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Summary

Although species of the genus _Fusarium_ are widespread in their distribution, the natural occurrence of the toxins elaborated by these moulds has not, until recent years, been the subject of a major study. This is partly because sensitive methods which can readily be applied to nationwide surveillance programmes have been difficult to develop for the trichothecene mycotoxins produced by the fusaria. The trichothecenes have an epoxide function as a common feature of their chemical structure, and this thesis describes the reaction of the trichothecenes with a nucleophilic reagent (sodium diethyldithiocarbamate) and the subsequent development of a method based on this reaction. This method was then applied to the examination of food, and feeding stuffs and to studying the fate of deoxynivalenol through a food processing system. Although samples with levels of trichothecene contamination greater than 20μg/kg were also assayed by a different method of detection, levels below this could not be confirmed because of the lack of a suitable alternative method of adequate sensitivity. In common with the data generated from other surveillance programmes, it was found that the trichothocenes occurred occasionally at very high levels, especially in cereals.

There were clear indications that the nature of the reaction occurring between trichothecenes and sodium diethyldithiocarbamate (DDTC) was not analogous to that which was reported in work on an epoxide containing anti-cancer drug and DDTC, indicating nucleophilic substitution of the epoxides. Some 18 months later, independent research work proved that indeed nucleophilic substitution was not occurring, and that the reaction product being estimated was the result of a change in the DDTC reagent which was not mediated by mycotoxins. Apart from the results confirmed by alternative techniques therefore, the surveillance data must be viewed with strong reservations.
A review of the current situation regarding analytical method development for the trichothecenes indicates that considerable efforts are being made in many areas. As yet, however, there is little published data on the incidence of trichothecenes in food, and therefore still a lack of information available on which to base assessments of whether fusarial toxins pose any risk to human health.
ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisors Mr W B Chapman and Dr M O Moss for their support, and to the Food RA and MAFF for making this research possible.

I would also like to thank Dr A W Holmes for allowing me the facilities for presenting this thesis and my colleagues for assisting in producing it.

Finally I would like to thank my family, especially my mother, my husband Alan and my son Andrew for all their support and encouragement.
CHAPTER 1

INTRODUCTION

Food may be invaded by moulds at many stages in its production, and indeed mould may be deliberately inoculated on to food, or allowed to proliferate, to give a ripened and distinctive character as in the case of some cheeses or sausages. Unfortunately however, most of the mould proliferation which we find on food is not associated with desirable effects. The growth of moulds on foods can be a significant cause of illness, especially if the moulds elaborate physiologically active toxic agents or mycotoxins.

There are several main stages during which moulds may infect foods (Moreau, 1974).

i. Pre-harvest e.g. by growing on grain ears. The species associated with this type of mould invasion are termed field fungi and include the genera *Alternaria*, *Fusarium*, *Helminthosporium* and *Cladosporium*.

ii. During storage of grain prior to use - the "storage fungi" which principally comprise the genera *Aspergillus* and *Penicillium*.

iii. During food processing; at this stage moulds may be deliberately added to the food. Thus mould ripened cheeses are inoculated with strains of *Penicillium roqueforti* or *Penicillium camemberti* in order to produce characteristically flavoured and moulded blue or white cheeses.
During storage of the finished product. This may be actually encouraged in some types of ripened foods such as continental sausages but more commonly, the mould is not a desirable component of the food.

This succession of moulds, largely depends on ecological conditions determining which genera are favoured. Two major factors affecting mould growth are moisture content and temperature. Several workers (Del Prado & Christensen, 1952; Iizukah, 1958; Tsunoda, 1953; Tsunoda, 1963; Tuite & Christensen, 1955; Tuite & Christensen, 1957) have reviewed the critical role played by the moisture content in determining which moulds are likely to invade cereals. Many of the studies have been by Japanese scientists studying the flora of rice (Kurata et al. 1968; Naito, 1953), but considerable work has also been done on other cereals such as wheat, corn and barley (Tuite & Christensen 1957; Christensen & Gordon, 1948). Since the moisture content and temperature are partially dependant on geographical location, the predominant types of moulds invading food vary throughout the globe. Thus in temperate climates Penicillium and Fusarium are the most abundant genera whereas aspergilli are more favoured by tropical conditions (Moreau, 1974). In addition, variations in the 'local' micro-climate which can exist within a large grain storage silo, may give rise to pockets of localised mould growth. The growth of moulds on foods and the health hazards they may present have been recognised for many years. Indeed Schoental (1980) reviews the biblical connections between avoidance of such health hazards by recognition of particular mould types and the Mosaic proscriptions. This entertaining view which is certainly feasible, would precede the
earliest recorded illness now known to be associated with consumption of mouldy food, that of St. Anthony's Fire. This disease, which has afflicted man since the Middle Ages, arises from the toxic ergot alkaloids produced by the ergot fungus *Claviceps purpurea*; these alkaloids were identified in the 1930-40's. However the episode which aroused the current interest in mycotoxins occurred in 1960 as a result of an economic disaster. Thousands of turkey poults died after consumption of food containing Brazilian ground-nut meal; this episode, called Turkey X disease, culminated in the isolation and identification of aflatoxin B$_1$ as the toxic principle. Our current interest in toxic metabolites of moulds, the mycotoxins, has derived largely from this time, and most of the scientific studies therefore are confined to the last two decades. The indications are that mycotoxins still contribute to human disease, especially in third world countries, where poor nutrition may necessitate the consumption of food which has become mouldy. In developed countries, it may be that mycotoxins are implicated in some chronic diseases; the working party on mycotoxins of MAFF is currently investigating the incidence of mycotoxins in UK food (Anon, 1980). The presence of moulds on foods does not necessarily indicate that mycotoxins will be present, since not all moulds are toxigenic, and even for toxigenic moulds specific conditions only favour mycotoxin production. Conversely a foodstuff may apparently be free from mould, but may contain high levels of toxins (Cooper, Wood and Chapman, 1982). For aflatoxins, the environmental conditions resulting in the maximum yield have been studied in depth by various workers (Mateles & Wogan, 1967; Hesseltine *et al.*, 1970; Diener & Davis, 1966).
In a survey of mould damaged food at the Food Res. Association some samples were completely mould-ridden and visually unacceptable but contained no detectable mycotoxins. Some samples, for example, one batch of peanuts, were not visibly mouldy although they did have an unsavoury appearance. These samples contained up to 8,000 parts per billion aflatoxins. For some food commodities, the type of mycotoxins detected fell into a pattern; thus aflatoxins were the predominant mycotoxin in nuts, and patulin was associated with fruit products. This is undoubtedly related to the dominant mould type for these products. Composite commodities, such as meat pies, did not have a predominant type of mycotoxin present.

Many moulds are toxigenic when cultured in the laboratory, but may not produce mycotoxins in a food such as cheese. Thus *Penicillium roqueforti* and *Penicillium camemberti* can elaborate many different mycotoxins. (Lafont, et al. 1979; Lafont et al. 1976; Wei & Liu, 1978) but some of these toxins are not stable in the food. PR toxin, an acutely toxic metabolite of *Penicillium roqueforti* is thus unstable in cheese, breaking down to PR imine, whose toxicity is considerably less than that of PR toxin. (Scott & Kanhere, 1979). Leistner and Pitt (1977) did a comprehensive review of *Penicillium* isolates from meat products in order to establish how many of the species isolated were toxigenic, which mycotoxins these isolates elaborated and what factors affected the mycotoxin formation in food. In summary, 73% of the isolates were toxigenic, and a third of these toxigenic isolates elaborated mycotoxins. Mycotoxins could be detected only in the upper 0.3 cm layer of meat products. Isolation of moulds to species level may
therefore be invaluable in estimating the likelihood of contamination of foods by mycotoxins although the research work at the Food RA (Cooper et al. 1982) has indicated that a positive correlation between the moulds identified and the mycotoxins detected occurred in only approximately 50% of samples. The genera Cladosporium and Penicillium were very commonly identified in mould-spoiled food samples; Fusarium was not isolated at all. Fusarium moulds generally occur as field fungi and are very widespread in nature. Many surveys of cereals and feeding stuffs have indicated the frequency of species of Fusarium invading these commodities, and since many of these moulds are toxigenic, they are of great importance from the veterinary and human health point of view. Thus in a survey in Canada of corn, the mycotoxins produced by Fusarium spp were the most frequently isolated toxins (Andrews, Thompson & Trenholm, 1981), and in 1980 (Trenholm et al. 1981; Scott, Lau & Kanhere, 1981) contamination of the wheat crop by fusarial toxins was virtually 100%. A survey of the 1973 crop (Eppley et al. 1974) had indicated similar contamination and other surveys of feedstuffs (Mirocha et al. 1976) confirm this high incidence of contamination. Animal feeds and cereals are susceptible to invasion by Fusarium both before harvesting and during storage in silos. As yet, little is known specifically about the environmental conditions which permit lethal levels of mycotoxins to be produced, but considerable research is in progress in this area. Strangely enough, although the growth of the fusaria is encouraged by wet climatic conditions, surveillance of the Canadian crop (Trenholm et al. 1981) indicates that mycotoxin production may occur under drier conditions than is generally believed. However,
high rainfalls in the year 1980 had probably contributed to a very poor crop for that year, and indeed some feeds prepared from this crop did cause several outbreaks of illness in pigs. Surveillance of animal feeding stuffs conducted by ADAS in this country (Benham, 1982) did not indicate a significant incidence of contamination by fusarial toxins; this may partly be accounted for however, by the analytical method of detection used.

Species of the genus *Fusarium* produce several toxic metabolites. These metabolites include the closely related group of toxins called the trichothecenes, and a macrocyclic phenolic metabolite: zearalenone. In addition, several species can also produce moniliformin and butenolide: Figs 1.1, 1.2 show the structures of these latter two toxins. Of 13 isolates tested by Rabie et al. (1978) for moniliformin production, all were highly toxic. The ability to produce moniliformin is therefore widespread, but the quantities of toxin produced varied greatly amongst isolates. Screening of isolates of fusaria for toxic activity has been conducted in several surveys by monitoring of biological activity (Yagen & Joffe 1976, Yates, Tookey & Ellis, 1970), but this is a rather non-specific measure of toxicity. Whilst the toxic metabolites moniliformin and butenolide are elaborated by fusaria, the study presented here is confined to the incidence of trichothecenes and zearalenone in foodstuffs and animal feeds, and the fate of these toxins through processing systems.

Although zearalenone and the trichothecenes are both products of the secondary metabolism of *Fusarium*, they are entirely dissimilar in terms
of chemical structure and toxic activity, and are derived from different biosynthetic pathways. Fig. 1.3 shows the chemical structure of zearalenone which is a straight-forward polyketide metabolite. This toxin shows considerable oestrogenic activity, being only slightly less potent when administered sub-cutaneously than oestradiol-17β or DES (diethylstilbestrol) (Hobson et al. 1977). The most susceptible farm animal to the effects of zearalenone in the feed is the pig, although hyperoestrogenism and abortion have been reported in cattle and other farm animals (Mirocha, Pathre & Christensen, 1977).

The trichothecenes are sesquiterpene alcohols or esters derived from the mevalonate pathway, and are characterised by an olefinic double bond between C9 and 10 and by (with one exception), an epoxide function at C12 and 13: Fig. 1.4 shows the principal structure of the trichothecenes. It is usually convenient to classify trichothecenes into groups according to their chemical characteristics and Ueno (1977) classifies them as follows:-

Group A members have no carbonyl at the C8 position. This group includes T-2 toxin, HT-2, diacetoxyscirpenol and neosolaniol as the more commonly occurring trichothecenes.

Group B members have a carbonyl group at C8 and include nivalenol, deoxynivalenol (vomitoxin) and fusarenon X.

Group C are characterised by a macrocyclic lactone linking $R_3$ and $R_4$; this group includes the verrucarins, roridins and satratoxins. (It should be noted that satratoxins are not produced by species of Fusarium but by Stachybotrys and are not included in this study.)
Fig. 1.1  Moniliformin

\[
\begin{array}{c}
\text{O} \\
\text{O-R} \\
\text{R = Na}^+ \text{ or } K^+ \\
\end{array}
\]

Fig. 1.2  Butenolide

Fig. 1.3  Zearalenone
Fig. 1.4 The Structure of the Trichothecone skeleton

<table>
<thead>
<tr>
<th></th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$R_4$</th>
<th>$R_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5-2$ toxin</td>
<td>$\text{OCC} \cdot \text{CH}_2 \cdot \text{CH} \cdot (\text{CH}_3)_2 \cdot \text{H}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
</tr>
<tr>
<td>$5-2$ toxin</td>
<td>$\text{OCC} \cdot \text{CH}_2 \cdot \text{CH} \cdot (\text{CH}_3)_2 \cdot \text{H}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
</tr>
<tr>
<td>Dicistrosporin</td>
<td>$\text{OH}$</td>
<td>$\text{H}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OH}$</td>
</tr>
<tr>
<td>Neosolanol</td>
<td>$\text{OH}$</td>
<td>$\text{H}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OH}$</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>$\text{C} = \text{O}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>$\text{C} = \text{O}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
</tr>
<tr>
<td>Fusorinon x</td>
<td>$\text{C} = \text{O}$</td>
<td>$\text{OAC}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
<td>$\text{OH}$</td>
</tr>
</tbody>
</table>

Verucorin A: Macrocyclic lactone linking $R_3:R_4$; $\text{CH} = \text{CH}$, $\text{CH} = \text{CH}$.
Group D of which there is only one member, crotocin, has a second epoxide at C7 and 8.

The chemical distinction between Group A and Group B type trichothecenes confers many marked differences in both their behaviour during chemical analysis and in terms of their toxicity. Pathre and Mirocha (1977) classified the trichothecenes in order of their polarity and solubility in solvents; whilst this classification is valuable for determining the most appropriate method of analysis, it does not lend itself to grouping according to the chemical characteristics most commonly used for analysis and for distinguishing the different types of trichothecenes in a sample extract. Thus using Ueno's classification, the Group B type including deoxynivalenol give a yellow colour on reaction with p-anisaldehyde, while the Group A type give a purple colour. Other spray reagents for TLC will distinguish between class members; thus p-dimethylamino-benzaldehyde in concentrated HCl is reported by Dawkins (1966) to give a violet colour with diacetoxyscirpenol but not with T-2 toxin, both of which are Group A type trichothecenes. The distinctions in Group A and Group B trichothecenes also results in large differences in the Rf values on silica gel TLC plates. In practice, these differences in Rf value can be usefully applied by chromatographing extracts several times in solvents which are appropriate for each group. A systematic method of optimising the detection of Fusarium mycotoxins by utilising the differences in polarity and chromatographic behaviour has been devised by Kamimura et al. (1981).
The toxins are extracted with a very polar solvent (methanol/water) and then fractionated from an amberlite chromatography column. A four-step TLC analysis comprehensively detects nivalenol, deoxynivalenol, fusarenon X, diacetoxyscirpenol, neosolaniol, T-2 toxin, HT-2 toxin, butenolide, moniliformin and zearalenone by utilising their functional properties.

The biological activity of the Group A and Group B trichothecenes is markedly different. Thus comparing the skin-necrotising dose induced in experimental animals, for T-2 toxin the minimum effective dose for guinea-pigs is 0.2 µg/spot, whereas for nivalenol it is 10 µg/spot (Ueno et al. 1970); similarly comparing inhibition of protein synthesis in rabbit reticulocytes, the inhibitory dose for T-2 toxin is 0.03 µg/ml, but for nivalenol is 3.0 µg/ml. (Ueno et al. 1973).

The biosynthesis of the trichothecenes is comprehensively reviewed by Tamm and Breitenstein (1980) in Steyn and by Manitto (1981). Structurally similar, they are all derived from a common tricyclic skeleton-trichothecane, and mechanisms have been proposed for their biosynthesis. Amongst the macrocyclic group (the "roridin" group) of trichothecenes, several compounds containing further epoxides in the macrocyclic grouping have been isolated (Matsumoto et al. 1977). A triepoxide-baccharin- has been isolated from Baccharis megapotamica (Kupchan et al. 1976) and is the first reported isolation of a trichothecene derivative in higher plants. It is probable, however, that this compound is of fungal origin (deriving from fungi in the soil). Zearalenone is biosynthesised from the polyketide pathway
(Steele, Leiberman & Mirocha, 1974). There are several reports of both trichothecenes and zearalenone being elaborated by Fusarium species; thus Vesonder and co-workers (Vesonder, Ellis & Rohwedder, 1981) report both zearalenone and vomitoxin being produced by all 16 strains belonging to F. graminearum and F. culmorum. Mirocha et al (1976) report the detection of diacetoxyscirpenol, deoxynivalenol and zearalenone from feeding stuffs contaminated with F. roseum.

