

Research Article

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Effect of Ozone Gas on Degradation of Aflatoxin B₁ and *Aspergillus Flavus* Fungal

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Abstract

Wheat (*Triticum aestivum*) is one of the most important agricultural crops. Requirements, wheat must be produced free of hazardous contaminants. However, previous investigations showed that wheat could be contaminated by aflatoxins above the limits that may be critical for health. In this study, use of the high oxidising power of ozone achieved degradation of aflatoxin B₁. Samples were subjected to ozonation at various ozone concentrations (20, 40ppm) and exposure time (5, 10, 15, 20 min). The reduction percentages of aflatoxin B₁ in artificially contaminated wheat 10 µg/kg were 84.1 and 86.75% after exposures to 20 and 40 ppm ozone for 20min respectively, and 86.7 and 96.66 % with 20 µg/kg after exposures 20 and 40 ppm ozone for 20 min, respectively. The percentage of inhibition zone from *Aspergillus flavus* was 46.4 % to 87.8 % after ozonation using concentration 20 ppm for 5 and 20 min respectively. While with 40 ppm ozone inhibition zone was 65.6% and 95.6 % with 5 and 20 min respectively. Exposure of strain to 20 ppm ozone inhibition of product of AFB₁ percentage of 40.94%, 52.5%, 59.32% and 60.4 % for exposure time 5, 10, 15 and 20 min, respectively. However, observed increase on inhibition of AFB₁ with topping up ozone dose to 40 ppm attained 55.2%, 64%, 74.5% and 77.2% with 5, 10, 15 and 20 min exposure time respectively.

Keywords: Aflatoxin B₁; Degradation; Ozone; *Aspergillus flavus*; Wheat

Introduction

Aflatoxins (AFs) are a group of highly carcinogenic mycotoxins produced primarily by the fungus *Aspergillus flavus*. Within the group of AFs, Aflatoxin B₁ (AFB₁) is the most toxic, and it is known for its harmful effects on humans and animals. AFs contamination is a worldwide problem, especially in warmer climates, and the toxin can enter the food chain through contaminated food and feed products. They are considered an unavoidable food and feed contaminant [1].

AFB₁ is the most potent of the four naturally occurring AFs and because of its remarkable hepatotoxicity and carcinogenicity, this feed contaminant has been the focus of considerable research since its discovery [2].

According to Cardwell et al. [3] AFs contamination of agricultural crops causes annual losses of more than \$750 million in Africa. Jolly et al. [4] also reveal that post-harvest losses of crops are greater than the improvements made in primary production. In the US, it was reported that income losses due to AFs contamination cost an average of more than US\$100 million per year to US producers [5]. Toxicogenic fungi infect agricultural crops both in the field and in storage. Conditions favoring the development of mycotoxins in cereals before and after harvest are important to grain exporting countries concerned with marketing high-quality products. In post-harvest situations, crop spoilage, fungal growth, and mycotoxin formation result from the interaction of several factors in the storage environment [6-8]. These factors include: moisture, temperature, time, insect vectors, damage to the seed, oxygen levels, composition of substrate, fungal infection level, prevalence of toxigenic strains of fungi, and microbiological interactions. An understanding of the interactions involved would facilitate prediction and prevention of mycotoxin development, particularly in newly developed cereal cultivars. *Aspergillus flavus* and *A. parasiticus*, the most important.

A variety of chemical, physical, and biological treatments have been tested for their ability to reduce or eliminate the AFs in contaminated

feeds and foods [9,10]. Ozonation, an oxidation method, has recently been developed for the detoxification of AFs in foods [11]. Ozone, or triatomic oxygen (O₃), is a powerful disinfectant and oxidising agent [12]. Ozone treatment of grain is generally applied in silos or vessels. Prior to ozone application, it is necessary to characterise the dynamics of ozone movement through the various grain types to optimise ozone generators for use on large commercial storage bins [13]. Ozone either completely degrades mycotoxins or causes chemical modifications, reducing their biological activity [12,14,15]. Ozone reacts across the 8, 9 double bond of the furan ring of AFB₁ through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids [16]. There are many methods for the production of ozone, such as electrical discharge in oxygen, electrolysis of water, or thermal, photochemical or radiochemical methods. For industrial use ozone is generated mainly from pure oxygen or atmospheric oxygen in a corona discharge process [12,15]. In corona discharge, air or pure oxygen is fed into a unit that converts the oxygen to ozone using high voltage. The attractive aspect of ozone is that it decomposes rapidly (half-life of 20–50 min) to molecular oxygen without leaving a residue [17]. Several research studies have been undertaken to evaluate the effects of ozone gas in reducing AFs levels in contaminated agricultural products. Maeba H et al. [18] reported that AFB₁ and AFG₁ were sensitive to ozone and easily degraded with 1.1 mg/L of ozone within 5 min at room temperature. On the other hand, AFB₂ was resistant to ozone, requiring

