

**THE CHROMATOGRAPHIC DETERMINATION OF *FUSARIUM* TOXINS IN
MAIZE ASSOCIATED WITH HUMAN OESOPHAGEAL CANCER**

by

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Dedicated to my mother, the
memory of my late father
and especially to Gill and
Shaun for their continuous
love and encouragement

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ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
ATA	Alimentary toxic aleukia
DON	Deoxynivalenol
ECD	Electron capture detector
ELISA	Enzyme-linked immunosorbent assay
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FID	Flame ionization detector
GC	Gas chromatography
GC/MS	Gas chromatography - mass spectrometry
GGT ⁺	Gamma-glutamyl-transpeptidase-positive
HPLC	High performance liquid chromatography
IR	Infra-red
LEM	Equine leukoencephalomalacia
MON	Moniliformin
MS	Mass spectrum
MRC	South African Medical Research Council
NIV	Nivalenol
NMR	Nuclear magnetic resonance
RIA	Radio-immunoassay
TCA	Tricarballic acid
TLC	Thin layer chromatography
UV	Ultra-violet
ZEA	Zearalenone

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CHAPTER 1

INTRODUCTION

1.1 AIM OF THE STUDY

Interest in the role played by a number of food-borne mycotoxins in the etiology of animal and human diseases and/or syndromes, has intensified over the last 25 years. It is known that these mycotoxins are as diverse in their chemical nature as are the fungi that produce them.

Long before the Christian era food was subject to legislation and inspection. An example of early food 'legislation' was the law, promulgated by the King of the Hittites (Schuller et al., 1983), the first part of which reads "Thou shalt not taint the fat or the bread of thy neighbour, neither shalt thou bewitch the fat or the bread of thy neighbour". These days, the food laws not only prohibit the introduction, delivery for introduction, or receipt in commerce of adulterated and misbranded food, but often includes specific legislation that imposes limits or tolerances on the concentrations of specific contaminants in food. These contaminants can be of industrial or natural origin. The importance of mycotoxins to animal and human health might then be reflected in the fact that of the natural contaminants, the mycotoxins are the most recent to be considered for legislative regulation.

The necessity to obtain accurate and reliable data pertaining to the range and/or levels of mycotoxin contamination in a variety of food and feed substrates, intended for human or animal consumption, has therefore, become important. An integral part of this study will be the provision of the most suitable analytical methods for the determination of selected *Fusarium* mycotoxins, in maize. The culmination will be the application of those selected methodologies to a series of Transkeian maize samples associated with human oesophageal cancer-risk.

1.2 BACKGROUND

Fungi or moulds are members of the lower plant species that do not contain chlorophyll and which reproduce by means of spores. Due to the absence of chlorophyll, they are unable to synthesize carbohydrates via the process of photosynthesis, as do the higher plant species. Consequently, fungi need to obtain their food by growing either parasitically on the living tissues of plants, animals or man, or saprophytically on dead organic matter. It is due to the process by which they obtain their food that fungi in general pose a potential hazard to human health. During their growth stage, numerous fungi have the ability to produce a diverse range of secondary metabolites (mycotoxins), which can be poisonous when ingested by animals or man (Marasas, 1988).

Studies concerning the death of 100,000 turkeys in Britain in

1960 from a disease first called "turkey X disease" might be regarded as the catalyst for modern research into mycotoxins and their role in related diseases. Isolation and characterisation of the causative agent for the "turkey X disease" led to the discovery of the aflatoxins (Austwick, 1978), and the name of the disease was hence changed to "aflatoxicosis". The impact of the discovery of "aflatoxicosis" as a distinct disease syndrome, and the subsequent implication of aflatoxins as potential carcinogens in the human health chain (LeBreton et al., 1962; Kraybill and Shimkin, 1964), had international implications. Although major research efforts centered on the aflatoxins, attention rapidly turned to other mycotoxins concerning their cause and effect relationship with a number of idiopathic diseases. Hence, the worldwide concern for "aflatoxins" and "aflatoxicosis" became a broader one for "mycotoxins" and "mycotoxicosis".

1.3 FUNGI AND *FUSARIUM* SPECIES IN PARTICULAR:

CLASSIFICATION

Fungi and moulds have on the grounds of morphology been grouped into specific genera. This study will deal exclusively with the genus *Fusarium*. Morphological differences have also been used to divide the different species within the genus *Fusarium* into subsections. The correct taxonomy and nomenclature are of importance, and since several classification systems have been used worldwide, discrepancies with regard to nomenclature of numerous species by different workers are

to be found in the literature. These differences have, therefore, led to a degree of confusion.

The genus *Fusarium* was first described by Link in 1808 (Ueno, 1983). In 1935, the genus was divided into 16 sections, 55 varieties and 22 forms (Wollenweber and Reinking, 1935). In 1945 this classification system was modified and the number of species was reduced to 9 (Snyder and Hansen, 1945). This system has been extensively used in several parts of the world. Toussoun and Nelson (Toussoun and Nelson, 1968) published an illustrated guide as an aid to the identification of the fusaria based on the Snyder and Hansen classification system.

Due to the confusion aroused within the literature, Nelson and co-workers (Nelson et al., 1983) published an illustrated guide to the identification of the genus, based predominantly on the classification system of Wollenweber and Reinking, (1935). However, the authors also incorporated certain characteristics of other classification systems. This attempt to establish a single universal system was augmented by the publication of a book encompassing the identity and mycotoxicological data of the toxigenic *Fusarium* species (Marasas et al., 1984). In this study the system of Nelson et al., (1983) will be used when reference is made to any *Fusarium* species.

1.4 HUMAN AND ANIMAL MYCOTOXICOSIS

Since an integral part of this study concerns the prevalence of three specific *Fusarium* species (ie *F. graminearum* Schabe of the Section *Discolor* and *F. moniliforme* Sheldon and *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas of the Section *Liseola*), the mycotoxicological data pertaining to these species will be dealt with separately in sections 1.5 to 1.7.

Mould induced food poisoning has posed a threat to human health for more than 2000 years. It has been suggested that an association exists between food-borne fungi and the plagues of Egypt, during biblical times (Schoental, 1984).

The oldest known outbreak of human mycotoxicosis is that of "ergotism". A disease occurred sporadically throughout central Europe during the Middle Ages, which was characterised by a sensation of cold hands and feet which progressed to an intense burning sensation. In advanced cases the extremities of the victim became gangrenous and necrotic. Belief on the part of affected victims that relief from the intense burning sensations could be sought by visiting the shrine of St Anthony, resulted in the disease adopting the popular name "St Anthony's fire". The disease became associated with the ingestion of bread made from rye infected with the fungus *Claviceps purpurea* (Shank, 1978). The fungus produced ergots, but it was only in the seventeenth century that

alkaloids produced by the ergots, were found to be responsible for the disease.

In the years 1942-1947 septic angina or alimentary toxic aleukia (ATA), a serious and often fatal disease, accompanied by extreme leukopenia, sepsis and the depletion of bone marrow, occurred widely in the Orenburg district of the USSR (Joffe, 1978). It is estimated that ATA claimed the lives of approximately 10% of the population of the region (corresponding to hundreds of thousands of people), following the ingestion of overwintered grain infected with *Fusarium poae* Peck and *Fusarium sporotrichioides* Sherb. Initially, ATA was thought to be due to a deficiency of vitamins B₁ and C and riboflavin. However, later work resulted in the isolation of the potent trichothecene T-2 toxin from cultures of *F. poae* and *F. sporotrichioides* isolated from the overwintered grains (Bamburg and Strong, 1971; Mirocha and Pathre, 1973). Using the isolated toxin, the characteristic symptoms of ATA were reproduced in cats (Lutsky et al., 1978), thereby substantiating the role of T-2 toxin in the etiology of ATA.

Cases of animal mycotoxicoses have been well documented (Christensen, 1979; Marasas et al., 1984). Many of these outbreaks have been related to the consumption of feeds contaminated with a wide range of mycotoxins produced by various genera of toxigenic fungi. Distinct diseases or syndromes emanating from specific genera of fungi or group of mycotoxins have been collectively named (ie Fusariotoxicosis is

the poisoning of animals resulting from the consumption of feed contaminated by toxin(s) produced by fungi of the genus *Fusarium*). Similarly, aflatoxicosis and ochratoxicosis relate to syndromes having their etiology linked to the presence of aflatoxins and ochratoxin, respectively.

A specific example of fusariotoxicosis is that of the haemorrhagic syndrome (mouldy maize toxicosis) which is associated with the consumption of mouldy cereals contaminated with *F. sporotrichioides*. Outbreaks of the syndrome are characterised by bloody diarrhoea, necrotic oral lesions, haemorrhagic gastro-enteritis and extensive haemorrhages in many organs in animals such as cattle, pigs and poultry (Marasas et al., 1984). The characteristic pathological changes have been reproduced experimentally with crystalline trichothecenes isolated from cultures of toxic strains of *F. sporotrichioides* associated with outbreaks of the syndrome (Marasas et al., 1984). In the United States, Australia and New Zealand, sporadic outbreaks of a disease in cattle, known as "fescue foot", has been characterised by lameness, loss of weight, arched back, elevated body temperature and dry gangrene involving the hind feet, tail tip and ears. Only one of the characteristic features of this disease (ie necrosis of the tip of the tail) has been reproduced experimentally by the administration of butenolide, a toxin known to be produced by *F. sporotrichioides* (Grove et al., 1970; Kosuri et al., 1970). However since butenolide has never been found to occur naturally in toxic fescue hay, the

causative role of butenolide and *F. sporotrichioides* in fescue foot of cattle has not been proven (Marasas et al., 1984).

1.5 *FUSARIUM GRAMINEARUM* SCHWABE

1.5.1. Incidence and Distribution

Fusarium graminearum belongs to the Section Discolor (Nelson et al., 1983). It has a worldwide distribution as a pathogen that causes root-, foot-, crown-, stem-, and ear rot as well as head blight (scab) of cereals (Marasas et al., 1984). In Australia, *F. graminearum* is the predominant *Fusarium* fungus associated with crown rot of wheat (Burgess et al., 1975). Two populations of the fungus have been differentiated (Francis and Burgess, 1977) and designated as *F. graminearum* Group 1 and Group 2. Members of Group 1 are normally associated with diseases of the crowns of plants, whereas those members of Group 2 are associated with diseases of the aerial parts of the plants.

1.5.2. Association of *F. graminearum* with human diseases

Outbreaks of "scabby grain" intoxication or "Alkakabi-byo" in humans have been reported in both the USSR and Japan. In the USSR, victims suffered headaches, chills, nausea, vertigo, vomiting and visual abnormalities (Dounin, 1926), following

the ingestion of bread prepared from "scabby" rye infected with *F. graminearum*. Several outbreaks of the syndrome have occurred in Japan and Korea (Yoshizawa, 1983), where the disease is more commonly known as "Akakabi-byo". In two separate incidents, a number of people in Hokkaido, Japan complained of headaches, chills and abdominal pains amongst other symptoms, following the consumption of noodles prepared from red-mould contaminated flour (Kurata, 1978). *F. graminearum* was the predominant species isolated from Japanese barley, oats, rye and rice (Tsunoda, 1970; Yoshizawa and Morooka, 1977). A large scale cereal scab epidemic in Korea (caused by *F. graminearum*), resulted in 80% losses in the barley yield (Yoshizawa, 1983). Similar clinical symptoms as previously described resulted from the ingestion of food prepared from the infected grain. The possibility that *F. sporotrichioides* was also involved in Akakabi-byo has been reported (Marasas et al., 1984). Although both nivalenol and deoxynivalenol (two type B trichothecenes amongst other mycotoxins known to be produced by *F. graminearum* and *F. sporotrichioides*) have frequently been reported to occur naturally in Japanese grains and grain products (Tanaka et al., 1985a, 1985b), neither of these potentially potent trichothecenes have been implicated in any case of human mycotoxicoses.

1.5.3. Association of *F. graminearum* with animal diseases

Many animals, particularly pigs, have refused to eat *F. gra-*

minearum infected grain, whilst those animals ingesting even small amounts of the scabby grain were found to vomit. The active compound was eventually isolated and characterised (Vesonder et al., 1973). The compound was given the trivial name "vomitoxin", which is still used extensively, although it is more correctly termed "deoxynivalenol".

Hyperestrogenism (vulvo-vaginitis), a disease normally associated with pigs, is characterised by a number of clinical signs including pseudo-estrus and swollen vulvas in young gilts, prolapse of the vagina, enlargement of the mammary glands and infertility (Nelson et al., 1973; Aucock et al., 1980). In extreme cases death can result from septicemia following necrosis of the exposed prolapsed parts. McNutt and co-workers (McNutt et al., 1928), were the first to associate hyperestrogenism with the consumption of mouldy maize. The disease was induced with maize cultures of *F. graminearum*, isolated from fungally contaminated feed implicated in an outbreak of porcine hyperestrogenism (Stob et al., 1962). Using a bioassay technique the causative compound was isolated and given the trivial name F-2 toxin (Christensen et al., 1965). The chemical structure of F-2 toxin was determined (Urry et al., 1966) and the name of the toxin was changed to "zearalenone".

1.5.4. Mycotoxins produced by *F. graminearum*

The following mycotoxins have been reported to be produced by

F. graminearum in culture (Marasas et al., 1984):

butenolide, deoxynivalenol, deoxynivalenol monoacetate, deoxynivalenol diacetate, diacetoxyscirpenol, fusarenon-X, HT-2 toxin, monoacetoxyscirpenol, neosolaniol, nivalenol, T-2 toxin and zearalenone.

1.6 *FUSARIUM MONILIFORME* SHELDON

1.6.1. History and distribution

Fusarium moniliforme belongs to the section *Liseola* (Nelson et al., 1983) and is one of the most prevalent fungi associated with human and animal dietary staples, occurring on a wide variety of plant hosts. Saccardo (Saccardo, 1881) first described the fungus, found on Italian maize kernels, naming it *Oospora verticillioides*. This fungus was implicated as a possible cause of the disease "pellagra" due to its prevalence in maize associated with the disease in Italy and Russia (Tiraboschi, 1905; Von Deckenbach, 1907). Widespread outbreaks of an animal disease in the United States were reported (Peters, 1904). The fungus most commonly associated with the maize consumed by the animals was identified as *F. moniliforme* Sheldon (Sheldon, 1904). It was later shown that *F. moniliforme* was identical to *O. verticillioides* (Manns and Adams, 1923).

1.6.2. Association of *F. moniliforme* with human diseases

In southern Africa, the highest human oesophageal cancer rate occurs in the south-western districts of the Transkei, while the rate is relatively low in the north-eastern region of the Transkei (Marasas et al., 1981, 1988a), where maize is the dietary staple in both areas. In a comparative study of the mycoflora of the local home-grown maize of the two areas, a significantly higher incidence of *F. moniliforme* was found to be present in the maize produced in the high-rate area of the Transkei (Marasas et al., 1981, 1988a). Several isolates of *F. moniliforme* taken from maize emanating from the high-rate area have been found to be acutely toxic to ducklings. The acute and long term effects in experimental animals of two of these isolates (MRC 826 and MRC 602) have been determined and although these isolates have been shown to affect a variety of organs in different animal species (Marasas et al., 1984), no experimental evidence has been obtained to indicate that they can cause oesophageal cancer. In China, *F. moniliforme* is one of the most frequently encountered fungi associated with foodstuffs in the Lin Xian county in Henan province, which is one of the highest oesophageal cancer risk areas of the world (Li et al., 1979, 1980).

1.6.3. Association of *F. moniliforme* with animal diseases

Equine leukoencephalomalacia (LEM) is a neurotoxic disease of

horses, donkeys and mules. It is characterised by liquefactive necrotic lesions in the white matter of the cerebral hemispheres (Marasas et al., 1984). Mortalities in horses, normally preceded by marked nervous symptoms prior to death, have been recorded since 1850. The disease has been reproduced experimentally in horses on a number of occasions, by feeding naturally contaminated mouldy maize (Butler, 1902; Biester et al., 1940). It was only in 1971 that *F. moniliforme* was identified as the causative fungus (Wilson, 1971; Wilson and Maronpot, 1971). Culture material of *F. moniliforme* MRC 826, isolated from maize intended for human consumption in the Transkei, has also been shown to induce LEM in horses (Kriek et al., 1981). Clinical signs similar to those of equine LEM have also been reported in "bean hulls poisoning", a disease of horses which is prevalent in the Hokkaido district of Japan (Ueno et al., 1972). However, as yet it is not known whether this disease is identical to LEM.

Efforts to isolate and characterise the *F. moniliforme* mycotoxin responsible for LEM has been in progress for approximately 20 years. Using a bioassay technique as a monitor for cancer-promoting principles, the fumonisins were isolated from culture material of *F. moniliforme* MRC 826 (Gelderblom et al., 1988). The major compound isolated was given the trivial name "fumonisin B₁" - (FB₁). LEM was reproduced experimentally in a horse by the intravenous injection of FB₁ isolated from MRC 826 (Marasas et al., 1988b).

1.6.4. Mycotoxins produced by *F. moniliforme*

The following mycotoxins have been reported to be produced by *F. moniliforme* in culture (Marasas et al., 1984, Gelderblom et al., 1988):

fusaric acid, fusarin C, fusariocins, moniliformin, zearalenone, fumonisin B₁, fumonisin B₂.

1.7 *FUSARIUM SUBGLUTINANS* (WOLLENWEBER & REINKING) NELSON, TOUSSOUN AND MARASAS

1.7.1. Incidence and distribution

Fusarium subglutinans belongs to the Section Liseola (Nelson et al., 1983). Relatively little information is available on the incidence and distribution of *F. subglutinans* due to its apparent misidentification as *F. moniliforme* (Booth, 1971). It has been suggested that *F. subglutinans* occurs on a wide range of hosts, in many areas of the world (Marasas et al., 1984). Booth (Booth, 1971) claimed that *F. subglutinans* had a lower optimum temperature for growth and that it would be more predominant in more temperate climates than *F. moniliforme*.

1.7.2. Association of *F. subglutinans* with human diseases

Fusarium subglutinans is one of the most prevalent fungi associated with home-grown maize produced in the high oeso-

ophageal cancer risk areas of the Transkei (Marasas et al., 1981). One of the isolates of *F. subglutinans* from Transkeian maize was shown to be highly toxic to ducklings and rats and to produce large quantities of the *Fusarium* mycotoxin moniliformin, in culture (Kriek et al., 1977). There is, however, no evidence that either *F. subglutinans* or moniliformin are involved in the etiology of oesophageal cancer.

1.7.3. Mycotoxins produced by *F. subglutinans*

The following mycotoxins have been reported to be produced by *F. subglutinans* in culture (Marasas et al., 1984):
fusaric acid, moniliformin

1.8 MYCOTOXINS RELEVANT TO THIS STUDY

1.8.1. Selected mycotoxins

Although the fusaria in general, and the species outlined in sections 1.5 to 1.7 in particular, have been shown to produce a diverse range of secondary metabolites, only a few of these metabolites occur naturally (the majority occurring only in cultures). For the purpose of this study, the presence of 7 of these mycotoxins (deoxynivalenol, diacetoxyscirpenol, fumonisin B₁, moniliformin, nivalenol, T-2 toxin and zearalenone) will be taken into consideration. The chemical characteristics of each of these toxins will be

discussed in the following sections (1.8.2 to 1.8.10).

1.8.2. The trichothecenes - introduction

The trichothecenes are a chemically related group of mycotoxins produced by various strains of *Fusarium*, *Myrothecium*, *Trichoderma* and other genera of imperfect fungi. The first known trichothecene, trichothecin, was isolated from a culture of *Trichothecium roseum* Link (Freeman and Morrison, 1948). Phytotoxic metabolites were isolated from *F. scirpi* Lambotte & Fautr. (Brian et al., 1961), the major metabolite being named diacetoxyscirpenol, based on the fungal name and the presence of two acetyl groups present in the structure. Within several years, T-2 toxin, nivalenol and its acetylated derivative, fusarenon-X, had been isolated (Bamburg et al., 1968, Morooka and Tatsuno, 1970). Although to date, in excess of 70 trichothecenes have been isolated and chemically characterised, the vast majority of these only occur in cultures under controlled laboratory conditions. Only a few of the trichothecenes (including nivalenol, deoxynivalenol, diacetoxyscirpenol and T-2 toxin) have been found to occur naturally on food and feedstuffs (Ueno, 1983; Kurata and Ueno, 1983), and it is these four trichothecenes which will be included in this study.

1.8.3. The trichothecenes - structural classification

The collective name for the structure of a tricyclic skeleton

composed of cyclohexane, cyclopentane, 6-membered oxirane rings and four methyl groups was proposed as "trichothecane" (Godtfredsen et al., 1967). However, most of the naturally occurring compounds contain an epoxide ring and a double bond at C-12,13 and C-9,10, respectively, and are therefore, characterised by the 12,13-epoxytrichothec-9-ene ring system, hence the name "trichothecene", the basic structure of which is shown in Fig. 1.1.

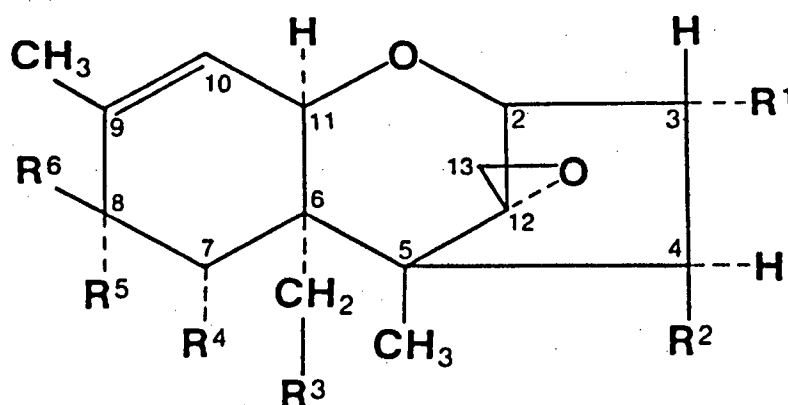
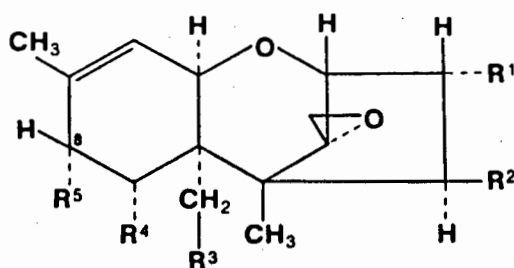


FIG. 1.1 Basic structure of the 12,13-epoxytrichothec-9-enes

Based on a number of chemical characteristics, the trichothecenes have been divided into 4 distinct groups (or types), namely A, B, C and D (Ueno, 1983). The four trichothecenes included in this study are members of groups A and B. Group A trichothecenes (diacetoxyscirpenol and T-2 toxin) lack a carbonyl at the C-8 position and have hydroxyl or acetyl groups at R^1 , R^2 , R^3 and R^4 (Table 1.1). Group B trichothecenes

(deoxynivalenol and nivalenol) have a carbonyl at the C-8 position and mostly hydroxyls at R^1 , R^2 , R^3 and R^4 (Table 1.2).

TABLE 1.1 CHEMICAL STRUCTURES OF GROUP A TRICOTHECENES INCLUDED IN THIS STUDY

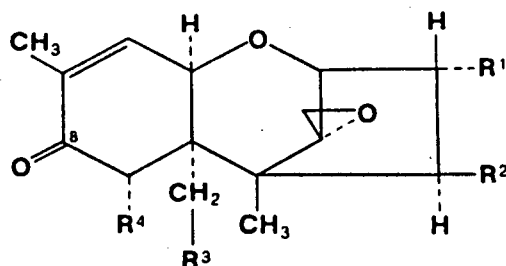


<u>Trichothecene</u>	<u>R^1</u>	<u>R^2</u>	<u>R^3</u>	<u>R^4</u>	<u>R^5</u>
Diacetoxyscirpenol	OH	OAc	OAc	H	H
Neosolaniol monoacetate	OH	OAc	OAc	H	OAc
T-2 toxin	OH	OAc	OAc	H	Oiv

OAc = Acetate ($-O-COCH_3$)

Oiv = Isovalerate ($-O-COCH_2CH(CH_3)_2$)

TABLE 1.2 CHEMICAL STRUCTURES OF GROUP B TRICHOTHECENES
INCLUDED IN THIS STUDY



<u>Trichothecene</u>	<u>R¹</u>	<u>R²</u>	<u>R³</u>	<u>R⁴</u>
Nivalenol	OH	OH	OH	OH
Fusarenon-X	OH	OAc	OH	OH
Deoxynivalenol	OH	H	OH	OH

1.8.4. The trichothecenes - physico-chemical properties

The trichothecenes are generally colourless, crystalline compounds, which are soluble in moderately polar organic solvents such as ethanol, acetone, ethyl acetate and chloroform, but only slightly soluble in water (Ueno, 1980). Due to the absence of any conjugated double bonds in their

structures, the group A trichothecenes do not exhibit UV absorbance in either the ultraviolet or visible region of the spectrum, nor any fluorescence characteristics. Conversely, the presence of carbonyl group at the C-8 position present in the structures of the group B trichothecenes results in an absorbance between 215-226 nm.

1.8.5. Zearalenone - introduction and structure

Although zearalenone is referred to as a mycotoxin it should be classified as a non-steroidal hormone as it is non-toxic and induces estrogenic activity in animals, especially pigs, at relatively low dietary levels (Christensen, 1979). The chemical structure of zearalenone was elucidated by Urry et al., (1966) as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β -resorcylic acid- μ -lactone. Zearalenone exists in equilibrium between the *cis* and *trans* isomers (Fig. 1.2), however under ultraviolet irradiation the equilibrium is moved in the direction of the *cis* isomer.

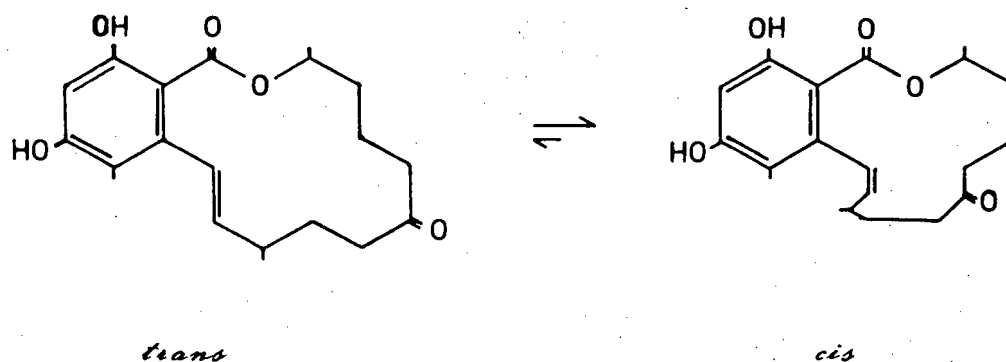


FIG. 1.2 Chemical structure of zearalenone isomers

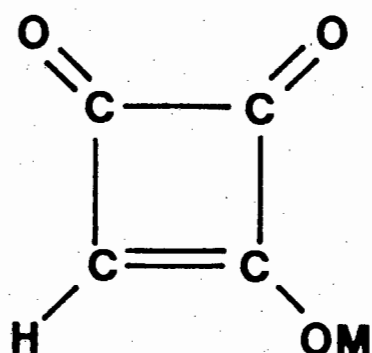
Several derivatives of zearalenone have been reported, but the majority of these have only been detected in culture or after microsomal metabolism by mammalian systems (Mirocha et al., 1977).

1.8.6. Zearalenone - physico-chemical characteristics

Zearalenone is an optically active, white, odourless crystalline compound with a melting point range of 161-164°C, and a molecular mass of 318 (Pohland et al., 1982). The compound is soluble in ethanol, benzene and dichloromethane but only partially soluble in water. The ultraviolet spectrum exhibits absorption maxima at 236 nm, 274 nm and 316 nm (Pohland et al., 1982). Although zearalenone exhibits fluorescence characteristics, some confusion exists in the literature concerning the corresponding fluorescence data (Trenholm et al., 1984; Bennett et al., 1985; Bagneris et al., 1986).

1.8.7. Moniliformin - introduction and structure

Moniliformin (Fig. 1.3) was first isolated from *F. moniliforme* and identified by x-ray crystallography as the potassium salt of 3-hydroxycyclobut-3-ene-1,2-dione (Cole et al., 1973; Springer et al., 1974). However the free acid had previously been chemically synthesized (Hoffman et al., 1971; Pinske, 1972).



M = Na or K

FIG. 1.3 Chemical structure of moniliformin

1.8.8. Moniliformin - physico-chemical characteristics

Moniliformin is soluble in water and exhibits absorption maxima at 229 nm and 254 nm in the ultraviolet region of the spectrum (Cole and Cox, 1981). Isolated from a number of *Fusarium* species as the potassium, sodium, or a mixture of both salts, it decomposes without melting at temperatures up to 350°C.

1.8.9. Fumonisin (B₁ and B₂) - introduction and structure

Using a short-term cancer initiation-promotion bioassay with diethylnitrosamine-initiated rats and the induction of gamma-glutamyl-transpeptidase-positive (GGT+) foci as endpoint, the fumonisins were isolated from maize culture material of *F. moniliforme* MRC 826 (Gelderblom et al., 1988). Two distinct compounds were isolated and given the trivial names "fumoni-

most probably exists as the salt form in nature. It does not exhibit any absorbance or fluorescence characteristics in the ultraviolet or visible regions of the spectrum.

1.9 *FUSARIUM* MYCOTOXIN ANALYTICAL METHODOLOGY AND OBJECTIVES OF THE STUDY

A number of analytical methods have been developed and published for the determination of single or groups of mycotoxins in a number of different matrices. Although desirable, methods for the co-determination of several unrelated mycotoxins (so called multi-mycotoxin methods), have been reported and reviewed (Steyn, 1981), but on the whole, these methods have been found to be impractical. The majority of these methods have utilized numerous and often laborious pre-chromatographic sample preparation procedures followed by thin layer chromatographic (TLC) separation. Due to the diversity of the mycotoxins with regard to their respective chemical characteristics, these multi-mycotoxin methods have suffered from highly variable recoveries for the separate mycotoxins.

Consequently, since the early 1980's, there has been a perceptual shift in emphasis, away from the multi-mycotoxin approach towards the development of methods aimed at the determination of singular or groups of closely related mycotoxins, in food substrates. Several books dealing with mycotoxin methodology have been published, a most recent and com-

prehensive addition being that edited by Dr R.J. Cole (Cole, 1986).

It is interesting to note that TLC is still used extensively for the estimation of mycotoxin contamination. In a recently published survey (Van Egmond, 1989), 66 countries responded to enquiries concerning existing or proposed legislation and methodology, pertaining to mycotoxin contamination of food. With regard to the aflatoxins (the most regulated of the mycotoxins), 64% of the countries reported having regulations and official methods of analysis, while 36% of the countries reported that regulations were either currently being considered, proposed, or were in fact non-existent. Only 28% of those countries having official methods, however, used analytical techniques other than TLC (Van Egmond, 1989).

