QUANTITATIVE DETERMINATION OF FUMONISIN B_1 IN BIOLOGICAL MATERIAL

by

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Submitted in partial fulfilment of
the requirements for the degree of

MASTER OF MEDICAL SCIENCE

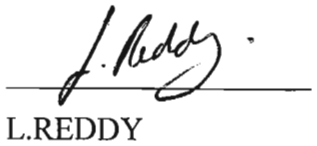
in the
Department of Physiology
University of Natal
Durban

1999

DECLARATION

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

The research described in this thesis was carried out under the supervision of Professor M.F.Dutton.



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PUBLICATION AND PRESENTATIONS

PUBLICATION

Title: Serum Fumonisin B_1 Levels in African Black Women with Pre-eclampsia

Authors: Reddy L., Dutton M.F., Chuturgoon A.A., Moodley J. and Moodley D.

Journal: *British Journal of Obstetrics and Gynaecology* (with supervisors)

CONFERENCE PRESENTATION

1. Production, purification and detection of fumonisin B_1 in culture material.
   Reddy L., Nair A., Odhav B. and Dutton M.F.
   Poster presented at **Biotech SA '97** in January 1997, Grahamstown.

2. HPLC analysis of fumonisin B_1. Reddy L., Dutton MF.
   Oral presentation at **Regional SASM/ Biochemistry Conference** in October 1997, Durban.

3. HPLC analysis of fumonisin B_1 in serum.
   Reddy L. and Dutton M.F.
   Poster presented at **International Conference on Frontiers in Biotechnology** in November 1997, Trivandrum, India.

4. HPLC analysis of serum of pre-eclamptic / eclamptic women for the presence of fumonisin B_1.
   Reddy L., Ramsunder K. and Dutton M.F.
   Poster presented at **1998 International Symposium on Technology Education in June 1998**, Cape Town.

5. HPLC analysis of serum of pre-eclamptic / eclamptic women for the presence of fumonisin B_1.
   Reddy L., Ramsunder K. and Dutton M.F.
   Poster presented at **10th Biennial Conference of SASM** in July 1998, Durban.
   Conference abstracts published in South African Journal of Science **95**, May 1999.

6. Fumonisin B_1 found in sera of eclamptic women in South Africa. Reddy L., Moodley D., Moodley J. and Dutton M.F.
   Poster presented at the **1999 Gordon Research Conference on Mycotoxins and Phycotoxins** in June 1999, Plymouth, U.S.A.

WORKSHOP

Attended the **Satellite Workshop to the Gordon Conference** on **Detection Methods for Phycotoxins and Mycotoxins** in June 1999, Salsbury Cove, Maine, U.S.A.

SUPPORTING SERVICES

In this research the statistical planning and analyses, and recommendations arising from these analyses, have been done with the support of the Institute for Biostatistics and the Pregnancy Hypertension Unit of the Medical Research Council.

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to the following individuals for their assistance in the preparation of this thesis:

Professor M.F. Dutton, supervisor and Head of the Department, and Mr. A. Chuturgoon, Senior Lecturer, of the Department of Physiology, University of Natal, for their expert guidance and constructive criticism.

Professor J. Moodley and Dr. D. Moodley of the MRC Pregnancy Hypertension Unit, University of Natal, for permission to use sera from the Obstetric clinic at the King Edward VIII Hospital.

Ms. D.R. Naidoo and Dr. B. Odhav, Head and Acting Head of the Dept.of Biological Sciences respectively, ML Sultan Technikon, for their encouragement and the granting of study leave.

My spiritual master (Bhagavan Sri Sathya Sai Baba), parents, husband and two little sons for their support, encouragement and patience throughout this research endeavour.

The FRD for their continued financial support during my research without which it would have been a huge struggle to complete a part-time Masters degree.

Merck NT (PTY) Ltd, especially Gopal Pillay, Shona Gander and Janet Sharp for their prompt assistance when required.

Professor G. Shephard, PROMEC, MRC, Tygerberg, for his kind telephonic assistance.

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ABSTRACT

The mycotoxin, fumonisin B_1 is produced by the mould *Fusarium moniliforme,* a common contaminant of maize and maize products. Small doses (mg/kg) of ingested fumonisin B_1 have been shown to cause diseases and even death in animals, including non-human primates. Thus highly sensitive methods have been employed to detect fumonisin B_1 presence in foods, feeds and in animals. This study comprised two parts.The initial part focussed on establishing reliable extraction, purification and quantitation of fumonisin B_1 using high-performance liquid chromatography (HPLC) on culture extracts. The second part was to analyse sera of Black African women with pre-eclampsia for the presence of fumonisin B_1 using HPLC.

Maize patty cultures and broth cultures were inoculated with *Fusarium moniliforme* PPRI 1059 and incubated. Fumonisin B_1 was extracted and purified by centrifugation strong anion exchange chromatography (SAX). Eluents from SAX cartridges were analysed using Thin - layer chromatography (TLC) and fluorescence HPLC after o-phythadialdehyde (OPA) derivatisation. Fumonisin B_1 standards on HPLC gave a retention time of 7.5 minutes using methanol /0.1 M sodium dihydrogen phosphate (68 + 32, pH 3.3) as mobile phase and a 25 cm C8 column. Patty cultures produced the highest yields of fumonisin B_1. In the case of serum samples, a double-blind study was carried out using women attending the obstetric clinic at a large city teaching hospital. The population comprised normal, pre-eclamptic and eclamptic women. On HPLC analysis a significantly higher mean concentration of fumonisin B_1 concentration was found in the eclamptic group ($P< 0,005$) as compared to the other two groups.Thus fumonisin B_1 may have a role to play in eclampsia for which the aetiology is still unknown.

INTRODUCTION

The world is plagued with various types of contaminations. There are physical, chemical, microbiological, emotional and many other types of contaminating agents or processes. These affect us to some degree or other in our daily lives. One such contamination, is mycotoxins, an example being fumonisin B_1 (FB_1), which is produced by the mould *Fusarium moniliforme* (Sheldon) (Gelderblom *et al.,* 1988.). This mould commonly contaminates maize, maize products and various other foods and feeds worldwide (Doko *et al.,* 1995, Sydenham *et al.,*1991, Yoshizawa *et al.,* 1994, Fazekas and Tothe 1995, Murphy *et al.,* 1993). Thus people and animals who consume maize frequently are at high risk of fumonisin intoxication (Bondy *et al.,* 1995; Campbell, 1996; Espada *et al.,* 1994; Marasas *et al.,* 1993).

Animal studies have already shown this toxin to cause serious animal diseases, such as hole-in-the-head disease or equine-leucoencephalomalacia (ELEM) in horses (Marasas *et al.,* 1988, Kellerman *et al.,* 1990, Pellegrin *et al.,* 1990), pulmonary oedema in pigs (Gelderblom *et al.,* 1992, Mirocha *et al.,* 1992, Colvin and Harrison, 1992) and hepatocarcinoma in rats (Gelderblom *et al.,* 1992). Epidemiological studies have suggested that fumonisins may be partially responsible for the high incidence of oesophageal cancer (OC) in the Transkei region of South Africa (Sydenham *et al.,* 1991, Rheeder *et al.,* 1992).

The fumonisins are long hydroxylated hydrocarbon chains with an amino group and two tricarboxylic acid groups attached via ester bonds (Caldas *et al.,* 1995). The latter groups make fumonisins water soluble but insoluble in non-polar organic solvents. Thus, although production of fumonisins in culture media by *Fusarium* spp. is somewhat simple,

extraction and purification of the toxin is tedious (Cawood *et al.,* 1991). Many authors relate the problems encountered with regard to the production, extraction and purification of fumonisins in solid and liquid cultures. Fumonisin found in foods, feeds and culture material may occur in relatively low levels (μg /kg). Thus sensitive analytical techniques, such as high-performance liquid chromatography (HPLC) (Thiel *et al.,* 1993) and enzyme-linked immunoassay (Schneider *et al.,* 1995), are being used to detect and quantitate fumonisins in biological material.

The fumonisin molecule shows a strong structural resemblance to the phospholipid, sphingosine (Wang *et al.,* 1991) and has been found to disrupt sphingolipid biosynthesis (Shephard *et al.,* 1996). This could result in numerous pathophysiological effects in animals including man (Brown *et al.,* 1996; Reddy *et al.,* 1996; Bondy *et al.,* 1997).

Fumonisins have been shown to produce many serious pathological effects in many different animals, animal and human cell lines, plants and microorganisms that have been experimentally injected with the toxin. A thorough literature search has shown that many of the symptoms produced in animals by fumonisin are similar to that of a disease in pregnancy, called pre-eclampsia (current study). Some of these symptoms are pulmonary oedema, cerebral oedema, foetal malformations and liver and kidney damage.

AIMS AND OBJECTIVES

1. Production of FB_1 standard.

2. Setting up of suitable and sensitive analytical procedures for the quantitation of FB_1.

3. Analysis of serum from patients with pre-eclampsia for the presence of FB_1 using HPLC.

CHAPTER 1

1. 0 LITERATURE REVIEW : FUMONISINS AND DISEASE

1.1 Isolation and Structure of Fumonisins

Fumonisins are a group of mycotoxins (poisonous secondary metabolites of fungi) that are produced by the mould *Fusarium.* The toxins were originally isolated from *Fusarium moniliforme* MRC 826 by the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) group of South African researchers directed by Professor WFO Marasas (Bezuidenhout *et al.,* 1988; Gelderblom *et al.,* 1988) . Fumonisin was also isolated from Transkeian maize cultures and designated fumonisin B_1 (FB_1) (Laurent *et al.,* 1989; Haschek *et al.*, 1992). Today *F. moniliforme* remains the most important producer of FB_1 followed by *F. proliferatum, F. nygamai* and *F. napiforme* (Nelson *et al.,* 1992), probably because of their association with maize and millet. Other species that produce the toxin are *F. subglutinans, F. anthophilum, F. dlamini, F. oxysporum* var *redolens, F. polyphialidicum, F. globusum* and *Alternaria alternata* (Thiel *et al.,* 1991; Norred *et al.,* 1993; Bullerman and Tsai, 1994; Abbas and Ocamb, 1995; Seo and Lee, 1996; Sydenham *et al.*, 1997).

To date, seven forms of fumonisins have been discovered, namely, fumonisin B_1 (FB_1), B_2, B_3, B_4, A_1, A_2 and C_1 (Wilkes and Sutherland, 1998). Their structures are based on a 20 carbon hydroxylated hydrocarbon chain (pentahydroxycosane) with two hydroxyls esterified to two propane- 1,2,3-tricarboxylic acid molecules and an amino group at one

end (Fig.1) (Dutton,1996; Gelderblom *et al.*,1992; Poche *et al.*, 1994; Badria *et al.*, 1995; Wilkes and Sutherland, 1998). The molecular formula is $C_{34}H_{59}NO_{15}$ with a molecular weight of 721D.

Structural and purity verification of fumonisins have been possible with nuclear magnetic resonance (NMR) spectroscopy (Bezuidenhout *et al.*,1988). Fumonisin B_1 differs from B_2 in that it has an extra hydroxyl at position 10. A stereochemical model of fumonisins (Beier and Stanker, 1997) shows a globular cage-like feature of the backbone and acid side-chains of FB_1 and FB_2 which suggest their potential as chelators. Their exposed surfaces were found to be hydrophobic. Gas chromatography-mass spectrometry (GC-MS) experiments indicate that alanine, a precursor in the biosynthesis of FB_1, is directly incorporated into FB_1 at C-1 and C-2 (Fig.1) (Branham and Plattner, 1993; Melcion and Richard-Molard, 1997; Plattner and Shackleford, 1992).

Fumonisins are hydrolysed on heating with 6 M HCl or 0.05 - 2 M KOH bringing about the removal of the two tricarballylic acid R groups (Fig.1) leaving the corresponding aminopentol (Jackson and Bennett, 1990; Sydenham *et al.*, 1995). Another toxin showing a similar chemical structure is the AAL toxin which is produced by the *Alternaria* spp. (Boyle and Kishi, 1995).

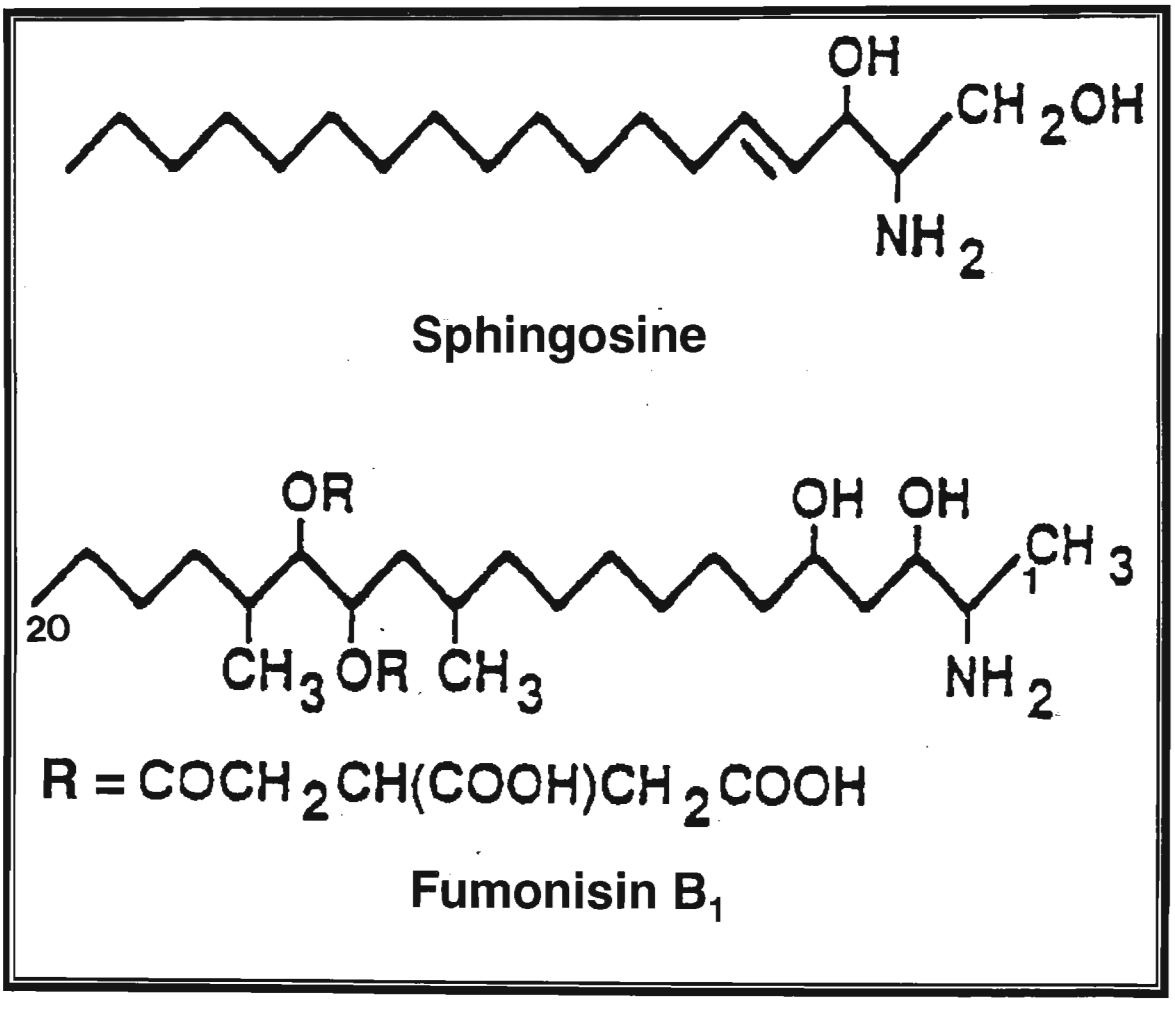


Fig.1 Structure of fumonisin B_1 and sphingosine *(Wang 1992)*

1.2 Occurrence of Fumonisin B_1

The most common food contaminated by *Fusarium* spp. is maize and it is here that the toxin is produced under certain host stresses, such as drought followed by heavy rains and damage by insects (Vleggaar,1989) (Fig. 2). The fungus however grows endophytically and shows no symptoms for quite a while. Thus although the maize may appear healthy it may possess minute, though sufficient toxin to cause pathological damage to consumers.

The toxin has also been found to occur in other foods like rice, oats and sorghum (Dawlatana *et al.,* 1995; Sydenham *et al.,* 1992A; Doko *et al.,* 1995). Contaminated maize is often used as animal feed and thus feed is an indirect source of fumonisins (Sanchis *et al.,* 1995, Ueno *et al.,* 1993). Fumonisins have been found to co-occur with zearalenone and deoxynivalenol, two other mycotoxins produced by *Fusarium* spp., in the maize of Transkei (Marasas *et al.,* 1979).

Maize and maize-based foods and feeds contaminated by fumonisin B_1 (μg /kg) worldwide are summarised in Table 1. Healthy maize is that which was destined for human consumption and was found to contain an average concentration of FB_1 ranging from 80 to 2 380 μg /kg (Sydenham *et al.,*1990, 1991; Rheeder *et al.,* 1992; Sanchis *et al.,* 1994). Plain maize (maize samples in general) was found to contain FB_1 with levels ranging from 40 to 3 500 μg/kg in fifteen studies carried out (Bhat *et al.*, 1997; Shetty *et, al.*, 1997; Patel *et al.*, 1997). Maize meal was also extensively studied, being the diet staple of many countries. A particular type of maize meal found in the USA, blue maize meal, was found to contain the highest level of fumonisins at a level of 8 484 μg/kg (Pestka *et al.*, 1994).

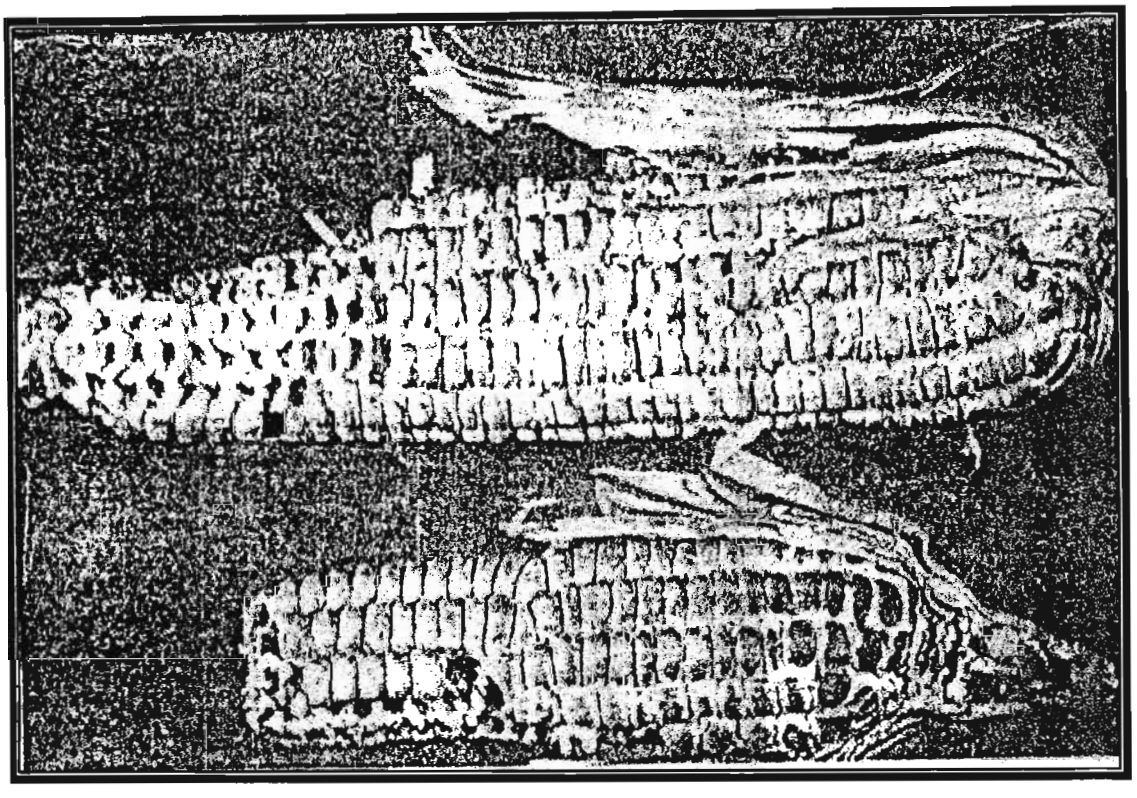


Fig. 2 Maize cobs contaminated with a fungus which produces toxic fumonisins. A toxin responsible for hole-in-the-head disease in horses and donkeys was isolated.

(Vleggar 1989)

Other maize meal had fumonisins ranging from 50 to 5 824 μg/kg (Doko *et al.*, 1995; Sydenham *et al.,* 1991). Maize rice (grits), tortilla and popcorn were found to contain a similar FB_1 concentration with a maximum of 1 410 μg/kg (Ueno *et al.*,1993; Pittet *et al.*, 1992). However, three out of four studies showed that cornflakes, corn noodles and corn cookies contain no FB_1 (Sydenham *et al.*, 1991). This is perhaps to be expected as these products are dry heat-processed (Dupuy *et al.*, 1993; Jackson *et al.*, 1997). One study carried out on puffed maize, which is processed differently, gave a relatively high level of FB_1, namely, 61 000 μg/kg (Doko and Visconti,1994). Canned sweet corn contained 235 μg/kg and frozen sweet corn gave a higher concentration (Trucksess *et al.*, 1995).

Table 1. Occurrence of FB_1 in foods and feeds worldwide.

