DEVELOPMENT AND APPLICATION OF AN ELISA METHOD OF ANALYSIS FOR FUMONISINS

BY

PATRICIA MAY BIDEN

B.Sc. (Hons.), University of the Witwatersrand

Submitted in partial fulfillment of the requirements for the degree of Masters in Medical Science in the Department of Physiology, Faculty of Medicine, University of Natal, Durban

2000

ABSTRACT

Fumonisins, mycotoxins produced by the fungus, *Fusarium moniliforme*, which grows on maize, are a major worldwide agricultural problem. Consumption of contaminated maize feeds causes a wide variety of toxic effects in animals depending on the species of animal. In humans, high concentrations of fumonisins have been shown to correlate with increased incidence of oesophageal cancer (OC). Most analyses for fumonisins are done using high performance liquid chromatography (HPLC) which requires time-consuming extraction and clean-up prior to preparation of a fluorescent derivative. Enzyme-linked immunosorbent assays (ELISA), which are sensitive and specific, are a viable alternative but commercially available antibodies and kits are extremely expensive.

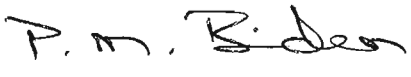
Polyclonal antibodies against fumonisin B_1 (FB_1) were raised in chickens and rabbits; all animals produced antibodies from week 2 onwards, the highest titre was at week 8 from one of the chickens. Cross-reactivities with FB_1 analogues were checked. A sensitive, quantitative competitive indirect ELISA (CI-ELISA) was developed and optimised; range 0.2 to 20 ng/ml (in buffer), detection limit 0.2 ng/ml (in buffer), intra-assay coefficient of variation (CV) was 5.33 % and inter-assay 7.04%.

This method was adapted to analyse human plasma and urine samples. After removal of proteins by boiling, the range of recoveries of FB_1 were 94.7% to112.4% at 4 ng/ml; and 94.6% to 108.7% at 8 ng/ml. Blood and urine samples from patients with OC (40 plasma, 17 urine), controls (21 plasma, 12 urine) and patients with other forms of cancer (20 plasma, 10 urine) were collected from hospitals in the Durban Metropolitan area and analysed for fumonisins. Detectable levels (>0.4 ng/ml) were found in 86.9% of plasma samples and 94.9% of urine samples. Statistical evaluation showed a highly significant difference between plasma results for OC and controls ($p<0.0001$) but no significant difference between the urine results. Comparison of other forms of cancer and controls showed no significant differences for either the plasma or the urine samples. However, there was a highly significant difference between the OC and other forms of cancer results for both plasma ($p<0.005$) and urine ($p<0.05$) samples. Some samples (9 plasma, 8 urine) were checked by HPLC. For plasma samples there was correlation between the ELISA and HPLC methods ($r = 0.656$, $p<0.005$) but not for urine samples.

AUTHOR'S DECLARATION

The experimental work presented in this thesis represents the original work by the author, and has not been submitted in any form to any other university. Where use was made of the work of others, it was duly acknowledged in the text.

The research described in this study was carried out under the supervision of Prof. M. F. Dutton and Mr. A. A. Chuturgoon in the Department of Physiology, University of Natal Medical School, Durban during the period April 1996 to September 2000.



P. M. Biden (Mrs.)

DEDICATION

This thesis is dedicated to my family, Christopher, Katherine and Nicola. Without their love, support and encouragement this study would not have been completed.

The Haunting

You're my ghosts, my shades: I wear you like necklaces
Fused in my flesh and bound in my bones.

You give me direction and guide me through life.
You're my maps, my compass,
My line-of-sight reckoning.

But maps can mislead, and compasses fail,
So you are replaced yet remain:
You expand.

I am a crucible, a deep distillation
Of those who have known me, of all I have known.
I am a puzzle of pieces of you
That I have selected.
(And some are my own)

You who have made me, you haunt me eternally
Filling my choices, enchanting my dreams.
Yet though you have changed me
I am still my own
For though you're my ghosts,
I haunt you too, it seems.

(Katy Biden, 1998)

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Biden, P.M., Chuturgoon, A.A., Coetzer. T.H.T. and Dutton, M.F.

Enzyme immunoassay for fumonisin B_1 using polyclonal antibodies raised in chickens and rabbits.
Submitted to *Toxicon*, July 2000.

Biden, P.M., Chuturgoon, A.A., Dutton, M.F. and Nevines, E.

Fumonisin B_1 in plasma and urine of patients with oesophageal cancer.

In preparation for submission to *Carcinogenesis.*

PRESENTATIONS

Production of polyclonal antibodies against fumonisin in chickens.

Biden, P.M., Chuturgoon, A.A., Coetzer. T.H.T. and Dutton, M.F.

14th National Society of Medical Laboratory Technologists of South Africa (SMLTSA) Congress, Bloemfontein, 3-5 May, 1997.

Production of polyclonal antibodies against fumonisin in chickens and rabbits.

Biden, P.M., Chuturgoon, A.A., Coetzer. T.H.T. and Dutton, M.F.

Joint KwaZulu-Natal Biochemistry and Microbiology symposium, 15-17 October, 1997.

Development of an ELISA method of analysis for fumonisin using a polyclonal antibody raised in chickens.

Biden, P.M., Chuturgoon, A.A.and Dutton, M.F.

SMLTSA, KwaZulu-Natal branch Mini-seminar, 3 October, 1998.

Development of an ELISA method of analysis for fumonisins.

Biden, P.M., Chuturgoon, A.A and Dutton, M.F.

15th National SMLTSA Congress, Port Elizabeth, 27-30 April, 1999.

ACKNOWLEDGEMENTS

My heartfelt thanks to the following people:-

Professor M.F. Dutton and Mr. A.A. Chuturgoon for giving their time and expertise to assist and encourage me with the experimental work, presentation of papers, submission of papers for publication and not least in the preparation and presentation of this manuscript.

Dr. T.H.T. Coetzer for giving freely of her time and knowledge to help me raise antibodies. Without her input there would have been no antibodies and no thesis.

Mrs. E. Nevines for her assistance with the high performance liquid chromatography analyses. Without her expertise the problems encountered would not have been solved.

Mr. R. Parkinson, Quality Control Officer at the Natal Blood Transfusion Service for his expert knowledge on the vagaries of quantitative ELISA's and his encouragement that I was on the right track. My thanks, too, for making their plate reader available to confirm my quantitative method.

Mr. D. Singh, statistician, for his endless patience and ability to understand the statistical needs of a biochemist without confounding me with theory. Thanks, too, for his invaluable quick lesson on how to use Quattro Pro as a statistical tool.

Mr. A. Peterson, for being unfailingly cheerful and willing to help me at a moment's notice with any equipment problems.

Mrs. M. Hurley and Mrs. N. Perumal for their pleasant and efficient manner on the telephone and willingness to take messages.

Mr. S. Naidoo of the Department of Pharmacology for his assistance with using the Model 3550 Plate reader.

Mrs. K. Mudaly for her help with the collection and storage of the blood and urine samples when I was not able to be in the Department.

Mr. P. Chelule, a fellow older student and medical technologist, who always encouraged me to keep on trying and assisted me with the extraction for chromatography.

My fellow students who despite the disparity in our ages made me feel welcome and part of the group.

The staff in the Department who gave me positive moral support.

My thanks to the Foundation for Research and Development for their financial support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	antibody
Ab_1	primary or first antibody
Ab_2	second antibody
Ab_2^E	second antibody with enzyme label
Ag	antigen
Ag^E	antigen with enzyme label
ABTS	2, 2′-azino-bis (3-ethyl)benzthiazoline-6-sulphonic acid
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP_1	aminopentol $_1$
AST	aspartate aminotransferase
B	bound fraction in immunoassay
Bo	bound fraction for zero point on standard curve
BSA	bovine serum albumin
Ca	calcium
CD-ELISA	competitive direct enzyme-linked immunosorbent assay
CI-ELISA	competitive indirect enzyme-linked immunosorbent assay
CK	creatine kinase
CoA	co-enzyme A
CT	cholera toxin
CV	coefficient of variation
CZE	capillary zone electrophoresis
DBD-F	4-(*N,N*-dimethyl-aminosulphonyl)-7-fluoro-2,1,3 -benzoxadiazole
E	enzyme
ECG	electrocardiograph
EDTA	ethylenediaminetetraacetic acid
ELEM	equine leukoencephalomalacia
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
ESI-MS	electrospray ionisation – mass spectrometry
FA_1	fumonisin A_1
FA_2	fumonisin A_2

FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FB_4	fumonisin B_4
FB_1-BSA	fumonisin B_1- bovine serum albumin conjugate
FB_1-HRPO	fumonisin B_1- horseradish peroxidase conjugate
FB_1-KLH	fumonisin B_1- keyhole limpet hemocyanin conjugate
FB_1-OVA	fumonisin B_1- ovalbumin conjugate
FC_1	fumonisin C_1
FITC	fluorescein isothiocyanate
FMOC	9-fluorenylmethyl chloroformate
F. moniliforme	*Fusarium moniliforme*
FP_1	fumonisin P_1
FP_2	fumonisin P_2
FP_3	fumonisin P_3
GC-MS	gas chromatography – mass spectrometry
GGT	γ-glutamyltranspeptidase
HC	high control
HFB_1	hydrolysed fumonisin B_1
HFB_2	hydrolysed fumonisin B_2
HPLC	high performance liquid chromatography
HPLC-ESI-MS-MS	high performance liquid chromatography electrospray-ionisation , tandem mass spectrometry
HPTLC	high performance thin layer chromatography
HRPO	horseradish peroxidase
HSA	human serum albumin
IC_{50}	concentration at 50% inhibition
ID_{50}	dosage at 50% inhibition
IgG	immunoglobulin G
IgY	immunoglobulin Y
IUPAC	International Union of Pure and Applied Chemistry
KLH	keyhole limpet hemocyanin
LC	low control
LC-ESI-MS	liquid chromatography-electrospray ionisation-mass spectrometry

LD / LDH	lactate dehydrogenase
LDL	low density lipoprotein
mAb	monoclonal antibodies
Mg	magnesium
MRC	Medical Research Council
MS	mass spectrometry
MWCO	molecular weight cutoff
NADP	nicotinamide adenine dinucleotide phosphate
NAG	*N*-acetyl-β-D-glucosaminidase
NBD-F	4-fluoro-7-nitrobenzofurazan
NBTS	Natal Blood Transfusion Service
nd	no data
NDA - KCN	naphthalene-2,3-dicarboxaldehyde- potassium cyanide
NK	natural killer
NOEL	no observed effect level
OC	oesophageal cancer
OPA	o-phthaldialdehyde
OPD	o-phenylenediamine dihydrochloride
OVA	ovalbumin
pAb	polyclonal antibodies
PBS	phosphate buffered saline
PEG	polyethylene glycol
PGST	placental glutathione S-transferase
PH_1	partially hydrolysed fumonisin B_1
ppb	parts per billion (i.e. μg/kg or ng/g or ng/ml)
PKC	protein kinase C
PPE	porcine pulmonary oedema
ppm	parts per million (i.e. mg/kg or μg/g or μg/ml)
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
RIA	radioimmunoassay
RSD	relative standard deviation (same as coefficient of variation)
RT	room temperature
S	sorbent
Sa	sphinganine

SAX	strong anion exchange
SD	standard deviation
SE	standard error
SFE	supercritical fluid extraction
SMLTSA	Society of Medical Laboratory Technologists of South Africa
So	sphingosine
SPE	serum protein electrophoresis
TCA	tricarballylic acid
tCO_2	total carbon dioxide
TLC	thin layer chromatography
UV	ultraviolet
χ^2	chi square statistic

CHAPTER 1

INTRODUCTION

1.1 Fumonisin Mycotoxicoses

Mycotoxins are secondary metabolites produced by filamentous fungi which are harmful to animals and humans. Normally, the production of mycotoxins is restricted to specific species (Dutton, 1996) and may occur in the field but more commonly during poor storage of commodities (Sydenham *et al.*, 1990B). The ingestion of mycotoxins, usually through food and feed, causes mycotoxicoses which manifest with a wide variety of disease symptoms (Nelson *et al.*, 1993). Fumonisins are mycotoxins produced by the fungus, *Fusarium moniliforme* Sheldon [recently renamed *Fusarium verticillioides* (Kriek *et al.*, 1981; Marin *et al.*, 1999; Blackwell *et al.*, 1999)] which is a common contaminant of maize and to a lesser extent other cereals such as wheat and barley.
[In overseas publications, the term corn is used for maize (*Zea mays)*].

Fumonisins are a major worldwide agricultural problem as the consumption of contaminated feeds can cause a wide variety of signs in animals depending on the species of animal, concentration of mycotoxin present and the duration of exposure to the contaminated feed. Fumonsins have been associated with various syndromes and mycotoxicoses including equine leukoencephalomalacia (ELEM) (Kellerman *et al.*, 1990; Marasas *et al.*, 1988A); porcine pulmonary oedema (PPE) (Haschek *et al.*, 1992; Riley *et al.*, 1993) and hepatocarcinoma and nephrotoxicity in rats (Gelderblom *et al.*, 1991; Voss *et al.*, 1993, 1995A). In humans, high concentrations of fumonisins have been shown to correlate with increased incidence of oesophageal cancer (OC) in the Transkei (Marasas *et al.*, 1988B; Sydenham *et al.*, 1990A, 1990B) and China (Chu & Li, 1994; Yoshizawa *et al.*, 1994).

1.2 Chemistry

Chemically the fumonisins are a group of related, polar molecules based on a long hydroxylated hydrocarbon chain (pentahydroxyicosane) with attached methyl, amino and carboxyl groups (propane-1,2,3-tricarboxylic acid moieties esterified to two hydroxyl groups). The B series have a free amino group; the A series are *N*-acetyl derivatives.

The configuration of the fumonisins is a cage-like structure suggesting they might act as chelators and may interact with divalent cations (Sauviat *et al.*, 1991; Beier *et al.*, 1995; Beier & Stanker, 1997).

The major fumonisin is fumonisin B_1 (FB_1) but fumonisin B_2 (FB_2), fumonisin B_3 (FB_3) and fumonisin B_4 (FB_4) are also produced by *F. moniliforme* (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988, 1992B). Structurally the fumonisins are similar to the long chain bases sphinganine (Sa) and sphingosine (So), components of sphingolipids which play important roles in cell regulation including the control of normal growth (DNA synthesis) and differentiation; as well as roles in the structure and functions of cell membranes (Merrill *et al.*, 1995B, 1996).

1.3 Mechanisms

Using primary rat hepatocytes, it was found that FB_1 is a potent competitive inhibitor of the enzyme, ceramide synthase (sphingosine *N*-acyltransferase) required for formation of complex sphingolipids. It is believed that at a molecular level many of the effects of FB_1 toxicity are due to interference with sphingolipid metabolism and an increase in the amount of free sphinganine (Sa) (Wang *et al.*, 1991). Animals consuming feed contaminated with FB_1 showed an increase in free Sa and the ratio of So to Sa in sera and tissues (liver, lung, kidney). These changes provide an early marker of exposure to fumonisins since they were seen before detectable lesions or elevations of enzymes (Wang *et al.*, 1992; Riley *et al.*, 1993).

Experiments done by dosing animals with unlabelled or radiolabelled FB_1 and FB_2, showed that they are poorly absorbed from the gut, and are rapidly eliminated mainly via the faeces, bile and urine (Shephard *et al.*, 1992A, 1992C, 1994A, 1994B, 1994C).

1.4 Analysis

At present most analyses for fumonisins are done using high performance liquid chromatography (HPLC) which requires time consuming extraction and clean-up prior to formation of a fluorescent derivative (Shephard *et al.*, 1990). Although simpler, thin layer chromatography (TLC) also requires prior extraction, clean-up and derivatisation and is not as sensitive (Rottinghaus *et al.*, 1992).

Shephard *et al.* (1992B, 1994A, 1994B, 1995A, 1995B) succeeded in developing HPLC methods to measure FB_1 and FB_2 in urine, plasma, bile and faeces; Shetty & Bhat (1998) optimised a HPLC method to analyse urine and Chelule *et al.* (2000) analysed human faecal samples for FB_1. Other investigations of the absorption and excretion of fumonisins in animals used the HPLC methods (Shephard *et al.*, 1992A; Smith & Thakur, 1996) or radiolabelled FB_1 and FB_2 (Norred *et al.*, 1993; Prelusky *et al.*, 1994; Shephard *et al.*, 1992C, 1994C; Vudathala *et al.*, 1994).

Immunoassays, which are sensitive and specific, are a viable alternative to chromatography and recent groups have developed assay methods using antibodies to FB_1 and an enzyme-linked immunosorbent assay (ELISA) format. Although antibodies and kits (Veratox ® Fumonisin Kit and Fumonisin Agri-Screen) are now commercially available, they are not widely used because of high cost (Azcona-Olivera *et al.*, 1992A, 1992B; Usleber *et al.*, 1994; Abouzied *et al.*, 1996). Furthermore, methods that have been developed are primarily used for the measurement of fumonisins in maize and other agricultural products (Azcona-Olivera *et al.*, 1992A, 1992B; Pestka *et al.*, 1994; Shelby *et al.*, 1994; Usleber *et al.*, 1994; Ware *et al.*, 1994; Schneider *et al.*, 1995; Abouzied *et al.*, 1996; Sydenham *et al.*, 1996A, 1996B; Thompson & Maragos, 1996; Yeung *et al.*, 1996; Yu & Chu, 1996; Maragos, 1997) or milk (Maragos & Richard, 1994; Hammer *et al.*, 1996) or beer (Scott *et al.*, 1997). There are no recorded ELISA methods suitable for analysis of physiological fluids for fumonisins.

1.5 Health Risks

Maize is a staple food for many people in South and Southern Africa. Contamination of maize with *F. moniliforme* and fumonisins is common. By eating this food humans expose themselves to the risk of acquiring fumonisin-related mycotoxicoses. The health risk associated with exposure to fumonisins is unknown. Safe concentrations of fumonisins for human or animal feed have not been determined (Marasas *et al.*, 1988B; Sydenham *et al.*, 1990B; Rheeder *et al.*, 1992; Thiel *et al.*, 1992; Badria *et al.*, 1996; Doko *et al.*, 1996; Rava *et al.*, 1996).

"Maximum allowable levels of FB_1, FB_2, and FB_3 in maize exported from and imported into Africa and in maize-based human foods and animal feeds should be specified. Important risk assessment factors in determining these tolerance levels include the following: hazard assessment by determining toxic and carcinogenic levels of fumonisins in animal experiments and exposure assessment by determining naturally occurring levels of fumonisins in human foods and animal feeds. A great deal of research remains to be done however, on the risk assessment of the fumonisins for human health in Africa." (Marasas *et al.*, 1993).

1.6 Objectives

The aims of this study were:

1. To raise polyclonal antibodies against FB_1 in chickens and rabbits and check these for titre and cross-reactivity.
2. To use the antibodies to develop and optimise a quantitative, sensitive and economical ELISA to analyse human blood and urine samples.
3. To analyse samples from controls, patients with OC and patients with other types of cancer for FB_1, calcium and magnesium.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Fumonisins are produced by a limited variety of fungi, members of the genus *Fusarium* in the section Liseola; predominantly *F. moniliforme* Sheldon, now called *F. verticilliodes*, and *F. proliferatum* (Kriek *et al.*, 1981, Thiel *et al.*, 1991A; Ross *et al.*, 1990; Marin *et al.*, 1999; Blackwell *et al.*, 1999). Only the A and D mating populations of *F. moniliforme* that share the teleomorph (sexual stage) *Gibberella moniliformis*, produce fumonisins; and different mating populations vary considerably in the amount of fumonisin produced (Leslie,1996). These fungi also produce moniliformin and fusarins (Nelson *et al.*, 1993).

Fusarium moniliforme is an endophyte found distributed over the pericarp of maize kernels and within the kernel with hyphae found in roots, stems, leaves and cobs. Disease signs are found with symptom-inducing strains where hyphae colonise the interior of the kernel including the embryo. Of more concern for animal and human health is the symptomless infection where the fungus may be located in vegetative parts (e.g. root or shoot) (Bacon & Williamson, 1992). Studies on the incidence of seedborne fungi in commercial South African maize showed the predominant fungi were *F. subglutinans* and *F. moniliforme* (Rava *et al.*, 1996).

2.2 OCCURRENCE of FUMONISINS

Sydenham *et al.* (1990A) were the first to conclusively report the natural occurrence of FB_1 in maize (*Zea mays*) by analysing mouldy home-grown maize collected from the Butterworth district in the Transkei. In the1989 and 1990 South African harvests, yellow maize had higher levels of *F. moniliforme* but lower levels of fumonisins than white maize. In the 1993 harvest fumonisins were found in all samples analysed with levels being higher in yellow maize than white (FB_1>FB_2>FB_3) (Rheeder *et al.*, 1995; Rava *et al.*, 1996).

Fumonisin producing strains of *F. moniliforme* have been found in a variety of samples from different countries. Strains were isolated from maize seeds from Mexico (Desjardins *et al.*, 1994) and poultry feed, maize, soybean, pea and wheat samples from Spain (Castellá *et al.*, 1996).

Samples of maize, wheat, barley, sorghum and mixed feed from Italy, Spain, Poland and France had highest levels of FB_1 in isolates from maize (average 1 259 μg/g) followed by wheat (average 769 μg/g) and barley (320 μg/g) (Visconti & Doko, 1994B).

Musser & Plattner (1997) examined 22 cultures of *Fusaria* for total fumonisin composition. The major fumonisins in all cultures were FB_1, FB_2, FB_3, fumonisin A_1 (FA_1) and fumonisin A_2 (FA_2); but seven other fumonisins of the A, P and C series were identified at levels <10% of the FB_1 produced. In contrast, Shetty & Bhat (1997A) tested the ability of different maize varieties to support production of FB_1 by inoculating with *F. moniliforme*. All varieties tested showed good fungal growth with a wide range of concentrations of FB_1 (159 μg/g to 1 824 μg/g of dried culture material) but there was no correlation between fungal growth and the amount of FB_1 produced.

Natural co-occurrence of fumonisins and other toxins has been reported. Analysing mouldy maize samples from the Transkei, Sydenham *et al.* (1990A, 1990B) found high concentrations of $FB_1$and FB_2, but also deoxynivalenol, moniliformin, nivalenol and zearalenone.
In South African maize products for the 1994/1995 season, Rava *et al.* (1996) and Rava (1996), found both fumonisins, deoxynivalenol and nivalenol in maize bran and maize screenings; zearalenone and fumonisins in sifted, special and super maize meal for human consumption; and ochratoxin A and fumonisins in maize germ meal for animal consumption. Highest mean levels of fumonisins were found in maize bran (1 788 μg/kg) and maize screenings (8 878 μg/kg); levels in animal feed products were higher (1 993 μg/kg) than in yellow commercial maize for humans (865 μg/kg).

In China, co-occurrence of fumonisins with type-A trichothecenes (e.g. T-2 toxin) were found in mouldy corn but levels of aflatoxin were low (Chu & Li, 1994). However, Shetty & Bhat (1997B) found both FB_1 and aflatoxin B_1 in normal and rain-affected sorghum, maize and poultry samples in India. In Italy, fusaproliferin and beauvericin were found associated with FB_1 in 22 samples of preharvest maize ears infected with *Fusarium* (Ritieni *et al.*, 1997).

In several countries, various maize and maize-based products for human and animal consumption have been analysed for fumonisins (Tables 2.1, 2.2 and 2.3).

TABLE 2.1 OCCURRENCE OF FUMONISINS IN FOODS/FEEDS IN AFRICA

Country	Type of Sample	Fumonisins (µg/kg)	Reference
South Africa	White maize (1993 maize crop)	FB_1: 30 (0 - 5 637) FB_2: 55 (0 - 1 430) FB_3 : 27 (0 - 400)	Rava *et al.*, 1996
	Yellow maize (1993 maize crop)	FB_1: 677 (0 - 11 773) FB_2: 220 (0 - 5 690) FB_3 : 106 (0 - 1 963)	Rava *et al.*, 1996
	White maize (1994/1995 maize crop)	FB_1: 637 (0 - 12 963) FB_2: 118 (0 - 4 187) FB_3 : 83 (0 - 3 110)	Rava, 1996
	Yellow maize (1994/1995 maize crop)	FB_1: 664 (0 - 5 062) FB_2: 148 (0 - 2 000) FB_3 : 54 (0 - 1 431)	Rava, 1996
	Maize meal	FB_1: 138 (0 - 475) FB_2: 83 (0 - 131)	Sydenham *et al.*, 1991
	Maize-grits	FB_1:125 (0 - 190) FB_2: 85 (0 - 120)	Sydenham *et al.*, 1991
Botswana	Maize meal	FB_1: 35 – 255 FB_2: nd - 85 FB_3 : nd - 40	Doko *et al.*, 1996
	Maize kernels	FB_1: 350 FB_2: 105 FB_3 : 70	Doko *et al.*, 1996
	Sorghum meal	FB_1: 20 FB_2 and FB_3: nd	Doko *et al.*, 1996
Egypt	Corn-based human foodstuffs	FB_1: 2 380 (1 780 - 2 980) FB_2: 595 (470 – 780)	Sydenham *et al.*, 1991
Kenya	Maize kernels	FB_1: 780 FB_2: 275 FB_3 : 130	Doko *et al.*, 1996
Malawi	Maize kernels	FB_1: nd - 115 FB_2: nd - 30 FB_3 : nd	Doko *et al.*, 1996
Mozambique	Maize kernels	FB_1: 240 - 295 FB_2: 75 - 110 FB_3 : 25 - 50	Doko *et al.*, 1996
Tanzania	Maize kernels	FB_1: nd - 165 FB_2: nd - 60 FB_3 : nd	Doko *et al.*, 1996
Uganda	Maize kernels	FB_1: 605 FB_2: 155 FB_3 : 85	Doko *et al.*, 1996
Zambia	Maize meal	FB_1: 740 FB_2: 380 FB_3 : 85	Doko *et al.*, 1996
Zimbabwe	Maize meal	FB_1: 55 - 1 910 FB_2: nd - 620 FB_3 : nd - 205	Doko *et al.*, 1996
	Maize kernels	FB_1: nd - 125 FB_2: nd - 40 FB_3: nd	Doko *et al.*, 1996

TABLE 2.2 OCCURRENCE OF FUMONISINS IN FOODS/FEEDS IN EUROPE and ASIA

Country	Type of Sample	Fumonisins (µg/kg)	Reference
EUROPE			
Italy	Maize	FB_1: up to 5 310 FB_2: 1 480	Doko & Visconti, 1994
	Puffed corn	FB_1: up to 6 100 FB_2: 520	Doko & Visconti, 1994
	Corn-grits/ cornflour /polenta	FB_1: 420 – 3 760 FB_2: 80 - 910	Doko & Visconti, 1994
	Sweetcorn	FB_1: 60 - 790 FB_2: nd	Doko & Visconti, 1994
	Popcorn	FB_1: up to 60 FB_2: up to 20	Doko & Visconti, 1994
	Tortilla chips	FB_1: up to 60 FB_2: up to 10	Doko & Visconti, 1994
	Corn flakes	FB_1: 10	Doko & Visconti, 1994
Sardinia	Infected maize ear kernels	FB_1: up to 250 000	Bottalico *et al.*, 1995
Switzerland	Maize-based food for humans	FB_1: 55 - 790 FB_2: 50 - 160	Pittet *et al.*, 1992
	Poultry feed	FB_1: 235	Pittet *et al.*, 1992
United Kingdom	Corn snacks	FB_1: 11 - 220	Patel *et al.*, 1997
	Breakfast cereals	FB_1: 11 - 194	Patel *et al.*, 1997
	Popping corn & microwaveable popcorn	FB_1: 14 - 784	Patel *et al.*, 1997
	Polenta	FB_1:16 - 2 124	Patel *et al.*, 1997
ASIA			
China	Maize kernel	FB_1: 6 800 (5 300 - 8 400) FB_2: 3 300 (2 300 - 4 300)	Ueno *et al.*, 1993
	Maize flour	FB_1: 100 (60 - 200) FB_2: nd	Ueno *et al.*, 1993
India	Sorghum (rain affected	FB_1: 480 (70 - 8 000)	Shetty & Bhat, 1997B
	Sorghum (normal)	FB_1: 270 (150 - 510)	Shetty & Bhat, 1997B
	Maize (rain affected)	FB_1: 1 170 (40 - 65 000)	Shetty & Bhat, 1997B
	Maize (normal)	FB_1: 620 (10 - 5 000)	Shetty & Bhat, 1997B
	Poultry feed	FB_1: 100 (20 – 260)	Shetty & Bhat, 1997B
Japan	Corn-grits	FB_1: 500 (200 - 2 600) FB_2: 1 000 (300 - 2 800)	Ueno *et al.*, 1993
Korea	Maize kernels	FB_1: 53 - 1 327 FB_2: 69 - 680	Ung-Soo *et al.*, 1994
Nepal	Maize kernel	FB_1: 600 (50 - 4 600) FB_2: 1 600 (100 - 5 500)	Ueno *et al.*, 1993

Of maize-based food samples analysed in the United Kingdom, none were detected in food product samples from commercial maize processing although fumonisins were found in the original maize. Fumonisins were also not found in samples of barley, wheat, soya, oats, rice, corn syrup or oil, corn-on-the-cob, corn-fed chicken or wheat flour tortilla. Yet fumonisins (chiefly FB_1) were detected in 26% (76/291) of retail samples with highest levels being in samples of polenta (16 to 2 124 µg/kg); popping corn and microwaveable popcorn (14 - 784 µg/kg); corn snacks samples (11 - 220 µg/kg); and breakfast cereals (11 - 194 µg/kg) (Patel *et al.*, 1997) (Table 2.2).

In India, FB_1 was found in rain-affected maize (0.04 - 65 mg/kg) and sorghum (0.07 - 8 mg/kg) samples. However, FB_1 was also found in 5/14 poultry feed samples; 26/35 normal maize samples (0.01 – 5 mg/kg); and 2/43 normal sorghum samples (0.15 – 0.51 mg/kg) (Shetty & Bhat, 1997A, 1997B) (Table 2.2).

In samples from the 1988-1991 maize crops from Iowa, Wisconsin & Illinois (USA), Murphy *et al.* (1993) found levels of fumonisins to be about 10 times higher in maize screenings as compared to maize. In Columbia, a higher number of positive FB_1 samples (75%) were found in maize and maize-based products for animal intake than for human consumption (55%). The actual levels of FB_1 were also higher; average 694 µg/kg for animals as opposed to 218 µg/kg for humans. Percentage positive samples and levels of FB_2 were lower (58.3%, average 283 µg/kg and 35%, average 118 µg/kg for animal and human consumption respectively) (Perilla & Diaz, 1998) (Table 2.3).

The first report of FB_3 and hydrolysed fumonisin B_1 (HFB_1) in human foods (in the USA) was by Hopmans & Murphy (1993) who analysed yellow and white maize meal, canned corn, masa and tortilla chips; FB_3 was detected in 10/13 foods and HFB_1 in canned yellow maize, masa and tortilla chips. Fumonisins B_1, B_2 and B_3 were also found in dry dog and cat foods, and rat chow.

TABLE 2.3 OCCURRENCE OF FUMONISINS IN FOODS/FEEDS IN AMERICA

Country	Type of Sample	Fumonisins (µg/kg)	Reference
Georgia, USA	Maize	FB_1: 870	Chamberlain *et al.*, 1993
Iowa, Wisconsin &	Maize (1988)	FB_1: 0 - 14 900 FB_2: 700 FB_3 : 200	Murphy *et al.*, 1993
Illinois	Maize (1989)	FB_1: 37 900 FB_2: 800 FB_3 : 200	Murphy *et al.*, 1993
	Maize (1990)	FB_1: 0 - 19 100 FB_2: 900 FB_3 : 300	Murphy *et al.*, 1993
	Maize (1991)	FB_1: 0 - 15 800 FB_2: 800 FB_3 : 400	Murphy *et al.*, 1993
Columbia	Maize/maize-based products for humans	FB_1: 218 (24 - 2 170) FB_2: 118 (21 - 833)	Perilla & Diaz, 1998
	Maize/maize-based products for animals	FB_1: 694 (32 - 2 964) FB_2: 283 (44 - 987)	Perilla & Diaz, 1998
	Hominy feed	FB_1: 86 - 2 964 FB_2: 57 - 987	Perilla & Diaz, 1998
Costa Rica Brunca Huetar Norte Huetar Atlantica	Maize	FB_1: 2 500 3 580 1 810	Viquez *et al.*, 1996
Argentina	Maize	FB_1: 1 110 - 6 695 FB_2: 325 - 2 680 FB_3: 130 - 855	Sydenham *et al.*, 1993
Peru	Maize meal (human)	FB_1: 660 (0 - 660) FB_2: 135 (0 - 135)	Sydenham *et al.*, 1991
Canada	Maize meal (human)	FB_1: 50 (0 - 50) FB_2: 0	Sydenham *et al.*, 1991
USA	Maize meal (human)	FB_1: 1 048 (0 - 2 790) FB_2: 298 (0 - 920)	Sydenham *et al.*, 1991
	Corn-grits (human)	FB_1: 601 (105 - 2 545) FB_2: 375 (0 - 1 065)	Sydenham *et al.*, 1991
USA	Maize-based mixed feeds	FB_1: 1 300 - 16 800 FB_2: 100 - 6 500 FB_3: 0 - 2 650	Sydenham *et al.*, 1992
USA	Canned & frozen sweetcorn	FB_1: 0 - 82 (235:1/1 350: 1/1)	Trucksess *et al.*, 1995

Data in Tables 2.1, 2.2 and 2.3 show that the presence of fumonisins in food and feeds for both human and animal consumption is a worldwide problem. "Thus, contamination of maize with the foodborne carcinogenic mycotoxins, the fumonisins, is a Pan-African, as well as a global problem" (Marasas *et al.*, 1993).

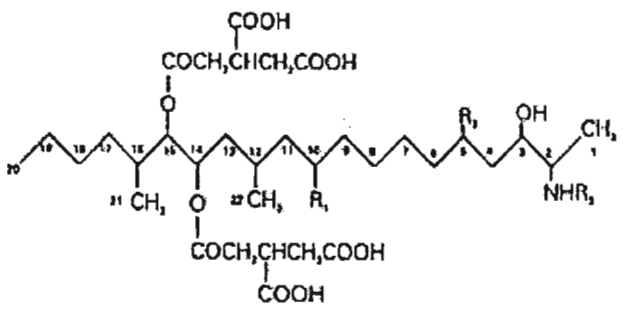
2.3 CHEMISTRY

Fumonisins were first isolated from *F. moniliforme* MRC 826, a toxigenic strain isolated from mouldy corn in the Transkei by extraction with ethyl acetate and methanol and purified using chromatographic methods (Kriek *et al.*, 1981; Gelderblom *et al.*, 1988).

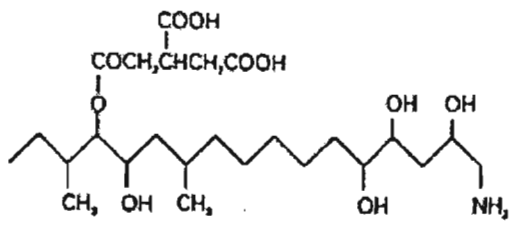
The structures were elucidated using liquid secondary ion mass spectrometry and nuclear magnetic resonance spectrometry. The backbone for the B series is 2-amino-12,16,dimethyl-3,5,10,14,15-pentahydroxyicosane joined in diester linkage with propane-1,2,3,-tricarboxylic acid [tricarballylic acid (TCA)] via hydroxyl groups at C-14 and C-15; and the terminal carboxyl group of TCA (Figure 2.1). The presence of a free amino group and four free carboxyl groups means the fumonisins are strongly polar and water soluble. They are also soluble in methanol and acetonitrile/water but are not soluble in non-polar solvents (Scott & Lawrence, 1992). Both the fumonisins and AAL toxin show structural similarities with sphingosine (Figure 2.1).

FUMONISINS

	R_1	R_2	R_3
FA_1	OH	OH	CH_2CO
FA_2	H	OH	CH_2CO
FB_1	OH	OH	H
FB_2	H	OH	H
FB_3	OH	H	H
FB_4	H	H	H



AAL TOXIN



SPHINGOSINE

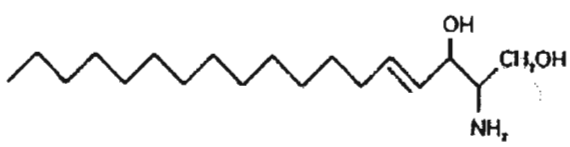


FIGURE 2.1 CHEMICAL STRUCTURES OF FUMONISINS, SPHINGOSINE AND AAL TOXIN (Norred, 1993)

The predominant fumonisin is FB_1 whereas FB_2, the C-10 deoxy analogue of FB_1 is less common. The B series have a free amino group which distinguishes them from the A series (FA_1 and FA_2) that are the *N*-acetyl derivatives (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988). Fumonisin B_3 and fumonisin B_4 (FB_4) (Figure 2.1), were later identified by Gelderblom *et al.* (1992B). Branham & Plattner (1993A) isolated a new fumonisin, fumonisin C_1(FC_1), from liquid cultures of *F. moniliforme* which differs from FB_1 in that it lacks the amino-end terminal-methyl group. In the P series (FP_{1-3}), 3-hydroxypyridinium replaces the amine at C-2; these new fumonisins can occur in amounts approximately one-third of the B series (Musser *et al.*, 1996). Recently, MacKenzie *et al.* (1998) reported the discovery of iso-fumonisin B_1 which has a hydroxyl group at C-4 instead of C-5.

Other chemical forms of the fumonisins can be prepared. Treatment with calcium hydroxide, i.e. hydrolysis, results in the sequential formation of a partially hydrolysed (removal of one of the two tricarballylic acid groups) and then a fully hydrolysed (removal of both groups) molecule or aminopentol [e.g. FB_1 to HFB_1 or aminopentol (AP_1)]. Partially hydrolysed FB_1 (PH_1) exists as an equilibrium mixture of the two possible monoesters, HFB_1 and hydrolysed FB_2 (HFB_2), which retain biological activity (Sydenham *et al.*, 1995B; Badria *et al.*, 1995).

2.3.1 Extraction and Purification

Extraction and purification of fumonisins from culture material is necessary so they may be used as standards; and for other analytical and toxicity experiments. Gelderblom *et al.* (1988) used ethyl acetate and aqueous methanol for extraction followed by XAD-2 adsorption chromatography, Sephadex LH-20 fractionation and a reverse phase C_{18} column for final clean-up to give a 92% recovery of two amorphous solids, pure FB_1 and pure FB_2. Cawood *et al.*, (1991) successfully purified fumonisins B_{1-4} and their *N*-acetyl derivatives from corn cultures by extracting with methanol:water (3:1), purification on an Amberlite XAD-2 column, then two silica gel columns and finally a reversed phase C_{18} column but the yield was low (40%). If methanol is used for extraction, mono- and dimethylesters are produced which interfere with the purification of individual fumonisins.

Using solid corn or rice cultures inoculated with *F. proliferatum* strain M5591, Dantzer *et al.* (1996) obtained 85% pure FB_1. Liquid cultures of the same strain gave better results - >95% purity and 37% recovery. Purification was by XAD-16 adsorption liquid

chromatography, C_8 partition liquid chromatography, DEAE-Sepharose (ion exchange liquid chromatography) and finally gradient C_{18} partition liquid chromatography.
Poling & Plattner (1996) working with an unusual strain of *F. moniliforme* (KSU 819) which mainly produces FB_3 and FB_4, obtained 95% recovery using solid phase C_{18}- and propylamine (NH_2)- bonded phases.

Liquid culture has made possible production of radiolabelled fumonisin by adding ^{14}C-acetate or L-[methyl-^{14}C] methionine to the medium to produce ^{14}C labelled FB_1 with the isotope at C-21 and C-22. These studies also showed that in the biosynthesis of fumonisins, methionine, glutarate and serine or alanine are added to the hydrocarbon backbone (Branham & Plattner, 1993B; Alberts *et al.*, 1993; Blackwell *et al.*, 1994).

2.3.2 Stereochemistry

Studies of the stereochemistry using molecular modelling techniques show the fumonisin molecules (FB_{1-4}) have a unique folded globular structure, similar to a peptide with internal H-bonding, and a predominantly hydrophobic surface. The folding of the amine backbone with the two esterified tricarballylic side chains forms a three-dimensional cage-like structure suggesting the molecules may act as chelators. Furthermore, there appears to be an internal space beginning on the electrophilic end, transversing the molecule, bifurcating and opening into two locations to either side of the lipophilic end (Beier *et al.*, 1995; Beier & Stanker, 1997). The authors suggest that, "These spaces may change in size as a result of changing hydrogen bond patterns. If the lipophilic end of FB_1 were imbedded into a membrane with the electrophilic portion protruding; the membrane may become leaky due to these spaces or holes within the fumonisin structure." This hypothesis seems likely in view of work done by Sauviat *et al.*, (1991) on frog heart muscle where they found FB_1 interacted with the calcium channel inhibiting conductance.

2.3.3 Stability

Several workers have investigated the heat stability of fumonisins in aqueous solution. Processing for 60 minutes at 125, 150 and 175 °C resulted in loss of <27%, 18 - 90% and >90% of 5 ppm FB_1 respectively. Fumonisin B_1 was most stable at pH 7, then pH 10 and least stable at pH 4 (Jackson *et al.*, 1996A). Similar results were obtained for FB_2 (Jackson *et al.*, 1996B).

In a 6 week storage experiment at four storage temperatures (-18, 4, 25, 40 °C), FB_1 and FB_2 were found to be most stable in acetonitrile:water (1:1) but decomposition and/or formation of fumonisin methyl esters occurred if stored in methanol. Extending the storage time to 6 months confirmed the stability of fumonisins in acetonitrile:water (Visconti *et al.*, 1994A).

2.4 ANALYSIS

2.4.1 Thin Layer Chromatography

This is a simple method but requires initial sample extraction. Rapid methods have been developed for FB_1, FB_2 and FB_3 in corn (Ackermann, 1991; Cawood *et al.*, 1991) and rice samples (Dawlatana *et al.*, 1995). After extraction with acetonitrile:water (1:1) and separation on normal phase silica with chloroform:methanol:acetic acid as the mobile phase, acidic anisaldehyde was used as the visualisation reagent. Alternatively C_{18} columns were used for further clean-up prior to separation on reverse phase C_{18} plates using methanol:4% aqueous potassium chloride as the developing solvent. Visualisation was achieved by spraying successively with 0.1M sodium borate buffer, fluorescamine and 0.01M boric acid. Examination under longwave ultraviolet light (UV) showed FB_1 (Rf 0.5) and FB_2 (Rf 0.1) as bright yellowish-green fluorescent bands (Rottinghaus *et al.*, 1992). Stockenström *et al.* (1994), found less interferences if strong anion exchange (SAX) columns were used instead of C_{18}.

Thin layer chromatography is a suitable method, particularly for analysing large numbers of samples, as it is faster and cheaper than HPLC; but results are not as accurate. Comparison of HPLC and a rapid TLC method (similar to the above) in maize samples showed good agreement ($r = 0.953$; $p<0.0005$) (Schaafsma *et al.*, 1998).

2.4.2 High Performance Liquid Chromatography - Fluorometric/UV detection

High performance liquid chromatography is the most frequently used method of analysis for fumonisins. However, fumonisins do not absorb UV light nor do they fluoresce so formation of a fluorescent derivative is essential; and to overcome matrix interferences, prior extraction and clean-up is required. Extractions have been done using methanol:water (3:1) or acetonitrile:water (1:1) followed by further purification using SAX or C_{18} columns. The SAX columns were washed with methanol:water (1:3 or 3:1) and pure methanol; elution was with methanol:water (3:1); or 5%, 1% or 0.5% acetic acid in methanol (Sydenham *et al.*, 1990A; Shephard *et al.*, 1990; Akiyama *et al.*, 1995; Velázquez *et al.*, 1995).

The C_{18} columns were washed with acetonitrile:water (1:4) and the eluant was acetonitrile:water (1:1 or 7:3) (Hopmans & Murphy, 1993). Bennett & Richard (1994) found SAX columns to be efficient in isolating fumonisins from crude extracts but for partial purification of hydrolysis products C_{18} columns were needed, because hydrolysed fumonisins are not retained by SAX columns (Stockenström *et al.*, 1994).

Alternatives to SAX and C_{18} columns are immunoaffinity columns (Fumonitest ^{TM}); elution is with methanol:buffer. These have been successfully applied to corn, milk, and canned and frozen sweetcorn (Scott *et al.*, 1994; Ware *et al.*, 1994; Trucksess *et al.*, 1995; Duncan *et al.*, 1998). However, they have a small capacity (1 μg FB_1) so are not suitable if large amounts of fumonisins are present, yet may be useful for measurements in tissues or plasma (Bennett & Richard, 1994).

Eluates from the extraction/clean-up procedure were evaporated to dryness at 60°C under nitrogen and redissolved prior to the formation of a fluorescent derivative and analysis by HPLC. Sydenham *et al.* (1990A) made a maleyl (UV absorbing) and a fluorescamine (fluorescent) derivative. Unfortunately, the maleyl derivative did not have the desired specificity or sensitivity. Successful HPLC for FB_1 and FB_2 was done with an o-phthaldialdehyde (OPA) derivative using a C_{18} reversed phase column and methanol:0.1 M sodium dihydrogen phosphate (pH 3.3) (80:20) as the mobile phase. The acidic pH is required to prevent ionisation of the TCA groups (Shephard *et al.*, 1990).

Since OPA derivatives are unstable and fluorescamine derivatives give two peaks, other methods were developed viz. 4-fluoro-7-nitrobenzofurazan (NBD-F) or naphthalene-2,3-dicarboxaldehyde- potassium cyanide (NDA-KCN) (Scott & Lawrence, 1992; Scott *et al.*, 1994). Other derivatising chemicals used were 9-fluorenylmethyl chloroformate (FMOC) (Holcomb *et al.*, 1993); naphthalene dicarboxaldehyde (NDA) (Bennett & Richard, 1994); 4-(*N,N*-dimethyl-aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) (Akiyama *et al.*, 1995); 6-amino-quinolyl *N*-hydroxy-succinimidylcarbamate (Velásquez *et al.*, 1995) and fluorescein isothiocyanate (FITC) (Maragos, 1995).

Of all the derivatising agents, OPA is now the one used most often (Shephard *et al.*, 1990; Sydenham *et al.*, 1992; Scott *et al.*, 1994; Trucksess *et al.*, 1995; Thakur & Smith, 1996; Rava *et al.*, 1996, Rava, 1996; Duncan *et al.*, 1998). It reacts with all free primary amines

(hence extensive clean-up is needed) and since the fluorescence intensity decays with time, timing between addition of the reagent and injection on to the column must be constant and accurately reproduced for each sample – usually one minute (Shephard *et al.*,1990; Sydenham *et al.*, 1992; Thakur & Smith, 1996).

A C_{18} column was used for separation with a mobile phase of methanol:0.05M sodium dihydrogen phosphate buffer (pH 3.5) alone or with acetonitrile:water (Scott *et al.*, 1994); or various proportions of acetonitrile:water:acetic acid (Trucksess *et al.*, 1995; Thakur & Smith, 1996). Sydenham *et al.* (1992) used a C_8 column and a mobile phase of methanol: 0.1M sodium dihydrogen phosphate (pH 3.35) to simultaneously analyse FB_1, FB_2, and FB_3.

TABLE 2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF FUMONISINS

Sample	Recovery	Detection Limits	Reference
Maize	99.5% (FB_1) 85.9% (FB_2)	50 ng/g FB_1 100 ng/g FB_2	Shephard *et al.*, 1990
Maize	66.3% (FB_1 at 100 µg/g)	10 µg/g	Sydenham *et al.*, 1990A
Maize	94% (FB_1) 80% (FB_2)	100 ng/g Range (125 - 5 000 ng/g)	Scott & Lawrence, 1992)
Maize	96.8%(FB_3)	50 ng/g	Sydenham *et al.*, 1992
Rodent feed	83% (FB_1 at 2-20 ppm)	200 ng/g	Holcomb *et al.*, 1993
Maize	116% , 119%, 110% (FB_1 at 250, 500, 1 000 ng/g)	25 ng/ml FB_1 standard 50 ng/ml FB_2 standard	Hopmans & Murphy, 1993
Milk	87 - 109% (FB_1 at 5 - 50 ng/ml) 69 - 83% (AP_1 at 50 - 100 ng/ml)	3 ng/ml (FB_1 and FB_2) 20 - 25 ng/ml (AP_1)	Scott *et al.*, 1994
Maize	85.4% (FB_1) 87.1% (FB_2)	10 ng/g FB_1 4 ng/g FB_2	Ware *et al.*, 1994
Maize	92% (FB_1) 90% (FB_2) (0.5 µg/g)	0.01 µg/g	Akiyama *et al.*, 1995
Canned & frozen sweetcorn	76 - 88% (FB_1) (50 - 200 ng/g)	4 ng/g	Trucksess *et al.*, 1995
Maize	no data	0.26 µg/g	Velázquez *et al.*, 1995
Maize	80 - 85%	20 µg/kg (FB_1, FB_2, FB_3)	Rava *et al.*, 1996, Rava, 1996
Maize	83%	0.016 µg/g Range 0 - 10 µg/g	Duncan *et al.*, 1998

Successful HPLC analysis was done on a variety of samples – maize and maize-based products but also on milk and rodent feed (Table 2.4).

Recoveries varied from 66.3% to 119% and detection limits for FB_1 from 4 to 260 ng/g (in maize); and for FB_2 from 3 ng/ml (in milk) to 100 μg/g (in maize).

An International Union of Pure and Applied Chemistry (IUPAC) inter-laboratory comparative study analysing maize samples by a specified method gave intra-laboratory relative standard deviations (RSD) of 7.7 to 25.5% for FB_1 (200 to 2 000 ng/g) and 12.5 to 36.8% for FB_2 (70 to 740 ng/g). Inter-laboratory RSD's were 18.0 to 26.7% for FB_1 and 28.0 to 45.6% for FB_2. Statistical comparisons (using the ratio of the actual inter-laboratory RSD with that predicted from the known concentration) to evaluate the method showed that the results were acceptable and the method is suitable for adoption as an official method (Thiel *et al.*, 1993).

In a European study intra-laboratory RSD's were 15% for FB_1 and FB_2, while inter-laboratory RSD's were 11% (FB_1) and 13% (FB_2) (Visconti *et al.*, 1996A). These results confirm the role of HPLC as a viable, reproducible and widely used method for analysis of fumonisins (Shephard,1998).

2.4.3 Other Methods

Liquid chromatography used with mass spectrometry (MS) and no derivatisation gave detection limits of 0.01 μg/g for both FB_1 and FB_2 (Akiyama *et al.*, 1998).
Both positive and negative electrospray ionisation mass spectrometry (ESI-MS) and liquid chromatography /electrospray ionisation mass spectrometry (LC-ESI-MS) have been successful in analysis of FB_1, FB_2 and FB_3 in corn-grit, maize meal, rodent feed and forage grass (Mirocha *et al.*, 1992; Caldas *et al.*, 1995; Lukacs *et al.*, 1996; Josephs, 1996; Churchwell *et al.*, 1997).
Thermospray mass spectrometry and capillary liquid chromatography-fast atom bombardment mass spectrometry have been used to analyse HFB_1, HFB_2 and $PHFB_1$ and FA_2 (Thakur & Smith, 1996; Xie *et al.*, 1997).
A capillary gas chromatography - mass spectrometry method identified the TCA moieties after acid hydrolysis and esterification of FB_1 (Sydenham *et al.*, 1990A).

Supercritical fluid (carbon dioxide) extraction (SFE) of maize, maize dust and *F. moniliforme* culture samples followed by HPLC analysis gave a sensitive, fast and reproducible method for analysing FB_1 in low concentrations (Selim *et al.*, 1996).

Using fluorescein isothiocyanate (FITC) derivatives of FB_1, FB_2 and HFB_1, Maragos (1995) achieved good correlation when comparing capillary zone electrophoresis (CZE) and HPLC.

The most sensitive method was high-performance liquid chromatography – electrospray-ionisation, tandem mass spectrometry (HPLC-ESI-MS-MS) with a detection limit of 0.8 ng/g in maize (Lukacs *et al.*, 1996); the least sensitive (with detection limits of 0.25 μg/g for both), were fluorometry for maize (Duncan *et al.*, 1998) and high-performance thin-layer chromatography (HPTLC) for rice (Dawlatana *et al.*, 1995) (Appendix 1, Table 2.5).

2.4.4 Immunoassay

Because of its sensitivity and specificity, use of immunoassays particularly ELISA's for analysis of mycotoxins is an obvious choice. Several workers have developed methods using both polyclonal and monoclonal antibodies raised in various animals (rabbits, mice, hens, ascites fluid) to analyse for different mycotoxins (zearalenone, aflatoxin B_1, aflatoxin M_1, ochratoxin A, citrinin, T-2 toxin, cyclopiazonic acid, roridin A) in a variety of samples (maize, maize meal, wheat, barley, cereals, peanuts, Job's tears, animal and human serum and urine, pig feed, chicken meat, milk, dairy products) (Table 2.6).

Since fumonisins are of low molecular weight, they must first be conjugated to a protein to form an immunogenic molecule. The most common method is by linking the C-2 amine of FB_1 to a glutaraldehyde activated protein and stabilising the linkage with sodium borohydride (Chu, 1996). The carrier proteins used have included cholera toxin (CT) (Azcona-Olivera *et al.*, 1992A, 1992B; Yeung *et al.*, 1996); keyhole limpet hemocyanin (KLH) (Usleber *et al.*, 1994; Fukuda *et al.*, 1994; Yu & Chu, 1996; Abouzied *et al.*, 1996); bovine serum albumin (BSA) (Yu & Chu, 1996; Hammer *et al.*, 1996); human serum albumin (HSA) (Yeung *et al.*, 1996) and ovalbumin (OVA) (Fukuda *et al.*, 1994).
By protecting the amino group prior to carbodiimide coupling of FB_1 to HSA and then deprotecting before immunisation, Yeung *et al.* (1996) managed to produce high affinity and avidity antibodies.

The initial step in the assay is to coat microwell plates with FB_1 and for this another conjugate was made e.g. FB_1-ovalbumin (FB_1-OVA)(Azcona-Olivera *et al.*, 1992A, 1992B; Yu & Chu, 1996; Abouzied *et al.*, 1996; Yeung *et al.*, 1996) or FB_1-bovine serum albumin (FB_1-BSA) (Fukuda *et al.*, 1994).

TABLE 2.6 IMMUNOASSAYS OF VARIOUS MYCOTOXINS

Mycotoxin / Type of Antibody	Methodology	Substance analysed	Reference
Zearalenone			
mAb	CI-ELISA	Porcine urine	MacDougald *et al.*, 1990
mAb (ascites)	CD-ELISA	Milk	Azcona *et al.*, 1990
mAb	Line immunoblot assay	Maize	Abouzied & Pestka, 1994
mAb	CD-ELISA	Maize, wheat, pig feed	Bennett & Nelsen, 1994
mAb (ascites fluid of mice)	CI-ELISA	Barley, Job's tears	Tanaka *et al.*, 1995
Aflatoxin B_1			
pAb (rabbits)	CI-ELISA	Serum (blood donors)	Wilkinson *et al.*, 1988
pAb	CD-ELISA	Maize, peanuts	Park *et al.*, 1989
pAb	RIA	Human sera	Fukal & Reisnerova, 1990
EZ-Screen test kit	CD-ELISA	Urine (human and animal)	Stubblefield *et al.*, 1991
mAb	Line immunoblot assay	Maize	Abouzied & Pestka, 1994
mAb	Fiber–optic biosensor	Peanuts and maize meal	Carter *et al.*, 1997
Aflatoxin M_1			
pAb (rabbits)	CD-ELISA	Dairy products	Fremy & Chu, 1984
Ochratoxin A			
mAb (mice)	CI-ELISA	Chicken meat, wheat flour, porcine plasma, bovine serum	Kawamura *et al.*, 1989
pAb	RIA	Human sera	Fukal & Reisnerova, 1990
pAb (hens)	CI-ELISA	Swine finisher diets	Clarke *et al.*, 1993
mAb	CD-ELISA	Cereals	Barna-Vetró *et al.*, 1996
Citrinin			
pAb (rabbits)	CI-ELISA	Wheat flour	Abramson *et al.*, 1995
T-2 toxin			
mAb	Dipstick CD-ELISA	Wheat	de Saeger & van Peteghem, 1996
Cyclopiazonic acid			
mAb (mice)	Sandwich ELISA	Fungal mycelia and cheese	Hahnau & Weiler, 1993
mAb	CD-ELISA	Maize, peanuts, mixed feed	Yu & Chu, 1998
Roridin A			
pAb (rabbits)	CI-ELISA		Märtlbauer *et al.*, 1988

Key to Table 2.6

mAb	monoclonal antibodies
pAb	polyclonal antibodies
CI-ELISA	competitive indirect ELISA
CD-ELISA	competitive direct ELISA
RIA	radio-immunoassay
EZ-Screen test kit	aflatoxin test kit (Environmental Diagnostics Inc., Burlington, NC)

Both polyclonal (pAb) and monoclonal (mAb) antibodies against fumonisins (usually FB_1) have been raised in a variety of animals and both competitive direct ELISA (CD-ELISA) and competitive indirect ELISA (CI-ELISA) methods developed. Monoclonal antibodies were IgG_1 kappa (Azcona-Olivera *et al.*, 1992B; Fukuda *et al.*, 1994).
The enzyme used most often was horseradish peroxidase (HRPO) either attached to the second antibody (CI-ELISA) (Yu & Chu, 1996); or to FB_1 (CD-ELISA) (Azcona-Olivera *et al.*, 1992B; Usleber *et al.*, 1994; Abouzied *et al.*, 1996; Yu & Chu, 1996).

Different antibodies/methods have different detection limits, ranges and cross-reactivities with FB_1, FB_2, FB_3, HFB_1 and TCA (Table 2.7).

TABLE 2.7 ELISA METHODS FOR FUMONISINS

Antibody / ELISA Method	Detection limit (buffer)	ID_{50} (ng/ml)	Range (ng/ml)	Cross-reactivity	Reference
pAb ; BALB/c mice; ascites fluid; CI-ELISA	100 ng/ml	FB_1 - 260 FB_2 - 300 FB_3 - 650	no data	FB_2 - 87% FB_3 - 40% TCA - 0%	Azcona-Olivera *et al.*, 1992A
mAb; BALB/c mice; CD-ELISA	50 ng/ml	FB_1 - 630 FB_2 - 1 800 FB_3 - 2 300	50 to 5 000	FB_2 - 38% FB_3 - 33% TCA - 0% HFB_1 - 0%	Azcona-Olivera *et al.*, 1992B
mAb; mice ; CI-ELISA		FB_1 - 144 FB_2 - 56	10 to 1 000 to 5 000	FB_2 - 290%	Fukuda *et al.*, 1994
pAb; rabbits; CD-ELISA	FB_1 - 167 pg/ml FB_2 - 1 200 pg/ml FB_3 - 460 pg/ml	FB_1 - 0.623	0.02 to 20	FB_2 - 24% FB_3 - 55%	Usleber *et al.*, 1994
pAb; sheep; CD-ELISA	<0.1 ng/ml	FB_1 - 5.5 FB_2 - 23 FB_3 - 18	0.1 to 30 ng/ml	FB_2 - 24% FB_3 - 30%	Abouzied *et al.*, 1996
pAb; rabbits; CI-ELISA	0.05 ng/ml	FB_1 - 0.66	0.05 to 0.75	FB_2 - 62% FB_3 - 14% HFB_1 - 11% HFB_2 - 3%	Yeung *et al.*, 1996
pAb; rabbits; CD-ELISA	no data	FB_1 - 0.45 FB_2 - 0.72 FB_3 - 25	0.01 to 1 000	FB_2 - 62% FB_3 - 1.8%	Yu & Chu, 1996
pAb; rabbits; CI-ELISA	no data	FB_1 - 1.33 FB_2 - 1.66 FB_3 - 28	0.01 to 1 000	FB_2 - 80% FB_3 - 4.8%	Yu & Chu, 1996

Cross-reactivities with FB_1 (Table 2.7) were 100% in each case. Studies of the cross-reactivities suggest that the epitope lies in the C-11 to C-20 region of FB_1 (Azcona-Olivera *et al.*, 1992A, 1992B) or antibodies may recognise a completely different region (Fukuda *et al.*, 1994).

The ID_{50} is the concentration of mycotoxin required for 50% inhibition of antibody binding and is an indication of affinity; the lower the value the greater the affinity. High affinity antibodies give better sensitivity in immunoassays (Yu & Chu, 1996). Detection limits in buffer varied from 0.05 ng/ml to 100 ng/ml (Table 2.7).

The monoclonal antibodies raised by Azcona-Olivera *et al.*(1992B) were used to develop the Fumonisin Agri-Screen kits (Neogen); and Abouzied *et al.*, (1996) used their polyclonal antibodies to develop the Veratox ® Fumonisin Kit (Neogen).

To improve sensitivity and specificity, it was found necessary to perform some cleanup/extraction before application to a microwell plate as different matrices caused interference (Table 2.8).

TABLE 2.8 EXTRACTION AND RECOVERY IN ELISA METHODS FOR FUMONISIN B_1

Method	Sample	Extraction	Detection limit	Recovery	Reference
CD-ELISA	Maize	50% acetonitrile and dilution of supernatant	5 µg/g	103%	Azcona-Olivera *et al.*, 1992B
CD-ELISA (Agri-Screen kit)	Milk	None	100 - 300 ng/ml	no data	Maragos & Richard, 1994
CD-ELISA	Maize	Methanol:water (75:25)	10 ng/g	59.7% (50 ng/g) 73.2% (500 ng/g)	Usleber *et al.*, 1994
CD-ELISA (Veratox ® kit)	Maize	Methanol: water 70% (v/v); filtration, dilution	0.1 ng/ml	74 - 91%	Abouzied *et al.*,1996
CI-ELISA	Maize	Methanol: water 75% (v/v); filtration, dilution	5 ng/g	75.5% - 90.3%	Yeung *et al.*, 1996
CD-ELISA	Maize	Acetonitrile:water (1:1) and dilution in buffer or C_{18} column clean-up	10 – 50 ng/g	85.9% (no clean-up) 70.5% (with clean-up)	Yu & Chu, 1996
CD-ELISA	Beer	None	0.1 ng/ml	98.7 - 102.8%	Scott *et al.*, 1997

Recoveries ranged from 59.7% to 103% and detection limits from 0.1 ng/ml (beer and maize) to 500 ng/g (maize) (Table 2.8). Recoveries are similar to those for HPLC but ELISA methods are more sensitive (Table 2.4 and 2.8).

In comparing TLC and competitive ELISA for screening of large numbers of samples, the ELISA method gave higher values which was attributed to lack of specificity of the antibodies, or loss of sample during the more extensive clean-up done prior to TLC (Shelby *et al.*, (1994).

Comparison of results obtained from ELISA and HPLC methods showed higher values for the ELISA methods but generally good correlation, $r = 0.967$, $p<0.001$ (Sydenham *et al.*, 1996B). Better correlation in analysing maize and wheat-based food products is found between gas chromatography – mass spectrometry (GC-MS) and HPLC ($r = 0.946$, $p<0.01$) than between ELISA and HPLC ($r = 0.512$, $p<0.05$) (Pestka *et al.*, 1994).
When analysing maize samples, Yu & Chu (1996) surprisingly found better correlation between ELISA and HPLC for samples without clean-up ($r = 0.955$, $p<0.001$) than for samples with clean-up ($r = 0.811$, $p<0.01$). Similarly, for beer samples, Scott *et al.* (1997) found better correlation between liquid chromatography (LC) and ELISA at low levels of FB_1 (0 -10 ng/ml) for samples with no clean-up compared to those extracted on an immunoaffinity column. In contrast to other workers, they found ELISA results were lower than HPLC. Using an in-house immunoaffinity column prior to analysis of maize samples by both immunoassay and HPLC, Maragos (1997) found good agreement between immunoassay and HPLC results at low levels of fumonisins (< 2 μg/g) but at higher levels the HPLC results were lower.

