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Aflatoxin evaluation in ready-to-eat brazil nuts using reversed-phase liquid chromatography and post-column derivatisation

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A high-performance liquid chromatography-fluorescence (HPLC-FD) method for aflatoxin quantification in brazil nuts was developed. Samples of brazil nuts collected in Brazilian markets were extracted with methanol:water and cleaned using an immunoaffinity column. Aflatoxins were eluted with methanol and a post-column derivatisation was performed with bromine, using a Kobra Cell system. The optimised method for total aflatoxins was sensitive, with detection and quantification limits of 0.05 and 0.25 μg kg^{-1}, respectively. The method was accurate, with recovery values of 87.6%; 85.3% and 85.0% for 0.5, 5.0 and 14.6 μg kg^{-1} spiked levels, respectively. It was shown that the method was applicable to brazil nuts. From a total of 95 brazil nut samples analysed from 21 São Paulo supermarket samples and 51 Manaus and 23 Belém street markets samples, 37.9% showed detectable levels of aflatoxins and three exceeded the recommended Codex Alimentarius limit of 10 μg kg^{-1} for ready-to-eat brazil nuts.

Keywords: brazil nuts; aflatoxins; HPLC; immunoaffinity column

Introduction

Aflatoxins are the most significant and toxic mycotoxins in food, mainly in tropical and sub-tropical regions, causing damaging effects to animal and human health. The hepatotoxic, teratogenic, mutagenic and carcinogenic properties of aflatoxins have resulted in restrictive regulations for these contaminants in foods (International Agency for Research on Cancer 1993). CAC (2010a) and Brazilian Regulations (ANVISA 2011) have recommended a maximum level for aflatoxins in brazil nuts of 15 and 10 μg kg^{-1} for further processing and ready-to-eat, respectively. Several studies have reported the presence of aflatoxigenic fungi and aflatoxins in brazil nuts (*Bertholletia excelsa*), which is one of the most important products produced in the Amazon region (Gilbert & Sheperd 1985; Arrus et al. 2005; Olsen et al. 2008; Pacheco & Scussel 2009; Baquião et al. 2012, 2013; Reis et al. 2012; Calderari et al. 2013). The extractivism way of harvesting, and the hot and humid climate from the forest (ranging up to 26°C and 80% relative humidity) are factors that favour growth of aflatoxin-producing fungi.

Currently more than 25 species have been described belonging to *Aspergillus* section *Flavi* and 15 are considered aflatoxin producers: *A. arachidicola*, *A. bombycis*, *A. flavus*, *A. minisclerotigenes*, *A. nomius*, *A. parasiticus*, *A. parvisclerotigenes*, *A. pseudocaelatus*, *A. pseudonomius*, *A. pseudotamarii*, *A. togoensis*, *A. mottae*, *A. sergii*, *A. transmontanensis* and *A. novoparasiticus* (Varga et al. 2011; Gonçalves et al. 2012; Soares et al. 2012; Taniwaki et al. 2012; Baquião et al. 2013). Olsen et al. (2008) reported *A. nomius* as the main aflatoxin producer in brazil nuts. On the other hand, Calderari et al. (2013) reported the presence of five aflatoxigenic species belonging to *Aspergillus* section *Flavi* in brazil nuts: *A. flavus*, *A. nomius*, *A. bombycis*, *A. arachidicola* and *A. pseudotamarii*, but *A. nomius* and *A. flavus* showed higher levels of incidence. Recently, Taniwaki et al. (2012) described a new non-aflatoxigenic species of *Aspergillus* section *Flavi* called *A. bertholletius* from brazil nut and soil samples, showing the biodiversity of this section in the Amazon rainforest.

