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EFFECT OF MODIFIED ATMOSPHERE PACKAGING ON THE GROWTH AND AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS* UNDER TROPICAL ENVIRONMENTAL STORAGE CONDITIONS

by

William Otoo Ellis

Department of Food Science and Agricultural Chemistry
Macdonald Campus, McGill University
Montreal, Quebec

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March, 1993.

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Suggested short title:

EFFECT OF MAP ON AFLATOXIN PRODUCTION

To my dear wife, Florence and my beloved daughters, Emmanuella and Theodora-Ann.

TO

WILLIAM ELLIS

"A man cannot be measured by the colour of his skin, or by his speech, or by his clothes and jewels, but only by his heart"

from SINUE THE EGYPTIAN
by Mika Waltari

FOREWORD

This thesis is submitted in the form of original papers suitable for journal publications. The first two sections is a general introduction and a literature review presenting the theory and previous knowledge on this topic. The next seven sections represent the body of the thesis (each is a complete manuscript). The last section is a summary of the major conclusions. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines Concerning Thesis Presentation, section 7 "Manuscripts and Authorship" which are as follows:

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Although all the work reported in this thesis is the responsibility of the candidate, the project was supervised by Dr. James P. Smith, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

ABSTRACT

The combined effect of Modified Atmosphere Packaging (MAP) involving gas packaging, oxygen absorbent and other environmental factors to control aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* in both synthetic media and peanuts were studied using a process optimization technique termed Response Surface Methodology (RSM). Regression analysis of the data indicated that water activity (a_w), pH, storage temperature, initial concentration of headspace oxygen and inoculum level were all highly significant factors ($p < 0.001$) in controlling aflatoxin production by *A. flavus* and *A. parasiticus* in both synthetic media and packaged peanuts. Toxin production was usually higher than recommended levels of safety (20 ng/g) in studies using higher inoculum levels (10^2 - 10^4 spores/g). At lower inoculum levels (10^1 spores/g), aflatoxin production was always less than 20 ng/g irrespective of the packaging/storage conditions. Subsequent studies with peanuts inoculated with high levels of aflatoxigenic mold spores (10^4 spore/g) and packaged under various modified atmospheres showed that the level of residual oxygen in the package headspace played a critical role in controlling the level of aflatoxin detected. Generally, the level of aflatoxin production by *A. flavus* and *A. parasiticus* was well below the recommmended safety level when inoculated media/peanuts were stored under low oxygen atmospheres obtained by oxygen absorbent technology compared to similar products packaged under a CO_2:N_2 (60:40) enriched atmosphere. However, the effectiveness of all MAP products was reduced at higher storage temperatures due to changes in the barrier characteristics of high barrier films used in the study irrespective of the packaging conditions

Studies were done to examine the effects of storage temperature and relative humidity on the gas transmission rates and permeability coefficients of high, medium and low barrier films to oxygen, carbon dioxide and water vapour. These studies confirmed earlier observations in MAP storage studies that the barrier characteristics of all three films are reduced, particularly at high storage temperatures and high relative humidity (> 90%). These changes in the barrier characteristics influenced the headspace gas composition within the product and under modified atmospheres hence the level of aflatoxin detected in these stored products.

In conclusion, this study has shown that the combined effect of several "barriers" can be used in conjunction with low oxygen modified atmosphere and high barrier packaging films to inhibit or reduce aflatoxin to safe and acceptable levels, particularly at abusive temperatures encountered during storage.

RESUME

L'effet combiné de l'emballage sous atmosphère modifiée (EAM) en présence de piège à oxygène et d'autres facteurs environnementaux pour contrôler la production d'aflatoxines, par *Aspergillus flavus* et *Aspergillus parasiticus*, en milieu synthétique et sur des arachides fut étudié par la méthode des réponses de surface (MRS). Une analyse de régression a montré que l'activité de l'eau (a_w), le pH, la température de conservation, la concentration initiale en oxygène ainsi que la quantité d'inoculum étaient des facteurs hautement significatifs ($p < 0.001$) pour contrôler la production d'aflatoxine par *A. flavus* et *A. parasiticus* dans les deux types de milieu de culture et les arachides emballées. Le niveau de production de toxine était généralement plus haut que la limite permise (20ng/g) en utilisant les quantités d'inoculum les plus élevées (10^2 - 10^4spores/g). Quand des quantités réduites d'inoculum (10^1spores/g) étaient utilisées, la production d'aflatoxine était toujours inférieure au 20ng/g quelque soit les conditions d'emballage et d'entreposage. Des études réalisées avec des arachides inoculés avec de fortes quantités de spores (10^4spores/g) et différentes conditions d'emballage ont montré que la quantité d'oxygène résiduel joue un rôle important dans le contrôle du niveau d'aflatoxine detecté. Des études comparatives sur la quantité d'aflatoxine produite par *A. flavus* et *A. parasiticus* sur les deux milieux de cultures (milieu synthétique et arachides) en présence de divers types d'atmosphère modifiées ont été réalisées. La production d'aflatoxine par les deux germes, sur les deux types de milieu, était beaucoup plus faible que le niveau recommandé quand les deux milieux de culture étaient conservés en présence d'un piège à oxygène, pour diminuer la quantité d'oxygène présent dans l'emballage, ainsi que lorsque l'atmosphère modifiée avait pour composition enrichie CO_2/N_2

(60:40). Cependant, l'efficacité de tous les types d'emballages sous atmosphère modifiée était réduite pour les plus hautes températures de conservation, à cause de changements induits dans les caractéristiques des films à hautes barrières utilisés dans cette étude. Des études furent réalisées pour connaître les effets de la température de conservation et l'humidité relative sur la vitesse d'échange des gaz et la perméabilité (haute, moyenne ou faible) des films barrière par rapport à l'oxygène, au dioxide de carbonne et aux vapeurs d'eau. Ces expériences ont confirmé les observations déjà connues sur l'entreposage sous atmosphère modifiée, montrant que les caractéristiques de barrière des trois types de film sont réduites, en particulier à haute températures de conservation et haute humidité relative (100%). Ces changements dans les caractéristiques des barrières influencent la composition en gaz du produit emballé et favorisent la production d'aflatoxine dans le produit emballé.

En conclusion, cette étude a montré que l'effet combiné des films d'emballage de haute barrière et d'une atmosphère pauvre en oxygène inhibent ou réduisent la production d'aflatoxine à un niveau non dangereux et acceptable en particulier à des températures extrêmes de conservation.

PREFACE

CLAIM OF ORIGINAL RESEARCH

(1) The use of Response Surface Methodology (RSM) to study the combined effects of several environmental/storage conditions on aflatoxin production by *Aspergillus flavus* in synthetic media and peanuts.

(2) The use of novel methods of atmosphere modification involving oxygen absorbers, oxygen absorbers/carbon dioxide generators to control aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* in peanuts.

(3) The effect of temperature and relative humidity on barrier characteristics of films used in MAP and the influence of changes in these barrier characteristics on the level of aflatoxin production by *A. flavus* and *A. parasiticus* in peanuts packaged under various modified atmospheres.

(4) The development of models to predict the effect of various storage conditions on the gas barrier properties of films and to predict the levels of environmental/storage conditions to reduce the level of aflatoxin production to safe and acceptable levels (20 ng/g), particularly at tropical environmental storage conditions.

Part of this work has been published as follows:

Ellis, W. O., Smith, J. P., Simpson, B. K. and Oldham, J. H. (1991) Aflatoxins in food: Occurrence, biosynthesis, effects on organisms, detection and methods of control. *CRC Crit. Rev. in Food Sci. and Nutrit.* **30**, 403-439.

Ellis, W. O., Smith, J. P., Simpson, B. K., Khanizaden, S. and Oldham, J. H. (1993) Control of growth and aflatoxin production of *Aspergillus flavus* under modified atmosphere packaging (MAP) conditions. *Food Microbiol.* **10**, 9-21.

Ellis, W. O., Smith, J. P., Simpson, B. K. and Ramaswamy, H. (1993) Effect of inoculum level on aflatoxin production by *Aspergillus flavus* under modified atmosphere packaging (MAP) conditions. *Food Microbiol.* (submitted)

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I would like to express my sincere appreciation to my family especially my parents for their support throughout my studies. My deepest gratitude goes to my beloved Emmanuella Esi Ahima Ellis, for her sacrifice, my little darling Theodora-Ann Esi Frempomaa Ellis and to my dear wife, Florence, for the encouragement and strength to achieve this task. Above all, the glory and the honour goes to the Almighty God, who has made all this possible.

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CHAPTER 1

1. INTRODUCTION

Aflatoxins are a group of closely related heterocyclic compounds formed by *Aspergillus flavus* and *Aspergillus parasiticus* (Buchi and Rae, 1969; Ellis et al., 1991). They are potent hepatotoxins and carcinogens affecting a wide variety of animal species. Both *A. flavus* and *A. parasiticus* are ubiquitous in nature and, under favourable environmental conditions, can grow and produce aflatoxins on a wide variety of substrates. Numerous studies have been done to prevent the growth of, and aflatoxin production by *A. flavus* and *A. parasiticus* by controlling factors affecting mold growth. These factors include temperature (Holmquist et al., 1983), water activity (Koehler et al., 1985), mold inhibitors (Marshall and Bullerman, 1986), pH (Rusul and Marth, 1987), competitive inhibition through the addition of lactic acid bacteria (Karunaratne et al., 1990) and Controlled Atmosphere Storage (CAS) conditions involving reduced oxygen levels in conjunction with elevated levels of carbon dioxide with or without gases such nitrogen and carbon monoxide (Clevestrom et al., 1983; Buchanan et al., 1985; Wilson et al., 1985; Paster, 1991). The application of these and other factors to control aflatoxigenic molds has been extensively reviewed by Ellis et al., (1991).

Recently, Modified Atmosphere Packaging (MAP) has become increasingly popular as a method of food preservation. The growing consumer concerns about the use of chemical additives and preservatives and the increasing energy cost associated with alternative methods such as freezing and drying coupled to increasing advancement in packaging technology, have led to the wide application of modified atmosphere packaging in the shelf-life extension of food products and food preservation. MAP

has been defined as "the enclosure of food products in high gas barrier materials in which the gaseous environment has been changed to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage with the intent of extending shelf life (Young et al., 1988). The technology has been used to extend the shelf life and keeping quality of muscle foods, bakery products, snack foods, pizza and sandwiches. However, despite the antimicrobial effect of CO_2 enriched atmospheres on the growth of aerobic spoilage microorganisms, several studies have shown that molds can grow in the presence of elevated levels of CO_2 if O_2 is present. While both CAS and MAP mean that the gaseous atmosphere around a product differs from air, CAS has a more precise gas compositional control than MAP and is generally used for bulk storage of products (Smith et al., 1990a,b). MAP is mainly used for retail distribution of small pre-packed units and the concentration of CO_2 used in these products is generally much higher than the levels used for products under CAS conditions (Smith et al.,1990a,b). Most studies to date have focused on the use of CAS storage to control/inhibit the growth of aflatoxigenic mold species (Clevestrom et al., 1983; Buchanan et al., 1985; Wilson et al., 1985; Paster, 1991). However, there is a paucity of data on the ability of these molds to grow and produce aflatoxin under MAP conditions.

The rationale of this research was to use statistically based predictive modelling techniques to study the effects of Modified Atmosphere Packaging and other environmental factors on the growth of, and aflatoxin production by, aflatoxigenic molds under various environmental storage conditions.

CHAPTER 2

LITERATURE REVIEW

2.1. *Historical background*

Aflatoxins date back to the 1960s when there was a severe outbreak of the Turkey "X" disease killing about 100,000 turkeys and other farm animals. The cause of this disease was traced to a feed component, peanut meal, which was heavily infested with *Aspergillus flavus*. On analysis of the feed, it was discovered that a series of fluorescent compounds, later termed aflatoxins, were responsible for the outbreak (Buchi and Rae, 1969). Aflatoxins are mycotoxins produced by molds, specifically *A. parasiticus* and *A. flavus*. Molds are ubiquitous in nature and all types of organic matter are suitable substrates for their growth (Concon, 1988). Molds are beneficial to mankind in many ways; they play significant roles in the production of cheese, antibiotics, vitamins, enzymes and glycerol (Davies and Diener, 1987). However, many molds are also harmful, causing spoilage of food, while others produce chemical substances known as mycotoxins (Table 1). The term mycotoxins stems from two Greek words: "mykes" meaning fungus and "toxicum" meaning poison (Goldblatt, 1972). These compounds are poisonous and show toxic symptoms when food or feed containing them are ingested by humans and animals. The word aflatoxin is formed from the following set up, the first letter "A" for the genus *Aspergillus*, the next set of three letters, "FLA" for the species flavus, and the noun "TOXIN" meaning poison (CAST, 1979).

Table 1. Examples of mycotoxins produced by molds

Molds	Mycotoxin	Reference
A. flavus	Sterigmatocystin	(Holzapfel et al., 1966)
	Aflatoxins	(Diener & Davies, 1969)
	Aspergillic acid	(White, 1940)
	Kojic acid	(Yabuta, 1924)
	Aspertoxin	(Rodricks et al., 1968)
A. fumigatus	Gliotoxin	(Menzel et al., 1944)
A. niger	Oxalic acid	(Reiss, 1975)
A. ochraceus	Ochratoxins	(Hutchinson & Holzapfel, 1971)
	Penicillic acid	(Wilson, 1976)
A. versicolor	Sterigmatocystin	(Bullocks et al., 1962)
P. citrinum	Citrinin	(Kuehn & Gunderson, 1963)
P. expansum	Patulin	(Sommer et al., 1974)
P. patulum	Patulin	(Harwig et al., 1973)
P. viridicatum	Ochratoxin	(Van Walbeek et al., 1969b)
	Citrinin	(Scott et al., 1972)

2.2. *Classification of Aspergillus*

The genus *Aspergillus* belongs to a class of fungi known as the Hyphomycetes, which belongs to the subdivision of fungi termed as the Deuteromycotina. This subdivision includes conidial states of organisms whose perfect states are unknown. The Hyphomycetes consist of organisms with conidia produced directly on mycelia or on single or clustered conidiophores (Beuchat, 1987). Figure 1 shows an outline of the classification.

2.3. *Characteristics and properties of aflatoxin*

Aflatoxins are classified chemically, as difuranocoumarin derivatives (Buchi and Rae, 1969). Presently, 18 different types of aflatoxins have been identified with aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2 being the most common (Beuchat, 1987) (Figure 2). Of these, aflatoxins B_1 and G_1 occur most frequently with B_1 being the most potent. The chemical and physical properties of the six common ones are shown in Table 2. Aflatoxins like other known mycotoxins are relatively small organic compounds. They are not infectious, since they are not living organisms and are also not antigenic. Aflatoxins do not accumulate in the fat tissues of the body as do many pesticides (Wilson and Romer, 1990). The letters B and G refer to the fluorescent colors observed under long-wave ultraviolet light and the subscripts 1 and 2, to the seperation patterns of these compounds on thin layer chromatographic plates (Bullerman, 1979). Figure 2 shows the structures of the common aflatoxins.

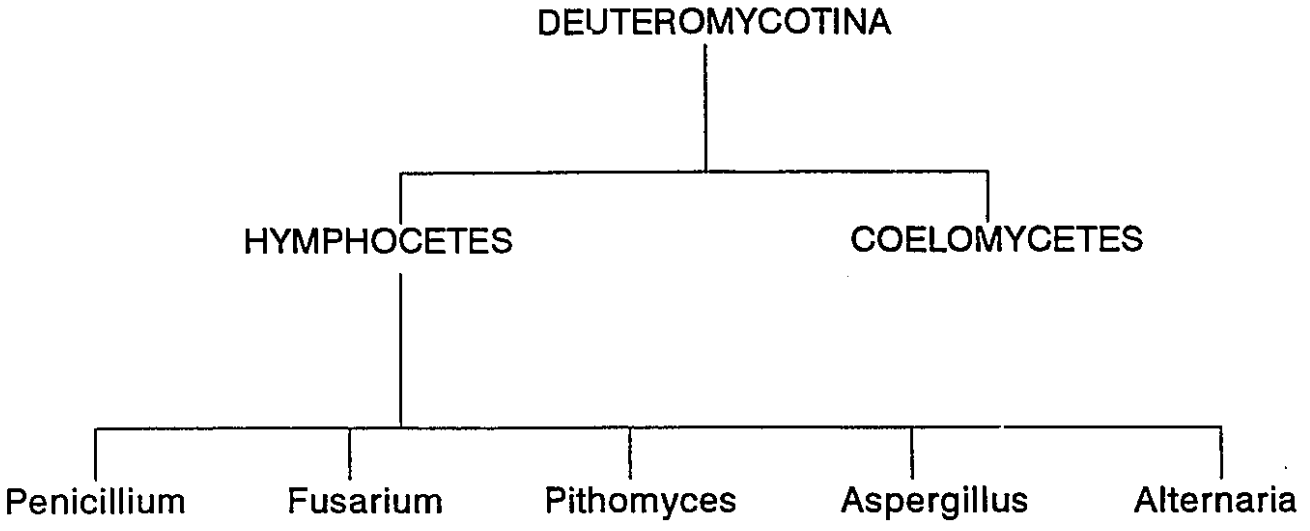


Figure 1 Classification of Molds

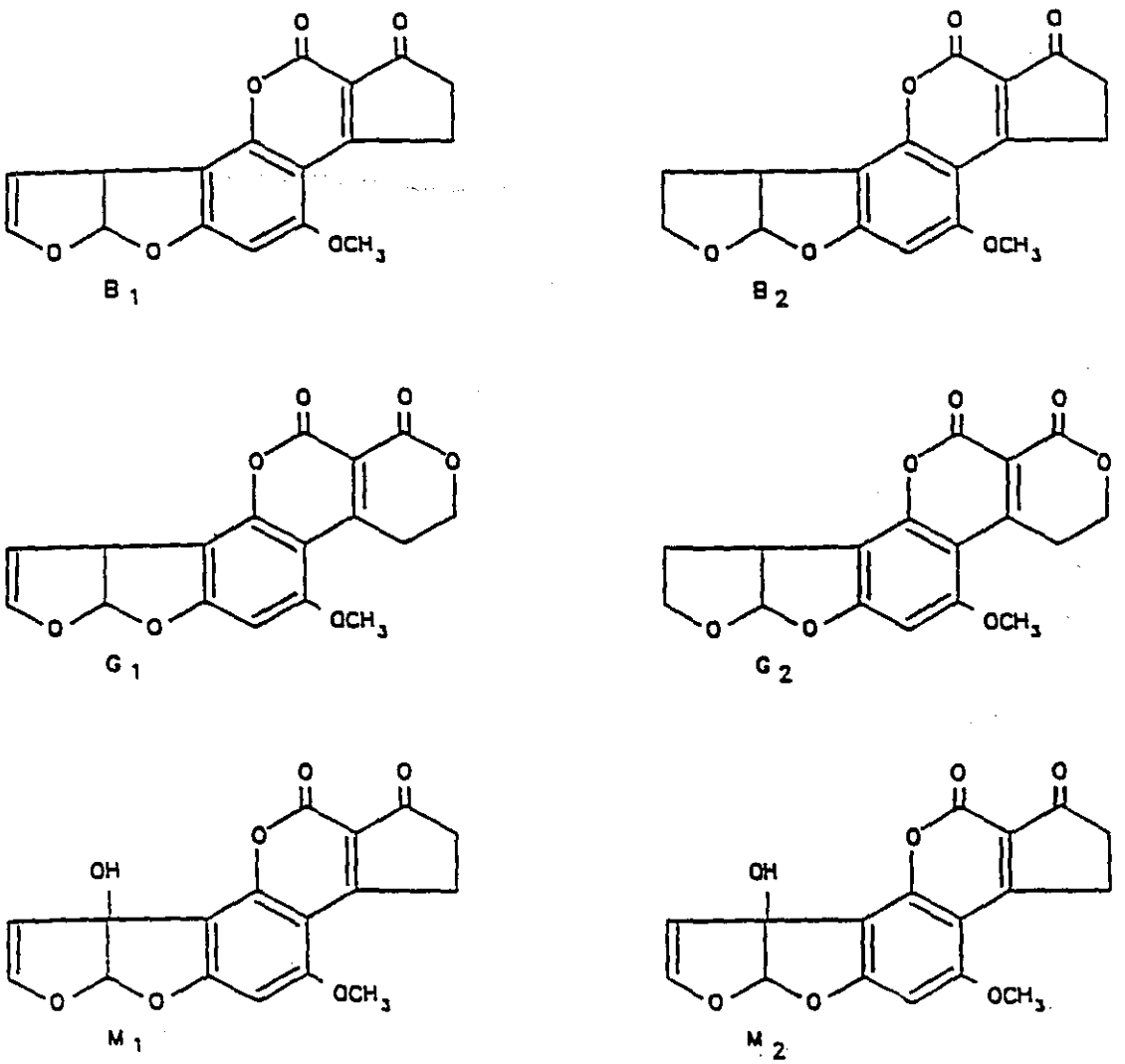


Figure 2. Structures of common aflatoxins.

Table 2. Properties of the common aflatoxins

Types of aflatoxin	Mol. formula	Mol. weight	Melting point
B_1	$C_{17}H_{12}O_6$	312	267(d)[a]
B_2	$C_{17}H_{14}O_6$	314	303 - 306(d)
G_1	$C_{17}H_{12}O_7$	328	257 - 259
G_2	$C_{17}H_{14}O_7$	330	237 - 240
M_1	$C_{17}H_{12}O_7$	328	299(d)
M_2	$C_{17}H_{14}O_7$	330	293(d)

[a](d)- Decomposition. (Adapted from Betina, 1989)

2.4. *Occurrence*

Fungi form part of the indigenous microflora of air and soil and are found on both living and dead plants and animals. Most molds, which are capable of producing aflatoxins, are also frequent contaminant of food and agricultural commodities. *Aspergillus* species are capable of growing on a variety of substrates and under a variety of environmental conditions. Therefore, most foods are susceptible to invasion by aflatoxigenic *Aspergillus* species at some stages of production, processing, transportation, and storage. Commodities from which aflatoxigenic molds have been isolated are shown in Table 3. However, the presence of aflatoxigenic molds on a substrate does not automatically mean the presence of aflatoxin. Conversely, the absence of aflatoxigenic molds does not guarantee the absence of aflatoxins, since the toxins may persist long after the mold growth has disappeared. The natural contamination of commodities is likely to persist whenever warm and moist weather conditions, faulty or inadequate storage facilities, and human error combine to produce circumstances favourable for fungal growth and toxin production (Diener and Davies, 1969).

The occurrence of aflatoxin is worldwide, particularly in products such as corn, peanuts and peanut butter. The results of international surveys, conducted by the Food and Agricultural Organisation (FAO), the World Health Organisation (WHO) and the United Nations Environmental Programme (UNEP) from 1976 to 1983 to assess the level of aflatoxin in both domestic and imported corn, peanuts and peanut butter are shown in Tables 4 and 5, respectively (Jelinek et al., 1989). It is evident from Table 4, that corn from the U.S.A., the Soviet Union, Kenya, and Guatemala had concentration levels of aflatoxin greater than the regulatory limit of 5 to 20 ug/kg. Similar results were obtained from surveys of peanuts and peanut products.

Table 3. Commodities from which aflatoxigenic molds have been isolated

Flour	Cocoa
Corn meal	Cheese
Peanut	Sausage
Meat pies	Bread
Milk	Marcaroni
Cottonseed	Copra
Cassava	Cooked meat
Refrigerated and frozen pastries	
Pistachio nuts	Oilseeds
Rice	Pumpkin seeds

Table 4. Concentration of aflatoxins in corn

Country	Year	No. of samples	90th percentile ug/kg*
Brazil	1981	228	< 8.0
Canada	1976	25	< 4.0
U.K.	1978	29	8.0
Guatemala	1976 - 79	231	4 - 360
Kenya	1978 - 79	78	30 - 1920
Mexico	1979 - 80	96	< 2.5 - 30
U.S.A.	1978 - 83	2633	10 - 700
Soviet Union	1981 - 82	219	< 1 - 662

* Level below which 90% of findings occur in a given survey.

FAO/WHO/UNEP monitoring program data (1976 - 83).

(Adapted from Jelinek et al., 1989.).

Table 5. Concentration of aflatoxins in peanut and peanut butter

Country	Year	No. of samples	90th percentile ug/kg*
Brazil	1979 - 83	1044	30 - 5000
Ireland	1977 - 82	61	300 - 4000
U.K.[a]	1982 - 83	77	38 -535
Guatemala	1977	13	150
Mexico	1980	29	700
Switzerland	1980	11	338
U.K.	1978	159	75
U.S.A.	1983	120	24
Soviet Union	1982	21	329

* Level below which 90% of findings occur in a given survey

[a] Data for peanut butter.

FAO/WHO/UNEP monitoring program data (1977 - 83).

(Adapted from Jelinek et al., 1989).

Table 6. Concentration of aflatoxins in other nuts and seeds

Product	Country	Year	No. of samples	90th percentile ug/kg*
Pistachios	Canada	1981	23	110.0
Pumpkin seeds	Canada	1981	69	63.0
Brazil nuts	U.K.	1978	32	65.0
Brazil nuts	U.S.A.	1976 - 77	235	40 - 65
Pistachios	U.S.A.	1976 - 79	1146	15 - 67

* Level below which 90% of findings occur in a given survey

FAO/WHO/UNEP monitoring program data (1976 - 81)

(Adapted from Jelinek et al., 1989).

Table 7. Occurrence of aflatoxins in feedstuffs and oilseeds

Location	Samples	Incidence
Africa	Peanut cake	1594/2965
Asia	Feeds	237/415
Asia	Oilseeds	24,840/25,565
Europe	Feeds	62/547
Europe	Oilseeds	7371/8133
Australia	Feeds	35/84
South America	Oilseeds	258/461
South America	Feeds	1/23
U.S.A.	Feeds	94/278
Canada	Feeds	1/100

Tropical Products Institute data (1970 - 76)

Nearly all countries surveyed within the period shown had levels of aflatoxin in these commodities greater than the recommended level of 20 ug/kg (Table 5). The occurrence of aflatoxins in other products such as pistachio nuts, brazil nuts, pumpkin, seeds, animal feedstuff, and oilseeds is shown in Tables 6 and 7, respectively. It is evident from Tables 4 to 7 that the occurrence of aflatoxins in food commodities at levels greater than the recommended level of 20 ug/kg of product in the food commodities surveyed is worldwide and that these products may pose a potential public health risk to humans through direct and indirect consumption of contaminated food products.

2.5. *Biosynthesis*

Aflatoxins are secondary metabolites and their synthesis occurs through the polyketide pathways, the major pathway of secondary metabolism in molds. All forms of life require exogenous materials to build into biomass. These organic compounds are used for both anabolic and catabolic processes, termed primary metabolism. Secondary metabolism is distinct from primary metabolism in the following ways: (1) it occurs optimally after a phase of balanced growth and is often associated with morphogenetic changes in molds, (2) secondary metabolite production is restricted to a small number of species, therefore it may be species or even strain specific (Moss and Smith, 1985).

The polyketide process involves the condensation of an acetyl unit with three or more malonyl units with the loss of CO_2 (Bennett and Lee, 1979). Polyketide intermediates are thought to be built up in a cyclic process similar to fatty acid biosynthesis (Sedgwick and Morris, 1980), but omitting the reduction-elimination-reduction sequence, resulting in the loss of the acetate oxygen (Hsieh et al., 1976) (Figure 3). During fatty acid synthesis, ketonic groups, formed with the addition of successive malonyl CoA units, are reduced continuously to give a paraffin chain structure of the final product. Failure of these ketonic groups to be reduced results in a highly reactive precursor, which can undergo a series of

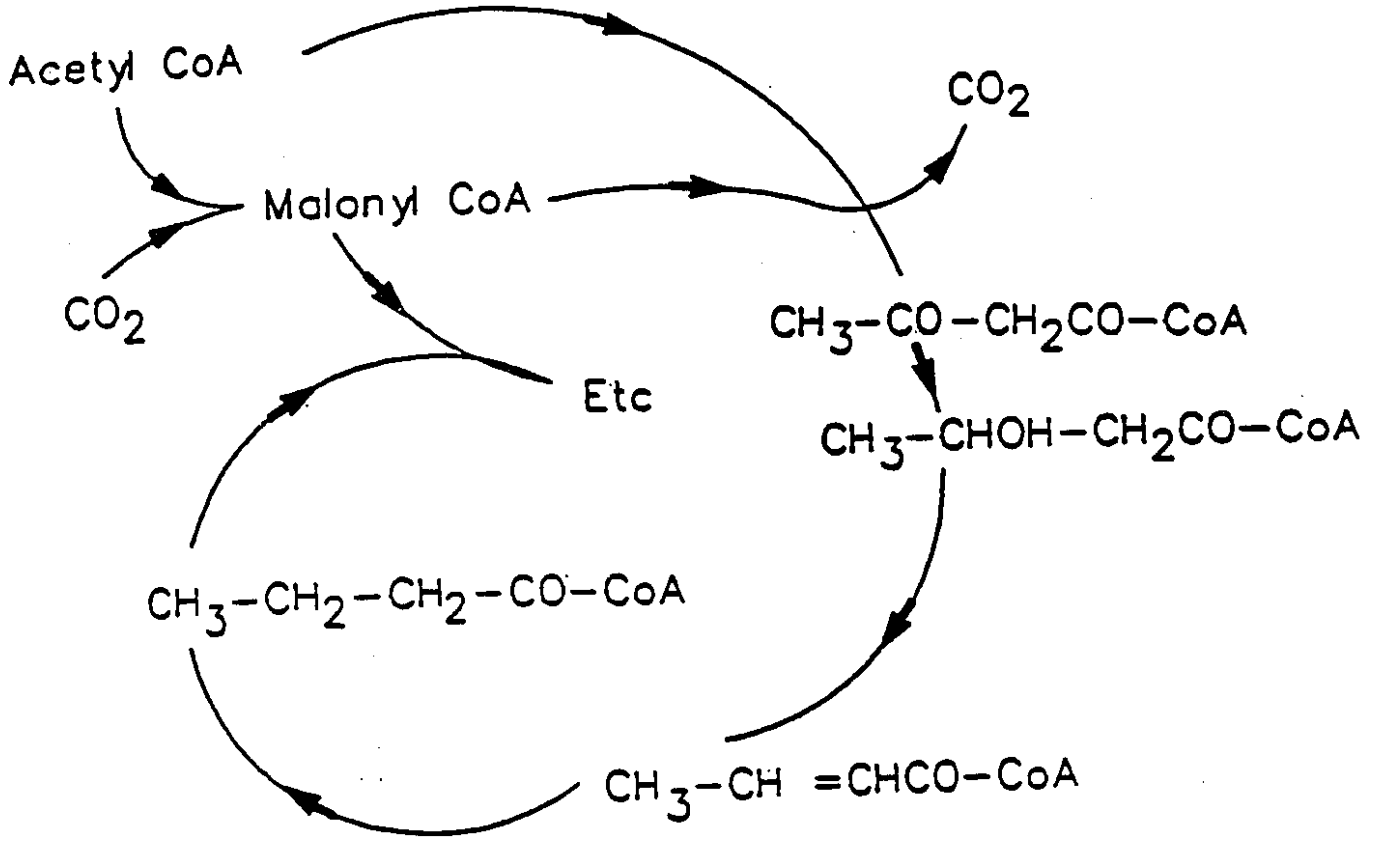


Figure 3. Fatty Acid Biosynthetic Cycle

condensation reactions leading to the formation of polyketides (Moss and Smith, 1985). Polyketides are classified as triketides, tetraketides, pentaketides etc. based on the number of repeating two carbon units that have contributed to the biosynthesis of the particular compound (Bennett and Lee, 1979). Even though aflatoxins are nonaketides, their biosynthesis occurs through decaketide-derived intermediates (Hsieh et al., 1976). Biosynthesis is presumed to start from acetate in the activated form as acetyl CoA that reacts with malonate in the form of malonyl CoA (Hsieh and Mateles, 1971). Activation of both acetate and malonate occurs through an ATP-mediated formation of thiol esters, either coenzyme A or pantetheine (Betina, 1989). The sequence involves acetyl CoA reacting with two units of malonyl CoA through the full reduction-elimination-reduction sequence similar to fatty acid biosynthesis to produce hexanoate, which is enzyme bound. Hexanoate then reacts successively with seven malonate units (Bennett and Christensen, 1983), and by omitting the reduction sequence, yields the requisite intermediate or polyketide chain of the general type:

$$R - CO - CH_2 - CO - CH_2 - CO - CH_2 - CO - CH_2 - CO - SCoA$$

where R indicates the radicals of the different starter units (Betina, 1989), as shown in Figure 4.

Vederas and Nakashima (1980) reported that the precursor undergoes direct cyclization and aromatization to form anthrone, which is then oxidized to give norsolorinic acid as shown in Figure 5. Norsolorinic acid undergoes several metabolic conversions involving several decaketides intermediates to form aflatoxin B_1 (Figure 6). The intermediates are enzyme bound, while modifications such as cyclization, alkylation, and reduction occur and metabolites are released (Simpson, 1985). The pattern of folding and condensation of the initial polyketide chain is fairly specific for any particular strain of mold and occurs on

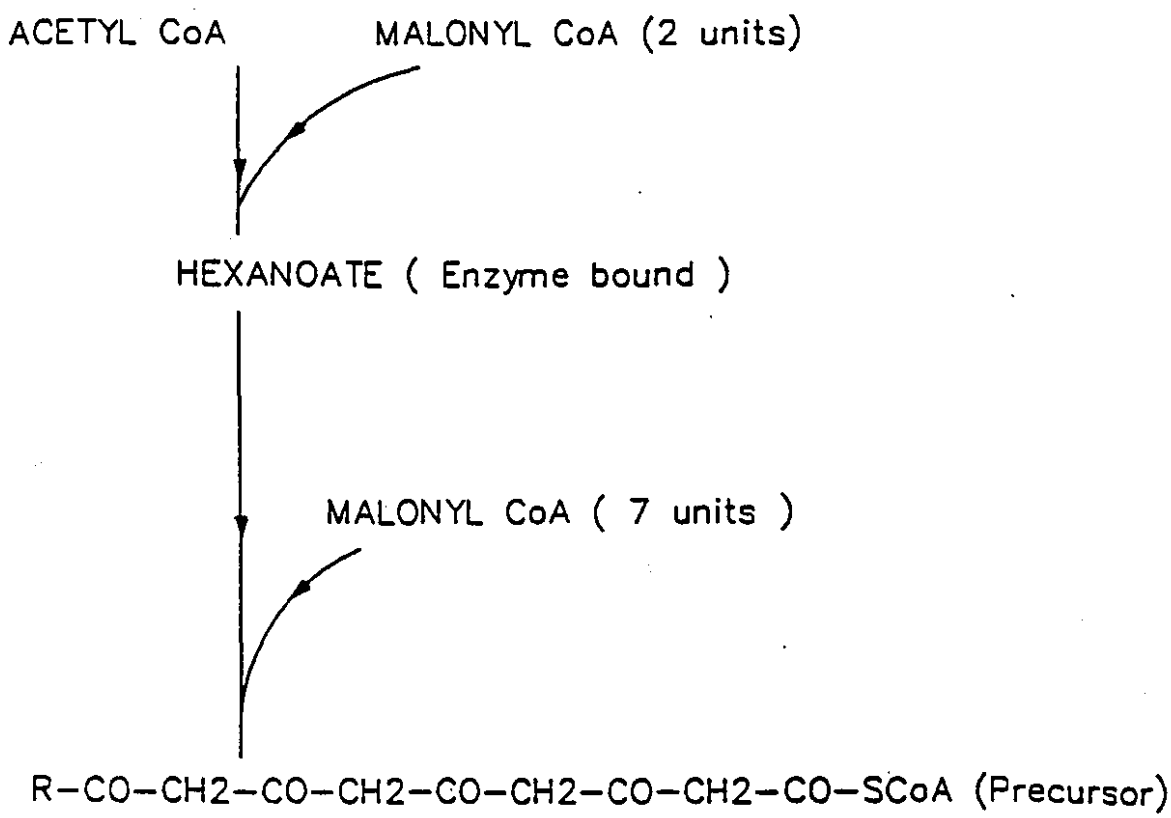


Figure 4. Formation of a polyketide precursor

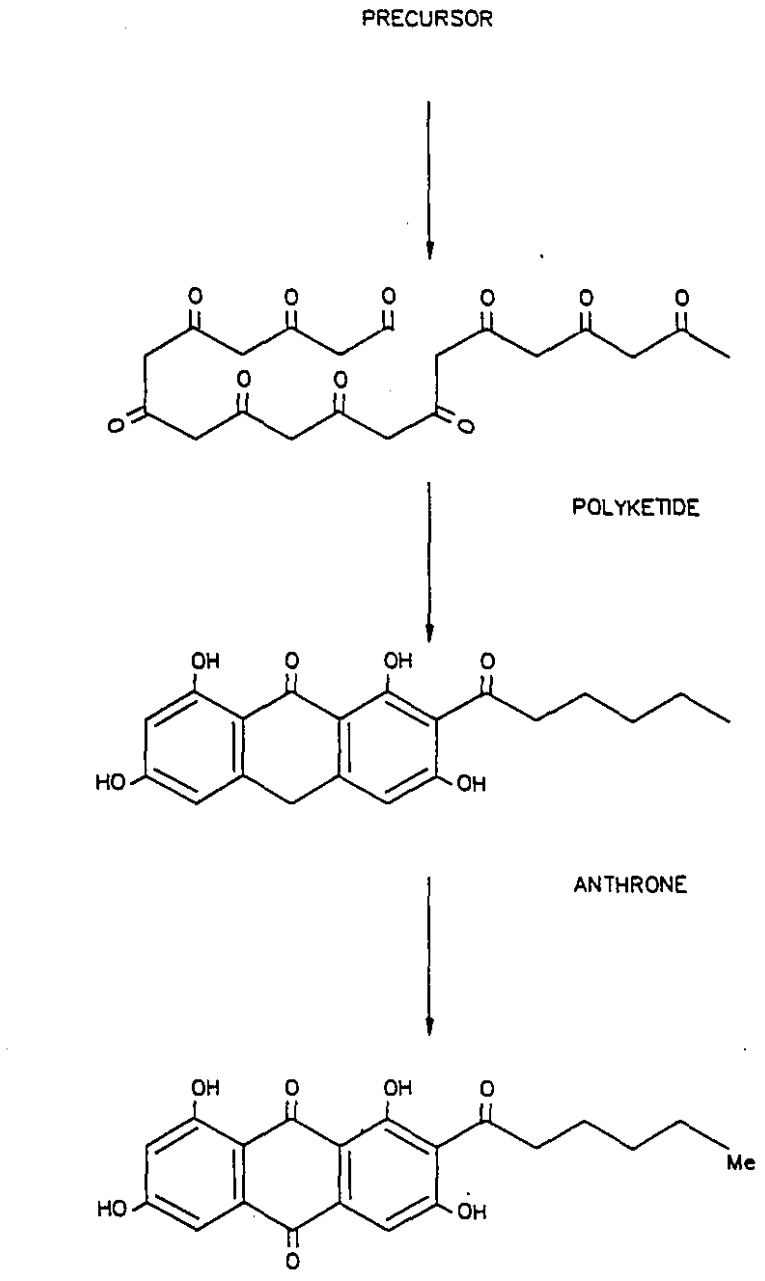


Figure 5. Synthesis of norsolorinic acid

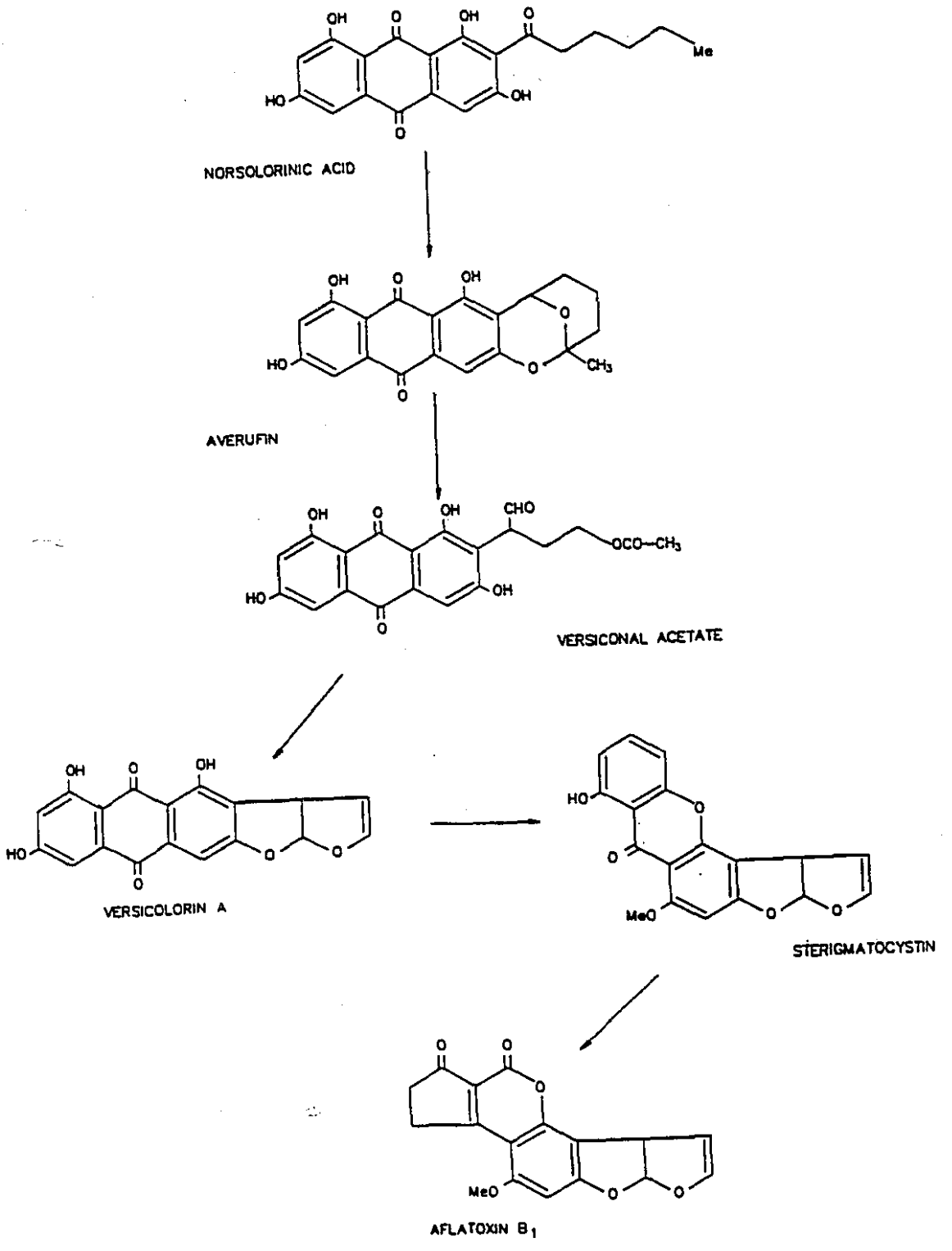


Figure 6. Biogenesis of aflatoxin B_1 from norsolorinic acid.

specific enzyme surfaces (Moss and Smith, 1985). Due to the inherently unstable nature of the intermediates, these processes are mediated by divalent metal ions such as Ca^{2+}, Mg^{2+} or Zn^{2+} thereby stimulating synthesis (Lee et al., 1966; Simpson, 1985). After aflatoxin B_1 formation, interconversion of aflatoxin B_1 to B_2, G_1, and M_1 can occur (Heathcote et al., 1976).

2.6. *Factors affecting aflatoxin biosynthesis*

Several biological, chemical and environmental factors affect the biosynthesis of aflatoxins as shown in Table 8.

2.6.1. *Biological factors*

2.6.1.1. *Strain variability*

Aflatoxins have been shown to produced by *A. flavus* and *A. parasiticus* species. However, the types of toxin produced are species specific. *A. flavus* produces mainly aflatoxins B_1 and B_2, while *A. parasiticus* produces aflatoxins B_1, B_2, G_1 and G_2. Furthermore, not every species or strain produces aflatoxin (Hesseltine et al., 1970) as the genotype of each strain determines whether or not it is aflatoxigenic (Moss and Smith, 1985). If a strain has the genetic potential to produce aflatoxins, the level of production will depend on several other factors, such as the effect of competing mold microflora.

Table 8. Factors affecting aflatoxin biosynthesis

Biological factors	Chemical factors
Strain variability	Type of substrate
Competing microflora	Type of nutrients
Inoculum level	Antifungal agents

Environmental factors

Temperature

Water activity (Aw)

Atmospheric gases

Light intensity

pH

2.6.1.2. *Competing microflora*

Competing mold microflora affect aflatoxin production through competition for substrate and through the production of inhibitory metabolites. It has been observed that while *A. parasiticus* grew well in the presence of *A. candidus*, and *A. chevalieri*, aflatoxin production was inhibited (Boller and Schroeder, 1973; 1974). It has been shown that *A. niger* can reduce the substrate pH to near 3 through citric acid production, a pH that is sufficient to inhibit aflatoxin formation. In maize, *A. niger* produced a water-soluble component that was inhibitory to aflatoxin formation (Horn and Wicklow, 1983). Therefore, competing mold microflora may either metabolize aflatoxin produced, alter the metabolism of *A. flavus*, compete for substrates necessary for aflatoxin formation, or render conditions unfavorable for aflatoxin production (Moss and Frank, 1985).

2.6.1.3. *Inoculum level*

Spore inoculum size also affects aflatoxin biosynthesis and production *in vitro*. Jinks (1969) reported that aflatoxin production in laboratory medium was due to mycelial branching and differentiation. Therefore, tip extension and hyphal fusion would be enhanced the larger the initial population of spores. Nutrient depletion and release of staling substances has been shown to influence lateral branching (Gottlieb, 1971). With smaller populations these limitations are reduced, resulting in more lateral branching, leading to increased synthesis and higher yields of aflatoxins (Sharma et al., 1980). Karunaratne and Bullerman (1990) reported that maximum aflatoxin production occurred at spore loads of 10^3 spores/ml. Inoculum levels less or greater than 10^3 spore/ml resulted in decreased aflatoxin production at optimum growth temperatures.

2.6.2. *Chemical factors*

2.6.2.1. *Substrate*

The type of substrate also affects aflatoxin synthesis and the level of production. Substrates with substantial concentrations of carbohydrates and fatty acids enhance toxin production, as observed in the high yields of aflatoxin from fresh coconut (Arseculeratue et al., 1969). Substrates high in proteins and low in carbohydrates do not enhance aflatoxin production by *A. parasiticus*. However, *A. flavus* can utilize low amounts of carbohydrates and produce substantial amounts of aflatoxin (Park and Bullerman, 1983). With prolonged incubation at temperatures of approximately 25^{o}C, proteins are degraded to amino acids by fungal proteases. Some of these amino acids serve as nitrogen sources, while others serve as a source of carbon, if other carbon sources are limited. When amino acids are used as carbon sources, large amounts of ammonia may be liberated, which affects aflatoxin production, as ammonia and other readily metabolized nitrogen sources repress or inhibit aflatoxin production (Diew and Demain, 1977). Other studies have shown that optimum aflatoxin production occurs on solid substrates rich in carbohydrate such as coconut, wheat, rice, and cottonseed (Detroy et al., 1971).

2.6.2.2. *Nutrients*

Aflatoxin biosynthesis and level of production is influenced by the nutrient composition of the substrate. Simple sugars such as glucose, fructose and sucrose are the preferred carbon sources for aflatoxin biosynthesis by *A. flavus* (Davies and Diener, 1968). Mannose and xylose have been shown to stimulate aflatoxin production by *A. parasiticus*. However, these two sugars have been shown to inhibit aflatoxin production by *A. flavus* (Mateles and Adye, 1965). Certain amino acids such as glycine, glutamate, proline, aspartate, alanine,

glutamine and minor nutrients such as Cd^{2+}, Mg^{2+} and Zn^{2+} have all been found to stimulate aflatoxin production (Davies et al., 1967; Lee et al., 1966). Zinc, which is essential for cellular growth and metabolism, is also essential for aflatoxin biosynthesis. The influence of Zn^{2+} is thought to be due to regulation of the formation of intermediate compounds, such as versicolorin A and C (Bennett et al., 1979) involved in aflatoxin biosynthesis. Thamine and other B group vitamins, with the exception of riboflavin, have also been observed to stimulate aflatoxin biosynthesis (Basappa et al., 1967).

2.6.2.3. *Antifungal agents*

Certain compounds, which inhibit fungal growth, also affect aflatoxin biosynthesis. Propionic acid is an effective antifungistatic agent against *A. flavus*, but its activity is influenced by pH and certain feed ingredients (Dixon and Hamilton, 1981). Sorbic acid is also a food preservative and its salts have been shown to inhibit growth and aflatoxin production by *A. flavus* and *A. parasiticus* (Yousef and Marth, 1981). However, subinhibitory levels of sorbic acid have been reported to stimulate aflatoxin production, but the mechanism of this reaction has not been elucidated yet. Yousef and Marth (1983) suggested that sorbic acid inhibited aflatoxin biosynthesis by inhibiting the transfer of substances from the growth medium into the cell. Garcis et al., (1984) proposed that the stimulatory effect of the subinhibitory levels of sorbic acid may be due to inhibition of the tricarboxylic acid cycle that leads to an accumulation or increase in acetyl CoA concentration, a precursor required for aflatoxin biosynthesis.

2.6.3. *Environmental factors*

2.6.3.1. *Temperature*

The optimum temperature range for fungal growth and aflatoxin production is 25°C to 30°C (Northolt et al., 1977). However, in nature, temperatures are seldom constant due to seasonal variations or through spontaneous heating in stored products or food commodities, such as grain. As a result of temperature variation, the yield of aflatoxins can vary considerably. Generally, *A. flavus* is classified as a mesophilic fungus having cardinal growth temperatures as follows, minimum around 8°C, optimum about 30°C and maximum around 40°C and above but these temperatures are dependent on other biological, chemical and environmental factors. At low temperatures, approximately equal amounts of aflatoxins B and G are formed, while at higher temperatures aflatoxin B production predominates relative to aflatoxin G. Studies has shown that while aflatoxin B_1 and G_1 were produced in equal amounts at 15°C to 18°C on rice, they were produced at a ratio of 12:1 at higher storage temperatures of 32°C (Diener and Davies, 1969). The decrease in aflatoxin G_1 production has been attributed to the accelerated catabolism of G_1 at higher storage temperatures (Schroeder and Hein, 1967).

2.6.3.2. *Water activity* (a_w)

Water activity (a_w) is the ratio of water vapour pressure of the substrate to the vapor pressure of pure water at the same temperature and pressure. At low a_w, water is bound by salts, sugars, proteins and other solutes, therefore growth of molds cannot occur since water is not present in an available form (Northolt et al., 1976). Aflatoxin production ceases or decreases at a_w values below 0.85. However, fungal growth can still occur at a_w values as low as 0.78 to 0.80. The minimum a_w for growth of *A. flavus* has been reported as

0.78 to 0.84, while the minimum a_w for toxin production was found to be 0.84. *A. parasiticus* has a minimum a_w of 0.84 for growth but a minimum a_w of 0.87 for aflatoxin production (Diener and Davies, 1970). Based on the range of a_w values given *A. flavus* and *A. parasiticus* are classified as mesophytes. With respect to sporulation, the minimum a_w is 0.85. The optimum a_w for aflatoxin production by both *A. flavus* and *A. parasiticus* is reported to be in the range of a_w 0.95 to 0.99 (Diener and Davies, 1967).

2.6.3.3. *Atmospheric gases*

Fungi are aerobic organisms, but the relationship between oxygen and carbon dioxide requirements vary considerably among species and strains. Low concentrations of CO_2 have been shown to be beneficial to spore germination and are involved in fungal metabolism and in the synthesis of proteins, nucleic acids and intermediates of the tricarboxylic acid cycle (TCA). Carbon dioxide has also been shown to catalyze the malonyl CoA system involved in fatty acid synthesis as well as other natural compounds. Also CO_2 is an essential factor for the initiation of germination of *Aspergillus* conidia. However, concentrations of CO_2 greater than 20% inhibit mold spore germination, while more than 10% CO_2 suppresses toxin production (Tabak and Cooke, 1968). A decrease in atmospheric oxygen (O_2) to less than 20%, or increase in O_2 concentration to 90% or higher, have also been shown to inhibit aflatoxin formation (Landers et al., 1967; Shih and Marth, 1973). An increase in nitrogen concentration has also been shown to suppress aflatoxin formation (Epstein et al., 1970). The maximal production of aflatoxin therefore depends not only on the mold strain but also on the appropriate concentrations of +headspace CO_2 and O_2. This is due to the fact that aflatoxin formation is neither associated with maximal concentrations of O_2 present nor with maximal mycelial growth (Shih and Marth, 1973).

2.6.3.4. *Light*

Light is essential to many mold species for the induction of and completion of sporulation. It influences both the vegetative and growth and aflatoxin production of toxigenic molds in both liquid and solid media. With respect to species, the role of light may be either inhibitory or stimulatory due to photochemical effects on the medium (Carlile, 1970). The effects of light on aflatoxin production by *A. flavus* and *A. parasiticus* has been studied by Bennett et al., (1978). They reported that while conidiospores were produced in both light and dark conditions, conidiospores were more abundant when molds were exposed to light. Bennett et al., (1978) observed that the blue region of white light was most effective in eliciting fungal photoresponses. Bennett et al., (1981) also reported that photoresponses of molds was influenced by temperature, which affected the amount of aflatoxin production. They showed that aflatoxin production was inhibited by light at either high or low temperatures but not at intermediate temperatures between 20°C to 25°C. The type of substrate also affected photoresponses and aflatoxin production. Joffe and Lisker (1969) observed that aflatoxin production was completely inhibited in Czapek's medium in the presence of light, while Reiss (1975) showed that light had no effect on aflatoxin production in bread. Light intensity also plays an important role in the destruction of aflatoxins. Nkama et al., (1987) reported an increased rate of aflatoxin B_1 destruction with increasing light intensity.

2.6.3.5. *pH*

While the pH of the growth medium has a minimal effect on the primary metabolic patterns of fungi, it appears to have a more pronounced effect on aflatoxin production. During fungal growth, pH may fluctuate to pH values of 4 to 5 as a result of fungal

activities (Moreau and Moss, 1979). *A. flavus* and *A. parasiticus* are both able to grow over a wide range of pH values with optimum growth occuring in the pH range of 5 to 8. Lie and Marth (1968) reported that *A. flavus* and *A. parasiticus* were able to grow over a range of pH values from 1.7 to 9.34, with optimum growth occurring between pH 3.24 and 5.47. However, aflatoxin production did not occur at all pH levels. Therefore, while molds in general can tolerate more acidic conditions, these acidic conditions inhibit aflatoxin production (Northolt et al., 1977).

2.7. *Effects of aflatoxins*

Aflatoxins can have a serious effect on the health of living organisms. These effects can be subdivided into two; (1) biochemical effects, and (2) biological effects.

2.7.1. *Biochemical effects*

Aflatoxins may be considered as biosynthetic inhibitors *in vivo* and *in vitro*, with large doses causing total inhibition of biochemical systems and lower doses affecting different metabolic systems (Moreau and Moss, 1979).

2.7.1.1. *Energy metabolism*

It has been shown that aflatoxins B_1, G_1 and M_1 inhibit oxygen uptake in whole tissues by acting on the electron transport chain system. They inhibit the activity of the enzyme adenosine triphosphatase to varying degrees, resulting in decreased production of adenosine triphosphate (Moss and Smith, 1985).

2.7.1.2. *Carbohydrate and lipid metabolism*

Several studies hace also shown that hepatic glycogen levels are reduced due to aflatoxin action. This may be due to the effect of aflatoxin on (1) the inhibition of glycogenesis, (2) depression of glucose transport into liver cells, and (3) the acceleration of glycogenolysis. However, both lipid synthesis and transport across cells are enhanced (Moss and Smith, 1985).

2.7.1.3. *Nucleic acid and protein metabolism*

Aflatoxins may bind with DNA affecting its activity. Aflatoxin B_1 hasbeen shown to bind more strongly with DNA than aflatoxin G_1 and G_2. Aflatoxin B_1 can also be converted to its epoxide form which binds to DNA, preventing transcription (Clifford et al., 1967; Swensen et al., 1977). It can also bind to RNA, inhibiting protein synthesis (Swensen et al., 1977). Aflatoxin B_1 also forms an adduct with serum albumin in a dose dependent manner by binding to the lysine component of this protein, resulting in the formation of Lysine-Aflatoxin B_1, which has been used to assess the level of exposure to aflatoxin B_1 in humans (Sabbioni, 1990). Aflatoxin B_1 can also be converted to one of its metabolites, aflatoxin B_{2a}, which reacts readily with free amino groups of functional proteins. Aflatoxin B_{2a} is not generally regarded as a mycotoxin and is believed to be in equilibrium with its dialdehyde, which reacts with the free amino groups to form Schiffs bases, resulting in reduced enzyme activity (Figure 7) (Moreau and Moss, 1979).

2.7.2. *Biological effects*

Biological effects can be subdivided into the following categories; 1. Carcinogenecity, 2. Mutagenicity, 3. Teratogenicity, 4. Hepatotoxicity and 5. Aflatoxicosis.

The biological effects of aflatoxins are influenced by species variation, sex, age, nutritional status and the effect of other chemicals. In addition, the dose level and period of exposure of the organism to the toxin are very important. Different species respond differently to the effects of aflatoxin as shown in Table 9. The LD_{50} value is the lethal dose of aflatoxin that will cause acute toxicity in 50% of the target population (Moss and Smith, 1985). The variation in response to aflatoxin is due to differences in metabolism among species.

Sex differences and interactions with other chemicals also influence the effect of aflatoxin. For example, male rats are less resistant to aflatoxin B_1 compared to female rats. This may be due to differences in metabolism as the concentration of microsomes was found to be twice as high in males rats compared with female rats. Thus, male rats have the capacity to metabolize and activate aflatoxins more readily than female rats (Gurtoo and Motycka, 1976). Furthermore, microsomal activity between male and female rats has been shown to be under the control of sex hormones, androgens and estrogens, respectively. Studies have shown that enzyme activities are decreased by castration in males. However, the administration of androgens to castrated males increased the activities of these sex hormone-dependent enzymes (Hodgson and Levi, 1987). Chemicals or drugs, such as phenobarbital, also affect an organism's response to aflatoxin. It has been shown that in rats treated with phenobarbital, the rate of conversion of aflatoxin B_1 into its mutagenic form was faster than in nontreated rats (Busby and Wogan, 1981). The nutritional status of the organism also determines its susceptibility to aflatoxins (Rubin and Lieber, 1968). Protein deficiency in the diet has been shown to influence the *in vivo* binding of aflatoxin B_1 to the macromolecules of rat liver (Preston et al., 1976).