Although there are many well documented reports of outbreaks of animal disease after consumption of mouldy feedstuffs (Ueno, 1977) it is only comparatively recently, (mainly within the last decade) that such outbreaks have been attributed to mycotoxins. Zearalenone, by virtue of its oestrogenic activity is associated with hyperoestrogenism in farm animals and more particularly with vulvo-vaginitis in pigs. The trichothecenes are now thought to have been implicated in ATA (alimentary toxic aleukia) which killed 10% of the population in Orenburg near Siberia in 1944. This illness which gave rise to symptoms of vomiting, diarrhoea, multiple haemorrhage and exhaustion of the bone marrow, followed the consumption of over-wintered wheat, contaminated with Fusarium sporotrichioides and F. poae.

The most recent claims concerning the toxicity of the trichothecene mycotoxins occur in reports of "Yellow Rain". It has been suggested that trichothecenes are being used as biological warfare weapons and these suggestions are supported by the analysis of clinical samples, which have indicated that 16 out of 33 samples contained T-2, HT-2 or diacetoxyscirpenol (Anon, 1982). Other claims for the notoriety of the
trichothecenes have included comments by Schoental (1982) that mycotoxins may be implicated in the disorders associated with consumption of alcoholic beverages. For example "Cobalt Beer Drinkers Cardiomyopathy", several outbreaks of which have occurred in the winter of 1965-1966 in Quebec, Omaha, Minnesota and Belgium, may be related to the presence of mycotoxins in the beer. There is also circumstantial evidence, namely that fatalities in cattle occurred in Wisconsin in the same year after consumption of mouldy fodder contaminated with Fusarium tricinctum. Similarly, "fetal alcohol syndrome" may be associated with the cheaper grade beer consumed by the lower socioeconomic groups. In Table 1.1, some of the data linked with trichothecene toxicosis is given. Since many of the incidents occurred several decades ago, it has generally not been possible to obtain suitable samples for analysis, although reconstruction of the historical data and analysis of similar foodstuffs has clearly indicated that several fatal outbreaks of intoxications were almost certainly associated with trichothecene poisoning. Clinical symptoms have been reported in a number of cases after consumption of mouldy feed or feed contaminated with trichothecenes (Yagen & Joffe 1976; Yates, Tookey & Elliss 1970; Mirocha et al. 1976).

It is pertinent to note here that clinical toxicity in experimental animals may not necessarily reproduce the effects observed in nature. Thus pure toxins alone, or extracts of the toxins may give few, if any of the symptoms elicited by contaminated feed. Different animal species also show different responses to trichothecenes. Hence pigs will refuse barley infected with 5% of seeds infected with Fusarium graminearum, but
<table>
<thead>
<tr>
<th>Year</th>
<th>Geographical Location</th>
<th>Species Affected</th>
<th>Disease</th>
<th>Moulds</th>
<th>Possible Mycotoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1890</td>
<td>Siberia</td>
<td>Man, farm animals</td>
<td>Taumelgetreide toxicosis</td>
<td>Gibberella, Cladosporium, Fusarium</td>
<td>Trichothecenes</td>
</tr>
<tr>
<td>1942/7</td>
<td>Orenburg, USSR</td>
<td>Man</td>
<td>ATA</td>
<td>F. sporotrichioides, F. poae, C. epiphyllum</td>
<td>Trichothecenes</td>
</tr>
<tr>
<td>1931</td>
<td>USSR, Hungary</td>
<td>Horses, swine calves, poultry</td>
<td>Stachybotryotoxicosis</td>
<td>Stachybotrys alternans</td>
<td>Satratoxins</td>
</tr>
<tr>
<td>1929</td>
<td>USA</td>
<td>Pig, cow</td>
<td>Mouldy corn toxicosis</td>
<td>F. tricinctum</td>
<td>Trichothecenes</td>
</tr>
</tbody>
</table>

*After Venko (1977)*
if they do consume it, they vomit and become somnolent. Ruminants however can tolerate a diet containing 56% infected grain (Roche & Bohstedt, 1931); in poultry, a drop in egg production may result from consumption of contaminated feed.

Since the fusaria are principally field fungi, many cereal crops are liable to contamination by trichothecenes. Furthermore, the trichothecenes are very stable and because they are unlikely to be degraded by many food processes, may persist into foodstuffs. A greater understanding of the causal relationships between environmental factors and toxin production is essential to control the quality of raw materials and feedstuffs. Although the information is so incomplete, Table 1.2 gives some data on the substrates from which species of Fusarium have been isolated and some of the toxic effects documented for these substances. The toxic effects of fusarial toxins are diverse, but the knowledge of diseases now linked with ingestion of these toxins has centred on retrospective studies of the acute rather than chronic symptoms. Little is known of the effects of sub-lethal levels of trichothecenes in the diet, be they derived directly from consumption of cereals or derived from products of animals consuming trichothecenes in their feedstuffs to give a food chain-effect. There is a paucity of published data for chronic studies. Ohtsubo and Saito (1977) concluded from experimental trials on mice that trichothecenes could not be considered carcinogenic substances. Unusual tumours were obtained in the experimental group after dosage with fusarenon X, but these were not apparently dose-related. Therefore, whilst fusarenon X does inhibit growth and resistance to infection, the carcinogenicity was not proven.
TABLE 1.2
Some of the substrates from which Fusaria have been isolated and associated diseases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mould species isolated</th>
<th>Animal Species affected</th>
<th>Disease type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds</td>
<td><em>Fusarium nivale</em>&lt;br&gt;<em>F. tricinctum</em>&lt;br&gt;<em>F. roseum</em>&lt;br&gt;<em>Trichothecium roseum</em>&lt;br&gt;<em>Trichoderma viride</em></td>
<td>Pig, Horse, sheep</td>
<td>Gastro-intestinal disorders</td>
</tr>
<tr>
<td>Straw, Cereals</td>
<td><em>F. sporotrichioides</em>&lt;br&gt;<em>Stachybotrys atra</em></td>
<td>Man, horse</td>
<td>Haemorrhagic</td>
</tr>
<tr>
<td>(incl. millet)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cereals</td>
<td><em>F. nivale</em></td>
<td>Man</td>
<td>Neurotoxic</td>
</tr>
<tr>
<td>Maize</td>
<td><em>F. graminearum</em></td>
<td>Pig, poultry</td>
<td>Oestrogenic-abortive</td>
</tr>
</tbody>
</table>
Schoental (1982) comments on the possible carcinogenicity of the trichothecenes in alcoholic beverages, especially since deaths from cobalt beer drinkers cardiomyopathy have been associated with gastro-intestinal abnormalities; these symptoms could not be accounted for by the cobalt content of the beer, even when considering the possible nutritional status of the beer-drinkers. Nutritional status is of importance in experimental induction of aflatoxin injury, and is likely to be so for trichothecene toxicosis.

In aflatoxin-induced disease, malnutrition and a high incidence of liver cancer are related (Newberne & Gross, 1977). More particularly it would seem that aflatoxin and pyridoxine deficiency in the diet result in liver damage. Considerable data is reviewed by Newberne and Gross (1977) presenting this and detailed information on the role of nutrition in liver damage by aflatoxin. In the case of trichothecenes, there are indications that poor nutritional status was involved in A.T.A. and the provision of a good diet was essential for recovery of victims.

The acute effects of trichothecenes and zearalenone are now well documented, largely as a result of concentrated toxicological studies on these mycotoxins. Many of the acute effects of the trichothecenes have been used to some advantage in the design of assay techniques, particularly bioassays and these effects fall into several broad categories.

1. Emetic and haemorrhagic; this effect includes the "feed refusal syndrome" which is considered to be characteristic of deoxynivalenol contamination of food. Haemorrhagic bowel
syndrome and bloody stools are further pathological conditions arising from trichotheccene poisoning. Mirocha et al. (1976) isolated diacetoxyscirpenol, deoxynivalenol and zearalenone from feedstuffs associated with emetic and haemorrhagic diseases in farm animals. "Vomiting doses" have been used to define the order of toxicity of the trichotheccenes expressed as a 'minimum emetic dose' (Sato & Ueno, 1977; Yoshizawa & Morooka, 1974; Ueno et al 1971).

A typical vomiting dose for the duckling, which is a sensitive animal test for this effect, is 0.1 mg/kg s.c. for T-2 toxin, and 0.4 mg/kg s.c. for fusarenon X. This order of toxicity follows that observed for other biological effects and is related to the grouping of the trichotheccenes, as defined by Ueno (1977). The emetic principle has been associated with grain infected with Fusarium graminearum (Christensen & Kernkamp, 1936; Dounin, 1926) and with F. culmorum and F. avenaceum (Prentice, Dickson & Dickson, 1954).

2. Cutaneous irritation or dermatitic reactions. These effects of trichotheccenes have been used as a screening method for toxic isolates in the rabbit or rat skin patch test assay. The intensity of the skin reaction can be used as a crude guide to concentration of the toxin. Eppley in 1975 (Eppley, 1975) considered skin irritation to the rabbit to be the most promising biological assay, and many workers have used this type of test as a guide to the toxicity of crude extracts from mouldy feeds and Fusarium isolates, (Eppley, 1975; Hayes & Schiefer, 1979; Yagen et al. 1977; Ghosal et al. 1982).
3. Inhibition of protein synthesis. The trichothecenes are potent inhibitors of protein synthesis in eucaryotic cells. This effect has been used to advantage in biological assays for trichothecenes; notably the rabbit reticulocyte cell system assay (Terao & Ueno, 1978). They demonstrated inhibition of protein synthesis by nivalenol and fusarenon X. McLaughlin et al. (1977) studied the mechanism of action of the trichothecenes in their inhibitory action. Their findings indicated that the trichothecenes worked by one of two mechanisms; one group inhibited the initiation of synthesis (which includes T-2 toxin and diacetoxyscirpenol), whilst the other group inhibited termination or elongation by binding to the ribosome and inhibiting peptidyl transferase. The protein synthesis inhibition is thus specific and sensitive. (Ueno & Shimada, 1976)

4. Oestrogenic: this effect is confined to zearalenone and is associated with abortion and vulvo-vaginitis in pigs fed contaminated feedstuff. Reports of this effect of zearalenone are numerous and many surveys of food and feeding stuffs have established that zearalenone has been detected frequently in maize throughout the world. (Eppley et al., 1974; Andrews et al., 1981; Shotwell, 1977; Anon, 1980; Stoloff, Henry & Francis, 1976). The anabolic steroid-like activity of zearalenone has been used for fattening livestock. Indeed there are commercial patents for the preparation of zearalenone and derivatives in the United States. Hobson (Hobson et al., 1977) devised a model to predict the effects of low level exposure of man to zearalenone, in view of the high incidence of zearalenone, particularly in cereals.
From the world-wide view point, there is little information on the distribution of toxigenic fusaria in both food and feeding stuffs, and under what climatic and specific environmental conditions these fusaria produce toxins. Similarly, our knowledge concerning the occurrence and levels of fusarial toxins in the normal food comprising our diets is lacking. In 1980 (Anon, 1980) MAFF recommended that further work be carried out on raw materials, particularly maize, since this cereal is susceptible to contamination by a wide range of mycotoxins. Lack of surveillance data for the trichothecenes is undoubtedly due to the absence of suitable methodology which would permit appropriate large scale surveys of foods and feeding stuffs. At present, food surveillance programmes exist to monitor selected food for a range of mycotoxins, and generally include aflatoxins, ochratoxin, sterigmatocystin, zearalenone and a few toxins produced by the genus *Penicillium*. For these mycotoxins, adequate methodology of suitable sensitivity has been developed. The considerable interest in the epidemiological data linking diseases in man with ingestion of mycotoxins has generated the impetus for detailed analytical investigations in the case of these toxins. Thus primary liver cancer in Kenya linked with aflatoxin contaminated food (Anon, 1969) and Balkan nephropathy with ochratoxin A contaminated food (Krogh, 1977) have arisen from such detailed investigation. As for the toxins produced by fusaria, there are several cases associating lethal toxicosis with trichothecenes, particularly in animals, but for the most part these are retrospective investigations into the acute and dramatic effects of trichothecenes. The toxic effects of trichothecenes are therefore
observed after catastrophic events have occurred, rather than a methodical approach of surveillance to identify potential problem areas in advance. A clear need still remains for suitable methodology as an essential undertaking for comprehensive studies in the immediate future.

From the analyst's viewpoint, the trichothecenes present a difficult problem. They are not amenable to direct spectroscopic or fluorimetric analysis, except at very high concentrations. Thin layer chromatography has poor limits of detection, especially in naturally contaminated samples. Good resolution of individual trichothecenes can however, be obtained by this means. Some bioassays can be very sensitive to specific trichothecenes, but the sensitivity is very dependant on the group type of trichothecene (Ven0, 1977). Gas chromatographic methods have been used, usually with success on trichothecenes possessing a free hydroxyl group. Generally the trimethylsilyl (TMS) or trifluoracetyl (TFA) derivatives are prepared for analysis. Mass-spectrometry is unfortunately the only reliable means of identifying each trichothecene. Liquid chromatography can be used for the satratoxins (Stack & Eppley, 1980), and may be used for detecting deoxynivalenol at 215 nm. This wavelength does however, require a UV detector with rather exceptional properties, especially since most solvents absorb at this wavelength (See Table 1.5).

Liquid chromatography also provides a sensitive assay for zearalenone, as detailed by Chaytor (1981). Other assays for trichothecenes have included an enzymic assay (Foster, Slater & Patterson, 1975) in which the trichothecene is reacted with GSH in the presence of glutathione
S-epoxidetransferase; the residual GSH is then reacted with 5, 5
dithiobis- (2-nitrobenzoic) acid and the rate of formation of the
coloured ion 5-mercaptop, 2 nitrobenzoic acid measured. Of the more
promising recent developments in analytical methodology, capillary
column gas chromatography with electron capture detection is sensitive
and selective, and can be used to detect several trichothecenes
simultaneously. Szathmary et al. (1980) show how this analysis can be
used to achieve successful resolution of several trichothecenes. In
Canada, where the high incidence of crop contamination by deoxynivalenol
has necessitated surveillance and regulatory practice, GC with ECD and
mass spectrometry are routinely used. Tables 1.3-1.5 summarising the
current state of the art of analytical method development and indicating
typical detection limits are given below.

Table 1.3 shows typical detection limits for some of the assay methods.

Table 1.4 shows some of the most commonly used spray reagents for
detecting trichothecenes by TLC.

Table 1.5 indicates some of the latest developments in analytical
methodology together with comments on these methods where possible.