For the presence of aflatoxins in brazil nuts, the reported results vary, ranging from not detected to 17 926 μg kg^{-1} (Gilbert & Shepherd 1985; Arrus et al. 2005; Pacheco & Scussel 2009; Baquião et al. 2012; Martins et al. 2012; Reis et al. 2012; Calderari et al. 2013). Some studies have pointed out some critical steps associated with the presence of aflatoxin in brazil nuts. Johnsson et al. (2008) described that, firstly, aflatoxin formation is favoured by storage at 97% relative humidity and, secondly, it increases 40–90 days before drying at the processing plant. Calderari et al. (2013) reported that sorting in processing plants after shelling is effective at decreasing aflatoxin contamination in brazil nuts. Both studies show that the samples that do not go through processing plants and are sold directly to the markets are a concern.

Several methods for aflatoxin analyses in brazil nuts are described, including the use of antibody-based, immunoassay and packed mini-column clean-up methods, followed by derivatisation, liquid chromatography or mass

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spectrometry detection (Trucksess et al. 1994; Sobolev & Dorner 2002; Sobolev 2007; Pacheco & Scussel 2009; Martins et al. 2012). The use of reversed-phase liquid chromatography post-column derivatisation is an effective method and has been used for aflatoxin determination. Regardless of the method used for aflatoxin quantification, they all need to be submitted to a validation test to ensure reliable results, since they are found at low concentrations in the food and influenced by the matrix effect, which is generally organic and complex. The validation is important as it proves that the analytical method is suitable for its purpose.

In this study a method for aflatoxin analysis in brazil nuts using reversed-phase liquid chromatography and post-column derivatisation was tested and optimised in-house. The occurrence of aflatoxins in ready-to-eat samples of brazil nuts collected from the Brazilian market is also reported.

Material and methods

Brazil nut samples

A total of 95 brazil nut samples each of approximately 2 kg of in-shell and 1 kg of shelled nuts were collected at supermarkets in São Paulo and at street markets in Manaus (AM) and Belém (PA), in Brazil. Samples in-shell were manually shelled before milling, using a specific instrument to remove the shell. All samples were ground using an Ika A11 mill and stored at –20°C until the aflatoxin analyses were performed.

Aflatoxin determination

Aflatoxin analyses were carried out on kernels based on Stroka et al. (2000) methodology.

Standard preparation

A working standard aflatoxins solution was prepared in the laboratory by diluting the stock solution (Sigma, lot 71K4031, part number A6636) with toluene:acetonitrile (99:1). Concentrations of 1.41, 0.57, 0.31 and 0.46 µg/ml^{-1} of B1, B2, G1 and G2 of aflatoxins were obtained respectively and calculated based on absorbance values of each aflatoxin in the spectrophotometer. The working standard solution was diluted in order to obtain the calibration curve, reaching the range of 0.005–1.61 ng µl^{-1} for aflatoxin B1, 0.002–0.73 ng µl^{-1} for aflatoxin B2, 0.001–0.45 ng µl^{-1} for aflatoxin G1 and 0.002–0.59 ng µl^{-1} for aflatoxin G2. Amber volumetric flasks were used. Five-point calibration curves were plotted.

Sample extraction and clean-up

Samples (25 g) of finely ground kernels were added to NaCl (2 g) and extracted with methanol:water solution (100 ml; 8:2, v/v) for 3 min at high speed (10,000 rpm) using an Ultra-Turrax homogeniser (Polytron, Luzern, Switzerland). The homogenised solution was filtered through Whatman No. 2 filter paper and Whatman A-H glass microfiber filter (Whatman, Maidstone, UK). The filtrate (10 ml) was diluted in phosphate-buffered saline (60 ml) and applied to an Aflatest WB immunoaffinity column (Vicam, Milford, MA, USA) at a flow rate of 2–3 ml min^{-1}. The column was then washed with distilled water (30 ml) and aflatoxins eluted with methanol (1250 µl) and diluted with Milli-Q water up to 3 ml.

