FUMONISINS: CHROMATOGRAPHIC METHODOLOGY AND THEIR ROLE IN HUMAN AND ANIMAL HEALTH

BY

ERIC WILLIAM SYDENHAM

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Promotors: Prof Klaus R Koch (University of Cape Town)
Dr Pieter G Thiel (Medical Research Council)

Especially dedicated to my wife
Gill and son Shaun

DECLARATION

I, Eric William Sydenham, hereby declare that this thesis represents my own original work and that the contents have not previously been submitted, either in their entirety or in part, to any other University for a degree. Pertinent extracts of the research work of others, have been acknowledged and are fully referenced.

Signed by candidate

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(Date)

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PUBLICATIONS

Data presented in this thesis have already been incorporated into the following scientific papers and presentations.

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ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
AP_1	Aminopentol
BCR	European Central Reference Bureau
C	Celcius
Ca	Calcium
$CaCl_2$	Calcium chloride
$CHCl_3$	Chloroform
$CH_3COOC_2H_5$	Ethyl acetate
CH_3COOH	Acetic acid
CH_3CN	Acetonitrile
CH_3OH	Methanol
cm	Centimetre(s)
CSID	Chemiselective immobilisation & detection
DEN	Diethylnitrosamine
DnsCl	Danzyl chloride
DON	Deoxynivalenol
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked-immunosorbent assay
ES	Electrospray
FAB	Fast atom bombardment
FA_1	Fumonisin A_1
FA_2	Fumonisin A_2
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FC_1	Fumonisin C_1
FID	Flame ionization detector (-tion)
FP	*Fusarium proliferatum*
g	Gram(s)
GC	Gas chromatography
GGT+	Gamma-glutamyltranspeptidase positive foci
HCl	Hydrochloric acid
HORRAT	Horwitz equation
HPLC	High-performance liquid chromatography

HPTLC	High-performance thin layer chromatography
H_2O	Water
H_2SO_4	Sulphuric acid
Hz	Hertz
IARC	International Agency for Research on Cancer
ip	*Intraperitoneal*
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure & Applied Chemistry
iv	*Intravenous*
K	Potassium
kg	Kilogram(s)
KCl	Potassium chloride
KOH	Potassium hydroxide
LEM	Leukoencephalomalacia
LC	Liquid chromatography
M	Molar
Mg	Magnesium
$MgCl_2.6H_2O$	Magnesium chloride
mm	Millimetre(s)
MRC	Medical Research Council
MS	Mass spectrometry/Mass spectrum
Na	Sodium
NA	Not analysed
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium hydrogen carbonate (bicarbonate)
NaH_2PO_4	Sodium dihydrogen phosphate
$Na_2B_4O_7$	Sodium tetraborate
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NBD-F	7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole
ND	Not detected
ng	Nanogram(s)
nm	Nanometre(s)
NIV	Nivalenol
NMR	Nuclear magnetic resonance spectroscopy
NR	Not reported
OC	Oesophageal cancer
ODS	Octadecasilane

OPA	*o*-phthaldialdehyde
p	Probability factor
PH_1	Partially hydrolysed moiety of FB_1
ppb	Parts per billion
ppm	Parts per million
PPO	Porcine pulmonary oedema
PROMEC	Programme on Mycotoxins & Experimental Carcinogenesis
r	Correlation coefficient
R_f	Retention factor
RIA	Radio-immunoassay
RSD_r	Within-laboratory relative standard deviation
RSD_R	Between laboratory relative standard deviation
SAMB	South African Maize Board
SAX	Strong anion exchange
SL	Sample lost
SPE	Solid phase extraction
S_r	Within-laboratory standard deviation
S_R	Between-laboratory standard deviation
TCA	1,2,3-propane tricarboxylic acid
TFAI	Trifluoroacetylimidazole
TLC	Thin layer chromatography
v/v	Volume/volume
µg	Microgram(s)
µm	Micrometre(s)
µl	Microlitre(s)
USA	United States of America
UV	Ultra violet
W	White
Y	Yellow
ZEA	Zearalenone

CONTENTS

ABSTRACT

Fumonisins: Chromatographic Methodology and Their Role in Human and Animal Health

Eric William Sydenham

Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa

The fumonisins consist of a group of 7 structurally related mycotoxins originally isolated from *Fusarium moniliforme*, a fungal contaminant of maize worldwide. The incidence of *F. moniliforme* in home-grown maize, has been associated with human oesophageal cancer (OC) risk in the Transkei and China. Fumonisin B_1 (FB_1), the major fumonisin analogue, exhibits both cancer-initiating and -promoting activities, and has been shown to induce a number of disease syndromes in different animal species. Two other fumonisin analogues, fumonisins B_2 (FB_2) and B_3 (FB_3) also exhibit cancer-initiating potentials, similar to those observed for FB_1.

A method, developed at PROMEC, for the analytical determination of FB_1 and FB_2 in maize, based on ion-exchange purification of crude extracts, derivatisation, reversed-phase liquid chromatography separation and fluorescence detection, was subjected to an international collaborative study involving 11 laboratories from 6 countries. Although the results established that the method was highly reproducible, alterations were made in order to reduce analysis time, identify and eliminate potential sources of error and include the co-determination of FB_3.

Both methods were used, in conjunction with confirmatory techniques, to determine the extent of animal and human exposure to the fumonisins. Naturally occurring fumonisin levels in animal

feeds, were used in conjunction with hazard assessment data, to establish fumonisin tolerance guidelines for selected animal species. The results indicated that combined fumonisin concentrations in feeds of 10 and 100 μg/g (ppm) should be regarded as potentially harmful to horses and swine, respectively.

Human exposure assessment was based on data from various sources, including the 1989 and 1990 South African maize crops, maize imported into South Africa, retail maize-based foods from 14 countries, and home-grown maize from the Transkei. The data indicated that fumonisin contamination occurs worldwide, while the levels to which populations are exposed differ considerably. A statistical association was established between fumonisin contamination of home-grown maize, and the prevalence of human OC in the Transkei, where humans can be exposed to fumonisin levels that would be deemed harmful to both horses and swine.

Physical removal of fine particulate matter from maize shipments, prior to further processing, resulted in reductions in fumonisin levels by between 26 and 69%. Chemical treatment, with aqueous calcium hydroxide [$Ca(OH)_2$], resulted in the base hydrolysis of the fumonisins, to yield their corresponding aminopolyol and tricarballylic acid moieties. The fate of FB_1 was monitored during ambient temperature $Ca(OH)_2$-treatment of maize. Following treatment, FB_1 levels in the residual maize were reduced by between 88 and 95%, with the bulk of the toxin being transferred to, and monitored in, the aqueous fraction as its aminopentol moiety. Base hydrolysis of FB_1 proceeds via a partially hydrolysed moiety, which exists in equilibrium between two possible monoesters. These moieties were isolated and identified using liquid chromatography-electrospray mass spectrometry.

CHAPTER 1: INTRODUCTION TO STUDY

1.1 *MOTIVATION*

Numerous studies have confirmed the popular public belief/perception that the term "*natural*", especially when applied to food, is associated with healthy, wholesome and safe (Morgan & Fenwick, 1990). The active components in natural products (and herbal remedies) are perceived to be disassociated from chemicals, which in turn are commonly regarded as being man-made, damaging to the environment, and in the context of food, both hazardous and potentially carcinogenic. It is perhaps therefore an enigma that food is probably the most chemically complex substance to which the public is exposed. It has been estimated that there are more than half a million naturally occurring compounds in fresh plant foods; while more are undoubtedly formed during either food preparation or processing procedures (Morgan & Fenwick, 1990). These natural compounds to a greater extent account for the texture, appearance and flavour of the foods, as well as for their nutritional value and physiological effects. By contrast, there are less than a few thousand approved additives and agrochemicals, the majority of which have been subjected to extensive and rigorous biological evaluation prior to their approval (Morgan & Fenwick, 1990). Similar toxicological scrutiny is almost unknown for naturally occurring food compounds, even though many clearly possess varying degrees of biological activity.

There exists, therefore, a substantial number of naturally occurring dietary compounds about which there is incomplete evidence concerning their long-term effects in both animals and man. It has been suggested that were these compounds to be presented as synthetic chemicals to any of the current appropriate government committees, their use in foods would not even be considered (Morgan & Fenwick, 1990). Wodika (1971) used onset, severity and prevalence of symptoms to rank food hazards in order of importance, and classified microbiological contam-

ination as the most important risk factor followed by nutritional imbalance; the third was environmental contamination, followed by natural toxicants. Of least importance according to Wodika (1971) were the risks associated with agrochemicals and additives. It would therefore appear that scientific investigations (and public concern) should be based on the bioactivity of a chemical rather than its origin. It is also evident that in order to make an assessment of the risk to either human or animal health, posed by a selected chemical component, information is required concerning the concentrations at which it occurs in the diet, so that calculations on exposure can be made.

Both plants and fish have been identified as dietary sources of natural toxicants. Mycotoxins - compounds emanating from the fungal contamination of foods also merit inclusion as natural toxicants, since under many circumstances their presence in foods is almost inevitable.

1.2 *GENERAL BACKGROUND TO MYCOTOXINS AND MYCOTOXICOSES*

Fungi are members of the lower plant species that do not contain chlorophyll as do the higher plant species. Due to the absence of chlorophyll, fungi are unable to synthesise carbohydrates via photosynthesis, and accordingly they have to obtain their nutrients by growing either parasitically on the living tissues of plants, animals or man, or saprophytically on dead organic matter. It is due to the process by which they obtain their nutrients that fungi in general pose a potential threat to both animal and human health. During their growth stage, numerous fungi have the ability to produce a quite diverse range of secondary metabolites (mycotoxins), which can be toxic and/or carcinogenic if ingested by animals or man (Marasas, 1988). The term "secondary metabolites" was introduced into microbial biochemistry to differentiate those compounds such as alkaloids, terpenes, flavonoids and other plant products that were considered to be non-essential for the growth of the plants themselves (Bu'Lock, 1961). Conversely,

amino acids, fatty acids, saccharides, nucleic acids and proteins; compounds essential for all living organisms were termed "primary metabolites".

Mycotoxicoses has been defined as "*the intoxication of animals and man caused by the intake into the organism of mycotoxins*" (Krogh, 1969) and also as "*the illness or death of man, or his domesticated animals, following consumption of food contaminated with mycotoxins*" (Smith & Moss, 1985).

During the early months of the year 1960, significant losses of turkey poults were reported in the United Kingdom (Blount, 1961). The observed syndrome was termed "*turkey X disease*", although large scale losses of ducklings and pheasants were also reported. The disease was characterised by the rapid deterioration in the condition of the birds, followed by subcutaneous haemorrhages and death. Pathological examinations revealed livers that were pale in colour, fatty with extensive necrosis and biliary proliferation (Butler, 1974). The disease was eventually traced to the consumption of animal feed prepared from groundnut meal imported into the United Kingdom from Brazil. Mycological investigation of the implicated feeds resulted in the identification of *Aspergillus flavus* as the major fungal contaminant. Investigations eventually led to the isolation and characterisation of the causative agents which were termed the "aflatoxins", which in turn led to the disease being renamed "aflatoxicosis" (Austwick, 1978). The impact of the discovery of "aflatoxicosis" as a distinct disease syndrome, and the subsequent implication of aflatoxins as potential carcinogens in the human health chain, had international implications (LeBreton et al., 1962; Kraybill & Shimkin, 1964). Although major research efforts centered for some time on the aflatoxins, attention rapidly turned to alternative mycotoxins and their possible cause/effect relationship with other idiopathic diseases. Hence, the worldwide concern for aflatoxins and aflatoxicoses became a broader one of "mycotoxins" and "mycotoxicoses".

Although the outbreak of "aflatoxicoses" in the United Kingdom

might be regarded as the catalyst for modern mycotoxin research programmes, outbreaks of human mycotoxicoses can be traced back to the Middle Ages in Europe. These outbreaks were associated with the metabolic products of various species of parasitic moulds belonging to the genus *Claviceps*. The alkaloids produced by this class of fungi were responsible for the mass poisoning of both animals and man, over a period of several centuries. The disease was caused by the ingestion of bread prepared from rye flour contaminated with ergot sclerotia of *Claviceps purpurea* (Shank, 1978; Blunden et al., 1991). The disease was characterised by the onset of epileptic fits, vomiting and symptoms of insanity. Due to the constriction of arteries and veins, victims complained of the sensation of extreme cold in both their hands and feet, which was then followed by the burning sensations associated with ischaemia. In advanced cases the extremities of the victim became gangrenous and necrotic. Belief on the part of the victims that relief from the intense burning sensations could be sought by visiting the shrine of Saint Anthony, resulted in the disease adopting the popular name "*Saint Anthony's fire*". It was only in the seventeenth century that the alkaloids, produced by the ergot sclerotia of the implicated fungus, were identified as being responsible for the disease.

Outbreaks of confirmed and suspected animal and human mycotoxicoses associated with the specific *Fusarium* fungal species encountered in this study, will be introduced and discussed in each of the the appropriate chapters.

1.3 *FUSARIUM SPECIES AND THE CLASSIFICATION OF FUNGI*

This study will deal almost exclusively with the genus *Fusarium* and the recently identified mycotoxins the fumonisins. The genus *Fusarium* was first described by Link in 1808 (Ueno, 1983). In 1935, the genus was divided, on morphological grounds, into 16 sections, 55 varieties and 22 forms (Wollenweber & Reinking, 1935). The system was subsequently modified and the number of species reduced to 9 (Snyder & Hansen, 1945).

This latter system formed the basis of an illustrated guide to the identification of the fusaria (Toussoun & Nelson, 1968). Due to confusion within the literature with respect to nomenclature, an illustrated guide to the identification of the genus, based predominantly though not wholly on the classification system of Wollenweber & Reinking (1935), was published by Nelson et al. (1983). This latter system will be used throughout this study when reference is made to any *Fusarium* species.

1.4 *FUSARIUM TOXINS*

The fusaria in general have the ability to produce a diverse range of mycotoxins, but many of these are produced only under culture conditions, whilst far fewer are produced naturally. The structures of some of the major naturally occurring mycotoxins are illustrated in Figures 1.1 to 1.5, and they clearly indicate the chemical diversity of this class of compounds. It is these naturally occurring mycotoxins, and their association with important veterinary and human fusariotoxicoses that are cause for concern. Marasas (1991) identified *F. sporotrichioides, F. poae, F. equiseti, F. graminearum* and *F. moniliforme* as the five most important toxigenic *Fusarium* species. Prior to 1988, the four main groups of *Fusarium* mycotoxins produced by the five major species were the trichothecenes, zearalenone, moniliformin and butenolide (Marasas, 1991).

1.4.1 *Trichothecenes*

The trichothecenes (the basic structure of which is shown in Figure 1.1) are a chemically related group of mycotoxins produced by various *Fusarium* species, as well as by other genera of imperfect fungi. Trichothecin, isolated from *Trichothecium roseum* Link (Freeman & Morrison, 1949), was the first trichothecene mycotoxin identified. Phytotoxic compounds were isolated from *F. scirpi* Lamotte & Fautr. (Brian et al., 1961), the major metabolite being named diacetoxyscirpenol (based on the name of the fungus from which it was isolated and

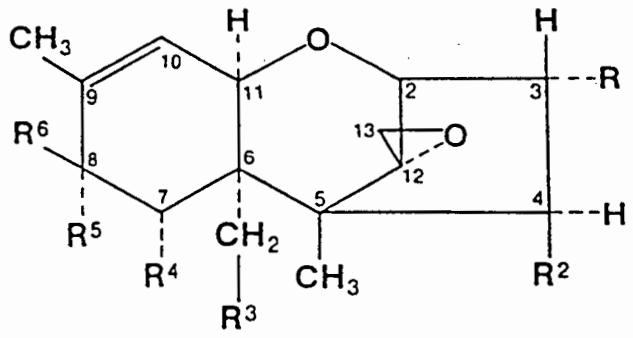


Figure 1.1 Basic chemical structure of the 12,13-epoxytrichothec-9-enes

the presence of two acetyl groups in its structure). Within several years, T-2 toxin, nivalenol (NIV) and its acetylated derivative, fusarenon-X, had been isolated (Bamburg et al., 1968; Morooka & Tatsuno, 1970). Although in excess of 70 trichothecenes have thus far been identified, the vast majority only occur in cultures under controlled laboratory conditions. Relatively few have been found to occur naturally on food and feedstuffs (Ueno, 1983; Kurata & Ueno, 1983), but their toxicological importance has been well established (Marasas et al., 1984b).

1.4.2 *Zearalenone*

The chemical structure of zearalenone (ZEA - Figure 1.2) was first elucidated by Urry et al. (1966). Although ZEA is referred to as a mycotoxin, it might better be classified as a

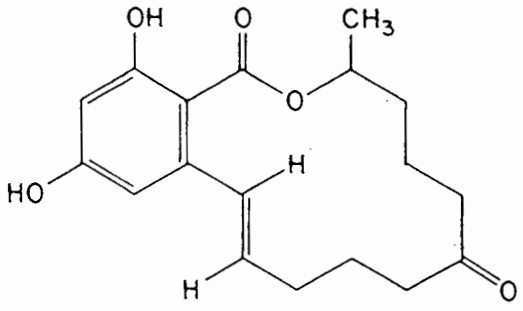


Figure 1.2 Chemical structure of zearalenone

non-steroidal hormone as it is non-toxic and induces estrogenic activity in animals, especially pigs, at relatively low dietary levels (Christensen, 1979).

1.4.3 *Moniliformin*

Moniliformin (Figure 1.3) is a highly polar, common metabolite produced by many *Fusarium* species. It has been shown to be extremely toxic to rats, ducklings, mice and chicks (Kriek et al., 1977; Burmeister et al., 1979). Moniliformin has been found to occur at high levels under natural conditions but its occurrence has not yet been associated with field outbreaks of fusariotoxicoses in either animals or man (Marasas, 1991).

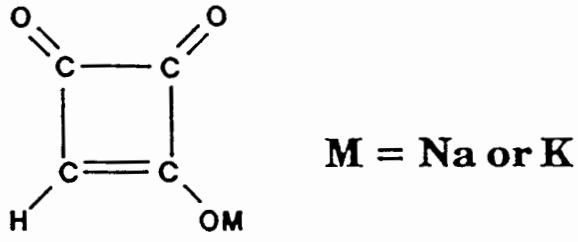


Figure 1.3 Chemical structure of moniliformin

1.4.4 *Butenolide*

Butenolide (Figure 1.4) was first isolated, both simultaneously and independently from culture material of *F. sporotrichioi-*

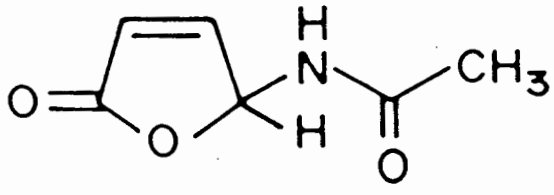


Figure 1.4 Chemical structure of butenolide

des (Yates et al., 1967) and *F. equiseti* (White, 1967). Isolates of *F. equiseti*, taken from toxic fescue hay associated with a disease syndrome of cattle known as "fescue foot", were found to produce butenolide (Burmeister et al., 1971). Similar syndromes of cattle have also been reported from India and China (Marasas, 1991), but the role of butenolide in these gangrenous diseases of cattle remains unresolved (Marasas, 1991).

In 1988, the isolation of a new group of *Fusarium* mycotoxins, the fumonisins (Gelderblom et al., 1988a), was of major importance to the field of mycotoxin research. An introduction to the isolation and characterisation of these toxins will be dealt with in sections 1.5 and 1.6, while the development of the initial analytical methods for their determination in cereal commodities will be discussed in sections 1.7.2 - 1.7.4.

1.5 *FUMONISIN MYCOTOXINS*

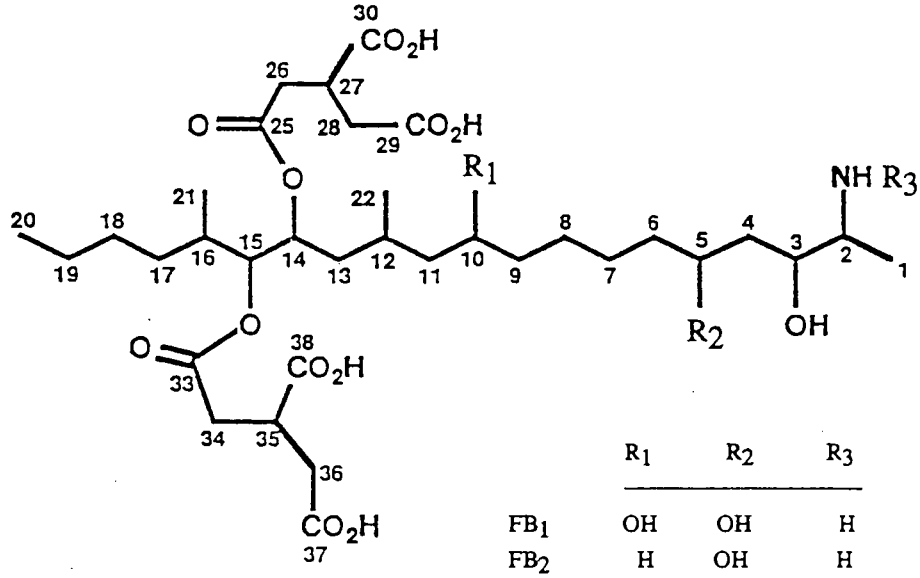
Fusarium moniliforme Sheldon [perfect state: *Gibberella fujikroi* (Swanda) Wollen.] has been classified in the section Liseola by Nelson et al. (1983). It occurs worldwide on a variety of plant hosts and has been found to be one of the most prevalent fungi associated with maize (*Zea maydis* L.) (Booth, 1971). The occurrence of *F. moniliforme* has been implicated in a number of animal and human diseases (Marasas et al., 1984b). Various strains of the fungus have been shown to be highly toxic to a number of animal species including horses, primates, pigs, sheep and rats (Kriek et al., 1981a,b), as well as carcinogenic to rats (Marasas et al., 1984a; Jaskiewicz et al., 1987a,b). Since, in addition, *F. moniliforme* had also been shown to be statistically associated with human oesophageal cancer risk in the Transkei, southern Africa (Marasas, 1982; Marasas et al., 1981, 1988a) and in China (Li et al., 1980; Yang, 1980), investigations have focused on the isolation and characterisation of the toxic/carcinogenic principles produced by *F. moniliforme*.

The mutagenic activities exhibited by various strains of *F. moniliforme* in the *Salmonella typhimurium* mutagenicity test, resulted in the isolation and characterisation of a group of four related compounds, which were initially given the trivial names fusariogenins A, B, C, and D. The major analogue (fusariogenin C) was subsequently renamed fusarin C, with the reported molecular formula of $C_{23}H_{29}O_7$ (Weibe & Bjeldanes, 1981; Gelderblom et al., 1984; Gaddamidi et al., 1985). However, the lack of carcinogenicity of fusarin C (Gelderblom et al., 1986) made it unlikely that the mutagen was involved in the carcinogenic effects attributed to the fungus from which it was isolated. Subsequent observations revealed that several strains of *F. moniliforme* exhibited cancer-promoting activity in a short-term cancer initiation/promotion bioassay in rats, using diethylnitrosamine (DEN) as a cancer initiator and the induction of gamma-glytamyltranspeptidase positive foci (GGT+) in the liver as end point (Gelderblom et al., 1988b). Utilizing the bioassay as a monitor for cancer-promotion principles, the fumonisins were isolated (Gelderblom et al., 1988a) and chemically characterized (Bezuidenhout et al., 1988) from culture material of *F. moniliforme* strain MRC 826. Strain MRC 826 had initially been isolated from home-grown maize collected from a high oesophageal cancer risk area of the Transkei, southern Africa, and culture material of the strain had previously been shown to be hepatocarcinogenic to rats (Marasas et al., 1984a; Jaskiewicz et al., 1987a).

1.6 *CHEMICAL STRUCTURES AND PROPERTIES OF THE FUMONI-FUMONISINS*

The recently described fumonisin mycotoxins are a group of 7 structurally related secondary metabolites originally isolated from culture material of *F. moniliforme* (Bezuidenhout et al., 1988; Gelderblom et al., 1988a; Cawood et al., 1991, Branham & Plattner, 1993). Isolation from the culture material was achieved by solvent extraction, followed by extensive fractionation of the extract using combinations of macroreticular polystyrene resin (XAD-2), reversed-phase Sephadex LH-20 and silica gel chromatography. Structurally elucidated by

both mass spectrometry (MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, the structure of the major analogue, fumonisin B_1 (FB_1), was shown to be the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane, in which the C-14 and C-15 hydroxy groups are esterified with the terminal carboxy group of propane-1,2,3-tricarboxylic acid (tricarballylic acid - TCA). In addition to FB_1, three other structurally similar fumonisin B toxins have been characterised and given the trivial names fumonisins B_2 (FB_2), B_3 (FB_3) and B_4 (FB_4) (Gelderblom et al., 1988a; Cawood et al., 1991), while two other analogues have been given the trivial names fumonisins A_1 (FA_1) and A_2 (FA_2) (Bezuidenhout et al., 1988). The structures of the FA_1 and FA_2 correspond to the *N*-acetyl derivatives of FB_1 and FB_2, respectively. Recent studies by Branham & Plattner, (1993) resulted in the isolation of fumonisin C_1 (FC_1), a seventh analogue, from liquid culture medium of *F. moniliforme*. The authors characterised FC_1 as being structurally similar to FB_1, with the absence of the end terminal-methyl group of the FB_1 analogue. The chemical structures of the



	R_1	R_2	R_3
FB_1	OH	OH	H
FB_2	H	OH	H
FB_3	OH	H	H
FB_4	H	H	H
FA_1	OH	OH	CH_3OH
FA_2	H	OH	CH_3OH

Figure 1.5 Structures of the fumonisin A and B analogues

fumonisin A and B analogues are given in Figure 1.5.

Cawood et al. (1991) reported that of the characterised fumonisin analogues, only three (FB_1, FB_2 and FB_3) were produced in significant quantities under culture conditions. Short-term carcinogenesis studies in a rat liver bioassay indicated that of the five additional fumonisin A and B analogues, only FB_2 and FB_3 exhibited toxicological and cancer-initiating properties similar to those observed for FB_1 (Gelderblom et al., 1991b). Hence, this study has centered on these three major biologically active fumonisin analogues.

Fumonisins B_1, B_2 and B_3 exist as colourless compounds that are soluble in polar solvents such as water (H_2O), methanol (CH_3OH) or acetonitrile (CH_3CN) and insoluble in non-polar solvents (ie. hexane). Fumonisin B_1 has a molecular formula of $C_{34}H_{59}NO_{15}$ and a molecular mass of 721, while both FB_2 and FB_3 contain one oxygen atom less, having similar molecular formulae of $C_{34}H_{59}NO_{14}$ and molecular masses of 705 (Bezuidenhout et al., 1988; Cawood et al., 1991). None of the fumonisin mycotoxins possess chromophores, and therefore they do not absorb either ultra-violet (UV) or visible light, nor do they fluoresce (Sydenham, 1989).

1.7 *THE DEVELOPMENT OF FUMONISIN ANALYTICAL METHODOLOGY*

Major problems exist with respect to the analytical determination of mycotoxins in foods and feeds. As previously stated (Morgan & Fenwick, 1990), natural foods are in general highly complex matrices, and the chemical characteristics of many mycotoxins are similar to numerous other naturally co-occurring compounds present in the food/feed substrates.

1.7.1 *Mycotoxin methodology in general*

Mycotoxins regularly occur in nature, and exhibit physiological effects in animals and man, at concentrations in the microgram to nanogram per gram range [ie. parts per million (ppm) to

parts per billion (ppb)] (Marasas, 1988). Even with the advent of advanced technology and improved detection systems, it is still necessary to develop analytical methods that can significantly increase the relative concentrations of the analyte of interest. Solvent extraction has traditionally been used for the initial concentration of the mycotoxins from various substrates. Chromatography has been used extensively in the predeterminative sample preparation (ie. clean-up) steps of methods, which have become important in the determination of low concentrations of mycotoxins.

Attempts have previously been made to develop analytical procedures for the co-determination of several mycotoxins (termed "*multi-mycotoxin*" methods), and these have been reviewed (Steyn, 1981). In general, these methods have been found to be impractical, dealing as they have, with a number of structurally different mycotoxins, possessing a diverse range of chemical characteristics. These differences have led to highly variable recoveries for the individual mycotoxins. Consequently, there has been a shift in emphasis, away from the multi-mycotoxin approach, towards the development of methods aimed at the sensitive and reproducible determination of single, or groups of structurally related mycotoxins (Cole, 1986).

Thin Layer Chromatography (TLC) is still used extensively for the analytical estimation of a number of mycotoxins in various food and feed substrates. Its continued use is no doubt due to its speed, versatility and relatively low cost when compared with other instrumental chromatographic techniques. A number of mycotoxins (ie. the aflatoxins - Austwick, 1978) possess chromophores which allow them to fluoresce under longwave UV light (365 nm) resulting in a range of specific coloured spots/bands. Aflatoxins B_1 and B_2 fluoresce blue, while the spots/bands for aflatoxins G_1 and G_2 are green in colour. These differences with respect to colour generation led to the adoption of the trivial nomenclatures of the "B" and "G" forms, respectively, for the four major naturally occurring aflatoxin analogues. Hence, TLC can provide not only a retention factor (R_f) for a given compound (relative to its position with

respect to the solvent front) and intensity, both of which may be compared with analytically pure standards, but the colour of the resultant spot/band can also be used as confirmatory evidence. The worldwide popularity of TLC in the field of mycotoxin methodology was confirmed in a survey published by Van Egmond (1989). In the survey, respondents from over 60 countries were asked to reply to questions concerning existing and proposed legislation, and analytical methodology pertaining to mycotoxin contamination of food in their own countries. With regard to the aflatoxins (the most regulated of the mycotoxins), 64% of respondents reported having both regulations and official methods of analysis. However, only 28% of those countries having official methods used analytical techniques other than TLC (Van Egmond, 1989).

Recent editions of the Official Methods of Analysis of the Association of Official Analytical Chemists International (AOAC Int, 1984), list predominately TLC methods of analysis for the determination of each mycotoxin within the section devoted to Natural Poisons. Regular reviews of the literature pertaining to mycotoxin methodology are published annually by the General Referee for mycotoxins of the AOAC. Recent reports (Scott, 1990, 1991, 1992, 1993) have indicated the increased use of instrumental chromatographic techniques, such as high performance liquid chromatography (HPLC) and gas chromatography (GC). Subsequent to suitable validation, it is likely that these techniques will eventually be adopted by the AOAC, if not as replacements for TLC techniques, then as complementory techniques. Radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques have also been developed and used for several mycotoxins (Park et al., 1989; Scott, 1992, 1993).

A review of the analytical chromatographic techniques previously developed within the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), for the analytical determination of the fumonisin mycotoxins, will be discussed in sections 1.7.2 - 1.7.4.

1.7.2 *Application of TLC to the determination of the fumonisins*

TLC was initially used by Gelderblom et al. (1988a) as a screening procedure during the isolation of FB_1 and FB_2 from culture material of *F. moniliforme* strain MRC 826. Sydenham et al. (1990a) modified the method, which was applied to naturally contaminated food samples. Briefly, the procedure consisted of aqueous methanolic extraction of maize followed by solvent partitioning with chloroform ($CHCl_3$). The aqueous phase was subsequently reduced in volume and aliquots further purified on C_{18} cartridges. Following sample preparation, the purified extracts were applied to silica gel 60 TLC plates, which were then developed in a solution of $CHCl_3$:CH_3OH:acetic acid (CH_3COOH). The plates were sprayed with a 0.5% solution of *p*-anisaldehyde in CH_3OH:CH_3COOH:sulphuric acid (H_2SO_4), heated and visually assessed. Under the prescribed conditions, FB_1 showed as a brown-purple spot with an R_f value of 0.25. Alternatively, ninydrin spray solution was used which resulted in the appearance of a pink-purple spot. Visual assessment could only be obtained following spraying/heating due to the reaction of the reagent with the amino group present at the C-2 position in the structure of the fumonisins (Figure 1.5). Although the method was suitable as a screening procedure for fungal cultures, where relatively high concentrations are encountered, the detection limit of the method [approximately 500 μg/g (ppm)] was considered to be unsuitable for the screening of naturally contaminated maize samples (Sydenham, 1989; Sydenham et al., 1990a).

1.7.3 *Application of HPLC to the determination of the fumonisins*

Resolution, when applied to chromatography, may be expressed as the measure of the ability of a system to fully separate two closely eluting compounds (Bristow, 1976). The resolving power of any chromatographic system is dependant on a number of factors, an important one being the characteristics of the stationary phase. With care, the resolution obtained on high

performance TLC (HPTLC) plates can approach that of HPLC. Analyte quantification in HPTLC is however restricted, while a wide range of instrumental detectors are available for use with HPLC. Due to the absence of a chromophore in the structure of the fumonisins (Figure 1.5), it was necessary to prepare suitable derivatives in order to utilize standard post-column spectrophotometric UV or fluoresence detectors.

1.7.3.1 *Maleic anhydride derivatisation*

Siler and Gilchrist (1982) prepared maleyl derivatives for the HPLC determination of a host-selective phytotoxin (TA toxin), produced by *Alternaria alternata* f. sp. *lycopersici*, by the chemical attachment of the UV absorbing maleyl group at the

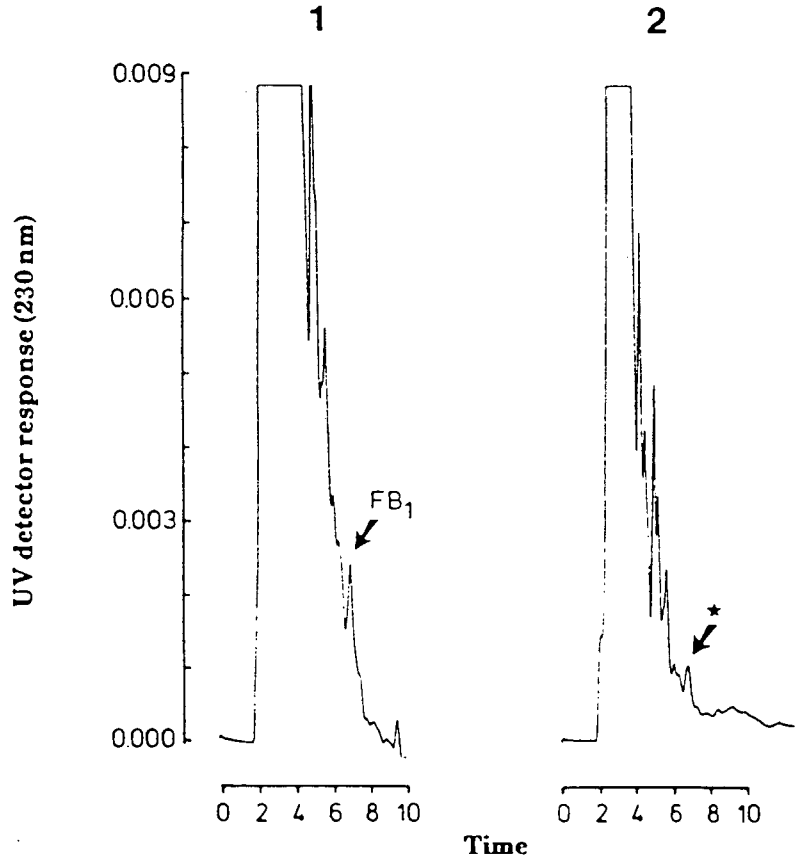


Figure 1.6 HPLC chromatograms of the maleyl derivative of (1) a naturally fumonisin-contaminated maize sample and (2) a control sample.

primary amine present in the structure of toxin. The fumonisins are structurally similar to TA toxin, the latter differing from the former by the absence of one TCA group bound to an aliphatic aminopolyol moiety having three less carbon atoms. Consequently, the derivatisation procedure of Siler and Gilchrist (1982) was, with modifications, applied to maize extracts prepared according to the clean-up procedure outlined in section 1.7.2. The derivatisation procedure (Sydenham, 1989; Sydenham et al., 1990a) involved the purified extracts being dissolved in aqueous sodium carbonate (Na_2CO_3 - pH 9.2) which was treated with an excess of a solution of maleic anhydride in dioxane. Subsequent to the completion of derivatisation, the pH of the solution was altered to between 6 and 7, the solvent evaporated to dryness and the residue redissolved in CH_3OH:H_2O.

HPLC separations of the maleyl derivatives were performed on a C_{18} reversed-phase column and the eluent was monitored at 230 nm using a variable wavelength UV detector. The detection limit of the method was found to be approximately 10 μg/g (ppm) - a substantial improvement over the TLC procedure (Sydenham et al., 1990a).

Figure 1.6.1 illustrates the resultant chromatogram of a naturally contaminated food sample obtained from the Transkei, southern Africa, while Figure 1.6.2 shows a similar chromatogram obtained from a control maize sample (which was subsequently shown to be free of contamination with the fumonisins). Base-line separation of the peak corresponding to FB_1 could not be obtained due to the presence of substrate matrix interferences (Figure 1.6.1). In Figure 1.6.2 the degree of matrix interference was substantially lower, however a minor peak was observed in the chromatographic position of FB_1 (identified by the asterisk). Subsequent GC analysis of the same sample (as described in section 1.7.4) indicated the absence of the TCA moiety, suggesting that the identified peak observed in Figure 1.6.2 was not FB_1. The presence of the peak suggested that it was an interfering compound found intrinsically in maize. The presence of matrix-related factors demonstrated the limitations of the maleyl derivative procedure for the determination of the

fumonisins in naturally contaminated maize samples. These limitations could to some extent be attributed to the insufficient pre-chromatographic purification of sample extracts, although the non-specificity and/or sensitivity of the UV detection system, to the maleyl derivatives of the fumonisins, may also have been a contributory factor.

1.7.3.2 *Fluorescamine derivatisation*

Fluorescence tends to be both a more sensitive and selective detection system than UV detection. The essential problem associated with detection of the fumonisins by fluorescence was the lack of intrinsic fluorecent characteristics. A number of fluorescent labels are commercially available, and several have been used successfully for the derivatization of primary amines (Perrett, 1985; Rosenthal, 1985).

A number of fluorescent labels were initially evaluated including dansyl chloride (Dns-Cl), *o*-phthaldialdehyde (OPA), 7-

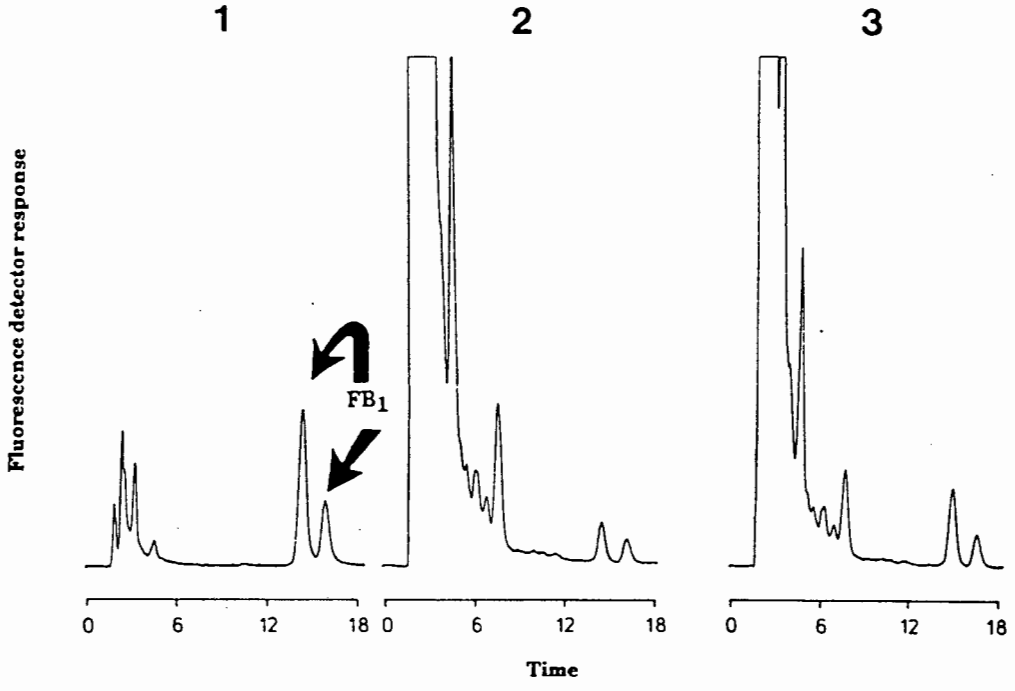


Figure 1.7 HPLC chromatograms of the fluorescamine derivatives of (1) a FB_1 standard, (2) a naturally contaminated sample, and (3) the latter sample spiked with derivatised FB_1

fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), and fluorescamine (4-phenylspiro [furan-2(3H)yl-1-phthalan]-3,3'-dione) (Sydenham, 1989). Fluorescamine is a compound that lacks intrinsic fluorecence (unlike other labels such as NBD-F), but it reacts rapidly at room temperature with primary amines ($t_{½}$ = 500 milliseconds; Perrett, 1985) to form stable fluorophores, whereas Dns-Cl and OPA derivatives tend to be less stable.

Due to matrix effects, an alternative sample preparation procedure was developed based on aqueous CH_3OH extraction and

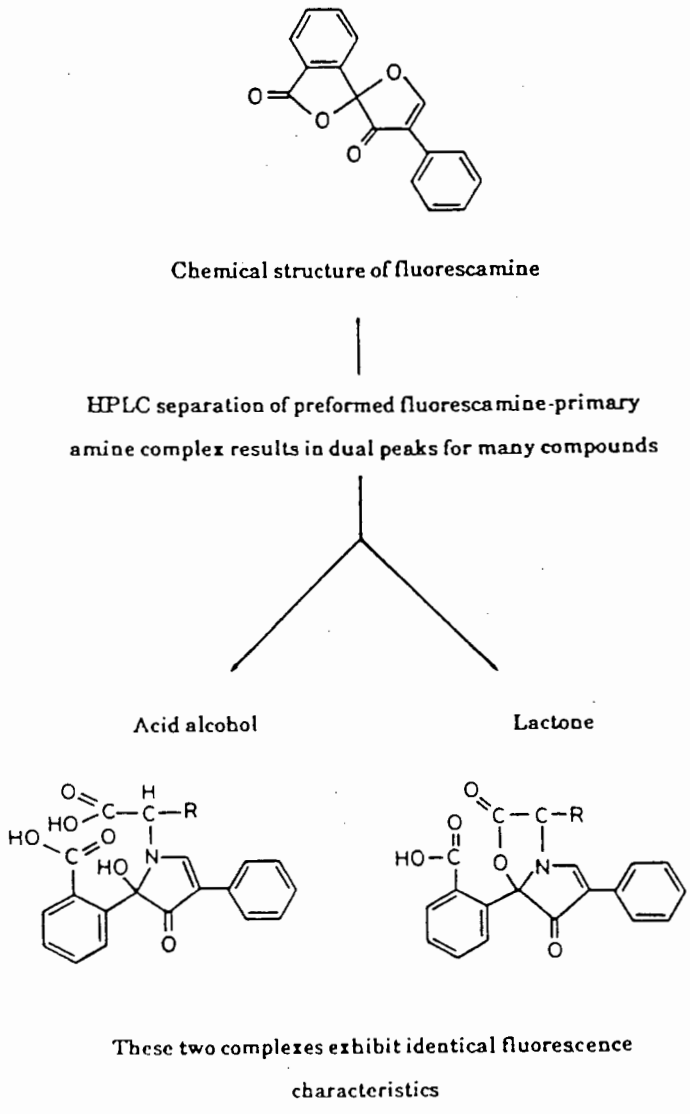


Figure 1.8 Diagram of a preformed fluorescamine-primary amine complex which results in the formation of 2 derivatives

purification on normal phase silica gel (Sydenham, 1989). The purified residue was derivatised by dissolving in sodium bicarbonate ($NaHCO_3$ - pH 8.6) and reacting it with a solution of fluorescamine in acetone at room temperature (Sydenham et al., 1990a). Aliquots of the derivatives were subsequently analysed by reversed-phase HPLC, utilizing flow-through fluorescence detection with excitation and emission wavelengths of 390 nm and 475 nm, respectively.

Figure 1.7.1 shows the chromatogram obtained for an FB_1 standard, illustrating the presence of two well defined peaks eluting after 14.3 and 16.5 minutes, respectively. Figures 1.7.2 and 1.7.3 shows the resultant chromatograms obtained for a naturally contaminated sample and the same sample spiked with FB_1, respectively.

The HPLC separation of stable fluorescamine derivatives can often result in the formation of two chromatographic peaks, corresponding to the acid alcohol and lactone forms of the fluorescent derivative (as shown in Figure 1.8), both of which exhibit identical fluorescent characteristics (Rosenthal, 1985). The chromatographic system itself does not influence the distribution of the derivative between the two forms. The presence of two peaks was however considered to be inappropriate for the analytical determination of the fumonisins in maize, although attempts were made to develop a post-column fluorescamine derivatisation procedure, where only a single chromatographic peak was observed. The chromatographic system developed was also inappropriate, since it required the use of a three pumps, two reaction coils and pulse dampeners for each pump (Sydenham, 1989). Ross et al. (1991a) further developed the basic pre-column fluorescamine method to co-determine FB_1 and FB_2 in animal feeds, using a binary gradient HPLC system.

1.7.3.3 *OPA derivatisation*

The major obstacle to the determination of low levels of the fumonisin mycotoxins in maize, was the presence of matrix related contaminants. An alternative pre-chromatographic sample

clean-up procedure was developed based on the use of silica gel, chemically modified to include strong anion exchange (SAX) sites on the silica backbone (Shephard et al., 1990). The method relied on the dissociation of the TCA groups present in the structures of the fumonisins, and was applied to the co-determination of the major characterised fumonisin B toxins then available (ie. FB_1 and FB_2).

Briefly, the method involved the application of aqueous CH_3OH extracts of maize samples to anion exchange solid phase extraction (SPE) cartridges, containing 500 mg amounts of sorbent. The sorbent bed was then washed to remove matrix interferences and the fumonisins selectively eluted with a CH_3OH:CH_3COOH solution. The eluate was subseqently evaporated to dryness at 60°C under nitrogen, and the residue redissolved in CH_3OH.

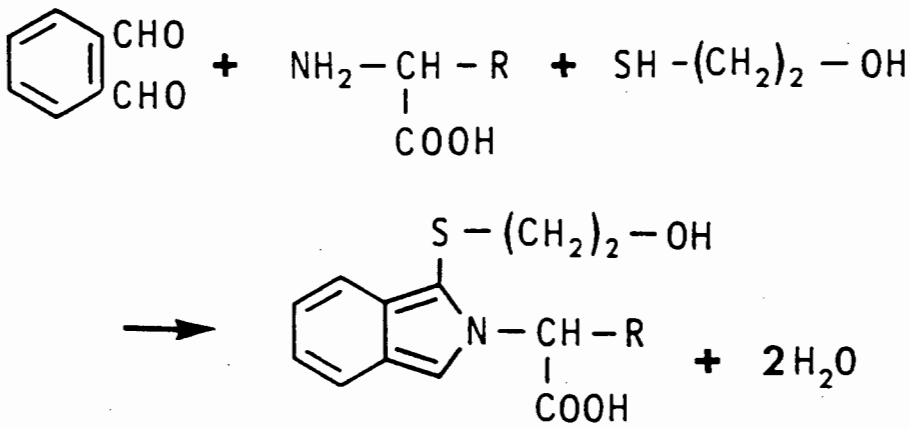


Figure 1.9 Formation of OPA derivatives with primary amines

Aliquots of the residue were derivatised with a solution of OPA in CH_3OH:sodium tetraborate ($Na_2B_4O_7$), containing 2-mercapto-ethanol as a catalyst. The formation of a new heterocyclic species (Figure 1.9), a substituted isoindole (Joseph & Marsden, 1986), allowed for the reversed-phase HPLC separation of the fumonisin derivative, which was monitored by fluorescence detection at the wavelength maxima of 335 nm and 440 nm, excitation and emission, respectively.

The method was applied to numerous naturally contaminated maize samples. Figure 1.10.1 shows the chromatogram of fumonisin standards, clearly illustrating the presence of two well defined peaks, identified accordingly. Figure 1.10.2 shows the chromatogram obtained for a naturally contaminated maize-based feed sample, with peaks corresponding to both FB_1 and FB_2 present in the purified extract.

The improved sample preparation technique coupled with the separation of OPA derivatives, allowed for the HPLC determination of FB_1 and FB_2, using a single (isocratic) mobile phase.

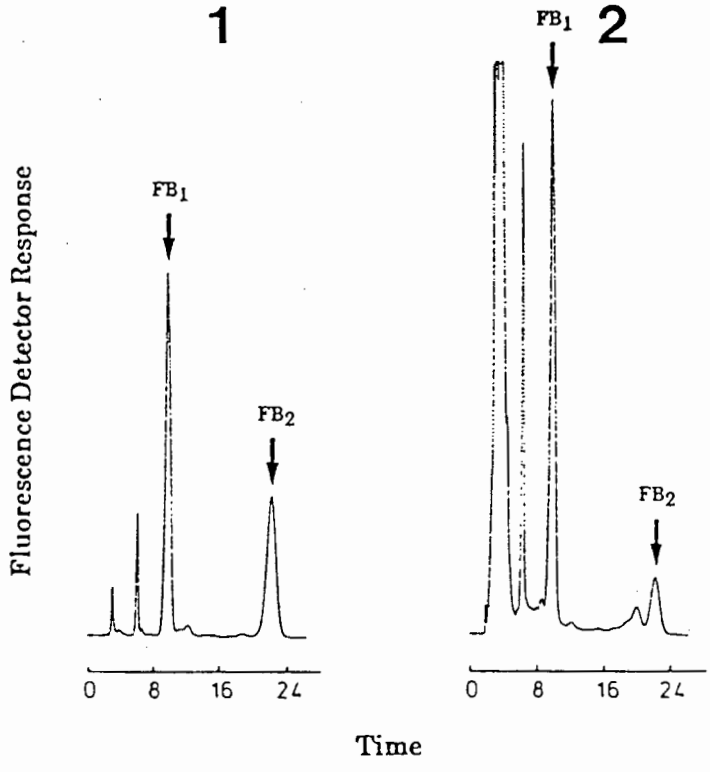


Figure 1.10 Chromatograms of OPA derivatives of (1) FB_1 and FB_2 standards and (2) a naturally contaminated maize sample

The detection limit of the method (based on a signal to noise ratio of 7:1) was found to be approximately between 50 and 100 ng/g (ppb) for each fumonisin toxin, and the recoveries were in the order of 94% and 89% for FB_1 and FB_2, respectively. Based on these results, the method was considered to be adequate for

the determination of naturally occurring fumonisin levels in maize intended for both animal and human consumption.

1.7.4 *Application of GC to the determination of the fumonisins*

The fumonisins are relatively large, highly polar molecules, containing a number of different functional groups, including hydroxyl and carboxyl groups and a primary amine (Figure 1.5). The presence of these functional groups was detrimental to the volatilisation of the fumonisins, and their chromatographic determination by means of capillary GC analysis. Several derivatisation procedures were evaluated, each of which were aimed

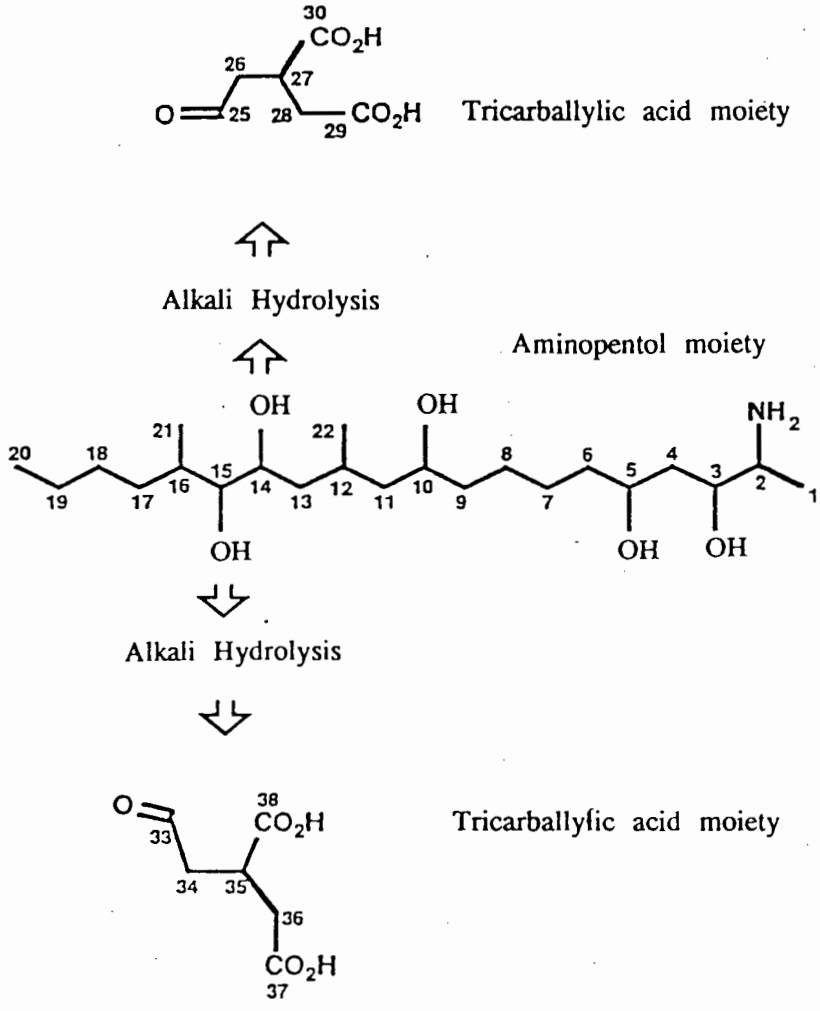


Figure 1.11 The hyrolysis products of FB_1 - the formation of the aminopentol and TCA moieties

at improving the volatility and thermal stability of the fumonisin molecules (Sydenham, 1989). However, subsequent analyses did not result in the identification of chromatographic peaks that could be ascribed to the fumonisin molecules.

