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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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DISCUSSION PAPER ON AFLATOXIN CONTAMINATION IN BRAZIL NUTS

Prepared by Brazil

BACKGROUND

1. The Codex Committee on Contaminants in Food (CCCF), at its First Session held in April 2007, agreed that the Discussion Paper on Aflatoxin Contamination in Brazil Nuts would be updated by the Delegation of Brazil, incorporating additional data that would become available on the contribution of the shell to aflatoxin contamination of Brazil nuts, for consideration at Second Session of the Committee (ALINORM 07/30/41 paragraph 66).

2. This Discussion Paper was also updated taking into account the 68th JECFA evaluation on the impact of different hypothetical limits of aflatoxins (AFs) in tree nuts, including Brazil nuts, on the dietary intake.

INTRODUCTION

3. AF contamination can be a potential problem in tree nuts and other products such as maize, groundnuts, oilseeds, cocoa products, dried fruits and spices. This Discussion Paper is applicable only to Brazil nuts which is the only crop among the main traded tree nuts that uses the extrativism activity for production.

4. Brazil nuts are seeds of *Bertholletia excelsa* Humb. & Bompl., trees that grow wild on the Amazon rainforest. They can reach up to 60 meters, take 12 years to bear fruits and live up to 500 years. The trees occur in groves of 50-100 individuals and the groves are separated by up to 1 km; pollination occurs by wild large-bodied bees, especially *Euglossinae* bees (Wadt *et al*., 2005).

5. The Amazon rainforest has multiple ecosystems with a huge biodiversity. It has an important role in the global weather balance and is the shelter of many native ethnicities. The equatorial climate is hot and humid, with an average temperature of 26ºC and relative humidity 80-95%.

6. The Brazil nuts extractivism represents an important activity for the native people in the growing countries, stimulating a sustainable use of renewable natural resources while conciliating social development with preservation. It does not involve the destruction of forest nor threat the ecological balance and the environment.

7. The number of collectors and processors living from the Brazil nut industry is about 1.2 million in Brazil, 600,000 in Bolivia and 200,000 in Peru. In 2006, the total world production reached 20100 metric tons, being about 64 % from Bolivia, 24 % from Brazil and 12 % from Peru (INC, 2007).

8. A Code of Practice for the Prevention and Reduction of AFs Contamination in Tree Nuts was adopted by the CAC at its 28th Session; an Appendix specific for Brazil nuts was adopted by the CAC at its 29th Session. INC information indicated that industries and producers have been making considerable efforts in the last 15 years to minimize mould growth and aflatoxin production in tree nuts. Particularly, in the case of Brazil nuts, the climatic conditions on the Amazonian environment and the characteristics of the extrativism cannot be controlled, exerting direct or indirect effects on the toxigenic fungi and aflatoxin production

9. Ready-to-eat Brazil nuts are nuts which are not intended to undergo an additional processing/treatment that has proven to reduce aflatoxin levels; they also refer to shelled nuts or kernels. Brazil nuts for further processing are intended to undergo an additional processing/treatment to reduce aflatoxin levels, including shelling, sorting by size, specific gravity, colour or damage; they can be referred to in-shell or shelled nuts.

10. This Discussion Paper considers many aspects related to the AFs in Brazil nuts, including occurrence and dietary intake estimation.

CHEMICAL STRUCTURE

11. AFs are a group of structurally related compounds produced under favourable conditions by ~~some strains~~several species of *Aspergillus* *including A. flavus*, *A. parasiticus,* and more rarely by *A. nomius*, *A. toxicarius, A. parvisclerotignenus* and *A. pseudotamarii* (Frisvad *et al*., 2006, Ito~~TO~~ *et al*., 2001). The most known naturally occurring AFs are named B_1, B_2, G_1, and G_2. AFB_1 occurs in the highest amounts in contaminated commodities; AFB_2, AFG_1, and AFG_2 are generally not reported in the absence of AFB_1. Total aflatoxins (AFT) refer to the sum of the four related compounds.

