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Development of a Novel Monoclonal Antibody-based Immunoaffinity Column for the Detection of Aflatoxins

by

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Abstract

1G7-1E2 is a novel monoclonal antibody, created at the University of Strathclyde in Glasgow. Several IG7-1E2 antibodies were produced by poly ethylene gycol fusion of mouse myeloma cells with sensitised spleen cells isolated from Balb c/NZB F1 hybrid mice immunised intraperitoneally with aflatoxin B_1 and ochratoxin A protein conjugates. The main characteristic of these monoclonal antibodies is their ability to bind sufficiently better to all four main aflatoxins and ochratoxin A than other antibodies already on the market. Past studies have shown how useful these antibodies can be when used as diagnostic tools, such as in an enzyme linked immunoabsorbent assay, (Candlish, 1984, Prasertsilpa, 1999), however, they have not been tried and tested in other applications to date.

In this study, a set of immunoaffinity columns were produced in order to achieve one main aim; to produce a diagnostic tool that has the ability to compete with others already established on the immunodiagnostic market, whilst making use of 1G7-1E2. The results provide evidence that 1G7-1E2 has the ability to meet this aim. The immunoaffinity columns produced capture a higher amount of aflatoxin B_1, G_1 and G_2 than current columns on the market. As an additional experiment, a set of enzyme linked immunoabsorbent assays were produced in an attempt to discover the component responsible for the antibody's affinity towards other mycotoxins found in foodstuffs and feed. The outcome of this is still undetermined.

Abbreviations

$A_{240/280}$	Absorbance at 240/280 nm
Ab	Antibody
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFB_1-oxime	Aflatoxin B_1-1- (O-carboxymethyl) oxime
Ag	Antigen
APS	Ammonium persulphate
ATA	Alimentary Toxic Aleukia
ß-CD	Beta Cyclodextrin
BSA	Bovine serum albumin
DMSO	Dimethyl sulphoxide
EDAC	1-ethyl-3-(3-dimethylaminopropyl)
ELISA	Enzyme linked immunosorbant assay
FP	Fluorescence polarization
em	Emmision wavelength
ex	Excitation wavelength
FCS	Foetal calf serum
GIPSA	Grain Inspection, Packers and Stockyards Administration
HAT	Hypoxanthine-aminopterin-thymidine
HCl	Hydrochloric acid
HEPES	(N-[2-Hydroxyethyl]piper-azine-N'-[2-ethanesulfonic acid])
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HT	Hypoxanthine-thymidine
IAC	Immunoaffinity column
Ig	Immunoglobulin

kD	kilodalton
LN_2	Liquid Nitrogen
MAb	Monoclonal antibody
M	Mol
mins	Minutes
mM	Milli Mols
Nanopure	Purified water
$(NH_4)_2SO_4$	Ammonium sulphate
nm	Nanometer
nM	Nano Mols
OTA	Ochratoxin A
PAT	Patulin
PBS	Phosphate Buffered Saline
PLL	Poly-L-Lysin
ppb	Parts per billion
RPMI 1640	Cell culture medium
SD	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TMB	3,3',5,5'-Tetramethyl-benzidine
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
ZEAR	Zearalenone
µl/pw	Microlitres per well
°C	Degrees Celsius
x g	G-force

CHAPTER I

AIMS and OBJECTIVES
and
INTRODUCTION

Introduction

Some moulds which grow on foods and foodstuffs are responsible for the production of toxic secondary metabolites, mycotoxins. The name mycotoxin was derived from the Greek word "Mykes" which means fungus and the Latin word "toxicum" which means toxin or poison (Bennett and Klich, 2003). Toxic syndromes can arise in both humans and animals if these compounds are ingested and ingestion of mycotoxins is likely even in the modern world as they are occasionally found in cereals that are used to make bread and bakery products, breakfast cereals, snacks, beer and animal feeds. Many case studies involving mycotoxins have been reported worldwide. The study of toxigenic fungi and fungal toxins has taken place since the 1960's after it was discovered that a disease that killed over 100,000 turkeys in England was caused by Aflatoxin (AF), a toxin produced by *Aspergillus flavus (parasiticus)* in peanuts used to produce peanut meal (Sargeant *et al*, 1961). It became apparent with the outbreak of this disease (known as Turkey X Disease) that extensive research into mycotoxins is required to prevent disease and death in animals, and humans.

I. The Mycotoxins

After the discovery of AFs, numerous moulds were tested within laboratories for the production of toxic metabolites. Hundreds of mycotoxins have been produced within the laboratory environment since. However, only around 20 of them that occur naturally in foods and feeds produce enough toxin with sufficient frequency to cause concern in food safety. There are five taxonomic genera of moulds that are of most concern, *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps* (Smith & Moss, 1985).

In Europe and America alike, the major mycotoxins that are presently being studied include the AFs, trichothecenes, deoxynivalenol (DON), zearalenone and Ochratoxin A (OTA), which will be discussed in more detail later (www.mycotoxins.org).

Aspergillus species are known to produce toxins such as AFB_1, B_2, G_1, G_2, M_1 and M_2 and OTA, amongst others. OTA is also produced by the *Penicillium* species, as are toxins like patulin and citrinin. Species such as *Aspergillus* and *Penicillium* are generally saprophytic and often attack cereal grains and nuts when in storage, however, they can also invade in the field (Prange *et al*, 2005). Fusarium species produce trichothecenes, for example, DON, zearalenone, fumonisins and moniliformin. *Alternaria* differs slightly from the other species mentioned; it produces biologically active compounds that have questionable toxicity within mammals. These compounds are tenuazonic acid, alternariol and alternariol methyl ether. *Claviceps* toxins are the ergot alkaloids, found in ergot parasitized grasses and small grains (Tudzynski et al, 1999).

II. Disease and Mycotoxins

Various toxic effects can arise from mycotoxins when they enter the biological systems of microorganisms, plants, animals, or humans. The actual effects produced depend on the dose of toxin. A high level of toxin can result in death, a lower level in reduced egg and milk production and a low level of toxin received over a long period of time can result in fertility problems, liver damage and even cancer. This disease is called mycotoxicosis when in animals. Economically, mycotoxicosis can cause a reduced rate of growth as well as increased reproductive problems in food producing animals and livestock.

When exposed to short-term (acute) doses, AFs can cause liver damage and death in animals. Young animals such as calves, chicks and swine are more susceptible than mature ruminants and chickens. As with many diseases, young animals are more susceptible to the effects of mycotoxicosis, but older animals may also be affected. The clinical signs of chronic (and sub-acute) exposures of AFs to animals are gastrointestinal problems, reproductive problems (infertility and mutation of the embryo), anemia and jaundice (a direct result of liver damage).

A common disease associated with swine is mycotoxic nephropathy, caused by OTA. OTA is classified as a carcinogen and is thought to be immunosuppressive. It can also cause liver damage, necrosis and hemorrhage when animals are exposed to high doses. It is a known fact that ruminants are more resistant to OTA than swine, possibly because of degradation in the rumen (Miller, 1979).

The trichothecenes include DON, a toxin that generally causes gastroenteritis, feed refusal, necrosis and hemorrhage in the digestive tract, destruction of bone marrow, suppression of blood cell formation, and suppression of the immune system (Bennett and Klich, 2003). Clinical symptoms shown after ingestion of the toxin are gastrointestinal problems, loss of appetite, vomiting, bloody diarrhea, reproductive problems, abortions and death. In poultry, mouth lesions frequently develop as well as extensive hemorrhaging in the intestines.

Fumonisins are not as carcinogenic as AFs are; however, they are often found on corn and have been known to cause high incidences of human esophageal cancer in areas where corn is part of the main diet (Norred and Voss, 1994). Several other diseases are caused by fumonisins, such as, equine leukoencephalomalacia, porcine pulmonary edema and liver cancer in rats (Bird *et al*, 2002).

Zearalenone causes many reproductive problems, particularly when high doses are exposed to swine. It disrupts estrus cycles and causes vulvovaginitis in females and feminization of males (Lawlor and Lynch, 2001 & Bristol and Djurickovic, 1971). Monogastric animals are more susceptible to the effects of zearalenone than ruminants are, again, possibly due to ruminants' ability to degrade materials in the rumen.

Wide ranges of adverse effects caused by mycotoxins in animals have been documented. The range includes embryonic death, inhibition of fetal development, abortions and deformities in developing embryos (teratogenicity). Dysfunctions of the nervous system have also been noted, including, tremors, weakness of limbs,

uncoordinated movement, staggering, muscular collapse, and brain tissue destruction (which leads to loss of comprehension, GIPSA, 2006). Symptoms such as seizures, excess salivation, gangrene of limbs, ears and tails have been reported also (Semple *et al* 1989). Cancer of the liver, kidney, urinary tract, digestive tract and lungs are caused by several mycotoxin (Carlson and Ensley, 2003 & WHO, 1976).

It is more difficult to find documentation of the effects of mycotoxins on humans than it is on animals. Mouldy bread and rice grains have been known to cause many cases of food poisoning in the past, resulting in the death of children (WHO, 1999). Some toxins produced by the fungi in the genera *Claviceps* and *Fusarium* have caused diseases like ergotism (Pitt & Hawking, 1986) and alimentary toxic aleukia (ATA (Matossian, 1981)) in humans. Large numbers of people have contracted these diseases previously and their symptoms could be readily observed (Matossian, 1981). For example, ATA was observed in Russia during World War II. Because of the shortage of food, people were forced to eat mouldy grains of cereal (Abbott, 2002). The disease causes bone marrow synthesis to cease (Peraica, *et al*, 1999), damage to the hematopoietic system and loss of blood-making capacity, severe hemorrhaging, anemia and death.

