

Nuclear Magnetic Resonance Approach in Brazil Nut Oil and the Occurrence of Aflatoxins

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Abstract: Carcinogenic metabolites of fungi such as aflatoxins play a toxic role in some tree nuts and need to be monitored in their by-products, such as oil. In this context, Brazil nut (*Bertholletia excelsa*) oil, which is a commodity of great economic importance to Brazil, requires attention to monitor the presence of these toxic agents. Therefore, this study aimed to evaluate the presence of aflatoxins in Brazil nut oil and relate it to the presence of fatty acids in the oil as a surveillance tool for food safety. Brazil nut oil samples (n= 25) were acquired in northern Brazil as (a) non-branded products (n= 07) produced by local farmers using artisanal methods from nuts to be discarded by the industry and (b) industrialized products (n= 18). The samples were analyzed for total aflatoxin content by high-performance liquid chromatography and fatty acid content by nuclear magnetic resonance imaging. Seven (28%) samples were positive for the aflatoxin fractions (B1 + B2 + G1 + G2), ranging from undetected (<2.32) to 50.87 µg/kg. Of the aflatoxin positive samples evaluated by NMR analysis, it was not possible to state that the presence of a particular fatty acid can interfere or influence aflatoxin contamination. This was the first study with data on aflatoxin occurrence in Brazil nut oil. Nevertheless, further research is required to relate saturated or unsaturated fatty acid content with aflatoxin levels. We also suggest the implementation of systems to prevent contamination of the raw materials (seed) and detoxification of the oil to guarantee the product's safety and quality.

Key words: *Bertholletia excelsa*, lipids, acidity, peroxide

1 Introduction

Tree nut oils are present in diets worldwide due to their clinical properties, including antioxidant, anti-inflammatory, and potential antitumor effects¹. Brazil nut (*Bertholletia excelsa*) oil is known for its high nutritional value and unsaturated fatty acids (UFA), which are mainly composed of oleic and linoleic acids². Moreover, Brazil nut oil is commonly used as a functional food ingredient, pharmaceutical product, and skincare^{3,4}. The oil extracted from Brazil nut seeds has already been tested against diseases such as cancer, atherosclerosis, inflammatory disorders, and in terms of their benefits for human and animal metabolism⁵⁻⁷. In addition to the lipid fraction, it is a source of phenolic compounds, carotenoids, selenium (Se), and vitamin E, which has antioxidant properties against free radical damage and premature aging⁸. Regarding the recommended amount of Se intake, the estimated average requirement

for adults is 45 mg per day, and the maximum tolerable intake cannot exceed 400 mg per day⁹.

The amount of Se in Brazil nuts may vary according to geographical regions, and not all seeds are sources of Se¹⁰. This can be resolved during the industrial processing of the seeds, as factories generally mix different batches of Brazil nuts in natura. Despite being nutritious, Brazil nuts have been associated with mycotoxin contamination, primarily aflatoxins (AFL), a fungal metabolite with mutagenic, teratogenic, and carcinogenic activity. Among AFL, aflatoxin B1 (AFB1) is the most toxic form for humans, according to the International Agency for Cancer Research¹¹. Codex alimentarius (2011)¹² established tolerance limits for aflatoxins in products for human consumption, albeit there are still no limits for AFL in Brazil nut oil. In the Amazon rainforest, Brazil nuts are harvested almost exclusively from nature, and their trade represents income for numerous in-