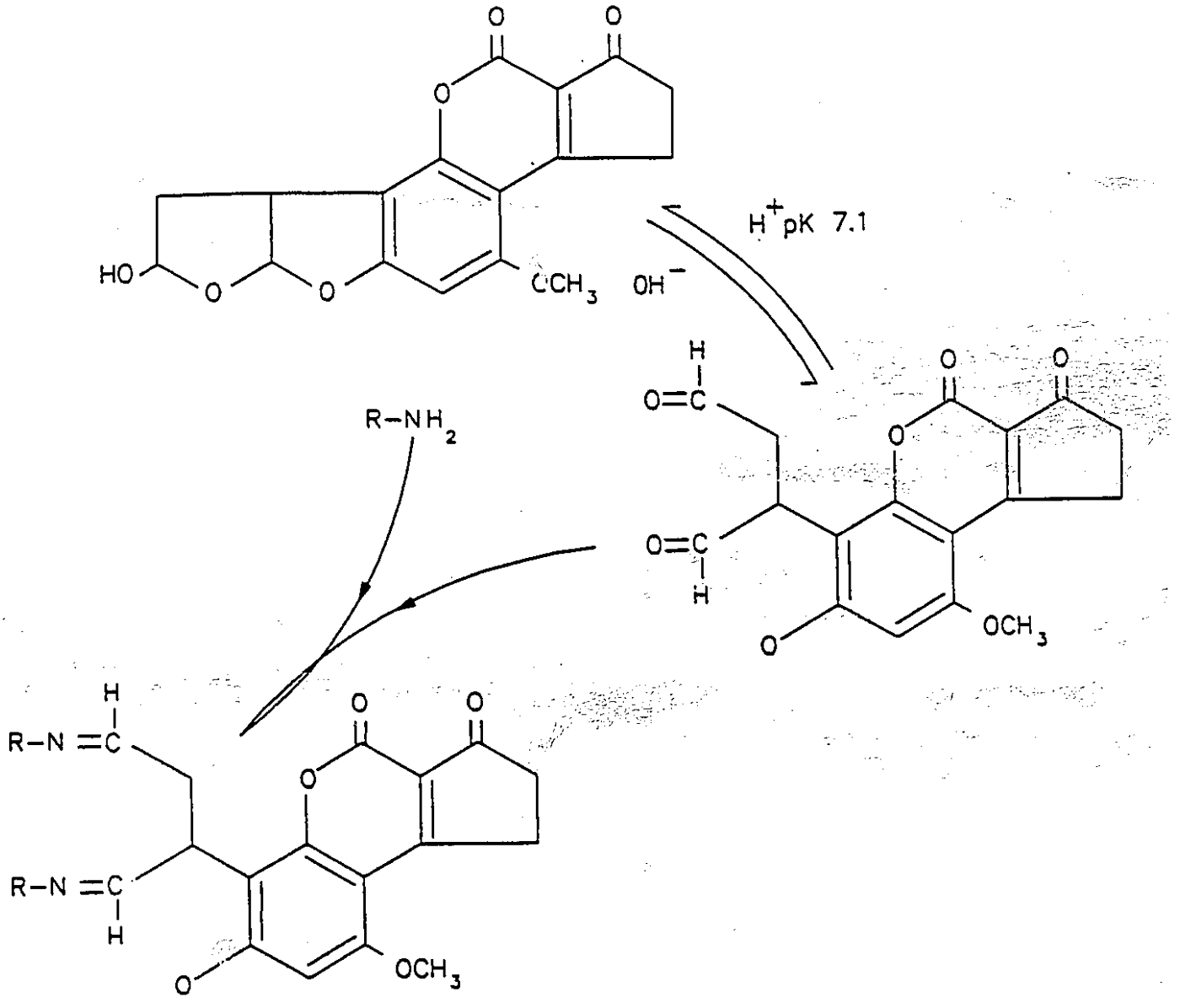


Figure 7. Schiff base formation by Aflatoxin B_{2a}

Table 9. Toxicity of aflatoxin B_1 in different Species

Species	LD_{50} mg/kg body weight	Route of administration
Rabbit	0.3	Oral
Cat	0.55	Oral
Rainbow trout	0.8	Oral
Dog	0.5 - 1.0	Oral
Guinea pig	1.4 - 2.0	Oral
Baboon	2.0	Oral
Chicken	6.3	Oral
Rat (male)	5.5 - 7.2	Oral
Rat (female)	17.9	Oral
Mouse	9.0	Oral

2.7.2.1. *Carcinogenecity*

Aflatoxins are known to be potent carcinogens and have been shown to cause cancer of the liver, colon and kidney in some animals, such as the rats, ducks and monkeys (Table 10). Aflatoxin B_1 is the main hepatocarcinogen in animals, but B_2 and G_1 have also been shown to cause cancers, but with reduced potency relative to B_1 (Moss and Smith, 1985). Evidence that aflatoxins may be carcinogenic to man arises from epidemiological studies and studies based on the development of biochemical epidemiological methods for aflatoxin analysis (Groopman et al., 1988; Shank et al., 1972), and also from reports of cases of primary liver cancer in primates such as Rhesus monkeys (Adamson et al., 1976). The toxicological effects of aflatoxin B_1 occur after the metabolic activation of the molecule by the microsomal mixed function oxidase system (Sarasin et al., 1977). These enzymatic reactions involve metabolism and detoxification. The metabolic phase leads to the formation of highly reactive intermediates, one of which is 2,3-epoxy-aflatoxin B_1 (Moss and Smith, 1985) (Figure 8). D'Andrea et al., (1978) reported that adenine and guanine may serve as targets for binding of microsomally activated aflatoxin B_1 as this specificity does not occur with denatured DNA. Aflatoxins B_1, B_2, G_1 and G_2 can also be converted to their reactive form by photoactivation with Near ultraviolet light (Misra et al., 1983). Binding of these reactive intermediates to DNA results in disruption of transcription, leading to abnormal cell proliferation.

2.7.2.2. *Mutagenicity*

Aflatoxin B_1 has also been found to be a potent mutagen. The mutagenic effects commmence by microsomal activation of the mixed function oxidase to the epoxide form (Sarasin et al., 1977).

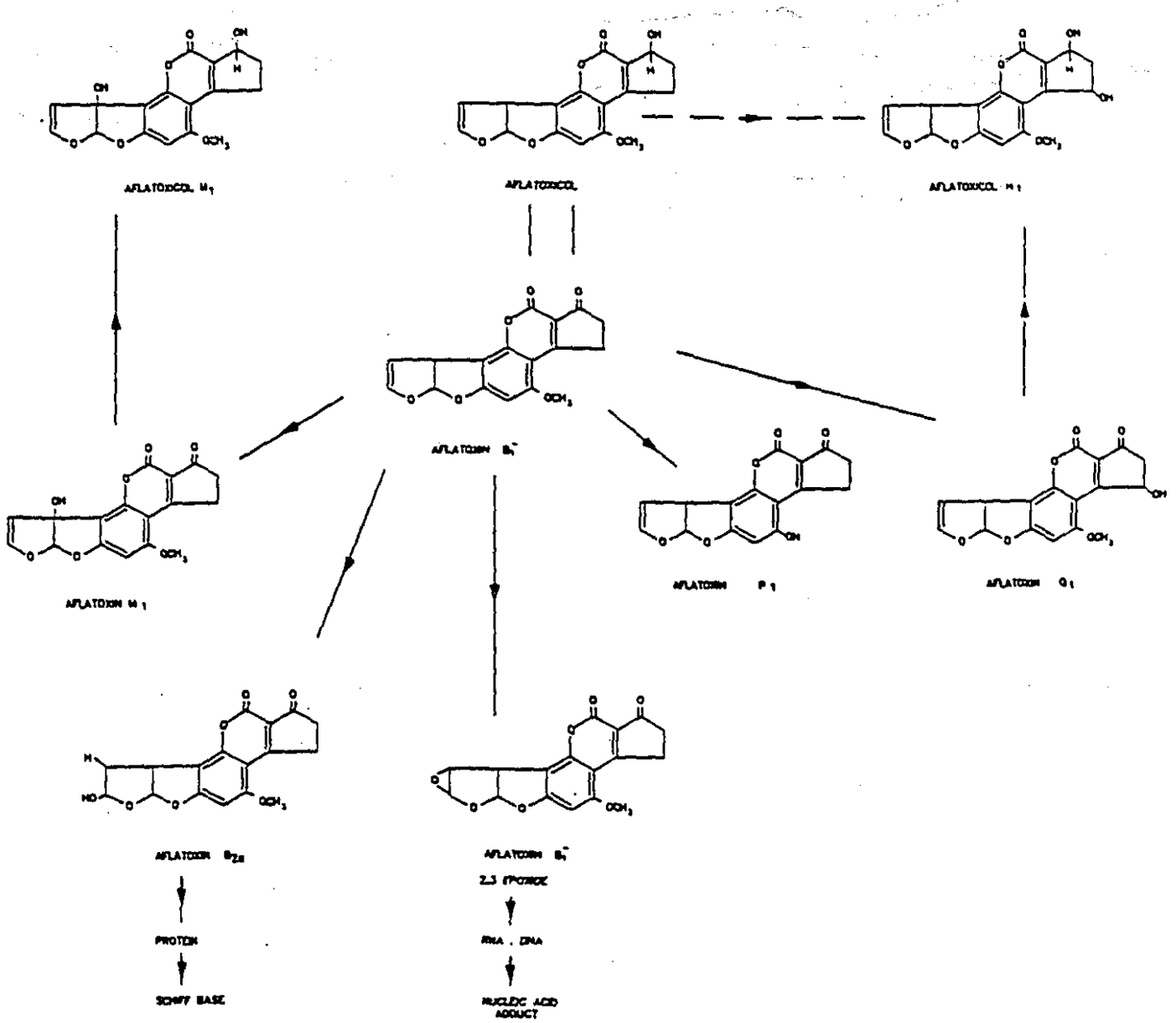


Figure 8. Metabolism of Aflatoxin B_1 in the liver

Table 10. Carcinogenity of aflatoxin B_1 depicting dosage and period of oral exposure

Species	Dosage per Kg body weight	Period of exposure	Tumor frequency (%)
Duck	30 ug	14 months	8/11 (72%)
Trout	8 ug	1 year	27/65 (40%)
Monkey	100 - 800 ug	> 2 years	3/42 (7%)
Rat	100 ug	54 - 88 weeks	28/28 (100%)
Mice	150 ug	80 weeks	0/60 (0%)

Table adapted from Moss and Smith, (1985).

Mutagenicity studies on bacteria suggest that the possible mechanism of mutagenesis may be initiated by an aflatoxin B_1-DNA binding process, leading to the formation of single stranded gaps (Stark, 1986) as a result of inhibition of DNA polymerase activity at DNA binding sites (Wragg et al., 1967). This action stimulates an error-prone repair system that may induce mutation by one of the following means;

(1) insertion of erroneous nucleotides opposite spontaneously occuring apurinic sites or (2) through errors during filling of single-stranded gaps that do not contain additional DNA lesions (Stark, 1986).

2.7.2.3. *Teratogenicity*

Aflatoxin B_1 has been suggested to be teratogenic due to its prenatal effects on certain animals. Since it is a potent inhibitor of protein synthesis in eukaryotic cells, it impairs differentiation in sensitive primordial cells (Moss and Smith, 1985). Susceptibility to teratogens varies greatly during the course of gestation, although, in general, the embryo is most susceptible during the early stages of morphological differentiation. Therefore, aflatoxin B_1 is not teratogenic at all dosage levels (Gourley, 1971). For example, a single intraperitoneal injection of aflatoxin B_1 at 4 mg/kg body weight, administered to a hamster on the 8th day of pregnancy, caused a high proportion of malformed and dead or reabsorbed fetuses (Moreau and Moss, 1979).

2.7.2.4. *Hepatotoxicity*

Hepatic tissues of the liver absorb toxic substances from the bloodstream and thus from circulation. Aflatoxins, specifically aflatoxin B_1, is eventually secreted in the liver where it has been shown to be toxic to cells (Moreau and Moss, 1979). Aflatoxin in the liver is degraded in two phases by (1) biotransformation to a more toxic product and (2)

detoxification to a less toxic and easily excretable product (Hodgson and Levi, 1987).

In phase 1, aflatoxin B_1 is metabolized to its reactive form by the microsomal mixed function oxidase by means of oxidation, reduction and hydroxylation. In this phase, there is an increase in enzyme levels and the rapid metabolism of aflatoxins (Chou Wing-Wu and Tung Ta-Chang, 1969).

In phase 2, the reactive intermediate is detoxified into a less toxic metabolite and then excreted from the body. This process occurs through any of the mechanisms of glucuronidation, sulphation, acetylation, or reaction with glutathione, depending on the type of organism. Biotransformations are not as rapid in phase 2 compared with phase 1. Reactive intermediates, such as 2,3-epoxy-aflatoxin B_1, react with macromolecules of the liver cells, resulting in fatty and pale livers, moderate to extensive necrosis and hemorrhage. For instance, aflatoxins could be detected in cow's milk and urine within forty eight hours after consumption of 0.5 mg/kg contaminated feedstuff containing aflatoxins B_1 (44%), G_1 (44%) and B_2 (2%). However, no toxin could be detected in milk or urine after four days or in feaces after six days (Allcroft et al., 1968).

2.7.2.5. *Aflatoxicosis*

This is the major toxicity syndrome associated with aflatoxins and it can be subdivided into (1) primary aflatoxicosis and (2) secondary aflatoxicosis.

Primary aflatoxicosis can be further subdivided into (a) acute and (b) chronic aflatoxicosis. Acute primary aflatoxicosis results when high to moderate concentrations of aflatoxin are consumed, usually resulting in death of the animal. It is therefore expressed as the death of the animal in a time dependent on the particular sensitivity. The syptoms of the acute primary aflatoxicosis include:

i. fatty, pale and decolorized livers

ii. derangement of normal blood clotting mechanisms resulting in hemorrhages

iii. reduction in total serum proteins of the liver

iv. increases in certain serum enzymes of the liver

v. accumulation of blood in the gastrointestinal canal (Newberne, 1973).

The kidney may also show lesions such as glomerular nephritis, and there may be congestion in the lungs (Moreau and Moss, 1979).

Chronic primary aflatoxicosis occurs when moderate to low concentrations of aflatoxin are consumed. Changes associated with chronic aflatoxicosis are very different and the symptoms associated with chronic aflatoxicosis include:

i. congested liver with hemorrhagic and necrotic zones

ii. proliferation of the hepatic parenchyma and epithelial cells of the bile duct

iii. Kidneys are congested and show occasional hemorrhagic enteritis (Newberne, 1973) .

The results of chronic aflatoxicosis, in most cases, is a reduction in growth rate and reproductive efficiency (Moss and Smith, 1985).

Secondary aflatoxicosis occurs when low concentrations of aflatoxins are consumed. The biological effects associated with secondary aflatoxicosis are impairment of native resistance and immunogenesis. Impairment of native resistance occurs through the reduction of phagocytic effectiveness of macrophages and nonspecific humoral substances, such as complements. Impairment of immunogenesis involves the cell-mediated immune system and therefore reduces the effectiveness of elective vaccination setups in the animals (Piers et al., 1979).

2.8. *Detection of aflatoxin*

Methods of detecting aflatoxin are broad and conplex. This complexity is due to the diverse nature of the commodities contamunated with aflatoxins. Several assay methods specific for commodities, such as peanuts, cottonseed, corn, green coffee and mixed feeds have been developed based on the inherent nature of the commodity. Methods of detection are divided into main groups: physicochemical methods and biological methods. The various divisions and subdivisions of detection methods for aflatoxin are shown in Table 11. Whatever the method used, the analytical procedures involved include:

Sampling
Sample preparation
Extraction
Purification / clean-up
Development / separation
Quantitation / confirmation

2.8.1. *Sampling/Sample preparation*

Sampling involves selection of a representative sample from sample lot or population for analysis. It is the most important aspect of assaying for aflatoxins since improper sampling leads to inaccurate results. Aflatoxins are rarely uniformly distributed in a product. They are usually found in high concentrations at the sites of invasion of the toxigenic mold (Moss and Smith, 1985) and these sites may form a relatively small percentage of the product. Analysis of agricultural products such as cereals, nuts and feed involve reduction in particle size by grinding or milling for efficient extraction, (Bullerman, 1987) since this increases the surface area and decreases the mean free path of the extraction solvent.

Table 11. Physicochemical methods of detection of aflatoxin

A.	Chromatographic methods
	1. Thin-layer chromatography
	2. High-performance liquid chromatography
	3. Capillary gas chromatography
B.	Instrumental methods
	1. Fluorodensitometry
	2. Spectrophotometry
C.	Rapid methods
	1. Blue-green-yellow fluorescence
	2. Minicolumn detection

The sample is then effectively mixed for homogeneity and subsampled for analysis. It has also been recommended that a large number of finely ground subsamples are randomly taken, mixed well and a representaive sample taken from this for analysis (Stoloff, 1972). For free-flowing powders, liquids and pastes, thorough mixing is required before subsampling (AOAC, 1984). The main difficulty in sampling for aflatoxins arises from the heterogenous nature of toxin distribution in contaminated unprocessed commodities. Therefore, the larger the individual food particle, the greater the problem with adequate sampling (Moss and Smith, 1985).

2.8.2. *Extraction*

Extraction involves the removal of aflatoxin from the sample for quantitation. Therefore, extraction procedures must be efficient, quantitative and must not alter or have any effect on the toxin. Early methods were based on defattening of sample prior to extraction. However, it has since been shown that aflatoxin extraction is not affected by the presence of lipids and that interfering substances, such as fats and pigments, are simpler and faster to remove from the extract than aflatoxins. Due to the diverse nature of commodities that may be contaminated, no single method of extraction is adequate for all commodities. Commodities with high lipid and pigment content require a different treatment relative to those with a low content of these components. Most of the interfering substances are often soluble in the same solvents as aflatoxin, therefore selective extraction or extensive purification methods are required to produce pure extracts. Therefore, the nature of the sample and the properties of aflatoxins reflect the type of extraction procedure to be used. Aflatoxins in general, are soluble in slightly polar solvents and insoluble in completely nonpolar solvents.

Table 12. Extraction solvents used for aflatoxin analysis

Commodity	Extraction solvent
Corn, cottonseed	Acetone : Water (85:15)
Green coffee, beans, soyabeans, coconut copra	Chloroform : Water (91:9)
Peanuts, pistachio nuts	Chloroform : Water (91:9)
	Methanol : Water (55:45)
	Acetonitrile : Water (90:10)
Powdered milk	Acetone : Water (70:30)

Practically, all aflatoxins are extracted using mixtures of organic solvents such as acetone, chloroform, methanol or acetonitrile in combinations with small amounts of water (Bullerman, 1987). Aqueous solvents more easily penetrate hydrophilic tissues and enhance aflatoxin extraction. The presence of fats, lipids and pigments in extracts reduces the efficiency of the seperation techniques, therefore addition of fat solvents, such as hexane to the extraction solvents results in many fats and lipids being partitioned into the hexane portion of the solvent, which is subsequently discarded (Moss and Smith, 1985). Types of solvents commonly used in extraction of aflatoxins are shown in Table 12.

2.8.3. *Purification and clean-up*

When extracts containing aflatoxins are very clean or if only quanlitative results are required, then the above step can be omitted. However, in most cases a purification atep is required. The objective of the clean-up step is to remove co-extracted substances in order to reduce the chemical complexity of the final extract used for identification and quantitation (Moss and Smith, 1985). Purification and clean-up involves liquid-liquid partitioning and precipitation of impurities, then removal using preparative thin-layer chromatography or column chromatography. Liquid-liquid partitioning can also occur during extraction when equilibrium extraction systems are used. Recent development in bonded phase clean-up methods using disposable cartridges offer the possibility of rapid and automated clean-up for aflatoxin assay procedures with reduction in solvent volume. The bonded phase may either be polar (Si cartridge) (Trucksess et al., 1984) or nonpolar (C18, C8, C2, CH [cyclohexyl], PH [phenyl] cartridges (Tomlins et al., 1989). In addition, selective modern clean-up tools involving the use of immunoaffinity columns, such as monoclonal antibody preparative affinity columns, permit efficient and rapid isolation and quantitation of aflatoxins in food and milk samples (Groopman and Donahue, 1988). Precipitation of interfering substances, such as pigments, lipids and fatty acids can also be

achieved using clarifying agents, such as lead acetate. Concentration of the extracted aflatoxin solution may be achieved using one of the following processes:

1. rotary evaporation under reduced pressure.
2. steam bath evaporation.
3. use of an enclosed hot plate to evaporate solvent.

Steps (2) and (3) are kept under a gentle stream of nitrogen to prevent loss of aflatoxin through oxidation (Bullerman, 1987).

2.8.4. *Development/Separation*

This step is achieved using either physicochemical methods or biological methods. The physicochemical methods involve chromatographic processes mainly thin-layer chromatography and high-performance liquid chromatography, and spectrophotometry including fluorodensimetry.

2.8.4.1. *Physicochemical methods*

2.8.4.1.1. *Thin-layer chromatography* (TLC)

Thin-layer chromatography (TLC) involves coating a glass plate with silica gel and applying a concentrated sample of aflatoxin on a baseline. Seperation occurs by solvent migration through the sorbent layer by capillary action effecting separation into individual spots perpendicular to the baseline followed by drying and characterization of the resultant spots (Pomeranz and Meloan, 1987). Recent techniques in TLC for aflatoxin analysis involve a two dimensional process. The sample is developed in one direction with a given solvent, dried and then developed in a perpendicular direction to the first with a subsequent

solvent. This technique has been found suitable for samples with high amounts of co-extracted substances. Development in the first direction serves as a clean-up step, while the second is the actual detection or quantitation (Moss and Smith, 1985). Detection is based on the fluorescent properties of aflatoxin. Quantitation can be achieved by several methods, with one of the most common methods being visual estimation. This involves comparison of aflatoxin standards of known R_f values with the colour and intensity of fluorescence of sample over a range of concentrations of standards. Recent methods involve the use of the fluorodensitometer, in which the TLC plates are examined under ultraviolet light and scanned with a photometer that allows the exact location of the position of the fluorescent spots as well as a precise measurement of the intensity of their fluorescence, to be determined.

2.8.4.1.2. *High-performance liquid chromatography* (HPLC)

High performance liquid chromatography (HPLC) is now used increasingly for the analysis of aflatoxin and other toxins due to its increased sensitivity and improved accuracy compared to the TLC method. The technique involves the seperation of sample constituents, followed by their detection and quantification. Separation is achieved by a competitive distribution of the sample between a mobile liquid phase and a stationary liquid or solid phase that is supported in a column (about 25 cm by 4 mm internal diameter). Efficiency of seperation depends on the optimization of the column parameters, especially particle size. The mobile phase moves under pressure by use of a pump and passes through a column that contains the extract and then flows to an ultraviolet absorption fluorescence detector. A change in electrical output is produced, which is recorded on a moving chart to give a chromatogram. The retention time for aflatoxin is constant under fixed conditions. A comparison of the retention times with those of the

standards enables results to be compared on a quantitative basis as the area under each peak on the chromatogram is proportional to the concentration of the particular type of aflatoxin. Extremely low levels of aflatoxin can be detected by attaching sensitive detection and sophisticated data retrieval equipment to the HPLC (Moss and Smith, 1985). HPLC has been used in assaying aflatoxin in many foods, such as cottonseed (AOAC, 1984), peanut products, figs, corn (Pons and Franz, 1977), milk and milk products for aflatoxin M_1 (Moss and Smith, 1985) and in blood of mammals (Thiel, 1985).

2.8.4.1.3. *Capillary gas chromatography*

Gas chromatography has been used in the analysis of various mycotoxins, such as trichothecenes, patulin and zearalenone. However, gas chromatography has been considered unsuitable for the analysis of aflatoxins due to the high polarity, molecular weight, low volatility and thermal instability of the aflatoxin molecule (Beaver, 1986). The advent of fused silica capillary columns and the use of mass spectrometer as a detector resulted in capillary gas chromatography using on-column injection, being used to chromatograph aflatoxin B_1 standard (Friedli, 1981). Trucksess et al., (1984) used this technique to detect aflatoxin B_1 in corn and peanut butter using a methyl silicone fused silica column. Rosen et al., (1984) used bonded-phase fused silica capillary column (15 m x 0.32 mm id) coated with 0.25 *um* thick film of 5% phenylmethylsilicone (DB-5) to detect aflatoxins B_1 and B_2 in peanuts. Recently, success has been achieved using a flame ionization detection (FID) method for the analysis of aflatoxins. Goto et al., (1988) using a FID method with a capillary on-column injector and a fused silica capillary column, was able to seperate aflatoxins B_1, B_2, G_1 and G_2. The sensitivity was much lower for G_1 and G_2 relative to B_1 and B_2 when longer columns were used.

2.8.4.1.4. *Fluorodensimetry*

Aflatoxins not only absorb ultraviolet light but they also remit part of the energy of the absorbed ultraviolet light and thus they can easily be detected and quantified. Since the intensity of fluorescence can be used as a measure of concentration, aflatoxins can be quantitized more accurately using a densitometer and comparing to known standards. Fluorodensitometry is usually applied to TLC determinations and is a useful and reliable research tool (Heathcote and Hibbert, 1978).

2.8.4.1.5. *Spectrophotometry*

This method is based on the absorption characteristics of aflatoxins at 363 nm in the ultraviolet region. Purified aflatoxins extracts, chromatographed on silica gel TLC plates are scraped off from plates and eluted from the gel with methanol. The solution obtained is made up to a standard volume and the optical density measured at 363 nm. With known extinction coefficients, concentrations of aflatoxins are then calculated (Heathcote and Hibbert, 1978). However, this method is not often used for detection of aflatoxins and has been preempted by other more sensitive and accurate methods.

2.8.4.1.6. *Rapid methods*

Since TLC methods are time-consuming, rapid methods for the detection of aflatoxins are now being developed. Examples of rapid methods for aflatoxin detection include the bright greenish-yellow (BGY) fluorescence test and the minicolumn detection technique.

2.8.4.1.6.1. *Bright-Green Yellow (BGY) fluorescence test*

This test is based on the fluorescence of cottonseed or corn colonizied by *A. flavus* when viewed under ultraviolet light. The seeds usually show a BGY fluorescence on exposure to ultraviolet. Although fluorescence is not due to aflatoxins, it has been shown that there is a correlation between the appearance of BGY fluorescence and the presence of aflatoxin (Heathcote and Hibbert, 1978). Bright-green yellow (BGY) fluorescence is thought to be due to kojic acid produced by molds, which is converted to a fluorescing substance by plant tissue peroxidases (Marsh et al., 1969). Its correlation to aflatoxin is attributed to the fact that most *A. flavus* and *A. parasiticus* toxin-producing strains generally produce kojic acid. Fluorescence occurs in the interior of the kernel, therefore, kernels are broken to enable observation of the characteristic fluorescence. Bright-green yellow fluorescence is only a presumptive test for aflatoxin presence (Bullerman, 1987). Therefore, there is the likelihood of obtaining inaccurate results, since most biological materials contain fluorescent compounds. In view of this, results need to be read properly and only the BGY fluorescence recorded (Marsh and Simpson, 1984).

2.8.4.1.6.2. *Minicolumn detection*

This is a rapid, qualitative assay method used for the detection of total aflatoxins. The method is of importance for screening large numbers of food and feed samples and has been used in the screening of total aflatoxins in commodities such as almonds, peanuts, cottonseeds and mixed feeds (Moss and Smith, 1985). The method involves chemical extraction and chromatography in short columns of silica gels in combination with florisil and alumina layers, with the subsequent separation of aflatoxin as a single fluorescent band (Heathcote and Hibbert, 1978). The sample is extracted using methanol to give a

Table 13. Summary of physicochemical methods for detection of aflatoxin in food products

Methods	Detection level(ng/g)	Advantages	Disadvantages	Commodity
TLC	>10	Versatile, compact, rapid	Tedious, poor sensitivity.	Peanuts,cocoa, corn,coconut, copra
HPLC	>1	High precision, good recovery, high sensitivity, versatile and automated.	Expensive	Milk, cottonseed, peanut products.
GLC	N/I	Rapid and automated.	Low sensitivity, expensive.	
Mini-column	> 5	Fast, easy to handle, good for routine analysis.	Very low precision	Feeds, peanuts, cottonseed, corn
BGYF	N/I	Rapid method	Less accurate	Corn,[a] barley, sorghum[b]

[a] Mainly used for corn

[b] Botharst and Hesseltine, 1975.

N/I - Not identified

methanol/toxin solution, which is then applied to a minicolumn with slight pressure to enhance mobility through the column (Bullerman, 1987). It is eluted with chloroform:acetone (9:1) with alumina removing the coloured impurities, while calcium sulfate acts as a drying agent, resulting in aflatoxin being firmly attached to the florisil layer (Heathcote and Hibbert, 1978). A similar column is developed for aflatoxin standards and these are compared with the test samples under ultraviolet light. A distinct blue fluorescing band in the florisil layer is a positive result for aflatoxin. Table 13 shows a summary of the physicochemical methods for the detection of aflatoxins, the advantages and disadvantages of each method.

2.8.4.2. *Biological methods*

Biological methods for the detection of aflatoxin involve mainly bioassays and immunoassays.

2.8.4.2.1. *Bioassays*

Numerous biological methods have been investigated as potential bioassay procedures for detection and quantitation of aflatoxins. These include cell and tissue cultures and the use of laboratory animals and microorganisms. Bioassay methods can be used as toxicity tests for aflatoxins.

2.8.4.2.2. *Cell and tissue cultures*

(i) *Chick embryo*

The chicken embryo bioassay method is classified as a tissue culture system because it is done on an embryo. Eggs from fertile inbred single-comb White Leghorn hens are usually used, although other strains may also be considered (Moss and Smith, 1985). After candling, the air cell is marked and the surface sterilized prior to injection by drilling a hole into the shell covering the center of the air cell. The toxin is dissolved in the appropriate solvent and then injected into the egg either prior to or several days after incubation. Injection can be done either in the air cell or the yolk sac. However, the air cell method is easier and requires less skill. Injection after incubation ensures development of the live embryo, but time of injection is critical since embryos that are too old may be resistant to aflatoxin. Injection prior to incubation is probably a more desirable method, especially in cases of high fertility rate eggs. Death of the embryo is counted as positive test and background mortality is also taken into account. If the percentage mortality is equal to or less than the background mortality, the extract is considered to be nontoxic or the test invalid. Dead embryos should be examined for abnormalities usually involving the beak, wings, legs or feet. This may indicate teratogenic effects. This method is relatively inexpensive, simple and sensitive but is nonspecific in its response, since other factors may be responsible for the mortality (Bullerman, 1987). The chick embryo bioassay method is also used as an official method for the confirmation of the toxicity of aflatoxin B_1 (AOAC, 1984).

2.8.4.2.3. *Animals*

(i) *Brine shrimp larvae*

The brine shrimp, *Artemia salina*, has been used widely as a convenient and inexpensive means of assaying for aflatoxins (Brown et al., 1968). The eggs are easily stored under laboratory conditions and will hatch out in saline water at 27°C after 24 hours. The larvae are phototropic and can be easily separated from the eggs. In this method, 30 to 50 larvae are placed in a solution of aflatoxin, allowed to incubate for 24 hours at 37.5°C and the percentage dead calculated (Moss and Smith, 1985). The major disadvantage with the brine shrimp bioassay method is that some naturally occuring fatty acids are also toxic to shrimp (Curtis et al., 1974) Other organisms sensitive to the test are the zebra fish larvae, crustacean *Cyclops fuscus*, brown planaria (Llewellyn, 1973) and the eggs and larvae of the amphibian *Triturus alpestris* (Bullerman, 1987).

(ii) *One day old ducklings*

The use of the one day old duckling was one of earliest method of biological assays for testing aflatoxin presence in food and feed samples (Sargeant et al., 1961). Samples were introduced into the gizzard of the duckling by way of plastic tubes and the ducklings died within 7 days after exposure to high dose levels of aflatoxins. Chronic and sublethal levels of toxicity can cause proliferation of the bile duct, and this is used as a semiquantitative index. This test has been used for the detection of aflatoxins B_1, B_2, G_1, G_2 and M_1.

(iii) *Trout*

Among the larger animals, trout is one of the most sensitive fish species to aflatoxin and exhibits characteristic pathological changes in their liver. However, the main disadvantage of using trout is the high maintenance cost (Moss and Smith, 1985).

2.8.4.2.4. *Microorganisms*

Many bacteria have been used as bioassays due to their sensitivity to aflatoxins as well as other mycotoxins. Results of tests show that *Bacillus megaterium* and a strain of *B. brevis* were the most sensitive bacteria to aflatoxins (Burmeister and Hesseltine, 1966). The effect of aflatoxin B_1 on *B. megaterium* has been incorporated into a rapid confirmatory test using the disc assay method (Clements, 1968). The test involves adding known quantities of toxin to paper discs, placing them on nutrient agar seeded with a suspension of the test organism. A standard curve may be constructed, similar to those for antibiotic assays (ug of aflatoxin/disc vs. zone of inhibition) from which quantitative results can be obtained (Moss and Smith, 1985). Other microorganisms that have been investigated for rapid bioassay methods include *Escherichia coli*, *Bacillus mycoides*, *Brevibacterium* species and *Bacillus subtilis* (Eka, 1972). Bioassays are relatively simple but are nonspecific and used to complement physicochemical methods of analysis.

2.8.4.2.5. *Immunoassays*

Immune responses occur when an organism forms antibodies in response to specific antigens. Antigens are substances that when introduced into the body will give rise to the formation of antibodies. These can be proteins, lipoproteins, nucleoproteins,

polysaccharides and most polypeptides. Small molecules or haptens become antigenic when they are linked to proteins or to synthetic polypeptides. Antibodies are highly specific and sensitive compounds and are capable of detecting trace levels of specific molecules. Aflatoxins are nonantigenic but are able to cause antibody response in test animals (Moss and Smith, 1985). Immunoassay methods are of two types:

(1) radioimmunoassay methods (RIA) and

(2) enzyme-linked immunosorbent assay methods (ELIZA).

Both techniques are based on competition of binding between unlabeled aflatoxin in the sample and labeled aflatoxin in the assay system for specific binding sites of antibody molecules present in limited concentration (Chu, 1984).

2.8.4.2.5.1. *Radioimmunoassay* (RIA)

In the RIA method, antigen and specific antibody form a reversible soluble antigen-antibody complex as shown

$$Ag + Ab \text{ ------ } AgAb$$

$$Ag^{*} + Ab \text{ ------ } Ag^{*}Ab \text{ (labeled)}$$

Since aflatoxins are nonantigenic, proteins-aflatoxin conjugates have been developed to immmunize animals to produce specific antibodies for aflatoxins. Aflatoxins do not have a suitable reactive group and therefore derivatives are used. The most successful derivative used is aflatoxin B_1-1-oxime (O-carboxy methyl form), with conjugation occuring through the cyclopentane portion of molecule (Chu and Ueno, 1977). This conjugate has a free carboxyl group suitable for coupling to bovine serum albumin (BSA), which can then be used for subsequent immunization (Moss and Smith, 1985). The antibodies generally recognize the furanofuran portion of the molecule. The conjugate is dissolved in sterile

saline solution, emulsified with complete adjuvant and injected into rabbits. Antibodies are obtained 5 to 7 weeks after initial immunization. The RIA method involves simultaneous incubation of the unknown sample or known standard dissovled in phosphate buffer with a constant amount of labeled toxin and specific antibody. Free aflatoxins and bound aflatoxins are then seperated, and using scintillation counters, radioactivity levels determined. Aflatoxin concentration of the unknown sample is determined using a standard curve of ratio of radioactivity of bound and free aflatoxin vs. $\log_{10}$ conc. of unlabeled standard toxin (Chu, 1984). Seperation of free and bound aflatoxin can be achieved by either:

1. precipitation with $(NH_4)_2SO_4$
2. double antibody techniques or
3. solid phase RIA.

Precipitation with $(NH_4)_2SO_4$ is inadequate compared with the other two methods. Solid phase RIA involves the conjugation of immunoglobulins (IgG) to CNBr-activated sepharose gel (Thean et al., 1980), and seperation is accomplished, after incubation by filtration followed by a radioactive count.

2.8.4.2.5.2. *Enzyme-Linked immunosorbent assay* (ELISA)

Two types of ELISA are commonly used for aflatoxin detection:(i) homogenous ELISA and (ii) heterogenous ELISA (Maggio, 1980). In the homogenous ELISA method, enzyme activity is altered after binding to specific antibodies and it is not necessary to seperate free and bound form of the enzyme-ligand conjugate in the assay. In the heterogenous ELISA method, enzyme activity remains unaltered and seperation of the free and bound enzyme-ligand conjugate is necessary. The heterogenous ELISA method is most commonly used for the analysis of aflatoxin (Lawellin et al., 1977). The enzyme used in most systems is horseradish peroxidase, except in the case of aflatoxin B_1-DNA adduct system, where

Table 14. Summary of biological methods for detection of aflatoxin in food products

Method	Detection level (ng/g)	Advantages	Disadvantages
Cell and Tissue	-	Simple, sensitive, inexpensive	Non-specific response, less accurate.
Animal			
Trout	-	Sensitive, specific	Expensive,slow, requires well trained staff.
Duckling	-	Specific, sensitive	Less accurate.
Brine-shrimp	-	Fast and simple	Less accurate.
Microorganisms	N/D	N/D	
RIA	>5	Highly specific, sensitive	Radioactive hazards, expensive.
ELISA	>0.25	Highly specific, sensitive and fast.	

N/D - Not well developed.

alkaline phosphatase is used. In the heterogenous ELISA method, coating of specific antibodies occurs on two solid phases using either (1) a microplate (Pestka et al., 1981) or (2) a polystyrene tube method (Lawellin et al., 1977). Antibodies are coated on the solid phase using glutaraldehyde or bicarbonate (Pestka et al., 1983), and when dried, washed with buffer before use. Sample solution or standard toxin solution is generally incubated simultaneously with the enzyme conjugate (Pestka et al., 1981) or seperately in two steps. The plate is washed and the residual enzyme, bound to the solid phase, determined by incubation with a substrate solution containing hydrogen peroxide and appropriate chromogens. The resulting colour is then measured spectrophotometrically or by visual comparison with standards. Oxidizable chromogens used are O - toluidine and 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS). This method has some advantages when compared with RIA method, by being more rapid, less expensive and with no radioactive hazards (Chu, 1984). A brief summary of the advantages and disadvantages of the biological methods is shown in Table 14.

2.9. *Control of aflatoxin*

The toxic effects of aflatoxins on man and animals arise from the ingestion of food and feedstuff contaminated with aflatoxins. Due to the increasing number of reports on the toxic nature of this chemical, there is a need to prevent contamination of products by aflatoxin-producing molds or to control mold growth by manipulation of their microenvironment. Other control methods should be directed at either reducing the concentration of aflatoxins to safe levels or to producing nontoxic degradation products without reducing the nutritional value of the treated commodities (Doyle et al., 1982). Methods of control can therefore be classified into two main categories: (i) detoxification of toxic products and (ii) prevention of mold contamination and growth.

2.9.1. *Detoxification of toxic products*

Detoxification processes involve degrading, destroying, or inactivating aflatoxins in commodities by physical, chemical, or biological methods. For a successful detoxification system, the process should satisfy the following important criteria;

1. It must be economical.
2. It must be be capable of eliminating all traces of toxin and leave no harmful residues.
3. The nutritional quality of commodity must not be impaired (Heathcote and Hibbert, 1978).

2.9.1.1. *Physical methods of detoxification*

Physical methods of detoxification involves extraction with solvent, heat inactivation, irradiation and adsorption.

2.9.1.1.1. *Extraction*

Solvent extraction is one of the most effective means of removing aflatoxins from contaminated products. Solvents used for extracting the toxins include 95% ethanol, 90% aqueous acetone, 80% isopropyl alcohol, 90% aqueous acetonitrile, hexane-ethanol and hexane-methanol. Studies have shown that extraction with organic solvents can be used to remove all traces of aflatoxin from oilseed meals with no formation of toxic byproducts or reduction in protein content or nutritional quality of the meal (CAST, 1979). The major problems associated with solvent extraction methods are the cost of additional processing, the need for special extraction and solvent removal equipment, loss of some nutrients from residual meals, mainly carbohydrate and the disposal of the extract. In many ways materials treated in this way are only suitable for animal feed (Moss and Smith, 1985).

2.9.1.1.2. *Heat (Inactivation temperatures)*

Aflatoxins are relatively heat stable and are only inactivated at temperatures as high as 250^{o}C. Therefore, the use of dry heat, such as roasting temperatures approaching 250^{o}C, need to be used for effective degradation of aflatoxin (Peers and Linsell, 1975). The moisture content of the heated product and the increase in pressure resulting from heating enhances the amount of aflatoxin destroyed by heat (Davies and Diener, 1987). It has been shown that increasing the moisture content of cottonseed meal resulted in increased rates of aflatoxin degradation when temperatures and heating times were held constant. For example, when meal containing 30% moisture was heated for 2 hours at 100^{o}C, 85% of the aflatoxin was degraded compared with only 50% degradation when a meal of 6.6% moisture content was heated under similar temperature and time conditions (Mann et al., 1976). Roasting techniques have been found to result in a 45 to 83% reduction in aflatoxin content of peanuts, depending on the conditions of roasting and the initial level of aflatoxin in the raw peanuts (Goldblatt, 1971). Roasting has also been used to reduce aflatoxin levels in commodities such as pecans and corn (CAST, 1979). Although heat treatments lower aflatoxin levels, it is not an economically viable method since it may also alter the nutritive value of commodities.

2.9.1.1.3. *Irradiation*

Aflatoxins are sensitive to ultraviolet light (Kiermeier and Hemmerich, 1947). However, the use of ultraviolet light as an effective means of detoxification is questionable. Fenell (1966) reported that exposing aflatoxin contaminated peanut meal to ultraviolet light for an 8 hour period resulted in liver lesion in ducklings fed with the treated meal. Treatment with gamma rays at a dose level of 2.5 Mrad resulted in similar lesions. The ineffectiveness of this treatment may be explained by the fact that aflatoxins are not often attacked by

gamma rays directly. However, the presence of water is likely to be a major factor in determining the effectiveness of this process, since radiolysis of water or other simple compounds yield free radicals which react with organic molecules (Doyle et al., 1982). Priyadarshim and Tulpule (1967) in their work on cereals and root vegetables, suggested that the susceptibility of products to invasion and aflatoxin production by *A. flavus* and *A. parasiticus* may even be increased by irradiation. Frank and Grunewald (1970) in their work with soft X-rays and electron irradiation on aflatoxin concluded that dose levels required to effectively destroy aflatoxin would also destroy the irradiated commodity.

2.9.1.1.4. *Adsorption*

Bentonite is a clay with adsorptive properties. Masimango et al., (1978) observed that aflatoxin B_1 in solution was adsorbed by bentonite when it was added to the solution. Removal of the bentonite resulted in removal of almost all aflatoxins present. The ability of bentonite to adsorb and retain aflatoxin was dependent on the particle size and the degree of heat treatment. Similar studies by Marth and Applebaum (cited in Doyle et al., 1982) resulted in the adsorption of aflatoxin M_1 from milk. However, more research is required to determine the practical application of using bentonite for aflatoxin in milk and other fluid products.

Other studies have been done on other chemical substances such as alumina, silica and aluminosilicates based on their adsorptive properties. From these studies, it was found that hydrated sodium calcium aluminosilicate (HSCAS) had a high affinity for aflatoxin B_1 through the formation of a stable association with aflatoxin B_1. Philips et al., (1988) in their studies using HSCAS reported that, *in vitro*, HSCAS adsorbed more than 80% of the available aflatoxin B_1 from aqueous solutions with very little desorption (less than 10%) occuring, even in a series of eluotropic solvents. *In vivo* studies, using one day old male leghorn chicks also showed that HSCAS had protective effects on the gross hepatic

changes arising from aflatoxin B_1. Therefore, it was suggested that HSCAS could modify the toxicity of aflatoxin B_1 in chickens through a sequestering effect resulting in reduced bioavailability of aflatoxin B_1.

2.9.1.2. *Chemical methods of detoxification*

Chemical inactivation appears to be a promising method of removing aflatoxin from food commodities. A wide range of chemicals have been studied as reagents for the degradation of aflatoxins. These include acids, bases, oxidizing agents, aldehydes, several gases and bisulfites (Moss and Smith, 1985). The ability of acids to destroy aflatoxins especially B_1 and G_1, is achieved by conversion of the aflatoxins to their corresponding hemiacetal forms, B_{2a} and G_{2a}, through incorporation of water (Pons et al., 1972). Although strong acids can effectively degrade aflatoxins, these methods are too drastic and change the properties of the product. Another disadvantage of this process is that acids have little or no effects on aflatoxin B_2 and G_2.

Several studies have shown that inorganic or organic bases are an efficient and relatively inexpensive means of degrading aflatoxins. Sodium hydroxide has been used in refining oils to reduce aflatoxin to very low levels. Calcium hydroxide treatment has also been used to reduce aflatoxin content in peanut and cottonseed meals to very low levels. Aldehydes, such as formaldehyde, have also been reported to be effective in reducing aflatoxin levels in peanut and cottonseed meals (Goldblatt and Dollear, 1977). Of these bases, ammonia gas seems to be the most effective and economically feasible for the reduction of the aflatoxin content of a variety of feedstuffs (Brekke et al., 1977). When used as an anhydrous gas at elevated temperatures and pressures, a 95 to 98% reduction in total aflatoxin concentration in peanut meal has been reported. Ammonia is thought to act by opening the lactone ring of aflatoxin B_1, forming an ammonium salt with the resulting hydroxyacid. Decarboxylation of the *B*-keto acid results in the formation of aflatoxin D_1 at

high temperatures and pressures. This technique is used mainly for the detoxification of animal feedstuff (Moss and Smith, 1985), and is now used on a commercial scale in Senegal, France and the United States. However, the disadvantage of this process is that it lowers the protein efficiency ratio of the product and enhances the production of off-flavors and odors in the product. Volatile bases, such as methyamine and ethylamine, have also been found to reduce aflatoxin levels (Mann et al., 1971). This method is not gaining acceptance due to formation of toxic by-products.

2.9.1.2.1. *Oxidizing agents*

The terminal double bond of the dihydrofuran ring of aflatoxin B_1, G_1 and M_1 are susceptible to attack by oxidizing agents and reactive forms of oxygen. Aflatoxins B_2, G_2 and M_2 whichlack this double bond are more resistant to oxidation (Heathcote and Hibbert, 1978). Dwarakanath et al., (1968) compared ozone, oxygen and air as oxidizing agents for aflatoxin degradation in cottonseed and peanut meals under similar heating conditions (100^{o}C for 2 hours) and moisture content of 22%. Storage in ozonized air resulted in complete destruction of aflatoxins B_1 and G_1 within one hour. However, these conditions had no effect on aflatoxin B_2 and it also decreased the amino acid, lysine, as well as the protein efficiency ratio of the meals (Dollear et al., 1968).

Hydrogen peroxide (H_2O_2) is another oxidizing agent acceptable in foods and has the potential to destroy aflatoxins. It has been used in defatted peanut meals, resulting in 97% destruction of aflatoxin, while having no effect on protein efficiency ratio (PER) of wheat and other feeds mixed with these treated meals (Sreenivasamurthy et al., 1967). Hydrogen peroxide in conjunction with riboflavin, resulted in a 98% reduction of aflatoxin M_1 in milk, but the mechanism of this combined action is unknown. When hydrogen peroxide was used alone, only a small amount of aflatoxin was reduced (Applebaum and Marth, 1980). The proposed mechanism for the combined action of hydrogen peroxide and riboflavin on

the destruction of aflatoxin M_1 is thought to be due to the production of singlet oxygen, which reacts with the terminal bond of the furanofuran ring of aflatoxin M_1. Riboflavin is photosensitive to visible or near-UV light and becomes electronically excited. In this state, it may react with oxygen to produce singlet oxygen through the action of lactoperoxidase in the presence of chloride ions, both of which are present in milk (Korycka-Dahl and Richardson, 1978). Doyle and Marth (1978) have shown that lactoperoxidase in the presence of hydrogen peroxide and chloride ions can degrade aflatoxin B_1 and G_1.

2.9.1.2.2. *Bisulfite*

Bisulfite is commonly used as a food preservative in beverages, fruits and vegetables. It is used in foods for several purposes, such as inhibition of browning, as an antioxidant, reducing agent and to inhibit microbial growth (Roberts and McWeeny, 1972). It has also been used to degrade aflatoxin B_1 and G_1. Bisulfite reacts with aflatoxins B_1 and G_1 resulting in loss of fluorescence. The reaction is a first-order reaction, with the rate depending on the concentration of bisulfite, but the reaction rate of aflatoxin G_1 is faster than that of aflatoxin B_1. It has been shown that there is a marked increase in aflatoxin degradation with increasing temperature (Doyle and Marth, 1978). Moerck et al., (1980) used different concentrations of sodium bisulfite with contaminated corn and concluded that at concentrations of 0.5% and 1.0% sodium bisulfite was more effective in degradation of aflatoxin compared with similar concentrations of aqueous ammonia solutions.

2.9.1.3. *Biological degradation*

Many microorganisms, including bacteria, actinomycetes, yeasts, molds and algae can degrade aflatoxins. The most active organism discovered is *Flavobacterium aurantiacum* NRRL B-184, which in aqueous solution can irreversibly metabolize and degrade aflatoxins B_1, G_1 and M_1 (Ciegler et al., 1966; Lillehoj et al., 1971). Other microbes capable of converting or transforming aflatoxin B_1 to aflatoxicol are shown in Table 15. However, the conversion of aflatoxin B_1 to aflatoxicol is a very slow and incomplete process and can take 3 to 4 days to transform 60% of aflatoxin B_1 to aflatoxicol (Detroy and Hesseltine, 1969). Karunaratne et al., (1990) have shown that *Lactobacillus acidophilus*, *L. bulgaricus* and *L. plantarum* could be used to either prevent mold growth or to degrade aflatoxins. The prevention of mold growth was attributed to a pH effect and mocrobial competition. Molds capable of producing aflatoxins may also degrade them. It has been shown that production of aflatoxin by *A. parasiticus* and *A. flavus* reaches a maximum and then decreases during continuous incubation of the culture. Conditions influencing degradation of aflatoxins by these molds are;

1. Age of mycelia - 8 to 10 day old mycelia are very effective for the degradation of aflatoxin B_1.
2. Disrupted mycelia - fragmented mycelia are more effective compared to the intact mycelia.
3. Substrate type - substrate supporting substantial mycelia growth yield mycelia with greater potential for degradation.
4. Strain of mold - strains producing larger amounts of aflatoxins generally degrade more aflatoxins.
5. Amonut of mycelia in media - increased quantity of mycelia in the growth medium increases the rate of aflatoxin degradation.

Table 15. Microorganisms capable of converting aflatoxin B_1 to aflatoxicol

Corynebacterium rubrum	*Aspergillus niger*
Trichoderma viride	*Mucor ambigus*
Dactylium denroides	*Mucor griseo-cyanus*
Absidia repens	*Mucor alternans*
Rhizopus arrhizus	*Rhizopus oryzae*
Rhizopus stolonifer	*Tetrahymena pyriformis*

Doyle et al., 1982.

6. Temperature - maximum degradative activity occurs at 28°C.

7. pH - maximum degradative activity is around pH of 5 to 6.5.

It is thought that these degradation reactions occur through enzymatic activity and that these enzymes produce end products or by-products that react with aflatoxins. Peroxidase was speculated to be one such enzyme since it catalyzes the decomposition of hydroperoxides to produce free radicals (Richardson, 1976), which may then react with aflatoxins. Furthermore, certain peroxidases, such as myeloperoxidases, produce hypochlorite and singlet oxygen in the presence of hydrogen peroxide and chloride ions (Allen, 1975). Doyle and Marth (1979) have shown that *A. parasiticus* is capable of producing peroxidase and degrading aflatoxin B_1. They also showed a direct correlation between the amount of peroxidase produced and the amount of aflatoxin degraded. However, further research needs to be done in this area.

2.9.2. *Prevention and control of mold contamination*

Prevention of mold contamination and or mold growth can be achieved by one or more of the following methods outlined below;

1. Improved farm management
2. Antifungal agents
3. Genetic engineering approaches
4. Control of environmental conditions:
   a. Moisture
   b. Temperature
   c. Gaseous atmosphere

2.9.2.1. *Improved farm management*

Recent studies have shown that fungal attack occurs in the field, therefore, contamination of plants with aflatoxin usually occurs in the growing plant. This has been observed for certain commodities such as peanuts, corn and cottonseed growing under stress conditions. To prevent contamination, good management practices, such as using sound, fungus-free seeds for planting, controlling insects and plant disease and controlling irrigation practices, should be employed. In addition, harvesting plants at maturity and proper adjustment and operation of harvesting equipment to avoid damage to crops should also be employed. Good storage facilities with sound environmental conditions can be used to prevent contamination by aflatoxin-producing molds (CAST, 1979).

2.9.2.2. *Antifungal agents*

Certain short-chain fatty acids have been shown to inhibit the growth of fungi. Propionic acid has been shown to be an effective fungistatic agent against *Aspergillus flavus*. It is a volatile fatty acid that has been used to preserve high moisture corn without reducing its value as animal feed (Vandegraft et al., 1975). The activity of propionic acid is enhanced by acid pH and certain feed ingredients (Dixon and Hamilton, 1981). Salts of sorbic acid have been shown to inhibit growth of *Aspergillus flavus* and *Aspergillus parasiticus* (Yousef and Marth, 1981). The compound dichlorvos has been found to inhibit aflatoxin biosynthesis by *Aspergillus parasiticus* even though it has no effect on fungal growth (Hsieh, 1973).

2.9.2.3. *Genetic engineering approaches*

The idea of genetic engineering to develop varieties that may be resistant to mold attack or inhibit toxin production was believed to be an ideal solution. Lillehoj and Zuber (1975) in

several laboratory studies and in field work have shown varietal differences in corn with respect to resistance to *Aspergillus flavus* and aflatoxin production. Vesonder et al., (1978) from a survey of a series of corn hybrids concluded that "evaluation and selection will probably be more difficult for plant resistance to infection by *Aspergillus flavus* and aflatoxin production than it has been for plant resistance to diseases".

2.9.2.4. *Rapid screening techniques*

Aflatoxins in contaminated seeds are generally found in only a relatively small number of kernels. In order to ensure that only high quality food material enters the food system, rapid detection methods and removal of contaminated seeds are required (CAST, 1979). Manual, mechanical and electronic methods have been used to exclude damaged or discolored peanuts from food stocks and these methods have reduced the aflatoxin content to negligible levels in processed peanuts (Heathcote and Hibbert, 1978). Other techniques involve the use of low power microscopy, which is based on the identification of *Aspergillus flavus* mold growth as being a positive sign for aflatoxin and therefore the rejection of the kernels (Dickens and Welty, 1979). The BGY fluorescence test is also used as a rapid screening test for corn and cottonseed and is based on the BGY fluorescence under ultraviolet radiation as a positive sign of aflatoxin production. This test requires further confirmation by chemical analysis (CAST, 1979)). Other methods of potential value are "air classification". This is based on the principle that less dense or lighter nuts are more likely to be invaded by *Aspergillus flavus* and thus possibly contaminated with aflatoxin. Separation has therefore been thought of as a useful tool for reducing aflatoxin contamination in peanuts from experimental results (Goldblatt, 1970). Density segregation has also been used as a rapid technique in terms of buoyancy. For example, aflatoxin contaminated corn has been seperated from sound corn on the basis of its buoyancy in water and three concentrations of sucrose solutions of 20, 30 and 40% respectively (Huff,

1980; Huff and Hagler, 1982). Although these methods have low precision, they have been employed to reduce aflatoxin contamination or mold contamination in certain commodities, particularly corn.

2.9.2.5. *Control of environmental conditions (Storage)*

Storage conditions play an important role in the physicochemical and microbiological quality of commodities. The specific fungal species that develop in a given environment depend on moisture, temperature, presence of competing microorganisms and the nature and physiological state of the commodity. These and other factors influence mold metabolism and their capacity to utilize food material for growth and metabolite production (Davies and Diener, 1987).

2.9.2.5.1. *Moisture*

Moisture levels and temperature are the most important factors in the protection of stored grains against mold growth and aflatoxin production (Heathcote and Hibbert, 1978). It has been shown that *Aspergillus flavus* will not invade grain and oilseeds when the moisture content is in equilibrium with a relative humidity of 70% or less. At this relative humidity, the moisture content of wheat is about 13% and approximately 7 to 10% for commodities rich in oil, such as peanuts and cottonseed (Golumbic and Kulik, 1969). An increase in the moisture content of stored commodities, especially corn, has been found to result in a tenfold increase in aflatoxin production. This increase in moisture content at localized areas in the stored commodity is due to inadequate aeration that may cause significant variation in temperature within the stored crop. This may result in pockets of high moisture in the cooler areas of the crop (wet spots), resulting in mold growth (Heathcote

and Hibbert, 1978). Therefore, there is a need for continuous and adequate aeration and a constant check on the moisture content of the stored commodities and temperature of the storage environment.

2.9.2.5.2. *Temperature*

Temperature is one of the most critical environmental factors influencing mold growth and aflatoxin production. Several studies have shown that aflatoxin can be produced at temperatures as low as 7.5^{o}C to 10^{o}C (Schroeder and Hein, 1967; Van Walbeek et al., 1969a). Low-temperature storage can be very suitable for controlling the growth of *Aspergillus flavus*, however, temperature should be reduced to 5^{o}C as quickly as possible throughout the product, especially perishable commodities. Modern practices of storing or enclosing commodities, especially perishables in airtight plastic containers or self-sealing plastic foils before storing under refrigerated conditions may provide a potentially hazardous situation. The airtight conditions may keep the products moist, particularly on the surface of commodities, thereby providing favourable conditions for the growth of *Aspergillus flavus* (Heathcote and Hibbert, 1978). Therefore, even though refrigeration temperature may be an ideal means of controlling mold growth, care should be taken not to turn it into an ideal environment for aflatoxin production by maintaining temperatures preferably at 0^{o}C. However, the use of cold temperatures for large scale storage of agricultural crops is generally not economically feasible.

2.9.2.5.3. *Modified gaseous atmosphere*

A modified atmosphere is one in which the normal composition of air has been changed or "modified". This modification usually results in a reduction in the oxygen of the air, while

increasing the level of carbon dioxide and nitrogen in the atmosphere. Major terms used to describe this atmosphere modification are controlled atmosphere storage (CAS) and modified atmosphere packaging (MAP). In CAS, the gaseous atmosphere is modified to a desired level and controlled at this level within strict limits, throughout the storage period (Kader et al., 1989). CAS is usually applied to bulk storage of products and is used to extend the shelf life of apples that can be "put to sleep" and kept fresh for up to 6 to 9 months using the correct gas mixture in conjunction with temperature and humidity control. CAS has been used to control growth of and aflatoxin production by *Aspergillus flavus* in bulk storage of corn, peanuts (Diener and Davies, 1972), wheat (Fabbri et al., 1980). For instance, growth of *A. flavus* was prevented on high-moisture peanuts in an atmosphere of 80% CO_2 and 20% O_2 (Landers et al., 1967). Wells and Payne (1980) also reported significant decrease in fungal colonies isolated from pecan kernels when stored in an atmosphere of 1% O_2 and 30% CO_2. They observed that *Penicillium* spp. were also inhibited for 5 months during storage in 30% CO_2 and 21% O_2 and under the same conditions *Fusarium* spp. were also inhibited for 4 months. CAS has also been used to completely inihibit the production of sterigmatocystin by *A. versicolor* (Orth, 1976) and to control T-2 toxin production by *Fusarium tricinctum* (Paster et al., 1986) and patulin production (Paster and Lisker, 1985; Orth, 1976). Furthermore, CAS has been used to control insect infestation in stored grains, thereby preventing mold infection of stored products (Harein and Press, 1968).

MAP is the enclosure of the food product in gas barrier materials, in which the gaseous environment has been changed to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage. MAP results in reduced O_2 content of the air within package headspace and increased CO_2 and N_2 levels (Smith et al., 1990). However, there is little or no data with respect to aflatoxin production in foods packaged under MAP.

2.10. *Principles of experimental designs*

Experimental designs were first used in agricultural experimentations after its introduction in 1925, but since then, experimental designs have found applications in practically all fields of research and development. It has been defined in several ways by several researchers. Ostle and Mensing (1975) defined it as a complete sequence of steps taken ahead of time to ensure that the appropriate data will be obtained in a way that will permit an objective analysis leading to valid inferences with respect to the stated problem. This definition implies that the person formulating the design clearly understands the objective of the proposed investigation. It also implies that a statistical design must come before experiment is begun.