With reference to AFs, acute doses of these toxins in humans can cause liver damage, edema and death. In areas where liver cancer and hepatitis B have been found to be endemic, high levels of AF have been found (Peers *et al*, 2006). The human kidney disease Balkan Endemic Nephropathy has been associated with OTA (Tatu *et al*, 1998). Zearalenone was associated with an outbreak of precocious pubertal changes in young children in Puerto Rico and is also thought to be one possible cause of human breast cancer (Arukwe *et al*, 2009; Dees *et al*, 1997). It is also a known endocrine disrupter. *Fusarium moniliforme* and some fumonisins have been linked to esophageal cancer in parts of Africa, Italy and China (Clements *et al*, 2003). In some parts of China, moniliformin is known to have contaminated corn used for human consumption. It is thought that Keshan Disease, a degenerative heart disease is the result of the consumption of contaminated corn (Sørensen *et al*, 2007). The disease involves myocardial necrosis. Immunotoxicity is another affect caused

by *Fusarium* species. The T2 Toxin has often been found to cause diseases such as leukemia as it is highly immunosuppressive. It is commonly found where there is a *Fusarium* species present **(**Hsia *et al*, 1983**).** DON can cause elevated immunoglobin A (IgA) levels in mice, which leads to kidney damage (Jia *et al*, 2004**).**

It is because of this, that the human disease glomerulonephritis or IgA nephropathy is considered a possible outcome of DON exposure (Pestka, 2003).

It is not as easy in humans as it is in animals, to find evidence that toxins are the causative factors of certain diseases, however, studies are always being carried out in this field. Suspected cases of mycotoxicosis, for example, can be confirmed by reproducing the clinical disease during a feeding trial using the suspected ration, or by detection of a known mycotoxin in the ration or tissues of animals consuming the ration. However, this is a slow and expensive process and other more rapid and cheaper methods are always being sought, for example, by using Enzyme Linked Immunoaffinity Assays (ELISA) to detect the toxins in feed prior to its release to consumer groups (Carlson and Ensley, 2003). Considerable funds are made available for monitoring, awareness programs, specific risk projects, genetic research and biological research. An integrated project approach of "farm to table" is possible with the co-operation of a number of countries and various organizations, including universities, government institutions, food and safety organizations, etc. The collaboration of numerous projects form 'cluster' projects such as "Concerted Action: Quality Control Measures in the Production and Processing Chain to Reduce *Fusarium* Mycotoxin Contamination of Food and Feed Grains" (Clark, 2003).

III. Toxigenic Fungi

The production of any mycotoxin is affected by various factors, including, temperature, level of moisture, humidity, pH, competitive growth with other microorganisms and stress on plants such as drought and damage to seeds from insects and farming appliances. Corn, cottonseed, peanuts, wheat and barley are just a few commodities that are frequently contaminated by mycotoxins (Diekman & Green, 1992).

Aspergillus flavus, *Aspergillus parasiticus* and *Aspergillus nomius* all produce AFs (*Aspergillus flavus* also produces cyclopiazonic acid (Hedayati *et al*, 2007)). OTA can be produced by *Aspergillus ochraceus*, *Aspergillus niger* and *Penicillium verrucosum* (O'Callaghan *et al*, 2003). Patulin can be produced by *Penicillium expansum*, as well as other *Penicillium* and *Aspergillus* species (Paster *et al*, 1995). Depending on the geographical origin of a strain, zearalenone, DON and nivalenol can all be made by *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium crookwellense* (Sydenham *et al*, 1991). The fumonisins are produced by *Fusarium moniliforme*, *Fusarium proliferatum* and *Fusarium subglutinans* (Nelson *et al*, 1992).

As it is impossible to completely protect crops from contamination of moulds, it is necessary to have methods available for detecting and quantifying the toxigenic fungi. Detection methods may focus on either a particular toxin, or the mould from which it is produced. Detecting the presence of a mould first may help to decide if the quality of the commodity in question is of an acceptable level for animal or human consumption. Early detection of moulds within foods and feeds can be a good indicator of the mycotoxins that may be encountered.

The European Mycotoxin Awareness Network (www.mycotoxins.org) gives information on European legislation with regards to mycotoxins, specifically AFs and OTA. Each mycotoxin detected within feeds and foods must be under a certain

level for the foodstuffs to be deemed safe for consumption. European countries and the USA have laws which try to prevent outbreaks of mycotoxicosis within their countries, however, there are many countries (third world countries in particular) that still do not possess the technology or "know how" to apply such laws of their own. The Table below is the result of information provided by the World Health Organization (www.who.int, 1997). It gives an example of toxin levels allowed in some foods and feeds.

Table 1.0 Toxin levels in foods and feedstuffs, adapted from information provided via the World Health Organisation

Aflatoxin	Tolerance Level (μg/Kg)	Food/Feed
B_1	5.00	Feed for Dairy Cattle
M_1	0.05	Milk
$B_1+G_1+B_2+G_2$	15.00	Raw Peanut for Human Consumption
$B_1+G_1+B_2+G_2$	10.00	Processed Peanut for Human Consumption

There are regulations set already for contamination levels of certain mycotoxins produced on various types of nuts and dried fruits, however, there is an ongoing investigation (Murphy *et al*, 2006) into what would be considered a safe level of contamination within foods such as apples and spices, and in drinks such as milk and coffee.

Since high levels of exposure to the previously mentioned mycotoxins have been found to cause a range of adverse health effects in laboratory animals, there is a concern that similar effects may occur in humans through long-term consumption of foods and beverages that contain relatively high levels of these contaminants.

Legal limits for their presence in infant foods apply only to foods intended for infants or young children up to three years old and comply with nutritional requirements set down in European Union law. Tolerable Daily Intakes (TDIs) have been set by scientific committees such as the former SCF (EC Scientific Committee for Food)

and are based on threshold levels, identified during toxicological studies, below which the toxins are considered to not cause an adverse effect (Boon *et al*, 2009). A TDI represents an estimate of the amount of a contaminant, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risks (WHO, 2004).

Figure 1.0, overleaf, is a graph showing the worldwide total AF limits and a map (Figure 1.1) showing the countries that employ regulations on mycotoxin levels in foods and feedstuffs.

Figure 1.0 Number of countries that have mycotoxin regulations

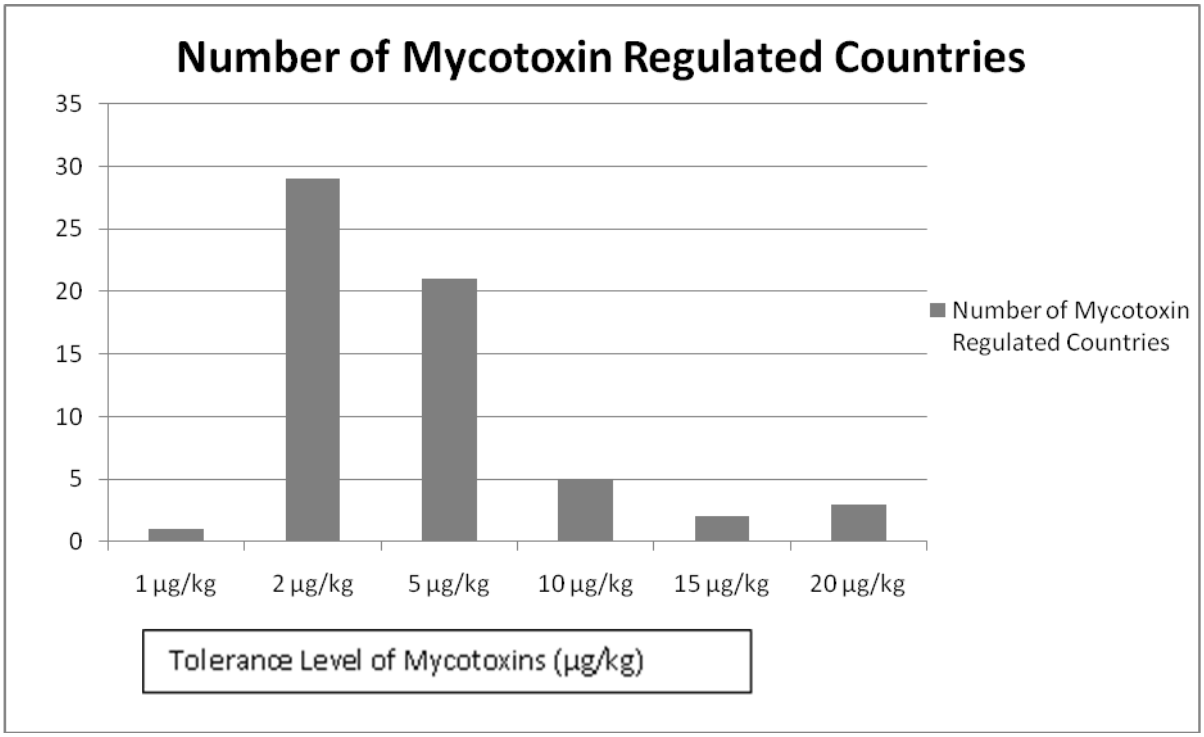
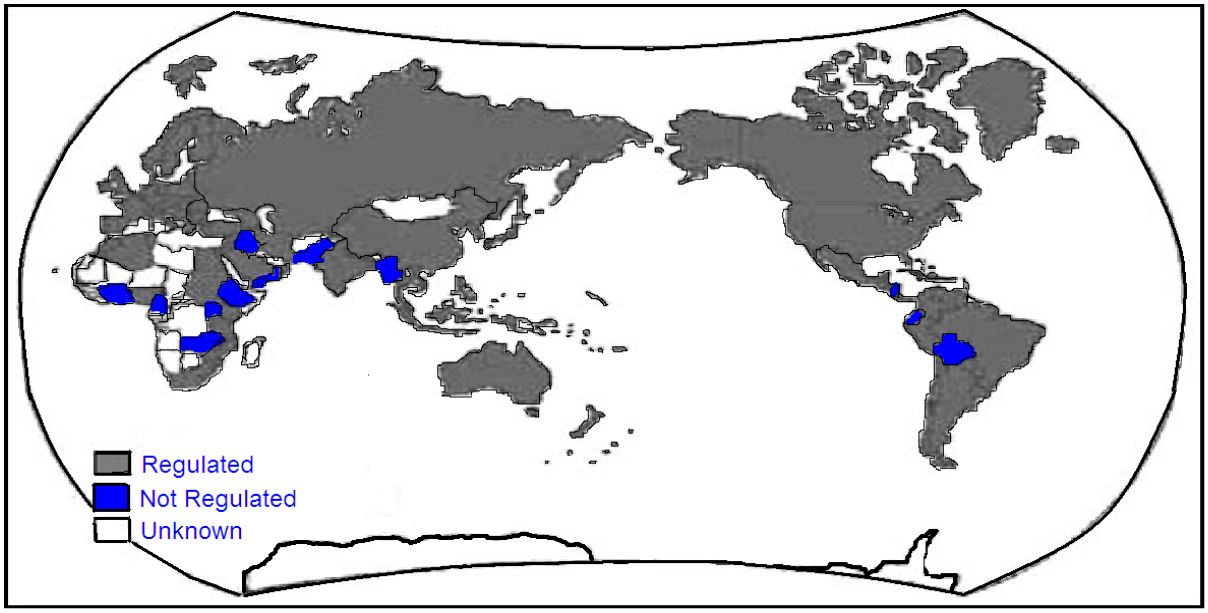