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digenous and riverine communities¹³). Nevertheless, seeds can be affected by high ambient temperatures ($>30^{\circ}\text{C}$), relative humidity (RH%; $>70\%$), unsatisfactory sanitary storage conditions, and water activity (aw) (>0.70)¹⁴. Limited technology in Brazil nut production and inadequate storage conditions are known to favor AFL formation and cause restrictions on commercialization¹⁵. In this context, some food facilities in the Amazon region have been producing Brazil nut oil as an alternative product, and extraction is done by hot or cold pressing and the crude oil at room temperature ($25\text{--}35^{\circ}\text{C}$). Factors such as exposing the nuts to air, light, heat, and temperature variations contribute to lower final product quality¹⁶. However, there is little data on the safety of Brazil nut oil and toxic agents such as AFL, and contamination of edible vegetable oils is a global concern, which has led to method validations of olive oil, peanut oil, and sesame oil have been developed¹⁷. As for oil obtained from other vegetables, the peanut (*Arachis hypogea*) is likely the most widely studied oilseed due to the presence of contaminating fungi and their mycotoxins, especially AFL¹⁸. In peanut oil samples randomly collected from households in western Guangdong (China) in 2016, AFB1 was quantified in 22.5% of samples with $15.4\text{--}49.9\text{ }\mu\text{g/kg}$ ¹⁹. The contaminated samples exceeded the European Union limit of AFL in peanut oil, with the maximum levels of AFB1 being $2\text{ }\mu\text{g/kg}$ ²⁰. In this context, this study aimed to evaluate AFL presence in Brazil nut oil from local outlets and producers and relate it to the presence of fatty acids in the oil as a surveillance tool for food safety.

2 Materials and Methods

2.1 Sampling

Brazil nut oil samples ($n=25$) were purchased in local markets in Manaus (Amazonas State, Brazil). They included: (a) unbranded products ($n=07$), which are produced by local farmers using artisanal methods with raw materials discarded from industrial companies, and (b) industrialized ($n=18$) samples produced in Brazil in their original packaging (plastic or glass), ranging from 20 to 250 ml. All samples were stored according to the information on the label at 25°C . Of the positive samples, numbers 12 and 14 had enough volume for NMR analysis after AFL quantification.

2.2 Aflatoxin assay

Samples were weighed, solubilized with a buffered solution and methanol/water, and subjected to mechanical shaking for 2 min. Extraction was performed using the Afla Star TM immunoaffinity column and performed according to the manual's instructions using a glass syringe and vacuum pump (6 mL/min.). The extract was stored in a freezer and protected from light until high-performance liquid chromatography (HPLC) analysis. Aflatoxins B1, B2, G1, and G2 were quantified for total aflatoxin (Total AFL) content using the method described by AOAC 994.08 (2016)²¹ via HPLC. Samples were submitted to derivatization to enhance natural fluorescence. Standards were prepared at known concentrations. A standard pool of AFL (B1, B2, G1, and G2) was used for chromatographic comparison with the analyzed samples. The LOQ method was defined by 6 times the signal-to-noise ratio. Five points were used to build an analytical curve, to obtain the R values for LOQ. Each point corresponded to a mean of five injections of each extract. The limits of quantification (LOQ) were

