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# Occurrence of fungi and aflatoxins B in nuts and products marketed the Brazilian northeastern regions

# Ocorrência de fungos e aflatoxinas do tipo B em castanhas e produtos comercializados no Nordeste brasileiro

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**ABSTRACT**

Aflatoxin contamination has been considered as a public health problem, especially in tropical countries, including Brazil. In order to investigate the presence of type B aflatoxins in products marketed in the city of Fortaleza, 23 samples were analyzed by thin layer chromatography. Visible fungal contamination in food was identified according to their macroscopic and microscopic features. The contamination by aflatoxins was detected in 8.7 % of 23 analyzed samples, and 12.5 % of Brazilian nuts samples were positive for AFB1 (<8 µg/kg) and for AFB2 in a contamination rate above the allowed value (16 µg/kg). Among the peanut samples, 33.3 % were positive to AFB1 and AFB2, also in a contamination rate (317.1 µg/kg) which was higher than that recommended by ANVISA. The isolation and morphological characteristics of fungi detected mainly in Brazilian nuts with peel showed the occurrence of the following species: *Aspergillus flavus, Aspergillus niger, Mucor* sp, *Cladosporium sphaerospermum, Cladosporium cladosporoides, Fusarium* sp, *Aspergillus terreus* and bacteria of *Actinomycetes* phylum. These findings indicate that it is needed to apply the mostly effective quality monitoring of food available to consumers.

**Keywords.** Brazil nuts, peanuts, aflatoxins, chromatography, thin layer.

**RESUMO**

A contaminação por aflatoxinas tem sido considerada um problema de saúde pública principalmente em países tropicais, incluindo o Brasil. Com o intuito de investigar a presença de aflatoxinas do tipo B em produtos comercializados na cidade de Fortaleza, 23 amostras foram analisadas por meio de cromatografia em camada delgada. Os fungos visivelmente presentes em alimentos foram identificados de acordo com suas características macroscópicas e microscópicas. A contaminação por aflatoxina foi detectada em 8,7 % das 23 amostras analisadas; e 12,5 % das amostras de castanha-do-Brasil apresentaram positividade para AFB1 (<8 µg/kg) e para AFB2 com taxa de contaminação acima do valor permitido (16 µg/kg). Quanto às amostras de amendoim, 33,3 % apresentaram positividade para AFB1 e AFB2, também com nível de contaminação (317,1 µg/kg) acima do preconizado pela ANVISA. O isolamento e a caracterização morfológica dos fungos encontrados principalmente nas castanhas-do-Brasil com casca revelaram a presença das espécies: *Aspergillus flavus*, *Aspergillus niger*, *Mucor* sp, *Cladosporium sphaerospermum*, *Cladosporium cladosporoides*, *Fusarium* sp, *Aspergillus terreus* e bactérias do filo *Actinomicetos*. Estes resultados demonstram que há necessidade de fiscalização mais efetiva da qualidade dos alimentos oferecidos aos consumidores.

**Palavras-chave.** castanha-do-Brasil, amendoim, aflatoxinas, cromatografia em camada delgada.

## INTRODUCTION

The importance of fungi and mycotoxins has been growing, not only from a scientific point of view, but also in terms of economics and public health. Mycotoxins are secondary metabolites produced by fungi of various species, with divergent chemical properties (structure and molecular weights) and with no apparent function in the fungal metabolism, which contaminate food pre and post-harvest, therefore threatening the health of humans and animals that eat grains and cereals contaminated[1].

Aflatoxins (AF) are the main class of mycotoxins produced by fungi of the genus *Aspergillus*, especially the species *A. flavus* and *A. parasiticus*, which commonly develop in oilseeds, mainly at high temperature and moist environments. Among the main types of aflatoxins (B1, B2, G1 and G2), AFB1 has been considered one of the largest concerning mycotoxins worldwide, due to its carcinogenic potential[2]. The toxic effects of aflatoxin varies from liver tissue changes, which can trigger hepatitis, cirrhosis, hepatic necrosis and hepatocellular carcinoma, to immunosuppressive activities in cell-mediated responses. Besides, it can cause infertility due to changes in morphology, motility and number of spermatozoa. In women, it has been associated with low weight fetus, contaminated breast milk, and in addition it can produce teratogenic and mutagenic effects[3].