The 1984 edition of the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC, 1984) lists predominantly TLC methods of analysis for the determination of each mycotoxin within the section concerning Natural Poisons. The general referee for mycotoxins, Dr P.M. Scott, regularly reviews the literature pertaining to mycotoxin research including methodology. In the last three reports (Scott, 1987, 1988, 1989), publications concerning mycotoxin methodology have included the increased use of more sophisticated separation and detection techniques, such as high performance liquid chromatography, gas chromatography and mass spectrometry, and it is foreseen that these

techniques will be adopted by the AOAC, if not as replacement techniques for TLC analysis, then as confirmatory techniques. The use of radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques, as screening procedures (predominantly for the analysis of the aflatoxins) also appear to have gained a great deal of attention (Park et al., 1989).

This investigation deals with the optimization of the conditions for the determination of 7 selected *Fusarium* mycotoxins in food, through the following objectives:

1. The screening and comparison of, where possible, existing toxin methodology.
2. Where necessary, the optimization of existing methodology.
3. Where necessary, the development of innovative methodology.
4. The application of the most suitable methods for the determination of the selected toxins in a series of home-grown maize samples from different human oesophageal cancer risk areas of the Transkei, southern Africa.
5. Where possible, the verification of the presence or absence of the selected toxins determined in objective

number 4.

6. The statistical correlation between the toxin analytical and previously generated mycological results, for those samples covered in objective number 4.

An overview of the parameters covered in this investigation is represented schematically in Figure 1.5.

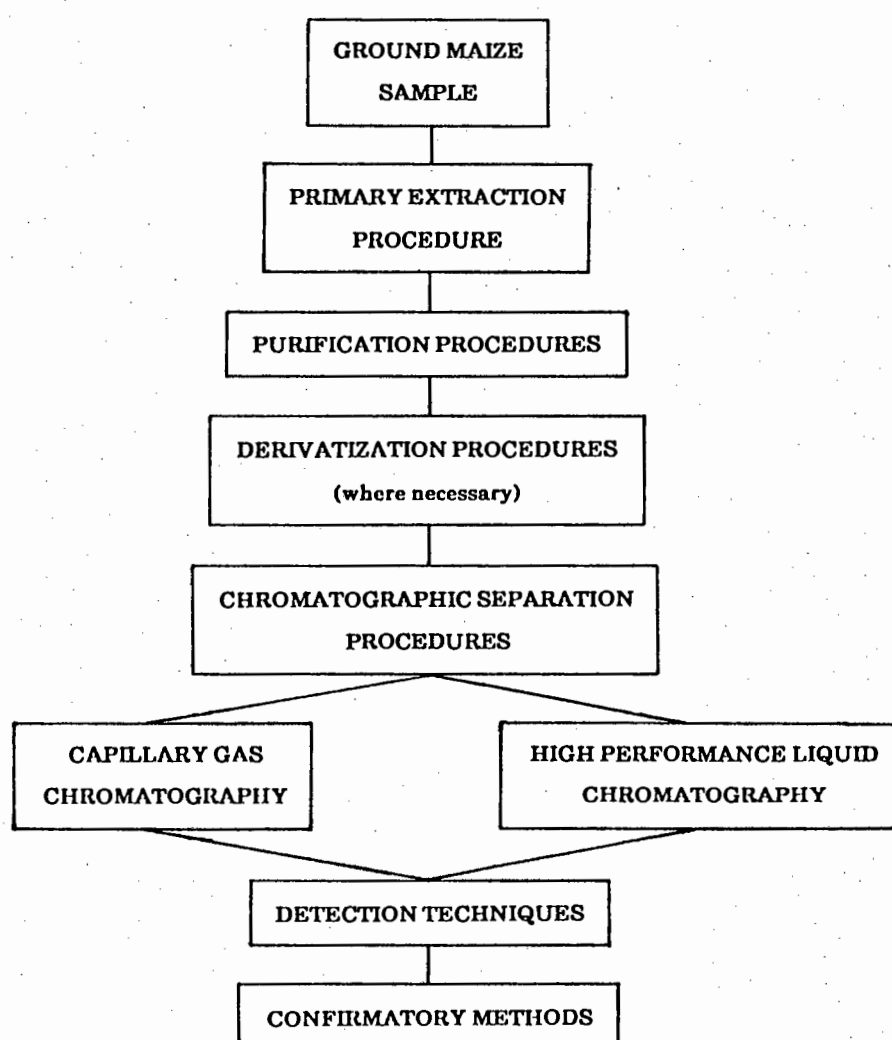


FIG. 1.5 Schematic representation of investigation

CHAPTER 2

EXPERIMENTAL

2.1 SAMPLE MATERIALS

2.1.1 Control maize

First grade, yellow maize kernels were used as a control matrix to evaluate a number of the analytical methods for the determination of the *Fusarium* mycotoxins listed in Sections 1.8.1-1.8.10. The kernels were dried at 50°C before being finely ground in a laboratory mill and the material was maintained at 5°C prior to analysis. Recovery experiments were conducted using this control maize, to which authentic mycotoxin standards of known concentration were added prior to extraction and analysis.

2.1.2 Fungal cultures

Samples of maize kernels on which selected fungi had been grown were also used for the evaluation of the analytical methods for the determination of the trichothecenes diacetoxyscirpenol and T-2 toxin, and for FB₁ and FB₂. Lyophilized conidia of *F. armeniacum* (MRC 4481) and *F. moniliforme* (MRC 826) from the collection of the South African Medical Research Council (MRC), were used to inoculate autoclaved yellow maize kernels (400 g maize and 400 ml water) in 2-

litre glass fruit jars. The cultures were incubated at 25°C in the dark for 21 days, after which the material was dried (50°C for 24 hours) and finely ground in a laboratory mill. The dried fungal culture material was stored at 5°C prior to analysis.

2.1.3 Transkeian maize sample - evaluation of fumonisin B₁ methodology

A sample of mouldy maize ears of the 1978 crop was also used to evaluate the fumonisin B₁ methodology. The sample was obtained from a farm in the Butterworth district of Transkei, during July 1978 (Thiel et al., 1982). Visibly *Fusarium* infected ears were selected and hand shelled. The kernels (a mixture of *Fusarium*-infected, healthy, and kernels infected with other fungi) were retained as sample M-84 (Thiel et al., 1982; Gelderblom et al., 1984). Two subsamples, one containing predominantly healthy kernels and the other predominantly *Fusarium*-infected kernels, were selected from sample M-84 and retained as samples M-84/C and M-84/F, respectively. Each sample was ground in a laboratory mill and stored at 5°C prior to analysis.

2.1.4 Transkeian maize samples - collection and mycology

The maize samples described in sections 2.1.1.-2.1.3, were used primarily for the evaluation of the *Fusarium* mycotoxin analytical methodology. The final phase of this study was the

application of the most suitable methods for each mycotoxin, to a series of home-grown maize samples collected from areas of the Transkei.

Samples of home-grown maize of the 1985 crop intended for human consumption were collected from 12 households each in the Kentani and Bizana areas of the Transkei (Marasas et al., 1988). The maize ears had previously been separated and stored by the housewife of each household into "good" ears (intended for porridge making) and "mouldy" ears (for beer making and animal feed). The ears were hand shelled and the fungi isolated from 100 surface-sterilised kernels per sample as previously described (Marasas et al., 1981). *Fusarium* species were identified according to the system of Nelson and co-workers (Nelson et al., 1983). Kernels harvested from the "mouldy" maize ears were finely ground in a laboratory mill, stored at 5°C and used for the purpose of this study. Mycological data pertaining to these samples will be presented in the results section for statistical purposes, although the mycological data itself was generated by Prof W.F.O. Marasas (Marasas et al., 1989).

2.2 ANALYTICAL STANDARDS

The analytical standards used in this investigation were either obtained from commercial sources or were previously isolated within the Institute. The identity and purity of each standard was assessed using a number of techniques in-

cluding: thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), melting point, infra-red (IR), ultra-violet (UV), fluorescence and nuclear magnetic resonance spectroscopy, and mass spectrometry (MS). The results generated were, where possible compared with those previously published (Cole and Cox, 1981; Pohland et al., 1982).

2.3 EVALUATION OF EXISTING METHODOLOGY

2.3.1 General overview

Several published methods for the determination of the *Fusarium* toxins previously mentioned in sections 1.8.1 - 1.8.10, in maize and other substrates, were evaluated. Sample preparation and chromatographic separation of these methods will be dealt with separately in sections 2.3.2 - 2.6.3.

2.3.2 Analytical methodology - type A trichothecenes

Two methods for the simultaneous determination of diacetoxyscirpenol and T-2 toxin in maize were evaluated (Cohen and Lapointe, 1984; Sydenham and Thiel, 1987).

2.3.2.1 Sample preparation - (Cohen and Lapointe, 1984)

A subsample of ground maize (50 g) was extracted with methanol/water (1:1, 250 ml) by blending at high speed for 5

minutes. The mixture was then centrifuged for 5 mins at 2000 rpm, followed by filtration. An aliquot of the filtrate (50 ml) was mixed with 30% aqueous ammonium sulphate (100 ml) and Celite (20 g), stirred for 2 mins and filtered. The filtrate was partitioned with ethyl acetate (4 x 100 ml) which was then dried with anhydrous sodium sulphate, re-filtered and evaporated to dryness under vacuum. The residue, dissolved in dichloromethane (2 ml) was transferred to a silica gel Sep-pak cartridge, the flask being washed with further aliquots of dichloromethane (3 x 5 ml) which were quantitatively transferred to the same cartridge. The eluate (first 5 ml) from the cartridge was discarded, the remaining eluate being collected. The cartridge was then washed with methanol/dichloromethane (5:95, 15 ml), the eluate being added to that already collected. The combined eluates were subsequently evaporated to dryness under vacuum, the residue redissolved in chloroform/hexane (1:1, 2 ml) and transferred to a Bond-Elut cyano extraction column. The toxins were eluted from the column with chloroform/hexane (1:1, 13 ml) and the eluate evaporated to dryness under vacuum. The residue represented a 10 g equivalent sample weight. A 2 g equivalent sample weight of the residue was placed in a second vial and retained for chromatographic analysis.

2.3.2.2 Sample preparation - (Sydenham and Thiel, 1987)

This method was initially developed for the analysis of fungal cultures, but was also found to be suitable for the de-

termination of diacetoxyscirpenol and T-2 toxin in grain samples.

A subsample of ground maize (25 g) was extracted with methanol/water (1:1, 100 ml), by agitating at high speed for 30 minutes on a wrist action shaker. An aliquot (20 ml) of the filtered extract was transferred to an Extrelut 20 column and left for several minutes for adsorption purposes. The toxins were eluted with cyclohexane/dichloromethane (1:3, 40 ml), the eluate being collected and evaporated to dryness. The dried residue, dissolved in chloroform/hexane (1:3, 8 ml) was transferred to a silica gel Sep-pak cartridge. The cartridge was washed with benzene (6 ml) and acetone/benzene (5:95, 8 ml). The purified toxins were eluted from the cartridge with diethyl ether (6 ml), the eluate being collected and evaporated to dryness under vacuum. This residue represented a 5 g equivalent sample weight.

2.3.3 Analytical standards - diacetoxyscirpenol and T-2 toxin

The second method evaluated (Sydenham and Thiel, 1987) incorporated the use of another type A trichothecene (neosolaniol monoacetate - Table 1.1) as an internal standard prior to chromatographic separation. Diacetoxyscirpenol, T-2 toxin and neosolaniol monoacetate stock solutions were prepared in glass-distilled methanol ($500 \mu\text{g ml}^{-1}$). Aliquots (20 μl) of the diacetoxyscirpenol and T-2 toxin stock solutions were placed in one vial while a similar aliquot of the neosolaniol

monoacetate stock solution was placed in a separate vial. The contents of each vial were evaporated to dryness and derivatized as outlined in section 2.3.4. Following derivatization, 5, 10, 15 and 20 μl aliquots of the organic phase containing the standard toxin esters were placed in each of four separate vials to which 15 μl of the organic phase containing the ester of the internal standard were added. The contents were evaporated to dryness under nitrogen, redissolved in hexane (1 ml), capped, mixed and analysed by capillary gas chromatography. A calibration curve (covering the range 50 - 200 ng ml^{-1} diacetoxyscirpenol and T-2 toxin with 150 ng ml^{-1} internal standard) was constructed for each toxin by plotting the ratio of the toxin peak areas to that of the internal standard against toxin concentration.

2.3.4 Derivatization procedure - type A trichothecenes

Both methods required the formation of the heptafluorobutyryl derivatives of the purified extracts prior to capillary GC separation

The residues (transferred to 4 ml vials) were dissolved in toluene/acetonitrile (95;5, 1 ml) and heptafluorobutyrylimidazole (HFBI, 100 μl) was added. The vials were capped, the contents mixed and heated at 60°C for 1 hour. After cooling, sodium phosphate buffer (0.1M, pH 6.0, 1 ml) was added (to neutralize any excess HFBI) and the contents mixed. The

phases were allowed to separate and a suitable aliquot of the upper organic phase was removed, evaporated to dryness and redissolved in hexane (1 ml), prior to analysis by capillary gas chromatography. All samples were chromatographed on the same day as derivatization.

2.3.5 Chromatographic separation - type A trichothecenes

Samples were chromatographed using a Carlo Erba Model 5300 high resolution gas chromatograph, equipped with a ^{63}Ni electron capture detector (ECD) used in the constant frequency mode, split injector and a 25 m x 0.32 mm (i.d.) fused silica capillary column coated with a 0.25 μm film thickness of PS 255. Data were collected using a Waters 745 data module. The exact chromatographic conditions are given in Table 2.1.

2.4 ANALYTICAL METHODOLOGY - TYPE B TRICHOTHECENES

Two methods for the simultaneous determination of nivalenol and deoxynivalenol in maize were evaluated (Tanaka et al., 1985a; Scott et al., 1986).

2.4.1.1 Sample preparation - (Tanaka et al., 1985a)

A subsample (20 g) was extracted with acetonitrile/water (3:1, 200 ml) by shaking for 30 minutes on a wrist action shaker. The mixture was then filtered and an aliquot of the filtrate (125 ml) was partitioned with hexane (100 ml). The

TABLE 2.1 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF DIACETOXYSCIRPENOL AND T-2 TOXIN

Column	PS-255 25 m x 0.32 mm (i.d.)
Film thickness	0.25 μ m
Carrier gas (flow rate)	Helium at 35 cm sec ⁻¹
Make-up gas (flow rate)	Nitrogen at 30 ml min ⁻¹
Detector	ECD (⁶³ Ni)
Attenuation	64
Injection volume	1 μ l
Split ratio	1 : 10
Injector temperature	200°C
Detector temperature	300°C
Temperature profile	(1) 180°C - 220°C at 4°C min ⁻¹ (2) 220°C - 250°C at 15°C min ⁻¹ (3) 250°C for 5 min

upper hexane layer was discarded and ethanol (120 ml) was added to the aqueous acetonitrile layer and evaporated to dryness under vacuum at 45°C. The residue was dissolved in methanol (5 ml) and a 4 ml aliquot was transferred to the top of a wide bore chromatography column (30 cm x 2.2 cm i.d.), containing Florisil (20 g) packed between two layers of anhydrous sodium sulphate (2 x 10 g), in toluene. The column was washed with hexane (200 ml) and the toxins eluted with chloroform/methanol (9:1, 250 ml). The eluate was evaporated to dryness and redissolved in methanol (2 ml). An aliquot of the extract (1 ml) was mixed with chloroform (1 ml) and

hexane (50 ml), and placed on a silica-gel Sep-Pak cartridge. The cartridge was washed with n-hexane (50 ml) and the toxins eluted with ethyl acetate/toluene/methanol (20:5:1, 100 ml). The eluate was collected, evaporated to dryness, redissolved in methanol (2 ml) and an aliquot (1 ml) was reserved for derivatization and separation by capillary GC.

2.4.1.2 Sample preparation - (Scott et al., 1986)

A milled subsample (50 g) was extracted with methanol/water (7:3, 150 ml) by blending at high speed for 5 minutes in a Sorvall omnimixer. The "puree" was transferred to a centrifuge tube and centrifuged for 15 minutes at 2000 rpm. An aliquot of the clear supernatant (30 ml) was mixed for 2 minutes with 10% aqueous ammonium sulphate (120 ml) and diatomaceous earth, using a magnetic stirrer. The contents were then filtered and an aliquot of the filtrate (10 ml) transferred to an Extrelut 20 column. Following a period of time for absorption ($\pm$ 5 minutes), the toxins were eluted with ethyl acetate (160 ml), the eluate being collected and evaporated to dryness under reduced pressure. The residue was dissolved in dichloromethane/methanol (3:1, 0.5 ml) and placed on a small diameter chromatography column (10 mm i.d.) containing silica gel (2 g) embedded between two layers of anhydrous sodium sulphate (2 x 1 g), in toluene. The column was washed successively with toluene (15 ml) and hexane (15 ml), before the purified toxins were eluted with dichloromethane/methanol (9:1, 60 ml). The latter eluate was collected, evaporated to

dryness under reduced pressure, transferred to a 4 ml capacity vial and dissolved in dichloromethane/methanol (3:1, 1.33 ml). The solution represented a 0.5 g ml^{-1} sample equivalent weight. An aliquot of the solution (0.5 ml) was removed to a second vial and evaporated to dryness prior to derivatization and separation by capillary GC.

2.4.2 Analytical standards - nivalenol and deoxynivalenol

Nivalenol and deoxynivalenol stock solutions ($50 \mu\text{g ml}^{-1}$) were prepared in glass distilled methanol. Aliquots of the stock solutions ($50 \mu\text{l}$) were placed in a vial and evaporated to dryness prior to derivatization (section 2.4.3). Following derivatization, 2.5, 5, 10 and 15 μl aliquots were placed in separate vials and diluted to 500 μl with glass distilled hexane to give a concentration range of 25 - 150 ng ml^{-1} per toxin. A calibration curve for each toxin was constructed by plotting the peak area of each toxin against toxin concentration.

2.4.3 Derivatization procedure - nivalenol and deoxynivalenol

The type B trichothecenes also require derivatization due to their chemical characteristics.

Tri-Sil TBT (a commercially available mixture of N-trimethylsilylimidazole: N,O-bis(trimethylsilyl)acetamide: Trime-

thylchlorosilane, 3:3:2, v/v, 50 μ l) was added to each vial which was then capped, mixed and allowed to stand for 15 minutes at room temperature. Hexane (500 μ l) was then added to each vial followed by phosphate buffer (0.1M, pH 7, 2 ml). The contents of each vial were mixed, the layers allowed to separate, and a suitable aliquot of the upper organic phase removed to a second vial where the volume was made up to 500 μ l with hexane.

TABLE 2.2 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF NIVALENOL AND DEOXYNIVALENOL

Column	DB-5 30 m x 0.32 mm (i.d.)
Film thickness	0.25 μ m
Carrier gas (flow rate)	Helium at 35 cm sec ⁻¹
Make-up gas (flow rate)	Nitrogen at 48 ml min ⁻¹
Detector	ECD (⁶³ Ni)
Attenuation	32
Injection volume	1 μ l
Splitless (on time)	0.5 min
Injector temperature	180°C
Detector temperature	300°C
Temperature profile	(1) 80°C for 1 min (2) 80°C - 275°C at 10°C min ⁻¹

2.4.4 Chromatographic separation - nivalenol and deoxynivalenol

All samples were chromatographed using the instrumentation outlined in section 2.3.5. However, the capillary column used was a 30 m x 0.32 mm i.d fused silica DB-5 (0.25 μ m thickness). The exact chromatographic conditions are given in Table 2.2.

2.5 ANALYTICAL METHODOLOGY - ZEARALENONE

Three methods for the determination of zearalenone in maize were evaluated (Association of Official Analytical Chemists (AOAC), 1984; Chang and DeVries, 1984; Bagneris et al., 1986). The methods differed considerably with respect to their extraction and clean-up methodologies.

2.5.1.1 Sample preparation - (AOAC, 1984)

A subsample (50 g) was extracted with chloroform/water (10:1, 270 ml), to which diatomaceous earth (25 g) had been added, by shaking for 30 minutes on a wrist action shaker. The mixture was filtered and an aliquot of the filtrate (50 ml) was placed onto a chromatography column packed with silica gel (10 g) placed between layers of anhydrous sodium sulphate (2 x 5 g), in chloroform. the column was washed successively with hexane (150 ml) and benzene (150 ml) before the toxin was eluted with acetone/benzene (5:95, 250 ml). The latter

eluate was collected and evaporated to dryness under reduced pressure. The residue, redissolved in hexane (40 ml) was transferred to a small separating funnel. Zearalenone was partitioned into acetonitrile (3 x 10 ml), this layer being collected, evaporated to dryness and transferred to a vial in benzene (500 μ l).

2.5.1.2 Sample preparation - (Chang and DeVries, 1984)

A subsample (50 g) was extracted with dichloromethane/water (10:1, 270 ml) to which cupric carbonate (10 g) was added, by shaking for 30 minutes. The mixture was filtered and an aliquot of the filtrate (50 ml) was evaporated to dryness under reduced pressure. The residue was redissolved in acetonitrile (80 ml) and partitioned with petroleum ether (100 ml). The acetonitrile layer was collected and evaporated to dryness under reduced pressure. The residue was subsequently redissolved in methanol/water (4:1, 2 ml), prior to separation by HPLC.

2.5.1.3 Sample preparation - (Bagneris et al., 1986)

A subsample (30 g) was extracted with chloroform/water (12.5:1, 270 ml) by shaking for 15 minutes. The mixture was then centrifuged for 3 minutes at 2000 rpm. The extract was filtered and an aliquot of the filtrate (50 ml) transferred to a separatory funnel. Saturated sodium chloride solution (10 ml) and aqueous sodium hydroxide solution (2%, 50 ml) were then

added to the funnel, the contents shaken and the two layers allowed to separate. The lower organic layer was discarded and the aqueous layer partitioned with chloroform (50 ml). The organic layer was once more discarded and citric acid solution (0.5M, 50 ml) added to the funnel. Zearalenone was extracted from the solution by partitioning with dichloromethane (2 x 50 ml). The dichloromethane extracts were dried with anhydrous sodium sulphate, collected and evaporated to dryness under reduced pressure. The residue was redissolved in methanol/water (7:3, 500 μ l), prior to separation by HPLC.

2.5.2 Analytical standard - zearalenone

Zearalenone stock solution (50 μ g ml^{-1}) was prepared in methanol/water (7:3). Aliquots (0.25, 0.5, 0.75 and 1 ml) were diluted to 10 ml in separate volumetric flasks to yield a concentration range of 1.25 - 5 μ g ml^{-1} . These concentrations were verified by recording their UV absorptions at 236 nm and calculating the concentration using the molecular weight and molar extinction coefficients previously published (Cole and Cox, 1981) and the formula:

$$\text{Zearalenone concentration } (\mu\text{g ml}^{-1}) = \frac{\text{Abs (@ 236 nm)} \times 1000 \times \text{molecular wt. (318)}}{\epsilon \text{ (@ 236 nm)} = 29700}$$

2.5.3 Chromatographic separation - zearalenone

Reverse phase separations of 20 μ l aliquots of standards and samples were performed on a 4- μ m Nova-Pak C₁₈ column (15 cm x 3.9 mm i.d.). Mobile phase was delivered by means of a Waters Model 510 liquid chromatographic pump. Peak detection was performed on a Perkin-Elmer 650S fluorescence detector. The prevailing chromatographic conditions are presented in Table 2.3. Data were collected on a Waters 745 data module and quantitative determinations were by comparison of toxin peak areas against a calibration curve.

TABLE 2.3 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF ZEARELENONE

Column	Nova-Pak RP C ₁₈ (4- μ m) 15 cm x 3.9 mm (i.d.)
Mobile phase	Methanol : Water (7:3)
Flow rate	0.5 ml min ⁻¹
Injection volume	20 μ l
Detector	Fluorescence
Detector settings	274 nm (Excitation) 465 nm (Emission)
Slit widths	15 nm

2.6 ANALYTICAL METHODOLOGY - MONILIFORMIN

Two methods for the determination of moniliformin in maize were evaluated (Thiel et al., 1982b; Scott and Lawrence, 1987).

2.6.1.1 Sample preparation - (Thiel et al., 1982b)

Sample extracts were prepared by extracting a subsample (3 g) with distilled water (40 ml) in a centrifuge tube for 1 hour. The contents were then centrifuged at 10,000 g, and the supernatant extracts filtered through Millipore HA filters (0.45 μ m). The filtered extracts were retained for HPLC separation.

2.6.1.2 Sample preparation - (Scott and Lawrence, 1987)

Extracts were prepared by extracting a subsample (10 g) with acetonitrile/water (95:5, 100 ml) by blending at high speed for 5 minutes. The samples were filtered and partitioned with hexane (150 ml). The hexane layer was discarded and an aliquot (50 ml) of the remaining phase evaporated to dryness under reduced pressure. The residue was redissolved in methanol (2 ml). An aliquot (250 μ l) of the partially purified extract was placed on a pre-conditioned Sep-pak C₁₈ cartridge and moniliformin was eluted with distilled water (2 ml). The eluate was collected, evaporated to dryness and the residue dissolved in 500 μ l of the mobile phase (Table 2.4). The solution was passed through a small prepacked alumina/Millipore

HA filter column by syringe pressure, the eluate being collected and retained for HPLC analysis.

2.6.2 Analytical standard - moniliformin

Moniliformin stock solution ($50 \mu\text{g ml}^{-1}$) was prepared in distilled water. Aliquots (0.5, 1.0, 1.5, and 2.0 ml) of the stock solution were placed in separate 10 ml volumetric flasks and diluted to the mark with distilled water. The concentrations were verified by measuring their absorbances at 229 nm and applying the formula given in section 2.5.2, substituting the values of molecular weight and molar extinction coefficient for moniliformin (Cole and Cox, 1981).

TABLE 2.4 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF MONILIFORMIN (PAIRED ION CHROMATOGRAPHY)

Column	Nova-Pak RP C ₁₈ (4- μm) 15 cm x 3.9 mm (i.d.)
Mobile phase	0.005 M Tetrabutylammonium hydrogen sulphate in 4% methanol/0.1 M phosphate buffer (pH 7.0)
Flow rate	1 ml min ⁻¹
Injection volume	20 μl
Detector	UV
Detector setting	229 nm
Attenuation	0.02 AUFS

2.6.3 Chromatographic separation - moniliformin

Purified extracts were separated on a 4- μ m Nova-Pak C_{18} column (15 cm x 3.9 mm i.d.). Mobile phase was delivered by means of a Waters Model 510 liquid chromatographic pump and peak detection was performed on a Waters Model 481 variable wavelength U.V. detector. Data were collected with a Waters 745 data module. The exact chromatographic conditions are given in Table 2.4. Quantitative measurements of moniliformin were obtained by the comparison of toxin peak areas in each sample to a calibration curve of standard peak areas covering the range 2.5 - 10 μ g ml⁻¹.

2.7 INNOVATIVE METHODOLOGY

2.7.1 Sample preparation - fumonisin B₁

Sample extracts were prepared by extracting a subsample (25 g) of maize (5 g for culture material) with methanol/water (3:1, 50 ml) by blending at high speed for 5 minutes and filtering. An aliquot of the filtrate (25 ml) was evaporated to dryness at 50°C, redissolved in methanol/water (1:3, 25 ml) and partitioned with chloroform (2 x 50 ml). The organic phase was discarded and the aqueous phase evaporated to dryness and redissolved in methanol/water (1:3, 10 ml; 50 ml for cultures). Aliquots of these extracts (2 ml for HPLC and 1 ml for GC) were further purified by application to a Waters Sep-pak C_{18} cartridge, washing with methanol/water (1:3, 10 ml)

and eluting the toxin with methanol/water (3:1, 10 ml). The eluate was evaporated to dryness and retained for derivatization prior to chromatographic separation.

2.7.2 Derivatization for HPLC separation

Maleyl derivatives of the purified extracts were prepared and analysed by HPLC according to a previously published method (Siler and Gilchrist, 1982), with minor modifications. The residues were dissolved in sodium carbonate buffer solution (0.05M, pH 9.2, 5 ml) and treated with an excess of 1M maleic anhydride solution in dioxane (3×10 μ l). The pH of the solution was constantly monitored while the solution was agitated by means of a magnetic stirrer. The pH was adjusted to >9 using sodium hydroxide solution (0.1M), following each addition of maleic anhydride. Following a reaction time of 10 minutes, the pH of the solution was adjusted to between 6 - 7 using dilute HCl (0.1M). The derivatized extracts were then evaporated to dryness under reduced pressure at 50°C, redissolved in methanol/water (1:1, 2 ml) and retained for HPLC separation.

2.7.3 Hydrolysis, derivatization and GC separation

Purified sample extracts were hydrolysed (under nitrogen) with HCl (6M, 2 ml) at 95°C for 3 hours. Each sample was then cooled, centrifuged at 2000 rpm and an aliquot of each subjected to a previously published esterification procedure

(Labadarios et al., 1984). Briefly an aliquot (500 μ l) of each hydrolysate was transferred to an appropriate tube and the excess acid removed under reduced pressure. Esterification of the residue was performed under nitrogen using isobutanol containing 3M HCl (250 μ l) and heating at 100°C for 45 minutes. The acidified isobutanol was removed under reduced pressure and the residue acylated with heptafluorobutyric anhydride (100 μ l) by heating for 10 minutes (under nitrogen) at 150°C. The samples were then cooled in ice, freeze-dried, dissolved in ethyl acetate (100 μ l) and transferred to small vials prior to separation by capillary GC.

2.7.4. Secondary extraction and derivatization - fumonisin B₁

A secondary technique for the determination of fumonisin B₁ was investigated which involved extraction, fluorecamine derivatization and HPLC separation.

Samples of maize (25 g) were extracted with methanol/water (3:1; 50 ml) by shaking for 30 minutes. The extracts were filtered and an aliquot of the filtrate (5 ml) was evaporated to dryness at 50°C. The residue was partially redissolved in methanol/water (7:3, 1 ml) and fully solvated following the addition of methanol (1 ml). The solution was transferred to a prepared chromatographic column (10 mm i.d.) containing activated silica gel (2 g; 70-230 mesh) suspended between two layers of anhydrous sodium sulphate (2 x 1 g), in methanol.

The column was washed with methanol (15 ml) the toxins eluted with 0.1% acetic acid/methanol (20 ml) and the eluate was evaporated to dryness. The purified residues were redissolved in 0.05M sodium hydrogen carbonate (pH 8.6; 1 ml) and an aliquot (25 μ l) was diluted to 500 μ l in a plastic microfuge tube (1.8 ml capacity) with the same solution. While vortex mixing, fluorescamine (50 μ l; 2 mg 100 μ l⁻¹ in acetone; Aldrich) was added to the tube. Mixing was continued for 1 minute and the mixture was then centrifuged for 1 minute at 9,000 g. The derivatized extracts were retained for HPLC separation coupled with fluorescence detection.

2.7.5 Analytical standard - fumonisin B₁

A stock solution of fumonisin B₁ was prepared in distilled water (2.5 mg ml⁻¹). An aliquot of the stock solution (200 μ l) was used to prepare the standard maleyl derivative as outlined in section 2.7.2, to give a final concentration of 250 μ g ml⁻¹. A second aliquot of the same stock solution was used to prepare the fluorescamine derivative (as outlined in section 2.7.4), to give a final concentration of 3.25 μ g ml⁻¹.