12. AFs are highly oxygenated heterocyclic compounds and have closely related structures. AFs contain a coumarin nucleus fused to a bifuran: aflatoxins B (AFB) contain a pentanone structure attached to the coumarin nucleus and aflatoxins G (AFG) contain a six-membered lactone (Salunkhe *et al*., 1987).

TOXICOLOGICAL EVALUATION

13. At its 49th Meeting, the JECFA (1998) considered the carcinogenic potency of AFs and the potential risks associated with their intake. No tolerable daily intake was proposed since these compounds are genotoxic carcinogens. The potency estimates for human liver cancer resulting from exposure to AFB_1 were derived from epidemiological and toxicological studies.

14. The JECFA reviewed a wide range of studies conducted with both animals and humans that provided qualitative and quantitative information on the hepatocarcinogenicity of AFs. The carcinogenic potency of AFB_1 is substantially higher in carriers of hepatitis B virus (about 0.3 cancers/year/100,000 persons/ng of AFB_1/kg bw/day), as determined by the presence in serum of the hepatitis B virus surface antigen ($HBsAg^+$ individuals), than in $HBsAg^-$ individuals (about 0.01 cancers/year/100,000 persons/ng of AFB_1/kg bw/day). The JECFA also observed that vaccination against hepatitis B virus would reduce the number of carriers of the virus, and thus reduce the potency of the AFs in vaccinated populations, leading to a reduction in the risk for liver cancer (JECFA, 1998).

SAMPLING

15. Although the incidence of AF contamination in tree nuts is low, the AF levels are quite variable and high levels can be found in a small percentage of nuts (Schade *et al*., 1975; Schatzki, 1995; Schatzki, 1996). Consequently, an appropriate sampling plan is critical.

16. Most of the sampling procedures in place for AF in tree nuts (CAC, 2001; EU, 1993; FAO, 1993) have been derived from sampling plans developed for AFs in groundnuts, the most evaluated commodity.

17. The 36th CCFAC agreed that a working group led by the United States, with the assistance of Argentina, Brazil, Iran, European Community and the INC, would prepare sampling plans for AFs in almonds, Brazil nuts, hazelnuts and pistachios.

18. The Proposed Draft Sampling Plan was presented at the 37th CCFAC and retained at Step 4 by the Committee at its 38th Meeting, waiting for the outcome of the Discussion Paper on Maximum Levels of AFs in tree nuts. The Committee also agreed that the sampling plan for tree nuts should include Brazil nuts, unless the data indicated the need for a specific plan for this product.

19. The CCCF, at its First Session, agreed to return the Proposed Draft to Step 2 for redrafting by an electronic Working Group led by USA to be considered at the next Session at Step 4.

ANALYTICAL METHODS

20. There are a number of analytical methods available for the determination of aflatoxins in nuts, although most of them are not fully validated for Brazil nuts specifically. In general, the methods include a solvent extraction step and sample clean-up using either liquid-liquid partition or solid phase extraction sorbents such as silica, florisil, C18, aluminium oxide and immunosorbents. Methods for identification and quantification normally used are thin layer chromatography (TLC or HPTLC) or high performance liquid chromatography (HPLC), with UV or fluorescence detection. More recently, HPLC with mass spectrometry detection has been extensively used, with the advantage that there is no need for the clean-up and confirmation steps. LOQ for each aflatoxin depend on the clean-up and detection method and normally are within the 0.1 to 1 µg/kg range (Gilbert and Vargas, 2003, Marklinder *et al*., 2005, Solobov, 2007).

21. Antibody-based test kits for AF analysis are mostly used for screening purposes. The AOAC International website (AOAC, 2005) lists different kit formats for AFB_1 and AFT, with antibodies coated onto cups, ELISA plates, columns, cards and tubes. However, few kits have been validated by a full interlaboratory collaborative study (Gilbert and Vargas, 2003).

22. The CCFAC has established a performance criteria for methods of analysis for AFT in food (CX/CF 07/1/6). General method performance characteristics for aflatoxins have been also established by the European Committee for Standardization (EC 401, 2006).

