

Effect of gas barrier characteristics of films on aflatoxin production by *Aspergillus flavus* in peanuts packaged under modified atmosphere packaging (MAP) conditions

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The effects of the gas barrier characteristics of three films (ASI, ASII and ASIII) and storage temperature on the growth of, and aflatoxin production by, *Aspergillus flavus* in peanuts packaged in air and under a modified gas atmosphere of CO₂:N₂ (65:35) were investigated. Mold growth was barely visible in air packaged peanuts using high-medium barrier films (ASI and ASII) and stored at 20°C with more extensive growth occurring in air packaged peanuts stored at 25 and 30°C. Extensive mycelial growth and sporulation occurred in all air packaged peanuts in a low barrier film (ASIII), especially at 30°C. Gas packaging inhibited mold growth in peanuts packaged in a high gas barrier film at 20°C. However, mold growth occurred in gas packaged peanuts packaged in film ASII at higher storage temperatures while extensive mycelial growth was observed in all peanuts packaged in film ASIII irrespective of storage temperature. Levels of aflatoxin greater than the regulatory limit of 20 ng/g were detected in all air packaged peanuts with the highest level of aflatoxin (~76 ng/g) being detected in peanuts packaged in a high gas barrier film ASI. Aflatoxin production was inhibited in gas packaged peanuts using a high barrier film. However, higher levels of aflatoxin were detected in all gas packaged peanuts in medium-low gas barrier films (ASII and ASIII), particularly at higher storage temperatures. This study has shown that MAP using a CO₂:N₂ (65:35) gas mixture was effective in controlling aflatoxin production by *A. flavus* in peanuts to levels less than the regulatory limit of 20 ng/g. However, the antimycotic effect of low O₂-high CO₂ atmospheres is dependent on the gas barrier characteristics of the packaging films, especially at higher storage temperatures.

Keywords: Aflatoxin, peanuts, barrier films, modified atmosphere packaging.

INTRODUCTION

Modified Atmosphere Packaging (MAP) involving gas packaging is rapidly becoming the food preservation technology of the future. The technique involves packaging of products in high gas

barrier films in a mixture of gases, specifically, CO₂ and N₂ (Smith *et al.*, 1992). Recently, MAP involving gas packaging has been investigated as a means of controlling the growth of, and aflatoxin production by, *Aspergillus flavus* (Ellis *et al.*, 1993a). This aflatoxigenic mold is ubiquitous in nature and is associated with a wide variety of agricultural products. The presence of aflatoxins in these products is of public health concern due to their carcinogenic, mutagenic and teratogenic effects on living organisms (Ellis *et al.*, 1991).

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The success of gas packaging as a preservation technique is due to a number of inter-related factors specifically, the level of microbial contamination, storage temperature, level of residual oxygen in the package headspace, packaging integrity and gas barrier characteristics of the packaging material. This latter parameter is important since the oxygen transmission rate (OTR) of barrier films is influenced by the storage environment, particularly relative humidity and storage temperature (Ellis, 1993). Furthermore, many aerobic spoilage bacteria and molds have been shown to grow in relatively low concentrations of oxygen (0.5–1.0%) even in the presence of inhibitory levels of carbon dioxide (Dallyn & Everton, 1969; Smith *et al.*, 1986). These low levels of headspace oxygen can readily be reached in the package headspace due to permeation of oxygen through the packaging film (Smith *et al.*, 1986). However, there is a lack of data on the influence of the OTR of films on aflatoxin production in products packaged under MAP conditions.

Therefore, the objective of this study was to determine the effect of three films of different gas barrier characteristics on the growth of, and aflatoxin production by, *A. flavus* in peanuts packaged under a CO₂ enriched atmosphere.

MATERIALS AND METHODS

Packaging films

Three laminated polymeric films, coded ASI, ASII and ASIII were used in this study. The films had an Oxygen Transmission Rate (OTR) of 3–6, 50 and 4000 cc/m²/day, respectively at 23°C and 0% relative humidity (RH). Film ASI was obtained from Cryovac, Montreal, QC, while films ASII and ASIII were obtained from Winpak, Winnipeg, MN, Canada.