Tricarballic acid (TCA) standard stock solution was prepared in glass distilled methanol (10 mg ml⁻¹). An aliquot of this solution (100 μ l) was used to prepare a standard esterified derivative as outlined in section 2.7.3. Aliquots of the derivative in ethyl acetate (2.5, 5, 7.5 and 10 μ l) were transferred to separate vials and diluted with ethyl acetate

(1 ml) to give a concentration range of 25 - 100 $\mu\text{g ml}^{-1}$ TCA as its butyl ester.

2.7.6 Chromatographic conditions - fumonisin B₁ (as its maleyl derivative) by HPLC

HPLC separations of the preformed maleyl derivatives were performed on a 4- μm Nova-pak C₁₈ column. Solvent delivery and peak detection was performed using the equipment outlined in section 2.6.3. The prevailing chromatographic conditions used are given in Table 2.5. Quantitative determination of fumonisin B₁ was by comparison of peak areas in the samples to that of the peak area of a similar derivatized fumonisin B₁ standard.

TABLE 2.5 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF FUMONISIN B₁ (AS ITS MALEYL DERIVATIVE) BY HPLC

Column	Radial-Pak RP C ₁₈ (4- μm) 10 cm x 8 mm (i.d.)
Mobile phase	Methanol : 0.05M phosphate buffer (7:3) adjusted to pH 3.5 with concentrated HCl
Flow rate	1.5 ml/minute
Injection volume	5 or 10 μl
Detector	UV
Detector setting	230 nm
Attenuation	0.02 AUFS

2.7.7 Chromatographic separation - fumonisin B₁ (as its fluorescamine derivative) by HPLC

Aliquots of the preformed fluorescamine derivatives were separated on a 7 μ m Phenomenex Ultracarb ODS 30 C column. Solvent delivery was performed using the equipment outlined in section 2.6.3 and peak detection was performed using a Perkin-Elmer 650S fluorescence detector. The prevailing chromatographic conditions used are detailed in Table 2.6.

TABLE 2.6 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF FUMONISIN B₁ (AS ITS FLUORESCAMINE DERIVATIVE)

Column	Phenomenex Ultracarb ODS 30 (7 μ m) 25 cm x 4.6 mm (i.d.)
Mobile phase	0.1M Acetate buffer (pH 4.0) : Acetonitrile (1:1)
Flow rate	1 ml/min
Injection volume	20 μ l
Detector	Fluorescence
Detector settings	390 nm (Excitation) 475 nm (Emission)
Slit widths	20 nm

2.7.8 Chromatographic conditions - TCA by capillary GC

The butyl esters of standards and samples were separated on a DB-5 capillary column. The Carlo-Erba Model 5300 gas chromatograph used was equipped with a splitless injector and a flame ionization detector (FID). The exact chromatographic conditions are given in Table 2.7. Data were collected with a Waters 745 data module, and quantitative determinations of butyl esters of TCA present in the samples, was by comparison of peak areas to that of a calibration curve of the peak areas of similarly derivatized standards.

TABLE 2.7 CHROMATOGRAPHIC PARAMETERS FOR THE DETERMINATION OF TRICARBALLYLIC ACID

Column	DB-5	30 m x 0.32 mm (i.d.)
Film thickness	0.25 μm	
Carrier gas (flow rate)	Helium at 35 cm sec^{-1}	
Detector	FID	
Attenuation	8	
Injection volume	1 μl	
Splitless (on time)	0.5 min	
Injector temperature	140°C	
Detector temperature	280°C	
Detector fuel gases	Air at 300 ml min^{-1}	
	Hydrogen at 30 ml min^{-1}	
Temperature profile	(1) 50°C for 3 min	
	(2) 50°C - 280°C at 15°C min^{-1}	

2.8 CONFIRMATORY TECHNIQUES

2.8.1 Improvement to chromatographic data

In order to improve or verify some of the chromatographic observations concerning the presence of the selected *Fusarium* mycotoxins in the series of Transkeian maize samples, various alterations and/or additional confirmatory techniques were used.

Possibly the easiest of these techniques (spiking) was used in each analysis. Briefly, mixtures were prepared containing aliquots of the sample extracts together with aliquots of similarly prepared (ie. derivatized where necessary) authentic target toxin standards. Aliquots of these mixtures were then re-chromatographed, and the resultant chromatogram was compared to that obtained for the sample extract. Other techniques and/or improvements used in this investigation are presented in sections 2.8.2 - 2.8.6.

2.8.2 Chromatographic confirmation of nivalenol and deoxynivalenol

Neither method evaluated (Tanaka et al., 1985a; Scott et al., 1986) made use of an internal standard. A type B trichothecene, fusarenon-X, whose characteristics and structure are similar to both nivalenol and deoxynivalenol (Table 1.2) was incorporated as an internal standard for this study. A

Tri-Sil TBT derivative of fusarenon-X was prepared as previously described (section 2.4.3) and an aliquot (corresponding to a final concentration of $600 \mu\text{g ml}^{-1}$) was added to each sample vial prior to chromatographic separation.

The presence of nivalenol and deoxynivalenol in several samples extracts was confirmed by capillary GC/mass spectrometry (GC/MS) using a Finnigan Model 4500 GC/MS and the conditions outlined in Table 2.8.

TABLE 2.8 CHROMATOGRAPHIC PARAMETERS FOR THE CONFIRMATION OF TRICARBALLYLIC ACID, NIVALENOL AND DEOXYNIVALENOL

Column	DB-5 60 m x 0.32 mm (i.d.)
Film thickness	0.25 μm
Carrier gas (flow rate)	Helium at 30 cm sec^{-1}
Detector	Finnigan 4500 MS
Injection volume	1 μl
Splitless (on time)	0.5 min
Injector temperature	180°C
Detector temperature	300°C
Temperature profile	(1) 80°C for 1 min (2) $80^{\circ}\text{C} - 280^{\circ}\text{C}$ at $15^{\circ}\text{C min}^{-1}$
Mass range	100 - 400 m/z

2.8.3 Chromatographic confirmation of zearalenone

Extracts of a number of samples that indicated relatively high levels of zearalenone were re-chromatographed (using the chromatographic conditions detailed in Table 2.3) and monitored at three different UV wavelengths simultaneously, using a Hewlett-Packard 1040A diode array detector. The wavelengths monitored corresponded to the three wavelength maxima recorded for the UV spectrum of a zearalenone standard (Figure 3.3). The measured ratios of the wavelengths of the zearalenone peak in the sample extract were then compared with the same ratios measured in the standard. In addition, the extracts were also monitored over the wavelength range 200-400 nm and computer software accompanying the instrument was used to display the data as a 3 dimensional representation.

2.8.4 Chromatographic confirmation of moniliformin

In addition to the paired ion chromatographic method for the determination of moniliformin in sample extracts (Table 2.4), several of the extracts were also chromatographed using ion exchange chromatography (the prevailing chromatographic conditions are given in Table 2.9). Sample extracts, separated by both techniques were also monitored using the diode array detector as previously described (section 2.8.3). The wavelength maxima monitored were conspecific with those determined from the UV spectrum of authentic moniliformin (Figure 3.2).

TABLE 2.9 CHROMATOGRAPHIC PARAMETERS FOR THE CONFIRMATION OF
MONILIFORMIN BY ION EXCHANGE CHROMATOGRAPHY

Column	Whatman RAC II SAX (5 μ m) 10 cm x 4.6 mm
Mobile phase	0.001M Sodium dihydrogen phosphate
Flow rate	1.5 ml min ⁻¹
Injection Volume	20 μ l
Detector	UV
Detector setting	229 nm
Attenuation	0.02 AUFS

TABLE 2.10 CHROMATOGRAPHIC PARAMETERS FOR THE CONFIRMATION
OF FUMONISIN B₁ (AS ITS MALEYL DERIVATIVE) BY
HPLC

Column	Phenomenex Ultracarb ODS 30 (7 μ m) 25 cm x 4.6 mm (i.d.)
Mobile phase	0.05M Acetate buffer (pH 3.5) : methanol (35:65) pH maintained at 3.5 with concen- trated HCl
Flow rate	1 ml min ⁻¹
Injection volume	20 μ l
Detector	UV
Detector setting	230 nm
Attenuation	0.02 AUFS

2.8.5 Chromatographic confirmation of fumonisin B₁

Extracts prepared by the maleyl derivative procedure were separated on a second reverse phase column using an acetate buffer mobile phase system (details are given in Table 2.10).

2.8.6 Chromatographic confirmation of TCA

Butyl esters of several sample extracts, prepared as previously described (section 2.7.3) were subjected to GC/MS analysis. Confirmation of the presence of TCA as its butyl derivative in purified sample extracts was by comparison of their MS to that of the MS of a similarly prepared TCA analytical standard. The GC/MS used was the same as that described in section 2.8.2., and the prevailing chromatographic conditions were the same as those for the confirmation of the presence of nivalenol and deoxynivalenol (Table 2.8).

2.9 STATISTICAL ANALYSES

Statistical analyses of the data were performed by Dr D.J. van Schalkwyk via the analysis of variance, followed by the Student-Neuman-Keuls multiple comparison method. Since a number of the variables included either percentages or the means of percentages, it was expected that they might not be normally distributed. As a precaution, both parametric and non-parametric analyses were performed. Pearson parametric analyses of the data were done by the analysis of variance

and, where necessary, followed by the Student-Neuman-Keuls multiple comparison method. In addition, Spearman non-parametric analyses were also performed. The results were consistent and the probability levels reported are the most conservative (ie least significant) obtained.

CHAPTER 3

RESULTS AND DISCUSSION

A: STANDARDS AND EXISTING METHODOLOGY

3.1 ANALYTICAL STANDARDS

3.1.1 Importance of authentic standards

Several factors have to be taken into consideration to establish a precise and accurate analytical determination, such as the degree to which the subsample used for the analysis is representative of the bulk of the sample. The availability of an "authentic" analyte standard is, however, an absolute prerequisite, especially in quantitative chromatographic techniques. This may be illustrated by the fact that in the absence of sophisticated monitoring techniques (i.e. MS) at the final chromatographic stage of an analysis, the presence or absence and concentration of a particular analyte, in an often complex matrix, is solely based on the retention time and size of a chromatographic peak, and its comparison to the retention time and size of a similarly chromatographed "authentic" standard.

Some of the standards used in this study were obtained from commercial sources, but several (including deoxynivalenol,

neosolaniol monoacetate, moniliformin and fumonisin B₁) were previously isolated within the Institute. In order to verify the identity and purity of the analytical standards used, several analytical parameters were measured on each toxin (detailed in sections 3.1.2 - 3.1.8). Where possible the results were compared with those previously published (Cole and Cox, 1981; Pohland et al., 1982). It was not possible to collect all data on each toxin due to insufficient quantities of a number of toxin standards.

3.1.2 Melting points

Where possible, approximately 20 mg of each toxin was dried in an oven at 110°C for 1 hour, removed and stored in a desiccator. Several crystals of each toxin standard were placed between two cover slides and their melting points determined using a Reichert melting point variable heating stage, fitted with a single lens microscope. A large glass plate was placed over the heating stage, following the placement of each sample, so as to ensure an enclosed environment within the heating block. The temperature of the stage was increased gradually and the temperatures at which the crystals of each toxin appeared to start melting and were completely molten, were recorded. The melting points were performed in triplicate and the melting point ranges recorded are given in Table 3.1. The accuracy of the equipment used to determine the melting points was checked against the melting point of benzoic acid.

TABLE 3.1 MELTING POINTS OF SELECTED MYCOTOXIN STANDARDS

Toxin	Melting Point (°C)	Published Melting Point (°C)
Deoxynivalenol	152 - 155	151 - 153 ¹
Diacetoxyscirpenol	163 - 165	162 - 164 ¹
Neosolaniol mono-acetate	188 - 189	190 - 190.5 ²
T-2 toxin	146 - 149	151 - 152 ¹ 145 - 149 ²
Zearalenone	163 - 165	164 - 165 ¹ 161 - 164 ²

¹ Cole and Cox, (1981)

² Pohland et al., (1982)

3.1.3 Thin layer and high performance liquid chromatography

Toxin standard solutions were screened by both TLC and HPLC techniques on either silica gel TLC plates or reverse phase HPLC columns, as previously described (Tables 2.3 and 2.4). A number of solvent systems were used for TLC separations and the chromatographed plates were sprayed (if necessary) with

p-anisaldehyde (0.5% v/v in methanol:acetic acid:sulphuric acid, 85:10:5) solution as well as with sulphuric acid solution. Where spraying of the plates was necessary, the plates were heated in an oven at 110°C prior to visual inspection. Plates which did not require spraying were viewed under either longwave (366 nm) or shortwave (254 nm) UV light. In each case, only a single spot (TLC) or peak (HPLC) could be detected.

3.1.4 Capillary GC analyses of HFBI and Tri-Sil TBT derivatives

HFBI and Tri-Sil TBT derivatives of each trichothecene standard used in this study were prepared as described in sections 2.3.4. and 2.4.3., respectively, at concentrations of approximately 100 µg ml⁻¹. Splitless injections of each derivative were separated on a Carlo-Erba GC fitted with an apolar Machery-Nagel fused silica capillary column (25 m x 0.32 mm i.d.) coated with a 0.25 µm thickness of SE-30. The prevailing chromatographic conditions used were the same as those previously described in Table 2.2. Using the FID as a universal detector, only a single chromatographic peak could be detected in each of the derivatives.

3.1.5 Infra-red absorption spectra

Infra-red spectra of a number of toxin standards were collected using a Perkin-Elmer 983 IR spectrophotometer. The reso-

lution was 3 cm^{-1} , and each sample was scanned over the range $4000 - 180\text{ cm}^{-1}$. Each toxin standard was thoroughly ground with spectroscopic grade potassium bromide (KBr) at a mass fraction of approximately 1.25 mg g^{-1} KBr. The ground mixtures were pressed into 13 mm discs prior to analysis. The IR spectra obtained were compared to those previously published (Cole and Cox, 1981, Pohland et al., 1982). Excellent agreement was obtained for each sample analysed which included deoxynivalenol, nivalenol, T-2 toxin, diacetoxyscirpenol, neosolaniol monoacetate, moniliformin and zearalenone. Figure 3.1 shows the comparison of the spectrum obtained for the diacetoxyscirpenol standard to that reported in the literature (Pohland et al., 1982).

3.1.6 Ultra-violet absorption spectra

Standard solutions of toxins were prepared in methanol, water or a mixture of the two solvents. The spectra were recorded on a Beckman 5260 double beam recording spectrophotometer over the range 200-350 nm at a scan speed of 2 nm sec^{-1} and a chart speed of 20 nm inch^{-1} . Spectra were once again compared with those reported in the literature (Cole and Cox, 1981; Pohland et al., 1982). In each case excellent agreement was obtained between the generated spectrum and that published. Figures 3.2 and 3.3 show the spectra obtained for moniliformin and zearalenone, respectively. The concentrations of the toxins in their respective solutions are given in the legends to each figure.

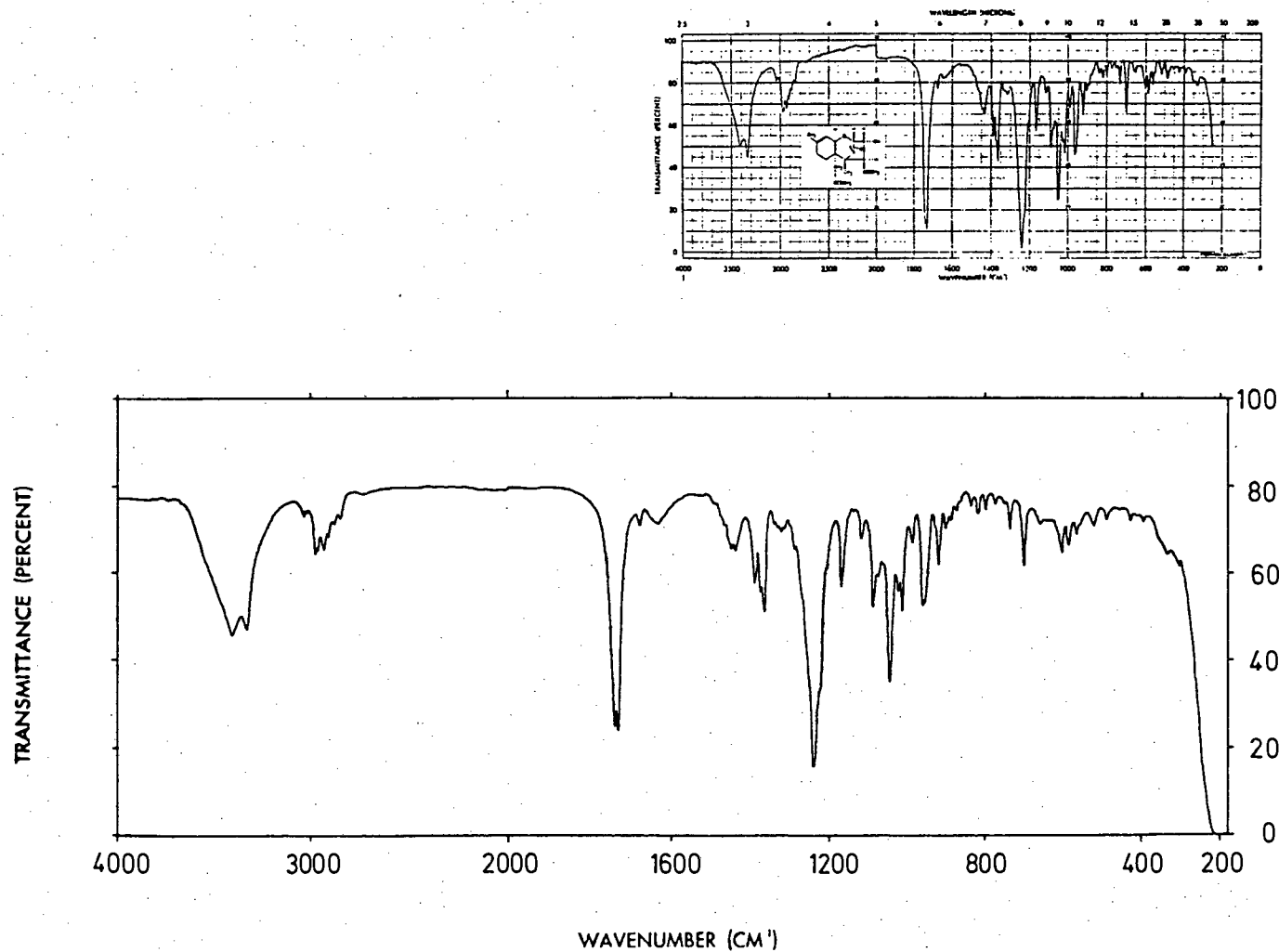


FIG. 3.1 Infra-red absorption spectrum of diacetoxyscirpenol with insert - published spectrum (Pohland et al., 1982)

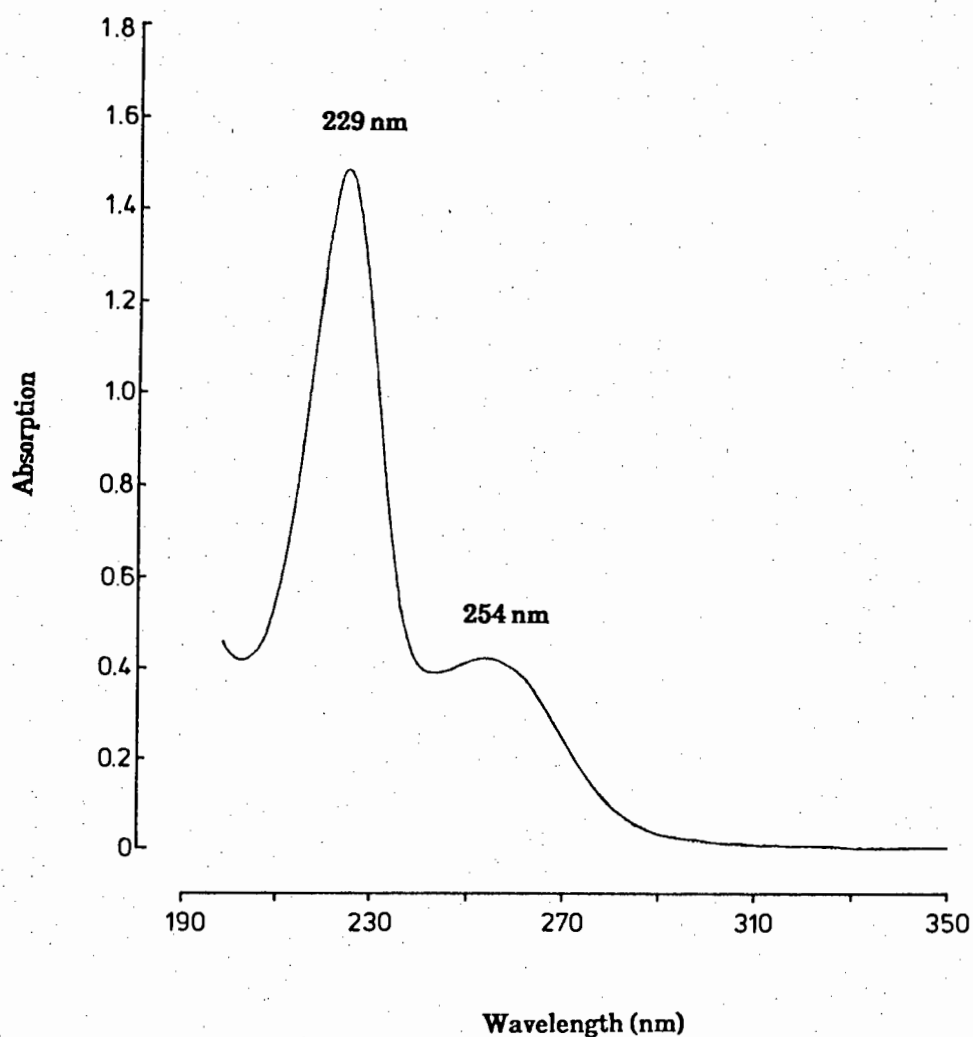


FIG. 3.2 Ultra-violet spectrum of moniliformin [$9.25 \mu\text{g ml}^{-1}$] in water

3.1.7 Fluorescence spectroscopy

Fluorescence characteristics were measured using a Perkin-Elmer 650S fluorescence spectrophotometer. The excitation spectra (200 - 450 nm) were recorded by setting the emission spectral bandpass (slit width) to 15 nm and the emission dial to zero order. The excitation bandpass was set at approxi-

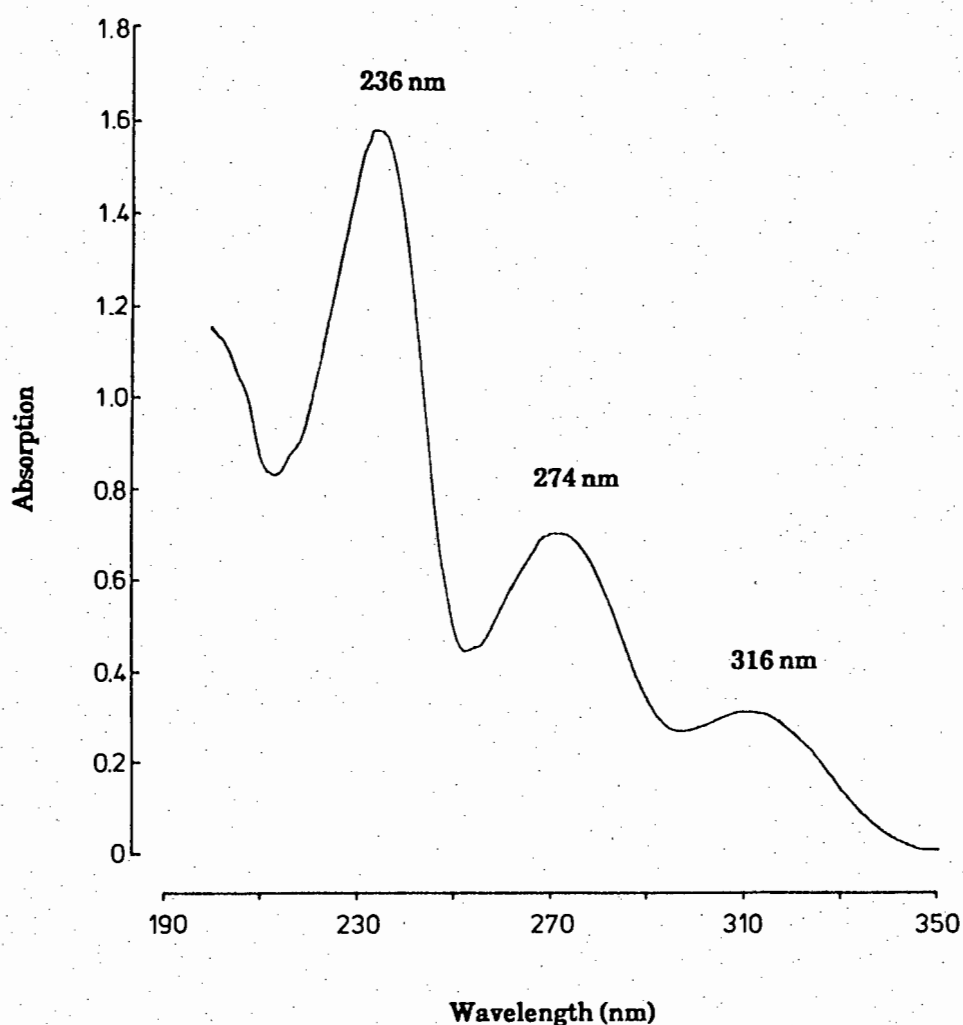


FIG. 3.3 Ultra-violet spectrum of zearalenone [$17 \mu\text{g ml}^{-1}$] in methanol:water (7:3)

mately 3 nm and spectra were recorded at a scan rate of $60 \text{ nm minute}^{-1}$. Emission spectra (350-550 nm) were recorded at the same scan rate, by setting the excitation wavelength to that corresponding to the excitation maxima and the spectral band-passes were reversed. Of the available standards, only zearalenone displayed any fluorescence characteristics which are illustrated in Figure 3.4.

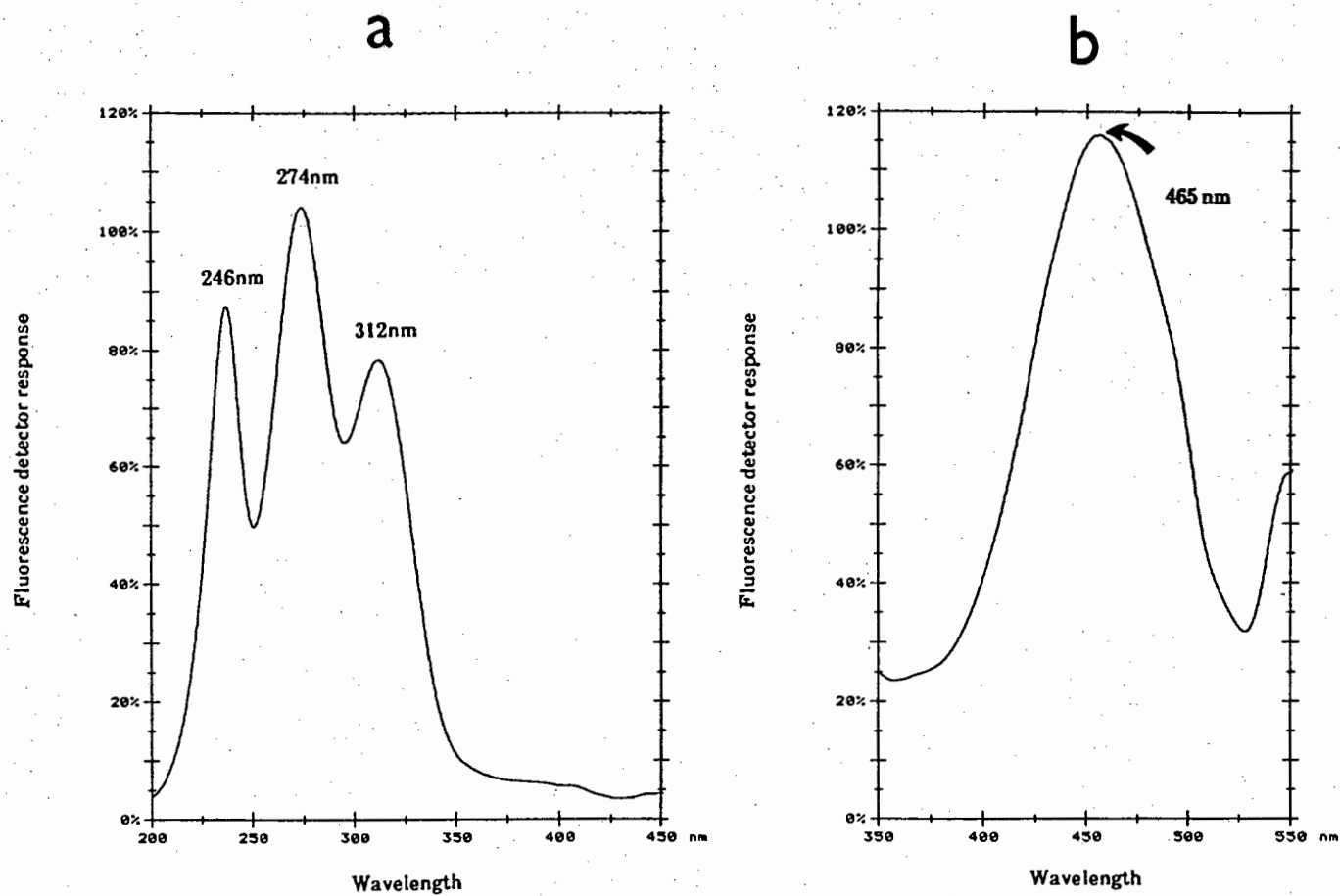


FIG. 3.4 (a) The fluorescence excitation scan of zearalenone [$3 \mu\text{g ml}^{-1}$] in methanol:water (7:3) and (b) the fluorescence emission scan of the same solution at an excitation wavelength of 274 nm

3.1.8 Nuclear magnetic resonance and mass spectrometry techniques

The identity of two of the toxin standards (neosolaniol monoacetate and fumonisin B₁), which had previously been isolated within the Institute, were confirmed by MS and ¹H and ¹³C N.M.R. spectroscopy, by Dr R Vleggaar, CSIR, Pretoria. The results for the toxins were compared with those previously published (Cole and Cox, 1981; Bezuidenhout et al., 1988).

3.1.9. Analytical standards - conclusion

Using the techniques described in sections 3.1.2 - 3.1.8, the identities of the analytical standards were confirmed. The absence of contaminants, as determined using a number of methods, in each of the standards analysed, appeared to indicate a high degree of purity. Some of the physico-chemical data generated for the standards used in this study will be incorporated into an ongoing IUPAC Food Commission project dealing with the provision of such data on mycotoxin standards.

3.2 COMPARISON OF TYPE A TRICHOTHECENE METHODOLOGY

3.2.1 Extraction procedures

The extraction procedures of the two methods evaluated, though similar, differed with regard to the primary purifica-

tion step. While the first method (Cohen and Lapointe, 1984) used solvent partitioning of the toxin from an aqueous phase into ethyl acetate the latter (Sydenham and Thiel, 1987) made use of a hydrophilic (Kieselguhr) matrix. Use of the Kieselguhr column resulted in a partially purified eluate free from emulsions, a problem often encountered with solvent-solvent partitioning procedures. Both methods used a short silica gel column for the secondary purification step, however, in addition, the method of Cohen and Lapointe, (1984) also used a "cyano" column as a tertiary sample preparation step. Difficulty in obtaining the specified cyano column meant that a similar substitute column (cyano Bond Elut) had to be used. Both sample preparation methods were found to be rapid and simple.