Several possible explanations for these differences have been proposed including differences in extraction procedures and lower recoveries from samples prepared for HPLC; interference (inhibition) in the ELISA methods by components in the sample (matrix differences, possibly lipid based); completely different methodology principles; and precursors or metabolites produced by *F. moniliforme* that cross-react with the antibodies or interfere in the immunoassay (Pestka *et al.*, 1994; Usleber *et al.*, 1994; Schneider *et al.*, 1995; Tejada-Simon *et al.*, 1995; Abouzied *et al.*, 1996; Sydenham *et al.*, 1996A, 1996B; Yu & Chu, 1996). Most antibodies (including monoclonal antibodies) are not specific for FB_1 but rather measure total fumonisins (Table 2.7).

Usleber *et al.* (1994) suggested differences in the ratio of the open chain and cyclic forms of the TCA side chains may have affected results as this ratio is pH dependent and different extraction procedures gave different pH values of the extracts.

The TCA groups are believed to be important for antibody binding (Azcona-Olivera *et al.*, 1992A, 1992B). Use of higher dilutions for samples with high concentrations of fumonisins can overcome the matrix effect (Usleber *et al.*, 1994; Sydenham *et al.*, 1996A).

In addition to ELISA's, immunoassays with different formats have been developed mainly to provide rapid visual screening tests suitable for use in the field. These include a line immunoblot assay using monoclonal antibodies, detection limit 500 ng/ml (Abouzied *et al.*, 1994); dipstick immunoassay using polyclonal antibodies, detection limits 40 - 60 ng/g in maize samples (Schneider *et al.*, 1995); fiber-optic immunosensor with fluorescence detection using monoclonal antibodies, detection limit 3.2 μg/g in spiked maize from diluted methanolic extracts and 0.4 μg/g from affinity column purified extracts (Thompson & Maragos,1996; Maragos, 1997) and a surface plasmon resonance immunosensor using polyclonal antibodies, detection limit 50 ng/ml in buffer (Mullett *et al.*, 1998). None of these methods are as sensitive as ELISA assays.

2.4.5 Analysis of Physiological Samples

Most of the methods of analysis have been applied to maize and maize-based products and very little work has been done on analysis of fumonisins in physiological samples. Shephard *et al.*, (1992, 1995B) developed methods for analysing FB_1 and FB_2 in physiological samples from rats injected intraperitoneally with FB_1. Urine samples were diluted in distilled water, extracted with methanol, cleaned up on a SAX column, and measured as OPA derivatives on reverse phase HPLC (as for maize samples). Plasma samples for FB_1 were de-proteinised by addition of methanol, centrifuged and the supernatant applied to a SAX column and analysed as for urine. For FB_2, acetonitrile was used for deproteinisation of plasma samples and acetonitrile and acetonitrile/water (1:1) for washing the SAX columns; elution and analysis was as for FB_1. Recoveries were 91% and 94% (FB_1 in urine); 86% and 87% (FB_1 in plasma); 101.5% (FB_2 in urine) and 90.3% (FB_2 in plasma). Detection limits were about 50 ng/ml and relative standard deviations <5%.

Freeze-dried faecal samples were ground to a powder and fumonisins extracted nine times with 0.1M ethylenediaminetetraacetic acid (EDTA) (pH 5.2):methanol (4:1). Supernatants were combined, acidified to pH 3.1 and an aliquot applied to a C_{18} cartridge conditioned with methanol and then water. After washing with water, methanol:water (1:3) and (1:1), samples were eluted with methanol. The dried residue was redissolved in methanol prior to

derivatisation and HPLC. Extraction recoveries were 90% for FB_1 and 86.4% for FB_2 (Shephard *et al.*, 1994A, 1995B). Using this method, Chelule *et al.* (2000) analysed human faecal samples and found FB_1 in 35% of samples from rural dwellers and in 9% of samples from urban dwellers; concentrations ranged from 790 to 19 560 ng/g of freeze-dried faeces.

To improve sensitivity and remove interfering solutes in human urine samples, Shetty & Bhat (1998) used an Amberlite XAD-2 non-ionic polymeric resin column prior to the SAX column. For 100 ml spiked samples, 10 to 500 ng/ml FB_1, recoveries were 93.6% to 94.4%, RSD <5% and detection limit <8 ng/ml. Using a commercial immunoaffinity column for clean-up, horse serum spiked with FB_1 was derivatised with FITC and analysed by CZE. Recoveries at 100 ng/ml were 76% (range 67.0-85.6%; CV 10.6%) but levels below 100 ng/ml could not be quantitated because of interference by a component in the unspiked serum which had a similar migration time to FB_1 (Maragos, 1995).

2.5 TOXIC EFFECTS OF FUMONISINS IN HUMANS

2.5.1 Oesophageal Cancer

Maize and maize products are vitally important in the daily diet of humans in Southern Africa (Rava *et al.*, 1996). However, fungal infection of maize with production of mycotoxins is associated with the risk of oesophageal cancer (OC). Common factors in regions with high incidence of OC are mineral poor soil with excessive alkalinity and low annual rainfall resulting in stressed plants susceptible to heavy fungal contamination (including *F. moniliforme*) with the production of potentially carcinogenic mycotoxins. Also, the diet of people living in high risk areas is based on maize or wheat whereas, in low risk areas, more nutritious staples like sorghum, millet, cassava and peanuts are consumed (Rose, 1982; van Rensburg, 1985).

As early as 1957, in a study done on 104 OC cases from the local Bantu population attending the out-patients department of Frere Hospital, East London, it was concluded that the most likely reason for the high prevalence of OC was consumption of the local brew (*cidiviki)* which was shown to contain both cancer initiators and cancer promoters. Possibly, the rural Xhosa in the past had intuitive knowledge of possible deleterious effects of drinking homebrewed beer. "There is mounting evidence that even the 'raw', rural Xhosa has an insight into *umhlaza wombiza*, 'a sore inside the gullet which never heals'.

This affliction is attributed to having drunk, at a stranger's kraal, kaffir-beer which was 'poisoned' by someone intent upon avenging an old score." (Burrell, 1957).

In a case-control study of the urban black population of Soweto where several risk factors were considered, the major possible causes of OC were identified as smoking pipe tobacco and consumption of traditional beer brewed from malted sorghum and maize, alcohol content about 3%. The risk with drinking large quantities of beer may derive from its poor nutritional qualities (decreased B group vitamins) and cancer promoting effects of alcohol rather than a direct carcinogen (Segal *et al.*, 1988). Dietary deficiencies of zinc, molybdenum, selenium, magnesium, riboflavin and nicotinic acid are believed to increase susceptibility to carcinogens (van Rensburg, 1985). Deficiencies in molybdenum, zinc, iron, copper, and magnesium were found in the soil of areas with a high rate of OC in the Transkei (Rose, 1982).

The strong link between OC and fumonisins is due to the work done at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) group studying low (Bizana and Lusikisiki) and high (Butterworth and Kentani) risk areas in the Transkei. Over a period of 15 years, for people aged 0 to 70 years, the incidence rate was 33.58/100 000 for males and females in Kentani and 2.52/100 000 in Bizana (Rose, 1982; Jaskiewicz *et al.*, 1987B). During 1985 and1986, using brush biopsy capsules, cytological examinations were done on adults in 12 households in a low, intermediate and high OC rate area. Mild and advanced cellular changes occurred more frequently in occupants of high OC rate households. Analysis of samples of both good and mouldy homegrown maize from the same households showed a significantly higher prevalence of *F. moniliforme* ($p<0.001$) in households whose members showed cytological abnormalities (Marasas *et al.*, 1988B; Rheeder *et al.*, 1992). Significantly higher levels of FB_1 and FB_2 were found in healthy maize samples from the high OC rate area compared with the low rate area; including some of the highest recorded levels from naturally infected maize viz. 117 520 ng/g FB_1 and 22 960 ng/g FB_2 (Sydenham *et al.*, 1990B; Rheeder *et al.*, 1992).

In China, too, a higher incidence of contamination with fumonisins was found in maize from households in a high risk area for human OC compared with a low risk area (Yoshizawa *et al.*, 1994). Strains of *F. moniliforme*, isolated from mouldy maize from OC regions in China, produced various nitrosamines in addition to fumonisins which, with other mycotoxins, are probably factors in carcinogenesis (Chu & Li, 1994; Mingxin *et al.*, 1980).

A survey of animal epidemiology in high and low risk OC areas in China revealed that the incidence of pharyngeal and OC in chickens paralleled the human cancer rate; rats were also found with hyperplastic lesions but they are short-lived compared to hens. Attempts to induce OC in domestic fowls by nitrosamines were unsuccessful and the conclusion was that other factors in both the human and animals' diet were responsible. Could these be the fumonisins ? (Mingxin *et al.*, 1980). Lim *et al.* (1996) induced oesophageal cell proliferation in rats after a single injection of FB_1 but no tissue injury. In contrast, Gelderblom *et al.* (1991) found oesophageal basal cell hyperplasia in rats after feeding them culture material of *F. moniliforme* but administering pure FB_1 did not cause any oesophageal lesions.

TABLE 2.9 AVERAGE LEVELS OF FUMONISINS IN MAIZE FROM LOW AND HIGH RISK AREAS FOR OESOPHAGEAL CANCER

Country	Area	Risk for OC	FB_1 (ng/g)	FB_2 (ng/g)	Reference
South Africa	Butterworth; Kentani	High (good maize)	1 600	610	Rheeder *et al.*, 1992
		High (mouldy maize)	23 900	7 550	Rheeder *et al.*, 1992
	Bizana; Lusikisiki	Low (good maize)	375	83	Rheeder *et al.*, 1992
		Low (mouldy maize)	6 520	2 500	Rheeder *et al.*, 1992
China	Cixian; Linxian	High (good maize)	35 300		Chu & Li, 1994
		High (mouldy maize)	74 000		Chu & Li, 1994
China	Linxian	High	872	448	Yoshizawa *et al.*, 1994
	Shanqui	Low	890	330	Yoshizawa *et al.*, 1994
USA		High	105 - 1 915	70 – 460	Sydenham *et al.*, 1991

2.5.2 Diarrhoea

In India in 1995, there was an outbreak of a foodborne disease later traced to being caused by consumption of rain affected mouldy sorghum and maize samples infested with *F. moniliforme* and producing FB_1. The characteristics of the disease were abdominal pain, borborygmi and diarrhoea; the diarrhoea was reproduced in day old cockerels fed contaminated grains from affected households. Levels of FB_1 in affected households were 0.14 - 7.8 mg/kg (sorghum) and 0.25 - 64.7 mg/kg (maize) and in unaffected households were 0.07 - 0.36 mg/kg (sorghum) and 0.05 - 0.24 mg/kg (maize). The disease was self limiting and symptoms ceased when non-mouldy grain was eaten. Thus, fumonisins can cause gastrointestinal effects in humans as well as their association with OC (Bhat *et al.*, 1997).

2.6 TOXIC EFFECTS OF FUMONISINS IN ANIMALS AND PLANTS

2.6.1 Rodents

Marasas *et al.*, (1984) fed freeze- or oven-dried culture material of *F. moniliforme* MRC 826 to male BD IX rats in a life-long feeding experiment. Hepatotoxicity and 100% mortality occurred at a level of 8%; levels of 4% (later reduced to 2%) caused hepatocellular and ductular liver carcinomas - the incidence of carcinomas increased with time.

Using a short-term cancer initiation-promotion bioassay in rats as the monitoring system, the cancer-promoting compounds were isolated viz. the fumonisin mycotoxins. The bioassay was checked using purified FB_1 (0.1%) which caused a marked reduction in weight gain, induction of γ-glutamyl transferase (GGT) positive foci and toxic hepatitis (Gelderblom *et al.*, 1988). Elevated serum enzyme activities [alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP)] and microscopic liver lesions (indicating liver damage) were found after feeding rats aqueous and chloroform/ methanol extracts of *F. moniliforme* MRC 826. Toxic diets had concentrations of 93 - 139 ppm (FB_1) and 82 - 147 ppm (FB_2); non-toxic ≤ 22 ppm (FB_1) and ≤ 65 ppm (FB_2) (Voss *et al.*, 1990).

After the development of methods to purify fumonisins, several authors administered the mycotoxins themselves rather than culture material extracts either in the rats' diets or by injection which rapidly induces hepato-and nephrotoxicity (Bondy *et al.*, 1995).
A variety of symptoms of fumonisin toxicity in rats have been recorded (Table 2.10).

TABLE 2.10 LEVELS OF FUMONISINS FED TO RATS AND TOXIC EFFECTS

Fumonisin B_1	Toxic Effect	Reference
50 mg/kg diet for 26 months	↑ AST, GGT, creatinine, conjugated bilirubin macro- and micronodular cirrhosis, cholangiofibrosis, hepatic carcinoma chronic interstitial nephritis	Gelderblom *et al.*, 1991
0, 15, 50, 150 ppm (μg/g or mg/kg) diet for 4 weeks NOEL < 15 ppm	↑ ALT, ALP, triglycerides, cholesterol ↑ sphingolipids, free Sa ↓kidney weight microscopic liver lesions nephrosis	Voss *et al.*, 1993 Riley *et al.*, 1994 Voss *et al.*, 1995A
7.5 or 10 mg/kg injected intraperitoneally for 4 days	↓ body, kidney, liver, spleen, thymus weights, food consumption ↓ glucose, amylase ↑ urine volume, relative brain weights, ALT, AST, cholesterol, calcium, total protein, albumin, globulins, urea, erythrocytes, haemoglobin Lesions in liver, kidney, thymus	Bondy *et al.*, 1995
7.5 or 10 mg/kg injected peritoneally for 4 days	↓ food intake, body weight, absolute kidney weight, urine osmolality ↑ urine volume, urine proteins, urine GGT, LDH, NAG	Suzuki *et al.*, 1995
0, 1, 3, 9, 27, 81 ppm diet for 13 weeks NOEL 3 ppm (mice 27 ppm)	↓ kidney weight ↑ serum creatinine microscopic kidney lesions	Voss *et al.*, 1995B
0, 1.25 mg/kg injected intravenously	↑ cholesterol, urea ↓ glutathione (hepatic and renal) Cell proliferation, apoptosis and necrosis in kidney outer medulla Cell proliferation in liver and oesophagus	Lim *et al.*, 1996
0, 15, 50, 150 ppm diet for 28 days NOEL for hepatotoxicity in rats is 163 ppm (F); 234 ppm (M)	↑ serum creatinine, free Sa and Sa/So ratio in liver and kidney ↓ kidney wt., tCO_2 Kidney lesions (liver lesions only at 150 ppm)	Voss *et al.*, 1996B
0, 1, 10, 55 ppm diet for 18 weeks	↑ALT, AST, ALP, urea, creatinine, bilirubin, cholesterol ↓ tCO_2, body weight gain, liver and kidney weights Mild renal lesions	Voss *et al.*, 1996C
50, 100, 250 mg/kg diet for 21 days	↑ cholesterol (liver & serum), phosphatidylethanolamine (liver) ↓ sphingomyelin (liver)	Gelderblom *et al.*, 1997
1, 10, 25 mg/kg diet fed for 2 years	↑ phosphatidylethanolamine (liver)	Gelderblom *et al.*, 1997
69.3 μmol/kg diet fed for 4 weeks	↑ cholesterol, ALT, prostaglandins ($PGF_{2\alpha}$, PGE_2) GGT- and PGST- positive hepatic foci ↓ NK cell activity, body weight	Lu *et al.*, 1997

(Key to Table 2.10 is on the next page).

Key to Table 2.10

↑	increase	↓	decrease
LDH	lactate dehydrogenase	NAG	*N*-acetyl-β-D-glucosaminidase
NK	natural killer	NOEL	no observed effect level
PGST	placental glutathione S-transferase	tCO2	total serum carbon dioxide

Decreased food intake and body weight gain, decreased organ weights (absolute/relative), increased free Sa and Sa/So ratio in urine, serum, liver and kidney indicate exposure to fumonisins (Voss *et al.*, 1993, 1995A, 1996B; Riley *et al.*, 1994). Increased bilirubin, AST, ALT, GGT, cholesterol, triglycerides, and microscopic liver lesions to cirrhosis, carcinoma indicate hepatotoxicity (Gelderblom *et al.*, 1991; Voss *et al.*, 1993). Increased excretion of high molecular weight proteins, enzymuria [GGT, lactate dehydrogenase (LDH), *N*-acetyl-β-D-glucosaminidase (NAG)], increased creatinine and urea, increased urine volume and decreased osmolality, nephrosis, microscopic kidney lesions, apoptosis and necrosis in outer medulla indicate nephrotoxic effects (Suzuki *et al.*, 1995; Bondy *et al.*, 1995; Voss *et al.*, 1995B, 1996B, 1996C; Lim *et al.*, 1996; Lu *et al.*, 1997).

Nephrotoxic effects in rats occur at lower concentrations of FB_1 than hepatotoxic effects. (Lim *et al.*, 1996; Voss *et al.*, 1996B). There was also a sex-related difference; no observed effect level (NOEL) for nephrotoxicity was 27 ppm for female rats but 3 ppm for males; hepatotoxicity 163 ppm for females but 234 ppm for males (Voss *et al.*, 1995B, 1996B). Fumonisin B_1 causes alterations in the phospholipids (sphingomyelin, phosphatidylcholine, phosphatidyl-ethanolamine) and their fatty acid components in the blood and liver of rats. The actual effects depend on the level and duration of treatment with FB_1; changes in lipid biosynthesis may be mediators of hepatic effects of fumonisin B_1 (Gelderblom *et al.*, 1997).

Mice are less sensitive to FB_1 than rats. Feeding various concentrations of FB_1 to both rats and mice, Voss *et al.* (1995B, 1996B) found significant interspecies differences in that mice showed mild hepatic injury while rats showed minimal to mild nephrosis. After feeding mice 1 to 75 mg FB_1/kg body weight /day for14 days, Bondy *et al.* (1997) found increased serum cholesterol and ALT, single cell necrosis, mitosis and anisokaryosis in the liver and minor histopathological changes in the kidneys of females.

To investigate the effects of FB_2 and FB_3, Voss *et al.* (1998B) used culture material from a strain of *F. moniliforme* that did not produce FB_1. The toxicological and histopathological effects were similar to FB_1 viz. decreased body weight gains and kidney weights, increased serum liver function test results, increased Sa/So ratios, and apoptosis of hepatocytes and

epithelium in kidney tubules. Some animals, after being fed the control diet (no fumonisins) for a further 3 weeks, showed almost complete recovery from these effects.

2.6.2 Equidae

Equine leukoencephalomalacia (ELEM) is a neurotoxic disease characterised by liquefactive necrosis of white matter in one or both cerebral hemisheres, associated with consumption of mouldy maize contaminated with *F. moniliforme* (Badiali *et al.*, 1968; Wilson & Maronpot, 1971; Kellerman *et al.*, 1972; Buck *et al.*, 1979; Kriek *et al.*, 1981; Meireles *et al.*, 1994). In a breakthrough study, Marasas *et al.* (1988A) provided experimental evidence that FB_1 was the causative agent of ELEM. Dosing two horses with culture material of *F. moniliforme* MRC 826 caused brain oedema and hepatosis.

After extracting and purifying FB_1 from the culture material, it was injected into a horse (0.125 mg FB_1/ kg body weight/ day for 7 days) and caused symptoms typical of ELEM viz. apathy, nervousness, a wide-based staring stance, shuffling gait, inco-ordination and bumping into objects, trembling, ataxia, reluctance to move, paresis of the lower lip and tongue, inability to eat or drink with principal lesions being severe oedema of the brain and focal necrosis in the medulla oblongata. Other authors confirmed these findings (Wilson *et al.*, 1990; Kellerman *et al.*, 1990; Ross *et al.*, 1991A,1991B; Thiel *et al.*, 1991B; Wilkins *et al.*, 1994). After this discovery, levels of fumonisins associated with field outbreaks of ELEM were recorded by several authors; others did studies on the amounts of FB_1 administered to induce ELEM (Tables 2.11 and 2.12).

TABLE 2.11 CONCENTRATIONS OF FUMONISINS IN FEEDS ASSOCIATED WITH OUTBREAKS OF EQUINE LEUKOENCEPHALOMALACIA

Feed (number of samples)	Location	Concentration of fumonisin (µg/g)	Reference
White maize (3)	Arizona	FB_1: 37 - 122 FB_2: detected	Wilson *et al.*, 1990
Maize, screenings, sweet feeds, commercial pellets (45 and 98)	USA	FB_2 : <1 - 126	Ross *et al.*, 1991A, 1991B
Maize and commercial feed (14)	Southeastern USA	FB_1: 1.3 - 27 FB_2: 0.1 - 12,6 FB_3: 0 - 2.65	Thiel *et al.*, 1991B; Sydenham *et al.*, 1992
Maize corn	USA	FB_1: 370 FB_2: 105 FB_3: 41	Wilkins *et al.*, 1994

TABLE 2.12 CONCENTRATIONS OF FUMONISIN B_1 CAUSING EQUINE LEUKOENCEPHALOMALACIA

Concentration of FB_1 administered	Duration	Number of animals Positive/Total	Reference
29.7 mg/kg body wt. 42.1 mg/kg body wt.	33 days 29 days	1/1 1/1	Kellerman *et al.*, 1990
< 1 – 22 ppm feed 8 ppm (mg/kg feed)	225 days 180 days	1/4 5/5	Wilson *et al.*, 1992

Fumonisins can cause ELEM or hepatotoxicosis depending on the dose; effects are time and concentration dependent. Abnormal blood parameters are elevations in total bilirubin, AST, GGT and total protein (Kellerman *et al.*, 1990; Wilkins *et al.*, 1994).

Riley *et al.* (1997) found that FB_2 was able to induce symptoms of ELEM in ponies but FB_3 did not even though both caused changes in sphingolipids. It has been hypothesised that disruption of sphingolipid metabolism is linked with development of ELEM.

2.6.3 Pigs

In pigs, consumption of feed contaminated with fumonsins is associated with PPE. Symptoms of PPE include acute onset of dyspnea, lethargy, weakness, increased respiratory rate, cyanosis and death; abortions; concurrent hydrothorax and pulmonary oedema (and in some cases icterus). Concentrations of fumonisins in feed associated with outbreaks of PPE ranged from 7 to 330 μg/g while feed from control farms showed very low or undetectable levels. The risk of PPE increased as the concentration of fumonisins increased; farms with ≥ 20 ppm fumonisins were at increased risk (Ross *et al.*, 1991B; Bane *et al.*, 1992; Osweiler *et al.*, 1992).

Injecting pigs intravenously with FB_1 or feeding them naturally or artificially contaminated corn screenings, caused decreased feed consumption, increased total bilirubin, cholesterol and liver enzymes (GGT, ALP, ALT, AST, arginase); mild to severe respiratory distress and interstitial pulmonary oedema with increased respiration rate and marked cyanosis as well as hepatic changes – disorganisation and necrosis of hepatocytes, loss of sinusoidal microvilli; pancreatic acinar cell degradation and the presence of membranous material in hepatic sinusoids and pulmonary intravascular macrophages. Increased free Sa and Sa/So ratios in the liver, lung and kidney before histopathological or serum biochemistry changes were observed. The hypothesis is that fumonisins cause altered sphingolipid metabolism, which damages the liver resulting in release of membranous material into the circulation.

This is phagocytosed by pulmonary macrophages triggering the release of mediators resulting in pulmonary oedema. (Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Colvin *et al.*, 1993; Riley *et al.*, 1993; Motelin *et al.*, 1994).

Since pulmonary oedema can be caused by congestive heart failure due to increased capillary pressure (Colvin *et al.*, 1993), investigations of cardiovascular function were done. Pigs fed culture material containing ≤ 20 mg/kg fumonisins daily for 7 days showed a significant increase in mean pulmonary artery pressure, decreased heart rate, cardiac output and decreases in both arterial and mixed venous oxygen tension but the electrocardiograph (ECG) was normal. (Smith *et al.*, 1996A, 1996B).

At lower doses (23 - 101 ppm or <16 mg FB_1/kg/day by intubation or 200 mg FB_1/ kg feed), the effect was of hepatic disease while at higher doses (175 ppm or >16 mg FB_1/kg/day by intubation) acute pulmonary oedema occurred. In some cases of liver failure, mild to moderate renal tubular necrosis occurred; reversibility of the hepatotoxic effects was also observed (Colvin *et al.*, 1993; Becker *et al.*, 1995). The NOEL for elevation of free Sa was 1- 10 ppm feed; based on liver enzymes was <12 ppm; based on liver histopathology was < 23 ppm and pulmonary oedema at 175 ppm. (Motelin *et al.*, 1994; Rotter *et al.*, 1996). These are within the range of FB_1 concentrations found in swine feed.

2.6.4 Poultry

Overall, poultry appear to be less susceptible to toxic effects of fumonisins than other animals. Day-old broiler chicks fed diets containing 100 to 400 or 75 to 525 mg FB_1/kg feed for 14 to 21 days showed gross effects of diarrhoea, significant decreases in body weights and average daily gain (or feed conversion) and increases in relative weights of liver, kidney, gizzard and proventriculus. Biliary hyperplasia, multifocal hepatic necrosis, muscle necrosis, intestinal goblet-cell hyperplasia and rickets occurred (Brown *et al.*, 1992; Ledoux *et al.*, 1992; Weibking *et al.*, 1993; Espada *et al.*, 1994).

Measured blood parameters showed increases in serum calcium, cholesterol, AST, GGT, LDH, creatine kinase (CK) but a decrease in triglycerides, uric acid and ALP (Ledoux *et al.*, 1992; Espada *et al.*, 1994). Mean cell haemoglobin, free Sa and Sa/So ratio (at 75 mg FB_1/kg feed) increased (Weibking *et al.*, 1993). Other workers found cytotoxic effects on

lymphocytes and erythrocytes (poikilocytosis) after feeding rations with fumonisins and moniliformin - possibly an additive or synergistic effect (Dombrink-Kurtzman *et al.*, 1993).

The higher the dose and/or the longer the feeding schedule, the more severe the effect. Lowest toxic doses are 10 mg/kg feed of pure FB_1 and 30 mg /kg of *F. moniliforme* culture material (Espada *et al.*, 1994). However, feeding lower levels (4 mg FB_1/kg feed) to laying hens for 28 days resulted in no significant lesions (Vudathala *et al.*, 1994). Inoculation of fertile eggs with pure FB_1 or culture material caused 50 to100% mortality, which was time and dose dependent. (Javed *et al.*, 1993). Zacharias *et al.* (1996) found a dose and time dependent correlation between the amount of fumonisins injected into the yolk sac and an increase in the ratio of Sa/So at levels of FB_1 >72 μg/egg for three or more days.

An outbreak of an unusual disease in laying hens characterised by diarrhoea, severe reduction of food intake, body weight and egg production with 10% mortality was concluded to have occurred due to the FB_1 (5.2 to 8.5 mg/kg) in the diet. Livers of affected hens were pale yellow with peripheral congestion, mild haemorrhage in the proventriculus and watery accumulations in the intestine; withdrawal of the contaminated diet resulted in a return to normal (Prathapkumar *et al.*, 1997).

Effects on turkey poults were also dose-dependent and similar to those on broiler chicks i.e. decreases in feed intake and body weight gains but increases in liver and pancreas weights and Sa/So ratios (≥ 25 mg FB_1/kg diet). Hepatocellular (all levels of FB_1, 25 to 475 mg/kg diet) and biliary (150 to 300 mg FB_1/kg diet) hyperplasia was evident (Weibking *et al.*, 1995; Kubena *et al.*, 1995, 1997; Ledoux *et al.*, 1996).

2.6.5 Other Animals

Diverse effects of fumonisins have been found in other animal species. **Baboons** fed culture material of *F. moniliforme* developed acute congestive heart failure and hepatic cirrhosis (Kriek *et al.*, 1981). **Vervet monkeys** developed hepatitis as shown by increased liver enzymes and histopathological changes (Jaskiewicz *et al.*, 1987A). Monkeys fed a low fat carbohydrate diet plus added fumonisins developed an atherogenic lipid profile [increased cholesterol, low density lipoprotein (LDL)-cholesterol, apoprotein B, enzymes (AST, ALT, GGT, ALP), albumin, factor VII and fibrinogen] probably secondary to chronic liver toxicity (Fincham *et al.*, 1992).

Of all animals **cattle** seem to be the least affected by fumonisins. After consuming diets containing fumonisins (148 or 400 μg/g FB_1; 130 μg/g FB_2) for 30 days they showed some signs of hepatobiliary change (increased cholesterol, bilirubin and enzymes) but no change in feed intake, body weights or temperature. However, the animals were languid and visibly distressed and ate their food sluggishly (Osweiler *et al.*, 1993; Smith & Thakur, 1996).

As in equidae, fumonisins caused central nervous system changes in **rabbits**. Doses of 1.75 mg/kg/day diet resulted in leukoencephalomalacia and haemorrhage in the brains of pregnant rabbits; hepatic and renal changes (predominantly apoptosis) were also observed (Bucci *et al.*, 1996). Orally dosed culture material with total fumonisins 11.1 to 45.5 mg/kg body weight caused hepato- and nephro-toxicity in **lambs** (Edrington *et al.*, 1995). Liver and kidney abnormalities were also found in **mink** (Restum *et al.*, 1995) and channel **catfish** (Lumlertdacha *et al.*, 1995).

2.6.6 Plants

Fumonisins are phytotoxic causing mosaic like patterns on leaves and inhibiting growth; higher concentrations caused plants to wilt and die. Other effects were electrolyte leakage, autolysis and photodegradation of chlorophyll; effects were light and concentration dependent. The A series were less toxic; the TCA groups seem to be necessary for herbicidal activity (Abbas *et al.*, 1991, 1992A, 1992B, 1993; Tanaka *et al.*, 1993). Addition of a100 ppm solution of FB_1 to germinating maize kernels inhibited radicle elongation of the maize seedlings by up to 75%. The mechanism appears to involve inhibition of amylases in the endosperm (Doehlert *et al.*, 1994).

2.7 TOXIC MECHANISMS

2.7.1 Carcinogenic

As shown by Lim *et al.* (1996) fumonisins can cause both cell proliferation and apoptosis *in vivo*; these two are key elements in carcinogenesis of nongenotoxic substances. Fumonisin B_1 also induced development of primary hepatocellular carcinoma and positive GGT and PSGT foci in rat liver proving it is a complete carcinogen (Gelderblom *et al.*, 1991,1996A; Lu *et al.*, 1997). Fumonisin B_1 is a poor cancer initiator but is not genotoxic. Its cancer-initiating ability is dependent on both dosage and duration of exposure and a balance exists between compensatory cell proliferation due to hepatotoxicity and inhibition of cell growth i.e. a critical dosage level exists for cancer initiation. An intact molecule and the presence of

a free amino group are required for cancer initiation. Cancer promotion (10 - 50 mg/kg diet) occurs at lower levels than initiation (100 - 250 mg/kg) and hepatotoxicity (Gelderblom *et al.*, 1992A, 1993, 1994, 1996A).

In studies of the mechanism of inhibition of cell proliferation *in vitro*, concentrations of 150 to 300 μM FB_1 for 44 hr in primary rat hepatocytes caused up to 90% inhibition of epidermal growth factor-induced DNA synthesis. Fumonisin B_1 exerted its mitoinhibitory effect during late G_1 phase and/or during S-phase at levels below those causing cytotoxicity; removal of FB_1 reversed the effect. (Gelderblom *et al.*, 1993, 1995, 1996A).

2.7.2 Sphingolipids

Sphingolipids are found in cellular and subcellular membranes of animals, plants and some lower eukaryotes e.g. yeast. The term sphingolipid covers the biochemical entities of sphingomyelins and glycosphingolipids (cerebrosides and gangliosides); all of which are derivatives of the base So. Sphingosine has a double bond between C-4 and C-5; Sa is dihydro-sphingosine; ceramides are precursors of sphingolipids. Fumonisins are structurally similar to the sphingoid bases (Figure 2.1).

Synthetic pathways are as follows:

(1) Palmitoyl-SCoA + Serine → 3-Ketosphinganine → Sphinganine + CoA-SH + CO_2

(2) Sphinganine + $NADP^+$ → Sphingosine + NADPH + H^+

(3) Sphinganine/sphingosine + RCO-SCoA → Dihydroceramide/ceramide + CoA-SH

Reaction (3) is catalysed by the enzyme ceramide synthase (sphinganine/sphingosine *N*-acyl transferase) which catalyses the addition of a fatty acid to the sphingoid base as part of the *de novo* synthesis of sphingolipids or in the re-acylation of long-chain bases from turnover of complex sphingolipids. Dietary sphingolipids are broken down to So and ceramides which may be absorbed or remain in the gastrointestinal tract; So and Sa are lipid soluble so can readily cross cell membranes and be found in the plasma or urine.

Sphingolipids are of crucial biological significance. Their functions include structural components of membranes giving rigidity (with cholesterol); regulation of normal cell growth and differentiation; increase intracellular calcium; possibly mediators in apoptosis; inhibitors/ activators of protein kinase C and other kinases/phosphatases and are responsible

for cell-cell and cell-matrix interactions with bioactive substances such as bacterial toxins, glycoprotein hormones, interferons, viruses (Bohinski, 1987; Yoo *et al.*, 1992; Merrill *et al.*, 1995B, 1996).

High performance liquid chromatography methods using OPA derivatives have been developed and optimised for measuring free So and free Sa as well as total sphingolipids in serum, urine, kidney and liver of rats. Complex sphingolipids were estimated as total minus free bases (Merrill *et al.*, 1988; Riley *et al.*, 1994). Since these methods are time-consuming and labour intensive, rapid methods which reduced the number of steps in extraction and clean-up; and also improved precision and accuracy were developed for analysis of Sa and So in cell cultures and serum (Yoo *et al.*, 1996B; Solfrizzo *et al.*, 1997; Castegnaro *et al.*, 1998).

Cell culture is a useful technique for studying the toxic effects of mycotoxins. Several workers using different cell lines investigated the effect of fumonisins on sphingolipids. In all cases, it was found that FB_1, even at levels as low as 1μM, inhibited ceramide synthase resulting in increased levels of free Sa and increased ratio of Sa/So. (Appendix 1, Table 2.13). The implications of cell culture studies are that toxic effects of fumonisins may occur by disrupting sphingolipid biosynthesis which affects the rigidity and function of cell membranes; this disruption may be a critical event in the toxicity of fumonisins. However, the effects of fumonisins were reversible as, after removal of the toxin, cells showed normal growth and morphology.

Experiments using β-chloroaniline, an inhibitor of serine palmitoyl transferase, only partially prevented the effects of fumonisin on cell growth and death because it exacerbated the depletion of more complex sphingolipids (Yoo *et al.*, 1996A). Working with Swiss 3T3 fibroblasts, Schroeder *et al.*, (1994) concluded that fumonisin B_1 stimulates DNA synthesis by disruption of sphingolipid metabolism and accumulation of sphingoid bases to a critical concentration for mitogenesis. Thus both increased sphinganine concentrations and decreased complex sphingolipid concentrations contribute to the cytotoxicity of FB_1 but the actual intracellular concentration of Sa, which is cytotoxic, has not been established *in vivo* (Voss *et al.*, 1996B).

The effects of FB_2, FB_3 and FB_4 were found to be similar to FB_1 but hydrolysed fumonisins lacking the TCA side chains, were less effective inhibitors while *N*-acetylated FB_1 did not block ceramide synthase (Norred *et al.*, 1992, 1997). In contrast, Voss *et al.*, (1998A) showed HFB_1 had the same effects as FB_1 on sphingolipid metabolism *in vivo* in rats. Measurement of sphingoid bases can be used as a biomarker for exposure to fumonisins as the changes in sphingolipids occurred before other markers e.g. elevation of serum liver enzymes or changes in tissue morphology (Wang *et al.*, 1992; Riley *et al.*, 1993, 1997; Voss *et al.*, 1995A, 1996B,1998A). Because of a significant correlation between the Sa/So ratio and the number of cells in the urine, Voss *et al.* (1996B) suggested using urine samples to check for recent or ongoing exposure to fumonisins.

Increased levels of free Sa and an increase in the ratio Sa/So was found in various animals and tissues; serum of **ponies** fed diets with FB_1 (15 - 44 μg/g), FB_2 and FB_3 (Wang *et al.*, 1992; Riley *et al.*, 1997); serum, liver, lung and kidney of **pigs** fed FB_1 and FB_2 (5 - 23 μg/g diet) (Riley *et al.*, 1993); urine, serum, liver and kidney of **rats** fed FB_1 (15-150 μg/g diet, 8-71 μg/g) (Riley *et al.*, 1994; Voss *et al.*, 1995A, 1996B, 1998A) and brain (FB_1 0.8 mg/kg/day) (Kwon *et al.*, 1997A, 1997B); also precision-cut liver (0.1 μM) and kidney slices (1 μM) (Norred *et al.*, 1996). Also in serum of **turkey poults** and day old broiler **chicks** fed FB_1 (75 μg/g diet) (Weibking *et al.*, 1993, 1995).

2.7.3 Other Mechanisms

Although disruption of sphingolipid metabolism appears to be the major route of fumonisin action at a molecular level, how this translates into its toxic and carcinogenic effects is not clear. Workers have investigated the influence of fumonisins on various enzymes and proteins but whether the effect is directly due to fumonisins or due to increased concentrations of sphingoid bases alone or in conjunction with fumonisins is not known. The results of several experiments are summarised below.

Fumonisin B_1 may alter signal transduction pathways since it inhibits **protein kinase C** (PKC) activity in monkey kidney CV-1 cells but stimulates transient and rapid activation of **mitogen-activated protein kinase** in Swiss 3T3 fibroblasts. Sphingosine is a potent inhibitor of PKC which phosphorylates nuclear proteins (Huang *et al.*, 1995; Wattenberg *et al.*, 1996). By inhibiting dephosphorylation of the enzymes, FB_1 inhibited five **protein serine/threonine phosphatases**.

It is postulated this leads to genetic alterations (Fukuda *et al.*, 1996). Fumonisins and sphingoid bases interacted differently with **GTP-binding proteins** in rat brain plasma membranes and bovine retina rod outer segments. The binding was calcium dependent which suggests a role of calmodulin-calcium in the interactions. (Ho *et al.*, 1996). In monkey kidney CV-1 cells but not monkey kidney COS-7 cells, FB_1 affected **cell-cycle regulatory proteins** (Ciacci-Zanella *et al.*, 1998).

Fumonisin is mitogenic or inhibitory to cell proliferation i.e. can cause stimulation or inhibition of DNA synthesis, as has been shown both *in vivo* and *in vitro*. The presence of insulin augments stimulation of DNA synthesis in Swiss 3T3 cells both by fumonisin as well as by Sa and So. This led Schroeder *et al.* (1994) to postulate that mitogenesis is due to direct action of the sphingoid bases whose intracellular concentration has been increased by the action of fumonisin. The bases might act via inhibition of PKC or phosphatidic acid phosphohydrolase or Na/K-ATPase or activation of epidermal growth factor receptor kinase or other protein kinases (Schroeder *et al.*, 1994; Lim *et al.*, 1996).

Fumonisins can affect **lipid biosynthesis** and alter the pattern and types of lipids produced *in vivo* in livers of rats. Gelderblom *et al.* (1997) found serum cholesterol and liver cholesterol and phosphatidylethanolamine were increased; liver sphingomyelin was decreased and there were changes in the types of fatty acids synthesised. Alterations in the lipids of yeast cells appear to be mediated by sphingoid bases as these inhibited key enzymes in the lipid biosynthetic pathways (Wu *et al.*, 1995).

2.8 ABSORPTION AND EXCRETION

Since it is known that consumption of feed contaminated with fumonisins causes effects at molecular levels, fumonisins must be absorbed and excreted. Cytotoxicity studies led Gelderblom *et al.*, (1993) to surmise that the TCA moiety is needed for absorption of fumonisins; and the biological activities of the fumonisins depends on their polarity, the presence/absence of a free amino group and possibly the specific location of a hydroxyl group. In studies using a variety of animals and feeding them unlabelled or radiolabelled FB_1 (or FB_2) the toxicokinetics were investigated (Tables 2.14 and 2.15).

In **rats** FB_1 was rapidly absorbed after intraperitoneal injection, with rapid equilibrium of FB_1 between body tissues and rapid elimination from plasma via the bile (67% of the total dose); 88% of the bile excretion occurred in the first 4 hr persisting for up to 24 hr. Oral dosing resulted in far less of the toxin appearing in the urine which implies either poor absorption from the gut or excretion of FB_1 by the liver (biliary) as a first pass effect. Most of the FB_1 was excreted in faeces and urine within 24 hr with no major retention in the tissues (Table 2.14).

The recovered FB_1 was unmetabolised; it seemed that after oral administration there was little enterohepatic circulation. In male BD IX rats excretion followed first order kinetics; the calculated t½ was18 min for FB_1 and 26 min for FB_2. The toxicokinetics of FB_2 were similar to FB_1 – both were cleared rapidly after intraperitoneal injection; the FB_2 recovered was unmetabolised (Shephard *et al.*, 1992A, 1992C, 1994B, 1995A, 1995B). After intragastric dosing to fasting rats, Norred *et al.* (1993) concluded that FB_1 was absorbed from the gut and was retained in target tissues (liver and kidney) possibly by binding to an enzyme (perhaps ceramide transferase).

Unlike rats, after intravenous injection of unlabelled FB_1 to **vervet monkeys,** both unmetabolised (11.7% and 19%) and partially hydrolysed (27.4% and 9.6%) FB_1 was recovered in the faeces (<0.1% as the aminopentol) and only unmetabolised FB_1 in the urine. The t½ was 40 min. Seventy-two hours after oral administration, most of the labelled FB_1 was found in the faeces as both unmetabolised (35.2% and 39.2%) and partially (27.1% and 27.8%) and fully hydrolysed FB_1 (Table 2.15). The partially hydrolysed FB_1 was an equilibrium mixture of the two mono-esters and was formed either in the liver (biliary excretion) or by intestinal enzymes or micro-organisms. After oral dosing only 76% of total radioactivity could be accounted for; and after intravenous injection < 50%.(Shephard *et al.*, 1994A, 1994C).

In **swine**, after intravenous injection of radiolabelled FB_1 (total recovery 79.5% to 88.4%), large amounts were recovered in the bile of cannulated animals but for others excretion was mainly via the faeces. The calculated half-lives were $t_{½\alpha}$ 2.2 min for initial phase; $t_{½\beta}$ 10.5 min for distribution phase; and $t_{½\gamma}$ 182.6 min for elimination phase. After intragastric dosing, (total recovery 91.4% to 91.7%), most of the FB_1 was excreted in the faeces and only a small amount in the bile of cannulated pigs.

The authors concluded that although FB_1 has poor oral availability, a fraction was absorbed, distributed and remained in the tissues for an extended period despite enterohepatic cycling. The implications are that FB_1 may accumulate in the tissues if consumed over a period of time (Prelusky *et al.*, 1994) (Table 2.15).

TABLE 2.14 EXCRETION OF FUMONISIN B_1/FUMONISIN B_2 IN RATS

Animal	Route of administration of FB_1/ FB_2	Recovery	Reference
Male BD IX rats	Intraperitoneal injection, Unlabelled FB_1	Urine 16.6% (24 hr) Urine 15.3% (48 hr)	Shephard *et al.*, 1992A
Male BD IX rats	Oral (gavage), Unlabelled FB_1	Urine <0.4% (24 hr)	Shephard *et al.*, 1992A
Male BD IX rats	Intraperitoneal injection, ^{14}C-FB_1	Faeces 66% (24 hr) Urine 32% (24 hr) Liver 1± 0.4% (24 hr) Kidney, erythrocytes <1% Plasma, heart, brain <0.5%	Shephard *et al.*, 1992C
Male BD IX rats	Gavage, ^{14}C-FB_1	Faeces 101%	Shephard *et al.*, 1992C
Male Sprague-Dawley rats	Intragastric (stomach tube), ^{14}C-FB_1	Faeces 80% (48 hr) Urine 2 - 3% (24 hr) Liver 0.5% (4 hr) Liver, blood, kidney - trace (96 hr)	Norred *et al.*, 1993
Male Sprague-Dawley rats	Intravenous (tail vein) injection, ^{14}C-FB_1	Faeces 35% Urine 10% Plasma 2% (96 hr) Liver 25% (96 hr) Kidney 10 - 12% (96 hr)	Norred *et al.*, 1993
Male Wistar rats	Intraperitoneal injection, ^{14}C-FB_1	Bile 67% (24 hr)	Shephard *et al.*, 1994B
Male Wistar rats	Gavage, ^{14}C-FB_1	Bile 0.2% (24 hr)	Shephard *et al.*, 1994B
Male BD IX rats	Intraperitoneal injection, Unlabelled FB_2	Faeces 84.1% (24 hr) Urine 1.2% (24 hr) Plasma – undetectable (24 and 72 hr)	Shephard *et al.*, 1995A, 1995B
Male BD IX rats	Oral (gavage), Unlabelled FB_2	Urine - trace (24 hr) Plasma - undetectable (detection limit 20 ng/ml)	Shephard *et al.*, 1995A, 1995B
Male BD IX rats	Gavage, ^{14}C-FB_2	Faeces 98.9% Urine 1%	Shephard *et al.*, 1995A, 1995B
Pregnant female Sprague-Dawley rats	Intravenous injection (dorsal metatarsal vein), ^{14}C-FB_1	Gastro-intestinal tissues and contents 45% (1 hr) Blood 1.2% (1 hr) Liver 14.5% (1 hr) Kidney 4% (1 hr) Uterus 0.24 - 0.44% (1 hr) Placenta 0.13 - 0.22% (1 hr) Fetuses ≤ 0.01% (1 hr) Skin, fat, skeletal muscle - variable but high	Voss *et al.*, 1996C

TABLE 2.15 EXCRETION OF FUMONISIN B_1 IN VARIOUS ANIMALS

Animal	Route of administration of FB_1	Recovery	Reference
Pigs (Yorkshire barrows)	Intravenous injection (jugular vein); ^{14}C-FB_1	Faeces 58.3% (72 hr) Urine 21.2% (72 hr)	Prelusky *et al.*, 1994
Pigs (Yorkshire barrows)	As above but gall bladder cannulated	Faeces 1.5% (72 hr) Urine 16.2% (72 hr) Bile 70.8% (72 hr) Liver 10% (72 hr) Kidney <1% (72 hr) Muscle 6.5% (72 hr.	Prelusky *et al.*, 1994
Pigs (Yorkshire barrows)	Intragastric; ^{14}C-FB_1	Faeces 90.8% (72 hr) Urine 0.6% (72 hr)	Prelusky *et al.*, 1994
Pigs (Yorkshire barrows)	As above but gall bladder cannulated	Faeces 89.8% (72 hr) Urine 0.4 - 12% (72 hr) Bile 0.7 - 1.7% (72 hr)	Prelusky *et al.*, 1994
White Leghorn laying hens	Oral (crop intubation); ^{14}C-FB_1	Excreta (faeces and urine) 96.6%	Vudathala *et al.*, 1994
White Leghorn laying hens	Intravenous (wing vein); ^{14}C-FB_1	Excreta (faeces and urine) 98.6%	Vudathala *et al.*, 1994
Holstein steers	Feeding for 30 d (Detection limit 30 ng/ml)	Faeces ≥ 80% Urine trace Blood 0.005% (30 min) Liver 2 100 ng/g Kidneys 23.4 ng/g Muscle 97 ng/g	Smith & Thakur, 1996
Two female vervet monkeys	Intravenous injection; Unlabelled FB_1	Faeces (72.6%; 72.0%) Urine (5.9%; 20.3%)	Shephard *et al.*, 1994A, 1994C
Two female vervet monkeys	Gavage; ^{14}C-FB_1	Faeces 61% (3 d) Urine 1.2% (3 d) Bile 0.1% Contents of intestine 12% Liver 0.4% Skeletal muscle 1% Bile, plasma, kidney, heart, erythrocytes, 0.1% each Brain 0.2%	Shephard *et al.*, 1994A, 1994C

After both oral dosing and intravenous injection of **laying hens**, most of the FB_1 was found in the excreta (faeces and urine). The pharmacokinetic parameters ($t_{½\alpha}$ 2.5 min for distribution; and $t_{½\beta}$ 48.8 min for elimination) indicated limited systemic absorption and poor but rapid distribution and clearance from the plasma (only trace levels detected 3 hr after dosing). Most of the toxin was excreted between 2 - 6 hr post dosing and 92 - 104% was eliminated after 24 hr. In contrast to other animals tissue residues were low (Vudathala *et al.*, 1994) (Table 2.15).

As with other animals most of the FB_1 fed to **cattle** were eliminated unmetabolised via the faeces and urine (Table 2.15). *In vitro* experiments showed 35% degradation of FB_1 in rumen fluid over 9 hr; possibly by deamination or engulfment by protozoa since no partially or fully hydrolysed metabolites of fumonisin were found. The authors postulated that fumonisins were absorbed through the rumen wall and eliminated rapidly through the biliary system. Lack of de-esterification means fumonisins remain negatively charged and not lipophilic, and hence difficult to absorb through the gut walls. The relative low susceptibility of cattle to FB_1 may be because it is diluted in the rumen by the large volume and copious amounts of saliva (Smith & Thakur, 1996). Fumonisins were not excreted in the milk of lactating cows (Hammer *et al.*, 1996; Richard *et al.*, 1996).

2.9 DE-TOXIFICATION

Because of the risk of fumonisin-related mycotoxicoses in humans and animals, various methods of de-toxification of infected corn have been investigated. Using cell culture techniques, it was found that the less polar the molecule, the higher its cytotoxicity i.e. $FB_2 > FB_3 > FB_1$ and the *N*-acetyl derivatives were less cytotoxic than parent molecules (indicating a cytotoxic role for the free amino group) while the hydrolysis products after removal of the TCA group were the most cytotoxic of all. These structural differences should be taken into account when devising decontamination strategies (Abbas *et al.*, 1993; Gelderblom *et al.*, 1993).

Lu *et al.* (1997) after reacting the amine group of FB_1 with **fructose,** found FB_1-induced hepatotoxicity in rats was prevented and liver-associated natural killer cell activity was increased. This adds further evidence to the need of the amino group for toxicity.

In investigations of feed associated with ELEM and PPE, the highest levels of FB_1 and the highest rate of mycotoxicosis comes from corn screenings (Ross *et al.*, 1991B; Osweiler *et al.*, 1992). Simply **removing "fines"** from bulk shipments of maize gave a 26.2 to 69.4 % reduction in total fumonisin levels (Sydenham *et al.*, 1994). Other possible preventative strategies would be breeding ***F. moniliforme* resistant corn** or use of **uncontaminated seed** (Badria *et al.*, 1996); or use of **antagonistic rhizobacteria** applied to corn kernels at planting (Bacon & Williamson, 1992).

Atmospheric ammoniation is ineffective as a decontaminant because although it reduced FB_1 levels by 45% in naturally contaminated corn and by 30% in culture material, they were still toxic to rats (Norred *et al.*, 1991; Voss *et al.*,1992). In contrast, Park *et al.*(1992), found 79% reduction in FB_1 levels after ammonia treatment at high pressure/ambient temperature and/or low pressure/high temperatures but reaction products were not investigated for toxicity.

Alkaline hydrolysis (nixtamalisation) is also ineffective as a de-toxification method because although fumonisin-contaminated corn treated with 0.1 M calcium hydroxide gave a 74.1% transformation of FB_1 to AP_1 (Sydenham *et al.*, 1995A, 1995B), hydrolysed fumonisins also inhibit ceramide synthase *in vitro* (Merrill *et al.*, 1993A) and *in vivo* (Voss *et al.*, 1998A). Nixtamalised *F. moniliforme* culture material containing 58 ppm of hydrolysed FB_1 proved to be hepatotoxic and nephrotoxic to rats (Voss *et al.*, 1996A, 1998A).

Water extraction (washing of freeze-dried culture material with deionised water) was more effective. The FB_1 content was reduced from 1 420 to 271 ppm and only induced mild hepatopathy in one rat; otherwise mild to moderate nephropathy. Water extracted culture material was less toxic and unaltered culture material was more toxic than the nixtamalised material (Voss *et al.*, 1996A, 1998A).

Heat is another possibility for decontamination but FB_1 is thermostable at temperatures used in the main drying processes of corn (75 to150°C) and the usual conditions of baking or frying (Dupuy *et al.*, 1993; Jackson *et al.*, 1997). Heating corn meal at 220°C for 25 min caused complete loss of FB_1 and FB_2 (possibly due to chemical decomposition) but 60 min at 190°C resulted in recoveries of 40% (Scott & Lawrence, 1994A). Heating milk at 62°C for 30 min (to imitate pasteurisation) did not significantly reduce the measured concentrations of FB_1 or FB_2 (Maragos & Richard, 1994). In an aqueous model system (to remove matrix effects), 90% of FB_1 was decomposed to hydrolysis products after 60 min at $\geq$ 175°C regardless of pH. Fumonisin B_2 gave similar results. (Jackson *et al.*,1996A, 1996B).

Gamma irradiation of maize flour decreased fumonisin content by only about 20% and fumonisins were stable in irradiated maize for 4 weeks at 40°C and at least 6 months at 25°C (Visconti *et al.*, 1996B).

More effective were **activated carbons** which removed about 95% of FB_1 and about 99% of aflatoxin B_1 from an aqueous extract of naturally contaminated corn (Galvano *et al.*, 1997).

2.10 HEALTH RISKS

The public are largely ignorant about mycotoxins yet they might be responsible for many unexplained diseases including cancer, especially in countries with hot, humid conditions favouring fungal growth; and inadequate storage facilities. The potential for exposure of animals and humans to fumonisins is a world-wide problem since maize and maize based products are a dietary staple for humans and animals particularly in developing countries. Poor people are often forced to eat mouldy grain or starve as they do not have the money to purchase good grain. Daily maize intakes in Eastern and Southern Africa average 200 g/person/day (up to 470 g) which translates to 245 μg of fumonisins/day or 14 μg FB_1/kg body weight/day (Thiel *et al.*, 1992; Badria *et al.*, 1996; Doko *et al.*, 1996).

Fusarium moniliforme (which is tasteless and not always visible) and fumonisins are found on both mouldy and physically sound maize so visual assessment followed by separation of ears into two groups does not ensure that maize used for human consumption is free of mycotoxins. Drinking beer made from mouldy maize probably results in exposure to high levels of fumonisins (Sydenham *et al.*, 1990B). Fusaric acid has a synergistic toxic response with FB_1 implying that the combination of these toxins may be causing enhanced and unpredicted toxicity in animals and humans (Bacon *et al.*, 1995). Generally whole grain and products that undergo little/mild processing retain highest levels of fumonisins (Patel *et al.*, 1997) and products for animal consumption have higher levels and a higher incidence of fumonisins than those for humans (Rava, 1996; Perilla & Diaz, 1998). Some of the highest recorded levels of FB_1 from naturally infected corn are 117.52 μg/g (South Africa) and 250 μg/g (Sardinia) (Rheeder *et al.*, 1992; Bottalico *et al.*, 1995).

Levels of fumonisins (FB_1 and FB_2) in commercially available maize-based foods for human consumption ranged from <100 to 600 ng/g in South Africa but from <1000 to 4 000 ng/g in the USA (Thiel *et al.*, 1992).

TABLE 2.16 HIGHEST NATURALLY OCCURRING LEVELS OF FUMONISIN B_1

Type of Sample	Country	FB_1 (µg/g)	Reference
Rain affected maize	India	65.0	Shetty *et al.*, 1997B
Maize	USA	37.9	Murphy *et al.*, 1993
Maize-based mixed feeds	USA	16.8	Sydenham *et al.*, 1992
White/yellow maize	South Africa	12.963	Rava , 1996
Maize screenings	South Africa	8.878	Rava *et al.*, 1996A, Rava, 1996
Maize kernel	China	8.4	Ueno *et al.*, 1993
Maize	Argentina	6.695	Sydenham *et al.*, 1993
Puffed corn	Italy	6.1	Doko & Visconti, 1994
Maize	India	5.0	Shetty *et al.*, 1997B
Maize-based products for humans	Egypt	2.98	Sydenham *et al.*, 1991
Maize-based animal feed	Columbia	2.964	Perilla & Diaz, 1998
Maize meal for humans	USA	2.79	Sydenham *et al.*, 1991
Maize kernels	Zimbabwe	2.735	Doko *et al.*, 1996
Corn-grits	Japan	2.6	Ueno *et al.*, 1993
Corn-grits	USA	2.545	Sydenham *et al.*, 1991
Polenta	United Kingdom	2.124	Patel *et al.*, 1997
Yellow maize animal feed	South Africa	1.993	Rava *et al.*, 1996, Rava, 1996
Maize meal	Zimbabwe	1.91	Doko *et al.*, 1996
Maize kernels	Kenya	0.78	Doko *et al.*, 1996
Sorghum meal	India	0.51	Shetty *et al.*, 1997B
Poultry feed	India	0.26	Shetty *et al.*, 1997B
Corn-grits	South Africa	0.19	Sydenham *et al.*, 1991

(For the purpose of comparison, values for concentrations of FB_1 (Table 2.16) were adjusted to µg/g).

TABLE 2.17 LOWEST TOXIC DIETARY LEVELS OF FUMONISIN B_1

Animal	Effect	FB_1 (µg/g)	Reference
Humans (South Africa)	High risk area for OC	1.6 (0.05 - 7.9) good corn 23.9 (3.45 - 46.9) mouldy corn	Rheeder *et al.*, 1992
Humans (China)	High risk area for OC	35.3 (20 - 60) good corn 74 (18 - 155) mouldy corn	Chu & Li, 1994
Humans (USA)	High risk area for OC	0.1 - 1.9	Sydenham *et al.*, 1991
Rodents	Hepatotoxicity	15	Voss *et al.*, 1993, 1995A
Rodents	Nephrotoxicity	3 - 27	Voss *et al.*, 1995B
Equidae	ELEM	1.3 - 27	Thiel *et al.*, 1991B
Pigs	Hepatotoxicity	12 - 23	Motelin *et al.*, 1994
Pigs	PPE	7 – 55	Bane *et al.*, 1992
Poultry	Diarrhoea; Hepatic abnormalities	5.2	Prathapkumar *et al.*, 1997

Comparing the highest naturally occurring levels of FB_1 with the lowest toxic dietary levels (Tables 2.16 and 2.17) clearly shows that worldwide humans and animals are at risk of developing fumonisin-related mycotoxicoses. Concentrations of FB_1 in corn/ maize and corn-based products ranged from 0.26 to 37.9 μg/g in asymptomatic samples. Toxic dietary concentrations of FB_1 ranged from 0.1 to 60 μg/g . Although there are different toxic effects in different animals, all animals gave symptoms of liver damage. In 75% of ELEM cases and 71% of PPE, the level of FB_1 was >10 μg/g but these may not be the lowest levels causing the mycotoxicosis (Pestka *et al.*, 1994).

In assessment of potential toxicity, it is important to take into account the time of exposure as well as the concentration. The effective dosage level of FB_1 for cancer initiation in rats was 30.8 mg FB_1/100 g body weight for 21 days; whereas 29.7 mg FB_1/100 g body weight for 7 days did not initiate cancer (Gelderblom *et al.*, 1994).

Because fumonisins have been found in commercially available corn-based foodstuffs for human consumption in many countries in the world, humans are at risk of toxic effects through ingestion of these products (Ueno *et al.*, 1993; Doko & Visconti, 1994; Rava,1996). However, reported results for corn-based foods should be treated with caution as recoveries differ with different matrices; control recoveries should be done for each type of food (Scott & Lawrence, 1994). In measuring levels to elucidate health risks, care must be taken not to underestimate (possible by HPLC) or overestimate (possible by ELISA) the fumonisin concentration – bearing in mind that FB_2, FB_3 and hydrolysis products are also toxic (Gelderblom *et al.*, 1993; Voss *et al.*, 1998A).

In addition to the risk of OC, consumption of fumonisins seems to pose a risk of secondary vascular disease caused by elevated LDL-cholesterol and apoprotein B (Fincham *et al.*, 1992). Hence, lipid profiles should probably be done in people consuming food contaminated with fumonisins.

Strategies to reduce human (and animal) exposure to fumonisins include prevention by use of resistant maize varieties (Nelson *et al.*, 1993), improving agricultural and storage practices, detoxification and fortifying the staple food viz. maize with appropriate minerals and vitamins (Rose, 1982; Marasas *et al.*, 1993). In experiments with various cell lines, Badria *et al.*, (1996) concluded that the only structural features needed for biological activity

are a long alkyl chain, a free amino group and probably a hydroxyl group on an adjacent carbon atom.

This means that the fumonisin molecule can be changed extensively without destroying its toxicity and normal cooking procedures are unlikely to reduce the potential threat of toxic effects.

Concerns may be raised that fumonisins ingested by animals could be passed on to humans or animals. The carry-over rate of fumonisins into milk was negligible and neither FB_1, AP_1 nor their conjugates were detected in milk from cows dosed orally or intravenously with FB_1 (Scott *et al.*, 1994B; Hammer *et al.*, 1996).

Various biomarkers to detect exposure to fumonisins are used including serum enzymes associated with liver abnormalities (GGT, LD, AST), total cholesterol (a relatively easy assay), So and Sa and the Sa/So ratio. However, Castegnaro *et al.* (1998) found no significant differences in Sa and So results between controls and OC patients nor between untreated and treated rats (oral daily dose of 1 mg FB_1/kg body weight 5 days a week for 5 weeks). Interestingly, healthy South African Asian females had higher serum So levels and lower ratios than their French counterparts; also, there were large inter-individual variations in the ratio. Thus, it seems that genetic and environmental factors are of importance in interpreting results.

"The Pan African and worldwide contamination of maize with the fumonisins makes it imperative that realistic, scientifically sound and economically reasonable tolerance levels for maize should be established as soon as possible" (Marasas *et al.*, 1993).
At present recommended amounts are <5 μg/g in the non-roughage portion of feed for horses and <10 μg/g for pigs (Miller *et al.*, 1996). For humans there are no current regulations but levels > 1 μg/g are of concern (Perilla & Diaz, 1998).

CHAPTER 3

PRODUCTION OF POLYCLONAL ANTIBODIES TO FUMONISIN B_1

3.1 INTRODUCTION

Antibodies against fumonisin (as opposed to kits for analysis) are not readily commercially available so the first step was to attempt to raise them. Antibodies are produced when a complex non-self or foreign molecule (the immunogen) stimulates the innate humoral immune response mechanism in an animal. The immunogen is processed by the lymphoid system; macrophages ingest and enzymatically degrade the immunogen to smaller fragments. A small amount of immunogenic material is retained or received at the macrophage cell surface and on contact activates B-cell (or T-cell) lymphocytes. The activated B-cells give rise to effector cells (which remain in the lymph node) and memory cells (which circulate in the blood). Individual B-cells proliferate and differentiate to form a clone of plasma cells that produce identical antibodies (immunoglobulins) with specificity for a particular epitope. An epitope is the smallest biochemical unit capable of stimulating formation of a specific antibody; complex molecules like proteins contain many different epitopes so a heterogeneous collection of antibody molecules will be produced in an immune response i.e. polyclonal antiserum. Smaller molecules, termed haptens (molecular weight less than 5 000 Daltons), have fewer epitopes so are not immunogenic in themselves but have to first be covalently linked to a larger more complex carrier molecule – usually a protein.

Animals are injected with the immunogen and then given booster injections at suitable time intervals. Initially the antibodies are immunoglobulin M (IgM) but later molecules of the G class (IgG) are produced (Ritchie, 1986; Crowther, 1995).

Since FB_1 is a hapten (molecular mass 721.8 Daltons) it was necessary to first link it to a carrier protein, KLH, to make an immunogenic molecule. Keyhole limpet hemocyanin is an oxygen carrying copper protein derived from an invertebrate, the mollusc *Megathura crenulata* with a molecular mass of 3 - 7.5 x 10^6. It is often used as a carrier as it is immunogenic in its own right but is also easy to conjugate to haptens since it contains many lysine residues. It has good solubility in water and buffers. Glutaraldehyde is a bifunctional reagent linking through an amine group – as is found in FB_1. The reaction mechanism is poorly understood (Boehringer Mannheim, Germany, package insert; Merck Index 1996; http://www.applied biosystems.com).