23. The 36th CCFAC noted that there was no need for further development of methods of analysis for AFs in tree nuts. Upon request, the Codex Committee on Methods of Analysis and Sampling (CCMAS) could consider additional methods in the future.

FACTORS AFFECTING THE PRESENCE OF AFS IN BRAZIL NUTS

24. Several environmental factors influence fungal growth and AF production, but temperature and relative humidity are considered the most critical. Other factors include water activity, moisture, substrate composition, storage condition and insect damage (Arrus *et al*., 2005).

25. Baymam *et al.* (2002) have shown that most of the *Aspergillus* species infection in Brazil nuts is internal. At present, there is no evidence that infection with aflatoxigenic fungi occurs before the pods fell from the tree.

26. Fungus development and toxin production in Brazil nuts occurs mostly on the soil, during harvesting, transport and storage, and are favoured by the climatic conditions of the Amazon rainforest, mainly during the rainy season (Cartaxo *et al*., 2003; Wadt *et al*., 2005; Pacheco & Scusel, 2007).

27. Aspergillus *flavus* and *A. niger* were isolated in Brazil nuts collected up to 60 days after the pods fall in the forest (Cartaxo *et al*., 2003) and during processing (Souza *et al*., 2003). In addition to these species, *Aspergillus parasiticus* was also isolated in defective Brazil nuts (Brazil, 2003; unpublished data).

28. The effects of relative humidity (r.h.) and temperature on AF production were evaluated in Brazil nuts in-shell and shelled (whole and half kernels) inoculated with aflatoxigenic *Aspergillus* spp (Arrus *et al*., 2005). Maximum production of AFs was found in nuts stored at 25-30 ºC and 97% r.h.. Half kernels showed the highest level of AFB_1 (4483 µg/kg) and AFT (6817 µg/kg) while in-shell nuts contained the lowest AFB_1 and AFT levels (49 and 93 µg/kg, respectively). AFs were not produced at either 10 ºC (97% r.h.) or at 30 ºC (75% r.h.). This suggests that fungal growth after harvest can be prevented by an adequate control of temperature and water activity, which is an important strategy to prevent AF production in Brazil nuts.

29. According to Arrus *et al*. (2005), the limiting moisture content and water activity (a_w) required to control AF production (<4 µg/kg) at 30 ºC for up to 60 days of storage were: 4.57 % (a_w 0.70) for in-shell nuts and 4.50 % (a_w 0.68) and 5.05 % (a_w 0.75) for shelled nuts (whole and half kernels, respectively). Above these values, AF production may increase significantly. ~~Fungal growth is always predicted by a_w and not by moisture content.~~ The availability of water needed to allow fungal growth is best expressed as water activity (a_w), which is a measure of unbound water in the crop/food. From the data of Arrus et al. (2005) the adsorption isotherms of Brazil nuts (in-shell, shelled) were obtained and shown in Figure 1.

