African Journal of Food Science Vol. 5(8) pp. 513 – 521, August 2011
Available online http://www.academicjournals.org/ajfs
ISSN 1996-0794 ©2011 Academic Journals

Full Length Research Paper

Comparative analysis of detecting ochratoxin A in cocoa powder samples using high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA)

C. O. Jayeola[1]*, A. Oluwadun[2], L. E. Yahaya[1]*, L. N. Dongo A. A. Ajao[1] and F. C. Mokwunye[1]

[1]Crop Processing and Utilization Division, Cocoa Research Institute of Nigeria, P. M. B., 5244, Ibadan, Oyo State, Nigeria.
[2]Department of Medical Microbiology and Parasitology, Obafemi Awolowo College of Health Science, Olabisi Onabanjo University Sagamu, Ogun State, Nigeria.

Accepted 15 June, 2011

Different cocoa powder (CP) samples were analyzed for mycoflora contaminants as a means of ensuring food safety. A total of 360 samples of 24 brands of CP were purchased between April and November, 2007. Ten-fold serial dilutions of the cocoa samples in sabouraud dextrose broth (SDB) were plated on potato dextrose agar (PDA) for yeast and mould counts. Colonies of yeasts and moulds, isolated from the cocoa samples were identified by standard mycological methods. The Ochratoxin A (OTA) extraction and detection from the samples was determined by high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA). The common fungi isolated were *Aspergillus niger*, *Saccharomyces cerevisae*, *Penicillium chrysogenum* while the least encountered fungi were *Aspergillus melleus* and *Aspergillus ochraceous* in the tested cocoa powder samples. This study showed that CP, which forms the bulk ingredient of cocoa-based beverages, is a possible source of microbial contamination to the beverages, though the values obtain from the use of HPLC is below the recommended value of 5 µg/kg as specified by European Union (EU), thus making the cocoa powder produced from these areas safe for consumption.

Key words: Cocoa powder, mycoflora, beverages, contaminants, food safety.

INTRODUCTION

The mycoflora and the intrinsic factors that might contribute to the spoilage or deterioration of the powder are very important in order to ensure food safety (Stannard, 1997).

Food safety is usually determined by the absence or presence of pathogenic organisms, or their toxins, and the number of pathogens, with their expected or destructive agents (Ogunledun, 2007). The level of spoilage microbes reflects the microbial quality, wholesomeness, of a food product as well as the effectiveness of measures used to control or destroy such microbes (Pierson and Smoot, 2001).

Food borne disease and microbial spoilage of food result from the failure of or inability to control micro organisms at one or more stages of food chain, from raw material production to consumption of the final product. Specifically, the microbiological tools are used to assess the safety of food, adherence to good manufacturing practices (GMPs), the keeping quality (shelf life) of certain perishable foods and the utility (suitability) of a food or ingredient for a particular purpose (NRC, 1985).

Ochratoxin A (OTA) is a type of mycotoxin which are known to be secondary metabolites produced by fungi in the *aspergillus* and *penicillium* general in food and feeds and upon an ingestion, it can result in the illness or death of animals and humans. These natural food contaminants are found in different kinds of foods, spices beverages, cereals, beans, dried fruits, coffee, cocoa, wine, beer, spice, juice and milk (Bennet and Klich, 2003). The occurrence of OTA in agricultural staples has been a

*Corresponding author. E-mail:eugeneyah@yahoo.co.uk.

topic of concern in food assurance due to human and animal health hazard. The toxicological properties of OTA include nephrotoxicity, teratogenicity, citrotoxicity and genotoxicity (IARC, 1993). The EU has recently introduced maximum permissible limits for OTA to reduce risk to the consumer. In cereals, these limits are 5 µg/kg for whole grain and 3 µg/kg for processed products (European Commission Regulation, 2002). The present of OTA in food is of great concern due to chronic effects at low levels of exposure, in humans; severe dietary exposure to Ochratoxin A has been associated with chronic, progressive, Balkan endemic nephropathy which is a kidney disease.

There has been great awareness on the health benefit of cocoa especially in treating hypertension and diabetics, (Olubamiwa, 2007) also there is an increased public health concern on the quality of these products especially those packaged as eruku oshodi- a popular cocoa drink that is produced from the mixture of cocoa, sugar and milk powder, as food poison outbreaks have often been traced to the consumption of unhygienically handled food products (Ogunledun, 2007; Chukwuka, 1997; Badru, 2005). The consumption of cocoa products contributes to human nutrition through provision of lipids, sugars, minerals (potassium, magnesium, copper and iron) and antioxidants principally polyphones (Hollman et al., 1999). Tea and red wine have long been known for their high content of polyphenolic substances, but it is less known that cocoa bean, as well as cocoa derived products also present a rich source of polyphenols which exhibit equal or even higher antioxidants capacity than some fruit or vegetables (Lee et al., 2003). Regarding the fact that contamination of Ochratoxin A in commodities is unavoidable, there is need to monitor the level in food in order to avoid health hazards that can be caused to human. HPLC is the most commonly used method for ochratoxin determination (Blesa et al., 2004;Dall'Asta et al., 2004) but it requires extensive clean-up of sample extract, a trained staff and it is time-consuming in addition to its high cost and use of harmful solvents (Sydenham and Shepherd, 1996). While immunoassays provide an attractive and promising alternative for ochratoxin detection in food due to high specificity, sensitivity, simplicity, potential for automation and possibility of use under field conditions(Hefle, 1995). The aim of this study was to identify fungal population in cocoa powder circulating in Nigeria in order to evaluate the quantity of OTA produced in them. In order to determine the OTA levels produced by toxigenic spps, adequate validated methods are required for obtaining reliable data so as to assist in correct decision making.