Experimental design and statistical analysis

A randomized complete block design comprising of three films (ASI, ASII and ASIII), three temperatures (20, 25 and 30°C) and three replicates per film was used as the experimental design in this study. Statistical analyses (analysis of variance, least square means and regression analysis) were computed using the Statistical Analysis System (SAS, 1982) program on the McGill University mainframe.

Preparation of spore inoculum

Aspergillus flavus ATCC strain No. 16872 obtained from ATCC, Rockville, MD, USA, was used throughout the study. The mold was grown on Czapek agar and subcultured onto slants of Malt Extract Agar (MEA) (Difco, MI, USA) for storage at 5°C. Mold inoculum was prepared by growing *A. flavus* on MEA slants for 7–10 days until complete sporulation. Spores were harvested by washing the surface of the slants with sterile 0.1% (w/v) peptone solution. The suspension was filtered twice using Whatman No. 1 filter paper and the spore load determined using the surface plating technique on Potato Dextrose Agar (PDA, Difco) and incubating plates at 30°C for 2 h. Based on the count obtained (10⁷ spores/ml), decimal dilutions were prepared to obtain the desired inoculum concentration of 10⁴ spores/ml used throughout the study. This spore load was chosen since this level of spores resulted in high amounts of aflatoxin production in previous studies on synthetic media (Ellis *et al.*, 1993b).

Preparation and inoculation of peanuts

Fresh whole peanuts were obtained from Provigo, Montreal. The peanuts were usually inspected for defects, e.g. discoloration, mold growth and defective peanuts were removed from the batch. The sound peanuts were then shelled and 250 g amounts placed in autoclavable desiccators containing glycerol solutions of known water activity (0.96). This was then sterilized by autoclaving for 15 min at 121°C. Peanuts were then allowed to equilibrate at room temperature until the desired equilibrium water activity was reached. Water activity measurements of adjusted peanuts were done on each batch of peanuts using a previously calibrated Decagon water activity meter (Decagon Device Inc. Pullman, WA, USA). All measurements were done at 25°C and had an accuracy of $\pm 0.5\%$. Random samples of the sterilized peanuts were checked for sterility using Potato Dextrose Agar (PDA, Difco). All peanuts tested negative for mold growth. Fifty gram amounts of the sterile peanuts were then weighed into 150 $\times$ 15 mm petri dishes and inoculated with 10⁴ spores of *A. flavus*. For each packaging condition, three inoculated plates and one non-inoculated plate (control) were packaged.

Packaging

Peanuts were packaged in two atmospheres; (i) air, and (ii) CO₂:N₂ (65:35% v/v). Packaging in air

was done by placing plates in 210 x 210 mm bags in each of three packaging films and sealing with a pulse heat sealer. Packaging and sealing of packages in a CO₂ enriched atmosphere was done using a Multivac vacuum/gas packaging unit (model AG500, W.R. Grace and Co., Ajax, ON, Canada). Each package was filled with a 65:35 ratio of CO₂:N₂ gas mixture. A Smith's proportional mixer (Tescom, Minneapolis, MN) was used to give the desired proportions of CO₂ and N₂ in each package. Packaged plates were incubated 25 and 30°C in a Precision Gravity Convection incubator (model 2EG, CanLab, Montreal, QC, Canada) and at 20°C in a Labconco Scientific incubator (CanLab, Montreal, QC, Canada) for 15 days.

Headspace gas analysis

Headspace gas analysis was done immediately after packing to ensure that the desired concentration of gases had been attained and throughout the storage period. Gas samples (0.5 ml volume) were withdrawn using a 0.5 ml gas-tight Pressure-lok syringe, through silicon seals attached to the outside of each package. Headspace gas was analysed using the Varian gas chromatograph (model 3300, Varian Canada Inc., Montreal, Canada) fitted with a thermal conductivity detector and using Porapak Q (80–100 mesh) and Molecular Sieve 5A (80–100 mesh) columns in series. Helium was used as the carrier gas with a flow rate of 30 ml/min. The column oven temperature was set at 150°C, the injector and detector temperatures were set at 160 and 200°C, respectively. Peaks were recorded and analysed with a Hewlett Packard integrator (model 3390A).