Generally speaking, most routine methods offer either specificity or
sensitivity. Bioassays for example are sensitive, but usually
non-specific, whilst TLC is specific but comparatively insensitive. The
GC methods, being the most sensitive, suffer from considerable sample
interferences, and therefore usually require an extensive and lengthy
### TABLE 1.3

**Typical Detection Limits for Some Trichothece Assay Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Trichothecene</th>
<th>Detection Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Dimensional TLC</td>
<td>T-2</td>
<td>0.07-0.10µg/spot (after spraying)*</td>
</tr>
<tr>
<td></td>
<td>DAS</td>
<td>0.5-0.10µg/spot (after spraying)*</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>1.0-1.5µg/spot (after spraying)*</td>
</tr>
<tr>
<td>2-Dimensional TLC</td>
<td>DON</td>
<td>0.01µg/spot (after spraying)*</td>
</tr>
<tr>
<td>GC. of TMS ester</td>
<td>T-2</td>
<td>0.03-0.05µg/µl</td>
</tr>
<tr>
<td></td>
<td>DAS</td>
<td>0.05µg/µl</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>0.02µg/µl</td>
</tr>
<tr>
<td>GC - MS + (in SIM mode)</td>
<td>T-2</td>
<td>0.02µg/µl</td>
</tr>
<tr>
<td></td>
<td>DAS</td>
<td>0.007-0.01µg/µl</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>0.007µg/µl</td>
</tr>
<tr>
<td>Rat Patch test</td>
<td>T-2</td>
<td>70-100µg</td>
</tr>
<tr>
<td></td>
<td>DAS</td>
<td>35-75µg</td>
</tr>
<tr>
<td>oBHK Cells</td>
<td>HT-2</td>
<td>0.01µg</td>
</tr>
</tbody>
</table>

* See table 1.4
+ SIM = Selective ion monitoring
° BHK = Baby Hamster kidney

After Samré and Mirecha (1977)
**TABLE 1.4**

Spray Reagents for TLC "Visualisation" of Trichothecenes Under UV

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Type of trichothecene</th>
<th>Colour (after spraying)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{SO}_4$</td>
<td>T-2 type</td>
<td>Blue/green</td>
</tr>
<tr>
<td></td>
<td>DON type</td>
<td>Yellow/brown</td>
</tr>
<tr>
<td>Alcoholic AlCl$_3$</td>
<td>DON type</td>
<td>Purple/blue</td>
</tr>
<tr>
<td>p-Anisaldehyde</td>
<td>T-2 type</td>
<td>Purple</td>
</tr>
<tr>
<td></td>
<td>DON type</td>
<td>Yellow</td>
</tr>
<tr>
<td>Ehrlich's Reagent (p-dimethylaminobenzaldehyde in conc.HCl)</td>
<td>DAS</td>
<td>Intense violet</td>
</tr>
<tr>
<td></td>
<td>T-2</td>
<td>No colour</td>
</tr>
</tbody>
</table>

*After Fannek Hiroea (1977)*
### TABLE 1.5

Current Developments in Analytical Methodology for Trichothecenes

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Detection Limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. G.C. with capillary column</td>
<td>50ppb for most trichothecenes</td>
<td>Bijl et al. (1982)</td>
</tr>
<tr>
<td>2. HPLC/UV @ 215nm +</td>
<td>25ppb DON</td>
<td>Bohm, Schuh &amp; Leibetseder (1982)</td>
</tr>
<tr>
<td>3. &quot;Kit&quot; incorporating* TLC Spray Reagent</td>
<td>Stated to be 0.5ppm DON</td>
<td>'Myco-chek' (LSB Products).</td>
</tr>
<tr>
<td>5. ELISA</td>
<td>2.5 p.g. T-2</td>
<td>Pestka et al. (1981)</td>
</tr>
</tbody>
</table>

+ The majority of UV detectors cannot be used to monitor compounds with $\lambda$ max at 215nm.

* This kit is designed as a qualitative test, assuming that levels of below 1.0 ppm deoxynivalenol (and fusarenon X type compounds) are safe to use. The kit is currently being evaluated at the Food RA but is basically a TLC/colorimetric method.
sample clean-up. A method, defined by the following parameters is clearly needed;

a. Specific for trichothecenes (and preferably readily applicable to several trichothocenes).

b. Sensitive - ideally to a detection limit of 10 ppb.

c. Rapid enough to permit screening of large numbers of samples.

d. Selective enough to suffer minimal interferences in sample matrices.

e. Utilises standard laboratory equipment available to most laboratories and not prohibitively expensive.

An examination of the chemical structures of the trichothecenes reveals the most outstanding common feature of the group to be the epoxide ring at C12, 13. A derivatisation reagent which was selective for epoxides could provide a selective method, thus meeting criterion (a.). If the derivatisation product were amenable to spectroscopic detection, e.g. by UV absorption, (e.) would also be met. The following experimental data are the result of a detailed investigation of the reactions of a nucleophilic epoxide derivatising reagent with the trichothecenes, in a search for the development of a suitable method.

It should be noted that experiments may not necessarily be in strict chronological order. They are categorised by number in order to present the experimental data in a logical format.
CHAPTER 2

MATERIALS

Reagents

Unless otherwise stated, all reagents are 'AR' Grade

Glacial Acetic acid

Propan-2-ol

Hexane (DISTOL grade)

Methanol

Chloroform

Dichloromethane

Peroxide free anhydrous diethyl ether

Benzene

Sodium diethyldithiocarbamate

EDTA - 0.1M Solution in water

Epoxypropane

Acetone

Universal Buffer :

6.008g citric acid )

3.893g potassium )

dihydrogen phosphate ) made up to

1.769g boric acid ) 2 litres water

5.266 g diethyl )

barbituric acid )
Buffer Solutions pH 7.7: (a) 100ml universal buffer + 58 ml 0.2M Sodium hydroxide
(b) 9 vol M/15 sodium phosphate (Na₃H₂PO₄)
+ 1 vol M/15 potassium phosphate (KH₂PO₄)
Sodium metabisulphite 2%(m/v) in water
Hypochlorite Solution: 5 ml industrial Chloros diluted to 100 ml with water
Sodium sulphate - anhydrous
Silica Gel - Merck 7734
Standards

Deoxynivalenol: Initially donated by Dr. B. Osborne, FMBRA and then purchased from Myco Labs USA.
Dianhydrogalactitol: Kindly donated by MRC.

Apparatus

Sep-Paks (Waters Associates)
Millipore single use filtration units 0.45µm: Millex SLSR-025-NS
Filter papers: Whatman No. 4. 11cm and 24cm diameter
Rotomixer-variable speed vortex mixer
Gilson Pipetman: P200µl
Scintillation vials - 20 ml capacity (Camlab Ltd)
Syringes - 5ml and 10ml glass with Luer fitting
Measuring Cylinders
Conical flasks - 250ml capacity with Quickfit neck
Glass funnels - 1cm and 5cm diameter
Chromatography columns: 60cm x 2cm approx with interflon stopcock
Separating funnels - 100ml with interflon stopcock
Silverson sealed blender with 1" tubular head; 16oz oval blending bottles
Waring explosion proof blender and 1 litre stainless steel blending assemblies
Water bath - thermostatically controlled
Bolton and Revis extraction apparatus
Pye SP 800 Recording spectrophotometer
HPLC Equipment

Altex Dual Pump Solvent Delivery System controlled by Model 420 programmer

Column: 25cm x 0.6cm i.d. Amino-propyl silyl bonded silica

Detector: Cecil CE 2112 variable wavelength UV Detector

Data Retrieval: Spectra Physics SP4000 Computing Integrator

Samples

a) General surveillance samples: Samples of breakfast cereals and muesli ingredients were purchased from local retail outlets.

b) Feeding stuffs: These samples were derived from either member companies of the Food RA which had been submitted for analysis, or from ADAS regional laboratories.

c) Mould damaged foods were submitted to the RA for a special MAFF project.

d) Corn grits for cornflake manufacture were supplied by a member company.
CHAPTER 3

METHODS AND RESULTS OF METHOD DEVELOPMENT

The following chapter describes the development of a method utilising a derivatisation technique since the trichothecenes do not have any useful UV absorbance alone.

Experiment 1

Preliminary Investigations of the Reactions of Diethyl-dithiocarbamate (sodium salt) (DDTC) with standard solutions of T-2 toxin

DDTC had been reported by Munger et al. (1973) to be a nucleophilic reagent selective for epoxides. In their experimental work, 5% aqueous DDTC reacted at pH 7.0 with dianhydrogalactitol (an anti-cancer drug) directly in blood plasma to yield a bis-dithiocarbamoyl ester (see Fig 3.1). This ester was amenable to liquid chromatography/UV detection at 254nm. They found it possible to detect 50ng/ml dianhydrogalactitol in blood plasma by derivatisation with DDTC. Fig. 3.2 shows a typical chromatogram obtained by this technique. In their method, to 1 ml of plasma, buffered with the addition 0.5ml of 0.1M potassium phosphate, 0.5ml of 5% aqueous DDTC was added, plus 2ml of water. The reaction mixture was allowed to stand at room temperature for 1 hour, then extracted with chloroform, discarding the aqueous layer. The chloroform layer was washed with sodium chloride, analysing the final extract by HPLC at 254nm; chloroform was found to give optimum extraction of the derivative from the aqueous phase. They characterised the derivatisation reaction as nucleophilic attack on the
Figure 3.1
The Reaction of Sodium Diethylthiocarbamate with dianhydrogalactitol
HPLC chromatograms of the bis (diethyldithiocarbamoyl) ester of DAG
(a) obtained from 1ml plasma containing 250ng DAG and
(b) blank plasma
epoxides of dianhydrogalactitol to give the product shown in Fig 3.1, reacting wholly with carbons 1 and 6, and not C_2 or C_5, to give a single product. Unfortunately, a UV curve of the derivative was not given in the paper. Using similar conditions to those described by Munger et al. (1973) T-2 toxin was exposed to DDTC and the resulting solution analysed. The conditions used were as follows:

To 1ml of T-2 standard containing 100ng T-2 add 0.5ml of phosphate buffer and 0.5ml of 5% aqueous DDTC. Stand for 2 hours at room temperature. Extract with 10 ml of chloroform. Wash extract with 2 x 3ml portions of 33% aqueous sodium chloride. Dry extracts on a water bath (<40°C) under nitrogen, then take up in chloroform prior to analysis. The derivatised extracts were analysed by adding 3ml of chloroform, transferring the solution to a cuvette, and then running a UV scan. The results obtained showed that 30ng T-2 toxin/ml CHCl_3 gave a double peaked UV curve with maxima at 284 and 265 nm. Underivatised T-2 toxin did not give any absorption at this wavelength. The reagent blank gave some absorption at these maxima, but not to the same extent. Using the reagent blank in the reference cuvette, 2.5ng of T-2 toxin/ml CHCl_3 gave a significant UV absorption. Figs. 3.3 and 3.4 illustrate typical UV curves obtained.

Conclusions

1. A solution of T-2 toxin reacts with DDTC to give a product amenable to UV spectroscopy, with a detection limit of approximately 2ng/ml T-2 toxin.
Figure 3.3
UV curves of T-2 derivatised with DDTC and a Reagent Blank

Wavelength (nm)
Figure 3.4
UV curves of T-2 derivatised with DDTC - Reagent Blank as reference

Absorbance

Wavelength (nm)

2.5ng T-2v. Reagent Blank
2. Reagent blanks give a residual absorption which can readily be deducted from the absorption due to trichothecene derivatives by using a reagent blank preparation in the reference cuvette.

3. The UV curve shows a large maximum at 265nm and a smaller peak at 284nm.
Experiment 2

Derivatisation Reactions of T-2 toxin added to samples at various levels

The preliminary investigations, as described in experiment 1, had indicated that DDTC reacted with pure solutions of T-2 toxin. It was now pertinent to know whether T-2 toxin in samples reacted similarly, and whether the sample matrix interfered with the reaction to a significant extent. Samples of processed cereals were ground to a fine powder, and 50 gm portions were spiked with the addition of T-2 toxin solution. After permitting the solvent to evaporate, the samples were then analysed by the method of Eppley as given in Appendix 1. The spiking levels are given in Table 3.1. In summary, the toxins from 50gm of sample are extracted with 250ml of chloroform, and 50ml of the filtered extract 'cleaned up' on a silica gel chromatographic column. Eppley indicated that the 'T-2 fraction' was partially eluted from the silica gel column with anhydrous peroxide-free diethyl ether. (Note: It is important to store diethyl ether in the dark in a cool place to prevent the spontaneous formation of peroxides. These storage conditions are always observed for any mycotoxin analysis using diethyl ether.) Sample blanks (i.e. sample extracts which were underivatised) were prepared for reference. The diethyl ether fractions were evaporated to small volume, on a rotary evaporator, transferred to a clean sample vial (of 20ml capacity) and evaporated to dryness under nitrogen on a steam bath. The extracts (both derivatised and underivatised) were analysed by running UV absorption curves, and the % recovery of
T-2 toxin assessed by comparison with derivatised standard solutions of T-2 toxin, calculating from the absorbance both at 265nm and 284nm. Derivatised sample extracts were reacted as described in Experiment 1 at pH 7.0 for 2 hours at room temperature, diluting the final residue to 3ml. Some of the sample blanks (i.e. underivatised extracts) gave a relatively high absorption, dependent on how processed the cereal was, i.e. mueslis containing a high proportion of little-processed ingredients, such as fruit and nuts, gave measurable absorption both at 265nm and 284nm. This absorption was deducted from the absorption due to the T-2 derivatisation in order to calculate recoveries. In Table 3.1, the spiking levels and the % recoveries of T-2 toxin calculated at 265nm and 284nm are given. Fig. 3.5 shows a typical UV curve from a derivatised spiked sample extract.

Conclusions

1. Sample matrices give some interfering absorption which can be circumvented by always including an identical reference underivatised preparation and deducting this absorption from that due to trichotheccene derivatisation.

2. T-2 toxin added to processed cereal commodities can be analysed by the method of Eppley and subsequently the extracts derivatised with DDTC to give good recovery of the toxin.

3. Recoveries calculated at 284nm are less prone to interference and more reliable than those calculated at 265nm.

4. The sensitivity of this analysis is considerably less than 10ppb.
Figure 3.5
UV curve of Derivatised Sample Extract Containing 7.5ng T-2/ml chloroform
5. The sample matrix does not apparently modify the derivatisation product as indicated by UV curve.
TABLE 3.1

Recoveries of T2 toxin from processed cereals

<table>
<thead>
<tr>
<th>Commodity</th>
<th>ng T-2 toxin added to 50gm sample</th>
<th>Equivalent to ng T-2/ml final solution</th>
<th>ng T-2 determined at 265nm</th>
<th>284nm</th>
<th>% recovery 265nm 284nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puffed rice product</td>
<td>225</td>
<td>7.5</td>
<td>9.8</td>
<td>7.6</td>
<td>131 101</td>
</tr>
<tr>
<td>Puffed rice product</td>
<td>90</td>
<td>3</td>
<td>4.4</td>
<td>2.9</td>
<td>146 97</td>
</tr>
<tr>
<td>Rice Product</td>
<td>225</td>
<td>7.5</td>
<td>9.0</td>
<td>6.5</td>
<td>120 87</td>
</tr>
<tr>
<td>Rice Product</td>
<td>225</td>
<td>7.5</td>
<td>13.5</td>
<td>8.3</td>
<td>180 111</td>
</tr>
</tbody>
</table>

*Note: The filtrate for column-chromatographic clean-up is divided into 8 x 25 ml portions.
Experiment 3

Effects of defatting samples on trichothecene determination

Several samples had been submitted to the laboratory for bioassay testing by the brine-shrimp bioassay. The trichothecene fraction of the Eppley analysis is eluted with diethyl ether (as described in Experiment 2) and therefore contains many lipids. These lipids can result in high mortality of the brine shrimps. Trichothecene fractions eluted with diethyl ether and subjected to bioassay were less liable to give erroneous high mortality if samples were initially defatted by refluxing 50 gm portions of ground sample in a soxhlet type extraction apparatus with hexane for 3 hours.

Diethyl ether fractions after initial hexane-defatting were also noticeably cleaner for derivatisation treatment, and gave lower sample blanks. Samples were therefore always subjected to an initial defatting with hexane prior to Eppley analysis. Analysis of the hexane used for defatting indicated that T-2 toxin was not lost in the hexane. Fig. 3.6 shows UV curves for derivatised sample extracts, both with and without an initial hexane defatting.

Conclusions

1. Defatting samples with hexane improves the final sample extract by removal of lipids.

2. Trichothecenes are not lost in the hexane used for defatting.
Figure 3.6
The effects of sample defatting with hexane on derivative UV absorption curve
3. Defatting extracts give a sharper-peaked UV curve compared to samples which had not been given an initial defatting.
Experiment 4

Interferences from metallic ions which react with DDTC

It had been noted that occasionally, derivatised extracts were yellow in colour which was always associated with high UV absorption. DDTC is used as a reagent for the sensitive colorimetric determination of copper and also reacts with several other metallic ions such as nickel and zinc. Aqueous solutions containing approximately 10ppm of nickel, zinc and copper were allowed to react with DDTC for 1 hour at room temperature, buffered to pH 7.0, and the resulting solutions extracted with chloroform and analysed by UV scan. Typical UV curves are shown in Fig. 3.7.

A 0.1M solution of EDTA was therefore added with the derivatisation reagent to chelate metallic complexes which would grossly mask any reaction occurring between DDTC and trichotheccenes.
Figure 3.7
UV curves of the derivatisation products of DDTC with copper, nickel and zinc
EDTA was generally used successfully to remove these interferences, but occasional yellow coloured extracts were still obtained. These preparations were discarded, and the analysis repeated since a large UV absorption was almost invariably obtained from these. The interference from copper reactions became significantly more frequent when the mycotoxin section moved to new premises. This was associated with the water supply which was found to contain high levels of copper. Residues left on washed glassware may contribute to the interferences. Similarly, glass vials if they have been washed and re-used are a common source of metal interference, since they are particularly difficult to wash and rinse completely effectively.