MATERIALS AND METHODS

Acetonitrite, methanol and chloroform were of analytical grade (Mallinckradt Baker Inc Philipsburg, New Jersey, USA. Immunoaffinity columns for OTA (Ochratest^{TM}) and filter paper were from Vicam Inco. (Watertown, USA). OTA standard were purchased from Sigma Chemical Co (St Louis) MO, USA. Micro titre plates (96 wells) were from Corning (USA). 360 of 24 brands cocoa powder samples were obtained from cocoa producing companies while some are purchased at major modern markets in Southwest Nigeria. The samples were stored in air tight containers under room temperature.

Proximate analysis was carried out according to the method of AOAC (2000). Yeast and mould counts were determined using McFaddin (1986) methods. Direct plating of the samples on agar media was carried out by aseptically plating 1 g of each cocoa powder sample into 9 ml of sterile distilled water. This was thoroughly shaken and from the suspension, 1 ml was transferred to another tube containing 9 ml of sterile distilled water and thoroughly mixed again. This dilution procedure was further repeated thrice so that there were series of five tubes giving a serial dilution of 10-1 to 10-5. An aliquot of 1 ml was pipette at each dilution into Petri dishes and were overlaid with cooled molten potato dextrose agar (PDA). The plates were incubated under room conditions (28±2°C) and examined after 7 days under a stereoscopic binocular microscope for the presence of fungi. The number of fungal colonies that appeared in a plate was multiplied by the dilution factor to obtain the number of colony forming units per gram (cfu/g) of cocoa powder samples. Colonies of fungi that appeared on agar plates were repeatedly sub cultured on fresh PDA until pure culture of each isolate was established. Identification of fungi was by observing the growth habits and morphological characteristics under a wide binocular microscope. Wet mount of hyphae/asexual structures stained with lactophenol in cotton blue were viewed under compound microscope and identified with reference to standard texts (Barnett and Hunter, 1987). Characterisation of the fungi was done based on the colour of the colony, appearance, conidiophore, mycelium, arrangement of conida on sterigmata. The pure culture of fungi got was prepared on a clean glass slide and stained with cotton blue in lactophenol. Observation was done under ×40 oil immersion objective lens.

OTA extraction

Preparation of crude extract of OTA was according to Fujii (2002) with little modification. 1 g of cocoa powder was mixed with 10 ml acetonitrite-water v/v. The suspension was sonicated (ultrasonic cleaner unique® Brazil) for 20 min shaken at 150 rpm for 10 min. The crude extract 10 ml was mixed with 10 ml chloroform and 10 ml 0.5% NaCl solution and was shaked at 150 rpm for 10 min. The chloroform layer 10ml was evaporated to dryness (under a stream of nitrogen, at 40°C) dissolved in methanol-1%, sodium bicarbonate (70:30v/v)

Ochratoxin A analysis by HPLC

OTA was analyzed using the method of Pittet (1996) with modification. This was by a reversed – phase HPLC system using a nucleosil C_{18} column 25 cm×3.0 mm and Ultra Violet (UV) detector (S5ODS2). The flow rate was at 1.0 ml/min and was injected at 20 µl. The detector limit was at 232 nm and the mobile phase used was methanol: water (1:1) OTA concentration was determine using a calibration curve ranging of 1 to 10 ng/ml.

Ochratoxin A analysis by ELISA

The method as described by the manufacturer (Vicam) was strictly followed. 20 g of cocoa powder sample were weighed into a clean jar. 10 ml of 70:30 (v/v) methanol: water extraction solution was added. The mixture was shaken for 3 min after which the samples were allowed to settle. The top layer of the extract was then filtered

through a Whatman No 1 filter and the filtrate was collected. 30 number of blue –bordered dilution strips were placed in a microwell strip holder since one dilution well was used for each standard 0, 2, 5, 10, 20, 40 µl as well as the 24 cocoa powder samples. Using an 8-channel pipette, 200 µl of conjugate was dispensed into each dilution well. With the use of a single channel pipettor, 100 µl of each standard and sample was added into appropriate dilution well containing 200 µl of conjugate. The mixture was thoroughly mixed by pipetting each up and down 3 times and 100 µl of the content for each dilution wellwere transferred into a corresponding antibody coated microwell. These were incubated at room temperature for 10 min. The content of the microwell strips were emptied into a waste container and each microwell were washed with distilled water for five minutes and absorbent paper towels were used to expel as much residual water as possible as well as the bottom of the microwells. 1 ml/strip amount of substrate were measured from the blue-capped bottle into separate container. 100 µl of the substrate was pipette into each microwell strip and was incubated for 5 min at room temperature. 100 µl of stop solution was pipette into each microwell, the colour changed from blue to yellow. The strips were read in a microwell reader using a 450 nm filter with a differential filter of 630 nm. Each OD reading for each microwell was recorded. OD values were expressed as a percentage of the OD of the zero standards. A dose- response curve was constructed using the five standards. The unknown was measured by interpolation from the standard curve. OTA concentration was expressed in ppb.

