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Effect of Ozone Gas on Brazil Nut (*Bertholletia excelsa* H. B. K.) Mycoflora and Aflatoxin Reduction

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Abstract: Raw Brazil nuts grow and are harvested in the wild of the Amazon forest. At post-harvest they are submitted to two storage stages prior to their drying process. The first storage is in the forest (on pallets) and the second in cities near the Amazon River or its tributaries to be subsequently send to the factories by boat. They are kept in wooden silos inside suspended stalls to keep them away from the environment. Despite of that, the relative forest humidity and temperature are high and suitable to fungi proliferation. The main biological factor that can affect in-shell nuts' quality during storage is fungi (deteriorating and aflatoxigenic strains) apart from forest termites. This work reports on an evaluation of ozone (O_3) gas influence on Brazil nut fungi load and its effect on aflatoxins (AFLs). Groups of *in – shell* Brazil nuts (14kg) from the year 2006 harvest, AFL contaminated with 5.62 (g/kg, collected in the Brazilian Amazon were submitted to O_3 treatment at different concentrations and conditions. After the gas exposure period, nuts were submitted to mycology tests, moisture and AFL analysis. Total fungi count was carried out utilizing malt extract agar and the aflatoxigenic fungi identification with *A. flavus* and *Parasiticus* agar. The nuts' moisture was determined by gravimetry and AFB_1 by high performance liquid chromatography with fluorescence-detection. As expected, the mycological tests showed that O_3 treatment affected mycoflora growth, lowering their cfu/g count and so the moisture content (from 8.2% to 5.6 %). The O_3 treatment applied within 5 hours at 31 mg/L was able to successfully destroy nuts' fungi contamination (initial cfu/g: 40×10^4). Fungi reduction just after harvesting by applying O_3 will certainly reduce the possibility of further fungi proliferation and so AFL formation. From a food quality and safety point of view, prevention is a better strategy than detoxification which is much more complicated and so are the implications towards human and animal health.

Key words: Brazil nut, ozone, post – harvest, mycoflora, aflatoxin

Introduction

Brazil nut (*Bertholletia excelsa* Humb. and Bonpl.) is native to the Amazon forests of South America and represents some of the oldest living tree species on earth. Many of these trees date back more than 1 100 years[12]. Harvesting of Brazil nuts, a major non-timber forest product, not only helps in preserving the Amazon rainforest but also creates an economy on which thousands of local people depend[3,25,26]. Brazil nut is widely recognized as the cornerstone species of the Amazonian extractive economy, and is the only internationally traded nut collected almost entirely from natural populations in mature forest[7,24]. The occurrence of aflatoxins (AFLs) produced by *Aspergillus flavus* Link, in Brazil nuts has been confirmed in several studies[6,36,15,5,25]. In many instances, the presence of the mycotoxins were detected on the surface of shelled nuts exhibiting visible mold growth and/or inside shriveled, cracked, or brown spotted nuts[15,8].

Several environmental factors are known to influence AFL production, but temperature and relative humidity (r. h.) are considered to be the most critical. Studies performed on hazelnuts and pistachios suggested that optimum temperature and r. h. for AFL production is 25℃ to 30℃ and 97% to 99%, respectively[9,10,34,22,35]. Additional factors such as water activity, moisture content, substrate composition[31], storage time, insect damage[18,33], and presence of a shell[4] also influence fungal growth and AFLs production. It is also important to recognize, however, that the interaction of all these factors may provide for varying results in regards to fungal growth and mycotoxin production even on identical substrates. The presence of AFLs is a serious concern for exporters of Brazil nuts especially since 1998, when the European Community decreased the maximum tolerance limit of total and B1 AFLs to 4 and 2 ng/g, respectively[11]. Moreover,

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since temperature and r. h. are important factors for AFLs production, it is of interest to evaluate the effect of these parameters on AFLs production during storage of Brazil nuts[24,26]. The main problems of Brazil nuts that reduces its quality and safety are fungi, AFB_1. and lipid oxidation. Since export companies must provide documentary evidence of laboratory analyses for AFB_1 and microorganisms, primary control for each nut lot is performed by sampling at the reception stage[23].