Conclusions

1. Metal ions in the sample extracts will react with DDTC.

2. The addition of EDTA (0.1M) will usually prevent interference from the metal ions by chelating the ions and rendering them not extractable by CHCl₃.

3. Care is still needed with preparation of reagents, where glassware has been hand washed by domestic staff.

4. It is preferable to use new vials for each preparation to minimise interference.

5. Derivatised sample extracts which are obviously yellow in colour have possibly become contaminated with trace metals and should be freshly prepared.
The initial experimental work on the reactions of DDTC with trichothecenes had been assessed by monitoring UV absorption curves. Since a suitable UV monitor which could be coupled to a liquid chromatography system became available, it was appropriate to evaluate conditions permitting HPLC analysis of derivatives. The UV absorption curve of T-2/DDTC derivatives shows two main peaks at 284nm and 265nm. Initially, recoveries of T-2 toxin from samples were calculated at both 284 and 265 nm and the results compared as in Table 3.1. This showed that recovery at 284nm was more reliable, and so this wavelength was chosen for the HPLC work.

The following HPLC operating conditions were found to be suitable for elution of derivatives; typical HPLC chromatograms are shown in Fig 3.8 for a solution of T-2 toxin standard derivatised, and a reagent blank.

Column : S5NH amino-propyl silyl column 5μm. 25cm x 6mm i.d.
Mobile Phase : 5% B in A where B = AR Propan-2-ol
A = 10% v/v AR Glacial Acetic acid in Hexane.
Flow Rate : 2ml/min
Detector : Cecil CE 2112 variable wavelength monitor set at 284nm.
Scale Expansion : x1
Injection aliquot : usually 10μl taken from a final derivative volume of 200μl chloroform.
Figure 3.8
HPLC chromatograms of T-2 toxin derivative and a Reagent Blank
Conclusions

1. HPLC analysis of derivatised extracts can readily be performed giving a peak at a retention time of approximately 184 sec.

2. The reagent blank gives a small peak at a similar retention time to the T-2 toxin derivative peak.
Experiment 6

Linearity of the derivatisation response of T-2 toxin for sub-nanogram levels as determined by HPLC.

The linearity of the derivatisation response of T-2 toxin with DDTC over the range 0.1-2.0ng T-2 toxin in 10μl injection aliquots was determined by HPLC. The calibration curve obtained is shown in Fig 3.9.

Further checks of the linearity of the derivatisation response of standards derivatised alongside samples (e.g. 1, 2, 5, 10ng T-2) indicate that good linearity can be obtained up to 1μg toxin.

Conclusions

1. T-2 toxin gives an approximately straight line relationship between peak height of the derivative and ng toxin over the range determined.

2. Linearity checks performed alongside all sample analyses confirm this relationship.
Figure 3.9  
Calibration curve for T-2 toxin
The derivatisation of T-2 with DDTC was originally accomplished at pH 7 using a 1 hour reaction time. The reactions of other trichothecenes, which is discussed in the following experiments, and the results obtained on some samples, indicated that sometimes derivatisation was incomplete as judged by the presence of additional shoulder peaks on the chromatogram. The derivative peak is coincident with, or slightly later (a few seconds) in retention time than the reagent blank when only one trichothecone is present. The conditions giving maximum response for T-2 toxin were therefore evaluated, by studying the effect of variation of pH, reaction time and temperature.

i) pH: The effect of pH on T-2 toxin derivatisation was investigated by altering the pH over the range 6.6-8.0 using a Universal Buffer Mixture.

ii) Time: The initial time for reaction was 1 hour, but a range of times from 1 hour to 5 hours were studied at room temperature.

iii) Temperature: This was studied in conjunction with the time effect.

The results of these experiments are shown in Figs. 3.10, 3.11, 3.12 and 3.13.
Figure 3.10
The effect of pH on HPLC peak height
Figure 3.11
The effect of time of derivatisation at room temperature on HPLC peak height
Figure 3.12
The effect of time of derivatisation at 60°C on HPLC peak height
Figure 3.13
The effect of temperature on different HPLC peaks
From these figures it can be seen that pH 7.7 is the optimal pH, whilst the best temperature and time regime is 2 hours 30 minutes at room temperature, or 30 minutes at 60°C.

Conclusions

1. The optimal conditions for derivatisation of T-2 toxin with DDTC are at pH 7.7, 2 hours 30 minutes at room temperature or 30 minutes at 60°C.

2. It was found more convenient to use 2 hours 30 minutes at room temperature, since at 60°C very careful adherence to time control is necessary in order to prevent further peaks appearing. It is more difficult to control the exact timing of 12 or more samples, when each has to be extracted with chloroform within a short space of time. Additionally, interferences were more commonly encountered (usually noticeable as discolouration of extracts) when the 30 minutes at 60°C regime was used.

3. Since nucleophilic substitution generally attacks the less substituted carbon, in this case carbon 13, the effects seen on the HPLC chromatogram as the temperature was increased, suggest that either carbon atom 12 or 13 may be substituted under differing conditions.

4. The effect of time of derivatisation at room temperature on HPLC peak height (Fig. 3.11) is very unusual and totally unlike that obtained in Munger et al.'s (1973) work, which showed that
reaction was completed in 1 hour giving a plateau after this time. The rapid drop in 'response' after 3 hours suggests that the derivative may be unstable. This instability is also suggested by the chromatograms observed for derivatisation at 60°C against time; similarly the derivatives were found to break down when stored in a refrigerator for more than 48 hours giving many peaks on the chromatogram and often being yellow coloured and sulphurous smelling.
Recoveries of T-2 toxin from samples by HPLC quantification were determined by spiking sample extracts with T-2 toxin and derivatising these extracts. This would evaluate the suitability of HPLC analysis for determining quantitatively the recovery of T-2 toxin in sample extracts as opposed to pure standard solutions, and whether sample extracts contained compounds giving interfering peaks. Sample extracts were spiked as given in Table 3.2, and the recoveries calculated by comparison of peak heights with those of standard T-2 solution. Fig 3.14 shows typical HPLC traces of a derivatised sample extract, a sample blank, and for comparison a standard preparation.
Recovery of T-2 toxin from spiked sample extracts as determined by HPLC (Detection at 284nm)

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Spike: ng T-2 toxin added to sample extract (derived by the epipley method of analysis)</th>
<th>Expected ng T-2 toxin in 10 μl injection aliquot</th>
<th>T-2 recovered from sample injection aliquot (ng)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornflake</td>
<td>2.0</td>
<td>0.1</td>
<td>0.095</td>
<td>95</td>
</tr>
<tr>
<td>Type</td>
<td>2.0</td>
<td>0.1</td>
<td>0.096</td>
<td>96</td>
</tr>
<tr>
<td>Weetabix</td>
<td>4.0</td>
<td>0.2</td>
<td>0.19</td>
<td>95</td>
</tr>
<tr>
<td>Type</td>
<td>4.0</td>
<td>0.2</td>
<td>0.19</td>
<td>95</td>
</tr>
<tr>
<td>Inst. Porridge</td>
<td>10.0</td>
<td>0.5</td>
<td>0.553</td>
<td>107</td>
</tr>
<tr>
<td>Type</td>
<td>10.0</td>
<td>0.5</td>
<td>0.37</td>
<td>74</td>
</tr>
<tr>
<td>Rice Krispies</td>
<td>15.0</td>
<td>0.75</td>
<td>0.74</td>
<td>99</td>
</tr>
<tr>
<td>Type</td>
<td>15.0</td>
<td>0.75</td>
<td>0.68</td>
<td>91</td>
</tr>
<tr>
<td>Shreddies</td>
<td>20.0</td>
<td>1.0</td>
<td>0.95</td>
<td>95</td>
</tr>
<tr>
<td>Type</td>
<td>20.0</td>
<td>1.0</td>
<td>0.77</td>
<td>77</td>
</tr>
<tr>
<td>Muesli</td>
<td>30.0</td>
<td>1.5</td>
<td>1.13</td>
<td>76</td>
</tr>
<tr>
<td>Type</td>
<td>30.0</td>
<td>1.5</td>
<td>lost</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.14
HPLC traces of a derivatised spiked sample extract, a sample blank, an uncontaminated sample extract and a standard solution of T-2
Conclusions

1. The sensitivity of HPLC detection is equally as good for sample extracts as for standard solutions.

2. The quantity of T-2 toxin in a sample extract can be obtained by deducting the sample blank peak height from that attributable to T-2 toxin and comparing the peak height with standards.

3. Measurement of peak height at 284nm is preferable (since more interferences are encountered at 265nm).

4. HPLC analysis can be used to detect as little as 0.1ng T-2 toxin in the 10µL injection aliquot of a sample extract.
Experiment 9

Reactions of other mycotoxins with DDTC

Since it is possible (although unlikely) that traces of other mycotoxins may be co-extracted in the diethyl ether fraction of the Eppley analysis, it is essential to know whether other mycotoxins will react with DDTC. Aliquots of standard solutions of penicillic acid, patulin, zearalenone, citrinin, ochratoxin A, aflatoxin B₁ and sterigmatocystin were dried down, and derivatised with aqueous DDTC for 1 hour as in Experiment 1 and then analysed by HPLC as in Experiment 5. Penicillic acid, patulin, zearalenone, citrinin and ochratoxin A did not give any detectable reaction. Aflatoxin B₁ and sterigmatocystin derivatised to give a peak approximately half the size of the equivalent quantity of T-2 toxin. This reaction may be the result of aflatoxin B₁ and sterigmatocystin forming epoxides in solution. Although their reactions are potentially useful confirmatory techniques, they are an interfering reaction when determining trichothecenes.

In order to remove the possibility of AFB₁ and SMN reacting, all sample extracts were exposed to a weak solution of hypochlorite (5 vol. of commercial hypochlorite solution/100 ml H₂O) for a period of 2 hours 30 minutes. After this period of time, excess hypochlorite was removed, by the addition of a 2% aqueous sodium metabisulphite solution to the extract. This solution was then extracted by shaking out in a 50ml separating funnel with chloroform
and evaporating to dryness under nitrogen before proceeding with derivatisation. Weak hypochlorite did not diminish the response of T-2 toxin with DDTC. Typical results are shown in Figs. 3.15 and 3.16. The traces show the derivatised peak height for aflatoxin B₁, sterigmatocystin, and for T-2 toxin with and without a 2 hour 30 minute 'reaction' period with sodium hypochlorite. Sample extracts spiked with T-2 toxin and subjected to a 2 hour 30 minute hypochlorite treatment still showed a similar recovery of T-2 toxin as shown in Table 3.2.

Conclusions

1. Aflatoxin B₁ and sterigmatocystin react with DDTC - a potential interfering reaction.

2. The potential reaction of aflatoxin B₁ and sterigmatocystin can be removed by exposing extracts to weak hypochlorite solution.

3. The reaction response of T-2 toxin is not diminished by hypochlorite treatment.

4. The recovery of T-2 toxin from spiked extracts treated with hypochlorite is not affected.
Figure 3.15
Reaction of aflatoxin B<sub>1</sub>, Sterigmatocystin and T-2 toxin without the addition of sodium hypochlorite solution
Figure 3.16
Reaction of aflatoxin B₁, sterigmatocystin and T-2 toxin after 2.5hr exposure to weak hypochlorite (5vol/100ml H₂O)
Experiment 10

Reactions of other trichothecenes with DDTC

All work so far reported on the reactions of trichothecenes with DDTC have concentrated on T-2 toxin. Since it is quite likely that samples may be contaminated with other trichothecenes, it is necessary to know how other trichothecenes react with DDTC. The derivatisation response of several trichothecenes was therefore examined using the conditions optimal for the reaction of T-2 as described in Experiment 7. Some months later, with an increase in interest in deoxynivalenol contamination, the linearity of the response of DON over the range 200ng-1μg DON was determined. The results of the initial work on several trichothecenes are given in Table 3.3, whilst a typical calibration curve for deoxynivalenol obtained some months later is given in Fig. 3.17.

TABLE 3.3
Reactions of several trichothecenes with DDTC as originally determined

If T-2 response (for 10ng T-2, or 0.5ng in the HPLC system) = 1, then for a comparable result it should be possible to normalise for mol. wt.

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>Pk ht for 0.5ng determined by HPLC</th>
<th>Mol.wt.</th>
<th>M. wt. T-2 M. wt. x</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-2</td>
<td>1.1 x T-2 pk ht</td>
<td>424</td>
<td>1.1</td>
</tr>
<tr>
<td>DAS</td>
<td>1.25 x T-2 pk ht</td>
<td>366</td>
<td>1.27</td>
</tr>
<tr>
<td>DON</td>
<td>1.4 x T-2 pk ht</td>
<td>296</td>
<td>1.57</td>
</tr>
<tr>
<td>Verrucarin A</td>
<td>0.6 x T-2 pk ht</td>
<td>502</td>
<td>0.93</td>
</tr>
<tr>
<td>Roridin A</td>
<td>0.5 x T-2 pk ht</td>
<td>532</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Figure 3.17
Calibration curve for Deoxynivalenol
The peak heights obtained by HPLC for the initial work on the derivatisation of other trichothecenes agreed approximately when normalised for molecular weight for HT-2, DAS and DON with the equivalent responses as measured. The differences noted in the response of the macrocyclics verrucarin A and Toridin A may lie partially in the steric effects of the molecules of these trichothecenes.

A major discrepancy however became apparent some months later when working exclusively on DON. The peak height regularly obtained for DON derivatives was approximately 1/10th of that usually obtained for T-2 toxin, rather than the factor of 1.5 times originally found. This size of peak has remained of similar proportions throughout all recent work. In the initial work with other trichothecenes it may be that the conditions used were slightly different resulting in a different response being obtained. The figures in Table 3.3 refer to the initial work, whereas Fig. 3.17 shows a DON calibration curve obtained after work on T-2 toxin had ceased, and when this 'diminished' response was that typically found.

Several attempts were made to achieve chromatographic separation of derivatives of individual trichothecenes when prepared as mixtures. This was not possible, surprisingly, despite the use of a range of mobile phases and stationary phases. The best separation of individual derivatives obtained showed inflected peaks or close shoulder peaks. Fig. 3.18 illustrates chromatograms obtained from mixtures of derivatised trichothecenes.
Figure 3.18
"Mixed" Derivatised Trichothecenes - HPLC chromatograms
The individual trichothecenes were run individually by HPLC first in order to assign the small shoulder and plateau peaks to each trichothecene. However, the retention times are so closely similar that in a sample extract containing a mixture of trichothecenes it would be very difficult to accurately assign peaks with any degree of confidence. It seems remarkable that the macrocyclic trichothecenes have similar retention times in view of their different characters compared to deoxynivalenol. This could indicate that the DDTC reaction is not a straightforward nucleophilic attack as suggested by the original work by Munger et al. (1973) on DAG. Since separation of the derivatives was not possible with the available apparatus it was decided to choose conditions which resulted in a single peak, and the results calculated as "total trichothecenes" expressed as T-2 toxin. This work was not extended to the determination of other trichothecenes in samples apart from the rapid method developed in Experiment 11 and for deoxynivalenol in samples. The chromatograms derive from derivatised pure solutions only.

Conclusions

1. Several other trichothecenes were found to react with DDTC to give detectable products with some differences in reaction response.

2. The response of each trichothecene appeared to be correlated with its molecular weight relative to T-2 toxin.
3. Chromatographic separation of derivatives was difficult and thus conditions giving a single peak were chosen and results calculated as "total trichothecenes".

4. Deoxynivalenol gives a straight line relationship between peak height of the derivative and ng toxin over the range determined.

