Research Article

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Efficacy of ozone as a fungicidal and detoxifying agent of aflatoxins in peanuts

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Abstract

BACKGROUND: Peanut contamination by fungi is a concern of processors and consumers owing to the association of these micro-organisms with quality deterioration and aflatoxin production. In this study the fungicidal and detoxifying effects of ozone on aflatoxins in peanuts was investigated. Peanut kernels were ozonated at concentrations of 13 and 21 mg L^{-1} for periods of 0, 24, 48, 72 and 96 h.

RESULTS: Ozone was effective in controlling total fungi and potentially aflatoxigenic species in peanuts, with a reduction in colony-forming units per gram greater than 3 log cycles at the concentration of 21 mg L^{-1} after 96 h of exposure. A reduction in the percentage of peanuts with internal fungal populations was also observed, particularly after exposure to ozone at 21 mg L^{-1}. A reduction in the concentrations of total aflatoxins and aflatoxin B1 of approximately 30 and 25% respectively was observed for kernels exposed to ozone at 21 mg L^{-1} for 96 h.

CONCLUSION: It was concluded that ozone is an important alternative for peanut detoxification because it is effective in controlling potentially aflatoxigenic fungi and also acts in the reduction of aflatoxin levels in kernels.

Keywords: ozonation; peanuts; food safety; potentially aflatoxigenic fungi

INTRODUCTION

Peanut contamination by fungi is a concern of processors and consumers owing to the association of these micro-organisms with quality deterioration and mycotoxin production.[1] Of the various types of mycotoxin, aflatoxins stand out as presenting high acute and chronic toxicity in animals, including humans; they can also cause liver damage, such as cirrhosis and hepatocellular carcinoma, in addition to teratogenic effects.[2] These compounds are produced mainly by the species *Aspergillus flavus* and *Aspergillus parasiticus* and also by *Aspergillus nomius*.[3–5] While *A. flavus* predominantly produces aflatoxins B1 and B2, *A. parasiticus* is able to additionally synthesise aflatoxins G1 and G2.[6]

The potentially aflatoxigenic species *A. flavus* and *A. parasiticus* can be found both in soil and in the air and are classified as field and storage fungi, indicating that they are capable of infecting kernels during pre- and postharvest. In the field, temperatures in the range 28–34 °C associated with water stress during peanut formation are favourable for infection by fungi and production of aflatoxins.[7,8] During storage, aflatoxin production is affected by several factors, among which temperature and water activity are especially relevant. Aflatoxin synthesis by *A. flavus* occurs between 15 and 37 °C, being most pronounced in the range 20–30 °C.[9] The optimal temperature and water activity for aflatoxin synthesis by *A. flavus* are 30 °C and 0.996 respectively.[10]

Since the 1960s the processes of prevention and control of several types of mycotoxin, especially aflatoxins, have not presented a safe and effective model with a definite solution.[11] In this context, there are two basic processes for the reduction of mycotoxin levels in food: decontamination and detoxification.[12] Chemical methods of removal have been widely tested owing to their high capability of aflatoxin degradation and inactivation.[13] However, despite the significant power of chemical substances to eliminate aflatoxins, in the majority of cases such methods are technically and economically unfeasible. An alternative that has been presented for the prevention and control of aflatoxins in food is ozone gas.[14,15]

Ozone gas can act in the inactivation or inhibition of micro-organism development. In the literature there are several reports that describe the effects of ozone on micro-organisms, including fungi of the genera *Aspergillus, Fusarium, Geotrichum, Myrothecium, Alternaria* and *Mucor*[16–18] as well as viruses, protozoans and bacteria.[19–21] The inactivation of micro-organisms by ozone is

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mainly attributable to disruption of the cell membrane and subsequent dispersion of cytoplasmatic contents because of the high oxidising power of this gas.[22] This power of inactivating or inhibiting micro-organism development is crucial with respect to food safety because it can represent a method for the control of potentially aflatoxigenic fungi and consequently the prevention of aflatoxin synthesis.