Fumonisin B_1 was subsequently subjected to a hydrolysis step

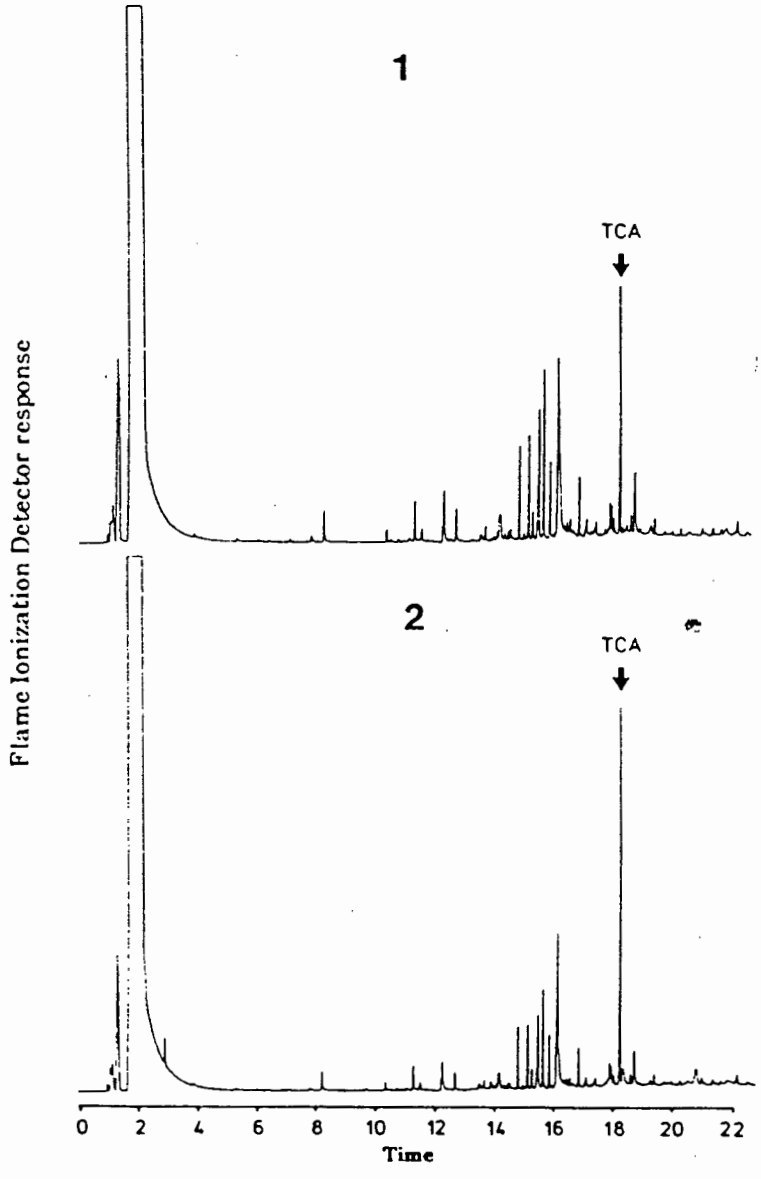


Figure 1.12 Capillary GC-FID chromatograms of (1) a hydrolysed esterified extract of a naturally contaminated sample showing the presence of a peak corresponding to the isobutyl ester of TCA at 18.3 minutes, and (2) the same sample spiked with esterified TCA

which yielded two major individual moieties present in the structure of FB_1 (Figure 1.11). An aliquot of the hydrolysate was esterified and acylated, using a procedure initially developed for the capillary GC analysis of amino acids (Labadarios et al., 1984). Chromatographic separation of the derivative using flame ionization detection (FID), resulted in the observation of a single peak, rather than the two peaks expected. Further investigations suggested that, due either to the derivatisation or chromatographic procedures used, only the peak corresponding to the esterified TCA could be observed.

The detection limit of the method for the determination of the TCA moiety (based on a signal to noise ratio of 10:1) was found to be in the order of 500 ng/g (ppb), and it was subsequently applied to naturally contaminated food samples. Figure 1.12.1 illustrates the GC-FID chromatogram obtained for a naturally contaminated maize sample (as its isobutyl ester), while Figure 1.12.2 shows the same extract spiked with similarly derivatised TCA. Well resolved peaks corresponding to the esterified TCA could be observed at 18.3 minutes. Further confirmation of the presence of TCA was obtained by the application of similarly prepared derivatives to combined GC-MS (Sydenham, 1989; Sydenham et al., 1990a).

Sydenham (1989) suggested that the determination of the TCA moiety might be used as an indicator to monitor total fumonisin contamination in maize. Plattner et al. (1990) also used hydrolysis in conjunction with silylation to determine both the TCA and aminopentol moieties of FB_1, in horse feed samples. However, silylation of the extracts did result in the observation, on occasions, of doublet peaks for the aminopentol moiety (Plattner, 1990).

1.8 *CONCLUSIONS*

It may be concluded then, that the majority of the methods developed for the determination of the fumonisins in maize exhibited a number of problems. These problems were primarily

associated with:

(a) the insufficient sample preparation (clean-up) of extracts leading to poor method detection limits,

(b) the formation of more than one derivative leading to the need to determine both peaks for quantitative purposes, and

(c) the necessity (in the case of GC analysis) to hydrolyse the molecule in order to determine its TCA moiety.

One method (Shephard et al. 1990) was however shown to give excellent recoveries for FB_1 and FB_2 from maize. The detection limit of the method indicated that it could be used for the determination of the low concentrations (50-100 ng/g - ppb) that might be expected to occur under natural conditions. However, validation of the method (ie. the reproducibility and accuracy characteristics, amongst others) had been assessed only in an intra-laboratory excercise. Wider acceptance of the method (by other laboratories and agencies) would ideally require that the method be validated in a inter-laboratory collaborative study.

In summary, the fumonisins are the most recently identified group of *Fusarium* mycotoxins. Their natural occurrence in maize, a dietary staple for both animals and man, may be cause for concern. The purpose of this study will be to develop further, suitably validated methods to monitor and evaluate possible human and animal exposure to the fumonisins. Based on the observations, it is intended to include proposals to limit exposure to these mycotoxins. The objectives of the study are listed in section 1.9.

1.9 *STUDY OBJECTIVES*

This study will centre on several factors associated with the occurrence of the fumonisins. The following chapters will deal with:

(a) The study of the reproducibility characteristics of the HPLC method, developed by Shephard et al. (1990) for the analytical determination of the fumonisins in maize, as their OPA derivatives.

(b) The further development of improved fumonisin analytical methodology and confirmatory techniques.

(c) The application of suitable methods to the analyses of maize-based human foods and animal feeds, as part of a fumonisin-exposure assessment programme - an essential component of overall risk assessment studies.

(d) The development of suitable procedures in order to reduce both animal and human exposure to the fumonisin mycotoxins.

Each chapter will be preceded by its own short introductory section, followed by experimental and results and discussion sections. The separate chapters may therefore be considered as individual dissertations, but each should be regarded as representative of a logical progressive step in the research investigations associated with the fumonisin mycotoxins.

CHAPTER 2: VALIDATION OF A HPLC METHOD FOR THE DETERMINATION OF FUMONISINS IN MAIZE

2.1 *INTRODUCTION*

In former years, discrepancies in analytical results obtained from different laboratories were often ascribed to indifference or incompetence on the part of one or more of the laboratories. With the observation that these discrepancies occurred within a single laboratory came the realisation that there existed, in all measurements, an inherent variability which is a function, *inter alia*, of the magnitude of the characteristic or property being measured (Horwitz, 1983a,b; 1993). It was also shown that this variability could be statistically estimated.

The accuracy of a chemical analysis is judged on the basis of how close the actual assay is to the true value. Yet as the concentration of an analyte decreases, so it becomes increasingly difficult to know what its true value is. At high analyte concentrations (at 1% and above), the inherent variabilities of the measurements are relatively small; typically in the order of 1-2% of the analytical value (Horwitz, 1993). Therefore, variabilities emanating from external sources, which result in a difference of several percent in the final value of the analyte, can easily be distinguished against a background of between 1-2% of analytical variability. However, in trace analyses (where analyte concentrations are measured in the ppm to ppb range), the analytical variabilities are of the order of 10-30% of the analytical value. Consequently, even to be noticed, external influences would have to precipitate variabilities in excess of the 10-30% analytical variance (Horwitz, 1983a,b; 1993).

Systematic errors emanating from the use of instruments or the analyte standards themselves, tend to be compounded by those problems associated with the prcision of results, as the concentration of the analyte of interest decreases. Analysts

will individually be able to demonstrate a reasonable degree of reproducibility (precision). In trace analyses, however, it becomes important that the performance of the individual and their methods, be judged relative to other laboratories rather than relative to themselves (Horwitz, 1983a).

The increase in the worldwide trade of commodities has led to the implementation of legislation in many countries, that now require that test portions of samples be made available to several different laboratories for replicate analyses. These directives in turn require governmental bodies, agencies and commercial laboratories to operate within an interlaboratory environment. It is important, therefore, that these laboratories appreciate and understand the limitations and constraints associated with working in such an environment.

Organisations such as the International Union of Pure and Applied Chemistry (IUPAC), the International Organisation for Standardisation (ISO) and the AOAC Int, recognised the need to conduct collaborative studies in order to determine the attributes of methods used for the analytical determination of hazardous compounds. Collaborative studies have therefore become an integral part of the assessment and improvement of analytical methods (Horwitz & Albert, 1991). Official acceptance of a method is only granted once its performance characteristics have been shown to comply with certain criteria.

Validated methods are essential in order to determine compliance with economic specifications, and for the enforcement of regulatory requirements. Such compliance with specifications and standards, require the presentation of analytical methods that are both accurate and reproducible, to the extent that they are able to produce the same result in different laboratories (Horwitz & Albert, 1991). The term "*same*" in the above context does not imply that the results are "*identical*", but rather that they are "*statistically equivalent*". The major characteristic of any repetition of measurement is that it inherently encompasses with it an associated degree of error, which in turn virtually ensures that the results of no two

measurements need be identical.

The ISO, IUPAC and AOAC Int, have independently developed protocols and guidelines for the co-ordination of collaborative studies. In addition they have developed their own statistical models for the evaluation of the data generated by the studies. These differences have, in the past, created confusion for those involved in all aspects associated with the regulation of harmful substances. Attempts have been made to create a degree of protocol harmonisation within the major governing bodies, for the adoption of standardised analytical methods (Horwitz, 1983b, 1986; Parkany, 1986; Taylor, 1986; Pocklington, 1990; Kelly, 1990). Co-operation between the governing bodies has resulted in an agreement on the minimum criteria required for the design, conduct and interpretation of collaborative studies. Some of these criteria are presented in sections 2.1.1 to 2.1.3.

2.1.1 *Collaborative studies - criteria for consideration*

Possibly the most important consideration is that of defining the purpose of a proposed study. There are several different kinds of interlaboratory studies, depending upon the variables that are held constant (ie. laboratories (analysts), materials or methods). At a recent IUPAC harmonisation workshop (Horwitz, 1993), it was agreed that the definition of a collaborative study would be "*a study in which selected characteristics of a method of analysis are to be determined*" (ie. the same method and materials are used by a random set of laboratories). A method-performance study is therefore different from a proficiency (laboratory-performance) study, in which the performance of laboratories or analysts in conducting an analysis are determined (ie. the same materials are analysed by a random set of laboratories using their own methods of choice).

It becomes necessary to consider both the scope of the method (screening or definitive) and its intended purpose (surveillance, monitoring, research or quality control). Consideration also has to be given to the relative importance of the various

method attributes such as bias, precision, specificity and limit of detection. Each of these variables can influence the results of a collaborative study. Therefore, an initial, concise definition of the purpose of the study, especially at its planning stage, will assist in its optimum design.

An important recommendation, dealing with the preliminary aspects of a collaborative study, is to conduct interlaboratory trials with methods that have received thorough testing within a single laboratory. Hence, the *intra*laboratory attributes of a candidate method should be determined prior to its selection for *inter*laboratory trials. The attributes for consideration would include an estimation of the measurement range of the method, its applicability to commodities of interest, as well as its precision and accuracy characteristics. Clearly, a method that does not perform satisfactorily within a single laboratory, cannot be expected to perform any better when submitted for interlaboratory evaluation.

A method of analysis (or protocol) is a written set of instructions on how to conduct a specific biological, chemical or physical measurement. Methods in general have been described as the most visible, and consequently, the most adjustable aspects of biological and/or chemical experimental systems (Horwitz, 1993). It is therefore necessary to prepare a clearly worded description/protocol and explanation of each step of the analytical procedure. This description should specify any performance-related reagents that would be required. A carefully worded protocol will discourage deviations from the method. In cases where the candidate method involves a new method of analysis, it has been recommended that it first be submitted for assessment in a "pilot study" involving at least three laboratories, prior to its final selection, (AOAC, 1989).

The selection of participants should take into consideration the availability of (1) personnel experienced in the basic techniques used, and (2) the necessary equipment required for the successful completion of the study. It is also important

that the participants know the purpose and extent of the study. To this end, guidelines listing the information and data that must be supplied by the participants, to the study co-ordinators, should be clearly stated.

2.1.2 *Design of the collaborative study*

Since the study is intended for international consideration, several laboratories from different countries should be invited to participate. The study should be extensive enough to ensure that sufficient data is generated. The minimum number of laboratories submitting valid data has been set at 8 (AOAC Int, 1989). One has to consider, however, the possibility of laboratories being unable to complete the study, or of laboratories submitting non-valid data. It is therefore advisable that the study be undertaken with at least 10 participating laboratories. Valid data may still be obtained even in cases where 2 laboratory failures are observed in a 9-laboratory study (AOAC Int, 1989).

It is crucial that there be a homogeneous distribution of the analyte of interest within the test materials submitted to the participants of the study. Any nonhomogeneity will result in an increased variance in the analytical data, that is itself not part of the method variability. The concentration range of the analyte within the test materials should reflect the dynamic range of interest, and where necessary, blank materials (containing none of the analyte) should be included. At least 5 test materials should be submitted for analyses, and in cases where the within-laboratory variability characteristic is an important attribute, "*blind*" replicates (ie. two or more subsamples of the same material) or "*split levels*" should be included. A "*split level*" consists of two materials of slightly different analyte concentrations, which are however, sufficiently close that the measurements can be considered as having the same variance (Horwitz, 1983b). Each test portion should be coded at random, so that there is no preselection of the samples by the participants, based on the order of material presentation.

2.1.3 *Statistical considerations*

The statistical evaluation and interpretation of data generated in collaborative studies, has been an issue of concern for all regulatory agencies. However, following consultation, the guidelines set by these agencies have been specific, restricting statistical treatment of submitted data to a minimum (Horwitz, 1993). It has been suggested that adherence to these criteria will permit a single collaborative study, conducted under the auspices of one organisation, to serve all others, while allowing each to interpret the results according to its own specific needs (Horwitz, 1993).

In a single laboratory environment, it is assumed that the average of a series of measurements of the same analyte, is the best estimate of its true concentration. The variability of the individual within-laboratory measurements, that compose an average, provides an indication of the reliability of the estimate of that average. This within-laboratory variability consists of numerous smaller components which include:

(**a**) variability from different analysts in the same laboratory,

(**b**) between and within day variability of results, and

(**c**) between and within calibration curve variability.

These sources of variability form a sometimes overlapping and complex error structure. Under these conditions it is often impossible to clearly identify single sources of error, and even statistical models can fail to assist in the interpretation of data. These potential sources of variability have been known for many years, and their practical implications on the interpretation of data from interlaboratory studies was well demonstrated as far back as 1951 (Wernimont, 1951).

It is therefore easier to combine and classify these sources of error in the single estimator of "*within*" or intralaboratory (or repeatability) standard deviation.

These problems are compounded when data from additional laboratories are taken into consideration. Each laboratory will produce a similar, though individual, mean and within-laboratory variability. This between or interlaboratory variability will be larger and more complex than the intralaboratory variability, because the variability determined for the former can only be measured by a process that includes the variability factors determined for the latter.

Statistical analysis of collaborative data must therefore be used to determine both the within-laboratory standard deviation (S_r) and the measured between-laboratory standard deviation (S_L), which when added together as variances, give the overall standard deviation by the following equation:

$$S_R = (S^2_r + S^2_L)^{\frac{1}{2}}$$

These parameters, better expressed as the corresponding percentage relative standard deviations, RSD_r and RSD_R, are useful summary statistics that can be used to predict the performance of methods used in the interlaboratory environment.

The permitted statistical treatment of data involves only that data which is considered valid (ie. bias free). An important aspect to consider is the treatment of "outliers". Outliers are values that are far outside the bulk of the concentration estimates, and are identified when a computation indicates that it is highly improbable that the value belongs with the bulk of the data. However, care has to be taken to distinguish between outlier and invalid data. Invalid data are results from laboratories that, for example, did not follow the instructions, or that reported malfunctioning equipment (ie. these are suspect data that can be traced to a specific cause).

Data are tested for outliers on an assay-by-assay basis. The data should first be evaluated by the Cochran extreme variance test (AOAC, 1989), that discards results from laboratories reporting poor variability, when compared with the variability obtained from the other participating laboratories. Data should

then be removed from laboratories that show extreme values by the single value Grubbs test (AOAC, 1989), and in the event of there being more than one extreme outlier, a paired Grubbs test (Horwitz et al, 1993).

2.1.4 *Collaborative studies involving mycotoxins*

Numerous collaborative studies have been undertaken involving the determination of several mycotoxins (ie. aflatoxins, ZEA and deoxynivalenol - DON) in various matrices such as cheese (Francis et al., 1987), maize (Park et al., 1990; Trucksess et al., 1991), peanuts and peanut butter (Campbell et al., 1984; Patey et al., 1991), and animal feeds (Park et al., 1989; Van Egmond et al., 1991).

Based on the recovery results determined for the HPLC method developed by Shephard et al. (1990), for the determination of FB_1 and FB_2 in maize, a collaborative study aimed primarily at determining the reproducibility characteristics of the method was initiated. The study was undertaken under the sponsorship of the Commission of Food Chemistry of IUPAC, and was developed and executed under the "*Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis*" (AOAC, 1989). The guidelines required that the method initially be evaluated in a pilot study, the results of which would ultimately determine the methods' suitability for further evaluation in a full collaborative study.

2.2 *EXPERIMENTAL*

2.2.1 *Evaluation of alternative milling preparations*

Maize kernels (approximately 250 g each) were ground either in a Bizerba large capacity grinder set to grind to a fine meal, or a Hobart hammer mill fitted with frits that were identified as "coarse", "fine" and "ultra-fine". Each ground meal was then subjected to particle size distribution analysis using a series of 8 Tyler screens covering the range 2000 μm - 106 μm.

2.2.2 *Preparation of samples for pilot and full collaborative studies*

Numerous maize samples were screened for FB_1 and FB_2 levels according to the method of Shephard et al. (1990). Based on these results, several were selected for use in either the pilot or full collaborative studies. The selected samples (approximately 1 kg each) were ground to a meal using the Hobart hammer mill fitted with the "fine" frit, and subsequently passed through two consecutive sieves/screens (an 850 μm screen connected to a 250 μm screen). Those fractions retained between the two screens were subjected to extensive and repeated riffling and blending to improve homogeneity, and re-analysed according to the the method of Shephard et al. (1990). Based on the second series of analytical results, the ground meals were further blended and mixed together to obtain 3 test samples (each of 500 g in mass) having FB_1 levels ranging from approximately 200 to 2000 ng/g (for the pilot study). The same procedures were used to obtain 5 similar samples used for the full collaborative study (each of ± 1200 g in mass), having approximately the same range of FB_1 levels. The FB_2 levels were, in each case, approximately one third of the corresponding FB_1 levels. An additional sample, having FB_1 and FB_2 levels below 50 ng/g was prepared for inclusion into the full collaborative study. Two other samples, identified as "A" and "B" were also prepared from the balance of the unused ground maize meals, for use by the participants in the full collaborative study, for practice purposes and for the optimisation of the participants' chromatography. The blended and homogenised meals were dried in an oven at 100°C for 12 hours and, following cooling, 30 g quantities of each were transferred to clean 100 g capacity plastic bottles. The ground meal samples were then "purged" with dry nitrogen prior to being sealed and labelled accordingly.

2.2.3 *Preparation of fumonisin standards*

Analytically pure standards of FB_1 and FB_2 were isolated from culture material of *F. moniliforme*, in accordance with a

previously published method (Cawood et al., 1991). The identity of the individual toxins was confirmed by NMR and MS analysis, while HPLC analysis of the OPA derivatives of the individual toxin standards resulted in the observation of a single chromatographic peak for each. A stock solution of FB_1 and FB_2 in CH_3OH was prepared to give concentrations of 250 and 125 μg/ml for FB_1 and FB_2, respectively. Aliquots (200 μl) of the stock solution were placed in the base of individual 4 ml capacity amber coloured vials. The solvent was removed under nitrogen at 60^{o}C, and the vials were capped and labelled accordingly.

2.2.4 *Submission of collaborative test materials*

2.2.4.1 *Pilot study materials*

Each of 4 invited laboratories were supplied with packages containing the following items:

(a) Six coded, blind duplicates of 3 naturally contaminated maize meals.
(b) One vial identified as "Fumonisin standards".
(c) Ten strong anion exchange (SAX) SPE cartridges, as specified in the method.
(d) A copy of the HPLC method to be used.
(e) A list of instructions on how to proceed with the study.
(f) A report sheet.

2.2.4.2 *Full collaborative study materials*

Although only 11 collaborators were invited to participate in the interlaboratory study, 15 similar packages, containing items a - g (listed below) were prepared. The contents of each package were as follows:

(a) Twelve coded, blind duplicates of 6 naturally contaminated maize meals.
(b) Two practice maize samples identified as "A" and "B", naturally contaminated with FB_1 levels estimated at <600 ng/g

and >600 ng/g, respectively.

(c) One vial identified as "Fumonisin standards".

(d) Fifteen strong anion exchange (SAX) SPE cartridges.

(e) A copy of the HPLC method to be used.

(f) An appendix containing information on suggested alternative reversed-phase HPLC columns and mobile phases, together with chromatograms obtained under the specified conditions.

(g) A report form for the submission of analytical data as well as for the presentaion of any criticisms and/or suggestions.

2.2.5 *Statistical evaluation of data*

Statistical treatment of the submitted data were performed in accordance with the guidelines specified by the AOAC (1989). Outliers were removed based on the Cochran and Grubbs tests (Grubbs & Beck, 1972; AOAC, 1989). In addition, the HORRAT ratios were determined for FB_1 and FB_2 in each of the submitted test materials, according to the following formula:

$$RSD_R = 2^{(1-0.5 \log C)}$$

where C is equal to the concentration expressed as a decimal fraction (Horwitz & Albert, 1991).

2.3 *RESULTS AND DISCUSSION*

2.3.1 *Evaluation of milling processes*

The efficiency of extraction of target compounds from complex matrices can be dependant on a number of factors. The relative chemical characteristics of the analyte of interest, and of the matrix in which it occurs, are of major concern. One factor that is often discounted is the initial physical state of the matrix itself, prior to extraction. A broad distribution of particulate size may result in an equally wide distribution of solvent extraction efficiencies for a given analyte from a

matrix.

In order to determine the optimum particulate size distribution for the samples to be submitted to participants in the collaborative study, ground maize meals were prepared using different mills and frits. The results (Table 2.1) were then compared with a maize sample, previously obtained as part of an inter-laboratory study involving the analytical determination of the aflatoxins, organised under the auspices of the International Agency for Research on Cancer (IARC).

The use of the hammer mill equipped with the "fine" frit resulted in a fairly narrow distribution of particulate size for the ground meal, with almost 91% of the applied sample being retained between the 500 - 250 μm screens. In excess of 12% of the applied ground maize was retained by the 850 μm screen when using the "coarse" frit, whereas use of the "ultra-

Table 2.1 Comparison of particle size distribution for maize prepared by grinding in different mills

Mesh range (μm)	Percentage of maize retained by screens				
	Hobart hammer mill			Bizerba mill	IARC sample
	Coarse	Fine	Ultra-fine	Fine	
>2000	0.0	0.0	0.0	0.7	0.0
850-2000	12.1	0.6	1.4	28.9	1.7
500-850	39.2	27.5	11.5	30.8	25.1
335-500	17.9	34.3	26.9	21.3	23.8
250-335	24.8	29.1	47.6	8.6	16.9
180-250	3.6	5.6	8.8	8.6	22.1
150-180	1.4	2.1	2.7	1.0	8.7
106-150	0.7	0.8	0.7	0.1	1.7
<106	0.3	0.0	0.4	0.0	0.0

fine" frit resulted in a almost 50% of the sample being retained in a single screen (250 - 335 μm). Use of the Bizerba mill resulted in the retention of predominately larger particles.

The IARC maize sample was found to contain particles predominately retained between the 500 μm and 180 μm screens. Comparison of the results indicated that use of the hammer mill, equipped with the "fine" frit, would result in the size distribution of the majority of maize, within a range that would be similar to that previously used in the IARC study.

2.3.2 *Pilot study results*

The participants were requested to prepare one extract from each of the six test samples, purify the individual extracts according to the prescribed method and perform duplicate HPLC analyses on the extracts. The participants were then requested to report both duplicate results for each sample extract, to comment on the method and its description, and to make any other suggestions or criticisms that they considered to be valid. The raw data received from the participants, as well as the average values obtained by each, are presented in Table 2.2.

The values obtained by laboratory 2 for FB_1 were considerably higher than those obtained by the other participants, especially at the lower concentrations. The higher levels for FB_1 appeared to be due to the non-optimisation of the chromatographic separation of extracts.

Figure 2.1.1 illustrates the chromatographic separation of test sample number 2 achieved by laboratory 2, clearly showing that FB_1 had not been fully resolved from contaminants eluting in the solvent front, whereas Figure 2.1.2 shows the optimised separation of the an extract of the same sample which was achieved by laboratory number 4.

Table 2.2 Results - Pilot Study - FB_1 and FB_2 in maize

Lab No	Sample number 1 x	1 y	1 z	2 x	2 y	2 z	3 x	3 y	3 z
				Fumonisin B_1					
1a	155,157	156	146	604,598	601	592	1737,1814	1776	1824
1b	139,133	136		580,583	582		1895,1847	1871	
2a	433,305	369	309	575,650	613	627	2445,2476	2461	2123
2b	250,247	249		633,648	641		1773,1797	1785	
3a	163,153	158	139	466,468	467	546	1590,1550	1570	1605
3b	121,118	120		625,625	625		1600,1680	1640	
4a	109, NR	109	122	536,636	586	545	2204, NR	2204	2182
4b	136, NR	136		488,504	496		2160, NR	2160	
				Fumonisin B_2					
1a	46, 38	42	39	150,161	155	157	726,803	765	761
1b	38, 34	36		170,145	158		740,771	756	
2a	68, 65	67	57	162,159	161	153	851,880	861	806
2b	57, 37	47		184,105	145		710,781	746	
3a	NR	NR	NR	NR	NR	NR	NR	NR	NR
3b	NR	NR		NR	NR		NR	NR	NR
4a	<20, NR	<20	<20	144,220	182	145	668, NR	668	770
4b	<20, NR	<20		108,108	108		872, NR	872	

Key to table 2.2 - see overleaf

Key to symbols in Table 2.2

x = Individual values for corresponding fumonisins.

y = Mean of duplicate HPLC analyses performed on a single extraction.

z = Mean of duplicate values from 2 extractions.

a,b = The values obtained for the separate extracts of blind duplicate subsamples of test samples 1, 2 and 3, respectively.

NR = Result not reported

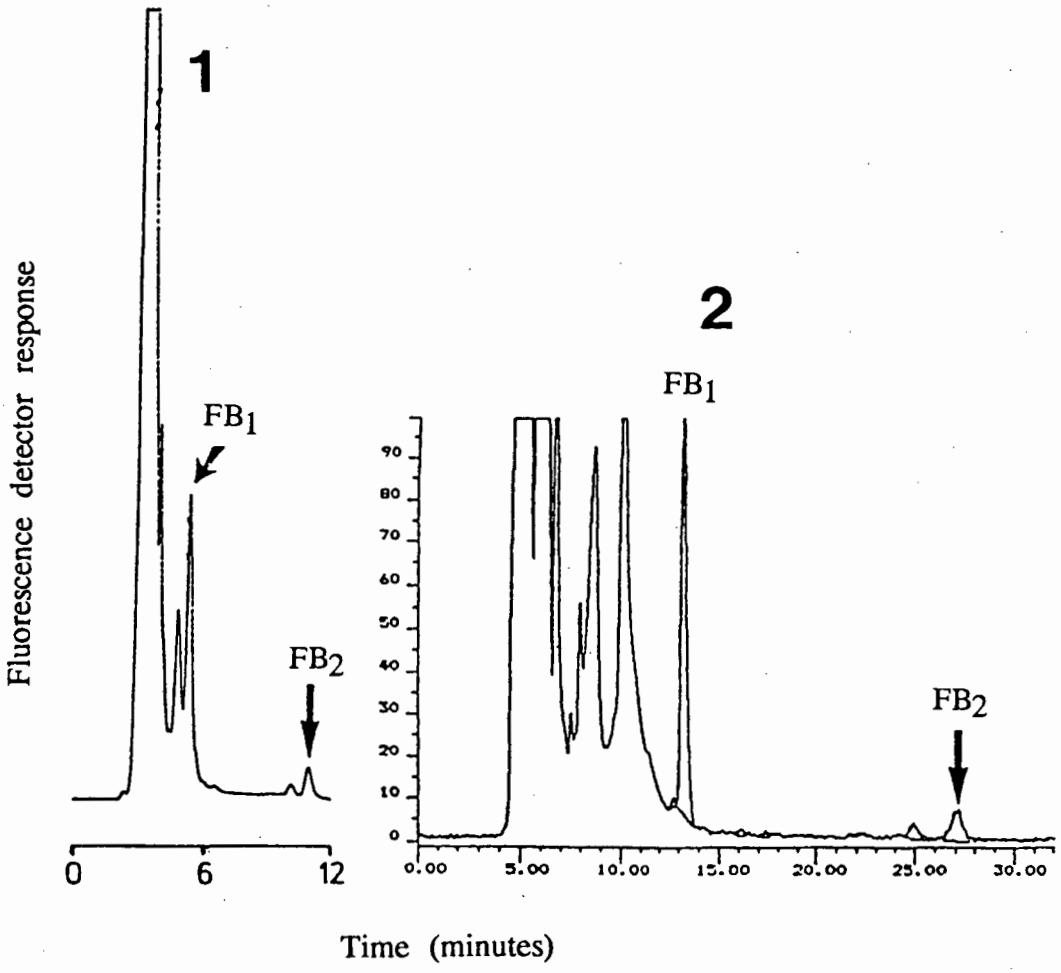


Figure 2.1 HPLC chromatograms of pilot study test sample number 2 returned by (1) laboratory number 2 and (2) laboratory number 4

Laboratory 3 did not report any values for FB_2. This participant claimed that the supplied reference standards were found to be unstable, and used his own reference standard of FB_1 for completion of the study. The participant was unable to report values for FB_2 due to the lack of a suitable standard. Laboratory 4 failed to detect any FB_2 in sample number 1, but this may have been due either to the non-optimisation of the fluorescence detector, or the inherent lack of sensitivity of the detector itself.

Analyses of the data presented in Table 2.2 indicated that there was a significant difference between the values obtained for both FB_1 and FB_2, in the three test samples. In addition, there were no statistically significant differences between the blind duplicate samples, indicating that the method for the preparation of the duplicate test portions dispatched to the participants, would be suitable for application to the full collaborative study.

2.3.2.1 *Comments of participants in the pilot study*

The participants were requested to comment of several aspects of the method and its description, and these are summarised in sections 2.3.2.2 - 2.3.2.3.

2.3.2.2 *Method description*

It was suggested that more information be included concerning the preparation of standard curves, and that the limits of detection should be specified. One participant added that the method should be re-written to comply with the format of the AOAC, and that a formula for the calculation of results should be included.

2.3.2.3 *Method*

One participant considered that the elution and washing volumes from the SAX cartridges should be reduced, and that the use of more stable fluorescent derivatives should be investigated, as

this would enhance the method and allow the use of auto-samplers/injectors in integrated HPLC systems. Other comments included the suggestion that the chromatographic "run-time" was too long, and that an alternative mobile phase should be used to reduce the analysis time. Another participant claimed that recoveries of the fumonisins from SAX cartridges were suspect, and suggested that practice samples should have been included. It was also stated that "*spiked*" samples should be included in a full collaborative study, in order to determine the accuracy (recovery) characteristics of the method.

2.3.3 *Action taken based on the results and comments from the participants in the pilot study*

Within the limitations of a pilot study, the data indicated that with few exceptions, the method showed good reproducibility characteristics for both FB_1 and FB_2, which in turn justified commencement of the full collaborative study.

The stability problems associated with the use of OPA for derivatisation of the fumonisins were acknowledged. However, the data indicated that reproducible results could be obtained if the derivatives were injected into the HPLC system within 1 minute of derivatisation (as specified in the method). The use of alternative derivatives would also have necessitated the use of a binary gradient HPLC system, to co-determine FB_1 and FB_2. It was felt that prescribing the use of a gradient system to participants in a collaborative study, most of whom would be unfamiliar to the fumonisins, would cause them problems with the optimisation of their chromatography - a problem already highlighted in the pilot study in which a basic isocratic system had been used (Figure 2.1).

No explanation for the apparent instability of the supplied reference standards (reported by one of the participants) could be given, and none of the other participants encountered similar problems. Therefore, reference standards for the full collaborative study would be supplied in the same way as for the pilot study.

The aim of the collaborative study was initially defined as the determination of the repeatability & reproducibility characteristics of the method. It was felt that these characteristics could best be assessed by using naturally contaminated maize samples rather than "spiked" samples. The screening of numerous maize samples had also failed to result in the collection of sufficient quantities of relatively fumonisin-free maize that could be used, on a collaborative basis, for the determination of the accuracy (recovery) characterisitics of the method.

The description of the method was altered to comply with the format of the AOAC Int and a formula for the calculation of fumonisin concentrations was included. As suggested, two practice samples were supplied to the participants of the collaborative study.

2.3.4 *Full collaborative study results*

The participants were requested to prepare one extract per sample (following the prescribed method as closely as possible) and to make one HPLC determination per sample. Participants were also requested to submit chromatograms and to complete the report form giving comments, criticisms, and notations detailing any deviations they may have made from the prescribed method.

Each of the 11 invited collaborators completed the interlaboratory study. One laboratory (number 9) experienced technical problems while attempting to analyse the test samples. The problems involved malfunctioning instrumentation, but the laboratory concerned completed the study on a second batch of samples. This highlighted the necessity to prepare an additional number of test sample packages, over and above the number required for each participant, during the preparation stage of any collaborative study. The raw data received from the participating laboratories is tabulated in Table 2.3

The results reported in Table 2.3, and the chromatograms returned by the participants demonstrated that all of the

laboratories (with the exception of laboratory 3) experienced few problems in following the instructions for the method, nor

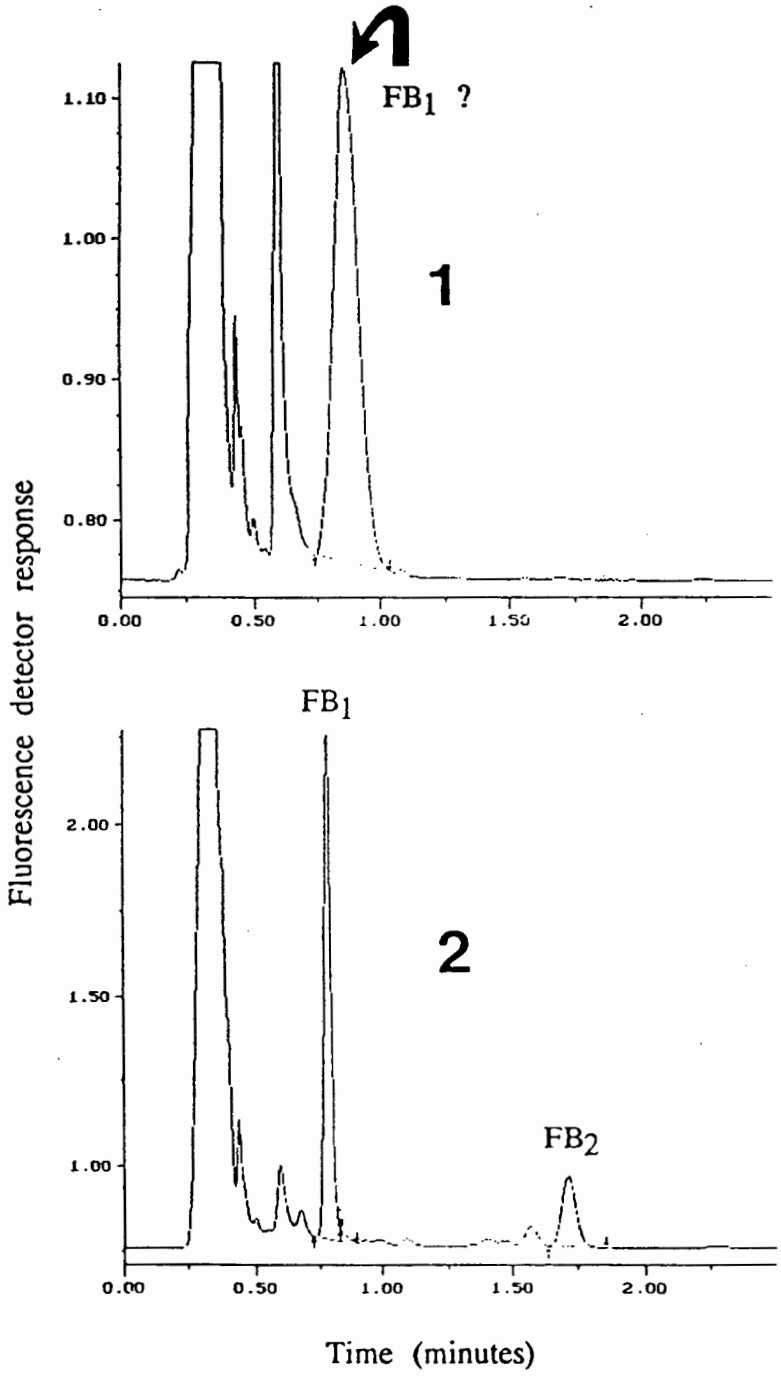


Figure 2.2 HPLC chromatograms of the purified extracts prepared by laboratory 5 of (1) test sample 1 (coded 10) and (2) test sample 6 (coded 8), as their OPA derivatives

Table 2.3 Collaborative study - FB_1 and FB_2 in maize

Sample No	Code No	Participant/Laboratory number 1[a]	2	3	4	5	6	7	8	9	10	11
		Fumonisin B_1										
1	2	SL[b]	0	0	0	152[c]	0	7	0	16	29	20
	10	0	0	9	15	1903[c]	0	18	19	15	27	13
2	6	234	185	2	242	268	171	214	299	174	267	254
	11	219	168	51	228	240	181	258	290	155	346	221
3	1	SL	276	28	462	451	274	375	308	233	394	418
	9	281	298	29	342	144	353	369	227	264	453	371
4	3	514	500	29	628	699	534	631	514	424	744	631
	7	493	510	30	617	538	490	597	555	350	733	602
5	5	1034	902	73	1421	1233	1078	1165	1430	417	1502	1452
	12	1032	865	247	1168	1145	960	1468	1771	819	1406	1116
6	4	1739	1489	77	1966	2019	1842	2181	2073	1529	2425	1993
	8	1715	1543	198	1930	2277	2058	2097	2846	944	2904	2080
		Fumonisin B_2										
1	2	SL	0	0	0	93	0	0	0	15	0	0
	10	0	0	0	0	0	0	0	0	15	0	0
2	6	69	67	0	79	80	65	93	52	47	121	77
	11	32	56	14	77	75	66	84	54	57	158	66
3	1	SL	72	8	19	159	105	125	79	68	172	145
	9	67	94	0	91	31	118	122	55	89	195	113
4	3	182	222	8	242	296	250	293	161	179	405	292
	7	219	233	0	316	227	236	274	237	165	425	250
5	5	348	267	20	469	439	434	435	393	142	733	510
	12	327	289	89	365	401	369	566	535	278	695	406
6	4	568	533	25	692	686	790	840	525	507	1202	826
	8	572	533	65	607	794	790	800	959	313	1507	778

Sample number

[a]Laboratory number

[b]Sample lost by participant

[c]Invalid data (see section 2.3.4)

did they appear to have difficulty in executing the steps in the procedure. All the participants, with the exception of laboratory 5, reported values below 50 ng/g for test sample 1.

Figure 2.2.1 shows the chromatogram returned by laboratory 5 for test sample 1 (coded 10 - Table 2.3), while Figure 2.2.2 illustrates the chromatogram of test sample 6 (coded 8 - Table 2.3), also analysed by laboratory 5. The peak eluting in the chromatographic position of FB_1 for test sample 1 (Figure 2.2.1) has a broad base peak width that is far wider than that of the FB_1 peak observed in Figure 2.2.2. This suggests that the major peak eluting in test sample 1 was erroneously identified as FB_1, leading to an overestimation of the FB_1 concentration reported by the participant (Table 2.3). The width of the peak suggests that it may have been a late eluting compound-peak from a prior injection.

The results of the statisical evaluation of the raw data presented in Table 2.3 are listed in Table 2.4. These data include the within-laboratory repeatability values (r), corresponding standard deviations (Sr) and relative standard deviations (RSD_r), and between-laboratory reproducibility values (R), corresponding standard deviations (S_R) and relative standard deviations (RSD_R). Table 2.4 also contains the HORRAT ratios calculated for both FB_1 and FB_2 at the 5 concentration levels tested.

The HORRAT is the ratio of the RSD_R found in the actual assay, conducted in the interlaboratory study, to that predicted from the determined or known concentration (Horwitz & Albert, 1991). The HORRAT equation stems from a review of over 700 AOAC method-performance studies, which involved the determination of numerous analytes in a variety of matrices (Horwitz, 1993). The author summarised his findings in a curve that related the coefficient of variation among laboratories (RSD_R), to the concentration expressed as a power of ten. The Horwitz equation states that the RSD among laboratories doubles for every decrease of two orders of magnitude in analyte concentration.

Table 2.4 Statistical evaluation of the data supplied by the 11 participants in the collaborative study, for the HPLC determination of FB_1 and FB_2 in maize

Sample No	Mean (ng/g)	S_r (ng/g)	RSD_r %	S_R (ng/g)	RSD_R %	Outliers No of labs	HORRAT ratio
Fumonisin B_1							
1[a]	<50	-	-	-	-		
2	226	33.2	14.7	55.7	24.6	1	1.2
3[a]	337	84.5	25.5	86.2	26.0	1	1.4
4	565	43.5	7.7	101.5	18.0	1	1.0
5	1169	170.1	14.6	311.8	26.7	1	1.7
6	1983	255.1	12.9	457.3	23.1	1	1.6
Fumonisin B_2							
1[a]	<50	-	-	-	-		
2	74	12.6	17.1	27.8	37.7	1	1.6
3[a]	101	37.1	36.8	46.1	45.6	1	2.0
4	255	32.0	12.5	71.3	28.0	1	1.4
5	421	66.2	15.7	144.4	34.3	1	1.9
6	741	130.9	17.6	270.0	36.4	1	2.2

[a]One sample lost - see Table 2.3

Statistical analyses were not performed on the values reported for test sample 1 because the levels were below that which was conservatively considered to be the detection limit of the method (ie. 50 ng/g - based on a signal to noise ratio of 7:1). All values supplied by laboratory 3 were excluded from the statistical evaluation of the data, based on the fact that the participant made major changes to the prescribed derivatisation procedure (see section 2.3.4.1). None of the other results had to be rejected on the basis of the Grubbs test, for the removal of extreme values (Grubbs & Beck, 1972).

Comparison of the blind duplicate values reported by each participant (for test samples 2 - 6) indicated that all participants (with the exception of laboratory 3) had no difficulty in repeating their results. The acceptable degree of within-laboratory precision is reflected in the relative RSD_r values reported in Table 2.4, which varied from 7.7 to 25.5%, and from 12.5 to 36.8% for FB_1 and FB_2, respectively. The mean values for the 5 fumonisin-positive test samples ranged from 226 to 1983 ng/g for FB_1, and from 74 to 741 ng/g for FB_2. The satisfactory repeatability of the method is further demonstrated, because any lack of homogeneity (with respect to the distribution of the analytes in the test materials), in the blind duplicates submitted to the individual participants, would have been reflected in the reported RSD_r values. The data would therefore appear to indicate that of the submitted test samples, number 3 was the least homogeneous while number 4 was the most homogeneous, since the highest and lowest relative standard deviation values (RSD_r) were found for both FB_1 and FB_2 in the two test samples, respectively (Table 2.4).

As might be expected, the between-laboratory variability of the data was higher than the corresponding within-laboratory variability (Table 2.4). The RSD_R values for the individual test samples varied, for FB_1, from 18.0 to 26.7%, and from 28.0 to 45.6% for FB_2. These results were similar to those previously obtained for other collaborative studies of HPLC methods involving the determination of aflatoxins in various matrices (Park et al., 1990; Patey et al., 1991). The results are also in accordance with the findings of Horwitz (1983a), in which a relationship between interlaboratory precision and concentration was established (based on data collected from almost 200 interlaboratory studies which involved various analytes, matrices and analytical techniques). Comparison of the results displayed in Table 2.4, with those generated by Park et al. (1990) are particularly apt, since both studies concerned the determination of mycotoxins in maize, and both involved solvent extraction of the analytes, SPE clean-up of extracts, precolumn derivatisation and HPLC/fluorescence detection. The overall RSD_R values reported by Park et al.

(1990) were between 15.8 and 38.4% for aflatoxin B_1, which are similar to those observed for FB_1 and FB_2 (Table 2.4). It should however be noted that the total aflatoxin levels used in the study of Park et al. (1990) (between 13 and 130 ng/g) were lower than those used in present fumonisin study (from 226 to 1983 ng/g for FB_1 and from 74 to 741 ng/g for FB_2 - Table 2.4).

The acceptability of the method is also reflected in the HORRAT ratios reported in Table 2.4. Horwitz & Albert (1991) stated that they "*found this ratio to be the best single index of acceptability of method performance*" and that "*in general, values above 2 are considered unacceptable*". All of the HORRAT ratios calculated for FB_1 were lower than 2, and only one value for FB_2 was found to be higher than 2 (Table 2.4).

Plattner et al. (1991) conducted a short collaborative study of a method for the determination of FB_1 in maize. The method of Ross et al. (1991a), which involved the purification of extracts on C_{18} material and derivatisation with fluorescamine, was used by the laboratories for the determination of FB_1 levels. The study was conducted in 4 laboratories in the USA and involved the analyses of 5 test samples having mean FB_1 levels ranging from 2 to 1557 μg/g. Values for RSD_R for the naturally contaminated samples were found to range from 20.8 to 85.3%. The concentration range for FB_1 in the positive samples (excluding the fungal culture) used in the study of Plattner et al. (1991) (mean values: 25 - 209 μg/g) were far higher than those used for the study described in this Chapter (combined FB_1 and FB_2 levels ranged from 0.3 to 2.6 μg/g - Table 2.4). In this respect, it would appear that the results and data of the current collaborative study were superior to those reported by Plattner et al. (1991). It is likely, however, that the results presented by Plattner et al. (1991) were affected by the participants' being allowed to alter the prescribed method at their own discretion. Two of the participants also analysed the samples according to the capillary GC-MS procedure of Plattner et al. (1990), and a comparison of the results with those generated by the HPLC procedure appeared to be favourable.

2.3.4.1 *Comments from participants in the collaborative study*

All participants confirmed that the supplied instructions and method description were easily followed and that they experienced no difficulty during the analyses. One participant found the chromatographic analysis time to be too long, while two other participants commented on the large solvent volumes used for both the washing and elution of the ion-exchange cartridges, which also led to long evaporation times for the eluate.

Laboratory 3 commented that "*when the standard, following derivatisation, was left past 1.5 minutes, the peak areas went up rather dramatically*" and that "*after 20 minutes the peak areas were 20 to 50% higher than at 1.5 minutes*". These observations are contrary to all experience with OPA derivatives (Seiler & Demisch, 1977), including the fumonisins (Shephard et al., 1990). Laboratory 3 admitted that a significant alteration had been made to the method "*in order to get results on our system*". The alteration involved the use of a "*0.1M borate buffer in the derivatisation step instead of the cited 0.1M borate solution*". Derivatisation with OPA requires that the reaction take place in the presence of a catalyst at a pH of between 10 and 12 (Seiler & Demisch, 1977). Laboratory 3 did not supply any details concerning the characteristics of the buffer solution they used (ie. pH), however the results and chromatograms submitted by this laboratory (Table 2.3) were clearly not in agreement with those from the other 10 participating laboratories. Accordingly, their data were excluded from the statistical evaluation process on the grounds of their alteration to the method.

Participant number 1 commented that "*in general our results for FB_2 were lower than those obtained by the other laboratories*", and sought an explanation for his observations.

Virtually all integrators use a series of Savitsky-Golay algorithms as part of their data processing procedures (Dyson, 1990). There are a number of parameters available to the

analyst that can affect the measurement of peak areas, heights and retention times recorded by the integrator. It is essential that the analyst understand the relative influences of at least the major parameters, since a number of them (ie. peak width and slope sensitivity) are interrelated. Ideally, correct integration could best be obtained by the analyst selecting the correct parameters for each individual chromatographic peak. This approach is somewhat impractical, and hence a situation arises where the selection of integrator parameters, by the analyst, can best be described as a "*compromise*".

The "*peak width*" integrator parameter is possibly the most important parameter selected by the analyst. This parameter sets the rate at which the detector signal is digitised for integration and storage. Peaks are detected because the detector signal amplitude changes more rapidly when peaks elute. Integrators monitor the detector signal, and observe the change in slope which occurs when a peak emerges. Smaller changes are allowed to pass as baseline fluctuations. There is a threshold below which peaks are not detected, and this value is set by the second most important integrator parameter, the "*slope sensitivity*". The ability of the integrator to correctly determine the end of a chromatogrphic peak is set by the "*baseline drift tolerance*" parameter. The purpose of this parameter is to allow the integrator to select a new baseline after a peak, or a group of peaks has eluted, when the detector fails to return to its original level. This parameter is often confused with "*slope sensitivity*", yet there is a distinct difference; the drift parameter determines how "*far*" the baseline may drift from its original position, while the slope sensitivity parameter determines how "*fast*" it may drift away (Dyson, 1990).

An integrator will monitor all peaks during an analysis, however, since peak widths increase with retention time (assuming that the chromatographic system is isocratic or, in the case of GC, isothermal), the integrator parameters that were suitable for early eluting peaks may not be appropriate for those eluting later in the analysis. All integrators, through the

"*timed events*" programme, allow the analyst to alter and further optimise any of the integrator parameters.

A useful option available on most integrators is that of "*peak markers*". These may be displayed on the chromatogram and define the points at which the integrator (based on its programmed parameters) observes the beginning and end of all chromatographic peaks. The beginning of a peak is usually

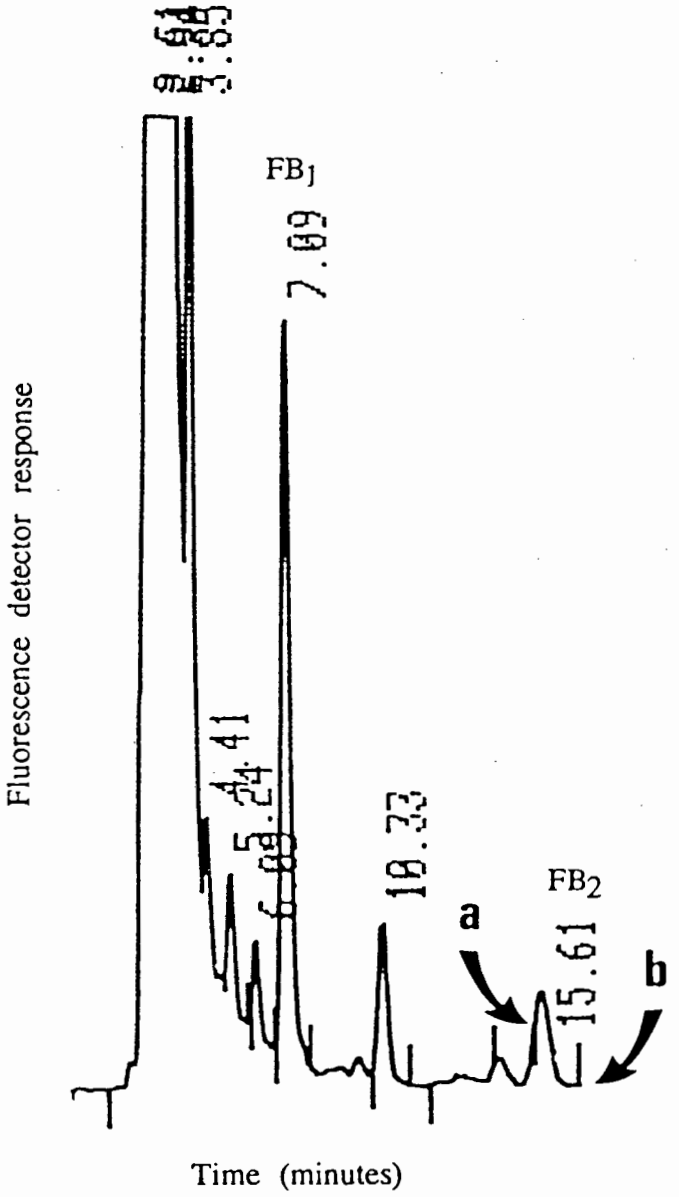


Figure 2.3 The HPLC chromatogram of coded sample number 11 supplied by participant number 1

denoted by a mark ("*tick-mark*") displayed in a negative direction, and conversely, the end of a peak is denoted by a similar mark in the positive direction.

