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Assessment of Poultry Feed Contamination Level by Aflatoxin B1: Quantification by Two Chromatographic Analysis Methods

Rokhaya Gueye[1*], Viviane Chatchueng Sandefo[1,2], Babacar Beye[2], Elhadji Ousmane Faye[1], Amadou Diop[1], Serigne Omar Sarr[1], Bara Ndiaye[1], Yérim Mbagnick Diop[1]

[1]Laboratoire de Chimie Analytique et Bromatologie, Faculté de Médecine, de Pharmacie et d'Odontologie-Stomatologie, Université Cheikh Anta DIOP, Dakar, Sénégal
[2]Laboratoire de Mycotoxines, Institut de Technologie Alimentaire, Route des Pères Maristes, Dakar, Sénégal
Email: *rokhaya2.gueye@ucad.edu.sn

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Abstract

Aflatoxin B1 is a mycotoxin that can contaminate a wide feedstuffs variety. Ingestion of contaminated feed by poultry can lead to impaired health and zootechnical performances but also a human diet safety problem related to residues presence in animal origin products. Aflatoxin B1 contamination of poultry feed samples marketed in Dakar city and in peri-urban areas (Gorom, Sangalkam) was studied. A total of 15 samples were collected from Dakar city markets as well as from poultry farms in Gorom and Sangalkam areas. Aflatoxin B1 quantification was performed by high performance liquid chromatography and thin-layer chromatography. HPLC results showed that all samples were contaminated with levels ranging from 0.15 to 22 ppb, 0.099 to 2.05 ppb and 0.099 to 4.95 ppb respectively for Gorom, Sangalkam and Dakar. Only the finishing feed from Gorom had an aflatoxin B1 level above the maximum limit set by regulations. TLC is a suitable method for aflatoxins detection. However, it was associated with overestimation for aflatoxin B1 quantification. Results suggest that poultry feed represents a real source of human diet contamination. In addition, HPLC remains the most reliable quantification technique for quality control.

Keywords

Poultry Feed, Aflatoxin, High Performance Liquid Chromatography, Thin-Layer Chromatography, Dakar

1. Introduction

The term mycotoxin is generally restricted to chemicals synthesized by fungi

that are poisonous to mammals in low concentrations [1] [2]. They are secondary metabolites of fungi like *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium* and *Stachybotrys* and contaminate agricultural commodities before, during or after harvest [3] [4]. Mycotoxins can cause diseases or death in humans and domestic animals, including birds, when ingested, inhaled or absorbed through the skin [5]. They are also of great concern in agro-economics context [6]. Most relevant mycotoxins found in animal feed are aflatoxin B1 (AFB1), ochratoxin A (OTA), fumonisin B1 (FB1), deoxy-nivalenol (DON), T-2 and HT-2 toxins as well as zearalenone (ZEN) [7]. AFB1 belongs to aflatoxins family which is considered as most important mycotoxins in human food and animal feedstuffs, on a worldwide scale, because of their hepatotoxicity and carcinogenicity [8]. These compounds were first discovered in the early 1960s following "Turkey X disease" epidemic when over 100,000 turkeys suddenly became ill and died in England. The disease was associated with Brazilian groundnut meal affected by *Aspergillus flavus*. Thus, toxin was named *A. flavus* toxin or aflatoxin [9] [10]. Eighteen *Aspergillus* species can produce aflatoxins, though *A. flavus* and *A. parasiticus* are of the greatest importance owing to their widespread occurrence and high toxigenicity [11]. It has been estimated that 4.5 billion people in developing countries are at risk of exposure to uncontrolled aflatoxin levels [12]. Besides serious health threats to humans and livestock, aflatoxins also pose a significant economic burden, causing annually at least 25% of world's food crop destruction [13]. There are nearly 20 different forms of aflatoxins that structurally all contain a coumarin ring and an unsaturated lactone moiety. AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 are the most important forms. AFM1 and AFM2 are hydroxylated metabolites produced from AFB1 and AFB2, respectively [14] [15]. Among these forms, AFB1 is the most toxic and potent inducer of acute and chronic liver injury as well as hepatocellular carcinoma. Thus, it has been classified by the International Organization for Research in Cancer (IARC) as a Class 1A substance with confirmed epidemiological evidence like causative agent of human hepatocellular carcinomas [16] [17]. It also has been shown to possess teratogenic, immunosuppressive, and mutagenic properties. AFB1 has been the subject of regulation in food and feed in many countries [18] [19]. In addition, it is the most dangerous aflatoxin type that can impair poultry productivity even in low concentrations [20]. Scientific nomenclature for AFB1 is (6aR-cis) (2,3,6a,9a)-tetrahydro-4-methoxycyclopenta[c]furo[2,3-h] [1] benzopyran-1,11-dione. Its structure is represented in **Figure 1**. In natural conditions, aflatoxin B1 contaminates cereals, legumes, various nuts, oil seeds, cocoa and coffee, animal feed as well as other food products [21] [22].