The ozonation process is also proposed as a technology capable of degrading mycotoxins, including aflatoxins, fumonisins, ochratoxin, patulin, deoxynivalenol and zearalenone.[14,23] McKenzie *et al*.[24] introduced the mechanism of aflatoxin B1 degradation by ozone, the final products of the reaction being aldehydes, ketones, acids and carbon dioxide. According to these authors, ozone reacts with the double C8–C9 units of the furan terminal of aflatoxin B1. It is important to highlight that the toxigenic, carcinogenic and teratogenic effects of aflatoxin B1 are associated with the presence of the furan terminal.[25,26] The power of ozone gas to eliminate aflatoxins in corn kernels was evaluated by McKenzie *et al*.,[24] who fed turkeys a diet composed of corn contaminated with mycotoxin, treated or untreated with ozone gas. The efficacy of ozone in concentrations from 148 to 179 mg L^{-1} for the degradation of aflatoxins was also evaluated by Prudente and King,[15] who observed 92% reduction in the aflatoxin content of corn kernels.

Considering that ozonation represents an alternative for the control of aflatoxins in foods, since ozone may act as a fungicidal agent and aid in reducing aflatoxin levels, and that the presence of these mycotoxins in peanuts is considered important from the point of view of public health, the purpose of this study was to evaluate the fungicidal and detoxifying effects of ozone gas on aflatoxins in peanut kernels.

MATERIALS AND METHODS

For evaluation of the fungicidal and detoxifying effects of ozone gas, peanut kernels (variety Tatu) with an average moisture content of 80 g kg^{-1} were used containing potentially aflatoxigenic fungi and aflatoxin levels lower than 20 μg kg^{-1}, which is the maximum limit established by Brazilian legislation for peanuts.[27] For acquisition of kernels with aflatoxin contamination levels greater than 20 μg kg^{-1}, a modified version of the method proposed by Prudente[28] was used. Peanut kernels were stored for 10 days in an acclimatisation chamber at a temperature of 28 ± 3 °C and a relative humidity of 95 ± 2%, conditions considered optimal for growth of aflatoxigenic fungi and production of aflatoxins.[29,30] At the end of this process, in addition to quantification of aflatoxins (200 μg kg^{-1}), the moisture content of the kernels was adjusted to 90 g kg^{-1} by means of drying. Determination of moisture content in the peanuts was performed using an oven with forced air circulation at a temperature of 130 ± 1 °C for 6 h.[31]

Ozone gas was obtained by means of an ozone generator based on the dielectric barrier discharge method developed in the Department of Physics, Technological Institute of Aeronautics, São José dos Campos, SP, Brazil. In the process of ozone generation, oxygen supplied by a Mark 5 Plus Oxygen Concentrator (Nidek Medical Products Inc., Birmingham, USA) with a purity of 90 ± 3% and no humidity was used. Ozone concentration was determined by the iodometric method[32] before each application of gas.

In the ozonation process, 1 kg samples of previously contaminated peanuts were stored in 3 L glass flasks at 25 °C and ozonated at concentrations of 13 and 21 mg L^{-1} (6151 and 9936 μL L^{-1} respectively) at a gas flow rate of 1 L min^{-1} for periods of 0, 24, 48, 72 and 96 h.

Evaluation of fungicidal effect of ozone gas in peanuts

For evaluation of the fungicidal effect of ozone gas in peanuts, two methods of quantification of fungi were used: dilution plating and direct plating.[33]

