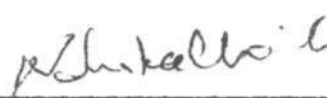


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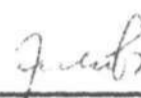
This thesis by Lucy Jumeiyi Ogbadu meets the regulations governing the degree, Master of Science of Ahmadu Bello University and is approved for its contribution to Scientific knowledge.

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INFLUENCE OF PRESERVATIVES ON THE
GROWTH AND TOXIN PRODUCTION 9V
ASPERGILLUS FLAVUS I.A.R. 586

BY

LUCY JUMEYI OGBADU

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C O N T E N T

<u>Title</u>		<u>Page</u>
THESIS TITLE	1
ACKNOWLEDGEMENTS	ii
TABLES	vi
FIGURES & PLATES	vii
SUMMARY	viii
INTRODUCTION	1
LITERATURE REVIEW	4
Morphology	4
Structure Physical & Chemical Properties of aflatoxin	4
Factors affecting aflatoxins production	6
Biosynthesis of aflatoxins	12
Natural occurrence of aflatoxins	15
Assay of aflatoxins	17
Metabolism	22
Metabolites of aflatoxins	24
Biochemical effects of aflatoxins	24
Biological effects of aflatoxins	28
Control of aflatoxins	30
Aflatoxins detoxification	32
Preservation of foods	33

				<u>Page</u>
MATERIALS AND METHODS	35
Organism	35
Growth substrates	35
Mycelial dry weight	40
Removal of pigment	40
Amount of substrate in extraction	42
Caliberation of the toxin meter	43
Analysis of aflatoxin	44
Bioassay	47
RESULTS	49
DISCUSSION	61
REFERENCES	71

TABLES

	<u>PAGE</u>
1. Metabolites of aflatoxin	25
2. Trace elements and sugar content of pawpaw fruits	37
3. Composition of fish	38
4. Effect of sodium benzoate on growth and aflatoxin production by <u>Aspergillus flavus</u> (IAR 586) ..	49
5. Effect of O-hydroxybenzoic acid (salicylic acid) on growth and aflatoxins production by <u>Aspergillus</u> <u>flavus</u> (IAR 586)	51
6. Effect of p-aminoethyl benzoate on growth and aflatoxins production by <u>Aspergillus flavus</u> (IAR 586)	53
7. Effect of inhibitory levels of salts and aflatoxins on chicks in ten days	55
8. Effect of woodsmoke on aflatoxin production by <u>Aspergillus flavus</u> (IAR 586)	58
9. Constituents of woodsmoke	68.

FIGURES AND PLATES

<u>FIGURE</u>		<u>Page</u>
1.	Smoking unit	39
2.	Diagram of Microcolumn	46
<u>PLATES</u>		
1.	Fluorotoxin meter	45
2.	Photomicrograph of liver from chick intubated with sodium benzoate supplemented diet ..	50
3.	Photomicrograph of liver from chick intubated with <i>O</i> -hydroxybenzoic acid supplemented diet	52
4.	Photomicrograph of liver from chick intubated with <i>p</i> -amino ethylbenzoate supplemented diet	54
5.	Photomicrograph of liver from chick intubated with aflatoxin containing diet	56
6.	Photomicrograph of liver from chick intubated with aflatoxin containing diet	57
7.	Inoculated smoked and unsmoked fish ..	59
8.	Inoculated pairs of smoked and unsmoked fish for different times	60

SUMMARY

The influence of three benzoic acid derivatives - sodium benzoate, O-hydroxybenzoic acid and p-aminoethyl benzoate on the growth and aflatoxins production by *A. flavus* was investigated on mashed fruits of *Carica papaya* (pawpaw) with an initial pH of 5.3 as the sole growth substrate.

These salts were inhibitory to both growth and aflatoxins production, with variation in the effective concentrations. At complete inhibition, the percentage contents of the salts stood at 0.33 for sodium benzoate, 0.17 for O-hydroxybenzoic acid and 0.10 for p-aminoethyl benzoate. Investigations on the safety of these complete inhibitory levels were carried out on day old Babcock cockerels by crop intubation with diets containing a total of 600mg sodium benzoate, 300 mg O-hydroxybenzoic acid and 180mg p-aminoethyl benzoate for a period of ten days. The histopathological results revealed normal liver with no toxic effects. Lesions were observed in chicks fed on diet inoculated without any salts incorporated.

The influence of woodsmoke on growth and aflatoxins production by *A. flavus* was investigated on smoked and unsmoked fish (*Tilapia* spp). There was more growth and toxins production

on the unsmoked fish, inhibitory action of the woodsmoke was increased with increase in the time of smoking and this influence of the woodsmoke was more marked on toxins production than on mycelial growth.

I N T R O D U C T I O N

Fungi are ubiquitous in their distribution and there has long been the awareness of their contamination on feeds and foodstuffs. The association of fungi with toxic metabolic products has been known for centuries. The effect of ergot, a toxic secondary metabolite of Claviceps purpurea was known a thousand years ago as "St. Anthony's Fire" when thousands of people died of ergotism (Berger, 1931). The awareness increased over the years but it was not until early 1960 that the possible toxic effect of other moulds was appreciated, due to an outbreak of "Turkey X" disease. Thousands of Turkey poults in the United Kingdom died after feeding on mould contaminated Brazilian groundnut as feed supplement. A pure culture of Aspergillus flavus was isolated and the disease was linked to the toxic metabolites of this fungus which showed to be toxic to a number of domestic animals (Asplin, 1961). Subsequently the name "aflatoxin" was adapted to refer to toxic metabolites of Aspergillus species.

In the past decade a lot of research has been done and reported on aflatoxins. The report of the Joint Food and

Agricultural Organisation (FAO), World Health Organisation (WHO) and United Nations Environment Programme (UNEP) conference on mycotoxins shows that aflatoxins are universally distributed in the environment and aflatoxin contamination in foods and feeds has been detected in all parts of the world. Aflatoxin has been extracted from a wide variety of foods ranging from plant products like fruits to animal products like meat and fish.

Aflatoxicosis (Disease caused by ingestion of aflatoxins) has been reported in animals, plants and even possibly man (Belchetz and Cohen, 1976). Since the awareness of the toxicity of aflatoxin, much attention has been given to prevention of aflatoxin contamination. This essentially is a problem of *Aspergilli* which entails either drying the growth substrate to levels of moisture that would prevent fungal colonization or direct application of chemicals that would inhibit growth of the fungus provided such chemicals do not lower or alter the quality of the substrate nor produce any toxic or side effects. Both methods have been employed in the forms of smoking and chemical incorporation into foods respectively.

Smoking has been used traditionally in the past, without the awareness of its chemical effect. In this country there

is the traditional practice of smoking "bush meat" (meat from wild animals) and fish for the purpose of preservation. This is a feasible method for cheap effective control of aflatoxin contamination particularly in the developing world.

Benzoic acid has been known to occur naturally in some foods. It is an accepted preservative in certain foods (Jay 1978). However publications on use of benzoic acid derivatives to inhibit growth and aflatoxin production of toxigenic strain of Aspergillus flavus is limited.

The objectives of this study therefore are:

(1) To investigate the influence of Benzoic acid derivatives on aflatoxin production by a toxigenic strain of Aspergillus flavus on paw paw fruits (Carica papaya).

(2) To investigate the safety of the complete inhibitory levels of these salts on this substrate by use of day-old chicks.

(3) To investigate the influence of hard wood smoke on growth and aflatoxin production by the same fungus on fish (Tilapia spp).

The molecular formula of aflatoxin B₁ was established as C₁₇H₁₂O₆ and of aflatoxin G₁ as C₁₇H₁₂O₇; aflatoxins B₂ and G₂ were found to be the dihydro derivatives of the parent compounds, C₁₇H₁₄O₆ and C₁₇H₁₄O₇ (Hartley et al., 1963).

Structures of aflatoxins B₁ and G₁ proposed based on interpretation of spectral data (Asao et al., 1963; Asao et al., 1965). Chang et al. (1963) proposed the structure of aflatoxin B₂ and G₂ shortly after. The proposed structure for G₁ has been supported by X - ray crystallography (Chaung and Sim, 1964).

The aflatoxins are high substituted coumarins and the presence of the furocoumarin configuration places them among a large group of naturally occurring compounds with many pharmacological activities (Soine, 1964). The bifuran structure, however has previously been encountered in sterigmatocystin a metabolite of A. versicolor (Bullock et al., 1962).

Complete catalytic hydrogenation of aflatoxin B₁ has been shown to require 3 moles of hydrogen with the production of the tetrahydroxy derivative (Asao et al., 1963). If there is any interruption of the hydrogenation process after the uptake of 1 mole of hydrogen, aflatoxin B₂ is produced in quantity. (Chang et al., 1963). Andrellos and Reid (1964) reported that aflatoxin B₁ reacts additively with a hydroxyl group to form the hemiacetal, the reaction being catalysed by a strong acid.

With formic acid - thionyl chloride, acetic

acid - thionyl chloride or trifluoacetic acid addition products of greatly altered chromatographic properties are formed.

Factors Affecting Aflatoxin Production

Temperature

Aflatoxins production is affected by temperature. West et al. (1973) observed that increasing the initial temperature for A. parasiticus from 15 to 21^oC after 24 hours of incubation and then to 28^oC after 48 hours resulted in a four fold increase in total aflatoxin, over the usual fermentation which is held constant at 28^oC for six days. Increase in aflatoxin B₁ was also observed. Diener and Davies (1966) however, showed that highest levels of aflatoxin are produced between 25 - 30^oC with a decrease at 35^oC and slight to trace amount at 40^oC. A.flavus produces aflatoxin over a temperature range from 12 - 41^oC with optimum production at 30^oC, the limits depending on the substrate and specific experimental conditions (Davies and Diener, 1968). Under laboratory conditions at 25 - 30^oC aflatoxins have developed within 48 hours on moistened ground-

nuts, rice and cottonseed whereas a minimum of 4 to 5 days on wheat has been reported.