3.2.2 Chromatographic procedures

Greater sensitivity was achieved following the formation of the heptafluorobutyryl derivatives of the type A trichothecenes than their corresponding trimethylsilyl derivatives. The method of Cohen and Lapointe, (1984) employed a splitless injection of a preformed purified sample derivative and separation of the extract on a DB-5 capillary column. The retention times for diacetoxyscirpenol and T-2 toxin (using the conditions outlined by Cohen and Lapointe, 1984) were in the order of 41 and 50 minutes, respectively. The method of Sydenham and Thiel, (1987) used a split injection technique and separation of similarly prepared derivatives on a slightly

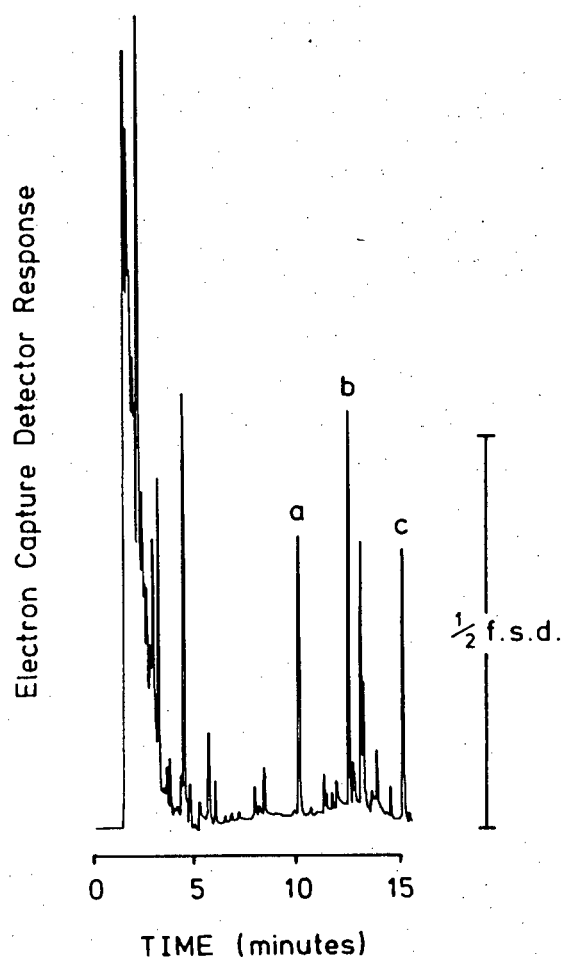


FIG. 3.5 Chromatogram of a spiked control maize sample extract (prepared according to the method of Sydenham and Thiel, 1987) showing the chromatographic positions of (a) diacetoxyscirpenol, (b) neosolaniol monoacetate and (c) T-2 toxin, as their respective heptafluorobutyrate esters

more polar phase column (Table 2.1), giving retention times of approximately 10 and 15 minutes for diacetoxyscirpenol and T-2 toxin, respectively. Although the introduction of sample extract via a splitter, onto a chromatographic column, can

result in reproducibility problems, the use of neosolaniol monoacetate as an internal standard in the method (Sydenham and Thiel, 1987) reduced these errors to a minimum (Table 3.2). The suitability of neosolaniol monoacetate as an internal standard could be attributed to the fact that it is also a type A trichothecene (Table 1.1), with a structure and chemical properties similar to those of diacetoxyscirpenol and T-2 toxin. Using the prevailing chromatographic conditions, neosolaniol monoacetate eluted equidistant between diacetoxyscirpenol and T-2 toxin (Figure 3.5).

TABLE 3.2 RECOVERIES OF DIACETOXYSCIRPENOL AND T-2 TOXIN (AND ESTIMATES OF ERROR¹) USING THE PROCEDURE OF SYDENHAM AND THIEL, (1987)

	Diacetoxyscirpenol	T-2 toxin
Recovery (%)	88.2	88.0
Error (%) pooled within run	1.7	1.6
CV (%)	1.9	1.8
Error (%) between run	4.7	4.1
CV (%)	5.3	4.7
Error (%) total	5.0	4.4

¹ Estimates of error were calculated according to Krouwer and Rabinowitz, (1984). Data were compiled from 10 separate extractions with triplicate determinations per extraction, on control maize spiked at a level of 1200 ng g⁻¹ diacetoxyscirpenol and T-2 toxin

3.2.3 Toxin recoveries and method detection limit

Using the chromatographic conditions outlined in Table 2.1, the two methods were evaluated by analysing control maize to which 1200 ng g^{-1} of each target toxin had been added. Triplicate recoveries using the method of Cohen and Lapointe, (1984) averaged 82.3 and 79.4% for diacetoxyscirpenol and T-2 toxin, respectively, and were within the range of recoveries reported by the authors. The recoveries of diacetoxyscirpenol and T-2 toxin using the method of Sydenham and Thiel, (1987) were found to be slightly higher at 88% and 88.2%, respectively (Table 3.2). The total errors (Table 3.2) for the latter method were extremely low, with the major source emanating from the "between-run" factors. The detection limit of each method was approximately 100 ng g^{-1} (parts per billion), as determined using spiked control maize. Each method was also assessed by analysing a fungal culture (*F. armeniacum* MRC 4481 - section 2.1.2) previously found to produce 440 ng g^{-1} T-2 toxin. Figure 3.6.1 shows the chromatogram obtained from the fungal culture extract, following purification procedures according to Sydenham and Thiel, (1987). Diacetoxyscirpenol was not detected in the extract, however a well resolved peak corresponding to T-2 toxin, eluted after approximately 15 minutes. These observations were verified by spiking the sample with a similarly derivatized T-2 toxin standard. The chromatogram illustrates that T-2 toxin can be successfully extracted and identified from a fungal culture matrix, at relatively low concentrations, without undue interference from co-eluting contaminants. Figure 3.6.2 shows the chromatogram

obtained from the same culture, following the procedure described by Cohen and Lapointe, (1984). T-2 toxin was not recovered, however, the use of non-specified materials during the sample purification stage of the method may have contributed to this observation.

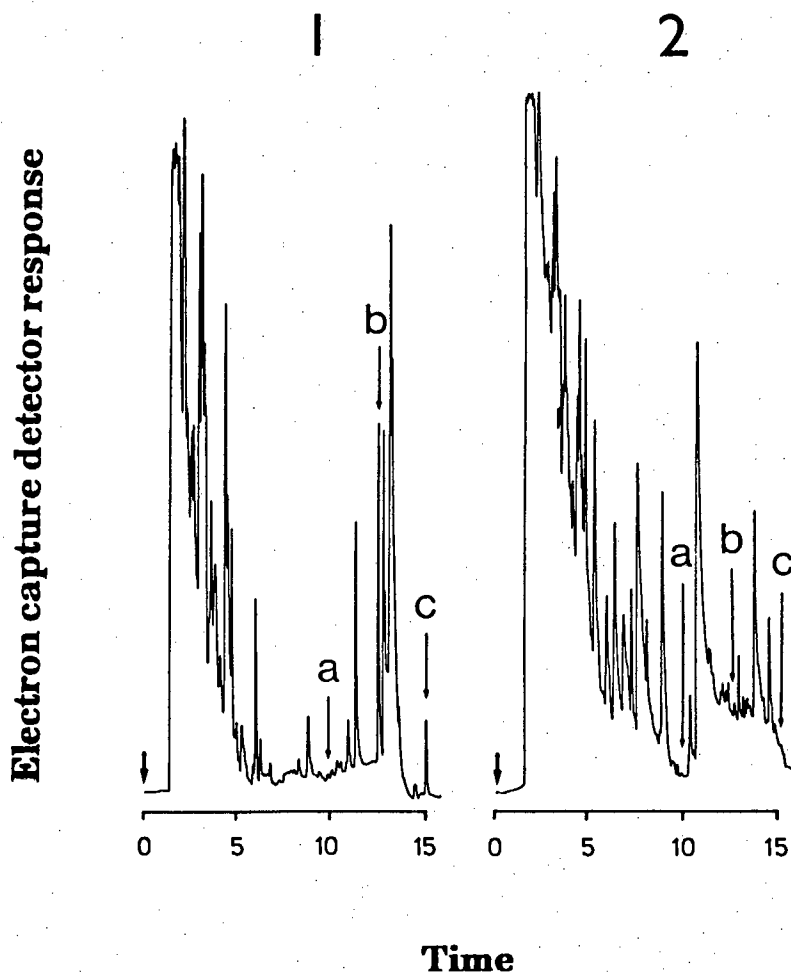


FIG. 3.6 Chromatograms of the purified extracts of a maize culture (MRC 4481) analysed according to the methods of (1) Sydenham and Thiel, 1987 and (2) Cohen and Lapointe, 1984. The alphabetical notations a, b and c correspond to the chromatographic positions of diacetoxyscirpenol, neosolaniol monoacetate and T-2 toxin, respectively

3.2.4 Type A trichothecene methodology - summary

When analysing control maize, the methods compared favourably. However, the use of neosolaniol monoacetate as an internal standard in the method of Sydenham and Thiel, (1987) contributed significantly to the quality of the recorded data and resulted in the elution of the toxins within 15 minutes. It should be noted that the method would also be suitable for the extraction and chemical determination of neosolaniol monoacetate itself. This was demonstrated in a recent publication (Sydenham et al., 1989c) on the production of neosolaniol monoacetate by an undescribed *Fusarium* species where, with minor modifications, the same method was used.

Neosolaniol monoacetate has never been reported as a natural contaminant of grain samples and is itself a rare trichothecene, having been isolated only from three *Fusarium* species and four Russian isolates belonging to the section *Sporotrichiella* (Sydenham et al., 1989c).

3.3 COMPARISON OF TYPE B TRICHOTHECENE METHODOLOGY

3.3.1 Extraction procedures

The extraction and purification procedures of the two methods evaluated, differed considerably. According to the publication of Tanaka and co-workers (Tanaka et al., 1985a), the use of acetonitrile/water (3:1) as an extraction solvent, resul-

ted in superior deoxynivalenol and nivalenol recoveries than other solvent blends examined (including several methanol/water blends). The authors also reported that the acetonitrile/water (3:1) solvent blend resulted in extracts containing fewer "interfering components" than the corresponding methanol/water blends. The method subsequently involved the removal of some interferences via solvent partitioning with hexane followed by two separate chromatographic purification steps on Florisil and disposable silica gel columns, respectively. Although this method was found to be time consuming (especially at the Florisil column stage), using relatively large volumes of organic solvents, it did result in a purified extract containing the two target toxins.

The second method evaluated (Scott et al., 1986), which is a modification of an earlier method (Scott et al., 1981), involved extraction with methanol/water, precipitation with ammonium sulphate solution, followed by partitioning of the toxins on a hydrophylic matrix and final purification on a short silica gel chromatographic column. This method was found to be relatively rapid, using minimal volumes of solvents and resulted in a visibly "less contaminated" extract than that resulting from the sample preparation procedure method of Tanaka et al. (1985a).

3.3.2 Chromatographic procedures

Although it is known that packed column GC inherently allows

for maximum column capacity (ie the highest sample equivalent weight that may be applied to an analytical GC column), the superior resolution attainable on capillary GC columns make their use highly desirable, especially when confronted with complex matrices. Therefore, extracts prepared by both analytical procedures were analysed by capillary GC, even though one of the methods (Tanaka et al., 1985a) specified packed column GC as the chromatographic separation technique.

Both analytical procedures required the formation of trimethylsilyl derivatives of the purified sample extracts prior to chromatographic separation. Scott (Scott et al., 1986) suggested that the preparation and separation of HFBI derivatives of samples (as used for the type A trichothecenes) could be used for confirmation purposes. Tri-Sil TBT was used as the silylation reagent (Scott et al., 1986) and it was found to form stable derivatives within a short reaction time (15 minutes), which exhibited excellent electron capturing characteristics and resulted in a far less complex capillary chromatogram than that obtained from a corresponding HFBI derivative. Scott et al. (1986) reported that the chromatographic separation of the nivalenol heptafluorobutyrate resulted in the appearance of two peaks on certain specified packed GC columns, as opposed to a single peak on capillary columns.

Figure 3.7.1 shows the chromatogram (corresponding to a 100 μg mass sample weight) obtained from a control maize sample spiked with 1000 ng g^{-1} deoxynivalenol and nivalenol (prior

to extraction), prepared by the method of Tanaka et al. (1985a). Figure 3.7.2 shows a similar extract prepared according to the method of Scott et al. (1986). In each case, fusarenon-X (a type B trichothecene similar in both chemical

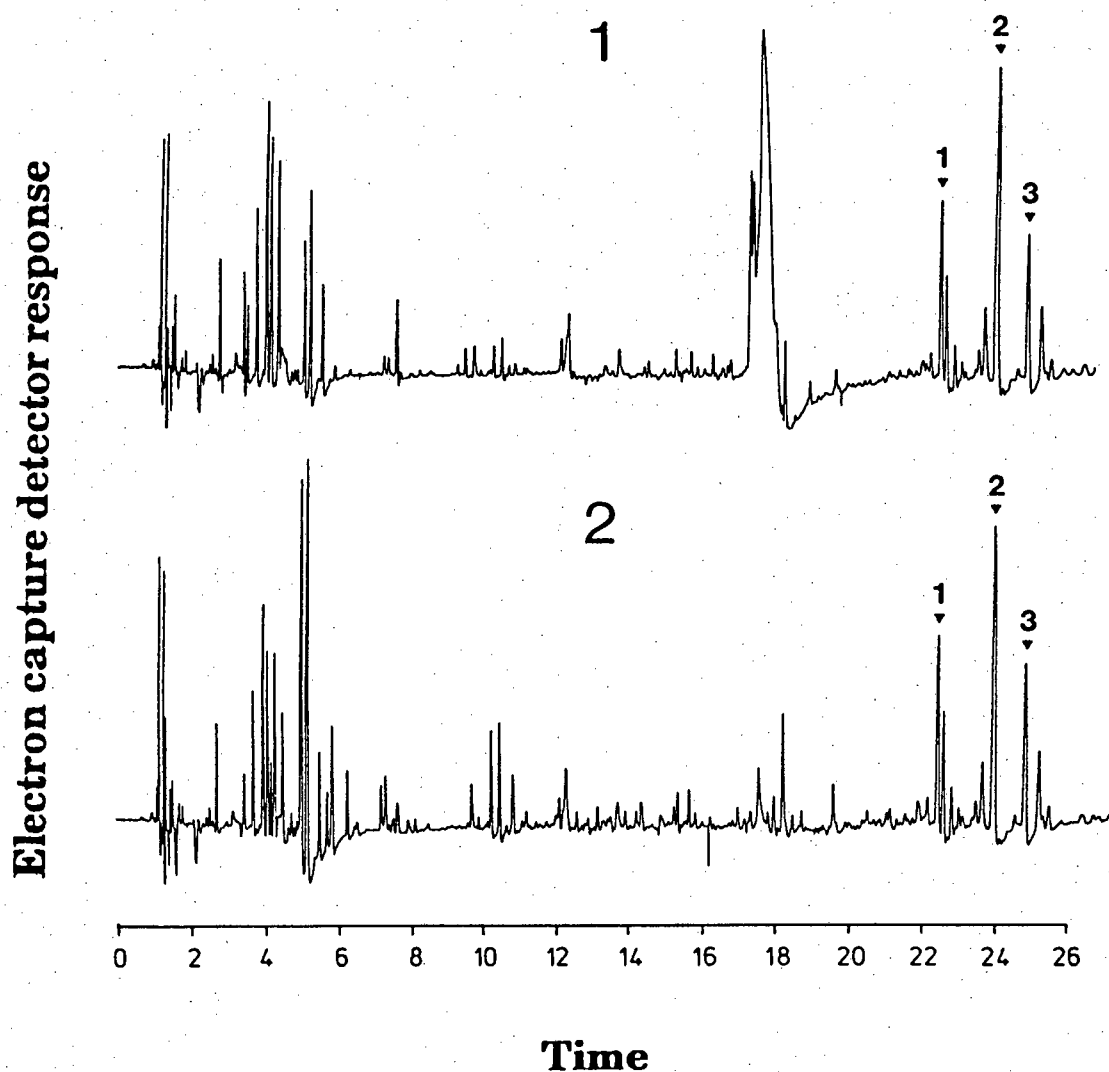


FIG. 3.7 Chromatograms of control maize spiked with deoxynivalenol and nivalenol (at 1000 ng g^{-1}) and extracted according to the methods of (1) Tanaka et al. 1985a and (2) Scott et al. 1986. The numerical notations 1, 2 and 3 correspond to the chromatographic positions of deoxynivalenol, fusarenon-X (internal standard) and nivalenol, respectively

structure and characteristics to deoxynivalenol and nivalenol - Table 1.2) as its similarly prepared trimethylsilyl derivative (designated peak #2), was used as an internal standard. Fusarenon-X was found to elute equidistant between deoxynivalenol and nivalenol (designated peaks #1 and #3, respectively). The major chromatographic difference between the two methods was the presence of a major component eluting at ± 17.5 minutes (Figure 3.7.1) in the extract prepared according to the method of Tanaka et al. (1985a). However, the presence of the peak did not compromise the resolution of the deoxynivalenol, fusarenon-X and nivalenol peaks, which eluted at ± 22.6 , 24.1 and 25.1 minutes, respectively.

3.3.3 Toxin recoveries, reproducibility and method detection limit

Using the chromatographic conditions outlined in Table 2.2, control maize extracts spiked with deoxynivalenol and nivalenol (at 1000 ng g^{-1} each toxin) were analysed in triplicate. Recoveries, using the method of Tanaka et al. (1985a) were found to average 89% (SD = 2.6) and 83% (SD = 2.5) for deoxynivalenol and nivalenol, respectively. Slightly higher average recoveries of 92% (SD = 1.3) and 88% (1.6%) for deoxynivalenol and nivalenol, respectively, were obtained using the procedure of Scott et al., (1986).

The detection limit of each method was found to be approximately 40 ng g^{-1} for each toxin, using the prevailing chroma-

tographic conditions. This compared with the detection limits of 20 ng g⁻¹ and 2 ng g⁻¹, reported by Scott et al. (1986) and Tanaka et al. (1985a), respectively. Differences in the chromatographic parameters used by the authors as opposed to those used in this study may have contributed to these apparent anomalies with regard to method sensitivity.

The use of fusarenon-X as an internal standard improved the reproducibility as shown by the standard deviations calculated on 6 injections each of derivatized control maize extracts including and excluding fusarenon X (SD = 7.8 without internal standard reduced to 0.83 with internal standard). The use of an internal standard was necessary since a manual splitless injection system was used. However, had an automatic injection system been available, the reproducibility of injection would have been even better, and would no doubt have been reflected in lower standard deviation values than those reported above.

3.3.4 Type B trichothecenes - summary

The presence of deoxynivalenol as a major contaminant of head blighted or "scabby" wheat has been reported in many countries worldwide. Its detection in grains from Canada and the United States (Trenholm et al., 1981, 1983; Romer, 1983; Scott, 1983; Scott et al., 1984; Eppley et al., 1984; Hagler et al., 1984; Seitz and Bechtel, 1985; Shotwell et al., 1985) gave rise to the rapid development of methods for its deter-

mination, predominantly by scientists in those countries. Conversely, nivalenol (the toxicity of which is higher than that of deoxynivalenol) appears to be the major contaminant of Japanese grains, prompting extensive method developments for the co-determination of deoxynivalenol and nivalenol in grains, by Japanese scientists (Kamimura et al., 1978, 1981; Yoshizawa, 1984; Tanaka, 1985a). Poor recoveries of nivalenol inherent in the methods used by Canadian scientists have since been rectified and Scott et al. (1986) and Lauren and Greenhalgh, (1987), have reported excellent recoveries for nivalenol. The latter method (Lauren and Greenhalgh, 1987) is a HPLC procedure, however, since capillary GC was the preferred chromatographic technique (because of its superior resolution over HPLC), the method was not included for evaluation purposes.

A brief attempt to further reduce the sample preparation time of the method according to Scott et al. (1986), by replacing the short silica gel column with a similarly packed disposable cartridge, resulted in unacceptably lower recoveries of both target toxins (approximately 10% reduction). In summary, of the two analytical methods evaluated, the speed of sample preparation coupled with the excellent recoveries of both target toxins, made the method of Scott et al. (1986) preferable.

3.4 COMPARISON OF ZEARALENONE METHODOLOGY

3.4.1 Extraction procedure

Subsamples of control maize were extracted according to the three procedures outlined in sections 2.5.1.1 - 2.5.1.3, which differed considerably with respect to their methodologies. The AOAC method (1984) involved extraction of the maize with chloroform/water followed by chromatographic separation from lipid-type material on a silica gel column, using large volumes of organic solvents. Final purification involved partitioning of the target toxin into acetonitrile. Although the method specified TLC as the chromatographic separatory technique, an aliquot of the purified extract was separated by HPLC and monitored by fluorescence detection (due to the increased resolution and sensitivity of the technique when compared to TLC). The use of a large chromatographic column (silica gel step), used in the AOAC procedure, resulted in this method being the most time consuming of the three evaluated. The most rapid sample preparation method was found to be that of Chang and DeVries, (1984) which involved extraction with dichloromethane/water, precipitation of proteinaceous material with cupric carbonate and partitioning of the zearalenone into acetonitrile. The third method (Bagneris et al., 1986), which is a modification of that adopted by the AOAC, following successful collaborative studies reported by Bennett et al. (1985), involved extraction with chloroform/water followed by primary partitioning into a base solution

and secondary partitioning into dichloromethane. The sample extracts prepared by the latter method were visibly the least complex, being colourless or slightly opaque, whilst those extracts prepared by the previous methods were yellow in colour.

3.4.2 Chromatographic procedures

Although the presence of zearalenone in sample extracts may be monitored by HPLC using UV detection at several different wavelengths (Figure 3.3), fluorescence detection affords greater sensitivity and selectivity for the determination of zearalenone. Sample extracts were, therefore, monitored by fluorescence detection, using the excitation and emission wavelengths as determined in section 3.1.7 (Figure 3.4). The literature concerning the choice of fluorescence detector wavelengths for the determination of zearalenone is, however, somewhat confusing. Figure 3.8 (a-e) shows a series of chromatograms of a single sample extract screened under identical chromatographic conditions and instrument sensitivity settings, but monitored at a range of wavelengths cited in the literature (Sydenham et al., 1988). With all the combinations of excitation and emission wavelengths (Figure 3.8 a-e), both zearalenone and alternariol monomethyl ether (AME - an *Alternaria* mycotoxin present in the samples screened, but which itself does not form part of this study) could be detected. The choice of excitation and emission wavelengths substantially influenced the sensitivity of detection. In

this study, the recorded fluorescence wavelength maxima (Figure 3.4) were similar to those previously published (Chang and DeVries, 1984).

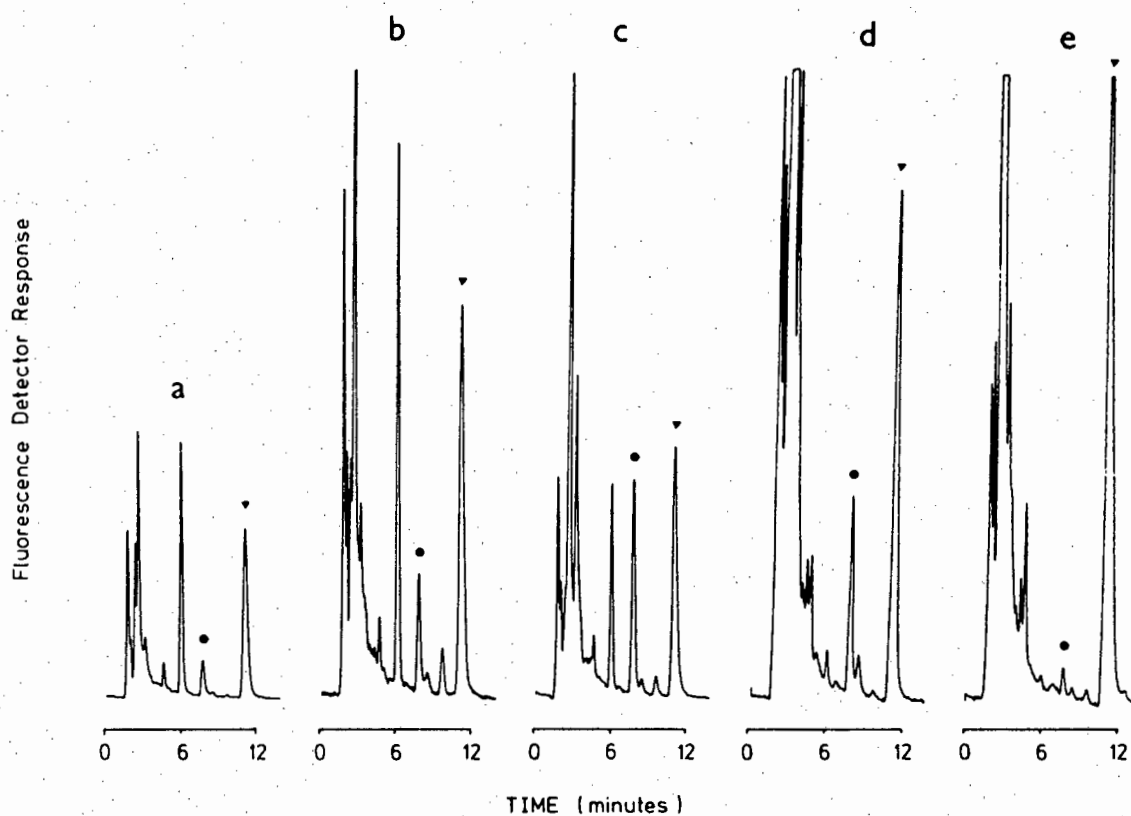


FIG. 3.8 Chromatograms of a sample extract monitored at various wavelengths (excitation wavelength (nm), emission wavelength (nm), reference): (a) 236, 418, Trenholm et al., 1984; (b) 274, 418, Bennett et al., 1985, Bagneris et al., 1986; (c) 280, 465, Chang and DeVries, 1985; (d) 320, 445, Sydenham et al., 1988; (e) 335, 404, Sydenham et al., 1988. Key: ●, zearalenone; ▼ alternariol monomethyl ether

Figure 3.9 shows the chromatogram of an unspiked control maize sample, prepared according to the AOAC method (1984), using chromatographic conditions similar to those cited in Table 2.3 (except that a flow rate of $0.5 \text{ ml minute}^{-1}$ was used). The chromatogram represents a 120 mg sample equivalent weight injected onto the chromatographic column. Whilst the chromatographic position of zearalenone (approximately 9 minutes - designated by an asterisk) was clearly contaminant free, major component peaks were found to elute after 18 and 43 minutes.

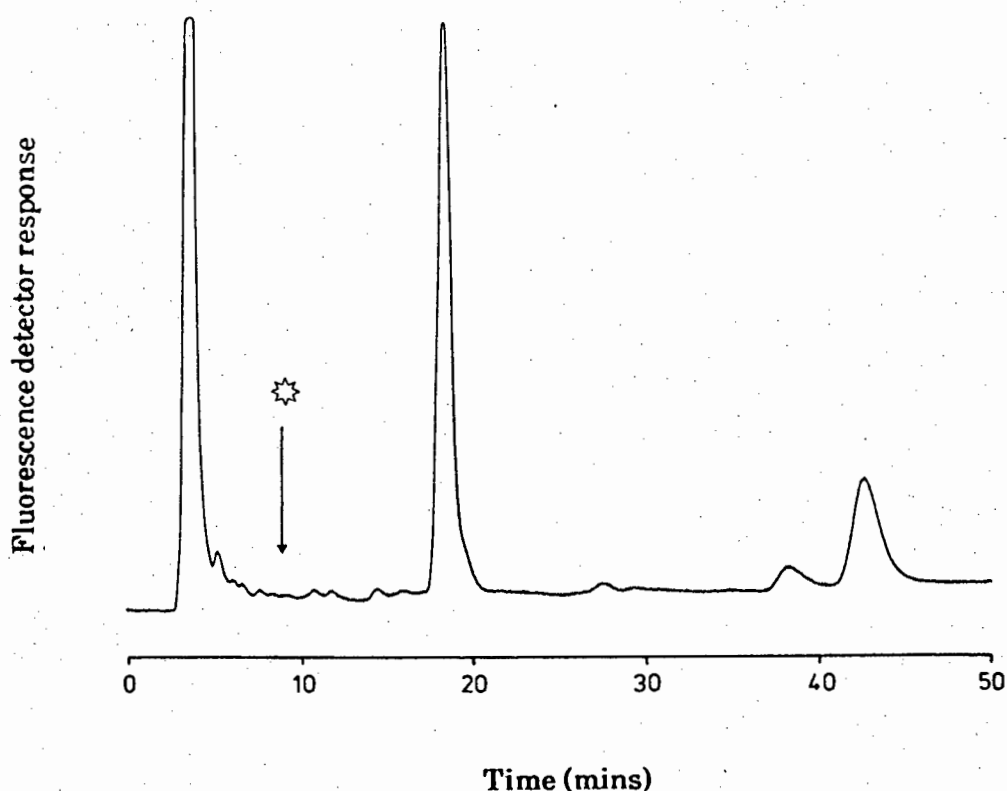


FIG. 3.9 Chromatogram of an unspiked control maize sample extracted according to the method of the AOAC, 1984. The chromatographic position of zearalenone is designated by the asterisk

Figures 3.10.1 and 3.10.2, show the chromatograms obtained from similar extracts prepared according to the methods of Bagneris et al. (1986) and Chang and DeVries, (1984), respectively. Using these methods, all components were found to elute from the analytical column within 12 minutes. No contaminant peaks were observed in the chromatographic position of zearalenone in the extract prepared by the method of Bagneris et al. (1986). However, minor contamination could be seen in the same chromatographic area in the latter extract (Chang and DeVries, 1984; Figure 3.10.2).

3.4.3 Toxin recoveries and method detection limit

Subsamples of control maize spiked with authentic zearalenone standard (at 150 ng g^{-1}) were prepared and used to determine toxin recoveries (in triplicate), for the three methods. Average recoveries and coefficients of variation (given in brackets) of 84% (± 8.7), 82% (± 7.2) and 78% (± 6.0) were recorded for the methods according to the AOAC (1984), Bagneris et al. (1986) and Chang and DeVries, (1984), respectively. Using control maize, and in the absence of interfering compounds, the detection limit of the methods was found to be in the order of $10\text{-}20 \text{ ng g}^{-1}$.