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50-60 min to degrade them completely with 34.3 mg/l of ozone. Inan et al. [19] used of ozone in the detoxification of AFB₁ in red pepper. The process reduced the AFB₁ content in flaked red pepper by as much as 80% after ozonation for 60 min. Initial AFB₁ level of 32 ppb, was reduced by as much as 93% after exposure to gaseous ozone for 60 minutes. Allen et al. [20] used gaseous ozone to inactivate fungi in barley grain. They found that at an ozonation rate of 0.01 mg/g/min 39% of fungal spores were inactivated after 5 min of treatment. As the ozone rate increased, the level of spore inactivation increased; 78% inactivation at a rate of 0.07 mg/g/min and a 96% inactivation at a rate of 0.16 mg/g/min. A rate of 0.98 mg/g/min did not result in further inactivation. Wu et al. [21] reported that gaseous ozone was very effective in the inactivation of fungi associated with the wheat within 5 min of ozonation, 96.6% of the fungal spores were inactivated by applying 0.33mg of ozone (g wheat)⁻¹min⁻¹. Wheat, which is a very sensitive product for *Aspergillus spp* and AFs formation under unsuitable processing conditions, is an important product for the economy and import of Egypt. The objective of this work was to study the effect of ozone gas on degradation of AFB₁ in wheat grain and evaluate the effects of exposure to ozone gas on *Aspergillus flavus* strain producer of AFB₁.

Material and Methods

Chemicals and reagents

Aflatoxin B₁ from *Aspergillus flavus* was purchased from Sigma Chemical Co. (St. Luis, MO 63118, U.S.A.). The immunoaffinity column AflaTes[®] HPLC were obtained from VICAM (Watertown, MA, USA). Methanol, trifluoroacetic acid, and sodium chloride, were purchased from Sigma chemical Co. (St. Louis, MO, U.S.A.). All solvents were of HPLC grade. The water was double distilled with Millipore water purification system (Bedford, M A, USA). Potato dextrose agar (PDA) and Yeast extract and peptone were purchased from Sigma-Aldrich, France.

Fungal strain: Toxigenic strain of *Aspergillus flavus* (ATCC 28542) was purchased from MIRCEN, (Microbial Research Center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt).

Wheat samples

Wheat samples were obtained from the South Cairo Mills Company, Cairo, Egypt.

Method

Preparation of artificially wheat grain samples

Five replicates of each whole wheat samples were spiked with 10 and 20 µg/kg AFB₁ and followed by shaking to 10-15 min. In addition samples control with spiked without treatment (O₃) and samples control without spiked with treatment. Standard stock was prepared according to the AOAC [22] method [23].

Preparation samples treatment

Whole wheat was transferred into a 500mL flask and the sample flask was plugged with a silicone stopper with 2 holes in it. One hole was for the ozone line and the other was for tubing connected to the ozone destruct unit. The wheat was treated for 5, 10, 15 and 20 min at room temperature with two different ozone concentrations (20 and 40 ppm). Ozone gas was produced from air using ozone generator Model OZO 6 VTTL OZO Max Ltd, Shefford, Quebec Canada (<http://www.ozomax.com>).

Preparation of potato dextrose agar (PDA) and yeast extract sucrose (YES)

The (PDA) was prepared according to the manufacturers directions by adding 40 g of the dehydrated PDA to 1000 ml of distilled water. The culture media was then mixed and autoclaved at 121°C for 15 min. YES culture was carried out according to the method of Munimbazi and Bullerman [20] at following (2% yeast extract and 15% sucrose / liter distilled water) were transferred into 500 Erlenmeyer flask, and autoclaved at 121°C for 15 min.