Modified Atmosphere

Growth of storage fungi such as Aspergilli in natural substrates is dependent not only on availability of moisture and favourable temperature but also on atmospheric conditions surrounding the substrate. Replacement of air by various mixtures of oxygen, carbon dioxide or oxygen and nitrogen gases has been shown to influence toxin production by A. parasiticus. Shih and Marth (1973) reported that formation of toxin is suppressed in the presence of these gases. Complete inhibition was observed at atmospheres of 100% carbon dioxide or 100% nitrogen gas. Wilson and Jay (1975) observed that corn stored in modified atmospheres did not accumulate over 15 ng of aflatoxin B₁ per kg and 20 ng of total aflatoxins per kg corn in the presence of high CO₂ (61.7% CO₂, 8.7% O₂ and 29.6% N₂) went mouldy at four weeks. With high Nitrogen (99.7% N₂ and 0.3% O₂) and controlled atmosphere (13.5% CO₂, 0.5% O₂, 84.8% N₂) treatments, there was no toxin production but on removal from these modified atmosphere the corn deteriorated rapidly and was soon contaminated with aflatoxins.

pH

The influence of pH on aflatoxin production has been investigated. Davies et al. (1966) have shown that initial pH has little or no effect on aflatoxin yield from yeast extract sucrose medium. Buchanan and Ayres (1976) observed that maximal growth, aflatoxin production and aflatoxin per unit of growth occurred at initial pH levels of 5.0, 6.0 and 7.0 respectively. They also observed that initial pH levels less than pH 6.0 favoured production of the B toxins whereas levels greater than pH 6.0 favoured production of the G toxins. Reddy et al. (1971) reported that pH 4.5 produced optimal aflatoxin accumulation in SL medium. Lie and Merth (1968) reported that when a casein substrate was used, maximum aflatoxin production occurred at both acidic and alkaline pH extremes. The influence of pH on aflatoxin production appears to be dependent on the mode of culture and composition of the medium.

Media

Various raw and processed foods have been shown to produce aflatoxins when inoculated with toxigenic strains of Aspergillus. A potent A. flavus isolate was reported to be a consistent

producer of aflatoxins on all natural substrates which supported the growth of the mould (Wildman et al., 1967). These substrates ranged from solid foods to commercially prepared fruit juices, peanuts, bread, cocoa, beans, potatoes, cheese, beef and grape juice, orange juice, pineapple juice, tomato juice and cranberry drink respectively. Bassir and Adekunle (1972) reported the production of aflatoxin B₁ in many Nigerian fruits; pawpaw, citrus fruits, banana, plantain onion, tomato, okra and mango and they reported highest yield of toxin on the pawpaw. Osiyemi and Adekunle (1973) recovered aflatoxin B₁ from a culture of A. flavus grown on sterile pulp of Carica papaya var. West Indian. Effect of gamma irradiation on aflatoxin production by A. flavus was investigated on many Nigerian foods (Oghadu, 1979). Among these foods was fruit mash of Carica papaya. Codner et al. (1963) reported high yield of aflatoxin on sterilized peanuts with A. parasiticus. Others include rice sorghum, corn, soybean, wheat rye, oats (Hesseltine et al., 1966). Toxin yield varied among the different substrates (Wildman et al., 1967 ; Hesseltine et al. 1966 and Bassir and Adekunle, 1972). Suspension of ground smoked fish in water has also supported the mycelial growth of, and aflatoxin production by Aspergillus flavus (Oghadu, 1979) This yield was observed to decrease or vary with different doses of gamma irradiation.

Moisture

Laboratory studies show that a moisture content in equilibrium with relative humidity of about 85 \pm 1% is the lower limit for growth of A. flavus and production of aflatoxin on natural substrates. This corresponds to a moisture content of 18 to 18.5% in wheat, corn and sorghum grains; 16.5% in paddy rice and 17.5% in polished rice; 17 - 18% in soybeans and 9 - 10% in peanuts, Brazil nuts, other nuts and copra (Joint FAO/WHO/UNEP, 1977). Calderwood and Shroeder (1968) reported that 305 - 750 μ g/kg of aflatoxins were produced in polished rice with a moisture content of 24 - 26 % in seven to twenty one days. At a moisture content of 19 - 20 percent at 25^oC, Van Warmelo et al. (1968) observed maximum aflatoxin production within two to three weeks in corn naturally infested with toxigenic strains of A. flavus. At a moisture content between 15 and 30% at 32^oC production of aflatoxin was observed in two days while at a moisture content between 20 and 31% at 21^oC production of the toxin was observed in four days in freshly dug peanuts (Dickens and Patten 1966).

Carbon and Nitrogen Sources

The effects of both carbon and nitrogen sources on aflatoxins production have been studied. Visser (1967) observed that carbohydrates were generally better carbon sources than amino acids for A. flavus. He also reported that a high C: N ratio was a prerequisite in a substrate for good growth of the fungus. Bassir and Adekunle (1972) reported high C: N ratio for pawpaw as 2.09.

Adye and Mateles (1964) found that the best carbon sources were glucose, sucrose and fructose whereas little or no aflatoxins was produced with lactose, maltose, sorbose acetate succinate and malate. Bassir and Adekunle (1972) reported Fructose, Glucose and Sucrose content of pawpaw to be as high as 27.04×10^{-5} , 24.81×10^{-5} , 2.95×10^{-5} mole/100 mg respectively while maltose and lactose were found absent. Mateles and Adye (1965) reported that casamino acids are the preferred nitrogen source. Ammonium sulphate and potassium nitrate are good inorganic nitrogen sources for aflatoxin production (Eldridge, 1965; Schroeder, 1966; Davies et al., 1967).

Biosynthesis of Aflatoxin

Aflatoxins which are a group of closely related compounds are produced as a result of secondary metabolism of certain species of Aspergillus. Several biogenetic pathways have been suggested (Moody, 1964; Holker and Underwood, 1964; and Heathcote et al., 1975) and there is abundant experimental evidence which support some of these schemes. Some of these have revealed that the entire carbon skeleton of aflatoxin B₁ molecule is derived from acetate (Biolaz et al., 1970). They investigated the label distribution of ¹⁴C - labelled aflatoxin synthesized from (1 - ¹⁴C) and (2 - ¹⁴C) acetate while Hsieh and Matales (1971) provided evidence for the polyketide hypothesis:

acetate → anthraquinone → xanthenes → coumarins.

Experiments with mutants of A. parasiticus impaired in aflatoxins biosynthesis have led to the finding that anthraquinones like averufin, norsolorinic acid and versicolorin A are accumulated in place of aflatoxins (Lee et al., 1971). This suggests that these compounds are likely to be intermediates in aflatoxin biosynthesis.

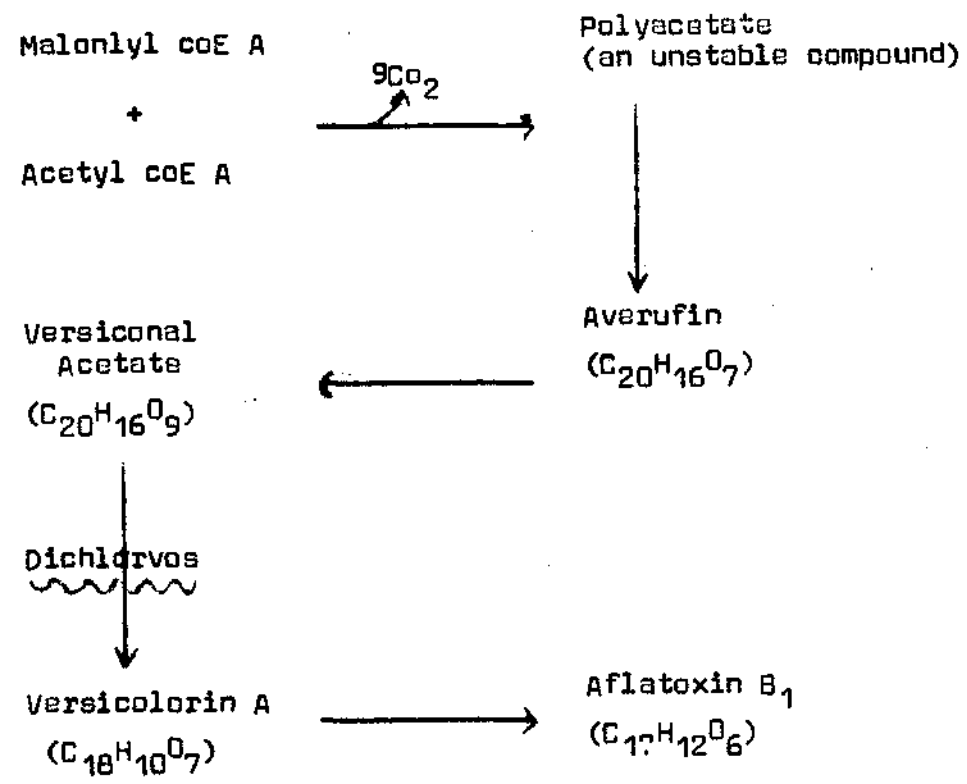
Gupta et al. (1975) reported the involvement of (1 - ¹⁴C) acetate and (2 - ¹⁴C) malonate in the biosynthesis of aflatoxin by resting mycelia of A. parasiticus. More of the malonate (4.3%) than acetate (1.6%) entering the mycelia of the fungus was incorporat

into aflatoxins. Their result suggested that malonate is an intermediate in aflatoxin biosynthesis and that it can be incorporated without being converted to acetate.

An orange compound has been observed by various workers in the process of aflatoxin synthesis and was identified as versiconal acetate (Yao and Hsieh, 1974; Uraih *et al.*, 1977). They reported that dichlorvos (dimethyl - 2, 2 dichlorovinyl phosphate) an organophosphate inhibits a step which lies beyond averufin but before sterigmatocystin (a xanthone) at the formation of versiconal acetate. Sterigmatocystin can also be converted to aflatoxin B₁ as well as averufin (Lin *et al.*, 1973).

With the knowledge of these anthraquinones being possibly biogenetic precursors of aflatoxins B₁, Singh and Hsieh (1977) attempted to show the sequence and the interrelationship between them in the biosynthetic pathway of aflatoxins. They observed that versicolorin A producing mutant incorporated acetate, averufin and versiconal acetate into versicolorin A but in the presence of dichlorvos the major product being versiconal acetate indicating that dichlorvos inhibits conversion of versiconal acetate to versicolorin. Conversion of versicolorin A and sterigmatocystin

to aflatoxin is unaffected by dichlorvos confirming that point of action of dichlorvos must be before formation of versicolorin A.



Natural Occurrence of Aflatoxins

The ubiquitous nature of the *Aspergilli* explains the wide occurrence of aflatoxins on almost all types of foodstuffs (Joint FAO/WHO/UNEP, 1977).

Blount (1961) discovered the presence of these toxins in a Brazilian groundnut meal supplement as exotoxins of a fungus, *A. flavus*. Subsequently Sargeant *et al.* (1961) identified the toxin as a by-product of *A. flavus* metabolism in grains gathered from India and many parts of Africa.

