Reduction of in-shell Brazil nut (*Bertholletia excelsa* H.B.K.) aflatoxin contamination by ozone gas application during storage

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Abstract

The susceptibility of the in-shell Brazil nut mycoflora and aflatoxins (AFLs) contamination to ozone (O_3) gas during storage is reported. In-shell Brazil nuts obtained from retail market were submitted to O_3 gas atmosphere at different concentrations immediately before to be stored. Samples were collected just after the gas exposure and every 30 days during the storage period to carry on mycological tests and AFLs analysis. A sensorial evaluation by descriptive quality analysis was carried out to check treated nuts sensory attributes according to consumer acceptance after gas exposure. The O_3 treatment applied within 5 h at 31 mg/L was able to successfully inhibit the viability of fungi of the nut-contaminating microflora and so the toxigenic *Aspergillus species* from the day of application. AFLs were totally degraded in all samples whatever O_3 concentration applied. No significant changes on sensory attributes were observed that could affect nut acceptability after the O_3 treatments and storage conditions applied in the present experiment. This procedure is tentatively applied at an Amazon State nut factory for checking its potential in mycotoxin risk contamination of in-shell Brazil nuts safeguarding under the Amazon region environment.

Keywords: In-shell Brazil nut, Ozone, Mycoflora, Aflatoxin, Storage, Sensory evaluation.

1. Introduction

Prevention of aflatoxin (AFL) contamination in tree nuts by controlling the environment conditions for toxigenic fungi growth has not always been effective. The risk of AFL contamination in Brazil nuts (*Bertholletia excelsa* H.B.K.) can occur, either in the forest or during storage and it can vary with the year of harvest (Pacheco and Scussel, 2006; 2009). Nut contamination by fungi, especially the aflatoxigenic species of *Aspergillus*, are directly related to the climatic conditions (high humidity and warm conditions) in the Amazon forest during the period of harvesting (at wet season: December to May) (Arrus et al., 2005; Pacheco and Scussel, 2006; 2007; 2009; Olsen et al, 2008). The presence of AFLs is a concern for exporters of Brazil nuts according to European Community (EC) reduction the maximum tolerance limit of aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2) and AFB_1 to 4 and 2 µg/kg, respectively (ECC, 2006) either in-shell or shelled. Thus, there is a need for the development of safe technologies at the forest environment level enabling to control fungi proliferation and reduce toxin contamination.

Fungi can growth both, on the shell and inside the shell on the nuts when shells are cracked or through its opercule. Thus, AFLs have been detected on the surface of shelled nuts and/or inside of cracked, or brown spotted, in-shell nuts (De Mello and Scussel, 2007; 2009). Recently, it was found that the main site of the in-shell nut contaminated by AFLs is located between the shell and the the nut peel (Conforcast, 2008). Several environmental factors are known to influence fungi growth and AFL production, being temperature and relative humidity (r.h.) the most critical (Mangan and Aldred, 2007). Ozonation, an oxidation method has been studied for detoxification of AFLs in foods (Shamarajeewa et al., 1990). The oxidation decreases AFL concentration over time (McKenzie et al., 1997). O_3 modified atmospheres have been developed for dried figs, barley, pistachio among other foods (Oztekin et al., 2006). The attractive aspect of O_3 is that it decomposes rapidly (half-life of 20–50 min) to molecular oxygen after application without leaving a residue (Mason et al., 1997; Frazier and Westhoff, 1988; Maeba et al., 1988; Perez et al., 1999; Kells et al., 2001; Yesilcimen and Murat, 2006; Inan et al., 2007; Olmez et al., 2009). It can be applied to food as a gas or dissolved in water.