In the dilution plating method, 25 g of peanuts were combined with 225 mL of 1 g L^{-1} peptone water in a sterile polyethylene bag and pummelled with a stomacher for 2 min. In plating, dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were used. In the direct plating method, ten peanuts were used, previously disinfected with sodium hypochlorite. In the standard disinfection procedure, 20 g of sample was immersed in 100 mL of 4 g L^{-1} sodium hypochlorite solution and mixed with sterile forceps for 2 min. The sodium hypochlorite solution was then drained off and the treated grain was shaken vigorously to dislodge any excess solution. For quantification of total fungi in peanuts, in both the dilution plating and direct plating methods, a culture medium of potato dextrose agar (PDA) acidified with 100 g L^{-1} tartaric acid was used and the plates were incubated for 5 days in acclimatisation chambers at 25 °C.[34] For quantification of potentially aflatoxigenic species *A. flavus* and *A. parasiticus*, a culture medium of *A. flavus* and *A. parasiticus* agar (AFPA) was used and the plates were incubated for 42 h at 30 °C.[35] Results from the dilution plating method were expressed as colony-forming units per gram (CFU g^{-1}), while those from the direct plating method were expressed as percentage of kernels infected by fungi.

The effect of ozone on the fungi was analysed with an Olympus BX 50 optical microscope (Olympus Corporation, Tokyo, Japan) with a clear chamber and coupled imaging system, using an objective of 40×.

Evaluation of detoxifying power of ozone gas on aflatoxins in peanuts

In order to evaluate the detoxifying power of ozone gas, the concentrations of total aflatoxins and aflatoxin B1 were quantified by high-performance liquid chromatography (HPLC) in peanuts ozonated at two concentrations (13 and 21 mg L^{-1}) and exposed to the gas for periods of 0, 24, 48, 72 and 96 h.

Triturated peanut samples weighing 50 g obtained from each combination of gas concentration and exposure period were placed in 250 mL Erlenmeyer flasks. After adding 100 mL of methanol/water solution (80 : 20 v/v), the flasks were placed on an TE-141 agitator table (Tecnal Laboratory Equipment Ltd, Piracicaba, Brazil) for 30 min. Subsequently, the samples were filtered through Whatman no. 4 filter paper, 2 mL of the filtrate was transferred to a 50 mL beaker and 14 mL of phosphate buffer (pH 7.2) was added. The mixture (sample + buffer) was passed through an AflaStar™ (3 mL) immunoaffinity column (Romer Labs Inc., Union, USA). Next the column was washed with 20 mL of phosphate buffer, and finally aflatoxin elution with 1.5 mL of methanol and 1.5 mL of Milli-Q water was performed.

Chromatographic analyses were performed according to the methodology described by Blesa *et al*.[4] An LC-10AT VP (Shimadzu Corporation, Kyoto, Japan) device with a fluorescence detector (excitation 362 mm, emission 465 nm) and a C-18 column (5 μm, 150 mm × 4.6 mm) was used. The mobile phase was water/methanol/acetonitrile (60 : 20:20 v/v/v) at flow rate of 1 mL min^{-1}, and 100 μL of sample was injected. Post-column derivatisation was performed with a Kobra electrochemical cell

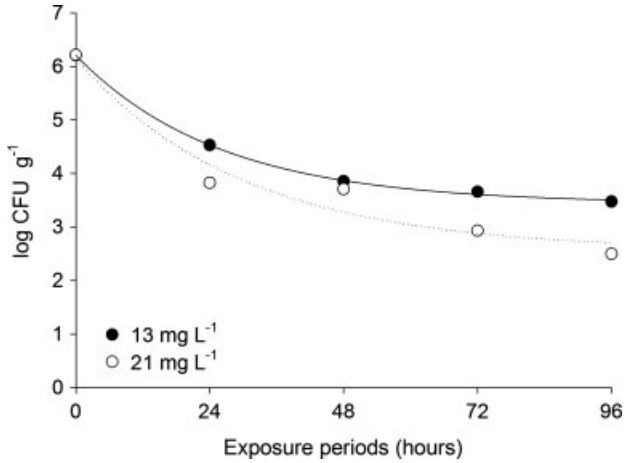

Figure 1. Colony-forming units per gram (CFU g^{-1}) of total fungi in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h.