COMMODITY **FOODS**	**COUNTRY**	**AVERAGE CONC. μg/kg** (parts/billion)	**REFERENCE**
Maize			
healthy	Egypt	2 380	Sydenham *et al.*,1991
	Egypt	+ ve	Allah, 1998
	Transkei	1 600	Sydenham *et al.*,1990
	Transkei	1 530	Rheeder *et al.*,1992
	USA	1 048	Sydenham *et al.*,1991
	Spain	80	Sanchis *et al.*,1994
flour	China	100	Ueno *et al.*,1993

plain maize	China	+ ve	Yoshizawa *et al.*, 1994
	-	+ ve	Bhat *et al.*, 1997
	India	+ ve	Shetty *et al.*, 1997
	Southeast Asia	+ ve	Yamashita *et al.*, 1995
	European countr.	3 500	Doko *et al.*, 1995
	Pol,Rum.,Croat.	40	Doko *et al.*, 1995
	USA	+ ve	Patel *et al.*, 1997
	Zambia	1 710	Doko *et al.*, 1995
	Italy	2 250	Logrieco *et al.*, 1995
	South Africa	+ ve	Dutton *et al.*, 1993
	Poland	+ ve	Chelkowski *et al.*, 1995
	Uruguay	+ ve	Pineiro *et al.*, 1997
	Italy	+ ve	Ritieni *et al.*, 1997
	Taiwan	+ ve	Tseng and Liu, 1997
	Transkei	1 990	Rheeder *et al.*, 1992
	Transkei	563	Vesonder *et al.*, 1990
meal (blue)	USA	8 484	Pestka *et al.*, 1994
meal (yellow)	USA	5 824	Pestka *et al.*, 1994
	USA	1 050	Sydenham *et al.*, 1990
	-	910	Stack and Eppley, 1992
	Zimbabwe	740	Doko *et al.*, 1995
	Peru	660	Sydenham *et al.*, 1991
	Canada	50	Sydenham *et al.*, 1991
	Switzerland	85	Pittet *et al.*, 1992
	South Africa	138	Sydenham *et al.*, 1991
rice (grits)	Japan	500	Ueno *et al.*, 1993
	Switzerland	260	Pittet *et al.*, 1992

	USA	1 048	Sydenham *et al.*,1991
cornflakes	USA	0	Sydenham *et al.*, 1991
	USA	27	Pestka *et al.*, 1994
	Switzerland	55	Pittet *et al.*, 1992
tortilla /popcorn	USA	1 410	Hopmans *et al.*, 1993
	USA	500	Pestka *et al.*, 1994
corn cookie/ noodle	Switzerland	0	Pittet *et al.*, 1992
commercial foods	USA	635	Sydenham *et al.*, 1991
	USA	+ ve	Rumbeiha *et al.*, 1997
puffed maize	Italy	61 000	Doko and Visconti, 1994
sweet corn	Switzerland	70	Pittet *et al.*, 1992
(frozen)	USA	350	Trucksess *et al.*, 1995
(canned)	USA	235	Trucksess *et al.*, 1995
screenings	USA	37 900	Murphy *et al.*, 1993
bran	USA	0	Pestka *et al.*, 1994
	Taiwan	2 500	Tseng and Liu, 1997
	-	200	Stack and Eppley, 1992
	-	+ ve	Katta *et al.*, 1997
kernels	Moz, Bots,Mal.	20	Doko *et al.*, 1995
	China	6 800	Ueno *et al.*,1993
	Switzerland	+ ve	Dowling *et al.*, 1997
	USA,SA,Argent.	1 600	Ueno *et al.*, 1993
seeds	France	5 000	Logrieco *et al.*, 1995
pre-harvest (earrot)	Sardinia	250 000	Bottalico *et al.*, 1995

farm stored	Honduras	3 000	Julian *et al.*, 1995
mouldy	Hungary	16 735	Fazekas and Tothe,1995
Oats (black)	Brazil	+ ve	Sydenham *et al.*, 1992A
Rice	-	5 000	Dawlatana *et al.*, 1995
	-	+ ve	Desjardin *et al.*, 1997
Wheat	Poland	+ ve	Chelkowski *et al.*, 1995
Barley	Poland	+ ve	Chelskowski *et al.*, 1995
Rye	Egypt	+ ve	Fadl-Allah *et al.*, 1997
Sorghum	Bots/Malawi	20	Doko *et al.*,1995
(mouldy)	-	+ ve	Bhat *et al.*, 1997
	Korea	650	Soo *et al.*, 1994
	India	+ ve	Shetty *et al.*,1997
Millet	Korea	650	Soo *et al.*, 1994
Milk	USA	< 5 ng/ml	Maragos and Richard,1994
	-	+ ve	Hammer *et al.*, 1996
FEEDS			
Forage grass	New Zealand	+ ve	Mirocha *et al.*, 1992
Mouldy maize	Transkei	29 300	Sydenham *et al.*, 1990
	Transkei	53 740	Rheeder *et al.*, 1992
Feed	USA	12 100	Price *et al.*, 1993
(ELEM)	USA	632 462	Sydenham *et al.*, 1992A
	Brazil	19 000	Sydenham *et al.*, 1992A
	Switzerland	235	Pittet *et al.*, 1992
(poultry)	India	+ ve	Shetty *et al.*, 1997
(rodent)	-	+ ve	Churchwell *et al.*, 1997

	Spain	400	Sanchis *et al.*, 1995
Gluten-based	USA, unknown	1 100	Ueno *et al.*,1993
Dog/cat food	USA	859	Hopmans *et al.*, 1993
Maize kernels	Korea	506	Soo *et al.*,1994

Maize screenings after sieving gave a high level of toxin 37 900 μg/kg (Murphy *et al.*, 1993). Toxin levels in bran ranged from 0 to 2 500 μg/kg (Pestka *et al.*, 1994). Maize kernels in Africa and the Far East gave levels ranging from 20 to 1600 μg/kg (Doko *et al.*, 1995 ; Ueno *et al.*, 1993). Maize seeds tested in France (Logrieco *et al.*, 1995) gave a concentration of 5 000 μg/kg fumonisins. Ear rot pre-harvest maize had a very high level of FB_1 (250 000 μg/kg). Farm stored maize had 3 000 μg/kg fumonisins and mouldy maize had 16 735 μg/kg fumonisins. Other foods that contained FB_1 were black oats, rice, wheat, barley, sorghum and millet with FB_1 levels 20 to 5000 μg/kg (Sydenham *et al.*, 1992A; Dawlatana *et al.*, 1995; Chelkowski *et al.*, 1995; Doko *et al.*, 1995; Soo *et al.*, 1994) . Sorghum and maize are used in the making of traditional beer and the toxin is often transfered to the beer (Marasas *et al.*, 1993). Milk showed negligible levels of toxin (< 5 ng/ml) derived from cows fed fumonisin contaminated feed (Maragos and Richard, 1994). Feeds were also extensively surveyed and fumonisins were found in relatively high levels with a maximum of 632 462 μg/kg. Gluten-based foods had 1 100 μg/kg FB_1. Fumonisins were also found in dog and cat food (859 μg/kg).

Fumonisins, unlike most other mycotoxins, are not restricted to certain countries. They were found in all continents of the world thus indicating that their prevalence is not climate dependant (Table 1). Egypt and the European countries showed high levels of the toxin in healthy and plain maize (Sydenham *et al.*, 1991; Doko *et al.*, 1995). Blue and yellow

maize meal, maize rice and tortilla showed highest levels of fumonisins in the USA. Puffed maize had a high level of the toxin in Italy. The USA showed very high levels of toxin in maize screenings confirming their possible presence in maize based foods.The Far East countries had high levels of toxin in maize kernels (Ueno *et al.,* 1993). Studies of maize from the Transkei by Sydenham *et al.* (1990), where ELEM was found to occur showed the highest level of FB_1 in plain maize (Rheeder *et al.*,1992). In Sardinia ear-rot maize showed an extremely high level of the toxin (Bottalico *et al.,* 1995). Farm stored maize in Honduras showed high levels of toxin. Pittet *et al.*(1992) in Switzerland found the highest levels of FB_1 in his country to be in maize rice. Of the many African countries, from which Doko *et al.* (1995) analysed maize, he found that Zimbabwe showed the highest level of fumonisins in maize meal. In Hungary high levels of toxin was found in mouldy maize (Fazekas and Tothe, 1995). Feeds showed extremely high levels of toxin in the USA, Transkei and Brazil.

1.3 Production of Fumonisins

1.3.1 Solid culture

Fumonisins can be readily produced by strains of *F. moniliforme, F. proliferatum* and related species on autoclaved maize kernels (Nelson *et al.*, 1992). For large-scale production, 500 g of yellow maize kernels and 500 ml of distilled water are added to a 30.5 x 61 cm autoclavable polyethylene bag. An autoclavable bag would avoid contamination of kernels during transfer to another container. The maize is inoculated by drawing an aqueous suspension from a lyophilised culture (*F. moniliforme*) into a sterile 5 ml syringe fitted with a 19-gauge needle and injecting 1 ml through the side of each bag.

Bags of inoculated maize are incubated in the dark at 20 to 22 ^{0}C in a closed cupboard for four weeks. Seven to eight days after inoculation, holes are punched near the tops of the bags to promote aeration. After the incubation period, cultures are soaked overnight in chloroform/ acetone (50/50, v/v) in 4-litre flasks to kill the fungus. This process is followed by filtering through 1 mm nylon mesh screens and air drying the culture material for 24 to 48 hours. The most important factors in the production of fumonisins in bulk maize cultures are temperature control, moisture and aeration using a shaking incubator. By this method yields of 2-4 g fumonisins per kg of culture material can be routinely obtained (Nelson *et al.*, 1992, 1993). Cracked maize is also used to produce fumonisins, but may be problematic in that the clumping of maize in culture may prevent uniform distribution of inoculum and thereby lead to reduced concentrations of toxin being produced.

The biosynthesis of mycotoxin labelled with ^{14}C methionine by *Fusarium* spp. has been carried out with both solid an liquid media. Although production of mycotoxins is much higher in solid media, isotopic labeling in solid media yields compounds with low specific activities (Alberts *et al.,* 1993A). The production of fumonisins in solid maize culture begins after 14 days whereas it takes only 3 days for toxin to be produced in liquid culture. This is too long for specific incorporation of precursors. The kinetics of growth and production of fumonisin by *F. moniliforme* MRC 826 in maize patty cultures were investigated (Alberts *et al.,* 1993A). Production time was reduced to 2 days. Incorporation of the isotope was enhanced by adding 50 mg of unlabelled L- methionine and 200 μCi of L- *methyl* ^{14}C methionine to a 30 g maize patty over 9 days yielding a specific activity of 36 μCi / mmol.

1.3.2 Liquid culture

Early studies of liquid culture of *Fusarium* spp. produced lower yields of fumonisins than solid culture. *Fusarium proliferatum* gives better yields of fumonisins in liquid culture. More recently, fumonisins have been produced in 10 litre stirred liquid cultures, where yields comparable to solid culture were claimed with simpler extraction and clean-up procedures (Miller *et al.*, 1994). Ion-exchange chromatography using a DEAE Sephadex column was carried out to extract fumonisins from filtered broth. The toxin was then purified on a silica gel column. Various liquid culture media using different carbon sources were also investigated since they are metabolised by micro-organisms during growth via the glycolytic pathway and Krebs tricarboxylic acid cycle to acetate, a precursor of fumonisin biosynthesis (Alberts *et al.*, 1994). Optimal fungal growth and production of fumonisins were obtained in medium A, B, and C primarily containing glucose, sucrose and galactose respectively.

1.3.3 Clean-up methods

Extraction and purification of fumonisins can be unexpectedly difficult and time consuming. Fumonisins contain a free primary amine and multiple free carboxyl groups that can carry positive and negative charges. Subtle changes in pH or ionic strength in the complex extracts from which they are purified can sometimes affect chromatographic behaviour. A common solvent mixture used for extraction of fumonisins from maize is methanol/ water (3:1). The PROMEC group of researchers and others carry out sample preparation for HPLC analysis of FB_1 with the Bond-elut strong anion exchange (SAX)

column (Shephard *et al.*,1990; Scott and Lawrence, 1994). An alternative method of extraction uses the C18 Sep-Pak Vac (SAX) columns (Holcomb *et al.*, 1993) or the Fumonitest immunoaffinity columns.

1.4 Analytical Methods

1.4.1 Thin-layer chromatography (TLC)

Many TLC systems have been developed for qualitative analysis of fumonisins. Ackerman's (1991) systems are the most widely used with chloroform/ methanol/ acetic acid (60:35:10) run on normal phase silica with visualisation accomplished by acidic anisaldehyde reagent, because of a lack of a suitable chromatophore in the molecule. Another TLC method by Rottinghaus *et al.* (1992), used methanol/ 4% KCl (3:2) as mobile phase on a Whatman LKC18-K reverse phase TLC plate. High-performance TLC (HPTLC) in normal phase was also carried out for quantitative determination of fumonisin in rice (Dawlatana *et al.*, 1995).

1.4.2 High-performance liquid chromatography (HPLC)

The fumonisins do not absorb UV or visible light, nor do they fluoresce, thus fumonisin must be derivatised for detection purposes. Many derivatising agents have been used to improve detection and quantitation of fumonisins, but with varying degrees of efficiency. Gelderblom *et al.* (1988) used maleic anhydride coupled with reversed phase LC and UV detection for the quantitation of FB_1 and FB_2 in culture material of *F. moniliforme.* Other

derivatives used were fluorescamine (Sydenham *et al.,* 1990), naphthalene 2,3 dicarboxyaldehyde (NDA) (Bennett and Richard, 1994), maleyl derivatisation (Alberts *et al.*, 1993B) and o-phthaldialdehyde (OPA) (Shephard *et al.,* 1990; Akiyama *et al.*, 1998).

Fluorescamine was used in pre-column derivatisation of the primary amino group of fumonisins for their determination by reverse phase LC with fluorescence detection, however, the method is inconclusive, as 2 peaks are formed of acid/alcohol and lactone derivatives (Ross *et al.,* 1991). Scott and Lawrence's (1994) method, using 4- fluoro-7-nitrobenzofurazan (NBD-F), gave moderately stable derivatives and as little as 1 ng FB_1 can be detected with a linear response up to at least 50 ng injected. In this method reversed-phase LC (C18 column) was carried out with a mobile phase of methanol/ 0,05 M sodium dihydrogen phosphate, adjusted to pH 5 (1:1). The mobile phase was altered after 5 minutes by introducing acetonitrile/water v/v (8:2) at a ratio 1:1. A detection limit of 100 ng/g was obtained in the analysis of maize samples. Quantitation of fumonisins in maize using the NBD-F and NDA methods was in agreement (Scott and Lawrence, 1994). Holcomb *et al* (1993) used (9-fluoroenylmethyl) chloromate (FMOC) to derivatise fumonisins with gradient elution and fluorescence HPLC and found the method to be more reliable than the above methods. Akiyama *et al* (1995) used pre-column derivatisation with 4-(N,N-imethylaminosulphonyl) -7-fluoro-2,1,3 benzoxadiazole for fumonisins. Maragos (1995) used fluorescein 5-isothiocyanate (FITC "isomer 1") derivatisation with gradient elution and fluorescence detection. This method is unique in that the same derivatives may be used on capillary electrophoresis.

The OPA derivatives have proved most sensitive, accurate and reproducible with a

detection limit of 50 ng/g (Shephard *et al.,* 1990). Thiel *et al.,* (1993) reported an inter-laboratory comparative trial with OPA with contaminated maize, which gave relative standard deviation (RSD) values varying from 18 to 27 % amongst laboratories. The only disadvantage of the technique is that the OPA derivative is unstable after approximately 2 minutes. The OPA derivative of fumonisin B_1 was also prepared in the presence of 2-methyl-2-propanethiol and analysed on HPLC with fluorescence and electrochemical (EC) detection. The EC response of the derivative was unstable, but that with fluorescence was stable for up to 30 minutes giving a detection limit of 30 ng/ml as compared to 250 ng/ml for ECD. Plasma and urine have also been analysed for FB_1 presence using OPA derivatisation (Shephard *et al.*, 1992A) .

Evaporative light scattering detection with HPLC (HPLC-ELSD) carried out on a gradient of tri-fluoroacetic acid buffer (pH 2.7) and acetonitrile gave good separation and purification of 4 fumonisins, without derivatisation.

1.4.3 Mass spectroscopy (MS)

Fumonisins were also analysed using thermospray (TSMS), fast-atom bombardment (FABMS) and electrospray mass spectrometry (ESMS)(Korfmacher *et al.,*1991). Thermospray MS was not found to be suitable for the analysis of ng quantities of fumonisins, however both FABMS and ESMS provided useful data. Plattner *et al.* (1991) has analysed maize for FB_1 presence using gas chromatography with MS. More recently Plattner (1999) has achieved sensitive detection limits for fumonisin analysis, using HPLC ESMS detection, especially of the N-acetylated fumonisin that are not otherwise easily

detected. On-line capillary LC with FABMS has also been used to detect fumonisins (Xie *et al.*, 1997). High-performance liquid chromatography coupled with ESMS was also used to quantitate fumonisins (Churchwell *et al.*,1997; Josephs, 1996; Wilkes and Sutherland, 1998).

1.4.4 Immunoassay (IA)

In order to avoid extreme costs and time requirements necessary for the above analytical techniques, alternatives for screening cereals for mycotoxins were looked at by various workers (Pestka *et al.,* 1994; Chu and Li , 1994; Usleber *et al.,* 1994). A commercial test (Fumoni Test, Vicam) uses monoclonal antibodies in an affinity column for fumonisin detection at 1 ppm level. The quantitation of FB_1 in the above may be carried out using a fluorometer. Many EIA methods have been developed to quantitate down to ppb levels (Usleber *et al.*, 1994; Schneider *et al.*, 1995; Scott *et al.*, 1997). Enzyme immunoassay compared well with HPLC results having recoveries close to 100 %. A computer assisted multianalyte system has also been developed by Abouzied and Pestaka. (1994) using the line immunoblot method. Another ELISA kit is available from Neogen Corporation called the VERATOX kit for quantitating nanogram amounts of FB_1 in commodities.

1.4.5 Capillary Electrophoresis (CE)

More recently CE has been developed as a complementary method of analysis to HPLC. This method depends upon a high voltage (20-30 kV) being applied down a fused silica capillary of 1 μm diameter filled with a suitable buffer and cooled to avoid heating effects.

Not only ionised molecules of the correct charge pass down the capillary but also uncharged and opposite charge due to ion flow created in the buffer. Fumonisins, having ionisable groups, would seem to be highly suitable to this method, which has the advantage of a very high resolving power, although the sensitivity at the moment is not as good as fluorescence detection used with HPLC. A method has been developed using this instrumentation by Maragos (1995) which also employs a derivatizing reagent, flourescein 5-isothiocyanate and another with ESMS as a detection method (Hines *et al.,* 1995). Capillary electrophosis has also been combined with immunofluorescence for the detection of FB_1 (Maragos, 1997).

1.5 Pathophysiology of fumonisin intoxication

1.5.1 Effects on animals

Horses:

Horses were found to be affected by ELEM as early as 1979 (Buck *et al.,* 1979). The disease (ELEM) has also been shown by numerous researchers to be directly related to intake of *Fusarium* contaminated feed (Kellerman *et al.*, 1990; Wilkins *et al.,* 1994; Riley *et al.*, 1997) and was found to occur especially during the winter months. The literature on the ELEM can be divided into parts, i.e., the natural occurrence of the disease and experimentally induced disease with toxigenic strains of *F .moniliforme* or pure FB_1.

In earlier reported cases of ELEM (Wilson *et al.,*1971; Kellerman *et al.,* 1990; Marasas *et al.,* 1976; Buck *et al.,* 1979; Wilson *et al.,* 1992; Domenech *et al.,* 1984) common elements are observed as follows : the animal develops inappetance after a period of eating

contaminated feed; being lethargic; and as neurotoxic effects become apparent, develops uncoordinated movement and aimless walking with blindness and violent blunderings into the front of stalls and walls. In general the animal will become difficult to handle and ill tempered. In some cases, death can occur without any nervous symptoms and in others, liver related symptoms are seen such as swelling of the lips and nose, severe icterus, with petechial haemorrhages in the mucous membranes and cyanosis.

Two main types of histopathological signs are found on autopsy, those involving the brain where oedema in the form of an accumulation of clear fluid under the meninges is seen and liquefaction of areas within the cerebral hemispheres is found, causing lesions that might range in size from microscopic to one occupying most of the lobe. In severe cases there may be a large liquified cavity within the white matter of the right cerebral hemisphere with the cerebrum posterior to the cavity enlarged and oedematous with congested blood vessels (Fig.3).

The other organ affected was the liver which often showed a mild swelling with a change in colour (yellow brown). In more severe cases gross liver lesions may be seen with fibrosis of the centrilobular area. Hepatocytes on the edge of the fibrotic area have large fatty globules in their cytoplasm.

These observations are in general supported by field outbreaks of ELEM where fumonisins were determined in the feed (Thiel *et al.*, 1991; Ross *et al.*, 1991; Wilson *et al.*, 1992) or by experiments using fumonisins in added cultured material (Kellerman *et al.*, 1988; Ross *et al.*, 1992) or administered as purified compounds (Marasas *et al., 1988;* Laurent *et al.*,

Fig. 3 Toxic fumonisins cause liquefaction of the brain cells of animals, resulting in the fatal hole-in-the-head disease.

1989; Kellerman *et al.*, 1990). In addition to ELEM, the role of *F. moniliforme* cultures was also linked to duodenitis and proximal jejunitis in horses (Schumacher *et al.*, 1995). It was thought that this condition which is characterized by a copious reflux of gastric juice did not have a bacterial cause and might be related to FB_1 and four other mycotoxins that cause neurotoxicological symptoms. For a correct diagnosis the specific mycotoxin has to be isolated and identified from the feed.

Donkeys:

In examining the effects of maize inoculated with a toxigenic strain of *F. moniliforme* (derived from an outbreak of ELEM) on 3 horses and 3 donkeys, Kellerman *et al.* (1972) observed varying results. One horse and one donkey were unaffected, one donkey developed a transient pruritus and the remaining animals died. Observation on autopsy of the animals were similar, the gross lesions being severe cardiac haemorrhages, oedema, icterus and liver damage. No brain lesions, other than small peri-vascular haemorrhages were found. With hindsight it seem very likely that these effects were caused by a combination of *Fusarium* toxins. Other studies involving feeding donkeys with infected material were done by Badiali *et al.* (1968) and Haliburton *et al.* (1979). In all these studies the animals were found to develop ELEM in varying degrees.

Ponies:

Ponies were given feed containing a maize culture of a strain of *F .moniliforme* associated with an outbreak of ELEM. Of the five experimental ponies, one developed clinical signs of toxicity and on autopsy was found to have brain lesions consistent with ELEM. Following their observation that fumonisins can alter sphingosine metabolism Wang *et al.* (1992) gave ponies feed containing 15 to 44 mg/kg FB_1. They found, in addition to liver cytotoxicity, encephalopathy, death and elevation in free sphingosine to sphinganine blood

levels occurred. The latter effects rapidly appeared and it was suggested that such measurements could be used as early markers for exposure to fumonisin. Similar symptoms were also observed by Ross *et al.* (1992) in a feeding trial with FB_1 on 4 ponies.

Another study was done by Wilson *et al.* (1992) to determine the minimum toxic dose of FB_1 on ponies. A group of 4 ponies were fed rations with 1-22 mg/kg and a second group of 5 animals 8 mg/kg. One pony in the first group died with ELEM after 225 days feeding (last 22 days of the diet contained 22 mg/kg) the other animals having various degrees of lesion mainly associated with liver and cerebral hemispheres. The second group of ponies showed mild transient clinical signs and autopsy revealed mild brain lesions.

Pigs:

An outbreak of disease in pigs in Georgia, USA in 1989 was investigated by Harrison *et al.* (1990). On autopsy the animals had pulmonary oedema and hydrothorax with the thoracic cavities being filled with a yellow liquid. The problem was traced to maize screenings and *F. moniliforme* was isolated leading to the suspicion that a mycotoxicosis was involved. Four pigs were injected in the cranial vena cava (blood system of the brain) with various amounts of FB_1 and FB_2. The one that received the highest dose, 0. 4 mg/kg body weight for four days, a total of 11.3 mg, died on the fifth day and exhibited similar lesions to the pigs involved in the field outbreak.

Another outbreak of disease in Georgia, USA was characterized by foetal mortality, and in older animals by respiratory disorders (Osweiler *et al.,* 1992). Feed samples were later taken from outbreaks of disease in Illinois and Iowa and analysed for fumonisin, which were found to be positive. Fumonisin B_1 was found to range from 20 - 330 mg/kg.

Several studies link, what has been called porcine pulmonary oedema syndrome (PPE) with outbreaks of ELEM (Ross *et al.,* 1991) and it soon became accepted that the two diseases had a common cause, i.e., fumonisin.

Experimental feed studies of pigs were carried out using either naturally contaminated feed with fumonisins at known levels (Casteel *et al..,* 1993; Haschek *et al.,* 1992; Colvin *et al.,* 1992,1993) or by injection with quantities of purified fumonisin (Harrison *et al.,* 1990). In general animals fed high levels of FB_1, died with pulmonary oedema and those surviving on lower levels, had evidence of subacute hepatotoxicosis. From the limited evidence, animals receiving pure toxin were more likely to develop pulmonary oedema, whereas those on naturally contaminated feed had both lesions. In one study pigs that were fed with 100-190 mg/kg FB_1 developed nodular hyperplasia of the liver and tissue damage to the oesophageal mucosa. Synergistic effects are known between other mycotoxins, including FB_1 with fusaric acid (Bacon *et al.,* 1995).

Feeding FB_1 to lactating sows did not affect suckling pigs and there was not evidence of the toxin present in the milk (Becker *et al.,* 1995). This agrees with other studies on cow's milk (Scott *et al.,* 1994).

As with other animal studies the uptake of ^{14}C labelled FB_1 administered intra-gastrally to pigs was poor (3-6%). When fumonisin was given intraperitoneally (ip), elimination was rapid and 80 % of the dose was recovered within 3 days in the excreta with the remainder being associated with the liver and kidney (Prelusky *et al.,* 1994). Riley *et al .(*1993) found alterations in sphinganine to sphingosine ratios in pigs fed diets containing FB_1 and FB_2

and also suggested this as an early biomarker for exposure to the toxin.

Rats:

Prior to the discovery of the fumonisins, several trials had been done where rats were dosed with feed infected with *F. moniliforme* related to outbreaks of ELEM (Voss *et al.*, 1998) or to OC (Jaskiewicz *et al.*, 1987; Gelderblom *et al.*, 1988). In general these studies indicated the presence of an hepatocarcinogen in these cultures with the development of hepatic nodules, adenofibrosis, hepatocellular carcinoma - ductular carcinoma and cholangiocarcinoma.

Several studies on the rat were conducted by the workers at PROMEC (Gelderblom *et al.*, 1992; Shephard *et al.*, 1994) exploring the absorption and excretion of fumonisins and its effects on the animals.