Finney (1960) defined experimental design in a different context to mean;

1. the set of treatments selected for comparison;
2. the specifications of the plots to which the treatments are to be applied;
3. the rules by which the treatments are to be allocated to plots and;
4. the specifications of the measurements or other records to be made on each plot.

In simple terms, a good design must (i) answer the right questions, (ii) be realistic, cost effective and should not be robust. The basic principles of experimental design comprise mainly randomization, replication and local control. Some examples of experimental designs are simple comparative experiments, randomized complete block design, factorial experimental designs and response surface designs.

2.10.1. *Response surface methodology (RSM)*

Response surface methodology (RSM) can be defined as a statistical method which uses quantitative data from appropriate experimental designs to determine and simultaneously solve multivariate equations (Giovanni, 1983). RSM is widely used in process optimization studies (Henika, 1982; Giovanni, 1983). RSM, therefore encompasses;

(i) Setting up a series of experiments that will yield adequate and reliable measurement of the response of interest.

(ii) Determining a mathematical model that best fits the data collected from the design chosen in (i) by conducting appropriate tests of hypothesis concerning the model's parameters and,

(iii) Determining the optimum settings of the experimental factors that produce the maximum (or minimum) value of the response (Khuri and Cornell, 1987).

Giovanni (1983) stated that RSM performs 3 main functions;

1. to determine the combination of factors which yield the optimum response;
2. to determine how the response is affected by a given set of factor levels and;
3. to describe the interrelationship among the test variables.

Consequently, the term response surface has been associated with experiments intended to identify or evaluate one or more response variables as a function of the independent variables.

2.10.2. *Response surface designs*

Response surface experiments (RSE) are carried out when a specific statistical model for the response is known. Generally, RSE attempts to identify the output or response of a

system as a function of the explanatory variables. Therefore most RSE designs focuses on polynomial models, with emphasis on the first and second order designs (Thomsons, 1982).

2.10.2.1. *First order model*

First order models are useful for screening experiments or are designs set up for the purpose of collecting data for fitting such models. The purpose of screening is to identify the most significant variables. The design most commonly used to fit first order models are the 2k factorial designs (Gacula and Singh, 1984). This design is a full factorial experiment with K variables each at two levels. This notation does not only define the experiment but indicates the number of experimental units or points (Thomsons, 1982). In addition to the 2^k design points, a center point is also frequently incorporated into the design to permit improved estimation of the experimental errors. Thomsons (1982) also reported that, for experiments with four or more explanatory variables, fractional factorial replications of factorial experiments are recommended for first order designs. Gacula and Singh (1984) stated that first order models are often inadequate and provide a poor description of the geometric shape of the response surface because for a better description, the model must include both the interaction and quadratic effects and this requires designs with three or more levels to be run for each factor.

2.10.2.2. *Second order model*

This is a higher order model relative to the first order model and it is formed by adding higher order terms to the first order model. The second order models can be in several designs, such as ; (i) orthogonal second order models, (ii) rotatable second order designs and (iii) composite designs. Most of the second order RSE utilize the central composite designs.

2.10.2.2.1. *Central composite designs*

This design was introduced by Box and Wilson (1951) and it mainly consists of;

1. A complete or fractional 2^k factorial design, where the factor levels are coded to the usual -1, +1 values. This is termed as the factorial portion of the design.
2. N center points (N > 1).
3. Two axial points on the axis of each design variable at a distance alpha from the design center. This is termed as the axial portion of the design. Therefore, the total number of design points or treatment combination in a central composite design is given by $2^k + 2k + N$ (Khuri and Cornell, 1987). A full factorial is utilized if the number of explanatory variables, K, is less than 5. If K is between 5 and 7, a 1/2 factorial is recommended and if K is 8 or greater, a 1/4 factorial is recommended (Dykstra, 1960).

Generally, the value of the axial portion is selected to make the design rotatable. A rotatable design has uniform variance at any given radius from the center of the design. The center points provide a means for estimating the experimental error and provide a measure of lack of fit with one degree of freedom. The number of center points to be included is sometimes selected for experimenter's convenience or to ensure adequate degrees of freedom in the estimate of the experimental error (Thomsons, 1982).

2.10.2.2.2. *Coded variables*

In RSE, variables are usually coded to facilitate computational analysis of data. The use of coded variables instead of the original input variables when fitting the polynomial models has some advantages. Two of the most obvious advantages are;

1. Computational ease and increased accuracy in estimating the model coefficients.
2. Enhanced interpretability of the coefficient estimates in the model (Khuri and Cornell,

1987). Also by using coded levels for each variable, the designs are dependent only on the number of variables and the selected response equation. The center point for each explanatory variable level is given by a code of zero. The highest and lowest levels of interest for each variable are coded plus (+) or minus (-) one respectively for three level designs. Designs having more than three independent variables, the highest and lowest levels of interest are given a maximum and minimum code respectively (Thomsons, 1982).

2.10.3. *Response surfaces*

Generally, the results of response surface experiments are either reported as a mathematical model or used to optimize the system response. Therefore, the response can be thought of as a surface over the explanatory variables' experimental space. In view of this, the term response surface has been associated with experiments intended to identify or evaluate one or more response variables as a function of the independent variables. When the fitted response function is graphed as a function of independent variables, the resulting graph is called a Response Surface plot or Contour map (Gacula and Singh, 1984). These plots may be either three-dimensional or two-dimensional in nature. Giovanni (1983), reported that response surfaces can be prepared in a wide variety of shapes, the most commonly generated ones being the cradle (or bowl) and the saddle point. For the cradle, the optimum response lies along the top edges, while the saddle point has the optimum response along the sides or in each of the four corners.

2.11. *Modified atmosphere packaging*

The natural composition of air is 78% N_2, 21% O_2 and 0.03% CO_2 and each of these gases has different characteristics. A modified atmosphere can simply be defined as any gas atmosphere where composition differs from air. Atmosphere modification can be achieved

through various techniques;

(1) Vacuum packaging which strictly is a form of MAP (Elworthy et al., 1989). Vacuum packaging can be defined as the packaging of a product in a high barrier package from which air is removed to prevent growth of aerobic spoilage organisms, shrinkage, oxidation and color deterioration (Genigeorgis, 1985).

(2) Controlled atmosphere packaging (CAP) which is the enclosure of food products in variable gas-barrier materials, in which the gaseous environment has been changed and is selectively controlled to increase shelf life,

(3) Modified atmosphere packaging (MAP) which is the enclosure of the food products in high gas-barrier materials, in which the gaseous environment has been changed once to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage with the final effect of lengthening shelf life (Koski, 1988; Young et al., 1988). MAP differs from CAS which involves maintaining a precisely defined atmosphere in the storage chamber, and this is achieved by means of a system which continuously compensates for atmospheric changes caused by product or microbial respiration or container permeability and

(4) Use of oxygen absorbents/gas generators which are placed inside a packaged product and actively modifies the gas. They are defined as " a range of chemical compounds introduced into the MAP package (not the product) to alter the atmosphere within the package (Agriculture Canada, 1990). The compounds remove O_2 or add CO_2 into the package environment. O_2 absorbers in general act as a compliment to MAP, reducing O_2 level to approximately 0.0001% (Smith, 1993). The capacity of these absorbers have definite boundaries, the efficiency and length of control is determined to a large extent by the transmission rates of the packaging material and the rate of gas production by the

product (Young, 1987). There are various types of O_2 absorbers, most of them in the form of small satchets containing various reducing agents such as powdered iron oxide, ferrous carbonate, ferrous compounds or metallic platinum (Wagner and Vaylen, 1990) or ascorbic acid and its associated salts packed in air permeable materials. The organic compounds lessen problems associated with metal detectors or the potential for metallic taste and odours that may be imparted to the food products (Smith, 1993). The various types of O_2 absorbers, classified based on the type of material, reaction style, reaction period, application and function are shown in Table 16. O_2 absorbers play a very significant role in that they produce an oxygen-free environment (less than 0.01% O_2), ensuring a longer shelf life for foods by preventing the growth of molds and aerobic bacteria, insect damage and oxidation of unsaturated fatty acids (Harima, 1990). It is usually used in conjunction with an O_2 indicator called the ageless eye. Its function is based on a chemical reaction used an indicator. It is pink when there is no O_2 in the environment (0.1% or less) and blue in the presence of O_2 (0.5% or more) (Smith, 1993).

Oxygen absorbers have been used in the shelf life extension of pizza crust, sponge cake and other bakery products (Mitsubishi gas company reports). Studies by Smith et al., (1986), showed the usefulness of O_2 absorbers in extending the shelf life of crusty rolls to more than 60 days. Powers and Berkowitz (1990) also used O_2 absorbers to extend the shelf life of MRE (meals ready-to-eat) pouched bread for 13 months. Generally, MAP is thought of as an alternative to vacuum packaging but on the other hand vacuum packaging is a form of MAP because the removal of air is in itself a modification of the atmosphere (Hintlian and Hotchkiss, 1986). Vacuum packaging is considered a form of MAP in the sense that elevated levels (10 - 20%) of CO_2 are produced within the vacuum packages by microorganisms on the food as they utilize the residual O_2 (Silliker and Wolfe, 1980) or by respiring produce.

Table 16 Classification of oxygen absorbers

A. Classification according to material
1. Inorganic-iron powder
2. Organic-ascorbic acid, catechol
B. Classification according to reaction style
1. Self-reaction type
2. Moisture-dependent type
C. Classification according to reaction period
1. Immediate effect type
2. General effect type
3. Slow effect type
D. Classification according to use
1. For very moist foods
2. For moderately moist foods
3. For low-water foods
4. For extra dry foods
E. Classification according to function
1. Single function type
2. Composite function type
a. Oxygen absorption and carbon dioxide generation
b. Oxygen absorption and carbon dioxide absorption
c. Oxygen absorption and alcohol generation
d. Oxygen absorption and others

(Harima, 1990).

2.11.1. *Elements of MAP*

Certain factors (elements) directly influence the safety and shelf-life of MAP products. These factors are;

(i) Nature of the product

(ii) The gaseous environmwent

(iii) The package

(iv) Storage temperature

(v) Packaging process.

2.11.1.1. *Nature of the product*

There is no universal MAP formula, each product or product category has its own characteristics and unique needs. Each will respond in a different way and to a different extent to the application of MAP (Elworthy et al., 1989). The critical factors include the product pH and water activity values, the presence of antimicrobial additives or preservatives and the respiration rates of the product itself. It is also very important that both the quantity and types of pathogenic and spoilage microorganisms expected to occur on the product be known (Hotchkiss, 1988).

2.11.1.2. *Gaseous environment*

The gases used for MAP are mainly carbon dioxide, oxygen and nitrogen and their concentrations play an important role with respect to each individual product.

2.11.1.2.1. *Carbon dioxide (CO_2)*

Carbon dioxide is found in the atmosphere in trace amounts and is also a by-product of respiration, including human respiration. Its effect on microorganisms varies with the type of organism. Some require small amounts while others are inhibited or killed (Hotchkiss, 1989). Carbon dioxide is important because of its biostatic activity against many spoilage organisms which grow at refrigeration temperatures (Enfors and Molin, 1980) and its inhibitory effect on product respiration (Kader, 1980). To be effective, CO_2 must dissolve into the liquid phase of the food product (Hotchkiss, 1988) and it should be applied at relatively high concentrations to ensure its availability for extended period of time. The mechanism of inhibition is not known but it is speculated that, inhibition may be due to a simple lowering of pH within the cells of some organisms or it may inhibit specific metabolic pathways (Daniels et al., 1985). Inhibition is optimal at a higher CO_2 concentrations but if the concentration is too high, this may result in discolouration and a sharp acid taste (due to the carbonic acid produced when CO_2 dissolves in the water contained in the food). The time of application, the storage temperature and the concentration of gas all contribute to the degree of inhibition. CO_2 also has the advantage of being relatively nontoxic to humans.

2.11.1.2.2. *Nitrogen (N_2)*

Nitrogen is found in the atmosphere. It is inert, tasteless and almost insoluble in water and does not react directly with microorganisms or food products. N_2 has two roles in gas packaging, first, it displaces or replaces reactive gases such as O_2 and CO_2 and thus reduces oxidative reactions such as lipid oxidation and rancidity in foods and help prevent off-flavors. N_2 can also indirectly influence microbes in perishable foods by displacing O_2, preventing growth of aerobic organisms, whilst anaerobes proliferate. Secondly, it acts as

an inert filler to keep the package from collapsing as CO_2 dissolves into the product (Hotchkiss, 1989).

2.11.1.2.3. *Oxygen (O_2)*

Oxygen forms about 21% of the air's composition and it is known for its reactivity. Oxygen is necessary for many forms of life at atmospheric concentrations, but relatively small deviations from this concentration can be toxic for these same organisms. Oxygen inhibits the growth of anaerobic pathogens therefore its inclusion in a package can be a safety factor but on the other hand, most spoilage microorganisms require O_2 and therefore its exclusion makes sense from the standpoint of spoilage (Hotchkiss, 1989). In many cases, O_2 does not directly extend shelf life (Hotchkiss, 1988). Much deterioration in flavor and colour, oxidation and mold generation proceeds in the presence of small amounts of residual O_2 (Harima, 1990). Oxygen is desirable for the preservation of the red colour of fresh beef. On the whole, the reliability of the gas mixture should not be an assumption but must be a quality control factor (Hotchkiss, 1988).

2.11.1.3. *Storage temperature*

Temperature plays an important role in most reactions as well as metabolic processes, therefore, temperature control is a critical factor when MAP is used. MAP is not a replacement or even a backup for proper storage temperature (Ogrydziak and Brown, 1982). The effectiveness of MAP is decreased as the storage temperature increases because the solubility of a gas in a liquid (or product) also decreases. For example, for respiring products, increasing the temperature also increases the rate of respiration resulting in a decrease in shelf life. Therefore, the effects of temperature abuse are particularly important from a safety standpoint (Hotchkiss, 1988).

2.11.1.4. *The package*

Factors of importance with respect to package include headspace, permeability, form and intergrity. The amount of gas and the time that the gas is available for microbial inhibition is affected by the package headspace and permeability. Higher barrier packages with larger headspace yield longer shelf-life . For respiring products, the permeability characteristics of the film determine to a large extent, the equilibrium gas concentration achieved in the package (Hotchkiss, 1988). The goal of MAP is to allow sufficient gaseous contact or interaction with product (since dissolution of the gas into product is what extends the shelf-life). For this reason, package design is very important (Hotchkiss, 1988).

The basic MAP systems used are;

1. Flexible thermoform with vacuum or gas flush,
2. In-line-formed or preformed rigid trays with lid stock or gas flush,
3. Vacuum skin packaging and master-pack/bulk packaging of many non-barrier tray overwrapped packages in a barrier pouch with vacuumizing/backflushing,
4. Heat-sealable bags (films) with vacuum or gas flush (Lawlis and Fuller, 1990).

The films used for gas packaging must adhere to certain criteria such as having low vapour transmission rates and preventing changes in moisture content. Therefore, for the selection of packaging film, certain factors should be taken into consideration such as the gas and moisture barrier properties, the antifog properties, the mechanical strength, sealability, machinability and cost. Polymers commonly used for gas packaging of foods include polyester (nylon), polypropylene, polyvinylidene chloride (PVDC), ethylenevinyl alcohol (EVOH) and polyethylene (PE). Since all of the desired characteristics of a packaging film are seldom found in one polymer, individual polymers are laminated to one another to produce films with the desired characteristics for gas packaging of both non-respiring and respiring products. Examples of laminated structures for non-respiring products used

include nylon - PE, nylon - PVDC - PE and nylon - EVOH - PE. Strength is provided by the outermost layer of nylon, gas and moisture vapour impermeability is provided by EVOH or PVDC and heat sealability is provided by PE (Smith et al., 1990b).

2.11.1.5. *The Process*

Modification of the atmosphere within the package can be done through one of several methods including (1) snorkel vacuum and backflush (2) chamber vacuum and flush (3) use of engineered permeability films or (4) use of interactive inedible components. The last two depends on the interaction of the product through respiration, the permeability of the film and the addition of O_2, CO_2 and moisture absorbents to the package (Hotchkiss, 1988). The first two factors simply involves removing air from the package and replacing it with a mixture of gases. The pressure of the gas inside the package usually approaches one atmosphere (Smith et al., 1990b).

2.11.1.6. *Applications of MAP*

MAP has been used extensiveiy in (1) extending the shelf-life of several food products, (2) prevention of oxidation (of lipids), discoloration, fading and protection of nutrients, (3) prevention of microbial spoilage, (4) preservation of red oxymyoglobin color for red meat and (5) physical protection of contents. The effectiveness of modified atmospheres in the area of food packaging and preservation depends in part on the nature of the food product. Food products can be grouped into two major categories, respiring products such as fruits and vegetables and non-respiring products such as cooked foods, complex multi-ingredient systems. Table 17 shows some of the applications of MAP for shelf life extension of food.

Table 17. Examples of gas mixtures for selected food products

Product	Temp.^{o}C	Gas concentration(%)		
		O_2	CO_2	N_2
Fresh meat	0 - 2	70	20	10
Cured meat	1 - 3	0	50	50
Poultry	0 - 2	60 - 80	20 - 40	0
Fish - fatty	0 - 2	0	60	40
Fish - white	0 - 2	30	40	30
Fresh poultry	0 - 2	0	50	50
Fresh sausage	0 - 2	40	60	0
Cheese	1 - 3	0	60	40
Apples	4 - 6	2	1	97
Tomatoes	5 - 10	4	4	92
Baked product	RT	0	60	40
Pizza	RT	0	60	40
Dry snacks	RT	0	20 - 30	70 - 80

RT - Room temperature (Adapted from Smith et al., 1990b)

2.12 *RESEARCH OBJECTIVES*

The control of aflatoxin production by *Aspergillus flavus* and Aspergillus parasiticus in food and feedstuffs is of regulatory concern both nationally and internationally. This objective is even more critical in view of recent recommendations to lower the standard of acceptable safety (in food) of aflatoxin from 20ng/g to 10-15ng/g. Many studies have been done to examine the effect of various environmental storage factors to control the growth of and aflatoxin production by *A. flavus* and *A. parasiticus* in agricultural products. The experimental approach used in these studies has usually been the conventional "one variable at a time approach". The limitations of this experimental approach are (i) it is time consuming and laborious, (ii) it generates a lot of data which may be difficult to interpret, (iii) it does not take into account the main factors and their interactions on toxin production and (iv) it does not generate any mathematical equation to predict the levels of aflatoxin which can be produced under various storage conditions.

Recently MAP, involving vacuum packaging, gas packaging, the use of oxygen absorbents and more recently the use of oxygen absorbents and carbon dioxide generators is becoming more popular as a method of food preservation. The success of this technology is dependent on a number of inter-related factors, specifically the headspace gas composition within the package and the effect of films of various gas transmission rates in maintaining the headspace gas composition. However, there is a paucity of data on the use of MAP technology to control the growth of aflatoxigenic molds in foods, particularly under tropical environmental storage conditions. To overcome the limitations of the one variable at a time experimental approach and to predict the combined effect of several barriers to control the level of aflatoxin production by *A. flavus* and *A. parasiticus*, the objectives of this study were;

(1) To determine the combined effect using a multifactorial experimental design termed

Response Surface Methodology (RSM) of water activity, pH, headspace oxygen concentration and storage temperature and inoculum levels on the growth of and aflatoxin production by *A. flavus* on synthetic media and peanuts.

(2) To determine the effect of temperature and relative humidity on the gas and vapor transmission rates and permeability of high, medium and low barrier films to oxygen, carbon dioxide and water vapour.

(3) To determine the effect of films of different gas transmission properties on aflatoxin production by *A. flavus* under various MAP/environmental storage conditions.

(4) To determine the effect of novel methods of atmosphere modification involving Ageless oxygen absorbents (Type S) and Ageless oxygen absorbents/carbon dioxide generators (Type G) to control aflatoxin production by *A. flavus* and *A. parasiticus*.

(5) To predict the levels of factors to control the level of aflatoxins to safe and acceptable levels particularly under tropical environmental storage conditions.

CHAPTER 3

CONTROL OF GROWTH AND AFLATOXIN PRODUCTION OF *ASPREGILLUS FLAVUS* UNDER MODIFIED ATMOSPHERE PACKAGING (MAP) CONDITIONS

3.1 INTRODUCTION

Modified Atmosphere Packaging (MAP) is becoming increasingly popular as a method of food preservation. MAP has been defined as "the enclosure of food products in high gas barrier materials in which the gaseous environment has been changed to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage with the intent of extending shelf life (Young et al., 1988). While both Controlled Atmosphere Storage (CAS) and MAP mean that the gaseous atmosphere around a product differs from air, CAS has more precise gas compositional control than MAP and is generally used for bulk storage of products (Smith et al., 1990a,b). MAP is mainly used for the retail distribution of small pre-packed units and the concentration of CO_2 used in these products is generally much higher than the levels used for products stored under CAS conditions (Smith et al., 1990a,b).

MAP involving various gas mixtures of CO_2, O_2 and N_2 has been used to extend the chemical and microbiological shelf life of meat (Clark and Lentz, 1969), peanuts and pecans (Holaday et al., 1979), fish (Banks et al., 1980), rice (Ory et al., 1980), and bakery products (Ooraikul et al. 1984). However, despite the antimicrobial effect of CO_2 enriched atmospheres on the growth of aerobic spoilage microorganisms, several studies have shown that molds can grow in the presence of elevated levels of CO_2 if oxygen is present. Tomkins (1932) found that the inhibitory effect of CO_2 was independent of the oxygen concentration. He showed that the extent of mold growth in 20%, 40% and 60% CO_2 was

the same whether the oxygen concentration was 20% or 5%. Dallyn and Everton (1969) reported that *Xeromyces bisporus*, a xerophilic mold which cause food spoilage in food products of low water activity, grew in an atmosphere of 90% CO_2 and 10% O_2. These authors also reported the growth of *Aspergillus* species in 85% CO_2 and 3% O_2. Smith et al., (1986) also found that *Aspergillus niger* could grow in a gas packaged bakery product containing 60% CO_2 and low oxygen levels (<1% O_2).

Most studies to date have focused on the use of CAS storage conditions involving reduced oxygen levels in conjunction with elevated levels of carbon dioxide to control/inhibit the growth of aflatoxigenic mold species (Clevestrom et al., 1983; Buchanan et al., 1985; Wilson et al., 1985; Paster, 1991). However, there is a paucity of data on the ability of these molds to grow and produce aflatoxin under MAP conditions. The objectives of this study were to determine the combined effect of CO_2 enriched atmospheres containing various levels of headspace oxygen and environmental storage conditions to control the growth of, and aflatoxin production by, *Aspergillus flavus* on synthetic medium. To overcome the limitations of the one variable at a time experimental approach and to adequately quantify the effect of several factors simultaneously on the growth of, and aflatoxin production by *A. flavus* a mathematical modeling technique term Response Surface Methodology (RSM) used by Smith et al., (1988) was used as the experimental design.

3.2 MATERIALS AND METHODS

3.2.1 *Experimental design*

The factors and levels of each factor used in this study included a_w (0.94-0.98), pH (5-9), temperature (15-35^{o}C) and level of O_2 in the package headspace (0-20%) in a balance of CO_2 and N_2 (60:40). The values of each level of environmental factor selected were based on previous studies with *Aspergillus flavus* (Diener and Davies, 1970, Landers et al., 1967; Northolt et al., 1977; Shih and Marth, 1973; Wheeler et al., 1991). To determine the effect of each environmental factor simultaneously on the growth of, and aflatoxin production by *A. flavus*, a 4 factor, 5 level central composite rotatable design (CCRD) of Box et al., (1978) was used (Table 18). In the CCRD, variable levels were coded -2, -1, 0, +1, +2 as descibed by Box et al., (1978). The coded and actual values of levels used in the CCRD are shown in Table 19. All experimental runs were done in duplicate.

3.2.2. *Preparation of Spore Inoculum*

An aflatoxigenic strain of *Aspergillus flavus* (ATCC strain No. 16875), used throughout this study was obtained from the American Type Culture Collection in Rockville, Maryland, U.S.A. The mold was grown on Czapek agar and subcultured onto slants of Malt Extract Agar (MEA) (Difco) for storage at 5^{o}C. Mold inoculum was prepered by growing *A. flavus* on MEA slants for 7 to 10 days at 25^{o}C until sporulation. A spore suspension was prepared by washing the surface of the agar slants with sterile distilled water, followed by filtration through Whatmans No. 1 filter paper. Spores were enumerated using an Improved Neubauer bright line hemacytometer (Cambridge Instruments Inc. Buffalo, New York, U.S.A.). Appropriate dilutions were made from the stock spore suspension using sterile 0.1% peptone solution as diluent to obtain the desired inoculum level of 4×10^1 cells/ml.

Table 18. Coded level combinations for a 4 variable CCRD for growth of and aflatoxin production by *A. flavus*.

Run No.*	Variables**			
	X_1	X_2	X_3	X_4
1	-1	-1	-1	-1
2	1	-1	-1	-1
3	-1	1	-1	-1
4	1	1	-1	-1
5	-1	-1	1	-1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	-1
9	-1	-1	-1	1
10	1	-1	-1	1
11	-1	1	-1	1
12	1	1	-1	1
13	-1	-1	1	1
14	1	-1	1	1
15	-1	1	1	1
16	1	1	1	1
17	-2	0	0	0
18	2	0	0	0
19	0	-2	0	0
20	0	2	0	0
21	0	0	-2	0
22	0	0	2	0
23	0	0	0	-2
24	0	0	0	2
25	0	0	0	0

* Each run replicated twice for a total of 50 runs.

** $X_1 = a_w$; X_2 = pH; X_3 = Temp.^{o}C; X_4 = Headspace O_2 (Balance CO_2:N_2).

Table 19: Values of coded levels used in the CCRD for *A. flavus*.

Variables	Levels				
	-2	-1	0	1	2
a_w (X_1)	0.94	0.95	0.96	0.97	0.98
pH (X_2)	5	6	7	8	9
Temp.^oC (X_3)	15	20	25	30	35
Headspace * O_2 (X_4)	0	5	10	15	20

* Balance CO_2:N_2 (60:40)

3.2.3 *Preparation and Inoculation of media*

Malt extract agar (MEA) was used as the basal media throughout the study. Adjustment of a_w was done using glycerol and measurement of a_w of the adjusted media was done using the Decagon CX-1 equipment (Decagon Devices, Inc. Pullman, WA. U.S.A.). Media pH was adjusted by the addition of appropriate amounts of 1M NaOH to the pre-sterilized media and pH measurement was done using a previously calibrated Corning pH meter (model 240). All plates (100 x 15mm) containing known weight of media were inoculated with 0.5ml (2×10^1 cells) of the prepared inoculum using a surface plating technique, based on the principle that each colony arises from a single cell. For each experimental run, two inoculated plates and one non - inoculated plate (control) were packaged in each bag.

3.2.4. *Packaging*

Packaging and sealing of packages was done using a Multivac type vacuum/gas packaging unit (model AG 500) (W. R. Grace and Co., Ajax, Ont. Canada). Plates were packaged in 210 x 210mm Cryovac bags (O_2 transmission rate 3 - 6 cc/ m^2 / 24hr, atm @ 4.4°C, 0% RH) under various gas atmospheres. Each package was initially filled with 60:40 ratio of CO_2:N_2 gas mixture. A Smith's proportional mixer (Tescom, Minneapolis, Minnesota) was used to give the desired proportions of CO_2 and N_2 in each package. Specific volumes of O_2 gas, obtained from a previously constructed calibration curve, were injected through silicone seal attached to each bag to give the desired headspace O_2 concentration in the experimental design. Anaerobic conditions (0% O_2) were obtained and maintained within the package headspace by placing an Ageless type S - 100 oxygen absorbent (Mitsubishi Gas Chemical Co., Japan) inside the appropriate packages prior to sealing. Packaged plates were incubated at 25°C and 35°C in a Precision Gravity Convection incubator (model 2EG), at 20°C in a Forma Scientific incubator and at 15°C in a Fisher Scientific

Low temperature incubator (model 307) for 15 days. Unadjusted control plates (a_w 0.995, pH 7.0) were inoculated as described, packaged in air and stored at 20, 25 and 30^{o}C. All plates were examined daily for visible signs of mold growth. Mold colonies, when observed, were measured daily for increse in colony diameter (mm) until the end of the storage period (Day 15) using the method of Northolt et al., (1977).

3.2.5 *Headspace gas analysis*

Headspace gas analysis was done immediately after packing to ensure that the desired concentration of gases had been attained and maintained throughout the 15 day storage period. Gas samples were drawn using a 0.5ml gas-tight Pressure-lok syringe (Precision Sampling Corp., Baton Rouge, La), through silicone seals attached to the outside of each package. Headspace gas was analysed using a Varian gas chromatograph (model 3300, Varian Canada Inc.) fitted with a thermal conductivity detector and using a Porapak Q (80-100 mesh) and molecular Sieve 5A (80-100) columns in series. Helium gas was used as the carrier gas with a flow rate of 80 ml/min. The column oven temperature was initially set at 50^{o}C, then programmed to increase by 50^{o}C/min to reach 150^{o}C. The injector and detector temperatures were set at 80^{o}C and 100^{o}C. Peaks were recorded and analysed with a Hewlett Packard intergrator (model 3390A, Hewlett-Packard Co. Avondale, PA.).

3.2.6 *Extraction and analysis of aflatoxin B_1*

After 15 days incubation, the contents of each plate showing mold growth were carefully transferred into 125ml flask, 40ml of chloroform added and the mixture shaken in a precision water bath shaker (Precision Scientific Inc. Chicago, Illinios, U.S.A.) for 1 hour. The mixtures were then fitered twice using 24cm Whatman's No.1 filter paper and the filtrate collected in a 50ml flask. The filtrate was then evaporated under a gentle stream of

N_2 in a water bath at 40°C to reduce the volume to less than 10ml. Contents of each flask were carefully transferred into 7ml vials (amber coloured) and evaporated to complete dryness under a gentle stream of N_2. Derivatization and quantitation of the extracted aflatoxin B_1 was done based on the method of Park et al., (1990) using trifluoroacetic acid as the derivatizing agent. The derivatized solution was filtered prior to liquid chromatographic analysis using a 0.22 *um* Millex-GV millipore filter unit (Millipore Canada Ltd., Mississauga, Ontario). Quantitation of the derivatized extracts for aflatoxin B_1 was done using a Beckman System Gold programmable module 126. Aflatoxin B_1 was detected by a Beckman 157 fluorescence detector, with a 7-60 excitation filter and a 450nm (40nm BW) emission filter attached to a Beckman System Gold Analog Interface module 406. Detection was done at the least sensitive range of 1.00 R.F.U (Relative fluorescence unit) and the solvent flow set at 1.00 ml/min. Concentration of the aflatoxin B_1 was calculated by comparison to standard peaks of known concentration of aflatoxin B_1 as outlined in the Beckman System Gold methodology.

3.2.7 *Statistical analysis*

Statistical analysis (regression coefficients, analysis of variance and correlation coefficients) were computed using the Statistical Analysis System (SAS, 1982). All 3 dimensional graphs and 2 dimensional contour plots were done using the SAS/Graph program on a McGill University mainframe computer and a Zeta plotter.

3.3 RESULTS AND DISCUSSION

The combined effect of a_w, pH, temperature and initial headspace O_2/CO_2 concentration on the growth of, and aflatoxin production by *A. flavus* on MEA plates packaged in a high gas barrier film after 15 days is shown in Table 20. Mold growth was either completely inhibited or was visible after 1-2 days. Generally, growth was more extensive in plates packaged in 10-20% O_2 (54-48% CO_2) and stored at 25-35°C. Growth of *A. flavus* was inhibited in certain treatment combinations stored at 25°C and completely inhibited in all plates stored at 15-20°C. Holmquist et al., (1983) and Karunaratne and Bullerman (1990) found that maximal growth of *A. flavus* occurred at 33-35°C and decreased as storage temperature was reduced. Furthermore, *A. flavus* has been reported to grow over a temperature range of 12-43°C (Ayerst, 1969) and a pH range of 3.9-9.1 (Lie and Marth, 1968). However, it is widely accepted that microorganisms show greatest tolerance to a single environmental factor, such as CO_2 concentration, storage temperature or pH when all other conditions conditions are optimal for growth (ICMSF, 1980). Conversely, two or more simultaneous environmental conditions or "barriers" will be far more inhibitory than each barrier considered seperately, a synergism which has been well documented in several studies with aflatoxigenic mold species. Landers et al., (1967) and Yackel et al., (1971) reported that the inhibitory effect of a CO_2 enriched atmosphere on the initial growth and mycelial development of *A. flavus* was enhanced at lower storage temperatures. Holmquist et al., (1983) also demonstrated a synergism between a_w and temperature on the growth of *A. flavus* and *A. parasiticus*. Mold growth occurred at 22, 27 and 33°C and decreased linearly as a_w was reduced from 0.99 to 0.85. At 15°C, mold growth was observed but only at a_w values of 0.95 or above. Magan and Lacey (1984) found that aflatoxigenic field and storage fungi were tolerant to low O_2 concentrations (0.14%) and to elevated CO_2 levels (>15%) at high a_w levels (0.98). However, a reduction in both storage temperature and a_w level increased the sensitivity (measured by linear growth) of the fungi to high CO_2 levels

Table 20. Effect of water activity, pH, storage temperature and gas atmosphere on growth of and aflatoxin production by *A. flavus*.

	Variables				Extent of growth[a]	Aflatoxin B_1(ng/g)[b]
Run #	a_w	pH	T°C	O_2%		
1	0.95	6	20	5	-	-
2	0.97	6	20	5	-	-
3	0.95	8	20	5	-	-
4	0.97	8	20	5	-	-
5	0.95	6	30	5	4.50(0.42)	1.35(0.20)
6	0.97	6	30	5	3.60(0.33)	2.19(0.15)
7	0.95	8	30	5	2.00(0.18)	1.69(0.07)
8	0.97	8	30	5	5.60(0.30)	1.64(0.10)
9	0.95	6	20	15	-	-
10	0.97	6	20	15	-	-
11	0.95	8	20	15	-	-
12	0.97	8	20	15	-	-
13	0.95	6	30	15	7.40(0.12)	1.61(0.09)
14	0.97	6	30	15	7.50(0.26)	2.19(0.12)
15	0.95	8	30	15	4.10(0.18)	1.60(0.12)
16	0.97	8	30	15	6.70(0.41)	1.82(0.04)
17	0.94	7	25	10	-	-
18	0.98	7	25	10	6.20(0.43)	0.83(0.03)
19	0.96	5	25	10	-	-
20	0.96	9	25	10	-	-
21	0.96	7	15	10	-	-
22	0.96	7	35	10	9.50(0.25)	1.08(0.08)
23	0.96	7	25	0	-	-
24	0.96	7	25	20	6.20(0.43)	2.60(0.46)
25	0.96	7	25	10	9.80(0.15)	1.74(0.04)

[a] Colony diameter in mm after 15 days.

[b] Mean of 4 replicates after 15 days.

- No growth.

( ) Standard deviation.

with most species being inhibited by low CO_2 concentrations (<5%). The inhibition of mold growth at 15-20^{o}C and in certain treatments at 25^{o}C in this study can also be attributed to the synergistic effects of several "hurdles" or "barriers" as extensive growth of *A. flavus* was observed in all unadjusted MEA plates (a_w 0.995, pH 7.2) packaged in air after 2-4 days 20-25^{o}C.

In all cases where growth occurred, the level of aflatoxin detected was less than the current regulatory standard of 20ng/g. The low level of aflatoxin observed can again be attributed to the combined inhibitory effects of storage temperature, a_w, pH and CO_2 enriched atmospheres on the growth of and aflatoxin production by *A. flavus*. Cuero et al. (1988) reported that aflatoxin production by *A. flavus* decreased from a maximum level of 54 ng/g in maize with a_w of 0.95 and stored at 16^{o}C to 0 ng/g in maize with an a_w 0.90 at the same storage temperature. Epstein et al., (1970) observed a maximum concentration of aflatoxin of 2 ug/g after 12-13 days at 60^{o}F (16^{o}C) in corn stored under a controlled atmosphere of 10% CO_2, 1.8% O_2 and 88.2% N_2. However, the higher concentration of aflatoxin detected by Epstein et al. (1970) i.e. approximately 1000X the maximum concentration detected in our study, was probably due to the low level of CO_2 (10%) used in their study. Studies have shown that low CO_2 concentrations may even stimulate aflatoxin production (Gibb and Walsh, 1980 ; Magan and Lacey, 1984). Landers et al., (1967) concluded that, under optimal conditions of temperature and moisture for mold growth, a high CO_2 concentration (20-70%) was far more inhibitory to aflatoxin production than a low headspace O_2 concentration. Another factor contributing to the low level of aflatoxin observed (Table 20), may be the low inoculum level used (1 spore/g) in this present study. Karunaratne and Bullerman (1990) observed that an inoculum level of 10^3 spore/g resulted in the highest amount of aflatoxin being formed (380 ug/g) at 28^{o}C while lower and higher inoculum levels (10^1 and 10^7 spores/g) produced comparatively lower levels of aflatoxin. However, at higher storage temperatures (33^{o}C), the lowest spore level (10^1 spores/g)

produced the highest amount of aflatoxin (68 ug/g) (Karunaratne and Bullerman, 1990).

The changes in headspace O_2, CO_2, and N_2 for selected treatments showing mold growth are shown in Figure 9. In all cases, headspace O_2 decreased to less than 1% within 1-10 days depending on the initial O_2 concentration and storage temperature. Headspace CO_2 increased initially throughout storage as a result of mold metabolism and then gradually decreased due either to its absorption by the media, its loss through the packaging film or possibly its incorporation into the synthetic pathway for aflatoxin production (Hsieh and Mateles, 1971). There appeared to be a trend between changes in headspace CO_2 and concentration of aflatoxin detected. For example, in experimental treatments 6 and 22 (Table 20), headspace CO_2 decreased by approximately 15% and 8% respectively over the 15 day storage period resulting in 2.19 ng/g and 1.08 ng/g of aflatoxin being detected. Both Magan and Lacey (1984) and Gibb and Walsh (1980) observed a stimulatory effect of 5-10% CO_2 on mold growth at low O_2 concentration (<1%). These authors concluded that the beneficial action of CO_2 was due to carbon dioxide fixation resulting from the enhanced nutritional requirements of fungi under virtually anaerobic conditions. Statistical analysis of the data showed no significant correlation between changes in headspace CO_2 with time and the level of aflatoxin at the end of the 15 day storage period. However, a significant correlation was evident between the extent of mold growth and aflatoxin production (Figure 10). Regression analysis of aflatoxin production and mold growth showed a curvilinear relationship as shown by Equation 1.

$$\mathbf{Y = 0.017 + 0.060\ Growth - 0.048\ Growth^2} \quad \text{(Equation 1)}$$

The analysis of variance (ANOVA) for Y, aflatoxin production (ng/g) indicated that the model was highly significant ($p < 0.001$) with an R^2 value of 0.86 and a correlation coefficient of 0.83. The curvilinear relationship between aflatoxin production (Y) and

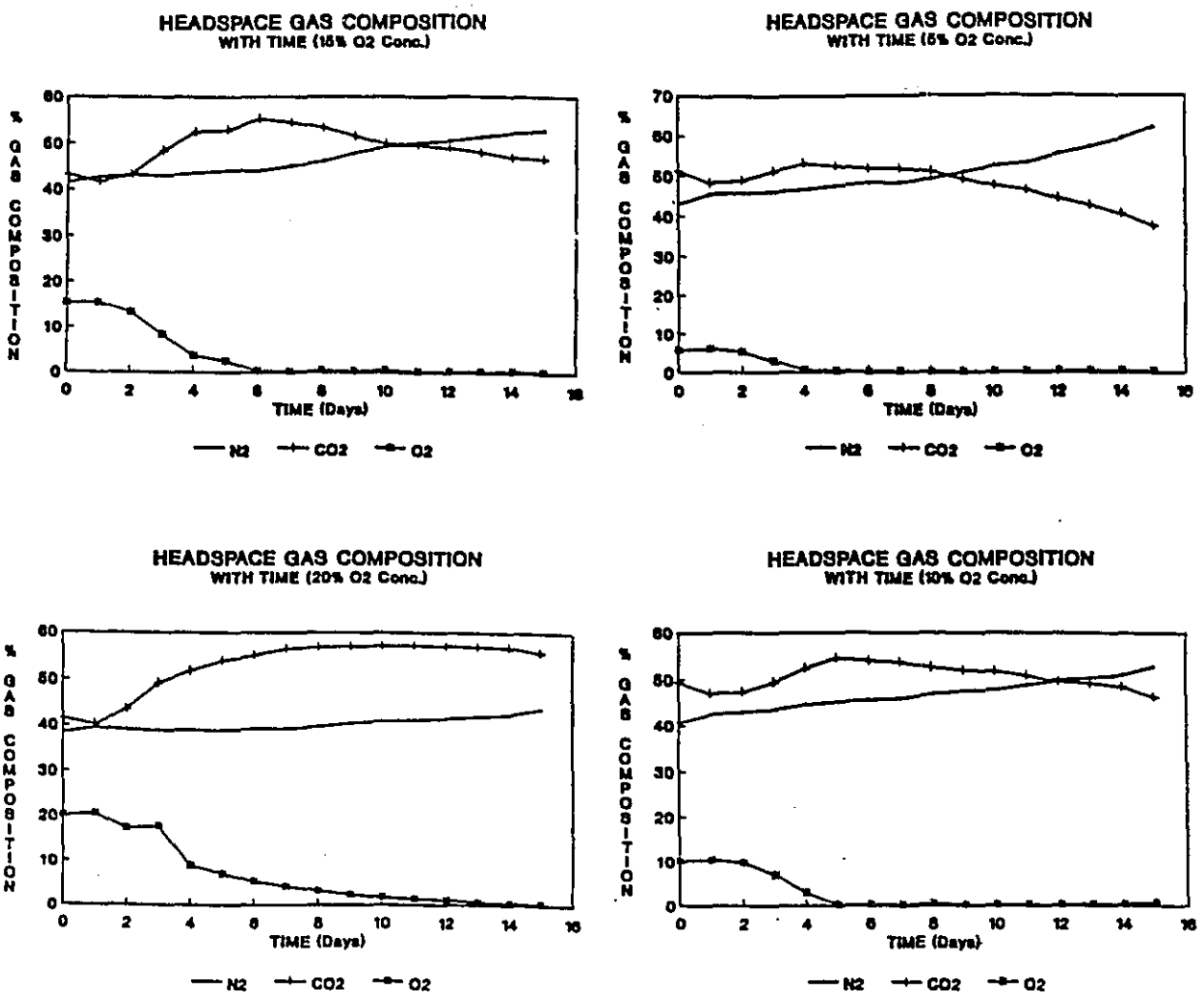


Figure 9. Headspace gas changes in inoculated malt extract agar plates packaged under various gaseous consitions

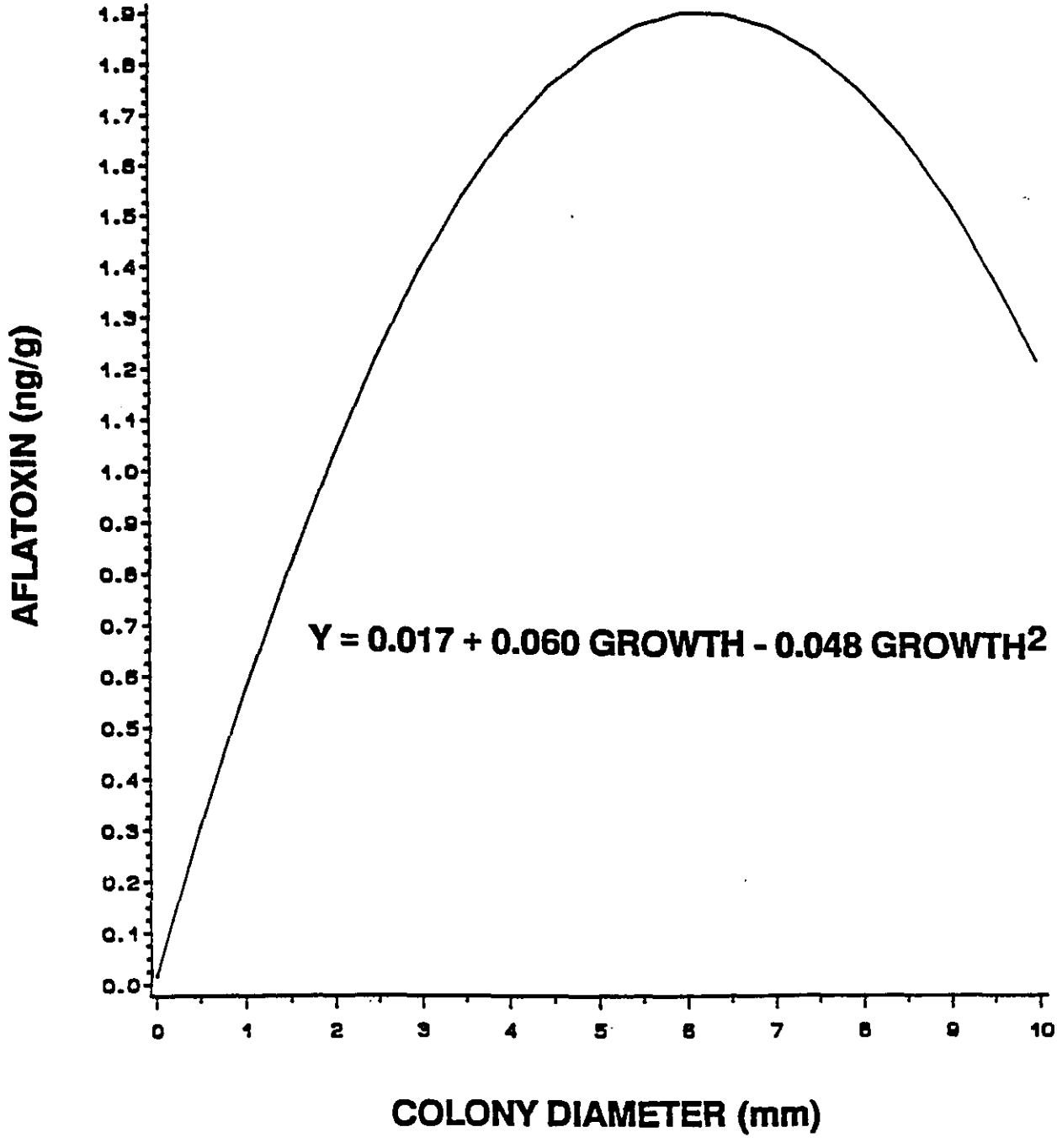


Figure 10. Relationship between colony diameter and aflatoxin production

growth (colony diameter, (mm)) indicates that aflatoxin production increases up to a specific colony diameter (approximately 7 mm for this set of experimental conditions) and then decreases with increasing colony diameter. This may be due to to a depletion of available substrate for further mycelial development and mold growth (Doyle et al., 1982). Karunaratne and Bullerman (1990) also demonstrated a curvilinear relationship between aflatoxin production and spore inoculum. Maximum aflatoxin production was observed at lower inoculum levels (10^3 spores/ml) while at higher inoculum levels (10^7 spores/ml) mold mycelia were capable of degrading the pre-formed aflatoxin at a faster rate than the rate at which the toxins were formed (Karunaratne and Bullerman, 1990). Buchanan et al., (1985) also reported that aflatoxin production by *A. flavus* accumulated rapidly after 1-2 days in product packaged in air or with 2% O_2. Thereafter, aflatoxin production decreased rapidly and remained at a constant level (<2 ug/mg dry weight) for all gaseous treatments. These results indicate that the rate of aflatoxin production is greatest in young mycelium and, as colonies enlarge, less physiologically active mycelia contribute more to colony dry weight.

To determine the combined synergistic effects of a_w, pH, storage temperature and elevated levels of CO_2 in the presence of various levels of oxygen on the growth of, and aflatoxin production by, *A. flavus* an Response Surface Method (RSM) approach was used. The advantage of this approach is that mathematical models can be generated to predict the packaging/environmental storage conditions which would control aflatoxin production by *A. flavus* particularly at temperature abuse storage conditions. The second order polynomial model generated from multiple regression of the uncoded results (Table 20) is shown in Equation 2.

$$Y = -14356 + 29961X_1 - 5.29X_2 - 3.22X_3 - 0.11X_4 - 15812X_1^2 - 2.39X_2^2 - 0.046X_3^2 - 0.06X_4^2 + 42.5X_1X_2 + 6.5X_1X_3 + 1.5X_1X_4 - 0.06X_2X_3 - 0.07X_2X_4 + 0.02X_3X_4 \quad \text{(Equation 2)}$$

Regression analysis of this model showed that the fitted model was highly significant ($p < 0.001$) and accounted for 92% of the total variation after being corrected for the mean. Examination of the fitted model indicated that all linear terms (a_w, X_1; pH, X_2; temperature (^oC), X_3 and headspace O_2, X_4) and their corresponding quadratic terms (a_w^2, X_1^2; pH^2, X_2^2; temperature^2 (^oC), X_3^2 and headspace O_2^2, X_4^2) were highly significant ($p < 0.001$) while all cross product terms (a_w.pH, X_1X_2; a_w.T^oC, X_1X_3; a_w.O_2, X_1X_4; pH.T^oC, X_2X_3; pH.O_2, X_2X_4 and T^oC.O_2, X_3X_4) were non-significant. These results are in agreement with Holmquist et al., (1983) who founds that a_w, pH and storage temperature were all highly significant factors ($p < 0.01$) in controlling the growth of *A. flavus* and *A. parasiticus* on synthetic media. Smith et al., (1988) also showed that a_w, pH, headspace O_2 and CO_2 and storage temperature were the most important factor ($p < 0.01$) influencing the growth of *A. niger* in a gas packaged bakery product.

The significant linear and quadratic terms influencing mold growth (Equation 2) were subsequently used to generate 3 dimensional response surface graphs and 2 dimensional contour plots. Examples of response surface graphs of a_w versus pH on the growth of *A. flavus* with temperature and headspace O_2 levels held constant at 30^oC and oxygen levels at 5% and 10% are shown in Figures 11-12. As these figures illustrate, a decrease in product a_w-pH or decrease in a_w and increase in pH resulted in a decrease in colony diameter. However, an increase in a_w at all pH values resulted in an increase in colony diameter. The inhibitory effect of both a_w and pH on mold growth was more enhanced when plates were packaged initially in 5% headspace O_2 (Figure 11) compared to plates packaged in 10% O_2, balance CO_2:N_2 (Figure 12). The results are again consistent with previous observations of the synergistic effects between a_w, pH, storage temperature and headspace gas composition on growth of, and aflatoxin production by, aflatoxigenic molds species.

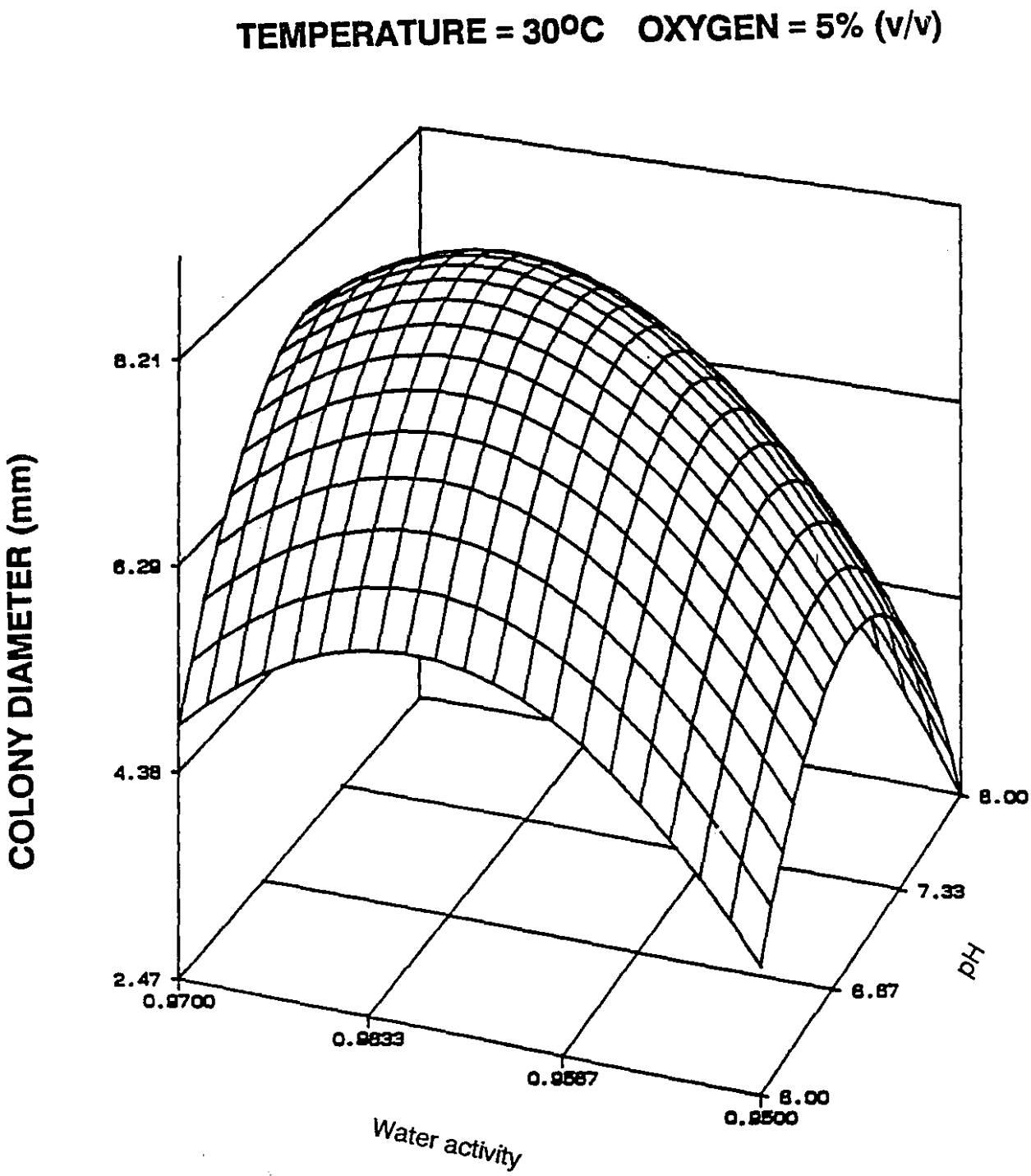


Figure 11. Three dimensional Responce Surface Graph showing effect of a_w and pH on colony diameter (mm) of *A. flavus* at 30^0C and 5% O_2 concentration.

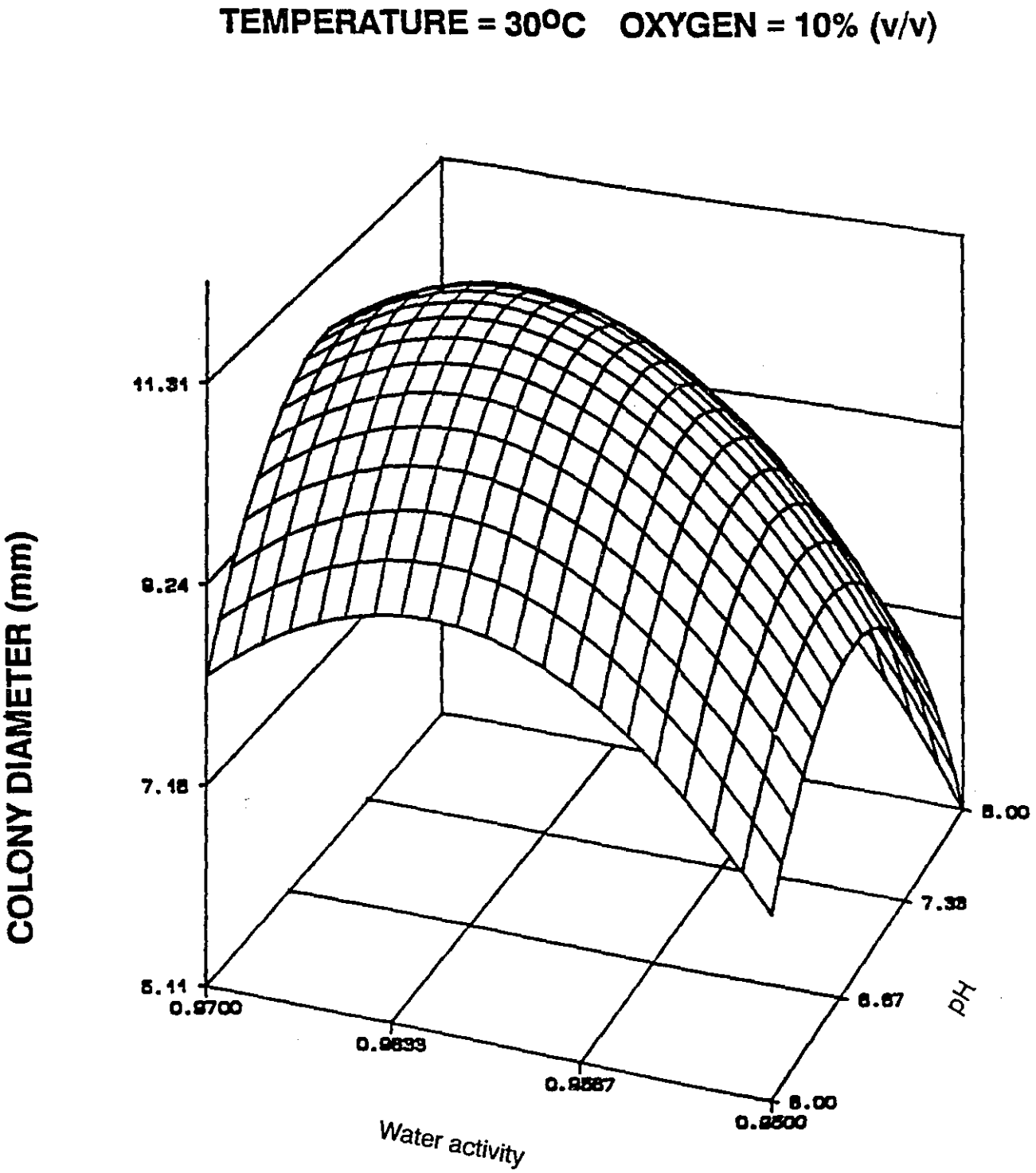


Figure 12. Three dimensional Response Surface Graph showing the effect of a_w and pH on colony diameter (mm) of *A. flavus* at 30°C and 10% O_2 concentration.

The advantage of these 3 dimensional graphs is that they assist in determining the level of factors which could be used to control the growth of and aflatoxin production by *A. flavus* in food of similar a_w and pH values and packaged under similar gaseous conditions. They also graphically show the nature of the fitted surface as maximum, minimum or saddle point. Canonical analysis for this set of experimental data indicates that the stationary point on the fitted surface is maximum. The values of variables, which would give a maximum diameter of 11.6 mm are shown in Table 21. The actual colony diameter observed for this set of experimental conditions was 11 mm and indicates the accuracy of the fitted model to predict the extent of mold growth. However, it is difficult to determine the actual levels of a_w and/or pH which could be used to control the growth of and aflatoxin production by *A. flavus* from 3 dimensional graphs. This can be more readily achieved from 2 dimensional contour plots of the same variables (Figures 13-14). For example, the growth of *A. flavus* could be reduced to less than 2 mm at either pH 6 or 8 and a_w of 0.95 in a MAP product packaged with 5% O_2 (balance CO_2:N_2, 60:40) and stored at 30°C (Figure 13). However, if the headspace O_2 concentration is increased to 10%, the colony diameter would increase to approximately 5 mm for similar a_w and pH levels (Figure 14). On the basis of the significant correlation between the extent of mold growth and level of aflatoxin production (Equation 1), the predicted level of aflatoxin B_1 for these colony diameters would be 0.7 - 1.6 ng of aflatoxin B_1/g of product. By referring to these contour plots, the manufacturer can use appropriate combinations of high pH and low a_w or low pH and low a_w to completely inhibit mold growth or to control the level of aflatoxin production by *A. flavus* in food packaged under MAP conditions particularly with residual oxygen levels. Residual headspace oxygen could readily occur through improper evacuation/gas packaging of product or through packaging of product in a low - medium barrier film. The growth of *A. flavus* could be controlled under such conditions through judicious reformulation of a product's a_w and pH. However, if this were not possible from a sensory viewpoint or from a product viewpoint, e.g., peanuts, then the product can be

Table 21: Actual values of variables at stationary point Xo* (Point of optimum colony diameter (mm).