In Senegal, aflatoxins analysis capacities are relatively limited and concentrated in Dakar where almost all services are provided by the mycotoxins Laboratory of *Institut de Technologie Alimentaire* (ITA: Institute of Food Technology). In addition to research works initiated by ITA, other aflatoxins analyses carried out come mainly from industrialists (manufacturers of chocolate, peanut

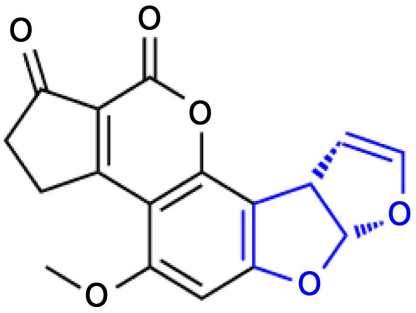

Figure 1. Aflatoxin B1 chemical structure.

paste, animal feed, etc.) [23]. To safeguard animal and population health, feedstuffs quality must be ensured. Thus, we investigated aflatoxin B1 contamination levels in poultry feed distributed in Dakar city and two peri-urban areas of Dakar region. Comparative quantification was carried out through high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

1.1. Experimental

Solvents and chemicals of analytical or HPLC grade, double distilled or ultrapure water as well as class A glassware were used during our investigations.

1.2. Poultry Feed Samples

Feed used during poultry rearing, at different growth stages, were collected from Dakar city markets and poultry farms in Dakar region peri-urban areas (Gorom and Sangalkam) in 2016 (**Figure 2**). Sampling points were selected randomly among sites: Gorom (3 points), Sangalkam (1 point) and Dakar (4 markets) (**Table 1**).

Collected samples were packaged, labelled, and sent to laboratory. After grinding to fine powder and repackaging, aflatoxin B1 contents determination was carried out through HPLC and TLC.

1.3. Aflatoxin Extraction

Sample (50 g) was weighed in a conical flask before successive addition of celite (25 g), chloroform (250 ml) and distilled water (25 ml). Mixture was stirred mechanically for 30 minutes then filtered using Whatman paper.

1.4. Double Purification of Aflatoxin Extract

Glass column—Florisil cartridge (Sep-Pak® 51960, Waters) assembly was conditioned with chloroform (10 mL) before extract (25 mL) loading. Impurities elution was performed with chloroform (5 mL) then methanol (20 mL). Aflatoxin B1 was then eluted using acetone/water (98:2) binary mixture. This first extract was then purified through a C18 cartridge (Sep-Pak® 51910, Waters) previously conditioned with methanol (10 mL) and water (10 mL). Final elution was carried out with methanol (2 mL) before evaporation under nitrogen gas.

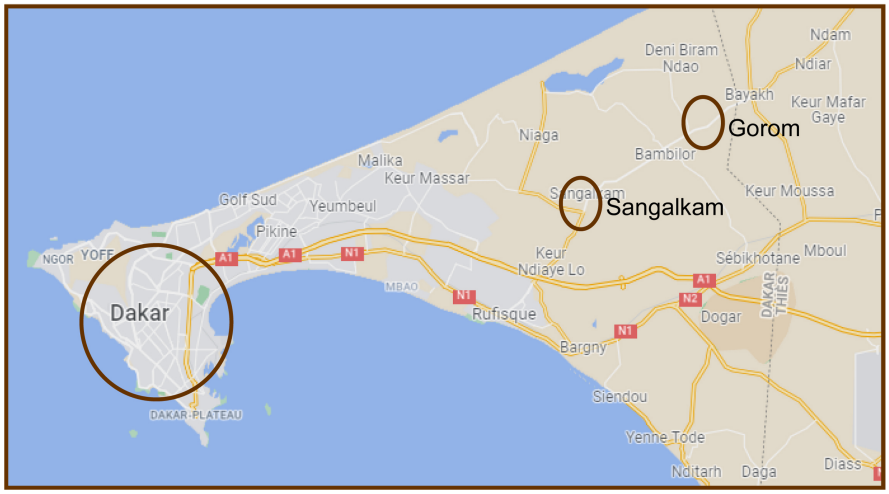

Figure 2. Poultry feed samples collection areas.

