ANALYSIS OF THE MYCOTOXINS OF FUSARIUM

BY

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ABSTRACT

Fusarium contamination of maize has been shown to be a problem in Zambia. In this study it was decided to test for the presence of five toxins produced by Fusarium: Zearalenone, T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON) and nivalenol, in maize and maize products. This was the first major study involving the analysis of trichothecenes in Zambia, and thus several methods of extraction and determination of the four trichothenes and Zearalenone in maize and mixed feeds were tested, in order to arrive at the most suitable methods for use in our Laboratory. The methods finally adopted gave 80, 80, 86, 50 and 60 per cent recoveries of added Zearalenone, DON, nivalenol, T-2 toxin and DAS respectively. TLC was used for qualitative as well as quantitative determination of the toxins and as low as 50 µg/kg Zearalenone, 80 µg/kg DON and nivalenol, and about 2000 µg/kg T-2 toxin and DAS could be determined.

Mouldy maize, rejected by NAMBOARD but mostly used by farmers as animal feed supplement or in the brewing of local opaque beer, was collected from farmers around Lusaka and analysed for the five Fusarium toxins. This maize was found to contain only Zearalenone and DON in the range of 0.08-6.0 mg/kg and 0.5-16 mg/kg respectively.

A year long survey of mixed animal feeds from National Milling Company was initiated and 148 samples were analysed for Zearalenone, DON and nivalenol. 17 per cent of the samples contained Zearalenone (0.05-0.6 mg/kg) and 1.4 per cent of the samples contained DON (1.0 mg/kg). No nivalenol was detected in any of the samples. The Zearalenone and DON positive samples were further tested for T-2 toxin and DAS but none was detected. This is the first report of the natural occurrence of Zearalenone and DON in mixed feed samples in Zambia.

An isolate of F. graminearum was grown at 26°C and 16°C corresponding to average maximum and minimum temperatures often recorded near the end of the rainy season. Zearalenone production was found to be favoured by low temperatures (16°C) while DON production was found to be favoured by high temperatures (26°C).

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 ZAMBIAN CLIMATE

Zambia is a landlocked country and lies between latitudes 8 and 18 degrees south and between longitudes 22 and 34 degrees east. It covers approximately 752,619 square kilometres. Generally the altitudes lie between 1067 and 1372 metres above sea level.

Rainfall is the most important element in seasonal differentiation in Zambia but other climatic factors like temperature, winds, humidity and sunshine also have a marked seasonal rhythm. When these are put together, they define Zambia's three seasons, namely the warm-wet season, the cool-dry season and the hot-dry season (Archer, 1976).

The warm-wet season extends from November to April. Humidity begins to rise near the end of October reaching a maximum in January-February (early morning values of around 95 per cent). Mean annual rainfalls are around 1300 mm in the north and 800 mm in the south. Rains gradually die out by the end of March in the south and around April in the north. Fungal growth on crops during this season is a common occurrence.

The cool-dry season starts in April and may go on until late July or early August. The sun is overhead in the northern hemisphere and the lowest temperatures are experienced. They usually fall to between 7 and 10^{o}C (or lower) at night. Relative humidities in July range from 20 to 40 per cent during the afternoon and between 60 to 80 per cent in the early morning. Mean relative humidity decreases steadily from the end of the

rainy season, usually reaching a minimum in September.

The hot-dry season sets in in late August when the temperature begins to rise rapidly. October is the hottest month, although November may be hotter if the rains are delayed. The highest maxima occur in the low-lying regions in the south and stations in the lower Luangwa valley may have mean daily maximum temperatures of over 38°C (Archer, 1976).

1.2 MAIZE PRODUCTION IN ZAMBIA

There are five cereal crops grown in Zambia today namely maize, wheat, rice, sorghum and millet of which maize is the principle staple food for over 80 per cent of the population in the country. Table 1.1 shows the marketed productions of the five cereal crops.

Maize (Zea mays) is the major cash crop and principle food. Being rain fed, maize production is basically determined by favourable rainfall as well as other weather conditions. The crop is adversely affected by unevenly distributed or excessive rainfall. It requires annual rainfalls of between 700 - 900 mm and summer monthly mean temperatures of 22.5°C and above. In Zambia, rainfall is fairly reliable, despite having been heavy and excessive during some seasons and poorly distributed and erratic during other seasons. Maize can be grown practically everywhere in Zambia as shown in Figure 1.1.

Maize is grown by two groups of farmers, the traditional subsistence (small scale) and the commercial (large scale) farmers. As can be seen from Table 1.2, a substantial percent-

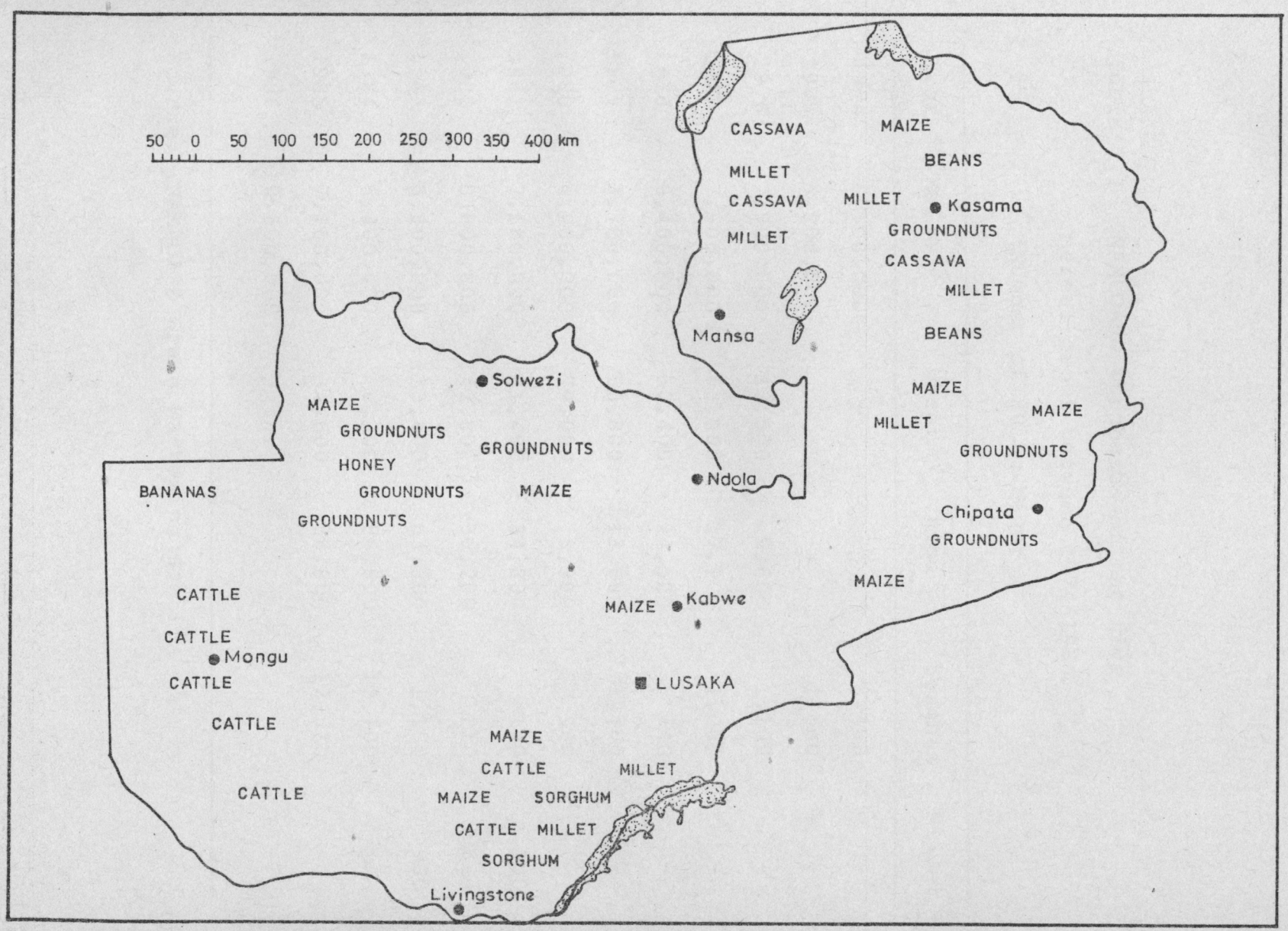


Figure 1.1: Small Scale and Large Scale Farming in Zambia

Table 1.1 NAMBOARD purchases of the five cereal crops between 1974-1986 (Number of 90 Kg bags)

Year	Maize	Wheat	Rice	Sorghum	Millet
1974	6,100,000	-	-	3,400	-
1975	5,900,000	-	-	1,500	-
1976	7,900,000	38,500	9,800	1,100	-
1977	7,600,000	63,000	17,000	1,000	-
1978	6,400,000	50,400	24,500	740	-
1979	3,700,000	56,800	13,600	1,700	-
1980	4,200,000	79,000	8,300	980	-
1981	7,600,000	81,400	34,800	140	2,200
1982	5,600,000	142,800	36,200	210	620
1983	5,900,000	113,400	63,300	1,100	900
1984	6,400,000	6,300	86,800	12,700	-
1985	7,100,000	34,600	88,800	12,510	-
1986	10,600,000				

Source: NAMBOARD Grain Marketing Section

Table 1.2 Maize purchases by NAMBOARD from Large and small scale farmers (Chella, 1983)

Harvest year	Large scale producers (X1000 bags)*	Small scale producers (X1000 bags)	Total (X1000 bags)
1971-72	2,511	1,640	4151
1972-73	3,799	2,558	6357
1973-74	2,083	2,352	4435
1974-75	3,052	3,485	6534
1975-76	2,860	3,357	6217
1975-76	2,860	3,357	6217
1976-77	3,749	4,584	8333
1977-78	3,405	4,333	7738
1978-79	2,779	3,684	6463
1979-80	1,568	2,165	3733
1980-81	2,094	3,013	5107
1981-82	3,148	4,722	7870

* 1 bag = 90 Kg

age of the marketed production of maize comes from small scale farmers. The contribution of each farmer is negligible as compared to the national total but there are many such farmers in this category so that their total production put together is around 60 per cent of the total marketed maize. Most of of the production by subsistence farmers is done basically to feed themselves on the farms and they only sell the surplus. The large scale farmer on the other hand produces maize for sale and will usually have a large area under cultivation. Although the number of commercial farmers is small, 600 from 1981 statistics (Chella, 1983), they now account for around 40 per cent of all the marketed maize.

The main maize growing areas in Zambia are the Southern, Central and Eastern Provinces. The three provinces account for over half the total marketed maize (see Table 1.3). Central and Southern Provinces have the largest concentration of commercial farmers and this may explain the fact that more maize is marketed in these two provinces.

Total maize production in Zambia is difficult to determine as the present information fails to show fully how much is consumed on the farm and how much is sold through the private marketing channels. The only figures reported are those of quantities from the official marketing associations, the National Marketing Board (NAMBOARD), and the Cooperative Marketing Unions.

1.2.1 MAIZE CONSUMPTION

In Zambia, maize consumption can be divided into direct

Table 1.3 NAMBOARD maize purchases by province

(Number of 90 Kg bags)

Year	Northern (X1000)	Lusaka (X1000)	Central (X1000)	Southern (X1000)	Copperbelt (X1000)	Luapula (X1000)	North Western (X1000)	Western (X1000)	Eastern (X1000)	Total (X1000)
1969-70	57	x	903	461	7	20	15	10	16	1,489
1970-71	64	x	2,604	1,504	33	25	33	28	15	4,306
1971-72	72	x	4,197	2,115	29	29	39	36	335	6,852
1972-73	72	x	2,307	1,043	35	22	31	22	408	3,940
1973-74	59	x	2,510	1,172	134	16	30	15	506	4,442
1974-75	67	x	3,786	1,970	25	16	29	30	612	6,535
1975-76	113	x	2,844	2,336	33	18	29	74	770	6,217
1976-77	184	x	3,948	3,073	72	25	38	80	912	8,332
1977-78	212	413	2,865	3,077	70	32	40	86	942	7,737
1978-79	203	254	2,164	2,911	51	34	35	39	772	6,463
1979-80	121	184	1,234	1,554	42	18	32	34	517	3,736
1980-81	159	132	1,519	1,554	35	19	16	14	740	4,188
1981-82	328	325	2,679	3,679	38	29	42	43	1,182	8,345

x : Included in Central Province

Source : NAMBOARD Grain Marketing Section

human consumption and commercial use (Chella , 1983). The former accounts for over half the maize produced. It is estimated that of the maize retained on the farms, 67 per cent is for food, 10 per cent for beer, 5-10 per cent for stock feed and 1-2 per cent for seed. In the case of maize entering the official marketing channel, known as marketed production, it is estimated that 83 per cent is for food, 10 per cent for beer, 7 per cent for stock feed and a negligible amount for processed cereal foods. The consumption of maize in Zambia is higher than for any other staple food.

Maize is consumed on the cob while it is green, but most of it is consumed after it has been processed into flour. The maize flour (maize meal or mealie meal) is then used to make "Nsima" a form of thickened porridge to be eaten with a side dish or relish, and it is in this form that most of the maize is consumed.

1.3 FUNGAL GROWTH

Microfungi, also known as moulds are the primary agents of decay in many plant and animal materials. Over a hundred species of fungi have been reported from cereal grains (Christensen and Kaufmann, 1974). They thrive in materials within warehouses, elevators, mills, processing plants, and homes as well as out doors. In general, the fungi that grow on and in seeds have been divided into two groups: field fungi and storage fungi, this division being based mainly on the ecology of the fungi concerned and the moisture requirements.

1.3.1 FIELD FUNGI

These fungi invade the developing or maturing seed while it is still on the plant or after the grain is cut and swathed, but before it is threshed. Maize stored moist on-the-cob in cribs may also be attacked and decayed by typical field fungi. The major genera of field fungi are Alternaria, Helminthosporium, Cladosporium, Fusarium, Diplodia and Chaetomium (Christensen, 1957). All require a substrate with a high moisture content to grow i.e. a moisture content in equilibrium with relative humidity of 90-100 per cent, which in cereals means a moisture content of 22-23 per cent wet-weight basis or 30-33 per cent dry-weight basis (Christensen and Kaufmann, 1974). These may survive for years on dry grain but die off quite rapidly in grains held at moisture content in equilibrium with relative humidity of 70-75 per cent. All these fungi have a wide host range and are able to remain in the soil and old vegetation so that the potential for infection is always present.

Species of Fusarium are common and widespread in nature, occurring as saprophytes in the soil and in decaying vegetation of all kinds and as parasites of wild and cultivated plants, in which they cause a variety of diseases such as wilts, blights and rots. Fusarium can cause severe economic losses in major cereal crops like rice, wheat, barley, millet, sorghum and maize. "Scab" of barley and wheat is a worldwide problem.

For example, in 1980, about 20 per cent of the wheat crop in the wheat growing region of Ontario and Quebec, Canada was affected (Mirocha and Christensen, 1984). Cob rot of maize infected in the field or when stored in open cribs on the farm is a serious problem and Fusarium species are the major fungi involved.

1.3.2 STORAGE FUNGI

These are able to develop on and within the seed at the moisture content of the seeds often found in storage. The conditions of harvest, and to a greater extent those of storage of agriculture commodities and their products, have considerable influence on the growth of moulds. The major storage fungi are Aspergillus and Penicillium (Christensen, 1957). Fungal growth during storage depends on a number of factors:

(1) Temperature. Temperature plays an important role in the growth of mycelium and in the formation and germination of spores. Most fungi develop at temperatures from 5 to 35°C with optimum being around 25 to 35°C (Panasenko, 1967), see also Table 1.4.

(2) Humidity and moisture content of the substrate. Humidity affects not only the growth of moulds but the germination of spores as well. Humidity of over 70 per cent is required. The moisture content of the grain being stored also plays some role in determining the type of fungi that would grow on them. A moisture content of between 13-17

Table 1.4 Common storage fungi on grain with their approximate minimum, optimum and maximum temperatures for growth

(Christensen and Kaufmann, 1974)

Fungus	Temperature for growth °C		
	minimum	optimum	maximum
Aspergillus restrictus	5-10	30-35	40-45
A. glaucus	0-5	30-35	40-45
A. candidus	10-15	45-50	50-55
A. flavus	10-15	40-45	45-50
Penicillium	-5-0	20-25	35-40

per cent is mostly required (Moreau, 1979).

(3) Nutritional requirements. The exact nutritional requirements for each species differ. Most fungi grow at a pH in the range 4-8 and most of them are aerobic. Low levels of CO_2 (generally 0.03-1 per cent) are required for growth (Paster and Lister, 1985).

(4) The time of storage plays some role in the formation of moulds, but this is closely tied with the moisture content and temperature. The higher the moisture content and temperature within the limits of the fungus involved, the shorter the permissible time of storage (Christensen and Kaufmann, 1974).

1.3.3 EFFECTS OF FUNGI ON GRAINS AND SEEDS

Invasion of grains and seeds by fungi can result in decreased germination potential of the seeds. This is of particular importance when dealing with barley meant for malting and also in seed meant for planting (Christensen, 1957).

Both field and storage fungi cause discolouration of whole seeds or part of the seeds. The germ and embryo are the main targets of invasion. This may result in a direct loss of income by the farmer as well as decreased processing quality in wheat and maize meant for milling.

Storage fungi can cause the heating of stored grain, mustiness, caking and at times total decay. The last three are all final stages of grain spoilage.

Apart from the above effects, fungi may produce dangerous toxins which may cause serious diseases in both animal and man consuming the toxic grain.

1.3.4 MOULDY MAIZE PROBLEM IN ZAMBIA

Although the incidence of cob rot (mouldy or diseased maize) has been seen to vary over the past few decades in Zambia, it appears that 1969 was the first year in which the effect of the disease was evaluated (NCSR, 1976). The incidence fell to very low levels in the next four years and only caused a problem on a national scale in 1974. Slightly more that 7 per cent of the country's marketed maize failed to meet the acceptable standard required by the National Marketing Board (Table 1.5).

According to the grading system in existence at the time, NAMBOARD only bought maize containing 2 per cent diseased maize, which was graded as A, B and C depending on the quality (Table 1.6). However, due to the widespread incidence of diseased maize in the 1974 season, the Government introduced a revised grading system in which grades D and E were to cater for maize with 2 to 5 per cent and 5 to 10 per cent diseased maize respectively, and these were also bought by NAMBOARD. Grade E was the inferior grade fetching the lowest price.

Upon the introduction of the new grades, NAMBOARD undertook the task of cleaning the infected maize in order to recover the healthy grain. Six cleaning machines (Eden gravity separators) were installed at the Lusaka depot in 1975. The

Table 1.5 NAMBOARD total purchases of maize and mouldy maize between 1974 - 1981

(Number of 90 Kg bags)

Year	Total intake (All grades)	Mouldy maize (Grades D and E)
1974	6,100,000	437,000
1975	5,900,000	137,500
1976	7,900,000	57,500
1977	7,600,000	NIL
1978	6,400,000	23,000
1979	3,700,000	330
1980	4,200,000	8
1981	7,600,000	NIL

Source: NAMBOARD Grain Marketing Section

Table 1.6 Standards of classification, quality and moisture content.

	A	B	C	D
Maximum % moisture	12.5	12.5	12.5	12.5
Maximum % extraneous matter	0.5	0.75	1.0	1.0
Maximum % trash	0.05	0.05	0.125	0.125
Maximum % chipped grain	8.0	-	-	-
Maximum % insect damaged grain	3.0	5.0	25	25
Maximum % defective grain	5.0	10	20	40
Maximum % other coloured grain included with defective grain	2.0	4.0	10	10
Maximum diseased grain included with defective grain	2.0	2.0	2.0	-
Condition	A, B and C; Fresh and plump D; Marketable			
The colour of the grain should be white or yellow but unmixed				

Source: The Laws of Zambia, chapter 356, National Agriculture Marketing.

Rots by Nigrospora sphaerica are less common and can be identified by the presence of black fungal growth at the base of the shrivelled grains.

These fungi will only grow while the crop moisture content is fairly high and by the time the cob is dry enough to harvest, no further damage would be caused. However, in some seasons (e.g. 1974 season), the rains continue when the cobs should be drying and it is during such seasons that the disease is most severe (Logan, 1974).

1.4 DISEASES OF ANIMALS CAUSED BY FUNGI AND THEIR METABOLITES.

Fungi may be implicated in three kinds of illness in man and animal, namely, mycoses, allergies or toxicoses (Moreau, 1979).

1.4.1 MYCOSES

In this case, the fungi invade the living tissues by either penetrating directly into a healthy organ or penetrate following a lesion of some other kind. The fungi involved are infectious and often contagious. Examples of these are otitis (infection of the ear) and keratitis (infection of the nails). Others may infect the bronchia and the lungs and are referred to as bronchiomycoses and pneumomycoses respectively. Attacks by Aspergilli are among the most serious. Some mycoses may be lethal as in the case of those involving Coccidioides immitis, Cryptococcus neoformans and Aspergillus fumigatus (Moreau, 1979).

1.4.2 ALLERGIES

Some examples of these are conjunctivitis, dermatitis, bronchial asthma e.t.c. (Moreau, 1979). Farmer's Lung, a respiratory disorder caused by the inhalation of spores of mould and actinomycetes present in hay, has been found to be caused by thermophilic actinomycetes such as Thermoactinomyces vulgaris and Micropolyspora faeni (Gregory and Lacey, 1963; Moreau, 1979).

1.4.3 TOXICOSES (MYCOTOXICOSES)

The major difference between mycoses and toxicoses is that the fungi associated with toxicoses are not contagious or infectious (Moreau, 1979). In mycoses, the disease is a direct result of action of the fungi while in toxicoses, it is the toxic fungal metabolites which are responsible for the disease. As the toxic moulds grow on the food and food products, they produce toxins which diffuse into the food so that the consumer suffers the results. These mycotoxins (myco meaning fungi and toxins meaning poisonous) are poisonous to animals (zootoxins), plants (phytotoxins), and some act against other microorganisms e.g. bacteria (antibiotics).

Mycotoxicoses is the illness resulting from the consumption of such food - borne toxins, and they are named in two main ways. One is based on aetiology while the other is based on the symptoms of the disease (Moreau, 1979). The former is on the basis of the causative agent e.g. Aspergillotoxicosis, Stachybotryotoxicosis, Fusariotoxicosis

etc, corresponding to toxicoses due to Aspergillus, Stachybotrys, and Fusarium respectively. Toxicoses can also be named after the substrate on which the toxic mould was growing e.g. mouldy corn toxicoses of swine, forage poisoning, mouldy rice poisoning etc.

The other form derives its name from the symptoms produced by the toxin in animal or man, e.g. facial eczma of ruminants, alimentary toxic aleukia (ATA), cardiac ulceration in pigs, hyperestrogenic syndrome in swine, haemorrhagic syndrome of poultry etc. Sometimes provisional names such as Turkey 'X' disease have been used.

1.5 MAJOR MYCOTOXINS

1.5.1 ASPERGILLUS TOXINS

Although a few reports on the occurrence of mycotoxins had been reported before, it was not until after 1960 that a lot of attention was drawn to mycotoxins. This was as a result of the death of thousands of turkeys aged between 3-6 weeks in the south and east of England. The disease being unknown at the time was called Turkey 'X' disease (Blount, 1961). Similar symptoms were later observed in chickens, cows, pheasants and other animal species in many parts of the world (Goldblatt,1969). Sargeant et al.(1961a) working on groundnuts from East Africa identified the fungus Aspergillus flavus, and an isolate from A. flavus contaminated nuts produced lesions similar to those produced by Turkey 'X' disease. A. flavus is normally classified as a storage fungus but there is

evidence that contamination can occur under field conditions as well (Stoloff, 1977).

The name aflatoxin was given to toxic metabolites of A. flavus before it was realised that it was a complex mixture of compounds. The aflatoxins are a group of bis-furano-isocoumarin secondary metabolites produced by A. flavus and A. parasiticus. The aflatoxin derivatives most commonly associated with A. flavus and A. parasiticus are aflatoxin B_1, B_2, G_1, and G_2. Aflatoxins B_1 and B_2 fluoresce blue and aflatoxins G_1 and G_2 fluoresce green when viewed under ultraviolet light (365nm), hence the designation B for blue and G for green (Nesbitt et al., 1962) (see Figure 1.2).

Aflatoxins are known to produce acute necrosis, cirrhosis and carcinoma of the liver in a variety of animal species (Newberne and Butler, 1969; Siller and Osler, 1961). The liver is the primary target in most animals although cancerous lesions have been observed in the kidney, colon, lung and lacrinal gland of various animals employed in toxicological studies (Stoloff, 1977).

