DETERMINATION OF EXPOSURE OF HUMANS TO SELECTED MYCOTOXINS WITH PARTICULAR REFERENCE TO AFLATOXINS

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ABSTRACT

Mycotoxins are poisonous secondary metabolites commonly produced by fungi and are involved in human disease conditions known as mycotoxicoses. There is evidence to show that food eaten by the rural Black population of Southern Africa is contaminated with mycotoxins. A tenuous relationship exists between the occurrence of mycotoxins in foods and certain disease conditions in humans.

In order to verify this relationship, efforts have, in the past, been made to detect mycotoxins and their metabolites in physiological fluids and tissues. The difficulty with this approach is that mycotoxins in the body have short half lives, being rapidly excreted or metabolised to other forms. More recently it has been shown that aflatoxin B_1, as its activated epoxide, can conjugate with macromolecules such as nucleic acids and proteins. These survive for much longer than the free toxins and by suitable methods can be isolated and measured. This allows for a much better estimate of exposure of the individual to aflatoxin.

This study reviews and evaluates screening methods for the detection and analysis of mycotoxin contamination in rural foodstuffs such as maize and groundnuts. Methods for the production of aflatoxin-lysine and protein adducts are motivated and developed then used in the identification of naturally occurring adducts in humans. Isolation and quantitative analysis techniques are proposed to routinely screen patients for evidence of aflatoxin exposure.

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this study was carried out in the Department of Physiology, Medical School, University of Natal, under the supervision of Professor M.F. Dutton.

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ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

absorbance	Abs
aflatoxin B_1	AFB_1
aflatoxin B_2	AFB_2
aflatoxin G_1	AFG_1
aflatoxin G_2	AFG_2
aflatoxin M_1	AFM_1
aflatoxin Q_1	AFQ_1
aflatoxicol	AFL
Analar grade	AR
N,O-bistrimethylsilyl acetamide	BS
dimethyl sulphoxide	DMSO
bovine serum albumin	BSA
m-chloroperbenzoic acid	MCPBA
deoxyribonucleic acid	DNA
diethylstilbestrol	DES
enzyme labelled immunosorbent assay	ELISA
emission	Em
ethylenediamine-tetra acetic acid	EDTA
excitation	Ex
fumonisin B_1	FB_1
gas chromatography mass spectrometry	GCMS
gas liquid chromatography	glc
gel permeation chromatography	GPC
glutathione-S-transferase	GST
hepatocellular carcinoma	HCC
hepatitis B virus	HBV
hepatitis B virus antigen	HBVAg
high performance liquid chromatography	HPLC
immuno-affinity column	iac
King Edward VIII Hospital	KEH
leukoencephalomacia	LEM
mass to charge ratio	m/z
mass spectrometer	MS
mixed function oxidase	MFO

moisture content	m/c
monoclonal antibody	MAb
4-(4-nitrobenzyl) pyridine reagent	NBP
nucleoside adenosine diphosphate	NADPH
nuclear mass resonance	NMR
oesophageal cancer	OC
polyacrylamide gel electrophoresis	PAGE
protein energy malnutrition	PEM
relative humidity	RT
ribonucleic acid	RNA
strong anion exchange	SAX
temperature	T
tris-borate-ethidamide	TBE
tris-ethidamide	TE
ultra violet	UV

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

As we approach the end of the 20th century, mankind faces a number of problems not the least of which is the growing population. With overcrowding on earth, man uses sophisticated technology to explore outer space and assess the possibilities of colonizing space stations. Despite the modern technology which renders space exploration feasible, millions of tonnes of cereals are lost annually due on earth to spoilage of various sorts. Famine and malnutrition are the by-products of the population explosion, especially in the poorer countries.

There is increasing global concern regarding the world's food supply and thus the conditions under which food is stored must be optimized. To minimise losses, hazardous conditions, such as mycotoxin contamination of cereals and groundnuts must be considered. Mycotoxin contamination is not only a problem for the third world as it has been encountered in the United States, a country with advanced agricultural food marketing systems (Roos, 1986).

Toxigenic fungi and mycotoxin contamination of foodstuffs, together with the diseases that are thought to be related to their ingestion, also occur worldwide. It has been estimated by the Food and Agriculture Organisation of the United Nations, that a quarter of the world's food crop is spoiled annually by the production of mycotoxins. In 1986, Mannon and Johnson, found that, in Africa and the Far East alone, between 10% and 50% of cereal crops were contaminated. Since much of these contaminated crops are nevertheless ingested by both animals and man, the effects of mycotoxins, both on man and economically valuable livestock, are important and motivate research in this direction.

Mycotoxin ingestion is particularly prevalent in third world rural areas where populations subsist on diets derived largely from poorly stored commodities such as maize. Maize is the staple diet of the majority of the Black population of South Africa. Since grain production is seasonal and consumption is continuous, the grain must be stored until it is needed for consumption. Storage facilities range from sites directly on the ground, piles of bagged grain and underground pits, to storage bins of many sizes, shapes and types of construction (Beck, B., pers. comm.[1]). To ensure uncontaminated storage, the grain must be protected from extreme weather conditions, moulds, other micro-organisms, rodents and insects (Beck *et al., 1991)*. For safe storage, the highest moisture content, (m/c), and extremes of temperature must be considered, since these will influence how fast storage fungi will develop and the extent to which damage may occur (Christensen and Kaufmann, 1969).

Mycotoxicoses has been documented since ancient Egyptian times and is typified by the medieval affliction known as St Anthony's Fire. These conditions were later shown to have been related to the ingestion of food materials contaminated with toxins produced by the fungi invading the food (van Rensburg and Alternkirk, 1974). It is currently suspected that other disease conditions are also related to the ingestion of such toxins.

In the determination of exposure of humans to mycotoxins, it is important to develop mycotoxin screening methods which may indirectly prevent the development of such diseases or reverse the symptoms of those conditions that may have already occurred. There have been a number of studies worldwide which have attempted to resolve these issues (Mirocha *et al.*, 1977; Ueno, 1977: Burmeister *et al*, 1979; Patterson, 1981; Gelderblom *et al.*, 1988). One important area of such research is the correlation between the presence of mycotoxins and mycotoxin-protein adducts in physiological fluids and the incidence of disease conditions such as kwashiorkor.

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[1] Mr. B. Beck, Ministry of Agriculture and Forestry, KaNgwane

This study is structured around interrelated, but not identical research regions, in three areas of Southern Africa namely, Durban, Eastern Cape and KaNgwane all of which have high temperatures and relative humidity during summer and autumn. These areas are also subjected to cyclical droughts, developed by the El Nino phenomenon, which are likely to cause crop stress and fungal susceptability. In areas such as these, which are conducive to fungal growth, contamination of stored grain e.g. by windborne spores, may occur, or fungal contaminants already present in poorly stored feed and foodstuffs will proliferate with high generation of mycotoxins. It is during summer and autumn that children suffering from kwashiorkor pass most frequently through King Edward VIII Hospital (KEH) in Durban. In 1990, Ramjee found that Zululand mealiemeal was contaminated with *Aspergillus flavus*, while a large number of kwashiorkor patients had free aflatoxin (AFB_1) in their blood. Earlier Wild *et al.* (1990a), developed a method to screen blood for AFB_1-lysine adducts in an attempt to determine the exposure levels to the toxin.

In South Africa, particularly in the Transkei adjacent to the Eastern Cape, the rate of oesophageal cancer (OC), a form of squamous carcinoma, has reached almost epidemic proportions and may be one of the major single causes of adult death. The incidence of OC in the Transkei cannot be explained by smoking and alcohol abuse within the community. A study of the staple diets of the people in this region was made and it was deduced that nutrient deficiencies were important in the aetiology of the disease (van Rensburg, *et al.*, 1980). The disease was first thought to be aggravated by a carcinogen such as nitrosamine or polycyclic aromatic hydrocarbon but was later explained by the high incidences of fungi in foodstuff of high-risk areas. The fungi produce a carcinogen which may play a role in OC (van Rensburg *et al*, 1980).

Kriek *et al.* (1977) noted that the incidence of *Fusarium moniliforme* was higher in areas where OC occurred and suggested that this fungus may be part of the aetiology of the disease. The presence of zearalenone, (ZEA) (Marasas and van Rensberg, 1979; Thiel *et al.*, 1982) and deoxynivalenol (DON) was reported in this area (Marasas and van Rensberg, 1979).

While fumonisins have also been detected in naturally contaminated maize in the Transkei (Sydenham *et al.*, 1990), Thiel *et al.*, (1991), found that people in this region may be exposed to fairly high levels of fumonisin B_1 (FB_1) which have been shown to have adverse effects on experimental animals.

Plate 1: Typical rural scene in KaNgwane

There are a number of accounts of unidentified aetiology reported in KaNgwane. These illnesses include elevated levels of congestive cardiac failure and OC, among others (Campbell, *et al.*, 1990). KaNgwane, a rural homeland located in the Eastern Transvaal of South Africa (Plate 1), shares common borders with the Transvaal, Mozambique and Swaziland (Figure 1).
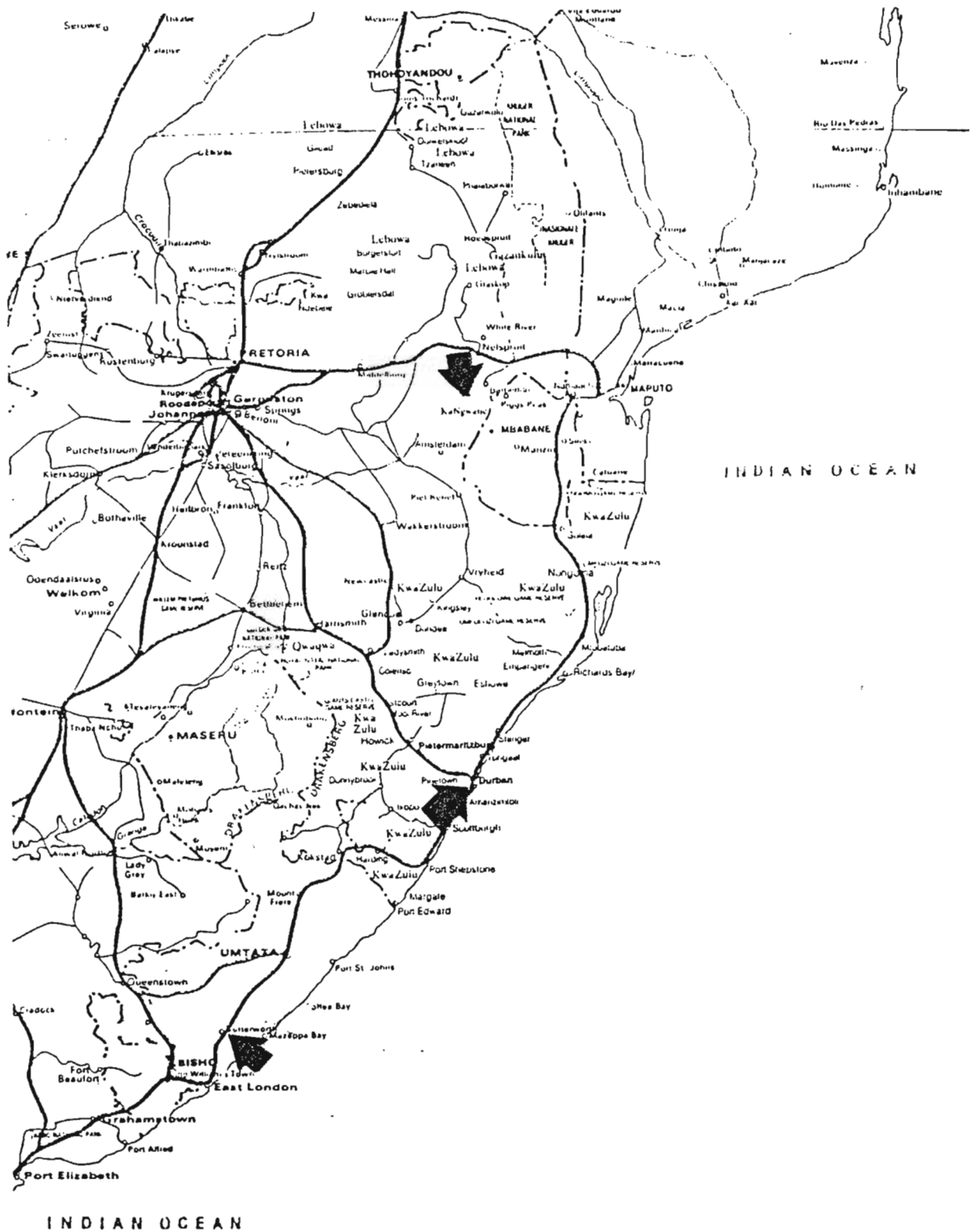
Figure 1: Map of South Africa showing Durban, Eastern Cape and KaNgwane (Rockhaven Press)

A large portion of the KaNgwane population (86%) live in the rural areas where the housing is traditionally mud huts. Approximately 7 people occupy each hut (Kritzinger and Oosthuizen, 1987). The kwashiorkor patients admitted to Temba Hospital (Plate 2) usually come from large families with low incomes.

Plate 2:   **Temba Hospital, KaNgwane**

In a 1992 study of this region, Hean found that the major fungal contaminant of maize was *Fusarium moniliforme*, although *Aspergillus* and *Penicillium species* were also detected in lower frequencies. The mycotoxins FB_1, moniliformin, DON and ZEA were detected. The work by Hean (1992), suggested that it would worthwhile undertaking a further analysis of maize and groundnut samples, together with a closer examination of the diets and exposure of the rural people to mycotoxins.

Monitoring human exposure to aflatoxins can be accomplished indirectly by the analysis of raw agricultural commodities and food products ready for consumption. This approach, however, has certain important shortcomings in that it relies on dietary recall for the amounts and types of food consumed, or laborious sampling and analysis of individual foods prepared for individual differences in consumption. The disposition of the carcinogen subsequent to investigation has also to be taken into consideration.

The presence of carcinogenic mycotoxins in rural KaNgwane maize (chapter 3) and the presence of free AFB_1 in KEH Paediatric Patients, led to the investigation of mycotoxin adducts (chapter 5) as a useful means of determining exposure to AFB_1 as proposed by Wild *et al.* (1990b) and Wild *et al.* (1992) in studies in The Gambia and China. The AFB_1-lysine adduct is not readily available in South Africa and this study investigates the possibilty of local production (chapter 4). Chapter 4 of this study details in vitro experiments to produce AFB_1-adducts by the incubation of AFB_1 and albumin or lysine and five alternative production methods are critically reviewed. As aflatoxins are potent carcinogens, the binding of DNA to the epoxide was also investigated. Work by Ramjee (1990) found that children suffering from kwashiorkor showed the presence of aflatoxins in their blood. This study develops a method for the quantitisation of the AFB_1-lysine adduct levels in human blood.

Maize from the KaNgwane area was screened and Gel Permeation Chromatography (GPC) and dialysis "clean-up" methods compared, each of which were followed by Thin Layer Chromatography (TLC). Gas Permeation Chromatography Mass Spectrometry (GCMS) and High Performance Liquid Chromatography (HPLC) were used to identify the presence of trichothecenes and AFB_1 in groundnuts.

This project concentrates on the methods used to screen samples for mycotoxins and their adducts so that future long term studies can be directed to correlate the incidence of certain disease conditions in man with the ingestion of foodstuffs contaminated by mycotoxins.

CHAPTER 2

LITERATURE SURVEY

2.1 AN INTRODUCTION TO THE MYCOTOXINS

There are two classes of metabolites produced by fungi; primary and secondary metabolites. The primary metabolites play a role in the functioning of the fungal system while the secondary metabolites, have no obvious function in cell growth and are synthesized by cells that have stopped growing (Weinberg, 1970).

Secondary metabolites are produced upon activation of the secondary metabolic enzymes. This is during the idiophase of the fungal life cycle, when normal growth of the fungus has stopped and differentiation has begun (Bu'Lock, 1975) and may occur once an essential growth factor, such as nitrogen or carbon, is exhausted. This may be at the end of the growth period (Behal *et al.*, 1986). The fungus *Penicillium notatum* produces penicillin, a useful metabolite: however, those secondary metabolites investigated in this study are particularly toxic.

Secondary metabolites are often assembled in a manner similar to common precursors (Weinberg, 1970) such as malonate. They have been found to be synthesized during morphogenesis. The role of mycotoxins, with both anti-bacterial and anti-fungal properties has not been fully established. They may play a role in morphogenesis or in a type of chemical fungal warfare against other organisms for nutrients as shown by Waring and Mullbacher, (1990).

Among the factors found to influence mycotoxin production are low ambient oxygen concentrations (< 1%) and /or increased concentrations of carbon dioxide or nitrogen which prevent the development of mould on grain and inhibit the production of selected mycotoxins, e.g. aflatoxins, ochratoxin, patulin, penicillic acid and

Trichothecene T-2 toxin. However, those levels of CO_2 needed to inhibit mould growth are much higher than those required for the inhibition of mycotoxin production. The degree of inhibition achieved by elevated CO_2 concentration is dependent on other environmental factors, such as relative humidity (RH) and temperature (T). Nevertheless, the biosynthetic pathways for mycotoxin production are merely blocked, but not damaged by high CO_2 levels. Another factor influencing mycotoxin production is radiation (Paster and Bullerman, 1988): however, the differing effects this has on AFB_1 production will not be dealt with in this project.

Mycotoxin production occurs when a toxigenic fungus finds not only a suitable substrate upon which it can grow, but also a favourable environment (Smith *et al.*, 1982). Substrates differ in their ability to support mycotoxin production, with more AFB_1 being produced on maize, wheat and rice than on sorghum or soya beans (Lacey, 1986). This is due to the differing carbohydrate contents of the cereal which support fungal growth to varying degrees. Thus subsistence farmers in the KaNgwane district are encouraged to farm alternative crops such as ground nut and mung beans.

There is a general lack of data on occurrence of mycotoxins in food commodities in African countries. Difficulties with regard to sampling and obtaining representative samples need to be addressed.

Notwithstanding the above, mycotoxin research has included:

(i)   Determination of the occurrence of all mycotoxins in African food commodities.

(ii)   Identification of the different fungi present on African food commodities.

This narrows the field for identification of the mycotoxins likely to be present and an analysis for those mycotoxins can then be performed. More knowledge concerning geographical and seasonal variations, as well as commodity contamination is required, as well as the identification of the occurrence of mycotoxin-related diseases. There is generally little information on mycotoxins in the aetiology of certain

disease conditions. Up to now research on markers of exposure to aflatoxins has been conducted and this should be extended to other mycotoxins. There are only a few epidemiological studies on mycotoxins and human health and a significant limitation is the lack of markers of individual human exposure. Appropriate biomarkers may include:

(i)   direct markers of exposure such as mycotoxins and their metabolites in human body fluids

(ii)   indirect markers of exposure including mutation spectra in target genes, common chromosomal abnormalities; polymorphisms in enzymes metabolizing mycotoxins.

Further development of research into the mechanism of action of mycotoxins;

(i)   interaction between co-occurring mycotoxins

(ii)   interaction with biological agents (parasites and viruses)

(iii)   immunotoxicity

as well as the consideration of intervention measures to reduce mycotoxins which are appropriate at the local level are necessary (Lehtinen et al., 1993).

Under conditions of high temperature and relative humidity, storage fungi invade stored grain and proliferate. However, it has been reported that these fungi also invade the plant in the field (Mycock, et al, 1990) thus it may be necessary to spray the growing plants with appropriate fungicides. The fungicide chosen should pose no threat to plant growth, grain production or the ultimate consumer. To date, such a fungicide does not exist and this would appear to be an area for development. Careful handling of the seeds after harvest should prevent surface damage and air-borne contaminants from entering the seed through any wounds or opening in the surface. The peduncle and from there the micropylar, remain a natural portal of entry for any invading fungus (Mycock, et al., 1988). Fungal contamination is likely, however, particularly under the storage facilities utilised by the subsistence farmer and other rural populations.

Spores of seed fungi may be transported by insects or wind. Since storage fungi are opportunistic invaders and saprophytes, the routes of entry into plant tissue are normally those of least resistance such as wounds on the surface, or stomata (Christensen and Kaufmann, 1974). There are three principal genera associated with mycotoxin production. These are *Aspergillus, Penicillium,* and *Fusarium* (Ueno *et al.*, 1983). The fungi associated with cereal grains are generally divided into 3 ecological groups (Figure 2) (Christensen and Kaufmann, 1969; 1974; Christensen and Sauer, 1982). Field fungi have, however, also been found in storage. Mycock and Berjak, (1992) suggest that the term "seed associated fungi" be used to embrace the entire spectrum of fungi found on and within seeds.

FIELD FUNGI

e.g. *Fusarium spp., Cladosporium spp., Curvularia spp.*

S
P
E
C
I
E
S   INTERMEDIATE FUNGI

e.g. *Verticillium lecani spp., Rhizopus spp.*

S
U
C
C
E
S   STORAGE FUNGI
S
I   e.g. *Penicillium spp., Aspergillus spp.*
O
N
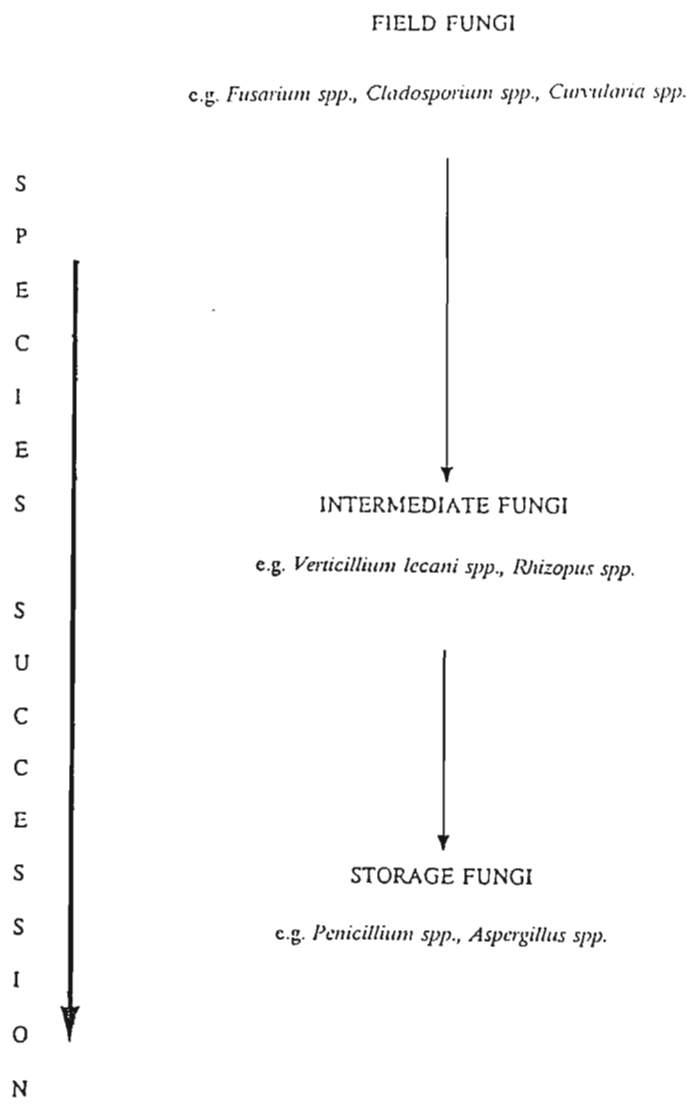
Figure 2:   Spectrum of fungi that infect the parent plant and subsequently the stored grain

Proliferation of fungi is dependent on temperature and water availability (Ramjee, 1990). Field fungi (Figure 2) constitute the species that invade the developing grain before harvest and are represented by the genera *Cladosporium, Curvularia, Epicoccum, Fusarium, Alternaria* and *Verticillium* among others (Christensen and Kaufmann, 1969; 1974). As the prevalence of field fungi declines with the decrease in m/c of the seed after harvest as well as during storage, fungal species of the intermediate group, e.g. *Cladosporium, Mucor, Rhizopus, Verticillium*, may grow during short - term storage (Pelhate, 1979; 1981). Storage fungi are represented by 10 - 15 group species of *Aspergillus* and several species of *Penicillium*.

Chapter 3 deals with multi-screening methods of cereals for mycotoxin contamination. When developing any monitoring and control strategy it is necessary to continue surveillance in order to establish an overview of the nature and extent of any potential contamination problem. The result of the surveillance test may indicate the possibility of a problem area, and where appropriate remedial action may be taken. A more pro-active approach is to attempt to identify the cause of the problem and introduce improved practices. In the case of mycotoxins contamination this might involve improvements in storage or post-harvest drying of cereals. Actions might involve introducing quality assurance procedures, for example screening of commodities to identify contaminated parts of a lot. A further possible innovation is a process of wet-heat treatment, which minimises seed infection without affecting germination (Berjak, 1990). An alternative to improved practices is to attempt control through the introduction of regulations. It is, however, necessary to have the means and resources for effective enforcement.

Specific food surveillance establishes an overview of the nature and extent of any contamination problems. An alternative way of obtaining an overview of the situation is by assessing average consumer exposure to a contaminant in the food chain rather than determining specific contamination of individual commodities. This approach is more applicable to contaminants that are uniformly distributed throughout a number of food commodities rather than, for example to mycotoxins that tend to be associated with only specific commodities. In the past both total and duplicate diet study approaches have been used to assess intake of contaminants.

Following consumption the medical importance of exposure of mycotoxins in man has stimulated much research. It is thought that a number of disease conditions, including Cardiac Beri-Beri and kwashiorkor are either caused or exacerbated by mycotoxins, particularly where chronic exposure is prevalent.

Interest in mycotoxins first began in South Africa in 1918 when a neurotoxic disease (diplodiosis) was diagnosed in cattle (the causal agent was *Diplodia maydis* (Mitchell, 1918). Laboratories in this country involved in mycotoxin research include the Veterinary Research Institute at Onderstepoort, the Plant Protection Research Institute in Pretoria, the Chemical Research Laboratory also in Pretoria, as well as the National Research Institute for Nutritional Diseases, Tygerberg, and the Department of Agriculture and Forestry of the Government of KaNgwane (Kriek and Marasas, 1983).

The potential for exposure of humans to AFB_1, particularly in tropical regions, has been well documented. Shank *et al.* (1972), found that *Aspergillus flavus* was the predominant species occurring in contaminated food products such as rice, beans and corn in Thailand and Hong Kong.

More recently Dutton and Westlake (1985) have reported similar findings for peanuts and maize in Africa. This project concentrates on mycotoxins which were screened for in maize, namely the trichothecenes, ZEA, fumonisin B_1, DON and aflatoxins. Groundnuts were also screened for aflatoxins and trichothecenes, while blood was analysed for bound AFB_1 and free AFB_1 and ZEA.

2.2 THE TRICHOTHECENES

There are two groups of trichothecenes; simple and macrocyclic trichothecenes (Jarvis *et al.*, 1985). All trichothecenes are sesquiterpene. Figure 3 shows their common tetracyclic 12, 13 trichothec-9-ene ring structure. Trichothecenes may have very different polarities. This may range from the relatively non polar T-2 toxin, with no free hydroxy groups, to the highly polar DON with four hydroxyl groups.
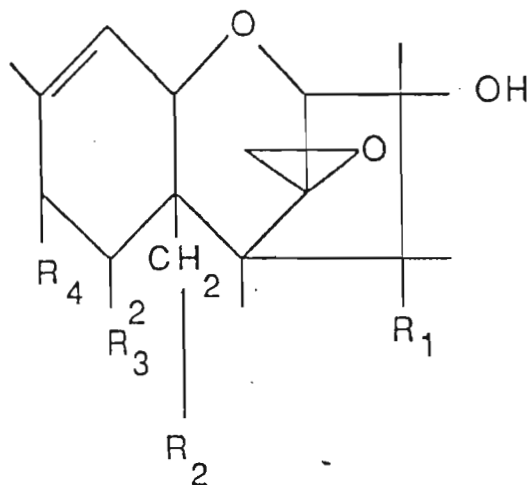
COMPOUND	R_1	R_2	R_3	R_4
T-2 Toxin	OAc	OAc	H	$OC(O)CH_2CH(CH_3)_2$
HT-2 Toxin	OH	OAc	H	$OC(O)CH_2(CH3)_2$
Deoxynivalenol	H	OH	OH	O

Figure 3: Structures of some of the trichothecenes (from Roos, 1986)

Most of the trichothecenes, 45 of which occur naturally, are produced by various fungi including members of the genera *Fusarium, Stachybotrys, Trichothecium, Verticimonosprium* and *Cephalosporium* (Ueno, 1983). The most well known fungal trichothecenes including: DON (produced by *Fusarium graminearum, F. cumorum),* nivalenol, monoacetoxyscripenol, diacetoxyscripenol, HT-2, T-2 toxin (produced by *F. sorotrichioides)* and neosolaniol (Gilbert, 1989). Exposure to the trichothecenes results in several symptoms which include skin irritations, vomiting, anorexia, haemorrhaging, convulsions and death (Ueno, 1983) and DON which is detected frequently, is one of the least toxic of the trichothecenes (LD50 = 70 mg/kg in mice). It has been found that T-2 toxin is more toxic (LD50 = 0,5 mg/kg) but its occurrence is less common (Ueno, 1983; Gilbert, 1989).

Work by Holt and Deloach, (1988) has shown that trichothecenes have a number of different properties, including antibiotic, antibacterial, antiviral and cytotoxic properties and that T-2 toxin can modulate the immune response. Feurstein *et al.* (1985) have found that antibodies raised against T-2 toxin neutralise this adverse effect and protect laboratory animals against the toxic effects induced by T-2. In 1990 Hunter *et al.*, showed that these monoclonal antibodies cause an efflux of the intracellular T-2 toxin from the cell into the plasma and protein synthesis within the cell is restored. It has been found in rats that the lethal effects of the toxin can be avoided if the rat is injected with the antibody within 30 minutes of toxin administration.

Work by Bamburg *et al.* (1971) with T-2 toxin shows the detrimental effects of this toxin. The use of monoclonal antibodies in initiating steps towards therapy for T-2 toxicosis is an area receiving much attention. Table I shows animal and human mycotoxicoses possibly caused by trichothecenes.

TABLE I: PROBABLE TRICHOTHECENE MYCOTOXICOSES (FROM UENO,1977)

TOXICOSIS	LOCATION	AFFECTED SPECIES	SYMPTOMS	FUNGUS
Alimentary Toxic Aleukia	USSR	human, horse,pig	vomiting,diarrhoea, skin inflammation, leukopaenia, angina	*Fusarium sporotrichioides*
Bean-hull Toxicosis	Japan	horse	convulsioncyclic movement.	*Fusarium solani*
Dendrochio-Toxicosis	Europe	horse	skin inflammation	*Dendrochium toxicum*
Mouldy Corn Toxicosis	USA	pig, cow	vomiting, haemorrhage, refusal of feed	*Fusarium tricinctum*
Red Mould Toxicosis	Japan	human, horse, pig, cow	vomiting, diarrhoia, abortion	*Fusarium graminearum*
Stachybotryo-toxicosis	Europe	horse	shock, dermal necrosis, leukopaenia	*Stachyborys atra*
"Taumelgetriede" Toxicosis	USSR (Siberia)	human, horse, pig, fowl	headaches, chills, nausea, vomiting	*Gibberella saubinetti*

2.3   ZEARALENONE

The name Zearalenone is derived from *Zea mays*, the latin name for maize, and refers to the lactone and ketone functions in this compound.   This mycotoxin is a   [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-B-resorcyclis   acid   μ   lactone]   with   a molecular weight of 318.   Figure 4 shows the structure of ZEA as being a polyketide-derived metabolite.   The synthesis of this compound involves the reaction of 8 malonyl coenzyme A molecules with a molecule of acetyl coenzyme A (CoA)(Figure 5).   This is almost identical to fatty acid biosynthesis requiring a multi-enzyme system to function (Moss and Neal, 1982).
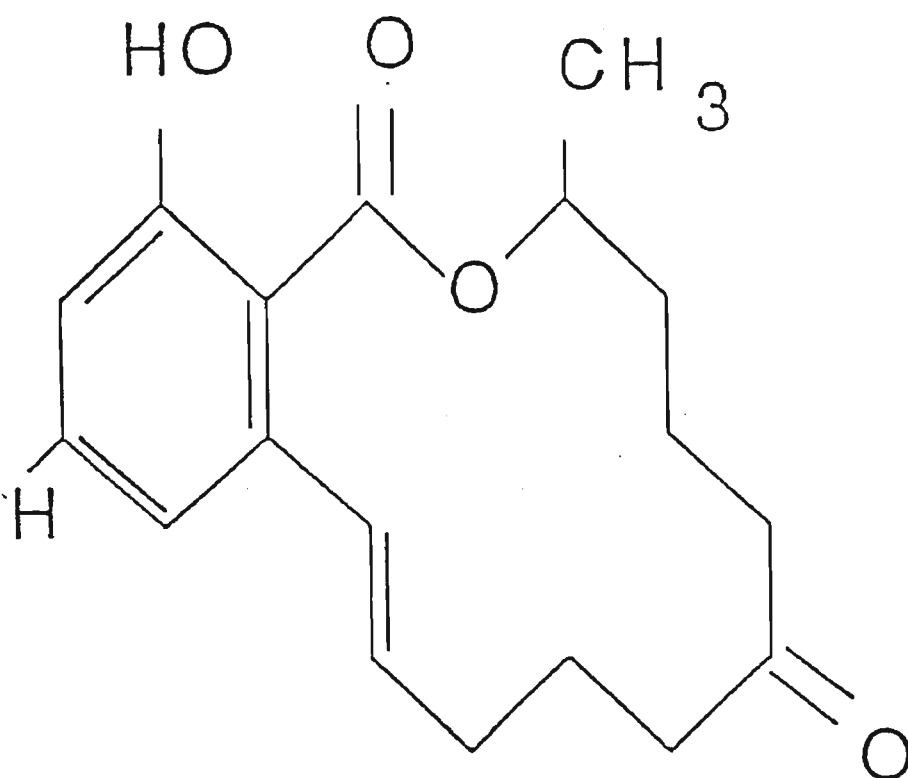
Figure 4:   The structure of zearalenone (from Moss and Neal, 1982)
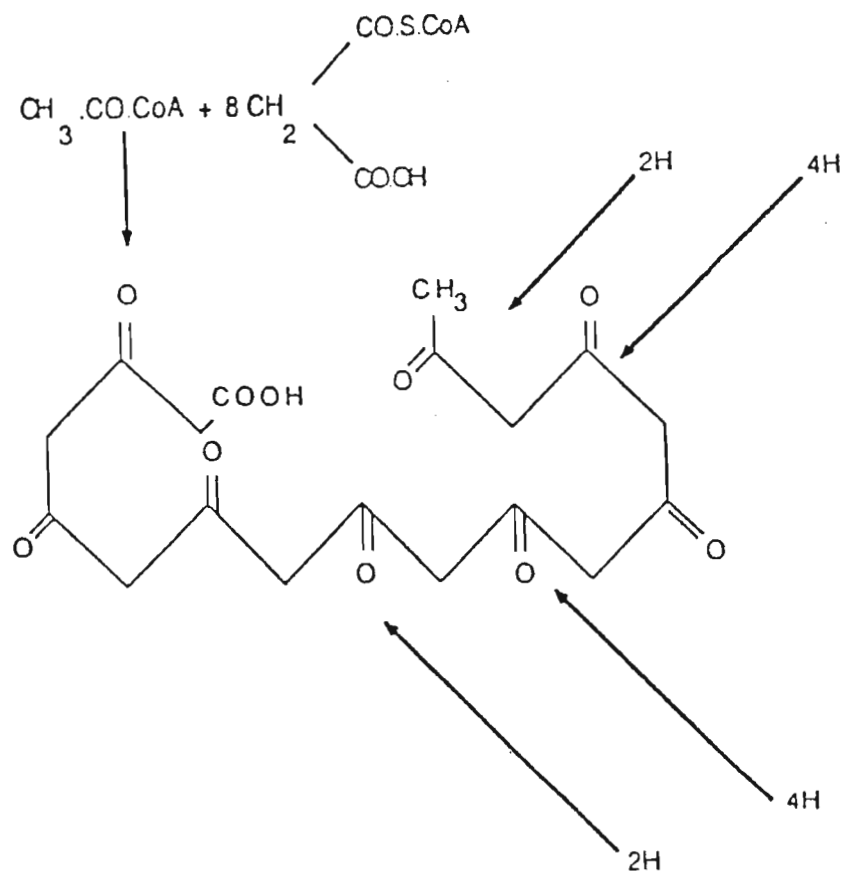
Figure 5: Formation of zearalenone from acetyl coenzyme A and malonyl coenzyme A (from Moss and Neal, 1982)

The presence of a secondary methyl group which retards nucleophilic attack on the lactone carbonyl makes ZEA a very stable compound which is not rapidly degraded by harsh chemicals such as lactic acid and sulphur dioxide solutions and it is able to withstand a temperature of 120 °C for 4 hours, thus making it highly heat stable (Bennett *et al.*, 1983; Gilbert, 1989). Two isomers of ZEA exist, i.e. *cis* and *trans*, the latter being the form that occurs naturally, while the former is more active biologically (Mirocha *et al.*, 1978). Recent studies have raised the suggestion that the *cis* isomer has a higher binding affinity for the specific oestrogenic receptor protein, than its *trans* counterpart (Mirocha *et al.*, 1978).

The hydroxy derivatives of ZEA, α- and β- zearalenol, are more biologically active than ZEA (Mirocha *et al.*, 1978; Gilbert, 1989). It is thought that as ZEA is often detected along with trichothecenes in analysed samples, a possible synergism may exist between these toxins. This synergism may make the diagnosis of mycotoxin

related diseases more difficult (Mirocha *et al.*, 1977). *Fusarium roseum* was the first fungus initially found to produce this toxin (Christensen and Kaufmann, 1969). Since then a number of other fungi have been found capable of ZEA production. These include *F. tricinctum, F. roseum "Culmorum", F. roseum" Equiseti", F. rosaeum "Gibbsum", F. reseum" Graminearum", F. sporotrichioides, F. oxysporum,* and *F. moniliforme* (Caldwell *et al.*, 1970; Mirocha *et al.*, 1976a). The distribution of ZEA is widespread and its occurrence has been reported in 23 different countries including Australia, China, South Africa, U.S.A., Mexico and Northern Europe (Kuiper-Goodman *et al.*, 1987). This mycoxin occurs in many agricultural products including corn, sorghum and barley. Ingestion of ZEA may have a number of different effects, particularly in pigs which exhibit a hyperoestrogenic syndrome with an enlarged vulva, uterus and mammary glands, by functioning similarly to oestrogen.

2.4 THE FUMONISINS

The structure of fumonisin B_1 (FB$_1$) (Figure 6) (Gelderblom *et al.*, 1988) shows it is a polar compound. Several fungi, including *F. moliforme, F. proliferatum* and *F. nygamai* produce fumonisins. (Thiel *et al.*, 1991).

Figure 6:   **Structure of fumonisin B$_1$ (from Gelderblom et al., 1988)**

Fumonisins form a family of related compounds, namely fumonisin B_1(FB1), B_2(FB$_2$), B_3(FB$_3$), A_1(FA$_1$) and A_2(FA$_2$). There are also a number of acetylated derivatives of FB$_1$ and FB$_2$. (Gelderblom *et al.*, 1991a). The most prevalent fumonisin is FB$_1$ (Thiel *et al.*, 1991) but this is dependent on the fungal isolate. The ratio of FB$_1$ to FB$_2$ production may also vary, but normally the following ratio exists: FB$_1$:FB$_2$ = 3:1 and FB$_1$:FB$_3$ = 12:2. There are also some isolates, that produce FB$_2$ in very much larger quantities (Ross *et al.*, 1991). The fumonisins, especially FB$_1$ about which the most is known, have numerous medical effects which include the stimulation of liver cancers. Gelderblom *et al.* (1991b) has suggested that the fumonisins may be the cause of cholangiocarcinomas. In short periods of fumonisin exposure, it was shown that FB$_1$, FB$_2$ and FB$_3$ cause the formation of hepatic nodules.

The fumonisins are also responsible for the equine neurotoxic disease, leukoencephalomacia (LEM) or "hole in the head" disease (Gelderblom *et al.*, 1988; Thiel *et al.*, 1991). This disease is associated with the consumption of *F. moniliforme* contaminated material. Horses suffering from this disease show various nervous signs, blindness and also paralysis of the pharynx and periods of delirium. Colvin and Harrison (1992) concluded that the clinical signs of poisoning by fumonisins depend on the length and level of exposure to this toxin. The fumonisins may also pose a potential threat to humans as they have been detected in human food and have been shown to be statistically correlated with the prevalence of human oesophageal cancer in Transkei (Sydenham *et al.*, 1990).

Another disease associated with the fumonisins is Mystery Swine Disease. This disease is characterised by prenatal and neonatal mortality, as well as respiratory failure in older animals. First reports of this disease occurred in 1988 and 1989 and it was thought to be linked to the consumption of fumonisins (Bane *et al.*, 1991).

Methods of isolation and purification of fumonisins have only recently been developed and so the full extent of the potency of these mycotoxins has not yet been fully characterised. Scott and Laurence (1994) investigated the fumonisins stability to corn-based foods. In these studies, distinctions were made between real losses, binding

and any matrix-related method problems. They showed that recovery rates of FB_1 and FB_2 varied depending on whether the extraction solvent used was methanol-borate buffer (pH 9,2) (3+1) or methanol-water (3+1).

The awareness of the presence of low levels of fumonisins in apparently uncontaminated corn, and the potential of high levels of this family of mycotoxins to cause severe animal losses, led to an increase in the analyses for fumonisins in corn and corn products. In addition to their natural occurrence in corn-based animal feeds and in home-grown African corn used for food, fumonisins are frequently found in commercial corn-based foods.

Fumonisins are moderately heat-stable. No effective means of decontamination has yet been developed for contaminated feeds (Scott *et al.*, 1993). Electron microscopical investigations have shown that FB_1 causes intracellular membrane degeneration and plasma membrane changes. This suggests that the cell membrane may be an early target of these toxins. It was further suggested that FB_1 may induce abnormalities in membrane lipid turnover (Scott *et al.*, 1993).

This theory was supported and extended by the work of Norred *et al.* (1991) and Wang *et al.* (1991) who reported that FB_1 disrupted sphingolipid metabolism in rat liver hepatocytes. Another area where FB_1 may be implicated is the aetiology of OC and this is discussed in section 1.1, page 3.

Work by Gelderblom *et al.* (1994) suggests that a balance exists between the compensatory cell proliferation due to hepatotoxicity induced by FB_1 and the inhibitory effect on the subsequent hepatocyte cell proliferation. A threshold level for cancer initiation exists which, as a function of time, will be determined by the dosage used and the subsequent inhibitory effect on cell proliferation. A number of other studies have been aimed at establishing which substances, produced by *F. moniliforme* or other fungi, might play roles in causing OC. In Natal, levels of 41×10^{-6} propagules/g *Fusarium* have recently been recorded in animal feed, while FB_1 has been found in

chicken feed at levels of 800-1000 ppb (Kinsey, A., pers. comm.[2]). Work undertaken in 1994 by the Analytical Unit, Physiology Department, Natal Medical School, using HPLC, showed levels of FB_1 up to 1300 ppb (Wagner, M., pers. comm.[3]) in KwaZulu maize samples. In an analysis of mycotoxin content in maize samples from KaNgwane by Hean (1992), FB_1 showed the highest incidence, occurring in 15/21 samples.

2.5   THE AFLATOXINS

It was known for some time that certain oilseed based animal feeds could be toxic to animals (Moreau and Moss, 1979). It was, however, only after the widespread mortality of turkeys from "Turkey X Disease" in the UK, as well as the death of ducklings in Hungary and Uganda, and chickens in Spain, that the source was isolated to contaminated groundnuts (Moreau and Moss, 1979). The toxic effects of aflatoxins on man were subsequently studied. The toxic substance was extracted with chloroform and called AFB_1 with the disease being known as aflatoxicosis (Moreau and Moss, 1979).

Since the discovery of the aflatoxins in the early 1960s, the study of mycotoxins has moved from an area of vague inter-relationship with nutrition-related diseases to an expanding multidisciplinary research effort focusing on many fungal metabolites. The research endeavours are ongoing and not least with respect to the studies on AFB_1, the most commonly occurring of the aflatoxins, together with cyclopiazonic acid.

One of the most significant discoveries with respect to AFB_1 was its carcinogenic properties. Butler (1974) showed that of all the naturally occurring carcinogens, the rat exhibited the greatest susceptibility to AFB_1. The importance of this discovery was the implication as to the role of the toxin in human disease. It may be that aflatoxins together with other environmental agents such as hepatitis B virus, will provide a model which may explain the origins and mechanisms of cancers that are dependent on an interaction between both environmental and genetic factors.

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Aflatoxins are heterocyclic compounds containing either a bisfuran or dihydrofuran moiety which may be responsible for biological activity. The first four major aflatoxins discovered were designated AFB1, aflatoxin B_2, (AFB$_2$), aflatoxin G_1 (AFG$_1$) and aflatoxin G_2, (AFG$_2$) (Figure 7) (Moreau and Moss, 1979). The aflatoxins B and G are named after their blue and green fluorescence, respectively, in ultraviolet light and their relative R_f values on chromatographic plates.
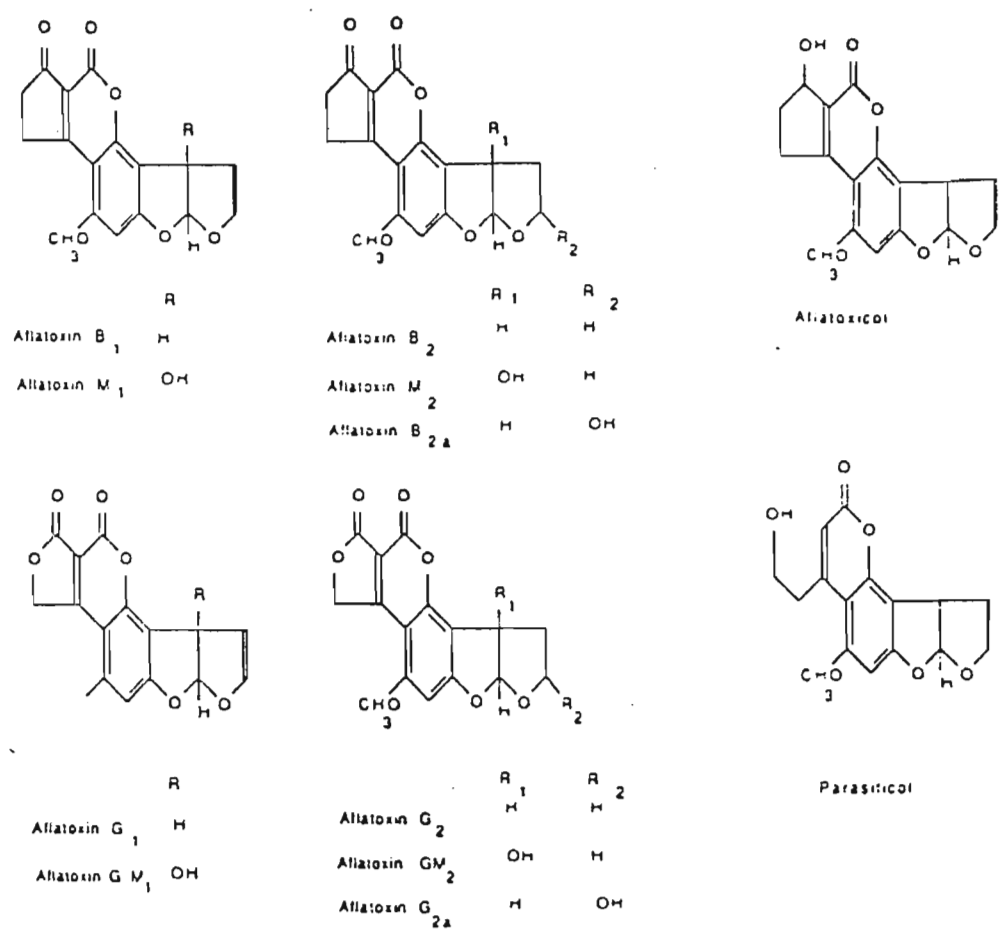
Figure 7: Structure of the aflatoxins and two of the related metabolites: aflatoxicol and parasiticol (from Dutton, 1988)

The synthesis of AFB$_1$ resembles fatty acid biosynthesis, lacking in only the dehydration and reduction steps (Moule, 1984). The biosynthesis of the most common AFB$_1$ has been the most researched. Nuclear magnetic (NMR) studies (utilising isotopes) and the use of mutant *A. parasiticus* strains blocked at known stages of the biosynthetic pathway (Figure 8) gives intermediates within this pathway (Dutton, 1988), and have revealed more about the structure of this mycotoxin. Figure 8 also illustrates the number of biosynthetic enzymes involved in the pathway as well as the sites at which the pathway is blocked in some of the *A. parasiticus* mutants. Work remains to be done to establish a better understanding of AFB$_1$ biosynthetic pathway.
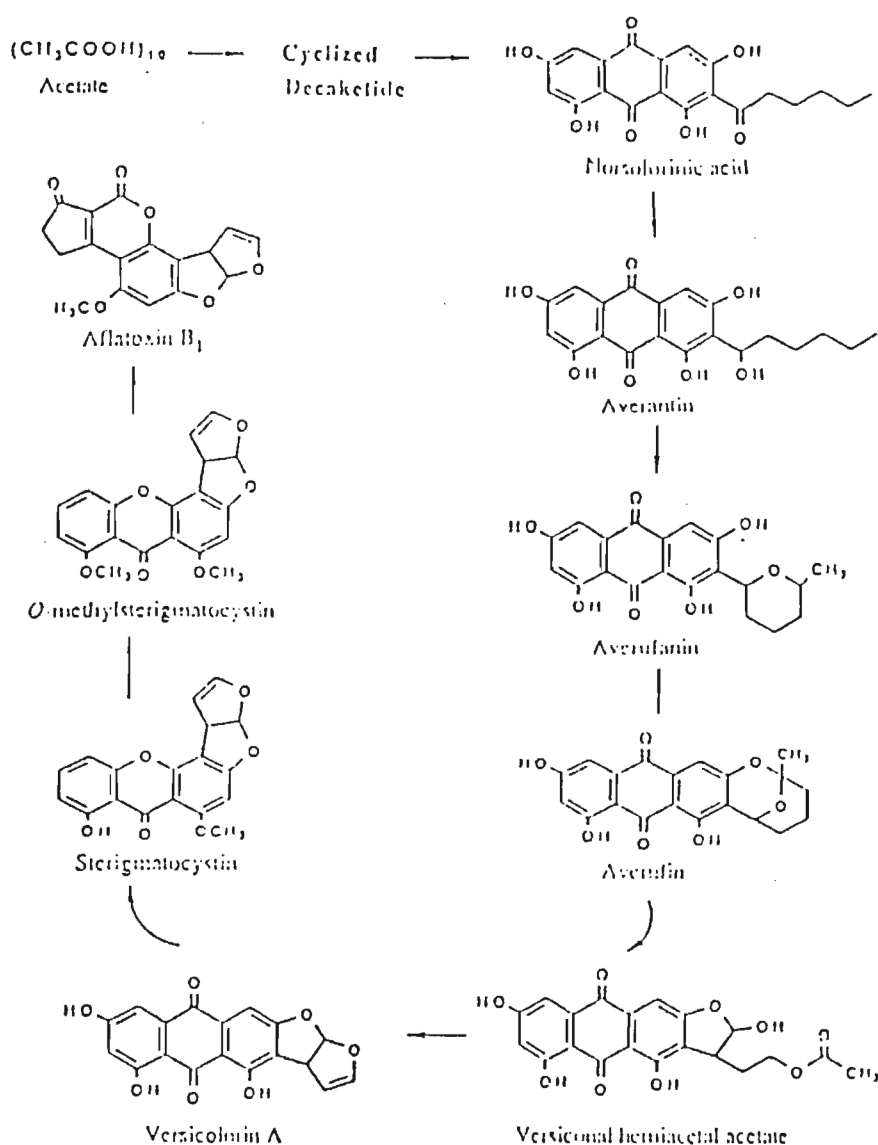
Figure 8: Intermediates within the aflatoxin B$_1$ biosynthetic pathway (from Dutton, 1988)

The production of AFB_1 is optimal in a relative humidity between 80% and 90% and a mean temperature of about 27 °C (range 12 - 40 °C). (Goldblatt, 1969). In addition, there are genetic and physiological factors that influence aflatoxicosis (Figure 9).

GENETIC FACTORS   PHYSIOLOGICAL   ENVIRONMENTAL
                          FACTORS   FACTORS

Species,   Age   Climatic conditions
breed & strain   Hormonal status   Chemicals
                          Nutrition   Husbandry & management
                       Intestinal microflora
                       Infection & parasitism

                                    ⎧ Absorption
                    Aflatoxin   ⎪ Distribution
                    metabolism   ⎨ Biotransformation
                                    ⎪ Excretion
                                    ⎩

                        Toxicity

                    Biochemical defect

                    Functional defect

                 Microscopic anatomical defect

                 Macroscopic (grossly visible) defect

                          Death

Figure 9: **Simplified representation of some general relationships in aflatoxicosis (Redrawn from Bryden, 1982)**

To date about 18 aflatoxins have been discovered and characterised. The biological activity of aflatoxins show a potency series $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (Busby and Wogan, 1981). The sensitivity, however, of the different species to aflatoxins varies enormously. The metabolic effects of aflatoxins in man and other animals can be serious as it may include the inhibition of DNA and RNA synthesis as well as the inhibition of protein and lipid synthesis (Busby and Wogan, 1981). Other effects include the reduction of miscellaneous enzyme activities, the depression of glucose metabolism, the inhibition of clotting factor synthesis and immunosuppression (Richard *et al.*, 1978). The principle target for toxicity is the liver and pathological

here include fatty infiltration, biliary proliferation, acute toxic necrosis and portal fibrosis (Figure 10).
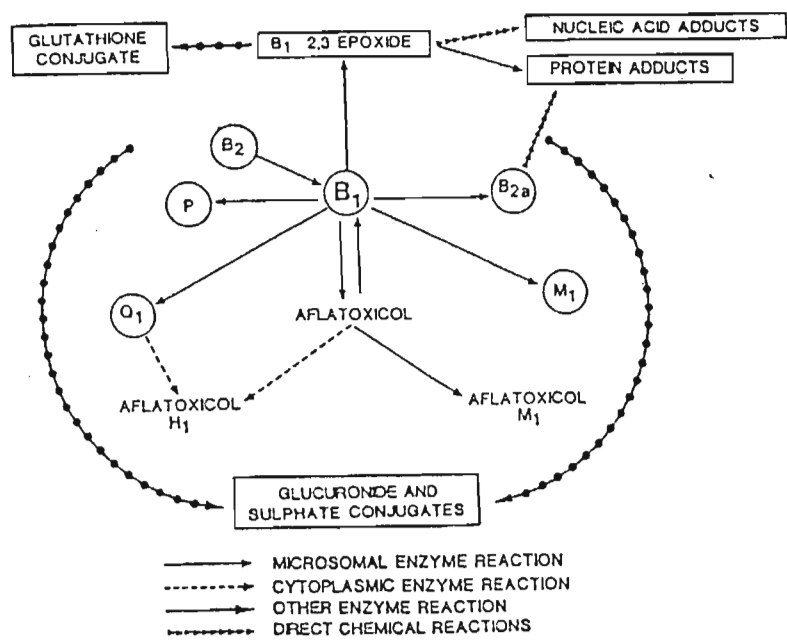
Figure 10:   Metabolism of aflatoxins in the liver (Hendrickse and Lamplugh, 1987)

When discussing biological activity and metabolic effects of AFB_1 it is important to understand the liver's metabolism of the mycotoxin. The toxicity of the metabolite may be modified by the induction or inhibition of the mixed function oxidase system (MFO) (Gumbmann and Williams, 1970). AFB_1 requires metabolic activation to exert its toxicity. Several competing pathways in the target hepatocyte influence its toxicity. With one exception all primary transformation of AFB_1 implicate its conversion by the MFO to hydroxylated metabolites which are conjugated with sulphate or glucuronic acid and readily excreted in urine or bile. These mechanisms usually detoxify the parent compound. The exception is the conversion of AFB_1 to aflatoxicol (AFL). The

metabolism in the liver of AFB_1 to aflatoxicol and via AFQ_1 to AFH1 (Figure 10) are catalysed by a cytoplasmic NADP - dependent dehydrogenase (Patterson, 1981). The conversion of AFB_1 to aflatoxicol is a reversible reaction and may be inhibited by 17 - keto - steroid sex hormones. The reversibility of this reaction may provide a store of intracellular AFB_1, thus enhancing its toxic effects. During metabolism, products can also be formed which have the capacity to react covalently with various nucleophilic centres in cellular macromolecules such as DNA, RNA and protein (Patterson, 1981). A summary of metabolic transformations is presented in Figure 10.

The impact of the aflatoxins is primarily on the liver and is referred to as hepatitis X. Cows fed AFB_1 and AFB_2 excrete in their milk aflatoxins which are similar to, but less toxic than their parent compounds and are designated AFM_1 and AFM_2 respectively. The significance of mycotoxin assimilation in the contamination of milk and milk products has aroused much interest. The two possible pathways contaminating milk and milk products with mycotoxins are either the secretory or post-secretory route. It has been found that a secretory contamination can only occur with such mycotoxins, which undergo no complete degradation through their passage into the milk. From the mycotoxins present in cow's feed; virtually only AFB_2 yields a milk borne metabolite, the AFM_1 (Moreau and Moss, 1979).

The biotransformation of AFB_1 involves its conversion to the hydroxylated metabolite by the microsomal MFO. This is a complex organisation of cytochrome - coupled O_2 and NADPH - dependent enzymes. The result is detoxification of the parent compound to hydroxylated metabolites which are conjugated with sulphates or glucuronic acid to form water-soluble glucuronides or sulphate esters which are excreted in urine or bile. AFB_1 is transformed to less toxic AFP_1, AFQ_1 and AFM_1 (Bassir and Osiyemi, 1967). The latter is found in milk and is a hydroxylated derivative formed in humans by cytochrome P450 IA2 (Faletto, *et al.*, 1988), carcinogenic for both the liver and colon in rats.

Other mycotoxins which will not be dealt with in this study include ochratoxin A, fusarins, moniliformin, citrinin, penicillic acid and kojic acid.

2.6 BIOCHEMICAL AND MOLECULAR ASPECTS OF MYCOTOXINS

Mycotoxins at a physiological level are potentially teratogenic, mutagenic, carcinogenic and embryotoxic (Hayes, 1980). At a biochemical level they affect the basic metabolic processes. This is important when considering their carcinogenic properties and their effect on protein and nucleic acid biosynthesis. Figure 11 illustrates the site of action of various mycotoxins (Kiessling, 1986).

DNA TEMPLATE

aflatoxins
sterigmatocystin
patulin
penicillic acid
luteoskyrin
pslorens

TRANSCRIPTION   RNA POLYMERASE

RNA (mRNA,tRNA,rRNA)

aflatoxins B_1 and G_1
sterigmatocystin
patulin
citreoviridin

TRANSLATION   RIBOSOMES

POLYSOMES

aflatoxin B_1
trichothecenes
ochratoxin A

PROTEINS

METABOLIC REACTIONS

aflatoxin B_1 and G_1
patulin
trichothecenes
citreoviridin
ochratoxin A
cyclochlorotine
moniliformin

Figure 11: Sites of mycotoxin action (redrawn from Ramjee, 1990)

AFLATOXINS B₁, G1, M₁

AFLATOXINS B₁,

AFLATOXINS B₁,

PROTONS

ELECTRON TRANSPORT

NADPH → UBIQUINONE → CYTOCHROME B → CYTOCHROME → CYTOTOXIN

2H + ½O

H₂O

PHOSPHORYLATION

ADP + P   ATP   ADP + P   ATP   ADP + P   ATP

→ INHIBITION OF ELECTRON TRANSPORT

----• UNCOUPLING OF OXIDATIVE PHOSPHORYLATION

AFLATOXINS B₁ G1, M₁

LUTEOSKYRIN
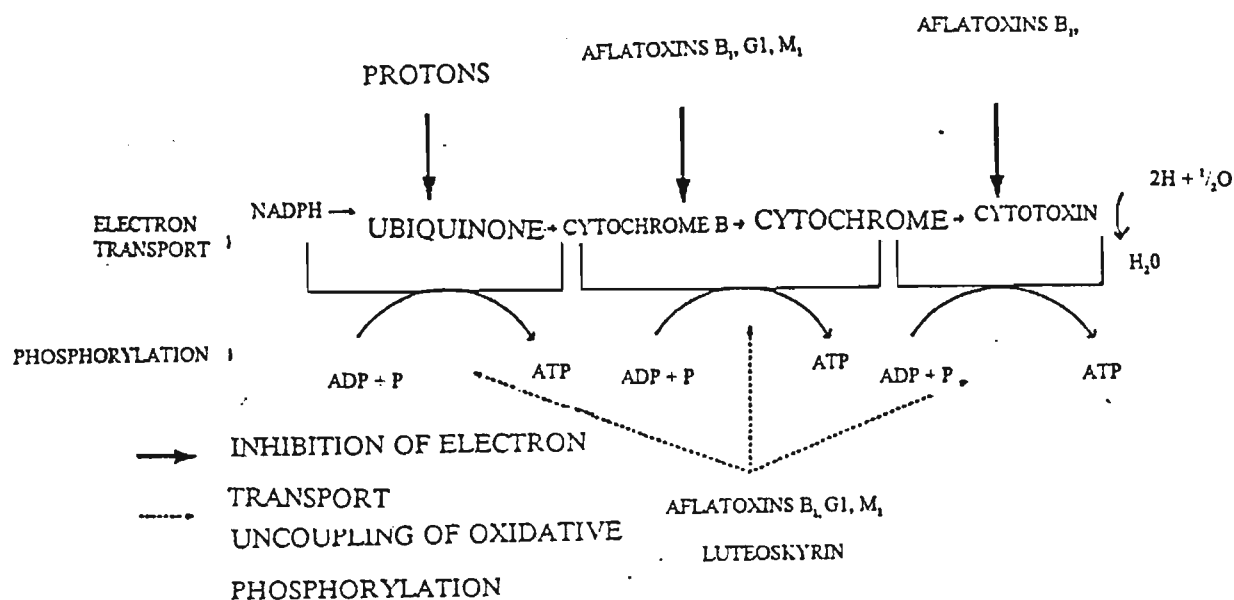
Figure 12: The sites of interaction of some mycotoxins in the oxidative phosphorylation pathway (from Kiessling, 1986)

During AFB₁ metabolism reactive metabolites are formed which have the capacity to react covalently with nucleic acids and proteins. The covalent incorporation of the AFB₁ moiety into nucleic acids and protein may be considered to be an important mechanism by which toxicity and carcinogenicity are initiated. AFB₁ albumin adducts are found in peripheral blood after exposure to AFB1 and the measurement of these adducts is potentially a useful tool in the aetiology of liver cancer and aflatoxicosis.