Figure 1.1 Map of Mycotoxin Regulatory Regions



It is possible to grow some of the toxin producing fungi on selective media, which makes the colonies visible to the naked eye. Several media are available for the detection of toxigenic fungi (e.g. Sabouraud Broth, RPMI 1640, Dulbeccos' Modified Eagle Medium (DMEM), along with nutrients such as glucose and yeast) particularly some of those within the *Aspergillus* species. To obtain a sample of mycotoxin, however, various analytical methods can be used. To obtain a mycotoxin from a sample of food, for example, an organic solvent, solvent mix or sometimes just purified water can be used for extraction purposes. Techniques such as immunoaffinity chromatography may be used to obtain purified toxins.

Currently, there are three types of Immunochemical methods available for detection of AFs: ELISAs, IACs and Radio Immunoassays (RIA). There is also Thin Layer Chromatography (TLC) which can detect AF levels as low as 1ng/g (European Mycotoxin Awareness Network; www.mycotoxins.org). Some TLC methods require a two step toxin extraction technique to be carried out prior to AF detection. This usually involves a mix of solutions (ether, methanol and water for the first step, and chloroform and acetone for the second step). One group of scientists, Dunne *et al* (2003), took the more unusual approach of using dichloromethane-1 M hydrochloric acid (10:1) for the purpose of multi-toxin extraction, with gel permeation chromatography being used for the clean-up.

After extraction from foodstuffs, AF must be 'cleaned-up' to get a pure a sample as possible. This can be done by using solid phase extraction (SPE) with IACs, C_{18} cartridges, or other silica columns, to name but a few. There are a number of manufacturers now who produce these SPEs, each with their own slight differences, never the less, they all serve the same purpose; purification of toxin. HPLC can be used to determine the toxins extracted from foodstuff. There are two phases of HPLC, normal and reversed-phase. Since some AFs (e.g. AFB_1 and AFG_1) are low emitters of fluorescent light (which is what HPLCs detect and quantify), these AFs must be boosted with fluorescence enhancers prior to using an HPLC. Fluorescence enhancers include Trifluroacetic acid (TFA), Bromine and β-cyclodextrine (β-CD). The columns used in these HPLCs normally contain C_{18} material (stationary phase),

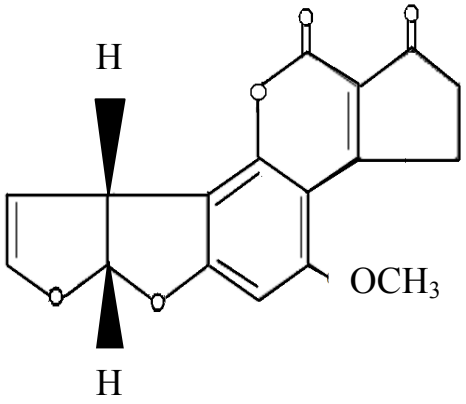
and a mobile phase (normally a solvent/water mix, e.g. methanol or acetonitrile) is used to push the toxin through the column, allowing it to be quantified via fluorescent light detection.

Mass spectroscopy (LC-MS = combination of Liquid Chromatography and Mass Spectometry) can also be used to detect AFs. Legal limits for foodstuffs, such as peanuts, were established using a mass spectrometer as they are highly specific and sensitive enough to pick up low level concentrations of toxins. However, they are expensive to use and require much expertise when being used (European Mycotoxin Awareness Network; www.mycotoxins.org, Wilson *et al*, 2002).

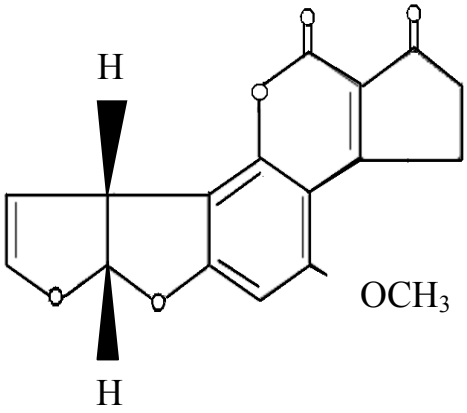
IV. Aflatoxins

There are four major AFB_1, B_2, G_1 and G_2, plus two additional secondary metabolites, M_1 and M_2, that are produced in lactating animals affected by AF (Van Egmond, 1989). AFB_2 and G_2 are the dihydroxy derivatives of AFB_1 and G_1. AFM_1 is 4-hydroxy AFB_1 and AFM_2 is 4-dihydroxy AFB_2. Shown overleaf are the structures for some of the various AFs discussed.

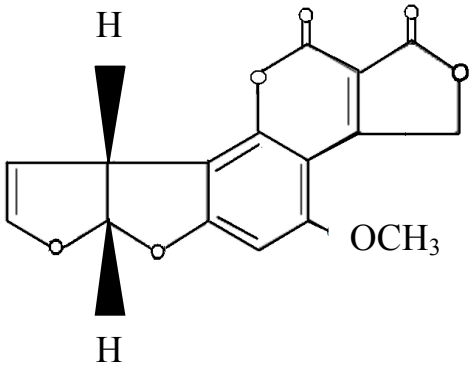
Figure 1.2 Structure of Aflatoxins; AFB_1, AFB_2 , AFG_1 , AFG_2 and AFM_1.



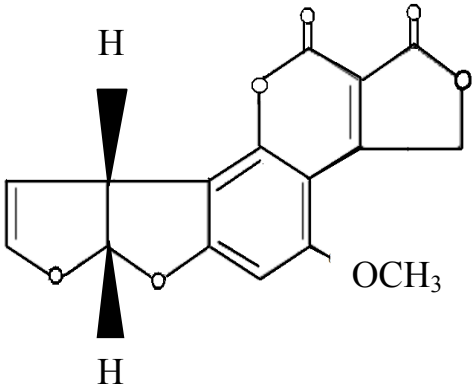
(a) Aflatoxin B_1



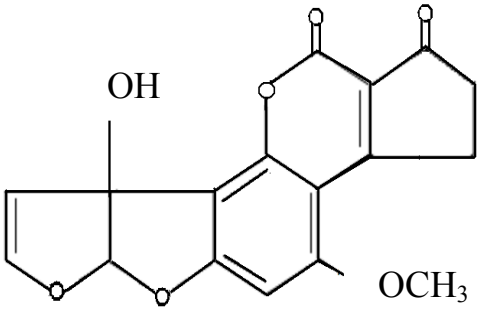
(b) Aflatoxin B_2



(c) Aflatoxin G



(d) Aflatoxin G_2



(e) Aflatoxin M_1

Out of all the AFs, AFB_1 is the most carcinogenic and estrogenic. Predominately, it is known to cause cancer of the liver in both man and animals (Barrett, 2000).

AFB_1 is a hepatocarcinogen, which makes it easy to penetrate the skin. Because of this, and the fact that it is easy to grow *Aspergillus* species readily, as well as producing and storing AF cheaply, AF is considered a bioterrorism agent (McGovern *et al*, 1999). It could have dramatic affects on crops, causing economic problems and general ill health and even death among the contaminated populations.

It is known that the cytochrome P450 can convert AF into an epoxide, which then reacts with nucleic acids. This then leads to depurination of DNA and RNA as the epoxide reacts with guanine. Protein and DNA synthesis is then inhibited in the most active tissues, such as the liver, the intestines and in the bone marrow (Aflatoxins, essential data, 1999).

The Food and Drug Administration (FDA) has established levels of AF that are allowed to be present in animal foodstuffs (table 1.1). Any toxins found to be above these levels must not be used for animal consumption (GIPSA, 2006).

Table 1.1 Tolerance levels of Aflatoxin in Europe (GIPSA, 2006)

Tolerance Level (ppb)	Feed and Animal
20	For corn and other grains intended for immature animals (including immature poultry) and for dairy animals, or when its destination is not known
20	For animal feeds, other than corn or cottonseed meal
100	For corn and other grains intended for breeding beef cattle, breeding swine, or mature poultry
200	For corn and other grains intended for finishing swine of 100 pounds or greater
300	For corn and other grains intended for finishing (i.e. feedlot) beef cattle and for cottonseed meal intended for beef cattle, swine or poultry.