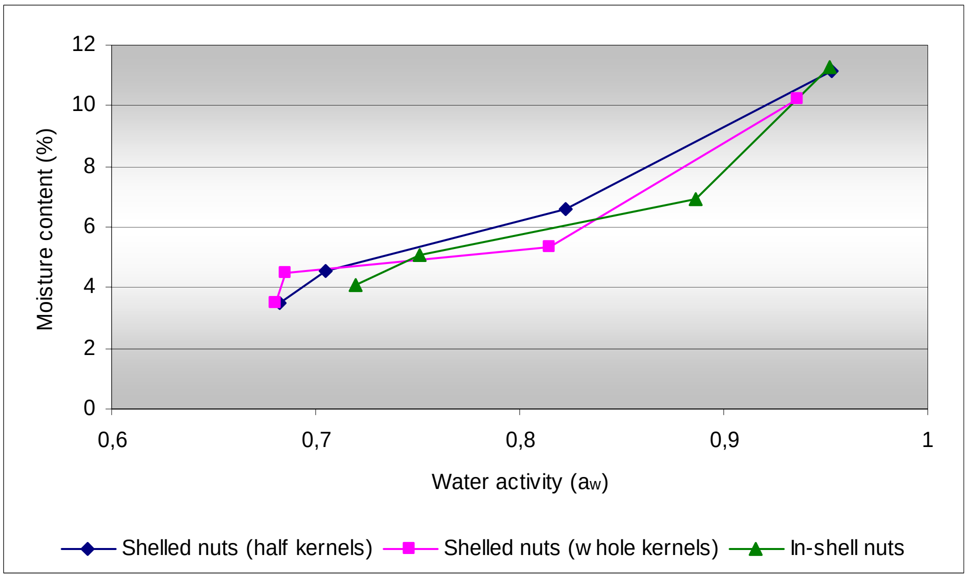

Fig. 1. Adsorption isotherms of in-shell and shelled (whole and half kernels) Brazil nuts at 30 ºC after 60 days of storage.

OCCURRENCE OF AFS IN BRAZIL NUTS

30. Several countries have been studying the occurrence of AFs in Brazil nuts. From 176 samples analyzed in the United States, 11 % were contaminated with AFs at levels ranging from traces up to 20 µg/kg, and 6 % had levels above 20 µg/kg. The maximum level detected was 619 µg/kg (Pohland, 1993).

31. In Japan, 70 of the 74 Brazil nuts samples analysed were not contaminated and only 2 samples contained AFs above 10 µg/kg. The maximum detected level was 123 µg/kg (JECFA, 1998).

32. From 51 Brazil nuts samples analysed in the Republic of Cyprus, 10 samples were contaminated with AFs at levels ranging from 8.3 to 20 µg/kg for B_1, ND to 1.1 µg/kg for B_2 and 2.3 to 9.4 µg/kg for G_1 (Ioannou-Kakouri *et al*., 1999).

33. A survey was conducted by the UK Food Standard Agency between November 2003 and March 2004 in a variety of nuts and nut products. Four of the 21 samples of Brazil nuts analysed contained levels above the EC and UK regulatory limits of 4 µg/kg for AFT (Food Standards Agency, 2004).

34. A survey conducted in Brazil from 1998 to 2004, analysed 500 (302 shelled and 198 in-shell) Brazil nut samples. No AFs were detected (<0.6 µg/kg) in 71.8 % of the shelled and 41.4 % of the in-shell nuts analysed. AFB1 levels were <2 µg/kg in 69.4 % and <10 µg/kg in 80 % of the samples (shelled + in-shell). About 70 % and 80 % of all samples had levels <4 µg/kg and <20 µg/kg, respectively. The median concentrations of AFT were 1.85 and 0.8 µg/kg in in-shell and shelled Brazil nuts, respectively (CAC, 2005a).

35. Data presented by the Brazilian Ministry of Agriculture refers to the occurrence of aflatoxins in Brazil nuts samples collected from lots to be exported and lots rejected by importing countries during the years of 2005 and 2006. In all cases only the edible portion (kernels) were analyzed. About 85 % of the 294 samples (lots) analysed had no detectable levels of AFB1 (< 0.6 or 1µg/kg). AFT levels in positive samples (lower bound) ranged from 0.4 to 242 µg/kg and only 13 samples (4.4 %) had levels > 20 µg/kg (Brazil, 2006; unpublished).

36. In another study conducted in Brazil, 9 out of 109 in-shell Brazil nuts samples analysed for AFs were contaminated at levels ranging from 183.4 to 924.2 µg/kg. No AFs were detected in 30 shelled samples analysed (Pacheco *et al*., 2006).

37. A study to evaluate the consumers' ability to discriminate AF contaminated in-shell Brazil nuts was carried out in Sweden (Marklinder *et al*., 2005). The median and 95th percentile level of AFs in the edible portion of 132 samples collected before panel sorting were 1.4 and 557 µg/kg, respectively. After sorting these levels were 0.4 and 56 µg/kg, respectively. The study concluded that Brazil nuts may be one of the few nuts that consumers may visually separate the edible from the inedible contaminated nut during the shelling process before eating and thus protect themselves from exposure to high levels of AFs.