Extraction and quantitation

Extraction of aflatoxin B₁ was done using the Mycosep Multifunctional Cleanup (MFC) column technique of Wilson and Romer (1991). The contents of each plate after 15 days of incubation was extracted using 100 ml acetonitrile–water (9:1) in a 250 ml blender. Blending was carried out for 3 min at high speed after which the contents were filtered using Whatman's No. 1 filter paper and a 4.25 cm Buchner filter funnel under minimal vacuum. Aliquots of 5 ml of the filtrate were collected into 10 ml culture tubes and the flanged-end of the Mycosep MFC column pushed into the culture tubes until ca. 0.5 ml extract is purified. Two hundred µl of the purified extract was derivatized

with 700 µl derivatizing agent (10 ml reagent grade trifluoroacetic acid, 5 ml reagent grade glacial acetic acid and 35 ml distilled water). The derivatized solution was filtered prior to liquid chromatographic analysis using 0.22 µm Millex-GV millipore filter unit (Millipore Canada Ltd., Mississauga, ON). Detection and quantitation of the derivatized extract for aflatoxin B₁ was done using a Beckman System Gold liquid chromatographic system, consisting of a Beckman System Gold programmable module 126. Aflatoxin B₁ was detected using a Beckman 157 fluorescence detector, with a 7–60 excitation filter and a 450 nm (40 nm BW) emission filter attached to a Beckman System Gold Analog Interface module 406 (Beckman Instruments, Mississauga, ON, Canada). Detection was done at 0.5 R.F.U. (Relative fluorescence unit) and the solvent flow rate set at 2.0 ml/min. Concentration of aflatoxin B₁ was calculated by comparison to standard peaks of known concentration of aflatoxin B₁ as outlined in the Beckman System Gold methodology.

RESULTS

Mold growth

The combined effect of storage temperature and packaging film type on the extent of visible growth of *Aspergillus flavus* in peanuts packaged in air and under a modified atmosphere (65% CO₂:35%N₂) after 15 d is shown in Table 1. A 15 d storage period was chosen for comparison with previous inoculation studies (Ellis, 1993; Ellis *et al.*, 1993a,b). Visible growth, i.e. to the naked eye, was chosen as the most natural and practical means of assessing mold spoilage of packaged peanuts by consumers. Mold growth was not observed in any of the uninoculated plates indicating that peanuts had not been contaminated by airborne mold spores during inoculation. For air packaged peanuts in a high gas barrier film ASI, (~OTR 3–6 cc/m²/d) or a medium barrier film ASII, (OTR ~50 cc/m²/d) mold growth and sporulation was barely visible after 15 d storage at 20°C (Table 1). However, when peanuts were packaged in these films under similar gaseous conditions and stored at 25 and 30°C, mold growth was observed after only 3–5 d (Table 1). Extensive mycelial growth and sporulation occurred in all air packaged peanuts in a low barrier film ASIII (OTR 4000 cc/m²/d) after 1–5 d with growth and

Table 1. Effect of packaging films and storage temperature on the extent of visible mold growth in peanuts packaged in air and in a modified gas atmosphere (65% CO₂:35% N₂)

Film type	Storage temp (°C)	Packaging conditions	Days to visible mold growth ^a	Extent of visible growth at 15 d ^b
ASI	20	Air	~12	±
	25	Air	3-5	1+
	30	Air	2-4	1+
ASII	20	Air	~14	±
	25	Air	3-4	2+
	30	Air	2-4	2+
ASIII	20	Air	4-5	3+
	25	Air	2-3	3+
	30	Air	1-2	4+
ASI	20	CO ₂ :N ₂	>15	—
	25	CO ₂ :N ₂	~15	±
	30	CO ₂ :N ₂	12-14	±
ASII	20	CO ₂ :N ₂	>15	—
	25	CO ₂ :N ₂	13-14	1+
	30	CO ₂ :N ₂	~14	1+
ASIII	20	CO ₂ :N ₂	7-8	2+
	25	CO ₂ :N ₂	5-6	3+
	30	CO ₂ :N ₂	3-4	4+

^a Mean of three replicates.