In vitro studies of AFB₁ binding with plasma albumin suggest a possible *in vivo* transport mechanism for this toxin as an albumin complex (Rao *et al.*, 1968). Recently rats and mice treated with AFB₁ showed a difference in sensitivity, with the former being sensitive and the latter resistant. The frequency of chromosomal aberrations and micronuclei in the bone marrow was measured and compared to the level of AFB₁ bound covalently to albumin in the peripheral blood. At the highest dose the level of chromosomal abberations was about 10 times higher in rats than in mice. The results

also suggest that AFB_1-albumin may reflect the level of genetic alterations resulting from the initial binding of this carcinogen to cellular DNA. Thus the adduct used as a biomarker in studies of human exposure to aflatoxins may provide information not only on exposure but also on the risk of genetic alterations consequent to that exposure (Anwar *et al.*, 1994) A number of workers have studied the outcome of the incubation of rat S"9" fraction with macromolecules (section 4.3.4.1, page 111).

Imaoka and co-workers (1992) investigated the genotoxic and mutagenic activation of AFB_1 on *Salmonella typhimurium* by rat hepatic, renal and pulmonary microsomal fractions and purified cytochrome P-450 enzymes. Hepatic microsomes displayed the greatest mutagenic activation, while renal microsomes had the lowest activation. It would appear that the greater ability of hepatic microsomes (as compared with pulmonary and renal equivalents) to bio-activate AFB_1 is dependent on the different classes of cytochrome P-450s present in different tissues. Ames *et al.* (1973) have shown that the carcinogen AFB_1 is activated by liver homogenates to form potent frame shift mutagens and suggest, that these carcinogens cause cancer by somatic mutation. They used rat liver homogenate for carcinogen activation in their study as well as a set of *Salmonella* histidine mutants for mutagen detection. The *in vitro* binding has a strict requirement for NADPH and oxygen, suggesting that it has basic functions in common with the mixed oxidase system responsible for the N- and C-oxygenation of aromatic amines. Cytochromes P-450 and P-448 may be the predominant species in liver microsomes from rats treated with phenobarbital and 3-methylcholanthrene, which are necessary for binding to occur (Imaoka *et al.*, 1992).

Monitoring human exposure to aflatoxins can be accomplished indirectly by analysis of raw agricultural commodities and food products ready for consumption. Wild *et al.* (1990a) found that AFB_1-lysine conjugates are an excellent molecular dosimeter for exposure assessment. They also performed other experiments with isolated perfused rat liver and their results showed that AFB_1 metabolites covalently react, not only with albumin in the hepatocyte, but also with circulating proteins in the perfusate suggesting that a reactive AFB_1 metabolite secreted by the liver may form serum albumin adducts in circulating blood. Shang Gan *et al.* (1988) compared AFB_1-

serum albumin adducts in the blood of residents of Guangxi Province, China with AFB_1 intake and excretion of AFM_1 in urine. A high correlation was observed.

Human exposure to airborne or particle-borne chemical compounds can be determined either by ambient air monitoring or by the detection of the compounds or their metabolites in body fluids. The method of Wild *et al.* (1990) has the advantage of measuring the amount of toxin entering the bloodstream without suffering the problems of rapid metabolism: however analysis of human samples have progressed slowly as a consequence of the instability of the adduct.

The initial AFB_1-lysine adduct (Figure 13) is formed by condensation of the dialdehyde tautomer of 8,9-dihydro-8,9-dihydroxy-AFB_1 with the ε-amino group of lysine, to form a Schiff's base, and that the Schiff's base undergoes an Amadori rearrangement to an α-amino ketone. The pyrolinone ring is formed by condensation of the amino group with the remaining aldehyde to yield the final product. The purified product is relatively stable, but decomposed to a certain extent under isolation (Sabbioni and Wild, 1991).
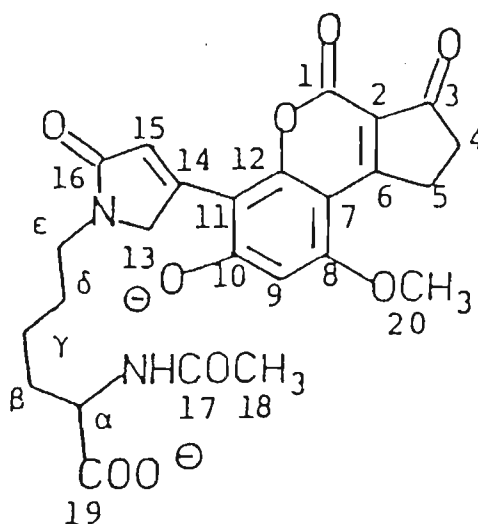
Figure 13: Structure of aflatoxin B_1-lysine adduct

Thus serum-albumin is a readily accessible target protein for AFB_1. Wild and co-workers (1990) reported the development of a sensitive method to quantify AFB_1-lysine by combined immunoaffinity chromatography/high-performance liquid chromatography (iac/HPLC) with fluorescence detection.

For this method serum is digested with pronase and the adducts are purified by monoclonal antibody iac and quantified by HPLC. Enzymatic proteolysis of AFB_1-adducted serum albumin produces a monomeric adduct composed of lysine and an AFB_1 residue in which the AFB_1-coumarin ring system is intact (Sabbioni, et al., 1987). Intact serum albumin bearing an AFB_1 adduct is not recognized by the antibody and will not antigenically bind to the antibody when it is immobilized on a solid support. Thus it is possible to isolate, through iac, at least some of the AFB_1-derived products from proteolysis of modified serum albumin (Groopman et al., 1988).

Although the AFB_1 epoxide has never been detected in biological systems, its role as a reactive intermediate has been inferred from the structures of adducts with DNA and other bioreceptors. The level of macromelecular binding is correlated with the likelihood of tumour formation (Miller et al., 1978; Moreau and Moss, 1979). It is thus important to identify products formed through the covalent binding of AFB_1 to cellular macromolecules in order to identify target molecules and to study further cellular responses (e.g., repair, mutation) to these interactions.

The detection of AFB_1 has been shown to form a covalent adduct with DNA primarily at the N^7 atom of guanine both in vitro and in vivo (Croy and Wogan, 1981). The major AFB_1-DNA adduct found is 2,3,-dihydro-2-(N^7-guanyl)-3 hydroxy AFB_1 (Figure 14). These adducts consist of 60%-90% of the covalently bound mycotoxin to DNA. The structure of the major adduct suggest that the C_2-C_3 unsaturated bond is the reactive moiety of aflatoxins and supports the exo-2,3 oxide as being the reactive (ultimate) metabolite. Two types of interactions were found to occur between aflatoxins and nucleic acids: non-covalent, weak and reversible binding which requires the mere presence of mycotoxin and nucleic acid and irreversible covalent binding requiring mammalian metabolizing systems.
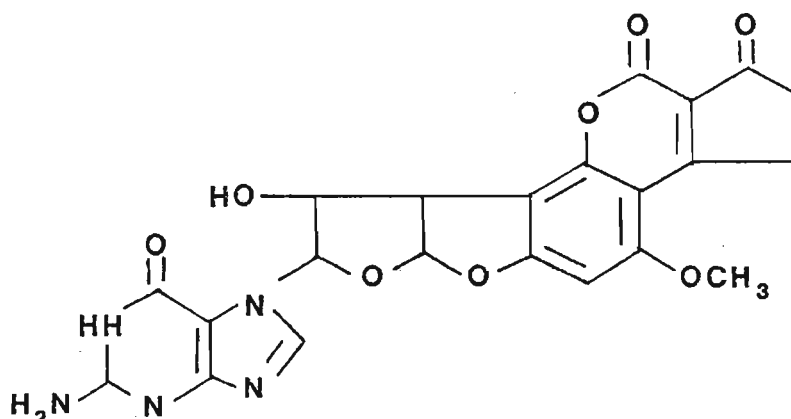
Figure 14: Structure of 2,3-dihydro-2-(N^7-guanyl)-3 hydroxy aflatoxin B$_1$ (Croy and Wogan, 1981)

The DNA replication is inhibited by AFB$_1$, as reported by Rogers and Newberne, (1967). The formation of DNA-AFB$_1$ adducts depends on activation by a number of cytochromes including in decreasing order P-450IA2, P-40IIA3, P-450IIIA4 and P-450IIB (Aoyama *et al.*, 1990) and P-450IA enzymes which metabolise the detoxification of AFB$_1$ to AFM$_1$ (Koser *et al.*, 1988), while cytochrome P-450 isoenzyme are responsible for the conversion of AFB$_1$ to other less toxic metabolites for example AFQ$_1$ (in man, by P-450IIIA (Forrester *et al.*, 1990), and AFP$_1$ (Hayes *et al.*, 1991a).

In 1993 Choy suggested that the dose-response of DNA adduct formation in both ingestion and injection studies is linear. While Choy, (1993) has warned that extrapolation of this data to humans should be viewed with caution, as human AFB$_1$-DNA adduct data are incomplete, Groopman *et al.* (1992) on the other hand concluded from their study that urine AFB$_1$-guanine adduct is a particulary good non-invasive marker for exposure to AFB$_1$ and also the risk of genetic damage. It may thus be inferred that the metabolic activation which leads to the formation of both DNA and SA adducts also occurs in humans.

Two factors of activation *in situ* and bi-functionality affect frame shift potency. It would appear that the carcinogen reaches specific tissues in their unreactive form and are only activated once they are inside the target cell. A ring system which is capable of a stacking interaction with DNA results in the molecule having more of an affinity for DNA than an alkylating agent. Work by Miller *et al.* (1978) and Miller (1970) suggests that carcinogenic derivatives are electrophilic reactants and react with nucleophilic cellular constituents.

The biologically effective dose of AFB_1 for any individual would not be expected to reflect only food intake, but also the host associated and environmental variants, including tobacco and alcohol consumption (Harris and Sun, 1986). Much work has been done by Garner *et al.* (1979) on the binding of AFB_1 or AFG_1 by microsomal or peracid oxidation *in vitro*. Rat liver microsomes, BSA or DNA and were incubated for 90 minutes at 37 °C with vigorous shaking (100 strokes/minutes). The DNA or BSA was recovered, hydrolysed or digested accordingly and subjected to HPLC. A radioactive adduct was formed by incubating [^{14}C] AFB_1 with *m*-chloroperbenzoic acid to form the epoxide, which then bound to DNA. The two phases were left to react overnight at room temperature with vigorous stirring. The phases were then separated and the aqueous phase extracted three times with chloroform. The DNA was precipitated with 3 volumes of ethanol, wound out on a glass rod and re-dissolved in sodium chloride and sodium citrate (pH 6,5). An aliquot was taken to hydrolyse in 0,5 M perchloric acid at 70 °C for 20 minutes and the amount of radioactivity measured in Triton X-100 scintillant DNA was measured. Detection and quantification of these adducts have been suggested as alternative methods to detect human exposure to aflatoxins (Autrup *et al.*, 1987).

Behroozikha *et al.* (1992), however found that *in vitro* epoxidation of AFB_1 was determined using liver microsomes from rats of different ages (as measured by adduct formation with calf thymus DNA, newborn rats were capable of minimal AFB_1-DNA binding when compared with adults. Those workers found that levels of the AFB_1-glutathione conjugate were similarly low in neonatal rats. The results of their experiments suggest that the immature liver is less efficient than the mature organ at

activating and detoxifying foreign chemicals. Schlemper *et al.* (1991) found that various hepatic cells populations (hepatocytes, Kupffer and endothelial) have been found to differ in their AFB_1-DNA binding when compared with those found in adults.

Kitamura *et al.* (1992) suggested that when cytochrome P-450IIIA7 was expressed the formation of reactive AFB_1 metabolites was promoted. Environmental agents might influence the susceptibility to AFB_1-mediated hepatocarcinogenesis. These environmental agents may effect the expression of individual P-450 enzymes by either activating or detoxifying AFB_1 (Kolars, 1992).

The cytochrome P-450IA2 is the most important P-450 isoenzyme which prompts AFB_1 binding to DNA (Aoyama *et al.*, 1990). Raney *et al.* (1992) proposed the P-450IIIA4 cytochrome is the dominant enzyme in human liver microsomes. It is involved in the oxidation of AFB_1 to its epoxide, the active form of AFB_1 and AFB_1 to AFQ_1. It has been suggested that P-450IA enzymes may be induced during smoking and promote metabolism of AFB_1 to AFM_1 which is a detoxification reaction, rather than activation to more reactive metabolites by other P-450 enzymes (Lin *et al.*, 1991).

It has been found that animal *species* vary in their sensitivity to the effects of AFB_1. Variation in DNA repair may be responsible for this. Not very much is known about the removal of covalently bound AFB_1 from mammalian cells. Leadon *et al.* (1981) have reported that the AFB_1N^7-Gua adduct is removed spontaneously and enzymatically in fibroblasts, probably by nucleotide excision repair mechanisms. The AFB_1N^7-Gua adduct may be converted to a repairable persistent lesion, the AFB_1-formamidopyrimidine (AFB_1-FAPY) and this remains an area for possible future research.

Tjalve *et al.* (1992) found that microsomal preparations of bovine olfactory mucosa are more effective than liver microsomes to induce covalent binding of AFB_1 to calf thymus DNA and microsomal proteins. When glutathione was added to these preparations less DNA bound to AFB_1. It has been suggested that the nasal olfactory mucosal tumours prevalent in cattle in developing countries may be caused by the

activation of the AFB_1 by bovine olfactory mucosa (i.e P-450 involvement) and is also influenced by lower levels of GST activity (Tjalve et al., 1992), high levels of which would have a counter effect. The potential carcinogenic properties of AFB_1 have raised the question as to the number of doses which aggravate tumour induction. Following a single dose with AFB_1, maximum liver DNA adduct levels were measured after 2 hours. After 24 hours, 88% of the AFB_1-DNA adducts were removed (Wogan et al., 1980; Croy and Wogan, 1981).

Looking at the broader picture, susceptibility and resistance to the toxic and carcinogenic effects of AFB_1 may depend on the age, and general nutritional status of the patient, while the biochemistry involves expression of bio-activating cytochrome P-450s; detoxifying P-450s; glutathione S-transferase activity (McLellan et al., 1994); effective removal of AFB_1 detoxification products from the cells and the ability for excision of AFB_1 adducts from DNA and repair of damage to nucleic acid.

It has been suggested that GST activity plays an important role in regulating AFB_1 activity sources, which in turn enhance GST activity. Several food extracts have been found to enhance GST activity and hence AFB_1-glutathione conjugation. (Groopman et al., 1992). Similarly Kensler et al. (1992) reported that male rats fed 1,2-dithiole-3-thione (0,001%-0,003%) all showed signs of elevated hepatic GST activities. Work by Kirby et al. (1993) suggests that in view of the lack of significant glutathione-S-transferase (GST)-mediated protection against AFB_1 in human liver, variations in expression of hepatic P450, due either to genetic polymorphisms or to modulation by environmental factors, may be important determinants in the risk of liver cancer development in AFB_1-exposed population.

Another factor which results in lower levels of DNA adduction after adult exposure to AFB_1 is neonatal diethylstilbestrol (DES) (Zanger et al., 1992). Gopalan et al. (1992) found that when in vitro studies were performed, the highest catalytic activity with microsome-mediated AFB_1-epoxide conjugation was observed with GST 3-3. However, GST 4-4 appeared to be important for synthetic AFB_1-epoxide conjugation. Rat in vitro generated AFB_1-epoxide may have been inactivated by α-

GST. Two types of efflux pumps regulate the transport of chemicals out of the cell and are involved in elimination of AFB_1. The P-glycoprotein pump is specific for the removal of hydrophobic compounds and the glutathione-S-conjugate carrier is specific for the removal of drug-glutathione conjugates (Hayes *et al.*, 1991a). Although most research into AFB_1 detoxification is still in the early stages of development, the general opinion is that a protective effect is afforded by GSTs in different tissues, as measured by decreased AFB_1-DNA adduct formation with increased GST activity (Hayes et al., 1991a/b). The chemically unstable AFB_1-guanine adduct may be lost spontaneously from DNA to yield apurinic sites. Mutations may arise at this stage (Hayes *et al.*, 1991a).

It is thought that substances provided by the diet inactivate transcription of cytochrome genes, the products of which may be responsible for epoxide formation of several xenobiotic chemicals. Since a number of potential mutagens and carcinogens are diet-related, it would be impossible to eliminate them completely. For a long time free radicals have been thought to be involved in tumour development (Mavelli and Rotillio, 1984). The generation of free radicals appears to be a more or less universal phenomenon in tumour development. It would seem that any compound that can scavenge the oxygen and hydroperoxide, thereby preventing their oxidation of cellular components, is of importance. A number of workers including Bailey and Williams (1993) and Ueno (1993) have reviewed the concept of diet/toxin interaction and have emphasized the protective role of free radical scavengers.

Other dietary factors (eg selenium, vitamin E, sulphur-containing amino acids, copper and zinc) are known to affect the antioxidative ability of an organism (Huan and Fwu, 1993). Other factors include protein deficiency which impairs the antioxidative defense system (Chow, 1988). This is particularly important when considering the status of the kwashiorkor patients screened in this study. Recently Huang and Fwu (1993) found that the degree of protein deficiency in rats affects the extent of depression of the activities of the antioxidative enzymes (glutathione peroxidase, superoxide dimutase) and hence the degree of tissue lipid peroxidation.

Besides dietary protein, the metabolism and toxicity of xenobiotics are instrumental in regulating the activities of activation and detoxification metabolism (Yang *et al.*, 1993), with cytochrome P-450 enzymes being differentially activated (Yoo *et al.*, 1992).

As well as affecting animals and man, mycotoxins have also been found to be phytotoxins affecting plants: however these effects will not be dealt with in this study.

2.7 DISEASES CAUSED BY MYCOTOXIN INGESTION

Mycotoxicosis is not transmissible directly from one animal to another and vitamins and antibiotics have no effect on its severity. Recently Hendrickse and Lamplugh, (1987) detected AFB_1 in certain heroin batches, a possible path of exposure by intravenous drug users. Nutritional deficiencies may play a role in the expression of a mycotoxicosis as discussed later in this study (van Rensberg, *et al.*, 1985). A living organism can be exposed to mycotoxins in a number of ways (Hsieh, 1983; Dutton and Westlake, 1985) including through the consumption of foodstuffs contaminated with toxigenic fungi that have produced toxin, as well as through consumption of meat, milk or eggs from livestock or poultry that have eaten contaminated feed (Figure 15). Other sources of contamination include inhalation of contaminated dust, or direct contact in the laboratory. Mycotoxicosis may assume three levels:

(i) Acute primary mycotoxicosis

This occurs when high to moderate levels of toxin have been consumed and a target organ is affected. Although damage to other organs and tissues may also occur (Hayes, 1980), the disease is classified according to the primary lesions in the target organ (e.g., the liver in aflatoxicosis).

(ii) Chronic primary mycotoxicosis

This results from moderate to low intake of a mycotoxin and may be seen as slower growth rates, reduced reproductive efficiency and inferior

market qualities in stock animals. The more severe effects of mycotoxicosis may not be manifested.

(iii) Secondary mycotoxic diseases

These are the result of low levels of toxin intake. The overt symptoms of mycotoxicosis are not expressed. The victim becomes predisposed to other infections due to an impaired immune response (Pier *et al.*, 1980).

PLANT PRODUCT

FUNGAL INFECTION

MYCOTOXIN FORMATION

CONSUMPTION BY MAN OR FARM ANIMAL

SECRETION IN MILK

BINDING IN

MILK PRODUCTS

PRIMARY MYCOTOXICOSIS

MEAT PRODUCTS

CONSUMPTION BY MAN

SECONDARY MYCOTOXICOSIS
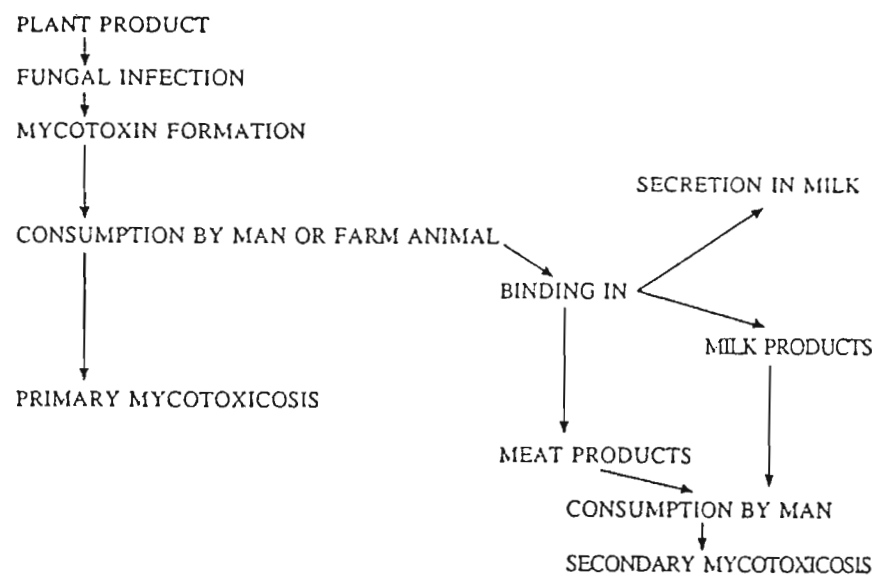
Figure 15: Mechanism of exposure to mycotoxins. Primary and secondary mycotoxicosis (Redrawn from Pier *et al.*, 1980)

The results of Ozturk (1991) support the work of van Rensberg *et al.* (1985) which suggests an aetiological role for AFB_1 as a procarcinogen in the development of liver cancer. Kolars (1992), on the other hand, believes that HBV (hepatitis B_1 virus) (or other agents of chronic liver disease endemic to particular areas) may be a prerequisite for AFB_1-mediated hepatocellular carcinoma (HCC). While Hsing *et al.* (1991) recognise the importance of dietary and environmental factors, they are of the

opinion that HBV infection has a role in liver cancer mortality in China. During (1991) Zhang and co-workers investigating the presence of AFB_1-DNA adducts and of HBVAg (hepatitis B_1 virus antigen) in Taiwanese HCC patients, concluding that both AFB_1 and HBV may be involved in HCC development in Taiwan. More recently, in 1993, Yap *et al.* concluded that both HBV and AFB_1 have a cumulative effect on HCC development. Those authors comment further that while HBV increases the likelihood of HCC, it is not essential for the development of HCC.

Mycotoxins elicit a wide spectrum of toxicologic effects on humans (Table II) and other animals (Pestka and Bondy, 1990). Mycotoxicoses have been reported as far back as 460 B.C. when it was found the consumption of rye and other cereals, contaminated with the fungus *Claviceps purpures* caused gangrene and paralysis, (van Rensburg and Alternkirk, 1974). Table II and Table III show examples of human mycotoxicoses and examples of mycotoxins suspected of being involved in human mycotoxicosis.

Several human mycotoxicoses have been due to the ingestion of identified mycotoxins. Table II shows a summary of this relationship (Smith and Moss, 1985). Mycotoxicoses have been linked with other nutritional diseases including protein energy malnutrition (PEM).

TABLE II: SUMMARY OF IDENTIFIED RELATIONSHIPS

DISEASE	MYCOTOXIN	FUNGUS
Alimentary Toxic Aleukia	Trichothecenes	*Fusarium spp*
Balkan Nephropathy	Ochratoxin	*Aspergillus spp*
Celery Harvester's Disease	Psoralen derivatives	*Sclerotina spp*
Egotism	Ergotamine etc.	*Clavicepes spp*
Kashin Beck Disease	Trichothcenes	*Fusarium spp*
Yellow Rice Toxicosis	Leuteoskyrin	*Penicillium spp*

TABLE III: EXAMPLES OF MYCOTOXINS SUSPECTED OF BEING INVOLVED IN HUMAN
DISEASE

DISEASE	MYCOTOXIN	FUNGUS/REF
Cardiac Beri-beri	Citreoviridin	*Penicillium spp* (Smith and Moss, 1985)
Kwashiorkor	Aflatoxin	*Aspergillus spp* (Hendrickse and Lamplugh, 1987)
Liver Cancer	Aflatoxin	*Aspergillus spp* (Smith and Moss 1985)
Oesophageal Cancer	Fumonisin	*Fusarium spp* (Marasas *et al.*, 1988)
Reye's Syndrome	Aflatoxin	*Aspergillus spp* (Shank *et al.*, 1972)
Precocious Development	Zearalenone	*Fusarium spp* (Smith and Moss, 1985)

2.8   PROTEIN ENERGY MALNUTRITION (PEM)

The term protein energy malnutrition in children refers to conditions characteristed by deficiency of protein and chemical energy. It applies to a range of conditions including the underweight child, and symptoms of kwashiorkor, marasmic-kwashiorkor and marasmus.

Kwashiorkor was first described by Hinojosa in Mexico in 1865. His patients were between 1 - 2 years of age and had diarrhoea followed by oedema, as well as lesions of the skin (Waterlow, 1991). The condition of PEM occurs worldwide, but is endemic to a variable extent in all third world countries. It may become particularly severe when natural catastrophies such as floods or drought, or man-made disasters such as war, disrupt food supplies. This condition is more noticeable in children, since their protein and energy requirement is much greater per kg of body mass than that of adults (Coovadia and Loening, 1984). This study looks only at kwashiorkor patients, as these patients have been shown to have difficulties with AFB_1 metabolism (Ramjee, *et al.*, 1990). Children with kwashiorkor are found in South Africa mainly in the Black

population, generally the lower income group, as the children are fed a low protein, high cereal (maize), diet which is often deficient in essential vitamins and minerals.

2.8.1 Classification of PEM

Marasmus is described as a severe nutritional disturbance characterised by emaciation and weakness of the body (Hansen, 1991). Waterlow (1948) distinguished between marasmus and kwashiorkor by comparing the degree of fatty liver, oedema, wasting and serum albumin concentration, although generally classification is largely according to the Wellcome Trust (1970). It is based on the presence, or absence, of oedema and low body weight. The salient features of kwashiorkor, which set it apart from marasmus, are the presence of hypoalbuminaemia and gross fatty infiltration of the liver, and its appearance in specific geographical and climatic locations (Hendrickse and Lamplugh, 1987). Kwashiorkor tends to be a disease of warm, humid climates and whenever a seasonal prevalence has been reported, it has been associated with wet rather than dry seasons. Marasmus is more commonly found in drier and desert climates (Poskitt, 1972). Clinical features, as stated by Golden (1987), are, oedema, enlarged fatty liver, progressive skin changes, straightening and bleaching of hair, development of jowls, atrophy of the lymphatic tissue and immunoparesis, especially of the cell-mediated immune system. Infection and lack of inflammatory response and apathy, with irritability when disturbed are typical clinical symptoms.

The clinical presentation of PEM shows enormous variations, and is influenced by a number of factors including duration and degree of food deprivation, age, associated specific vitamin or mineral deficiencies, and underlying or complicating disease.

2.8.2 Biochemical Alterations in PEM

The biochemical changes characterising PEM include:

(i)   A reduction in plasma proteins that is greater in the kwashiorkor child than in the marasmic child (Alleyne et al., 1977).

(ii)   Low serum albumin in kwashiorkor (Whitehead *et al.,* 1977).

(iii)   A reduction in serum transferrin in kwashiorkor patients (Whitehead *et al,* 1977).

(iv)   Fatty infiltration of the liver in the kwashiorkor child, (Golden, 1987).

2.8.3   Aetiology of PEM

Factors which determine whether a malnourished child will exhibit the features of kwashiorkor are uncertain. Golden (1987) found that the disease was dispelled on provision of a protein diet with no change in plasma albumin concentrations. In 1968, Gopalan found no nutritional differences in the diets of those children in the same community that develop kwashiorkor and those that develop marasmus. It thus would appear that PEM is not the only cause of kwashiorkor.

The geographical distribution, seasonal incidence and biochemical derangement observed in kwashiorkor are similar to those recorded in epidemiological and animal studies on aflatoxins (Hendrickse and Lamplugh, 1987).

2.9   AFLATOXINS AND KWASHIORKOR

Aflatoxicol, has been detected in kwashiorkor and marasmic kwashiorkor groups, but not in controls in studies carried out in Zimbabwe, Transkei, Liberia (Hendrickse and Lamplugh, 1987), Ghana (De Vries and Lamplugh, 1989) and Kenya (Apeaggei, *et al.,* 1986). Recent research to support the hypothesis that aflatoxins may play an important part in the aetiology of kwashiorkor were inconclusive. In 1987, Golden reported that sera from Jamaican children suffering from kwashiorkor did not show significant levels of AFB_1, while Hendrickse and Lamplugh (1987), in a study on Sudanese children, found higher levels of AFB_1 in the sera from kwashiorkor patients. Hendrickse and his colleagues (1987), have explained their findings by suggesting that the aflatoxins in kwashiorkor build up because of inability of the malnourished liver

to metabolise them. Thus after the liver has been damaged by a low protein diet and depressed lipid synthesis, a vicious circle results with the serum toxin concentrations rising as the ability to metabolise the toxin declines.

Any biotransformation of AFB_1 involves its conversion to the hydroxylated metabolite by the microsomal MFO system, a complex organisation of cyclic-coupled O_2 and NADPH-dependent enzymes. The final result is detoxification of the parent compound to hydroxylated metabolites. These, in turn, are conjugated with sulphates, or glucuronic acid, to form water soluble glucuronides or sulphate esters which are excreted in the urine or bile (Gumbmann and Williams, 1970; McLean and Marshall, 1971; Nebert, 1983).

Studies carried out by De Vries and Lamplugh, (1989) confirmed that aflatoxins cross the human placenta. The possibility of infants already exposed to aflatoxins at birth does therefore exist. Moreover, the neonatal liver does not function fully at birth (Adhikari, 1990) and this could possibly cause an accumulation of the toxin, perhaps having an effect in the longer term. Low birth weight, high perinatal and neonatal mortality and high incidence of neonatal jaundice, often unexplained, characterise the new-born in the tropics (De Vries and Lamplugh, 1989; Adhikari, 1990). Pier, *et al.* (1980) reported that piglets born to sows given AFB_1 in the diet during pregnancy had low serum protein pools. It may be proposed that aflatoxins and other mycotoxins could contribute to the similar problem encountered in the human population in South Africa and other tropical and sub-tropical countries.

Ramjee *et al.* (1990) undertook afaltoxins analyses on 74 children diagnosed, at KEH in Durban, as cases of kwashiorkor. The control group comprised of 35 age-matched, well nourished patients. Aflatoxins were detected in serum and/or urine from all groups, including the controls, while the serum/urine ratio was significantly higher in the kwashiorkor group than in the other groups.

The control group had a higher proportion of urine aflatoxins than the kwashiorkor group. These findings were interpreted in terms of impaired liver

function in kwashiorkor and it was suggested that the consumption of poorly stored maize is likely to be accompanied by ingestion of particular mycotoxins depending on the origin of the seed and the geographic location and climatic conditions of the storage area. Toxic stress induced by aflatoxins and other mycotoxins, poor diet and possibly other environmental factors are all likely to play a role in the pathogenesis of kwashiorkor.

It is probable that a greater knowledge of aflatoxins and other mycotoxins, environmental agents and HBV, will contribute to a better understanding of the origins and mechanisms of cancers that are dependent on an interaction between environmental and genetic factors.

This Chapter has reviewed the far reaching effects of toxigenic fungi and the mycotoxins they produce, with the aim of demonstrating the importance of mycological and mycotoxin studies.

CHAPTER 3

ANALYTICAL METHODS FOR THE ANALYSIS OF MYCOTOXINS

3.1 GENERAL INTRODUCTION

Considerable progress has been made in the development of methods for the analysis of mycotoxins, particularly multi-screening methods that are convenient and capture some quantitisation. These methods can have a high specificity and also detect low levels of mycotoxins. Basic to all areas of mycotoxin research is the development of specific, sensitive and economical analytical methods. The availability of good analytical methods is important in assessing the extent of, and eventually controlling the risk to, human health associated with the contamination of food and feeds by mycotoxins. The interest in mycotoxin methodology is important, particularly for mycotoxins other than aflatoxins, as this is an area less researched. To investigate cases of suspected mycotoxicosis, studies on the natural occurrence of mycotoxins in materials or for backing-up multi-toxin surveillance programmes, reliable, sensitive and proven methods are required. The purpose of the studies described in this chapter is the evaluation of analytical techniques used in mycotoxin analysis. These techniques include multimycotoxin extraction, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gel permeation chromatography (GPC), gas chromatography-mass spectrometry (GCMS) and immunoassay. Some of these techniques have been used in subsequent studies reported in this study.

The most difficult and time-consuming step in the analysis of a natural substrate is often the purification of the analyte, i.e., the "clean-up" of the extract. This chapter compares two "clean-up" methods: the dialysis method (Roberts and Patterson, 1975) and GPC (Scudamore and Hetmanski, 1992). In December 1993 and April-May 1994, two separate batches of KaNgwane groundnuts were "cleaned-up" using the dialysis

method. The fifteen extracts in the first batch were analysed by HPLC for AFB_1, and the sixteen extracts in the second batch were analysed by GCMS for trichothecenes. For the detection of AFB_1 HPLC is the preferred technique, while GCMS is more suited to trichothecene detection.

3.2. MULTISCREENING METHODS FOR MYCOTOXIN DETECTION

3.2.1 Introduction

Methods for the determination of low levels of mycotoxins in cereals and animal feed stuffs are commonly complex, lengthy and costly to carry out. The dialysis "clean-up" method by Roberts and Patterson (1975) has been widely used in the UK and elsewhere; Buckle (1983) has reported data obtained using this method for the occurrence of a number of mycotoxins determined in samples received at the laboratories of the UK Ministry of Agriculture, Fisheries and Food during 1979. The dialysis method was also used successfully by Dutton (1993) on grain samples sent to the University of Natal for routine analysis.

A recently developed multimycotoxin screen is based on GPC. In this method a column of gel particles or porous glass granules is in equilibrium with a suitable solvent for the molecules to be separated. Large molecules which are completely excluded from the pores will pass through the interstitial spaces, while smaller molecules will be distributed between the solvent inside and outside the molecular sieve and will then pass through the column at a slower rate. Figure 16 shows a diagrammatic representation of separation by exclusion chromatography.

The solvent absorbed by a swollen gel is available to a solute to an extent which is dependent upon the porosity of the gel particle and the size of the solute molecules. Thus the distribution of a solute in a column of a swollen gel is determined solely by the total volume of solvent, both inside and outside the gel particles, which are available to it.
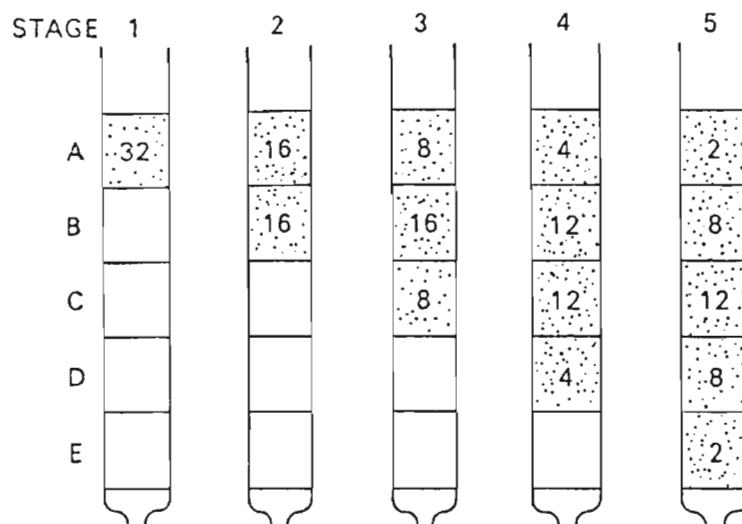
Figure 16: Principle of column chromatographic separation

For a given type of gel, the distribution coefficient K_d, of a particular solute between the inner and outer solvent is a function of its molecular size. If the solute is large and completely excluded from the solvent within the gel, $K_d = 0$, whereas if the solute is sufficiently small to gain complete accessabitly to the inner solvent, $K_d = 1$. Due to variation in pore size for a given gel, there is some inner solvent which will be available and some which will not be available to solutes of intermediate size, hence K_d values vary between 0 and 1. It is this complete variation of K_d between these two limits which makes possible separation of solutes within a narrow molecular size range for a given gel.

The elution volume, V_e of a given solute depends on the void volume, V_o, the distribution coefficient and the volume inside the gel matrix itself, V_i,

Thus: $$V_e = V_o + K_d V_i$$

The inner volume, V_i, can be calculated from the dry weight of the gel, and the water regain value, W_r since:

$$V_i = a W_r$$

The numerical value of V_e for a given solute will vary with the size of the column, whereas K_d is a characteristic value for the solute and is independent of the geometry of the gel bed. For two substances of different molecular weight and K_d values $K_{d'}$ and $K_{d''}$, the differences in their effluent volumes, V_s is given by:

$$V_s = V_{e'} - V_{e''} = (V_o + K_{d'}V_i) - (V_o + K_{d''}V_i)$$

therefore:

$$V_s = (K_{d'} - K_{d''})V_i$$

Thus for complete separation of the two substances, the sample volume must not be larger than V_s. In practice, deviations from ideal behaviour, for example due to poor packing of the column, make it advisable to reduce the sample volume below the value of V_s since the ratio between sample volume and inside gel volume affects both the sharpness of the separation and the degree of dilution of the sample.

When "cleaning-up" mycotoxins it is important to remember that these compounds usually fall into the 200 - 600 molecular weight range and should thus be separated from high molecular weight food constituents extracted along with the mycotoxins.

The Milton Roy Spectronic 3000 Array Spectrophotometer is a single-beam spectrophotometer that should enable spectral data to be obtained quickly and precisely. The instrument offers a variety of features including a built in computer, advanced photodiode array optics and unique automatic sample-positioning platform.

For this study, twelve maize samples were collected in the early part of 1993. One sample was from KwaZulu, seven from KaNgwane and four from the Eastern Cape. A mycological analysis (Appendix 1) and mycotoxin analysis, using the dialysis "clean-up" method (Roberts and Patterson, 1975) referred to as method A described below, was performed on each of these samples. The maize samples were also "cleaned-up" in a parallel analysis using method B, the GPC procedure (Scudamore and Hetmanski, 1992), in order to compare, by HPLC, the recoveries of this latter method with those of the dialysis method. The same dialysis "clean-up" method was performed by Mrs. R. McLaren (S.A.I.M.R) at the Rob Ferreira Hospital on maize samples collected over the period of February 1993 - September 1993 from the KaNgwane region. The TLC plate results from the Rob Ferreira Hospital, were sent to the University of Natal Medical School for comparison purposes.

3.2.2 Materials

Equipment for method A comprised a 250 ml conical flask, shaker (Brunswick), rotary evaporator, Buchner apparatus, 30 ml glass vials, separating funnel. and Whatmann No. 1 filter paper and dialysis tubing, 25 cm lengths (Sigma D-9277 Lot 30$^+$10674). Reagents of Analar (AR) grade were as follows: aqueous potassium chloride (4%), acetonitrile/potassium chloride solution (4%), sodium bicarbonate solution, 1 M sulphuric acid solution, iso-octane, chloroform, aluminium chloride (20%), sulphuric acid spray (20%), anisaldehyde spray, dianisidine spray, 4-4(-nitrobenzyl)pyridine dipping solution and tetrapentamine spray solution. The make-up of these solutions is detailed by Dutton (1993).

Equipment for method B comprised a 500 mm x 25 mm internal diameter glass column (Sigma Chemical Company St Louis, USA) fitted with a 40-60 μm porous bed support and adjustable plunger was packed with Bio-Beads S-X3 gel (Bio-Rad Watford, UK) and interconnecting tubing between column and fraction collector (Gilson FC) and between column and solvent tank. Reagents of AR grade were as follows: dichloromethane, ethyl acetate and 99% formic acid.

3.2.3 Method (A) Dialysis "clean-up" method (Robert and Patterson, 1975)

The method employed was modified from that used by Roberts and Patterson (1975). Milled maize samples (25 g) were vigorously shaken (2 hr) in an acetonitrile/potassium chloride (4%) solution (100 ml). The extract was filtered on a Buchner apparatus, washing the residue with 10 ml of the solvent mixture. Lipids were removed by partitioning the extract with iso-octane; (2 x 100 ml). The iso-octane fraction was discarded and a sodium bicarbonate solution (50 ml) was added to the remaining defatted aqueous extract. This was partitioned with chloroform (50 ml). The separating funnel was shaken gently to avoid the formation of an emulsion. The chloroform layer (bottom) was run through a small bed of anhydrous sodium sulphate 5-10 g in a folded 11 cm filter paper in a small filter funnel placed in the neck of a 250 ml rotary evaporator flask. This is the neutral fraction. The aqueous phase was

extracted with a further three 25 ml portions of chloroform. Both the aqueous (acid fraction) and the chloroform layers (neutral fraction) were retained.

The combined chloroform extracts were evaporated to dryness on the rotary evaporator with the water bath at a temperature of 65 °C. Dialysis tubing, which had been soaked in water for at least one hour was knotted at one end to form a closed sac and the tube was filled with water to check for leaks. This was then emptied and drained. The residue in the flask was dissolved in 2 ml of acetonitrile, and then transferred into the sac using a transfer pipette fitted with a rubber teat. The flask was washed out with a further 1 ml acetonitrile and transferred to the sac. After removing as much air as possible a knot was tied at the top of the sac and the sac placed in a boiling tube (15 x 25 cm) which contained 50 ml of aqueous acetone (30% v/v). The top knot of the tube was looped over the lip of the tube, and a piece of aluminium foil crimped over the top and secured with a rubber band. The tube was left in a shaker overnight.

The following day the dialysate was transferred to a 250 ml separating funnel and the boiling tube washed out with acetone which was added to the dialysate. The sample was extracted with three 25 ml portions of chloroform and each extract was passed through a clean bed of anhydrous sodium sulphate into a clean rotary evaporation flask. The sample was evaporated on a rotary vacuum evaporator to dryness. A volume of 2 ml of chloroform was added and the residue transfered using a long transfer pipette fitted with a teat to a 1 dram screw cap vial. The sample was dried under a stream of nitrogen. When dry the sample was sealed with a screw cap. (It is necessary to store the sample in the dark at -20 °C if is not to be immediately examined by TLC).

A volume of 50 ml of 1M sulphuric acid was slowly added to the retained aqueous fraction (from which the neutral fraction had been extracted). After the effervescence had subsided, the acidified solution was extracted with three 25 ml portions of chloroform. The extracts were run through a bed of anhydrous sodium sulphate, pooled and treated the same way as the neutral extract, but with the omission of the dialysis step. This constituted the acid fraction.

3.2.4 Method (B) Analysis using the gel permeation chromatography "clean-up" method (modified from Scudamore and Hetmanski 1992).

A glass column, 500 mm x 25 mm id, (Sigma Chemical Company, St. Louis, USA) fitted with a 40-60 μm porous bed support and adjustable plunger was packed with Bio-Beads S-X3 gel. The gel (60 g) was suspended in 120 ml of dichloromethane: ethyl acetate: formic acid (49,9:49,9:0,2 v/v/v) for 1 day at 5 °C. The mixed gel suspension was poured into the glass column and after the gel had settled, the plunger was depressed to give a column height of approximately 350 mm. Elution solvent was pumped through the column for 2 hours before use.

A 25 g portion of finely ground sample was weighed into a 500 ml Erlenmeyer flask with 12,5 g of sand 12,5 ml of 1 M hydrochloric acid and 125 ml of dichloromethane. The flask was stoppered with an aluminium foil wrapped rubber bung and shaken (New Brunswick Scientific Co. Inc) for 30 minutes before filtering the sample through a 27 cm Whatman No 1 fluted filter paper into a 250 ml round-bottom flask. The residue in the filter paper was rinsed three times with 25 ml portions of dichloromethane, which was then added to the filtrate in the 250 ml flask. The combined-filtrate and washings were evaporated to near dryness by rotary evaporation at 30 °C and the residue in the flask transferred to a 10 ml volumetric flask using four rinses of dichloromethane, approximately 1 ml each time. A mixture of 5 ml of ethyl acetate and 0,02 ml of formic acid were added and the solution made up to the mark with dichloromethane. All flasks were wrapped in aluminium foil to exclude light and so avoid breakdown of light-sensitive compounds.

After column equilibration, 5 ml portions of standards or spiked sample were loaded onto the column. Using AFB_1 as the model 50 ng, 100 ng, 250 ng and 500 ng, were prepared in the GPC eluent solution (dichloromethane: ethyl acetate: formic acid (49,9 : 49,9 : 0,2 v/v/v)), and loaded onto the column. The flow rate was set at 4 ml/min and the eluent collected in 5 ml portions (Gilson FC Fraction Collector).

As a second sample, a mixture of AFB_1 (10 µg/µl), DON (10 µg/µl), FB_1 (10 µg/µl), and ZEA (10 µg/µl) was prepared in the GPC eluent solution, and loaded onto the GPC column and the fractions collected. The fractions were combined as described below.

Secondly, uncontaminated maize was spiked with the same mycotoxins at the same concentrations as mentioned above, prior to extraction with hydrochloric acid and dichloromethane as described. A 5 ml portion of spiked sample extract solution was loaded onto the column and then eluted as above. In order to determine in which fractions mycotoxins appeared, each successive 5 ml portion eluted was collected and tested separately using qualitative one-dimensional TLC (section 3.3). Using a micropipette 20 µl of each second sample was added to methanol and the absorbance between 200-700 nm recorded using a Milton Roy Spectronic 3000 Array Spectrophotometer.

A volume of 20 µl of every other fraction eluted from the column (5 ml) was spotted onto a TLC plate and then developed in toluene : ethyl acetate : acetone : acetic acid (80 : 30 : 20 : 1) and then viewed under UV light. Those plates which showed fluorescence were sprayed with 50 % ethanolic H_2SO_4 and heated. Those fractions showing fluorescence on TLC were combined in a separating funnel with 10 ml of water, stoppered and shaken well. The lower organic layer was run off through a fluted filter paper holding anhydrous sodium sulphate, into a 100 ml round-bottom flask. The sodium sulphate was rinsed with 15 ml of dichloromethane and the combined filtrate and washings were evaporated to near dryness at 30 °C. The residue in the flask was transferred to a smaller container with four rinses of 1 ml dichloromethane and the solution evaporated to dryness at room temperature under a stream of nitrogen.

3.3   THIN LAYER CHROMATOGRAPHY (TLC)

3.3.1   Introduction

The technique of thin layer chromatography (TLC) is simple, quick and cheap, and allows several samples to be studied concurrently when used for analytical and preparative purposes. The technique was a natural development from the earlier paper chromatograph with the advantage that in TLC the matrix may be varied. By using cellulose powder, a layer equivalent to that of paper may be spread, or, if silica gel is used, a more polar layer is obtained which is suitable for the separation of less polar compounds, that may prove troublesome on paper. In the case of polar compounds, these would tend to bind very strongly to matrices such as silica gel and would therefore need a more polar solvent to move them, which would not be desirable. In this case less polar layers such as cellulose are preferred.

Layers are usually prepared by using a slurry of finely powered matrix material often with water as the solvent. This is spread over glass plates to a preset thickness using a TLC spreader. The thickness does not generally exceed 1 mm and the plates may vary from 5 cm x 20 cm to 20 cm x 20 cm in size. Once the solvent has dried off the layer is ready for use. Cellulose binds naturally into a firm layer but materials like silica gel require binding agents such as gypsum added at around 10%. Material treated in such a manner has the letter G added, e.g. silica gel G. Commercially prepared plates are preferred, as these generally have consistent matrix layers, but would be more expensive than those spread in the laboratory.

The choice of solvent systems used depends very much upon the polarities and solubilities of the compounds to be separated in a mixture. As already mentioned more polar compounds require polar solvents. Normally solvent mixtures are used to optimise separating conditions and their selection is usually a matter of both trial and error and experience.

A grading of solvent polarities would be:

paraffins e.g. hexane->toluene->chloroform->acetone->alcohol->water

increasing polarity----------------->

The amino acids, which are fairly polar, are separated using mixtures of alcohols and water plus a weak acid or base, whereas a non-polar compound like a carotene would be separated by toluene.

A number of factors are important in performing TLC. Plate activation is an important point as this depends on the amount of water absorbed by the layer. Silica gel layers may be activated by pre-heating to over 100 °C to drive off the water, while storing in desiccators does not help as the plate has to be spotted in the open laboratory and the time spent doing this is quite sufficient for equilibrium to be reached. When running plates in the two dimensional method, the TLC plate is dried after the first run, turned through 90° and run again in a second solvent (Figure 17). Good separation between compounds and contaminants is important. The two dimensional TLC technique provides a better separation of the compound than a uni-dimensional run, since no two compounds for separation are likely to move the same distance in two different solvent systems.

Direction 1 solvent
chloroform:ethyl acetate:
propano-2-ol (90:5:5)

Direction 2 solvent
toluene:ethyl acetate:
formic acid (6:3:1)
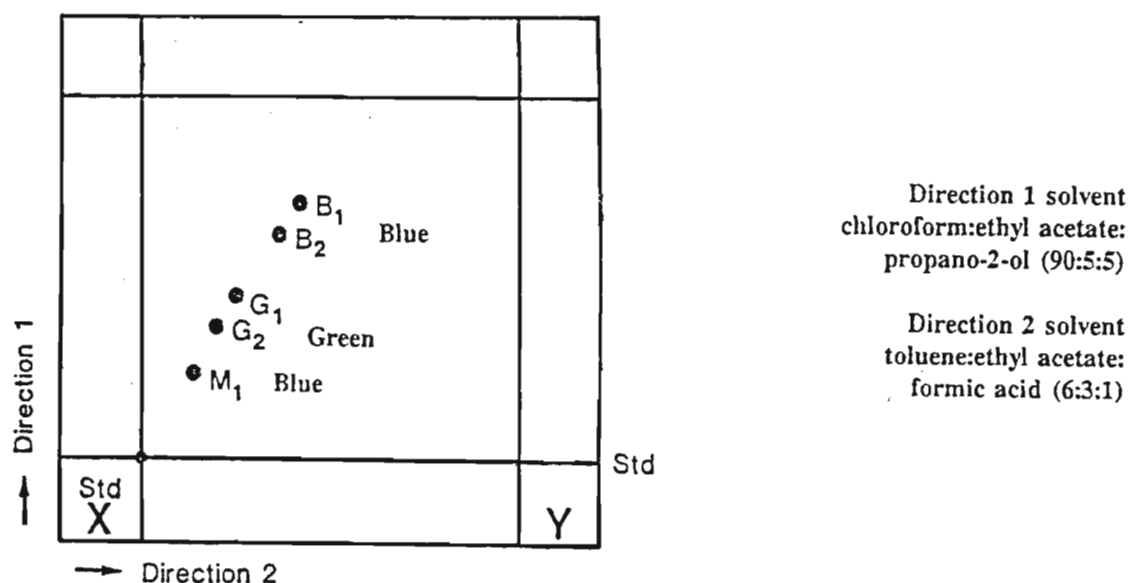
Figure 17: Schematic representation of 2-dimensional TLC of aflatoxin B_1, B_2, G_1, G_2, M_1 spots on plate

In effect, the development of the TLC plate in one direction is a "clean-up" step while development in the second direction is the actual separation-detection step. Its major drawback is that it is time-consuming, as only one sample can be applied per TLC plate (Pohland, *et al.,* 1990). The chromatograms are then viewed under good daylight, visible spots marked with a pencil and identified with a suitable code (Appendix 5.0). The distance migrated by the solvent (DS), which is the distance from the baseline drawn from the origin to the edge of the plate, is measured, as is the distance moved from the baseline by each spot (DC). The R_f value for this spot in the first dimension is determined using: $R_f = DC/DS$. This ratio will always be less than 1. R_f values can also be expressed as a percentage.

It may be necessary to spray the plates in order to identify some mycotoxins. It is important that known standard plates are also run so that the presence of a specific mycotoxin can be confirmed. New spots which appear are suitably coded and the R_f values calculated. It is important that spots which develop are immediately dotted around with pencil and recorded as to colour and R_f value. This is necessary because many colours quickly fade or change, e.g. those with the trichothecené spray reagents (Dutton, 1993).

It is important in analysis to distinguish the mycotoxin from interfering compounds such as maize pigments, and other fungal metabolites within the sample. This can be achieved by comparison with standards, which can either be run externally or internally. A standard can be spotted onto a second plate, which can be run under exactly the same conditions as the test plate, or the plate is spotted as normal and then the standard is spotted on top. The standard will then co-chromatograph with the same component in the extract. The plate is compared with a similar run without the spiked standard.

It is important to maintain a standard initial spot size by spotting the same amounts. To obtain consistent R_f values, running conditions should be standardised. The tank atmosphere varies in its saturation with solvent vapour. The poorer the saturation the longer the running time and the further the distance migrated by the

spot. This results in higher R_f values. Consistency of run parameters with regard to temperature and tank saturation must be ensured.

In some instances plates may be run in unsaturated tanks to gain better resolution of the spots but reproducibility of R_f values will be sacrificed. The plates are put in the tank immediately after the solvent so that the same conditions of unsaturation appertain in all cases.

The R_f values of identical standards which have been run on the same plate may differ according to their position on the plate. Those closest to the edge of the plate generally have a higher R_f value. This is termed the "edge effect" and can be on a one dimensional chromatograph with a series of the same standard spotted along the origin as a "bowed" line of spots. A variability of the solvent composition may also cause changes in R_f value. Solvents, AR grade, are recommended, even though this does increase expense. After performing a run it is inadvisable to keep the same solvent mixture in a tank for a prolonged period of time, as its composition will change due to the preferential evaporation of the more volatile components, until a constant boiling mixture is attained. This may well be far from the starting composition. It is also possible that the components may actually react with each other.

If temperature varies markedly, there will be a corresponding change in R_f value. This factor can be overcome by placing the tanks in an incubator at a constant temperature of 18 °C. Over-heating can cause background colour to develop and in the case of sprays containing sulphuric acid, extensive charring. Sprays may be suitably stored in 100 ml Quickfit conical flasks with B14 joints and stoppers; these can be labelled and kept in the refrigerator. Some sprays have to be prepared as required e.g. Pauly's reagent: these are described in detail below.

It is important when working with mycotoxins, especially aflatoxins that all glassware is carefully washed. Although sodium hypochlorite is usually used in the detoxification of AFB_1 controversy over its use exists (Costegnaro et al., 1981). These workers have found that sodium hypochlorite solution which has been in use for a

long time as a reagent to treat laboratory wastes and equipment contaminated with AFB_1, has been shown by TLC and HPLC and mass spectrometry to lead to the formation of products which include its carcinogenic 2,3-dichloro derivative. A modification to the method of decontamination using sodium hypochlorite, based on the stability of the 2,3-dichloro derivative in the presence of acetone, is suggested.

3.3.2   Materials

The equipment for these experiments comprised silica aluminium backed TLC plates (Merck Art 5553), solvents, chloroform, butanol, propan2-ol, Viewing cabinet, developing tanks, drier and sprays.

3.3.3   Method

Following the dialysis "clean-up" method, the extract was dissolved in 200 µl of chloroform-acetone (1:1 v/v).   An aluminium backed TLC plate (20 x 20 cm) precoated with silica gel G was cut into 4 equal 10 x 10 cm plates. A sample of the extract (20 µl) was spotted in 2 µl portions onto the origin of the plate and carefully dried at each addition with a stream of air. Three plates were spotted with the neutral extract and two with the acid extract. A cross was lightly pencilled 1 cm from one corner (origin). In the top corner diagonally opposite the origin the identification of the sample to be run was pencilled in e.g. 1A 1/2 would refer to sample 1, acid extract, solvent 1 followed by solvent 2.

The plate was placed with the origin in the bottom left hand corner of the tank containing 20 ml of the first solvent system. In order to obtain good separations, specific solvent systems given in Appendix 3.0 for AFB1, DON, FB_1, ZEA were used.

The plates were sprayed with the appropriate stains (Appendix 4.0). The plates were propped against a large piece of glass with both plates and glass standing on paper towels in a fume hood. The spray gun was held about 50 cm from the plates and the entire surface of the plate sprayed with reagent. In some cases it was

necessary to heat the plates in a standard laboratory oven preset to the correct temperature (120 °C). Where dipping techniques were employed the plate was held with forceps and dipped evenly and quickly into the dipping solution. The plates were viewed under both long and short wave (UV) light. Any fluorescing or absorbing spots were marked with a suitable colour code (Appendix 5.0).

The prescence of **DON** was identified by spraying the plate with sulphuric acid, followed by heating at 110 °C for 10 minutes (Abbas, *et al.*, 1986).

The presence of **FB₁** was identified by spraying the plate with anisaldehyde, followed by heating at 110 °C for 10 minutes (Bezuidenhout *et al.*, 1988).

The presence of **ZEA** was identified by spraying the plate with diazotized dianisidine. Half an hour was permitted for the colour to develop (Balzar *et al.*, 1978)

The **Trichothecene epoxides** were identified by dipping the plate in 4-(4-nitrobenzyl) pyridine reagent (NBP Reagent) and spraying with tetrapentamine spray (Takitani, *et al.*, 1979).

3.4   HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

3.4.1   Introduction

The aim of chromatography is to separate components of a sample within a reasonable period of time into separate bands or peaks as they migrate through the column. The resolution, R, between two peaks can be quantitatively measured as shown by:

$$R_s = \frac{2\,(t_{RB} - t_{RA})}{\varphi_A + \varphi_B}$$

where t_{RB} and t_{RA} are retention times of the retained components measured at the peak maximum and At is the difference between t_{RB} and t_{RA}. The peaks for φ_B and φA are the peak widths in units of time measurement at the base.

It can be shown that when $R_s = 1,5$ the separation of the two peaks is 99,7% complete. In most practical cases an R_s value of 1,0, corresponding to 98% separation, is satisfactory. According to the definition of resolution, R_s-1 peaks fit between any two peaks in question. Peak resolution is determined by three factors: selectivity which measures the discriminatory power of the system, retention which measures the retentive power of the system and which is related to the partition coefficients, K_d, of the compounds and efficiency which measures the relative narrowness of the peaks in terms of the ratio of retention time to peak width.

The number of theoretical plates (plate number) (N) involved in the elution of a particular compound is given by:

$$N = 16\left(\frac{t_R}{\varphi}\right)^2$$

$$\text{or} \quad N = 5,54\left(\frac{t_R}{\varphi}\right)^2$$

where φ = the peak base width and is equal to $4°\sigma$
φh = the peak width at half the peak height and is equal to $2,355°\sigma$
and the constants 16 and 5,54 are derived from 4^2 and $2,355^2$ respectively.

The value of N can therefore very easily be calculated. The plate number can be increased by increasing the column length (L) but there is a limit to this since the retention time and peak width increase proportionally with L whilst the peak height decreases as the square root of N. Whilst N is a measure of the efficiency of the column, the plate height, which is also called the Height Equivalent to a Theoretical Plate $(HETP)$, is useful for comparative purposes such as operating the column under different conditions. It can be shown that:

$$HETP = \frac{L}{N} = H$$

The maximum number of peaks that can be separated by a specific chromatographic system is called the peak capacity (n). It is related to the retention volumes of the first and last peaks ($V\alpha$ and $V\varphi$) respectively and to the plate number:

$$n = 1 + \sqrt{\frac{N}{16}} \left(\ln \frac{V_\varphi}{V\alpha} \right)$$

Peak capacity is determined by acceptable separation times and by detection sensitivity. In practice it can be increased by the procedure of gradient elution as in liquid chromatography (Wilson and Golding, 1986).

In ordinary column chromatography a glass column is packed with a chromatographic matrix and components are separated by elution from the column with suitable solvents. The separated components are then collected in fractions from the bottom of the column. In the modern version of this technique, high performance liquid chromatography (also called high pressure liquid chromatography) the same principle is used, except that the solvent is pumped through the column at high pressures (up to 200 atmospheres). The advantage of this is that the column can be packed with much finer particles of matrix allowing much better resolution (separation) of components and therefore much shorter and narrower columns are used. The technique of HPLC is popular as it enables the analysis of a wide variety of compounds, including compounds that are easily degraded by heat, light, or air. The methods can be adapted to confirmatory procedures. It allows the potential for automation and offers the flexibility for development of increasingly sensitive UV, fluorescence and electrochemical detectors and high-resolution normal and reverse phase columns.

The choice of column is important for a given application. In normal phase liquid-liquid chromatography, the stationary phase is a polar compound such as alkyl

hexane. For reverse-phase chromatography, the stationary phase is a non-polar compound such as a C(8) or C(18) hydrocarbon and the mobile phase a polar solvent such as water/acetonitrile or water/methanol/methanol mixtures.

The entire instrument was computer controlled and may have an automated sampling system. The computer automatically runs pre-selected solvent systems which may be changed in composition during the run. Solvent flow, detection parameters, quantification, recording of full absorption spectra and automatic sampling will also be controlled from this point.

The solvents must be of HPLC grade and the HPLC grade water must be prefiltered through a Millipore Norganic cartridge (or equivalent). This is to prevent blockage of the column. There are two types of elution: gradient and isocratic elution. Gradient elution is defined as increasing the strength of the mobile phase during a chromatographic analysis. The net effect of gradient elution is to shorten the retention time of compounds strongly retained on columns. The fundamentals of gradient elution have been described by Snyder, (1965). Gradient elution offers several advantages; the overall resolution per unit time of a mixture is increased, the peak shape is improved (less tailing), effective sensitivity is increased since there is little variation in peak shape. Gradients may be stepwise or continuous. An isocratic mobile phase will run at the same ratio of solvents throughout the run.