38. The EFSA Scientific Panel evaluated the data on occurrence of AF in tree nuts and other products from 2000 to 2006 submitted by 22 EU Member States. The samples were related to import, market or company control and it was not specified which samples were ready-to-eat or for further processing. From 622 Brazil nuts samples analysed, 56.4~~7~~% had AFT levels ~~were~~ below the LOD (0.1 – 0.2 µg/kg), ~~78.5~~22% between LOD and ~~had total levels up to~~ 4 µg/kg, 2.4 % between 4 and 10 µg/kg ~~-~~and 19.1 % ~~had levels~~ above 10 µg/kg (EFSA, 2007).

39. At its dietary intake evaluation, the 68th JECFA used data on AF contamination from producing countries. The mean concentration of AFT in Brazil nuts (shelled) was 20 µg/kg (WHO, 2007).

40. In general, the shell represents about 50 to 60 % of the Brazil nut total weight. Marklinder *et al* (2005) have shown that in most cases, AFT levels were lower in shells than in kernels from the same sample. Pacheco and Scusel (2007) found a in-shell/shelled AFT mean ratio (n= 80) of 1.1 when analysing samples collected directly from the silos, without any type of sorting. Arrus *et al*. (2005) inoculated Brazil nuts with *A. flavus* and *A. parasiticus* and the nuts were incubated for 30 or 60 days at 25-30 °C and 97 % r.h. In this *in vitro* situation, in-shell nuts contained much lower AFB1 and AFT levels than the shelled nuts.

DIETARY INTAKE

41. Cereals (mainly corn), groundnuts, oilseeds, tree nuts, dried figs, spices and copra are the main products contaminated with AFs. The most important dietary sources of AFs are corn, groundnuts and their products, which form an essential part of the diet in some countries (CAC, 2005b).

42. At its 49th Meeting, the JECFA evaluated the potential impact of two hypothetical standards for AF contamination on peanuts (10 or 20 µg/kg) on sample populations and their overall risk. It was concluded that reducing the maximum permitted level (ML) of AFT in peanuts from 20 µg/kg to 10 µg/kg would not result in any observable difference in rates of liver cancer (JECFA, 1998).

43. The dietary intake of AFs by the Swedish population was estimated to be 0.6 and 0.7 ng/kg bw for the average and the high consumers (95th percentile), respectively (Thuvander, 2001). The estimated Brazil nuts consumption was 0.3 g/day for both the average and the 95th percentile consumers. The body weights of the two populations were not reported. In another study conducted in Sweden, ~~for~~ assuming~~a~~ a 70 kg b.w consumer and ~~a yearly~~ consumption of Brazil nuts of 0.3 kg during Christmas time, the median AFs intake was ~~estimated as~~ 0.73 ng/kg bw and the 95th percentile 1~~.1~~ 10 ng/kg ~~bw~~ bw. Smoothing the consumption over a full year, the figures would be 0.002 and 0.3 ng/kg bw (Marklinder *et al*., 2005).

44. At its 68th Meeting, the JECFA evaluated the impact to human health from a dietary exposure to AFs from the consumption of the tree nuts edible parts (ready-to-eat) and dried figs (WHO, 2007). Using the 13 GEMS/Food Consumption Cluster diets (WHO, 2006) and assuming a body weight of 60 kg, the Committee evaluated the impact on dietary exposure to AFs of setting hypothetical MLs of 4, 8, 10, 15 or 20 µg/kg for AFT in almonds, Brazil nuts, hazelnuts, pistachios and dried figs.