Factor	Actual Value
a_w (X_1)	0.964
pH (X_2)	6.86
Temperature ^oC (X_3)	31.7
Level of O_2 (X_4)**	12.9

* Predicted colony diameter at stationary point 11.6mm.

** Balance CO_2:N_2 (60:40)

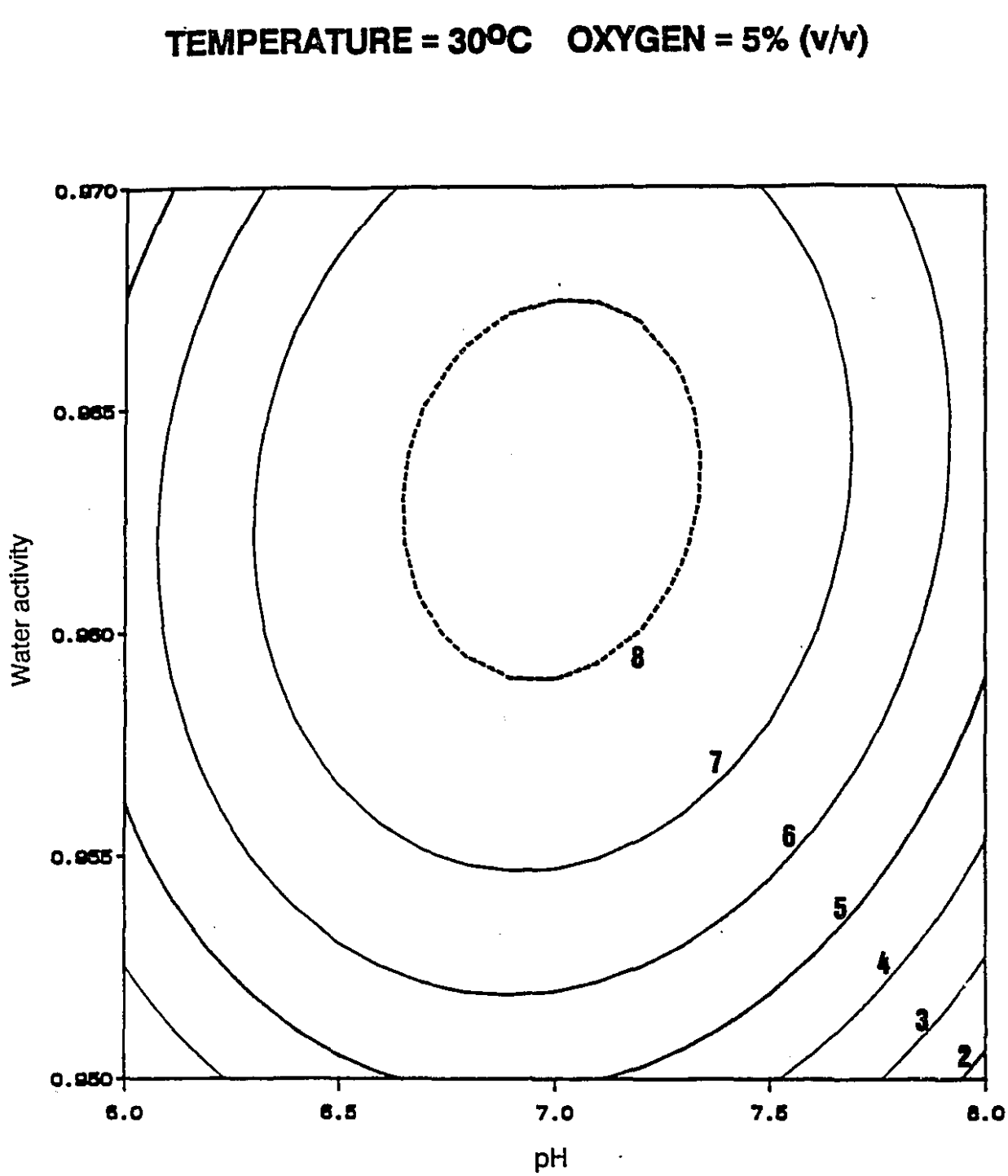


Figure 13. Two dimensional Contour plot showing the effect of a_w and pH on colony diameter (mm) of *A. flavus* at 30°C and 5% O_2 concentration.

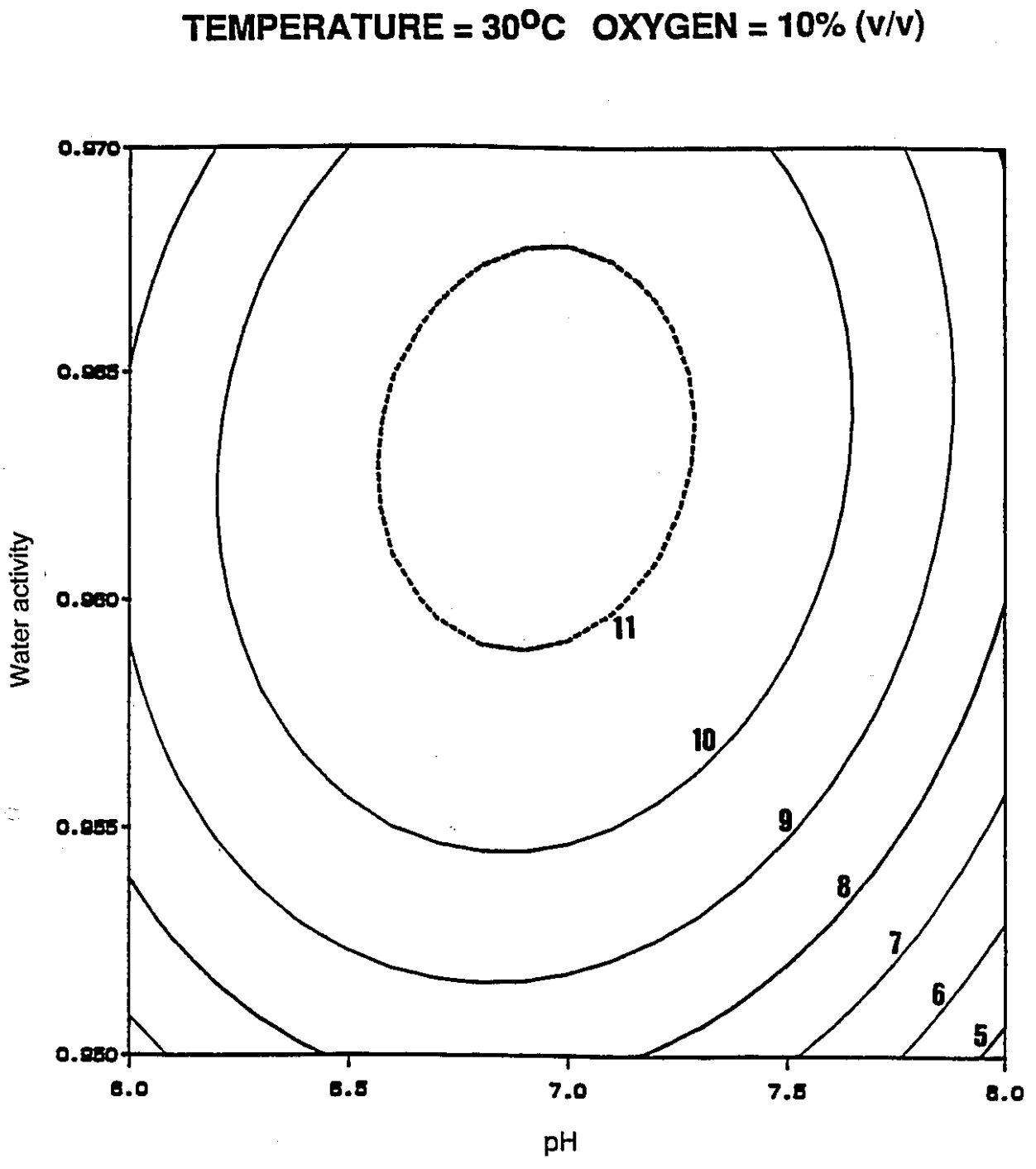


Figure 14. Two dimensional Contour plot showing the effect of a_w and pH on colony diameter (mm) of *A. flavus* at 30°C and 10% O_2 concentration.

packaged under completely anaerobic environment using oxygen absorbent technology.

3.4 CONCLUSION

In conclusion, this study has shown that RSM is a powerful and useful experimental technique when several variables are to be examined simultaneously. The advantages of this approach compared to the conventional "one variable at-a-time" approach are that (i) it is less time consuming and less expensive than the conventional approach, (ii) it quantifies the effects of the main factors and important interactions between variables and (iii) it generates data in an easy to interpret graphical form. The disadvantages of the technique are that the conclusions are only valid for *A. flavus* and the experimental conditions used in this study. For example, *A. parasiticus* may produce different results for similar experimental conditions. However, Holmquist et al., (1983) showed that the growth response of both *A. flavus* and *A. parasiticus* were similar for all a_W, pH and temperature levels used in their study. This study also emphasizes the combined synergistic effects of several barriers to either completely inhibit mold growth or to reduce aflatoxin production in MAP products containing various levels of headspace oxygen to safe and acceptable levels (<20 ng/g), particularly at temperature abuse storage conditions. However, the effect of inoculum levels remain unclear and still warrants further investigation.

CHAPTER 4

EFFECT OF INOCULUM LEVEL ON AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* UNDER MODIFIED ATMOSPHERE PACKAGING (MAP) CONDITIONS

4.1 INTRODUCTION

Modified Atmosphere Packaging (MAP) is a rapidly expanding food preservation technology being used to extend the shelf life and keeping quality of muscle foods, bakery products, snack food products, pizza and sandwiches (Smith et al., 1991). Recently, MAP involving reduced oxygen levels in conjunction with elevated levels of carbon dioxide was used to control aflatoxin production by *Aspergillus flavus* to safe and acceptable levels (<20 ng/g) on synthetic media, particularly at temperature abuse storage conditions (Ellis et al., 1993). The low level of aflatoxin observed was attributed to the combined inhibitory effects of water activity (a_w), pH, storage temperature and headspace gas composition on mold growth and the low inoculum level of *A. flavus* (1 spore/g) used in this study (Ellis et al., 1993). This inoculum level was chosen since (i) it is representative of mold contamination in products packaged under good manufacturing conditions, and (ii) there is a consensus of opinion that the lower the level of contamination the safer the product. However, the effect of inoculum levels on aflatoxin production by *A. flavus* or *A. parasiticus* is unclear. Karunaratne and Bullerman (1990) observed that an inoculum level of 10^3 spores/g resulted in the highest amount of aflatoxin being formed at 28°C while lower and higher inoculum levels produced comparatively lower levels of aflatoxin. However, at higher storage temperatures (35°C), the lowest spore inoculum (10^1 spores/g) produced the highest amount of aflatoxin.

In view of these conflicting observations, the specific objectives of this study were (i) to determine the effect of inoculum levels on aflatoxin production by *A. flavus* on synthetic media packaged under modified atmospheres, and (ii) to determine the packaging/storage conditions which could be used to control aflatoxin production by *A. flavus* to safe and acceptable levels, particularly at temperature abuse storage conditions.

4.2 MATERIALS AND METHODS

4.2.1 *Experimental design*

The factors and levels of each factor used in this study included a_w (0.95-0.97), inoculum level (10^2-10^4 spore/g), temperature (20-30°C) and level of O_2 in the package headspace (5-15%), balance of CO_2 and N_2 (60:40). The values of each level of environmental factor selected were based on previous studies with *Aspergillus flavus* (Diener and Davis, 1970; Karunaratne and Bullerman, 1990; Northolt et al., 1977; Ellis et al., 1993; Shih and Marth, 1973). To determine the effect of each environmental factor simultaneously on the growth of, and toxin production by *A. flavus*, a two level full factorial design augmented by a central point, as described by Box et al., (1978) (Table 22), was used as the experimental design. The coded and actual values of levels used in the experimental design are shown in Table 23. All experimental runs were done in duplicate.

4.2.2 *Preparation of spore inoculum*

An aflatoxigenic strain of *A. flavus* (ATCC strain 16875) used throughout the study was obtained from the American Type Culture Collection in Rockville, Maryland, U.S.A.. Preparation and harvesting of the spores were done as described in section 3.2.2 of chapter 3. However, enumeration was determined on Malt Extract Agar (MEA) using the surface plating technique. Based on this initial spore count (10^6 spores/ml), decimal dilutions were prepared to obtain spore suspensions containing 10^2 to 10^4 spores/ml for this study.

Table 22. Uncoded level combination of a (2^k+1) factorial design to determine effect of inoculum level on growth and aflatoxin B_1 production by *A. flavus* under MAP conditions.

	Variables			
Run No.	X_1	X_2	X_3	X_4
1	0.95	10^2	20	5
2	0.97	10^2	20	5
3	0.95	10^4	20	5
4	0.97	10^4	20	5
5	0.95	10^2	30	5
6	0.97	10^2	30	5
7	0.95	10^4	30	5
8	0.97	10^4	30	5
9	0.95	10^2	20	15
10	0.97	10^2	20	15
11	0.95	10^4	20	15
12	0.97	10^4	20	15
13	0.95	10^2	30	15
14	0.97	10^2	30	15
15	0.95	10^4	30	15
16	0.97	10^4	30	15
17	0.96	10^3	25	10

$X_1 = a_w$ X_2 = Inoculum level X_3 = Temperature X_4 = Headspace Oxygen

Table 23. Values of coded levels used in the (2^k+1) factorial design for *A. flavus*.

	Levels		
Variables (K)	-1	0	+1
Aw	0.95	0.96	0.97
Inoculum level	10^2	10^3	10^4
Temperature (^oC)	20	25	30
Headspace O_2 (%)*	5	10	15

* Balance CO_2:N_2 (60:40)

4.2.3 *Preparation and inoculation of media*

Malt extract agar was again used as the basal medium throughout the study. Water activity adjustment and inoculation of plates with appropriate dilutions of *A. flavus* were as described in section 3.2.3 of chapter 3.

4.2.4 *Packaging*

Packaging of plates in Cryovac barrier bags under various gas atmospheres were done according to the method described in section 3.2.4. of chapter 3. Packaged plates were incubated at 25^{o}C and 30^{o}C in a Precision Gravity Convection incubator (model 2EG) and at 20^{o}C in a Mocon incubator (Lab-line instrument Inc) for 15 days. All plates were examined daily for changes in growth. Four packages were prepared for each experimental run. Two packages were analysed for aflatoxin while the remaining two were evaluated for mold growth.

4.2.5 *Headspace gas analysis*

Headspace gas analysis was done immediately after packaging to ensure that the desired concentration of gases had been attained, and to monitor changes in the headspace gas composition throughout the 15 day storage period. Gas sampling and analysis by gas chromatography were done as described in section 3.2.5 of chapter 3.

4.2.6 *Growth measurement*

The extent of growth (mycelial dry weight (mg/g)) was determined after 15 days incubation by melting the contents of each plate in boiling water (100^{o}C) and filter collecting the mycelium by washing with hot water (80^{o}C) using a 4.25 cm Buchner separating funnel under minimum vacuum with a pre-weighed Whatman's No.1 filter paper. The filter paper containing the mycelia was then dried at 100^{o}C for 4 hours, cooled in a dessicator and then re-weighed. Mycelial weight was determined by difference and expressed as mg/g.

4.2.7 *Extraction and analysis of aflatoxin* B_1

After 15 days of incubation, the contents of test plates were extracted and analysed for aflatoxin according to procedures described in section 3.2.6 of chapter 3.

4.2.8 *Statistical analysis*

Statistical analysis of the data were computed using the Statistical Analysis System (SAS, 1982) and the SAS program on a McGill University mainframe computer.

4.3 RESULTS AND DISCUSSION

The combined effect of a_w, inoculum level, temperature and initial headspace oxygen concentration on the growth of, and aflatoxin production, by *A. flavus* on synthetic media packaged in a high gas barrier film after 15 days is shown in Table 24. Generally, mold growth was evident after 2-3 days in all plates stored at 25-30^{o}C, but delayed until day 4-5 in plates stored at 20^{o}C. These results are in agreement with previous observations of Ayerst (1969), who reported that growth of *A. flavus* occurred over a temperature range of 12-43^{o}C. However, the results were contrary to observations of Ellis et al., (1993) who observed that the growth of *A. flavus* on synthetic medium was inhibited in certain treatment combinations stored at 25^{o}C and completely inhibited in all plates stored at 20^{o}C. This inhibition was attributed to the synergistic effects of a_w, storage temperature, headspace gas composition and low inoculum level (1 spore/g) (Ellis et al., 1993). Since similar levels of factors were investigated in this study, it can be concluded that the effectiveness of additional barriers to ensure product quality and safety is influenced by inoculum size and that the combined effect of a_w, storage temperature and headspace gas composition is less inhibitory against higher spore inoculum levels at lower storage temperatures (20^{o}C), as indicated by the amount of growth and aflatoxin production by *A. flavus*. From a manufacturing viewpoint, this implies that the shelf life quality and safety of a product will be enhanced when produced under good manufacturing conditions compared to products produced under poor manufacturing conditions and contaminated with a higher inoculum level of mold spores.

The analysis of variance (ANOVA) for Y, i.e., growth (mycelial dry weight) indicated that the model was highly significant ($p<0.001$) with an R^2 value of 0.93, i.e., accounting for 93% of experimental variation after being corrected for the mean. Examination of the data showed that all main factors, i.e., a_w (X_1), inoculum level (X_2), storage temperature (X_3)

Table 24. Combined effect of water activity, inoculum level, storage temperature and headspace oxygen on growth of and aflatoxin production by *A. flavus* using a $2^k + 1$ factorial design.

Run#	Variables				Extent of Growth (mg/g)* [a]	Aflatoxin B_1 (ug/g) [a]
	a_w	Inol	T°C	O_2%		
1	0.95	10^2	20	5	2.753 (0.03)	2.401 (0.01)
2	0.97	10^2	20	5	1.246 (0.04)	1.341 (0.02)
3	0.95	10^4	20	5	1.537 (0.07)	0.708 (0.02)
4	0.97	10^4	20	5	1.780 (0.04)	0.503 (0.03)
5	0.95	10^2	30	5	2.300 (0.19)	0.223 (0.01)
6	0.97	10^2	30	5	2.037 (0.12)	0.049 (0.01)
7	0.95	10^4	30	5	1.009 (0.03)	0.442 (0.01)
8	0.97	10^4	30	5	1.372 (0.03)	0.666 (0.02)
9	0.95	10^2	20	15	3.842 (0.03)	2.297 (0.02)
10	0.97	10^2	20	15	2.946 (0.02)	1.402 (0.01)
11	0.95	10^4	20	15	4.169 (0.02)	3.023 (0.05)
12	0.97	10^4	20	15	2.765 (0.05)	1.610 (0.04)
13	0.95	10^2	30	15	2.149 (0.02)	1.317 (0.02)
14	0.97	10^2	30	15	2.614 (0.02)	1.980 (0.05)
15	0.95	10^4	30	15	3.510 (0.07)	1.936 (0.01)
16	0.97	10^4	30	15	2.543 (0.04)	0.118 (0.01)
17	0.96	10^3	25	10	3.916 (0.11)	3.449 (0.13)

* Mycelial weight (mg) after 15 days.

[a] Mean of two replicates.

() Standard error.

and headspace oxygen (X_4) were all significant variables influencing mold growth (Table 25). Furthermore, the two factor interactions, a_w*temp. (X_1X_3), a_w*oxygen (X_1X_4), inoculum level*oxygen (X_2X_4) and temp*oxygen (X_3X_4) and all three factor interactions also significantly affected mycelial dry weight. Based on the positive regression coefficients of the main factors and their higher order interactions, it was expected that the amount of mold growth would increase as the level of each factor increased. Conversely, a decrease in the level of each factor would result in a decrease in mycelial growth. However, while this trend was apparent in certain treatment combinations it was not a consistent trend as shown in Table 24 and Figures 15 and 17 (a-d) which demonstrates the significant contribution both inoculum level and headspace oxygen play in mycelial development and aflatoxin production. For example, mold growth (mg/g) was generally more extensive in plates packaged in 10-15% O_2 at 20-30^oC compared to plates packaged at 5% O_2 and stored at similar temperatures (Table 24, Figures 15a-d). For plates packaged in 10-15% O_2, higher inoculum levels consistently resulted in greater mycelial growth (Figures 15a & c) with the exception of two experimental treatments (Runs #9 & 10) where lower inoculum levels resulted in greater mycelial growth. The effect of both a_w and storage temperature on growth of *A. flavus* was pronounced in plates packaged in high O_2 concentrations. Mycelial growth was generally greater in plates of lower a_w (0.95) compared to plates of a_w value 0.97 when packaged in 15% O_2 (Figures 15a-d). Furthermore, at these oxygen levels, growth was consistently higher in plates stored at 20^oC irrespective of the a_w or inoculum level (Figure 15a-c).

These results were surprising since it was expected that mycelial growth would always be higher in plates inoculated with a higher inoculum level and packaged under the most favourable conditions for growth as indicated by the regression coefficients. For plates packaged in 5% headspace O_2, the opposite trend was observed, i.e., mold growth was generally greater in plates with a low inoculum level (10^2 spores) compared to plates

Table 25. Analysis of variance for (2^k+1) factorial design of factors influencing growth of *A. flavus*.

Source	dF[a]	Sum of squares	Mean square	F
Model	16	59.40	3.71	45.78***
A_w (X_1)	2		6.31	77.77***
Inoculum (X_2)	1		0.39	4.87*
Temperature (X_3)	1		3.17	39.04***
Headspace O_2 (X_4)	1		27.30	336.60***
A_w*Inol (X_1*X_2)	1		0.04	0.44ns
Inol*Temp (X_2*X_3)	1		0.01	0.10ns
Inol*O_2 (X_2*X_4)	1		4.04	49.79***
Aw*Inol.*Temp.	1		1.11	13.64***
Aw*Inol.*O_2	1		4.77	58.88***
Inol.*Temp.*O_2	1		1.40	17.22***
Aw*Inol.*Temp.*O_2	2		0.02	0.26ns
Residual (error)	51	4.14	0.08	
Total	67	63.54		
R^{2b}		0.93		

[a] Degrees of freedom; [b] Coefficient of determination.

Level of significance*** P<0.001; Level of significance ** P<0.01.

Level of significance * P<0.05; ns = not significant.

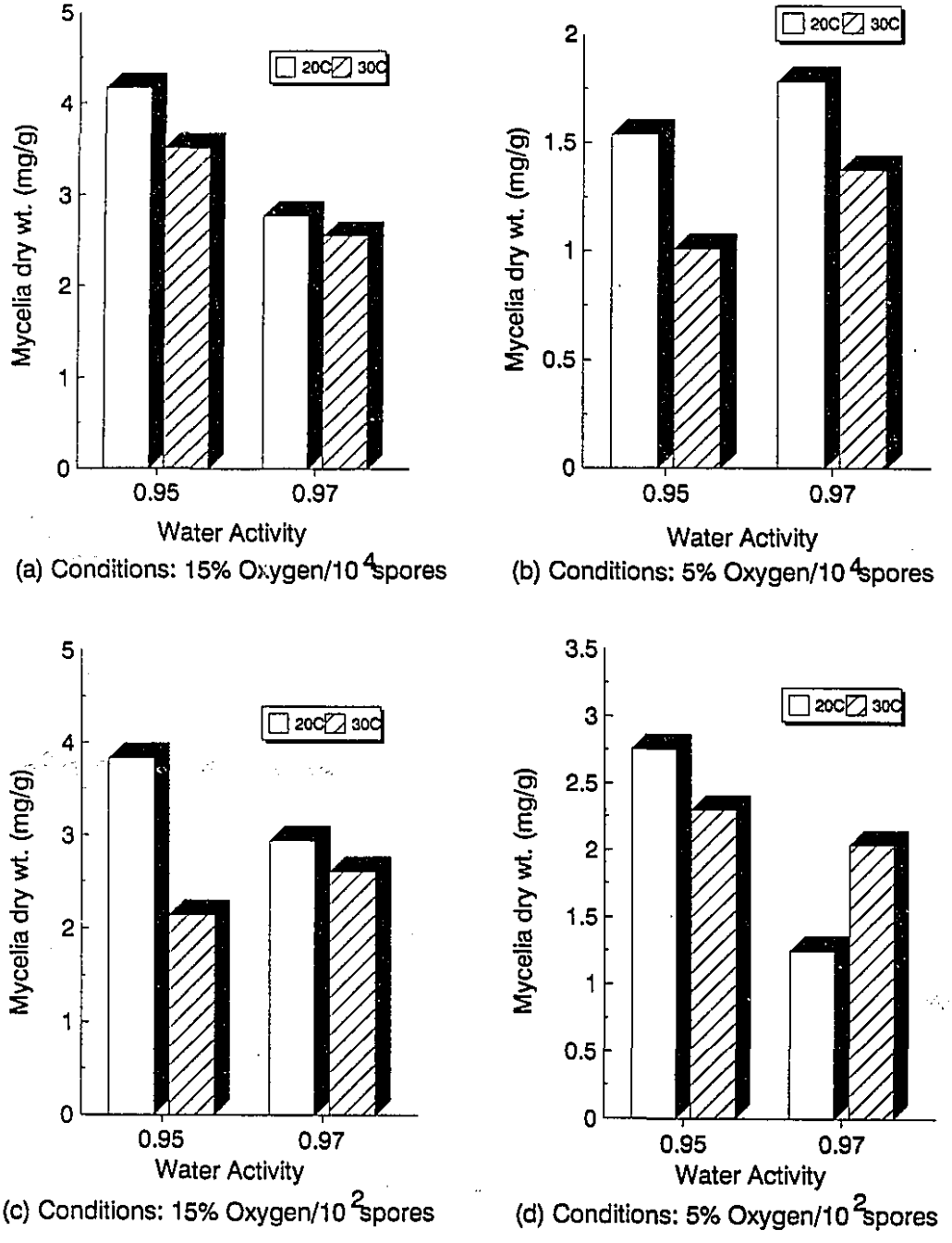


Figure 15 Effect of inoculum level, water activity, headspace O_2 conc. and storage temp. on growth of *A. flavus*

spiked with higher inoculum levels (10^4 spores) irrespective of storage temperature (Table 24, Figure 15b,d). Therefore, low headspace O_2 would appear to have a more pronounced effect on the growth of higher inoculum levels of *A. flavus*. For plates packaged in 5% O_2, growth of *A. flavus* was consistently greater in plates of higher a_w (0.97) when inoculated with higher spore levels, irrespective of the storage temperature (Figure 15b). However, the opposite trend was observed for plates with lower inoculum levels of *A. flavus*, and packaged in 5% O_2 again irrespective of storage temperature (Figure 15d). Therefore, both lower O_2 and lower a_w levels could be used effectively to control the growth of higher inoculum levels of *A. flavus*.

However, while growth was higher in plates inoculated with higher inoculum levels and packaged in higher O_2 levels, statistical analysis of the growth means indicated that there was no significant difference between inoculum level and final mycelial dry weight. This observation confirms the work of Sharma et al., (1980) who reported that the growth of *A. parasiticus* was independent of inoculum size. Odamtten et al., (1987) also reported that the growth of non-irradiated cells of *A. flavus* was independent of inoculum size while Karunaratne and Bullerman (1990) observed that mycelial dry weight were comparable irrespective of the initial inoculum size.

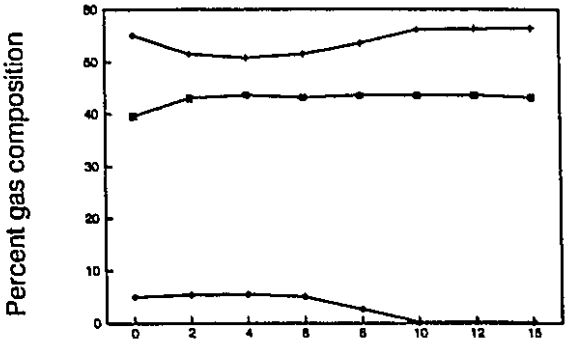
Most plates showing growth displayed some degree of sporulation which varied according to packaging and storage conditions. At 20°C, sporulation was barely noticeable. However, in plates stored at 25-30°C, sporulation occurred faster and was more intense, particularly in plates with higher spore loads (10^4/plate). Sharma et al., (1980) also observed that the on-set and intensity of sporulation was directly proportional to the spore inoculum.

Changes in headspace gas composition for selected inoculum levels/packaging treatments are shown in Figure 16. In all cases, headspace O_2 decreased to less than 1% within 5-14

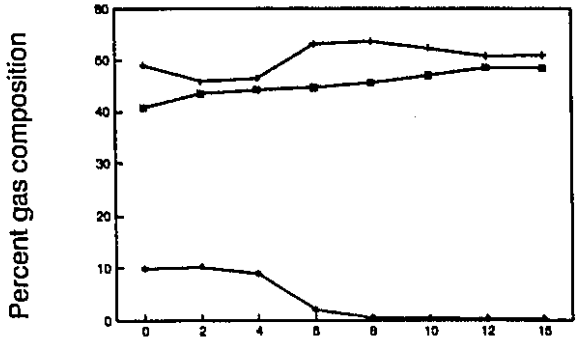
days depending on the initial headspace O_2 concentration, the spore inoculum and storage temperature. An initial increase in headspace CO_2 was observed throughout storage as a result of mold metabolism and then a gradual decrease due either to its absorption by the media, its loss through the packaging film or possibly its incorporation into the biosynthetic pathway for aflatoxin production (Hsieh and Mateles, 1971). However, there was no correlation between changes in headspace CO_2 and aflatoxin production confirming previous observations of Ellis et al., (1993).

The results for aflatoxin production by *A. flavus* are summarized in Table 24. In all cases where growth occurred the level of aflatoxin was greater than the current regulatory standard of 20 ng/g. The higher concentrations of aflatoxin detected in this study can again be attributed to the higher inoculum levels since the level of aflatoxin detected in plates inoculated with a lower inoculum level (10^1 spores/g) and packaged and stored under similar gaseous and environmental conditions was always less than the regulatory limit of 20 ng/g (Ellis et al., 1993). Aflatoxin production on a synthetic medium has been attributed with mycelial branching and differentiation. With increasing spore inoculum, hyphal fusion is faster resulting in a greater depletion of nutrients and release of staling substances which eventually influences lateral branching (Gottlieb, 1971).

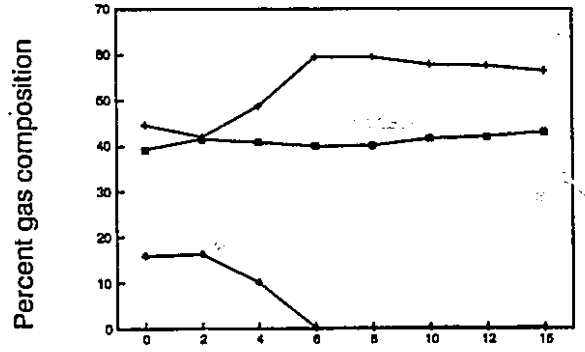
Statistical analysis of the data (Table 24) indicated that the model was highly significant ($p<0.001$) with an R^2 value of 0.97 (Table 26). Examination of the data shows that all main factors (a_w, inoculum level, temperature and headspace O_2 concentration) and most of the 2 and 3 factor interactions (with the exception of inoculum*temperature (X_2X_3)) significantly affected aflatoxin production (Table 26). The combined effects of inoculum levels, packaging and storage conditions on aflatoxin production by *A. flavus* are shown in Table 24 and Figures 17a-d. Similar trends were observed between inoculum levels and mold growth, and inoculum levels and aflatoxin production. Higher concentrations of



(a)

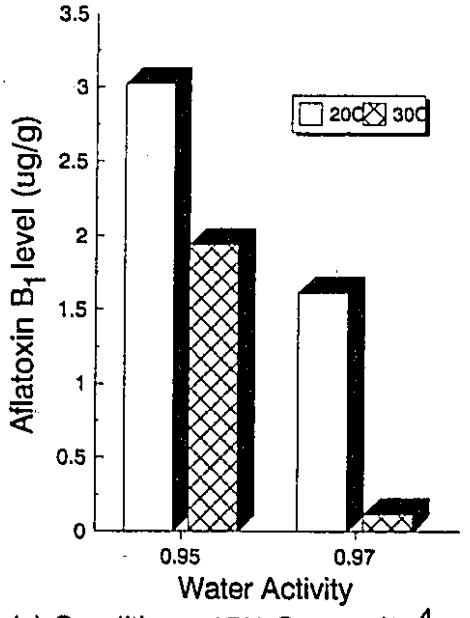


(b)

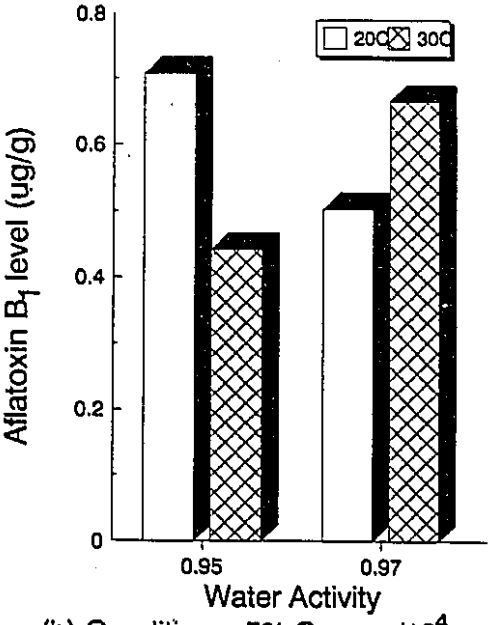


(c)

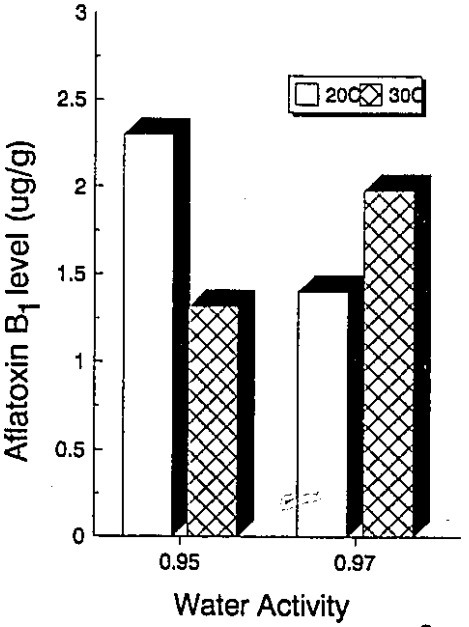
Figure 16 Changes in headspace gas composition of selected samples with time



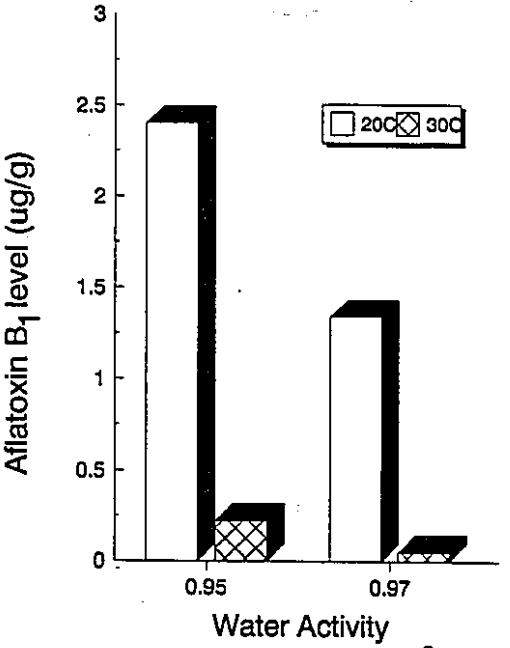
(a) Conditions: 15% Oxygen/10^4spores



(b) Conditions: 5% Oxygen/10^4 spores



(c) Conditions: 15% Oxygen/10^2spores



(d) Conditions: 5% Oxygen/10^2spores

Figure 17 Effect of inoculum level, water activity, headspace O_2 conc. and storage temp. on aflatoxin B_1 production

Table 26. Analysis of variance for a (2^k+1) factorial design of factors influencing aflatoxin production by *A. flavus*.

Source	dF[a]	Sum of squares	Mean square	F
Model	16	67.79	4.24	121.52***
A_w	2		11.87	340.48***
Inoculum	1		1.03	29.67***
Temperature	1		10.63	304.83***
Headspace O_2	1		13.61	390.28***
A_w*Inol.	1		0.79	22.70***
Inol.*Temp.	1		0.37	10.62***
Inol.*O_2	1		0.50	14.41***
Aw*Inol.*Temp.	1		1.43	41.07***
Aw*Inol.*O_2	1		4.45	127.70***
Inol.*Temp.*O_2	1		7.76	222.63***
Aw*Inol.*Temp.*O_2	2		0.30	8.56***
Residual (error)	51	1.78	0.03	
Total	67	69.57		
R^{2b}		0.97		

[a] Degrees of freedom; [b] Coefficient of determination.

Level of significance *** P<0.001; ns = not significant.

aflatoxin were generally detected when plates of lower a_w (0.95-0.96) were inoculated with higher levels of *A. flavus* packaged in 10-15% O_2 and stored at lower storage temperatures (20-25^oC) (Table 24; Figures 17a & c). For plates packaged in 5% O_2, aflatoxin production was consistently higher in plates inoculated with a lower spore inoculum. This trend for aflatoxin production confirms previous results for mold growth, i.e., low headspace O_2 limits aflatoxin production by higher inoculum levels of *A. flavus*. In most cases, the level of aflatoxin detected was generally lower in plates stored at 30^oC, irrespective of water activity, inoculum level or headspace O_2 concentration. The lower concentration of aflatoxin at 30^oC can be attributed to its more rapid breakdown as an additional substrate for mold growth at this temperature.

The highest concentration of aflatoxin B_1 was produced by inoculum level of 10^3 spores/g and packaged in 10%O_2 and stored at 25^oC. These results confirm the observations of Karunaratne and Bullerman (1990), who found that 10^3 spores/g resulted in the highest amount of aflatoxin being formed (380 ug) at 28^oC while lower and higher inoculum levels (10^1 and 10^7 spores/g) produced comparatively lower levels of aflatoxin. However, the levels of aflatoxin B_1 detected in this study were substantially lower than the levels detected in other studies with *A. flavus* (Karunaratne and Bullerman, 1990; Odamtten et al., 1987). This again can be attributed to the combined additive effects of storage temperature, a_w, and headspace CO_2:O_2 concentration on mold growth and aflatoxin production. Nevertheless, the concentrations observed in this study were far greater than the regulatory limits of 20 ppb, indicating the importance of the level of mold contamination in food products.

4.4 CONCLUSION

In conclusion, while higher inoculum levels resulted in greater growth of, and aflatoxin B_1 production *by A. flavus*, particularly in plates packaged in higher O_2 concentration, the effect of growth on aflatoxin production was not statistically significant. However, the levels of aflatoxin produced were significantly different from the earlier study by Ellis et al., (1993) when plates were inoculated with only 10^1 spores/g of *A. flavus* and packaged and stored under similar environmental conditions used in this study. While the combined synergistic effects of a_w, storage temperature and level of headspace CO_2:O_2 could be used to reduce the level of aflatoxin to safe and acceptable levels (<20 ppb) in plates spiked with low inoculum levels (10^1 spores/g), similar headspace gases and environmental storage conditions failed to achieve this objective when plates were challenged with higher spore levels. Therefore spore levels greater than 10^1 spores/g will reduce the shelf life and safety of products packaged under MAP conditions. Conversely, low levels of contamination by aflatoxigenic mold species will ensure the quality and safety of MAP products. However, this level of contamination would be difficult to achieve, particularly in tropical countries where spore levels ranging from 10^2-10^7 spores/g have been reported for a wide range of products (Ellis et al., 1991). Possibly the only means of controlling the growth of and aflatoxin production by spore levels > 10^1 spores/g is to package the product under completely anaerobic conditions using oxygen absorbent technology.

CHAPTER 5

MODELLING STUDIES TO PREDICT LEVELS OF FACTORS TO CONTROL THE GROWTH OF AND AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* IN PEANUTS PACKAGED UNDER MAP CONDITIONS

5.1 INTRODUCTION

Aflatoxins formed by *Aspergillus flavus* and *Aspergillus parasiticus* are of considerable importance in foods and animal feeds due to their toxic nature. Numerous studies have been done to prevent the growth of, and aflatoxin production by *A. flavus* and *A. parasiticus* by controlling factors affecting their growth. More recently, Modified Atmosphere Packaging (MAP) involving reduced oxygen levels in conjunction with elevated levels of carbon dioxide were used in conjunction with other environmental factors to control aflatoxin production by *A. flavus* to safe and acceptable levels (<20 ng/g) in synthetic media particularly at temperature abuse conditions (Ellis et al., 1993). The low level of aflatoxin observed was attributed to the combined synergistic effects of water activity (a_w), pH, storage temperature, headspace gas composition and low inoculum levels (1 spore/g). However, there is a paucity of data as to the application of MAP to control/inhibit the growth of aflatoxigenic molds species on natural substrates such as maize, peanuts, cottonseed etc.

The objectives of this study were to (1) determine the combined effect of CO_2 enriched atmospheres containing various levels of headspace oxygen and environmental storage conditions to minimize the growth of, and aflatoxin production by *Aspergillus flavus* in peanuts and (ii) to determine the packaging/storage conditions to ensure the public health safety of peanuts particularly at temperature abuse storage conditions.

5.2 MATERIALS AND METHOD

5.2.1 *Experimental design*

To determine the effect of water activity (a_w), storage temperature and elevated levels of CO_2 in the presence of various levels of O_2 simultaneously on the growth of, and aflatoxin production by *Aspergillus flavus* in peanuts, a 3 factor, 5 level central composite rotatable design (CCRD) of Box et al., (1978) was used (Table 27). The levels of each factor used in the study included a_w (0.91-0.97), temperature (16.6-33.4) and headsapce O_2 (1.6-18.4%) in a balance of CO_2 and N_2 (60:40). The values of each level of environmental factor selected were based on previous studies with *A. flavus* (section 3.2.1 (chapter 3); section 4.2.1 (chapter 4). In the CCRD, variable levels were coded -1.682, -1, 0, +1, +1.682 as described by Khuri and Cornell (1987). The coded and actual values of levels used in the CCRD are shown in Table 28. All experimental runs were done in triplicate.

5.2.2 *Preparation of spore inoculum*

The aflatoxigenic strain of *Aspergillus flavus*, its preparation, harvesting and enumeration of spores were as described previously in section 4.2.2 (chapter 4). Based on an initial count obtained (10^7 spores/ml), appropriate dilutions were then prepared to obtain the desired inoculum level of 10^4 spores/ml used for the study. This spore level was chosen in this study since this level of spores resulted in high amounts of aflatoxin production in previous studies on synthetic media.

Table 27. Coded level combinations for a 3 variable CCRD for growth of and aflatoxin production by *A. flavus* on peanuts

Run No.*	Variables**		
	X_1	X_2	X_3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.682	0	0
10	+1.682	0	0
11	0	-1.682	0
12	0	+1.682	0
13	0	0	-1.682
14	0	0	+1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

* Each run replicated thrice for a total of 60 runs.
** $X_1 = a_w$; X_2 = Temp.^{o}C; X_3 = Headspace O_2 (balance CO_2:N_2)

Table 28. Values of coded levels used in the CCRD for growth of and aflatoxin production by *A. flavus* on peanuts

	Levels				
Variables	-1.682	-1	0	+1	+1.682
Aw (X_1)	0.91	0.92	0.94	0.96	0.97
Temp.(oC) (X_2)	16.6	20	25	30	33.4
Headspace* O_2 (X_3)	1.6	5	10	15	18.4

* Balance CO_2:N_2 (60:40)

5.2.3 *Preparation and inoculation of peanuts*

Peanuts were obtained from Provigo, Montreal. The peanuts were visually inspected for defects, e.g., discoloration, mold growth and defective peanuts were removed from the batch. The sound peanuts were then shelled and 250g amounts placed in autoclavable dessicators containing glycerol solutions of known water activity levels. They were then sterilized by autoclaving at 121°C for 15min cooled and then equilibrated until they reached the desired water activity levels. Water activity measurements were done on each batch of peanuts using a previously calibrated Decagon water activity meter (Decagon Device Inc. Pullman, WA, U.S.A.). Fifty gram amounts of the sterile peanuts were then weighed into 150 x 15mm petri dishes and inoculated with 10^4 spores/g of *A. flavus*. For each packaging condition, six inoculated plates and two non-inoculated plates (control) each packaged. Three of the packaged plates were used for growth studies while the remaining three were used for aflatoxin analysis.

5.2.4 *Packaging and headspace gas analysis*

Packaging of inoculated peanuts in the appropriate gas mixtures and analyses of headspace gas composition of packaged plates throughout storage were done as described previously in sections 4.2.4 and 4.2.5 of chapter 4.

5.2.5 *Growth measurement*

Measurement of growth was based on a colony plate count. At each storage period the contents of each plate was transferred into stomacher bags, 100ml of sterile 0.1% peptone was then added and the mixture stomached for 2 min. Appropriate dilutions of the spore suspension formed were then prepared using 0.1% peptone solution as diluent. Fifty

microliter (*ul*) of the appropriate dilution was then inoculated onto Potato Dextrose Agar (PDA) (BDH). Plates were incubated at 25°C in a Precision gravity convection incubator for 24 to 48 hours. Colonies formed were then counted using a Quebec colony counter. Based on the count obtained, total colony count per gram of peanut was then determined for every packaging/storage combination.

5.2.6 *Extraction and quantitation*

Extraction of aflatoxin was done using the Mycosep Multifunctional Cleanup (MFC) column technique of Wilson and Romer (1991). The contents of each plate after storage was extracted using 100ml acetonitrile-water (9 : 1) in a 250 ml blender. Blending was carried out at high speed for 3 min and the resulting mixture filtered using Whatman's No.1 filter paper, using a 4.25 cm Buchner funnel under minimal vacuum. A 5 ml volume of the filtrate was collected into 10 ml culture tubes and the flanged-end of the Mycosep MFC column pushed into the tubes until ca. 0.5 ml extract is purified. A 200 *ul* volume of the purified extract was derivatized with 700 *ul* derivatising 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 chromatographic analysis using a Millex-GV millipore filter unit. Detection and quantitation of the derivatized extracts for aflatoxin B_1 was done using a Beckman System Gold programmable module 126 as described previously (section 4.2.7 chapter 4). Detection was done at 0.5 R.F.U (Relative fluorescence unit) with a solvent flow rate set at 2.00 ml/min. Concentration of aflatoxin B_1 was calculated by comparison to standard peaks of known concentration of aflatoxin B_1 as outlined in the Beckman System Gold methodology.

5.2.7 *Statistical analysis*

Statistical analysis (regression coefficients, analysis of variance and correlation coefficients) were computed using the Statistical Analysis System (SAS, 1982). All 3 dimensional graphs and 2 dimensional contour plots were done using the SAS/Graph program on a McGill University mainframe and a Lotus Freelance Graphic program.

5.3 RESULTS AND DISCUSSION

The combined effect of a_w, storage temperature and headspace O_2 over a 3 week period on the growth of, and aflatoxin production by *A. flavus* on sterile peanuts packaged in high gas barrier film is shown in Tables 29-30. Mold growth was extensive during the first week of storage in all treatment combinations with a 1.5 to 4.0 fold increase in growth. However, with the exception of treatments 1, 5, 8, 10 and 12 which showed continous growth during the 3 week storage period, mold growth in most of the treatment combinations either increased slightly, remained constant or decreased during the second and third week of storage (Figure 18a,b,c). Maximum mold growth was observed with peanuts having high a_w levels, with no significant difference in growth during weeks 2 and 3 for most of the treatment combinations. Most of the packaged peanuts produced unpleasant odors, after 2 to 3 weeks of storage. This was more significant with peanuts having high a_w levels and packaged in high O_2 concentrations and can be attributed to oxidative rancidity.

The trend in growth changes with time can be attributed to the significant effect of a_w, temperature and headspace O_2 concentration on *A. flavus*. The extensive growth of *A. flavus* during the first week of storage is due to the availability of O_2 during the first few days of storage thereby enhancing active metabolism and multiplication. A similar observation was made by Shih and Marth (1974) in their study with *A. parasiticus* in static and agitated glucose-salt medium for 15 days. They observed that maximal growth occurred at day 5 of storage. Karunaratne and Bullerman (1990) also observed in their study with *A. flavus* on sterile rice, that maximum growth occurred within 3 to 5 days of incubation at higher temperatures (35^{o}C) and within 5 to 7 days at 28^{o}C after which the mold entered the stationary phase of growth.

Table 29. Effect of a_w, temp.,gas atmosphere and storage time on growth of *A. flavus* on packaged peanuts.

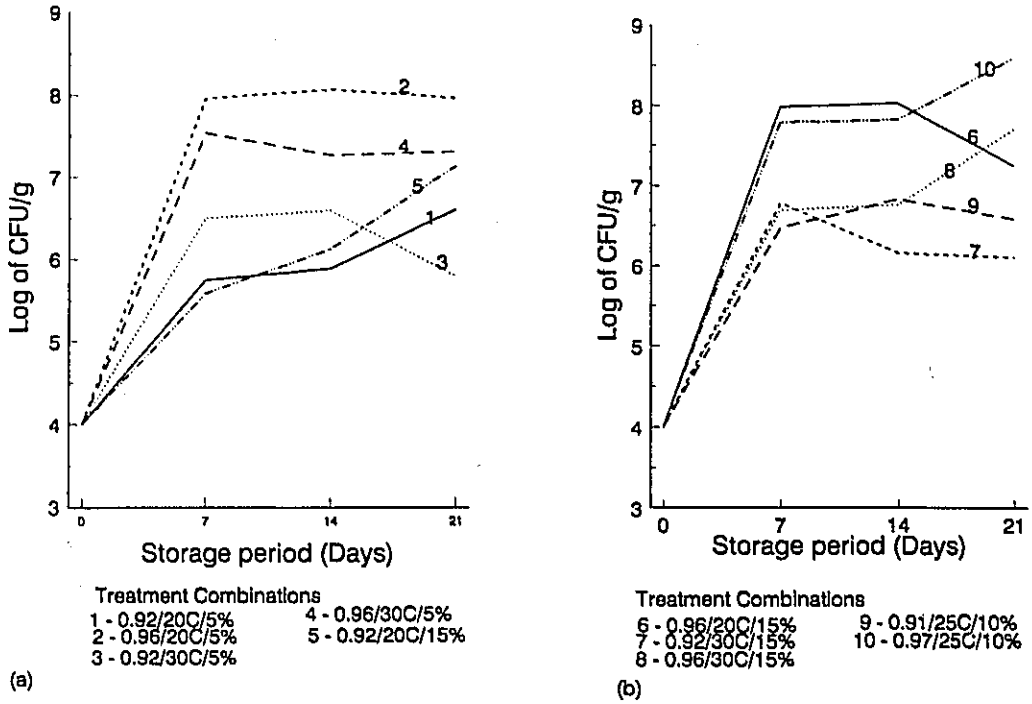
				Extent of Growth (Log CFU/g)**		
	Variables			Incubation period (days)		
Run #	a_w	T°C	O_2%	7	14	21
1	0.92	20	5	5.75 (0.03)	5.88 (0.04)	6.60 (0.04)
2	0.96	20	5	7.96 (0.03)	8.06 (0.02)	7.96 (0.01)
3	0.92	30	5	6.50 (0.05)	6.59 (0.10)	5.78 (0.07)
4	0.96	30	5	7.53 (0.06)	7.26 (0.10)	7.30 (0.02)
5	0.92	20	15	5.58 (0.06)	6.12 (0.02)	7.13 (0.04)
6	0.96	20	15	7.98 (0.04)	8.03 (0.01)	7.24 (0.03)
7	0.92	30	15	6.78 (0.21)	6.16 (0.06)	6.10 (0.04)
8	0.96	30	15	6.70 (0.01)	6.76 (0.04)	7.69 (0.04)
9	0.91	25	10	6.47 (0.05)	6.83 (0.07)	6.58 (0.11)
10	0.97	25	10	7.79 (0.03)	7.82 (0.01)	8.58 (0.05)
11	0.94	16.6	10	8.29 (0.08)	7.35 (0.02)	7.25 (0.03)
12	0.94	33.4	10	5.78 (0.03)	6.83 (0.04)	7.29 (0.13)
13	0.94	25	1.6	7.79 (0.04)	7.62 (0.09)	7.65 (0.05)
14	0.94	25	18.4	8.17 (0.08)	7.20 (0.02)	7.53 (0.10)
15	0.94	25	10	5.78 (0.03)	6.07 (0.01)	5.61 (0.01
16	0.94	25	10	5.37 (0.19)	6.07 (0.01)	5.40 (0.84)
17	0.94	25	10	5.18 (0.20)	6.04 (0.10)	5.57 (0.05)
18	0.94	25	10	5.21 (0.19)	6.05 (0.02)	5.63 (0.02)
19	0.94	25	10	5.48 (0.20)	6.08 (0.02)	5.60 (0.01)
20	0.94	25	10	5.19 (0.20)	5.75 (0.09)	5.50 (0.02)

** Mean of 3 replicates. () Standard deviation.

Table 30. Effect of a_w, temp.,gas atmosphere and storage time on aflatoxin B_1 production by *A. flavus* on packaged peanuts.

				Aflatoxin B_1 level (ng/g)**		
	Variables			Incubation period (days)		
Run #	a_w	T°C	O_2%	7	14	21
1	0.92	20	5	13.95 (0.34)	25.11 (1.17)	40.00 (1.53)
2	0.96	20	5	37.46 (0.61)	36.86 (0.21)	8.85 (0.62)
3	0.92	30	5	13.64 (0.79)	4.35 (0.33)	9.24 (0.19)
4	0.96	30	5	45.50 (1.80)	24.87 (1.10)	36.69 (1.64)
5	0.92	20	15	25.04 (1.80)	6.53 (0.34)	6.43 (0.42)
6	0.96	20	15	17.99 (0.38)	6.58 (0.03)	6.33 (0.33)
7	0.92	30	15	21.75 (0.49)	24.32 (0.97)	11.68 (0.17)
8	0.96	30	15	26.89 (0.21)	23.15 (0.85)	12.97 (0.39)
9	0.91	25	10	10.79 (0.25)	6.57 (0.12)	5.18 (0.22)
10	0.97	25	10	34.35 (0.69)	20.63 (0.67)	20.77 (0.21)
11	0.94	16.6	10	24.27 (0.63)	21.03 (0.57)	8.16 (0.18)
12	0.94	33.4	10	14.67 (0.14)	11.19 (0.06)	9.03 (0.05)
13	0.94	25	1.6	15.33 (1.02)	6.07 (0.30)	12.00 (0.81)
14	0.94	25	18.4	36.66 (0.51)	17.29 (0.22)	4.21 (0.01)
15	0.94	25	10	21.37 (0.25)	23.65 (0.87)	48.66 (1.80)
16	0.94	25	10	20.92 (0.30)	22.92 (0.50)	44.68 (1.03)
17	0.94	25	10	23.69 (1.47)	24.02 (0.77)	46.91 (1.42)
18	0.94	25	10	21.73 (0.34)	21.74 (0.40)	42.47 (0.73)
19	0.94	25	10	25.47 (1.48)	25.47 (1.32)	47.56 (1.50)
20	0.94	25	10	23.96 (1.27)	23.63 (0.80)	46.75 (1.20)

** Mean of 3 replicates. () Standard deviation.



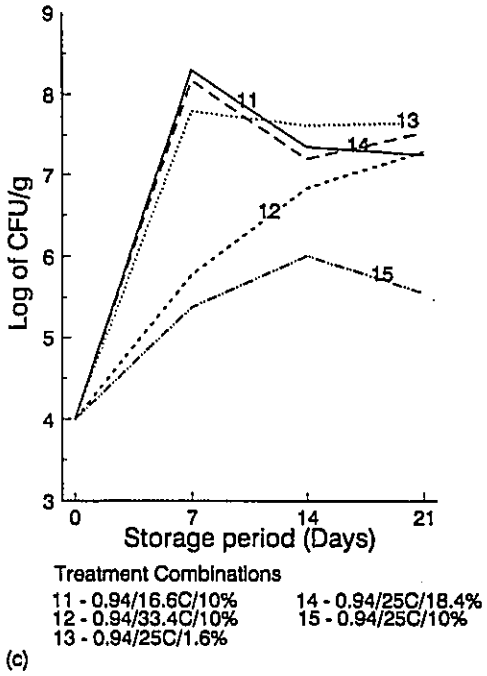


Figure 18 Trend in growth of *A. flavus* with storage time under different packaging conditions

Generally, the rate of O_2 consumption and CO_2 production depended on 4 major factors, the level of a_w, environmental temperature, microbiological condition of the substrate and the gas transmission properties of the packaging film. Each of these factors can significantly influence the growth of *A. flavus*, with a corresponding effect on toxin production. Magan and Lacey (1984) found that aflatoxigenic field and storage fungi were tolerant of low O_2 concentrations (0.14%) and to elevated CO_2 levels (>15%) at high a_w levels. However, a reduction in both storage temperature and a_w level increased the sensitivity of the fungi to high CO_2 levels with most species being inhibited by low CO_2 concentration.

Diawara et al., (1986) found that the biological O_2 demand for a grain/microorganism ecosystem was very high for grains with high a_w levels, with the demand decreasing with a decrease in the a_w level of the substrate. A similar trend was also observed with CO_2 production, with an increased CO_2 production at high a_w and a decrease in production at reduced a_w levels. The evolution in gas changes greatly depended however on the environmental temperature. The decrease in growth after week 1, can be attributed to the synergistic effects of high CO_2 concentration produced as a result of mold metabolism (>55%) and reduced O_2 level in conjunction with the storage temperature and a_w level.