Extracts to be injected are usually dissolved in a solvent compatible with the solvent system. Standards (from Sigma and C.S.I.R) are chromatographed in the system so that it may be calibrated with respect to retention time and amount of the mycotoxin of interest. The samples are then injected. AFB_1 and AFG_1 may be derivatised (section 3.4.3, page 63). This is to enhance the fluorescence of the compounds. A more sensitive response is obtained using a fluorometer, which changes the retention time of the compounds by a fixed amount thus acting as a reference. The wide applicability, speed and sensitivity of HPLC (Figure 18) have resulted in it becoming the most popular form of chromatography and virtually all types of biological molecules have been purified using this approach. Reverse phase partition

biological molecules have been purified using this approach. Reverse phase partition HPLC is particularly useful for the separation of polar compounds such as drugs and their metabolites; peptides among others. Prior to the advent of this form of chromatography, the separation of such polar compounds was not easily accomplished and often required pre-derivatisation to less polar compounds. The technique is particularly widely used in clinical and pharmaceutical work as it is possible to apply biological fluids such as serum and urine directly to the column, preferably using a guard column. The separation of some highly polar compounds such as amino acids which are difficult to resolve adequately by reverse phase chromatography can be improved by either ion-suppression, in which the ionisation of the compound is suppressed by chromatographing at an appropriately high or low pH, or by ion-pairing.
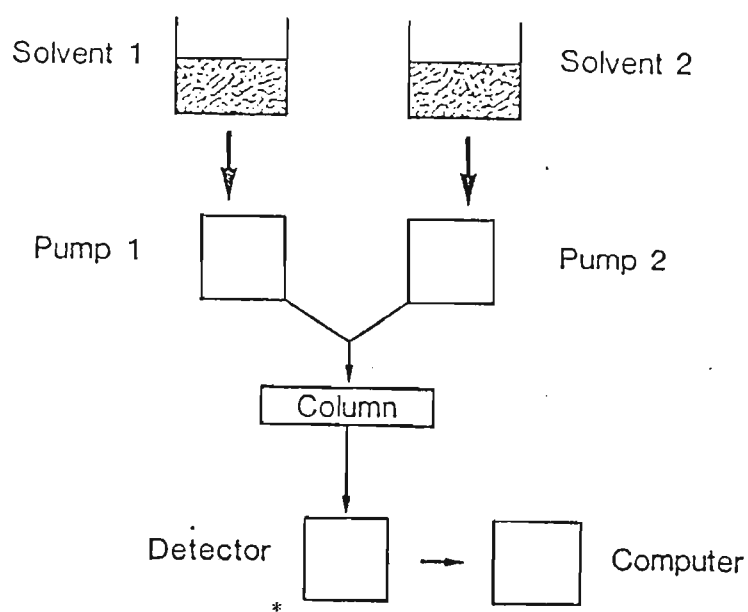
Figure 18: Schematic representation of HPLC

* either UV or fluorescence (redrawn from Ramjee, 1990)

Repeated application of highly impure samples such as sera, urine, plasma or whole blood, which may have been deproteinated, may eventually cause the column

to lose its resolving power. To prevent this occurrence a guard column is installed between the injector and the analytical column. This guard column is a short (2 to 10 cm) column of the same internal diameter and packed with a similar material to that present in the analytical column. The packing of the guard column can be replaced at regular intervals.

3.4.2 Materials

A total of 15 KaNgwane groundnut extracts (from groundnuts harvested in December 1993) were sent to the Physiology Department, Medical School, University of Natal, for AFB_1 screening and quantitation using HPLC. Test equipment comprised a Spectra-Physics FL2000 fluorescence detector, a reverse phase column (Merck) measuring 12,5 cm x 4,6 mm, an alternate reverse-phase column (Spheri-Sorb) 25 cm x 4,6 mm and a millipore filter cartridge. The solvent; acetonitrile, acetic acid, water, and methanol were of HPLC grade. Trifluoroacetic acid (TFA) for derivatisation.

3.4.3 Method

The method reported here is that devised by Berry *et al.* (1984). The instrument was set to pump at a flow rate of 1 ml/minute using an isocratic solvent system consisting of acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 1% acetic acid 40%, which had been filtered through a millipore cartridge prior to injection to avoid blockage of the column.

Two different columns were connected to the Spectra-Physics fluorescence detector. The reverse phase (Merck) column was used for the quantitation of recoveries of spiked maize samples "cleaned-up" using either dialysis or GPC "clean-up" methods. The reverse-phase column (Spheri-Sorb) was used for the quantization of AFB_1 in groundnut extracts.

To enhance the fluorescence of the compounds, the standards and extracts were first derivatised with TFA following the method described below. The sample was

redissolved in chloroform and 200 μl removed and evaporated to dryness under a stream of nitrogen. To the dried extract 100 μl TFA:Water (9:1) were added and the vial vortexed. This was left for 15 minutes, before being evaporated to dryness under a stream of nitrogen (no heat). Methanol was added to increase the rate of evaporation. A volume of 2 ml injection solvent (mobile phase) was added and 20 μl used for injection. The Excitation wavelength (Ex) was set at 360 nm and Emission wavelength (Em) at 440 nm. Derivatised samples and standards were used within 24 hours. Samples were injected in duplicate.

3.5 RESULTS OF "CLEAN-UP" PROCEDURES

TABLE IV: ELUTION ORDER OF MYCOTOXINS FROM GPC COLUMN AS DETERMINED BY TLC

Mycotoxin	Fractions (5 ml) containing mycotoxins (formic acid strength 0,2 %)
Zearalenone	2-30
Deoxynivalenol	2-40
Aflatoxin B$_1$	12-30

(1)

(2)

(3)
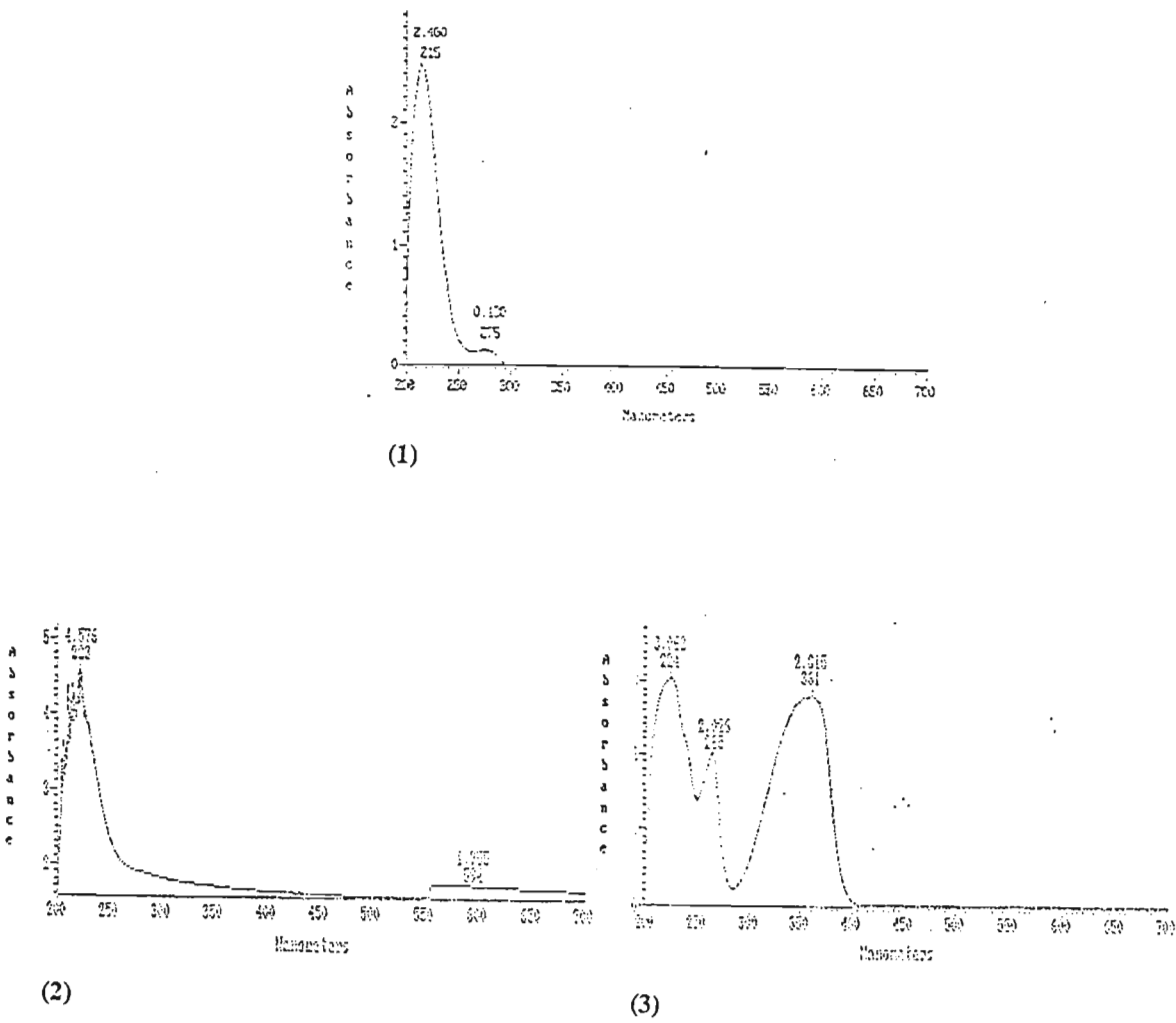
Figure 19: Scans (200-700 nm) of maize extract following "clean-up" using GPC (1) Zearalenone, (2) Deoxynivelenol, (3) Aflatoxin B$_1$

Four standards of AFB$_1$, 50 ng, 100 ng, 250 ng and 500 ng, were injected onto a RP C(18) column and the HPLC chromatographs are shown in Figures 20-23.
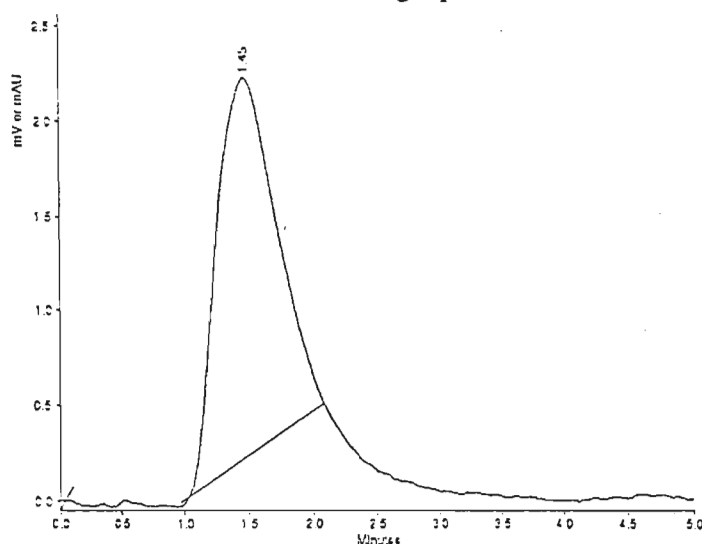
Figure 20: HPLC chromatograph of aflatoxin B$_1$ (50 ng), RP (C18) column (Merck), 1ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 0,4 % acetic acid , Ex: 360 nm, Em: 440 nm
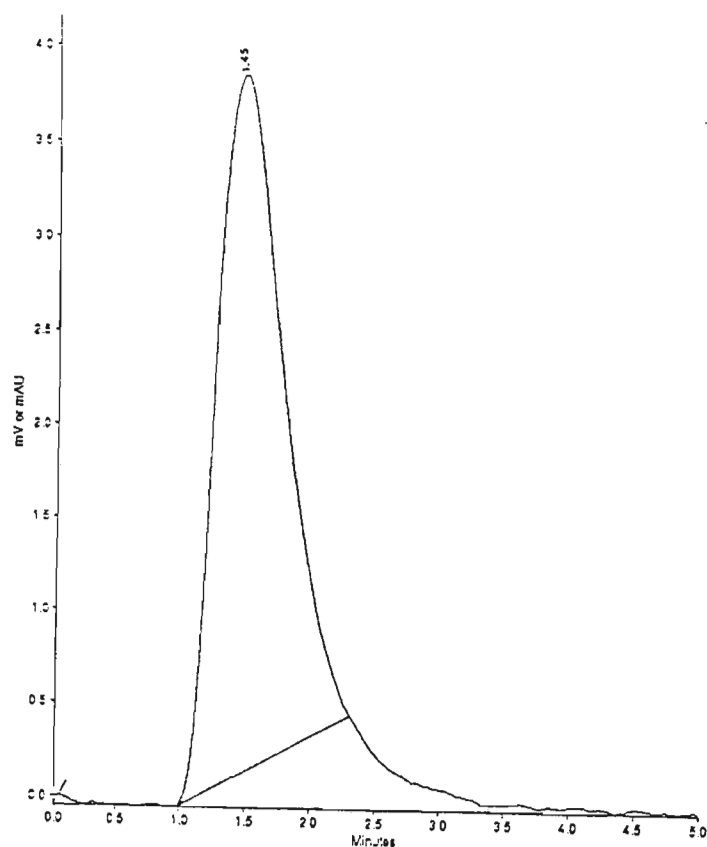
Figure 21: HPLC chromatograph of aflatoxin B1 (100 ng), RP (C18) column (Merck), 1ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 0,4 % acetic acid, Ex: 360 nm, Em: 440 nm
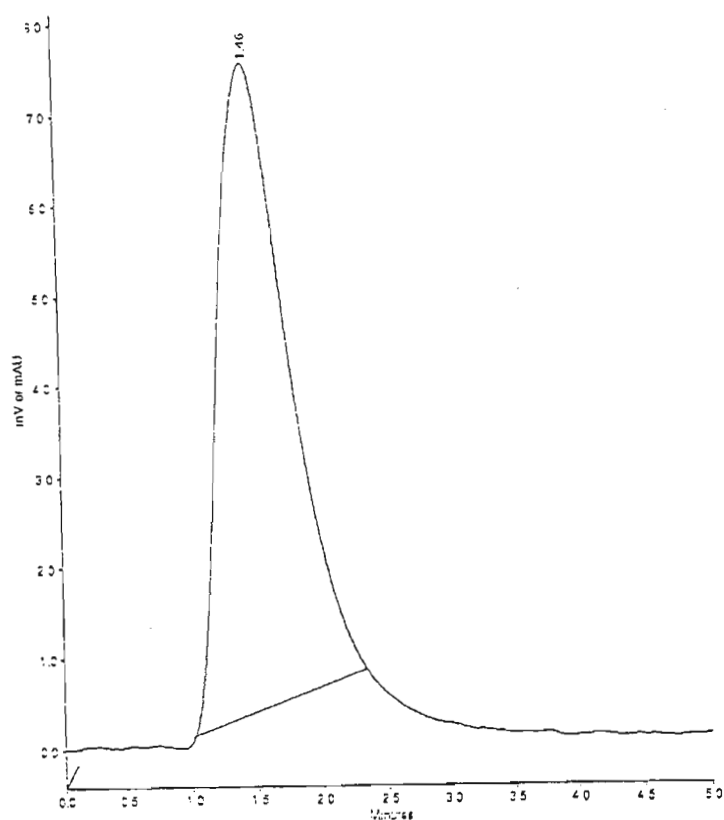
Figure 22: HPLC chromatograph of aflatoxin B1 (250 ng), RP (C18) column (Merck), 1ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 0,4% acetic acid, Ex: 360 nm, Em: 440 nm
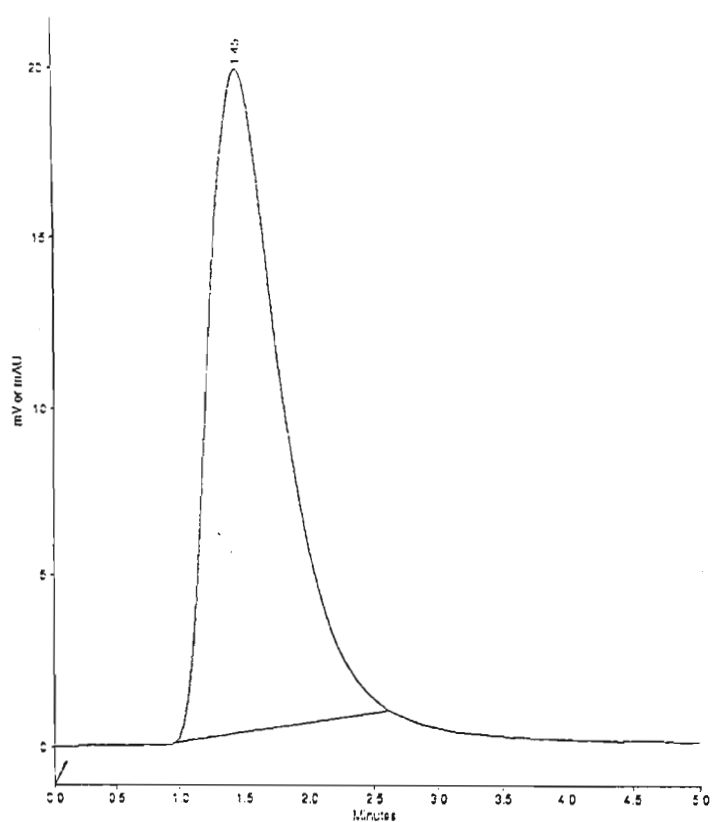
Figure 23: HPLC chromatograph of aflatoxin B_1 (500 ng), RP (C18) column (Merck), 1ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 0,4% acetic acid, Ex: 360 nm, Em: 440 nm
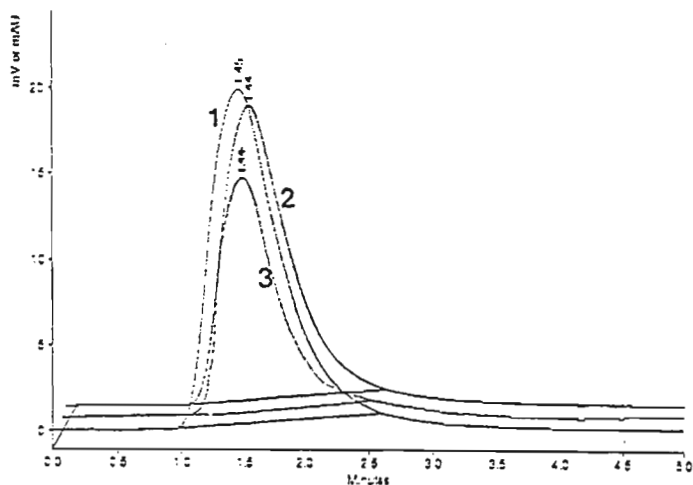
Figure 24: HPLC chromatograph of (1) aflatoxin B₁ (500 ng); (2) recovery of aflatoxin B₁ (500 ng) after dialysis "clean-up", (3) after GPC "clean-up", RP (C18) column (Merck), 1ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 0,4% acetic acid, Ex: 360 nm, Em: 440 nm

TABLE V: AMOUNTS OF AFLATOXIN B₁ RECOVERED FROM SPIKED MAIZE; AS DETERMINED BY HPLC AFTER DIALYSIS OR GPC "CLEAN-UP"

METHOD	Recovery at spike conc. 50 ng/25 g	Recovery at spike conc. 100 ng/25 g	Recovery at spike conc. 250 ng/25 g	Recovery at spike conc. 500 ng/25 g
DIALYSIS	23 ng	68 ng	155 ng	400 ng
GPC	10 ng	47 ng	163 ng	341 ng
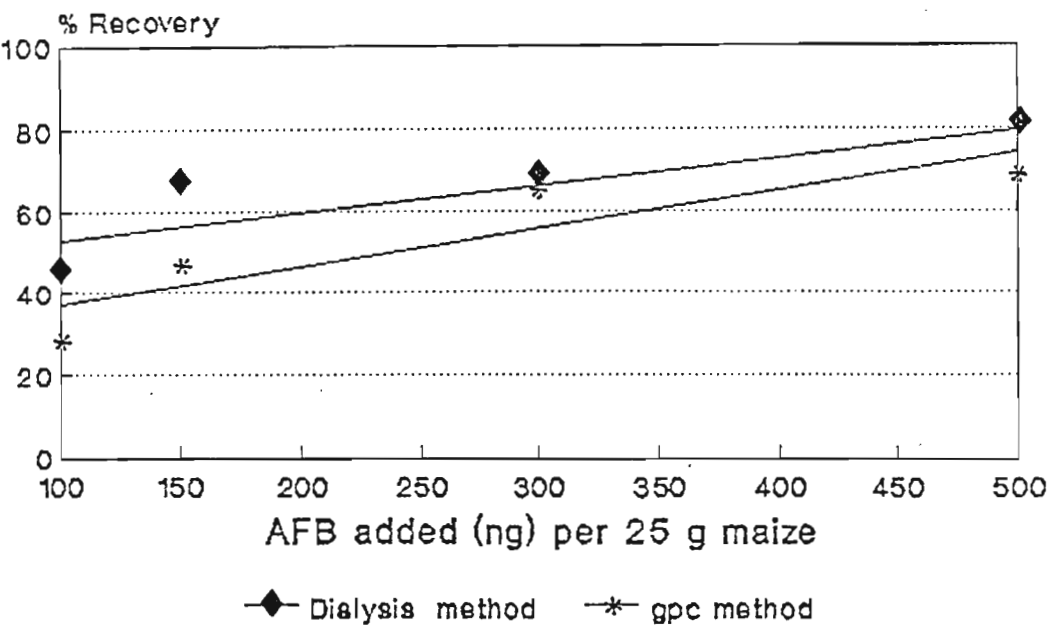
Figure 25: Comparative recovery of aflatoxin B₁ from "spiked" maize samples using dialysis and GPC "clean-up" methods with quantitation by HPLC

Plates (1) and (2) 1D solvent chloroform:ethyl acetate:isopropanol (90:5:5), 2D solvent toluene:ethyl acetate: formic acid (6:3:1), plate (3) 1D solvent carbon tetrachloride:ethanol (98:2), 2D solvent cycloheaxane:ether (3:1)
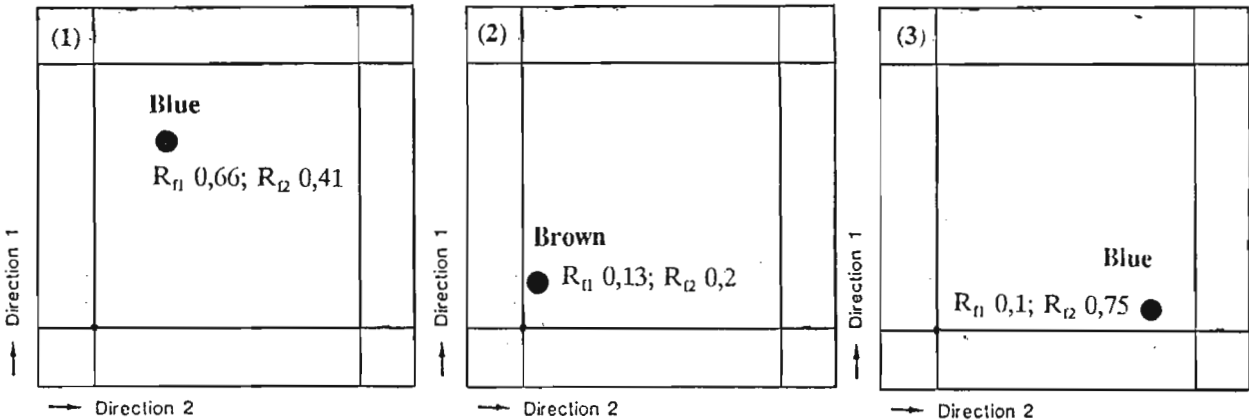
Figure 26: Thin layer chromatogram of sample 2 (GPC) viewed under UV 360 nm showing (1) aflatoxin B_1, (2) deoxynivalenol after spraying with sulphuric acid and heating, (3) zearalenone after spraying with aluminium chloride and heating

TABLE VI: DETECTION OF NATURALLY OCCURING MYCOTOXINS BY TLC AFTER DIALYSIS AND GPC METHODS OF "CLEAN-UP"

DIALYSIS METHOD A		GPC METHOD B	
MAIZE SAMPLE No.	MYCOTOXINS	MAIZE SAMPLE No.	MYCOTOXINS
1	-	1	Zearalenone
2	Aflatoxin B_1	2	Aflatoxin B_1 Deoxynivalenol Zearalenone
3	Aflatoxin B_1	3	Aflatoxin B_1
4	Zearalenone	4	Zearalenone
5	Aflatoxin B_1 Zearalenone	5	Aflatoxin B_1 Zearalenone
6	Deoxynivalenol	6	Deoxynivalenol Zearalenone

- indicates absence of any mycotoxins

Direction 1 solvent
chloroform:ethyl acetate:
· propano-2-ol (90:5:5)

Direction 2 solvent
toluene:ethyl acetate:
formic acid (6:3:1)
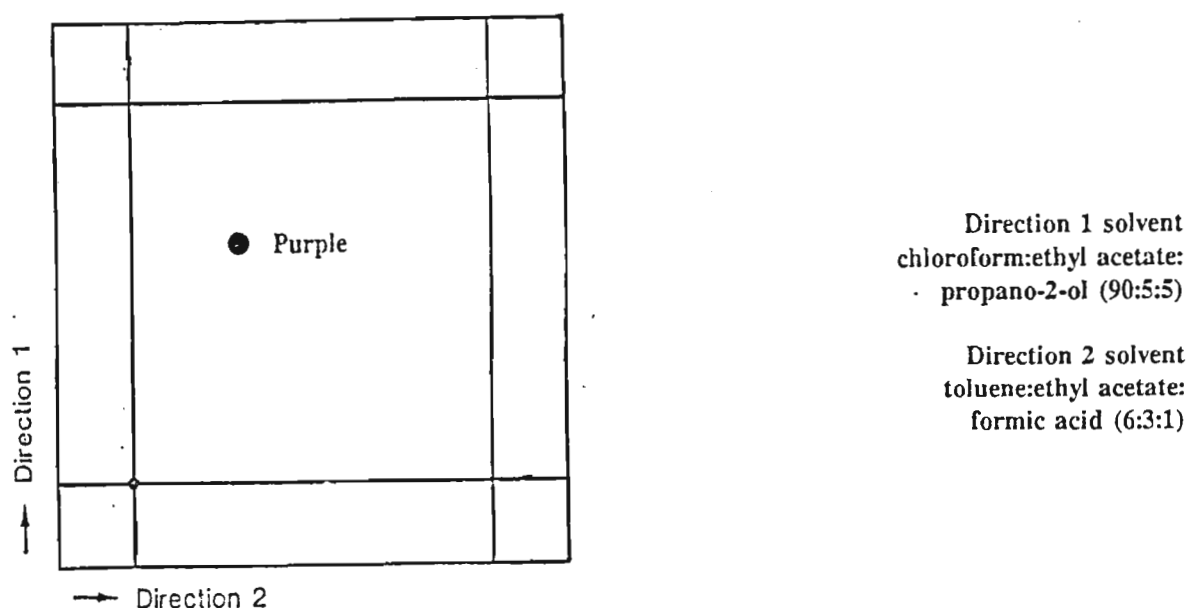
Figure 27: Thin layer chromatogram run in two dimension CEI/TEF, viewed under UV 360 nm, showing aflatoxin B_{2a} fluorescence following conversion of aflatoxin B_1 standard by GPC mobile phase

3.6 DISCUSSION

Analysis of Spectroscopy scans and Thin Layer Chromatography Plates after Dialysis and Gel Permeation Chromatography

The purpose of this study is to evaluate GPC (method A) as a means of mycotoxin "clean-up" when compared with the dialysis method (method B) as monitored by TLC and quantified using HPLC. The dialysis method of "clean-up" is well established and used commercially, while the GPC method (Scudamore and Hetmanski, 1992) is a recent development and has not been fully evaluated. "Clean-up", using GPC, is based on the elution of higher molecular weight plant components followed by the mycotoxins.

When testing new methodology, it is important that the samples tested are contaminated with specific mycotoxins at known and easily detectable levels. This necessitates the spiking of an extract with known concentrations of a specific toxin to quantify the levels following the "clean-up" procedure. Subsequent tests can then be run on naturally contaminated samples.

Of twelve maize samples screened by the dialysis method (method A), five positive and one negative for the prescence of mycotoxins (Table VI and Figure 26) were examined using the GPC method (method B).

Every second fraction eluted from the GPC column for each sample was scanned between 200-700 nm, using a spectrophotometer to detect mycotoxins with UV absorbance, i.e., ZEA, DON and AFB_1 (see appendix 13.0 for standard spectra). Examples of scanned fractions are shown in Figure 19, indicating peaks at 215 (1,833) for ZEA, 223 (4,576) for DON and 224 (3,062), 265 (2,026), 361 (2,815) for AFB_1. In fractions 2-30 ZEA was detected, DON was detected in fractions 2-40 and AFB_1 in fractions 12-30. On examination by TLC the results were confirmed (Figure 26).

These results do not agree with those quoted by Scudamore and Hetmanski (1992), who state that most mycotoxins elute over the range 81-130 ml and can be collected as a single fraction. These authors did, however, mention that exact reproducibility with their method was difficult, particularly with extracts from maize. This may explain the discrepancy which could be compensated for by adequate column eluent monitoring.

Another problem which came to light during the course of analysis using method B was the appearance of a purple fluorescent spot on the TLC plates at lower R_f values than AFB_1 (Figure 27). This spot corresponded to AFB_{2a} and was not present in AFB_1-containing extracts derived by method A. It seems highly likely that exposure of AFB_1 to the acid conditions used in method B cause its conversion to AFB_{2a}. This observation must be taken into account when using method B to analyse for AFB_1 and steps should be taken to minimise the conversion.

Samples of maize meal obtained from Durban and the Eastern Cape were shown to be free of detectable mycotoxins using the dialysis method. These were then spiked with different levels of AFB_1, split into portions and analysed using methods A and B. The amount of recovered toxin was determined by HPLC (Figure 24) and by visual estimation using TLC, but the latter method only gives estimates and is

limited at lower levels, since the detection by the human eye is 0,4 ng (Coomes *et al.*, 1965). Patterson and Roberts, (1975) give detection limits for AFB_1, ZEA and DON as 3 ppb, 1000 ppb and 1000-4000 ppb respectively, using method A.

Evaluation of recoveries of Aflatoxin B_1 following Dialysis and Gel Permeation "Clean-up"

Figure 25 gives a comparison between AFB_1 recoveries achieved by the two methods. In general, recoveries increase as the level of spiking goes up, i.e., for method A, from 47% recovery derived from a spiking rate of 50 ng/25 g, to 83% at 500 ng/25 g. This is in keeping with usual analytical practice as small loses are a bigger fraction of lower levels than larger. Recoveries were also better, on average, for method A over method B, although these are marginal and cannot be statistically validated due to lack of data. A further consideration is that the recoveries of method B may have been influenced by the conversion of AFB_1 to AFB_{2a} in significant quantities.

From the results of analyzing naturally contaminated maize, it would seem that the efficiency of recovery demonstrated by spiking with AFB_1 cannot be extended to ZEA and DON. Application of method B shows that ZEA and DON were present in samples 1, 2 and 6, which was not the case on analysis by method A. The obvious deduction is that method B yields better recoveries for the two mycotoxins, however, this conclusion needs to be confirmed by similar spiking trials as used for AFB_1.

For each method, the cost of consumables was calculated and is shown, together with a capital cost comparison of equipment used, in Table VII.

TABLE VII: COMPARISON OF COSTS OF "CLEAN-UP" USING DIALYSIS OR GPC METHOD

	Dialysis	GPC
Equipment	Separating funnel, funnel, Buchner apparatus, shaker, rotary evaporator, glass vials	Glass column (500 mmx25 mm), shaker, Gilson Fraction Collector, glass test tubes (200), Milton Roy Spectronic Spectrophotometer
CAPITAL COST	R9 000,00c	R78 000,00c
Consumables	Anhydrous sodium sulphate, chloroform, iso-octane, dialysis tubing, acetonitrile, filter paper	Bio-Beads S-X3 gel, dichloromethane, ethyl acetate, formic acid
CONSUMABLE COST/SAMPLE	R15,74c	R13,74c

TABLE VIII: COMPARISON OF EFFECTIVENESS; METHODS A AND B

Parameter	Method A	Method B	Preferrred method
Time taken per sample	24 hours	5 hours	B
Labour	more intensive	less intensive	B
Cost per sample	R15,74	R13,74	B
Recovery aflatoxin B_1	better	poorer	A
Recovery zearalenone	poorer	better	B
Recovery deoxynivalenol	poorer	better	B
Skill required	less	more	A
Equipment	less costly	more costly	A
Power consumption	more	less	B
Safety	manual open system	auto-closed system	B
Environmental	noxious fumes	noxious fumes	comparable

The cost of consumables was calculated per sample for each of the "clean-up" methods. The dialysis method is more labour intensive and the cost of consumables is marginally higher. The capital cost of the equipment required for the GPC method, as shown in Table VII is significantly higher than that of the equipment needed for the dialysis method. The break-even point, neglecting labour, would appear to be around thirty five thousand samples, above which the GPC method becomes more economical. In practical terms, however, this would extend beyond the useful life of the equipment and comparative labour costs would have to be taken into account to give a more realistic assessment. Budget constraints precluded the purchase of ancillary equipment for the GPC and consequently this method was not pursued further.

From Table VIII it appears that while the capital cost of the GPC "clean-up" method is greater than that of the dialysis "clean-up" method, the former offers greater sensitivity for ZEA and DON, long term cost advantages together with a quicker turn around time for sample analysis. Clearly, however, the capital cost of GPC and skill levels associated with setting up the instrument would be key factors in any selection decision. Dialysis, meanwhile, has the advantage of comprising relatively simple equipment, necessitating little technical support and offering a straightforward operating prodecure.

The limited scope of this study does not allow an unequivocal association to be made between any particular fungus or toxin and the diseases reported in KaNgwane that are thought to be mycotoxin related, but it does show that mycotoxins are found in maize used for food and that they are the same mycotoxins as those previously reported by Hean (1992). The occurrence of these mycotoxins in human blood after ingestion of contaminated food is, however, discussed in section 5.5, page 145.

3.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND GAS CHROMATOGRAPHY MASS SPECTROMETRY (GCMS) OF GROUNDNUT SAMPLES

3.7.1 Introduction

The techniques of HPLC and GCMS were applied in the analysis of groundnut samples from KaNgwane. Although HPLC methods are available for separating and detecting most mycotoxins, it is not possible to incorporate them all into one sensitive detection procedure and in certain instances GCMS is the preferred technique.

The technique of GCMS is based upon the partitioning of compounds between a gas and a liquid phase and is widely used for the qualitative and quantitative analysis of a large number of compounds, since it has a high sensitivity, reproducibility and speed of resolution. A stationary phase of liquid material such as a silicone grease is supported on an inert granular solid, packed into a narrow coiled glass or steel column 1 to 3 m long and 2 to 4 mm internal diameter through which an inert carrier gas (the mobile phase) such as nitrogen, helium or argon is passed. The column is maintained in an oven at an elevated temperature which volatilises the compounds to be separated. The basis for the separation is the difference in the partition coefficients of the volatilised compounds between the liquid and gas phases as the compounds are carried through the column by the carrier gas. As the compounds leave the column they pass though a detector, linked via an amplifier to a chart recorder which records a peak as a compound passes through the detector.

Mass spectrometry is widely used to elucidate the molecular structure of biological compounds. It is based upon the property of a moving ion to be deflected by a magnetic field to an extent that is dependent upon its mass and velocity. Ions of a larger momentum are deflected less than those of lower momentum, whilst a mixture of ions of different mass but constant velocity will be deflected in proportion to their individual masses. The method is very sensitive and uses as little as 10^{-6} to 10^{-9}g of material. A mass spectrum is a plot of the abundance of the fragment and molecular

ions against mass. The majority of ions produced during the initial ionisation procedure have a single positive charge, i.e. one electron is removed from the molecule or fragment so that the mass to charge ratio (m/z) is numerically equal to the molecular weight. Thus the ions produced differ only in their mass. Occasionally, however, molecules lose more than one electron and multicharged ions are produced. When producing ions by electron bombardment the degree of fragmentation of the molecule depends on the energy of the bombarding electrons. At low energies (1 to 2×10^{-18} J), only one electron is removed from the molecule. The resulting positively charged molecular ion has a m/z value corresponding to the molecular weight of the parent compound. At the usual operating electron beam energy of 10^{-17}J, the molecules are cleaved into positively charged fragments of different masses. The way in which the compound fragments gives a mass spectrum characteristic of the compound being analysed and is called the fragmentation pattern. From this pattern the structure of the molecule can be deduced.

The interpretation of the structure of any compound by mass spectrometry necessitates having the compound in a relatively pure state. It is, therefore particularly advantageous to couple a mass spectrometer to a gas-liquid chromatograph (glc). A mixture of compounds may be applied to the glc and separated into the individual components. As each compound emerges after a different time interval from the glc, it is fed into the mass spectrometer, analysed and its fragmentation pattern recorded in the usual manner for identification purposes as used in this study.

KaNgwane groundnut samples (December 1993 to September 1994) were "cleaned-up" using the dialysis method by Mrs. R. McLaren at the Rob Ferreira Hospital and extracts were examined by TLC. The plates sent to the Physiology Department, Medical School, Durban where they were sprayed and screened by viewing under UV. Fifteen selected extracts from samples, previously shown to have *Aspergillus spp* contamination of 10^2 to 10^6 propagules/g), (McLaren, R., pers. comm.[4]), were analysed for AFB_1 using HPLC.

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[4] Mrs. R. McLaren, S.A.I.M.R., Rob Ferreira Hospital, Nelspruit

During 1994, KaNgwane groundnut was available and sixteen samples were collected from seven plots, as indicated in Table IX and "clean-up" by the dialysis method, as well as detection of *Fusarium spp* (10^2 to 10^6 propagules/g) by mycological analysis, was also undertaken in Nelspruit. The extracts were sent to the Physiology Department, Medical School, University of Natal for analysis by GCMS. As GCMS techniques facilitate rapid simultaneous analysis of a number of trichothecenes, this technique was adopted in all trichothecene analyses performed.

3.7.2 Materials

The materials for HPLC are described in sections 3.4.2.

Test equipment for GCMS comprised an Hewlett Packard 5971A mass spectrometer detector and Hewlett Packard 5890A gas chromatographer. The standards used included 3-acetyldeoxynivalenol, fusaric acid, deoxynivalneol, acetyldiacetoxyscirpenol, diacetoxyscirpenol, nivalenol, scirpentriol, roridin A, neosolaniol, helvolic acid, fusarenone-X, acetyl T-2 toxin, trichothecin, T-2 tetraol, tetra-acetate, T-2 toxin, T-2 triol, zearalenone, trichothecolone and derivatising agent N,O-Bistrimethylsilylacetamide (Janssen Chimica) (BS).

3.7.3 Methods

The method for HPLC analysis is described under section 3.4.3.

The injector and detector temperatures on the gas chromatographer and mass spectrometer were set at 280 °C. Column pressure was as follows:

Column pressure	0,34 bar
Split ratio	10:1
Carrier gas (He)	60 ml/min
Flow	0,9 ml/min

Known amounts of each toxin standard (Makor) (10 µg) were dissolved in ethyl acetate (20 µl) and dried before 50 µl of BS were added to the standard and incubated for one hour at 60 °C. This was redissolved in 10 µl ethyl acetate and 5 µl used for injection. The sample was dissolved in 500 µl ethyl acetate and 100 µl were removed and dried before 100 µl BS were added and incubation in a 60 °C water bath allowed to proceed for an hour. After derivatisation, BS was evaporated under a stream of N_2 and 100 µl ethyl acetate added, 5 µl of which were injected.

After injection of the standards these were incorporated in a library on the Hewlett packard Software (HP G1034C ms ChemStation) against which peaks in the samples could be compared.

3.7.4 Results

TABLE IX: DETECTION OF TRICHOTHECENES IN GROUNDNUT BY GAS CHROMATOGRAPHY

Plot number	Extraction 1 from plants harvested in mid April 1994	Extraction 2 from plants harvested at the end of April 1994	Extraction 3 from plants harvested in mid May 1994	Extraction 4 from plants harvested at the end of May 1994
1	-	unavailable	unavailable	unavailable
2	-	nivalenol	-	unavailable
3	nivalenol	nivalenol	unavailable	unavailable
4	-	-	-	-
5	nivalenol	-	unavailable	unavailable
6	nivalenol	-	unavailable	unavailable
7	nivalenol	-	unavailable	unavailable
TOTAL	4/7	2/6	0/2	0/1

- indicates the absence of trichothecenes
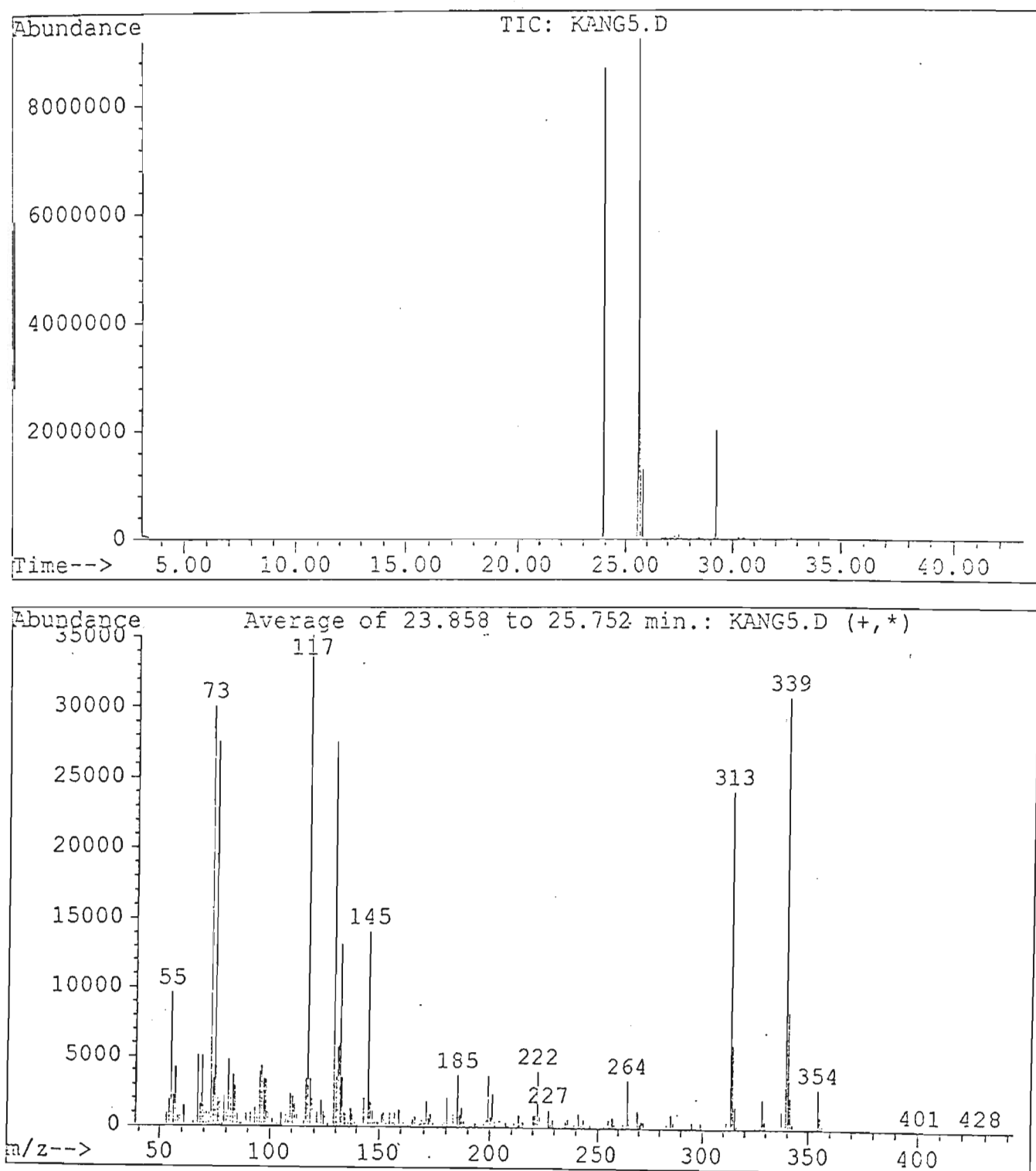
Figure 28 : Chromatograph of extract from groundnut sample for trichothecenes using Ion flow in mass spectrometry as detection and derivatised by BS (sample 5), conc. 0,56 µg/µl extract (calculated from results and std.)
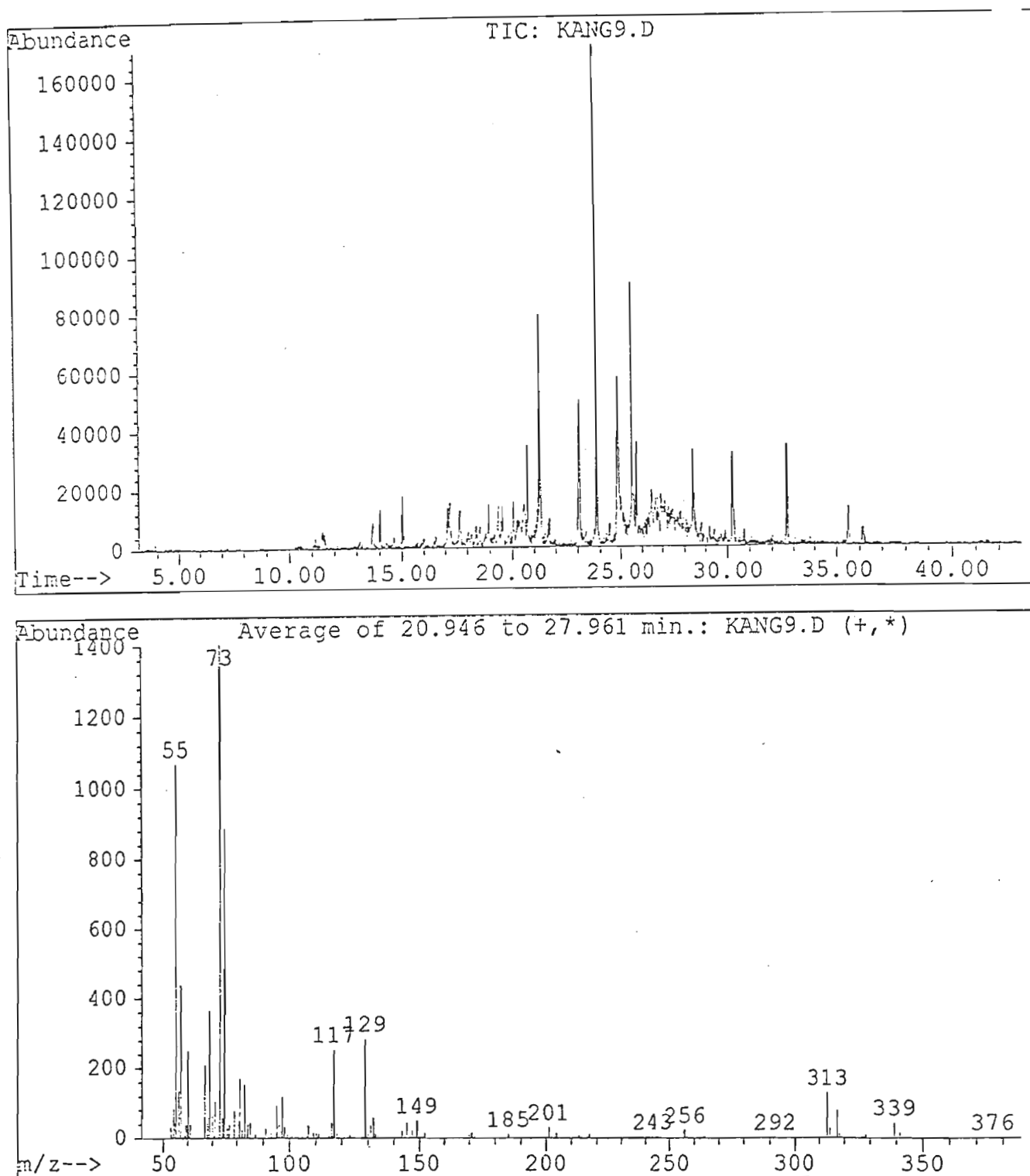
Figure 29: Chromatograph of extract from groundnut sample for trichothecenes using Ion flow in mass spectrometry as detection and derivatised by BS (sample 9), conc. 0,001 μg/μl extract (calculated from results and std.)
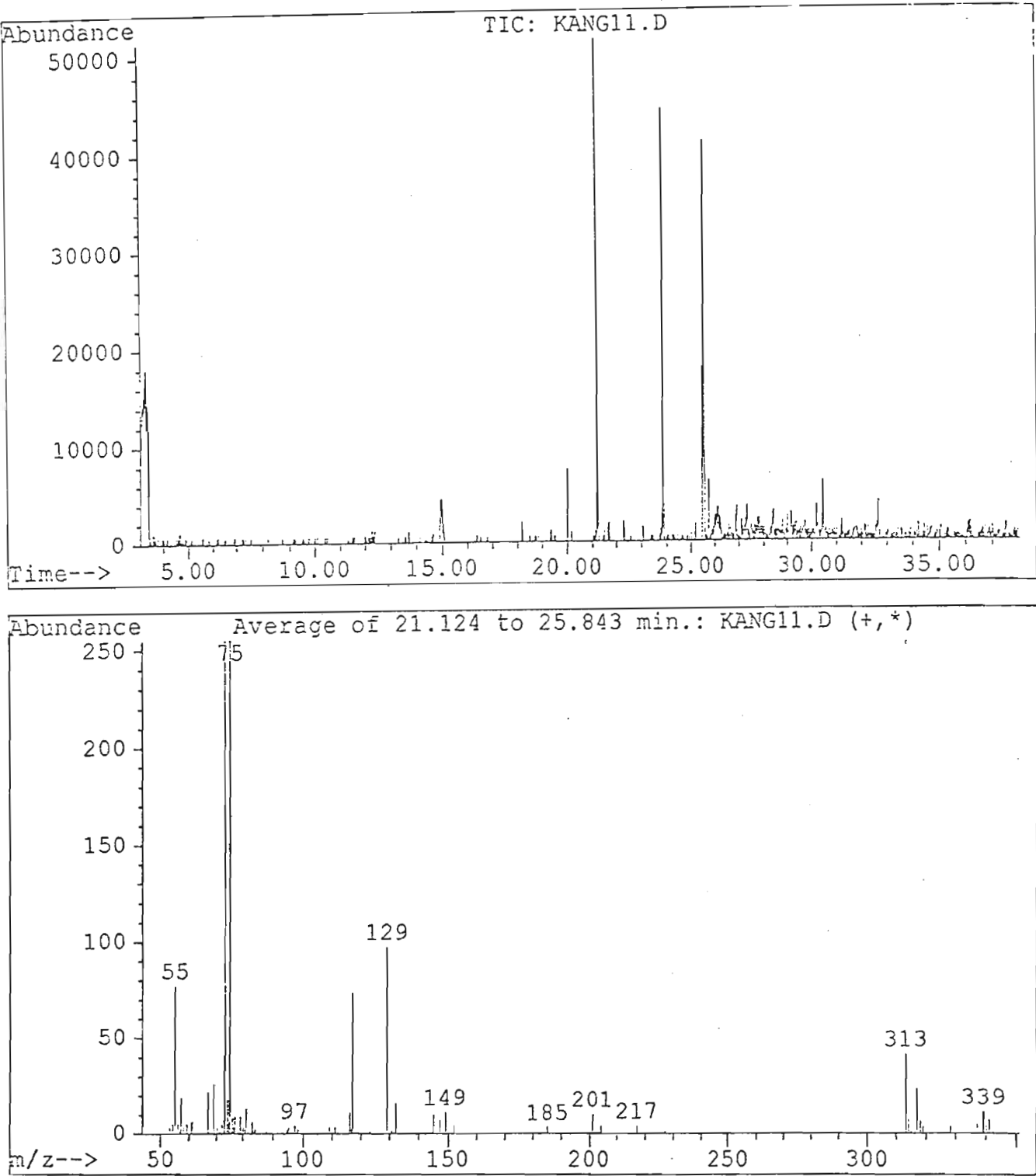
Figure 30: Chromatograph of extract from groundnut sample for trichothecenes using Ion flow in mass spectrometry as detection and derivatised by BS (sample 11), conc. 0,004 µg/µl extract (calculated from results and std.)
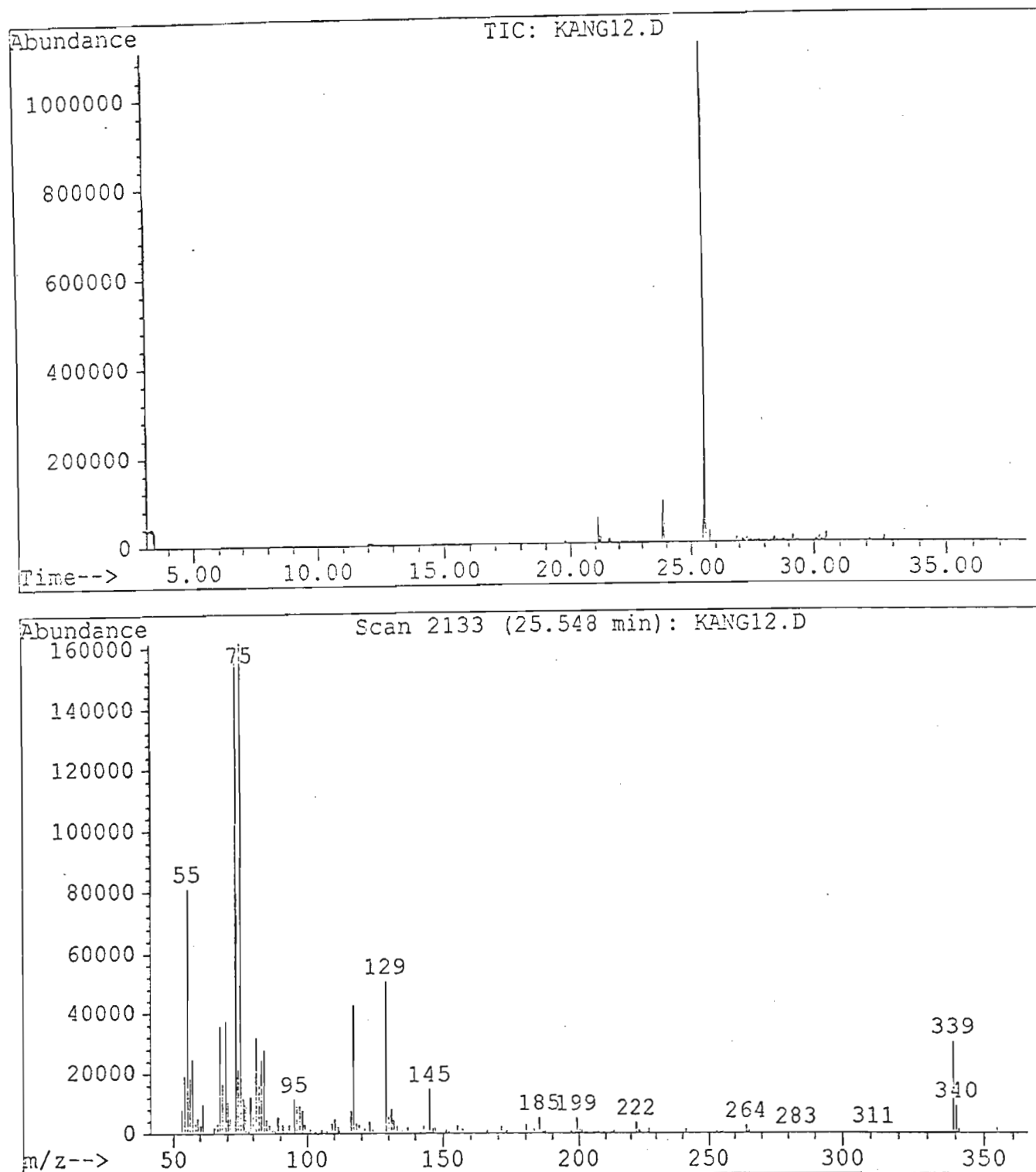
Figure 31: Chromatograph of extract from groundnut sample for trichothecenes using Ion flow in mass spectrometry as detection and derivatised by BS (sample 12), conc. 0,068 µg/µl extract (calculated from results and std.)
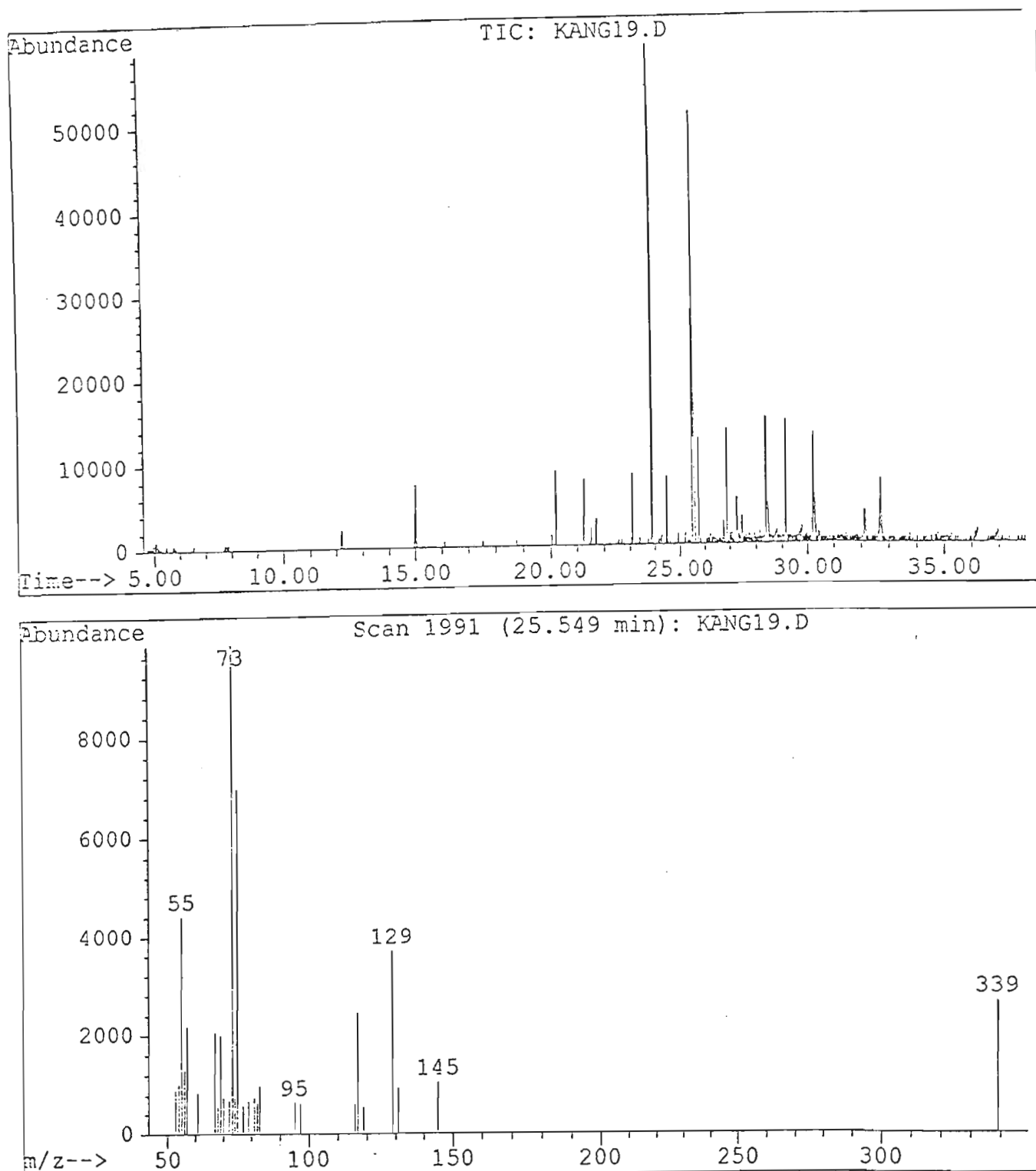
Figure 32: Chromatograph of extract from groundnut sample for trichothecenes using Ion flow in mass spectrometry as detection and derivatised by BS (sample 19), conc. 0,004 µg/µl extract (calculated from results and std.)
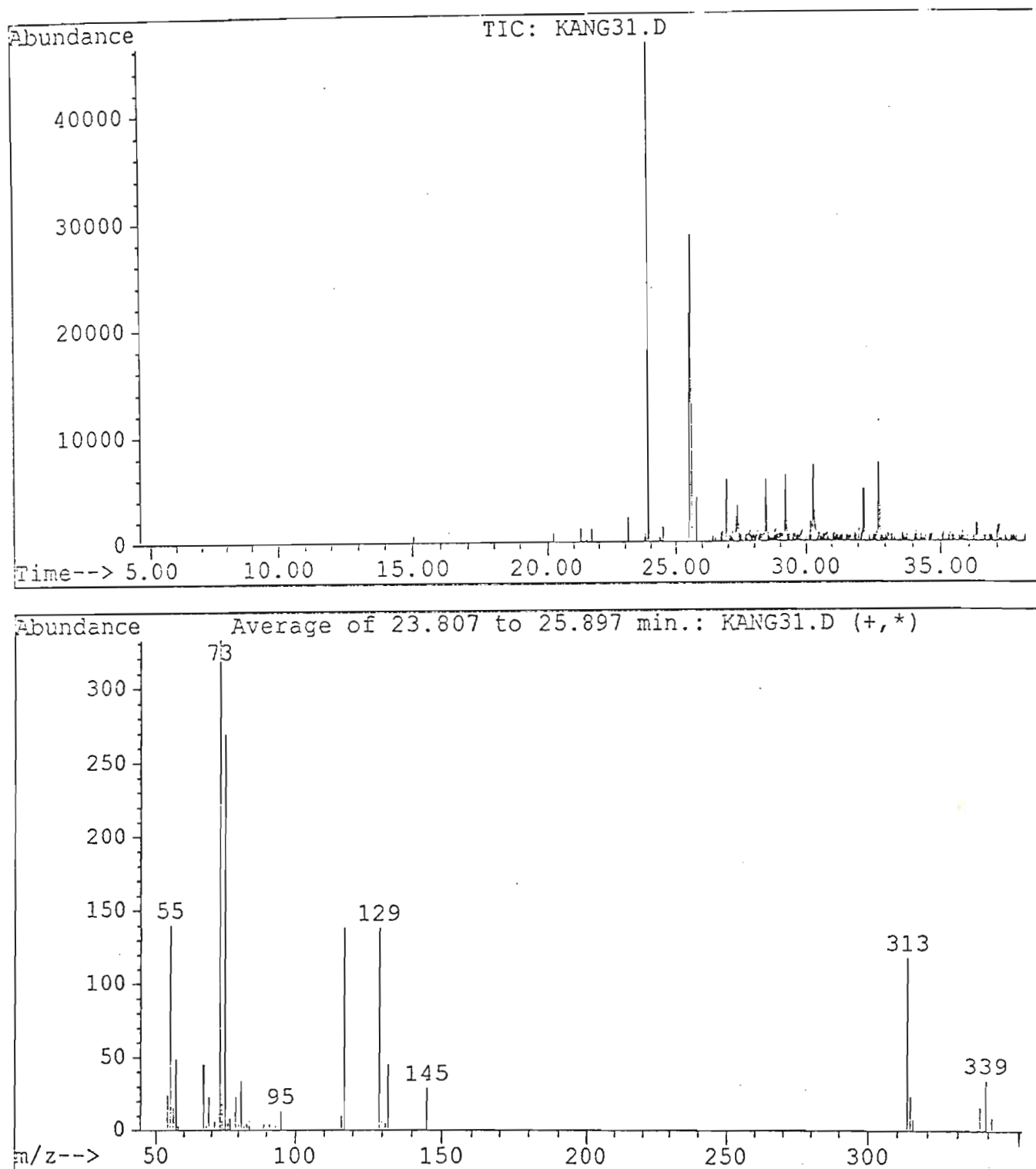
Figure 33: Chromatograph of extract from groundnut sample for trichothecenes using Ion flow in mass spectrometry as detection and derivatised by BS (sample 31), conc. 0,003 µg/µl extract (calculated from results and std.)