Both polyclonal and monoclonal antibodies against fumonisins (usually FB_1) have been raised. To form the immunogen, FB_1 was linked to various carrier proteins (CT, KLH, BSA and OVA) usually by the glutaraldehyde method (Azcona-Olivera *et al.*, 1992A, 1992B; Fukuda *et al.*, 1994; Usleber *et al.*, 1994; Abouzied *et al.*, 1996; Chu, 1996; Yeung *et al.*, 1996; Yu & Chu, 1996). Briand *et al.*, (1985) found that using 1% glutaraldehyde gave stable conjugates but the coupling reaction may affect the activity of the antigen.

3.1.1 Objectives

1. To prepare a FB_1-KLH conjugate to use as an immunogen.
2. To immunise chickens and rabbits and collect eggs and blood samples respectively.
3. To extract immunoglobulin Y (IgY) from the eggs and immunoglobulin G (IgG) from serum and measure the protein concentrations of all batches.
4. To optimise an indirect ELISA method to check for antibody titre.
5. To check all IgY and IgG extracts for antibody titre.

3.1.2 Ethical Approval

Ethical approval for working with animals (chickens and rabbits) was obtained from Dr. T.H.T. Coetzer, convenor of the Research Committee for Animal Ethics, University of Natal, Pietermaritzburg.

At all times the animals were handled humanely and under the supervision of Dr. Coetzer.

3.2 MATERIALS

3.2.1 Chemicals

Fumonisin B_1, Freund's Adjuvant-Complete, Freund's Adjuvant-Incomplete, Horseradish Peroxidase conjugated Rabbit Anti-Chicken IgG, Horseradish Peroxidase conjugated Goat Anti-Rabbit IgG and ABTS (2,2'-Azino-bis (3- ethyl)benzthiazoline-6-sulphonic acid) were purchased from Sigma Chemical Co., USA.

Keyhole limpet hemocyanin, Precinorm U and bovine serum albumin, Fraction V, were purchased from Boehringer Mannheim, Germany.

Polyethylene glycol 6000, casein, Tween 20 and Parafilm were from Merck and H_2O_2 from Polychem.

Serum Protein Electrophoresis kit was purchased from Beckman; Humatrol N and Humatrol P from S.A. Scientific.

Pre-immunisation rabbit IgG and a horseradish peroxidase enzyme-labelled second antibody for chickens were kindly donated by Dr. T.H.T. Coetzer, Department of Biochemistry, University of Natal, Pietermaritzburg.

Other chemicals (all Analar grade) were from Saarchem.

3.2.2 Buffers/Solutions

The following buffers were used in the production of polyclonal antibodies:

0.1M Sodium carbonate buffer (pH 8.5)	(Appendix 2, 2.1)
Phosphate buffered saline (PBS) (pH 7.2) (10x)	(Appendix 2, 2.2)
0.1M (100mM) Sodium phosphate buffer (pH 7.6), 0.02% sodium azide	(Appendix 2, 2.3)
Borate buffered saline (pH 8.6)	(Appendix 2, 2.4)
0.1M Carbonate buffer (pH 9.6)	(Appendix 2, 2.5)
0.15M Citrate-phosphate buffer (pH 5.0)	(Appendix 2, 2.6)
Stopping buffer: 0.1% sodium azide in citrate-phosphate buffer	(Appendix 2, 2.7)

3.2.3 Fumonisin B_1 Stock Standards

Fumonisin B_1 (1 mg) was dissolved in 10 ml PBS (pH 7.2) to give a final concentration of 100 µg/ml. Aliquots (125 µl, 10 µg/100 µl) were stored in Eppendorf micro test tubes (AEC Amersham) and sealed with Parafilm (Merck) at either 4°C or – 20°C.

3.3 METHODS

3.3.1 Preparation of Immunogen viz. Fumonisin B_1-Keyhole limpet hemocyanin conjugate (FB_1-KLH)

This was based on the method of Usleber *et al.* (1994). 33.3 mg of KLH (30% protein) was dissolved in 1ml 0.1 M sodium carbonate buffer (pH 8.5) and the concentration checked by measuring absorbance at 280 nm (Milton Roy Spectronic 3 000 Spectrophotometer). The solution was dialysed (Spectra/Por cellulose membrane dialysis tubing, molecular weight cut-off (MWCO) 12 000 Daltons or greater, capacity approximately 10 ml/ft., 10 mm flat width, 6 mm diameter, Sigma Chemical Co., USA) against 2 x 500 ml PBS (pH 7.2) first for 2 hr then overnight at 4°C and the protein concentration checked again. 20 µl glutaraldehyde (1% in water) was added to 1 mg FB_1 and incubated at room temperature (RT) for 2 hr. To this, 800 µl of KLH solution was added, mixed and incubated at RT for 3 hr, mixing every 30 min. The conjugate was dialysed against PBS (3 x 1 000 ml) at 4°C. The protein concentration was determined by reading absorbance at 280 nm of a 1:50 dilution (in PBS).

The conjugate was aliquoted to give 250 μg per aliquot, and stored in sealed Eppendorf tubes at –70°C.

3.3.2 **Immunisation of Chickens and Rabbits**

Two White Amberlink chickens (Ukulinga Research Farm, University of Natal, Pietermaritzburg) and two female rabbits of mixed breed, one brown and one black (supplied by the Biochemistry Department, University of Natal, Pietermaritzburg) were used to raise antibodies. All animals were kept in cages, treated humanely and given free access to food and water at all times (Figures 3.1, 3.2 and 3.3).

FIGURE 3.1 CHICKEN 1 (SPECKLED) & CHICKEN 2 (WHITE)

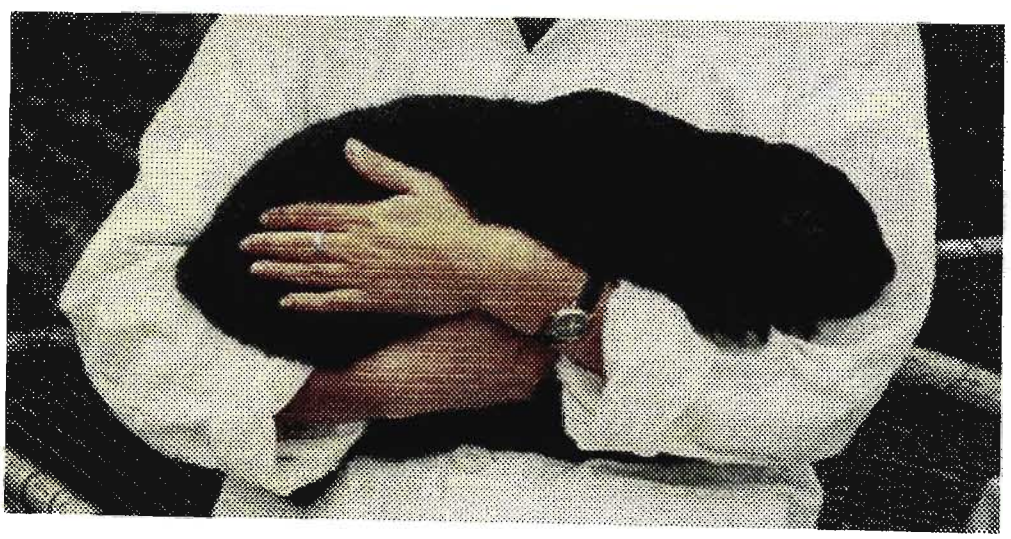


FIGURE 3.2 RABBIT 1 (BLACK)

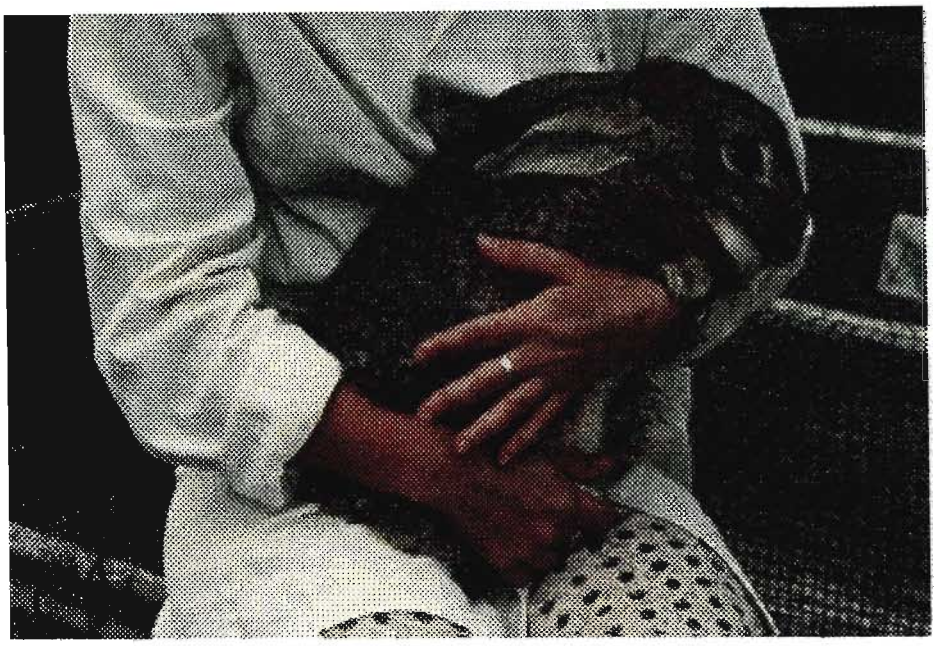


FIGURE 3.3 RABBIT 2 (BROWN)

The same immunisation schedule was followed for chickens and rabbits viz. initial injection at week 0 and boosters at weeks 2, 6 and 10. Emulsions (about 2 ml for two animals) of KLH-FB_1 and adjuvant were freshly prepared; animals were injected with about 1 ml each. The emulsions contained 2 aliquots (500 µg) of the immunogen plus 1.2 ml Freund's adjuvant i.e 250 µg KLH-FB_1 per animal per injection. At week 0 complete Freund's adjuvant was used and at weeks 2, 6 and 10 incomplete Freund's adjuvant. Chickens were injected intramuscularly on either side of the breastbone; rabbits were injected subcutaneously behind the neck.

Eggs were collected daily, dated and stored at 4°C. Blood was collected from the ear vein of the rabbits at weeks 4, 8 and 12. After standing overnight at 4°C serum was separated by centrifuging twice for 10 minutes at 1 410 g (3 000 rpm) at RT. For pre-immunisation samples, eggs and blood were collected during the week prior to the first injection.

3.3.3 Extraction of Immunoglobulin Y from Egg Yolks

Eggs were sorted into weekly batches (3 - 7 eggs/week) and extraction of IgY was done per batch. Egg white was separated from yolks, the yolks washed with distilled water and the combined egg yolk volume measured. Two volumes of 100 mM sodium phosphate buffer, pH 7.6, were added and polyethylene glycol 6 000 (PEG) 3.5% (m/v) was dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation at 4 420 g for 30 min at RT (Beckman J2-21 refrigerated centrifuge with JA-17 rotor).

After slowly filtering the supernatant through absorbent cotton wool (to give a clear solution free of lipids) PEG (8.5% m/v) was added (giving a 12% solution) which was mixed thoroughly and centrifuged at 12 000 g for 10 min at RT. The pellet was redissolved in the buffer in a volume equal to the volume obtained after filtration. Polyethylene glycol 6 000 (12% m/v) was added, mixed and centrifuged again at 12 000 g for 10 min at RT. The final pellet was dissolved in 1/6 of the original egg yolk volume using 100 mM sodium phosphate buffer (pH 7.6).

For final clarification, the extract was filtered through cotton wool again before storing at 4°C in scintillation vials (Analytical and Diagnostic Production) sealed with Parafilm.
The extracts are stable under these conditions for a number of years but must not be frozen (Dr. T.H.T. Coetzer, personal communication; Polson *et al.*, 1985).
The batches of extract were numbered according to the chicken and the week; e.g. C1/6 is week six for chicken one ; C2/P is pre-immunisation for chicken two.

3.3.4 Extraction of Immunoglobulin G from Serum

After measuring rabbit serum volumes, two volumes of borate buffered saline (pH 8.6) and 14% (m/v) PEG were added and dissolved by gentle mixing. The mixture was centrifuged at 12 000 g for 10 min at RT. The pellet was re-dissolved in the original serum volume using 100 mM sodium phosphate buffer, pH 7.6. Again 14% (m/v) PEG was added, mixed and centrifuged at 12 000 g for 10 min at RT. The final pellet was dissolved in ½ the original serum volume using 60% (v/v) glycerol in the sodium phosphate buffer. Aliquots (125 µl) of the extracts were stored in sealed Eppendorf tubes at –20°C (Dr. T.H.T. Coetzer, personal communication; Polson *et al.*, 1964).
The batch numbering system for the extracts was according to the rabbit and the week e.g. R1/4 is week four for rabbit one.

3.3.5 Measurement of Protein Concentration

Duplicate 1:40 dilutions of each extract (chicken and rabbit) were made in 0.1 M sodium phosphate buffer (pH 7.6) and the absorbances read at 280 nm. Using correct extinction co-efficients viz. E = 1.25 for IgY (Coetzer, 1985) and E = 1.43 for IgG (Hudson & Hay, 1989), protein concentrations in mg/ml were calculated. For IgY the blank was buffer alone; for IgG it was 60% glycerol in sodium phosphate buffer. Absorbance at 280 nm is mainly due to aromatic rings of tyrosine, tryptophan and phenylalanine; this content varies from protein to protein (Bohinski, 1987).

The IgY concentrations were checked (in duplicate) using the biuret method (Cobas Mira Plus Selective Automated Analyser, Boehringer Mannheim). Precinorm U was used as the standard and Humatrol N and Humatrol P for method controls. Because of insufficient serum, protein concentrations of IgG antibodies were not checked by the biuret method. The biuret method involves a formation of a complex between cupric ions in alkaline medium and peptide linkages. The absorbance of the complex is proportional to the number of peptide linkages and hence the concentration of protein (Koller, 1984).

3.3.6 Electrophoresis

To check the efficacy of the extraction procedure, protein electrophoresis was carried out on two of the IgY and one of the IgG extracts (at various stages of the extraction procedure) using the Beckman serum protein electrophoresis (SPE) kit and the Beckman Paragon system. The egg samples analysed were aliquots (200 µl) from the redissolved pellet solutions after the addition of 3.5%, 8.5% and 12% PEG. The blood samples analysed were the original serum and aliquots (200 µl) after the first and the second 14% PEG precipitations. In addition a normal (human) serum sample was run as a method control.

In the Beckman Paragon system, all reagents except acetic acid and methanol were supplied with the SPE kit. The support medium was agarose gel and the electrophoresis buffer was a barbital buffer (pH 8.6). The samples were diluted 1:5 in barbital buffer and 3 - 5 µl of each applied to designates lanes on the gel using the template supplied. The gel was electrophoresed for 25 min at 100 volts. The proteins were fixed by dipping the gel into an acid alcohol solution (20% glacial acetic acid and 30% methanol in de-ionised water). The gel was dried and stained with Paragon Blue stain prepared according to the manufacturer's instructions. Excess stain was removed by dipping the gel into 5% acetic acid solution.

The gel was dried and scanned using the Beckman Appraise Densitometer System. The electrophoresis was a non-denaturing procedure.

3.3.7 Indirect ELISA for Antibody Titre

The format for the indirect ELISA was as shown below:

$SAg + Ab_1 \rightarrow SAg\text{-}Ab_1 + Ab_2^E \rightarrow SAg\text{-}Ab_1\text{-}Ab_2^E$

The antigen (Ag), is attached to the solid phase (S) i.e. the wells of the microtitre plate. Then the primary antibody (Ab_1) is added and allowed to bind to the Ag; after incubation excess Ab_1 is removed by washing. In a second incubation, an enzyme-conjugated second antibody against primary antibody (Ab_2^E) reacts with Ab_1 to form the final complex (Voller & Bidwell, 1986; Crowther, 1995).

For application of this format, the Ag was FB_1, Ab_1 was the antibody from chickens/rabbits and the relevant Ab_2^E was obtained commercially.

Since RT in the laboratory can vary by 10°C, only temperatures of 37°C (incubator) or 4°C (refrigerator) were used for incubation.

For optimisation of the method various experimental parameters were checked using checkerboard titrations i.e. varying different conditions each time to assess which was the best. A decision as to the best result was based on which gave the steepest curve or the lowest value for the blank and maximum absorbance of 0.8 to 1.0 (Crowther, 1995).

For each parameter, the corresponding well for the pre-immunisation extract and for the non-specific binding blank (no Ab_1) was included. Extracts C1/8, C1/P and C2/8, C2/P were used for the optimisation and results checked with rabbit antibody R/P and R1/12, R1/14. All wells were done in duplicate. The ABTS solution (a clear light green) was prepared just before use and kept covered with aluminium foil since it is light sensitive.

In the summary below the final optimised method is shown first and the tested alternatives for each step are shown underneath in Italics.

1. The wells of the microtitre plate (Nunc 96 well Maxisorb, AEC Amersham) were coated directly with FB_1, 100 ng/ml in PBS, 150 µl per well. The plate was covered and incubated for 18 hr at 4°C.

   Use of PBS (pH 7.2) or 0.1M carbonate buffer (pH 9.6).

   Concentration of FB_1 – 1 000, 100, 10, 1, 0.1 ng/ml.

2. The wells were blocked with 1.0% casein (m/v) in PBS, 200 µl per well, and incubated for 1 hr at 37°C.

   Blocking agent was PBS plus 0.5% BSA or 2% or 1% or 0.5% casein.

3. The plate was washed three times with 0.1% (v/v) Tween 20 in PBS.

4. Dilutions of first antibody (Ab_1), 100 µl in 0.5% BSA(m/v) in PBS, were added and incubated for 2 hr at 37°C.

   Concentrations of Ab_1 – 1 000, 100, 10, 1 µg/ml made up in PBS plus 0.5% BSA or 0.5% or 1% or 2% casein.

5. The plate was washed three times with 0.1% (v/v) Tween 20 in PBS.

6. The second antibody, (Ab_2^E) anti-chicken IgG (or anti-rabbit IgG) conjugated with horseradish peroxidase, 1:2 000, 120 µl per well, was added and incubated for 1 hr at 37°C.

   Ab_2^E (from Dr. Coetzer) 1:350 dilution for chickens and rabbits.

   Ab_2^E (from Sigma) 1:500 or 1:1 000 or 1:2 000 or 1:5 000 or 1:10 000 or 1:20 000 or 1:30 000 for chickens.

   Ab_2^E (from Sigma) 1:1 000 or 1:2 000 or 1:5 000 or 1:10 000 for rabbits

7. The plate was washed three times with 0.1% (v/v) Tween 20 in PBS.

8. 150 µl of substrate, 7.5 mg ABTS plus 7.5 µl H_2O_2 in 15 ml citrate-phosphate buffer (pH 5) was added to each well. The colour was allowed to develop for 15 min in the dark at RT.

9. 50 µl stopping buffer was added to each well.

10. The absorbance of each well was read at 405 nm (Bio-Rad Model 550 Plate Reader).

3.3.8 Measurement of Antibody Titre

Using the optimised method, all batches of IgY and all batches of IgG at concentrations of 1 000, 500, 250, 100, and 25 µg/ml were tested for titre. On each plate the relevant pre-immune sample (at the same concentrations) and a non-specific binding (no Ab_1) blank were run.

Results for antibody titre were calculated by taking the means of duplicate absorbance readings, subtracting the mean of the relevant pre-immune absorbance value and the absorbance value for non-specific binding (i.e. no Ab_1 blank). Duplicates were only considered acceptable if differences were <10% preferably <5%.

3.4 RESULTS

3.4.1 Immunogen and Immunisation

The recommended loading ratio for formation of the immunogen is 1 000:1 (hapten:carrier) and the protein concentration of KLH (mg/ml) = dilution factor x absorbance (at 280 nm) x 0.388 (Package insert for KLH).

Concentration of KLH before dialysis = 7.60 mg/ml (absorbance 0.392, dilution 1:50)

Concentration of KLH after dialysis = 6.65 mg/ml (absorbance 0.343, dilution 1:50)

800 µl of dialysed KLH i.e. 5.32 mg was added to 1 mg FB_1.

Molecular Mass of KLH is 3 - 7.5 x 10^6 (average 5.25 x 10^6) and for FB_1 is 721.8

No. mmoles KLH = $5.32 \div 5.25 \times 10^6$ = 1.013×10^{-6}

No. mmoles FB_1 = $1 \div 721.8$ = 1.385×10^{-3}

Ratio $1.385 \times 10^{-3} : 1.013 \times 10^{-6}$

Loading achieved 1 367:1

Concentration of FB_1-KLH after dialysis = 5.30 mg/ml (absorbance 0.273, dilution 1:50). Thus to give 250 µg per aliquot needed 47 µl of conjugate plus 400 µl PBS to make up to a suitable volume for injection.

All four animals were healthy and showed no ill effects for the entire period of immunisation and egg/blood collection. The chickens were allowed to live for their allotted life span; the rabbits were given away as pets.

3.4.2 Immunoglobulin Y and Immunoglobulin G Extracts

TABLE 3.1 SUMMARY OF EXTRACTION OF IMMUNOGLOBULIN Y (CHICKEN 1)

Batch number of extract	Number of eggs	Yolk volume (ml)	Final volume (ml)	Appearance of final extract	Protein concentration (280 nm) (mg/ml)	Protein concentration (biuret) (mg/ml)
C1/P	4	67	11.2	Opalescent	25.09	24.79
C1/0	7	123	20.5	Pale yellow	19.87	20.52
C1/1	5	86	14.3	Pale yellow	26.72	27.84
C1/2	5	80	13.3	Pale yellow	21.89	22.88
C1/3	5	84	14.0	Light yellow	31.30	30.79
C1/4	3	48	8.0	Pale yellow	31.71	32.34
C1/5	9	160	26.7	Pale yellow	30.08	30.99
C1/6	6	110	18.3	Pale yellow	27.58	27.29
C1/7	6	110	18.3	Pale yellow	31.10	31.62
C1/8	6	107	17.8	Pale yellow	39.42	39.17
C1/9	6	99	16.5	Pale yellow	34.62	35.67
C1/10	6	115	19.2	Light yellow	34.40	34.25
C1/11	5	90	15.0	Pale yellow	33.79	34.22
C1/12	6	112	18.7	Pale yellow	36.19	36.78
C1/13	7	129	21.5	Pale yellow	30.72	32.14
C1/14	5	100	16.7	Pale yellow	30.21	30.85

Hen 1 produced more fatty yolks than hen 2, whilst hen 2 had more albumin.

TABLE 3.2 SUMMARY OF EXTRACTION OF IMMUNOGLOBULIN Y (CHICKEN 2)

Batch number of extract	Number of eggs	Yolk volume (ml)	Final volume (ml)	Appearance of final extract	Protein concentration (280 nm) (mg/ml)	Protein concentration (biuret) (mg/ml)
C2/P	4	67	11.2	Opalescent	17.50	18.27
C2/0	6	100	16.7	Pale yellow	27.68	28.32
C2/1	3	47	7.8	Pale yellow	27.74	28.84
C2/2	1	15	2.5	Pale yellow	29.79	29.54
C2/3	1	15.5	2.6	Yellow	42.56	44.55
C2/4	1	16	2.7	Yellow	41.41	41.84
C2/5	6	108	18.0	Pale yellow	33.79	36.61
C2/6	6	103	17.2	Pale yellow	34.05	34.70
C2/7	5	78	13.0	Pale yellow	39.87	40.80
C2/8	7	127	21.2	Light yellow	37.06	38.38
C2/9	7	124	20.7	Light yellow	37.34	38.10
C2/10	6	115	19.2	Pale yellow	33.12	33.56
C2/11	4	72	12.0	Pale yellow	34.05	35.47
C2/12	8	145	24.2	Pale yellow	36.51	38.31
C2/13	7	122	20.3	Pale yellow	36.51	37.79
C2/14	5	93	15.5	Pale yellow	32.35	33.11

Statistics using a one-way analysis of variance (ANOVA) showed there was no difference in the protein results as measured by the two methods.

Generally the chickens produced 4 to 7 eggs per week (about one egg per day) except for week 4 for chicken 1 (only 3 eggs); and for chicken 2 week 1 (3 eggs) and weeks 2, 3 and 4 (1 egg per week). The batches from the weeks when fewer eggs were laid gave smaller final volumes of extract (2.5 to 8.0 ml) compared to other weeks (11.2 to 21.5 ml).

The highest final volume (26.7 ml) was from the batch for week 5 for chicken 1 when 9 eggs were laid; the highest final volume for hen 2 was 24.2 ml from week 12 (8 eggs).

Protein concentrations for the two chickens were similar and ranged from 19.87 to 39.42 mg/ml for chicken 1 and from 17.50 to 42.56 mg/ml for chicken 2 (Tables 3.1 and 3.2).

TABLE 3.3 SUMMARY OF EXTRACTION OF IMMUNOGLOBULIN G

Batch number of extract	Serum volume (ml)	Final volume of extract (ml)	Protein concentration (280 nm) mg/ml
R/P			16.25
RABBIT 1			
R1/4	4.55	2.28	11.19
R1/8	6.10	3.05	10.35
R1/12	7.10	3.55	11.24
R1/14	32.10	16.05	11.94
RABBIT 2			
R2/4	3.30	1.65	12.25
R2/8	3.15	1.58	24.34
R2/12	3.45	1.73	13.90
R2/14	12.10	6.05	15.41

The highest final volumes for both rabbits were from week 14 but the volume for rabbit 1 (16.05 ml) was greater than for rabbit 2 (6.05 ml). Smaller volumes of serum and smaller volumes of extracted IgG were obtained from rabbit 2 compared to rabbit 1.

The protein concentrations were similar for both rabbits and ranged from 10.35 to 11.94 mg/ml for rabbit 1 and from 12.25 to 24.34 mg/ml for rabbit 2. The highest protein concentration was from rabbit 2 for week 8 (Table 3.3).

Overall larger volumes of extracted immunoglobulins were obtained from the two chickens compared to the rabbits (Tables 3.1, 3.2 and 3.3). The IgY extracts also had higher protein concentrations compared to the IgG extracts.

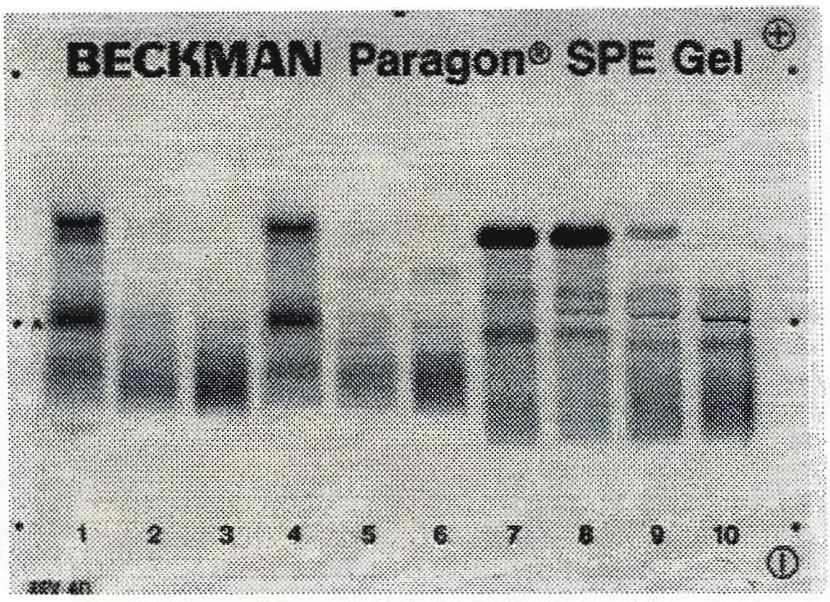


FIGURE 3.4 PROTEIN ELECTROPHORETOGRAM OF SAMPLES AT DIFFERENT STAGES OF EXTRACTION OF IMMUNOGLOBULIN Y AND IMMUNOGLOBULIN G

Key to Figure 3.4

Lanes 1, 2, 3 chicken 1 Lanes 4, 5, 6 chicken 2 Lane 7 control serum Lanes 8, 9, 10 rabbit 1
For the chickens, lanes 1 and 4 were after addition of 3.5% PEG; lanes 2 and 5 were after addition of 8.5% PEG (to make up to 12%); lanes 3 and 6 were after final addition of 12.0% PEG.
Lane 8 was rabbit serum, lane 9 after first addition of 14% PEG and lane 10 after final addition of 14% PEG.

Since the control serum gave a normal pattern, it was concluded that the electrophoresis had been done correctly. The albumin band travelled the fastest and was closest to the anode; the gamma globulin band was closest to the cathode. The progressive steps of purification of IgY and IgG with different concentrations of PEG 6 000 gave a reduction in the albumin bands but an increase in the gamma-globulin bands (Figure 3.4)

3.4.3 Optimisation of Indirect ELISA for Antibody Titre

Step 1.

The buffers commonly used for coating the microwell plates with antigen are PBS(pH 7.2) and carbonate (pH 9.6) (Crowther, 1995).

TABLE 3.4 COMPARISON OF TWO COATING BUFFERS USING TWO DIFFERENT IMMUNOGLOBULIN Y EXTRACTS

	PBS (pH 7.2)				Carbonate (pH 9.6)			
	C1/8				C1/8			
FB_1 (ng/ml)	**1000**	**100**	**10**	**1**	**1000**	**100**	**10**	**1**
Ab_1 (µg/ml)								
1000	2.586	2.341	2.295	2.423	2.586	2.268	1.910	2.164
100	2.557	2.056	1.365	1.122	2.415	1.407	0.952	1.058
10	1.846	1.131	0.671	0.458	1.433	0.651	0.408	0.581
	C2/8				C2/8			
FB_1 (ng/ml)	**1000**	**100**	**10**	**1**	**1000**	**100**	**10**	**1**
Ab_1 (µg/ml)								
1000	2.591	2.427	2.292	2.297	2.591	2.313	1.959	1.996
100	2.499	1.957	1.424	1.275	2.118	1.581	0.978	1.045
10	1.730	0.955	0.706	0.580	1.407	0.693	0.449	0.63

Key to Table 3.4

Results are means of duplicate absorbance values at 405 nm after subtraction of the mean of the non-specific binding (no Ab_1) blank.

Coating with PBS gave similar results to coating with carbonate buffer at the same coating concentrations of FB_1 (Table 3.4). It was decided to use PBS since then the same buffer could be used for coating and for other steps in the method.

TABLE 3.5 COMPARISON OF CONCENTRATIONS OF FUMONISIN B_1 FOR COATING MICROWELL PLATES

FB_1 (ng/ml)	**100**	**10**	**1**	**0.1**
Ab_1 (µg/ml)				
1000	1.281	0.643	0.624	0.395
100	1.098	0.144	0.114	0.000
10	0.411	0.081	0.009	0.000
1	0.000	0.000	0.000	0.000

Key to Table 3.5

Results are means of duplicate absorbance values at 405 nm after subtraction of the mean of the no Ab_1 blank. Batch number C2/8 of Ab_1 was used.

At concentrations of FB_1 < 100 ng/ml for coating, the antibody does not titrate out and curves were flat (Table 3.5). Similar results were obtained for batch C1/8.

Step 2.

After coating with antigen (FB_1), wells were blocked to prevent non-specific binding of $Ab_1$and Ab_2. The no Ab_1 blank (which must be run for each coating concentration of FB_1) estimates non-specific binding of either antibody Ab_2 or HRPO. The no Ab_2 well was a reaction blank effectively giving the lowest absorbance for the substrate solution and can be omitted. It was run at the highest concentration of Ab_1.

TABLE 3.6 COMPARISON OF REACTION BLANKS WITH DIFFERENT BLOCKING AGENTS

Blocking Agent	No Ab_1 Blank	No Ab_2 Blank
0.5% BSA in PBS	0.113	0.082
2% casein in PBS	0.078	0.076
1% casein in PBS	0.082	0.076
0.5% casein in PBS	0.083	0.079

Key to Table 3.6
Results are means of duplicate absorbance values at 405 nm.

Bovine serum albumin at a concentration of 0.5% was not an effective blocking agent as both blanks gave higher absorbance readings than casein. Casein at concentrations of 0.5% or 1% or 2% casein give similar and better blanks than BSA. It was easier to make up 1% than 2% casein which was difficult to dissolve. The results for 1% casein were better than for 0.5% (Table 3.6).

Step 4.

TABLE 3.7 COMPARISON OF DILUENTS FOR PRIMARY ANTIBODY

Diluent	0.5% BSA	0.5% casein	1% casein	2% casein
Ab_1 (µg/ml)				
1000	1.340	0.884	0.935	1.002
100	1.185	0.313	0.310	0.471
10	0.507	0.000	0.000	0.005

Key to Table 3.7

Results are means of duplicate maximum absorbance values at 405 nm after subtraction of the mean of the no Ab_1 blank. All diluents were made up in PBS.

Bovine serum albumin at a concentration of 0.5% proved to be the best diluent for antibodies since casein gave low absorbance values at lower concentrations of antibody; also the titration graph was steeper for BSA than for casein (Table 3.7).

Initially wide ranges of concentrations of Ab_1 (10 times differences) were used to find the region in which a suitable reaction was obtained. The range of 100 to1 000 µg/ml gave satisfactory results. (Tables 3.4, 3.5 and 3.7).

Step 6.

Below is an example of a plate set-up for checkerboard titrations. This one was used to check which concentration of Sigma Ab_2^E to use for rabbit antiserum (Table 3.8).

TABLE 3.8 SET-UP OF A PLATE FOR CHECKERBOARD TITRATION TO DETERMINE THE DILUTION OF THE SECOND ENZYME-LABELLED ANTIBODY (SIGMA) TO USE FOR ANTIBODY TITRE OF RABBIT IMMUNOGLOBULIN G

Dilution of Sigma Ab_2^E		**1:1 000**			**1:2 000**			**1:5 000**			**1:10 000**		
Batch Number		**R1/ P**	**R1/ 12**	**R1/ 14**	**R1/ P**	**R1/ 12**	**R1/ 14**	**R1/ P**	**R1/ 12**	**R1/ 14**	**R1/ P**	**R1/ 12**	**R1/ 14**
Ab_1 (µg/ml)		1	2	3	4	5	6	7	8	9	10	11	12
1000	A												
1000	B												
500	C												
500	D												
100	E												
100	F												
25	G												
	H	No Ab_1	No Ab_1	No Ab_2	No Ab_1	No Ab_2	No Ab_1	No Ab_2	No Ab_1	No Ab_1	No Ab_2	No Ab_1	No Ab_1

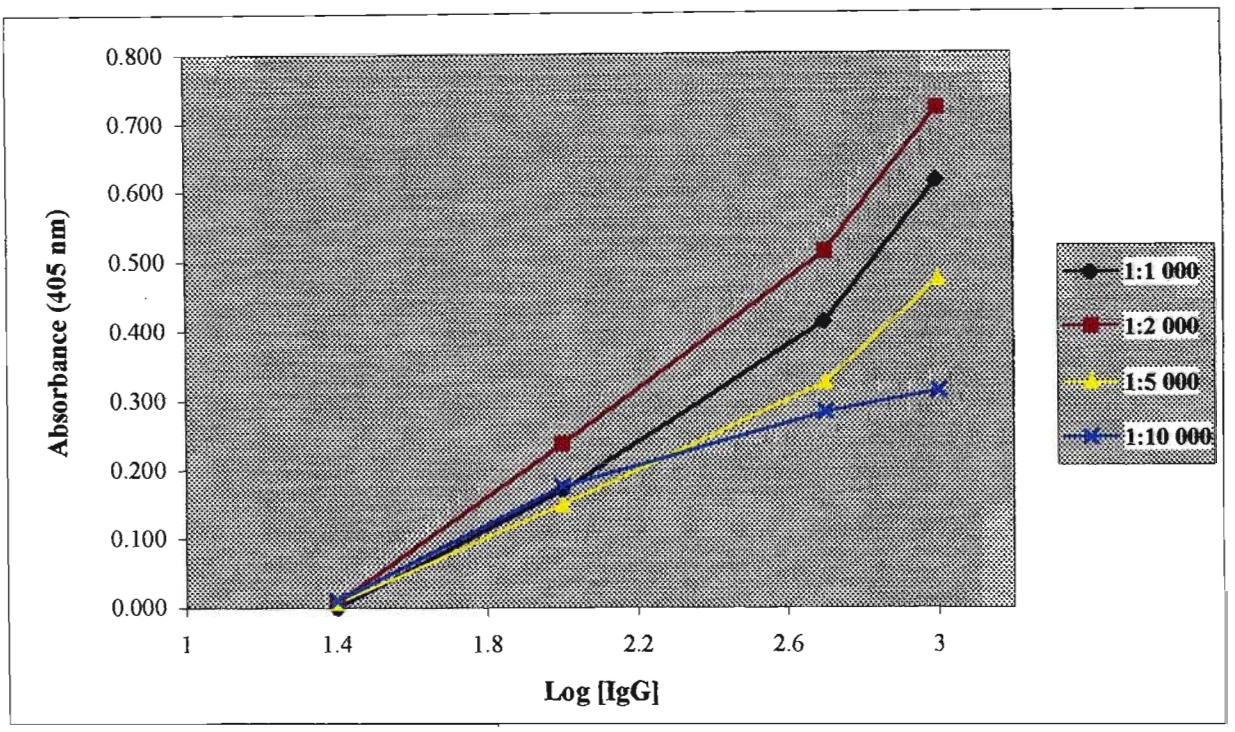


FIGURE 3.5 RESULTS OF CHECKERBOARD TITRATION TO DETERMINE THE DILUTION OF THE SECOND ENZYME-LABELLED ANTIBODY (SIGMA) TO USE FOR ANTIBODY TITRE OF RABBIT IMMUNOGLOBULIN G USING BATCH R1/14

Figure 3.5 clearly shows a 1:2 000 dilution of the Sigma Ab_2^E is the best to use as indicated by the steepest graph and suitable absorbance value (Crowther, 1995). The 1: 1 000 dilution gave a steep curve but lower absorbance values; the curves for the 1:5 000 and 1:10 000 dilutions were too flat. (Data used to generate Figure 3.5 is in Appendix 1, Table 3.9).

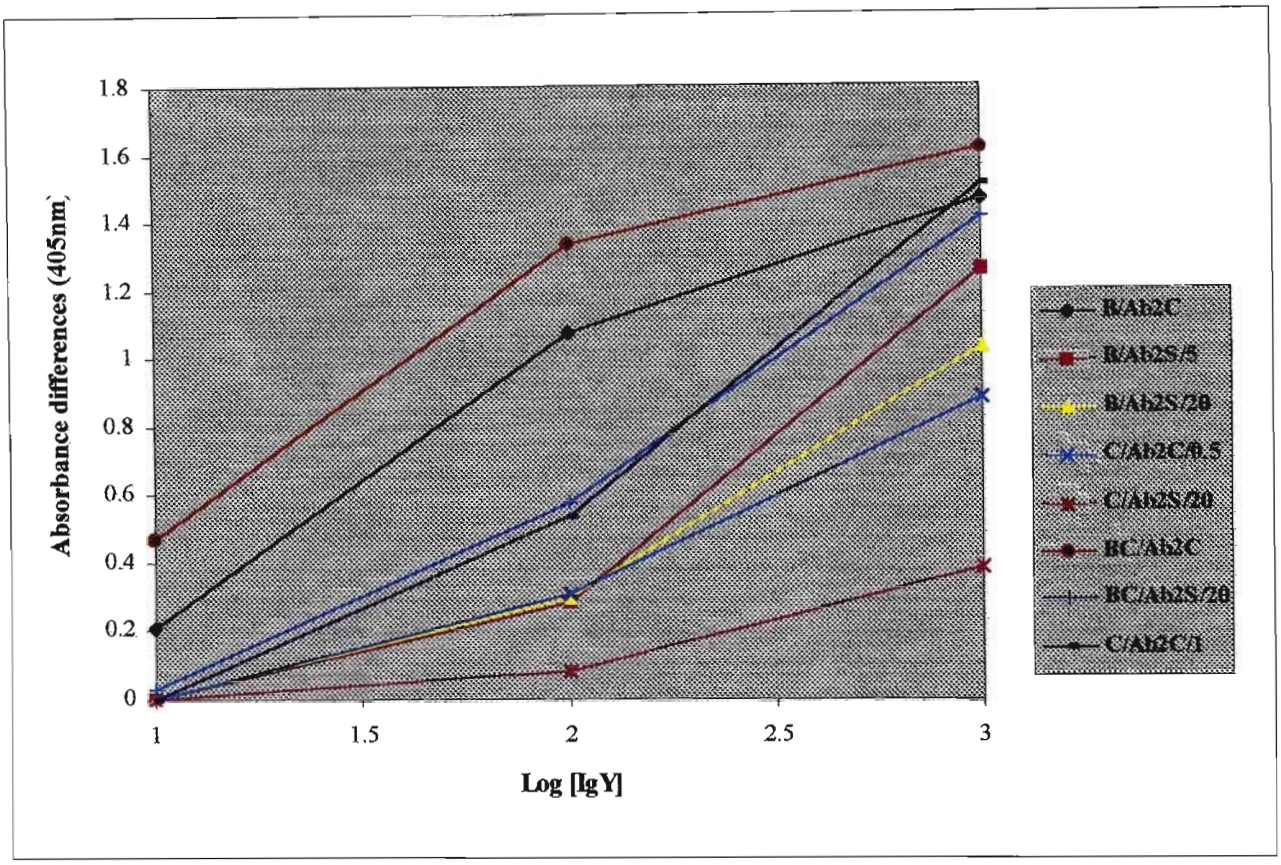


FIGURE 3.6 SUMMARY OF RESULTS OF EXPERIMENTS TO DETERMINE OPTIMUM BLOCKING AGENT, DILUENT AND DILUTION OF SECOND ENZYME-LABELLED ANTIBODY FOR TITRE OF CHICKEN IMMUNOGLOBULIN Y

Key to Figure 3.6

Code	Diluent	Blocking Agent	Ab_2^E
B/Ab_2C	0.5% BSA	0.5% BSA	Dr. Coetzer 1:350
B/ Ab_2S/5	0.5% BSA	0.5% BSA	Sigma 1:500
B/ Ab_2S/20	0.5% BSA	0.5% BSA	Sigma 1: 2 000
C/ Ab_2C/0.5	0.5% casein	0.5% casein	Dr. Coetzer 1: 350
C/ Ab_2S/20	1% casein	1% casein	Sigma 1: 2 000
BC/ Ab_2C	0.5% BSA	1% casein	Dr. Coetzer 1: 350
BC/ Ab_2S/20	0.5% BSA	1% casein	Sigma 1: 2 000
C/ Ab_2C/1	1% casein	1% casein	Dr. Coetzer 1: 350

(Data used to generate Figure 3.6 are in Appendix 1, Table 3.10).

The best results were using the second antibody supplied by Dr. Coetzer, 0.5% BSA as diluent and either 1% casein or 0.5% BSA as blocking agent (BC/ Ab_2C and B/ Ab_2C). However, as there was unfortunately insufficient of this antibody, the Sigma antibody diluted 1: 2 000, 1% casein for blocking and 0.5% BSA for diluent was used (BC/ Ab_2S/20) (Figure 3.6).

Use of other combinations of diluent and blocking agent gave unsuitably low absorbance values for the highest concentration of primary antibody, 1 000 µg/ml (Table 3.11).

TABLE 3.11 SUMMARY OF MAXIMUM ABSORBANCE VALUES USING DIFFERENT DILUENTS, BLOCKING AGENTS AND DILUTIONS OF SECOND ENZYME-LABELLED ANTIBODY

Dilution of Sigma Ab_2^E	Absorbance	Diluent	Blocking agent
1:500	0.509	0.5% BSA	0.5% BSA
1:2 000	0.660		
1:10 000	0.257		
1:20 000	0.264		
1:30 000	0.217		
1:1 000	0.347	1% Casein	1% Casein
1:2 000	0.397		
1:5 000	0.310		
1:10 000	0.256		
1:20 000	0.207		

Key to Table 3.11

Results are means of duplicate absorbance values at 405 nm after subtraction of the mean of the relevant pre-immune absorbance value (C1/P) and the absorbance value for the no Ab_1 blank. Fumonisin B_1 at a concentration of 100 ng/ml was used for coating and Ab_1 was batch number C2/8.

3.4.4 Antibody Titre

Table 3.12 shows an example of a typical set-up of a plate to check antibody titre.

TABLE 3.12 EXAMPLE OF A PLATE TO CHECK ANTIBODY TITRE (CHICKEN 2 EVEN WEEKS)

Ab_1 (µg/ml)		1 000 No Ab_2	1 000	1000	500	500	250	250	100	100	25	25	No Ab_1
		1	2	3	4	5	6	7	8	9	10	11	12
C2/P	A												
C2/2	B												
C2/4	C												
C2/6	D												
C2/8	E												
C2/10	F												
C2/12	G												
C2/14	H												

The pre-immune extract (C2/P) was run at the same protein concentrations (1 000, 500, 100 and 25µg/ml) as the other batches (C2/2, C2/4, C2/6, C2/8, C2/12 and C2/14).

Column 1 was for the no Ab_2 blank; column 12 for the no Ab_1 blank. The plate set-up was similar for all other titre experiments.

The final colour of the plate (Table 3.12) is shown in Figure 3.7.

1 2 3 4 5 6 7 8 9 10 11 12

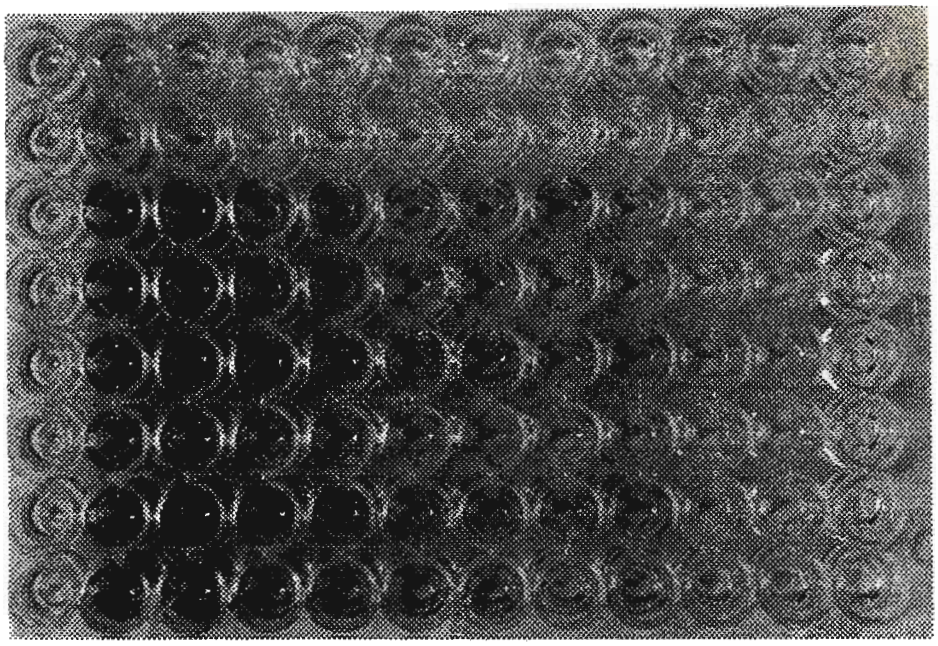


FIGURE 3.7 PHOTOGRAPH OF PLATE TO CHECK ANTIBODY TITRE FOR CHICKEN 2 (EVEN WEEKS)

In Figure 3.7 the darker the colour the higher the amount of antibody. The blank columns viz. no Ab_1 in column 1 and no Ab_2 in column 12 were clearly lighter than other columns.

TABLE 3.13 DATA TO CHECK ANTIBODY TITRE FOR CHICKEN 2 (EVEN WEEKS)

Batch number	1 000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	25 µg/ml	No Ab_1	No Ab_2
C2/P	0.311	0.232	0.154	0.119	0.092	0.083	0.089
C2/2	0.255	0.114	0.078	0.000	0.000		
C2/4	1.569	1.053	0.643	0.342	0.083		
C2/6	1.484	1.070	0.689	0.304	0.041		
C2/8	1.698	1.330	1.034	0.648	0.168		
C2/10	1.129	0.876	0.616	0.330	0.030		
C2/12	1.487	1.284	0.961	0.616	0.080		
C2/14	1.471	1.180	0.723	0.340	0.023		

Key to Table 3.13

Except for C2/P, no Ab_1 and no Ab_2, results are the means of duplicate absorbance readings at 405 nm after subtracting the mean of the relevant pre-immune absorbance value (C2/P) and the mean of the absorbance value for non-specific binding (no Ab_1). Duplicates differed by <5%.

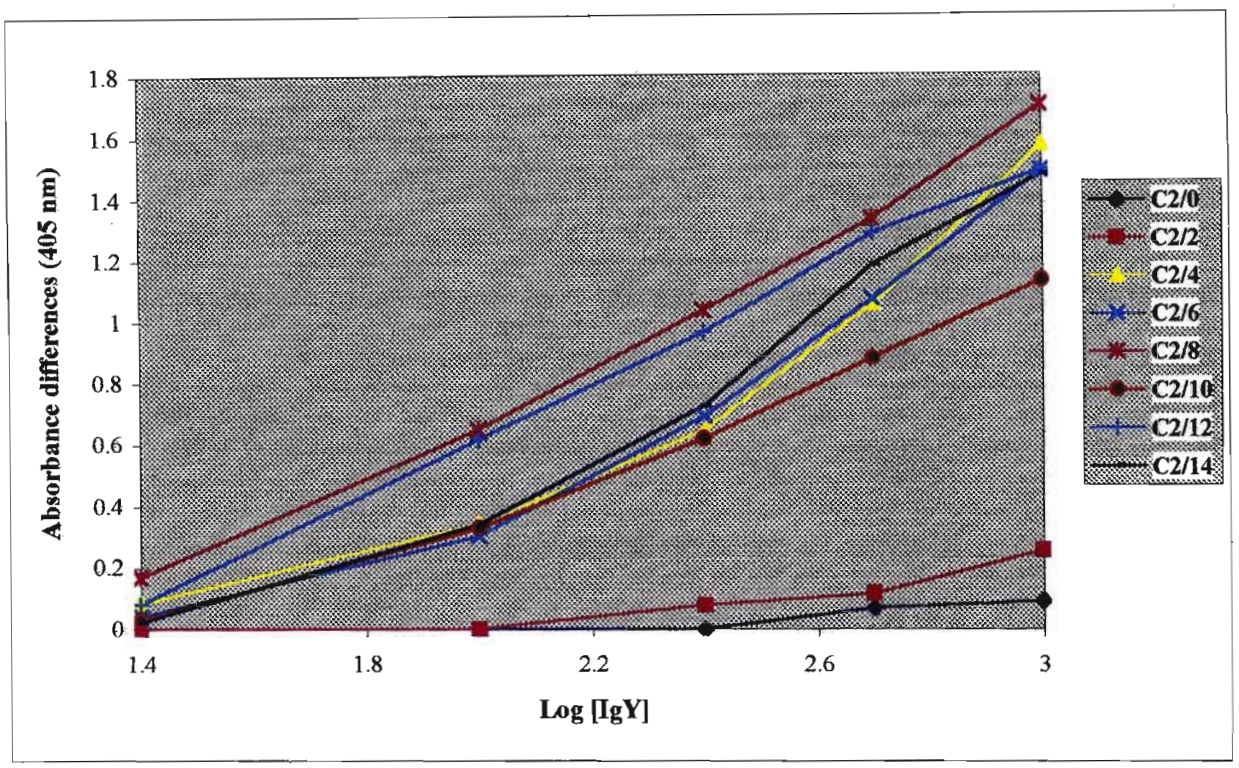


FIGURE 3.8 ANTIBODY TITRE FOR CHICKEN 2 (EVEN WEEKS)

Results for all other batches of IgY and IgG are shown in Figures 3.9 to 3.11

(The data used to generate Figures 3.9, 3.10, 3.11 is in Appendix 1, Tables 3.14, 3.15 and 3.16)

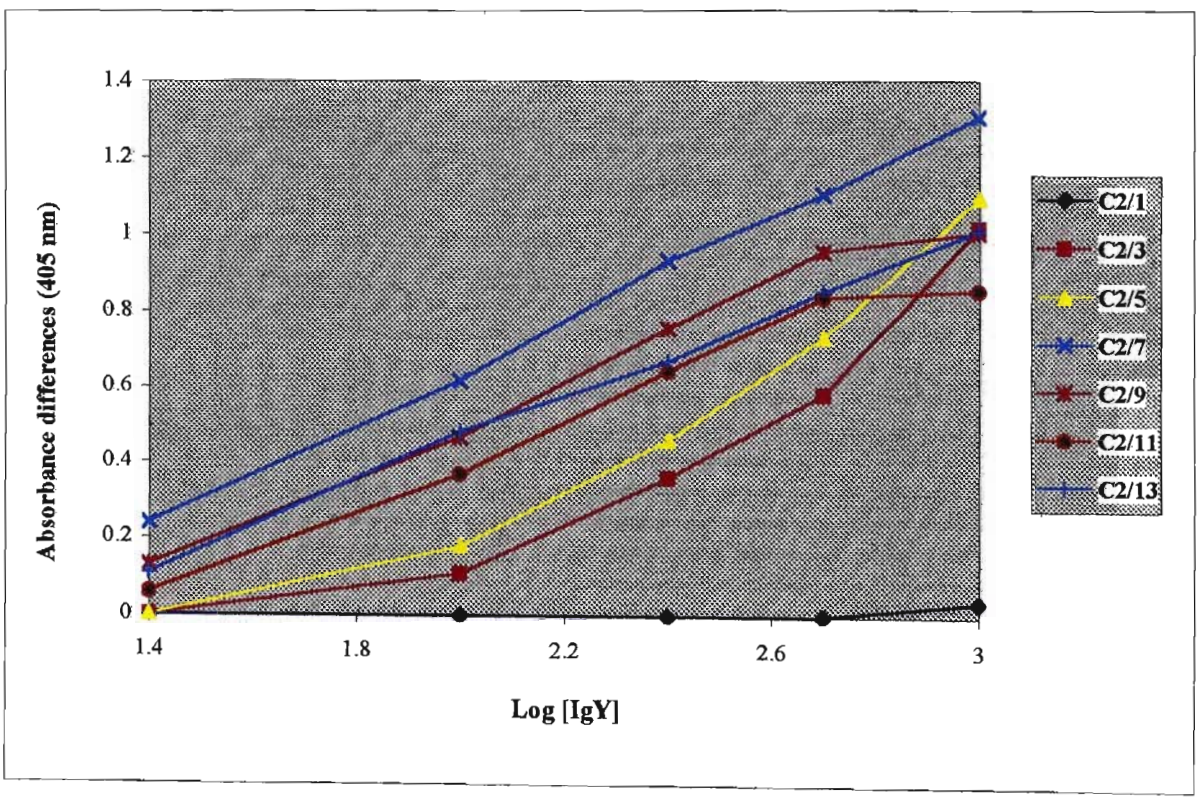


FIGURE 3.9 ANTIBODY TITRE FOR CHICKEN 2 (ODD WEEKS)

The highest titres for chicken 2 are batches from weeks 7, **8** and 12.

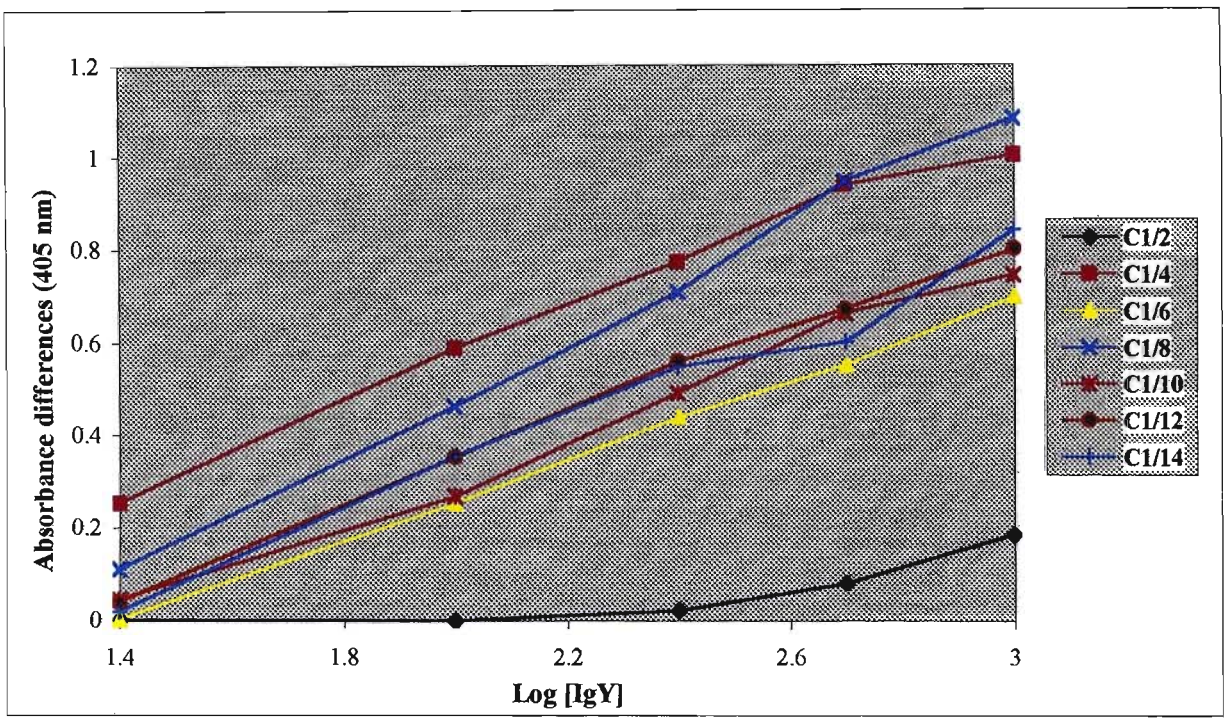


FIGURE 3.10 ANTIBODY TITRE FOR CHICKEN 1 (EVEN WEEKS)

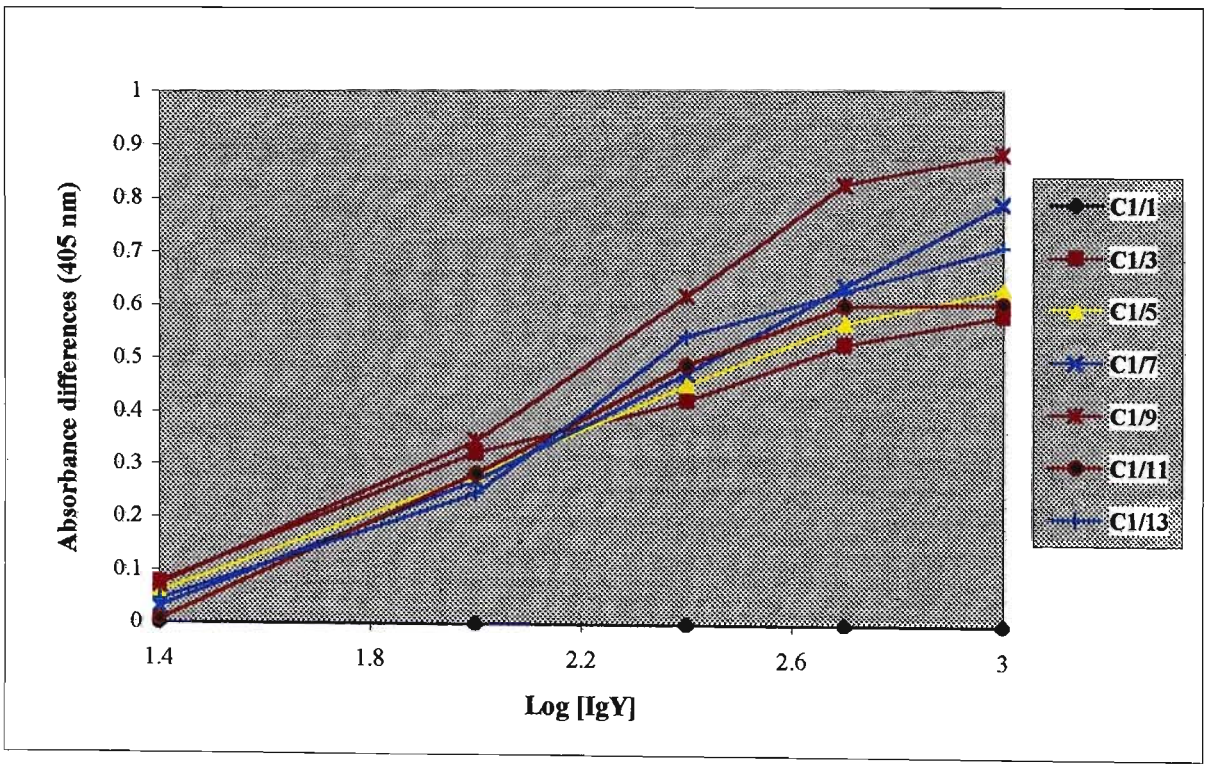


FIGURE 3.11 ANTIBODY TITRE FOR CHICKEN 1 (ODD WEEKS)

The highest titres for chicken 1 are from batches for weeks 4, **8** and 9.

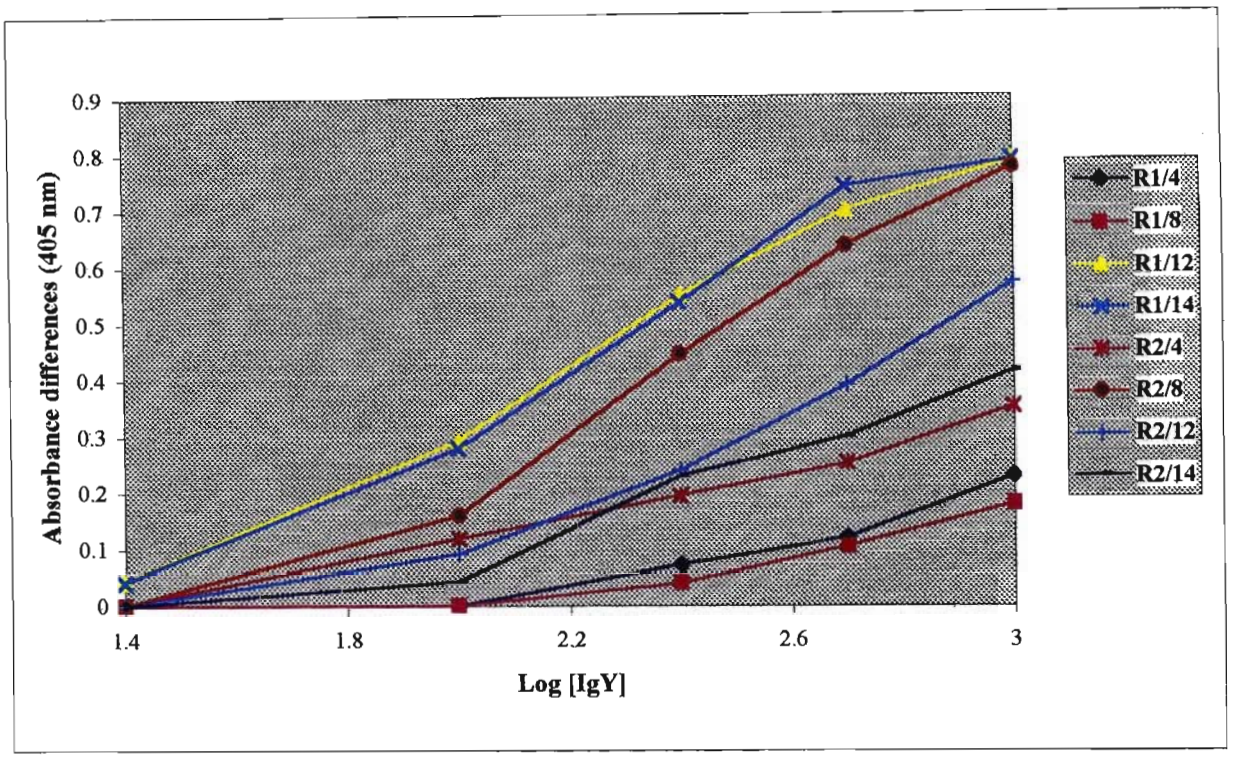


FIGURE 3.12 ANTIBODY TITRE FOR RABBITS 1 AND 2

The highest titres for rabbit 1 are from batches for weeks 12 and **14**;
and for rabbit 2 from week **8**.