Figure 2.3 shows the printed integrator chromatogram of coded sample number 11 submitted by participant 1. The chromatogram shows the excellent separation of the peaks corresponding to FB_1 and FB_2, from other contaminant peaks. The *"tick-marks"* are clearly displayed in Figure 2.3. Close scrutiny of the peak eluting after 15.61 minutes (corresponding to FB_2 - Figure 2.3) shows that the integrator measured the start of the peak approximately halfway on the up-slope of the peak (the position of the appropriate mark is identified by an "a" - Figure 2.3), while the end of the peak is clearly defined (marked by a "b" - Figure 2.3). This would have resulted in the integrator recording, for the FB_2 peak, only that area demarcated by the markers "a" and "b", resulting in an underestimation of the area under the peak and hence a similar underestimation of its concentration. The selected integrator parameters affected only the later eluting compounds, since the peak markers shown for the peak corresponding to FB_1 (retention time 7.09 minutes - Figure 2.3) were adequate. Therefore, the underestimation of the FB_2 concentrations reported by participant number 1, can be attributed to the incorrect programming of integrator parameters. This illustrates one of the sources of error that can be encountered, that is itself unrelated to the performance characteristics of the analytical method.

One participant (laboratory 7) independently compared the prescribed method with a procedure that used a C_{18} cartridge clean-up step. Using a single feed sample, the participant noted that the recovery of FB_1 and FB_2 from the SAX cartridge was about 50% lower than that obtained using the C_{18} cartridge. The results of this participant in the collaborative study were, however, in excellent agreement with those from the other 9 laboratories used for the statistical evaluation of the method. Possible explanations for these observations, concerning lower recoveries from SAX cartridges, are discussed in Chapter 3.

2.4 *CONCLUSIONS*

The interlaboratory collaborative study indicated that the reproducibility characteristics of the method were such, that it would be suitable for adoption as an official AOAC method, provided the accuracy characteristics of the method were also assessed collaboratively. The comments of collaborators who participated in both the pilot and full collaborative studies were taken into consideration and incorporated into an optimised method that is addressed in Chapter 3. The results of this interlaboratory collaborative study have been published (Thiel et al., 1993).

CHAPTER 3: OPTIMISED FUMONISIN ANALYTICAL METHODOLOGY AND THE DEVELOPMENT OF CONFIRMATORY METHODS

3.1 *INTRODUCTION*

In Chapter 2, the HPLC method of Shephard et al., (1990) for the determination of FB_1 and FB_2 in maize, was subjected to an interlaboratory collaborative study, in which the reproducibility characteristics of the method were assessed. Comments from participants in the study (section 2.3.4.1), in addition to observations made by the candidate, formed the basis of the re-evaluation and further optimisation of the method. Due to the subsequent isolation and identification of a third toxicologically important fumonisin analogue - FB_3 (Cawood et al., 1991), its incorporation into the optimised method (together with FB_1 and FB_2), was considered desirable. The parameters covered as part of this optimisation process are described in sections 3.2.1 - 3.2.7.

Chemical measurements are based primarily on the presence of a particular "*characteristic*", rather than its absence. In chromatographic systems, this "*characteristic*" may be a charged particle, an aggregate of particles, or electromagnetic waves of specific length. All of these effects are subsequently amplified, interpreted instrumentally and displayed into a visible concept (ie. the chromatogram). The chromatogram therefore represents the recording of a response of a detection system, to some physicochemical property of the molecules that are separated by the column. The chromatogram gives little information concerning the identity of the compounds eluting from the chromatographic column. Consequently their identification requires the use of separate techniques, to enable either the partial or unequivocal confirmation of chromatographic observations. The development and application of confirmatory techniques for the determination of fumonisins will be described in section 3.2.8, and discussed separately.

3.2 *EXPERIMENTAL*

3.2.1 *Solvent extraction of the fumonisins*

A 500 g maize sample, previously shown to be naturally contaminated with FB_1 and FB_2 according to the method of Shephard et al (1990), was prepared by grinding in a laboratory mill to pass a 840 μm sieve. The homogeneity of the sample was improved by riffling and blending. Subsamples (4 by 25 g) were extracted with 50 ml CH_3OH:H_2O (3:1) for 1, 3 and 5 minutes, respectively, using a Polytron homogeniser set at 60% full speed, while the fourth was extracted for 5 minutes using a Sorvall Omnimixer. An additional three 50 g subsamples were extracted with 100 ml of the same extraction solvent, for 1, 3 and 5 minutes using a Sorvall Omnimixer, set at 60% full speed. A single 50 g subsample was also extracted with 100 ml extraction solvent for 60 minutes on a wrist action shaker set at high speed. The extracts were subsequently analysed for FB_1 and FB_2 according to the HPLC method of Shephard et al. (1990).

3.2.2 *Application of extracts to SAX cartridges*

Maize extracts were prepared by the solvent extraction of a 50 g subsample using the Sovall Omnimixer (as cited in section 3.2.1). Aliquots (10 ml each) were applied to four separate SAX solid-phase extraction (SPE) cartridges, mounted on a vacuum manifold, at flow rates measured at 1, 2.1, 3.1 and 8.2 ml/minute. The fumonisin levels were then determined according to the method of Shephard et al. (1990).

3.2.3 *Aspects associated with the pH of extracts*

Maize extracts were prepared using the Sorvall omnimixer. The pH of several aliquots were altered by the addition of 0.01M hydrochloric acid (to pH values of 6.0, 5.8, 5.6, 5.4, 5.2 and 5.0). Each aliquot was then applied to its own pre-conditioned SAX cartridge, and the fumonisin levels were determined according to the method of Shephard et al. (1990).

3.2.4 *Elution of fumonisins from SAX cartridges*

A second series of maize extracts were prepared as cited in section 3.2.1., and 10 ml aliquots were applied to four separate SAX cartridges, at a flow rate accurately maintained at 2 ml/minute. The fumonisins were then eluted from the cartridges with 14 ml 0.5% (v/v) CH_3COOH in CH_3OH at flow rates of 1, 2.5, 4.1 and 6.7 ml/minute. The fumonisin levels were again determined as previously described (Shephard et al., 1990).

An extract of a naturally contaminated maize sample, previously shown to contain predominantly FB_1, was similarly prepared, and aliquots (10 ml) were applied to two SAX cartridges at a flow rate of 2 ml/minute. The fumonisins were eluted from one of the cartridges with 14 ml 0.5% (v/v) CH_3COOH in CH_3OH, and from the other with 14 ml 1.0% (v/v) CH_3COOH in CH_3OH, at flow rates measured at 0.8 ml/minute. For each cartridge, the first 4 ml fraction followed by 5 subsequent 2 ml fractions were collected separately. Each fraction was evaporated to dryness at 60°C under dry nitrogen, in separate vials, and the fumonisin concentrations were determined (Shephard et al., 1990).

3.2.5 *Stability of fumonisin-OPA derivatives*

A 50 µg/ml standard solution of FB_1 in CH_3CN:H_2O (1:1) was prepared, and aliquots (50 µl) were used to form eight similar FB_1-OPA derivatives, as described by Shephard et al. (1990). Each derivative was prepared in a vial which was then capped and stored on the bench under white light for periods of 0.5, 1, 2, 4, 8, 16, 32 and 64 minutes. Aliquots of each derivative were subsequently injected into the HPLC, and the areas under the peaks corresponding to FB_1 were recorded.

3.2.6 *Regeneration and re-use of SAX cartridges*

An extract was prepared by blending 100 g of fumonisin-contaminated maize with 200 ml CH_3OH:H_2O (3:1), using a Sorvall Omnimixer as described in section 3.2.1. Aliquots (10 ml) were applied to four separate SAX cartridges at flow rates of 2 ml/

minute, and the fumonisin concentrations were determined by the method of Shephard et al. (1990). The used cartridges were subsequently treated with 5 ml 0.1N HCl followed by 8 ml H_2O. The cartridges were conditioned with $CH_3OH:H_2O$ (3:1), and additional 10 ml aliquots of the original sample extract were reapplied to each of the regenerated/conditioned cartridges. The fumonisin levels were assessed as previously described (Shephard et al., 1990).

3.2.7 *Recoveries of FB_3*

Fumonisin-free maize was spiked with a solution of FB_3 in CH_3OH at a concentration of 1000 ng/g. A 100 g subsample of the maize was extracted with 200 ml $CH_3OH:H_2O$ (3:1) in a Polytron homogeniser (for a period of 3 minutes), as described in section 3.2.1. Suitable aliquots were then applied to 5 individual cartridges and the FB_3 levels were determined in accordance with the method specified in sections 3.4.1 - 3.4.4.

3.2.8 *Confirmation of the fumonisins*

Two methods aimed at the development of confirmatory methods are presented in sections 3.2.8.1 - 3.2.8.2.

3.2.8.1 *Use of capillary GC-MS*

Extracts purified according to the method described in sections 3.4.1 - 3.4.4 were dissolved in 2 ml 2N potassium hydroxide (KOH) and heated for 60 minutes at 70°C. The solutions were allowed to cool and the hydrolysate was then acidified with 1M HCl to pH 2 (Plattner et al., 1990). The extracts were applied to C_{18} Sep-pak cartridges, pre-conditioned with 5 ml H_2O. The cartridges were washed with an additional 8 ml H_2O, and the aminopolyol moieties of the fumonisins were subsequently eluted from the cartridges with 8 ml CH_3OH. The hydrolysates were transferred to individual glass vials and the solvent removed under nitrogen at 60°C.

The hydrolysate residues were derivatised according to a modi-

fication of the procedure of Sydenham & Thiel (1987), by the addition of 50 µl of trifluoroacetylimidazole (TFAI) to each vial, which was then capped and heated to 60°C for 40 minutes. Following cooling, 450 µl of toluene was added to the vials followed by 500 µl 0.1M phosphate buffer (pH 6.0). The contents were then mixed and the upper organic layer was removed and analysed by capillary GC-MS, using the conditions outlined in Table 3.1.

Table 3.1 Chromatographic parameters for the GC-MS confirmation of the aminopolyol moieties of the fumonisins

Column	HP1 10 m x 0.25 mm id (0.25 µm) film thickness
Carrier	Helium at 25 cm/second
Instrument	HP 5890 GC coupled to a HP 5970B mass selective detector
Splitless	0.5 minutes (on time)
Injector	Temperature 180°C
Detector	Temperature 300°C
Temperature	Profile (1) 80°C for 1 minute
	(2) 80°C-250°C at 6°C/minute
Mass range	90-200 m/z

3.2.8.2 *Use of TLC*

Rottinghaus et al. (1992) utilized fluorescamine as a spray reagent for the visualisation of the fumonisins, following separation of purified sample extracts on reversed-phase TLC plates. The separation and derivatisation procedure of Rottinghaus et al. (1992) was applied to a series of maize extracts prepared according to the optimised procedure presented in sections 3.4.1 - 3.4.4. The resultant TLC chromatograms were compared with those extracts prepared from the same maize samples, using the pre-chromatographic preparation procedure defined by Rottinghaus et al. (1992).

The method of Rottinghaus et al. (1992) involved extraction of the extracts with $CH_3CN:H_2O$, aliquots of which were mixed with 1% KCl. The solutions were then passed through pre-conditioned C_{18} cartridges, which were washed to remove interferences. The fumonisins were eluted with a solution of $CH_3CN:H_2O$ (7:3), the eluates being collected, evaporated to dryness and reconstituted in a small volume (100 μl) of $CH_3CN:H_2O$ (1:1).

Aliquots of purified extracts prepared by both clean-up procedures were applied to the pre-concentration zones of Whatman KLC_{18} reversed-phase plates. The plates were then developed in a solution of CH_3OH:4% KCl (4:1), air dried and sprayed with 0.1M $Na_2B_4O_7$ buffer (pH 8.6), followed by fluorescamine (0.4 mg/ml in CH_3CN). After a period of 1 minute, the plates were sprayed with a solution of 0.01M boric acid in CH_3CN (4:6), dried at room temperature and viewed under longwave UV light (365 nm).

3.3 *RESULTS AND DISCUSSION*

The results of the optimisation criteria of the HPLC method will be presented and discussed in sections 3.3.1 - 3.3.5, while the results of the confirmation procedures will be dealt with separately in sections 3.5.1.- 3.5.2.

3.3.1 *Optimisation of sample purification*

Previous work by the candidate in association with colleagues at PROMEC resulted in the publication of an HPLC method for FB_1 and FB_2 in maize (Shephard et al., 1990). The authors optimised the solvent system necessary for the initial extraction of the fumonisins from the maize matrix, evaluating the use of different blends of H_2O, CH_3OH and CH_3CN. A solvent blend of $CH_3OH:H_2O$ (3:1) was found to give recoveries that were similar to those obtained with $CH_3CN:H_2O$ (1:1). This latter solvent system was found to be highly selective, resulting in a clear extract free from emulsions. However, the high cost of CH_3CN and the problems associated with its safe disposal were

considered to be prohibitive to its continued use in the laboratory. Prolonged exposure of the fumonisins to CH_3OH, under acidic conditions, has been reported to cause esterification of the fumonisins, and the formation of mono- and di-methyl esters (Cawood et al., 1991). In addition, Visconti et al. (1993) compared the stability FB_1 and FB_2 in solutions of $CH_3OH:H_2O$ and $CH_3CN:H_2O$, and observed limited stability for both fumonisin analogues when exposed to CH_3OH. It was therefore suggested that following initial solvent extraction, purification of the samples should be effected within the shortest possible time.

With $CH_3OH:H_2O$ (3:1) as solvent, the use of a Polytron homogeniser, for the intial extraction of the fumonisins from maize-based matrices, was evaluated against similar treatment with a Sorvall Omnimixer and shaking on a wrist-action shaker. The results obtained are displayed in Table 3.2.

No significant differences in fumonisin recoveries were observed when extracting 25 g subsamples with 50 ml aliquots of

Table 3.2 Effect of extraction technique on the recovery of FB_1 and FB_2 from contaminated maize

Extraction technique	Grams maize/ ml solvent	Time period for extraction (minutes)	Fum. conc. (ng/g)	
			FB_1	FB_2
Polytron	25g / 50ml	1	5786	2555
Polytron	25g / 50ml	3	6066	2612
Polytron	25g / 50ml	5	5957	2415
Sorvall	50g /100ml	1	4062	1842
Sorvall	50g /100ml	3	5243	2165
Sorvall	50g /100ml	5	5913	2365
Sorvall	25g / 50ml	5	5360	2048
Wrist	50g /100ml	60	4405	1688

solvent, for either 1, 3 or 5 min, using the Polytron homogeniser (relative standard deviations of less than 4.0% were obtained). Similar recoveries were observed, using the Sorvall Omnimixer, only when a two fold increase in sample weight and solvent volume were used, and a 5 minute time period for extraction was allowed (recoveries corresponded to between 94-99% of the results obtained for the Polytron). The lower recoveries obtained for the Sorvall Omnimixer, when extracting a 25 g subsample with 50 ml solvent, was probably due the geometry of the extraction vessel. Under these conditions a relatively large proportion of the subsample was, in the process of extraction, thrown onto the inside surface of the vessel, to the extent that there was an insufficient volume of solvent to wash the material back into solution. This resulted in only partial extraction of the maize subsample, and hence lower recoveries (Table 3.2). Extraction on the wrist-action shaker for a period of 60 minutes resulted in recoveries of between 67 and 74% of the Polytron results.

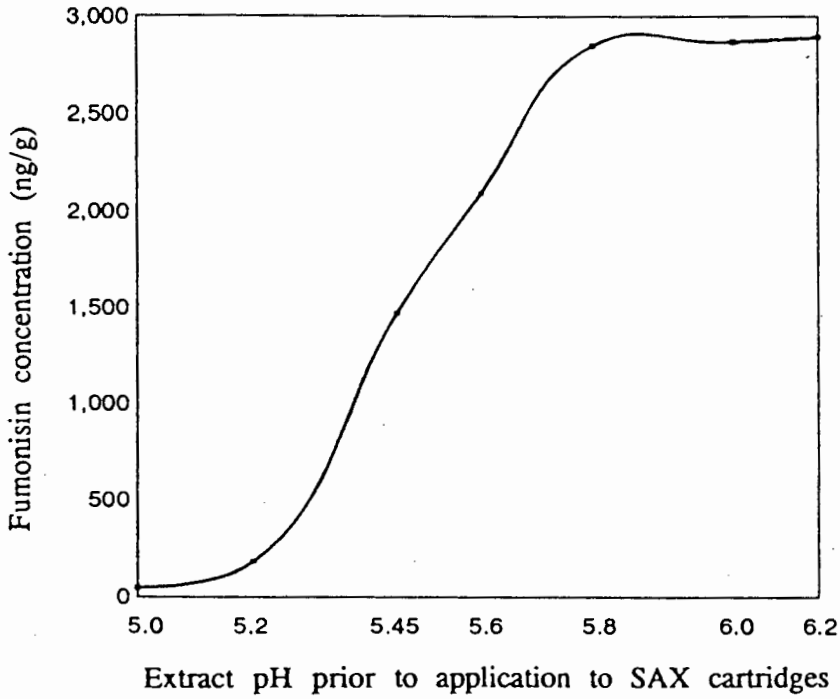


Figure 3.1 Effect of initial pH of extracts on the recovery of FB_1 from SAX cartridges

The results indicated that the Polytron blender gave the most consistent and reproducible recoveries of the three procedures (Table 3.2). Sorvall extraction using larger sample weights and solvent volumes gave comparable results, but required longer extraction times. Due to the low recoveries, the use of a wrist-action shaker for the initial extraction of the fumonisins from maize-based matrices would not be recommended.

3.3.2 *Application to and elution from SAX cartridges*

In ion exchange chromatography, separation takes place as a result of the competition that exists between ions in the eluent and the solute, for oppositely charged sites present on the stationary phase (Bristow, 1976). The separation mechanism on ion exchange materials can be complex, and several types of mechanism can be operating simultaneously (Bristow, 1976). Factors such as pH, the ionic strength of solvents and flow rates can significantly affect either the retention or elution profiles of solutes.

It is also important to realise that major differences exist between the base silica and chemical processes used for its chemical modification, by the different manufacturers of SPE cartridges (Makro & Radová, 1991). Hence the optimised conditions pertaining to the cartridges used in this study may well be applicable only to the specified cartridges. Conversely, the use of similar cartridges, supplied by other manufacturers may work equally as well as those specified, but their use would probably require additional validation with respect to the parameters discussed in this section.

The pH values of crude extracts prepared from the majority of naturally contaminated maize-based matrices, have previously been measured at between 6.0 - 6.5. In solution, the fumonisin species will be affected by the pH, in that the toxins may be differently charged (ie. under alkaline conditions the carboxylic acid groups will be ionised, while under acidic conditions, ionisation will be suppressed).

Figure 3.1 illustrates the importance of the initial pH of the crude extracts, prior to their application to the specified SAX cartridges. The pH of the extract prepared for this study was 6.2, but as shown in Figure 3.1, at pH values below 5.8, there is an appreciable drop in fumonisin recoveries. Therefore, it is crucial that the pH of sample extracts should be measured, and if necessary altered to above 5.8, prior to their application to the SAX cartridges. In the analyses of mixed feeds, many initial extracts have been found to have pH readings of 5.7 or less. It is therefore possible that the observation of a low recovery from a single feed sample (reported by one participant in the collaborative study - section 2.3.4.1) may have been pH-related.

Table 3.3 Effect of sample extract application rate to SAX cartridges

Application flow rate (ml/minute)	Fumonisin concentration (ng/g)	
	FB_1	FB_2
1.0	564	213
2.1	563	207
3.1	604	220
8.2	603	232

The effect of the application flow rate of sample extracts, on the recovery of the fumonisins is shown in Table 3.3. The data indicate that retention of the fumonisins by the ion exchange stationary phase, is not a function of the initial rate of application. Slight increases in recoveries were recorded between flow rates of 1.0 and 8.2 ml/minute, but these differences were less than that inherent in the method.

Conversely, Table 3.4 illustrates the effect that the elution

flow rate had on the recovery of the fumonisins, using 14 ml 0.5% (v/v) CH_3COOH in CH_3OH as prescribed by Shephard et al. (1990). Fumonisin recoveries from the cartridges reduced substantially at elution flow rates of between 2.5 and 6.7 ml/minute, when compared with the recoveries obtained at an

Table 3.4 Effect of elution flow rate of fumonisins from SAX cartridges, using 0.5% (v/v) CH_3COOH in CH_3OH

Elution flow rate (ml/minute)	Fumonisin concentration (ng/g)	
	FB_1	FB_2
1.0	1517	605
2.5	1154	500
4.1	957	428
6.7	858	407

Table 3.5 Effect of solvent strength on the elution profiles of FB_1 from SAX cartridges at flow rates of 0.8 ml/minute

Fraction	Volume (ml)	Percentage Fumonisin B_1	
		0.5% CH_3COOH in CH_3OH	1.0% CH_3COOH in CH_3OH
1	4	91.8	97.9
2	2	4.2	1.6
3	2	2.4	0.5
4	2	1.4	0.0
5	2	0.2	0.0
6	2	0.0	0.0

elution flow rate of 1 ml/minute.

The equilibrium between the elution solvent, the fumonisins, and the ion exchange material would appear to be critical. Therefore, the elution flow rate of 2 ml/minute recommended by Shephard et al. (1990) required further consideration.

The elution profiles for FB_1 using different solvent strengths, and eluted from SAX cartridges at a constant flow rate of 0.8 ml/minute, are compared in Table 3.5. More than 90% of the FB_1 eluted from the SAX cartridge within the first 4 ml, when the elution solvent was 0.5% (v/v) CH_3COOH in CH_3OH. Thereafter, the residual FB_1 "*streaked*" from the cartridge, and was only completely recovered after 12 ml of the eluate was collected. By contrast, the use of a 1% (v/v) solution of CH_3COOH in CH_3OH resulted in complete elution of the FB_1 in only 8 ml of solvent; with more than 99% being recovered in the first 6 ml.

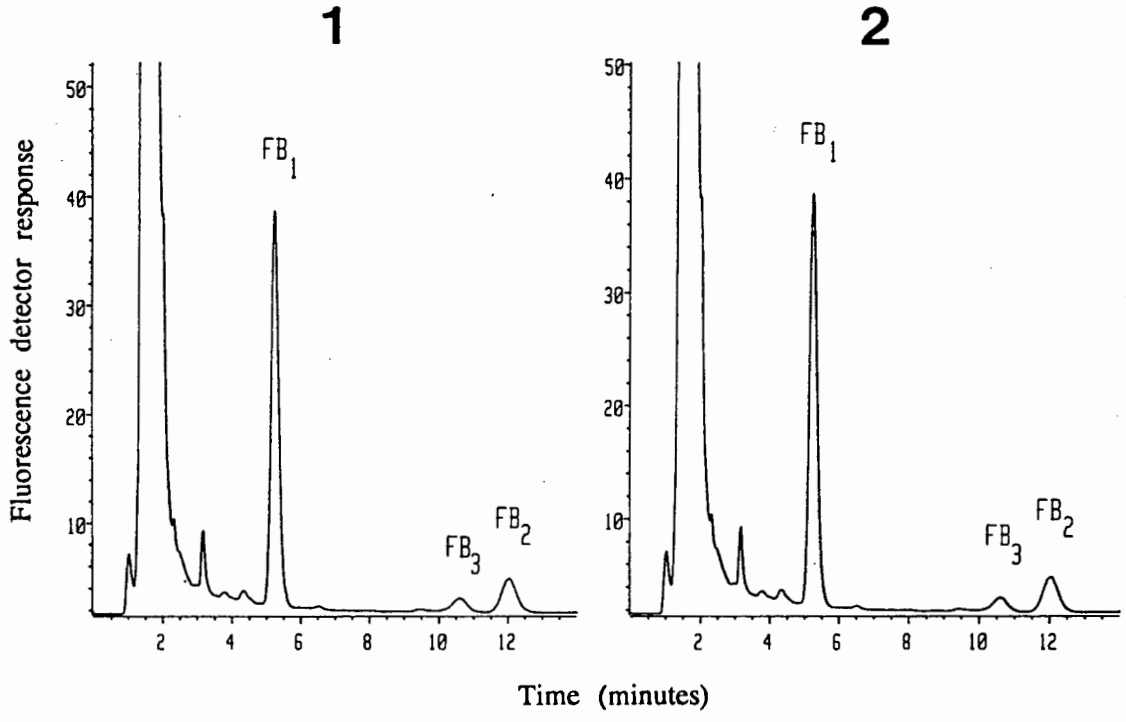


Figure 3.2 Chromatograms of OPA derivatives of a single maize sample purified according to the methods of (1) Shephard et al. (1990) and (2) section 3.4.1 - 3.4.4

Figure 3.2.1 illustrates the chromatogram obtained for a naturally contaminated sample purified according to the method of Shephard et al. (1990), while Figure 3.2.2 shows the chromatogram obtained for the same sample using the optimised HPLC method described in sections 3.4.1 - 3.4.4

Comparison of the two chromatograms clearly indicates that the use of 1% (v/v) CH_3COOH in CH_3OH as the elution solvent, did not result in the additional co-elution of maize intrinsic compounds that interfered with the chromatographic determination of the fumonisins.

Although use of the optimised method increased the overall time required for the complete elution of the fumonisins, the use of the stronger elution solvent significantly reduced the volume of eluate, which in turn reduced the time required for solvent evaporation of the eluate.

3.3.3 *Stability of fumonisin-OPA derivatives*

Derivatisation of the fumonisins with OPA occurs under alkaline conditions (pH 10) in the presence of 2-mercaptoethanol. The use of OPA for derivatisation, presents both advantages and disadvantages. One of the advantages includes the fact that OPA is a relatively cheap reagent. In addition, OPA does not react with H_2O, unlike dansyl chloride or fluorescamine, and derivatives can be prepared at room temperature to yield single products (Joseph & Marsden, 1986).

The stability of various fluorescent derivatives has been reviewed (Perrett, 1985; Rosenthal, 1985), and those of OPA have been reported to be less stable than others. Joseph & Marsden (1986) claimed that the stability of OPA derivatives was compound-dependant, and that some derivatives exhibited greater stability than others. To assess the stability of OPA-fumonisin derivatives, standard solutions of FB_1 were reacted with OPA, and left to stand under white light for increasing periods of time, prior to injection in to the HPLC system, and the results are presented graphically in Figure 3.3.

No major differences were observed for those standards injected within 4 minutes of addition of the OPA reagent. However, after a period of 8 minutes the response was approximately 95% of that previously observed, and following 64 minutes it decreased to 48% of the initial readings. Similar results have been

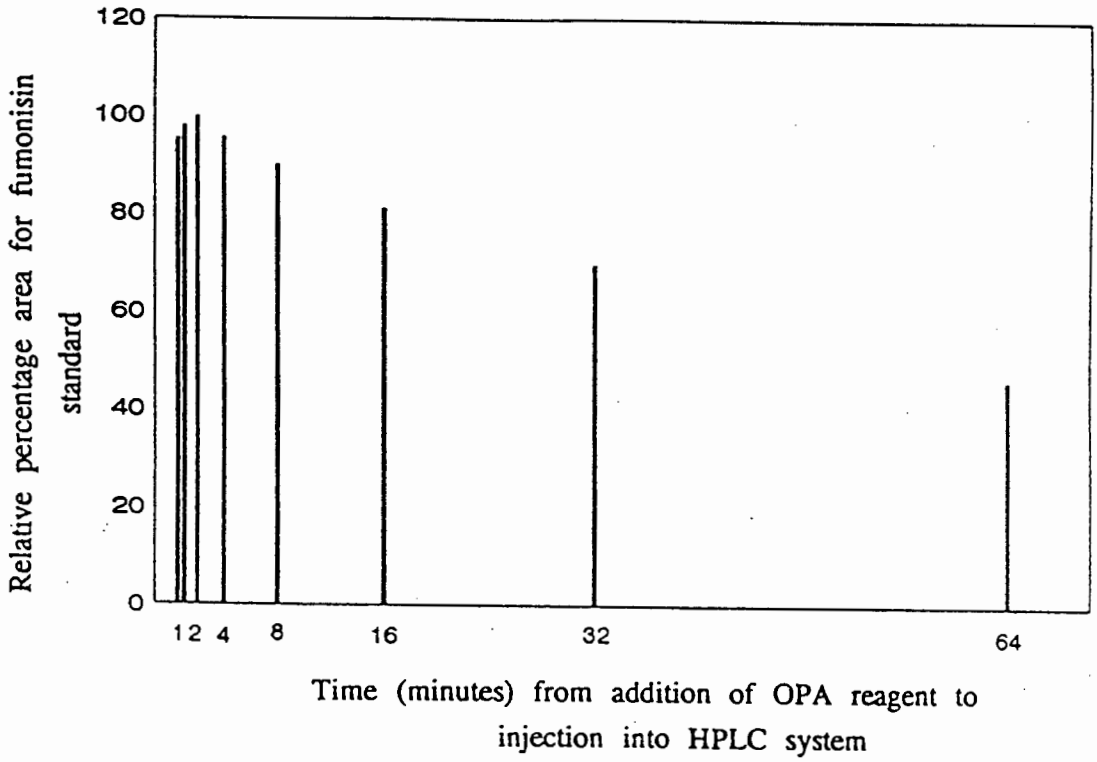


Figure 3.3 Stability of FB_1-OPA derivatives

observed during the interlaboratory study of the stability of FB_1-OPA derivatives, organised by the Community Beureau of Reference (BCR) (Van Egmond, 1992). These data would appear to indicate that OPA-fumonisin derivatives are light sensitive, however the OPA stability experiment was repeated whereby the derivatives, once formed, were screened from the light source prior to injection. No difference in derivative stability was observed between the two sets of derivatives.

The data suggested that provided HPLC injections of the preformed derivatives are made reproducibly, and within 4 minutes following the addition of the OPA reagent, no appreciable derivative stability problems should be encountered. With the advent of automated systems, the pre-column derivatisation of

the fumonisins with OPA could be performed automatically.

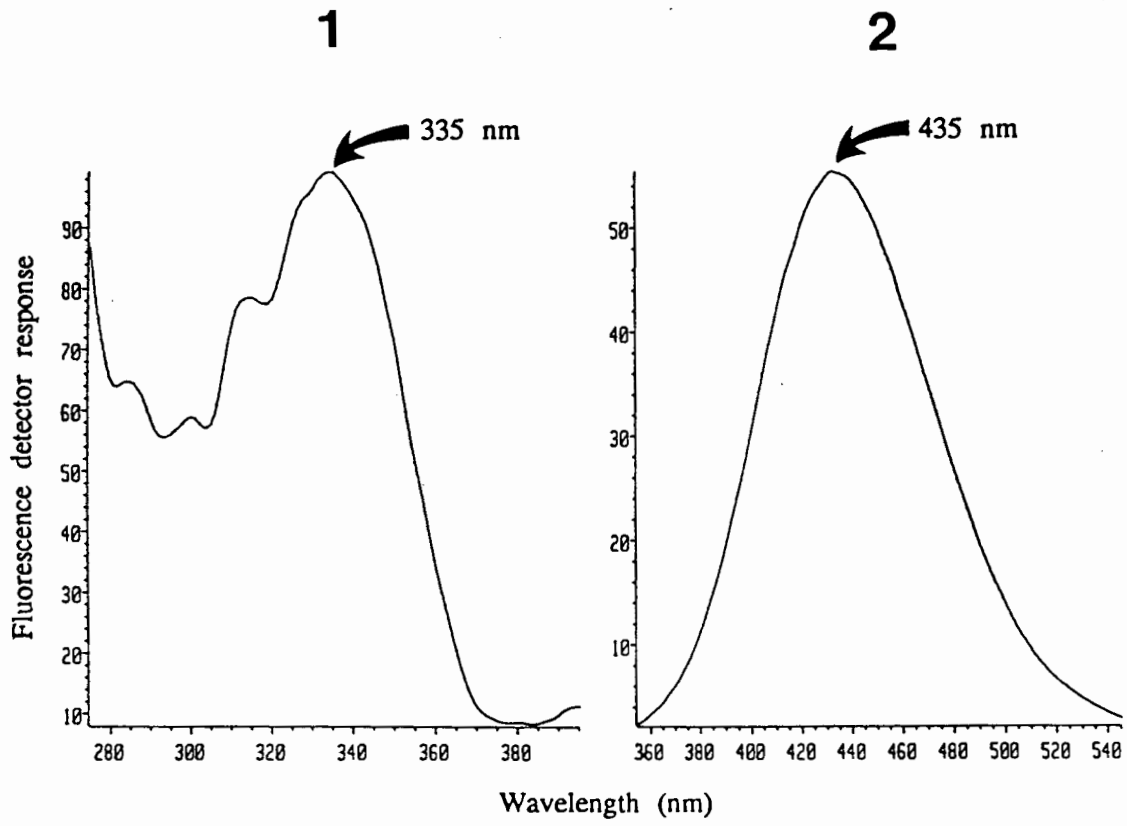


Figure 3.4 The excitation (1) and emission (2) spectra recorded for an FB_1-OPA derivative

The fluorescence excitation and emission wavelength maxima used for the determination of primary amine-OPA derivatives have been reported to be 335 nm and 440 nm, respectively (Perrett, 1984). The excitation and emission maxima for an FB_1-OPA derivative were measured at 230 nm and 394 nm, respectively. However, use of these wavelengths was found to be impractical, resulting in a high fluorescence background and limited selectivity.

Figure 3.4 shows the excitation and emission spectra recorded for an FB_1-OPA derivative (from 280 nm and above), where greater selectivity was observed. While the excitation maximum was found to be 335 nm, the emission maximum was repeatedly recorded at 435 nm. Under these conditions, a 1.5% increase in fluorescence was obtained, when compared to that observed at an emission wavelength of 440 nm.

3.3.4 *Re-use of SAX cartridges*

In order to acertain whether the SAX cartridges could be re-used, extracts of a fumonisin-contaminated sample were applied to four separate cartridges both before and after regeneration, and the results are presented in Table 3.6.

No significant differences were observed between the fumonisin concentrations recorded for the two series of determinations; RSD values were 0.85% for FB_1 and 0.35% for FB_2 in the first series, and 1.0% and 0.25% for FB_1 and FB_2, respectively, for the second series. The results suggested that where necessary, SAX cartridges can be regenerated and reused without loss of analyte recoveries.

Table 3.6 Fumonisin levels obtained from SAX cartridges both before and following regeneration

	Fumonisin concentration (ng/g)			
Column number	Before regeneration		After regeneration	
	FB_1	FB_2	FB_1	FB_2
1	3600	910	3680	1020
2	3635	875	3720	965
3	3440	940	3495	985
4	3550	960	3655	1000
Mean	3550	920	3640	995
RSD (%)	0.85	0.35	1.00	0.25

3.3.5 *Recovery of FB_3*

Based on 5 separate determinations, using fumonisin-free maize spiked with FB_3 at a concentration of 1000 ng/g, the mean recovery from the SAX cartridges was found to be 96.8%, with an RSD of 2.3%. These results were complementary to those previ-

ously recorded for both FB_1 and FB_2, which were reported to be 99.4% (RSD = 1.9%) and 85.9% (RSD = 4.7%), respectively (Shephard et al., 1990). The limit of detection for FB_3 was also found to be similar to those of FB_1 and FB_2 (ie. 50 ng/g).

3.4 *OPTIMISED HPLC METHOD FOR THE DETERMINATION OF FB_1, FB_2 AND FB_3*

Following evaluation (sections 3.3.1 - 3.3.5), the parameters affecting the performance of the HPLC method were incorporated into the optimised method which is detailed in the sections 3.4.1 - 3.4.4. This method has been published in the Journal of the Association of Official Analytical Chemists International (Sydenham et al., 1992b).

3.4.1 *Apparatus*

3.4.1.1 *Liquid chromatograph*

Waters M-510 HPLC pump fitted with a Waters U6K manual injector (Waters, Milford, USA).

3.4.1.2 *HPLC column*

Stainless steel (12.5 cm x 4 mm i.d.) packed with either Lichrosorb or Lichrosphere 5 μm C_8 reversed-phase material (Merck, Darmstadt, Germany).

3.4.1.3 *Fluorescence detector*

Either a Perkin-Elmer 650S fitted with an 18 μl flow cell and set at 335 nm (excitation) and 435 nm (emission) and slit widths of 12 nm (Perkin-Elmer, Norwalk, USA) or a Hewlett-Packard 1046A detector fitted with a 5 μl flow cell, set at the same wavelengths as above, with 4 mm slit widths, and operated at 220 Hz (Hewlett-Packard, Palo Alto, USA).

3.4.1.4 *Data system*

Either a Waters Model 745 integrator or a Hewlett-Packard 9000 computing system.

3.4.1.5 *Blender*

Polytron mixer (Kinematica, Luzern, Switzerland).

3.4.1.6 *Solid-phase extraction (SPE) cartridges*

Bond-Elut strong anion exchange SPE cartridges, 10 ml capacity, each containing 500 mg sorbent (Varian, Harbour City, USA).

3.4.1.7 *SPE vacuum manifold*

Twelve port SPE tube manifold (Supelco, Bellefonte, USA).

3.4.1.8 *Sample evaporator*

Six port Silli-therm module (Pierce, Rockford, USA).

3.4.1.9 *pH meter*

Beckman (California, USA) Model 70 calibrated with solutions of pH 4 and 7.

3.4.2 *Reagents*

3.4.2.1 *Solvents and reagents*

Analytical grade CH_3CN, CH_3OH, *o*-phosphoric acid (>85%), CH_3COOH, OPA, 2-mercaptoethanol, KOH, NaH_2PO_4, $Na_2B_4O_7$.

3.4.2.2 *HPLC mobile phase*

Prepare CH_3OH:0.1M NaH_2PO_4 (68:32), and adjust to pH 3.35 with *o*-phosphoric acid. Filter mobile phase through a 0.45 μm Waters HV membrane and pump at a flow rate of 1 ml/minute.

3.4.2.3 *OPA solution*

Dissolve 40 mg OPA in CH_3OH (1 ml) and dilute with 5 ml 0.1M $Na_2B_4O_7$. Add 50 µl 2-mercaptoethanol and mix. The solution may be stored for up to one week at room temperature in a dark, capped amber vial.

3.4.2.4 *Fumonisin standard solution*

Standard solutions of FB_1, FB_2 and FB_3, isolated according to the methods of Gelderblom et al. (1988) and Cawood et al. (1991) were prepared in $CH_3CN:H_2O$ (1:1), at a concentration of 50 µg/ml. The solution was stored at 4°C.

3.4.3 *Extraction and sample purification*

A subsample (25 g) of maize or maize-based product, previously ground and blended to pass a 840 µm sieve, was placed into a 100 ml glass centrifuge bottle and homogenised for 3 minutes with 50 ml $CH_3OH:H_2O$ (3:1), using a Polytron blender set at 60% full speed. The extract was then centrifuged for 10 minutes at 500 x g at 4°C, and the supernatant filtered through a fluted Whatman No.4 filter paper. The pH of the filtered solution was monitored, and if necessary adjusted to between 6.0 and 6.5 with 0.1M KOH.

The SPE manifold was connected to a vacuum source (ie. water pump) and a single SAX cartridge per sample was placed on the manifold (it is possible to process up to six samples simultaneously). Each cartridge was conditioned by washing successively, first with 5 ml CH_3OH followed by 5 ml $CH_3OH:H_2O$ (3:1). While maintaining a flow rate of no more than 2 ml/min, aliquots (10 ml each) of the filtered, pH adjusted extracts were applied to the individual cartridges. Each cartridge was then washed with 8 ml $CH_3OH:H_2O$ (3:1), followed by 3 ml CH_3OH (to remove interferences) and the washings were discarded. The fumonisins were eluted and collected in 20 ml capacity vials, by the addition of 10 ml aliquots of a solution of CH_3COOH: CH_3OH (1:99). The flow rate during the elution stage was

maintained at no more than 1 ml/min. The eluates were transferred to individual 4 ml capacity vials and evaporated to dryness on the Silli-therm module under a stream of nitrogen at 60°C. Each collection vial was washed with an additional 1 ml CH_3OH, and the washings were added to the corresponding 4 ml vial. The additional CH_3OH was evaporated to dryness under nitrogen, and the dried residues were retained at 4°C prior to HPLC analyses.

3.4.4 *Derivatisation and HPLC analyses*

3.4.4.1 *Preparation of standard derivative*

An aliquot (25 µl) of the fumonisin standard solution was transferred to the base of a small test tube. OPA reagent (225 µl), was added to the tube which was then mixed. Within 1 minute following the addition of the OPA, a 10 µl aliquot of the derivative was injected into the HPLC system. The sensitivity of the fluorescence detector was adjusted such that the peak corresponding to the FB_1-OPA derivative (equivalent to 50 ng injected onto the HPLC column) gave at least an 80% recorder response.

3.4.4.2 *Maize and maize-based sample extracts*

The purified extracts were redissolved in CH_3OH (200 µl). An aliquot (25 or 50 µl) of each extract was transferred to the base of a test tube and OPA reagent (225 or 200 µl, respectively) was added. The solutions were mixed and aliquots (10 µl) were injected into the HPLC column within 1 minute following the addition of the OPA solution.

3.5 *CONFIRMATION TECHNIQUES*

The retention time of a peak in chromatography gives only indirect evidence of the presence of a given compound. In basic chromatographic systems, both the retention time and peak area may be compared with standards, but no structural information is obtained from the chromatogram.

Spiking of sample extracts, and their subsequent re-chromatographic separation has been used to assist in the identification of peaks, however additional evidence of the identity of a peak should be sought elsewhere. In cases where the compound of interest has other specific chemical characteristics, such as a well defined UV spectrum (ie. ZEA - a mycotoxin produced by, amongst others, the fungus *F. graminearum*), multi-wavelegth diode array detectors can be used to record the complete spectrum of a compound eluting from a HPLC column. However, the most reliable technique for the unequivocal identification of a compound is the observation of its mass spectrum (MS).

The MS of a compound may be considered as its *"fingerprint"*, which is both highly specific and equally reproducible. Alter-

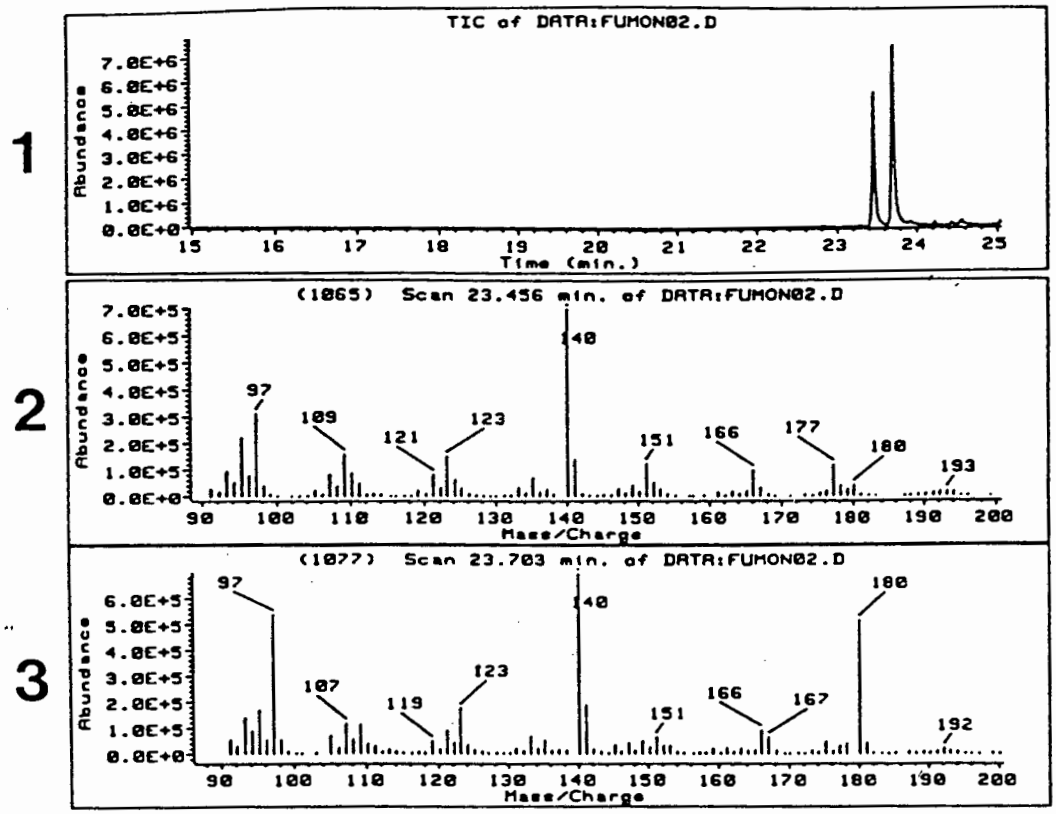


Figure 3.5 Total ion chromatogram (TIC) of the acylated hyrolysates of authentic FB_1 and FB_2 (1). Partial mass spectrum of the aminotetrol moiety derived from FB_2 (2) and the similar mass spectrum derived from the aminopentol moiety of FB_1 (3)

natively, additional evidence for the presence of a compound could be obtained the using an alternative analytical technique to that used for quantitative purposes. Both approaches were applied to the analyses of the fumonisins, and the results are presented and discussed in sections 3.5.1 - 3.5.2.

3.5.1 *Application of capillary GC-MS for confirmation purposes*

Attempts to volatilise the highly polar fumonisins, as the intact molecules, for capillary GC separation and subsequent mass spectral investigation were undertaken, but these failed to generate any chromatographic peaks that could be attributed to the fumonisins (Sydenham, 1989; Sydenham et al., 1990a). However, hydrolysis of the fumonisin molecules followed by acylation with TFAI, resulted in the observation of peaks corresponding to the aminopolyol moieties present in the structures of the fumonisins (Figures 3.5.1 and 3.5.2).

Figure 3.5.1 shows the total ion chromatogram (TIC) of the hydrolysed, acylated products of FB_1 and FB_2 standards. The two chromatographic peaks eluting after 23.45 and 23.70 minutes, corresponded to the aminotetrol and -pentol moieties of FB_2 and FB_1, respectively. Figures 3.5.2 and 3.5.3 show the partial mass spectra of the two peaks (90 to 200 *m/z*).

Unfortunately, due the absence of suitable instrumentation, the mass spectra presented in Figures 3.5 and 3.6 were obtained from an alternative facility. Only partial mass spectra were obtained, resulting in difficulty in the interpretation of the fragmentation patterns. These problems were compounded by the fact that the mass spectra were of the complex TFAI-derivatives of the aminopolyol moieties of the fumonisins. Spiteller (1989) maintained that the mass spectral interpretation of even basic methylated or silylated derivatives, could be difficult.

The method was applied to extracts prepared from a series of samples, including a number of fungal cultures of various *Fusarium* species. Figure 3.6.1 illustrates the TIC of the

hydrolysed and acylated extract of *F. nygamai* strain MRC 4003, prepared according to the method of Sydenham et al. (1992b). Figures 3.6.2 and 3.6.3 illustrate the partial mass spectra observed for the two major peaks seen in Figure 3.6.1, which eluted at retention times of 23.45 and 23.68 minutes, respectively.

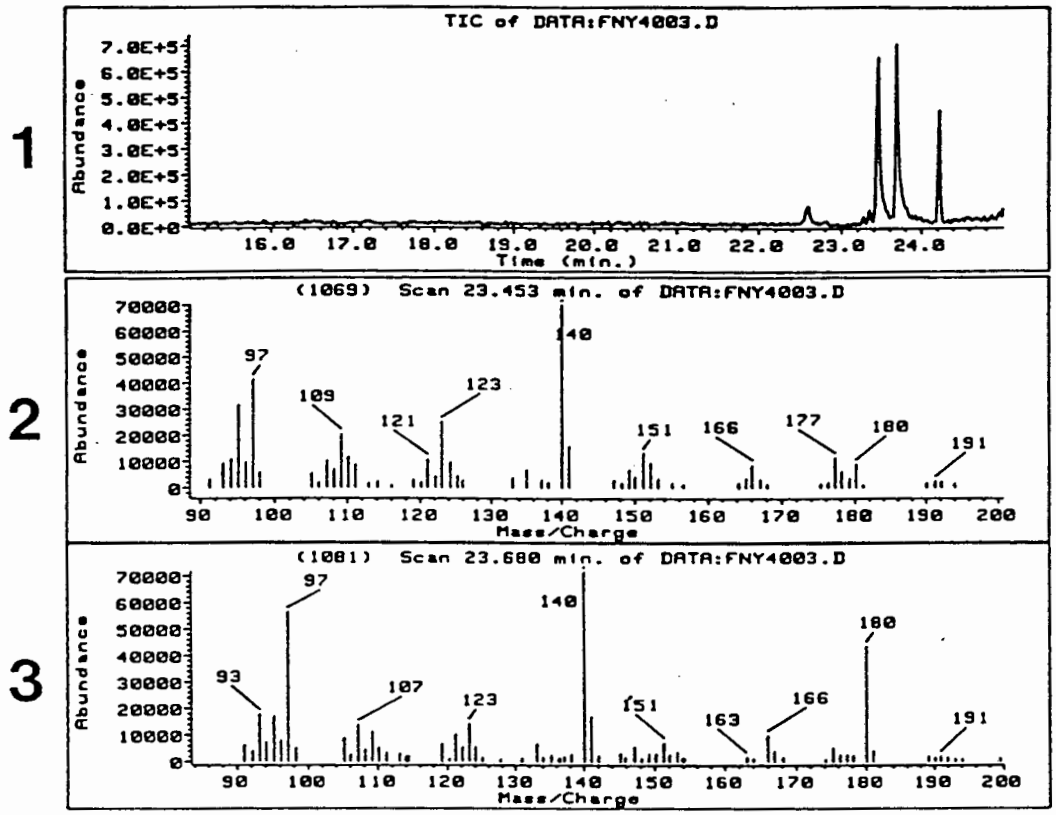


Figure 3.6 TIC of the acylated hyrolysate of an extract of *F. nygamai* (1). Partial mass spectrum of the peak eluting after 23.45 minutes (2), and similar mass spectrum of peak eluting after 23.68 minutes (3).

The excellent agreement between the retention times and mass spectra observed in Figures 3.5.1 - 3.5.3 and 3.6.1 - 3.6.3 confirmed the production of both FB_1 and FB_2 by *F. nygamai* strain MRC 4003.

In addition to the analyses of the aminopolyols by capillary

GC-MS (as their acylated derivatives), it was also possible to prepare the OPA derivatives of the hydrolysates, and analyse them using the same HPLC chromatographic conditions as used for the separation of the non-hydrolysed fumonisins (Sydenham et al., 1992b).

Figure 3.7.1 shows the HPLC chromatogram of a naturally contaminated maize sample analysed for the fumonisins using the method and chromatographic conditions described by Sydenham et al. (1992b). Figure 3.7.2 illustrates the resultant chromatogram of a portion of the same sample extract following hydrolysis and derivatisation with OPA. The major difference observed between the Figures 3.7.1 and 3.7.2 is that of the increase in retention times for the hydrolysed forms of FB_1, FB_2 and FB_3.

