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Aflatoxins intake from peanut candy marketed in Rio de Janeiro city, Brazil

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Abstract

Paçoca is a very popular product of indigenous origin that is widely consumed by children, especially during the annual folkloric celebrations in June and July in Brazil. One of the main risks of this peanut candy is contamination by aflatoxins. Aflatoxins are natural contaminants produced mainly by the aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*, and have important toxic effects for humans, such as, acute aflatoxicosis and risk of cancer development. This work aimed to determine the levels of aflatoxins in 36 different brands of *paçoca* samples marketed in Rio de Janeiro, Brazil, during the period of folkloric celebrations in 2012, and to estimate the aflatoxins intake by children and adolescents using a Food Frequency Questionnaire applied to 157 individuals. The aflatoxins were quantified by High Performance Liquid Chromatography with Fluorescence Detection in 72 *paçoca* samples (100%). Twenty-seven samples (37.4%) were positive for aflatoxins and ten (13.8%) showed aflatoxins levels higher than the limit established by Brazilian legislation for peanut products (20 µg/kg). The overall average corresponded to 4.9 µg/kg and the highest value found was 39.6 µg/kg. The Probable Daily Intake (PDI) of aflatoxins through the consumption of *paçoca* corresponded to 1.37 ng/kg body weight (b.w.). Children aged from 8 to 11 were the most exposed to these mycotoxins, which may pose a significant risk to their health. This is the first report to be made showing levels of aflatoxinas in a peanut product marketed in Rio de Janeiro, Brazil.

Introduction

The annual Brazilian folkloric celebrations, which occur in winter, during the months of June and July, are commemorated with typical songs, dances, foods and drinks (Chianca and Aldé, 2009). In this period, there is a high consumption of candies made with peanuts, both homemade and industrialized. Among these is *paçoca*, which is a Brazilian product of indigenous origin, and is very popular and widely consumed by children. *Paçoca* is basically made of ground peanuts, sugar, salt and corn or cassava flour.

The quality of the peanuts used in the production of *paçoca* is of great importance and, one of the main health risks is the possible contamination of the peanuts with toxins produced by filamentous fungi, called mycotoxins (Magrine *et al*., 2011; Chang *et al*., 2013). In 2011, new limits for the presence of mycotoxins in foods were set in Brazil by the Ministry of Health that established stricter levels for aflatoxins in different kinds of foods, such as, cereals, beans, nuts, almonds, dry fruits, spices and dairy products. For peanuts and peanut products, the maximum limit for total aflatoxins ($B_1+B_2+G_1+G_2$) is 20 µg/kg (Brazil, 2011).

Aflatoxins are natural contaminants with important toxic effects for human and animal health that are produced mainly by the aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus*. Aflatoxins represent a public health problem because they remain partially stable even during industrial processes that foods are subjected to (EFSA, 2013; Milani and Maleki, 2014). The International Agency of Research on Cancer (IARC) classified aflatoxins as carcinogens agents in Group 1 (carcinogenic to humans) (IARC, 2002) and the aflatoxin B_1 has the

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highest carcinogenic potential among the known mycotoxins (EFSA, 2013). The contamination of peanuts produced in Brazil is a concern because the climatic conditions prevailing in the country, allied to the traditional practices of harvest, drying and storage, allow the infestation of the kernels by *Aspergillus* spp and the consequent production of aflatoxins (Gorayeb *et al*., 2009; Souza *et al*., 2014).

Therefore, the monitoring of peanut kernels and their derivatives is very important to assess any possible human exposure to aflatoxins. One way to indirectly estimate such exposure is through a Food Frequency Questionnaire (FFQ), which is a checklist based on the consumption of such foods that may be contaminated, over a specific period of time (Magrine *et al*., 2011; Jager *et al*., 2013). In this estimation, the degree of exposure is based on the average level of contamination, and it is measured in terms of Probable Daily Intake (PDI), expressed in ng/kg of body weight (b.w.). Given the fact that aflatoxins are genotoxic and carcinogenic substances, there is no acceptable daily intake and, it is recommended that the levels of these mycotoxins present in foods be as low as reasonably possible (Brazil, 2011).