3.4.4 Zearalenone methodology - summary

Although, at a spiking level of 150 ng g^{-1} in control maize, the AOAC method (1984) gave the highest toxin recovery, a

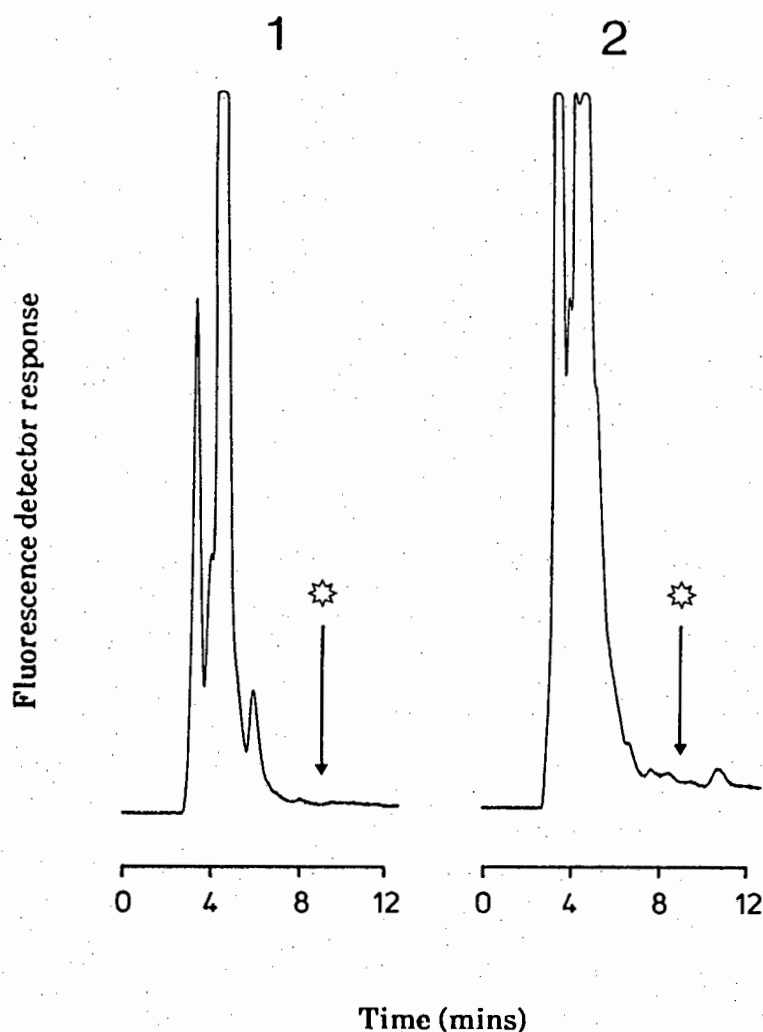


FIG. 3.10 Chromatograms of unspiked control maize extracted according to the methods of (1) Bagneris et al., 1986 and (2) Chang and DeVries, 1984. The chromatographic position of zearalenone is designated by the asterisk

number of inherent method characteristics, including sample preparation time, the use of large volumes of organic solvents (including the carcinogen benzene) and the necessity for prolonged analysis times (to elute all components), combined to make the method the least desirable. The method of

Bagneris et al. (1986) gave the second highest toxin recovery, whilst it also gave both visibly and chromatographically, the least complex sample extract of the three methods.

Sydenham and co-workers (Sydenham et al., 1988) also reported that the three methods evaluated in this study also extracted a totally unrelated toxin (ie AME), from a series of mixed feed samples. The limitations of TLC as a separatory technique was also demonstrated (Sydenham et al., 1988), since the zearalenone and AME co-contaminants of the feed samples could not be fully resolved by TLC, but were so by HPLC. The importance of the choice of excitation and emission wavelengths in fluorescence detection were demonstrated in the same publication.

3.5 COMPARISON OF MONILIFORMIN METHODOLOGY

3.5.1 Extraction procedures

The method of Thiel et al. (1982b) was found to be an extremely simple three step procedure, consisting of (a) extraction of the toxin from the matrix with distilled water followed by (b) centrifugation and (c) supernatant filtration prior to HPLC separation. Although the method required minimal sample handling, the major time consuming step was the one hour required for toxin extraction. The method of Scott and Lawrence, (1987) appeared to be far more involved, consisting of extraction with acetonitrile/water, a defatting process via solvent partitioning with hexane and two succes-

sive cleanup stages on small C₁₈ and neutral alumina columns, respectively. However, not only was the extraction procedure found to be more rapid than that of Thiel et al. (1982b), it also resulted in an extract that was visibly less contaminated than the opaque extract resulting from the method of Thiel et al. (1982b).

3.5.2 Chromatographic procedures

The UV spectrum of moniliformin (Figure 3.2), clearly identifies two absorption maxima that might be used for monitoring the presence of moniliformin (229 nm and 254 nm). When monitoring moniliformin, the 229 nm wavelength was found to give a threefold increase in sensitivity over the 254 nm wavelength. However, the two wavelengths could be used in conjunction with each other for confirmation purposes, where the maximum sensitivity was not a prerequisite (Figure 4.6).

Paired ion chromatography was used by both methods for the separation of the purified extracts, however Thiel et al. (1982b) also used ion exchange chromatography for confirmatory purposes. Using the specified HPLC conditions the moniliformin peak eluted at a retention time of approximately 8 minutes.

Under the specified analytical conditions, the greatest difference between the methods was the sample equivalent weight applied to the analytical column (ie 1.5 mg for the method of Thiel et al. 1982b, as opposed to 25 mg for the method of

Scott and Lawrence, 1987). To a large extent the attainable sensitivity of any analytical method is dependant upon the efficiency of the sample cleanup, since this is the limiting factor with respect to the mass equivalent of matrix that can

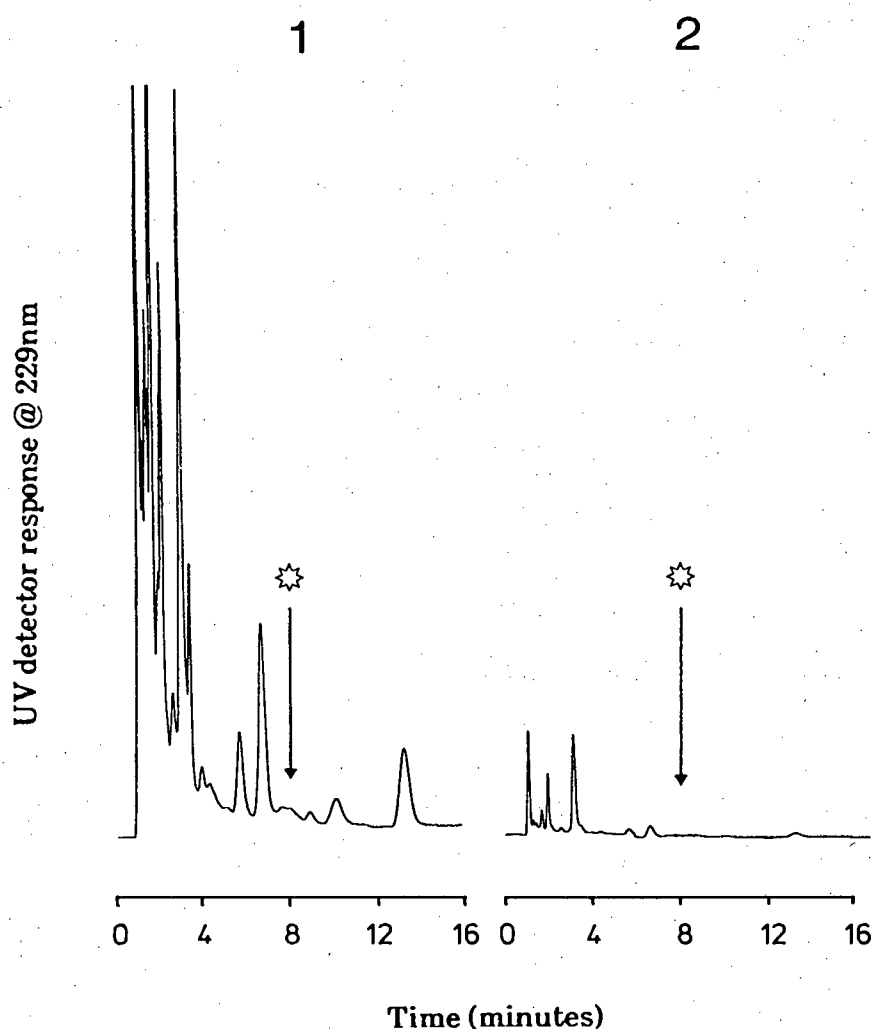


FIG. 3.11 Chromatograms representing 17 mg mass sample equivalents injected onto the analytical column, of unspiked control maize extracted according to the methods of (1) Thiel et al., 1982 and (2) Scott and Lawrence, 1987. The chromatographic position of moniliformin, in both chromatograms, is designated by the asterisk

be injected onto any chromatographic column. The methods of Thiel et al. (1982b) and Scott and Lawrence (1987), were therefore altered slightly so as to compare the chromatograms obtained from each method, following the injection of the same sample mass fraction onto the HPLC column. Figures 3.11.1 and 3.11.2 illustrate a 17 mg sample equivalent weight extracted from a control maize sample, according to the methods of Thiel et al. (1982b) and Scott and Lawrence, (1987), respectively. The figures clearly demonstrate the degree of contamination present in the extract prepared according to the method of Thiel et al. (1982b - Figure 3.11.1), whilst the absence of this contamination in the extract prepared according to the method of Scott and Lawrence, (1987), is similarly illustrated. In both cases, the chromatographic position of moniliformin is indicated by an asterisk.

3.5.3 Toxin recoveries and method detection limit

Taking into account the fact that different sample equivalent weights are injected onto the column, different spiking levels were necessary for the two methods. Two subsamples of control maize, spiked with 50 and 3 $\mu\text{g g}^{-1}$ moniliformin, respectively, were prepared, extracted and analysed in triplicate according to the methods of Thiel et al. (1982b) and Scott and Lawrence, (1987) - average recoveries of 80% and 96% were obtained, respectively. The detection limit (using control maize and the chromatographic conditions outlined in Table 2.4) for the method of Thiel et al. (1982b) was found

to be in the order of $10 \mu\text{g g}^{-1}$, whereas the detection limit for the method of Scott and Lawrence, (1987) was found to be 200 ng g^{-1} . By altering the detector sensitivity, the detection limits (using control maize) could be reduced by factors of approximately 2.5 and 10, respectively. This difference was due primarily to excessive matrix interferences encountered in the method according to Thiel et al. (1982b), which were substantially reduced in the method of Scott and Lawrence, (1987).

3.5.4 Moniliformin methodology - summary

The overwhelming superiority of the method according to Scott and Lawrence, (1987) over that according to Thiel et al. (1982b), was demonstrated by a number of observations. The former method not only gave a less complex extract, it also exhibited a higher target toxin recovery and greater sensitivity, than the latter method. A third method for the determination of moniliformin in maize was initially considered for evaluation purposes (Shepherd and Gilbert, 1986). Problems were encountered with regard to toxin recovery at the secondary cleanup stage of the method. Due to the non-availability of some of the specified materials, alternatives were used. These alterations to the method may well have been the cause of the low recoveries encountered. Due to these problems, it was not possible to fully evaluate the method, and therefore, for the purposes of this study it was excluded from the evaluation procedure.

B: INNOVATIVE METHODOLOGY

3.6 TLC DETERMINATION OF THE FUMONISINS

TLC has been used as a monitoring system for the isolation of fumonisins B₁ and B₂ from culture material of *F. moniliforme* MRC 826 (Gelderblom et al., 1988). The fumonisins could be detected using silica gel 60 TLC plates developed in chloroform : methanol : acetic acid (6:3:1), followed by spraying with p-anisaldehyde solution and visual inspection (as described in section 3.1.2). However, as the limit of detection (for the TLC method) was found to be in the order of 500 µg g⁻¹ for each fumonisin toxin, it was considered unsuitable as a technique for the determination of the fumonisins (FB₁ or FB₂) in naturally contaminated samples.

3.7 HPLC DETERMINATION OF FUMONISIN B₁ (AS ITS MALEYL DERIVATIVE)

Siler and Gilchrist, (1982) used a derivatization procedure for the determination of a host-selective phytotoxin (TA toxin), produced by *Alternaria alternata* f. sp. *lycopersici*, by the introduction of the UV absorbing maleyl group onto the primary amine present in the structure of the toxin. Figures 3.12.1 and 3.12.2 illustrate the structures of TA toxin and fumonisin B₁, respectively, and clearly show the structural similarity of the two compounds which are produced by separate, totally unrelated genera of fungi.

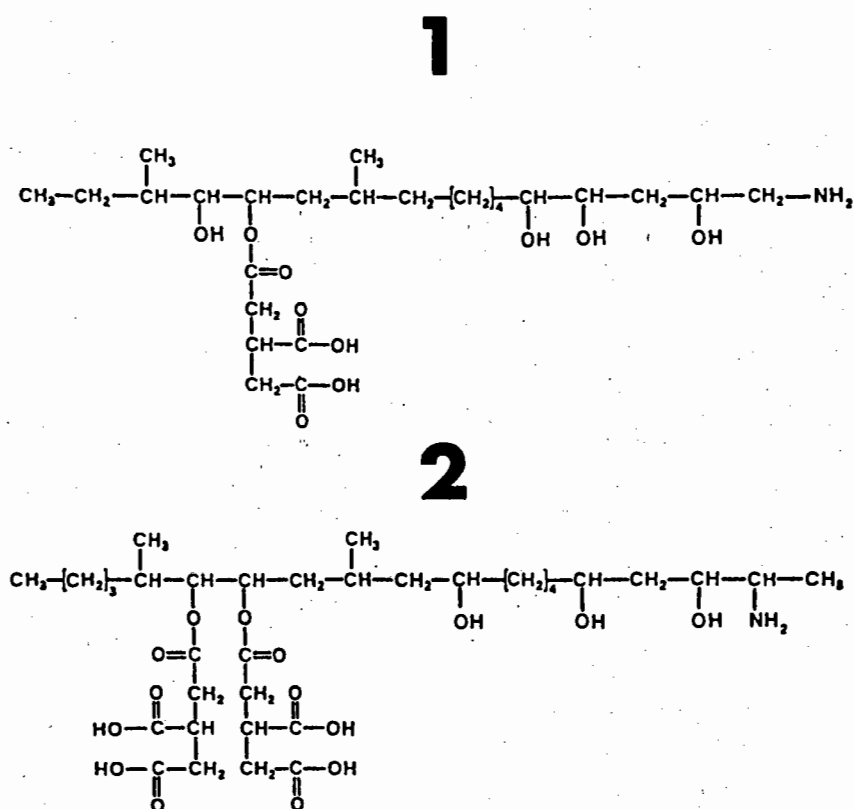


FIG. 3.12 Chemical structures of (1) the *Alternaria* phyto-toxin TA toxin and (2) the *Fusarium* mycotoxin fumonisin B₁

The difference between the two toxins is the absence of one TCA group and a C₃ shorter aliphatic aminopentol moiety in the structure of the Al toxin. Due to this structural similarity, the derivatization procedure (Siler and Gilchrist, 1982) was, with minor modifications, applied to a fumonisin B₁ standard. Figures 3.13.1 and 3.13.2 show the chromatograms obtained from a derivative blank and a fumonisin B₁ standard, respectively. The derivatized fumonisin B₁ eluted at a reten-

tion time of ± 6.0 minutes (Figure 3.12.2). Using control maize spiked with fumonisin B₁ (at $100 \mu\text{g g}^{-1}$), triplicate recoveries were performed. The average corrected recovery was found to be 66.3% (SD = 1.48%) and the limit of detection was in the order of $10 \mu\text{g g}^{-1}$.

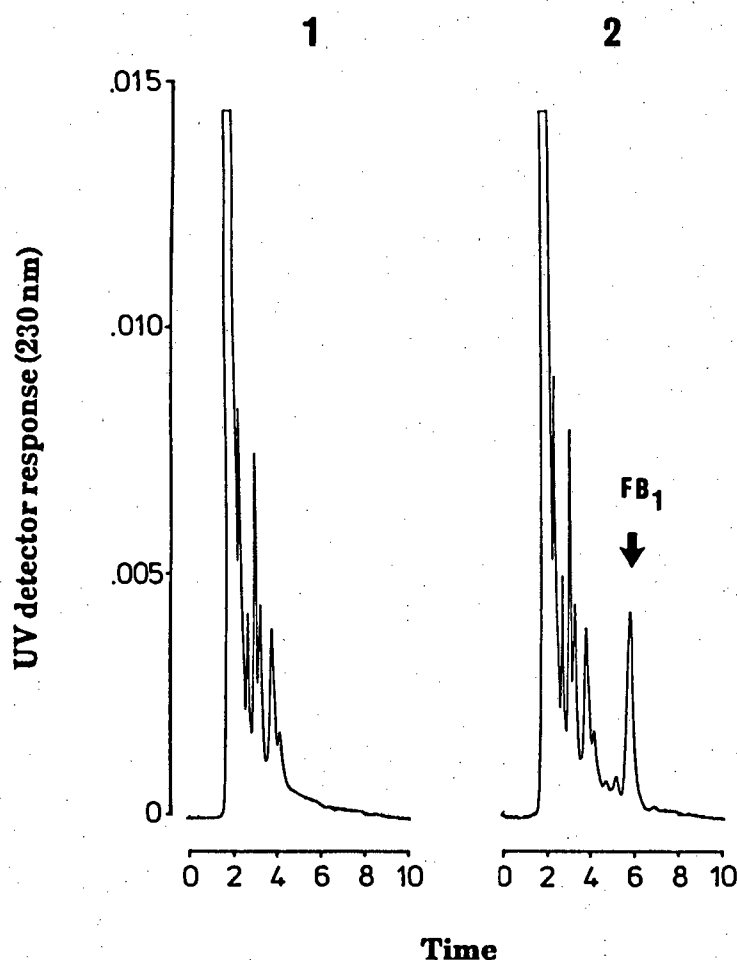


FIG. 3.13 HPLC chromatograms of (1) a maleyl derivative blank and (2) a similarly derivatized fumonisin B₁ standard

The extraction, derivatization and chromatographic procedures for the determination of fumonisin B₁ (as its maleyl derivative) in maize (sections 2.7.1, 2.7.2 and 2.7.6), were applied to two samples of control maize, three subsamples of home-grown Transkeian maize (detailed in section 2.1.3) and a sample of culture material (*F. moniliforme* MRC 826 - section 2.1.2). The results are given in Table 3.3.

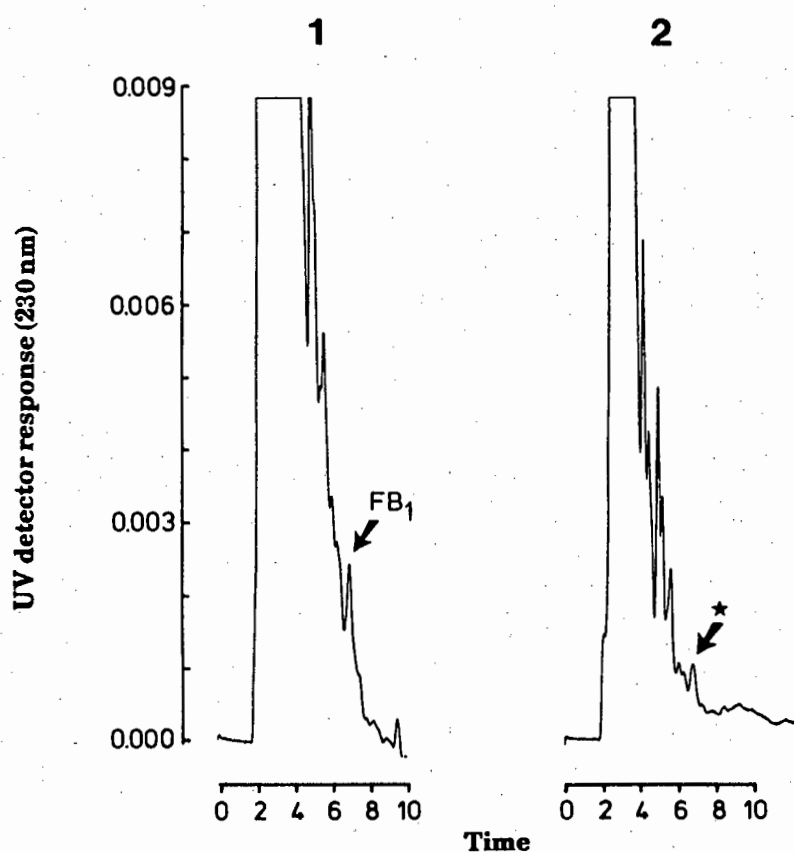


FIG. 3.14 HPLC chromatogram of (1) the maleyl derivative of sample M-84 and (2) the chromatogram of a similarly prepared extract of hand-selected maize kernels, showing the presence of a contaminant peak (★), which elutes in the chromatographic position of fumonisin B₁ (FB₁)

Figure 3.14.1. shows the chromatogram obtained from a 5 μ l aliquot of the maleyl derivative of an extract of the Trans-keian maize sample M-84. Baseline separation of the fumonisin B₁ peak was not achieved, due to the presence of matrix interferences from the maize substrate. Resolution problems were encountered (especially when analysing naturally contaminated maize samples), following the chloroform partitioning step during the primary clean-up stage of the extraction procedure. The residue could not be fully dissolved in methanol: water (1:3). A gelatinous methanol soluble precipitate was normally encountered.

Figure 3.14.2 details the chromatogram obtained from a similarly derivatized extract of a control maize sample. Whilst the degree of matrix interference is much lower than that observed in Figure 3.14.1, a small peak could be seen eluting at the chromatographic position of fumonisin B₁ (corresponding to $<10 \mu\text{g g}^{-1}$ fumonisin B₁ - Table 3.3). A similar peak was also observed in a sample of commercially available maize meal and subsequently in 3 other maize samples. The presence of this peak in several maize samples indicated the possibility that it was an interfering compound found intrinsically in maize.

Figure 3.15 illustrates the chromatogram obtained from an extract of a fungal culture (*F. moniliforme* MRC 826) and clearly shows the reduced matrix interferences encountered chromatographically (when compared to Figure 3.14.1).

TABLE 3.3 LEVELS OF FUMONISIN B₁ (FB₁) AND TRICARBALLYLIC ACID (TCA) ASSAYED AS THEIR MALEYL AND BUTYL ESTER DERIVATIVES, RESPECTIVELY¹

Sample ²	FB ₁ measured as the maleyl derivative ³ ($\mu\text{g g}^{-1}$)	TCA calculated as present due to FB ₁ (maleyl derivative results) ($\mu\text{g g}^{-1}$)	TCA measured as the butyl ester deriva- tive ⁴ ($\mu\text{g g}^{-1}$)
Hand selected control corn	<10 ⁵	<4.9	<0.5
Commercial corn meal	<10	<4.9	13
M-84/C	<10	<4.9	21
M-84	44	22	101
M-84/F	83	41	164
MRC 826	9 280	4 530	6 400

¹ Results currently in press (Sydenham et al., 1989a)

² M-84/C = Healthy maize kernels

M-84 = Mouldy maize kernels

M-84/F = *Fusarium*-infected maize kernels

MRC 826 = Culture material of *Fusarium moniliforme* MRC 826

³ Determined by HPLC/UV; detection limit = approximately $10\mu\text{g g}^{-1}$

⁴ Determined by GC/FID; detection limit = $0.5\mu\text{g g}^{-1}$

⁵ Subsequent analysis indicated that this was a contaminant peak
eluting in the position of FB₁

Figure 3.15 also shows that fumonisin B₂ can be monitored by the same derivatization technique.

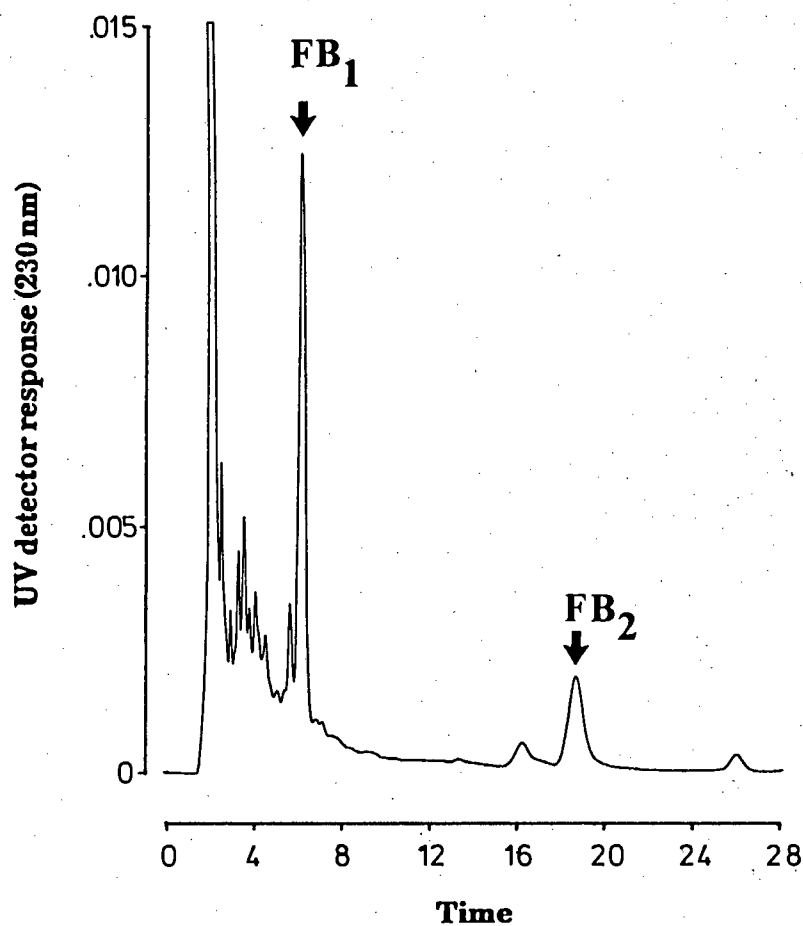


FIG. 3.15 Chromatogram of the maleyl derivative of an extract of *F. moniliforme* (MRC 826), showing the presence of fumonisin B₁ (rt = 6.0 minutes) and fumonisin B₂ (rt = 24.0 minutes)

Therefore, a number of matrix related factors demonstrated the limitations of the maleyl derivative procedure for the

determination of fumonisin B₁ in naturally contaminated maize samples. However, when analysing culture samples, matrix interferences, at both the extraction and chromatographic stages of the method, were substantially reduced. This may be explained by the fact that due to the far higher concentrations of the fumonisins present in fungal culture samples, a far lower sample equivalent weight is injected onto the HPLC column (up to 50 times less), significantly reducing matrix effects.

3.8 HPLC DETERMINATION OF FUMONISIN B₁ (AS ITS FLUORESCAMINE DERIVATIVE)

It was necessary to consider the use of detection techniques other than UV, for the determination of fumonisin B₁ in naturally contaminated maize, due to the relatively poor detection limit ($10 \mu\text{g g}^{-1}$) obtained by the maleyl derivatization/UV procedure. Fluorescence detection tends to offer superior sensitivity and selectivity than UV for numerous compounds. In the case of fumonisin B₁ it was necessary to introduce a fluorescent species (or label), due to the molecules' lack of intrinsic fluorescence characteristics. A number of fluorescent labels are commercially available and several have been used extensively for the derivatization of primary amines, especially in the area of amino acid analyses (Perrett, 1985; Rosenthal, 1985).

Dansyl chloride (Dns-Cl, 1-dimethylaminonaphthalene-5-sulpho-

nyl chloride), ortho-phthalaldehyde (OPA) and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) were three fluorescent labels which were initially evaluated. Derivatization of fumonisin B₁ (with these reagents) required reactions to be carried out at various pH, reaction time and temperature settings. Initial HPLC separations of the preformed derivatives (using a Waters 4- μ m C₁₈ column - as used for both zearalenone and moniliformin analyses) resulted in pronounced chromatographic tailing effects.

Fluorescamine (4-phenylspiro [furan-2(3H)yl-1-phthalan]-3,3'-dione), another fluorescent label that has been used for the analyses of amino acids, was also evaluated. It is a compound which lacks intrinsic fluorescence (unlike other labels - ie NBD-F), but it reacts rapidly at room temperature with primary amines ($t_{1/2}$ = 100-500 milliseconds - Perrett, 1985), to form stable fluorophors (Dns-Cl and OPA derivatives tend to be unstable). A fluorescamine - fumonisin B₁ derivative complex was also found to exhibit chromatographic tailing. This effect was reduced, to a large extent, by using a HPLC column having high carbon loading and extensive end-capping features.

Control maize and the Transkeian maize subsamples were analysed according to the method outlined in section 2.7.6. Figure 3.16.1 shows the chromatogram of a fluorescamine derivatized fumonisin B₁ standard (65 ng), where two well resolved peaks may be seen at the retention times of 14.3 and 16.5 minutes,

respectively. Figure 3.16.2 illustrates the chromatogram obtained from a similarly derivatized extract of M-84/F and Figure 3.16.3, the same extract spiked with derivatized fumonisin B₁.

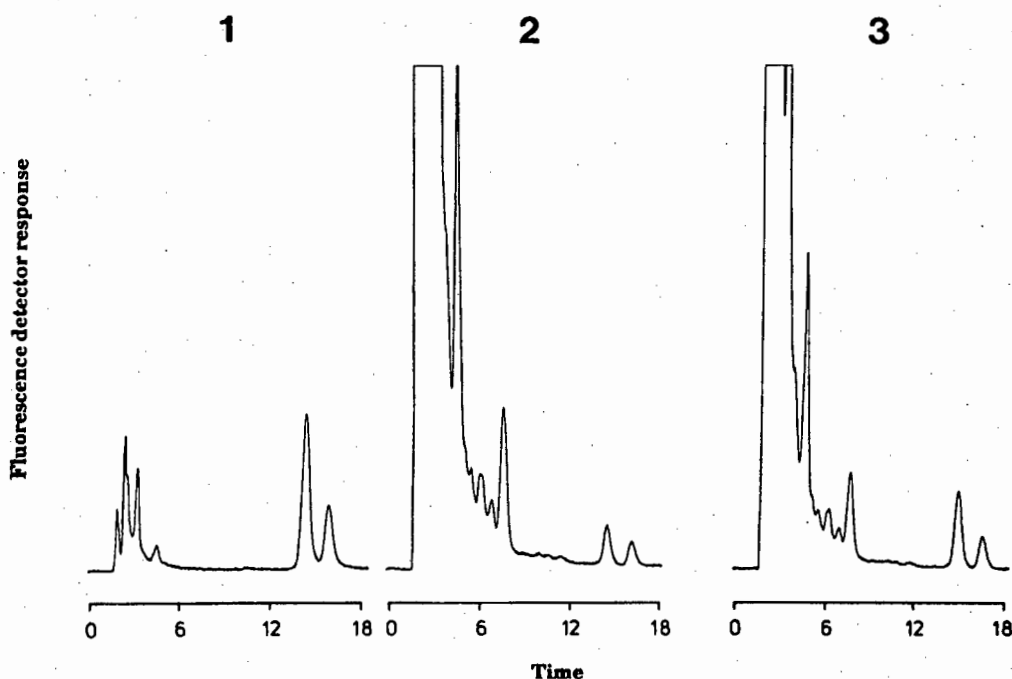


FIG. 3.16 HPLC chromatograms of the fluorescamine derivatives of (1) 65 ng fumonisin B₁, (2) sample M-84/F and (3) the chromatogram of the same extract spiked with derivatized fumonisin B₁

When used as part of a post column detection system, a single fluorophore is chromatographed. However, HPLC separation of a preformed fluorescamine - primary amine complex often results in a dual peak for primary amines, as was the case with fumo-

nisin B₁ (Figure 3.16.1). The two peaks result from the acid alcohol and lactone forms of the fluorescent complex which exhibit identical fluorescent characteristics (Rosenthal, 1985), as shown in Figure 3.17.