OTA recovery

Cocoa powder which were OTA negative by HPLC (< 0.80 ng/g), were spiked with OTA (5.0, 10.0, 25.0, and 50.0 ng/g) dissolved in methanol. The samples were extracted after standing for 16 h at 25°C. OTA quantitation and recovery tests were carried out by HPLC and ELISA.

Statistical analysis

The level of significance was determined at 95%. Data are presented as mean ± standard deviation of five measurements. Sensitivity test, correlation coefficient and scattered diagram were used to determine the relationship between HPLC and ELISA analyses. Data analysis was carried out with statistical package for social sciences (SPSS version 15).The Student's test (t-Student) and Mc Neman test were applied to determine the sensitivity of OTA detection by HPLC and ELISA methods.

RESULTS AND DISCUSSION

Cultural and microscopic features of fungal isolates are shown in Table 1 with *Aspergillus niger* and *Saccharomyces cerevisae* having isolation rates of 25% each, *Penicilium chrysogenum* 16.7%, *Aspergillus melleus* 8.3% and *Aspergillus ochraceous* 2.5%. The presence of *Aspergillus spp* is of great public health importance (Ogunledun, 2007). The presence of *Aspergillus species* and *Penicillium chrysogenum* a lipolytic and toxigenic moulds (Uraih and Ugbadu, 1980) should be viewed with great concern since in recent years food poisoning outbreak have been traced to contamination of food products by these organisms. The presence of mould is an indication that most of these food borne fungi exhibit the potential to produce toxic metabolites. There is sufficient strong evidence to conclude that, naturally occurring aflatoxins and ochratoxin are carcinogenic to animal and humans (IARC, 1993).

Aspergilli are among the most abundant and widely distributed organisms on earth (Bennet and Klich, 2003. Virtually all the common *aspergilla* have been recovered at same time from agricultural products (Samson et al., 2004). The main impact on agriculture is in saprophytic degradation of products before and after harvesting and in production of mycotoxins (Domsch et al., 1980). Members of the genus *Aspergillus* have been reported to be more heat tolerant and xerophilic than most other fungal general (Pitt and Hocking, 1997). These unique attributes must have enhanced their survival, despite the drying and roasting processes and also in the presence of high osmotic pressure in the cocoa powder brands. The isolation rate of the fungi showed that *A. niger and A. Ochraceus* has 25% each while *P. chrysogenum* is 16.7% and *A. melleus* is 8.3%.

Mycotoxins seem able to cause serious disease of the liver, kidney and blood forming organs in extremely low quantities. In human mycotoxins have been implicated in a form of encephalopathy observed in Thailand and in a particular nephropathy rather frequently seen in the Balkans (Betina, 1989). Despite the danger of food poisoning caused by mycotoxins producing fungi, they also utilize the nutrient found in the food. To improve quality and prevent spoilage at various aw, it was suggested by Mossel and Shennan (1976) that if the a_w is below 0.65 and the product is maintained at this level, during storage, problems arising due to microbial spoilage are rare, irrespective of the number of contaminating organism present. It was noted that mycotoxins production ceases or become very low at aw below 0.85 (Pierier and Davis, 1969).

Few studies have been carried out on the incidence of OTA in cocoa beans and not on cocoa powder. Miraglia and Brera (2002) reported a study in which none of their 96 cocoa bean samples had OTA levels exceeding 2 µg/kg). The draft limit of 5 µg/kg proposed by Codex for Ochratoxin A in cereals and cereal products with the appropriate safe exposure levels, the provisional tolerable weekly intakes (PTWIs), recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was used as standard .

Figure 1 showed a great variation in the OTA samples present in cocoa powder samples as analysed by ELISA methods. The value varies between 0.05 to 27.5 µg. Figure 2 showed a little variation in the OTA content present in cocoa powder samples and this varies between 0.65 to 3.28 µg/kg. These values were still below the recommended value of 5 µg/kg by the EU.

Figure 3 depicts relationship between OTA levels (µg/kg) detectable in cocoa as were assessed by both HPLC and ELISA techniques. Although, a positive correlation (r = + 0.305) was observed between the two techniques, the relationship however, found to be

Table 1. Identification of fungal species in cocoa powder according to morphological characteristics.

Cultural and Microscopic Features	Possible Isolates	N	n	Isolation rate %
Blackish-brown often with yellow mycelium. Reverse greenish-yellow to yellow-or Its head globose, splitting with age. Its metulae is long, closely packed and brownish.	*Aspergillus niger*	12	3	(25)
White to yellow mycelium. Yellow, buff to brown sclerota. Pale yellow /gold or cream coloured conidia. Uncoloured exudates when present	*Aspergillus melleus*	12	1	(8.3)
Yellow-buff coloured colonies, small and nearly smooth conidia, pink to purple sclerotia. Uncoloured, yellow or dull red exudates when present.	*Aspergillus ochraceus*	12	3	(2.5)
The texture is sulicete and velvetious. green to (dark) green observed. Its reverse is yellow (occasionally creamish). It has a short smooth strupe. The penicillin is terverni-culete, phialides ampulli form, collula very short, both Divergent and appressed branched. The conidia is allipsoidal to spherical, smooth and greenish.	*Penicillium chrysogenum*	12	2	(16.7)
It is creamish in colour, obverse and oval in shape (spore). The cellular is smooth and very small. It has branched cells (spores)	*Saccharomyces cerevisae*	12	3	(25)

N = Number of samples investigated,n= number with positive culture.

insignificant ($p > 0.05$).

