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External and internal characteristics of *in-shell* brazil nuts and their relation to aflatoxin contamination analyzed by LC-MS/MS

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Abstract

Brazil nut (*Bertholletia excelsa* H B K), belongs to *Lecythidaceae* family and is naturally grown in the Amazon Region. It is an important product for the Brazilian Northern States economy and for some of them, the main export product. Aflatoxin (AFL) contamination has been of concern for Brazil nut exporters, since 1998, when the European Community (EC) reduced the maximum residue level of total AFLs (AFB₁ + AFB₂ + AFG₁ + AFG₂) to 4 µmg kg⁻¹ (E.C, 1998; Newing and Harrop, 2000) and rejected contaminated batches from Brazil.

Some studies have been carried out on Brazil nut AFL contamination also on *visual* external characteristics and related them to the quality of edible Brazil nuts, however, none has actually *measured* those different external characteristics (dimensions, weight and chromacity). Therefore a study was carried out to measure the external and internal characteristics of *in-shell* Brazil nuts. They were evaluated according to their following external shell characteristics: (a) dimensions [length, faces width] (b) weight and (c) chromaticity. They were also analyzed for (d) moisture content (mc) and (e) AFL contamination by LC-MS/MS. According to their sizes Brazil nuts were classified in three groups I, II and III, corresponding to Big, Medium and Small respectively. From the external characteristics of *in-shell* Brazil nuts it was possible to establish some parameters as length: 53.49, 43.89 and

36.40, weight: 12.14, 8.64 and 6.08 g, and out-shell chromaticity components: L* 26.94, a* 5.38 and b* 15.08, for the three Groups, respectively. Moreover, the nuts classified as Small Group (III) presented AFB₁ levels of 5.616 µg kg⁻¹, while AFLs was not detected in other Groups (I and II). Therefore the external characteristics of Brazil nut can indicate nut deterioration, although indirectly and it can be used as an important tool for Brazil nut sorting.

Key words: Brazil nuts, aflatoxins, dimensions, chromaticity.

Introduction

The Brazil nut tree is a typical species of the Amazon Region distributed especially at the Brazilian, Bolivian and Peruvian Amazon (Embrapa, 2003). It has an average height of 50 meters. The fruit is a wooden sphere that holds ca. 12-25 seeds (Brazil nuts) inside (Bonelli et al., 2001). Brazil nut is one of the most important species of the Amazon Region with thousand tons exported each year and the Brazilian production represents 80-90 % of the world production (Cardarelli and Oliveira, 2000; Mehlenbacher and Janick, 2003).

The risk of AFLs contamination can occur, either in the forest or during the storage of Brazil nut. Their contamination by fungi, especially the species *Aspergillus flavus* that produces

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aflatoxins (AFLs) is directly related to the (a) climatic conditions (high humidity and heat) in the Amazon forest during the period of harvesting (wet season: December to May). The other factors that contribute to the proliferation of fungi in the forest are: (b) time that hulls stay on the wet ground waiting for collection, the (c) primary nut storage conditions in the forest and (d) precarious conditions of river transport (boats) leading to a long time to reach the processing site (Pacheco and Scussel, 2006). These factors don't take action alone but together and some of them has more influence than others.

Despite of that, some characteristics of Brazil nuts, which can be noted *visually* by consumers, such as visible mould, slimy and soft consistency, low weight discoloration, irregular forms and if the nut is rattled or rattle inside in the shell can help on selecting the none deteriorated/contaminated nuts (Marklinder et al., 2005). Other characteristics that may be selected *visually* by consumers are their shell color variation, light weight and small sizes. These could be also an indirect way of sorting nuts with possible AFL contamination. Although some studies have been carried out on AFL contamination and also on *visual* external characteristics, none have measured and related them directly to safety (AFLs). Therefore, this study was carried out to (a) establish the external parameters of *in-shell* Brazil nuts and their relation to AFL contamination and (b) compare TLC and LC-MS/MS performance for Brazil nut matrix. (c) This is preliminary work for the development of a Brazil nuts sorting machine.