Preparation of inocula

Preparation of spore suspensions: The fungal culture was grown on (PDA) slants at 28°C for about 14 days or until good sporulation was observed. Spores were harvested by adding growing the *A.flavus* by adding 10 ml of sterilized aqueous solution of Tween 80 (0.05% v/v) to cultures Ramakrishna et al. [24] Spore suspensions were then centrifuged at 20,000 rcf for 5 min and the Supernatants discarded. The spore concentrations were adjusted to yield a final count of 10⁵ spores/ml and the ensuing preparations were used as spore inoculum.

Effect of ozone on fungal growth from mycelia inocula

To determine the effect of ozone on mycelia inocula of the *A. flavus*, (PDA) plates were spread with aliquots of 50 µl of mycelial suspensions. After 14 days on inoculated plates were exposed to two ozone concentrations of 20 and 40 ppm for 5, 10, 15 and 20 min each three replicate for concentration with time exposure. The ozone exposed plates were compared with controls which were not treated.

Preparation of fungi for estimation of total biomass

One hundred ml of (YES) were transferred into 500 Erlenmeyer flasks, and autoclaved at 121°C for 15 min. The YES medium was inoculated with 1ml of wet biomass of *A.flavus* strain was incubated at 28°C for 14 days after treated with ozone concentrations of 20 and 40 ppm for 5, 10, 15 and 20 min each three replicate for concentration with time exposure. The biomass was harvested by centrifuging the culture broth and decanting the supernatant. The wet biomass was used as the inoculum to study the effect of ozone on overall biomass production. The percent mycelia inhibition was calculated.

Inhibition zone percentage = [(Area of fungi dead (cm) / all growth area (cm)] X 100.

Inhibition of production AFB₁ percentage = C-T/C

Where: C is sample control and T is sample treated with ozone

Extraction of AFB₁

Sample extraction: Weigh 50g sample with 10g salt sodium chloride and place in blender jar. Add to jar 200 ml methanol: water (80:20). Cover blender jar and blend at high speed for 1 minute. Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution: Pipet or pour 10 ml filtered extract into a clean vessel. Dilute extract with 40 mL of purified water. Mix well. Filters dilute extract through glass microfiber filter into a glass syringe barrel using markings on barrel to measure 4 ml.

Immunoaffinity chromatography: Pass 4 mL filtered diluted extract (4 mL = 0.2g sample equivalent) completely through AflaTest[®]-P affinity column at a rate of about 1-2 drops/second until air comes through column. Pass 5 mL of purified water through the column at a

rate of about 2 drops/second. Elute affinity column by passing 1.0 mL HPLC grade methanol through column at a rate of 1-2 drops/second and collecting all of the sample eluate (1mL) in a glass vial. Evaporated to dryness under stream of nitrogen and was determination of HPLC.

Extraction of AFB₁ of liquid media

AFB₁ was extracted according to the method described by El-Banna et al. [25] Extraction was carried out using 20 ml of chloroform (twice with 10 ml media), and homogenization for 3 min in a separation funnel. The chloroform phase was filtered through Whatman no. 3 filter paper and concentrated to dryness under a nitrogen steam.

Determination of AFB₁ by HPLC

Derivatization: The derivatives of samples and standard were done as follow:100 µl of trifluoroacetic acid (TFA) was added to samples and mixed well for 30 s and the mixture stand for 15 min. 900 µl of water: acetonitrile (9:1 v/v) were added and mixed well by vortex for 30 s. and the mixture was used for HPLC analysis.

Apparatus: 1. The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Watres 2475 Multi- Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C18 (250 x 4.6 mm i.d.), 5 µm from Waters corporation (USA). An isocratic system with water: methanol: acetonitrile 240:120:40 [26]. The separation was performed at ambient temperature at a flow rate of 1.0 mL/min. The injection volume was 20 µL for both standard solutions and sample extracts. The fluorescence detector was operated at wavelength of 360 nm for excision and 440 nm for emission.

Statistical analysis: All data were statistically analyzed using the General Linear Model of the SPSS var. 19. The significance of the differences among different methods was determined by Waller-Duncan k-ratio [27]. All statements of significance were based on probability of p< 0.05.

Results and Discussion

Effect of ozone gas on AFB₁ in artificially contaminated wheat grain

Data presented in Figure 1 showed content of AFB₁ in wheat grain samples were artificially contaminated with 10 and 20 µg/kg. AFB₁ was degraded to 3.74 ± 0.035 and 1.59 ± 0.009 µg/kg after 5 and 20 min exposure to ozone at 20 ppm respectively. While observed increases of degraded with 40 ppm ozone, degraded to 2.80 ± 0.47 and 1.33 ± 0.014 after ozonation for 5 and 20 min respectively. Wheat grains samples were artificially contaminated with 20 µg/kg AFB₁ degraded to 6.72 ± 0.039 and 2.65 ± 0.039 µg/kg after 5 and 20 min exposure to ozone at 20 ppm respectively. During ozonation at 40 ppm, the AFB₁ was degraded to 3.77 ± 0.014 and 0.668 ± 0.010 after 5 and 20 min respectively.

