

Full Length Research Paper

Quantification of aflatoxin B₁ in ready-to-use food thickeners in South-east geo-political zone in Nigeria

G. I. Okwu¹, Premila N. Achar^{2*} and Santosh K. Sharma³

¹Department of Microbiology, Ambros Alli University Ekpoma, Nigeria.

²Department of Biology and Physics, Kennesaw State University, Kennesaw, GA 30144, USA.

³Department of Botany, Rajasthan University, Jaipur, India-302004, India.

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A total of 150 ready to use food thickeners were randomly collected from various markets in both urban and rural settings. Four of the samples tested Achi (*Brachystegia eurycoma*), Akpalata (*Afzelia africana*), Ofor (*Detarium microcarpum*), and Ukpo (*Mucuna flagellipes*) were contaminated with fungal flora. The most common and prevalent fungi observed on incubated powdered form of food thickeners on media, were the *Aspergillus* group namely *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus niger*. Pure isolates of *A. flavus* and *A. parasiticus* from various food thickeners from open markets in Nigeria were screened for their potential to produce aflatoxin B₁ (AFB₁) on agar media. Ultraviolet (UV) light, a standard procedure was used to differentiate the toxin from non-toxin form of *Aspergillus* species. Further, aflatoxin quantification was done using thin layer chromatography (TLC) followed by fluorometry. *A. flavus* was more prevalent than *A. parasiticus* in all samples. Toxin and non-toxin isolates were grouped as per bright greenish-yellow fluorescence (BGYF) presumptive test under UV light. The amounts of AFB₁ from *A. flavus* isolates ranged between 0.94 to 3.83 µg/g of agar and all positive *A. parasiticus* ranged from 0.22 - 2.87 µg/g of agar. Analysis of food thickeners also revealed a high incidence and alarming levels of naturally produced aflatoxin. The levels of AFB₁ ranged between 4.0 and 95 µg/g in various food thickeners tested. That the presence of aflatoxin in food thickeners poses a potential health threat to consumers in this part of Nigeria and elsewhere is discussed.

Key words: Ready-to-use food thickeners, *Aspergillus* sp., aflatoxin B₁, thin layer chromatography, fluorometry.

INTRODUCTION

The problem of food and feed contamination with toxigenic moulds, especially *Aspergillus* species, is of current concern and has received a great deal of attention during the last three decades (Rustom, 1997). These fungi are capable of growing on a great variety of food commodities and animal feed materials when the conditions of temperature, relative humidity and product moisture are favorable (Iamanaka et al., 2007; Rosi et al., 2007). Mainly, *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins B₁, B₂, G₁, G₂ in different ratio. Aflatoxin B₁ is the most commonly occurring

among mycotoxins and is known to contaminate agricultural commodities such as peanuts, corn, animal feed. The contamination of foods and animal feed with these mycotoxins is controlled worldwide by legal limits and depending on the toxicity of these mycotoxins, countries under the European Union (EU), equal limits are valid for aflatoxins: namely 2 µg/kg for AFB₁ and 4 µg/kg for all aflatoxins in total (Van Egmond, 1995). Due to their toxicity, including carcinogenic activity, aflatoxins affect not only the health of humans and animals but also the economics of agriculture and food (Hwang et al., 2004). They are probably the best known and most intensively researched mycotoxin in the world because of their toxic and carcinogenic potentials to humans and animals (Chu, 2002). Aflatoxins have also been associated with

*Corresponding author. E-mail: pachar@kennesaw.edu.

various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world. The EU has established demanding regulatory limits for controlling aflatoxins B₁, B₂, G₁ and G₂, in cereals, nuts, nut products and dried fruit, aflatoxin M₁ in milk, and ochratoxin A in cereals (Gilbert and Anklam, 2002).

Several methods have already been proposed and reviewed for AFB₁ determination in food and agricultural products (CAST, 2003; Kriska et al., 2005; Zheng et al., 2006). These analytical techniques have been used either singly or in combination: TLC and HPLC [(High performance liquid chromatography): Dilara et al., 2002; Sapsford et al., 2006; Calleri et al., 2007; Rosi et al., 2007; Tekinsen and Ucar, 2008; Kastner et al., 2010)], Fluorometry (Richard, 1999), enzyme linked immunosorbent assay (ELISA) (Ardic et al., 2008; Rosi et al., 2007; Tekinsen and Ucar, 2008). Dilara et al. (2002) described a comparative study of three different methods for determination of aflatoxins in Tahini, high-performance liquid chromatography (HPLC), fluorometry and enzyme-linked immunosorbent assay (ELISA). Rosario et al. (1999) reported aflatoxin determination in corn and corn products by HPLC and minicolumn method and reported this technique to be an efficient screening tool for aflatoxin detection in corn and its commercial products.

