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Mycological and toxicological analysis of peanuts and derivatives

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ABSTRACT

The filamentous fungi *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* produce toxins, which are secondary metabolites called aflatoxins. These toxin-producing species grow rapidly on peanuts and cereals in favorable conditions of temperature and humidity. Their toxins can either cause acute effects, and even be lethal or accumulate in the organism, resulting in liver cancer in the long term. Based on the health risks of aflatoxins in food, a research was conducted on peanuts and derivatives sold in Alfenas, MG, Brazil, to evaluate the presence of *Aspergillus* sp. and aflatoxins. The samples were randomly collected at "popular stores", from November 2008 to May 2009, to assess the occurrence of aflatoxins B_1, B_2, G_1 and G_2 by thin layer chromatography (TLC). The mycological analysis revealed the presence of fungi in 50% of the samples: *Penicillium* sp. (53.85%), *A. flavus* (19.23%), *A. niger* (15.38%) and *A. fumigatus* (11.54%), and 63.64% of these showed the presence of aflatoxins : B_1 (43.14%), B_2 (25.49%), G_1 (23.53%) and G_2 (7.84%). It is concluded that the results reported here are a cause for concern, given the harmfulness of these cumulative and carcinogenic toxins.

Keywords: Aflatoxin. *Aspergillus sp*. Mycotoxin. Peanuts. Contamination.

INTRODUCTION

Chemical contaminants in food are all the substances that are not natural to it but have become part of it during either the productive process or storage, owing to natural or artificial factors, and present health risks to those who ingest them. Their presence beyond the quality and safety limits can lead to toxicological risk, which is enough for the contaminated food to be taken off the market (Miller, 1991).

Aflatoxins (AFs) are toxic metabolites produced by fungi, notably *Aspergillus flavus* and *Aspergillus parasiticus*, which grow mainly on peanuts and cereals. In the human body, their effects depend on the dose and frequency of ingestion, and can be acute (lethal or not) or subacute (Fonseca, 1998a). When subacute, they are cumulative and can, in the long term, cause liver neoplasias and many other diseases. The Brazilian sanitation, food and drug agency, ANVISA (Brasil, 2009), is highly concerned about such food contamination and has been working with industry to control the levels of AF in peanuts and derivatives. This requires, among other measures, special attention to harvesting, storing, insect, rodent and humidity control (Brasil, 2009).

Brazilian legislation stipulates a maximum allowed level for the detection of mycotoxins in foods. The Ministry of Health has established a limit of 30 μg/kg $AFB_1 + AFG_1$ in human food, and the Ministry of Agriculture, Livestock and Food Supply has put a limit of 20 μg/kg total AF on the raw material of food and rations. According to the ANVISA regulation RDC 7, February 2011, there are new established maximum permissible levels (MPL) for mycotoxins that should come into force in 2012. In peanuts (shelled or peeled, raw or roasted) or peanut butter, for aflatoxin, the MPL is 20μg/kg (Brasil, 2011). These limits are comparable to those established by other countries and recommended by the World Health Organization and the Food and Agriculture Organization (WHO/FAO, 1998).

Peanuts frequently offer ideal conditions for fungal growth, when rain in the post-harvest drying period increases the humidity. The highest incidence of fungi occurs when the peanuts are threshed, packaged and stored under damp conditions, humidifying again the kernels which had already been dried (Pitt et al., 1991; Fernandez et al., 1997; Almeida et al., 1998; Bruno et al., 2000).

Control became more effective when it was discovered that various types of food are susceptible to contamination, and that AFB_1 is the most potent carcinogenic

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agent yet known, causing liver tumors in various laboratory animals, and also other diseases in humans and animals (Fonseca, 1998a; Burguera, 1986; IDEC, 1998; Murillo et al., 1994).

In addition, there is a proven relationship between the above pathogens and the incidence of hepatitis B and kwashiorkor, a form of childhood protein-energy malnutrition characterized by lethargy, mental retardation, anemia, anorexia, skin pigmentation disorders, hair loss and discoloration (Burguera, 1986; Wong, 1998). In Brazil, high contamination levels have been found in food for human and animal consumption (Prado, 1983; Sylos, 1998). Mycological and toxicological analyses of peanuts and derivatives sold in the city of Alfenas, MG, Brazil, were carried out to detect the presence of fungi, notably *Aspergillus* sp. and others, and also the presence of aflatoxins (B_1, B_2, G_1 and G_2).