The legal toxin levels for foodstuffs for human consumption for Europe can be obtained via the European Mycotoxin Awareness Network website (www.mycotoxins.org). Transient effects of AFB_1 can be produced when less than 10 μg/Kg of toxin per day is ingested. From epidemiological studies of human outbreaks of aflatoxicosis, it has been shown that a minimum of 50 μg/Kg of toxin per day is required to show significant clinical effects (Aflatoxins, essential data, 1999). Technologies such as HPLC, reverse-phase liquid chromatography and fluorescence polarization (FP) immunoassays can be used to quantify the amount of AF in certain foodstuffs (Nasir & Jolley, 2002, Chiavaro; *et al*, 2001 and Sartir and Zakhia, 2004 and Maragos, 1997). MAbs against AF have been produced for research purposes and used to develop Immunoaffinity Columns (IAC's) and ELISAs, (Candlish, 1987; Wang *et al*, 2001).

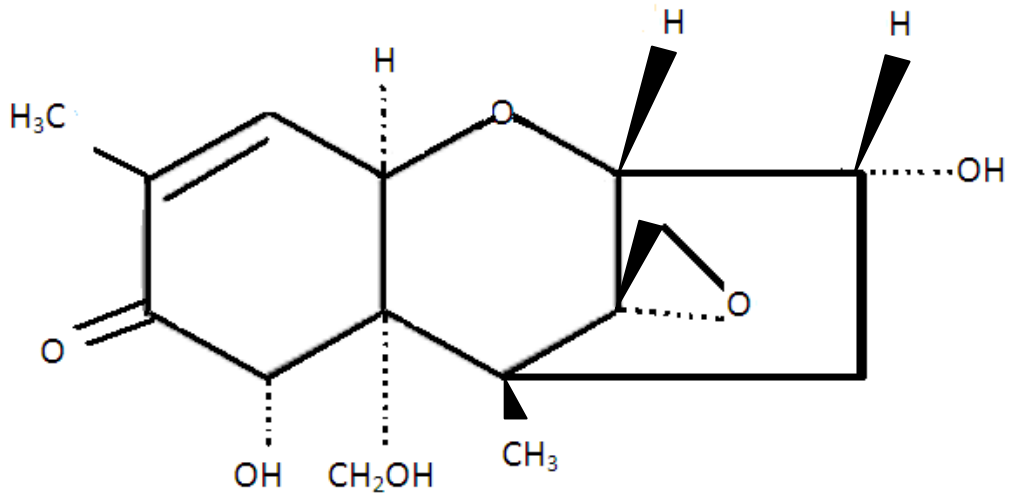
There was a project carried out by Maragos *et al* (2004) to compare FP assays with ELISAs, using DON as the test toxin. FP assays do not require a reaction with an enzyme and separation of the bound and free label is not required, as they are with ELISAs. Consequently, FP assays can eliminate the separation (wash) required of many ELISAs and can be performed as solution phase assays. In FP assays, the rate of rotation of a fluorophore is measured rather than fluorescence intensity. Polarization has the advantage that it is only minimally affected by solution opacity or colour as it is not directly dependant on the concentration of the fluorophore as it is a dimensionless process.

However, the size of the fluorophore does affect the results and, similar to ELISA methods, the smaller molecules tend to produce a signal that is inversely proportional to the toxin content. When the DON FP assay was compared to HPLC, the results from each experiment agreed with each other, suggesting that HPLC and FP assays are as good as each other for use in the laboratory (Maragos, Jolley and Nasir, 2001). As for IACs, the advantages of using them are that they allow for the direct coupling with HPLC, are highly selective and offer efficient isolation, concentration and purification of the test toxin.

V. Deoxynivalenol

DON is also known as vomitoxin as it induces vomiting in swine (Johnson *et al*, 1997). It is a low molecular weight metabolite (figure 1.3) of the tricothecene group, which is often found in barley, wheat and maize. DON is highly toxic and has been noted to cause many symptoms as well as inducing vomiting (Pestika, Islam and Amuzie, 2008).

Figure 1.3 Structure of DON

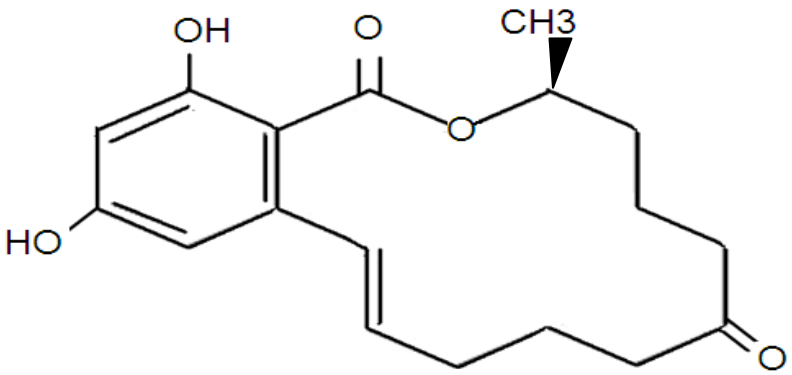


As with AFs, MAbs are available for DON, as are techniques to quantify the toxin as it is one of the major causes of loss of business within the agricultural industry (Maragos and Plattner, 2002, Maragos and McCormack, 2002, and De Saeger *et al*, 2002).

VI. Zearalenone

Zearalenone is associated with hyperestrogenism and other reproductive disorders in animals (Richardson *et al*, 1984). It can be found in many food products and drinks such as beer (increasing estrogen levels in the male population that more commonly drinks this sort of beverage and inducing feminization in regular drinkers). It is a toxin from a resorcylic acid lactone group, making it a good structure to bind protein to. The structure of zearalenone is shown in figure 1.4.

Figure 1.4 Structure of Zearalenone

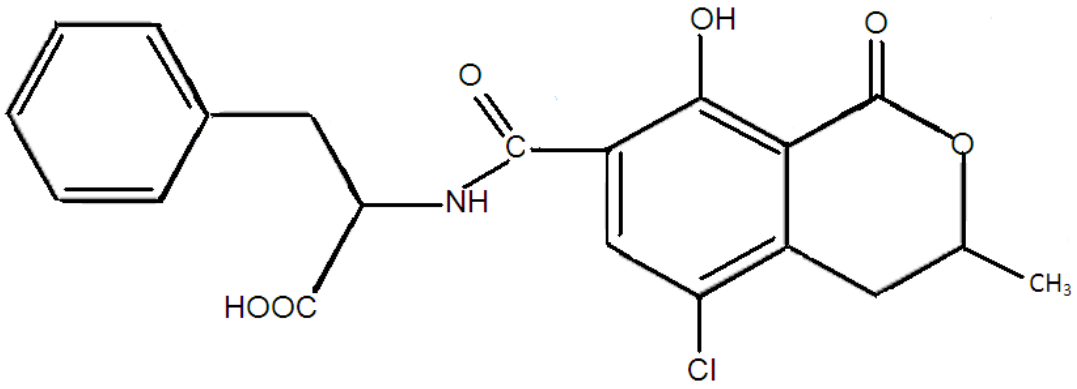


Similar diagnostic tests have been produced for zearalenone as have for the other mycotoxins mentioned (Maragos and Kim, 2004, Kruger *et al*, 1999, De Saeger et al, 2002).

VII. Ochratoxin A

OTA is a known nephrotoxin and carcinogen (Mally *et al*, 2007). It can be found in a wide range of commodities; however, it is mainly found in cereal products. Cereals account for between 50% and 80% of the average consumers intake of OTA (Zohair & Salim, 2006). Again, much the same tests are available commercially for OTA as for DON and the AFs, with the corresponding antibodies (Abs) (Shim; *et al*, 2004, Checovich, *et al*, 1995, De Saeger; *et al*, 2002).