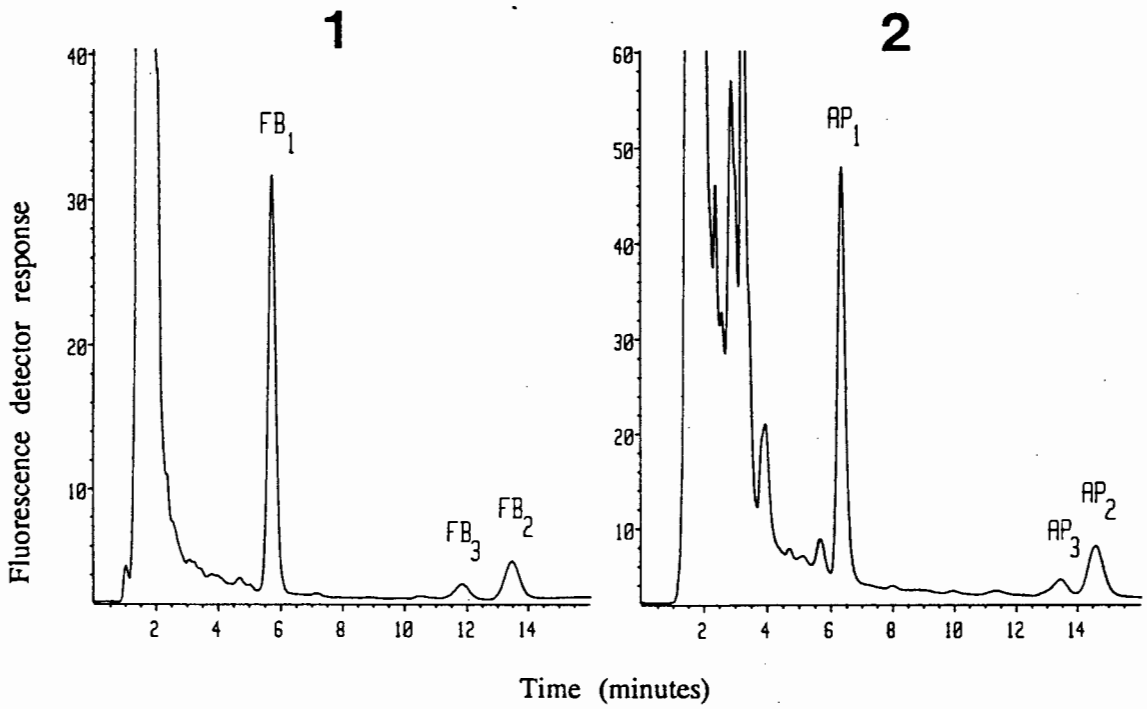


Figure 3.7 HPLC chromatogram of a naturally contaminated maize sample (1) as its OPA derivative, and (2) the chromatogram of the same sample as its hyrolysed form, showing the corresponding fumonisin aminopolyol (AP) moieties

However, HPLC separation of the later derivatives provides additional information concerning the identity of the fumonisins.

3.5.2 *Application of TLC for confirmation purposes*

Rottinghaus et al. (1992) developed a C_{18} pre-chromatographic purification procedure, in the preparation of animal feed samples, for the analytical estimation of the fumonisins. Following separation by TLC, the plates were sprayed with a solution of fluorescamine in CH_3CN, prior to quantification by visualising the plate under longwave (365 nm) UV light. The use of fluorescamine as a spray reagent was applied to extracts of a series of naturally contaminated maize samples prepared

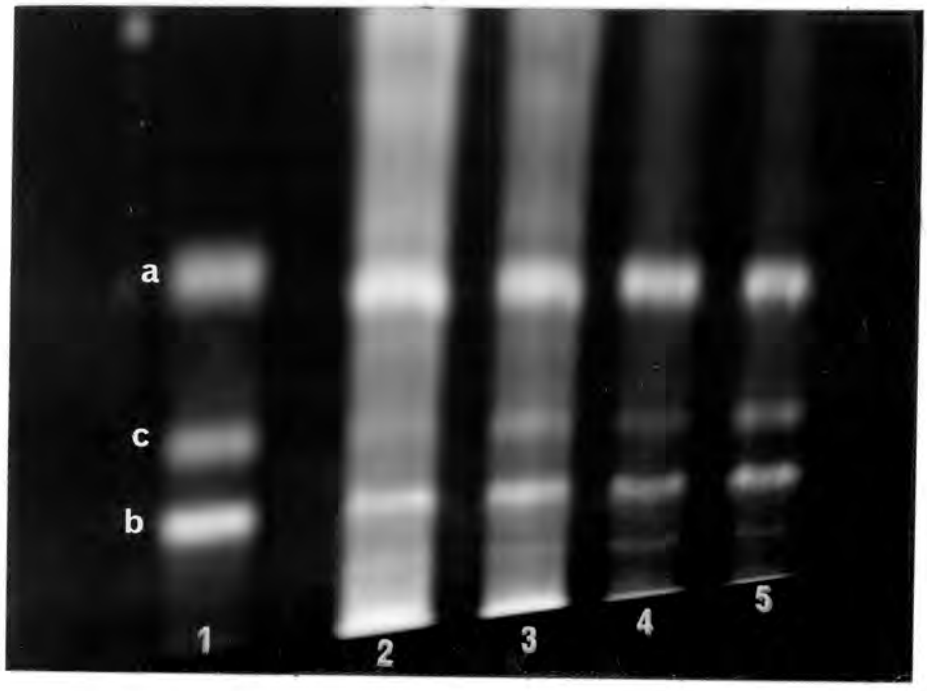


Figure 3.8 TLC separations of (1) 1000 ng each of (a) FB_1, (b) FB_2 and (c) FB_3, (2) maize extract prepared by the method of Rottinghaus et al. (1992), (3) the extract spiked with fumonisin standards, (4) sample prepared by the method of Sydenham et al. (1992b), and (5) the spiked extract of that shown in (4)

according to the method of Rottinghaus et al. (1992), and the results were compared to those obtained for the same sample extracts prepared according to the method of Sydenham et al. (1992b).

Figure 3.8 shows the black and white photograph of a thin layer chromatogram of the separation of fumonisin standards and maize extracts. The chromatographic bands corresponding to FB_1, FB_2 and FB_3 (Figure 3.8.1) were observed as bright yellowish bands having R_f values of 0.47, 0.19 and 0.29, respectively. Figure 3.8.2 shows the chromatogram obtained for a naturally contaminated maize sample, prepared according to the method of Rottinghaus et al. (1992), while Figure 3.8.3 illustrates the same extract "*spiked*" with fumonisin standards. The chromatogram illustrated in Figure 3.8.4 is that of the same maize sample prepared by the method of Sydenham et al. (1992b), and Figure 3.8.5 is the corresponding "*spiked*" extract. Identical sample equivalent weights were applied to the plate (Figures 3.8.2 and 3.8.4).

Comparison of the TLC chromatograms obtained by both sample preparation methods (Figures 3.8.2 and 3.8.4) illustrates the greater selectivity obtained when using ion-exchange chromatography for extract clean-up (Sydenham et al., 1992b). Problems of toxin band discernment and the appearance of excessive contamination can clearly be observed in the extract prepared by the method of Rottinghaus et al. (1992) (Figure 3.8.2). These were particularly noticable when attempting to determine fumonisin levels at relatively low concentrations (ie. ± 1 μg/g). Following extraction of the fumonisins from maize, and the addition of 1% KOH (in accordance with the method of Rottinghaus et al., 1992), problems associated with the formation of precipitates were encountered which, on many occasions, led to sedimentary blocking of the C_{18} cartridges used as part of the clean-up procedure.

While these observations suggest that the sample preparation procedure of Rottinghaus et al. (1992) is inferior to that prescribed by Sydenham et al. (1992b), little difference

Table 3.7 Fumonisin concentrations in three maize sample extracts purified according to two methods and analysed by HPLC, as their OPA derivatives

Sample number	Fumonisin concentration (ng/g)		
	FB_1	FB_2	FB_3
1[a]	13920	4060	1100
1[b]	14880	4780	1220
2[a]	1065	345	ND[c]
2[b]	1250	465	90
3[a]	505	160	55
3[b]	405	165	50

[a]Prepared according to the method of Rottinghaus et al. (1992)
[b]Prepared according to the method of Sydenham et al. (1992b)
[c]Not detected (<50 ng/g)

in fumonisin recoveries were recorded between the two methods. Using the two procedures, fumonisin levels in three different maize samples were determined by HPLC, and the results are presented in Table 3.7.

The data presented in Table 3.7 suggest that little difference with respect to fumonisin recoveries are experienced between the methods. From this data (Table 3.7), and the observations of the thin layer chromatograms, it would appear that TLC can be used both quantitatively and qualitatively for the determination of fumonisin levels in maize. However, if restricted to TLC for quantitative purposes (and in cases where low fumonisin levels are encountered - ≤ 1 µg/g), the use of the pre-chromatographic clean-up procedure of Sydenham et al. (1992b) would be recommeded.

3.6 *OTHER RECENT ADVANCES IN FUMONISIN METHODOLOGY*

The HPLC method detailed in sections 3.4.1 - 3.4.4 (Sydenham et al., 1992b) represents the most recent addition to analytical methodology emanating from the activities within PROMEC. However, several groups in addition to PROMEC have also been involved in the development of analytical methods for the determination of the fumonisins in maize and other matrices.

Ackermann (1991) made minor alterations to the initial TLC method of Gelderblom et al. (1988a) which involved the use of alternative mobile phases and reversed-phase TLC plates. No major improvement in the sensitivity of the TLC technique was observed, since *p*-anisaldehyde was also used for visualisation of the separated spots/bands corresponding to FB_1 and FB_2. The method was applied only to the analysis of fumonisins in culture material (Ackermann, 1991). Pittet et al., (1992) applied aliquots of purified maize extracts to reversed-phase TLC plates and used vanillin for visualisation purposes. The development of blue-purple spots for the fumonisins was used for the qualitative confirmation of HPLC observations, and the authors reported that the detection limit of the method was in the order of 250 ng/g (Pittet et al., 1992).

The use of HPLC for the analytical determination of the fumonisins, has also received attention from other research groups. In addition to the method developed by Ross et al. (1991a), which was based on pre-chromatographic clean-up on C_{18} material followed by derivatisation with either OPA or fluorescamine, Scott & Lawrence (1992) used the basic clean-up procedure on SAX cartridges, but prepared derivatives using a solution of naphthalene-2,3-dicarboxaldehyde/potassium cyanide. The resultant derivatives were reported to be more stable than those of OPA, but required binary gradient HPLC for elution. Similarly, Holcomb & Thompson (1992, 1993) prepared stable derivatives with 9-fluorenylmethyl-chloroformate which also required gradient HPLC for effective separation of the fumonisins.

An alternative technique, chemiselective immobilisation and

detection (CSID), has been developed by Phillips et al. (1992). The technique involves the use of a variety of inorganic sorbents, layered in a small tube so as to form a narrow column. Maize extracts, partially purified on C_{18} material, are derivatised with fluorescamine and then applied to, and passed through the column. The fumonisin-fluorescamine derivatives are selectively retained on the column as narrow bands which can be visualised under longwave-UV light. The detection limit of the method has been estimated to be 50 ng/g [the same as for the HPLC method of Sydenham et al. (1992b)], but the method does not distinguish the individual fumonisin analogues (Phillips et al., 1992).

Korfmacher et al. (1991) and Holcomb et al. (1993) demonstrated the use of thermospray, fast atom bombardment (FAB) and electrospray (ES) mass spectrometry (MS) (in conjunction with HPLC) for the determination of fumonisin B_1. They concluded that FAB-MS and ES-MS could provide useful data for the characterisation of the fumonisins. Both FAB-MS and ES-MS are "soft" ionisation techniques that result in molecular weight information (similar to chemical ionization), rather than the fragmentation patterns normally associated with electron impact-MS. Although not part of this study, the potential of ES-MS may be demonstrated by observing prelininary studies, the results of which are illustrated in Figures 3.9.1 to 3.9.3.

The TIC (between 170 and 800 *m/z*) of a non-derivatised urine extract, collected from a rat that had been subjected to treatment with FB_1 (Shephard et al. 1992a,b), following HPLC separation is shown in Figure 3.9.1, while Figure 3.9.2 shows the extracted 722 *m/z* ion profile. A major peak, corresponding to FB_1, was observed at a retention time of 6.18 minutes (Figures 3.9.1 and 3.9.2), the mass spectrum of which is shown in Figure 3.9.3.

As shown in Figure 3.9.3, a strong base peak could be observed at *m/z* 722, corresponding to protonated molecular ion $[MH]^+$ of FB_1, while *m/z* 744, 760 and 766 probably corresponded to the $[MNa]^+$, $[MK]^+$, and the $[M+2Na]^+$ ions, respectively. The

technique of ES-MS was found to be very sensitive (20 pg FB_1 gave a signal to noise ratio of 10:1). Electrospray-MS appears to offer a number of advantages over other HPLC detection systems, in that it (a) demonstrates increased sensitivity, (b) provides structural information and (c) does not require derivatisation for detection purposes. The technique could be

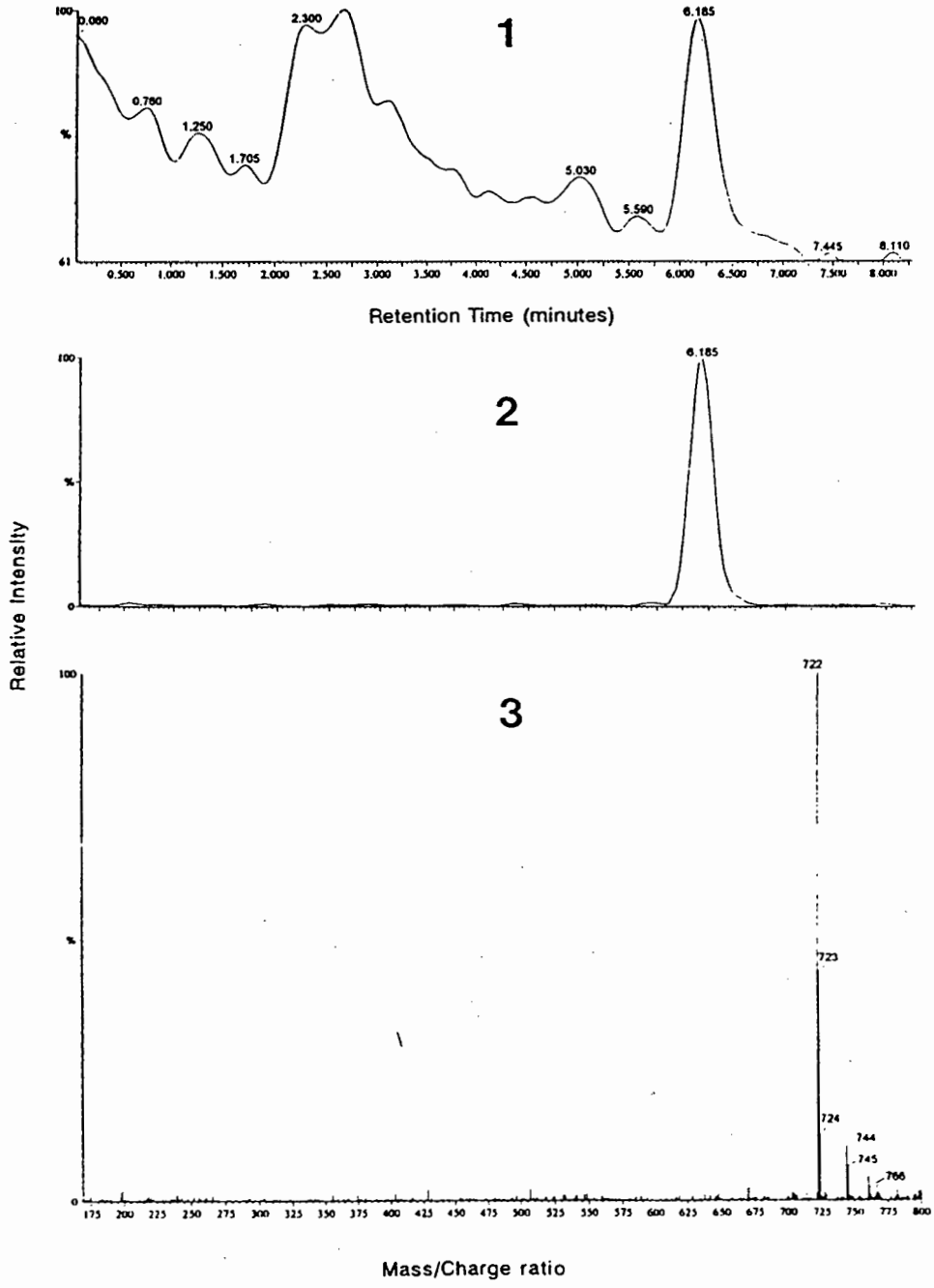


Figure 3.9 (1) TIC of a urine extract, (2) extracted *m/z* 722 profile and (3) the mass spectrum of (2)

most useful, especially where greater sensitivity and component identification are required.

The production and isolation of mono- and polyclonal antibodies to the fumonisin mycotoxins has been a major development in the field of fumonisin research (Azcona-Olivera et al., 1992a,b), and has led to the establishment of an ELISA method for the determination of the fumonisins (Miller, 1992a). The ELISA method has been developed to give a reaction to maize extracts containing combined FB_1, FB_2 and FB_3 levels in excess of 5000 ng/g, and it is therefore aimed at the screening of animal feeds, rather than human foodstuffs (see Chapters 4 and 5). Preliminary observations have, however, suggested that there may be some degree of antibody cross-reactivity resulting in the over-estimation of fumonisin levels in naturally contaminated samples (personal observations). One area that may benefit from the development of antibodies to the fumonisins, will be that of sample preparation. The development and use of fumonisin "*affinity*" columns (Hansen, 1993) will result in high selectivity for the concentration of the fumonisins from crude solvent extracts. Purified extracts could then be used in conjunction with existing HPLC derivatisation and separation methodologies, for the determination of fumonisin concentrations.

3.7 *CONCLUSIONS*

In conclusion, numerous methods have been developed for the analytical determination of the fumonisins. Of these methods, advanced HPLC-MS techniques exhibit excellent sensitivity, but the high cost of the instrumentation is likely to restrict their use. Conversely, HPLC is an instrumental technique that has been used extensively for the determination of various other mycotoxins. Most of the methods thus far developed for the HPLC determination of the fumonisins, require extensive sample purification, derivatisation of the amino group present in the fumonisin structure, and fluorescence detection.

In the present study, aspects of a method (that could give rise to erroneous results) were further evaluated and where necessary altered to improve the overall "*ruggedness*" of the method. This optimised method (Sydenham et al., 1992b) allows for the co-determination in maize, of the three toxicologically important fumonisin mycotoxins. Although the use of OPA results in the formation of derivatives that are less stable than other fluorophors, the OPA-fumonisin derivatives can be separated within 15 minutes using isocratic elution. The optimised method is similar to that for which the precision characteristics were successfully assessed collaboratively (Chapter 2). Therefore, it is likely that the precision characteristics of the optimised method will be similar, if not superior to those of the previous method (Thiel et al., 1993). Intralaboratory studies have indicated the optimised method to be precise and accurate, although these aspects would also preferably have to be assessed collaboratively. Accordingly, a protocol has been registered with, and accepted by the Food Commission of IUPAC, aimed primarily at determining collaboratively, the accuracy characteristics of the optimised method of Sydenham et al., (1992b).

CHAPTER 4: FUMONISINS - IMPLICATIONS FOR ANIMAL HEALTH

4.1 *INTRODUCTION*

The provision of accurate and sensitive methods for the quantitative determination of the fumonisins in maize-based matrices (Shephard et al., 1990; Sydenham et al., 1992b), was an essential prerequisite for the evaluation of the association between the occurrence of fumonisins in feeds or foods, and possible toxicological/pathological effects in either animals or humans.

4.1.1 *F. moniliforme and animal disease syndromes*

Associations between fungal contamination of feeds in general (and with *Fusarium* species in particular), and various animal disease syndromes may be traced back to the latter part of the 19th century, and the early years of the 20th century (Marasas et al., 1984b). Widespread field outbreaks of a disease in animals were reported to have occurred throughout the United States (Peters, 1904). The disease, which was associated with the ingestion of mouldy maize, was characterised by the appearance of sloughed hooves of cattle and horses, and the loss of bristles and feathers from pigs and chickens, respectively. A substantial number of the affected animals also developed signs of convulsions which, in many cases, was followed by death (Peters, 1904). The fungus most commonly isolated from the feeds implicated with these disease syndromes was described as *F. moniliforme* Sheldon (Sheldon, 1904).

Equine leukoencephalomalacia (LEM), a neurotoxic disease of horses, donkeys and mules is characterised clinically by apathy, nervous disorders, partial paralysis of the lower lip, an inability to eat and mobility problems; and pathologically by multifocal liquefactive necrotic lesions predominately in the white matter of the cerebral hemispheres - symptoms from which the name equine LEM was derived (Marasas et al. 1984b).

Butler (1902) experimentally reproduced equine LEM in horses by feeding them naturally contaminated mouldy maize. These findings were subsequently confirmed by other workers in China, Egypt and the United States (Marasas et al., 1984b). Although identified as a mycotoxic syndrome, the causative fungus responsible for equine LEM was only identified in 1971, when the disease was experimentally reproduced in donkeys with culture material of *F. moniliforme* (Wilson, 1971; Wilson & Maronpot, 1971), while later work in South Africa using horses confirmed these findings (Kriek et al., 1981b; Marasas et al., 1976).

Equine LEM is the one animal disease syndrome for which the causative role of *F. moniliforme* had been established beyond doubt (Bridges, 1978; Buck et al., 1979; Kriek et al., 1981b; Wilson, 1971; Wilson & Maronpot, 1971). Deaths of equine species due to LEM, have been observed since the year 1850, especially in the maize growing regions of the United States where maize has traditionally been used for animal feeds (Biester et al., 1940; Butler, 1902; Schwarte et al., 1937). Thousands of horses died in Maryland around the turn of the century (Buckley & MacCullum, 1901) and in several Midwestern states during the 1930's (Schwarte et al., 1937). During the 1978-79 season, large numbers of horses died throughout the United States (Buck et al., 1979), while as recently as 1989-90, similar numbers also suffered the same fate (Ross et al., 1992; Wilson et al., 1990). The disease has also been known to occur in Argentina (Rodriguez, 1945), Brazil (Sydenham et al., 1992a), China (Iwanoff et al., 1957), Egypt (Badiali et al., 1968) and South Africa (Kellerman et al., 1972; Marasas et al., 1976).

Isolates of *F. moniliforme* have also been shown to cause a fatal hepatic syndrome in horses (Kellerman et al., 1972, Kriek et al., 1981b). The hepatic lesions were characterised pathologically by fatty changes, fibroplasia and biliary statsis, among others (Kellerman et al., 1972). An association between the onset of equine LEM and the hepatotoxic syndrome was observed, since the applied dosage rate of the culture

material could precipitate either lesion (Marasas et al., 1976). Clinical signs similar to those of equine LEM have also been reported to occur in horses in Hokkaido, Japan (Konishi & Ichijo, 1970). The major pathological lesions associated with the disease, known as "*bean-hulls poisoning*", have been described as the "*degenerative change of the nerve cells of the cerebral cortex and acute circulatory disturbance*" (Ueda et al., 1967). Although the disease has been reproduced experimentally in Japan by the feeding of mouldy soya bean hulls to horses (Konishi & Ichijo, 1970), toxigenic strains of *F. moniliforme* isolated from feed substrates associated with pathologically confirmed outbreaks of the disease, have not yet been used to successfully reproduce the syndrome. It is therefore, not yet known whether "*bean hulls poisoning*" is identical to equine LEM.

Associations between animal diseases and the occurrence of *F. moniliforme* have not however been restricted to members of the equidae family. Sharby et al. (1973) identified *F. moniliforme* as one of the major fungal contaminants of commercially produced poultry feeds. Maize cultures prepared from the isolates were subsequently shown to reduce both weight gain and feed conversion efficiency in broiler chicks, which was followed by abnormal bone development and the appearance of severe leg deformities (Sharby et al., 1973). Similarly, *F. moniliforme* contaminated feeds have been implicated in outbreaks of diseases that occurred in France, that were characterised by nervousness and paraplegia in pigs, and by paralysis and mortality in poultry (Moreau, 1974, 1979). It has been postulated that fusaric acid, a known chelating reagent produced by *F. moniliforme*, might precipitate the ricket-type disease in animals due to phosphorous deficiency, while the fungus is also known to produce a thiaminase, which could possibly induce thiamine deficiency and the onset of paralysis and death (Moreau, 1974, 1979). Similar observations have also been noted in Germany (Gedek et al., 1978).

Kriek et al. (1981b) reported the deaths of two baboons from acute congestive heart failure, while a third was found to

exhibit severe cirrhosis of the liver, following the incorporation of culture material of *F. moniliforme* into their diet.

Kriek et al. (1981a) observed acute toxicity in rats fed diets containing various proportions of culture material of *F. moniliforme*. Strain MRC 602, originally isolated from maize collected in the Transkei, was shown to cause the death of all test animals (number = 20) which received diets containing 16% and above, of the culture material. Cirrhosis and nodular hyperplasia of the liver were, amongst others, the principal characteristic lesions. Several strains including MRC 826 (also isolated from Transkeian maize) were subsequently shown to be noticeably more toxic to rats than MRC 602 (Kriek et al., 1981b). Li et al. (1980) and Yang (1980) observed the formation of tumours in several different organs of experimental rats fed maize innoculated with isolates of *F. moniliforme* collected from the Linxian County in the Henan Province of China. Several nitrosamines were identified in maize innoculated with the Chinese isolates (Li et al., 1980), but it is not known whether their presence was responsible for the carcinogenic potential observed in the rats.

The acute toxicity to mice of four strains of *F. moniliforme* isolated from feeds associated with suspected outbreaks of animal mycotoxicoses was reported by Korpinen & Ylimaki (1972). Hemorrhages in the mucosa of the gastro-intestinal tract together with slight degeneration of the liver were the major pathological observations. Van Rensburg et al. (1971) reported relatively low toxicity during a 20-day experiment, which resulted in the death of one of ten adult male mice fed a diet containing culture material of *F. moniliforme*. The nine surviving experimental animals however exhibited severe loss in weight (Van Rensburg et al., 1971).

In addition to these studies, the standard rabbit skin test, involving the application of alcohol extracts, and the subsequent dermatoxic reaction, has been used to screen *F. moniliforme* strains for toxicity (Joffe et al., 1973), while limited studies have also been carried out on pigeons (Ghosal

et al. 1978) pigs and sheep (Kriek et al., 1981b). More recently, Brown et al. (1992) induced multifocal hepatic necrosis and biliary hyperplasia in broiler chicks fed culture material of *F. moniliforme*.

4.1.2 *Experimental aims*

Substantial evidence exists concerning the fungal contamination of feeds with *F. moniliforme*, and specific animal diseases (section 4.1.1). However, while the responsible fungus has been identified, little evidence existed concerning the resposible toxin(s) (Marasas et al., 1984b). Subsequent to the isolation and identification of the novel *F. moniliforme* mycotoxins, the fumonisins (Gelderblom et al., 1988a), it will be essential to determine both the extent of animal exposure to these cancer-promoting compounds, and if necessary, establish tolerance levels based on risk-assessment stategies. Assessment studies must take into account not only the toxicity of potentially harmful compounds, but also the levels to which certain animal (or human) populations may be exposed.

One approach to determining the extent of possible mycotoxin exposure is to screen isolates of the dominant fungal species that might commonly occur in suspected food or feed substrates, for their ability to produce the toxins. An alternative approach is to determine toxin levels present in substrates that have been associated with outbreaks of mycotoxicoses, the clinical and pathological signs of which, suggest that the observed syndromes might have had their aetiology associated with the contamination of the implicated feeds, with the responsible mycotoxin(s).

Both approaches, aimed towards the determination of the extent of animal exposure to the fumonisins were investigated. The experimental details and subsequent results and discussions are presented in sections 4.2 to 4.4, respectively.

4.2 *EXPERIMENTAL*

4.2.1 *Fusarium isolates - survey of fumonisin production*

Each of the 40 *Fusarium* isolates used for the study were obtained from the fungal culture collection of the Medical Research Council (MRC), South Africa. Culture material of each isolate had already been shown to be toxic to ducklings, causing four out of four deaths when fed to 1-day old Pekin ducklings, over a period of 14 days (Marasas et al., 1979).

Lyophilized conidia of each *Fusarium* isolate was suspended in sterile H_2O and used to inoculate yellow maize kernels (400 g) in H_2O (400 ml), in autoclaved glass jars. The cultures were incubated in the dark at 25°C for 21 days, following which they were dried at 45°C (for 12 hours) and subsequently ground to a fine meal in a laboratory mill. The cultures were stored at 4°C prior to analyses.

Subsamples (10 g) of each isolate were analysed for the presence of FB_1 and FB_2 according to the HPLC method of Shephard et al. (1990).

4.2.2 *Feed samples*

4.2.2.1 *Samples collected from the United States of America*

Fourteen feed samples (10 of which were commercially prepared mixed feeds and the balance maize), were obtained over a 4-year period between 1983 to 1986. The feed samples originated from localities in the south-eastern regions of the USA, and were representative of feeds that had been consumed by horses that had subsequently developed equine LEM between 1 and 3 weeks following consumption of the feed. In each case, equine LEM had been confirmed as the cause of death, by a combination of clinical signs, gross pathology and histopathology (Wilson et al., 1990). The ten most toxic *F. moniliforme* strains, of over 100 isolated from the feed samples, were also analysed for FB_1, FB_2 and FB_3 production (Sydenham et al., 1992b). Toxicity

of the isolates was based on duckling tests (Marasas et al., 1979).

4.2.2.2 *Samples collected from Brazil and Argentina*

Twenty-one maize or maize-based mixed feeds were collected over a 5-year period between 1985 and 1990, from farms in the State of Paraná, Brazil. In each case, the feeds had been associated with outbreaks of confirmed and suspected mycotoxicoses in various animal species. Two samples associated with a confirmed field outbreak of equine LEM were also obtained from Argentina. Twenty-six *F. moniliforme* isolates from the 21 Brazilian feed samples were prepared as cited in section 4.2.1, and subsequently analysed for FB_1 and FB_2 according to the method of Shephard et al. (1990).

4.3 *RESULTS*

4.3.1 *Fumonisin production by Fusarium species*

The study involved the screening of 40 toxic *Fusarium* isolates that were representative of 27 taxa in 9 sections of the genus *Fusarium*, according to the classification system of Nelson et al. (1983). In addition, two recently described *Fusarium* species; *F. nygamai* (Burgess & Trimboli, 1986) and *F. napiforme* (Marasas et al., 1987), which have not yet been classified into sections, were also included in the study.

The origin of each isolate, the substrate from which it was isolated, the *Fusarium* taxon to which it is classified and the FB_1 and FB_2 levels produced, are presented in Table 4.1 for those species classified in sections Spicarioides, Sporotrichiella, Roseum, Arthrosporiella, Gibbosum, Elegans and Lateritium. Corresponding data for those species classified in sections Discolor and Liseola, together with recently described *Fusarium* species, are presented in Table 4.2.

Each of the isolates of *F. moniliforme* and *F. proliferatum*

Table 4.1 Fumonisin production by *Fusarium* species

Section, MRC No.[a] and species	Origin	Substrate	Fumon. conc. µg/g FB_1	FB_2
Spicarioides				
2801 *F. decemcellulare*	South Africa	Avocado	ND[b]	ND
Sprotrichiella				
43 *F. sporotrichioides*	France	Maize	ND	ND
3424 *F. poae*	South Africa	Barley	ND	ND
1577 *F. tricinctum*	United States	Ivy	ND	ND
Roseum				
3302 *F. avenaceum*	South Africa	Medicago	ND	ND
Arthrosporiella				
2905 *F. semitectum*	South Africa	Maize	ND	ND
4815 *F. camptoceras*	Costa Rica	Cocoa	ND	ND
3520 *F. camptoceras*	South Africa	Soil	ND	ND
Gibbosum				
2330 *F. equiseti*	United States	Cereal	ND	ND
4463 *F. equiseti*	Transkei	Soil	ND	ND
3311 *F. acuminatum*	South Africa	Medicago	ND	ND
4028 *F. acuminatum*	South Africa	Soil	ND	ND
3687 *F. scirpi*	Australia	Soil	ND	ND
3528 *F. longipes*	South Africa	Soil	ND	ND
4458 *F. longipes*	New Guinea	Soil	ND	ND
Elangans				
1492 *F. oxysporum*	South Africa	Groundnut	ND	ND
Lateritium				
1925 *F. lateritium*	Zimbabwe	Coffee	ND	ND

Table 4.2 Fumonisin production by *Fusarium* species

Section, MRC No.[a] and species	Origin	Substrate	Fumon. conc. µg/g FB_1	FB_2
Discolor				
3307 *F. sambucinum*	South Arica	Medicago	ND[b]	ND
4666 *F. sambucinum*	South Africa	Potato	ND	ND
1115 *F. graminearum*	Transkei	Maize	ND	ND
4917 *F. graminearum*	South Africa	Barley	ND	ND
5052 *F. graminearum*	South Africa	Wheat	ND	ND
3636 *F. reticulatum*	South Africa	Medicago	ND	ND
1386 *F. compactum*	South Africa	Groundnut	ND	ND
Liseola				
826 *F. moniliforme*	Transkei	Maize	7100	3000
1065 *F. moniliforme*	Transkei	Maize	85	10
4315 *F. moniliforme*	Transkei	Maize	2645	325
4317 *F. moniliforme*	Transkei	Maize	205	40
4318 *F. moniliforme*	Transkei	Maize	105	ND
4319 *F. moniliforme*	Transkei	Maize	180	40
4321 *F. moniliforme*	Transkei	Maize	1330	140
2059 *F. proliferatum*	South Africa	Sorghum	20	160
2301 *F. proliferatum*	United States	Maize	870	450
2302 *F. proliferatum*	United States	Maize	290	65
2383 *F. proliferatum*	Sierra Leone	Maize	660	200
1077 *F. subglutinans*	Transkei	Maize	ND	ND
3823 *F. anthophilum*	South Africa	Oats	ND	ND
Newly described species				
4033 *F. nygamai*	South Africa	Soil	605	530
4150 *F. nygamai*	Namibia	Millit	ND	ND
4134 *F. napiforme*	Namibia	Sorghum	ND	ND

Key to Tables 4.1 and 4.2: [a]MRC = Medical Research Council; [b]ND = not detected (<1 µg/g)

produced FB_1 at levels ranging from 20 to 7100 μg/g, with FB_2 being produced by each isolate, at levels ranging from 40 to 3000 μg/g (with the exception of MRC 4318). The only other identified fumonisin producing isolate was *F. nygamai* MRC 4033 which produced 605 μg/g and 530 μg/g FB_1 and FB_2, respectively (Thiel et al., 1991a).

4.3.2 *Fumonisin levels in feeds associated with confirmed outbreaks of equine LEM*

The 14 USA feed samples together with 13 of the 21 Brazilian, and 2 Argentinian feeds, associated with confirmed field outbreaks of equine LEM, were analysed for the presence of FB_1, FB_2 and in the case of 13 of the 14 USA samples, FB_3. The results of the analyses are presented in Table 4.3.

All of the samples, a substantial number of which were commercially prepared feeds, contained FB_1 at levels ranging between 0.2 to 38.5 μg/g, and 28 of the 29 samples also contained detectable levels of FB_2 at concentrations ranging from 0.1 to 12.6 μg/g. In a number of cases, limited quantities of the implicated feeds were available, and due to the lack of an analytically pure FB_3 standard, it was not possible to determine the levels of this latter toxin in each of the feeds. Levels of FB_3 were, however, monitored in 13 of 14 USA feeds at levels of between <0.05 to 2.65 μg/g.

The data also indicated that FB_1 is the major naturally occurring fumonisin analogue, accounting for between 49.6 to 100% of the total fumonisin concentrations (FB_1+FB_2+FB_3) monitored in the samples. Similarly FB_2 levels accounted for between 6.9 to 33.3%, and FB_3 between 2.7 and 18% of the total fumonisin levels recorded in the 29 and 14 samples, respectively.

4.3.3 *Fumonisin concentrations associated with field outbreaks of porcine pulmonary oedema (PPO)*

Only three of the 21 Brazilian feeds were associated with con-

Table 4.3 Fumonisin concentrations in animal feeds associated with field outbreaks of equine leukoencephalomalacia

Sample number	Source	Fumonisin conc. (μg/g)						
		FB_1	FB_2	FB_3	Total	$\%FB_1$	$\%FB_2$	$\%FB_3$
1	USA	4.6	1.1	0.15	5.85	78.5	18.8	2.7
2	USA	4.4	0.7	0.20	5.30	83.0	13.2	3.8
3	USA	3.7	0.6	0.15	4.45	83.7	13.6	2.7
4	USA	8.0	4.1	2.65	14.75	54.2	27.8	18.0
5	USA	6.0	2.4	0.75	9.15	65.7	26.3	8.0
6	USA	5.8	1.6	0.25	7.65	75.6	20.9	3.5
7	USA	4.5	1.0	0.15	5.65	79.5	17.7	2.8
8	USA	1.3	0.1	0.50	1.45	89.6	6.9	3.5
9	USA	9.6	3.1	0.30	13.00	73.9	23.9	2.2
10	USA	19.8	6.5	0.70	27.00	73.4	24.1	2.5
11	USA	7.1	6.3	0.90	14.30	49.6	44.0	6.4
12	USA	6.2	1.3	0.50	8.00	77.6	16.3	6.1
13	USA	3.2	0.5	ND[a]	3.70	86.5	13.5	-
14	USA	27.0	12.6	NA[b]	39.60	68.2	31.8	-
15	Brazil	24.2	8.3	NA	32.50	74.5	25.5	-
16	Brazil	38.5	12.0	NA	50.50	76.2	23.8	-
17	Brazil	20.8	6.6	NA	27.40	75.9	24.1	-
18	Brazil	0.6	0.1	NA	0.70	85.7	14.3	-
19	Brazil	2.6	0.7	NA	3.30	78.8	12.2	-
20	Brazil	4.5	0.8	NA	5.30	84.9	15.1	-
21	Brazil	19.2	8.0	NA	27.20	70.6	29.4	-
22	Brazil	7.4	3.7	NA	11.10	66.7	33.3	-
23	Brazil	0.8	0.2	NA	1.00	80.0	20.0	-
24	Brazil	22.9	7.9	NA	30.80	74.4	25.6	-
25	Brazil	0.9	0.1	NA	1.00	90.0	10.0	-
26	Brazil	1.9	0.3	NA	2.20	86.4	13.6	-
27	Brazil	200	ND	NA	200	100.0	-	-
28	Argentina	28.7	9.3	2.1	40.10	71.6	23.2	5.2
29	Argentina	4.4	1.2	0.4	6.00	73.5	20.0	6.5

Key to Table 4.3 [a]**ND = not detected (<0.05 μg/g);** [b]**NA = not analysed.**

firmed outbreaks of PPO, and the results of the fumonisin analyses are presented in Table 4.4.

Table 4.4 Fumonisin concentrations in Brazilian feeds associated with confirmed field outbreaks of PPO

Sample number	Fumonisin concentration (μg/g)	
	FB_1	FB_2
1	8.5	1.9
2	11.1	3.2
3	10.4	3.2
Mean	10.0	2.8

The FB_1 and FB_2 levels determined in the three samples fell within a relatively narrow range, with mean concentrations of 10 and 2.8 μg/g, FB_1 and FB_2 respectively, being recorded.

4.3.4 *Fumonisin concentrations in feeds associated with other field outbreaks of suspected animal mycotoxicoses*

The remaining five Brazilian feeds were associated with outbreaks of suspected mycotoxicoses in different animal species. The relevent data together with the FB_1 and FB_2 levels recorded in the five feeds are listed in Table 4.5.

Table 4.5 Fumonisin concentrations in Brazilian feeds associated with outbreaks of suspected animal mycotoxicoses

Animal	Clinical signs and/or disease syndrome	Fumon. concn. (ng/g) FB_1	FB_2
Piglets	elevated temperature	0.2	ND[a]
Pigs	estrogenic effects	7.0	1.5
Horses	unconfirmed early LEM	0.6	0.1
Rabbits	nervous signs/feed refusal	ND	ND
Chickens	diarrhoea	5.1	1.2

[a]ND = not detected (< 0.05 μg/g)

4.3.5 *Fumonisin production by cultures of F. moniliforme isolated from feeds associated with field outbreaks of confirmed and suspected animal mycotoxicoses*

A total of 36 *F. moniliforme* isolates, collected from the feed samples associated with field outbreaks of animal mycotoxicoses in Brazil and the USA were analysed for the production of FB_1 and FB_2, and the results are presented in Tables 4.6 and 4.7, respectively.

The FB_1 concentrations determined in the *F. moniliforme* cultures isolated from the implicated Brazilian feed samples ranged from 65 to 4420 μg/g, while FB_2 concentrations ranged between 5 and 1380 μg/g. Combined fumonisin levels ranged from 70 to 5880 μg/g, with the FB_1 levels accounting for between 60 and 93% of the combined fumonisin levels (Table 4.6). Similarly, each of the *F. moniliforme* cultures isolated from the USA feeds produced FB_1 at levels ranging from 160 to 3800 μg/g, and FB_2 at levels ranging from 20 to 950 μg/g. Total fumonisin levels were found to range from 180 to 4690 μg/g, and the FB_1 levels corresponded to between 80 and 90% of the total fumonisins levels (Table 4.7).

Table 4.6 Fumonisin B_1 and B_2 levels produced by cultures of *F. moniliforme* isolated from feed samples associated with field outbreaks of animal mycotoxicoses in Brazil

	Fumonisin concentration (μg/g)		
Isolate number[a]	FB_1	FB_2	Total[b]
MRC 6063	1140	350	1490
MRC 6064	1320	430	1750
MRC 6065	2260	520	2780
MRC 6066	3660	970	4630
MRC 6073	65	5	70
MRC 6076	1040	180	1220
MRC 6078	530	120	650
MRC 6079	720	210	930
MRC 6082	640	80	720
MRC 6084	1490	980	2470
MRC 6086	4420	1380	5800
MRC 6087	3650	910	4560
MRC 6088	1220	230	1450
MRC 6159	1635	290	1925
MRC 6160	285	100	385
MRC 6161	2280	710	2990
MRC 6163	2230	540	2770
MRC 6164	3380	825	4205
MRC 6165	625	200	825
MRC 6166	910	160	1070
MRC 6167	270	40	310
MRC 6168	1385	200	1585
MRC 6169	1980	260	2240
MRC 6170	690	65	755
MRC 6171	1795	290	2085
MRC 6173	1570	280	1850

[a]Accession number of the isolate held in the culture collection of the Medical Research Council

[b]Total= Combined FB_1 and FB_2 concentrations

Table 4.7 Fumonisin B_1 and B_2 levels produced by cultures of *F. moniliforme* isolated from feed samples associated with field outbreaks of animal mycotoxicoses in the USA

	Fumonisin concentration (μg/g)		
Isolate number[a]	FB_1	FB_2	Total[b]
MRC 4056	1200	150	1350
MRC 4057	1120	170	1290
MRC 4058	1150	150	1300
MRC 4059	470	20	490
MRC 4060	3710	950	4660
MRC 4061	3800	890	4690
MRC 4062	3290	540	3830
MRC 4063	520	60	580
MRC 4064	160	20	180
MRC 4065	270	40	310

[a]Accession number of the isolate held in the culture collection at the Medical Research Council

[b]Total = combined FB_1 and FB_2 concentrations

4.4 *DISCUSSION*

4.4.1 *Fumonisin production by Fusarium species*

The data presented in Tables 4.1 and 4.2 indicate that the isolates were collected from various countries, and from a variety of substrates. The results of the analyses indicated that, with the exception of one isolate of *F. nygamai* (MRC 4033 - Table 4.2), fumonisin production was restricted to two species classified in the section Liseola. With the exception of *F. moniliforme* MRC 4318 (which produced only FB_1), all other *F. moniliforme* and *F. proliferatum* isolates produced both major fumonisin toxins (Table 4.2). The highest producer of both FB_1 (7100 μg/g) and FB_2 (3000 μg/g) was *F. monili-*

forme MRC 826, the strain from which the fumonisins were originally isolated (Gelderblom et al., 1988a) and characterised (Bezuidenhout et al., 1988).

The observation that each of the *F. proliferatum* isolates included in the study were found to produce the fumonisins, substantiates the reports by Ross et al. (1992), and more recently Nelson et al. (1992). One isolate each, of two other *Fusarium* species classified within the section Liseola *(F. subglutinans* MRC 1077 and *F. anthophilum* MRC 3823), did not produce detectable levels of either FB_1 or FB_2 (ie <1 μg/g). Nelson et al. (1992) also observed the non-production of fumonisins by 23 isolates of *F. graminearum*, but did detect relatively low levels of FB_1 (<600 μg/g) produced by 3 of 17 isolates of *F. anthophilum*. The fact that toxin production may be restricted to selected strains of fungal species, illustrates the need to screen numerous strains in order to be able to confirm the production of a toxin by fungal species.

In addition to the two fumonisin-producing species within the section Liseola (identified in the present study), only one other isolate (*F. nygamai* MRC 4003) was found to produce detectable levels of FB_1 (605 μg/g) and FB_2 (530 μg/g) (Table 4.2). The identities of FB_1 and FB_2 produced by *F. nygamai* were confirmed by base hydrolysis of purified sample extracts, followed either by derivatisation with OPA and HPLC analysis (according to the method in section 3.5) or by acylation with TFAI and GC-MS analysis (see section 3.5). The mass spectra obtained for the aminotetrol and aminopentol moieties of FB_2 and FB_1, respectively, during the confirmation process are illustrated in Figure 3.6.

Fusarium nygamai is one of a series of recently described species (Burgess & Trimboli, 1986). It is morphologically related to those of the section Liseola, but has thus far been excluded from categorisation within the section, due to the production of chlamydospores. The strain *F. nygamai* MRC 4003 is one of the authentic isolates cited in the original description of the species (Burgess & Trimboli, 1986). Nelson et al.

(1992) confirmed the production of fumonisins by 10 of 27 isolates of *F. nygamai*.

Other recently described *Fusarium* species that have morphological similarities with those of the section Liseola, but which have also been excluded from the section include *F. dlamini* (Marasas et al., 1985), *F. napiforme* (Marasas et al., 1987) and *F. beomiforme* (Nelson et al., 1987). Of these three additional species, the two former (*F. dlamini* and *F. napiforme*) have also been shown to produce the fumonisins in 5/9 and 5/33 isolates tested, respectively (Nelson et al., 1992).

These data have therefore identified 6 fumonisin-producing *Fusarium* species, either classified in, or closely related to those of the section Liseola. Of these 6 *Fusarium* species, there is little doubt that *F. moniliforme* and *F. proliferatum* are the most significant, since they constitute the major fungal contaminants of maize worldwide (Marasas et al., 1984b), which is an important staple cereal for both animal and human consumption. Two other identified producers (*F. napiforme* and *F. nygamai*) may ultimately prove to be of importance, as they are known fungal contaminants of other staple cereals such as millet and sorghum (Nelson et al., 1992). Conversely, *F. anthophilum* and *F. dlamini* should be considered to be of minor importance, since they appear to have a limited geographical distribution and are not considered to be associated with the widespread contamination of grain commodities (Nelson et al., 1992).

Recent data by Chen et al. (1992) has however indicated that fumonisin production may not only be restricted to those species of the genus *Fusarium*. The presence of FB_1 was observed in liquid cultures of an isolate of *Alternaria alternata*, a host-specific pathogen of tomato plants which had previously been shown to produce a group of toxins (the AAL toxins) (Siler & Gilchrist, 1982), that are structurally similar to the fumonisins (Chen et al., 1992). Extensive studies have been undertaken at PROMEC, on the *A. alternata* strain reported by

Chen et al. (1992) to produce FB_1. However, these observations and others (Miller, 1992b) have failed to confirm the findings of Chen et al. (1992).

4.4.2 *Fumonisin levels: feeds associated with equine LEM*

Marasas et al. (1988b) experimentally reproduced LEM in a horse following the *intravenous* (iv) administration, over a seven day period, of 0.125 mg of the pure toxin/kg body mass/day. Clinical signs of the syndrome, which included nervousness followed by apathy, trembling, ataxia, reluctance to move, paralysis of the lower lip and an inability to eat or drink, began to appear on the eighth day. By the tenth day the animal, which by then was in a tetanic convulsion, was euthanased. The principal pathological lesions included severe oedema of the brain and early necrosis in the medulla oblongata (Marasas et al., 1988b). Kellerman et al. (1990) subsequently proved conclusively that FB_1 caused equine LEM, following studies involving the oral adminisration of the toxin to two horses. The first, a filly, received 21 doses of between 1.25 to 4.0 mg/kg body mass/day over a period of 33 days, whilst the second, a colt, received 20 doses of between 1.0 to 4.0 mg/kg body mass/day over 29 days. In each case, the experimental animals developed clinical signs, previously associated with the onset of equine LEM. Following euthanasia, characteristic necrotic lesions of LEM were observed in the brains of both test animals.

Fumonisin B_1 has therefore been identified as a causative factor in the development of equine LEM. The data presented in Table 4.3, would also indicate that FB_1 (and other fumonisin analogues) are present in feeds implicated with the onset of equine LEM. The range and mean FB_1 and FB_2 concentrations determined in the equine LEM-implicated feeds from Argentina, Brazil and the USA (Table 4.3) are compared in Table 4.8, with other published data obtained for feeds also implicated with confirmed outbreaks of equine LEM. Of the 110 implicated feeds, the mean combined concentrations of FB_1 and FB_2 have in general (but with the exception of 3 samples from the USA - Wilson et

al., 1990) been found to fall within a relatively narrow range of between 10 and 30 μg/g (10 - 30 ppm). Thus far, FB_3 levels have only been reported for selected animal feeds obtained from Argentina and the USA (Table 4.3), where mean concentrations of 1.25 and 0.5 μg/g were recorded, respectively.

Wilson et al. (1992) performed studies aimed at determining the minimum toxic dose of FB_1 necessary to precipitate equine LEM.

Table 4.8 Comparison of the range and mean FB_1 and FB_2 levels determined in feeds (Table 4.3) with similar reports concerning confirmed outbreaks of equine LEM[a]

Country	Number of samples	Fumonisin levels (μg/g)	
		FB_1	FB_2
Argentina	2	4.4 - 28.7 (16.6)	1.2 - 9.3 (5.9)
Brazil	13	0.2 - 38.5 (12.0)	<0.1 - 12.0 (4.1)
South Africa[b]	1	(8.9)	(3.0)
USA	14	1.3 - 27.0 (7.7)	2.0 - 12.6 (3.1)
USA[c]	3	37.0 - 122.0 (72.0)	2.0 - 23.0 (12.0)
USA[d]	77	2.0 - 126.0 (30.9)	NA[e]

[a]Mean levels given in brackets; [b]Published by Shephard et al. (1990); [c]Published by Wilson et al. (1990); [d]Published by Ross et al. (1991a,b, 1992); [e]NA= not analysed

They concluded that ponies fed diets containing 8 ppm FB_1, over a period of 180 days showed mild clinical signs of LEM. Following euthanasia, all experimental animals showed evidence of mild histopathological brain lesions associated with the onset of equine LEM (Wilson et al., 1992). The studies were based on the administration of FB_1, and so excluded the contribution to the development of equine LEM that could have been precipitated by the presence of other fumonisin toxins (Wilson et al., 1992). Recent studies (Nelson, 1993) have illustrated the induction of equine LEM following ingestion of culture material of *F. proliferatum* that produced only FB_2, suggesting that the second most predominant naturally occurring fumonisin toxin is also a contributory factor in the development of the syndrome. No data yet exist concerning the contribution that could be made by the natural occurrence of FB_3, although FB_3 and FB_2 have both been shown to exhibit cancer-initiating potentials, in the rat liver bioassay, similar to those observed for FB_1 (Gelderblom et al., 1992).

The data of Wilson et al. (1990) and others (Marasas et al., 1988b; Kellerman et al., 1990), would appear to indicate that feeds contaminated with the fumonisin toxins at 8 ppm and higher, should be considered to be possibly harmful to equine species. The concentration of FB_1 used by Wilson et al. (1992) (8 ppm) is close to the lower mean fumonisin levels generally observed in feeds associated with confirmed field outbreaks of equine LEM (10 ppm - Table 4.8). Within the limitations associated with errors emanating from the sampling of the implicated feeds, the exposure and toxicological assessments would appear to complement one another. The data therefore indicate that feeds contaminated with combined fumonisin concentrations of approximately 10 µg/g (10 ppm) should be considered to be possibly harmful to animals of the equine species.

4.4.3 *Fumonisin levels: feeds associated with PPO*

Harrison et al. (1990) observed pulmonary oedema and hydrothorax in pigs that died after consuming maize screenings heavily

contaminated with *F. moniliforme*. The syndrome was reproduced experimentally following the iv administration of 0.4 mg FB_1/kg body mass/day over a period of four days.

The fumonisin levels recorded for the Brazilian feed samples associated with field outbreaks of PPO (Table 4.4) are similar to those recorded for the feeds associated with LEM (Table 4.3). However, these levels differ significantly from the bulk of the data concerning similarly implicated feeds reported by American researchers, which are summarised in Table 4.9.

Table 4.9 Range and mean fumonisin concentrations in feeds associated with confirmed outbreaks of PPO in the USA

No of samples	Fumonisin concn. (µg/g)		Reference
	FB_1	FB_2	
4	92.0 - 166.0 (129.0)	4.8 - 28,0 (16.4)	Harrison et al., (1990) Haschek et al., (1992)
70	2.0 - 330.0 (65.0)	NA[a]	Ross et al., (1991a,b) Ross et al., (1992)
29	3.0 - 330.0 (75.0)	NA	Osweiler et al., (1992)

[a]NA = not analysed

The bulk of the USA implicated feeds were analysed for the presence of FB_1, and concentrations of between 2 and 330 µg/g have been recorded (2 - 330 ppm - Table 4.9). Mean concentrations were found to range from 65 and 129 µg/g (65 - 129 ppm). Only 4 samples were analysed for the presence of FB_2, with lower mean levels of around 16.4 ppm being recorded. Comparison of the data presented in Tables 4.4 and 4.9 would

indicate that the mean combined fumonisin levels, recorded in the 3 Brazilian feeds, were approximately between 5 to 10 fold lower than those recorded for the more than 100 corresponding USA samples.