^b — = No visible growth; ± = barely visible growth; 1+ = light growth; 2+ = medium growth; 3+ = heavy growth; 4+ = extensive growth.

sporulation being most extensive in peanuts stored at 30°C (Table 1).

Mold growth was inhibited in all peanuts packaged in a CO₂:N₂ (65:35) gas mixture in film ASI and ASII at lower storage temperatures (Table 1). However, barely visible to light mold growth was observed in all gas packaged peanuts in these films after 12-15 d at 25-30°C. Medium to extensive mycelial growth occurred in all gas packaged peanuts in film ASIII after 2-8 d (Table 1).

Aflatoxin B₁ production

The effect of the various packaging and storage conditions on aflatoxin B₁ production by *A. flavus* are shown Figures 1-2. Levels of aflatoxin B₁, greater than the regulatory limit of 20 ng/g or 20 ppb, were detected in all air packaged peanuts with the highest level of aflatoxin (~76 ng/g) being detected in peanuts packaged in film ASI at 20°C (Fig. 1). Lower levels of aflatoxin B₁ were detected in air packaged peanuts in more permeable films to O₂ and CO₂, i.e. film ASII and ASIII, particularly at higher storage temperatures (Fig. 1). Statistical analysis of the results for air packaged

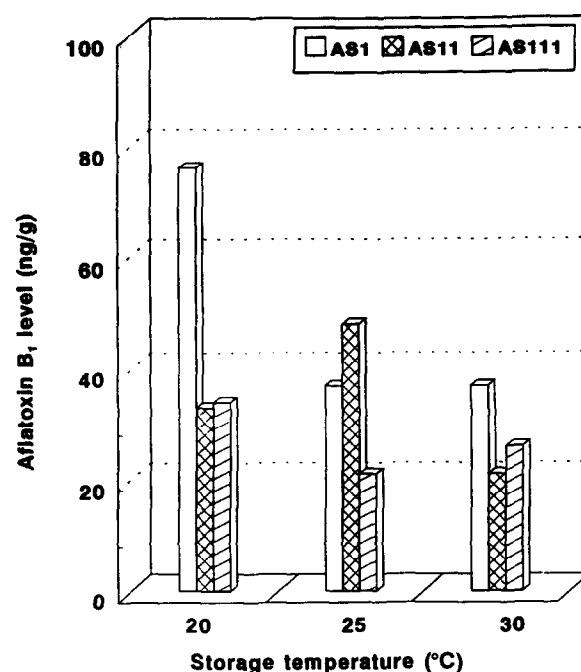


Fig. 1. Effect of storage temperature and packaging film on aflatoxin B₁ production in air packaged peanuts.

peanuts showed that the model used was highly significant ($P \leq 0.001$) with a coefficient of determination of 0.91. The results also showed that storage temperature, film type and the interaction between these two variables had a significant effect ($P \leq 0.001$) on the level of aflatoxin B₁ produced

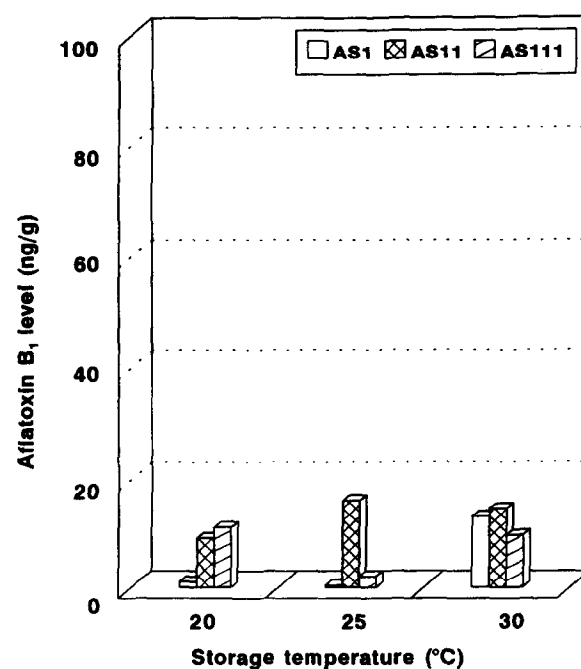


Fig. 2. Effect of storage temperature and packaging film on aflatoxin B₁ production in gas packaged (65% CO₂:35% N₂) peanuts.

by *A. flavus* in air packaged peanuts. A comparison of the means of the three films showed that film ASI was significantly different ($P \leq 0.001$) from film ASII and ASIII with respect to aflatoxin production in air packaged peanuts.