MATERIAL AND METHODS

The samples were obtained at "popular stores" in Alfenas, MG, Brazil. Each sample was taken and stored in accordance with the methods recommended in the literature to prevent its contamination and alteration (Busta et al., 1984).

Forty-four 500-g samples of peanuts and derivatives - salty, sugar-coated and caramelized peanuts, peanut brittle (*pé de moleque*) and peanut crumbly candy (*paçoca*) - were randomly collected from grocery and supermarket shelves and analyzed in no more than 12 hours.

For the toxicological analysis, 50g aliquots (in duplicate) were taken from the samples, homogenized for 5 minutes in a blender with 270 mL methanol and 30 mL of 4% KCl. The extract was filtered through Whatman filter paper.

To 150 mL of the filtrate, 150 mL of a clarifying solution ($CuSO_4$) and 50 mL of powdered milk were added; the mixture was homogenized with a glass rod and filtered. In a 500 mL separating funnel, a chloroform-saturated atmosphere was created; then 150 mL of the purified filtrate was transferred to the funnel and 150 mL distilled water and 10 mL chloroform were added and the whole shaken strongly for 3 minutes. The systems were allowed to stand for 5 minutes, after which the (lower) chloroform phase was drained off. The process was repeated once more and the chloroform phases pooled to give the chloroform extract (approx. 20 mL), which was evaporated in a water bath at 65ºC.

Subsequently the residue was resuspended in 500 mL chloroform and 10 µL was applied to a thin-layer chromatographic plate (silica gel on TLC-PET foils: DC-Folien, Kieselgel) with a micro-syringe (Hamilton Microliter® Syringes, 10 µL), together with the aflatoxin standards (Sigma – Aldrich Laborchemikalien, standards B_1 (2 µg/mL) and G_1(2µg/mL), and Pool – Sartorius standards: B_1(0.875 µg/mL) B_2 (0.808 µg/mL) G_1(0.800 µg/mL) and G_2 (0.871 µg/mL)), and the TLC was performed with toluene-ethyl acetate-formic acid (50:40:10) as the mobile phase. The plates were observed under ultraviolet light to assess their respective hRf (retention factor):

- Aflatoxin B_1: blue fluorescence and hRf = 0.50;
- Aflatoxin B_2: blue fluorescence and hRf = 0.45;
- Aflatoxin G_1: green fluorescence and hRf = 0.39;
- Aflatoxin G_2: green fluorescence and hRf = 0.35.

To confirm the presence of aflatoxin on the TLC, a drop of 20% H_2SO_4 was added to the fluorescent site on the chromatographic plate and the AFs exhibited a yellow color (Rocha et al., 2008).

To search for and isolate the fungal microbiota, 25 g of the sample was mechanically triturated. Then 225 mL of peptone saline (10^{-1} dilution) was added to the powder and, from the stirred mixture, successive decimal dilutions were prepared, down to 10^{-3}. Sabouraud dextrose agar pour plates were prepared from these dilutions and incubated at 25ºC for 3-5 days. Subsequently, the colony-forming units (CFU) were counted (Silva et al., 2007).

After the development of characteristic colonies, a sample was transferred with a sterile platinum loop on to the middle of a plate of Sabouraud dextrose agar and incubated in a light-tight chamber at room temperature for 3-10 days (Silva et al., 2007).

The microscopic study of the fungi was based on the general morphology of the colony, aspect, fluorescence, texture, color (pigment diffusion), odor, growth speed, diameter, days of culture and other morphological features inherent to each fungus (Riddell, 1950; Barnett & Hunter, 2004; Suassuna & Nóbrega, 2004). Next, microcultivation was used for the microscopical analysis. Identification of fungi was based on comparison of fungal structures (conidia, vesicles and phialids) (Barnett & Hunter, 2004; Suassuna & Nóbrega, 2004).

For the analysis of water activity (Aw) and temperature (Tº C), samples of the assayed food products were tested in a specific apparatus (Pawkit – Water Activity Meter, BrasEq – Brasileira de Equipamentos Ltda.).