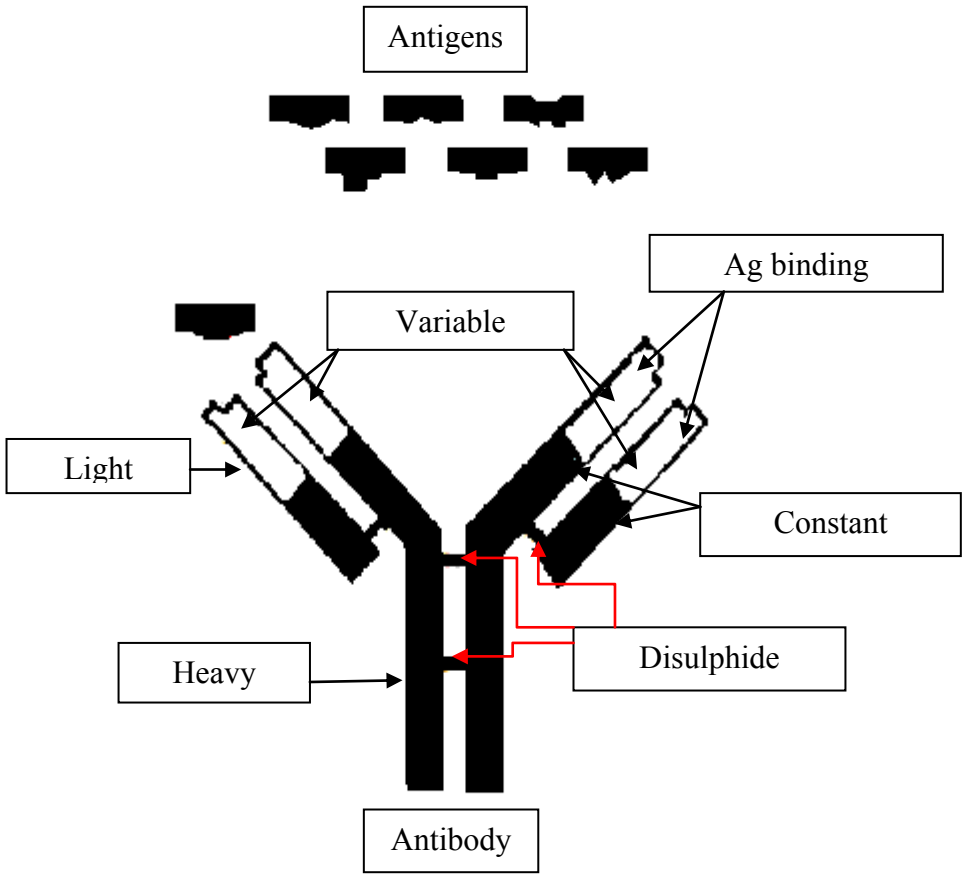
Figure 1.5 Structure of OTA



VIII. Anti-aflatoxin antibodies

Abs are considered to be the indispensable tools of biomedical research. They are widely used for experimental and therapeutic purposes as they have an extraordinary specificity for binding antigen (Ag), which is matched with their ease of use and adaptability (Barrett, 2000 & Yu *et al*, 1988). Abs are glycoproteins, termed immunoglobulins (Ig), which are circulated around the body via plasma cells. They are produced as part of an immunological response to the introduction of foreign bodies into the recipients body. There are five classes of immunoglobulins, IgA, IgG, IgM, IgD and IgE. The Abs are made of two heavy chains and two light chains (figure 1.6), which are bound by disulphide bonds. The amount of Ab production at any one time is the direct result of an antigens (Ags) immunogenicity, which is dependent on the size of a molecule. For example, haptens, such as mycotoxins (molecules with a molecular weight lower than 1000) are not large enough to be antigenic, however, they can attach to a larger protein carrier, making themselves immunogenic (Xiao *et al*, 1995). Just the right amount of haptens attached to such a carrier can illicit an immune response. On the surface of the antigenic molecules lie epitopes. It is these regions that the Ab recognises and binds to in a non-covalent manner. It is essential, therefore, that mycotoxins are covalently bound to a protein carrier. The conjugation also prevents any kind of toxic action against the immune system of the immunised animal.

Figure 1.6 Structure of an Antibody; Each Ab contains two heavy and two light chains. The variable regions are what make each Ab different (via somatic recombination of the V, D and J genes). The Disulphide bonds hold the Ab structure together. The Ag binding fragment is the region that binds to the specific Ag (the immune system works towards producing an Ab for each Ag).



(i) Antibodies and their uses

All Abs are extremely specific. Each and every Ab binds to, and attacks a particular Ag. When Abs become activated by the occurrence of a disease, they continue to show resistance against that disease. For example, the Abs to *Varicella zoster*, which causes chicken pox (Talukder *et al*, 2005).

MAbs are particularly valuable to modern medicine. It is the specificity of these Abs that make them so versatile in their uses. For example, they do not only help protect against disease (e.g. via vaccines), but they also help to diagnose disease such as Hepatitis, Human Immunodeficiency Virus (HIV) and cancer (via diagnostic tools such as ELISAs), and can be used to detect the presence of drugs, alcohol, viral and bacterial products in the blood (Takahashi *et al*, 2005; Husson *et al*, 1990; Ward *et al*, 2006 and Diamandis, 1990).

Disease therapy can involve the use of "targeted" MAb therapy. This specific therapy is used to treat diseases such as rheumatoid arthritis, (Feldmann & Maini, 2001) multiple sclerosis (Doggrell *et al*, 2003) and many forms of cancer, including non-Hodgkin's lymphoma (Plosker and Figgitt, 2003) colorectal and breast cancer (Vogel *et al*, 2001). Unlike drugs, MAbs cause very little or no side effects due to them having such specific targets.

Some diseases (or more specifically, immune deficiencies) cause a person, or animal to have only a small number of Abs, if any at all. This includes X-linked agammaglobulinemia and hypogammaglobulinemia **(**Pahwa *et al* 1987**).** One way of treating these types of diseases is to pool MAbs from serum and transfer these to the patient, providing them with what is termed as passive immunity. MAbs are very much the focus of many research laboratories, because of their diversity of uses. To obtain MAbs in a pure state is quite a task; however, it is not impossible. For example, by injecting a laboratory animal (e.g. a mouse) with an Ag and then, collecting the Abs formed from the blood antiserum. However, there are a couple of problems with this method; it provides only a small amount of Ab and the antiserum they come from contains unwanted proteins.

Large amounts of pure Abs can be produced by obtaining cells that produce Abs naturally. Also, there are cells in existence that can grow continually in cell culture. Yet, another method of creating MAbs is to produce a hybrid that combines the characteristic of immortality with the ability to produce the sought after protein (Nickerson *et al*, 1983).

A hybridoma is a tumour cell that has been fused with an Ab producing mammalian cell. Tumour cells have the capability of consistently replicating. MAbs are so called because they come from only one type of cell, the hybridoma. Abs derived from preparations containing a mixture of cells are termed polyclonal abs (associated with more conventional methods).

In 1984, Köhler, Milstein and Jerne were awarded a Nobel Prize for their work, that took place in 1975, involving the use of mice to produce MAbs (Cole *et al*, 1984; www.nobelprize.org). However, the first MAb to reach the market was Johnson & Johnson's Orthoclone OKT3 (muromonab) in 1986 (Business Insights Report, www.piribo.com, reported 2006).

An example of how to create MAbs, is to take a myeloma cell line (tumour of the bone marrow) and fuse its cells with Ab producing mammalian spleen cells (figure 1.7). The resulting hybridoma's are capable of producing large amounts of pure MAbs.

Figure 1.7 Overview of MAb production; A mouse is immunized by injection of Ag X to stimulate the production of Abs targeted against X. The Ab forming cells are then isolated from the mouse's spleen. MAbs are produced by fusing single Ab-forming cells to tumour cells grown in culture (hybriomas). Each hybridoma produces relatively large quantities of identical Abs. By allowing the hybridoma to multiply in culture, it is possible to produce a population of cells, each of which produces identical Abs. These Abs are called "monoclonal antibodies" because they are produced by the identical offspring of a single, cloned Ab producing cell. Once a MAb is produced, it can be used as a specific probe to track down and purify the specific protein (Ag) that induced its formation.

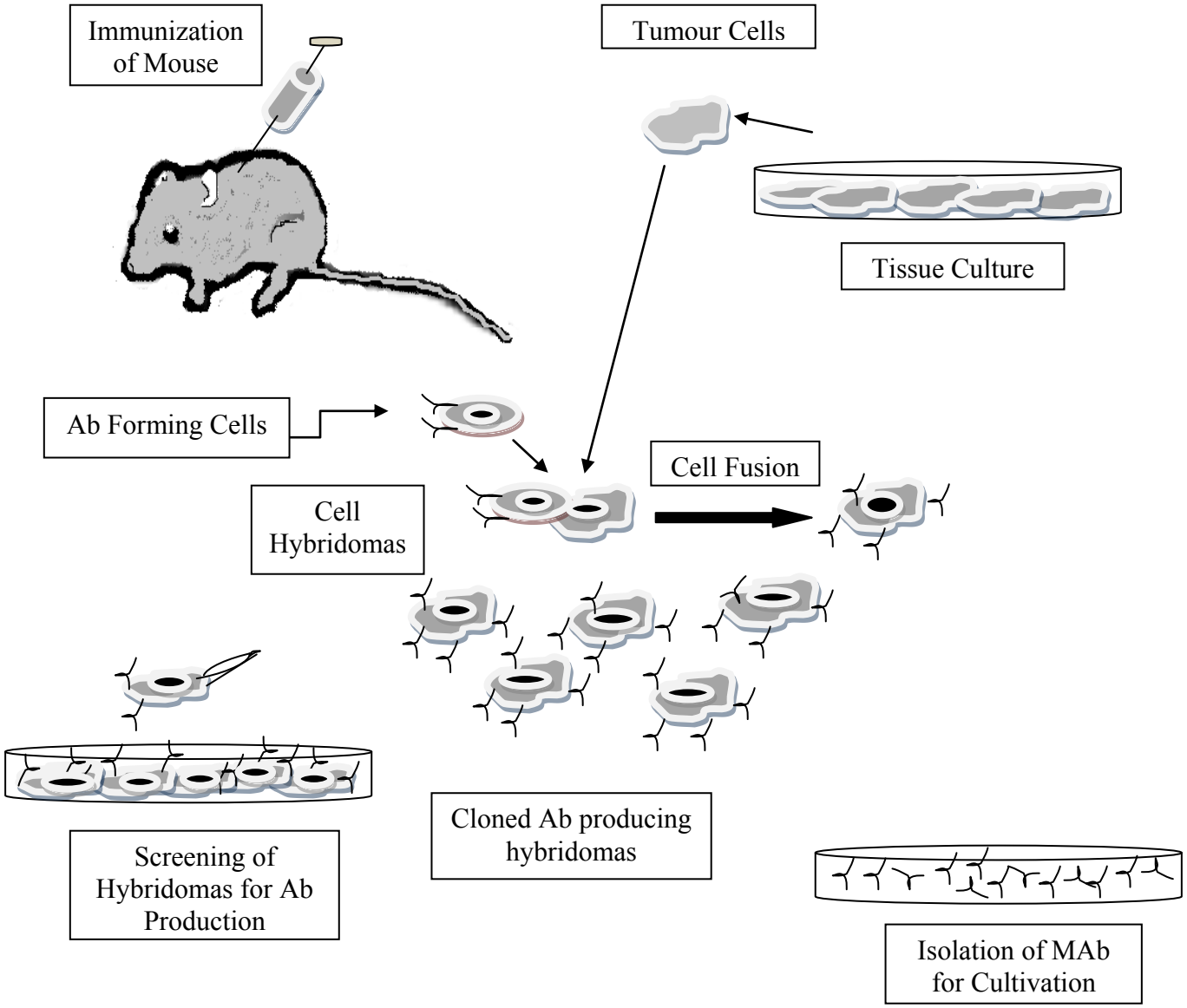
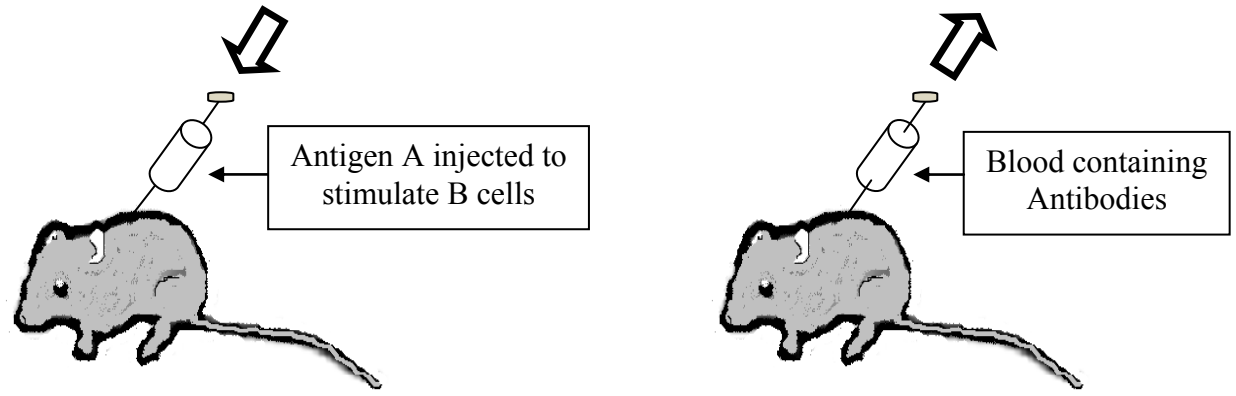
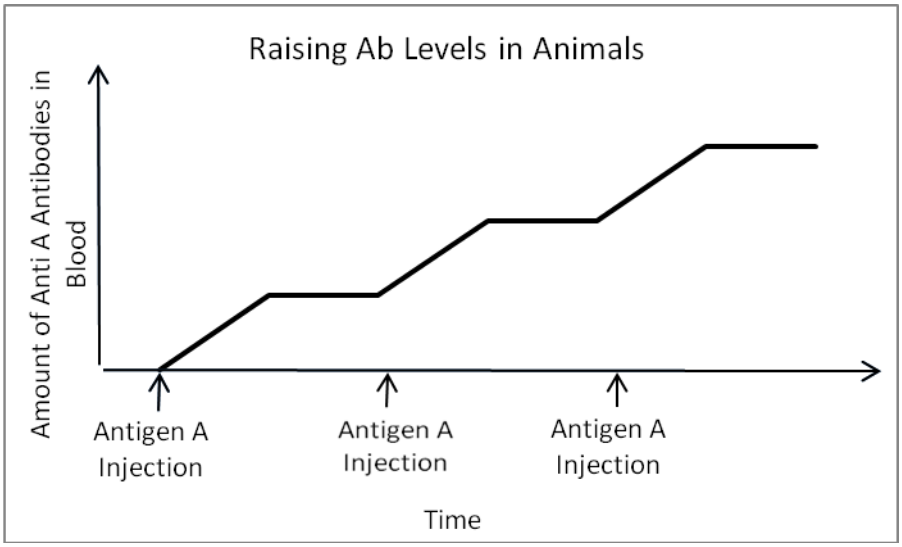