Aflatoxins have been reported to occur in human food samples in Zambia. A study by Njapau et al. (1985) revealed an incidence of 7 per cent contamination in human food samples collected from Katete, Chipata and Lusaka districts (maize based, beans and milk). The contamination ranged from 1 to 50 µg/kg with a mean lying between 16.38-32.92 µg/kg.
Aflatoxin M_1 and Aflatoxicol have also been detected in urine from patients admitted at the University Teaching Hospital

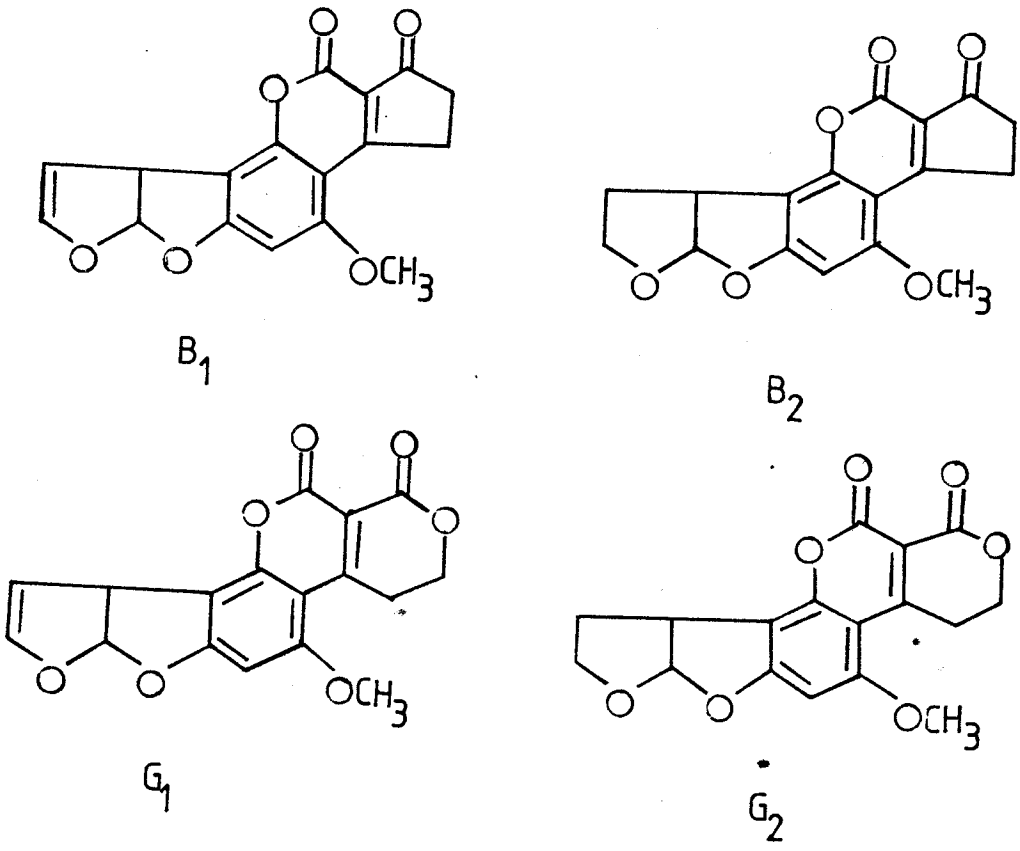


Figure 1.2 The Aflatoxins B_1, B_2, G_1, and G_2

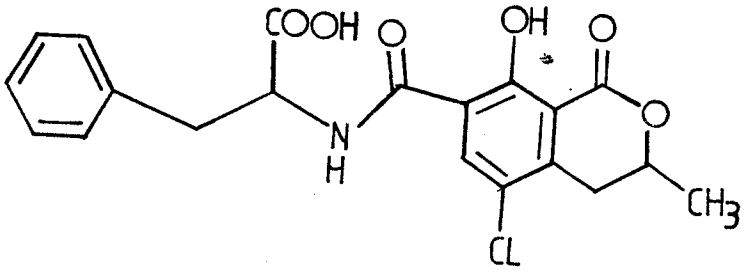


Figure 1.3 The structure of ochratoxin A

(Lovelace et al., 1983; Kaggwa et al., 1985).

Species of Aspergillus produce other toxins like sterigmatocystin and ochratoxins. Sterigmatocystin is a derivative of difuranoxanthone and a metabolite of A. versicolor and some species of Penicillium. It is carcinogenic and similar to the aflatoxins in chemistry and biological activity (Hamasaki and Hatsuda, 1977). The ochratoxins are a group of closely related compounds produced by Aspergillus and Penicillium species e.g. A. ochraceus and P. viridicutum. Seven metabolites are included in the ochratoxin group but only ochratoxin A (Figure 1.3) has been found to be widespread as a natural contaminant. The ochratoxins affect many different kinds of animals and the kidney appears to be the major target organ. They have been implicated in cases of porcine nephropathy and as a possible disease determinant in Balkan endemic nephropathy in humans (Krogh, 1977).

1.5.2 PENICILLIUM TOXINS

Toxic substances associated with the consumption of rice contaminated with P. islandicum are known under the collective name of Islanditoxicosis (Moreau, 1979). P. islandicum has been isolated from rice, sorghum, millet and barley and is considered to be a widespread member of the soil flora. Islandicin was the first toxic substance isolated from P. islandicum but the toxins luteoskyrin and islanditoxin have also been isolated from the same fungus. Islanditoxin (Figure 1.4) appears to be the more toxic metabolite. Acute toxicity results

in rapid death associated with vacuolar degeneration of liver cells followed by a progressive destruction of these cells and haemorrhages especially in the periportal region.

Citrinin (Figure 1.5) is another toxin produced by Penicillium species e.g. P. citrinum. It was initially shown to have antibacterial properties but it has been found to be too toxic to be of any pharmacological use. It mainly affects the liver and kidneys and is particularly responsible for kidney lesions in which there is considerable enlargment of the collective tubules (Morean, 1979).

Patulin and rubratoxin are other toxins of importance produced by several species of penicillium.

1.6 FUSARIUM TOXINS

1.6.1 TAXONOMY OF THE GENUS FUSARIUM

The taxonomy of the genus Fusarium is complex and difficult to apply. This is because of the different taxonomic systems in use in different countries and also because of the extreme variability of Fusarium species in culture. Fusarium species mutate and degenerate rapidly, particularly under conditions of repeated subculturing on common laboratory media (Marasas et al., 1985). This has led to difficulties in relating toxicological studies in different laboratories.

A number of ways of classifying Fusarium have been proposed. Wollenweber (1913) and later Wollenweber and Reinking (1935) divided the genus into groups or sections.

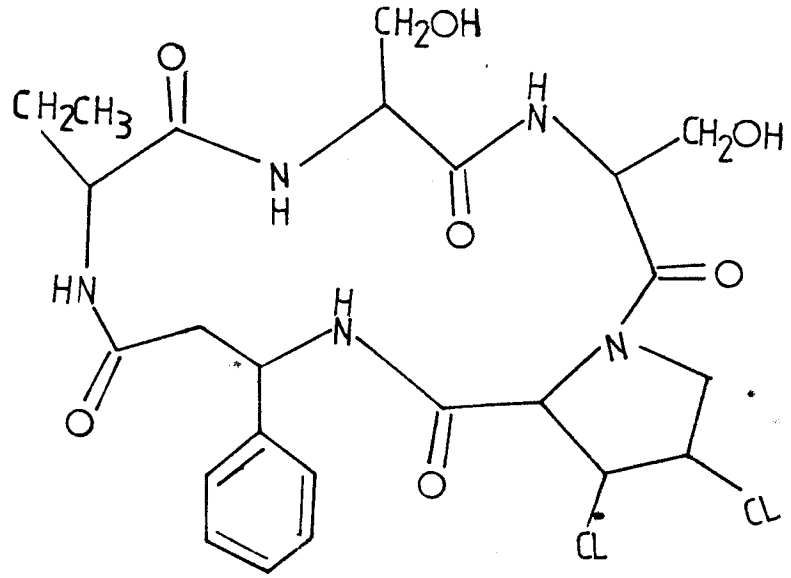


Figure 1.4 The structure of islanditoxin

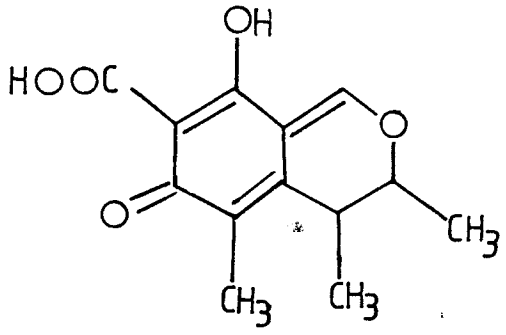


Figure 1.5 The structure of citrinin

They recognised 142 species, varieties and forms which they distributed into 16 groups. Some later classifications by Gordon (1952), Booth (1971) and Joffe (1974a) have been based largely on Wollenweber and Reinking's earlier system.

Snyder and Hansen (1940, 1941, and 1945) proposed a revision of the classification which resulted in a fundamentally different and simplified concept of Fusarium taxonomy. They suggested that Wollenweber's species reflected a large degree of genetic variability of the species and they reduced the number of species to nine. Their work was based largely on morphological variability. They stated that species should be based only on highly constant morphological characteristics and that all other characteristics should serve only for intraspecific distinction. Some of the other characteristics include all physiological activities especially pathogenicity to crop plants. This concept was accepted by many plant pathologists because it simplified the classification of Fusarium. However, it has not been universally accepted by professional mycologists (Joffe, 1977a). The system of Snyder and Hansen grouped all the species which were previously in the sections Roseum, Arthrosporiella, Gibbosum and Discolour into one section, Fusarium roseum. Strains which were pathogenic to cereals were labelled F. roseum f. sp. cerealis. The cultivar concept was later introduced in which morphologically different strains were given cultivar names. For example, morphologically different strains of F. roseum were given cultivar names such

as F. roseum "Graminearum" and if pathogenic to cereals, F. roseum, f. sp. cerealis cultivar "Graminearum", "Culmorum, or "Avenaceum". This introduced a more complex nomenclature for Fusarium species.

Recently Messiaen and Cassini (1968) proposed another system based on Snyder and Hansen's system of classification in which a number of varieties were adopted. These include, sambucinum, culmorum, graminearum and avenaceum which were made varieties of F. roseum.

Nelson et al. (1983) proposed another system of classification and based on this system, Marasas et al. (1984) gave a detailed review of the 20 toxigenic species of Fusarium. A worldwide collection of published toxigenic strains of Fusarium, known as the International Toxic Fusarium Reference Collection (I T F R C) has been established at The Pennsylvania State University. The 20 toxigenic species are given in Table 1.7 together with the section under which they fall.

It has been known for a long time that grain invaded by some species of Fusarium, under certain conditions, might be toxic. Fusarium toxication of humans and livestock, such as dogs, horses, pigs, cattle and poultry has been reported from a number of countries. However, clinical conditions are quite complex and exact diagnosis is considered difficult. A summary of some of the major outbreaks of poisoning is presented in Table 1.8. The widespread nature of the toxin-producing Fusarium in cereals, feeds and vegetables ranks

Table 1.7: Toxigenic *Fusarium* strains in the International Toxic *Fusarium* Reference Collection (ITFRC) (Marasas *et al.*, 1984)

Section	Species
Eupionnotes	*F. merismoides*
Arachnites	*F. nivale* *F. larvarum*
Sporotrichiella	*F. sporotrichioides* *F. chlamydosporum* *F. poae* *F. tricinctum*
Roseum	*F. avenaceum*
Arthrosporiella	*F. semitectum*
Gibbosum	*F. equiseti* *F. acuminatum*
Discolor	*F. sambucinum* *F. culmorum* *F. graminearum*
Liseola	*F. moniliforme* *F. proliferatum* *F. subglutinans* *F. anthophilum*
Elegans	*F. oxysporum*
Martiella	*F. solani*

Table 1.8 Trichothecene Mycotoxicoses (Ueno, 1977)

TOXICOSIS	DISTRICTS and AFFECTED SPECIES	SYMPTOMS	FUNGUS
"Taumelgetreide" Toxicosis	Siberia man, horse, pig, fowl	headaches, chills, nausea, vomiting	G. saubinett
Alimentary Toxic Aleukia	USSR man, horse, pig,	vomiting, diarrhea, skin inflammation leukopenia, angina	F. sporotrichioides
Stachibotryotoxicosis	Europe horse	shock, somatitis dermal necrosis, leukopenia	Stachybotrys atra
Bean-hull Toxicosis	Japan horse	convulsion, cyclic movement	F. solani
Dendrodochiotoxicosis	Europe horse	skin inflammation	Dendrodochiu toxicum
Moldy Corn Toxicosis	U.S.A. pig, cow,	emesis, hemorrhage, refusal of feed	F. Tricinctum
Red-mold Toxicosis	Japan man, horse, pig, cow,	vomiting, diarrhea, emesis, abortion	F. graminarum

the toxins produced by these fungi among the most important mycotoxins. Zearalenone and the trichothecenes are the most important toxins produced by Fusarium. Among the trichothecenes, the major toxins are T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON) and nivalenol. These four trichothecenes and zearalenone, have attracted a lot of attention among researchers as causes of poisoning in humans and farm animals. In this section, a discussion of some of their properties, structures, biological activities and their natural occurrence is given.

1.6.2 ZEARALENONE

Zearalenone (also known as FES, RAL or F-2) is a natural metabolite produced by the following species and subspecies of Fusarium: F. roseum, F. tricinctum, F. oxysporum, F. moniliforme, F. graminearum, F. equiseti and F. culmorum (Cadwell and Tuite, 1970a and 1970b; Mirocha and Christensen, 1974). Stob et al.(1962) first isolated the toxin after an unusually high incidence of estrogenic signs in swine. Christensen et al. (1965) working independently isolated the same estrogenic metabolite from F. roseum and named it F-2.

Zearalenone belongs to a group of natural products called resorcylates and was characterised as 6(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone (Figure 1.6) by Urry et al.(1966). It is a white crystalline compound with molecular formula $C_{18}H_{22}O_5$, molecular weight (M.W.) 318, melting point (m.p.) 164-165°C and has ultraviolet (u.v.)

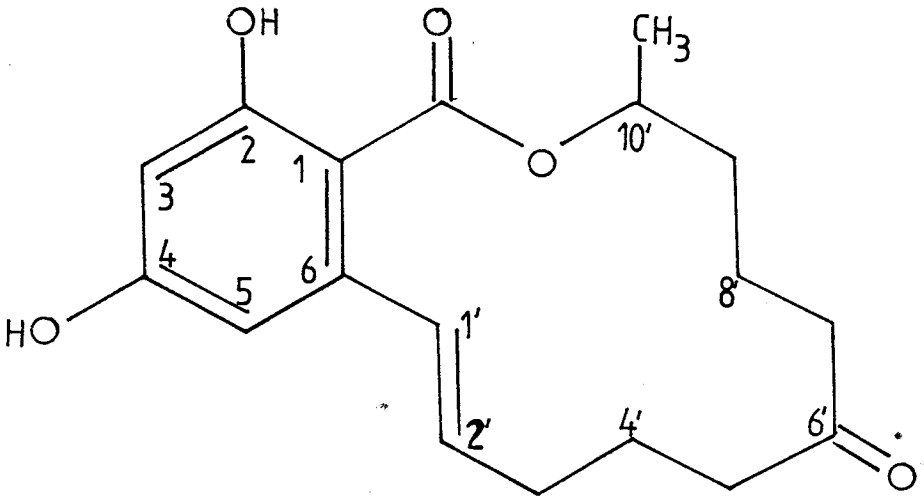


Figure 1.6 The structure of zearalenone

maxima at 236 (ε= 29,700), 274 (ε= 13,909), and 316 nm (ε= 6,020). It fluoresces blue/green when irradiated by short wavelength u.v. (260 nm). This property is made use of in the determination of zearalenone. It also exhibits some infra-red (i.r.) absorption peaks at 3300 (OH), 1645 (Lactone carbonyl), 1688 (C6' carbonyl group), and 970 cm^{-1} (trans disubstituted olefinic absorption). It is insoluble in water, carbon disulphide and carbon tetrachloride but soluble in aqueous alkali, diethyl ether, chloroform, dichloromethane, ethyl acetate, acetonitrile and alcohols and slightly soluble in petroleum ether (BP 30-60^{o}C) (Mirocha et al., 1977).

Zearalenone can be produced in the laboratory by using high yielding isolates of fungi and seeding autoclaved moist polished rice or maize kernels at moisture content of 60 and 45 per cent respectively. The cultures are incubated at 24-27^{o}C for one week followed by incubation at 12-14^{o}C for 4-6 weeks (Eugenio et al., 1970). Normally, the low temperature (12-14^{o}C) is necessary to induce high yields of zearalenone. Sherwood and Peberdy (1972) investigating the effects of moisture content, incubation period and temperature on zearalenone production by F. graminearum, found that low temperatures (12^{o}C) after a period of incubation at 25^{o}C enhanced zearalenone production. However, considerable amounts of zearalenone were still produced at 25^{o}C showing that incubation at 12^{o}C did not appear to be essential. Zearalenone production was

found to be retarded at moisture content of 23 per cent while maximum zearalenone production was obtained at 37 per cent moisture content.

1.6.2.1 BIOLOGICAL ACTIVITY OF ZEARALENONE

An extensive review on the effects of zearalenone on animals has been given by Mirocha and Christensen (1974). Some of the animals affected include swine, dairy cattle, poultry, rats, mice, guinea pigs and monkeys.

(a) Estrogenism in swine was first reported by Buxton (1927 and although he did not know the cause at the time, he described the signs in young gilts as consisting of swelling and eversion of the vagina. McNutt et al. (1928) first associated the disease with consumption of mouldy maize but attributed the outbreak of estrogenism as being indirectly due to an unusually wet autumn in northeast Iowa. The sows developed enlarged vulvas and mammary glands and at times prolapsed vaginas or rectums. Pullar and Lerew (1937) reported a similar outbreak of hyperestrogenism in swine in Australia among pigs 2-3 months old. They attributed the condition to mouldy corn resulting from harvesting and storing corn with high moisture content. In addition to the signs described above, they found that the lumen of the vagina was filled with a thin catarrhal exudate. Similar signs in swine were reported in Ireland and mouldy barley was implicated (McErlean, 1952). The fungus most frequently isolated was F. graminearum and it was suspected that this fungus produced some toxins responsible for hyperes-

trogenism. After a number of reports of swine herds exhibiting vulvar hypertrophy and hyperestrogenic symptoms, investigations were initiated at Purdue University by Stob et al. (1962) where the compound zearalenone was isolated and found to produce symptoms similar to those found in the field.

Mirocha and Christensen (1974) divided the effects of the mycotoxins on swine into three groups:

(1) the outward signs of hyperestrogenism,

(2) infertility characterised by lack of production, and

(3) abortion. However, Chang et. al., (1979) pointed out that zearalenone does not cause abortion but contributes to decreased litter size and infertility. Based on the response obtained in test animals, concentrations between 1 and 5 mg/kg is considered physiologically significant (Mirocha and Christensen, 1974).

(b) Zearalenone toxic effects on poultry are not as pronounce as on swine. Speers et al. (1971) fed maize infected by F. roseum and pure zearalenone to growing chicks and laying hens. In the growing chicks, the body weight showed a quadratic response to increasing levels of zearalenone. Growth appeared to be stimulated by rations containing 20 per cent F. roseum infected corn. During the feeding period, no outward pathological or physiological changes other than body weight were observed. When the birds were necropsied at the end of the experiment, a high incidence of cystic

development on the genital tract was observed. The highest incidence and the largest cysts were observed in birds fed on 20 per cent Fusarium corn. Feeding mature hens with Fusarium infested corn (10 and 20 per cent) and zearalenone (250 and 500 mg/kg) had little influence on the birds, only a decline in the egg shell quality was observed. Sherwood and Peberdy (1973) found that incorportion of 40 mg/kg of zearalenone into the food of male chicks for a period of 10 days had little effect on the weight gain or efficiency of food conversion, but caused significant increase in weight of the testes and comb.

(c) Vulvovaginitis has often been reported by veterinarians in dairy herds but no conclusive evidence that zearalenone is responsible has been given. Mirocha et al. (1968) found 14 mg/kg of zearalenone in a bad lot of hay implicated in causing decreased fertility in dairy cattle.

(d) The effects of zearalenone on humans is not known, but data from studies on non-human primates indicate that hormonal effects do occur (Hobson et al., 1977). Zearalenone administered subcutaneously at 14 µg/kg body weight, depressed levels of serum luteinizing hormone in Rhesus monkeys but it was found to be less potent when given orally.

1.6.2.2 NATURAL OCCURRENCE OF ZEARALENONE

Zearalenone has been detected in maize, sorghum and barley from many countries throughout the world (Table 1.9 and 1.10).

Table 1.9 Natural Occurrence of Zearalenone (Shotwell, 1977)

Commodity or product	Examined because of:	Country	Zearalenone (mg/kg)
Hay	Infertility in dairy cattle	England	14.0
Feed	Infertility in cattle and swine	Finland	25.0
Corn	Hyperestrogenism in farm animals	France	2.3
Animal feed	Hyperestrogenism in cattle and swine	United States	0.1-2,900
Corn		Yugoslavia	18
Corn	Poisoning in swine	Yugoslavia	2.5-35-6
Corn	Severe mold damage swine refusal	Yugoslavia	0.7-14.5
Barley	Stillbirths, neonatal mortality, and small litters in swine	Scotland	0.5-0.75
Corn (freshly harvested)	Gibberella zeae damage	United States	0.1-1.5
Corn (stored)	*A. flavus* damage	United States	ND-92[1]
Barley	Death in swine	Scotland	"traces"
Feed	Field problems in animals	United States	Not stated
Grain sorghum	Head blight in sorghum	United States	Not stated
Corn	Swine hyperestrogenism	Yugoslavia	35.6
Pig feed	Swine hyperestrogenism	United States	50.0
Corn	Swine hyperestrogenism	United States	2.7
Sorghum	Cattle abortion	United States	12.0
Corn	Swine abortion	United States	32.0
Silage		United States	87.3
Corn		England	306.0
Corn	Swine feed refusal	United States	2.5
Dairy ration	Cattle feed refusal, lethargy, anemia	United States	1.0
Pig feed	Swine internal hemorrhaging	United States	0.1
Pig feed	Swine hyperestrogenism	Yugoslavia	0.5
Pig feed	Swine infertility and abortion	United States	0.01

[1] Samples taken from several parts of the bin rather than blending entire lot.

Table 1.10: Coexistence of zearalenone and the trichothecenes.

Mycotoxin	Concentration (µg/kg)	Source	Reference
DON Zearalenone	1800 250	Maize Kernels (U.S.A.)	Mirocha et al. (1977)
DON Zearalenone	40-60 3600	Commercially pelleted feed (U.S.A.)	"
T-2 toxin Zearalenone	76 700	Mixed feed (U.S.A.)	"
DON Zearalenone	1000 500	Mixed feed (U.S.A.)	"
Nivalenol DON T-2 toxin Zearalenone	1.18-4.28 0.14-0.6 0.02 2.5-10	Maize Kernels (France)	Jemmali et al. (1978)
DON Zearalenone	2.5 6.4	S. African Maize Kernels	Marasas et al. (1977)
DON Zearalenone	7.4 12.8	Zambian Maize Kernels	"
Nivalenol DON		Cereal grains (Japan, Korea, Taiwan)	Ueno et al. (1985)

The presence of zearalenone has mainly been established as a result of investigating field outbreaks of mycotoxicoses or from screening of grains at dispersal points in the marketing system. Zearalenone has also been detected in Zambia (Lovelace and Nyathi, 1977). Levels of 0.1-0.8 mg/kg were detected in 1974 maize used to produce opaque beer. In this study both home and commercial brewed beers were examined and found to contain as high as 4.6 mg/L with an average of 0.92 mg/L.

1.6.3 THE TRICHOTHECENES

The trichothecenes are a group of sesquiterpenoids produced by species of Trichothecium, Trichoderma, Myrothecium, Cephalosporium, Stachybotrys, Verticimunosporium, Fusarium, and Cylindrocarpon (Ueno and Ishii, 1985). These fungi have been established as plant-pathogens invading various agricultural products and plants. Since Fusarium and other related fungi infect important foodstuffs, they are associated with human and animal intoxication throughout the world. Mycotoxicoses such as alimentary toxic aleukia in Russia, mouldy corn toxicoses in USA and red mould disease in Japan were reported to be caused by fungi of the genus Fusarium. A summary of reported mycotoxicoses is given in Table 1.8. Toxins elaborated by species of Fusarium have been implicated in most of these mycotoxicoses. Some of the toxins which have been isolated as causes of food-borne intoxication are, T-2 toxin *(Bamburg et al., 1968)*, *nivalenol* and deoxynivalenol *(DON)*

(Tatsuno et al., 1968; Yoshizawa and Morooka, 1973). Diacetoxyscirpenol (DAS) was first isolated as a phytotoxic trichothecene (Brian et al., 1961) but has also been implicated in food-borne intoxication (Mirocha et al., 1976).

The trichothecenes contain only carbon, hydrogen and oxygen and are normally esters of sesquiterpene alcohols possessing the tricyclic trichothecane skeleton (Godtfredsen et al., 1967). They are colourless, mostly cystalline, optically active solids and are generally soluble in moderately polar solvents like chloroform, acetone, alcohols and ethyl acetate. They contain an olefinic double bond at the C-atom 9 and 10 and an epoxy group at C-atom 12 and 13 and are therefore characterised as 12, 13-epoxytrichothecenes.

The I.R. spectrum of the trichotheneces does provide some useful information regarding the presence of various functional groups in the molecule, although it is not very diagnostic. The acetyl group displays strong absorption at 1720 (C=0) and 1250 (C-O) cm^{-1}. The i.r. spectrum is sometimes useful to detect the presence of the epoxide group that absorbs at 4545 and 6060 cm^{-1}. The absence of conjugated unsaturation in most of the trichothecenes explains the absence of u.v. absorption. However, those belonging to the roridin and verrucarin series do give characteristic u.v. spectra.

Naturally occurring trichothecenes have been classified into four groups according to their chemical character as groups A, B, C and D (Ueno, 1977). Group B differs from

group A in that group B trichothecenes possess a carbonyl group at C-8 while group A trichothecenes possess either an hydroxyl group or hydrogen at the same position. Group C comprises the macrocyclic trichothecenes while crotocin, a member of group D possesses a second epoxide group. Pathre and Mirocha (1977) used the solubility properties to classify the trichothecenes into two groups: groups A and B. Group A consists of T-2 toxin, HT-2 toxin, DAS, neosolaniol, verrucarins, roridin and verrucarol with more or less the same polarity and are highly soluble in most aprotic solvents such as ethyl acetate, acetone, chloroform, dichloromethane, and diethyl ether. These toxins are usually extracted from substrates with the same aprotic solvents. Group B trichothecenes are highly hydroxylated and are relatively polar making them soluble in polar or protic solvents like methanol or ethanol. These include DON, nivalenol and T-2 tetraol. They are frequently extracted with methanol, aqueous methanol, aqueous acetonitrile or water. More than 80 derivatives of the trichothecenes have been reported as fungal and plant metabolites (Ueno, 1985).