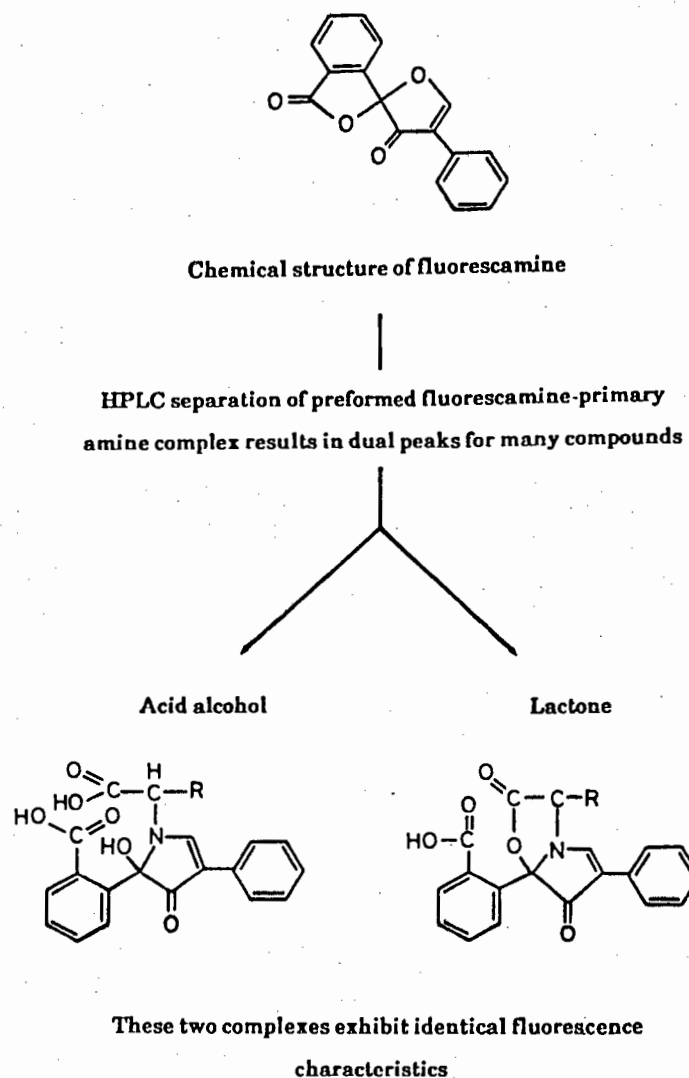


FIG. 3.17 Flow diagram of a preformed fluorescamine-primary amine complex which results in the formation of 2 derivatives

The clean-up and derivatization procedure gave excellent re-

coveries (in excess of 90%) for the determination of pure fumonisin B₁. However, when extracts of control maize spiked with fumonisin B₁ were analysed, the observed recoveries were far lower (< 50%). The cause for the anomaly appeared to be the presence of other primary amine containing compounds in the control maize sample, which were also derivatized by the available fluorecamine. Methods to remove these interferences and/or the utilization of a post column fluorecamine derivatization procedure were investigated, but still require further optimization. Therefore, due to inherent matrix effects, the procedure could only provide qualitative data pertaining to the presence of fumonisin B₁ in maize.

3.9 CAPILLARY GC OF FUMONISIN B₁

Fumonisin B₁ is a large molecule, having a molecular weight of 721. Attempts were made to chromatograph the complete molecule intact, which required derivatization of hydroxyl, carboxyl and primary amine groups. Several derivatization procedures (Blau and King, 1977) were evaluated. These procedures including the use of diazomethane, HFBI, Tri Sil-TBT, N,O-bis(trimethylsilyl)acetamide (BSA) and N-methyl-N-(tert-butyl-methylsilyl)-trifluoroacetamide (MTBSTFA). In each case, a fumonisin B₁ and a derivative blank were prepared for comparative chromatographic analysis. Using these derivatization procedures (or the prevailing chromatographic conditions) no chromatographic peaks could be detected.

Fumonisin B₁ was subsequently subjected to a hydrolysis, esterification and acylation procedure used for the capillary GC separation of amino acids (sections 2.7.3 and 2.7.8, Labadarios et al., 1984). Chromatographic separation of the derivative (using the conditions outlined in Table 2.7) resulted in the elution of a single peak. This peak could not be detected when the acylation step alone was performed on an aliquot of the fumonisin B₁ hydrolysate. However, esterification of an aliquot of the same hydrolysate (without the subsequent acylation step), resulted in the detection of the same single chromatographic peak. These observations suggested that the hydrolysis had successfully hydrolysed the fumonisin B₁, but that in the process (or using either the derivatization or chromatographic parameters outlined) only the esterified tricarballylic acid (TCA) moiety of the fumonisin B₁ could be detected, and not the anticipated aminopentol moiety. This supposition was confirmed upon esterification and GC analysis of an authentic TCA standard, which resulted in a single peak being detected at a retention time identical to that observed for the esterified extract of the fumonisin B₁ hydrolysate. Using the prevailing chromatographic conditions (Table 2.7), the detection limit for TCA was found to be in the order of 500 ng g⁻¹.

Using the extraction/hydrolysis and esterification procedure outlined in section 2.7.1, 2.7.3 and 2.7.8, the levels of the TCA moiety present in the two control maize, three Transkeian maize and fungal culture samples (used for the evaluation of

the HPLC-maleyl derivative procedure) were determined by capillary GC (as its iso-butyl ester), and the results obtained are given in Table 3.3

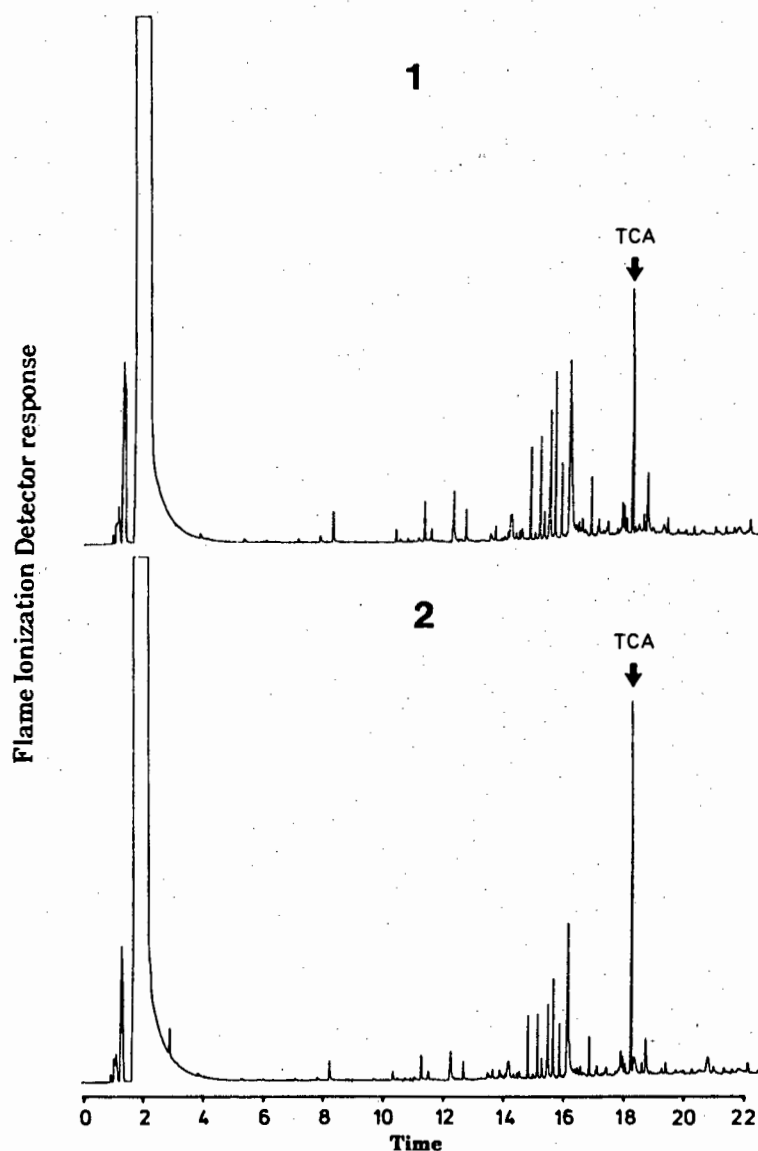


FIG. 3.18 Capillary GC/FID chromatograms of (1) a hydrolysed esterified extract of M-84/C showing a major peak eluting at 18.3 minutes (Tricarballic acid - TCA), and (2) the same sample extract spiked with esterified TCA

Figure 3.18.1 shows the capillary GC-FID chromatogram obtained from a hydrolysed-esterified extract of M-84/C, whilst Figure 3.18.2 shows the same extract spiked with a similarly esterified fumonisin B₁ standard. A well resolved peak corresponding to the isobutyl ester of TCA eluted at a retention time of 18.3 minutes (Figures 3.18.1 and 3.18.2).

The TCA contribution due to the fumonisin B₁ (determined as its maleyl derivative), present in each sample, was calculated and the results are given in Table 3.3. The levels of TCA determined experimentally (by the GC-FID procedure) were invariably higher in each sample than the corresponding TCA content contributed by the fumonisin B₁. In the case of the hand-selected control maize sample, the peak observed in the chromatographic position of fumonisin B₁, as its maleyl derivative (Figure 3.14.2) was undoubtedly a contaminant peak since no corresponding TCA was detected in the sample. Since a similar sized peak was also observed in the commercial maize meal and M-84/C sample extracts, the levels of TCA determined experimentally in these samples could not be directly attributed to the presence of fumonisin B₁.

The fact that the TCA levels in 5/6 samples were higher than could be explained by the presence of fumonisin B₁, was not unexpected, as it has already been shown that TCA containing compounds other than fumonisin B₁ (such as fumonisin B₂) are produced by *F. moniliforme* (Gelderblom et al., 1988). Another TCA containing fumonisin (fumonisin B₃) has also recent-

ly been isolated from *F. moniliforme* (Gelderblom - personal communication). In the case of the MRC 826 culture material, the fumonisin B₁ accounted for >70% of the total TCA detected experimentally in the hydrolysed-esterified extract, which agrees with the observation that fumonisin B₁ is the major fumonisin produced by *F. moniliforme* in culture (Gelderblom et al., 1988). In samples M-84 and M-84/F, fumonisin B₁ contributed less to the total TCA than in the culture material, implying that more of the other fumonisins were present in these samples.

A paper detailing the natural occurrence of fumonisin B₁ in the Transkeian maize subsamples, based on the chromatographic evidence presented in sections 3.7 - 3.9 was recently accepted for publication (Sydenham et al., 1989a)

3.10 SUMMARY OF FUMONISIN B₁ METHODOLOGY

The chromatographic investigations covered in sections 3.6 - 3.9 resulted in a number of observations listed below:

1. That TLC was an inappropriate analytical method other than as a screening procedure for the presence of the fumonisins in culture material.
2. That the maleyl derivative method was useful as a quantitative procedure for the analysis of the fumonisins B₁ and B₂ in culture material, but that the detection limit (10

$\mu\text{g g}^{-1}$) was insufficient for the detection of low concentrations that would be present in naturally contaminated samples.

3. That the fluorescamine derivative-fluorescence detection procedure, whilst potentially allowing superior sensitivity, still required optimization with respect to potential sample matrix effects.

4. That at present, using the hydrolysis and subsequent derivatization procedures outlined, only the TCA moiety present in the structures of the known fumonisins, can be detected.

These points therefore suggested that until methods are available for the determination of the separate fumonisins, the hydrolysis-esterification procedure for the determination of the TCA moiety, might be used as a chromatographic indicator of the potential extent of the total fumonisin contamination of naturally contaminated maize samples.

CHAPTER 4

APPLICATION OF *FUSARIUM* MYCOTOXIN METHODOLOGY TO A SERIES
OF HOME-GROWN TRANSKEIAN MAIZE SAMPLES ASSOCIATED WITH HUMAN
OESOPHAGEAL CANCER RISK

4.1 OESOPHAGEAL CANCER IN THE TRANSKEI

It has for many years been suggested that an association exists in Africa, between the occurrence of human oesophageal cancer and maize cultivation (Cook, 1971). In Africa, oesophageal cancer rate is highest in the south-western districts of the Transkei (ie Kentani - Figure 4.1), whilst the rate in the north-eastern region (ie Bizana, Figure 4.1) is relatively low (Rose, 1973, 1982; Rose and McGlashan, 1975; Jaskiewicz et al., 1987). Maize is the staple diet in both regions.

An integral part of an ongoing mycotoxicological investigation, concerning maize gathered from the two regions, has been the implementation of comparative studies of the dominant mycoflora of the maize. The most consistent difference in the mycoflora in the home-grown maize during each of 3 years, has been the significantly higher incidence of *Fusarium moniliforme* Sheldon in maize produced in the high rate area (Marasas et al., 1981). This fungus has also been associated with foodstuffs in the Lin Xian district in the Henan province of China, which is one of the highest oesophageal cancer risk areas in the world (Li et al., 1979, 1980; Yang, 1980).

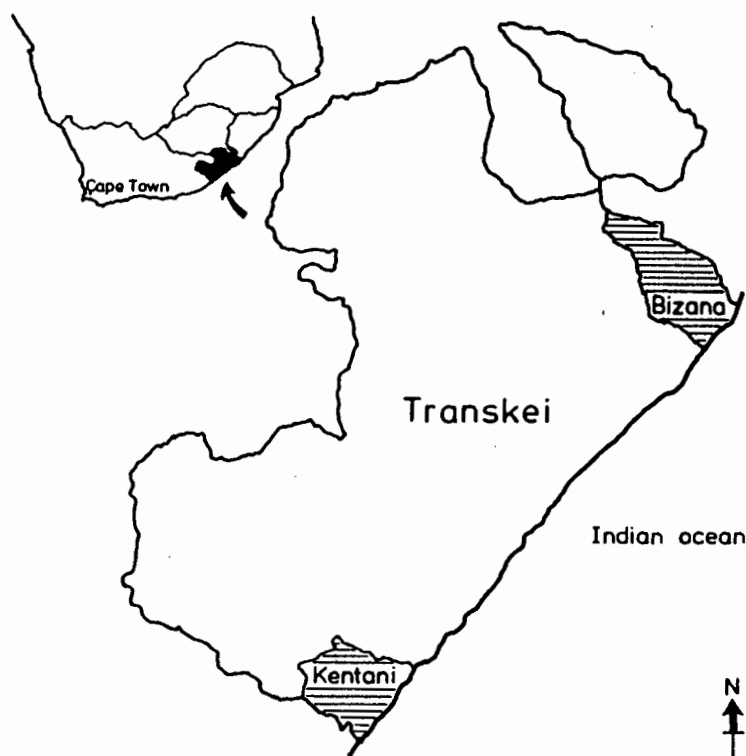


FIG. 4.1 Map detailing the position of Transkei within southern Africa, illustrating the locations of the low (Bizana) and high (Kentani) oesophageal cancer risk regions

Previous reports concerning the correlation of the presence of *F. moniliforme* and oesophageal cancer risk rates in Transkei, were based on the random sampling of households in the various geographical areas (Marasas et al., 1981), but no information was available concerning the incidence of oesophageal cancer in the occupants of the households.

In a subsequent study (Marasas et al., 1988a) the prevalence of human oesophageal cytological abnormalities were determi-

ned by means of brush biopsy capsules in adult occupants in each of 12 households in a low, an intermediate and a high oesophageal cancer rate area, in Transkei. Mild cellular changes (folic acid deficiency, atypia and mild dysplasia) and advanced changes (dysplasia and cancer), occurred more frequently in the occupants of households in high, than in intermediate or low oesophageal cancer rate areas. A study of the dominant fungi present in home-grown maize samples collected from each of the households included in the cytological screening survey, showed *F. moniliforme* was significantly higher in maize from cytologically "affected" households in the high oesophageal cancer rate area than from "unaffected" households in the low oesophageal cancer rate area (Marasas et al., 1988a). Therefore, whereas the previously established correlation was between *F. moniliforme* and oesophageal cancer rate, the results provided evidence for an association between the fungus and oesophageal cytological abnormalities in living individuals.

4.2 *FUSARIUM* MYCOTOXIN ANALYSES

The last part of this investigation is therefore, the application of the most suitable methodology for the determination of *Fusarium* mycotoxins (discussed in Chapter 3), to the home-grown maize samples which were collected from the households of individuals involved in the cytological survey detailed in section 4.1 (Marasas et al., 1988a).

TABLE 4.1 METHODS USED FOR THE DETERMINATION OF *FUSARIUM* MYCOTOXINS
IN HOME-GROWN TRANSKEIAN MAIZE SAMPLES

<i>Fusarium</i> mycotoxin	Separation and Detection technique	Extraction and purification (References)
Diacetoxyscirpenol and T-2 toxin	Capillary GC-ECD	Sydenham and Thiel, 1987
Nivalenol and Deoxynivalenol	Capillary GC-ECD	Scott et al., 1986
Zearalenone	HPLC-Fluorescence	Bagneris et al., 1986
Moniliformin	HPLC-UV	Scott and Lawrence, 1987
Fumonisin	Capillary GC-FID	Sydenham et al., 1989a

TABLE 4.2 TRANSKEIAN HOME-GROWN MAIZE SAMPLES (1985), MYCOLOGICAL AND
CHEMICAL RESULTS¹

District	F. <u>mon.</u> ²	F. <u>sub.</u> ³	F. <u>gram.</u> ⁴	Mon ⁵ $\mu\text{g g}^{-1}$	Zea ⁶ $\mu\text{g g}^{-1}$	Niv ⁷ $\mu\text{g g}^{-1}$	Don ⁸ $\mu\text{g g}^{-1}$	TCA ⁹ $\mu\text{g g}^{-1}$
BIZANA								
1	67	5	12	0.64	0.79	1.02	0.09	8.0
2	17	1	61	0.38	1.78	4.09	0.40	5.0
3	22	0	82	0.89	1.26	2.42	4.39	13.1
4	34	6	47	0.94	0.54	1.87	7.73	6.3
5	43	1	61	1.21	2.94	15.20	0.55	10.2
6	50	10	29	7.51	1.74	SL ¹⁰	SL	20.8
7	26	27	16	8.55	ND ¹¹	0.97	0.05	19.2
8	35	9	0	1.69	ND	SL	SL	16.9
9	20	15	25	2.51	0.31	3.82	12.10	14.3
10	54	26	14	11.57	1.36	SL	SL	SL
11	32	8	9	4.97	ND	1.53	0.32	16.5
12	14	13	63	1.51	3.28	10.64	0.56	6.5
KENTANI								
1	51	6	1	1.80	ND	0.08	0.09	16.7
2	94	1	5	0.52	ND	SL	SL	46.2
3	66	4	33	0.65	1.43	3.39	0.72	20.1
4	89	2	3	0.35	ND	SL	SL	57.4
5	51	11	0	0.48	ND	0.90	0.90	32.4
6	34	0	0	1.12	ND	SL	SL	31.6
7	72	8	1	0.56	ND	0.50	0.90	36.2
8	61	2	3	0.95	0.12	0.92	9.16	SL
9	76	3	12	1.04	0.15	0.75	0.14	24.5
10	38	8	36	1.17	2.39	8.73	1.00	25.8
11	87	3	1	0.48	ND	0.14	0.11	518.0
12	93	8	1	0.91	0.23	1.59	0.25	46.1

KEY TO TABLE 4.2

- 1 No diacetoxyscirpenol or T-2 toxin were detected in any of the maize samples (detection limit = 100 ng g^{-1})
- 2 F. mon. - Kernels infected with Fusarium moniliforme (%) (Marasas et al., 1988a)
- 3 F. sub. - Kernels infected with Fusarium subglutinans (%) (Marasas et al., 1988a)
- 4 F. gram. - Kernels infected with Fusarium graminearum (%) (Marasas et al., 1988a)
- 5 Mon - Moniliformin (detection limit = 200 ng g^{-1})
- 6 Zea - Zearalenone (detection limit = 20 ng g^{-1})
- 7 Niv - Nivalenol (detection limit = 40 ng g^{-1})
- 8 Don - Deoxynivalenol (detection limit = 40 ng g^{-1})
- 9 TCA - Tricarballic acid (detection limit = 500 ng g^{-1})
- 10 SL - Sample lost
- 11 ND - Not detected (see relevant toxin detection limits above)

Maize samples (12 each) from the high (Kentani) and low (Bizana) oesophageal cancer rate areas were analysed for the presence of the *Fusarium* mycotoxins using the methods detailed in Table 4.1. The previously reported mycological results (Marasas et al., 1988a) and the generated toxin results for each of the 24 samples, are given in Table 4.2.

4.3 CHROMATOGRAPHIC ANALYSES AND CONFIRMATION

4.3.1 Type A trichothecene analyses

The presence of diacetoxyscirpenol and T-2 toxin was not detected in any of the maize sample extracts (at a detection limit of 100 ng g^{-1}). Figure 4.2.1 illustrates the capillary GC chromatogram obtained from a maize sample extract as its heptafluorobutyryl ester, containing neosolaniol monoacetate (as the internal standard). Figure 4.2.2 shows the chromatogram of the same extract spiked with similarly derivatized diacetoxyscirpenol and T-2 toxin standards.

4.3.2 Type B trichothecene analyses

Nivalenol and deoxynivalenol were detected in each sample analysed, using a capillary GC-ECD procedure. Initial verification of the presence of deoxynivalenol and nivalenol in the sample extracts, was by the spiking (and subsequent chromatographic separation) of the extracts with similarly derivatized standards.

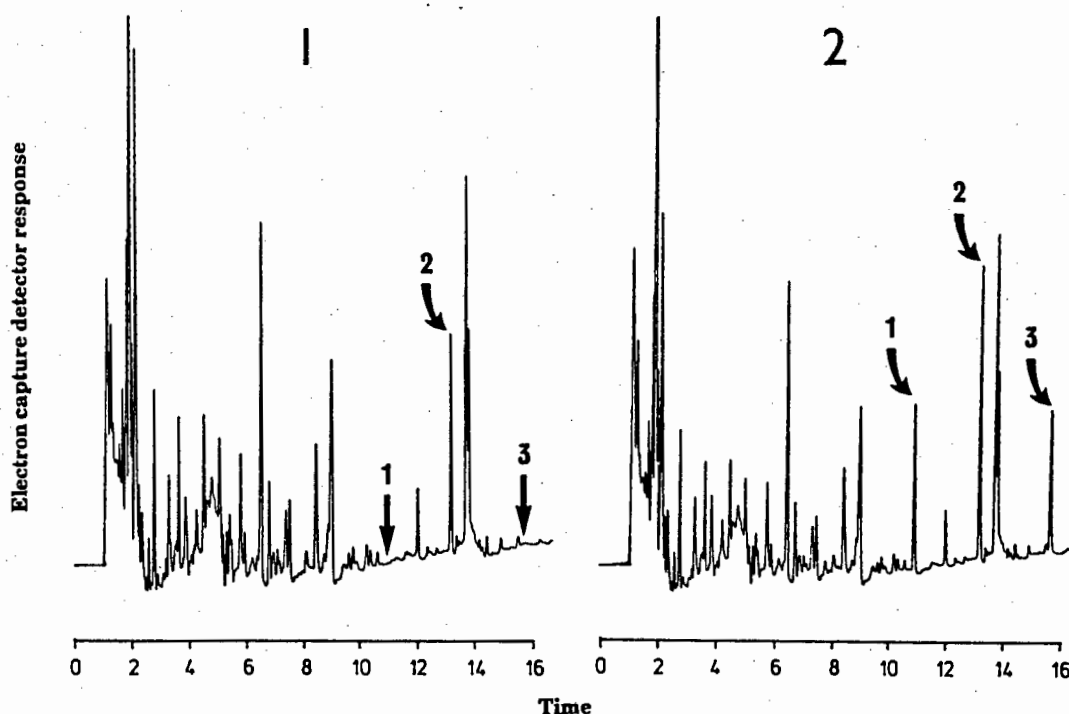


FIG. 4.2 Capillary GC chromatograms of (1) an extract of a Transkeian maize sample as its heptafluorobutyryl ester and (2) the same extract spiked with the target type A trichothecenes. The numerical notations 1, 2 and 3 correspond to the chromatographic positions of diacetoxyscirpenol, neosolaniol monoacetate (internal standard) and T-2 toxin, respectively

Due to problems with the cold storage facilities in which the maize samples were maintained, several of the samples were severely damaged and subsequently excluded from the analyses. In order to verify the identity of nivalenol and deoxynivalenol in a number of maize samples, extracts were analysed and monitored by capillary GC-MS using the conditions outlined in section 2.8.2.

Figure 4.3.1 shows the capillary GC-MS chromatogram of a maize sample extract, as its TMS derivative (Bizana sample number 9), previously shown to contain $12.1 \mu\text{g g}^{-1}$ deoxynivalenol (Table 4.2). The chromatographic position of deoxynivalenol (DON) was found to be approximately 24.8 minutes. The mass spectrum (100-400 m/z) of this peak is shown in Figure 4.3.2. The excellent agreement between the retention times and the mass spectra of the DON peak in the maize sample and that of a similarly prepared authentic DON standard (as its corresponding TMS derivative - Figure 4.3.3), provided unequivocal evidence for the presence of deoxynivalenol in the maize extract.

Similarly, Figure 4.4.1 shows the capillary GC-MS chromatogram of Bizana sample number 5, which had been found to contain $15.2 \mu\text{g g}^{-1}$ nivalenol (NIV). The presence of nivalenol, in the sample, was confirmed via the agreement of the retention times and mass spectra of the NIV peak in the sample and that obtained for authentic nivalenol (Figures 4.4.2 and 4.4.3, respectively).

Several other sample extracts contaminated with various concentrations of both *Fusarium* toxins were also analysed by capillary GC-MS, and in each case the retention times and mass spectra compared favourably.

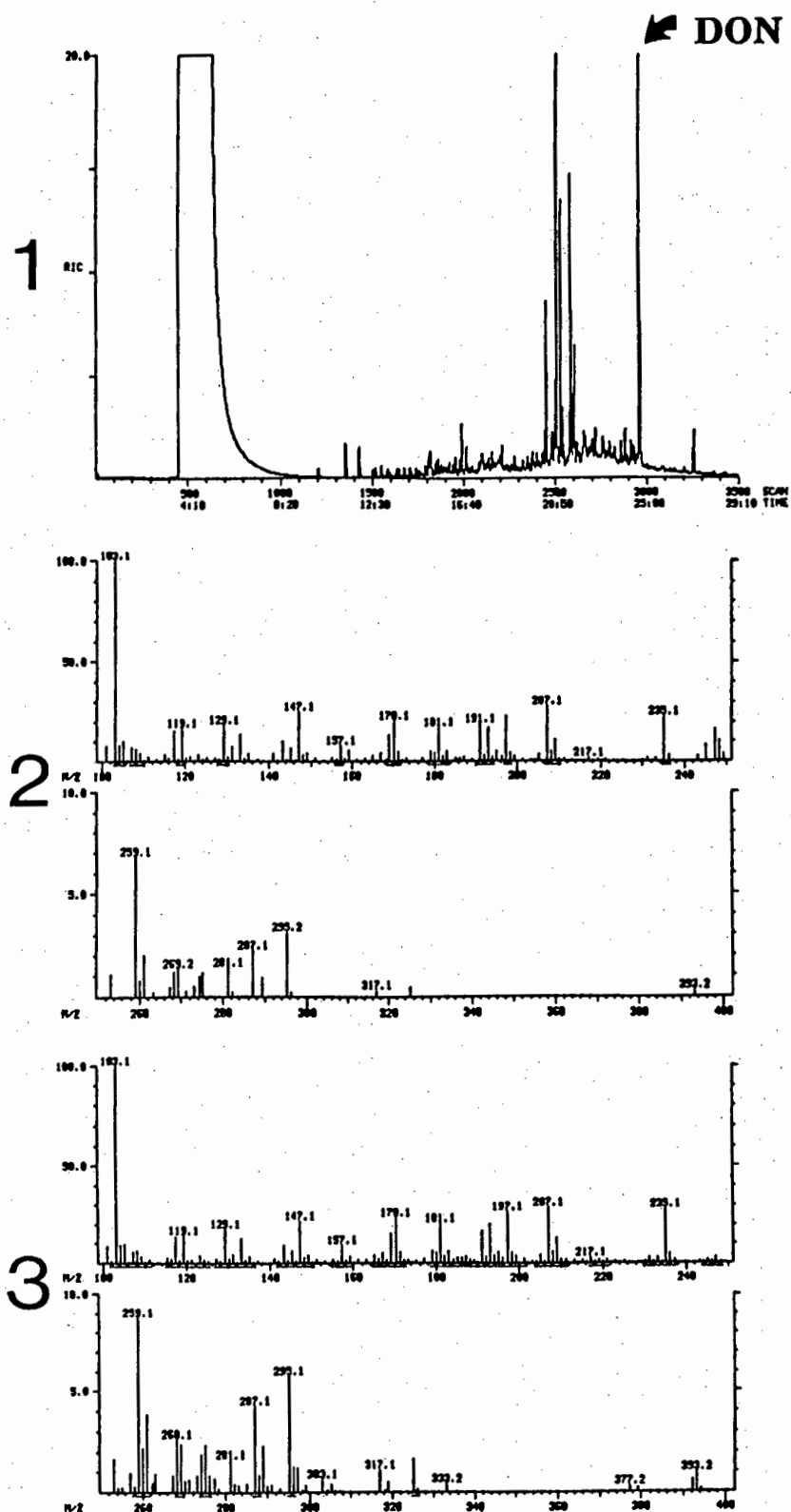


FIG. 4.3 (1) Capillary GC-MS chromatogram of a sample extract (Bizana #9) as its trimethylsilyl (TMS) derivative, (2) mass spectrum of the deoxynivalenol (DON) peak (rt = 24.8 minutes) and (3) the mass spectrum of an authentic DON-TMS derivative

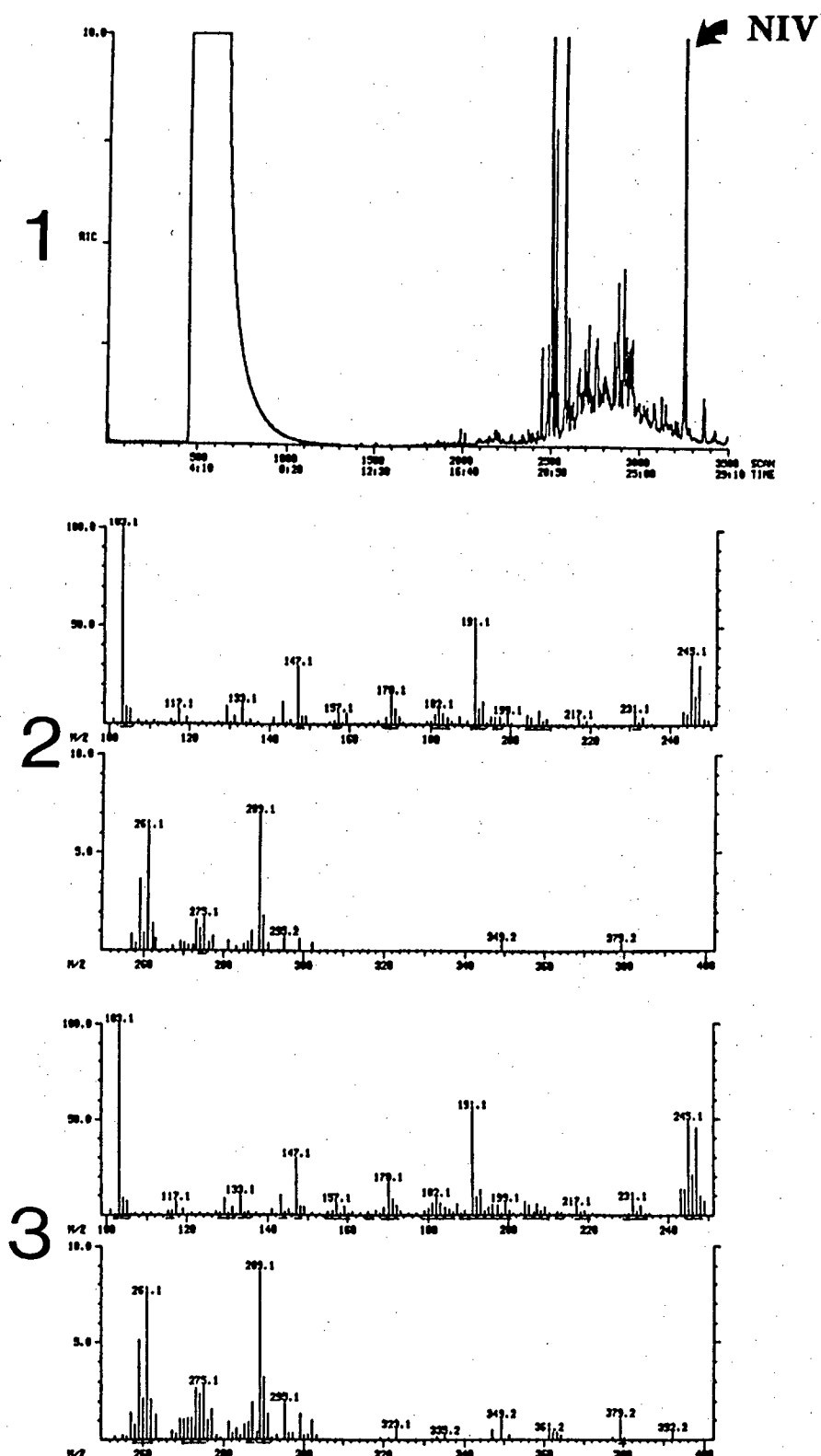


FIG. 4.4 (1) Capillary GC-MS chromatogram of a sample extract (Bizana #5) as its trimethylsilyl(TMS) derivative, (2) the mass spectrum of the nivalenol (NIV) peak (rt = 27.7 minutes) and (3) the mass spectrum of an authentic NIV-TMS derivative

4.3.3 Moniliformin analyses

Moniliformin was detected in each sample analysed at levels of between $0.35 - 11.57 \mu\text{g g}^{-1}$. Figure 4.5.1 shows the HPLC chromatogram of Bizana sample number 11 which was found to contain $4.97 \mu\text{g g}^{-1}$ moniliformin. Figure 4.5.2 shows the chromatogram of the same extract spiked with moniliformin standard. The observation of a single peak in the chromatographic position of moniliformin in the spiked extract (Figure 4.5.2) provided evidence for the presence of moniliformin.