Figure 1.8 Developing Antibodies in Animals; Ag A is injected into the animal (often a mouse) to produce Abs. Anti-A Abs are produced in abundance when repeated injections of the same Ag are given over several weeks; thereby stimulating specific B cells, which in turn start Ab production. The blood will contain a variety of Anti-A Abs because various B cells will be stimulated. Each Anti-A Ab will bind A in a different way. The graph shows how, over a period of time, Ab production increases. As it suggests, there can be many Abs produced in response to Ag, however, some will bind better than others to the target Ag. By singling these Abs out using purification methods, it is possible to obtain a good quality Ab for use in diagnostic tools.





(ii) History of Antibodies

The study of Abs goes as far back as 1890 when Emil Von Behring and Shibasaburo Kitasato described Ab activity against diphtheria and tetanus toxins. They derived the theory of humoral immunity, and proposed that a mediator in serum could react with a foreign Ag (Canadian Medical Association Journal, 1931). This theory encouraged Paul Ehrlich to propose the side chain theory for Ab and Ag interaction in 1897. His hypothesis was that receptors (side chains) on the surface of cells could specifically bind to toxins, in a kind of "lock-and-key" interaction and that this binding reaction was the trigger for the production of Abs (Winau *et al*, 2004). However, other scientists believed that Abs existed freely in the blood. In 1904, for example, Almroth Wright suggested there was a process (he called opsonization) whereby soluble Abs were coated in bacteria as a manner of labelling them for the purpose of phagocytosis (Silverstein, 2003).

In the 1920s, Michael Heidelberger and Oswald Avery observed that Ags could be precipitated by Abs. They later showed that Abs were made of protein (Van Epps, 2006). It was John Marrick, in the late 1930's who examined the Ag-Ab interactions more thoroughly, paying particular attention to biochemical detail (Marrack, 1938). However, it was not until the 1940's, before the next main step was achieved...Linus Pauling confirmed the lock-and-key theory proposed by Ehrlich back in 1897. He did this by showing that the Ag-Ab interactions are dependant on their shape, more so than their chemical composition (Pauling, 1940). It was Astrid Fagreaus who discovered that B cells (in the form of plasma cells) were responsible for producing Abs in 1948 (Silverstein, 2004).

Gerald Edelman and Joseph Gally discovered (in the early 1960s) that Abs have a light chain in their structure (Edelman *et al*, 1962) much the same as the protein described by Henry Bence-Jones in 1845 (Stevens *et al*, 1991). Although, it was Gerald Edelman who went on to discover that Abs are composed of heavy AND light chains, linked by disulphide bonds. Ab binding (Fab) and Ag binding regions (Fc) of IgG were described by Rodney Porter (Raju, 1999). Jointly, these scientists were awarded the 1972 Nobel prize for characterizing the structure of IgG (Raju, 1999).

Other Igs were researched in the 1960s (Tomasi, 1992; Barra *et al*, 2000; Johansson, 2006). Somatic recombination of Ig genes were identified by Susumu Tonegawa in 1976 (Hozumi and Tonegawa, 1976), which showed the vast diversity of Abs.

(iia) Antibody Structure

The Greek letters: α, δ, ε, γ, and μ are used to represent the five types of mammalian Ig heavy chain. The type of heavy chain (IgA, IgG, IgM, IgD, or IgE) defines the class of Ab. Heavy chains differ in size and composition, for example, α and γ contain approximately 450 amino acids, while μ and ε have approximately 550 amino acids (Janeway *et al*, 2001).

The heavy chains γ, α and δ have a constant region of three Ig domains, as well as a hinge region (for flexibility). The μ and ε chains have a constant region composed of four Ig domains. As for the variable region of heavy chains, these differ in Abs produced by different B cells, but for Abs produced by a single B cell (or a B cell clone), they are the same. These regions are composed of a single Ig domain, and are around 110 amino acids in length (Van de Winkel & Capel, 1993; Janeway *et al*, 2001).

There are two types of light chain in mammals, lambda (λ - located on chromosome 22 (Erikson *et al*, 1981)) and kappa (κ - located on chromosome 2 (Malcolm *et al*, 1982)), each with one constant and one variable domain. These chains are around 211 to 217 amino acids in length. Each and every Ab contains two identical light chains, but only one type of light chain, κ or λ, is present per Ab.

The Fab (Fragment antigen binding) region is the site that binds antigen. It is has one constant and one variable domain from each Ab heavy and light chain (Putnam *et al*, 1979). The F_V region (or paratope) is the most significant part for Ag to Ab binding process. There are three variable loops (complementarity determining regions, or CDRs) on each of the heavy and light chains which also partake in the Ab binding to the Ag.

The Fc (Fragment, crystallizable), which is at the base of the Ab, plays a role in modulating immune cell activity and is composed of two heavy chains that, depending on the class of Ab, have either two or three constant domains (Janeway *et al*, 2001). The Fc region can bind to various proteins on an Ag, soliciting an immune response (Barclay, 2003), for example, binding to proteins such as cell receptors and complement components allows for processes such as cell opsonization, lysis and degranulation of leukocytes (Woof & Burton,2004; Heyman B, 1996). When producing direct-conjugated antibodies, the Fc region is the part of the Ab that is labeled with an enzyme, or fluorophore. It is also the region that anchors the Ab to the plate in an ELISA, as well as the part that is seen by secondary Abs in immunoprecipitation, immunoblots and immunohistochemistry processes.

IgM is the first Ig to illicit an immune response. This is because its Fc portions are capable of activating the classical complement pathway by binding to the C1 component, the initial enzyme in the pathway responsible for specific Ab activation. IgM is found on the surface of B lymphocytes and B cell receptors, in monomeric form (Grey *et al*, 1971).

IgA is primarily formed of mucosal associated lymphoid tissues (MALT) and is mostly found in body secretions, such as saliva, mucous and tears (Cesta, 2006). Internal body surfaces are protected from the environment (bacteria and viruses) by these mucous membranes. IgA is a dimer, with four epitope binding sites, and a secretory component to help protect it from digestive enzymes in secretions. It is the Fc portion of this Ab that binds to mucus components and assists in trapping microbes trying to gain access to the body. IgA can activate the Mannan-binding lectin complement pathway (similar to the classical complement pathway, but with a protein that differs in activation to C1q (Roos *et al*, 2001)), as well as the alternative complement pathway (only specific antigens can activate this pathway, but it does not require specific antibodies).

IgD is a serum Ab and a monomer, with two epitope binding sites. These Abs are generally seen on the surface of B lymphocytes as a B cell receptor and are thought to play a role in eliminating B lymphocyte autoantibodies (Chen *et al*, 2009).

IgG is a monomer with two binding sites and is responsible for the secondary immune response. It protects the body from many kinds of pathogens (including viruses and bacteria) by several means, such as, opsonization, agglutination, classical complement activation and phagocytosis (Grey *et al*, 1971).