The level of aflatoxin B₁ detected in all gas packaged peanuts (65% CO₂:35% N₂) was always less than the regulatory limit of 20 ng/g (Fig. 2). However, higher levels of aflatoxin were consistently found in gas packaged peanuts in high-medium gas barrier films at higher storage temperatures or in film ASIII at all storage temperatures (Fig. 2). Statistical analysis of aflatoxin production in gas packaged peanuts also showed that film type, storage temperature and the interaction between these variables were again highly significant ($P \leq 0.001$) with respect to aflatoxin production in gas packaged peanuts. A comparison of the means showed that film ASI was significantly different ($P \leq 0.001$) from film ASII and ASIII and that it would be the most appropriate film to use in conjunction with gas packaging to control growth of, and aflatoxin production by *A. flavus* to < 20 ng/g, particularly under higher temperature storage conditions.

Changes in headspace gas composition

Changes in headspace gas composition for peanuts packaged in films ASI, ASII and ASIII are shown in Figures 3–4. Since the trends in headspace gas composition were similar for both air and gas packaged peanuts at all storage temperatures, only the changes in gas composition for peanuts stored at 30°C are shown. For air packaged peanuts in film ASI and ASII (Fig. 3), headspace O₂ decreased rapidly to $< 1\%$ within 1–2 d, with a concomitant increase in headspace CO₂ to $\sim 40\%$ as a result of mold metabolism. The gradual decrease in CO₂ concentration throughout storage may be due either to the adsorption of CO₂ into the pores of the peanuts (Holaday *et al.*, 1979), its loss through the packaging film, or its incorporation into the biosynthetic pathway for aflatoxin production (Hsieh & Mateles, 1971). Higher levels of residual O₂ (2–3%) and lower levels of CO₂ (10–20%) were consistently observed in air packaged peanuts in film ASIII at 30°C (Fig. 3). The changes in headspace gas composition for air packaged peanuts in film ASIII were significantly different ($P \leq 0.001$) than films ASI and ASII and can be attributed to the lower gas barrier characteristics of film ASIII.