One of the most important applications of O_3 in agriculture is in the post-harvest treatment of crops and the main purposes of gas application are: inactivation of bacterial growth, prevention of fungal decay, destruction of pesticides and chemical residues, and control of storage pests. Storage period can be doubled when some fruits and vegetables are held in an environment with O_3 (Frazier and Westhoff, 1988; Inan et al., 2007; Olmez et al., 2009). Regarding *fungi* and AFLS in different food commodities, Oztekin et al. (2006) reported a significant reduction of microorganism counts on dried figs at 5 mg/L of O_3, decreasing the total yeast/mould counts of 72% and Perez, et al. (1999) observed a fungal decay of strawberry after 4 days of storage under ozonation. Five mg/L of O_3 inhibited the surface growth, sporulation and mycotoxin production of cultures of *Aspergillus flavus* and *Fusarium moniliforme* (Mason et al., 1997). Yesilcimen and Murat (2006) studied pistachio exposure to O_3: 5.0, 7.0 and 9.0 mg/L and found AFB_1 and total AFLs reduction by 23 and 24% at 9 mg/L. Maeba et al. (1988) observed the destruction and detoxification of AFB_1 and AFG_1 with O_3 in model experiments.

We report here the application of O_3 gas at different times and concentrations during the storage of in-shell Brazil nut to improve nuts safety regarding fungi and AFLs contamination.

2. Materials and methods

2.1. Sample

Dry (processed) in-shell Brazil nuts (14 kg), export Medium Size Type 40-50 mm length (De Melo and Scussel, 2007). A naturally contaminated batch was chosen for the study with initial AFLs (AFB_1 / AFB_2 / AFG_1 / AFG_2) of 11.58 µg/kg obtained in the retail market. That level is allowed by regulations from USA, Canada, Brasil and Mecosur (15, 15, 30, 20 µg kg, respectively). Total fungi count: 4.83 log cfu/g and 9.43% and m.c.

2.2. Storage

*S*even vertical silos, build with vinyl polychloride (PVC) with the following dimensions 80 x 15 x 0.2 cm for height, diameter and width, respectively; containing an upper lid and two apertures (top and lower part of the silos) for sample collection and O_3 application, respectively; connected to an ozone generator (Megazon™) (Fig. 1).

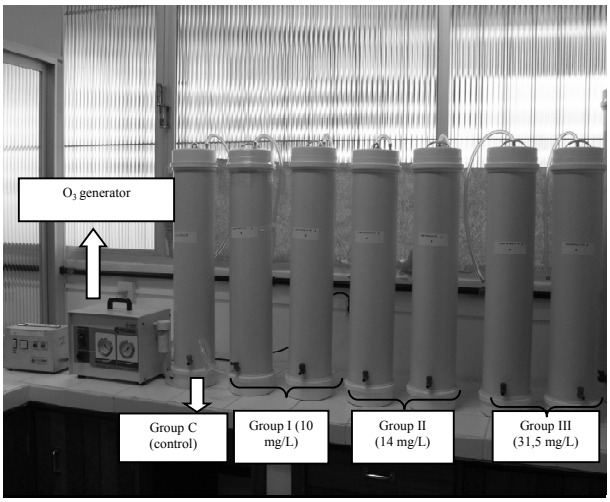

Figure 1 Brazil nut ozone treatment and storage system: silos (n=7) and O_3 generator

2.3. Chemicals, reagents and culture media

The reagents, potassium iodine, sulphuric acid, sodium thiosulfate, 2-thiobarbituric acid (TBA), trichloroacetic and butylated hydroxytoluene were of Analytical grade (Vetec) and utilized for the TBA test. The solvents were methanol, ethanol, acetonitrile, benzene and toluene from Carlo Erba, starch indicator from Synth and the ultrapure water from MilliQ system, Millipore. The standards of AFL

(AFB_1, AFB_2, AFG_1 and AFG_2) were obtained from Sigma. Malt extract agar-MEA was purchased from Himedia™; *A. flavus* and *parasiticus* agar-AFPA from Fluka™, peptone agar from Himedia™ and Tween 80 from CRQ™.