Data presented in Figure 2 showed the reductions of AFB₁ in artificially contaminated wheat 10 µg/kg after ozonation for 20 min at 20 and 40 ppm ozone gas were 84.1 and 86.75% respectively, While observed increase on reduction with 20 µg/kg AFB₁, it was 86.7 and 96.66 % after ozonation for 20 min at 20 and 40 ppm ozone respectively.

These results are in agreement with the available literature on AFs degradation by ozone [11,12,16,18,19,28] in which the observed reductions of AFB₁ contents of contaminated foods varied from 56% to 95%. It is proposed that the degradation begins with attack of ozone at the C₈₋₉ double bond with the net addition of two atoms of oxygen

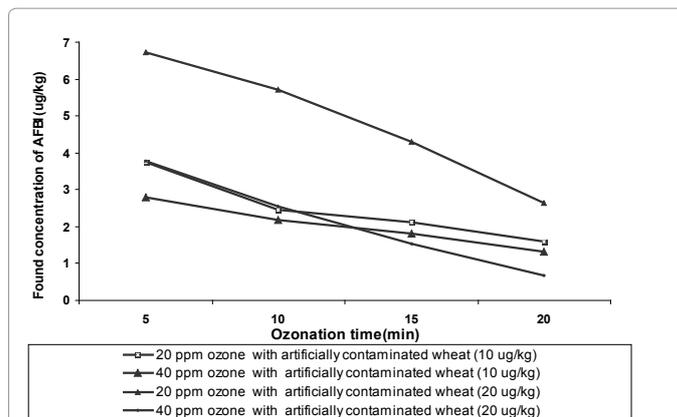


Figure 1: Effect of ozone gas on degradation of AFB₁ in artificially contaminated wheat.

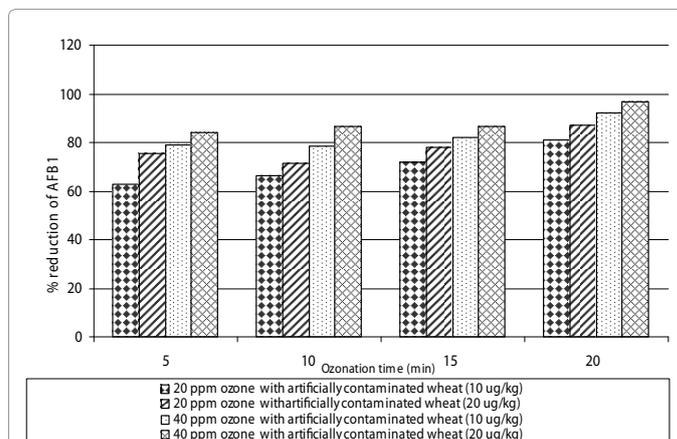


Figure 2: Effect of ozone treatment on reduction of AFB₁ in artificially contaminated wheat.

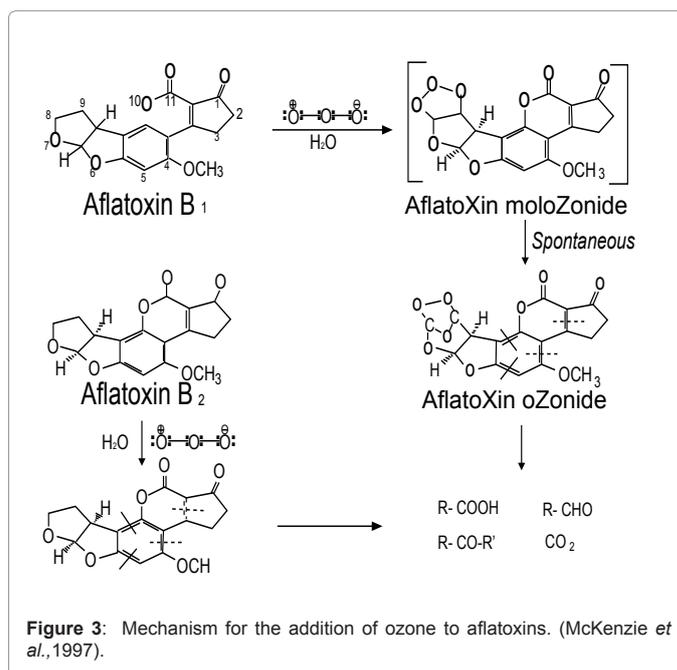


Figure 3: Mechanism for the addition of ozone to aflatoxins. (McKenzie et al., 1997).