Food thickeners are consumed by millions of people in Nigeria. Thickening agents or thickeners are substances which when added to a mixture increase its viscosity without substantially modifying other properties such as taste and aroma.

They increase stability and also improve suspending action of a mixture. Currently, they are produced on a cottage industry scale, which varies from one ethnic group and locality to another. Moreover, practice of aseptic technique during harvest, storage, transport, processing and packaging from buying to selling point is minimal. The processing methods in particular, vary from one site to another, thus, the microbiological quality of the products also varies.

In general, after processing into flour, the thickeners are spread on a mat or metal trays, for cooling before packaging in unsterilized bags. In addition, in the open markets, unpackaged food thickeners are displayed in open basins or bowls for sale. With surrounding environment usually hot and humid, these practices are potential sources of microbial contamination by microorganisms, which may predispose the product to public health hazards. In our initial study, using light microscopy, the prevalence of *Aspergillus* species in ready-to-use food thickeners in South-East geo-political zone in Nigeria has been established (Okwu, 2008, 2010). Present investigation aims at screening a large collection of food thickeners from open markets, contaminated by *Aspergillus* species and quantification of AFB₁ using TLC and fluorometry.

MATERIALS AND METHODS

Isolation of *A. flavus* and *A. parasiticus* from food thickeners

Ready-to-use thickeners (Achi, Akpalata, Ofor and Ukpo) were obtained from several open markets from Abia, Anambra, Ebonyi, Enugu and Imo States in Nigeria. Samples were collected from these markets in major towns, cities and villages. A total of 150 samples were collected with the aid of sterile polyethylene bags and transported to the laboratory aseptically for microbiological evaluations. Isolates were named after the States and market place where samples were collected.

Samples were finely grounded with a dry Brawn coffee grinder. Powdered form of the above food thickeners were equally distributed on Potato Dextrose Agar (PDA) plates and incubated for 7 days at room temperature ($27 \pm 2^\circ\text{C}$). After incubation period, based on morphology and colony character, potential colonies of *A. flavus* and *A. parasiticus* were screened and identified. Both species were isolated from incubated samples and were further purified individually by sub-culturing in PDA slants.

They were then identified according to Raper and Fennell (1965) and Klich (2002). Pure identified cultures were stored at 4°C during the duration of the study. Further, monoconidial colonies from pure cultures were visually analyzed using optical microscopy then transferred into PDA plates and incubated at $25 \pm 2^\circ\text{C}$ in the dark. Pure fungal cultures of *A. flavus* and *A. parasiticus* were used to test for AFB₁ production in agar media.

Extraction of AFB₁ from *A. flavus* and *A. parasiticus*

Petri dishes each containing, yeast extract sucrose (YES) agar media, were inoculated with 1 ml of conidial suspension, from above, containing 10^5 conidia. 3 replications were maintained for each isolate and incubated for 7 days at $25 \pm 2^\circ\text{C}$. Plates were observed daily for mycelial growth and after incubation period, cultures were exposed to the bright greenish-yellow fluorescence (BGYF) presumptive test under UV light at 360 nm, in which fluorescence indicates the presence of aflatoxin. The non-aflatoxin cultured plates were separated from the toxin producing ones based on the fluorescence.

Extraction and analysis of AFB₁ was done using methods described by Criseo et al. (2001) with slight modifications. Moldy agar (20 g) was placed into the blender waring blender containing 50 ml chloroform. The blender was run at a low speed for 2 - 3 min to homogenize the mixture. The mycelial mat was separated by passing through whatman no. 1 filter paper. The filtrate was transferred to a separating funnel and the chloroform layer filtered through anhydrous sodium sulphate. The extraction procedure was repeated twice using 50 ml of chloroform each time.

Identification of AFB₁ on TLC from *A. flavus* and *A. parasiticus* extracts from food thickeners

Extracts were combined and evaporated to dryness in a Rotavapor in N₂ chamber. A preliminary visual determination of AFB₁ was done by TLC (20 x 20 cm, Merck, USA) and stored at 4°C . The residue of each sample was re-dissolved in 10 μl chloroform and applied to the TLC plates. The solvents, toluol, ethylacetate and acetic acid (50: 30: 4 vol.) were used. The developed plates were viewed under longwave UV (365 nm) as blue spots and compared with standard aflatoxin (Biological Carolina, USA) spotted on the same plate. Two replicates were analyzed by spotting the crude extract of aflatoxins from the media.