Many physical and chemical methods such as microwave heating, treatments with ozone (O_3) (ozonation) or ammonia have been recommended for detoxification of AFLs contaminated food[13,37,29]. Ozonation, an oxidation method, has recently been developed for the detoxification of AFLs in foods[32]. O_3 or triatomic oxygen, is a powerful disinfectant and oxidizing agent[20]. It reacts across the 8,9 double bond of the furan ring of AFLs through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids[28]. The attractive aspect of O_3 is that it decomposes rapidly (half-life of 20 50 min) to molecular oxygen without leaving a residue[17]. As a disinfectant, O_3 is 1.5 times stronger than chlorine and is effective over a much wider spectrum of micro-organisms[37]. Several research studies have been undertaken to evaluate the effects of O_3 gas in reducing AFLs levels in contaminated agricultural products. Maeba et al. (1988)[19] have confirmed the destruction and detoxification of AFB_1 and AFG_1 with O_3. AFB_1 and AFG_1 were sensitive to O_3 and degraded with 1.1 mg/L of O_3 in 5 min in model experiments. O_3 is used to preserve the quality of fruit and vegetables after harvest. Frazier and Westhoff (1988)[14] reported that the storage period can be doubled when strawberry, raspberry, currant and apples are held in an environment including 2 – 3 mg/kg of O_3.

The objective of this research was to determine the influence of O_3 gas treatment on the mycoflora, moisture content and AFLs reduction in Brazil nuts.

Materials and Methods

Material

(*a*) ***Sample***: in-shell Brazil nuts (14 kg), 2005/2006 harvest, supplied by a Brazil nuts factory, located in Manaus city, Amazonas State (AM), Brazil. The AFL contamination was 5.62 g/kg.

(*b*) ***Storage***: (b. 1) seven vertical *silos*, made with vinyl polychloride (PVC) with 80 cm × 15 cm × 0.2 cm for height, diameter and width, respectively containing a lid and two apertures i. e. , top and lateral – inferior of the silos, for sample collection and O_3 application, respectively. (b. 2) ozoniser, Megazon (b. 3).

(*c*) ***Mycology tests***: (*c.* 1) glassware: Erlenmeyer (2 000mL), test tubes, Petri plates, microbiological pipettes (1, 10mL), automatic pipette 100, 1 000 L tips, microscope slides, Drigalski agar; (*c.* 2) culture media: malt extract agar (MEA), *A. flavus* and *A. parasiticus* agar (AFPA), peptone media, tween 80. (*c.* 3) equipment: autoclave, oven, microscope, incubator set at 20 – 25℃, scale, scissors, microscope stereoscope, colonies counter and tubes racks.

(*d*) ***Moisture content***: dissectors, microbiological oven, Fanen; analytical scale, Mettler; semi – analytical, CAB and industrial Brazil nuts cracker provided by CIEX, Manaus, AM.

(*e*) ***Aflatoxin analysis***: LC with isocratic pump and fluorescence detector, Gilson.

Methods

(*a*) ***Sample preparation for O_3 application***: in – shell Brazil nuts were weight and portions of 2 kg were aseptically added into the silos for O_3 treatment. Samples were collected from each silo for the following analysis: mycological, moisture content and AFLs.

(*b*) ***Preparation of the silos***: the silos (total = 7), after cleaned up with sulphite hypochloride, were filled with the 2 kg of nuts and had tightly closed the upper part with the lid. They were divided into 4 Groups for O_3 application at different concentrations: Group I (Control = no O_3 application), Group II (O_3 = 10mg/L), Group III (O_3 = 14 mg/L) and Group IV (O_3 = 31.5 mg/L), n = 2.

(*c*) ***Ozone application***: after closing the upper part of the silos, O_3 gas was applied through a lower lateral aperture by means pf a vacuum pump to get the following concentration in each silo: 10, 14 e 31.5 mg/L (n = 2) during 1, 3 and 5 hours and closed. The O_3 concentrations were measured utilizing the iodinemetrical method of APHA (1980).

(*d*) ***Storage***: after O_3 application, silos were placed in a room with temperature and UR monitored for up to 6 months. Brazil nuts were monitored for mycological tests, moisture con-

tent as well as R. H. and temperature.

(*e*) ***Sample collection for analysis***: samples were aseptically collected for mycology, moisture content and Afls from the top silo aperture, de-shelled and ground.

(*f*) ***Analysis***: (*f*. 1) *Mycology*: 225 mL of peptone media (0. 1% com Tween 80) were added to 25 g portions of ground Brazil nuts, shake and 0. 1 mL applied on the surface of MEA media. After their incubation at 25℃ for 7 days the fungi total colonies count was carried out. The fungi identification were carried out utilizing AFPA media and their strains toxigenicity checked utilizing the Machida & Saito (1999) method. (*f*. 2) *Moisture content*: by gravimetry. 5 g of each Group of Brazil nuts were taken to a drying oven with temperature of 105℃ up to constant weight (AOAC, 2005). (*f*. 3) *Relative humidity and temperature*: temperature and r. h. were monitored utilizing thermometer and hygrometer, respectively. (*f*. 4) *Aflatoxins*: by high performance liquid chromatography with fluorescence-detection HPLC/FD – (AOAC, 2005).