2.4. Equipment and apparatus

The equipment and apparatus utilized for sample preparation, microbiological and AFL analysis were homogenizer from IKA T 25-Ultra Turrax; water bath from Quimis-Dubnoff™ Q226D; autoclave from Phoenix™; microscope from 100-400x PZO; incubator set at 20-25°C from Dist™; microscope stereoscope from Carl Zeiss Iena™ (Germany); colonies counter from Phoenix™ and microbiological oven from Fanen™. Analytical cales from Mettler™ and the semi-analytical one from CAB™. Industrial Brazil nut cracker was provided by CIEX™ (Manaus, AM, Brazil). The spectrophotometer model E005 was from Hitachi™. The liquid chromatograph comprised an isocratic pump model 305 and fluorescence detector model 121 from Gilson™. The column was of C_{18} with 15 cm length, 4.6 mm diameter, and particle size of 5 µm from Phenomenex™. Thermometer and hygrometer utilized for environment temperature and moist readings were from CE™.

2.5. Sample preparation for O_3 application

In-shell Brazil nuts previously analysed for fungi load, m.c. and AFLs contamination were aseptically weighted into portions of 2 kg to be added into each silo for the O_3 treatment.

2.6. Preparation of the storage Silos, ozone application and iodinemetry

Silos were filled with nuts and tightly closed. They were divided into 4 groups for O_3 application: Group C (Control = no O_3 application), Group I (O_3 conc. = 10 mg/L), Group II (O_3 conc. = 14 mg/L) and Group III (O_3 conc. = 31.5 mg/L) (Fig. 1). Each treatment was carried out in duplicate (n = 2) except for Group C. After closing the upper part of the silos, O_3 gas was applied through the lower lateral aperture to get the following concentration in each silo: 10, 14 e 31.5 mg/L, with 1, 3 and 5 h exposure time, (n = 2) for silo Groups 2, 3 and 4, respectively. Those concentrations were checked by iodinemetrical analysis (APHA, 1980). The storage time was expanded on a 180-d period (May to October).

2.7. Sample collection for analysis

Individual 200 g samples of Brazil nuts were aseptically collected from each silo at day-one and every 30 d. This sampling was carried out from the top silo aperture. Samples collected for analysis were made in duplicate (n = *2*).

2.8. Mycology, m.c., r.h., temperature, AFLs and sensory analysis

For total fungi count the method used was from Pit and Hocking (1997). For fungi toxigenicity (for AFLs) the method was from Machida and Saito (1999). The identification of fungi in genus and species was carried out according to the keys of Samsom et al. (2004). The strains aflatoxigenicity was checked utilizing the AFPA method developed by Pitt et al. (1983). The m.c. was carried out by a gravimetric method (AOAC, 2005). Relative humidity and temperature were monitored daily utilizing hygrometer and thermometer, respectively. In parallel, data on r.h. and temperature was obtained from the National climatic recordings database EPAGRI/SC (May to October). AFLs analysis was performed by liquid chromatography with fluorescence-detection, with a limit of quantification (LOQ) at 0.50, 0.17, 0.50, and 0.17 µg/kg for AFB_1, AFB_2, AFG_1 and AFG_2, respectively (Sobolev et al., 2007).

2.9. Sensory evaluation

The descriptive quantitative analysis by Stone and Sidel (1993) was conducted using a team of 18 trained (specifically for Brazil nut taste) panelists and four sessions (n = 4). The hedonic scale was separated into 5 degrees as follows: 1: dislike very much, 2: dislike, 3: neither like nor dislike, 4: like, and 5: like very much. Six sensory attributes of the Brazil nuts were recorded: shell appearance, nut appearance, strange odor, roasted flavor, rancidity and firmness.

2.10. Statistical analysis

The results were expressed as the mean values and standard errors. Statistical analysis was performed by analysis of variance (ANOVA) and included the Turkey´s test to evaluate significant differences among the means ($p < 0.05$).