Toxicity due to the trichothecenes is difficult to diagonise. However, acute or lethal toxicological effects of trichothecenes are similar in all animal species tested. The amount of each specific trichothecene needed to induce acute or chronic toxicosis varies from species to species. Clinical effects produced by the trichothecenes can be grouped into

five categories:

(1) feed refusal, (2) dermal necrosis, (3)gastroenteric effects, (4) coagulopathy, and (5) immunological dysfunction (Osweiler et al., 1985). Clinical signs of trichothecene intoxication of livestock may depend on route of exposure (Pier, 1981).

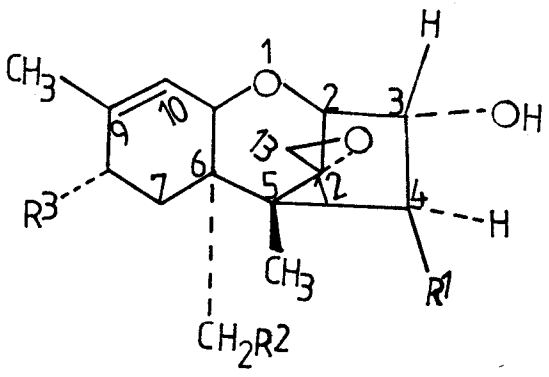
1.6.3.1 T-2 TOXIN AND DIACETOXYSCIRPENOL

T-2 toxin and DAS are produced by F. sporotrichioides (F. tricinctum), F. poae, F. equiseti, F. acuminatum, F. roseum and F. solani (Brian et al., 1961; Ueno et al., 1972; Marasas et al., 1984) Most of the above species produce both T-2 toxin and DAS.

Bamburg et al. (1968) isolated and characterised T-2 toxin as 4 ,15-diacetoxy-8 - (3-methylbutyryloxy)-12,13-epoxytrichothec - 9 - en - 3 - ol (Figure 1.7), molecular formula $C_{24}H_{34}O_9$, M.W. 466, m.p. 151 - 152°C. It exhibits strong I.R. absorptions at 3400, 2940, 1720, 1365 and 1240 cm^{-1}. It only exhibits u.v. end absorptions due to the epoxide group.

DAS was first isolated as a phytotoxic compound (Brian et al., 1961), and its structure has been characterised as 4, 15 - diacetoxyscirp - 9 - en - 3 - ol (Figure 1.7) (Dawkins, 1966), molecular formula $C_{19}H_{26}O_7$, M.W. 366, m.p. 161 - 162°C.

The toxigenicity of the DAS and T-2 toxin producing fungi is greatly influenced by ecological factors such as



Compound	R^1	R^2	R^3
T-2 toxin	$OCOCH_3$	$OCOCH_3$	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	$OCOCH_3$	$OCOCH_2CH(CH_3)_2$
Neosolaniol	$OCOCH_3$	$OCOCH_3$	OH
Diacetoxyscirpenol	$OCOCH_3$	$OCOCH_3$	H

Figure 1.7 Some of the group A trichothecenes

humidity,temperature and nutrients of substrates where the species is isolated. Joffe (1962) pointed out that the toxigenicity of F. sporotrichioides was much greater when the fungus was cultivated at low temperatures and this explains the fact that severe toxicosis develops after ingestion of overwintered grains. Bamburg and Strong (1969) reported that F. tricinctum produced the largest amounts of T-2 toxin and DAS when the fungus was grown at 8 ^{o}C. When grown at 24^{o}C, the same fungus produced HT-2 toxin in addition to T-2 toxin and DAS. Joffe (1974b) working with F. poae and F. sporotrichioides found that the two species produced the highest toxicity (using the rabbit skin test) when the fungi was grown at 5 and 8^{o}C. However, more vigorous growth of the two species was observed at 18-24^{o}C where toxicity was moderate. He also pointed out that overall toxin production was greater on starch substrates.

1.6.3.1.1 BIOLOGICAL ACTIVITIES OF T-2 TOXIN AND DAS

The toxicity of T-2 toxin and DAS has been studied in many laboratories using laboratory and farm animals such as rats, guinea pigs, rabbits, cats, dogs, pigs, ducklings, chicks and cattle. These two toxins are the principle trichothecene compounds of veterinary interest from group A (Ueno, 1977 nomenclature). They are skin and mucous membrane irritants. Experimental animals usually develop diarrhoea, rectal haemorrhaging and necrotic lesions of the oral cavity following their adminstration. T-2 toxin and DAS have been shown

to be major immunosuppressive agents. They have been associated with alterations in serum proteins and immunoglobulin profiles, reduced antibody formation, thymic aplasia, generally reduced cell mediated immune responsiveness with enhanced delayed cutaneous hypersensitivity and impairment of bacterial clearance and acquired immunity (Pier and McLoughlin, 1985).

(a) Young swine when fed a ration incorporated with 1,2,4 and 8 mg/kg T-2 toxin for 8 weeks, showed no gross microscopic or biochemical changes. When the level was increased to 16 mg/kg, feed consumption was reduced and when the level was further increased to 24 and 32 mg/kg, the feed was rejected by the swine (Weaver et al., 1978a). In an other experiment, Weaver et al. (1978b) found that T-2 toxin produced fertility problems in three proven sows when fed 12 mg/kg of the toxin in standard pig ration for 220 consecutive days. Small piglets and small litters were produced. When T-2 was administered intravenously at levels of 0.21-0.41 mg/kg to three pregnant sows, all of them aborted but no lesions were produced (Weaver et al., 1978c).

The LD_{50} for T-2 toxin given intravenously was found to be 1.21 mg/kg body weight, and that for DAS has been found to be 0.376 mg/kg body weight (Weaver et at., 1981).

(b) The trichothecenes T-2 toxin and DAS have been found to have pathological effects on poultry also. Lesions include caseous, necrotic lesions of the oral cavity especially the

palate and underside of the tongue, fatty degeneration and haemorrhaging in internal organs together with marked reduction in leukocyte and thrombocyte count (Chi and Mirocha, 1978).

Toxicity tends to vary with age of the birds and duration of exposure to the toxin. For orally administered T-2 toxin, the LD_{50} was found to be 5.25, 4.97 and 6.27 mg/kg for one day old chicks, 8 day old chicks and laying hens respectively (Chi and Mirocha, 1978). When one day old chicks were fed 5 mg/kg of DAS or T-2 toxin for 3 weeks, they developed yellowish plaque-like lesions on the beak, tongue and angle of the mouth. They also showed poor weight gains with those fed DAS showing the least weight gains (Chi et al., 1977). Effects on the laying hens include decreased feed consumption, egg production and egg shell thickness. Ellison and Kotsonis (1973) found T-2 toxin to be the emetic factor in mouldy maize fed to pigeons at non-lethal concentration when an extract from F. poae was administered orally or intravenously.

(c) Field outbreaks of Fusarium toxicoses in cattle have been shown to involve the following symptoms, inappetance, ataxia, scouring and marked decrease in milk yields in dairy cattle (Fisher et al., 1967). Hsu et al. (1972) reported the identification of T-2 toxin in mouldy maize associated with lethal toxicosis in dairy cattle. Seven out of 35 cattle died within a 5 month period after prolonged ingestion

of ground mouldy corn. These animals showed typical haemorrhagic syndrome associated with mouldy corn toxicosis. Examination of original feed revealed the presence of T-2 toxin at a concentration of 2 mg/kg.

Mirocha et al. (1974) isolated T-2 toxin from commercially prepared cattle ration and in mouldy maize associated with haemorrhaging and poor weight gains in cattle. T-2 toxin was also detected in stored brewers grain associated with decreased appetite, decreased milk yield, bloody diarrhoea and multiple petechial haemorrhages of mucous membrane (Petrie et al., 1977). Osweiler et al. (1985) reported the depression of serum immunoglobulin M and A by T-2 toxin and thereby altering some aspects of humoral immunity.

(d) The trichothecene T-2 toxin has also been implicated in human mycotoxicoses. Outbreaks of the dreadful disease ATA occured in parts of Russia during 1942-1947. This disease was caused by eating of overwintered toxic grain. A description of the disease is given by Forgacs and Carll (1962) and more recently by Joffe (1974b). The first signs are discomfort of the mouth, throat and stomatch, which are followed by inflamation of the intestinal mucosa. Damage to the mucosa membrane results in vomiting and diarrhoea. With more consumption of the toxic grain, damage to the bone marrow and haemopoetic system occurs followed by anaemia and a drop in erythrocyte and platelet counts. Blood capillary walls become

weakened and haemorrhaging frequent. Those areas originally affected become necrotic and dead tissues become infected with bacteria. With repeated ingestion of the toxic grain, death resulted about 6-8 weeks after the first symptoms. Analysis of an extract from the grain implicated in the toxicoses, revealed the presence of T-2 toxin, T-2 tetraol, neosolaniol and zearalenone (Mirocha and Pathre, 1973). T-2 toxin and DAS have also been implicated in the suppression of the immune system of the body against general bacteria and viral infection (Joffe, (1974b).

1.6.3.1.2 NATURAL OCCURRENCE OF T-2 TOXIN AND DAS

The natural occurrence of the trichothecenes T-2 toxin and DAS has been reported from several countries. The danger is greatest in those countries where the toxins would be produced in the colder seasons of the year. As already pointed out in section 1.6.2.1, toxin production is favoured by temperatures below those supporting optimal mycelial growth i.e. 6-12^{o}C for T-2 toxin and DAS production as compared to optimal mycelial growth temperatures of 18-25^{o}C. However, some amounts of T-2 toxin and DAS are produced even at temperatures such as 25^{o}C (Bamburg et al., 1968), making it possible for toxicosis to occur even in the subtropical countries like Zambia. Neither T-2 toxin nor DAS have as yet been reported in Zambia although some of the fungi which produce the two toxins have been identified here (NCSR, 1976 ; Marasas et al., 1978; Kapooria et al., 1981).

1.6.3.2 DEOXYNIVALENOL AND NIVALENOL

Deoxynivalenol (DON) and nivalenol are structurally related compounds which only differ in their substitution at C-4. Nivalenol has a hydroxyl group while DON has a hydrogen atom at C-4. According to both Pathre and Mirocha (1977), and Ueno (1977) nomenclature, the two toxins belong to group B trichothecenes. The two toxins are produced by several species and subspecies of Fusarium. These include F. graminearum, F. nivale, F. roseum, F. equiseti and F. oxysporum (Ichinoe et al., 1983; Tatsuno et al., 1969; Yoshizawa and Morooka, 1973; Marasas et al., 1984). So far, the two toxins have not been reported to be produced by the same fungal isolate although the two toxins have been isolated from the same sample of naturally infected cereals. Ichinoe et al. (1983) working with 113 isolates of F. graminearum, found that the isolates could be subdivided into two chemotaxonomic groups: DON producers and nivalenol producers, with no cross production of the two types of trichothecenes in the isolates.

DON was first isolated from barley infected with Fusarium spp. and was given the trivial name Rd toxin (Morooka et al., 1972). The structure was later reported to be 3α, 7α, 15-trihydroxy - 12, 13-epoxytrichothec - 9 - en - 8 - one by Yoshizawa and Morooka (1973). Vesonder et al. (1973) working independently isolated and characterised the same toxin under the trivial name, vomitoxin. DON forms fine

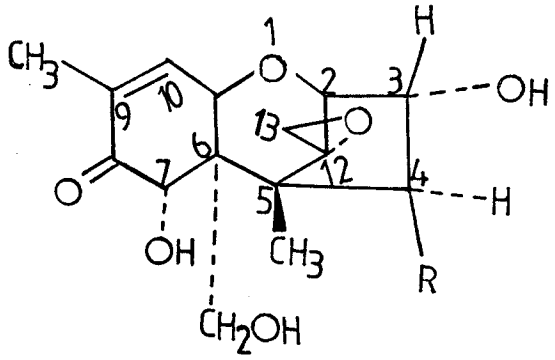
needles when recrystallised from ethyl acetate-petroleum ether with m.p. 151-153°C, M.W. 296 and molecular formula of $C_{15}H_{20}O_6$ (see Figure 1.8).

Nivalenol was first isolated from rice infected with F. nivale by Tatsuno et al. (1968) and the structure was later reported as 3α, 4β, 7α, 15-tetrahydroxyscirp-9-en-8-one (Tatsuno et al., 1969). Nivalenol forms fine needles as well when recrystallised from methanol, m.p. 224-226°C, M.W. 312 and molecular formular $C_{15}H_{20}O_7$ (see Figure 1.8).

The production of DON and nivalenol is also influenced by geographical as well as ecological factors. Ueno et al. (1972) reported that fungal strains which produce trichothecenes DON and nivalenol were mostly found in the southern districts of Japan where the climates are warmer. The production of the two toxins is generally greater at higher temperatures (25-28°C) than at lower temperatures (6-12°C) (Ueno et al., 1970; Yoshizawa and Morooka., 1977). Apart from the temperature, the type of substrate can also influence the production of the toxins. Ueno et al. (1970), working with F. nivale, reported that moist rice grains were the most suitable substrate for the production of nivalenol while the same fungus preferentially produces Fusarenon-X (4-0-acetyl-nivalenol) on peptone-Czapek liquid medium.

1.6.3.2.1 BIOLOGICAL ACTIVITIES OF DON AND NIVALENOL

The "red mould disease '(Akakabi-byo)" in Japan causes severe damage to cereal crops and often affects a large portion



Compound	R
Nivalenol	OH
Fusarenon-X	$OCOCH_3$
Deoxynivalenol (DON)	H

Figure 1.8 Some of the group B trichothecenes

of the national cereal production. It is caused by Fusarium invasion of the crops when the rainy season coincides with the blooming, maturing and harvesting of the crops. If these mouldy cereals are used as food or animal feed, serious diseases are seen to develop and the symptoms include vomiting, and diarrhoea in people, refusal of feed and congestion or haemorrhages on the lung, adrenal, intestine, uterus, vagina and brain of animals (Ueno, 1977). F. roseum and related fungi have been found to be the major fungi causing the damage (Yoshizawa and Morooka, 1977).

As already pointed out, Fusarium is the major cause of cob rot in Zambia, with F. graminearum being the most important. DON and nivalenol are two of the toxins usually isolated from infected grains in Japan. DON causes emesis naturally in swine and experimentally in dogs, cats, and ducklings (Pier, 1981). It is also known to cause feed refusal in swine and some other animals. DON is now considered to be an important toxin because of the many instances of contamination of food by this toxin which have been reported.

Little information is available on the toxicity of nivalenol to livestock and humans. However, in experiments with laboratory animals, nivalenol has been shown to be more toxic than DON (see Tables 1.11, 1.12, and 1.13).

(a) Swine appear to be the most sensitive to DON. Many studies have been carried out to relate the involvement of DON in causing emesis and feed refusal. Vesonder et al. (1973)

first isolated it as the mycotoxin responsible for vomiting in swine. The minimum emetic dose to swine weighing 9-10 kg was found to be 0.05 mg/kg body weight intraperitoneally and 0.1 - 0.2 mg/kg orally (Forsyth et al., 1977). In the same experiments, it was found that purified DON reduced feed consumption by 20 per cent at 3.6 mg/kg level and by 90 per cent at 40 mg/kg level. However, feed refusal of naturally infected maize blended with sound maize was found to be much greater than for DON spiked maize containing the same amount of DON. This suggests that other metabolites present in the mouldy maize may also be active as feed refusal factors. Trenholm et al. (1981) reported that swine diets containing 0.3 and 0.7 mg/kg DON resulted in decreased feed consumption and weight gains.

(b) Unlike swine, there is evidence to show that poultry and cattle are more resistant to DON (Wyllie and Morehouse (ed), 1977). It has been found that maize refused by swine can be accepted by cattle or poultry.

(c) DON does not seem to have any apparent ill effect on man (Mirocha et al., 1985). However, DON has been found in maize used by humans in Transkei where the incidence of oesophageal cancer is very high (Thiel et al., 1982 b).

1.6.3.2.2 NATURAL OCCURRENCE OF DON AND NIVALENOL

The natural occurrence of nivalenol, sometimes together with DON, has been reported in grains from Japan (Yoshizawa and Morooka, 1977; Yoshizawa and Hosakawa, 1983; Kamimura

et al., 1981), South Africa (Thiel et al., 1982a), France (Jemmali et al., 1978), and more recently in Taiwan, and the Soviet Union (Ueno et al., 1985). However, it has not as yet been reported from Zambian grains. The natural occurrence of DON on the other hand, has been reported from maize, wheat, and rice in many parts of the world (Morooka et al., 1972; Vesonder et al., 1973; Mirocha et al., 1976; Trenholm et al., 1981)

DON has been reported to occur in Zambian maize. Marasas et al. (1977) reported the isolated of DON from hand-selected naturally infected maize kernels.

1.6.3.3 COMPARATIVE TOXICITIES OF THE TRICHOTHECENES

The trichothecenes as a whole possess similar chemical and biological features. In this section, a comparison of the toxicological activities of the four trichothecenes of interest is considered.

A number of experiments have been performed by different authors to investigate the following biological activities:

(1) the acute toxicity in terms of half lethal doses (LD_{50}),
(2) minimum effective doses (MED) to induce skin necrotisation and vomiting in experimental animals,
(3) cytotoxicity to cultured cells, and
(4) their inhibition of protein synthesis.

The acute toxicity of trichothecenes has been studied using laboratory and farm animals. LD_{50} of many trichothecenes

have been determined with mice and rats, making it possible to compare their toxicities. T-2 toxin and nivalenol have approximately the same toxicity to mice when administered intraperitoneally (Table 1.11) (Ueno and Ishii, 1985). DON is much less toxic (70.0 mg/kg, i.p.) than T-2 toxin or nivalenol.

Several trichothecenes cause skin necrotisation when applied on the back of experimental animals. T-2 toxin, HT-2 toxin and DAS induce a skin reaction on guinea pigs at a dose of 0.2 µg. As compared to guinea pigs, mice appear to be less sensitive, 1 µg of T-2 toxin and HT-2 while 10 µg of DAS is required. The group B toxins are less reactive and 75 µg of DON is required for a reaction. The group A trichothecenes are more effective in causing vomiting in ducklings than are the group B toxins, for example, the MED for inducing vomiting is 0.1 µg for T-2 toxin and 13.5 µg for DON when the toxins are administered subcutaneously. Table 1.12 (Ueno and Ishii, 1985) gives MED for inducing skin necrotisation and vomiting.

The trichothecenes have been shown to be cytotoxic to eukaryotic cells including those of fungi, plants and animals. Table 1.13 (Tanaka et al., 1977) shows the half inhibition concentration values of trichothecenes in three lines of cultured cells. T-2 toxin appears to be the most effective.

In biochemical studies on the cytotoxicity of the trichothecenes, Ueno et al. (1969) reported that to inhibit protein

Table 1.11: LD_{50} values (mg/kg) of trichothecenes (Ueno and Ishii, 1985).

Compounds	Mice				Rats			
	i.v.	i.p.	s.c.	p.o	i.v.	i.p.	s.c.	p
T-2 toxin		5.2		10.5				5
HT-2 toxin		9.2						
Diacetoxyscirpenol	12.0	23.0			1.3	0.75		
8-Acetylneosolaniol						1.0		
Neosolaniol		14.0						
NT-1		28.5						
Nivalenol		4.1					0.9	
Fusarenon-X	3.4	3.4	4.2	4.5			0.5	
Deoxynivalenol		70.0		46.0				
3-Acetyldeoxynivalenol		49.0		34.0				

Table 1.12: Minimum effective doses for inducing skin necrotization and vomiting (Ueno and Ishii, 1985

Trichothecenes	Skin necrotization (μg)		Vomiting (mg/kg, s.c.)
	Guinea pigs	Mice	Ducklings
T-2 toxin	0.2	1	0.1
HT-2 toxin	0.2	1	0.1
Diacetoxyscirpenol	0.2	10	0.2
Neosolaniol	1	10	0.1
Nivalenol	10	100	1.0
Fusarenon-X	1	10	0.4
Deoxynivalenol		(75)*	13.5
3-Acetyldeoxynivalenol		(>1000)*	13.5

( )* Data obtained with rats.

synthesis in rabbit reticulocytes (see Table 1.14), T-2 toxin and DAS have equal toxicity (o.03 µg/ml), and nivalenol and DON are less toxic (3 and 2 µg/ml respectively).

1.6.4 CO-EXISTENCE OF ZEARALENONE AND THE TRICHOTHECENES

Many toxigenic species of Fusarium have been shown to produce more than one toxin. F. sporotrichioides produces several toxins including T-2 toxin, DAS and zearalenone, while F. poae has been shown to produce T-2 toxin and DAS (Marasas et al., 1985), F. graminearum has been shown to produce either DON and zearalenone or nivalenol and zearalenone (Ichinoe et al., 1983).

Apart from single species producing more than one toxin, several species of fungi can occur on the same sample. For example, F. graminearum has been isolated from samples together with F. moniliforme and F. equiseti (Marasas et al., 1977).

Reports on the presence of more than one toxin as natural contaminants in cereal grains have appeared in the literature. Mirocha et al. (1977) reported the occurrence of DON and zearalenone together in maize kernels and mixed feeds in the United States. The same authors reported the occurrence of T-2 toxin and zearalenone in mixed feeds. Marasas et al. (1977) reported the occurrence of DON and zearalenone in South African and Zambian maize while Jemmali et al. (1978) reported the natural occurrence of nivalenol, DON, T-2 toxin and zearalenone in France. The co-existance of nivalenol, DON and zearalenone has been reported from Japan, Korea and Taiwan (Ueno et al., 1985) (Table 1.10).

Table 1.13: Cytotoxicity of trichothecenes to cultured cells (Tanaka et al., 1977)

Trichothecenes	IC_{50} (µg/ml)		
	HeLa	HEK	HL
Acetyl T-2 toxin	1.0	0.8	0.03
T-2 toxin	0.01	0.02	<0.0003
HT-2 toxin	0.01	0.1	0.01
Neosolaniol	0.1	0.06	0.05
Diacetoxyscirpenol (DAS)	0.01	0.01	<0.001
Monoacetoxyscirpenol	0.1	0.1	0.3
Nivalenol	0.3	1.0	0.3
Fusarenon-X	0.1	1.0	0.3
Deoxynivalenol	1.0	3.0	0.5
Monoacetyldeoxynivalenol	10	10	10

Table 1.14: Inhibition of protein synthesis in rabbit reticulocytes by trichothecene mycotoxins (Ueno, 1977)

Type	Trichothecenes	ID_{50}[1] (µg/ml)
A	T-2 toxin	0.03
	HT-2 toxin	0.03
	Diacetoxyscirpenol (DAS)	0.03
	Neosolaniol	0.25
	7-Hydroxy-DAS	0.4
	7,8-Dihydroxy-DAS	0.6
B	Nivalenol	3.0
	Fusarenon-X	0.25
	Diacetylnivalenol	0.10
	Tetraacetylnivalenol	10
	Deoxynivalenol	2.0

[1] 50% inhibition dose in the "whole cell" assay system.

1.7 ANALYTICAL METHODS

The discovery of aflatoxin has revolutionised research on moulds and mould metabolites because of its potency as a toxin and carcinogen. For quite a long time the name aflatoxin was synonymous with the term mycotoxin and aflatoxin has served as a model for research on and control of other mycotoxins that might otherwise have been unrecognised or neglected. Method development for the analysis of other mycotoxins has followed closely the stages used in aflatoxin analysis. Analytical methods for aflatoxin analysis have tended to follow the following sequence:

(1) Sampling and sample preparation,

(2) extraction of the aflatoxins from samples,

(3) extract purification,

(4) chromatographic separation of the aflatoxins,

(5) measurement of aflatoxins and,

(6) comfirmation of their identity. A similar sequence has been used in the analysis of other mycotoxins including zearalenone and the trichothecenes.

1.7.1 SAMPLING AND SAMPLE PREPARATION

The sampling plans which are in existance at the moment have been devised for aflatoxin contamination, but so far there has been no evidence that contamination by other mycotoxins is any different from aflatoxin contamination (Campbell et al., 1986). Therefore, this information

presented here can be considered to apply to other mycotoxins.

High concentrations of aflatoxins have been found in individual kernels of maize, peanut and cottonseed, and aflatoxins were often concentrated in only a small percentage of the seed within a lot. There is a large variation in the aflatoxins concentration of the sample as compared to the lot and determination of true aflatoxin concentration of the lot is difficult. Cucullu et al. (1966) demonstrated that aflatoxin contamination in suspect lots of mould damaged peanuts is non-uniform among individual kernels. In the analysis of 20 individual kernels from a 2 kg suspect sample, 12 were found to contain aflatoxin B_1 ranging from 300 - 1 million µg/kg and 8 were free from detectable aflatoxin B_1. The rest of the 2 kg sample was finally ground and on analysis was found to contain no detectable aflatoxins. Therefore, in order to obtain a representative sample, it is necessary to take a relatively large number of particles from a number of sites in the lot. It is difficult to make a general recomendation about the size of the sample that should be used in surveys. Large samples increase accuracy, but the cost of transporting the samples may be limiting. For shelled maize, a 5 kg (10Lb) sample is probably sufficient for most survey purposes (Davis et al., 1980). However, much larger samples of cotton-

seed, shelled peanut and possibly other products may be needed for the same accuracy.

Samples may be taken from crops growing in the field, during handling, during storage or points in the production, marketing and processing. When feasible, samples should be taken after a lot has been reduced to smaller particulate size. For example, it is better to sample shelled maize than ear maize, better still to sample ground maize than shelled maize (Davis et al., 1980). A sampling plan for aflatoxin analysis of peanuts has been described by Dickens (1977).