Materials and methods

Material

(a) **Samples:** *in-shell* Brazil nut (1,647 nuts = ca. 15 kg) from the year 2005 harvest (November and December) collected in the state of Amazonas.

(b) **Equipments:** digital caliper (6 inches), Lee; semi-analytical Kern 440-53 and analytical

Mettler Toledo/AB204-S scales; nut cracker; liquid chromatographer model 1100, Agilent; mass/mass detector API 4000, Applied Biosystems MDS SCIEX and sphere spectrophotometer with DRS (dynamic rotational sampling), model SP60, X-rite.

(c) **Aflatoxin standards:** standards AFB₁, AFB₂, AFG₁ and AFG₂. All from Sigma.

Methods

(a) **External characteristics:** the *in-shell* nuts were measured for length, width (three *faces*), weight and shell chromaticity as follows:

(a.1) **Length:** nuts were separated into 3 Groups (Group I, II and III for Big, Medium and Small, respectively) by measuring their length as follows: length of each nut was measured with a caliper from top to bottom extremities and reported in millimeters (mm). See Figure 1.a. The length range was established for each Group. Group I: higher than 50 mm; Group II: from 40 to 50 mm and Group III: lower than 40 mm.

(a.2) **Face width:** the three *faces* (B, C and D) of each nut had their width measured at the half of each *face* length and reported in millimeters (Figs 1.a and 1.b).

(a.3) **Weight:** the nuts previously divided into three Groups, were weighted individually and data registered in grams (g).

(a.4) **Shell chromaticity analysis:** the chromaticity values (achromatic component L* - relative darkness or lightness; chromatic component a* - green to red; chromatic component b* - blue to yellow) were obtained by photocolometric readings (opening diameter 8mm) on the three *faces* of each nut: B, C and D. That was carried out measuring two points of each *face* (40 nuts per Group). *Faces* were divided in two halves and color measured in the center of each half (point one and point two) and the average calculated. See Figure 2.

(b) **Internal characteristics:** apart from the external measurements, nuts were analyzed for moisture content (mc) and aflatoxins (AFLs)

(b.1) **Mc:** by gravimetry (art. 925.40, AOAC, 1995).