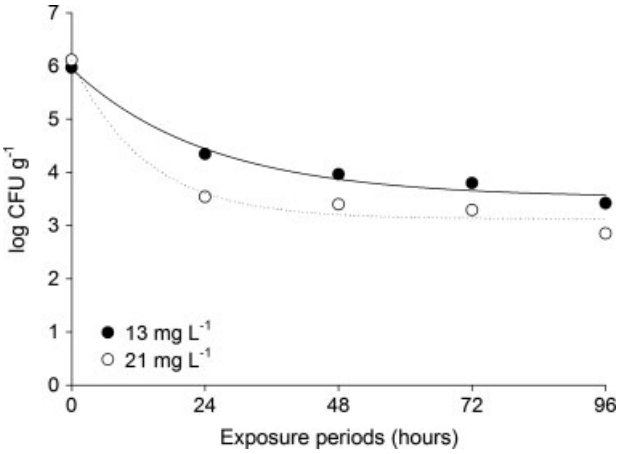

Figure 2. Colony forming units per gram (CFU g^{-1}) of *Aspergillus flavus* and *Aspergillus parasiticus* in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h.

(R-Biopharm Rhône Ltd, Glasgow, Scotland). Quantification of aflatoxins was performed by calculating the peak area of each type of mycotoxin using CLASS-VP Version 6.12 (Shimadzu Corporation, Kyoto, Japan).

Statistical analysis

The experiment employed a split-plot design, with plots representing the concentrations of ozone (13 and 21 mg L^{-1}) and subplots representing the periods of exposure to the gas (0, 24, 48, 72 and 96 h) in a completely randomised design with three replications. Initially an analysis of variance at 5% probability was performed and subsequently a regression analysis. In the variance analysis, SAEG Version 9.1 was used.[36] SigmaPlot 2001 (SPSS Science, Chicago, IL, USA) was used for acquisition of the regression equations and plotting of the graphs.

RESULTS

The effect of ozone gas at concentrations of 13 and 21 mg L^{-1} for different exposure periods on the CFU g^{-1} of total fungi and of *A. flavus* and *A. parasiticus* in peanuts is presented in Figs 1 and 2 respectively. A significant decrease ($P < 0.05$) in the count of total fungi occurred (Fig. 1) as the exposure period of peanuts to ozone gas was increased. The reduction in the count of total fungi was 2 and 3 log cycles greater when peanuts were ozonated at concentrations of 13 and 21 mg L^{-1} respectively after an exposure period of 96 h.

Similar behaviour was observed in the count of potentially aflatoxigenic species in the ozonated peanuts. A significant reduction ($P < 0.05$) in the CFU g^{-1} of *A. flavus* and *A. parasiticus* was obtained (Fig. 2) by increasing the ozone concentration and exposure period. The decrease in the CFU g^{-1} of *A. flavus* and *A. parasiticus* was approximately 3 log cycles when peanuts were ozonated at a concentration of 21 mg L^{-1} for a period of 96 h.

Table 1 shows the adjusted regression equations and their respective coefficients of determination referring to the CFU g^{-1} of total fungi and of *A. flavus* and *A. parasiticus* in peanuts exposed to ozone gas at concentrations of 13 and 21 mg L^{-1} during different periods of exposure.

Figures 3 and 4 present the percentage of kernels infected by total fungi and by *A. flavus* and *A. parasiticus* respectively during different periods of exposure to ozone gas at concentrations of 13 and 21 mg L^{-1}.