When a representative sample has been obtained, the sample is then prepared for analysis. In general this will involve mixing and blending the material, coarse grinding to reduce particle size and, mixing again to obtain uniformity. The sample is then subdivided to get a portion for further grinding to produce a flowable material which can be subdivided to the specific size of the analytical sample; e.g. 25-100 g (Campbell et al., 1986, Trenholm et al., 1985). Several pieces of equipment for sample preparation have been used. The Hobart Vertical Cutter Mixer (HVCM) can simultaneously grind and blend a 12 kg (25 Lb) in-the-shell sample of Brazil nuts to produce analytical size samples in 2-3 minutes. The Dickens subsampling mill can comminute and subsample peanut kernels at a rate of about 3 kg per minute. In the case of cottonseeds, a Bauer disc mill is used to dehull the seeds which are then ground by the Dickens sub-

sampling mill. Other equipment used in sample preparation are; the hammer mills, grinders, food choppers, twin shell blenders and planetary mixers (Dickens et al., 1979; Horwitz (ed). 1975; Campbell et al., 1986).

1.7.2. EXTRACTION METHODS

Different procedures have been proposed for the extraction of mycotoxins from solid samples. Earlier methods involved the use of Soxhlet extraction especially in the extraction of aflatoxin from peanuts (Sargeant et al., 1961a and 1961b, Coomes and Sanders, 1963). However, Nabney and Nesbitt (1965) showed that while Soxhlet extraction for 6h completely removed aflatoxin, some 1.5-2.0 per cent of aflatoxin B_1 present was decomposed during each hour of extraction. The use of high speed blenders with more efficient extraction solvents was proposed in the extraction of aflatoxins (Nesheim, 1964). Equilibrium extraction using a mechanical shaker for removal of aflatoxin from cottonseeds, peanuts and other agricultural materials was suggested by Pons and Goldblatt (1965). Similar procedures have been proposed for the extraction of zearalenone and the trichothecenes from solid samples. Liquid-liquid partition has been used for liquid samples.

Several solvent systems for the extraction of toxins from samples have been described and these have varied depending on the type of sample and toxins being analysed. Some

toxins are more polar than others, the more polar toxins are extracted with polar solvent systems like methanol while the less polar ones require solvent systems such as chloroform.

Ethyl acetate has been used in the extraction of some toxins. Ellison and Kotsonis (1973) described a method in which T-2 toxin was extracted with ethyl acetate from mouldy maize by continuous stirring for 46h at 25°C. A Soxhlet apparatus was used to extract zearalenone and aflatoxins from mixed feeds or maize (Mirocha et al., 1974), and T-2 toxin and DAS from feedstuff (Mirocha et al., 1976). The same solvent can be used in liquid-liquid partition for the extraction of T-2 toxin from urine or serum (Lee and Chu, 1981a).

Methanol is one solvent which has been used widely as the extraction solvent especially in the extraction of polar toxins like the trichothecenes. Aqueous solutions tend to perform better than methanol on its own when dealing with more polar compounds. Romer et al. (1978) used aqueous methanol (1 + 1, v:v) for the extraction of T-2 toxin and DAS. The same solvent system has been used successfully for the extraction of DON (Scott et al., 1981). In both cases, a high speed blender was used. A mixture of methanol and water (7 + 3, v + v) has been used to extract DON and nivalenol from cereals by use of a high speed blender (Health Protection Branch, 1985; Scott et al.,

1986). Very high recoveries, 89 per cent for nivalenol and 106 per cent for DON, were obtained for the two toxins by the above method. In the method of Kamimura et al. (1981), several mycotoxins including seven trichothecenes and zearalenone were extracted with methanol - water (95+5, v:v) by continuous shaking on a wrist-action shaker.

Acetonitrile on its own is not an efficient extraction solvent, but in combination with water, it gives cleaner extracts and better recoveries than aqueous methanol in the extraction of DON and nivalenol (Tanaka et al., 1985; Yoshizawa, 1984). Tanaka et al. (1985) reported the best recoveries (over 90 per cent) when acetonitrile - water (3+1, v:v) was used, and the sample shaken on a wrist-action shaker for 30 minutes. The use of acetonitrile - water (84+16, v:v) has also been reported in the extraction of DON using a wrist-action shaker (Trucksess et al., 1984; Eppley et al., 1984). Roberts and Patterson (1975) described a multi-toxin extraction procedure in which acetonitrile - 4 per cent KCl (90+10, v:v) as the extraction solvent was used. In another multi-toxin screening method (Van Egmond et al., 1979), acetonitrile - 0.1 M H_3PO_4 (125+12.5, v:v) was used and a wrist-action shaker employed.

Zearalenone is less polar than DON and nivalenol and solvents like chloroform or dichloromethane have been used for its extraction. Mirocha et al. (1967) used the Soxhlet extractor to extract zearalenone from stored maize with

dichloromethane (methylene chloride). Mouldy maize was adjusted to 30 per cent moisture content before extracting for 16h. The following year, Eppley (1968) described a screening method for zearalenone, aflatoxins and ochratoxins using chloroform as the extraction solvent. This method was applied in a survey of 1967 maize from commercial markets for aflatoxins, zearalenone and ochratoxins by Shotwell et al. (1970). Other reports in which either chloroform or dichloromethane has been used have appeared in the literature (Sherwood and Peberdy, 1972; Malaiyandi et al., 1976; Bennett et al., 1985).

1.7.3 CLEAN-UP PROCEDURES

Lipids and other substances like carbohydrates and pigments are co-extracted with mycotoxins and they may interfere with subsquent chromatography. For samples containing more than 2 per cent lipids (Moreau, 1979), it is essential to remove the lipids. Thus, it is usual to employ a clean-up step.

(a) Liquid-liquid partition has been employed to remove interfering lipids, carbohydrates and pigments. The difference in the solubility of toxins in two immiscible solvents is made use of in Liquid-Liquid partition and is usually used in conjunction with other clean-up procedures. Mirocha et al. (1976) used petroleum ether (b.p. 60-70^{o}C) to remove interfering substances from acetonitrile in the determination

of T-2 toxin and DAS from maize and feedstuff. Roberts and Patterson (1975) employed iso-octane in a multi-toxin screening method and used partition with a mixture of acetonitrile - 4 per cent potassium chloride (90+10, v:v). Another solvent which has been used widely to remove interferences is n-hexane (Trucksess et al., 1984). Bennett et al. (1985) described a method in which liquid-liquid partition was used solely as the clean-up step in the analysis of zearalenone and α-zearalenol in maize. The corn was extracted with chloroform, the toxins taken up in 2 per cent sodium hydroxide, 10.6 per cent citric acid added to the sodium hydroxide layer and the toxins re-extracted into either chloroform or dichloromethane.

(b) Purification of the extract by column chromatography has been extensively used in the isolation and analysis of the trichothecenes and zearalenone. An adsorption material is packed into a glass column, a reduced volume of the extract is added and toxins are allowed to be adsorbed. The toxins are then selectively eluted from the column using suitable solvent systems leaving most of the interferences on the adsorbent. A number of materials have been reported. Silica gel is most commonly used. It has been used in the screening of zearalenone, aflatoxins and ochratoxins (Eppley, 1968; Shotwell et al., 1970), the analysis of T-2 toxin and DAS (Romer et al., 1978), DON (Scott et al., 1981) and

nivalenol (Health Protection Branch, 1985; Scott et al., 1986). Florisil has also been reported as suitable adsorption material (Kamimura et al., 1981; Tanaka et al., 1985). Ueno et al. (1973) used charcoal for the purification of extracts in the analysis of several trichothecenes while Truckesess et al. (1984) proposed a column packed with a mixture of charcoal-alumina-celite in the clean-up procedure for DON. Ambelite XAD-4 resin was used in a multi-toxin screening method (together with florisil) by Kamimura et al. (1981). Commercially prepared columns are now being employed e.g. a column packed with a hydrophilic matrix has been used in the analysis of DON (Health Protection Branch, 1985; Scott et al., 1986). Sep Pak silica cartridges have also been used in the analysis of DON and nivalenol (Tanaka et al., 1985), T-2 toxin, HT-2 toxin and DAS (Cohen and Lapointe, 1984).

(c) Roberts and Patterson (1975) reported a membrane clean-up procedure in a multi-toxin screening method. An acetonitrile extract from a mixed feed was equilibrated against 30 per cent aqueous acetone in a Visking dialysis tubing and interference from lipids and pigments was reduced. This method has been used successfully by Howell (1983) and Roberts et al. (1983).

(d) Preparative thin layer chromatography (TLC) has also been employed as a clean-up step for extracts before analysis

by gas chromatography (GC) and mass spectrometry (MS) (Yamamoto, 1975; Brumley et al., 1985).

1.7.4 DETECTION AND QUANTITATION

A number of procedures for the detection and quantitation of zearalenone and the trichothecenes have been employed. The choice of a particular method will depend mainly on the degree of sensitivity required and the availability of instruments.

(a) The use of TLC for the separation and analysis of complex mixtures has grown rapidly in recent years. Mycotoxins include a broad range of compounds and TLC is ideal for handling a great variety of compounds. With careful choice of mobile phases and the use of two-dimensional TLC, separation of many compounds is made possible. TLC is simple and non-destructive so that compounds may be recovered for further analysis. Silica gel has been used almost exclusively in the analysis of mycotoxins (Nesheim and Trucksess, 1986).

In TLC, the extract is spotted on a glass or aluminium plate coated with silica gel (thickness ranging from 0.2-0.5 mm), the plate is then developed in a chosen solvent system. A number of solvent systems have been reported for the development of zearalenone and the trichothecenes on TLC plates as shown in Table 1.15.

After development, the toxins separated on the plate

Table 1.15: Rf values of the mycotoxins in different solvent systems

MYCOTOXIN	SOLVENT SYSTEM											
	A	B	C	D	E	F	G	H	I	J	K	L
Zearalenone	0.90	0.44	0.41	0.50	0.90	0.78	0.88	0.80	0.50	0.54		0.62
T-2 toxin	0.67	0.22	0.20	0.45	0.29	0.68	0.92	0.60	0.15	0.27	0.56	0.42
DAS	0.67	0.20	0.20	0.45	0.29	0.66	0.90	0.60	0.15	0.23	0.53	0.36
DON	0.35					0.47	0.56	0.40		0.22	0.05	0.15
Nivalenol	0.03	0.12	0.00	0.00	0.00	0.20	0.30	0.06		0.05		

Solvent systems,

A: diethyl ether - methanol - water (94 + 4.5 + 1.5); B: ethyl acetate - n-hexane (1 + 1);
C: benzene - chloroform - acetone (45 + 40 + 15); D: chloroform - acetone - n-hexane (7 + 2 + 1);
E: Chloroform - acetone (9 + 1); F: chloroform - isopropanol - acetone (85 + 20 + 15);
G: Chloroform - methancl (4 + 1); H: chloroform - isopropanol - acetone (8 + 1 + 1);
I: diethyl ether - cyclohexane (75 + 25); J: toluene - ethyl acetate - 90% formic acid (6 + 3 + 1); K: chloroform - methanol (95 + 5); L: ethyl acetate - toluene (3 + 1)

The mycotoxins were developed in lined equilibrated tanks on silica gel pre-coated plates (E. Merck).

are made visible and quantitated. This is usually based on the measurement of fluorescence, colour or the ultraviolet (u.v.) absorption of the toxin or its derivative. Fluorescence determination is preferred in that it is more specific and sensitive. The fluorescence properties of aflatoxins under long wavength u.v. light have provided the basis for extremely sensitive analytical methods. Zearalenone fluoresces blue/green under long and short wavelength u.v. light (365 and 254 nm) (Eppley, 1968) but due to their lack of extended conjugation, except for verrucarins and roridins, trichothecenes exhibit no fluorescent properties, nor do they absorb appreciably in the ultraviolet region.

Treatment of the TLC plate with some chemicals has however been shown to make some of the trichothecenes visible or to fluoresce under u.v. light. Spraying the TLC plate with sulphuric acid and heating makes group A trichothecenes (e.g. T-2 toxin and DAS) fluorescent under u.v. light (365 nm) (Scott, 1980). Group B trichothecenes can be made to fluoresce under u.v. light (365 nm) by spraying and heating the plate with aluminium chloride solution. Naoi et al. (1974) reported fluorodensitometric determination of Fusarenon-X by spraying the plate with 50 per cent aluminium chloride solution and heating at 130°C for 10 minutes. Fusarenon-X gave a blue fluorescent spot under u.v. light (360 nm).

Kamimura et al. (1981) reported a method in which both group A and B trichothecenes could be visualised. Group B toxins are first detected by spraying with 20 per cent aluminium chloride and heating at 110°C for 10 minutes, then group A toxins are detected by spraying the same plate with 20 per cent sulphuric acid and heating for 10 minutes at 110°C. Observation of the toxins was made under u.v. light (365 nm) in both cases.

A method which detects all trichothecenes which have a characteristic 12, 13-epoxy group was described by Takitani et al. (1979). The plate was sprayed with 3 per cent 4-(p-nitrobenzyl) pyridine, dried and heated for 30 minutes at 150°C, then sprayed with tetraethylene pentamine. The blue/violet colour was then measured densitometrically. Sano et al. (1982) also described a method in which all 12, 13-epoxy-substituted trichothecenes can be converted to a fluorescent derivative by treatment of the TLC plate with nicotinamide and 2-acetylpyridine. The detection limits for the five trichothecenes examined were in the range of 20-50 ng per spot by visual inspection under u.v. light (360 nm).

Methods using p-anisaldehyde in acidic methanol as chromogenic reagent have been reported in the determination of zearalenone and the trichothecenes (Scott et al., 1970; Durackova et al., 1976). The plate is sprayed with p-anisaldehyde solution and the colours developed by heating

Table 1.16 Detection Limits of different mycotoxins using different sprays

Spray	Toxin	detection limit (ng)	Reference
Aluminium chloride, heat (u.v)	DON	20	Eppley et al. (1984
P-anisaldehyde	T-2 toxin	250	Scott (1980)
	DAS	"	
Fast violet B salt	Zearalenone	5	Scott et al. (1978)
4-(p-nitrobenzyl) pyridine	12,13 epoxy-trichothecenes	20-200	Takitani et al. (1979)
nicotinamide/ 2-acetylpyridine (u.v.)	"	20-50	Sano et al. (1982)
Bis-diazotised benzidine	Zearalenone	2	Malaiyandi et al. (1976)
u.v. (254 nm)	"	20	Scott et al. (1980)
Ferric chloride/ potassium ferricyanide	"	100	Mirocha et al. (1974)
H_2SO_4, heat (u.v.)	T-2 toxin/DAS	50	Scott et al. (1980)

The Flame Ionisation Detector (FID) and the Electron Capture Detector (ECD) are the main detectors used in the determination of mycotoxins. Trimethyl silyl (TMS) ether derivatives of the toxins have widely been used in the determination of toxins using FID, for example, Kamimura et al. (1981) used a mixture of N-trimethylsilylimidazole, trimethylchlorosilane and ethyl acetate. The minimum detectable concentrations in test materials were; 2 µg/kg for the group B trichothecenes, 80 µg/kg for the group A trichothecenes and 10 µg/kg for zearalenone. Sensitivity was improved with the use of ECD. A good review on the optimum conditions for trimethysilylation of mycotoxins, and the dangers posed by incomplete reaction between reagents and toxins in quantitation work with FID is given by Gilbert et al 1985. N-Methylbis (trifluoroacetamide) (TFA) esters of group A trichothecenes have also been used for quantitation using FID (Gareis et al; 1985). Dimethoxy and methyl oxime derivatives have been used in GC analysis of zearalenone (Mirocha et al., 1974), which are readily detected by FID.

ECD has been reported to be more sensitive than FID in the determination of TMS ethers of both type A and B trichothecenes. This is even more apparent in the determination of type B trichothecenes due to their conjugate carbonyl group (Scott, 1982). Tanaka et al 1985 proposed

a method in which DON and nivalenol in cereals could be determined as TMS ethers. Derivatisation was done by use of N-trimethylsilylimidazo-trimethylchlorosilane-ethyl acetate. The detection limit reported was 2 µg/kg. Heptafluorobutyrate (HFB) derivatives of trichothecenes however, have been reported to give very good sensitivity when analysed by ECD. A number of methods have been described; Romer et al.(1978)reported detection limits of 100 ng T-2 toxin and 25 ng DAS per gram of feedstuff, Scott et al. (1981)reported detection limits of 10 ng DON/gram of wheat. It is recommended that for GC analysis of the trichothecenes, ECD be used whenever available. ECD has also been used in the detection of zearalenone as in Holder et al. (1977), where zearalenone was detected as a pentafluoropropionyl derivative.

(c) The combination of GC and mass spectrometry (MS) has been used in the detection and confirmation of mycotoxins. However, the use of such methods is limited due to the absence of a suitable MS in many laboratories i.e. a MS capable of monitoring at high m/z values (Scott, 1982). Mirocha et al. (1976) described a method in which TMS ethers of several trichothecenes and zearalenone were determined using computer controlled GC-MS operating in the selected ion monitoring (SIM) mode. For each ion analysed, intensities of a set of nine characteristic ions were monitored as

each component of the sample eluted from the column. Tanaka et al. (1985) reported an improved methodology for the simultaneous detection of DON and nivalenol using GC-MS (SIM) GC-MS operating in the multiple ion monitoring mode has also been used to detect DON (Trenholm et al., 1981). Scott et al (1981) reported improved sensitivity over GC-ECD when GC-MS (SIM) was used. Some of the samples analysed contained false positives of DAS when analysed by GC alone but were clearly negative by GC-MS (SIM). The HFB derivatives of the trichothecenes were shown by the same authors to be readily detected by GC-MS (SIM). The use of GC-MS is of great value as a quantitative confirmatory technique especially in new method development on the trichothecenes.

(d) Liquid chromatographic (LC) determination of the trichothecenes is difficult because of the absence of u.v. absorption by most of them. However, DON has a u.v. absorption maximum at 225 nm and Chang et al. (1984) reported a rapid method by LC using a sensitive u.v. detector which used a Cadmium lamp with a u.v. filter at 229 nm. The results were shown to be comparable to those obtained by Scott et al. (1981) using GC-ECD. Zearalenone on the other hand has u.v absorptions and can therefore be determined by LC methods using either u.v. or fluorescence detection (Cohen and Lapointe, 1980; Ware and Thorpe, 1978). Bennett et al. (1985) reported an LC method for the determination of zearalenone and α-zearalenol in maize. High-performance liquid chromato-

graphy (HPLC) with either u.v. or fluorescence detection was proposed and fluorescence detection was found to give improved sensitivity and selectivity. Both toxins could be detected successfully at 50 µg/kg.

(e) Immunochemical assay methods have also been described for the analysis of some trichothecenes. Radioimmunoassay (RIA) was first reported for the determination of T-2 toxin by Chu et al. (1979), the detection limits being 3 ng per assay. This method has been applied for the determination of T-2 toxin in cereal grains and biological fluids (Lee and Chu, 1981a and 1981b). The toxin was first converted to the T-2-hemisuccinate by reacting the toxin with succinic anhydride. This was then coupled with bovine serum albumin (BSA) to form the T-2-HS-BSA conjugate. Antisera specific for T-2 toxin were then prepared by injecting rabbits with the T-2-HS-BSA immunogen and the resulting hyperimmune serum purified with ammonium sulphate for RIA.

Pestka et al. (1981) proposed a sensitive enzyme-linked immunosorbent assay (ELISA) for T-2 toxin. This type of assay has the advantage of not requiring radio-isotopes or expensive scintillation counters, so may be of use in smaller laboratories.

Reports in the literature on the incidence of Fusarium toxins have been scarce mainly because of lack of suitable methods of analysis. This is especially true

of countries in the tropical regions, including Zambia, although spoilage of cereals by Fusarium on a wide scale here has been reported (MacDonald and Raemaekers, 1974, NCSR, 1976). But as to whether Fusarium toxicoses is a serious problem is yet to be established. Methods of routine analysis chosen in countries in Sub-Sahara Africa must be inexpensive, involve instrumentation which can be easily maintained and involve a minimum of solvents, which are difficult to transport. It appears that TLC and ELISA, and in some cases GLC will be the methods of most use for food surveys in countries such as Zambia.

1.8 PURPOSE OF THE PRESENT STUDY

Fusarium contamination of maize has been shown to be a problem in Zambia especially in years of high rainfall. It was therefore decided to test for the presence of Fusarium toxins in maize and maize products meant for human and animal consumption. Five toxins, zearalenone, deoxynivalenol, nivalenol, T-2 toxin, and diacetoxyscirpenol were chosen.

The first part of the study will involve the development of suitable methods for use in the analysis of these toxins. A few methods have been used in Zambia for analysis of aflatoxins and one method for zearalenone. This is the first major study in Zambia involving analysis of trichothecenes. So

it was necessary to test a variety of methods to look at their suitability for use in survey work in Zambia.

In previous years, mouldy maize was bought from farmers by NAMBOARD who processed the maize to recover the good grain. But this is no longer the case. Farmers who accumulate a lot of mouldy maize are forced to use the maize themselves or sell locally. Most of this maize ends up either as animal feed supplement or is used in the brewing of local opaque beer. In this study, samples of mould maize from farmers will be analysed for Fusarium toxins to see whether they pose a danger to human and animal health. Samples of mouldy maize will be collected from small scale farmers in areas on the outskirts of Lusaka.

To determine the extent of the contamination of these toxins on a wider scale, it was decided to take a complete year long survey of samples from National Milling company. Maize from different farms is mixed and used for animal feed preparation, which is bought by farmers all around Lusaka. Any contamination may enter animal products and this may be linked with occassional outbreaks of disease in chickens and pigs for which the cause has not been identified.

Since Fusarium graminearum is one of the most common causes of cob rot in Zambia, an isolate of F. graminearum will be tested for toxin production on maize at temperatures commonly encountered near the end of the rainy season in Apr

Maximum temperatures in April average 26°C while minimum temperatures in April average 16°C. The effect of the two temperatures on toxin production will be studied.

CHAPTER TWO

EXPERIMENTAL

2.1 SAMPLE COLLECTION

2.1.1 FIELD SAMPLES

Maize samples were collected between May and July 1986, which covered the end of the rainy season and the beginning of harvesting. Samples were collected from three different areas within a radius of 100 Km of Lusaka. The areas covered were, (1) Chipapa area, about 45 Km south of Lusaka, (2) Chief Mungule's area, about 65 Km north west of Lusaka, and (3) Chinkuli area,about 50 Km north east of Lusaka. In each of these areas, samples were collected from farmers within a radius of 20 Km. An average of eleven samples were collected from each area representing about eleven farmers. Most of the farmers in question were small scale farmers, see section 1.2.

Most of the maize samples collected had just been harvested and were being dried further in the sun. In a few cases, the maize was being harvested on the actual day the farm was visited so that the maize collected was straight from the field. The maize collected was visibly mouldy (mostly pink or brown cob rot) and is harvested mainly for use as animal feed and in the brewing of beer. About 2 Kg of the shelled maize was collected and the samples were stored frozen ($-20^{o}C$) in a deep freezer until they were analysed. The date of harvest and the type of storage to be used were noted. Maize meant for human consumption on these farms is stored in cribs

with grass thatched walls and roof. July usually marks the end of the harvesting season for most farmers in the areas visited.

The farmer's surplus maize is sold to NAMBOARD or the Provincial Cooperative Marketing Unions. NAMBOARD purchases most of the maize marketed in the country (see section 1.2) and storage is mainly in three ways: (1) the shelled grain is put in bags which are then stacked together on concrete slabs and the bags covered with tarpaulin, (2) loose grain is put in silo-type bins made of steel or aluminium, and (3) the loose grain is put in grain elevators made of concrete.

The samples were analysed for zearalenone, T-2 toxin, DAS, DON and nivalenol.

2.1.2 MIXED FEED SAMPLES

Animal feed in Lusaka is ground and mixed by the National Milling Company, Marambo Road Branch. Maize and its by-products (maize bran and maize meal No. 3) and wheat bran (wheatings) account for over 50 per cent of the raw materials used in the production of the five mixed feed samples analysed in our laboratory (see Table 2.1). The maize is bought from NAMBOARD and only grades A,B and C are used in animal feed production. It was therefore decided to analyse these raw materials in addition to the mixed feeds (pig meal, layers mash, broiler starter mash, broiler finisher mash and growers mash) to determine the possible source of any

Table 2.1 The major raw materials used in the production of selected feeds at National Milling Company.

	Kg/1000Kg of feed								
	Lime	Maize Meal No 3	White Maize	Wheatings	Maize bran	Sunflower cake	Cottonseed cake	Lucern	Molasses
Growers mash	26	312	155	233	100	35	-	-	50
Broiler starter mash	18	413	-	170	-	196	30	-	37
Broiler finisher mash	24	280	140	200	-	191	30	-	30
Layers mash	90	266	140	250	-	130	-	15	50
Pig meal	-	300	-	-	400	125	25	120	30

Source: National Milling Company

mycotoxin contamination found in the feed.

Sampling of the raw materials and the feeds was done by use of a probe or trier and only samples from the same production line were taken for each feed sample. A number of bags were sampled by inserting the probe into the bag and withdrawing a few grammes. About 1 Kg of each animal feed sample was collected and stored frozen (-20^{o}C) until it was analysed.

The samples were collected once every fortnight (feeds usually spend a maximum of one week in the warehouse) from November 1985 to November 1986. Variations in toxin concentrations with seasonal changes were to be noted. The samples were analysed for zearalenone, T-2 toxin, DAS, DON and nivale

2.1.3 LABORATORY INFECTED SAMPLES

A few lightly infected maize kernels (depending on the discolouration shown by the kernels) were used in the isolation of F. graminearum.

The kernels were washed in running tap water and then soaked in commercial bleach containing 5 per cent sodium hypochlorite for 10 minutes. The kernels were rinsed in sterile water and soaked in Penicillin solution (60mg/200ml) for 10 minutes (Kapooria et al., 1981).