5. The response of deoxynivalenol obtained in calibration curves prepared some months after the initial work on trichothecenes was only 1/10th of an equivalent T-2 reaction.
Several samples of Canadian wheat (two of which were known to be contaminated with deoxynivalenol) became available for testing at the Research Association. It was felt desirable to analyse samples whose contamination level had already been determined (but not revealed to the RA) to validate the method. These samples were analysed as described in Experiments 2 and 3 (i.e. by the method of Eppley 1968, after an initial defatting). Results obtained were found to be only 10% of the reported level. The quantification step alone, using DDTC derivatisation on sample extracts supplied by the same member who submitted the wheat, indicated that this stage was satisfactory. It was therefore apparent that the method of extraction of the toxin from the sample was not suitable. The chloroform extraction, and diethyl ether elution was satisfactory for Type A trichothecenes such as T-2 toxin, but not for Type B, such as DON. This latter type requires a more polar extraction solvent; most methods extract DON with methanol or methanol:water, with a methanolic elution from a silica gel clean-up column. A method capable of extracting all types of trichothecenes was therefore required. In addition, the method of Eppley, after a defatting treatment was very time-consuming, requiring 3 days elapsed time before obtaining the results. A method utilising rapid clean up by silica Sep-Pak cartridges (for a note on Sep-Paks
see page 67) was tried. The toxins were extracted from 10 gm of sample; the extracts were filtered, passed through a Sep-Pak and eluted by a selective solvent. Evaluation of the solvents suitable for initial extraction of the toxins, and the elution of the toxins from the Sep-Pak, was aimed at obtaining appropriate conditions for the analysis of all types of trichothecenes, since in general, one would not know which a sample contained. 10 gm portions of ground sample were spiked with solutions of trichothecenes and extracted by the use of 50 ml portions of several different extraction solvents and 10 ml portions of Sep-Pak elution solvents. 20 ml of filtrate were applied to each Sep-Pak, and the elution solvent consisted of 10 ml of various solvents. The recoveries obtained are given in Table 3.3.
TABLE 3.3

Effect of different solvents on the extraction of trichothecenes and their elution from silica Sep-Paks.

<table>
<thead>
<tr>
<th>Commodity (10gm)</th>
<th>Extraction solvent (50ml)</th>
<th>Sep-Pak elution solvent</th>
<th>*see key Toxin</th>
<th>ng Toxin added to 10gm sample</th>
<th>ng recovered</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornflakes</td>
<td>MeOH : H₂O 1 : 1</td>
<td>CHCl₃ : MeOH 97 : 3</td>
<td>DON</td>
<td>20</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T-2</td>
<td>20</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VER</td>
<td>20</td>
<td>28</td>
<td>140</td>
</tr>
<tr>
<td>Bran</td>
<td>MeOH : H₂O 1 : 1</td>
<td>CHCl₃ : MeOH 97 : 3</td>
<td>DAS</td>
<td>20</td>
<td>18</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DON</td>
<td>20</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T-2</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oats</td>
<td>CHCl₃</td>
<td></td>
<td>DON</td>
<td>20</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VER</td>
<td>20</td>
<td>6.6</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T-2</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weetabix</td>
<td>CHCl₃</td>
<td></td>
<td>HT-2</td>
<td>20</td>
<td>10.2</td>
<td>51</td>
</tr>
<tr>
<td>Rice cereal</td>
<td>CHCl₃ : MeOH 1 : 1</td>
<td></td>
<td>T-2 + DON</td>
<td>10 + 10</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ROR</td>
<td>40</td>
<td>5.9</td>
<td>15</td>
</tr>
</tbody>
</table>

DAS : Diacetoxyscirpenol  VER : Verrucarin A  ROR : Roridin A
From this table, extraction with chloroform:methanol 1:1 gives a good recovery for both Type A and Type B trichotheccenes. Some anomalies are apparent from this table, also. Thus, the 85% recovery from oats of deoxynivalenol with chloroform alone is rather high. Similarly, the recovery of 85% from Weetabix of T-2 + DON using chloroform alone is also high. These anomalies may be partly attributed to the difficulties of satisfactorily spiking samples. Samples spiked by the addition of pure toxin solution in solvent behave in terms of recoverable toxin differently from naturally contaminated samples. Uneven distribution of toxin and the absence of the toxin being thoroughly bound to the food may therefore give rather optimistic results. A true measure of the efficiency of the analysis is accordingly rather difficult to estimate. Using chloroform: methanol 1:1 extraction and chloroform:methanol 97:3 Sep-Pak elution, good agreement of the quantities of deoxynivalenol in contaminated samples already determined by GC-MS was obtained for some of the samples. The comparative results are given in Table 3.4. Results showing poor agreement may be partially attributable to sampling problems inherent to all mycotoxin analysis. Thus, sample (2) of the second set was subsequently found to be poorly mixed.
## TABLE 3.4

Results of DDTC Method compared with the GC-MS Method for DON in naturally contaminated samples of wheat

<table>
<thead>
<tr>
<th>Samples</th>
<th>MAFF Results (GC-MS) ppb</th>
<th>Food RA (DDTC) ppb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Set</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWRS/P</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>CWS/P</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>OSWT1/81</td>
<td>240</td>
<td>205</td>
</tr>
<tr>
<td>OSWT</td>
<td>307</td>
<td>135</td>
</tr>
<tr>
<td><strong>2nd Set</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>ND (&lt;10)</td>
<td>ND (&lt;1)</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>90</td>
</tr>
</tbody>
</table>

The rapid method finally devised and used for some of the analyses reported in the following chapters is given in Appendix II.

**Conclusions**

1. The method of Eppley (1968) is not appropriate for analysing samples for all types of trichothecenes, being satisfactory for Type A trichothecenes (such as T-2) only.

2. A general method for trichothecenes requires the use of a more polar solvent extraction and elution if Type B trichothecenes are present.
3. Chloroform:methanol (1:1) extraction; and chloroform:methanol (97:3) elution from a silica Sep-Pak provide a suitable combination for both Type A and Type B trichothecenes from spiked samples.

4. Spiked samples may not necessarily give a completely accurate evaluation of recoverable toxin.

5. Some measure of agreement was obtained using the rapid method to determine deoxynivalenol in samples naturally contaminated with DON; poor agreement could be partially attributable to unevenly distributed toxin.

A Note on Sep-Paks

Sep-Paks are small disposable cartridges containing silica gel (with or without bonded phases), florisil or alumina of 3 pH values. Their function is to provide rapid clean-up of small volume filtrates, and essentially fulfil the same role as a large column containing the same absorbent material. They do however, have slightly different absorption properties compared to a large column. Conditions for absorption and elution of toxins in conjunction with Sep-Paks must therefore always be evaluated (as must any methodology).
Discussion of Analytical Method Development

Despite the apparent successes of the method development reported, there were many drawbacks still affecting the method. The most disturbing feature was the considerable operator dependence. The success rate of the method was very variable, depending on which analyst undertook the experimental work. In an attempt to check whether successful operation of the method originated in the slight differences in technique used by individual analysts, a trial was conducted within the laboratory. Four experienced analysts were given 10 different solutions of mycotoxins (including aflatoxin B₁) and asked to determine the levels of mycotoxins in each. For this purpose, five solutions were treated with hypochlorite and five were not, before derivatisation (from this treatment, the presence of aflatoxin B₁ could be determined). The analysts were not given any indication as to the contents of their test vials. The results of this trial are given in Table 3.5.

TABLE 3.5
Results of the Laboratory Trial of the DDTC Method

<table>
<thead>
<tr>
<th>Analyst</th>
<th>Results of tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>* Satisfactory *</td>
</tr>
<tr>
<td>SC (author)</td>
<td>7</td>
</tr>
<tr>
<td>WBC 2 (2nd trial)</td>
<td>4</td>
</tr>
<tr>
<td>GMW 1 (1st trial)</td>
<td>2</td>
</tr>
<tr>
<td>GMW 2 (2nd trial)</td>
<td>5</td>
</tr>
<tr>
<td>MBP 1 (1st trial)</td>
<td>5</td>
</tr>
<tr>
<td>MBP 2 (2nd trial)</td>
<td>9</td>
</tr>
</tbody>
</table>

Key: * Satisfactory: Estimated no. of ng's within 20%.
     * Doubtful: Estimated no. of ng's > 20%.
     * Incorrect: Estimate totally incorrect.
It is apparent from these results that there was considerable variation in the number of correct estimations obtained by different analysts. It is possible that slight differences of technique greatly effect the results obtained; these differences were not apparent despite careful scrutiny of each operator whilst at work. The operator dependence of the method has not been resolved, largely because the project funding for this method development was terminated, which curtailed further work. It did however become possible to perform an intensive days experimental work in conjunction with Unilever at Colworth. Samples of pure toxin solutions and contaminated wheat extracts were derivatised simultaneously by the RA analyst and by an experienced analyst from Unilever at the research laboratories. The results of these experiments indicated that it was possible for the Unilever analyst to obtain results comparable to the author's when working alongside the author. Arising from this work, Unilever prepared a report, which included suggestions that close attention to detail and strict adherence to these details should be observed; these suggestions closely parallel the requirements of any 'trace' analysis. These requirements include the use of freshly prepared analytical grade reagents and glassware appropriate to trace analysis. For example: separating funnels should be fitted with interflon stopcocks, rather than glass stopcocks which have been greased. All these suggestions have now been incorporated into the method and are given in Appendix II.
Further validation of the method has been afforded by the collaborative work of the Working Party on Mycotoxins of MAFF. (See also p. 97 et seq., Chapter 7) Thus the results reported in Table 3.4 showed reasonable agreement between the DDTC method and the GC-MS method for deoxynivalenol as used by MAFF for some samples. The Analytical Panel of the Mycotoxins Working Party was set up in Jan. 1982 to examine the analytical methodology of all organisations submitting data to the Mycotoxins Working Party. This panel is continuing to evaluate the validity of panel members' methodology by conducting appropriate collaborative trials for several mycotoxins. This panel is of great value in providing assessment of analytical methods and the dissemination of analytical expertise. The first deoxynivalenol collaborative trial indicated that the Gas Chromatography with Electron-Capture Detection Method for trichothecenes could give rise to erroneous high positive results. Thus one participating laboratory obtained a figure for deoxynivalenol ten times higher than GC-MS determination had given. Considerable further work was clearly necessary on the DDTC method to determine not only the exact mechanism involved, but also to provide a clearer understanding of why the current method was operator sensitive, and suffered both from sporadic interferences and erratic sensitivity on occasions.

Experimental work aimed at elucidating the reaction mechanisms has included derivatisation reactions of simple epoxides, such as epoxypropane, analysis by MS of derivatives, and derivatisation of dianhydrogalactitol as prepared originally by Munger et al. (1973).
The results of these investigations have not provided much additional information and are discussed below.

**Derivatisation of simple epoxides**

Solutions containing 21 - 212 ng of epoxypropane in methanol were exposed to DDTC using the conditions optimal for the derivatisation of T-2 toxin. HPLC chromatograms obtained from the products of these reactions are shown in Fig. 3.19. The scale expansion of the UV detector was x 0.5 (which is 2 x more sensitive than used for T-2 determination). The reagent blank contained a small quantity (100 μl) of methanol to give an equivalent preparation to the test solutions. Methanol was chosen as an appropriate solvent for the simple epoxides because of its miscibility with water; the concentration of the epoxypropane solutions was adjusted to give 100 μl methanol/test vial. Sample solutions of epoxypropane gave peaks at RT 230 and RT 620 which are not seen with the trichothecene preparations. The size of these peaks was not apparently proportional to the concentration of epoxypropane. These peaks were present whether old GPR grade epoxypropane or fresh AR grade was used, and are therefore unlikely to be the product of derivatisation of impurities. Clearly, some reaction had occurred to give a compound of different components to those of trichothecene derivatives, and of unexpected complexity. The large peak at 222 sec in the 44ng solution of epoxypropane may represent underivatised material adhering to the HPLC column as it appears to be a compound which is retained for a long time period.
Figure 3.19
HPLC chromatograms of derivatised epoxypropane
Analysis by Mass - Spectrometry of Trichothecene Derivatives

Derivatives of deoxynivalenol were prepared both with and without hypochlorite treatent and submitted to MAFF. The samples comprised a blank preparation, "DON 1" containing 35μg of derivatised DON with no hypochlorite treatment and "DON 2" containing 23μg derivatised DON subjected to a hypochlorite treatment (Expt. 9). Significantly larger peaks were obtained by HPLC on DON 1 and DON 2 than for the blank. These peaks were then 'trapped' after HPLC, and evaporated to dryness for direct probe MS. The mass spectra obtained are shown in Figs 3.20, 3.21, 3.22. MAFF commented that they could not detect any significant differences between the two DON samples and the reagent blank, and that there did not appear to be any evidence of an analogous product to that described in the Munger et al. (1973) paper. The presence of residual DDTC reagent in all three samples caused analytical difficulties because it complexed with tin from a previous analysis giving an intense tin thiocarbamate spectrum.

Examination of the mass spectrum shows that the control blank contains an ion at 296, which could be of underivatised deoxynivalenol. The blank was prepared in a clean vial and theoretically contained only the reagents. It is possible that shared equipment (e.g. separating funnels, HPLC syringes, pasteur pipettes for nitrogen) could have transferred a low level of deoxynivalenol to the blank preparation. This would appear to be the only logical explanation apart from contamination having occurred at the MAFF laboratory. The mass-spectrum for hypochlorite treated DON contains ions at masses 276 and 279 which are not
apparent in the non-treated DON. Both the control bank and the hypochlorite treated DON have many low ion mass peaks; these may derive from compounds in the hypochlorite solution, although many other ions are not common to these two preparations. Underivatised deoxynivalenol may be present in the spectrum for non-hypochlorite treated DON. There are several differences between the treated and non-treated DON spectra, particularly the presence of ions at 279, 276 and 194 in treated DON which are completely absent from the non-treated trace; the nearest equivalent ions to those in the non-treated DON are at 296 and 205 (i.e. smaller ions in treated DON). This suggests that the hypochlorite treatment may have reacted with the DON. The ions at 205 however are present in the control blank; this may represent cross contamination again. It does appear that DON reacted with DDTC does not produce a direct nucleophilic substitution as DAG does with DDTC. Since a direct reaction is not apparently occurring considerably more work would clearly be necessary to characterise the reaction product. The instability of the reaction product and the atypical reaction response curves resulting from variations in pH of reaction mixture and temperature all suggest that the trichothecene:DDTC reaction is far more complicated than originally supposed. This may in some part explain the difficulties experienced in characterising the reaction mechanism and in obtaining good reproducibility of the DDTC method. A major commitment to study this reaction would seem to be indicated. Termination of funding has unfortunately curtailed any further developments and work in this area.
Figure 3.21 DON 1 - Mass-spectrum = Derivatised Deoxynivalenol - no hypochlorite treatment
Figure 3.22 DON 2 – Mass-spectrum = Hypochlorite treated DON
Derivatisation of Dianhydrogalactitol (DAG)

A small quantity of DAG was kindly donated by MRC. Test solutions containing 25ng/ml DAG were derivatised using standard T-2 conditions. The reaction products were then analysed both by UV absorption curve and by HPLC. The results of these experiments are shown in Figs. 3.23 and Fig. 3.24. The UV curve shows one peak only at 260nm, with no inflection peak at 284 nm as observed for trichothecenes. The reaction product is therefore obviously different in character than for T-2 toxin. The HPLC chromatogram shows 2 peaks of similar RT to that of derivatised T-2. It could be that under optimal conditions for T-2 analysis, the pH is too high for optimal derivatisation of DAG. It is interesting to note that in common with the trichothecene HPLC traces, a similar retention time is still obtained, despite the reported bis-dithiocarbamoyl ester being formed with DAG.