The changes in headspace gas composition for selected packaging conditions are shown in Figure 19. In all cases, headspace O_2 decreased to less than 1% within 1-3 days depending on the initial headspace O_2 concentration, the a_w and the storage temperature. An initial increase in headspace CO_2 was observed throughout storage as a result of mold metabolism and then a gradual decrease due either to its adsorption into the pores of the peanut (Holaday et al., 1979), its loss through the packaging film or possibly its incorporation into the biosynthetic pathway for aflatoxin production (Hsieh and Mateles, 1971). However, the extent of decrease in CO_2 concentration depends also on the

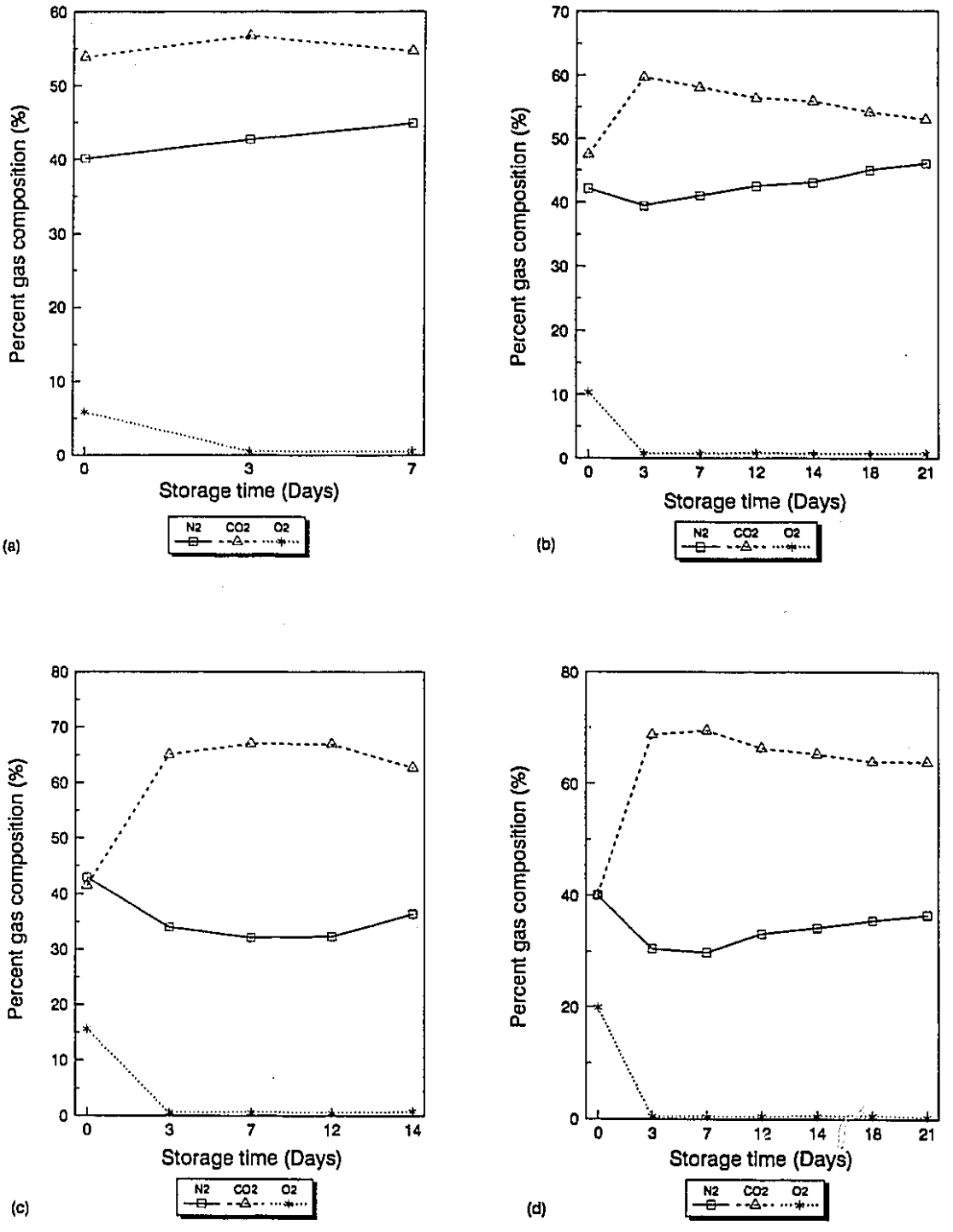


Figure 19 Changes in headspace gas composition of selected samples with time

incubation period. The extent of decrease is greater in packages stored for 21 days relative to packages stored for 7 days. The results for aflatoxin production is shown in Table 30 and Figure 20. A similar trend in aflatoxin B_1 production with time is observed as in the growth of *A. flavus*, with optimum levels being produced during the first week of storage for most of the treatment combinations and in week 2 for certain treatment combinations. With the exception of treatments 1 and 15, which showed continous toxin production throughout the 21 day storage period, there was a significant decrease in the level of toxin in most other treatments to less than 20 ppb (with the exception of treatment 4). However, treatments 3, 6, 9, 12 and 13 produced aflatoxin B_1 levels far below the regulatory limit of 20 ppb, with most of the other treatments producing optimum aflatoxin levels in excess of the regulatory limit of 20 ppb.

The optimum level of aflatoxin B_1 detected during the first week of storage may be due to the presence of available nutrients and suitable environmental conditions for toxin production. However, the decrease in toxin level with time may be due a depletion of available substrate for toxin production or the breakdown of aflatoxin as a substrate for further mycelial development and mold growth (Doyle et al., 1982). Buchanan et al., (1985) found that aflatoxin production by *A. flavus* accumulated rapidly after 1-2 days in products packaged in air or 2% O_2 and then aflatoxin production decreased rapidly and remained at a constant level (<2 ug/mg dry weight) for all gaseous treatments. Karunaratne and Bullerman (1990) observed that at higher inoculum levels (10^7 spores/ml) mold mycelia were capable of degrading the preformed aflatoxin at a faster rate than that at which the toxins were formed. Ellis et al., (1993) also observed a curvilinear relationship between aflatoxin production and growth of *A. flavus* with toxin production increasing to an optimum and then decreasing with increasing growth.

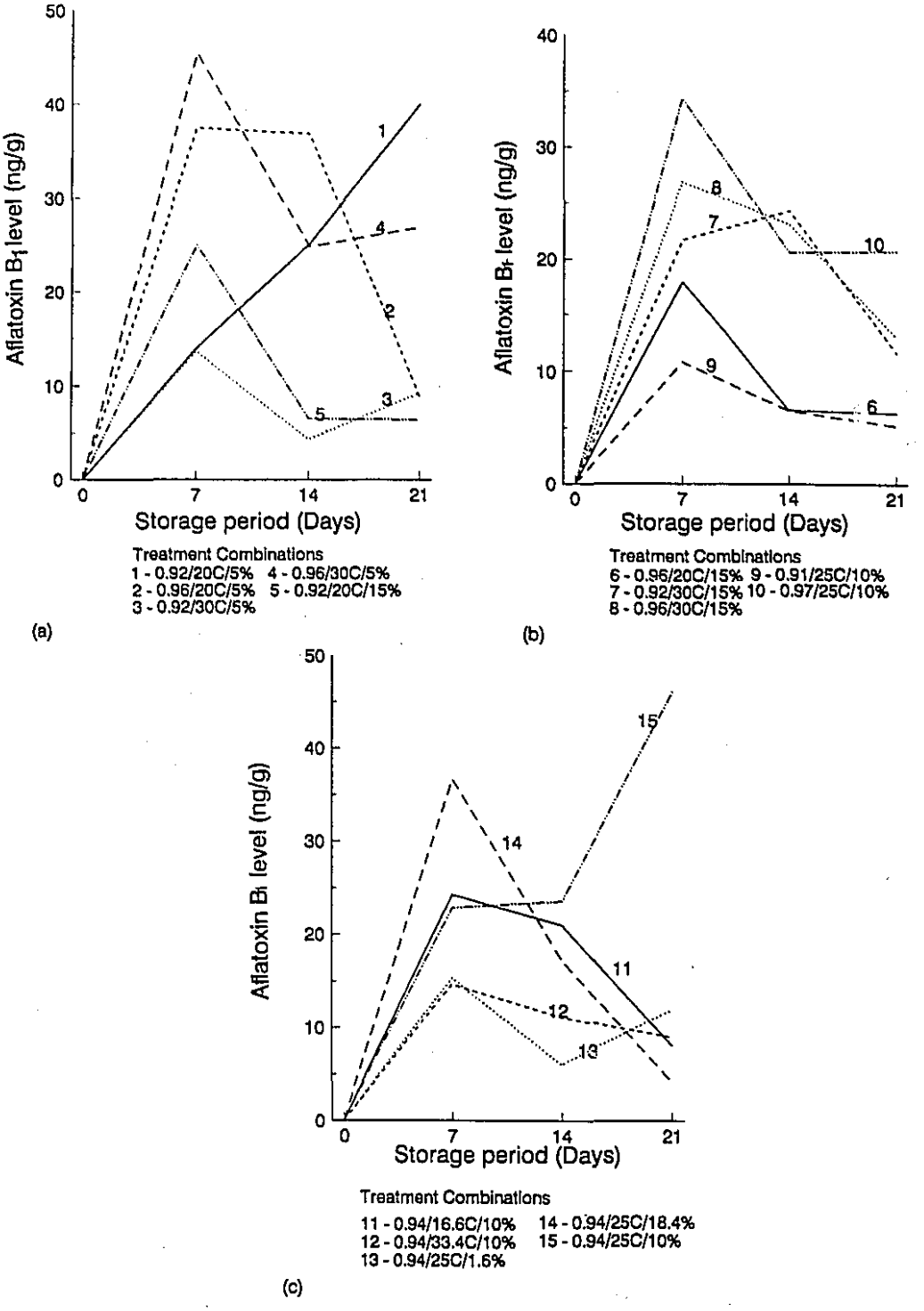


Figure 20 Trend in aflatoxin B_1 production by *A. flavus* with storage time under different packaging conditions

There appeared to be a trend between the extent of mold growth and the level of aflatoxin B_1 produced. However, statistical analysis of the data for the 14 day storage showed no significant correlation between changes in mold growth with time and the level of aflatoxin produced. To determine the combined synergistic effect of a_w, storage temperature and elevated levels of CO_2 in the presence of various levels of oxygen on the growth of, and aflatoxin production by *A. flavus* in sterile peanuts for a 14 day storage period, a Response Surface Methodology (RSM) approach was used. This method has the added advantage of enabling the generation of mathematical models to predict the packaging/environmental storage conditions which could be used to control aflatoxin production by *A. flavus*. The second order model resulting from the multiple regression of the uncoded results for growth (Table 29) is shown in Equation 1.

$$\mathbf{Y = 772.96 - 1735.36X_1 + 2.82X_2 + 0.23X_3 + 986.71X_1^2 + 0.01X_2^2 + 0.01X_3^2 - 3.52X_1X_2 - 0.43X_1X_3 - 0.01X_2X_3} \quad \textbf{(equation 1)}$$

Regression analysis of the model showed that the fitted model was highly significant ($p < 0.001$) and accounted for 84% of the total variation after being corrected for the means. Examination of the fitted model indicated that all linear terms (a_w, X_1; temperature (^oC), X_2 and headspace O_2, X_3), quadratic terms (a_w^2, X_1^2; temperature^2(^oC), X_2^2 and headspace O_2^2) and cross product terms ($a_w.T^oC$, X_1X_2; $a_w.O_2$, X_1X_3 and $T^oC.O_2$, X_2X_3) were highly significant. These results are in agreement with Smith et al., (1988) who showed that a_w, pH, headspace O_2 and CO_2 and storage temperature were the most important factors ($p < 0.01$) influencing the growth of *A. niger* in a gas packaged bakery product. Ellis et al., (1993) also found that a_w, pH, temperature and headspace O_2 were all highly significant factors ($p < 0.001$) in controlling the growth of *A. flavus* on synthetic media. Holmquist et al., (1983) also found that a_w, pH and storage temperature were all highly significant factors ($p < 0.01$) in controlling the growth of *A. flavus* and *A. parasiticus*

on synthetic media. The significant linear, quadratic and cross product terms influencing mold growth (Equation 1) were used to generate 3 dimensional response surface graphs and 2 dimensional contour plots. Figure 21 shows an example of a response surface graph of a_w versus O_2 on the growth of *A. flavus* with temperature held constant at 20°C. As the figure illustrates, a decrease in a_w or O_2 or a decrease in a_w and an increase in O_2 resulted in a decrease in growth. However, an increase in both a_w and O_2 resulted in an increase in mold growth. When temperature was increased to 30°C, a similar trend was observed, however, the extent of growth was much more extensive (Figure 22).

Canonical analysis for the set of experimental data indicates that the stationary point on the fitted surface is a minimum. However, it is difficult to determine the actual levels of a_w and/or O_2 which could be used to control the growth of *A. flavus* from the 3 dimensional graphs. This can be achieved from 2 dimensional contour plots of the same variables. For example, the growth of *A. flavus* can be reduced to less than $\log_{10}$ 5.39 at O_2 concentrations of 5.5% and an a_w of 0.90 in a MAP packaged peanut stored at 20°C (Figure 23). However, if the temperature is increased to 30°C, growth (log CFU/g) increases to 6.24 under the same conditions of a_w and headspace O_2 concentration (Figure 24). These results are consistent with previous observations by Ellis et al., (1993) of the synergistic effects between a_w, pH, storage temperature and headspace gas composition on growth of, and aflatoxin production by aflatoxigenic mold species. However, the stationary point observed in that study was a maximum.

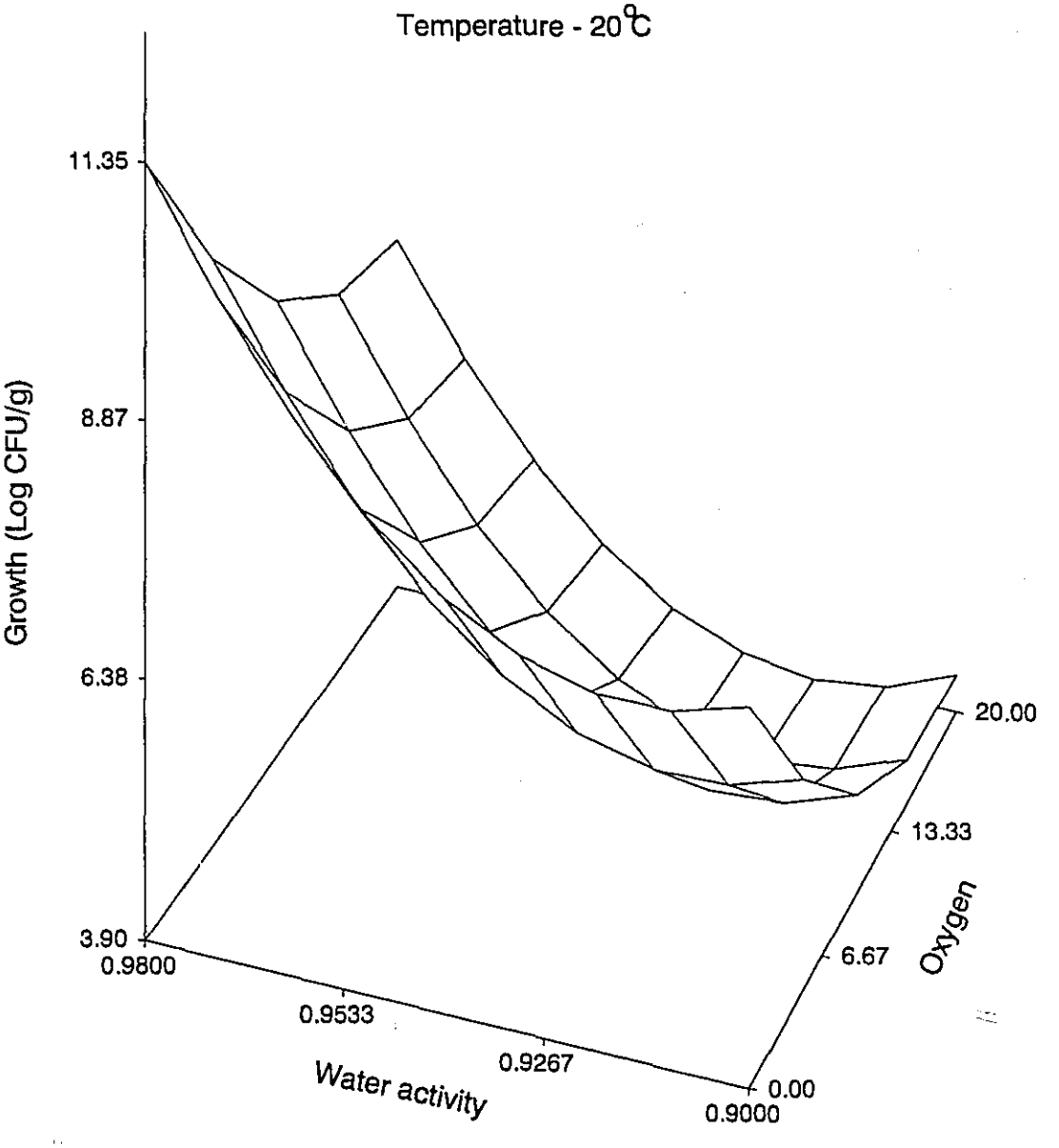


Figure 21 Three dimensional response surface graph showing the effect of water activity and oxygen on growth (log. CFU) of *A. flavus* at 20°C

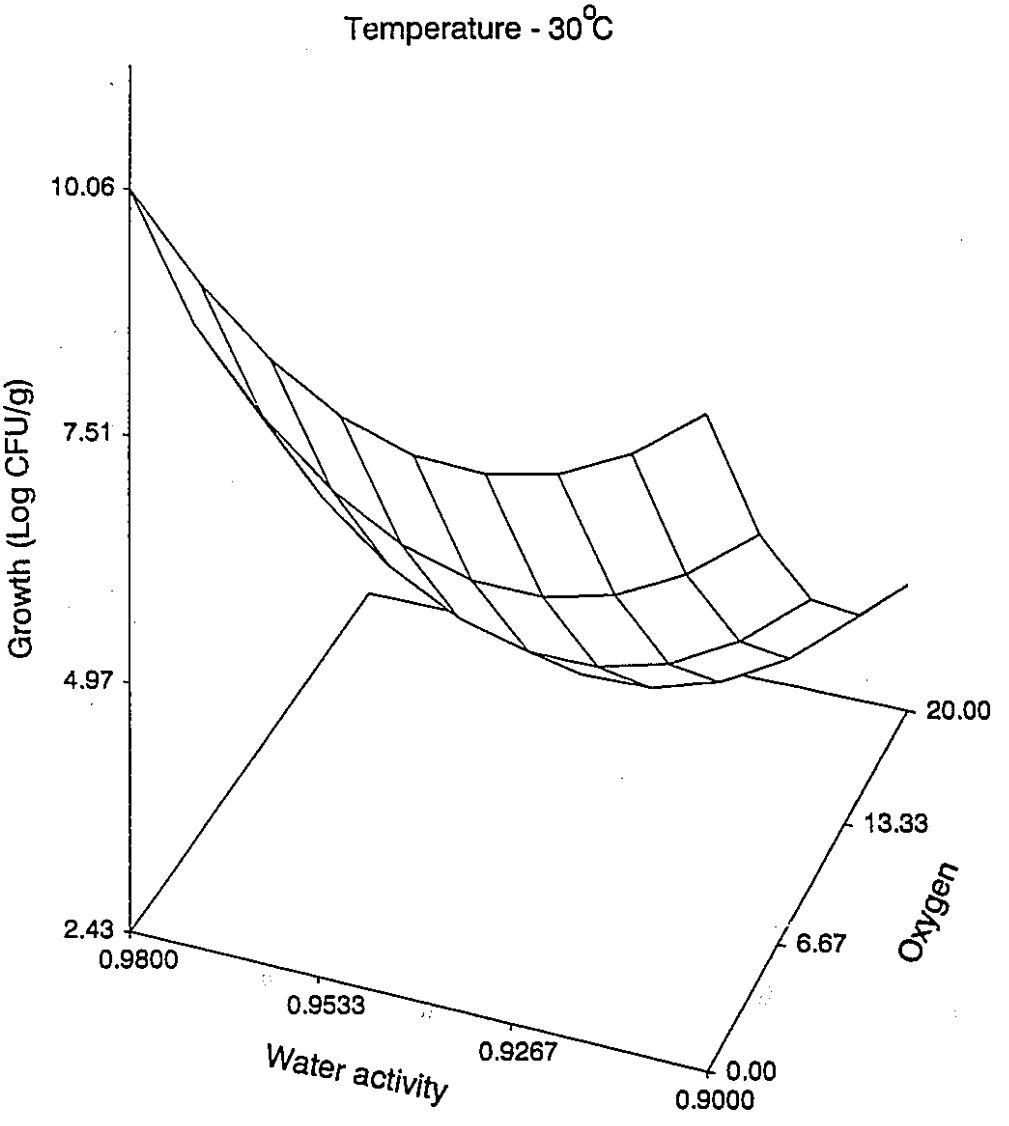


Figure 22 Three dimensional response surface graph showing the effect of water activity and oxygen on growth (log. CFU) of *A. flavus* at 30°C

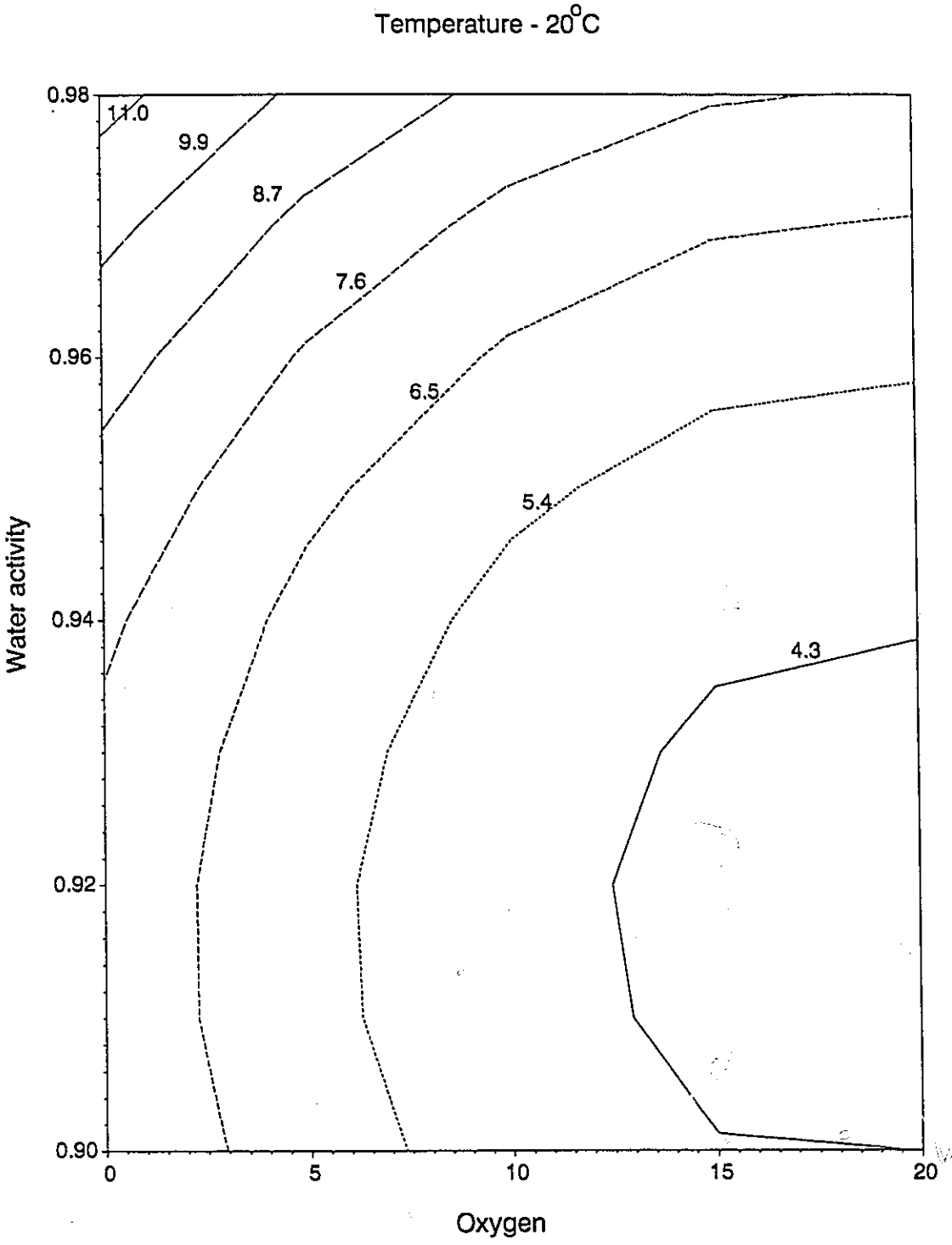


Figure 23 Two dimensional contour plot showing the effect of water activity and oxygen on growth of *A. flavus* at 20°C

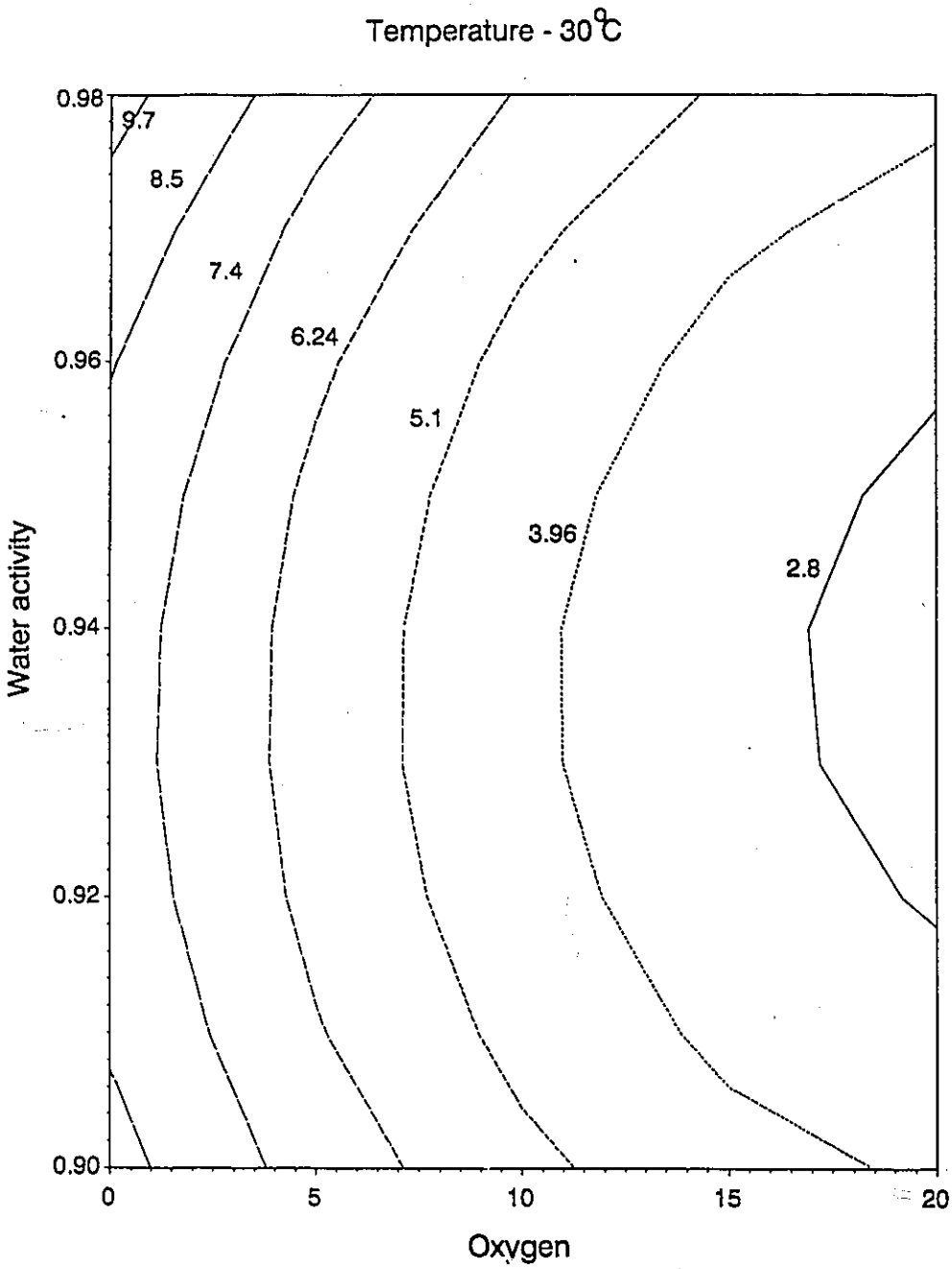


Figure 24 Two dimensional contour plot showing the effect of water activity and oxygen on growth of *A. flavus* at 30°C

Multiple regression of the uncoded data for aflatoxin B_1 (Table 30) for packaged peanuts stored for 14 days was used to generate a second order polynomial model shown in Equation 2 to determine the combined effect of a_w, temperature and headspace O_2 on toxin production.

$$Y_1 = -7163.32 + 14985X_1 - 10.31X_2 + 32.79X_3 - 7746.87X_1^2 - 0.04X_2^2 - 0.10X_3^2 + 9.42X_1X_2 - 41.76X_1X_3 + 0.33X_2X_3 \text{ (equation 2)}$$

Regression analysis of the above model showed that the fitted model was highly significant ($p< 0.001$) and accounted for 72% of the total variation after being corrected for the means. Examination of the fitted model indicated that all linear terms (a_w, X_1; temperature, X_2 and headspace O_2, X_3) and their corresponding quadratic terms (a_w^2, X_1^2; temperature^2 (^oC), X_2^2 and headspace O_2^2, X_3^2) were highly significant while the cross product terms (a_w.T^oC, X_1X_2; a_w.O_2, X_1X_3 and T^oC.O_2, X_2X_3) were also significant. The significant linear, quadratic and cross product terms influencing aflatoxin B_1 production (Equation 2) were used to generate 3 dimensional response surface graphs and 2 dimensional contour plots. Examples of response surface graphs of a_w versus O_2 on aflatoxin B_1 production by *A. flavus* with temperature held constant at 20^{o}C and 30^{o}C is shown in Figures 25 and 26. As these figures illustrate, an increase in substrate a_w and package headspace O_2 resulted in a decrease in toxin level, whilst an increase in a_w and a decrease in O_2 or decrease in a_w and increase in O_2 resulted in increased toxin level. These results are consistent with the previous findings of Ellis et al., (1993) of the synergistic effects between a_w, pH, storage temperature and headspace gas composition on growth of, and aflatoxin production by *A. flavus*.

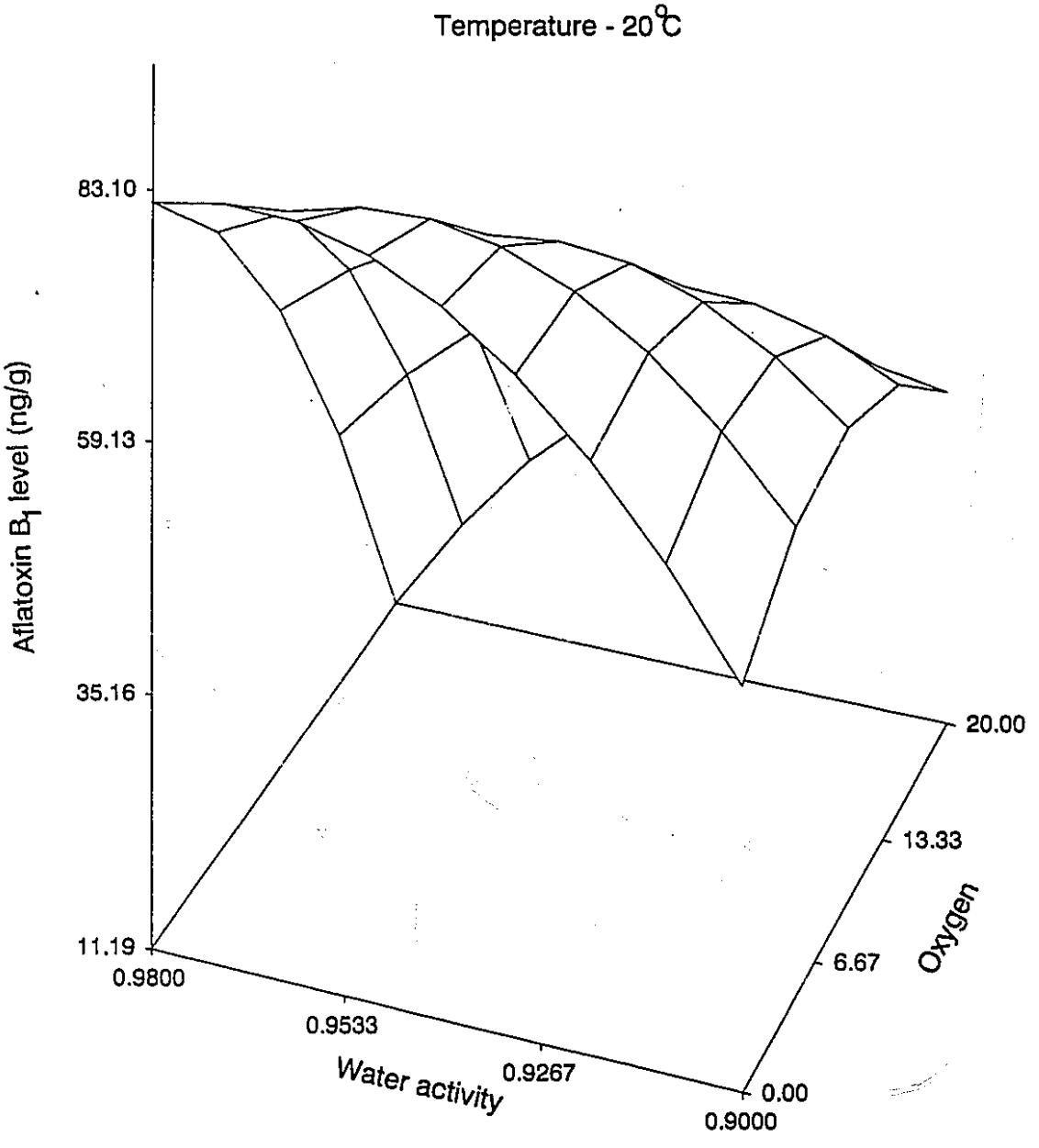


Figure 25 Three dimensional response surface graph showing the effect of water activity and oxygen on aflatoxin B_1 production at 20°C

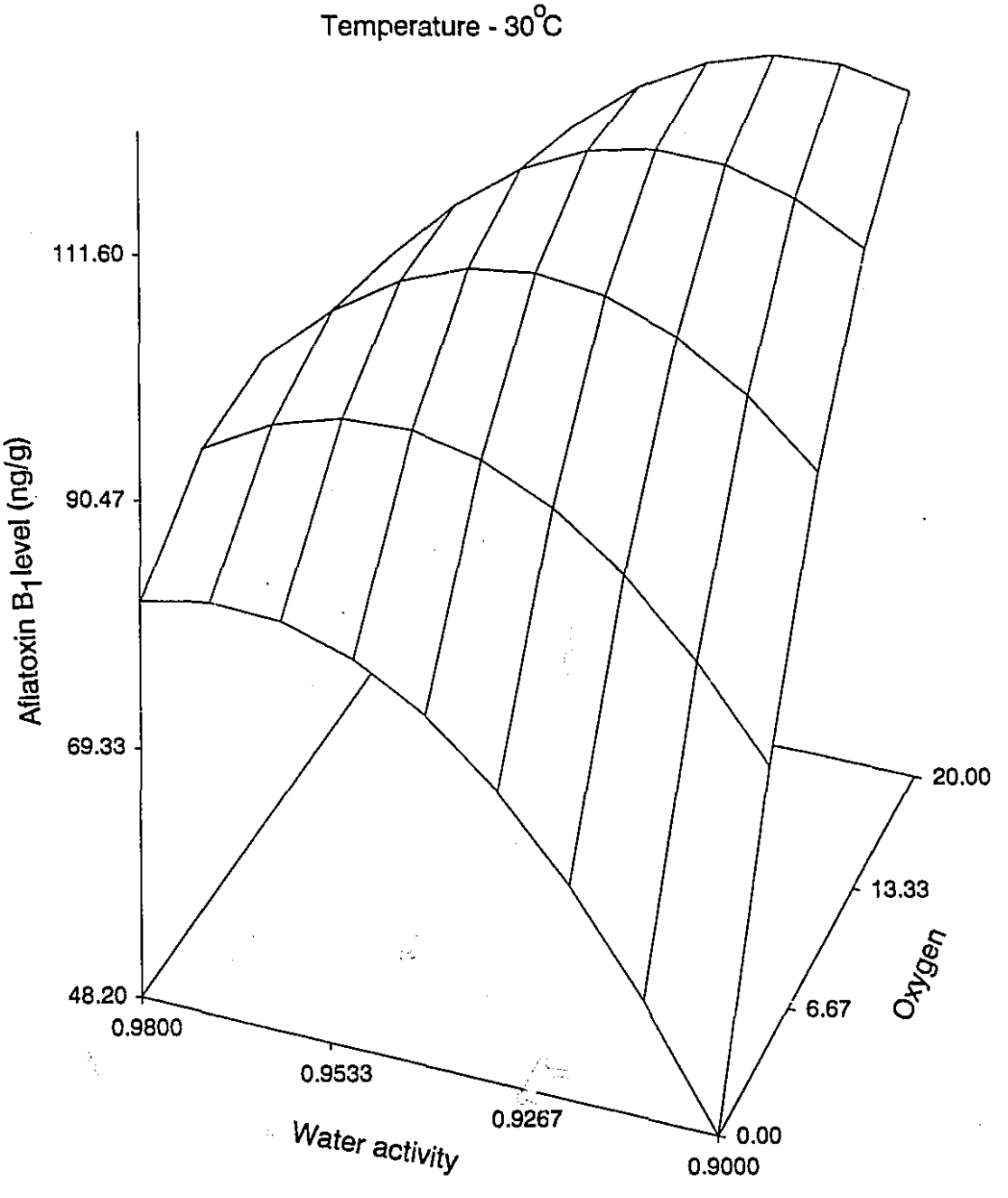


Figure 26 Three dimensional response surface graph showing the effect of water activity and oxygen on aflatoxin B_1 production at 30°C

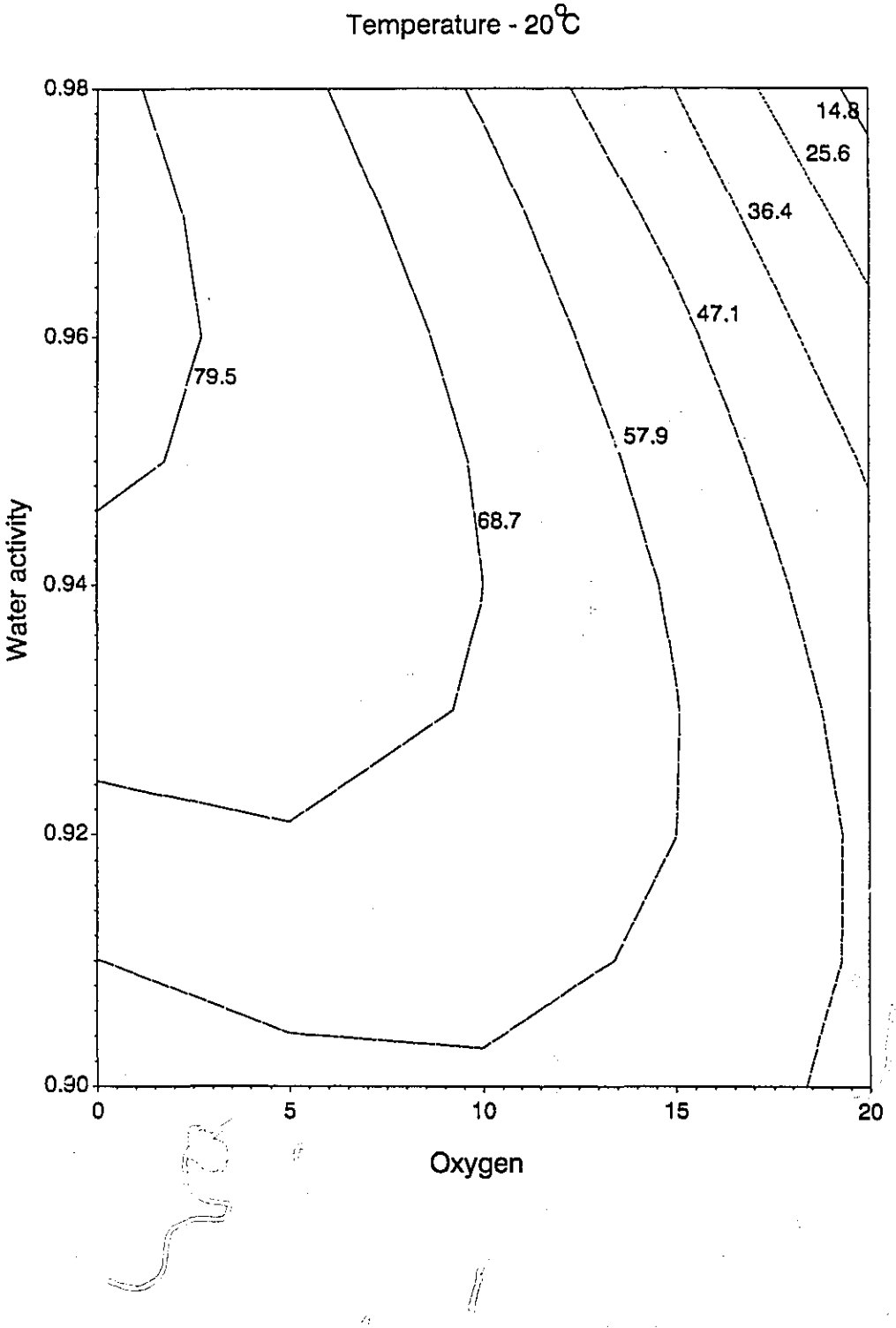


Figure 27 Two dimensional contour plots showing the effect of water activity and oxygen on aflatoxin B_1 production at 20°C

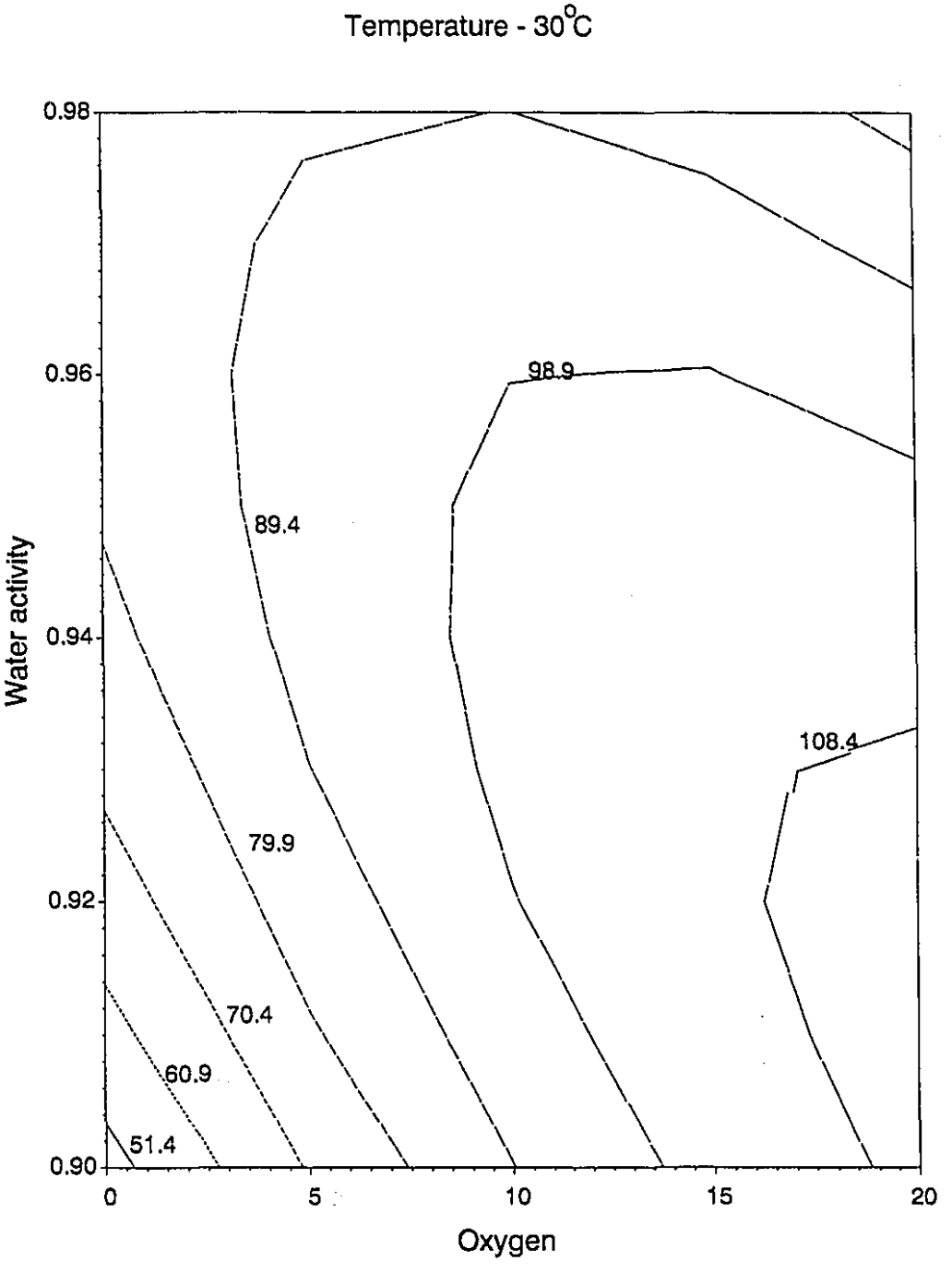


Figure 28 Two dimensional contour plot showing the effect of Water activity and oxygen on aflatoxin B_1 production at 30°C

Canonical analysis for the experimental data indicates that, the stationary point is a saddle point, i.e., neither a minimum nor a maximum. To determine the actual values of a_w and O_2 which could be used to control toxin production by *A. flavus* from the 3 dimensional graphs, 2 dimensional contours plots of the same variables are used (Figures 27 and 28). From the experimental conditions used in this study, it implies that for example, peanuts of a_w level 0.90 when packaged with headspace O_2 of about 17% in a CO_2 enriched atmosphere and stored at 20°C may result in the production of 50 ng/g aflatoxin B_1 (Figure 27). Under the same packaging conditions, an increase in storage temperature to 30°C, would result in an increase in aflatoxin B_1 level to about 108 ng/g (Figure 28).

The results of this study shows that for the effective control of the growth of, and aflatoxin production by *A. flavus* in food packaged under MAP conditions, there should be an effective means of controlling residual O_2 in the package either through the combined use of high and medium barrier films in conjunction with O_2 scavengers. Also it is essential to reduce product a_w level prior to packaging.

5.4 CONCLUSION

In conclusion, the study once again emphasizes the combined synergistic effects of several barriers to either completely inhibit mold growth or control aflatoxin production in MAP products containing various levels of headspace oxygen, especially at higher temperatures to levels well below the regulatory limits of 20 ppb.

CHAPTER 6

STUDIES ON THE GAS TRANSMISSION PROPERTIES OF POLYMERIC FILMS FOR APPLICATION IN THE CONTROL OF AFLATOXIN PRODUCTION UNDER MODIFIED ATMOSPHERE PACKAGING (MAP) CONDITIONS

6.1 INTRODUCTION

Aflatoxin contamination of foods and feeds is a worldwide problem which is of serious economic and public health concern (Fabbri et al., 1980). Several techniques have been used to prevent or limit the production of aflatoxins by *Aspergillus flavus* and *Aspergillus parasiticus* in bulk storage of foods and feeds. One such technique is Controlled Atmosphere Storage (CAS), involving the use of reduced oxygen levels in conjunction with elevated levels of carbon dioxide (Ellis et al., 1991) Recently, however, Modified Atmosphere Packaging (MAP) has become popular as a method of food preservation. MAP has been defined as "the enclosure of food products in high gas barrier films in which the gaseous environment has been changed to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage with the intent of extending shelf life (Young et al., 1988). The successful application of MAP depends on a number of inter-related factors, specifically the level of mold contamination, the concentration of residual oxygen in the package, package integrity and packaging film permeability. Generally, high barrier films such as Polyvinylidene chloride (Saran) or intermediate barrier films such as Nylon/Low Density Polyethylene are commonly used as packaging materials with MAP products. Ellis et al., (1993) reported that the growth of, and aflatoxin production by *A.*

flavus could be inhibited by 60:40 ratio of CO_2:N_2 and 0% O_2 using Ageless oxygen absorbent in plates packaged in high barrier films. However, when the atmosphere contained 5% O_2, aflatoxin was produced at levels greater than the regulatory limit of 20 ppb. Indeed, several studies have shown that mold growth is possible in lower concentrations of oxygen even in the presence of elevated levels of other antimicrobial gases, e.g., CO_2.

Dallyn and Everton (1969) reported the growth of *Aspergillus* species in 85% CO_2 and 3% O_2. Smith et al., (1986) also found that *Aspergillus niger* could grow in a gas packaged bakery product containing 60% CO_2 and low oxygen levels (<1% O_2). These studies clearly demonstrate that molds can tolerate and even grow in very low concentrations of headspace oxygen. This level of residual oxygen in an MAP product could be achieved through a number of factors such as (i) inadequate evacuation and gas flushing, (ii) leakage of air through poor sealing, (iii) ability of food to entrap air, (iv) oxygen permeability of packaging material. This latter parameter is very important since the gas transmission properties of packaging materials are a function of the storage environment, particularly temperature and relative humidity. This problem is of particular significance in MAP foods stored under tropical conditions, i.e., high temperature, high relative humidity. However, little is known about the effect of such environmental storage conditions on the gas transmission properties of barrier films. Therefore the objectives of this study were;

(i) to determine the gas transmission rates and permeability coefficients for three polymeric films stored under subambient and tropical environmental conditions.

(ii) to develop equations to predict the gas transmission rates under various environmental storage conditions.

6.2 MATERIALS AND METHODS

6.2.1 *Test films*

The three polymeric films used in the study were Cryovac films, PAXE and E_{50} films. Cryovac films were obtained from Cryovac (Canada Ltd.) while PAXE and E_{50} were obtained from Winpak, Winnipeg. The actual composition, oxygen transmission rate (O_2TR), water vapor transmission rate (WVTR) and the coded names of each film used in this study are shown in Table 31.

6.2.2 *Experimental design and statistical analysis*

The experimental design used in the study was a randomized complete block design (Box et al., 1978), comprising of three samples per film were each tested for oxygen, carbon dioxide and water vapor transmission at four different temperatures and 100% relative humidity. Statistical analysis (GLM, ANOVA) were computed using the Statistical Analysis System on a McGill University mainframe.

6.2.3 *Measurement of gas transmission rates*

The gas transmission rates of each film for oxygen, carbon dioxide and water vapour were each measured at four temperatures ($T^{o}C$ = 10, 23, 30 and 38) and at 100% relative humidity. For oxygen and carbon dioxide transmission rates, 100% O_2 and CO_2 were used as the permeant gas respectively. An external bubbler (Mocon P/N 021-001) with distilled

Table 31. Composition and oxygen transmission rates of test films

Film	Code	Composition	O_2T.R.[a]
Cryovac	ASI	Nylon/EVOH/PE	3-6[b]
PAXE	ASII	Nylon/PE	50[c]
E_{50}	ASIII	EVA/PE	4000[c]

[a] cc / m^2 / day.

[b] 4.4°C / 0% relative humidity.

[c] room temperature / 0% relative humidity.

water was added to the system for humidification purposes. Atmospheric pressure and laboratory temperature were recorded continuously. The permeability of the two films AS1 and AS11 to O_2 and CO_2 were measured directly by cutting a uniform size film and placing it directly in the test cell. However, due to the high permeability of AS111, the film was placed between two aluminium masks (Mocon 130-015), made of heavy 50 *u*m aluminium foil and adhesive backed with a precision die-cut hole in the centre to reduce the exchange surface area. The exchange surface of the mask was equivalent to 5 cm^2. The thickness of each film was measured 9 times and recorded using a Digimatic indicator 543 coupled to a Digimatic Mini-processor DP-1 264, from Mitutoyo M & G Co., Ltd, Japan. Mylar (polyester) 25 *u*m thick was used as the standard film, with a blank (foil) to measure any leakage and to determine the correction factor. All films were conditioned at the test temperature and 100% relative humidity for 24 to 48h prior to testing.

For the oxygen diffusion tests, an OX-TRAN 10/50 (Mocon, Minnesota, USA) with a data acquisition and logging system (Model DL-200) was used. The operation and apparatus are recognized as the standard test method for O_2 transmission rates (ASTM D3985-81). Moisture vapour transmission rate (MVTR) tests were measured with the Permatran W6 (Mocon, Minnesota, USA) also fitted with a data acquisition and logging system (Model DL-100) and corresponds to the standard test method for the MVTR of flexible barriers (ASTM F 372-73). Finally, for carbon dioxide diffusion, a Permatran C-IV (Mocon, Minnesota, USA) equipped with a paper chart recorder (Mocon BD 4004/05) was used. All three systems were equipped with a water-circulating bath (Neslab RT 3-5 B) to control the temperature of the test cells. The CO_2 transmission rate was calculated from the measured

signal of a known volume of CO_2. This volume was corrected for atmospheric and room temperature conditions. The dynamic accumulation mode (15 min) was used.

The cells of each system were divided in two chambers (top and bottom) separated by the test material. For the oxygen transmission tests, humidified oxygen was passed through the upper chamber and humidified carrier gas (99% nitrogen and 1% hydrogen) passed through the bottom chamber to sweep the permeant gas to the sensor. A scanning automatic valve sent the sample gas, oxygen to a specific coulometric sensor detector. The detector gives a current output directly proportional to the rate of oxygen arrival at the sensor. Thus, the oxygen flux across the film is dynamically measured. For carbon dioxde transmission rate, humidified carbon dioxide was passed through the upper chamber and a humidified carrier gas (100% nitrogen) was passed through the bottom chamber to sweep the permeant to the detector.

6.3 RESULTS AND DISCUSSION

Permeability can be defined as the amount of penetrant (mil or cc) diffusing through a polymer of standard thickness (25 *u*m or 1 mil). The mechanism of permeation includes (i) absorbtion of gas at the high pressure side (ii) diffusion through a film of uniform thickness and (iii) desorbtion at the low pressure side of film as shown in Figure 29. Permeability coefficient (**P**) can therefore be expressed as;

$$\mathbf{P} = \frac{\text{(Quantity of permeant) (film thickness)}}{\text{(Area) (Time) (Pressure drop across film)}}$$

In general, gas transmission rate gives the actual rate of diffusion per unit of surface for a penetrant where the thickness and the partial pressure gradient are not normalized. Therefore, Figure 29 and the following equations will explain briefly the difference between the two terms GTR and **P**.

$$\text{GTR} = \frac{dQ}{dt} = \frac{D\,A\,dc}{dx} \qquad (1)$$

where

$$\frac{\text{Volume}}{\text{Time}} \times \frac{1}{\text{Surface}} = \text{Diffusion constant} \times \frac{\text{Concentration gradient}}{\text{Thickness}}$$

If Henry's law is applied, c = Sp and assuming

$$\frac{dQ}{dt} = \frac{D\,S\,A\,(c_1 - c_2)}{dx}$$

By combining Fick's law with Henry's law for gas pressure and solubility we obtain the relationship shown in equation 2.

$$\frac{dQ}{dt} = \frac{D\,S\,A\,(p_1 - p_2)}{dx} \qquad (2)$$

or

$$\frac{dQ}{dt} = P\,A\,\frac{dp}{dx} \qquad (3)$$

where **P** (= DS) is the permeability coefficient for the polymer; D is the diffusion constant; S is the solubility of the gas in the polymer; c (= Sp) is the concentration of gas in the membrane; A is the exchange surface; x is the thickness; p is the partial pressure exerted; dp ($p_1 - p_2$) is the partial pressure gradient and dc ($c_1 - c_2$) the concentration gradient on both sides of the film (driving force) (Doyon et al., 1991). Therefore the permeability coefficient is represented by equation 4;

$$\mathbf{P} = \frac{Q.X}{A.t.dp} \qquad (4)$$

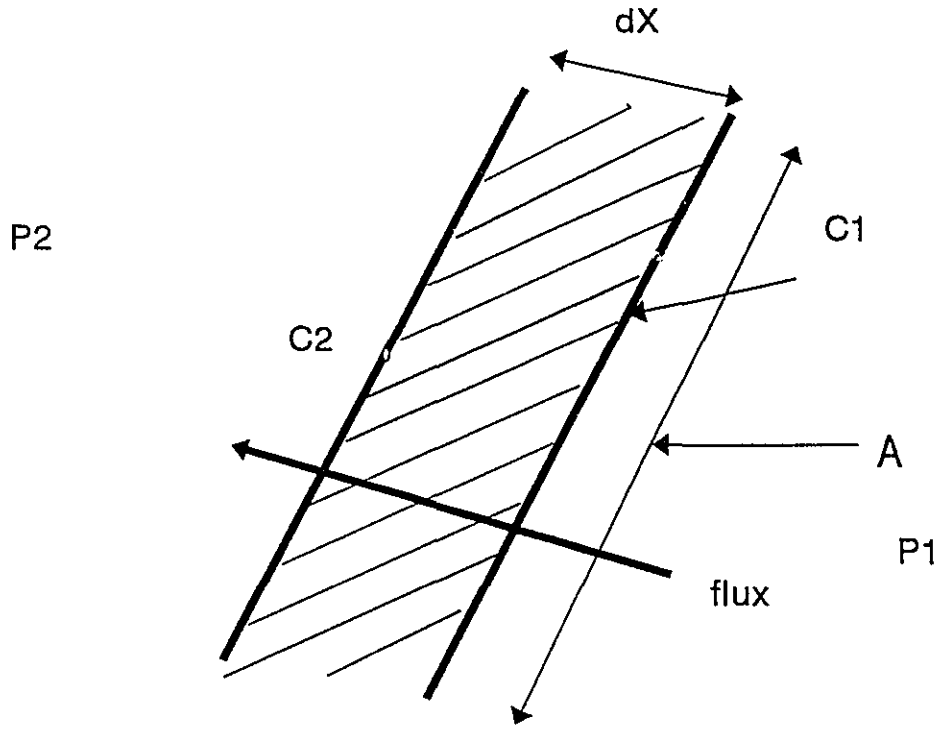


Figure 29. Schematic representation of "activated diffusion" across a packaging film.

The Mocon instrument generates the oxygen transmission rate (O_2TR), i.e., Q/A.t or permeance of the gas. The permeability is obtained by adjusting measured O_2TR or permeance with the pressure of permeant and the thickness of the material under the test conditions.

A summary of the results of the gas transmission rates/permeability to O_2, CO_2 and water vapour for the films under investigation at various storage temperatures is presented in Tables 32-37. The oxygen transmission rate (O_2TR) and permeability to oxygen of the various films are shown in Tables 32 and 35. It is evident that, film AS111 was the most permeable film to oxygen while film AS1 was the least permeable (at all temperatures under investigation). The order of both O_2TR and permeability of film to oxygen was AS111 > AS11 > AS1 (Table 32 and 35). Temperature played an important role in the O_2TR and permeability of the films to oxygen at constant RH (100%). Both O_2TR and P (permeability coefficient) increased as the storage temperature increase. This can be attributed to the changes in the diffusion and solubility coefficients.

Temperature had a similar effect on the transmission and permeability of CO_2 and water vapor through the packaging materials (Tables 33, 34, 36 & 37). However, a slightly different trend was observed for the transmission/permeability of the films to CO_2 and water vapor compared to oxygen. With respect to CO_2, this gas is the largest of the three gas molecules, while it has the lowest diffusion coefficient, its solubility (S), however, is much greater than for the other gases. Normally, carbon dioxide permeates four to six times faster than oxygen and oxygen four to six times faster than nitrogen. On the basis of the large solubility coefficient of CO_2 and the trend in the transmission/permeability of the films to oxygen, it was expected that CO_2 transmission/permeability would follow a similar trend, i.e., AS111 > AS11 > AS1. While this trend was apparent at lower temperatures (10^oC), it changed as temperature increased.

Table 32. Effect of temperature on oxygen transmission rate of three different polymeric films [a]

Temp.(°C)	Transmission rate (cc/m^2 day)		
	AS-1	AS11	AS111
10	2.6 (0.03)	10.8 (0.13)	1906 (3.4)
23	10.9 (0.06)	56.4 (0.80)	3208 (11.7)
30	27.9 (0.11)	81.6 (0.50)	4645 (12.1)
38	120.7 (1.18)	217.3	8755 (27.9)

Film thicness: AS1 - 64 +/- 0.003 *u*m; AS11 - 77 +/- 0.003 *u*m; AS111 - 50 +/- 0.002 *u*m.
[a] Values are means of 9 replicates obtained with 100% O_2.
( ) Standard error.