Figure 3. It is noted here to increase the percentage of reduction with an increased concentration. Also found to increase the percentage of reduction with increasing dose of ozone this makes sense. HPLC chromatograms for AFB₁ (20 µg/kg) the artificially wheat grain samples were presented in Figure 4 for exposure times (5, 10, 15 and 20 min) after ozonation at 20 ppm.

The analysis of variance showed higher significant differences between the concentrations of AFB₁ extracted after treated with different dose of ozone. These results clearly indicated that the exposure time higher significantly than the dose of ozone. Ozone moves through grain slowly because the gas reacts with the chemical constituents present in the outer layer of grain (seed coat). Diffusion of ozone into the grain depends upon the grain characteristics [29]. Ozone adsorption in the grain layer depends on ozone concentration in the feed gas, duration of exposure, gas flow rate, temperature, grain characteristics and the presence of other organic matter such as insects and surface microbial status of the grain. Presence of moisture also plays an important role in ozone reactivity with grain because water solubilizes ozone and increase contact between gas and grain. These results were below the limit of the Egypt Food Codex, which select the level of last AFB₁ in food by 5 µg/kg.

Inhibitory effect of ozone against mycelial growth of *A.flavus*

Data presented in Figure 5 showed the percentage of inhibition zone from fungal growth was 46.4 to 87.8 % after ozonation at 20 ppm from 5 to 20 min respectively. Ozonation at 40 ppm the percentage of inhibition was 65.6 to 95.6 % with 5 to 20 min respectively. It stated that the percentage of inhibition increases with increasing time exposure and dose of ozone used. These results are consistent with; Allen et al. [20] they found that at an ozonation rate of 0.01 mg/g/min 39% of fungal spores were inactivated after 5 min of treatment. As the ozone rate increased, the level of spore inactivation increased; 78% inactivation at a rate of 0.07 mg/g/min and a 96% inactivation at a rate of 0.16 mg/g/min. Also Wu et al. [18] reported that gaseous ozone was very effective in the inactivated of fungi associated with the wheat within 5 min of ozonation, 96.6% of the fungal spores were inactivated by applying 0.33 mg of ozone (g wheat)⁻¹min⁻¹. Figure 6 appear killed zone in on PDA.

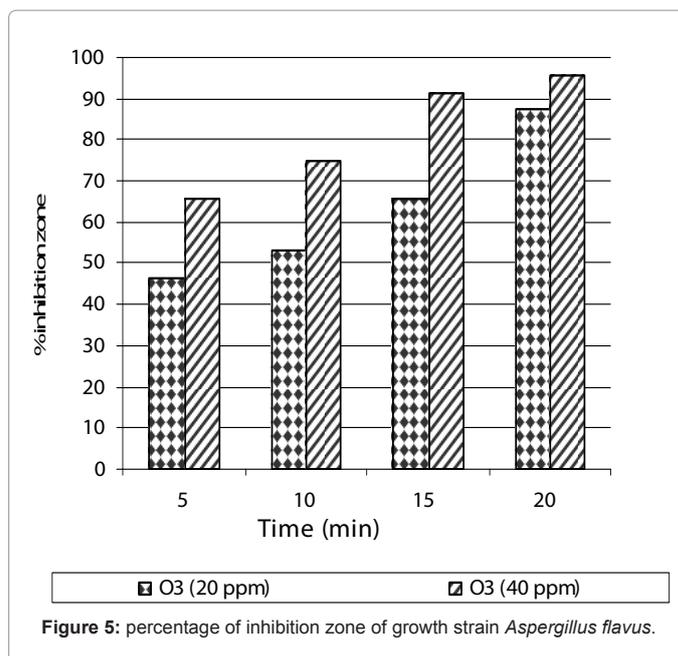


Figure 5: percentage of inhibition zone of growth strain *Aspergillus flavus*.