The treated kernels were transferred to moistened blotters in disposable Petri-dishes and incubated for one week at room temeprature (23-25^{o}C). The pinkish fungal

growth was identified as F. graminearum at Mount Makulu Government Agricultural Research Station, Chilanga, Zambia. It was subcultured onto a Petri-dish containing potato dextrose agar (PDA) in a zig zag streak using asceptic technique and incubated at 25°C for one week. The fungal growth was scraped off the surface of the plate and suspended in 10 ml sterile distilled water for inoculation.

A sample of healthy maize grains (containing no detectable amounts of zearalenone, T-2 toxin, DAS, DON and nivalenol) was ground using a coffee grinder (Moulinex Type 241.2.00, France). 300 g of the ground maize was transferred to 1 litre glass flasks and autoclaved at 120°C for one hour. The moisture content of the maize was then adjusted to 37 per cent (wet-weight basis) with sterile distilled water.

Using a sterile pipette 10 ml of the F. graminearum suspension was added to each flask under ultraviolet light using asceptic technique. The flasks were then incubated in duplicates at 16°C and 26°C for 10 weeks as shown in Table 2.2.

Samples (50g) of the mould infected maize were withdrawn from each flask every 2 weeks using a spatula and asceptic technique and analysed for zearalenone, T-2 toxin, DAS, DON and nivalenol. The sample was taken in a different position in the flask each time, including top and lower layers.

2.2 SAMPLE PREPARATION

In the case of field samples, where maize kernels were collected, the whole amount (about 2 Kg) was finely ground

Table 2.2 Conditions under which *Fusarium graminearum* was grown.

Flask No.	Incubation period (weeks) 26°C	16°C
1	10	
2	5*	5
3		10
4	5+	5

* 5 weeks at 26°C followed by 5 weeks at 16°C

+ 5 weeks at 16°C followed by 5 weeks at 26°C

using a coffee grinder (Moulinex, Type 241.2.00, France). The grinding process thoroughly mixed the sample as well. The amount used for toxin analysis was then removed.

The samples collected from National Milling Company had been previously ground and blended whilst manufacturing the animal feeds. The sample used in toxin analysis was removed after stirring the sample with a glass rod. The maize kernel samples were treated in the same way as the field samples above.

The laboratory inoculated samples were ground in a coffee grinder (this was mainly for the purpose of obtaining a uniformly mixed sample). The 50 g sample was then divided into two portions (25 g each) for toxin analysis.

2.3 EXTRACTION AND DETERMINATION METHODS

Several methods of extraction and determination of the five toxins were investigated in order to arrive at the most suitable methods for use in this project. A number of factors were considered, the main ones being (1) the amount of interference in the final extract which could cause problems with visualisation of the toxins when the TLC plate has been developed, (2) the efficiency i.e. the percentage recovery of added zearalenone or trichothecene, (3) the amount and complexity of chemicals used. When one-dimensional TLC did not give good resolution of the toxin spot from the background interference, two-dimensional TLC was tried. Recoveries equivalent to 70 per cent or greater were considered

satisfactory. Furthermore, if five or more samples could be extracted by a particular method in a day, this was taken to be reasonable (excluding the time needed for TLC).

The various methods tried out in our Laboratory are briefly discussed below while the method finally adopted is given in more detail at the end of each section.

2.3.1 ZEARALENONE DETERMINATION

(a) The method of Mirocha et al. (1974) with some modification by MacDonald (1975) as given by Lovelace et al. (1977), was tried for the extraction and estimation of zearalenone. 20 g sample was extracted with ethyl acetate for 9 h using a Soxhlet apparatus. The ethyl acetate was evaporated off and the residue dissolved in chloroform. The extract was cleaned by extracting zearalenone into 1M sodium hydroxide the pH adjusted to 9.5 with 2M H_3PO_4 and zearalenone re-extracted into chloroform for TLC analysis. Silica gel G plates (0.5 mm thick) were used with either (1) chloroform-ethanol (97+3, v:v) or (2) 12.5 per cent iso-propanol in n-hexane, as developing solvents. Zearalenone appeared as a blue/green fluorescent spot (Rf ≈ 0.5 in either solvent) under short and long wavelength (254 and 365 nm). Zearalenone was also made visible by spraying the plate with 50 per cent sulphuric acid in methanol and heating at 120°C for 10-20 minutes, zearalenone appeared as a yellow to brown spot.

A mixed feed sample (Pig Meal) to which zearalenone at

2 mg/kg had been added, was extracted according to the above method. The final extract was dissolved in 200 µl of chloroform, 10 µl was spotted on a TLC plate along with zearalenone standards and developed in either of the above solvent systems. When the plate was observed under u.v. light (254 and 365 nm) interference occurred, yellow fluorescence occurring at the same R_f as zearalenone, which made it difficult to determine the presence of the toxin. Two dimensional TLC was carried out with either of the above solvent systems as the first solvent system and diethyl ether - cyclohexane (75 + 25, v:v) as the second solvent system. There still remained one large yellow spot spread over a large area including the zearalenone position, making it rather difficult to determine the recovery of the method. The spot remained visible even after spraying with 50 per cent sulphuric acid but turned gray upon heating at 120°C. Because of the interference, this method could not be used for the extraction of zearalenone from mixed feeds. The duration of the extraction (9 h) was another factor which made the method unsuitable.

(b) Roberts and Patterson (1975) and Patterson and Roberts (1979), reported a multi-toxin screening method for the detection of 12 mycotoxins including zearalenone. 25 g sample was extracted with acetonitrile - 4 per cent potassium chloride (90 + 10, v:v) on a wrist-action shaker for 30 minutes. The extract was defatted

with iso-octane, distilled water added and the toxins extracted into chloroform. The chloroform layer was transfered to an 8/32 Visking dialysis tubing and equilibrated against 30 per cent aqueous acetone for 16 h to extract the neutral toxins. (The acidic toxins were extracted by acidifying the aqueous - acetonitrile layer followed by partitioning with chloroform). The aqueous acetone was partitioned with chloroform and the chloroform analysed by TLC. The solvent systems used were (1) diethyl ether-cyclohexane (75 + 25, v:v) and (2) acetone-chloroform (10 + 90, v:v). The TLC plate was sprayed with 20 per cent aluminium chloride in 95 per cent ethanol and observed under long wavelength (365 nm).

This method was used for the extraction of zearalenone from a mixed feed sample to which zearalenone had been added (2 mg/kg). The final volume of the extract was 200 µl and 10 µl of the extract spotted on a TLC plate. The plate was developed in either of the above solvent systems. Diethyl ether-cyclohexane gave better resolution of the toxin from interfering fluorescent spots. The two extracts (one containing acidic toxins and the other containing neutral toxins) were analysed for zearalenone and both were found to contain the toxin instead of the one containing neutral toxins only. An attempt was made to extract all the zearalenone from the aqueous acetonitrile into chloroform by doubling the amount of chloroform

used in the partition step. This did not remove all the toxin as some of it could still be observed in the acidic extract. The other difficulty was that the method included dialysis for 16 h, a problem when dealing with many samples and therefore, the method could not be used.

(c) The multi-toxin screening method of Van Egmond et al. (1979) was also tested. In this method, a 25 g sample was extracted with a 150 ml mixture of acetonitrile - 0.1M H_3PO (125 + 12.5, v:v) on a wrist-action shaker for 30 minutes. Acetonitrile was added to the extract followed by distilled water (containing 1.25 per cent sodium bicarbonate and 3 per cent sodium chloride). The mixture was defatted with iso-octane and the iso-octane layer discarded to leave two layers. The acetonitrile layer was acidified with 2M sulphuric acid and the neutral toxins taken up in chloroform. The aqueous layer was also acidified with 2M sulphuric acid and the acidic toxins taken up in chloroform. The two extracts were analysed by TLC using solvent systems (1) chloroform-acetone (95 + 5, v:v) and (2) hexane-diethyl ether-acetic acid (75 + 25 + 15, v:v:v). The plate was observed under long wavelength (365 nm).

The clean-up procedure for this method was not successful as a lot of interference remained when an extract from a mixed feed spiked at 2 mg/kg level was tested. An intense yellow/brown spot with the same Rf value as zearalenone was observed. This spot covered quite a large area making obser-

vation for zearalenone extremely difficult. On spraying the plate with 50 per cent sulphuric acid in methanol, no improvement was observed. The same problem was encountered when the extract was run in two dimensional TLC, so it was felt that this method was not suitable.

(d) The method of Kamimura et al.(1981), used in the extraction of ten Fusarium toxins, was also tested. In this method a 100 g sample was extracted with 200 ml methanol-water (95 + 5, v:v) on a wrist-action shaker for 30 minutes. The extract was cleaned by an amberlite XAD-4 column with zearalenone and the trichothecenes were eluted with methanol. This was further cleaned by passing through a florisil column. The extract was then analysed by TLC using chloroform-methanol (93 + 7, v:v) as the solvent system. The plate was observed before and after spraying with 20 per cent aluminium chloride solution in 95 per cent ethanol. It was then heated at 110°C for 10 minutes and again observed under long wavelength (365 nm). Zearalenone appeared as a blue fluorescent spot.

This method was assessed to be the best method for this project in that it extracted all the five toxins being studied. However, when the method was tried out using a spiked Pig Meal sample (2 mg/kg), a few problems were encountered. The final extract was oily and made spotting on TLC plates rather difficult. The spot tended to spread out leaving a large spot. The problem was further compounded during the

development of the plate as the spots tended to diffuse further. It was not possible to observe zearalenone when the plate was developed in only one direction due to other fluorescent spots with same R_f.

Two dimensional TLC was carried out with diethyl ether-cyclohexane (75 + 25, v:v) as the second solvent system. Most of the fluorescent spots were removed from the zearalenone position but no zearalenone was detected. Kamimura et al. (1981) reported recoveries of 88 per cent from corn spiked with 2 mg/kg according to this method. This method could not be used on our mixed feed samples due to the amount of oily materials which made spotting difficult and no zearalenone could be detected. It is possible that some zearalenone may have been present in the final extract but with the amount of interference, most fluorescing bright yellow/brown and with the same Rf as zearalenone, it was impossible to observe the zearalenone.

(e) A method based on Bennett et al. (1985) and modified so that TLC could be used in the determination step (Mirocha, personal communication) was finally adopted. 25 g sample was mixed with 12.5 g diatomaceous earth (Celite 545, Koch-Light Laboratories Ltd. UK) and 10 ml distilled water. This was transferred to a 250 ml glass-stoppered extraction flask. 125 ml chloroform was added to the flask, stoppered tightly and shaken on a wrist-action shaker (Griffin flask

shaker REGD. DESN No 896331 & 896332, Griffin and George, UK) for 30 minutes.

The mixture was filtered through a fluted filter paper (Whatman No 1) and 50 ml collected. The filtrate was transferred to a 250 ml separatory funnel and 10 ml saturated sodium chloride solution added and mixed well. To the mixture were added 50 ml 2 per cent sodium hydroxide and shaken vigorously for about 1 minute. The layers were allowed to separate completely. The lower chloroform layer with as much of the sediments as possible was discarded. A second 50 ml portion of chloroform was added to the separatory funnel and the contents shaken for about 1 minute. The chloroform layer was discarded as before. 50 ml of 10.6 per cent citric acid solution were added to the separatory funnel and the contents mixed well. Zearalenone was then taken up in two 50 ml portions of dichloromethane, allowing the two layers to separate completely before drawing out the lower dichloromethane layer.

The dichloromethane layers were dried by passing them through a layer of 10 g anhydrous sodium sulphate and the sodium sulphate was washed with a further 10 ml dichloromethane. The combined extracts were evaporated to near dryness at 40^{o}C water bath temperature on a rotary evaporator (Rotavapor RE120, Buchi Laboratoriums-Technik AG, Switzerland). The residue was transferred to a teflon-lined screw cap vial and

the dichloromethane evaporated to dryness under a gentle stream of nitrogen at 40°C on a heating block (Techne Dri-Block DB-1, Duxford Cambridge,England). The residue was dissolved in 200 µl chloroform for TLC estimation.

Thin Layer Chromatography was carried out on 0.2 mm silica gel 60 plates (No 5553, E. Merck) in lined equilibrated tanks over a distance of 12 cm. 10 µl extract was spotted on the plate together with 1, 2, 4, 6 and 10 µl zearalenone standard (Sigma Chemical Co. Ltd., U.K) solution (50 µg/ml chloroform). The extract and the standards were spotted on TLC plates using mycrosyringes (Hamilton, Bonaduz, Switzerland).

The plate was developed in the solvent system, diethyl ether-cyclohexane (75 + 25, v:v) and observed under long wavelength (366 nm) (29010 CAMAG Duluxe UV lamp, Muttenz/Schweiz) before and after spraying with 20 per cent aluminium chloride solution in 95 per cent ethanol. The fluorescence intensity of the zearalenone spot increased on spraying and appeared as a blue/green fluorescent spot. When the plate was heated at 110°C for 5 minutes, zearalenone appeared as an intense blue spot under long wavelength (366 nm). Appropriate dilutions were made for extracts with a higher concentration than the most concentrated standard spot until the concentration fell within the standard's range.

The solvent system used gave very good separation (Rf = 0.5) and the detection limit under long wavelength (366 nm) was

25 ng per spot. Recoveries averaged 80 per cent at 2 and 4 mg/kg spiking levels.

2.3.2 DEOXYNIVALENOL AND NIVALENOL DETERMINATION

A number of methods were tried out for the extraction and estimation of DON and nivalenol. Two of the methods were specifically for the extraction and estimation of DON, one was a multi-toxin extraction method and the one which was finally adopted was given for the extraction of both DON and nivalenol.

(a) Trucksess et al. (1984) described a TLC method for the extraction of DON in cereals. A 50 g wheat or corn sample was extracted with acetonitrile-water (84 + 16, v:v) and defatted by hexane partition. The extract was cleaned by a charcoal-alumina-celite column and analysed by TLC. Silica gel 60 plates were used and developed in chloroform-acetone-isopropanol (8 + 1 + 1, v:v:v). After spraying with 20 per cent aluminium chloride solution in 95 per cent ethanol, followed by heating at 120°C for 7 minutes, DON on the plate could be quantitated by visual comparison (under u.v. light 365 nm) or by densitometer.

This method was used to extract and estimate DON in a mixed feed sample (Spiked with 2 mg/kg DON). The final product was dissolved in 200 µl chloroform-methanol (3 + 1, v:v) and 10 µl spotted on the plate. This method gave very clean extracts which were free from interfering spots with the

same Rf as DON. When 2-dimensional TLC was used, visualisation was made even easier. The only problem encountered was that of the low recoveries obtained by this method. The recomended 20 ml acetonitrile-water followed by 10 ml of the same solvent mixture for the removal of DON from the adsorbent column was found to be inadequate. This only removed 30-50 per cent of the DON. The volume was increased to use 20 ml followed by 30 ml of the acetonitrile-water. This amount removed about 85 per cent of DON from the column.

The overall recovery of the method was then found to average 60 per cent. This method was tried for the extraction and estimation of nivalenol but recoveries were very low, 30 per cent recoveries obtained at 2 and 4 mg/kg spiked levels. This method was therefore found to be unsuitable for use on mixed feed samples due to the low recoveries.

(b) Mirocha (1981) reported a method for the extraction and estimation of DON in corn. A 50 g sample was extracted with methanol-water (45 + 55, v;v) containing 1 per cent potassium chloride. The extract was defatted by petroleum ether (b.p. 60-70^{o}C) partition. DON was taken up in chloroform which was then cleaned by passage through Sep Pak C_{18} cartridge. The extract was analysed by TLC (GC could also be used). The plate was developed in chloroform-ethanol (90 + 10, v:v) or chloroform-methanol (90 + 10, v:v). Then it was sprayed

with freshly prepared p-anisaldehyde solution, heated at 120°C for 5 minutes and DON appeared as a yellow spot.

This method was used on a mixed feed sample which was spiked with DON at 2 mg/kg level. The method gave relatively clean extracts. Spraying with p-anisaldehyde solution tended to give a number of yellow spots with the same Rf as DON making quantitation difficult. At the same time p-anisaldehyde required rather high detection limits (about 100 ng per spot). This was improved by replacing the p-anisaldehyde with 20 per cent aluminium chloride solution and heating at 120°C for 7 minutes.

Recoveries for this method averaged 62 per cent for DON. However, it was discarded because the method finally chosen gave higher recoveries and could extract both DON and nivalenol.

(c) The method of Kamimura et al. (1981) described above (see 2.3.1 (d)) was used on a DON and nivalenol spiked sample. The mixed feed sample was spiked with either 1 or 2 mg/kg DON and nivalenol. Again the oily nature of the extract made spotting rather difficult just as in the case of zearalenone. Developing the plate in one solvent system chloroform-methanol (93 + 7, v:v) did not give clear results. A second solvent system chloroform-acetone-isopropanol (8 + 1 + 1, V:v:v) was used in 2-dimensional TLC, and this removed most of the interference from the

DON and nivalenol position making quantitation easier.

Recoveries for DON and nivalenol were found to average 30 per cent and 20 per cent respectively. Due to the amount of interference and the low recoveries obtained, this method could not be used on our samples for the extraction and estimation of DON and nivalenol.

(d) The method given by Health Protection Branch (1985) was finally adopted (see also Scott et al., 1983 (Method 2) and Scott et al., 1986). A few modifications were included so that TLC could be used in the determination step.

To 25 g sample was added 100 ml methanol-water (1 + 1, v:v) and the mixture blended for 3 minutes at full speed using a Waring blender (Waring Commercial blender, Waring Products Division, New Hartford Connecticut 06057). The sample was filtered under suction through Whatman No 1 filter paper. 30 ml of the filtrate was transferred to a 250 ml beaker and 120 ml aqueous ammonium sulphate (10%) added. This was followed by addition of 5 g of diatomaceous earth (Celite 545, Koch-Light Laboratories Ltd. UK) and the mixture stirred for about 2 minutes on a magnetic stirrer (Gallenkamp and Co Ltd, UK).

The mixture was filtered through Whatman No 1 fluted filter paper. 10ml of the filtrate were transferred to a Chem Tube column containing a hydrophilic matrix (Chem Tube

Hydromatrix Lot. No CE6A263-1, Analytichem International Harbor City CA 90710) and left to stand for 3 minutes for the aqueous solution to be absorbed onto the matrix. The column was eluted with 8 x 20 ml portions of ethyl acetate, allowing each portion to drain to the top of the matrix after each addition. The eluates were collected in a 300 ml pear shaped flask and the ethyl acetate evaporated off on a rotary evaporator (Rotavapor RE120) with water bath temperature of 40°C. The residue was transferred to a 4 ml vial with 3 x 1 ml portions of dichloromethane-methanol (3 + 1, v:v). the solvents were evaporated off under nitrogen on a heating block (Techne Dri-Block DB-1) at 40°C.

A chromatographic column (1.2 x 30 cm) was prepared by plugging the bottom with glass wool and 1 g of anhydrous sodium sulphate added. The column was half filled with toluene and 2 g silica gel 60 (Art. No 7754, E. Merck) as a slurry in toluene added. Anhydrous sodium sulphate (1 g) was added on top of the silica gel and toluene drained to about 1 cm above the upper sodium sulphate layer.

The sample extract was dissolved in 0.5 ml dichloromethane-methanol (3 + 1, v:v) by mixing on a vortex mixer (Spinmix, Gallenkamp and Co. Ltd, UK). This was transferred to the top of the column with a pasteur pipette the vial was rinsed with a further 0.5 ml dichloromethane-methanol (3 + 1, v:v) which was also transferred to the column. The solvents

were allowed to drain to the top of the upper sodium sulphate layer. The column was washed with 15 ml of toluene which was followed by 15 ml n-hexane. DON and nivalenol were eluted with 60 ml dichloromethane-methanol (9 + 1, v:v) into a 250 ml round bottomed flask. The solvents were evaporated off to near dryness on a rotary evaporator with a water bath temperature of 40°C. The residue was transferred to a 4 ml vial, the flask was rinsed with 3 x 1 ml portions of dichlomethane-methanol (3 + 1, v:v) which were also transferred to the vial. The solvents were evaporated to dryness under a gentle stream of nitrogen on a heating block at 40°C (Techne Dri-Block DB-1). The residue was dissolved in 200 µl of dichloromethane-methanol (3 + 1, v:v) for TLC.

Thin Layer Chromatography was carried out on 0.2 mm silica gel 60 (No 5553, E. Merck) plates in lined equilibrated tanks. 10 µl of the extract was applied to the plate alongside 1, 5 and 10 µl of standard DON (Sigma Chemical Co. Ltd., U.K) and nivalenol (Wako Chemicals, W. Germany) (DON and nivalenol standard concentrations were 20 and 23 µg/ml in chloroform-methanol (3 + 1, v:v) respectively). The plate was developed in chloroform-acetone-isopropanol (8 + 1 + 1, v:v:v) for approximately one hour. The solvents were allowed to evaporate from the plate at room temperature in the dark after which the plate was sprayed with an even layer of 20 per cent aluminium chloride. The plate was examined briefly under long wavelength (366 nm) (29010 CAMAG

Duluxe u.v. lamp) for possible blue fluorescing interference. The plate was then heated for 7 minutes in an upright position at 120°C in an oven. After cooling, the plate was observed under long wavelength (366 nm). Both DON and nivalenol appeared as blue fluorescent spots (DON Rf = 0.4 and nivalenol Rf = 0.06).

For better quantitation, two-dimensional TLC was used with diethyl ether-methanol-water (94 + 4.5 + 1.5, v:v:v) as the second solvent system (DON Rf = 0.25 and nivalenol Rf = 0.03). Quantitation was by comparison with standard spots. Recoveries according to the above method from a mixed feed sample (Pig meal) to which either 2 mg/kg or 4 mg/kg of DON and nivalenol had been added averaged 80 and 86 per cent respectively.

2.3.3 T-2 TOXIN AND DIACETOXYSCIRPENOL DETERMINATION

In the extraction and determination of T-2 toxin and DAS it was not possible to get a method with recoveries of > 70 per cent. The method which was finally adopted gave rather low recoveries for both T-2 toxin and DAS of 50 and 60 per cent respectively. No percentage recoveries could be obtained for the other methods which were tried for T-2 toxin and DAS due to a number of problems encountered. Two dimensional TLC was used in all determinations.

(a) An attempt was made to extract T-2 toxin and DAS from a mixed feed sample to which these two toxins had been

added at 4 mg/kg level using the method of Roberts and Patterson (1975) and Patterson and Roberts (1979) as described in 2.3.1 (b). The extract was analysed by TLC for T-2 toxin and DAS. Two dimensional TLC was used with the following solvent systems (1) toluene-ethyl acetate - 90 per cent formic acid (60 + 30 + 10, v:v:v) and (2) chloroform-isopropanol-ethyl acetate (95 + 5 + 5, v:v:v). The plate was sprayed with 20 per cent sulphuric acid in 95 per cent ethanol, heated at 105°C until standard spots began to char (5-10 minutes).

The plate was cooled and observed under long wavelength (366 nm). T-2 toxin appeared as a blue/green fluorescent spot and DAS as a yellow/brown fluorescent spot. No T-2 toxin or DAS were observed in the extract although a number of spots with Rf values very close to those of T-2 and DAS were observed. These could not be considered as T-2 toxin or DAS because of the difference in the colours they gave under long wavelength. Most of them had a yellow/green colour before and after spraying with sulphuric acid which later turned to brown on heating at 105°C. Apart from the fact that this method could not extract detectable T-2/DAS, the duration of the clean-up step was another factor which made the method unsuitable just as for zearalenone.

(b) The multi-toxin detection method of Kamimura et al (1981) (see 2.3.1 (d)) was another method tried out on T-2

toxin and DAS Spiked samples. DAS and T-2 toxin were added to a mixed feed sample at 2 mg/kg level (as given by Kamimura et al., 1981) as well as at 4 mg/kg level.

Just as in the case of Zearalenone, DON and nivalenol extraction, the major problem was to do with the oily nature of the final product which made spotting difficult. One dimensional TLC of the extract gave a lot of interfering spots even before spraying with 20 per cent sulphuric acid, and after spraying, toxin spots could not be distinguished. Two - dimensional TLC was attempted, which produced improved separation but no T-2 toxin or DAS were observed in the extracts. This method could therefore not be used due to its non-extraction of detectable amounts of T-2 toxin and DAS

(c) The method of Van Egmond et al. (1979) though not specifically for T-2 toxin or DAS, was tried out to see whether it could be used in the extraction of T-2 toxin and DAS from spiked samples (4 mg/kg). The extracts were developed in two directions in the solvent systems given in 2.3.3 (b). The plate was sprayed with 20 per cent sulphuric acid and heated at 105°C for 5-10 minutes. When the plate was observed under long wavelength (366 nm) no T-2 toxin nor DAS were observed. Both Extract (1) and Extract (2) were analysed for the two toxins but with no success. The method was originally given for the extraction of zearalenone, aflatoxin B_1, ochratoxin A, patulin, sterigmatocystin, penicillic acid, citrinin and α-cyclopiazonic acid in feed stuffs.

(d) The method of Romer et al.(1978) was used with modification as given by Gareis et al (1985). TLC was used for quantitation.

A 50 g sample was mixed with 25 g diatomaceous earth (Celite 545, Koch-Light Laboratories Ltd, UK) in a 500 ml conical flask. 200 ml methanol-water (1 + 1, v:v) was added, the flask tightly stoppered and shaken for 30 minutes on a wrist-action shaker (Griffin flask shaker, Griffin and George, UK). The mixture was filtered under suction through a Whatman No 1 filter paper. 60 ml of the filtrate were transferred to a 500 ml beaker, 100 ml diatomaceous earth were added, followed by 240 ml 30 per cent ammonium sulphate. These were mixed for about 2 minutes on a magnetic stirrer (Gallenkamp and Co. Ltd). The mixture was filtered through Whatman No 2 fluted filter paper. 200 ml of the filtrate were transferred to a 250 ml separatory funnel. 10 ml chloroform were added and the mixture shaken vigorously for about 1 minute. The layers were allowed to separate and the lower chloroform layers passed through anhydrous sodium sulphate (5 g) to dry the chloroform. A second 10 ml portion of chloroform was added to the separatory funnel and the contents shaken as before and the chloroform layer collected. The combined chloroform was evaporated to about 10 ml and 30 ml n-hexane added.