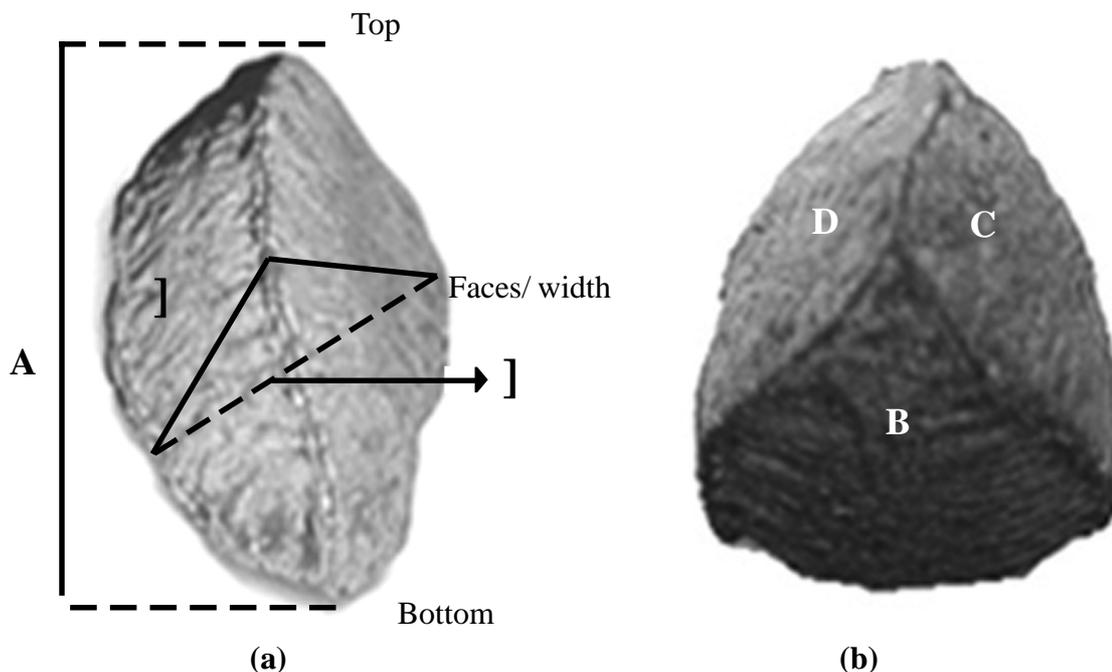


Figure 1. (a) dimensions of *in-shell* Brazil nuts: length (A) *faces* (B, C and D) from [lateral view]; (b) identification of three Brazil nut *faces* (B, C and D) from [top view]

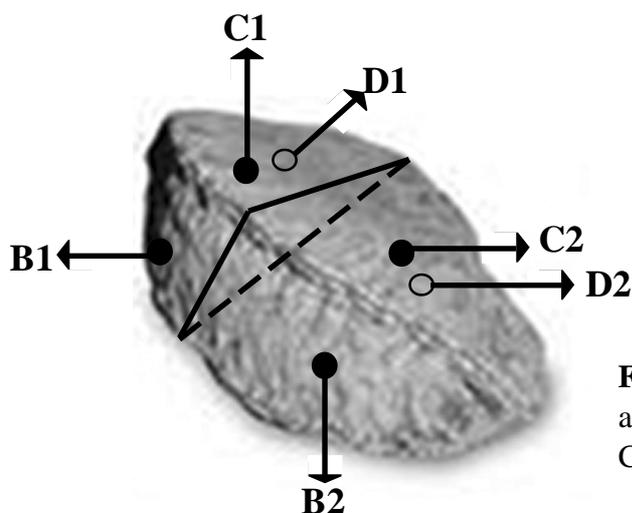


Figure 2. Points taken for the shell of chromaticity analysis: *face* B (points: B1 and B2); *face* C (points: C1 and C2) and *face* D (points: D1 and D2).

(b.2) **AFLs:** a pool of (a) *shelled* [triplicate] and (b) *in-shell* nuts of each Group was submitted to AFL analysis by (a) TLC with UV detection at 365nm (AOAC, 2000) and (b) LC-MS/MS (Spanjer et al, 2003; Xavier and Scussel, 2006). The LOD / LOQ for (a) TLC were $1 \mu\text{g kg}^{-1}$ / $2 \mu\text{g kg}^{-1}$ for the total AFLs ($\text{AFB}_1 + \text{AFB}_2 + \text{AFG}_1 + \text{AFG}_2$) and for (b) LC-MS/MS they were: 0.04, 0.045, 0.05 and $0.06 \mu\text{g kg}^{-1}$ / 0.08, 0.09, 0.1 and $0.12 \mu\text{g kg}^{-1}$ for each AFL, respectively.

Results and discussion

External characteristics

As far as *in-shell* Brazil nut external characteristics are concerned, the dimensions measured allowed to classify them into three Groups: I, II and III. Their respective average dimensions [length (*faces* width B, C and D)] were as follows: Group I: 53.5 mm (*faces*: 21.1,

29.1 and 29.2 mm); Group II: 43.9 mm (*faces*: 19.7, 26.4 and 26.2 mm); Group III: 36.4 mm (*face*: 18.6, 23.4 and 23.4 mm). See Table 1 and Figure 3 for more details. Considering their *face width* dimensions, it was observed that *face B* had always lower average values (19.81 mm) when compared

to C and D (26.32 and 26.25 mm, respectively). Important to emphasize that 74.1 mm was the maximum Brazil nut length obtained in this study, and the shortest (minimum length) was 14.02 mm. The RSD obtained for length were 6.45; 6.04 and 7.94 %, respectively.

Table 1. Dimension measurements and weight of *in-shell* Brazil nuts.