Table 1. Adjusted regression equations referring to colony-forming units per gram (CFU g^{-1}) of total fungi and of *Aspergillus flavus* and *Aspergillus parasiticus* in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h

Species	Concentration of ozone (mg L^{-1})	Adjusted equation	R^2
Total fungi	13	$y = 3.438 + 2.769e^{-0.039x}$	0.99
	21	$y = 2.560 + 3.584e^{-0.033x}$	0.96
A. flavus and *A. parasiticus*	13	$y = 3.524 + 2.427e^{-0.041x}$	0.98
	21	$y = 3.125 + 2.980e^{-0.075x}$	0.98

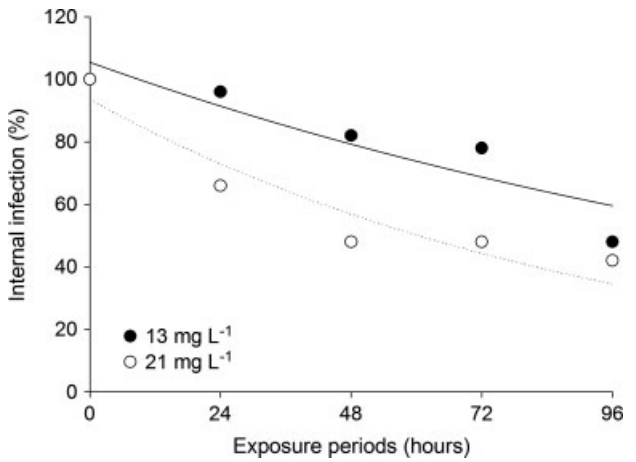

Figure 3. Percentage of internal infection by total fungi in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h.

The percentage of kernels infected by total fungi and by *A. flavus* and *A. parasiticus* was significantly reduced ($P < 0.05$) as the exposure period was increased at both 13 and 21 mg L^{-1}. The reduction in the percentage of infected kernels was more pronounced in peanuts ozonated at a concentration of 21 mg L^{-1}.

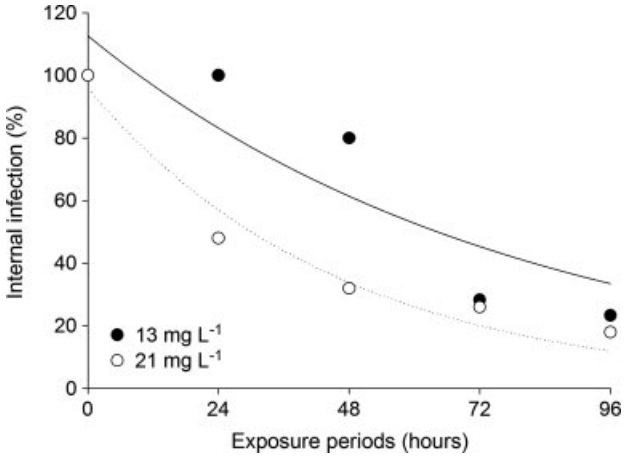

Figure 4. Percentage of internal infection by *Aspergillus flavus* and *Aspergillus parasiticus* in peanut kernels exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h.

Table 2. Adjusted regression equations referring to percentage of peanuts infected by total fungi and by *Aspergillus flavus* and *Aspergillus parasiticus* as a result of ozone application at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h

Species	Concentration of ozone (mg L^{-1})	Adjusted equation	R^2
Total fungi	13	$y = 105.489e^{-0.006x}$	0.84
	21	$y = 93.646e^{-0.010x}$	0.90
A. flavus and *A. parasiticus*	13	$y = 112.668e^{-0.013x}$	0.79
	21	$y = 95.957e^{-0.028x}$	0.96