Radio-labelled FB_1 studies using ip administration of ^{14}C - FB_1 was carried out on tissues from the kidney of the rat. Of the material given orally 101 % of fumonisin was found in the faeces. The extra 1% may have been previously present in the system of the rat.A further study (Shephard *et al.*, 1994) clarified how FB_1 ended up in the faeces of animals dosed intra-peritoneally (ip). Sixty seven percent of the dose in this latter study was found in the bile of the rat.

Evidently only a small portion of the doses (<1%) are absorbed from the gastrointestinal tract (GIT) which is understandable considering their high polarity and presumable lack of membrane transport systems. This may explain why relativity high levels of contamination of feed are required >5 mg/kg to produce symptoms in animals but it also indicates that

the fumonisins must be highly active once internalised.

Other work reported by the PROMEC group shows that the fumonisins are hepatotoxins and carcinogens in the rat (Gelderblom *et al.*, 1997). Culture material from *F. moniliforme* fed to rats produced micro and macronodular cirrhosis in animals that died, cholangiofibrosis and primary adenofibrosis (Gelderblom *et al.,* 1991; Wilson *et al.,* 1992). Tubular nephrosis was found in the renal cortex of animals fed material infected with *F. moniliforme* associated with outbreaks of ELEM (Voss *et al.,* 1998). Inhibition of protein synthesis in hepatocytes was also found (Norred *et al.,* 1990). Later work, where purified FB_1 was fed to rats, showed that sphingolipid metabolism in the kidney is more susceptible to the toxin than in liver (Riley *et al.,* 1994). Various markers of nephrotoxicity were measured in rats dosed with FB_1 (Suzuki *et al.,* 1995). The use of ^{14}C- labelled FB_1 as a single dose to rats, killed at time intervals gave recoveries of the label in faeces and urine of over 80 % (Norred *et al.,* 1993). The balance, however, remained in the blood, liver and kidney for the duration of the experiment, i.e., up to 96 hours, which does not quite agree with the PROMEC results, in that larger quantities remained bound in the animal.

Lebepe-Mazur *et al.* (1995) showed that FB_1 affected the foetus in pregnant rats, causing low litter weights and impaired foetal bone development, as compared to untreated controls, and found that placental glutathione S-transferase was a more useful marker in this case than glutamyl transferase in detecting hepatic cancer foci (Ferguson *et al.*, 1997).

Other Rodents :

Pregnant mice fed culture material containing known amounts of fumonisin were shown to

have lowered body weights, increased morbidity and mortality in a a dosed response way (Gross *et al.*, 1994). Other observations were, liver damage and ascites and a reduction in the number of live offspring.

Similar results were found in another investigation which indicated that FB_1 fed at < 81 ppm to female mice caused hepatotoxicity and doses at 27 ppm or less had no effect, which is in contrast to the rat where nephrosis was observed at 9 ppm (Voss *et al.*, 1998). Fumonisin B_1 fed to Syrian hamsters at a rate of 18 ppm also resulted in increased foetal deaths but without evidence of maternal intoxication (Floss *et al.*, 1994).

Poultry:

Maize based feeds for chicken have been found to be often contaminated with *F. moniliforme* (Bragulat *et al.*, 1995). Day old broiler chicks were fed doses of FB_1 ranging from 0 to 400 mg/kg for 21 days and 300 mg/kg for 2 weeks (Brown *et al.*, 1996). Body weight gain was gradually reduced, hepatic necrosis, biliary hyperplasia and thymic cortical atrophy were noted along with dairrhoea and rickets. A further investigation (Weibking *et al.*, 1993) revealed increased sphinganine and sphinganine:sphingosine ratios in young poultry treated with culture material containing FB_1. Turkeys also showed pathology due to fumonisin and moniliformin (Bermudez *et al.*, 1997; Kubena *et al.*, 1997B; Ledoux and Rottinghaus., 1996).

Abnormal erythrocyte formation and lymphocyte cytotoxic effects were also found in poultry (Javed *et al.*, 1993). Chicken embroyos exposed to FB_1 showed a mortality of a 100 % when dosed at 100 μM. Pathological changes were found in liver, kidney, heart, lungs, musculoskeletal system, intestines, testes and brain (Javed *et al.*, 1993). Espada *et al.*

(1994) showed that 10 mg/kg feed for 6 days was toxic to young chicks. Laying hens (Vudathala *et al.,* 1994) also showed poor oral uptake and rapid excretion of the ip dose, although no FB_1 residues were found in the eggs.

Chatterjee *et al.*(1994) examined the effects of FB_1 on chicken peritoneal macrophages and found them to exhibit breakdown of the nucleus with increasing numbers in a dose response manner (6-18 μg/ml). This suggests impaired disease immunity in chickens (Marijanovic *et al.,* 1991). Vitamin A was depleted in the serum of broiler chicks (Hall *et al.,* 1995). Combined effects of mycotoxins were investigated in broiler chicks by Kubena *et al.*

(1997A). Ducklings had slightly swollen and reddened livers with low body fat. Turkey poults showed variations in body and organ weight increase. Generalised hepatocellular hyperplasia was found in all the treated poults. Biliary hyperplasia and turkey cerebral encephalomalacia were also noted in turkey. The combined feeding of aflatoxin and fumonisin to turkey poults showed a variation in body and organ weights (Weibking *et al.*, 1994 ; Kubena *et al.*, 1997B).

Primate Studies:

Prior to isolation of the fumonisins, as part of a study on the effect of toxic extracts of *F. moniliforme* on animals, baboons were found to develop hepatic cirrhosis and intraventricular thrombosis (Kriek *et al.,* 1981). In another investigation 10 young vervet monkeys were fed *F. moniliforme* culture material (Jaskiewicz *et al.,* 1987). This was found to cause toxic hepatitis of varying degrees related to the amount fed with the release of liver enzymes into the serum paralleling the liver damage. In another trial four male and seven female vervet monkeys were fed cultured material containing FB_1, FB_2 and FB_3

(Fincham *et al.,* 1992). Days of feeding ranging from 40 to 573 on a "high" dose regime (total fumonisin ranging from 784-3257 μg/vervet/day). The unexpected result was that the appearance of hyper-cholesterolaemia, all treated animals having statistically significant increased serum cholestrol over that of controls and with raised plasma fibrinogen and activity of factor V11 which would enhance atherogenesis. In addition chronic hepatotoxicity was observed, which is in keeping with the findings in other animal species.

Further work on primates (Shephard *et al.,* 1994) was aimed at investigating their absorption and excretion of fumonisin as previously studied in rats (Shephard *et al.,* 1992B). In this case, however, extraction from faeces was effected using EDTA, which presumably allowed the release of the fumonisins from metal chelaters. In one experiment two vervet monkeys were given radio-labelled FB_1 at a rate of 8 mg/kg body weight. The bulk of the radio-label FB_1 was found in the faeces, both unchanged and as partially hydrolysed monoesterase derivatives.

In a later more detailed investigation using four vervet monkeys (Shephard *et al.*, 1994B) radio-labelled toxin was administered by gavage or ip injection. From two animals treated by ip injection, 47 % of the dose was recovered in urine and faeces over 5 days, as FB_1 or its partially hydrolysed derivatives whereas 61 % was recovered from those dosed by gavage. A further 15 % was accounted for in the latter animals, mainly as intestinal contents with small amounts in skeletal muscle, liver, brain, kidney, heart, erythrocytes and bile. This result is intriguing as a quarter of the fed material could not be accounted for which is in contrast with the experiment with rats.

Other Animals:

One case of ELEM symptoms in white tailed deer has been described in the USA (Howerth *et al.,* 1989) which seems to have been caused by the animal finding a source of infected maize. Calves given feed containing FB_1 up to 148 mg/kg were unaffected in terms of feed intake or weight gain, although certain blood enzymes were elevated along with cholesterol (Osweiler *et al.,* 1993).

Lambs dosed with fumonisin showed evidence of liver and kidney damage. Those that received 44.5 mg/kg fumonisin died (Edrington *et al.,* 1995). Liver and kidney damage were also observed in mink given chronic doses of fumonisins (Restum *et al.,* 1995).

1.5.2 Humans

Fumonisin and oesophageal cancer (OC):

The discovery of the fumonisins derived directly from the investigation by the PROMEC group of human oesophageal cancer (OC) in the Transkei in S.Africa. Oesophageal cancer occurs in geographical pockets around the world e.g., China (Yang., 1980) and was first reported in the Transkei in 1957 (Burrell., 1957) an observation that was amply confirmed (Jaskiewicz *et al.*, 1987; Rose, 1973). As in most rural communities in Southern Africa the main staple diet is maize. It was well established that maize grown in this region was susceptible to infection by several fungi, in particular *Fusarium* spp. (Marasas *et al.,* 1979; 1988; Segal *et al.*, 1988) and of these *F. moniliforme* and *F. subglutinans* were the most prevalent with *Diplodia maydis* being third. Because of the high incidence of these fungi together with their mycotoxins they were investigated in outbreaks of OC. Burrell (1957) found OC in the Bantu in South Africa which may been caused by dietary exposure to

fumonisins.

A significant difference in the levels of fumonisin in maize and maize used for making home brewed beer (Rheeder *et al.*,1992; Marasas *et al.*, 1993) between high and low OC areas was found. A similar distribution was found in China (Chu and Li, 1994; Yoshizawa *et al.*, 1994) where the incidence of fumonisin in high risk areas was thrice that of low risk.

1.5.3 Tissues and cell culture

The fumonisins are cytotoxic (Norred *et al.*, 1996) and the effects of FB_1 and FB_2 were tested on several mammalian cell lines including rat hepatoma and dog kidney epithelial cells. Fumonisin B_1 was also found to inhibit protein synthesis (Norred *et al.*, 1990) and inhibit the secretion of ceramide into very low density lipoprotein (Merrill *et al.*, 1995). Chick embroyos and brine shrimp bioassay showed toxicity from fumonisin B_1 (Hlywka and Bullerman, 1997).

Turkey lymphocytes were used in an MTT-based cytotoxicity assay by Dombrink-Kurtzman *et al.* (1994) and the 50 % inhibitory dose for FB_1 or FB_2 was 0.4-0.5 μg/ml medium (Smith, 1996). Cells exposed to the toxins exhibited vascuolization and were unable to proliferate. Chicken chondrocytes in culture were exposed to FB_1 which was inhibited by levels as low as 25 μg. Baby hamster kidney cells also showed pathology (Abeywickrama and Bean, 1992).

The yeasts, *Saccharomyces cerevisiae* (Wu *et al.,* 1995) and *Pichia* spp developed abnormalities in their sphingolipid metabolism showing that lower organisms are equally affected by the toxin. The bioluminescent genotoxicity test using *Vibrio fischeri* revealed that FB_1 was directly mutagenic. This was in contrast to the Ames test, as FB_1 exhibited cytotoxicity but no mutagenicity activity of *F. moniliforme* isolates.

In green monkey kidney cells treated with FB_1 the toxin was found to repress expression of protein kinase C, whereas it stimulated the promoter of a cyclic AMP receptor (Huang *et al.*, 1995). LaBorde *et al* .(1997) showed no embryo toxicity to FB_1 in New Zealand white rabbits.

1.5.4 Mode of Action

Wang *et al* .(1991) showed that FB_1 disrupted sphingolipid biosynthesis in rat hepatocytes. A further investigation using rat hepatocytes and pig kidney epithelial cells (Norred *et al.,* 1997) confirmed that the fumonisins were potent inhibitors of sphingolipid biosynthesis in both types of cell, killing the renal cells after 3 days at a level of 70 μM of FB_1. This effect was further studied in mouse cerebellar neurons (Merrill *et al.,* 1993, 1996; Rother *et al.,* 1992) cells, where it was shown that FB_1 specifically inhibited the conversion of sphinganine to dihydroceramides. The inhibitory effect on neuron cultures of FB_1 was demonstrated where treated hippocampal neuron axonal growth was completely suppressed as was short term axonal branching (Schwartz *et al.*, 1995).

It was pointed out by Wang *et al.* (1991), that the structure of the backbone of the

fumonisin molecule, was similar to the important phospholipid, sphingosine (Fig.1). Hence FB_1 may exert its action by interfering with its metabolism and processes mediated by this type of molecule, such as the inhibition of protein kinase C, and via ceramides, regulation of cell growth, differentiation and apotosis (Huang *et al.,* 1995*).* Ramasamy *et al* .(1995) have shown that FB_1 increases the rate of albumin transfer across endothelial cell monolayers derived from pulmonary artery, in addition to elevation of sphinganine levels, the latter possibly explaining the loss of endothelial barrier function.

Morgan *et al.*(1997) investigated the effect of FB_1 on Swiss 3TS fibroblasts, and found an accumulation of sphinganine, which was greatly promoted by the presence of insulin. This led to an increase in DNA synthesis. This mitogenic nature of fumonisin also gives a lead to the mechanism of tumour promotion by FB_1 by the stimulation cell proliferation. In addition the possible influence of dietary sphingolipid and fumonisin on the role of fat in disease has been reviewed by Merrill *et al.* (1995,1996). Morgan *et al* .(1997) found an increase in the free sphingosine to sphinganine ratio in urine and liver. A HPLC method to quantitate sphingosine has also been developed (Merrill *et al.*, 1988; Solfrizzo *et al.*, 1997).

1.5.5 Pre-eclampsia and Fumonisin

Both pre-eclampsia in humans and FB_1 intoxication in animals produce a large number of pathophysiological effects involving most of the major organs of the body such as liver, kidney and brain. Bermudez *et al* .(1997) and Bondy *et al* .(1995) found that intake of FB_1 caused decreased foetal body weight gains in pregnant rats with diffuse hepatocellular

hyperplasia and nephrotoxicity . This was confirmed by Suzuki *et al.* (1995) . Table 2 shows some of the common effects caused during pre-eclampsia and fumonisin toxicoses. Often perioperative seizures have to be managed in pre-eclampsia (Cheng and Kwan, 1997). Blood plasma in eclampsia shows a high free radical trapping ability (Jendryczko and Tomala, 1995). Magnesium sulphate is often used in seizure prevention in pre-eclampsia (Anthony *et al.*, 1996; Handwerker *et al.*, 1995). Magnesium also combines well with FB_1 which is a good metal chelator. Magnesium may thus be instrumental in removing free FB_1 in pre-eclamptic women thus reducing the severity of the disease. The role of nutritional factors, including agricultural products, in pre-eclampsia and eclampsia was discussed by Vasiljevic *et al* .(1996). The role of obesity and the risk of toxaemia in pregnancy was found to contribute to pregnancy disorders (Fields *et al.,* 1994).

Insulin inhibits the symptoms of pre-eclampsia (Maharaj and Moodley, 1994) and fumonosis by increasing sphinganine levels in fibroblasts (Morgan *et al.*,1997). Another study showed that FB_1 is detoxified by fructose (Lu *et al.*, 1997) indicating that the presence of carbohydrates in animals or humans may decrease fumonosis and pre-eclampsia.

Table 2. Pathophysiological effects common to pre-eclampsia/eclampsia (PE) and animal fumonosis (F).

ORGAN / SYSTEM	PATHOPHYSIOLOGICAL EFFECTS OF *PE* AND *F*	REFERENCES & CORRESPONDING EFFECTS (Column 2)
Immune system Blood system	1. Abnormal ratio of prostoglandin to thromboxane 2. Increased platelet level 3. Vasoconstriction, increased blood pressure 4. Blood coagulation 5. Increased epinephrine/norepinephrine 6. Increased amino acids and ammonia 7. Increased sphingolipid conc. 8. Increased plasma ascorbic acid 9. Immuno-compromised	Moodley, 1991 - PE/1,3 Martinova and Merrill, 1985 - F/2 Goel *et al., 1996* - F/7 Halim *et al.,* 1995 - PE/4 Khatun *et al., 1997* - PE/5 Li *et al., 1996* - PE/6 Ozan et al., 1997 - PE/8 Tryphonas *et al.,* 1997 - F/9
Placenta/foetus	1.Immunological defect 2.Low birth weight 3.Foetal distress/death	Moodley, 1991 - PE/1,3 Ferguson *et al.*, 1997 - F/2 Lebepe-Mazur *et al., 1995* - F/3 McCowan *et al., 1996* - PE/3 Voss *et al.*, 1998 - F/2 Floss *et al.*, 1994 - F/2,3

Kidney	1. Proteinuria 2. Renal failure 3. Histopathology	Moodley, 1996 - PE/1,2 Bondy *et al., 1995* - F/2 Beek *et al., 1998* - PE/1,2 Edrington *et al., 1995* - F/2 Gurel *et al.,* 1998 - PE/1,2 Naicker *et al.,* 1997 - PE/3 Randeree *et al.,* 1995- PE/2 Riley *et al.*, 1994 - F/2 Shaarawy *et al.,* 1997 - PE/3 Bucci *et al.*, 1998 - F/2
Liver	1. Hepatocellular damage 2. Liver enzyme elevation 3. Acute fatty liver 4. Liver cancer	Moodley, 1991 - PE/1,2,3 Cawood *et al.,* 1994 - F/1 Gelderblom *et al.,* 1995; 1996A,B- F/1 Marasas *et al.,* 1984 - F/1,4 Knasmuller *et al.*, 1997 - F/1 Norred *et al.,* 1990,1997 - F/1 Sharma *et al.,* 1997 - F/1 Edrington *et al.*, 1995 - F/1 Voss *et al.*, 1998 - F/1 Gross *et al.*, 1994 - F/1 Ueno *et al.*, 1997 - F/4

Hematological	1. Hemolytic anaemia 2. Thrombocytopenia 3. Erythrocyte fragility	Moodley., 1991 - PE/1 Srivastava *et al.*, 1995 - PE/2 Ozan *et al.*, 1997 - PE/3
Heart	1. Heart arrhythmias	Moodley ,1991 - PE/1 Casteel *et al.*, 1994 - F/1
Lungs	1. Pulmonary oedema	Moodley, 1991 - PE/1 Casteel *et al.*, 1994 - F/1 Colvin *et al.*, 1993 - F/1 Guzman *et al.*, 1997A;B - F/1 Rotter *et al.*, 1996 - F1
Brain	1. Seizures 2. Cerebral oedema 3. Encephalopathy 4. Haemorrhage 5. Altered sphingolipid	Moodley, 1991 - PE/1,2,4 Badiali *et al.*, 1968 - F/2 Caramelli *et al.*, 1993 - F/2 Cheng *et al*, 1997 - PE/1 Beek *et al.*, 1998 - PE/1,2 Howerth *et al.*, 1989 - F/2 Ito *et al.*, 1995 - PE/2 Witlin *et al.*, 1997 - PE/2 Manfredi *et al.*, 1997 - PE/3 Bucci *et al.*, 1996 - F/4 Plumlee and Galey, 1994 - F/2 Kwon *et al.*, 1997 - F/5

CHAPTER 2

2.0 PRODUCTION FUMONISIN B_1

2.1 Introduction

Fungi of the genus *Fusarium* are found in plant debris and crop plants worldwide (Marasas *et al.*,1993). Several species from this genus are economically important because, in addition to their ability to infect important crops, such as maize, wheat and other small grains in the field, they produce mycotoxins in the crops in the field and in storage.The presence of high levels of mycotoxins in grains poses a significant economic threat because animals that consume contaminated grains may perform poorly or even become sick and die. The threat of mycotoxins entering the human food supply through contaminated grain products and exposed animals is also a concern for regulatory officials.

Fumonisins are produced by the fungus *F. moniliforme* and some closely related species in section *Liseola*. Fumonisin B_1 is generally the most abundant member of the family of fumonisins. This study sought to investigate the growth of the fungus, *F. moniliforme*, and its potential to produce FB_1 in solid and liquid culture. Toxin production was analysed qualitatively using thin-layer chromatography.

2.2 Materials and Methods

2.2.1 Preparation of inoculum

Fusarium moniliforme PPR1 1059 (Mercen culture collection) was stored at -20° C. The organism was sub-cultured on Sabouraud Dextrose Agar (SDA) McCartney slants and on petri dishes and incubated for 14 days at 28 °C until complete sporulation occurred. The slants were stored long-term at 4 °C. Spores on the petri dishes were harvested by washing the surface of the agar plates with 1ml sterile distilled water to prepare a conidial suspension. The suspension was quantitated using a 0.5 Mc Farland standard (0.5% Barium solution) comparing turbidity and stored at 4 °C until use.

2.2.2 Reagents and Solvents

All the salts and solvents were of analytical grade and were purchased from Merck NT.

2.2.3 Broth culture and extraction of fumonisin B_1

The broth used for the production of FB_1 consisted of glucose, 90g; KH_2PO_4, 2g; $(NH_4)_2$ SO_4, 3,5g; Mg SO_4, 0.3g; $CaCl_2$, 0.4g; $MnSO_4$, 0.016g in 1 litre deionised water and corresponded to Alberts's *et al.*(1994) broth culture A. Broth F, a non-producer of FB_1 (Alberts *et al.*,1994) was used as a control. Three 1 litre volumes of broth A media were dispensed in Erlenmeyer flasks (2 litre), sterilized by autoclaving at 121 °C for 15 minutes, inoculated with spore suspension (1 ml) and incubated on a shaking incubator at

26° C for 28 days. The broths were sampled and analysed by TLC every 2 days to check for FB_1 production. The cultures were harvested after 28 days incubation.

The broths were centrifuged at 12 000 RPM for 15 minutes in a Beckman floor centrifuge and the supernatant was filtered on a Buchner. One hundred grams of Amberlite IRA beads in 500 ml methanol was poured into a 100 x 2.5 cm long glass column giving a packed bed length of 60 cm (Fig.4). A tap at the bottom of the glass column was used to regulate the flow rate of the solvents. The column was pre-conditioned with 350 ml methanol and 350 ml methanol/ water v/v (3:1). The filtrate was run onto the column thereafter at 2 ml/min. The column was washed with 350 ml methanol/ water and 350 ml methanol. Fumonisin B_1 was eluted with 700 ml of 1% acetic acid in methanol. Thirty fractions (10 ml) were collected in McCartney bottles, and were later analysed by TLC (2.2.5).

2.2.4 Maize patty culture of fumonisin B_1

A mix of maize meal and deionised water (1.2 ml/g) was prepared and 30 g was added to each of 30 glass petri dishes. These were immediately autoclaved for 1 hour at 15 psi, and re-autoclaved on the following day. Cooled plates were then inoculated with a 1 ml spore suspension of *F. moniliforme* PPR1 1059. The inoculum was kept constant for all patties. The plates were then incubated in the dark at 26 °C (incubator) for 6 weeks.