Figure 4 showed OTA peak standard with other peak detected using HPLC analysis while Figure 4 showed OTA concentration when ELISA kit was used.

Figure 5 showed the relationship between OTA levels (µg/kg) detectable in cocoa were assessed by both HPLC and ELISA techniques. Although, a positive correlation ($r = +0.305$) was observed between the two techniques, the relationship however, found to be insignificant ($p > 0.05$).

In Figure 6, relationship between OTA level (µg/kg) in cocoa powder and their moisture contents (%) was determined. Though, a positive correlation was observed between the two parameters($r = +0.089$), but the relationship was however, found not to be significant ($p > 0.05$).

In Table 2, the result indicated that OTA detection was more sensitive in HPLC (92.0%) analysis than that of ELISA analysis (83.33%) the result of Mc Neman's chi square showed no significant difference $\chi^2MN = 18.01$, $p<0.05$. The result showed that the sensitivity of ELISA and HPLC were adequate for detecting OTA in cocoa powder samples.

Table 3 revealed a lot of differences in the mean values of OTA as detected by the two methods of analysis but there was no significant difference with $p<0.147$ also it showed the correlation coefficient (which is the strength of relationship) between HPLC and ELISA. The table revealed weak positive correlation (0.3048); because it is less than 0.5.

The performance OTA detection in cocoa powder as shown in Table 4 between ELISA and HPLC were evaluated using artificially contaminated cocoa powder (5 to 50 ng OTA/g). Immunoassay recorded higher values of OTA (0.5 to 27.5 µg) than HPLC (0.65 to 3.28 µg) in

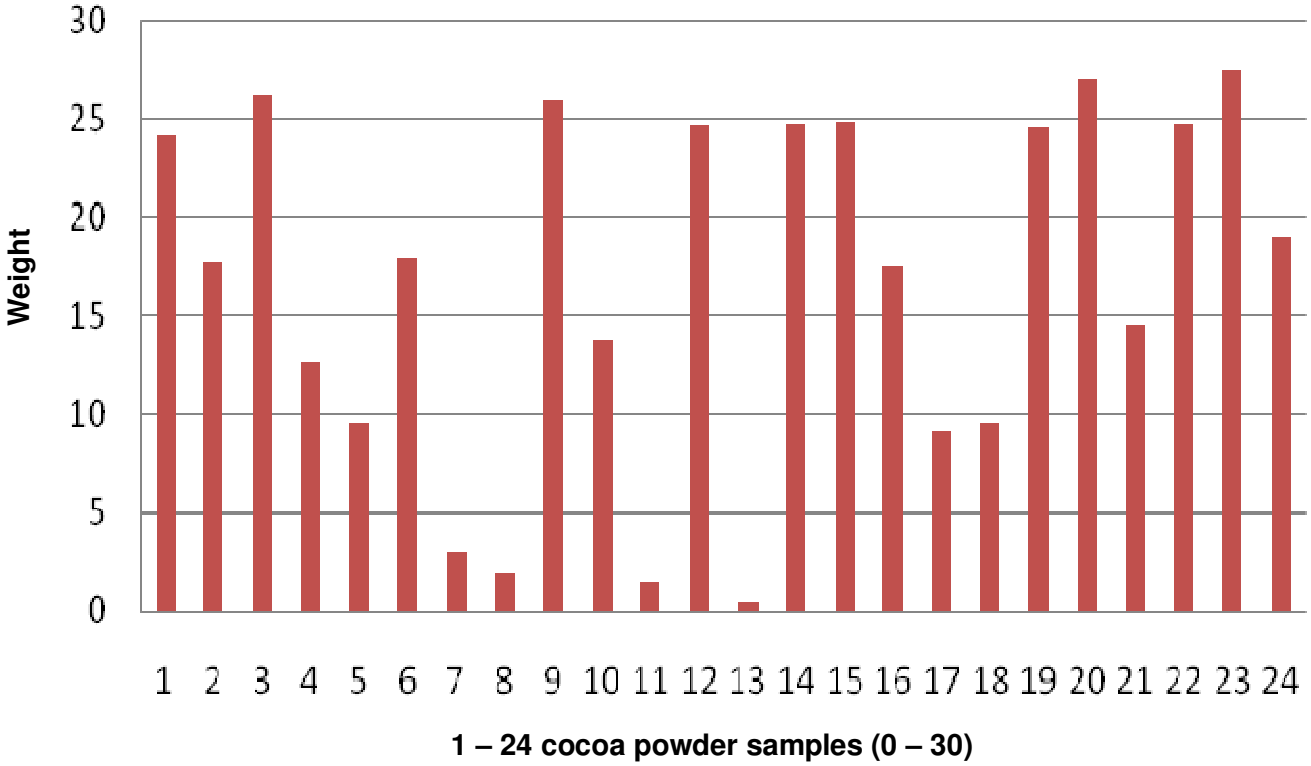

Figure 1. OTA analysis of cocoa powder samples using enzyme-linked immunosorbent assay (ELISA).