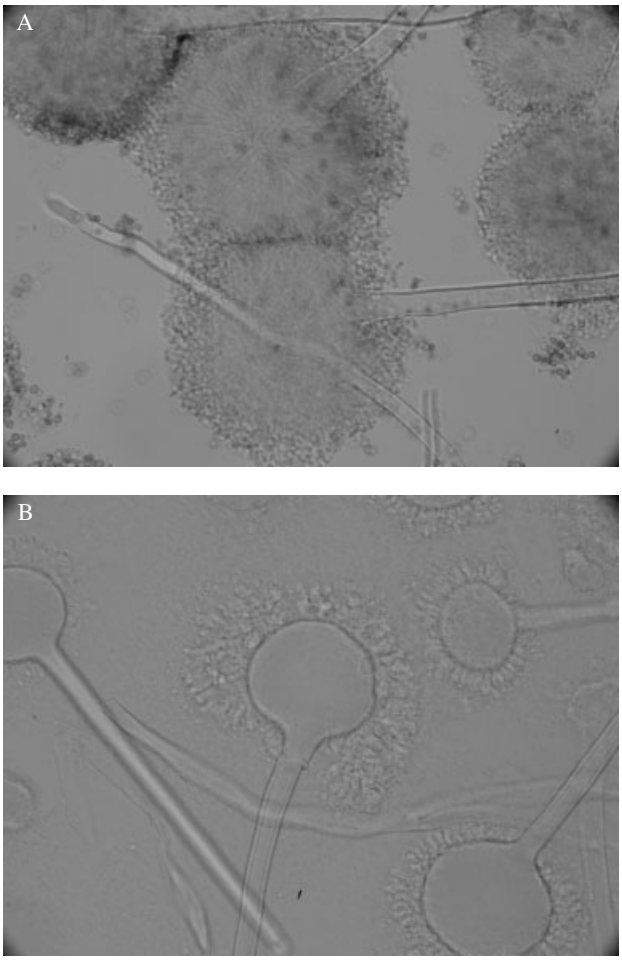

Figure 5. Morphological aspects of *Aspergillus* spp. (A) before and (B) after exposure to ozone at a concentration of 21 mg L^{-1} for 96 h, obtained by optical microscopy with an objective of 40×.

The percentage of kernels infected by total fungi (Fig. 3) and by *A. flavus* and *A. parasiticus* (Fig. 4) was 40 and 20% respectively when the exposure period was 96 h.

The adjusted regression equations and their respective coefficients of determination referring to the percentage of kernels infected by total fungi and by *A. flavus* and *A. parasiticus* during different periods of exposure to ozone gas at concentrations of 13 and 21 mg L^{-1} are shown in Table 2.

Figure 5 presents the morphological aspects, obtained by optical microscopy, of fungi from the genus *Aspergillus* collected from the peanuts exposed or unexposed to ozone gas at a concentration of 21 mg L^{-1} for 96 h. Depigmentation of fungal colonies, associated with disorganisation in the micro-organism structure, was observed. This behaviour occurred for all combinations of gas concentration and exposure time. The colonies of fungi in the untreated kernels maintained a green colour. In the kernels exposed to ozone gas at different concentrations for different periods, the colonies of fungi had a white colour.

The concentrations of total aflatoxins and aflatoxin B1 in peanuts ozonated at concentrations of 21 and 13 mg L^{-1} for different exposure periods are presented in Figs 6 and 7 respectively. A significant reduction ($P < 0.05$) in the concentrations of total aflatoxins and aflatoxin B1 was observed with increasing exposure time, with a more pronounced decrease in peanuts ozonated at a concentration of 21 mg L^{-1}. In peanuts exposed to ozone gas at a concentration of 21 mg L^{-1} for 96 h, there was a reduction in the concentrations of total aflatoxins and aflatoxin B1 of approximately 30 and 25% respectively.

Table 3 shows the adjusted regression equations and their respective coefficients of determination referring to the concentrations of total aflatoxins and aflatoxin B1 in peanut kernels ozonated at concentrations of 13 and 21 mg L^{-1} for different exposure periods.