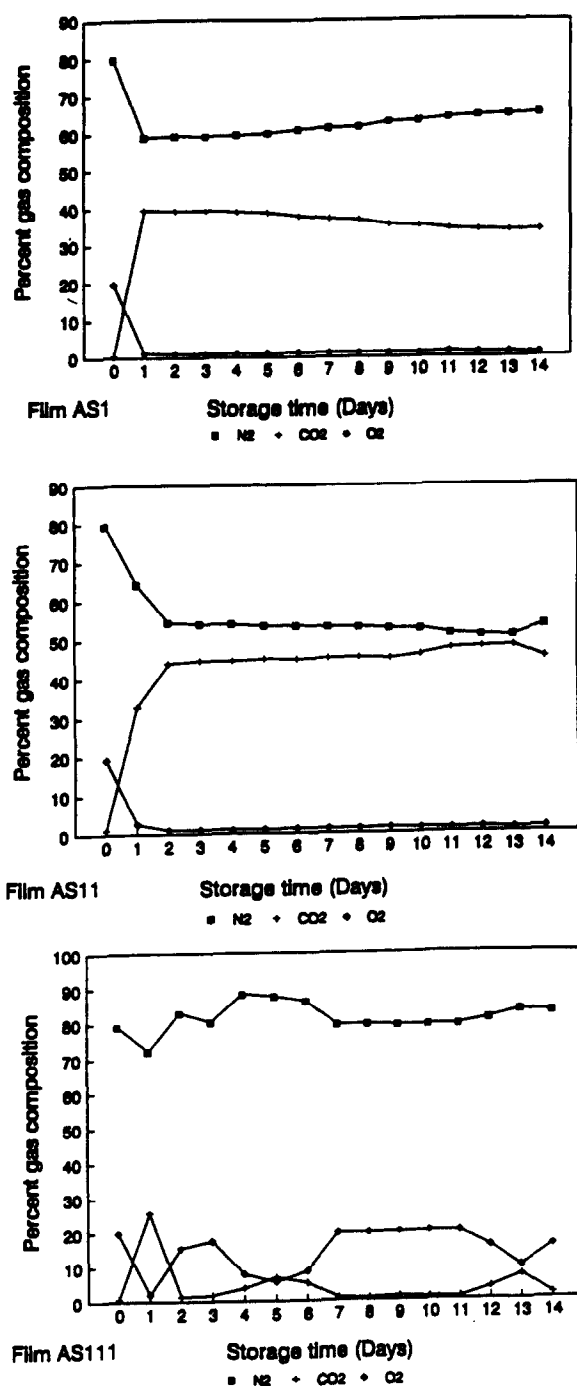


Fig. 3. Changes in headspace gas composition for air packaged peanuts in films ASI, ASII and ASIII and stored at 30°C.

Changes in the headspace composition for gas packaged peanuts at 30°C are shown in Figure 4. Similar trends were observed for changes in headspace gases using films ASI and ASII, i.e. an initial decrease in N₂ to $\sim 20\%$ and a gradual increase in headspace CO₂ to $\sim 80\%$, levels which were maintained throughout the storage period (Fig. 4). For gas packaged peanuts in film ASIII, headspace O₂ fluctuated between 2 and 8%

throughout storage while headspace CO_2 decreased from 60% to 10–20% with a corresponding increase in headspace N_2 (Fig. 4). Changes in the headspace gas composition for gas packaged peanuts in film ASIII were again significantly different ($P \leq 0.01$) from film ASI and ASII (Fig. 4). These changes can again be attributed to the lower barrier properties of film ASIII to both CO_2 and O_2 , particularly at higher storage temperatures (Ellis, 1993).