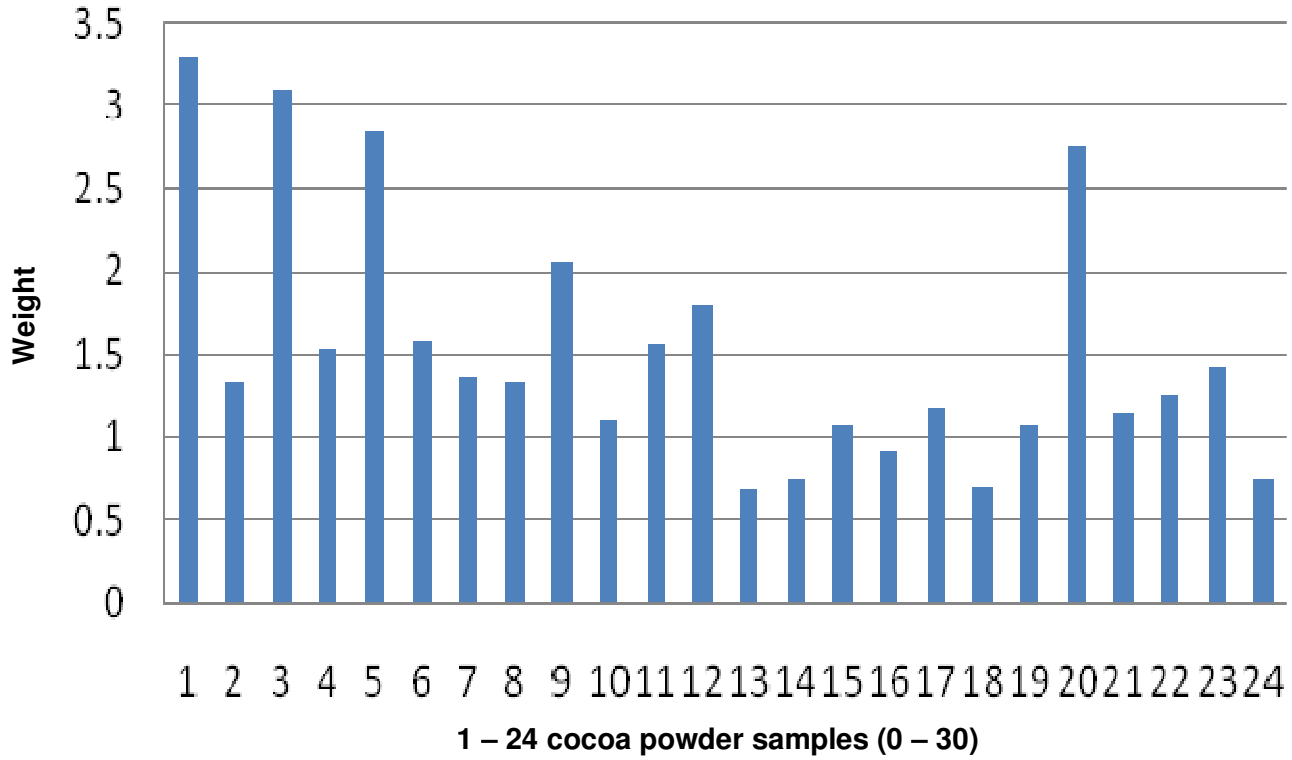

Figure 2. OTA analysis of cocoa powder samples using High performance liquid chromatography (HPLC).

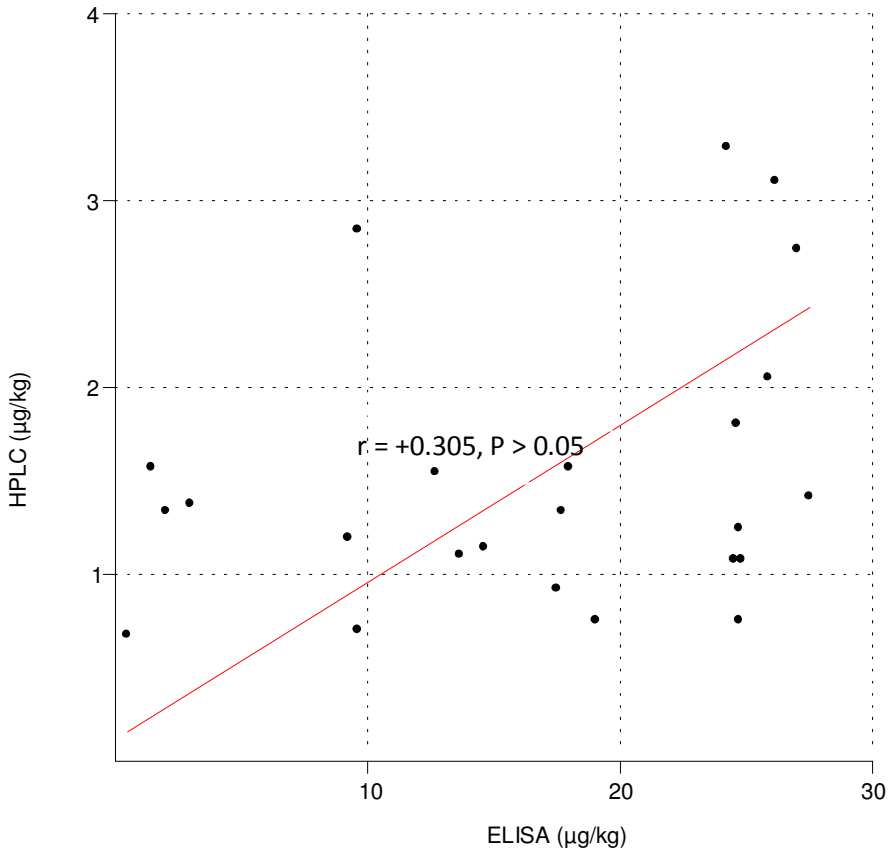

Figure 3. Competitive detection of OTA using enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC).