Both chickens started producing antibodies at week 2 and continued to week 14 when collection of eggs ceased. Both rabbits produced antibodies for every bleed viz. weeks 4, 8, 12 and 14.

Checks of the blanks done on all the plates for checking antibody titre gave inter-plate CV of 6.33% (n = 42, mean 0.079, SD 0.005) for no Ab_1 and 6.58% (n = 40, mean 0.076, SD 0.005) for no Ab_2.

Figure 3.13 shows a comparison of highest titres for each animal.

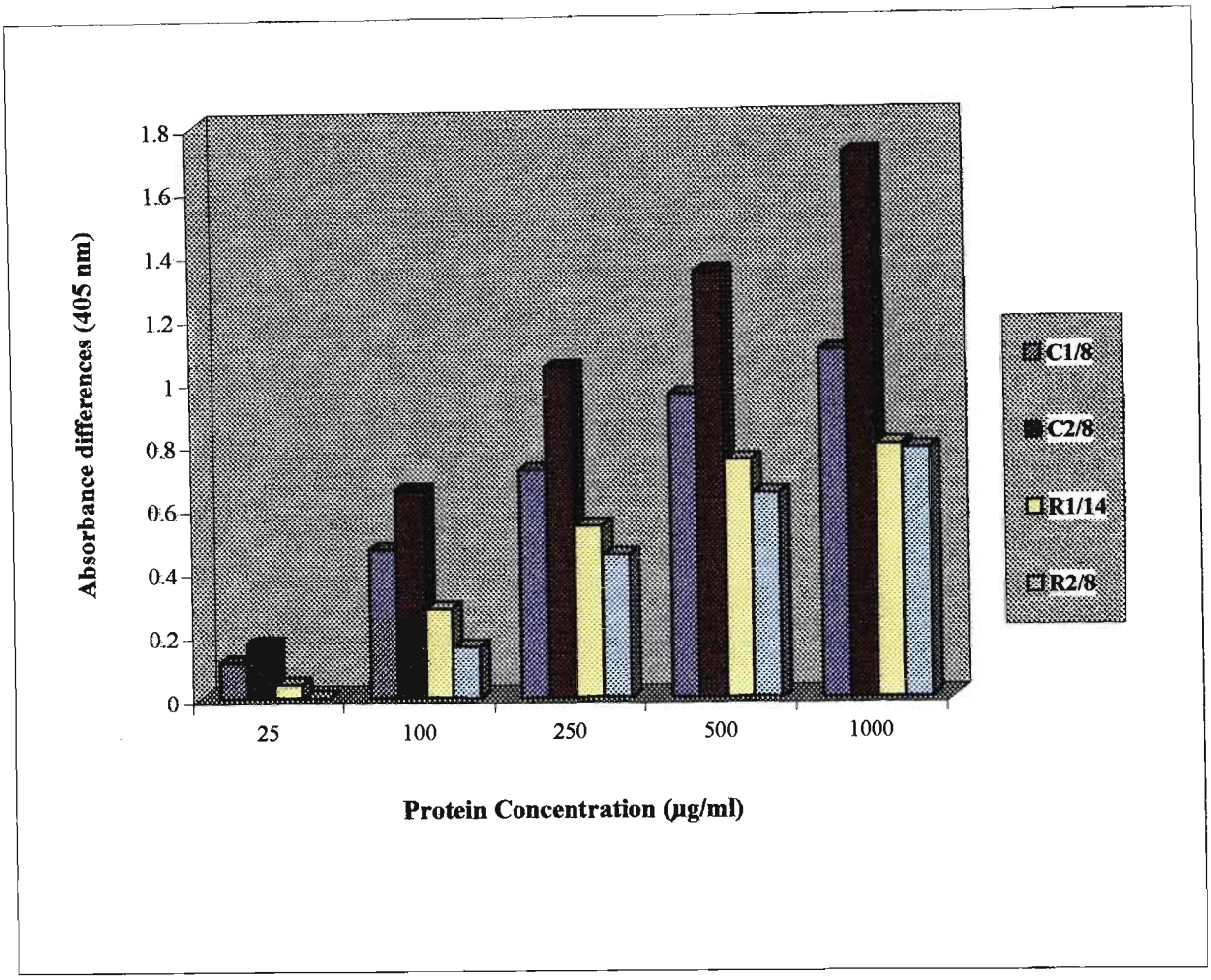


FIGURE 3.13 COMPARISON OF HIGHEST ANTIBODY TITRES

The highest titres in order are from chicken 2 week 8, chicken 1 week 8, rabbit 1 week 14 and rabbit 2 week 8.

3.5 DISCUSSION

3.5.1 Immunogen and Immunisation

Successful production of antibodies is largely a matter of chance. Certain factors can be controlled and others e.g. genetic make-up and humoral response of a particular animal are beyond the researcher's control (Ritchie, 1986). Since mycotoxins are not immunogenic it was necessary to conjugate them to a carrier by first introducing a reactive group (Chu, 1985; Chu, 1996). Fumonisin B_1 has two reactive carboxyl and hydroxyl groups and a primary amine (Chu 1996; Yeung *et al.*, 1996; Yu & Chu, 1996).

In this study, conjugation of FB_1 to KLH was presumed successful as antibodies were produced. Commercial antibodies from Neogen could have been used to check if the immunogen was only KLH or FB_1-KLH but this was too expensive.
Although it was not possible to check whether or not the FB_1 had bound to the KLH (as there were no readily available methods with sufficient sensitivity), a suitable loading ratio of hapten to carrier (1 367:1) was achieved (the recommended ratio was 1 000:1). Usleber *et al.* (1994) used FB_1: KLH at 700:1 while Azcona-Olivera *et al.* (1992A, 1992B) and Fukuda *et al.* (1994) used 50:1. Using FB_1 linked to KLH, Abouzied *et al.* (1996) produced polyclonal antibodies in sheep; Usleber *et al.* (1994) and Yu & Chu (1996) in rabbits; and Fukuda *et al.* (1994) produced monoclonal antibodies in mice.
It was a long 8 month wait until checks for antibody could be done.

Chickens (laying hens) are seldom the first choice of animal for raising antibodies yet they were easy to handle and to inject. Collection of eggs was a non-invasive and easier procedure than the collection of blood from the ear vein of the rabbits; also the volumes of egg yolks with which to begin the extraction of IgY were greater than the volumes of serum obtained from the rabbits (Tables 3.1, 3.2 and 3.3). Clarke *et al.* (1993) successfully produced antibodies against Ochratoxin A in laying hens (using a BSA conjugate as immunogen and injecting intramuscularly into the pectoral muscles) but this study is the first report of antibodies against fumonisin being produced in hens.

3.5.2 Extraction of Immunoglobulin Y and Immunoglobulin G

It is preferable to perform some type of purification of antibodies (at the least the removal of albumin) as the amount of specific antibody in serum is only 1-5% of the total protein (Crowther, 1995). Ammonium sulphate precipitation is an acceptable method to fractionate antisera (Hebert *et al.*, 1973) or antibodies can be purified by ammonium sulphate precipitation followed by use of an affinity column (CH-Sepharose 4B) conjugated with FB_1 (Chu *et al.*, 1995) but the affinity column used 4 mg of FB_1 which was far too costly for this study. Usleber *et al.* (1994) purified rabbit antibodies using 70% ammonium sulphate; Yu & Chu (1996) used 35% while Yeung *et al.* (1996) did no purification but simply diluted rabbit sera with an equal volume of 50% glycerol in water and stored aliquots at –20°C.

Using PEG 6 000 as a precipitant was relatively easy and cheap. The extraction of IgY required good technical skills and careful work to ensure effective precipitation and complete removal of interfering albumin and lipids.

It is important to use PEG with the correct molecular mass otherwise the extraction does not work (Polson *et al.*, 1985; Dr. T.H.T. Coetzer, personal communication). Extraction of IgG was easier except for working with smaller volumes. The volumes of the final IgY extracts were on the whole larger than for the IgG extracts (with the exception of batch R1/14) because of larger starting volumes. This is another advantage of using chickens/eggs over rabbits/serum.

The rabbit antibodies (IgG) are stable for long periods if stored at –20°C with 60% (v/v)glycerol but the hen antibodies (IgY) are denatured by freezing (Dr. T.H.T. Coetzer, personal communication). During the time period of this study, it was noted that the IgY extracts were stable for at least 4 years at 4°C.

3.5.3 Electrophoresis

The electrophoretogram (Figure 3.4) clearly showed a progressive reduction in protein concentration in the albumin and α1-, α2- and β-globulin regions with an increase in the gamma globulin regions indicating successful purification.

3.5.4 Protein Concentration

Measurement of protein concentration was a simple procedure but again needed careful work to get correct and reproducible results. From the results for protein concentration (Tables 3.1, 3.2 and 3.3) and the statistical evaluation, the two different methods were in good agreement. Measurement of the protein concentration was necessary to be able calculate the amounts of Ab_1 to use for titre experiments.

Because the extracted IgY and IgG will contain antibodies other than those against FB_1 (e.g. antibodies against KLH), a high protein concentration did not necessarily indicate a high antibody titre. For example, chicken 2 had highest protein concentration at week 3 (when only one egg was laid) yet the highest titre was for week 8 yet for the other animals the batch with highest titre also had the highest protein concentration viz. week 8 for chicken 2, week 14 for rabbit 1 and week 8 for rabbit 2 (Tables 3.1, 3.2 and 3.3 and Figure 3.13).

3.5.5 Optimisation of ELISA for Antibody Titre

Checkerboard or chess board titrations are an accepted method of optimising ELISA methods. Good results are indicated by a steep curve, low values for no Ab_1 and no Ab_2 blanks (less than lowest absorbance value for other wells), suitable maximum absorbance values (< 2 or between 1.0 and 1.5) and low results for pre-immune sera compared to post-immunisation sera (Dr. T.H.T. Coetzer, personal communication, Crowther, 1995).

Antigens or antibodies adhere to plates by adsorption / hydrophobic interactions (Crowther, 1995). Some authors (Abouzied *et al.*,1996; Azcona-Olivera *et al.*, 1992A, 1992B; Yeung *et al.*, 1996 Yu & Chu, 1996) used FB_1-OVA or FB_1-BSA conjugate (Fukuda *et al.*, 1994) to coat microtitre plates but since FB_1 is like a large amino acid and charged in solution at pH 7, coating directly with FB_1 was tried. Fumonisin B_1 at a concentration of 100 ng/ml in PBS attached readily to the microwell plates (Table 3.5). Optimal antigen concentrations were 1-10 μg/ml to saturate the available sites on the plate (Crowther, 1995).

Casein at a concentration of 1% in PBS was a cheap and effective blocking agent although it was not as easy to dissolve as BSA (Table 3.6). Blocking agents used by other authors were 1% polyvinyl alcohol in PBS (Abouzied *et al.*, 1996), 2% instant skim milk powder in PBS (Clarke *et al.*, 1993), 0.1% non-fat dry milk (Fukuda *et al.*, 1994) or 0.1% gelatin in PBS (Yu & Chu, 1996).

For stability, it was necessary to have a protein in the diluent for antibody solutions. Bovine serum albumin at a concentration of 0.5% in PBS proved to be the best (Table 3.7). The range of primary antibody concentrations in titre experiments should be 100 to 500 μg/ml (Dr. T.H.T. Coetzer, personal communication) and the use of 25, 100, 500 and 1 000 μg/ml gave good results. For the second incubation, the enzyme-labelled second antibody from Sigma at 1:2 000 dilution gave the best results (Figure 3.6).

In conclusion, for all titre experiments coating was done with 100 ng/ml FB_1 in PBS, blocking with 1% casein, washing with 0.1% Tween 20 in PBS, antibody diluent was 0.5% BSA in PBS, concentrations of Ab_1 were 25 to 1 000 μg/ml and Ab_2^E (Sigma) at 1:2 000.

3.5.6 Antibody Titre

Antibody titre was checked for all batches of IgY and IgG. Good inter-plate CV values were obtained for no Ab_1 and no Ab_2 blanks from all plates run for titre indicating good reproducibility of the method. Although antibodies were successfully produced in both chickens and rabbits, the chickens produced higher titres as shown by high maximum absorbance values and steep curves on the graphs. The highest titre was from chicken 2 for week 8 (Figure 3.13). Highest titre has been assessed as the maximum dilution (>1:50 000) to give absorbance 2 x pre-immune (Azcona-Olivera *et al.*, 1992A; Abouzied *et al.*, 1996) or taken as the steepest curve and highest absorbance plotting A vs. dilution of antisera (Yu & Chu, 1996).

The chickens produced antibodies as early as two weeks after initial immunisation and continued for the 14 week period of the study; the rabbits produced antibodies at every bleed (4, 8, 12 and 14 weeks). Abouzied *et al.* (1996) produced successful antibodies 8 weeks after initial injection whereas Yeung *et al.* (1996) obtained highest titres after 5 to 7 months. Usleber *et al.* (1994) detected antibodies 4 weeks after initial exposure and Yu & Chu (1996) found antibodies at 5 weeks with highest titre at 12 weeks; and better titre with a FB_1-KLH conjugate than FB_1-BSA.

3.6 CONCLUSION

Keyhole limpet hemocyanin linked to FB_1 proved to be an effective immunogen as polyclonal antibodies against fumonisin B_1 were successfully raised in both chickens and rabbits. Antibodies were extracted from egg yolks (IgY) for chickens and from blood for rabbits (IgG). Electrophoresis showed the extraction to be efficient giving relatively pure gamma globulin fractions.

Using checkerboard titrations, an indirect ELISA method was successfully optimised for measurement of titre. Thereafter the titre of all batches of IgY and IgG was measured as the steepest titration curve. Chickens had higher titres than rabbits – probably due to different genetic make-up. The highest titre was from chicken 2 for week 8.

CHAPTER 4

COMPETITIVE DIRECT ELISA

4.1 INTRODUCTION

Immunoassays such as ELISA methods, because of their sensitivity and specificity, are an obvious choice for analysis of mycotoxins as mycotoxins and their metabolites are usually present in small amounts. Generally ELISA methods are ten to a hundred times more sensitive than radio-immunoassays (RIA) when purified toxins are used (Chu, 1985).

Quantitative ELISA methods to measure antigen can be direct or indirect competitive, or can use a capture antibody. Because of their small size (low molecular weight), sandwich assays are not suitable for fumonisins. Sandwich assays are used for large antigens with at least two antigenic determinants (Crowther, 1995). Below is the equation for a direct competitive format using enzyme-labelled antigen.

$$S\text{-}Ab + Ag + Ag^{E} \leftrightarrow \ S\text{-}Ab\text{-}Ag + \ S\text{-}Ab\text{-}Ag^{E}$$

The primary antibody (Ab) is attached to the solid phase (S). Labelled (Ag^{E}) and unlabelled antigen (Ag) compete for binding to the Ab. Separation is by washing which removes any unreacted reagents. The amount of antibody coated to the solid phase and the amount of labelled antigen is constant while the amount of unlabelled antigen varies – being either standards or samples. Only the S-Ab- Ag^{E} can be measured by addition of a suitable substrate. By the law of mass action, the more Ag present the more S-Ab-Ag will be formed and the less S-Ab-Ag^{E} i.e. there is an inverse relationship between the amount of coloured product formed (measured as absorbance) and the amount of Ag present (Crowther, 1995, Voller & Bidwell, 1986).

For application of this format to fumonisins, the primary antibody (Ab) was that raised in chickens and rabbits (as described in Chapter 3), the solid phase was the wells of the microtitre plate and the antigen was FB_1.

In ELISA assays two enzymes are commonly used viz. alkaline phosphatase and horseradish peroxidase. Horseradish peroxidase [Hydrogen-peroxide oxidoreductase (EC 1.11.1.7)] is a glycoprotein with a molecular mass of 40 000 Daltons. Since it has both a carbohydrate moiety and amino groups, it can be linked to other proteins using sodium periodate or carbodiimide. Sodium periodate forms aldehyde groups on the carbohydrate moiety by oxidation. The aldehyde forms Schiff bases with α or ε amino groups; sodium borohydride stabilises the Schiff base (Nakane & Kawaoi, 1974). As FB_1 has a free amino group, the periodate method can be used to link it to HRPO.

Other workers successfully prepared FB_1-HRPO conjugates using the sodium periodate method (Azcona-Olivera *et al.*, 1992B; Usleber *et al.*, 1994; Abouzied *et al.*, 1996). They then used the conjugates to develop CD-ELISA methods to measure fumonisins in maize/food samples. However, none of their methods were adapted for physiological samples.

Usleber *et al.* (1994) produced a successful CD-ELISA with detection limits of 0.17 ng/ml in buffer and 10 ng/g in maize, a standard range of 0.1 to 5.0 ng/ml, maximum absorbance of 1.1, %B/Bo (absorbance of bound fraction expressed as a percentage of the absorbance for the zero point on the standard curve) from 20 to 80%, intra-assay CV of 7.5%, inter-assay CV of 14% and ID_{50} of 0.62 ng/ml. Coating was done using 100 µl of polyclonal antibodies overnight at RT in carbonate buffer(pH 9.6).

In the method of Abouzied *et al.* (1996), sample extracts or standards were mixed with FB_1-HRPO in mixing wells prior to being transferred to reaction wells already coated with polyclonal antibodies. The range of the standard curve was from 0 to 15 ng/ml, detection limits were <0.1 ng/ml FB_1 in 10% methanol in water, intra-assay CV (same day) was 3.8% and inter-assay CV (different days) was 7.6%. Recovery from maize was 74 to 91% and detection limits in corn were < 0.2 – 6 µg/g. This method is now marketed as the Veratox ® Fumonisin Kit (Neogen Corporation).

Using monoclonal antibodies, Azcona-Olivera *et al.*, (1992B), coated overnight at 40°C in carbonate-bicarbonate buffer (pH 9.6), then added 50 µl standard or sample followed by 50 µl FB_1-HRPO (2 µg/ml) in 1% OVA in PBS. The maximum absorbance values were 1.2 in PBS,

1.3 in 27% methanol and 1.3 in13% acetonitrile. Standard curves were similar for all three solvents with a range from 50 to 5 000 ng/ml. Detection limits were 200 ng/g in maize, recovery from maize samples was103%, intra-assay CV was 11.5% and inter-assay CV was 14.8%. This method is also commercially available as Fumonisin Agri-Screen kits (Neogen Corporation).

In their method, Yu & Chu (1996) used FB_1-HRPO (50 µl, 25 ng/ml) prepared by the carbodiimide method. They coated with antibody overnight at 4°C in PBS (100 µl, 6 µg/ml), blocked with 0.1% BSA in PBS and then added 50 µl FB_1/ well followed by). The range of the standard curve was from 0.01 to 100 ng/ml, maximum absorbance was 1.8, ID_{50} was 0.45 ng/ml and detection limits were 0.05 ng/ml in buffer and < 50 ppb in maize.

4.1.1 Objectives

1. To prepare the antigen-enzyme conjugate, FB_1 linked to HRPO.
2. To use the antibodies already raised in chickens to develop a CD-ELISA.

4.2 MATERIALS

4.2.1 Chemicals

Sodium periodate ($NaIO_4$), and sodium borohydride ($NaBH_4$) were purchased from Sigma Chemical Co., USA.

Horseradish Peroxidase was purchased from Boehringer Mannheim, Germany.

Ethanol was purchased from Merck and glycerol from Saarchem.

Coomassie Brilliant Blue viz. Serva Blue G (Research Grade) was obtained from Serva Feinbiochemica; 89% o-phosphoric acid from M & B laboratory chemicals.

Primary antibody was prepared as described in Chapter 3.

FB_1 stock standards were prepared as described in Chapter 3, section 3.2.3.

For all other chemicals refer to Chapter 3, section 3.2.1.

4.2.2 Buffers/Solutions

The following buffers were used in the CD-ELISA:

Phosphate buffered saline (PBS) (pH 7.2) (Appendix 2, 2.2)

0.1M (100 mM) Sodium phosphate buffer (pH 7.6), 0.02% sodium azide (Appendix 2, 2.3)

0.15M Citrate - phosphate buffer (pH 5.0) (Appendix 2, 2.6)

Stopping buffer : 0.1% sodium azide in citrate-phosphate buffer (Appendix 2, 2.7)

1 mM Sodium acetate buffer (pH 4.4) (Appendix 2, 2.8)

200 mM Sodium carbonate buffer (pH 9.5) (Appendix 2, 2.9)

0.1M Carbonate/bicarbonate buffer (pH 9.6) (Appendix 2, 2.10)

4.3 METHODS

4.3.1 Preparation of Fumonisin B_1 – Horseradish peroxidase conjugate (FB_1-HRPO)

The method was based on that of Usleber *et al.* (1994) and Nakane & Kawaoi (1974). Four milligrams (4 mg) of HRPO (copper coloured crystals) were dissolved in 1 ml ultra-pure water (reverse osmosis and de-ionised). To activate the enzyme 200 µl of freshly prepared sodium periodate (100 mM, 0.2139 g in 10 ml ultra-pure water) was added and the resulting green solution mixed at RT for 20 min. The solution (1 170 µl) was dialysed at 4°C overnight (Spectra/Por cellulose membrane dialysis tubing, MWCO 12 000 to 14 000 Daltons, 10 mm flat width, 6 mm diameter, capacity approximately10 ml/ft., Sigma Chemical Co., USA) against one litre of 1mM sodium acetate buffer (pH 4.4). The pH of the activated HRPO was adjusted to 9.5 using 200 mM sodium carbonate buffer (pH 9.5) (giving a yellow-brown solution,) and immediately 1 mg FB_1 dissolved in 1 ml ultra-pure water was added and mixed.

After incubation for 2 hr at RT, 100 µl of freshly prepared sodium borohydride (41.7 mg in 10 ml ultra-pure water) was added, mixed and incubated at 4°C for 2 hr. The solution (2.55 ml) was dialysed for 3 x 12 hr at 4°C against one litre PBS, pH 7.2. The dialysate (2.3 ml) was diluted 1:1 with 60% glycerol and stored in 55 µl aliquots in sealed Eppendorf micro test tubes (AEC Amersham) at – 20°C.

4.3.2 Measurement of Protein Concentration of Fumonisin B_1 – Horseradish peroxidase conjugate using the Bradford Assay

Fifty ml of 89% o-phosphoric acid and 23.5 ml ethanol were added to 50.3 mg Serva Blue G and the solution was made up to 500 ml with distilled water. After mixing for 1 hr at RT, it was filtered through Whatman No. 1 filter paper and used the same day (Bradford, 1976; Read & Northcote, 1981; Compton & Jones, 1985). Since there was insufficient HRPO to use as a standard, a stock protein standard was made by dissolving 50.7 mg BSA, fraction V, in 50 ml 30% glycerol in PBS (to have the same diluent as the FB_1-HRPO conjugates).

The concentration was checked by reading absorbance at 280 nm (Milton Roy Spectronic 3 000 Array Spectrophotometer).

From this stock solution a range of standards were prepared viz. 800, 600, 400, 200, 100 and 50 µg/ml in 30% glycerol in PBS. Duplicate 50 µl volumes of standard or FB_1-HRPO conjugate were pipetted out, 2.5 ml Bradford reagent was added and mixed.

After 5 minutes (and within 1 hr) absorbances were read at 595 nm against a blank of 30% glycerol in PBS.

4.3.3 Measurement of Protein Concentration of Primary Antibody

Chicken antibodies had given the highest titres and volumes (Chapter 3, section 3.4.4) ImmunoglobulinY batches C2/8 and C2/12 from chicken 2 were combined to use as primary antibody. A 1:40 dilution of the combined extracts was made in 0.1M sodium phosphate buffer (pH 7.6) and the absorbance was read at 280 nm. Using an extinction coefficient, E, of 1.25 (Coetzer, 1985), the protein concentration in mg/ml was calculated.

4.3.4 Development of Competitive Direct ELISA Method

The method was based on that of Usleber *et al.*(1994). For development and optimisation of the method various parameters were checked using checkerboard titrations. Certain experiments required the use of a second enzyme-labelled antibody. In the summary below the tested alternatives for each step are shown in Italics.

1. The wells of the microtitre plate (Nunc 96 well Maxisorb, AEC Amersham) were coated directly with primary antibody, 150 µl per well.
   The plate was covered and incubated for 18 hr.
   Incubation temperature – RT or 4°C.
   Concentrations of Ab_1 – 500, 400, 300, 200, 100, 10, 1, 0.8, 0.6, 0.4, 0.2, 0.1 µg/ml.
   Buffer – PBS (pH 7.2) or 0.1M carbonate/bicarbonate buffer (pH 9.6)
2. The wells were blocked, 200 µl per well.
   0.5% BSA or 1% casein in PBS, for 1 hr at RT or 37°C.
3. The plate was washed three times with 0.1% (v/v) Tween 20 in PBS.

4. 50 µl of FB_1 standards were pipetted out in duplicate; 50 µl FB_1-HRPO was added per well and incubated.

   Concentration of FB_1 standards – 0, 1, 5, 10, 25, 50 ng/ml in PBS.

   Concentration of FB_1-HRPO – 10, 25, 50, 100, 250, 500 ng/ml and 1, 2, 5, 10 µg/ml in 0.5% BSA or 1% casein in PBS.

   Incubation for 1 or 2 hr at RT or 37°C.

5. The plate was washed three times with 0.1% (v/v) Tween 20 in PBS.
6. 150 µl of substrate, 7.5 mg ABTS plus 7.5 µl H_2O_2 in 15 ml citrate-phosphate buffer, pH 5.0 was added to each well. The colour was allowed to develop for 15 min in the dark at RT.
7. 50 µl stopping buffer was added to each well.
8. The absorbance of each well was read at 405 nm (Bio-Rad Model 550 Plate Reader).

4.4 RESULTS

4.4.1 Protein Concentration of Fumonisin B_1 – Horseradish peroxidase conjugate

Duplicate absorbance readings of the BSA stock standard at 595 nm were 0.674 and 0.671; the mean was 0.673.

The extinction coefficient of BSA at 280 nm for a 1% solution (1 g/100 ml) i.e. E_{280} 1% is 6.67 (Hudson & Hay, 1989).

Concentration of BSA stock standard = (0.673 x 10) ÷ 6.67 = 1.009 mg/ml.

The correct concentration of the BSA stock standard was 1 009 µg/ml.

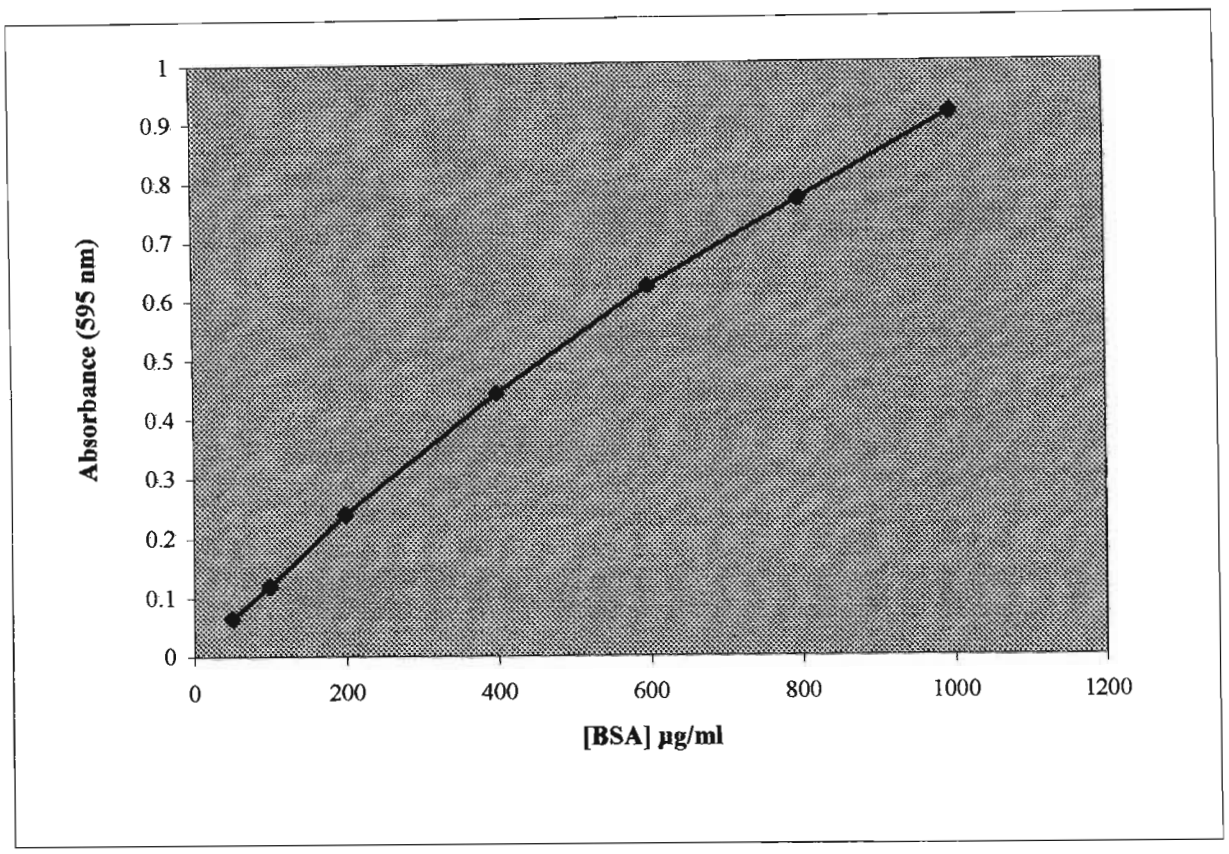


FIGURE 4.1 PROTEIN STANDARD CURVE USING BOVINE SERUM ALBUMIN (BRADFORD ASSAY)

(The data used to generate Figure 4.1 is in Appendix 1, Table 4.1).

The equation for the graph was $y = 0.0009x + 0.0482$.

Duplicate absorbance readings of FB_1-HRPO conjugate samples at 595 nm were 0.433 and 0.426; the mean was 0.430.

Protein concentration of FB_1-HRPO = $(0.430 - 0.0482) \div 0.0009 = 424$ µg/ml.

4.4.2 Protein Concentration of Primary Antibody

Duplicate absorbance readings of 1:40 dilutions of combined IgY extracts at 280 nm were 1.149 and 1.153; the mean was 1.151.

Protein concentration of Ab_1 = $(1.151 \div 1.25) \times 40 = 36.83$ mg/ml.

4.4.3 Competitive Direct ELISA Method

Step1.

Initial experiments were done to check the coating buffer [PBS (pH 7.2) or carbonate/ bicarbonate (pH 9.6)] and the coating temperature (RT or 37°C) using two blocking agents (1% casein or 0.5% BSA in PBS).

TABLE 4.2 COMPARISON OF COATING BUFFERS AND COATING TEMPERATURES

PBS for coating	**Absorbance at RT**		**Absorbance at 4°C**	
Blocking Agent	**1% casein**	**0.5% BSA**	**1% casein**	**0.5% BSA**
No Ab_1 blank	0.087	0.085	0.094	0.091
Ab_1 (µg/ml)				
0.1	0.778	0.624	0.669	0.718
1	2.583	2.448	1.959	1.944
10	>3.000	>3.000	2.353	2.323
100	2.842	2.844	2.349	2.309
Carbonate/bicarbonate buffer for coating	**Absorbance at RT**		**Absorbance at 4°C**	
Blocking Agent	**1% casein**	**0.5% BSA**	**1% casein**	**0.5% BSA**
No Ab_1 blank	0.103	0.110	0.114	0.102
Ab_1 (µg/ml)				
0.1	0.576	0.666	0.678	0.776
1	1.660	1.668	2.130	2.208
10	2.396	2.461	2.684	2.714
100	2.490	2.628	2.641	2.635

Key to Table 4.2

Absorbance values at 405 nm are the means of duplicates after subtraction of no Ab_1 blank.
Other experimental conditions were blocking for 1 hr at 37°C, no FB_1 or FB_1-HRPO but addition of 120 µl Ab_2^E, 1:2 000 incubated for 1 hr at 37°C.

The best conditions for coating the plate with Ab_1 were using PBS at RT (and 1% casein for blocking) as these gave the highest absorbance values for each antibody concentration with a low absorbance reading for the no Ab_1 blank (Table 4.2). Even though the RT varies throughout the year, it was more constant at night and incubation was done overnight.

Step2.

Next experiments were done to decide on the blocking agent (1% casein or 0.5% BSA in PBS), and blocking temperature (1 hr at RT or 37°C) using various dilutions of primary antibody in PBS.

TABLE 4.3 COMPARISON OF BLOCKING AGENTS AND BLOCKING TEMPERATURES

	Absorbance at RT		Absorbance at 37°C	
	1% casein	0.5% BSA	1% casein	0.5% BSA
No Ab_1 blank	0.106	0.107	0.105	0.105
Ab_1 (µg/ml)				
0.1	1.030	0.924	1.028	0.959
0.2	1.644	1.495	1.605	1.453
0.4	2.305	2.146	2.239	2.141
0.6	2.490	2.341	2.354	2.291
0.8	2.593	2.451	2.466	2.347
1.0	2.824	2.605	2.398	2.342

Key to Table 4.3
Absorbance values at 405 nm are the means of duplicates after subtraction of the no Ab_1 blank.
Other experimental conditions were no FB_1 or FB_1-HRPO but addition of 120 µl Ab_2^E, 1:2 000 incubated for 1 hr at 37°C.

As a blocking agent, 1% casein gave higher absorbance results with lower values for the no Ab_1 blank than 0.5% BSA. This was fortunate since casein is cheaper than BSA. Results at the two temperatures (RT and 4°C) were similar (Table 4.3). The range of concentrations of antibody for this and other experiments was selected to cover a wide range. To saturate available sites on the plate, Crowther (1995) recommended using 50 - 500 ng/well in a volume of 50 µl (but the concentration could be increased if a larger volume were used).
However, for efficient use of Ab_1 it would also be preferable to use the lowest possible concentration that gave satisfactory results.

Step 4.
Having established coating and blocking conditions, to assess the viability of the enzyme triplicate dilutions of various concentrations of FB_1-HRPO conjugate were reacted with substrate.

TABLE 4.4 CHECK FOR ACTIVITY OF ENZYME IN FUMONISIN B_1-HORSERADISH PEROXIDASE CONJUGATE

FB_1-HRPO	10 ng/ml	25 ng/ml	50 ng/ml	100 ng/ml	200 ng/ml	500 ng/ml	1 000 ng/ml
Absorbance	0.493	1.155	2.068	> 2.5	> 2.5	> 2.5	> 2.5

Key to Table 4.4
Absorbance values at 405 nm are the mean of duplicates after subtraction of substrate blank.

The high absorbance values confirmed that the enzyme was still active (Table 4.4).
At a concentration of 25 ng/ml for FB_1-HRPO, a suitable maximum absorbance value was achieved. The maximum absorbance value should be <2 , preferably between 1.0 and 1.5 (Crowther 1995).

Experiments were then done to check the zero point on the standard curve i.e. no FB_1 standards but at varying concentrations of antibody and FB_1-HRPO.

TABLE 4.5 ZERO POINT ON STANDARD CURVE AT HIGHER ANTIBODY CONCENTRATIONS

FB_1-HRPO	**25 ng/ml**	**50 ng/ml**	**100 ng/ml**
Total	1.310	2.242	> 2.3
No Ab_1 blank	0.078	0.078	0.082
Ab_1 (µg/ml)			
10	0.102	0.112	0.118
100	0.106	0.113	0.122
500	0.102	0.104	0.108

Key to Table 4.5

The results are means of duplicate absorbance values at 405 nm. Other experimental conditions were coating at RT overnight, blocking with 1% casein for 1 hr at 37°C, incubation with 50 µl PBS and 50 µl FB_1-HRPO for 2 hr at 37°C. Total tubes were included to give a measure of the maximum possible absorbance for FB_1-HRPO.

In another experiment, different concentrations of Ab_1 and FB_1-HRPO were used and the incubation reaction between the conjugate and the antibody were done at RT and at 37°C.

TABLE 4.6 ZERO POINT ON STANDARD CURVE AT LOWER ANTIBODY CONCENTRATIONS

FB_1-HRPO	10 ng/ml		100 ng/ml		1 ug/ml		10 µg/ml	
Total	0.441		1.487		2.573		>3.0	
	RT	37°C	RT	37°C	RT	37°C	RT	37°C
No Ab_1 blank	0.085	0.070	0.081	0.077	0.088	0.087	0.171	0.144
Ab_1 (µg/ml)								
0.1	0.082	0.076	0.080	0.081	0.084	0.090	0.140	0.155
0.2	0.080	0.074	0.081	0.080	0.088	0.090	0.147	0.147
0.4	0.087	0.079	0.087	0.089	0.096	0.099	0.173	0.151
0.6	0.084	0.082	0.088	0.088	0.097	0.104	0.167	0.157
0.8	0.090	0.086	0.098	0.111	0.110	0.112	0.161	0.160
1.0	0.090	0.088	0.100	0.099	0.112	0.117	0.193	0.179

Key to Table 4.6

The results are the means of duplicate absorbance values at 405 nm.

Other experimental conditions were coating at RT overnight using PBS as coating buffer, blocking with 1% casein for 1 hr at 37°C, incubation with 50 µl PBS and 50 µl FB_1-HRPO. Total tubes were included to give a measure of the maximum possible absorbance for FB_1-HRPO.

Further experiments using different concentrations of antibody and FB_1-HRPO also gave disappointing results.

TABLE 4.7 ZERO POINT ON STANDARD CURVE USING DIFFERENT ANTIBODY AND FUMONISIN B_1-HORSERADISH PEROXIDASE CONCENTRATIONS

FB_1-HRPO	50 ng/ml	100 ng/ml	250 ng/ml	500 ng/ml	1 µg/ml	2 µg/ml
Total	1.013	1.823	2.715	2.940	2.890	2.868
No Ab_1 blank	0.093	0.102	0.096	0.095	0.102	0.128
Ab_1 µg/ml						
1	0.107	0.097	0.115	0.109	0.096	0.101
5	0.113	0.102	0.112	0.103	0.103	0.151
10	0.108	0.110	0.118	0.137	0.118	0.117
50	0.109	0.105	0.108	0.148	0.162	0.143
100	0.128	0.105	0.108	0.133	0.116	0.116

Key to Table 4.7

The results are the means of duplicate absorbance values at 405 nm.

Other experimental conditions were coating at RT overnight using PBS as coating buffer, blocking with 1% casein for 1 hr at RT and incubation for reaction of conjugate and antibody for 1 hr at 37°C.

TABLE 4.8 ZERO POINT ON STANDARD CURVE USING HIGHER FUMONISIN B_1-HORSERADISH PEROXIDASE CONCENTRATIONS

FB_1-HRPO	50 ng/ml	100 ng/ml	500 ng/ml	1 µg/ml	2 µg/ml	5 µg/ml
Total	0.827	1.520	2.617	2.383	1.156	1.006
No Ab_1 blank	0.090	0.093	0.102	0.129	0.115	0.204
Ab_1 (µg/ml)						
1	0.098	0.098	0.100	0.122	0.113	0.187
10	0.100	0.103	0.109	0.118	0.118	0.136
100	0.105	0.104	0.107	0.112	0.108	0.136
500	0.100	0.100	0.101	0.107	0.102	0.104

Key to Table 4.8

The results are the means of duplicate absorbance values at 405 nm.

Other experimental conditions were coating at RT overnight using PBS as coating buffer, blocking with 1% casein for 1 hr at RT and incubation for reaction of conjugate and antibody for 1 hr at 37°C.

The FB_1-HRPO was made up in 0.5% BSA or 1% casein in PBS. Results in Table 4.8 were for 0.5% BSA in PBS; results for 1% casein were similar i.e. low absorbance values.

In the experiments to establish the zero point of the standard curve (which should give the highest absorbance value) results were disappointing as at a variety of concentrations of antibody and FB_1-HRPO, maximum absorbance values were only between 0.096 and 0.187. These results were too close to the values for the no Ab_1 blank which varied from 0.070 to 0.204. The total and zero point absorbances increased with increasing concentrations of FB_1-HRPO but so did the results for the no Ab_1 blank. The highest value obtained (0.187) was using 5 µg/ml per well of FB_1-HRPO but the blank was 0.204.

The variation in results for total tubes was due to the light sensitivity of the ABTS. The plate reader used was located in a different department so there was an unavoidable delay between adding the stopping buffer and reading the plate which caused a drop in the total absorbance values particularly at higher readings (Tables 4.5, 4.6, 4.7 and 4.8).

Using RT or 37°C for the incubation temperature for the competition step gave similar results (Table 4.6). Since no satisfactory result was obtained for the zero point, no attempt was made to run a standard curve.

Since this ELISA format was unsuccessful, a sandwich experiment was done to see if the FB_1-HRPO conjugate would bind by initially coating the plate with FB_1. Thus the final sandwich was $FB_1 - Ab_1 - FB_1$-HRPO.

TABLE 4.9 ZERO POINT ON STANDARD CURVE USING SANDWICH ASSAY

FB_1 for Coating	**10 ng/ml**			**100 ng/ml**			**200 ng/ml**		
FB_1-HRPO	**2 ng/ml**	**10 ng/ml**	**50 ng/ml**	**2 ng/ml**	**10 ng/ml**	**50 ng/ml**	**2 ng/ml**	**10 ng/ml**	**50 ng/ml**
Total	0.530	1.030	2.508	0.533	1.049	2.483	0.544	1.070	2.473
No Ab_1 blank	0.079	0.087	0.102	0.083	0.082	0.092	0.078	0.087	0.084
Ab_1 (µg/ml)									
10	0.082	0.081	0.080	0.083	0.085	0.081	0.078	0.081	0.077
100	0.084	0.084	0.083	0.091	0.083	0.093	0.076	0.083	0.092
200	0.080	0.085	0.082	0.082	0.089	0.098	0.082	0.091	0.110

Key to Table 4.9

The results are means of duplicate absorbance values at 405 nm.

Other experimental conditions were coating FB_1 overnight at 4°C, blocking with 1% casein in PBS for 1 hr at 37°C, Ab_1 incubated for 2 hr at 37°C, FB_1-HRPO (no FB_1 standard) for 2 hr at 37°C.

4.5 DISCUSSION

For effective use in a CD-ELISA, the enzyme-antigen conjugate must retain both enzymatic and immunological activity (Voller & de Savigny, 1981). This appears not to have been the case in the experiments described in this chapter. Mycotoxin-enzyme conjugates can have stability problems with the enzyme (Chu, 1985).

Coating with Ab_1 at 0.8 or 1 µg/ml and using 10 µg/ml FB_1-HRPO gave the best results but differences between absorbance for the zero point and no Ab_1 blank were still only ± 0.022 to 0.035 which was unacceptably low (Table 4.6). Using high concentrations of conjugate was not acceptable as there would then have been sufficient to run only a few plates before making a new batch. This would not be economical as both FB_1 and HRPO are costly items. Also, increasing the concentration of coating antibody (Ab_1) or antigen-enzyme conjugate (FB_1-HRPO) would decrease the sensitivity of the method (Schneider *et al.*, 1995).

The similarity in results between the zero point and no Ab_1 blanks indicated that the conjugate is probably not binding to the antibody (Tables 4.5, 4.6 and 4.7). There could have been a problem with the conjugate; either the FB_1 did not attach to the HRPO or it could have linked in most of the conjugates at the same or adjoining sites required to bind antibody causing steric hindrance. The little colour obtained could be from non-specific binding of HRPO to the plate. Possibly the BSA or casein in the diluent were preventing binding of FB_1-HRPO to Ab_1. Another possibility is incomplete blocking or washing but this is unlikely as these techniques had been successful with the method for antibody titre (Chapter 3).

The sandwich assay to check binding of the FB_1-HRPO conjugate to antibody was also unsuccessful as again absorbance values were close to those for no Ab_1 blanks (Table 4.9). Again this could be because the FB_1 did not attach to HRPO and the conjugate extract was only HRPO with the FB_1 having been removed during dialysis. Alternatively, assuming the conjugate to have been correctly prepared, the antibody may not have two binding sites for FB_1; or there was steric hindrance.

Briand *et al.*, (1985) suggested that if coupling is unsuccessful with one agent another should be tried. Possibly use of the carbodiimide method as used by Yu & Chu (1996) should have been attempted but due to limited funding, purchase of more FB_1 was not possible.

Alternatively a different ELISA format using antibody labelled with HRPO could have been attempted. However, this would have required purification of the primary antibody to remove anti-KLH and other antibodies by use of immunoaffinity methods. Again these would require purchase of more FB_1 which was not possible on the limited budget.

4.6 CONCLUSION

Despite 4 months careful work, this method was not successful. The problem seemed to be the fumonisin-enzyme conjugate which did not bind to the antibody. It was decided to attempt an indirect competitive method.

CHAPTER 5

COMPETITIVE INDIRECT ELISA

5.1 INTRODUCTION

For a quantitative method, an indirect competitive ELISA (CI-ELISA) can be used instead of a direct competitive method. Indirect competitive ELISA methods usually have better sensitivity and use ten to a hundred times less antibody than a direct competitive method but they are more costly (need for a second enzyme-labelled antibody bought commercially) and more time consuming (second incubation with second antibody). The second antibody is from a different animal to the first antibody e.g. Rabbit anti-chicken; goat anti-rabbit. conjugate is used to coat the plate (Chu, 1985).

The format for a CI-ELISA is as follows:-

$$S\text{-}Ag + Ab_1 + Ag \leftrightarrow S\text{-}Ag\text{-}Ab_1 + Ag\text{-}Ab_1$$

$$S\text{-}Ag\text{-}Ab_1 + Ab_2^{E} \rightarrow S\text{-}Ag\text{-}Ab_1\text{-}\ Ab_2^{E}$$

In the first reaction, antigen (Ag) bound to the microtitre plate competes with free antigen (standard or sample) for binding to the primary antibody (Ab_1). After washing to remove unreacted free antigen and antibody as well as the antigen-antibody complex in solution, a second enzyme-labelled antibody (Ab_2^{E}), anti-Ab_1, is added. By the law of mass action, the more free Ag is present the less S-Ag-Ab_1 and hence the less S-Ag-Ab_1- Ab_2^{E} will be formed i.e. there is an inverse relationship between the amount of free Ag present and the amount of coloured product formed (Crowther, 1995; Voller & Bidwell, 1986).

Optimisation of a CI-ELISA method is also more complex because of more steps in the assay method. All the following need to be optimised:

* coating buffer and concentration of antigen for coating the microtitre plates
* concentration of primary antibody for competition to cover suitable range of antigen concentrations
* buffer/diluent, blocking agent, incubation times
* concentration of second antibody to give best results

For application of this format to analysis of fumonisins, the antigen was FB_1, the primary antibody was from chickens or rabbits (prepared as described in Chapter 3), the solid phase was the wells of the microtitre plate and the second enzyme-labelled antibody was rabbit anti-chicken IgG-HRPO or goat anti-rabbit IgG-HRPO (Sigma Chemical Co., USA).

Competitive indirect ELISA methods were successfully developed (Azcona-Olivera *et al.*, 1992A; Fukuda *et al.*, 1994; Shelby *et al.*, 1994;Yeung *et al.*, 1996; Yu & Chu, 1996) but these were primarily to check for cross-reactivity with FB_1 analogues (Azcona-Olivera *et al.*, 1992A; Fukuda *et al.*, 1994; Yu & Chu, 1996). Only Shelby *et al.* (1994) and Yeung *et al.* (1996) adapted their methods to analyse for fumonisin in maize samples.

5.1.1 Objectives

1. To use HPLC to check the stability of the FB_1 stock standards that had been kept at 4°C (refrigerator) and –20°C (freezer) for close to 2 years.
2. To use the chicken antibodies (combination of IgY extracts C2/8 and C2/12) to develop a sensitive CI-ELISA for analysis of fumonisins.
3. To investigate methods of data reduction to obtain the most accurate quantitative results.
4. To check the cross-reactivity of the rabbit (IgG extract R1/14) and chicken antibodies to FB_2, FB_3 and So.
5. To determine range, detection limits, intra-assay and inter-assay CV of the method.

At all times the over-riding factors were that the method must be sensitive so that it could be used for analyses in physiological fluids and it must be relatively easy to perform and not too expensive.

5.2 MATERIALS

5.2.1 Chemicals

D-sphingosine and o-phthaldialdehyde were purchased from Sigma Chemical Co., USA..

Methanol (HPLC grade) and acetonitrile (HPLC grade) were from BDH.

FB_1 stock standards (for ELISA) were prepared as described in Chapter 3, section 3.2.3.

Anti-FB_1 antibodies previously raised in chickens and rabbits (Chapter 3).

FB_2 and FB_3 were kindly donated by Dr. M. Castegnaro, IARC, France.

For all other chemicals refer to Chapter 3, section 3.2.1.

5.2.2 Buffers/Solutions

The following buffers were used in the CI-ELISA:

Phosphate buffered saline (PBS) (pH 7.2)	(Appendix 2, 2.2)
0.15M Citrate-phosphate buffer (pH 5.0)	(Appendix 2, 2.6)
Stopping buffer : 0.1% sodium azide in citrate-phosphate buffer	(Appendix 2, 2.7)
0.1M Sodium phosphate buffer (pH 3.35)	(Appendix 2, 2.11)

5.3 METHODS

5.3.1 High Performance Liquid Chromatography

This was done to check the aqueous standards and was based on the methods of Shephard *et al.* (1992B), Thiel *et al.* (1993) and Duncan *et al.* (1998).

A stock fumonisin B_1 standard for HPLC was made by dissolving 1 mg FB_1 in 1 ml acetonitrile:water (1:1). Suitable dilutions of the stock standard were made in acetonitrile:water (1:1) to give standards of 50, 100 and 200 µg/ml.
The mobile phase was composed of 0.1M sodium phosphate buffer (pH 3.35) : methanol (27:73). It was degassed for 15 min/300 ml at 20°C and 220V (UMC 10 waterbath, Ultrasonic Manufacturing Co.)

The Spectra-Physics HPLC instrument (Thermo Separation Products, SMM Instruments, South Africa) had a C_{18} guard column (Hichrom) and a C_{18} analytical column (Nova-Pak stainless steel column, 3.9 x 150 mm, 4 µm packing material, Waters). For fluorometric detection (xenon lamp), the excitation wavelength was 336 nm and the emission wavelength was 440 nm; the flow rate was 1 ml/min and the pressure was ±1800 psi.

Two100 µl aliquots of PBS and four 100 µl aliquots of aqueous FB_1standards (two kept at 4°C and two kept at –20°C) were pipetted into Eppendorf micro-test tubes (AEC Amersham) and freeze dried. The powders were re-dissolved in acetonitrile:water (1:1). Just before analysis, 25 µl of re-constituted FB_1 standard or buffer was added to 225 µl o-phthaldialdehyde (OPA) (Appendix 2, 2.13), and mixed for exactly 1 minute. Using a 100 µl Hamilton syringe, 40 µl of the mixture was injected through the septum; 20 µl was transferred to the column via the injection loop. Each sample/standard was derivatised and injected in duplicate. The time from derivatisation to loading on to the column was 2 min.

Each standard/sample was run for 6 min in case there was any FB_2 or FB_3 present (These elute after FB_1). Calculations of peak area were done using PC-1000 software (Thermo Separation Products). The same experiment was repeated twice.

5.3.2 Competitive Indirect ELISA

In the summary below the final optimised method is shown first and the tested alternatives for each step are shown underneath in Italics. Unless otherwise stated experiments were done using chicken antibodies.

Checkerboard titrations were done to ascertain the optimum concentration of coating antigen (10 to 1 000 ng/ml), the concentration of first antibody (100 to 500 µg/ml) to give a suitable range for the standard curve, the diluent for antibody solutions (containing BSA or casein) and incubation times and temperatures .

Since room temperature in the laboratory could vary by 10°C, only temperatures of 4°C (refrigerator) or 37°C (incubator) were used for incubation.

1. The wells of the microtitre plate (Nunc 96 well Maxisorb, AEC Amersham) were coated directly with FB_1, 200 ng/ml in PBS, 100 µl per well. The plate was covered and incubated for 18 hr at 4°C.
   Concentrations of FB_1 - 50, 100, 150, 200, 250 ng/ml.
2. The wells were blocked with 1.0% casein (m/v) in PBS, 250 µl per well and incubated for 1 hr at 37°C.
3. The plate was washed three times with 0.05% (v/v) Tween 20 in PBS.
   0.05% or 0.1% Tween 20 in PBS.
4. FB_1 standards (0, 1, 2, 3, 5, 8, 10, 20 ng/ml in PBS) were pipetted in triplicate into relevant wells, 50 µl per well.
   Concentration of FB_1 standards - 0, 1, 2, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50 ng/ml.
5. The primary antibody (Ab_1) was added to relevant wells, 300 µg/ml in 0.5% BSA in PBS, 50 µl per well.
   Concentration of primary antibody – 100, 200, 300, 400, 500 µg/ml.
6. The plate was mixed by gentle tapping and incubated for 1 hr at 37°C.
   1 or 2 hr at 37°C.

7. The plate was washed three times with 0.05% (v/v) Tween 20 in PBS.
   0.05% or 0.1% Tween 20 in PBS.
8. The second antibody (Ab_2^E), anti-chicken IgG (or anti-rabbit IgG) conjugated with horseradish peroxidase was added, 1:4 000, 120 µl per well and incubated for 1hr at 37°C.
   Dilution of second antibody – 1:2 000, 1:3 000, 1:4 000, 1:5 000
   100 or 120 µl per well.
9. The plate was washed three times with 0.05% (v/v) Tween 20 in PBS.
   0.05% or 0.1% Tween 20 in PBS.
10. 150 µl of substrate, 7.5 mg ABTS plus 7.5 µl H_2O_2 in 15 ml citrate-phosphate buffer (pH 5.0) was added to each well. The colour was allowed to develop for 15 min in the dark at RT.
11. 50 µl of stopping buffer was added to each well.
12. The absorbance of each well was read at 405 nm (Bio-Rad Model 550 Plate Reader).

In all assays, the following were done:

* All glassware was washed thoroughly with detergent, rinsed 5x in tap water then 3x in glass distilled water and air dried.
* New plates and new tips were used every time.
* PBS (10x), citrate-phosphate buffer and stopping buffer were prepared every 2 weeks.
* PBS and PBS-Tween were prepared freshly the day before every run.
* 1% casein in PBS was prepared the day before any run and allowed to stand overnight at 4°C to dissolve.
* All other reagents were prepared 15 to 20 min before use except ABTS where the H_2O_2 was added just before use.
* Plates were mixed by tilting and gentle tapping.
* The plate was covered with a lid.
* For incubation at 37°C, plates were placed in a sealed constant humidity box in the incubator.
* All samples/standards/controls were assayed in triplicate.
* Triplicate absorbance results were only acceptable if CV ± 5%.

* A no Ab1 blank (which gives measure of non-specific binding) was included on every plate.
* Washing steps were done for 3 x 30 sec.

5.3.3 Calculations

Various alternative ways of calculating quantitative results using suitable software were investigated. This was necessary as the plate reader (Bio-Rad Model 550) only gave absorbance readings.

Initially a curve fit was done to determine the parameters in the selected equation (curve type) for the standard curve graph and then backfitting to solve the equation for x using the values obtained for the parameters.

A suitable curve type has to be selected viz. linear regression, linear interpolation, logit-log, 2^{nd} order polynomial, 3^{rd} order polynomial or 4-parameter non-linear regression equation. Scales on the axes can be linear or logarithmic. For a partly sigmoid curve (as obtained with an indirect competitive ELISA), the recommended equation is a 2^{nd} order polynomial ($y = ax^2 + bx + c$) or a 4-parameter non-linear regression ($y = (a-d)/(1 + (x/c)^b) + d$. Curve fitting is done more than once (iterations) until the best fit is obtained. If the curve fit is good, the deviation between known concentrations and measured concentrations are small; deviations must be randomly distributed over the entire curve. In a 4-parameter curve, parameter a is the upper asymptote, b is the slope, c is the concentration at the inflection point of the curve and d is the lower asymptote. In a 2^{nd} order polynomial parameter c is the y intercept, a and b are constants for that curve. (ELISA+ booklet, Meddata Inc., New York; Sigmaplot instruction book, Jandel Scientific; Rodgers, 1986).

The backfit equation for a 2^{nd} order polynomial is $x = (- b - (b^2 - 4a(c - y))^{0.5})/2a$

The following options for curve fitting were examined:-

	y-axis	x-axis	Equation
Method 1	Absorbance	$[FB_1]$ ng/ml	2^{nd} order polynomial
Method 2	Absorbance	Log $[FB_1]$ ng/ml	2^{nd} order polynomial
Method 3	%B/Bo	$[FB_1]$ ng/ml	2^{nd} order polynomial
Method 4	%B/Bo	Log $[FB_1]$ ng/ml	2^{nd} order polynomial

Method 5	Absorbance	[FB_1] ng/ml	4-parameter equation
Method 6	Absorbance	Log [FB_1] ng/ml	4-parameter equation
Method 7	%B/Bo	[FB_1] ng/ml	4-parameter equation
Method 8	%B/Bo	Log [FB_1]	4-parameter equation
Method 9	Absorbance	[FB_1] ng/ml	4-parameter equation
Method 10	%B/Bo	[FB_1] ng/ml	linear regression
Method 11	%B/Bo	[FB_1] ng/ml	logit-log

In methods 6 and 8, parameter dependencies for c and d were close to 1 and very close to each other, so the equation was altered to remove parameter d (as recommended in the Sigmaplot instruction book, Jandel Scientific). These were recorded as methods 6A and 8A.
Method 9 was a check on the calculations using ELISA software (ELISA +, Meddata, Inc. New York) linked to the plate reader at the Natal Blood Transfusion Service (NBTS) with the kind assistance of Mr. R. Parkinson.
Some methods were tried with and without weighting viz. 3, 4, 7, 8 but the results were no better. Furthermore, if using weighted methods the standard error and coefficient of variation of the parameters have no meaning (Sigmaplot).

Absorbance values were means after subtraction of the mean value for the no Ab_1 blank.
%B/Bo was the ratio of the mean absorbance value expressed as a percentage of the mean value for the zero point on the standard curve (Crowther, 1995).

In addition to standard curves, results from low (± 4 ng/ml) and high (± 8 ng/ml) method controls (FB_1 made up in PBS, n = 20) were used to decide on the best method of data reduction.

Criteria for an acceptable method of data reduction were as follows:-

* low values for the chi square statistic (χ^2 <0.01)
* low values for SE (<0.1) and CV of parameters (<10%)
* low value for norm of fit results (Sigmaplot)
* low values for CV of low and high controls

Acceptable standards at NBTS are $\chi^2 < 0.01$ and number of iterations < 9 (Mr. R. Parkinson, personal communication).

To further check the correctness of selected method of calculation, method control results were compared with those obtained using ELISA+ software for calculation.
Also, plasma sample results (Chapter 6) were compared with those from a Bio-Rad Model 3550 Plate Reader with dedicated software. This calculated results using a quadratic curve fit and plotted absorbance vs. concentration. Unfortunately this plate reader was not available for regular use.

5.3.4 Method Parameters

The range of the standard curve was established by running a series of standards in triplicate viz. 1, 2, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50 ng/ml.
Intra-assay (same plate) and inter-assay (different plates) reproducibility were calculated using the fumonisin method controls (± 4 and ± 8 ng FB_1 /ml in PBS).
Ruggedness was estimated as the reproducibility of the concentration of FB_1 giving 50% inhibition in the competition (i.e. ID_{50} value).
Detection limits were calculated using Student's two sample t-test on results of diluted FB_1 standards (0.5, 0.4, 0.3, 0.2, 0.1, 0.0 ng/ml, n = 11 for each).

5.3.5 Cross-reactivity

The cross-reactivity of chicken and rabbit antibodies with FB_2, FB_3 and So was done by constructing standard curves at concentrations of 2, 3, 5, 8, 10, 20 and 40 ng/ml (in triplicate). The concentration of Ab_1 for both chicken (combined IgY extracts C2/8 and C2/12) and rabbit (IgG extract R1/14) antibodies was 300 µg/ml. Graphs were plotted as %B/Bo vs. log [FB_1] . Concentrations of FB_1, FB_2, FB_3 and So at 50% binding were used to calculate ID_{50}.
Cross-reactivity was calculated as ID_{50} for FB_1 / ID_{50} for analogue x 100.

5.4 RESULTS

5.4.1 High Performance Liquid Chromatography

TABLE 5.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CHECK ON STORED FUMONISIN B_1 STANDARDS

Sample	Retention time	Peak area	FB_1 (µg/ml)
PBS (i.e. OPA)	1.433	N/A	
Standard 50 µg/ml	2.381	10012	
Standard 50 µg/ml	2.447	9079	
Standard 200 µg/ml	2.443	37025	
FB_1 standard stored at 4°C	2.431	18883	100.5
FB_1 standard stored at – 20°C	2.419	18384	97.8
PBS (ie. OPA)	1.479	N/A	
Standard 50 µg/ml	2.360	16490	
Standard 50 µg/ml	2.371	16517	
Standard 100 µg/ml	2.363	26068	
Standard 100 µg/ml	2.362	32746	
FB_1 standard stored at 4°C	2.353	30693	98.7
FB_1 standard stored at – 20°C	2.360	27663	88.9

The PBS samples only show a peak for OPA i.e. no fumonisin present. Using the peak areas of the standards, the concentrations of the stored fumonisin standards were calculated.

Mean at 4°C = 99.6 µg/ml.

Mean at – 20°C = 93.4 µg/ml.

The expected result was 100 µg/ml.

5.4.2 Competitive Indirect ELISA

The initial checkerboard titration experiment was to determine the concentration of FB_1 to use for coating and a suitable concentration of Ab_1 to give a workable standard curve. In the titre experiments it had already been found that FB_1 in PBS coated directly to the microtitre plate (Chapter 3, section 3.4.3).

The no Ab_1 blank gives an indication of non-specific binding of Ab_2^E to the plate; the no Ab_2 blank is a substrate blank for ABTS and an indicator of non-specific binding of ABTS to bound Ab_1.

The absorbance values for no Ab_1 and no Ab_2 blanks (Table 5.2) were about the same therefore Ab_2^E is not binding to coated FB_1 and/or casein.

Even though the checkerboard did not titrate out, results already looked more promising than those for the competitive direct method.

TABLE 5.2 CHECKERBOARD TITRATION FOR CONCENTRATION OF FUMONISIN B_1 FOR COATING AND CONCENTRATION OF PRIMARY ANTIBODY

Coating concentration of FB_1	**10 ng/ml**		
No Ab_1 blank	0.098		
No Ab_2 blank	0.101		
Concentration of Ab_1	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**
FB_1 standards (ng/ml)			
0	0.111	0.158	0.207
5	0.110	0.145	0.181
10	0.108	0.154	0.182
50	0.111	0.150	0.180
100	0.105	0.141	0.187
250	0.112	0.145	0.204
500	0.109	0.142	0.174
Coating concentration of FB_1	**100 ng/ml**		
No Ab_1 blank	0.103		
No Ab_2 blank	0.098		
Concentration of Ab_1	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**
FB_1 standards (ng/ml)			
0	0.150	0.328	0.492
5	0.116	0.161	0.252
10	0.122	0.157	0.208
50	0.133	0.163	0.194
100	0.114	0.151	0.186
250	0.158	0.172	0.192
500	0.130	0.155	0.191

Key to Table 5.2

The results are the means of duplicate absorbance values at 405 nm.

Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; incubation for competition for 1 hr at 37°C; 120 µl per well of Ab_2^E at 1:2 000 dilution, incubated for 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2).

Step 1.

The next experiment was to determine the concentration of FB_1 for coating. Lower concentrations of FB_1 standards were used as the objective was to develop a sensitive ELISA assay.