RESULTS

The mycological analysis revealed fungi in 50% of the samples: *Penicillium sp* (53.85%), *A. flavus* (19.23%), *A. niger* (15.38%), *A. fumigatus* (11.54%); and aflatoxins in 63.64%: B_1 (43.14%), B_2 (25.49%), G_1 (23.53%), G_2 (7.84%). The values for water activity (Aw) were: highest = 0.78; lowest = 0.35; mean = 0.61; the average temperature (T° C) was 26.68°C, ranging from 24.3°C to 28.8°C

Table 1: Mycological and toxicological profile of peanuts and derivatives sold in "popular stores" in Alfenas, MG, Brazil.

Sa	Colonies (CFU/g)	FUNGI				AFLATOXINS hRf				A_w	T ºC
		A. flavus	*A.fumigatus*	*A.niger*	*Penicillium sp*	B1	B2	G1	G2		
1	< 10	-	+	-	+	-	0.47	0.40	0.33	0.73	27.3
2	< 10	+	-	-	-	0.50	-	-	-	0.43	28.5
3	< 10	+	-	-	+	-	-	-	-	0.35	27.2
4	-	-	-	-	-	0.50	0.45	0.38	-	0.72	26.9
5	-	-	-	-	-	-	0.50	0.45	0.37	0.71	26.3
6	-	-	-	-	-	0.56	0.47	0.41	0.35	0.57	25.9
7	-	-	-	-	-	-	-	-	-	0.41	25.8
8	-	-	-	-	-	0.60	0.52	-	-	0.47	25.4
9	-	-	-	-	-	0.55	0.47	0.40	-	0.77	25.2
10	-	-	-	-	-	-	-	-	-	0.37	25.6
11	< 10	-	-	-	+	-	-	-	-	0.77	25.7
12	-	-	-	-	-	0.55	0.50	-	-	0.54	25.9
13	< 10	-	+	-	-	-	-	-	-	0.76	25.4
14	< 10	-	-	-	+	-	-	-	-	0.77	25.5
15	-	-	-	-	-	-	-	-	-	0.77	25.5
16	< 10	-	-	-	+	-	-	-	-	0.66	25.9
17	< 10	-	-	-	+	-	-	-	-	0.67	26.2
18	-	-	-	-	-	0.51	-	-	-	0.54	26.8
19	< 10	+	-	-	-	0.52	-	0.37	-	0.66	27.4
20	< 10	+	-	-	-	-	-	0.37	-	0.77	27.7
21	< 10	-	-	-	+	-	-	-	-	0.65	27.6
22	< 10	-	-	+	+	-	-	-	-	0.63	28.3
23	< 10	+	-	-	-	0.57	-	-	-	0.60	28.4
24	< 10	-	-	-	+	0.57	-	-	-	0.63	28.7
25	< 10	-	-	-	-	0.57	-	-	-	0.63	28.8
26	< 10	-	-	-	+	0.55	-	-	-	0.56	28.7
27	< 10	-	-	+	-	0.58	-	-	-	0.61	28.0
28	-	-	-	-	-	0.54	0.47	-	-	0.50	28.1
29	< 10	-	-	-	-	0.56	0.51	-	-	0.47	27.7
30	< 10	-	-	-	-	0.56	-	-	-	0.46	24.7
31	-	-	-	-	-	0.55	-	0.43	-	0.69	24.3
32	-	-	-	-	-	-	-	0.36	-	0.75	25.1
33	< 10	-	-	+	-	-	0.46	0.38	0.33	0.78	25.1
34	-	-	-	-	-	0.53	0.48	0.45	-	0.74	25.8
35	< 10	-	+	+	-	0.56	-	-	-	0.48	25.9
36	-	-	-	-	-	-	-	-	-	0,70	25,9
37	< 10	-	-	-	+	-	-	-	-	0.71	26.4
38	-	-	-	-	-	0.51	-	-	-	0.43	26.1
39	< 10	-	-	-	+	-	-	-	-	0.41	26.2
40	< 10	-	-	-	+	-	0.46	-	-	0.74	26.3
41	-	-	-	-	-	-	-	-	-	0.38	26.8
42	< 10	-	-	-	+	0.53	-	-	-	0.40	28.7
43	-	-	-	-	-	0.50	0.47	0.42	-	0.71	28.0
44	-	-	-	-	-	-	-	-	-	0.62	28.4

Sa – Samples; CFU/g – Colony-forming unit per gram; hRF – Retention factor; Aw – Water activity; TºC – Temperature in degrees Celsius.