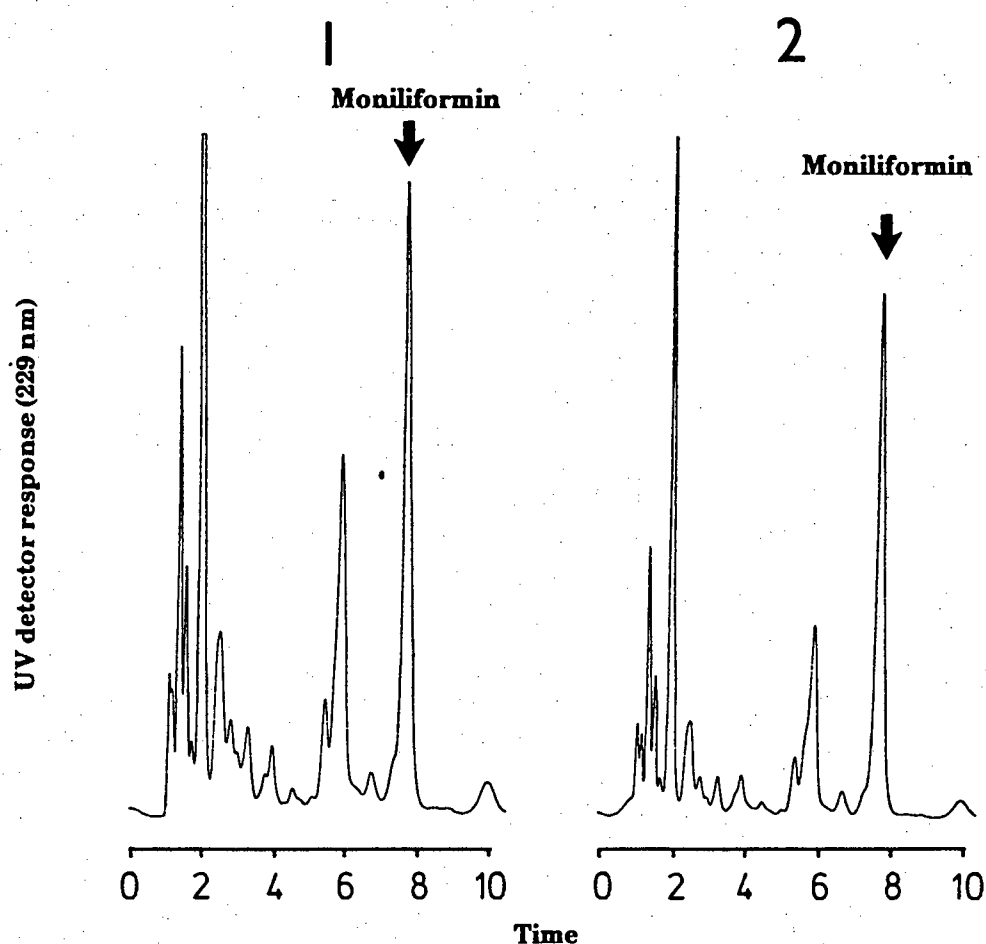


FIG. 4.5 (1) HPLC-UV chromatogram of a sample extract (Bizana #11) and (2) a similar chromatogram of the same extract spiked with moniliformin standard

As detailed in section 2.8.4, extracts were also monitored at two separate wavelengths simultaneously (229 nm and 254 nm), using a diode array detector. The two wavelengths chosen corresponded to the two wavelength maxima observed in the UV spectrum of a moniliformin standard (Figure 3.2). Figure 4.6 shows the two separate chromatograms obtained from an extract of Bizana sample number 7 (Table 4.2). A ratio of 3.5:1 (229 nm:254 nm) was obtained for the two chromatographic peaks. This ratio was identical to that observed for a similarly treated moniliformin standard and provided evidence that the compound eluting in the chromatographic position of moniliformin, in the sample extract, had the same wavelength ratio characteristics as the moniliformin standard.

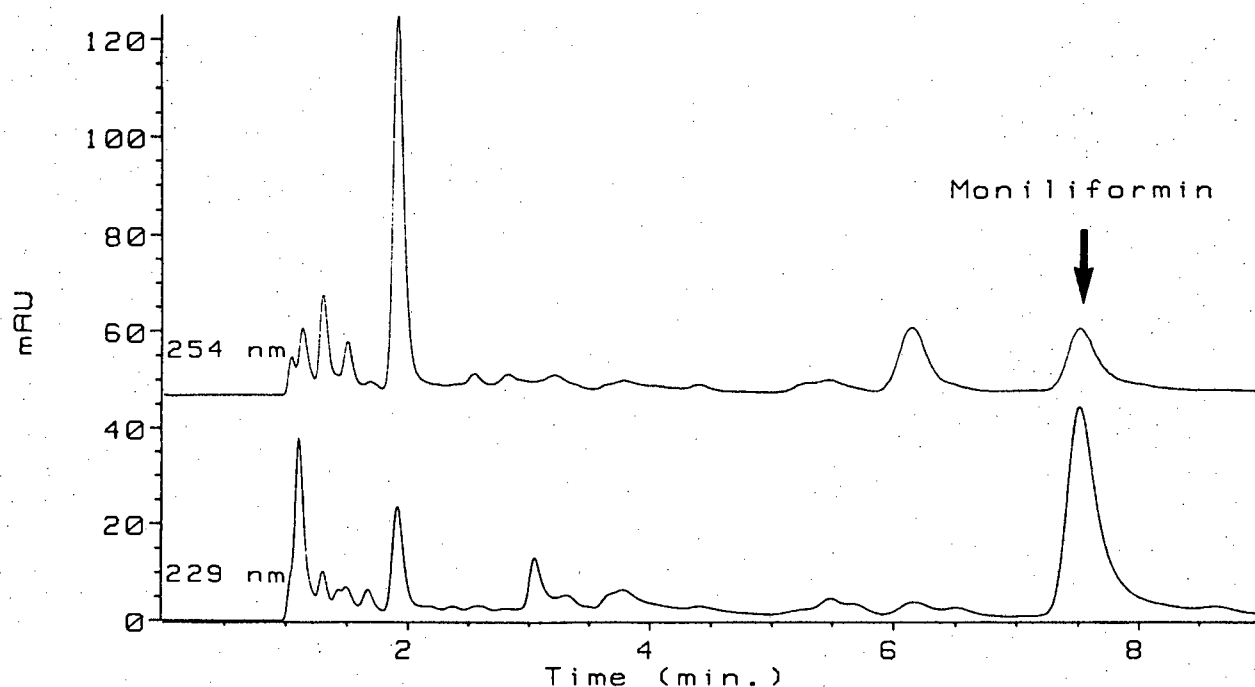


FIG. 4.6 Paired ion chromatograms of a sample extract (Bizana #7) monitored at two separate wavelengths (229 nm and 254 nm) simultaneously

A number of sample extracts were also separated on an ion exchange HPLC column (section 2.8.4), using the chromatographic conditions given in Table 2.9. Figure 4.7 shows the ion exchange chromatogram (monitored at 229 nm) of an extract of Bizana sample number 11 (Table 4.2), showing a peak eluting at 8.1 minutes, the UV spectrum of which is shown in the insert. The characteristic UV spectrum (200-400 nm) was found to be identical to that observed for a moniliformin standard (Figure 3.2), and clearly provided conclusive evidence of the presence of moniliformin in the sample extract.

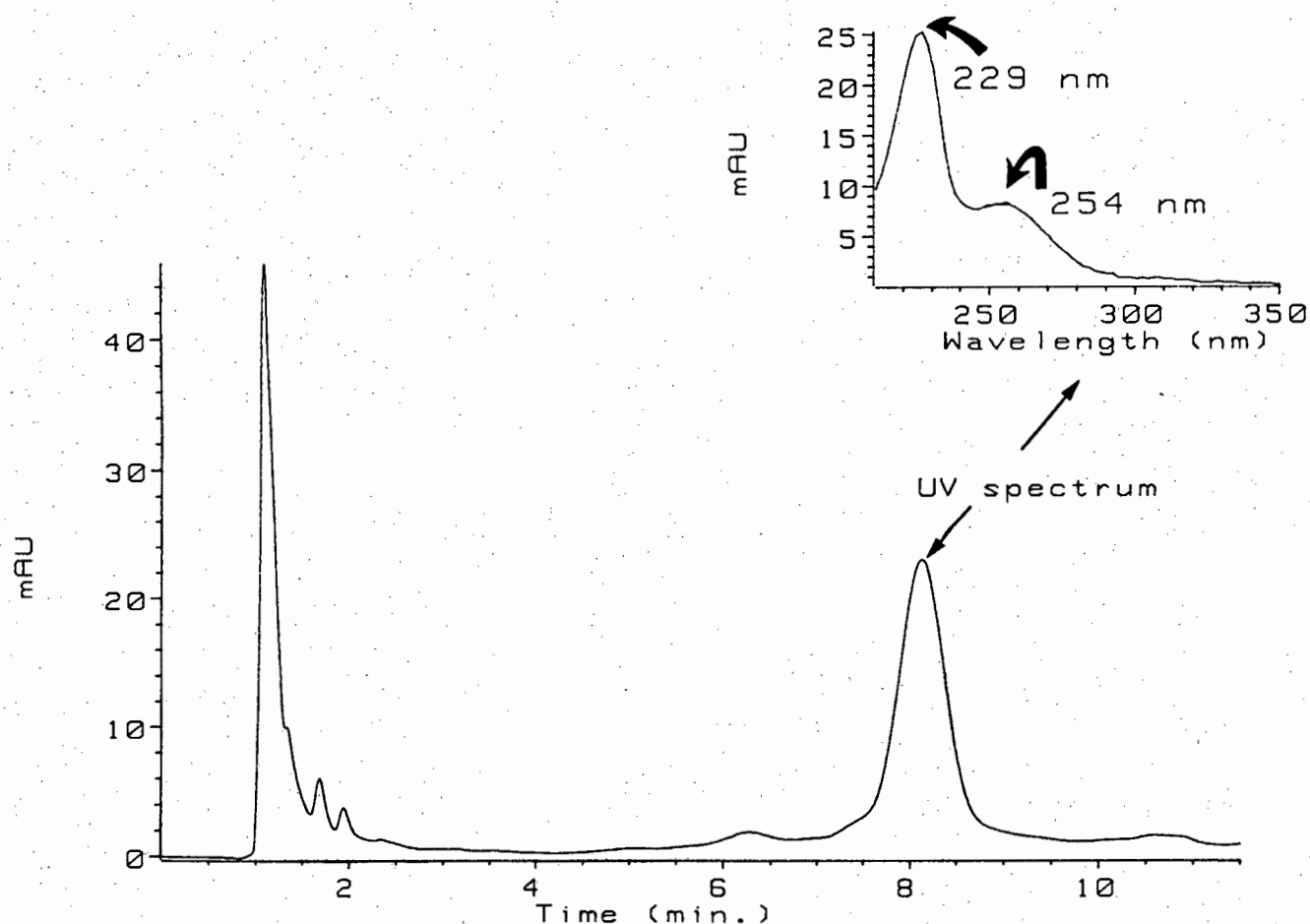


FIG. 4.7 Chromatogram of a sample extract (Bizana #11) separated on an ion exchange HPLC column, showing a moniliformin peak eluting after 8.1 minutes (the UV spectrum of which is shown in the insert)

4.3.4 Zearalenone analyses

Using the chromatographic conditions detailed in Table 2.3, the presence of zearalenone was detected in $\pm 66\%$ of the Bizana and $\pm 42\%$ of the Kentani maize sample extracts. Figure 4.8.1 displays the chromatogram of an extract prepared from Kentani sample number 1, whilst Figure 4.8.2 illustrates the

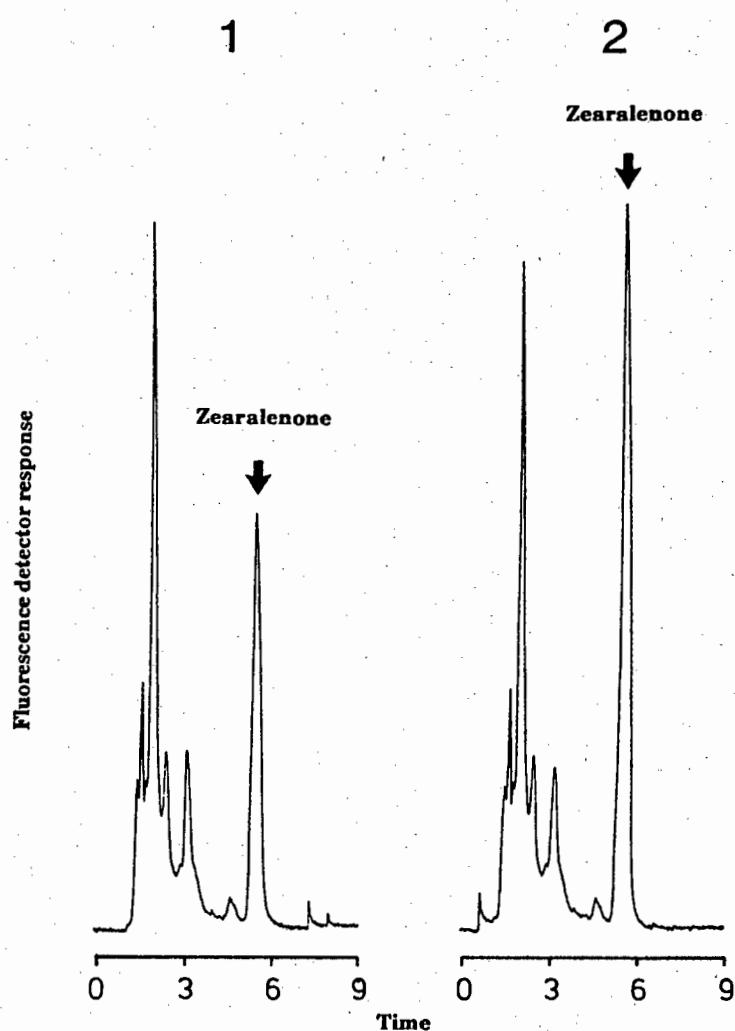


FIG. 4.8 HPLC-fluorescence chromatograms of (1) a sample extract (Kentani #1) and (2) the same extract spiked with authentic zearalenone

chromatogram of the same extract spiked with zearalenone standard. Although in this case a successful "spike" is accompanied by an increase in peak height (and area), it should be stressed that the observation of a single chromatographic peak (rather than a closely eluting secondary or shoulder peak), may be considered as confirmatory evidence for a compounds' presence.

As described in section 2.8.3, the presence of relatively high levels of zearalenone, in a number of the maize extracts was also monitored using UV detection at the three separate wavelength maxima for zearalenone (Figure 3.3). Figure 4.9 shows the three chromatograms, recorded simultaneously, of a maize sample extract (Bizana sample number 5 - Table 4.2).

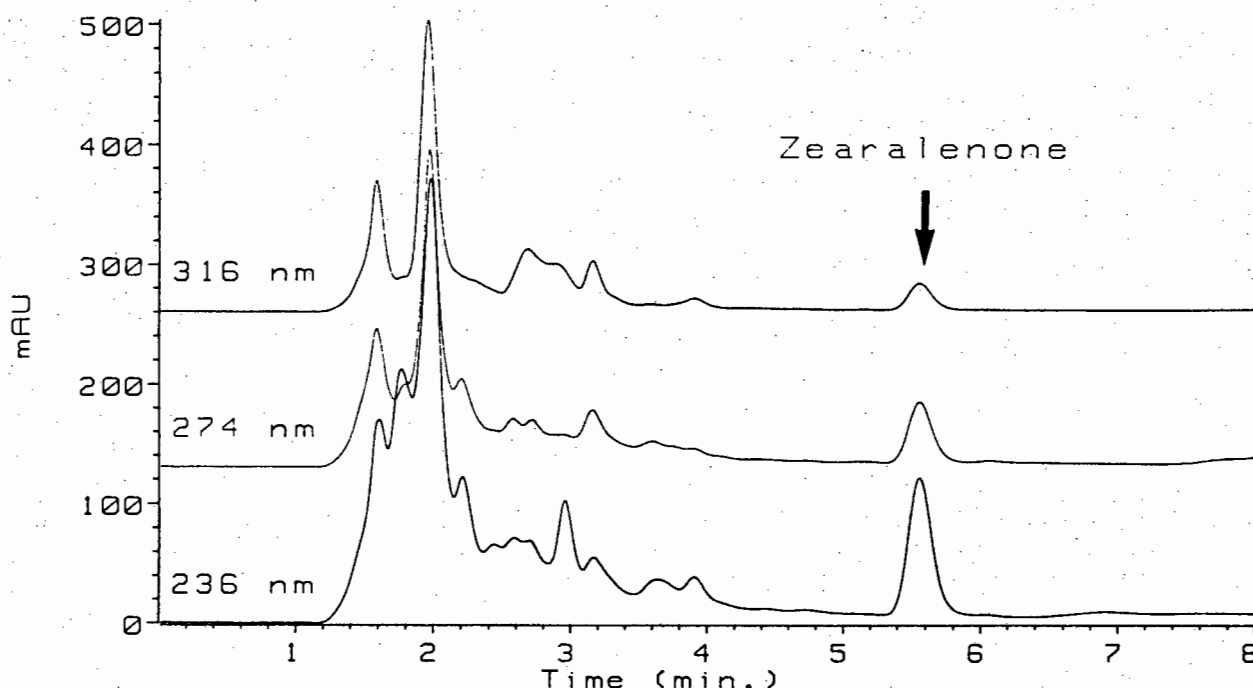


FIG. 4.9 HPLC-UV chromatograms of an extract of Bizana sample #5 monitored at three wavelengths (236 nm, 274 and 316 nm) simultaneously

Table 4.2). A ratio of 5.2:2.3:1 was calculated from the three chromatograms obtained at the different wavelengths (236:274:316 nm). This ratio was found to be identical to that observed for a zearalenone standard, thereby supplying additional confirmatory evidence for the presence of zearalenone in the sample extract.

The data collected on Bizana sample number 5, via the diode array detector, could also be displayed as a 3 dimensional representation (Figure 4.10). The characteristic UV spectrum for zearalenone can be seen at a retention time of 5.5 minutes.

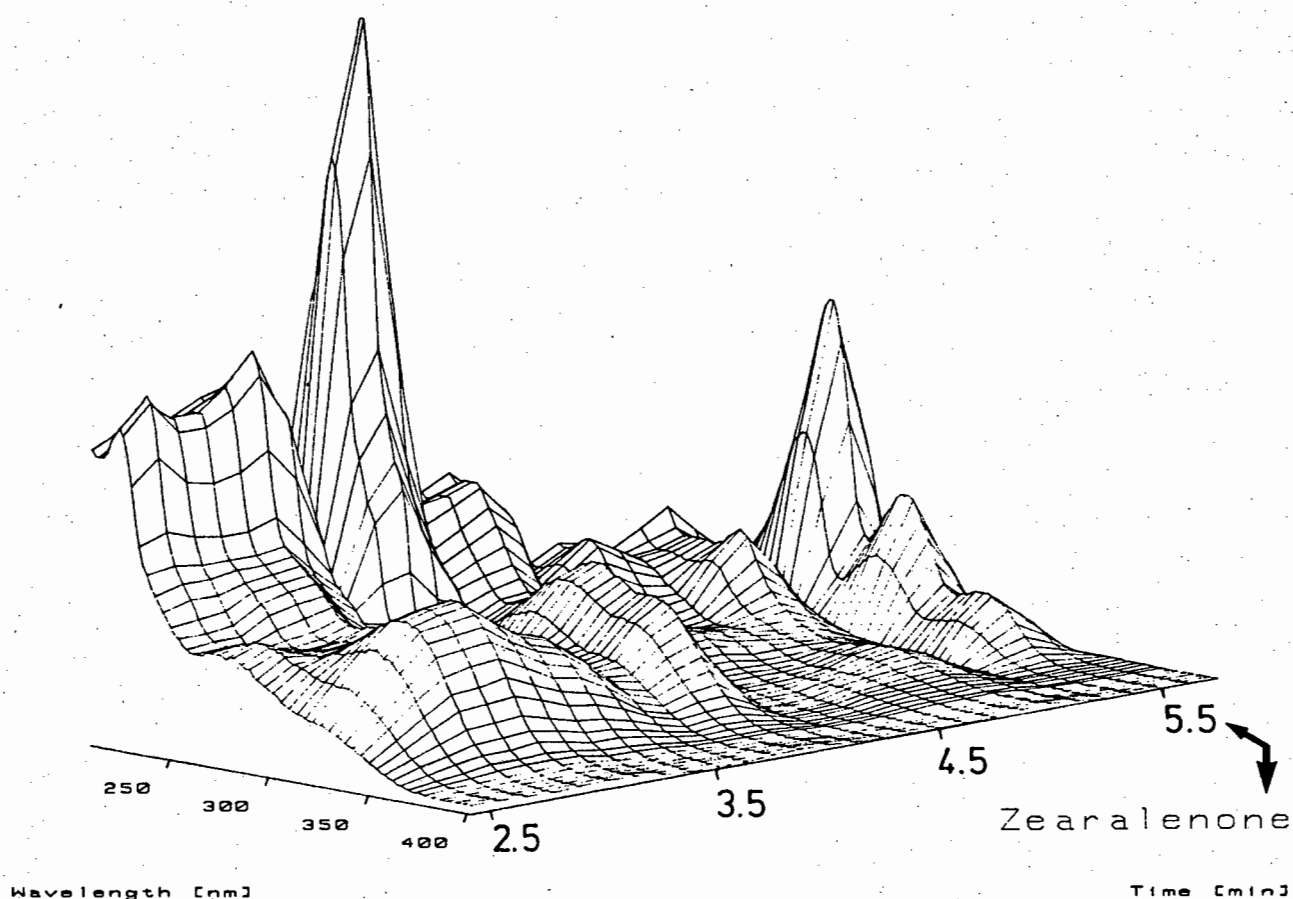


FIG 4.10 Three dimensional representation of a HPLC-UV chromatogram of Bizana sample #5

4.3.5 Tricarballylic acid analyses

As discussed in sections 3.9 - 3.10, an indication as to the possible extent of fumonisin contamination in maize samples, might be obtained by the determination of the tricarballylic acid (TCA) moiety, present in the structures of the known fumonisins. TCA was detected in each Transkeian maize sample

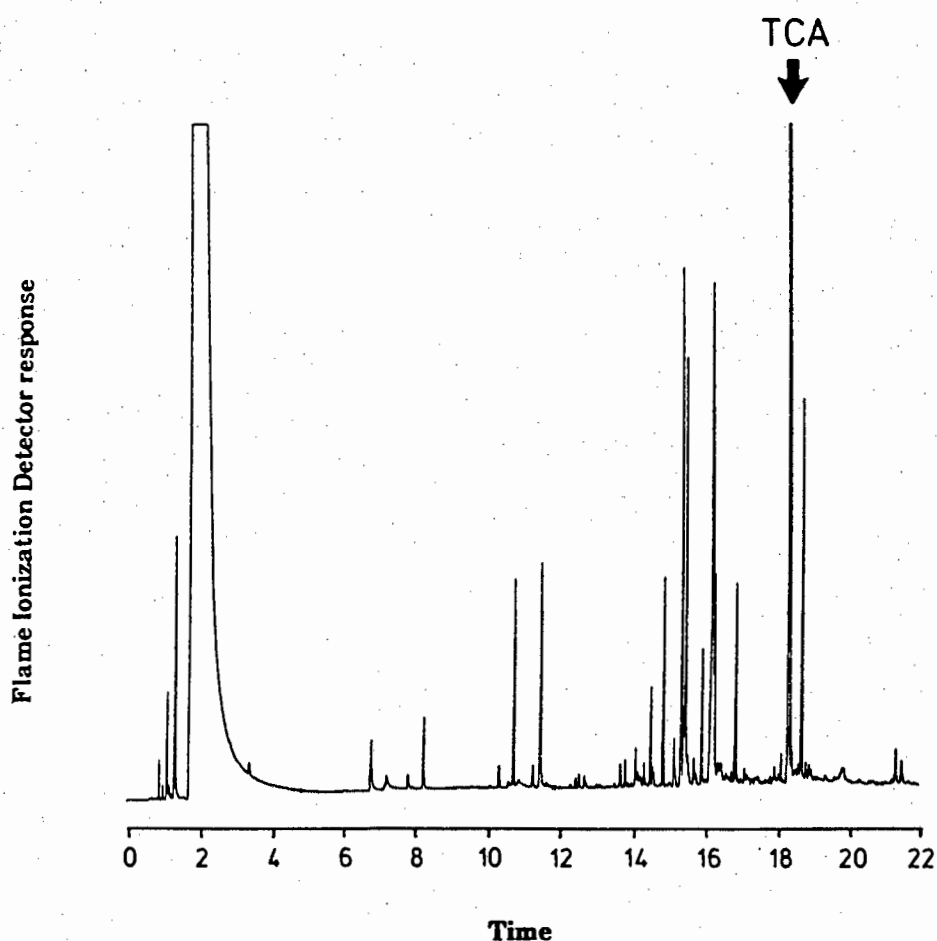


FIG. 4.11 Capillary GC-FID chromatogram of a hydrolysed-esterified extract of Kentani sample #11, showing a major peak eluting at 18.3 minutes, corresponding to the chromatographic position of an esterified tricarballylic acid (TCA) standard

analysed using the capillary GC-FID quantitative procedure outlined in section 2.7.3. Figure 4.11 displays the chromatogram of a hydrolysed-esterified extract of Kentani sample number 11 (Table 4.2). A major peak, corresponding to the chromatographic position of a similarly derivatized TCA standard, can be seen at a retention time of 18.3 minutes.

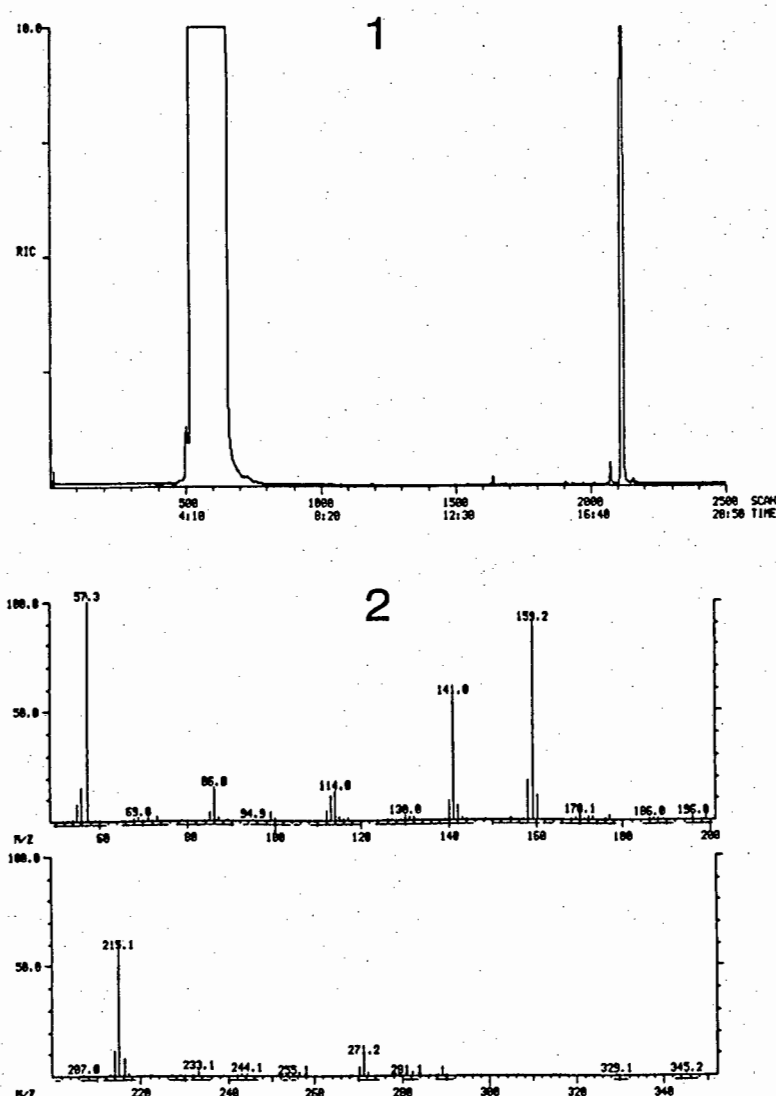


FIG. 4.12 (1) Capillary GC-MS chromatogram of an esterified tricarballic acid (TCA) standard showing a major peak eluting at 17.3 minutes, (2) the mass spectrum of the peak

In order to verify the presence of TCA in the hydrolysed-esterified maize sample extracts, several derivatives were analysed by capillary GC-MS, as outlined in section 2.8.6. Figure 4.12.1 shows the reconstructed ion chromatogram (RIC) of TCA as its iso-butyl ester. A major peak eluted at a retention time of 17.3 minutes, the mass spectrum of which is shown in Figure 4.12.2.