Table 1. Poultry feed samples information.

Sample code	Growth stage	Sampling area	Collecting point
G1	Starter	Gorom poultry farms	1
G2	Grower		2
G3	Finisher		3
G4	Egg-laying		1
G5	Chicken		2
S1	Starter	Sangalkam poultry farm	1
S2	Grower		1
S3	Finisher		1
S4	Egg-laying		1
S5	Chicken		1
D1	Starter	Dakar city markets	1 (*Fass*)
D2	Grower		2 (*Castors*)
D3	Finisher		3 (*Tilène*)
D4	Egg-laying		2 (*Castors*)
D5	Chicken		4 (*Grand-Yoff*)

1.5. Quantification by High Performance Liquid Chromatography

Extracts were analysed on a Waters liquid chromatograph (Empower software) equipped with reverse-phase column (150 mm × 4.6 mm × 5 µm), Waters 2707 autosampler, 1525 pump and 2475 multi fluorescence detector (λ ex et em: respectively 335 and 465 nm). Eluent system consisted of water/methanol/acetonitrile (1300:700:400) ternary mixture containing potassium bromide (240 mg) and 4 M nitric acid (350 µL) at 1 mL/min flow rate [24]. Aflatoxin B1 was used as external standard. Elution time was 16 minutes.

1.6. Quantification by Thin-Layer Chromatography

Dry residue obtained after purification on C18 cartridge was dissolved in chloroform (100 µL). Sample extract and standard were deposited as spots on glass plates covered with a 0.25 mm thickness silica gel stationary phase (60 F 254). Aflatoxin B1 standard was deposited in increasing volumes and concentrations interspersed with samples. Development system consisted of acetone/chloroform (5:45). Plate reading was performed under UV light at 366 nm. Comparison of AFB1 spots from sample and standard allowed quantification [25].

2. Statistical Analysis

XLSTAT 6.1.9 software was used for data statistical analysis through ANOVA at 5% probability level.

3. Results and Discussion

Present work aimed to contribute at animal health and food safety by determining levels of AFB1 contamination in poultry feed. A standard is applicable for AFB1 determination in feedingstuffs through HPLC method. It has been described by International Organization for Standardization (ISO) [24]. However, in laboratory, HPLC chain is sometimes out of order due to a breakdown. In this case, quantification of AFB1 after extraction and purification is performed by TLC using the Association of Official Analytical Chemists (AOAC) protocol [25]. Thus, we were interested in a comparative study between these two chromatographic techniques. Fifteen (15) samples were collected from Dakar city markets as well as in Gorom and Sangalkam peri-urban areas poultry farms. These feedstuffs were used for different growth stages: starter, grower, finisher, egg-laying and chicken. HPLC results indicated AFB1 presence in all samples. However, in some cases, it was in trace amounts. According to Food and Drug Administration (US FDA), poisonous or deleterious AFB1 level for animal feed is 20 ppb (parts per billion or µg/kg) [26]. This maximum level is also recommended in European Union (EU) [27] [28]. All feed complied with this regulation, by containing AFB1 level in the range 0.0990 to 4.95 ppb, except finishing sample collected in Gorom (22.1 ppb) (**Table 2** and **Figure 3**). AFB1 detection was not surprising as these feed contain peanuts, maize and/or derivatives which present high susceptibility to infestation by toxigenic moulds [28] [29] [30]. Low contamination associated with almost all feed could be explained by AFB1 elimination through physical and/or chemical detoxification during manufacture processes using various techniques such as: heating inactivation, irradiation, oxidation, acidification and alkalinisation [31] [32]. For non-compliant sample, a re-contamination may have occurred during conservation, as its storage period could be longer (poultry feed for last growth phase). Inadequate storage and/or warehousing conditions lead to aflatoxin biosynthesis [15]. AFB1 contamination of poultry feed available in Dakar region raises thorny issues of animals and consumers health through food chain (residues in chicken tissues and eggs) [33]

Table 2. AFB1 contents (ppb) in poultry feed through HPLC.