The aim of this study was to determine the levels of aflatoxins in *paçoca* samples marketed in Rio de Janeiro, Brazil, during the period of folkloric celebrations in 2012, and to estimate the aflatoxins intake due to the consumption of this product by children and adolescents through a Food Frequency Questionnaire.

Material and Methods

Sampling

During June and July 2012, 72 *paçoca* samples were acquired from local markets in Rio de Janeiro city, Brazil (Figure 1). Thirty-six different brands of *paçoca* were identified in the city and 1 kg samples were collected from 2 different batches of each brand, totaling 72 samples, which were transported to the laboratory and stored at -10ºC until analysis.

Chemicals and reagents

The aflatoxins standards, B_1 (5000 µg), B_2, G_1 and G_2 (1000 µg) were purchased from Sigma (St. Louis, MO, USA). Individual stock solutions (50 µg/mL) and working solutions (2 µg/mL) were prepared in methanol and their concentrations were confirmed by absorption in UV light with a Shimadzu UV-1201 spectrophotometer (Kyoto, Japan), according to the Association of Official Analytical Chemists (AOAC, 2000).

HPLC-grade acetonitrile and methanol were purchased from Tedia (São Paulo, SP, Brazil). The other solvents and reagents used were of analytical grade, and were obtained from Merck (Rio de Janeiro, RJ, Brazil). The deionized water used was obtained using the Milli-Q® purification system (Millipore, MA, USA). Whatman filter paper (qualitative, 1) and a 0.45 µm PVDF membrane (Durapore® 13 mm, Millipore) were used for filtration.

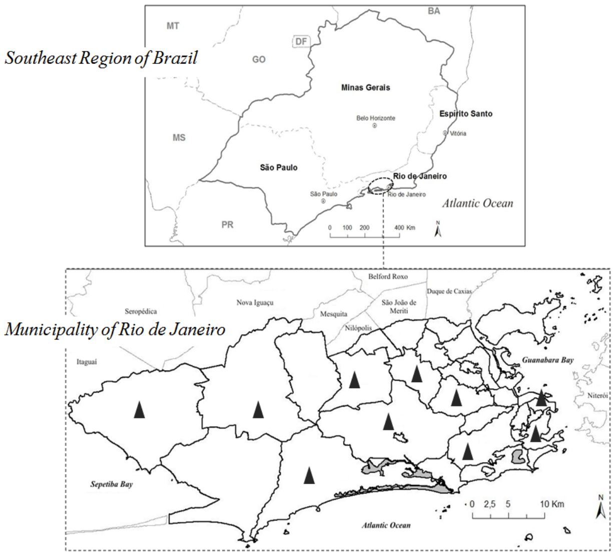

Figure 1. Rio de Janeiro, Brazil. The triangles represent the neighborhoods where the paçoca samples were acquired

Aflatoxins extraction and derivatization

Each of the 1 kg samples (n= 72) was homogenized and then 25 g was collected for analysis. The mycotoxin analyses were performed in triplicates. The methodology used for extraction and purification was performed according to the method proposed by Soares and Rodriguez-Amaya (1989) with minor adaptations, as follows.

An aliquot of the sample (exactly 25 g) was put into an Erlenmeyer flask and 7.5 mL of KCl aqueous solution (4% w/v) and 67.5 mL of methanol were added to the flask and then agitated in a horizontal Shaker for 30 min. Then, 75 mL of $CuSO_4$ aqueous solution (10%, w/v) and 25 mL of diatomaceous earth were added to the flask and mixed for 1 min. After giving sufficient time for precipitation (approximately 5 min), the mixture was filtered and 75 mL collected. This solution was transferred to a funnel and 25 mL of hexane was added to the funnel and stirred vigorously for 1 min. After stirring, the hexane phase (upper) was discarded and this step was repeated with another 25 mL of hexane. Subsequently, 25 mL of chloroform was added to the funnel with vigorous shaking for 1 min and the chloroform phase (bottom) was collected. This step was repeated with another 25 mL of chloroform and then evaporated until dryness in a water bath at 65°C, under N2 flow.