Thin layer chromatography (TLC), among the analytical methods used in the detection of aflatoxins, was taken as the reference technique because it presents low cost, easy implementation and reliability[1]. Because of its tropical weather, Brazil offers favorable conditions for the development of aflatoxigenic fungi, increasing this issue in terms of public health actions and food security. Since there are few studies investigating the presence of aflatoxin and fungi in products marketed in northeastern Brazil, the goal of this research was to detect the presence of B aflatoxins (AFB) in food samples marketed in Fortaleza city by TLC.

## MATERIAL AND METHODS

### Sampling

A total of 23 samples of the following products were analyzed: (i) cashew nuts, (ii) peanuts (iii) nougat: the cashew nuts and peanuts, (iv): Brazil nuts, (v): linseed meal, (vi): chia flour (vii): granola and (viii): oats. Except for a sample of shelled Brazil nuts purchased in bulk at room temperature, all other products were obtained in air-conditioned environment and had brands and separate lots (there was no two similar products with the same brand). Samples were taken at different shops of the city of Fortaleza - CE, all within the expiration date, during the months from July to December 2013.

### Extraction of aflatoxin

The extraction methodology of aflatoxins was done as described by Singh et al[4] with some modifications. In short, 50 g of each sample was weighted on a precision scale (Shimadzu®), these being crushed and sieved. At that time was added 250 mL of methanol-water solution (60:40) and the mixture homogenized for 30 minutes on a magnetic stirrer (Fisatom®). After decanting, the material was filtered and the liquid part added to 30 mL saturated NaCl solution and hexane. When decanted, it was separated in separator funnel and 50 mL chloroform was added. Finally, the sample was added anhydrous sodium sulfate PA. The mixture was then filtered and obtained the test solution, which solvent was evaporated in rotary evaporator (Quimis®) and the sample was subsequently eluted 0.1 mL of chloroform.

### Preparation of Aflatoxin Standards

The standards (Sigma®, USA) were established by following an AOAC official method (Official Method 970.44, 2005)[5] in an adapted way, using the following process: aflatoxins were individually diluted in chloroform, generating solutions with concentrations of 20 µg/mL, and packed under protection from light and chilled at -20 °C in vertical freezer until use.



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### Analysis and quantification

Detection and quantification of aflatoxins were performed by TLC as described by Vieira et al[6]. Summing up, aflatoxin B1 and B2 were separated into chromatoplates TLC plate AL on silica gel 60 g, 20 x 20cm F254 (Merck, Germany), using as mobile phase toluene: ethyl acetate: formic acid, purity 99.8 % (Vetec, Brazil) at a ratio of 6: 4: 1. Different amounts of the extract were used from each sample and YμL standards samples B1 and B2 Sigma® brand in 20 μg/mL concentrations in chromatoplates 20x20 cm (Y= 2 μL AFB1 and 4 μL AFB2 for Brazil nuts and 8 μL AFB1 and B2 for peanut). Then the plate with the applied chromatographic material was placed in a glass cell with the same eluent until it stayed 2 cm from the top edge. After elution of the mobile phase plates, they were dried at room temperature and examined visually under ultraviolet radiation type A flashlight emission 365 nm and 254 nm C (trademark Boitton Instruments). The quantification was performed by comparing fluorescence intensities of samples and standards. The centers and the size of the spots were marked. Once the samples reached fluorescence intensity similar to the patterns, the following formula was applied:

$$\frac{S \times Y \times V}{W \times Z} = \mu L/kg = ppb$$ , where:

**S** = aflatoxin standard volume (S μL)
**Y** = concentration of aflatoxin standard (Y μg/mL)
**V** = volume of solvent used for dilution of the final sample (1000 μL)
**Z** = sample volume applied on the plate (Z μL)
**W** = sample weight (50 g)

### Detection and quantitation limit

The detection limit corresponds to the lowest measuring and stating with 95 % to 99 % confidence of the substance which concentration is bigger than zero, while the limit of quantification is the lowest concentration of analyte that can be determined with an acceptable level of precision and accuracy[7].