3 Results and discussion

3.1. Effect of O_3 on the in-shell Brazil nut mycoflora

The total fungi load, aflatoxigenic *Aspergillus* species and levels of AFLs variation during the storage of in-shell Brazil nuts under O_3 atmosphere are shown in Table 1. There was a clear reduction on the total fungi count, AFLs and m.c. when compared to the Control Group. However this reduction trend was dependent upon the O_3 concentration used and time of storage.

Table 1 Fungi, aflatoxigenic *Aspergillus* species and aflatoxins levels in Brazil nut stored under ozone at room temperature

Storage			Brazil nut										
	O_3 treatment		Fungi				m.c./loss (%)		Aflatoxins (in-shell / after shelling) (µg/kg)[a]				
days			total count (log cfu/g)		*Aspergillus* aflatoxigenic species		in-shell	after shelling					
	group	conc. (mg/L)	in-shell	after shelling	in-shell	after shelling			∑AFLs	AFB_1	AFG_1	AFB_2	AFG_2
1	C	0[b]	4.83	2.54	*A. flavus, A. parasiticus*	ng ng	9.43 (NA)	5.14 (NA)	11.58/6.61	3.48/1.16	3.57/1.89	2.21/2.02	2.32/1.74
	I	10	3.5	ng	*A. flavus, A. parasiticus*	Ng ng	7.72 (6.61)	3.97 (6.25)	3.01/ND	3.01	ND	ND	ND
	II	14	3.3	ng	*A. flavus, A. parasiticus*	ng ng	7.71 (7.58)	3.96 (6.89)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.68 (8.56)	3.95 (7.52)	ND	ND	ND	ND	ND
30	C	0	4.84	2.57	*A. flavus, A. parasiticus*	ng ng	9.57 (NA)	5.28 (NA)	12.06/8.01	3.69/1.37	3.78/2.73	2.23/2.05	2.36/1.86
	I	10	ng	ng	ng	ng	7.67 (10.22)	3.95 (8.04)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.66 (10.60)	3.94 (8.67)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.64 (11.74)	3.93 (9.29)	ND	ND	ND	ND	ND
60	C	0	4.86	2.60	*A. flavus, A. parasiticus*	ng ng	9.63 (NA)	5.32 (NA)	12.24/7.95	3.83/1.16	3.82/2.83	2.22/2.08	2.37/1.88
	I	10	ng	ng	ng	ng	7.63 (11.65)	3.93 (9.03)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.61 (12.59)	3.90 (9.65)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.58 (13.34)	3.88 (10.28)	ND	ND	ND	ND	ND
90	C	0	4.88	2.62	*A. flavus, A. parasiticus*	ng ng	9.84 (NA)	5.46 (NA)	12.34/8.03	3.86/1.14	3.86/2.86	2.25/2.10	2.37/1.93
	I	10	ng	ng	ng	ng	7.56 (14.65)	3.87 (11.38)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.54 (15.75)	3.87 (12.29)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.51 (16.11)	3.60 (12.50)	ND	ND	ND	ND	ND
120	C	0	4.89	2.65	*A. flavus, A. parasiticus*	ng ng	9.89 (NA)	5.51 (NA)	12.49/8.08	3.94/1.14	3.88/2.88	2.27/2.11	2.40/1.95
	I	10	ng	ng	ng	ng	7.47 (16.15)	3.85 (12.23)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.43 (17.78)	3.84 (13.14)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.41 (17.96)	3.82 (13.34)	ND	ND	ND	ND	ND
180	C	0	4.91	2.69	*A. flavus, A. parasiticus*	ng ng	9.93 (NA)	5.63 (NA)	12.55/8.17	3.95/1.13	3.90/2.91	2.28/2.17	2.42/1.96
	I	10	ng	ng	ng	ng	7.37 (18.82)	3.80 (12.99)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.35 (19.89)	3.78 (13.79)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.32 (19.89)	3.76 (14.19)	ND	ND	ND	ND	ND