After incubation the contaminated patties were discarded and 14 patty cultures were labelled A to N. These were oven dried at 45 °C for 48 hours. Liquid extraction was carried out on 5 g samples of each plate, by blending with 100 ml of methanol/ water v/v

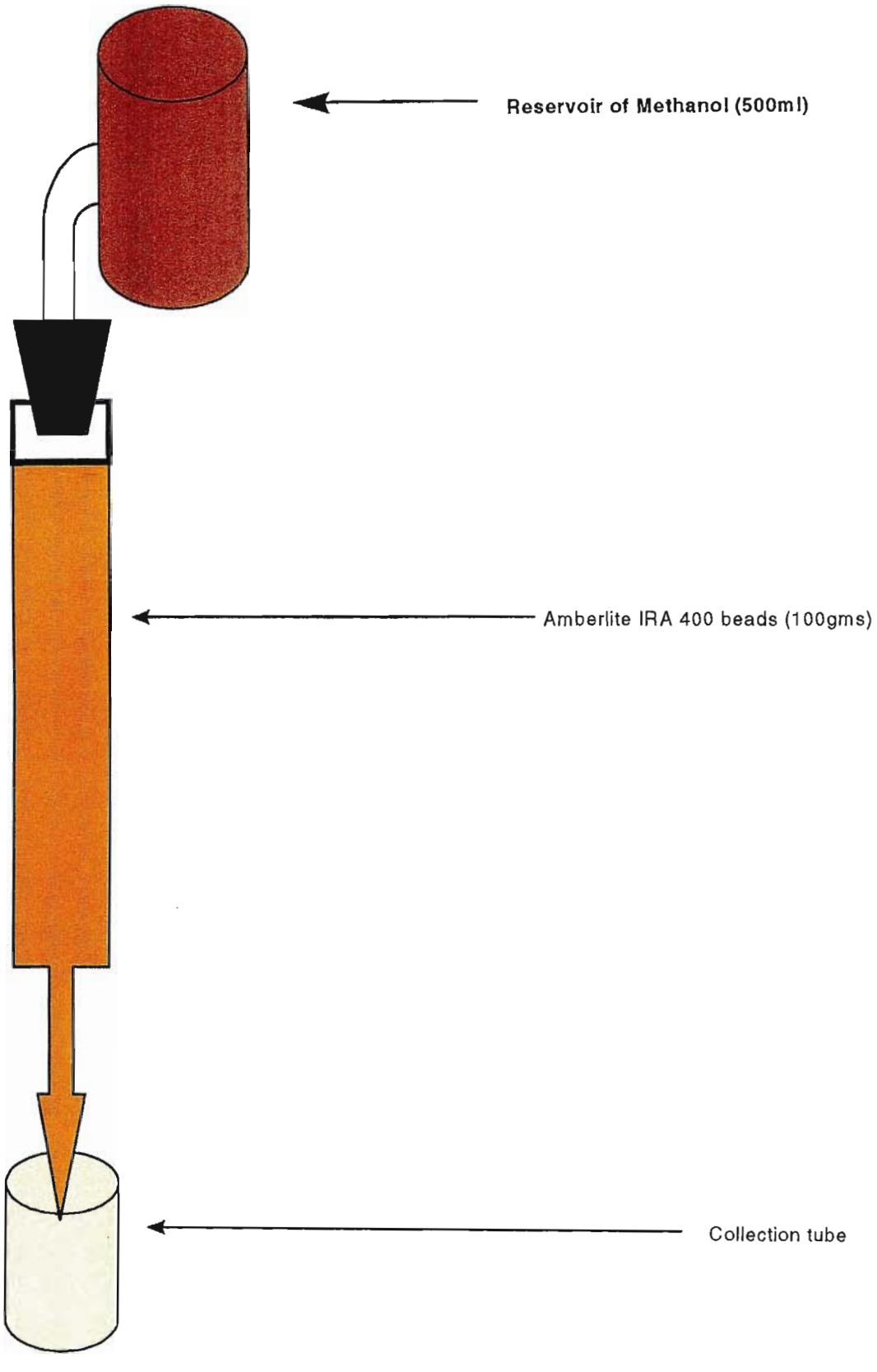


Fig. 4 **Amberlite IRA beads used to clean-up *F. moniliforme* brot culture.**

(3:1) for 3 minutes at 60% of the full speed. The material was added to a 250 ml centrifuge bottle and spun at 500 *g* for 10 minutes at 4 °C. The supernatant was filtered through fluted No.4 paper and the filtrate was adjusted to pH 5.8.

Strong anion exchange columns (SAX)(Bond-elut, Varian) were used to purify the FB_1 from the patty culture extract. The columns were attached to a Vac-elut system (Fig.5) connected to vacuum and a pressure gauge. The columns were pre-conditioned with 5 ml methanol (Analar grade, Merck) and 5 ml methanol/ water v/v (3:1) at a flow rate of 1 ml/min. The filtered extract (10 ml) was then applied. The column was washed with 5 ml methanol/ water followed by 3 ml methanol. The sample was eluted with 10 ml 1 % acetic acid in methanol at a flow rate of 1 ml/min. The eluent was collected in a sterile Bijou bottle and dried under nitrogen gas until required for TLC and HPLC analysis.

2.2.5 Thin-layer chromatography (TLC)

2.2.5.1 Apparatus

TLC plates - Silica gel 60 plates (20 x 20 cm, Merck NT).

TLC developing tank - glass tank (20 x 20 cm).

TLC spray bottles - plastic bottle with fine spray nozzle.

Micro-capillary tubes - graduated 10 μl.

2.2.5.2 Solvents

Butanol, acetic acid, acetonitrile and methanol were required. These were of analytical grade (Merck NT).

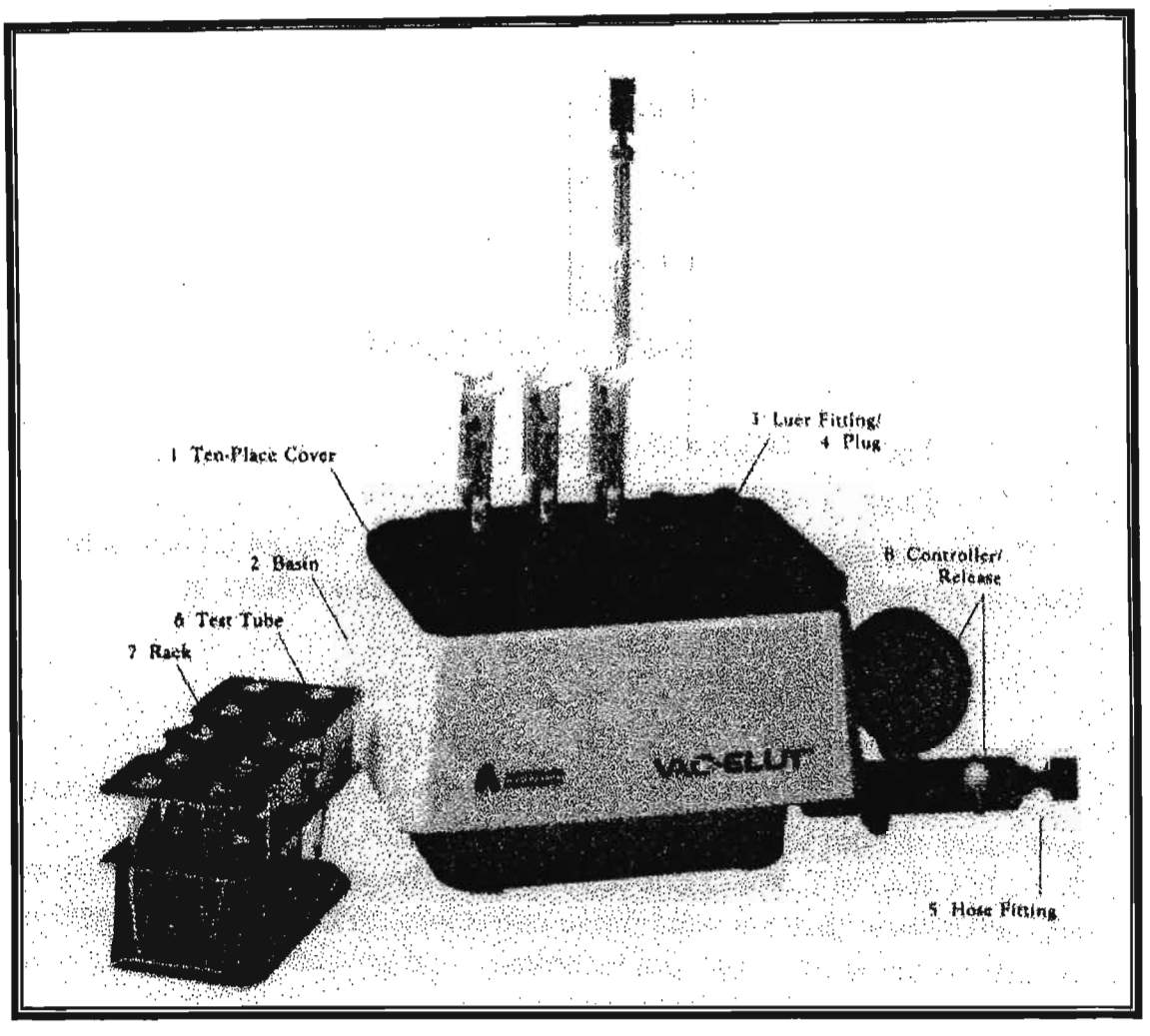


Fig. 5 Using the Bond Elut / Vac Elut™ system.
The components of the BOND ELUT / VAC ELUT system are pictured above. A 10-place molded cover (1) with a foam polyethylene gasket precisely fits the top of the vacuum basin (2). BOND ELUT columns are inserted into luer fittings (3) which are an integral part of the cover. Unused spaces are capped with plugs (4) supplied with the system. Vacuum is applied through a 1/8" NPT hose fitting (5). Sample eluants are collected in test tubes (6) which are held under the columns in several types of stainless steel removable racks (7).

2.2.5.3 Mobile phase

100 ml solution of butanol/ methanol/ acetic acid (BMA) v/v/v (6 :1.5 :2.5) was prepared and allowed to equilibrate in TLC tank.

2.2.5.5 Fumonisin B_1 standards

One milligram of FB_1 (Sigma) was dissolved in acetonitrile/ water v/v (1:1) to make a stock solution of 50 μg/ml.

2.2.5.6 Spray reagent

The reagent was prepared by mixing 85 ml methanol with 5 ml concentrated sulphuric acid and allowed to cool. Glacial acetic acid (10 ml) was added thereafter and finally 0.5 ml p-anisaldehyde (Sigma) was added. The reagent was added to an amber bottle and kept in the dark until use. The reagent was used for one week and discarded thereafter.

2.2.5.7 TLC analyses of broth and patty extracts

The 1 ml of broth sampled every 2 days, and the 30 fractions of broth eluents collected after Amberlite IRA extraction were spotted on the TLC plate in the following way. Ten μl samples of broth extract were spotted approximately 1 ml apart along the pre-concentration zone of a TLC plate and labelled appropriately. The spots were dried and the plate was placed in the TLC tank and left to develop in the mobile phase. Upon developing, the solvent front was drawn and the plate was left to air dry. Patty culture extracts A to N, were spotted as above alongside standards.

2.2.5.8 Derivatization and visualization

The dried plate was sprayed with the p-anisaldehyde spray reagent using the spray bottle to ensure an even covering of the plate. The plate was then heated in an oven at 120 °C for 5 minutes. Visual intensity of the broth spots were compared to the purple spots of the standard, FB_1. The Rf values of the broth spots were calculated using the following equation:

$$\text{Rf of } FB_1 = \frac{\text{Distance travelled by } FB_1 \text{ concentrates}}{\text{Distance of solvent front}}$$

2.3 Results

2.3.1 Broth

The triplicate A broths turned dark pink-peach 17 days after inoculation with *F. moniliforme* (Fig.6) as compared to the F broth, which produced only white mycelium. Thin-layer chromatography analysis indicated the production of FB_1 from 17 days after inoculation and at 28 days, showed maximum production of FB_1. When FB_1 was extracted from broth using the amberlite column, only the first 12 of the 30 fractions eluted FB_1 positive (Fig.7). The Rf value of FB_1 was found to be 0.92. The 12 positive fractions were pooled and kept for analysis on HPLC.

2.3.2 Maize patty culture

The maize patty cultures of *F. moniliforme* grew well over a 6 week period. They turned

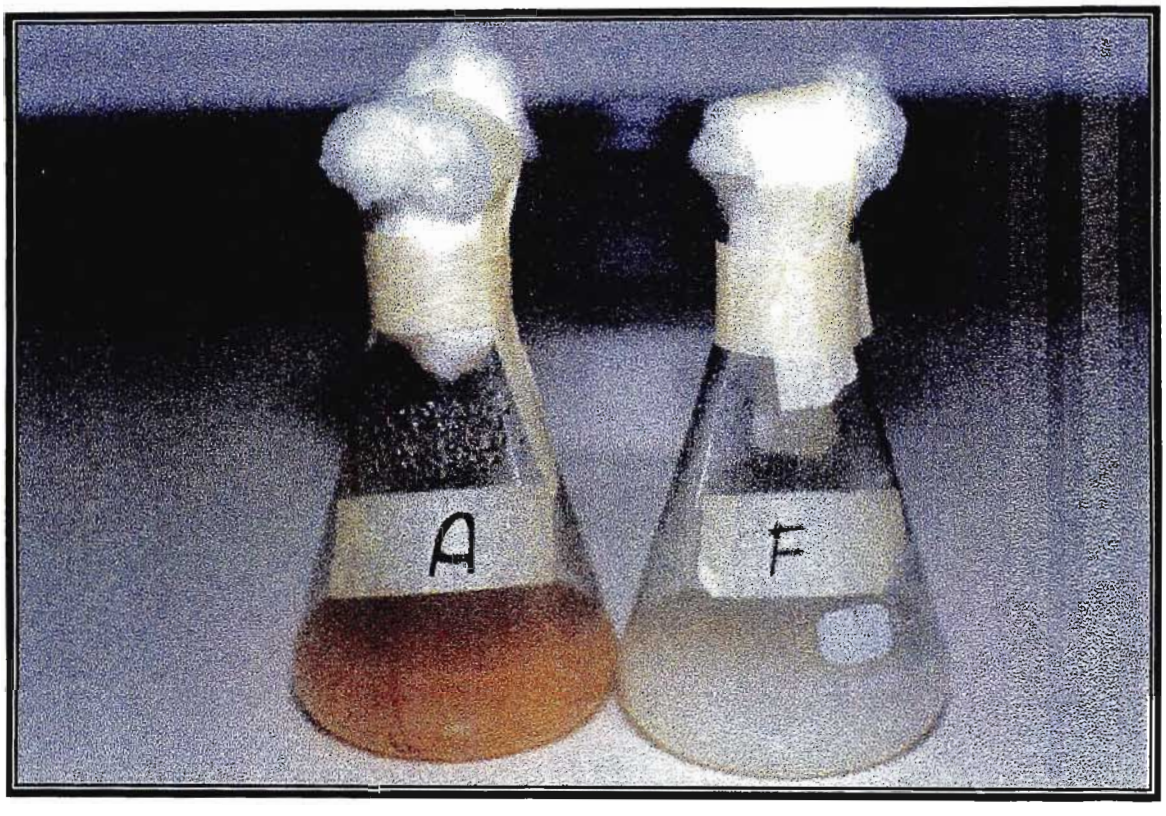


Fig. 6 Broth cultures of Fusarium moniliforme PPR1 1059 after 17 days (Broth A : fumonisin production; Broth F : no fumonisin).

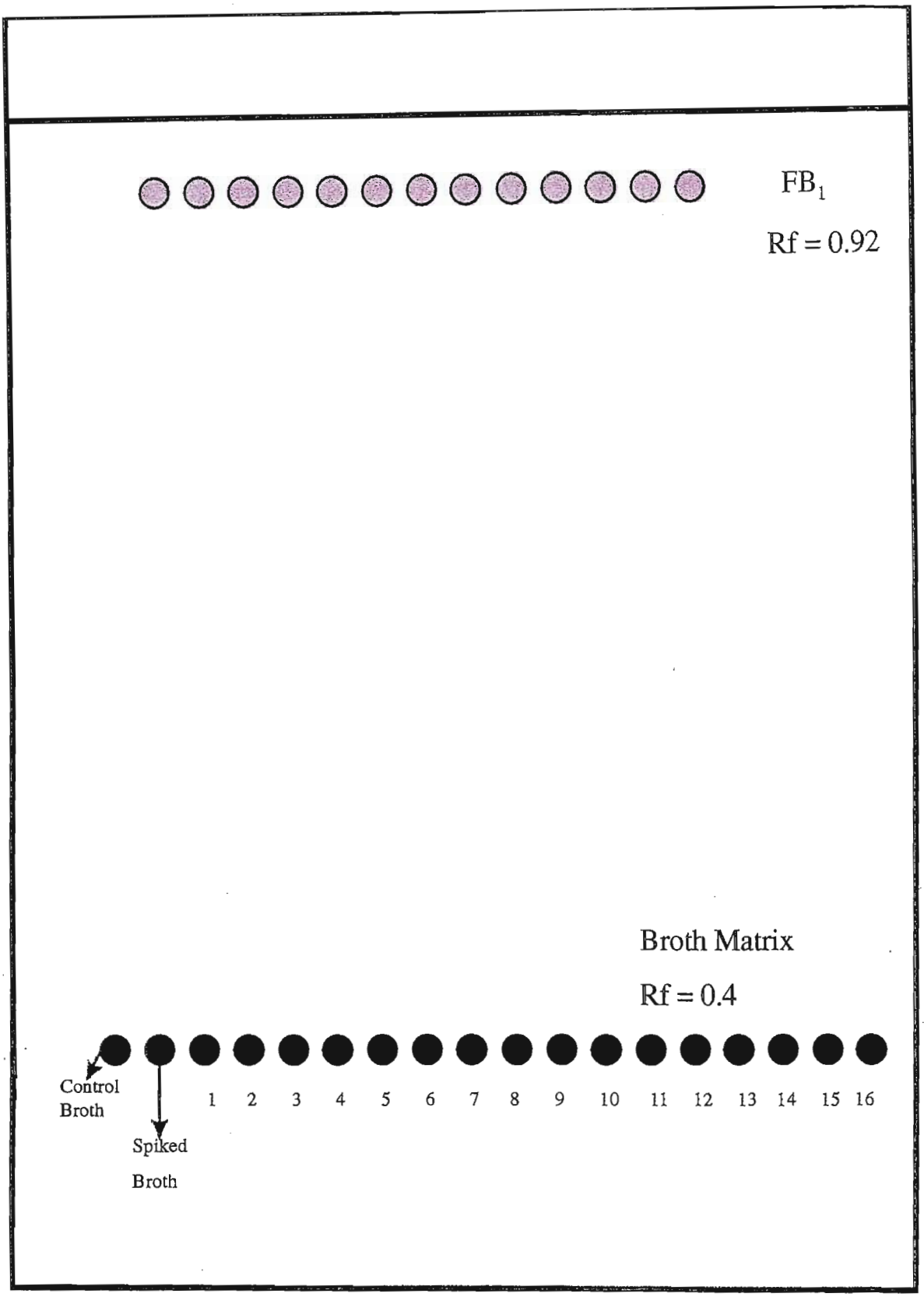


Fig. 7 TLC analysis of broth culture fractions eluted from amberlite column after 28 days.
[Solvent system: BMA (6:1.5: 2.5), using Silica gel 60 plates]

from white to pink to purple (Fig. 8), a similar colour produced on SDA medium after 10 days (Fig. 9) but with a more even and dense growth. At the end of the incubation period the colour turned black and on drying gave a burnt appearance. This, however, did not affect the isolation of FB_1, and examination of extracts by TLC showed the presence of FB_1 in all 14 patty cultures (Fig. 10).

Quantitative analysis of the extract was carried out later using fluorescence HPLC and is reported in chapter 3 (section 3.3).

Fig.8 Patty culture of *F. moniliforme* PPR1 1059 at 26°C for 14 days

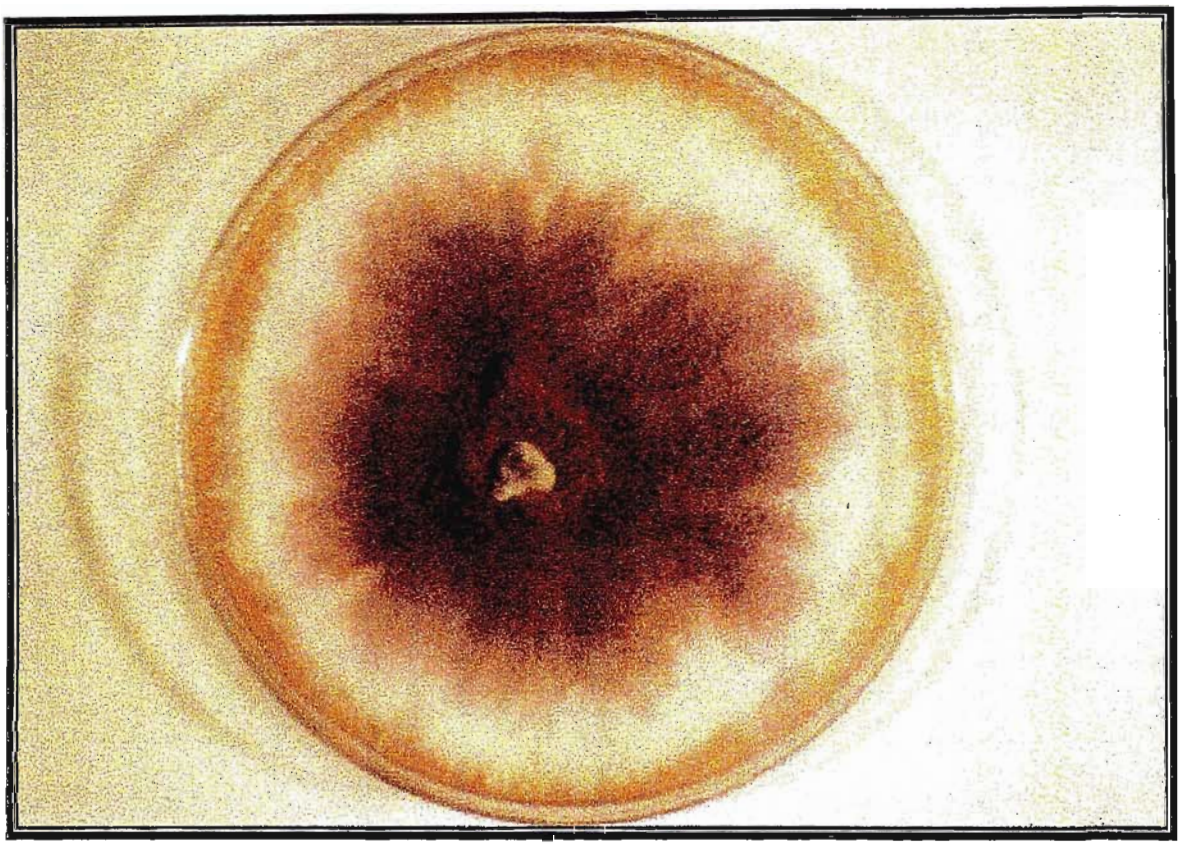


Fig. 9 F. moniliforme PPR1 1059 on Sabourand agar at 26°C for 1 week

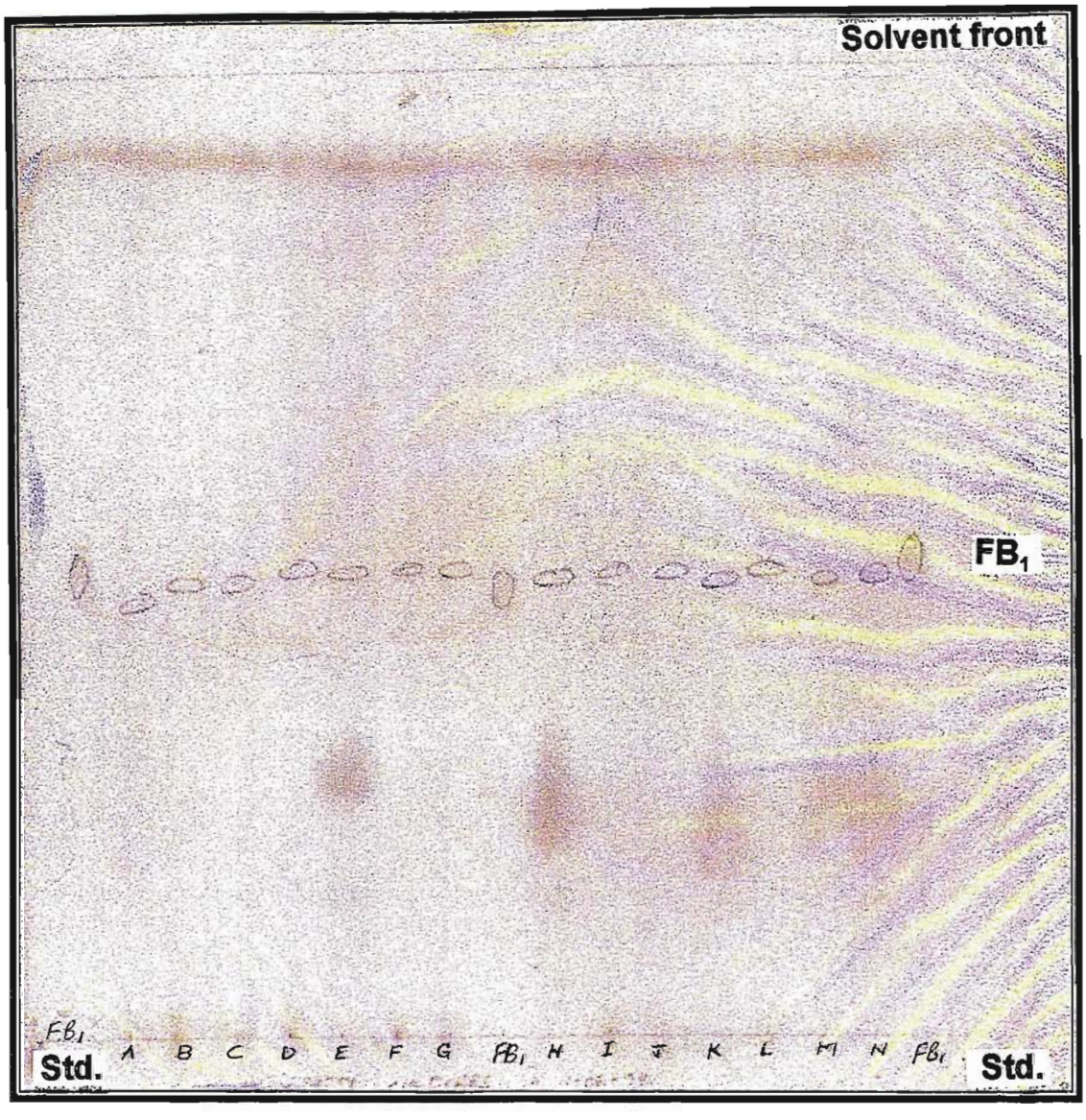


Fig.10 TLC analysis of maize patty cultures (A-N) after clean-up with Bond- Elut cartridges, with Rf values of FB_1 ranging from 0.5 to 0.53.
[Solvent system: chloroform / methanol / acetic acid (6 :1.5 : 2.5), using Silica gel 60 plates (Ackerman, 1991)]

2.4 Discussion

Fumonisin B_1 production

Broth A was found to be a good producer of fumonisins in keeping with the finding of Alberts *et al.* (1994). As in Jackson and Bennett's (1990) experiments, the production of FB_1 steadily rose from 17 to 28 days . Figure 7 shows a display of distinct purple spots (FB_1) away from the broth matrix spots. The purple spots were confirmed as being FB_1 due to a similar pattern being produced by a FB_1 spiked broth culture.