TABLE 5.3 COMPARISON OF COATING CONCENTRATIONS OF FUMONISIN B_1

	Coating concentrations of FB_1 (ng/ml)				
	50	100	150	200	250
FB_1 standards (ng/ml)					
0	0.360	0.905	0.984	1.180	1.297
1	0.338	0.862	0.912	1.035	1.091
2	0.318	0.664	0.886	0.969	1.023
5	0.290	0.595	0.735	0.715	0.713
10	0.273	0.440	0.457	0.492	0.539
20	0.307	0.330	0.397	0.412	0.336
50	nd	0.303	0.479	0.385	0.345

Key to Table 5.3

Results are means of duplicate absorbance values at 405 nm after subtraction of the mean of the no Ab_1 blank. Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; [Ab_1] 300 µg/ml, incubation for competition was 1 hr at 37°C; 120 µl per well of Ab_2^E at 1:2 000 dilution, incubated for 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2).

A coating concentration of 50 ng FB_1/ml did no titrate out; at 100 and 150 ng/ml the standard curves were not steep enough at low concentrations of FB_1 standards (Appendix 1, Figure 5.1). Results for coating with 200 or 250 ng/ml FB_1 were similar but it was more economical to use 200 ng/ml (Table 5.3 and Appendix 1, Figure 5.1).

Step 3.

Using 0.05% Tween 20 for washing gave less foaming than 1% Tween 20; the presence of foam can cause errors as the next reagent may not be able to react with reagents already bound to the plate (Crowther, 1995).

Step 5.

The optimum concentration for Ab_1 was checked for both hen and rabbit antibodies.

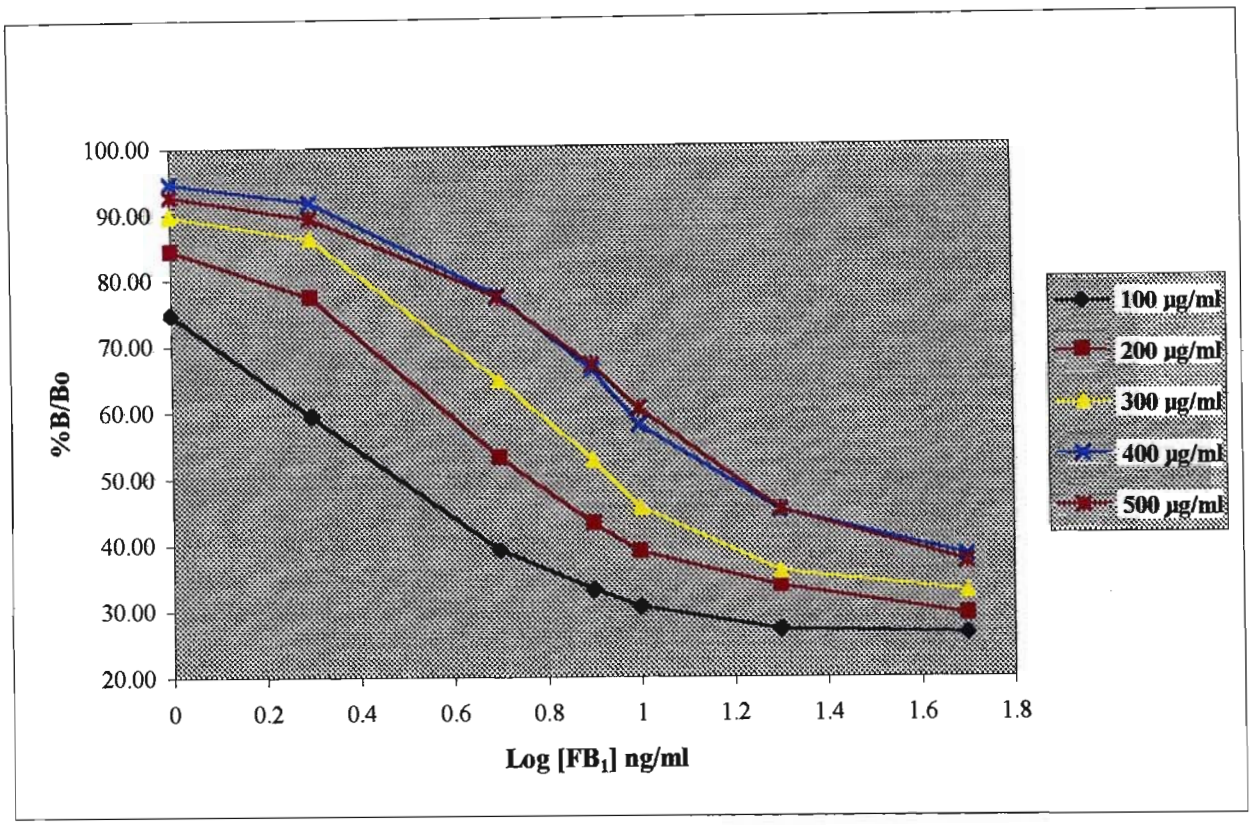


FIGURE 5.2 OPTIMUM CONCENTRATION OF PRIMARY ANTIBODY (CHICKEN ANTIBODIES)

Key to Figure 5.2

The results are the means of duplicate absorbance readings at 405 nm after subtraction of the mean of the no Ab_1 blank. Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; Ab_1 was a combination of IgY extracts C2/8 and C2/12; incubation for competition was 1 hr at 37°C; 120 µl per well of Ab_2^E at 1:2 000 dilution, incubated for 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2). The data used to generate Figure 5.2 is in Appendix 1, Table 5.4.

Plotting results as %B/Bo instead of absorbance takes into account inter-plate variation and any time differences between adding stopping buffer and reading on the plate reader.

The absorbance values for the curve at 100 µg/ml were too low (0.199 to 0.475). The curves using 400 and 500 µg/ml were not steep enough at low concentrations of FB_1. The best results were using 300 µg/ml which gave a steep graph at low concentrations of FB_1 (<10 ng/ml) and a reasonable absorbance value (0.764) for the zero point on the standard curve (Appendix 1, Table 5.4).

The results for the experiment to determine the optimum concentration of Ab_1 for rabbit antibodies (IgG extract R1/14) are in Appendix 1, Figure 5.3 and Table 5.5.
Optimisation of Ab_1 concentration using rabbit antibodies also showed 300 μg/ml gave the best results even though the zero absorbance (0.702) was low. The curves for the rabbit antibodies showed an upturn from the 20 to the 50 ng/ml standard probably due to insufficient Ab_1 at higher concentrations of FB_1 to combine with the FB_1 coated on to the plate i.e. most of the antibody has combined with the free FB_1 and been removed by washing (Appendix 1, Figure 5.3 and Table 5.5).

Step 6.

Incubation for competition studies was done at 37°C because the temperature could be controlled by using an incubator. The time of incubation (1 or 2 hr) was checked.
The curves (Figure 5.4) had a similar shape but the shorter incubation time (1 hr) had a wider range of %B/Bo and its shape was closer to the correct sigmoid shape for a competitive ELISA.

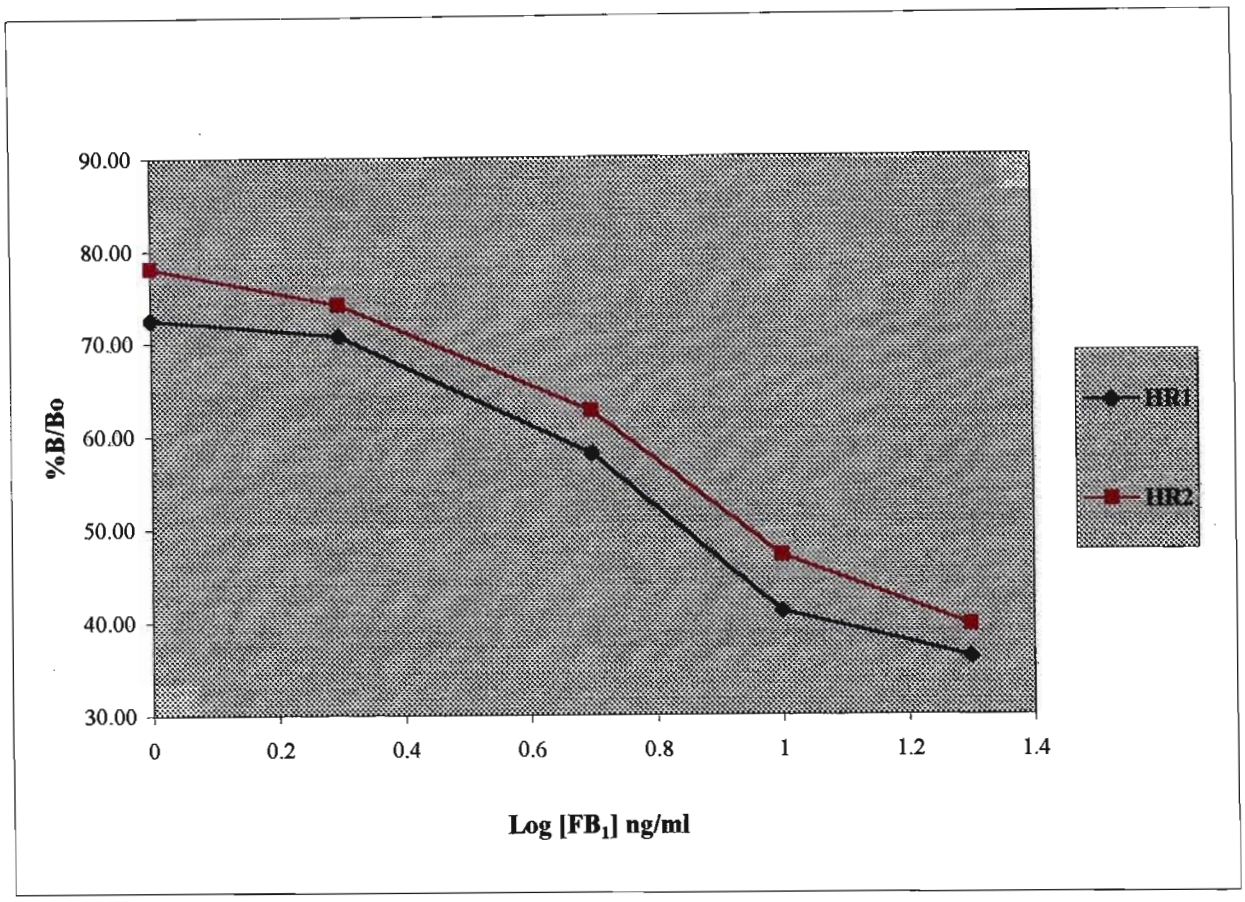


FIGURE 5.4 COMPARISON OF INCUBATION TIMES FOR COMPETITION STEP IN COMPETITIVE INDIRECT ELISA METHOD

Key to Figure 5.4

The results are the means of duplicate absorbance readings at 405 nm after subtraction of the mean of the no Ab_1 blank. Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; Ab_1 was a combination of IgY extracts C2/8 and C2/12; 120 µl per well of Ab_2^E at 1:2 000 dilution, incubated for 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2).

HR1 – incubation for 1 hr HR2 – incubation for 2 hr

The data used to generate Figure 5.4 is in Appendix 1, Table 5.6.

Step 8.

The final checkerboard titrations were done to optimise the concentration and volume of Ab_2^E.

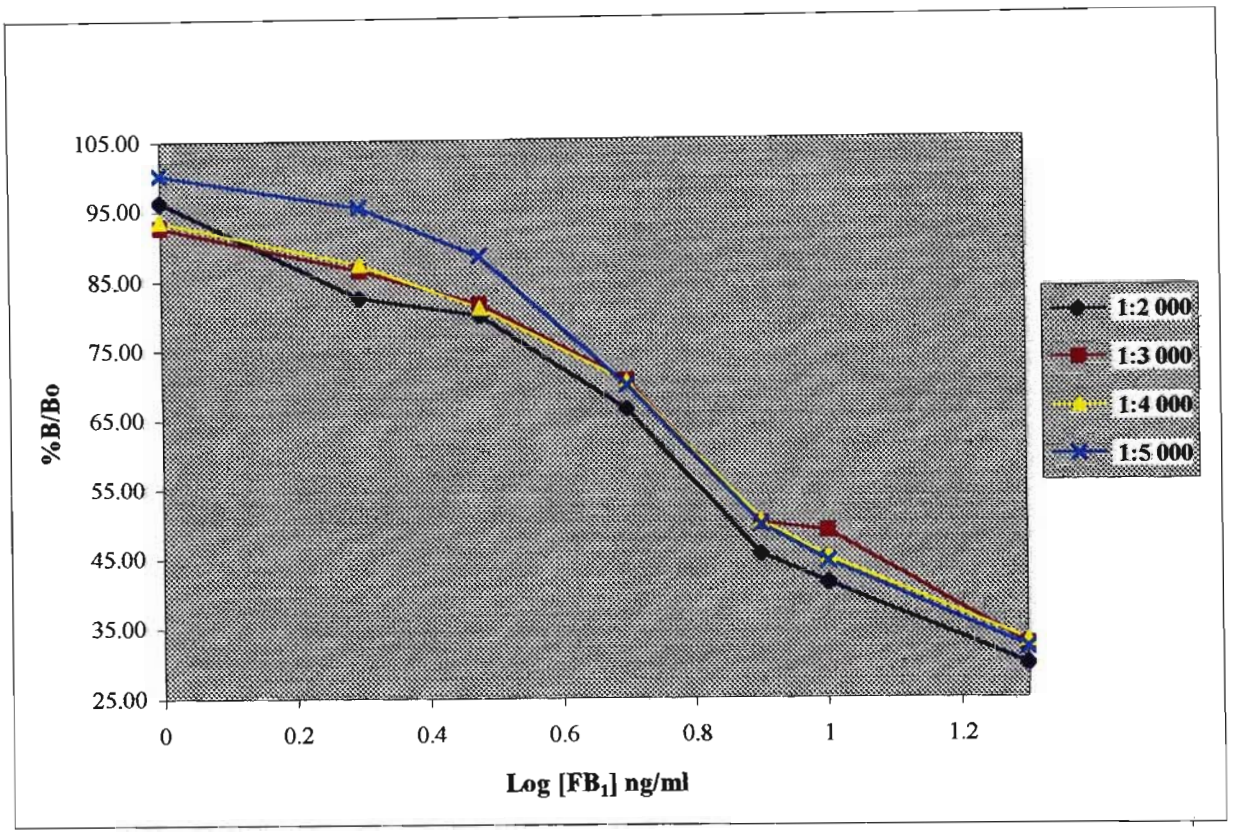


FIGURE 5.5 SELECTION OF OPTIMUM DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (CHICKEN ANTIBODIES)

Key to Figure 5.5

The results are the means of duplicate absorbance readings at 405 nm after subtraction of the mean of the no Ab_1 blank. Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; Ab_1 was a combination of IgY extracts C2/8 and C2/12; incubation for competition was 1 hr at 37°C; 120 µl per well of Ab_2^E, incubated for 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2). The data used to generate Figure 5.5 in Appendix 1, Table 5.7.

The 1:5 000 dilution (Figure 5.5) did not give a sufficiently steep slope at low concentrations of FB_1. The curves for the other dilutions were very similar with the 1:3 000 and the 1:4 000 dilution being almost identical. Therefore the last optimisation experiment was to decide between using a 1:2 000 dilution (as used in the titre method, Chapter 3, section 3.3.7) or a 1: 4 000 which would be more economical. The checkerboard also checked if it was possible to use a smaller volume per well of Ab_2^E viz. 100 µl instead of 120 µl.

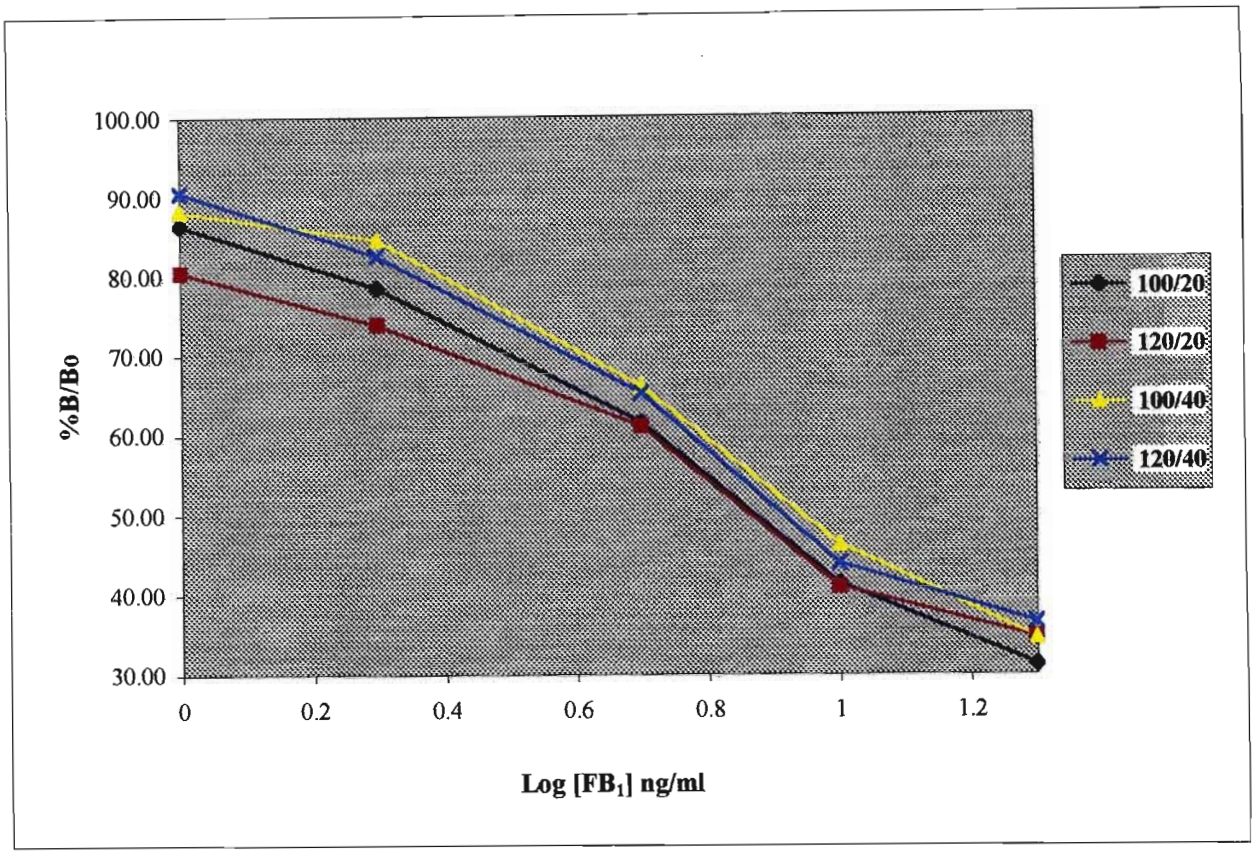


FIGURE 5.6 COMPARISON OF VOLUME AND DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (CHICKEN ANTIBODIES)

Key to Figure 5.6

100/20 - 100 µl of 1:2 000 dilution **120/20** - 120 µl of 1:2 000 dilution

100/40 - 100 µl of 1:4 000 dilution **120/40** - 120 µl of 1:4 000 dilution

The results are the means of duplicate absorbance readings at 405 nm after subtraction of the mean of the no Ab_1 blank. Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; Ab_1 was a combination of IgY extracts C2/8 and C2/12; incubation for competition was 1 hr at 37°C; incubation for second antibody was 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2).

The data used to generate Figure 5.6 is in Appendix 1, Table 5.8.

Using 120 µl of the 1:4 000 dilution (Figure 5.6) gave the best results.

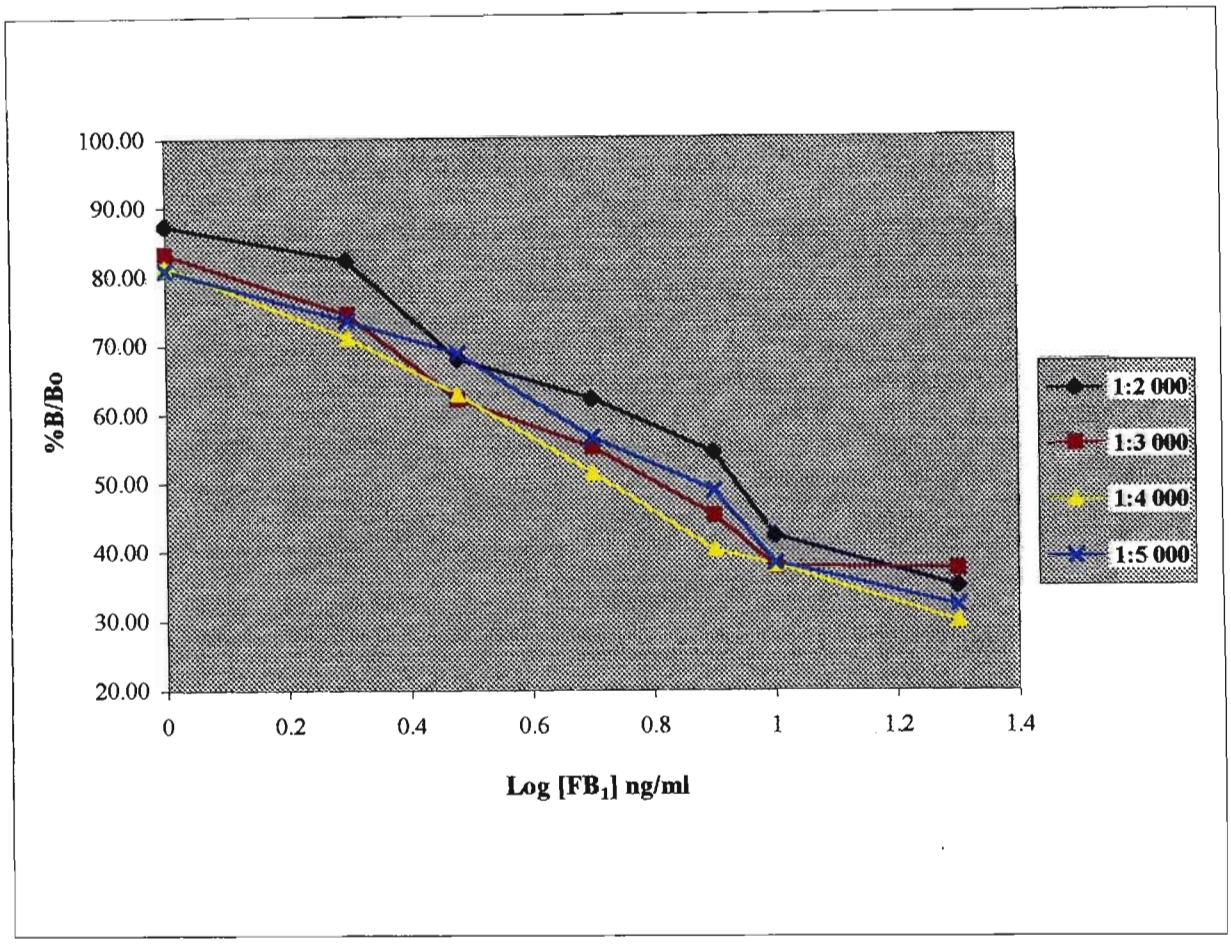


FIGURE 5.7 SELECTION OF OPTIMUM DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (RABBIT ANTIBODIES)

Key to Figure 5.7

The results are the means of duplicate absorbance readings at 405 nm after subtraction of the mean of the no Ab_1 blank. Other experimental conditions were coating with 1% casein in PBS for 1 hr at 37°C; Ab_1 was IgG extract R1/14 (300 µg/ml) and incubation for competition was 1 hr at 37°C; incubation for second antibody was 1 hr at 37°C and substrate reaction as described in steps 9 to 12 (section 5.3.2). The data used to generate Figure 5.7 is in Appendix 1, Table 5.9.

Results for rabbit antibodies (Figure 5.7) also showed that a 120 µl of 1:4 000 dilution was the best as the graph gave almost a straight line between 0 and 8 ng/ml with a maximum absorbance of 1.188.

5.4.3 Calculations

TABLE 5.10 DATA TO DECIDE BEST METHOD OF CALCULATION / CURVE FITTING

Method	1	2	3-nw	3-W	4-nw	4-W	5	6	
Norm	0.02899	0.06204	3.01194	0.38047	6.43408	0.888105	0.03714	0.044436	
SE/P									
a	0.001219	0.07089	0.01232		8.042		0.01712	0.02485	
b	0.00252	0.09584	0.2713		11.74		0.1937	0.5977	
c	0.008527	0.02843	1.055		3.875		0.9115	0.6171	
							0.06256	0.5592	
CV/P									
a	6.371	60.52	6.163		93.45		1.771	2.715	
b	3.662	31.56	3.795		32.68		13.52	29.66	
c	0.8739	3.04	1.046		3.98		14.2	49.06	
d							26.0	338	
χ^2	0.001398	0.007519	0.1462	0.002456	0.784077	0.014329	0.00263	0.003637	
Mean/HC	8.412	9.257	8.404	8.373	9.251	9.11	8.485	8.599	
CV/HC	5.33	7.11	5.33	5.32	7.11	7.41	6.53	7.04	
Mean/LC	4.055	3.651	4.054	4.046	3.649	3.529	3.901	3.802	
CV/LC	11.61	13.1	11.6	11.56	13.09	12.97	10.92	10.66	
Method	**6A**	**7-nw**	**7-W**	**8-nw**	**8-W**	**8A**	**9**	**10**	**11**
Norm	0.045406	3.86369	0.52729	4.6096	0.61643	4.70727			0.363639
SE/P							SD		
a	0.01989	2.368		2.578		2.547	0.016803	2.687	0.1174
b	0.2236	0.2318		0.601		0.2326	0.19029	3.424	0.1496
c	0.03461	0.7919		0.6119		0.03639	0.88497		
D		5.821		57.37			0.060944		
CV/P									
a	2.184	2.378		2.727		2.709	1.7362	2.699	4.181
b	9.959	15.55		29.78		10.32	13.2847	7.287	5.505
c	3.205	12.75		48.82		3.379	13.7985		
d		22.01		343.8			25.2147		
χ^2	0.003555	0.276149	0.005122	0.379394	0.0067	0.006683	0.019944	1.007921	0.293084
Mean/HC	8.473	8.479	8.386	8.591	8.436	8.43	8.479	8.903	9.11
CV/HC	7.01	6.53	6.5	7.04	6.98	6.97	6.53	8.25	5.79
Mean/LC	3.837	3.899	3.904	3.801	3.829	3.832	3.899	3.32	4.219
CV/LC	10.23	10.91	10.68	10.64	10.21	10.19	10.91	12.49	11.75

All curve fitting met the requirement of all converged and tolerance satisfied (Sigmaplot).

Key to Table 5.10 is on the next page.

Key to Table 5.10

Norm	value for norm of fit
SE/P	standard error of parameters
CV/P	coefficient of variation of parameters (%)
χ^2	chi square value
Mean/HC	mean value for high control in ng/ml
HC/CV	coefficient of variation of high control (%)
Mean/LC	mean value for low control in ng/ml
LC/CV	coefficient of variation of low control (%)

Using the criteria as set out in section 5.3.3, the best method was 1 (plotting absorbance vs. concentration of FB_1 and using a 2nd order polynomial for curve fitting. The next best was 6A (plotting absorbance vs. log [FB_1] and a 4-parameter equation for curve fitting (Table 5.10). For method 1 the norm of fit value was 0.02899, the standard errors of the parameters were all less than 0.01, the CV of the parameters were all less than 6.4%, the chi square value was 0.001398 and the CV for the high control was 5.33%. The only criterion that was not met was the CV of the low control which was 11.61%.

For method 6A, the norm of fit value was 0.045406, the standard errors of two of the parameters (a and c) were less than 0.04, the CV of the parameters were all less than 10%, the chi square value was 0.003555 and the CV of the high and low controls were 7.01% and 10.23% respectively. In both methods the number of iterations was <5.
In all further experiments if χ^2 >0.01 or number of iterations >5 the run was repeated.

Parameter values for methods 5 and 9 were as follows:-

Parameter	Method 5	Method 9
a	0.9665	0.96778
b	1.4329	1.4324
c	6.4211	6.4135
d	0.2407	0.2417

Comparison of the parameter values from methods 5 and 9 show good agreement i.e. the method of calculation using Sigmaplot rather than dedicated software attached to a plate reader is acceptable. (Method 5 used Sigmaplot for curve fitting and method 9 used ELISA+ software).

TABLE 5.11 COMPARISON OF CALCULATION OF RESULTS OF METHOD CONTROLS

Control Number	FB_1 (ng/ml) Sigmaplot	FB_1 (ng/ml) ELISA +
Low Control 1	4.426	4.234
Low Control 2	4.235	4.058
Low Control 3	4.311	4.128
Low Control 4	3.841	3.702
Low Control 5	3.633	3.517
Low Control 6	4.140	3.972
Low Control 7	4.146	3.977
Low Control 8	4.673	4.462
High Control 1	8.441	8.505
High Control 2	8.368	8.415
High Control 3	8.496	8.574
High Control 4	8.413	8.471
High Control 5	8.690	8.819
High Control 6	8.738	8.879
High Control 7	8.514	8.597
High Control 8	8.653	8.772

The same means of triplicate absorbance readings (405 nm) were used to calculate results by both methods.

Statistical evaluation of the results (Table 5.11) using both a paired Student's t test and a one-way ANOVA showed there was no significant difference between the two methods of calculation.

TABLE 5.12 COMPARISON OF CALCULATION OF RESULTS OF PLASMA SAMPLES

Sample Number	FB_1 (ng/ml) Sigmaplot	FB_1 (ng/ml) Plate Reader Model 3550	Sample Number	FB_1 (ng/ml) Sigmaplot	FB_1 (ng/ml) Plate Reader Model 3550
1	3.725	3.949	26	7.133	7.112
2	8.088	8.811	27	0.350	0.366
3	4.375	5.385	28	2.415	2.400
4	8.390	9.737	29	4.062	4.117
5	3.991	4.331	30	3.990	4.042
6	4.835	5.315	31	9.644	10.103
7	7.004	7.971	32	7.027	7.239
8	9.652	11.007	33	4.438	4.508
9	8.416	9.645	34	3.045	3.498
10	9.183	9.338	35	4.125	4.802
11	9.533	10.847	36	2.751	3.430
12	17.667	18.941	37	3.045	3.736
13	16.495	13.119	38	4.872	5.526
14	18.670	17.046	39	4.827	5.489
15	22.549	17.478	40	5.949	6.605
16	19.866	21.823	41	7.180	7.822
17	4.356	4.721	42	4.508	4.987
18	3.748	3.988	43	3.393	3.700
19	10.049	11.418	44	2.606	2.888
20	10.710	10.887	45	3.282	3.580
21	12.114	13.538	46	3.307	3.609
22	11.674	13.023	47	4.605	4.926
23	5.244	5.212	48	2.728	3.015
24	4.133	4.103	49	3.983	4.285
25	11.224	11.325	50	3.951	4.257

The same means of triplicate absorbance readings (405 nm) were used to calculate results by both methods.

Statistical evaluation of the results (Table 5.12) using a one-way ANOVA showed there was no significant difference between the two methods of calculation.

5.4.4 Standard Curves

In analyses done after the CI-ELISA method had been optimised, the typical set up of a plate for a run included standards, controls and samples (Table 5.13).

TABLE 5.13 EXAMPLE OF A TYPICAL PLATE FOR COMPETITIVE INDIRECT ELISA ANALYSES

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	NA1	NA1	NA1	S6	S6	S6	S14	S14	S14
B	1	1	1	LC	LC	LC	S7	S7	S7	S15	S15	S15
C	2	2	2	HC	HC	HC	S8	S8	S8	S16	S16	S16
D	3	3	3	S1	S1	S1	S9	S9	S9	S17	S17	S17
E	5	5	5	S2	S2	S2	S10	S10	S10	S18	S18	S18
F	8	8	8	S3	S3	S3	S11	S11	S11	S19	S19	S19
G	10	10	10	S4	S4	S4	S12	S12	S12	S20	S20	S20
H	20	20	20	S5	S5	S5	S13	S13	S13	S21	S21	S21

Key to Table 5.13

Columns **1, 2, 3** and rows **A to H** are fumonisin standards

NA1 no Ab 1 blank

HC high method control (± 8 ng/ml)

LC low method control (± 4 ng/ml)

S1 to S21 samples 1 to 21

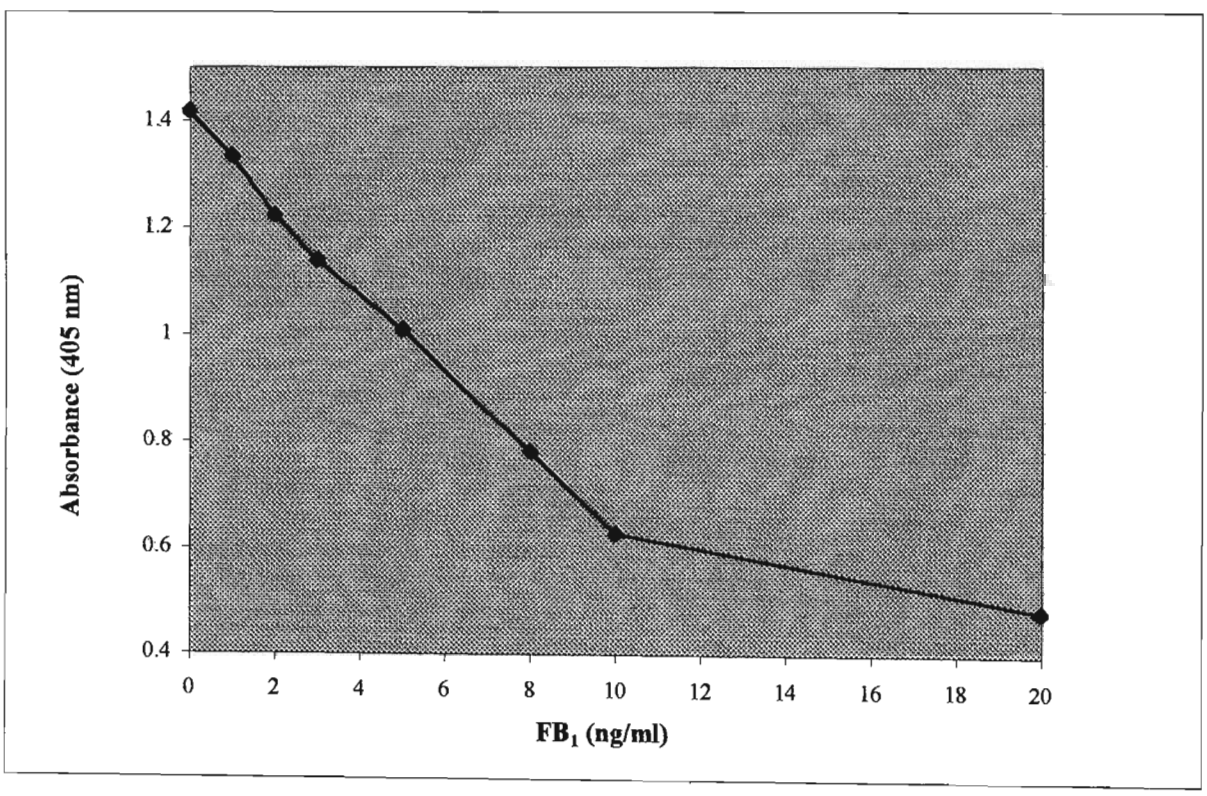


FIGURE 5. 8 TYPICAL STANDARD CURVE FOR FUMONISIN B_1 (CHICKEN ANTIBODIES)

The data used to generate Figure 5.8 is in Appendix 1, Table 5.14. Similar results were obtained with the rabbit antibodies (Appendix 1, Figure 5.9 and Table 5.15).
Both standard curves have a suitable value for the zero point viz. 1.418 for chicken antibodies and 1.083 for rabbit antibodies. Also they both have a steep slope over the range 0 to 20 ng/ml i.e. a sensitive assay.

5.4.5 Method Parameters

5.4.5.1 Range of the Standard Curve

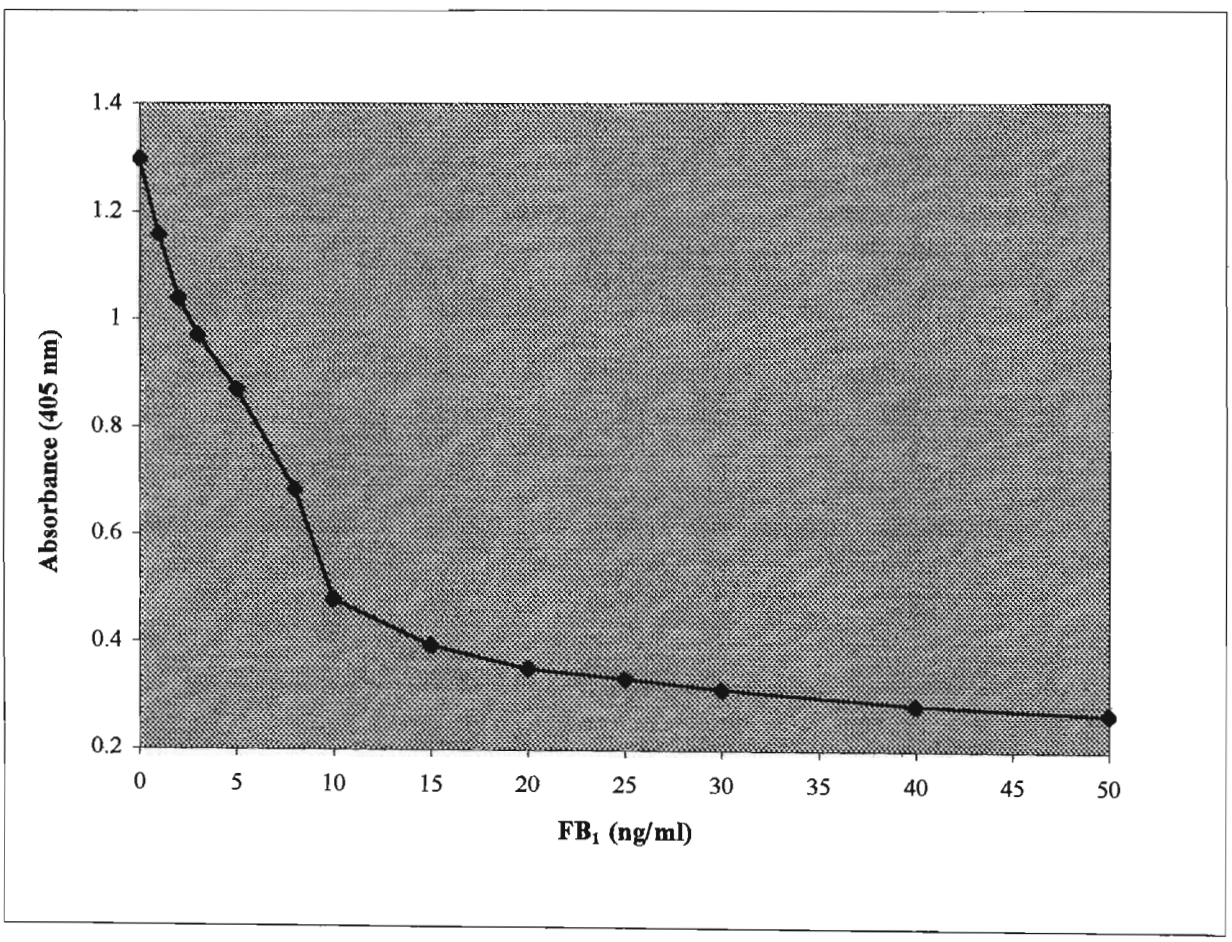


FIGURE 5.10 RANGE OF STANDARD CURVE (CHICKEN ANTIBODIES)

Key to Figure 5.10
Absorbance values were the mean of triplicates at 405 nm after subtraction of the mean of the no Ab_1 blank. The data used to generate Figure 5.11 in Appendix 1, Table 5.16.

The working range of the standard curve was from 0 to 20 ng/ml as above 20 ng/ml the graph levelled out (Figure 5.10).

5.4.5.2 Reproducibility

The intra-assay reproducibility was done on same microtitre plate on the same day; the inter-assay reproducibility was done on different plates and different days over 18 months.

TABLE 5.17 REPRODUCIBILITY OF COMPETITIVE INDIRECT ELISA

	Mean FB_1 (ng/ml)	CV	n
HIGH CONTROL			
Intra-assay	8.412	5.33 %	20
Inter-assay	7.548	7.41 %	31
LOW CONTROL			
Intra-assay	4.102	10.47 %	20
Inter-assay	4.077	7.37 %	30

5.4.5.3 Ruggedness

The ruggedness of the assay was shown by a low average coefficient of variation, 12.2% for ID_{50}, over a year.

5.4.5.4 Detection Limits

The detection limit in buffer was 0.2 ng/ml, calculated as the concentration of FB_1 that gave absorbance readings significantly different from zero (Thompson, 1984).

Mean absorbance readings (n = 11) for 0, 0.1 and 0.2 ng/ml FB_1 were 1.541, 1.509 and 1.483 respectively. The difference between 1.541 and 1.483 was significant ($p<0.05$) but there was no significant difference between 1.541 and 1.509.

5.4.6 Cross-reactivity

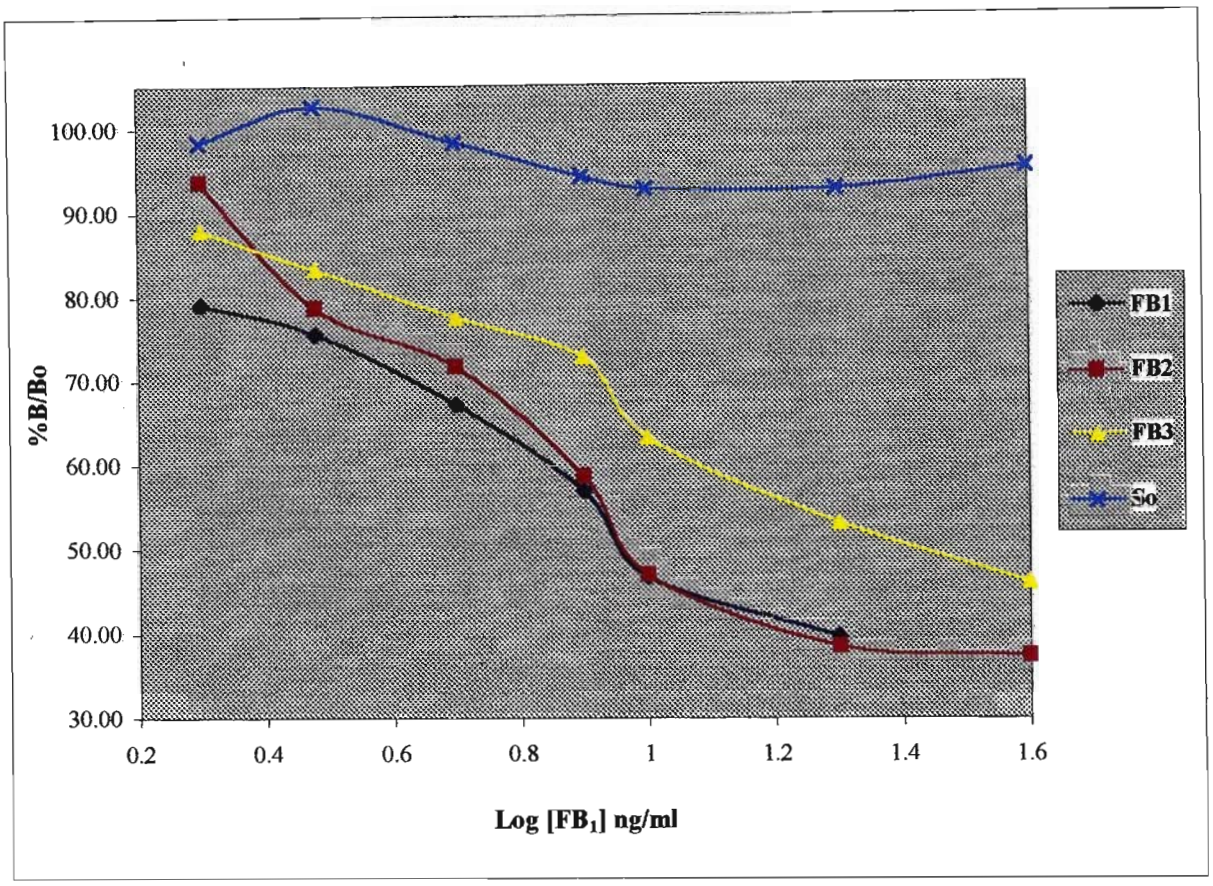


FIGURE 5.11 COMPETITIVE INDIRECT ELISA STANDARD CURVES FOR CROSS-REACTIVITY (CHICKEN ANTIBODIES)

Key to Figure 5.11

Results are the means of duplicate absorbance readings at 405 nm after subtraction of the no Ab_1 blank and then expressed as a percentage of the value for the zero point. Data used to generate Figure 5.12 is in Appendix 1, Table 5.18.

The concentration at 50% (ID_{50}) for each analyte was calculated using curve fitting and Sigmaplot software.

There was no cross-reactivity with So.

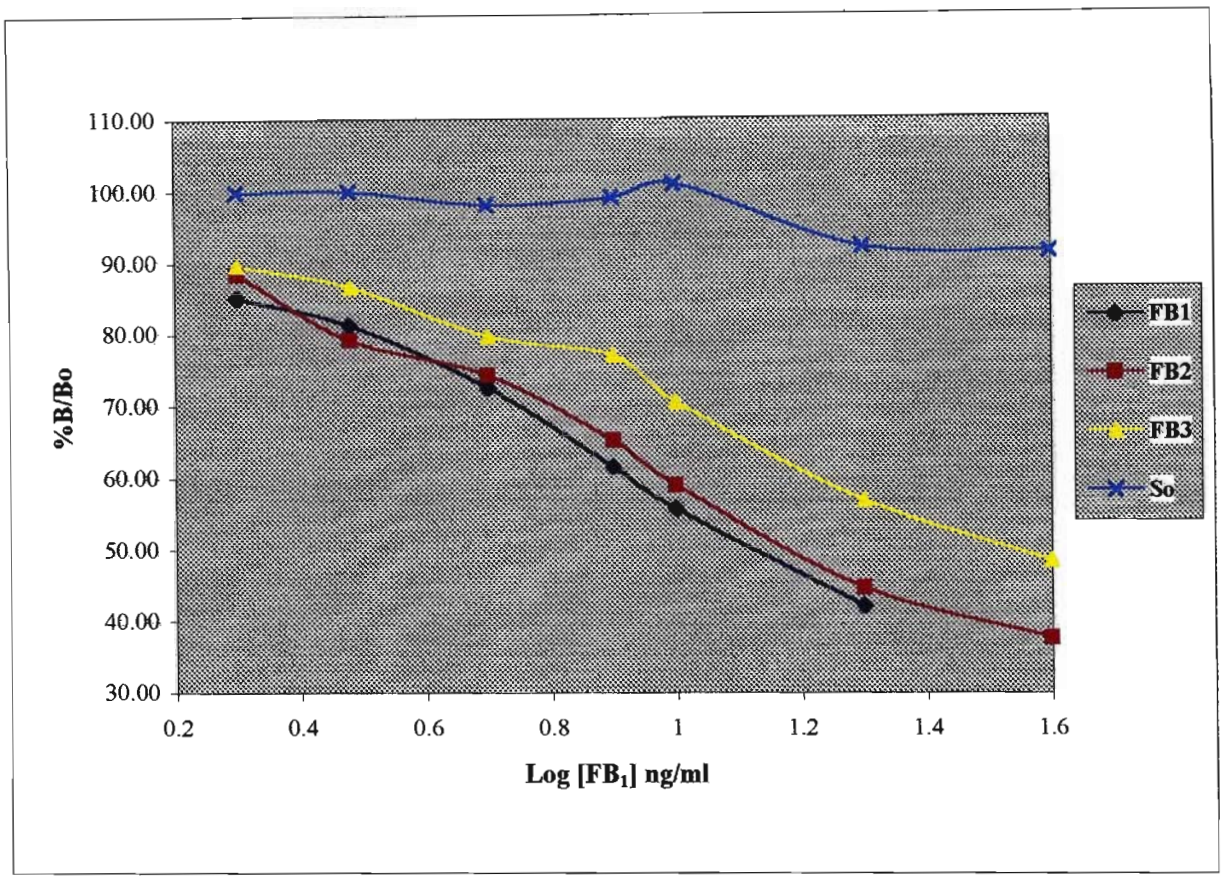


FIGURE 5.12 COMPETITIVE INDIRECT ELISA STANDARD CURVES FOR CROSS-REACTIVITY (RABBIT ANTIBODIES)

Key to Figure 5.13

Results are the means of duplicate absorbance readings at 405 nm after subtraction of the no Ab1 blank and then expressed as a percentage of the value for the zero point. Data used to generate Figure 5.13 is in Appendix 1, Table 5.19.

The concentration at 50% (ID_{50}) for each analyte was calculated using curve fitting and Sigmaplot software.

There was no cross-reactivity with So.

TABLE 5.20 CROSS-REACTIVITY OF ANTIBODIES WITH FUMONISIN B_2 AND FUMONISIN B_3

	ID_{50} (ng/ml)	Cross-reactivity
CHICKEN ANTIBODY		
FB_1	8.91	100.0 %
FB_2	9.12	97.7 %
FB_3	26.92	33.1 %
RABBIT ANTIBODY		
FB_1	13.18	100.0 %
FB_2	15.14	87.1 %
FB_3	34.67	38.0 %

Cross-reactivity (Table 5.20) was calculated as the ratio of the concentration at 50% binding (ID_{50}) for FB_1 to that of FB_2 or FB_3 and expressed as a percentage.

5.5 DISCUSSION

5.5.1 High Performance Liquid Chromatography

The fumonisin standards stored in PBS for 2 yr at 4°C and – 20°C had minimal deterioration (Table 5.1). Some of the fumonisin B_1 may have been lost during freeze-drying. Since storage at 4°C gave better stability than at –20°C, henceforth only stock standards stored at 4°C were used. Other authors have stored their stock standards, 1 and 0.1 mg/ml in acetonitrile:water (3:1) at 4°C (Yeung *et al.*, 1996)

5.5.2 Competitive Indirect ELISA

Good results in checkerboard titrations are indicated by a steep curve with no plateau region at high absorbances, low absorbance values for the no Ab_1 blank (< value for highest standard), absorbance value for the zero point on the standard curve < 1.6 and a steep sigmoid curve (Crowther, 1995). These were used as guidelines for all decisions made from checkerboard titrations.

In optimising the CI-ELISA method in this study, method parameters that had already been checked in the titre experiments (Chapter 3, section 3.3.7) were not altered viz. coating with FB_1 in PBS directly overnight at 4°C, blocking with 1% casein in PBS, using 0.5% BSA in PBS as diluent for antibody solutions and the ABTS reaction.

The optimal concentration of 200 ng FB_1/ml for coating gave a suitably steep standard curve at low concentrations of FB_1 and was more economical than 250 ng/ml (Table 5.3 and Appendix 1, Figure 5.1). However, to reduce costs, a smaller volume of FB_1 (100 µl) was used as compared to titre experiments (150 µl). To compensate the volume of 1% casein used for blocking was increased from 200 to 250 µl to fill the wells to capacity (section 5.3.2).

Other groups coated with FB_1 bound to a protein, either FB_1-OVA (Azcona-Olivera *et al.*, 1992A;Yu & Chu, 1996) or FB_1-BSA (Fukuda *et al.*, 1994; Shelby *et al.*, 1994). For blocking a variety of agents was used e.g. 1% OVA in PBS (Azcona-Olivera *et al.*, 1992A); 0.1% gelatin in PBS (Yu & Chu, 1996); 0.1% non-fat dry milk powder in borate buffer (pH 6.5) (Fukuda *et al.*, 1994) while Shelby *et al.* (1994) omitted a blocking step. For washing, either 0.02%, 0.05% or 0.5% Tween 20 in PBS was used (Azcona-Olivera *et al.*, 1992A; Shelby *et al.*, 1994;Yu & Chu, 1996).

In the competition step 300 µg/ml for the primary antibody for both chicken and rabbit antibodies was selected as it gave steep graphs at low concentrations of FB_1 (<10 ng/ml) and acceptable readings for the absorbance of the zero point on the standard curve (Figure 5.2 and Appendix 1, Table 5.4, 5.5 and Figure 5.3). It was found possible to use shorter incubation times viz.1 hr instead of 2 hr (Figure 5.4 and Appendix 1, Table 5.6). Further refinements of this method should include checks to use shorter times for other steps of the assay but due to time restraints these were not done. Instead the same times as for the titre experiments were used for blocking, the second antibody and the ABTS reaction.

For the competition step, Shelby *et al.* (1994) used 50 µl of maize sample extracts or FB_1 standards with 50 µl of mAb (1 µg/ml in 0.5% Tween 20 in PBS) and incubated for 15 min at RT. In contrast, Yeung *et al.* (1996), first added 1 ml of a 1:30 000 dilution of Ab_1 (in 0.1% BSA in PBS) to 25 µl of maize samples/standard and incubated for 1 hr at 4°C. Then 200 µl of the mixture was added to coated plates and incubated for a further 30 min at 4°C.

The main objectives in developing this CI-ELISA method were to produce a sensitive, easy and cost-effective method able to measure low concentrations of FB_1 in physiological fluids.

Hence more than one experiment was done to check the optimal dilution and volume of Ab_2^E so that a minimum amount of this expensive reagent was used. The selected dilution of the second antibody (1:4 000) gave a steep titration curve and a maximum absorbance of between 1.0 and 1.4 as recommended (Crowther, 1995) (Figures 5.5, 5.6 and 5.7; and Appendix 1, Tables 5.7, 5.8 and 5.9). Other groups used 100 µl of the second enzyme-labelled antibody (1:500 to 1:10 000 dilutions) and incubated for 15 min at RT or 30, 45 or 60 min at 37°C (Azcona-Olivera *et al.*, 1992A; Fukuda *et al.*, 1994; Shelby *et al.*, 1994; Yu & Chu, 1996).

The ABTS reaction had already been optimised (Dr. Coetzer, personal communication) to give zero order kinetics for the enzyme reaction so it would have been foolish to alter the concentration of ABTS. Even though the optimum pH for reaction of HRPO and ABTS is 4.0, use of pH 5.0 gives a slower reaction but does not affect the linearity of the kinetics (Crowther, 1995). Any change in the volume of ABTS used would have necessitated a change in the volume of stopping buffer. Pipetting of substrate solution and stopping buffer must be accurate so for this a multi-channel pipette (Finpipette, 8 channel, 300 µl, AEC Amersham) was used. A fixed time was used for the ABTS incubation to reduce inter-plate variability.

In conclusion, for all experiments done using this CI-ELISA method, coating was done with 200 ng/ml FB_1 in PBS, blocking with 1% casein, washing with 0.05% Tween 20 in PBS, antibody diluent was 0.5% BSA in PBS, concentration of Ab_1 was 300 µg/ml, standards were 0, 1, 2, 3, 5, 8, 10 and 20 ng FB_1 /ml, and Ab_2^E was diluted 1:4 000. Incubation for coating was overnight at 4°C; for the competition step and for the second antibody reaction incubation was for 1 hr at 37°C. Every run included a no Ab_1 blank and FB_1 method controls.

5.5.3 Calculations

Since ELISA methods give sigmoid curves a statistically sound curve-fitting programme is necessary to obtain quantitative results. The formula chosen should have as small a number of parameters as possible; it is iteratively fitted to the data obtained using non-linear least squares methods (Rodgers, 1986). For a partially sigmoid curve as obtained with the CI-ELISA, a 2nd order polynomial is the recommended curve type; a 4-parameter equation is best suited to a fully sigmoid curve. (Elisa+ , Meddata Inc., New York).

Statistically the chi square value and the sum of squares give a measure of the goodness of fit with low values showing a good curve fit. The sum of squares is the sum of the squares of differences between observed and expected values and the chi square test statistic is this sum divided by the expected values (Sigmaplot instruction book, Jandel Scientific).

Both Sigmaplot and Elisa+ use the Levenberg-Marquardt algorithm to fit the curves. The parameters derived from this algorithm minimise the sum of squares. In the iterative process the parameters are checked again and again until convergence is reached i.e. the differences between the residual sum of squares no longer decreases significantly (Sigmaplot instruction book).
Generally it is better to have three parameters rather than four (Mr. D. Singh, personal communication). The norm value is an index of closeness of fit and decreases as the fit improves (Sigmaplot instruction book).

It is vitally necessary to select valid and statistically sound computation methods that are sufficiently flexible, easy to interpret geometrically and having as small a number of parameters as possible. Differences in data-processing methods must be taken into account in comparative studies and data-processing errors are not negligible. Commercial packages sold as an adjunct to a plate reader generally have good instructions for use but minimal documentation on the numerical methods employed. It is also essential to run proper control samples with every ELISA assay and to use these results to validate the method and sample results (Rodgers, 1986).

The calculation method finally selected met the criteria for an acceptable method of data reduction viz. plotting absorbance vs. [FB_1] ng/ml and using a 2^{nd} order polynomial for curve fitting (Table 5.10). The backfit equation was used in Excel (Microsoft Windows 98) to enable quantitative results to be computed easily. The validity of this approach is shown by the good agreement in results (no significant difference) between fumonisin controls calculated by this method and by ELISA+ (Table 5.11). The number of iterations was 5 and the norm of fit value was low. Further verification of this approach is that sample results calculated by this method and results from dedicated software on the Model 3550 Plate Reader also owed no significant difference. However, it is interesting to note that different methods of calculation gave different answers (Table 5.12).

Other methods of calculation have also been used. Shelby *et al.* (1994) used a regression equation and plotted absorbance at 490 nm vs. log ppm FB_1 while Yeung *et al.* (1996) used a least squares plot of logit of absorbance vs. log FB_1 concentration. Other workers plotted %B/Bo vs. log FB_1 and used specific software with a cubic spline function to calculate results (Usleber *et al.*, 1994).

5.5.4 Method Parameters

5.5.4.1 Standard Curves

Criteria for selection of an acceptable standard curve are the steepest slope and straightest line (Dr.Coetzer, personal communication). A suitable range for absorbances for a standard curve are 0.025 to 1.3 (Mr.R. Parkinson, personal communication). The standard curve must also have a working range to meet the proposed applications of the method.

The absorbance values for the zero point were within acceptable range [1.0 to 1.6, (Crowther, 1995)] and the no Ab_1 blanks were low (0.129 and 0.105). The actual values varied with every plate run but remained within designated limits. In the CD-ELISA established by Usleber *et al.*, (1994), the absorbance for the zero point, Bo was 1.1.
The CV of quadruplicate points on their standard curve was 1.2 - 7.3%. An acceptable CV of triplicates is <10% (Mr.R. Parkinson, personal communication). The CV of triplicates for the chicken antibody standard curve was 0.36 - 5.91% and for the rabbit antibody 1.79 - 5.40% (Appendix 1, Tables 5.14 and 5.15).

Good standard curves covering the range of 0 to 20 ng/ml were obtained with both chicken and rabbit antibodies (Figures 5.8 and 5.10 and Appendix 1, Figure 5.9). Shelby *et al.*, (1994) in a CI-ELISA used serial 1:2 dilutions of FB_1 to give a standard range from 0.1 to 100 ppm (µg/ml) while Yeung *et al.*, 1996 had a very limited but sensitive range of 0.05 to 0.75 ng/ml. In between are the ranges of 0.1 to 30 ng/ml (Abouzied *et al.*, 1996) and 50 to 5 000 ng/ml (Azcona-Olivera *et al.*, 1992B). The latter two were both competitive direct ELISA methods.

5.5.4.2 Reproducibility

An acceptable intra-plate CV at NBTS is < ± 10% (Mr.R. Parkinson, personal communication). Yeung *et al.* (1996) obtained intra-assay CV <10% and inter-assay varying from 7.3 to 17.8% depending on the concentration of added FB_1 (lower CV's for higher concentrations).

In their CD-ELISA methods, Usleber *et al.* (1994) obtained intra-assay CV below 7.5% and inter-assay below 14%; Abouzied *et al.* (1996) obtained 3.8% for intra-assay and 7.6% for inter-assay; Azcona-Olivera *et al.*(1992B) obtained 11.55% for intra-assay and 14.85 % for inter-assay.

Thus the results of 5.33 % (high control) and 10.47% (low control) for intra-assay coefficients of variation and 7.41% (high control) and 7.37% (low control) for inter-assay obtained in this CI-ELISA method (Table 5.17) are similar to other workers and acceptably low. This shows a good method and good technical skills.

5.5.4.3 Detection Limits / Sensitivity

The ID_{50} value gives an indication of the affinity of the antibody; the lower the value, the higher the affinity and the greater the sensitivity of an assay. The ID_{50} is the concentration of FB_1 causing 50% inhibition of binding to antibody (Chu, 1996). The chicken antibodies are of higher affinity (ID_{50}, 8.91 ng/ml) than the rabbit antibodies (ID_{50}, 13.18 ng/ml) and compare favourably with results from other workers 260 ng/ml (Azcona-Olivera *et al.*, 1992A); 630 ng/ml (Azcona-Olivera *et al.*, 1992B); 144 ng/ml (Fukuda *et al.*, 1994); 5.5 ng/ml (Abouzied *et al.*, 1996) but not as good as an affinity as Yu & Chu (1996) with 0.45 and 1.33 ng/ml or Usleber *et al.* (1994) with 0.623 ng/ml or Yeung *et al.*, (1996) with an ID_{50} of 0.66 ng/ml.

Reproducibility of the ID_{50} gives a measure of the ruggedness of an assay and the CV result of 12.2% over a year compares favourably with the 8.5% obtained by Yeung *et al.* (1996) over one month.

With a detection limit of 0.2 ng/ml this is a sensitive CI-ELISA which compares well with the work of Usleber *et al.* (1994) whose CD-ELISA method had detection limits of 0.17 ng/ml. Other sensitive methods with detection limits of <1 ng/ml (Abouzied *et al.*, 1996) and 0.05 ng/ml (Yeung *et al.*, 1996) contrast with that of Azcona-Olivera *et al.* (1992A) whose detection limit in a CI-ELISA with polyclonal antibodies was 100 ng/ml and for monoclonal antibodies was 50 ng/ml (Azcona-Olivera *et al.*, 1992B).

5.5.5 Cross-reactivity

The cross-reactivities of the antibodies against FB_2 and FB_3 viz. 97.7% and 33.1% respectively for chickens and 87.1% and 38.0% respectively for rabbits (Table 5.20 and Figures 5.11 and 5.12) are similar to those for the pAb produced by Azcona-Olivera *et al.* (1992A) in mice. In their CI-ELISA there was 87% cross-reactivity with FB_2 and 40% with FB_3 but no cross-reactivity with TCA or the hydrolysed backbone of fumonisin.

In a CD-ELISA using mAb, cross reactivity with FB_2 was 38% and with FB_3 was 33% (Azcona-Olivera *et al.*, 1992B). They suggested the epitope lies in the C-11 to C-20 region of the fumonisin B_1 molecule near the linkage of the TCA moieties. Because of similar values for cross-reactivity, it seems possible that the chicken and rabbit antibodies in this study recognised the same region. Unfortunately suitable pure preparations of other fumonisins (FB_4, A and C series) and metabolites (partially and fully hydrolysed fumonisins) were not available to check their cross- reactivities. Other workers produced pAb with varying cross-reactivities (Chapter 2, Table 2.7).

5.6 CONCLUSION

A sensitive CI-ELISA method of analysis for fumonisin with excellent reproducibility was successfully developed and optimised. The method is rapid, easy to perform and not expensive. Standard curves with a good range for low concentrations of FB_1 were achieved using both the chicken and the rabbit antibodies raised as described in Chapter 3. Furthermore, it was established that FB_1 for use as a standard can be stored for more than two years in PBS at 4°C. Investigation of a range of alternative ways of calculating the CI-ELISA data came up with a simple and accurate method to obtain quantitative results.