[a] nuts were evaluated for fungi whole (in-shell + edible part) and after shelling (only edible part); nuts Groups: [b] C = control (no O_3 treatment); I (O_3 conc. = 10 mg/L), II (O_3 conc. = 14 mg/L) and III (O_3 conc. = 31.5 mg/L); NT = not treatment ; NA = no applicable; ng = no growth; mc = moisture content; total fungi load initial = $6.9x10^4$; initial mc= 9.43%/ ND = not detected; [c] = concentration AFLs µg kg in duplicate; LOD 0.25; 0.08; 0.25 and 0.08 µg/kg e LOQ 0.50; 0.17; 0.50 and 0.17 µg/kg, to AFB_1, AFB_2, AFG_1 and AFG_2 respectively; [d] $AFB_1 + AFG_1 + AFB_2 + AFG_2$. Temperature: 20°C (16.6 – 20.6°C); r.h.: 80% (75.8 – 85)

3.1.1 Total fungi load

As expected, the in-shell Brazil nuts ozonation showed to be effective on the fungi/spores destruction during the period of storage. A reduction of total fungi count was registered as soon as the first day after O_3 application in all treated nut Groups. However, the complete destruction of fungi (no growth: 'ng' label) was reached at different stages of storage depending upon the gas concentration applied. Total fungi destruction started at the first day when the highest O_3 concentration was applied (31.5 mg/L) and after 30 days of storage, when O_3 exposure was achieved at a lower concentration i.e., at 14.0 and 10 mg/L. Thus, no fungi growth was registered in all O_3 treated nut Groups after a month of storage up to the end of the six month. From the original (untreated nuts) total fungi load of 4.83 log cfu (Control), it went down at day one of O_3 treatment to 3.5 and 3.3 log cfu/g for groups I (O_3: 10.0 mg/L) and II (O_3: 14.0 mg/L), respectively; and no fungi growth was detected in the nuts treated with the highest O_3 concentration (Group III – O_3: 31.5 mg/L). These figures were very different of those observed with the

nuts of control groups, where the fungi load remained somewhat stable with an insignificant increase during the whole period of storage i.e., from -zero/-one (4.83 log cfu/g) to180 d (4.91 log cfu/g). Similar situation happened to those nuts when analyzed after being shelled, however in a lower extend from D-one (2.54 log cfu/g) to 180 d (2.69 log cfu/g) – control group) which was expected, as the edible part was protected by the shell. Some works have reported the effect of ozonation in food and nuts and they have similar findings for fungi load reduction as obtained in the present experiments, however applying lower O_3 concentrations. We utilized higher O_3 concentrations due to the fact that our aim was more than just fungi disinfection. We wanted to obtain a significant AFLs degradation too (to be discussed in the next Session) and for that purpose the O_3 concentration should be higher. Zhao and Cranston (1995) studied the effect of O_3 on black pepper at a lower gas concentration (6.7 mg/L) than in our study. The authors reported fungi load reduction from 7 to 4 log cfu/g. In another study carried out in dried figs (Oztekin et al., 2006), O_3 reduced the total fungi load from initial 1.46 to 1.00, 0.57, and 0.40 log cfu/g using O_3 at 1, 5, and 10 mg/L, respectively. In a work carried out on Brazil nut, however treating the nuts with aqueous O_3 solutions (0, 1, 10 and 20 mg/L) the authors showed that these treatments were effective to fungi control reaching an inactivation rate of 100% for *A. parasiticus* and 96% for *A. flavus*. However O_3 application in water can lead to an increase of moisture, reducing crunchy and firmness (Freitas and Venancio, 2008).