The original report of an outbreak of PPO in the USA (Harrison et al. 1990) involved the consumption of "*corn screenings*" (maize fines) by pigs on two separate farms. Fumonisin B_1 levels in the two implicated feeds were found to be 105 and 155 µg/g (ppm), respectively. The lowest level of fumonisin necessary for the induction of PPO has not yet been assessed. However, based primarily on the evidence from American researchers, it seems reasonable to assume that swine exhibit a higher fumonisin tolerence level than do members of the equidae family. The evidence further suggests that additional work is required concerning the levels that may induce PPO. Essentially however, based on the levels observed in the implicated feeds, it is proposed that feeds containing combined fumonisin concentrations of 100 ppm or greater should be considered to be potentially harmful to pigs.

4.4.4 *Fumonisin levels: other outbreaks of suspected animal mycotoxicoses*

A low level of FB_1 was recorded in the feed consumed by young piglets under the age of 70 days, causing the deaths of 15 animals. The only clinical signs observed in the animals were an elevation in temperature. Casteel et al. (1993) monitored the effects of FB_1 in rations fed to 5 week-old weanling pigs. The levels that induced nodular hyperplasia in the livers of the test animals corresponded to between 100 - 190 µg/g FB_1 in the diets. These levels were significantly higher than those found in the implicated Brazilian feeds. It is doubtful that, provided the implicated feed sample was representative of that consumed by the affected piglets, the very low levels of FB_1 would have precipitated early signs of PPO. It is therefore impossible, based on the information available, to ascribe the observed symptoms to a conclusive outbreak of mycotoxicoses.

A second sample associated with an outbreak of suspected mycotoxicoses in pigs contained both FB_1 and FB_2, at levels of 7.0 µg/g and 1.5 µg/g, respectively. Consumption of the implicated feeds did not result in any deaths, and the principal clinical signs were estrogenic in nature. Such effects have previously been correlated with the presence of ZEA, which is not normally regarded to be a mycotoxin produced by *F. moniliforme* (the dominant fungus isolated from the implicated feed - Sydenham et al., 1992a). It has however been suggested that a group of plant growth promoters, the "*gibberellins*" (that have been reported to be produced by *F. moniliforme*), can induce estrogenic effects in pigs (Marasas et al., 1984b). It is also worthy to note that the implicated feed sample, that was contaminated with combined fumonisin levels close to those determined in the PPO-implicated feeds from Brazil (Table 4.4), did not induce any symptoms of PPO. This observation further calls into question the validity of the feed samples submitted as being representative of those consumed by the animals that exhibited confirmed symptoms of PPO.

The three remaining Brazilian feed samples were associated with an unconfirmed, though suspected outbreak of equine LEM as well as suspected outbreaks of mycotoxicoses in rabbits and chickens (Table 4.7). Based on the analysis, it is unlikely that the fumonisin levels present would have precipitated early signs of equine LEM, while no FB_1 or FB_2 were detected in the feed sample associated with the nervous signs and feed refusal symptoms observed in the rabbits. One must therefore conclude that the toxic principle(s) responsible for the clinical signs observed in the rabbits is as yet unknown.

The disease that affected the chickens was characterised by diarrhoea and hemorrhagic lesions in the gastrointestinal tract. Ledoux et al. (1992) monitored the effects in young broiler chicks fed diets containing up to 400 µg/g FB_1, for 21 days. They observed dramatic decreases in body weight in those animals fed higher doses of FB_1. Diarrhoea, hepatic necrosis, biliary hyperplasia and rickets were present in those animals fed FB_1, indicating that it is toxic to young chicks (Ledoux et

al., 1992). In an additional study, Weibking et al. (1993) monitored the effects of dietary FB_1 in young turkey poults. Day-old poults were fed diets containing between 0 and 200 mg/kg FB_1, which was consumed for 21 days. Decreases in feed conversion were found to be linear with increasing dietary FB_1, while target organ weights tended to decrease. The authors summarised that turkey poults appeared to be more sensitive to FB_1 than broiler chicks (Weibking et al., 1993). It is therefore possible that some, if not all, of the observed symptoms in the chickens (Table 4.5) could have been induced by the presence of the fumonisins. However, it is apparent that further research is necessary in order to more fully evaluate the role, if any, that the fumonisins may play in the mycotoxicoses of different animal species.

4.4.5 *Fumonisin levels: production of fumonisins by fungal cultures isolated from implicated feeds*

It is a common practice in many mycotoxicological laboratories, to determine the specific toxin-producing ability of fungal cultures, isolated from foods and/or feeds associated with field outbreaks of mycotoxicoses. Ten and 26 *F. moniliforme* strains, isolated from the USA and Brazilian implicated feeds, respectively, were tested for toxicity to ducklings, and analysed for their ability to produce the fumonisin toxins. The results of these analyses are compiled in Tables 4.6 and 4.7.

It is perhaps significant that each of the *F. moniliforme* cultures isolated from the USA and Brazilian feeds had the ability to produce both FB_1 and FB_2, although conflicting results with respect to toxicity to ducklings, for the individual fungal strains of *F. moniliforme* were observed (Thiel et., 1991b, Sydenham et al., 1992a). However it is important to understand that the concentrations of the fumonisin mycotoxins, produced by the individual isolates under culture conditions, bears no reflection to the fumonisin producing ability of those same isolates under natural conditions. This was demonstrated by the fact that, of the 26 Brazilian isolates, the 3 highest fumonisin producers were isolated from feeds that were natu-

rally contaminated with relatively low levels of the toxins (Sydenham et al., 1992a). Therefore, studies concerning fumonisin production by fungal cultures should be used to demonstrate the overall ability of a fungal species to produce the toxins. Conversely, the data should not be presented as evidence to suggest that laboratory-based studies are representative of the situation that occurs in nature.

Preliminary studies concerning the fumonisin-producing ability of *F. moniliforme* strains, isolated from feeds and foods have also been carried out in Spain (Sala et al., 1993; Cabanes, et al., 1993) and France (Le Bars et al., 1993). The results substantiated that significant numbers of the isolates tested produced the fumonisins under culture conditions.

4.5 *OTHER TOXICOLOGICAL STUDIES INVOLVING FUMONISIN B_1*

Gelderblom et al. (1991a) fortified a maize-based diet with 50 µg/g purified FB_1 (± 90% pure). The diet was fed to 25 rats over a period of 26 months, with a control group receiving the diet without the added toxin. Five rats in each group were euthanased after 6, 12, 20 and 26 months of exposure. The liver appeared to be the major target organ in the FB_1-treated rats while the hepatic pathological changes were identical to those previously observed in rats fed diets containing culture material of *F. moniliforme* (Gelderblom et al., 1991a). Primary hepatocellular carcinoma was observed in >60% of animals that survived for 18 months or longer, and metastases to the heart, lungs or kidneys was observed in four of the test animals (Gelderblom et al., 1991a). The authors concluded that FB_1 was responsible for the hepatotoxic and hepatocarcinogenic effects in rats, previously ascribed to *F. moniliforme* strain MRC 826.

Osweiler et al. (1993) monitored feed consumption, weight gain and several serum chemistry profiles in feeder calves fed diets containing combined fumonisin (FB_1, FB_2 and FB_3) levels ranging from 15 to 148 µg/g over a period of 31 days. Mild liver

lesions were observed in two calves consuming the highest concentration of fumonisins, but overall, only serum chemistry profiles were significantly altered. The authors concluded that cattle appeared to be less susceptible to the fumonisins than other species (ie. horses and pigs) (Osweiler et al., 1993).

Sphingolipids (which are structurally similar to the fumonisins) are highly bioactive molecules that are thought to affect cell growth, differentiation and behaviour (Merrill, 1991). Fumonisin B_1 has been shown to inhibit sphingolipid biosynthesis in rat liver hepatocytes (Wang et al., 1991), and the administration of feed containing between 15 and 44 $\mu g/g$ FB_1 to ponies was recently found to significantly disrupt sphingolipid metabolism (Wang et al., 1992). Alterations in tissue and serum sphingolipid profiles have also been monitored in pigs (Riley et al., 1993) fed fumonisin-containing feed. Clearly, the alteration of sphingolipid profiles might be the initial molecular target of the fumonisin mycotoxins (Norred et al., 1992).

Initial studies aimed at the determination of the toxicokinetics of FB_1 in rats have indicated that it is rapidly absorbed in plasma, exhibiting a half-life of approximately 18 minutes (Shephard et al., 1992a). Both "*intraperitoneal*" (ip) and "*per os*" (po) dosing of ^{14}C-labelled FB_1 resulted in the observation of relatively low levels in the urine of animals, with the bulk of the toxin being excreted in the faeces (Shephard et al., 1992b).

4.6 *SUMMARY*

The data presented in this chapter presents evidence for, and quantitative estimations of, the extent of animal exposure to the fumonisin toxins. It is concluded that several *Fusarium* species have the ability to produce the fumonisins, that could potentially harm animals if ingested. Evaluation of the toxicological evidence and assessment of the extent of fumonisin exposure to various animal species, indicate that horses

and pigs are particularly susceptible to diseases associated with the ingestion of fumonisin-contaminated feeds. It is also possible that poultry may be susceptible to fumonisin levels, that occur under natural conditions. It is clear, however, that further evaluation is required, both on the toxicological and exposure assessments of the role that the fumonisins may play in animal health problems. It is recommended that combined fumonisin levels of 10 μg/g (ppm) and 100 μg/g (ppm) should be considered to be potentially dangerous to horses and pigs, respectively. Further work will undoubtedly assist in the optimum utilization of fumonisin-contaminated animal feeds, while preventing exposure of particularly susceptible species to the harmful consequences of the fumonisin toxins.

CHAPTER 5: FUMONISINS - IMPLICATIONS FOR HUMAN HEALTH

5.1 *INTRODUCTION*

Maize constitutes one of the major dietary grain crops consumed by both animals and humans worldwide. In Chapter 4, contamination of maize with *F. moniliforme* was shown to be responsible for various animal disease syndromes (ie. equine LEM and PPO). Indeed, in the case of equine LEM, *F. moniliforme* had been identified as the causative agent as far back as 1978 (Bridges, 1978; Buck et al., 1979). Human consumption of maize contaminated with *F. moniliforme* has not been scientifically established as the causative factor responsible for any human disease or syndrome. A partial exception has, however, been the statistical association between the incidence of contamination of home-grown maize with *F. moniliforme* and human oesophageal cancer (OC) risk.

5.1.1 *Human oesophageal cancer (OC)*

Cancer of the oesophagus was, until a few decades ago, a relatively rare disease of the South African population. However a dramatic increase in human OC has been monitored in the Transkei, since 1950 (Rose, 1973).

Transkei is situated on the eastern (Indian ocean) coast of southern Africa (Figure 5.1). The geographical distribution, and relationship of human OC to other neoplasms has been monitored in Transkei, and other parts of Southern Africa (Rose, 1973; Rose & McGlashan, 1975; Rose & Fellingham, 1981; McGlashan, 1977, 1981; Kibblewhite et al., 1984; Jaskiewicz et al., 1987a; Sumeruk, 1992). These studies have indicated that the incidence of human OC has increased, and is at present the commonest cancer in black males in many parts of South Africa, especially in the Transkei (Rose & Fellingham, 1981; Van Rensburg et al., 1983; Jaskiewicz et al., 1987a).

Figure 5.1 Map of Transkei showing the low human OC districts of Bizana and Lusikisiki, and the high human OC districts of Butterworth and Kentani

During the first major epidemiological survey period (carried out in Transkei between 1955 and 1969 - Rose, 1973), human OC rates ranged from 50 to 116 per 100,000 per annum for females and males, respectively in the south-western districts of Butterworth and Kentani, to only 2 and 3 per 100,000, respectively, in the north-eastern district of Bizana (Figure 5.1). Later extensive surveys (Rose & McGlashan, 1975; Rose & Fellingham, 1981) confirmed these initial findings.

Similar trends in human OC incidence have since been reported in the Caspian littoral of Iran (Mahboubi et al., 1973), and in China (Yang, 1980), where the major high-risk areas are located in Henan, Hebei and Shanxi provinces, with Linxian County in Henan being the highest OC risk area (Hsia, 1983, 1984).

5.1.2 *Etiological factors associated with human OC*

In epidemiological investigations conducted in developed countries, major emphasis has predominantly been placed on the role

of both alcohol and tobacco, in the etiology of human OC (Tuyns, 1982). Nutritional factors have also been considered in some of these studies (Ziegler et al., 1981; Tuyns et al., 1987). However in several developing countries, where the prevalence of human OC is high (ie. China, central Asia and northern Iran), the use of alcohol and/or tobacco products are restricted, and hence their association with OC is to a large extent less convincing than it might appear to be in developed countries (Jaskiewicz et al., 1987a). Epidemiological studies in some of the developing countries that exhibit high OC risk, have identified low socio-economic status, poor agricultural conditions, nutritional deficiencies and high single cereal diets of either wheat or maize, as potential OC risk factors (Groenewald et al., 1981; Kmet et al., 1981; Kolycheva, 1980; Van Rensburg, 1981).

Specific nutritional deficiencies that have been identified include those of vitamins A and C (Van Rensburg et al., 1983; Richter et al., 1984; Turham et al., 1985; Yang et al., 1982). Jaskiewicz et al. (1988) used an oesophageal brush biopsy technique to establish a relationship between oesophageal cytological changes (including oesophgitis, signs of folic acid deficiency, dysplasia and cancer) and blood micronutrients (vitamins A, E, B_{12} and folic acid), in patients from high human OC risk areas in Transkei. Cytological abnormalities and blood micronutrient deficiencies were less prevalent in those patients from low-OC risk areas. Dietary habits revealed that while maize formed the dietary staple in both areas, intakes of vegetables, fruits and animal-proteins were lower in people from the high risk areas (Jaskiewicz et al., 1988) . In a follow-up study Jaskiewicz (1989) reported that mild oesophageal lesions were inversely related to the frequency of the intake of vegetables, fruits and animal-proteins, but directly related to alcohol and tobacco usage. In addition, low blood selenium levels were correlated with the degree of cytological abnormalities.

Alcohol consumption in South Africa has also been identified as a potential human OC risk factor, but unlike western studies,

the alcohol consumed has been home-brewed beer prepared predominantly from maize (Segal et al., 1988). Additional studies by McGlashan et al. (1982) and Sagar & Ellwood (1987) have tended to confirm this supposition. Morton (1991) disputed the suggestion that the preparation and consumption of maize-beer was a potential OC risk factor, citing that dark sorghum is the dominant cereal used for the preparation of native beer. While sorghum is indeed used in the preparation of a local beer in South Africa, maize is the dietary staple grown, consumed and used in the Transkei for beer-production. Conversely, a recent epidemiological survey by Sammon (1992) claimed that the consumption of traditional beer was not a risk factor. It would, therefore, appear that the role of maize-based home-brewed beer in the etiology of human OC cancer, in Transkei and other developing countries, requires additional investigation.

Home-grown maize constitutes the major human dietary (and nutrient source) staple food in Transkei, and other parts of South Africa. Home-grown maize in both high and low human OC risk areas of the Transkei is normally separated into "healthy" and "mouldy" fractions, which are then prepared for human consumption either as a porridge or a traditionally brewed beer, respectively (Sydenham et al., 1990b). Mycological surveys have consistently shown that the prevalence of *F. moniliforme* in home-grown maize kernels is correlated with human OC rate in Transkei (Marasas, 1982; Marasas et al., 1979 & 1981). In a survey conducted in Transkei during 1985 and 1986, Marasas et al. (1988a) again confirmed previous observations with respect to the significantly higher prevalence of *F. moniliforme*-contamination of maize from high OC rate areas. In addition, individuals living in both high and low risk areas were also screened cytologically, by means of oesophageal brush biopsy, and the results provided evidence for an association between the prevalence of *F. moniliforme* in home-grown maize, and cytological abnormalities in living individuals (Marasas et al., 1988a).

In China, *F. moniliforme* has also been identified as the major fungal contaminant of maize collected in the high OC risk

areas (Li et al., 1980; Yang, 1980; Zhen, 1984), where the maize is normally consumed as a steamed cake known as *"wotou"* (Zhu & Jeffrey, 1992).

5.1.3 Additional evidence concerning the role of *F. moniliforme* in human OC

There is no experimental proof of a causative relationship between OC and the consumption of *F. moniliforme*-contaminated maize. Fungal cultures of *F. moniliforme* have, however, been shown to be hepatocarcinogenic to rats (Marasas et al., 1984a) and to cause a significant enhancement of (site-specific) nitrosamine-induced OC in rats. Papilloma and carcinoma of the forestomach, and hyperplasia of the epithelium of the oesophagus and glandular stomach, have been induced in rats with maize-bread inoculated with a strain of *F. moniliforme* isolated from foodstuffs obtained from a high OC risk area of China (Li et al., 1980, 1982). Four nitrosamines have been detected in maize-bread, inoculated with Chinese isolates of *F. moniliforme*, in the presence of sodium nitrite (Lu et al., 1979 & 1980). One of these, N-methyl-N-benzylnitrosamine, is a known site-specific oesophageal carcinogen in rats (Lu et al., 1980). These results perhaps suggest that carcinogenic metabolites of *F. moniliforme*, and nitrosamines in mouldy maize, may act synergistically to cause cancer of the forestomach in rats (Li et al., 1980, 1982).

5.2 *CHAPTER OBJECTIVES*

With the isolation of the fumonisins, and the realisation that they may act both as cancer-initiating and -promoting agents, the aim of this part of the study is to determine the extent to which human population groups may be exposed to the fumonisin toxins, through the consumption of maize or maize-based foodstuffs. Data of this type is essential for overall risk-assessment studies involving the fumonisins.

5.3 *EXPERIMENTAL*

5.3.1 *Commercial maize production in South Africa*

Subsamples (500 g each) of 372 and 363 samples of commercial maize harvested during 1989 and 1990, respectively, were collected by personnel of the South African Maize Board (SAMB) and submitted to the PROMEC laboratories, where they were stored at 4°C prior to analyses. The samples comprised of varieties of both white (W) and yellow (Y) maize of three grades, which were coded W1, W2, W3, and Y1, Y2, Y3, respectively. The maize samples were selected from the five most important maize production areas in South Africa (ie. Northern Orange Free State, Eastern Orange Free State, Natal, Western Transvaal and Eastern Transvaal). Random samples were selected for mycological investigation and fumonisin analysis (FB_1 and

Table 5.1 Sampling and analysis design for South African commercial maize samples of the 1989 and 1990 crops

Maize grade	Fumonisins B_1, B_2 (1989)		Fumonsins B_1, B_2, B_3 (1990)	
	Samples/Area	Total No	Samples/Area	Total No
W1	5	25	5[a]	23
W2	5	25	5[a]	23
W3	All samples	18	All samples	20
Total (W)		**68**		**66**
Y1	5	25	5	25
Y2	5	25	5[a]	22
Y3	All samples	3	All samples	15
Total (Y)		**53**		**62**
Total (W + Y)		**121**		**128**

[a]Not possible to obtain 5 samples/area

FB_2 only during 1989, with the addition of FB_3 during 1990), in accordance with the sampling design presented in Table 5.1.

5.3.2 *Argentinian field-trial maize samples*

During February 1991, field trials were conducted in the "9 de Julio" and "Pergamino" districts of the Province of Buenos Aires, Argentina, using commercial and experimental maize hybrids. At harvest, randomized maize samples were withdrawn from the trials, and subsamples (n = 17) of approximately 1 kg each, were submitted to the PROMEC laboratories where they were stored at 4°C prior to analysis.

5.3.3 *Bulk consignments of maize*

5.3.3.1 *Imported maize samples*

Bulk shipments of maize (≥ 48,000 tonnes) imported into South Africa from Argentina and the USA (2 consignments each) were sampled by SAMB personnel at entry ports in Cape Town and Durban. In each case, samples were withdrawn pneumatically from the individual holds of each ship. Sampling of each hold involved the removal of (where possible) 3 subsamples, each of 5 kg in mass, representative of the top, middle and bottom thirds (on the vertical axis) of each sampling point, at three independant sampling sites selected laterally across each hold. This ensured that for each full hold, 9 subsamples each of 5 kg mass were withdrawn.

All samples (n = 57) withdrawn from one of the two maize consignments from the USA were submitted to the PROMEC laboratories. Composite subsamples (n = 21, 22 and 26), corresponding to the lateral layers withdrawn from the individual holds of the other three consignments, were prepared by SAMB personnel. These latter samples were submitted as coarsely ground maize kernels of approximately 150 g mass each.

5.3.3.2 *Exported maize samples*

Maize kernel samples (n = 36) of approximately 1 kg mass each, collected by SAMB personnel at the points of origin within South Africa, were designated as "A" samples, and submitted to the PROMEC laboratories. The samples were a subset of every fourth sequential sample (of a larger number of samples of the 1989 South African maize crop), that formed a consignment of maize intended for export to Taiwan. A series of "B" samples (n = 32) also of approximately 1 kg in mass (from the same consignment of export maize) were collected from the end-point distributors in Taiwan.

All kernel samples were blended and riffled to improve homogeneity, and 500 g subsamples of each were withdrawn, coded and stored at 4°C prior to analysis.

5.3.4 *Commercial maize products*

From May 1990 onwards, a total of 189 maize products intended for human consumption were either purchased from retail outlets or supplied by co-workers in Austria (n = 3), Botswana (n = 3), Bulgaria (n = 7), Canada (n = 2), China (n = 3), Egypt (n = 2), France (n = 1), Hungary (n = 1), Kenya (n = 4), New Zealand (n = 12), Peru (n = 4), South Africa (n = 104), USA (n = 41) and Zimbabwe (n = 2). All samples were stored at 4°C prior to analysis.

5.3.5 *Home-grown maize from Transkei*

Over the 1985-1986 season, 48 samples of home-grown maize cobs (ears), previously separated into visibly healthy (intended for human consumption) and mouldy (intended for the brewing of traditional beer) fractions, by a member of each household, were selected from 12 households each in the Bizana and Kentani districts of the Transkei, southern Africa. The healthy maize samples were randomly collected, whereas the samples of mouldy maize were biased samples, whereby only *Fusarium* infected cobs were selected.

During 1989, samples of healthy (n = 8) and mouldy (n = 7) home-grown maize cobs were collected from the Bizana district, whereas 6 samples of each type were similarly collected from households in the district of Kentani. All maize cobs were hand shelled, resulting in approximately 1 kg of kernels for each sample. The samples were stored at 4°C and retained for mycological investigation and the determination of FB_1 and FB_2.

5.3.6 *Transkeian beer samples*

A single sample of traditionally prepared maize-based beer was obtained from a household in Transkei. Three additional beer samples were prepared at the PROMEC laboratories (using home-grown maize collected during 1992 from a household in Transkei), in accordance with the traditional brewing process. Since the prepared beer contained both liquid and solid fractions, the samples were first homogenised and filtered. The pH of the filtrates (found to be *ca* 3.5) were altered to 6.0 and aliquots (10 ml) applied to SAX columns. Fumonisin levels were determined according to the method of Sydenham et al. (1992b) (sections 3.4.1 to 3.4.6).

5.3.7 *Mycological screening*

Subsamples (100 g each) of the 1989 and 1990 South African maize crops (section 5.2.1), and of the Transkei samples (section 5.2.5), were surface-disinfected for 1 minute in a solution containing 3.5% sodium hypochlorite. The kernels were then washed with sterile water and 100 kernels (5 kernels per plate) transferred to 1.5% malt extract agar containing 150 mg novobiocin/litre. The plates were incubated at 25°C in the dark for between 5 and 7 days, after which the cultures of fungi that developed from the kernels were identified and counted. *Fusarium* species were identified according to the classification system of Nelson et al. (1983).

5.3.8 *Fumonisin analyses*

The kernels of each maize sample were ground to a fine meal in a laboratory mill. The ground maize was then blended and riffled to improve homogeneity, and subsamples were analysed according to the methods of either Shephard et al. (1990) or Sydenham et al. (1992b) (sections 3.4.1 - 3.4.6). All data was rounded to the nearest 5 ng/g.

5.3.9 *Statistical analyses*

5.3.9.1 *Analyses of 1989 and 1990 maize crops*

Statistical analyses of the mycological and chemical data were performed using the Statistical Analysis System (SAS) programme package on a IBM mainframe computer. Analyses were performed on log-transformed data.

5.3.9.2 *Analyses of Argentinian field-trial and Transkeian home-grown maize*

These analyses were also performed on log-transformed data. To determine the significance of the difference in fumonisin levels between field-trial regions (Argentinian samples), high and low OC rate areas and between healthy and mouldy maize (Transkeian samples), analyses of covariance were performed with the occurrence of *F. moniliforme* as the covariate. The probability levels reported are the most conservative (ie. least significant) obtained.

5.4 *RESULTS*

5.4.1 *Commercial maize production in South Africa during two seasons (1989 & 1990)*

The raw chemical and mycological data obtained for 249 of the 735 maize samples collected during 1989 and 1990 are tabulated in Appendix 1, however the numbers of samples found to contain

Table 5.2 Range of total fumonisin concentrations recorded for the South African maize crops harvested in 1989 and 1990

Fumonisin range (ng/g)	1989[a]		1990[b]	
	No of samples	%	No of samples	%
0 - 500	91	75.2	99	77.3
501 - 1000	14	11.6	17	13.3
1001 - 1500	5	4.1	7	5.5
1501 - 2000	3	2.5	0	0.0
>2000	8	6.6	5	3.9

[a]1989 = FB_1 + FB_2 concentrations in 121 maize samples
[b]1990 = FB_1 + FB_2 + FB_3 concentrations in 128 maize samples

Table 5.3 Mean incidence of *F. moniliforme* and fumonisins in maize samples of different grades of the 1989 South African crop[a]

Characteristic	Type	Maize grade		
		First	Second	Third
F. moniliforme (%)	White	8.3	12.4	12.6
F. moniliforme (%)	Yellow	13.5	10.6	13.0
Fumonisin B_1 (ng/g)	White	694	230	872
Fumonisin B1 (ng/g)	Yellow	150	208	270
Fumonisin B_2 (ng/g)	White	250	64	274
Fumonisin B_2 (ng/g)	Yellow	35	72	37

[a]No significant differences between grades (p = >0.05)

combined fumonisin levels (between selected ranges) are compiled in Table 5.2. The degree of fungal infection of the 1989 crop with *F. moniliforme* and of concurrent contamination with FB_1 and FB_2 is compared in Table 5.3, for both white and yellow maize samples.

No significant differences were observed between the three grades of maize, however significantly higher ($p = <0.05$) contamination with both FB_1 and FB_2 was recorded in the white maize, than in the corresponding yellow maize samples (Table 5.3). Overall, the incidence of *F. moniliforme* infection in both white and yellow maize was correlated with concurrent contamination with FB_1 ($r = 0.770$; $p = <0.001$) and FB_2 ($r = 0.490$; $p = <0.05$). Table 5.4 shows similar data obtained for the 1990 maize crop (which included data for FB_3 levels in

Table 5.4 Mean incidence of *F. moniliforme* and fumonisins in maize samples of different grades of the 1990 South African crop[a]

		Maize grade		
Characteristic	**Type**	**First**	**Second**	**Third**
F. moniliforme (%)	White	8.6	10.0	11.2
F. moniliforme (%)	Yellow	11.5	12.9	12.9
Fumonisin B_1 (ng/g)	White	496	328	308
Fumonisin B_1 (ng/g)	Yellow	121	179	268
Fumonisin B_2 (ng/g)	White	192	104	117
Fumonisin B_2 (ng/g)	Yellow	50	76	84
Fumonisin B_3 (ng/g)	White	52	28	40
Fumonisin B_3 (ng/g)	Yellow	11	18	37

[a]No significant differences between grades ($p = >0.05$)

Table 5.5 Fumonisin concentrations in maize samples harvested from field trials conducted in Argentina

Sample number	*F. moniliforme* propagules/g ($X10^3$)[a]	Fumon. concn. (ng/g)			
		FB_1	FB_2	FB_3	Total
9 de Julio district					
1	11	1745	775	205	2725
2	300	1900	775	230	2905
3	60	2735	935	410	4080
4	120	2320	795	290	3405
5	10	1820	725	275	2820
6	60	2210	1910	440	4560
7	100	2880	925	145	3950
8	10	1655	545	110	2310
9	140	4235	1750	540	6525
10	700	3960	1545	640	6145
11	20	1740	600	130	2470
12	40	2385	845	330	3560
Mean		2459	1010	312	3781
RSD (%)		34.9	45.3	53.6	36.3
Pergamino district					
13	50	1110	325	150	1585
14	50	2530	735	315	3580
15	400	5450	2680	800	8930
16	80	6695	2440	855	9990
17	100	3535	1030	450	5015
Mean		3864	1442	514	5820
RSD (%)		57.9	73.1	59.5	61.1

[a]Fungal counts from published data (Sydenham et al., 1993)

addition to those of FB_1 and FB_2).

The mean levels of FB_1, FB_2 and FB_3 were consistantly higher in first, second and third grade white maize that in corresponding grades of yellow maize ($p = <0.05$). Combined data showed that the incidence of *F. moniliforme* was correlated with contamination with FB_1 ($r = 0.284$; $p = <0.01$), FB_2 ($r = 0.300$; $p = <0.01$) and FB_3 ($r = 0.222$; $p = <0.05$).

5.4.2 *Argentinian field trial maize*

The degree of fungal infection with *F. moniliforme* and concurrent fumonisin concentrations in the 17 Argentinian maize trial samples, are presented in Table 5.5.

Combined fumonisin levels were found to range from 2310 to 6525 ng/g and from 1585 to 9990 ng/g in the samples collected from the "9 de Julio" and "Pergamino" districts, respectively. With the exception of one sample (number 6 - Table 5.5) in which the FB_1 and FB_2 levels were similar, the proportion of FB_1 and FB_2 in the samples (expressed as percentages of total fumonisin concentrations) ranged between 61.0 to 72.9% and from 20.5 to 30.0%, respectively. The FB_3 levels accounted for between 3.7 and 10.4% of the total fumonisins in the samples.

The degree of *F. moniliforme* contamination of the 17 maize trial samples was significantly correlated with the levels of FB_1 ($r = 0.596$; $p = <0.05$), FB_2 ($r = 0.550$; $p = <0.05$), FB_3 ($r = 0.597$; $p = <0.05$) and total fumonisins ($r = 0.612$; $p = <0.01$). The levels of FB_1 in the samples were highly significantly correlated with the concurrent levels of both FB_2 ($r = 0.887$; $p = <0.001$) and FB_3 ($r = 0.846$; $p = <0.001$), while FB_2 levels were similarly correlated with those of FB_3 ($r = 0.864$; $p = <0.001$). Although numerical differences in mean fumonisin concentrations were observed between the two regions (Table 5.5), no statistically significant differences for either the individual or combined fumonisin concentrations were obtained.

Table 5.6 Range and mean fumonisin concentrations in maize imported into and exported from South Africa[a]

Source	No of samples	Fumonisin concentrations (ng/g)			
		FB_1	FB_2	FB_3	Range
USA[b]	57	2229	612	239	1230 - 5265
USA	22	2652	815	287	2155 - 5365
Argentina	21	229	73	30	50 - 1210
Argentina	26	354	116	62	105 - 1490
SA[c]	36	57	25	NA[d]	<50 - 880
SA[e]	32	146	13	NA	<50 - 985

Table 5.7 Number of positive samples and relative standard deviations recorded for imported and exported maize consignments[a]

Country	Fumonisin B_1		Fumonisin B_2		Fumonisin B_3	
	positive/ samples	RSD (%)	positive/ samples	RSD (%)	positive/ samples	RSD (%)
USA	57/57	33.3	57/57	34.1	57/57	47.7
USA	22/22	22.5	22/22	22.6	22/22	19.2
Argentina	21/21	73.8	16/21	99.4	11/21	29.3
Argentina	26/26	49.0	25/26	74.7	17/26	111.2
SA[c]	10/36	225.4	6/26	243.7	-	-
SA[e]	14/32	170.4	4/32	271.7	-	-

Key to Tables 5.6 and 5.7

[a]Calculations based on all results; [b]USA = United States of America; [c]SA = South Africa (samples collected in South Africa); [d]NA = Not analysed; [e]SA = South Africa (samples collected in Taiwan)

5.4.3 *Imported maize samples*

The fumonisin levels in the 126 and 68 samples, representative of 4 imported and 1 exported bulk maize shipments, respectively, are given in Appendix 2. The results are however summarised in Table 5.6.

All of the samples obtained from the two USA shipments were found to contain detectable levels of FB_1, FB_2 and FB_3 (Table 5.6), with FB_1 levels corresponding to between 70.6 and 72.4% of the total fumonisin concentrations. Similarly, the concurrent levels of FB_2 and FB_3 accounted for between 19.9 and 21.7%, and between 7.6 and 7.7 % of the total concentrations, respectively (Table 5.6).

Fumonisin concentrations appeared to be narrowly distributed within the USA shipments, as seen from the %RSD results (Table 5.7). The fumonisin levels determined in the Argentinian and South African maize consignments were lower than those obtained for the USA consignmets (Table 5.6), with fewer samples contaminated with the individual fumonisin toxins, and a wider distribution in fumonisin levels, within the individual maize samples (ie. an increase in %RSD results - Table 5.7).

5.4.4 *Commercially available maize-based products*

The fumonisin levels in the 189 individual maize-based human foodstuffs, obtained from retail outlets in 14 countries are tabulated in Appendix 3 and summarised (according to product type) in Table 5.8 . The majority of the samples obtained were

Table 5.8 Range and mean fumonisin concentrations in maize-based commercial human foodstuffs obtained from retail outlets in various countries[a]

Country	Product type	Number of samples	Fumonisin concn. (ng/g)	Mean concn. (ng/g)[b]
Austria	Meal	2	70 - 115	93
Botswana	Meal	3	265 - 620	405
Bulgaria	Meal	7	50 - 1260	291
Canada	Meal	2	<50 - 50	50
China	Meal	3	<50	<50
Egypt	Meal	2	2190 - 3760	2975
France	Meal	1	1810	1810
Hungary	Meal	1	<50	<50
Kenya	Meal	4	<50 - 300	195
Peru	Meal	2	<50 - 795	795
SA[c]	Meal	67	<50 - 625	156
USA[d]	Meal	21	<50 - 4530	1313
NZ[e]	Meal	12	<50	<50
SA	Grits[f]	22	<50 - 535	206
USA	Grits	10	<50 - 3610	872
Peru	Alkali[g]	2	<50	<50
USA	Alkali	3	<50 - 50	50
Austria	Misc[h]	1	<50	<50
SA	Misc	15	<50 - 90	83
USA	Misc	7	<50 - 3470	1149
Zimbabwe	Misc	2	1420 - 4765	3093

[a]Range of total fumonisin levels (ie $FB_1+FB_2+FB_3$); [b]Mean concentrations in positive samples only; [c]SA = South Africa; [d]USA = United States of America; [e]NZ = New Zealand; [f]Grits = maize products classified as rice, samp or grits; [g]Alkali-treated products (ie. tortilla preparations); [h]Misc = various maize-based samples (ie. maize-flour, cornflakes etc.)

maize meals, where a wide range in fumonisin levels was observed, although the highest combined FB_1 and FB_2 concentration was recorded in one of two samples purchased in Egypt (3760 ng/g - Table 5.8). A significant number of samples (n = 32) were identified as "*grits*". These comprised of maize samples that were coarsely ground rather than fine meals. The fumonisin levels in these "*grits*" samples were similar to the levels observed in corresponding meal samples collected from the same countries.

Five of the samples had been subjected to treatment with lime water as part of the industrial and traditional process involved with the preparation of tortillas. Only one of these five tortilla preparations contained a detectable level of FB_1 (at 50 ng/g - ie. the detection limit of the analytical method - Table 5.8).

Several of the samples classified in the miscellaneous section were "cornflakes" samples, and none of these contained detectable levels of the fumonisins. The highest recorded fumonisin level detected in a maize-based sample occurred in a health breakfast cereal-product from Zimbabwe, which had a combined fumonisin concentration of 4765 ng/g.

5.4.5 *Transkeian home-grown maize*

The fumonisin concentrations (FB_1 and FB_2) in the 1985 and 1989 maize samples collected from low and high OC risk regions of the Transkei are tabulated in Appendix 4. The range and mean fumonisin levels recorded in the samples are however summarised in Table 5.9.

Fumonisins were recorded in only 3 of 12 healthy maize samples collected from the 1985 crop, from the low OC rate area, at levels ranging from <50 to 550 ng/g and from <50 to 150 ng/g for FB_1 and FB_2, respectively. Fumonisin levels in the corresponding samples collected from the high OC rate area ranged between 50 and 7900 ng/g FB_1 and between 0 and 2250 ng/g FB_2 in 12/12 and 10/12 samples, respectively. Far higher fumonisin

Table 5.9 Mean and range of fumonisin concentrations in healthy and mouldy home-grown maize collected from low and high OC risk areas of the Transkei

Range of fumonisin concentrations (ng/g)

		Healthy maize		Mouldy maize	
Year	Toxin	Low OC	High OC	Low OC	High OC
1985	FB_1	<50 - 550	50 - 7900	460 - 18900	3450 - 46500
1985	FB_2	<50 - 150	<50 - 2250	150 - 6750	900 - 16300
1989	FB_1	<50 - 3310	<50 - 5300	110 - 11340	3020 -117520
1989	FB_2	<50 - 970	<50 - 1320	<50 - 3700	750 - 22960

Mean fumonisin concentrations (ng/g)[a]

		Healthy maize			Mouldy maize		
Year	Toxin	Low OC	High OC	P[b]	Low OC	High OC	P[b]
1985	FB_1	375	1600	<0.001	6520	23900	<0.01
1985	FB_2	83	610	<0.001	2500	7550	<0.01
1985	FB_1+FB_2	333	2100	<0.001	9010	31500	<0.01
1989	FB_1	667	1840	N/S[c]	4050	53740	<0.005
1989	FB_2	515	508	N/S	1277	13680	<0.005
1989	FB_1+FB_2	630	1960	N/S	5150	67410	<0.005

[a]Means are based on the number of positives only

[b]P = Probability values were calculated based on the complete data given in Appendix 4

[c]NS = Not significant (>0.05)

levels were recorded in the mouldy maize fractions (Table 5.9), however the overall mean fumonisin levels were significantly higher ($p = <0.001$ for healthy maize and $p = <0.01$ for mouldy maize), in those samples collected from the high OC rate area, than in corresponding samples obtained from the low OC rate area.

In the 1989 samples, the combined (mean) fumonisin levels were numerically higher in the healthy maize samples collected from the high OC rate area than in corresponding samples from the low OC rate area, but not significantly so. The FB_1 and FB_2 levels were, however, significantly higher in the mouldy maize fractions collected from the high OC rate area ($p = <0.005$) than in the corresponding samples from the low OC rate area. Mean combined fumonisin concentrations in the mouldy fractions were 5150 ng/g (5.1 ppm) and 67410 (67.4 ppm) ng/g for the low- and high OC rate areas, respectively (Table 5.9). The highest combined FB_1 and FB_2 concentration in a single sample was found to be >140,000 ng/g (140 ppm - Appendix 4)

5.4.6 *Beer samples*

The individual and combined fumonisin levels in the four maize-

Table 5.10 Fumonisin levels in maize-beer samples, prepared according to traditional Transkeian methods

	Fumonisin concentrations (ng/ml)			
Sample origin	**FB_1**	**FB_2**	**FB_3**	**Total**
Transkei	610	255	75	940
PROMEC[a]	1345	350	185	1880
PROMEC	1585	555	315	2455
PROMEC	1655	555	250	2460

[a]Programme on Mycotoxins and Experimental Carcinogenesis

beer samples are given in Table 5.10.

All three naturally occurring fumonisin analogues were recorded in each of the beer samples. A combined fumonisin level of 940 ng/g was observed in the single sample collected from the Transkei, while combined fumonisin levels of between 1880 and 2460 ng/g were recorded in the beer samples prepared under laboratory conditions, from maize collected in the Transkei.

5.5 *DISCUSSION*

5.5.1 *Commercial maize production in South Africa and Argentina*

The distribution of fumonisin levels recorded in the South African maize crops of 1989 and 1990 are illustrated graphic-

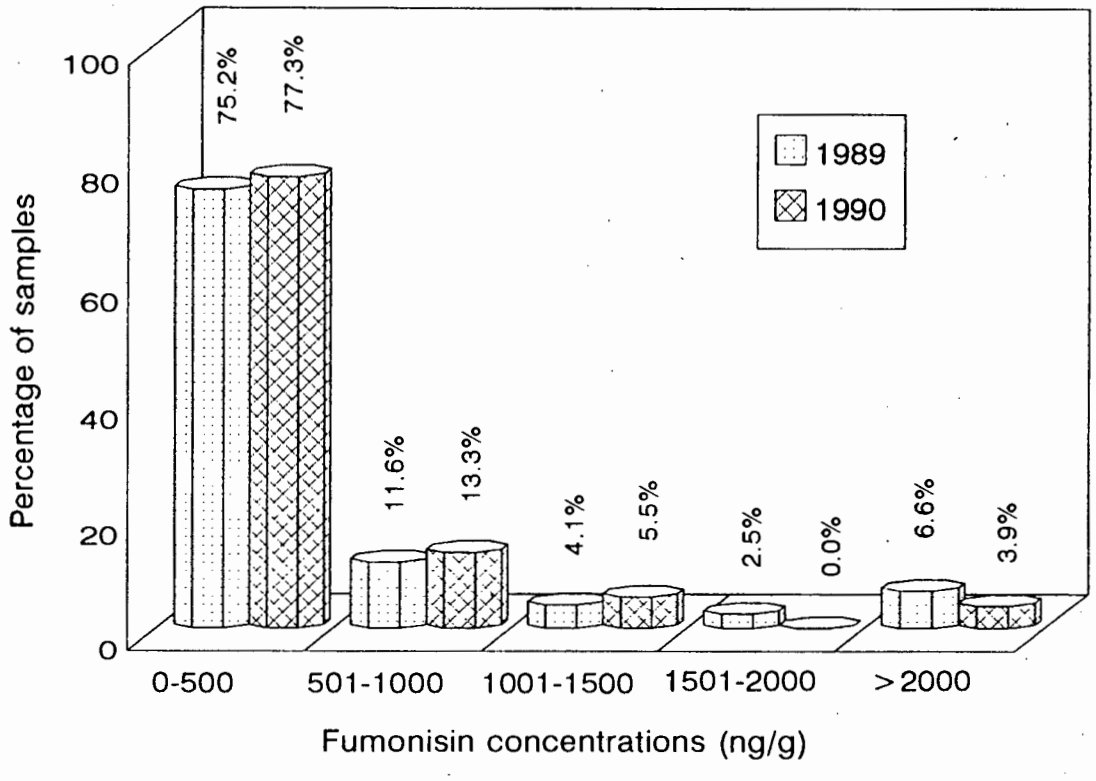


Figure 5.2 Fumonisin levels in the 1989 and 1990 South African maize crops

ally in Figure 5.2.

In the 1990 crop, fumonisins were detected in 83% of the samples (106 out of 128) compared with 68% of the samples of the 1989 crop. The fumonisin levels determined in both crops were similar, although in general the levels in the 1990 crop were slighly lower than those recorded in the 1989 crop (though not significantly so). Fewer samples of the 1990 crop contained combined fumonisin levels in excess of 2000 ng/g (3.9%) as opposed to the 1989 crop (6.6%), and this despite that fact that the 1990 data included FB_3 levels, which occurred in 47 of the 128 samples (36.7%). A significant proportion of the samples representative of both seasons, contained combined fumonisin concetrations below 1000 ng/g (ie. 85.9% in 1989 and 90.6% in 1990) (Figure 5.2).

The incidence of *F. moniliforme* contamination tended to be higher in the yellow maize than in the corresponding white maize samples, but not significantly so. Conversely, the mean concentrations of FB_1 and FB_2 were significantly higher in white than in yellow maize (Marasas & Thiel, 1991). This anomaly is somewhat difficult to explain, although lower incidences of all fungi (except that of *F. graminearum*) have been observed in white maize than in yellow maize (Marasas & Thiel, 1991). It is possible that, under field conditions, this situation could result in less competition for *F. moniliforme*, enabling the production of higher levels of the fumonisins. It is also possible that white maize is a superior substrate to yellow maize, for fumonisin production.

Although no significant differences were observed in the degree of fungal infection with *F. moniliforme*, between the three grades of either white of yellow maize, combined mean fumonisin concentrations were significantly higher in third grade maize than in first grade, over both growing seasons. Marasas & Thiel (1991) noted that the incidence of the fungus *Diplodia maydis* increased significantly from first to second to third grade, and concluded that infection with *D. maydis* was mainly responsible for the down-grading of commercial South African

maize, although infection with *D. maydis* would not account for the high levels of the fumonisins in the lower grade maize.

The surveys of the 1989 and 1990 crops indicated that (with individual exceptions - Appendix 1), fumonisin contamination was relatively low. However, it is important that surveillance should continue particularly in climatologically abnormal years, such as a dry summer followed by a wet autumn. Such a season occurred during 1989/1990, resulting in wide-spread contamination of the USA commercial maize crop with *F. moniliforme* and concurrent high fumonisin levels. Consumption of the harvested maize caused the deaths of large numbers of horses and pigs (Ross et al., 1991a,b, 1992). Although a substantial number of data have been published concerning fumonisin levels in selected animal feeds from the USA, little information has

Table 5.11 Comparison of the range and mean fumonisin levels recorded in maize crops from South Africa and the USA[a]

Year	Country & No of samples	Fumonisin concentrations (µg/g)		
		FB_1	FB_2	FB_3
1988	USA (22)	0-14.9 (2.5)	0- 5.7 (0.7)	0-2.1 (0.2)
1989	USA (44)	0-37.9 (2.9)	0-12.3 (0.8)	0-4.0 (0.2)
1989	SA (121)	0- 5.4 (0.4)	0- 1.6 (0.1)	NA[b]
1990	USA (59)	0-19.1 (3.3)	0- 6.1 (0.9)	0-2.8 (0.3)
1990	SA (128)	0- 5.0 (0.3)	0- 1.7 (0.1)	0-0.04 (<0.1)
1991	USA (50)	0-15.8 (2.9)	0- 4.4 (0.8)	0-2.3 (0.4)

[a]Mean concentrations are given in brackets

[b]NA = Not analysed

been published on similar fumonisin levels in the USA maize crop. However, recently Murphy et al. (1993) monitored fumonisin levels in maize samples obtained from the states of Iowa, Wisconsin and Illinois, over a period of 4 growing seasons. The data of Murphy et al. (1993) are compared in Table 5.11 with the present data for the 1989 and 1990 South African maize crop samples.

Table 5.11 clearly illustrates the difference in both the range and mean fumonisin levels recorded in the commercial maize samples from the two countries. The mean combined fumonisin concentrations in the USA maize ranged from 3.5 to 5.0 μg/g (ppm), while the mean combined levels in the corresponding South African samples were between 7 and 10-fold lower (at 0.5 μg/g). These results are particularly important, in view of the suggestion that fumonisin levels of 10 ppm and above should be considered dangerous to horses. It is probable that humans have consumed maize produced in the USA, containing combined fumonisin levels that were only between 2 to 3 times lower than that which could conceivably induce equine LEM.

This type of data will be essential for overall human risk assessment studies involving the fumonisins. Legislation aimed at reducing human exposure to the fumonisin mycotoxins, will have to take into consideration the enormous economic consequences associated with the establishment of suggested tolerance levels. Clearly, if a tolerance level of 1 ppm total fumonisins (one tenth the level suggested for horses and other members of the equidae family) were to be set for humans, a substantial proportion of the USA maize crop would be deemed unsuitable for human consumption, though such a level would have a far less dramatic effect for the South African maize industry (based on the available data), whereby ± 90% of the crop would have been graded as suitable for human comsumption.

The fumonisin levels recorded in the USA maize crop (Murphy et al., 1993) are similar to the levels recorded for the Argentinian field-trial samples (Table 5.5). Although the 17 Argentinian samples were representative of selected field-

trials, they may be compared with maize crop data, rather than with other sample types (ie. animal feeds). The mean combined fumonisin levels recorded for the Argentinian samples from both field-trial sites, ranged from 3.8 to 5.8 µg/g (ppm), while several samples from both districts, had individual fumonisin levels (Table 5.5) similar to those previously shown to be associated with outbreaks of equine LEM (Thiel et al., 1991b; Sydenham et al., 1992b), a disease that has occurred in some South American countries including Brazil and Argentina (section 4.2.2.2). Of the 17 Argentinian samples, only 1 had a combined fumonisin level of less than 2000 ng/g (2 ppm). These results should be of concern to Argentinian authorities, since maize is one of the major crops grown in Argentina. Although human dietary consumption of maize in Argentina is relatively low (Gallo et al., 1993), the vast majority of maize grown in the country is used either as animal feed or for export purposes.

5.5.2 *South Africa and the importation/exportation of maize*

Although South Africa has in the past, been able to produce a surplus of maize (over and above that used for its own consumption), a period of drought over the past few years (ie. from 1990 onwards) has necessitated the importation of large quantities of maize to supplement the shortfall.

The data presented in Appendix 2 and summarised in Tables 5.6 and 5.7, correspond to a series of samples collected from 4 consignments of maize imported into South Africa from Argentina and the USA, during 1992. The 2 USA shipments contained relatively higher levels of all three major naturally occurring fumonisin toxins, than did the corresponding shipments imported from Argentina. The fumonisin levels in the USA shipments were similar in magnitude to the levels reported by Murphy et al. (1993), that were representative of commercial crops from 3 states of the USA, grown between 1988 and 1991. However, no data was available concerning the age or source of the 2 USA maize consignments, and it is therefore not possible to

critically compare the results. It is, however, interesting to note that all of the USA samples (from both shipments) contained detectable levels of each fumonisin B mycotoxin at levels that were narrowly distributed (Table 5.7). These results indicated a high degree of homogeneity. The finding that FB_1 was the major naturally occurring fumonisin analogue was in agreement with other data (section 5.5.1 - Appendix 1).

These results were in contrast to the maize consignments imported into South Africa from Argentina, which in general had fewer samples contaminated with FB_2 and FB_3. The concentrations of all three naturally occurring fumonisin analogues were lower than observed in the USA samples, and appeared to have a wider distribution in levels, between the individual samples (Table 5.7). In general, the fumonisin levels in the imported Argentinian maize were not in accordance with the levels determined in the field-trial samples (Table 5.5). It is possible that the field-trial samples may not reflect the true situation, with respect to fumonisin distribution within the Argentinian commercial maize industry, although it has been suggested that storage and transportation of fumonisin contaminated maize may result in a lowering of fumonisin levels (Resnik, 1990).

During 1989, shipments of maize were exported from South Africa to Taiwan. The results of the South African export maize differed again from both the USA and Argentinian imported shipments. The recorded fumonisin levels were very low, being similar in magnitude to the levels recorded for the South African 1989 yellow maize crop. The mean FB_1 concentration in the "A" samples (collected in South Africa) was significantly lower ($p = <0.05$) than the corresponding level recorded in the "B" samples (collected in Taiwan). This significant increase in the mean level of FB_1 contamination of the "B" samples is difficult to explain, except that the sampling procedures at both sites may have contributed to the effect, especially since the homogeneity (of fumonisin levels throughout the consignment) was poor (RSD data - Table 5.7). The reverse observation was made with respect to the FB_2 concentrations obtained from the "A" and "B" samples, with numerically higher FB_2 levels

being recorded in the "A" samples than in the corresponding "B" samples, although the difference was not found to be significant.

The present data is therefore insufficient to either confirm or refute the suggestion that fumonisin levels may be increased during transportation (Resnik, 1993). However, the data substantiate that South African production maize, in the recent past, contained less fumonisins, than maize produced in Argentina or the USA.

5.5.3 *Commercial maize-based retail products*

The majority (>68%) of the 189 commercial samples collected from the continents of Africa (Botswana, Egypt, Kenya, South Africa), Asia (China), Australasia (New Zealand), Europe (Austria, Bulgaria, France, Hungary) and the Americas (Canada, Peru and the USA), consisted of ground maize meals. In the majority of cases, only a few samples were obtained, although in general the data indicated that occurrence of the fumonisins may be a worldwide phenomenon (Table 5.8). One particular exception would appear to have been the 12 selected maize samples obtained from New Zealand, none of which contained detectable levels of either FB_1, FB_2 or FB_3. Similarly, 3 samples from China, 2 from Canada and 1 from Hungary contained negligible levels of fumonisins. Although based on relatively few data, it is possible that maize grown in those countries having relatively cool, damp climates (ie. New Zealand, Canada) might be expected to be less susceptible to fungal contamination with *F. moniliforme* (and concurrent contamination with the fumonisins), that those countries with warm, dry climates (ie. Egypt, Zimbabwe).