Figures 34 and 35 depict HPLC chromatographs following analyses for AFB₁.
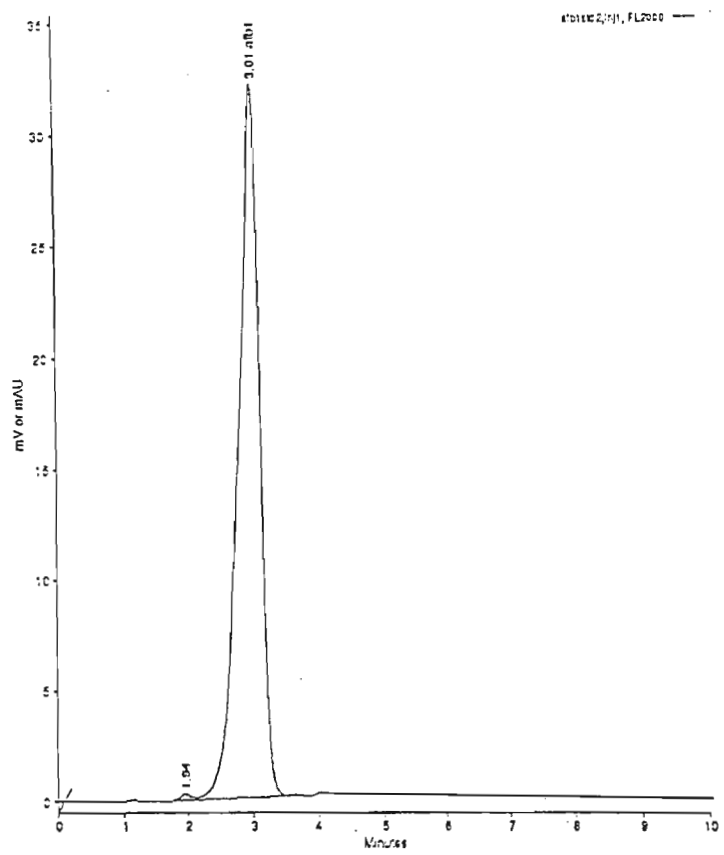
Figure 34:   HPLC of aflatoxin B₁ standard, column (Spherisorb), 1 ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 1% acetic acid 40%, Ex: 360 nm, Em: 440 nm
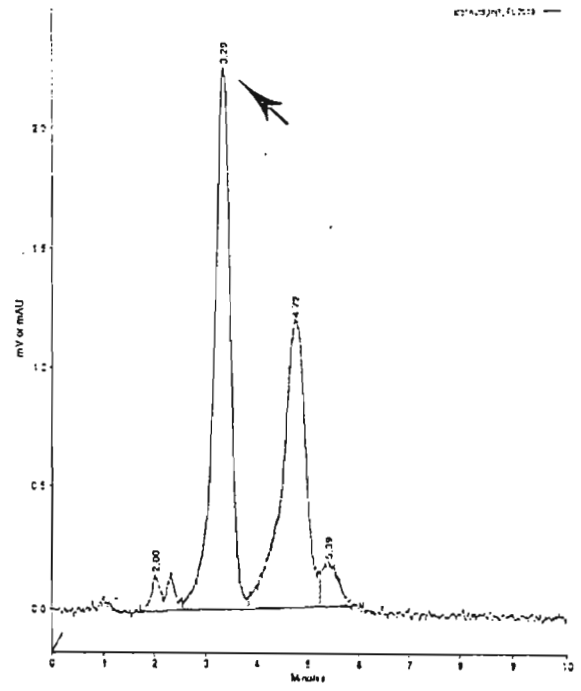
Figure 35:   HPLC chromatograph of aflatoxin B₁ in groundnut sample (Spherisorb), 1 ml/min, acetonitrile-glacial acetic acid:methanol (180:10:10) 60% and water plus 1% acetic acid 40%, Ex: 360 nm, Em: 440 nm

3.7.5   Discussion

In May 1994, samples of harvested groundnuts were found to be contaminated with *Fusarium spp*, (10^2 to 10^6 propagules/g) and extracts from these samples on TLC showed evidence of contamination by trichothecenes (McLaren, R., pers. comm.[4]) These extracts were further examined by GCMS in order to confirm the prescence of a trichothecene and its identity.

The extracts came from groundnuts that were harvested from seven plots, and where possible, samples were taken at half monthly intervals. Unfortunately, the number of samples available dropped with time due to depletion of material in the field (Table IX). On analysis of the extracts by GCMS, mass spectra were obtained which were characteristic of a trichothecene. Figures 28 to 33 show the presence of an m/z ratio comparable to the profile for nivalenol indicated in Appendix 14.12. While the chromatographs do not exactly replicate those in Appendix 14.12, nivalenol was selected by the library in the software as being the contaminant. Discrepancies between the chromatographs of the samples and the nivalenol standard may be explained by the low concentration of the nivalenol concentration indicated in the samples, when compared with the concentration of the standard. The mass spectra show a series of peaks or lines corresponding to the m/z values of the positive ions produced from the compound. The height of the peaks corresponds to the relative abundance of ions. For example carbon dioxide is ionised and fragmented to yield CO^+_2, CO^+, O^+_2, O^+ and C^+, resulting in a mass spectrum with major peaks at m/z values of 44, 28, 32, 16 and 12 respectively. Figures 29 to 33 showed the occurrence of a repeated peak at m/z 129 and this may be associated with a common impurity tri-n-butyl acetyl citrate. Peaks with an m/z ratio of 73 and 75 indicate the possible presence of $C_4H_9O^+$ and $C_2H_5C(0H_2)^+$ respectively.

The GCMS results (Table IX) show that nivalenol contamination occurred from mid April to early May with a change over time with a chi-square analysis for trend of p = 0,0173 which shows an acceptably high degree of significance.   This was unexpected, as groundnut is not usually contaminated by trichothecenes but by AFB_1.

An explanation for this result is that the groundnuts were contaminated with *Fusarium spp* and not the more usual *Aspergillus flavus* and *Aspergillus parasiticus*, which produce AFB_1. Evidently field conditions favoured infection by *Fusarium*, which probably is dependent upon availability of moisture. No incidences to date, in the literature, have been reported on nivalenol contamination in groundnuts. It is interesting to note that Table IX shows nivalenol contamination in plot 2 after an earlier sample from that plot had been shown to be uncontaminated, while plots 5, 6 and 7 gave opposite results and contamination was not evident in the later sample after having been found in the earlier. The derivatisation method for GCMS preparation of the trichothecenes used BS, which derivatises hydroxyl groups, while Gilbert *et al.* (1985) suggest the use of bis-trimethyltrifluoroacetamide (BSTFA), trimethylcholorosilane (TMCS) as a more reliable reagent. Use of BS has been queried by Gilbert *et al.* (1985) who stated that derivatisation is incomplete if this reagent is used alone. However, the use of strictly standardised conditions for derivatisation and qualification and quantitification by comparison with a standard mixture prepared under identical conditions allows correction for efficiency of derivatisation. Such reproducibility was observed in this study. Duplicate injections were done and all contaminated samples agreed within the range incorporated in the instrument software.

Analysis of Groundnut Extracts by High Performance Liquid Chromatography

Of the 15 extracts analysed using HPLC only one contained AFB_1 at a concentration of 450 ng. A follow up groundnut extraction was performed a month later on other samples from the same plot giving a negative HPLC result. Environmental conditions, such as intermittent rainfall, unreliable irrigation, high temperature, humidity and poor storage sites, could be associated with a change in fungal growth and metabolism (Cole and Dorner, 1993).

Hudson *et al.* (1992) showed, by HPLC, that groundnut sources had the highest level of contamination; mean 162 ppb (range 18 to 943 ppb), while the contamination level in the maize had a mean of 9,7 ppb (range 2 to 35 ppb).

The HPLC was calibrated with 50 ng, 100 ng, 300 ng and 500 ng of derivatised AFB_1. The length of column used has an influence on the retention time (RT) of the AFB_1. The RT of the AFB_1 standard, as well as the extracts from the GPC and dialysis "clean-up" (section 3.5, page 65), was 1,45 using the 12,5 cm x 4,6 mm column.

The RT of the AFB_1 standard and AFB_1 found in the groundnut sample was, 3,01 and 3,29 minutes, respectively (Figures 34 and 35) as a 250 mm x 4,6 mm column, having more theoretical plates, was used. Since RT is a function of column length for similarly packed columns of the same diameter, then RT of the AFB_1 found in the groundnut sample, based on column length of 12,5 cm, is half that of the 25 cm column, namely 1,47 which correlates well with the AFB_1 standard RT of 1,45. Use of the longer column for AFB_1 analysis is preferred as peak resolution is better and tailing, as seen in Figure 23, is avoided. The peaks with RT of 2,20; 4,59; and 5,29 indicated in Figure 35 may be attributable to impurities in the extract.

The findings discussed in this chapter indicate that the sensitive methods of HPLC and GCMS can be used to confirm marginal cases of contamination or to detect contamination in those samples deemed to be uncontaminated when previously checked by TLC. In the case of samples taken for commercial purposes, this would minimise the risk of cross contamination in storage with uncontaminated groundnuts.

3.8   GENERAL DISCUSSION

A number of collaborative organisations including Eco-Link, Agricura, Natal University and KaNgwane Ministry of Agriculture and Forestry are involved in the Malekutu, KaNgwane, Dryland Project, co-ordinated by Brian Beck from the Ministry. The aim of the Dryland Project is to optimise agricultural output in the area. The combined efforts of the organisations involved is directed, in part, towards minimising fungal growth and contamination. A Farmers' Day, held in March 1993 at Malekutu, was aimed at bringing local farmers together and advising them on storage facilities for harvested maize, as well as the use of agro chemicals and the cultivation of mung beans and groundnut as alternative crops to maize (Plate 3). Among the topics discussed were the use of herbicides and insecticides, as well as the production of new insect and drought resistant crop strains. Following the Farmers' Day, an opportunity arose to arrange with Doctor Kobeyer, Paediatric Department, Temba Hospital, for the collection of paediatric blood samples. These samples were analysed for AFB_1-lysine as described in section 5.3, page 138.

Plate 3:   Experimental plot in KaNgwane to test suitability of different cultivars of maize and ground nut in the area

Plate 4: One of the groundnut cultivars from the KaNgwane region tagged with plot number

The multiscreening assays of maize samples cultivated by subsistence-level farmers in KaNgwane, showed that ZEA, DON, and AFB_1 were the most prevalent mycotoxins. The incidences of these compounds is given in Table VI. The ZEA levels were consistent with the elevated levels of *F. moniliforme* found contaminating the maize in this area.

Much of the routine screening of TLC plates for extracts from Rob Ferreira Hospital was undertaken at the Natal Medical School, as part of this project, and approximately 800 plates were viewed and sprayed accordingly for the presence of mycotoxins. Those extracts which showed possible T-2 contamination were sent to The Animal Sciences Department, Agricultural Faculty, University of Natal, Pietermaritzburg for further analysis by Mrs. A. Kinsey. Appendix 7.0 shows the negative results of the KaNgwane maize samples screened for mycotoxins following dialysis "clean-up" from January 1993 to November 1993. Wider collection of maize samples would be essential in the Nsikazi area to give a more accurate assessment of the methods to determine the extent of mycotoxin contamination and give a better and more significant statistical analysis of the dialysis and "clean-up" methods.

Appendix 8.0 shows the results of mycotoxin screening in groundnuts during December 1993. Further analyses were performed by Mrs. R. McLaren, on Random Eco-link groundnut samples, supplied by Mr. B. Beck, KaNgwane Ministry of Forestry and Agriculture (Appendices 9.0 and 10.0). Microfloral analyses of these samples revealed that *Aspergillus flavus*, 10^2, *Penicillium spp*, 10^4, and *Fusarium spp* contamination were present. In certain cases a correlation can be made between the presence of the toxins and the fungi that were detected in the microfloral analyses (Appendix 10.0). The climate, the types of maize grown, the presence of insect vectors and deficiency of the soil in elements such as molbdenum, manganese, boron, copper and cobalt (Kibblewhite, *et al.*, 1984) may influence fungal growth.

KaNgwane maize was found to be aflatoxin free (Appendix 7.0), supporting the findings of Hean (1992), while groundnuts from the same area were found in certain instances to be contaminated (section 3.7.4, page 78). The occurrence of these toxins has been associated with liver cancer in areas comparatively close to KaNgwane (Swaziland and Mozambique), (Keen and Martin, 1971a).

In the KaNgwane region the trichothecenes may play a role in abnormalities associated with brain and heart tissue (Lee and Chu, 1972). More recently Daynes (1991) reported on neuromycotoxicoses and the presence of trichothecenes in samples from the household stores of two patients who experienced sudden schizophrenia, indicating possible areas for future research into storage.

The findings of this study indicate that ongoing research into routine analysis of maize and groundnut samples should be refined and expanded as far as practical to enhance the process of rural development and minimise the risk of mycotoxin contamination.

CHAPTER 4

IN VITRO SYNTHESIS OF AFLATOXIN B_1-MACROMOLECULE ADDUCTS

4.1   INTRODUCTION

The importance of the biochemical aspects of AFB_1, protein and DNA adducts has already been discussed in section 2.6, pages 27-37. The appropriately employed HPLC methods to identify the adduct (section 5.3, page 138) may help clarify the role of AFB_1 in the aetiopathogenesis of HCC and other diseases (Wild and Montesano, 1990). The commercially available AFB_1-lysine adduct produced by Sigma (Chu and Ueno, 1977) is made by the preparation of the AFB_1-oxime, and thus could not be used as a standard for this study which focuses on the AFB_1 epoxide (Figure 36).
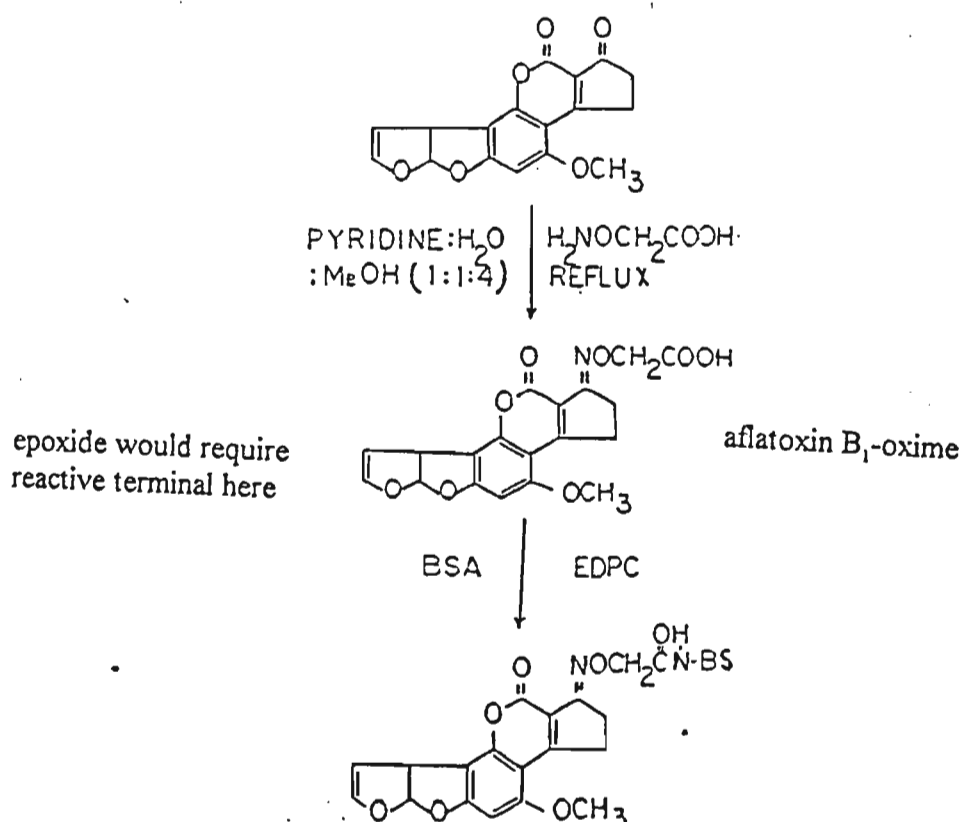
Figure 36:   Diagram showing the difference between aflatoxin B_1-oxime and aflatoxin B_1 epoxide (Chu and Ueno, 1977)

This chapter reviews five possible methods to produce AFB_1-lysine adduct in albumin hydrolysates, the principal product from the reaction of AFB_1 with albumin, to act as a standard in the HPLC analysis of adduct levels. The five methods for adduct synthesis included; viewing the position of AFB_1 fluorescence and albumin bands after Poly Acrylamide Gel Electrophoresis (PAGE), producing the epoxide using either *m*- chloroperbenzoic acid or rat microsomal fraction which gave a low yield of product, a bromo-derivative method and a dioxirane method from which better yield were obtained.

Proteins are important structural and functional cellular components (Hsieh, 1987). Those which bind mycotoxins reversibly may act as reservoirs of the toxin, thus prolonging exposure to the toxin, or may serve as carriers in the transport of reactive metabolites (Ch'ih and Devlin, 1984; Hsieh, 1987). The structures and activities of proteins that bind with mycotoxins in a nonspecific-irreversible-covalent manner may be altered as a result of conformational change resulting in denaturation, blocking of binding sites as well as specific-reversible-noncovalent (competitive) binding. At this stage the interaction of the albumin binding and transporting AFB_1 in blood is unclear and little is known about the exact nature of the noncovalent interactions between mycotoxins and protein.

Ch'ih and Devlin, (1984) and Ch'ih *et al.* (1993) proposed that AFB_1 is translocated (non-covalently bound) to microsomes where it is activated to the epoxide. Part of the activated AFB_1 may be translocated to various subcellular sites where covalent binding occurs, first, to cellular macromolecules and then later to DNA. Covalent binding of AFB_1 to nuclear membrane proteins may reduce uptake of thymidine and other precursor nucleotides necessary for DNA synthesis. The majority of the epoxide is detoxified as glutathione bound toxin and removed from the cell as water-soluble, polar metabolites (Kunimoto *et al.*, 1974). A number of workers have tried to bind AFB_1 to macromolecules and the results of studies based on their methods are described below.

4.2 BINDING OF AFLATOXIN BY PLASMA ALBUMIN *IN VITRO* (Based on method of Rao *et al.*, 1968)

4.2.1 Introduction

Rao and co - workers (1968) collected blood samples from healthy individuals and after separating the plasma undertook toxin binding studies. A volume 1 ml AFB_1 solution, containing 0,2 mg of toxin was dried in a beaker under reduced pressure to remove most of the solvent (chloroform). To the dry toxin in the beaker, 1 ml of plasma (12 mg/ml) was added and the toxin-protein interaction was allowed to proceed for two hours at 37 °C. The mixture was agitated occasionally. At the end of the incubation period the material in the beaker was carefully transferred to a cellophane dialysis bag and the mixture was dialysed against 0,005 M barbitone buffer pH 8,6 overnight at 4 °C. The material inside the dialysis bag was subjected to paper electrophoresis in 0,05 M barbitone buffer pH 8,6 at 220 V and 4 mA current strength for three hours.

The results showed that AFB_1 incubated with plasma albumin fluoresced blue in the area corresponding to plasma albumin when the unstained electrophoretogram was viewed under ultraviolet light.

In this study gel electrophoresis was used rather than paper electrophoresis. Separation of charged molecules in the gel resulted from both differences in charge and size. More highly charged species move more rapidly in an electric field; larger molecules and those less spherical in shape, are retarded by the gel to a greater extent. These two effects may work together or in opposition. The choice of gel system will maximise the differences in electrophoretic and/or gel retardation to give the best possible resolution of particular macromolecules.

When the size range of the sample components is too wide to be sieved by a gel of a single pore size, a gradient gel may be used. Electrophoresis of proteins may be carried out under either denaturing or non-denaturing conditions. Non-denaturing buffers are required whenever biological activity must be retained. They are also used

when charge differences are known to exist which may give greater resolution than separation on the basis of size. Under non-denaturing conditions, the pH must be carefully selected to maximize the charge differences. It is useful to know the isoelectric points of the proteins of interest in order to choose the best pH. In contrast, only the molecular size is relevant to separation of proteins denatured with sodium dodecyl sulphate (SDS). This anionic detergent unfolds the native protein, binds to it, and confers a charge proportional to the length of the polypeptide chain. The charge density and the electrophoretic mobility are nearly constant for most proteins. Separation on an SDS-containing gel is therefore based on gel sieving alone and the pH of the buffer in a continuous system is not very critical.

Two types of electrophoresis were performed. The SE'250' "Mighty Small II" was used for PAGE analysis and a Paragon gel electrophoresis kit was used as a parallel check. The SE'250' "Mighty Small II", Slab Gel Electrophoresis Unit, (Hoefer Scientific Instruments, San Francisco) is a miniature vertical slab gel unit intended for the rapid electrophoresis of protein or nucleic acid samples of small volume using either rapid polyacrylamide or agarose gels.

The Paragon SPE Kit, on the other hand, provides for the electrophoretic separation of proteins in a buffered agarose gel. After electrophoresis, the proteins in the gel are immobilized in a fixative solution and the gel is dried to a film. The protein pattern is visualized by staining the film with a protein-specific stain.

4.2.2 Materials and Methods

Blood samples were collected in additive free vacutainers from healthy individuals. These were placed in a 37 °C water bath and centrifuged to separate the serum. Toxin binding studies were carried out as follows: 1 ml of chloroform containing 0,2 mg of AFB_1 was dried under a stream of nitrogen to remove the solvent (chloroform). To the dry toxin in the vial 1 ml of serum was added and the toxin-protein interaction was allowed to proceed for 2 hours at 37 °C. The mixture was agitated occasionally. At the end of the incubation period the material in the was dialysed for 24 hours at 4 °C against Paragon tank buffer.

The incubated proteins were subjected to polyacrylamide gel electrophoresis (PAGE) using the SE '250'-"Mighty Small II" with reagents as listed in Appendix 15.0 Two sandwiches, each constructed with a glass plate (12 cm x 10 cm), spacers and a notched alumina plate to transfer heat, were mounted against a vertical inner core. Two separate upper buffer chambers, each holding about 75 ml are formed when the sandwiches are clamped. Between the two upper buffer chambers, the core itself serves as a heat exchanger through which coolant may be circulated.

The inner core with its attached gel sandwiches, was simply snapped into place across the center of the lower buffer chamber. The lower buffer chamber held 150 ml and was moulded from clear acrylic plastic. An acrylic lid fitted neatly over the entire unit. Agarose (1%) was melted in running gel buffer and kept at 60 °C. The core assembly was set upright on the glass plate. Melted agarose was run down one spacer into the first assembled sandwich, using a Pasteur pipette. The whole unit was tilted slightly to spread the agarose evenly across the bottom of the sandwich. As it hardened, it formed a plug of gel, sealing the bottom of the sandwich. This step was repeated once the plate had been turned around so that the other sandwich faced the worker. To pour an acrylamide gel, the monomer solution was prepared, was deaerated and polymerization initiator and catalyst were added at the last minute. The volume required for a 1,5 mm thick gel poured to the height of the notch in the alumina plate was approximately 9,3 ml. A comb was inserted into the gel to form sample wells. If a stacking gel was being prepared the top of the gel was overlaid with running gel buffer or water saturated n-butanol to prevent a meniscus from forming. The gel was allowed to polymerize and the overlay poured off. A volume of 5 ml of a stacking gel monomer solution was deaerated, initiator and catalyst were added and the gel poured using a Pasteur pipette. A comb was inserted into the stacking gel to form sample wells and the stacking gel allowed to polymerize. The comb was removed and the wells rinsed with distilled water.

To slow down gelation it was necessary to pour the agarose quickly without warming the sandwich. After the plug of gel had been poured as described above, the assembled sandwiches and core were placed in a 60 °C oven. After a few minutes the

assembly was removed from the oven and each sandwich filled with melted agarose, using a warm Pasteur pipette. This was inserted to a comb to a depth of about 5 mm. It is also possible to pour agarose quickly in the sandwich first. The comb was positioned slightly to one side, leaving room along the opposing spacer to insert the tip of a ten ml pipette with the warm agarose solution. Before liquid samples were loaded the gel sandwiches were placed in the lower buffer chamber. The central core was positioned across the center of the buffer chamber and pushed down gently. The core was held by its sides, not by the sandwiches. This was to avoid breaking the glass plates. The core was snapped into place. The cooling connectors for the coolant hung over the rim of the lower buffer chamber on either side. The SE 250 comes with a "well location decal" to facilitate sample loading. The decal, a clear plastic square adheres to glass when wet. The outline of three different combs, corresponding to 5, 10 and 15 wells respectively, was marked along three separate edges of the decal. The decal was wetted and placed against the front of the sandwich with the appropriate edge outlying the sample wells. When a preparative comb was used, the side wells for standards corresponded to the outer-most wells formed by the 10-well comb. The sample wells and the upper buffer chamber were filled with buffer, each chamber holding about 75 ml. The samples were prepared with 10% glycerol or sucrose to increase the density. A Hamilton syringe was used to underlay 10 µl of each sample in a well with bromophenol blue added as a marker. Table X shows the samples loaded into each well.

TABLE X: LOADING OF AFLATOXIN B_1 AND SERUM INTO ELECTROPHORESIS WELLS

Well No.	1	2	3	4	5	6	7
Aflatoxin B1 conc. (mg/ml)	1	0,2	0,5	0	0,2	0,5	0,2
Bovine serum albumin conc. (mg/ml)	0	0	0	0	0	0	1,0
Normal serum volume (ml)	0	1,0	1,0	1,0	1,0	1,0	0

The tubing from the inner core was connected to a cold water faucet or refrigeration unit and coolant circulated. The safety lid was placed on the unit so that the leads were correctly attached to the power supply (red to anode).

Gels were normally run at constant voltage, but for higher resolution a lower voltage setting is advisable. When the tracking dye reached the bottom of the gel (below the lower buffer), the power supply was turned off.

The flow of coolant through the inner core was stopped and the tubing disconnected. The inner core was removed from the lower buffer chamber and the buffer was poured out. The side clamps were removed and each gel sandwich lifted off the core. An extra spacer was used to prise open the gel sandwich from the bottom to avoid breaking the ears of the notched alumina plate. The gel will usually stick to the alumina plate. The spacers were removed and the gel peeled off the plate into a tray of stain or fixative. The positions of aflatoxins were identified by viewing the gel under ultraviolet light after the gel was run for 40 minutes at 40 mA.

The mycotoxins; AFB_1, FB_1 (0,2 mg/ml) were incubated with serum from a healthy patient. A volume of 1 ml of each of the mycotoxin and serum mix was loaded into wells on a Paragon gel. One well was loaded with serum from a liver cancer patient. The gels were run and prepared in accordance with the Paragon instruction manual.

4.2.3 Results and Discussion

The results of the electrophoresis are indicated in Plates 5, 6 and 7.
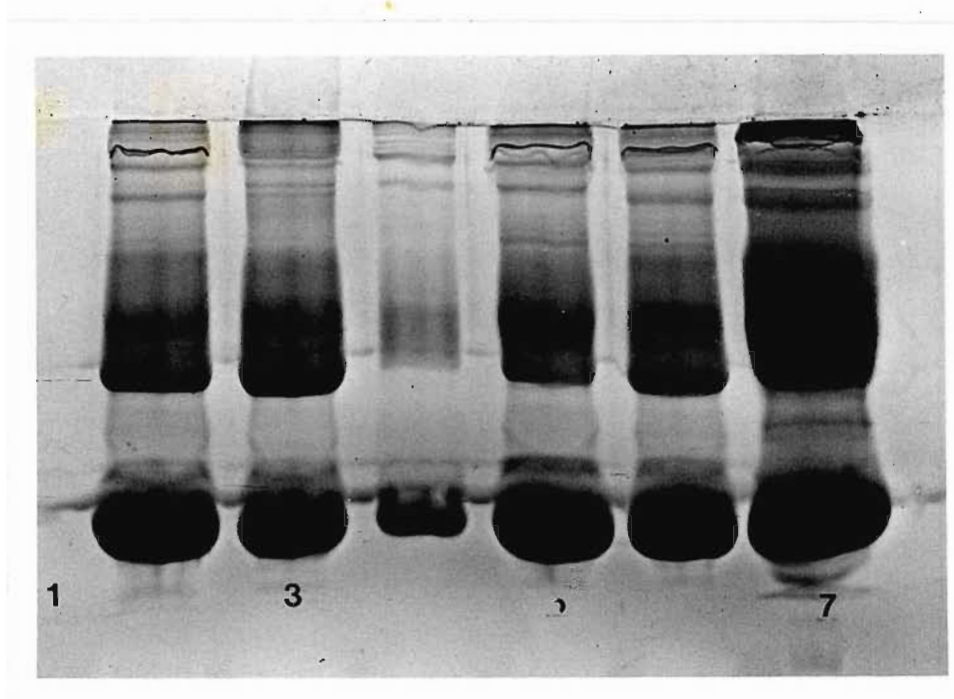
Plate 5: PAGE Gel of serum incubated with different concentrations of aflatoxin B_1

Well No.   1-1,0 mg/ml aflatoxin B_1 standard,
2-1,0 mg/ml aflatoxin B_1, 1,0 ml normal serum
3-0,5 mg/ml aflatoxin B1, 1,0 ml normal serum
4-1,0 ml normal serum,
5-0,2 mg/ml aflatoxin B_1, 1,0 ml normal serum
6-0,5 mg/ml aflatoxin B_1, 1,0 ml normal serum
7-0,2 mg/ml aflatoxin B_1, 1,0 mg/ml bovine serum albumin
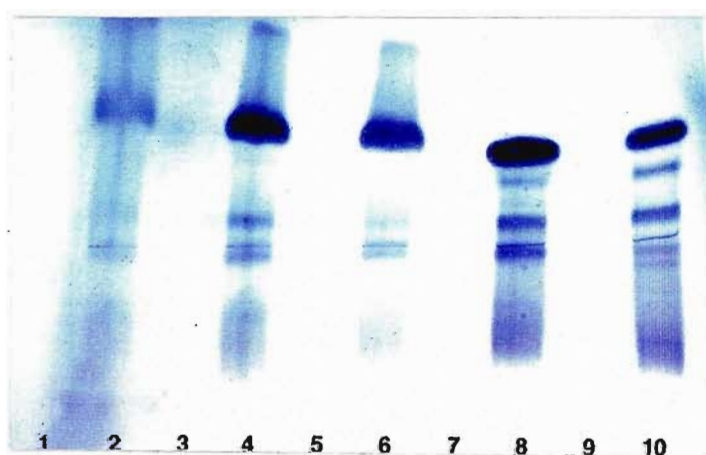
Plate 6: Paragon gel showing wells loaded with serum incubated with different mycotoxins

Well No.   1-1,0 mg/ml aflatoxin B_1 standard
2-1,0 ml kwashiorkor serum
4-0,2 mg/ml sterigmatocystin, 1,0 ml normal serum
6-0,2 mg/ml fumonisin B_1, 1,0 ml normal serum
8-1,0 ml normal serum
10-liver cancer serum

Plate 7: Scan of protein peaks in Paragon gel using IS 1000 Digital Imager (Alpha Inotech Corporation) to confirm gel indications

The electrophoresis results illustrated in this chapter do not agree at all with those of Rao and co-workers (1968) as no fluorescence was observed in the area corresponding to plasma albumin when the unstained electrophoretogram was viewed under UV light after either PAGE (Plate 5) or Paragon gel kit (Plate 6) had been carried out, suggesting the adduct had not been formed. After PAGE slight fluorescence could be observed in lane 1 (Plate 5), which contained the AFB_1 standard, but this was not the case with the first lane in the Paragon gel (Plate 6) which also contained AFB_1 standard. The viewing of a gel under UV is not a commonly practised technique and it may be that fluorescence is masked by the gel, thus preventing detection at low levels. The concentrations of AFB_1 were lower in lanes 1, 2, 3, 5 and 7 than that of the standard in lane eight (Table X). Plate 7 shows the protein peaks in the Paragon gel when scanned with IS-1000 Digital Imaging System. A possible refinement to the test procedure might be to use the image enhancement facility of the IS-1000 in conjunction with UV light to lower the threshold of fluorescence detection in the gel.

The techniques of electrophoresis, PAGE and the Paragon gel system, have superseded paper electrophoresis as used by Rao, (1968) and are more reliable. As

shown in Plates 5 and 6, the techniques can be used successfully to separate serum proteins into bands: however, do not appear suitable for the production of an adduct.

Garner *et al.* (1975), as mentioned in section 2.6, page 33 suggest that AFB_1 must be activated to the epoxide before it can bind to macromolecules and this may explain why the presence of an adduct was not detected. Synthesis of the adduct was attempted following an adapted method of Garner and Martin *et al.* (1979) and is described in section 4.3 following. For ongoing tests in this study TLC was used as the method for fluorescence detection: however, this has the drawback that protein band separation cannot be seen. As subsequent tests concentrated primarily on epoxide formation and detection, using TLC, protein band separation was not critical.

4.3 PRODUCTION OF AFLATOXIN B_1, S"9" FRACTION AND DNA; AND THEIR REACTIONS

4.3.1 Introduction

Recently it has been found that in order for AFB_1 to exert effect, it must be converted to its reactive epoxide. This occurs *in vivo* by the action of the mixed function oxygenase enzyme systems (MFO). Cytochrome P-450, the CO-binding pigment of liver microsomes plays a vital role in the metabolism of drugs, carcinogens, pesticides and other foreign compounds. The possibility that different CO-binding pigments occur in liver microsomes has been one of the most extensively studied aspects of this versatile MFO system (Mannering *et al.*, 1969). Only once it has been converted to its epoxide can the AFB_1 bind to macromolecules such as protein and nucleic acids. The experiments which follow in section 4.2.3 are based on the method by Garner *et al. (1975)*, discussed in section 2, page 33.

4.3.2 Production of Aflatoxin B_1 and radiolabelled [^{14}C] Aflatoxin B_1

Both AFB_1 and radiolabelled [^{14}C] AFB_1 were produced *in vitro*, according to the method described below, for use in AFB_1-lysine and AFB_1-DNA adduct synthesis. Leucocyte DNA was extracted, following an adapted method (Kormut, E., pers. comm.[5]), for incubation with AFB_1 to produce on hydrolysis AFB_1-guanyl adducts.

Radioisotopes provide a convenient method of ascertaining turnover times for particular compounds. They have also been widely used in studying the mechanisms and rates of absorption, accumulation and translocation of inorganic and organic compounds by both plants and animals, as well as for the location of metabolites and drugs. There are two types of scintillation counting. Solid or external scintillation counting and liquid or internal scintillation counting. The former was not used and will therefore not be discussed in this project. In the latter method, the sample is mixed with a scintillation cocktail containing a solvent and one or more fluors, the fluorescence of which can be monitored. This method is used to quantify soft ß-emitters such as ^{3}H, which is often used in biological work. Unstable isotopes or as they are more commonly known, radioisotopes, are generally produced artificially, but some do occur in nature. Radioisotopes emit particles and/or electromagnetic radiation as a result of changes in the composition of the atomic nucleus. These processes, known as radioactive decay, result, either directly, or as a result of a decay series, in the production of a stable isotope.

4.3.2.1 Materials

Aspergillus parasiticus NIX derived from (CMI 910196) was used in this project as it produces good yields of all four aflatoxins i.e., AFB_1, AFB_2, AFG_1, AFG_2.

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[5] Mr. E. Kormut, Dept. Chemical Pathology, Medical School, University of Natal, Durban

4.3.2.2 Methods (based on Williams, 1987)

Culture was preserved as a conidia preparation on silica gel. To do, this 1 ml of concentrated spore suspension was added to 50 g gel after preheating to 200 °C sterilisation then cooling. This was stored at -20 °C until it was needed. In the meantime, culture plates were prepared, by melting potato dextrose agar (PDA), allowing it to cool and then pouring it into a Petri dish which was then flamed to remove bubbles. The dishes were left overnight on the bench and then checked for contamination. Some of the sterile silica gel was spread over the surface and the plates were placed in the incubator (28 °C) for 6 days.

An inoculum was prepared from the culture by adding 5 ml sterile 0,01 % SDS (w/v) solution and titrating with a sterile glass triangle. The suspension was transferred with a sterile disposable pipette into a sterile screw cap tube and whirly mixed. It was run through sterile glass wool in a funnel into another sterile screw cap tube and then centrifuged at 9000 x g. The conidia were resuspended in sterile distilled water and whirly mixed. The turbidity at 660 nm was checked; the optic density (OD) of $1 = 5 \times 10^6$ spores per ml. In this case OD = 1,408, therefore tube contains $5 \times 10^6 \times 1,4 = 7 \times 10^6$ total conidia.

A liquid mixture medium (Williams, 1987) was prepared by dissolving L asparagine (10 g) in 250 ml distilled water. Sucrose (86 g), ammonium sulphate (4 g), potassium dihydrogen orthophosphate (0,75 g), magnesium sulphate septahydrate (0,35 g), calcium chloride dihydrate (0,075 g), zinc sulphate septahydrate (0,01 g), manganous chloride quadrahydrate (0,005 g), ammonium molybdate (0,002 g), disodium tetraborate decahydrate (0,002 g), ferrous sulphate septahydrate (0,002 g) were added and the total volume made up to 1 L with distilled water. A volume of 100 ml was dispensed in 250 ml Erhlenmeyer flasks which were autoclaved at 121 °C for 15-20 minutes. Once cooled each flask was inoculated with 1 ml of the spore suspension. The flasks were checked for bacterial contamination by means of a gram stain and incubated in a controlled environment shaker (New Brunswick Scientific Co. Inc.) at 27 °C and 200 rev/minute for 6 days. On termination of incubation, gram

stains were once again performed to check for bacterial contamination. If contamination occurs the procedure (inoculation of the medium) must be repeated.

The mycelia were broken up using a glass rod and the AFB_1 was extracted by adding acetone (150 ml) to each conical flask; the flasks were then shaken at 200 revs/minute for one hour in a shake incubator. The liquid was decanted and chloroform (150 ml) was added to each of the flasks, which were agitated for twelve hours in the Brunswick shaker. The resultant mixture was filtered through Whatmann No. 1 paper with the aid of celite on a Buchner apparatus. The remaining solid material in the Buchner funnel was washed with two further portions of chloroform (50 ml). All the chloroform extracts were combined and run through a bed of anhydrous sodium sulphate into 500 ml rotary evaporator flasks, and evaporated almost to dryness on a rotary evaporator in a water bath at a temperature not exceeding 50 °C. The residue was quantitatively transferred to a vial and evaporated to dryness on a hot plate under a stream of dry nitrogen before storage at 4°C prior to purification.

The same procedure for the production of [^{14}C] AFB_1, as that used in the production of unlabelled AFB_1 was followed: however, after inoculation, incubation at 27 °C (200 revs/minute) proceeded for only 48 hours. After this time the mycelia were filtered off on No. 1 Whatmann paper in a Buchner apparatus and washed with cold sterile distilled water. The mycelia were then treated by the following method.

The washed mycelia were transferred to plugged sterilized 250 ml conical flasks containing sterile replacement medium (70 ml). A filter-sterilized solution of U-[^{14}]C sodium acetate (200 μl = 0,04 mCi) was added to each of the flasks in three equal portions of 200 μl per application at twelve hour intervals, starting at the time of inoculation. One of the culture flasks containing [^{14}]C-labelled sodium acetate was equipped with a stopper containing a Bunsen valve to allow air in and an outlet to pass expired air through a carbon dioxide trap containing barium hydroxide solution. The traps were replaced at 24 hour intervals and the resultant precipitated radio-labelled barium carbonate was filtered off, washed with distilled water (200 ml),

and the residue and paper dried overnight in an oven at 40 °C. The filter paper was then cut into squares, placed in glass scintillation vials and counted. The concentration of both the aflatoxin produced and the activity of the barium carbonate produced, were plotted as a function of time.

The AFB_1 was isolated and purified by preparative TLC, described below, and quantised by UV spectroscopy as follows: Glass plates for TLC were thoroughly washed with detergent, clean hot water, distilled water and finally acetone. The plates were placed in the spreading base and the spreader gate adjusted to 0,3 mm using a feeler gauge. A slurry was prepared by mixing silica gel G60 (30,0 g) with distilled water (70 ml). The consistency was such that the slurry coated the spatula. The slurry was poured into the spreader which was used to cover the plates. The layer was allowed to set and the plates placed in a rack and air dried. The AFB_1-containing extracts previously prepared were redissolved in chloroform (1 ml) and streaked in a line along the origins, 2 cm from the edge of 6 chromatoplates, using a glass capillary tube. Chloroform was removed from the base line under a gentle stream of cold air. The chromatoplates were developed using acetone-chloroform (1:9 v/v) in saturated glass TLC tanks, until the solvent front reached the top of the tanks, air dried and viewed under long wavelength ultra violet (UV) light (365 nm). The fluorescent zones of silica gel G60 containing the various aflatoxins, were then scraped individually from the chromatoplates into separate sintered glass funnels and the AFB_1 eluted with 40% acetone in chloroform (100 ml). The resultant extract was evaporated on a rotary evaporator and transferred as described above. Two further purification steps were made for each extract, using preparative TLC with diethyl ether-methanol-water (94:4,5:1,5 v/v) as the solvent system. The pure dry aflatoxins were stored in vials at 4 °C.

The purity of each of the aflatoxins was determined by two dimensional TLC (section 3.3.1, page 53) identification being made from the R_f values of UV fluorescent spots (365 nm). The amounts isolated, were calculated from their absorbances at 365 nm in known volumes of methanol and published extinction coefficients (Cole and Cox, 1981). Typical absorption spectra for the isolated mycotoxins are depicted in

Appendices 13.1 to 13.4. Two dimensional TLC was performed according to the method described in section 3.3.1, page 54.

All ^{14}C - labelled metabolites were counted in glass scintillation vials using the Beckman H# method (Beckman Liquid Scintillation Systems) on a Beckman LS 3801 liquid scintillation counter with an Epson RX - 80 recorder. The substance to be counted was dissolved in a volatile solvent and made to a specific volume. A measured amount of this solution was dispensed into a glass scintillation vial and the solvent was removed in a stream of dry nitrogen with heating to 50 °C. Scintillation cocktail (20 ml, 40% Lumax in toluene) was added and the sealed vial stored in the dark, prior to counting.

4.3.2.3 Results
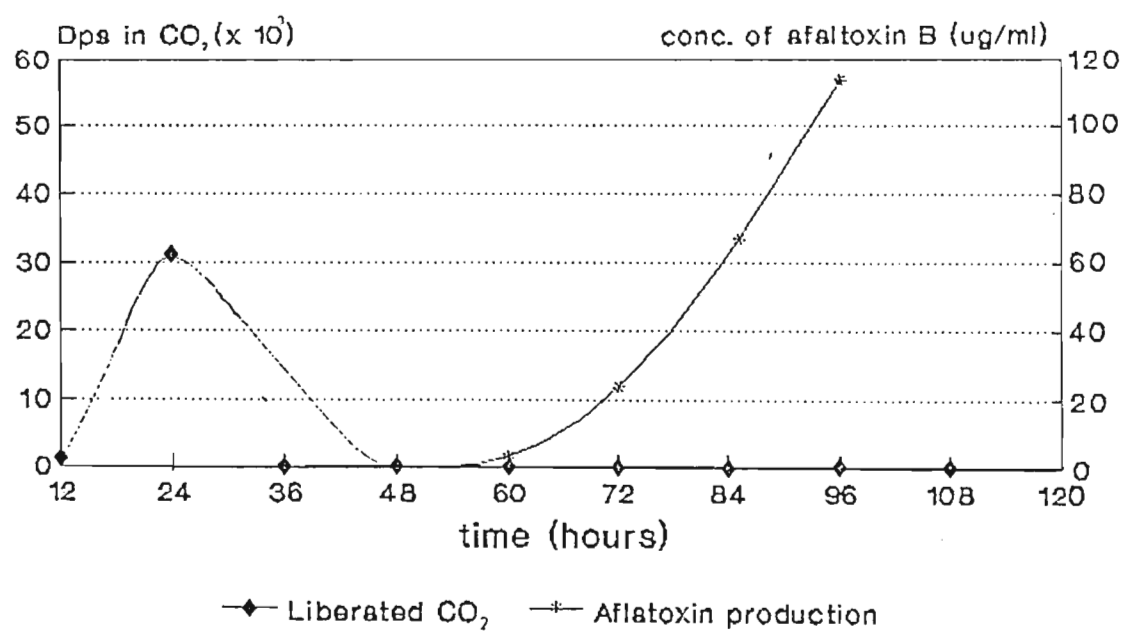
Figure 37: Production of [^{14}C] aflatoxin B_1

4.3.2.4 Discussion

Preparative TLC was used to purify the toxin. The mass of AFB_1 produced was 1500 µg. A portion of this AFB_1 was used as described in sections 4.2.3, and 4.4, in the preparation of the AFB_1-lysine. The results shown in Figure 37 indicate that the incubation period of 96 hours gave a disappointingly low yield of [^{14C}] AFB_1 and insufficient for further experimental work with the Garner *et al.* (1979) method. Production, nevertheless, was shown to increase rapidly with time after 72 hours. The graphical results from Williams, (1987) suggest that a better yield might have been achieved with an incubation period of 120 hours: however, cost constraints precluded a repeat of the experiment. It is noted that the CO_2 generation peak in Figure 37 precedes the detection of AFB_1 by approximately 48 hours and a possible explanation for this might be the time lag between depletion of nutrients in the supporting medium and the introduction of stress to, and consequent AFB_1 production by, the fungus. The purpose of the experiment was to explore the possibility of producing [^{14}C] AFB_1 for use in the method proposed by Garner *et al.* (1979) and serve as an alternative means of detection.

4.3.3 Extraction of DNA

4.3.3.1 Introduction

As mentioned in section 2.6 pages 27-37, the level of AFB_1 binding with DNA may be correlated with tumour formation. The use of commercially available imported DNA was considered: however, in-house extraction of DNA from leucocytes was selected as a more cost effective solution for on-going work.

4.3.3.2 Materials and Methods adapted from Kormut, (1993)

A sample of whole blood (1 ml) was combined with 60 µl 0,5 M ethylenediamine tetra-acetic acid (EDTA) (Boerhingher Mannheim), and 1 ml 6% Dextran in a test tube and left to stand at 37 °C for 30 minutes. The sample was

washed with 0,9% NaCl (2 ml) and spun at 4000 x g for 10 minutes at 4 °C. The supernatant was discarded and the tube flicked to disintegrate the pellet before the second wash of 0,9 % NaCl was added. The pellet was spun at 2500 x g for 15 minutes. After discarding the supernatant, samples 1, 2, 5 and 6 were treated with 600 μl 0,9% NaCl, 600 μl 0,5 M EDTA, 150 μl 5,0 M $NaClO_4$. Samples 3, 4, 7 and 8 were treated with 600 μl 50 mM TRIS, 600 μl 0,5 M EDTA, 150 μl 5,0 M $NaClO_4$.

The contents of the tubes were mixed by inversion and shaken gently for 20 seconds. A sample (80 μl) of w/v 10% SDS was added and the contents mixed by inversion and the vials left to stand for 10 minutes. To this 1,5 ml chloroform - isoamyl alcohol (24:1) were added to each vial which was then shaken for 20 minutes. The tubes were spun at 4000 x g at 4 °C for 15 minutes. The supernatant from each sample was transferred to a clean Eppendorf tube. To the samples 1, 2, 5 and 6 containing 600 μl NaCl (or chloroform), 80 μl SDS were added. The tubes were shaken and spun for 10 minutes at 4000 x g at 4 °C, the supernatant removed and 1 vol propan-2-ol was added and gently mixed with the residue. The leucocyte DNA was removed with the aid of a glass hook which had been prepared by forming the end of a pasteur pipette in a bunsen burner flame. The DNA was suspended in 70% ethanol (to improve solubility of MMPP) and allowed to stand for two minutes. The DNA was removed using the glass hook and the strand was transferred to an Eppendorf tube containing 60 μl tris-ethidamide (TE) buffer.

The supernatant was removed from samples 3, 4, 7, and 8 and 1 ml propan-2-ol was added to each with gentle inversion. The strands of DNA can be seen at this stage were removed with the aid of a glass pipette and suspended in 70% ethanol and allowed to stand for two minutes. The DNA clinging to the glass hook, was transferred to an Eppendorf tube that contained 60 μl TE buffer.

The Eppendorf tubes with DNA were placed in a 37 °C water bath. To an equivalent number of empty Eppendorf tubes, 990 μl of distilled water was added. Then 10 μl of DNA sample were added to each Eppendorf tube containing water. A blank sample of distilled water was prepared. The purity of the DNA was measured on Beckman Dc - 5 Spectrophotometer.

In a separate purity check an agarose gel was run. The instructions for setting up the 'SE 250' "Mighty Small" can be found in section 4.2.1, page 94. To make the gel, agarose (0,6 g) was dissolved in 75 ml tris-borate ethadamine (TBE) (Boeringher Mannheim) buffer (54 g/l tris base and 27,5 g/l boric acid) and the wells prepared. Loading buffer was prepared by adding 2 g ficoll 400 in 3,2 ml 0,5 M EDTA together with 20 mg bromophenol blue in 8 ml of distilled water, and 100 μl ethidium bromide (10 mg/ml).

To seven clean Eppendorf tubes, 9 μl DNA sample and 1 μl loading buffer were added. The Eppendorf tubes were spun for a few seconds. The total volume (10 μl) was removed and pipetted into the well. The gel was run for 4 hours at 70 V.

4.3.3.3 Results

TABLE XI: CONCENTRATION OF DNA EXTRACTED FROM HUMAN LEUCOCYTES

Sample	ABS at 280 nm	ABS at 260 nm	Purity ratio 260:280 nm	Conc/(μg/μl)
1	0,046	0,098	2,1	0,49
2	0,019	0,040	2,1	0,2
3	0,102	0,169	1,68	0,85
4	0,039	0,058	1,49	0,29
5	0,034	0,070	2,06	0,35
6	0,012	0,020	1,67	0,10
7	0,030	0,058	1,93	0,29
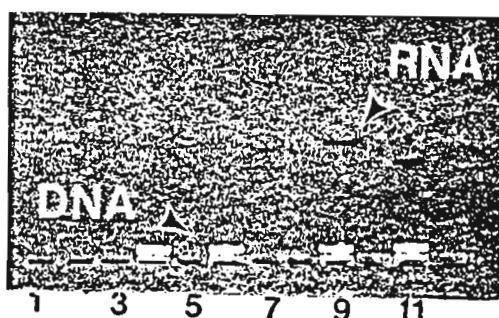
Figure 38: Agarose Gel with DNA of different purities and concentrations from 0,1 μg/μl to 0,85 μg/μl viewed under UV

4.3.3.4 Discussion

Table XI shows the purity ratios for seven DNA extractions. The ideal ratio expressed as 260:280 is between 1,8-2,0: however, a ratio less than 1,8 indicates the presence of protein and a ratio greater than 2,0 indicates the possible presence of RNA.

Table XI shows that an average of 0,4 µg/µl DNA was extracted, the high yield confirming the efficiency of the extraction process. The test procedure was found to be a viable alternative to the commercially available DNA extraction kit, (GENOMIX Talent, Trieste, Italy). The results show that one sample had ideal purity, while protein was present in three samples and RNA contamination occurred in the remaining three. In order to enhance the purity of the six contaminated samples it would have been necessary to repeat the NaCl + SDS steps. For the purpose of this study, the purity was, however, considered adequate and it was not necessary to repeat the "salting out" steps.

The purity of the DNA was checked visually by running an agarose gel (Figure 38) and also confirmed by determining the purity ratio (Table XI). The separation of double stranded linear nucleic acid fragments is entirely based on size, since the charge density is the same for pieces of different length. The same is true for single-stranded nucleic acid fragments under denaturing conditions. Conformational differences can be used to resolve polynucleotides under special circumstances. The RNA contaminants can be viewed in lanes 3, 5, 8 and 9. For the purposes of purity verification, the use of a molecular weight marker is not necessary.

It is worthy of note that the DNA extraction was performed on whole blood. An alternative method would be to separate, according to the method described in Appendix 11.0 the white blood cells before DNA extraction. It is probable that this method would lead to DNA extraction of greater purity.

4.3.4 Interaction of Aflatoxin B_1 and DNA with the S"9" fraction from rat liver

4.3.4.1 Introduction

The epoxidation of AFB_1 in *A. flavus* is catalysed by cytochrome p-450 (Bhatnagar, *et al.*, 1982). Microsomes of mammalian liver have been found to be active in the epoxidation of AFB_1 (Allameh, *et al.*, 1984). A number of researchers have investigated the binding of AFB_1 with protein using S"9" fraction from rat liver as discussed in section 2.6, pages 27-37. Microsomal studies have shown AFG_1 to bind to exogenous DNA when co-incubated with liver mixed function oxidases and a NADPH-generating system (Gurtoo and Dave, 1975). An early report using ^3H - labelled AFG_1 of low radiochemical purity showed it to bind covalently to rat liver macromolecules after intraperitoneal injection (Lijinsky *et al.*, 1970).

Rat liver for this project was made available as a by-product of other experiments (Biomedical Resource Centre, University of Durban Westville). The S"9" fraction was prepared using the procedure of Garner *et al.* (1979).

4.3.4.2 Materials and Methods

All steps were performed at 0 - 4 °C with cold and sterile solutions and glassware. The rat liver (22,39 g) was washed in an equal volume of 0,15 M KCl, minced with sterile scissors in three volumes of 0,15 M KCl (3 ml/g of wet liver), and homogenized with a Dounce homogenizer. The homogenate was centrifuged (Beckman Model J2-21) for 10 minutes at 9000 x g, and the supernatant, which is called the S"9" fraction, (also known as fraction "9") was decanted and frozen in liquid nitrogen and stored at -80 °C. As required, sufficient S"9" fraction was thawed (at room temperature) and kept on ice for later incubation with AFB_1 or DNA.

With the ingredients; 0,15 M potassium chloride, 33 mM potassium chloride, 8 mM magnesium chloride, 4 mM nicotinamide diphosphate (NADPH), 100 mM pH 7,4 disodium hydrogen phosphate, a mixture was prepared with 100 µl KCl (33 mM), 150 µl $MgCl_2$, 100 µl NADPH. To vials one, three and four 10 µl AFB_1 and 300 µl

S"9" were added to the mixture and made up to 3 ml with NaPO4 buffer. In vial two, 10 μl AFB$_1$ and 300 μl S"9" fraction were added to the vial without the mixture and made up to 3 ml with NaPO$_4$ buffer. Vial mixtures were incubated as described in Table XII.

TABLE XII: **INCUBATION OF AFLTOXIN B$_1$ WITH S"9" FRACTION**

Sample	AFB$_1$, S"9"	AFB$_1$, S"9" + mixture	AFB$_1$, S"9" + mixture (x2)
1	-	+	-
2	+	-	-
3	-	-	+
4	-	+	-

+ substances added to vial
- substances excluded from vial
mixture denotes KCl, MgCl$_2$, NADPH

These were incubated for 20 minutes at 37 °C. The vials containing different substances were incubated for 24 hours with 1 ml of serum in each vial. Following incubation, 50 μl were removed from each vial and to each, 50 μl of chloroform were added. This was vortexed and centrifuged. An aliquot (10 μl) of the lower chloroform layer of each sample was spotted onto a silica gel TLC plate and developed in 10% acetone in chloroform. An aliquot of S"9" (10 μl) was spotted to check the homogenate and act as control. Samples (10 μl) of S"9" AFB$_1$ and spiked S"9" AFB$_1$ were spotted onto TLC plates and developed in 10% acetone in chloroform. The albumin was precipitated (section 5.3, page 138) from those samples incubated with the serum and this was digested with pronase (0,67 g/ 2 mg albumin). From this, 2 ml were removed from the original vial and 3 ml of chloroform were added and the vial shaken. Following centrifugation, the chloroform layer was removed and evaporated to near dryness and a sample was spotted on a TLC plate. This was compared with a standard spotted on a TLC plate and also developed in 10% acetone and chloroform.

A further experiment was performed but in this case double volumes of the components of the S"9" mixture, 10 µl AFB$_1$, and either 0,5 mg BSA or 0,5 mg DNA were incubated for 24 hours and 1 ml was removed after 2 hours, 4 hours and 24 hours incubation. Before incubation, 1 ml of reaction mixture (S"9" with NADPH), (that is without AFB$_1$) was extracted three times with 2 ml of chloroform. The absorbance of the supernatant was read on the spectrophotometer (Milton Roy Spectronic 3000 Array) which had been preset with absorbance (Abs) = 1 and wavelength = 340 nm. As the samples contained a cloudy residue it was necessary to centrifuge the tubes before taking the absorbance.

After 2 hours incubation with AFB$_1$ 1 ml of the reaction mixture in the vial, described above, was extracted and read at 340 nm. This was repeated for the remaining incubated samples. The chloroform extract was dried under a stream of nitrogen; 200 µl of chloroform were added to the extract and 20 µl were spotted onto a TLC plate and the plate was developed as above. An AFB$_1$ standard was also run. A further experiment was performed with the above volumes and 20 µg of DNA, extracted as described in section 4.3.3.3. Volumes each of 1 ml were removed after 2 hours, 4 hours and 24 hours incubation. Those samples which showed fluorescence in TLC were analyzed using HPLC.

4.3.4.3 Results
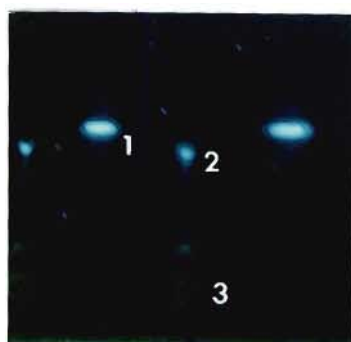
(developed in 10% acetone in chloroform)

Plate 8: Fluorescent spots on TLC plates viewed under UV (365 nm), following incubation of aflatoxin B1 and rat S"9" fraction,
(1) aflatoxin B$_1$ standard (unreacted),
(2) Non-reacted aflatoxin B$_1$,
(3) Light fluorescence
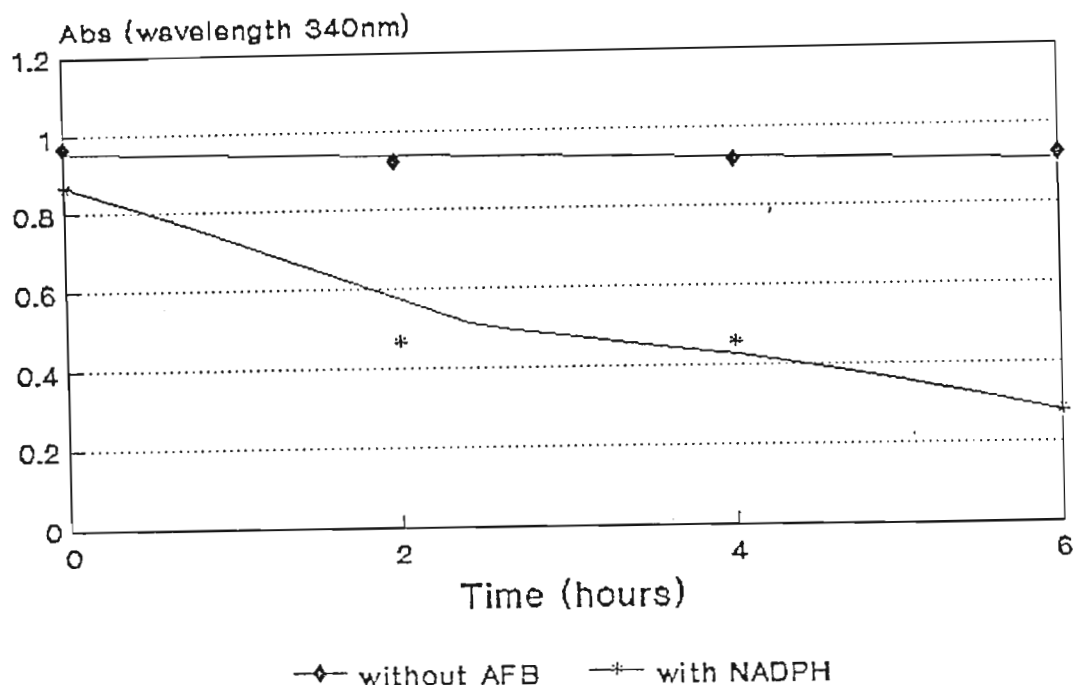
Figure 39: Monitoring of NADPH concentration by its absorbance at 340 nm over a 6 hour period at 2 hour intervals during incubation with aflatoxin B_1 and S"9" fraction

4.3.4.4 Discussion

Hepatic metabolism of xenobiotics utilizes various integral proteins located within the smooth endoplasmic reticulum of hepatocyes. Mixed function oxidases, including P-450, play an integral role in the metabolism of most xenobiotics (Pelkonen and Nebert, 1982). The liver microsomal enzymes responsible for activating AFB_1 require NADPH and it is customary when performing *in vitro* studies to add an NADPH-generating system such as NADP, glucose-6 phosphate and glucose-6 phosphate dehydrogenase, as well as phosphate buffer, $MgCl_2$ and KCl (Garner, *et al.*, 1979).

It was important to monitor the NADPH levels and AFB_1 florescence levels on TLC plates at two hourly intervals. The results showed a decrease in NADPH level (Figure 39) and reduced fluorescence of AFB_1 on the TLC plate with slight fluorescence around the origin on the TLC plate (Plate 8) suggesting utilization of NADPH in the production of the epoxide, which stuck to the origin on the TLC plate, hence the decrease of the AFB_1 fluorescence. (It is important to distinguish between AFB_1 fluorescence and any liver metabolites which may also show up under UV). When DNA was incubated with rat microsomal fraction there did not appear to be a change in the NADPH levels and there was no fluorescence detected on the TLC plates suggesting little utilization of NADPH and that the DNA absorbs the fluorescence under UV light.

Little is at present known about the chemical nature and the mechanisms by which the reactive compound, presumably the epoxide, is formed. Although it is known that the reaction has a strict requirement for both NADPH and O_2, confirmation that it is the epoxide which sticks to the origin of the TLC plate, as seen in Plate 8, can be made using Nuclear Magnetic Resonance (NMR) Spectroscopy.

The incubated S"9" fraction and AFB_1 (plus mixture) was run on a Paragon gel. No fluorescence was detected suggesting that the albumin and AFB_1 did not bind. Adminstration of [^{14}C] AFB_1 to Rhesus monkeys and subsequent gel electrophoresis and autoradiography of their blood, showed that radioactivity was associated with albumin (Dalezios and Wogan, 1972).

Allameh et al. (1984) found a differential effect of butylated hydroxytoluene on AFB_1 in vitro by liver and lung microsomes. Rubano et al. (1990) found that orally administered glucose had an effect on hamster S"9"-mediated mutagenesis, metabolism and DNA binding of AFB_1. It is important to consider the effects of carbohydrate administration on liver metabolism, especially in relation to the nutrition levels of kwashiorkor patients, as discussed in section 5.5, pages 146 and 152.

Shortcomings of this method are that animal tissue is required and the time taken for the experiment is about 48 hours. These practical restraints might limit the commercial applications. An alternative method is that proposed by Iyer and Harris (1993) and described in section 4.4 following.