Table33. Effect of temperature on carbon dioxide transmission rate of three different polymeric films [a]

Temp.(°C)	Transmission rate (cc/m^2 day)		
	AS1	AS11	AS111
10	11.5 (0.12)	14.6 (0.17)	9244 (18.84)
23	68.7 (0.26)	66.7 (0.93)	15527 (65.50)
30	138.1 (0.29)	93.1 (0.22)	19911 (70.45)
38	316.8 (1.71)	156.8 (0.63)	36715 (236.19)

Film thickness: AS1 - 64 +/- 0.003 *u*m; AS11 - 77 +/- 0.003 *u*m; AS111 - 50 +/- 0.002 *u*m.
[a] Values are means of 9 replicates obtained with 100% CO_2..
( ) Standard error.

Table 34. Effect of temperature on water vapour transmission rate of three different polymeric films [a]

	Transmission rate (g/m^2 day)		
Temp.(^oC)	AS1	AS11	AS111
10	0.30 (0.003)	0.54 (0.004)	0.54 (0.002)
23	1.29 (0.01)	2.12 (0.01)	2.08 (0.01)
30	2.64 (0.02)	3.75 (0.02)	3.84 (0.01)
38	6.40 (0.04)	8.32 (0.10)	7.46 (0.01)

Film thickness: AS1 - 64 +/- 0.003 *u*m; AS11 - 77 +/- 0.003 *u*m; AS111 - 50 +/- 0.002 *u*m.
[a] Values are means of 9 replicates obtained with saturated N_2.
( ) Standard error.

Table 35. Permeability values for oxygen of three polymeric films[a]

	Permeability (cc. 25*u*m/m^2.d.atm.)		
Temp. (^oC)	AS1	AS11	AS111
10	6.0	30.5	3828
23	23.7	182.4	6435
30	70.3	272.9	9025
38	312.6	676.7	17456

[a] Values are means of 9 replicates.

Table 36. Permeability values for carbon dioxide of three polymeric films[a]

	Permeability (cc. 25um/m^2.d.atm.)		
Temp. (^{O}C)	AS1	AS11	AS111
10	55.5	44.3	18526
23	161.8	207.0	30868
30	354.0	294.9	39027
38	762.9	487.2	73870

[a] Values are means of 9 replicates.

Table 37. Permeability values for water vapour of three polymeric films[a]

	Permeability (g. 25 um/m^2.d)		
Temp. (^{O}C)	AS1	AS11	AS111
10	0.710	1.652	1.099
23	3.135	6.521	4.134
30	6.360	11.630	7.448
38	15.320	25.788	15.249

[a] Values are means of 9 replicates.

While film AS111 had the highest transmission/permeability to CO_2 at all temperatures, film AS1 had a greater transmission/permeability to CO_2 than film AS11, particularly at the higher storage temperatures (30-38^{o}C). The higher permeability of film AS1 to CO_2 at 30-38^{o}C can be attributed to the different types of polymeric chains and the influence of the polymer functional groups on the solubility of the gases and vapors in the amorphous portion of each polymer. Cryovac film, a high barrier film, is comprised of three distinct layers; an outer layer of nylon for strength, a middle layer of ethylene vinyl alcohol (EVOH) as a barrier and an innermost layer of low density polyethylene (LDPE) as the heat sealant layer. EVOH is an extremely hydrophilic film and its gas transmission properties change considerably as the RH increases due to the plasticizing effect of the absorbed moisture vapor. This change in the physical structure of the film, i.e., more "voids" per unit area, coupled with the high solubility coefficient (S) of carbon dioxide would enhance its diffusion across film AS1 compared to film AS11 which comprises of nylon and LDPE only.

The effect of temperature on the water vapor transmission rate (WVTR) and permeability to water vapor of the three different polymeric films are shown in Tables 34 and 37. Similar trends were observed for WVTR compared to O_2TR and CO_2TR, i.e., the transmission rates of all films increased as temperature increased. It is evident from Table 34 that the order of the WVTR of the films was AS11 > AS111 > AS1, i.e., film AS11 was most permeable to moisture vapor while film AS1 was the least permeable. Generally, high barrier films to fixed gases, such as O_2 and CO_2 are also high barrier film to moisture as in the case of film AS1. However, some films may be a low barrier to gases but a high barrier to moisture vapor, e.g., LDPE or PVC. Such films with selective barrier characteristics are used in wrapping of fresh meat where a high O_2 permeability is required to maintain the fresh meat color or "bloom". Conversely, certain films can be medium barriers to O_2 and CO_2 but low barriers to water water vapor, e.g., nylon, ethylene vinyl acetate (EVA)

(Smith et al., 1991). It is clear that only the WVTR of film AS1 can be related to its transmission rate to O_2 and CO_2. Film AS11 is less permeable to O_2 and CO_2 compared to film AS111 but more permeable to water. The selective permeability of laminated films is due to a number of inter - related factors including (i) the thickness and position of each component in the laminated structure, (ii) the ratio of the amorphous/crystalline regions in each film and (iii) the mobility of each region as a function of temperature and relative humidity.

The transmission data obtained were used to generate Arrhenius plot of rates of transmission and permeability changes with respect to temperature (Figures 30-32). The arrhenius plots, where the natural log. (Ln) or log. of the transmission properties (O_2, CO_2 and water vapor) is plotted against the reciprocal of the temperature (K) is a useful method for estimating tha activation energies and for predicting the O_2TR, CO_2TR and WVTR at other temperatures (Karel, 1975). It is is evident from Figures 33-35, that there is a linear relationship between the log of permeability and temperature (^oC). Another important parameter is the CO_2/O_2 permeability ratio or the selectivity factor (P) of films in relation to temperature change (Table 38). This factor is essential in the selection of films since it reflects the extent of transmission of gases (CO_2 and O_2) through the same material. For all three film, the ratio decreased with an increase in temperature. As temperature increased from 10^oC to 38^oC, the ratio for AS1 decreased by approximately 3.8-fold and for AS111 and AS11 approximately 1.1 to 1.5-fold respectively. This confirms the findings of Tolle (1971) and Doyon et al., (1991), who found that the selectivity ratio of CO_2/O_2 was not constant for a given film but increased as the decreased. Tables 39-41 shows the regression data and constants for the test films (not normalized to 25 *um*) The results of the study shows that, Ea_{tr}, was highest for O_2TR of Film AS1 (24.73 Kcal/g.mol) and lowest for CO_2TR of Film AS111 (8.71 Kcal/g.mol).

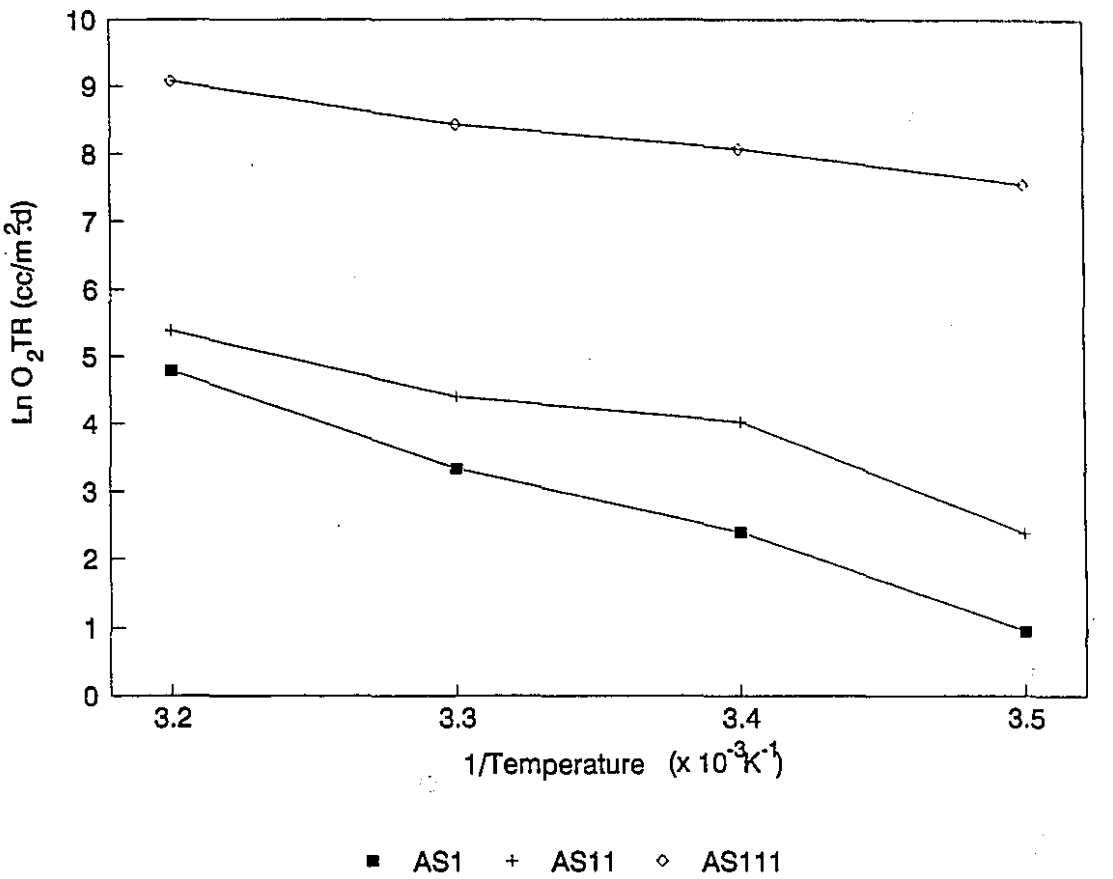


Figure 30 Arrhenius plot of oxygen transmission rate versus temperature change

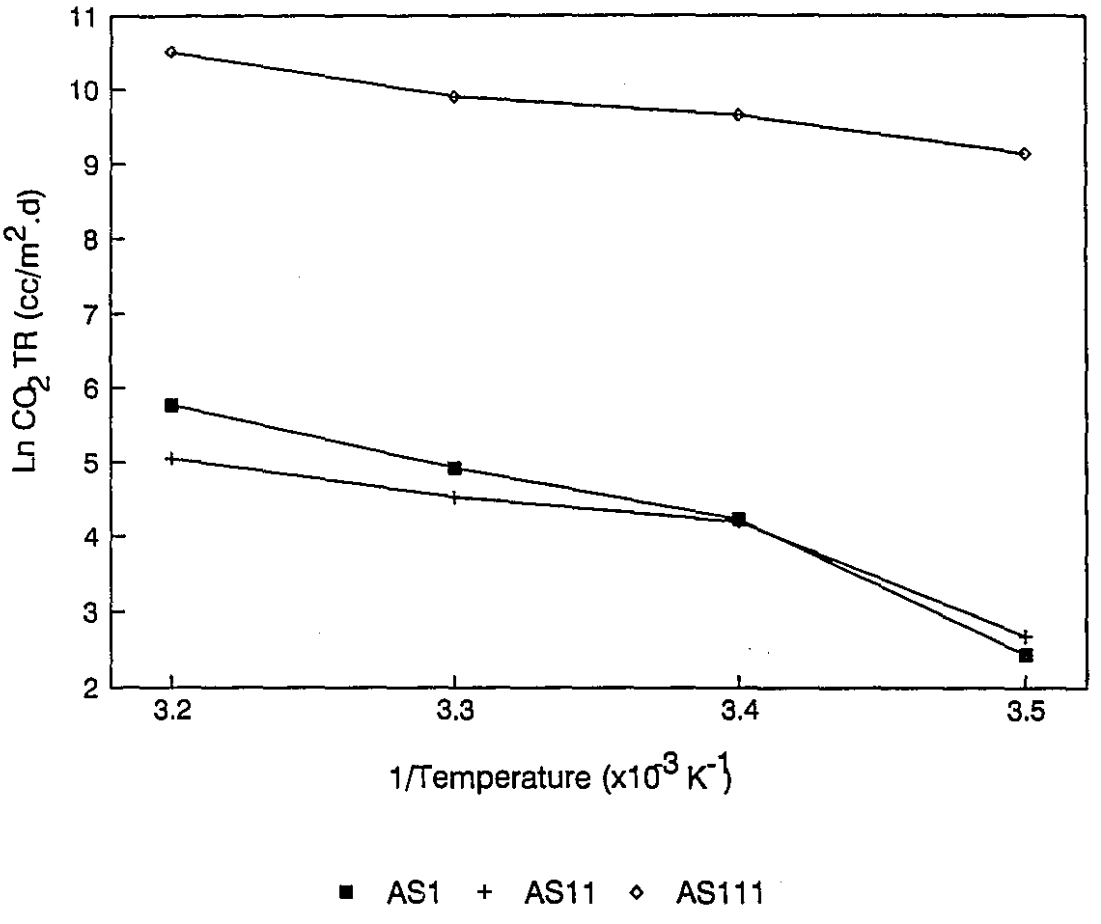


Figure 31 Arrhenius plot of carbon dioxide transmission rate versus temperature change

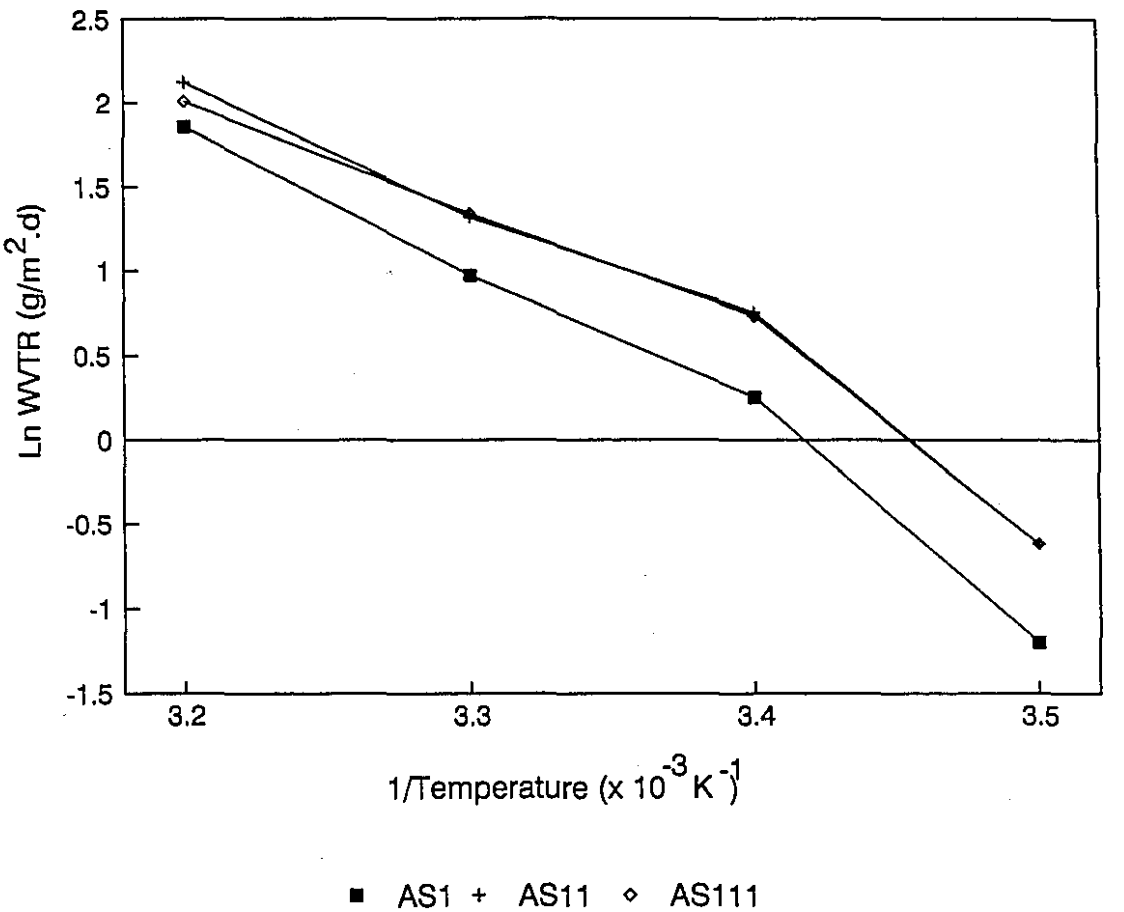


Figure 32 Arrhenius plot of water vapour transmission rate versus temperature changes

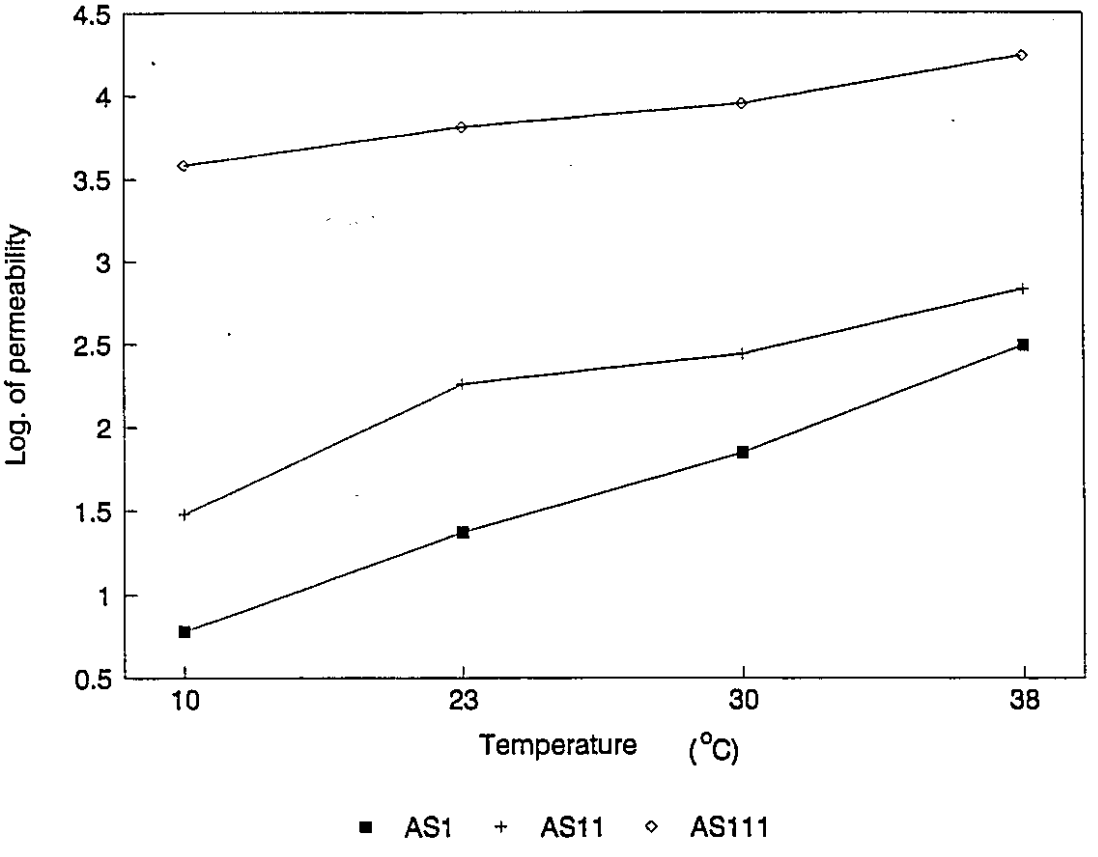


Figure 33 Graph of log. of oxygen permeability versus temperature change

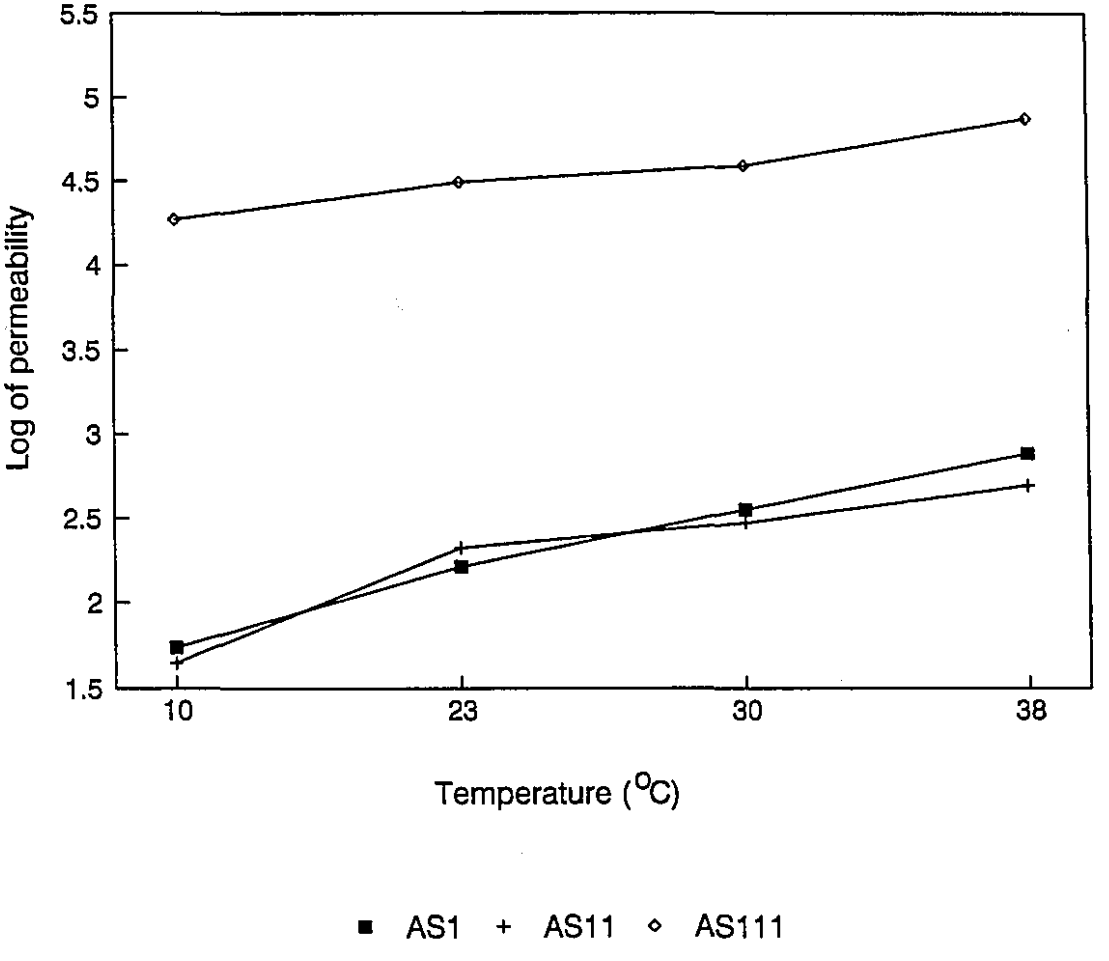


Figure 34 Graph of log. of carbon dioxide permeability versus temperature change

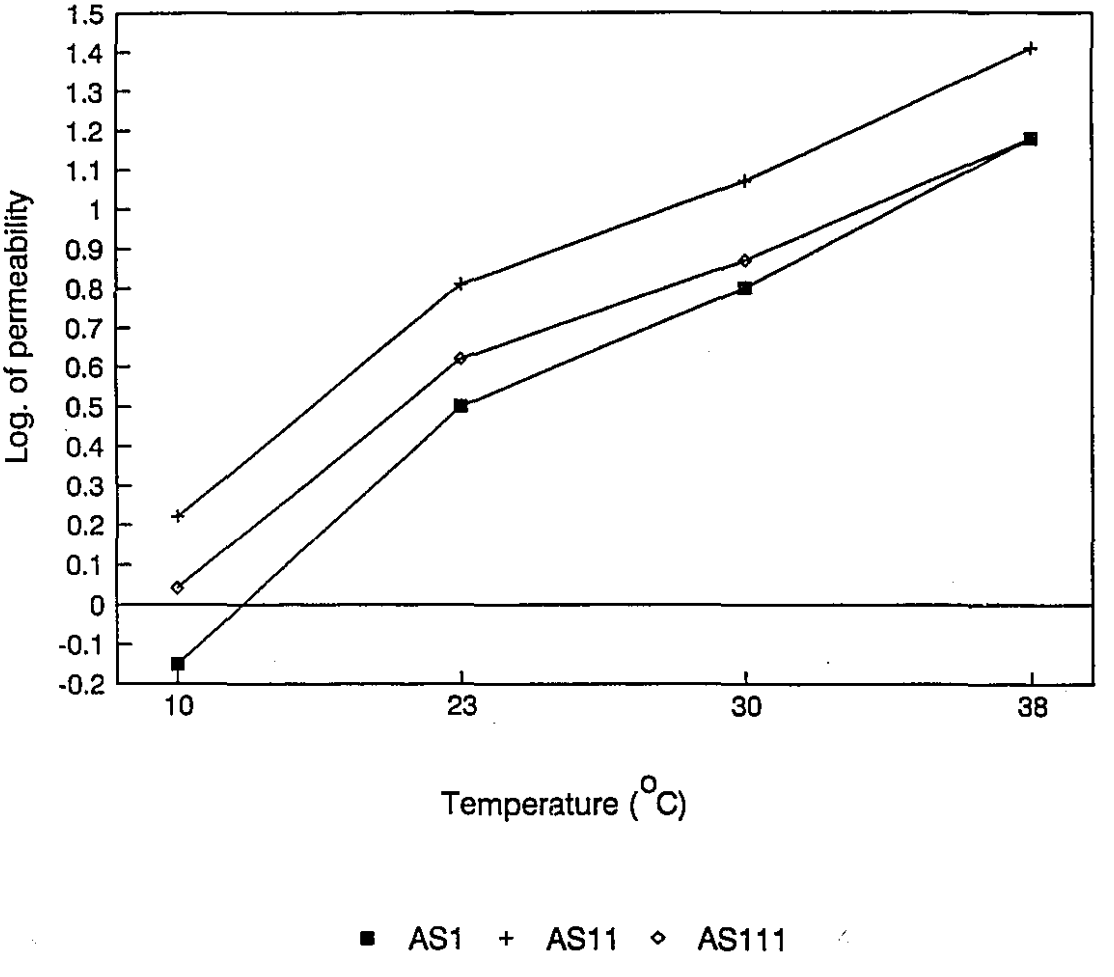


Figure 35 Graph of log. water vapour permeability versus temperature change

Table 38. Gas permeability ratios for test films

	CO_2/O_2		
Temp. (^oC)	AS1	AS11	AS111
10	9.20	1.45	4.83
23	6.83	1.13	4.79
30	5.04	1.08	4.32
38	2.44	0.72	4.23

Table 39. Regression data and constants for transmission rates of test films versus temperature

	Arrhenius plot (*AP*)		
Film	O_2	CO_2	WV
AS1	R^2 = 0.996	R^2 = 0.996	R^2 = 0.987
	a = 44.57	a = 33.21	a = 33.69
	b = -12450.66	b = -8565.85	b = -9919.03
	Ea = 24.73	Ea = 17.01	Ea = 19.70
AS11	R^2 = 0.967	R^2 = 0.944	R^2= 0.982
	a = 35.61	a = 29.064	a = 30.31
	b = -9420.38	b = -7446.43	b = 8780.94
	Ea = 18.71	Ea = 14.79	Ea = 17.44
AS111	R^2 = 0.995	R^2 = 0.988	R^2 = 0.981
	a = 24.73	a = 24.49	a = 29.18
	b = -4909.27	b = -4386.14	b = -8448.82
	Ea = 9.75	Ea = 8.71	Ea = 16.78
Fit		Lny = $a + bx$	

Ea = Kcal/g.mol (obtained by multiplying (R) 1.986 cal/g.mol to the gradient)

Table 40. Regression data and constants for transmission rate data of test films versus temperature

Film	Transmission rates		
	O_2	CO_2	WV
AS1	$R^2 = 0.992$	$R^2 = 0.998$	$R^2 = 0.999$
	a = 0.583	a = 8.622	a = 0.101
	b = 0.135	b = 0.093	b = 0.109
AS11	$R^2 = 0.983$	$R^2 = 0.981$	$R^2 = 0.998$
	a = 4.049	a = 7.326	a = 0.211
	b = 0.105	b = 0.084	b = 0.097
AS111	$R^2 = 0.985$	$R^2 = 0.983$	$R^2 = 0.998$
	a = 1040.42	a = 5430.57	a = 0.223
	b = 0.053	b = 0.047	b = 0.094
Fit		$Y = ac^{bx}$	

Table 41. Regression data and constants for permeability of test films versus temperature

	Permeability		
Film	O_2	CO_2	WV
AS1	R^2 = 0.998	R^2 = 0.996	R^2 = 0.986
	a = 20.44	a = 14.92	a = 15.02
	b = -5617.08	b = -3754	b = -4309.91
AS11	R^2 = 0.939	R^2 = 0.942	R^2 = 0.983
	a = 18.24	a = 13.26	a = 13.72
	b = -4759.62	b = -3276.75	b = -3833.23
AS111	R^2 = 0.993	R^2 = 0.989	R^2 = 0.982
	a = 11.01	a = 10.96	a = 13.01
	b = -2123.64	b = -1912.68	b = -3681.84
Fit		Log y = $a + bx$	

Table 42. Analysis of variance for a randomized complete block design for oxygen transmission rate

Source	dF[a]	Sum of squares	Mean squares	F
Model	23	246065067.4	10698481.2	1791.14***
Film (F)	2	166563176.6	83281588.3	13943.0***
Temp(^{o}C)	3	29060093.7	9686697.9	1621.75***
Block	2		1277.8	0.21^{ns}
Film*Temp	6	50401159.4	8400193.2	1406.36***
Film*Blk	4		859.8	0.14^{ns}
Temp*Blk	6		5773.8	0.97^{ns}
Error	12	71676	5973.0	
Total	35	246136743.4		
R^{2b}		0.99		

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p<0.001$.

ns = not significant.

Table 43. Analysis of variance for a randomized complete block design for carbon dioxide transmission rate.

Source	dF[a]	Sum of squares	Mean square	F
Model	23	13560915217	589605009	1212.16***
Film	2	9827352136	4913676068	10101.93***
Temp.	3	1284357146	428119049	880.16***
Block	2		35125	0.07ns
Film*Temp	6	2447679439	407946573	838.69***
Film*Blk	4		35691	0.07ns
Temp*Blk	6		218914	0.45ns
Error	84	40858419	486410	
Total	107	13601773636		
R^{2b}		0.99		

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p<0.001$.

ns = not significant.

Table 44. Analysis of variance for a randomized complete block design for water vapour transmission rate

Source	dF[a]	Sum of squares	Mean square	F
Model	23	250.64	10.89	1087.66***
Film	2	7.17	3.58	357.88***
Temp.(°C)	3	240.63	80.21	8005.75***
Block	2		0.01	1.36ns
Film*Temp	6	2.59	0.43	43.18***
Film*BlK	4		0.01	1.18ns
Temp*Blk	6		0.03	2.78ns
Error	12	0.12	0.01	
Total	35	250.76		
R^{2b}		0.99		

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p<0.001$.

ns = not significant.

Statistical analysis of the data shows that the model was highly significant (R^2 - 0.99) for the testing of the gas transmission rate across the three films for each of the gases (Tables 42-44). Furthermore, temperature was a highly significant variable affecting the transmission of the films to O_2, CO_2 and water vapor.

6.4 CONCLUSION

Films are classified as high, intermediate and low barriers based on the degree to which they transmit the specific material (gas or vapour) under consideration. Tables 45 and 46 show the range of values for oxygen and water vapour transmission rates and the corresponding barrier classification. For effective control of aflatoxin production under MAP conditions, films of high barrier properties to both O_2 and CO_2 would be most suitable since aflatoxigenic molds are aerobic and inhibition of growth and toxin production depends on the antimicrobial effect of elevated levels of CO_2 or low oxygen concentration using an oxygen absorbent technology. This study shows that for the three films investigated, there was a significant change in their WVTR with temperature. Comparing to standard values, the films can all be classified as high barriers (0.1-10 g/m^2/day) to moisture over a wide temperature range . On the other hand, their response to both oxygen and carbon dioxde transmission varied with temperature. The O_2TR values for AS1 shows that at low temperatures (< 23^oC) the film acts as a high barrier (0.1-10 cc/m^2/day). However, with increasing temperature it exhibited medium barrier properties (10-100 cc/m^2/day) while at very high temperatures (> 35^oC) it became a low barrier film (100-200 cc/m^2/day). A similar trend was exhibited for the CO_2 transmission of this film. However, film AS11 which is classified as a medium barrier, maintains its O_2TR and

Table 45 Classification ranges for O_2TR of films and flat stock.

Description	$cc/m^2/day$
Very low barrier	200 - 600
Low barrier	100 - 200
Medium barrier	10 - 100
High barrier	0.01 - 10
Very high barrier	0.001 - 0.01

Table 46 Classification ranges for WVTR of films and flat stock.

Description	$g/m^2/day$
Very low barrier	1,000 - 5,000
Low barrier	100 - 1,000
Medium barrier	10 - 100
High barrier	0.1 - 10
Very high barrier	0.01 - 0.1

CO_2TR properties over a wider range of temperature change. Film AS111 is a very low barrier material at all temperatures for both oxygen and carbon dioxide which is in agreement with literature values. From the results, films AS1 and AS11 would be appropriate barriers for use in MAP to control aflatoxin production in stored foods. However, film AS1 would be the most suitable of the three films studied based on the fact that the average temperature range and relative humidity for the tropics is about 20-35^{o}C and 70-90% respectively. At these storage temperatures/relative humidity conditions, film AS1 would have superior oxygen and water vapor barrier characteristics and therefore be useful for foods packaged in high CO_2/low O_2 concentrations using either gas packaging or oxygen absorbent technology. If such a film is used in gas packaging, the product should be flushed with a higher CO_2 concentration (80-90%) to ensure a residual headspace CO_2 concentration of 50-60% due to higher permeability to CO_2 at higher temperature and relative humidity.

CHAPTER 7

EFFECT OF PACKAGING FILMS OF DIFFERENT GAS TRANSMISSION RATES ON AFLATOXIN B_1 PRODUCTION BY *ASPERGILLUS FLAVUS* IN PEANUTS PACKAGED UNDER MODIFIED ATMOSPHERE PACKAGING (MAP) CONDITIONS

7.1 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 CO_2 and N_2 (Smith et al., 1991). Gas packaging has been shown to slow down aerobic microbiological spoilage and oxidative rancidity problems in food and has been used to extend the shelf life of muscle foods, rice, bakery products, pizza and sandwiches (Smith et al., 1991). 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., 1993). These aflatoxigenic molds are ubiquitous in nature and are 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).

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, the concentration of residual oxygen in the package headspace, packaging intergrity and barrier characteristics of the packaging material. This latter parameter is very important since the oxygen transmission rate (O_2TR) and carbon dioxide transmission rate (CO_2TR) of barrier films are a function of the storage environment, particularly relative humidity and storage temperature (chapter 6).

Furthermore, many spoilage bacteria and mold have been shown to grow in relatively low concentrations of oxygen (0.1-20%) even in the presence of inhibitory levels of carbon dioxide (Dallyn and Everton, 1969; Smith et al., 1986). However, there is a lack of data with respect to the effect of packaging film permeability on aflatoxin production in products packaged under MAP conditions and stored under subambient and tropical temperature conditions.

The objectives of this present study were to monitor the effects of three different films, with different gas transmission properties, to control aflatoxin production in peanuts stored under a CO_2 enriched atmosphere and under tropical temperature conditions.

7.2 MATERIALS AND METHODS

7.2.1 *Experimental design*

Three multi-layered polymeric films, Cryovac films, PAXE and E_{50} coded AS1, AS11 and AS111 respectively were used in the study. Cryovac films were obtained from Cryovac, Montreal and PAXE and E_{50} films from Winpak, Winnipeg. The composition and oxygen transmission rates (O_2TR) of these films are shown in Table 31 (chapter 6). A randomized complete block design comprising of 3 films (AS1, AS11 and AS111), 3 temperatures (20^oC, 25^oC and 30^oC) and 3 replicates per film per temperature was used as the experimental design in this study.

7.2.2 *Preparation of spore inoculum*

Aspergillus flavus ATCC strain No. 16872 obtained from ATCC, Rockville, Maryland was used throughout the study. The mold was grown on Czapek agar and subcultured onto slants of Malt Extract Agar (Difco, Michigan, U.S.A) for storage at 5^oC. Mold inoculum was prepared by growing *A. flavus* on MEA slants for 7-10 days until complete sporulation. Spores were harvested by washing surface of the slants with sterile 0.1% peptone solution. The suspension was filtered twice using Whatman's No.1 filter paper and the spore load determined using the surface plating technique. Based on the count obtained (10^7 spores/ml), decimal dilutions were prepared to obtain the desired inoculum concentration of 10^4 spores/ml used throughout the study.

7.2.3 *Preparation and inoculation of peanuts*

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 250g amounts placed in autoclavable dessicators containing glycerol solutions of known water activity (0.96). This was then sterilized by autoclaving for 15 min at 121^{o}C. It was then allowed to equilibrate at room temperature until the desired equilibrium water activity was reached. Water activity measurements of adjusted peanuts were done as described previously in section 5.2.3 of chapter 5. Fifty gram amounts of the sterile peanuts were then weighed into 150 x 15 mm petri dishes and inoculated with 10^4 spores/g of *A. flavus*. For each packaging condition, 3 inoculated plates and one non-inoculated plate (control) were packaged.

7.2.4 *Packaging*

Packaged peanuts were in two atmospheres, (i) in air and (ii) a high CO_2-N_2 atmosphere (60:40). Packaging in air was done by placing plates in 210 x 210 mm bags in each of 3 packaging films and sealing with a pulse heat - sealer. Packaging and sealing of packages in a CO_2-enriched atmosphere was done using a Multivac type vacuum/gas packaging unit (model AG500) (W.R. Grace and Co., Ajax, Ont. Canada). Each package was filled with a 60:40 ratio of CO_2:N_2 gas mixture. A Smith's proportional mixer was used to give the desired proportions of CO_2 and N_2 in each package. Packaged plates were incubated 25^{o}C and 30^{o}C in a Precision Gravity Convection incubators (model 2EG) and at 20^{o}C in a LabConco Scientific incubator (CanLab, Montreal, Canada) for 15 days.

7.2.5 *Headspace gas analysis*

Headspace gas analysis was done immediately after packing to ensure that the desired concentration of gases had been attained, in the gas packaged samples and to monitor changes in headspace gas composition throughout the 15 day storage period. Gas samples were drawn 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.) fitted with a thermal conductivity detector and using a Porapak Q (80-100 mesh) and Molecular Sieve 5A (80-100) columns in series. Helium was used as the carrier gas with a flow rate of 80 ml/min. The column oven temperature was initially set at 50^{o}C, then programmed to increse by 50^{o}C/min to 150^{o}C. The injector and detector temperatures were set at 80^{o}C and 100^{o}C respectively. Peaks were recorded and analysed with a Hewlett Packard intergrator (model 3390A).

7.2.6 *Color measurement*

After 15 days of storage, samples were evaluated visually for changes in color of the peanuts for each package using a 5-point scale (+1= Excellent color; +5= Extremely discolored). Fresh peanuts were used as a reference color control immediately after sterilization.

7.2.7 *Extraction and quantitation*

Extraction of aflatoxin B_1 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 separating funnel under minimal vacuum. Five ml of the filtrate was 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 *u*l of the purified extract was derivatized with 700 *u*l 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 *u*m Millex-GV millipore filter unit (Millipore Canada Ltd., Mississauga, Ontario.). Detection and quantitation of the derivatized extracts for aflatoxin B_1 were done using a Beckman System Gold liquid chromatographic system, consisting of a Beckman System Gold programmable module 126. Aflatoxin B_1 was detected by a Beckman 157 fluorescence detector, with a 7-60 excitation filter and a 450nm (40nm BW) emission filter attached to a Beckman System Gold Analog Interface module 406 (Beckman Instruments, Mississauga, Ont., 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 B_1 was calculated by comparison to standard peaks of known concentration of aflatoxin B_1 as outlined in the Beckman System Gold methodology.

7.2.8 *Statistical analysis*

Statistical analysis (analysis of variance and least square means) were computed using the Statistical Analysis System (SAS) programme on a McGill University mainframe.

7.3 RESULTS AND DISCUSSION

The transfer of mass between product, packaging material and the external and internal package environment can have significant consequences on the microbial activities associated with packaged foods (Bureau, 1985). This inter-relationship, specifically the combined effect of temperature and packaging material on aflatoxin production by *Aspergillus flavus* in peanuts packaged in both air and under modified atmosphere conditions is shown in Table 47. For peanuts packaged in air in films AS1 and AS11, there was no sign of visible mold growth after 15 days storage at 20°C. However, mold growth was visible in air packaged peanuts in the same films after 3-4 days at 25°C-30°C. Mycelial growth was evident in all peanuts packaged in film AS111 after 2-5 days at all three incubation temperatures with growth being most intense in plates stored at 30°C. It is evident from the changes in headspace gas composition (Figures 36-37) and the level of aflatoxin B_1 formed under these packaging conditions (Table 47) that mold growth was occurring. The lack of visible signs of mold growth for the air packaged peanuts at 20°C may be attributed to the high headspace CO_2 produced as a result of mold metabolism throughout the 15 day storage period and the high - medium barrier properties of these films to CO_2 at 20°C (chapter 6). Similar observations were reported by Wilson et al., (1985) who showed that the superficial contamination by *A. flavus* decreased in product stored in a 60% CO_2 atmosphere. Ingram (1962) also showed that the growth of *Pseudomonas* was self inhibited due to the formation of high levels of CO_2 within a high barrier film to O_2 and CO_2.

For peanuts packaged in a CO_2:N_2 (60:40) gas mixture in films AS1 and AS11, there was no signs of visible mold growth at 20°C and 25°C throughout the 15 day storage period. However, mold growth was observed at 30°C in all gas packaged peanuts using film AS1 and AS11. Medium - heavy mycelial growth was observed for all gas packaged plates in

Table 47. Effect of temperature and packaging film on aflatoxin production by *A. flavus* under MAP conditions.

Film	Temperature	Packaging condition	Color score[b]	Aflatoxin B_1 conc. (ng/g)[b]
AS1	20°C	Air	1.8 (0.1)	75.57 (1.90)
	25°C	Air	2.0 (0.0)	36.50 (3.80)
	30°C	Air	2.8 (0.1)	36.69 (2.43)
AS11	20°C	Air	2.0 (0.0)	33.04 (0.46)
	25°C	Air	2.3 (0.1)	48.21 (2.46)
	30°C	Air	2.7 (0.2)	21.16 (1.08)
AS111	20°C	Air	2.0 (0.0)	33.97 (0.94)
	25°C	Air	2.7 (0.1)	20.71 (1.04)
	30°C	Air	3.8 (0.1)	25.40 (1.46)
AS1	20°C	CO_2 / N_2	1.5 (0.2)	1.19 (0.01)
	25°C	CO_2 / N_2	1.7 (0.1)	0.36 (0.07)
	30°C	CO_2 / N_2	2.7 (0.2)	13.09 (0.87)
AS11	20°C	CO_2 / N_2	1.8 (0.1)	9.05 (0.40)
	25°C	CO_2 / N_2	2.0 (0.0)	16.20 (2.59)
	30°C	CO_2 / N_2	2.8 (0.1)	14.52 (0.70)
AS111	20°C	CO_2 / N_2	2.4 (0.1)	11.29 (0.43)
	25°C	CO_2 / N_2	3.0 (0.0)	1.96 (0.01)
	30°C	CO_2 / N_2	3.0 (0.0)	9.47 (0.17)

[b] Values in brackets represent the standard errors.

film AS111 at all incubation temperatures. The lack of visible signs of growth of *A. flavus* in air packaged peanuts with film AS1 and AS11 at 20°C does not imply that mold growth did not occur nor that the product was safe. The visible growth of *A. flavus* on gas packaged peanuts in high barrier (AS1) and medium barrier (AS11) films can be attributed to the residual headspace O_2 in these packages which was between 0.5-1% and/or the fact that the barrier properties of these films decreases as temperature increases. Miller and Golding (1949) showed that molds required very low oxygen concentrations for growth and reported that the growth of *Aspergillus* and *Penicillium* spp. was inhibited when concentration of oxygen was < 0.5%. Similar observations have been made by Wilson et al., (1975), who showed that the growth of *A. flavus* and *Fusarium moniliforme* was not inhibited in corn stored under a controlled atmosphere containing only 0-5% oxygen, although deterioration of the grain was delayed under these conditions. Furthermore, several studies have shown that molds can grow in relatively low concentrations of oxygen (0.5-2%) even in the presence of inhibitory levels of CO_2 (Smith et al., 1986).

7.3.1 *Changes in headspace gas composition*

Changes in headspace O_2, CO_2 and N_2 for peanuts packaged in air for the three different films at three different temperatures are shown in Figures 36-38. In air packaged samples, headspace O_2 decreased to 1% or less than 1% within 1-2 days depending on the storage temperature and type of film. Headspace CO_2 increased initially throughout storage as a result of mold metabolism and then decreased gradually, particularly in films AS1 and AS11 stored at 25-30°C. The gradual decrease may be due either to its adsorption 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 and Mateles, 1971). There was no significant difference in the trend for films AS1 and AS11 for changes in headspace gas composition at 20°C and 25°C but significant differences at 30°C.

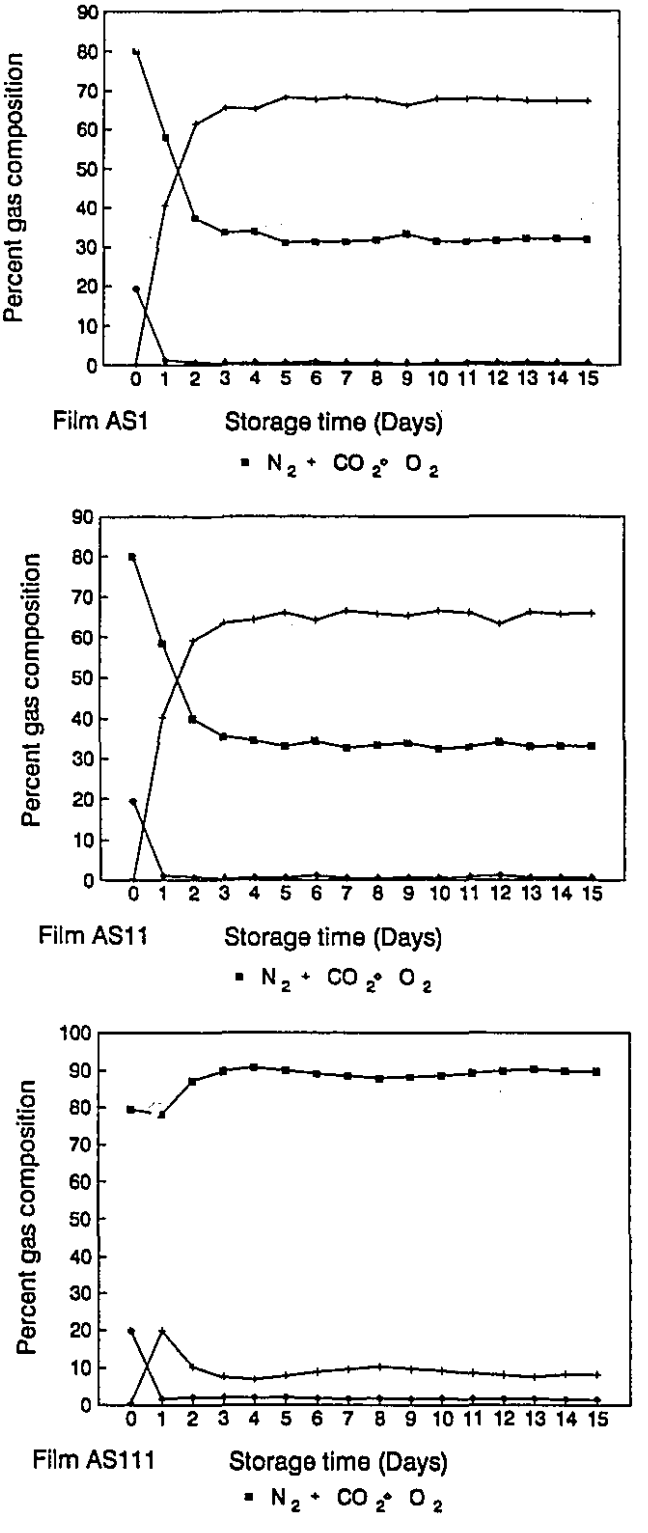


Figure 36 Headspace gas changes with time for three packaging films at 20℃ in air

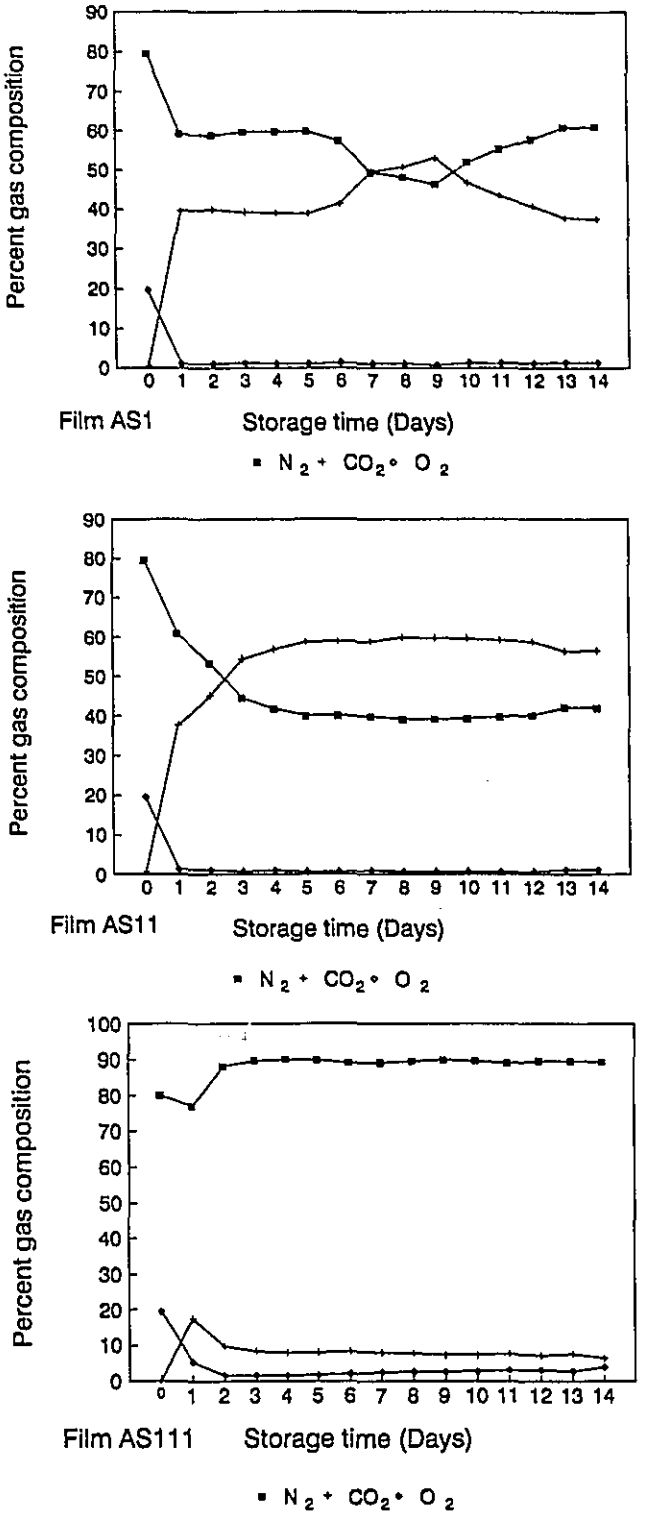


Figure 37 Headspace gas changes with time for three packaging films at 25°C in air

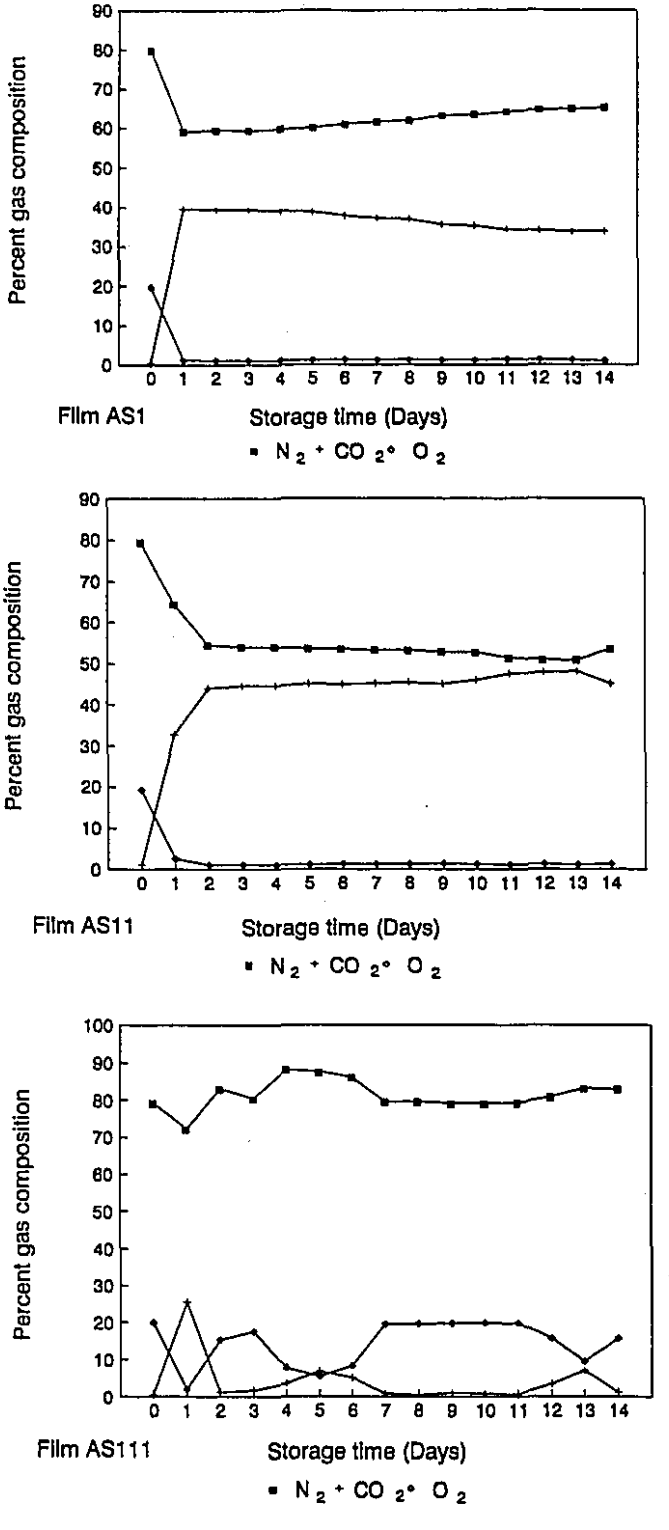


Figure 38 Headspace gas changes with time for three packaging films at 30°C in air

However, the changes in headspace gas composition for film AS111 were significantly different compared to films AS1 and AS11 at all three temperatures. The differences in the headspace CO_2 and O_2 composition between films AS1, AS11 and AS111 are due to the different gas transmission properties of these films to CO_2 and O_2, particularly at higher storage temperature. Studies in chapter 6, showed that, film AS111 was a low barrier at all these temperatures, while film AS11 maintained its medium barrier properties over a wide range of temperature. Film AS1 showed high barrier properties below ambient temperature (< 25°C) but above this temperature it exhibited medium barrier properties. The low barrier characteristics of film AS111 explains why headspace O_2 was 2-3% throughout storage and CO_2 levels were only 10-20% compared to the higher levels (40-60%) observed in air packaged peanuts in films AS1 and AS11.

Changes in headspace composition for peanuts packaged in a CO_2:N_2 (60:40) mixture in the three films AS1, AS11 and AS111 at 20°C, 25°C and 30°C are shown in Figures 39-41 respectively. For peanuts packaged in 60% headspace CO_2/balance N_2, similar trends were observed for changes in headspace CO_2 for films AS1 and AS11 at 20°C and 25°C with an initial decrease in CO_2 levels due to its adsorption by the peanuts then it remained fairly constant throughout the storage period. At 30°C, there was a greater depletion of CO_2 due to its loss through the film which becomes more permeable to CO_2 at higher temperatures. Changes in headspace gas composition for peanuts packaged in film AS111 were different from the other two films (Figures 39-41). In this film, there was a gradual increase in headspace O_2, particularly in products stored at 30°C and then a sharp decrease in headspace CO_2 to about 10-20% with a corresponding increase in headspace N_2. These changes can also be attributed to the lower barrier properties of film AS111, particularly at higher storage temperatures to both CO_2 and O_2 or the adsorption of CO_2 by the peanuts or its incorporation into the biosynthetic pathway for toxin production.