A chromatographic column was prepared as in 2.3.2 (d) using chloroform in place of toluene.

The sample extract was added to the column and the solvents allowed to drain until the solvents were just above the upper sodium sulphate layer. The column was washed with 30 ml toluene followed by 40 ml Toluene - acetone (38.5 + 1.5, v:v). T-2 toxin and DAS were eluted with 30 ml diethylether (anhydrous). The diethylether was evaporated off to dryness on a rotary evaporator at water bath temperature of 40°C. The residue was transferred to 4 ml vial with 3 x 1 ml portions of chloroform. The chloroform was evaporated off under a gentle stream of nitrogen on a heating block (Techne Dri-Block DB-1) at 40°C. The residue was dissolved in 100 µl of chloroform.

Silica gel 60 TLC plates (No 5553, E. Merck) were cut to obtain 10 x 10 cm plates and two-dimensional TLC was used throughout. 10 µl of the extract were spotted along with with 5 and 10 µl of T-2 and DAS standards (Sigma Chemical Co. Ltd., U.K), 25 µg/ml in chloroform. The plate was developed in solvent systems (1) toluene-ethyl acetate - 90 per cent formic acid (60 + 30 + 10, v:v:v) and (2) chloroform-methanol (95 + 5, v:v) in lined equilibrated tanks. The plate was dried in the dark at room temperature (about 25°C) and sprayed with 20 per cent sulphuric acid in 95 per cent ethanol. The plate was heated for 5 minutes at 110°C in an oven, cooled in the dark and observed under short and long wavelength u.v. light (254 and 366 nm). T-2 toxin appeared as a blue/green fluorescent

spot and DAS as a yellow/brown fluorescent spot under long wavelength. The fluorescence intensity of the T-2 toxin spot increased when observed the following day.

The Rf values for T-2 toxin and DAS in solvent system (1) were 0.27 and 0.23 respectively while in solvent system (2) they were 0.56 and 0.53 respectively. Quantitation was by comparison with the standards. Recoveries from mixed feed samples spiked with 2 mg/kg of T-2 toxin and DAS averaged 50 and 60 per cent respectively.

2.4 CONFIRMATION OF POSITIVE SAMPLES

Gas chromatography equipped with a flame ionisation detector was used to confirm the presence of the toxins in all the positive extracts. The method of Kamimura et al. (1981) was used for both zearalenone and DON.

Apparatus:

(a) Gas chromatography: (Hitachi model 263-50, Japan) equipped with flame ionisation detector.

Operating conditions:

(i) For zearalenone determination,

Carrier gas (N_2)	30 ml/min,
Hydrogen (H_2)	40 ml/min,
Air	400 ml/min,
Range	10
Attenuation	128
Column temperature	250°C

Injector and	
Detector temperature	280°C
Chart speed	5 mm/min

(ii) For DON determination, same as that for zearalenone determination except,

Carrier gas	60 ml/min
Chart speed	20 mm/min

(b) Recorder (Hitachi model 561, Japan)

(c) Chromatographic column:

2 m x 5 mm i.d., packed with

2 per cent OV - 17 on 80 - 100 mesh Uniport.

Reagents:

(a) Derivatising reagent:

N-trimethylsilylimidazole (Sigma Chemical Co. Ltd, UK) -Trimethylchlorosilane (Sigma Chemical Co. Ltd, UK) - Ethyl acetate (Analytical grade, BDH Chemicals Ltd, U.K), (1 + 0.2 + 9, v:v:v). Freshly prepared each day.

(b) Mycotoxin reference standards:

(i) Zearalenone, 50 µg/ml in chloroform

(ii) DON, 20 µg/ml in chloroform.

Method:

The extract remaining after TLC determination was evaporated to dryness at 40°C on a heating block under a

gentle stream of nitrogen. The residue was dissolved in 500 µl of the derivatising reagent. The vial was sealed tightly and the contents mixed gently by swirling and left to stand at room temperature for about 15 minutes to complete the reaction.

The standards were also prepared as above, 200 µl of zearalenone (10 µg) and 500 µl of DON (10 µg) standard solutions were evaporated to dryness. 500 µl of the derivatising reagent was added to each residual toxin and left to stand at room temperature for about 15 minutes to complete the reaction.

After the reaction, 2 µl of the TMS - derivatives were injected directly into the gas chromatograph. Quantitation was by comparison of the peak areas of samples to those of the standard peaks (Area = ½ x base x height). Retention times for zearalenone and DON were 11.2 and 1.2 minutes respectively. Little background interference was obtained except for the solvent.

CHAPTER THREE

RESULTS

3.1 METHODOLOGY

The method of Bennett et al.(1985) gave 80 per cent recovery for Zearalenone whilst that of the Health Protection Branch (1985) gave recoveries of 80 per cent for DON and 86 per cent for nivalenol when feed samples were spiked at 2 mg/kg and 4 mg/kg. This was considered satisfactory considering recoveries reported by other authors. Gimeno (1982) reported recoveries of between 93 and 96 per cent for zearalenone while Eppley et al. (1984) reported recoveries of over 80 per cent for DON.

T-2 toxin however could only be recovered at 50 per cent and DAS at 60 per cent using the method of Romer et al. (1978). Other authors have reported higher recoveries. Gimeno (1982) reported recoveries of over 85 per cent while Romer (1986) reported recoveries of over 90 per cent for both T-2 toxin and DAS.

After extraction the toxins were quantified using separation on TLC and visual comparison with standards. Zearalenone and DON were the only Fusarium mycotoxins detected in the samples.

Figure 3.1 shows a thin layer chromatogram of four Zearalenone positive samples after development in diethyl ether - cyclohexane (75+25, v:v). The photograph was taken under long wavelength u.v. light (366 nm) after the plate had been sprayed with 20 per

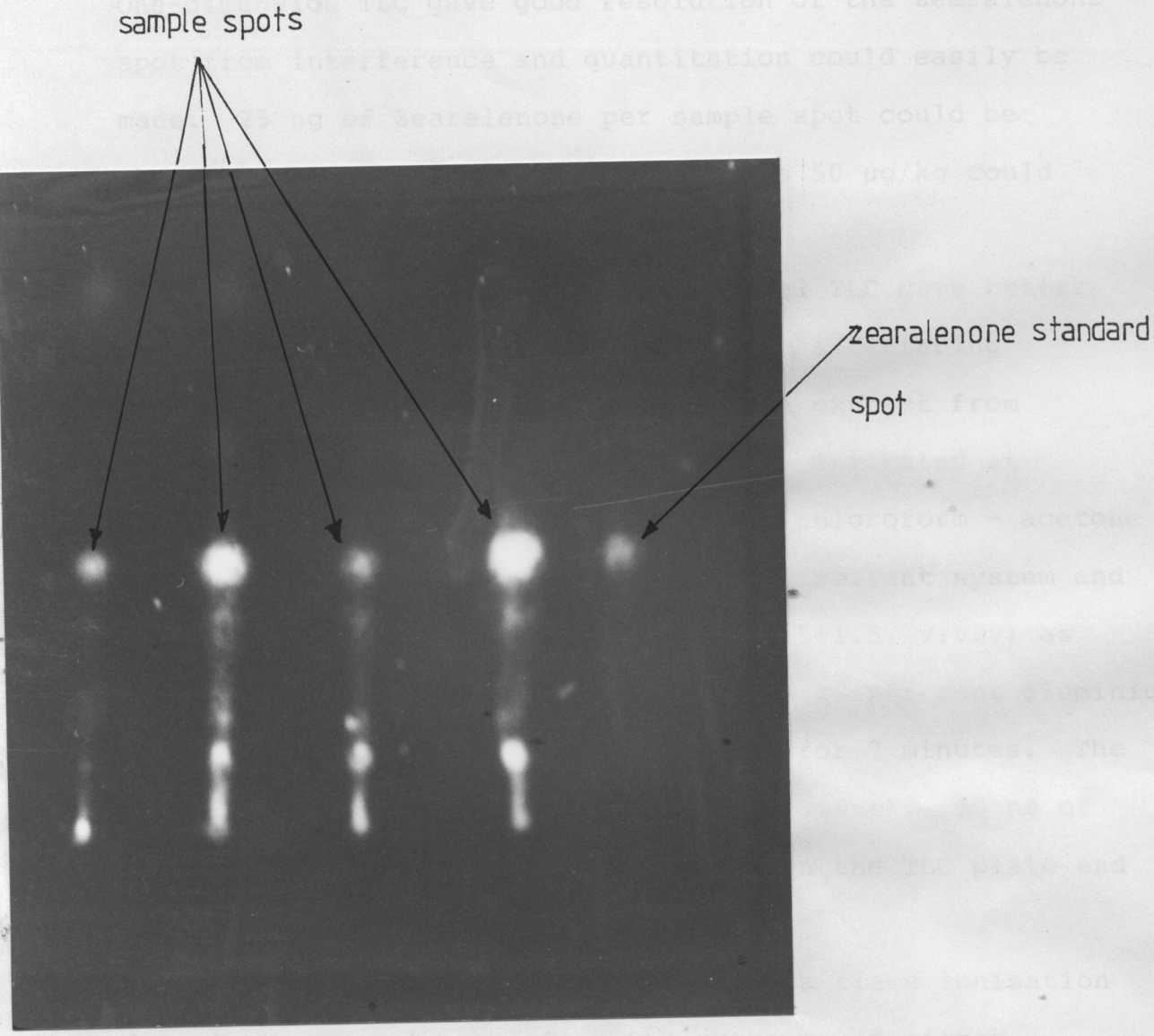


Figure 3.1 One-dimensional TLC of four zearalenone positive samples and standard . Solvent system : Diethyl ether - cyclohexane (75+25 , v:v)

cent aluminium chloride solution but before it was heated. One-dimension TLC gave good resolution of the Zearalenone spot from interference and quantitation could easily be made. 25 ng of Zearalenone per sample spot could be observed on the TLC plate and as low as 50 µg/kg could be determined.

In the case of DON, two-dimensional TLC gave better resolution of the toxin spot from other interfering fluorescent spots. Figure 3.2 shows an extract from naturally infected maize containing DON estimated at 9.6 mg/kg. The plate was developed in chloroform - acetone - isopropanol (8+1+1, v:v:v) as the first solvent system and diethyl ether - methanol - water (94+4.5+1.5, v:v:v) as the second solvent system, sprayed with 20 per cent aluminium chloride solution, and heated at 120°C for 7 minutes. The photograph was taken under u.v. light (366nm). 50 ng of DON per sample spot could be observed on the TLC plate and as low as 80 µg/kg could be determined.

A gas chromatograph equipped with a flame ionisation detector was used to confirm the presence of either Zearalenone or DON. Representative gas chromatograms are shown in Figures 3.3 - 3.8. The levels of mycotoxins obtained were comparable to those obtained by TLC but were slightly lower in almost all cases. For example, Figure 3.4 and Figure 3.5 show gas chromatograms of Zearalenone positive samples contaminated with 3 and 240mg/kg Zearalenone. However, these same samples

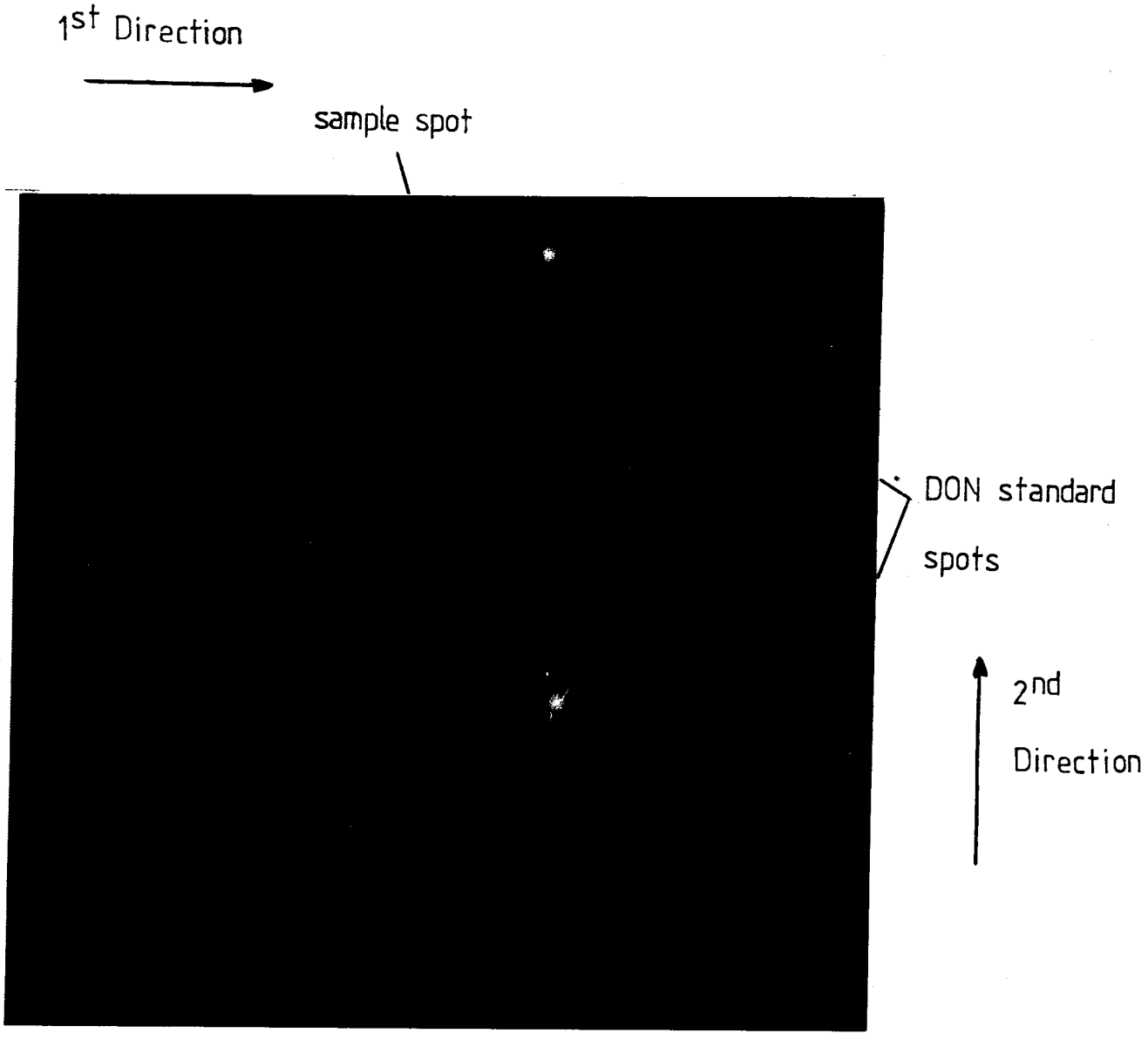


Figure 3.2 Two-dimensional TLC of a DON positive sample containing 9·6 mg/kg DON . Solvent system 1: chloroform-acetone-isopropanol (8 +1+1, v:v:), solvent system 2 : diethyl ether - methanol-water (94+4·5+1·5, v: v: v) .

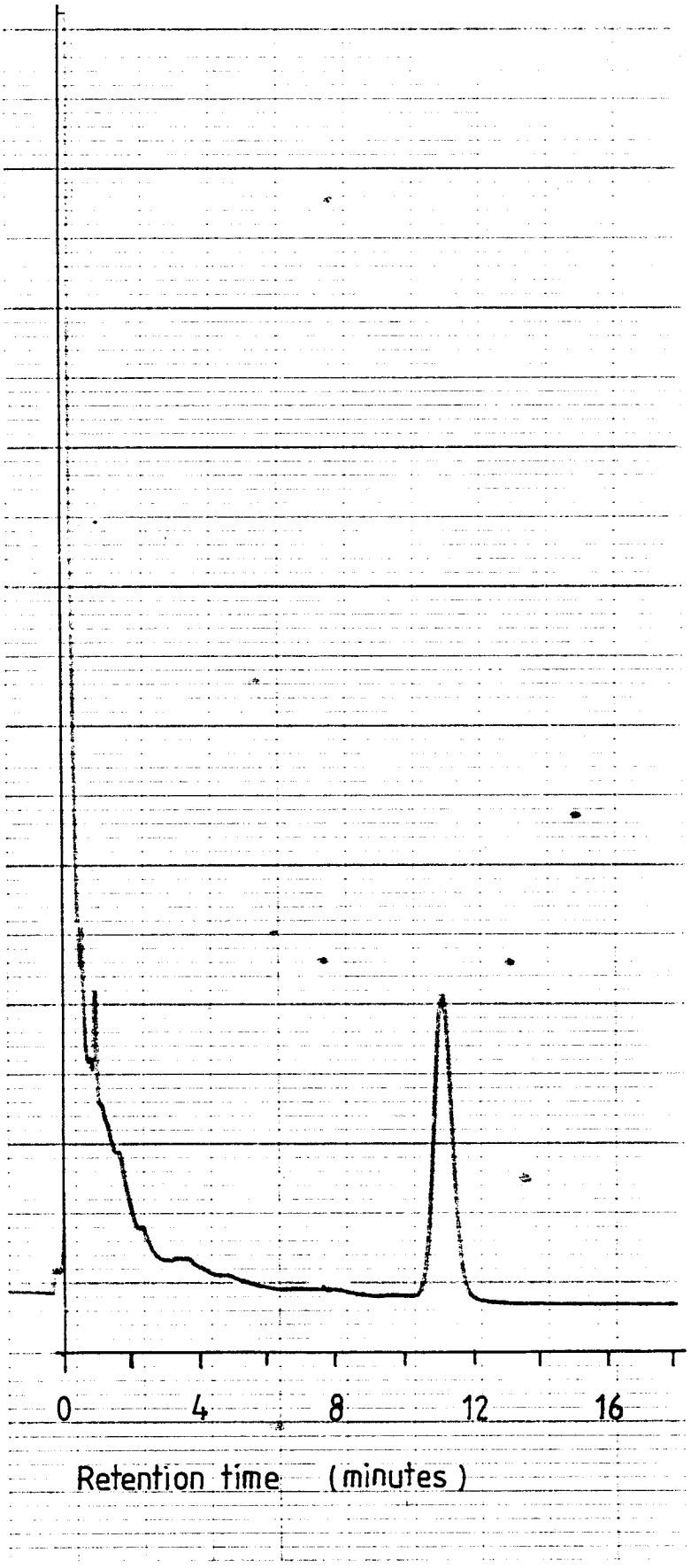


Figure 3.3 Gas chromatogram of TMS-derivative of 0.2 µg zearalenone on a 2 m column with 2% OV-17 on Uniport using FID .

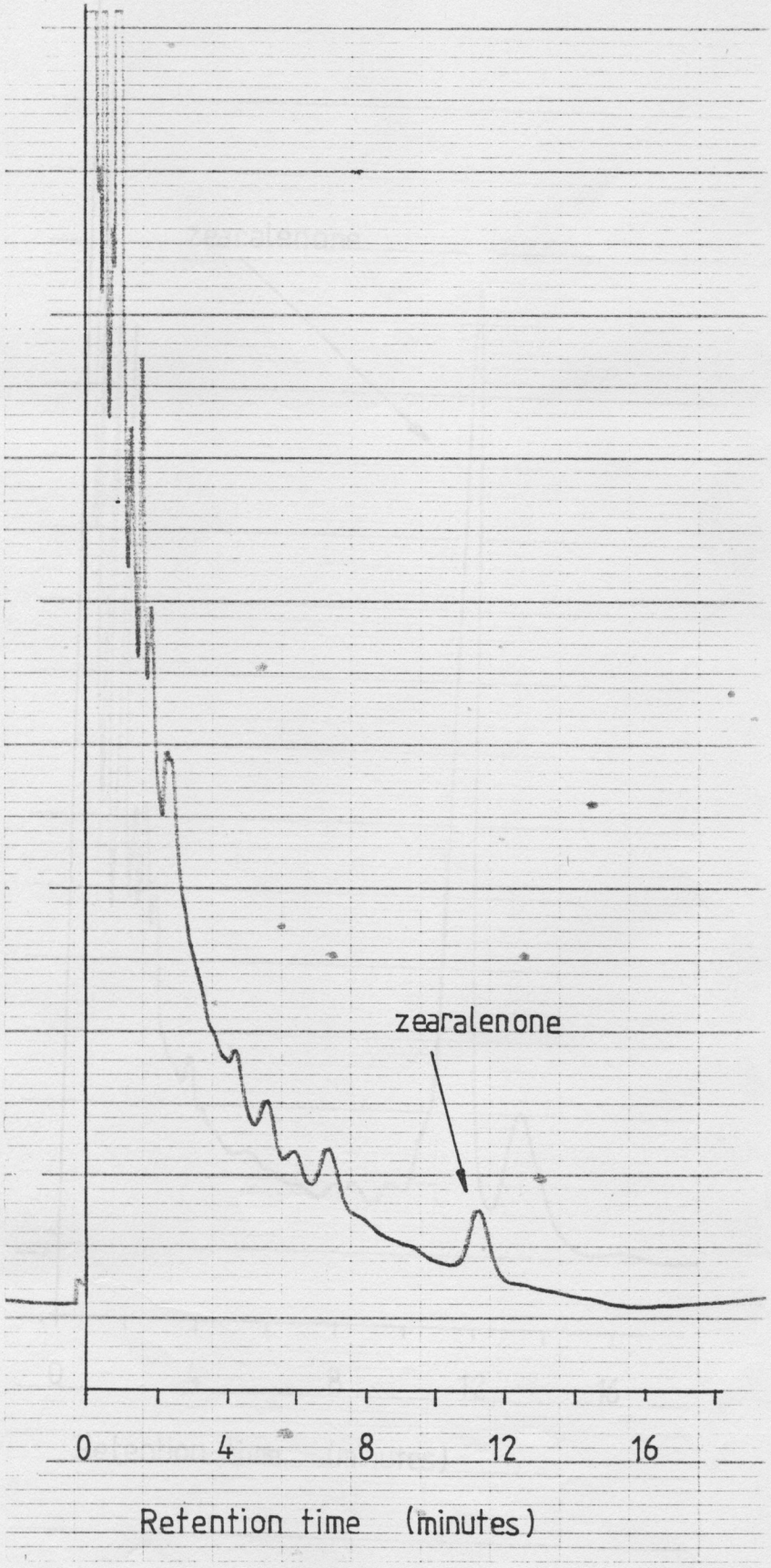


Figure 3.4 Gas chromatogram of silylated extract from naturally infected maize contaminated with 3 mg/kg zearalenone .

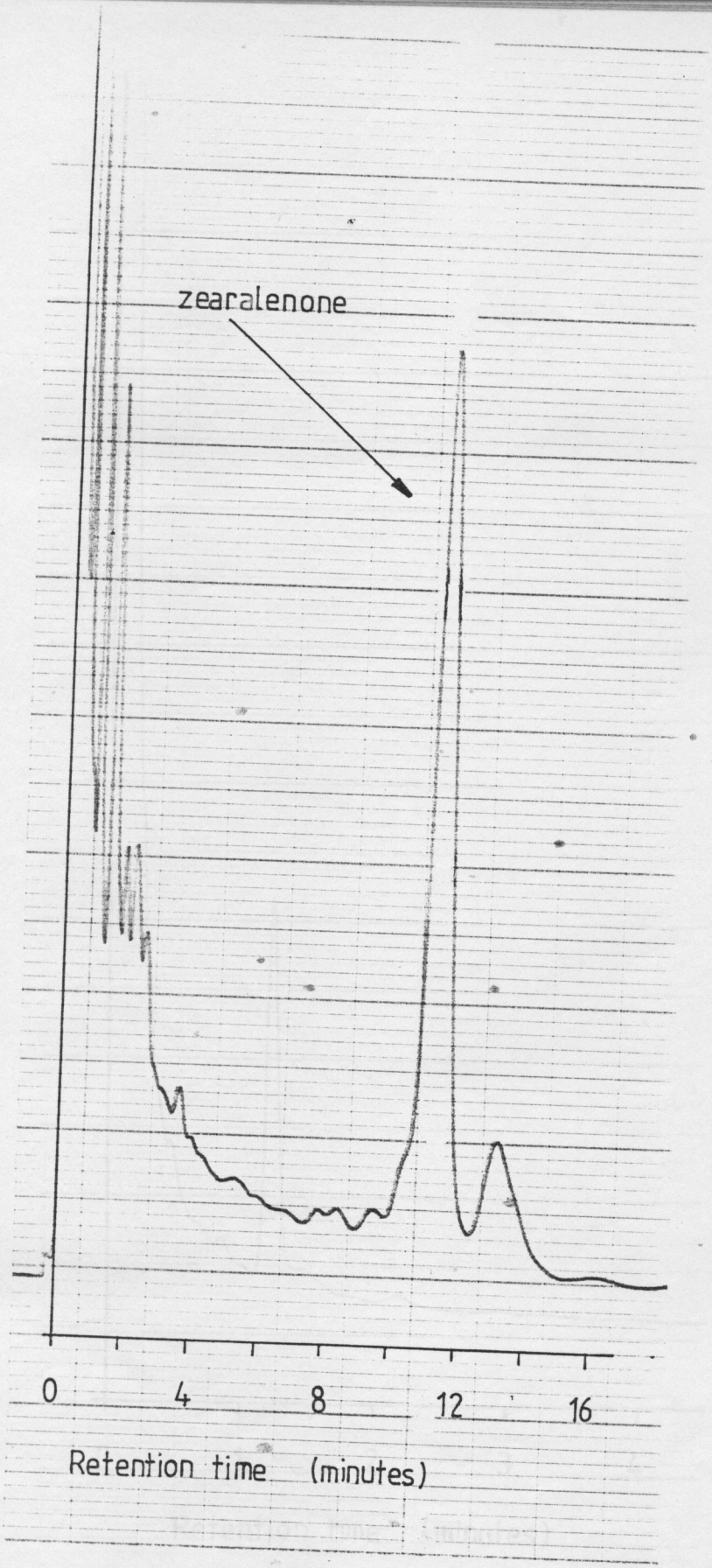


Figure 3·5 Gas chromatogram of silylated extract from laboratory infected maize containing 240 mg/kg zearalenone .