The chicken and rabbit antibodies cross-reacted mainly with FB_2 and to a lesser extent with FB_3 but there was no cross-reactivity with So. It is intended that this method be applied to analyses in physiological fluids.

CHAPTER 6

ANALYSIS OF PLASMA and URINE SAMPLES

6.1 INTRODUCTION

Very little work has been done on analysis of fumonisins in physiological samples. Most of the immunoassay and HPLC methods of analysis have been applied to maize and maize-based products; also beer and milk. In application of ELISA methods, no prior extraction was done for beer and milk, whereas, for maize/maize-based samples, extraction with acetonitrile or methanol and water in various combinations was needed (Chapter 2, Tables 2.4, 2.5 and 2.8).

Chromatographic methods for analysing FB_1 and FB_2 in plasma, urine, faeces and bile samples from rats and vervet monkeys who had been given FB_1or FB_2 by intraperitoneal injection or by gavage were developed. These involved a final extraction into organic solvents suitable for application to HPLC i.e. except for an initial extraction of faecal samples into 0.1M EDTA (pH5.2), a purely aqueous medium was not used (Shephard *et al.*, 1992A, 1992B, 1992C, 1994A, 1994B, 1994C, 1995A, 1995B).

In studies on the absorption and excretion of FB_1 and FB_2, unlabelled or radiolabelled fumonisins were fed to a variety of animals. It was found that in rats fumonisins were poorly absorbed from the gut, and rapidly eliminated unmetabolised mainly via the faeces, bile and urine. After oral administration of labelled FB_1 to vervet monkeys, within seventy-two hours, most of it was excreted in the faeces and urine as unmetabolised, partially and fully hydrolysed FB_1 (Shephard *et al.*, 1994A, 1994C). Laying hens and cattle also excreted FB_1 unmetabolised, mainly in the faeces and urine (Vudathala *et al.*, 1994; Smith & Thakur, 1996).

Even though fumonisins are poorly absorbed orally and then rapidly excreted, some studies have concluded that fumonisins are retained in the tissues. Prelusky *et al.* (1994) concluded that, in swine, a fraction of orally administered FB_1 was absorbed, distributed and remained in the tissues for an extended period implying that FB_1 may accumulate in the tissues if consumed over a period of time.

Smith & Thakur (1996) after feeding Holstein steers feed containing FB_1 found that although it was excreted mainly in the faeces, small amounts were retained in the liver, kidneys and muscle. Working with primates (vervet monkeys), Shephard *et al.*, (1994A, 1994C) found that after oral administration of labelled FB_1, in addition to being found in the faeces and urine, fumonisin was found in the contents of the intestine, liver, skeletal muscle, plasma, bile, kidney, heart, erythrocytes and brain (Chapter 2, Table 2.15). So it may be possible to detect fumonisins in the blood.

Minimal studies have been done on natural excretion of fumonisins by humans and nothing is known about routes and rates of elimination. Recently, naturally occurring levels of FB_1 in human faeces was measured using HPLC. Concentrations ranged from 790 to 19 560 ng/g of freeze-dried faecal sample (Chelule *et al.*, 2000).

For humans in Southern Africa, maize and maize products are a major component of their daily diet (Rava *et al.*, 1996). However, consumption of maize contaminated with FB_1 is associated with the risk of OC. This was shown in the work done by the PROMEC, Medical Research Council (MRC) group studying low and high risk areas in the Transkei, South Africa (Burrell, 1957; Marasas *et al.*, 1988B; Rheeder *et al.*, 1992; Sydenham *et al.*, 1990B).

6.1.1 Objectives

1. To investigate aqueous extraction methods so that the CI-ELISA method (Chapter 5) could be used to analyse blood and urine samples for fumonisins.
2. To analyse blood and urine samples from control patients, patients with OC and patients with other types of cancer for fumonisins.
3. To analyse some of the same samples by HPLC to confirm the ELISA results.
4. To analyse serum samples from control patients, patients with OC and patients with other types of cancer for calcium and magnesium.

6.1.2 Ethical Approval

Ethical approval for use of human blood and urine samples was granted by the Ethics Committee, Faculty of Medicine, University of Natal (Protocol 031/96).

6.2 MATERIALS

6.2.1 Chemicals

Calcium and Magnesium kits were purchased from Boehringer Mannheim, Germany.

Level 1 and Level 2 control sera were purchased from Randox, United Kingdom.

For all other chemicals refer to Chapter 3, section 3.2.1 and Chapter 5, section 5.2.1.

6.2.2 Buffers/Solutions

The following buffers were used in analysis of plasma and urine samples:

Phosphate buffered saline (PBS) (pH 7.2)	(Appendix 2, 2.2)
0.15M Citrate-phosphate buffer (pH 5.0)	(Appendix 2, 2.6)
Stopping buffer : 0.1% sodium azide in citrate-phosphate buffer	(Appendix 2, 2.7)
1M Sodium phosphate buffer (pH 3.35)	(Appendix 2, 2.11)

6.3 METHODS

6.3.1 Competitive Indirect ELISA

1. The wells of the microtitre plate (Nunc 96 well Maxisorb, AEC Amersham) were coated directly with FB_1, 200 ng/ml in PBS, 100 µl per well. The plate was covered and incubated for 18 hr at 4°C.
2. The wells were blocked with 1.0% casein (m/v) in PBS, 250 µl per well and incubated for 1 hr at 37°C.
3. The plate was washed three times with 0.05% (v/v) Tween 20 in PBS.
4. FB_1 standards (0, 1, 2, 3, 5, 8, 10, 20 ng/ml in PBS) were pipetted in triplicate into relevant wells, 50 µl per well.
5. The primary antibody (Ab_1) was added to relevant wells, 300 µg/ml in 0.5% BSA in PBS, 50 µl per well.
6. The plate was mixed by gentle tapping and incubated for 1 hr at 37°C.
7. The plate was washed three times with 0.05% (v/v) Tween 20 in PBS.
8. The second antibody (Ab_2^E), anti-chicken IgG conjugated with HRPO was added, 1:4 000, 120 µl per well and incubated for 1 hr at 37°C.
9. The plate was washed three times with 0.05% (v/v) Tween 20 in PBS.

10. 150 µl of substrate, 7.5 mg ABTS plus 7.5 µl H_2O_2 in 15 ml citrate-phosphate buffer (pH 5.0) was added to each well. The colour was allowed to develop for 15 min in the dark at RT.
11. 50 µl of stopping buffer was added to each well.
12. The absorbance of each well was read at 405 nm (Bio-Rad Model 550 Plate Reader).

6.3.1.1 Extraction Methods for Fumonisins in Plasma and Urine

All recovery experiments for CI-ELISA included a positive control (serum/plasma/urine from a non maize eater spiked with FB_1 to give concentrations of 5 or 10 ng/ml, later 4 or 8 ng/ml); a buffer control (PBS spiked with FB_1 at the same concentration as the positive controls); and a negative control (serum/plasma/urine from a non maize eater with a volume of PBS added equivalent to the added volume of FB_1). The extraction procedure was done on all controls. The buffer control indicated any loss of FB_1 due to the extraction process without the complication of matrix interferences.

Four or more positive controls were done for each experiment. All CI-ELISA analyses were done in triplicate; a standard curve and method controls were included on every plate and fumonisin concentrations were calculated as described in Chapter 5.

Recovery results were calculated by first subtracting the result for the negative control from that of the spiked sample and then expressing it as a percentage of the buffer control.

The samples used were plasma obtained using lithium heparin or EDTA as anticoagulant, serum and urine. Criteria for an acceptable extraction method were a low value for the negative control, recovery > 80 %, and ease of use.

Pipetting serum/urine directly or simply diluted in buffer did not give good recoveries so various simple methods of clean-up (mainly to remove proteins) were tried.

Initial extraction experiments used **organic solvents** viz. methanol (1:5) or acetonitrile (1:3.3). After mixing, samples were centrifuged, the supernatant dried under N_2 at 60°C and re-dissolved in 500 µl PBS. Other experiments used addition of 10%, 20% or 50% methanol followed by centrifugation and analysis of the supernatant.

As an alternative, precipitation with **30% PEG 6 000** using a 1:1 dilution of serum was attempted. Prior experiments using a 7.5% solution of BSA in PBS were done to ascertain the concentration of PEG 6 000 to use to precipitate all the protein. Suitable weights of PEG were added to duplicate 500 µl volumes of the BSA solution to give a range of 18% to 30% PEG solutions. These were mixed for 1 min, centrifuged for 10 min (Hettich Mikroliter centrifuge) and the supernatant was analysed for protein by the biuret method (Chapter 3, section 3.3.5). (The average concentration of proteins in blood is 75 g/l).

Finally a simple **boiling method** using a 1:1 dilution of the sample in PBS gave excellent recovery results. 200 µl EDTA plasma / urine and 200 µl PBS were pipetted into Eppendorf micro test tubes (AEC Amersham), sealed, mixed and allowed to stand for 10 min.
The tubes were heated in a boiling water bath for 15 min, removed and cooled at RT for 5 min and then for 15 min at –20°C. Finally, they were centrifuged for 10 min at 15 624 g (Hettich Mikroliter centrifuge). Triplicate 50 µl volumes of the supernatant were used in the ELISA method.

6.3.1.2 Samples

Blood and urine samples for CI-ELISA were collected from hospital and clinic patients in hospitals in the Durban Metropolitan area. Unfortunately it was not possible to get both types of samples on all patients. There were three groups of samples viz. patients with OC, controls (no cancer) and patients with other types of cancer (i.e. not oesophageal). Since this was only a preliminary study, patients were not matched for age or sex.

In the OC group there were 40 blood and 17 urine samples; in the control group 21 blood and 12 urine samples. The 20 blood samples from the group of patients with other types of cancer were comprised of 9 with breast cancer, 4 with cervical cancer, 2 each with cancer of the stomach and rectum, and 1 each with cancer of the pancreas, thyroid and prostate. The 10 urine samples were from 6 patients with breast cancer, 2 with cervical cancer and 1 each with cancer of the stomach and the larynx.

Blood was collected into EDTA tubes (Vacutainer, Becton Dickinson) to give plasma for fumonisin analysis; serum was used for calcium and magnesium measurements. Random (not 24 hr) urine samples were collected into suitable containers. Separated plasma/serum and urine samples were given laboratory numbers and stored at –20°C until analysed.

Extractions were done in duplicate and ELISA assays in triplicate. Every run included a standard curve, wells for non-specific binding (no Ab_1), low and high FB_1 method controls (in buffer) and serum/urine negative controls (no FB_1) and serum/urine positive controls (spiked with FB_1 at 4 ng/ml).

The mean of the triplicate absorbance values (minus the average absorbance for non-specific binding) was used to calculate fumonisin concentrations as described in Chapter 5, section 5.4.3).

Runs were only accepted if method controls were within their acceptable limits (mean ± 2SD).

The mean value for the relevant negative serum/urine control was subtracted from sample results. The final fumonisin result was the average of the results for the two extractions multiplied by 2 (for the initial 1:1 dilution).

If the extraction concentrations differed by more than 1 ng/ml, the sample was repeated.

Total plasma fumonisins were calculated using a blood volume of 3.5 litres (Kleinman & Lorenz, 1996).

Statistical evaluation was done by one-way analysis of variance (ANOVA) using Corel Quattro Pro 8.

6.3.2 High Performance Liquid Chromatography

6.3.2.1 Extraction of Fumonisin B_1 from Plasma and Urine Samples

For extraction of plasma samples, 3 ml methanol was added to 500 µl plasma, mixed and centrifuged at 10°C for 10 min at 1 200 g.

For extraction of urine samples, 1 ml urine plus 7.5 ml methanol was used.

Strong anion exchange (SAX) columns (Varian Bond-Elut, 500 mg, SMM Instruments, South Africa) were pre-conditioned with 5 ml methanol followed by 5 ml methanol:water (3:1). The supernatant from the methanol extraction of plasma/urine was applied to the column, washed with 5 ml methanol:water (3:1) and then 5 ml methanol. Elution was with 10 ml 5% acetic acid in methanol. Flow rate was 1 ml/min and columns were not allowed to run dry.

The eluted extracts were dried under nitrogen at 60°C and stored sealed at 4°C until HPLC analysis. Each sample was extracted in duplicate (Shephard *et al.*, 1992B; Thiel *et al.*, 1993; Duncan *et al.*, 1998).

6.3.2.2 Analysis of Plasma and Urine Samples for Fumonisin B_1

The mobile phase was composed of 0.1M sodium phosphate buffer (pH 3.35):methanol (27:73).
The buffer was filtered before use through a 0.45 µm nylon filter (Lida, Chrom Tech Inc.).
The mobile phase was degassed by sonication for 15 min/300 ml at 20°C and 200V (UMC 10 waterbath, Ultrasonic Manufacturing Co.)
The HPLC system consisted of a Spectra SYSTEM P2 000 binary pump, a Spectra SYSTEM AS3 000 autosampler equipped with a 100 µl injection loop and a Spectra SYSTEM FL2 000 fluorescence detector (Thermo Separation Products, SMM Instruments, South Africa). The C_{18} analytical column (Waters Nova-Pak stainless steel column, 3.9 x 150 mm, 4 µm packing material, MicroSep, South Africa) was preceded by a HIRPB-10C guard column (Hichrom Ltd., SMM Instruments, South Africa). The guard column was replaced daily.

A stock FB_1 standard (1 mg/ml in 1:1 acetonitrile:water) was stored at 4°C.
Working standards were prepared daily by dilution of the stock standard to give concentrations of 50 and 20 µg/ml, and 500, 400, 200, 100, 50, 20 and 10 ng/ml.
Dried plasma/urine samples were re-dissolved in 100 µl acetonitrile:water (1:1).
For derivatisation, 40 µl sample or standard was added to 80 µl OPA solution (Appendix 2, 2.13) and mixed for exactly 10 sec before loading into the autosampler. The injection volume was 50 µl . Each sample/standard was derivatised and injected in duplicate. The time from derivatisation to loading on to the column was 2 min. The run time was 6 min. Analyses were done over three days.

The flow rate was 1 ml/min, the pressure 2 425 psi and temperature 23°C. For fluorometric detection the excitation wavelength was 230 nm and emission 440 nm. To avoid interference from impurities that gave high peaks beyond the limit of the photomultiplier tube, the emission wavelength was set at 600 nm for the first 2.5 min and switched automatically to 440 nm from 2.5 to 6.0 min. Calculations of peak area were done using Spectra SYSTEM PC-1000 software (Thermo Separation Products, SMM Instruments, South Africa).

Detection limits were calculated as a signal to noise ratio of 3:1.
Four serum and four urine samples were spiked at 16 ng/ml FB_1. These were extracted and analysed as for patient samples. Recoveries were calculated as the percentage of the measured result divided by 16.

6.3.2.3 Samples for High Performance Liquid Chromatography

Nine plasma and eight urine samples with high fumonisin concentrations as measured by CI-ELISA were selected for HPLC analysis. (Due to cost constraints it was not possible to analyse all the ELISA samples by HPLC).
Sample results (mean of duplicate injection peak areas) were calculated from the equation for the straight line standard curve for that day and corrected for dilution to give values in ng/ml of plasma or urine. The final result was the average of the results for the two extractions for each sample.

6.3.2.4 Statistics

A comparison of results from CI-ELISA and HPLC was done using a one-way ANOVA using Corel Quattro Pro 8.

6.3.3 Calcium and Magnesium

Calcium and magnesium levels were measured on all ELISA samples for which serum or heparinised plasma was available. (EDTA plasma is not suitable for calcium and magnesium analyses; results on random urine samples are meaningless). Analysis was done on a Cobas Mira Plus Selective Automated Analyser (Boehringer Mannheim) using kits. Calcium was analysed using o-cresolphthalein and magnesium using xylidyl blue. Precinorm U was used as calibrator; Level 1 and Level 2 control sera were used as method controls and run every 30 samples. Analyses were done in duplicate with an acceptable error < 0.1 mmol/l difference between duplicates.
Statistical evaluation was done by one-way ANOVA using Corel Quattro Pro 8.

6.4 RESULTS

6.4.1 Recoveries of Fumonisins from Blood and Urine (Competitive Indirect ELISA)

6.4.1.1 Organic Solvents

Undiluted and unextracted spiked serum and plasma samples gave low absorbance readings (0.194 to 0.399, lower than the 20 ng/ml standard 0.557) showing interference with the antigen-antibody reaction. (In the CI- ELISA absorbance is inversely related to concentration).
Results for urine were slightly better (0.907 to 0.989) indicating that the interference is probably from proteins in the sample. The only recovery result was for urine (44.9%, n = 1 at 5 ng/ml).

Diluting samples 1:5 with PBS also gave low absorbance readings. The three recovery values that could be calculated were unacceptable; 277.2% for EDTA plasma (n = 2 at 5 ng/ml) and 179.7% for urine (n = 1 at 5 ng/ml).

Extraction into methanol 1:5 gave better results but some results could not be calculated because the absorbance values were lower than that for the 20 ng/ml standard. Thus, there was still some variable interference with the antigen-antibody reaction. Recoveries that could be calculated were as follows:-

Serum	77.4% (n = 2 at 5 ng/ml)	54.3% (n = 2 at 10 ng/ml)
EDTA plasma	104.3% (n = 2 at 5 ng/ml)	86.7% (n = 2 at 10 ng/ml)
Heparin plasma	No result	19.8% (n = 1 at 10 ng/ml)
Urine	184.5% (n = 2 at 5 ng/ml)	No result

Since EDTA plasma seemed to give better results, a buffer solution containing 0.18% EDTA in PBS (i.e. the same concentration as used in Vacutainer blood collection tubes as an anti-coagulant) was tried as a diluent for samples. The pH had to be adjusted to 7 since the presence of the normally acidic (pH 4.82 in water, pH 5.98 in PBS before adjusting) EDTA disrupted the antigen-antibody reaction so that a flat standard curve was obtained.
Diluting serum samples 1:1 with PBS before extraction into methanol gave 64.8% recovery and using EDTA/PBS gave 59.8% (n = 1 at 10 ng/ml). These are no better than the results using undiluted serum.

Extraction with both methanol and acetonitrile gave unacceptably high values for the negative control i.e. the absorbance values (405 nm) were higher than those for the 10 ng/ml and sometimes the 20 ng/ml standard due to interference in the antigen-antibody reaction..
Even after dilution with PBS or PBS/EDTA , extraction with methanol left a fatty deposit in the tube which gave a turbid solution on reconstitution with PBS i.e. there was probably still some matrix related substance left which interfered with the antigen-antibody reaction giving poor recoveries. Extraction with acetonitrile (1:3.3) gave a smaller whiter pellet and clearer supernatant than with methanol but also poor recoveries, 79.4% for serum diluted with PBS (n = 1) and 33.4% if diluted with PBS/EDTA (n = 1).

Adding 10% or 20% methanol to a diluted serum sample (1:1 with PBS or EDTA/PBS) precipitated the proteins which were then removed by centrifugation; analysis was done on the supernatant. The presence of methanol affected the antigen-antibody reaction giving high recoveries (107 to 206%) but low values for the buffer control which should be 5 ng/ml.

TABLE 6.1 EFFECT OF ADDITION OF METHANOL ON BUFFER CONTROL

Diluent	No methanol	10% methanol	20% methanol
PBS	5.073 ng/ml	3.496 ng/ml	2.161 ng/ml
PBS/EDTA	4.787 ng/ml	3.924 ng/ml	2.846 ng/ml

Adding 50% methanol gave no results, i.e., no reaction taking place.

6.4.1.2 Polyethylene Glycol 6 000

TABLE 6.2 DETERMINATION OF PEG 6 000 CONCENTRATION NEEDED TO PRECIPITATE PROTEINS IN SERUM

PEG 6000	PROTEIN (g/l)
18%	37.08
20%	16.78
22%	8.05
24%	5.54
26%	2.35
28%	1.34
30%	0.00

Recovery results from serum were low; dilution with PBS gave only 19.5% recovery and dilution with PBS/EDTA gave 14.6% recovery (n = 1 at 5 ng/ml for both dilutions). These poor results were due to high values for the negative controls but the buffer controls were correct (5.329 ng/ml in PBS and 5.262 ng/ml in PBS/EDTA at 5 ng/ml).

6.4.1.3 Boiling

The best recovery results were obtained by using heat (boiling) to denature and precipitate the proteins (Table 6.3). This was also the quickest and easiest method of extraction; and gave acceptably low values for the negative controls. Boiling solutions of FB_1 in buffer had no effect on the fumonisin (unboiled standard 5.511 ng/ml, boiled standard 5.775 ng/ml).

TABLE 6.3 RECOVERIES OF FUMONISINS FROM SERUM/PLASMA/URINE (COMPETITIVE INDIRECT ELISA)

Level of FB_1 in spiked sample	Serum	EDTA Plasma	Heparin Plasma	Urine
4 ng/ml	112.37 %	99.11 %	94.65 %	97.73 %
N	5	7	5	6
CV	15.1 %	6.1 %	14.7 %	19.8 %
8 ng/ml	108.62 %	97.58 %	108.67 %	94.55 %
N	5	7	5	8
CV	6.8 %	5.5 %	9.5 %	4.8 %

(Results in Table 6.3 were calculated as described in section 6.3.1.1).

6.4.2 Results for Fumonisins on Patient and Control Samples (Competitive Indirect ELISA)

6.4.2.1 Method Controls

The mean value for the low FB_1 method control was 4.077 ng/ml, CV 7.4 % and acceptable range (mean ± 2SD) was 3.477 – 4.677 ng/ml.

The mean value for the high FB_1 method control was 7.548 ng/ml, CV 7.4 % and acceptable range 6.340 – 8.576 ng/ml. These did not undergo the extraction procedure.

The mean value for the positive plasma control was 4.378 ng/ml, CV 8.0 % and acceptable range 3.676 – 5.080 ng/ml.

The mean value for the positive urine control was 4.440 ng/ml, CV 15.1 % and acceptable range 3.100 – 5.780 ng/ml.

The latter two were treated like samples i.e. underwent the extraction process.

6.4.2.2 Detection Limits

The detection limit of FB_1 in buffer was 0.2 ng/ml (Chapter 5, section 5.4.5.4) which would theoretically be 0.4 ng/ml in plasma/urine since samples were diluted 1:1.

The mean of the negative control was 0.637 ng/ml (n = 12) which confirms a detection limit of less than 1 ng/ml.

6.4.2.3 Plasma and Urine Samples

TABLE 6.4 COMPETITIVE INDIRECT ELISA RESULTS FOR CONTROL PATIENTS

	PLASMA			URINE		
Patient Number	**Appearance**	**Fumonisins (ng/ml)**	**Total fumonisins (µg)**	**Colour**	**Clarity**	**Fumonisins (ng/ml)**
156		4.202	14.71	Y	C	6.192
158	± H	3.043	10.65			
160	+ H	5.429	19.00			
177		4.200	14.70			
178		9.226	32.29			
182		6.050	21.18			
183		4.272	14.95			
191		1.439	5.04	Y	+ T	10.936
192	+ H	2.188	7.66			
197	+ H	1.120	3.92			
198		0.130	0.46	Y	++ T	21.156
203	+ H	1.592	5.57			
205	++++ H	0.370	1.30			
206	++++ H	1.306	4.57	Y	C	17.852
210	++ H	1.032	3.61	Y	± T	10.636
222		5.949	20.82	PB	++ T	36.212
224		4.508	15.78			
226		2.606	9.12			
227		3.282	11.49	B	C	24.926
251	++ I	0.494	1.73	DB	+++++ I	22.552
252	+ H	0.000	0.00	Y	± T	12.499
258				Y	+ T	43.232
259				Y	C	23.535
261				Y	C	8.835

Key to Table 6.4

H indicates degree of haemolysis of sample from slight (±) to grossly haemolysed (+++);

I indicates icteric; T indicates degree of turbidity; Y – yellow; B – brown; PY – pale yellow;

C – clear; W – watery. Results were calculated as explained in section 6.3.1.2.

TABLE 6.5 COMPETITIVE INDIRECT ELISA RESULTS FOR PATIENTS WITH OESOPHAGEAL CANCER

	PLASMA			URINE		
Patient Number	Appearance	Fumonisins (ng/ml)	Total fumonisins (µg)	Colour	Clarity	Fumonisins (ng/ml)
106		1.044	3.65			
108		0.000	0.00	Y	C	9.265
110		0.924	3.23			
124	+ I	4.778	16.72			
135	+ H	0.602	2.11			
139	± H	3.378	11.82			
140	+ H	5.892	20.62			
146		1.130	3.96			
148		7.190	25.17			
149		7.828	27.40			
152	+ H	3.800	13.30			
155		19.115	66.90			
157		7.543	26.40	B	+ T	19.528
159	+ H	4.993	17.48	Y	+ T	28.076
164		5.180	18.13			
165		5.884	20.59			
166		7.749	27.12	Y	C	11.123
168		9.491	33.22			
169	± H	6.188	21.66	B	+ T	23.608
170		3.928	13.75			
171		5.420	18.97			
172		3.626	12.69			
173	++ H	2.260	7.91			
175		5.039	17.64			
176				PY	W	0.000
179	± H	3.044	10.65			
180		3.740	13.09			
184		7.070	24.75			
190	+ H	0.947	3.31			
195		4.208	14.73	Y	++ T	20.928
202		3.614	12.65	B	++ T	28.487
215	++ H	0.962	3.37			
216	+ H	4.125	14.44	B	++ T	28.522
217		2.751	9.63			
219		3.045	10.66	Y	+ T	20.079
220		4.872	17.05	B	++ T	17.268
225		3.393	11.88			
237		2.728	9.55			
239		3.983	13.94	Y	C	8.883
240		3.951	13.83	Y	++ T	26.183
253	+ H	0.000	0.00	Y	+ T	15.362

TABLE 6.5 (CONTINUED)

	PLASMA			URINE		
Patient Number	**Appearance**	**Fumonisins (ng/ml)**	**Total fumonisins (µg)**	**Colour**	**Clarity**	**Fumonisins (ng/ml)**
260				Y	W	0.000
262				B	+ T	46.624
263				B	+ T	17.879

Key to Table 6.5

H indicates degree of haemolysis of sample from slight (±) to grossly haemolysed (+++);

I indicates icteric; T indicates degree of turbidity; Y – yellow; B – brown; PY – pale yellow;

C – clear; W – watery. Results were calculated as explained in section 6.3.1.2.

TABLE 6.6. COMPETITIVE INDIRECT ELISA RESULTS FOR PATIENTS WITH OTHER TYPES OF CANCER

	PLASMA			URINE		
Patient Number & Cancer Type	**Appearance**	**Fumonisins (ng/ml)**	**Total fumonisins (µg)**	**Colour**	**Clarity**	**Fumonisins (ng/ml)**
Breast						
136	+ H	1.068	3.74	B	+ T	21.370
150	+ H	8.874	31.06			
154	+ H	8.644	30.25			
181		9.883	34.59			
204		0.936	3.28			
207		0.298	1.04	B	++ T	23.312
208	+ H	0.380	1.33			
211		2.860	10.01	Y	+ T	18.178
228		3.307	11.57	Y	+ T	16.335
244				Y	± T	9.496
264				B	+ T	37.320
Cervix						
151	+ H	6.249	21.87			
162		6.126	21.44			
193	± H	1.328	4.65	Y	C	6.655
236		4.605	16.12			
265				PY	C	8.289
Larynx						
242				Y	+ T	12.316
Pancreas						
153	+++ I	14.186	49.65			
Prostate						
134	+++ H	0.510	1.79			
Rectum						
209		0.132	0.46			
223		7.180	24.13			
Stomach						
137		2.011	7.04			
189		3.053	10.69	B	+ T	21.077
Thyroid						
221		4.827	16.89			

Key to Table 6.6

H indicates degree of haemolysis of sample from slight (±) to grossly haemolysed (+++);

I indicates icteric; T indicates degree of turbidity; Y – yellow; B – brown; PY – pale yellow;

C – clear; W – watery. Results were calculated as explained in section 6.3.1.2.

TABLE 6.7 SUMMARY OF COMPETITIVE INDIRECT ELISA RESULTS FOR FUMONISINS IN PLASMA AND URINE

Fumonisins (ng/ml) in EDTA Plasma				Fumonisins (ng/ml) in Urine		
Oesophageal Cancer		Controls	Other Cancers	Oesophageal Cancer	Controls	Other Cancers
Average	**4.385**	**2.973**	**4.323**	**18.930**	**19.910**	**17.435**
1.044	5.420	4.202	1.068	9.265	6.192	21.370
0.000	3.626	3.043	8.874	19.528	10.936	23.312
0.924	2.260	5.429	8.644	28.076	21.516	18.178
4.778	5.039	4.200	9.883	11.123	17.852	16.335
0.602	3.044	9.226	0.936	23.608	10.636	9.496
3.378	3.740	6.050	0.298	0.000	36.212	37.320
5.892	7.070	4.272	0.380	20.928	24.926	6.655
1.130	0.947	1.439	2.860	28.487	22.552	8.289
7.190	4.208	2.188	3.307	28.522	12.499	12.316
7.828	3.614	1.120	6.249	20.079	43.232	21.077
3.800	0.962	0.130	6.126	17.268	23.535	
19.115	4.125	1.592	1.328	8.883	8.835	
7.543	2.751	0.370	4.605	26.183		
4.993	3.045	1.306	14.186	15.362		
5.180	4.872	1.032	0.510	0.000		
5.884	3.393	5.949	0.132	46.624		
7.749	2.728	4.508	7.180	17.879		
9.491	3.983	2.606	2.011			
6.188	3.951	3.282	3.053			
3.928	0.000	0.494	4.827			
		0.000				

Table 6.7 is a summary of results in Tables 6.4, 6.5 and 6.6.

6.4.2.4 Statistical Evaluation

The one-way ANOVA showed a highly significant difference between plasma results for OC and controls ($p<0.0001$). Even if the one high result of 19.115 ng/ml was omitted the difference was still highly significant ($p<0.0001$). There was no significant difference between the urine results.

Comparison of other cancers and control samples showed no significant differences for either the plasma or the urine samples.

However, there was again a highly significant difference between the plasma results for OC and other cancers ($p<0.005$), which was maintained even if the high oesophageal cancer result of 19.115 ng/ml was excluded ($p<0.05$); and also if both this high result and the high result of 14.186 ng/ml for other cancers was excluded ($p<0.001$).

Interestingly the urine results also showed a significant difference between these two groups ($p<0.05$).

6.4.3 High Performance Liquid Chromatography

The usual injection volume was 20 µl but was increased to 50 µl to improve sensitivity.

For the three days on which analyses were done, the average retention times were 4.364 ± 0.071 min (CV 1.63%); 3.879 ± 0.053 min (CV 1.37%) and 4.379 ± 0.054 min (CV 1.23%).

Peaks at high concentrations of FB_1(chiefly higher standards) had a good shape but lower concentrations gave peaks with a shoulder.

TABLE 6.8 RESULTS FOR STANDARDS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Day 1		Day 2		Day 3	
Standard FB_1 (ng/ml)	**Peak Area**	**Standard FB_1 (ng/ml)**	**Peak Area**	**Standard FB_1 (ng/ml)**	**Peak Area**
		10	6116	10	6115
20	21775	20	10327	20	12194
50	44133	50	22090	50	24864
100	72215	100	51109	100	53785
200	127334	200	98452		

The equations for the standard curves on the three days of analysis were:-

Day 1	$y = 577.01x + 12991$	Regression	0.9974
Day 2	$y = 492.47x + 191.14$	Regression	0.9981
Day 3	$y = 523.19x + 696.05$	Regression	0.9958

The peak for the 50 ng/ml standard was clearly defined and had a retention time of 4.424 min (Figure 6.1)

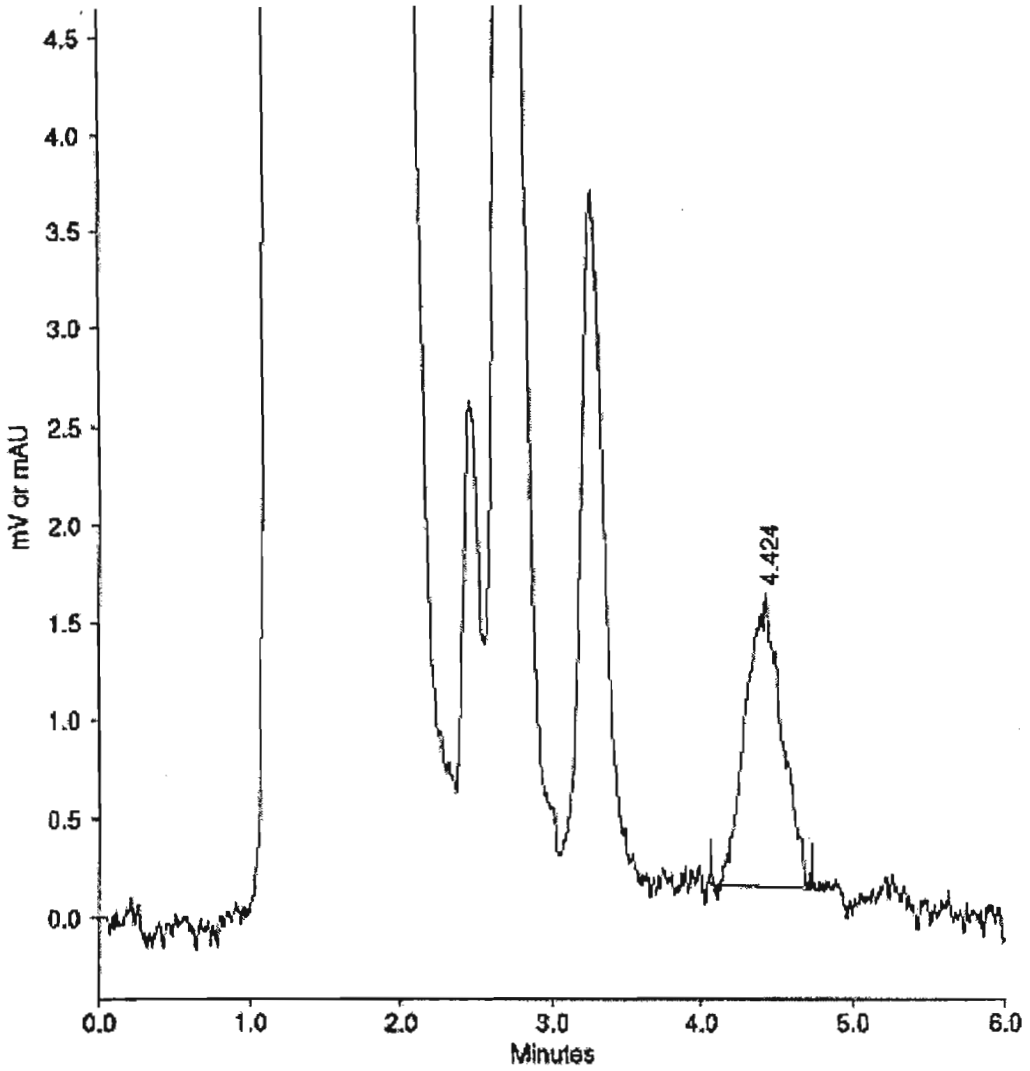


FIGURE 6.1 CHROMATOGRAM OF 50 NG/ML FUMONISIN B_1 STANDARD (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

Retention time for 50 ng/ml FB_1 standard (Figure 6.1) was 4.424 min.

6.4.3.1 Detection Limits

TABLE 6.9 DETECTION LIMITS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

FB_1 Standard (ng/ml)	Signal:noise	Average
20	5.6	4.2
20	3.3	
20	3.6	
10	3.1	3.3
10	3.4	

The detection limit was taken as 10 ng/ml FB_1 standard in acetonitrile/water which translates into 1 ng/ml for urine samples and 2 ng/ml for serum sample.

6.4.3.2 Recoveries

TABLE 6.10 RECOVERIES FOR FUMONISIN B_1 FROM PLASMA/URINE (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

FB_1 (ng/ml)	% Recovery	Average recovery
Plasma		69.62%
15.36	96.01	
14.64	91.48	
9.11	56.97	
5.44	34.00	
Urine		48.13%
8.20	51.28	
7.40	46.25	
7.08	44.26	
8.11	50.71	

Results (Table 6.10) were calculated as described in section 6.3.2.2.

6.4.3.3 Plasma and Urine Samples (High Performance Liquid Chromatography)

After re-dissolving in acetonitrile:water (1:1), the serum samples were mostly colourless but the urine samples were highly coloured (yellow, dark yellow, orange-brown, light yellow). These gave high fluorescence readings with a high interfering peak at 1-2 min which was avoided by changing the initial emission wavelength (section 6.3.2.2).

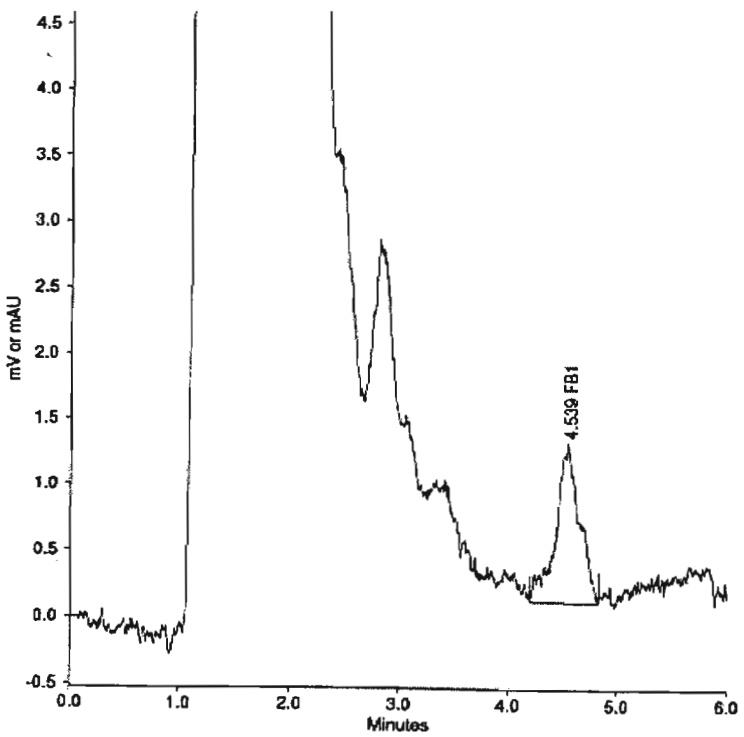


FIGURE 6.2 CHROMATOGRAM OF PLASMA SAMPLE 163 (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

Retention time for plasma sample 163 (Figure 6.2) was 4.539 min.

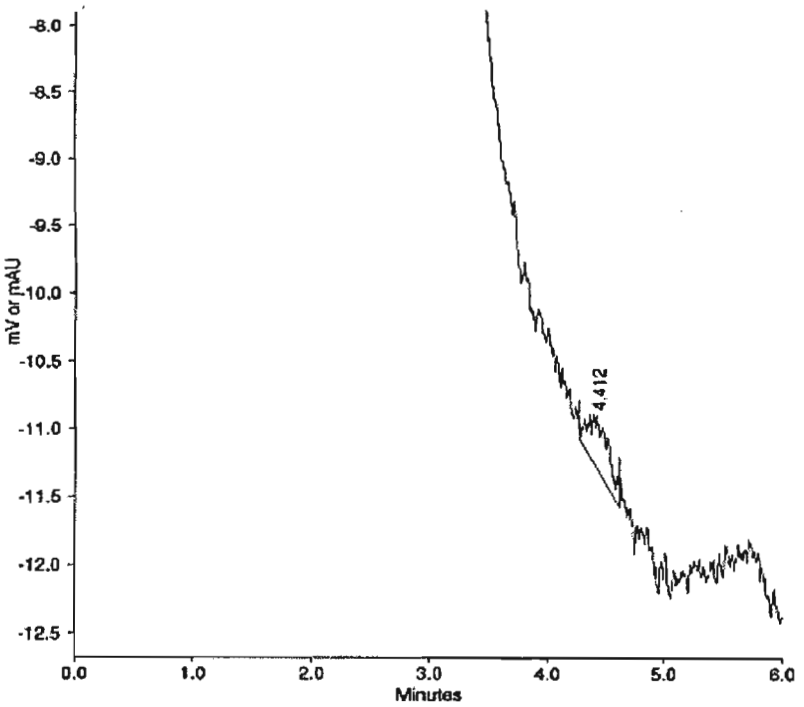


FIGURE 6.3 CHROMATOGRAM OF URINE SAMPLE 219 (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

Retention time for urine sample 219 (Figure 6.3) was 4.412 min.

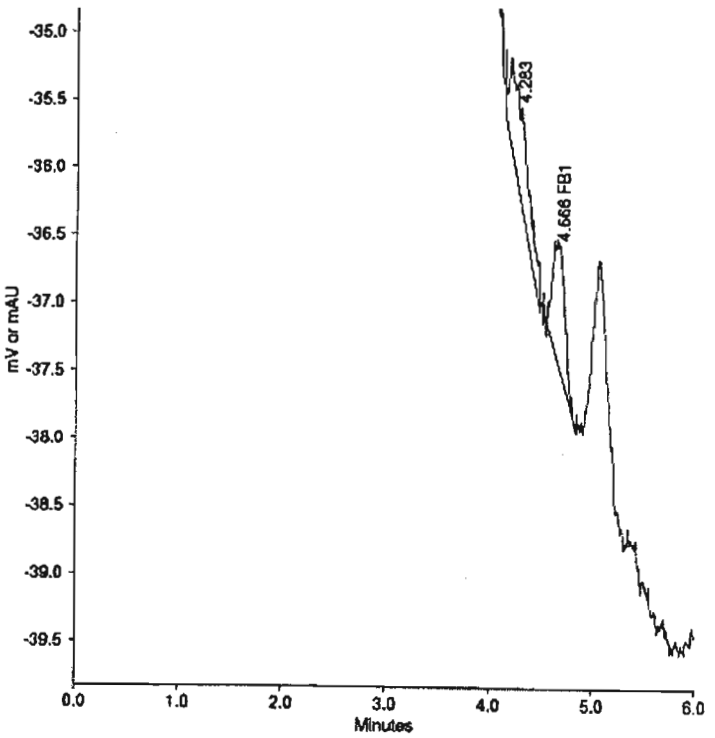


FIGURE 6.4 CHROMATOGRAM OF URINE SAMPLE 222 (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

Retention time for urine sample 222 (Figure 6.4) was 4.666 min.

Comparison of the plasma (Figure 6.2) and urine chromatograms (Figures 6.3 and 6.4) shows more clearly defined peaks and better results for plasma samples. In some samples FB_1 appeared as a shoulder on a larger peak (Figure 6.3); in others the presence of a peak with a retention time of about 5 min was clearly seen (Figure 6.4).

TABLE 6.11 COMPARISON OF PLASMA AND URINE RESULTS ANALYSED BY COMPETITIVE INDIRECT ELISA AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Plasma concentration of FB_1 (ng/ml)			Urine concentration of FB_1 (ng/ml)		
Sample Number	**ELISA**	**HPLC**	**Sample Number**	**ELISA**	**HPLC**
149	7.828	6.50	157	19.528	0.97
157	7.543	12.38	202	28.487	9.01
162	6.126	14.34	206	17.852	4.17
165	5.884	16.45	219	20.079	2.30
168	9.491	3.46	222	36.212	1.76
169	6.188	12.55	258	43.232	2.20
181	9.883	8.31	262	46.624	10.28
184	7.070	3.74	264	37.320	2.06
223	7.180	7.98			
Average	**7.466**	**9.523**		**31.167**	**4.09**

6.4.3.4 Statistical evaluation

A one-way ANOVA showed no statistically significant differences between the two methods for plasma samples. Correlation and regression were $r = 0.656$, $y = 25.44 - 2.13x$ ($p<0.005$). However, there was a highly significant difference between the two methods for urine results ($p<0.001$).

6.4.4 Calcium and Magnesium

The method controls (Level 1, Lot. No. 128UN and Level 2, Lot No. 062UE) were always within their limits.

Calcium	Level 1	Target value 2.26 mmol/l	Range 1.92 – 2.60 mmol/l
Calcium	Level 2	Target value 3.20 mmol/l	Range 2.88 – 3.52 mmol/l
Magnesium	Level 1	Target value 0.89 mmol/l	Range 0.80 – 0.98 mmol/l
Magnesium	Level 2	Target value 1.23 mmol/l	Range 1.06 – 1.40 mmol/l

TABLE 6.12 SUMMARY OF CALCIUM AND MAGNESIUM RESULTS ON SERUM SAMPLES

Calcium (mmol/l)				Magnesium (mmol/l)			
Oesophageal Cancer		Controls	Other Cancers	Oesophageal Cancer		Controls	Other Cancers
2.40	2.85	2.53	2.49	0.76	1.00	0.88	1.06
2.43	2.85	2.34	2.96	0.82	1.18	0.85	0.99
2.02	2.61	2.56	2.78	0.76	1.02	0.92	1.08
2.81	2.47	2.48	2.50	1.09	1.00	0.89	0.92
1.55	3.13	2.11	2.63	1.08	0.96	0.80	0.93
2.73	2.42	2.57	2.38	1.10	0.79	0.92	0.85
2.68	2.68	2.55	2.16	1.07	0.94	0.97	0.81
2.66	2.85	2.62	2.64	0.95	0.94	0.98	0.93
2.34	2.77	2.52	2.38	0.94	0.96	0.94	0.91
2.75	2.72	2.41	2.77	0.95	0.91	0.99	0.92
2.47	2.74	2.61	2.68	0.95	1.05	1.04	0.95
2.55	2.31	2.59	2.71	1.03	1.12	0.98	1.08
2.65	2.49	2.19	2.33	0.91	0.88	1.23	1.05
2.79	3.22	2.73	2.44	0.96	1.03	0.94	0.97
2.41	2.81	2.80	2.65	0.87	0.94	0.91	1.00
2.59	2.34	2.72	2.65	0.84	0.88	0.95	0.96
2.57	2.42	2.61	2.83	0.88	00.93	0.97	0.97
2.62	2.64		2.56	1.03	1.00		0.91
2.33			2.63	0.84			0.96

Results (Table 6.12) are the means of duplicates; the difference between duplicates < 0.1 mmol/l.

For further details of serum samples analysed for calcium and magnesium refer to Appendix 1, Tables 6.13, 6.14 and 6.15.

6.4.4.1 Statistical Evaluation

The one-way ANOVA showed a highly significant difference in calcium results between OC patients and controls ($p<0.0001$) which was maintained even if the lowest and highest values (a statistically acceptable approach) were excluded ($p<0.0001$). There was also a highly significant difference in magnesium results ($p<0.0001$) between these groups.
Comparison of other types of cancer and control patients showed no significant differences for both the calcium and the magnesium results.

However, there was again a highly significant difference between calcium and magnesium results from patients with OC and those with other types of cancer ($p<0.0001$ for both). The significant difference was unchanged even if the lowest and highest calcium result from the OC patients were excluded.

6.5 DISCUSSION

6.5.1 Extraction and Recoveries of Fumonisins from Blood and Urine (Competitive Indirect ELISA)

Recoveries from extraction of maize samples using organic solvents ranged from 59.7% to 103% (Azcona-Olivera *et al.*, 1992B; Maragos & Richard, 1994; Usleber *et al.*, 1994; Abouzied *et al.*, 1996; Yeung *et al.*, 1996; Yu & Chu, 1996). Without doing any extraction, Scott *et al.* (1997) obtained recoveries of 98.7 to 102.8% in beer samples. Scott & Lawrence (1994) in analysing maize-based foods concluded that control recoveries should be done for each type of food and different extraction methods are needed for different matrices.

Protein is an interferent in many analyses in physiological fluids, hence in this study the extraction methods were aimed at removal of protein. Dilution in buffer and extraction with organic solvents were not effective extraction techniques. Even though 30% PEG 6 000 precipitated the proteins in the sample, recoveries were low.

Boiling diluted samples proved to be a quick and easy method of extraction. Fumonisins are thermostable (Dupuy *et al.*, 1993; Maragos & Richard, 1994; Jackson *et al.*, 1997) but proteins are denatured by heat and can be removed by centrifugation.

Small differences in recoveries at two levels of FB_1 (Table 6.3) were found between blood samples collected without anticoagulant (112.4% and 108.6%) or using heparin (94.7% and 108.7%) or EDTA (99.1% and 97.6%). Urine samples also gave good recoveries (97.7% and 94.6%). Comparison of the coefficients of variation for the recoveries showed they were slightly better at the level of 8 ng/ml than 4 ng/ml.

The most consistent recovery results (CV 6.1% at 4 ng/ml and 5.5% at 8 ng/ml) were from plasma using EDTA as anticoagulant; hence, all further samples analysed by CI-ELISA were collected in EDTA tubes. Blood collected with EDTA was also used by Shephard *et al.* (1992B) in their HPLC experiments.

6.5.2 Plasma and Urine Samples analysed for Fumonisins (Competitive Indirect ELISA)

The patients in this study eat maize as a staple food. Studies of white and yellow maize in South Africa found the levels of fumonisins to range from 0 to12 963 μg FB_1/kg, 0 to 5 690 μg FB_2/kg and 0 to 3 110 μg FB_3/kg (Rava *et al.*, 1996; Rava, 1996). Thus, South Africans consuming maize daily are exposed to fumonisins and it might be expected to find them in their blood and urine. In this study, detectable levels of fumonisins (> 0.4 ng/ml) were found in both blood and urine of 86.9% and 94.9% of the samples, respectively.

This CI-ELISA method is not specific for FB_1 as there was cross-reactivity with FB_2 (97.7%) and FB_3 (33.1%) and possibly with hydrolysed fumonisins (Chapter 5, Table 5.20). Yet *F. moniliforme* produces predominantly FB_1 (Gelderblom *et al.*, 1988) so any person consuming contaminated maize or food will be ingesting mainly FB_1 and hence the major fumonisin expected to be found in physiological fluids (e.g. blood and urine) would be FB_1.
Excreted fumonisins in humans may be unmetabolised or partially or fully hydrolysed.

Haemolysis did not appear to affect results as similar values were found for unhaemolysed samples i.e. fumonisin was not sequestered inside the red blood cells.
The two watery urines had no detectable fumonisins (possibly a dilution effect) but for accurate quantitative analysis a 24 hr urine collection would be necessary. (This study used random urine samples).

Fumonisin levels in plasma samples from patients diagnosed with OC ranged from 0 to19.115 ng/ml, average 4.385 ng/ml (Table 6.5). In contrast, in the control patients, the highest level of fumonisin was 9.226 ng/ml, average 2.973 ng/ml (Table 6.4). Since both patients and controls consume a maize-based diet, it was not unexpected to find fumonisins in the plasma (and urine) of controls. There was a highly significant difference in fumonisin concentrations between patients with OC and controls ; even excluding the one high OC result, the difference was still highly significant indicating a possible relationship between raised levels of fumonisins in the blood and OC (Table 6.7).

In the group with other types of cancer, the highest plasma value (14.186 ng/ml) was from a patient with pancreatic cancer (Table 6.6). There was no significant difference between the plasma results of this group and the controls but again there was a highly significant difference between patients with other types of cancer and those with OC (Table 6.7). This may show that fumonisins are associated specifically with OC and not other forms of cancer.

Total fumonisins in plasma ranged from 0.00 to 32.29 μg in control patients and from 0.46 to 49.65 μg in patients with other types of cancer. For patients with OC the range was from 0.00 to 33.22 μg but one sample had 66.90 μg (Tables 6.4, 6.5 and 6.6). These results only reflect circulating fumonisins in the blood; total body levels may well be higher if fumonisin is sequestered in the tissues.

Levels of fumonisins in urine were higher than in plasma, ranging from 0 to 46.624 ng/ml in OC patients (Table 6.5); from 6.192 to 43.232 ng/ml in controls (Table 6.4) and from 6.655 to 37.320 ng/ml in patients with other types of cancer (Table 6.6). The higher levels of fumonisins in urine are to be expected since ingested fumonisins are rapidly excreted. There was no significant difference in urine results between patients with OC and controls suggesting that higher levels in the blood of OC patients are not due to poorer excretion. Yet there was a difference between OC patients and those with other types of cancer (Table 6.7) which reinforces the suggestion that fumonisins are associated only with OC.

6.5.3 High Performance Liquid Chromatography

Recoveries of FB_1 in serum and urine samples by HPLC averaged 69.6% and 48.1% respectively. Shephard *et al.* (1992B) obtained recoveries of 86% and 87% for FB_1 in plasma and 91% and 94% in urine. However, their samples contained much higher levels of fumonisins, viz. 1.4 μg/ml and 6.2 μg/ml in spiked plasma samples; and 1.0 μg/ml and 12 μg/ml in spiked urine samples i.e. about 1 000 times more FB_1 than in the present study where samples were spiked with 16 ng/ml FB_1. Shephard's HPLC method gave detection limits of about 50 ng/ml with a signal to noise ratio 4:1; this study gave lower detection limits for HPLC viz. 10 ng/ml at a signal to noise ratio of 3:1.

Better recoveries and more clearly defined peaks were found in plasma samples (Figure 6.2) compared to urine samples (Figures 6.3 and 6.4). In some of the urine samples there was an interfering peak which co-eluted with FB_1 or otherwise the FB_1 peak appeared as a shoulder on a larger peak (Figures 6.3 and 6.4). It was also noted that many of the urine samples showed another peak at a retention time of 5 min – eluting after FB_1 (which had average retention times of 3.879, 4.363 and 4.379 min for analyses done on three different days). The significance of this peak is not known. Maragos (1995) in analysing horse serum spiked with 100 ng/ml FB_1 also found interference by a component in the unspiked serum which had a similar migration time to FB_1.

There was also a problem in obtaining reliable results at such low levels of FB_1 – better peaks were obtained for standards at levels > 50 ng/ml but this would be below the level of naturally occurring FB_1 concentrations. To improve sensitivity and remove interfering solutes in human urine samples, Shetty & Bhat (1998) used an Amberlite XAD-2 non-ionic polymeric resin column prior to the SAX column and obtained recoveries of 93.6% to 94.4% (CV < 5%) with a detection limit of < 8 ng/ml. However, they were working with 100 ml of sample spiked at 10 to 500 ng/ml FB_1 - in contrast to 1ml spiked at 16 ng/ml in this study. Also, for routine analysis, use of an additional column would add to the cost.

6.5.4 Comparison of Results by High Performance Liquid Chromatography and Competitive Indirect ELISA on the Same Samples

There was no significant difference in plasma results by the two methods but urine results were significantly different. Statistically this comparison would have been improved by analysis of more samples. The correlation coefficient for plasma results was 0.656 ($p<0.005$).
Overall, plasma results were lower than urine results by CI-ELISA, whereas HPLC analysis gave lower urine than plasma results. Of the nine plasma samples, five gave higher results by HPLC than by ELISA and four gave lower results; all eight urine samples gave lower results by HPLC. The large interfering peak and inability to get a decent base-line contributed to the lower urine results.

The ELISA method was more sensitive with detection limits of 0.4 ng/ml for both plasma and urine samples in contrast with the HPLC method which had detection limits of 1ng/ml for urine and 2 ng/ml for plasma samples.

Recoveries for the ELISA method (99.11% and 97.58% for plasma, 97.73% and 94.55% for urine samples) were much better than for HPLC (69.62% for plasma and 48.13% for urine). Furthermore, the recovery experiments in CI-ELISA were done at lower spiked concentrations of FB_1 (4 and 8 ng/ml) than for HPLC (16 ng/ml).

No other workers have compared results between immunoassay and liquid chromatography for plasma or urine samples. However, in analysis of maize samples, ELISA methods usually showed higher values than HPLC but generally good correlation, $r = 0.967$, $p<0.001$ (Sydenham *et al.*, 1996B); $r = 0.955$, $p<0.001$ (Yu & Chu, 1996) and $r = 0.512$, $p<0.05$ (Pestka *et al.*, 1994). But these comparisons were at higher concentrations of FB_1 than those in physiological fluids viz. 0.05 to 5.44 μg/g (50 to 5 440 ng/g), <10 to 49 800 ng/g and 0.17 to 6.32 μg/g (170 to 6 320 ng/g) respectively, as measured by HPLC.
In contrast, using an in-house immunoaffinity column prior to analysis of maize samples by both immunoassay and HPLC, Maragos (1997) found good agreement between immunoassay and HPLC results at low levels of fumonisins (< 2 μg/g) but at higher levels the HPLC results were lower. For beer samples, Scott *et al.* (1997) also found ELISA results were lower than HPLC results.

Several possible explanations for differences between HPLC and ELISA results have been proposed including differences in extraction procedures and lower recoveries from samples prepared for HPLC; interference (inhibition) by components in the sample (matrix differences, possibly lipid based); completely different methodology principles; and precursors or metabolites produced by *F. moniliforme* that cross-react with the antibodies or interfere in the immunoassay (Pestka *et al.*, 1994; Shelby *et al.*, 1994; Tejada-Simon *et al.*, 1995; Abouzied *et al.*, 1996; Sydenham *et al.*, 1996B). Usleber *et al.* (1994) suggested that differences in the ratio of the open chain and cyclic forms of the TCA side chains may affect results as this ratio is pH dependent and different extraction procedures give different pH values of the extracts. The TCA groups are believed to be important for antibody binding (Azcona-Olivera *et al.*, 1992A, 1992B).

That the higher results for urine samples analysed by CI-ELISA compared to plasma samples are due to the measurement of other associated metabolites similar in structure to FB_1 seems likely since even taking differences in extraction recoveries into account, the concentrations of fumonisins in urine are lower by HPLC (measuring only FB_1) than by ELISA. Shephard *et al.* (1994A, 1994C) found that other primates excreted fully and partially hydrolysed fumonisins in urine.

The plasma samples probably contained lower concentrations of metabolites explaining the paradox that some samples gave higher results by HPLC and others lower results compared to HPLC. The cross-reactivity experiments (Chapter 5, section 5.4.6) reinforces the probability that the ELISA method is detecting other unmetabolised fumonisins and probably metabolic products.

6.5.5 Calcium and Magnesium

Since FB_1 is believed to have chelating characteristics (Beier *et al.*, 1995; Beier & Stanker, 1997) and is predominantly negatively charged at pH 7, it was decided to investigate calcium and magnesium levels in the blood samples of the three groups of patients used for this study.

The reference range for calcium in serum is 2.1 to 2.6 mmol/l and for magnesium is 0.65 to 1.05 mmol/l (Farrell, 1984). Only two calcium results, both on patients with OC, were below the reference range; however, 37 results were above the reference range (20 from the OC group, 6 from the control group and 11 from the group with other types of cancer – 4 with breast cancer, 3 cervical, 1 each for prostate, rectal, stomach and thyroid cancer). None of the magnesium results were below the reference range but six OC, one control and three patients with other types of cancer (2 with breast and 1 with cervical cancer) had results above the reference range (Table 6.12).

Highly significant differences for both calcium and magnesium results were found between OC patients cancer and controls with higher values for the OC patients. There were also highly significant differences between OC patients and those with other types of cancer but no significant differences between this latter group and control patients (section 6.4.4).

This raises the possibility that increased concentrations of divalent cations are involved in the mechanism of increased fumonisin levels in OC patients.
A possible hypothesis is that these divalent cations aid in the attachment of fumonisin to cell membranes by forming a link between negatively charged carboxyl groups on the fumonisin molecule and negatively charged proteins on the cell surface. Alternatively, the fumonisin may be chelating the cations, changing shape and so being retained in the blood longer.

6.6 CONCLUSIONS

A simple but effective method for extracting fumonisins from plasma and urine samples was developed. Fumonisins are thermostable and boiling to remove interfering proteins was found to be quick, cheap and easy. The method gave good and reproducible recoveries in serum, EDTA and heparinised plasma and urine in the CI-ELISA method.

The good agreement between plasma results for the CI-ELISA and HPLC methods confirmed the validity of the CI-ELISA method. The lack of correlation between the two methods for urine results was satisfactorily explained by the probable presence of fumonisin metabolites in the urine which are measured by CI-ELISA but not by HPLC. The extraction methods for plasma and urine samples for HPLC need to be optimised to improve the recoveries and to remove interfering substances, particularly in urine samples.

In analysis of fumonisins in plasma and urine, samples from three groups of patients viz. those with OC, controls (no cancer) and those with other types of cancer, a significant difference was found between the OC group and the other two. This implies an association between fumonisin levels in the blood and OC but it must be remembered that other factors may also be causative agents in the development of OC e.g. nitrosamines, tobacco, air pollutants (Burrell, 1957; Rose, 1982; van Rensburg, 1985). The significant differences in calcium and magnesium levels between the OC group and the other two indicated that these divalent cations could play a role in the fumonisin carcinogenicity in OC.

CHAPTER 7

GENERAL CONCLUSION

Polyclonal antibodies against fumonisins were successfully raised in chickens and rabbits using a FB_1-KLH conjugate as immunogen. This study was the first record of pAb against fumonisin being produced in chickens. Using PEG 6 000, IgY was extracted from the egg yolks and IgG was extracted from rabbit serum. (Chapter 3, sections 3.3.3 and 3.3.4).

Adaptation and optimisation of an existing method for estimating titre was done successfully. This study was the first record of coating microtitre plates directly with FB_1 i.e. it was not necessary to first prepare a FB_1- protein conjugate (Chapter 3, sections 3.3.7 and 3.4.3). Antibody titre was measured on all batches of IgY and IgG extracts from all four animals (Chapter 3, section 3.3.8). Both chickens produced antibodies every week from week 2 to week 14 when collection of eggs ceased. Antibodies were found for every bleed of the rabbits viz. weeks 4, 8, 12 and 14. The highest titres for each animal (in order from highest to lowest) were from week 8 for chicken 2, then chicken 1 at week 8, rabbit 1 at week 14 and rabbit 2 also at week 8 (Chapter 3, Figure 3.13).
Both the chicken and the rabbit antibodies showed cross-reactivity mainly with FB_2 but also with FB_3; there was no cross-reactivity with So (Chapter 5, sections 5.3.5 and 5.4.6).

Attempts to develop a CD-ELISA were not successful. Possibly the conjugation of FB_1 to HRPO using sodium periodate was not successful (Chapter 4, section 4.3.1) or the HRPO *did* bind to the FB_1 but caused steric hindrance so that the labelled FB_1 could not bind to Ab_1.

A CI-ELISA method was successfully developed and optimised using chicken antibodies. It was relatively inexpensive, rapid and easy to perform but could do with further refinements to reduce incubation times (Chapter 5, sections 5.3.2 and 5.4.2). The method also worked with the rabbit antibodies.
The method has good reproducibility (low intra-plate and inter-plate CV) and good sensitivity (range from 0 to 20 ng/ml). Internal quality control was done by running low and high method controls and non-specific binding blanks on every plate and checking they remained within acceptable limits (Chapter 5, sections 5.3.4 and 5.4.5).

A comprehensive check on various methods of quantitative calculation was done. The one finally selected was plotting absorbance (at 405 nm) against FB_1 concentration and using a 2^{nd} order polynomial curve fitting equation (Chapter 5, sections 5.3.3 and 5.4.3). Confirmation of the correctness of the results by this method of calculation was supplied by using the programme on the plate reader at NBTS (Chapter 5, Table 5.11) and a dedicated programme on a different model of plate reader to the one normally used (Chapter 5, Table 5.12).

For adaptation of the CI – ELISA to analyse blood and urine samples there was no information in the literature as how to proceed, as no other group had used an immunoassay method for physiological fluids or tissues.
Initial experiments indicated that the main interferent was protein as slightly better results were obtained with urine (lower protein content) compared with plasma/serum. After attempting several methods, heating by boiling to denature the proteins (which were then removed by centrifugation) proved to be a quick, cheap and easy method (Chapter 6, sections 6.3.1.1 and 6.4.1). Good recoveries were obtained with serum, EDTA plasma, heparinised plasma and urine (Chapter 6, Table 6.3). As a further part of the quality control, a positive and a negative plasma/urine control were included in every further run; these underwent the extraction process (Chapter 6, section 6.4.2.1).

The PROMEC group found a definite association between fumonisins and OC but no one had as yet shown the presence of fumonisins in the physiological fluids of patients with OC; possibly because the only readily available method to date has been HPLC which is less sensitive than ELISA (Chapter 6, section 6.1).