4.4 FORMATION OF THE EPOXIDE WITH *M* - CHLOROPERBENZOIC ACID (based on Iyer and Harris, 1993)

4.4.1 Introduction

Iyer and Harris (1993) report a method which was used for this study to prepare the AFB_1 8,9-epoxide using *m*-chlorperbenzoic acid (MCPBA). As an alternative to using liver microsomal enzymes, *m*-chloroperbenzoic acid can be used to form the AFB1 epoxide. The reaction is carried out in CH_2Cl_2 in contact with an aqueous phosphate buffer pH (7,2) to remove the by-product *m*-chloroperbenzoic acid, which would otherwise react with the epoxide. Excess *m*-chloroperbenzoic acid is removed with aqueous sodium thiosulphate. This method generated *exo*-and *endo* epoxide in a combined yield > 70 %.

4.4.2 Materials and Methods

As well as incubating AFB_1 with albumin (section 4.2.2) a study on the binding of AFB_1 with the amino acid lysine was performed. L-histidyl-L-lysine and poly-L-lysine at concentrations of 0,1 mg 0,5 mg and 1,0 mg in water were incubated for 36 hours at 37 °C with 0,1 mg, 0,25 mg and 0,5 mg AFB_1 respectively. Pronase (0,65 mg) was added to the vials after incubation. A sample (500 µl) was removed from each test tube and added to 500 µl of chloroform, the mixture being vortexed. The two layers separated and the aqueous layer was removed. A 10 µl aliquot of the chloroform layer and 20 µl of the aqueous layer were spotted onto cellulose aluminium backed TLC plates and run (1-dimension) in butanol, acetic acid, water (156 ml:36 ml:60 ml) for 1,5 hours. The plates were dried and viewed under UV light. To

compare the aqueous layers of both L-histidyl L-lysine and poly L-lysine, 50 µl of each were spotted onto cellulose aluminium TLC plates and the plates run in butanol, acetic acid, water (78: 18: 30). These were then sprayed with ninhydrin and the position of the amino acid established. A volume of 50 µl of the aqueous layer of the "adduct" was spotted onto silica aluminium backed TLC plates and run in chloroform: methanol (99 : 1).

m-Chloroperbenzoic acid (20 mg) (donated by Professor Green, University of the Western Cape) was dissolved in dichloromethane (1,5 ml) and purified *in situ* by washing with phosphate buffer pH 7,2 (4 x 2 ml). Fresh buffer (2 ml) was added to the dichloromethane solution. A solution of AFB_1 (5 mg) in 500 µl dichloromethane was then added and reaction was allowed to proceed at room temperature for 100 minutes with vigorous stirring. The aqueous layer was pipetted off. The organic layer was diluted with dichloromethane (500 µl) and washed with 0,5 M sodium thiosulphate solution (3 x 2 ml). The dichloromethane solution was diluted (1-2 ml of dichloromethane), dried over anhydrous sodium sulphate and filtered through a plug of sodium sulphate. Solvent was removed by evaporation under a stream of nitrogen. As the supply of MCPBA was limited, alternative preparations were carried out using magnesium monoperthalate (MMPP).

Four vials were incubated; a blank, one vial with 5 x MMPP, and two with the original amounts of reagents (AFB_1, dichloromethane). One of these vials was incubated for twice the time period recommended, and one with 5 % ethanol as well the recommended substances. A sample of 10 µl was removed every half hour, spotted onto a TLC plate, developed in 10 % acetone in dichloromethane and viewed under UV at 365 nm. The method was adapted and the dichloromethane and MMPP mixture was not washed with phosphate buffer as the MMPP may dissolve in the buffer, influencing its ability to activate the AFB_1.

4.4.3 Results
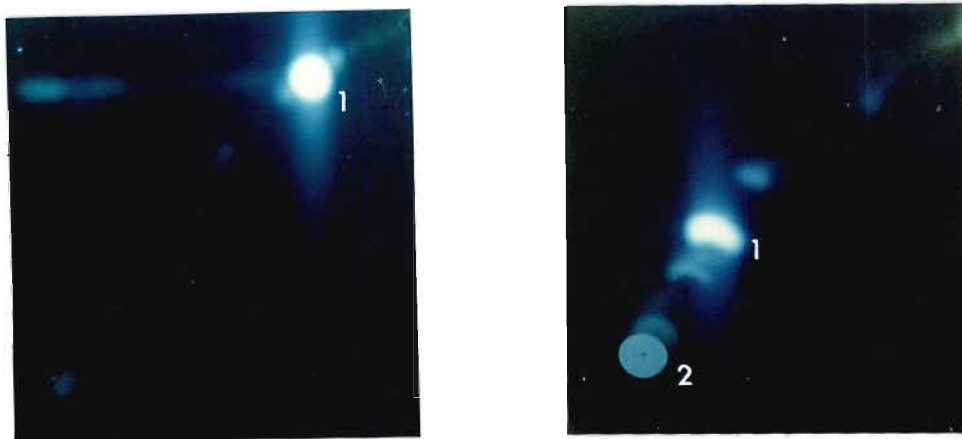
(developed in butanol:acetic acid:water, 78:80:30)

Plate 9: Fluorescent spots on TLC plate viewed under UV (365 nm) after incubation of aflatoxin B₁ with MCPBA (1) aflatoxin B₁ standard, (2) fluorescence on origin

4.4.4 Discussion

Thin layer chromatography revealed that incubation of AFB_1 with MCPBA had caused a chemical change (Plate 9). A definite fluorescent spot was observed on the origin of the silica plate spotted with the incubated mixture of MCPBA with AFB_1. When MMPP was used in place of MCPBA, almost no fluorescence was observed on the origin.

Problems with the yield of the epoxide were experienced and this may have been due to the MCPBA age as well as the fact that it was stored at a temperature, which exceeded the 0 °C recommended, during transport of the material. Although the results of this method appeared promising, further studies could not be persued because of a ban on MCPBA import into South Africa. This ban necessitated the use of MMPP (Merck), recommended as an alternative by Fieser, (1987): however, the

oxidising properties of MMPP were found to be inadequate for the purpose of this study. In the event that the import ban on MCPBA is lifted, further investigation into epoxide formation following this method would be justified. As a result of these problems, a further method of generating AFB_1 epoxide employing dioxirane was used.

4.5 PRODUCTION OF EPOXIDE USING DIOXIRANE SOLUTION (based on Murray and Jeyaraman, 1985)

4.5.1 Introduction

Dioxiranes are members of the smallest cyclic peroxide system, and are isomeric with carbonyl oxides, which are normally produced by ozonolysis. Dioxiranes have been postulated as intermediates in reactions involving peracids (Edwards, *et al.*, 1979). 2Synthesis of epoxide involved oxidation of AFB_1 with dimethyloxirane prepared from potassium peroxysulphate as a distilled 0,05 M solution in acetone. Approximately 1,5 equivalent dioxirane was added to AFB_1 dissolved either in acetone or methylene chloride. Mass spectral analysis supported the epoxide formulation. The low resolution spectrum had a prominent molecular ion at m/z 328, confirmed as $C_7H_{12}H_7$. Major fragments included m/z 270 (loss of $C_2H_2O_2$ from the epoxofuran ring, m/z 242 (a further loss of CO), and m/z 214 (loss of a second CO). The technique of NMR was used for confirmation of the results.

Figure 40: Scheme of mechanism of action of dioxirane on aflatoxin B₁

The presence of the epoxide was confirmed in this study using the epoxide spray (section 3.3.3, page 58). The spray NBP reacts with epoxides to yield methine dyestuffs (Brewer and Arnsberger, 1966) and with alkylating agents (R-X, X = e.g halogen) to yield coloured pigments (Friedman and Boger, 1961).

4.5.2 Materials and Methods

A 250 ml, three-necked round bottom flask, containing a mixture of water (30 ml), acetone (13 ml,), sodium bicarbonate (12 g) and a magnetic stirring bar, was fitted with an air condenser and solids funnel. A slight vacuum was pulled (180 torr). The receiver flask was cooled in dry ice and acetone. The flask was vigorously stirred and potassium monoperoxysulphate was added at room temperature. The acetone-dioxirane (6 ml) was collected in the receiver. A of solution 5 ml was added to 5 mg of AFB_1 and allowed to stand for 15 minutes at room temperature. Excess reagent was evaporated with a stream of nitrogen. The N-acetyl amino acid derivatives (1 mg), acetyl arginine (Ac-Arg), acetyl lysine (Ac-Lys), acetyl tryosine (Ac-Try), lysine (lys), histidine (his), were added to 1 mg of epoxide in acetone or dimethylsulphoxide (DMSO) and the mixture was allowed to stand overnight at room temperature. The N-acetyl amino acid derivatives (1 mg) serine (ser), were added to AFB_{2a} in an acid medium. Neutral (water pH 7), alkaline (0,1 M sodium hydroxide) and acid (0,1 M hydrochloric acid) medias were added to AFB_{2a} with lys and the mixtures allowed to stand overnight.

A sample of each of the mixtures (20 µl) was spotted onto cellulose TLC plates and run against a standard in BWA (butanol-water-acetic acid 12:5:3). An aliquot (20 µl) of the epoxide containing product was spotted on cellulose (10 cm x 10 cm) and run in dichloromethane. The plate was stained with a trichothecene dipping and spray solutions for the epoxides (section 3.3.3, page 58). A trichothecene standard was stained to check that the spray was effective. The epoxide was used to derivise DNA in buffer, (see below). After the N-acetyl amino acids had been incubated with AFB_1 "epoxide" and run on cellulose TLC, HPLC was performed as described in section 5.3, page 139. The adduct standard from the International Agency for Research on Cancer, Lyon, France was used. The AFB_1 "epoxide" was also analysed by HPLC (Mehta, *et al.*, 1992) after incubation with DNA. The DNA was dissolved in phosphate buffer pH 7 and adjusted to pH 1 with 0,1 N HCl and hydrolysed at 70 °C for 20 minutes. The sample was cooled, neutralised, made up to 5% in methanol and loaded on a methanol-prewashed RP C(18) Sep-Pak cartridge (Waters/Millipore Corp., Milford, MA). The column was then washed with 5% methanol to remove unhydrolysed DNA and eluted with 80% methanol to release the AFB_1-DNA adducts. The samples in 100 µl distilled water were injected onto a 12,6 cm x 4.6 mm RP C(18) column (Merck), isocratic mobile phase, run 35% methanol to 65% water, FR 1ml/min, Ex 363 nm, Em 417 nm.

4.5.3   Results
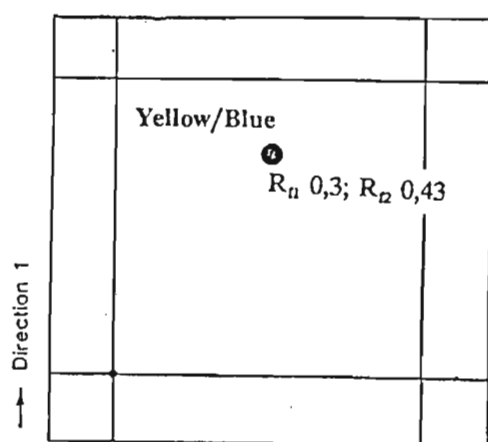
(developed in
butanol-water-acetic acid 12:5:3)

Figure 41: Cellulose TLC plate spotted with mixture of Ac-lys and aflatoxin B_1 after incubation with dioxirane and viewed under UV (365 nm), R_{f1} 0,3; R_{f2} 0,43

The proprietary adduct standard from the International Agency for Research on Cancer, Lyon, France, was found to have an HPLC retention time of 9,8 minutes, using a RP C(18) column (Merck) and a 12 minute gradient flow from 96% phosphate buffer to 60% methanol at 1 ml/minute (Figure 42).
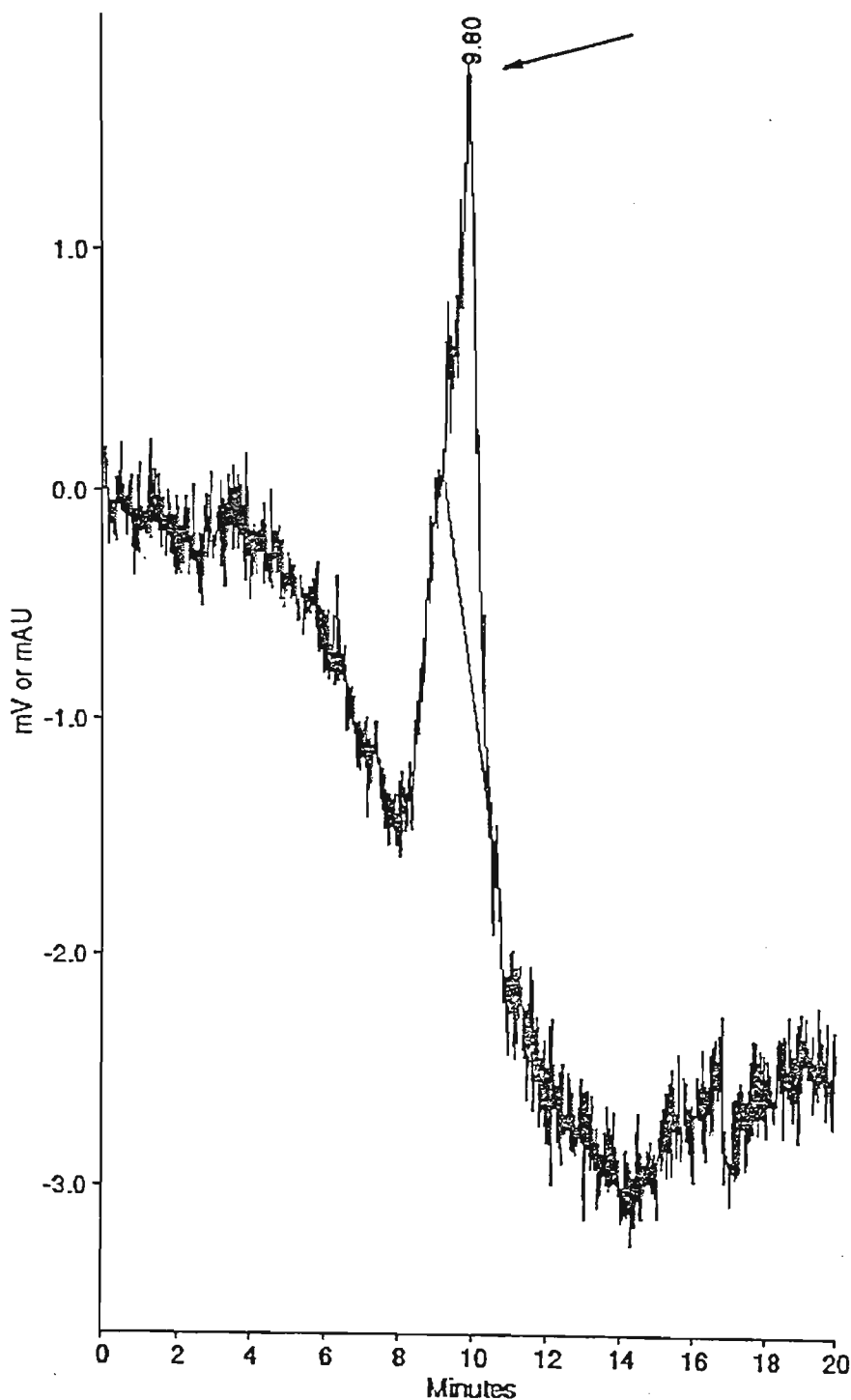
Figure 42: HPLC chromatograph of aflatoxin B_1-lysine adduct with retention time of 9,8 min, RP C(18) column (Merck) and a 12 minute gradient flow from 96% phosphate buffer to 60% methanol at 1 ml/minute.
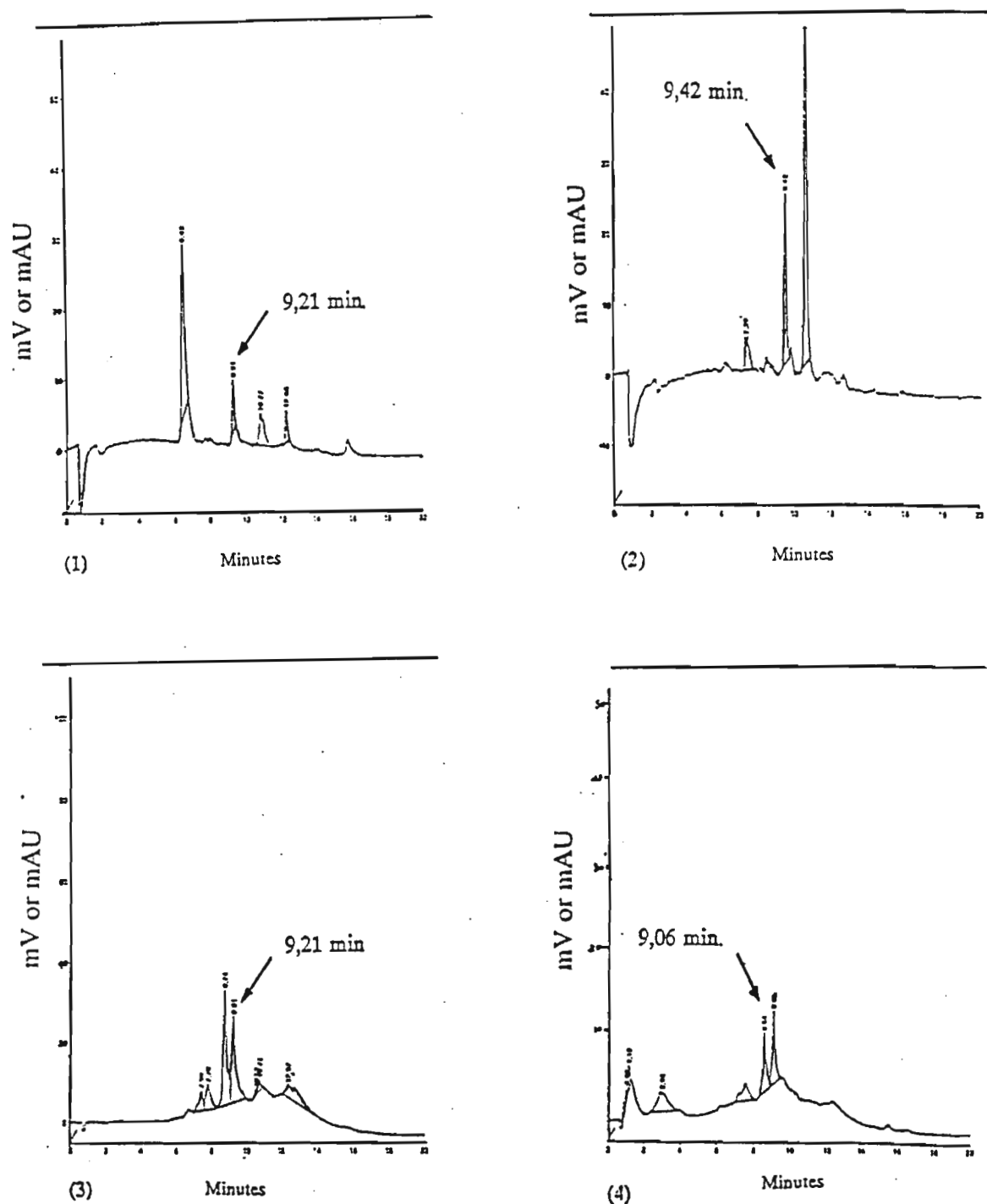
Figure 43:   HPLC chromatograph of N-acetyl amino acid derivatives with dioxirane, and aflatoxin B_1, RP C(18) column (Merck), gradient 12 minutes. Run 96% phosphate buffer to 60% methanol, FR 1 ml/min, Ex: 405 nm, Em: 470 nm

        (1) DMSO, Ac-lys, aflatoxin B1

        (2) DMSO, lys, aflatoxin B_1

        (3) Alkaline (0,1 M NaOH), lys, aflatoxin B_{2a}

        (4) Neutral, (0,1 M HCL), lys, aflatoxin B_{2a}
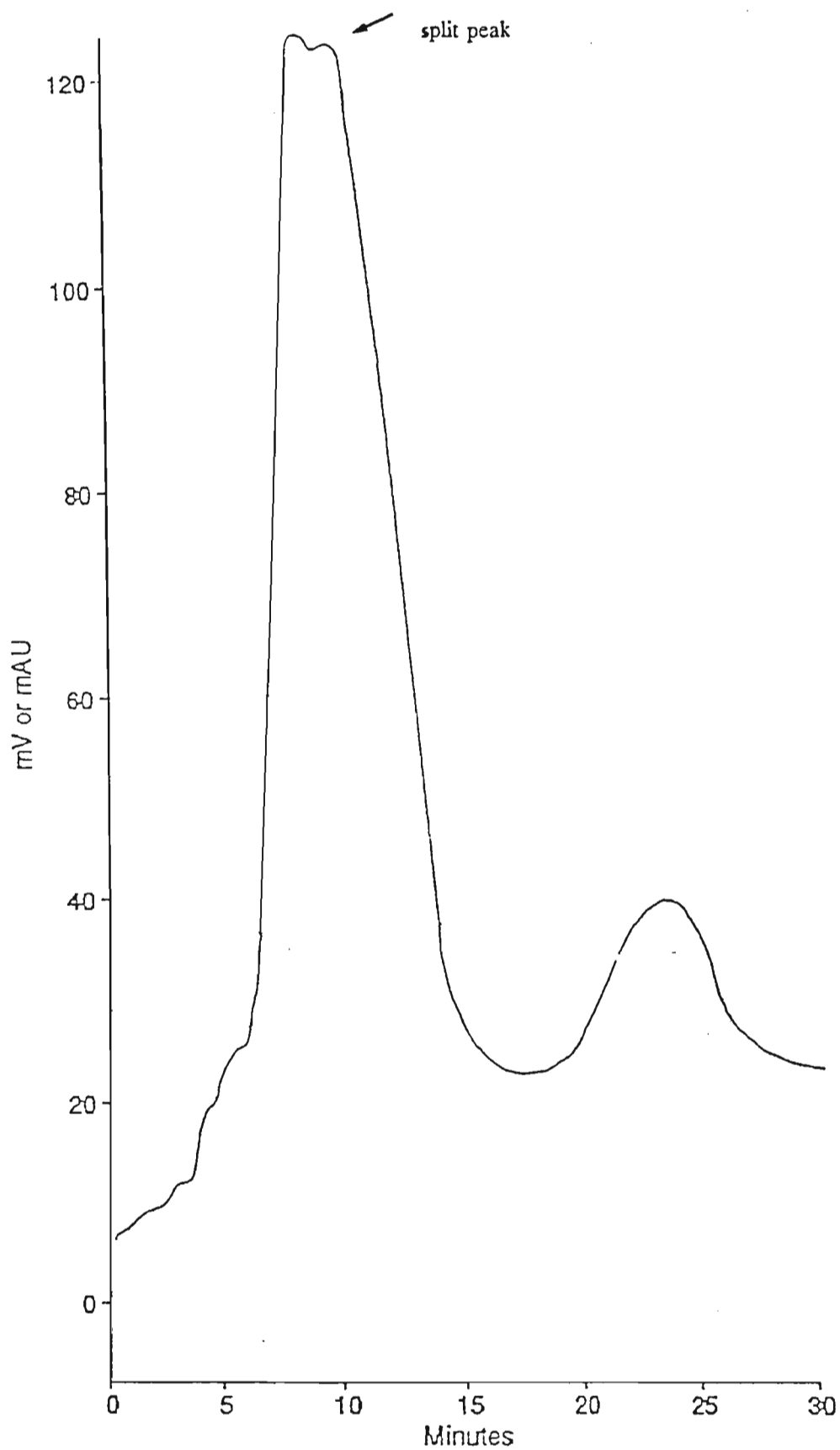
Figure 44: HPLC chromatograph of reaction of hydrolysed DNA with aflatoxin B_1 epoxide formed with dioxirane, RP C(18) column (Merck), isocratic mobile phase, run 35% methanol to 65% water, FR 1ml/min, Ex: 363 nm, Em: 417 nm

4.5.4 Discussion

A comparison was made of the HPLC retention times of other acetyl amino acid derivatives incubated with AFB_1 and dioxirane in order to establish whether any of these retention times were comparable to that of the imported standard.

The HPLC retention times shown in Figure 43 compare well with the 9,8 minutes retention time of the imported standard and of these four it was proposed that DMSO, Ac-lys incubated with AFB_1 and dioxirane could be used to produce a substitute standard. In the event insufficient quantities of the substitute were available and the imported standard was used for the purposes of this project.

The chromatograms shown in Figure 43 indicate that both DMSO, Ac-lys and DMSO, lys incubated with AFB_1 have retention times of 9,42 and 9,21 minutes respectively. This is similar to the 9,8 minute retention time of the imported adduct standard.

It is interesting to note that the retention times of alkaline medium, lys and neutral medium, lys incubated with AFB_{2a} also indicated in Figure 43 are also similar to the imported adduct standard. It is possible that the acid environment of the human digestive system is conducive to the conversion of ingested AFB_1 to AFB_{2a} and for this reason AFB_{2a} may have a significant role to play in the production of the adduct.

Exo Epoxide   Endo Epoxide
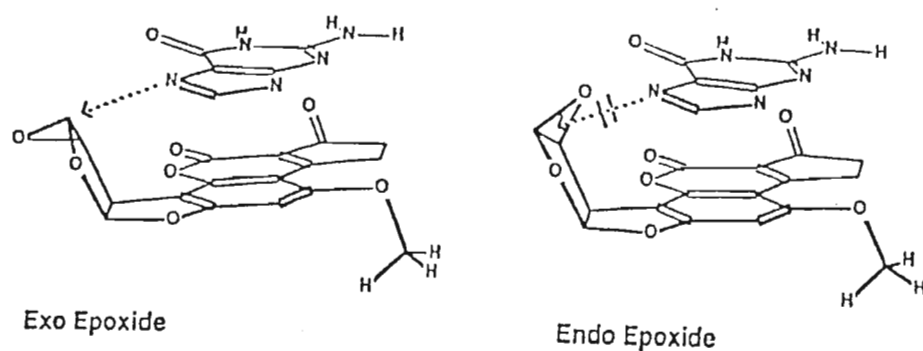
Figure 45: Synthesis of aflatoxin B_1-DNA adduct by oxidation with dioxirane

Details of AFB$_1$-DNA formation are discussed in section 2.6, pages 27-37. The results of the HPLC chromatograph in Figure 44 show a broad, split peak with RT of 9,00 minutes. Jennings *et al.* (1992) reported RT of 8,6 and 11,0 minutes for the hydrolysed DNA adduct (each peak representing an isomer). Further studies to refine the AFB$_1$-DNA adduct HPLC separation were outside the scope of this project. It is interesting, nevertheless, to note that, according to Iyer *et al.* (1994) the *exo* epoxide reacts efficiently with DNA, whereas the *endo* epoxide does not react at all. This observation is also consistent with the proposed biomolecular reaction mechanisms. Whereas intercalation of the planar portion of *exo* AFB$_1$ epoxide optimally orients the dioxirane moiety for attack of the N^7 position of guanine, similar intercalation of the *endo* epoxide places the CO bond of the dioxirane ring aiming towards guanine N^7 rather than away from it; these relationships are depicted above in Figure 45. Further analysis such as nuclear magnetic resonance (NMR) would have to be undertaken to confirm that the adduct has been formed in each of possible substitutes.

4.6 PREPARATION OF AFLATOXIN LYSINE ADDUCT VIA BROMO-DERIVATIVE (based on Sabbioni, *et al.*, 1987)

4.6.1 Introduction

The present work was undertaken to identify one or more serum albumin adducts which might prove useful in monitoring exposure. Work by Sabbioni *et al.* (1987), showed that the adduct was produced by the acylase-catalysed deacetylation of the reaction product of N acetyl-L-lysine with 8,9-dihydro- 8,9-dibromo-AFB$_1$.

4.6.2 Materials and Methods

A 1 mM bromine solution in methylene dichloride (1 ml) was added dropwise with stirring to AFB$_1$ (10 mg) in 1 ml methylene dichloride. After 20/30 minutes the solvent was evaporated off under nitrogen and the residue dissolved in 1 ml AR acetone. An aliquot (2 µl) was spotted on silica TLC plate and developed in acetone/chloroform 1:9 (CA) and viewed under UV. Fluorescent spots were marked.

The AFB$_1$ and bromine were added with stirring to a solution of acetyl lysine (250 mg) in 1 ml 0,1 M phosphate buffer pH 7 and left overnight. An aliquot (20 µl) was spotted on cellulose TLC plates and developed in 2 dimensions: (i) IPA-water-ammonia 7:2:1 (IWA) (ii) Butanol-water-acetic acid 12:5:3 (BWA). The plate was viewed under UV at each stage. The plate was sprayed with ninhydrin (0,2% in acetone) and positive spots were marked (Plate 10). It was then placed in a chlorination tank for 15 minutes, after which time excess chlorine was allowed to blow off in the fume cupboard for 10 minutes. When the plate no longer smelt of chlorine, a corner was sprayed with tolidine reagent to check for background reaction and if negative, the plate was sprayed and the spots were marked immediately. The acetone was removed from the reaction mixture using a stream of nitrogen. The sample was frozen solid and freeze dried. The residue was extracted twice with 2 ml acetone. The acetone was pooled and evaporated under nitrogen, dissolved in 1 ml acetone, and 20 µl were spotted on a silica gel plate and developed in 2 D (i) CA (9:1) (ii) CM (chloroform:acetone) (3:2). Any spots were marked.

A small cationic ion exchange column (12 cm x 3 cm) was prepared with Zeacarb resin. This was washed with 1 M sodium hydroxide (2 ml), then with water until the effluent was neutral, afterwards with 1 M hydrochloric acid, then water again until the eluent was neutral. At no time was the column allowed to run dry. The residue was dissolved in 2 ml water and applied to the column which removed the excess acetyl-lysine. The column was washed with water in 2 ml portions and the fractions were carefully collected. The pH was checked with pH paper and neutralised accordingly with sodium bicarbonate. Samples (20 µl) were spotted on cellulose TLC plates and run as above. The samples were frozen and freeze dried. Those fractions which showed staining upon spraying with tolidine spray reagent (detects amines and peptides) were digested at 25 °C overnight with 2 mg acylase (Sigma) in 1 ml phosphate buffer pH 7. The digest was then applied to the column and eluted with 4 M ammonia. The product of the digestion was then subjected to HPLC (section 5.3, page 139) and a resultant chromatograph is shown in Figure 46.

4.6.3 Results

The spots on the plates faintly fluoresced blue when sprayed with ninhydrin and stained green/yellow in a position with similar R_f values when sprayed with tolidine reagent (Plate 10).

Plate 10:   Cellulose plate after 2D development in IWA (7:2:1), BWA (12:5:3), spraying with 0,2 % ninhydrin, exposure to chlorine gas and tolidine reagent, R_n 0,3; R_2 0,5

The HPLC chromatograph shown in Figure 46 depicts a peak with a retention time of 9,1 minutes.
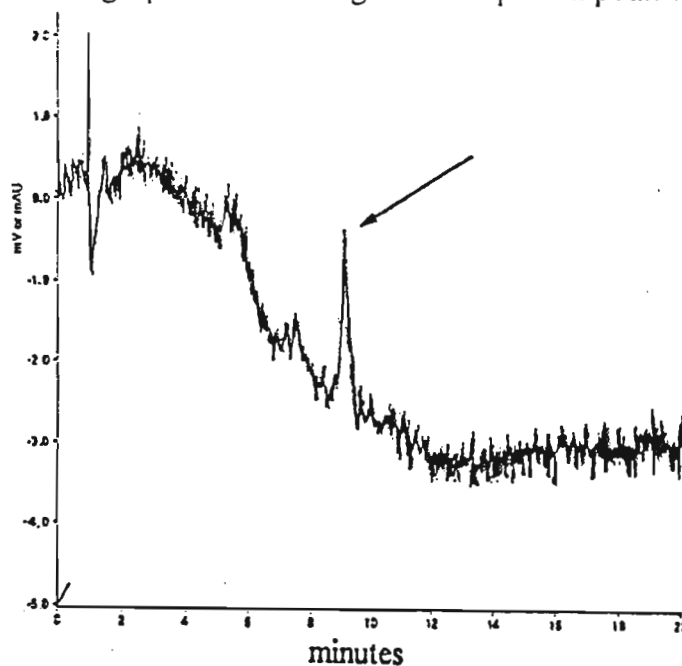
Figure 46:   HPLC chromatogram of brominated aflatoxin B_1 incubated with Ac-lys, RP C(18), 12 minute gradient 96% phosphate buffer to 60% methanol, FR 1 ml/min, Ex: 405 nm, Em: 470 nm.

4.6.4 Discussion

It is proposed that the initial adduct is formed by condensation of the dialdehyde tautomer of 8,9-dihyro-8,9hydroxy AFB_1, with the ϵ-amino group of lysine, to form a Schiff base and that the Schiff base undergoes an Amadori rearrangement to an α-amino ketone (Figure 47). The pyrrolinone ring is formed by condensation of the amino group of the remaining aldehyde to yield the final product.

R· o) -CH₂ICH₂l₃CHINH⁺₃)COO⁻

b) -CH₂ICH₂l₃CHINHCOCH₃)COO⁻
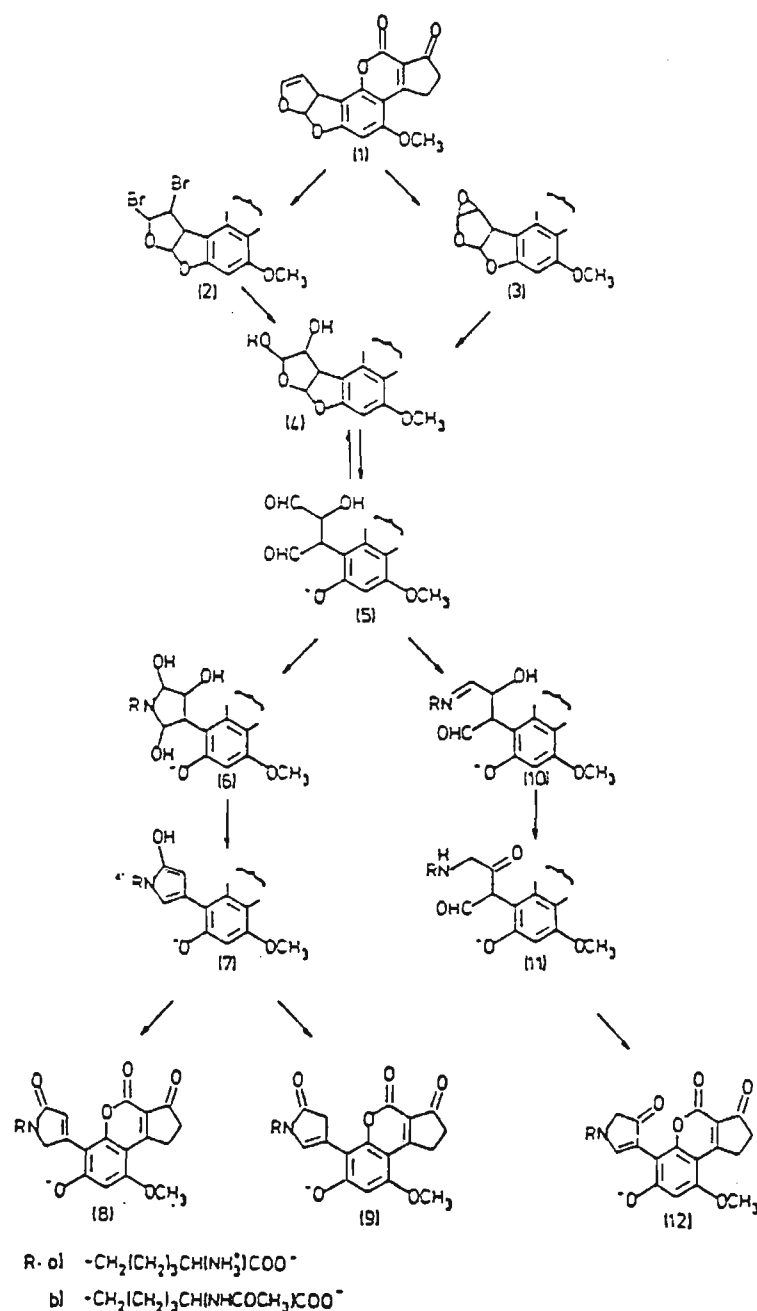
Figure 47.   Proposed mechanism for the formation of the aflatoxin B₁ - lysine adduct (Sabbioni, et al., 1987)

The TLC results showed that after spraying with tolidine reagent a green/yellow spot with an R_f value of 0,38; 0,38 was observed. This faded rapidly and it was necessary to mark the area on the TLC as soon as the spot appeared. When the digest was loaded onto the HPLC column (Merck) it eluted with an RT of 9,1 minutes, which is similar to that of the imported standard with an RT of 9,4 minutes. The concentration of the brominated AFB_1 adduct was very low, approximately 0,4 $f$mol/μl and it is recommended that subsequent AFB_1 bromination experiments should be performed with higher volumes of primary material in order to give an increased yield of adduct product.

Sabbioni, *et al.* (1987) produced an adduct using the bromination method. The adduct was analysed by HPLC on a Whatman ODS-3 Magnum-9 column eluted with isocratic mixture of 68% (0,02% acetic acid; 8% acetonitrile; 5% methanol in water) and 32% (8% acetonitrile in methanol). The RT under those conditions was 28, 5 minutes and the yield was 14%. A Whatman column similar to that used by Sabbioni and co-workers was not available for this study, thus it is not possible to compare the RT of the study with that found by Sabbioni, *et al.* (1987). Comparison of the RT of the brominated AFB_1-adduct from this study with the RT of the imported adduct standard was considered adequate.

4.7   GENERAL DISCUSSION

With regard to the mechanisms of carcinogenicity induced by the aflatoxins, a number of questions remain to be answered: in particular, how such unstable metabolites as epoxides react so extensively with proteins, rather than with other nucleophiles such as water. The AFB_1 epoxide is extremely labile under non-biological conditions and can be detoxified *in vivo* by conversion into the *trans*-diol by epoxide hydrase (Edwards and Wogan, 1970). The epoxide intermediate will therefore require protection in a hydrophobic environment during its migration to the site of action with genetic material (DNA) in the nucleus; alternatively epoxidation may occur in the nucleus (Garner and Wright, 1973). Peter (1985) reported that 1,2-epoxy-3,3,3-

trichloropropane, an epoxide hydrase inhibitor, does not affect the activation of AFB_1 by rat liver microsomes, while pretreatment of Wistar rats with phenobarbitone reduces the AFB_1 carcinogenicity (Garner *et al.*, 1975). Raj *et al.* (1975), observed the formation of the AFB_1 glutathione conjugate and found that the epoxidation of AFB_1 may represent not only an activation but also a detoxification mechanism.

Although, in this study, it does appear that AFB_1 is oxidised by rat microsomal fraction, this method has drawbacks in that it is time consuming (requiring two days) and animals are used. As with other TLC experiments mentioned in this chapter, it is likely that it is the epoxide which remains at the origin of the TLC plate, but there is no absolute proof of this. Literature suggests that NMR, HPLC and GCMS give a more definitive result (Croy and Wogan, 1981; Beije and Hultin, 1971 and Rubano, *et al.*, 1990). The adduct formed during experiments detailed in this chapter was identified by HPLC: however, difficulties with GCMS adduct analysis arose as a result of the properties of the incubation medium used for a number of experiments, particularly water, being unsuitable for GCMS analysis.

An alternative method of AFB_1 oxidation (requiring a half day) involves the use of *m*-chloroperbenzoic acid. Jacobsen and Humayun (1986) revealed that MCPBA induced DNA damage when mixed with AFB_1 and DNA. Jacobsen and Humayun (1986) determined that DNA damage could be avoided by first activating the AFB_1 with MCPBA, removing unreacted MCPBA and adding the active AFB_1 to DNA. This study aimed first to produce the epoxide, check for its presence and then incubate it with albumin and DNA. After the initial incubation, (before the addition of albumin or DNA) reduced AFB_1 fluorescence and slight fluorescence around the origin on the TLC plate could be detected when the TLC plate was viewed under UV (Plate 10). The drawback with this method is that the yield of epoxide was low. The drawback of this experiment was the yield of epoxide was low and supplies of MCBPA limited due to import restrictions.

Garner *et al.* (1975) found that the amount of binding *in vivo* or in microsomal activation of AFG_1 or AFB_1 with DNA does not appear to be associated with the

reactivity of the enol group or the stability of the epoxide. Using peracid oxidation as a means of generating the epoxide, no difference was seen in the amount of DNA binding. Investigations into the nature of the distribution co-efficients in various model membrane systems indicate than more AFB_1 than AFG_1 partitions into the model membrane suggesting that this could be one factor accounting for the difference in DNA binding. Research is still being done to establish how much of the removal of AFB_1 or AFG_1 bound to DNA is an enzymatic process and how much a chemical one. These problems are being studied by a number of different researchers worldwide. It may be that metabolism in the nuclear membrane of the liver is largely responsible for the DNA binding, whereas metabolism by the endoplasmic reticulum is responsible for RNA binding (Neal and Goday, 1976). Alternatively there may be a transportable AFB_1 metabolite which can break down in other organs to release a reactive electrophilic species.

Activation of AFB_1 with peroxy acids requires a two phase system. A single phase system is unacceptable due to the oxidative modifications of DNA and protein by the peroxy acid. The two phase system is limited to nonbiological systems because of the destructive effects of organic solvents on cells.

Chapter 4 describes five different techniques to produce AFB_1-albumin adduct for use as a standard. The bromination method emerged as the preferred method, although the dioxirane method also showed good results. The bromination method is simple, cost effective and, with a little refinement, is capable of producing high yields of adduct on a commercial basis. The dioxirane method, while giving excellent results, is costly, hazardous and the dioxirane itself is unstable if incorrectly stored.

According to literature, numerous attempts to produce the epoxide have failed (Gorst-Allman et al., 1977, Coles, et al., 1979, Garner et al., 1979, Miller, 1970) and this would indicate that further research into the epoxidation of AFB_1 as well as binding of other mycotoxins such as ZEA and DON to albumin, is justified.

CHAPTER 5

MONITORING OF AFLATOXIN B₁-LYSINE
ADDUCT LEVELS IN SERUM

5.1 INTRODUCTION

The ability to quantify human exposure to chemical carcinogens, at an individual level, should improve the sensitivity and specificity of epidemiological studies designed to identify environmental exposures, and to elucidate the multifactorial aetiology of human cancer. This approach of molecular or biochemical epidemiology defined by Perera and Weinstein, (1982) involves measuring the level of "exposure" to a chemical in the environment, or the "dose" entering the body. This may also be termed "the biologically effective dose" and is defined as the amount of the activated agent that has reacted with critical cellular targets such as protein, DNA or RNA. In the quantisation of macromolecular adducts it is important that the individual's capacity to absorb, metabolize and detoxify the compound prior to adduct formation, and the capacity to remove or repair the damage once formed, is taken into account. In addition, in the case of DNA adducts, the adduct formation may also be one of the determining events in the multistage process of carcinogenesis and could be informative in assessing the individual's risk of developing a tumour.

A key limitation in the early work done in this field of research (Munoz and Bosch, 1987; Yeh *et al.*, 1989; Campbell *et al.*, 1990) was the absence of individual markers of exposure to aflatoxins. Exposure assessment by measuring the mycotoxins in food samples and extrapolating to calculate average intakes at the population level was of low reliability. Several approaches, notably the measurement of AFB_1-nucleic acid adducts in the urine and AFB_1 bound to peripheral blood albumin (Groopman *et al.*, 1988, 1990; Wild and Montesano, 1990) are aimed at epidemiological studies in which these markers are appropriately employed to help clarify the role of the

aflatoxins in the aetiopathogenesis of HCC and other diseases, such as kwashiorkor (Wild and Montesano, 1990). In a study undertaken by Wild *et al.* (1992), a significant correlation (r = 0,55; P = < 0,05) was observed between the dietary intake and the level of albumin-bound aflatoxins.

In addition, a good agreement was obtained with the two analytical techniques which were used, namely ELISA and HPLC. A comparison of matched chronic hepatitis B surface antigen carriers with noncarriers did not reveal any difference in adduct formation for a given intake of aflatoxins. The measurement of aflatoxins bound to albumin is valuable for a number of reasons:

(i)   A significant percentage of ingested aflatoxins are covalently bound to albumin: in rat and man 1-3% of a single dose (Wild *et al.*, 1986; Sabbioni *et al.*, 1987; Shang-Gan *et al.*, 1988).

(ii)   The half-life of albumin (in man approximately 20 days) allows accumulation of AFB_1 adducts following repeated exposure and thus the adduct level gives an integration of recent past exposure (2-3 months) (Tannenbaum and Skipper, 1984).

(iii)   In rats the level of binding to serum albumin reflects the level of AFB_1 bound to liver DNA (Wild *et al.*, 1986). In humans a similar relationship is suggested by data demonstrating a correlation between AFB_1 nucleic acid adducts in the urine and AFB_1 bound to albumin (Groopman *et al.*, 1990). These observations are probably a result of the activation of AFB_1 in the hepatocyte and the subsequent binding to DNA or albumin in the same cells (Okoye *et al.*, 1990; Wild *et al.*, 1990).

(iv)   The structural identification of a major albumin adduct formed by AFB_1 (Sabbioni *et al.*, 1987) and AFG_1 (Sabbioni and Wild, 1991) has permitted the development of radioimmunoassays

(Shang-Gan *et al.*, 1988), enzyme-linked immunosorbent assays (ELISA) (Wild *et al.*, 1990a) and HPLC-fluorescence techniques (Wild *et al.*, 1990b) to measure the adduct.

(v) The ELISA has provided a simple, relatively inexpensive assay, compared with HPLC analysis, which is sensitive, specific and applicable to field studies (Wild *et al.*, 1990b). The ELISA technique has been designed to be applicable with a minimum of expensive equipment.

Exposure to aflatoxins and hepatitis B virus infection, have been implicated as major risk factors of HCC in high-incidence regions of the world. Investigations, carried out in areas such as The Gambia, Senegal, Kenya and The People's Republic of China using the assay of aflatoxins bound to peripheral blood albumin, have shown that exposure can occur throughout the lifespan of the individual, including during the perinatal period. The possibility of measuring exposure of aflatoxins at the individual level permits investigation of the putative mechanisms of interaction of these carcinogens with HBV in the aetiopathogenesis of HCC (Wild, *et al.*, 1993). High levels of HBV have been detected in a number of HCC patients. It is widely accepted that AFB_1 has a role in the aetiology of HCC and thus research in our department is currently aimed at the correlation of HBV and AFB_1-lysine adducts in HCC patients.

5.2 MATERIALS

A protocol for this study was submitted and approved by the University of Natal, Medical School Post-graduate (Ethics) Committee. Blood was collected, in additive-free vacutainers, from the Paediatrics Outpatients Departments at KEH in Durban. Batch one comprised blood from 15 children aged between 3 months and 5 years, while batch 2 comprised 16 samples from patients aged between 8 months and 10 years. In a third sampling 15 samples were collected from outpatient children aged between 9 months and 3 years. The first batch of samples was collected at KEH

between 7th May and 28th June 1993, while the second was collected between 9th August and 12th November 1993 and the third from 6th June 1994 to 28th November 1994.

The first batch of blood was, unfortunately, damaged by technical default. The batch of blood samples collected in the second half of 1993 was screened for free AFB_1 and ZEA. Each subsequent batch was screened for bound AFB_1-lysine adducts. In a separate sampling, blood was taken by Doctor Nigel Rollins from two kwashiorkor children upon admission to inpatients. Blood samples were drawn on two consecutive days following admission.

Blood samples were also taken in additive-free vacutainers from 7 adult HCC KEH patients from May 1993 to August 1994 to establish any possible correlation between the presence of HBV and AFB_1-lysine in these patients.

It was desirable to establish confirmation for this study at Temba Hospital. Blood samples were collected by Doctor Kobeyer from the paediatrics inpatients wards at Temba Hospital in KaNgwane from a total of 15 children aged between 1 month and 9 years and the second between the ages of 2 months and 4 years. A survey aimed at investigating the history and clinical symptoms of kwashiorkor patients from Temba Hospital was done (Questionnaire Aflatoxins\Kwashiorkor Study). This was performed in two sessions from 4th March to 28th June 1993 and 1st July to 23rd August 1993.

The loss of the first batch of blood limited the sample size to 16 from batch two. The blood was sent to the Rob Ferreira Hospital where the samples were centrifuged and the serum removed. The separated samples were packed on ice and transported by air (Link Air) to Durban together with the history of each patient (Appendix 16.0).

Controls at both hospitals were well nourished children in the same age group who had arrived at the hospital for other reasons, such as broken limbs. Children

were diagnosed as having kwashiorkor according to the Wellcome classification. Practical constraints did not facilitate urine collection from the children.

TABLE XIII: NUMBER OF BLOOD SAMPLES TAKEN BETWEEN 1993-1994

	KING EDWARD VIII HOSPITAL	THEMBA HOSPITAL
No. OF SAMPLES	34	16

5.3   METHODS

To extract free AFB_1 and ZEA, one ml of hexane was added to 1 ml of serum in a stoppered 10 ml test tube, mixed gently by shaking/swirling for 2 minutes, centrifuged for 10 minutes at 2000 x g and the upper hexane layer containing lipids removed and discarded. This procedure was repeated twice with the addition of 1 ml hexane, the hexane layer containing lipids being removed and discarded after each centrifugation.

Extraction of free aflatoxins from the lipid-free serum was carried out by adding 1 ml chloroform. The mixture was then shaken vigorously for 4 minutes, centrifuged for 10 minutes at 2000 x g and the lower layer (chloroform) in which the aflatoxins were dissolved, removed. This extraction was repeated three times using 1 ml of chloroform. The chloroform extracts were pooled, maintained in a warm bath and reduced to dryness, under a stream of nitrogen prior to analysis by TLC (section 3.3.1 page 56).

Due to the small quantities and number of blood samples drawn from the patients only the blood from Temba Hospital was screened qualitatively for free mycotoxins: AFB_1 and ZEA.

Serum samples from a kwashiorkor patient, a liver cancer patient and a healthy individual were run on a Paragon gel (section 4.2.3, page 99).

Each batch of blood was analysed for the presence of AFB_1-lysine adducts following an adapted method of Wild *et al.* (1990) as follows; an aliquot of saturated ammonium sulphate (750 µl) was added to 0,5 ml of serum with vortexing. Precipitated immunoglobulins were removed by centrifugation at 9000 x g for 15 minutes at 0 °C. The supernatant was removed and +/- 65 µl of 1 M acetic acid were added to adjust the pH to 5. A few drops of acetic acid were added and the pH checked between each addition. The precipitated albumin was collected by centrifugation for 10 minutes, as above. The supernatant was discarded. The albumin was redissolved in 1 ml 0,1 M Na-phosphate pH 7,4. This was vortexed. An aliquot (15 µl) was made up to 1 ml with buffer pH 7,4 (purity checked using Paragon gel kit) for the spectrophotometric protein assay.

Using both the Bradford assay and the spectrophotometric assay for protein the total protein concentrations of the albumin and that of the residues were measured. The method which offered the closest value equal to the total protein concentration employed. For the purpose of this study the spectrophotometric assay was employed.

The absorbance at $_{230}$ and $_{260}$ nm was measured. Bovine serum albumin was used as a standard 1 mg/ml and 1ml of buffer was used as a blank. Protein concentration (µg/ml) = 183 A_{230}-75,8A_{260}. The absorbances of a solution of protein and nucleic acid at 230 and 260 nm are given by:

$$A_{230} = E_{230}^{P}[P] + E_{230}^{N}[N]$$

$$A_{260} = E_{260}^{P}[P] + E_{260}^{N}[N]$$

where E_{230}^{N} is the absorption coefficient (millilitres per microgram per centimetre) chosen for protein at 230 nm, [N] is the nucleic acid concentration (micrograms per millilitre) and [P] is the protein concentration (micrograms per millilitre).

Albumin solutions (2 mg/ml in phosphate buffer pH 7,4) were digested with pronase (70,000 proteolytic units per gram weight Calibiochem) using 1 mg enzyme per 3 mg albumin. Digestion was for twelve hours at 37 °C. An immunoaffinity column (Vicam, Aflatest-10) was used for "clean-up". The method for "clean-up" was established, using a standard, as described below.

An aliquot (50 µl) AFB_1-lysine (40 $f$mol/µl), obtained from the International Agency for Cancer Research, Lyon, was diluted in 950 µl water. The affinity column was washed with 0,1 M phosphate buffer, pH 7,4 (1 ml). The AFB_1-lysine standard (1 ml) was added to the column, which was then washed with (i) phosphate buffer:methanol (1:1) or (ii) 100% methanol.

The eluent from (i) and (ii) was evaporated under a stream of nitrogen to a volume of 100 µl, and 20 µl of each was injected onto the HPLC column. (section 3.4.2, page 63 for HPLC model). Ultra Pure water (Millipore) was used and samples were filtered before injection onto the column. Separation was performed on a 25 cm x 4,6 mm RP C(18) (Spheri-sorb) column, a 25 cm x 4,6 mm SAX (Spheri-sorb) column and a 12,5 cm x 4,6 mm column (Merck) with an isocratic mobile phase, (phosphate buffer:methanol 90% : 10%) or 12 minute gradient of 96% phosphate buffer (0,02 M pH 7,2) to 60% methanol. Excitation was set at 405 nm and emission 470 nm and the flow rate was 1 ml/minute. The peak resolution using the different columns was compared.

5.4 RESULTS

TABLE XIV: DETECTION BY TLC OF FREE AFLATOXIN B$_1$ AND ZEARALENONE IN SERUM FROM TEMBA HOSPITAL PAEDIATRIC PATIENTS, (1993)

Sample No.	Aflatoxin B$_1$	Zearalenone
1	+	-
2	+	-
3*	+	+
4	-	-
5*	+	-
6	-	-
7*	+	-
8	-	-
9	-	-
10	-	-
11	+	+
12	+	+
13	-	+
14	-	+
15	-	+
16	-	-

- indicates absence of any fluorescence
  (zearalenone plates sprayed with aluminium chloride)
+ indicates presence of fluorescence
* denotes controls

TABLE XV: DETECTION BY TLC OF FREE AFLATOXIN B_1 AND ZEARALENONE IN SERUM FROM KING EDWARD VIII HOSPITAL HEPATOCELLULAR CARCINOMA PATIENTS (1993)

Sample No.	Aflatoxin B_1	Zearalenone
1	+	-
2	+	-
3	-	-

- indicates absence of free aflatoxin B_1 and zearalenone
+ indicates presence of free aflatoxin B_1 and zearalenone

TABLE XVI: AFLATOXIN B1-ADDUCT LEVELS IN SERUM FROM TEMBA HOSPITAL PAEDIATRIC PATIENTS, 1993, AS DETERMINED BY HPLC (CONCENTRATIONS)

Sample No.	Conc. of adduct ($f$mol/μl)
1	0
2	80
3*	0
4	0
5*	0
6	0
7*	0
8	0
9	0
10	0
11	50
12	0
13	35
14	30
15	0
16	0

* denotes controls; well nourished children admitted with other ailments

TABLE XVII: AFLATOXIN B_1-ADDUCT LEVELS IN SERUM FROM KING EDWARD VIII HOSPITAL PAEDIATRIC PATIENTS, JULY-AUGUST 1993, AS DETERMINED BY HPLC (CONCENTRATIONS)

Sample No.	Conc. of adduct ($f$mol/µl)
1	0
2	0
3	0
4	0
5	0
6	0
7	0
8	0
9	0
10	15
11	30
12	70
13	35
14	30
15*	0
16*	12,2
17*	6

* denotes control; well nourished children admitted for other ailments

TABLE XVIII: AFLATOXIN B_1-LYSINE ADDUCT LEVELS IN SERUM FROM KING EDWARD VIII HOSPITAL PAEDIATRIC PATIENTS, JUNE-NOVEMBER 1994, AS DETERMINED BY HPLC (CONCENTRATION)

Sample No.	Conc. of adduct ($f$mol/µl)
1	25,7
2	8,9
3	0
4	0
5*	22,8
6	43
7*	59
8	22,8

* denotes control; well nourished children admitted for other ailments

TABLE XIX: AFLATOXIN B₁-LYSINE LEVELS IN SERUM FROM KING EDWARD VIII
HOSPITAL HEPATOCELLULAR CARCINOMA PATIENTS, 1994, BY HPLC
(CONCENTRATION)

Sample No.	Conc. of adduct ($f$mol/µl)
1	57,1
2	17
3	0
4*	0
5	0
6	0

* denotes control patients
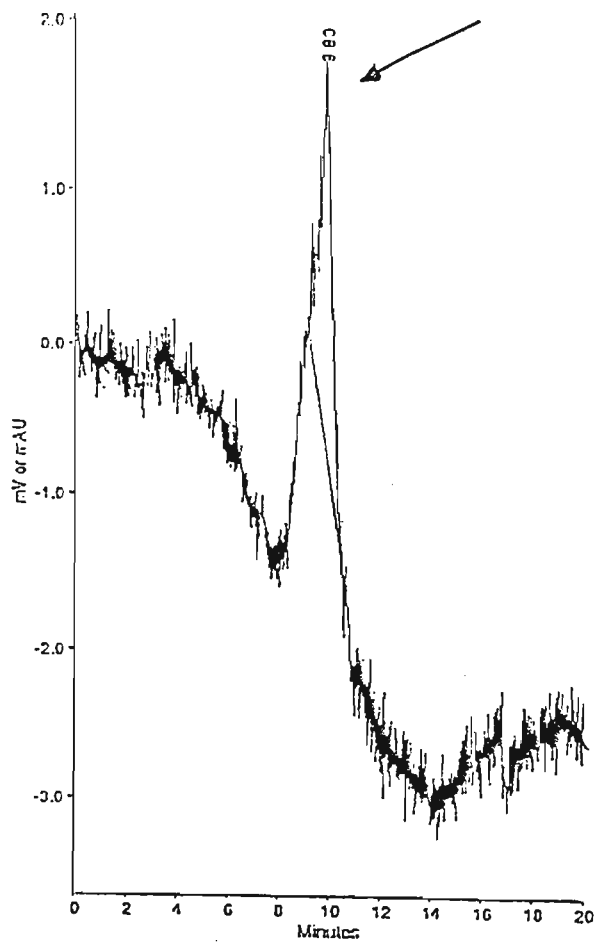
Figure 48:   HPLC chromatograph of aflatoxin B₁-lysine adduct, 12 cm x 4,6 mm
(Merck), Phosphate buffer : methanol, 12 minute gradient, 96% to 60% at
FR 1 ml/min, Ex:405 nm, Em: 470 nm
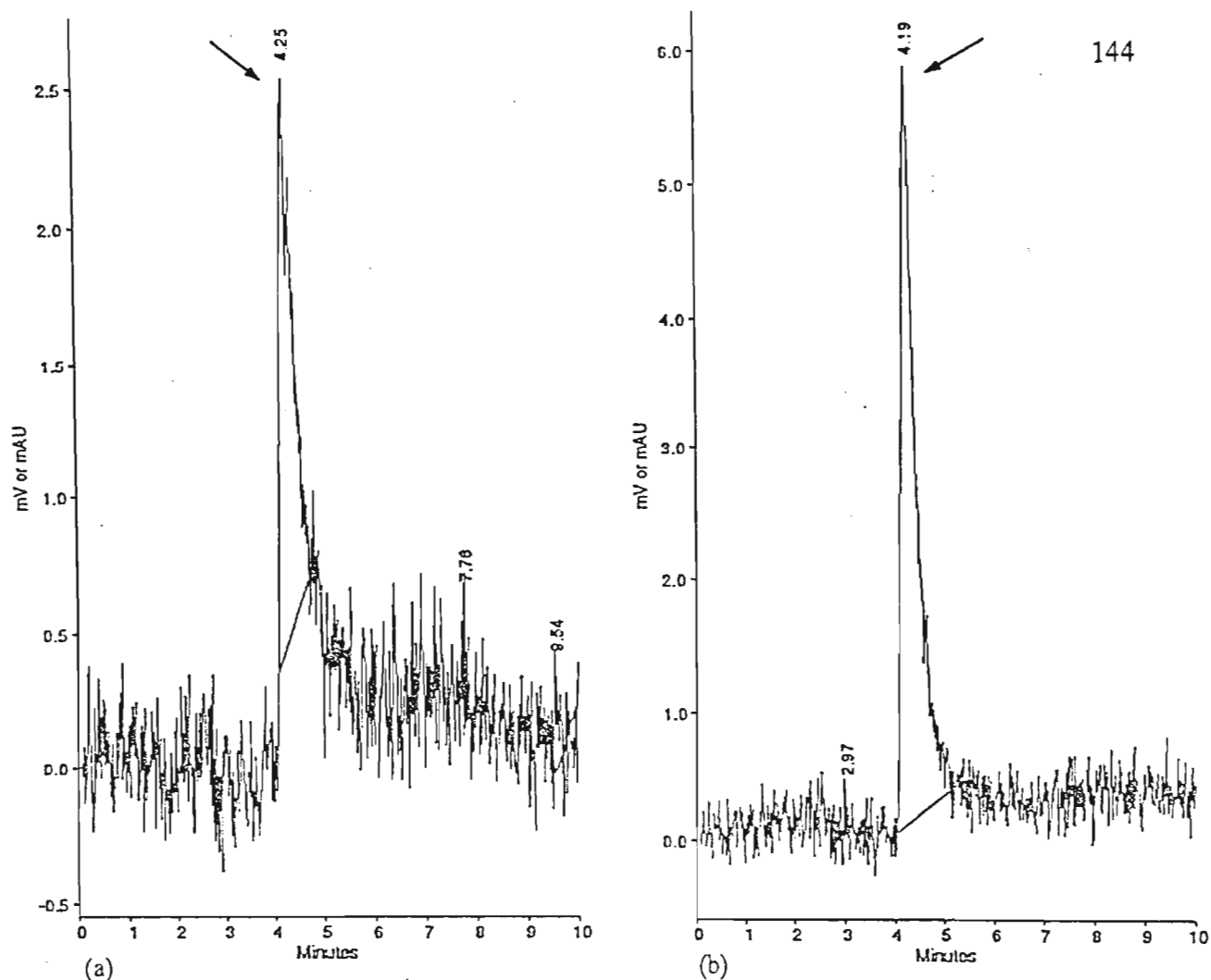
Figure 49: HPLC chromatograph of (a) aflatoxin B₁-lysine adduct, and (b) tryptophan, 25 cm x 4,6 mm   SAX column, Spheri-Sorb, Phosphate buffer:methanol, 90% : 10%, 1 ml/min, Ex: 405 nm, Em: 470 nm

5.5   DISCUSSION

The responses from a survey conducted to establish the diet of Temba Paediatric kwashiorkor patients suggest that, although maize is an important part of the diet, it is not the only dietary food group.   Maize is supplemented with beans, meat, eggs and vegetables (See Appendix 16.1) although males had a higher cereal intake, while females ate more vegetables and eggs.   As discussed in section 3.8, page 90, mycotoxins were found in maize and groundnut from KaNgwane.   The possibility that AFB_1-protein adducts exist in groundnuts was investigated (Appendix 17.0), but the results were negative for the adduct.