The maize patty cultures of F. *moniliforme* gave a very distinct purple hyphal growth. This was much more distinct as that described by other authors. The drying treatment of the culture at 45° C for 48 hours did not affect the presence of fumonisins. This indicates thermostability of FB_1.

The Bond-elut SAX cartridge (500 mg sorbent mass) is a convenient method of extraction because of its speed and ease of use as compared to preparing and using an amberlite IRA beads for the extraction of FB_1. However it may only be used to purify up to 10 ml extracts (ie. small scale). Larger scale extraction of FB_1, for example, one litre broth samples, require the manual preparation of larger columns. The 100 g Amberlite IRA bead column used in this experiment was successful in extracting the FB_1 from the broth extract. Fraction collection was time-consuming however. This may be overcome by the use of an automatic fraction collector.

The difficulty in selecting a single extracting and clean up method for fumonisins has been demonstrated above. Perhaps the development of a mycotoxin/matrix certified reference materials (CRM's) needs to be done as suggested by the European Commission in 1995. The accuracy precision and traceability of the extraction step in analytical procedures in particular to study the presence and/or effects of bound residues of mycotoxins needs to be set up for biological and biomedical matrices (Wilkes and Sutherland, 1998).

The TLC method used in the experiment was successful in indicating the presence or absence of FB_1, provided the plates were sprayed uniformly. However, differing Rf were obtained for FB_1 depending on the substrate from which FB_1 was being extracted and the clean-up method used. Broth and patty culture matrices differ greatly and thus may have contributed to the differing Rf values of fumonisins obtained in Fig. 7 and Fig. 10.
The diffused bands noted in Fig 10 may be due to incomplete clean up of those particular extract.
A comparison of yield of FB_1 produced in broth and patty cultures is done in chapter 3 (section 3.4).

CHAPTER 3

3.0 QUANTITATION OF FUMONISIN B_1

3.1 Introduction

In order to detect and quantitate low levels of FB_1 in agricultural commodities, feeds and other biological material highly sensitive analytical techniques are required. Fumonisins are not amenable to high temperature GC analysis without derivatisation. The mycotoxin class also lacks a strong UV chromophore and so cannot be directly determined by HPLC-UV. They are formed or sequestered in complex matrices (food, tissue, physiological fluids), so that analytical sensitivity is required to differentiate them from environmental co-extractants, yet their molecules do not contain distinctive moieties useful for selective detection. The fumonisins contain four carboxyl moieties which necessitate the use of a strong acid buffer, such as trifluoroacetic acid (TFA), for good reversed-phase HPLC peak shape. However, TFA degrades fumonisins when the sample is concentrated. The first derivatisation methods for HPLC of fumonisins used maleic anhydride with UV detection or fluorescamine with fluorescence detection (Sydenham *et al.*, 1990). Maleic anhydride produced a 10 μg/g detection limit which is not sensitive enough for incurred residues. Fluorescamine produced two separate peaks for FB_1 and was also not suitable for quantitation. Eventually reaction with o-phthaldialdehyde (OPA) was adopted as a more sensitive approach. This method has a 50 ng/g detection limit (Shepherd *et al.*, 1990). However, the OPA derivatives of the fumonisins are unstable. To obtain quantiatively rapid results samples must be injected onto the HPLC column at no later than 4 minutes

after the addition of the reagent. This method has been adopted as an Association of Official Analytical Chemists International (AOAC) approved method for the determination of FB_1, FB_2 and FB_3 (Sydenham *et al.*, 1997). Quantitative capillary electrophoresis (CE) results agreed closely with parallel HPLC results using pre-column 5-fluoro-isothiocynate (FITC) derivatisation (Maragos, 1995).

The OPA method was adapted for the present study, to realise an accurate and sensitive quantitation of FB_1 in culture material (Thiel *et al.*, 1993).

3.2 Method and Materials

3.2.1 High-performance liquid chromatography (HPLC) methodology (Bettina, 1993)

The HPLC became available for the analysis of foodstuffs nearly twenty years ago. The first published HPLC application for mycotoxin research dates from 1973. Since then, the trend has been towards increased use of HPLC for the ultimate separation, detection, and quantification of the mycotoxins in foods, after sufficient clean-up to remove interferences that could give rise to false positives. A typical HPLC system is shown in Fig. 11.

The important advantages of HPLC were its ability to handle thermally labile, poorly volatile, non-volatile, polar, and ionic compounds. The high resolving power between chemically similar compounds, the speed, increased sensitivity, accuracy and precision of

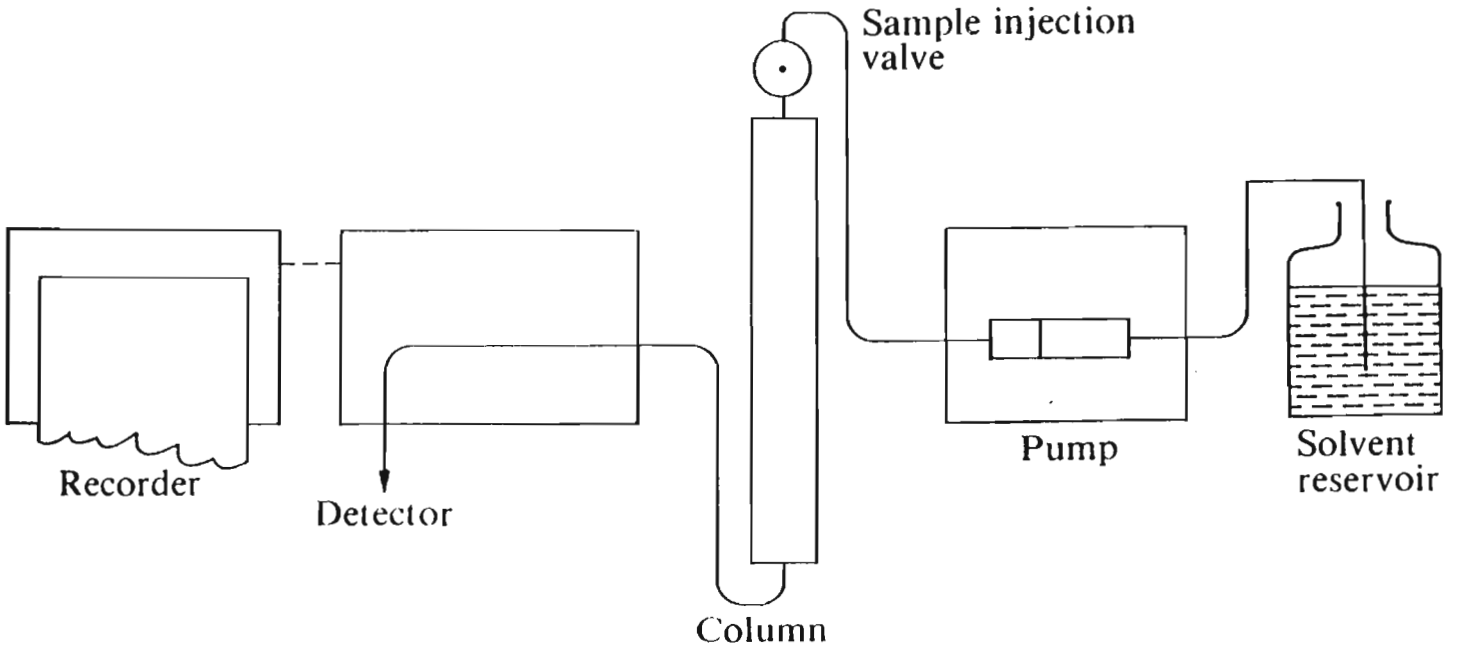


Fig. 11 A representation of the components of an HPLC system.

the method, and the variety of detection systems now available make modern HPLC a more suitable technique than other liquid chromatographic techniques. In addition, HPLC is a quantitative technique and is suited for on-line clean-up of crude sample extracts, and finally, it can be automated quite easily. However HPLC has limitations as well. The cost of modern HPLC equipment is quite high, and wide experience is necessary for obtaining the best possible benefit from the HPLC system.

Instrumentation and practice

In modern mycotoxin analyses, it will be profitable to use one of the three primary HPLC methods: reversed-phase (RP), iron-pair (IP), or normal-phase (NP) chromatography. RP-HPLC is nowadays the most commonly used method, and it is a potential technique for multimycotoxin analysis under gradient elution conditions.

Instrumentation

Modern liquid chromatographs, which vary widely in sophistication, can be assembled from modular units designed to work independently of each other, or purchased as a single, integrated unit. Each system has its own advantages and drawbacks.

Solvent reservoir

The solvent reservoir must hold a volume of solvent adequate for repeated analyses and it must be inert with respect to the solvents used, varying from aqueous buffers to hydrocarbons, depending on the mode of chromatography. Air bubbles are the major cause of problems with LC pumps. Problems with dissolved air are usually encountered with protic solvents such as water and alcohols. The best way to avoid bubbles is to thoroughly degas the solvents by heating, application of vacuum or ultrasound, or by sparging with helium. The latter is the most effective and convenient degassing method. After initial

(a few minutes) vigorous bubbling of helium through the solvents the helium flow is reduced to a trickle during the use of the LC system.

Pumping systems

The pump is one of the most important components in HPLC equipment, because its performance directly affects the reproducibility of retention times, quantitative analysis, and detector sensitivity. Nowadays reciprocating piston pumps provide the basis for most pumping systems. The pump must produce a wide range of flow rates for solvent delivery. Although most HPLC pumps operate at pressures of 300-400 bar, the operating pressures should be less than 50% of the maximum capability of the pump (typically < 100-50 bar) (Lachrom HPLC max. pressure 400 bars).

Injection device

Sample introduction is one of the critical steps in HPLC: even the best column will produce a poor separation if injection is not carried our carefully. The most widely used method uses an injection valve (e.g. Rheodyne Model 7010, 7125, or 7410), allowing reproducible volumes to be injected and good quantitative analyses. The sample volume must be about five times the volume of the loop (e.g. 100 μl of sample for a 20 -μl loop). This is important for quantitative analysis using an external standard method. An automated injection system (autoinjector) is generally an automated version of the six-port injection valve used for manual injections. With the change of precision syringes, the autoinjector offers a wide range of fully programmable injection volumes (Lachrom HPLC 0.5 - 400 μl). The 25 μl syringe provides the highest accuracy and precision. The autoinjector can be combined with the autosampler permitting unattended injection of samples into the liquid chromatography.

Column hardware

Columns are available in numerous different configurations and from about a hundred different suppliers. The columns vary in dimensions. They are typically 5-25 cm long when micro-particulate stationary phases of 3-10 μm are used. Most HPLC column blanks are made of stainless steel tubes and have compression fittings of various design (e.g. Swagelock,Valco) and steelfrits at either end of the column to retain the column packing material. Stainless steel is resistant to the pressures in HPLC and relatively inert to chemical corrosion.

Column temperature control

The role of temperature is usually neglected in HPLC, and most HPLC separations are carried out at ambient temperature without the aid of a column oven.

Normal-phase HPLC

When the stationary phase is more polar than the mobile phase, the HPLC mode is called normal-phase (NP) chromatography or often adsorption chromatography. NP-HPLC is carried out with inorganic solids such as silica or alumina and various polar bonded phases (e.g. cyano, amino, diol, nitro) which have been reviewed in many sources.
Mixtures of organic solvents are used as the mobile phase such as hexane and dichloromethane.

Reversed-phase HPLC

In the technique of the reversed-phase high-performance liquid chromatography (RP-HPLC) the mobile phase is more polar than the stationary phase which is the opposite of NP-HPLC. The RP methods have traditionally employed hydro-carbon-like stationary phases with polar aqueous organic mobile phases. Compounds are separated according to their relative hydrophobicity, the most polar compounds being eluted first and the non-

polar compounds being retained longer.

Stationary phase

The most commonly used stationary phases for RP separations have been and still are C-18 bonded phases, followed by C-8 and shorter n-alkyl, phenyl, or cyanopropyl bonded phases. Silica has been the most widely used base material for the aforementioned phases.

Mobile phase

It is generally accepted that retention in RP-HPLC is mainly controlled by the mobile phase, with the stationary phase playing the secondary role. Optimum selectivity is usually achieved by finding the right composition for the mobile phase. The preferred organic solvents for the RP-HPLC are methanol (MeOH), acetonitrile (ACN) and tetrahydrofurane (THF), used in binary, ternary, or sometimes in quaternary combinations with water. Organic solvents are strong and water is a weak solvent. In RP-HPLC solvent strength increases with the decrease in polarity. A change from methanol to acetonitrile or THF can result in significant selectivity changes for various sample solutes.

Isocratic elution is useful, only when toxins with similar retention behaviour are to be studied, whereas gradient elution is effective for the separation of samples containing compounds with a wide variety range of retention times.

Mobile-phase additives

Non-ionic compounds can usually be chromatographed in RP-HPLC in the absence of mobile-phase additives (acids, buffers, ion-pairing reagents, or triethylamine). The pH adjustment usually is performed by using acetic acid (AcOH), phosphoric acid, trifluoroacetic acid (TFA), or different buffers (e.g. sodium or potassium phosphate, ammonium acetate) as mobile phase modifiers.

Reporting retention data

There is yet no qualitative standard method of reporting retention data in HPLC. The methods most in use today are retention times (t_R), retention volumes (V_R), and capacity factors (k') (Eqn 1), which are all strongly sensitive to variations in the chromatographic parameters. Relative retention expressions such as relative retention times (r) (Eqn 2).

$$k' = (t_R - t_o) / t_o \qquad (\text{N.B. } t_o = \text{dead time}) \qquad (1)$$

$$r = t_{R\,(X)} / t_{R\,(st)} \qquad (\text{N.B. x = sample; st = standard}) \qquad (2)$$

Assessment of column efficiency

The ability of a chromatographic process to separate or resolve two similar compounds is measured as the resolution index (R_s) (Eqn 3). The greater the value of Rs the better the resolution of the two compounds. However, values of 1.5 are ideal (Holme and Peck, 1994).

$$\text{Resolution } (R_s) = \frac{\text{twice the distance between the two peaks}}{\text{sum of the base width of the two peaks}} \qquad (3)$$

$$= \frac{2\,(t_B - t_A)}{W_A + W_B}$$

The theoretical plate number (N) is an acceptable measure of the amount of zone broadening occurring in a column (Eqn 4). The greater the value of N the more efficient is the column.

$$\text{Number of theoretical plates (N)} = 5.54 \frac{(t_R)^2}{(W_{1/2})^2} \qquad (4)$$

The height equivalent to a theoretical plate (HETP) may be calculated from the value of N and the column length (L). The HETP is useful in assessing the varying efficiency of the same column under different conditions (Eqn 5).

$$HETP = L/N \quad (5)$$

Quantitative analysis

Although the response of the detector is usually proportional to the concentration of the test substance it is often impossible to define this relationship precisely and it is essential that the response to a series of standard solution is measured and a calibration factor or curve determined. For external standardization, replicate standards of known concentration of the pure substance are injected and the height or area of the resulting peaks measured.

Detection

The ideal HPLC detector possesses high sensitivity, low minimum detectability, wide linear dynamic range, good linearity and is predictable and fast. Thus far the most commonly used detectors in mycotoxin analysis have been the conventional UV-Vis and fluorescence detectors. The powerful combination of chromatography and spectroscopic techniques have become a reality in HPLC analysis of mycotoxins with the development of diode array detector (DAD) and other interfacing techniques especially thermospray (TSP) and dynamic fast atom bombardment (FAB) allowing HPLC to be coupled online with mass spectrometry (MS). Connecting MS to LC requires an interface device that will convert the liquid phase containing the analytes to a gas phase in the presence of a vacuum.

Compounds that naturally fluoresce or that can be made to fluoresce through chemical derivatisation can be detected with high selectivity and sensitivity by the fluorescence detector. It is about a thousand times more sensitive than a UV detector.

Laser induced fluorescence (LIF) detector offers the most promising application readily distinguishing toxins from a complicated matrix of compounds that do not fluoresce.

3.2.2 HPLC of fumonisin B_1 with OPA derivatisation (Sydenham *et al.*, 1992B)

3.2.2.1 Apparatus (see Fig. 12)

Liquid chromatograph - Lachrom HPLC (Merck NT)

Autosampler. - x, y, z configuration

Variable Fluorescence detector - Xenon lamp

Computer and printer - with Windows NT 4.0

Data system - HPLC System Manager (D-7 000 HSM).

Column - C 8 Lichrospher (5 μm) 25 cm x 4 mm (Merck NT).

3.2.2.2 Reagents/solvents

O-phosphoric acid, glacial acetic acid, o-phthaldialdehyde (OPA), 2 - mercaptoethanol, sodium dihydrogen phosphate and disodium tetraborate were analytical grade and obtained from Merck NT except for OPA which was obtained from Sigma. Methanol and acetonitrile were HPLC grade (Merck NT).

3.2.2.3 Mobile phase (MP)

The MP was made up of methanol/ 0.1 M sodium dihydrogen phosphate (68 : 32) and adjusted to pH 3.35 with o -phosphoric acid. Thereafter it was vacuum filtered using a 0.45 μm Waters HV membrane to remove particulate matter, and degassed by placing it in an ultrasonic bath for 10 minutes.

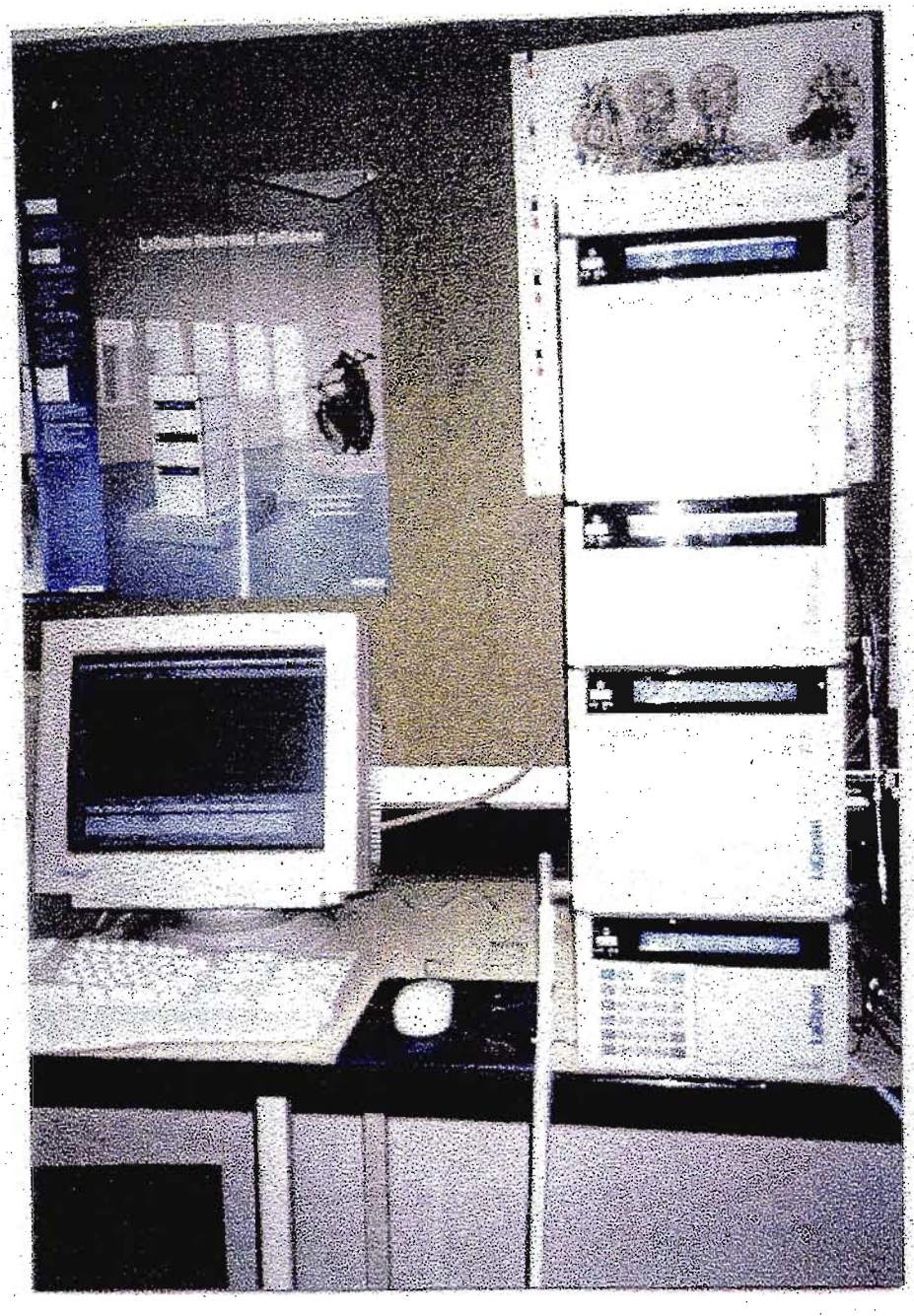


Fig.12 Lachrom HPLC (Merck NT) used for quantitation of FB_1. (Bottom right to top: pump, column,autosampler, UV detector, fluorescence detector, interface. Left: PC.)

3.2.2.4 Preparation of derivatising reagent

O-phthaldialdehyde (40 mg) was dissolved in 1 ml methanol and diluted with 5 ml 0.1 M disodium tetraborate. Fifty μl of 2-mercaptoethanol was then added. The reagent was mixed and kept in a capped amber bottle to be used for one week.

3.2.2.5 Preparation of standards and samples

A stock solution of 50 μg/ml FB_1 standard (Sigma) was made with acetonitrile:water (1:1) and kept in an Eppendorf tube at -20 °C. A series of dilutions of the stock solution was made, ranging from 4 ng to 0.1 ng/ml FB_1. Immediately prior to injection onto the HPLC, 50 μl of FB_1 standard was added to 450 μl OPA reagent and mixed. The injection volume was 20 μl.

Dried samples were rehydrated with 200 μl acetonitrile/ water (1:1). Fifty μl of the sample was derivatised by adding 450 μl OPA as above and injected immediately.

3.2.2.6 HPLC set-up

The Lachrom HPLC system was switched on from pump, autosampler, detector, interface and then the computer. The HPLC was initialised via the computer and the method was set up in the D-7000 HSM programme. The detector was set at a excitation of 335 nm and an emission of 440 nm. The system was purged with ultrapure water until the solvent lines were free of air bubbles. Thereafter the HPLC was flushed with water until a steady baseline and pressure was obtained. The pump was stopped and the solvent delivery line A was aded to the degassed mobile phase and the system was equilibrated at a flow rate of 1.2 ml/min for FB_1 analyses. A sample table was set up with an injection volume of 20 μl.