45. The JECFA decided to base the assessment on data provided by producing countries, noting that these better represent the materials in commerce and result in a robust estimate of dietary AFT exposure from tree nuts. The Committee noted that the majority of data included in the estimation of dietary AFT exposure from foods other than tree nuts and dried figs came from the EU and that these data do not reflect the actual mean values in other world regions. This probably results in an underestimate of dietary AFT exposure and overstates the relative contribution of dietary AFT exposure from tree nuts.

46. In the worst scenario, when no ML is in place, the intake of AFT from the consumption of tree nuts and dried figs contributed to more than 5% of the total dietary AFT exposure only for the GEMS/Food cluster diets B, C, D, E and M (24.6, 20, 45, 16.8 and 9.3 %, respectively).

47. If fully enforced, a ML at 20 µg/kg in almonds, Brazil nuts, hazelnuts, pistachios and dried figs would have an impact on the relative contribution to dietary AFT exposure only in these clusters, including high-level consumers of the tree nuts. This is due solely to the elevated AFT level in pistachios. For the tree nuts other than pistachios, the presence of a ML has no effect on total dietary AFT exposure.

48. The JECFA estimated that an enforced ML of 20, 15, 10, 8 or 4 µg/kg results in dietary exposures to AFT ranging from 0.12, 0.10, 0.08, 0.07 and 0.06 ng/kg bw per day in the cluster with the highest exposure (D) to 0.03, 0.02, 0.02, 0.02 and 0.01 ng/kg bw per day in the cluster with the lowest exposure (M).

49. The JECFA noted that the estimates for European clusters B, E and F, with MLs from 4 to 20 µg/kg for tree nuts were in the range of those reported in the EFSA opinion with MLs from 4 to 10 µg/kg for tree nuts, including high-level consumers.

50. The JECFA concluded that enforcing an ML of 15, 10, 8 or 4 µg/kg would have little further impact on the overall dietary exposure to AFT in all five of the highest exposed population groups, compared with setting an ML of 20 µg/kg. When the impact of the theoretical full enforcement of MLs scenarios for AFT was evaluated, the proportion of rejected samples by establishing a ML of 20 µg/kg for Brazil nuts was 11 %. This value increased by 63 % for a ML of 4 µg/kg.

CONCLUSIONS & RECOMMENDATIONS:

51. The present Discussion Paper on AFs in Brazil Nuts leads to the following conclusions and recommendations for consideration at the 2nd Session of the CCCF:

I) Brazil nuts production represents an important economic activity for the Amazonian population, contributing to the rainforest preservation.

II) Although the consumption of Brazil nuts leads to a low dietary daily intake of AFs, restrictive standard levels have been set in Europe, with a consequent impact on the population that depend economically on Brazil nut production. Therefore, there is a need for an international regulatory level, based on scientific evidence, aiming at the protection of human health with a minimum economical impact on international trade.

III) At its last Session, the JECFA has concluded that enforcing an ML of 15, 10, 8 or 4 µg/kg would have little further impact on the overall dietary exposure to AFT through the consumption of almonds, Brazil nuts, hazelnuts and pistachios in all five of the highest exposed population groups, compared with setting a ML of 20 µg/kg. Furthermore, for the tree nuts other than pistachios, the presence of a ML has no effect on dietary AFT exposure.

IV) Consumers may visually separate the edible from the inedible (highly contaminated) Brazil nut kernels during the shelling process before eating and thus protect themselves from exposure to high levels of AFs.

V) The data presented in this paper indicate that a ML for AFT in Brazil nuts ready-to-eat (shelled nut) of 20 µg/kg has no effect on total dietary AFT exposure, it is safe for consumers and has an acceptable economic impact on the producers.

VI) Brazil nuts can be in international trade both as for further processing (shelled and in-shell) and ready-to-eat (shelled nut). Therefore, MLs for both products are necessary.

VII) In agreement with studies showing that fungal infection is mostly internal, some studies have shown that the AF levels in the in-shell nuts are lower than what is found in the kernels. However, data presently available do not allow a conclusive in-shell/shelled nut ratio for AF levels. Brazilian Government is finalizing a study that might clarify this issue and should support a ML for in-shell nuts for further processing in a near future.

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