For the first time, this study found measurable levels of fumonisins in OC patients (Chapter 6, Tables 6.4 and 6.7). There was a significant difference in fumonisin levels in the blood and urine of patients with OC compared to controls and to patients with other types of cancer. Even though fumonisins were also found in the control group, this was not surprising since they too consume a maize-based diet. The fumonisins seemed to be associated specifically with OC and not with other types of cancer but larger numbers of samples need to be analysed to confirm these preliminary findings (Chapter 6, sections 6.3.1.2, 6.4.2.3 and 6.4.2.4).

To check the validity of the CI-ELISA results, samples with high levels of fumonisins were analysed by HPLC. The extraction step for HPLC was more time consuming and more expensive than the boiling method; also the sensitivity and recoveries were not as good as for the CI-ELISA (Chapter 6, sections 6.3.2.1, 6.4.2.2, 6.4.3.1 and 6.4.3.2). Adaptation of the actual HPLC analysis to cope with interferents gave improved sensitivity over previously published methods (Chapter 6, section 6.3.2.2 and 6.4.3.3). There was good correlation between results for plasma but not for urine samples (Chapter 6, Table 6.11 and section 6.4.3.4). This was explained by the fact that the ELISA method was probably measuring fumonisins plus metabolites which are more likely to be found in urine than in blood; also better peaks were obtained for plasma samples.

It was found that OC patients had significantly more calcium and magnesium in their serum compared to the control group and to patients with other types of cancer (Chapter 6, Table 6.12 and section 6.4.4.1). It was hypothesised that these cations might aid in the attachment of FB_1 to cell walls or may cause a delay in its excretion.

APPENDIX 1

TABLE 2.5 ANALYSIS OF FUMONISINS BY ALTERNATIVE METHODS

Method	Sample	Recovery	Detection Limits	Reference
Fluorometry	Corn	83%	0.25 μg/g Range 0 - 10 μg/g	Duncan *et al.*, 1998
HPLC-ESI-MS-MS	Corn grit and corn meal	no data	0.8 ng/g	Lukacs *et al.*, 1996
LC-ESI-MS	Rodent feed	68.9% (FB_1) 60.4% (FB_2) 74.3% (FB_3)	1.1 ppb (ng/g)	Churchwell *et al.*, 1997
TLC	Corn & corn-based feedstuffs	>80%	0.1 ppm (μg/g)	Rottinghaus *et al.*, 1992
HPTLC	Rice	81%	0.25 μg/g Range 0 - 5 μg/g	Dawlatana *et al.*, 1995
SFE/HPLC	Corn dust	no data	150 ppb	Selim *et al.*, 1996
CZE	Spiked horse serum	76%	100 ng/ml	Maragos, 1995

Key to Table 2.5

HPLC-ESI-MS-MS	high-performance liquid chromatography – electron spray- ionisation, tandem mass spectrometry
LC-ESI-MS	liquid chromatography electron spray-ionisation mass spectrometry
TLC	thin layer chromatography
HPTLC	high performance thin layer chromatography
SFE/HPLC	supercritical fluid extraction/high performance liquid chromatography
CZE	capillary zone electrophoresis

TABLE 2.13 EFFECTS OF FUMONISIN B_1 IN CELL CULTURE

Type of cells : FB_1 Concentration	Effect of fumonisin	Reference
Rat hepatocytes: 1 μM (1 hr)	Inhibition of incorporation of ^{14}C-serine into So Increased [free Sa]	Wang *et al.*, 1991
Primary rat hepatocytes: 10 mM (2 hr) 1 μM (4 d)	Inhibition of incorporation of ^{14}C-serine into So (IC_{50} 0.1 μM) Increased [free Sa]	Norred *et al.*, 1992
Pig kidney epithelial cells LLC-PK_1: 10, 35 μM (4 d)	Inhibition of incorporation of ^{14}C-serine into So (IC_{50} 35 μM) Decreased cell proliferation and cell death Increased [free Sa]	Norred *et al.*, 1992
Pig kidney epithelial cells LLC-PK: 10, 35 μM	Inhibition of proliferation (measured as increase in protein content) and cell death Decrease in incorporation of ^{3}H-serine into So Increased [free Sa] (IC_{50} 20 μM)	Yoo *et al.*, 1992
Mouse cerebellar neurons: 25 μM (1 d)	Inhibition of sphingolipid formation from ^{14}C-serine, ^{3}H-galactose, ^{3}H-sphinganine (IC_{50} 0.7 μM) Increased [free Sa]	Merrill *et al.*, 1993B
Swiss 3T3 fibroblasts: 10 - 50 μM (1 d)	Stimulated DNA synthesis Increased [free Sa]	Schroeder *et al.*, 1994
Chicken peritoneal macrophages: 6 - 18 μg/ml	Dose-dependent nuclear disintegration	Chatterjee *et al.*, 1995
Primary rat hepatocytes: 12.5, 25, 75 ,150, 300 μM	Inhibition of epidermal growth factor induced DNA synthesis measured as incorporation of ^{3}H-thymidine Increased [free Sa] at [FB_1] 1 μM	Gelderblom *et al.*, 1995
Rat hepatocytes: 10 μM	Inhibition of incorporation of ^{3}H-serine into sphingomyelin and ceramide	Merrill *et al.*, 1995A
Yeast cells (*Saccharomyces cerevisiae)* : 25, 50, 100, 200 μM	Inhibition of cell growth Inhibition of ceramide synthase Decrease in synthesis of various lipids Increased [free Sa] and [phytosphingosine]	Wu *et al.*, 1995
Primary rat hepatocytes: 100, 150 μM	Inhibition of incorporation of ^{14}C-palmitic acid into lipids Increased [free Sa]; Decreased [cholesterol] Increased [polyunsaturated fatty acids]	Gelderblom *et al.*, 1996B
African green monkey kidney cells CV-1: 1, 2, 5 μM (3 hr)	Induction of apoptosis Cell cycle arrest at G_1 phase	Wang *et al.*, 1996
Pig kidney epithelial cells LLC-PK1: 70 μM (3 d)	Increased [free Sa] Decreased [complex sphingolipids] Inhibition of cell growth; cell death	Yoo *et al.*, 1996A
Swiss 3T3 fibroblasts: 10, 20, 50 μM	Decreased synthesis of sphingomyelin and ganglioside GM_3 Inhibited cell proliferation and DNA synthesis	Meivar-Levy *et al.*, 1997

Key to Table 2.13

[ ]	indicates concentration	So	sphingosine
Sa	sphinganine	IC_{50}	concentration causing 50% inhibition

TABLE 3.9 DATA TO CHECK DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (SIGMA) TO USE FOR ANTIBODY TITRE OF RABBIT IMMUNOGLOBULIN G USING BATCH R1/14

Ab_1 (µg/ml)	Log [IgG]	1:1 000	1:2 000	1:5 000	1:10 000
1000	3.0	0.613	0.719	0.472	0.313
500	2.7	0.412	0.512	0.325	0.283
100	2.0	0.170	0.237	0.149	0.176
25	1.4	0.000	0.008	0.007	0.011

Key to Table 3.9

Results are means of duplicate (except for the 25 µg/ml) absorbance values at 405 nm after subtraction of the mean of the relevant pre-immune absorbance value (R1/P) and the absorbance value for the no Ab_1 blank.

TABLE 3.10 DATA FOR SUMMARY OF RESULTS OF EXPERIMENTS TO DETERMINE OPTIMUM BLOCKING AGENT, DILUENT AND DILUTION OF SECOND ENZYME-LABELLED ANTIBODY FOR TITRE OF CHICKEN IMMUNOGLOBULIN Y USING BATCH C2/8

Ab_1 (µg/ml)	Log [IgY]	B/Ab_2C	$B/Ab_2S/5$	$B/Ab_2S/20$	$C/Ab_2C/0.5$	$C/Ab_2S/20$	BC/Ab_2C	$BC/Ab_2S/20$	$C/Ab_2C/1$
1000	3	1.465	1.257	1.031	0.884	0.387	1.613	1.414	1.50
100	2	1.072	0.287	0.297	0.313	0.081	1.336	0.579	0.53
10	1	0.211	0.000	0.000	0.000	0.000	0.473	0.032	0.00

Key to Table 3.11

Code	Diluent	Blocking Agent	Ab_2^E
B/Ab_2C	0.5% BSA	0.5% BSA	Dr. Coetzer 1:350
$B/ Ab_2S/5$	0.5% BSA	0.5% BSA	Sigma 1:500
$B/ Ab_2S/20$	0.5% BSA	0.5% BSA	Sigma 1: 2 000
$C/ Ab_2C/0.5$	0.5% casein	0.5% casein	Dr. Coetzer 1: 350
$C/ Ab_2S/20$	1% casein	1% casein	Sigma 1: 2 000
BC/ Ab_2C	0.5% BSA	1% casein	Dr. Coetzer 1: 350
$BC/ Ab_2S/20$	0.5% BSA	1% casein	Sigma 1: 2 000
$C/ Ab_2C/1$	1% casein	1% casein	Dr. Coetzer 1: 350

Results are means of duplicate absorbance values at 405 nm after subtraction of the mean of the relevant pre-immune absorbance value (C1/P) and the absorbance value for the no Ab_1 blank.

TABLE 3.14 DATA TO CHECK ANTIBODY TITRE FOR CHICKEN 1

Batch number	1 000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	25 µg/ml	No Ab_1	No Ab_2
Log [IgY]	3.0	2.7	2.4	2.0	1.4		
EVEN WEEKS						0.078	0.085
C1/P	0.497	0.341	0.240	0.147	0.099		
C1/0	0.000	0.000	0.000	0.000	0.000		
C1/2	0.187	0.081	0.022	0.000	0.000		
C1/4	1.003	0.939	0.774	0.590	0.253		
C1/6	0.700	0.553	0.440	0.254	0.003		
C1/8	1.081	0.947	0.709	0.464	0.110		
C1/10	0.746	0.664	0.493	0.269	0.045		
C1/12	0.801	0.673	0.561	0.354	0.039		
C1/14	0.841	0.603	0.550	0.355	0.019		
ODD WEEKS						0.089	0.088
C1/P	0.394	0.243	0.177	0.134	0.129		
C1/1	0.000	0.000	0.000	0.000	0.000		
C1/3	0.580	0.526	0.422	0.322	0.077		
C1/5	0.630	0.566	0.451	0.279	0.060		
C1/7	0.789	0.635	0.472	0.269	0.031		
C1/9	0.881	0.825	0.617	0.344	0.072		
C1/11	0.603	0.599	0.488	0.282	0.007		
C1/13	0.709	0.625	0.541	0.245	0.044		

Key to Table 3.14

Except for C1/P, no Ab_1 and no Ab_2, results are the means of duplicate absorbance readings at 405 nm after subtracting the mean of the relevant pre-immune absorbance value (C2/P) and the mean of the absorbance value for non-specific binding (no Ab_1). Duplicates differed by <5%.

TABLE 3.15 DATA TO CHECK ANTIBODY TITRE FOR CHICKEN 2

Batch number	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	25 µg/ml	No Ab_1	No Ab_2
Log [IgY]	3.0	2.7	2.4	2.0	1.4		
EVEN WEEKS						0.083	0.089
C2/P	0.311	0.232	0.154	0.119	0.092		
C2/0	0.092	0.067	0.000	0.000	0.000		
C2/2	0.255	0.114	0.078	0.000	0.000		
C2/4	1.569	1.053	0.643	0.342	0.083		
C2/6	1.484	1.070	0.689	0.304	0.041		
C2/8	1.698	1.330	1.034	0.648	0.168		
C2/10	1.129	0.876	0.616	0.330	0.030		
C2/12	1.487	1.284	0.961	0.616	0.080		
C2/14	1.471	1.180	0.723	0.340	0.023		
ODD WEEKS						0.079	0.072
C2/P	0.323	0.233	0.172	0.122	0.093		
C2/1	0.037	0.000	0.000	0.000	0.000		
C2/3	1.017	0.581	0.359	0.108	0.000		
C2/5	1.096	0.732	0.459	0.179	0.000		
C2/7	1.305	1.104	0.932	0.616	0.239		
C2/9	1.006	0.956	0.755	0.466	0.129		
C2/11	0.855	0.836	0.642	0.368	0.058		
C2/13	1.011	0.852	0.668	0.481	0.108		

Key to Table 3.15

Except for C2/P, no Ab_1 and no Ab_2, results are the means of duplicate absorbance readings at 405 nm after subtracting the mean of the relevant pre-immune absorbance value (C2/P) and the mean of the absorbance value for non-specific binding (no Ab_1). Duplicates differed by <5%.

TABLE 3.16 DATA TO CHECK ANTIBODY TITRE FOR RABBITS 1 AND 2

Batch number	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	25 µg/ml	No Ab_1	No Ab_2
Log [IgY]	3.0	2.7	2.4	2.0	1.4		
RABBIT 1						0.095	0.088
R1/P	0.417	0.295	0.221	0.159	0.118		
R1/4	0.227	0.118	0.071	0.000	0.000		
R1/8	0.177	0.102	0.039	0.000	0.000		
R1/12	0.784	0.697	0.549	0.291	0.042		
R1/14	0.785	0.739	0.535	0.277	0.041		
RABBIT 2						0.116	0.095
R2/P	0.516	0.394	0.296	0.211	0.147		
R2/4	0.349	0.249	0.192	0.117	0.000		
R2/8	0.772	0.635	0.443	0.158	0.000		
R2/12	0.569	0.387	0.237	0.090	0.000		
R2/14	0.413	0.297	0.227	0.042	0.000		

Key to Table 3.16

Except for R1/P and R2/P, no Ab_1 and no Ab_2, results are the means of duplicate absorbance readings at 405 nm after subtracting the mean of the relevant pre-immune absorbance value (C2/P) and the mean of the absorbance value for non-specific binding (no Ab_1). Duplicates differed by <5%.

TABLE 4.1 DATA FOR PROTEIN STANDARD CURVE

Protein Concentration (µg/ml)	Absorbance	Absorbance	Mean
50	0.061	0.068	0.065
100	0.122	0.117	0.120
200	0.241	0.238	0.240
400	0.442	0.445	0.444
600	0.619	0.626	0.623
800	0.762	0.777	0.770
1000	0.919	0.908	0.914

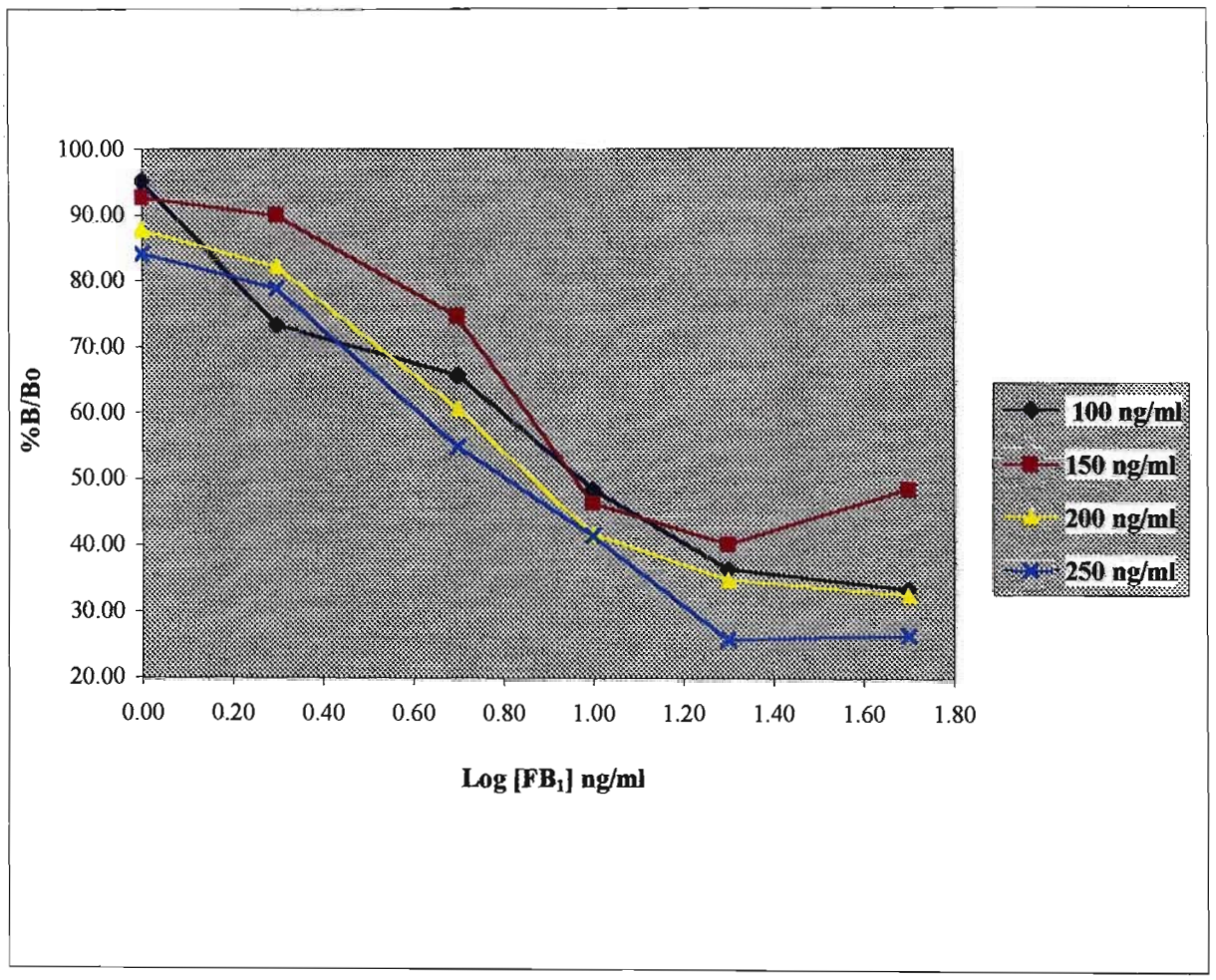


FIGURE 5.1 STANDARD CURVES AT DIFFERENT COATING CONCENTRATIONS OF FUMONISIN B_1

TABLE 5.4 DATA TO SELECT OPTIMUM CONCENTRATION OF PRIMARY ANTIBODY (CHICKEN ANTIBODIES)

	Ab_1 100 µg/ml							
FB_1 (ng/ml)	Absorbance at 405 nm		Mean	SD	CV	-No Ab_1	%B/Bo	Log[FB_1] ng/ml
0	0.475	0.474	0.475	0.001	0.15	0.377	100.00	
1	0.377	0.382	0.380	0.004	0.93	0.282	74.77	0.0
2	0.324	0.319	0.322	0.004	1.10	0.224	59.36	0.3
5	0.244	0.247	0.246	0.002	0.86	0.148	39.18	0.7
8	0.219	0.227	0.223	0.006	2.54	0.125	33.20	0.9
10	0.211	0.216	0.214	0.004	1.66	0.116	30.68	1.0
20	0.198	0.203	0.201	0.004	1.76	0.103	27.22	1.3
50	0.198	0.199	0.199	0.001	0.36	0.101	26.69	1.7
	Ab_1 200 µg/ml							
0	0.642	0.651	0.647	0.006	0.98	0.549	100.00	
1	0.551	0.571	0.561	0.014	2.52	0.463	84.41	0.0
2	0.519	0.525	0.522	0.004	0.81	0.424	77.30	0.3
5	0.391	0.389	0.390	0.001	0.36	0.292	53.24	0.7
8	0.333	0.337	0.335	0.003	0.84	0.237	43.21	0.9
10	0.306	0.317	0.312	0.008	2.50	0.214	38.92	1.0
20	0.281	0.286	0.284	0.004	1.25	0.186	33.82	1.3
50	0.255	0.265	0.260	0.007	2.72	0.162	29.54	1.7
	Ab_1 300 µg/ml							
0	0.761	0.766	0.764	0.004	0.46	0.666	100.00	
1	0.710	0.679	0.695	0.022	3.16	0.597	89.63	0.0
2	0.670	0.672	0.671	0.001	0.21	0.573	86.10	0.3
5	0.530	0.527	0.529	0.002	0.40	0.431	64.69	0.7
8	0.452	0.446	0.449	0.004	0.94	0.351	52.74	0.9
10	0.403	0.400	0.402	0.002	0.53	0.304	45.60	1.0
20	0.336	0.338	0.337	0.001	0.42	0.239	35.91	1.3
50	0.315	0.319	0.317	0.003	0.89	0.219	32.91	1.7
	Ab_1 400 µg/ml							
0	0.832	0.811	0.822	0.015	1.81	0.724	100.00	
1	0.784	0.781	0.783	0.002	0.27	0.685	94.61	0.0
2	0.756	0.766	0.761	0.007	0.93	0.663	91.64	0.3
5	0.660	0.657	0.659	0.002	0.32	0.561	77.47	0.7
8	0.579	0.575	0.577	0.003	0.49	0.479	66.21	0.9
10	0.518	0.516	0.517	0.001	0.27	0.419	57.91	1.0
20	0.422	0.428	0.425	0.004	1.00	0.327	45.20	1.3
50	0.374	0.377	0.376	0.002	0.56	0.278	38.36	1.7
	Ab_1 500 µg/ml							
0	0.948	0.979	0.964	0.022	2.28	0.866	100.00	
1	0.879	0.92	0.900	0.029	3.22	0.802	92.61	0.0
2	0.854	0.887	0.871	0.023	2.68	0.773	89.25	0.3
5	0.756	0.776	0.766	0.014	1.85	0.668	77.18	0.7
8	0.679	0.676	0.678	0.002	0.31	0.580	66.96	0.9
10	0.627	0.614	0.621	0.009	1.48	0.523	60.37	1.0
20	0.490	0.491	0.491	0.001	0.14	0.393	45.35	1.3
50	0.417	0.426	0.422	0.006	1.51	0.324	37.38	1.7

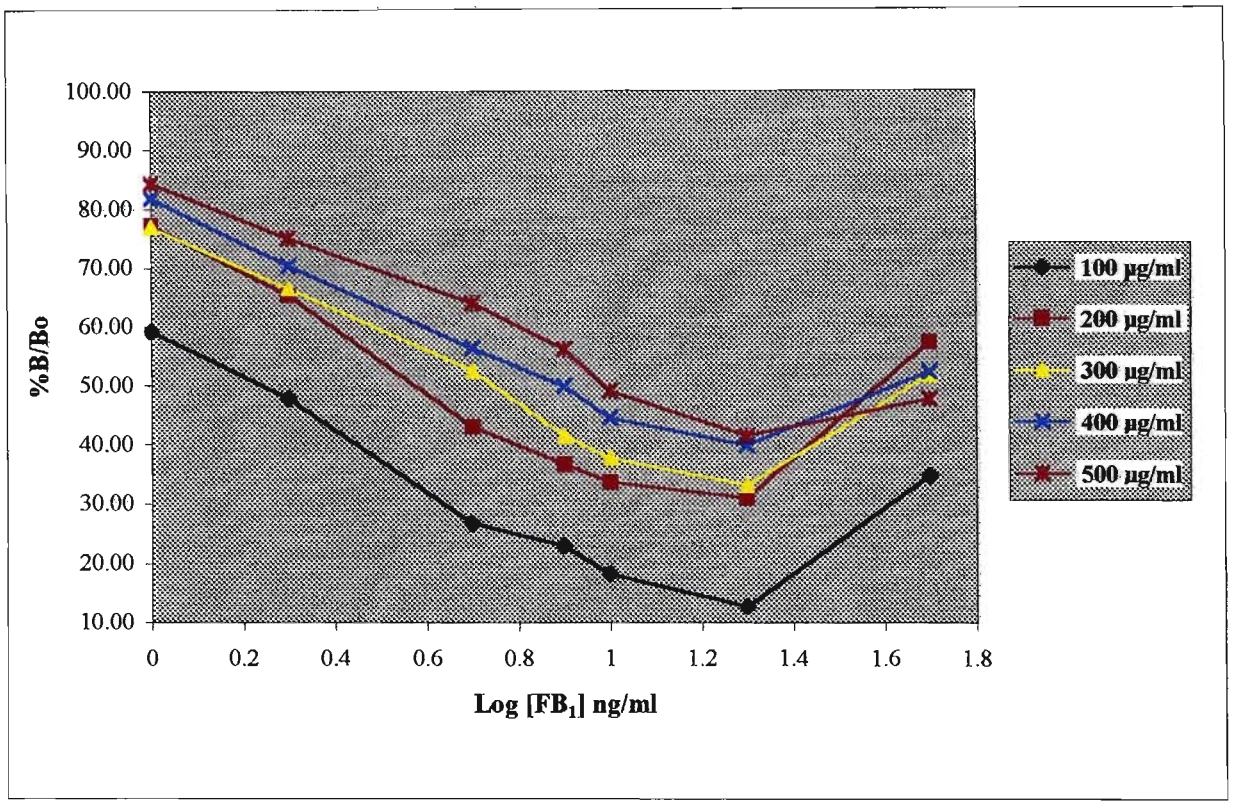


FIGURE 5. 3 OPTIMUM CONCENTRATION OF PRIMARY ANTIBODY (RABBIT ANTIBODIES)

TABLE 5.5 DATA TO SELECT OPTIMUM CONCENTRATION OF PRIMARY ANTIBODY (RABBIT ANTIBODIES)

	Ab_1 100 µg/ml							
FB_1 (ng/ml)	Absorbance at 405 nm		Mean	SD	CV	-No Ab_1	%B/Bo	Log [FB_1] ng/ml
0	0.429	0.497	0.463	0.048	10.39	0.273	100.00	
1	0.348	0.355	0.352	0.005	1.41	0.162	59.16	0.0
2	0.322	0.319	0.321	0.002	0.66	0.131	47.80	0.3
5	0.261	0.265	0.263	0.003	1.08	0.073	26.74	0.7
8	0.253	nd	0.253	0.000	0.00	0.063	23.08	0.9
10	0.248	0.232	0.240	0.011	4.71	0.050	18.32	1.0
20	0.218	0.232	0.225	0.010	4.40	0.035	12.82	1.3
50	0.285	nd	0.285	0.000	0.00	0.095	34.80	1.7
	Ab_1 200 µg/ml							
0	0.563	0.608	0.586	0.032	5.43	0.396	100.00	
1	0.457	0.534	0.496	0.054	10.99	0.306	77.24	0.0
2	0.447	0.450	0.449	0.002	0.47	0.259	65.36	0.3
5	0.361	0.360	0.361	0.001	0.20	0.171	43.11	0.7
8	0.339	0.331	0.335	0.006	1.69	0.145	36.66	0.9
10	0.323	0.324	0.324	0.001	0.22	0.134	33.75	1.0
20	0.312	0.314	0.313	0.001	0.45	0.123	31.10	1.3
50	0.418	0.415	0.417	0.002	0.51	0.227	57.27	1.7
	Ab_1 300 µg/ml							
0	0.705	0.699	0.702	0.004	0.60	0.512	100.00	
1	0.585	0.583	0.584	0.001	0.24	0.394	76.95	0.0
2	0.543	0.518	0.531	0.018	3.33	0.341	66.50	0.3
5	0.453	0.465	0.459	0.008	1.85	0.269	52.54	0.7
8	0.403	0.402	0.403	0.001	0.18	0.213	41.50	0.9
10	0.391	0.375	0.383	0.011	2.95	0.193	37.70	1.0
20	0.365	0.355	0.360	0.007	1.96	0.170	33.20	1.3
50	0.467	0.443	0.455	0.017	3.73	0.265	51.76	1.7
	Ab_1 400 µg/ml							
0	0.778	0.789	0.784	0.008	0.99	0.594	100.00	
1	0.666	0.685	0.676	0.013	1.99	0.486	81.80	0.0
2	0.591	0.625	0.608	0.024	3.95	0.418	70.43	0.3
5	0.515	0.534	0.525	0.013	2.56	0.335	56.36	0.7
8	0.486	0.487	0.487	0.001	0.15	0.297	49.96	0.9
10	0.451	0.458	0.455	0.005	1.09	0.265	44.57	1.0
20	0.429	0.425	0.427	0.003	0.66	0.237	39.93	1.3
50	0.507	0.495	0.501	0.008	1.69	0.311	52.40	1.7
	Ab_1 500 µg/ml							
0	0.809	0.824	0.817	0.011	1.30	0.627	100.00	
1	0.717	0.719	0.718	0.001	0.20	0.528	84.28	0.0
2	0.656	0.664	0.660	0.006	0.86	0.470	75.02	0.3
5	0.581	0.601	0.591	0.014	2.39	0.401	64.01	0.7
8	0.545	0.539	0.542	0.004	0.78	0.352	56.19	0.9
10	0.513	0.482	0.498	0.022	4.41	0.308	49.08	1.0
20	nd	0.450	0.450	0.000	0.00	0.260	41.50	1.3
50	0.507	0.471	0.489	0.025	5.21	0.299	47.73	1.7

TABLE 5.6 DATA TO COMPARE INCUBATION TIMES FOR COMPETITION STEP IN COMPETITIVE INDIRECT ELISA METHOD

HR 1			Mean	SD	CV	-No Ab_1	%B/Bo	Log[FB_1] ng/ml
FB_1 (ng/ml)								
0	0.526	0.493	0.510	0.023	4.58	0.422	100.00	
1	0.462	0.446	0.454	0.011	2.49	0.366	89.11	0.0
2	0.446	0.428	0.437	0.013	2.91	0.349	85.77	0.3
5	0.397	0.393	0.395	0.003	0.72	0.307	77.53	0.7
10	0.288	0.327	0.308	0.028	8.97	0.220	60.35	1.0
20	0.251	0.263	0.257	0.008	3.30	0.169	50.44	1.3
HR 2								
FB_1 (ng/ml)								
0	0.626	0.587	0.607	0.028	4.55	4.449	100.00	
1	0.497	0.452	0.475	0.032	6.71	6.608	78.24	0.0
2	0.458	0.406	0.432	0.037	8.51	8.413	71.23	0.3
5	0.374	0.361	0.368	0.009	2.50	2.403	60.59	0.7
10	0.326	0.306	0.316	0.014	4.48	4.377	52.10	1.0
20	0.254	0.261	0.258	0.005	1.92	1.824	42.46	1.3

TABLE 5.7 DATA TO SELECT OPTIMUM DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (CHICKEN ANTIBODIES)

	Dilution of Ab_2^E						1:2 000		
FB_1 (ng/ml)	Absorbance at 405 nm			Mean	SD	CV	-No Ab_1	%B/Bo	Log[FB_1] ng/ml
0	1.871	1.828	1.827	1.842	0.025	1.36	1.734	100.00	
1	1.743	1.811	nd	1.777	0.048	2.71	1.669	96.25	0.00
2	1.700	1.589	nd	1.645	0.078	4.77	1.537	82.44	0.30
3	1.549	1.527	1.405	1.494	0.078	5.19	1.386	79.91	0.48
5	1.322	1.231	1.226	1.260	0.054	4.29	1.152	66.42	0.70
8	0.928	0.869	0.897	0.898	0.030	3.29	0.790	45.56	0.90
10	0.867	0.77	0.844	0.827	0.051	6.13	0.719	41.46	1.00
20	0.625	0.61	0.645	0.627	0.018	2.80	0.519	29.91	1.30
	Dilution of Ab_2^E						1:3 000		
0	1.711	1.603	1.555	1.623	0.080	4.92	1.525	100.00	
1	1.581	1.489	1.462	1.511	0.062	4.13	1.413	92.63	0.00
2	1.428	1.401	1.417	1.415	0.014	0.96	1.317	86.38	0.30
3	1.382	1.348	1.293	1.341	0.045	3.35	1.243	81.51	0.48
5	1.185	1.169	1.169	1.174	0.009	0.79	1.076	70.58	0.70
8	0.843	0.821	0.915	0.860	0.049	5.72	0.762	49.97	0.90
10	0.845	0.840	nd	0.843	0.004	0.42	0.745	48.85	1.00
20	0.601	0.608	0.581	0.597	0.014	2.35	0.499	32.70	1.30
	Dilution of Ab_2^E						1:4 000		
0	1.650	1.565	1.614	1.610	0.043	2.65	1.515	100.00	
1	1.518	1.491	1.525	1.511	0.018	1.19	1.416	93.51	0.00
2	1.455	1.375	1.420	1.417	0.040	2.83	1.322	87.26	0.30
3	1.307	1.346	1.312	1.322	0.021	1.61	1.227	80.99	0.48
5	1.219	1.116	1.145	1.160	0.053	4.58	1.065	70.31	0.70
8	0.846	0.865	nd	0.856	0.013	1.57	0.761	50.21	0.90
10	nd	0.771	0.785	0.778	0.010	1.27	0.683	45.09	1.00
20	0.608	0.587	nd	0.598	0.015	2.49	0.503	33.18	1.30
	Dilution of Ab_2^E						1:5 000		
0	1.263	1.059	1.114	1.145	0.106	9.22	1.057	100.00	
1	1.191	1.159	1.089	1.146	0.052	4.55	1.058	100.09	0.00
2	1.114	1.079	nd	1.097	0.025	2.26	1.009	95.38	0.30
3	1.059	1.010	0.999	1.023	0.032	3.12	0.935	88.40	0.48
5	0.841	0.848	0.791	0.827	0.031	3.76	0.739	69.86	0.70
8	0.651	0.592	0.599	0.614	0.032	5.25	0.526	49.75	0.90
10	0.565	0.542	0.570	0.559	0.015	2.67	0.471	44.55	1.00
20	0.414	0.44	0.431	0.428	0.013	3.08	0.340	32.19	1.30

nd – no data

TABLE 5.8 DATA TO SELECT VOLUME AND DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (CHICKEN ANTIBODIES)

	Dilution and volume of Ab_2^E						100 µl, 1:2 000		
FB_1 (ng/ml)	Absorbance at 405 nm			Mean	SD	CV	-No Ab_1	%B/Bo	Log[FB_1] ng/ml
0	nd	1.041	1.031	1.036	0.007	0.68	0.850	100.00	
1	1.018	0.861	0.883	0.921	0.085	9.23	0.735	86.47	0.0
2	nd	0.831	0.876	0.854	0.032	3.73	0.668	78.53	0.3
5	0.763	0.698	0.722	0.728	0.033	4.52	0.542	63.73	0.7
10	nd	0.485	0.533	0.509	0.034	6.67	0.323	38.00	1.0
20	nd	0.450	0.453	0.452	0.002	0.47	0.266	31.24	1.3
	Dilution and volume of Ab_2^E						120 µl, 1:2 000		
0	1.074	0.973	1.072	1.040	0.058	5.55	0.839	100.00	
1	0.899	0.893	0.917	0.903	0.012	1.38	0.702	83.70	0.0
2	0.820	nd	0.799	0.810	0.015	1.83	0.609	72.56	0.3
5	0.739	0.729	0.658	0.709	0.044	6.23	0.508	60.53	0.7
10	0.544	0.576	0.557	0.559	0.016	2.88	0.358	42.69	1.0
20	0.514	0.471	0.526	0.504	0.029	5.74	0.303	36.09	1.3
	Dilution and volume of Ab_2^E						100 µl, 1:4 000		
0	0.838	0.816	0.804	0.819	0.017	2.10	0.708	100.00	
1	0.725	0.732	0.751	0.736	0.013	1.83	0.625	88.24	0.0
2	0.715	0.675	0.741	0.710	0.033	4.68	0.599	84.61	0.3
5	0.597	0.576	0.565	0.579	0.016	2.81	0.468	66.12	0.7
10	0.453	0.433	0.432	0.439	0.012	2.70	0.328	46.35	1.0
20	0.366	0.349	0.354	0.356	0.009	2.45	0.245	34.64	1.3
	Dilution and volume of Ab_2^E						120 µl, 1:4 000		
0	0.790	0.767	0.816	0.791	0.025	3.10	0.682	100.00	
1	0.736	0.705	0.740	0.727	0.019	2.64	0.618	90.62	0.0
2	0.670	0.673	0.674	0.672	0.002	0.31	0.563	82.60	0.3
5	0.571	0.523	0.568	0.554	0.027	4.85	0.445	65.25	0.7
10	0.406	nd	0.412	0.409	0.004	1.04	0.300	43.99	1.0
20	0.364	0.347	0.365	0.359	0.010	2.82	0.250	36.61	1.3

nd – no data

TABLE 5.9 DATA TO SELECT OPTIMUM DILUTION OF SECOND ENZYME-LABELLED ANTIBODY (RABBIT ANTIBODIES)

	Dilution of Ab_2^E					1:2 000			
FB_1 (ng/ml)	Absorbance at 405 nm			Mean	SD	CV	-No Ab_1	%B/Bo	Log [FB_1] ng/ml
0	1.322	1.399	1.344	1.355	0.040	2.93	1.193	100.00	
1	1.133	1.185	1.294	1.204	0.082	6.82	1.042	87.34	0.00
2	nd	1.175	1.114	1.145	0.043	3.77	0.983	82.36	0.30
3	0.942	0.971	1.006	0.973	0.032	3.29	0.811	67.98	0.48
5	0.930	0.924	0.859	0.904	0.039	4.35	0.742	62.22	0.70
8	0.853	0.753	0.830	0.812	0.052	6.45	0.650	54.48	0.90
10	0.683	0.617	0.700	0.667	0.044	6.58	0.505	42.30	1.00
20	0.629	0.548	0.560	0.579	0.044	7.55	0.417	34.95	1.30
	Dilution of Ab_2^E					1:3 000			
0	1.229	1.293	1.271	1.264	0.033	2.57	1.155	100.00	
1	1.064	1.104	1.044	1.071	0.031	2.85	0.962	83.24	0.00
2	0.965	0.990	0.954	0.970	0.018	1.90	0.861	74.50	0.30
3	nd	0.847	0.808	0.828	0.028	3.33	0.719	62.19	0.48
5	nd	0.765	0.729	0.747	0.025	3.41	0.638	55.22	0.70
8	0.635	0.654	0.610	0.633	0.022	3.49	0.524	45.35	0.90
10	0.535	0.548	0.557	0.547	0.011	2.02	0.438	37.88	1.00
20	0.549	0.556	0.520	0.542	0.019	3.52	0.433	37.45	1.30
	Dilution of Ab_2^E					1:4 000			
0	1.223	1.146	1.195	1.188	0.039	3.28	1.052	100.00	
1	1.011	0.976	0.988	0.992	0.018	1.79	0.856	81.34	0.00
2	0.919	0.859	0.875	0.884	0.031	3.51	0.748	71.13	0.30
3	0.780	nd	0.815	0.798	0.025	3.10	0.662	62.88	0.48
5	0.690	0.650	0.692	0.677	0.024	3.50	0.541	51.46	0.70
8	0.580	0.541	0.557	0.559	0.020	3.50	0.423	40.24	0.90
10	0.533	0.512	0.563	0.536	0.026	4.78	0.400	38.02	1.00
20	0.471	0.428	0.453	0.451	0.022	4.79	0.315	29.91	1.30
	Dilution of Ab_2^E					1:5 000			
0	0.966	nd	0.988	0.977	0.016	1.59	0.860	100.00	
1	0.823	0.806	0.809	0.813	0.009	1.12	0.696	80.89	0.00
2	0.767	0.728	0.754	0.750	0.020	2.65	0.633	73.57	0.30
3	0.721	0.701	0.705	0.709	0.011	1.49	0.592	68.84	0.48
5	0.590	0.619	0.602	0.604	0.015	2.41	0.487	56.59	0.70
8	0.537	0.537	0.538	0.537	0.001	0.11	0.420	48.88	0.90
10	0.450	0.451	0.441	0.447	0.006	1.23	0.330	38.41	1.00
20	0.408	0.394	0.381	0.394	0.014	3.42	0.277	32.25	1.30

nd – no data

TABLE 5.14 DATA FOR STANDARD CURVE (CHICKEN ANTIBODIES)

	Absorbance at 405 nm			Mean	SD	CV	-No Ab_1
No Ab_1	0.129	0.128	0.129	0.129	0.001	0.78	
FB_1 (ng/ml)							
0	1.473	1.518	1.649	1.547	0.091	5.91	1.418
1	1.498	1.444	1.445	1.462	0.031	2.11	1.333
2	1.380	1.354	1.323	1.352	0.029	2.11	1.223
3	1.269	1.272	1.263	1.268	0.005	0.36	1.139
5	1.132	1.166	1.118	1.139	0.025	2.17	1.010
8	0.933	0.868	0.934	0.912	0.038	4.15	0.783
10	0.730	0.764	0.780	0.758	0.026	3.37	0.629
20	0.616	0.610	0.609	0.612	0.004	0.62	0.483

TABLE 5.15 DATA FOR STANDARD CURVE (RABBIT ANTIBODIES)

	Absorbance at 405 nm			Mean	SD	CV	-No Ab_1
No Ab_1	0.107	0.110	0.099	0.105	0.006	5.40	
FB_1 (ng/ml)							
0	1.223	1.146	1.195	1.188	0.039	3.28	1.083
1	1.011	0.976	0.988	0.992	0.018	1.79	0.887
2	0.919	0.859	0.875	0.884	0.031	3.51	0.779
3	0.780	nd	0.815	0.798	0.025	3.10	0.693
5	0.690	0.650	0.692	0.677	0.024	3.50	0.572
8	0.580	0.541	0.557	0.559	0.020	3.50	0.454
10	0.533	0.512	0.563	0.536	0.026	4.78	0.431
20	0.471	0.428	0.453	0.451	0.022	4.79	0.346

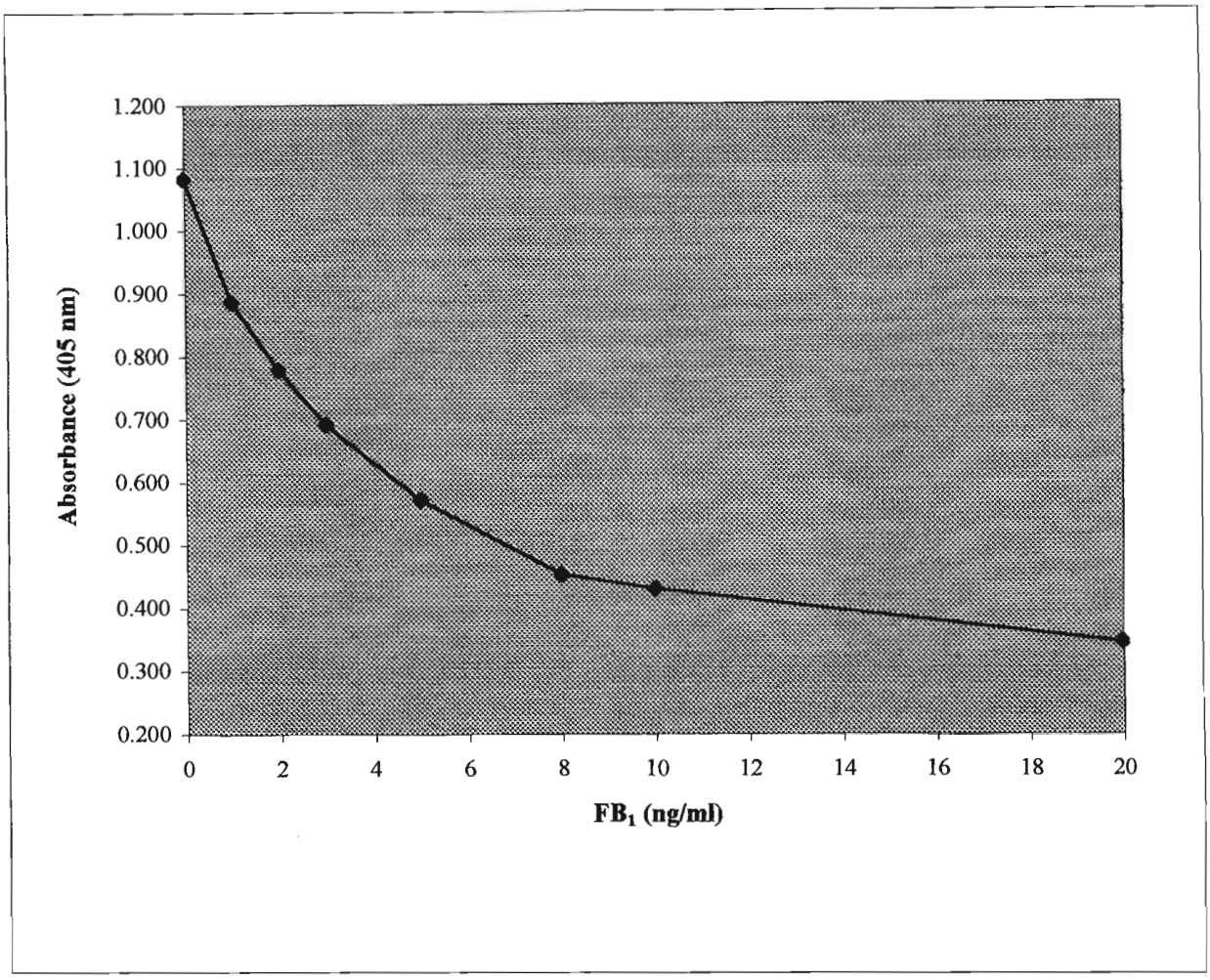


FIGURE 5.9 STANDARD CURVE FOR FUMONISIN B_1 (RABBIT ANTIBODIES)

TABLE 5.16 DATA FOR RANGE OF STANDARD CURVE

	Absorbance at 405 nm			Mean	SD	CV	-No Ab_1
No Ab_1	0.110	0.105	nd	0.108	0.004	3.29	
FB_1 (ng/ml)							
0	nd	1.373	1.437	1.405	0.045	3.22	1.297
1	1.294	1.236	1.267	1.266	0.029	2.29	1.158
2	1.146	1.150	1.144	1.147	0.003	0.27	1.039
3	1.074	1.118	1.041	1.078	0.039	3.58	0.970
5	0.951	0.987	0.995	0.978	0.023	2.40	0.870
8	0.783	0.795	0.792	0.790	0.006	0.79	0.682
10	0.594	0.584	0.579	0.586	0.008	1.30	0.478
20	0.460	0.456	0.461	0.459	0.003	0.58	0.351

TABLE 5.18 DATA FOR COMPETITIVE INDIRECT ELISA STANDARD CURVES FOR CROSS-REACTIVITY (CHICKEN ANTIBODIES)

		%B/Bo			
Concentration (ng/ml)	Log of Concentration	FB_1	FB_2	FB_3	So
2	0.30	79.11	93.76	88.03	98.41
3	0.48	75.68	78.80	83.35	102.70
5	0.70	67.20	71.81	77.61	98.37
8	0.90	57.00	58.80	72.82	94.31
10	1.00	46.84	47.10	63.22	92.82
20	1.30	39.84	38.72	53.14	92.75
40	1.60	nd	37.55	46.17	95.22

nd – no data

TABLE 5.19 DATA FOR COMPETITIVE INDIRECT ELISA STANDARD CURVES FOR CROSS-REACTIVITY (RABBIT ANTIBODIES)

		%B/Bo			
Concentration (ng/ml)	Log of Concentration	FB_1	FB_2	FB_3	So
2	0.30	85.02	88.46	89.60	99.80
3	0.48	81.21	79.17	86.58	99.95
5	0.70	72.46	74.21	79.62	97.96
8	0.90	61.43	65.09	76.87	98.97
10	1.00	55.61	58.88	70.42	100.88
20	1.30	42.08	44.65	56.74	92.08
40	1.60	nd	37.65	48.37	91.30

nd – no data

TABLE 6.13 CALCIUM AND MAGNESIUM RESULTS ON PATIENTS WITH OESOPHAGEAL CANCER

Patient No.	Ca (mmol/l)	Mg (mmol/l)	Appearance	Type	Patient No.	Ca (mmol/l)	Mg (mmol/l)	Appearance	Type
106	2.40	0.76		P	170	2.85	1.00	+ H	P
108	2.43	0.82		P	171	2.85	1.18		S
110	2.02	0.76		S	172	2.61	1.02		P
124	2.81	1.09	+ I	S	173	2.47	1.00	+ H	P
135	1.55	1.08	++++ H	S	175	3.13	0.96		P
139	2.73	1.10	+++ H	P	179	2.42	0.79	+ H	P
140	2.68	1.07	+ H	P	180	2.68	0.94		S
146	2.66	0.95		S	184	2.85	0.94		P
148	2.34	0.94	+ H	S	190	2.77	0.96	± H	S
149	2.75	0.95		P	195	2.72	0.91		P
152	2.47	0.95		S	202	2.74	1.05		S
155	2.55	1.03		P	215	2.31	1.12	+++ H	P
157	2.65	0.91	± H	S	216	2.49	0.88	++ H	P
159	2.79	0.96		S	217	3.22	1.03		P
164	2.41	0.87		P	219	2.81	0.94	± H	P
165	2.59	0.84		P	220	2.34	0.88		S
166	2.57	0.88		P	225	2.42	0.93	+ H	S
168	2.62	1.03		P	237	2.64	1.00		S
169	2.33	0.84	± H	S					

Key to Table 6.13
I – icteric; H – haemolysed; S – serum; P – heparin plasma

TABLE 6.14 CALCIUM AND MAGNESIUM RESULTS ON CONTROL PATIENTS

Patient No.	Ca (mmol/l)	Mg (mmol/l)	Appearance	Type	Patient No.	Ca (mmol/l)	Mg (mmol/l)	Appearance	Type
156	2.53	0.88		S	197	2.41	0.99	± H	S
158	2.34	0.85	+ H	S	203	2.61	1.04	+ H	P
160	2.56	0.92	± H	S	205	2.59	0.98	+ H	S
177	2.48	0.89		S	206	2.19	1.23	+++ H	P
178	2.11	0.80		S	210	2.73	0.94	+ H	S
182	2.57	0.92	± H	S	224	2.80	0.91		S
183	2.55	0.97		S	226	2.72	0.95		S
191	2.62	0.98	± H	S	227	2.61	0.97	± H	S
192	2.52	0.94	± H	S					

Key to Table 6.14
I – icteric; H – haemolysed; S – serum; P – heparin plasma

TABLE 6.15 CALCIUM AND MAGNESIUM RESULTS ON PATIENTS WITH OTHER TYPES OF CANCER

Patient No.	Ca (mmol/l)	Mg (mmol/l)	Appearance	Type	Patient No.	Ca (mmol/l)	Mg (mmol/l)	Appearance	Type
Breast					**Cervix**				
136	2.49	1.06	+ H	S	151	2.38	0.91	± H	S
150	2.96	0.99		S	162	2.77	0.92		P
154	2.78	1.08	++ H	S	193	2.68	0.95	+ H	P
181	2.50	0.92		S	236	2.71	1.08		P
204	2.63	0.93		S					
207	2.38	0.85	± H	S					
208	2.16	0.81	± H	S					
211	2.64	0.93		P					
Pancreas					**Prostate**				
153	2.33	1.05	+++ I	P	134	2.83	0.97	+ H	S
Rectum					**Stomach**				
209	2.44	0.97	+ I	S	137	2.56	0.91	± H	S
223	2.65	1.00		P	189	2.63	0.96		S
Thyroid									
221	2.65	0.96		S					

Key to Table 6.15
I – icteric; H – haemolysed; S – serum; P – heparin plasma

APPENDIX 2

Buffers/Solutions

2.1 0.1M Sodium carbonate buffer, pH 8.5

1.06 g anhydrous sodium carbonate (Na_2CO_3) was dissolved in 100 ml distilled water and 0.84 g anhydrous sodium hydrogen carbonate ($NaHCO_3$) was dissolved in 100 ml distilled water. The buffer was freshly prepared by mixing 10 ml Na_2CO_3 and 85 ml $NaHCO_3$. The pH was adjusted using 0.1M HCl and the volume was made up to 100 ml.

2.2 Phosphate buffered saline (PBS), pH 7.2 (10x)

80 g NaCl, 2 g KCl, 11.5 g disodium hydrogen phosphate (Na_2HPO_4) and 2 g potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 1 000 ml distilled water and stored at 4°C for maximum of three weeks. Daily a 1:10 dilution was made and adjusted to the correct pH using 0.1M HCl or 0.1M NaOH.

2.3 0.1M (100mM) Sodium phosphate buffer, pH 7.6, with 0.02% sodium azide

15.6 g sodium dihydrogen phosphate ($Na_2HPO_4.2H_2O$) and 0.2 g sodium azide (NaN_3) was dissolved in ± 850 ml distilled water, the pH adjusted using 2M NaOH and made up to 1 000 ml and stored at 4°C.

2.4 Borate buffered saline, pH 8.6

0.54 g boric acid, 0.548 g NaCl, 0.175 g NaOH and 0.155 ml 37% HCl were dissolved in 200 ml distilled water, the pH adjusted using 0.1M HCl or 0.1M NaOH, the volume made up to 250 ml and stored at 4°C.

2.5 0.1M Carbonate buffer, pH 9.6

1.06 g sodium bicarbonate (Na_2CO_3) was dissolved in 100 ml distilled water and 0.84 g sodium hydrogen carbonate ($NaHCO_3$) was dissolved in 100 ml distilled water. The buffer was freshly prepared by adding $NaHCO_3$ to 29.3 ml Na_2CO_3 until the volume was approximately 95 ml, adjusted to the correct pH using 0.1M HCl and made up to 100 ml.

2.6 0.15M Citrate-phosphate buffer, pH 5.0

2.1 g citric acid. H_2O was dissolved in 100 ml distilled water; 3.56 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) was dissolved in 100 ml distilled water. The citric acid was titrated with the sodium phosphate to pH 5.0 and stored at 4°C for a maximum of 2 weeks.

2.7 Stopping buffer: 0.1% sodium azide in citrate-phosphate buffer

0.1 g NaN_3 dissolved in 100 ml citrate phosphate buffer and stored at 4°C.
Sodium azide is a toxic chemical and must be handles with care.

2.8 1mM Sodium acetate buffer, pH 4.4

1 ml glacial acetic acid was added to about 900 ml distilled water and adjusted to the correct pH using 0.1M NaOH (0.4 g in 100 ml distilled water). The solution was made up to 1 litre and stored at room temperature.

2.9 200mM Sodium carbonate buffer, pH 9.5

1.68 g sodium hydrogen carbonate ($NaHCO_3$) was dissolved in 100 ml distilled water and 18.6 ml was pipetted into a beaker. 2.12 g Sodium carbonate (Na_2CO_3) was dissolved in 100 ml distilled water and was gradually added to the first solution until the pH was 9.5.

2.10 0.1M Carbonate/bicarbonate buffer, pH 9.6

0.795 g sodium carbonate (Na_2CO_3) was dissolved in 500 ml distilled water; 1.465 g sodium bicarbonate ($NaHCO_3$) was dissolved in 500 ml distilled water. The two solutions were combined and the pH checked and adjusted if necessary. The buffer was stored at 4°C.

2.11 0.1M Sodium phoshate buffer, pH 3.35

1.56g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) was dissolved in 100 ml deionised water, the pH was adjusted to 3.35 with o-phosphoric acid (about 2 drops) and the buffer was filtered under vacuum (Millipore system) through a 0.45µm, 47 mm nylon filter (Lida, Chrom Tech Inc.).

2.12 0.1M Borate buffer, pH 10.5

3.8 g sodium borate ($Na_2B_4O_7.10H_2O$) was dissolved in 80 ml deionised water, the pH adjusted using 1M KOH and made up to 100 ml.

2.13 o-Phthaldialdehyde (OPA)

21.2 mg OPA were dissolved in 600 µl methanol to which was added 20 µl mercaptoethanol and 15 ml 0.1M borate buffer (pH 10.5) . The reagent was stored for a maximum of a week in the dark at 4°C. Immediately before use the OPA was filtered through a 22 µm filter (Lida, Chrom Tech Inc.).

REFERENCES

Abbas, H.K., Boyette, C.D., Hoagland, R.E. and Vesonder, R.F. (1991) Bioherbicidal potential of *Fusarium moniliforme* and its phytotoxin, fumonisin. *Weed Sci.* **39**: 673-677.

Abbas, H.K., Paul, R.N., Boyette, C.D., Duke, S.O. and Vesonder, R.F. (1992A) Physiological and ultrastructural effects of fumonisin on jimsonweed leaves. *Can. J. Bot.* **70**: 1824-1833.

Abbas, H.K., Vesonder, R.F., Boyette, C.D., Hoagland, R.E. and Krick, T. (1992B) Production of fumonisins by *Fusarium moniliforme* cultures isolated from jimsonweed in Mississippi. *J.Phytopath.* **136**: 199-203.

Abbas, H.K., Gelderblom, W.C.A., Cawood, M.E. and Shier, W.T. (1993) Biological activities of fumonisins, mycotoxins from *Fusarium moniliforme*, in jimsonweed (*Datura stramonium* L.) and mammalian cell cultures. *Toxicon* **31(3)**: 345-353.

Abouzied, M. and Pestka, J.J. (1994) Simultaneous screening of aflatoxin B_1, and zearalenone by line immunoblot: a computer-assisted multianalyte assay system. *J. AOAC Int.* **77(2)**: 495-500.

Abouzied, M.M., Askegard, S.D., Bird, C.B. and Miller, B.M. (1996) Fumonisins Veratox ® A new rapid quantitative ELISA for determination of fumonisin in food and feed. *In*: Jackson, L., de Vries, J.W., Bullerman, L.B. (eds.) *Fumonisins in Food.* (Advances in Experimental Biology vol. 392). New York: Plenum Press pp. 135-144.

Abramson, D., Usleber, E. and Märtlbauer, E. (1995) An indirect enzyme immunoassay for the mycotoxin citrinin. *Appl. Environ. Microbiol.* **61(5)**: 2007-2009.

Ackermann, T. (1991) Fast thin-layer chromataographic systems for fumonisin isolation and identification. *J. Appl. Toxicol.* **11(6)** : 451.

Akiyama, H., Miyahara, M., Toyoda, M. and Saito, Y. (1995) Liquid chromatographic determination of fumonisins B_1 and B_2 in corn by precolumn derivatization with 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F). *J. Food Hyg. Soc. Jpn.* **36(1)**: 77-81.

Akiyama, H., Uraroongroj, M., Miyahara, M., Goda, Y. and Toyoda, M. (1998) Quantitation of fumonisins in corn by HPLC with *o*-phthalaldehyde postcolumn derivatization and their identification by LC/MS. *Mycopathologia* **140**: 157-161.

Alberts, J.F., Gelderblom, W.C.A., Vleggaar, R., Marasas, W.F.O. and Rheeder, J.P. (1993) Production of [^{14}C]fumonisin B_1 by *Fusarium moniliforme* MRC 826 in corn cultures. *Appl. Environ. Microbiol.* **59(8)**: 2673-2677.

Azcona, J.I., Abouzied, M.M. and Pestka, J.J. (1990) Detection of zearalenone by tandem immunoaffinity-enzyme-linked immunosorbent assay and its application to milk. *J. Food Prot.* **53(7)**: 577-580.

Azcona-Olivera, J.I., Abouzied, M.M., Plattner, R.D., Norred, W.P. and Pestka, J.J. (1992A) Generation of antibodies reactive with fumonisins B_1, B_2, and B_3 by using cholera toxin as the carrier-adjuvant. *Appl. Environ. Microbiol.* **58 (1)**: 169-173.

Azcona-Olivera, J.I., Abouzied, M.M., Plattner, R.D. and Pestka, J.J. (1992B) Production of monoclonal antibodies to the mycotoxins fumonisins B_1, B_2, and B_3. *J. Agric. Food Chem.* **40**: 531-534.

Bacon, C.W. and Williamson, J.W. (1992) Interactions of *Fusarium moniliforme*, its metabolites and bacteria with corn. *Mycopathologia* **117**: 65-71.

Bacon, C.W., Porter, J.K. and Norred, W.P. (1995) Toxic interaction of fumonisin B_1 and fusaric acid measured by injection into fertile chicken egg. *Mycopathologia* **129**: 29-35.

Badiali, L., Abou-Youssef, M.H., Radwan, A.I., Hamdy, F.M. and Hildebrandt, P.K. (1968) Moldy corn poisoning as the major cause of an encephalomalacia syndrome in Egyptian Equidae. *Am. J. Vet. Res.* **29(10)**: 2029-2035.

Badria, F.A., Abbas, H.K. and Shier, W.T. (1995) Chemical transformation of hydrolysed fumonisin B_1 to hydrolysed fumonisin B_2. *J. Agric. Food Chem.* **43**: 1989-1992.

Badria, F.A., Li S. and Shier, W.T. (1996) Fumonisins as potential causes of kidney disease. *J. Toxicol.* **15(3)**: 273-292.

Bane, D.P., Neumann, E.J., Hall, W.F., Harlin, K.S. and Slife, R.L.N. (1992) Relationship between fumonisin contamination of feed and mystery swine disease. *Mycopathologia* **117**: 121-124.

Barna-Vetró, I., Solti, L., Téren, J., Gyöngyösi, Á, Szabó, E. and Wölfling, A. (1996) Sensitive ELISA test for determination of ochratoxin A. *J. Agric. Food Chem.* **44**: 4071-4074.

Becker, B.A, Pace, L., Rottinghaus, G.E., Shelby, R., Misfeldt, M. and Ross, P.F. (1995) Effects of feeding fumonisin B_1 in lactating sows and their suckling pigs. *Am.J. Vet. Res.* **56(9)**: 1253-1258.

Beier, R.C., Elissalde, M.H. and Stanker, L.H. (1995) Calculated three dimensional structures of the fumonisin B_{1-4} mycotoxins. *Bull. Environ. Contam. Toxicol.* **54**: 479-487.

Beier, R.C. and Stanker, L.H. (1997) Molecular models for the stereochemical structures of fumonisin B_1 and B_2. *Arch. Environ. Contam. Toxicol.* **33**: 1-8.

Bennett, G.A. and Richard, J. L. (1994) Liquid chromatographic method for analysis of the naphthalene dicarboxaldehyde derivative of fumonisins. *J. AOAC Int.* **77(2)**: 501-506.

Bennett, G.A. and Nelsen, C. (1994) Enzyme-linked immunosorbent assay for detection of zearalenone in corn, wheat, and pig feed: collaborative study. *J. AOAC Int.* **77(6)**: 1500-1509.

Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C.P., Horak, R.M., Marasas, W.F.O., Spiteller, G. and Vleggaar, R. (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *J. Chem. Soc. Chem. Commun.* **11**: 743-745.

Bhat, R.V., Shetty, P.H., Amruth, R.P. and Sudershan, R.V. (1997) A foodborne disease outbreak due to the consumption of moldy sorghum and maize containing fumonisin mycotoxins. *Clin. Toxicol.* **35(3)**: 249-255.

Blackwell, B.A., Miller, J.D. and Savard, M.E. (1994) Production of carbon 14-labeled fumonisin in liquid culture. *J. Assoc. Off. Anal. Chem. Int.* **77(2)**: 506-511.

Blackwell, B.A., Gilliam, J.T., Savard, M.E., Miller, J.D. and Duvick, J.P. (1999) Oxidative deamination of hydrolyzed fumonisin B (1) (AP(1))) by cultures of *Exophiala spinifera*. *Nat.Toxins* **7(1)**: 31-38.

Bohinski, R.C. (1987) *Modern Concepts in Biochemistry* 5th Edition. Boston: Allyn and Bacon, Inc. pp.233-651.

Bondy, G., Suzuki, C., Barker,M., Armstrong, C., Fernie, S., Hierlihy, L., Rowsell,P. and Mueller, R. (1995) Toxicity of fumonisin B_1 administered intraperitoneally to male Sprague-Dawley rats. *Food Chem. Toxicol.* **33(8)**: 653-665.

Bondy, G.S., Suzuki, C.A.M., Fernie, S.M., Armstrong, C.L., Hierlihy, S.L., Savard, M.E. and Barker, M.G. (1997) Toxicity of fumonisin B_1 to B6C3F_1 mice: a 14-day gavage study. *Food Chem. Toxicol.* **35**: 981-989.

Bottalico, A., Logrieco, A., Ritien, A., Moretti, A., Randazzo, G. and Corda, P. (1995) Beauvericin and fumonisin B_1 in preharvest *Fusarium moniliforme* maize ear rot in Sardinia. *Food Addit. Contam.* **12(4)**: 599-607.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Branham, B.E. and Plattner, R.D. (1993A) Isolation and characterization of a new fumonisin from liquid cultures of *Fusarium moniliforme* . *J. Nat. Prod.* **56(9)**: 1630-1633.