3.2.2.7 Quantification

The fumonisin content of the sample is calculated from the chromatographic peak areas as shown below:

$$A\ (ng) = \frac{G \times S}{H}$$

where A= ng of FB_1 present in the sample injected onto the HPLC; G = peak area of the sample; H = peak area of the standard (50 μg/ml); S = amount of FB_1 standard injected onto the HPLC (ng).

$$C\ (ng/g) = \frac{A \times T}{I \times W}$$

where C = the concentration of the FB_1 present in the sample in ng/g or ng/ml;
A = as calculated above; T = total volume of derivatized sample solution (500 μl); I = the injection volume (20 μl); W = the sample equivalent amount derivatized (g or ml).
Simplified:

$$C\ (ng/g) = \frac{A \times 25}{W}$$

3.2.3 HPLC analysis of fumonisin B_1 with FITC derivatisation (Maragos, 1995)

3.2.3.1 Apparatus

As in section 3.2.2.1, except for fluorescence detection. Excitation was set at 250 nm and emission was 470 nm.

3.2.3.2 Reagents / solvents

Acetic acid, di-methyl sulphoxide (DMSO), 50 mM Borate Buffer pH 9.3 (Merck), 5 - fluoro-isothiocyanate (FITC), acetone and 10 mM Phosphate Buffer pH 7.5 were analytical grade. Acetonitrile and methanol were HPLC grade.

3.2.3.3 Mobile phase

Solvent delivery line A contained degassed methanol (HPLC grade) and solvent delivery line B contained 10mM phosphate buffer, with a pH of 7.5.

3.2.3.4 Preparation of FITC

A 1.3 mM FITC solution was prepared in acetone and kept in an amber bottle in the refrigerator.

3.2.3.5 Preparation of standards

Purified FB_1 were prepared in acetonitrile/water (1:1) (0.2 - 20 μg FB_1 into vials). Four ml methanol with 2% v/v acetic acid was passed through a SAX column and added to each vial above. Standards were dried with nitrogen at 60 °C and dissolved in 500 μl DMS0/ Borate buffer 50 mM, pH 9.5 (9:1). Thereafter 75 μl of FITC was added and the standard mixture was incubated at 60 °C for 90 minutes. This was then cooled to room temperature. Finally 4425 μl of methanol / Borate buffer 50 mM, pH 9.5 v/v (3:2) was added and the standard was ready for HPLC analysis.

3.2.3.6 Gradient elution

The column was equilibrated with 30 % A and 70 % B (see 3.2.3.3). After sample

injection solvent A was increased to 42 % over 7.2 mins., and held at this level until 12 mins. The column was then washed with 90 % A for 4 mins after which the mobile phase was returned to the starting concentration of 30 % A. A mobile phase flow rate of 1 ml/min was used throughout the run.

3.2.4 Capillary electrophoresis (CE) methodology

Capillary electrophoresis employs narrow-bore (20-200 μm internal diameter) capillaries to perform high efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages (> 500V/cm), which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary.

The tube is filled with buffer and subjected to an electric field. Under these conditions, the positive charges migrate toward the cathode at a rate determined by their mass-to-charge ratios. Detection procedures have been based largely on conventional UV absorbance methods, however visible absorbance, UV and fluorescence lasers, and electrochemical detection have been used. The basic instrumental configuration for CE is shown in Fig. 13.

3.2.5 Capillary electrophoresis (CE) of FB_1

3.2.5.1 Apparatus

The instrument used was a Beckman P/ACE System 5000 capillary electrophoresis system, equipped with an Argon-ion laser-induced fluorescence (LIF) detector (laser module 488).

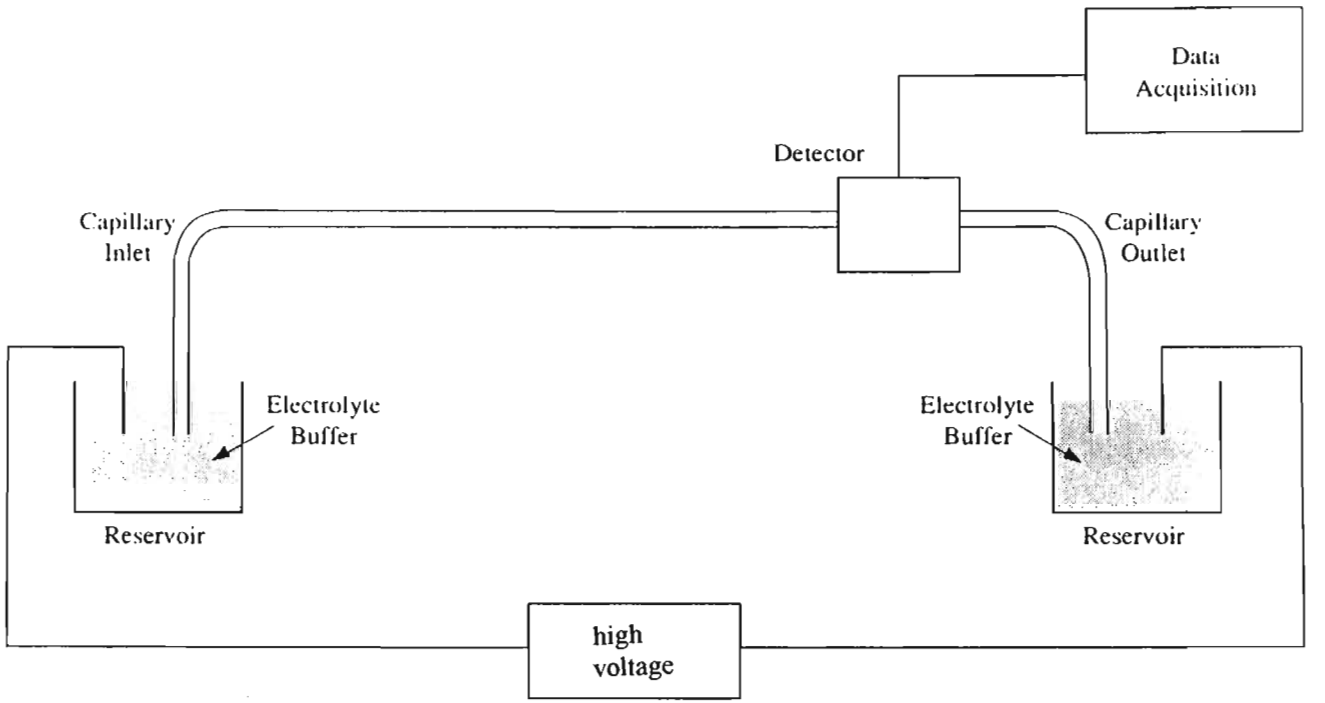


Fig. 13 Basic configuration of the P/ACE Capillary Electrophoresis System.

3.2.5.2 Cartridge

A Beckman 360 604 cartridge housing the capillary (50 cm x 75 μm i.d) was maintained at 35 °C.

3.2.5.3 Buffer

A 50 mM Borate buffer, pH 9.3 (Electropur grade, Merck NT) was used with a current of 250 μA provided by 27 kV.

3.2.5.4 Preparation of the FB_1 standard

The standard was prepared as above (3.2.3.5), but diluted at the end (1:20) in electrophoretic buffer.

3.3 Results

3.3.1 Chromatography of FB_1 standards

Chromatograms of standards and solvent blank are shown in Fig. 14 with retention time of FB_1 ranging from 7 to 9 minutes, which was well separated from reagent peaks. Table 3 and Fig.15 show standards analysed with OPA giving a linear response from 0.1 to 4 ng/ml FB_1 with a detection limit of 0.1 ng/ml. Chromatograms of HPLC analysis of FB_1 derivatised with FITC are shown in Fig.16 a . Figure 16 b is an electropherogram from capillary zone electrophoresis (CZE) analysis of FITC derivatised FB_1. The retention time of FB_1 was 16.99 minutes during a run time of 30 minutes. Table 4 shows the comparison of the different techniques used to quantitate

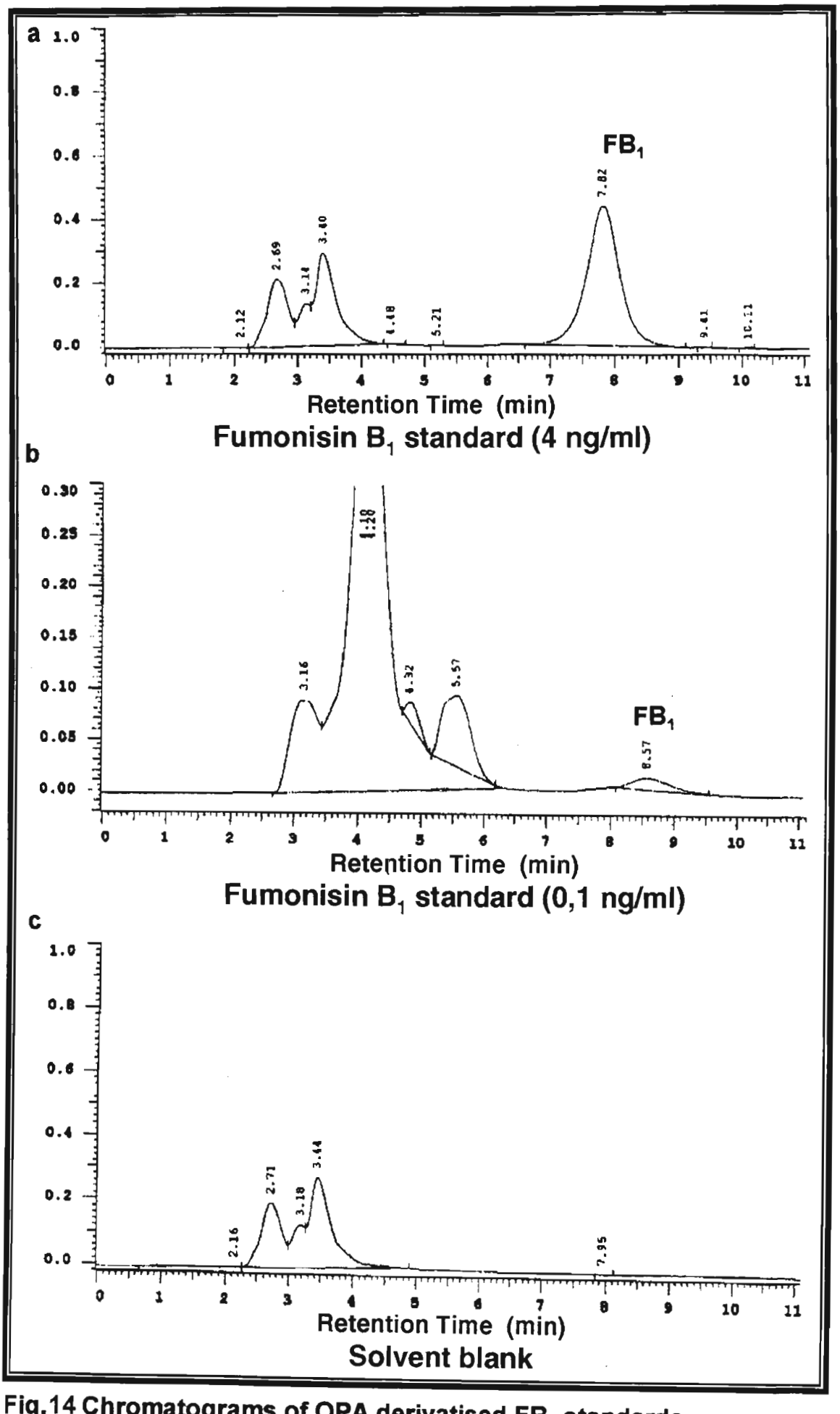


Fig.14 Chromatograms of OPA derivatised FB_1 standards

TABLE 3. FB_1 standards quantitated on HPLC using OPA derivatization

Vol. of stock solution (μl)	Retention time (min.)	Area ($x10^6$) (mean ± SD)	Concentration (ng/ml)
40	8.23	8.398 ± 0.45	4
30	8.56	6.175 ± 0.23	3
20	8.06	4.544 ± 0.31	2
10	7.97	2.083 ± 0.50	1
5	7.76	1.0 ± 0.66	0.5
1	7.76	0.205 ± 0.87	0.103
0.5	7.05	0.399 ± 0.09	0.05
0.1	6.98	0.266 ± 0.05	0.01

Fig. 15 Standard curve of OPA derivatised FB1

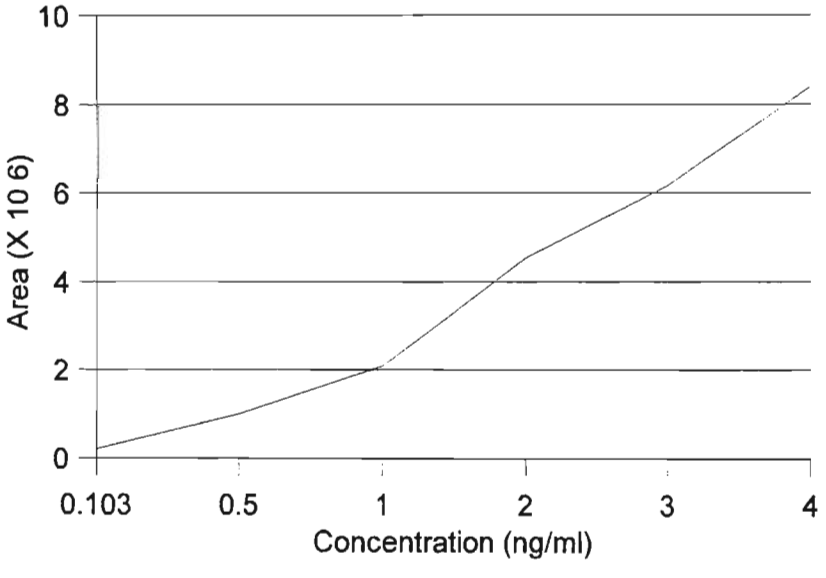
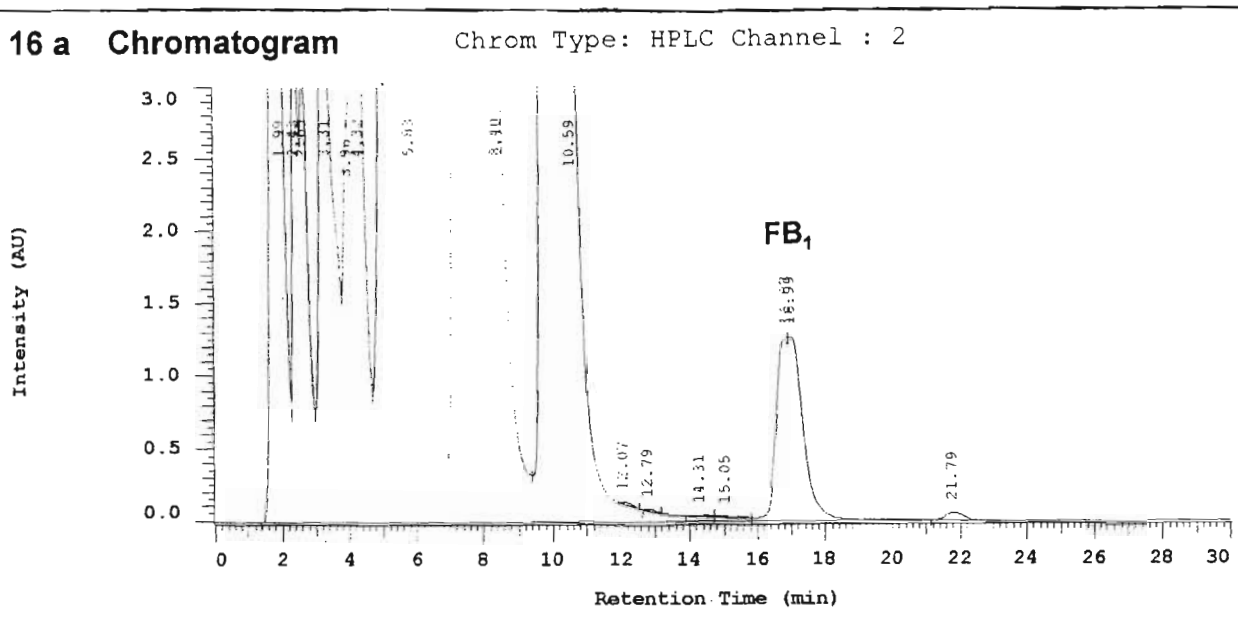


Fig. 16 HPLC and CZE analysis of FITC derivatised FB_1 (50 μg/ m

16 a Chromatogram



16 b Electropherogram

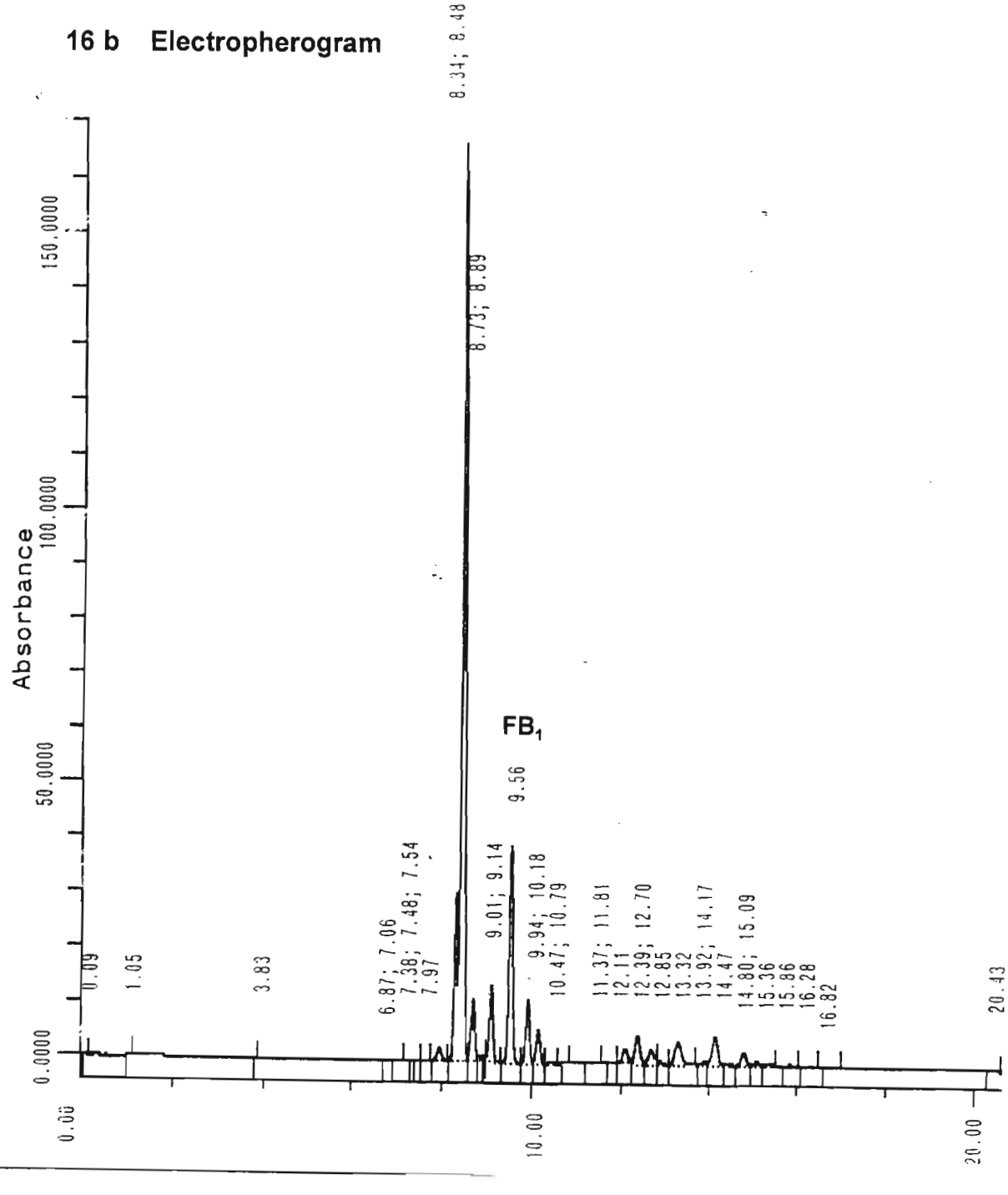


TABLE 4. Comparison of sensitivity of various procedures used to quantitate FB_1 standards.

CONC.(ng/ml.)	HPLC	HPLC	CZE
	OPA-FB_1	**FITC-FB_1**	**FITC-FB_1**
	Peak area	**Peak area**	**Peak area**
0.05	0.399	0	0
0.1	0.205	0	0
4	8.398	0	0.489
40	82.054	31.528	4.221
4000	8325.3	3364.9	461.34

fumonisin standards. Retention times and areas indicated in Table 3, are the averages of triplicate runs carried out at different stages of the research. The average pressure of the runs with OPA was 186 ± 7 bars. The pressure during the gradient FITC runs ranged from 170 to 210 bars.

3.3.2 Quantitation of FB_1 in broth culture

The FB_1 produced in chapter 2 were quantitated using HPLC. The positive fractions of broth extract eluted from the column were pooled and cleaned using the Bond-elut columns and then subjected to HPLC analysis as in chapter 2 (Fig. 17). The yield was calculated using the formula (section 3.2.2.7) and was found to be 1.69×10^7 ng/ml of FB_1.

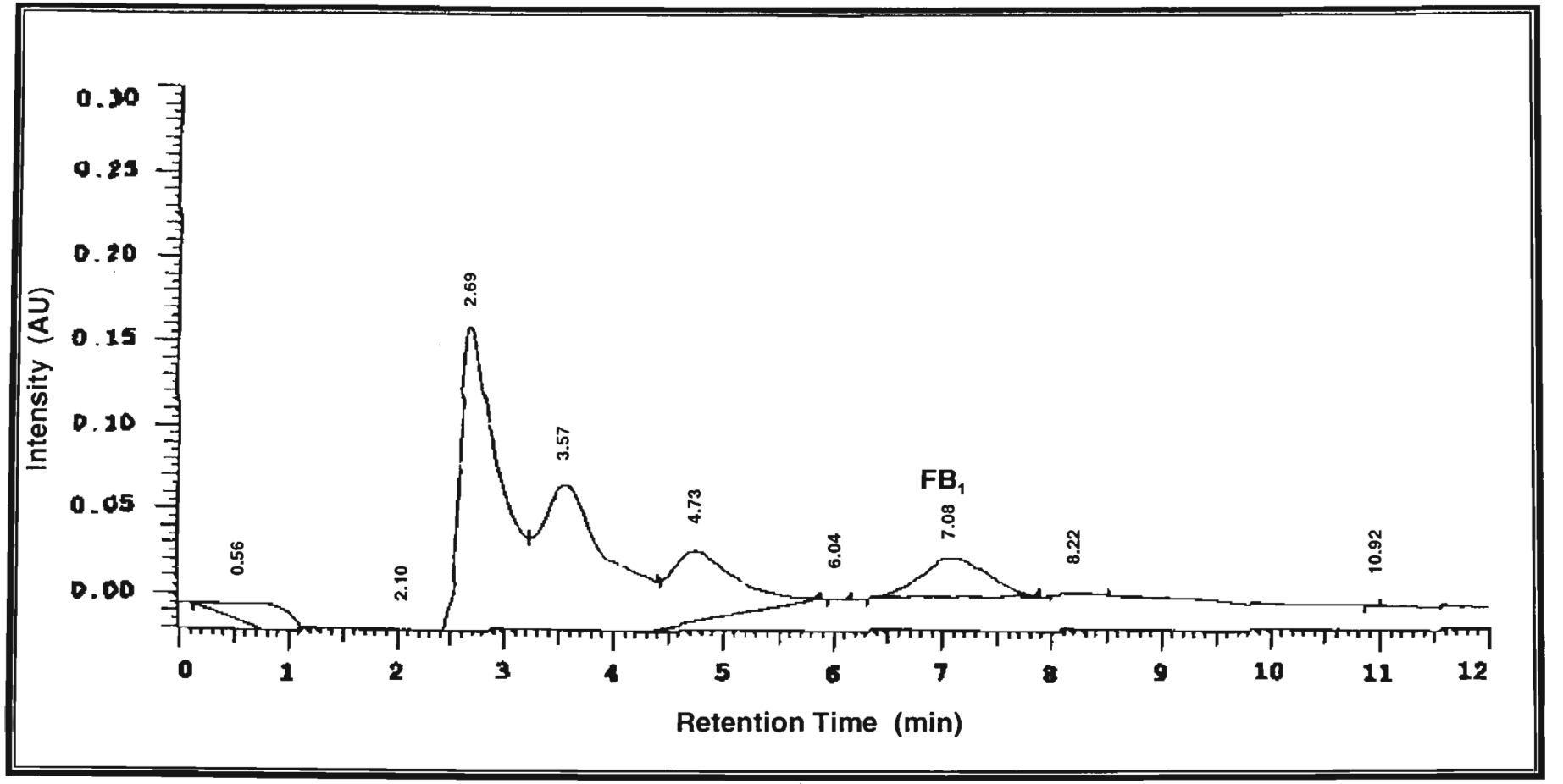


Fig. 17 HPL chromatogram of FB_1 produced in broth culture after 28 days. [OPA derivatisation using methanol / 0.1 M sodium dihydrogen phosphate (68 + 32) pH 3.3, C8 Licrospher (5 um), flow rate of 1.2 ml/min.]