Results and Discussion

As expected, the mycological tests showed that O_3 treatment affected mycoflora growth, lowering their cfu/g count and so the moisture content (from 8. 2% to 5. 6 %). The O_3 treatment applied in 5 hours at 31 mg/mL was able to successfully destroy fungi contamination in the nuts (initial cfu/g: 40×10^4). As far as aflatoxigenicity is concerned, according to Saito & Machida (1999) [30], in order to identify the trains toxigenicity when utilizing the AFPA media, its (the media) reverse should present an orange colour. In our experiment the media turned orange-aflatoxigenic fungi genera *Aspergillus* detected-only in the Control Group nuts. From the nuts further O_3 gas treated i. e., Groups T2 to T4 no aflatoxigenicity was detected in any of the isolated strains thus, showing that the gas treatment was efficiently able to destroy them. O_3 gas produces a progressive oxidation of the cell vital components leading to apoptosis[16]. Table 1 shows the different strains of *Aspergillus* and *Penicilium* as well other genera isolated from the nuts. No AFLs were also detected in the nuts samples after O_3 gas treatment. To reduce yeast/mould activity, O_3 could be applied either for longer periods at low concentration, or conversely for short period with higher concentrations. Literature studies show that low concentrations and long exposure times were usually preferred for O_3 applications. In the study of Palou et al. (2002)[27] with the peaches cultivars Elegant Lady, they were treated for a four week period by O_3 at 0. 3 mg/L concentration in cold storage conditions at 5℃ temperature and 90% r. h. Fungi reduction just after harvesting by applying O_3 will certainly reduce the possibility of further mycelia proliferation and so AFL formation.

As far as moisture content is concerned, variations were observed between Groups: the Control and the three treated Groups with different concentrations and exposition time to O_3. The treated Brazil nuts (Groups T1, T2 e T3) presented lower moisture content than the Control Group (Table 2), either in – shell or shelled ones, with an average of moisture reduction of 18. 13 to 21. 63 % and 22. 76 to 28. 59 % of the initial moisture content, respectively. Considering the shelled Brazil nuts, where the moisture loss is more intense due to the lack of shell protection, it was observed that although Groups: T1 (exposition of 2 hs at 10 mg/L of O_3) and the T2 (exposure of 3 hs at 14mg/L of O_3) presented much lower moisture content (3. 97%, 3. 94 %, respectively), the difference was more intense in Group T3 of 5 hs of exposure to the gas and 31. 5 mg/L of O_3, a higher concentration applied, reaching 3. 67 %. Fig. 1 shows clearly that a reduction on the moisture content by the O_3 treatment was observed from the third treatment (Group T3) onwards.

This work is part of a Research Project on "Methodology Development for Reduction and Control of AFLs in Stored Brazil Nuts" that has been developed in the Food and Technology Department of the Federal University of Santa Catarina, Brazil.

Conclusion

It can be concluded that a minimum of five hours O_3 treatment at 31. 5mg/L could be successfully used for reducing the microbial count of Brazil nuts. O_3 reduced fungal growth and so AFLs in Brazil nut, consequently, that treatment could be an effective method for reduction of nut deterioration and so the AFLs contamination risk in the market. By destroying yeast and moulds just after harvesting will certainly reduce the possibility of AFLs formation before the next processing steps. On the other hand sensitivity of fungi to O_3 could be influenced by

other factors including location of fungi in the nut and interactions among the different parameters. O_3 could be used in packaged nuts, as long as proper method such as hermetic or vacuum resistant materials can be applied. From a food quality and safety point of view prevention is a better strategy than detoxification which is much more complicated and so the implications to human and animal health. Despite of the findings, there is a need of more studies, especially in pilot plants and application in larger amounts of nuts under the environment of Amazon forest in order to establish the optimal and practical O_3 gas concentration and the time of exposure for maximum reduction either of deteriorating or aflatoxigenic fungi growth and so moisture content and AFLs contamination.