DISCUSSION

Peanut-derived candies are part of the popular Brazilian food culture, although so far they are not safe for consumption, owing to contamination, especially with AFs. In 2001, it was revealed that 40% of peanut crumbly candy (*paçoca*) on the market were contaminated with these toxins (Scussel, 2002). The rate was even higher in the present research, which found 78.57% of these candies contaminated by AFs. Increasing concern for the quality of the food supplied to the population has led several teaching institutions and government bodies to research the presence of food contaminants, such as AFs (Scussel, 2002). Many studies confirm that most products analyzed show some type of mycotoxin, as found herein: 63.64% of the samples were contaminated by AFs.

ANVISA has intensified the inspection of these products. In 2004, it prohibited 6 lots produced by 5 São Paulo and Paraná companies, because they showed contamination varying from 32.9 to 267.1 µg/kg, exceeding the levels accepted in Brazilian law. In 2005, it also prohibited one lot of raw peanuts produced by a plant in Goiânia, GO, which also had AF levels above those allowed by the sanitary legislation (Brasil, 2009). In 2008, three Minas Gerais brands were preventively prohibited, even without toxin assays, because preventive measures had to be taken to decrease contamination (Brasil, 2009).

In the period 1998 to 2000, in Brasília, aflatoxins were found in 60 samples of peanuts and derivatives, Brazil nut, popcorn and corn grains, and the incidence of contamination was 51.2% in peanut crumble and candies; 49.1% in raw peanuts; and 40% in toasted and sugar-coated peanuts (Caldas, 1998). Our results corroborate these, showing AF contamination in peanut brittles and mashed peanut candies (61.5%) and in toasted and sugar-coated peanuts.

From March 2000 to April 2002, in Rio Grande do Sul, out of 664 samples of peanuts and derivatives, 208 (31.3%) contained AFs (Mallmann, 2003). The difference between those data and ours may be due to differing harvest time, sample type, quality of the raw material, storage, handling and production system. In a study of whole peanuts and peanut crumbly candies carried out in Alfenas, MG, from June 2006 to February 2007, AFs were found in 38% of raw peanuts and 13% of candies (Rocha et al., 2008), whereas our results showed AF contamination in 78.57% of these candies.

On March 25, 2009, ANVISA published a notice of preventive prohibition (in Diário Oficial da União), for 90 days, of a certain brand, based on a finding of the Laboratório de Análises Micotoxicológicas of the Universidade Federal de Santa Maria, RS, Brazil, which detected AF levels above those permitted by the sanitary legislation (Brasil, 2009): lots 45 and 50 exhibited 80% AF contamination; out of 5 samples, sample 3 was negative, samples 4, 34 and 43 were positive for AFB_1, AFB_2, and AFG_1, and sample 5 was positive for AFB_2, AFG_1 and AFG_2.

According to our general results, of the 44 samples analyzed, 63.64% were contaminated by some type of AF. Although the levels were not quantified, this is cause for much concern, for it is known that all kinds of AF are harmful to the consumer. The mere confirmation of their presence in the samples reveals failure in one of the stages of food production: harvesting, raw material drying, storage and so forth.

The Brazilian Institute for Consumer Defense (IDEC, 1998) sent for analysis 40 industrialized peanut products, two of which had an AF concentration of 153.6 µg/kg, while the limit allowed in Brazil is only 30 µg/kg; three other products were approved, according to Brazilian legislation, but not according to Mercosul regulations. A second test was conducted on one of the rejected products, which found less than 1.5 µg/kg, 100 times less than the amount detected in the first lot. The companies claimed that the high rainfall in the growing region explained the high contamination levels (IDEC, 1998). This is not a valid reason because, according to Mixon et al. (1984), the contamination of peanuts by AFs can be prevented by good production practices, such as crop rotation, irrigation, mineral supplementation (e.g. limestone) and adequate procedures in harvesting, storage and transport. Two questions remain: is quality control is being done properly and at regular intervals? Is there real concern about ensuring the quality of the finished product?