It appears that males receive more food than females: however, the limitations of the survey must be borne in mind. Pure logistical problems prevented the accumulation of data on the levels of occurrence of kwashiorkor at Temba Hospital and indeed a number of problems have to be overcome when gathering data. In the rural areas transport may be a problem and not all patients go to hospitals for treatment. The sex ratio may be influenced by the movement of the men away from the region on migrant labour contracts. Traditional medicine is still practised in this area and may be an influencing factor, thus the limitations of the questionnaires must be considered.

Another factor which had to be considered was that the information on the diets of the people within the rural areas was not first hand and was recorded by a number of different people with varying backgrounds, skills and levels of understanding. The validity of information given by those questioned, cannot be therefore be taken as entirely accurate. These problems might be overcome with the first hand accumulation of information by a specialised team of researchers fluent in the language of the local people. Hean (1992) found that roasting or boiling of the maize was common practice. However, many of the toxins are heat-resistant and will survive this treatment (Bennett *et al.*, 1983; Gilbert, 1989). It is thus unlikely that the methods used to prepare food would detoxify contaminated maize. Another aspect is exposure to mycotoxins through the contaminated, mouldy maize used to make beer.

There was little data on the diets and patient's history available for the second batch of kwashiorkor blood samples from Temba Hospital.

The total admission of kwashiorkor patients at Themba hospital from January 1993 to September 1993 was 127. For KEH, the kwashiorkor intake from January 1993 to November 1993 was 454 patients and 233 from December 1993 to October 1994. The history and background of the KEH patients was not presented in this study, which concentrated on the development of a method for screening AFB_1-lysine in blood.

TABLE XX: FREE AND BOUND AFLATOXIN B₁ IN SERUM FROM TEMBA AND KING
EDWARD VIII HOSPITAL PATIENTS

Batch	Total No. patients	% positive	95% confidence %	adduct mean (fmol/μl)	range (fmol/μl)
Temba (free toxins) 1993	16	43,7	19,4-68,0	n/a	n/a
KEH HCC (free toxins) 1993	3	66,6	-	n/a	n/a
Temba (bound toxin) 1993	16	25,0	3,3-46,2	55,8	30,0-80,00
KEH (bound toxin 1993-1994)	25	52,0	32,4-71,5	28,3	6,0-70,0
KEH HCC (bound toxin) 1994	6	33,3	-	37,1	17,0-57,1

- indicates sample size too small for statistical analysis using the formula:
95% confidence interval = p +/- 1,96 $\sqrt{(^{pq}/_n)}$
where p = % positive, q = % negative and n = sample size.

For comparative purposes between controls, HCC and kwashiorkor patients, Paragon gels were run. These showed that the albumin level in the kwashiorkor patients was significantly lower than that of the controls. The HCC patients did not appear to suffer reduced albumin levels. The results of these tests support the hypothesis that kwashiorkor patients, with reduced albumin levels, are more susceptible to mycotoxicosis, with the binding of the AFB₁ epoxide to the reduced supply of available albumin, at source in the liver. Factors, as discussed in section 2.6, page 36, such as protein deficiency impair the antioxidative defence system (Chow, 1988).

The screening of blood for free AFB₁ from Temba Hospital kwashiorkor patients showed that 7/16 were positive for AFB1 and 7/16 positive for zearalenone.

Only two mycotoxins were screened for due to the small quantity of blood available for the method used for extraction of both toxins, also these mycotoxins were considered to be the most important contaminants in cereals from this area (section 3.5, page 69). It was not possible to perform this screening on patients from KEH VIII as only a single microtainer of blood was collected from each patient and this was used for the adduct analysis. Rather than repeat the work done by Ramjee *and co-workers in* (1990), this study was aimed at developing a method to detect the AFB_1-lysine adduct.

Haemoglobin which has a longer half life than the protein most commonly proposed for dosimetry, appears to be of little use for monitoring AFB_1 exposure since the fraction of dose which binds to the protein is very low (Tannenbaum and Skipper, 1984). The total protein concentration in the blood is 60-80 g/l and the albumin concentration is 38-48 g/l. A number of different researchers (whose work is described in section 2.6, pages 29-31 and section 4.6.4, page 130) have detected and measured AFB_1-lysine adducts using HPLC. Wild *et al.* (1990a) found that the pronase/albumin ratio of 3/1 gave the largest yield of adduct after 12 hours, and 24 hour hydrolysis did not alter the yield of adduct. Shang Gan *et al.* (1988) purified the adduct by applying the digest to a commercial aflatoxin immunoaffinity column (Aflatest-10, Vicam Corp., Medford, MA). The principle of this method is that the molecule is bound to the carrier and this bands for the recognition of the bound antibody for the aflatoxin moiety of the adduct. It is important that the Aflatest-10 (with antibody 2B11) affinity columns are used as the Aflatest affinity columns are designed for cereal analysis and are not efficient for the purpose of recognising the epoxide-lysine adduct (Figure 50).
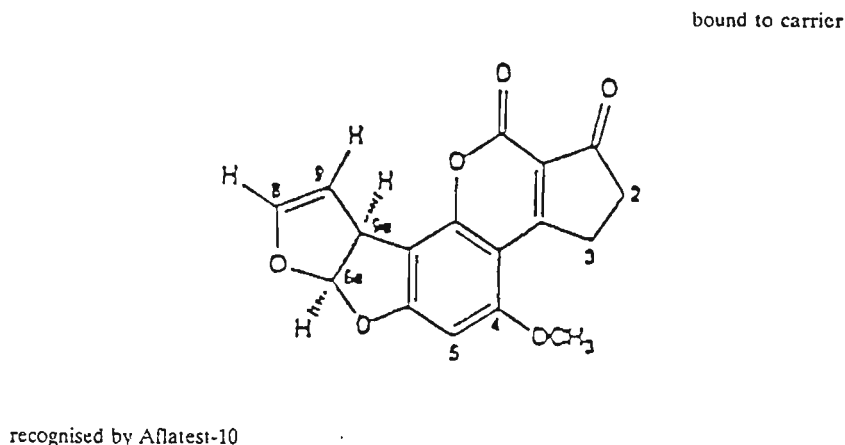
bound to carrier

recognised by Aflatest-10

Figure 50:   Diagram showing which part of aflatoxin B₁ binds to the antibody

Groopman *et al.* (1988) produced a column for "clean-up" of the free adduct which is recognised by the monoclonal antibody, 2B11, raised against AFB$_1$ coupled to bovine serum globulin (BGG) through its synthetic epoxide. The company Biocode Ltd (University Road, Heslington, York, England) does confirm that their columns can be used for adduct "clean-up": however, they would not divulge details on the type of antibody used in these columns.

The principle of separation of the SAX and RP C(18) columns used in this method are very different, that of the SAX depending on separation by charged particles and the zwitter ion, while separation on the RP C(18) depends on hydrophobicity. The use of different columns will obviously have a direct bearing on the retention time of the component of interest. The results of this study showed that when tryptophan, the internal standard, was injected onto a C(18) column (Merck), with a fluorescence detector set with Ex 406 nm, Em 440 nm, PT = 75, and a 12 minute gradient mobile phase from 96% buffer to 60% methanol with a flow rate 1,00 ml/min, the peak showed bad tailing with poor resolution. When a low concentration, due to limited amount available, of the adduct was injected onto the C(18) column, the peak was broadened and flatten out into the noisy baseline (Figure 48). A mobile phase of 100% methanol was run in an attempt to condition the column and to avoid baseline drift due to changing solvent composition.

This did not appear to reduce the noise and a Strong Anion Exchange Column (SAX) (Spheri-Sorb) column was substituted in the HPLC, but with little improvement as the gradient mobile phase gave a noisy drifting baseline. Because of this an isocratic mobile phase of 90% 0,02 M phosphate buffer pH 7,2, and methanol was used which gave an improved baseline (Figure 49) and this became the preferred method. As the SAX column is more polar, non-polar molecules would elute around max RT of +/- 5 if 100% phosphate buffer were used as the eluent. It is interesting to note that the RT of the adduct was 2,1 minutes, when injected onto the Merck column run with an isocratic mobile phase of 90% 0,02 M phosphate buffer pH 7,2.

A number of factors may be responsible for the noisy baseline. Adequate time must be given for the system to equilibrate and this was done. The system must be at temperature and mobile phase composition equilibrium to avoid baseline drift. Thoroughly degassed solvents must be used to avoid noise and spikes attributed to dissolved gases in the mobile phase. When using gradient pumps, the pumps must proportion the solvents properly as done by the Spectra-Physics HPLC System. A solvent spiked with UV-absorbing material and a gradient programme can help isolate composition inconsistencies. Adjustment of peak threshold made no difference to the noise level and the Spectra-Physics instrument does not have a software facility to change the attenuation.

There was not sufficient adduct standard to perform a recovery test on this material, but according to Wild et al. (1990) the recovery is 75-95%. Wild et al. (1990) injected 700 µl into the HPLC loop, probably in order to load a high concentration onto the column and increase the signal to noise ratio. It was not possible in this study to load more than 20 µl onto the column, due to the size of the loop which was available. This, therefore, is likely to be the cause of the rather poor chromatographs which can only be improved by injecting more material or carrying out preconcentration steps. As described in the methods of this chapter, 1 ml phosphate buffer:methanol (1:1) was found to be the optimum ratio for elution of the adduct from the affinity column. Practical problems with preconcentration of the eluent containing aqueous buffer were experienced. Wild et al. (1990) established a detection

limit of 5 pg AFB_1/mg albumin, whereas with this method using the Spectra-Physics HPLC System the detection limit was found to be 12 $fmol/\mu l$.

The second batch of blood samples from Temba and KEH were run on the HPLC SAX column. The resultant chromatographs showed that upon injection of affinity column eluent, which contained phosphate buffer and methanol (1:1), the absorbance was found to decrease into negative readings. Those samples which showed positive peaks for the AFB_1-lysine adduct on the SAX column were run on a C(18) 12,5 cm x 4,6 mm column (Merck). The reason for this was to confirm the identity of the adduct which has quite different characteristics on the C(18) column as compared with the SAX. A peak on the chromatograph was detected at the appropriate retention time for the AFB_1-lysine adduct injected on the same column, in spite of the very noisy baseline produced by the gradient mobile phase. It is important to note that the retention time of the two columns used differs due to the length, and thus number of theoretical plates, of the column, as well as the packing of the column. The use of capillary electrophoresis as an alternative technique, for confirmation of the presence of the adduct is a possibility. Access to such an instrument at the time was not practically possible.

The results of tests for the presence of free AFB_1 and AFB_1-lysine screening are shown in Tables XII and XIV. It is interesting to note that the results of this study showed that some Temba patients had free AFB_1 in their blood and no bound toxin, while others had bound and no free toxin present. This may be explained by the longer half-life of albumin bound to the AFB_1 as opposed to free toxin and the rate at which the epoxide forms from the free toxin after ingestion. In future studies it would be interesting to determine the concentration of free AFB_1 as well as bound AFB_1 in blood in all cases.

Clearly the above observations result from the previous history of toxin intake and indicate that deductions can be made about mycotoxin exposure. From the results of this study, we can conclude that measurement of the AFB_1-lysine adduct may be an excellent molecular dosimeter for the determination of exposure of humans to AFB_1

and used as a biomarker may reflect the level of genetic alterations from the initial binding of this carcinogen to cellular DNA.

Table XVII shows the concentrations of adduct in KEH HCC patients. Immunocytochemistry, performed on liver biopsies from the 1993 HCC patients, correlated well with the HPLC screening, with one patient being positive, one negative and the third having trace amounts of the adduct present in their blood. The KEH HCC HPLC results show that 33% of the patients were positive for the adduct, with a mean of 37,1 $f$mol/μl. These patients were tested for the HBV and 53% were found to be positive. Work is currently underway to perform ICC on liver biopsies from these patients. (Bux, S., pers. comm.[6]) The range reported by Sheabar *et al.* (1992) was 0-890 pmol AFB_1/g albumin, while Wild *et al.* (1990) reported levels of 11-455 and Shang-Gan *et al.* (1988) reported levels of 23-795 pmol AFB_1/g albumin.

When comparing the results from Temba Hospital and KEH for 1993 it is noticed that both the mean and the range of adduct levels differ considerably, being 55,8% at a range of 30-8 $f$mol/μl and 28,3% at a range of 6-70 $f$mol/μl respectively. The difference between the levels of adduct in blood from the two hospitals was not statistically significant (p = 0,325). Sample size was limited in the case of Temba hospital by the practical logistics arising from the remote location of the hospital and, in the case of KEH, by sporadic strike action which interfered with the collection programme.

The results for KEH patients in 1994 compared with the 1993 results show a higher incidence of adduct contamination possibly due to the start of the rainy season and thus proliferation of fungi in poorly stored foods.

In the case of the patients who were re-examined after admission there was a slight decrease of the levels from 22,8 $f$mol/μ to 20,8 $f$mol/μl of AFB_1-adduct. The small sample size of re-examined patients did not allow any statistical comparison to

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[6] Mrs. S. Bux, Electron Microscopy Unit, Dept. Physiology, Medical School, University of Natal, Durban

be made on any changes of adduct level after the patient had been on a hospital diet for 24 hours to several days. As discussed in section 4.3.4.4, page 115, glucose administered in the diet of the rat, and other dietary nutrients (section 2.6, page 36) have been shown to influence liver metabolism of AFB_1. In Jamacian kwashiorkor patients with low serum proteins, the presence or absence (or activation and inactivation) of a primer protein, is a form of control that can may override other controls that operate once polysaccharide synthesis has begun (Whelan, W.J., pers. comm.[7]). This is an area for possible future research, including the scaling of clinical symptoms and exploring the other mycotoxin adducts including ZEA and DON.

Assuming albumin is cleared from the body with first-order kinetics and that no specific mechanism exists for accelerating the removal of adducted albumin, then adduct clearance will be described by the function:

$$A(t) = A_c e^{-kt}$$

where $A(t)$ is the level at time t and Ac is the chronic exposure level. After time t, difference in values will be clearance:

$$Ac - A(t) \text{ or } A_c - A_c e^{-kt}$$

Under conditions of chronic exposure, the albumin adduct level will stabilize at a value such that the amount of adducts cleared per unit of time is equal to the amount of new adducts formed during the same interval. Letting $t = 1$ day and equating clearance with the daily production of new yields:

$$A_c - A_c e^{-k} = \text{(daily increment)}$$

Solving for A_c:

$$A_c = \text{(daily increment)} \times (1 - e^{-k})^{-1}$$

For man, $k = -0,035$ days^{-1} and $(1 - e^{-k})^{-1} = 29$.

Therefore, the chronic exposure albumin adduct level is expected to be 30 fold higher than the adduct level produced by a single dose. It is interesting to note that in a rat administered 0,5 µg of adduct, clearance of the adduct will take over 120 hours, by which time the level decreases to 12,2 pg AFB_1/mg plasma albumin with a half-life of the adduct of 55 hours.

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Clearly a single serum measurement of free AFB_1 provided by HPLC does not represent accurately how much AFB_1 has been consumed other than during the previous few hours, or which other toxins have been ingested (Denning *et al.*, 1990); Sabbioni *et al.*, 1988; Gan *et al.*, 1988).

Thus, it appears that AFB_1 is metabolically activated *in vivo* in humans to a form which binds to serum albumin, presumably the reactive intermediate being the 8,9-dihydro-8,9 epoxide, although this assumption has not been proven. Shang-Gan *et al.* (1988) found that the amounts of adduct measured agreed very closely with the values predicted by an animal model. It thus appears that the adducts formed with serum albumin are useful for determining exposure to AFB_1.

CHAPTER 6

GENERAL DISCUSSION

6.1 OVERVIEW

The availability of good analytical methods is the key to assessing the extent, and eventually controlling, the risk to human health associated with the contamination of foods and feed. There are to date a number of world-wide organisations currently investing significant resources in the development of accurate and rugged methods capable of detecting mycotoxins at low levels in foods, feeds and biological tissues. The first PAN-African Environmental Mutagen Society meeting on mycotoxins as mutagens and carcinogens: (Properties for disease prevention, Cairo, 23-26 January 1993) raised a number of issues including that of aflatoxins as a major problem both in food and feedstuffs. This is particularly relevant in developing areas, such as Latin America, China, Africa and India. These issues will be expanded at the 1996 meeting in Cape Town, South Africa.

A literature survey indicates that mycotoxin research in many developing countries focuses on the detection of the aflatoxins by using TLC as the primary method. There are many reasons for this, including the lack of trained personnel and appropriate sophisticated equipment (HPLC and GCMS) needed for work on the other toxins. More recently, emphasis on methods for the detection of aflatoxins specifically has decreased, while interest on methods for the detection of other mycotoxins has increased. This is particularly true of GCMS as demonstrated by the work carried out in this project.

Although this study concentrated primarily on the detection, quantitisation and confirmation steps of analysis this does not imply that sampling should be ignored. Any analytical result, irrespective of how accurately the determination has been

performed, is dependent on the quality of the original sample. Whether the sample is representative will be affected by factors including the number of samples taken, each sample volume and the sampling method. Information about the representative nature of the individual sample, or batch, or unit of material, as well as the relationship of those samples to the commodity as a whole, particularly with regard to national monitoring programmes will establish a broader understanding of the situation. It is important when analysing samples for mycotoxins to consider not only how representative the samples are, but also the accuracy of the analysis.

This study explored a number of techniques for the detection of mycotoxins, particularly AFB_1, in maize, groundnuts and blood. It evaluates these techniques and proposes certain methods as being suitable for ongoing development to commercial levels. The study has established that AFB_1 occurs in maize and groundnut and that it also occurs in blood from patients in the surrounding area. With time constraints and sample sizes available it was not possible to establish a definite link between the ingested mycotoxin and the occurrence of mycotoxicosis and further extensive sampling would be required to correlate this data irrefutably. This study, in isolating a suitable technique for the preparation of AFB_1-lysine adduct, has laid the firm foundation for future development and research.

Mycotoxins represent an extreme case in terms of sampling difficulty and the results of this study should be viewed in the context of regional differences such as variations in contamination levels in samples from different regions, seasonal variations and year-to-year differences. Surveys undertaken in 1993 may not necessarily be extrapolated to subsequent years, where climatic and other environmental factors that are frequently changing, are important in the formation of mycotoxins.

There are particular problems which occur with sampling for mycotoxin analysis. Aflatoxin contamination is frequently highly irregular within a batch of material and this distribution must be taken into account. A popular method used by Gilbert (1993) for analysing food combines all the samples and then analyses the combination for the presence of the specific contaminants. There are a number of

attractions to this approach in that it reduces the number of samples necessary to analyse and provides average data for a range of foods. The drawback, however, is that, if a single sample is contaminated, it may become very diluted and due to sensitivity problems, measurement of contamination in the group as a whole may be difficult.

A second potential source of error in any analytical procedure lies in the preparation and use of standards, the concentration and purity of which must be determined and checked frequently. A drawback in the development of analytical methods for mycotoxin detection is the cost and availability of the standards. This is exemplified by the experience of this study which determined that commercially available FB_1 and AFB_1 can be obtained in South Africa from one supplier only and, for 10 mg of each, the combined cost is in the region of R1000,00. Procurement of the AFB_1 adduct presents an even greater problem as this is not available off the shelf in South Africa. The adduct for use in this study was obtained from France after direct contact with Dr.C.P. Wild of the International Agency for Research on Cancer, Lyon. These circumstances then provided the motivation for the study to investigate techniques by which both AFB_1 and AFB_1-lysine adduct could be produced locally. The locally produced adduct could be used in screening procedures to determine human exposure to mycotoxins, particularly among HCC patients.

The results of this study show that "clean-up" of both cereals and blood are important steps leading to the final qualification and quantification by GCMS and HPLC of free and bound AFB_1. Significant progress was made in the evaluation of "clean-up" methods involving dialysis, GPC and immunoaffinity columns. Each of the techniques used in the "clean-up" of food has shortcomings, the dialysis method is labour intensive and exposes the compound of interest to possible undesirable changes during dialysis, while the second, GPC, requires the use of large volumes of volatile solvent which can be hazardous. Immunoassay procedures for the detection of various mycotoxins in biological fluids and tissue have attracted a great deal of interest world-wide. The technique is simple, rugged and specific where limited amounts of sample are available and where extremely low concentrations of toxins in large numbers of

samples are to be screened. This study made use of immunoaffinity columns packed with monoclonal antibodies to "clean-up" the AFB_1 lysine adduct before quantification by HPLC.

The technique of two-dimensional TLC is a powerful one, given the wide range of solvents available. Its major drawback is, however, that it is time-consuming, as only one sample can be applied to each TLC plate. An important feature of the analytical method of HPLC is confirmation of the identity of the compound measured. This is very important in routine screening. The HPLC technique can be easily adapted to confirmation procedure because it is generally non-destructive and because different detectors may be connected in series with little loss in peak resolution or sensitivity. A number of HPLC methods for mycotoxins other than the aflatoxins include a mass spectrometric confirmation step. The trichothecenes, for example, are difficult to detect and quantitate by either TLC or HPLC, because they lack chromogenic functional groups and are, therefore, more amenable to analysis by gas chromatography coupled with a mass spectrophotometer for confirmation of identity as applied in this study.

Maize forms the staple diet of many rural households in South Africa where subsistence farming is practised. Although ingestion of mycotoxin contaminated maize is a problem in rural South Africa, contamination occurrence has been reduced considerably (McLaren, R., pers. comm.[4]) as a result of storage training programmes. It is interesting to note that in India, neem leaves are placed in storage bins to inhibit bacterial and fungal contamination (Sharma, S., pers. comm.[8]). Prevention, is obviously better than cure, but rural maize growers do not have access to the more sophisticated methods of fungal control, resistant cereal strains and sophisticated screening methods. Researchers, therefore, continue to expended effort in establishing screening and exclusion methods aimed at eliminating mycotoxins and their adducts from commodities.

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Recent political developments in South Africa have focused on the upliftment of rural communities, while the economic climate, with an ever depreciating currency favours import substitution and exports. The local production of AFB_1-lysine adduct, as identified by the work in this study, is not only compatible with current political and economic thinking, but also offers real and exciting commercial prospects. This study, has clearly targeted AFB_1-lysine adduct production as a direction for future research and development by the University of Natal Medical School.

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APPENDICES

APPENDIX 1.0 :   **RINGER'S SOLUTION FOR IDENTIFICATION OF FUNGI**

Na Cl	2,250 g
KCl	0,105 g
CaCl2	0,060 g
sodium bicarbonate	0,050 g
sodium lauryl sulphate	0,100 g
distilled water	1,000 l

A volume of 9,9 ml of the above solution was added to bottles and autoclaved at 15 ilbs for 15 minutes.

OHIO Agricultural Experimental Station Agar (OAES)

glucose (dextrose)	5,000 g
yeast extract	2,000 g
sodium nitrate	1,000 g
magnesium sulphate	0,500 g
potassium phosphate	1,000 g
Oxoid oxbile dessicated	
Code L50	1,000 g
sodium propionate	1,000 g
Oxoid agar No3 Code L13	20,000 g
distilled water	1,000 l

The pH was adjusted to 6 and 200 ml of the solution dispensed into bottles and autoclave into 5 plates (0,-2,-4,-6 and control).

A volume of 1 ml of both sterile streptomycin sulphate and chloroamphenicol was added into each half full bottle, and 2 ml into the full bottle and the 0,5% solution made up. The bottles were autoclaved and the solution filtered into sterile bottles through sterile filter using 0,45. A volume of 1 ml of Ringer's was added to each plate.

APPENDIX 2.0: SOLVENT SYSTEMS FOR SEPARATING SPECIFIC MYCOTOXINS ON THIN LAYER CHROMATOGRAPHY

MYCOTOXIN	DIMENSION 1	R_n VALUE	DIMENSION 2	R_a VALUE
AFLATOXIN B$_1$	CEI	0,66	TEF	0,41
AFLATOXIN B$_2$	CEI	0,64	TEF	0,26
AFLATOXIN G$_1$	CEI	0,6	TEF	0,24
AFLATOXIN G$_2$	CEI	0,55	TEF	0,18
CITRININ	CEI	0,08	TEF	0,39
DEOXYNIVALENOL	CEI	0,13	TEF	0,2
DIACETOXYSCRIPENOL	CEI	0,59	TEF	0,39
FUMONISIN B$_1$	CMA	0,25	-	-
HT-2 TOXIN	CEI	0,14	TEF	0,14
KOJIC ACID	CEI	0,09	TEF	0,13
MONILIFORMIN	CM	0,51	BWA	0,61
OCHRATOXIN A	CEI	0,06	TEF	0,67
PENICILLIC ACID	CEI	0,39	TEF	0,47
STERIGMATOCYSTIN	CtE	0,35	ChE	0,2
T-2 Toxin	CEI	0,63	TEF	0,44
ZEARLENONE	CtE	0,1	ChE	0,75

APPENDIX 3.0: **SOLVENT SYSTEMS USED FOR TLC PLATE DEVELOPMENT**

1) CEI   = Chloroform-Ethyl Acetate-Propano-2-ol (90:5:5 v/v/v)
2) TEF   = Toluene-Ethyl Acetate-Formic Acid (6:3:1 v/v/v)
3) TEF2 = Toluene-Ethyl Acetate-Formic Acid (5:4:1 v/v/v)
4) EMW   = Ether-Methanol-Water (188-9-3 v/v/v)
5) CA   = Chloroform-Acetone (9:1 v/v)
6) CtE   = Carbon Tetrachlordie-Ethanol (98:2 v/v)
7) ChE   = Cyclohexane-Ether (3:1 v/v)
8) BE   = Benzene-Ethanol (95:5 v/v)
9) CM   = Chloroform-Methanol (95:5 v/v)
10) CMA   = Chloroform-Methanol-Acetic Acid (12:5:3 v/v/v)
11) CM2   = Chloroform-Methanol (3:2 v/v)
12) BWA   = Butanol-water-Acetic Acid (12:5:3 v/v/v)
13) HPB   = Hexane-Petroleum Ether (60/80)-Benzene-Chloroform-Acetone-
            Acetonitrile-Acetic Acid (1:1:1;2:1:1:3)
14) CAI   = Chloroform-Acetone-Propan-2-ol (85-10-5 v/v/v)
15) CM5   = Chloroform-Methanol (5:1 v/v)
16) CM10 = Chloroform-Methanol (9:1 v/v)
17) TE   = Toluene-Ethyl Acetate (3:1 v/v)

Thin layer plates developed in a typical analysis

Plate solvents fraction toxin detected

Plate	solvents	fraction	toxin detected
1	CEI/TEF	Neutral	Aflatoxins, Trihothecenes
2	CEI/TEF	Acidic	Cyclopiazonic, Penicillic, Kojic & Tenuazonic Acids, Ochratoxins & Citrinin
3	CA/TEF2	Neutral	Confirm aflatoxins, Patulin
4	CtE/ChE	Neutral	Sterigmatocystin, Zearalenone
5	CM2/BWA	M Fraction	Moniliformin, Fumonisin

APPENDIX 4.0:   SPECIFIC SPRAY REAGENTS FOR THE DETECTION OF MYCOTOXINS

MYCOTOXIN	VISUALISATION	TREATMENT	RESULT
Aflatoxin B_1	UV	-	Blue fluorescence
Aflatoxin B_2	UV	-	Blue fluorescence
Aflatoxin G_1	UV	-	Green fluorescence
Aflatoxin G_2	UV	-	Green fluorescence
Citrinin	UV	-	Yellow fluorescence
Deoxynivalenol	Sulphuric acid	Heat	Brown/charred visible and blue fluorescence
Fumonisin B_1	Anisaldehyde	Heat	Purple/brown visible and pink fluorescence
HT-2-toxin	Sulphuric acid	Heat	Blue fluorescence
Kojic Acid	UV Paul'y	- -	Adsorbent Red/orange visible
Moniliformin	UV DNPH	- Heat	Adsorbent Brown visible
Ochratoxin A	UV	-	Blue/green fluorescence
Penicillic acid	NH_3/UV Pauly's	- -	Blue fluorescence Yellow visible
Sterigmatocystin	UV Aluminium chloride	- Heat	Yellow/green fluorescence
T-2 Toxin	Sulphuric acid	Heat	Yellow/green fluorescence
Zearalenone	UV Aluminium chloride	- Heat	Blue fluorescence intensifies

APPENDIX 5.0:   COLOUR CODING USED FOR TLC SPOTS

A	=	Absorbent
B	=	Blue
Bk	=	Black
Bro	=	Brown
D	=	Dark
F	=	Fluorescent
G	=	Green
Int	=	Intensifies
L	=	Light
NF	=	Non-descript fluorescence
O	=	Orange
P	=	Purple
Pk	=	Pink
R	=	Red
V	=	Visible
UV	=	Ultra Violet
W	=	White
Y	=	Yellow

Aflatoxin	Formulae	Mol.Wt	Max(nm) (e...)
B$_1$	C$_{17}$H$_{12}$O$_6$	312	223(20800)266(12960)363(20150)
B$_2$	C$_{17}$H$_{14}$O$_6$	314	223(18120)266(12320)363(2310)
B$_{2a}$	C$_{17}$H$_{14}$O$_7$	330	228(17600)256(10300)363(20400)
M$_1$	C$_{17}$H$_{12}$O$_7$	328	225(2100)265(1100)360(21000)
M$_2$	C$_{17}$H$_{14}$O$_7$	330	222(19800)264(10000)358(21300)
A-toxicol	C$_{17}$H$_{14}$O$_6$	314	254(6790)261(10800)332(14100)
G$_1$	C$_{17}$H$_{12}$O$_7$	328	226(15730)243(11070)264(10670)363(17760)
G$_2$	C$_{17}$H$_{14}$O$_7$	330	220(21090)245(12400)265(100200)363(20030)
G$_{2a}$	C$_{17}$H$_{14}$O$_8$	346	223(18600)242(10100)262(8700)365(18000)
GM$_1$	C$_{17}$H$_{12}$O$_8$	344	235(21200)262(16300)358(12000)
GM$_2$	C$_{17}$H$_{14}$O$_8$	346	230(20000)263(14260)357(13020)
B$_3$	C$_{16}$H$_{14}$O$_6$	302	229(10000)253(7300) 262(7550) 326(9350)
Altenuene	C$_{15}$H$_{16}$O$_6$	292	215(35000)257(14000)253(6600)
Alternariol	C$_{14}$H$_{10}$O$_5$	258	218 258(3800) 302(330)
Citrinin	C$_{13}$H$_{14}$O$_5$	250	220(22300)253(8300)319(4700)
Cyclopiazonic acid	C$_{20}$H$_{20}$O$_3$N$_2$	336	225(39810)253(16595)284(2147)
Deoxynivalenol	C$_{15}$H$_{20}$O$_6$	296	218(4500)
Diacetoxyscirpenol	C$_{19}$H$_{26}$O$_7$	366	
Fusarenon X	C$_{17}$H$_2$O$_8$	354	
HT-2 Toxin	C$_2$H$_{32}$O$_8$	424	
Kojic acid	C$_6$H$_6$O$_4$	142	268(8080)
Moniliformin	C$_4$H$_2$O$_3$	98	229(19100)260(5600)
Neosolaniol	C$_{19}$H$_{26}$O$_8$	386	
Ochratoxin	C$_{20}$H$_{18}$O$_6$NCl	403	215(36800)333(6400)
Patulin	C$_7$H$_6$O$_4$	154	276(16600)
Penicillic acid	C$_8$H$_{10}$O$_4$	170	221(10500)
Rubratoxin	C$_{26}$H$_{30}$O$_{11}$	518	251(9700)
Sterigmatocystin	C$_{18}$H$_{12}$O$_6$	324	208(19000)235(24500)249(27500) 329(13100)
T-2 Tetraol	C$_{15}$H$_{22}$O$_6$	298	
T-2 Toxin	C$_{24}$H$_{34}$O$_9$	466	
Tenuazonic acid	C$_{10}$H$_{15}$O$_3$N	197	217(5248)277(13489)
Zearalcone	C$_{18}$H$_2$O$_5$	318	236(28700)274(13909)316(6020)

APPENDIX 7.0:   **MYCOTOXIN CONTAMINATION IN KANGWANE MAIZE SAMPLES 1993**

Sample	January	February	March	June	July	August	October	November
1	U	-	U	-	U	-	-	U
2	-	U	-	-	U	-	-	-
	-							
3	-	U	-	-	U	-	-	U
4	-	U	-	-	U	U	U	U
	-			-				
5	U	-	U	U	-	-	-	-
6	U	U	U	-	-	-	-	U
7	U	U	-	U	-	-	-	U
8	U	U	U	U	U	-	-	U
9	-	U	U	-	-	-	-	-
10	U	-	-	-	U	U	-	U
11	U	-	U	-	U	-	-	U
				-				
12	-	-	-	-	U	-	-	-
13	-	U	-	-	U	-	U	U
				-				
14	-	-	-	-	U	-	-	-
				-				
15	-	U	-	-	U	-	-	U
	-							
16	-	-	U	-	U	-	-	-
				-				
17	U	U	U	-	U	-	U	-
				-				
18	U	U	-	-	U	-	-	-
				-				
19	-	U	-	-	U	-	-	-
20	U	U	U	U	U	-	U	-

-indicates absence of fluorescence
U denotes unavailable

APPENDIX 8.0:   MYCOTOXIN CONTAMINATION IN KANGWANE GROUNDNUTS, DECEMBER 1993

Sample	Toxin
1	AFG_1, AFG_2
2	-
3	AFB_1, AFB_2, AFG_1
4	AFB_2, AFG_1, AFG_2, AFM_1, $_{AFM}2$
5	AFB_1, AFG_1, AFG_2
6	AFG_2, AFB_1, AFB_2
7	low AFB_1
8	AFB_1, AFB_2, AFG_1, AFG_2
9	AFG_1, AFG_2, AFM_1
10	AFB_1, AFB_2, AFG_1, AFG_2
11	-
12	-
13	-
14	AFB_1, AFB_2, AFG_1, AFG_2
15	AFB_1
16	AFB_1
17	AFB_1, AFB_2, AFG_2
18	-
19	-
20	AFB_1, AFB_2, AFG_1, AFG_2
21	AFB_1, AFB_2
22	AFB_1
23	-
24	-
25	AFB_1, AFB_2
26	-
27	AFB_1, AFB_2
28	-
29	AFB_1, AFB_2, AFM_1
30	AFB_2, AFG_1, AFG_1

-indicates absence of fluorescence.

APPENDIX 9.0:   **RANDOM ECO-LINK GROUNDNUT SAMPLES, 1994**

Sample	February	March	April	May
1	AFG_2	U	U	U
2	-	U	U	U
3	-	U	U	U
4	-	U	U	U
5	-	U	U	U
6	U	-	U	U
7	U	-	U	U
8	U	AFG_1, AFG_2	U	U
9	U	AFG_1, AFG_2	U	U
10	U	-	U	U
11	U	-	U	U
12	U	-	U	U
13	U	AFG_1, AFG_2	U	U
14	U	? AFG_2	U	U
15	U	-	U	U
16	U	-	U	U
17	U	U	U	U
18	U	-	U	U
19	U	-	U	U
20	U	-	U	U
21	U	-	U	U
22	U	U	U	-
23	U	U	U	-
24	U	U	U	-
25	U	U	U	-
26	U	U	U	-
27	U	U	U	-
28	U	U	U	-
29	U	U	U	-

-indicates absence of fluorescence.
U denotes unavailable

APPENDIX 10.0:   SUMMARY OF KANGWANE GROUNDNUTS SAMPLES SCREENED DURING 1994 (TLC)

Batch Data	PF	BF	G\GF	No F	Aflatoxin present	Fluor + toxin	PF + toxin	G\GF + toxin	ASP + toxin	Penn + toxin	Fus + toxin	A. flavus	A. niger	Fusarium	Penn	Other fungus
Fresh (wet) harvested 29/3/94-11/4/94	13	0	6	1	4	7	4	3	4	4	1	0	3 1 1 0	0 5 1 1	0 3 1 9	3 F. sambicinum 1 F. reticulatum 7 Mucor 1 ? contaminant 1 A. spp
Batch total:15	13	0	6	1	4	7	4	3	4	4	1	8	5	7	13	13
Dry 23/2-25/2 Batch total :5	4	0	1	2	1	4	1	0	1	0	1	1 1 1 2	1 - - 1	3 - - -	- - 2 2	4 Mucor 1 A. terreus
Dry 23/2/94:2	1	0	0	1	0	2	0	0	0	0	0	1 1 - -	1 - - -	- - - -	- - - -	2 Mocur 1 Nigraspora spp
Dry 6/12/93 Batch total:23	12	7	15	2	10	2	3	8	10	5	4	2 5 3 11	1 7 1 2	5 1 4 4	0 1 4 9	2 Aspergillus Spp 1 A. terreus 23 Mucor
Dry 6/12/93 Batch total 6	4	0	4	0	2	0	1	1	2	0	1	- 2 1 3	1 1 - 3	5 - - -	- - - 4	5 Mucor 1 Aspergillus spp
Dry 1/9-20/10 Batch total:5	2	0	2	2	2	1	1	2	2	1	2	- 1 - 3	1 - - 4	2 - - 2	- 1 1 2	5 Mucor
Dry 1/9/93 Batch total:20	-	-	-	-	4	-	-	-	4	2	2	5 5 1 2	4 3 2 4	4 3 2 1	1 1 5 6	3 Aspergillus spp 7 Mucor 3 Trychophyton spp 1 Nigrospora spp 1 contaminant
Fresh (wet) harvested nuts 4/5/94-20/5/94	17	0	5	1	1	16	1	0	1	1	1	83 2 3	1 3 3	5 2 4 5	1 3 2 7	11 Mucor 2 Yeast 3 Trichophytan 1 Microsporium 1 Curvulara spp 1 Phoma
Dry total:80	32	7	22	7	20	24	76	410	20	9	11	667	41	52	52	79
Grand total: 97	45	7	28	10	24	31	80	133	24	13	12	85	46	59	65	93

F denotes fluorescence; P denotes purple; B denotes blue· G denotes green·

APPENDIX 11.0:   **WHITE BLOOD CELL EXTRACTION**

Blood was collected in LiEp (green) tubes and the full blood count (FBC) checked. The blood was diluted down 2:1 in phosphate buffered saline (PBS) pH 7,2-7,4 and this was layeredover 5 ml Histopaque (Sigma) 1,077 (Density Gradient Medium). The sample was centrifuged for 1/2 hour raising the speed gradually until 2000 RPM.

The interface = monolayer population of cells, was extracted and washed three times with Phosphate buffered saline (PBS). The final concentration of 10×10^6 was established.

APPENDIX 12.0: **REACTION OF AFLATOXIN B_{2a} WITH LYSINE AND LYSINE DERIVATIVES**

PREPARATION OF AFLATOXIN B_{2A}

Crude fungal extract was dissolved in chloroform (concentrated solution) and streaked out along the origin of a silica thin layer on a glass plate (20x20 cm). It was developed in chloroform-acetone 85:15 and bands located under UV light and marked with scribe. The silica was scraped off and washed on a sintered funnel with acetone into a flask under vacuum. The eluant was evaporated to a small volume and a sample (20 µl) spotted on TLC plate to check purity.

A fraction containing aflatoxin B_1 was dissolved in 20 ml acetone. A volume of 30 ml 2M sulphuric acid was added and stired overnight at room temperature. The mixture was extracted three times with equal volumes of chloroform which was pooled dry by passing through sodium sulphate and evaporated to a small volume under nitrogen. The mixture was streaked out on prep-TLC plates and separated as before. The AFB_2 and aflatoxin B_{2a} were isolated as above.

REACTION OF AFLATOXIN B_{2A} WITH LYSINE

The extracted AFB_{2a} was dissolved in 10 ml acetone. The absorbance was checked at 365 nm and the amount of AFB_{2a} present calculated. Equimolar amounts of lysine (free acid) were dissolved in 10 ml water and stirred overnight. The acetone was blown off with nitrogen. Three washes of chloroform were used for extraction. This was pooled and dried over sodium sulphate. The chloroform was evaporated to a small volume (500 µl) and the purity checked by 2 dimensional chromatography on cellulose plates (aluminium backed 10x10 cm) using IWA/BWA. The plates were viewed under UV and sprayed with ninhydrin.

APPENDIX 13.0:   SCANS (200-700 nm) OF FOUR MYCOTOXIN STANDARDS

APPENDIX 13.1:   SCAN (200-700 nm) OF DEOXYNIVALENOL

deoxy.mrd (200.0 - 700.0) srd
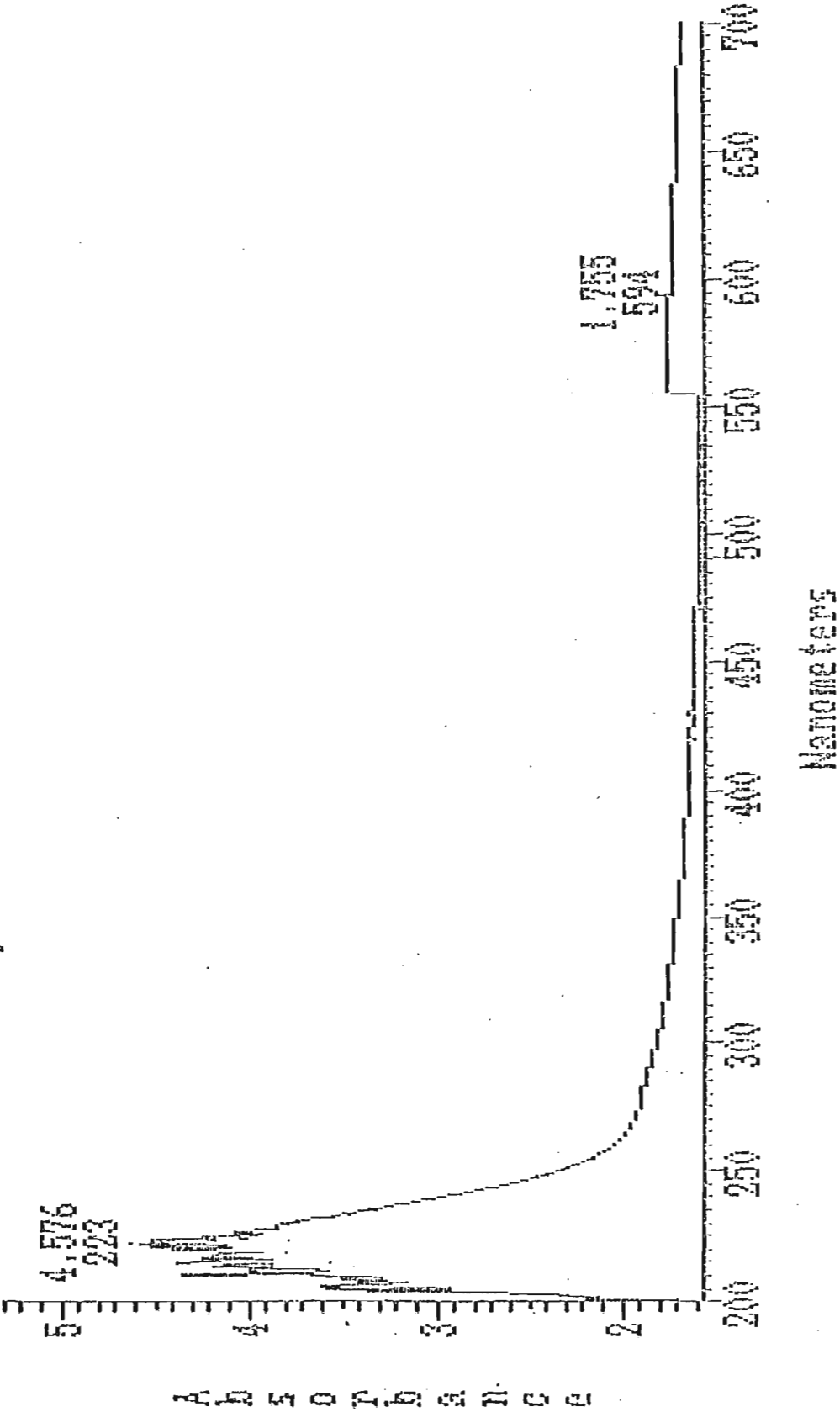
APPENDIX 13.2:   SCAN (200-700 nm) OF AFLATOXIN B₁
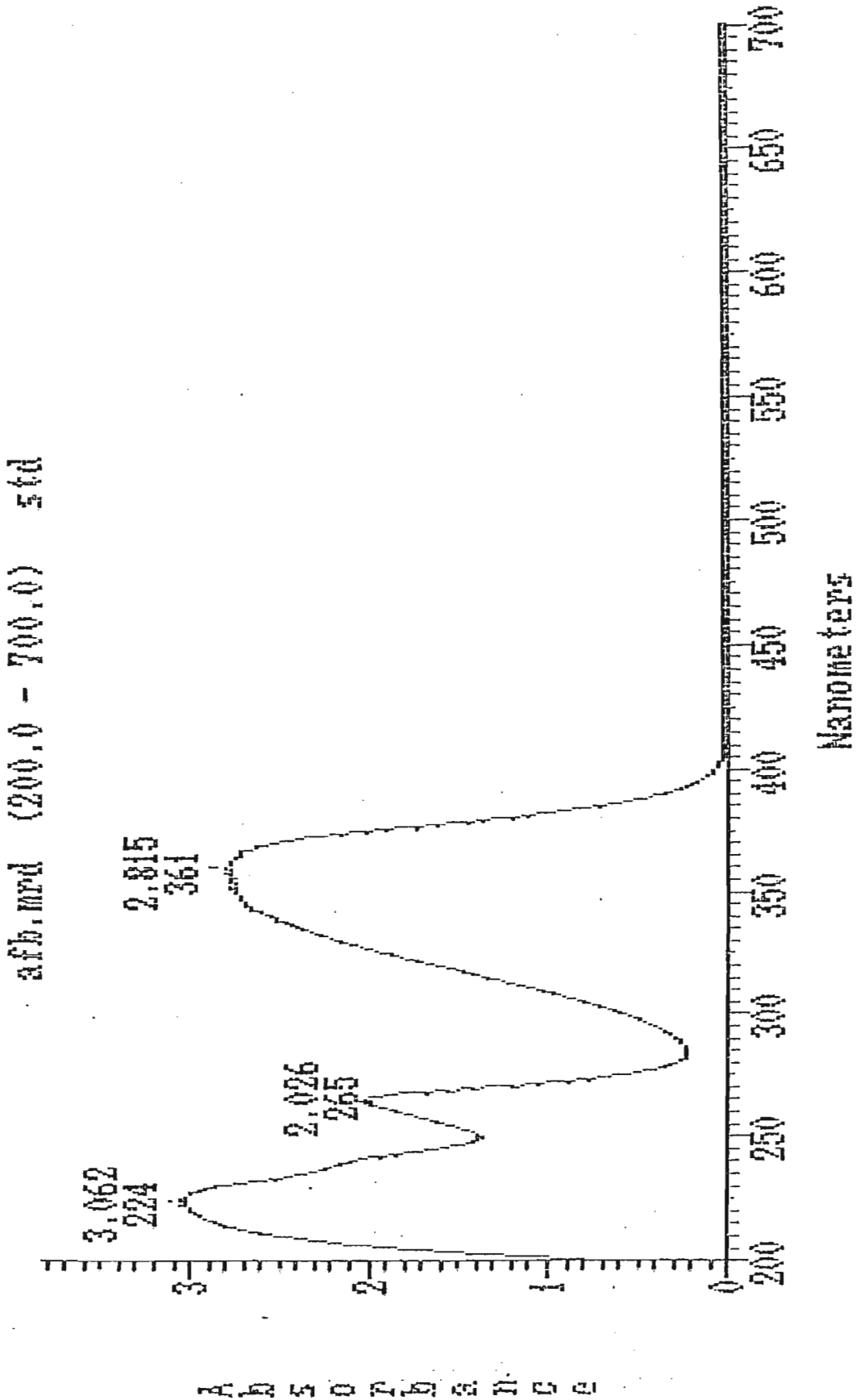
APPENDIX 13.3:   SCAN (200-700 nm) OF ZEARALENONE
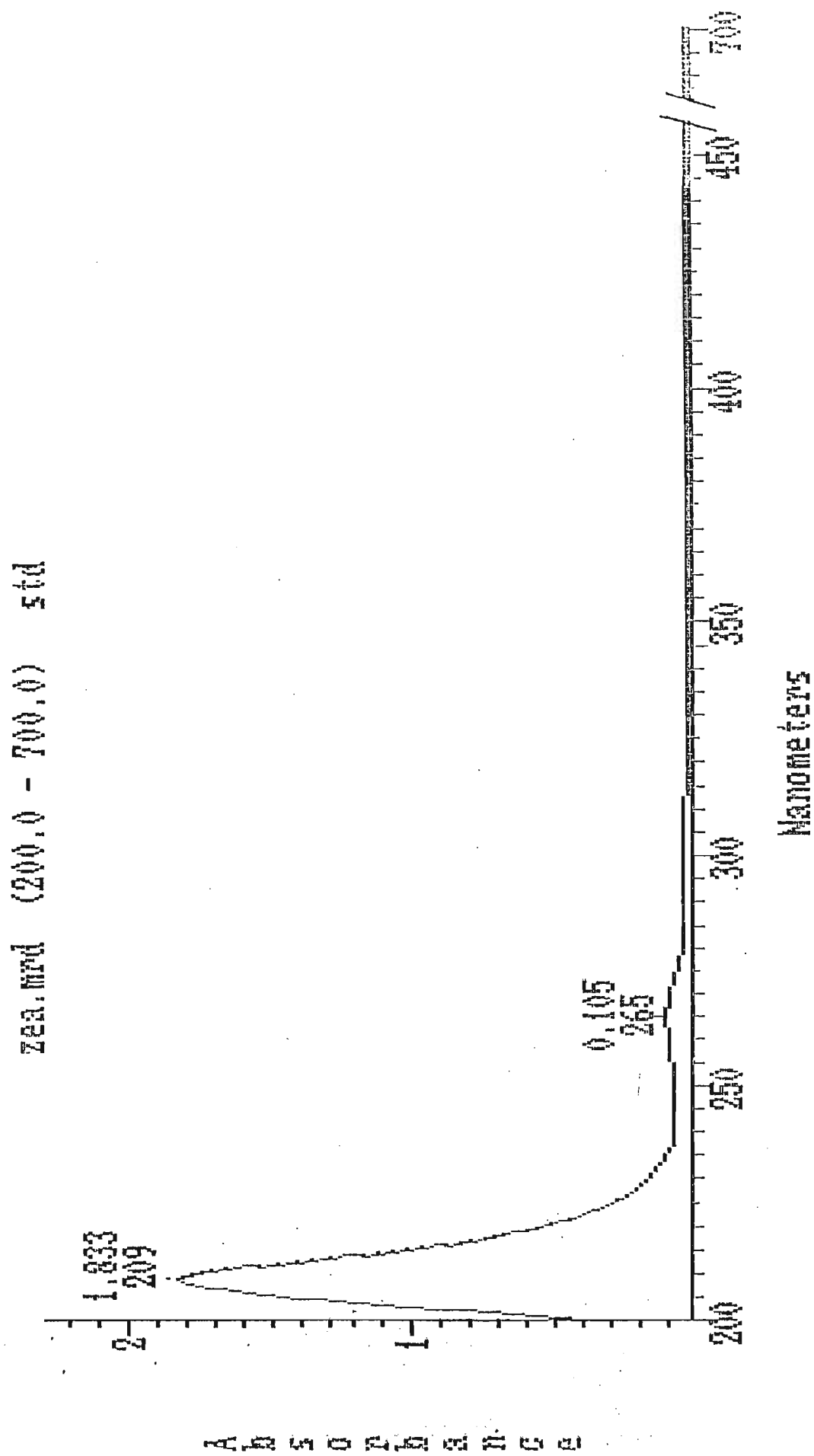
APPENDIX 13.4:   SCAN (200-700 nm) OF FUMONISIN
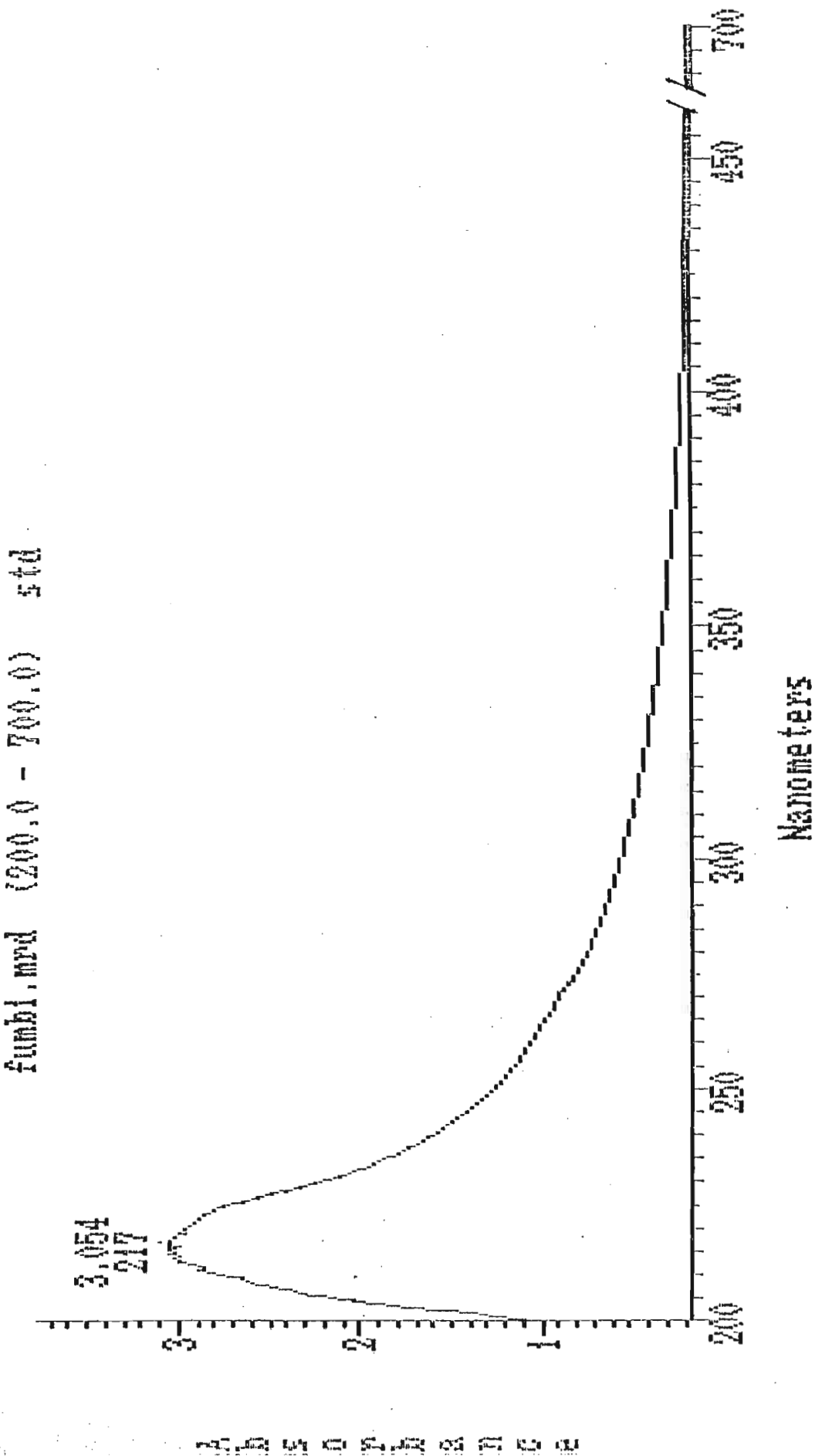
APPENDIX 13.5 :   SCAN (200-700 nm) OF METHANOL (BLANK)

(200.0 – 700.0)

Nanometers
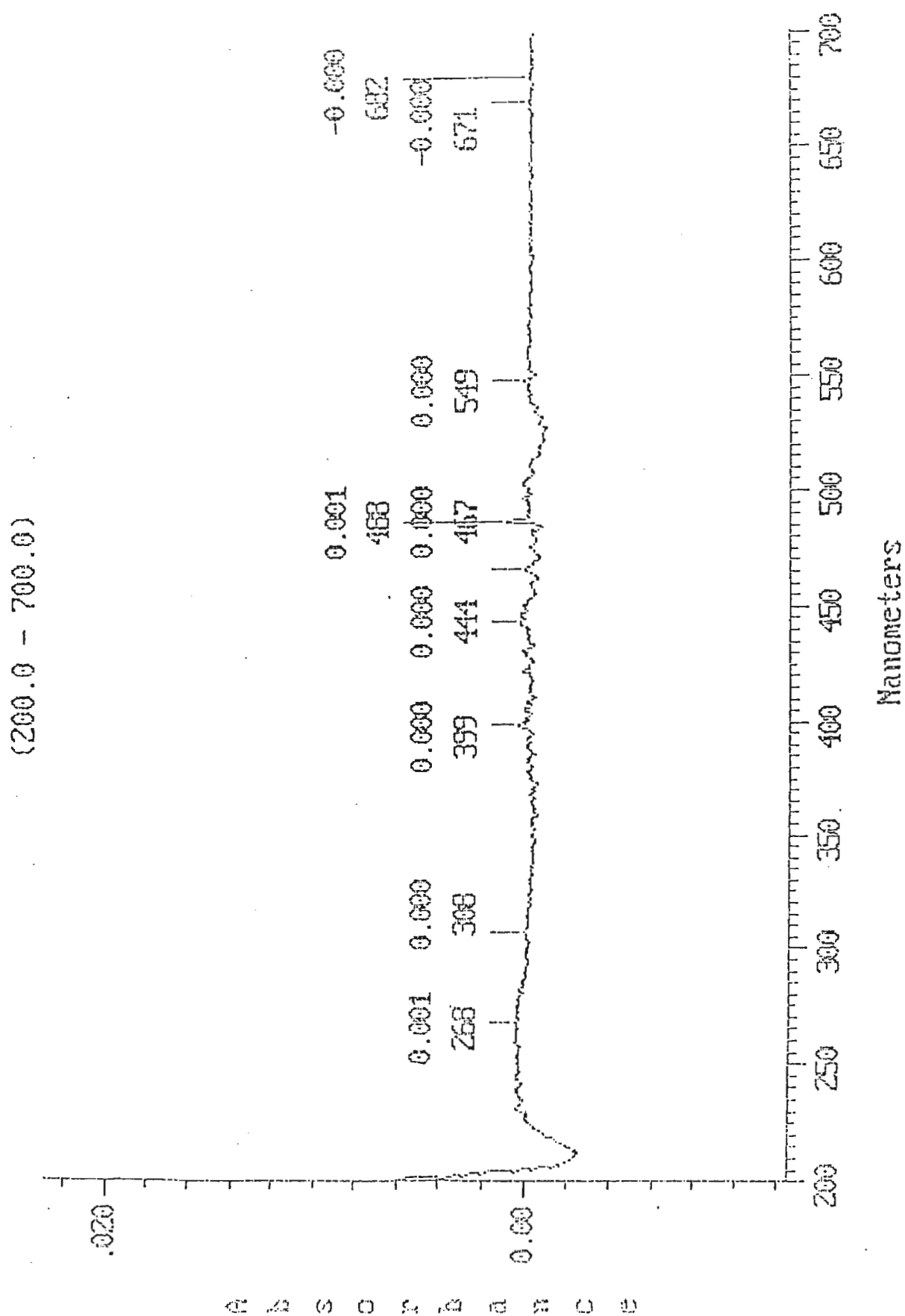
APPENDIX 14.0:   CHROMATOGRAPHS OF MYCOTOXIN STANDARDS DERIVATISED BY BSA AND ANALYSED BY GCMS