HPLC parameters

A Shimadzu LC-10VP HPLC system (Shimadzu, Kyoto, Japan) was used with a fluorescence detector set at 362 nm excitation and 455 nm emission for aflatoxins G1 and G2 and at 425 nm emission for aflatoxins B1 and B2. A Shimadzu CLC G-ODS (4 × 10 mm) guard column and Shimadzu Shimpack (4.6 × 250 mm) column were employed. The mobile phase used was water:acetonitrile: methanol (6:2:3, v/v/v) containing KBr (119 mg l^{-1}) and nitric acid (4 M, 350 µl l^{-1}). The flow rate was 1 ml min^{-1}. A post-column derivatisation of aflatoxins B1 and G1 was performed with bromine using a KobraCell (R-Biopharm Rhône, Glasgow, UK), with a flow rate of 1 ml min^{-1} and current of 100 µA. The injection volume was 100 µl.

In-house methodology optimisation

Criteria such as accuracy, precision, LOD, LOQ and linearity were established for an aflatoxin in-house validation method for brazil nuts.

For accuracy, blank shelled brazil nuts (n = 5) bought from a supermarket in Campinas (SP), previously prepared as showed above, were spiked with three different levels of total aflatoxins at 0.50 µg kg^{-1} (B_1: 0.20 µg kg^{-1}; B_2: 0.12 µg kg^{-1}; G_1: 0.08 µg kg^{-1} and G_2: 0.10 µg kg^{-1}), 5.0 µg kg^{-1} (B_1: 2.01 µg kg^{-1}; B_2: 1.24 µg kg^{-1}; G_1: 0.75 µg kg^{-1} and G_2: 1.0 µg kg^{-1}) and 14.6 µg kg^{-1} (B_1: 6.70 µg kg^{-1}; B_2: 3.40 µg kg^{-1}; G_1: 1.25 µg kg^{-1} and G_2: 3.29 µg kg^{-1}), and the recovery values were reported. Furthermore, a certified reference material FAPAS™ (Food Analysis Performance Assessment Scheme – T04134, UK) was analysed in parallel.

Precision, LOD and LOQ were calculated analysing eight brazil nut samples spiked with the aflatoxin standards and the standard deviation was calculated. Repeatability standard deviation (r) was calculated for B_1, B_2, G_1, G_2 and total aflatoxins in order to evaluate precision. Brazil nut samples (n = 5) for three different

levels (0.50, 5.0 and 14.6 μg kg^{-1}) were analysed using the same conditions in the laboratory and by the same analyst. For determination of the LOD, standard deviations were multiplied by the corresponding number listed in the Student's *t*-table for 99% significance. LOQ was determined by multiplying the standard deviation by 10 (Keith et al. 1983; Long & Winefordner 1983; Eurachem Guides 1998).

Five-point calibration curves of B_1, B_2, G_1, G_2 aflatoxins with a correlation coefficient of (r) > 0.99 were plotted to obtain the linearity.

Water activity

The water activity of brazil nut samples was determined using an Aqualab Series 3TE instrument (Decagon, Pullman, WA, USA) at 25 ± 1.0°C, in triplicate.

Results and discussion

Aflatoxin methodology in brazil nuts

The method described by Stroka et al. (2000) was implemented and optimised in the laboratory and showed satisfactory and accurate results for brazil nuts. Table 1 shows the results of recovery for aflatoxins B_1, B_2, G_1, G_2, total aflatoxins tested for three different spiked levels and the results of certified material.

The accuracy obtained using a reference material of brazil nuts (Fapas T04134) was satisfactory and all four aflatoxin results obtained were acceptable. The ranges of total aflatoxins and aflatoxin B1 expected were 4.87–12.53 and 2.30–5.90 μg kg^{-1} respectively and the sample result obtained was 11.19 μg kg^{-1} for total aflatoxin and 5.84 μg kg^{-1} for aflatoxin B1.