Ever since, several foodstuffs have been screened for possible contamination by these toxins. Allcroft *et al.* (1963) observed toxicity in maize meal. Presence of aflatoxins has also been reported in cottonseed cake by Loosemore *et al.* (1964), Bassir (1969) reported among other toxins, toxic conjugated fatty acids produced in palm juice by aflatoxin producing *A. flavus*. Kurata *et al.* (1968) isolated cultures of *A. flavus* and subsequently aflatoxins from soybeans, red bean and kidney bean flour in Japan. In Uganda, Alpert *et al.* (1971) isolated aflatoxins from beans. Flannigan and Hui (1976) reported presence of aflatoxins in ginger, Jamaica red and white peppers while tree nuts like pecans (Lillard *et al.*, 1970) and pistachio (Daniezl *et al.*, 1976) were reported to

contain aflatoxins. Reports from south-east Asia indicate the occurrence of aflatoxin contamination in some fish products. An average concentration of 166 mg/kg. of total aflatoxins in 5% of 139 samples of dried fish and shrimps was reported in a survey of Thailand foods (Joint FAO/WHO/UNEP, 1977).

Assay of Aflatoxins

The importance of aflatoxins in mycotoxicological research has led to having various methods for detecting and estimating the aflatoxins. These are assayed in two main ways; Physicochemical methods and Biological assay.

Physicochemical Methods

Isolation of the aflatoxins from toxic meals was greatly facilitated by the discovery that they fluoresce intensely under ultraviolet light. This characteristic of the aflatoxins has exposed a convenient means for monitoring of isolation and purification procedures. Allcroft et al. (1961) and Sargeant et al. (1961a) demonstrated that the aflatoxins were extractable with methanol. A variety of extraction procedures have since been developed for use with various natural products or mould cultures on natural substrates particularly in connection with chemical assays of agricultural commodities for aflatoxin contamination. These include the aqueous methanol method (Campbell et al., 1964) the aqueous acetone method (Pons and Goldblatt, 1964) and the hexane-acetone-water azeotrope (Goldblatt, 1965). These solvents are said to be efficient in extracting the compounds when the toxins are present in small concentration.

Aflatoxins are assayed visually by making a comparison of fluorescence intensities of standard samples with unknown extracts that are chromatographed under identical conditions (Nesheim, 1964; Rodricks and Stoloff, 1970). A modification of this method is by dilution of the extract to extinction until there is no more fluorescence such that when run on kieselgel G - plates the smallest concentration giving observable fluorescence is 0.0003 ng and 0.0004 ng for B₁ and G₁ respectively (Carnaghan et al., 1963). Pons (1976) has effectively adapted a method that utilises high pressure liquid chromatography for aflatoxins determination in most foodstuffs.

The ultraviolet spectrophotometric capability of the eluents of the aflatoxins separated on TLC plates have been utilised to provide a method for determining the concentration of aflatoxins (Nabney and Nesbitt, 1965). The aflatoxins absorb ultraviolet light maximally at 365 nm. This spectrophotometric method has been employed by various workers (Stoloff et al., 1971).

By use of fluorodensitometer it was observed that fluorescent peaks of both an unknown extract and a standard can be compared (Pons and Goldblatt 1964; Ayres and Sinnhuber, 1966).

This method tends to eliminate inaccuracies arising from the visual method. The emitted fluorescence as measured by peak areas has been reported to be linearly related to aflatoxin concentration over a range of at least 2×10^{-4} to 105×10^{-4} μg per spot of the four main aflatoxins. (Pons et al., 1966). A modification of this method has been reported by Bockwith and Stoloff (1968). Using their modified method they observed averagely $101 \pm 3 \%$ and $89 \pm 8 \%$ recovery of aflatoxins B_1 and B_2 respectively.

Fluorotoxin meter

The development of Velasco Fluorotoxin meter (P.45) by a Research Chemist James Velasco at the U. S. Department of Agricultural Research Service has provided a rapid, accurate and an alternative means of determining the concentration of aflatoxins in parts per billion in a matter of 10 minutes as opposed to the cumbersome chromatographic methods that depend on comparative visual colour areas. The fluorotoxin meter gives an instant recordable numerical values.

Principle of Operation

The system makes use of a glass microcolumn which is packed with florisil, sand, silica gel and alumina. The detection of aflatoxin is made by trapping it on this special florisil layer (pg. 46). The optical system is comprised of an ultraviolet light source - F4T4/9L fluorescent lamp, various filters and two photodetectors. The ultraviolet lamp with an energy output in the visible range is placed in a light tight enclosure with a special filter (F1) on one side so that the ultraviolet output passes through the filter before striking the glass microcolumn. This filter allows only energy between 310 and 410 nanometers to pass through and the energy from this particular ultraviolet lamp is concentrated primarily at 365 and 405 nanometers. The energy at 365 (λ) which is the desirable energy is unaffected by the F1 filter while that at 405 nanometers is severely attenuated. It is this unaffected energy - 365 nanometers that excites the florisil layer in the microcolumn. Once excited at 365 nanometers the florisil fluoresces at a second, longer wavelength, λ_2 . This wavelength can be either 430 or 460 depending on the particular aflatoxin fraction being measured. The energy output at λ_2 is proportional to both the original

excitation energy at λ_1 and the concentration in parts per billion of aflatoxin trapped by the florisil layer. Thus by holding the excitation energy constant, the energy output at λ_2 becomes a direct measure of the aflatoxin concentration in parts per billion. However since the fluorescence of the florisil layer is not completely uniform around the perimeter of the microcolumn, two photodetectors are used to intercept the λ_2 radiation from both sides of the microcolumn. Thus two photodetectors provide a more accurate determination of florisil activity and, hence of aflatoxin concentration (Instruction manual, Velsco fluorotoxin meter tm, 1974).

Biological Assay

The presence of aflatoxins is ~~mainly detectable~~ by the characteristic lesion it causes on the organs of the test animals, when fed on the toxin extract. Ducklings have been shown to be very sensitive to aflatoxins (Sargeant et al., 1961; Ambrecht and Fitzhugh, 1964). Bile duct proliferation is the characteristic lesion induced in day old ducklings.

With the chick embryo, Platt et al. (1962) reported LD₅₀ values of 0.048 ng per egg (when administered via the yolk) and 0.025 ng per egg (when administered via the air space).

before incubation. When administered via the chorion allantoic sac, the LD₅₀ for ten day old chick was observed to be 1.0 and 2.5 ug per egg.

The toxic effects of aflatoxins B₁ have also been investigated in several in vitro cell culture systems. Tissue cultures from the calf kidney has been affected by aflatoxins (Juhasz and Greczi, 1964). Aflatoxins concentrations of 1 to 5ug per ml of medium have destroyed human liver cells and Hela cells (Gablicks et al., 1965). Legator and Withrow, (1964) have shown that very small concentrations of aflatoxins B₁ inhibit the mitotic process in human embryonic lung cells.

The studies of Burmerster and Hesseltine, (1966), Uwaifo (1971) have demonstrated that aflatoxins can be determined by microbiological methods. Bacillus megaterium was inhibited by 1 ug of aflatoxin B₁ seven hours after incubation with well defined zones of inhibition after 18 hours of incubation (Clements, 1968).

Metabolism

The awareness of the toxicity and carcinogenic effects of aflatoxins has aroused the interest of various workers in the area of the metabolism of these toxins. Allcroft and Carnaghan

(1963b) isolated toxic metabolites from the milk of cattle fed rations containing high levels of aflatoxins. This finding heightened further interest in the metabolism of aflatoxins. This isolate when fed to ducklings was toxic to them and caused similar lesions to those of aflatoxin B₁ (De Iongh et al., 1964; Purchase, 1972). These metabolites were named aflatoxins M₁ and M₂ (Allcroft et al., 1966b). The structures of aflatoxins M₁ and M₂ have been reported (Holzapfel et al., 1966). They are 4-hydroxy forms of aflatoxins B₁ and B₂ respectively. Crude liver microsomal fractions prepared from the livers of chick, duckling, guinea pig and mouse were reported to have metabolized B₁ as rapidly as B₂ (Patterson and Roberts, 1970). The livers of chick, duckling, guinea pig and mouse were found to have metabolized aflatoxin B₁ almost completely under standard in vitro incubation conditions in contrast to those of the calf, goat, pig, rat and sheep (Patterson et al., 1969). An NADPH₂ - linked cytoplasmic enzyme system of duck liver was reported to be capable of reducing aflatoxin B₁ to the cyclopentenol, aflatoxicol (Patterson and Roberts, 1972). A reactive metabolite of aflatoxin B₁ binds covalently to tRNA when the nucleic acid is added to an aflatoxin B₁ metabolising system and more metabolite is bound when DNA is used in place of tRNA.

TABLE 1

TYPES OF AFLATOXIN

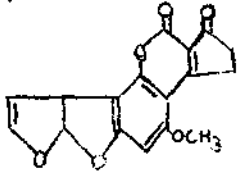
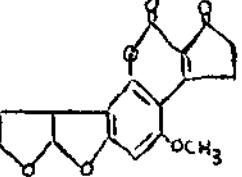
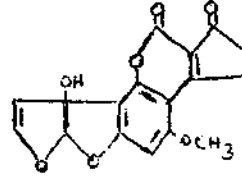
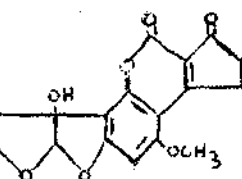
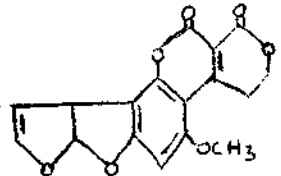
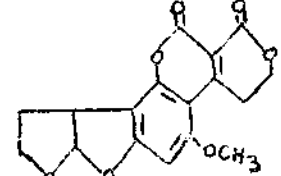
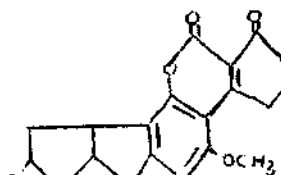
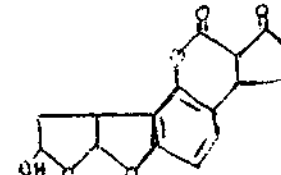
<u>Aflatoxin</u>	<u>Structure</u>	<u>Molecular Formula</u>	<u>References</u>
B ₁		C ₁₇ H ₁₂ O ₆	Allcroft & Carnaghan (1963)
B ₂		C ₁₇ H ₁₄ O ₆	Chang <u>et al.</u> (1963)
M ₁		C ₁₇ H ₁₂ O ₇	Holzappel <u>et al.</u> (1966)
M ₂		C ₁₇ H ₁₄ O ₇	Holzappel <u>et al.</u> (1966)

TABLE 1 CONT'D

<u>Aflatoxin</u>	<u>Structure</u>	<u>Molecular Formula</u>	<u>References</u>
G ₁		C ₁₇ H ₁₂ O ₇	Asao <u>et al.</u> (1963)
G ₂		C ₁₇ H ₁₄ O ₇	Asao <u>et al.</u> (1965)
G _{2a}		C ₁₇ H ₁₄ O ₈	Dutton & Heathcote (1968)
B _{2a}		C ₁₇ H ₁₄ O ₇	Dutton & Heathcote (1968)

entering the rat liver cell, aflatoxin passes to the nucleus where it binds to DNA and the RNA polymerase system. The reduced RNA synthesis involves reduction or inhibition of the messenger RNA which leads to reduced protein synthesis. Aflatoxin inhibited the DNA - dependent RNA polymerase system in rat liver nuclei soon after its administration in vivo interfering with gene transcription with levels of aflatoxin that were much lower than the rat LD₅₀ or that required to inhibit cytidine incorporation into RNA (Lilly, 1965).