APPENDIX 14.1:   CHROMATOGRAPH OF T2-TETRIOL
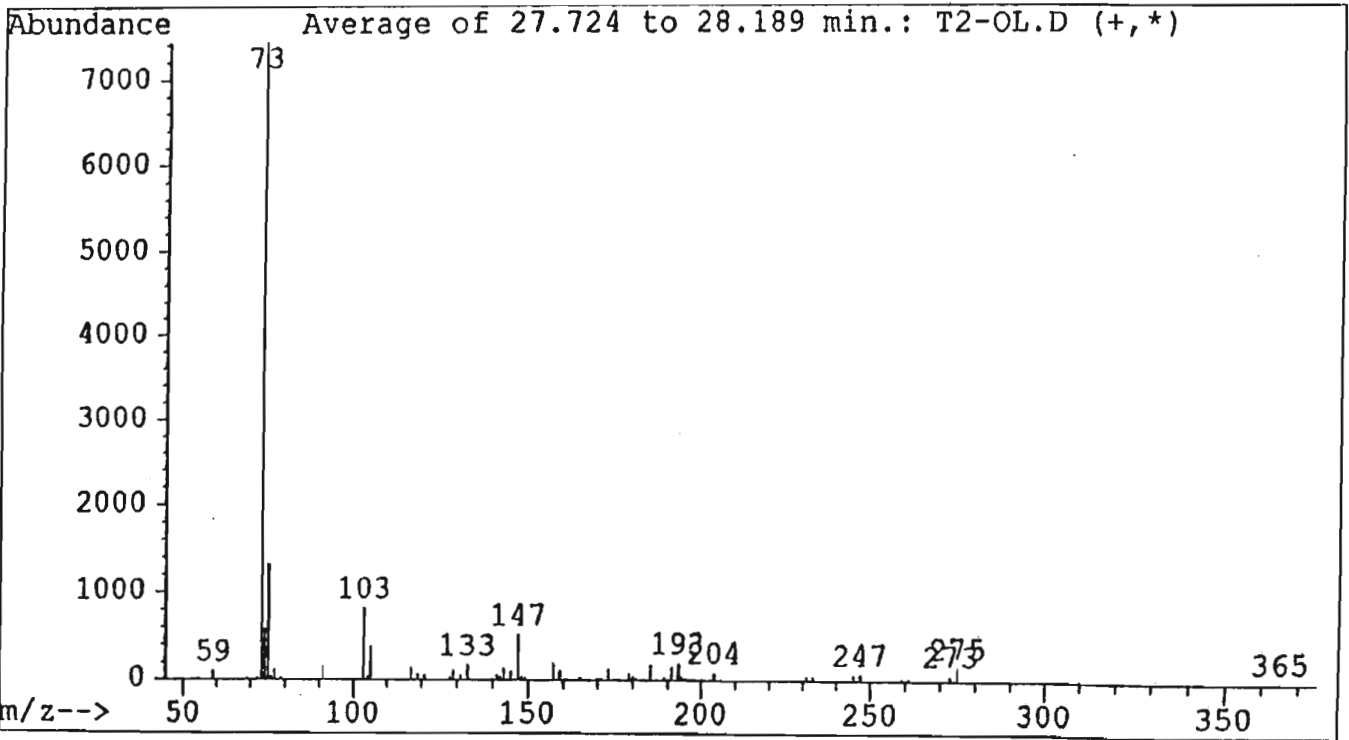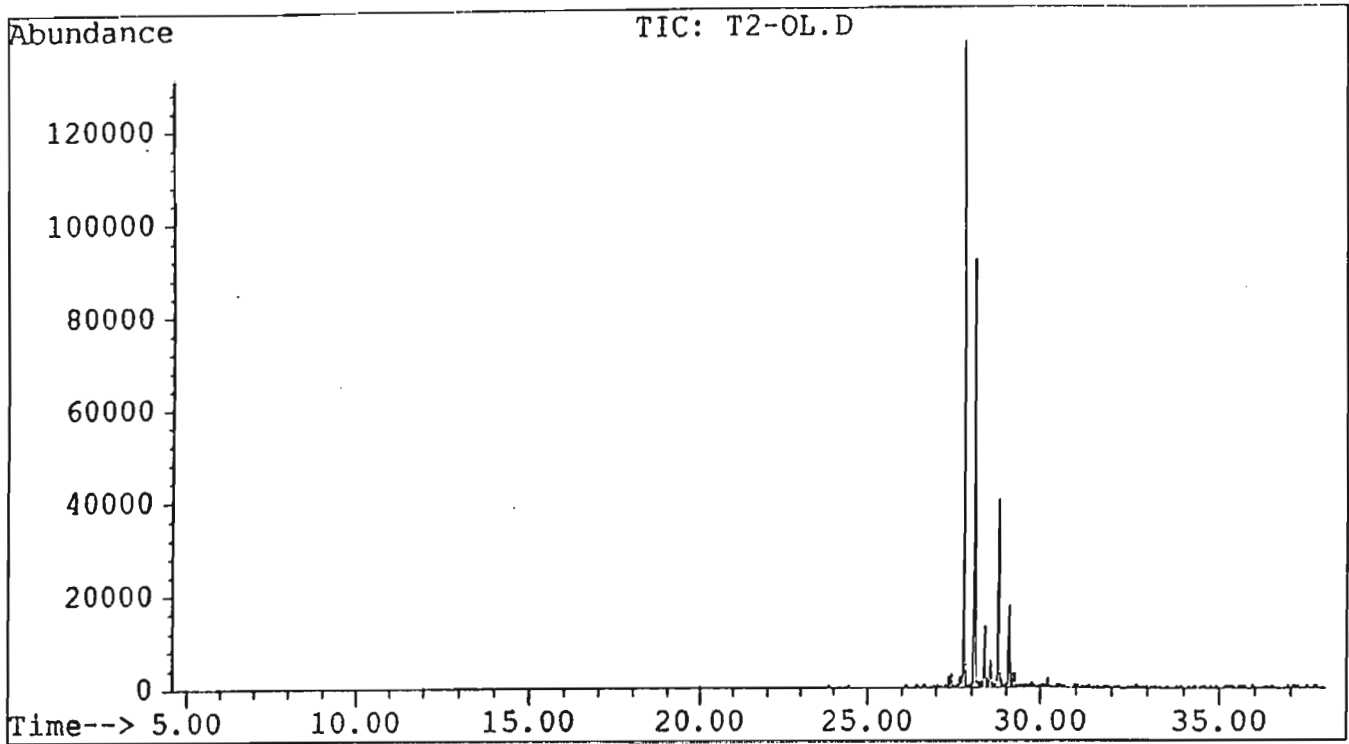
APPENDIX 14.2:   **CHROMATOGRAPH OF TETRA-ACETATE**
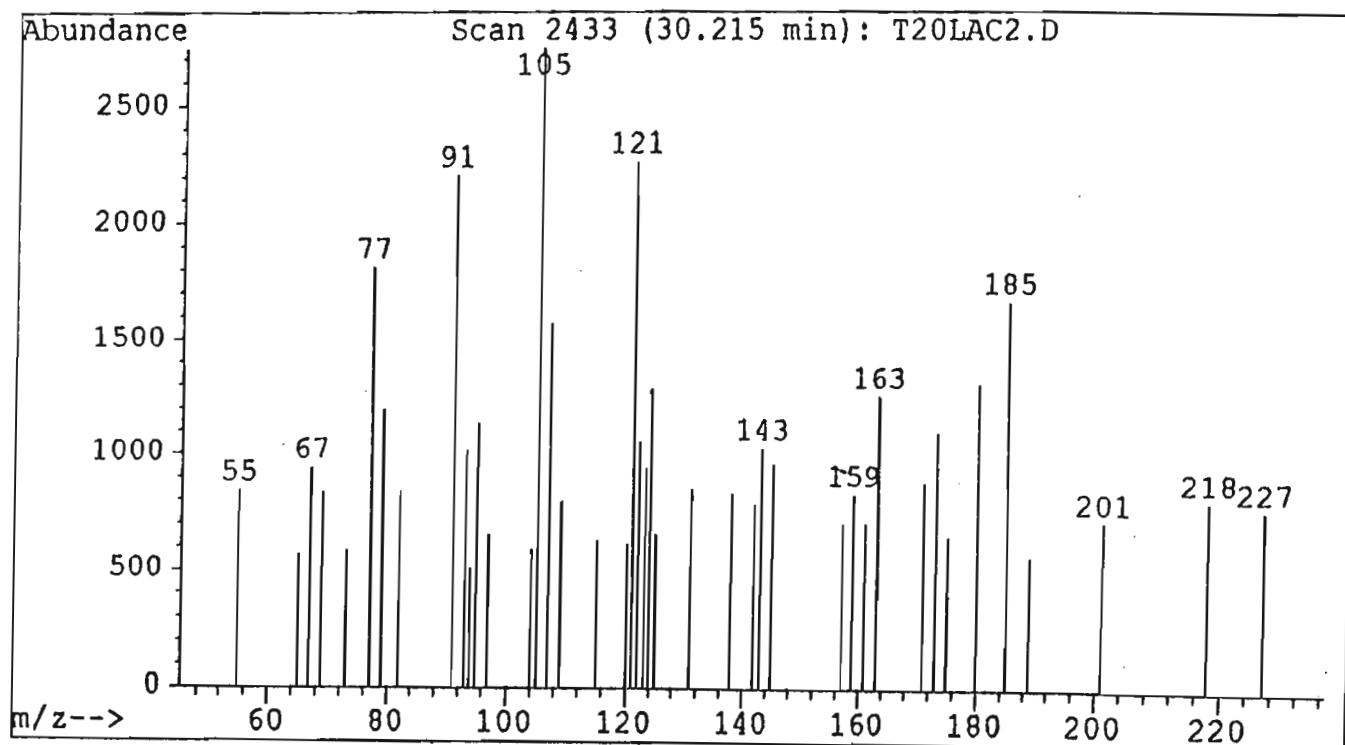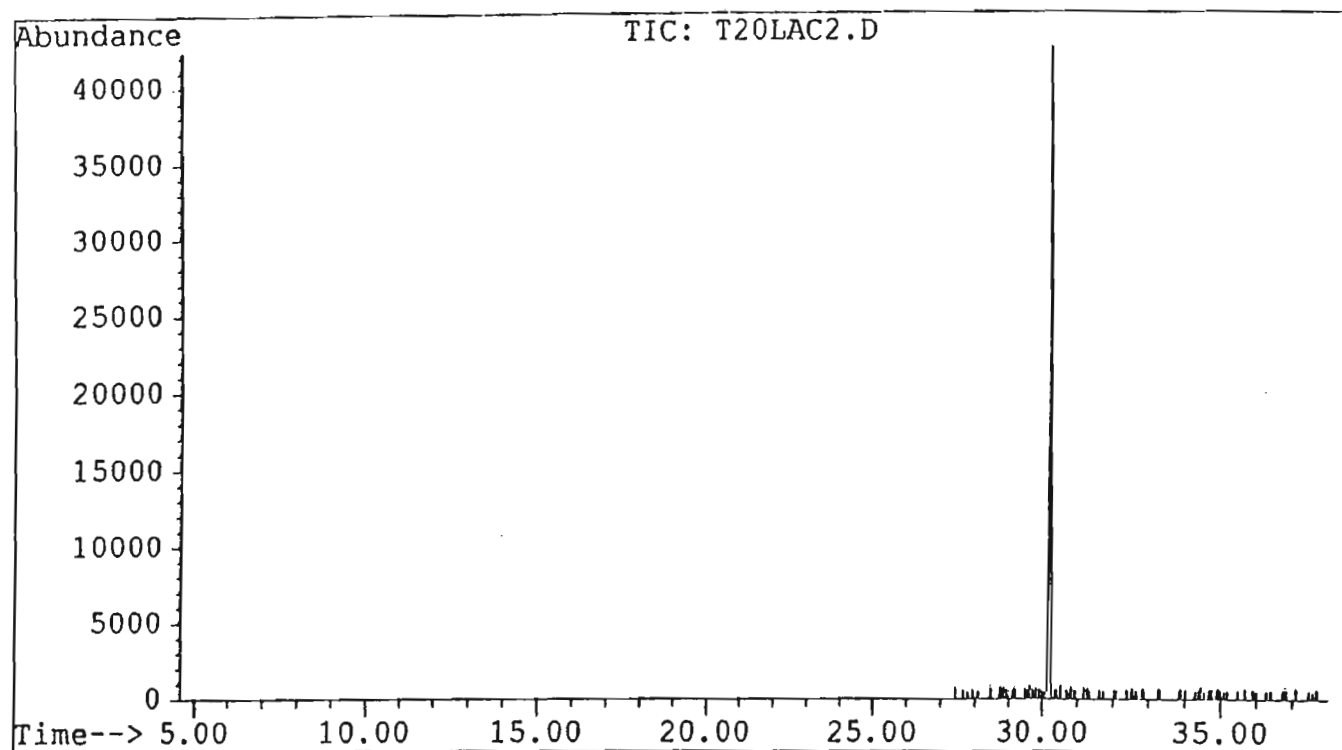
APPENDIX 14.3:   **CHROMATOGRAPH OF DIACETOXYSCIRPENOL**
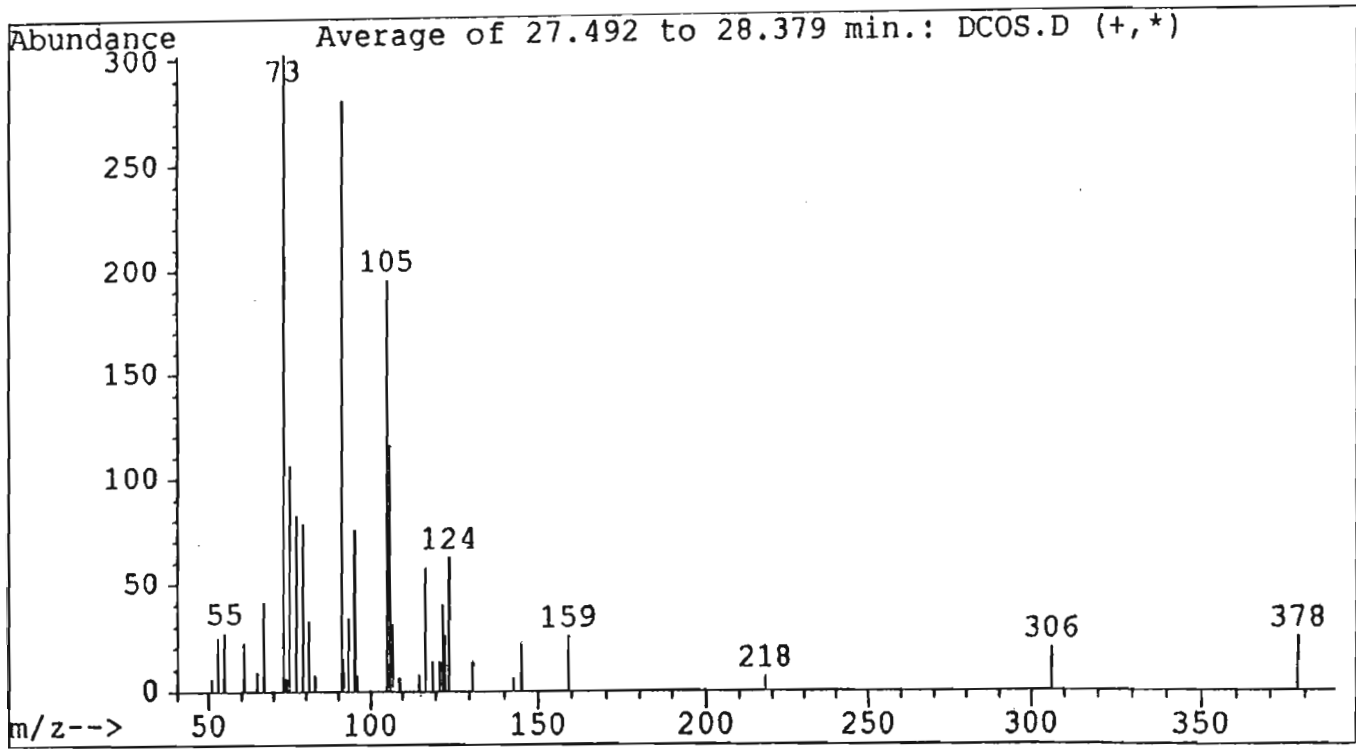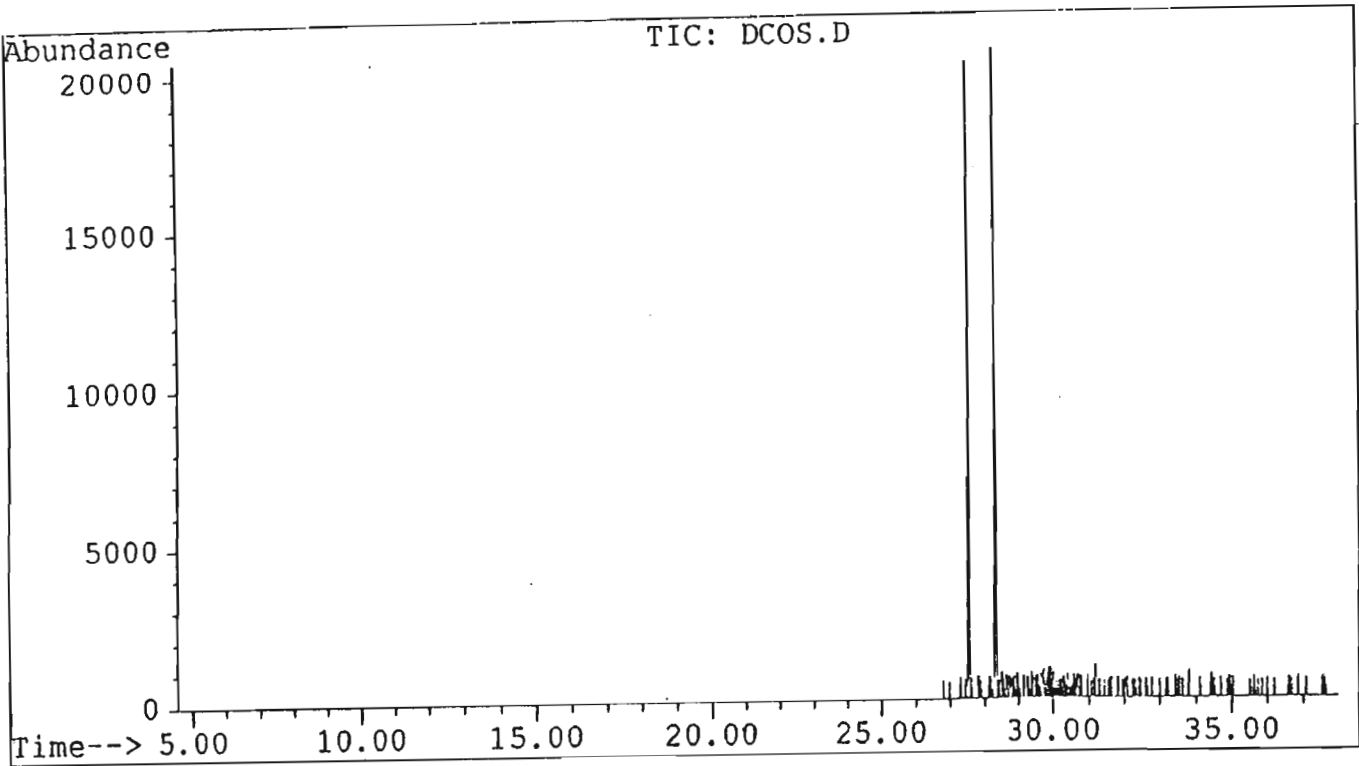
APPENDIX 14.4:   CHROMATOGRAPH OF T2-TETRAOL
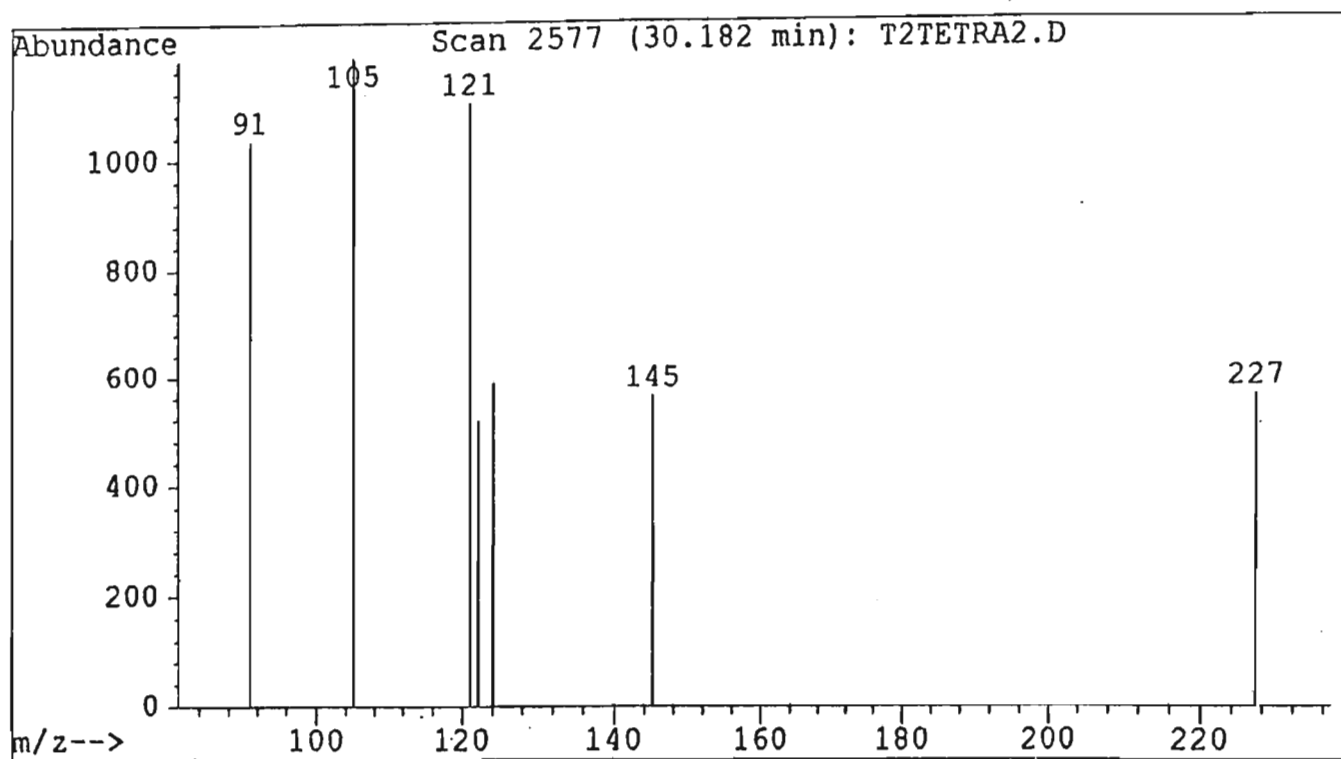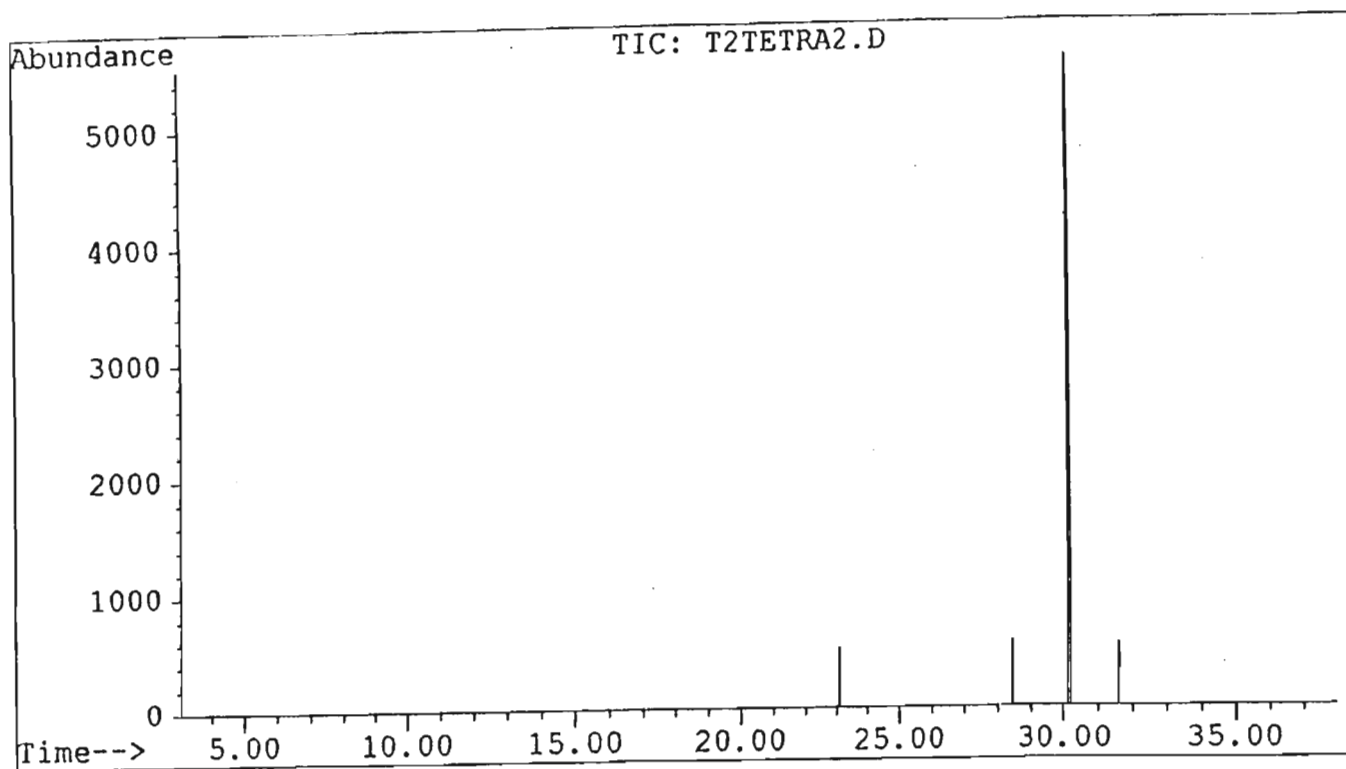
APPENDIX 14.5:   CHROMATOGRAPH OF NEOSOLANIOL
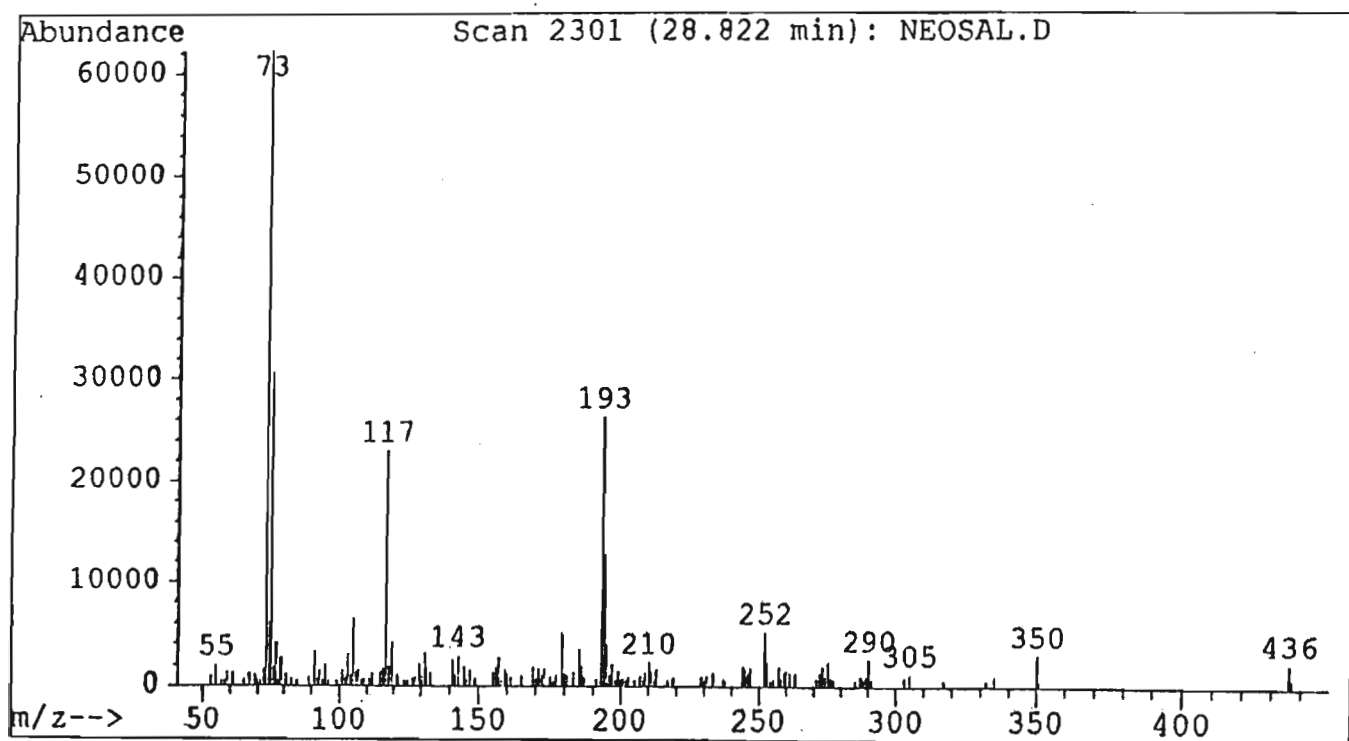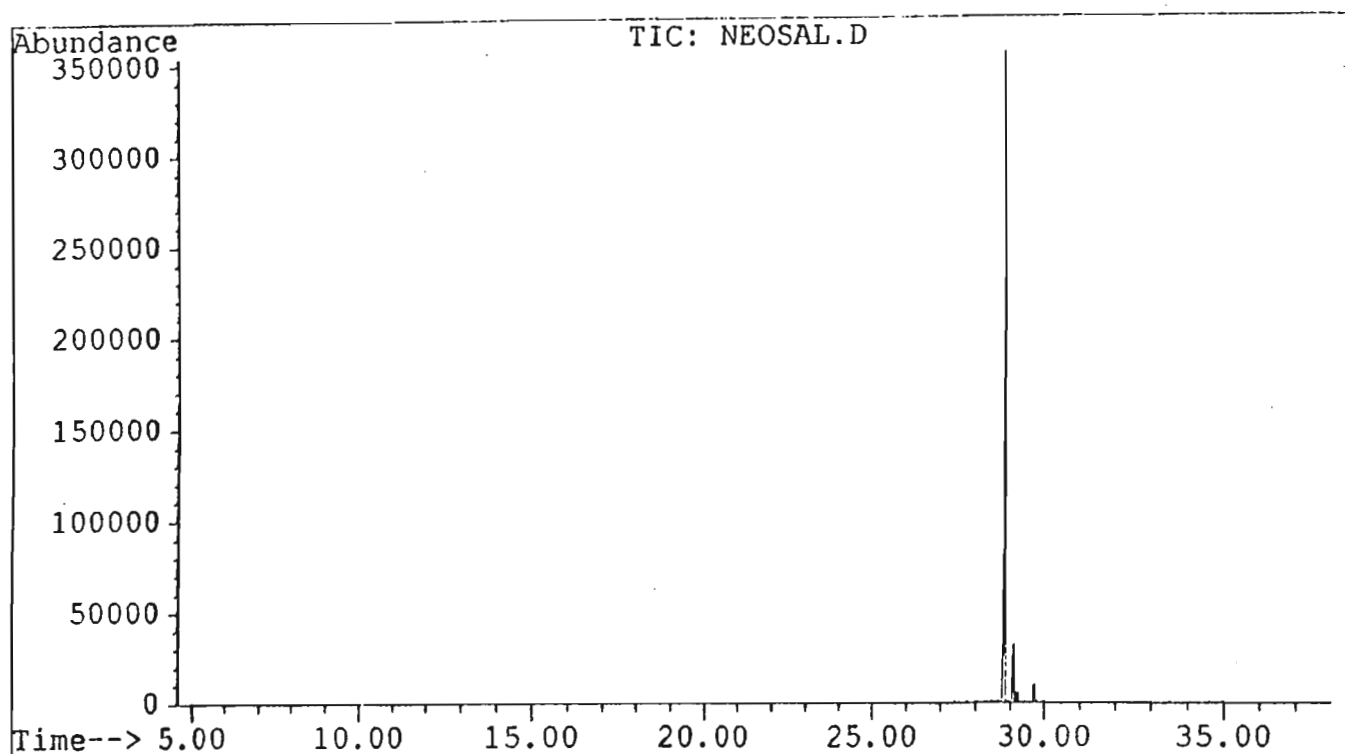
APPENDIX 14.6:   **CHROMATOGRAPH OF ACETYLDIACETOXYSCIRPENOL**