In humans, there are four sub-classes of IgG (IgG_1, IgG_2, IgG_3 and IgG_4 (Grey & Kunkel, 1974)). The functions of each IgG differs, for example, IgG_1 has the highest Fc receptor to phagocytic cell binding affinity, but IgG_3 is better at activating the complement pathway. The structure of these IgG subclasses are very similar, it is mainly the hinge regions that are different and allow for the various functions (Terry & Fahey, 1964).

Mice, on the other hand, have the isotypes IgG1, IgG2a, IgG2b and IgG3 (Ey *et al*, 1979). Like human IgG's, they differ in their half-lives and effector functions (Beenhouwer *et al*, 2007).

IgE is similar in structure to IgD in that it is a monomer and has two epitope binding sites. It mostly binds to basophils and mast cells via its Fc region. It is produced in response to allergens, parasitic worms and arthropods that invade/attack the body. It can illicit an inflammatory response by allowing IgG complement proteins and leukocytes to enter affected tissues, as well binding to mast cells and basophils to mediate allergic reactions. It is possible for the Fc portion of IgE to bind to eosinophils and prompt opsonization of attacking parasitic worms and arthropods. (Gould *et al*, 2002; Tlaskalová-Hogenová *et al*, 1984).

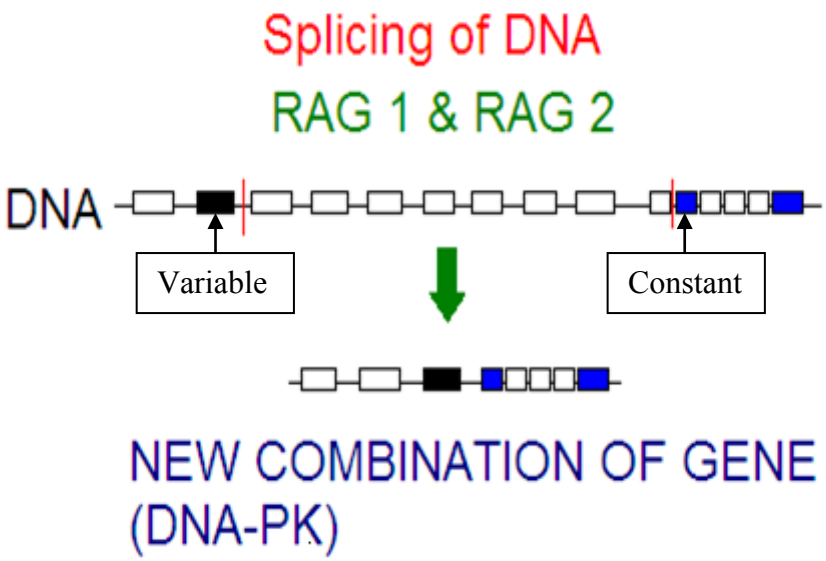
(iib) Class Switching

The process of class switching allows cells from the same activated B cell to produce different Ig isotypes for the purpose of eliminating foreign Ag's via the Ig effector functions. This process is triggered by cytokines (it depends on the type of cytokine as to which Ab is activated). The first Abs to be produced when an Ag is newly introduced to the body, is IgM and IgD. Following these, IgG, IgA or IgE is produced. The constant regions of the Ab heavy chains change during class

switching to activate the required Igs. The variable regions, that are Ag specific, remain unchanged (Stavnezer & Amemiya, 2004).

Class switch recombination (CSR) is the mechanism by which Ig class switching occurs. This is possible by Somatic Recombination, or VDJ Recombination (called after the genes involved in the process). These genes undergo intra-chromasomal recombination of Ab producing cells. In doing this, many, many variations of polypeptides can be produced. For example, the heavy chain region contains 65 Variable (V) genes plus 27 Diversity (D) genes and 6 Joining (J) genes (Li *et al,* 2004). The light chains also possess numerous V and J genes, but do not have D genes. Antibody DNA (Deoxyribonucleic acid) has, what is termed, switch (S) regions. A strand of DNA can be broken via a series of enzyme reactions (at two specific S-regions on the DNA), and then rejoined to a variable region (non-homologenous end joining (NHEJ)). The enzymes responsible for breaking the DNA into sections are called Recombination Activating Genes 1 and 2 (RAG-1 and RAG-2). DNA dependant Protein Kinase (DNA-PK) is responsible for repairing it, allowing the new combination of genes to occur. It is by this means that people, and animals, can fight against infection.

Figure 1.9 Process of Somatic Recombination by DNA splicing: The enzymes, RAG 1 and RAG 2 break-down the structure of DNA at the constant region, allowing a new combination of gene to be created with the aid of DNA-PK. The term somatic recombination refers to the editing and re-arranging of the immune system cells (which produce Abs). Prior to transcription, sections of the gene will act as transposons to re-arrange the coding exons (gene segments). Only some of these exons are copied. After transcription, introns are removed by editing the mRNA. Occasionally, at this point, the remaining exons re-arrange, prior to translation. Therefore, it only takes one gene to give rise to a multitude of different Abs (metaphorically, it is like having 3 decks of cards (V, J and D genes), dealing out different combinations of a card from each deck until a new combination of Ab is found to match an Ag).



(ii) Antibodies as diagnostic tools

Abs are commonly used to identify and locate intracellular and extracellular proteins and are now more frequently being used in flow cytometry to differentiate cell types by the proteins that they express. Proteins can also be detected and quantified with Abs, using ELISA and ELISPOT techniques (Reen, 1994; Kalyuzhny, 2005).

The need for purified Abs stems from the advantages gained from using them in diagnostic tools (the purer they are, the better they work as there are no extra proteins with which to compete for Ag binding). Both polyclonal and MAbs can be purified using Protein A, G or Ag-affinity chromatography. The term immunoprecipitation is used to describe the separation of proteins from any other molecules that are bound to them (co-immunoprecipitation (Williams, 2000)). Protein A is a 40-60 kDa microbial surface component which recognizes adhesive matrix molecules (or, MSCRAMM). It was originally discovered in the cell wall of the bacteria *Staphylococcus aureus*. Protein A can bind to human IgG_1, IgG_2, mouse IgG_{2a} and IgG_{2b} with a high affinity. As well as this, it can bind to human IgM, IgA, IgE, mouse IgG_3 and IgG_1 with moderate ability (Goodyear & Silverman, 2003, Löfdahl *et al*, 1983). It is often used in immunoprecipitation studies as it can be bound to beads that are then used to purify proteins indirectly through use of Abs against the protein/complex of interest. Similar to Protein A, but with differing specificities, Protein G is expressed in Streptococcocal bacteria (Groups C and G). Depending on the group of Streptococci it is found in, its size can be 58 kDa or 65 kDa (Sjobring *et al*, 1991). It can be used to purify Abs by binding to the Fc region, however, it also binds albumin (a contaminant in this instance); therefore some researchers prefer to use a recombinant version of Protein G in their studies (Björck *et al*, 1987).

To check for purity levels, Western blot and SDS PAGE are both well practiced methods often used in research. These both identify proteins separated by electrophoresis (Kurien and Scofield, 2006).

The mycotoxins are produced by saprophytic species of fungi and are found around the globe on many foodstuffs. They cause a major health concern, as many of them are known carcinogens, however, there are not really any rapid tests or preventative

methods for mycotoxicoses at present. As for the use of diagnostic kits for detecting the presence of mycotoxins in foodstuffs,there are a few, but there is a need for these tests to be improved with relation to speed, cost and sensitivity.

Aims and Objectives

The overall aim of this project was to use 1G7-1E2, a generic monoclonal antibody (MAb), to develop immunoaffinity columns (IACs) to capture aflatoxin B_1, B_2, G_1,and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) that produce higher recovery of total aflatoxins (AFs) than current columns on the market. To optimise column performance, various concentrations of gel and antibody (Ab) bound to gel; and Affi-Gel 10 versus cyanogen bromide (CNBr)-activated Sepharose 4B supports were tested. Since fluorescence enhancement is required for AFB_1 and G_1, alternative derivatization techniques were used to improve the signal produced by these toxins. The main objectives are given below:-

- Preparation and spectrophotometric quantification of AF standards for B_1, B_2, G_1 and G_2 from 0.5 ng/mL to 30 ng/mL in solvent.
- Fluorescence enhancement for AFB_1 and G_1 using β-cyclodextrin (β-CD).
- Fluorescence spectrophotometer determination of signals for AFB_1, B_2, G_1 and G_2.
- Preparation of standard curves of fluorescence reading versus concentration for all four toxins from fluorescence spectrophotometer readings.
- Preparation of ammonium sulphate precipitated anti-AF Ab 1G7-1E2.
- Coupling of semi-purified Ab to both Affi-Gel 10 and sepharose 4B at concentrations of 180 μg/mL of gel and 360 μg/mL of gel.
- Preparation of a batch of IACs using the above gels and Ab.
- Purchase of commercial IACs for comparative testing with laboratory-produced columns.
- Passing 30ng/mL AF standards through three columns to be compared (commercial column with 88 μg/mL of Ab, sepharose 4B column with 360μg/mL of Ab and an Affi-Gel 10 column with 360μg/mL of Ab).
- Purification of 1G7-1E2 by Protein-A, Protein-G and T-Gel columns.
- Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) testing to determine purity of purified Ab.

- Preparation and spectrophotometric quantification of AFB_1, B_2, G_1 and G_2 standards from 0.5 to 30 ng/mL in solvent for HPLC.
- Fluorescence enhancement of AFB_1 and G_1 with beta cyclodextrin (β-CD).
- Fluorescence enhancement of AFB_1 and G_1 with trifluoroacetic acid.
- HPLC assays of native AFB_2 and G_2.
- HPLC assays of fluorescence-enhanced AFB_1 and G_1.
- Preparation of standard curves for AFB_2, G_1 and G_2 from area data of HPLC chromatograms.