Aflatoxins also cause biochemical alterations in tissues of plant origin. Schoental and White (1965) showed that aflatoxins in concentrations of 25 ug/ml inhibit the germination of the seeds of cress, Lepidium sativum while smaller concentrations interfered with chlorophyll synthesis. Clerk and Caurie (1968) reported that aflatoxins and some other metabolites of Aspergillus species have a damaging effect on the root tuber of cassava Manihot esculenta crantz by altering the amino acid and soluble carbohydrate content both quantitatively and qualitatively and a lowering of the total nitrogen content was also reported. In the cowpea, Vigna sinensis, Adekunle and Bassir (1973) observed that aflatoxin B₁ inhibits chlorophyll formation and seed germination. Black and Altschul (1965) reported that gibberellic

acid - induced increases in lipase and amylase activity of the germinating cottonseed are inhibited by aflatoxin.

Biological Effects of Aflatoxins

Aflatoxins have been shown to be toxic to animals, plants and probably man. In all cases toxicity depends on the dose, period of exposure and the test organism.

Newberne (1964) reported that metabolic products of A. flavus are toxic to many animal species with induction of histopathologic lesions such as hepatic parenchyma, necrosis and proliferation of bile ductule cells. The severity of the damage increased with successive purification of the toxic compounds. Butler (1969) observed that aflatoxin has the highest toxicity for mammals, poultry and fish. Studies with several species of fish revealed that aflatoxin B₁ show acute toxicity on several species of fish. LD₅₀ of the toxin in rainbow trout was 0.5 mg per kg body weight. The coho salmon was 10-20 times more resistant to aflatoxin.

Studies by various workers have indicated that the target organ is the liver (Muller et al., 1970; Newberne et al., 1964). Van Zytveld et al. (1970) reported the toxin deposits in skeletal muscles and livers of affected chicken with manifestations of the lesions in these areas. Muller et al. (1970)

working with birds found that livers of grossly affected birds showed brown to tan and some varying degree of discolouration. In addition the kidneys were pale and swollen. Wogan (1965) reported that sensitivity of test rats to aflatoxins decreases with age. The female rat is less susceptible to the acute effects, even at 21 days of age with this sex difference becoming more marked as sexual maturity is approached.

Subacute toxic effects of the aflatoxins in monkeys have been reported (Tulpule et al., 1964). Young rhesus monkeys were fed either 0.5 or 1.0 mg of aflatoxin per day for 18 days, then 1.0 mg per day thereafter. All animals developed anorexia and died in 14 to 28 days. Liver lesions, biliary cirrhosis - as there was portal inflammation and fatty change were the results of histopathological findings.

Aflatoxins have also been shown to be carcinogenic. These toxins were found to induce liver tumour in 9 out of 11 rats fed on aflatoxin contaminated groundnuts over a period of 6 months (Lancaster et al., 1961). They further observed lung metastases caused by these toxins.

Aflatoxicosis has not been well reported in man. However of recent Belchetz and Cohen (1976) related pulmonary adenomatosis with aflatoxin inhalation. This was from a case study. Hepatic

dysfunction and also death has been reported in an Indian village (Krishnamachari et al., 1975).

Control of Aflatoxin

The undesirable effects of toxic metabolite of certain moulds has led to finding ways of controlling both the growth of the moulds concerned and the production of such metabolites. A lot of work has been done on prevention of aflatoxin contamination at either Federal, State, Municipal and even Private levels. Most of these methods are aimed at inhibiting the growth of the mould.

Applegate and Chipley (1973) observed that the growth and sporulation of 2 strains of A. flavus were greatly reduced in both wheat and synthetic media by exposure of these organisms to a dose of 300 krad, with complete inhibition at 400 or 600 krad. Chemicals have been employed a lot in the control of aflatoxin. Rao and Harein (1971) used dichlorvos, an organophosphate insecticide on stored grains and reported prevention of aflatoxin production when 20 ppm dichlorvos was used. Sodium borohydride has been used on aflatoxin B_{2a} (Ashoor and Chu, 1975). They reported production of a new derivative, a reduced aflatoxin RB_{2a}. Another chemical, sodium hypochlorite was used on groundnuts (Natarajan et al., 1975). This exhibited inhibitory affects.

Sodium chloride at concentrations equivalent or greater than 12g/100ml, sodium acetate (4g/100ml), malonic acid 50mM, benzoic acid (100mg/25ml) were found to effectively inhibit growth and aflatoxin production by A. flavus (Uraih and Chipley, 1976). Growth and aflatoxin production of A. parasiticus has been inhibited by 12-mercaptoethanol when added to liquid media by inhibiting $[1-^{14}C]$ acetate incorporation into both aflatoxin and neutral lipids indicating target of action is the early stage of aflatoxin biosynthesis (Gupta et al., 1976).

The conventional solid smoke aerosol process has been reported to provide meat products with protection against microbial growth (White et al., 1942). Smoking of hams was shown to limit growth of staphylococcus aureus (Lechowich et al., 1956). With the level of smoke obtained in smoke house treatment of goods, Arseculeratne and Weliana (1976) found a total suppression of fungal growth on the majority of palm kernels and in the few instances where growth occurred, it was marked by a lack of toxin product. Hungarian type Salamis (a type of ground pork and beef) was reported unable to produce aflatoxin at any temperature or humidity when it was inoculated after smoking.

Aflatoxin Detoxification

The awareness of the toxicity of aflatoxin has led to finding different ways of detoxification of the toxins and these ways have been reported. Ceigler et al. (1966) screened intensively for aflatoxin detoxifying microorganisms and reported Flavobacterium aurantiacum and different Rhizopus species as potent aflatoxin degrading micro-organism. Mann and Rehn (1975) found that Corynebacterium rubrum degraded more than 99% of aflatoxin in a liquid culture after four days. They also reported Mycobacterium phlei, Candida species and some moulds as being active at degrading aflatoxin. Such moulds as Cunninghamella echinulata, Stachybotrys labulata, penicillium islandicum, Aspergillus niger and Aspergillus ochraceus were found to degrade aflatoxin B₁.

In addition to the use of microorganism, chemicals have been used. Yang (1972) observed that both cultures of A. flavus and aflatoxins were destroyed by commercial bleach, chlorox with sodium hypochlorite as the active ingredients or an analytical reagent grade of sodium hypochlorite ($7.0 \times 10^{-3} M$) in five days. Animals injected with such a destroyed aflatoxin extract survived with no obvious liver or kidney damage. Similarly Fishbach and Campbell (1965) observed that aflatoxins extracts treated with 5% sodium hypochlorite for a few seconds lost their fluorescence

and toxicity. They also reported that chick embryos were unaffected by contaminated peanut meal which had been previously exposed overnight to 10% chlorine gas. Natarajan et al. (1975) reported limited reduction of aflatoxin B_{2a} with sodium borohydride at a neutral or slightly alkaline pH resulted in a new reduced derivative which was non-toxic to chicken embryos at levels 100 times the LD₅₀ of aflatoxin B₁. Aflatoxin inactivation has been observed by treating cottonseed meal with ammonia and methylamine (Mann et al., 1971).

Preservation of Foods

The importance of food has led to the finding of various ways of extending the shelf life of our foods, particularly the perishables and the semiperishables. Various preservative methods have been employed, ranging from heat treatment, drying, ionising radiation to chemicals (Frazier, 1976; Jay, 1978).

Heating and Drying

The success of heat treatment in preservation of foods is due to protein coagulation and inactivation of enzymes required for metabolism in the microorganisms. During smoking, a certain amount of heat is necessary to produce smoke as the smoke aerosol is produced by burning dry or dampened hardwood (Draudt, 1963).

Part of the shelf stability of smoked products is due to heat destruction of surface organisms as well to drying that occurs during heating (Jay, 1978). According to Pearson (1976) during curing of fish a loss in weight of about 5 - 25% is caused by the smoking process due to its drying effect. That the moisture content of smoked products is affected during smoking was reported by Sink and Hsu (1977) when they observed that the moisture content of Frankfurter was affected by different smoke treatment.

Preservation by Chemicals

The ideal preservative would be one which in addition to being antimicrobial is non toxic and does not impart any bad odour, flavour or taste to the food being preserved.

Benzoic acid and Related Compounds

Chemicals have been used with great success in preventing or delaying the spoilage of foods by microorganisms. Benzoic acid and related compounds have been accepted as preservatives for certain foods (Jay, 1978). The methyl and propyl esters of parahydroxybenzoic acid are commonly used in the food industry as antimicrobial agents (Chichester and Tanner, 1972).

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism

Aspergillus flavus strain 586 IAR used for this study was collected from culture collection of the Institute for Agricultural Research, Zaria. Stock cultures were grown on potato dextrose agar slants. Working stock cultures were obtained by subculturing mycelia from stock cultures. Culture for spore development and subsequent substrate inoculation were obtained from working stock cultures after fourteen days of incubation. All active cultures were maintained in loosely fitted screw - cap vials at room temperature ($27 \pm 1^{\circ}\text{C}$). Spore suspension were prepared by adding sterile distilled water to fourteen day-old sporulated cultures and spores were collected by suction filtration. Spores were washed twice using 50ml. of distilled water and then filtered aseptically.

Growth Substrates (Media)

Pawpaw fruits (carica papaya) and fish (Tilapia spp) were used solely as the growth media for Aspergillus flavus.

Pawpaw Fruit Medium (Table 2)

The fruits were obtained locally and peeled to remove the rind. They were cut into small pieces and ground into a fine watery mash. Thirty gram of the fruit mash was respectively weighed into 250 ml. Erlenmeyer flasks. Different levels of benzoic acid derivatives were added to these flasks (each level in quadruplicate) and autoclaved at 15 lb/sq inch for 15 minutes. Half a ml of the fungal spore suspension (10^5 spores/ml) was aseptically inoculated in quadruplicates, into each flask and incubated at room temperature ($27 \pm 1^\circ\text{C}$) for seven days.

Fish Medium (Table 3)

Fresh fish locally purchased were scaled, gutted and dried in the sunlight for one hour to produce a firm surface for further processing. They were selected into two equal groups on the basis of size. One half was smoked in the smoking unit (fig. 1). Some were smoked at $55 - 65^\circ\text{C}$ for six hours and some for eight hours to a brittle texture. The fish were spaced out in their arrangement on the wire mesh and turned at intervals to enable uniform smoking.