Brazil nut Group	Dimensions (mm)				Weight (g)
	Length A	B	C	D	
I					
Average	53.49	21.10	29.14	29.16	12.14
Maximum	74.10	26.40	34.86	35.41	18.00
Minimum	50.02	14.37	24.47	24.39	6.00
SD ¹	3.45	2.47	2.25	2.17	2.03
RSD ²	6.45	11.70	7.72	7.44	16.72
II					
Average	43.89	19.74	26.44	26.20	8.64
Maximum	49.97	33.77	39.88	37.58	17.00
Minimum	40.00	12.07	16.02	13.93	2.00
SD	2.65	2.55	2.88	2.85	2.04
RSD	6.04	12.92	10.89	10.87	23.61
III					
Average	36.40	18.61	23.38	23.41	6.08
Maximum	39.99	31.67	31.18	31.29	10.00
Minimum	14.02	11.72	15.01	13.22	1.00
SD	2.89	2.49	2.81	2.64	1.65
RSD	7.94	13.38	12.02	11.28	27.14

¹ Standard Deviation.

² Relative Standard Deviation (%).

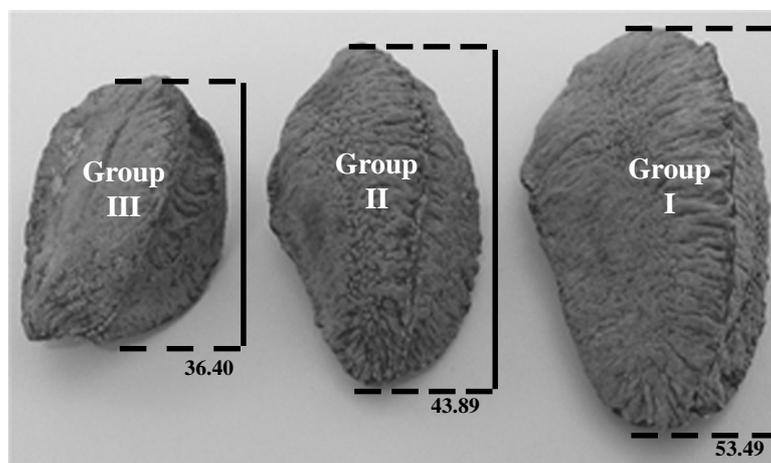


Figure 3. Average length (mm) of the three Groups (I, II and III) of *in-shell* Brazil nut.

As far as *weight* of the respective Groups is concerned, it was found some important data that could be related to fungi and AFLs. The maximum/minimum weight obtained for the Groups I, II and III were 18/6 g; 17/2 g and 10/1 g respectively. Although most of the largest nuts (Group I) had also the heaviest weight (18 g), some of them presented lighter weight (6 g) in the same Group. It was observed that a high percentage of light nuts were in the Group III as well as, the lightest ones. That was due to the fungi deterioration. Indeed, those nuts presented AFL contamination at levels of 5,616 mg kg⁻¹.

The shell chromaticity measurements

obtained in this study, for the achromatic component L*, the lightness (maximum) and darkness (minimum) values were 49.45 and 4.44, respectively (Table 3). The nuts of Group III were the ones that presented higher L* values corresponding to shell lighter chromaticity. In fact those nuts were contaminated by AFL as mentioned above. That data corroborates with the work reported by Marklinder et al. (2005), where the consumers *visually* selected, among different nut external characteristics, the light color and related them as not edible/safe. AFL wise, on the other hand, nuts classified in Group I had the lowest L* values and no AFLs detected.

Table 2. Chromaticity values of *in-shell* Brazil nuts.

Brazil nut group	Chromaticity value		
	L ¹	a ²	b ³
I			
Average	35.15	5.16	15.38
Maximum	41.78	6.90	21.33
Minimum	4.44	3.29	7.55
SD ⁴	4.37	0.76	2.68
RSD ⁵	12.43	14.72	17.42
II			
Average	36.22	5.42	16.37
Maximum	41.58	7.20	21.18
Minimum	29.36	3.66	11.09
SD	2.53	0.69	2.19
RSD	6.98	12.73	13.37
III			
Average	39.99	5.21	17.86
Maximum	49.45	7.69	22.95
Minimum	25.46	3.08	7.21
SD	4.65	0.83	2.91
RSD	11.63	15.93	16.29

¹ Achromatic component (relative darkness or lightness); ² Chromatic component (green to red); ³ Chromatic component (blue to yellow); ⁴ Standard Deviation; ⁵ Relative Standard Deviation (%).