The majority of the maize meal products (intended for human consumption) were purchased from outlets in South Africa (53%) and the USA (17%). Sixty-two of the 67 South African maize meal samples (93%) were contaminated with the fumonisins, but only 1 sample contained a combined fumonisin level of >600 ng/g. Similarly, 20/21 USA samples (95%) were contaminated with

the fumonisins, but at concentrations that were far higher than those in the South African samples, with only 5/20 (25%) having combined levels <600 ng/g. These data are reflected in the mean concentrations observed in the maize meals, which were 156 and 1313 ng/g in the South African and USA samples, respectively. This trend was also observed for the maize "*grits*" samples, where a mean concentration of 206 ng/g was recorded in 11 (58%) of the 19 South African samples, as opposed to a mean concentration of 872 ng/g in 9/10 (90%) of the corresponding samples collected in the USA. These data are therefore in agreement with the data concerning the maize crops produced in both countries (Table 5.11), as well as with other data pertaining to exported maize (Table 5.6).

Table 5.12 Other published data on the range of fumonisin levels determined in maize-based products[a]

Maize Sample	Toxin	Country							
		USA[b]		Switzerland[c]		Italy[d]		Asia[e]	
Meal	FB_1	70-2050	(17)	0-110	(7)	420-3730	(6)	60-200	(3)
Meal	FB_2	20-360		ND[f]		80-840		ND	
Grits	FB_1	140-270	(5)	0-790	(55)	3760	(1)	200-2600	(14)
Grits	FB_2	0-110		0-160		910		300-2800	
Flakes	FB_1	0-10	(6)	0-55	(12)				
Flakes	FB_2	ND		ND					
Tort[g]	FB_1	0-120	(2)	ND	(4)	0-60	(2)		
Tort	FB_2	ND		ND		ND			

[a]Number of samples tested are given in brackets; [b]Stack & Eppley (1992); [c]Pittet et al. (1992); [d]Doko & Visconti (1993); [e]Ueno et al. (1993) - countries include Japan and China; [f]ND = Not detected (<50 ng/g); [g]Tort = Tortillas

Five maize-based samples (2 from Peru and 3 from the USA), were treated with lime water (aqueous calcium hydroxide) as part of their manufacturing process (for tortilla preparations). Of these five samples, only 1 had a detectable level of FB_1 (50 ng/g) bordering on the detection limit of the method. Similarly, five of the miscellaneous samples were cereal samples (cornflakes), and none contained detectable levels of either FB_1 or FB_2. The production of cornflakes requires heat treatment, however Alberts et al. (1990) reported that FB_1 was heat stable, a conclusion that was recently supported by Doko & Visconti (1993), following the observation that fumonisins levels occurred in Italian extruded maize products. In view of the, on occasions, relatively high concentrations of the fumonisins in many maize-based products, their absence (or occurrence at low concentrations) in both cornflakes and calcium hydroxide-treated maize products warrant further attention.

Table 5.12 lists data pertaining to fumonisin levels in maize-based commodities, published by researchers in the USA, Switzerland, Italy and Japan. Only those data concerning specific product groups have been included, but the overall levels correspond well with the data presented in Table 5.8. Fumonisins were detected in both maize meal and maize grits samples, while the majority of the cornflakes and tortilla samples contained low fumonisin levels (Table 5.12). In addition to the data in Table 5.12, Scudamore and Chan (1993) reported FB_1 levels up to 4553 ng/g in maize-gluten samples.

While the present data illustrate that humans are undoubtedly exposed to the fumonisin toxins, at a wide range of concentrations, insufficient data yet exist on fumonisin levels in commercially available maize-based products.

5.5.4 *Fumonisin levels in Transkeian home-grown maize and beer samples*

Previous mycological studies (Rheeder et al., 1992) have indicated the statistically significant difference in the

distribution of *F. moniliforme*-contamination of home-grown maize in Transkei. These differences, which were monitored between 1976 and 1989, were in agreement with the geographical distribution of human OC rates reported for the period 1981 to 1984 (Jaskiewicz et al., 1987a,b). The fumonisin results (Appendix 4 and Table 5.9) corroborate the mycological findings for the 1985 and 1989 samples, for both the healthy and mouldy maize fractions (Sydenham et al., 1990b; Rheeder et al., 1992).

Sydenham et al. (1990b) determined the presence of other *Fusarium* toxins in the 1985 mouldy Transkeian maize samples. These included moniliformin (produced by *F. subglutinans*) and DON, NIV and ZEA (produced by *F. graminearum*), but all occurred at significantly higher levels in the maize samples collected from the low OC prevalence area, than in corresponding samples from the high OC prevalence area. The fumonisin results for the 1985 mouldy maize samples differed considerably, in that significantly higher levels ($p = <0.01$) of both FB_1, and FB_2 occurred in samples from the high than in the low prevalence area. These differences were also apparent in the healthy maize samples (Table 5.9). The data clearly indicated that the combined mean FB_1 and FB_2 levels in the fumonisin-contaminated healthy maize fractions were approximately 6 times higher in those samples from the high OC prevalence area, than in corresponding low OC prevalence area samples, while the difference for the mouldy fractions was approximately 3.5 times (Table 5.9).

The fumonisin results obtained for the 1989 Transkei samples tended to show a similar trend. While the mean combined fumonisin levels in healthy samples were numerically higher in those samples from the high OC rate area, than in corresponding low OC rate samples, they were not found to be significantly so. Conversely, the fumonisin results in the mouldy samples indicated that the combined mean FB_1 and FB_2 levels were significantly ($p = <0.05$) higher (in excess of 13 times higher), in those samples drawn from households in the high rate, than the low rate area.

The data concerning fumonisin levels in Transkeian home-grown maize clearly show that visual assessment, and subsequent separation of maize cobs into healthy and mouldy fractions by the housewife, does not ensure that the maize intended for human consumption, is free from fumonisin (or indeed other mycotoxin) contamination.

Since the mouldy fractions are separated and used for the preparation of traditional beer, it is likely that humans (and especially males) are exposed to additional levels of the fumonisins. In this respect, the data presented in Table 5.10 illustrates that the fumonisin mycotoxins can occur in beer prepared by traditional Transkeian methods. The prepared beer is neither filtered nor distilled prior to consumption, and hence its appearance resembles more a "*gruel*" or "*porridge*" rather than a clear liquid. Only one beer sample has thus far been obtained from the Transkei region. Additional beer samples need to be obtained and analysed in order to ascertain both the extent to which the fumonisins are retained in this beverage, as well as to determine the fumonisin levels to which humans may be exposed, through its consumption.

In addition, in times of environmental or financial constraints, it is probable that a significant proportion of the mouldy maize cobs are diverted from beer-production, and are used to supplement the basic human diet of maize porridge. Under these conditions, and based on the data in Table 5.9, humans may be exposed to combined fumonisin levels that should be considered as potentially dangerous to horses. In individual cases, humans could be exposed to even higher levels (>100 ppm), that could induce pulmonary oedema in pigs.

The situation with respect to fumonisin contamination of Transkeian home-grown maize, differs considerably to that which exists in the commercial maize growing industry in South Africa and elsewhere. Overall risk assessment strategies have to take into consideration not only the levels at which potentially toxic/carcinogenic compounds occur in various commodities, but also the final intake of those commodities. Human exposure to

the fumonisins in the Transkei (and other areas throughout Africa) is therefore of even greater concern, since maize constitutes a major proportion of the dietary nutrient intake of the local population (Thiel et al., 1992).

In Table 5.13, the estimated daily intakes of fumonisins which precipitated both natural and experimental equine LEM in horses, and the daily dose of FB_1 which caused hepatocarcino-

Table 5.13 Comparison of potential fumonisin intakes for various animal species and man

Exposure	Fumonisins (ng/g)	Intake (mg/kg/day)	Reference
LEM outbreak	72000	0.6 - 2.1	Wilson et al., 1990
LEM induction	-	1.0 - 4.0	Kellerman et al., 1990
Carcinogenesis[a]	50000	3.75	Gelderblom et al., 1991a
HUMAN EXPOSURE - LOW OC AREA[b]			
HM[c] (1985)	333	0.002	Table 5.9
HM (1989)	630	0.004	Table 5.9
MM[d] (1985)	9010	0.060	Table 5.9
MM (1989)	5150	0.034	Table 5.9
HUMAN EXPOSURE - HIGH OC AREA[b]			
HM (1985)	2100	0.014	Table 5.9
HM (1989)	1960	0.013	Table 5.9
MM (1985)	31500	0.21	Table 5.9
MM (1989)	67410	0.44	Table 5.9

[a]Rats - data based on a 200 g rat consuming 15 g feed per day; [b]Data based on a 70-kg person eating 460 g maize per day; [c]HM = Healthy maize; [d]MM = Mouldy maize

genesis in rats, are compared with the potential intakes of fumonisins by humans consuming Transkeian maize, contaminated with mean levels occurring in both the healthy and mouldy fractions (calculated from the 1985 and 1989 data - Table 5.9).

The estimated daily intakes (for humans) of fumonisins are consistently higher for both healthy and mouldy fractions, in maize consumed in the high OC rate area than in the corresponding low OC rate area. The mean fumonisin concentrations consumed would, in all cases, have been between 3 and 13 times higher in the high, than in the low OC rate area. The estimated daily intakes of fumonisins (by humans) are derived from studies by Langenhoven et al. (1988), which monitored nutrient and food intake patterns in local South African populations.

Therefore, the daily intakes of the fumonisin toxins by individuals consuming mouldy Transkeian maize of the 1989 crop (0.44 mg/kg/day), would appear to be exceedingly high when compared with the corresponding intakes that precipitated LEM in horses (0.6 to 4.0 mg/kg/day) and hepatocarcinogenesis in rats (3.75 mg/kg/day).

In addition, since previous studies have shown the presence of several other dominant *Fusarium* fungal species and their corresponding toxins in Transkeian maize samples, humans must be exposed to a diverse range of toxic principles, which raises the possibility of synergistic effects. It is important to consider, however, that the present data merely establishes a statistical relationship between the occurrence of the fumonisins and the incidence of OC. The results do not, in any way, imply that the fumonisins are the causative agents in the development or progression of OC in Transkei.

5.6 *CONCLUSIONS*

Several conclusions may be drawn from the results presented in this chapter. First, an interrelationship exists between the incidence of *F. moniliforme* in maize, and the degree of

contamination with the fumonisin mycotoxins. Secondly, consumers of maize and maize-based products in numerous countries are exposed to a wide range of fumonisin levels, and the toxicological significance of the reported levels needs to be assessed. Thirdly, while overall fumonisin levels in the Transkeian home-grown maize samples indicate that significantly higher concentrations are to be found in the high OC rate areas, fumonisin levels should also be monitored in other high OC risk areas (ie. China). The data do not imply that the fumonisins are causative agents in the development of human OC. Luo et al. (1990) have reported on the occurrence of *Fusarium* toxins in both maize and wheat samples from high and low OC areas of China, but these data concerned levels of various trichothecene mycotoxins and ZEA, but unfortunalely no data on the fumonisins.

With respect to dietary intake of micronutrients, results of a major 5-year vitamin/mineral intervention study in the Linxian county of China were recently reported. The authors concluded that, combinations of ß-carotene, vitamin E and selenium supplementation may effect a reduction in cancer risk in the Linxian population, but the results were not definitive (Blott et al., 1993).

The realities are that scientifically-based, economically viable and realistic tolerance levels will have to be established. At a recent meeting, the International Agency for Research on Cancer (IARC) designated the "*toxins derived from F. moniliforme*" as group 2B carcinogens (possibly carcinogenic to humans) (Vainio et al., 1993). Although the data compiled in Chapter 5 will assist in the overall human risk-assessment studies concerning the fumonisins, substantially more data will be required in order to establish tolerance levels. To this end, a project has been registered with the Food Commission of IUPAC (Sydenham & Thiel, 1993), aimed at the collection of pertinent data on fumonisin levels in various food and feed substrates, from research agencies and groups throughout the world.

CHAPTER 6: FUMONISIN DECONTAMINATION PROCEDURES

6.1 *INTRODUCTION*

In Chapters 4 and 5, the natural occurrence of the fumonisins in both animal feeds and human foodstuffs was demonstrated. The data substantiated that combined fumonisin levels of *ca* 10 and 100 µg/g should be considered as potentially hazardous to members of the equidae family and swine, respectively. Additional data clearly indicated that fumonisin levels of greater than 1 µg/g (and up to 100 µg/g in hand-selected home-grown maize) can occur in foods intended for human consumption. Hence, both animals and man are exposed to fumonisin concentrations that should be of concern to regulatory bodies.

However, the fumonisins are only one group of a diverse range of mycotoxins that have been estimated to contaminate up to 25% of the worlds' food/feed crops (Mannon & Johnson, 1985), while a report by the Council for Agricultural Science and Technology (1989) has described the enormous economic losses, and the occurrence of animal and human diseases, attributable to various mycotoxins. A substantial amount of research has therefore been conducted in an attempt to salvage mycotoxin-contaminated commodities, while avoiding the health risks associated with their ingestion (Beaver, 1991). Clearly, initial prevention of fungal and mycotoxin contamination of food and feedsuffs, is the most rational and economic approach in the control of mycotoxin-related problems. Prevailing production and storage conditions can however hamper these prevention strategies, leading to the inevitable fungal and mycotoxin contamination of field and storage crops.

Decontamination is therefore an alternative option for the recovery of already contaminated crop commodities. Successful decontamination procedures must meet selected criteria if they are to be considered acceptable (Park et al., 1988). These

criteria include the following:

(a) The specific mycotoxin should either be removed from the commodity, destroyed, or by some means inactivated.

(b) The food or feedstuff should retain its nutritive quality and remain palatable, leaving no harmful residues of chemical or microbial origin.

(c) The physical properties of the commodity should not be drastically altered.

(d) Fungal spores and mycelia should, where possible, be destroyed in order to prevent the production of the same/or alternative mycotoxins.

(e) The decontamination process should be economically viable (ie. the cost of the process should not exceed the value of the contaminated commodity).

Many approaches have been investigated for the mycotoxin-decontamination of grains and other commodities such as fruit. Procedures that have been shown to be successful include physical or mechanical removal of contaminated portions, extraction of mycotoxins with solvents, the biological control of mycotoxin production, and various chemical treatments of contaminated commodities. The application of these alternative approaches are reviewed in sections 6.1.1 to 6.1.4.

6.1.1 *Physical methods of decontamination*

Physical treatment of mycotoxin-contaminated cereals, fruits (etc.) are both useful and cost-effective procedures, for those commodities where the contaminated portions are easily identifiable. However these techniques may be only partially effective in the removal of secondary fungal metabolites, since many mycotoxins can diffuse throughout various commodities, and may not be associated exclusively with damaged, discoloured or malformed seeds, kernels or fruits (Beaver, 1991).

Techniques for sorting, on the basis of colour and other visual characteristics, have been used extensively for the control of aflatoxin contamination of peanuts (West & Bullerman, 1991). This technique has in the past been labour intensive, although suspect kernels can now be routinely and efficiently removed by means of electronic colour sorting methods (West & Bullerman, 1991).

Small peanut kernels have been shown to have higher aflatoxin contamination than larger ones (Tiemstra, 1977). This has led to some processors purchasing only large kernel grades, effectively separated from smaller grades by belt-screening methods (Beaver, 1991). Belt separation of peanuts having mean aflatoxin levels of 11.3 μg/kg, have resulted in the isolation of large kernels (containing 9.6 μg/kg aflatoxin) from undersized kernels (containing up to 48.9 μg/kg aflatoxin) (Beaver, 1991). The study also demonstrated that the aflatoxins were concentrated in the broken, immature or otherwise smaller nuts. Sieving and dehulling of barley, wheat, maize and rice has been shown to significantly reduce both DON and ZEA levels by between 40 and 100% (Trenholm et al., 1991).

Lee et al. (1987) examined the use of milling to reduce NIV, DON and ZEA levels in Korean wheat. Concentrations of the three *Fusarium* toxins were reduced by between 20 to 69%, in flour fractions intended for human consumption. Similar results were observed by both Young et al. (1984) and Scott et al. (1984) for DON contamination of Canadian wheat.

Physical and/or insect damage, and subsequent fungal contamination with various *Penicillium* species have been cited as the cause of patulin contamination of apples and their products. Lovett et al. (1975) observed that removal of the damaged portion of fruit, prior to further processing, could significantly reduce patulin concentrations in the end products.

In addition to these practices, *in situ* physical methods, including heat treatments such as roasting, baking, cooking and exposure to either UV or gamma radiation, have been shown to

reduce mycotoxin levels in various commodities (Samarajeewa, 1991).

6.1.2 *Solvent extraction of mycotoxins*

Although the removal of aflatoxins from crude edible oils during commercial processing is a successful method of detoxification, simple extraction of crude peanut oil with 10% aqueous sodium chloride (NaCl) at elevated temperatures (80^{o}C for 30 minutes) can reduce aflatoxin levels by 85% (Shantha & Sreenivasamurthy, 1975). Several organic solvent systems, especially those based on acetone and ethanol, have also been found to effectively remove aflatoxins from ground grain meals and flours (West & Bullerman, 1991).

Trenholm et al. (1992) investigated washing techniques for the removal of DON and ZEA from barley, maize and wheat. Exhaustive treatment of barley and maize with H_2O reduced DON concentrations by between 65 and 69%, and by between 2 and 61% for ZEA. Treatment with 1M Na_2CO_3 reduced DON and ZEA levels by between 72 and 74%, and by between 80 and 87%, respectively. The introduction of gentle stirring in the process caused reductions of DON levels in barley by between 80 to 95%, while the soaking of barley, maize and wheat in a 0.1M Na_2CO_3, for 24 and 72 hours, caused reductions in DON concentrations by between 42 and 100%.

6.1.3 *Biological treatments*

Numerous bacteria, yeasts and moulds are able to either degrade or remove aflatoxins from solution (Bhatnagar et al., 1991). Of 100 micro-organisms screened, Ciegler et al. (1966) identified *Flavobacterium aurantiacum* as one that could effectively remove aflatoxin B_1, while Lillehoj et al. (1971) showed that the same bacterium could remove aflatoxin M_1 from milk. Aflatoxicol (aflatoxin R_0 - a less active metabolite than the parent aflatoxin B_1), has been formed by the action of a variety of organisms, used during the treatment of aflatoxin B_1 contamination in solution (Bhatnagar et al., 1991).

The alcoholic fermentation of apple juice, in the presence of *Saccharomyces cerevisiae*, has resulted in a reduction of patulin levels in cider products by up to 90% (Burroughs, 1977).

The feeding of aflatoxin-contaminated grains to animals may also result in the biological destruction of the aflatoxins (Bhatnagar et al., 1991). Aflatoxin metabolites are often formed during metabolic processes, and these can be passed on to various animal products such as meat, milk or eggs. However, the levels of these metabolites in animal products tend to be significantly lower, and predominantly less toxic, than the parent compound consumed by the animals (Bhatnagar et al., 1991).

Several additional mycotoxins, including the trichothecenes, ochratoxin, ZEA and rubratoxin have been shown to undergo degradation (detoxification) within biological systems (Bhatnagar et al., 1991). Since these processes are generally considered to be enzymatic in nature, they tend to offer less harsh degradation routes than other decontamination processes. It has been suggested that the main application of biological detoxification techniques may be their use in integrated mycotoxin prevention strategies, rather than their present application as reclamation measures (Bhatnagar et al., 1991).

6.1.4 *Chemical decontamination procedures*

Due to their ubiquitous nature, relative chemical stability and extreme toxicity, the aflatoxins have been the subject of the majority of work associated with the development of chemical decontamination procedures (Pemberton & Simpson, 1991).

Since the toxicity of the aflatoxin hemiacetal derivative (aflatoxin B_{2a}) is approximately 1/200th that of aflatoxin B_1, efforts have centered on the efficiency of acidic treatments of aflatoxin-contaminated meals (Pemberton & Simpson, 1991). Although work by Lindenfelser & Ciegler (1970) measured significant reductions in aflatoxin B_1 levels (by up to 96%), follow-

ing treatment with 1M HCl, the resistance of the dihydro-aflatoxins (B_2 and G_2) to acidic treatment suggest that the process will be unable to reduce total aflatoxin levels in contaminated grains.

Bisulfite treatment of aflatoxin-contaminated maize indicated that only partial decontamination (± 50%) was induced (Hagler et al., 1982). This treatment process has however been used successfully to reduce patulin concentrations in apple juice samples (Burroughs, 1977). Patulin concentrations have also been reduced by the treatment of the pressed juice and juiced products with both ascorbic acid (Brackett & Marth, 1979) and charcoal (Van, 1989).

Alkaline treatments have been applied to the decontamination of aflatoxin-contaminated foods. Dollear et al. (1968) treated peanut meal with a concentrated (5.8%) solution of sodium hydroxide (NaOH) at 100^{o}C for 90 minutes. The resultant feed, upon drying, was shown to be non-toxic to ducklings or rats. Both Mann et al. (1970) and Moerck et al. (1980) noticed significant reductions in aflatoxin levels in both peanut and maize meals, respectively, when using 3% and 2% NaOH solutions in conjunction with moisture adjustments of the respective meals to around 20 and 22%. Lakshmirajam et al. (1984) observed aflatoxin reductions of 81 and 90% in samples of groundnut cake and maize, following treatment with 2% NaOH under pressure. In feeding trials, the treated meals did not suppress growth rates in broiler chicks.

Mann et al. (1970) also investigated the use of calcium hydroxide [$Ca(OH)_2$] for the decontamination of aflatoxin-contaminated peanut meal, although erratic results were obtained. Esproy (1972) stated that successful treatment with $Ca(OH)_2$, required the meal to have a mean particle size of 50 μm. The manufacture of tortillas requires that maize be treated with ± 1% aqueous $Ca(OH)_2$ in order to loosen the husks, however Price & Jorgensen (1985) observed only a 40% reduction in aflatoxin levels when contaminated maize was subjected to the $Ca(OH)_2$ treatment process.

The use of volatile amines has also been investigated. Dollear et al. (1968) added methylamine, as a 40% solution, to contaminated peanut meal and observed reductions in aflatoxin content to less than 5 ppb. Park et al. (1981) used methylamine together with $Ca(OH)_2$ to monitor the fate of ^{14}C-labelled aflatoxin B_1 added to peanut meal, which was then treated for 1 hour at 100°C. The process reduced aflatoxin levels by between 94 and 100%, but the majority of the radioactivity remained in the meal (69 to 80%), with the balance being retained in the H_2O soluble phase. Park et al. (1981) observed a number of by-products of the treatment, but most appeared to be significantly less toxic than the parent aflatoxin B_1 moiety.

Ammonia, either as ammonium hydroxide in solution or in a gaseous form, has been used in conjunction with various temperatures, times, moisture contents and pressures to reduce aflatoxin levels in various aflatoxin-contaminated feedstuffs (Pemberton & Simpson, 1991).

Peanut meal has been ammoniated on an industrial scale in a number of countries (Pemberton & Simpson, 1991), and has involved the heating of 15% moisture-corrected meals to 80°C for 20 minutes, with the injection of gaseous ammonia at 2 bars. Indian workers have achieved an 85% reduction in the aflatoxin content of peanut meal, using urea as a cheaper source of ammonia (Shantha et al., 1986). The feeding of ammoniated-meals to various animal species has generated favourable results. McKinney et al. (1973) failed to observe the production of aflatoxin M_1 (a metabolite of the parent B_1 analogue) in the milk of lactating cows fed diets consisting of ammoniated meals, suggesting that ammoniated feed could be safe for ruminants.

Brekke et al. (1977) made use of an aqueous ammonia source and atmospheric pressure to reduce aflatoxin contamination of maize, and observed that increased moisture and temperature favoured detoxification. The use of ammonia gas in place of the aqueous medium was also found to be effective under similar temperature and pressure conditions, resulting in a 100-fold

reduction of aflatoxin B_1 levels, from 1000 to 10 ppb (Brekke et al. (1978). Subsequent toxicity studies on ammonia-treated meals in trout (Brekke et al. 1977), rats (Norred & Morrissey, 1983) and chickens (Hughes et al., 1979; Hughes & Jones, 1979) have been encouraging.

The excellent results associated with the ammoniation treatment of aflatoxin-contaminated cereals has prompted investigations into the identification of the degradation products (Pemberton & Simpson, 1991). These studies have identified several intermediary phenol-based compounds, with the final step involving the base-induced lactone ring-opening of the coumarin system, present in the structure of the aflatoxins (Figure 6.1)

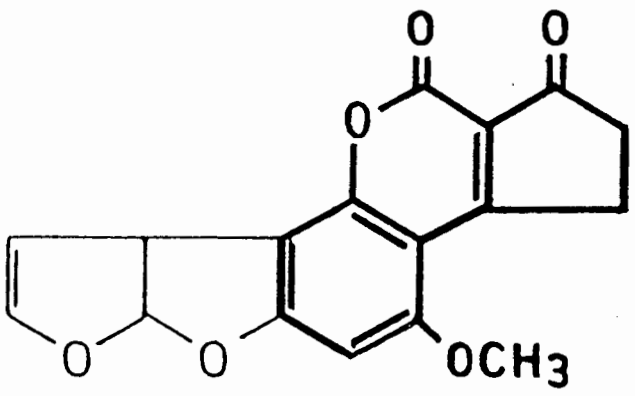


Figure 6.1 Structure of aflatoxin B_1, with the identification of the coumarin ring system (in bold)

While many of the chemical treatments are effective in the removal of aflatoxins from foodstuffs, ammoniation has additionally been shown to be both a practical and cost-efficient method. The treatment appears to degrade the coumarin ring structure, whereas the bisdihydrofuran structure, normally associated with the observed toxicity in animals, remains intact (Pemberton & Simpson, 1991). This observation would suggest that the presence of the coumarin system is essential

for toxicity, and implies that treatments that alter its structure are as useful as those targeted at the bisdihydrofuran structure (Pemberton & Simpson, 1991). It has been suggested that chemical treatments, and especially ammoniation, might be useful for the removal of several other mycotoxins such as the trichothecenes, ochratoxins and ZEA, from grain commodities (Pemberton & Simpson, 1991).

6.1.5 *Fumonisins - strategies for their removal from maize*

The realisation that the fumonisins appear to be ubiquitous contaminants of maize and maize-based products (Sydenham et al., 1991), has resulted in the investigation of potential fumonisin-decontamination procedures. Alberts et al. (1990) observed that heat treatment of fumonisin-contaminated culture material of *F. moniliforme*, did not result in either a reduction in fumonisin levels, or in the cancer-initiating potential of the heat-treated material. The heat stability of the fumonisins has subsequently been confirmed by Doko & Visconti (1993) and by Dupuy et al. (1993). Norred et al. (1991) and Park et al. (1992) used both atmospheric and high-pressure conditions together with various temperatures, to monitor the effect of ammoniation on fumonisin levels in maize. Essentially, Norred et al. (1991) recorded partial reductions in fumonisin levels, but the toxicity of the treated material was retained, although Park et al. (1992) observed FB_1 reductions by about 80%. Bothast et al. (1992) evaluated the use of fumonisin-contaminated maize for the production of ethanol. They recorded little degradation of FB_1 during processing, however most of the toxin was recovered in the distillers' grains, silage and soluble fractions, with none of the toxin being detected in either the distilled alcohol of centrifuge solids (Bothast et al., 1992). The authors concluded that ethanol fermentation of fumonisin-contaminated maize, coupled with further detoxification of the distillers' grains and silage, could be an effective and practical process for the reclamation of fumonisin contaminated maize (Bothast et al., 1992).

6.1.6 *Aims of the present study*

Based on observations related to the visual assessment of maize imported into South Africa, an evaluation of the effect on fumonisin levels, by the physical removal of selected fractions was investigated in the present study. In addition, based on the results of the analyses of commercially available alkali-treated maize products (section 5.4.4), an investigation into the effect of $Ca(OH)_2$ on fumonisin levels in maize, was also undertaken.

6.2 *EXPERIMENTAL*

6.2.1 *Physical decontamination procedure*

6.2.1.1 *Selection of maize samples*

A series of 54 maize samples, each of approximately 5 kg in mass, were collected from a bulk consignment of more than 48,000 tonnes, imported by ship into South Africa from the USA (section 5.3.3.1). The initial samples were withdrawn by pneumatic probe at 3 sampling sites selected laterally across each hold of the ship. Ten of the 54 samples were randomly selected and retained at 4°C prior to further treatment.

6.2.1.2 *Physical treatment of selected samples*

Each sample was fractionated by passing through a 3mm wire screen (sieve). The weights of the fractions which passed through the screen (termed "*fines*") as well as those that were retained (termed "*kernels*"), were recorded. The "*kernels*" fractions of each sample were mixed and riffled to improve homogeneity, after which 1 kg subsamples were removed and ground to fine meals in a laboratory mill, to pass a 840 μm sieve. The total "*fines*" fraction of each sample was ground in a laboratory mill, and similarly treated. All ground samples were stored at 4°C prior to analyses.

6.2.1.3 *Fumonisin analyses*

The ground maize samples were subjected to further mixing and riffling, prior to removal of 50 g subsamples which were used for the analytical determination of fumonisins, in accordance with the method of Sydenham et al. (1992b) (section 3.4).

6.2.2 *Chemical decontamination procedure*

6.2.2.1 *Effect of pH on the stability of FB_1*

Aqueous solutions (0.1M, 0.25M, 0.5M and 1.0M) were prepared [from solutions of NaH_2PO_4, NaOH, $Na_2B_4O_7$, disodium hydrogen phosphate (Na_2HPO_4), HCl and potassium chloride (KCl), of corresponding molarities] to give pH values equivalent to 7, 9, 11 and 13. Aliquots (150 μl), of the four solutions were placed in a series of individual vials. Aliquots (50 μl) of FB_1 in H_2O (50 μg/ml) were added to each of the vials which were then capped, mixed and placed on a heating block maintained at 100°C for 1 hour. The vials were subsequently removed and allowed to cool to room temperature.

6.2.2.2 *HPLC separation of FB_1 and its aminopentol moiety*

Aliquots (25 μl) of each solution (prepared as in section 6.2.2.1) were derivatised with OPA reagent (225 μl - section 3.4.2.3), and 10 μl aliquots were separated by HPLC on a Phenomenex column (250 x 4.6 mm i.d.) packed with 5 μm Ultracarb ODS 30 material. The mobile phase used was CH_3OH:0.1M NaH_2PO_4 (80:20) adjusted to pH 3.35 with *o*-phosphoric acid.

The resultant chromatograms were compared with those observed for FB_1 and its hydrolysed aminopentol moiety (AP_1), which was prepared in accordance with section 3.2.8.1.

6.2.2.3 *Effect of temperature and time on FB_1 breakdown at pH 13*

A 0.5M aqueous solution was prepared from 0.5M NaOH and 0.5M

HCl to give a pH of 13. Aliquots (150 μl) of FB_1 in CH_3OH (100 μg/ml) were placed in 7 separate vials, and the solvent was removed under nitrogen. The residues were reconstituted in 300 μl volumes of the pH 13 solution. The vials were capped, mixed and individually maintained at 20, 40, 60, 70, 80, 90 and 100°C for a period of 1 hour, after which the contents were allowed to cool to room temperature. Aliquots were withdrawn and screened by HPLC in accordance with the conditions specified in section 6.2.2.2.

Similar solutions (at pH 13) containing FB_1 were prepared, mixed, capped and maintained at 100°C for 10, 20, 30, 40, 50, 60, 70 and 80 minutes, prior to being removed, cooled and analysed by reversed-phase HPLC as in section 6.2.2.2.

6.2.2.4 *Effect of $Ca(OH)_2$ on the stability of FB_1, and a comparison of calcium, potassium and sodium hydroxides*

The effects of time and temperature on the stability of FB_1 (in accordance with the methods cited in section 6.2.2.3), were repeated using 0.01M $Ca(OH)_2$ (pH = 12.2), in place of the pH 13 solution. Aliquots (150 μl) of a solution of FB_1 in CH_3OH (100 μg/ml) were evaporated to dryness in 3 separate vials, and redissolved in 300 μl volumes of either 0.01M $Ca(OH)_2$, 0.02M NaOH or 0.02M KOH. The vials were capped and heated for 1 hour at 100°C.

An additional vial was similarly prepared, the FB_1 dissolved in 0.1M $Ca(OH)_2$, and the vial was left to stand for 24 hours at room temperature (23°C). All resultant solutions were analysed by HPLC as cited in section 6.2.2.2.

6.2.2.5 *Effect of $Ca(OH)_2$ on FB_2 and FB_3*

Solutions of FB_2 and FB_3 (100 μg/ml) were prepared in CH_3OH, and aliquots were added to 0.1M $Ca(OH)_2$ (300 μl - as in section 6.2.2.4). The solutions were allowed to stand at room temperature for 24 hours, before aliquots were removed and analysed by

HPLC in accordance with the conditions cited in 6.2.2.2.

6.2.3 *$Ca(OH)_2$ treatment of fumonisin-containing ground maize*

A 500 g quantity of maize, previously shown to be naturally contaminated with FB_1 at *ca* 8 μg/g, was ground to a fine meal and riffled/blended to improve homogeneity. Three 100 g subsamples were withdrawn and placed into 500 ml glass beakers. A slurried solution (400 ml) of 0.1M $Ca(OH)_2$ was added to each beaker together with a magnetic stirring bar. The beakers were covered and the solutions stirred slowly for 24 hours. Thereafter for each treated sample, the aqueous $Ca(OH)_2$ phase was separated from the maize meal by filtration. The meal was washed with 2 x 150 ml volumes of H_2O, filtered, and then dried at 110^{o}C for 4 hours before being ground in a laboratory mill. Both the meal and the aqueous $Ca(OH)_2$ phases were then analysed for FB_1 and AP_1, according to the methods in section 6.2.3.1 and 6.2.3.2, respectively.

6.2.3.1 *Determination of FB_1 and AP_1 in $Ca(OH)_2$-treated maize*

A 25 g subsample of the dried $Ca(OH)_2$-treated maize was extracted with 50 ml CH_3OH:0.01M ethylenediaminetetra acetic acid (EDTA) (1:1), by blending for 3 minutes in a Polytron homogeniser. The solution was centrifuged, filtered, and the pH of a 10 ml aliquot was adjusted to 2.5 with 0.1M HCl. Any resultant precipitate was separated by further centrifugation, prior to application to a pre-conditioned (5 ml H_2O) Chromabond C_{18}ec SPE cartridge, or its Bond Elut C_{18} equivalent (each containing 500 mg of sorbent). The cartridge was washed with H_2O (3 ml), followed by CH_3OH:H_2O (25:75, 5 ml) and the FB_1 and AP_1 were eluted with CH_3OH (5 ml for the Chromabond column and 15 ml for the Bond Elut equivalent). The solvent was removed under nitrogen at 60^{o}C, and the residues redissolved in CH_3OH (200 μl). An aliquot (50 μl) was derivatised with OPA solution (200 μl) and analysed by HPLC (see section 6.2.2.2).

6.2.3.2 *Determination of FB_1 and AP_1 in $Ca(OH)_2$ fraction following treatment of ground maize*

A 10 ml aliquot of the $Ca(OH)_2$ solution was adjusted to pH 2.5 with 1M HCl, and where necessary any precipitate was removed by centrifugation. The pH-adjusted $Ca(OH)_2$ solution was applied to either of the SPE columns specified in section 6.2.3.1, and the column was washed with H_2O (4 ml) followed by $CH_3OH:H_2O$ (1:1; 3 ml). The FB_1 and AP_1 were eluted with CH_3OH (5 ml for the Chromabond cartridge or 15 ml for the Bond Elut equivalent). The CH_3OH was evaporated to dryness, and the residue reconstituted in $CH_3OH:H_2O$ (75:25, 400 µl). The extracts were then analysed as their OPA derivatives, using the conditions cited in section 6.2.2.2.

Table 6.1 HPLC conditions for the separation of the unconfirmed partially hydrolysed moiety of FB_1

Column	: Ultramex 3 µm C_{18} (100 x 4.6 mm i.d.)
Mobile phase	: CH_3OH:0.1M NaH_2PO_4 (67:33)
pH	: Unadjusted at pH 6.0
Flow rate	: 1 ml/minute
Detector	: Fluorescence
Excitation	: 335 nm
Emission	: 445 nm

6.2.3.3 *$Ca(OH)_2$ treatment of maize kernels*

Maize kernels (100 g), collected from the Transkei (and known to be contaminated with 8200 ng/g FB_1), were placed in a beaker together with 300 ml 0.1M $Ca(OH)_2$. The contents were agitated by magnetic stirring for 24 hours, in a controlled environment at 25°C. The resultant mixture was filtered to separate the kernels from the aqueous phase. The kernels were washed with H_2O, and then separated by hand into those fractions showing

evidence of:

(a) partial removal of the outer pericarp layer, and

(b) total removal of the outer pericarp.

The separate fractions were dried in an oven at 100°C for 4 hours, ground to fine meals and analysed for FB_1 and AP_1 in accordance with the method cited in section 6.2.3.1.

6.2.4 *Isolation and purification of the partially hydrolysed moiety (PH_1) of FB_1*

6.2.4.1 *Preliminary study*

Culture material of *F. moniliforme* MRC 826 (10 g) was mixed with 200 ml H_2O, to which 1.48 g $Ca(OH)_2$ was added (to give a 0.1M slurry solution). The contents were constantly mixed, by magnetic stirring, and 1 ml fractions were removed every hour over a 9 hour period. Similar fractions were removed after 23 and 24 hours exposure, respectively. All fractions were centrifuged in a Beckman Mini-centrifuge, and 25 µl aliquots of the supernatants were removed, derivatised by the addition of 225 µl OPA reagent, and analysed by HPLC using the conditions cited in Table 6.1.

6.2.4.2 *Extraction of PH_1*

Thirty grams of culture material of *F. moniliforme* MRC 826 were added to a 1 litre beaker, to which 600 ml 0.1M $Ca(OH)_2$ were added. The contents were mixed by magnetic stirring for 4.5 hours at 27°C. The contents of the beaker were then transferred to several 250 ml capacity bottles, and centrifuged at 500 x g for 10 minutes. The combined supernatants were filtered under vacuum through a Whatman No 4 filter paper, and the pH of the solution was altered to 2.7 by the addition of 5M HCl.

6.2.4.3 *Initial purification of crude extract*

A large chromatography column (50 cm x 3 cm i.d.) was packed with Amberlite XAD-2 resin (previously washed sequentially with 400 ml diethyl ether, 400 ml CH_3OH and 1000 ml H_2O) in H_2O, to give a chromatographic bed of 22 cm. The $Ca(OH)_2$ solution was added to, and passed through the column at a flow rate of approximately 10 ml/minute. The column was then washed with H_2O (400 ml), followed by CH_3OH:H_2O (25:75, 400ml), and eluted with CH_3OH (800 ml). The CH_3OH eluate was collected and evaporated to dryness by rotary distillation under vacuum at 50°C.

6.2.4.4 *Three-stage silica gel chromatography*

A chromatography column (50 cm x 3 cm i.d.) was packed with Kieselgel 60 (0.063-0.2 mm dia. - Machery Nagel, Duren) in $CHCl_3$:CH_3OH:H_2O:CH_3COOH (55:36:8:1 - solvent A), to give a chromatographic bed of 42 cm. The residue from the Amberlite XAD-2 column was dissolved in 10 ml of solvent A, and applied to the top of the Kieselgel column. The column was eluted with solvent A and 20 ml-fractions were collected.

Each fraction was screened by normal-phase TLC using silica gel 60 TLC plates (Merck, Germany). The plates were developed with solvent A as the mobile phase, removed, dried, sprayed with a solution of 0.1% *p*-anisaldehyde and heated at 110°C for 10 minutes. The fractions were also monitored, as their OPA derivatives, by reversed-phase HPLC using the conditions in Table 6.1.

Those fractions containing PH_1 (#11-20) were combined and evaporated to dryness at 50°C. The resultant residue was subsequently dissolved in a 15 ml volume of ethyl acetate ($CH_3COOC_2H_5$):CH_3COOH:H_2O (12:6:1 - solvent B). The solution was added to the top of a second Kieselgel 60 column (of similar dimensions to those of the first), prepared in solvent B. The column was washed with 750 ml of solvent B, which was discarded, and eluted with 1000 ml solvent A. The eluate was collected and evaporated to dryness under vacuum at 50°C.

A third Kieselgel 60 column (15 cm x 3 cm i.d.) was prepared in solvent B. The dried residue from the second Kieselgel column was redissolved in 5 ml of solvent B, and applied to the top of the third column, which was eluted with the same solvent system at a flow rate of ± 5 ml/minute. Fractions (15 ml), were collected and screened by both TLC and HPLC. Those fractions containing PH_1 (#16-40) were pooled and evaporated to dryness under vacuum at 50°C.

6.2.4.5 *Two stage mini-column purification*

A short SAX chromatographic column (5 cm x 13 mm i.d.) was prepared by adding together the sorbent contents of six SAX cartridges (specified in section 3.4.1.6). The short column was then washed with CH_3OH (20 ml) followed by H_2O (40 ml). The residue from the third Kieselgel column was dissolved in CH_3OH:H_2O (75:25), and the pH of the solution was adjusted to 6.2 with 1M NaOH. This pH-adjusted solution was applied to the SAX column and washed with 30 ml CH_3OH. All eluates, including the application solution, were collected, combined and taken to dryness by rotary evaporation.

A second mini-column (Chromabond C_{18}ec - 5 cm x 13 mm i.d.) packed with 1 g sorbent, was used for final purification. The residue from the SAX mini-column was dissolved in 20 ml H_2O, and the pH adjusted to 2.9 with 3M HCl. The column was conditioned with H_2O, and the solution applied. The column was then washed with 40 ml H_2O followed by 20 ml CH_3OH:H_2O (1:3). The purified PH_1 was eluted with 40 ml CH_3OH:H_2O (1:1), and the eluate was concentrated to dryness in a 4 ml capacity vial under dry nitrogen. Residual solvent was removed under vacuum.

6.2.5 *Preparation of PH_1 moiety from a FB_1 standard*

Analytically pure FB_1 (2 mg) was dissolved in 0.1M $Ca(OH)_2$ (40 ml) and the solution was gently stirred, at room temperature, for 4.5 hours. The solution was acidified to pH 6.2 with 2M HCl, and applied to a small chromatographic column packed with ion exchange media (2 g - specified in section 3.4.1.6).

The column was washed with 40 ml H_2O, and the combined eluates (including the application solution) were collected and further acidified to pH 2.9. This solution was applied to a Chromabond C_{18}ec cartridge (specified in section 6.2.4.5), which was then washed with 40 ml H_2O. The PH_1 and AP_1 moieties were eluted with CH_3OH:H_2O (4:6). Fractions (2 ml) were sequentially collected and screened by HPLC as their OPA derivatives, using the conditions cited in Table 6.1.

In addition, TCA (tricarballylic acid; 1 mg) was dissolved in 0.01M $Ca(OH)_2$ (1 ml) and heated to 100°C for 1 hour, before being cooled and retained for further analysis.

6.2.6 *Confirmation of PH_1, AP_1 and TCA moieties*

Mass spectra (over the range 200 to 800 m/z) were recorded using a VG Platform, configured for electrospray operation, coupled to a Pharmacia LC pump operated at 10 µl/min. Mass spectra were obtained with low cone voltage settings, in order to reduce fragmentation, and aliquots of the individual moieties were introdcued into the mass spectrometer by flow injection analysis of approximately 200 ng per component.

6.3 *RESULTS AND DISCUSSION*

6.3.1 *Physical treatment of imported maize*

Visual assessment of the 10 selected maize samples was, in each case, characterised by a wide distribution with respect to the size of the particulate matter. Physical separation of the "*kernels*" fractions (Figure 6.2.1) from the "*fines*" fractions (Figure 6.2.2) by passing through a 3 mm screen, clearly showed the presence of chipped kernels, dust and chaff in the "*fines*" fractions.

The weights of the separate fractions (*kernels* and *fines*), expressed as a percentage of the total weight of each sample, together with the corresponding fumonisin levels, are presented

in Table 6.2.

Figure 6.2 Imported maize separated into (1) "*kernels*" fraction (>3 mm diameter) and (2) "*fines*" fraction (<3 mm diameter)

The combined fumonisin concentrations (FB_1+FB_2+FB_3) in the "*kernels*" fractions of the 10 selected samples, ranged from 530 to 1890 ng/g, and accounted for between 80.0 and 95.3% of the total mass of the samples (Table 6.2). Although the "*fines*" fractions accounted for between only 4.7 and 20.0% of the original mass of the samples, their combined fumonisin concentrations (measured at between 12340 and 27460 ng/g), were substantially higher than those recorded for each of the corresponding "*kernels*" fractions (Table 6.2). The combined fumonisin levels determined in each of the "*fines*" fractions were similar in magnitude to those levels previously associated with field outbreaks of equine LEM (Ross et al. 1991a,b, 1992; Thiel et al. 1991b; section 4.3). This is perhaps not surprising, since several reports involving field outbreaks of equine LEM have referred to the consumption of "*corn screenings*" by the affected animals (Harrison et al., 1990; Ross et al., 1992).

Table 6.2 Percentage by weight, fumonisin levels (and relative percentages of total fumonisins), recorded in the "*kernels*" and "*fines*" fractions of ten imported maize samples

Sample number	% By weight	Fumonisin concentrations (ng/g)						
		FB_1	(%)	FB_2	(%)	FB_3	(%)	Total
				"*Kernels*" fractions				
1	95.3	1270	(70.1)	410	(22.7)	130	(7.2)	1810
2	91.8	360	(67.9)	120	(22.6)	50	(9.5)	530
3	80.0	1210	(70.7)	360	(21.1)	140	(8.2)	1710
4	91.5	1250	(66.1)	430	(22.8)	210	(11.1)	1890
5	88.6	640	(70.3)	220	(24.2)	50	(5.5)	910
6	94.7	750	(69.4)	250	(23.2)	80	(7.4)	1080
7	92.4	510	(65.4)	190	(24.4)	80	(10.2)	780
8	92.7	1070	(67.3)	380	(23.9)	140	(8.8)	1590
9	93.6	460	(68.7)	140	(20.9)	70	(10.4)	670
10	94.7	550	(67.9)	190	(23.5)	70	(8.6)	810
				"*Fines*" fractions				
1	4.7	11500	(67.6)	3770	(22.2)	1740	(10.2)	17010
2	8.2	9340	(69.1)	3050	(22.5)	1130	(8.4)	13520
3	20.0	12850	(68.8)	4050	(21.7)	1770	(9.4)	18670
4	8.5	11070	(68.7)	3710	(23.0)	1340	(8.3)	16125
5	11.4	8660	(70.2)	2720	(22.0)	960	(7.8)	12340
6	5.3	21350	(77.7)	3660	(13.4)	2450	(8.9)	27460
7	7.7	13020	(71.5)	3720	(20.4)	1470	(8.1)	18210
8	7.3	13460	(70.0)	4160	(21.6)	1610	(8.4)	19230
9	6.4	18290	(68.0)	6320	(23.5)	2300	(8.5)	26910
10	5.3	14370	(68.2)	4770	(22.6)	1940	(9.2)	21080

No major differences in the distribution of the individual fumonisins were however observed in the separate fractions, since all three naturally occurring analogues co-occurred in each of the fractions. The distribution patterns for each fumonisin analogue, in both fractions, were similar for all samples (Table 6.2). As with previous observations (section 5.4.3) FB_1 was the major naturally occurring fumonisin analogue, accounting for between 65.4 and 77.7% of the total fumonisin concentrations, with the FB_2 and FB_3 levels accounting for between 13.4 and 24.4%, and between 5.5 and 11.1% of the total fumonisin levels, respectively (Table 6.2).

Problems (errors) associated with the collection of representative grain samples from bulk consignments have been highlighted (Garfield, 1989). A major source of error stems from the heterogeneous distribution of numerous contaminants, especially mycotoxins, in grain commodities such as wheat and maize (Horwitz, 1983a,b). Due to the wide variance in particulate size of the selected imported maize samples (Figure 6.2), initial stratification prior to analyses, improved the subsampling plan by enabling the analyses of subsamples that were more representative of the consignment.

From the data presented in Table 6.2, calculations could be made to indicate the corrected or "*true*" fumonisin levels in the 10 individual maize samples (Table 6.3). These *"calculated* values, when compared with the levels determined in the corresponding "*kernels*" fractions, reflect the effect that prior removal of the "*fines*" would have on the overall fumonisin levels.

The combined $FB_1+FB_2+FB_3$ "*calculated*" levels, found to range from 1450 to 4590 ng/g (Table 6.3), were in each case considerably higher than the levels determined in the corresponding "*kernels*" fractions. The data indicated that for the selected samples, physical removal of the "*fines*" resulted in substantial reductions in combined fumonisin concentrations, of between 26.2 and 69.4% (Table 6.3).

Table 6.3 Comparison of total fumonisin (FB_1+FB_2+FB_3) levels "*calculated*" in the maize samples, with those determined in the "*kernels*" fractions

Sample number	Fumonisin concn. (ng/g)		Percentage reduction in total fumonisin levels following removal of "*fines*"
	Calculated levels[a]	*Kernels* fractions[b]	
1	2520	1810	28.2%
2	1450	530	63.5%
3	4590	1710	62.7%
4	2560	1890	26.2%
5	1970	910	53.8%
6	2470	1080	56.4%
7	1900	780	58.9%
8	2440	1590	34.8%
9	2190	670	69.4%
10	1710	810	52.6%

[a]Determined values based on the data in Table 6.2
[b]Data presented in Table 6.2

Removal of fine particulate matter from bulk consignments might therefore be considered as a preliminary fumonisin decontamination procedure. With respect to other potential physical decontamination procedures, Viljoen et al. (1993) observed the progressive reduction in mean FB_1 and FB_2 levels in six successive fractions, which formed part of the milling process of South African production maize. They recorded combined fumonisin (FB_1+FB_2) levels ranging from greater than 3000 ng/g in maize screenings, to less than 160 ng/g in finely ground maize (Viljoen et al., 1993).

Although the "*fines*" fractions of the selected maize samples accounted for between 4.7 and 20.0% of the total weights, 8/10 contained "*fines*" corresponding to less than 10% of the total

mass of the individual samples (Table 6.2). Further studies will be necessary to determine the optimum sieve (screen) size required for the removal of "*fines*", although Murphy et al. (1993) reported that no significant size-associated segregation of FB_1 levels was observed for maize screenings passed through a series of 8 sieves. Ultimately, economic considerations and legislative action will determine the significance, if any, that the removal of fines might have on the overall reduction of both animal and human exposure to the fumonisin mycotoxins.

6.3.2 *Chemical treatment of maize*

6.3.2.1 *The effect of pH on the stability of FB_1*

Despite heating solutions to 100°C for 1 hour, FB_1 remained stable at pH values of 7, 9 and 11. Increasing the strength of the solution (from 0.1M through to 1.0M) had no effect on the stability of FB_1, suggesting that FB_1 is relatively stable over the pH range 7 to 11.

However, heating of FB_1 in a 0.1M solution at pH 13 (under similar conditions) resulted in the partial (13%) hydrolysis of FB_1 to its corresponding AP_1 and TCA moieties. The identity of the AP_1 was initially confirmed by "spiking" the hydrolysate with authentic AP_1 (prepared according to the method cited in section 3.2.8.1), and re-chromatographing the resultant OPA derivative. The degree of hydrolysis increased dramatically when FB_1 was similarly treated at pH 13, in solutions of higher molarity. An 85% conversion of FB_1 to AP_1 was observed in the presence of a 0.25M solution, while total conversion was observed following exposure of FB_1 to both 0.5M and 1.0M solutions, under the same temperature conditions and reaction time.

These results indicated that the stability of FB_1, under alkaline conditions, was dependant primarily on the pH of the solution (ie. hydrolysis occurred between pH 11 and 13), while solution strength also exhibited an effect on the extent of hydrolysis.

6.3.2.2 *Effect of temperature and time on the stability of FB_1 dissolved in a 0.5M NaOH/HCl solution at pH 13*

By maintaining a reaction time of 1 hour (using a 0.5M solution of NaOH/HCl at pH 13) and altering the temperature, the effect of temperature on the conversion of FB_1 to AP_1 could be monitored. The results are illustrated graphically in Figure 6.3.1. It was possible to observe the stoichiometric hydrolysis of FB_1 to AP_1, whereby a 50% conversion occurred at between 60 and 70°C. The degree of conversion increased such that complete hydrolysis was observed at between 90 and 100°C (Figure 6.3.1).

By changing the treatment time and retaining the temperature constant (ie. at 100°C), it was possible to monitor the effect that reaction time had on the hydrolysis process. Figure 6.3.2 displays the gradual (and stoichiometric) conversion of FB_1 to its aminopentol moiety. The results clearly show that complete hydrolysis required the temperature to be maintained for between 70 and 80 minutes.

6.3.2.3 *Effect of $Ca(OH)_2$ on the stability of FB_1*

In section 5.5.4, several maize-based human foodstuffs, treated with $Ca(OH)_2$ as part of the manufacturing process, were shown to contain no, or very low levels of the fumonisin mycotoxins. Only one of five samples contained a detectable level of FB_1 (at the detection limit of the method). These data were particularly significant in view of the fact that the vast majority of the other maize-based samples, obtained from the same regions as the alkali-treated samples, contained far higher fumonisin levels. The observation that $Ca(OH)_2$-treated maize products contained low fumonisin levels was subsequently confirmed by others (Pittet et al., 1992; Stack & Eppley, 1992; Doko & Visconti, 1993).