DISCUSSION

The efficacy of ozone as an antimicrobial agent has been studied by several authors, and this gas is considered one of the most powerful sanitisers known, since it is effective across a broad antimicrobial range, including the control of fungi, bacteria, viruses and protozoans as well as spores of fungi and bacteria.[12,18,37,38] Among species previously studied that were controlled by ozone, the bacteria *Salmonella typhimurium, Escherichia coli* and *Listeria monocytogenes*, the fungi *Aspergillus níger, A. parasiticus, Aspergillus fumigatus, Colletotrichum gloeosporioides* and *Fusarium oxysporum* and the protozoan *Cyptosporidium parvum*, among others, have been repeatedly characterised.[17,39–45] It is important to emphasise that every micro-organism shows some sensitivity to ozone, in such a way that bacteria are more sensitive than fungi and yeasts, Gram-positive bacteria are more sensitive than

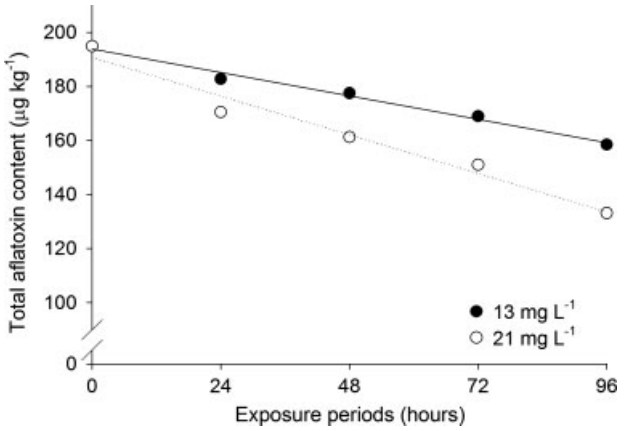

Figure 6. Total aflatoxin content in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h.

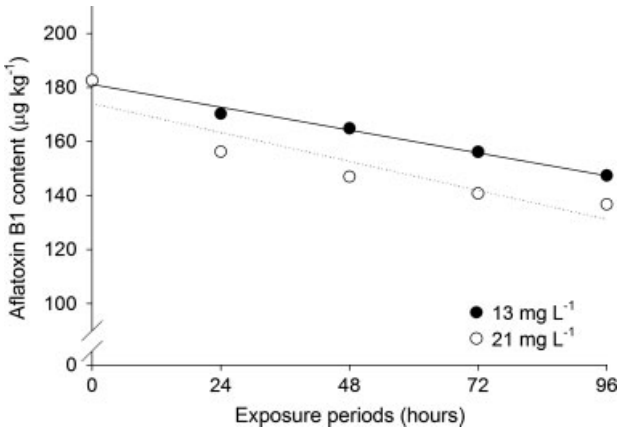

Figure 7. Aflatoxin B1 content in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h.

Table 3. Adjusted regression equations referring to total aflatoxin content and aflatoxin B1 content in peanuts exposed to ozone at concentrations of 13 and 21 mg L^{-1} after exposure for up to 96 h

Mycotoxin	Concentration of ozone (mg L^{-1})	Adjusted equation	R^2
Total aflatoxins	13	$y = 193.836 - 0.361^{**}x$	0.98
	21	$y = 190.734 - 0.596^{**}x$	0.96
Aflatoxin B1	13	$y = 181.123 - 0.352^{**}x$	0.98
	21	$y = 174.083 - 0.447^{*}x$	0.86

* Significant at 5% probability by *F* test.
** Significant at 1% probability by *F* test.

Gram-negative bacteria, and spores are more resistant than vegetative cells.[46]

The results obtained referring to the reduction in the CFU g^{-1} of total fungi and of *A. flavus* and *A. parasiticus* in the ozonated peanuts as well as the decrease in those infected (Figs 1–4) may be attributable to the high oxidative power of the gas. Ciccarese *et al.*[47] also observed a significant reduction in fungal infection in wheat, oats and peas due to exposure to ozone gas. Among the fungi evaluated by these authors, the genera *Aspergillus, Penicillium, Alternaria* and *Fusarium* were submitted to in-depth analyses.