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Table 1. Effect of different ozone gas concentrations and time of exposure on fungi development on in – shell and shelled Brazil nuts

Media	Fungi growth							
	Control In – shell	Treatment 1		Treatment 2			Treatment 3	
		Shelled	In – shell	Shelled	In – shell	Shelled	In – shell	Shelled
MEA								
10^{-1}	*A. flavus* (1) *A. parasiticus* (2) Yeasts (nbc)	*P. crustosum* (nbc) Yeasts (nbc)	*A. N ger* (1) Yeast (8)	Yeast (nbc)	Yeast (6) *P. nalgiovense* Laxa (1)	*Rhizopus stonifer* (Ehrenb) Lind. (1) *P. nalgiovense* Laxa (2)	Yeast (1)	Yeast (1)
10^{-2}	*Syncephalastrum recemosum* Cohn (1) *A. ochraceus* (1) Yeast (30)	*P. crustosum* (20) *A. versicolor* (25) Yeasts (15)	*A.. versicolor* (20) Yeasts (4)	Yeast (6)	Yeast (6)	*P. corylophilum* Dierckx (3)	*Byssochamy s nivea* Westling (1)	Yeast (1)
10^{-3}	Yeast (10)	Yeast (4)	Yeast (3)	Yeast (3)	Yeast (1)	*P. nalgiovense* Laxa (3)	NG	NG
10^{-4}	Yeast (4)	Yeast (2)	*Byssochamys nivea* Westling (1)	NG[a]	NG	NG	NG	NG
AFPA								
10^{-1}	*A. parasiticus* (4) *A. flavus* (2) *Cladosporium Spharospermum* Penzig (1)	*A. parasiticus*(1), *A. sydowii* (Bain. & Sart.) Thom & Church (2)	*A. parasiticus* (1)	*Cladosporium Spharospermum* Penzig (5)	*Cladosporium Spharospermum* Penzig (1)	NG	*Cladosporium Spharospermum* Penzig (1)	NG
10^{-2}	NG	*Cladosporium Spharospermum* Penzig (1)	NG	NG	*Cladosporium Spharospermu m* Penzig (1)	NG	NG	NG
10^{-3}	NG	NG	NG	NG	NG	NG	NG	NG
10^{-4}	NG	NG	NG	NG	NG	NG	NG	NG

[a] no microorganisms growth was observed

Table 2. Moisture content of Brazil nuts after ozone treatment

Group	O_3 treatment		Brazil nuts moiture content			
	Time (min.)	Concentration (mg/L)	In-shell (%)		Shelled (%)	
			Nuts	Reduction	Nuts	Reduction
C[a]	Zero	Zero	9.43	NA[b]	5.14	NA[b]
T1	120	10	7.72	21.63	3.97	22.76
T2	240	14	7.44	21.10	3.94	23.35
T3	300	31.5	7.39	18.13	3.67	28.60

[a] control [b] not applicable

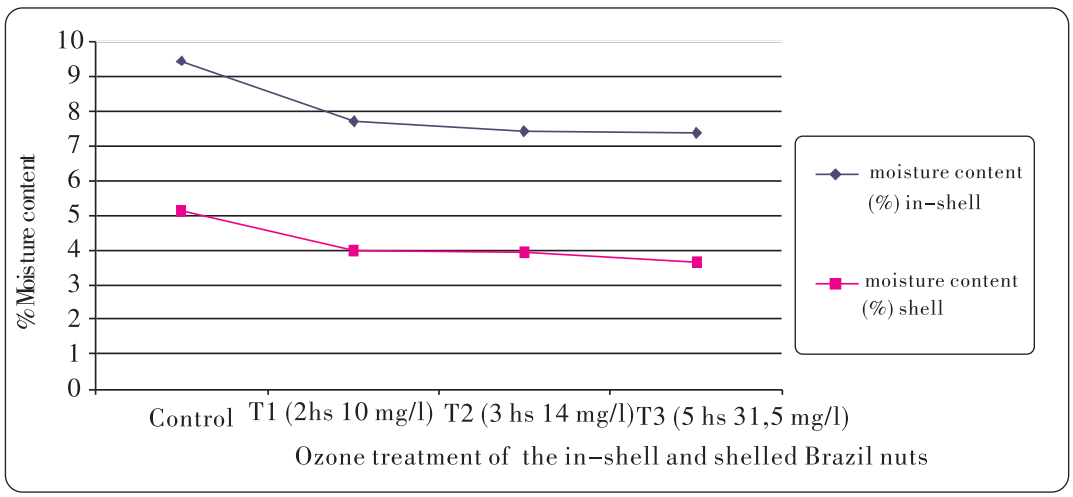

Fig. 1 Moisture content of in-shell and shelled Brazil nuts after treatment with different ozone concentrations