The ingestion of AFs by itself does not mean that the consumer will inevitably develop cancer but there is a risk. In African and Asian countries, where AF-contaminated foods are regularly consumed, the incidence of liver cancer is about 13 cases per 100,000 inhabitants (Mozambique) per year (Fonseca, 1998b). Some samples (22.73%) showed fungi without AFs, but their storage in favorable growing conditions of temperature and humidity may lead to the synthesis of mycotoxins. Other samples (27.27%) exhibited fungi and AFs, and the latter may have been produced either before or during storage. Still other samples (36.36%) showed only AFs, probably because the fungi did not resist the treatment given.

Some authors (Pereira et al., 2002; Afonso Júnior et al., 2003) report that an Aw of 0.80-0.99 is required for the growth of some toxigenic fungi (*A.flavus*), and 0.95-0.99 for the production of AFs. In one of the studies (Afonso Júnior et al., 2003), the results showed a mean Aw lower than 0.664 in coffee beans, regardless of the relative humidity, temperature and storage time, and no growth of toxigenic fungi. However, in the present study, the measurements obtained for Aw and temperature were respectively 0.610 and 26.68°C, with a relative humidity ranging from 30 to 80%, according to the season (CPTEC, 2012), and yet both fungal growth and AF production occurred.

The differences in results are possibly due to diversity in the foods studied and in the processing and storage conditions. Such inconsistencies can be expected, because the capacity for survival and multiplication of microorganisms present in food depends on a number of factors, both intrinsic, such as Aw, and extrinsic, such as temperature, which are considered critical for survival. This explanation involves the definition of Aw, which is the ratio between the vapor pressure of the food and that of pure water, at a given temperature (Pereira et al., 2002; Afonso Júnior et al., 2003; Franco & Landgraf, 2008).

Out of the 44 samples in this study, only 6 (13.64%) were negative for both fungi and aflatoxins, while 7

(15.91%) were positive only for a fungus of the genus *Penicillium*, which synthesizes ochratoxin.

In some of the samples, the packages were very fragile and easily ruptured, which would enhance food contamination by environmental fungi. It was also noted that the expiry dates of the primary and secondary packages did not match each other. Many of the samples did not show the lot number, or fabrication and validity dates, demonstrating a production quality error.

RESUMO

Análise micológica e toxicológica em amendoim e derivados

As espécies *Aspergillus flavus, Aspergillus parasiticus* e *Aspergillus nomius* produzem micotoxinas, que são metabólitos secundários denominados de aflatoxinas. Desenvolvem-se rapidamente em amendoins e cereais produzindo esta toxina, quando em condições favoráveis de temperatura e umidade. Essas toxinas podem produzir efeitos agudos, letais ou não, ou efeito cumulativo no organismo, que a longo prazo, podem causar, principalmente, lesões carcinogênicas hepáticas. Sabendo-se dos riscos à saúde, devido à presença de aflatoxinas nos alimentos, foi realizada uma pesquisa em amendoins e derivados comercializados na cidade de Alfenas – MG, Brasil, para avaliar a presença de aflatoxinas e de fungos do gênero *Aspergillus sp*. Amostras foram coletadas aleatoriamente em lojas populares no período de novembro de 2008 a maio de 2009 e avaliada a ocorrência de aflatoxinas B_1, B_2, G_1 e G_2, utilizando-se a técnica de Cromatografia de Camada Delgada (CCD). Na análise micológica foi constatada a presença de fungos em 50% das amostras, das espécies: *Penicillium sp*. (53,85%), *A. flavus* (19,23%), *A. niger* (15,38%), *A. fumigatus* (11,54%). Obteve-se como resultado para a presença de aflatoxinas 63,64 %, sendo: B_1 (43,14%), B_2 (25,49%), G_1 (23,53%), G_2 (7,84%). Concluiu-se que os números encontrados são preocupantes, tendo em vista a periculosidade desta substância.

Palavras-Chave: Aflatoxina. *Aspergillus sp*. Micotoxina. Amendoim. Contaminação.

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