Quantification of AFB₁ by fluorometer

The quantitative estimation of AFB₁ was done by laboratory fluorometer TD-700 with standard PMT (P/N 7000 - 009). The fluorescent blue spot with AFB₁ was scraped and dissolved in 3 ml methanol and then filtered through a microfibre (0.45 µm) filter. The clear solution was used to determine AFB₁ from *A. flavus* and *A. parasiticus*, respectively. The fluorometer was calibrated with the standard aflatoxin solution according to the user's instructions manual (Fischer scientific). 2 ml of the diluted filtrate from above extraction was passed through the aflatest affinity column (AflaTest®, VICAM) in order to absorb the aflatoxin produced and then the column was washed twice with 1 ml portions of deionized water. The aflatoxin was finally eluted with 1 ml HPLC-grade methanol into a cuvet to which 1.0 ml aflatest developer was added, thereafter, the corresponding fluorescence was recorded and the concentration of AFB₁ determined using standard calibration, from *A. flavus* and *A. parasiticus*, respectively.

Preparation and extraction of AFB₁ from food thickeners

Samples were finely ground with a dry Brawn coffee grinder as described above. Each sample was made into slurry by mixing with an equal amount of deionized water in a suitable container. The test portion (25 g) was placed in a waring blender jar, followed by addition of 100 ml of 60% (v/v) aqueous methanol/ water and 5 g NaCl (extraction solvent) and mixing for 5 min. The homogenized sample was then centrifuged at 3000 rpm for approximately 5 min. These portions contain pigments and lipids, were defatted twice by extraction with 25 ml portions of hexane. The blender was run at a low speed for 2 - 3 min to homogenize the mixture. The supernatant was filtered through a gravity filter paper (Whatman No. 1), and 10.0 ml of the filtrate was diluted in 40.0 ml deionized water. The diluted extract was filtered through a micro-fiber (0.45 µm) filter, while keeping the filtrate in a filtration cup (modified, Asis et al., 2002). 3 ml of filtrate was pipetted into a 10 ml graduated cylinder. With protection from light, the extract was centrifuged to remove particulate matter and the supernatant recovered. Extract from each sample was evaporated to dryness in a Rotavapor in N₂ chamber. Visual determination of AFB₁ in each sample was done by TLC, followed by quantification using fluorometer as described above.

Statistical analysis

The statistical presentation system software (SPSS) of microsoft (MS-Excel 03, complete data analysis) was used to calculate the means, standard errors and standard deviations. All the experiments were repeated thrice.

RESULTS AND DISCUSSION

AFB₁ production by *A. flavus* and *A. parasiticus* and its quantification in food thickeners

Aflatoxins, a group of mycotoxins mainly produced by *A. flavus* and *A. parasiticus*, have adverse health effects on humans and livestock that ingest aflatoxin-contaminated food products and feeds.

Aflatoxins as secreted by toxigenic strains of *A. flavus* and *A. parasiticus*, are amongst the most carcinogenic,

mutagenic and teratogenic substances found naturally in foods and feeds (Coulombe, 1991; Ahmed et al., 1997). Our investigation revealed that the food thickeners available to the general consumers in some region of Nigeria are contaminated by aflatoxin producing forms of *A. flavus* and *A. parasiticus* (Table 1), which raises serious concerns related to environmental safety, food quality, human and animal health. There have been several reports from underdeveloped and developing countries that aflatoxigenic forms of *Aspergillus* species are prevalent in food chain. From 1985 to 1994, over one hundred cases of mycotoxin food poisoning through consumption of contaminated food were reported in China every year (Annual Bulletin of Health Inspection, 1985 - 1994). In total, 471 food poisoning outbreaks occurred and 233 out of 6103 patients died. A study from 1997 to 2006 (Shih, 2006) revealed that 339 samples out of 1056 samples, including peanut candies, peanut butters, peanut meal, peanut rice soup and peanuts in Taiwan were tested positive for aflatoxins with a detected rate of 32.1%. In India, Vasanthi et al. (1997) studied aflatoxin intake and reported that aflatoxin intake through the consumption of cooked maize was assessed in 12 households in a rural population in southern India. Tabata (1998) reported aflatoxin contamination in food and food stuffs in Tokyo for 15 years during the period of 1982 - 1996 and detected aflatoxin in nuts, cereals, spices, beans and dairy products from commercial markets in Japan. Suprasert et al. (1999) studied mycotoxins contamination in food and feed in Bangkok and reported that aflatoxins were found in many kinds of food and feed such as peanut, corn, coix seed, rice, spice, milk and cheese. The occurrence of aflatoxin B₁ and ochratoxin A in maize harvested in Vietnam between 1995 - 1996 was reported by Chau et al. (1997). In the present study, both toxin and non-toxin formers were present in all samples tested but most importantly, many aflatoxin-producing isolates of *A. flavus* and *A. parasiticus* were detected indicating that the food thickeners are not only a good substrate for aflatoxin contamination but a threat to consumers' health considering the toxic nature of aflatoxins.

