies towards reducing the levels of OTA in human and animal feeds. In conclusion, the quality of cocoa beans produced in Nigeria can be improved in spite of the fact that 91.52% of samples were above the detection limit of 0.01 $\mu g\ kg^{-1}$. This can be achieved by putting in place effective weather forecasting systems where by a farmer could be advised on when to harvest for proper drying of the fermented beans. Since sun drying may be a difficult task due to the high rainfall at the time of harvest, artificial dryers can be provided at subsidized rates for the Cocoa farmers in Nigeria. A lot of work has been done on the design of solar and mechanical dryers for use by farmers in the tropics (Axtell and Bush, 1991). However, these dryers are not in use by farmers because of huge financial involvement. Cocoa farmers should be educated on the need to observe good agronomic practices as these have been shown to have profound effect on mycotoxin contamination of crops in the field (Avantaggio, 2002). Additionally, improvements of the industrial shelling process could prevent or reduce to the barest minimum OTA occurrence in cocoa final products.

Creation of awareness among the citizenry is very important as the problem posed to health and the economy is restricted among scientists. Lastly, there should be routine monitoring of OTA in cocoa beans and cocoa products to boost food safety. All these put in place will ultimately boost international trade and offer long-term health benefits to the consumers.

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