For derivatization, the extracts were resuspended in 0.6 mL of acetonitrile, submitted to an ultrasound bath for 30 sec and 1.2 mL of the derivatizing solution constituted by water: trifluoroacetic acid: glacial acetic acid (7: 2: 1, v/v/v) was added. The solution was maintained in a water bath at 65°C for exactly 9 min (AOAC, 2005). Finally, the extract was filtered through a 0.45 µm membrane and injected into the HPLC system.

HPLC-FL analysis

Aflatoxins were quantified using a High Performance Liquid Chromatography system (Agillent 1100 Series, Waldbronn, Germany), with a fluorescence detector (excitation at 365 nm and emission at 450 nm), a Rheodyne injector (20 µL), a C18 column (Ace, 250 mm x 4.6 mm, 5 µm) and an isocratic mobile phase, consisting of water: methanol: acetonitrile (6: 3: 1, v/v/v) at a flow rate of 0.6 mL/min. The results were expressed by the mean of the triplicates, in µg/kg.

Validation procedures

Linearity was evaluated by external standardization, using analytical curves built up from 7 different concentrations of each aflatoxin (B_1, B_2, G_1, G_2), ranging from 0.008 to 0.347 µg/mL. The coefficient of determination was considered adequate when r^2 was higher than 0.99. The limits of detection (LOD) and quantification (LOQ) were found by adding decreasing concentrations of standard solution containing the four aflatoxins in the samples, in quintuplicate. Then these samples were submitted to extraction and quantification until the lowest detectable concentration (LOD) and the lowest quantifiable concentration (LOQ), under suitable conditions of repeatability (n = 5, Coefficient of Variation less than 15%) were reached. Recovery was assessed by adding each aflatoxin (B_1, B_2, G_1, G_2) at concentrations of 5, 10 and 20 µg/kg in blank samples. Five (5) samples were submitted to extraction and quantification for each addition. The precision was expressed by the CV% for each level.

Food frequency questionnaire

A Food Frequency Questionnaire (FFQ) was elaborated for the assessment of the consumption of *paçoca* through June and July 2012. This questionnaire was applied at public schools in Rio de Janeiro city, to 157 individuals, between the ages of 8 and 17, who consume *paçoca* at least once a month. A portion of *paçoca* was considered to be 22.5 g, since this value represents the average portion of the product found for the 36 different brands collected for aflatoxins determination. The body weights of each individual were recorded in order to calculate the Probable Daily Intake (PDI) of aflatoxins, which was obtained using the following formula: PDI = (mean concentration of aflatoxins x average daily consumption of *paçoca*)/(each individual body weight). The PDI value was expressed as ng/kg b.w.

Statistical analysis

Statistical analyses were performed using Sisvar® 5.3 Build 77 (UFLA, Brazil). A probability value of 0.05 was used to determine the statistical significance in ANOVA.

Results and Discussion

Method performance

The LOD and LOQ values were 0.6 and 1.2 µg/kg, respectively, for each aflatoxin. The coefficients of determination for aflatoxins G_1, B_1, G_2 and B_2 in the analytical curves were 0.999, 0.993, 0.996 and 0.998, and the retention times were approximately 7.6, 10.3, 13.6 and 20.1 min, respectively (Figure 2). Mean recoveries and CV obtained in the five replicates were 94.5% (CV = 6.4%), 93.0% (CV = 4.2%) and 98.6% (CV = 5.9%) for the levels of 5, 10 and 20 µg/kg, respectively. The recoveries and CV values obtained using this methodology were satisfactory at the three addition levels studied, and they are in accordance with the European Commission Regulation No. 401/2006 (EC, 2006).