Recoveries of total aflatoxins obtained were 87.6%, 85.3% and 85.0% for spiking levels of 0.50, 5.0 and 14.64 μg kg^{-1}, respectively. Recoveries of aflatoxin B1 were 69.9%, 81.7% and 78.9% for spiking levels of 0.20, 2.01 and 6.70 μg kg^{-1}, respectively. The recovery values achieved were in accordance with European Community Directive EC No. 401/2006 that established values between 50% and 120% for aflatoxin levels lower than 1.0 μg kg^{-1}, between 70% and 110% for 1.0–10 μg kg^{-1}, and between 80% and 110% for higher than 10 μg kg^{-1}.

The LODs for B_1, B_2, G_1, G_2 and total aflatoxins were 0.02, 0.01, 0.01, 0.01 and 0.05 μkg and the LOQs were 0.1, 0.05, 0.05, 0.05 and 0.25 μg kg^{-1} respectively.

Five-point calibration curves of aflatoxins B_1, B_2, G_1 and G_2 are shown in Figure 1. Correlation coefficients obtained for each aflatoxin were higher than 0.99.

Figures 2 and 3 show the chromatograms of a brazil nut sample contaminated with aflatoxins and aflatoxin standards respectively.

Table 1. Level of aflatoxins (%) using three different aflatoxin contamination levels and certified reference material (Fapas) results.

Level of total aflatoxins (μg kg^{-1})	(n = 5)	B_1 (μg kg^{-1})	B_2 (μg kg^{-1})	G_1 (μg kg^{-1})	G_2 (μg kg^{-1})	Total aflatoxins (μg kg^{-1})
0.50	Average ± SD[a]	0.14 ± 0.02	0.11 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.42 ± 0.05
	Recovery ± SD (%)	74.70 ± 5.9	89.45 ± 4.83	112.33 ± 6.36	81.48 ± 6.45	87.58 ± 2.54
5.00	Average ± SD	1.66 ± 006	1.09 ± 0.01	0.87 ± 0.02	0.66 ± 0.02	4.27 ± 0.06
	Recovery ± SD (%)	81.71 ± 2.52	87.82 ± 1.03	115.23 ± 2.18	66.54 ± 1.67	85.28 ± 1.21
14.64	Average ± SD	5.28 ± 0.16	3.58 ± 0.10	1.42 ± 0.04	2.16 ± 0.16	12.44 ± 0.45
	Recovery ± SD (%)	78.87 ± 2.43	105.27 ± 2.93	113.65 ± 3.33	65.78 ± 4.75	85.03 ± 3.07
Fapas T04134						
Assigned value (range)		4.10 (2.30–5.90)	0.90 (0.50–1.30)	3.36 (1.88–4.84)	0.47 (0.26–0.68)	8.70 (4.87–12.53)
Laboratory results		5.84	0.86	4.17	0.32	11.19

Note: [a]SD, standard deviation (n = 5).

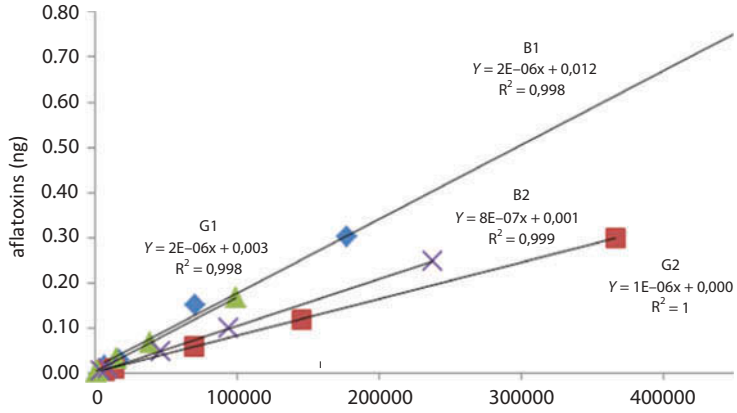

Figure 1. (colour online) Aflatoxin calibration curves.