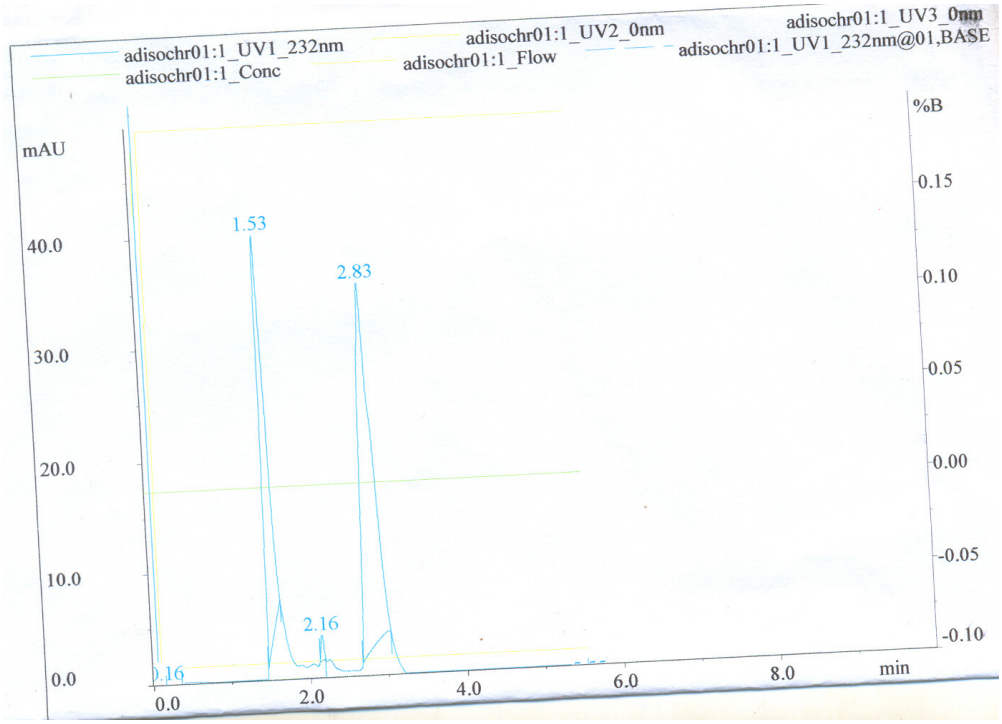

Figure 4. HPLC graph showing OTA detected peak.

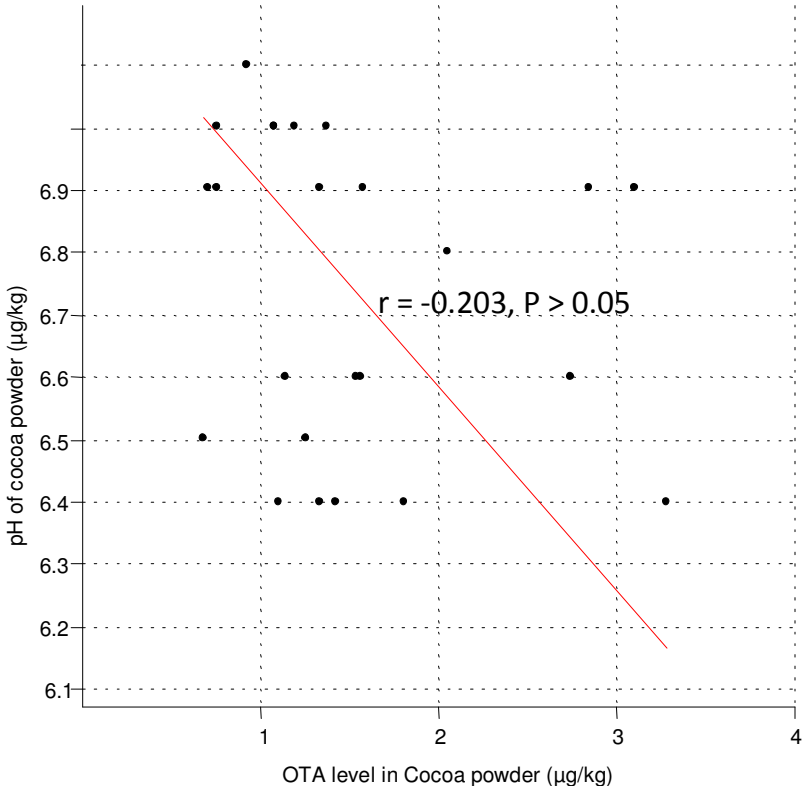

Figure 5. Effect of pH on OTA level in cocoa powder.