In each case, the products were (maize-based) tortilla preparations, in which the maize had initially been soaked and heated in the presence of $Ca(OH)_2$ (usually a 1% w/v solution) - a process often referred to as *nixtimilization* (Hendrich et

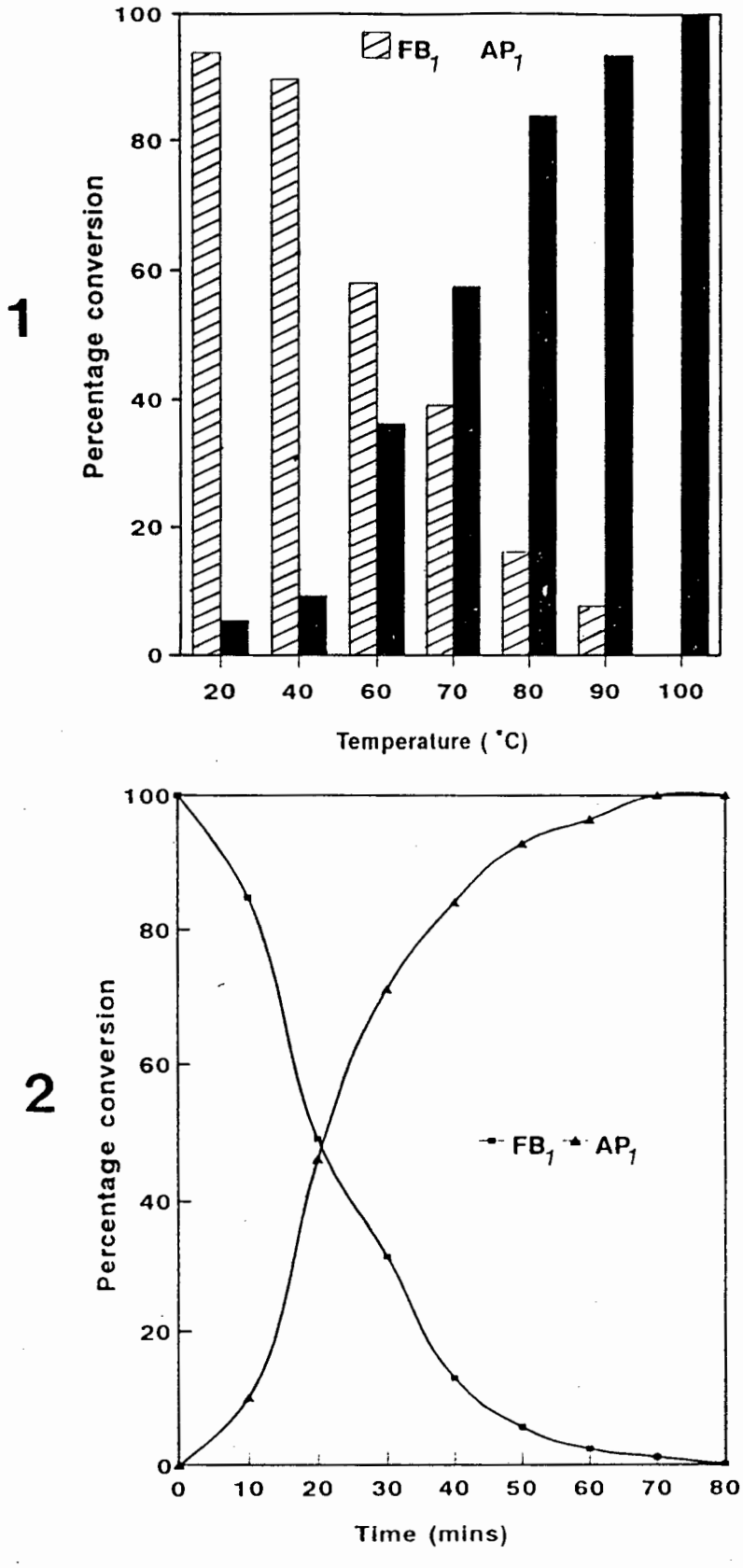


Figure 6.3 The effects of temperature (1) and time (2) on the hydrolysis of FB_1 to its corresponding AP_1 moiety, in the presence of a 0.5M NaOH/HCl solution at pH 13

al., 1993). End-product tortilla preparations therefore involve the processing of the $Ca(OH)_2$-treated maize, a commodity that is more commonly known as "*masa*" (ie. tortilla flour).

Calcium hydroxide is relatively insoluble with maximum solubility occurring at a concentration of 0.014M (which is approximately a 0.1% w/v solution). Therefore, treatment of maize in the production of "*masa*" must involve exposure of the kernels to a super-saturated (1%) $Ca(OH)_2$ solution, which might better be described as a "slurry". Exposure to a 1% slurried solution of $Ca(OH)_2$ for a period of 1 hour at 100°C, resulted in the hydrolysis of FB_1 to its AP_1 and TCA moieties. The same degree of hydrolysis was also observed when FB_1 was similarly treated with a 0.01M $Ca(OH)_2$ solution (the pH of which was 12.2). The effects of temperature and time on the stability of FB_1 in the presence of $Ca(OH)_2$, were determined using the 0.01M $Ca(OH)_2$ solution, and the results were compared with those previously observed for the 0.5M solution at pH 13 (Figure 6.3).

Using a standard reaction time of 1 hour, Figure 6.4.1 represents the effect that temperature had on the stability of FB_1 in 0.01M $Ca(OH)_2$. The stoichiometric conversion of FB_1 to its AP_1 moiety was similar to that observed for the pH 13 solution (Figure 6.3.1). Treatment of FB_1 with the $Ca(OH)_2$ did not, however, result in the complete hydrolysis of FB_1 to AP_1, as a 96% conversion was recorded at both 90 and 100°C. Increasing the temperature did not result in the complete disappearance of the residual chromatographic peak eluting in the position of FB_1.

The effect of reaction time on the stability of FB_1 in the presence of 0.01M $Ca(OH)_2$ was similarly monitored by maintaining the temperature at 100°C, and varying the exposure time. The results of this study are shown in Figure 6.4.2, which illustrates the rapid conversion of FB_1 to AP_1. The observed rate of hydrolysis was significantly higher than that previously recorded for FB_1 in solution at pH 13 (Figure 6.3.2), indicating that the calcium ion exhibited a major influence on the stability of FB_1 in solution.

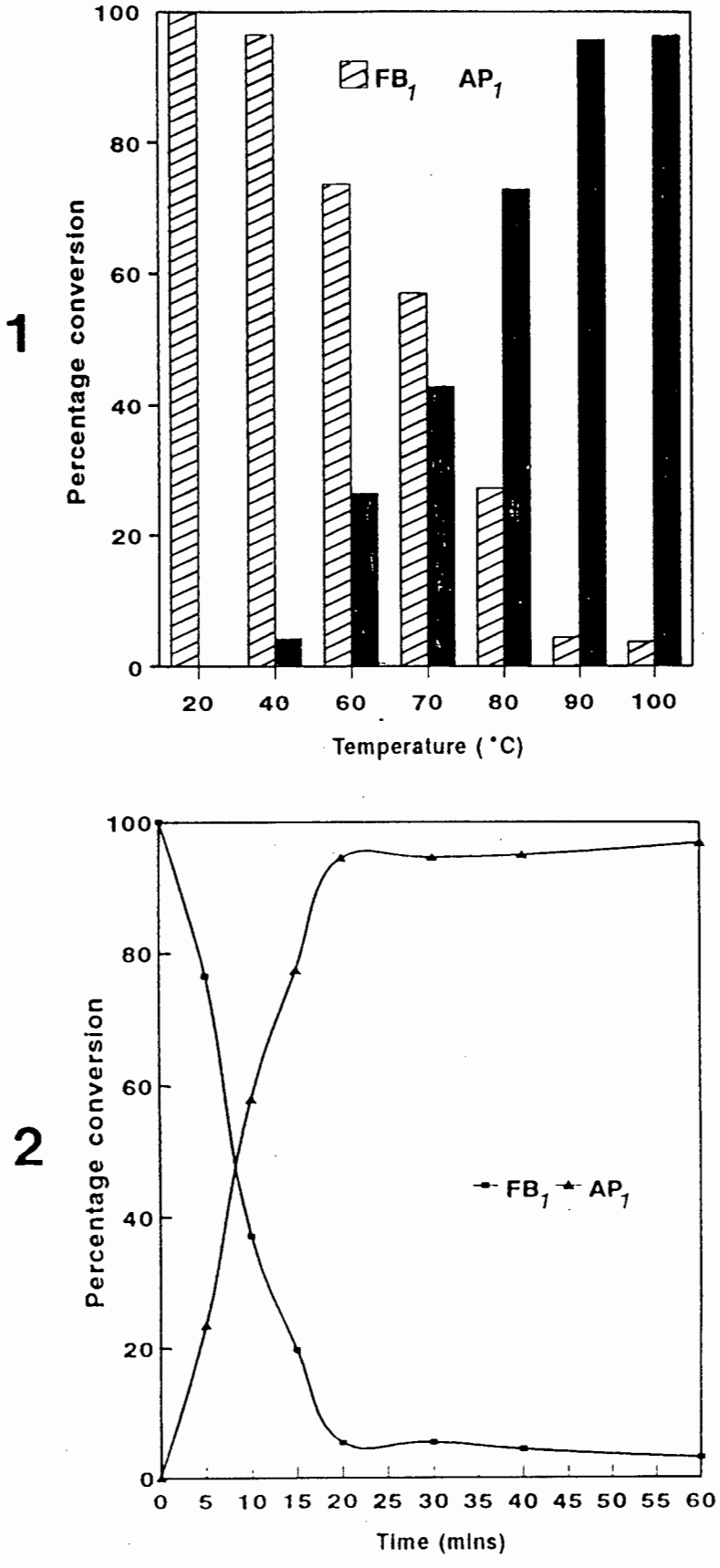


Figure 6.4 The effects of temperature (1) and time (2) on the hydrolysis of FB_1 to AP_1, in the presence of a 0.01M $Ca(OH)_2$ solution

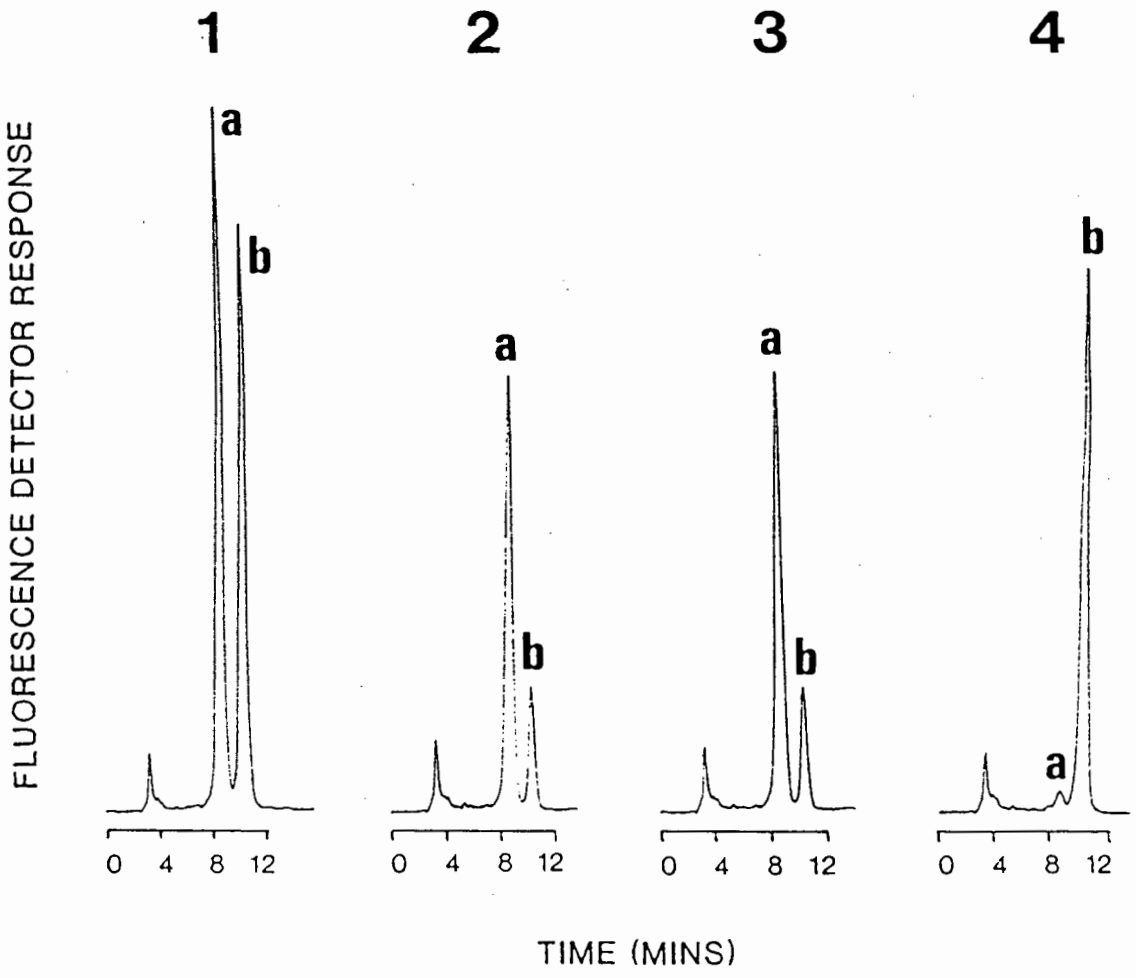


Figure 6.5 Chromatograms of (1) FB_1 {a} and AP_1 {b} standards, (2) FB_1 treated with 0.02M NaOH, (3) FB_1 treated with 0.02M KOH and (4) FB_1 treated with 0.01M $Ca(OH)_2$

Since $Ca(OH)_2$ is a relatively insoluble metal hydroxide it was considered pertinent to compare, under identical conditions, the action of $Ca(OH)_2$ on FB_1 with that achieved following exposure of FB_1 to other, more soluble, metal hydroxides such as NaOH and KOH. These latter solutions were prepared at a concentration of 0.02M, in order to obtain approximately the same solution pH (12.2) recorded for the 0.01M $Ca(OH)_2$ solution. Figure 6.5.1 shows the HPLC separation of FB_1 and AP_1 standards as their OPA derivatives. Figures 6.5.2 and 6.5.3, show the resultant chromatograms obtained for FB_1, heated for 1 hour at 100°C in the presence of 0.02M solutions of NaOH and KOH respectively, while Figure 6.5.4 shows the chromatogram

obtained for FB_1 similarly treated with 0.01M $Ca(OH)_2$. The results clearly show the partial (25%) hydrolysis of FB_1 to its AP_1 moiety, when heated in the presence of both NaOH and KOH. Conversely, almost total conversion was observed for FB_1 when it was reacted, under identical conditions, with 0.1M $Ca(OH)_2$. A similar degree of conversion of FB_1 to AP_1 (>96%) was also measured when FB_1 was allowed to react with a 0.1M slurried solution of $Ca(OH)_2$, following an exposure period of 24 hours at ambient temperature (23°C).

The unique influence of the calcium ion on the hydrolysis of FB_1 was additionally illustrated by comparing the effect that the ion had, when incorporated into the reaction solution in an alternative form. Fumonisin B_1 was heated for 1 hour at 100°C in the presence of 5 different solutions; *viz* 0.2M NaOH, 0.2 calcium chloride ($CaCl_2$), 0.2M NaOH containing 0.05M $CaCl_2$, 0.2M magnesium chloride ($MgCl_2.6H_2O$) and 0.2M NaOH containing 0.05M $MgCl_2.6H_2O$. As previously observed, the NaOH alone caused only partial hydrolysis of the FB_1, whereas no hydrolytic effect on FB_1 was observed when reacted with either the 0.2M $CaCl_2$ (pH = 9.3) or 0.2M $MgCl_2.6H_2O$ (pH = 6.6) solutions. However, incorporation of the calcium ion into NaOH (as $CaCl_2$) dramatically enhanced the conversion of FB_1 to AP_1 (to approximately 90%). Conversely, the addition of the magnesium ion to the NaOH solution, as $MgCl_2.6H_2O$, suppressed the degree of hydrolytic conversion. These results confirmed the supposition that the calcium ion exhibits a significant enhancement of both the rate and extent of hydrolysis of FB_1. This possibly involves the formation of a metal complex. It was unexpected that the magnesium ion (also a Group II metal) exhibited an inhibitory effect on the progression of FB_1 hydrolysis. This effect was unrelated to pH, since the combined NaOH:$MgCl_2.6H_2O$ solution pH was similar to that of the NaOH itself.

In separate experiments, both FB_2 and FB_3 were subsequently found to be similarly hydrolysed to their corresponding aminopolyol moieties, following exposure to $Ca(OH)_2$. These results suggested that each of the naturally occurring fumonisin analogues could be similarly hydrolysed in the presence of

$Ca(OH)_2$, or alternatively by the action of a combination of pH, and an external source of calcium.

6.3.2.4 *$Ca(OH)_2$ treatment of ground maize*

High-temperature treatment of ground maize in the presence of a 0.1M slurried solution of $Ca(OH)_2$, at 100°C for 15 mins, was found to be unsuitable since this led to the formation of a thick gelatinous mixture, that could not be effectively separated into the aqueous $Ca(OH)_2$ and solid maize fractions. Low temperature exposure (± 25°C) of the maize to 0.1M $Ca(OH)_2$, over a period of 24 hours was found to be effective, allowing for the separation of both fractions.

Based on the observations in section 6.3.2.3, the potential formation of the aminopentol moiety following $Ca(OH)_2$ treatment of maize, required the development of an alternative reversed-phase clean-up procedure of extracts. This was necessary due to the absence of the tricarballylic acid groups in the structure of the aminopentol moiety, resulting in its non-retention by anion exchange media.

In addition, Shephard et al. (1992a) suggested the possible formation of weakly soluble fumonisin salts in rat faeces,

Table 6.4 Recoveries of FB_1 and AP_1 from aqueous and solid fractions, following 0.01M $Ca(OH)_2$-treatment of ground maize

Moiety	Phase	Number of analyses	Spiking level	Mean recovery (%)	RSD (%)
FB_1	$Ca(OH)_2$	3	470 ng/ml	98.4	6.0
AP_1	$Ca(OH)_2$	3	450 ng/ml	96.3	7.2
FB_1	Maize	5	470 ng/g	93.6	4.7
AP_1	Maize	5	490 ng/g	94.7	2.1

following studies in which the metabolic fate of FB_1 was monitored. This suggestion stemmed from the need to use multiple extractions with EDTA in order to effectively remove the bound FB_1 from the rat faecal matter. Hence, EDTA was incorporated into the extraction solvent system for the analysis of $Ca(OH)_2$-treated maize. The presence of EDTA also necessitated the use of C_{18} material for sample clean-up, due to the high electrolyte concentration which resulted in low fumonisin recoveries from SAX media.

Recoveries of FB_1 and AP_1 from the separated aqueous and solid fractions were found to be excellent, as shown in Table 6.4. Recoveries ranged from 93.6 to 98.4%, at similar spiking concentrations, with RSD values of between 2.1 and 7.2%. These results suggested that the methods would be suitable to monitor the fate of FB_1 and subsequent formation of AP_1, during the $Ca(OH)_2$ treatment process of ground maize.

Initial results involving $Ca(OH)_2$ exposure of several 100 g quantities of FB_1-contaminated maize, for 24 hours at room temperature, repeatedly indicated large variabilities in the results obtained for the FB_1 and AP_1 moieties. The solid to liquid interface during the treatment process was not optimal, since for extended periods of time the maize separated, under gravity, from the aqueous $Ca(OH)_2$ phase. Hence, in an attempt to improve solid to liquid inter-action, mechanical mixing was introduced during the treatment process, which significantly reduced the variability of the results.

The maize used for the $Ca(OH)_2$ study was analysed for FB_1 content (in triplicate), and was found to be contaminated with a mean concentration of 8595 ng/g (RSD = 4.1%). Following treatment of the maize meal with $Ca(OH)_2$, the aqueous and solid fractions were separated, and each fraction was analysed for FB_1 and AP_1. The results of these analyses are tabulated in Table 6.5.

The temperature of the solutions, during the $Ca(OH)_2$-treatment process, were found to differ slightly for each sample (between

Table 6.5 Results of 0.1M $Ca(OH)_2$-treatment of ground maize meal contaminated with 8595 ng/g FB_1

Sample No	FB_1 (ng/g)[1]	% of orig. concn.	AP_1 (ng/g)[1]	FB_1 equiv. (ng/g)[1,2]	% of orig. concn.
			Aqueous $Ca(OH)_2$ fraction		
1 (29°C)	1055	12.2%	3275	5820	67.7%
2 (32°C)	ND[3]	ND	3420	6070	70.6%
3 (34°C)	ND	ND	3455	6135	71.4%
			Solid maize fraction		
1 (29°C)	ND	ND	510	910	10.6%
2 (32°C)	755	8.8%	790	1400	16.3%
3 (34°C)	ND	ND	825	1465	17.0%

[1]Mean values based on duplicate determinations
[2]FB_1 concentration calculated from the corresponding AP_1 level
[3]ND = Not detected (<100 ng/g)

29°C and 34°C - Table 6.5). This increase and difference in temperature (above that of ambient - 23°C) was probably due to the action of the mechanical stirring.

Only 1 of 3 $Ca(OH)_2$-treated maize fractions contained FB_1, corresponding to 12.2% of the original FB_1 concentration in the non-treated maize. The vast majority of the original FB_1 (between 67.7 and 71.4% - Table 6.5) was however present in the aqueous $Ca(OH)_2$ phase, as its fully hydrolysed aminopentol moiety. The data indicated that between 70.6 and 79.9% (mean = 74.0%) of the FB_1, initially present in the non-treated maize, was transferred to the easily separated aqueous $Ca(OH)_2$ phase.

Conversely, between 10.6 and 25.1% (mean = 17.6%) of the original FB_1 was retained in the solids after treatment. With

the exception of sample 2, this residual was present primarily as the AP_1 moiety (Table 6.5). Overall, between 7605 and 8225 ng/g FB_1 could be accounted for in the three trial samples, following treatment with $Ca(OH)_2$. This was equivalent to between 88.4 and 95.7% (mean = 91.6%) of the original FB_1 concentration determined in the non-treated maize.

Other chemical treatments of fumonisin-contaminated maize have been investigated. Norred et al. (1992) observed between a 30 and 45% reduction in FB_1 levels in culture material of *F. moniliforme*, following low temperature/atmospheric pressure ammoniation. Despite this reduction in fumonisin content, no significant reduction in toxicity to rats was observed following the ammoniation treatment (Norred et al., 1992). With the exception of the possible base-induced hydrolytic action of ammonia on the fumonisin molecule, consideration of the fumonisin structure would not indicate other likely sites for destruction of the toxin. However, Norred et al. (1992) did not observe the formation of the hydrolysed AP_1 moiety. The mode of action by which the ammoniation process reduces FB_1 concentrations is, therefore, presently unknown. However Norred et al. (1992) concluded that based on animal feeding trials, atmospheric ammoniation would appear to be an ineffective procedure for the successful decontamination of fumonisin-contaminated maize.

Conversely, Park et al. (1992) claimed that both high pressure/ambient temperature (60 psi/20^{o}C) and low pressure/high temperature (17 psi/175^{o}C) ammoniation of fumonisin-contaminated maize resulted in fumonisin reductions by an average 79% (ie. from 86 to 18 ppm), although the mode of action leading to the removal of the FB_1 from maize was not demonstrated. The data of Park et al. (1992) would therefore indicate that ammoniation leads to a reduction in FB_1 levels, and that the degree of reduction is similar to that observed for the $Ca(OH)_2$ treatment. Park et al., (1992) reported that the ammoniation process did not result in an increased mutagenic potential of the treated maize, however Gelderblom & Snyman (1991) had previously shown that the fumonisin B mycotoxins are non-mutagenic.

6.3.3.5 *$Ca(OH)_2$ treatment of maize kernels*

The development and/or adoption of a suitable decontamination procedure must take into account the issue of practicalities. Clearly, in the case of maize, it would be more practical to treat the intact kernels rather than the ground meal.

Table 6.6 Fumonisin B_1 levels in maize kernels before and after treatment with $Ca(OH)_2$

Fraction	FB_1 level (ng/g)	AP_1 level (corresponding FB_1 level) (ng/g)[a]	Total (ng/g)
Untreated	8200	ND	8200
Kernels with pericarp partially removed	1950	335 (595)	2545
Kernels with pericarp fully removed	105	175 (310)	415

[a]Corresponding FB_1 levels are given in brackets

Yellow maize kernels selected for $Ca(OH)_2$ treatment were shown to be naturally contaminated with 8200 ng/g FB_1 (Table 6.6). Following the treatment process, the aqueous $Ca(OH)_2$ fraction appeared to be very "cloudy" and yellow in colour. Conversely, the kernels could be visually separated into at least two major groups. Figure 6.6.1 shows kernels prior to treatment. However, following exposure to 0.1M $Ca(OH)_2$, the majority of the kernels showed signs of external damage. In most cases, kernels were characterised by either the partial (Figure 6.6.2) or total (Figure 6.6.4) removal of their outer cellulose *"pericarp"* (Figure 6.6.3). These distinct kernels fractions were separated by hand, ground to fine meals and analysed for FB_1 and AP_1, and the results are shown in Table 6.6.

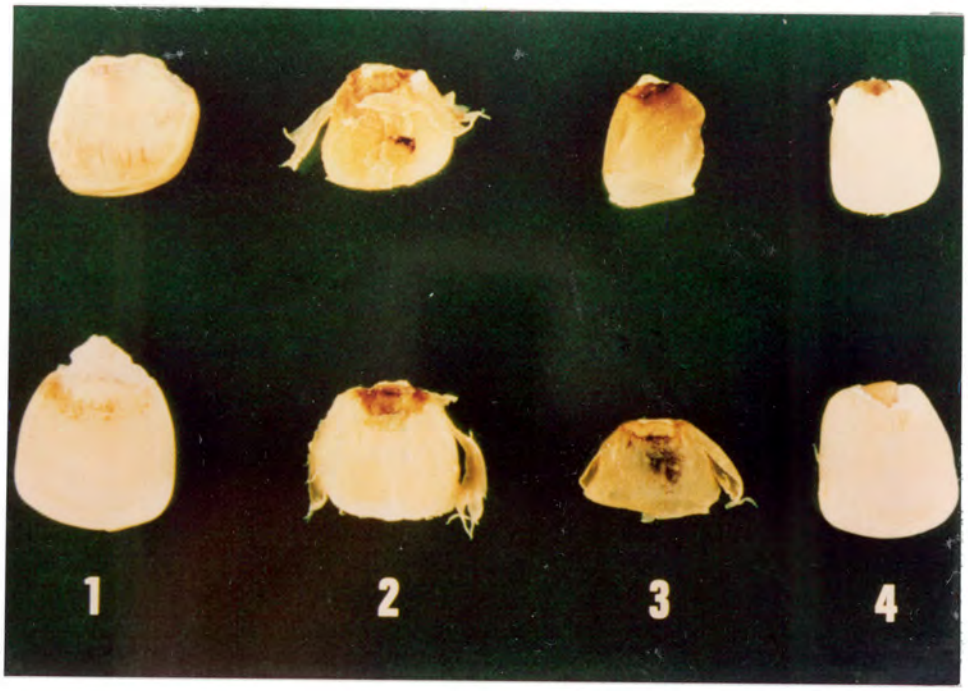


Figure 6.6 (1) Kernels prior to $Ca(OH)_2$ treatment, (2) partial removal of kernel pericarp, (3) pericarp removed from a maize kernel, (4) total removal of maize pericarp

From an initial FB_1 concentration of 8200 ng/g (and following the $Ca(OH)_2$ treatment), those kernel fractions exhibiting partial removal of their pericarp (Figure 6.6.2) retained 31% of the original FB_1 concentration, while those fractions in which the pericarp was totally removed retained only about 5% of the original FB_1 level. These data indicate that the fumonisin mycotoxins are concentrated in the outer layer(s) of maize kernels (Sydenham et al., 1992c). The results therefore substantiate, to an extent, the data presented in section 6.3.1 and Table 6.2, in which significantly higher fumonisin levels were recorded in the "*fines*" fractions separated by screening. It may be expected that the "*fines*" fractions would contain proportionally higher concentrations of both fine maize particles (possibly present due to the abrasive action of the kernels during handling processes) and chaff, derived from the pericarp layer of maize kernels.

Two of the major criteria associated with the development of a successful decontamination procedure are that:

(**a**) the treated commodity should not lose its nutritive value, and

(**b**) that the decontamination process should not result in the formation of an equally or more toxic by-product.

Maize has been found to have relatively poor nutritional qualities, being deficient in protein and certain essential elements (such as calcium) and vitamins (ie. riboflavin) (USDA, 1992). The carcinogenic potential of compounds has been shown to be dependant on nutritional status, such that improvement of fat, caloric or protein intake can promote carcinogenesis, while intake restriction of these essential components can suppress carcinogenic potential (Carroll & Kohr, 1975; Pariza & Boutwell, 1987; Youngman & Campbell, 1991).

The process of nixtamalization (heat and $Ca(OH)_2$ treatment) has been found to improve the nutritive value of maize (Carter & Carpenter, 1982). Hence the hydrolysis of a maize toxicant could interact with nutritional status to either reduce or enhance its carcinogenic potential. In this respect, Hendrich et al. (1993) reported that nixtamilization of maize culture material of *F. proliferatum* (FP) significantly reduced FB_1 content, to yield between 7 and 10 $\mu g/g$ AP_1 (from an original 50 $\mu g/g$ FB_1 in the corresponding non-nixtamalized material). In subsequent rat feeding trials, consumption of nixtamalized FP material still produced hepatic adenomas in DEN-initiated rats, but the degree of promotion appeared to be less than that observed for the non-nixtamalized FP material (Hendrich et al., 1993). However, they concluded that improvement in the nutritive value of nixtamalized maize, resulting in an increased consumption of food and body weight gain by the rats, indicated that the carcinogenic potential of nixtamalized maize was similar to that observed for the corresponding non-nixtamalized material. The authors suggested that treatment of maize with $Ca(OH)_2$ was not a potentially useful fumonisin-detoxification

strategy (Hendrich et al., 1993).

The cancer-promoting effects of pure AP_1 have to date not been reported, although Gelderblom et al. (1993) and Abbas et al. (1993) have reported on the cancer-initiation, cytotoxic and phytotoxic potentials of AP_1. In addition to the results of Hendrich et al. (1993), and in view of the evidence presented in sections 6.2.3.4 and 6.2.3.5, it will be necessary to study the relative cancer-promoting potential of $Ca(OH)_2$-treated maize, with that precipitated by the separated aqueous $Ca(OH)_2$ fraction itself, since this latter fraction would contain significantly more AP_1 than the maize.

6.3.3.5 *Isolation of PH_1 from culture material*

For some extracts (especially in the aqueous $Ca(OH)_2$ fractions following the treatment of ground maize), the chromatographic peak corresponding to FB_1 indicated the possible presence of an interfering compound. The presence of this additional compound is illustrated in Figure 6.7.1 which details the separation of FB_1 from AP_1. The peak corresponding to FB_1 is clearly not symmetrical. Further optimisation of the chromatographic conditions to those specified in Table 6.1, illustrated that the FB_1 peak in Figure 6.3.1 could be separated into 3 distinct peaks, with the additional peaks eluting between FB_1 and AP_1 (identified with a question mark in Figure 6.7.2). A similar series of chromatographic peaks could also be observed following low temperature $Ca(OH)_2$ treatment of an FB_1 standard (4.5 hours at 23°C - section 6.2.5).

Shephard et al. (1994) identified the presence of a new metabolite of FB_1, which was isolated from the faeces of a vervet monkey undergoing toxicokinetic studies. The metabolite was described as an equilibrium mixture of two structural isomers of partially hydrolysed FB_1. Its structure was considered to be the partial hydrolysis product of FB_1, in which one of the ester (TCA) groups had been preferentially hydrolysed. Shephard et al. (1994) claimed that *trans*-esterification of the resultant monoester, via an intramolecular mechanism, led to

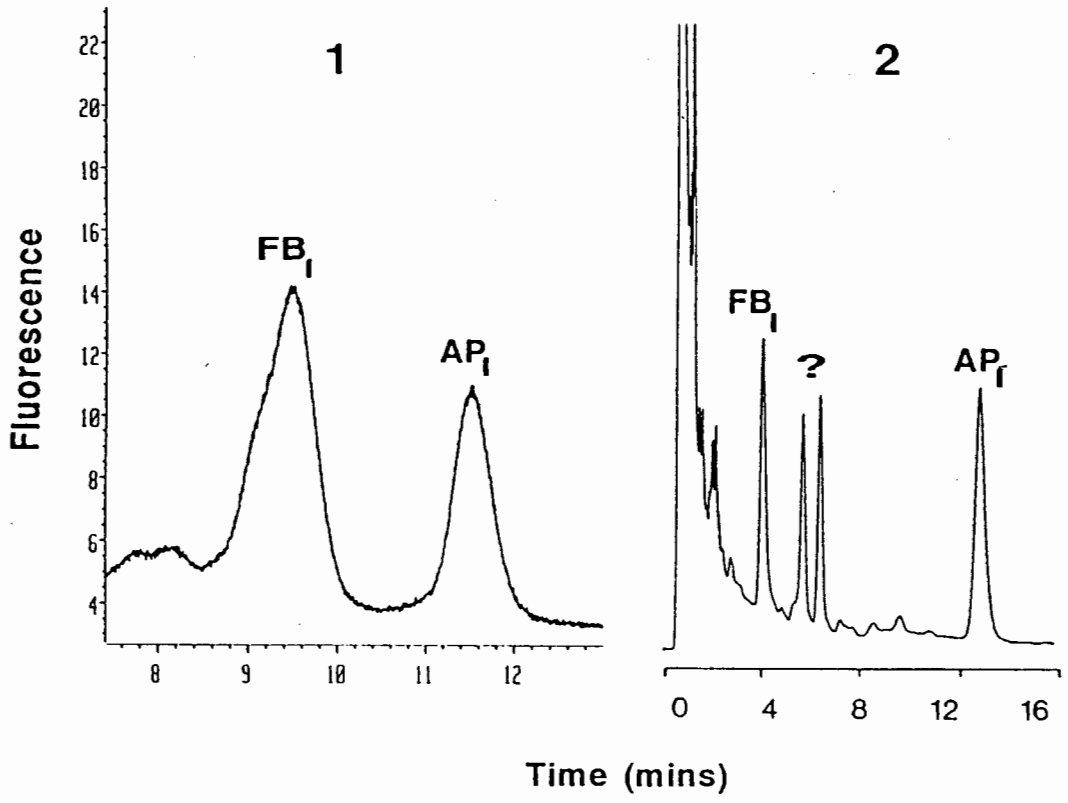


Figure 6.7 (1) Separation of a purified aqueous $Ca(OH)_2$ extract, as its OPA derivative, following nixtamalization of maize, and (2) the separation of the same extract using alternative chromatographic parameters

the formation of an equilibrium mixture of the two possible monoesters. The process is similar to the isomerization of the monoesters of glycerol, in which the acid moiety is transferred to a vicinal hydroxy group (Finar, 1967). Similar observations have previously been recorded for TA and TB toxins, produced by *A. alternata* (Shephard et al., 1993).

Based on the chromatographic evidence in Figure 6.7.2, culture material of *F. moniliforme* was used to monitor the progression of low-temperature (± 25°C) $Ca(OH)_2$ hydrolysis of FB_1 in maize. Figure 6.8 shows a series of selected chromatograms, obtained after reaction times of 0.05, 1, 3, 5, 7 and 9 hours, for the OPA derivatives of non-purified fractions of the cultture material. These fractions were removed from the reaction mixture at 1 hour intervals during treatment with 0.1M $Ca(OH)_2$. The absence of a purification step resulted in the inability to

fully separate FB_1 from other maize-intrinsic compounds. Following a reaction period of 1 hour it was possible to identify two peaks eluting between 6.5 and 8 mins (identified as PH_1, the retention times for which were similar to those previously observed in Figure 6.7.2), and the beginning of a peak corresponding to the fully hydrolysed AP_1 moiety (Figure 6.8). After 3 and 5 hours, the area of the unresolved peak of FB_1 was reduced, while the peaks corresponding to PH_1 and AP_1 had increased. From a reaction time of 7 hours onwards, only the AP_1 peak increased in area, and by 23 hours onwards (not illustrated) the peaks corresponding to FB_1 and PH_1 had diminished to such an extent that they could not be identified on the chromatograms.

The chromatograms in Figure 6.8 represent similar molar equivalents applied to the analytical HPLC column. Therefore, assuming that similar detector responses were obtained for the OPA derivatives of the different moieties, it was possible to draw some qualitative conclusions. The results could indicate that the two unconfirmed peaks (identified as PH_1 in Figure 6.8) are related "*intermediate compounds*" formed during the hydrolysis step. Their concentration appeared to increase with the corresponding decrease in FB_1, to an extent that following exposure for between 4 and 5 hours, they were larger in area than either the FB_1 or AP_1 moieties. Thereafter, an increase in the area of the peak corresponding to the fully hydrolysed AP_1 moiety was accompanied by an ever decreasing area for the two peaks corresponding to the "*intermediate compounds*" (PH_1). These observations prompted an attempt to isolate and confirm the identity of the PH_1 metabolites/moieties, from culture material of *F. moniliforme*.

The formation of PH_1 was carried out in accordance with the procedure followed for the initial trials illustrated in Figure 6.8. The hydrolysis reaction was stopped after 4.5 hours, by alteration of the pH. The time selected for reaction termination was based on the initial results (Figure 6.8), which indicated that the maximum concentration of PH_1 would be obtained after approximately 4.5 hours. Thereafter, extracts

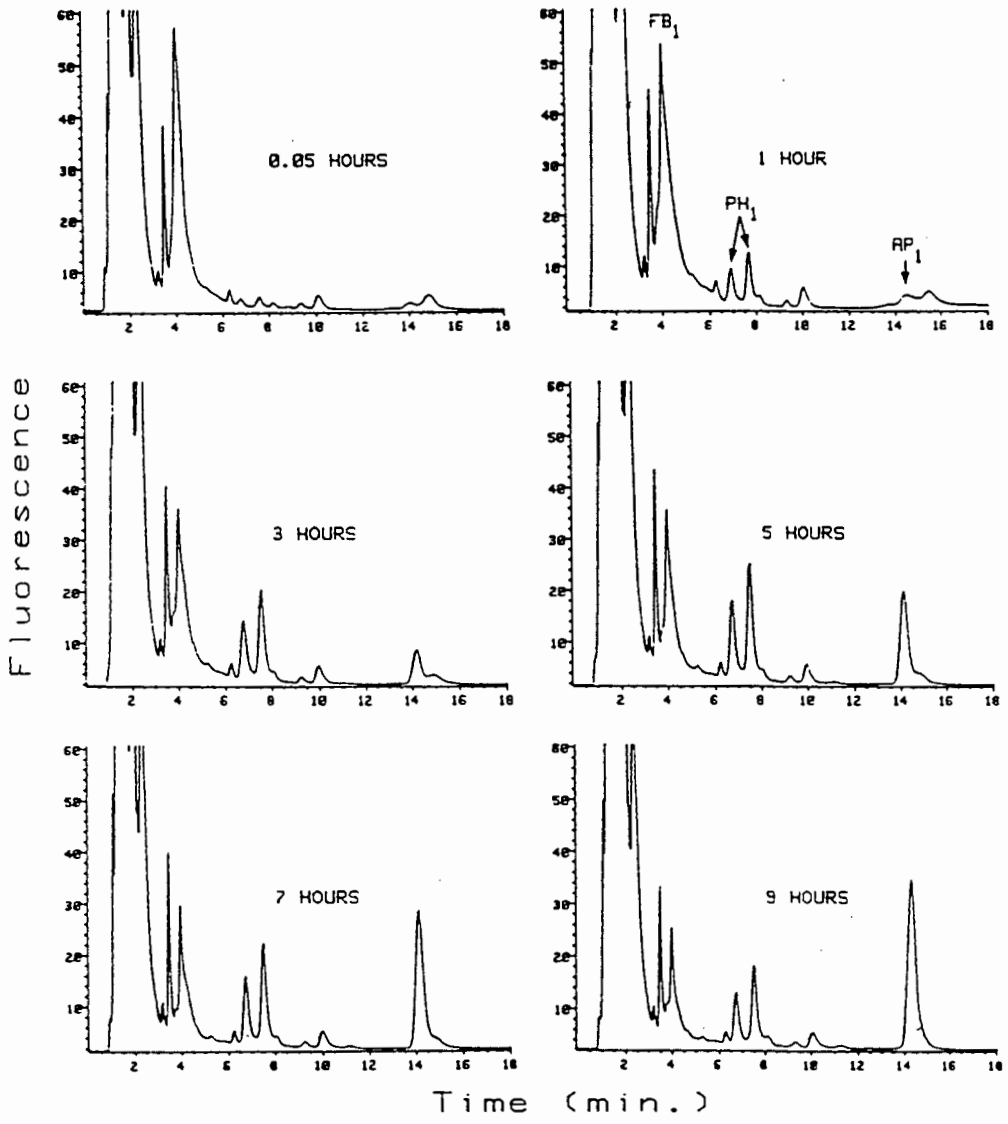


Figure 6.8 HPLC chromatograms of the OPA derivatives of fractions removed from $Ca(OH)_2$-treated *F. moniliforme* culture material, after periods of 0.05, 1, 3, 5, 7 and 9 hours of exposure

were further purified on large Amberlite XAD-2 and Kieselgel 60 chromatography columns. The isolation of the PH_1 was monitored by normal phase TLC, with two spots being observed for the unconfirmed PH_1 moiety. Final purification was achieved by purification on anion exchange and C_{18} media, prepared as short chromatography columns. Residual solvent was subsequently removed under vacuum to yield 42 mg of the purified compound. A portion of the purified compound was dissolved in CH_3OH, and assessed as its OPA derivative, and the resultant chromatogram

is shown in Figure 6.9.

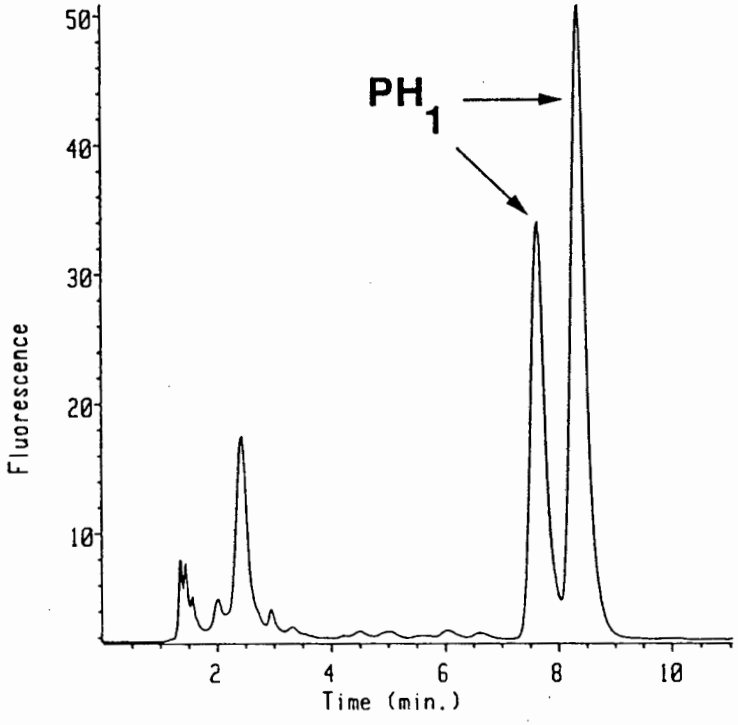


Figure 6.9 OPA derivative of the unconfirmed (purified) PH_1 moiety isolated from culture material of *F. moniliforme*, following treatment with 0.1M $Ca(OH)_2$

6.3.3.6 *Liquid chromatography-electrospray mass spectrometry studies*

The electrospray mass spectrum (ES-MS) (as a continuum format) of the PH_1 moiety, isolated from culture material of *F. moniliforme* is shown in Figure 6.10. The ES-MS was not baseline-corrected, but the protonated molecular ion was found to be 564.5 *m/z*. Other major masses were observed at 301.9, 309.3, 406.4 and 413.4 *m/z*. Since the instrument cone voltage settings were low, it was not expected that fragmentation would be induced. It was therefore possible that some of these masses could have originated from the background. Figure 6.10 however represents the ES-MS obtained for a dual compound, based on its OPA derivatisation which is illustrated in Figure 6.9. Further

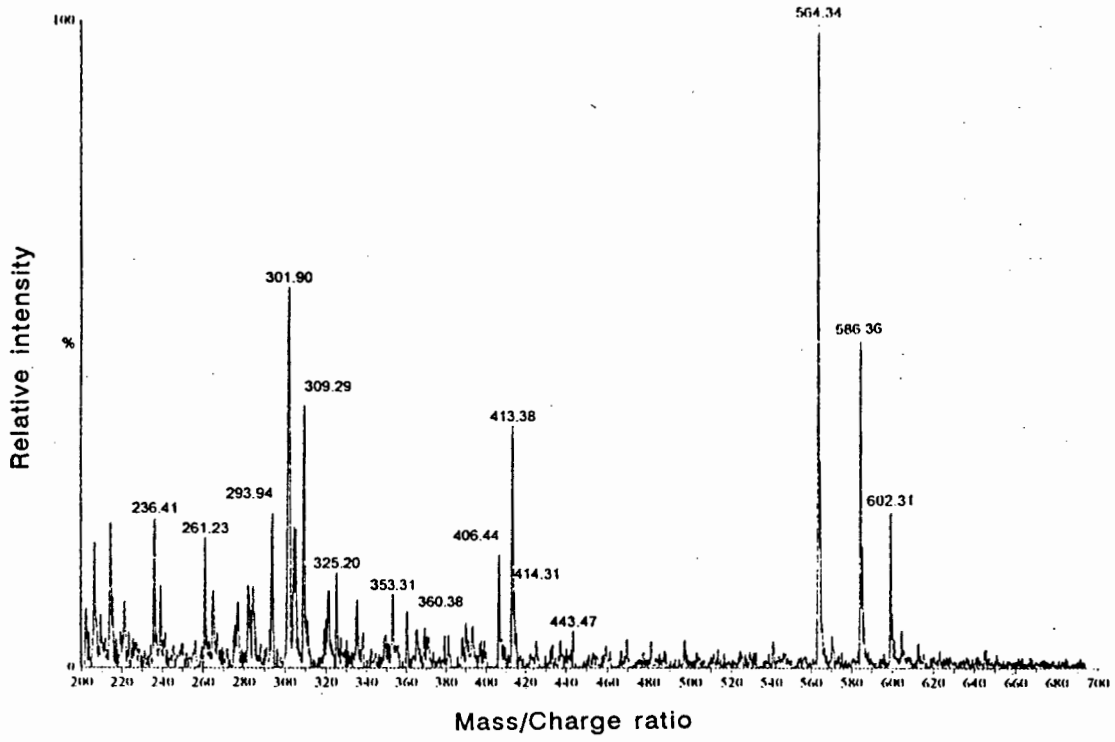


Figure 6.10 Continuum ES-MS of the PH_1 moiety isolated from culture material of *F. moniliforme*

proof of the identity of the two peaks was sought by treating FB_1 with $Ca(OH)_2$, followed by isolation and identification of the separate moieties. Following treatment of FB_1 with 0.01M $Ca(OH)_2$, the application of the resultant solution (adjusted to pH 6.2) to ion exchange media, resulted in the selective retention of any residual FB_1, partial retention of the PH_1 moiety, but no retention of the AP_1 moiety. Further purification of the eluent (containing the PH_1 and AP_1 moieties) on C_{18} reversed-phase material, resulted in the isolation of the two separate compounds corresponding to PH_1, and the single compound corresponding to AP_1.

Figure 6.11.1 shows the HPLC chromatogram, as its OPA derivative, of AP_1 eluting after 13.51 mins, while Figure 6.11.2 is the ES-MS (centroid form) of the AP_1 moiety. A strong protonated molecular ion $[MH]^+$ was measured at mass 406.3 *m/z*, which corresponds to a molecular formula of $C_{22}H_{47}NO_5$, and is consistent with the loss of the two TCA side chain moieties located

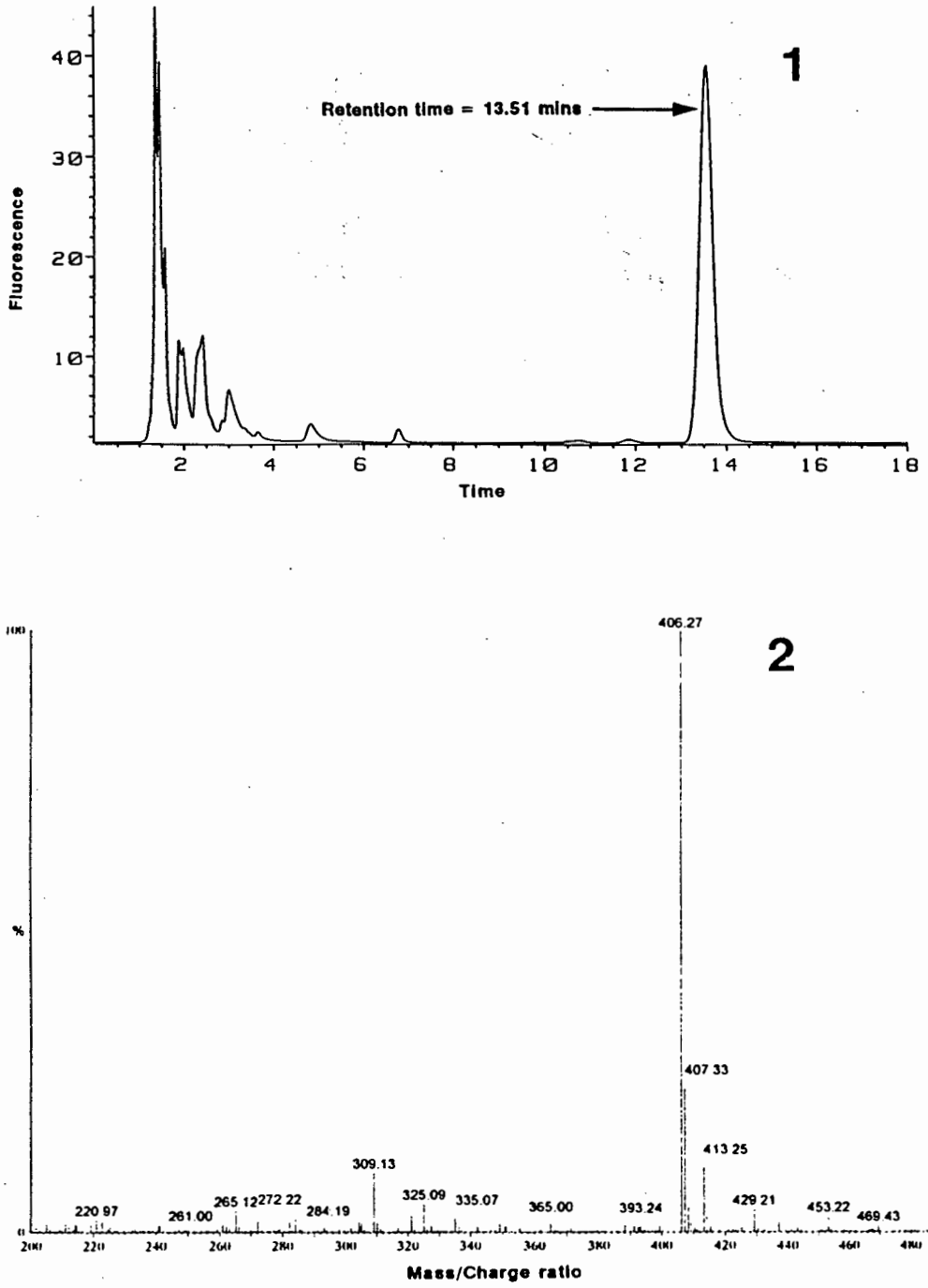


Figure 6.11 (1) the HPLC chromatogram of AP_1 eluting with a retention time of 13.51 mins, and (2) the ES-MS of the compound

at the C-14 and C-15 positions on the backbone of the fumonisin molecule (Figure 1.5). Mass 406 *m/z* is also present in the MS of the PH_1 moiety isolated from culture material of *F. moniliforme* (Figure 6.10). This suggests that even with low cone voltage settings, partial fragmentation may have been induced, and that the 406.4 *m/z* ion could correspond to AP_1 which may

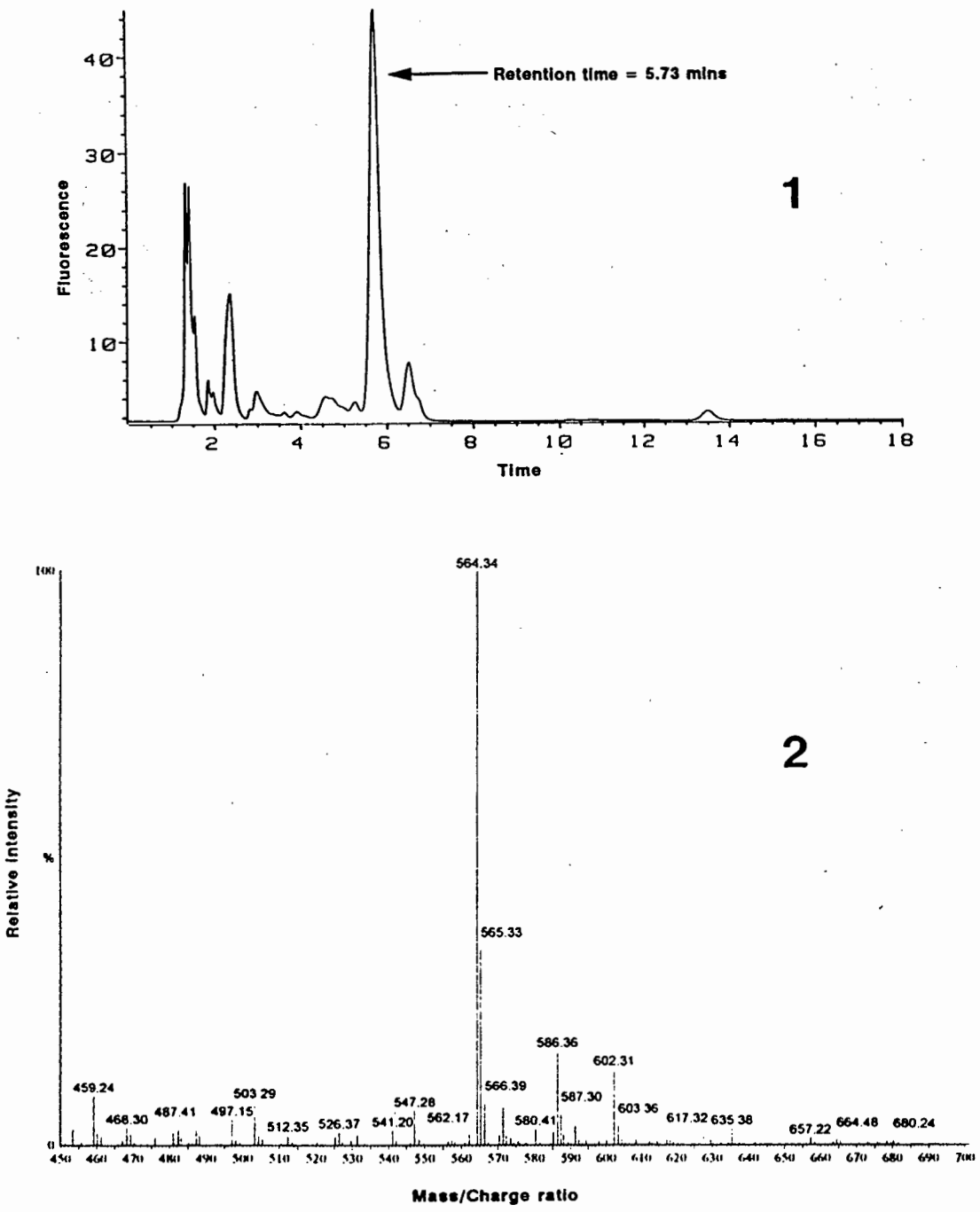


Figure 6.12 (1) the HPLC chromatogram of a fraction collected following $Ca(OH)_2$-treatment of FB_1, and isolated by reversed-phase chromatography, showing a major peak at 5.73 mins, and (2) the ES-MS obtained for the same fraction

be a fragment product of the PH_1 moiety.