Although Nigeria is still a developing country, her National Agency For Food and Drug Administration and Control (NAFDAC) is working auspiciously to instituting strict regulation on food, feed and aflatoxin contamination by mould. NAFDAC has recently given a permissible limit for AFB₁ of 4 - 5 µg/kg for beans and wheat (Makun et al., 2010). Qualitative analysis of AFB₁ from *A. flavus* isolates (Table 1) indicated that out of 20 isolates of *A. flavus* retrieved from various food thickeners, 15 isolates were qualified as positive, producing AFB₁ on TLC compared with standard at Rf 0.49.

Similarly, our study also revealed that out of 20 isolates of *A. parasiticus* tested, only 10 produced AFB₁. Furthermore, quantitative analysis by fluorometer,

Table 1. Concentration of AFB₁ (µg/g of agar) produced by *A. flavus* and *A. parasiticus* in food thickeners from various States and market place in East geo-political zone in Nigeria.

<i>A. flavus</i> * isolates from food thickeners	Market place sample collected	Concentration of AFB ₁ (µg/g of agar) in YES** medium	<i>A. parasiticus</i> * isolates from samples	Concentration of AFB ₁ (µg/g of agar) in YES medium
Akpa1	Abia	2.51 ± 0.258	Akpa11	0.39 ± 0.002
Akap2	Anambra	2.57 ± 0.012	Akap22	0.24 ± 0.002
Akap3	Ebonyi	2.73 ± 0.028	Akap33	-
Akpa 4	Enugu	-	Akpa 44	-
Offor1	Abia	2.58 ± 0.043	Offor11	0.37 ± 0.003
Offor2	Anambra	3.83 ± 0.03	Offor22	0.40 ± 0.002
Offor3	Ebonyi	2.74 ± 0.020	Offor33	-
Offor4	Enugu	2.68 ± 0.068	Offor44	-
Offor5	Imo	-	Offor55	-
Achi1	Abia	1.84 ± 0.020	Achi11	2.85 ± 0.026
Achi2	Anambra	1.54 ± 0.023	Achi22	-
Achi3	Ebonyi	1.85 ± 0.028	Achi33	2.74 ± 0.020
Achi4	Enugu	-	Achi44	-
Achi5	Imo	-	Achi55	-
Ukpo1	Abia	1.82 ± 0.026	Ukpo11	2.87 ± 0.011
Ukpo2	Anambra	1.74 ± 0.023	Ukpo22	2.81 ± 0.016
Ukpo3	Ebonyi	1.66 ± 0.031	Ukpo33	2.74 ± 0.026
Ukpo4	Enugu	0.97 ± 0.013	Ukpo44	-
Ukpo5	Imo	0.94 ± 0.026	Ukpo55	2.73 ± 0.03
Ukpo6	Imo	-	Ukpo66	-

± Standard Error *Isolates of *A. flavus* were coded after name of food thickeners collected from different States and open market place in Nigeria. Isolates from Akpalata was coded as Akpa1, Akpa2, Akpa3, Akpa4 and similarly isolates of *A. parasiticus* from Akpalata was coded as Akpa11, Akpa22, Akpa33, Akpa44, respectively). Codes were also given for the rest of the isolates from Offor, Achi and Ukpo similarly. **YES -yeast extract sucrose agar.

indicated that production of AFB₁ from all positive *A. flavus* isolates ranged from minimum 0.94 to 3.83 µg/g of agar and all positive isolates of *A. parasiticus* ranged from 0.22 - 2.87 µg/g of agar. Similar to our study, chromatographic methods have been used in earlier reports to determine aflatoxins and TLC is still being widely used because it is simple and less expensive. The separation and identification of aflatoxins and trichothecenes in submicrogram quantity by thin-layer chromatography/ fast atom bombardment (TLC/ FAB) mass spectrometry was reported by Tripathi et al. (1991). Mycotoxin contamination of feedstuffs in Thailand was studied by analyzing aflatoxin B₁, zearalenone and deoxynivalenol in raw materials of feeds and complete feeds collected in 2000 - 2003 with TLC method (Taivgmunkhong et al., 2004). Fluorometric analysis of aflatoxin, on the other hand, has also been used in corn, corn meal, popcorn, rice, wheat, cottonseed and peanuts and this method was found to be quantitative, inexpensive and very efficient (Richard, 1999). Time consuming, laborious and expensive methods like HPLC or gas chromatography, may not be economical for underdeveloped countries, however,

these techniques have been proven to be reliable in determining levels of aflatoxin in foods and feed commodities.