Aflatoxins determination in paçoca samples

Peanut grain may be contaminated by aflatoxins in the field and/or when improperly stored. This contamination can be effective controled when good agricultural practices (GAP) for growing, harvesting and storage are applied (Codex Alimentarius, 2014; Souza *et al.*, 2014). However, as it is not possible to fully eliminate their presence, monitoring their levels in the grains or in their derivatives products are very important.

In this study 72 samples of 36 different brands of *paçoca*, marketed in Rio de Janeiro city, Brazil, were analyzed and the results are presented in Table 1. Twenty-seven samples (37.4%) were positive for aflatoxins and ten samples (13.8%) showed aflatoxins levels higher than the limit established by Brazilian legislation for peanut products (20 µg/kg). The overall average was 4.9 µg/kg and the highest value found was 39.6 µg/kg.

This is the first report to be made showing levels of aflatoxins in a peanut product marketed in

Table 1. Total aflatoxins ($B_1+B_2+G_1+G_2$) in peanut candy *(paçoca)* marketed in Rio de Janeiro, Brazil in 2012

Range (µg kg^{-1})	Samples in the range	Percentage
Nd[a] to 5.0	50	69.4%
5.0 to 10.0	9	12.5%
10.0 to 20.0	3	4.2%
Higher than 20.0[b]	10	13.8%
Total	72	100%

[a] Nd: Not detected (< 1.6 µg.kg^{-1})
[b] Maximum permitted level adopted by Brazilian regulations

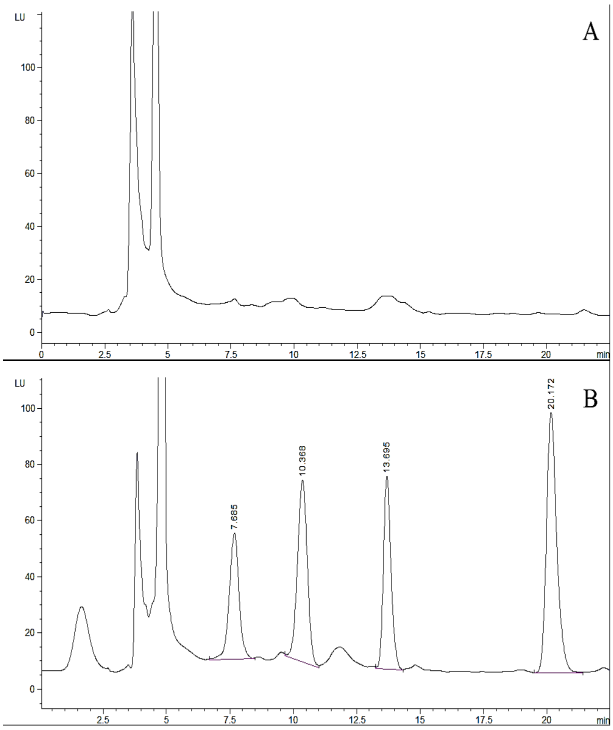

Figure 2. Chromatogram obtained by HPLC-FL in blank sample of peanut candy *(paçoca)* (A) and added with 5.0 µg kg^{-1} of each aflatoxin (B). Elution order: AFG_{2a} (derivatized form of AFG_1), AFB_{2a} (derivatized form of AFB_1), AFG_2, AFB_2

the state of Rio de Janeiro, Brazil. Similar research showing high levels of aflatoxins in peanut products, including *paçoca*, has been carried out in other regions of the country (Hoeltz *et al.*, 2012; Andrade *et al.*, 2013; Jager *et al.*, 2013) also showing high levels of contamination by aflatoxins. Oliveira *et al*. (2009) emphasized that Brazilian children are potentially exposed to aflatoxins, since they consume large quantities of peanut candies, and these products usually have the highest number of contaminated samples among peanut products.