	Breeding stages				
	Starter	**Grower**	**Finisher**	**Egg-laying**	**Chicken**
Gorom	0.450^{ab}	0.800^{ab}	22.1^{e}	0.150^{ab}	1.30^{abc}
Sangalkam	0.650^{ab}	1.00^{abc}	2.05^{bc}	0.0990^{a}	1.15^{abc}
Dakar	0.200^{ab}	4.95^{d}	0.200^{ab}	2.90^{c}	0.0990^{a}

Means with different letters in same row are significantly different.

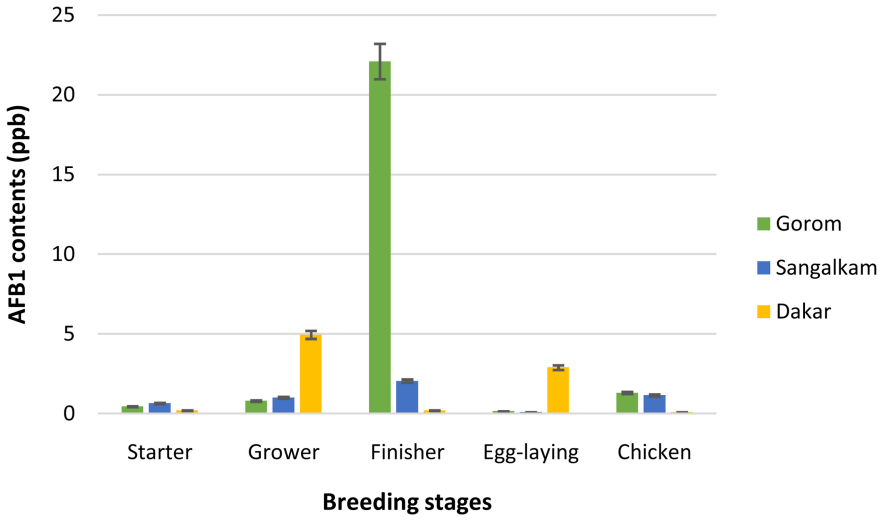

Figure 3. AFB1 contents distribution in poultry feed through HPLC.

[34].

AFB1 levels determined by TLC varied from 1 to 14 ppb (**Table 3** and **Figure 4**). All samples were contaminated but none reached maximum acceptable value specified by US FDA and EU. Highest AFB1 contents were associated with feed collected from Dakar markets. TLC results were overall higher than those obtained through HPLC method described by ISO. TLC assays were associated with AFB1 contents overestimations. Although less expensive implementation, TLC is a semi-quantitative technique more adapted for aflatoxins detection [30]. In order to improve TLC assays reliability, it should be coupled with densitometric detection [35].

However, in many works, classical TLC has also been used for AFB1 quantification in poultry feed. AFB1 levels determined by TLC on starter and finisher feed collected from local markets of Peshawar in Pakistan revealed substantial contaminant amounts up to 266.6 ppb [36]. Anjum *et al.* also carried out TLC and reported AFB1 contents up to 78 ppb in Pakistani poultry feed samples associated with a non-compliant 23.75 ppb average value (above 20 ppb) [37]. However, poultry feed from Indian livestock farms showed a 13.4 ppb average AFB1 content thanks to TLC determination. In this study, this mycotoxin was detected in 6 samples (35.2%) over a total of 17 which had been collected [38].

Table 3. AFB1 contents (ppb) in poultry feed through TLC.

Collection area	Breeding stages				
	Starter	Grower	Finisher	Egg-laying	Chicken
Gorom	7.00^{ab}	1.98^{a}	6.00^{ab}	1.98^{a}	8.00^{abc}
Sangalkam	1.98^{a}	1.00^{abc}	10.0^{bc}	1.98^{a}	1.15^{abc}
Dakar	12.0^{bc}	8.00^{abc}	1.98^{a}	14.0^{c}	8.00^{abc}

Means with different letters in same row are significantly different.