The inactivation/inhibition of micro-organism development by ozone through the oxidation of vital cell components is a complex process in which the gas acts on parts of the membrane and the cell wall, such as unsaturated fatty acids, as well as elements of the cell content, including enzymes and nucleic acids. Micro-organisms are inactivated by disruption of the cell as a result of the action of molecular ozone or free radicals during decomposition of the gas.[22,46,48] Victorin[49] affirmed that there are two mechanisms of ozone in the destruction of biomolecules. In the first mechanism the ozone oxidises sulfhydryl and amino acids groups of enzymes, proteins and peptides. In the second mechanism the gas acts as an oxidising agent in the conversion of polyunsaturated fatty acids to peroxiacids.

The discolouration of fungi in the ozonated peanuts may be attributed to the oxidation of micro-organism pigments. Zotti *et al.*[16] also observed discolouration of *A. niger* and *A. flavus* colonies as a result of exposure to ozone. These authors attributed the discolouration of *A. niger* to the destruction of melanin, a pigment of high molecular weight formed by oxidative polymeristion of phenolic compounds. Regarding *A. flavus*, discolouration was attributed to the destruction of pigments of the anthraquinone group, which are responsible for the yellow colour of the species and are intermediaries needed for aflatoxin synthesis.[50]

Ozone gas, in addition to being an alternative for the control of micro-organisms, has been proposed as an agent for the degradation of mycotoxins, among which are the aflatoxins.[14,15,24,51,52] It should be emphasised that, although there was a significant decrease in the concentrations of total aflatoxins and aflatoxin B1 as a result of treatment with ozone at both concentrations of gas used (Figs 6 and 7), the peanuts maintained mycotoxin levels above that allowed by Brazilian legislation, which is 20 µg kg^{-1} for total aflatoxins (B1 + B2 + G1 + G2).[27]

The reduction after 96 h of peanut exposure to ozone at a concentration of 21 mg L^{-1} was approximately 30 and 25% for total aflatoxins and aflatoxin B1 respectively. Comparatively, Proctor *et al.*[53] obtained a reduction in aflatoxin B1 equivalent to approximately 70% in peanuts after ozone application of 4.2% (~60 mg L^{-1}) at 25 °C for 15 min. It is important to state that the peanuts used by these authors was artificially contaminated and thus the mycotoxin was found only on the peanut surface. The distribution of aflatoxins in kernels is not uniform and, according to Cucullu *et al.*,[54] mycotoxins may be present throughout the entire cotyledon. There are two hypotheses to explain the distribution of this mycotoxin in kernels. The first hypothesis indicates that there is a diffusion of aflatoxins from the outer part to the inner part of kernels. The second hypothesis is that mycotoxin synthesis occurs inside the cotyledon. In accordance with the second hypothesis, aflatoxigenic fungi penetrate the kernels, with subsequent synthesis of aflatoxins.[55]

There are two other aspects related to the peanut ozonation process that need to be pointed out. The first is that oxygen with no humidity was used in the generation of ozone. Therefore the relative humidity during ozonation of peanuts may have influenced the gas toxicity over micro-organisms. The greater the relative humidity, the more efficient the ozonation process is,[56] and the use of a gas mixture with low air relative humidity implies a reduction in kernel moisture content and consequently affects the efficiency of ozone in controlling fungi.[17] The second important

aspect is that, despite the high oxidative capacity of ozone, the application of the gas to peanuts, in similar conditions to those used in this study, does not affect qualitative parameters of the kernels, such as electrical conductivity, or of the crude oil, such as free fatty acids, peroxide value and iodine index.[57]

CONCLUSIONS

From the obtained results it can be concluded that ozone gas at concentrations of 13 and 21 mg L^{-1} is effective as a fungicidal agent, controlling the potential aflatoxin-producing species *A. flavus* and *A. parasiticus*. Regarding the concentrations of total aflatoxins and aflatoxin B1, the most pronounced decrease was obtained in peanut kernels ozonated at a concentration of 21 mg L^{-1} for a period of 96 h. It was not possible to obtain peanuts with a concentration of total aflatoxins lower than the tolerance limit adopted by Brazilian legislation (20 μg kg^{-1}).

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