### Analytical quality control (recall limit)

Each sample analyzed included a recovery test. In the recovery test, the Sigma® standards were added to an uncontaminated sample the day before extraction. The standards additions were carried out so that the final concentrations of the samples were 10 μg/kg AFB1 and AFB2.

### Morphological identification of fungi

The samples with visible fungal contamination were grown and isolated to identify the gender level, being observed macroscopic characteristics of the colony (relief, edges, pigmentation, size) and microscopic (ontogeny of spores) of these fungi. This process was done through the cultivation and isolation on PDA (potato, D + anhydrous glucose [dextrose], agar agar type I) in Petri dishes. The samples were cultured for 7 days at 37 °C in incubator (Quimis®)[4].

## RESULTS AND DISCUSSION

In recent years, the issue of food safety, in particular to human exposure to mycotoxins, has generated a series of discussions between different government sections, in an attempt to ensure the population that products are not harmful to health.

**Table 1** shows an overall incidence of contamination by AFB of 8.7 % (2/23) of food samples obtained from different commercial establishments in Fortaleza-CE, through the thin layer chromatography method. Analysis by type of product shows that only lots of shelled Brazil nuts and peanuts showed the contamination. The AFB was detected in 12.5 % (1/8) of Brazil nuts samples with concentrations of 16 μg/kg for AFB2 and < 8 μg/kg for AFB1 and in 33.3 % (1/3) the peanut samples with levels of 317.1 μg/kg for AFB1 and AFB2, exceeding the limit permitted by Brazilian law. In Brazil, the maximum concentration of aflatoxins tolerated in shelled Brazil nuts and peanuts for human consumption is 10 μg/kg and 20 μg/kg respectively, and is considered the sum of the four types[8]. The quantification limits for all

aflatoxins (B1, B2) were 4 µg/kg. The detection limits for AFB1 and AFB2 were 0.5 µg/kg. The recovery limit for Brazil nuts was 86.17 % when aplied 10 µg/kg aflatoxin. For peanut, the limit was 91.29 %.

Aflatoxins has been detected in various products, especially peanuts, corn, nuts, beans, sunflower seeds, animal feed and condiments. Several studies conducted in Brazil, analyzing the occurrence of aflatoxins in peanuts and derivatives, report AFB1 levels above the limit allowed by country laws. Oliveira and Koller[9], analyzing 22 samples of peanuts and peanuts sweet marketed in the municipality of Porto Alegre, detected aflatoxins in 58 % of samples of peanuts and 60 % peanuts sweet, only peanuts *in natura* had levels of contamination above the allowed (126.18 µg/kg). Hoeltz et al[10] studying the contamination of peanuts and derivatives traded in the state of Rio Grande do Sul, showed a maximum level of contamination by AFB1 of 87.5 µg/kg. According to Silva et al[11], analyzes in foods marketed in Marília – SP, showed that 16 % of peanuts and derived grain samples showed contamination levels in raw peanuts samples ranging from 2 to 300 µg/kg and 9 to 466 µg/kg in tack samples.

On the other hand, Brazil nuts have been studied not only by the nutritional importance, but also by the high incidence of aflatoxins produced by fungi. In the paper by Reis et al[12], 11 % of 200 samples of Brazil nuts obtained from different states of the Brazilian Amazon region have contamination with AFB. Samples from Acre and from Amazonas showed AF concentrations above the tolerance limit (15 µg/kg - processed in shell nuts) established by the National Health Surveillance Agency (ANVISA) and Regulation of the European Commission (EC). Freitas-Silva et al[13] using the liquid chromatography technique of high efficiency with fluorescence detection (HPLC-FD) for AF detection, showed that the shelled nuts showed higher levels of contamination by AFB (35 µg/kg AFB1 and 0.2 µg/kg AFB2) when compared to in shell nuts.