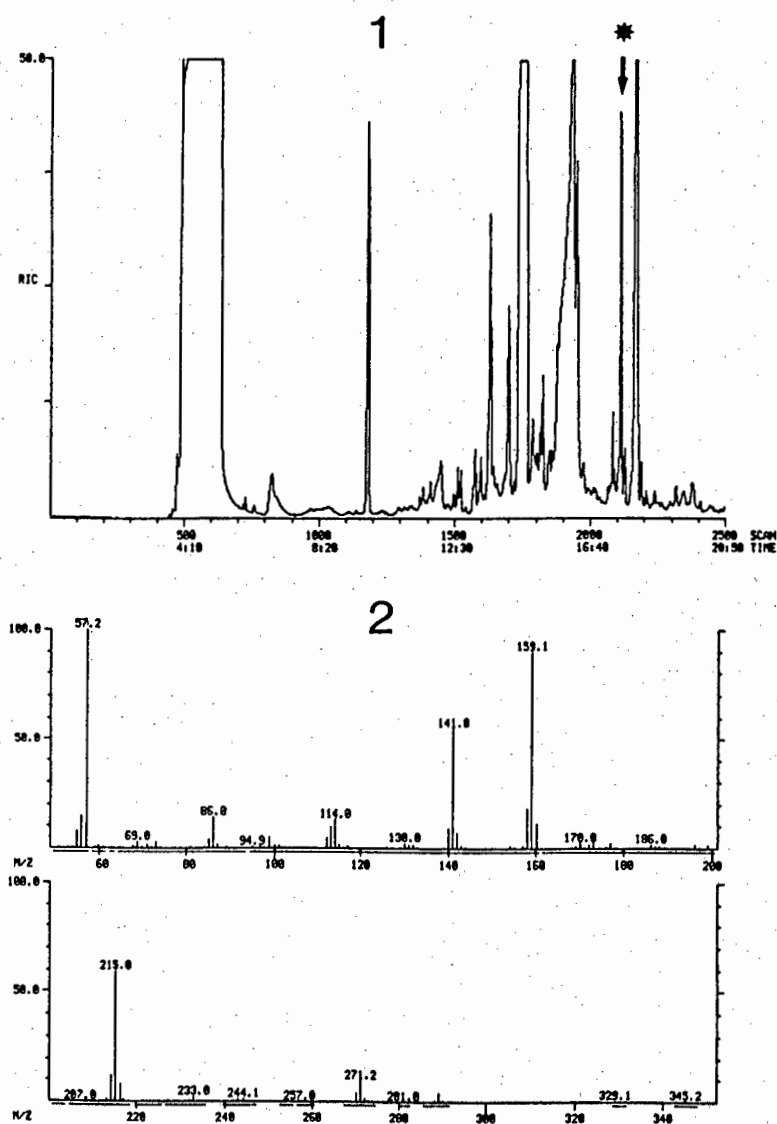


FIG. 4.13 (1) Capillary chromatogram of a hydrolysed esterified extract of Kentani sample #4, (the chromatographic position of tricarballic acid is indicated by an asterisk) (2) the mass spectrum of the peak

Figure 4.13.1 illustrates the RIC of a hydrolysed-esterified extract of Kentani sample number 4, found to contain in excess of $57 \mu\text{g g}^{-1}$ TCA (Table 4.2). A peak occurred at a retention time of 17.3 minutes (indicated by the asterisk). The mass spectrum of this peak is shown in Figure 4.13.2.

The excellent agreement between the mass spectra of the derivatized TCA standard (Figure 4.12.2) and the peak eluting at 17.3 minutes, in the similarly derivatized maize sample hydrolysate (Figure 4.13.2), provided unequivocal evidence for the presence of TCA in the sample extract. Derivatized extracts of several other samples were similarly analysed and excellent agreement between retention times and mass spectra were obtained.

In addition to the analysis of the sample extracts, by the capillary GC-FID quantitative method, attempts to qualitatively assess the contamination of maize samples with fumonisin B_1 , by the maleyl derivative procedure (detailed in section 2.7.2) were made. Those samples having TCA levels in excess of $30 \mu\text{g g}^{-1}$ (Kentani sample numbers 2,4,5,6,7,11 and 12), were analysed for the presence of fumonisin B_1 as its maleyl derivative. Of the seven samples analysed, only one sample extract (number 11) appeared to contain a detectable level of fumonisin B_1 . Figure 4.14.1 illustrates the chromatogram of fumonisin B_1 (1.25 μg) as its maleyl derivative. Figure 4.14.2 shows the chromatogram of a 5 μl aliquot of a similarly prepared sample extract of Kentani sample number

11, and Figure 4.14.3 shows the chromatogram of the same extract spiked with derivatized fumonisin B₁. The level of fumonisin B₁ determined in the sample was found to be in the order of 200 µg g⁻¹. However, this level (when converted) only accounted for approximately 20% of the total TCA determined in the sample by the capillary GC-FID procedure, which would suggest that the balance of the TCA present in this (as well as in the other samples) emanated from sources other than fumonisin B₁ (ie possibly fumonisins B₂ or B₃).

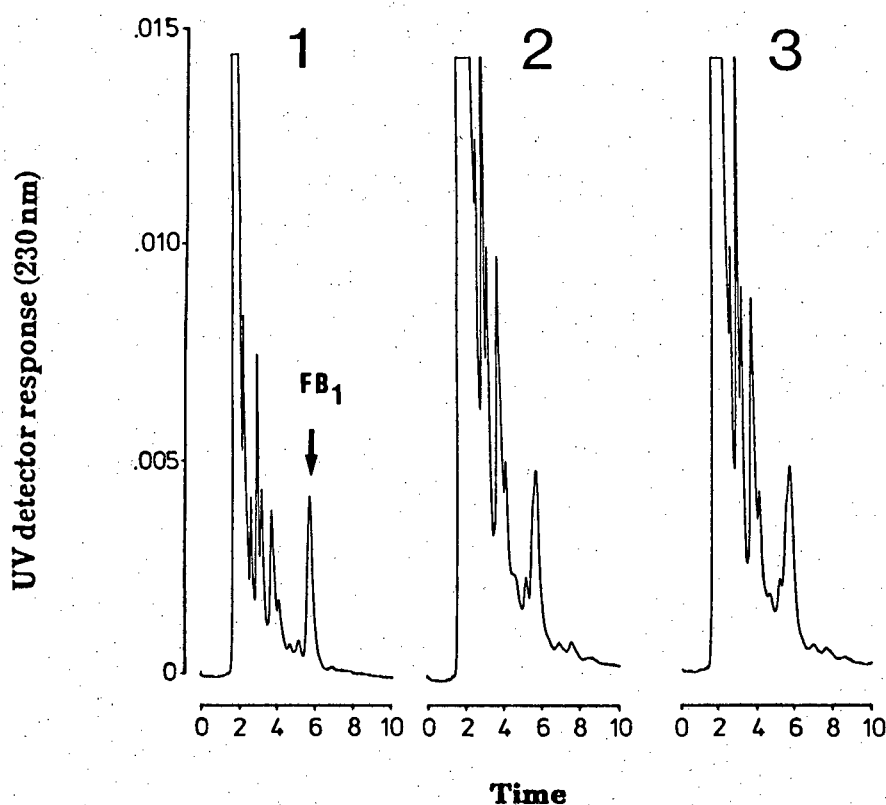


FIG. 4.14 HPLC chromatograms of (1) 1.25 µg fumonisin B₁ (FB₁), as its maleyl derivative, (2) the maleyl derivative of an extract from Kentani sample #11 and (3) the extract spiked with derivatized FB₁

The chromatogram of the maleyl derivative of the Kentani sample extract spiked with fumonisin B₁ (Figure 4.14.3) appeared to show the presence of a shoulder peak in the chromatographic position of fumonisin B₁ (retention time $\pm$ 6 minutes). Poor chromatographic separation of the fumonisin B₁ peak from earlier eluting interferences necessitated the development of a superior separatory system.

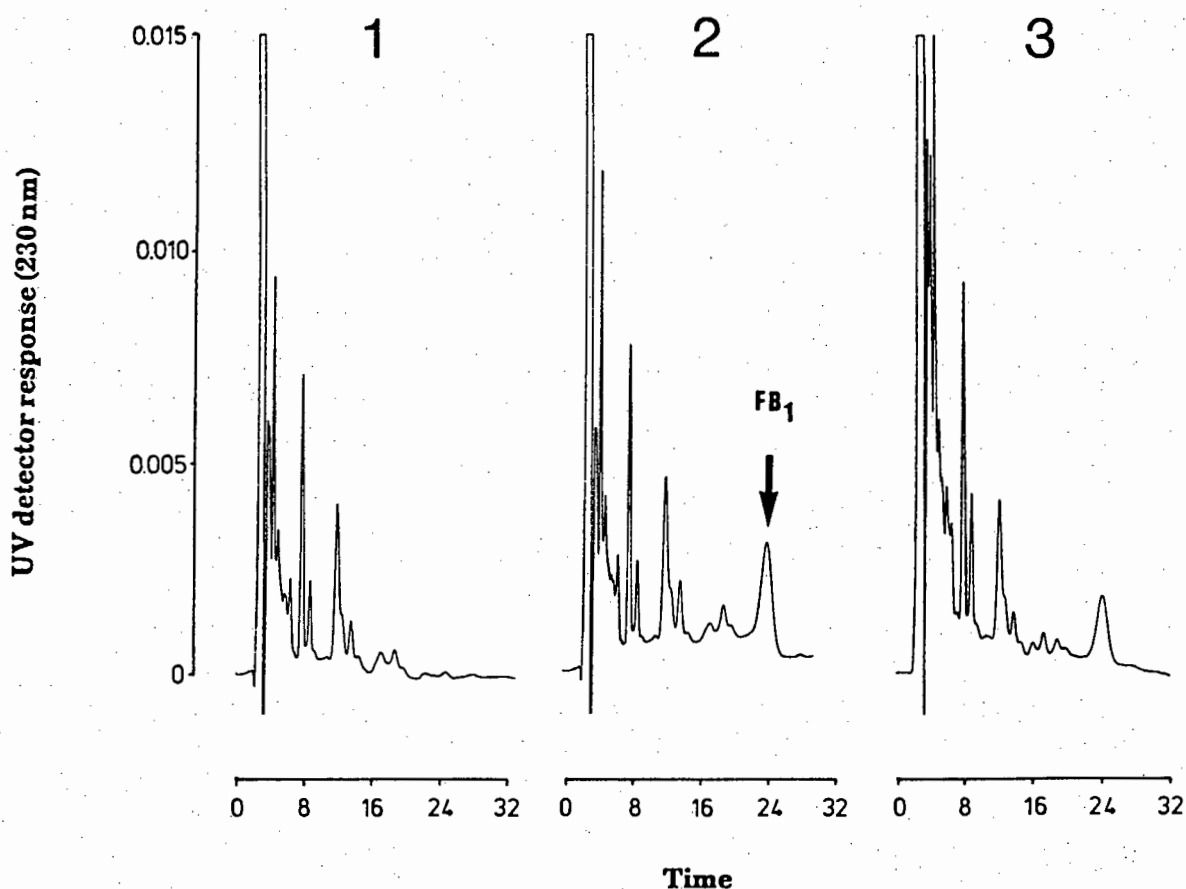


FIG. 4.15 HPLC chromatograms of (1) a maleyl derivative blank, (2) a fumonisin B₁ (FB₁) standard and (3) the maleyl derivative of Kentani sample #11, separated using an acetate buffer based solvent system and a Phenomenex ODS 30 HPLC column

Figure 4.15.1 illustrates the chromatogram of a maleyl derivative blank separated on a Phenomenex ODS 30 reverse phase column, using an acetate buffer:methanol mobile phase (detailed in section 2.8.5). Using the chromatographic conditions outlined in Table 2.10, fumonisin B₁ eluted after 24 minutes (Figure 4.15.2). Figure 4.15.3 shows the chromatogram obtained from the maleyl derivative of an extract of Kentani sample number 11, and clearly illustrates the superior chromatographic separation achieved compared to that previously obtained (Figure 4.14.3).

4.4 STATISTICAL ANALYSES OF THE CHEMICAL AND MYCOLOGICAL RESULTS

4.4.1 Overview

The correlations between the levels of the selected *Fusarium* mycotoxins determined in the Transkeian maize samples, and the degree of fungal infection present in the same maize samples by the dominant *Fusarium* species, is given in Table 4.3. Statistical correlations between the separate *Fusarium* species and the dominant toxins will be dealt with separately in sections 4.4.2 - 4.4.4.

4.4.2 Moniliformin and *Fusarium subglutinans*

Table 4.4 illustrates the mean results of *F. subglutinans* and moniliformin levels, determined in the mouldy maize samples collected from the two oesophageal cancer risk areas of the Transkei in 1985.

TABLE 4.3 CORRELATIONS BETWEEN *FUSARIUM* SPECIES AND TOXINS
IN MOULDY TRANSKEIAN MAIZE OF THE 1985 CROP

Toxin	<i>Fusarium</i> <i>moniliforme</i>	<i>Fusarium</i> <i>subglutinans</i>	<i>Fusarium</i> <i>graminearum</i>
Moniliformin	$r = - 0.312$	$r = + 0.603^1$	$r = + 0.228$
Zearalenone	$r = - 0.306$	$r = + 0.003$	$r = + 0.798^1$
Nivalenol	$r = - 0.539^2$	$r = - 0.171$	$r = + 0.855^1$
Deoxynivalenol	$r = - 0.512^2$	$r = - 0.176$	$r = + 0.650^1$
Tricarballic acid	$r = + 0.656^1$	$r = - 0.078$	$r = - 0.602^2$

¹ Significantly correlated ($p < 0.01$)

² Significantly negatively correlated ($p < 0.05$)

TABLE 4.4 INCIDENCE OF *FUSARIUM SUBGLUTINANS* AND MONILIFORMIN IN MAIZE FROM OESOPHAGEAL CANCER AREAS IN TRANSKEI, IN 1985¹

	Low Rate Area	High Rate Area	p^2
<i>F. subglutinans</i> (%)	10.1	4.7	NS ³
Moniliformin ($\mu\text{g g}^{-1}$)	3.53	0.78	< 0.01
<i>F. subglutinans</i> : Moniliformin, $r = + 0.603$ ($p < 0.01$) ⁴			

¹ Means based on 12 samples from each area

² Probability factor

³ Not significant

⁴ Correlations based on 24 samples

A higher mean value of kernels infected with *F. subglutinans* was observed in the Bizana district (low oesophageal cancer rate area) than in the high rate area (Kentani - Table 4.4), although the difference was not found to be statistically significant. A significantly higher mean value ($p < 0.01$) of moniliformin was recorded in the Bizana district than in the Kentani district, and the percentage kernels infected with *F. subglutinans* was significantly correlated with the moniliformin content ($r = + 0.603$, $p < 0.01$).

4.4.3 Zearalenone, nivalenol, deoxynivalenol and *Fusarium graminearum*

Fusarium graminearum was significantly correlated with zearalenone ($r = + 0.798$; $p < 0.01$ - Table 4.3), nivalenol ($r = + 0.855$; $p < 0.01$) and deoxynivalenol ($r = + 0.650$; $p < 0.01$).

The mean values of the percentage kernels infected with *F. graminearum* and the zearalenone, nivalenol and deoxynivalenol levels determined in the maize, were higher in those samples from the low oesophageal cancer risk area (Bizana) than the corresponding samples from the Kentani district (Table 4.5). With the exception of deoxynivalenol, these results were found to be significantly different, as reflected in the probability factor ($p < 0.05$).

TABLE 4.5 INCIDENCE OF *FUSARIUM GRAMINEARUM*, ZEARALENONE, NIVALENOL AND DEOXYNIVALENOL IN MOULDY MAIZE FROM OESOPHAGEAL CANCER AREAS IN TRANSKEI, IN 1985¹

	Low Rate Area	High Rate Area	p ²
<i>F. graminearum</i> (%)	34.9	8.0	<0.01
Zearalenone ($\mu\text{g g}^{-1}$)	1.15	0.36	<0.05
Nivalenol ($\mu\text{g g}^{-1}$)	4.62	1.75	<0.05
Deoxynivalenol ($\mu\text{g g}^{-1}$)	2.91	0.29	NS ³
<i>F. graminearum</i> : zearalenone, $r = + 0.798$ (p <0.01) ⁴			
<i>F. graminearum</i> : nivalenol, $r = + 0.855$ (p <0.01) ⁴			
<i>F. graminearum</i> : deoxynivalenol, $r = + 0.650$ (p <0.01) ⁴			

¹ Means based on 12 samples from each area

² Probability factor

³ Not significant

⁴ Correlations based on 24 samples

4.4.4 Tricarballic acid and *Fusarium moniliforme*

Table 4.6 displays the mean values of the percentage of kernels infected with *F. moniliforme* and the tricarballic acid determined in the samples, from the two oesophageal cancer rate areas of the Transkei.

TABLE 4.5 INCIDENCE OF *FUSARIUM GRAMINEARUM*, ZEARAELNONE, NIVALENOL AND DEOXYNIVALENOL IN MOULDY MAIZE FROM OESOPHAGEAL CANCER AREAS IN TRANSKEI, IN 1985¹

	Low Rate Area	High Rate Area	p ²
<i>F. graminearum</i> (%)	34.9	8.0	<0.01
Zearalenone ($\mu\text{g g}^{-1}$)	1.15	0.36	<0.05
Nivalenol ($\mu\text{g g}^{-1}$)	4.62	1.75	<0.05
Deoxynivalenol ($\mu\text{g g}^{-1}$)	2.91	0.29	NS ³
<i>F. graminearum</i> : zearalenone, $r = + 0.798$ (p <0.01) ⁴			
<i>F. graminearum</i> : nivalenol, $r = + 0.855$ (p <0.01) ⁴			
<i>F. graminearum</i> : deoxynivalenol, $r = + 0.650$ (p <0.01) ⁴			

¹ Means based on 12 samples from each area

² Probability factor

³ Not significant

⁴ Correlations based on 24 samples

4.4.4 Tricarballic acid and *Fusarium moniliforme*

Table 4.6 displays the mean values of the percentage of kernels infected with *F. moniliforme* and the tricarballic acid determined in the samples, from the two oesophageal cancer rate areas of the Transkei.

TABLE 4.6 INCIDENCE OF *FUSARIUM MONILIFORME* AND TRICARBALLYLIC ACID IN MOULDY MAIZE FROM OESOPHAGEAL CANCER AREAS IN TRANSKEI, IN 1985¹

	Low Rate Area	High Rate Area	p ²
<i>F. moniliforme</i> (%)	34.5	67.7	<0.01
Tricarballylic acid ($\mu\text{g g}^{-1}$)	12.4	77.7	<0.01
<i>F. moniliforme</i> : tricarballylic acid, $r = + 0.656$ ($p < 0.01$) ³			

¹ Means based on 12 samples from each area

² Probability factor

³ Correlations based on 24 samples

Far higher mean values for both tricarballylic acid and fungal infection with *F. moniliforme*, were recorded in the samples from the Kentani district (a high oesophageal cancer rate area) than in the corresponding low rate area (Bizana). These differences were found to be statistically significant ($p < 0.01$ - Table 4.6), and a significant correlation was found between the incidence of *F. moniliforme* and the levels of tricarballylic acid ($r = 0.656$; $p < 0.01$ - Table 4.3).

4.5 SUMMARY OF STATISTICAL ANALYSES

From previous studies involving the screening of fungal cul-

tures prepared from isolates taken from Transkeian maize samples, moniliformin has only been found to be produced by *F. subglutinans*. Similarly, zearalenone, nivalenol and deoxynivalenol have only been found to be produced by *F. graminearum*. Conversely, *F. moniliforme* isolates have never been found to produce any of these *Fusarium* mycotoxins. Therefore, the overall correlations as given in Table 4.3, agree with the toxin producing abilities of the *Fusarium* species, isolated from the series of samples, and clearly demonstrate the excellent compatibility that exists between the mycological enumeration and the quantitative analytical techniques used in this investigation.

Significantly negative correlations were found between the incidence of *F. moniliforme* and the levels of nivalenol and deoxynivalenol ($r = -0.539$; $p < 0.05$ and $r = -0.512$; $p < 0.05$, respectively - Table 4.3), as well as between *F. graminearum* and tricarballic acid ($r = 0.602$; $p < 0.05$). These results were indicative of a significantly negative correlation that was found between incidences of *F. moniliforme* and *F. graminearum* ($r = -0.683$; $p < 0.01$), and imply that samples having high levels of *F. moniliforme* containing tricarballic acid, normally had low levels of infection by *F. graminearum* and its metabolites, and vice versa.

Table 4.7 displays a similarly treated set of chemical and mycological results pertaining to studies carried out on

similar maize samples collected from the same oesophageal cancer risk areas in the Transkei, during 1979. These results have previously been presented (Thiel et al., 1982a).

TABLE 4.7 CORRELATIONS BETWEEN *FUSARIUM* SPECIES AND TOXINS IN MOULDY TRANSKEIAN MAIZE OF THE 1979 CROP^a

Toxin	<i>Fusarium</i> <i>moniliforme</i>	<i>Fusarium</i> <i>subglutinans</i>	<i>Fusarium</i> <i>graminearum</i>
Moniliformin	$r = + 0.108$	$r = + 0.611^1$	$r = - 0.557$
Zearalenone	$r = - 0.432$	$r = - 0.268$	$r = + 0.720^1$
Nivalenol	$r = - 0.363^2$	$r = - 0.434^2$	$r = + 0.823^1$
Deoxynivalenol	$r = - 0.013$	$r = - 0.031$	$r = + 0.158$

^a Results previously presented (Thiel et al., 1982a)

¹ Significantly correlated ($p < 0.01$)

² Significantly negatively correlated ($p < 0.05$)

Although the methods pertaining to the mycological investigations carried out on the 1979 maize samples, were the same as used on the 1985 series of samples, the chemical methodology differed considerably. Moniliformin and zearalenone levels were determined by HPLC and TLC, respectively (Thiel et al., 1982b). Nivalenol and deoxynivalenol levels were determined using a packed column GC-FID technique.

F. subglutinans was significantly correlated with monili-

formin content, while *F. graminearum* was similarly correlated with both zearalenone and nivalenol but not deoxynivalenol (Table 4.7). Similar correlations were therefore observed in the two sets of results. Figure 4.16 illustrates the distribution of *Fusarium moniliforme* and tricarballic acid levels determined in the 1985 Transkeian maize samples.

Figure 4.16 demonstrates the presence of two distinct populations (of results), corresponding to the samples emanating from the separate oesophageal cancer risk areas, of the Transkei.

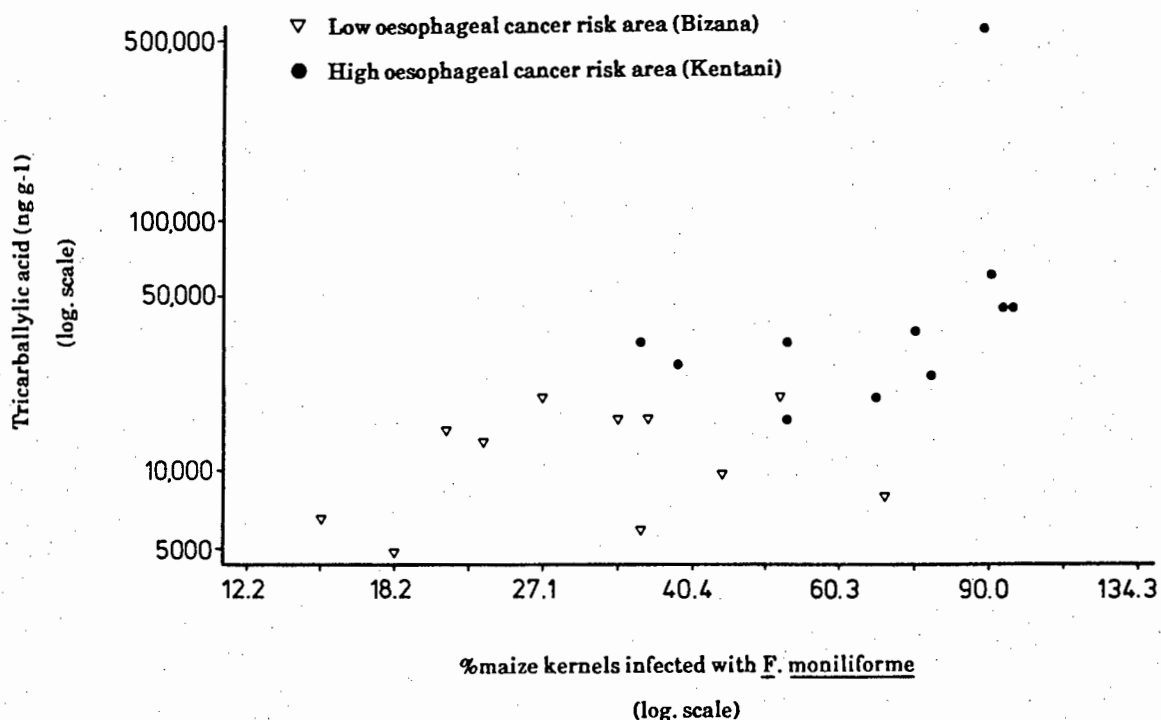


FIG. 4.16 Distribution of *Fusarium moniliforme* and tricarballic acid in mouldy maize from high and low oesophageal cancer risk areas of the Transkei, in 1985

It should be remembered that had the result of Kentani sample #11 ($518 \mu\text{g g}^{-1}$ - Table 4.2, represented in Figure 4.16 as the highest value) been within the range of results reported for the other Kentani samples, the correlation between *F. moniliforme* and tricarballic acid would have been superior to that reported ($r = + 0.656$ - Table 4.3).

The statistical evidence, from the present and previous studies, indicate that the populations of both the low and high oesophageal cancer risk areas of the Transkei, have been exposed to high levels of a number of food-borne *Fusarium* mycotoxins for several years. The evidence concerning the presence of tricarballic acid suggests that individuals residing in the high oesophageal cancer risk district of Kentani, are exposed to significantly higher levels of the potent cancer-promoting *Fusarium* mycotoxins, the fumonisins, than those residing in the Bizana district.

Whilst these results are certainly a cause for concern, they do not suggest that the fumonisins are responsible for oesophageal cancer, however they do imply that further investigations, concerning the role, if any, that the *Fusarium* mycotoxins play in the etiology of human oesophageal cancer, are warranted.

SUMMARY

Fungi or moulds are members of the lower plant species that can grow either parasitically on plants, animals or man or saprophytically on dead organic matter. During their growth stage, numerous fungi have the ability to produce a diverse range of secondary metabolites (mycotoxins), which can be poisonous when ingested by animals or man.

In several mycological surveys, *Fusarium moniliforme* Sheldon has been shown to be one of the three most prevalent *Fusarium* species isolated from mouldy home-grown maize from different oesophageal cancer areas of the Transkei, southern Africa. The incidence of this fungus has been correlated with human oesophageal cancer rates, both in Transkei and the Lin Xian county, in the Henan province of China, which are amongst the highest oesophageal cancer risk areas of the world. Recently a group of related compounds (the fumonisins) were isolated from a fungal culture of *F. moniliforme*, which itself was an isolate from a Transkeian maize sample. Fumonisin B₁ (FB₁), the major compound, was found to possess cancer-promoting abilities.

It has become important to develop and implement methodology for the determination of *Fusarium* mycotoxins in food samples, in order to assess the possible human exposure to these potentially dangerous, naturally produced, fungal metabolites. This study involved essentially two stages. The first stage being the provision of the most suitable analytical

procedures to determine the *Fusarium* mycotoxins. This was achieved via the evaluation of existing or development of innovative methodology for the chromatographic determination of the *Fusarium* mycotoxins, in a maize substrate. The acquisition and confirmation of authentic mycotoxin standards was a prerequisite for this stage of the investigation.

At least two existing methods for the determination of the trichothecenes (diacetoxyscirpenol, T-2 toxin, nivalenol, deoxynivalenol), moniliformin and zearalenone were evaluated. It was necessary to develop innovative methodology for the determination of FB_1 . Due to the absence of either UV or fluorescence characteristics, the preparation of FB_1 derivatives, prior to either HPLC or capillary GC analyses, was a prerequisite. HPLC-UV analysis of the maleyl derivative of FB_1 was found to be suitable for the determination of high concentrations of FB_1 (as encountered in fungal cultures), but was unsuitable for the screening of naturally contaminated samples. Matrix effects were found to cause problems during the formation of a FB_1 -fluorescamine derivative complex, prior to HPLC-fluorescence analysis. Acid hydrolysis and derivatization of FB_1 , followed by capillary GC-FID analysis, resulted in the determination of the tricarballylic acid (TCA) moiety, present in the structures of the fumonisins. During the evaluation of these FB_1 analytical techniques, using a Transkeian maize sample, it became apparent that other TCA containing compounds (possibly other known fumonisins) were present in maize samples. It was suggested

that the TCA moiety might be used as an indicator of the level of total fumonisin mycotoxin contamination of maize samples.

The second stage of this study involved the application of the most suitable selected *Fusarium* mycotoxin methods, to a series of Transkeian home-grown maize samples, collected in 1985. Excellent statistical correlations were obtained between the various toxin levels and the extent of *Fusarium* fungal contamination of the individual samples. The correlations agreed well with the toxin producing abilities of the dominant *Fusarium* species enumerated in the Transkeian maize samples, and suggested that both the analytical and mycological techniques used, were suitably compatible. The most relevant statistical correlation to be generated was that between the incidence of *F. moniliforme* and the concentration of TCA. If as suggested, TCA can be used as an indicator to monitor the total fumonisin contamination of maize, then the evidence suggests that humans in oesophageal cancer risk areas of the Transkei in southern Africa, were exposed to the cancer-promoting *F. moniliforme* mycotoxins, the fumonisins.

The isolation and characterization of other fumonisin compounds and the development of suitable analytical methodology for their determination, are continuing. Further investigation, concerning the role, if any, that the fumonisins and/or other *Fusarium* mycotoxins play in the etiology of human oesophageal cancer in the Transkei are envisaged.

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