TIC: ADS.D

Scan 2263 (28.420 min): ADS.D
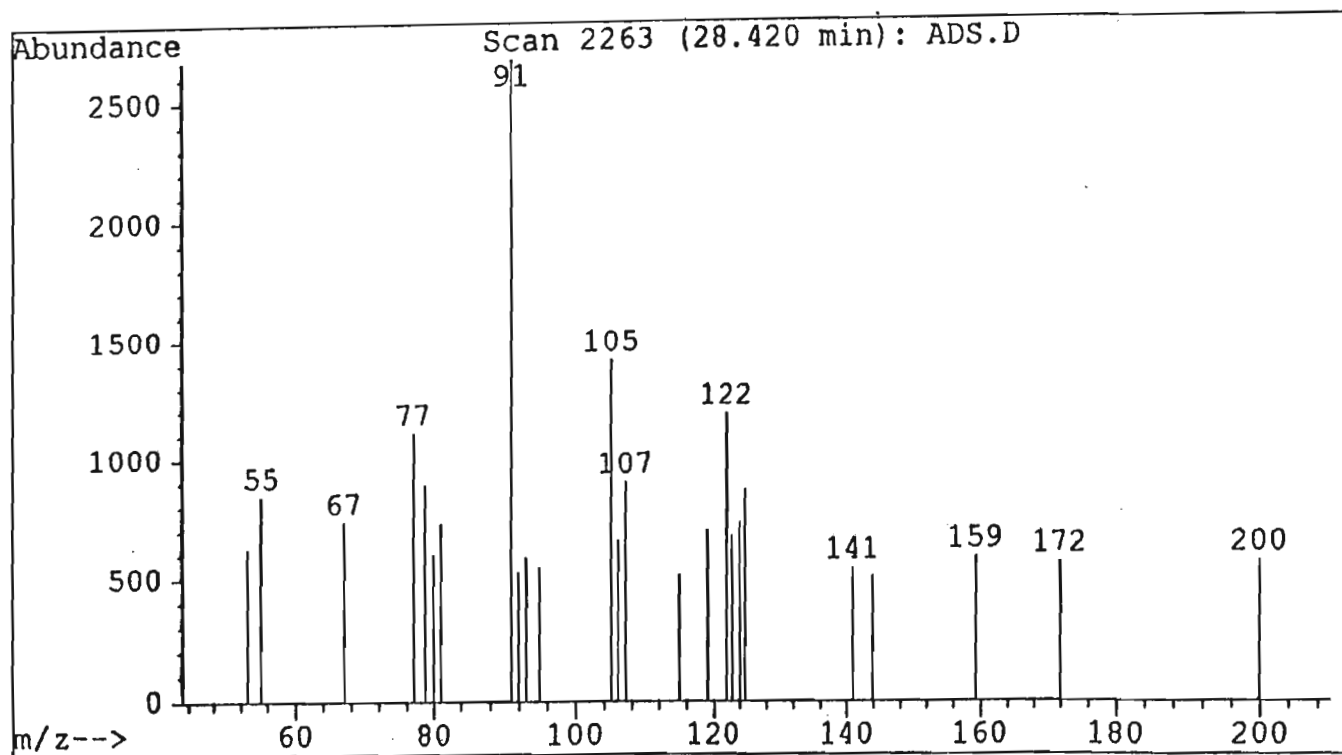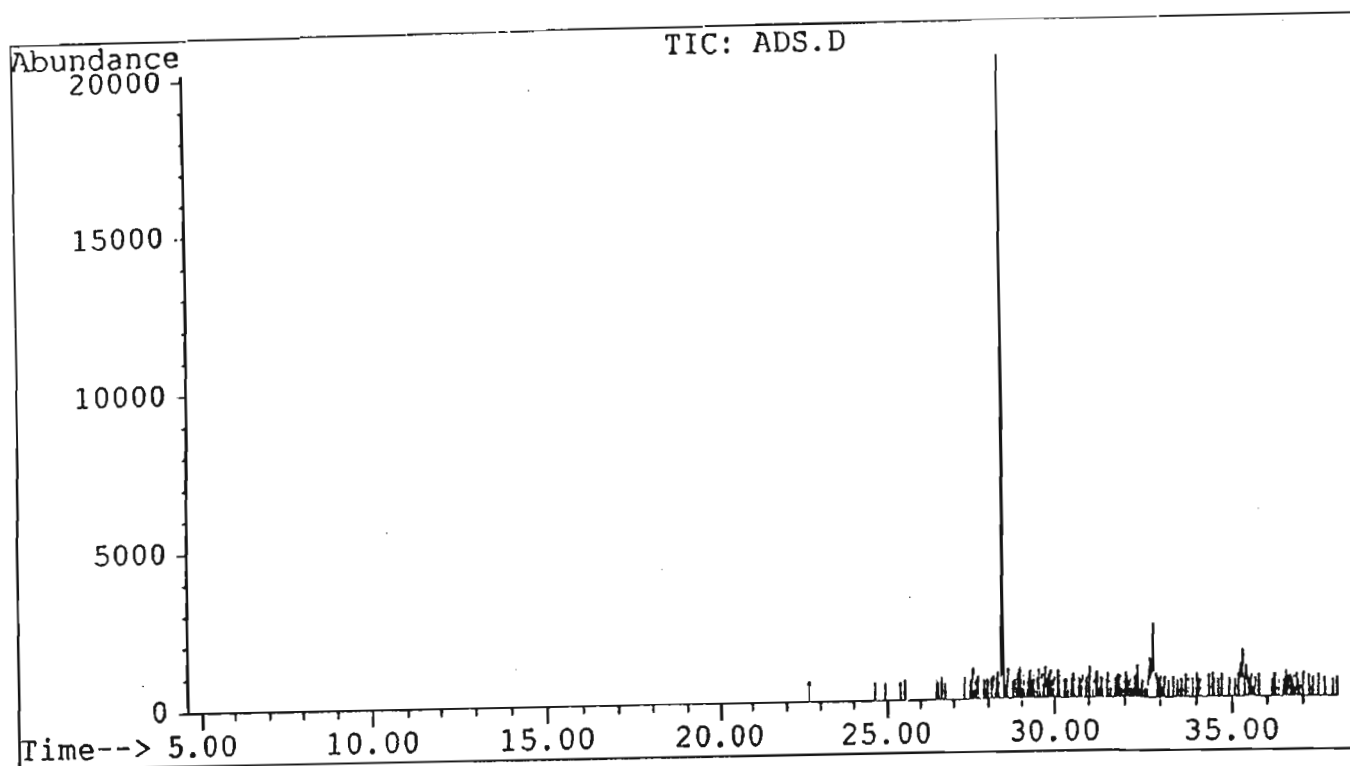
APPENDIX 14.7:   CHROMATOGRAPH OF FUSARIC ACID
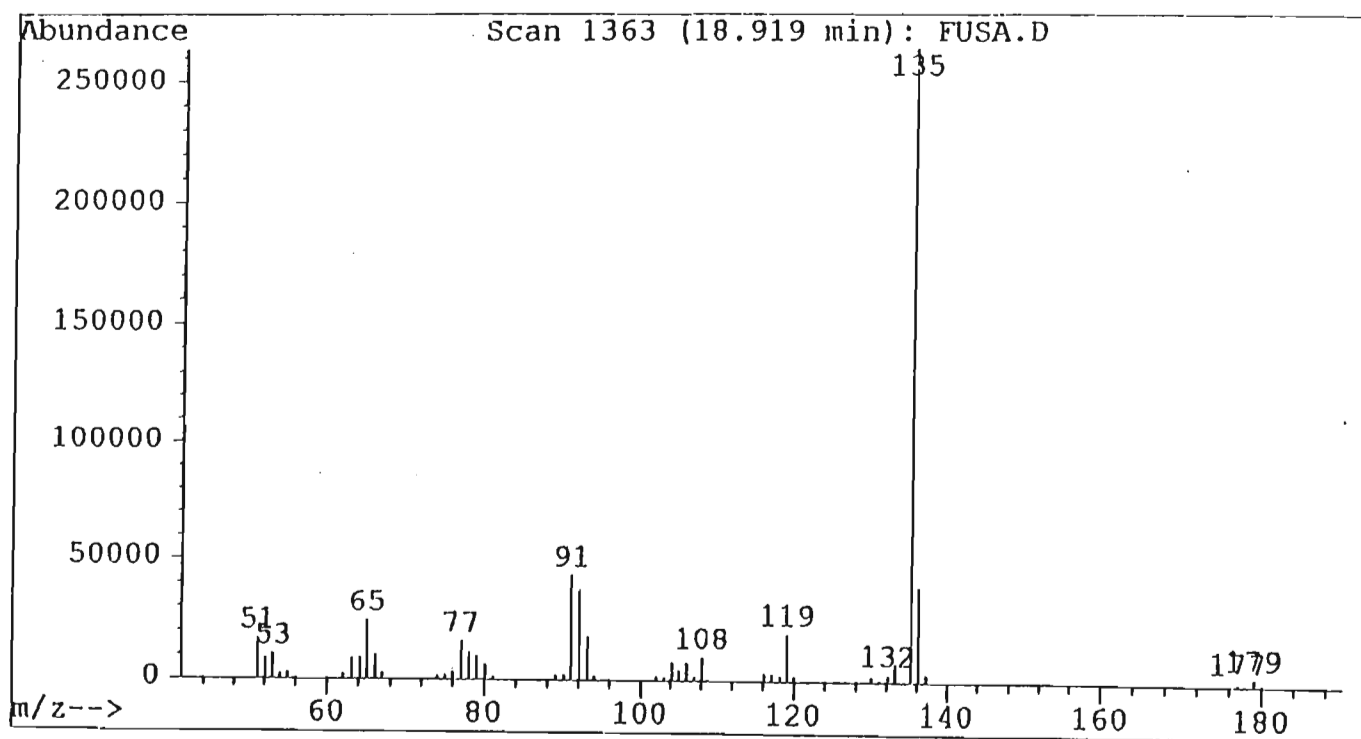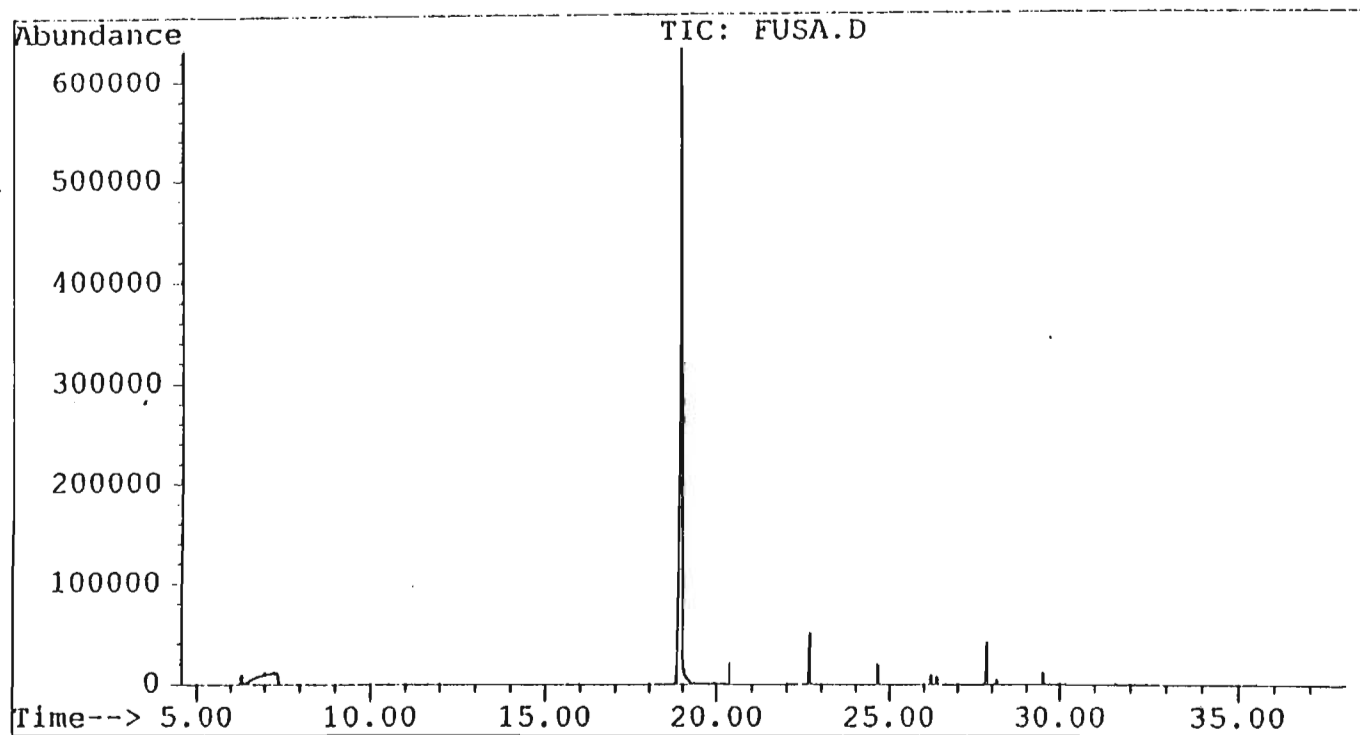
APPENDIX 14.8:   CHROMATOGRAPH OF ZEARALENONE
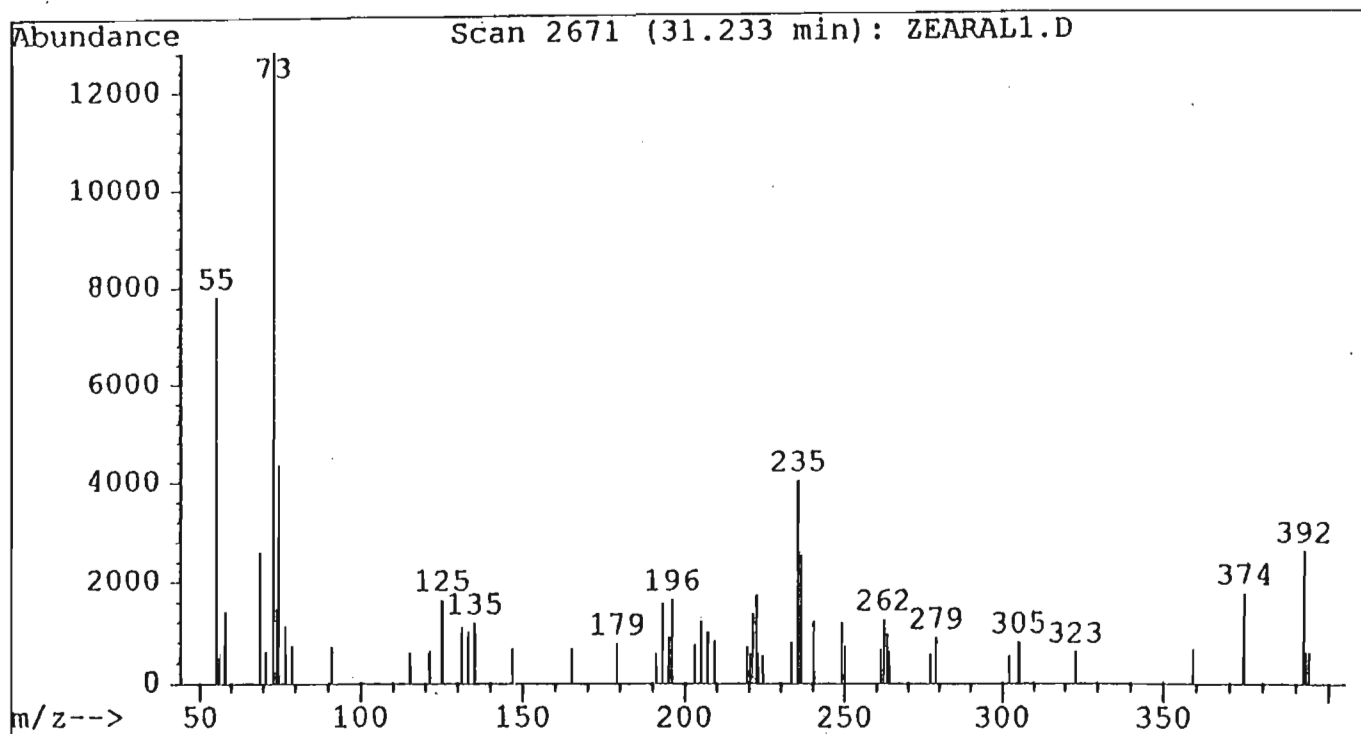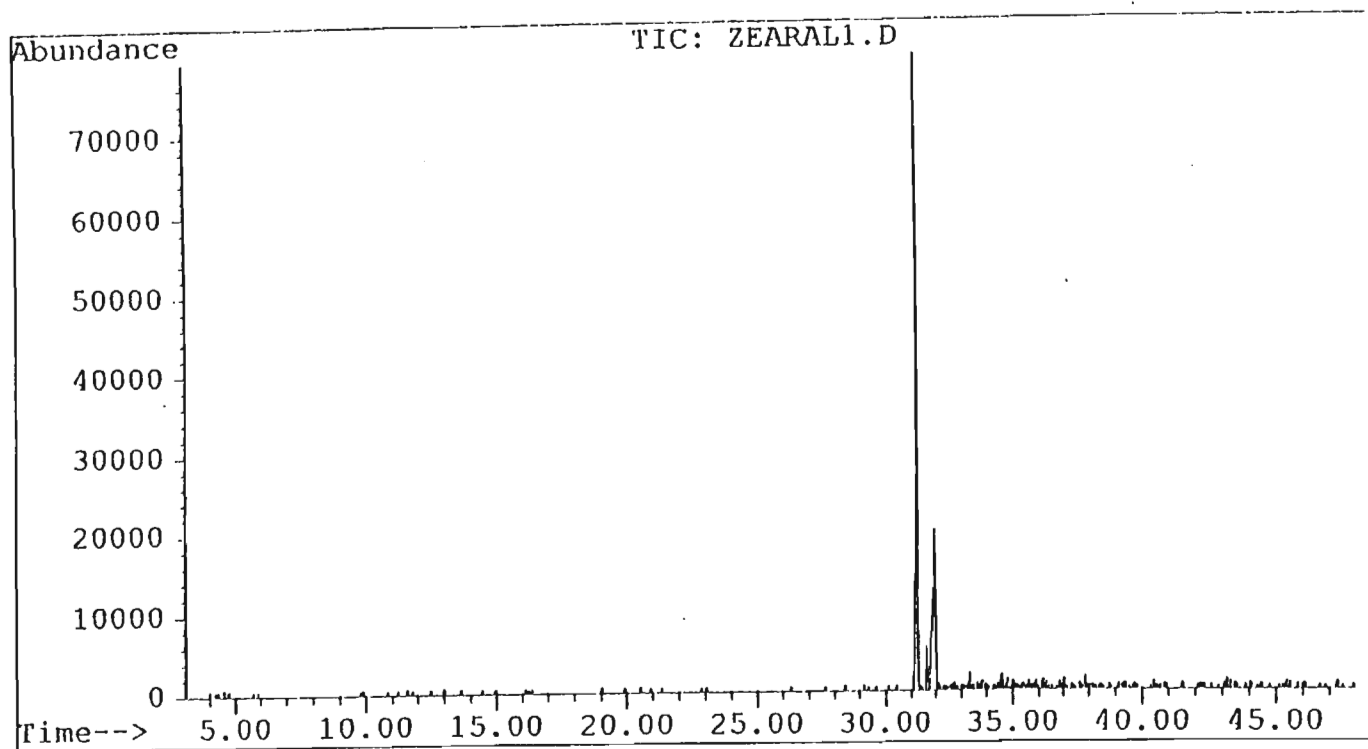
APPENDIX 14.9:   **CHROMATOGRAPH OF T2-TOXIN**
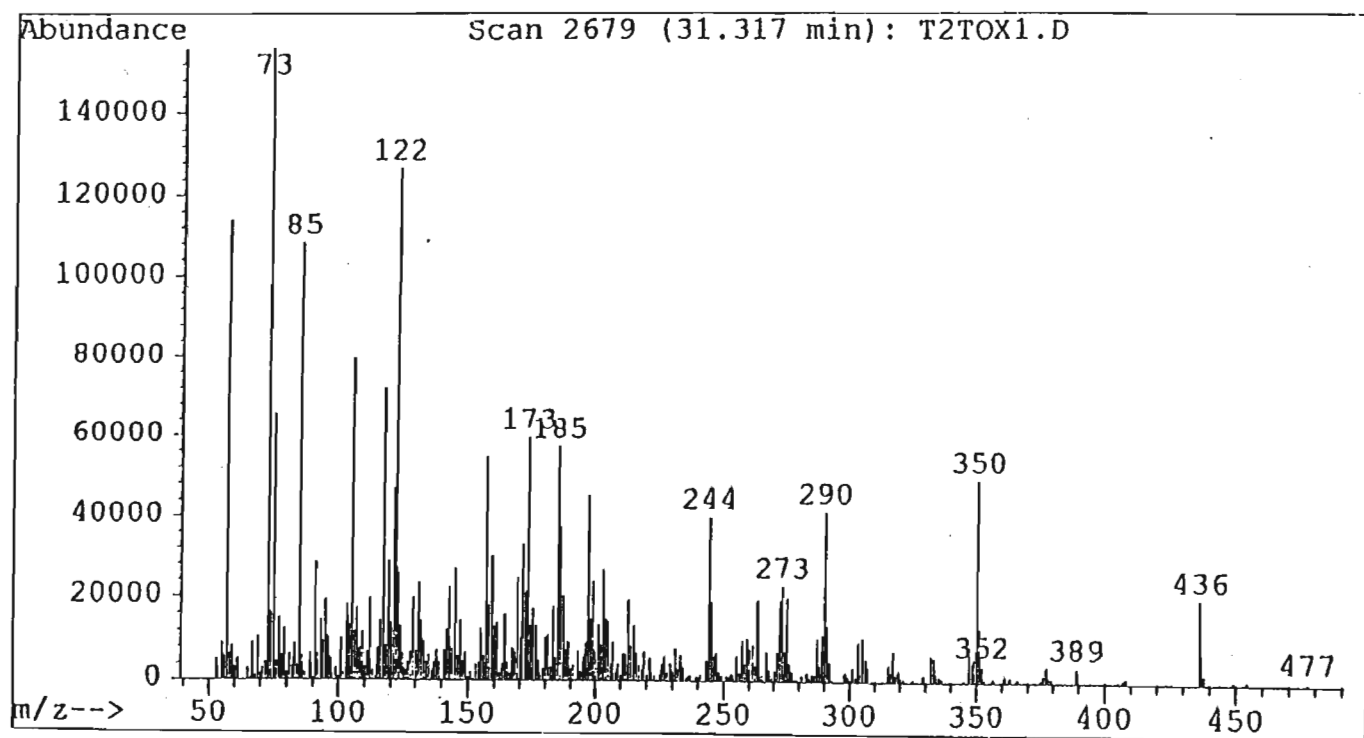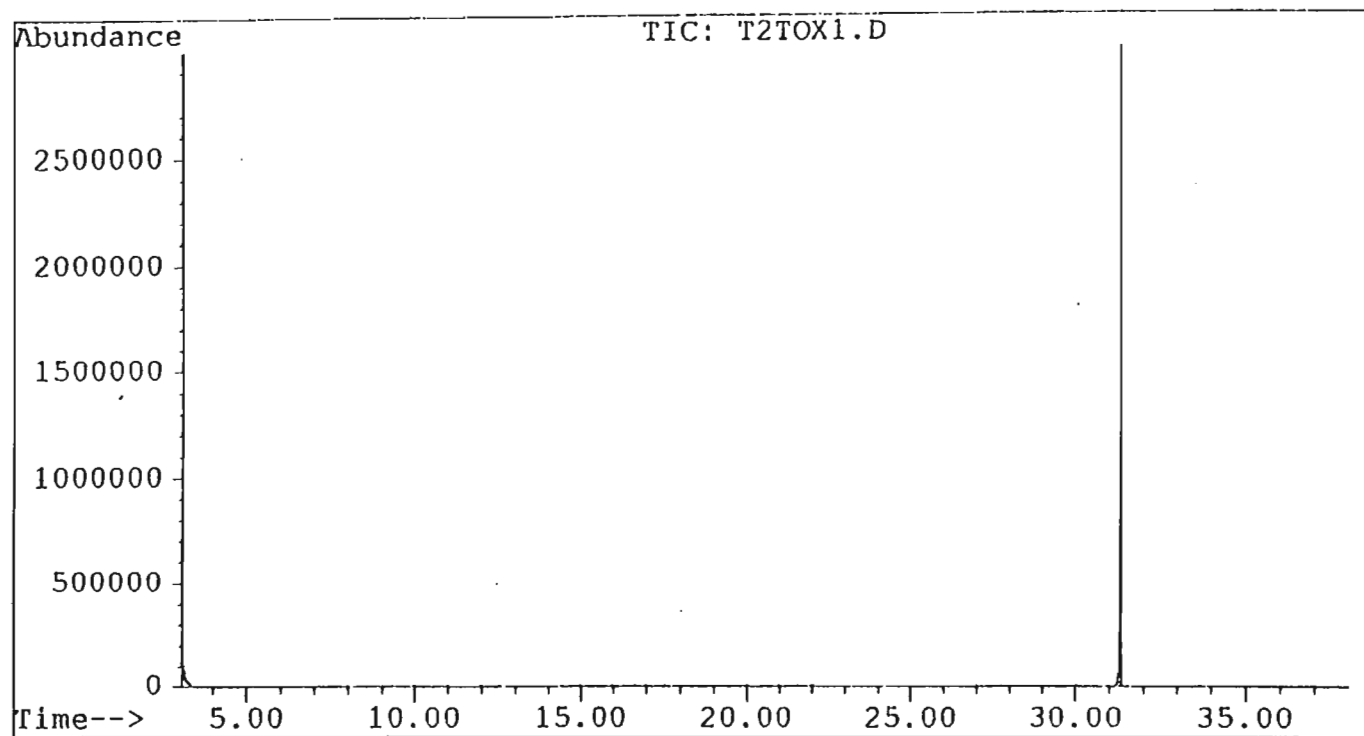
APPENDIX 14.10:   CHROMATOGRAPH OF HT2-TOXON
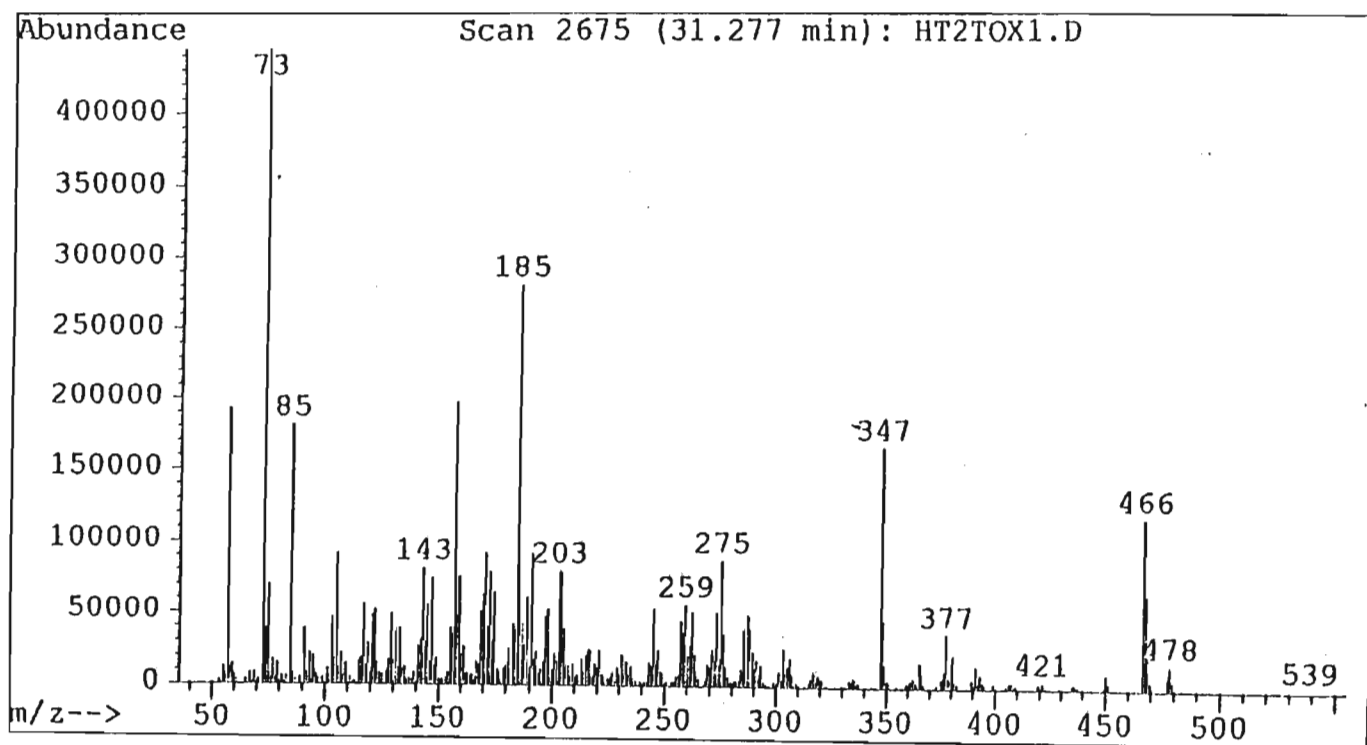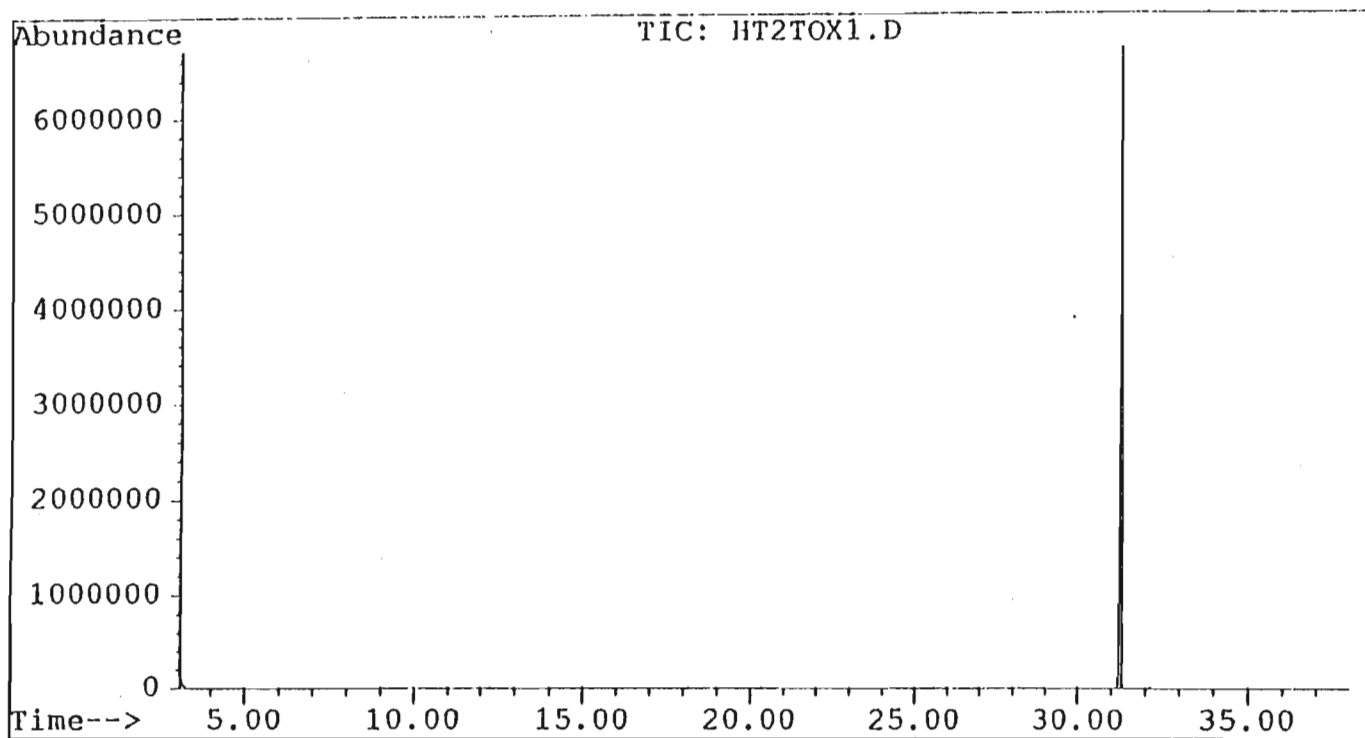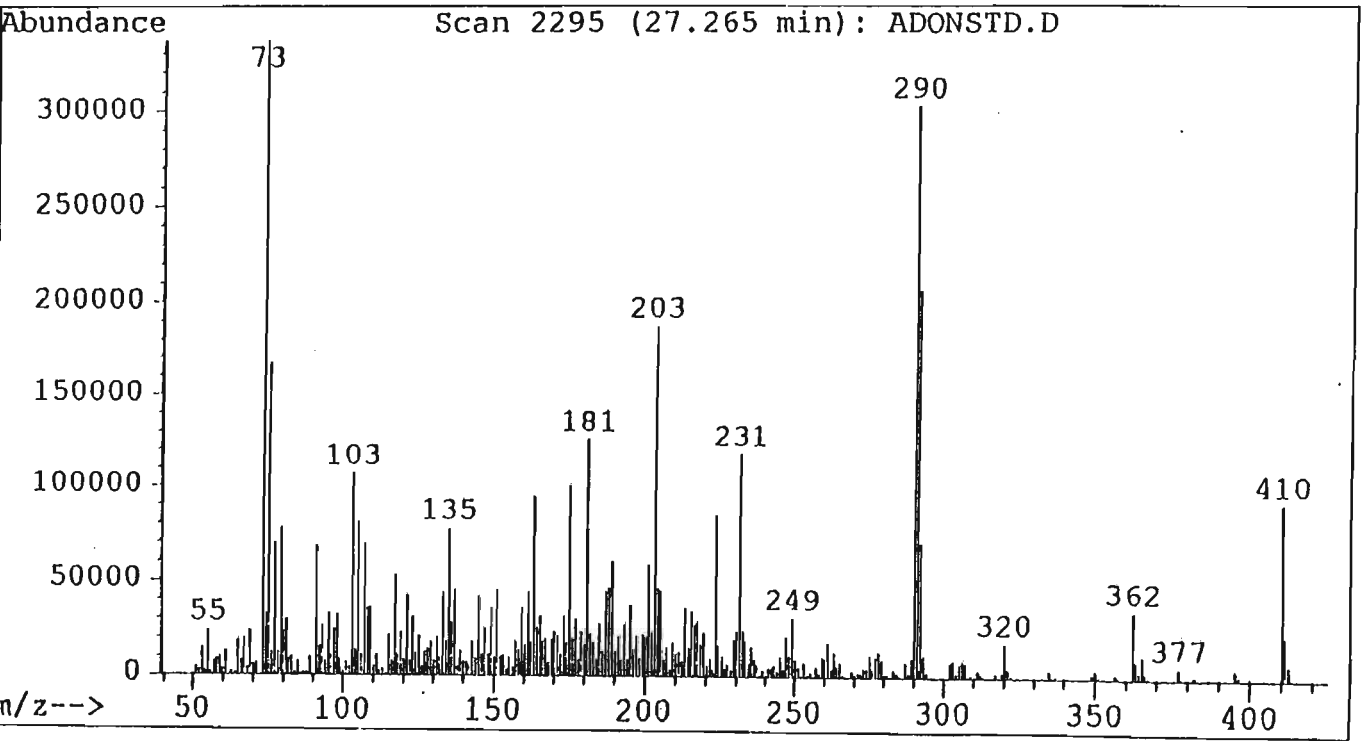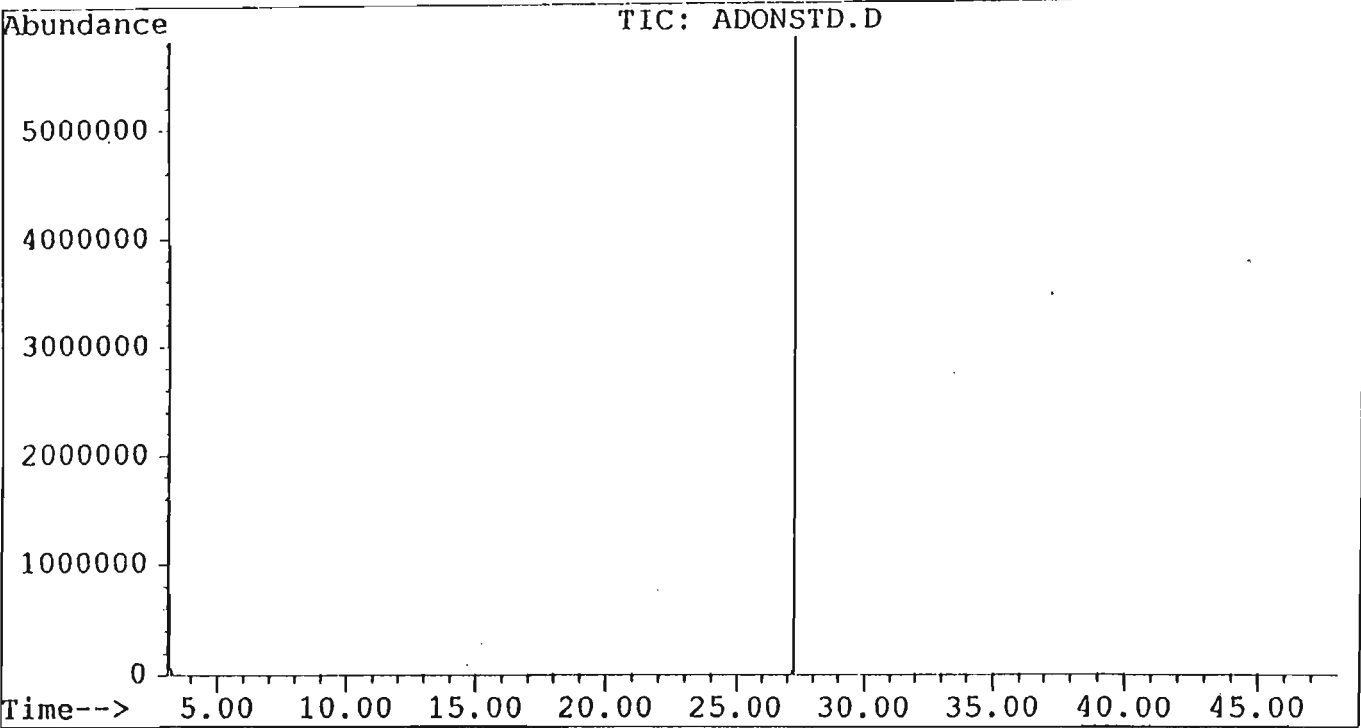
APPENDIX 14.11: **CHROMATOGRAPH OF SCIRPENTRIOL**
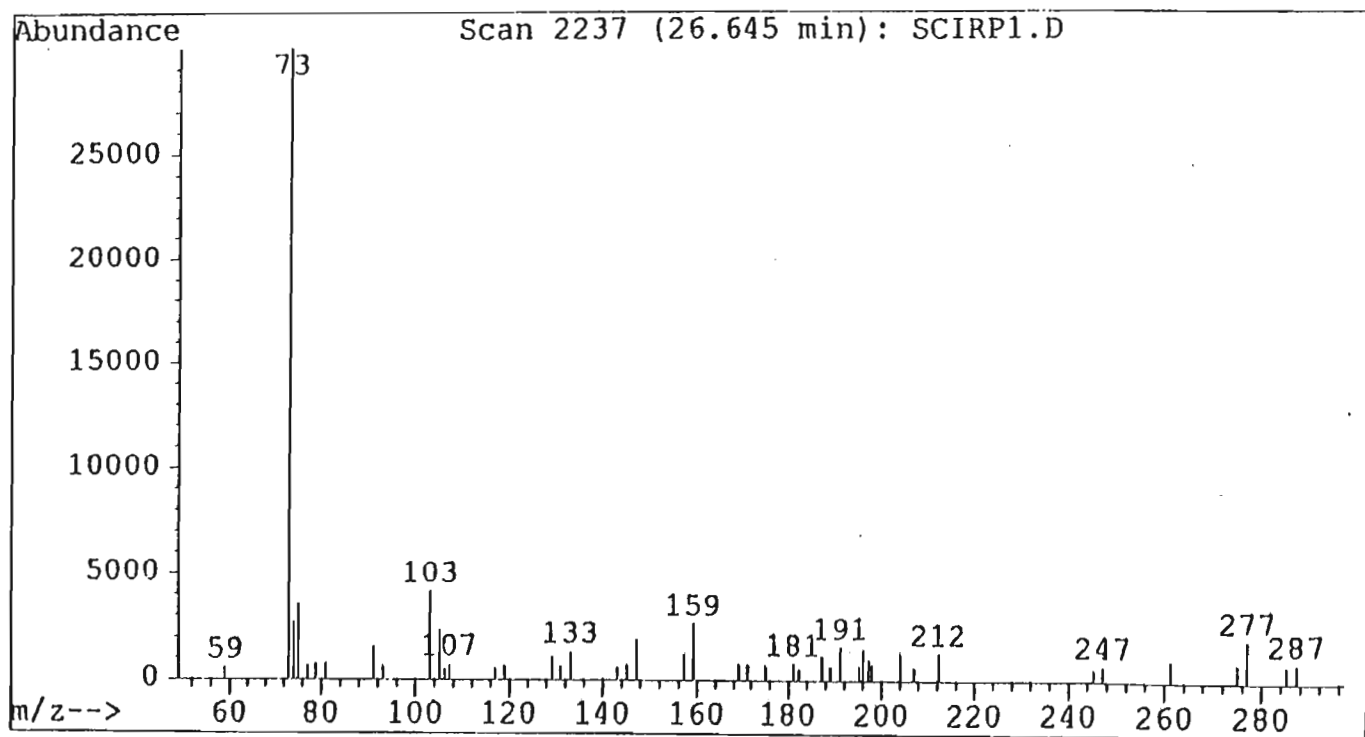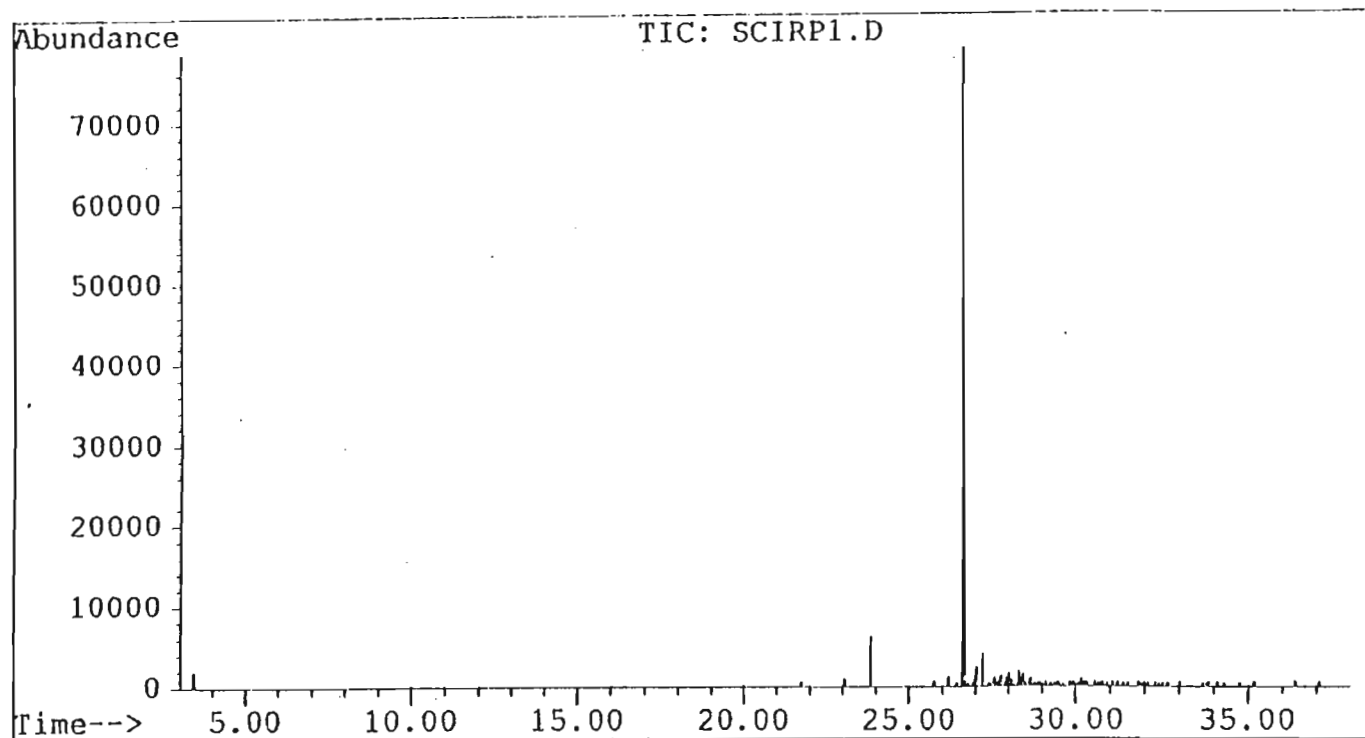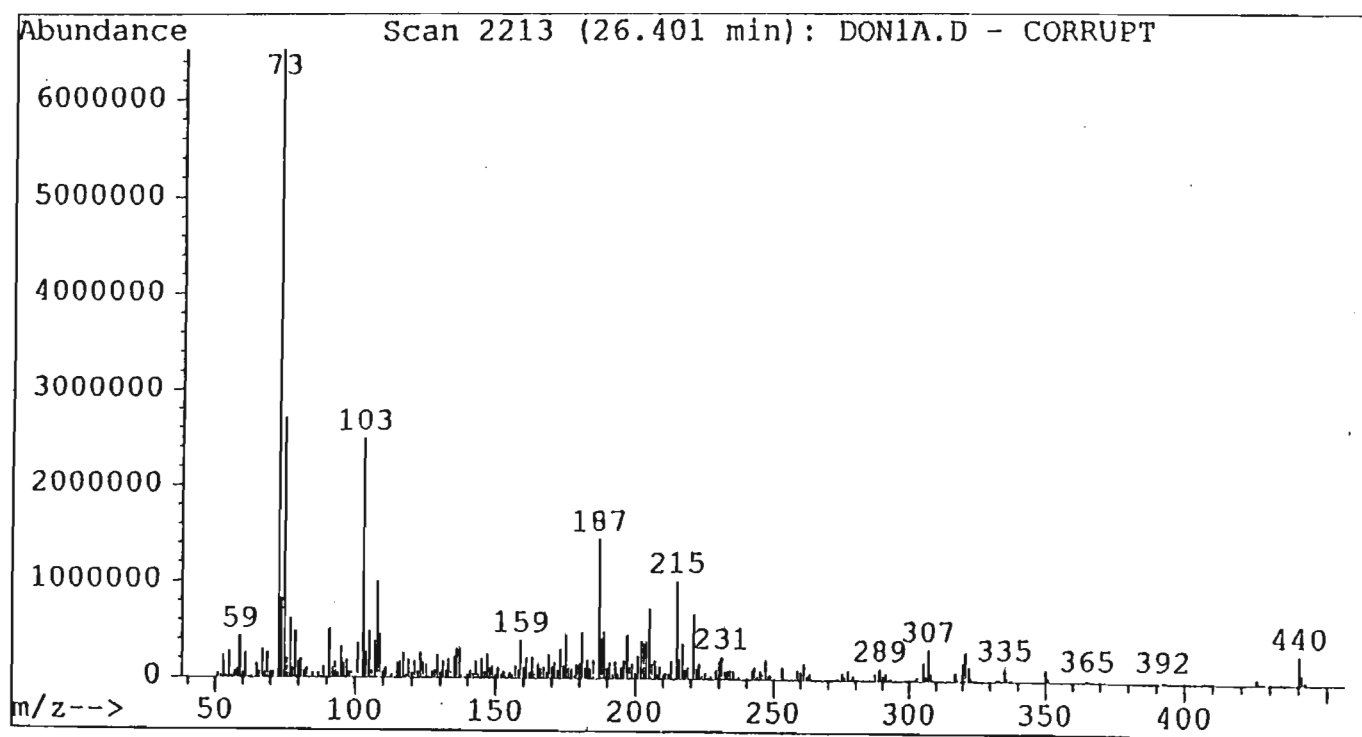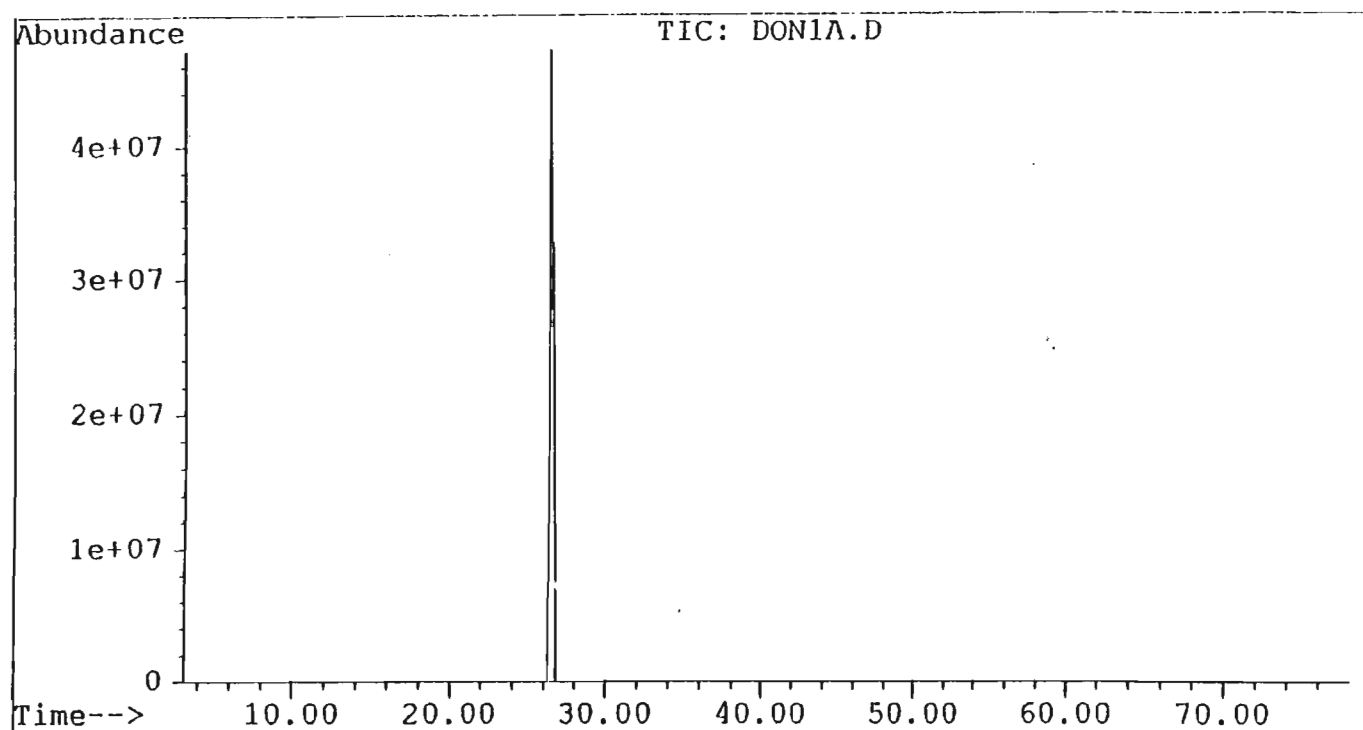
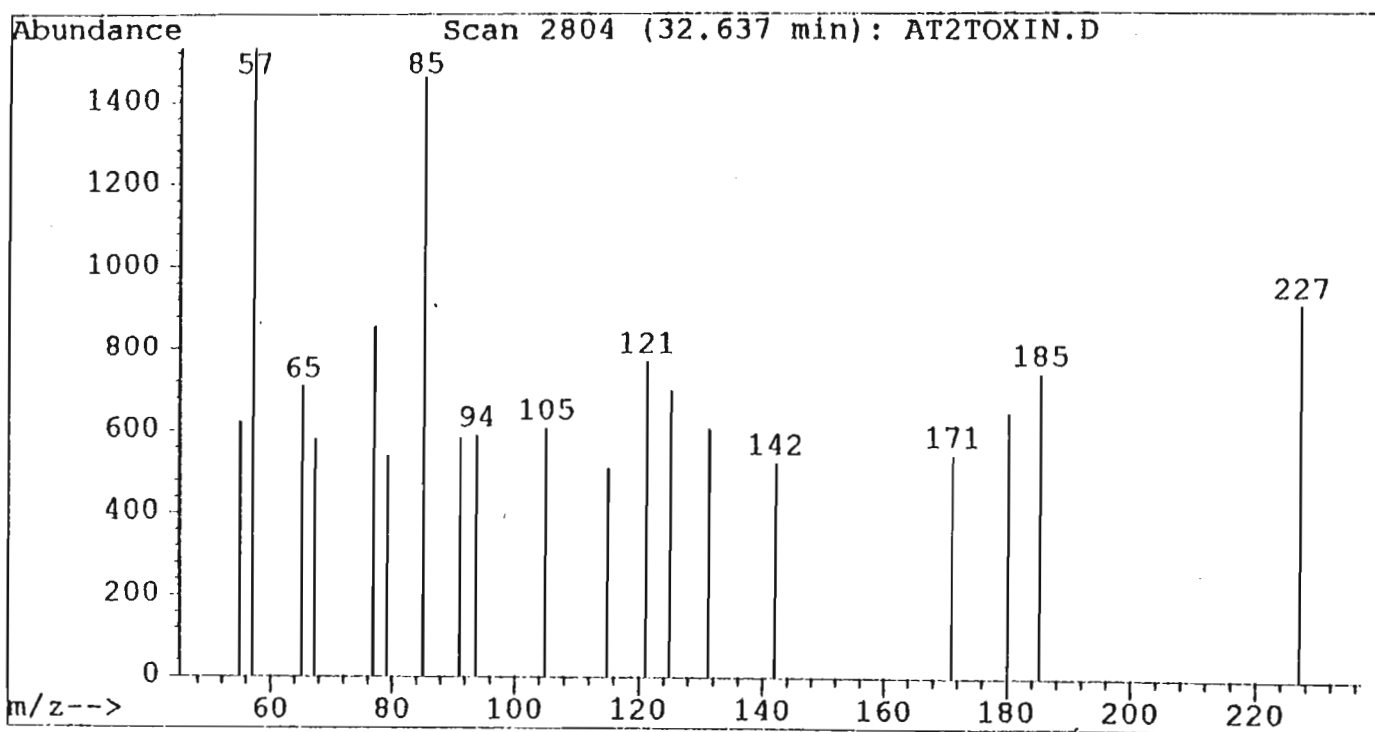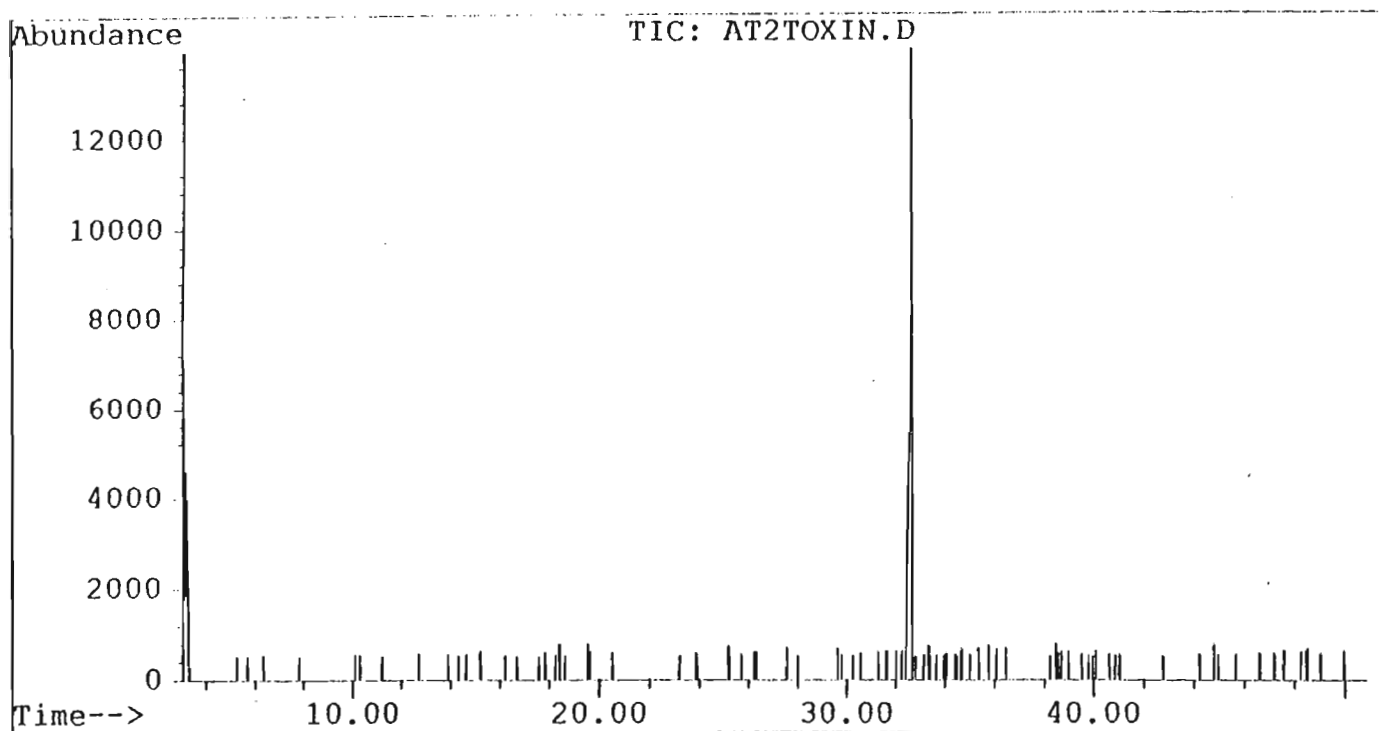APPENDIX 14.12:   CHROMATOGRAPH OF NIVALENOL
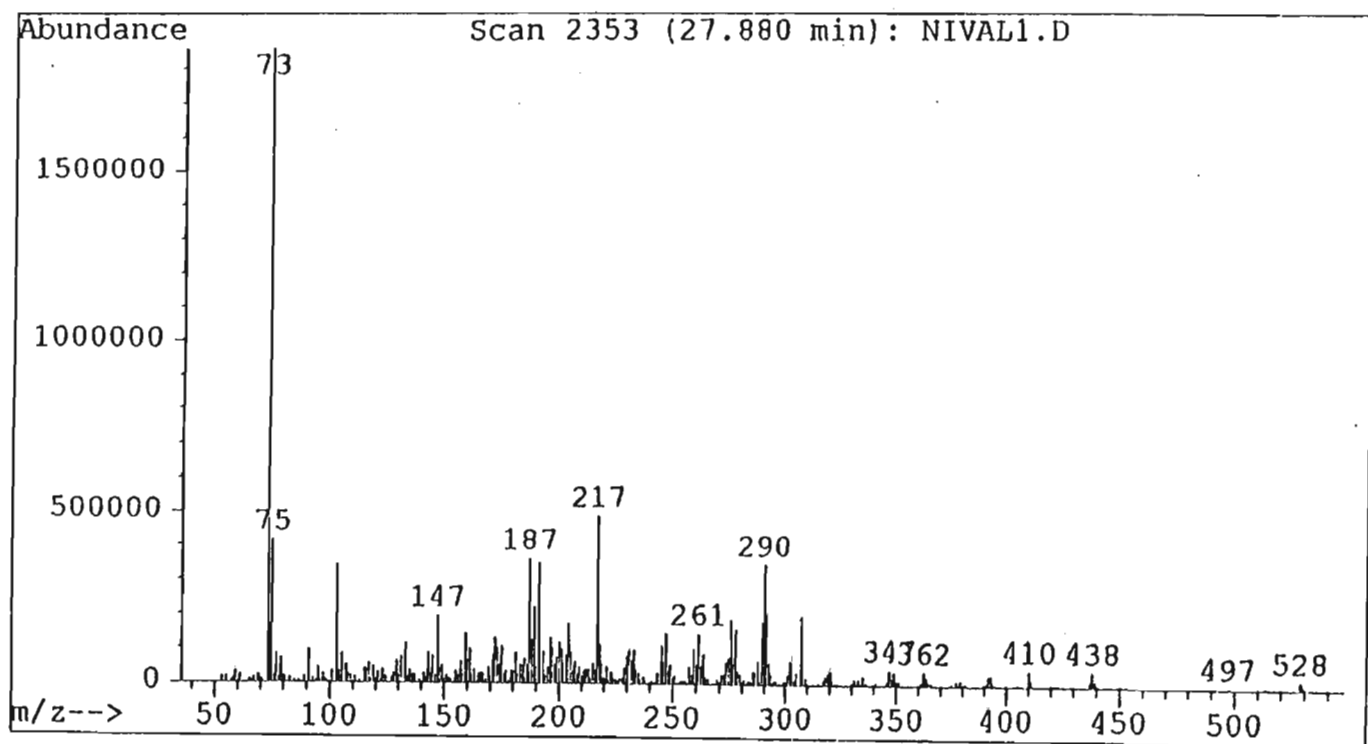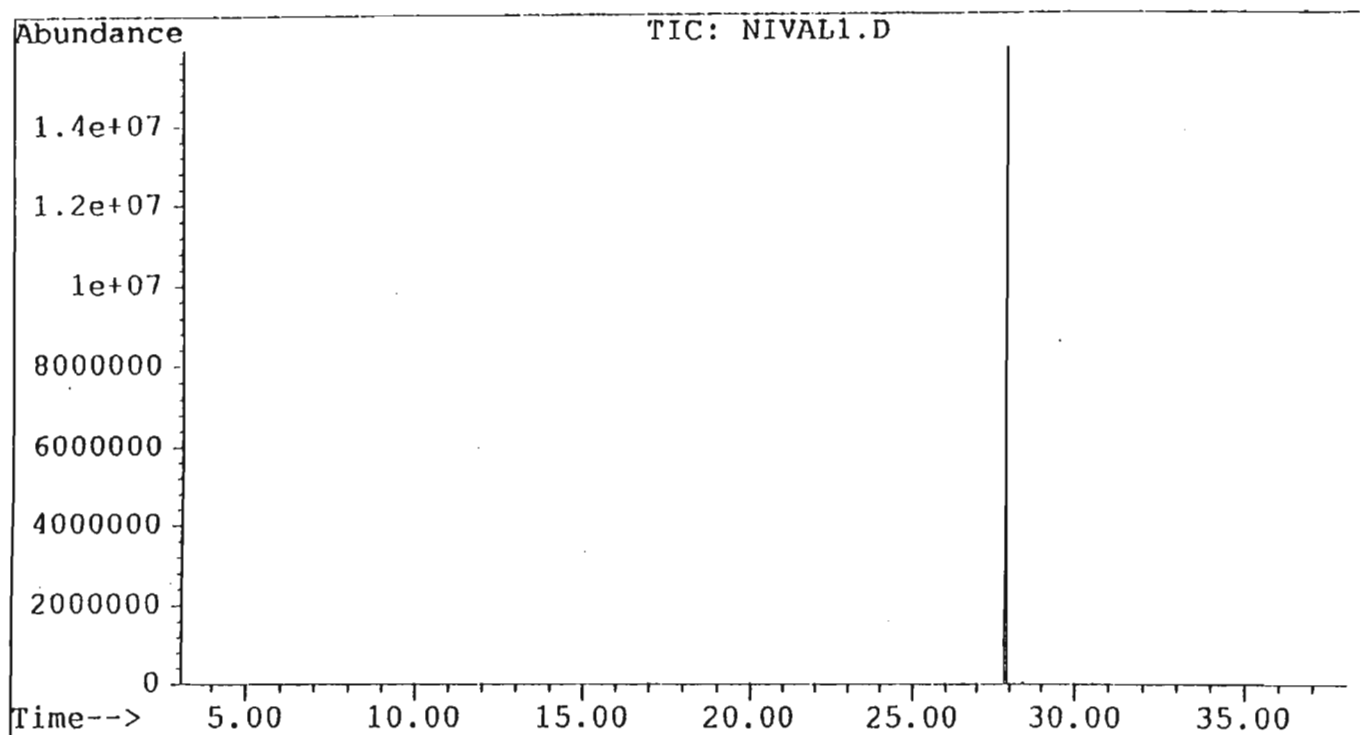
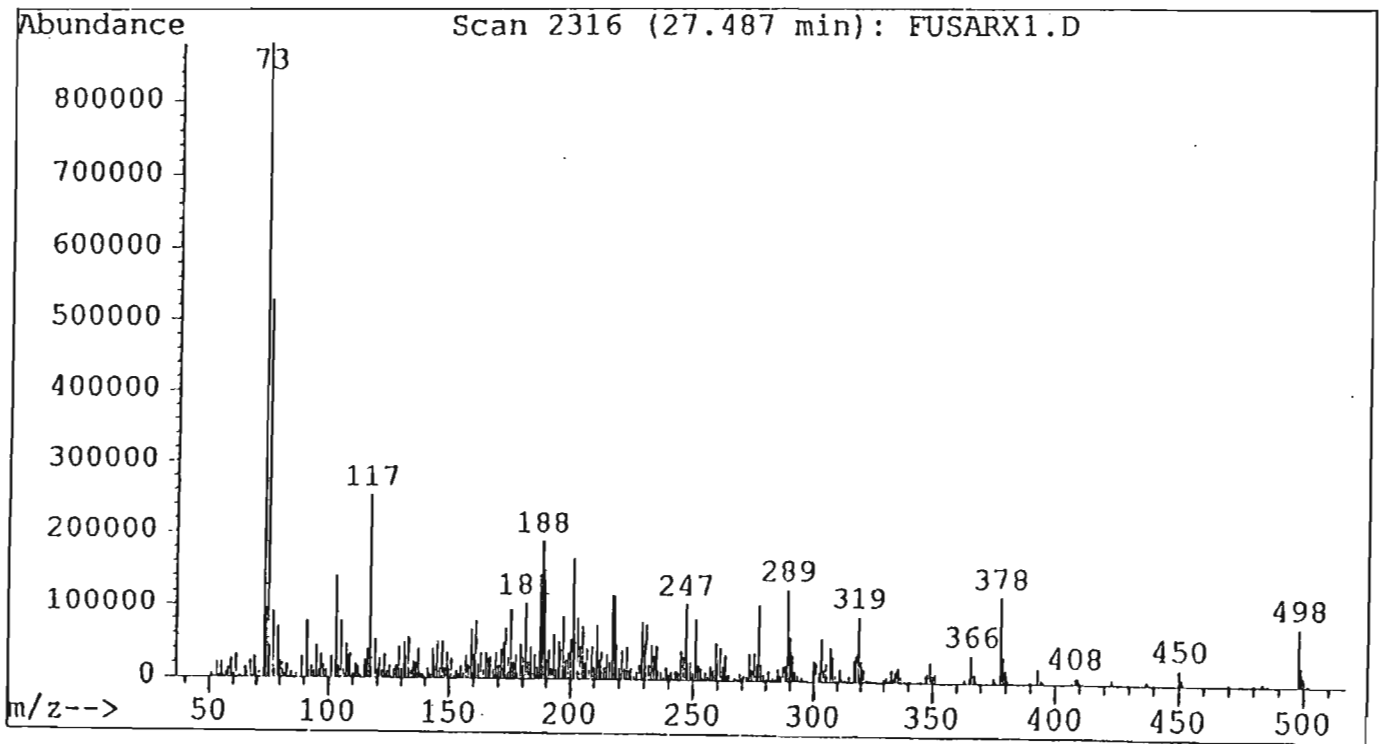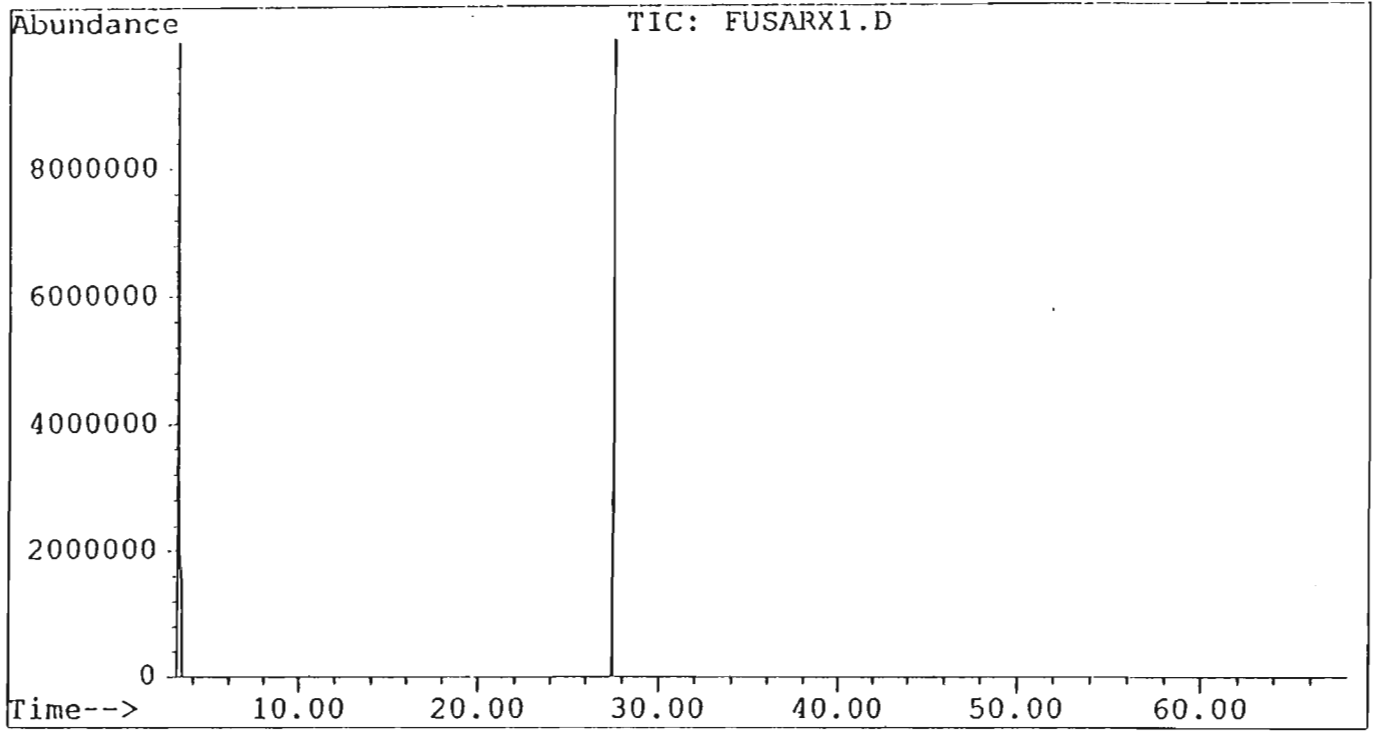**APPENDIX 14.13:** **CHROMATOGRAPH OF 3-ACETYLDEOXYNIVALENOL**

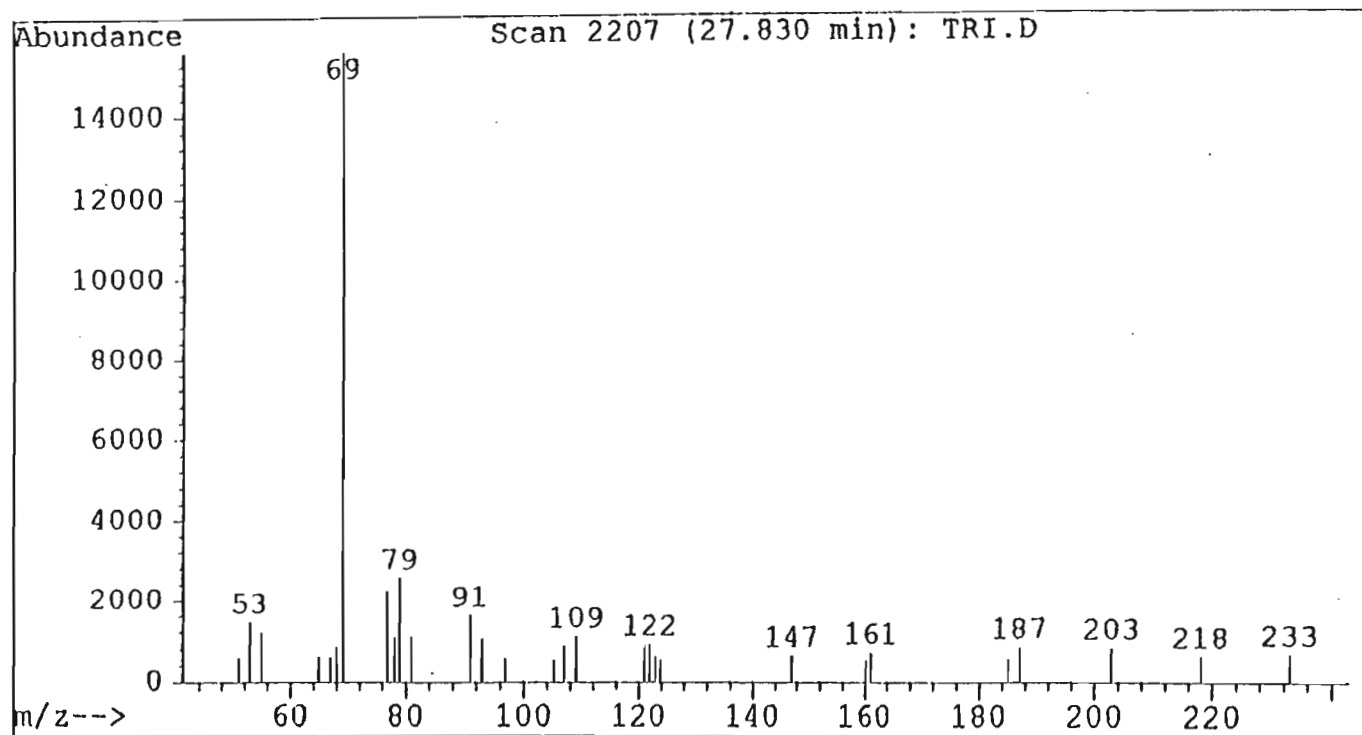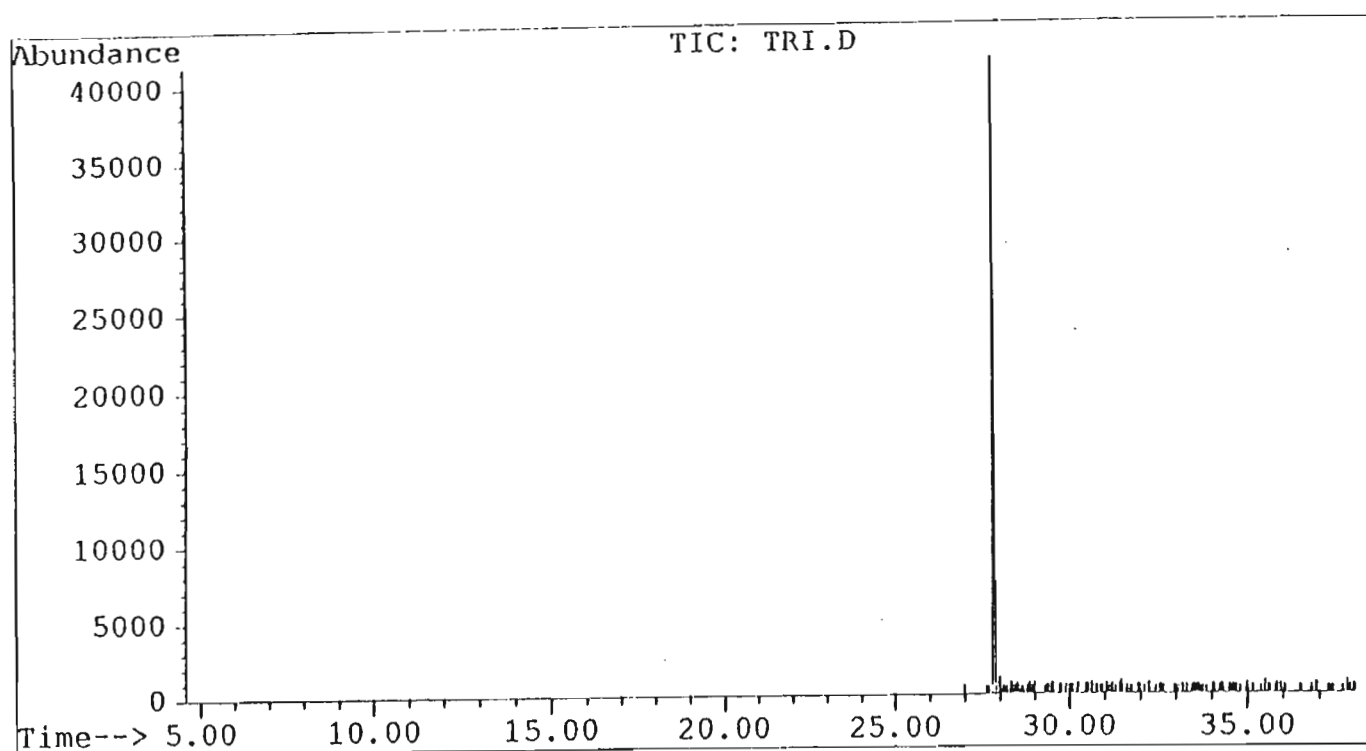**IAPPENDIX 14.14: CHROMATOGRAPH OF DEOXYNIVALENOL**

APPENDIX 14.15:   **CHROMATOGRAPH OF ACETYL T2-TOXIN**

APPENDIX 14.16:   **CHROMATOGRAPH OF FUSARENONE-X**

APPENDIX 14.17:   CHROMATOGRAPH OF TETRAOL

TIC: TRI.D

Scan 2207 (27.830 min): TRI.D

APPENDIX 15.0:   **PAGE REAGENTS**

STOCK SOLUTIONS (All solutions should be filtered.)

Monomer Solution (30% T; 2,7% C Bis)
*Acrylamide	58,40	g
Bis	1,60	g
H_2O	200,00	ml

Stored at 4 °C in the dark

Monomer Solution in 75% glycerol (30% T; 2,7% C)
*Acrylamide	58,40	g
Bis	1,60	g
75% glycerol	200,00	ml

(Stored at 4 °C in the dark)
*Acrylamide is neurotoxic and should be handled with care.

Resolving Gel Buffer (1,5 M Tris-Cl ph 8,8)
Tris	36,30	g
H_2O	200,00	ml

Stacking Gel Buffer (0,5 M Tris-Cl pH 6,8)
Tris	3,00	g
H_2O	50,00	ml

10% Sodium dodecyl sulphate (SDS)
SDS	50,00	g
H_2O	500,00	ml

Initiator (10% ammonium persulphate)
Ammonium persulphate	0,50	g
H_2O	5,00	ml

Resolving Gel Overlay (0,375 M Tris-Cl; pH 8,8; 0,1% SDS)
Tris	25,00	ml Solution (3)
SDS	1,00	ml Solution (5)
H_2O	100,00	ml

2x Treatment Buffer (0,125 M Tris-Cl; pH 6,8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol)
Tris	2,50	ml Solution (4)
SDS	4,00	ml Solution (5)
Glycerol	2,00	ml
2-mercaptoethanol	1,00	ml
H_2O	10,00	ml

Divided into aliquots and frozen.

Tank Buffer (0,025 M Tris; pH 8,3; 0,192 M glycine; 0,1% SDS)
Tris	12,00	g
Glycine	57,60	g
SDS	40,00	ml Solution (5)
H_2O	4,00	liters

Because the pH of this solution need not be checked, it can be made up directly in large 4,00 liters reagent bottles and 12-16 liters can be made up at a time.

Stain Stock (1% Coomassie Blue R-250)

Coomassie Blue R-250	2,00	g
H2O	200,00	ml

Stain 0,125% Coomassie Blue R-250, 50% methanol,
           10% acetic acid)

Coomassie Blue R-250	62,50	ml Stain Stock
Methanol	250,00	ml
Acetic acid	50,00	ml

Destaining Solution I (50% methanol, 10% acetic acid)

Methanol	500,00	ml
Acetic acid	100,00	ml
H2O	1,00	liter

Destaining Solution II (7% acetic acid, 5% methanol)

Acetic acid	700,00	ml
Methanol	500,00	ml
H2O	10,00	liters

PARAGON REAGENTS

B-2 Barbital Buffer; pH 8,6 (0,05 Ionic Strength):
The contents of the buffer bottle were dissolved into 1500 ml of deionized water.

Acid-Alcohol solution; 20% Acetic Acid-30% Methanol:
600 ml of methanol and 400 ml of glacial acetic acid were added to 1000 ml of deionized water, and mixed thoroughly.

Acetic Acid Solution, 5%: 150 ml of glacial acid was added to 2850 ml of deinoized water, and mixed thoroughly.

Paragon Blue Stain; 0,5%:
The contents of the stain bottle were dissolved in 1000 ml of 5% acetic acid solution and mixed thoroughly.

APPENDIX 16.0: **QUESTIONNAIRE: AFLATOXIN/KWASHIORKOR STUDY**

DEPARTMENT OF PHYSIOLOGY - FACULTY OF MEDICINE

UNIVERSITY OF NATAL

NAME: _____ . AGE: _____ ·GENDER: M/F

WEIGHT ON ADMISSION: _____ kg HEIGHT: _____ cm DATE: _____

A. FAMILY

Status of Mother

Single	
Married	
Divorced	
Separated	
Died	
Not Known	

Years of	Father	Mother
Schooling		
Occupation		

No of Siblings

No of Siblings with similar illness

B. HISTORY

Breast Fed
Yes
No

Total Duration of Breast Feeding

Duration of Illness

	Rice	Mealie Meal	Peanuts	Fruits/ Nuts	Fish	Meat	Eggs	Milk
Daily								
Frequently								
Sometimes								
None								
Not Known								

AFLATOXIN/KWASHIORKOR STUDY

C. CLINICAL EXAMINATION

	Dehydrated	Oedema	Anaemia
No			
Slight			
Moderate			
Severe			
Not Known			

Behaviour

Normal

Alert / Irritable

Apathy / Irritable

Drowsy

Not Known

	Mouth	CVS	Chest	Ears	Eyes
Normal					
Abnormal					
n/k					

	Jaundice	Ascites	Enlarged Liver	Enlarged Spleen
Yes				
No				
n/k				

Blood

HB ☐☐☐   Pev ☐☐☐

Protein

Total ☐☐☐   Alb ☐☐

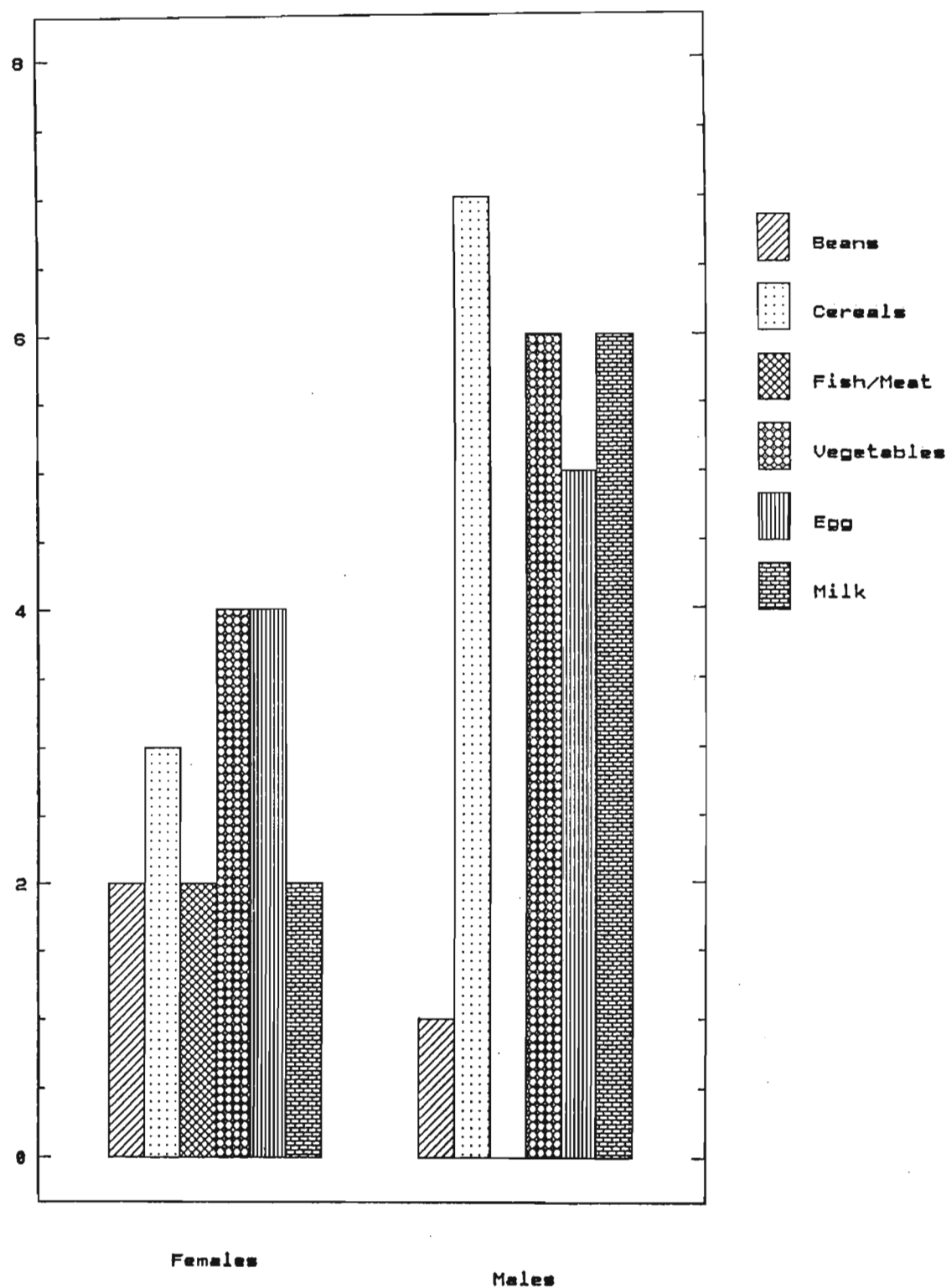☐☐☐   ☐☐

COMMENTS: _____

_____
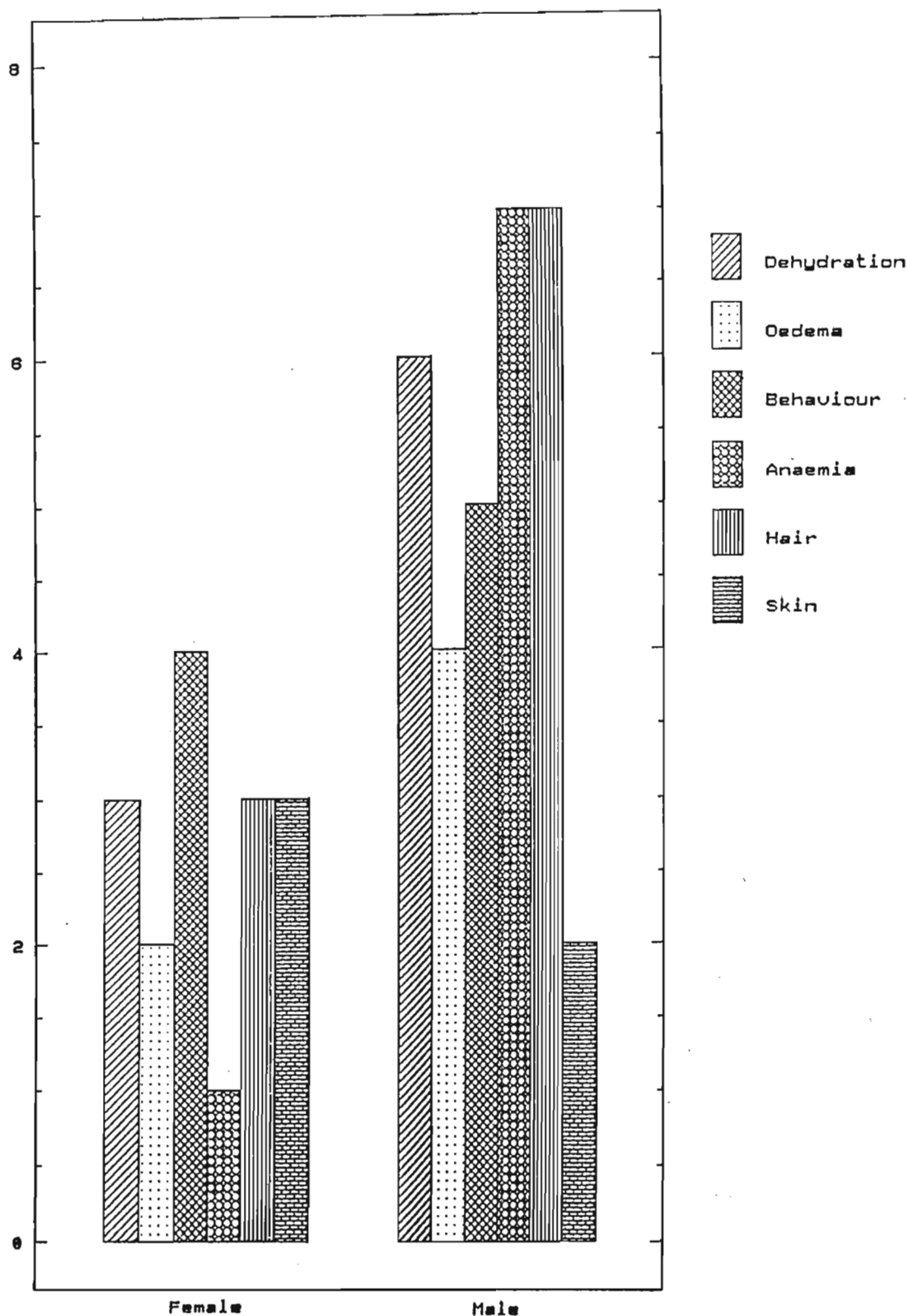
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APPENDIX 16.1:   RESULTS OF QUESTIONNAIRE

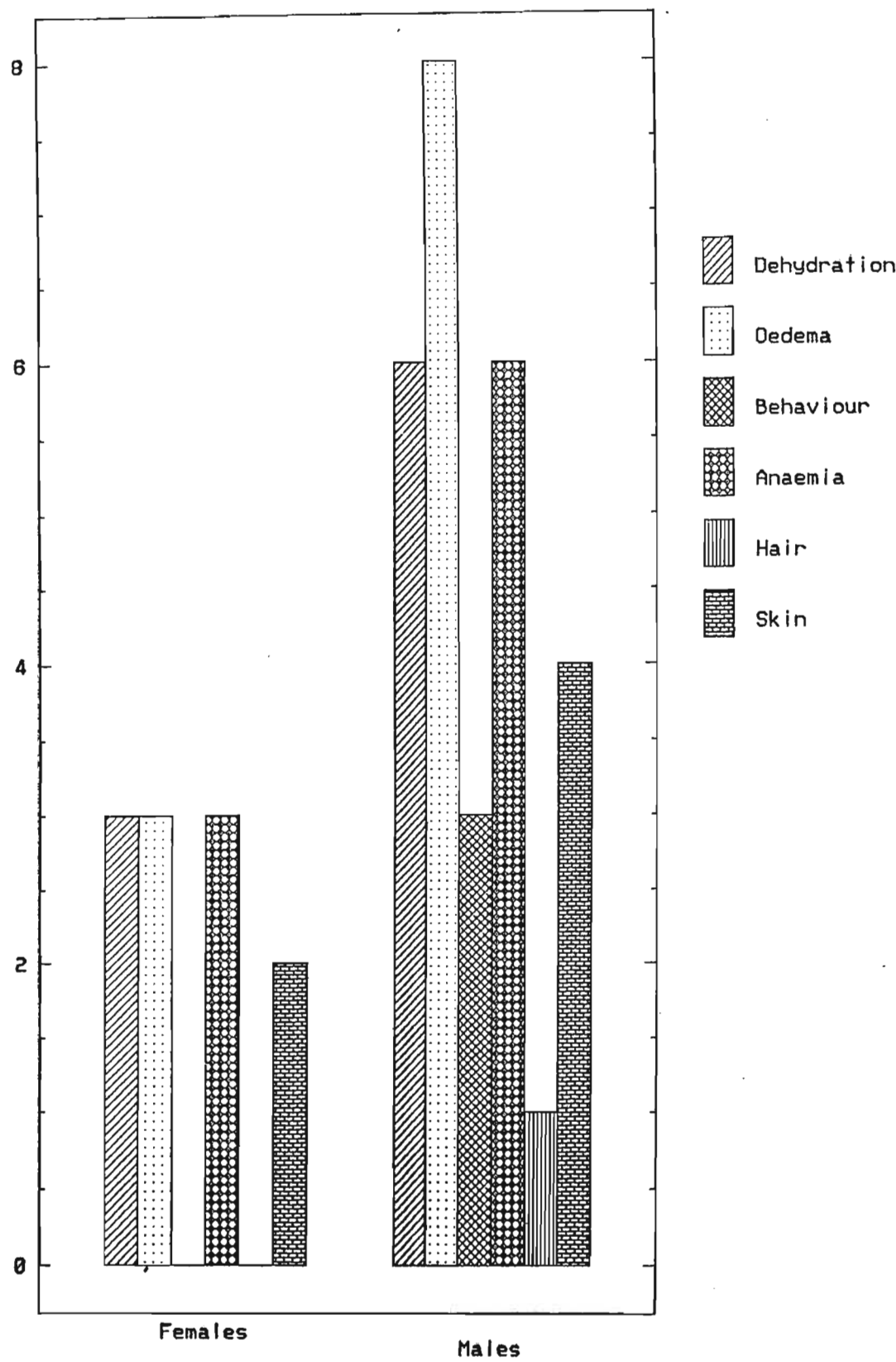ANALYSIS OF DIETS: TEMBA HOSPITAL PAEDIATRIC
PATIENTS, MARCH-JUNE 1993

APPENDIX 16.2:   RESULTS OF QUESTIONNAIRE

ANALYSIS OF CLINICAL SYMPTOMS: TEMBA HOSPITAL
PAEDIATIC PATIENTS, MARCH-JUNE 1993

APPENDIX 16.3:   RESULTS OF QUESTIONNAIRE

ANALYSIS OF CLINICAL SYMPTOMS: TEMBA HOSPITAL
PAEDIATIC PATIENTS, JULY-AUGUST 1993

APPENDIX 17.0:   ANALYSIS OF GROUNDNUT PROTEIN-MYCOTOXIN ADDUCTS

Groundnuts were first viewed under UV at 365 nm and those contaminated and which fluoresced blue were used for the following experiments. Groundnut splits were mechanically blanched to remove most of the cuticle. The splits were then ground using a pestle and mortar. The sample was dispersed in six parts (w/v) of deionized water (65 +/- 2 °C) while stirring with a magnetic stirrer. For the extraction of proteins, the pH of the dispersion was adjusted to 8,0 by adding 1N NaOH solution with constant stirring. In the case of the extraction of oil, the dispersion pH was adjusted to 4,0 by the addition of 1N HCl solution. After the pH adjustment, extraction was continued for 30 minutes in a temperature controlled water bath (60 +/-2 °C) with occasional stirring. The dispersion was then centrifuged at 4,000 x G for 30 minutes at 30 +/- 2 °C to separate the solid aqueous oil and emulsion phases, depending upon the experimental conditions. The amount of protein extracted was determined by the spectrophotometric method (section 5.3, page 142).

The equivalent of 2 mg of protein was digested with pronase and the same "clean-up" method with affinity columns was used before quantitation with HPLC.

PRESENTATIONS

National:

Department of Physiology, Medical School, University of Natal, Durban, March 1993

Department of Paediatrics, Medical School, University of Natal, Durban, July 1993

Natal Biochemistry Symposium, University of Durban Westville, October 1993

South African Biochemistry Conference, Stellenbosch University, January 1994

Natal Biochemistry Symposium, University of Natal, Pietermaritzburg, October 1994

International:

16th International Biochemistry and Molecular Biology Congress, New Dehli, India, September 1994 *(awarded prize for poster presentation in Young Scientist's Programme)*