Analyzed samples of shell Brazil nuts in this study showed no incidence of aflatoxins, however was observed a significant percentage of contamination by strains of *Aspergillus* sp, were isolated and characterized at species level. Among the isolated species is the aflatoxigenic *A. flavus* (21.4 %), *A. niger* (21.4 %) and *A. terreus* (7.1 %), the fungi *Mucor* sp (14.3 %), *C. sphaespermum* (14.3 %), *C. cladosporoides* (7.1 %), *Fusarium* sp (7.1 %) and bacteria of the *Actinomycetes* phylum (7.1 %) (**Figure 1**).

**Table 1.** Occurrence of aflatoxins in products marketed in Fortaleza – CE

| Samples | Contaminated Samples | Incidence (%) | AFB1 (µg/kg) | AFB2 (µg/kg) |
|---|---|---|---|---|
| Cashew nuts | 0/3 | 0 | nd | nd |
| Peanuts | 1/3 | 33.3 | 317.1 | 317.1 |
| Nougat | 0/2 | 0 | nd | nd |
| Brazil Nuts | 1/8 | 12.5 | <8.0 | 16.0 |
| Linsed meal and chia flour | 0/3 | 0 | nd | nd |
| Granola | 0/3 | 0 | nd | nd |
| Oatmeal | 0/1 | 0 | nd | nd |

*nd: not detected



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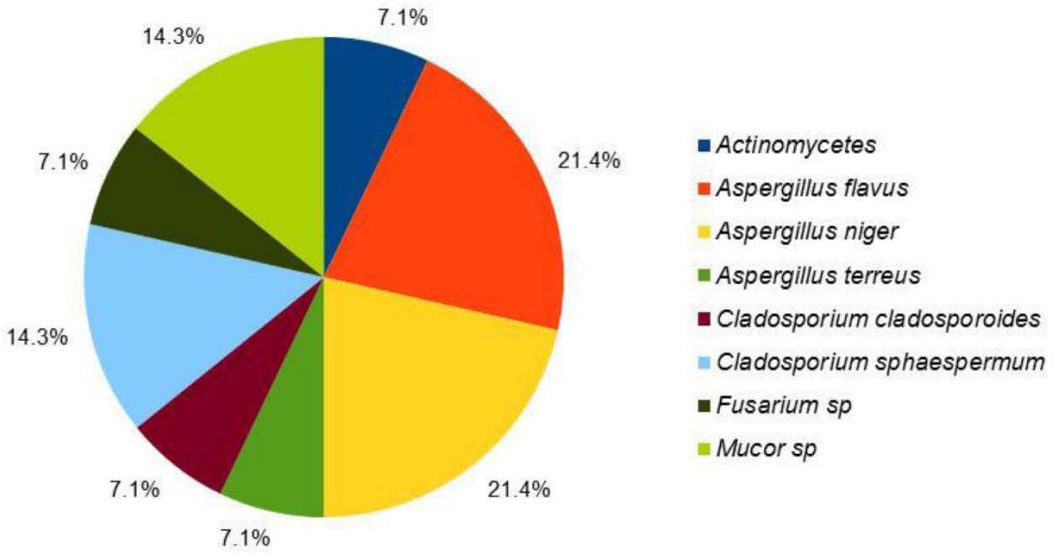


**Figure 1.** Percentage distribution of fungi isolation founded in shell Brazil nuts samples

Studies have shown *Aspergillus* as the main fungus present in the contamination of Brazil nuts. Freire et al[14] reported that among the 17 species of fungi found in Brazil nuts, *A. flavus* was predominant, followed by *A. niger, Penicillium citrinum and P. glabrum.* Analyzing the infection in the fruit, bark, almond, soil and air, before and after the ground contact, Baquião et al[15] showed the presence of potentially toxigenic fungi especially of the genera *Fusarium* sp, *Penicillium* sp and *A. flavus*, and exposure to soil as the main factor of contamination. These findings confirm that the shell does not protect the almond, on the contrary, facilitates fungal infection due to porosity.

## CONCLUSION

The results obtained in this study indicate a high susceptibility to fungal growth and the emergence of aflatoxins in Brazilian nuts and peanuts, reinforcing the need for constant monitoring by surveillance organs of food quality offered to consumers.

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