3.1.2. Aflatoxigenic species of Aspergillus

Regarding the isolation of aflatoxigenic species of *Aspergillus* (*A. flavus* and *A. parasiticus*) in the Brazil nuts just after the O_3 treatments (Group I, II, III), only the highest O_3 concentration treated nuts (Group III - 31.5 mg/L) did not allow them to growth since day one after gas application. The same occurred to the Groups of lower O_3 concentrations, but only after 30 d of storage. On the other hand, the Control Group was contaminated permanently by these species throughout the whole storage period. Similar situation happened when Mason et al. (1997) applied O_3 (5 mg/L) in cultures of *A. flavus* and *Fusarium verticillioides*. Ozone treatment in these conditions inhibited the growth, sporulation and mycotoxin production. Kells et al. (2001) studied the efficacy of O_3 as a fumigant to disinfest stored maize from insects. At a concentration of 50 mg/L, these authors observed a reduction of 63% of the kernel contamination level by *A. parasiticus*. It seems important to emphasize that the species *A. nomius* was not detected in the present experiments either due to the fact that after nut dehydration, fungi were destroyed by the heating temperature or fungi competition as nuts were purchased in the market in the South of Brazil, or also because the AFPA media does not give a clear response, *i.e.* not enhancing the characteristics of that *Aspergillus* species.

3.1.3. Environmental conditions after O_3 treatment: r.h. level

During the period of the in-shell Brazil nut storage in Southern Brazil were rather mild with average temperature of 20°C (min 16.6 and max 20.6°C) and r.h. of 80% (min 75.8 and max 85.3%).

3.1.4. Effect of O_3 on AFL contamination

In contrary to the nuts Control Group, a reduction on the AFL levels was detected throughout the whole storage period of the in-shell Brazil nuts O_3 treated (Table 1). Just after the O_3 treatments, either at gas concentration of 14 or 31.5 mg/L, the Brazil nuts did not present contamination by AFLs – up to the limit of quantification of the method used (0.50, 0.17, 0.50, and 0.17 µg/kg, for AFB_1, AFB_2, AFG_1 and AFG_2, respectively). Although the three concentrations applied were able to degrade the toxins, some AFLs were still detected: 3.01 µg/kg (74% reduction) at the lower O_3 concentration (10 mg/L) after the first month of storage, the toxins was able to be totally degraded after 30 d. As far as the storage period and the AFL degradation are concerned, all the groups did not present any AFLs contamination after one month of storage onwards. As expected with the Control Group, the nuts AFL level remained stable or slightly increased from the beginning to the end of storage (11.58 to 12.55 µg/kg, respectively). That could be explained by the controlled experiment environment and storage conditions applied, and additionally, by the mild/low temperatures in Southern Brazil. The fact that other AFLs were detected, i.e., AFB_2 and AFG_2 in the Control Group, was probably the consequence of the origin of the nuts which were purchased in the retail market of Southern Brazil and not in the Amazon region, thus exposed to different fungi species contamination through manipulation and different environments, from tropical to temperate climate down in the south, respectively.

3.1.5. O_3 nuts sensory attributes for quality acceptance

No significant changes ($P < 0.05$) were found between shell and nut appearances, strange odor, roasted flavor, rancidity and firmness scores of the ozonated Brazil nuts samples stored and they did not differ greatly among the concentrations used too. They were between 3 (indifferent) and 4 (like), different of the Control Group that received score 2 for most of the attributes except for nut firmness (score 3), showing that the treatment with O_3 and the period of storage of the in-shell Brazil nuts, did not affect their sensory quality attributes for all O_3 treated Groups. Also the shell received score 4, except for roasted flavor (score 3). Therefore, regarding sensory evaluation, the gas treatment kept nuts sensory attributes thus still palatable apart from being safer.

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 on application in pilot plants utilizing larger amounts of nuts under the Amazon forest environment (first and second storages) prior factory processing, in order to establish the optimal applicable O_3 gas concentration and time of exposure for maximum effectiveness utilizing the present findings. This work is being applied in a pilot plant at a Brazil nut factory in the Amazon State for checking its effectiveness under the Amazon region environment. Important to emphasize that gaseous O_3 decomposes to form O_2 and it does not affect the environment, nor leave residues in the nuts.

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