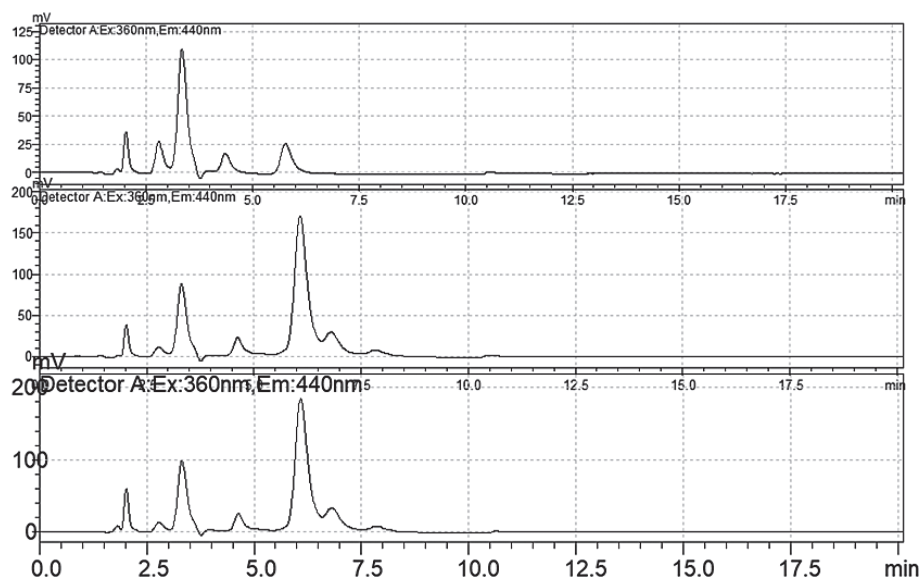


Fig. 1 AFL chromatogram.

2.32 µg/kg (AFB1 = 0.410, AFB2 = 0.410, AFG1 = 0.750, and AFG2 = 0.750).

2.3 Nuclear magnetic resonance assay

The obtained ¹H NMR spectra were processed using the software Topspin™ (version 4.0.8). Unsaturated, linolenic, linoleic, and oleic fatty acids and saturated fatty acids were estimated from the integration of the signals at δH 0.96 ppm (E), H δ2.75 ppm (A), δH 2.01 ppm (C), and δH 2.29 ppm (B), respectively, against the signal at δH 4.28 ppm (α) (Fig. 1), according to the method proposed by Barison (2010)²²⁾.

2.4 Statistical analysis

The AFL were evaluated by analysis of variance (ANOVA) and expressed as mean ± standard deviation.

3 Results and Discussion

3.1 Aflatoxins

The HPLC-FLD was efficient to AFL quantification in Brazil nut samples by other authors²³⁾ and it was to Brazil nut oil as well. Of the 25 samples analyzed, seven samples (28%) were contaminated with AFL (total), ranging from 8.8 to 50.87 µg/kg. The AFL positive samples were produced by local farmers using artisanal methods and classified as unbranded. In the industrialized samples, AFL were not detected. The chromatograms of positive and undetermined samples are shown in Fig. 1. According to Table 1, five of the positive samples (7%) were >2.0 µg/kg above EU limits and seven (100%) were >4.0 µg/kg for Total AFL. Brazilian legislation²⁴⁾ established the maximum tolerable limits (MTL) for AFL in Brazil nuts as: (a) shelled nuts for direct consumption (10 µg/kg) and (b) nuts for further processing (15 µg/kg). The European Union²⁰⁾ levels for peanuts and other oilseeds or their processed products (intended for human consumption or as an added ingredient in foods) were considered: the maximum levels set for AFB1 = 2 µg/kg. For Total AFL the maximum limit of 4.0 µg/kg was considered.

Based on these findings, it is crucial to consider the AFL

data for vegetable oils reported by Parker and Melnick²⁵⁾ and Brazil nut oil. The authors tested crude oils obtained by solvent extraction or hydraulic pressing of moldy peanuts, which contained only small fractions of the aflatoxins originally present in the peanuts. Conventional alkaline refining and washing of the oils reduced AFL content, and the bleaching operation eliminated the AFL from the oils. Idris *et al.*²⁶⁾ evaluated peanut oil and reported that unrefined oils were more susceptible to AFL contamination. Refining appears to eliminate AFL from oils obtained from moldy peanuts unfit for human consumption, and in this study, the oil from the branded samples was much more yellow or transparent than the unbranded samples.

Some unmarked samples contained small amounts of sediment in the bottle, suggesting the presence of the protein fraction. However, highly refined vegetable oils contain extremely low concentrations of residual protein from their source²⁷⁾. Based on these observations, it is plausible that industrialized samples have better processing, such as filtration, refining, or higher quality raw materials, even though they do not appear on the label. The unlabeled samples were obtained under artisanal conditions. Sometimes, local producers use “non-standard” Brazil nuts or those discarded from food facilities as raw materials to obtain the oil. In these cases, additional substances may be transferred to the oil (e.g., AFL), as previously reported in the “pie” (protein fraction resulting from the pressing of Brazil nuts). Sengul *et al.*²⁸⁾ studied whether AFL in highly contaminated hazelnuts transferred to hazelnut oil during production and confirmed small amounts in the oil, albeit almost all AFL content remained in the oil cake.