Whilst therefore several experiments have been undertaken to clarify the reaction mechanism and the product of the derivatisation, none have proved successful. In the following chapter the derivatisation method for quantifying trichotheccenes method has been applied to the surveillance of many samples. Detection of trichotheccenes at higher levels were confirmed by the use of TLC. The TLC methods do not permit good quantification, but they are useful confirmatory techniques for levels of T-2 toxin > 50ppb and for DON > 20-30ppb. Although 10ng of pure DON can be detected by 2D-TLC, in samples, interferences from the sample matrix generally limits the detection to 20-30ppb. Adequate confirmation of trichotheccenes below these limits is therefore not possible, since a method of comparable sensitivity is not available.
Figure 3.23
UV curve of derivatised DAG - 2.5ng/ml CHCl₃
Figure 3.24
HPLC chromatogram of derivatised DAG
CHAPTER 4

GENERAL SURVEILLANCE OF FOOD AND FEEDING STUFFS FOR TRICHOTHECENES

The "DDTC method" (as given in Appendix II) has been used in the surveillance of a large number of samples for trichothecenes; a wide variety of raw foods, cereals, processed cereal products and nuts have been analysed. Many of these samples, particularly the processed cereals were examined for aflatoxins, ochratoxins, sterigmatocystin and zearalenone also as part of the MAFF Working Party on Mycotoxins nationwide surveillance programme. Most of the animal feeds derived from ADAS laboratories following reports of veterinary problems thought to be associated with the animal feed. These samples had been previously analysed by a multi-mycotoxin screening method and were also tested by bioassay by the brine shrimp method, both by ADAS and at the RA. Several samples of mould-spoiled food were examined as part of a MAFF project concerning the incidence of mycotoxins occurring in mould-damaged foods. These samples were collected from a variety of sources including retailers, manufacturers and individual employees of the RA. Each item of mould-spoiled food was examined for identity of moulds and for appropriate mycotoxins. The balance of the samples comprised Food RA member requests for analysis; these requests were as an extension of their quality control practices, or when mould damage of raw materials and products had occurred, or occasionally, when clinical symptoms in livestock were reported. Considerably more data, mostly concerning the co-occurrences of the trichothecenes and zearalenone with other mycotoxins is not included
in the reported data, since the theme of this study is confined primarily to the incidence of fusarial toxins only. Approximately 98% of the analyses for trichothecenes were performed by the author. This is important in the light of the operator dependence of DDTC method. Table 4.1 summarises the incidence and levels of the trichothecenes in the commodities analysed. These are grouped by commodity to facilitate comparison. Table 4.2 indicates in how many of the samples, the presence or absence of trichothecenes was correlated with that of zearalenone in breakfast cereals only. Some of the information given in Table 4.2 is common to the "Breakfast Cereal" category of Table 4.1. However, not all the breakfast cereal samples were examined for zearalenone and could therefore not be included in Table 4.2.
### TABLE 4.1
Incidence and levels of trichothecenes in food and feeding stuffs

**KEY:**  
ND = none detected  
Trace = <1 µg kg⁻¹

<table>
<thead>
<tr>
<th></th>
<th>No. samples examined</th>
<th>No. with ND</th>
<th>No. with Trace</th>
<th>No. with &gt;trace (i.e. 1µg/kg or greater)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. MOULD-SPOILED FOOD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakery-raw</td>
<td>17</td>
<td>11</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Bakery-cooked</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>2. RAW MATERIALS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bran</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Oats</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Millet</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Wheat germ</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Barley</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cashew nuts</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Turkey feed</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20</td>
<td>1</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>% of totals</td>
<td>5</td>
<td>15</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td><strong>3. BREAKFAST CEREALS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornflake type</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Weetabix type</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Instant porridge</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Puffed Wheat</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rice Krispies</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Shredded Wheat</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Muesli ingredients</td>
<td>29</td>
<td>18</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Rice based</td>
<td>11</td>
<td>3</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Shreddies</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Bran based</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
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<tr>
<td><strong>Total</strong></td>
<td>78</td>
<td>35</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td>% of totals</td>
<td>45</td>
<td>32</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td><strong>4. PASTAS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of total</td>
<td>21</td>
<td>8</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>% of total</td>
<td>38</td>
<td>19</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td><strong>5. TAPIOCAS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of total</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>% of total</td>
<td>46</td>
<td>27</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td><strong>6. SEMOLINAS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of total</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>% of total</td>
<td>75</td>
<td>25</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

80
TABLE 4.2
Breakfast Cereals – MAFF Survey Only

Correlation between the occurrence of Trichothecenes and Zearalenone

<table>
<thead>
<tr>
<th>Cereal Type</th>
<th>No of Samples</th>
<th>No of samples where T2 + F2 either both present or absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornflake type</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Weetabix type</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Inst. Porridge type</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Puffed Wheat type</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Rice Krispies type</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Shredded Wheat type</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Diet type</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Muesli Ingredients (inc. cereals, nuts and dried fruits)</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>Bran based</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Muesli type</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Shreddies</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Discussion

In raw materials, a higher proportion of samples contained detectable levels of trichothecenes. The other commodity group with a fairly high incidence of positive samples is pasta. Pasta consists of wheat flour, water and semolina, which are mixed to form a stiff dough of about 30% moisture. This dough is extruded into various forms and then dried down to 10-12% moisture content. Slow drying is essential to retain pliability of the product. Since pasta is extruded and dried below pasteurisation temperature, and there is no cooking stage, there is the possibility of mycotoxins being formed by mould growth during the processing, or of mycotoxins contaminating raw materials persisting through to the final product.
In the breakfast cereals, in some commodities, a degree of correlation between detectable levels of zearalenone and trichothecenes was noted within the oat-and rice-based products. Thus for 8 samples of oat based foods (N.B. two of the oat-based foods are muesli ingredients) in the 6 samples of instant porridge type cereals both zearalenone and trichothecenes were either present or absent, and in the remaining 2/8 samples, (which were included in the muesli ingredients category) neither was detected. This correlation was noted to a lesser extent in cornflakes. It may therefore be possible in these commodities to infer that the presence of quantifiable levels of zearalenone suggests the likely presence also of trichothecenes. In many instances where a correlation is not apparent, this arises from considerable sample interferences preventing accurate and low level detection of zearalenone. Thus in many samples where no correlation was noted, zearalenone may have been present, but its identification and quantification was not possible. In only three of the breakfast cereal samples were possible significant levels (i.e. > 50 ppb) of zearalenone present in the absence of trichothecenes, and in two of these, interferences from sample extracts prevented confirmation by other techniques.

The majority of the mould-spoiled foods did not contain detectable levels of trichothecenes, although 6 bakery products contained zearalenone. Viable fusaria were not identified on any of these samples; it may be assumed therefore that either the raw cereal ingredients contained these toxins, or that fusarial growth had occurred and perished before mould identification. In general,
mould-damaged foods do not always show correlation of the mycotoxin content with the type of mould present. Some samples may be obviously very mouldy but no mycotoxins are detectable. This could be because the mould is non-toxigenic, or if the mould is toxigenic, it has not produced any toxins because either the storage condition or substrate did not facilitate toxin production. Other mould-damaged samples bore little or no obvious mould growth, but high levels of toxins were detected; in this instance it must be assumed that the mould growth had perished. Thus mould spoilage can on occasions result in mycotoxin contamination, but this is by no means always true.

Conclusions

1. The incidence of trichotheccenes in all the types of food and feeding stuffs examined was fairly high.

2. The greatest incidence occurred in unprocessed materials where 95% of the samples had detectable levels of trichotheccenes.

3. In the other commodities examined, a fairly high incidence was found (generally about 50% of samples contained detectable levels).

4. The lowest incidence of trichotheccenes in foods occurred in semolina.

5. Mould-damaged foods do not show a higher incidence of trichotheccenes; nor were any viable fusaria isolated.
6. In some commodities the presence and absence of zearalenone and trichothecenes showed a good correlation. This was particularly pronounced in rice- and oat-based products. This correlation is likely to be associated with the fact that some Fusarium spp. are reported to elaborate both zearalenone and trichothecenes. Vesonder et al. (1981) report that all 16 strains they tested belonging to F. graminearum and F. culmorum produced both zearalenone and vomitoxin, whilst Mirocha et al. (1976) have detected both zearalenone and trichothecenes in feeds contaminated with F. roseum. Unfortunately it was not known in these samples which trichothecene was present, since the method used for this surveillance study was suitable for determination of total trichothecenes only.
CHAPTER 5

SURVEILLANCE OF FOOD FOR DEOXYNIVALENOL

This surveillance, concentrating on deoxynivalenol only, was conducted in collaboration with ADAS. The interest in deoxynivalenol derives from the very high incidence of deoxynivalenol in cereals in Canada. Since much of the UK's wheat and maize is imported from Canada, a special survey, restricted to the occurrence of deoxynivalenol in wheat-and corn-based foods was conducted for MAFF. Breakfast cereal samples based on maize and wheat and popping corn were purchased from local retail outlets. Animal feedstuffs were supplied by ADAS, together with detailed reports of clinical symptoms occurring in animals which had consumed the feeds. These samples were usually associated with minor symptoms of debility in the animals. Wheat and wheat flour samples derived from member companies of the Food RA. All samples were ground to fine particle size and analysed by the DDTC method as given in Appendix II but modified by using 50ml methanol only as the extraction solvent (rather than chloroform : methanol 1 : 1) This modification was thought advisable since, although with spiked samples good recovery of deoxynivalenol was obtained, it was indicated that extraction of deoxynivalenol from naturally contaminated samples may be more difficult, requiring a more polar solvent. With the exception of the extraction solvent, the method as given in Appendix II was followed, DON standard solutions being used for quantifying by comparing peak heights in standards.
to samples. The results of this surveillance are given in Table 5.1. Individual results for samples containing levels of DON in excess of 1 µg/kg are given in brackets. Samples were quantified to a detection limit of 1 µg/kg (as in Chapter 3) since this is readily accomplished by the DDTC method.
TABLE 5.1

Deoxynivalenol Surveillance of Wheat and Corn Based Products

<table>
<thead>
<tr>
<th>Commodity</th>
<th>No Samples</th>
<th>None detected</th>
<th>Trace &lt;1 μg/kg</th>
<th>&gt;Trace+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn based processed food</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Popping corn</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Wheat based processed food</td>
<td>17</td>
<td>11</td>
<td>5</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Wheat and wheat flour</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>5 (2, 56, 41, 204, 137)</td>
</tr>
<tr>
<td>Maize based animal feeds</td>
<td>19</td>
<td>17</td>
<td>0</td>
<td>2 (3, 2)</td>
</tr>
</tbody>
</table>

+ Figures in brackets are the individual sample results.

*These figures were derived before the loss of sensitivity reported on P 58, hence the limit of detection was 1 μg/kg at this stage.
Discussion

The surveillance data from these samples indicates that there is a sporadic occurrence of high levels, especially in wheat samples. All the wheat samples were imported from Canada. This type of distribution, showing great variations occurring within the wheat crop from the same geographical location, was reported by Scott: (Scott et al. 1981) and it is mainly for this reason that the Canadian authorities have placed an acceptance limit of 300 ppb DON in wheat destined for human consumption. 300 ppb is an easily attainable limit of detection for both TLC and GC methods and can therefore be realistically applied in surveillance studies. The sporadic incidence of DON emphasises the need for continuing surveillance programmes, and quality control procedures suitable for screening cereal crops prone to contamination by fusaria. The 'Myco Chek' kit marketed by LSB products (Kansas, USA) is designed for quality control specifications, stating that "Any combination of vomitoxin, fusarenon X or other nivalenol type of mycotoxin known that occurs at less than 1 ppm can safely be used for animal feed. Food safety restrictions may be more strict in some countries, but generally, if the concentration is less than 0.5ppm, the grain or cereal product is safe for human consumption." They also state that "vomitoxin and fusarenon are not carcinogenic, and not lethal to livestock on feed." In Chapter 1, the lack of knowledge about the effects of consumption of low levels of trichotheccenes in foods was discussed, and it is certainly considered by some that there is not any clear cut evidence indicating that the trichotheccenes are not carcinogenic.
Several of the animal feeds examined were reported to be associated with a drop in food intake by livestock, or by a drop in egg production by poultry. Feed refusal is characteristic of contamination of food by deoxynivalenol, but the results obtained (in agreement with those obtained by ADAS) do not suggest that these feeds were contaminated with deoxynivalenol. In this context it may be pertinent to examine (as controls) feeds not associated with abnormal feeding patterns, and to examine all types of feeds for other fusarial metabolites, such as butenolide and moniliformin. The clinical toxicity of pure toxin solutions administered experimentally to animals differs markedly from the toxicity elicited by feeding experiments with naturally moulded feeds. This suggests that there may be synergistic effects of several toxic metabolites which can only occur when feeds are contaminated by inoculating the feeds with toxigenic moulds. Determination of the trichothecenes, and specifically deoxynivalenol, may not therefore wholly represent the agent(s) causing mycotoxicoses. More experimental studies aimed at investigating this aspect are therefore clearly indicated.

Conclusions

1. Wheat and wheat flour may be contaminated with significant levels of deoxynivalenol.

2. The incidence and level of deoxynivalenol varies greatly even in crops from the same geographical location.
3. The incidence of deoxynivalenol contamination for processed foods based on corn and wheat represents approximately 50%.
   (20/55 samples were positive) This incidence corresponds with the similar level of trichothecene contamination found in breakfast cereals as discussed in Chapter 4.

4. The significance of these levels of DON in maize and wheat is not known despite the viewpoint in the USA that up to 0.5ppm DON is a 'safe limit'.

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CHAPTER 6

THE FATE OF TRICHOThECENES IN FOOD PROCESSING SYSTEMS

The surveillance studies reported in Chapters 4 and 5 show that raw cereals contain a high incidence of trichothecenes. Similarly, the deoxynivalenol survey indicates the occurrence of relatively high levels in some unprocessed cereals. It is essential to know therefore if the trichothecenes are degraded by processing techniques, especially as a significant portion of our staple diets is cereal based in the form of bread, bakery products, snack products and cereal foods. Little work has been published on the fate of trichothecenes in food manufacture. At the Food RA, under the MAFF project of the fate of mycotoxins in food process operations, we have studied the fate of aflatoxin, ochratoxin A and deoxynivalenol through a laboratory simulated cornflake manufacturing process. Cornflake manufacture consists of the following stages.

1. Steam cooking of corn grits (de-germed maize produced by a wet milling process).

2. Drying and tempering to a particular moisture level.

3. Rolling of the grits to produce flakes.

4. Toasting to produce a crisp flake.
The operation as performed at the RA is shown as a flow diagram in Fig 6.1. Notes on the commercial method are included alongside for information. For production of deoxynivalenol contaminated corn grits, *Fusarium culmorum* was inoculated on to moistened grits (de-germed maize, supplied by a member of the Food RA) and incubated for 10 days at 25°C. The grits were treated with ethylene oxide immediately prior to processing; 50 gm portions of ground contaminated grits were analysed using the extraction method described in Appendix III. It was thought desirable to analyse larger portions of sample than the rapid method allowed, but this necessitated using a large column clean-up. The final extract was analysed by TLC.; at this stage (i.e. prior to ethylene oxide treatment and processing) it was sufficient to check the approximate level of deoxynivalenol only in order to assess whether manufacture could proceed, or to incubate for a longer period of time. Analysis indicated that approximately 250 ppb deoxynivalenol was present, which was sufficient for processing. This analysis also indicated that ethylene oxide treatment of the mouldy grits did not interfere with the analysis, nor did it result in any apparent loss of toxin. The 'spots' of DON visualised on TLC plates with aluminium chloride were entirely normal in appearance after ethylene oxide treatment, and a similar level was observed (approx. 250 ppb) both before and after treatment of the grits with ethylene oxide. DDTC analysis indicated that the retention time was normal after treatment of the grits and the level of DON corresponded with that determined by 2D TLC.
LABORATORY

STAGE 1

COOKING
600g grits + water + flavour
(supplied by a member of the food R.A.)
Seal in vacuum pouch and cook for 1¼ hrs at 19 psi.

Remove grits from pouch to flat trays

STAGE 2

DRYING & TEMPERING
Oven dry in fan assisted oven for 1 hr at 118°C to 18-20% moisture content
Temper in large screw capped jars overnight

Remove from jars

STAGE 3

ROLLING
Roll flakes to appropriate thickness through steel rollers

Transfer flakes (a cup full at a time) to toaster

STAGE 4

TOASTING
Toast flakes by rotating basket over gas jets for 30 seconds until crisped

Figure 6.1
Flow diagram of the process for laboratory manufacture of cornflakes

COMMERCIAL

Pre-steam grits for ½ hr
Cook at 18 psi for 1-2 hrs

Equilibrate in large container for 1-2 hrs until sticky to touch

As laboratory

As laboratory but much larger scale
The final product produced by the laboratory process was visually acceptable and was judged to be of satisfactory appearance and texture by the member of the Food RA who provided technical assistance throughout these trials.

50 gm portions were removed at each stage and analysed for deoxynivalenol as described in Appendix III, detecting and quantifying by both DDTC and 2DTLC. Results were not corrected for moisture content for each stage; the difference in moisture between cooked grits and dried grits is approx 15%. The results are given in Table 6.1. Fig 6.2 presents the results in histogram form.