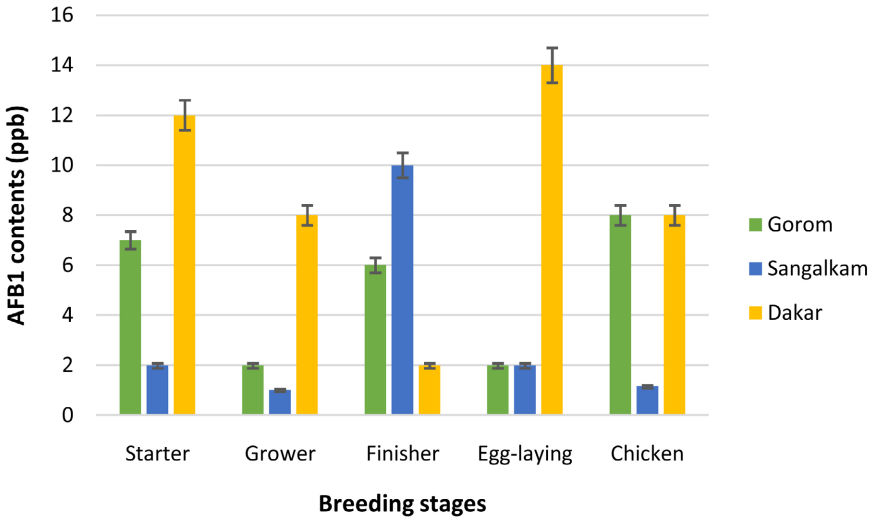

Figure 4. AFB1 contents distribution in poultry feed through TLC.

AFB1 quantification by Enzyme-Linked Immunosorbent Assay (ELISA) on Iranian farms feed showed compliant results relatively to international standards (between 6.44 and 18.34 ppb). Samples collected in autumn were more contaminated than those sampled during winter [39]. This technique also provided compliant results over Indian manufactures poultry feed (18.7 ppb maximum level) [40]. A Kenyan study revealed 93% positivity rate over 27 poultry feed samples with AFB1 levels in the range from 0.5 to 38.8 ppb thanks to liquid chromatography—tandem mass spectrometry (LC/MS/MS) [41]. This highly sensitive technique exhibited very high AFB1 contents on Nigerian feed from poultry farms (760 ppb with 74 ppb mean concentration; 83.3% positivity rate) [42].

Observed great variability in AFB1 contamination levels may be related to feed composition but also to environmental conditions. Indeed, responsible fungi can survive between 12˚C to 48˚C. However, optimum growth is generally at 28˚C - 37˚C with a high humidity of above 80% [43]. Other sources of recontamination during preservation are carbon, nitrogen, plant metabolites and sugars in substrates [8].

Face to HPLC absence, it would therefore be preferable to carry out aflatoxins quantification by means of other physico-chemical techniques providing more

reliable results than TLC like fluorimetry [44]. Bioanalytical methods, such as ELISA, can also be used [43] [45]. It would also be relevant to investigate this enzyme immunoassay method validity as described for chromatographic techniques [30] [46]. Indeed, simultaneous AFB1 quantification in Jordanian poultry feed samples using HPLC and ELISA gave different results (respectively 23.07% and 40% incidences of contamination associated with 39.41 ppb and 14.05 ppb maximum levels) [47]. A Guyanese study had also used these methods interchangeably for aflatoxins quantification in poultry feed [48]. Validate investigation would be executed to further ensure animal health but also safety of humans' food, since complete elimination of aflatoxins is almost impossible due to their thermal stability [46].

4. Conclusion

Poultry is consumed as a protein source in human diet. Its contamination with aflatoxin B1 can have dramatic consequences for animal and human health. To contribute to feeding and food safety, this mycotoxin was analysed in poultry feed samples collected from Dakar city markets and farms in two Dakar region peri-urban areas (Gorom and Sangalkam). Analysed samples showed acceptable contamination levels, only one contained aflatoxin B1 at an amount above international standard relative to animal feed. Both analytical methods (high performance liquid chromatography and thin-layer chromatography) allowed aflatoxins detection. For quantification, TLC is less accurate. Poultry feed is a real contamination source for humans. Following this preliminary study, it would be relevant to assess the possible correlation between aflatoxin levels and storage conditions (e.g., humidity) as well as season variability but also to extend sampling at the national level to carry out risk analysis upon animal and human health.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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