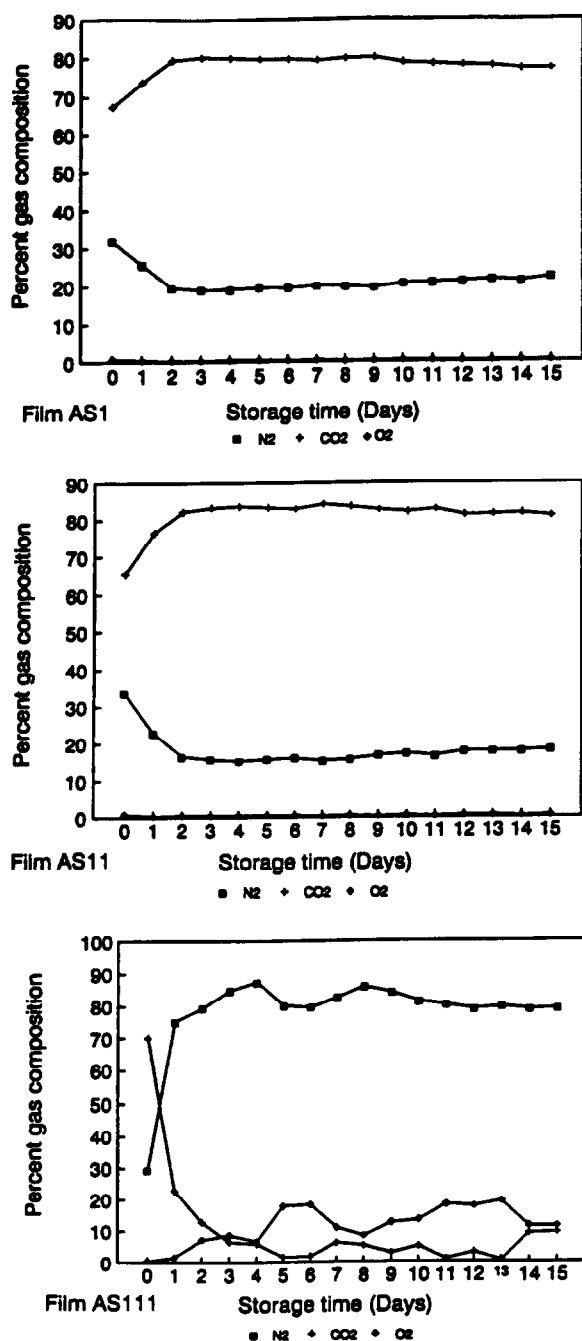


Fig. 4. Changes in headspace gas composition for gas packaged (65% CO_2 :35% N_2) peanuts in films ASI, ASII and ASIII and stored at 30°C.

DISCUSSION

The gas barrier properties of polymeric packaging films are classified as high, medium or low based on their transmission to O_2 and CO_2 under standard test conditions. On the basis of this classification, the OTR of the films used in this study was $\text{ASI} < \text{ASII} < \text{ASIII}$. Previous studies by Ellis (1993) showed that the OTR of these films to both O_2 and CO_2 increased as the storage temperature increased. For example, film ASI, a high barrier film to O_2 (10 $\text{cc/m}^2/\text{d}$) at ambient temperature ($< 25^\circ\text{C}$), exhibited medium barrier properties to O_2 (10–100 $\text{cc/m}^2/\text{d}$) at temperatures $> 25^\circ\text{C}$ while at even higher temperatures ($> 35^\circ\text{C}$), it became a low barrier film to O_2 (100–200 $\text{cc/m}^2/\text{d}$) (Ellis, 1993). These changes in film permeability are due to the greater mobility of the crystalline regions of the polymeric chains, and hence an increase in the size of the film pores at higher temperatures, thereby affecting the diffusion of gases through films (Ellis, 1993).

The extent of mold growth and aflatoxin production observed can be attributed to the different gas barrier characteristics of the films used in this study and to changes in headspace gas composition within the packaged products. The barely visible mold growth in the air packaged peanuts in films ASI and ASII at 20°C (Table 1) is due to the microenvironment within the packaged peanuts, i.e. it behaves as a modified atmosphere (Fig. 3). The low level of residual O_2 ($< 1\%$) and the elevated levels of CO_2 ($\sim 40\%$) as a result of mold metabolism would have a substantial effect on further hyphal growth and sporulation of *A. flavus*. Paster (1991) reported that the viability of *A. flavus* decreased when the levels of O_2 decreased and CO_2 increased within the package headspace. Similar observations were reported by Wilson *et al.* (1985) who showed that the growth of *A. flavus* decreased in products stored in a 60% CO_2 atmosphere. The more extensive mycelial growth and sporulation in all other air packaged peanuts, particularly in film ASIII, is due to the lower barrier properties of films to both O_2 and CO_2 at higher storage temperatures.

While gas packaging inhibited mold growth in peanuts packaged in high barrier films ASI and ASII at 20°C , growth of *A. flavus* was observed in gas packaged peanuts in these films at 25 and 30°C . The growth can be attributed to the residual headspace O_2 in peanuts packaged in films ASI and ASII, which ranged between 0.5 and 1% (Fig. 4).

The level of residual O_2 in the package headspace could be due to (i) inadequate evacuation and gas flushing; (ii) leakage through poor sealing; (iii) ability of product to trap air; and (iv) changes to the permeability of the film, particularly at higher storage temperatures. The extensive mold growth observed in all gas packaged peanuts in film ASIII can also be attributed to higher levels of residual O_2 (2–8%) and lower levels of headspace CO_2 (10–20%) as a result of the lower barrier properties of this film. Miller and Golding (1949) observed that molds require very low oxygen concentrations for growth and reported that the growth of *Aspergillus* and *Penicillium* spp. was only inhibited when the concentration of oxygen was $< 0.5\%$. Similar observations were made by Wilson *et al.* (1975), who reported the growth of *A. flavus* and *Fusarium moniliforme* in corn stored under a controlled atmosphere containing $< 5\%$ O_2 . Smith *et al.* (1986) also observed that molds can grow in relatively low concentrations of O_2 (0.5–2%) even in the presence of inhibitory levels of CO_2 .