Calleri (2007) used HPLC with fluorescence detection for AFB₁ determination in aqueous solutions. Kastner et al. (2010) also used HPLC method to analyse aflatoxin, ochratoxin A, and Deoxynivalenol (DON) in cassava. The AFs B₁, B₂, G₁ and G₂ were recovered from five fortified cassava samples with rates of 83.0 ± 27.5% which complies with EC recommendations (EC, 2006). The quantification limit was set to 0.3 µg/kg based on the first calibration point which showed a signal-to-noise ratio of >20:1 for aflatoxin B₁. In none of the samples were traces of aflatoxin B₁, B₂, G₁ or G₂ detected.

Furthermore, AFB₁ concentrations of red ground pepper were analysed by microtitre plate and enzyme linked immunosorbent assay (ELISA) method (Ardic et al., 2008) and they revealed that 72 of the 75 ground deep-red pepper samples contained AFB₁ in the range of 0.11 - 24.7 µg/kg. ELISA in conjunction with HPLC, has also been described for AFM₁ detection (Rosi et al., 2007;

Table 2. Degree of contamination by *A. flavus* and *A. parasiticus* in food thickeners from various states and market place in East geo-political zone in Nigeria.

Name of food thickeners	States samples collected*	No of samples	Markets place samples collected*	Presence of <i>A. flavus</i> **	Presence of <i>A. parasiticus</i> **	Conc. of AFB ₁ in all food thickeners (range) µg/g
Akpalata	Abia Anambra Ebonyi Enugu Imo	4	Umuahia (Isi-gate), Afor Nawfia, Ugwu- orie, Ogbete, Okigwe.	+++	++	8.5 - 95
Offor	Abia Anambra Ebonyi Enugu Imo	5	Umuahia (Isi-gate), Afor Nawfia, Ugwu- orie, Ogbete, Okigwe.	+++	++	8.0 - 90
Achi	Abia Anambra Ebonyi Enugu Imo	6	Umuahia (Isi-gate), Afor Nawfia, Ugwu- orie, Ogbete, Okigwe.	++	+++	4.5 - 56
Ukpo	Abia Anambra Ebonyi Enugu Imo	6	Umuahia (Isi-gate), Afor Nawfia, Ugwu- orie, Ogbete, Okigwe.	++	+++	4.0 - 50

* Food thickeners collected from different States and open market place in Nigeria. ** Degree of contamination by fungi: + (low), ++ (medium). +++ (high) and - (no contamination).

Tekinsen and Ucar, 2008). In our study, TLC in combination with fluorometry, were the methods of choice to quantify AFB₁ in food thickeners because they proved to be economical, less labor intensive, reliable and moreover, easily available. Developing countries like Nigeria can use these methods with low cost to analyze food thickeners and food samples against contamination by aflatoxin producing mold. If further quantification necessitates, then HPLC, gas chromatography and immunological technique may be used.

In addition, our results corroborates with earlier reports (Davis et al., 1966; Meimaroglou et al., 2009) that YES agar medium is easy to prepare, relatively inexpensive and is suitable for growth and aflatoxin production by *A. flavus* and *A. parasiticus*.

In areas of the world with warm and humid climates such as sub-saharan Africa and south east Asia, frequency of aflatoxin contamination of foods has been reported to be high (Shank et al., 1972; Peers et al., 1987; Yeh et al., 1989). Our analysis of natural occurrence of AFB₁ in food thickeners from open markets in this part of Nigeria further substantiate the probability of aflatoxin ingestion by the local people. The concentration ranged from 4.0 to 95 µg/g of all sample tested (Table 2). These findings provided evidence that there are aflatoxin problems in food thickeners which causes a potential threat to consumers' health. The high incidence

of naturally produced aflatoxin in food thickeners illustrates the hazards with which this community is confronted. Contaminated food is the main vehicle for aflatoxin ingestion and the general public is concerned about food safety and the prevention of mycotoxin contamination in cereal grains, corn and nuts that enter the food chain.

Present study throws light on the contamination of food thickeners by *A. flavus* and *A. parasiticus*, in open markets in the South-east geo-political zone in Nigeria and we also believe it is the first comprehensive report. Contaminated food thickeners can form an important source of inoculum and facilitate spread of *A. flavus* and *A. parasiticus* from one market to another and pose a threat to consumers in Nigeria.

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