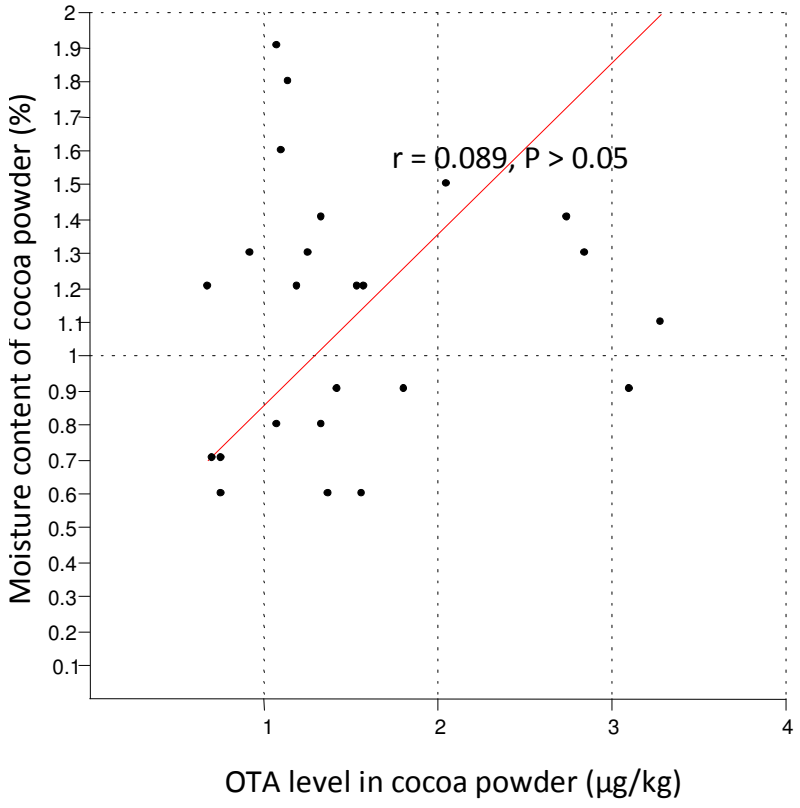

Figure 6. Effect of moisture content on OTA level of cocoa powder.

Table 2. Comparison of sensitivity of OTA detection in ELISA and HPLC.

OTA detection by ELISA (µg/kg)	OTA detection using HPLC (µg/kg)		
	Acceptable (<5.0)	Unacceptable (≥0.05)	Total
Acceptable (<5.0)	4	0	4
Unacceptable (≥5.0)	20	0	20
Total	24	0	24

χ^2MN = 18.01, P<0.05.Correlation coefficient (r) = 0.3048.

Table 3. Relationship between OTA in cocoa powder by HPLC and ELISA.

Methods	N	Mean ±SEM	P
ELISA	24	19.77± 0.01	0.147
HPLC	24	1.52 ±0.75	

Table 4. Recoveries of OTA added to cocoa powder from HPLC and ELISA.

Methods	OTA added (ng/g)[b]	OTA recovered (ng/g)	Recovery (%)	Recovery mean
HPLC[a]	5	4.76±1.22	95.21±1.01	72.28±1.82
	10	8.84±0.45	88.40±3.92	
	25	13.96±3.80	55.84±2.11	
	50	24.68±0.61	49.68±0.12	
ELISA[a]	5	3.81±2.01	76.20±0.21	63.09±0.73
	10	7.88±0.01	78.80±1.24	
	25	12.83±0.15	51.32±1.11	
	50	23.02±1.24	46.04±0.35	

Keys:[a]Mean ± SD of 2 repetition in triplicate.[b]Known quantities of OTA added.

most of the 24 cocoa powder tested indicating that HPLC is more sensitive and specific than ELISA this was because of high recovery means obtained from HPLC. When OTA was added to the positive samples, an increase in OTA peak area was observed. The assays exhibited linearity between the peak area (y) and the OTA concentration (x) in all of the intervals assayed. Moreover, an analysis of the three calibration samples for each interval and over the 3 days showed adequate values of precision and accuracy (RSD and RE, relative error <10%, respectively)

The recovery mean of OTA spiked with cocoa powder in HPLC analysis reveals 72.28±1.82 recovery mean while recovery mean for ELISA was 63.09±0.73. This could be attributable to the fact that HPLC is more sensitive than that of ELISA

These data are in line with those obtained in the past studies (Bellı´ et al., 2004; Esteban et al., 2006; Astoreca et al., 2007; Romero; 2007) and reveal that cocoa is not exempted from OTA contamination. There might be probability of detecting other compounds along with that of OTA in ELISA experiment. The values obtained for HPLC are below the recommended 5µg/kg as recommended by EU but higher in ELISA.

Conclusion

ELISA could be effectively applied to OTA screening of large quantites of cocoa powder detection, with advantages of simplicity, sensitivity, without cleanup or concentration steps. Though, HPLC is more sensitive and more accurate though cumbersome and also more expensive. The use of different preventive practices in stages previous to exporting and manufacturing processes are of great importance in minimizing the final OTA content in cocoa beans, and consequently, in cocoa derivatives. More specifically, it seems that drying is the most critical process in cocoa beans. Though the result obtained for all the samples are below the recommended

level as detected by HPLC.