The other half was dried at 100°C in an electrically heated oven, some for four hours and some for five hours.

TABLE 2

Trace elements and sugar content of pawpaw fruits
(Carica papaya Var West Indian) according to Bessir and
Adekunle (1972).

<u>Trace Elements</u>	
Element	g/g sample
Magnesium	250
Zinc	100
Iron	90
Molybdenum	50

<u>Sugar Content</u>	
Sugar	Concentration mole/g sample $\times 10^{-2}$
Fructose	27.04
Glucose	24.81
Sucrose	2.92
Maltose	Abs
Lactose	Abs

Abs = Absent

TABLE 3

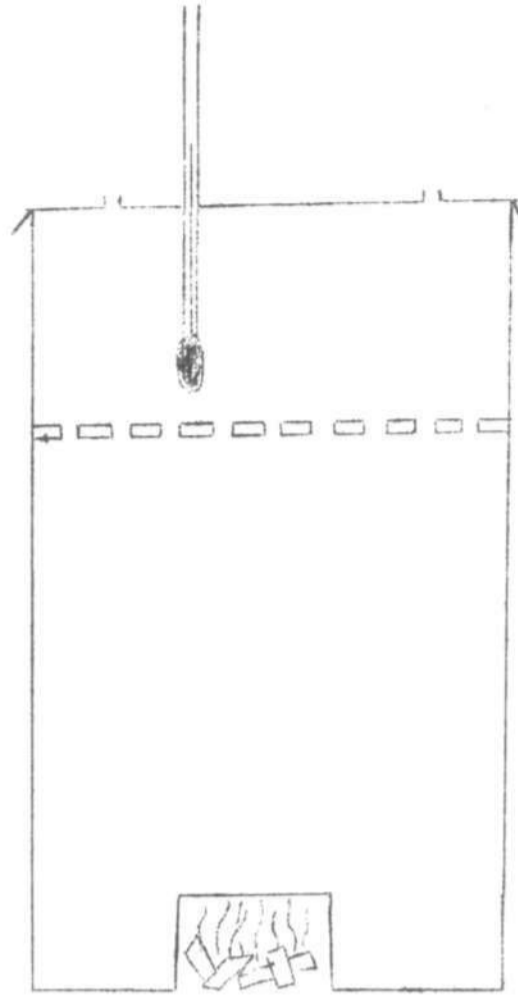
Composition of fish, 100 grams edible portion (Pellet and Shadarevian, 1970).

Water	} Main Constituents of fish	72.0g
Protein		19.0g
Fat		8.0g
Calcium		50mg
Phosphorus		250mg
Iron		1.1mg
Ash		
Thiamine		0.10mg
Riboflavin		0.20mg
Niacin		3.0mg
Waste		50g

Twenty gram of fish was placed in large petri dishes (140 diameter) and autoclaved at 15 lbs/sq inch for 15 minutes and inoculated with 0.5ml. of the fungal spore suspension. All the samples were incubated at room temperature $27 \pm 1^{\circ}$)

Figure 1

Smoking Unit



Mycelial Dry Weight

Whatman 1 filter paper (12.5cm) and Petri dishes (9.5cm diameter) were employed for this determination. Thirty gram watery fruit mash was respectively weighed into 250 ml Erlenmeyer flasks, and different levels of the salts incorporated into the flasks and autoclaved as previously described. The content of each flask was transferred aseptically into sterile Petri dishes. These were overlaid with sterile filter paper on top of which 0.5ml of the fungal spore suspension was inoculated. Incubation was for seven days at room temperature ($27 \pm 1^{\circ}\text{C}$) after which the plates were steamed to enable easy lifting of the filter paper/mycelial mat. They were dried in the hot air oven at 100°C for 30 minutes and weighed.

Removal of Pigment

Ten ml acetone water (85:15) was added to 30g portion of the substrate/fungus and shaken thoroughly for 3 minutes in a lab-line orbit environ-shaker (Lab line Instr., Inc.) at 200 rpm, filtered through 24cm filter paper (Whatman 2v) into 100ml graduated cylinder.

90ml filtrate was transferred into 250ml Erlenmeyer flask containing ferric gel prepared as follows; 10ml of 10% FeCl_3 solution was added to 100ml distilled water in 250ml Erlenmeyer flask. 15ml of 4.83% NaOH solution was also added swirled to mix and form a gel. After addition of the filtrate to the ferric gel the flask was stoppered and shaken vigorously for 45 seconds and filtered through 24cm filter paper (Whatman 2v) into 250ml graduated cylinder. 180ml of this filtrate was transferred to 500-ml separatory funnel containing 180ml distilled water.

Extraction of Aflatoxin

50ml chloroform was added to the separatory funnel and shaken vigorously for 45 seconds. The chloroform layer was drawn off into 250ml round-bottomed evaporatory flask and evaporated to 10ml using a rotary flash - evaporator (Buchler Instr. Inc.)

Amount of substrate involved in the extraction (Valesee
Fluorotoxin instruction manual).

30g Mashed fruit in stoppered
250ml conical flask

100ml
acetone-water
(85:15)

Shaken for 15 min and
filtered through Whatman
1 filter paper 0.3g/ml

90ml filtrate + 125 ml ferric gel

$(0.3 \times 90) \text{ g/ml substrate}$
 $\frac{27}{5}$

Shaken for 3 mins. and
filtered through Whatman
1 filter paper

180ml. filtrate

$180 \times 0.3 \times 90$
 $\frac{27}{5} \text{ g substrate}$

= 22.6g substrate

Calibration of the Toxin meter

The blank was prepared by wetting the prepared microcolumn with chloroform: methanol (96:4) from the base.

The instrument was calibrated using aflatoxin standard prepared as follows:

The preparation is a modification of the velasco Fluorotoxin manual instruction. To obtain an expected aflatoxin concentration of 20mg/ml appropriate dilution of the standard using chloroform as solvent was done and tested for the absorbance value on CE 505 cesil spectrophotometer at maximum absorption wavelength (365nm) from which the corresponding concentration was calculated using the formula:

$$\text{Concentration of aflatoxin} = \frac{A \times M.w \times 1000 \times C.F.}{E}$$

where A = Absorbance

M.w. = Molecular weight of aflatoxin type

E = Molar absorptivity of the aflatoxin type

C.F. = Correction factor for the instrument

= 0.90

(Rodricks and Stoloff, 1970).

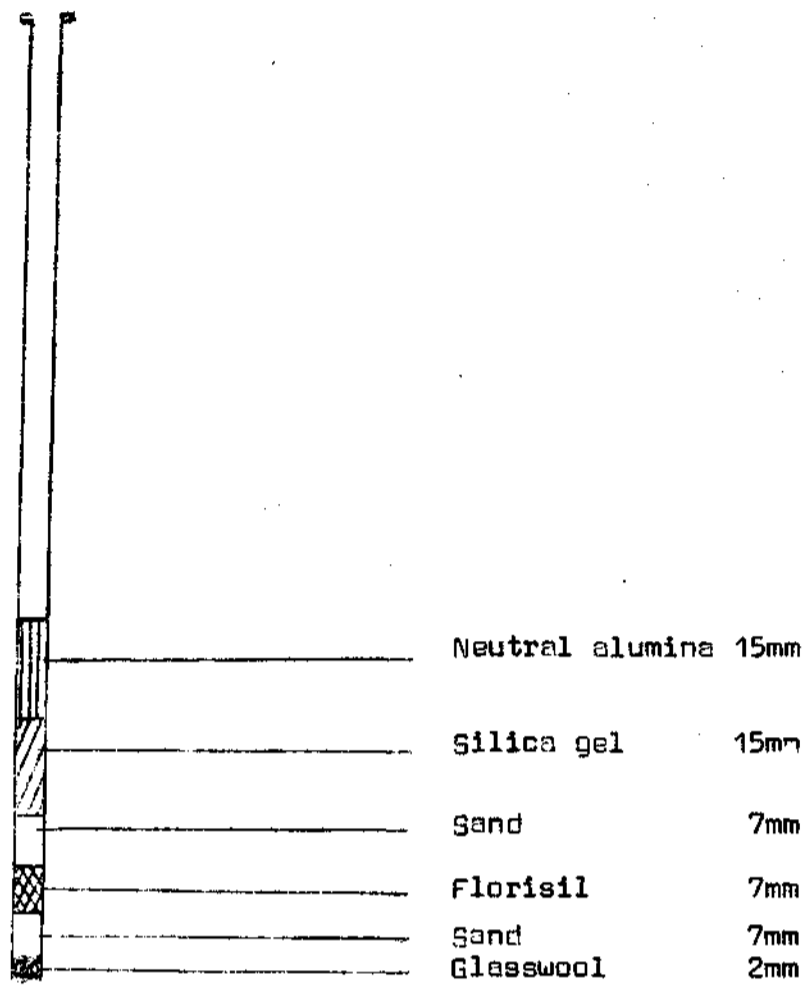


Plate 1: Fluorotoxin Meter.



Plate 1: Fluorotoxin Meter.

FIG. 2

Diagram of Microcolumn

Determination of Aflatoxin Concentrations

Using a glass syringe, 1ml of the sample pre-diluted with chloroform was injected into pre-wetted microcolumn and allowed to drain. One ml chloroform: methanol (94:4) was added on top and again allowed to drain. While the column was still wet, it was placed on the precalibrated velasco Fluorotoxin meter for reading.

Bioassay

Preparation of Diets

Three of the five diets contained minimal inhibitory levels of the salts used. The fourth diet contained aflatoxins and the fifth was the control - pawpaw and commercial feed only. Amounts of the different diets containing a total of 600mg sodium benzoate, 300mg O-hydroxybenzoic acid (salicylic acid), 180mg p-amino ethylbenzoate, all being minimal inhibitory levels, and 14.02mg aflatoxins were pooled in separate round bottomed flasks. They were freeze dried at -80°C using a freeze drier. Each diet was then dissolved in distilled water to give a semisolid paste.

Feeding

Day old white Sabcock cockerels were used for the feeding trial. The chicks were housed in four labelled cages in groups of ten per cage, in warm brooders at temperature of $31 \pm 1^{\circ}\text{C}$. In addition to the commercial feed, each group was fed on a diet corresponding to the label on the cage by crop intubation. Feeding was done twice daily for a period of 10 days, after which livers were removed weighed and sections were placed in 10% formalin to be processed for histopathologic study. Tissues were stained with haematoxylin and eosin.