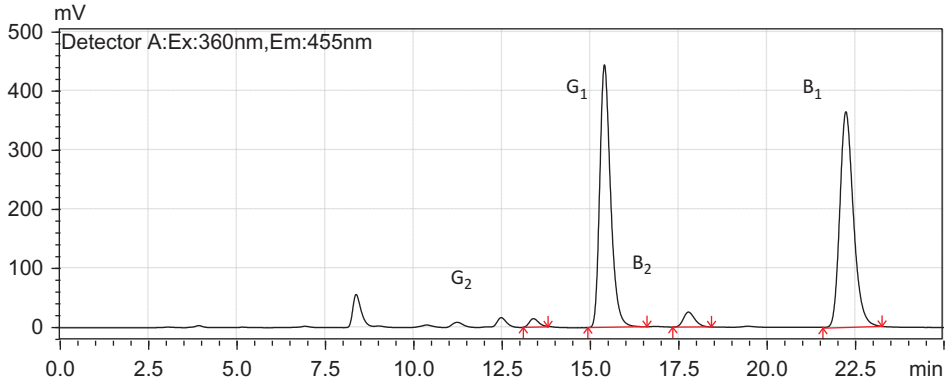

Figure 2. (colour online) Chromatogram of a naturally contaminated brazil nut sample: B1 = 17.0 μg kg^{-1}, B2 = 0.3 μg kg^{-1}, G1 = 14.06 μg kg^{-1} and G2 = 0.02 μg kg^{-1}.

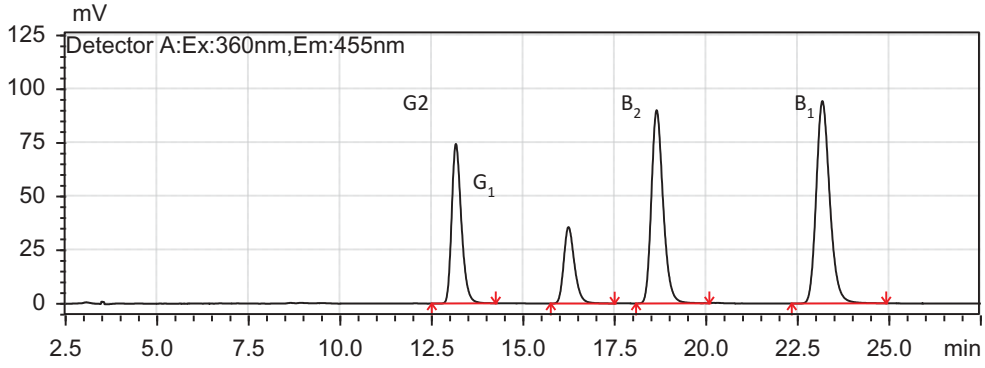

Figure 3. (colour online) Chromatogram of an aflatoxin standard: B1 = 0.16 ng $μl^{-1}$, B2 = 0.07 ng $μl^{-1}$, G1 = 0.04 ng $μl^{-1}$ and G2 = 0.06 ng $μl^{-1}$.

When comparing the original method (Stroka et al. 2000) that shows the results of a collaborative study of 16 laboratory participants in Europe and tested different matrices (peanut butter, pistachio, paste, dried fig paste and paprika powder) for aflatoxin detection using an immunoaffinity column, the results obtained for the methodology in our laboratory for brazil nuts were similar. The mean recovery values reported in the original article varied from 71% to 92% for total aflatoxins and from 82% to 109% for aflatoxin B1 (2.4 and 9.6 ng g^{-1}), while in the present study the results were 85.3% and 81.7% respectively for a similar contamination level (5.0 μg kg^{-1}). The LOD and LOQ obtained were low (aflatoxin B1 = 0.02 μg kg^{-1} and total aflatoxins = 0.05 μg kg^{-1})

compared with those reported in the original method. The methodology optimised in the laboratory was suitable for the purpose of analysing aflatoxins in brazil nuts.