ACKNOWLEDGEMENTS

We are grateful to the Executive Director of Cocoa Research Institute of Nigeria Prof. G. Iremiren for the permission given to publish this paper. We also appreciate Mrs Oyelami or her secretarial assistant during the course of this project.

REFRENCES

AOAC (2000). In Official Methods of Analysis of the AOAC International, 17th ed, vol. 11 Association of Official Analytical Chemist International, Washington, D.C.

Astoreca A, Magnoli C, Barberis C, Chiacchiera SM, Combina M, Dalcero A (2007). Ochratoxin A production in relation to ecophysiological factors by Aspergillus section Nigri strains isolated from different substrates in Argentina. Sci. Total Environ., 388: 16-23.

Badru MA (2005). Mycoflora and Ochratoxins in local cocoa based beverages in Lagos Nigeria. Msc. Theses, OOU Nigeria.

Bennet JW, Klich M (2003). Mycotoxins. Clin. Biol. Rev. 16: 497-516.

Blesa J, Berrada H, Sariano JM, Molto JC, Maries J (2004). Rapid determination of ochratoxin A in cereals products by liquid chromatography, J. Chromatogra., 1046: 127-131.

Barnett HL, Hunter BB (1987). Illustrated genera of imperfect fungi. Burgress Publishing Company, Minneapolis.

Betina (1989). Bioactive molecules. Mycotoxins chemical, biological and environmental aspects. Elselvier, Amster. The Netherlands.

Bellí N, Pardo E, Marín S, Farré G, Ramos AJ, Sanchis V (2004). Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. J. Sci. Food Agric., 84: 541-546.

Chukwuka B (1997). Bacteriology of cocoa powder drinks. B.sc.dissertation, University of Lagos, Lagos Nigeria.

Dall'Asta C, Galaverna G, Dossena A, Marchelli R (2004). Reversed-phase liquid chromatographic method for determination of ochratoxin A in wine. J. Chromatogr., 1024: 275-279.

Domsch KH, Gams W, Anderson TH (1980). Mycoflora of soil. Compendium of soil fungi, London UK. Academic Press, pp. 23-35

Pitt JI, Hocking AD (1997) Fungi and Food Spoilage. Blackie Academic and Professional, London.

Esteban A, Abarca ML, Bragulat MR, Caban˜es FJ (2006). Study of the effect of water activity and temperature on ochratoxin A production by Aspergillus carbonarius. Food Microbiol., 23: 634-640.

European commission Regulation (2002). No. 471/2002 of amending regulations (EC) No 466/2001 setting maximum levels for certain contaminants in food stuffs, 16(3): 18.

Fujii S (2002). Imunoensaio na detecção de ocratoxina A em café e efeito de cafeína sobre microbiota fúngica. Dissertação de Mestrado, Universidade Estadual de Londrina, Paraná, Brasil.

Hefle SL (1995). Immunoassay foundamentals. Food Technol., 49(2): 102-107

Hollman PCH, Katan MB (1999). Health effects and bioavailability of dietary flavonols. Free Rad. Res., 31(Suppl Dec): S75-S80.

IARC (1993). Ochratoxin A. In: IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 56, Lyon: IARC Press, pp. 489-521.

Lee KW, Kim YJ, Lee HJ, Lee CY (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem., 51: 7292-7295. [Medline]7.

Mossel DA, Shennan JC (1976). Microorganisms in dried food, their significance, limitation and enumeration. J. Food Technol., 11: 205-220.

Miraglia M, Brera C (2002). Assessment of dietary Intake of Ochratoxin A by the population of EU member States. Reports on tasks for scientific cooperation, Task 3.2.7 (Scoop Directorate-General Health and consumer protection http://europa.ev.int/comm/food/fs/scoop/3.2.7_en.pdf, pp. 69-86.

Mcfaddin JF (1986). Biochemical tests for the identification of medical bacteria 2nd edition. William and Walking New York, pp. 4-302.

NRC(1985). National Research Council. An evaluation of the microbiology criteria for food ingredient Washington DC National Academy Press, pp. 1-69.

Ogunledun DS (2007). Incidence of microbial contaminant and nutrient composition of selected cocoa based beverages in Ibadan, Nigeria, PhD Thesis, University of Ibadan.

Olubamiwa O (2007). Have you had your cocoa today? National Cocoa Development Committee. Feyisetan press Ibadan.

Pierson MD, Smooth LM (2001). Indicator micro-organisms and microbiological criterial in food microbiology MP, Doyle LP, Beuchat TJ, Maniville Edu Washington DC Asm, pp. 71-78.

Romero ED (2007). Bennet JW, Klich M (2003). Mycotoxins clinical biology review, 16: 497-516.

Samson RA, Hoekstra ES, Frisvad JC (2004b). Introduction to Food- and Airborne Fungi, 7nd edn. Centraalbureau Voorschimmelculturs, Utrecht, The Netherlands.

Sydenham EW, Shephard GS (1996). Chromatographic and allied methods of analysis for selected mycotoxins. In-Progress in Food Contaminants Analysis, ed. J. Gilbert. Blackie Academic and Professional, London, pp. 65-146.

Uraih W, Ogbadu G (1980). Incidence of aflatoxin in Nigerian Sorghum Microbiol., 10: 139-142.