R E S U L T S

TABLE 4

Effect of sodium Benzoate on growth and aflatoxins production by Aspergillus flavus (IAR 586)

Salt mg/30g Substrate	Mycelial dry weight g/30g Substrate	Percentage content of Salt	Aflatoxins µg/g substrate				
			B ₁	G ₁	B ₂	G ₂	Total
0	0.96	0	21.5	20.1	19.6	16.7	77.9
10	0.97	0.03	20.2	18.6	15.3	15.1	69.2
20	0.89	0.06	19.5	18.2	12.7	13.0	63.4
30	0.85	0.10	18.6	16.9	10.0	10.1	55.6
40	0.78	0.13	15.4	15.0	9.2	8.9	48.5
50	0.60	0.16	8.1	8.0	7.4	6.7	30.2
60	0.53	0.20	5.5	4.2	3.7	4.0	17.4
70	0.30	0.23	3.0	2.7	2.9	2.5	11.1
80	0.21	0.26	1.2	0.8	0.8	0.4	3.2
90	0.10	0.30	0.6	0.09	0.04	0.02	0.75
100	0	0.33	0	0	0	0	0

Plate 2: Photomicrograph of liver from chick incubated with sodium benzoate supplemented diet. Normal. Haematoxylin and eosin stain. Magnification: x 500.

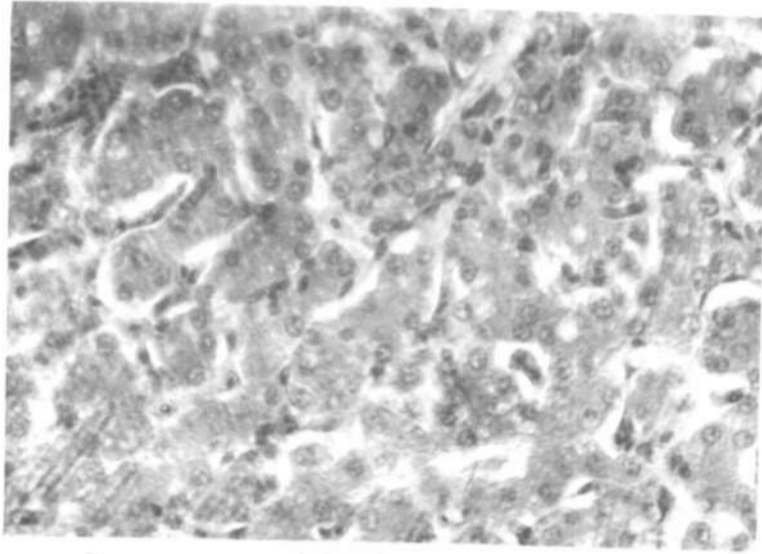


TABLE 5

Effect of *o*-hydroxybenzoic acid (Salicylic acid) on growth and aflatoxins production by Aspergillus flavus (IAR 586)

Salt mg/30g Substrate	Mycelial dry weight g/30g Substrate	Percentage content of Salt	Aflatoxins µg/g substrate				
			B ₁	G ₁	B ₂	G ₂	Total
0	0.96	0	21.5	20.1	19.6	16.7	77.9
10	0.73	0.03	19.2	18.0	18.4	17.1	72.7
20	0.61	0.06	18.1	19.6	16.3	11.3	65.6
30	0.42	0.10	14.5	13.0	10.0	9.8	48.2
40	0.21	0.13	6.1	2.5	2.1	0.3	11.0
50	0	0.16	0	0	0	0	0

Plate 3: Photomicrograph of liver from chick incubated with D-hydroxybenzoic acid supplemented diet. Normal. Haematoxylin and eosin stain. Magnification: X 500.

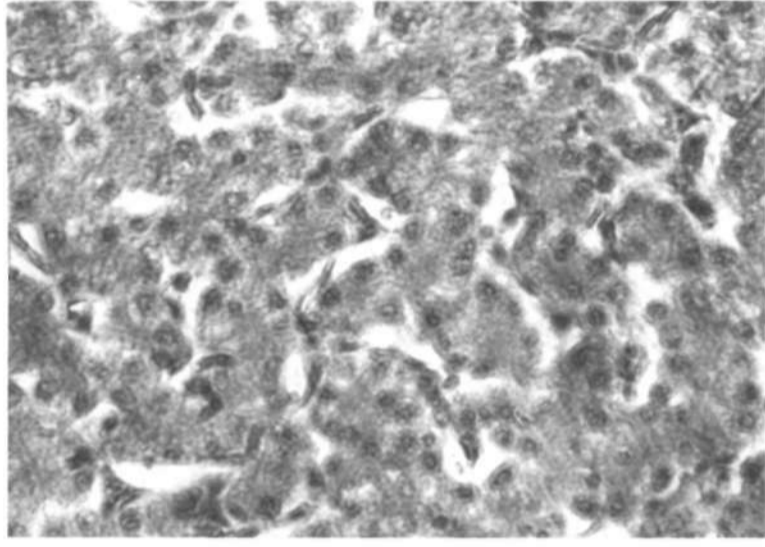


TABLE 6

Effect of p-amino ethyl benzoate on growth and
 aflatoxins production by Aspergillus flavus (IAR 586)

Salt mg/30g Substrate	Mycelial dry weight g/30g Substrate	Percentage content of Salt	Aflatoxins µg/g substrate				
			B ₁	G ₁	B ₂	G ₂	Total
0	0.96	0	21.5	20.1	19.6	16.7	77.9
10	0.54	0.03	13.2	12.9	10.4	9.1	45.6
20	0.17	0.06	2.5	2.0	1.9	2.0	8.4
30	0	0.10	0	0	0	0	0

Plate 4: Photomicrograph of liver from chick intubated with p-aminethybenzoate supplemented diet. Normal. Haematoxylin and eosin stain. Magnification: X 500.

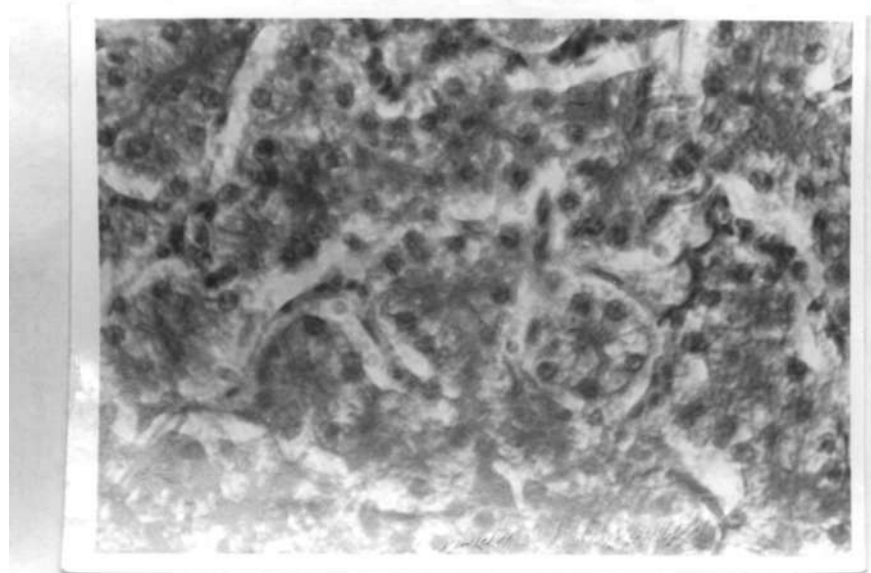


TABLE 7

Effect of inhibitory levels of salts and aflatoxins
on chicks in ten days

Diet Supplement	Initial weight (g)	Day 10 weight (g)	Live# Total	Weight % Body Weight	Histology result
0	35.0	63.4	3.0	4.7	Normal
Sodium Benzoate	33.1	58.5	2.5	4.2	Normal Pl. 2
p-aminethyl benzoate	34.6	61.0	2.7	4.4	Normal Pl. 4
O-hydroxy benzoic acid	32.0	60.0	2.6	4.3	Normal Pl. 3
Aflatoxin	33.0	45	1.5	3.37	Perivascular and cytoplasmic degeneration. Ductular cell proliferation.

Plate 5: Photomicrograph of Liver from Chick Intubated with
Aflatoxin containing diet. Ductular cells proli-
feration in rows. Haematoxylin and eosin stain.
Magnification: X 500.

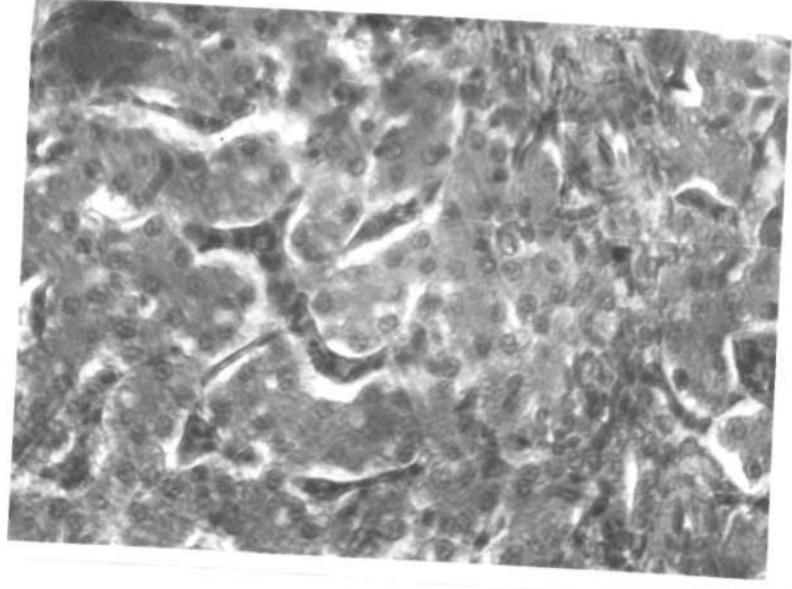


Plate 6: Photomicrograph of liver from chick intoxicated with aflatoxin containing diet. Parenchymal cell damage. Magnification: X 500.

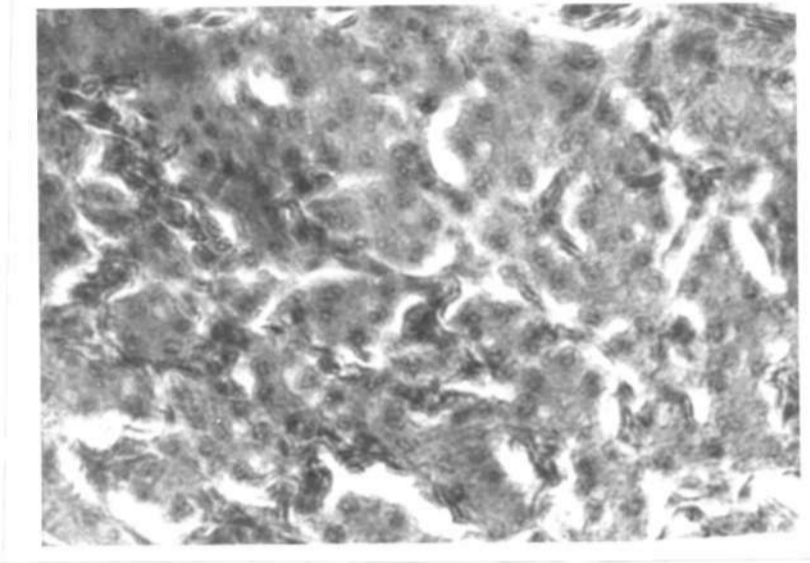


Plate 6: Photomicrograph of Liver from chick intubated with aflatoxin containing diet. Parenchymal cell damage. Magnification: X 500.