3.3.3 Quantitation of FB_1 in patty culture

On HPLC analysis of the 14 extracts of patties, extremely large peaks at retention time of FB1 (~9 min.) was found and an example of a chromatography is given in Figure 18. This gave a yield of 3.26×10^{8} ng/g.

3.4 Discussion

3.4.1 Development of methods of analysis for Fumonisin B_1

High performance chromatography of FB_1 derivatised with OPA and measured by fluorescence detection, showed a linear response from 0.1 to 4 ng/ml and a high level of sensitivity (0.05 ng/ml). Providing that the time taken from derivatisation to injection into the HPLC was constant, the results were reproducible in keeping with the studies done by Thiel *et al.* (1993). In contrast the FITC method of derivatisation was difficult and was not as compatible with HPLC. Phase separation was noted during the derivatisation stage and the HPL chromatograms were cluttered with reagent peaks, which masked the derivatised FB_1, making peak area measurements more difficult. In addition, the OPA derivative of FB_1 gave a larger response in the fluorimeter than the FITC derivative, of around a thousand fold.

On examining these two derivatives by CZE, however, the reverse was true. The FITC derivative of FB_1 gave a detection limit of as little as 4 ng, which agrees with the results of Maragos (1995). This was fifty times more sensitive than the result obtained with the FITC

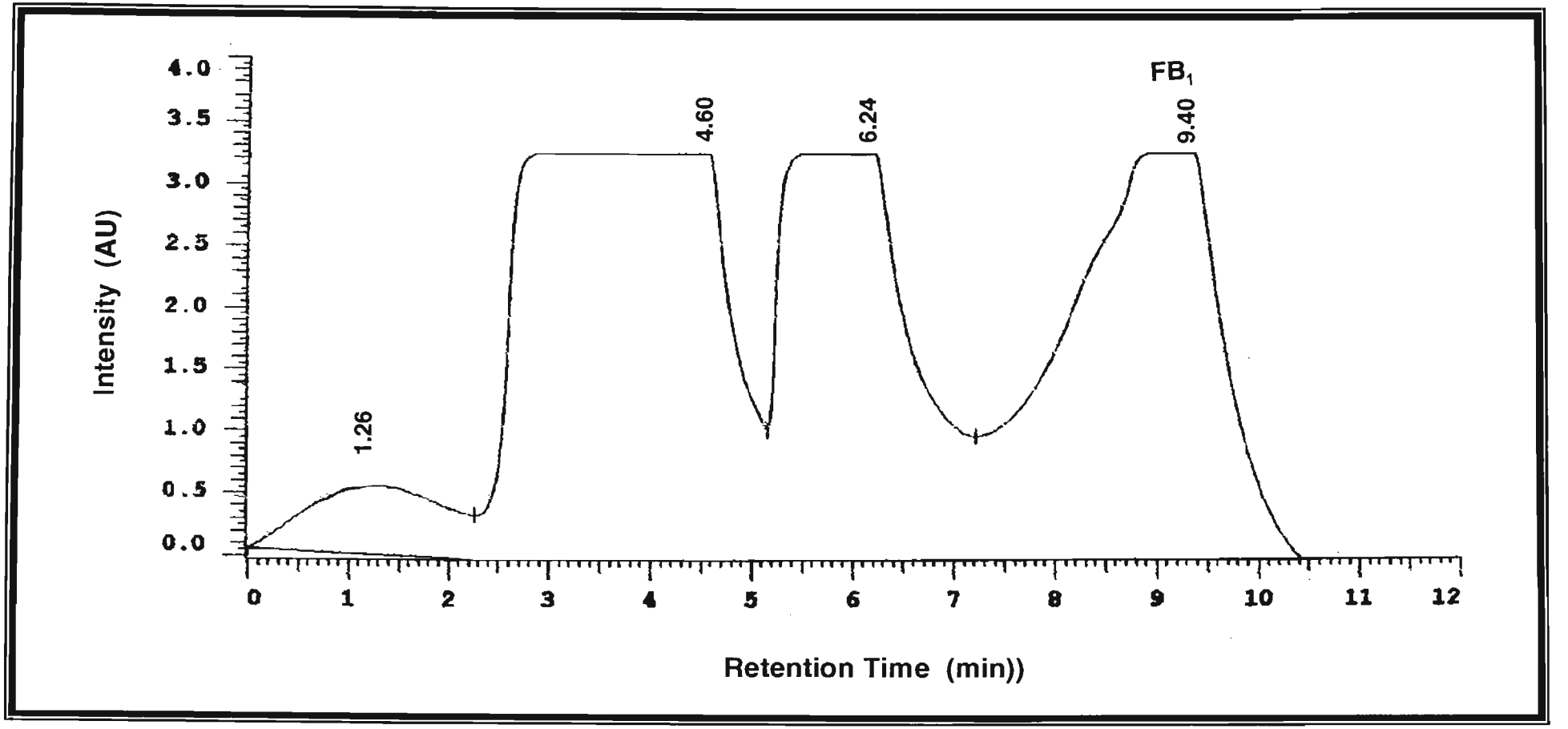


Fig. 18 Chromatogram of maize patty culture A (5g) showing presence of FB_1 [OPA derivatisation using methanol / 0.1 M sodium dihydrogen phosphate, (68 + 32) pH 3.3, C8 Lichrospher (5 µm), flow

derivative on HPLC. This is probably due to the laser-induced detection at 488 nm of the CE which is highly sensitive to FITC derivatives.

These results indicate that for CZE analysis the FITC derivative of FB_1 should be used and for HPLC, the OPA derivative. In this study the OPA derivatisation method was used with HPLC as this was the most sensitive and convenient method overall. The variation in retention times (eg.7-8 min. with OPA) may be attributed to conditions such as temperature, mobile phase or detector response differing from day to day. An internal control should have been used to work out intra-assay and inter-assay coefficients of variation.

3.4.2 Yield of FB_1 in Broth Culture

The yield of FB_1 was more than 100 times greater than that found by Alberts *et al.* (1994). This high yield of is probably due to the *Fusarium* strain and/or the method of extraction.

3.4.3 Yield of FB_1 in Patty Culture

The yield of FB_1 from maize patty culture was a hundred- fold greater than that produced by *F. moniliforme* PPRI 1059 that was grown on unmodified maize kernels (Nelson *et al.*, 1993; Alberts *et al.*, 1993A) and 10 fold greater than that produced after 13 weeks by the method by Alberts *et al.* (1990). The total amount of FB_1 produced in 420 g (14 plates) of maize patty culture was 137 g giving a yield of 3.26×10^{8} ng/g. Thus it can be concluded that the maize patty culture method for the production of FB_1 is better than the broth culture method.

CHAPTER 4

4.0 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF SERUM OF PRE-ECLAMPTIC/ ECLAMPTIC WOMEN FOR THE PRESENCE OF FUMONISIN B_1

4.1 Introduction

Pre-eclampsia is a disease involving hypertension during pregnancy. It is associated with proteinuria and oedema usually occurring after the 20 th week of gestation (Moodley, 1991). Eclampsia is the severest form of pre-eclampsia resulting in convulsions. Pre-eclamptic hypertension has been found to complicate approximately 7 % of all pregnancies worldwide (Moodley, 1991).

Approximately eighteen percent of all admissions to the King Edward VIII Hospital's obstetric unit have some degree of hypertension (140/90 mmHg) (Moodley, 1991; Maharaj and Moodley, 1994).

Pre-eclampsia is a multi-system disorder which can affect the maternal liver, kidneys, cardiovascular, central nervous and clotting systems as well as can cause impaired placental function (Moodley,1991). Currently the aetiology of pre-eclampsia is unknown, although various stresses on the patient, both physical and metabolic, have been hypothesised as contributing to the condition.

One possible source of metabolic stresses is related to diet, which can include an imbalance of nutrients and vitamins and the possible presence of toxic contaminants. Maize, the staple diet of most South African Black women, is often contaminated with mould such as *Fusarium moniliforme,* a mycotoxin producer. This mould abundantly produces one of six known fumonisin toxins, namely, fumonisin B_1 (FB_1). Global levels of FB_1 in maize, range from 30 to 334 000 ug/kg (Dutton, 1996). Animal models have shown FB_1 to be hepatotoxic and hepatocarcinogenic in rats (Gelderblom *et al.*, 1995), to cause leukoencephalomalacia (ELEM or 'hole in the head 'disease) in horses (Wilkins *et al.*, 1994; Thiel *et al.*, 1991) and pulmonary oedema in pigs (Tibor, 1995). Statistical analysis has associated FB_1 in home-grown maize among the rural population of the Transkei, South Africa, with oesophageal cancer (Sydenham *et al.*, 1990).

Fumonisin B_1 is the diester of propane -1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydoxicosane,in which the the C_{14} and C_{15} hydroxyl groups are esterified with one of the terminal carboxy groups of the tricarboxylic acid (Dutton, 1996). The structure of the backbone of the fumonisin molecule was found to be similar to the important phospholipid, sphingosine (Fig.1) (Wang *et al.*, 1992). Animal cell culture experiments have shown that FB_1 are potent inhibitors of sphingolipid biosynthesis (Norred *et al.*, 1997). Fumonisin B_1 was found to cause an accumulation of sphinganine and a paralleled increase in DNA synthesis in Swiss 3T3 fibroblasts. Fumonisin B_1 was also found to inhibit protein synthesis (Merrill *et al.*, 1996) and inhibit the secretion of ceramide into very low density lipoprotein (Merrill *et al.*, 1995).

Common symptoms of FB_1 intoxication in animals appear to be oedema (Harrison *et al.*,

1990), lesions (Kellerman *et al.*, 1990), haemorrhages (Bucci *et al.*, 1996) and other cellular changes in the liver (Norred et al., 1997; Voss *et al.*, 1998) and kidney(Bucci *et al.*, 1998). The foetus in pregnant rats were also affected causing low litter weights and fetal bone development (Ferguson *et al.*, 1997). Pregnant mice fed with fumonisins showed lowered body weights, increased morbidity and mortality in a dose response way (Gross *et al.*, 1994) . Liver damage and increased foetal deaths were also found (Voss *et al.*, 1998).

In vivo vervet monkey experiments indicate a lower level of excretion of FB_1 and its partly hydrolysed derivatives after intra-peritoneal (ip) injection than in the case of rats (almost 90%) (Shephard *et al.*, 1992A) . At least a quarter of the toxic material fed to primates could not be accounted for. Thus there is the possibility that the toxin may be absorbed by the blood in varying proportions in different species of animals, including man. The time-delay of excretion of toxin may also vary.

There is a high incidence of both consumption of FB_1 contaminated maize by Black women, and pre-eclampsia, in Kwa-Zulu Natal. Thus this study sought to hypothesize that an association exists between the incidence of FB_1 serum levels and pre-eclampsia. A double-blind study was carried out involving normotensive, pre-eclamptic and eclamptic patients of the King Edward V111 Hospital. Serum was analysed by fluorescence HPLC (Shephard *et al.*, 1992A).

4.2 Method

4.2.1 Study population and sample collection

This study was conducted at the King Edward V111 Hospital, Durban, South Africa following institutional ethical approval. The sample which was provided blind comprised normal pregnant women (16), pre-eclamptic (PEC) women (17) and eclamptic (EC) women (18) attending the obstetric clinic. Informed consent was obtained from the patients. The blood samples were collected intravenously and centrifuged with EDTA resulting in the separation of plasma from the serum supernatant. The sera were stored in cryovials at -20 ^{0}C .

4.2.2 Extraction and clean up of serum

A variety of extraction and clean-up methods for mycotoxins have been employed by several authors. Since mycotoxins occur in a wide variety of commodities and products, the extraction from a sample depends on the physicochemical properties of the sample as well as the toxin. In general, the sample is subjected to high- speed blending, mechanical shaking or centrifugation in the presence of the extraction solvent system. The slurry is then filtered and is ready for subsequent purification procedures. The most effeicient solvents for extracting mycotoxins are the relatively polar solvents such as methanol, acetone, acetonitrile, ethyl acetate, and chloroform. Fumonisins extraction is characteristically carried out in methanol/water (3:1) solvent mixtures. The water wets the substrate and increases penetration of the solvent mixture into the hydrophilic material.

The aqueous phase can include acid to break interaction between the toxin and proteins for example. Inorganic salts (eg. NaCl) may be added to minimize the formation of emulsions during the extraction.

Other clean-up methods include column chromatography, liquid-liquid extraction and commercially available solid-phase extraction (SPE) and chromatography cartridges. The final step prior to analysis of the sample involves concentration of the cleaned-up extract. This is performed using a rotary evaporator operating under reduced pressure or a steam bath under a stream of nitrogen for concentration of the sample.

Serum (1 ml) was de-proteinised by centrifuging with 7.5 ml methanol at 1 200 *g* for ten minutes at 20 ^{0}C. The supernatant was collected and applied to a solid- phase extraction cartridge, Bond-elut strong anion exchange (SAX) cartridge (Varian) at a flow rate of 1 ml/minute. The cartridge was pre-conditioned with 5 ml of methanol (HPLC grade, Merck) and 5 ml methanol/ water v/v (3:1) (Fig. 19) . The cartridge was washed with 5 ml of methanol/ water v/v (3:1) and 5 ml methanol. The sample was eluted with 10 ml of 5 % acetic acid (Analar grade, Merck) in methanol at a flow rate of 1,0 to 1,5 ml / minute. The eluate was dried under nitrogen at 60 ^{0}C.

4.2.3 Derivatisation and HPLC analysis

Standard and sample residues were derivatised using the o-phythaldialdehyde (OPA) reagent (Shephard *et al.*, 1994). A 50 μg/ml FB_1 standard solution in acetonitrile/water (1:1) was prepared and 50 μl was transferred to an autosampler vial. The purified sample

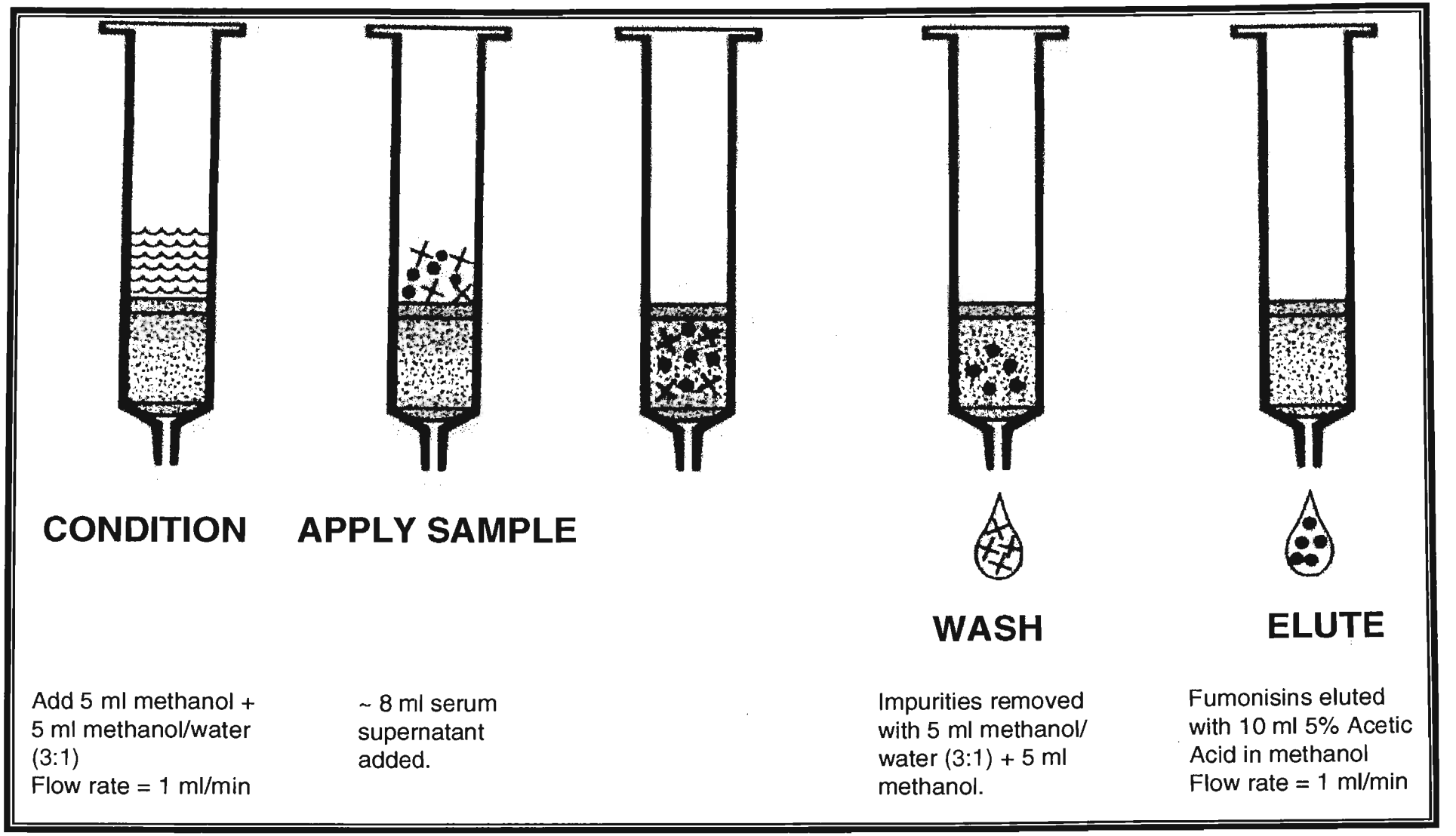


Fig. 19 Solid-phase extraction (Bond-elut) of fumonisins from serum

residue was redissolved in 200 μl of acetonitrile/ water v/v (1:1) and 50 μl of this was added to a vial. Both the standard and the samples were each derivatised by adding 450 μl of the OPA reagent. The solutions were mixed and subjected to immediate HPLC analysis (20 ul) on the Lichrosorb 5 μm C 8 reverse phase column (25 cm x 4 mm) (Merck). Peaks were detected using a Lachrom variable fluorescent detector (Merck) set at 335 nm (excitation) and 440 nm (emission).The mobile phase used was HPLC grade (Merck) methanol / 0.1 M sodium dihydrogen phosphate (68+32), pH of 3,3 held at a flow rate of 1,2 ml/minute. Each serum sample was analysed in duplicate. Quantification of FB_1 content of serum sample was calculated from the chromatographic peak areas using the formula described by the PROMEC group (Thiel *et al.*, 1993) (see section 3.2.2.7).

4.2.4 Recovery by Bond-elut SAX columns

Recovery studies were done where 1 ml of serum samples (not from Black women) , shown to be free of FB_1, were spiked with 50 μg/ml FB_1 standard solution (2 to 8 μl) and analysed by HPLC as above.

4.2.5 Verification of FB_1 by immunoaffinity columns

Immunoaffinity chromatography is a powerful and specific use for the separation and / or purification of biological compounds. The desired material binds specifically and reversibly to a ligand which has been fixed to an inert carrier. Ligands range from small molecules such as substrates for enzymes or mycotoxins to large molecules such as protein hormones. The introduction of a binding site with a ligand is determined by the overall size

and shape of the ligand as well as the number and distribution of complementary surfaces. These surfaces may involve a combination of charged and hydrophobic moieties and exhibit other short range molecular interactions such as hydrogen bonds and Vander Waals forces (Betina, 1993).

The method was similar to that of Ware *et al.* (1994). Selected serum samples (1 ml) were applied to immuno-affinity columns, the Fumonitests (Vicam) after spiking with fumonisin B_1 standard (Sigma). Eclamptic sera were also cleaned with the Fumonitest columns. After de-proteinisation, 5 ml of serum supernatant was passed through the column at a flow rate of 1 ml/min. Five ml of diluting solution (2.5 % NaCl, 0.5 % Na_2CO_3, 0.01 % Tween-20 in water) was passed through the column at a flow rate of 2-4 ml/min.. The column was washed with 5 ml of water. The absorbed FB_1 was eluted with 1 ml methanol (HPLC grade, Merck NT). The eluate was dried under nitrogen and analysed by HPLC as previously described.

4.2.6 Verification of the presence of FB_1 in serum by Mass spectroscopy

The Finnigan LCQ mass spectrometer (MS) was operated in the electrospray ionization (ESI) mode with a capillary temperature of 220 ^{0}C and the sheath gas was nitrogen at 80 ml/min. Fumonisin B_1 was detected in the positive ion mode using 1 % acetic acid in water at a flow rate of 3 μl/min. Qualitative analysis was carried out using the external standard method.

Serum samples were analysed for the presence or absence of FB_1. The serum samples were

treated with Bond-elut SAX as before. The samples were reconstituted in acetonitrile/water v/v (1: 1) before injection.

4.2.7 Statistical tests

Non-parametric tests were carried out at the Department of Biostatistics at the University of Natal in Durban, on the serum results and the clinical data of the women, owing to an absence of a normal distribution in the data.

4.3 Results

Fifty- one pregnant women comprised the study, and upon completion of the HPLC analysis, the study was unblinded. Three sample groups were identified according to their level of blood pressure. The clinical characteristics of the women are summarised in Table 5. No significant difference was found between the normal (29.8 ± 1.5; range 18-40 years) and pre-eclamptic (27.5 ± 1.9; range 18-45 years) groups with regard to age. However, a significant difference was noted between the pre-eclamptic and the eclamptic groups (19.8 ± 1.5; range 13-38 years). Parity and gravidity increased with increasing hypertension. Highly significant differences were noted in parity and gravidity between the normal and eclamptic groups (P = 0.001 and P = 0.000 4 respectively). Gestational age was similar in all groups.

Chromatograms indicate that the FB_1 standard (20 μg/ml) peak occurred at a retention time (t_R) of approximately 7.5 minutes (Fig.20 a). The FB_1 peak was absent in most of the

normal sera (Fig. 20 b). In the eclamptic sera 3 peaks were found to represent fumonisin, namely FB_1

Table 5. Obstetric and epidemiological characteristics of the three groups of women tested. Values are given as mean (SE).

	Normal	Pre-eclamptic	Eclamptic	P value		
Variable	**n=16**	**n=17**	**n=18**	**N/PE**	**N/E**	**PE/E**
Age (yrs.)	29.8(1.5)	27.5(1.9)	19.8(1.5)	0.3	0.0007	0.08
Parity	2.6(0.4)	1.4(0.4)	0.7(0.4)	0.05	0.001	0.1
Gravidity	3.8(0.4)	2.4(0.3)	1.7(0.4)	0.01	0.0004	0.1
Gestational age (years)	35.1(1.4)	33.8(1.9)	33.6(1.0)	0.6	0.15	0.3
DBP (mmHg)	80.0(3.0)	98.7(2.3)	115.6(3.3)			
SBP (mmHg)	113.2(1.7)	148.6(2.7)	183.2(8.1)			
Proteinuria	0.2(0.2)	0.6(0.3)	2.3(0.3)			

N = Normal, PE = Pre-eclamptic and E = Eclamptic.

DBP = diastolic blood pressure, SBP = systolic blood pressure.