Table 3. Levels of aflatoxins B₁, B₂, G₁ and G₂ of *in-shell* and *shelled* Brazil nuts

Brazil nut group	Method (mg.kg ⁻¹)	
	LC- MS/MS ^{1/2}	TLC ^{3/4}
I	ND ⁵	ND
II	ND	ND
III	5,616 (AFB ₁)	ND

¹ liquid chromatography mass/mass; ² *in-shell* nuts LOD/LOQ: 0.04, 0.045, 0.05, 0.06 µg kg⁻¹ / 0.08, 0.09, 0.1 µg kg⁻¹, 0.12 µg kg⁻¹, for the 4 AFLs, respectively; ³ thin layer chromatography; ⁴ *shelled nuts* LOD/LOQ: 1µg kg⁻¹ / 2 µg kg⁻¹ for the total AFLs (AFB₁, AFB₂, AFG₁ and AFG₂); ⁵ Not detected.

The average of chromatic components *a* (green to red) and *b* (blue to yellow) of the Brazil nut shell obtained were: 5.38 (min 3.08 and max 7.69) for *a* and 15.08 (min. 7.21 and max. 22.95) for *b*. Considering the nut brown color and the chromacity values *a** and *b** the data showed a more often average chromaticity mix-values on the Color System Chart of yellow + red = brown. In conclusion, the more often values considered average of brightness and brown color for the Brazil nuts studied were 4.44/49.45 ; 3.08/7.69 and 7.21/22.95 The RSD% were 12, 14 and 17 % For the chromacity components L*, *a** and *b**, respectively.

Internal characteristics

The average mc obtained from the nuts was 3.67 % g (3.58 min and 3.75 max), which is considered safe as far as fungi growth are concerned. That mc corresponds to Brazil nut dehydrated ready to be consumed.

Aflatoxin contamination: as expected, the results obtained from the two different methods, showed differences in the levels of AFL detected. When *shelled* nuts were analyzed by TLC the AFLs (AFB₁, AFB₂, AFG₁ and AFG₂) were not detected in any of the samples (up to the method LOQ: 2 µg kg⁻¹). However, when the *in-shell* Brazil nuts were analyzed by LC-MS/MS, aflatoxin AFB₁ was detected in the nuts of Group III at level of 5,616 µg kg⁻¹. In fact, the LOQ of LC-MS/MS for AFB₁ is much lower than the TLC ones

It was observed that the nuts of Group III which were contaminated with AFB₁, showed a slightly high mc. They also had the lowest weight (empty = hollow nuts) among the average of normal ones when compared to Groups I and II. Besides, those nuts presented more shell lighter chromaticity observed either, *visually* and especially through the chromaticity measurements.

From the external characteristics of *in-shell* Brazil nuts obtained it was possible to establish some parameters sizes for the three Groups as *length*: of 53.49, 43.89 and 36.40, *weight*: 12.14,

8.64 and 6.08 g, for Big, Medium and Small nuts (Group I, II and III) respectively. Also for *face* width B: 19.81, C: 26.32 and D: 26.25 mm. The out-shell chromaticity components: L* 26.94, *a** 5.38 and *b** 15.08. Moreover, the nuts classified as Small Group (III) presented AFL contamination (5,616 mg kg⁻¹ AFB₁), while aflatoxin was not detected in other Groups (I and II). About methodology: LC- MS/MS method for Brazil nut matrix: it showed being higher sensitive for *in-shell* nut matrix than when using *shelled* ones

Conclusions

External characteristics of Brazil nut such as size, weight and color may indicate *visually* nut deterioration, although indirectly. However, when using exact measurements of those characteristics, one can establish standard parameters for continuously selecting non-deteriorated and non-contaminated nuts. It was possible to establish a standard length for the three Groups of nuts which will be used as one of the parameters for the development of the Brazil nuts sorting machine as well as the chromaticity. More data are being gathered; especially of nuts measurements from different Amazons regions and harvesting periods in order to establish sorting standards.

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