Ozone was thought to kill microorganisms by oxidation of cellular components such as sulphhydryl groups and amino acids of enzymes, peptides, proteins and polyunsaturated fatty acids, and oxidation of the cell membrane [30-33]. Sensitivity of microorganisms to ozone was affected by several factors including the method of applied ozone, strain of the microorganism, physiological state of cells, growth level, pH of the medium, temperature, humidity and presence of other chemicals such as acids, surfactants and sugars. In pure suspensions of bacteria, yeasts, molds, viruses and parasites, low concentrations of ozone and short contact times were sufficient to inactivation. However, the ozone concentration of water was rapidly reduced in presence of organic material in foods [34].

Returns the capacity of ozone to kill fungus and bacteria that the cell wall of the fungus is made up of several layers containing 80 % carbohydrate and 10% protein and Glycoprotein also contains many cells on the wall of the bilateral ties of sulfur, which makes them subject to oxidation by ozone [35,36]. Also imbued with ozone gas through the cell wall into the cells and combines with cytoplasm, leading to an imbalance in the components of the cell and thus her death, and inability to perform the functions of natural and coerce the inability of this fungus to produce AFs after exposure to ozone [37,38] fungal mycelium from contaminated food may be directly responsible for spread of spoilage when stored with uncontaminated food.

Therefore, it is important to stop any growth of the fungus before storage or even to kill her. With the availability of food all the conditions of temperature and humidity in the warehouse, it is easy to produce AFs.

In vitro effect of ozone gas on AFB₁ production

Data in Figure 7 show amount of AFB₁ production by *A.flavus* after ozonation at 20 and 40 ppm ozone gas. Concentration of AFB₁ in media after 5, 10, 15 and 20 min with 20 ppm ozone were 109.65 ± 0.361, 88.19 ± 0.150, and 75.52 ± 0.133 and 73.54 ± 0.029 µg/100ml media respectively. While after ozonation at 40 ppm ozone gas were 83.21 ± 0.005, 66.71 ± 66.71, and 47.28 ± 0.032 and 42.38 ± 0.088 µg/100 ml for 5, 10, 15 and 20 min respectively but in media untreated was 185.6µg/100ml.