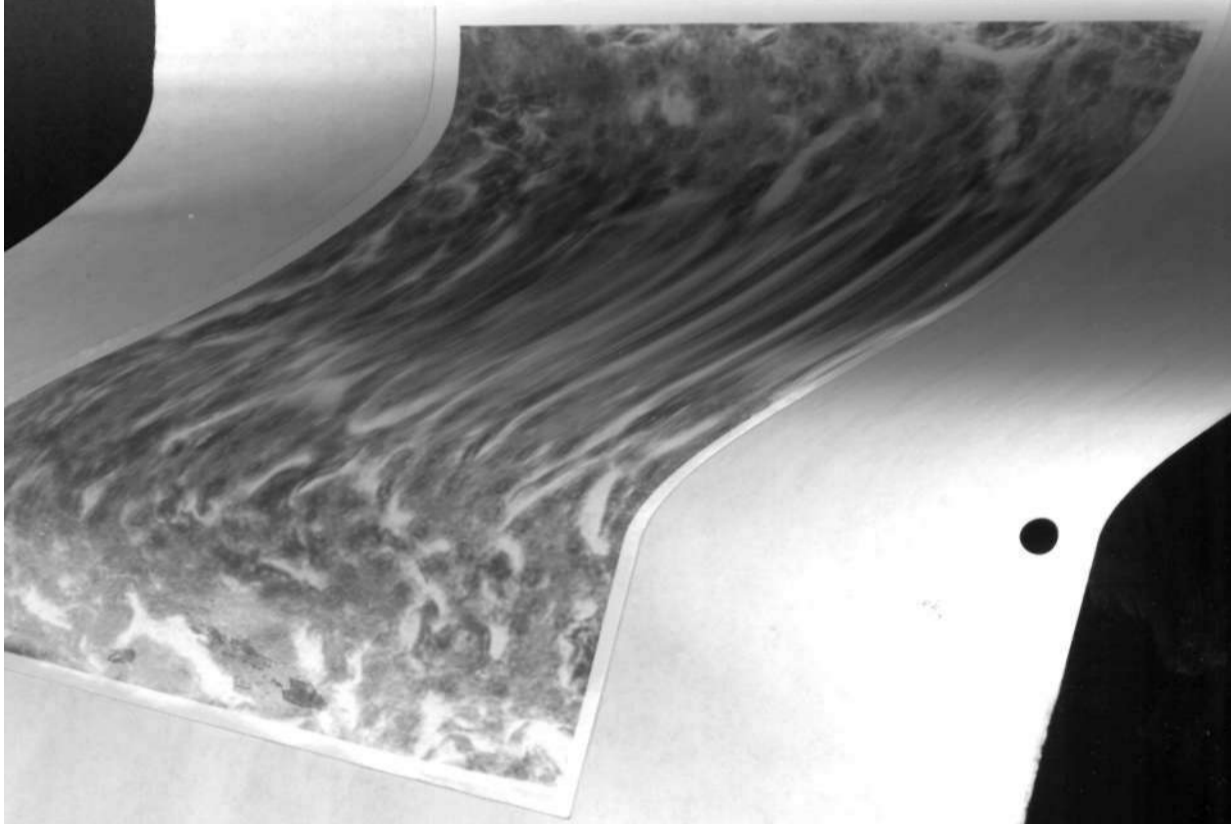


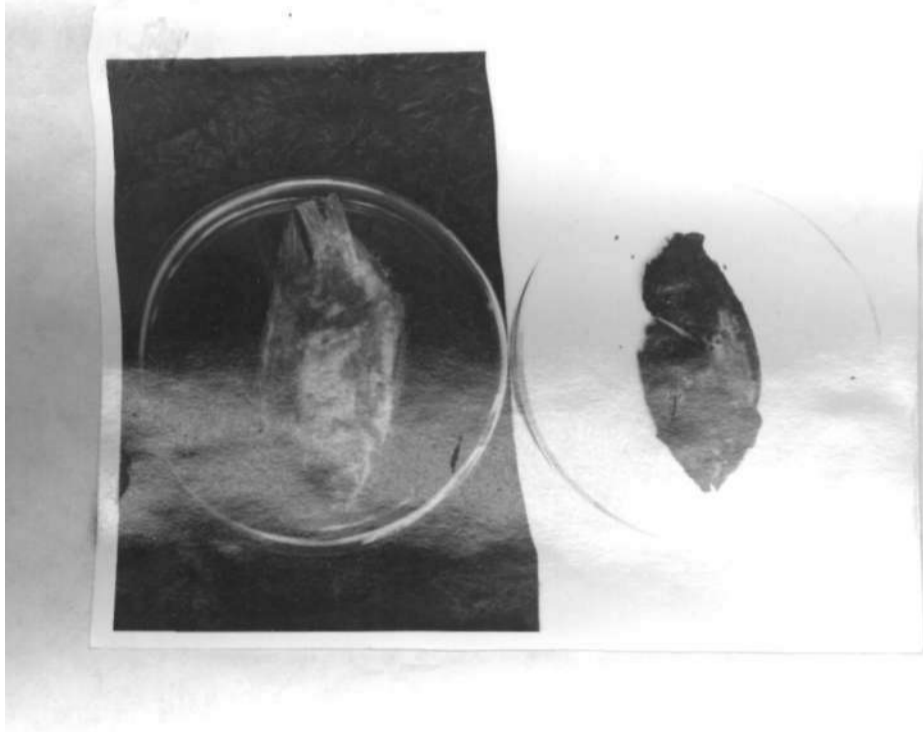
TABLE 8

Effect of wood smoke on aflatoxin production by
Aspergillus flavus (IAR 586)

	Aflatoxins Unsmoked		$\mu\text{g/g}$ Smoked	
	4 hours	5 hours	6 hours	8 hours
B ₁	1.65	0.54	0.022	0
G ₁	1.28	0.52	0.020	0
B ₂	0.81	0.39	0.016	0
G ₂	0.64	0.15	0.013	0
Total	3.38	1.6	0.071	0

Inoculated smoked (left) and unsmoked (right) fish after 7 days. Fluffy margin and general mouldy appearance indicative of the luxuriant growth on the unsmoked fish. Small patches of stunted growth on the smoked fish. Magnification: X 1/5.

Plate 7:



Inoculated smoked (left) and unsmoked (right) fish after 7 days. Fluffy margin and general mouldy appearance indicative of the luxuriant growth on the unsmoked fish. Small patches of stunted growth on the smoked fish. Magnification: X 1/6.

Plate 7:

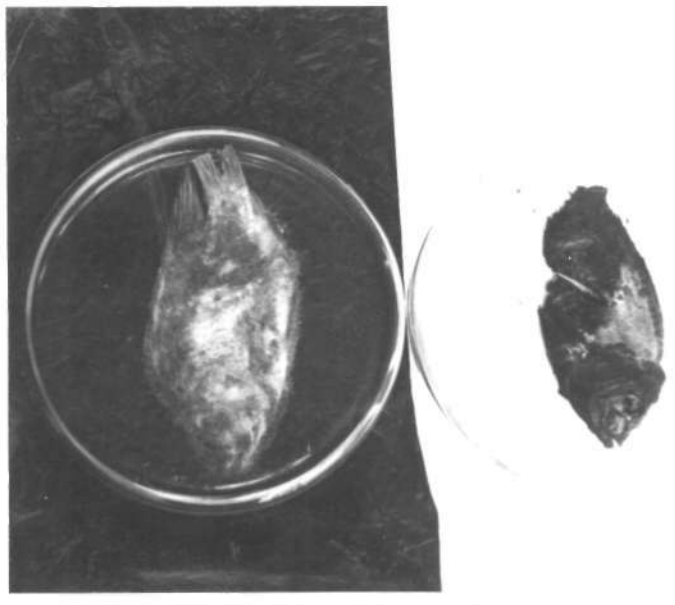


Plate 8: Inoculated pairs of smoked (left) and unsmoked (right) fish after 7 days. Bottom pair processed for a longer period - Note absence of growth as compared with top pair. Magnification: X 1/6.

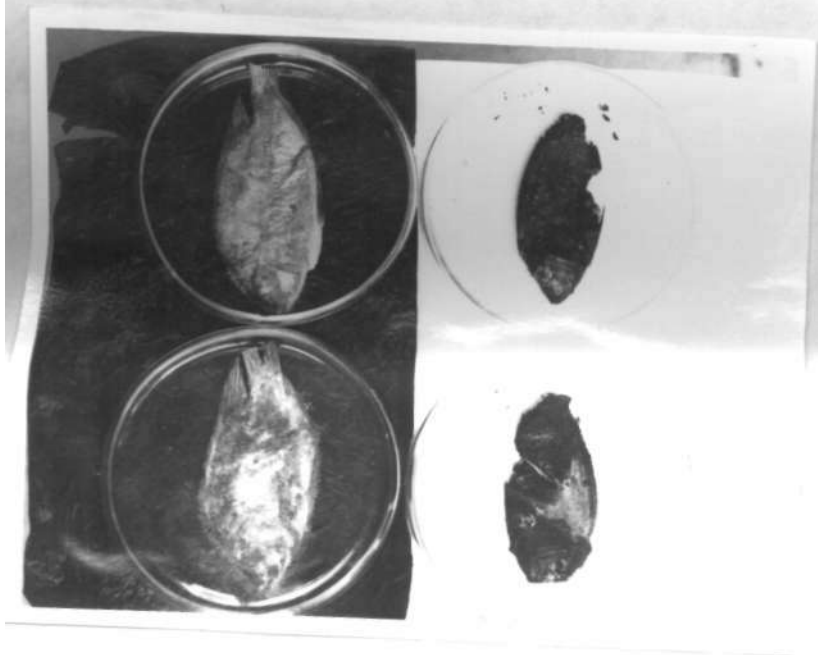
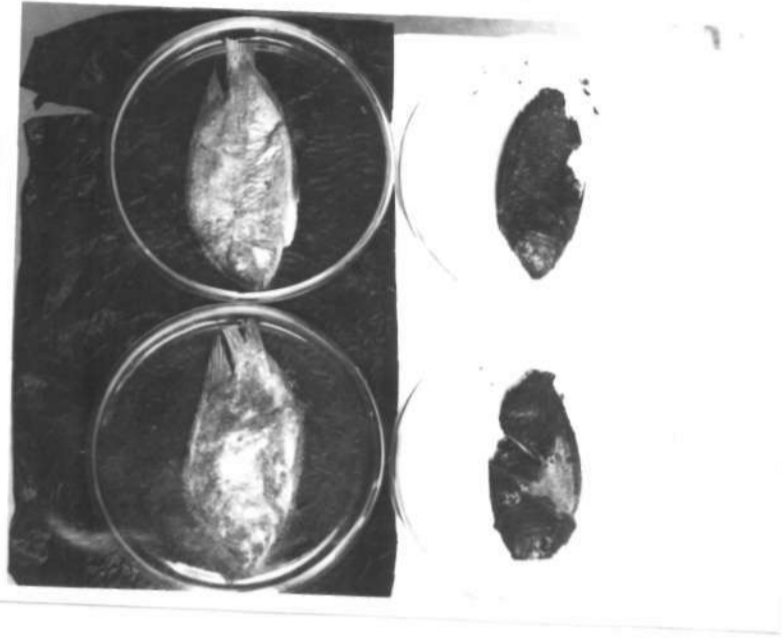


Plate 8: Inoculated pairs of smoked (left) and unsmoked (right) fish after 7 days. Bottom pair processed for a longer period - Note absence of growth as compared with top pair. Magnification: X 1/6.