In this study, the large number of brands of *paçoca* found in the region studied (36) is due to the period that the sampling was performed. In this period, several local industries produce this food due to the increased consumption, especially by children. The quality and the price of the product vary widely. There are those prepared only with roasted peanut flour, sugar and salt, or sweeteners in place of sugar, but there are also those that are made using cassava flour, corn flour, biscuit flour and other products of low value in the form of flours, besides the peanut flour. As there is no national legislation regulating the patterns of chemical or microbiological quality of *paçoca*, many ingredients may be used in the production, which can compromise the quality and the safety of this food.

Assessment of aflatoxin intake

The FFQ was used to interview 157 individuals with age from 8 to 17, and the results of the estimation of *paçoca* consumption and aflatoxins intake are presented in Table 2. *Paçoca* intake was calculated on a monthly basis and the mean of aflatoxins levels and each individual body weight were used to calculate the PDI of total aflatoxins. The highest PDI found corresponded to 2.47 ng/kg b.w. and the mean obtained was 1.37 ng/kg b.w. There was no significant difference ($p > 0.05$) in PDI between female and male consumers. Individuals aged from 8 to 11 had the highest PDI in the population studied. The consumption of *paçoca* by this age range was higher than the others, and, consequently, also the intake of aflatoxins. This result is very worrying because children have more immature neurologic and immune systems and so are more prone to develop complications when exposured to aflatoxins than adults (Khlangwiset *et al*., 2011; Owaga *et al*., 2011; Magnussen and Parsi, 2013).

The present study calculated the PDI of aflatoxins only with school age individuals (8 to 17 years old) and, it was based on a single peanut candy, during a specific period when there is an increase in the consumption of this food. Other studies conducted in Brazil have estimated the PDI of aflatoxins from the consumption of different types of foods, such as, peanut products, corn products, beans, milk products and nuts (Oliveira *et al*., 2009; Magrine *et al*., 2011; Jager *et al*., 2013; Andrade *et al*., 2013).

Since there is no Acceptable Daily Intake (ADI) for aflatoxins, as they are genotoxic and carcinogenic substances, and any level present in foods is considered a health risk. The PDI of total aflatoxins found in this work demonstrates that the population of Rio de Janeiro city, especially children, is exposed to high levels of aflatoxins through the consumption

Table 2. Estimative of peanut candy *(paçoca)* consumption and aflatoxins intake

Age range	*n*	Estimated consumption of *paçoca* (kg month^{-1})	PDI (ng kg^{-1} b.w. day^{-1})	
			Average	Variation
8 to 11	81	0.379	1.90	0.12 – 2.47
12 to 14	64	0.245	0.88	0.78 – 1.11
15 to 17	12	0.119	0.33	0.05 – 0.48
Total	157	0.311	1.37	0.05 – 2.47

n: Number of individuals that answered the Food Frequency Questionnaire.
PDI: Probable Daily Intake

of *paçoca*. However, more research evaluating the occurrence of aflatoxins and other mycotoxins in different foods is needed for a wider risk analysis, since such studies are inexistent in this region.

Conclusion

From a total of 72 *paçoca* samples (100%) of 36 different brands collected during the period of folkloric celebrations in 2012, 27 (37.4%) were positive for aflatoxins. Ten samples (13.8%) showed aflatoxins levels higher than the limit established by Brazilian legislation and the average contamination corresponded to 4.9 µg/kg. The Probable Daily Intake of aflatoxins through the consumption of *paçoca* by individuals aged from 8 to 17 corresponded to 1.37 ng/kg b.w. Children aged from 8 to 11 are the most exposed to these mycotoxins because they are the largest consumers of the product, which may pose a significant risk to their health. This is the first time that the levels of aflatoxins in peanut candy marketed in Rio de Janeiro, Brazil have been determined and reported.

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