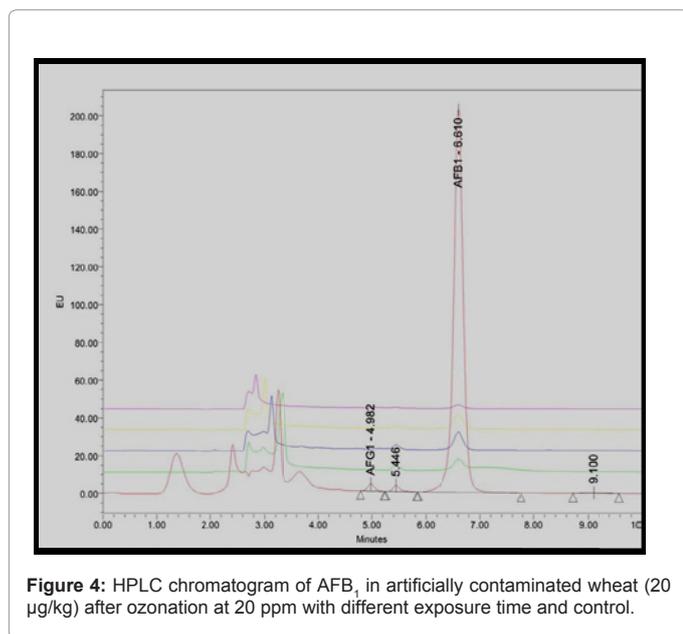
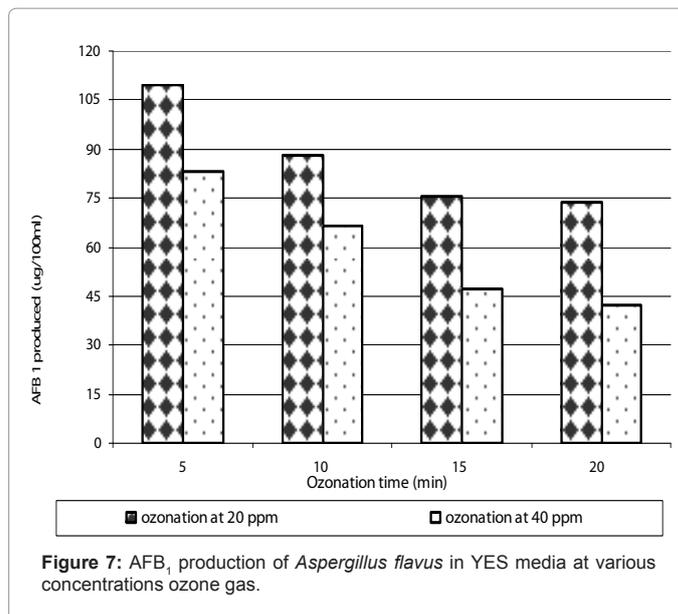
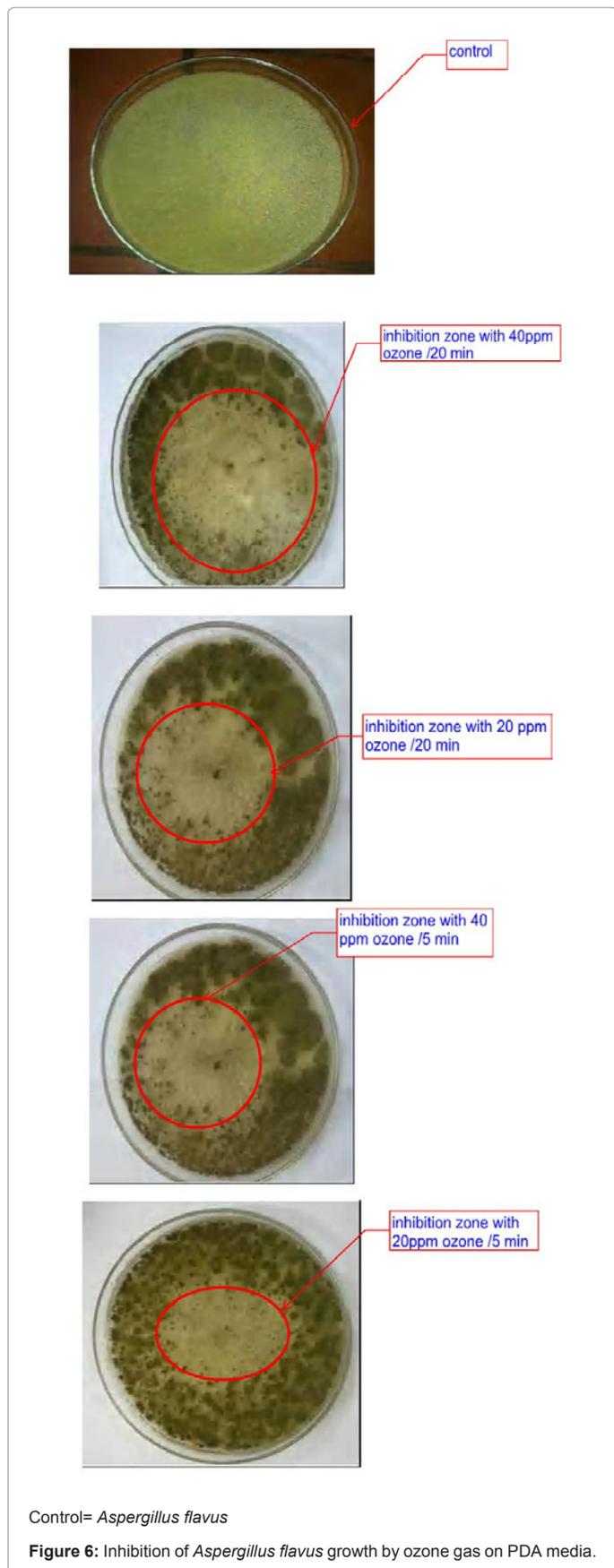


Figure 4: HPLC chromatogram of AFB₁ in artificially contaminated wheat (20 µg/kg) after ozonation at 20 ppm with different exposure time and control.



The mechanism of AFs inactivation synthesized by *A.flavus*, colonies using ozonation process. The anthraquinone pigments produced by the fungus may be help in storage for subsequent rapid conversion to AFs under this perspective; ozonation could destroy anthraquinonic intermediates, thus preventing the making of AFs [38].

Conclusion

Ozone degraded AFB₁ in wheat grains, and generally there was significant variation between ozonation time on reduction of AFB₁ and all one concentration of used ozone. Consequently, the treatment with ozone at 40 ppm for 20 min could be an effective method for the degradation of AFB₁ in wheat grain.

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