Branham, B.E. and Plattner, R.D. (1993B) Alanine is a precursor in the biosynthesis of fumonisin B_1 by *Fusarium moniliforme*. *Mycopathologia* **124**: 99-104.

Briand, J.P., Muller, S. and Van Regenmortel, M.H.V. (1985) Synthetic peptides as antigens: pitfalls of conjugation methods. *J. Immunol. Methods* **78**: 59-69.

Brown, T.P., Rottinghaus, G.E. and Williams, M.E. (1992) Fumonisin mycotoxicosis in broilers: performance and pathology. *Avian Dis.* **36**: 450-454.

Bucci, T.J., Hansen, D.K. and LaBorde, J.B. (1996) Leukoencephalomalacia and hemorrhage in the brain of rabbits gavaged with mycotoxin fumonisin B_1. *Nat. Toxins* **4**: 51-52.

Buck, W.B., Haliburton, J.C., Thilsted, J.P., Lock, T.F. and Vesonder, R.F. (1979) Equine leucoencephalomalacia: comparative pathology of naturally occurring and experimental cases. *Am. Ass. Vet. Lab. Diagn.* **22nd Annual Proceedings**: 239-258.

Burrell, R.J.W. (1957) Oesophageal cancer in the Bantu. *S.Afr. Med. J.* **31**: 401-409.

Caldas, E.D., Jones, A.D., Winter, C.K., Ward, B. and Gilchrist, D.G. (1995) Electrospray ionisation mass spectrometry of sphinganine analog mycotoxins. *Anal. Chem.* **67**: 196-207.

Carter, R.M., Jacobs, M.B., Lubrano, G.J. and Guilbault, G.G. (1997) Rapid detection of aflatoxin B_1 with immunochemical optrodes. *Anal. Lett.* **30(8)**: 1465-1482.

Castegnaro, M., Garren, L., Galendo, D., Gelderblom, W.C.A., Chelule, P., Dutton, M.F. and Wild, C.P. (1998) Analytical method for the determination of sphinganine and sphingosine in serum as a potential biomarker for fumonisin exposure. *J. Chromatogr. B* **720**: 15-24.

Castellá, G, Bragulat, M.R and Cabañes, F.J. (1996) Mycoflora and fumonisin-producing strains of *Fusarium moniliforme* in mixed poultry feeds and component raw material. *Mycopathologia* **133**: 181-184.

Cawood, M.E., Gelderblom, W.C.A., Vleggaar, R., Behrend, Y., Thiel, P.G. and Marasas, W.F.O. (1991) Isolation of the fumonisin mycotoxins: a quantitative approach. *J. Agric. Food Chem.* **39**: 1958-1962.

Chamberlain, W. J., Bacon, C.W., Norred, W.P. and Voss, K.A. (1993) Levels of fumonisin B_1 in corn naturally contaminated with aflatoxin. *Food Chem. Toxicol.* **31**: 995-998.

Chatterjee, D., Mukherjee, S.K. and Dey, A. (1995) Nuclear disintegration in chicken peritoneal macrophages exposed to fumonisin B1 from Indian maize. *Lett. Appl. Microbiol.* **20**: 184-185.

Chelule, P.K., Gqaleni, N., Chuturgoon, A.A. and Dutton, M.F. (2000) The determination of fumonisin B_1 in human faeces: a short term marker for assessment of exposure. *Biomarkers* **5 (1)**: 1-8.

Chu, F.S. (1985) Recent studies on immunochemical analysis of mycotoxins. In: *Mycotoxins and Phytotoxins A Collection of Invited Papers presented at the Sixth International IUPAC Symposium on Mycotoxins and Phytotoxins, Pretoria, Republic of South Africa, 22-25 July, 1985.* Steyn, P.S. and Vleggaar, R. (Eds.) Amsterdam : Elsevier Science Publishers B.V., 1986 p.277-292.

Chu, F.S. and Li, G.Y. (1994) Simultaneous occurrence of fumonisin B_1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Appl. Environ. Microbiol.* **60(3)**: 847-852.

Chu, F.S., Huang, X. and Maragos, C.M. (1995) Production and characterization of anti-idiotype and anti-anti-idiotype antibodies against fumonisin B_1. *J. Agric. Food Chem.* **43**: 261-267.

Chu, F.S. (1996) Immunochemical methods for fumonisins. *In*: Jackson, L., de Vries, J.W., Bullerman, L.B. (eds.) *Fumonisins in Food.* (Advances in Experimental Biology vol. 392). New York: Plenum Press pp. 123-133.

Churchwell, M.I., Cooper, W.M., Howard, P.C. and Doerge, D.R. (1997) Determination of fumonisins in rodent feed using HPLC with electrospray mass spectrometric detection. *J.Agric. Food Chem.* **45**: 2573-2578.

Ciacci-Zanella, J.R., Merrill, A.H.,Jr., Wang, E. and Jones, C. (1998) Characterization of cell-cycle arrest by fumonisin B_1 in CV-1 cells. *Food Chem. Toxicol.* **36**: 791-804.

Clarke, J.R., Marquardt, R.R., Oosterveld, A., Frohlich, A.A., Madrid, F.J. and Dawood, M. (1993) Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibodies from the yolk of the laying hen. *J. Agric. Food Chem.* **41**: 1784-1789.

Coetzer, T.H.T. (1985) Preparation and characterisation of antibodies against mouse Ig (all classes). Internal Report, Bioclones (Pty.) Ltd. Immunology Group, Stellenbosch.

Colvin, B.M., Cooley, A.J. and Beaver, R.W. (1993) Fumonisin toxicosis in swine: clinical and pathologic findings. *J. Vet. Diagn. Invest.* **5**: 232-241.

Compton, S.J. and Jones, C.G. (1985) Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem.* **151**: 369-374.

Crowther, J.R. (1995) *ELISA: Theory and Practice* 1st Edition Totowa: Humana Press Inc.

Dantzer, W.R., Hopmans, E., Clark, A., Hauck, C. and Murphy, P.A. (1996) Purification of fumonisin B_1 from liquid cultures of *Fusarium proliferatum*. *J.Agric. Food Chem.* **44**: 3730-3732.

Dawlatana, M., Coker, R.D., Nagler, M.J. and Blunden, G. (1995) A normal phase HPTLC method for the quantitative determination of fumonisin B_1 in rice. *Chromatographia* **41(3/4)**: 187-190.

De Saeger, S. and van Peteghem, C. (1996) Dipstick enzyme immunoassay to detect *Fusarium* T-2 toxin in wheat. *Appl.Environ. Biol.* **62(6)**: 1880-1884.

Desjardins, A.E., Plattner, R.D. and Nelson, P.E. (1994) Fumonisin production and other traits of *Fusarium moniliforme* strains from maize in northeast Mexico. *Appl. Environ. Microbiol.* **60(5)**: 1695-1697.

Doehlert, D.C., Knutson, C.A. and Vesonder, R.F. (1994) Phytotoxic effects of fumonisin B_1 on maize seedling growth. *Mycopathologia* **127**: 117-121.

Doko, M.B. and Visconti, A. (1994) Occurrence of fumonisins B_1 and B_2 in corn and corn-based human foodstuffs in Italy. *Food Addit. Contam.* **11(4)**: 433-439.

Doko, M.B., Canet, C., Brown, N., Sydenham, E.W., Mpuchane, S. and Siame, B.A. (1996) Natural co-occurrence of fumonisins and zearalenone in cereals and cereal-based foods from Eastern and Southern Africa. *J. Agric. Food Chem.* **44**: 3240-3243.

Dombrink-Kurtzman, M.A., Javed, T., Bennett, G.A., Richard, J.L., Cote, L.M. and Buck, W.B. (1993) Lymphocyte cytotoxicity and erythrocytic abnormalities induced in broiler chicks by fumonisins B_1 and B_2 and moniliformin from *Fusarium proliferatum*. *Mycopathologia* **124**: 47-54.

Duncan, K., Kruger, S., Zabe, N., Kohn, B. and Prioli, R. (1998) Improved fluorometric and chromatographic methods for the quantification of fumonisins B_1, B_2 and B_3. *J. Chromatogr. A.* **815**: 41-47.

Dupuy, J., Le Bars, P., Boudra, H. and Le Bars, J. (1993) Thermostability of fumonisin B1, a mycotoxin from *Fusarium moniliforme*, in corn. *Appl. Environ. Microbiol.* **59(9)**: 2864-2867.

Dutton, M.F. (1996) Fumonisins, mycotoxins of increasing importance their nature and their effects. *Pharmacol. Ther.* **70(2)**: 137-161.

Edrington, T.S., Kamps-Holtzapple, C.A., Harvey, R.B., Kubena, L.F., Elissalde, M.H. and Rottinghaus, G.E. (1995) Acute hepatic and renal toxicity in lambs dosed with fumonisin-containing culture material. *J.Anim.Sci.* **73**: 508-515.

Espada, Y., Ruiz de Gopegui, R., Cuadradas, C. and Cabañes, F.J. (1994) Fumonisin mycotoxicosis in broilers. Weights and serum chemistry modifications. *Avian Dis.* **38**: 454-460.

Farrell, E.C. (1984) Electrolytes (Chapter 55) *In*: Kaplan, L.A. and Pesce, A.J. (Eds.) *Clinical Chemistry theory, analysis, and correlation* St. Louis : C.V. Mosby Company pp.1051-1070.

Fincham, J.E., Marasas, W.F.O., Taljaard, J.J.F., Kriek, N.P.J., Badenhorst, C.J., Gelderblom, W.C.A., Seier, J.V., Smuts, C.M., Faber, M., Weight, M.J., Slazus, W., Woodrof, C.W., van Wyk, M.J., Kruger, M. and Thiel, P.G. (1992) Atherogenic effects in a non-human primate of *Fusarium moniliforme* cultures added to a carbohydrate diet. *Atherosclerosis* **94**: 13-25.

Fremy, J.M. and Chu, F.S. (1984) Direct enzyme-linked immunosorbent assay for determining aflatoxin M_1 at picogram levels in dairy products. *J. Assoc. Off. Anal. Chem.* **67(6)**: 1098-1101.

Fukal, L. and Reisnerova, H. (1990) Monitoring of aflatoxins and ochratoxin A in Czechoslovak human sera by immunoassay. *Bull. Environ. Contam. Toxicol.* **44**: 345-349.

Fukuda, S., Nagahara, A., Kikuchi, M. and Kumagai, S. (1994) Preparation and characterisation of anti-fumonisin monoclonal antibodies. *Biosci. Biotech. Biochem.* **58(4)**: 765-767.

Fukuda, H., Shima, H., Vesonder, R.F., Tokuda, H., Nishino, H., Katoh, S., Tamura., S., Sugimura, T. and Nagao, M. (1996) Inhibition of protein serine/threonine phosphatases by fumonisin B_1, a mycotoxin. *Biochem. Biophys. Res. Comm.* **220**: 160-165.

Galvano, F., Pietri, A., Bertuzzi, T., Bognanno, M., Chies, L., de Angelis, A. and Galvano, M. (1997) Activated carbons: in vitro affinity for fumonisin B_1 and relation of adsorption ability to physicochemical parameters. *J. Food Prot.* **60(8)**: 985-991.

Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R. and Kriek, N.P.J. (1988) Fumonisins - Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme* . *Appl. Environ. Microbiol.* **54** (7): 1806-1811.

Gelderblom, W.C.A., Kriek, N.J.P., Marasas, W.F.O. and Thiel, P.G. (1991) Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B_1, in rats. *Carcinogenesis* **12**: 1247-1251.

Gelderblom, W.C.A., Semple, E., Marasas, W.F.O. and Farber, E. (1992A) The cancer-initiating potential of the fumonisin B mycotoxins. *Carcinogenesis* **13(3)**: 433-437.

Gelderblom, W.C.A., Marasas, W.F.O., Vleggaar, R., Thiel, P.G. and Cawood, M.E. (1992B) Fumonisins: isolation, chemical characterisation and biological effects. *Mycopathologia* **117**: 11-16.

Gelderblom, W.C.A., Cawood, M.E., Snyman, S.D., Vleggaar, R. and Marasas, W.F.O. (1993) Structure-activity relationships of fumonisins in short-term carcinogenesis and cytotoxicity assays. *Food Chem. Toxicol.* **31(6)**: 407-414.

Gelderblom, W.C.A., Cawood, M.E., Snyman, S.D. and Marasas, W.F.O. (1994) Fumonisin B_1 dosimetry in relation to cancer initiation in rat liver. *Carcinogenesis* **15(2)**: 209-214.

Gelderblom, W.C.A., Snyman, S.D., van der Westhuizen, L. and Marasas, W.F.O. (1995) Mitoinhibitory effect of fumonisin B_1 on rat hepatocytes in primary culture. *Carcinogenesis* **16(3)**: 625-631.

Gelderblom, W.C.A., Snyman, S.D., Lebepe-Mazur, S., van der Westhuizen, L., Kriek, N.P.J. and Marasas, W.F.O. (1996A) The cancer-promoting potential of fumonisin B_1 in rat liver using diethylnitrosamine as a cancer initiator. *Cancer Lett.* **109**: 101-108.

Gelderblom, W.C.A., Smuts, C.M., Abel, S., Snyman, S.D., Cawood, M.E., van der Westhuizen, L. and Swanevelder, S. (1996B) Effect of fumonisin B_1 on protein and lipid synthesis in primary rat hepatocytes. *Food Chem. Toxicol.* **34**: 361-369.

Gelderblom, W.C.A., Smuts, C.M., Abel, S., Snyman, S.D., van der Westhuizen, L., Huber, W.W. and Swanevelder, S. (1997) Effect of fumonisin B_1 on the levels and fatty acid composition of selected lipids in rat liver *in vivo*. *Food Chem. Toxicol.* **35**: 647-656.

Hahnau, S. and Weiler, E.W. (1993) Monoclonal antibodies for the enzyme immunoassay of the mycotoxin cyclopiazonic acid. *J. Agric. Food Chem.* **41**: 1076-1080.

Hammer, P., Blüthgen, A. and Walte, H.G. (1996) Carry-over of fumonisin B_1 into the milk of lactating cows. *Milchwissenschaft* **51(12)**: 691-695.

Haschek, W.M., Motelin, G., Ness, D.K., Harlin, K.S., Hall, W.F., Vesonder, R.F., Peterson, R.E. and Beasley, V.R. (1992) Characterization of fumonisin toxicity in orally and intravenously dosed swine. *Mycopathologia* **117**: 83-96.

Hebert, G.A., Pelham, P.L. and Pittman, B. (1973) Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Appl. Microbiol.* **25(1)**: 26-36.

Ho, A.K., Peng, R., Ho, A.A., Duffield, R. and Dombrink-Kurtzman, M.A. (1996) Interactions of fumonisins and sphingoid bases with GTP-binding proteins. *Biochem. Arch.* **12**: 249-260.

Holcomb, M., Thompson, H.C., Jr. and Hankins, L.J. (1993) Analysis of fumonisin B_1 in rodent feed by gradient elution HPLC using precolumn derivatization with FMOC and fluorescence detection. *J. Agric. Food Chem.* **41**: 764-767.

Hopmans, E.C., and Murphy, P.A. (1993) Detection of fumonisins B_1, B_2, and B_3 and hydrolyzed fumonisin B_1 in corn-containing foods. *J.Agric. Food Chem.* **41**: 1655-1658.

Huang, C., Dickman, M., Henderson, G. and Jones, C. (1995) Repression of protein kinase C and stimulation of cyclic AMP response elements by fumonisin, a fungal encoded toxin which is a carcinogen. *Cancer Res.* **55**: 1655-1659.

Hudson, L. and Hay, F.C. (1989) *Practical Immunology* 3rd Edition. Oxford London: Blackwell Scientific Publications.

Jackson, L.S., Hlywka, J.J., Senthil, K.R., Bullerman, L.B. and Musser, S.M. (1996A) Effects of time, temperature, and pH on the stability of fumonisin B_1 in an aqueous model system. *J.Agric. Food Chem.* **44**: 906-912.

Jackson, L.S., Hlywka, J.J., Senthil, K.R. and Bullerman, L.B. (1996B) Effects of thermal processing on the stability of FB2 in an aqueous system. *J. Agric. Food Chem.* **44**: 1984-1987.

Jackson, L.S., Katta, S.K., Fingerhut, D.D., DeVries, J.W. and Bullerman, L.B. (1997) Effects of baking and frying on the fumonisin B_1 content of corn-based foods. *J. Agric. Food Chem.* **45**: 4800-4805.

Jaskiewicz, K., Marasas, W.F.O. and Taljaard, J.J.F. (1987A) Hepatitis in vervet monkeys caused by *Fusarium moniliforme*. *J. Comp. Path.* **97**: 281-291.

Jaskiewicz, K., Marasas, W.F.O. and Van Der Walt, F.E. (1987B) Oesophageal and other main cancer patterns in four districts of Transkei, 1981-1984. *S.Afr. Med. J.* **72**: 27-30.

Javed, T., Richard, J.L., Bennett, G.A., Dombrink-Kurtzman, M.A., Bunte, R.M., Koelkebeck, K.W., Cote, L.M., Leeper, R.W. and Buck, W.B. (1993) Embryopathic and embryocidal effects of purified fumonisin B_1 or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia* **123**: 185-193.

Josephs, J.L. (1996) Detection and characterization of fumonisin mycotoxins by liquid chromatography / electrospray ionization using ion trap and triple quadrupole mass spectrometry. *Rapid Comm. Mass Spectrom.* **10**: 1333-1344.

Kawamura, O., Sato, S., Kajii, H., Nagayama, S., Ohtani, K., Chiba, J. and Ueno, Y. (1989) A sensitive enzyme-linked immunosorbent assay of ochratoxin A based on monoclonal antibodies. *Toxicon* **27(8)**: 887-897.

Kellerman, T.S., Marasas, W.F.O., Pienaar, J.G. and Naude, T.W. (1972) A mycotoxicosis of Equidae caused by *Fusarium moniliforme* Sheldon. A preliminary communication. *Onderstepoort J. Vet. Res.* **39(4)**: 205-208.

Kellerman, T.S., Marasas. W.F.O., Thiel, P.G., Gelderblom, W.C.A., Cawood, M. and Coetzer, J.A.W. (1990) Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B_1. *Onderstepoort J. Vet. Res.* **57**: 269-275.

Kleinman, L.I. and Lorenz, J.M. (1996) Physiology and pathophysiology of body water and electrolytes. *In*: Kaplan, L.A., Pesce, A.J. and Kazmierczak, S.C. (Eds.) *Clinical Chemistry theory, analysis, and correlation* 3rd edition St. Louis : C.V. Mosby Company p. 442.

Koller, A. (1984) Total serum protein. *In*: Kaplan, L.A. and Pesce, A.J. (Eds.) *Clinical Chemistry theory, analysis, and correlation* St. Louis : C.V. Mosby Company pp. 1316-1319.

Kriek, N.P.J., Kellerman, T.S. and Marasas, W.F.O. (1981) A comparative study of the toxicity of *Fusarium verticillioides* (= *F. moniliforme*) to horses, primates, pigs, sheep and rats. *Onderstepoort J. Vet. Res.* **48**: 129-131.

Kubena, L.F., Edrington, T.S., Kamps-Holtzapple, C., Harvey, R.B., Elissalde, M.H. and Rottinghaus, G.E. (1995) Effects of feeding fumonisin B_1 present in *Fusarium moniliforme* culture material and aflatoxin singly and in combination to turkey poults. *Poult. Sci.* **74**: 1295-1303.

Kubena, L.F., Edrington, T.S., Harvey, R.B., Phillips, T.D., Sarr, A.B. and Rottinghaus, G.E. (1997) Individual and combined effects of fumonisin B_1 present in *Fusarium moniliforme* culture material and diacetoxyscirpenol or ochratoxin A in turkey poults. *Poult. Sci.* **76**: 256-264.

Kwon, O-S., Schmued, L.C. and Slikker, W., Jr. (1997A) Fumonisin B_1 in developing rats alters brain sphinganine levels and myelination. *NeuroToxicol.* **18(2)**: 571-580.

Kwon, O-S., Sandberg, J.A. and Slikker, W., Jr. (1997B) Effects of fumonisin B_1 treatment on blood-brain barrier transfer in developing rats. *Neurotoxicol. Teratol.* **19(2)**: 151-155.

Ledoux, D.R., Brown, T.P., Weibking, T.S. and Rottinghaus, G.E. (1992) Fumonisin toxicity in broiler chicks. *J. Vet. Diagn. Invest.* **4**: 330-333.

Ledoux, D.R., Bermudez, A.J. and Rottinghaus, G.E. (1996) Effects of feeding *Fusarium moniliforme* culture material, containing known levels of fumonisin B_1, in the young turkey poult. *Poult. Sci.* **75**: 1472-1478.

Leslie, J.F. (1996) Introductory biology of *Fusarium moniliforme*. *In*: Jackson, L., de Vries, J.W., Bullerman, L.B. (eds.) *Fumonisins in Food.* (Advances in Experimental Biology vol. 392). New York: Plenum Press pp. 153-164.

Lim, C.W., Parker, H.M., Vesonder, R.F. and Haschek, W.M. (1996) Intravenous fumonisin B_1 induces cell proliferation and apoptosis in the rat. *Nat. Toxins* **4**: 34-41.

Lu, Z, Dantzer, W.R., Hopmans, E.C., Prisk, V., Cunnick, J.E., Murphy, P.A. and Hendrich, S. (1997) Reaction with fructose detoxifies fumonisin B_1 while stimulating liver-associated natural killer cell activity in rats. *J. Agric. Food Chem.* **45**: 803-809.

Lukacs, Z., Schaper, S., Herderich, M., Schreier, P. and Humpf, H.-U. (1996) Identification and determination of fumonisin FB_1 and FB_2 in corn and corn products by high-performance liquid chromatography – electrospray-ionization tandem mass spectrometry (HPLC-ESI-MS-MS). *Chromatographia* **43(3/4)**: 124-128.

Lumlertdacha, S, Lovell, R.T., Shelby, R.A., Lenz, S.D. and Kemppainen, B.W. (1995) Growth, hematology, and histopathology of channel catfish, *Ictalurus punctatus,* fed toxins from *Fusarium moniliforme*. *Aquacult.* **130**: 201-218.

MacDougald, O.A., Thulin, A.J. and Pestka, J.J. (1990) Determination of zearalenone and related metabolites in porcine urine by modified enzyme-linked immunosorbent assay. *J. Assoc. Off. Anal. Chem.* **73(1)**: 65-68.

MacKenzie, S.E., Savard, M.E., Blackwell, B.A., Miller, J.D. and ApSimon, J.W. (1998) Isolation of a new fumonisin from *Fusarium moniliforme* grown in liquid culture. *J. Nat. Prod.* **61**: 367-369.

Maragos, C.M. and Richard, J.L. (1994) Quantitation and stability of fumonisins B_1 and B_2 in milk. *J. AOAC Int.* **77(5)**: 1162-1167.

Maragos, C.M. (1995) Capillary zone electrophoresis and HPLC for the analysis of fluorescein isiothiocyanate-labeled fumonisin B_1. *J. Agric. Food Chem.* **43**: 390-394.

Maragos, C.M. (1997) Measurement of mycotoxins in food with a fiber-optic immunosensor. *J. Clin. Ligand Assay* **20(1)**: 136-140.

Marasas, W.F.O., Kriek, N.P.J., Fincham, J.E. and Van Rensburg, S.J. (1984) Primary liver cancer and oesophageal basal cell hyperplasia in rats caused by *Fusarium moniliforme*. *Int. J. Cancer* **34**: 383-387.

Marasas, W.F.O., Kellerman, T.S., Gelderblom, W.C.A., Coetzer, J.A.W., Thiel, P.G. and Van der Lugt, J.J. (1988A) Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme*. *Onderstepoort J. Vet. Res.* **55(4)**: 197-203.

Marasas, W.F.O., Jaskiewiecz, K., Venter, F.S. and Van Schalkwyk, D.J. (1988B) *Fusarium moniliforme* contamination of maize in oesophageal cancer areas in Transkei. *S. Afr. Med. J.* **74**: 110-114.

Marasas, W.F.O., Thiel, P.G., Gelderblom, W.C.A., Shephard, G.S., Sydenham, E.W. and Rheeder, J.P. (1993) Fumonisins produced by Fusarium moniliforme in maize: foodborne carcinogens of Pan African importance. *Afr. Newslett. Occup. Health Saf. Suppl.* **2**: 11-18.

Marin, S., Sanchis, V., Sanz, D., Castel, I., Ramos, A.J., Canela, R. and Magan, N. (1999) Control of growth and fumonisin B_1 production by *Fusarium verticilliodes* and *Fusarium proliferatum* isolates in moist maize with propionate preservatives. *Food Addit. Contam.* **16(12)**: 555-563.

Märtlbauer, E., Gareis, M. and Terplan, G. (1988) Enzyme immunoassay for the macrocyclic trichothecene roridin A: production, properties, and use of rabbit antibodies. *Appl. Environ. Microbiol.* **54(1)**: 225-230.

Meireles, M.C.A., Correa, B., Fischman, O., Gambale, W., Paula, C.R., Chacon-Reche, N.O. and Pozzi, C.R. (1994) Mycoflora of the toxic feed associated with equine leukoencephalomalacia (ELEM) outbreaks in Brazil. *Mycopathologia* **127**: 183-188.

Meivar-Levy, I., Sabanay, H., Bershadsky, A.D. and Futerman, A.H. (1997) The role of sphingolipids in the maintenance of fibroblast morphology. *J. Biol. Chem.* **272(3)**: 1558-1564.

Merrill, A.H.,Jr., Wang, E., Mullins, R.E., Jamison, W.C.L., Nimkar. S. and Liotta, D.C. (1988) Quantitation of free sphingosine in liver by high-performance liquid chromatography. *Anal. Biochem.* **171**: 373-381.

Merrill, A.H., Jr., Wang, E., Gilchrist, D.G. and Riley, R.T. (1993A) Fumonisins and other inhibitors of *de novo* sphingolipid biosynthesis. *Adv. Lipid Res.* **26**: 215-234.

Merrill, A.H., Jr., van Echten, G., Wang, E. and Sandhoff, K. (1993B) Fumonisin B_1 inhibits sphingosine (sphinganine) N-acyltransferase and *de novo* sphingolipid biosynthesis in cultured neurons *in situ* . *J. Biol. Chem.* **268(36)**: 27299-27306.

Merrill, A.H., Jr., Lingrell, S., Wang, E., Nikolova-Karakashian, M., Vales, T. and Vance, D. E. (1995A) Sphingolipid biosynthesis *de novo* by rat hepatocytes in culture. *J. Biol. Chem.* **270(23)**: 13834-13841.

Merrill, A.H., Jr., Schmelz, E., Wang, E., Schroeder, J.J., Dillehay, D.L. and Riley, R.T. (1995B) Role of dietary sphingolipids and inhibitors of sphingolipid metabolism in cancer and other diseases. *J. Nutr.* **125**: 1677S-1682S.

Merrill, A.H., Jr., Liotta, D.C. and Riley, R.T. (1996) Fumonisins: fungal toxins that shed light on sphingolipid function. *Cell Biol.* **6**: 218-223.

Miller, M.A., Honstead, J.P. and Lovell, R.A. (1996) Regulatory aspects of fumonisins with respect to animal feed. *In*: Jackson, L.S., de Vries, J.W., Bullerman, L.B. (Eds.) *Fumonisins in Food.* (Advances in Experimental Biology vol. 392). New York: Plenum Press pp. 363-368.

Mingxin, L., Ping, L. and Baorong, L. (1980) Recent progress in research on esophageal cancer in China. *Adv. Cancer Res.* **33**: 173-248.

Mirocha, C.J., Mackintosh, C.G., Mirza, U.A., Xie, W., Xu, Y. and Chen, J. (1992) Occurrence of fumonisin in forage grass in New Zealand. *Appl. Environ. Microbiol.* **58(9)**: 3196-3198.

Motelin, G.K., Haschek, W.M., Ness, D.K., Hall, W.F., Harlin, K.S., Schaeffer, D.J. and Beasley, V.R. (1994) Temporal and dose-response features in swine fed corn screenings contaminated with fumonisin mycotoxins. *Mycopathologia* **126**: 27-40.

Mullett, W., Lai, E.P.C. and Yeung, J.M. (1998) Immunoassay of fumonisins by a surface plasmon resonance biosensor. *Anal. Biochem.* **258**: 161-167.

Murphy, P.A., Rice, L.G. and Ross, P.F. (1993) Fumonisin B_1, B_2, and B_3 content of Iowa, Wisconsin, and Illinois corn and corn screenings. *J. Agric. Food Chem.* **41**: 263-266.

Musser, S.M., Gay, M.L., Mazzola, E.P. and Plattner, R.D. (1996) Identification of a new series of fumonisins containing 3-hydroxypyridine. *J. Nat. Prod.* **59**: 970-972.

Musser, S.M. and Plattner, R.D. (1997) Fumonisin composition in cultures of *Fusarium moniliforme, Fusarium proliferatum,* and *Fusarium nygami. J. Agric. Food Chem.* **45**: 1169-1173.

Nakane, P.K. and Kawaoi, A. (1974) Peroxidase-labeled antibody a new method of conjugation. *J. Histochem. Cytochem.* **22(12)**: 1084-1091.

Nelson, P.E., Desjardins, A.E. and Plattner, R.D. (1993) Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annu. Rev. Phytopathol.* **31**: 233-252.

Norred, W.P., Voss, K.A., Bacon, C.W. and Riley, R.T. (1991) Effectiveness of ammonia treatment in detoxification of fumonisin-contaminated corn. *Food Chem. Toxic.* **29(12)**: 815-819.

Norred, W. P., Wang, E., Yoo, H., Riley, R.T. and Merrill, A. H., Jr. (1992) *In vitro* toxicology of fumonisins and the mechanistic implications. *Mycopathologia* **117**: 73-78.

Norred, W.P. (1993) Fumonisins – Mycotoxins produced by *Fusarium moniliforme*. *J. Toxicol. Environ. Health* **38**: 309-328.

Norred, W.P., Plattner, R.D. and Chamberlain, W.J. (1993) Distribution and excretion of [^{14}C] fumonisin B_1 in male Sprague-Dawley rats. *Nat. Toxins* **1**: 341-346.

Norred, W.P., Riley, R.T., Meredith, F.I., Bacon, C.W. and Voss, K.A. (1996) Time- and dose-response effects of the mycotoxin, fumonisin B_1 on sphingoid base elevations in precision-cut rat liver and kidney slices. *Toxicol. in Vitro* **10**: 349-358.

Norred, W.P., Plattner, R.D., Dombrink-Kurtzman, M.A., Meredith, F.I. and Riley, R.T. (1997) Mycotoxin-induced elevation of free sphingoid bases in precision-cut rat liver slices: specificity of the response and structure-activity relationships. *Toxicol. Appl. Pharmacol.* **147**: 63-70.

Osweiler, G.D., Ross, P.F., Wilson, T.M., Nelson, P.E., Witte, S.T., Carson, T.L., Rice, L.G. and Nelson, H.A. (1992) Characterization of an epizootic of pulmonary edema in swine associated with fumonisin in corn screenings. *J. Vet. Diagn. Invest.* **4**: 53-59.

Osweiler, G.D., Kehrli, M.E., Stabel, J.R., Thurston, J.R., Ross, P.F. and Wilson, T.M. (1993) Effects of fumonisin-contaminated corn screenings on growth and health of feeder calves. *J. Anim. Sci.* **71**: 459-466.

Park, D.L., Miller, B.M., Nesheim, S., Trucksess, M.W., Vekich, A., Bidigare, B., McVey, J. I. and Brown, L.H. (1989) Visual and semiquantitative spectrophotometric ELISA screening method for aflatoxin B_1 in corn and peanut products: follow-up collaborative study. *J. Assoc. Off. Anal. Chem.* **72(4)**: 638-643.

Park, D.L., Rua, S.M., Jr., Mirocha, C.J., Abd-Alla, E-S. A.M. and Ying Weng, C. (1992) Mutagenic potentials of fumonisin contaminated corn following ammonia decontamination procedure. *Mycopathologia* **117**: 105-108.

Patel, S., Hazel, C.M., Winterton, A.G.M. and Gleadle, A.E. (1997) Surveillance of fumonisins in UK maize-based foods and other cereals. *Food Addit. Contam.* **14(2)**: 187-191.

Perilla, N.S. and Diaz, G.J. (1998) Incidence and levels of fumonisin contamination in Colombian corn and corn products. *Mycotoxin Res.* **14**: 74-82.

Pestka, J.J., Azcona-Olivera, J.I., Plattner, R.D., Minervini, F., Doko, M.B. and Visconti, A. (1994) Comparative assessment of fumonisin in grain-based foods by ELISA, GC-MS and HPLC. *J. Food Prot.* **57(2)**: 169-172.

Pittet, A., Parisod, V. and Schellenberg, M. (1992) Occurrence of fumonisins B_1 and B_2 in corn-based products from the Swiss market. *J.Agric. Food Chem.* **40**: 1352-1354.

Poling, S.M. and Plattner, R.D. (1996) Rapid purification of fumonisins B_3 and B_4 with solid phase extraction columns. *J. Agric. Food Chem.* **44**: 2792-2796.

Polson, A., Potgieter, G.M., Largier, J.F., Mears, G.E.F. and Joubert, F.J. (1964) The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochim. Biophys. Acta* **82**: 463-475.

Polson, A., Coetzer, T., Kruger, J., von Maltzahn, E. and van der Merwe, K.J. (1985) Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol. Invest.* **14**: 323-327.

Prathapkumar, S.H., Rao, V.S., Paramkishan, R.J. and Bhat, R.V. (1997) Disease outbreak in laying hens arising from the consumption of fumonisin-contaminated food. *Br. Poult. Sci.* **38**: 475-479.

Prelusky, D.B., Locksley Trenholm, H. and Savard, M.E. (1994) Pharmacokinetic fate of ^{14}C-labelled fumonisin B_1 in swine. *Nat. Toxins* **2**: 73-80.

Rava, E. (1996) Mycotoxins in maize products of the 1994/95 marketing season. *Mycotoxin Res.* **12**: 25-30.

Rava, E., Viljoen, J.H., Kallmeyer, H. and de Jager, A. (1996) Fungi and mycotoxins in South African maize of the 1993 crop. *Mycotoxin Res.* **12**:15-24.

Read, S.M. and Northcote, D.H. (1981) Minimization of variation in the response to different proteins of the Coommassie blue G dye-binding assay for protein. *Anal. Biochem.* **116**: 53-64.

Restum, J.C., Bursian, S.J., Millerick, M., Render, J.A., Merrill, A.H., Jr., Wang, E., Rottinghaus, G.E. and Aulerich, R.J. (1995) Chronic toxicity of fumonisins from *Fusarium moniliforme* culture material (M-1325) to mink. *Arch. Environ. Contam. Toxicol.* **29**: 545-550.

Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S. and van Schalkwyk, D.J. (1992) *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology* **82(3)**: 353-357.

Rheeder, J.P., Sydenham, E.W., Marasas, W.F.O., Thiel, P.G., Shephard, G.S., Schlechter, M., Stockenstrom, S., Cronje, D.W. and Viljoen, J.H. (1995) Fungal infestation and mycotoxin contamination of South African maize harvested in 1989 and 1990. *S. Afr. J. Sci.* **91**: 127-131.

Richard, J.L., Meerdink, G., Maragos, C.M., Tumbleson, M., Bordson, G., Rice, L.G. and Ross, P.F. (1996) Absence of detectable fumonisin in the milk of cows fed *Fusarium proliferatum* (Matsushima) Nirenberg culture material. *Mycopathologia* **133**: 123-126.

Riley, R.T., An, N-H., Showker, J.L., Yoo, H-S., Norred, W.P., Chamberlain, W.J., Wang, E., Merrill, A.H., Jr., Motelin, G., Beasley, V.R. and Haschek, W.M. (1993) Alteration of tissue and serum sphinganine to sphingosine ratio: an early biomarker of exposure to fumonisin-containing feeds in pigs. *Toxicol. Appl. Pharmacol.* **118**: 105-112.

Riley, R.T., Hinton, D.M., Chamberlain, W.J., Bacon, C.W., Wang, E., Merrill, A.H., Jr. and Voss, K.A. (1994) Dietary fumonisin B_1 induces disruption of sphingolipid metabolism in Sprague-Dawley rats: a new mechanism of nephrotoxicity. *J. Nutr.* **124**: 594-603.

Riley, R.T., Showker, J.L., Owens, D.L. and Ross, P.F. (1997) Disruption of sphingolipid metabolism and induction of equine leukoencephalomalacia by *Fusarium proliferatum* culture material containing fumonisin B_2 or B_3. *Environ. Toxicol. Pharmacol.* **3**: 221-228.

Ritchie, R.F. (1986) Preparation of polyclonal antisera. *In*: Rose, N.R., Friedman, H. and Fahey, J.L. (Eds.) *Manual of Clinical Laboratory Immunology* 3rd Edition. American Society for Microbiology Washington D.C. pp. 4-8.

Ritieni, A., Moretti, A., Logrieco, A., Bottalico, A., Randazzo, G., Monti, S.M., Ferracane, R. and Fogliano, V. (1997) Occurrence of fusaproliferin, fumonsin B_1 and beauvericin in maize from Italy. *J. Agric. Food Chem.* **45**: 4011-4016.

Rodgers, R. P. C. (1986) Data processing of immunoassay results *In*: Rose, N.R., Friedman, H. and Fahey, J.L. (Eds.) *Manual of Clinical Laboratory Immunology* 3rd Edition. Washington, D.C. : American Society for Microbiology p.82-87.

Rose, E.F. (1982) Esophageal cancer in Transkei – the pattern and associated risk factors. In: *Cancer of the Esophagus* Volume 1 Ed. C.J.Pfeiffer, CRC Press Inc, Boca Raton pp.19-28.

Ross, P.F., Nelson, P.E., Richard, J.L., Osweiler, G.D., Rice, L.G., Plattner, R.D. and Wilson, T.M. (1990) Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Appl. Environ. Microbiol.* **Oct.**: 3225-3226.

Ross, P.F., Rice, L.G., Reagor, J.C., Osweiler, G.D., Wilson, T.M., Nelson, H.A., Owens, D.L., Plattner, R.D., Harlin, K.A., Richard, J.L., Colvin, B.M. and Banton, M.I. (1991A) Fumonisin B_1 concentrations in feed from 45 confirmed equine leukoencephalomalacia cases. *J. Vet. Diagn. Invest.* **3**: 238-241.

Ross, P.F., Rice, L.G., Plattner, R.D., Osweiler, G.D., Wilson, T.M., Owens, D.L., Nelson, H.A. and Richard, J.L. (1991B) Concentrations of fumonisin B_1 in feeds associated with animal health problems. *Mycopathologia* **114**: 129-135.

Rotter, B.A., Thompson, B.K., Prelusky, D.B., Trenholm, H.L., Stewart, B., Miller, J.D. and Savard, M.E. (1996) Response of growing swine to dietary exposure to pure fumonisin B_1 during an eight-week period: growth and clinical parameters. *Nat. Toxins* **4**: 42-50.

Rottinghaus, G.E., Coatney, C.E. and Minor, H.C. (1992) A rapid, sensitive thin layer chromatography procedure for the detection of fumonisin B_1 and B_2. *J. Vet. Diagn. Invest.* **4**: 326-329.

Sauviat, M-P., Laurent, D., Kohler, F. and Pellegrin, F. (1991) Fumonisin, a toxin from the fungus *Fusarium moniliforme* Sheld, blocks both the calcium current and the mechanical activity in frog atrial muscle. *Toxicon* **29(8)**: 1025-1031.

Schaafsma, A.W., Nicol, R.W., Savard, M.E., Sinha, R.C., Reid, L.M. and Rottinghaus, G. (1998) Analysis of *Fusarium* toxins in maize and wheat using thin layer chromatography. *Mycopathologia* **142**: 107-113.

Schneider, E., Usleber, E. and Märtlbauer, E. (1995) Rapid detection of Fumonisin B_1 in corn-based food by competitive direct dipstick immunoassay/enzyme-linked immunofiltration assay with integrated negative control reaction. *J. Agric. Food Chem.* **43**: 2548-2552.

Schroeder, J. J., Cranes, H. M., Xia, J., Liotta, D. C. and Merrill, A.H., Jr. (1994) Disruption of sphingolipid metabolism and stimulation of DNA synthesis by fumonisin B_1. *J. Biol. Chem.* **269(5)**: 3475-3481.

Scott, P.M. and Lawrence, G.A. (1992) Liquid chromatographic determination of fumonisins with 4-fluoro-7-nitrobenzofurazan. *J. AOAC Int.* **75(5)**: 829-834.

Scott, P. M. and Lawrence, G. A. (1994) Stability and problems in recovery of fumonisins added to corn-based foods. *J. AOAC Int.* **77(2)**: 541-545.

Scott, P.M., Delgado, T., Prelusky, D.B., Trenholm, H.L. and Miller, J.D. (1994) Determination of fumonisins in milk. *J. Environ. Sci. Health* **B29(5)**: 989-998.

Scott, P.M., Yeung, J.M., Lawrence, G.A. and Prelusky, D.B. (1997) Evaluation of enzyme-linked immunosorbent assay for analysis of beer for mycotoxins. *Food Addit. Contam.* **14(5)**: 445-450.

Segal, I, Reinach, S.G. and de Beer, M. (1988) Factors associated with oesophageal cancer in Soweto, South Africa. *Br. J. Cancer* **58**: 681-686.

Selim, M.I., El-Sharkawy, S.H. and Popendorf, W.J. (1996) Supercritical fluid extraction of fumonisin B_1 from grain dust. *J.Agric. Food Chem.* **44**: 3224-3229.

Shelby, R.A., Rottinghaus, G.E., and Minor, H.C. (1994) Comparison of thin-layer chromatography and competitive immunoassay methods for detecting fumonisin on maize. *J.Agric. Food Chem.* **42**: 2064-2067.

Shephard, G.S., Sydenham, E.W., Thiel, P.G. and Gelderblom, W.C.A. (1990) Quantitative determination of fumonisins B_1 and B_2 by high-performance liquid chromatography with fluorescence detection. *J. Liq. Chromatogr.* **13(10)**: 2077-2087.

Shephard, G.S., Thiel, P.G. and Sydenham, E.W. (1992A) Initial studies on the toxicokinetics of fumonisin B_1 in rats. *Food Chem. Toxic.* **30(4)**: 277-279.

Shephard, G.S., Thiel, P.G. and Sydenham, E.W. (1992B) Determination of fumonisin B_1 in plasma and urine by high-performance liquid chromatography. *J. Chromatogr.* **574**: 299-304.

Shephard,G.S., Thiel, P.G., Sydenham,E.W., Alberts, J.F. and Gelderblom, W.C.A. (1992C) Fate of a single dose of the ^{14}C-labelled mycotoxin, fumonisin B_1, in rats. *Toxicon* **30**: 768-770.

Shephard, G.S., Thiel, P.G., Sydenham, E.W., Vleggaar, R. and Alberts, J.F. (1994A) Determination of the mycotoxin fumonisin B_1 and identification of its partially hydrolysed-metabolites in the faeces of non-human primates. *Food Chem. Toxic* **32(1)**: 23-29.

Shephard, G.S., Thiel, P.G., Sydenham, E.W. and Alberts, J.F. (1994B) Biliary excretion of the mycotoxin fumonisin B_1 in rats. *Food Chem. Toxic.* **32(5)**: 489-491.

Shephard, G.S., Thiel, P.G., Sydenham, E.W., Alberts, J.F. and Cawood, M.E. (1994C) Distribution and excretion of a single dose of the mycotoxin fumonisin B_1 in a non-human primate. *Toxicon.* **32(6)**: 735-741.

Shephard, G.S., Thiel, P.G., Sydenham, E.W. and Snijman, P.W. (1995A) Toxicokinetics of the mycotoxin fumonisin B_2 in rats. *Food Chem. Toxic* **33(7)**: 591-595.

Shephard, G.S., Thiel, P.G. and Sydenham, E.W. (1995B) Liquid chromatographic determination of the mycotoxin fumonisin B_2 in physiological samples. *J. Chromatogr. A.* **692**: 39-43.

Shephard, G.S. (1998) Chromatographic determination of the fumonisin mycotoxins. *J. Chromatogr. A.* **815**: 31-39.

Shetty, P.H. and Bhat, R.V. (1997A) Differential production of fumonisin B_1 in maize inbreds and hybrids in laboratory. *Cereal Res. Commun.* **25(4)**: 1011-1015.

Shetty, P.H. and Bhat, R.V. (1997B) Natural occurrence of fumonisin B_1 and its co-occurrence with aflatoxin B_1 in Indian sorghum, maize, and poultry feeds. *J. Agric. Food Chem.* **45**: 2170-2173.

Shetty, P.H. and Bhat, R.V. (1998) Sensitive method for the detection of fumonisin B_1 in human urine. *J. Chromatogr. B.* **705**: 171-173.

Smith, G.W., Constable, P.D., Bacon, C.W., Meredith, F.I. and Haschek, W.M. (1996A) Cardiovascular effects of fumonisins in swine. *Fundam. Appl. Toxicol.* **31**: 169-172.

Smith, G.W., Constable, P.D. and Haschek, W. M. (1996B) Cardiovascular responses to short-term fumonsin exposure in swine. *Fundam. Appl. Toxicol.* **33**: 140-148.

Smith, J.S. and Thakur, R.A. (1996) Occurrence and fate of fumonisins in beef. *In*: Jackson, L.S., de Vries, J.W., Bullerman, L.B. (Eds.) *Fumonisins in Food.* (Advances in Experimental Biology vol. 392). New York: Plenum Press pp. 39-55.

Solfrizzo, M., Avantaggiato, G. and Visconti, A. (1997) Rapid method to determine sphinganine / sphingosine in human and animal urine as a biomarker for fumonisin exposure. *J. Chromatogr. B.* **692**: 87-93.

Stockenström, S., Sydenham, E.W. and Thiel, P.G. (1994) Determination of fumonisins in corn: evaluation of two purification procedures. *Mycotoxin Res.* **10**: 9-14.

Stubblefield, R.D., Greer, J.I., Shotwell, O.L. and Aikens, A.M. (1991) Rapid immunochemical screening method for aflatoxin B_1 in human and animal urine. *J. Assoc. Off. Anal. Chem.* **74(3)**: 530-532.

Suzuki, C.A.M., Hierlihy, L., Barker, M., Curran, I., Mueller, R. and Bondy, G.S. (1995) The effects of fumonisin B_1 on several markers of nephrotoxicity in rats. *Toxicol. Appl. Pharmacol.* **133**: 207-214.

Sydenham, E.W., Gelderblom, W.C.A., Thiel, P.G. and Marasas, W.F.O. (1990A) Evidence for the natural occurrence of fumonisin B_1, a mycotoxin produced by *Fusarium moniliforme*, in corn. *J. Agric. Food Chem.* **38(1)**: 285-290.

Sydenham, E.W., Thiel, P.G., Marasas, W.F.O., Shephard, G.S., Van Schalkwyk, D.J. and Koch, K.R. (1990B) Natural occurrence of some *Fusarium* mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, southern Africa. *J.Agric. Food Chem.* **38(10)**: 1900-1903.

Sydenham, E.W., Shephard, G.S., Thiel, P.G. , Marasas, W.F.O. and Stockenstrom, S. (1991) Fumonisin contamination of commercial corn-based human foodstuffs. *J. Agric. Food Chem.* **39**: 2014-2018.

Sydenham, E.W., Shephard, G.S. and Thiel, P.G. (1992) Liquid chromatographic determination of fumonisins B_1, B_2, and B_3 in foods and feeds. *J. AOAC Int.* **75(2)**: 313-318.

Sydenham, E.W., Shephard, G.S., Thiel, P.G. , Marasas, W.F.O., Rheeder, J.P., Peralta Sanhueza, C.E., Gonzalez, H.H.L. and Resnik, S.L. (1993) Fumonisins in Argentinian field-trial corn. *J.Agric. Food Chem.* **41**: 891-895.

Sydenham, E.W., Van der Westhuizen, L., Stockenstrom, S., Shephard, G.S. and Thiel, P.G. (1994) Fumonisin-contaminated maize: physical treatment for the partial decontamination of bulk shipments. *Food Addit. Contam.* **11(1)**: 25-32.

Sydenham, E.W., Stockenstrom, S., Thiel, P.G., Shephard, G.S., Koch, K.R. and Marasas, W.F.O. (1995A) Potential of alkaline hydrolysis for the removal of fumonisins from contaminated corn. *J. Agric. Food Chem.* **43**: 1198-1201.

Sydenham, E.W., Thiel, P.G., Shephard, G.S., Koch, K.R. and Hutton, T. (1995B) Preparation and isolation of the partially hydrolyzed moiety of fumonisin B_1. *J. Agric. Food Chem.* **43**: 2400-2405.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Bird, C. and Miller, B.M. (1996A) Determination of fumonisins in corn: evaluation of competitive immunoassay and HPLC techniques. *J.Agric. Food Chem.* **44**: 159-164.

Sydenham, E.W., Stockenstrom, S., Thiel, P.G., Rheeder, J.P., Doko, M.B., Bird, C. and Miller,B.M. (1996B) Polyclonal antibody-based ELISA and HPLC methods for the determination of fumonisins in corn : a comparative study. *J. Food Prot.* **59(8)**: 893-897.

Tanaka, T., Abbas, H.K. and Duke, S.O. (1993) Structure-dependent phytotoxicity of fumonisins and related compounds in a duckweed bioassay. *Phytochemistry* **33(4)**: 779-785.

Tanaka, T., Teshima, R., Ikebuchi, H., Sawada, J. and Ichinoe, M. (1995) Sensitive enzyme-linked immunosorbent assay for the mycotoxin zearalenone in barley and Job's-tears. *J. Agric. Food Chem.* **43**: 946-950.

Tejada-Simon, M.V., Maravatsanga, L.T. and Pestka, J.J. (1995) Comparative detection of fumonisin by HPLC, ELISA and immunocytochemical localization in *Fusarium* cultures. *J. Food Prot.* **58(6)**: 666-672.

Thakur, R.A. and Smith, J.S. (1996) Determination of fumonisins B_1 and B_2 and their major hydrolysis products in corn, feed, and meat, using HPLC. *J. Agric. Food Chem.* **44**: 1047-1052.

Thiel, P.G., Marasas, W.F.O., Sydenham, E.W., Shephard, G.S., Gelderblom, W.C.A. and Nieuwenhuis, J.J. (1991A) Survey of fumonisin production by *Fusarium* species. *Appl. Environ. Microbiol.* **57(4)**: 1089-1093.

Thiel, P.G., Shephard, G.S., Sydenham, E.W., Marasas, W.F.O., Nelson, P.E. and Wilson, T.M. (1991B) Levels of fumonisins B_1 and B_2 in feeds associated with confirmed cases of equine leukoencephalomalacia. *J. Agric. Food Chem.* **39**: 109-111.

Thiel, P.G., Marasas, W.F.O., Sydenham, E.W., Shephard, G.S. and Gelderblom, W.C.A. (1992) The implications of naturally occurring levels of fumonisins in corn for human and animal health. *Mycopathologia* **117**: 3-9.

Thiel, P.G., Sydenham, E.W., Shephard, G.S. and van Schalkwyk, D.J. (1993) Study of the reproducibility characteristics of a liquid chromatographic method for the determination of fumonisins B_1 and B_2 in corn : IUPAC collaborative study. *J. AOAC Int.* **76(2)**: 361-366.

Thiel, P.G., Sydenham, E.W. and Shephard, G.S. (1996) The reliability and significance of analytical data on the natural occurrence of fumonisins in food. *In*: Jackson, L., de Vries, J.W., Bullerman, L.B. (eds.) *Fumonisins in Food.* (Advances in Experimental Biology vol. 392). New York: Plenum Press pp. 145-151.

Thompson, S.G. (1984) Competitive-binding assays *In:* Kaplan, L.A. and Pesce, A.J. (Eds.) *Clinical Chemistry Theory, analysis, and correlation* 1st Edition St. Louis : C.V. Mosby p. 211.

Thompson, V.S. and Maragos, C.M. (1996) Fiber-optic immunosensor for the detection of Fumonisin B_1. *J. Agric. Food Chem.* **44(4)**: 1041-1046.

Trucksess, M.W., Stack, M.E., Allen, S. and Barrion, N. (1995) Immunoaffinity column coupled with liquid chromatography for determination of fumonisin B_1 in canned and frozen sweet corn. *J. AOAC Int.* **78(3)**: 705-710.

Ueno, Y., Aoyama, S., Sugiura, Y., Wang, D-S., Lee, U-S., Hirooka, E.Y., Hara, S., Karki, T., Chen, G. and Yu, S-Z. (1993) A limited survey of fumonisins in corn and corn-based products in Asian countries. *Mycotoxin Res.* **9**: 27-34.

Ung-Soo, L., Myong-Yur, L., Kwang-Sop, S., Yun-Sik, M., Chae-Min, C. and Ueno, Y. (1994) Production of fumonisin B_1 and B_2 by *Fusarium moniliforme* isolated from Korean corn kernels for feed. *Mycotoxin Res.* **10**: 67-72.

Usleber, E., Straka, M. and Terplan, G. (1994) Enzyme immunoassay for fumonisin B_1 applied to corn-based food. *J. Agric. Food Chem.* **42**: 1392-1396.

van Rensburg, S.J. (1985) Recent studies on the etiology of oesophageal cancer. *S. Afr. Cancer Bull.* **29**: 22-31.

Velázquez, C., van Bloemendal, C., Sanchis, V. and Canela, R. (1995) Derivation of fumonisins B_1 and B_2 with 6-aminoquinolyl *N*-hydroxysuccinimidylcarbamate. *J. Agric. Food Chem.* **43**: 1535-1537.

Viquez, O.M., Castell-Perez, M.E. and Shelby, R.A. (1996) Occurrence of fumonisin B_1 in maize grown in Costa Rica. *J. Agric. Food Chem.* **44**: 2789-2791.

Visconti, A., Doko, M.B., Bottalico, C., Schurer, B. and Boenke, A. (1994A) Stability of fumonisins (FB_1 and FB_2) in solution. *Food Addit. Contam.* **11(4)**: 427-431.

Visconti, A. and Doko, M.B. (1994B) Survey of fumonisin production by *Fusarium* isolated from cereals in Europe. *J. AOAC Int.* **77(2)**: 546-550.

Visconti, A., Boenke, A., Solfrizzo, M., Pascale, M. and Doko, M.B. (1996A) European intercomparison study for the determination of the fumonisins content in two maize materials. *Food Addit. Contam.* **13(8)**: 909-927.

Visconti, A., Solfrizzo, M., Doko, M.B., Boenke, A. and Pascale, M.(1996B) Stability of fumonisins at different storage periods and temperatures in γ-irradiated maize. *Food Addit. Contam.* **13(8)**: 929-938.

Voller, A. and de Savigny, D. (1981) Enzyme Linked Immunosorbent Assay (ELISA). *In*: Thompson, R.A. (Ed.) *Techniques in Clinical Immunology* 2nd Edition Oxford : Blackwell Scientific Publications p.157-169.

Voller, A. and Bidwell, D. (1986) Enzyme-Linked Immunosorbent Assay Section B: Immunoassay. *In*: Rose, N.R., Friedman, H. and Fahey, J.L. (Eds.) *Manual of Clinical Laboratory Immunology* 3rd Edition. Washington, D.C. : American Society for Microbiology p.99-109.

Voss, K.A., Plattner, R.D., Bacon, C.W. and Norred, W.P. (1990) Comparative studies of hepatotoxicity and fumonisin B_1 and B_2 content of water and chloroform/methanol extracts of *Fusarium moniliforme* strain MRC 826 culture material. *Mycopathologia* **112**: 81-92.

Voss, K.A., Norred, W.P. and Bacon, C.W. (1992) Subchronic toxicological investigations of *Fusarium moniliforme* – contaminated corn, culture material, and ammoniated culture material. *Mycopathologia* **117**: 97-104.

Voss, K.A., Chamberlain, W.J., Bacon, C.W. and Norred, W.P. (1993) A preliminary investigation on renal and hepatic toxicity in rats fed purified fumonisin B1. *Nat. Toxins* **1**: 222-228.

Voss, K.A., Chamberlain, W.J., Bacon, C.W., Riley, R.T. and Norred, W.P. (1995A) Subchronic toxicity of fumonisin B_1 to male and female rats. *Food Addit. Contam.* **12(3)**: 473-478.

Voss, K.A., Chamberlain, W.J., Bacon, C.W., Herbert, R.A., Walters, D.B. and Norred, W.P. (1995B) Subchronic feeding study of the mycotoxin fumonisin B1 in B6C3F1 mice and Fischer 344 rats. *Fundam. Appl. Toxicol.* **24**: 102-110.

Voss, K.A., Bacon, C.W., Meredith, F.I. and Norred, W.P. (1996A) Comparative subchronic toxicity studies of nixtamalized and water-extracted *Fusarium moniliforme* culture material. *Food Chem. Toxicol.* **34**: 623-632.

Voss, K.A., Riley, R.T., Bacon, C.W., Chamberlain, W.J. and Norred, W.P. (1996B) Subchronic toxic effects of *Fusarium moniliforme* and fumonisin B1 in rats and mice. *Nat. Toxins* **4**: 16-23.

Voss, K.A., Bacon, C.W., Norred, W.P., Chapin, R.E., Chamberlain, W.J., Plattner, R.D. and Meredith, F.I. (1996C) Studies on reproductive effects of *Fusarium moniliforme* culture material in rats and the biodistribution of [^{14}C] fumonisin B1 in pregnant rats. *Nat. Toxins* **4**: 24-33.

Voss, K.A., Riley, R.T., Bacon, C.W., Meredith, F.I. and Norred, W.P. (1998A) Toxicity and sphinganine levels are correlated in rats fed fumonisin B_1 (FB_1) or hydrolysed FB_1. *Environ. Toxicol. Pharmacol.* **5**: 101-104.

Voss, K.A., Plattner, R.D., Riley, R.T., Meredith, F.I. and Norred, W.P. (1998B) *In vivo* effects of fumonisin B_1–producing and fumonisin B_1–nonproducing *Fusarium moniliforme* isolates are similar: fumonisins B_2 and B_3 cause hepato- and nephrotoxicity in rats. *Mycopathologia* **141**: 45-58.

Vudathala, D.K., Prelusky, D.B., Ayroud, M., Trenholm, H.L. and Miller, J.D. (1994) Pharmacokinetic fate and pathological effects of ^{14}C-fumonisin B_1 in laying hens. *Nat. Toxins* **2**: 81-88.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T. and Merrill, A.H., Jr. (1991) Inhibition of sphingolipid biosynthesis by fumonisins: Implications for diseases associated with *Fusarium moniliforme*. *J. Biol. Chem.* **266**: 14486-14490.

Wang, E., Ross, P.F.,Wilson, T.M., Riley, R.T. and Merrill, A.H., Jr. (1992) Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moninilifórme*. *J. Nutr.* **122**: 1706-1716.

Wang, H., Jones, C., Ciacci-Zanella, J., Holt, T., Gilchrist, D.G. and Dickman, M.B. (1996) Fumonisins and *Alternaria alternata lycopersici* toxins: sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. *Proc. Natl. Acad. Sci. USA* **93**: 3461-3465.

Ware, G.M., Umrigar, P.P., Carman, A.S., Jr. and Kuan, S.S. (1994) Evaluation of fumonitest immunoaffinity columns. *Anal. Lett.* **27(4)**: 693-715.

Wattenberg, E.V., Badria, F.A. and Shier, W.T. (1996) Activation of mitogen-activated protein kinase by the carcinogenic mycotoxin fumonisin B_1. *Biochem. Biophys. Res. Commun.* **227**: 622-627.

Weibking, T.S., Ledoux, D.R., Bermudez, A.J., Turk, J.R., Rottinghaus, G.E., Wang, E. and Merrill, A.H., Jr. (1993) Effects of feeding *Fusarium moniliforme* culture material, containing known levels of fumonisin B_1, on the young broiler chick. *Poult. Sci.* **72**: 456-466.

Weibking, T., Ledoux, D.R., Bermudez, A.J., Turk, J.R. and Rottinghaus, G.E. (1995) Effects on turkey poults of feeding Fusarium moniliforme M-1325 culture material grown under different environmental conditions. *Avian Dis.* **39**: 32-38.

Wilkins, P.A., Vaala, W.E., Zivotofsksy, D. and Twitchell, E.D. (1994) A herd outbreak of equine leukoencephalomalacia. *Cornell Vet.* **84**: 53-59.

Wilkinson, A.P., Denning, D.W. and Morgan, M.R.A. (1988) Analysis of UK sera for aflatoxin by enzyme-linked immunosorbent assay. *Human Toxicol.* **7**: 353-356.

Wilson, B.J. and Maronpot, R.R. (1971) Causative fungus agent of leucoencephalomalacia in equine animals. *Vet. Record* **88**: 484-486.

Wilson, T.M., Ross, P.F., Rice, L.G., Osweiler, G.D., Nelson, H.A., Owens, D.L., Plattner, R.D., Reggiardo, C., Noon, T.H. and Pickrell, J.W. (1990) Fumonisin B_1 levels associated with an epizootic of equine leukoencephalomalacia. *J. Vet. Diagn. Invest.* **2**: 213-216.

Wilson, T.M., Ross, P.F., Owens, D.L., Rice, L.G., Green, S.A., Jenkins, S.J. and Nelson, H.A. (1992) Experimental reproduction of ELEM *A study to determine the minimum toxic dose in ponies. Mycopathologia* **117**: 115-120.

Wu, W-I., McDonough, V.M., Nickels, J.T. Jr., Ko, J., Fischl, A.S., Vales, T.R., Merrill, A.H., Jr. and Carman, G.M. (1995) Regulation of lipid biosynthesis in *Saccharomyces cerevisiae* by fumonisin B_1. *J. Biol. Chem.* **270(22)**: 13171-13178.

Xie, W., Mirocha, C.J. and Chen, J. (1997) Detection of two naturally occurring structural isomers of partially hydrolysed fumonisin B_1 in corn by on-line capillary liquid chromatography - fast atom bombardment mass spectrometry. *J. Agric. Food Chem.* **45**: 1252-1255.

Yeung, J.M., Prelusky, D.B., Savard, M.E., Dang, B.D.M. and Robinson, L.A. (1996) Sensitive immunoassay for fumonisin B_1 in corn. *J. Agric. Food Chem.* **44**: 3582-3586.

Yoo, H-S., Norred, W. P., Wang, E., Merrill, A.H., Jr. and Riley, R.T. (1992) Fumonisin inhibition of *de novo* sphingolipid biosynthesis and cytotoxicity are correlated in LLC-PK_1 cells. *Toxicol. Appl. Pharmacol.* **114**: 9-15.

Yoo, H-S., Norred, W.P., Showker, J. and Riley, R.T. (1996A) Elevated sphingoid bases and complex sphingolipid depletion as contributing factors in fumonisin-induced cytotoxicity. *Toxicol. Appl. Pharmacol.* **138**: 211-218.

Yoo, H-S., Norred, W.P. and Riley, R.T. (1996B) A rapid method for quantifying free sphingoid bases and complex sphingolipids in microgram amounts of cells following exposure to fumonisin B_1. *Toxicol. in Vitro* **10**: 77-84.

Yoshizawa, T., Yamashita, A. and Luo, Y. (1994) Fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. *Appl. Environ. Biol.* **60(5)**: 1626-1629.

Yu, F-H. and Chu, F.S. (1996) Production and characterisation of antibodies against fumonisin B1. *J. Food Prot.* **59(9)**: 992-997.

Yu, W. and Chu, F.S. (1998) Improved direct competitive enzyme-linked immunosorbent assay for cyclopiazonic acid in corn, peanuts, and mixed feed. *J. Agric. Food Chem.* **46**: 1012-1017.

Zacharias, C., Van Echten-Deckert, G., Wang, E., Merrill, A.H., Jr. and Sandhoff, K. (1996) The effect of fumonisin B_1 on developing chick embryos: correlation between de novo sphingolipid biosynthesis and gross morphological changes. *Glycoconjugate J.* **13**: 167-175.