Due to the lack of research on AFL in Brazil nut oils, several studies can be cited in which AFL were found in vegetable oils. Karunaratna *et al.*²⁹⁾ reported the status of AFL contamination in types of edible vegetable oils obtained in Sri Lankan markets: coconut oil, palm olein, sunflower oil, sesame oil, olive oil, soybean oil, and corn oil. The authors reported that improper practices may have caused AFL contamination (2.25–72.70 µg/kg) in coconut oil during copra (dried coconut kernel) production, thus promoting toxigenic fungi growth. Schwartzbord and Brown (2015)³⁰⁾ evaluated peanut butter samples in Port Prince

Table 1 Aflatoxin level in Brazil nut oil.

AFL ¹	Positive samples Number							Mean	SD ²	Min	Max
	2	4	5	6	7	12	14				
B1	13.25	10.37	1.12	9.45	1.12	6.56	10.04	7.42	4.72	1.12	13.3
B2	30.4	28.7	0.45	2.40	0.46	25.27	30.37	16.86	14.86	0.45	30.4
G1	2.49	2.4	1.29	4.35	3.04	2.16	1.97	2.53	0.96	1.29	4.35
G2	2.73	2.27	1.03	1.84	0.97	2.22	2.52	1.94	0.70	0.97	2.73
Σ AFL	50.87	47.74	8.88	24.04	12.59	37.21	47.90				

¹ Aflatoxin LOQ = AFB1 = 0.410, AFB2 = 0.410, AFG1 = 0.750, AFG2 = 0.750 µg/kg; ² Standard deviation

and reported AFL in 91% of the samples. In addition, 100% of peanut butter samples contaminated with AFL (335 µg/kg) were found in Cape Haitian in 2013. In Nigeria, peanut oil samples were evaluated by HPLC, and total AFL levels ranged from 0.11 to 45 µg/kg, and of the total samples evaluated, 4.8% contained levels above Nigerian limits (20 µg/kg), while 44.4% of samples had AFL concentrations > 2 µg/kg for AFB1 and 38.1% for a Total AFL of 4 µg/kg³¹⁾. In our study, 28% of the samples were > 4.0 for Total AFL, which reinforces that further research is required to assess the impact of daily consumption of Brazil nut oil and AFL on some consumer groups, such as vegans.

3.2 NMR for Brazil nut oil

Brazil nut oil analysis by ¹H NMR showed similar chemical profiles with characteristic triglyceride signals (Fig. 2). In the chemical composition of fatty acids, oleic and linoleic fatty acids were predominant (Table 2). From the ¹H NMR spectra, it was possible to observe: δH 0.87 ppm (m) and δH 1.26 ppm (m) referring to the methyl and methylene hydrogens of the aliphatic chains; δH 0.96 ppm (t, 7.5 Hz) referring to the methyl hydrogens of the linolenic acid; δH 1.59 ppm (m) referring to the methylene hydrogens of the car-

bon-carbon ester; δH 2.01 ppm (m) assigned to the β-carbon hydrogens of the double bond between the carbons; 2.2H 2.29 ppm (m) referring to the β-carbon hydrogens of the ester; δH 2.75 ppm (t, *J* = 6.6 Hz) referring to methylene hydrogens between the olefin carbons; δH 4.13 ppm (dd, *J* = 11.9 and 6.0 Hz) and δH 4.28 ppm (dd, *J* = 11.9 and 4.3 Hz) referring to α-hydrogens of glycerol; δH 5.25 ppm (m) referring to β-hydrogen of glycerol; and δH 5.33 ppm (m) referring to olefinic hydrogens.