TABLE 6.1
Deoxynivalenol content of corn grits through the laboratory cornflake manufacture

<table>
<thead>
<tr>
<th>Sample/Stage</th>
<th>ppb by DDTC method</th>
<th>ppb by 2DTLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control uncontaminated grits</td>
<td>ND+</td>
<td>ND</td>
</tr>
<tr>
<td>Contaminated grits</td>
<td>265</td>
<td>Approx 250</td>
</tr>
<tr>
<td>Cooked grits</td>
<td>101</td>
<td>Approx 100</td>
</tr>
<tr>
<td>Dried and tempered grits</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rolled flakes</td>
<td>175</td>
<td>Approx 150</td>
</tr>
<tr>
<td>Toasted flakes</td>
<td>66</td>
<td>Present; approx 50</td>
</tr>
</tbody>
</table>

+ ND = None detected
Figure 6.2
Concentrations of Deoxynivalenol through the laboratory cornflake manufacture
The dried and tempered sample was analysed twice, but no toxin could be detected on either analysis. (This sample was also very difficult to analyse for the other mycotoxins studied through the process). The grits at this stage are very sticky and firm, and impossible to grind to a small particle size. It could be therefore, that the extraction method was not appropriate for this type of sample. The results reported are not corrected for a constant moisture level after the dried and tempered stage; this may partly account for the apparent 'rise' in toxin concentration of the rolled flakes. Uneven distribution of the toxin (an inevitable result of 'patchy' mould growth) is perpetuated since each grit retains its identity throughout the process to become one flake. There is therefore no mixing inherent in the process.

Discussion

The apparent overall loss of deoxynivalenol from 265 ppb in the original grit to 66 ppb in the toasted flakes represents 75%. Neither the TLC analysis nor the DDTC analysis revealed the appearance of other compounds as the processing progressed. Comparing this food process with other food processes where the fate of mycotoxins have been studied, during the manufacture of cider, patulin is apparently 'lost' during the alcoholic fermentation. Thus in terms of its original chemical identity, patulin is (Bunagh, 1977) destroyed. However, there is a possibility that such a food process alters the mycotoxin chemically so that the analytical determination indicates loss, but the breakdown product is still biologically toxic, as determined by bioassays. This would seem to be true in
the case of the 'corn steeping' process which has been studied at
the Food RA (Wood, Cooper & Chapman, 1981). In this process, maize
is steeped in water containing numerous micro-organisms (principally
yeasts and lactobacilli). \( \text{SO}_2 \) is introduced into the steep, and
after approximately 2 days steeping in the commercial process, the
maize is ready for further processing to the various fractions (e.g.
cornflour, gluten, corn oil). A laboratory scale process of this
procedure had indicated that in the case of ochratoxin A, marked
cytotoxic effects were noted in some samples, (Wood et al.
1981). The level of ochratoxin A alone determined by HPLC was insufficient
to have caused the cytotoxic effect. In addition, the HPLC
chromatograms showed the presence of a considerable number of extra
compounds. This is then an example of a biological assay giving a
clear indication of toxic constituents, possibly breakdown products
of the original mycotoxin, which were not chemically identifiable.
In the case of cornflake manufacture, there was no evidence either
chemically or biologically, of breakdown products of
deoxyrvalenol. Since the level of deoxynivalenol in all the sample
extracts was not high enough to cause a biological effect on tissue
cultures it may be assumed that the processing had not modified the
deoxyrvalenol to a more toxic compound. The tissue culture method
for bioassay of corn extracts is given in Appendix IV.

Conclusions

1. The overall loss of deoxynivalenol during the laboratory
manufacture of cornflakes was 75%.
2. Small scale laboratory simulation of cornflake manufacture, whilst producing a visually acceptable product, was subject to considerable sampling variation in terms of toxin distribution.

3. The uneven distribution of the toxin is perpetuated throughout the process, since each corn grit retains its individual identity to become a cornflake; there is no mixing stage in the process which could distribute toxin more evenly.

4. The dried and tempered sample presented difficulties for analysis, in that it was too sticky to grind to a fine particle size, and it was not possible to extract any toxin.

5. There was no evidence for enhanced cytotoxic effects as assessed by tissue culture as a result of modification of deoxynivalenol by processing.
COLLABORATIVE WORK ON THE DDTC METHOD

The reproducibility of the DDTC method gives some cause for concern, but several measures have been taken both to attempt identification of the factors causing operator sensitivity, and to validate the method. In Chapter 3, the work on attempting characterisation of the derivatisation product was discussed. This work showed that the reaction mechanism originally proposed as analogous to that reported by Munger et al. (1973) was not apparently occurring. Participation in collaborative work on the method, both in MAFF conducted trials and in conjunction with other organisations has shown reasonable agreement on some occasions between the DDTC method and the other methods currently used for trichothecene determination. Results of this collaborative research are discussed below.

Joint work with Unilever

This work indicated that good results could be obtained when the author was present to oversee operations. The main conclusions from this work were that operator sensitivity rendered the method insufficiently robust for general use, and that more attention must be paid to specifying grades of solvent purity, types of glassware etc.
Collaborative Trials with MAFF

On Page 66, Table 3.4 details the first collaborative work in which the DDTC method was used. A second trial, conducted 6 months later was organised by MAFF. For this trial, spiked samples were supplied to each collaborator, together with a vial containing a standard of unknown concentration. The results of this trial are given in Table 7.1. The results were discussed at the Analytical Panel of the MAFF mycotoxins working party and led to the following conclusions.

a. The DDTC method did not give acceptable results for this trial.

b. The results for the unknown standard were very variable, which may reflect the method of spiking the vials. It was observed by one participant that if DON standard (the unknown standard) was corrected to 200 ng/g for each laboratory, and the results recalculated for sample DON 1 and DON 2, the range of results becomes very different. Thus for Lab. E, (the RA) sample DON 1 would become

$$\frac{200 \text{ (i.e. theoretical level of DON std)}}{33 \text{ (actual result)}} \times 54 = 327 \text{ and Lab E}$$

is not the outlier.

c. Several laboratories reported higher levels than the extracts were spiked with.

d. The standard supplied gave different results at the Food RA than the standard normally used, involving a factor of at least twice the response.
Arising from this trial, especially the disturbing range of results obtained for 'DON std', it was agreed to prepare an exercise to quantify standards only. Deoxynivalenol standards at this time could only be obtained from one source in the USA, and no criteria of purity are available. Without reference standards of known purity, quantitative analysis is virtually impossible. Workers in the mycotoxins field have experienced standards being only 50% pure, and containing interfering compounds. In the case of deoxynivalenol, mass spectrometry is the only reliable method of determining standard purity.

Further collaborative work is proceeding actively, and currently in all such trials the trichothecenes are determined both by DDTC and by 2D TLC. The work of the analytical panel of MAFF is thus providing an invaluable opportunity for continuing to evaluate the DDTC method. Further opportunities for validating the method are presented in the analysis of samples submitted by members of the Food RA.
<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Deoxynivalenol conc. (ng/g)</th>
<th>Method of Analysis</th>
<th>Limit of quantification (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DON 1</td>
<td>DON 2</td>
<td>DON B</td>
</tr>
<tr>
<td>Spiking Levels</td>
<td>250</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>373</td>
<td>124</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B</td>
<td>412</td>
<td>155</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>300</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td>D</td>
<td>250</td>
<td>38</td>
<td>&lt;5</td>
</tr>
<tr>
<td>E</td>
<td>54</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>92</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>
GENERAL DISCUSSION

The major factor in discussing the results presented in this thesis lies in the criticism of the method, that is to say the operator sensitivity. However, the overall picture of results obtained by applying the DDTC method to surveillance of samples parallels a similar pattern to that found by many workers engaged in surveillance for trichothecenes. The results indicate a sporadic occurrence of high levels of trichothecenes. This sporadic type of distribution is one of the most disturbing factors which emerge from this work, and that reported by other researchers, and indicates the unpredictable nature of trichothecene contamination. This is undoubtedly a reflection of the lack of knowledge concerning the ecological factors controlling trichothecene production, and has resulted in many laboratories in the United States and Canada conducting surveillance programmes to monitor the quality of the wheat and corn harvests. Only thus can manufacturers be alerted to the possible dangers. Where this surveillance differs, is in the presentation of low levels of trichothecenes; the results of this would suggest a fairly high incidence of relatively low levels in our diets and in many staple foods. In discussing the significance of this type of low-level contamination, it is difficult to assess what can be considered a 'safe' level likely to result in a nil effect to health. This is particularly difficult to evaluate for the trichothecenes. Clinical trials, where animals have been fed pure toxins only, or food contaminated with pure toxin, may not
result in the same observed effects as for trials where animals have been fed naturally contaminated feed. This may be because these latter type of feeds contain not only identifiable toxins, but other metabolites acting synergistically to produce clinical effects. Thus moniliformin or butenolide may be implicated on a greater scale than has been suggested. The results of clinical trials have therefore been rather inconclusive in some ways, being dependent on the exact route of administration of toxins, and the 'composition' of the toxic metabolites. This has led to some divergence of opinion regarding the actual clinical effects observed and reported in animals. For chronic toxicity, even less is known, and an assessment of a safe level is therefore impossible at present. Many of the results reported in this thesis were at very low levels (1μg/kg or less) and it would not seem likely that this would pose any risk to health. The Canadian limit of 300 ppb would however seem to be rather high in view of the stability of the trichothecenes chemically, although this would generally result in a level of 100 ppb or less in final products. The DDTC method provides a far greater measure of sensitivity than is probably therefore necessary. The original scope of the method development was aimed at obtaining a method capable of measuring approximately 10 ppb. The DDTC method met this criterion, but was incidentally found to be more sensitive than actually thought to be desirable. The results at the 1ppb level are therefore probably of academic interest rather than being of significance. The results obtained from any surveillance studies are very dependent on the method of sampling and un-representative samples could invalidate the data.
It is therefore essential to adhere to a recognised sampling plan. For groundnuts, the 'TPI' sampling plan exists for ensuring rejection of nuts contaminated with $>30$ ppb. Sampling plans do not exist for nuts other than peanuts or for obtaining suitable samples for surveillance studies. A similar problem exists for contamination of cereals by trichothecenes and although the distribution of zearalenone in maize has been investigated, little work has centered on the trichothecenes. At the RA samples are submitted by members and therefore it is not usually possible to specify how samples should be taken. In addition, many commodities are very valuable, and therefore a manufacturer may be unwilling to sacrifice several kilos for analytical examination. For mould damaged food or raw materials, the sampling problem may be somewhat different; thus a manufacturer may only wish to know whether the mouldy portions contain any mycotoxins, and if mycotoxins are present, whether they are likely to migrate to uncontaminated and mould free areas, and whether the levels of mycotoxins detected are "significant". The problem thus returns to one of assessing the 'safe' level for mycotoxin contamination.

Validation of new methodology is essential for surveillance work, and the trichothecenes still present a problem area. The analytical panel of the MAFF working party was set up with a view to providing a basis for validating the methods used by participating laboratories. This panel is specifically for practical analysts, and participation in this panel by the author has been (and continues to be) a useful basis on which to subject the DDTC method
to comparative testing. Whilst the method has apparently been successfully used in some collaborative work, there are still many aspects of the method which need further investigation. The erratic sensitivity of the method and operator dependence may be related to the nature of the reaction which is occurring. The unusual pH, temperature and time dependency of the reaction have all been considered as indicative of a complex reaction occurring, and not a typical nucleophilic substitution as once thought. The MS work which MAFF performed only served to confirm the view that the reaction was very complex, and not a normal reaction to produce a stochiometric reaction product. The derivatisation behaviour of epoxypropane, aflatoxin B₁ and sterigmatocystin, and the inability to separate individual DDTC derivatives all confirm this complexity. For the present, the only active work in this area is a continuation of comparison of results obtained by 2D-TLC and DDTC on member samples.

Recent developments in methodology now include the test kit for vomitoxin and fusarenon X-type compounds marketed by LSB products. This kit may prove a worthwhile 'field'test for deoxynivalenol-type trichothecene contamination, but our preliminary trials have indicated that the 1 ppm detection limit has not been attainable. Other new techniques may prove invaluable for the future. Immunoassays may be a useful method for many mycotoxin assays, being relatively rapid and specific. Preparation of a suitable antiserum is proving to be difficult for trichothecenes however, not least because the trichothecene group contains so many individual member
compounds. Although immunoassays are now gaining some recognition in the mycotoxins field, it is probably some time in the future before these methods will be fully accepted, and confirmation by an analytical technique of different principle is still desirable for confirmation of positives. As a rapid screening test, immunoassays are already being used (e.g. for ochratoxin A), alternative methods being used as confirmatory techniques.

Arising from this discussion, there are evidently many problems facing the worker in the mycotoxin field, but particularly the worker approaching the subject of trichothecenes. The lack of chemical characteristics amenable to the usual methods of detection and the abundance of different members all belonging to the one group are just small parts of the problem. Lack of reference standards available to the analyst or reliable means of producing these materials are another difficulty. Production of suitable reference standards is an essential priority, since, with the exception of deoxynivalenol, T-2, rosidin A, and verrucarin A, standards cannot be obtained. In this connection MAFF is liaising with workers in the USA, as a matter of urgency, and several programmes of research are now investigating this particular aspect. Although no data has yet been published, results appear to be promising. One of the major problems has been co-ordinating the work of several research establishments; thus it is quite common in the USA, for a particular laboratory to have prepared the one or two trichothecenes appropriate for their needs, but not to have necessarily published their findings. Similarly, many workers have
found it difficult to reproduce the conditions which have been reported to yield a particular trichothecene. This may in some part reflect the difficulty of correctly identifying *Fusarium* species. It seems likely that the species identified by one laboratory as a good toxin-producer may not be the same species as that used by another laboratory which is unsuccessful. Identification of the fusaria is still a difficult area, even for the trained mycologist. Work is, however, progressing on the preparation of reference materials and hopefully a greater variety of trichothecenes will become available. The Analytical Panel of the MAFF working party will be producing monographs for mycotoxins; these monographs will provide basic information on methodology, chemistry, spectra, purity (and appropriate means for checking this) and availability of standards as a reference document. Although the number of mycotoxins this is intended to cover is fairly restricted at present, concentrating on the more common mycotoxins, it should eventually be extended to provide a comprehensive service of great value to the analyst.

Considerable work is also in progress on elucidating the environmental controls involved in trichothecene production. This work may enable appropriate preventive measures to be taken in the future so as to avoid the sporadic occurrence of possibly dangerous levels of trichothecenes. It is reassuring to know that considerable effort is being extended to help solve some of the problems on an international scale, and in the UK, MAFF is active
in its participation. International meetings, such as those organised by the AOAC and IUPAC provide a useful forum for exchange of information and current trends. One of the most controversial subjects discussed in the latter years was that of the 'Yellow Rain'. Analysis of clinical samples obtained from Yellow Rain victims would suggest that T-2 toxin is metabolised to HT-2 toxin and excreted in the urine. However the paucity of samples, size of samples and general lack of available information have made this topic a difficult one, not least because of its political nature.

It is often believed that mycotoxins are not a major source of disease and that the problem should be ignored. One may hear the comment that, as mould and mycotoxins have been with the population through the centuries, they are not a cause for concern. Whilst this may be true for the majority of the world population, for the unfortunate few who fall victim to mycotoxin-mediated disease, this is no comfort. Recent reports from Kenya (Ngindu et al. 1982) serves only to remind us that fatalities still occur from mycotoxin contaminated feed, for all the sophisticated methods of testing and surveillance. Where the choice exists between contaminated food or starvation a radical change in approach is necessary; avoidance of contamination is clearly not a choice for Third World countries (unlike the developed countries) and detoxification programmes are essential. The ammoniation treatment for removal of aflatoxin shows some promise, and may perhaps be a worthwhile consideration for treatment of trichothecene contaminated cereals. There still remains a need for further studies: a rapid sensitive method for trichothecenes, continuing international surveillance, and above all, an understanding of the factors controlling trichothecene
production and hence a means of preventing contamination in the future. Although much work is in progress in this area, it is undoubtedly some way in the future before we can guarantee that our food is either free from mycotoxin contamination, or at least contains only those levels which we know will not result in disease in mankind either in the short or long term.
APPENDIX I

MYCOTOXIN LABORATORY METHOD

Method for the Extraction and Quantification of Aflatoxins, Ochratoxins, Zearalenone and Trichothecenes


A. PREPARATION OF SAMPLES
1. Grind the entire sample on Vertec grinder or in Kenwood as appropriate.
2. Mix the ground sample to ensure that it is of as uniform character as possible.