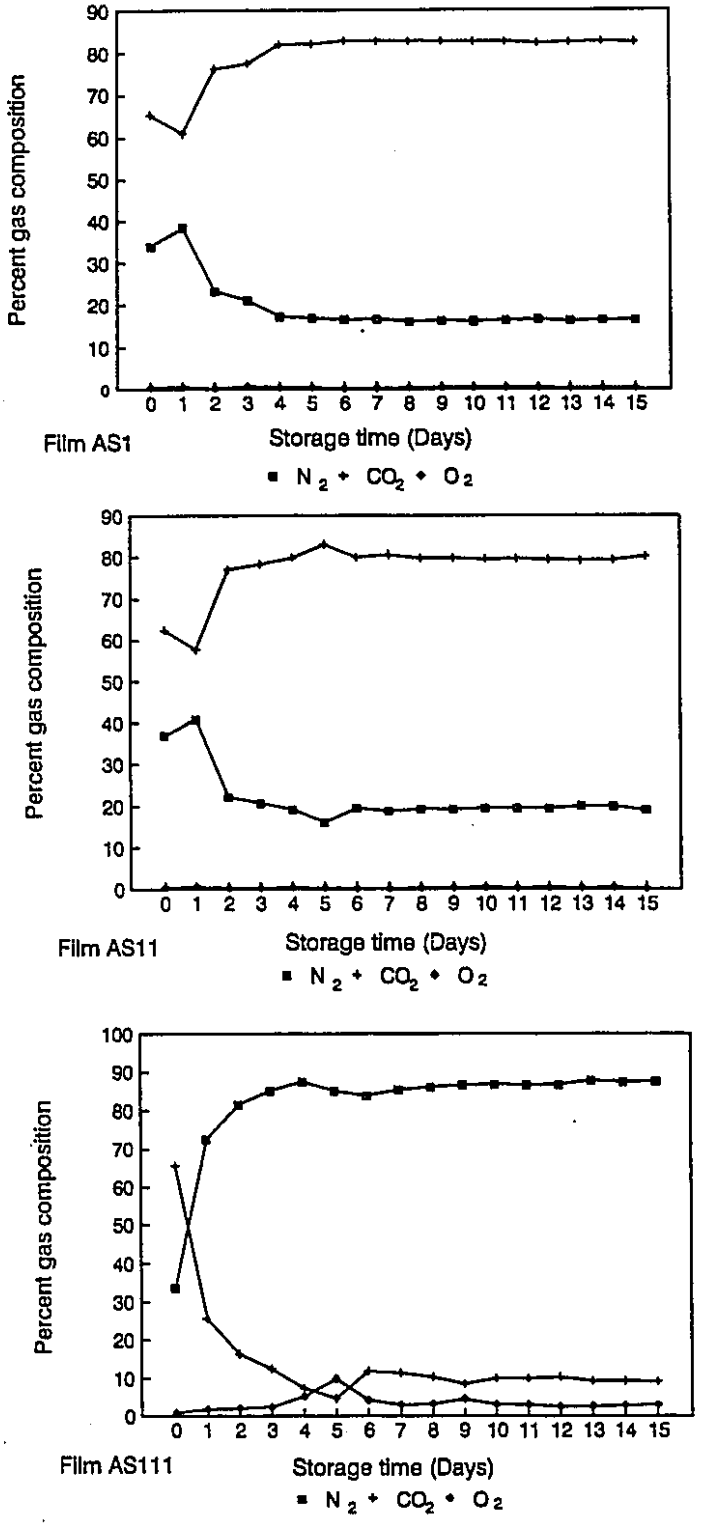


Figure 39 Headspace gas changes with time for three packaging films at 20°C in CO_2 /N_2

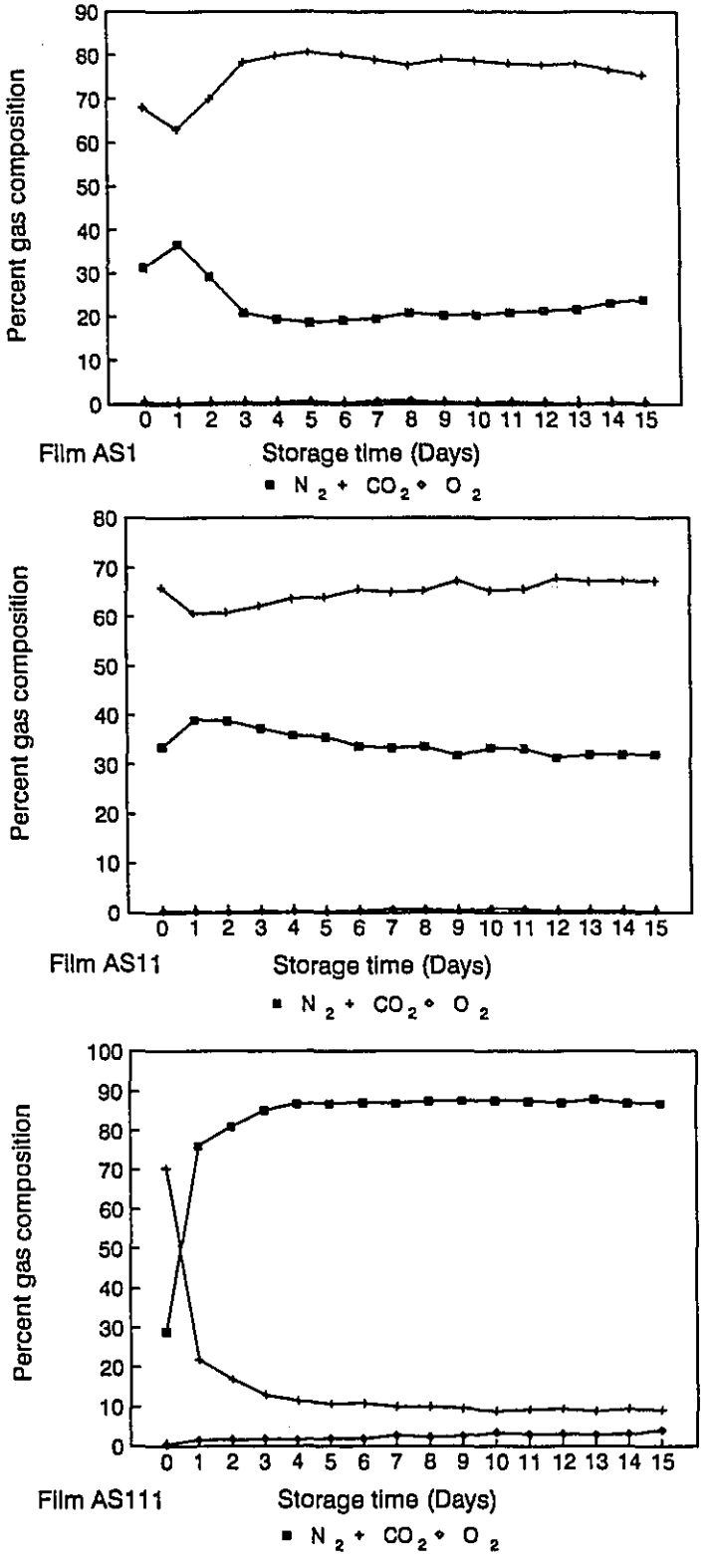


Figure 40 Headspace gas changes with time for three packaging films at 25°C in CO_2 /N_2

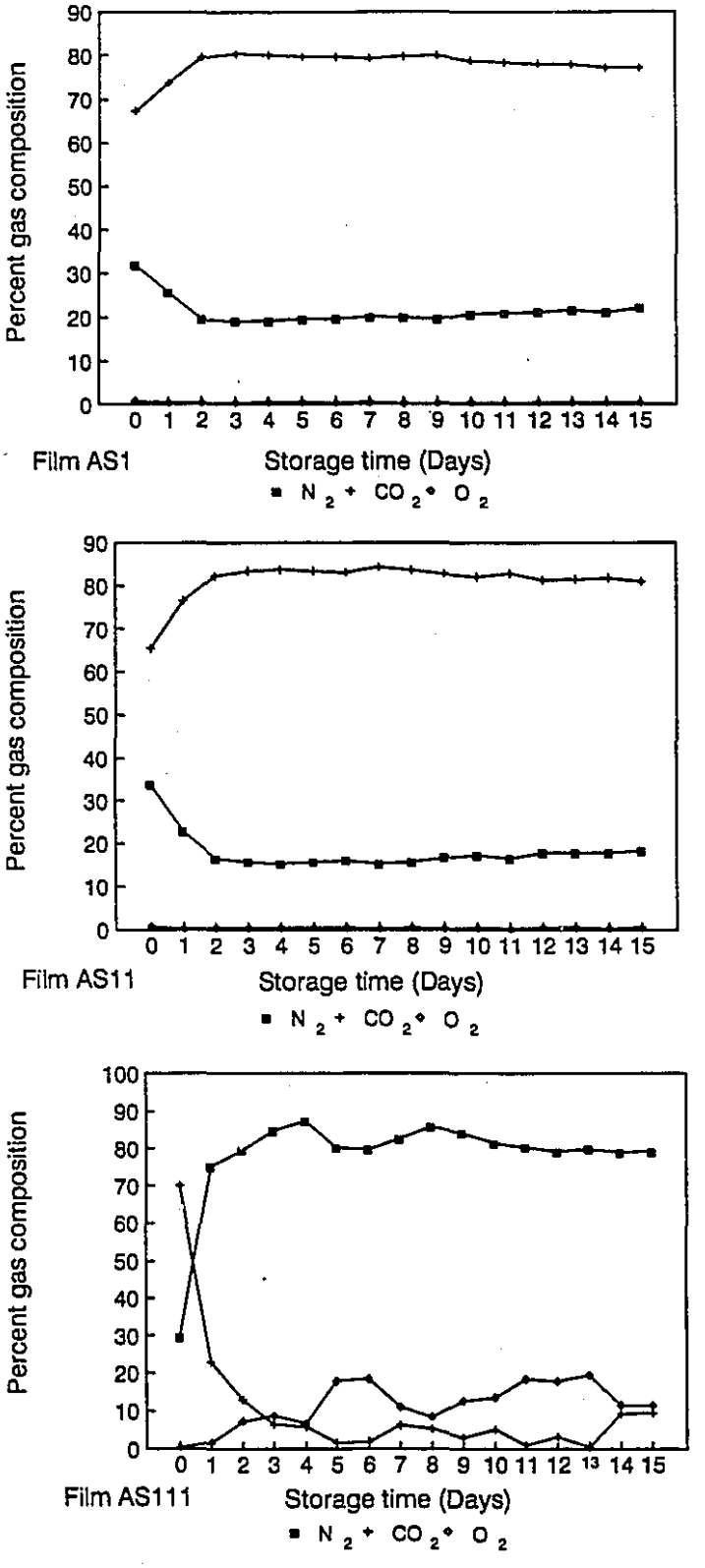


Figure 41 Headspace gas changes with time for three packaging films at 30°C in CO_2/N_2

7.3.2 *Color change*

Changes in the color of peanuts for the various packaging treatments are shown in Table 47. A color score of greater or equal to 2.5 was used as an arbitrary standard of an unacceptable product and hence termination of shelf life. Changes in the color score for air packaged peanuts in films AS1 and AS11 remained fairly constant at 20°C and 25°C. This can be attributed to the depletion of oxygen (Figures 36-38) and hence less oxidative rancidity of product. However, more intense color changes were noted for peanuts packaged in films AS1 and AS11 at 30°C. The greater color changes in these products can be attributed to the greater permeability of these packages to oxygen at 30°C. More severe color changes were observed for peanuts packaged in the low barrier film (AS111) particularly at 25°C and 30°C. Peanuts packaged in these films AS1 and AS11 and stored at 30°C and film AS111 stored at 25°C and 30°C were rejected on the basis of their color score (greater or equal to 2.5) at the end of the 15 day storage period.

Similar, but less severe trends in color scores, were noted for peanuts packaged in a CO_2 enriched atmosphere in film AS1, AS11 and AS111 at all storage temperatures. Once again peanuts packaged in high-medium barrier films and stored at 30°C or peanuts packaged in a low barrier film (AS111) and stored at 25°C and 30°C were rejected on the basis of their color score (> 2.5) at the end of the 15 day storage period.

Color scores could be a useful indicator of safety of air packaged products, particularly at temperatures commonly found in the tropics, i.e., greater than or equal to 25°C to 30°C since spoilage generally preceded toxigenesis in these products. Although gas packaged products were safe with respect to level of aflatoxin detected, i.e., < 20 ppb, nevertheless certain packaging treatments, particularly at the higher storage temperatures would be rejected on the basis of their color scores.

7.3.3 *Aflatoxin B_1 production*

The combined effects of the gaseous conditions, packaging film permeability and storage temperature on aflatoxin B_1 production by *A. flavus* is shown in Table 47 and Figures 42-43. It is evident that, the level of aflatoxin B_1 detected in all packaged samples was consistently greater than the regulatory limit of 20 ng/g or 20 ppb compared to gas packaged peanuts stored under similar conditions. The trend in the levels of aflatoxin production in air packaged samples is interesting. Higher levels of aflatoxin B_1 were detected in air samples packaged in the high barrier film AS1 with lower levels of aflatoxin B_1 being detected in air packaged samples packaged in the low barrier film to O_2 and CO_2, i.e., film AS111. These results imply that higher CO_2 concentrations may play a role in higher aflatoxin production due to its incorporation into the biosynthetic pathway. However, Ellis et al., (1993) reported no significant correlation between changes in headspace CO_2 with time and the level of aflatoxin detected on synthetic media. It could also be attributed to lower headspace O_2 concentrations and hence less oxidation and breakdown of pre-cursor products in aflatoxin production. Another possible explanation for the results is the observation by Shih and Marth (1974) that maximal yields of toxin were higher in stationary cultures than in agitated cultures. These authors observed the presence of greater Embden-Mayerhoff Pathway activity in cultures without agitation thus indicating a less aerobic environment, i.e., similar conditions to peanuts packaged in higher barrier film AS1. Acetate oxidation and NADPH oxidation or utilization are minimal under these stationary conditions, i.e., less oxygen and therefore more acetate, which is a precursor of aflatoxin and NADPH would increase, leading to an increase in aflatoxin biosynthesis. Conversely, in agitated cultures (equivalent to low barrier film AS111) there is a greater oxidation of acetate and NADPH and hence less available pre-cursors for aflatoxin production. Another possible reason for the lower level of aflatoxin detected in air packaged samples using the low barrier film AS111, is the higher level of N_2 formed in

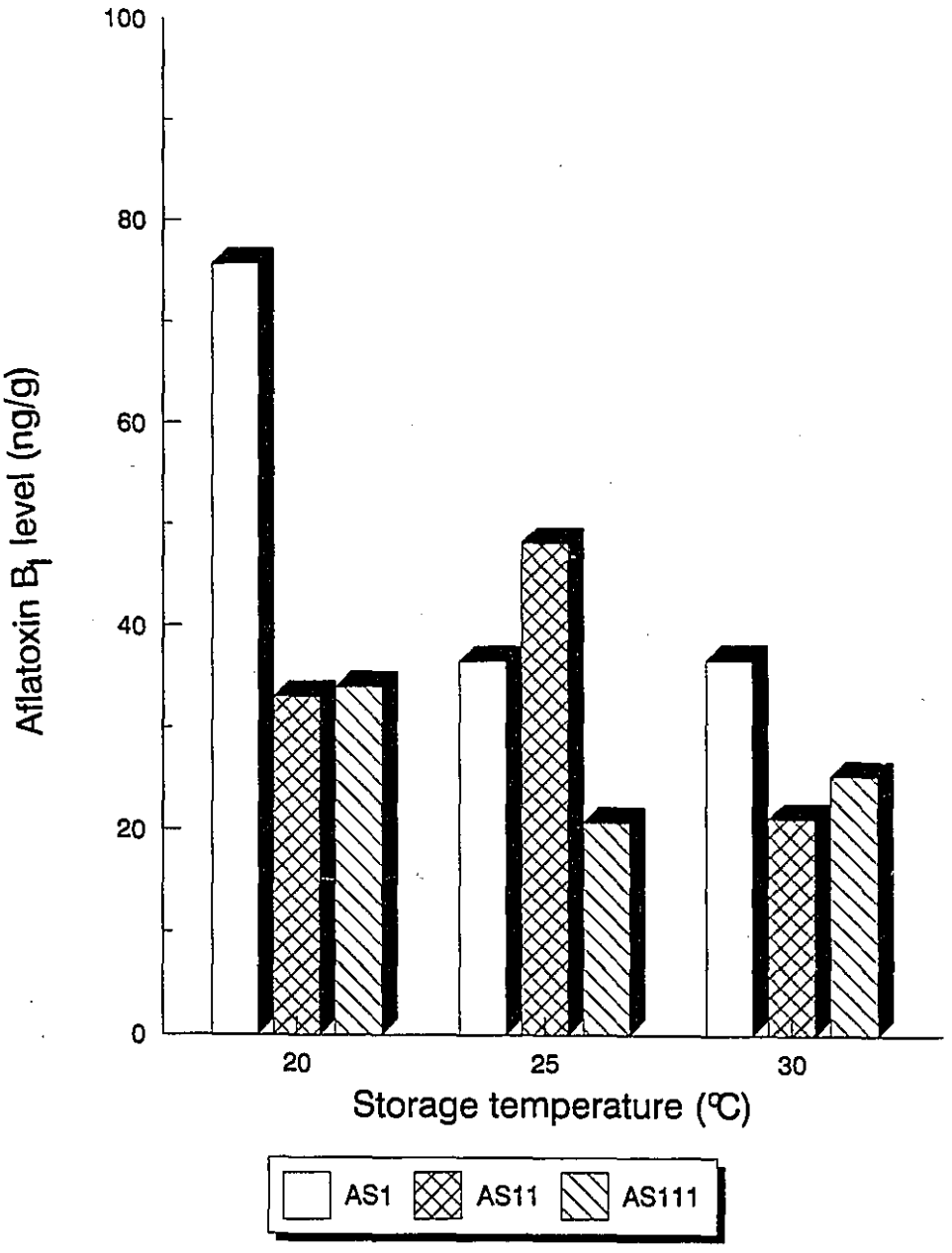


Figure 42 Effect of temperature and packaging film on aflatoxin B_1 production in air

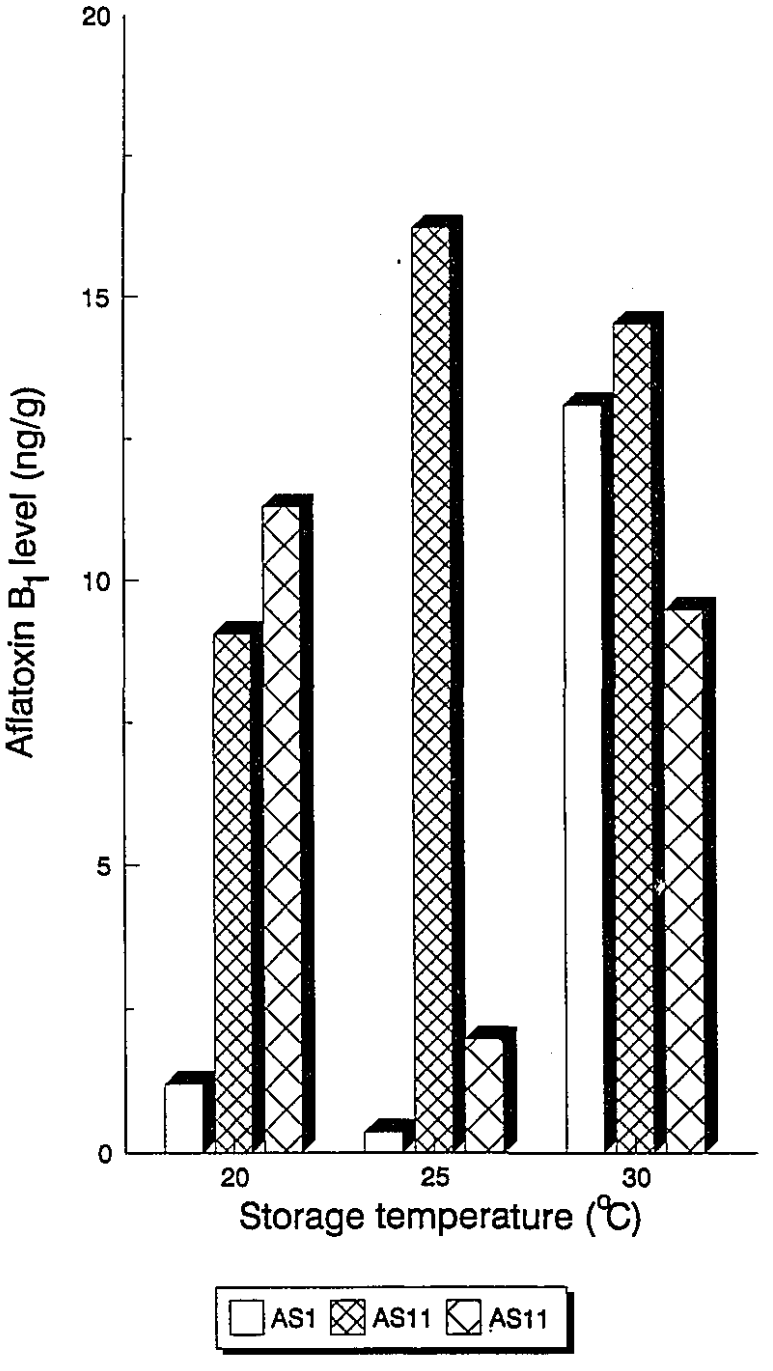


Figure 43 Effect of temperature and packaging film on aflatoxin B_1 production under MAP conditions

the package headspace at all temperatures but particularly 30°C (Figures 36-38). Fabbri et al., (1980) found that N_2 enriched atmosphere markedly inihibited the level of aflatoxin B_1 and B_2 detected in stored wheat. The depressant effect of N_2 on aflatoxin production was also reported by Jackson and Press (1967) on their studies with peanuts. It appears from these results that packaging film permeability plays a significant role in the level of aflatoxin production in air packaged samples. To minimize the risks of toxigenesis in air packaged peanuts, products should be packaged in low barrier films to both O_2 and CO_2, particularly in tropical storage conditions.

The level of aflatoxin B_1 detected in peanuts packaged in 60% CO_2 was well below the regulatory limit of 20 ppb. under all packaging conditions at all three storage temperatures. Extremely low levels of aflatoxin B_1 were detected in gas packaged peanuts using films AS1 and AS11 at 20°C and 25°C. However, higher levels of aflatoxin were detected at 30°C for all three films and in film AS11 at 20°C and 25°C.

It is evident from this study that gas packaging is an effective means of inhibiting aflatoxin B_1 production by *A. flavus*. To ensure the safety of products, gas packaged products should be packaged in low permeable films, i.e., AS1 and AS11. The levels of aflatoxin B_1 detected in this study were consistently lower than that of other studies (Wilson and Jay, 1975; Landers et al., 1967). This may be due to the nature of the different substrates used in the studies. However, it may also be due to the antimicrobial barrier effect of intact packaging materials (Fellows, 1988) as well as the inhibitory effects of increased levels of CO_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%. Wilson et al., (1977) also observed that low aflatoxin production in corn was due to the inhibitory effect of a high CO_2/low O_2 combination. Landers et al., (1967) also concluded that high concentrations of CO_2 rather than low concentrations of oxygen

Table 48. Analysis of variance for aflatoxin B_1 produced by *A. flavus* in air packaged peanuts.

Source	dF[a]	Sum of squares	Mean square	F
Model	8	6866.11	858.26	24.11***
Film	2	2454.37	1227.19	34.47***
Temp. ^{o}C	2	1797.51	898.75	25.24***
Film*Temp.	4	2614.23	653.55	18.36***
Residual(error)	18	640.86	35.60	
Total	26	7506.97		
R^{2b}	0.91			

[a] Degrees of freedom

[b] Coefficient of determination

Level of significance *** $p<0.001$

Table 49. Analysis of variance for aflatoxin B_1 produced by *A. flavus* in CO_2-enriched atmospheres

Source	dF[a]	Sum of squares	Mean square	F
Model	8	861.28	107.66	12.88***
Film	2	327.90	163.95	19.62***
Temp.^{o}C	2	197.60	98.80	11.82***
Film*Temp.	4	335.78	83.95	10.04***
Residual(error)	18	150.43	8.35	
Total	26	1011.71		
R^{2b}		0.85		

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p<0.001$.

Table 50. Least squares means (LSM) for the 3 films used in control studies for aflatoxin production by *A. flavus* on packaged peanuts

Film type	Packaging conditions	LSM	Std Err LSM
AS1	Air	49.59	1.99[a]
AS11	Air	34.14	1.99[a]
AS111	Air	26.69	1.99[a]
AS1	CO_2 / N_2	4.90	0.96[a]
AS11	CO_2 / N_2	13.25	0.96[a]
AS111	CO_2 / N_2	7.57	0.96[a]

[a] Level of significance (T-test) *** *P<0.001*

were the primary cause for inhibition of aflatoxin production under optimal conditions for mold growth.

Statistical analysis of the data (General linear model (GLM); Least square means (LSM)) on air packaged peanuts showed that the model used was highly significant ($P<0.001$) with a coefficient of determination of 0.91 (Table 48). The results also showed that temperature, film and the interaction between these two factors had a significant effect ($P<0.001$) on the level of aflatoxin B_1 produced by *A. flavus* in peanuts packaged in air. A comparison of the 3 films showed that, film AS1 was significantly different ($P<0.001$) from film AS11 and AS111 on its effect on toxin production in air packaged samples. However, the most suitable of the three films for lower aflatoxin B_1 production in air packaged samples was AS111 (Table 50).

Statistical analysis of the data (GLM, LSM) on CO_2 packaged peanuts show that, the effect of film, temperature and the interaction of these two factors on aflatoxin production were also significant ($P<0.001$) (Table 49). A comparison of the films showed that film AS1 differed significantly ($P<0.001$) from AS11, however there was no significant difference between films AS1 and AS111 with respect to aflatoxin production (Table 50). From the data, film AS1 is the most suitable of the three films under the experimental conditions studied to inhibit and limit the production of aflatoxin under a CO_2 enriched atmosphere.

7.4 CONCLUSION

This study shows that MAP conditions consisting of 60% CO_2:40% N_2 can be used to inhibit or limit the production of aflatoxin B_1 by *Aspergillus flavus* to levels below the regulatory limit of 20 ppb, particularly under high temperature storage conditions. However, it also demonstrates that the barrier performances of the packaging film plays an

important role in determining the activity of *Aspergillus flavus* on packaged peanuts. Furthermore, it confirms the fact that the efficiency of MAP is dependent on the barrier properties of the packaging material as was shown by the differences in the headspace gas compositions with time, specifically at higher storage temperatures.

CHAPTER 8

USE OF AGELESS OXYGEN ABSORBER TO CONTROL THE GROWTH OF AND AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* USING DIFFERENT PACKAGING FILMS

8.1 INTRODUCTION

Modified atmospheres have shown promise to control the growth and aflatoxin production by aflatoxigenic molds in packaged products. Atmosphere modification can be achieved through various techniques, such as vacuum packaging and gas packaging (Smith et al., 1991). These techniques are extensively used in the preservation and shelf life extension of several products such as pizza crust, sponge cake and other bakery products, crusty rolls (Smith et al., 1991) and pouched bread (Powers and Berkowitz, 1990). However, a novel technique for atmosphere modification involves the use of oxygen absorbents. These sachets, not unlike dessicators are placed inside the packaged product where they actively modify the headspace package composition. The ability of these absorbents to scavange oxygen depends on the free flow of oxygen around the absorbent and the permeability of the packaging film. This technology is simple and easy to use and does not demand a lot of complex packaging equipment. Hence it would be a useful technology for shelf life extension of products in developing countries, e.g., packaged peanuts. However, few studies have been done to determine the effects of oxygen absorbers on the growth of and the resultant toxin production by aflatoxigenic molds. Therefore, the objective of this study was to determine the effects of oxygen absorbers on growth and toxin production by *A. flavus* on peanuts using packaging films of different barrier characteristics.

8.2 MATERIALS AND METHOD

8.2.1. *Experimental design and statistical analysis*

Three multi-layered polymeric films, Cryovac film, PAXE and E_{50} films coded as AS1, AS11 and AS111 respectively were used in the study. Cryovac films were obtained from Cryovac, Montreal and PAXE and E_{50} films from Winpak, Winnipeg. The composition and oxygen transmission rates of these films have been reported in previous study, chapter 6. A randomized complete block design comprising of 3 films (AS1, AS11 and AS111), 3 temperatures (20°C, 25°C and 30°C) and 3 replicates per film per temperature was used as the experimental design in the study for each of the packaging conditions. Statistical analysis (analysis of variance and least square means) were computed using the Statistical Analysis System (SAS) programme on a McGill University mainframe.

8.2.2 *Preparation of spore inoculum*

Aspergillus flavus ATCC strain No.16872 was used throughout the study. The cultivation of the mold, harvesting and enumeration of spores were done according to section 7.2.2, chapter 7. Based on the count obtained (10^7 spores/ml), decimal dilutions were prepared to obtain the desired inoculum level of 10^4 spores/ml used in this study.

8.2.3 *Preparation and inoculation of substrate*

The preparation of peanuts, adjustment of a_w to 0.96 with glycerol and inoculation with *A. flavus* spores were done according to the procedures outlined in 7.2.3. of chapter 7.

8.2.4 *Packaging*

Peanuts were packaged under two different gaseous conditions, (1) in air and (2) air and an Ageless self reacting oxygen absorber (Type S-100). Packaging was done by placing plates containing peanuts in 210 x 210 mm bags of each of the packaging films and sealing with an impulse heat-sealer. For packages containing the Type S-100 oxygen absorber, two sachets per bag were placed in each bag prior to sealing. Packaged plates were then incubated at 25^{o}C and 30^{o}C in a Precision Gravity Convection incubators (model 2EG) (Baxter, Montreal Canada) and at 20^{o}C in a Labconco Scientific incubator (Mocon, Minneapolis, Minnesota) for 15 days.

8.2.5 *Headspace gas analysis*

At day 0 and after each storage interval, samples were analysed for changes in headspace gas composition. Gas sampling and analysis by gas chromatography were done as described in section 7.2.5. of chapter 7.

8.2.6 *Color measurement*

After 15 days of storage, peanuts were evaluated visually for changes in color of the peanuts for each package using a 5-point scale. Peanuts immediately after sterilization were again used as the reference standard.

8.2.7 *Extraction and quantitation*

The extraction and quantitation of aflatoxin B_1 at the end of the 15 day storage period was carried out according the procedures described in section 7.2.7 of chapter 7. Concentration of aflatoxin B_1 was calculated by comparison to standard peaks of known concentration of aflatoxin B_1 as outlined in the Beckman System Gold methodology.

8.3 RESULTS AND DISCUSSION

The combined effect of headspace gas composition, film permeability and storage temperature on the growth of and aflatoxin production by *A. flavus* are shown in Table 51 and Figures 44-51. No signs of mold growth were visible in peanuts packaged in air using film AS1 and AS11 at 20°C. However, growth was visible in peanuts packaged in these films stored at 25°C and 30°C. Visible growth was observed in all peanuts packaged in film AS111 in air samples, irrespective of storage temperatures with extensive mycelial growth occurring at 30°C.

Similar observations were reported for air and gas packaged samples in the previous study. This lack of visible growth was again attributed to elevated levels of headspace CO_2 produced during the aerobic metabolism of *A. flavus* in the air packaged samples and the high-medium barrier characteristics of film AS1 and film AS11 to CO_2, particularly at lower storage temperature.

When an O_2 absorbent was used with these films, no mycelial growth was observed at all storage temperatures, with the exception of peanuts packaged in film AS1 and incubated at 30°C. The inhibition of growth of *A. flavus* in peanuts packaged with an O_2 absorbent is due to the oxygen scavenging potential of the self working absorbent used (Type S) and the low residual concentration of headspace oxygen. However, the growth of *A. flavus* in peanuts packaged with an O_2 absorbent in film type AS111 can be attributed to residual O_2 present in the package (1-2%) due to the inability of the absorbent to scavenge the oxygen trapped in the peanuts and also due to the high permeability of the film to O_2. Several studies have shown

Table 51. Application of oxygen absorbers in the control of aflatoxin production by *A. flavus* under different packaging conditions using different packaging films

Film	Temperature	Packaging conditions	Color score[b]	Aflatoxin B_1 conc. (ng/g)[b]
AS1	20°C	Air	1.8(0.1)	70.75(2.00)
	25°C	Air	2.0(0.0)	48.25(2.80)
	30°C	Air	2.8(0.1)	58.96(2.43)
AS11	20°C	Air	2.0(0.0)	40.34(0.50)
	25°C	Air	2.3(0.1)	47.80(2.46)
	30°C	Air	2.7(0.2)	31.52(1.02)
AS111	20°C	Air	2.0(0.0)	43.68(0.84)
	25°C	Air	2.7(0.1)	37.40(1.20)
	30°C	Air	3.8(0.1)	42.70(1.52)
AS1	20°C	Air / O_2 absorber	3.0(0.29)	0.19(0.02)
	25°C	Air / O_2 absorber	3.0(0.0)	20.82(0.61)
	30°C	Air / O_2 absorber	3.1(0.06)	14.50(1.01)
AS11	20°C	Air / O_2 absorber	2.3(0.1)	0.29(0.03)
	25°C	Air / O_2 absorber	2.9(0.06)	20.94(2.75)
	30°C	Air / O_2 absorber	3.3(0.1)	0.99(0.19)
AS111	20°C	Air / O_2 absorber	3.8(0.1)	0.37(0.06)
	25°C	Air / O_2 absorber	3.7(0.1)	0.22(0.04)
	30°C	Air / O_2 absorber	4.7(0.2)	0.30(0.01)

[b] Values in brackets represent the standard errors

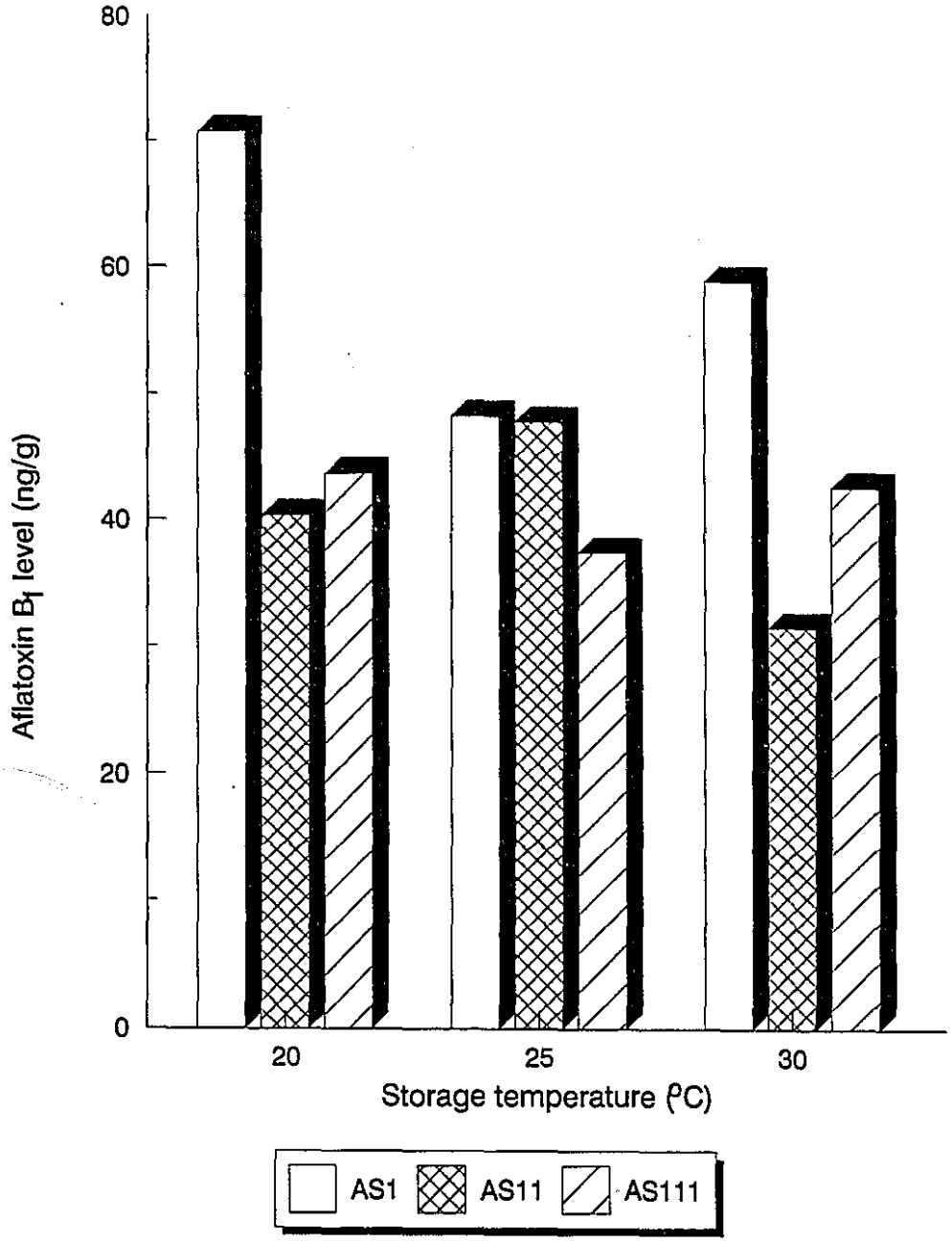


Figure 44 Aflatoxin B_1 level in air packaged peanuts

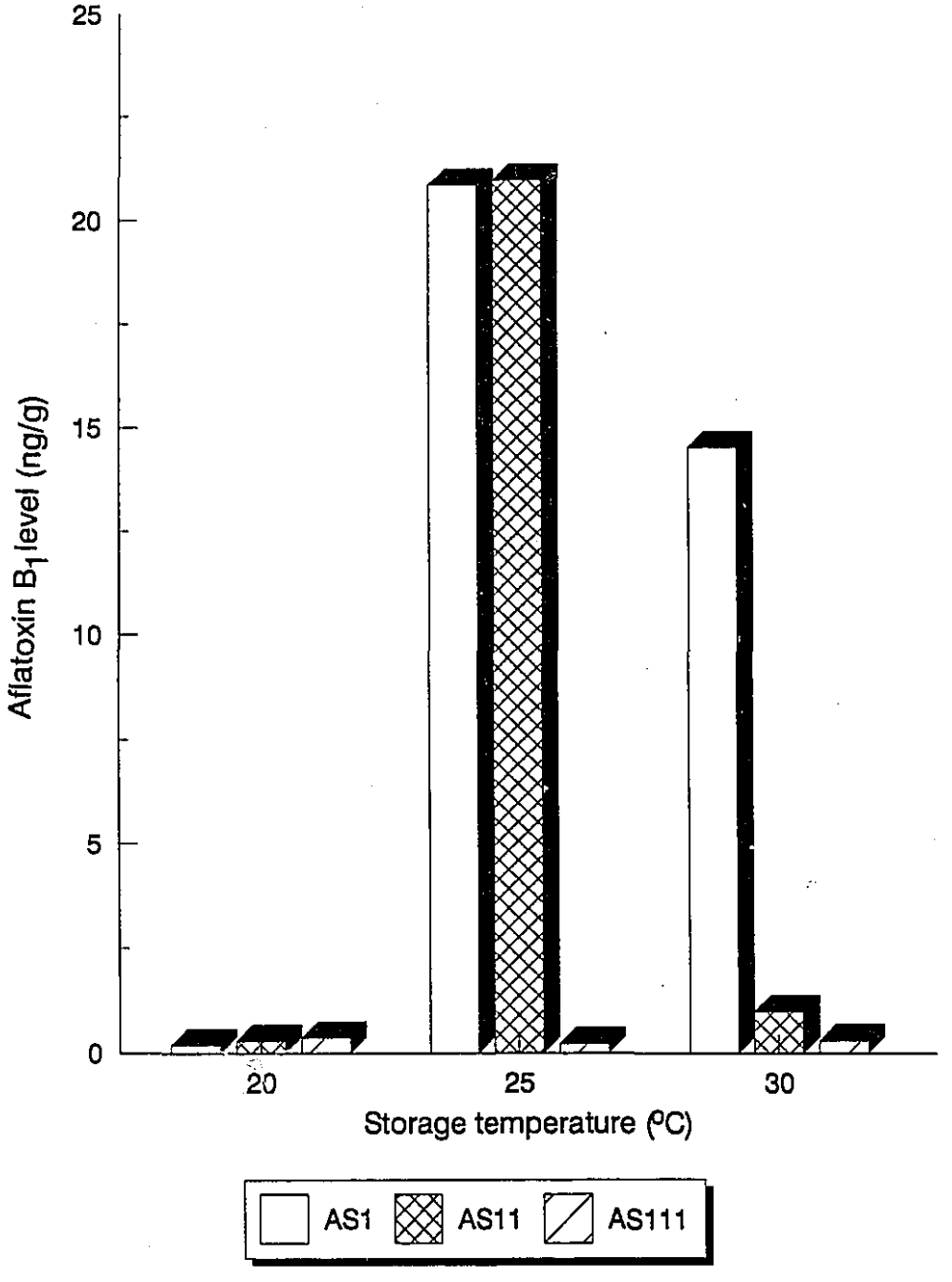


Figure 45 Aflatoxin B_1 level in absorbent packaged peanuts

that molds require very low residual oxygen levels to grow and produce aflatoxins (Miller and Golding, 1949; Wilson et al., 1975). Another possible reason may be that the absorbent was saturated and therefore had lost its absorbing capacity. Therefore, the continued influx of oxygen across the low barrier film, which is enhanced at higher storage temperatures, would result in gradual increase in haedspace oxygen creating favourable conditions for the growth of *A. flavus*, particularly at 30°C.

8.3.1 *Changes in headspace gas composition*

Changes in the levels of headspace O_2, CO_2 and N_2 for air packaged peanuts for the 3 different films at the 3 incubation temperatures are shown in Figures 46-48. Initial headspace O_2 decreased to 1% or less within 1-2 days depending on the storage temperature and the packaging film. While it remained at this level in peanuts packaged in film AS1 and AS11, it fluctuated in peanuts packaged in AS111 reaching levels of 10-15% in some cases. Headspace CO_2 increased initially throughout storage as a result of mold metabolism and remained at a fairly constant level of 40-60% in air packaged peanuts in films AS1 and AS11. There was no significant difference in changes in headspace gas composition for films AS1 and AS11 at each of the 3 storage temperatures. However, there was a significant difference in headspace gas composition for film AS111 compared to AS1 and AS11 at all the storage temperatures and a significant difference in the headspace gas composition of peanuts packaged in film AS111 at the various storage temperatures. The lower headspace CO_2 and slightly higher residual O_2 concentration in peanuts packaged in films AS111 is due most likely to the lower gas barrier characteristics of this film.

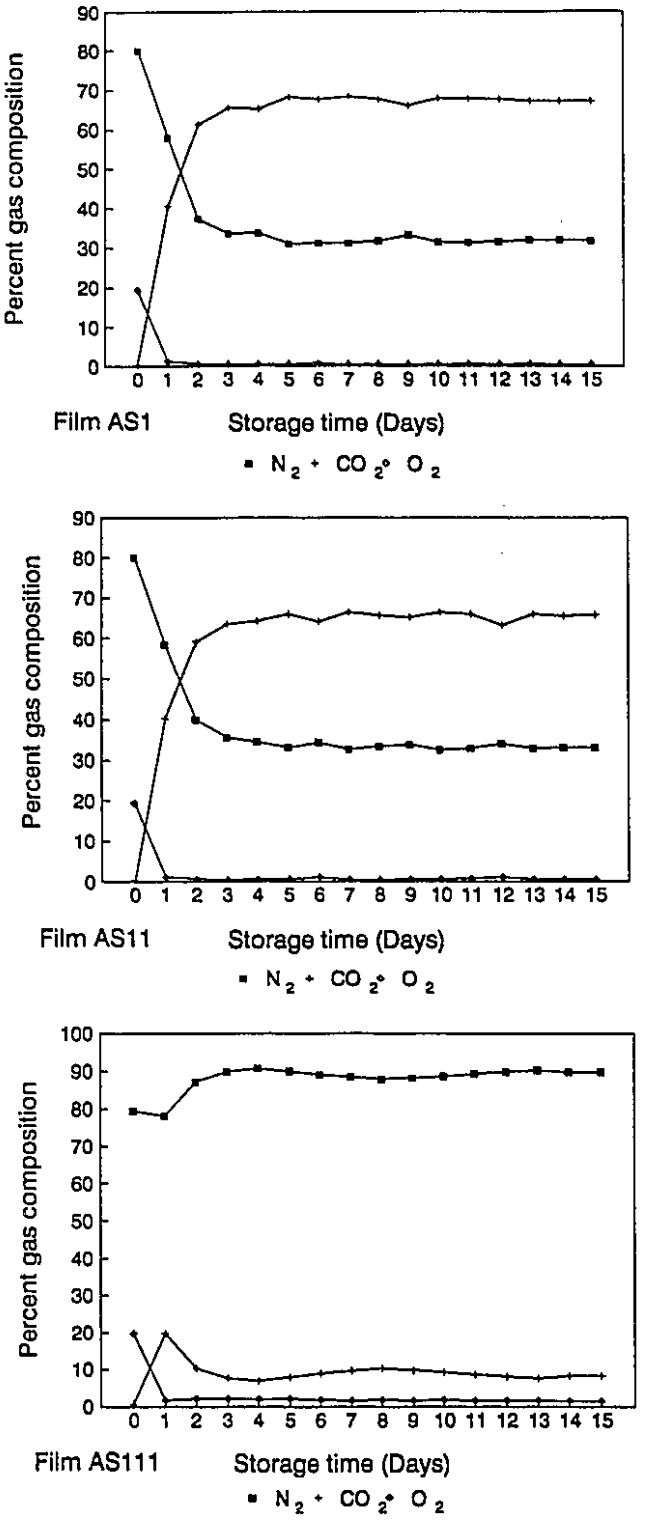


Figure 46 Headspace gas changes with time for three packaging films at 20℃ in air

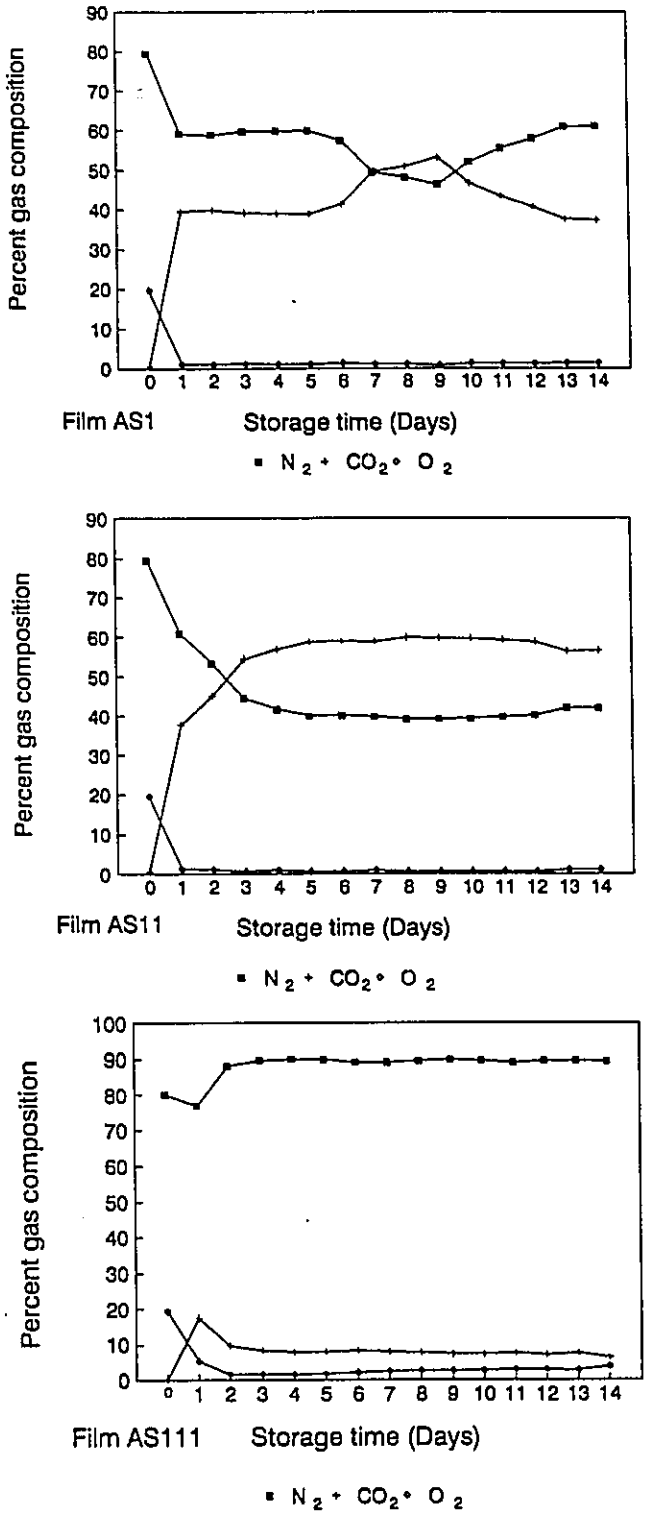


Figure 47 Headspace gas changes with time for three packaging films at 25°C in air

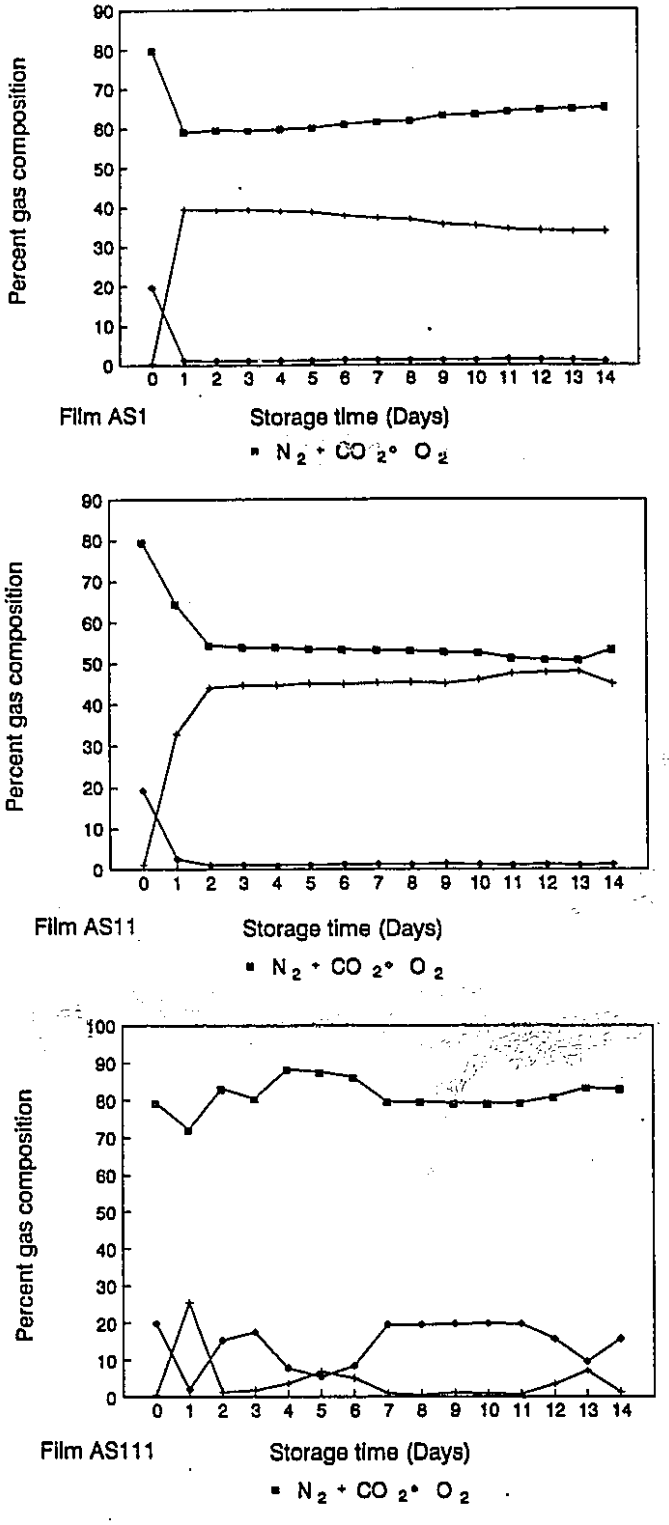


Figure 48 Headspace gas changes with time for three packaging films at 30°C in air

Headspace gas changes for peanuts packaged with an oxygen absorbent are shown in Figures 49-51. Headspace O_2 decreased to less than 1% or 1-2% within 24hrs with a gradual increase or no increase in headspace CO_2 depending on the packaging film and the incubation temperature (Figures 49-51). The decrease in headspace O_2 is again due to the oxygen scavenging capacity of the Type S-100 O_2 absorbers at 20-30^{o}C. There was a gradual increase in headspace CO_2 in film AS1 after 6 days and 1 day of storage at 20^{o}C and 30^{o}C respectively, followed by a gradual decrease in headspace CO_2 at 30^{o}C after day 8. The initial increase in CO_2 concentration may be due to the residual O_2 present in package resulting in mold metabolism. The decrease in CO_2 at 30^{o}C may be due to its loss through the packaging film or its incorporation in aflatoxin biosynthesis. Similar observations were noted at 25^{o}C and 30^{o}C for film AS11, with headspace CO_2 increasing to about 20% at 25^{o}C but only to 10% at 30^{o}C. With respect to film AS111, the trend was similar at 20^{o}C and 25^{o}C. However, a higher concentration of CO_2 was observed for film AS111 at 30^{o}C. In general, the results shows that in the application of O_2 absorbers, resulted in a low O_2 - N_2-rich atmosphere being created with headspace N_2 ranging between 70% and 95% after 24 to 48 hours.

8.3.2 *Color change*

The color change in packaged peanuts, are summarized in Table 51. A color score of > 2.5 was agian used as the standard of acceptability. A similar trend was observed for all air packaged samples in this study compared to results observed previously in gas packaging studies, i.e., peanuts were rejected on the basis of their color score when packaged in high-medium barrier films at higher storage temperature (30^{o}C) and at lower temperature (25^{o}C) in lower barrier films (AS111). However, the results with the oxygen absorbers were interesting. In all cases, a

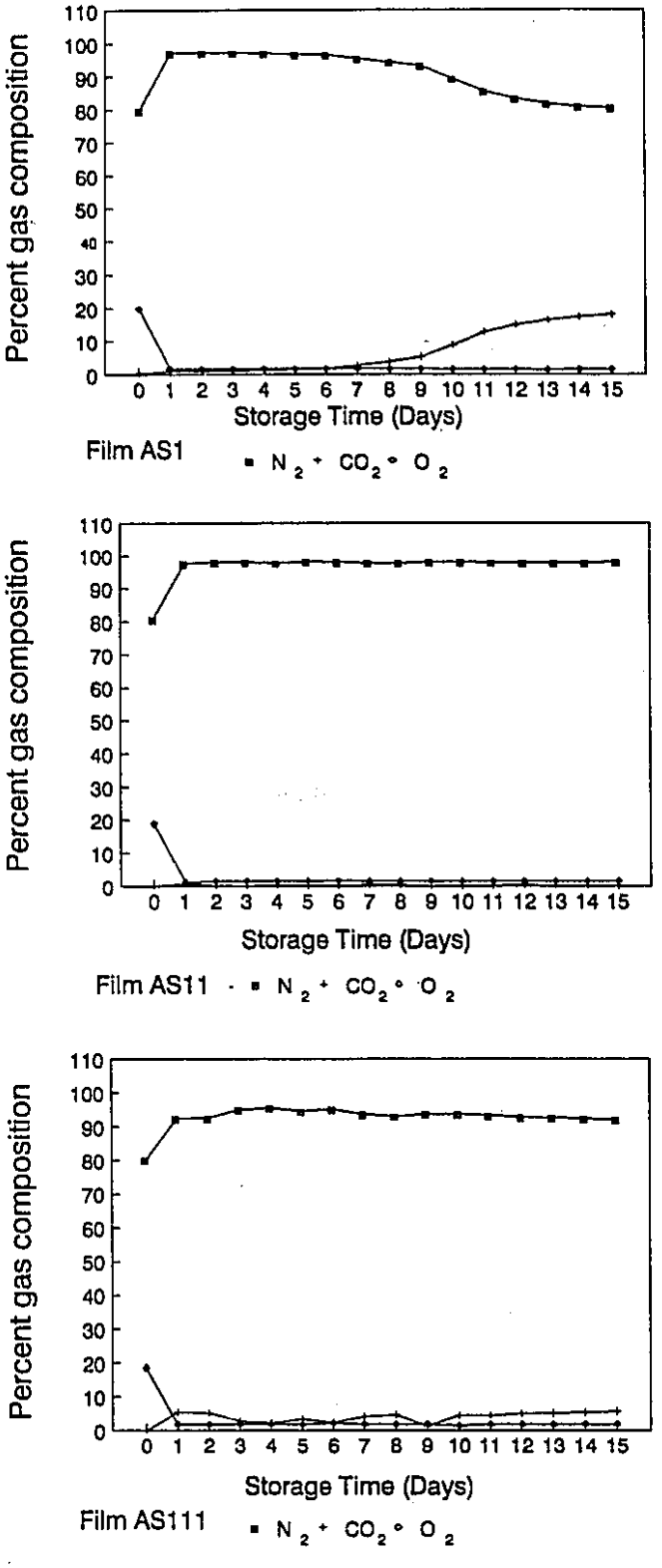


Figure 49 Changes in headspace gas composition with time for peanuts packaged with oxygen absorbent at 20°C.

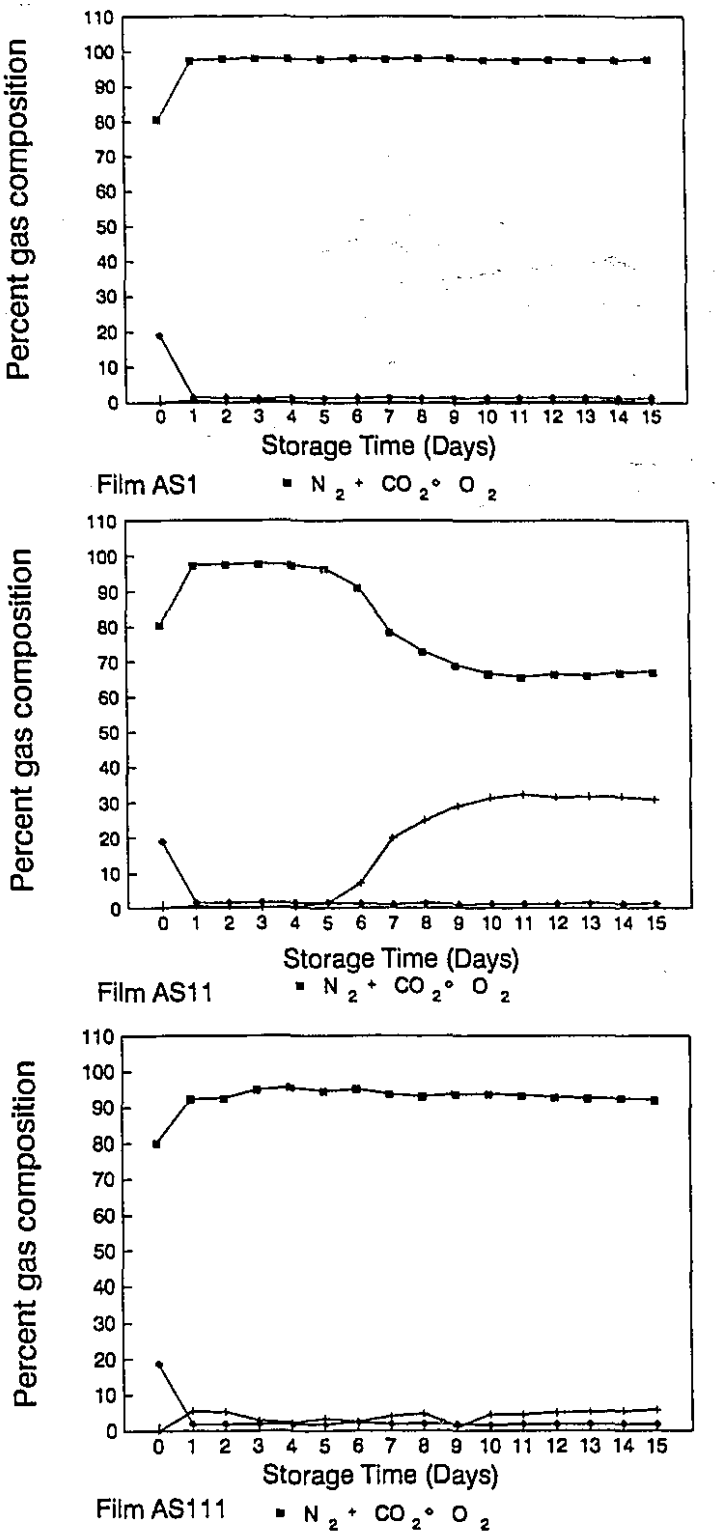


Figure 50 Changes in headspace gas composition with time for peanuts packaged with oxygen absorbent at 25°C.