The levels of aflatoxin detected in both air and gas packaged peanuts were also influenced by the extent of mold growth, headspace gas composition and the OTR of the three packaging films ASI, ASII and ASIII. The level of aflatoxin B_1 detected in air packaged peanuts was consistently higher than the upper regulatory limit of 20 ng/g or 20 ppb. Furthermore, there was an interesting trend in aflatoxin production in air packaged peanuts. Higher levels of aflatoxin B_1 were consistently detected in air packaged peanuts in a high barrier film (ASI) to both O_2 and CO_2 , with lower levels being detected in all air packaged peanuts in a low barrier film (ASIII) to these gases. These results imply that higher CO_2 concentrations, which were consistently found in all air packaged samples in films ASI and ASII (Fig. 3), may play a role in higher aflatoxin production due to its incorporation into the biosynthetic pathway. However, Ellis *et al.* (1993a) reported no significant correlation between changes in headspace CO_2 with time and the level of aflatoxin detected in synthetic media. The higher levels of aflatoxin concentration in peanuts packaged in films ASI and ASII could also be due to lower headspace O_2 in these packages and hence less oxidation and breakdown of pre-cursor products necessary for aflatoxin production (Shih & Marth, 1974). They observed that aflatoxin levels were always higher in stationary cultures i.e. cultures of *A. flavus* compared to agitated cultures. In stationary cultures i.e. similar to

conditions of peanuts packaged in the high barrier film ASI, there was greater Embden–Mayerhoff Pathway activity and less oxidation of acetate and NADH resulting in an increase in aflatoxin biosynthesis (Shih & Marth, 1974). Conversely, in agitated cultures (equivalent to low barrier film ASIII), a greater oxidation of acetate and NADH was observed resulting in less available precursors for aflatoxin production. Whatever the reason, it would appear from these results that packaging film permeability plays a significant role in the level of aflatoxin production in air packaged samples.

It is evident from this study that gas packaging is an effective means of inhibiting aflatoxin B_1 production by *A. flavus*, particularly in peanuts packaged in a high barrier film (ASI). This is due to the inhibitory effect of the residual O_2 ($< 1\%$) and high levels of CO_2 and N_2 initially present in the package headspace (Fig. 2). Landers *et al.* (1967) reported that aflatoxin production in groundnuts decreased as headspace CO_2 concentration increased from 0.03 to 100% or O_2 concentration decreased from 21% to 1%. Landers *et al.* (1967) concluded that high concentrations of CO_2 rather than low concentrations of oxygen were the primary cause for inhibition of aflatoxin production under optimal temperature conditions for mold growth. Fabbri *et al.* (1980) also found that a N_2 enriched atmosphere markedly inhibited the level of aflatoxin B_1 and B_2 detected in wheat. The depressant effect of N_2 on aflatoxin production was also reported by Serafini *et al.* (1980) in their studies with wheat stored in 100% N_2 . The higher levels of aflatoxin detected in gas packaged peanuts using film ASIII can be attributed to the gaseous conditions (~ 2 –8% O_2 and 10–20% CO_2) which are conducive to both mold growth and aflatoxin production. Magan and Lacey (1984) and Gibb and Walsh (1980) observed a stimulatory effect of 5–10% CO_2 on aflatoxin production at reduced O_2 levels. The authors concluded that the beneficial action of CO_2 was due to its fixation resulting from the enhanced nutritional requirements of molds under reduced oxygen conditions (Magan & Lacey, 1984; Gibb & Walsh, 1980).

In conclusion, this study has shown that MAP involving a $CO_2:N_2$ (65:35) gas mixture was effective in controlling the production of aflatoxin B_1 by *A. flavus* in peanuts to levels below the regulatory limit of 20 ng/g. However, the antimycotic effect of CO_2 enriched atmospheres is dependent on the barrier characteristics of the packaging films, especially at higher storage temperatures.

To ensure product safety, peanuts should be gas packaged in a high barrier film to both O₂ and CO₂, e.g. film ASI. Additional control could be achieved by incorporating an oxygen absorbent to scavenge any residual O₂ in the gas packaged product.

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