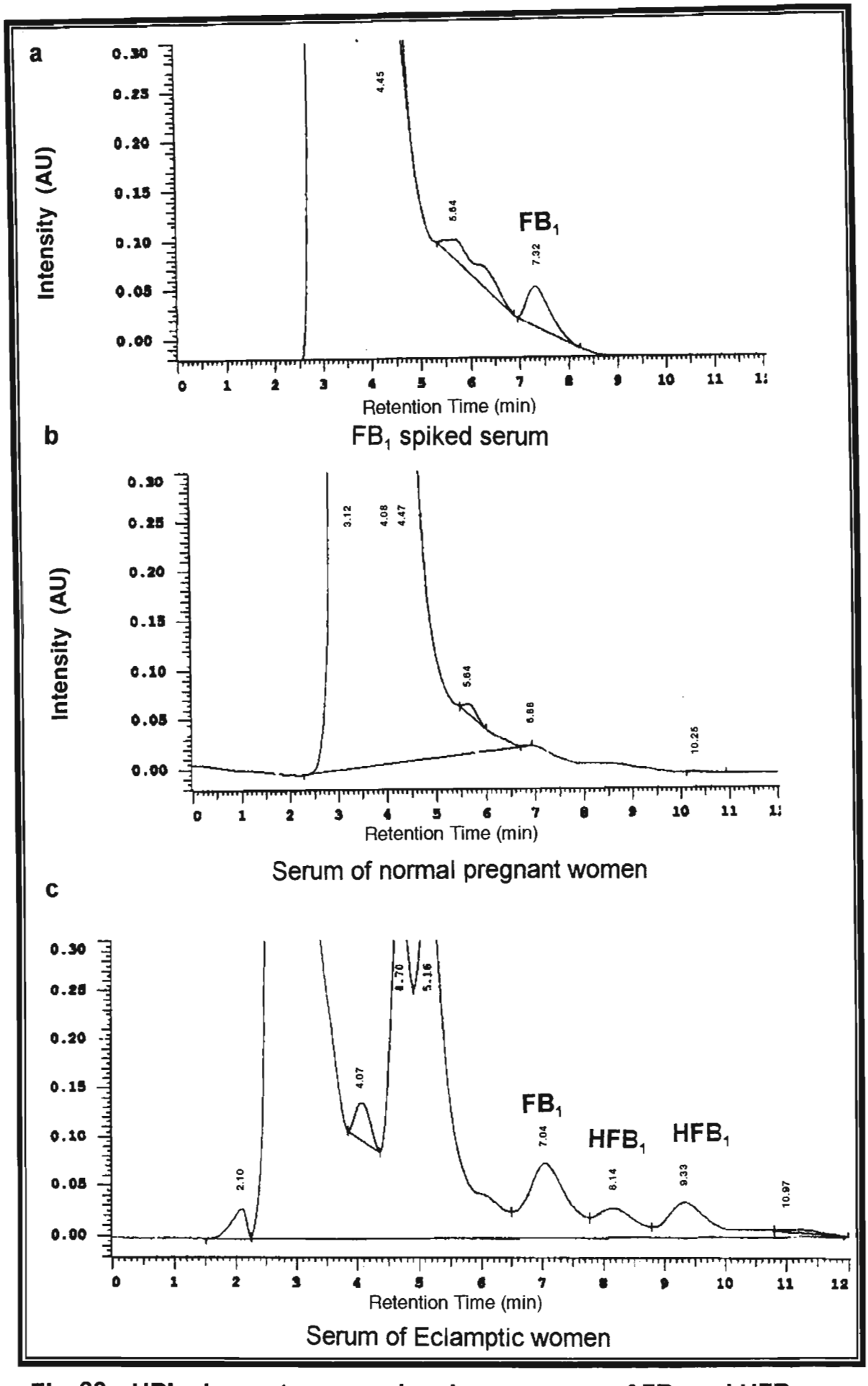


Fig. 20 HPL chromatograms showing presence of FB_1 and HFB_1. [OPA derivatisation using methanol / 0.1 M sodium dihydrogen phosphate (68 + 32) pH 3.3, C8 Lichrospher (5 μm),flow rate of 1.2 ml/min.]

(t_R = 7.04 minutes) and its 2 hydrolysed products (HFB_1), (t_R = 8.14 and 9.33 minutes, Fig. 20 c). The areas under the peaks were used to calculate the FB_1 concentration in sera (Table 6). An average recovery of 95 % was obtained for spiked sera over the range 0.1 to 0.4 μg/ml. The concentration of FB_1 was corrected for recovery (Table 6). The spiked sera passed through the Fumonitest columns gave peaks between 7 and 10 minutes. The calculated concentration of FB_1 was almost equivalent to that obtained when sera was cleaned with SAX columns (Table 7). Unspiked eclamptic sera that were cleaned with Fumonitest columns also produced FB_1 and HFB_1 peaks in the region 7-10 minutes .

Electrospray MS was an ideal technique to detect FB_1. Fumonisin B_1 tended to be ionic and produced abundant signals in the positive ion mode. In the positive mode, the protonated molecule (m/z 722 for FB_1) is the base peak in the mass spectrum (Fig.21). Analysis of serum samples are indicated in Table 8. The two maternal sera tested indicated the presence of FB_1 verifying the presence of FB_1 in pre-eclamptic women (Fig. 22).

Statistical tests indicate a significant difference between the normal (0.32 ± 0.08 μg/ml) and eclamptic (2.85 ± 0.80 μg/ml) groups and the pre-eclamptic (0.45 ± 0.17 μg/ml) and the eclamptic groups with regard to mean FB_1 concentration (Fig.23).

Table 6. Concentration of FB_1 in the three groups of sera tested.

	NORMAL (N = 16)	PRE-ECLAMPSIA (N = 17)	ECLAMPSIA (N = 18)
Concentration of FB_1 in serum samples (μg/ml)	0.03 0.55 0.6 0.06 0.09 0.68 0.65 0.86 0 0.06 0 0.63 0 0.01 0.31 0.37	0.3 0.09 0 0.63 2.19 0 2.23 0.11 0.11 0.19 0.13 0.07 0.5 0.09 0.08 0.27 0.2	3.41 4.58 3.17 0.45 0.6 1.2 1.4 3.8 13.29 7.15 5.35 0.96 1.22 0.51 0.16 0.72 0.62 0.07
Mean Conc. of FB_1 after SAX (μg/ml)	0.31	0.42	2.7
Mean Conc. +/- Std error of FB_1 corected for 95 % recovery (μg/ml)	0.32 +/- 0.08	0.45 +/- 0.17	2.85 +/- 0.8

Table 7. Comparison of clean-up procedures of FB_1 from sera.

Conc. of FB_1 spiked in sera (μg/ml)	SAX chromatography	Affinity chromatography
1.0	0.942	0.968
0.5	0.463	0.478
0.1	0.094	0.096

Table 8. Clinical data of serum samples tested on Mass Spectroscopy

SAMPLE	1	2	3	4
Source	placenta	placenta	maternal	maternal
Age (years)	24	23	24	19
Blood pressure	130/80	130/60	140/90	197/108
Parity / Gravidity	P2 G3	P0 G1	P0 G1	P0 G1
Pregnancy stage (weeks)	38	term	(C-section)	34
Fumonisin B_1	NO	NO	YES	YES

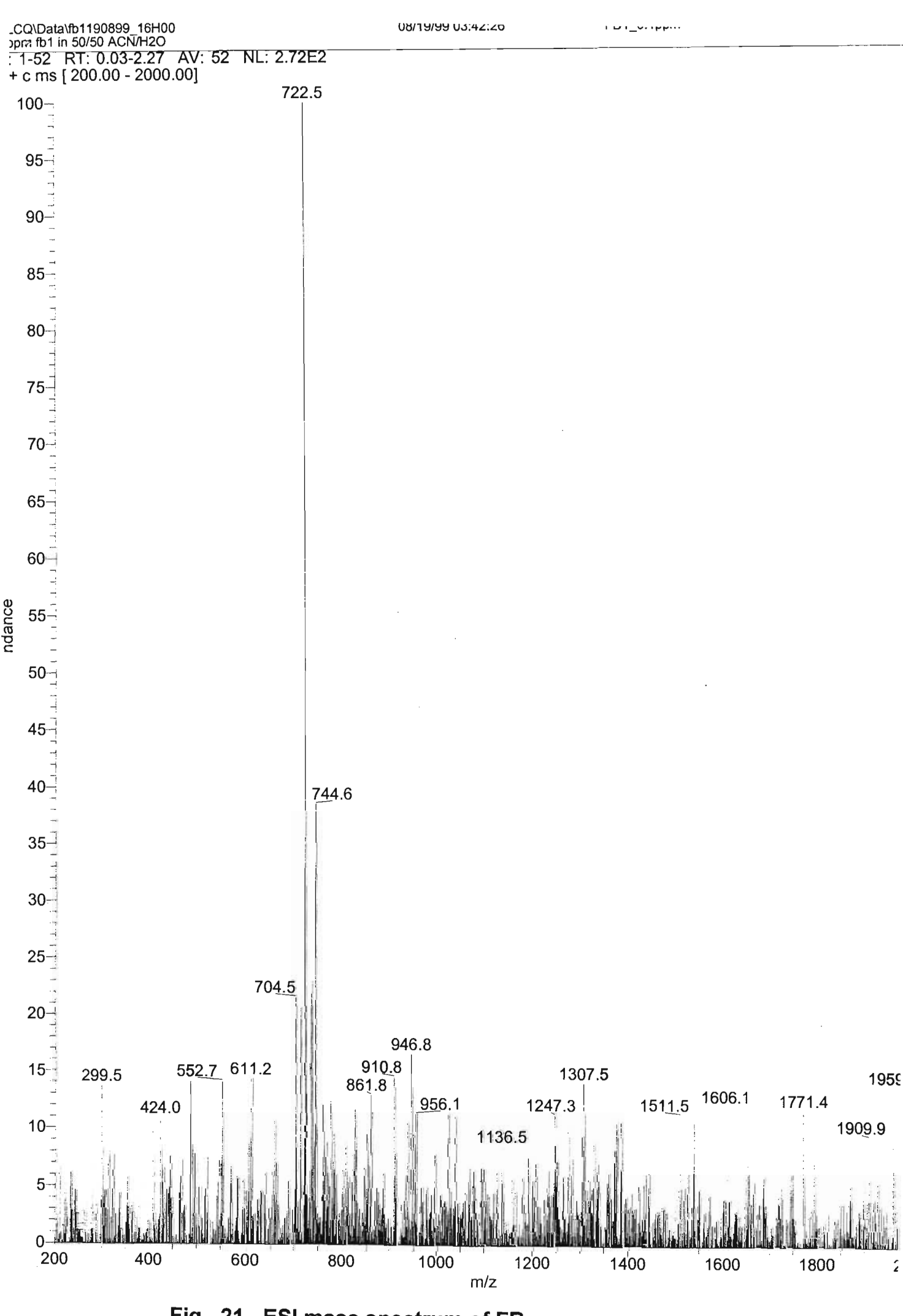


Fig. 21 ESI mass spectrum of FB_1.
(Note the base peak of FB_1 at m/z 722)

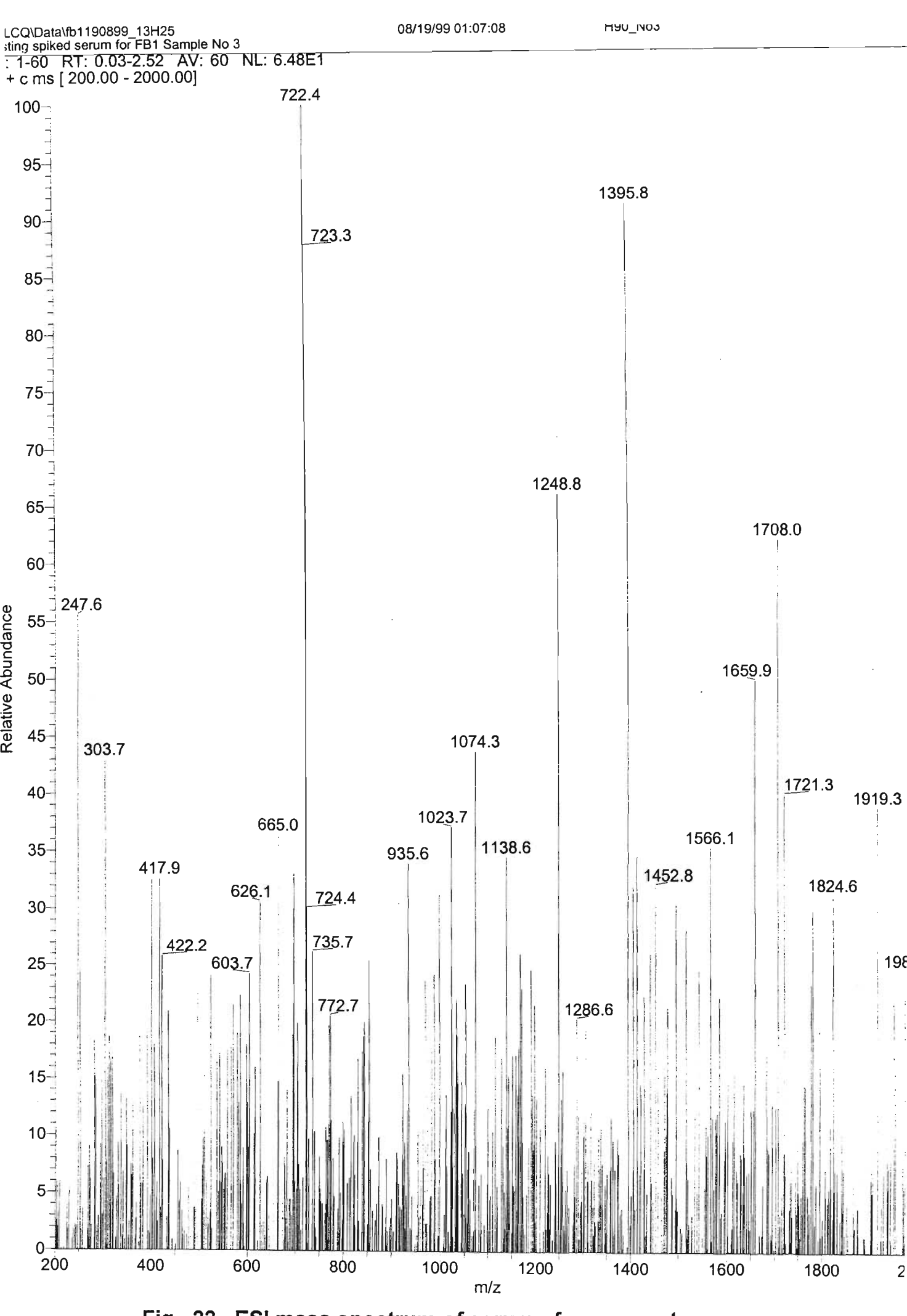


Fig. 22 ESI mass spectrum of serum of a pregnant woman. (Note the base peak of FB_1 at m/z 722)

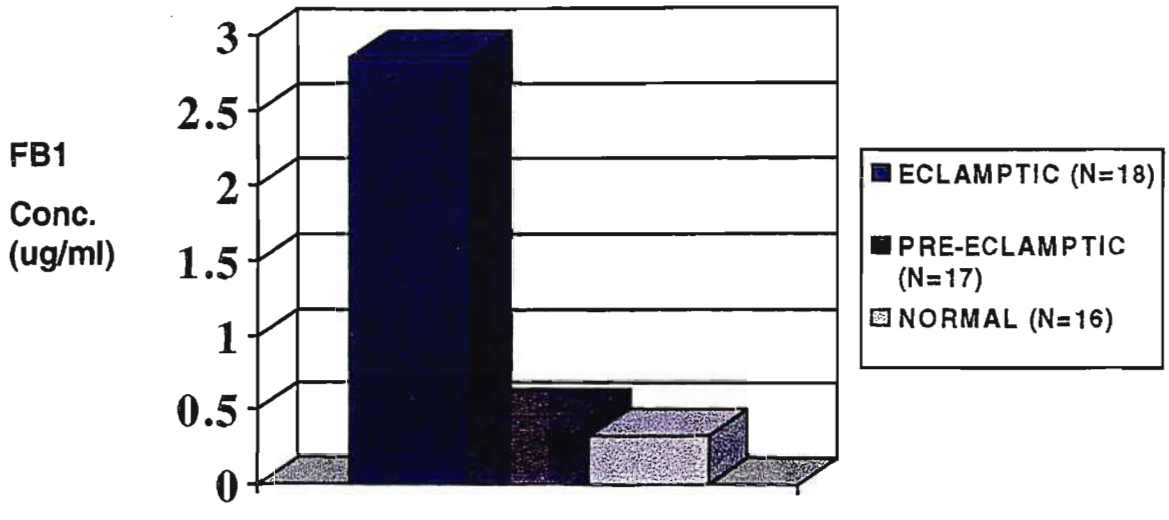


Fig. 23 Fumonisin B_1 concentration in sera of pregnant woman

4.4 Discussion

The results from this study are not only remarkable but unexpected in that animal studies have shown the absorption of fumonisins from the diet to be very low (1.0 %) (Shephard *et al.*, 1994A; Norred *et al.*,1996). Blood levels return to near zero after 48 hours from administration in the rat. This trend seems to be similar in other species investigated, e.g., in the monkey (Shephard *et al.*, 1994B) where fumonisin is rapidly excreted in the faeces together with unabsorbed material. If humans have a comparable absorption of FB_1 to the rat, then the levels in the food (assumed to be principally maize) that the patient has consumed would have to be μg/g. Furthermore this does not allow for time between ingestion of FB_1 and sampling of the blood which was done in the hospital. Analysis of rural and animal feed maize from Kwa-Zulu Natal does indicate the ubiquitous occurrence of FB_1 which is in over 50 % of the samples. The highest concentration measured was 120 μg/g which compares with that found by the PROMEC group (Sydenham *et al.*, 1991) in mouldy maize for beer brewing in the Transkei, South Africa in 1989. Continual ingestion of maize with levels of FB_1 of this order, coupled with malnourishment and vitaminosis could explain these results.

From the analytical point of view, the appearance of peaks in the chromatograms of serum extracts, subjected to the assay method , with the t_R of 7.04 minutes (Fig. 20 c) for FB_1 is not conclusive in that they cannot be assigned with absolute certainty as the toxin. Support of the identity of the peaks as being FB_1 was obtained when unspiked eclamptic serum samples showed that the FB_1 peaks were coincident with the peak t_R of approximately 7 minutes. As the immunoaffinity columns are specific for fumonisins, this result serves to

confirm the presence of FB_1 in the serum of the women investigated. The eluted FB_1 fraction from these gave comparable results to those from SAX columns (Table 7). Recovery experiments using spiked samples showed that the extraction of FB_1 from serum is good even at low concentrations. Some of the serum extacts were also subjected to an ELISA assay being developed in this laboratory (P.Biden). Although the ELISA method is not at a stage where quantitation of FB_1 in serum is precise, the results did broadly agree with those derived by HPLC thereby confirming the presence of FB_1 in eclamptic sera . The presence of FB_1 in sera of pregnant women was also confirmed by ESI MS (Fig. 21 and 22). It also happens that the two serum samples testing positive for FB1 on MS are of the lower age group (19-24 years) and also have a very high blood pressure (Table 8). This agrees with the results obtained with the HPLC analysis of the 51 sera of pregnant women tested.

As the results show a statistical difference between normal, pre-eclamptic and eclamptic sera, it is tempting to speculate that FB_1 has some role to play in the aetiology of eclampsia. The finding of higher levels of the toxin in women with eclampsia, in itself, is not proof of its aetiological effect . Other possibilities exist, not least that they are purely coincidental, e.g. a poor diet is the contributory factor and FB_1 is a marker of that. A major difficulty is to distinguish between cause and effect. Thus the results might be showing that women with pre-disposition to eclampsia poorly excrete the toxin and it accumulates and circulates in the blood system. Thus it is possible that high levels of FB_1 is due to other organ damage, e.g. the kidney and liver, as is suggested by patients records. Although 90% of fumonisin intake by animals is normally excreted (Shephard *et al.*,1994B) pre-eclamptic women, who often show renal and liver dysfunction (Randeree,1995), may have difficulty

with the ready excretion of the toxin. Thus the accumulating of FB_1 in the serum of pre-eclamptic women. The high toxin levels may be secondary to organ damage rather than a factor in the aetiology of pre-eclampsia or eclampsia.

If, however, it is accepted that FB_1 does have something to do with the disease, then explanations for two questions are possible. The first one is, why pre-eclampsia and eclampsia are so common in the eastern seaboard of South Africa? This is the area where maize is the main food staple and the conditions are right for extensive contamination of the crop by *F. moniliforme* and the production of FB_1 and other fumonisins. The second is that a standard treatment for pre-eclampsia is the administration of magnesium sulphate and calcium (van den Elzen *et al.,* 1996). Fumonisin is known to be polycationic and binds calcium and sodium, which in fact contributes to its poor uptake in the GIT (Shephard *et al.,* 1994). It is likely that magnesium and calcium ions administered after FB_1 uptake sequester it in the blood, perhaps rendering it more amenable to excretion by the kidney and also preventing it from binding to cells and being taken up by its normal site of action (van den Elzen *et al.,* 1995).

The toxicokinetic data generated by the intravenous injection of FB_1 in vervet monkeys show that the elimination of fumonisin from the plasma can be characterised by an initial distribution phase and a subsequent elimination phase with a mean half- life of 40 minutes. This is approximately twice that in rats. Hydrolysed products of fumonisins were also found in the liver gut and kidney of monkeys in varying proportions (Shepherd *et al.*, 1994B).

Since the patients were outpatients attending the clinic in the morning they were likely to

have consumed an early breakfast of contaminated maize meal. This may explain the high levels of fumonisins in their serum.

On the assumption that FB_1 is a factor in South African eclampsia, there is a need to speculate about its mode of action. Pre-eclampsia is relieved at birth and expulsion of the placenta, thus it may be speculated that the toxin interferes with sphingolipid biosynthesis, or signalling dependent upon sphingolipid in the placental tissue. Fumonisin is known to cause apoptosis in cells (Reddy *et al.*, 1996; Sharma *et al.*, 1997), which reflects interference in these mechanisms. Such activity within placental tissue would lead to pathological disturbances within the patient. In addition to the above, animal fumonisin intoxication and eclampsia share many common symptoms (Espada *et al.*, 1997; 1996; Barker *et al.,* 1995; Fields *et al.,* 1994), such as hepatic damage, cerebral oedema, pulmonary oedema, proteinuria, low serum albumin, foetal malformation and alteration of hemostasis.

4.5 Conclusion

Eclamptics have a higher level of FB_1 in serum than pre-eclamptics and normal pregnant women. This indicates that the presence of FB_1 in pregnant women may be a contributory factor towards eclampsia and perhaps pre-eclampsia. A thorough dietery profile of these patients needs to be done.to confirm the causal effect of fumonisin B_1 in the above diseases. In addition, sera should be analysed for a specific biomarker (sphinganine/ sphingosine ratio) for the possible implication of FB_1 in eclampsia and pre-eclampsia.

APPENDIX 1

LIST OF REAGENTS AND BUFFERS

1. o-phythaldialdehyde (OPA)

2. 5-fluoro-isothiocyanate 1.3 mM

3. Borate buffer 50 mM pH 9,3

4. Phosphate buffer 10 mM pH 7,5

5. 0,01% Tween -20 in water

APPENDIX 2

LIST OF ABBREVIATIONS

1. HPLC : high performance liquid chromatography
2. FB_1 : fumonisin B_1
3. TLC : thin layer chromatography
4. MS : mass spectroscopy
5. IA : immunoassay
6. CE : capillary electrophoresis
7. OPA : o-phythaldiadehyde
8. FITC : 5-fluoro-isothiocyanate
9. SE : standard error
10. SAX : strong anion exchange
11. BMA : butanol / methanol / acetic acid
12. PROMEC : programme on mycotoxins and experimental carcinogenesis

SUMMARY

The serious pathological effects of fumonisins on plants, animals and humans have been established by numerous researchers thus far. The case for human pathology caused by fumonisins has not however been strong, owing to the ethical and practical implications of such studies. This study has found a positive correlation between the amount of FB_1 in serum and the severity of pre-eclampsia. This may have been caused by dietary exposure to *Fusarium* contaminated maize, its various products and many other important foods. This is in keeping with findings by Shephard *et al.* (1994B) who found that although most of FB_1 dosed to primates were excreted within 48 hours, 25 % of the dosage was not accounted for. Perhaps they were absorbed and found their way to various parts of the body such as the liver. In this study significantly high levels of FB_1 were found in sera of eclamptic women. These women present various pathophysiological effects which are very similar to that found in animals intoxicated by fumonisins. HPLC analysis of culture material and sera were highly efficient in quantitating FB_1 at low levels and the OPA derivatisation procedure is recommended. However if a biomarker is required to confirm the pathophysiological effects of fumonisins in humans, then use must be made of HPLC techniques that quantitate sphingosine in biological fluids.

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