D I S C U S S I O N

DISCUSSION

The three benzoic acid derivatives employed in this study have proved to be inhibitory to both growth and aflatoxin production by the A. flavus on pawpaw fruits (Tables 4, 5 and 6). The inhibitory effects of benzoic acid and related compounds has been reported by various workers. Uraih and Chipley (1976) described the inhibitory effects of some benzoic acid derivatives on A. flavus grown on a synthetic medium. Benzoic acid has been shown to give a preservative effect in herring Salad (Heintze, 1978). Also a delay in the excystment of Schizopyrenus russeli cysts when treated with p-chloromercuric benzoate has been reported (Rastogi et al., 1969). The mode of action of these salts has been reported (Uraih et al., 1977). A study of a number of phenolic compounds such as benzoic acid derivatives and cinnamic acid with respect to their inhibitory effect on potato tuber lactate dehydrogenase isoenzymes, showed that the inhibition was the non competitive type (Rothe, 1976). The pH of each of the salt employed in this investigation in the medium,

at complete inhibition was 5.0, and the antimicrobial action and preserving effect of benzoic acid and its derivatives have been shown to be strongly dependent on the pH of the medium (Smith, 1962; Heintze, 1978). With as low pH value of between 2.3 to 2.4, Cruess and Richert (1929) reported that only 0.02 to 0.03 percent sodium benzoate was required to prevent growth of most fermentative organisms, however the much higher salt percentage (0.33) obtained in this study for sodium benzoate may possibly be due to the higher pH value.

A much higher concentration of sodium benzoate (100mg/30g substrate) and consequently a higher salt percentage of the fruit substrate was required to effect complete inhibition of both mycelial growth and aflatoxin production as compared with the lower concentrations of *o*-hydroxybenzoic acid and *p*-aminoethyl benzoate which are 50mg/30g substrate and 30mg/30g substrate respectively for the same effect. Factors other than pH may be responsible for this variation in the effective concentrations. Lynch and Geoghegan (1979) reported of a similar effect with a family of antibiotics which show differential toxicity between closely related species. This goes to suggest that mechanism of action is probably a function

of various chemical properties associated with the compounds, in which case the mechanism of inhibition of aflatoxin biosynthesis may be different for the different compounds as suggested by Rao et al. (1979) after a study of the uptake pattern of various labelled compounds in asparagina and zinc deficient A. parasiticus culture. The active agents in benzoic acid and its derivatives have been said to reside in the undissociated acid or in the substituted benzoate molecules (Hcintze, 1978). The salts used in this study have different substituted moieties. In addition the location of the carboxylic group in the aromatic ring is significant for the range of inhibition (Rothe, 1976).

At complete inhibition, the percentage content of the three salts stood at 0.33, 0.17 and 0.10 for sodium benzoate, *o*-hydroxy benzoic acid and *p*-aminoethyl benzoate respectively (Tables 4, 5 and 6). Except for the last, the first two salts exceed the legal limit (1000 parts/million in foods) for benzoic acid and related compound in the United States (Jay, 1978). The pH (5.3) of the pawpaw is probably responsible for these high values as benzoic acid and related compounds are used effectively within legal limits in high acid foods. Stern et al. (1979) reported that Trypticase Soy

broth containing various concentrations of butylated hydroxy-anisole and sodium chloride at pH of 5.0 and upward though effective against Staphylococcus aureus at the levels tested, became more effective in preventing growth as the pH of the medium was decreased.

The result of the feeding trial showed that sodium benzoate, O-hydroxybenzoic acid and p-aminoethylbenzoate are all non toxic to chicks at levels used (Plates 2, 3 and 4). This is in line with the expectation that an ideal preservative in addition to being microbicidal should be non toxic to the consuming public. In fact such phenolic compounds as benzoic acid, salicylate and p-hydroxybenzoate are produced by plant organs and thus occur naturally in plants (Lepold, 1964). The metabolism of benzoic acid has been studied. Chantrenne (1960) reported is rather over-used that benzoic acid is metabolised to form hippuric acid when conjugated with glycine in the system. Martin (1966) reported the excretion of hippuric acid in sheep fed various concentrations of benzoic acid. He further observed that the lethal dose for the sheep is close to 1g/kg body weight. This by far exceeds the amount used in this study. In another feeding trial with sixty-five

benzoic acid derivatives, it was observed that benzoic acid was non repellent to rodents. Tests with 300 other carboxy acids showed that there was no repellency connected with the carboxyl group itself, however when associated with other groups, compounds of high repellency were produced (Fearn and Dewitt, 1965). The non toxicity of these salts notwithstanding, an investigation into sensory evaluation of high moisture dried prunes preserved with sodium benzoate revealed that the panelist detecting benzoate, up to 950 parts/million. Although, they did not describe the flavour as objectionable a slightly different taste with a burning sensation was noted. A good preservative should not be taste perceived. From the results of these present studies and from a toxicological point of view, these salts could be used as preservatives for pawpaw.

The report of the existence of the phenomenon of taste blindness in respect of sodium benzoate (Fox, 1954) necessitates an investigation into the taste perception of these salts in pawpaw.

The aflatoxins gave histopathological lesions as shown in table 7 and plates 5 and 6. There is a general loss in

weight and also a lower value of total liver weight percent body weight as compared with those of the control chicks and chicks fed on salts supplemented diets. Studies of the metabolism of aflatoxins in different animals showed that once the toxin has reached the liver cell, the factor responsible for tissue injury in a particular animal species depends on the rate of and pattern of aflatoxin metabolism. When the toxin is metabolised slowly, untransformed toxin is the active part with chronic liver damage as the result. From the method of feeding used in this study, metabolism can be said to have been slow. However when it is metabolised rapidly the metabolites rather than the original toxin are involved, resulting in acute liver damage (Patterson, 1973). Huff et al. (1979) reported of cytoplasmic deposits of glycogen at liver lobe periphery of broiler chickens due to inhibition of glycogenolysis during ochratoxicosis. This probably explains for the cytoplasmic lesions observed. On the other hand inhibition of ribonucleic acid polymerase of rat liver and Escherichia coli and nuclear ribonuclease of rat liver and Tetrahymena pyriformis by various mycotoxins has been reported (Tashiro et al. 1979).

Investigations on wood-smoke have yielded the results in table 8, plates 7 and 8. Woodsmoke to a considerable extent has exhibited an inhibitory effect on mycelial growth and aflatoxin production by A. flavus. Although the definition of 'preservative' excludes substances added during smoking, the smoke given out during burning of wood is known to impart certain chemicals to products being smoked (Frazier, 1976; Jay, 1978; Sink, 1977) and these are observed to be microbicidal. Woodsmoke contains a large number of volatile compounds that differ in their bacteriostatic and bactericidal effect (Draudt, 1963). In addition to formaldehyde which is said to be most effective against microorganisms, other chemicals that are components of woodsmoke are shown in Table 9. This inhibitory effect of woodsmoke was more marked on toxin production (Table 8) than on mycelial growth of A. flavus (plate 7). Natorwicz et al. (1979) reported that aflatoxins were undetected in green and roasted regular coffee beans even though there was viable mould growth at moisture levels of 28% and 48% respectively. While only 0.071 g/g was produced on the smoked fish, 3.58 g/g was produced on the unsmoked fish. Arseculeratne (1976) reported a similar effect of smoke on smoked co-branuts.

Table 9Constituents of hardwood smoke
(Draudt, 1963).

<u>Constituents</u>	<u>Weight % Moist Wood</u>
Formaldehyde	0.12
Higher aldehydes	0.57
Ketones	0.67
Formic acid	0.38
Acetic and higher acids	1.71
Methyl alcohol	0.96
Tar	4.81
Water	2.42
Phenols	0.07
Resins	4.21

On the antimicrobial property of wood smoke, Lechowich et al. (1956) reported that growth of Staphylococcus aureus was limited on hams treated with wood smoke, while Tatini et al. (1976) observed and reported that pepperoni processed with smoking in two ways was not likely to be a health hazard from Staphylococcal enterotoxins as the growth of the toxin producing organism was hampered.

Smoking the fish to a brittle texture (for eight hours) resulted in an inhibition of both growth and aflatoxin production (Plate 8). This may be due to a reduction in the moisture content to the minimum through the heating and drying effects of smoking. Natorwicz et al. (1979) reported that even at moisture level of 17% and 34% for green regular and roasted regular coffee beans respectively, there was no visible mould growth while aflatoxins were detected in decaffeinated beans. Jensen (1943) reported that the moisture content of grains influences the bacteriostatic action of volatile fatty acids on the grains. Edelman (1939) reported the same effect for meat and Arseculeratne et al. (1976) for cobranuts. It is a common practice particularly in the local fish producing areas to find freshly smoked fish that contain reasonable amount of moisture. Nwokolo and Okonkwo

(1968) in a survey of aflatoxin load of common Nigerian foods reported that foods such as dried fish stored poorly for long periods carry unwholesome quantities of aflatoxin. If these fish are to be transported to non fish producing areas (which is larger, in this country) for sale, it is essential that they be smoked to a level of minimal moisture and further protected by being properly stored or redried from time to time to avoid the risk of being overgrown with fungi and toxins contamination.

In addition to the preservative effect of woodsmoke, the flavour and colour imparted during smoking (White et al. 1942) are desirable to the consuming public. However for economic and toxicological reasons a developing nation like ours would do well with such a cheaper and equally effective method of controlling aflatoxin contamination of food materials as the elaborate techniques in developed world.

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