Figure 6.12.1 shows the chromatogram of a fraction (as its OPA derivative), collected during the reversed-phase isolation of

$Ca(OH)_2$-treated FB_1. The major peak, corresponding to one of the two moieties of PH_1, can be observed at a retention time of 5.73 mins. A peak corresponding to the other PH_1 moiety was also present, eluting at 6.53 mins. The chromatogram also indicated the presence of AP_1, since a minor peak was seen with a retention time of approximately 13.5 mins (Figure 6.12.1). A partial ES-MS (centroided between 450 and 700 *m/z*) of the fraction is illustrated in Figure 6.12.2. The protonated molecular ion of the compound was 564.3 *m/z*, which would be indicative of the molecular structure $C_{28}H_{53}NO_{10}$. The data could be interpreted as being due to the loss of a single TCA group from the fumonisin molecule, which would therefore confirm the partial hydrolysis of FB_1. The masses 586.3 and 602.3 *m/z* most probably correspond to the $[MNa]^+$ and $[MK-H]^+$ ions, respectively.

The *trans*-esterification of the monoester (isolated following $Ca(OH)_2$ treatment of FB_1 - Figure 6.12), was confirmed by subjecting the monoester-fraction to a temperature of 70°C

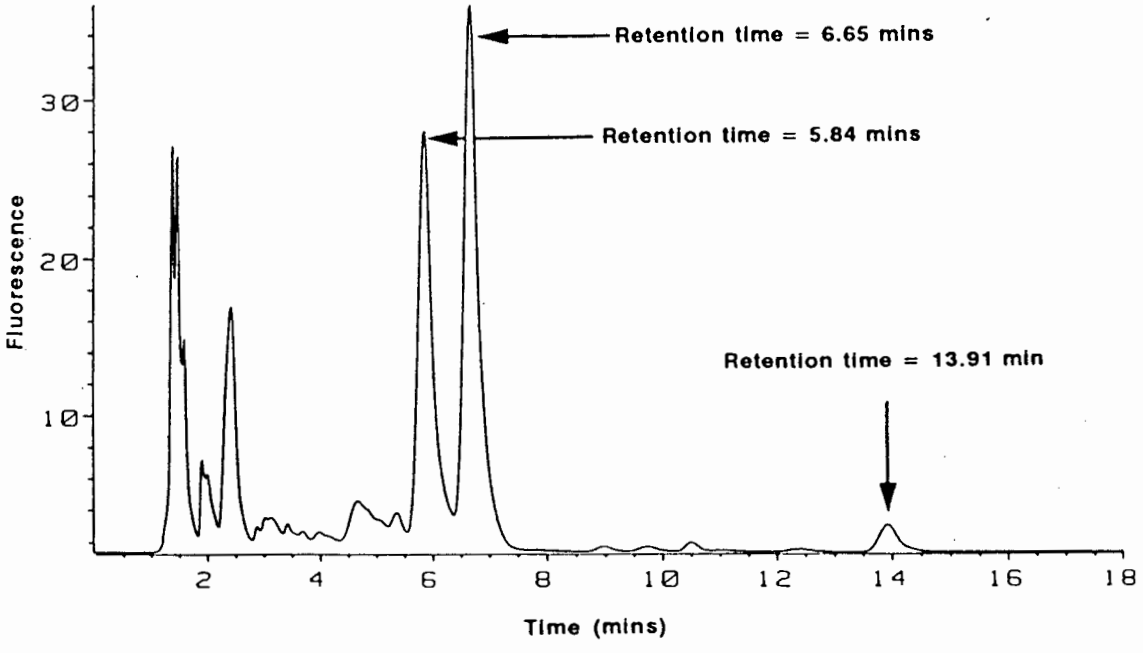


Figure 6.13 The HPLC chromatogram of the OPA derivative of the PH_1 fraction (shown in Figure 6.12.1) following heat exposure

for 3 hours. Figure 6.13 shows the resultant HPLC chromatogram obtained for the fraction following treatment, as its OPA derivative. Comparison of Figures 6.12.1 and 6.13 clearly show that the predominant peak observed in the former (rt = 5.73 mins) was replaced by two peaks (rt = 5.84 and 6.65 mins, respectively). In addition, a peak eluting at 13.91 mins (corresponding to a small concentration of AP_1) may also be seen in Figure 6.13, which is similar in size to that observed in Figure 6.12.1. The observed increase in retention times between the two chromatograms was probably due to minor differences in the mobile phases used for the generation of each.

These results would appear to indicate that heat treatment caused the *trans*-esterification of the monoester, but did not precipitate further degradation of the PH_1 moiety, to the fully hydrolysed AP_1 form. Indeed, subsequent to the heat treatment process, the relative ratio of the two chromatographic peaks in Figure 6.13, was similar to that observed for the PH_1 moiety isolated from culture material of *F. moniliforme* (Figure 6.9). Extended exposure to heat did not induce further *trans*-esterification, suggesting that the mixture had attained equilibrium. These results were in accordance with those suggested by Shephard et al. (1994), for a partially hydrolysed form of FB_1, isolated from the faeces of rats.

Conclusive proof of the presence of the two monoester forms of PH_1 was obtained following the isolation, and ES-MS analyses of the second monoester form. Figure 6.14.1 shows the HPLC chromatogram, also as its OPA derivative, of the second monoester (rt = 6.55 mins) isolated following the $Ca(OH)_2$ treatment of a FB_1 standard. The chromatogram also shows the presence of a smaller peak eluting at around 5.7 mins, corresponding to the primary monoester of PH_1. Figure 6.14.2 illustrates the corresponding partial centroid ES-MS, where again the major protonated molecular ion was recorded at 564.3 *m/z*. Comparison of the mass spectra obtained for both forms (Figures 6.12.2 and 6.14.2) indicated that they were identical compounds. The mass spectra were also identical to that isolated from the $Ca(OH)_2$-treated *F. moniliforme* culture material (Figure 6.10).

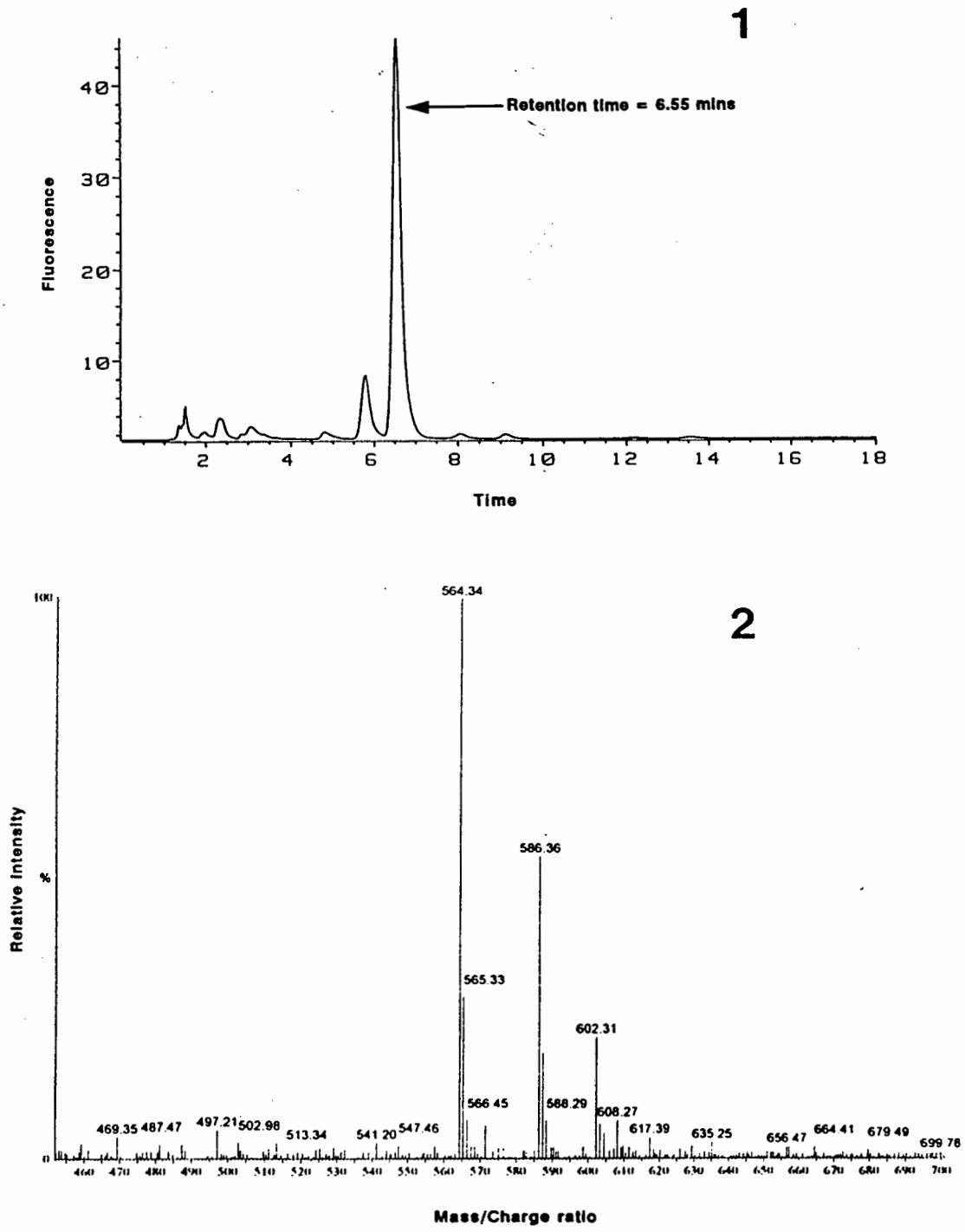


Figure 6.14 (1) the HPLC chromatogram of the second PH_1 moiety, and (2) its corresponding centroided ES-MS

The MS data obtained for the PH_1 moiety isolated from culture material of *F. moniliforme*, the individual monoesters of PH_1 isolated from $Ca(OH)_2$-treated FB_1, and the fully hydrolysed AP_1 form, showed a number of spectral similarties, especially between the mass range 200 to 400 *m/z*. Ion 309 *m/z* was obser-

ved in the AP_1 moiety, as well as in each of the PH_1 monoesters and the isolated diester PH_1 moiety. Similarly, ions 301.9 and 413.4 *m/z* (shown in Figure 6.10) were also present in both the individual monoester moieites. Background correction of the mass spectra indicated that these ions emanated from the compound, but careful consideration of the structures of both PH_1 and AP_1 did not result in the identification of possible fragments that could fully explain their presence. Ions 301.9 and 413.4 *m/z* were however major ions subsequently identified in the $Ca(OH)_2$ used for the chemical treatment process. It is therefore possible that these ions represent impurities in the $Ca(OH)_2$, that were carried over into the fractions during the treatment and isolation of the various $Ca(OH)_2$-hydrolysed moieties.

None of the mass spectral data concerning either the PH_1 or AP_1 moieties, indicated the presence of calcium. The formation of a FB_1-calcium complex had been considered as a possible explanation for its unique influence on the base-hydrolysis of FB_1. Studies were therefore carried out on TCA, the second major moiety produced by the base-hydrolysis of the fumonisin molecule. Solutions of TCA (purchased standard) in H_2O and 0.01M $Ca(OH)_2$ were prepared, and both were subjected to similar temperature conditions, prior to ES-MS analyses. An unexpected molecular ion of 240.5 *m/z* was observed for TCA (molecular weight = 176). One possible explanation is that the TCA was not present as a free acid, but as a copper salt, which would give rise to a molecular formula of $C_6H_6O_6Cu$ and a $[MH]^+$ of 239 *m/z*. However, copper salts are normally easily distinguised by their greenish-blue colour, which was not evident in the purchased TCA standard. Displacement of the copper ion with calcium would, however, result in a drop of 24 mass units to give a mass of 215 *m/z*, which corresponded to the molecular ion observed for the TCA moiety, following treatment in the presence of 0.01M $Ca(OH)_2$.

It is possible that the generation of ES-MS data, with high cone voltage settings on the instrumentation, would have induced greater fragmentation of the moities, which in turn may

have assisted in the interpretation of all the mass spectral data. Alternatively, the use of MS-MS techniques would also have improved interpretation. Unfortunaely, limited access to this type of advanced instrumentation prevented such studies being undertaken.

6.4 *CONCLUSIONS*

It would appear from the results of the physical and chemical treatments applied to fumonisin contaminated maize, that the fumonisin mycotoxins are concentrated in the outer layers of maize kernels. Significant reductions in combined fumonisin levels may be achieved, possibly economically, by the removal of maize "*fines*". Alternatively, chemical treatment [such as $Ca(OH)_2$], may also be used for the removal or destruction of fumonisins in maize. It is possible that both techniques could be incorporated into an integrated system, whereby chemical treatment could be targeted and applied to the maize "*fines*" themselves, prior to their subsequent use in animal feeds. However, the toxic and carcinogenic potential of the PH_1 and AP_1 moieites, formed during the $Ca(OH)_2$-treatment of maize, will have to be ascertained, in order to determine the safety, and potential use of $Ca(OH)_2$-treated maize products. Studies on the effect of the calcium ion, and its mode of action with respect to the hydrolysis of the fumonisin molecule (and especially its possible relationship with respect to the TCA moiety) are continuing.

THESIS CONCLUSION AND FUTURE STUDIES

Studies have repeatedly confirmed the public perception that the term "*natural*", especially when applied to foods, is associated with "*healthy, wholesome and safe*". The active components in natural products are perceived to be dis-associated from chemicals, which in turn are commonly regarded as being "*man-made, hazardous and potentially carcinogenic*". Food is, however, probably the most chemically complex sub-stance to which the public is exposed. There are a variety of naturally occurring, potentially harmful compounds that can contaminate food. One origin of contamination is that of fungal infection. Fungi are unable to synthesize carbohydrates via photosynthesis, and hence they have to obtain their nutrients by growing either parasitically on the living tissues of plants, animals or man, or saprophytically on dead organic matter. During their growth stage, numerous fungi exhibit the ability to produce a diverse range of secondary metabolites "*mycotoxins*", a number of which can be toxic and/or carcino-genic if ingested by animals or man.

In 1988, a novel group of structurally related mycotoxins, the fumonisins, were isolated from *Fusarium moniliforme*, a fungus that occurs worldwide and which is known to be one of the most prevalent fungi associated with maize; a dietary staple consumed by both animals and man. In addition, *F. moniliforme* has been shown to be the causative fungus responsible for a variety of animal disease syndromes, and the incidence of *F. moniliforme*-contamination of home-grown maize has been associated with an increase in human oesophageal cancer (OC) risk in both China and the Transkei.

Following the isolation of the fumonisins, FB_1 (the major analogue) was shown to be hepatotoxic and carcinogenic to rats, and to induce LEM and PPO in horses and swine, respectively. Further studies indicated that two other fumonisin analogues, FB_2 and FB_3, also exhibited cancer-initiating potentials

similar to those observed for FB_1. Clearly, the potential animal and human health problems associated with the ingestion of the fumonisins was cause for concern, which in turn necessitated the initiation of an integrated risk assessment programme. Data concerning the levels of fumonisins to which target groups or populations may be exposed, would be an essential component of a concerted risk assessment strategy. A prerequisite to the generation of this data, would be the provision of analytical methods that would enable the accurate and reproducible determination of fumonisins in maize and maize-based foods and feeds.

A method developed at PROMEC, which combined selective ion-exchange purification of crude maize extracts with OPA derivatisation, separation by HPLC and detection by fluorescence, was able to detect low levels of the fumonisins (down to 50 ng/g) and achieved excellent recoveries for FB_1 and FB_2 in maize. An initial aspect of this thesis was the validation of the method, and in particular, the establishment of its reproducibility characteristics. This was achieved by the initiation and co-ordination of an international collaborative study involving 11 invited laboratories from 6 countries in Africa, the Americas, Asia and Europe. The results of the study indicated that the method was highly reproducible, and its performance was such that it could, with additional supportive data, be suitable for adoption as an official AOAC method. The combined fumonisin levels selected for the samples used in the collaborative study (between ± 300 and 3000 ng/g) were based on the available data pertaining to maize intended for human consumption. Subsequent studies have indicated that commercially produced maize may be contaminated with combined fumonisin levels that are far higher than the upper 3000 ng/g level selected for the study. These results imply that these higher limits should be considered and selected in future collaborative trials.

Comments from study participants were, together with other observations, incorporated into an optimised method which addressed several important analytical parameters that could impact on the performance of the method. This optimised method

also incorporated the simultaneous determination of the third most abundant fumonisin analogue - FB_3, in addition to FB_1 and FB_2. This optimised method will be the subject of an additional collaborative study, in which the accuracy (and reproducibility) characteristics of the method will be established. The upper combined fumonisin level for the second study will be increased to ± 15000 ng/g, thereby covering the higher levels that can occur in commercially produced maize as well as the levels that could potentially occur in some animal feeds. This second collaborative study will be conduced under the auspices of both IUPAC and the AOAC. Fourteen laboratories from 8 countries have agreed to participate in the study which will be initiated in April 1994.

Procedures based on TLC and GC-MS techniques, that could be used for confirmation purposes, were also either developed and/or refined. The optimised analytical and confirmatory methods were subsequently applied to various matrices in order to assess both animal and human exposure to the fumonisin mycotoxins.

Over 40 *Fusarium* isolates were screened in order to determine their fumonisin-producing ability. This study identified two other *F. moniliforme*-related fumonisin-producing species (*F. proliferatum* and *F. nygamai*). These data extended to 6, the number of morphologically related *Fusarium* species that could produce the fumonisins. However of these, only *F. moniliforme* and *F. proliferatum* are of major importance due to their prevalence as contaminants of maize. Analyses of animal feeds implicated in field outbreaks of equine LEM and PPO that occurred in America, Brazil and Argentina, confirmed the presence of high fumonisin levels. Fumonisin B_1 was the major fumonisin analogue present in the feeds, followed by FB_2 and FB_3, respectively (at ratios that were similar to those observed in fungal cultures). Comparison of the toxicological data, with that of the fumonisin concentrations in the implicated feeds, resulted in recommendations that combined FB_1, FB_2 and FB_3 levels of 10 and 100 ppm or higher, should be considered potentially harmful to horses and swine, respectively.

Commercially produced maize of the 1989 and 1990 South African crops were analysed, and the results indicated that the vast majority of the 249 samples (approximately 90%) were contaminated with combined FB_1, FB_2 and FB_3 concentrations of <1 ppm. Conversely, maize imported into South Africa from America (during 1992) was contaminated with far higher fumonisin levels (mean levels were between 3 and 4 ppm). Commercially available maize-based retail products were also obtained from 14 countries. Fumonisin levels varied quite dramatically dependant on the source, but essentially the data illustrated that fumonisin contamination of maize and maize-based products is a worldwide phenomenon. Of particular concern, was the fact that selected mouldy home-grown maize samples collected from areas of the Transkei, were contaminated with combined mean (31 and 67 ppm over two seasons) and individual (up to 140 ppm) fumonisin levels that would certainly induce LEM in horses, and pulmonary oedema in swine. The data established a statistical association between the occurrence of the fumonisins in home-grown maize and human OC risk. It is important to reiterate, however, that the data in no way inferred that the fumonisins are the causative agents in the development of human OC.

Although these data will assist in overall human risk-assessment studies concerning the fumonisins, substantially more data will be required in order to establish scientifically based, economically viable and realistic tolerance levels. The data generated in this study will, however, form the basis of a PROMEC-initiated IUPAC project to be conducted during the latter part of 1994, aimed at the collation and interpretation of pertinent data on the worldwide occurrence of the fumonisins in foods and feeds.

Initial prevention of fungal, and subsequent mycotoxin contamination of food and feedstuffs, is the most rational and economic approach in the control of mycotoxin-related problems. Prevention of contamination is however often difficult, and therefore decontamination is an alternative option for the recovery of mycotoxin-contaminated crop commodities.

Visual assessment of maize imported into South Africa was characterised by a wide distribution in particulate size. Fractionation of selected samples, by passing through a 3 mm screen, resulted in the separation of maize "*fines*", which corresponded to between 4.7 and 20% of the total mass of each sample. These fractions were, in each case, contaminated with combined fumonisin levels that were significantly higher than the levels recorded in the corresponding "*kernels*" fractions (ie. those fractions having a diameter >3 mm). Physical removal of maize "*fines*", prior to further processing would have led to reductions in combined fumonisin levels by between 26-69%.

Treatment of maize with $Ca(OH)_2$ is used traditionally for the production of "*masa*" (tortilla flour). Several samples of tortilla preparations were shown to be contaminated with very low levels of the fumonisins. An evaluation of the effect, if any, of $Ca(OH)_2$ on FB_1 was therefore undertaken. In a preliminary study, pH-adjusted solutions were used to observe the effects of pH, time and temperature on the progression of hydrolysis of FB_1 to its corresponding aminopentol and TCA moieties. Each of these variables had an effect, but complete hydrolysis was only induced at a temperature of 100°C, maintained for 1 hour in the presence of a 0.05M solution at pH 13. By contrast (and under similar conditions) hydrolysis of FB_1 proceeded at an increased rate in the presence of 0.01M $Ca(OH)_2$. A similar degree of hydrolysis was observed for FB_1 when subjected to treatment with a slurried solution of 0.1M $Ca(OH)_2$, at ambient temperature for 24 hours. In a series of experiments, the Ca ion, in preference to K, Na or Mg, was shown to have a unique effect on the progression of hydrolysis. Both FB_2 and FB_3 were also shown to undergo base hydrolysis in the presence of $Ca(OH)_2$.

In experiments on both ground maize and intact kernels, the action of $Ca(OH)_2$ resulted in the transfer of bulk of the FB_1, from the maize to the aqueous phase, where it was converted to AP_1 and TCA moieties. Following $Ca(OH)_2$-treatment, fumonisin levels in intact maize kernels were reduced by approximately 95%.

Further chromatographic investigations revealed that during the low-temperature $Ca(OH)_2$-treatment process, hydrolysis of FB_1 to its AP_1 moiety progressed via an intermediate metabolite, PH_1. This metabolite was shown to be the partially hydrolysed moiety of FB_1, in which one of the two ester groups had been preferentially hydrolysed. *Trans*-esterification of the monoester, via an intramolecular mechanism, resulted in the formation of an equilibrium mixture of two possible monoesters. The PH_1 metabolite was isolated from $Ca(OH)_2$-treated culture material of *F. moniliforme*, and its identity was confirmed following by LC-MS studies. Given the influence that the Ca ion exhibited on the rate of hydrolysis, no evidence could be obtained that suggested the presence of a Ca-complex with either the PH_1 or AP_1 moieties. It is possible that the Ca ion may complex with the TCA moiety. Inconclusive evidence was obtained from the LC-MS studies of the TCA treated with $Ca(OH)_2$. Experiments are presently planned for proton NMR studies of the possible calcium-TCA complex formation and these will be compared with other possible metal complexes, such as K, Na and Mg.

It will also be essential to determine the cancer-promoting effects of residual maize and its corresponding aqueous phase, following the $Ca(OH)_2$-treatment process. A research protocol has been accepted by the MRC Ethics committee which aims to determine these attributes in the rat model, and the study will begin in June 1994, dependant on the availability of experimental animals.

APPENDIX 1

FUMONISIN AND FUSARIUM MONILIFORME CONTAMINATION IN MAIZE OF THE 1989 AND 1990 SOUTH AFRICAN CROPS

1989 CROP

Sample	Grade	*F. moniliforme* (% kernels infected)	Fumon. concn. (ng/g) FB_1	FB_2	Total
1	W1[a]	14	3850	1385	5235
2	W1	11	80	ND[b]	80
3	W1	10	140	ND	140
4	W1	7	2365	870	3235
5	W1	14	5420	1600	7020
6	W2[c]	95	205	65	270
7	W2	13	ND	90	90
8	W2	3	75	ND	75
9	W2	15	435	130	565
10	W2	18	150	ND	150
11	W3[d]	15	100	ND	100
12	W3	27	2525	685	3210
13	W3	7	2525	685	3210
14	W3	11	800	200	1000
15	W3	7	285	95	380
16	W3	28	1495	470	1965
17	W3	18	3210	850	4060
18	W1	2	70	ND	70
19	W1	2	ND	ND	ND
20	W1	1	135	115	250
21	W1	5	ND	ND	ND
22	W1	9	ND	ND	ND
23	W2	2	ND	ND	ND
24	W2	1	ND	ND	ND
25	W2	2	ND	ND	ND
26	W2	1	ND	ND	ND
27	W2	1	ND	ND	ND
28	W1	11	170	55	225
29	W1	10	95	ND	95
30	W1	2	ND	ND	ND
31	W1	7	230	330	560
32	W1	4	ND	ND	ND
33	W2	12	60	ND	60
34	W2	7	275	130	405
35	W2	11	ND	ND	ND
36	W2	7	255	85	340
37	W2	21	55	ND	55
38	W1	13	455	140	595
39	W1	7	ND	ND	ND
40	W1	12	160	55	215
41	W1	22	160	ND	160
42	W1	20	1310	435	1745
43	W2	16	85	ND	85
44	W2	14	ND	ND	ND
45	W2	9	180	85	265
46	W2	15	315	140	455

Appendix 1 (continued)

Sample	Grade	*F. moniliforme* (% kernels infected)	Fumon. concn. (ng/g) FB_1	FB_2	Total
47	W2	13	ND	ND	ND
48	W3	3	95	ND	95
49	W3	3	70	125	195
50	W3	14	130	110	240
51	W3	10	180	125	305
52	W3	18	345	160	505
53	W3	6	ND	ND	ND
54	W3	17	55	ND	55
55	W1	5	515	375	890
56	W1	12	210	85	295
57	W1	1	690	305	995
58	W1	6	1300	490	1790
59	W1	1	ND	ND	ND
60	W2	5	2110	380	2490
61	W2	7	90	ND	90
62	W2	6	ND	ND	ND
63	W2	13	350	125	475
64	W2	2	1120	370	1490
65	W3	5	575	270	845
66	W3	13	2520	870	3390
67	W3	16	600	170	770
68	W3	9	200	90	290
69	Y1[e]	3	ND	ND	ND
70	Y1	18	55	ND	55
71	Y1	16	435	120	555
72	Y1	16	60	ND	60
73	Y1	79	110	ND	110
74	Y2[f]	2	60	ND	60
75	Y2	14	ND	ND	ND
76	Y2	18	1120	335	1455
77	Y2	45	920	285	1205
78	Y2	32	75	ND	75
79	Y3[g]	3	ND	ND	ND
80	Y1	0	65	ND	65
81	Y1	4	65	ND	65
82	Y1	6	ND	ND	ND
83	Y1	4	ND	ND	ND
84	Y1	3	ND	ND	ND
85	Y2	2	ND	ND	ND
86	Y2	8	115	ND	115
87	Y2	0	ND	ND	ND
88	Y2	2	ND	ND	ND
89	Y2	0	ND	ND	ND
90	Y1	2	110	ND	110
91	Y1	5	355	100	455
92	Y1	5	ND	ND	ND
93	Y1	6	ND	ND	ND
94	Y1	8	ND	ND	ND
95	Y2	10	135	ND	135
96	Y2	3	80	ND	80
97	Y2	4	240	ND	240
98	Y2	8	95	55	150

Appendix 1 (continued)

Sample	Grade	*F. moniliforme* (% kernels infected)	Fumon. concn. (ng/g) FB_1	FB_2	Total
99	Y2	0	ND	ND	ND
100	Y3	9	380	ND	380
101	Y1	7	ND	ND	ND
102	Y1	16	ND	ND	ND
103	Y1	24	ND	ND	ND
104	Y1	32	430	90	520
105	Y1	31	115	ND	115
106	Y2	2	ND	ND	ND
107	Y2	20	700	695	1395
108	Y2	23	95	ND	95
109	Y2	21	635	350	985
110	Y2	19	345	ND	345
111	Y3	27	430	110	540
112	Y1	6	730	195	925
113	Y1	1	ND	ND	ND
114	Y1	38	1055	380	1435
115	Y1	2	155	ND	155
116	Y1	6	ND	ND	ND
117	Y2	4	ND	ND	ND
118	Y2	14	115	ND	115
119	Y2	12	355	85	440
120	Y2	2	110	ND	110
121	Y2	1	ND	ND	ND

		1990 CROP	FB_1	FB_2	FB_3	Total
1	W1	23	210	90	ND	300
2	W1	15	1300	950	160	2410
3	W1	8	90	ND	ND	90
4	W1	19	390	105	50	545
5	W1	7	ND	ND	ND	ND
6	W2	7	1490	465	200	2155
7	W2	34	240	110	ND	350
8	W2	21	50	ND	ND	50
9	W2	15	50	ND	ND	50
10	W2	10	ND	ND	ND	ND
11	W3	2	1020	400	80	1500
12	W3	22	275	80	ND	355
13	W3	18	300	70	50	420
14	W3	12	420	260	50	730
15	W3	4	100	50	ND	150
16	W3	7	50	ND	ND	50
17	W1	3	ND	ND	ND	ND
18	W1	10	2470	850	270	3590
19	W1	9	50	ND	ND	50
20	W1	6	ND	ND	ND	ND
21	W1	0	50	ND	ND	50
22	W2	1	50	ND	ND	50
23	W2	3	ND	ND	ND	ND
24	W2	3	50	ND	ND	50
25	W2	0	ND	ND	ND	ND
26	W2	0	ND	ND	ND	ND

Appendix 1 (continued)

Sample	Grade	*F. moniliforme* (% kernels infected)	Fumon. concn. (ng/g) FB_1	FB_2	FB_3	Total
27	W3	2	60	ND	ND	60
28	W3	2	ND	ND	ND	ND
29	W1	15	890	370	150	1410
30	W1	10	ND	ND	ND	ND
31	W1	14	310	220	130	660
32	W2	20	ND	ND	ND	ND
33	W2	11	ND	ND	ND	ND
34	W2	26	2910	1190	270	4370
35	W3	49	595	220	50	865
36	W3	11	335	140	50	525
37	W1	12	170	50	ND	220
38	W1	12	90	ND	ND	90
39	W1	8	50	ND	ND	50
40	W1	6	5030	1670	400	7100
41	W1	3	50	ND	ND	50
42	W2	13	180	110	ND	290
43	W2	25	50	ND	ND	50
44	W2	5	50	ND	ND	50
45	W2	2	60	ND	ND	60
46	W2	9	845	ND	ND	845
47	W3	3	230	260	80	570
48	W3	20	490	130	150	770
49	W3	15	400	145	60	605
50	W3	3	260	50	50	360
51	W3	26	590	250	70	910
52	W3	9	50	ND	ND	50
53	W3	11	140	ND	ND	140
54	W1	1	ND	ND	ND	ND
55	W1	5	ND	ND	ND	ND
56	W1	21	280	110	65	455
57	W1	3	ND	ND	ND	ND
58	W1	2	ND	ND	ND	ND
59	W2	7	960	330	150	1440
60	W2	9	200	90	ND	290
61	W2	10	300	95	ND	395
62	W2	2	100	ND	ND	100
63	W2	4	ND	ND	ND	ND
64	W3	0	ND	ND	ND	ND
65	W3	6	790	245	130	1165
66	W3	2	50	ND	ND	50
67	Y1	12	190	80	ND	270
68	Y1	18	ND	ND	ND	ND
69	Y1	33	90	50	ND	140
70	Y1	6	ND	ND	ND	ND
71	Y1	28	195	90	50	335
72	Y2	14	200	90	ND	290
73	Y2	5	50	ND	ND	50
74	Y3	15	ND	ND	ND	ND
75	Y3	2	ND	ND	ND	ND
76	Y3	1	55	ND	ND	55
77	Y1	3	ND	ND	ND	ND
78	Y1	5	490	120	ND	610

Appendix 1 (continued)

Sample	Grade	*F. moniliforme* (% kernels infected)	Fumon. concn. (ng/g) FB_1	FB_2	FB_3	Total
79	Y1	8	130	50	ND	180
80	Y1	1	550	235	80	865
81	Y1	3	280	220	ND	500
82	Y2	11	730	320	70	1120
83	Y2	17	200	230	50	480
84	Y2	27	190	180	50	420
85	Y2	3	ND	ND	ND	ND
86	Y2	10	ND	ND	ND	ND
87	Y3	30	ND	ND	ND	ND
88	Y1	2	50	ND	ND	50
89	Y1	3	ND	ND	ND	ND
90	Y1	1	50	ND	ND	50
91	Y1	7	ND	ND	ND	ND
92	Y1	3	115	90	50	255
93	Y2	13	ND	ND	ND	ND
94	Y2	24	50	ND	ND	50
95	Y2	13	50	ND	ND	50
96	Y2	20	380	130	50	560
97	Y2	4	50	ND	ND	50
98	Y3	13	400	155	50	605
99	Y3	7	ND	170	ND	170
100	Y3	6	50	ND	ND	50
101	Y1	19	50	ND	ND	50
102	Y1	15	190	65	50	305
103	Y1	11	ND	ND	ND	ND
104	Y1	36	110	50	ND	160
105	Y1	15	260	130	50	440
106	Y2	23	90	50	ND	140
107	Y2	23	630	170	70	870
108	Y2	37	300	100	50	450
109	Y2	7	160	55	ND	215
110	Y2	35	60	ND	ND	60
111	Y3	34	260	70	ND	330
112	Y3	9	950	315	70	1335
113	Y3	8	320	90	50	460
114	Y3	8	1060	320	115	1495
115	Y3	12	430	50	200	680
116	Y3	29	185	55	ND	240
117	Y3	11	225	55	50	330
118	Y1	61	ND	ND	ND	ND
119	Y1	1	ND	ND	ND	ND
120	Y1	22	ND	ND	ND	ND
121	Y1	0	215	120	50	385
122	Y1	3	50	ND	ND	50
123	Y2	16	50	ND	ND	50
124	Y2	11	185	85	ND	270
125	Y2	6	180	50	50	280
126	Y2	3	110	60	ND	170
127	Y2	4	340	180	50	570
128	Y3	8	50	ND	ND	50

Appendix 1 (continued)

Key to Appendix 1

[a]W1 = White maize grade 1
[b]ND = Not detected (<50 ng/g)
[c]W2 = White maize grade 2
[d]W3 = White maize grade 3
[e]Y1 = Yellow maize grade 1
[f]Y2 = Yellow maize grade 2
[g]Y3 = Yellow maize grade 3

APPENDIX 2

FUMONISIN LEVELS IN BULK CONSIGNMENTS OF MAIZE EXPORTED FROM AND IMPORTED INTO SOUTH AFRICA

Sample number	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
USA SHIPMENT NUMBER 1 (1992)				
1	1375	465	105	1945
2	3320	880	350	4550
3	2460	625	180	3265
4	3320	1000	400	4720
5	2950	620	255	3825
6	3375	755	300	4430
7	1975	485	150	2610
8	1790	465	170	2425
9	2985	660	230	3875
10	3215	765	295	4275
11	2545	590	205	3340
12	1865	470	135	2470
13	1765	515	245	2525
14	3335	870	495	4700
15	2405	605	215	3225
16	1745	510	150	2405
17	2780	705	250	3735
18	3100	875	290	4265
19	1690	390	150	2230
20	2280	610	185	3075
21	2330	665	210	3205
22	1575	420	195	2190
23	2690	760	275	3725
24	2550	720	370	3640
25	1590	400	165	2155
26	2235	685	530	3450
27	2765	765	375	3905
28	995	275	100	1370
29	2465	740	190	3395
30	1410	400	150	1960
31	1370	400	135	1905
32	1830	495	165	2490
33	3085	825	330	4240
34	1300	330	125	1755
35	3550	1105	460	5115
36	2075	565	225	2865
37	890	260	80	1230
38	1185	300	130	1615
39	2460	810	240	3510
40	1885	535	165	2585
41	3630	1085	550	5265
42	2570	755	220	3545
43	1120	350	105	1575

Appendix 2 (continued)

Sample number	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
44	2750	790	325	3865
45	2200	605	225	3030
46	1370	400	130	1900
47	2110	620	175	2905
48	2360	680	250	3290
49	1100	320	120	1540
50	1885	530	220	2635
51	2165	670	405	3240
52	965	265	140	1370
53	2335	625	275	3235
54	3370	935	470	4775
55	1495	410	135	2040
56	2170	590	205	2965
57	2950	925	310	4185
USA SHIPMENT NUMBER 2 (1992)				
1	2390	645	245	3280
2	1520	485	150	2155
3	3740	1165	385	5290
4	2855	735	265	3855
5	2370	745	255	3370
6	2645	815	290	3750
7	2720	910	335	3965
8	3270	1095	305	4670
9	3590	1105	390	5085
10	2015	630	260	2905
11	2265	780	315	3360
12	1990	635	265	2890
13	3855	1120	390	5365
14	2480	730	275	3485
15	1965	605	220	2790
16	2235	740	260	3235
17	2595	760	275	3630
18	2580	795	285	3660
19	3145	945	285	4375
20	3015	970	310	4295
21	2405	720	265	3390
22	2700	805	290	3795
ARGENTINIAN SHIPMENT NUMBER 1 (1992)				
1	155	60	50	265
2	210	65	50	325
3	50	ND[a]	ND	50
4	270	95	50	415
5	50	ND	ND	50
6	50	ND	ND	50
7	485	130	80	695
8	200	50	50	300
9	185	60	ND	245

Appendix 2 (continued)

Sample number	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
10	50	ND	ND	50
11	670	440	100	1210
12	105	50	ND	155
13	405	95	50	550
14	445	80	50	575
15	55	ND	ND	55
16	225	50	ND	275
17	115	50	ND	165
18	335	75	50	460
19	120	50	ND	170
20	345	95	50	490
21	330	80	50	460
ARGENTINIAN SHIPMENT NUMBER 2				
1	210	65	ND	275
2	90	50	ND	140
3	185	50	ND	235
4	170	55	ND	225
5	105	ND	ND	105
6	160	50	ND	210
7	140	50	ND	190
8	470	100	85	655
9	470	150	80	700
10	155	55	ND	210
11	565	160	65	790
12	460	105	80	645
13	515	140	70	725
14	455	190	75	720
15	530	160	65	755
16	720	175	95	990
17	495	495	500	1490
18	165	50	ND	215
19	675	180	85	940
20	350	85	50	485
21	465	125	65	655
22	475	110	75	660
23	390	115	50	555
24	500	130	65	695
25	310	75	50	435
26	445	110	55	610
SOUTH AFRICAN EXPORT SHIPMENT TO TAIWAN (1989)("A" SAMPLES - COLLECTED IN SOUTH AFRICA)				
1	90	ND	NA[b]	90
2	290	150	NA	440
3	ND	ND	NA	ND
4	ND	ND	NA	ND
5	ND	ND	NA	ND

Appendix 2 (continued)

Sample number	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
6	ND	ND	NA	ND
7	90	ND	NA	90
8	ND	ND	NA	ND
9	ND	ND	NA	ND
10	ND	ND	NA	ND
11	ND	ND	NA	ND
12	ND	ND	NA	ND
13	630	250	NA	880
14	250	170	NA	420
15	210	130	NA	340
16	ND	ND	NA	ND
17	ND	ND	NA	ND
18	ND	ND	NA	ND
19	ND	ND	NA	ND
20	ND	ND	NA	ND
21	ND	ND	NA	ND
22	70	90	NA	160
23	ND	ND	NA	ND
24	270	100	NA	370
25	ND	ND	NA	ND
26	ND	ND	NA	ND
27	ND	ND	NA	ND
28	ND	ND	NA	ND
29	60	ND	NA	60
30	ND	ND	NA	ND
31	ND	ND	NA	ND
32	90	ND	NA	90
33	ND	ND	NA	ND
34	ND	ND	NA	ND
35	ND	ND	NA	ND
36	ND	ND	ND	ND
SOUTH AFRICAN EXPORT SHIPMENT TO TAIWAN (1989) ("B" SAMPLES - COLLECTED IN TAIWAN)				
1	90	ND	NA	90
2	110	ND	NA	110
3	545	ND	NA	545
4	865	120	NA	985
5	ND	ND	NA	ND
6	595	ND	NA	595
7	550	ND	NA	550
8	ND	ND	NA	ND
9	ND	ND	NA	ND
10	ND	ND	NA	ND
11	775	ND	NA	775
12	160	ND	NA	160
13	ND	ND	NA	ND
14	ND	ND	NA	ND
15	50	75	NA	125
16	ND	ND	NA	ND

Appendix 2 (continued)

Sample number	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
17	ND	ND	NA	ND
18	ND	ND	NA	ND
19	ND	ND	NA	ND
20	290	110	NA	400
21	ND	ND	NA	ND
22	ND	ND	NA	ND
23	ND	ND	NA	ND
24	95	ND	NA	95
25	ND	ND	NA	ND
26	50	ND	NA	50
27	395	100	NA	495
28	ND	ND	NA	ND
29	ND	ND	NA	ND
30	ND	ND	NA	ND
31	ND	ND	NA	ND
32	115	ND	NA	115

[a]ND = Not detected (<50 ng/g)
[b]NA = Not analysed

APPENDIX 3

FUMONISIN CONCENTRATIONS IN MAIZE-BASED COMMERCIAL HUMAN FOODSTUFFS OBTAINED FROM RETAIL OUTLETS IN SEVERAL COUNTRIES[a]

MAIZE MEAL

Country	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
Austria	70	NDb	ND	70
Austria	115	ND	ND	115
Botswana	445	105	70	620
Botswana	210	55	ND	265
Botswana	280	50	ND	330
Bulgaria	95	60	ND	155
Bulgaria	1110	150	ND	1260
Bulgaria	60	ND	ND	60
Bulgaria	210	ND	ND	210
Bulgaria	120	ND	ND	120
Bulgaria	50	ND	ND	50
Bulgaria	180	ND	ND	180
Canada	50	ND	ND	50
Canada	ND	ND	ND	ND
China	ND	ND	ND	ND
China	ND	ND	ND	ND
China	ND	ND	ND	ND
Egypt	1780	410	NAc	2190
Egypt	2980	780	NA	3760
France	1240	390	180	1810
Hungary	ND	ND	ND	ND
Kenya	235	ND	ND	235
Kenya	50	ND	ND	50
Kenya	110	140	50	300
Kenya	ND	ND	ND	ND
Peru	ND	ND	NA	ND
Peru	660	135	NA	795
SA[d]	120	50	ND	170
SA	50	ND	ND	50
SA	50	ND	ND	50
SA	ND	ND	ND	ND
SA	65	ND	ND	65
SA	50	ND	ND	50

Appendix 3 (continued)

	Fumonisin concentrations (ng/g)			
Country	FB_1	FB_2	FB_3	Total
SA	455	120	50	625
SA	180	ND	ND	180
SA	140	55	NA	195
SA	55	ND	NA	55
SA	ND	ND	NA	ND
SA	215	60	NA	275
SA	425	100	NA	525
SA	85	50	NA	135
SA	110	ND	NA	110
SA	90	ND	NA	90
SA	105	ND	NA	105
SA	160	ND	NA	160
SA	210	65	NA	275
SA	90	50	NA	140
SA	165	60	NA	225
SA	340	95	NA	435
SA	200	50	NA	250
SA	105	ND	NA	105
SA	85	ND	NA	85
SA	60	ND	NA	60
SA	100	ND	NA	100
SA	85	ND	NA	85
SA	80	ND	NA	80
SA	90	ND	NA	90
SA	135	ND	NA	135
SA	65	ND	NA	65
SA	60	ND	NA	60
SA	100	ND	NA	100
SA	60	ND	NA	60
SA	130	50	NA	180
SA	105	ND	NA	105
SA	100	ND	NA	100
SA	ND	ND	NA	ND
SA	50	ND	NA	50
SA	110	50	NA	160
SA	70	ND	NA	70
SA	70	ND	NA	70
SA	280	ND	NA	280
SA	95	ND	NA	95
SA	355	115	NA	470
SA	55	ND	NA	55
SA	ND	ND	NA	ND
SA	110	ND	NA	110
SA	70	ND	NA	70
SA	50	ND	NA	50
SA	125	50	NA	175
SA	50	ND	NA	50
SA	110	ND	NA	110
SA	50	ND	NA	50
SA	475	130	NA	605
SA	110	ND	NA	110
SA	90	ND	NA	90

Appendix 3 (continued)

Country	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
SA	105	ND	NA	105
SA	160	ND	NA	160
SA	210	65	NA	275
SA	85	50	NA	135
SA	70	ND	NA	70
SA	ND	ND	NA	ND
SA	95	ND	NA	95
SA	370	95	NA	465
SA	105	ND	NA	105
USA[e]	3160	950	420	4530
USA	1215	355	155	1725
USA	2310	690	280	3280
USA	1030	300	145	1475
USA	995	280	NA	1275
USA	270	80	NA	350
USA	400	70	NA	470
USA	815	190	NA	1005
USA	625	90	NA	715
USA	1915	460	NA	2375
USA	405	100	NA	505
USA	790	210	NA	1000
USA	580	155	NA	735
USA	85	ND	NA	85
USA	205	50	NA	255
USA	1640	420	NA	2060
USA	1245	275	NA	1520
USA	685	155	NA	840
USA	ND	ND	NA	ND
USA	700	240	NA	940
USA	910	210	NA	1120
NZ[f]	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
NZ	ND	ND	ND	ND
MAIZE RICE, MAIZE SAMP, MAIZE GRITS				
SA	145	120	NA	265
SA	425	100	NA	525
SA	190	90	NA	280

Appendix 3 (continued)

Country	Fumonisin concentrations (ng/g)			
	FB_1	FB_2	FB_3	Total
SA	135	ND	NA	135
SA	185	65	NA	250
SA	140	ND	NA	140
SA	180	65	NA	245
SA	50	ND	NA	50
SA	50	ND	NA	50
SA	ND	ND	NA	ND
SA	ND	ND	NA	ND
SA	ND	ND	NA	ND
SA	ND	ND	NA	ND
SA	ND	ND	NA	ND
SA	190	90	NA	280
SA	185	ND	NA	185
SA	140	ND	NA	140
SA	65	ND	NA	65
SA	ND	ND	NA	ND
SA	ND	ND	NA	ND
SA	145	120	NA	265
SA	ND	ND	NA	ND
USA	ND	ND	NA	ND
USA	235	65	NA	300
USA	850	205	NA	1055
USA	2545	1065	NA	3610
USA	1360	470	NA	1830
USA	105	ND	NA	105
USA	140	ND	NA	140
USA	210	50	NA	260
USA	285	70	NA	355
USA	140	50	NA	190
ALKALI TREATED MAIZE PRODUCTS				
Peru	ND	ND	NA	ND
Peru	ND	ND	NA	ND
USA	ND	ND	NA	ND
USA	50	ND	NA	50
USA	ND	ND	NA	ND
MISCELLANEOUS MAIZE PRODUCTS				
Country	**Product**			
Austria	Flour	ND	ND	ND	ND
SA	Flour	ND	ND	NA	ND
SA	Flour	ND	ND	NA	ND
SA	Flour	ND	ND	NA	ND

Appendix 3 (continued)

Country	Product	Fumonisin concentrations (ng/g)			
		FB_1	FB_2	FB_3	Total
SA	Flour	ND	ND	NA	ND
SA	Flour	ND	ND	NA	ND
SA	Flakes	ND	ND	NA	ND
SA	Flakes	ND	ND	NA	ND
SA	Flakes	ND	ND	NA	ND
SA	Flakes	ND	ND	NA	ND
SA	Cereal[g]	90	ND	NA	90
SA	Cereal	75	ND	NA	75
SA	Cereal	ND	ND	NA	ND
SA	Cereal	ND	ND	NA	ND
SA	Popcorn	ND	ND	NA	ND
SA	Popcorn	ND	ND	NA	ND
USA	Cornmix	450	135	NA	585
USA	Flakes	ND	ND	NA	ND
USA	Flakes	ND	ND	NA	ND
USA	Cereal	700	240	NA	940
USA	Popcorn	85	50	NA	135
USA	Gluten	2560	665	245	3470
USA	Gluten	470	145	ND	615
Zimbabwe	Cereal	3625	910	230	4765
Zimbabwe	Cereal	1055	240	125	1420

[a]Fumonisin levels reflect the original source of the individual maize products rather than country from which they were purchased
[b]ND = Not detected (ie. <50 ng/g)
[c]NA = Not analysed
[d]SA = South Africa
[e]USA = United States of America
[f]NZ = New Zealand
[g]Cereal = maize-based breakfast cereal

APPENDIX 4

FUMONISIN LEVELS IN TRANSKEI HOME GROWN MAIZE SAMPLES

Sample number	Fumonisin concentrations (ng/g)					
	FB_1	FB_2	Total	FB_1	FB_2	Total
	"Healthy" samples			"Mouldy" samples		
LOW OC AREA (1985)						
1	195	50	245	4450	1800	6250
2	ND[a]	ND	ND	1350	550	1900
3	ND	ND	ND	7350	2400	9750
4	ND	ND	ND	3800	1350	5150
5	ND	ND	ND	NA[b]	NA	NA
6	ND	50	50	18900	6750	25650
7	ND	ND	ND	5200	1700	6900
8	ND	ND	ND	9000	2650	11650
9	ND	ND	ND	450	150	600
10	ND	ND	ND	10750	6500	17250
11	560	140	700	8750	2900	11650
12	ND	ND	ND	1700	700	2400
HIGH OC AREA (1985)						
1	520	160	680	3450	900	4350
2	7900	2240	10140	28900	10250	39150
3	1160	290	1450	11250	2550	13800
4	175	55	230	45700	12900	58600
5	730	230	960	20700	6100	26800
6	2590	880	3470	16600	6200	22800
7	155	50	205	18900	5400	24300
8	1510	450	1960	21700	8200	29900
9	2540	1390	3930	15400	4600	20000
10	60	ND	60	12600	4800	17400
11	900	275	1175	44600	12600	57200
12	90	ND	90	46900	16300	63200
LOW OC AREA (1989)						
1	100	ND	100	110	ND	110
2	50	ND	50	10510	2010	12520
3	3310	970	4280	2210	550	2760
4	ND	ND	ND	720	360	1080
5	180	ND	180	210	110	320
6	ND	ND	ND	11340	3700	15040
7	140	60	200	NA	NA	ND
8	220	ND	220	3280	930	4210
HIGH OC AREA (1989)						
1	440	150	590	37120	13530	50650
2	1890	600	2490	88480	20540	109020
3	210	60	270	45480	14650	60130
4	5380	1320	6700	117520	22960	140480
5	ND	ND	ND	3020	750	3770
6	1280	410	1690	30790	9640	40430

[a]ND = Not detected (<50 ng/g); [b]NA = Not analysed

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