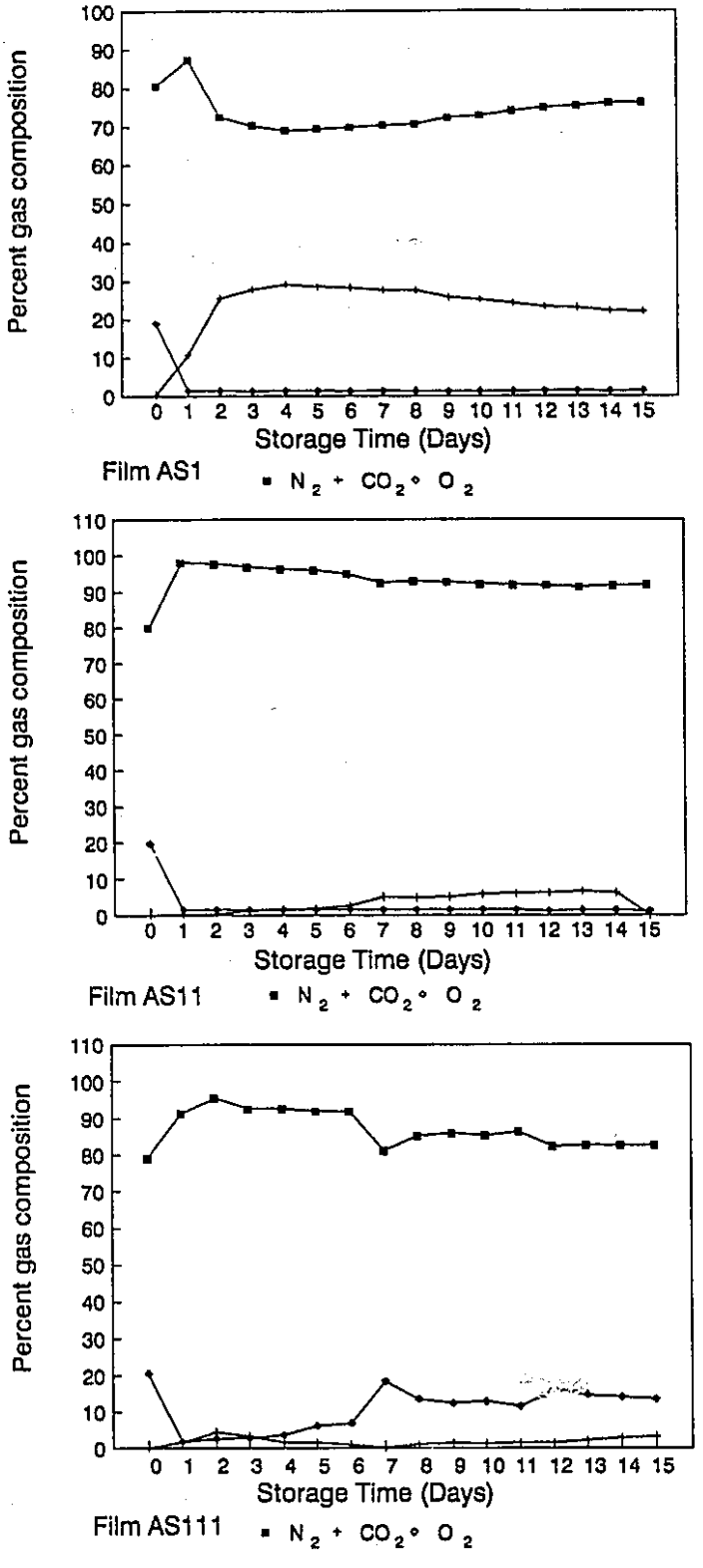


Figure 51 Changes in Headspace gas composition with Time for peanuts packaged with oxygen absorbent at 30°C

high degree of browning was observed in all peanuts, at all temperatures, particularly those stored in low barrier films. Indeed, all absorbent packaged peanuts were rejected on the basis of their color score (> 2.5). These results were opposite to the trend reported for peanuts packaged in CO_2:N_2 (60:40) gas mixture. Therefore, while atmosphere modification by gas packaging and oxygen absorbent, both controlled aflatoxin production to the same extent, gas packaging had a more pronounced effect in controlling discoloration than oxygen absorbents. One possible explanation is the dissolution of CO_2 in the peanuts which may reduce pH and slightly inhibit lipase activity. Another reason may be that the Type S oxygen absorbents also generate hydrogen gas and this may react with oxygen to produce water and increase product a_w. Studies have shown that browning rate is affected by available water in two ways (1) accelerating it by increasing the mobility of the substrates to the enzyme, or (2) decreasing the rate of browning due to dilution of the substrate. Between a water activity range of 0.65-0.85, the mobility factor predominates but above this value the dilution factor predominates, thereby reducing the rate of browning. However, these processes are affected by other factors such as the packaging conditions and the presence or absence of other deteriorative agents such as microorganisms.

8.3.3 *Aflatoxin B_1 production*

The levels of aflatoxin B_1 produced under the various gaseous/packaging/storage conditions is shown in Table 51 and Figures 44-45. The level of aflatoxin produced by *A. flavus* in air packaged peanuts was always greater than the regulatory limit of 20 ppb. The highest level of aflatoxin was again detected in film AS1 which had the highest barrier characteristics and the lowest level of aflatoxin was detected in peanuts packaged in films with low barrier

characteristics to oxygen and carbon dioxide (AS111) (Table 51; Figure 44). These results were similar to previous observations for air packaged control samples in gas packaging studies. The level of aflatoxin B_1 detected in peanuts packaged with an oxygen absorbent are shown in Table 51 and Figure 45. Low levels of aflatoxin were detected in absorbent packaged peanuts in films AS1 and AS11 at 20°C and 30°C respectively (Figure 45). Again, this is due to the low residual headspace oxygen and the nitrogen enriched atmosphere produced by the oxygen scavengers, conditions which are not conducive to aflatoxin production. However, absorbent packaged peanuts in high-medium barrier films AS1 and AS11 produced higher levels of aflatoxin at 25°C compared to 20°C. A similar trend was observed at 30°C for film AS1 under the same packaging conditions. These results were surprising and can be attributed to the presence of residual oxygen in these packages and the higher concentration of CO_2 produced as a result of mold metabolism. Magan and Lacey (1984) observed a stimulatory effect of 5-10% CO_2 on mold growth and aflatoxin production at low O_2 concentration (< 1%). These authors postulated that the beneficial action of CO_2 was due to carbon dioxide fixation resulting from the enhanced nutritional requirements of fungi under virtually anaerobic conditions. Peanuts packaged with oxygen absorbents in film AS111 produced the lowest levels of aflatoxin (< 1 ng/g) which was consistent with the trend for gas packaged peanuts in the low barrier film AS111. The low level of aflatoxin detected can be attributed to the saturation of the absorbent and the influx of oxygen resulting in the extensive mold growth observed in these peanuts during storage. Ellis et al., (1993) have shown a curvilinear relationship between growth and aflatoxin production implying that toxin production increases to an optimum and then starts to decrease with increasing mold growth due to the utilization of aflatoxin for further mycelial development. Studies have also shown that aflatoxin production is markedly inhibited in an N_2 enriched atmosphere, also observed in this study

Table 52. Analysis of variance for aflatoxin B_1 produced by *A. flavus* in oxygen absorbent packaged peanuts.

Source	dF[a]	Sum of squares	Mean square	F
Model	8	2014.43	251.80	16.22***
Film	2	518.66	259.33	16.70***
Temp. (^{o}C)	2	914.79	457.40	29.46***
Film*Temp.	4	580.98	145.24	9.36***
Residual(error)	18	279.44	15.52	
Total	26	2293.87		
R^{2b}	0.87			

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p < 0.001$.

Table 53. Least square means (LSM) for 3 films used in oxygen absorbent packaging of peanuts.

Film type	Packaging conditions	LSM	Std. Err. LSM
AS1	Oxygen absorbent	10.78	1.31[a]
AS11	Oxygen absorbent	7.53	1.31[a]
AS111	Oxygen absorbent	0.30	1.31[a]

[a] Level of significance (T - test) $p < 0.001$.

with film AS111 due to the depressant effect of nitrogen on aflatoxin production (Fabbri et al., 1980; Jackson and Press, 1967).

Statistical analysis of the data (GLM, LSM) showed that the type of film as well as the storage temperature had a significant effect ($P<0.001$) on the final concentration of aflatoxin B_1 produced by *A. flavus* in air packaged peanuts. The model used was highly significant with a correlation coefficient of 0.91. The data also showed that film AS1 was significantly different ($p<0.05$) from films AS11 and AS111 on its effect on aflatoxin production. However, the most suitable of the three films for lower toxin production in air packaged samples was film AS11. Similar results were obtained for absorbent packaged peanuts, with the model having a coefficient of determination of 0.87 (Table 52). However, the effect of film AS1 on toxin production was significantly different from that of film AS111 but was not significantly different from film AS11. Statistically, film AS111 was the most suitable for inhibiting aflatoxin production (Table 53). However, the results show that this film does not inhibit growth of *A. flavus* even in the presence of O_2 absorbent which may be a factor for lower toxin production. Therefore, for the inhibition of both growth and toxin production by *A. flavus* film AS11 would be the most suitable for application with oxygen absorbents.

8.4 CONCLUSION

In general, the level of aflatoxin produced using O_2 absorbent was either equal to the regulatory level or far below the regulatory limit of 20 ppb implying that the application of O_2 absorbents could be a simple and useful tool to control aflatoxin production. However, the results also show that the efficiency of O_2 absorbents to control aflatoxin production depends on the barrier characteristics of the film, the storage temperature and the tolerance of *A. flavus* to low levels of O_2 and CO_2.

CHAPTER 9

USE OF DIFFERENT TYPES OF OXYGEN ABSORBENTS TO CONTROL THE GROWTH OF AND AFLATOXIN PRODUCTION BY *ASPERGILLUS PARASITICUS* IN PACKAGED PEANUTS

9.1 INTRODUCTION

Aflatoxins are potent hepatotoxic and carcinogenic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus* (CAST, 1989). These molds invade, and grow on, a wide variety of food commodities such as peanuts, corn and cottonseed, with the resulting contamination of aflatoxin often making the commodity unfit for consumption. Several methods have been used to control growth and aflatoxin production by these molds. However, of these methods modified atmospheres have shown promise as a potential method to control the growth and consequently aflatoxin production by aflatoxigenic molds. Sanders et al., (1968) reported that little aflatoxin was found on inoculated peanuts maintained under modified atmospheres compared to the levels detected in peanuts stored in air at 25^{o}C and at 90% relative humidity. Similar results were reported by Landers et al., (1967). Wilson and Jay (1975) also reported lower levels of aflatoxin in high moisture corn stored under controlled atmosphere (13.5% CO_2, 0.5% O_2 and 84.8% N_2).

Atmospheric modification of the microenviroment surrounding a product can be achieved in several ways. Vacuum packaging and gas packaging are two methods commonly used to modify the atmosphere within a packaged food product. However, the limitation of these methods of atmosphere modification is that it is sometimes difficult to completely evacaute the air trapped insde a product resulting in low levels of residual oxygen in the package headspace. Several studies have shown that aerobic microorganisms, such as bacteria and

molds can tolerate and grow in low levels of O_2 resulting in spoilage problems. Possibly the only means to reduce the levels of oxygen sufficiently in products is to use oxygen absorbents. This is a technique which is gaining wide application. This is a unique technique for the preservation of food since deterioration in food quality is caused chiefly by the presence of oxygen, implying that the removal of oxygen can preserve food quality (Harima, 1990). This technique is less expensive, and easier to implement compared to vacuum and gas flushing techniques and could be a viable method to extend the shelf life and microbiological quality/safety of specific food products especially in tropical countries which lack a refrigerated distribution chain. Oxygen absorbents have been applied in the shelf-life extension of several food products such as cakes and snack foods (Minakuchi and Nakamura, 1990), pizza crust, salami, bakery products and MRE (meals ready to eat) pouch-packed breads (Powers and Berkowitz, 1990).

Recently, a new type of self working oxygen absorbent Ageless type G has been marketed in Japan. This absorbent in addition to scavenging oxygen also generates an equivalent amount of carbon dioxide. Therefore, in view of the previous observations, that color of peanuts was superior in gas packaged peanuts compared to peanuts packaged in oxygen absorbents alone, ageless type G absorbents may have the potential to inhibit aflatoxin production by *A. flavus* and *A. parasiticus* and at the same time enhance the color of the stored peanuts. However, few studies have been done in the application of oxygen absorbents or oxygen absorbent-carbon dioxide generators to control aflatoxin in food by *A. parasiticus*. Therefore the objective of this study was to determine the combined effect of two different types of oxygen absorbents, storage temperature and packaging film barrier characteristics on the growth of and aflatoxin production in peanuts by *Aspergillus parasiticus*.

9.2 MATERIALS AND METHODS

9.2.1 *Experimental design and statistical analysis*

Two multi-layered polymeric film, Cryovac film (high barrier) and E_{50} film (lower barrier) coded as AS1 and AS111 respectively were used throughout the study. Only two films were chosen in this study due to the similar barrier characteristics of the Cryovac film (AS1) and PAXE film (AS11) at higher temperatures. The composition and gas transmission rates of these films have been reported previously (chapter 6). A randomized complete block design comprising of 2 films (AS1 and AS111), 3 temperatures (20°C, 25°C and 30°C) and 3 replicates per film per temperature was used in the study for each of the packaging conditions. Statistical analysis (analysis of variance and least square means) were computed using the Statistical Analysis System (SAS) programme on a McGill University mainframe.

9.2.2 *Preparation of spore inoculum*

Aspergillus parasiticus ATCC strain No. 26804 obtained from ATCC, Rockville, Maryland, U.S.A. was used throughout the study. Cultivation of *A. parasiticus*, harvesting and enumeration of the mold spores were all done according to procedures described in section 8.2.2 of chapter 8. Based on the count obtained (10^8 spores/ml), decimal dilutions were prepared to obtain the desired inoculum of 10^4 spores/ml used throughout the study.

9.2.3 *Preparation and inoculation of substrate*

Peanuts were obtained from Provigo, Montreal. The peanuts were prepared, adjusted to a_w of 0.96 and innoculated with 10^4 spore/g of *A. parasiticus* as described previously.

9.2.4 *Packaging*

Peanuts were packaged under three different gaseous conditions, (1) in air, (2) in air with an immediate effect type oxygen absorber (Type S_{100}) and (3) in air with an oxygen absorber (Type G_{100}) which generates CO_2 equivalent to the amount of O_2 absorbed. Packaging was done by placing plates containing peanuts in 210 x 210mm bags of each of the packaging films and sealing with an impulse heat-sealer. For packages containing the Types S_{100} and G_{100} O_2 absorbents, two sachets per bag of each absorbent were placed in the each appropriate bag prior to sealing. Packaged plates were then incubated at 25°C and 30°C in Precision Gravity Convection incubators (model 2EG) and at 20°C in a Labconco Scientific incubator for 15days.

9.2.5 *Headspace gas analysis*

Headspace gas analysis was done immediately after packaging to determine the initial headspace gas composition and to monitor changes in its composition throughout the 15 days storage period. Gas sampling and analysis of gases by gas chromatography were as described in section 8.2.5 of chapter 8.

9.2.6 *Color measurement*

After 15 days of storage, peanuts were evaluated visually for changes in color for each package using a 5-point scale. Fresh peanuts were used as a reference standard after sterilization.

9.2.7 *Extraction and quantitation*

The extraction and quantitation of aflatoxin B_1 from peanuts were all done according to the procedures described in section 8.2.7 of chapter 8. Concentration of aflatoxin B_1 was calculated by comparison to standard peaks of known concentration of aflatoxin B_1 as outlined in the Beckman System Gold methodology.

9.3 RESULTS AND DISCUSSIONS

In general, oxygen is a major factor in the deterioration of food quality especially when the water activity of the food is high. Such food products are more susceptible to mold growth and spoilage. Therefore, the removal of oxygen from the microenvironment within a packaged product may be one method to inhibit the growth and resultant deterioration of food products by aerobic microorganisms, such as molds. Oxygen scavengers have proved to be effective in many ways in the preservation and shelf-life extension of most food products such as pizza crust, sponge cake and other bakery products and crusty rolls (Smith et al., 1991).

The results of the combined effect of packaging films, Ageless oxygen absorbents and oxygen absorbents/carbon dioxide generators on the production of aflatoxin B_1 by *A. parasiticus* is shown in Table 54. After 15 days incubation, there was no observable mycelial growth in air packaged peanuts with film AS1. However, under the same conditions using film AS111, all packaged peanuts showed extensive mold growth. Similar observations were made with peanuts packaged in air with the Type S and Type G oxygen absorbents for both films, i.e., no growth in peanuts packaged with film AS1 and visible mold growth in peanuts packaged in film AS111.

These observations can be attributed to the different gas transmission properties of the two packaging films used in the study. Previous studies have shown that at all the temperatures under study film AS111 has low barrier properties to oxygen and carbon dioxide while film AS1 is a high barrier below 23^{o}C, while above this temperature it behaves as a medium barrier film. The availability of air to the packaged peanuts has a significant effect on the growth state of *A. parasiticus* and can adversely affect the microbiological and ecological processes of the organism. Since *A. parasiticus* is aerobic, the presence of air enhances its

Table 54. Effect of different types of O_2 absorbers, packaging films and temperature on aflatoxin production by *A. parasiticus*.

Film type	Temperature	Packaging conditions	Color score[b]	Aflatoxin B_1 conc. (ng/g)[b]
AS1	20°C	Air	3.83 (0.1)	22.08 (0.41)
	25°C	Air	4.17 (0.1)	23.51 (2.10)
	30°C	Air	4.00 (0.1)	13.89 (0.50)
AS111	20°C	Air	4.83 (0.1)	52.95 (0.51)
	25°C	Air	5.00 (0.0)	18.23 (0.74)
	30°C	Air	5.00 (0.0)	17.59 (0.44)
AS1	20°C	Air / O_2 absorber	2.33 (0.1)	0.29 (0.08)
	25°C	Air / O_2 absorber	3.17 (0.2)	0.69 (0.23)
	30°C	Air / O_2 absorber	3.83 (0.1)	1.31 (0.43)
AS111	20°C	Air / O_2 absorber	3.67 (0.1)	2.07 (0.09)
	25°C	Air / O_2 absorber	4.00 (0.0)	20.01 (0.82)
	30°C	Air / O_2 absorber	4.75 (0.1)	15.94 (0.72)
AS1	20°C	Air / O_2 - CO_2 generator	1.83 (0.1)	17.59 (0.23)
	25°C	Air / O_2 - CO_2 generator	2.83 (0.1)	2.81 (0.52)
	30°C	Air / O_2 - CO_2 generator	3.00 (0.0)	0.28 (0.04)
AS111	20°C	Air / O_2 - CO_2 generator	5.00 (0.0)	12.61 (0.89)
	25°C	Air / O_2 - CO_2 generator	4.83 (0.1)	16.32 (0.54)
	30°C	Air / O_2 - CO_2 generator	5.00 (0.0)	1.94 (0.05)

() - Standard error.

[b] - Mean of 3 replicates.

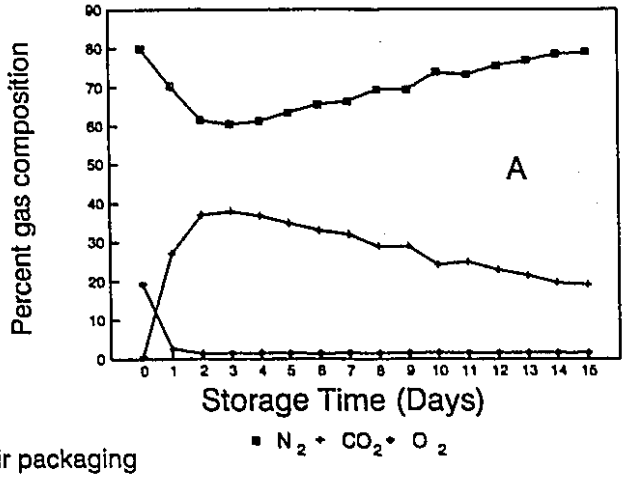
growth and hyphal development. If the amount of O_2 is reduced and CO_2 accumulates, the viability of *A. parasiticus*, and its ability to attack the peanut tissues is suppressed (Paster, 1990). In air packaged peanuts using film AS1, the high concentration of headspace CO_2 and N_2 may be the contributory factors for reduced visible growth observed. After 24hrs of incubation, the microenvironment within the package is different from air, i.e., it behaves as a modified atmosphere with a high headspace N_2 and CO_2 concentration with a low residual level of O_2. Wells and Payne (1980) in their studies with pecan kernels reported that the number of fungal colonies isolated in an atmosphere of 21%O_2 : 3%CO_2 at 21°C was significantly lower than in air. However, increasing the CO_2 level to 30% resulted in a further decrease in colony counts. A low Oxygen concentration of 1% alone had no effect on mold survival, however, when combined with 30% CO_2, the level of the fungal counts was significantly lowered.

For peanuts packages with Ageless Type S oxygen absorbents, the lack of visible growth in peanuts packaged in film AS1 is due to the depletion of the headspace oxygen within 24hrs by the Oxygen scavenger, creating a N_2 rich environment after 24hrs. Studies have shown that very low O_2 and high N_2 atmospheres have a fungistatic/delaying effect on the growth of some molds including *Aspergillus flavus* (Serafini et al., 1980). The observations with Ageless absorbent Type G is also due to the depletion of headspace O_2 by this scavenger absorbent and the high concentration of headspace CO_2 formed by this dual purpose absorbent. This synergism between atmospheric gases results in an extension of the latent period of mold growth in the packaged peanuts (Wilson and Jay, 1975). Peanuts packaged with film AS111 under all three packaging conditions showed observable growth at all incubation temperatures. Mold growth under these packaging conditions is probably due to the low barrier properties of the film AS111 and hence, the high level of residual O_2 (>1%) within the package headspace throughout the incubation period. When the oxygen absorbent becomes saturated, its oxygen absorbing capacity is lost. When this happens, any influx of oxygen through the film will result in an increase in the package headspace O_2

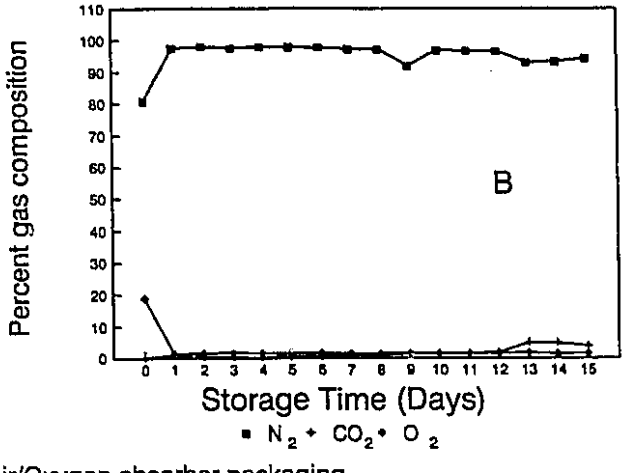
concentration. Wilson et al., (1975) reported that the growth of *A. flavus* and *F. moniliforme* on corn was not arrested in atmospheres containing 0.5%O_2 concentration although deterioration of the grain was delayed. Miller and Golding (1949) reported that the amount of dissolved O_2 required for normal growth of various species of molds is exceedingly low and that the growth of some *Aspergillus* and *Penicillium* spp. was affected only at <0.5% O_2.

9.3.1 *Changes in headspace gas composition*

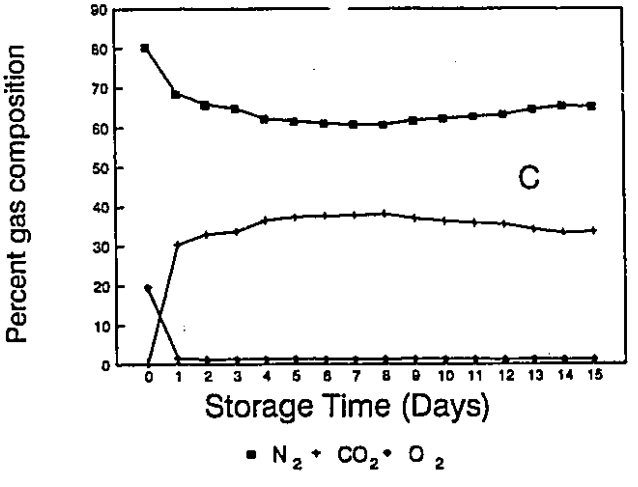
The changes in headspace O_2, CO_2 and N_2 composition for peanuts packaged in films AS1 and AS111 respectively at all the temperatures/packaging conditions under study are shown in Figures 52-57 respectively. For air packaged samples, headspace O_2 decreased to 1% or less within 1-2 days depending on the packaging conditions. At all three incubation temperatures, the trend in headspace gas changes for peanuts packaged in high barrier film AS1 or in air packaged with Ageless type G absorbent was similar. Headspace CO_2 increased initially during storage as a result of mold metabolism in air packaged samples (Figures 52-54A) and in samples packaged with Ageless Type G due to the release of CO_2 by the absorber and possibly mold metabolism since residual oxygen was about 1-2%. (Figure 52-54C). The gradual decrease in headspace CO_2 with storage time may either be due to its adsorption 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 and Mateles, 1971). For packages containing Ageless Type S oxygen absorbers, headspace CO_2 is maintained at approximately the same concentration (1-2%) throughout the incubation period due to the unavailability of O_2 for mold metabolism thereby suppressing CO_2 production by *A. parasiticus* (Figures 52-54B).



A - Air packaging

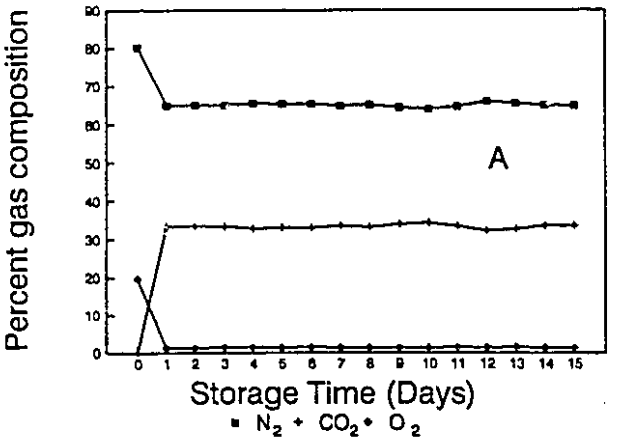


B - Air/Oxygen absorber packaging

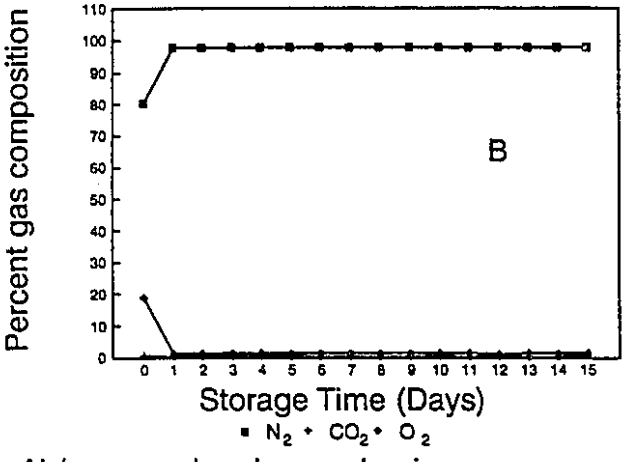


C-Air/Oxygen/Carbon dioxide generator absorber packaging

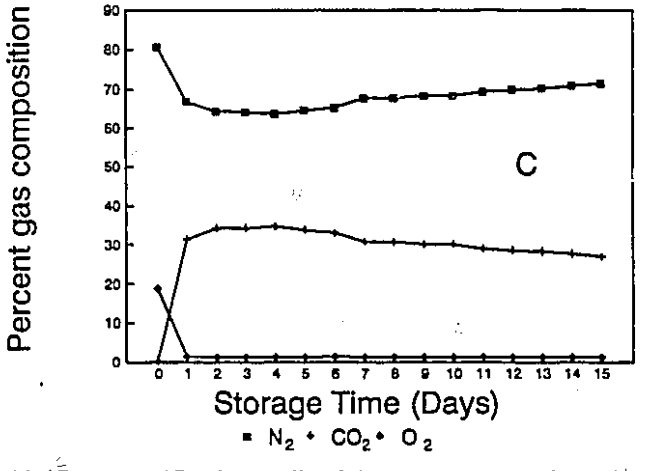
Figure 52 Changes in headspace gas composition with time in peanuts packaged with film AS1 at 20°C.



A - Air packaging

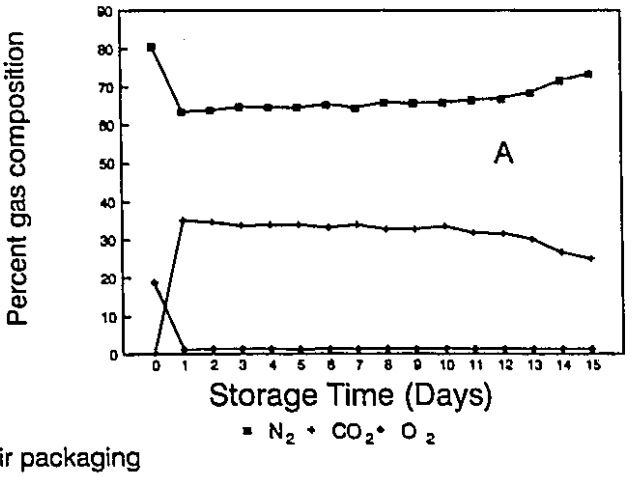


B - Air/oxygen absorber packaging

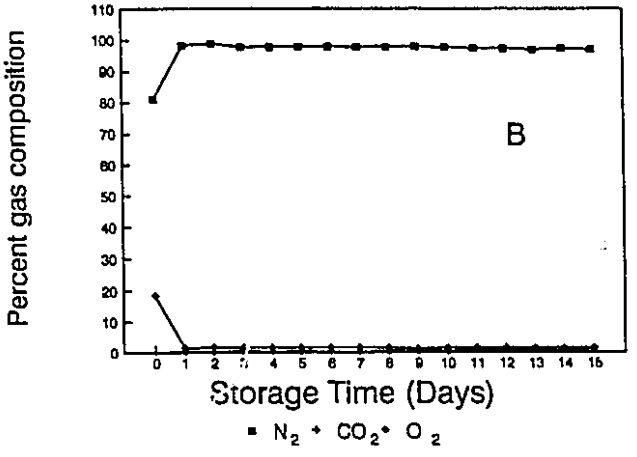


C-Air/Oxygen/Carbon dioxide generator absorber packaging

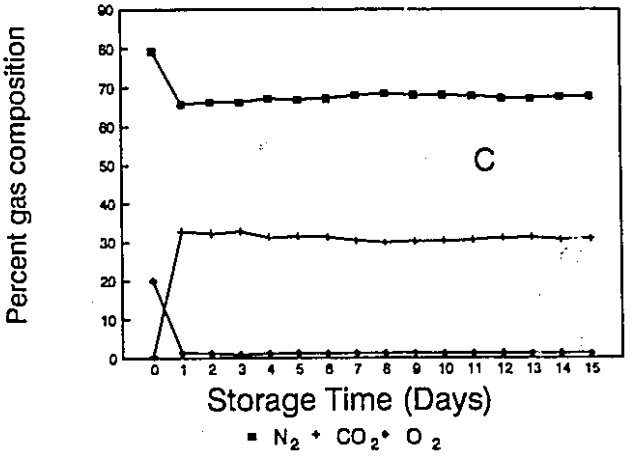
Figure 53 Changes in headspace gas composition with time in peanuts packaged with film AS1 at 25°C



A - Air packaging



B - Air/Oxygen absorber packaging



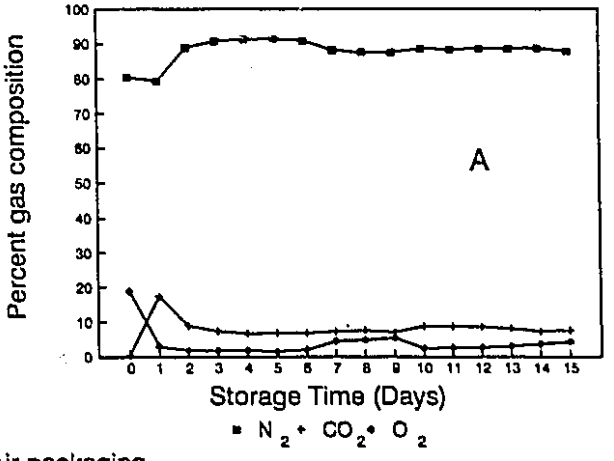
C-Air/Oxygen/Carbon dioxide generator absorber packaging

Figure 54 Changes in headspace gas composition with time in peanuts packaged with film AS1 at 30°C

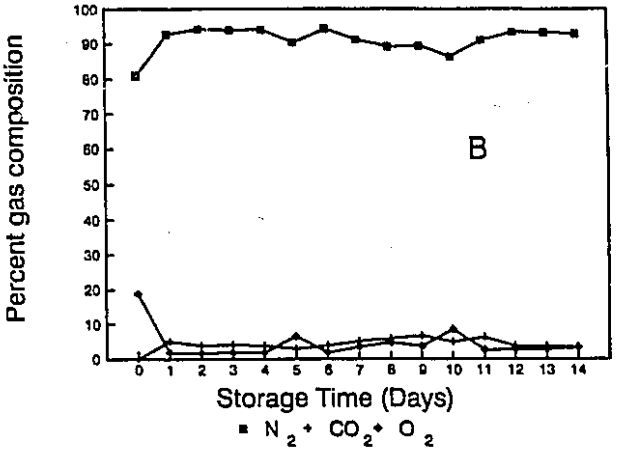
For peanuts packaged with film AS111, the trend in headspace gas changes is similar at all the 3 incubation temperatures. Headspace O_2 decreased initially to <1% with an increase in CO_2 after 24 hrs. This was followed by a gradual decrease in headspace CO_2 and a gradual increase in headspace O_2 to 1-1.5%. The gaseous atmosphere of these packages after 24hrs consisted mainly of N_2 with concentrations of N_2 ranging between 80-95%. It is evident from these results that Oxygen absorbents or Oxygen absorbents/Carbon dioxide generators can only be effective depending on the packaging material which are used to enclose the food products. The increase in headspace O_2 with Ageless Type S and Ageless Type G absorbents and the loss of CO_2 by Ageless Type G is attributed to the low barrier characteristics of film AS111 to these gases, particularly at higher storage temperatures.

9.3.2 *Color change*

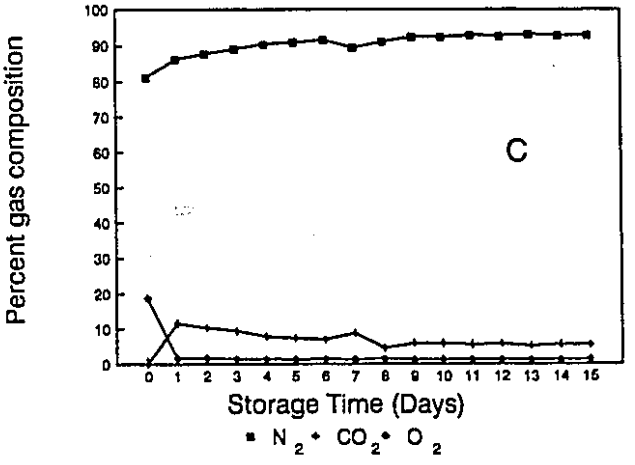
Color changes for peanuts packaged and stored under various conditions used in the study are shown in Table 54. Peanuts packaged with film AS111 showed a high degree of discoloration/browning especially at 25°C - 30°C compared to those of AS1. The highest degree of browning was observed with air packaged peanuts at higher storage temperatures and peanuts packaged with an oxygen absorbent/carbon dioxide generator in film AS111 at all temperatures. This discoloration is due to the combined effect of oxygen, the deteriorative action of mold growth and high storage temperatures. Similar observations were made by Holaday et al., (1979) in packaged pecans. With the exception of air packaged peanuts using film AS1, the other packaging treatments using film AS1 resulted in a reduced degree of browning, especially at 20°C (Table 54). Based on a color score of 2.5, it is obvious that most of the treatments failed to make this high standard with only peanuts packaged in air or preferably with oxygen absorbent or oxygen absorbent/carbon dioxide generator and packaged in a high barrier film (AS1) and stored at a low temperature could achieve this standard at the end of the 15 day storage period.



A - Air packaging

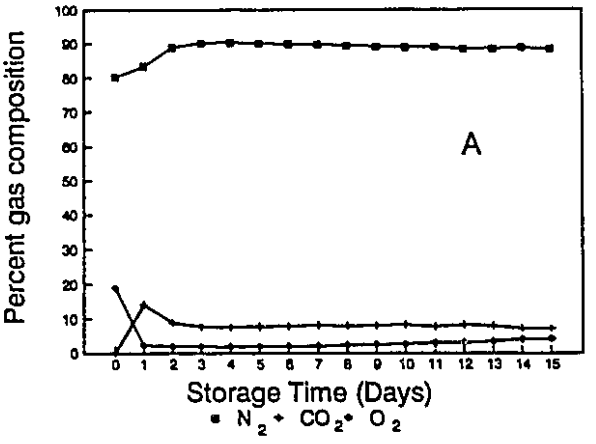


B - Air/Oxygen absorber packaging

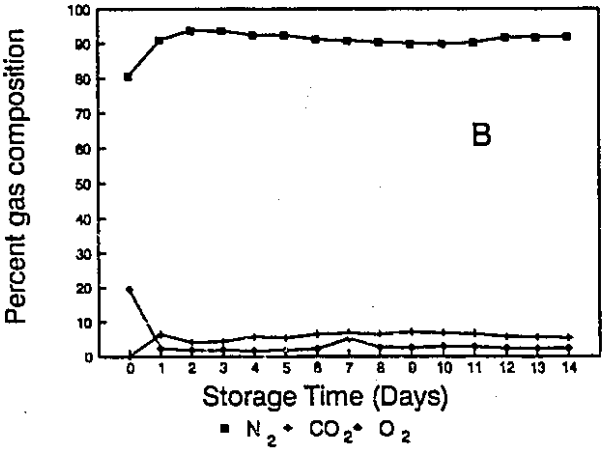


C-Air/Oxygen/Carbon dioxide generator absorber packaging

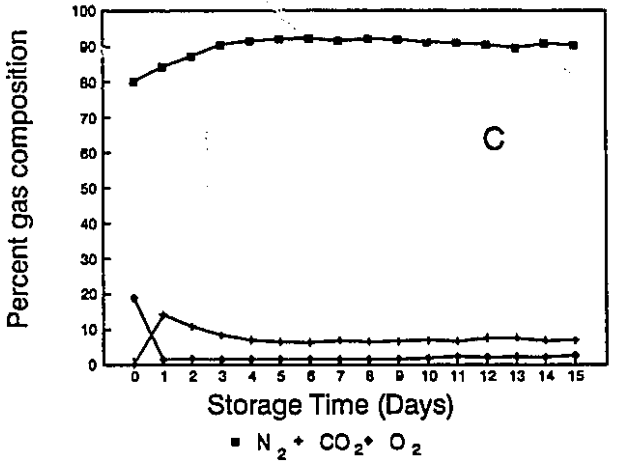
Figure 55 Changes in headspace gas composition with time in peanuts packaged with film AS111 at 20°C



A - Air packaging

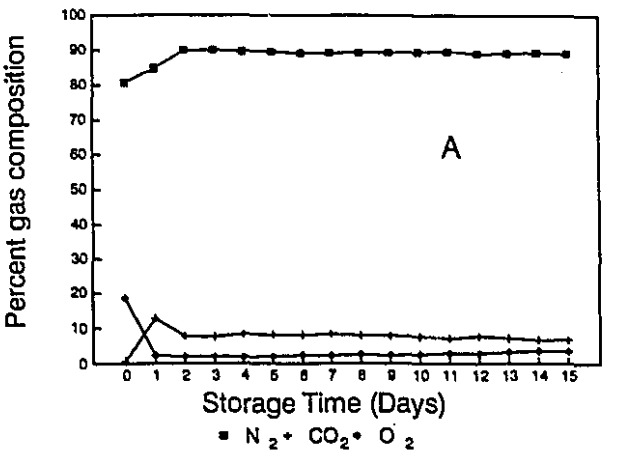


B - Air/Oxygen absorber packaging

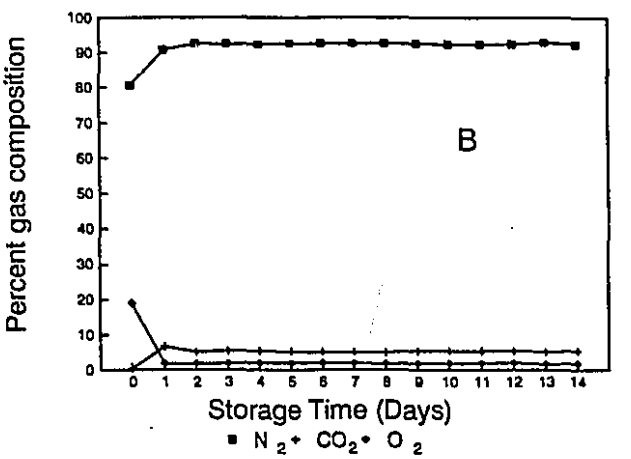


C-Air/Oxygen/Carbon dioxide generator absorber packaging

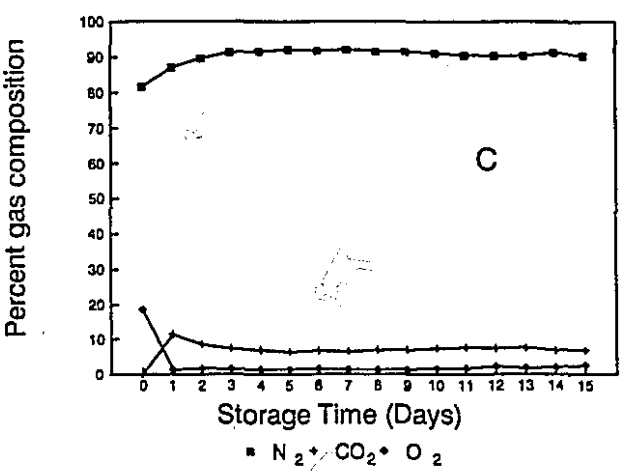
Figure 56 Changes in headspace gas composition with time in peanuts packaged with film AS111 at 25°C



A - Air packaging



B - Air/Oxygen absorber packaging



C-Air/Oxygen/Carbon dioxide generator absorber packaging

Figure 57 Changes in headspace gas composition with time in peanuts packaged with film AS111 at 30°C

9.3.3 *Aflatoxin B_1 production*

The effect of different types of gaseous conditions and packaging materials on aflatoxin B_1 production by *A. parasiticus* is shown in Table 54 and Figures 58-59. The results show that air packaging with films AS1 at 20°C and 25°C and AS111 at 20°C resulted in aflatoxin levels above the regulatory limit of 20 ppb. However, the level of aflatoxin B_1 detected for all the other packaged peanut conditions was below 20 ppb at all the incubation temperatures. The trend for aflatoxin production by *A. parasiticus* in air packaged peanuts was opposite to trend observed for *A. flavus* under the same packaging conditions, where toxin level was greater in film AS1 compared to peanuts packaged in film AS111. With the exception of peanuts packaged in film AS111 at 25°C, the level of aflatoxin B_1 detected in air packaged peanuts was consistently higher than peanuts packaged with absorbents. There was no significant difference in the levels of aflatoxin produced in air packaged peanuts using film AS1 at 20°C and 25°C compared to the lower amount produced at 30°C under the same packaging/storage conditions. However, for air packaged peanuts using film AS111, there was significant difference in the amount of aflatoxin detected at 25°C and 30°C compared to the level detected at 20°C.

There appeared to be a trend in the level of toxin produced in peanuts packaged in film AS1 using both Ageless Type S and Ageless Type G absorbents. A gradual increase in toxin level with increasing temperature was observed in packages containing Ageless Type S absorbents, even though the level of aflatoxin produced is still well below the regulatory limit of 20 ppb (Figure 58). However, the reverse trend was observed for peanuts packaged with Ageless Type G oxygen absorbents. With this absorbent, the level of aflatoxin decreased with increasing temperature (Figure 58). This may be attributed to the fact that increasing temperature increases the reaction speed of the absorber (Harima, 1990) and also the fact that the higher levels of headspace carbon dioxide generated by this sachet

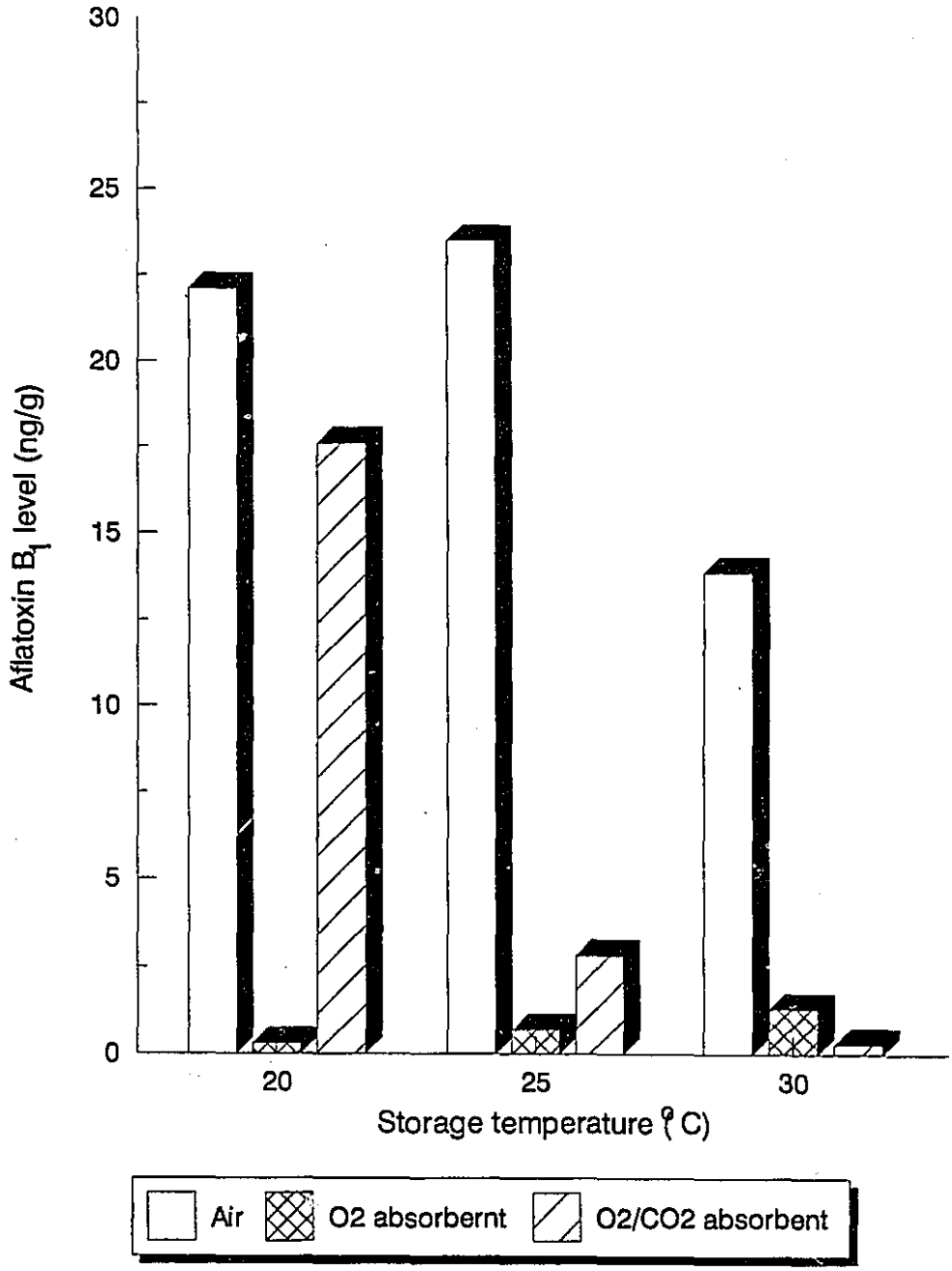


Figure 58 Aflatoxin B_1 level in peanuts packaged in film AS1 under different conditions

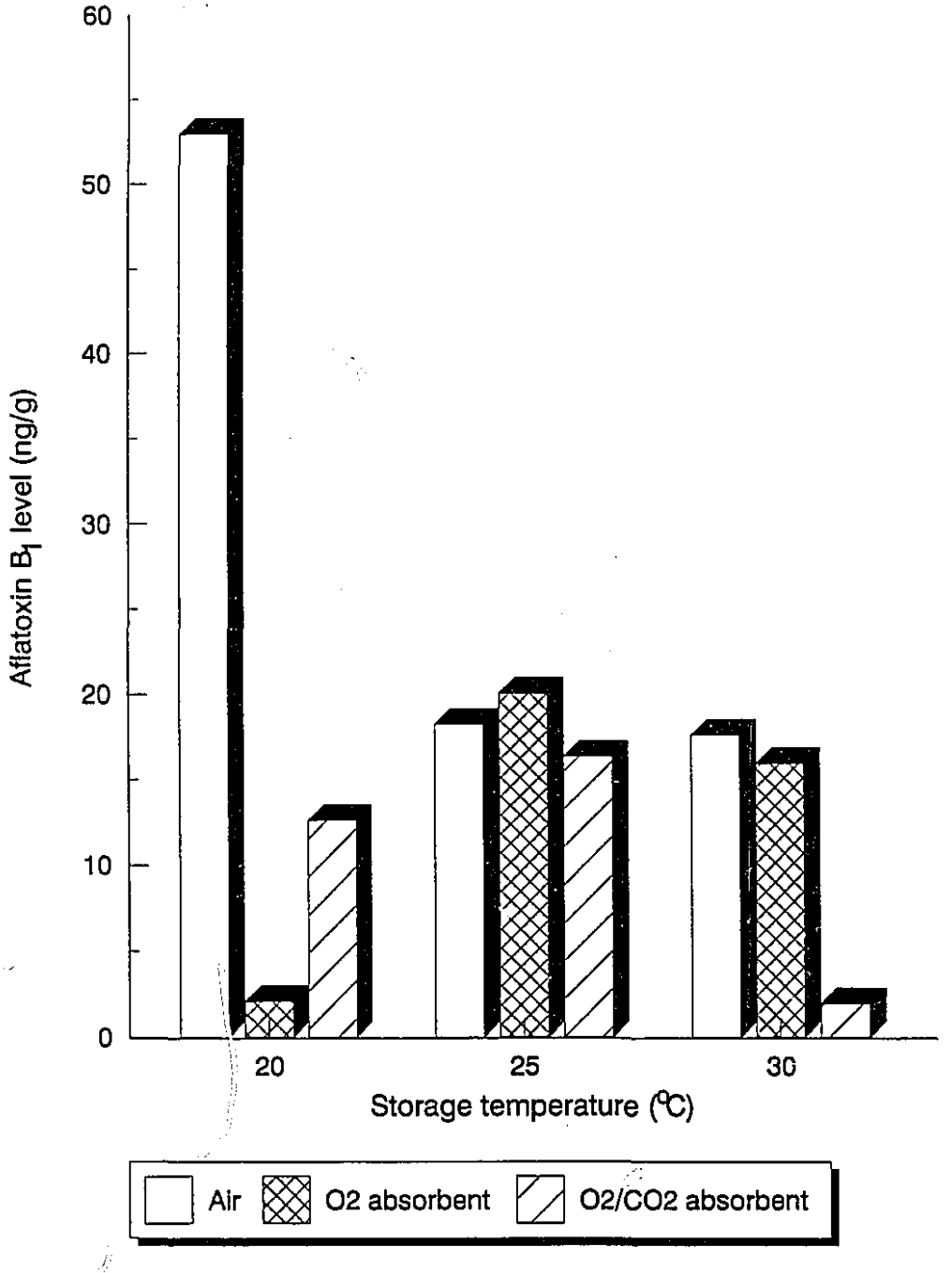


Figure 59 Aflatoxin B_1 level in peanuts packaged in film AS111 under different conditions

may have suppressed aflatoxin production. The low levels of aflatoxins observed with the two types of Ageless absorbents is due to their oxygen scavenging potential and, in the case of Ageless Type G, the CO_2 releasing potential thereby creating an atmosphere different from air and one which is inhibitory to the growth of and aflatoxin production by *A. parasiticus*. Diener and Davies (1972) and Pattee et al., (1966) reported that aflatoxin formation could be inhibited by reducing oxygen concentration. A decrease in toxin production was recorded when oxygen concentration was reduced (without CO_2) to 10% and below, with a marked decrease occuring after a reduction in oxygen level from 5 to 1%. Landers et al., (1967) also found that aflatoxin production in groundnuts decreased as CO_2 concentration increased from 0.03% to 100% or O_2 concentration decreased from 21% to 1%. Wilson et al., (1977) also reported the inhibitory effects of high CO_2 and low O_2 combination on aflatoxin production using corn.

The level of aflatoxin produced in peanuts packaged with absorbents in film AS111 were substantially higher compared to the levels detected using film AS1. Again this may be attributed to the high transmission rate of film AS111 to both O_2 and CO_2, thereby reducing the efficiency of these absorbents, particularly at higher temperature storage conditions. Nevertheless, despite the low barrier properties of film AS111, the level of aflatoxin produced was generally still less than the regulatory limit of 20 ppb. This may be due to the high N_2 concentration which produces a depressant effect on the formation of aflatoxin. Similar findings were reported by Jackson and Press (1967) in their studies with groundnuts and Fabbri et al., (1980) in their work with grains. It is interesting to note however, that the level of aflatoxin produced in peanuts packaged with oxygen absorbent or oxygen absorbent/carbon dioxide generator and packaged with film AS111 was much greater than previously detected in previous studies (chapter 8) (Figure 45). This may be due to the different mold species used in the study and the tolerance of the two species to the gases under the conditions of storage. Also, the higher level of aflatoxin produced by

Table 55. Analysis of variance for aflatoxin B_1 produced by *A. parasiticus* in air packaged peanuts.

Source	dF[a]	Sum of squares	Mean square	F
Model	5	2969.31	593.86	31.49***
Film	1	491.09	491.09	26.04***
Temp. (°C)	2	1499.52	749.76	39.76***
Film*Temp.	2	978.69	489.34	25.95***
Residual(error)	12	226.30	18.86	
Total	17	3195.61		
R^{2b}		0.92		

[a] Degrees of freedom

[b] Coefficient of determination

Level of significance *** $p < 0.001$.

Table 56. Analysis of variance for aflatoxin B_1 produced by *A. parasiticus* in oxygen absorbent packaged peanuts.

Source	dF[a]	Sum of squares	Mean square	F
Model	5	1169.84	233.97	107.43***
Film	1	638.07	638.07	292.98***
Temp. (°C)	2	284.58	142.29	65.33***
Film*Temp. (°C)	2	247.19	123.59	56.75***
Residual(error)	12	26.13	2.18	
Total	17	1195.98		
R^{2b}		0.97		

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p < 0.001$.

Table 57. Analysis of variance for aflatoxin B_1 produced by *A. parasiticus* in O_2 absorbent/CO_2 generator packaged peanuts.

Source	dF[a]	Sum of squares	Mean square	F
Model	5	910.52	182.10	85.46***
Film	1	51.85	51.85	24.33***
Temp. (^{o}C)	2	595.56	297.78	139.74***
Film*Temp. (^{o}C)	2	263.11	131.56	61.74***
Residual(error)	12	25.57	2.13	
Total	17	936.09		
R^{2b}		0.97		

[a] Degrees of freedom.

[b] Coefficient of determination.

Level of significance *** $p < 0.001$.

Table 58. Least square means (LSM) for two films used in air packaged and absorbent packaged peanuts for aflatoxin B_1 production by *A. parasiticus*.

Film type	Packaging conditions	LSM	Std. Err. LSM
AS1	Air	19.78	1.45[a]
AS111	Air	30.23	1.45[a]
AS1	Air / O_2 absorber	0.76	0.49[b]
AS111	Air / O_2 absorber	12.67	0.49[a]
AS1	Air / O_2 - CO_2 generator	6.89	0.49[a]
AS111	Air / O_2 - CO_2 generator	10.29	0.49[a]

[a] Level of significance (T - test) $p < 0.001$.

[b] Not significant.

the dual function absorber could be due to incorporation of CO_2 into the biosynthetic pathway for aflatoxin production (Hsieh and Mateles, 1971).

Statistical analysis of the data for all three packaging conditions, showed that the model was highly significant ($p<0.001$) with an R^2 of 0.92 for air packaged samples and 0.97 for absorbent packaged peanuts (Tables 55 & 57). The effects of the different films as well as their temperatures and interactions were also all highly significant ($p<0.001$). A comparison between the two films using Least square means (LSM) under all the three packaging conditions (Table 58) showed that film AS1 would be the most adequate film for packaging peanuts under modified atmospheres and to control aflatoxin production by *A. parasiticus* under these experimental conditions.

9.4 CONCLUSION

In conclusion, it is evident from the results obtained in this study that the application of O_2 absorbents and O_2 absorbent/CO_2 generators for the packaging of and preservation of peanuts against aflatoxin production by *A. parasiticus* is very effective and it would also be an economical and adaptable process for tropical developing countries. However, the efficiency of these absorbers to control toxin production is dependent to a large extent on the gas transmission properties of the packaging material, which is a function of storage temperature.

GENERAL CONCLUSION

Aflatoxins, especially aflatoxin B_1 continue to be compounds of major interest in food due to their carcinogenic, teratogenic and hepatotoxic effects. Controlling aflatoxin production in foods and feedstuffs has been of major concern both nationally and internationally. Such control is even more critical due to recent recommendations to reduce the standard of acceptable level of aflatoxins in food from 20 ppb to 10 - 15 ppb. Studies to date examining the effects of environmental factors on toxin production by aflatoxigenic molds have been done mainly by evaluating factors by the traditional one variable at a time approach. The disadvantage of this technique is that it is time consuming, and it generates large quantities of data which is often difficult to interpret. It also fails to measure interaction effects and hence generate mathematical models which can be used to predict the levels of factors required to inhibit mold growth and aflatoxin production.

In the application of Modified Atmosphere Packaging (MAP) for the preservation and shelf life extension of food products, there is a general consensus on the need to use mathematical models to determine the interaction effect of various factors or "barriers" on the activity of the microbes (Hotchkiss, 1988). Further attempts at understanding and modeling the dynamic processes in an MAP package are inadequate because most data on film permeability have been generated at a single temperature and relative humidity. There is also a paucity of data on the gaseous requirements of aflatoxigenic molds in packaged foods and the ability of these molds to grow and produce aflatoxins in foods packaged in films of various oxygen

permeabilities. Hence, this study was done in an attempt to address the limitations of previous studies on aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. This study was done in conjunction with the University of Science and Technology, Kumasi, Ghana, a country where aflatoxins are a major concern in many stored products, particularly peanuts. Furthermore, as the country continues to develop, more food products are being processed/packaged in response to consumer and export demands for such products. Therefore, the product chosen and the levels of factors selected in this study reflect the storage/environmental conditions associated with spoilage of many Ghanaian packaged food products.

The results of this study show that *Aspergillus flavus* can grow and produce aflatoxins in both synthetic media (malt extract agar) and in peanuts packaged in a carbon dioxide enriched atmosphere containing various residual levels of oxygen. Therefore, it is critical to control the level of residual oxygen in MAP foods if inhibition of mold growth is to be achieved. Furthermore, the extent of mold contamination is also critical in MAP food since high inoculum levels produce significantly higher levels of aflatoxin compared to low inoculum levels. However, while it may be difficult to control levels of contamination, headspace oxygen control can be achieved through the use of appropriate oxygen absorbent technology. This technology has been used alone or in conjuction with gas packaging to control aflatoxin levels below regulatory levels (20 ppb) even under temperature abuse condition. This is particularly important since it has been demonstrated that the barrier characteristics of films are influenced by both storage temperature and relative humidity.

In conclusion, this study has shown that MAP could be used to control the level of aflatoxins in packaged peanuts in developing countries such as Ghana. However, a major factor limiting the use of MAP technology is the cost of equipment and packaging films. This may partially be overcome by the use of oxygen absorbents which do not require the use of expensive evacuating/gas flushing equipment. One limitation of this technology is consumer resistance to satchets in the packaged product. This could be overcome by the use of "master packs" technology or the use of oxygen absorbent films. However, further research is warranted to determine the antimycotic effects of these new packaging technologies. While this study has generated interesting data on the growth of and aflatoxin production by *A. flavus* and *A. parasiticus* under MAP conditions, several questions arising from this research need to be addressed. This study was done using pure cultures of each mold species. However, studies need to be done to determine the effects of mixed cultures of these molds or other mold contaminants on aflatoxin production. Furthermore, the antimicrobial effects of naturally occuring lactic acid bacteria or added lactic acid bacteria needs further investigation to control aflatoxin production by *A. flavus* and *A. parasiticus*. Also the effects of natural antimicrobial agents such as chitosan and alpha macroglobulin need to be researched further. Further studies also needs to be done to determine the effects of microwave and irradiation on the growth of and aflatoxin production by aflatoxigenic molds under MAP conditions.

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