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

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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 microorganisms 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...
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 diabetes, (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; Chukwukwu, 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**

Acetonitrile, methanol and chloroform were of analytical grade (Mallinckrød Baker Inc Philsburg, New Jersey, USA). Immunoaffinity columns for OTA (Ochratest™) 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 over laid 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 stigmata. 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 acetonitrile-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 shaken 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 C18 column 25 cm×3.0 mm and Ultra Violet (UV) detector (SSOD52). 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 determined 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.
food borne fungi exhibit the potential to produce toxic contamination of food products by these organisms. The years food poisoning outbreak have been traced to should be viewed with great concern since in recent analyses. Data analysis was carried out with statistical package for presence of melleus Statistical analysis

HPLC and ELISA. OTA detection by HPLC and ELISA methods. Sensitivity test, correlation coefficient and scatter diagram were presented as mean ± standard deviation of five measurements. 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 McNeman 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, Penicillium 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 (Urach 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 aw 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 (Pierer 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.

<table>
<thead>
<tr>
<th>Cultural and Microscopic Features</th>
<th>Possible Isolates</th>
<th>N</th>
<th>n</th>
<th>Isolation rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>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.</td>
<td>Aspergillus niger</td>
<td>12</td>
<td>3</td>
<td>(25)</td>
</tr>
<tr>
<td>White to yellow mycelium. Yellow, buff to brown sclerotia. Pale yellow/gold or cream coloured conidia. Uncoloured exudates when present</td>
<td>Aspergillus melleus</td>
<td>12</td>
<td>1</td>
<td>(8.3)</td>
</tr>
<tr>
<td>Yellow-buff coloured colonies, small and nearly smooth conidia, pink to purple sclerotia. Uncoloured, yellow or dull red exudates when present.</td>
<td>Aspergillus ochraceus</td>
<td>12</td>
<td>3</td>
<td>(2.5)</td>
</tr>
<tr>
<td>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 aumplii form, collula very short, both Divergent and appressed branched. The conidia is allipsoidal to spherical, smooth and greenish.</td>
<td>Penicillium chrysogenum</td>
<td>12</td>
<td>2</td>
<td>(16.7)</td>
</tr>
<tr>
<td>It is creamish in colour, obverse and oval in shape (spore). The cellular is smooth and very small. It has branched cells (spores)</td>
<td>Saccharomyces cerevisae</td>
<td>12</td>
<td>3</td>
<td>(25)</td>
</tr>
</tbody>
</table>

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

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 $\chi^2{MN} = 18.01$, 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 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 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).
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.

<table>
<thead>
<tr>
<th>OTA detection by ELISA (µg/kg)</th>
<th>OTA detection using HPLC (µg/kg)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acceptable (&lt;5.0)</td>
<td>Unacceptable (≥0.05)</td>
</tr>
<tr>
<td>Acceptable (&lt;5.0)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Unacceptable (≥5.0)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Methods</th>
<th>N</th>
<th>Mean ±SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>24</td>
<td>19.77±0.01</td>
<td>0.147</td>
</tr>
<tr>
<td>HPLC</td>
<td>24</td>
<td>1.52±0.75</td>
<td></td>
</tr>
</tbody>
</table>

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

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

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

Conclusion

ELISA could be effectively applied to OTA screening of large quantities 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.

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