Several methods have been proposed to analyse mycotoxins in foods (Stroka et al. 2000; Krska et al. 2008; Soleimany et al. 2012; Streit et al. 2013; Varga et al. 2013; Koesukwiwa et al. 2014). Recently the use of LC-MS/MS has increased and its main advantage is to provide the simultaneous detection and quantification of a number of mycotoxins in food and feedstuffs. Varga et al. (2013) could validate 191 mycotoxins and fungi metabolites in different kinds of nuts. However, due to the high price of the equipment and its maintenance costs, many laboratories and companies are unable to obtain it. In this case the use of standard methods is recommended and a clean-up procedure is necessary, such as SPE, immunoaffinity columns and QuEChERS. These standard methods have also been used since they have shown satisfactory results, with LOD, LOQ and recovery results similar to LC-MS/MS. They are available for most laboratories that have carried out mycotoxin analyses. Martins et al. (2012) performed aflatoxin analyses in brazil nuts using liquid chromatography coupled with MS/MS obtaining LOD and LOQ of 0.3 and 0.85 μg kg^{-1} respectively and recovery values of 92.4%, 72.5%, 99.8% and 97.1% for aflatoxin B_1, B_2 G_1 and G_2 respectively. The LOD and LOQ found by these authors were higher than 0.05 and 0.25 μg kg^{-1} for total aflatoxins respectively obtained in our study.

Aflatoxin occurrence in ready-to-eat brazil nuts

Table 2 shows the results of total aflatoxins and water activity in shell and shelled brazil nut samples from three different regions. Most of the in-shell and shelled samples had no detected aflatoxin contamination or lower than the LOD.

The levels of aflatoxins and water activity varied among the samples from São Paulo, Manaus and Belém. Aflatoxins were present in 52.4% of samples from São Paulo, but the average of contamination was lower than other regions (average of 0.26 μg kg^{-1}). These samples were submitted to a process, such as drying and sorting, to be sold in São Paulo. Most samples showed low water activity, with an average of 0.47, which is an indication that they had been processed. The samples from Manaus (MA) and Belém (PA) showed higher levels of aflatoxins, with an average of 0.41 and 8.30 μg kg^{-1} respectively. These samples were collected from street markets where brazil nuts are sold at different stages of drying and conditions. The range of water activity in these samples was 0.422–0.994, showing a high variation.

In Manaus the average and median results of aflatoxin in shelled and in-shell samples were not detectable, indicating low contamination. The Belém samples a high average of total aflatoxin was found in shelled samples (average of 14.90 μg kg^{-1}) and it can be explained by the two samples with high aflatoxin contamination (32.1 and 140.0 μg kg^{-1}).

Three samples from Belém had high levels of aflatoxins, exceeding the limit recommended by CAC (2010a) and Brazilian regulation (ANVISA 2011) of 10 μg kg^{-1} total aflatoxins in ready-to-eat brazil nuts. These samples were visibly mouldy and showed total aflatoxins of 32.1, 48.2 and 140.0 μg kg^{-1}. The sample with the highest aflatoxin level (140 μg kg^{-1}) had water activity value of 0.569, indicating that this sample was submitted to critical conditions such as high humidity that provided the fungal growth and aflatoxin production.

In general brazil nuts sold in Brazilian markets did not show high contamination levels of aflatoxins and 62% of the total samples analysed were not contaminated (lower than the LOD). The average and mean values found from the total samples analysed were 2.29 μg kg^{-1} and lower than the LOD respectively. Various studies have shown the contamination of brazil nuts by aflatoxins with low levels being reported. Reis et al. (2012) evaluated 200 brazil nut samples from the Amazon region and found only 11% of the samples with detectable levels (LOD for each aflatoxin = 0.75 μg kg^{-1}). Pacheco and Scussel (2009) when analysing 171 brazil nut samples from the processing plants found 8% of the samples contaminated with >4.0 μg kg^{-1} aflatoxins. Baquião et al. (2012) when analysing brazil nuts from the tree and from the ground at different periods (0, 5, 10 and 15 days) in the forest found no detectable levels of aflatoxins in total samples analysed.