The fatty acid composition was similar for the AFL positive samples. Sample 6 (negative for AFL) showed similar fatty acid levels with the positive samples. Sample 8, also negative, differed from the others. It is worth noting that there were higher linolenic acid and linoleic acid contents in sample 8 (negative), in which an increase in unsaturated composition and a consequent decrease in saturated fatty acids was observed. Sample 6 did not show this behavior, so we cannot state whether the presence or absence of AFL alters the chemical composition. As for the chemical profile of the Brazil nut oils, sample 8 was the one that most closely resembled the Brazil nut oil analyzed by Cicero *et al.*³²⁾ compared to linolenic acid. The highest fatty acid content was found in samples 6, 12, and 14, rep-

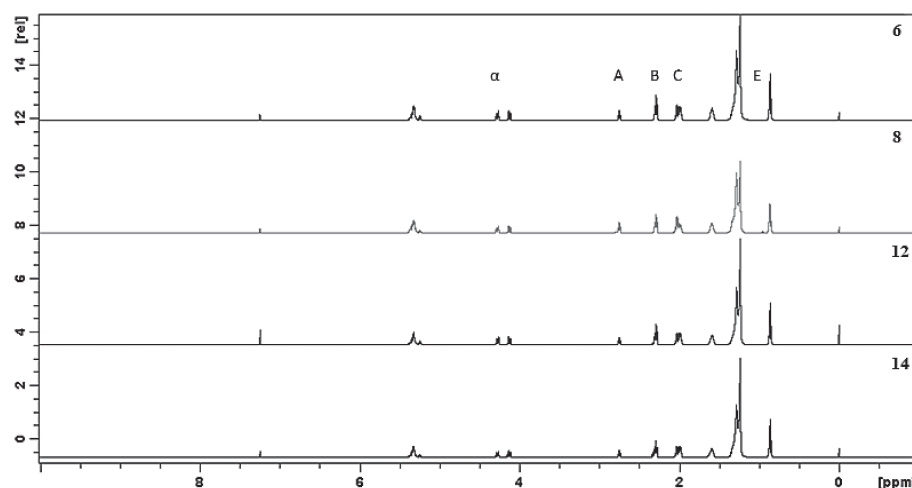


Fig. 2 ¹H NMR spectra (11.74 T, CDCl₃) of Brazil nut oils. Samples 6 and 8 were AFL negative and samples 12 and 14 were positive.

Table 2 Fatty acid composition in Brazil nut oil.

Sample Number	Fatty Acids (%)			
	Linolenic	Linoleic	Oleic	Saturated
<i>AFL negative</i>				
6	0.3	31.5	40.8	27.4
8	2.9	45.2	32.4	19.5
<i>AFL positive</i>				
12	0.1	30.9	41.0	28.1
14	0.2	29.6	42.9	27.3

resented by oleic acid followed by linoleic acid, as observed in the literature. Sample 8 presented the opposite profile, showing a higher number of unsaturated and linoleic acids followed by oleic acid.

4 Conclusion

The evaluation of AFL occurrence in Brazil nut oil was carried out, and positive samples were detected. As for NMR analysis, it was not possible to state whether the presence of a certain acid interferes with aflatoxin contamination, albeit it is plausible that there is more protection of oils with a higher fatty acid content, thus highlighting that more comprehensive studies are necessary. In our study, the samples were acquired from the retail market, and there was no information about the raw material available; therefore, we suggest future studies to compare the Brazil nuts suitable for consumption and those destined for disposal by food facilities (rotten or substandard) submitted to oil extraction. Another critical aspect to be studied in the future concerns the origin of the seeds (geographical area) and their nutritional content, which may explain the hypothesis of the transfer mechanisms of AFL from the protein fraction to the lipid one. In addition, further research is needed to assess the occurrence of the allergic fraction of the protein in Brazil nuts as a danger in Brazil nut oil retail. Due to the increasing development of oilseed products and their direct impact on consumer health, it is pivotal to monitor these products to contribute to population health risk data.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

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