B. DEFATTING OF SAMPLES

If the sample has a high fat content (eg. cocoa beans) it may be necessary to perform an initial de-fatting of the sample.
1. Weigh 50g of the sample into a large extraction thimble and plug the top of the thimble with glass wool
2. Extract for 3 hours with approximately 200ml SLR grade hexane in a straight-through extraction apparatus.
3. Invert the thimble into a 400ml beaker and leave to dry in a fume cupboard for approximately one hour. If analysis is to be completed on the following day, refrigerate overnight.

C. INITIAL EXTRACTION OF MYCOTOXINS

1. If the sample was not subjected to an intial defatting, weigh 50gm of the sample into a blending bottle, and add 25ml distilled water (or 25ml of 25% w/v AR silver nitrate solution for cocoa products) and 250ml AR chloroform. Proceed to step 4.
2. If the sample was defatted, transfer the sample quantitatively
to a blending bottle using a powder funnel, washing the funnel
with portions of the 250ml AR chloroform in (3). Add 25ml
distilled H₂O or 25ml of 25% w/v AR silver nitrate solution
for cocoa products.

3. Add the remainder of 250ml AR chloroform.

4. Blend for 3 minutes.

5. Filter the resultant mixture through a fluted 24cm Whatman No.
4 filter paper.

6. Collect 50ml of filtrate in a measuring cylinder and stopper to
prevent evaporation.

D. COLUMN "CLEAN-UP" AND FRACTIONATION PROCEDURE

1. Plug the bottom of a 22 x 300mm glass chromatography column
with glass wool. The column should be fitted with a tap.

2. Add approximately 5g of AR anhydrous sodium sulphate, wash down
the sides of the column with AR chloroform, and allow the
packing to settle. The level of the chloroform should be at
least 5cm above that of the sodium sulphate, in order to
prevent mixing when further packing is added.

3. Slurry 10-12gm silica gel (Kieselgel 60, 70 - 230 Mesh) with
chloroform, and pour gently into the column. Wash the sides
down with chloroform.

4. Allow the column packing to settle, and then add approximately
15g of anhydrous sodium sulphate. Wash down the sides of the
column with chloroform if necessary.

5. Run off the chloroform until the meniscus reaches the top of
the upper layer of sodium sulphate. (The column must not be
allowed to run dry).
6. Add 50ml of the sample filtrate to the column.

7. Allow the sample to pass through the column at a flow rate of 1 - 2ml/minute, until the meniscus reaches the top of the upper layer of sodium sulphate.

8. Add 150ml Distol grade hexane and allow this to pass through the column at the rate of 10 - 20ml/min.

For Zearalenone: (Note: If ochratoxin/aflatoxins only pass directly to step 13).

9. Add 150ml of AR grade Benzene and allow this to pass through the column.

10. Discard eluates from steps 7-9 above.

11. Elute zearalenone from the column with 150ml of benzene/acetone 95:5 v/v at a rate of 10-20ml/min. Collect eluate in a 500ml round bottomed flask (Zearalenone fraction).

12. Elute T-2 type trichothecenes by adding 150ml of AR anhydrous diethyl ether, and allow this to pass through the column at a rate of 10-20ml/min. Collect the eluate in 250ml flat-bottomed flask (Trichothecene fraction).

13. Elute the aflatoxins from the column with 150ml of chloroform/methanol 97:3v/v. Collect the eluate in a 250ml conical flask (or similar) (Aflatoxin fraction).

14. Elute the ochratoxins from the column with 150ml benzene/acetic acid 9:1 v/v at a rate of 10-20ml/min. Collect the eluate in a 500ml round bottom flask (Ochratoxin fraction). Note: this can be left to elute overnight if necessary.

15. Add a few anti-bumping granules to the aflatoxin fraction and the trichothecene fraction and evaporate to near dryness on a steam bath.
16. Evaporate the fractions containing ochratoxins and zearalenone to near dryness on a vacuum rotary evaporator. These fractions must not be heated to more than 35°C.

17. If vacuum rotary evaporation does not remove all of the solvent from the fractions, the remaining solvent can be evaporated under a vigorous stream of nitrogen on a water bath held below 35°C.

18. Quantitatively transfer the residues from the various fractions to 20ml glass vials using 10-15ml AR chloroform. (If the final analysis is to be by HPLC, filter the residues through millipore filters size 0.4 μm).

19. Evaporate the chloroform under a gentle stream of nitrogen. Heating will facilitate evaporation, but the temperature must not exceed 35°C for zearalenone.

20. Seal vials tightly with aluminium lined plastic caps. If samples are to be stored prior to TLC or HPLC analysis they should be stored in the dark, preferably at -18°C.

E. IDENTIFICATION AND QUANTIFICATION OF THE MYCOTOXINS BY HPLC

Aflatoxins

1. Dissolve the aflatoxin residue from D(19) above in 200μl of AR dichloromethane (agitate on a vortex mixer to ensure that the residue has been thoroughly dissolved).

2. Inject 10μl of this solution onto the HPLC column.
3. Conditions for HPLC analysis

Column: - Silica Gel 5μm particle size, length 20cm - Spherisorb 5SW.
Solvent A: - 10% AR glacial acetic acid in Distol grade hexane
Solvent B: - Isopropyl alcohol
% B in A: - 20%
Flow rate: - 2.0ml/min increasing to 4.0ml/min at 20 mins
Detector: - Fluorescence detector: either LDC or Perkin Elmer.
Excitation: - 365nm) Perkin Elmer
Emission: - 425nm ) Max. scale expansion
LDC 360nm Kit. Range x 5
Printer/Plotter x 5. Chart Speed 0.5cm/min

4. Also inject circa 10μl of mixed aflatoxin standard onto the HPLC column (circa 20ng of each of the aflatoxins, B₁, B₂, G₁ and 6ng G₂; the composition must be known exactly).

5. Identify the aflatoxins within the sample by comparing their retention times with those of the standards.

Ochratoxins

1. Dissolve the ochratoxin residue from D(19) above in 200μl of AR dichloromethane.
2. Inject 10μl of this solution onto the HPLC column.

Column: - (Normal phase) Amino-propyl-silyl column, 5μm particle size, 20cm long.
Solvents A & B: as for aflatoxins)

% Bin A: 20%

Flow rate: 3.0 mls/min

OR:

Column: (Reverse phase) Partisil 10 ODS.

Solvent: Acetonitrile/o.25N Phosphoric Acid 1:1 2ml/min.

Detector: Fluorescence detector: Perkin Elmer or LDC

Perkin Elmer:

Excitation: 340nm) Perkin Elmer Max. scale expansion.

Emission: 450nm

LDC 254nm kit Range x 5

Printer Plotter Atten. x 5 C/S 0.5 cm/min

4. Inject circa 25ng of ochratoxin A standard (also B if available) onto the HPLC column, if using Perkin Elmer detector. Inject 10ng of ochratoxin standard if using LDC detector.

5. Identify ochratoxin(s) in the sample by comparing their retention time(s) with those of the standards.

6. Quantify the ochratoxin(s) by comparison of peak areas with those of the standard(s).

Zearalenone

1. Dissolve the zearalenone residue from 419 above in 200 µl AR dichloromethane.

2. Inject 10 µl of this solution onto the HPLC column.

3. Conditions for HPLC analysis:

   Column: as for ochratoxins, normal phase

   Solvents A & B: as for aflatoxins)

   % B in A = 5%

   Flow rate = 2.0 ml/min
Detector: - Fluorescence Detector - Perkin Elmer

Excitation: - 310nm

Emission: - 435nm

Scale Expansion x 50

Printer/Plotter x 5. Chart Speed 0.5cm/min

4. Inject c. 50ng zearalenone standard on to the column.

5. Identify and quantify zearalenone as per aflatoxins and ochratoxins.

Trichothecenes: Prepare derivatised and underivatised sample extracts as described in Appendix III steps 11-15.

Note

The usual method for identification and quantitation for aflatoxins ochratoxins and zearalenone is by HPLC (as above). However, TLC is sometimes applied for additional confirmation. If positives or 'doubtfuls' are identified by HPLC, an appropriate quantity of standard (approx. the same amount as that identified in the sample) should be co-injected with the sample extract to check the retention time of the compound in question. Further work may then be indicated, such as TLC or other confirmation techniques.

Limits of Detection

HPLC as final quantification (dependent on commodity)

Aflatoxins 1.0μg/kg for each of the toxins
Ochratoxins 1-5μg/kg of each of ochratoxin A & B
Zearalenone 20μg/kg
Trichothecenes 1μg/kg
RAPID METHOD FOR TRICHOTHECENES

1. Grind entire sample and mix thoroughly.
2. Weigh 10gm into a Quickfitc-neck 250ml conical flask; add 50ml chloroform:methanol 1:1. Stopper and shake for 30 minutes on a wrist-action shaker.
N.B. For each sample prepare two extracts, one is a blank preparation.
3. Filter through a Whatman No. 4 filter paper (11cm) collecting 20mls.
4. Pass the extract onto a silica Sep Pak, using a 5ml syringe, discarding the eluate.
5. Wash the Sep Pak with 5ml Distol hexane and discard.
6. Elute the toxins with 10ml chloroform:methanol 97:3, collecting the eluate in a small flat-bottomed flask.
7. Add a few anti-bumping granules and evaporate to dryness on a steam bath.
8. Transfer the residue to a glass vial with chloroform; then dry down under nitrogen on a steam bath.
9. Derivatise the test samples as in Appendix III, steps 11-15.
APPENDIX III

METHOD FOR TRICHOTHECENES

Extraction

1. Homogenise or grind entire sample and mix thoroughly.
2. Weigh 50gm sample into a blending jar and add 250mls methanol.
   Blend for 3 minutes.
3. Filter through Whatman No. 4 filter paper collecting 100 mls.
4. Pack a chromatography column with 5gms sodium sulphate, 10gms silica gel (slurried in methanol) and 5 gms sodium sulphate;
   keep column in methanol until required.
5. Pass filtrate from (3) above through the column, collecting the eluate at a flow rate of 5 - 10mls/min. in a flat-bottomed pyrex flask.
6. Pass 100mls hexane through the column and discard.
7. Pass a further 100mls methanol through the column, bulking with the eluate from (5) above.
8. Evaporate the eluate to dryness on a steam bath.
9. Transfer to a vial with methanol, passing the residue through a silica sep pak if necessary.
10. Evaporate to dryness under nitrogen on a steam bath.
11. Expose dried extracts to 1ml weak hypochlorite solution (5 vol/100ml H₂O = 5 vol commercial Choros in 100ml H₂O) for 2.5 hours. Remove excess hypochlorite by adding 2mls 2% sodium metabisulphite. Shake extracts out gently with chloroform in a 50ml separatory funnel. Separating funnels fitted with
interflon stopcock are essential. Discard the aqueous layer; dry the chloroform layer on a steam bath under N\textsubscript{2}, briskly (preferably in <10 mins).

12 Derivatise one extract of each duplicate (=test solution) as follows (reserving one extract underivatised = sample blank preparations, which are analysed directly as in Step 15.)

Add 1ml Buffer pH 7.7 (9volM/15 \( \text{Na}_2\text{HPO}_4 \):1volM/15 \( \text{KH}_2\text{PO}_4 \))

1ml EDTA (0.1M aqueous)

1ml DDTC (2% aqueous diethyldithiocarbamate, sodium salt)

Buffer and DDTC must be prepared fresh daily.

Stand at room temperature for 2.5 hours.

13. Also prepare a calibration curve to cover the range

10 - 50ng in final injection aliquot of deoxynivalenol or 1 - 10ng in final injection aliquot of T-2.

14. Shake out extracts gently with chloroform using 50ml separating funnel with interflon stopcock, and evaporate the chloroform layer to dryness on a steam bath under N\textsubscript{2} briskly (in <40 mins).

15. Take up extracts in 200\( \mu \)l Dichloromethane and analyse 10\( \mu \)l aliquots by HPLC.

**HPLC Operating Conditions**

Cecil C E 2112 variable wavelength U.V. detector: 284nm; scale expansion. X 0.5 Altex Dual Pump Solvent Delivery System: 5% B in A where B = propan-2-ol and A = 10% acetic acid in hexane. 2mls/min.

Column: Spherisorb 5 \( \mu \)m amino-propyl silyl cross-linked silica 25cm x 0.6cm id

RT of T-2 approx 3 minutes.
Calculation

Subtract the peak height of the reagent blank from the height of the standard to give the peak height attributable to toxin only.

For samples subtract the peak height of the reagent blank or the sample blank whichever is the greater; by proportion with the standard express the concentration of the toxin $\mu$g$kg^{-1}$ in the sample.
APPENDIX IV

DETAILS OF BHK TISSUE CULTURE ASSAY METHOD

A. Preparation of cells

1. The cells are inoculated into growth medium in maintenance flasks and incubated at 37°C until confluent.

2. The cells are trypsinised as follows:
   (a) The medium is aseptically decanted from the flask.
   (b) 4mls of trypsin solution is added to the flask.
   (c) The flask is laid flat on the bench for 1 minute.
   (d) The trypsin is aseptically decanted off and the flask incubated at 37°C. There is sufficient residual trypsin to strip the monolayer from the surface of the flask.
   (e) After 5 - 10 mins. the monolayer is removed from the surface and with gentle shaking the cells are freed.
   (f) 5mls of growth medium is added to the flask, and the cells pipetted up and down to break up clumps.

3. The cells are counted in a haemocytometer. To 1ml of the cell suspension is added 0.2ml of Trypan blue (stains non-viable cells blue).

4. The cells are diluted in fresh growth medium to give a concentration of approximately $2 \times 10^5$ cells/ml.

5. 1ml of this cell suspension is inoculated into each Leighton tube to be used for the test. These tubes contain a glass coverslip on which the cells grow. Duplicate tubes are inoculated for each extract. The cells are inoculated at 37°C for 24 hours.
B. Toxicity testing

1. Sample extracts or toxin standards are evaporated to dryness and reconstituted in 100μl dimethyl sulfoxide (DMSO). 100μl of fresh growth medium is added for the first dilution. Further dilutions are made in growth medium.

2. The inoculated Leighton tubes are examined for cell growth. Any tubes showing sparse or abnormal cell growth are discarded.

3. After decanting off the growth medium the remaining tubes are inoculated in duplicate with 20μl of the sample extract. Two tubes are inoculated with DMSO only, and 2 tubes are left uninoculated. These are the control tubes.

4. The tubes are reinoculated with 1ml of fresh growth medium, and incubated at 37°C for 48 hours.

5. The cells are observed microscopically for any toxic effects.

6. The coverslips are fixed in methanol and then removed from the tubes and stained with haematoxylin and eosin.

7. The stained coverslips are observed microscopically and toxic effects evaluated.

GROWTH MEDIUM FOR BABY HAMSTER KIDNEY CELLS

Glasgow's modification of Eagle's Medium, containing lmM glutamine (Flow Laboratories)

10% foetal bovine serum

10% tryptose phosphate broth

10% antibiotic solution (containing penicillin and streptomycin)

20mM HEPES buffer
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GLOSSARY OF COMMONLY USED ABBREVIATIONS

NAMES OF MYCOTOXINS

Deoxynivalenol = DON
(also known as 'vomitoxin')
Diacetoxyscirpenol = DAS
Roridin A = ROR
Verrucarin A = VER
Aflatoxin B₁ = AFB₁
Sterigmatocystin = SMN
Zearalenone = F₂

CHEMICALS AND REAGENTS

Chloroform = CHCl₃
Distilled/Deionised Water = H₂O
Sodium Hypochlorite-Solution = NaOCl
Dianhydrogalactitol = DAG
Sodium diethyldithiocarbamate = DDTC
Ethylene diamine tetra-acetic acid - sodium salt = EDTA

LEVELS OF MYCOTOXINS

µg/kg = ppb = ng/g
µg = micrograms
ADDENDUM

Since completion of the work reported in this thesis, a further short period of research was performed at the Food RA (some 18 months later), but not by the author. In this work, the products of the reaction between DDTC and deoxynivalenol were studied in depth. Although the outcome of this research is confidential (not being divulged to the author), it is known that the compound being measured by UV curve and by HPLC was disulphuram. A change in the DDTC reagent occurs to form disulphuram, a change which is not mediated by deoxynivalenol. It has been stated in this thesis that the original reaction mechanism proposed was probably not occurring; this is now proved to be so. In view of this therefore, the quantitative results given in this thesis must be viewed with doubt, apart from those which were confirmed using a different technique.