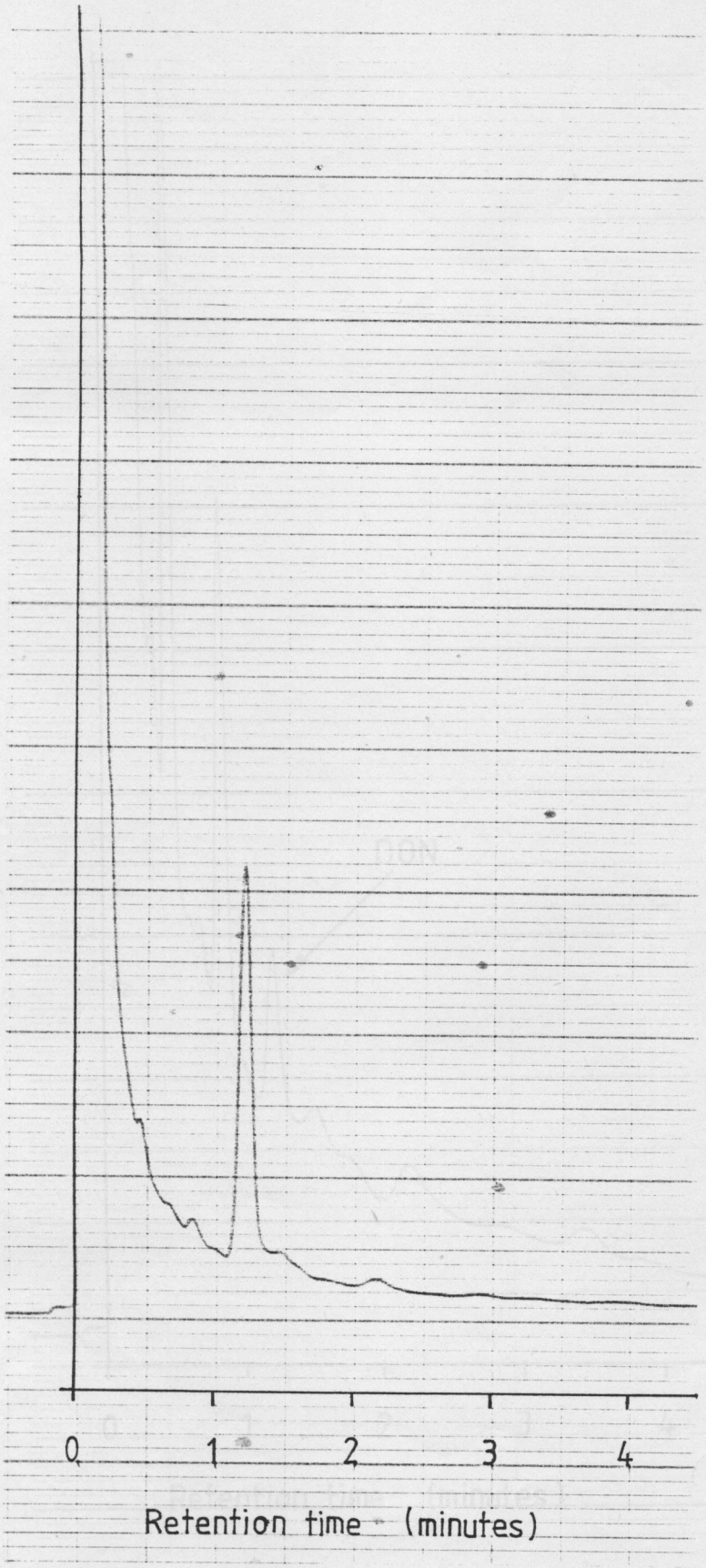


Figure 3.6 Gas chromatogram of TMS-derivative of 0.2 µg DON on a 2 m column with 2% OV-17 on 80-100 Uniport using FID.

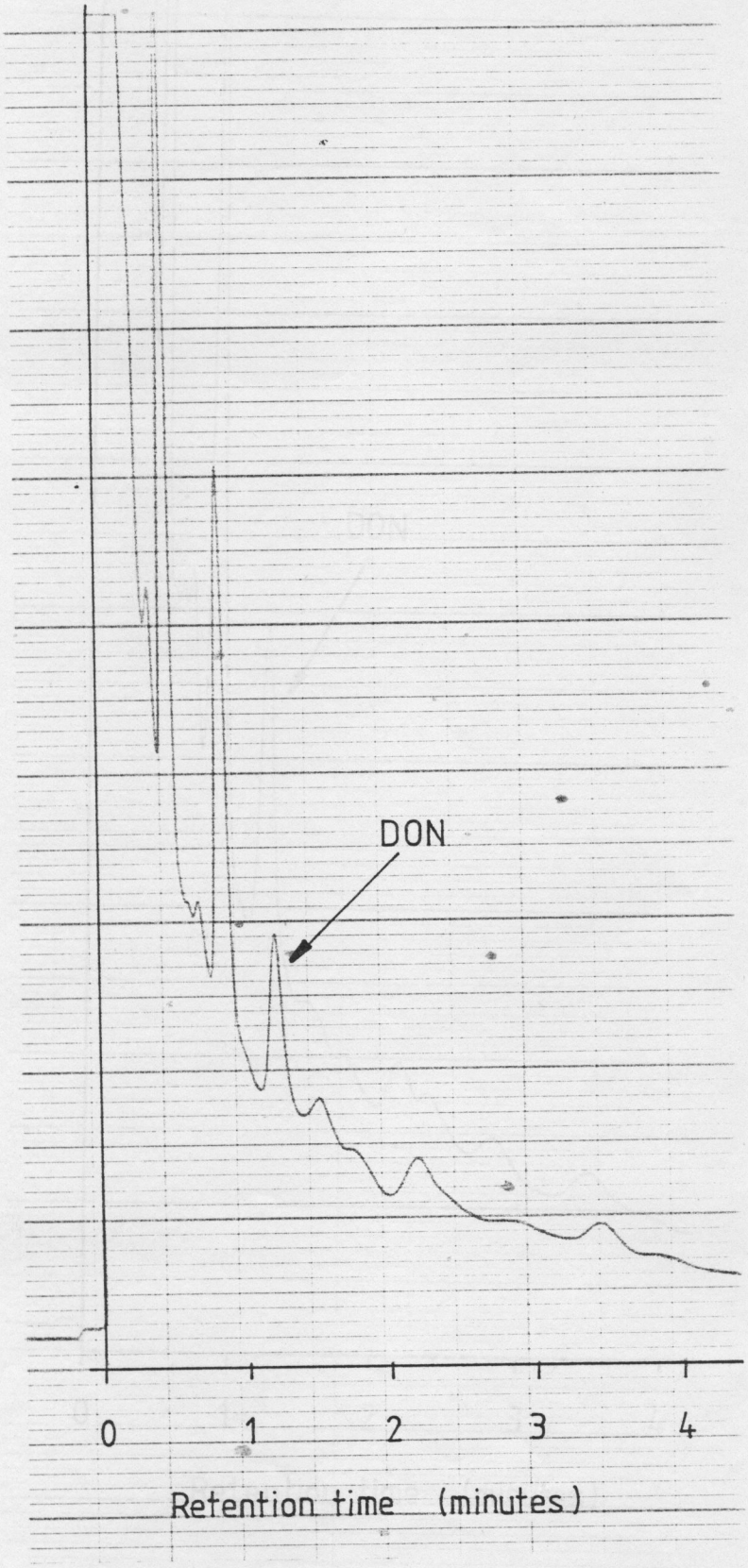


Figure 3.7 Gas chromatogram of silylated extract from naturally infected maize contaminated with 8·4 mg/kg DON .

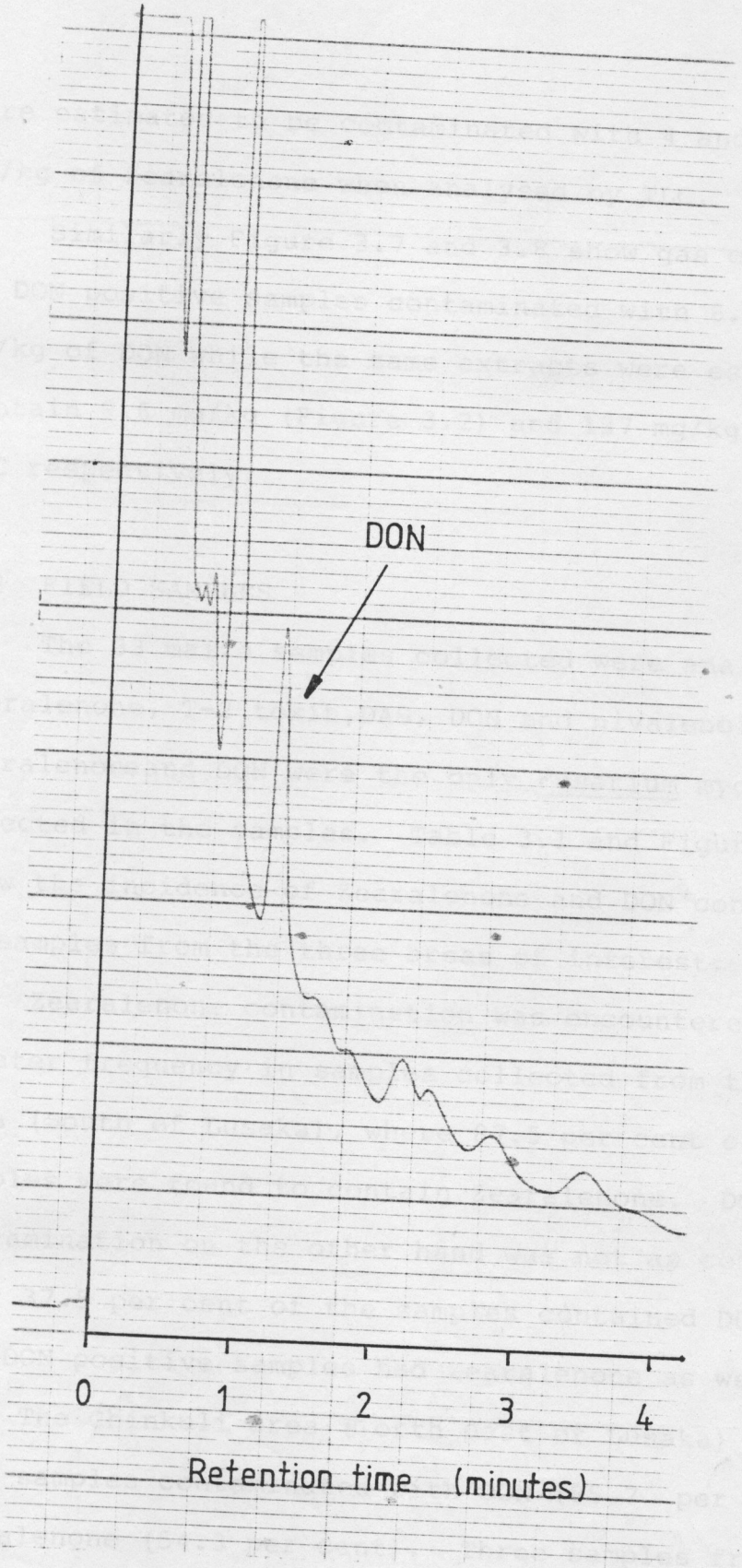


Figure 3.8 Gas chromatogram of silylated extract from laboratory infected maize containing 120 mg/kg DON.

were estimated to be contaminated with 4 and 260 mg/kg of Zearalenone when analysed by TLC.

Similarly Figure 3.7 and 3.8 show gas chromatograms of DON positive samples contaminated with 8.4 and 120 mg/kg of DON while the same extracts were estimated to contain 9.6 mg/kg (Figure 3.2) and 137 mg/kg of DON by TLC respectively.

3.2 FIELD SAMPLES

The 33 maize samples collected were analysed for Zearalenone, T-2 toxin,DAS, DON and nivalenol. Zearalenoneand DON were the only Fusarium mycotoxins detected in the samples. Table 3.1 and Figure 3.9 show the incidence of Zearalenone and DON contamination in samples from the three areas of interest.

Zearalenone contamination was encountered with greater frequency in samples collected from the Chipapa area (South of Lusaka), where 87.5 per cent of the samples were found to contain Zearalenone. DON contamination on the other hand was not as common, only 37.5 per cent of the samples contained DON. All the DON positive samples had Zearalenone as well.

The Chinkuli area (north east of Lusaka) had more samples contaminated with DON (85.7 per cent) than Zearalenone (54.3 per cent). Three samples from this area contained DON but no detectable amounts of Zearalenone while all samples contaminated with Zearalenone had DON as well.

Table 3.1 Zearalenone and DON contamination in maize samples from Chipapa, Chief Mungule and Chinkuli areas of Lusaka.

Area	No of samples	Zearalenone positive	DON positive	DON & Zearalenone positive	% Contamination (Zearalenone	% Contamination (DON)	Total % Contamination
Chinkuli	14	9	12	9	64.3	85.7	85.7
Chief Mungule	11	3	1	0	27.3	9.1	36.4
Chipapa	8	7	3	3	87.5	37.5	87.5
TOTAL	33	19	16	12	57.6	48.5	69.7

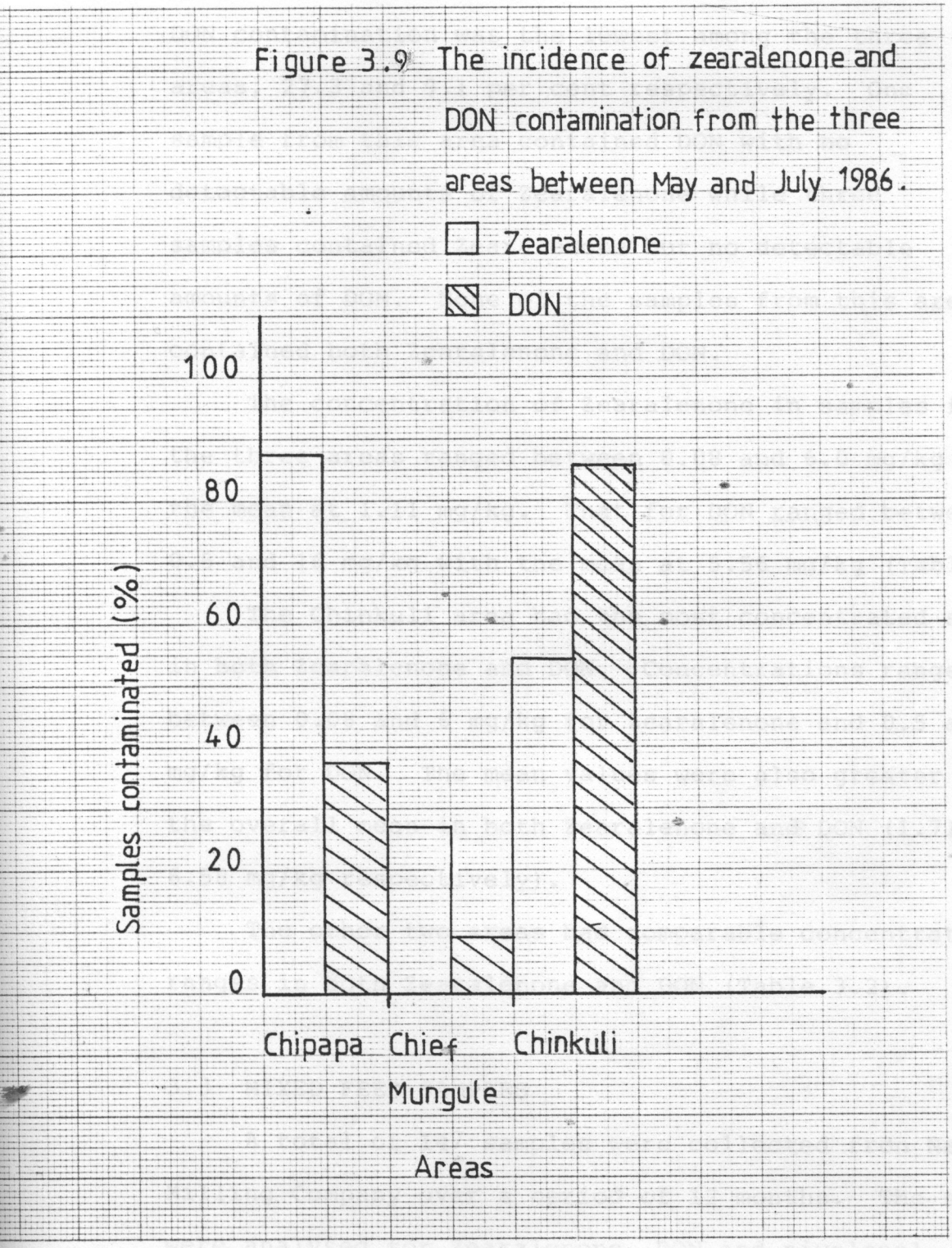


Figure 3.9 The incidence of zearalenone and DON contamination from the three areas between May and July 1986.

The least amount of contamination was found in samples collected from Chief Mungule's area (north west of Lusaka). The incidence of Zearalenone and DON contamination was the lowest among the three areas, 27.3 and 9.1 per cent respectively. One sample from this area contained DON with no detactable amounts of Zearalenone while three samples contained Zearalenone but no detectable amounts of DON. None of the samples from this area contained both Zearalenone and DON.

The concentration of Zearalenone in samples from the three areas ranged between 0.08 and 6.0 mg/kg with the mean at 1.11 mg/kg. That for DON ranged between 0.5 and 16 mg/kg with the mean at 5.56 mg/kg (Table 3.2).

The Chinkuli area had the most concentrated samples in both Zearalenone and DON. Concentrations ranged between 0.08 and 6 mg/kg for Zearalenone and 0.5 and 16 mg/kg for DON. The mean values were also greater than the overall mean in both Zearalenone and DON (1.71 and 6.58 mg/kg respectively).

The other two areas had comparable concentration ranges in both Zearalenone and DON (Table 3.2).

3.3 MIXED FEED SAMPLES

A total of 147 samples were collected fron National Milling Company over a period of 12 months. The samples were analysed for Zearalenone, DON and nivalenol.

Table 3.2 The range of zearalenone and DON concentrations in positive samples from Chipapa, Chief Mungule and Chinkuli areas of Lusaka.

(TLC results)

Area	Zearalenone (mg/kg)	Zearalenone mean (mg/kg)	DON (mg/kg)	DON mean (mg/kg)
Chipapa	0.4-1.6	0.73	0.5-5.0	2.0
Chief Mungule	0.08-0.16	0.11	4.0	4.0
Chinkuli	0.08-6.0	1.71	0.5-16	6.58
All positive samples	0.08-6.0	1.11	0.5-16	5.56

Zearalenone and DON were the only ones detected in the samples. The Zearalenone and DON positive samples were further analysed for T-2 toxin and DAS but none contained detectable T-2 toxin or DAS i.e. 2000 µg/kg and above.

The results obtained are given in Table 3.3 according to the month in which the samples were collected. The information is further presented in form of a histogram in Figure 3.10 to show the monthly variations.

Zearalenone was detected in 25 samples, representing a contamination of about 17 per cent. Only two samples contained detectable amounts of DON (1.4 per cent contamination).

The contamination of Zearalenone in the contaminated samples ranged from 0.05 to 0.6 mg/kg with the mean at 0.31 mg/kg. Two of the Zearalenone positive samples contained DON as well at 1.0 mg/kg.

In Table 3.4, the results are presented according to the type of sample analysed in order to show the amount of contamination in each type of sample. Maize bran samples had the highest incidence of Zearalenone and the most concentrated samples in Zearalenone.

The two DON positives were among the maize bran and maize meal No. 3 samples.

3.4. LABORATORY INOCULATED SAMPLES

Maize kernels (ground) were inoculated with

Table 3.3 Monthly variations in zearalenone and DON concentrations in mixed feed and raw material samples from National Milling Company. (TLC results)

Month	No of samples	No contaminated with Zearalenone	range of contamination (mg/kg)	mean (mg/kg)
Nov. 1985	14	1 (7%)	0.3	0.3
Dec. "	10	0		
Jan. 1986	9	0		
Feb. "	12	0		
Mar. "	28	5 (18%)	0.05-0.6	0.27
Apr. "	12	1 (8%)	0.6	0.6
May "	9	0		
Jun. "	9	2 (22%)*	0.05	0.05
July "	9	0		
Aug. "	9	8 (89%)	0.16-0.6	0.25
Sep. "	9	0		
Oct. "	9	0		
Nov. "	8	8 (100%)	0.1-0.5	0.25
Total	147	25 (17%)	0.05-0.6	0.25

* The two samples also contained DON at 1.0mg/kg.

Figure 3.10 Monthly variations in zearalenone concentration in mixed feeds and raw materials from National Milling Company

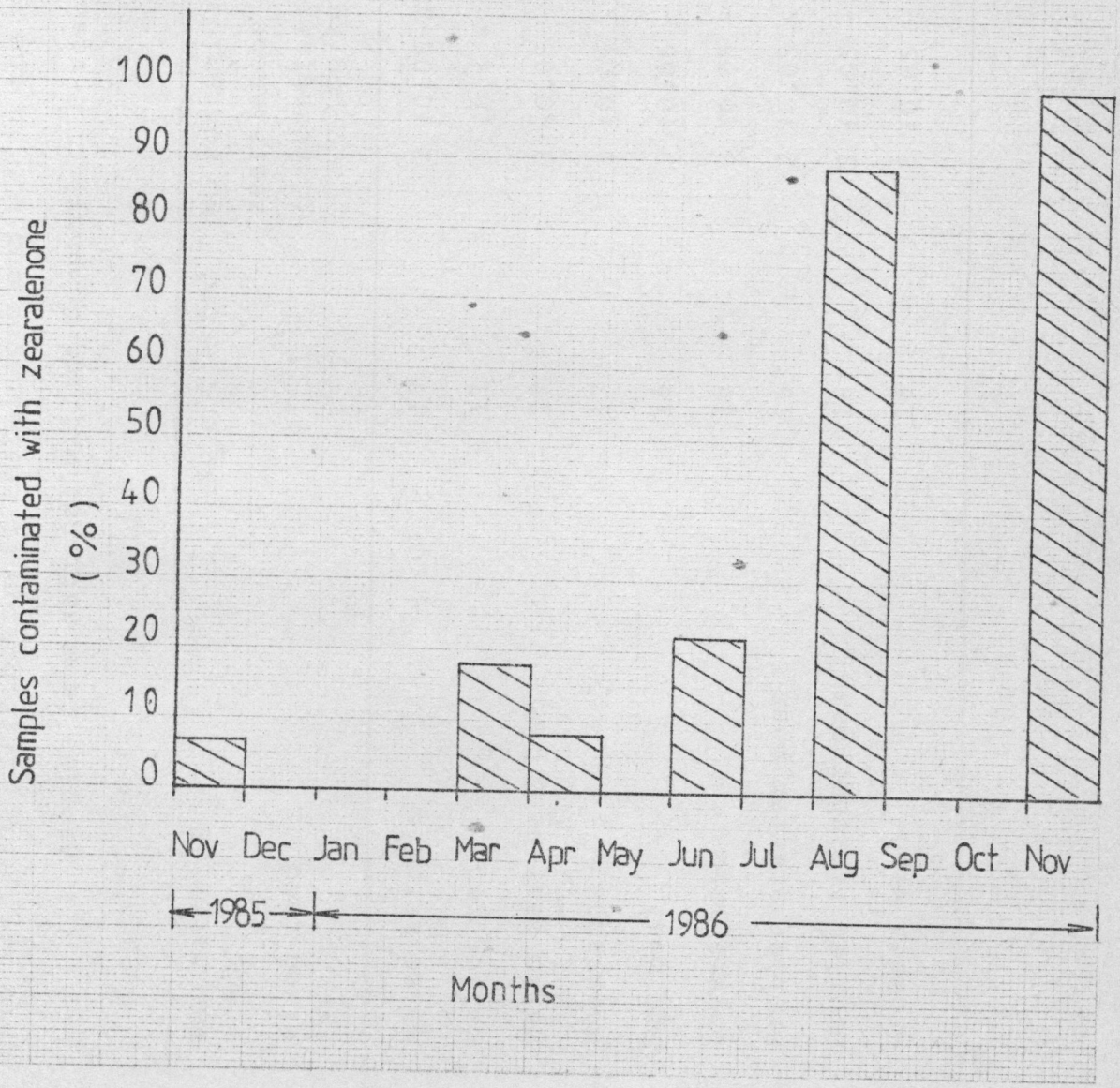


Table 3.4 The distribution of zearalenone and DON in the various samples from National Milling Company. TLC results)

Raw material or mixed feed	No collected	No contaminated with zearalenone (Concentration range mg/kg)	No contaminated with DON (conc. range mg/kg)
Maize kernels	17	2 (0.06 & 0.1)	-
Maize bran	17	5 (0.4 - 0.6)	1 (1.0)
Maize meal No 3	17	3 (0.05 - 0.6)	1 (1.0)
Wheatings	17	2 (0.05 & 0.5)	-
Pig meal	16	3 (0.05 - 0.5)	-
Layers mash	16	3 (0.05 - 0.5)	-
Broiler starter mash	16	2 (0.05 & 0.5)	-
Broiler finisher mash	16	3 (0.05 - 0.3)	-
Growers mash	16	3 (0.05 - 0.3)	-

Fusarium graminearum and treated in duplicate as shown in Table 2.2 to show the variations in toxin production at the two temperatures, 16°C and 26°C. Samples were removed at regular intervals and analysed for zearalenone, DON and nivalenol.