In spite of the majority of samples with low aflatoxin contamination, it is important to confirm the occurrence of a few samples located at Belém market contaminated with high levels of aflatoxins. Consumption of these contaminated brazil nuts, mainly by local peoples, can lead to undesirable aflatoxin effects.

The occurrence of aflatoxins in brazil nuts was investigated and showed that due to the complexity of the brazil nut production chain it cannot be completely eliminated easily. However, several national and international efforts, such as the establishment of maximum levels of total aflatoxins in brazil nuts by Brazilian Regulation (ANVISA 2011) and recommendation of maximum level by the CAC (2010a) and the Codex Code of Practice for handling brazil nuts by CAC (2010b) have been applied in order to offer safer products to consumers.

Conclusions

This research demonstrated that the use of an immunoaffinity column with reversed-phase liquid chromatography and post-column derivatisation is an easy and appropriate methodology to detect and to quantify aflatoxins in brazil nuts.

Table 2. Total aflatoxins (μg kg^{-1}) and water activity (aw) in shell and shelled brazil nuts from three different Brazilian cities.

Aflatoxins (μg kg^{-1}) Positive samples(%)		São Paulo (SP) 52.38		Manaus (AM) 39.20		Belém (PA) 47.82	
		Shelled (n = 17)	In shell (n = 4)	Shelled (n = 22)	In shell (n = 29)	Shelled (n = 12)	In shell (n = 11)
B1	Average	0.13	0.07	< LOD	0.18	11.03	2.72
	Median	0.06	0.02	n.d.	0.02	0.12	< LOD
	Range	n.d.–0.40	< LOD–0.22	n.d.–0.03	n.d.–2.29	n.d.–111.52	< LOD–24,83
B2	Average	< LOD	< LOD	n.d.	< LOD	0.16	0.42
	Median	n.d.	n.d.	n.d.	n.d.	0.01	n.d.
	Range	n.d.–0.04	n.d.–0.02	n.d.	n.d.–0.20	n.d.–1.04	n.d.–3.56
G1	Average	0.11	0.15	< LOD	0.62	2.54	2.03
	Median	0.01	0.11	n.d.	0.01	0.17	< LOD
	Range	n.d.–0.33	< LOD–0.35	n.d.–0.01	n.d.–4.58	n.d.–26.27	n.d.–17.93
G2	Average	< LOD	0.01	n.d.	0.01	0.16	0.31
	Median	n.d.	< LOD	n.d.	n.d.	n.d.	n.d.
	Range	n.d.–0.04	n.d.–0.04	n.d.	n.d.–0.33	n.d.–0.73	n.d.–1.89
Total	Average	0.26	0.25	< LOD	0.72	14.90	5.48
	Median	0.10	0.15	n.d.	< LOD	0.38	< LOD
	Range	n.d.–0.98	< LOD–0.66	n.d. to < LOD	n.d.–7.46	n.d.–140.00	n.d.–48.21
Aw	Average	0.478	0.531	0.918	0.817	0.530	0.828
	Median	0.488	0.548	0.969	0.919	0.532	0.806
	Range	0.273–0.586	0.431–0.597	0.603–0.994	0.583–0.985	0.422–0.595	0.618–0.981

Note: n.d., Not detected; LOD, detection limit of B1, B2, G1 and G2; and total aflatoxins: 0.02, 0.01, 0.01, 0.01 and 0.05 μg kg^{-1}.

In general, samples of brazil nuts analysed in this study showed aflatoxin contamination lower than the LOD, however some samples from street markets had high aflatoxin levels, showing the need for surveillance and control in these places.

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