Fungal growth was vigorous at both temperatures although more growth was observed at 26°C. By the end of the first week, the whole maize surface was covered by a white mycelial growth which later turned pink. By the end of the eighth week the whole substrate appeared to have been invaded by the fungi in all the flasks and the substrate in Flask No. 2 and No. 4 appeared to have been completely used up.

Analysis of the culture was done every two weeks and the results of one set of flasks are given in Table 3.5 for zearalenone and Table 3.6 for DON. The average of the duplicate flasks could not be taken due to uneven fungal growth in the duplicate flasks but the results of the duplicate flasks were comparable.

In the early stages of the experiment, more zearalenone was produced in flasks which were incubated at 26°C with only small amounts being produced at 16°C. As the experiment progressed, increasing amounts of zearalenone were detected in flasks at 16°C and by the time the experiment was stopped, large amounts of the toxin were being produced in flasks at 16°C i.e. Flask No. 3 and Flask No. 4. Overall, the flasks which remained at 16°C (Flask No. 3) for the duration of the experiment produced the largest amounts of zearalenone (Table 3.5 and Figure 3.11).

Table 3.5 Zearalenone production by Fusarium graminearum (TLC results)

Flask No.	Incubation Temperature	Zearalenone production (mg/kg) No of weeks from start of experiment 2	4	6	8	10
1	10 wks at 26°C	14	100	250	250	260
2	5 wks at 26°C & 5 wks at 16°C	10	100	130	175	260
3	10 wks at 16°C	5	28	100	860	1300
4	5 wks at 16°C & 5 wks at 26°C	5	28	150	750	750

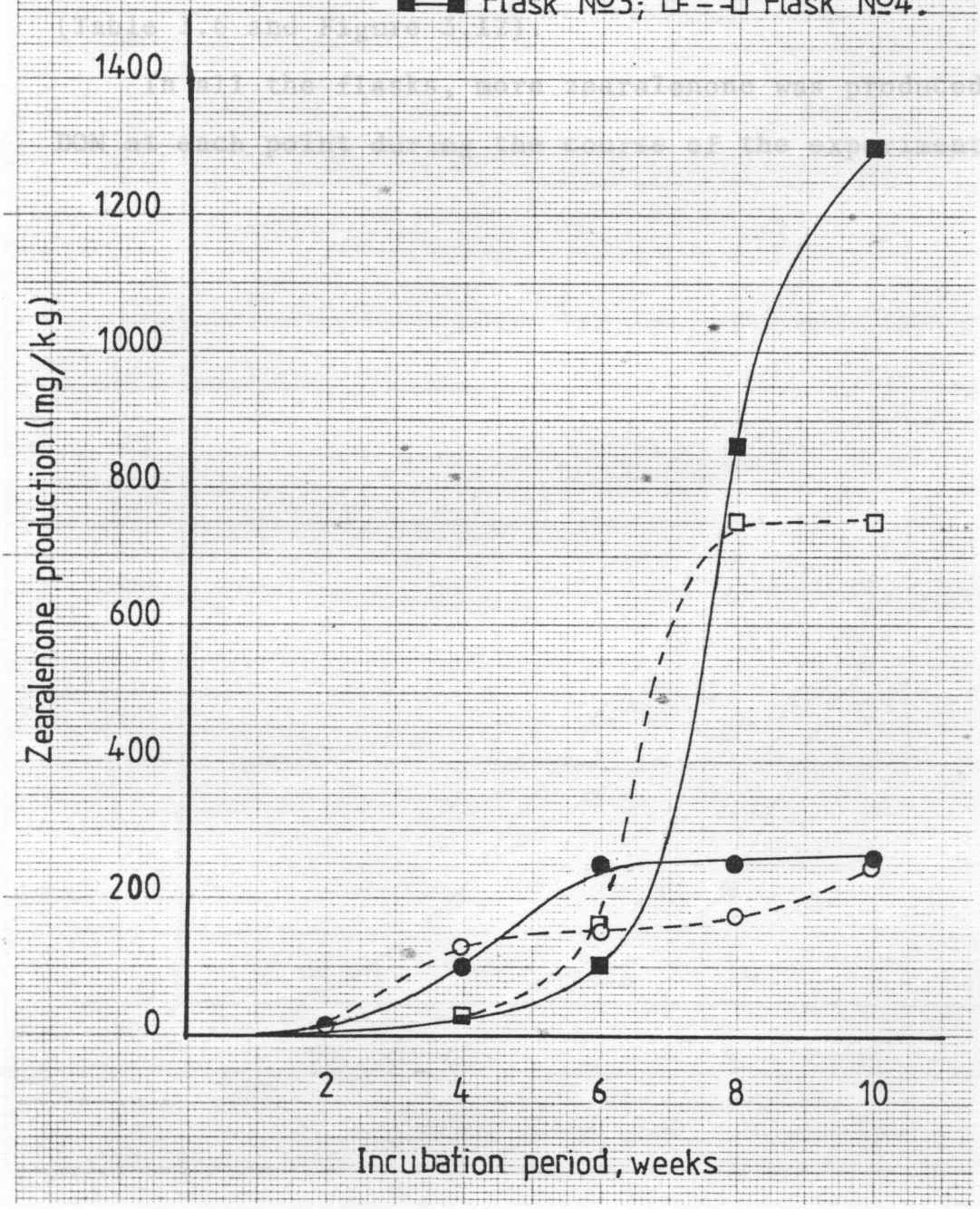
Table 3.6 DON production by Fusarium graminearum (TLC results)

Flask No.	Incubation Temperature	DON production (mg/kg) No of weeks from start of experiment 2	4	6	8	10
1	10 wks at 26°C	1	15	30	60	62
2	5 wks at 26°C & 5 wks at 16°C	1	-	30	40	44
3	10 wks at 16°C	5	5	5	16	50
4	5 wks at 16°C & 5 wks at 26°C	4	5	8	80	137

Figure 3.11 Effect of temperature and incubation period on zearalenone production.

●—● Flask №1; o- - -o Flask №2;

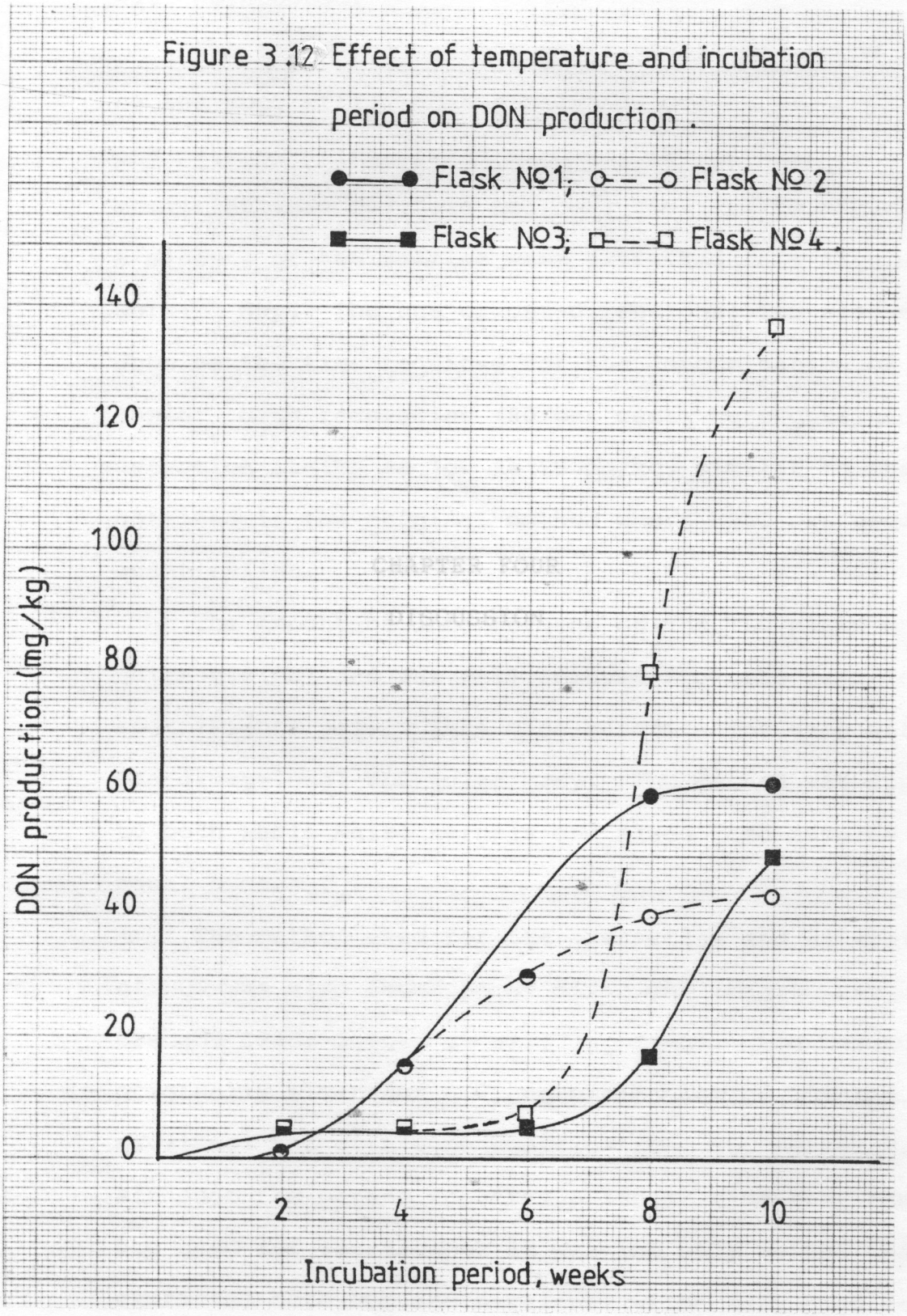
■—■ Flask №3; □- - -□ Flask №4.



In the case of DON, more was produced in the flasks which were later transferred to 26°C from 16°C i.e. Flask No. 4. The flasks which remained at 16°C for the duration of the experiment (Flask No. 3) produced the least amounts of DON. A considerable amount was produced in Flask No. 1 which was kept at 26°C for tne duration of the experiment (Table 3.6 and Figure 3.12).

In all the flasks, more zearalenone was produced than DON at each point during the course of the experiment.

Figure 3.12 Effect of temperature and incubation period on DON production.



CHAPTER FOUR

DISCUSSION

4.1 METHODOLOGY

The method of Bennett et al.(1985) was found to be the most efficient for this project in the extraction and determination of Zearalenone. It is simple, requiring less solvents and less time. With experience, a minimum of ten samples were extracted in a single working day. The use of one-dimensional TLC also cut down on the time required for the determination of the toxin. High recoveries (80 per cent) were obtained with detection limits as low as 50 µg/kg, similar to that obtained in the original method where HPLC was used for quantitation.

The amount of interference on the TLC plate was quite minimal and was well removed from the Zearalenone spot (Figure 3.1). This made quantitation even with one-dimensional TLC quite easy. The method was applied to both whole maize kernels and mixed feed samples with equal success.

The method used for the extraction and determination of DON was also found to be efficient (Health Protection Branch, 1985; Scott et al., 1986). The method was originally given for G.C. determination of DON and nivalenol but with some modification, TLC was used. Although the method required a longer time than the one used for Zearalenone it was the only method that extracted both DON and nivalenol with very high recoveries

(80 and 86 per cent respectively). The method gave very clean extracts so that one dimensional TLC could be used for detecting the presence of DON. However, due to background fluorescence, two-dimensional TLC gave the best results for quantitation.

Gas chromatography equipped with a flame ionisation detector was used to confirm the presence of the toxins in the positive samples. The extracts were prepared for injection into the GC according to the method of Kamimura et al.(1981). The results were comparable to those obtained by TLC although it was observed that TLC results were slightly higher than the GC results for both Zearalenone and DON. A similar trend was observed by Scott et al.(1978) when TLC results were compared with HPLC results in the determination of Zearalenone. There was overestimation of Zearalenone concentration on TLC plates. This was explained as being partly due to the enhancement of the fluorescence of the Zearalenone spot by the background. This calls for caution in the interpretation of TLC results when TLC is used on its own.

Similarly in the results given by Eppley et al.(1984) from GC and TLC determination of DON in six wheat samples, three of the samples gave TLC results which were slightly higher than those obtained by GC.

4.2 FIELD SAMPLES

The results obtained in the analysis of field samples are shown in Tables 3.1 and 3.2. Only Zearalenone and DON were detected in the field samples.

Although Zearalenone and DON were detected in samples from all the regions visited, it is evident that more samples from the Chipapa area were contaminated with Zearalenone (87.5 per cent) than the other two regions. DON on the other hand was found with greater frequency in samples from Chinkuli area (85.7 per cent) than the Chipapa (37.5 per cent) and Chief Mungule (9.1 per cent) areas. This indicates some geographical distribution of the two toxins in the areas visited. Ueno et al. (1972) reported the isolation of fungal strains which produce the group A trichothecenes from districts in Japan where climates are rather cold while fungal strains producing group B trichothecenes were isolated from districts where climates are warmer. However, in our case, it is most likely that the same fungus (Fusarium graminearum) produced the two toxins, Zearalenone and DON, found in the field samples so that temperature and humidity are the most likely determining factors instead of different species. This could possibly mean that temperatures in Chinkuli area were generally warmer (which favoured DON production) while the Chipapa area may have had cooler temperatures (which favoured Zearalenone production).

It is also important to note that most of the farms visited in the Chinkuli area had just started harvesting their maize while in the other two areas, harvesting was almost complete by that time. This would suggest that the rains may have been prolonged in Chinkuli area which would have delayed harvesting and possibly have allowed fungi to get established on the maize.

Chief Mungule's area had the lowest number of samples contaminated with either Zearalenone or DON. This region is generally dry and this could be seen from the fact that a large amount of cotton (a crop requiring less rainfall than maize) is grown in this region. Therefore, the question of excessive rainfall did not arise and hence the low incidence of Zearalenone and DON.

Slightly lower results than ours were obtained from a post-harvest survey of mouldy maize from the 1974 season for Zearalenone. It was found that maize containing over 12 per cent diseased grain had an average of 1.8 mg/kg Zearalenone (NCSR, 1976).

In another survey carried out on maize from the 1974 season, samples were taken from maize used in the brewing of beer. The maize was found to contain up to 0.8 mg/kg Zearalenone. Beer samples were found to contain up to 4.6 mg/litre Zearalenone (Lovelace and Nyathi, 1977). DON was not tested for but it is likely that some DON may have been present in some maize samples and the beer. DON has been detected in our

samples at very high concentrations (as high as 16 mg/kg). Since some of this maize is used for making beer, and since DON is quite stable to heat as in the making of Egyptian bread (El-Banna et al., 1983), it may not be destroyed by the brewing process and may end up in beer. More experiments should be carried out to determine the level of DON contamination in beer brewed from maize as this is one of the main uses of mouldy maize. This would give an approximate amount of DON consumed by an individual.

Thiel et al.(1982b) found moniliformin, DON and Zearalenone in maize intended for human consumption in Transkei, an area of high human oesaphageal cancer rate. Although there is no evidence at present to implicate any of the above toxins in esophageal cancer, caution should be excercised in the use of maize containing such high levels of Zearalenone and DON (as we have found) in the brewing of beer.

The implication of Fusarium toxins in general, and Zearalenone and DON in particular in human and animal mycotoxicoses have not been reported in Zambia. This could be due to the fact that little information is available on the natural occurence of these toxins here. At the same time, diagnosis in many cases of mycotoxicoses is not easy. However, some experiments have been performed here in order to determine the effect of mouldy maize on ducklings,pigs, steers and rats.

MacDonald and Raemaeker (1974) fed rations containing 0,5,10,25 and 50 per cent Fusarium diseased maize to ducklings. The ducklings fed 25 and 50 per cent diseased maize were found to show progressively lower food consumption and weight gains. Similar results were obtained with male rats. When pregnant rats were fed diets with Fusarium grain, small litters were produced all of which were either still-born or died within 24 hours.

Other experiments have been performed using pigs and steers (Progress Report on the Mouldy Maize Project, 1978-1982). Groups of pigs and steers were fed specially prepared rations to which 55 per cent grade A maize, grade E maize or Undergrade maize (this is maize spoiled during storage and contains over 10 per cent mouldy maize) had been added. The steers were fed in stalls at night and allowed to graze naturally in paddocks during the day. No significant differences in weight gains were observed for the duration of the experiment. When the pigs and steers were slaughtered at the end of six months, no gross lesions were noticed in any organ or tissue. This suggests that for short-term feeding of male animals as in fattening of pigs and steers, mouldy maize could be used with caution.

When the above experiment was repeated using breeding sows, it was noticed that in the gilts fed Undergrade maize, growth was retarded and puberty delayed. Only the animals fed grade A maize had littered (an average of 9

piglets per sow) by the end of six months unlike the other two groups. This means that feeding mouldy maize to sows is detrimental to their health and the reproductive system is clearly affected.

Similar results have been obtained elsewhere. Marasas et al.(1978) working with four samples of Zambian maize containing 1.8, 5.4, 13.0 and 20.3 per cent visually diseased maize kernels, found that none of the samples were lethal to either duckling or rats. No Zearalenone was detected in any of the samples. The dominant fungi isolated were F. moniliforme sheldon and Diplodia macrospora Earle. This could explain the absence of Zearalenone in the samples.

4.3 MIXED FEED SAMPLES

The results obtained are shown in Table 3.3. Only Zearalenone and DON were detected in the samples. As earlier pointed out in section 2.1.2, only grades A, B, and C (with a maximum of 2 per cent diseased grain) maize is used in feed manufacture. This is the same maize which is marketed for human consumption, therefore, the results obtained can be said to be true of the maize meant for human consumption.

This is the first report on the natural occurence of the toxins, Zearalenone and DON in mixed feeds in Zambia, although DON has been found in maize bran and maize meal.

An incidence of 17 per cent contamination was found for Zearalenone, with concentrations ranging between

0.05 and 0.6 mg/kg. This concentration range is consistent with that obtained from a post-harvest survey of 1974 mouldy maize. Maize containing 2-8 per cent diseased maize was found to contain between 0.2 and 0.8 mg/kg Zearalenone (NCSR, 1976). Comparable results were obtained in a survey of USA maize meant for human consumption, 0.2-0.5 mg/kg Zearalenone was detected (Eppley et al., 1974).

A greater incidence of Zearalenone was found in the outer portions of the maize, maize bran and maize meal No. 3 (mixture of maize bran and some maize flour), as compared to whole maize kernels. The two DON positive samples were also among the maize bran and maize meal No.3 samples, suggesting that DON is concentrated in the outer portions of the maize. Bennett et al.(1976) found that the grits fraction of maize contained the least amount of Zearalenone while the germ and feed fractions contained most of the Zearalenone in dry milled fractions of contaminated maize. Similarly Young et al.(1984), working on the effect of milling and baking on the DON content of Canadian wheat, found that milling led to a fractionation of DON in the various wheat fractions. Increased levels were found in the outer portions (e.g. bran) while decreased amounts were found in the inner flour portions.

From the data presented in Table 3.3 and figure 3.10, it is not easy to tell whether there were any seasonal variations in Zearalenone contamination. It appears that

Zearalenone contamination of maize occurs throughout the year with no apparent losses during storage. At the same time there is no information as to when particular maize samples used in feed manufacture were harvested. The maize supplied to the National Milling Company is randomly picked from NAMBOARD storage points, and this may explain the non-uniformity of the results. It is also very difficult to trace the source of the contaminated sample from the Feed Depot.

Mirocha and Christensen (1974) gave 1-5 mg/kg Zearalenone contamination as being physiologically significant when fed to test animals. This is much higher than was detected in these samples from National Milling Company (0.05-0.6 mg/kg). It can therefore be suggested that the levels detected in feed samples from National Milling Company are too low to be of major concern to farmers. However, low concentrations of Zearalenone have been detected in samples implicated in mycotoxicoses. 0.1 mg/kg Zearalenone was detected in a pig feed causing porcine internal haemorrhaging, 0.5 mg/kg was found in a pig feed sample causing porcine hyperestrogenism and 0.01 mg/kg was detected in a porcine gestation ration implicated in causing porcine infertility and abortion (Mirocha et al., 1974). It is likely that other toxins may have been involved as well in the above mycotoxicoses.

DON was only detected in two samples, maize bran and maize meal No. 3, from National Milling Company. The amount

detected was quite low (1.0 mg/kg) and this may explain why DON was not detected in the actual feeds. Maize bran and maize meal No. 3 make up about 50 per cent of the raw materials used in feed manufacture. This means that if the contaminated samples were used in feed manufacture, the final feed would have about 0.5 mg/kg DON which was still above our detection limits. However, no DON was detected in the mixed feeds suggesting further dilutions or losses in the manufacturing process.

In a survey of DON contaminated feed grain in the USA, Côté et al.(1984) found that 80 per cent of the samples contained DON ranging from 0.1-41.6 mg/kg (with a mean at 3.1 mg/kg). 39 per cent of the feed samples submitted had DON ranging from 0.1-22.0 mg/kg. This is much higher than what we have obtained for the National Milling Company samples.

Since an incidence of only 1.4 per cent was found, it can be said that mixed feed samples from National Milling Company were relatively free from DON during the period in question.

DON dietary levels of 5 mg/kg or higher have been suggested as being physiologically significant (Schuh et al., 1982). At lower concentrations, swine diets containing 0.3-0.7 mg/kg DON resulted in decreased feed consumption and weight gains (Trenholm, et al., (1981). No serious effects were noted in poultry fed similar levels of DON. Minimum emetic doses of DON given orally to swine were found to be 0.1-0.2 mg/kg body weight.

Feeding pigs with the two DON positive samples may result in their exhibiting decreased feed consumption and weight gains. However, the actual feeds were relatively free from DON.

4.4 LABORATORY INOCULATED SAMPLES

The results obtained from the Laboratory inoculated samples are given in Tables 3.5 and 3.6.

In the early stages of the experiment, more Zearalenone was produced in the flasks which were kept at 26°C than in those at 16°C. This could have come about as a result of the vigorous growth of the fungus at 26°C. However, as time progressed, the fungus in flasks at 16°C became established on the substrate and this coupled with the required low temperatures, brought about an increase in Zearalenone production (Figure 3.11).

Comparing Zearalenone production in Flask No. 1 (at 26°C for 10 weeks) and Flask No. 3 (at 16°C for 10 weeks), initially more Zearalenone was produced in Flask No. 1 while very little was produced in Flask No. 3. The substrate in Flask No. 1 was quickly used up as time progressed and by the end of the sixth week, Zearalenone production was almost negligible (Figure 3.11). This could have been as a result of the fungus losing viability due to lack of substrate. In Flask No. 3, growth appeared to be slow in the initial stages (and less Zearalenone was produced) but production greatly increased after the sixth

week. Similar results were given by Christensen (1979) when F. roseum was grown at 12°C and 25°C. Zearalenone production increased after six weeks when the fungus was grown at 12°C while production by the fungus kept at 25°C for the duration of the experiment reached its maximum after the sixth week.

Sherwood and Peberdy (1972) working on the factors affecting the production of Zearalenone by F. graminearum in grain found that growth at low temperatures (12°C) after a period at 25°C appeared to enhance Zearalenone production. Eugenio et al.(1970) had earlier recorded similar results. In further experiments this was found to be only the case when the fungus had become established on the grain at 25°C. This may explain why Zearalenone production in Flask No. 3 only increased greatly after the sixth week. They suggested that reduced growth rate of the fungus when transferred from 25°C to 12 °C resulted in the switching of carbon metabolism to other biosynthetic pathways leading to increased Zearalenone production.

The results obtained for Flask No. 2 (5 weeks at 26°C followed by 5 weeks at 16°C) and Flask No. 4 (5 weeks at 16°C followed by 5 weeks at 26°C) appear to be inconsistent with those obtained by the above authors. Zearalenone production in Flask No. 2 did not increase as expected when the flask was transferred to 16°C. On the other hand, Zearalenone production in Flask No. 4 increased upon transferring to 26°C.

A possible explanation of the results obtained in Flask No. 2 would be that most of the substrate may have been used up while the flask was still at 26°C resulting in the fungus losing viability so that the change in temperature had little influence on toxin production.

Two possible reasons can be given for the increase in Zearalenone production in Flask No. 4. One would be that the carbon metabolic pathways leading to increased Zearalenone production may have been stimulated before the flask was transferred to 26°C. This may explain why Zearalenone production in Flasks No.3 and No. 4 increased at almost the same time. The other reason could be that the increase in Zearalenone production was merely due to increased growth rate of the fungus i.e. there was more fungus producing Zearalenone but not necessarily at an increased rate. Alternatively, the increased growth rate at 26°C resulted in competition for the limited substrate which led to the switching of carbon metabolic pathways to increased Zearalenone production.

Naik et al. (1978) reported that Zearalenone production is influenced by both temperature and the strain of Fusarium graminearum used. Working with five strains of F. graminearum, they found that Zearalenone production was enhanced by temperature stress (reducing the incubation temperature to 10°C following a period of growth at 25°C) in only one of the strains tested. This may partly explain the results obtained in this study. Low temperatures (16°C) favour Zearalenone production in the strain of F. graminearum used in this study.

DON production on the other hand appeared to be favoured by high temperature (26°C). This is consistent with results obtained by Ueno (1977), and Yoshizawa and Morooka (1977). They found that DON production was higher at higher temperatures (22-28°C) than at low temperatures. This is even more marked in Flask No. 4 where DON production increased considerably when the flask was transferred from 16°C to 26°C. The least amount of DON was produced in Flask No. 3 which was kept at 16°C for 10 weeks.

Production of the two toxins, Zearalenone and DON, is stimulated by different temperatures. This may explain why they do not always appear together in the same sample even though they are produced by the same fungus.

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