CHAPTER II

MATERIALS and METHODS

Reagents and Materials

Affi-Gel, 10	BIO-RAD
AFB_1	SIGMA
AFB_2	SIGMA
AFG_1	SIGMA
AFG_2	SIGMA
Ammonium sulphate	BDH CHEMICALS LTD
ß-CD	SIGMA
BSA	SIGMA
CDI	SIGMA
Cryotubes – 1mL	NUNC
CNBr Activated Sepharose 4B	SIGMA
Dimethyl sulfoxide	SIGMA
Disodium hydrogen orthophosphate	BDH CHEMICALS LTD
DMEM	SIGMA
EDAC	SIGMA
Ethanol	SIGMA
Ethanolamine	BDH CHEMICALS LTD
Goat anti-mouse IgG	PIERCE BIOTECHNOLOGY
HPLC grade methanol	SIGMA
Hydrochloric acid	FISHER SCIENTIFIC
Hydrogen peroxide	SIGMA
Marvel® - Skimmed milk powder	PREMIER INTERNATIONAL FOOD UK
Methanol	BAMFORD LABORATORIES
Patulin	ACROS ORGANICS
Potassium chloride	BDH CHEMICALS LTD
Potassium dihydrogen orthophosphate	BDH CHEMICALS LTD

ProSieve®50 gel solution	LONZA
Sodium acetate anhydrous	BDH CHEMICALS LTD
Sodium azide	BDH CHEMICALS LTD
Sodium chloride	FISHER SCIENTIFIC
Sodium citrate	BDH CHEMICALS LTD
Sodium hydrogen bicarbonate	BDH CHEMICALS LTD
TMB	SIGMA
Tween 20®	SIGMA
Zearalenone	SIGMA

Cell Culture Reagents and Equipment

Amphotericin B	PAA LABORATORIES
Cell tissue culture flask, 75cm^2	GREINER BIO-ONE (COSTAR)
Centrifuge tubes - 15mL and 50mL	CORNING INC
Ethanol	FISHER SCIENTIFIC
FCS	HARLAN SERA-LAB LTD
L-Glutamine	CAMBREX
Pen – Strep, 2%	CAMBREX
RPMI 1640	GIBCO
Trypan Blue	SIGMA
Virkon®	ANTEC INTERNATIONAL

Methods

I. Aseptic Technique

All cell culturing procedures were implemented in a Class II lateral flow safety cabinet. Standard cell culture aseptic techniques and 70% (v/v) ethanol disinfectant were used.

II. Cell Culture Medium

The stock medium consisted of RPMI 1640 with 2% (v/v) L-glutamine, 1% (v/v) *Pen - Strep*, and 1% (v/v) amphotericin B. Foetal calf serum (FCS, 10% v/v) was added to produce complete RPMI 1640 medium.

III. Cell Line and Recovery from Cryo-preservation

The 1G7-1E2 hybridoma cells were produced and stored in LN_2 by the University of Strathclyde (Prasertsilpa, 1999). The vials of Ab were removed from storage and defrosted rapidly after transferring them to a water bath at 37°C. As a precaution, the vials were thawed from the LN_2 phase using a container with a lid in case the vials exploded. The Dimethyl sulphoxide (DMSO) that the cells had been stored in was then removed via centrifugation of cells (1000 rpm, for 7 minutes). The supernatant was removed and the pellet of viable cells left was re-suspended in 5mL complete RPMI 1640 media and inoculated into a $75cm^2$ cell culture flask with a further 15mL complete RPMI 1640.

IV. Cell Culture

The 1G7-1E2 cultures were held in an incubator at 37°C with 5% CO_2 in air, and inspected on a daily basis under an inverted microscope (Olympus, IM). The cells were fed daily by full exchange of medium. Any viable cells in the medium removed were recovered by centrifuging at 1000rpm for 7 minutes. The pellet was then re-suspended in 5mL of complete RPMI 1640, after which, the cells were inoculated into flasks with a minimum of 20mL complete RPMI 1640. The flasks were then incubated at 37°C, ready for the process to begin again, until enough cells were produced to harvest.

Viable cells can clearly be seen under a microscope. They are distinguished from dead cells by their colour, size and shape (large, round, smooth orange/brown cells, opposed to small, black irregular shaped cells). Trypan blue was used to make viable cells more visable for counting.

By introducing 0.5mL of 0.4% Trypan blue to 0.5mL of cell suspension, it became clear which cells were viable as intact cells exclude the dye, while dead cells become stained. A haemocytometer was used to count the number of viable cells in a suspension. The Trypan blue was handled with care as it is teratogenic, and possibly carcinogenic.

V. Harvesting of Antibody from Cells

After a 2 week incubation period (37˚C in a CO_2 incubator), the supernatant was ready for collection. To harvest the cells, the medium (containing free Ab, cells and debris) was removed from each flask and pipetted into centrifuge tubes. The medium was then centrifuged down to form a cell pellet (1000 rpm, for 7 minutes). This time, the medium was kept and the cell pellet was disposed of in Virkon® (a strong, multipurpose disinfectant). The supernatant was then syringe filtered (using a 0.22 micron porous membrane attached to a sterile syringe that was used to push the Ab through to a collection tube, and allowing larger, unwanted molecules to be filtered out) and frozen until required for further use.

VI. Ammonium Sulphate Precipitation

A solution of 100% saturated ammonium sulphate (approximately 4M $(NH_4)_2SO_4$) was used to precipitate the Abs from supernatant. After measuring at A_{280}, an equal volume of $(NH_4)_2SO_4$ was added dropwise to MAb-containing supernatants and left stirring overnight at 4˚C.

The mixture was then centrifuged at 10,000 x g for 30 minutes and washed twice with 50% saturated $(NH_4)_2SO_4$. The pellet was re-suspended in the same volume of 100mM HEPES buffer as original sample and the A^{280} measured. This was then

dialysed overnight against HEPES (100mM) at 4˚C. Insoluble precipitates were removed by centrifuging at 10,000 x g for 30 minutes. The Ab was measured (A_{280}) before it was purified by way of thiophilic adsorption chromatography.

VII. Purification using Thiophilic Adsorption Chromatography

Post ammonium sulphate precipitation, the 1G7-1E2 Ab preparation was put through a T Gel column (a commercial column used to purify Abs by using a ligand, that contains a sulfone group which is in close proximity to a thioether group, to bind to proteins), as follows. To every mL of 1G7-1E2 recovered, 87mg K_2SO_4 was added and gently mixed. The Ab was then centrifuged at 10,000 x g for 20 minutes to remove any unnecessary aggregates.

The clear solution containing Abs was then allowed to bind to the T-gel with the aid of 50mL binding buffer, 0.5M K_2SO_4 and 50mM Na_2HPO_4, pH 8.0.

Fractions (1mL) were collected and measured at 280nm on a spectrophotometer. The fractions were collected until the A_{280} values were at or close to 0. Once background had been reached, up to 13 column volumes of elution buffer (50 mM Na_2HPO_4 and 0.05% (w/v) NaN_3, pH 8.0, adjusted with HCl) was put through the column to elute any Ab. The Ab was collected in 1 mL fractions as before and kept at 4˚C until required.

A storage buffer was used to store the gel. This consisted of 0.5M Tris and 0.02% (w/v) NaN_3, pH 7.4 (adjusted with HCl). The column packed with gel and all buffers were kept in 10mM PBS plus NaN_3 (0.02% w/v) at 4°C in a cold room and only brought up to room temperature when required for the purification step.

VIII. Ultrafiltration of Antibody

Using an ultra-centrifugal filtration tube, produced by Millipore, the post purified 1G7-1E2 was spun down in a centrifuge to make a more concentrate Ab solution. Millipore filtration tubes are non-sterile, single use tubes that can hold up to 15mL of solution. 1G7-1E2 was centrifuged at 4000 rpm for 15 minutes until a concentrated volume of Ab was collected. The Ab was transferred to and from the tubes using a

pipette. Millipore's filtration tubes incorporate Ultracel®, a regenerated cellulose low binding membrane that produces a recovery rate of 90% of dilute protein concentrate with high concentration factors of up to 80-fold.

IX. Testing of Antibody Purification by SDS PAGE

The 10 mL of resolving gel required to run SDS PAGE was prepared using the following,

Components	**Volume required for 10% Resolving Gel**
Deionized H_20	5.3 mL
ProSieve® 50 gel (acrylamide) solution	2.0 mL
1.5M Tris-HCl, pH 8.8	2.5 mL
10% SDS Solution	100 µl
10% APS	100 µl
TEMED	4 µl

The gel was poured into the cassettes of the electrophoresis apparatus, covering ¾ of the area, then allowed to polymerise for 30 mins with an overlay of water before casting the stacking gel (prepared as below).

Components	**Volume required for 1 mL Stacking Gel**
Deionized H_20	750 µl
ProSieve® 50 gel solution	100 µl
1 M Tris-HCl, pH 6.8	130 µl
10% SDS Solution	10 µl
10% APS	10 µl
TEMED	1 µl

The overlay water was decanted prior to the addition of the stacking gel, using a pipette. A comb was then inserted into the stacking gel, and the gel was left again to polymerise for 30 mins.

Once the comb was removed, the white tape was also removed and the cassette was placed in the electrophoresis apparatus. The middle part of the chamber was filled with the running buffer, previous to the protein marker and protein samples being loaded.

Before the protein samples were loaded, they were combined with equal volumes of 2 x denaturing sample buffer (as below) in mini-centrifuge tubes and these were placed in a little boiling water for five minutes. Loading dye, 2 x (just enough to cover the bottom of each well), was added to the wells to make them easy to locate.

Denaturing Sample Buffer (x 2)	**Volume Required**
0.5 M Tris-HCl, pH 6.8	2.5 mL
Glycerol	2.0 mL
10 % (w/v) SDS solution	4.0 mL
2 - Mercaptoethanol	0.5 mL
0.1 % (w/v) Bromophenol Blue	0.5 mL
Deionized H_2O	10 mL

Running Buffer	**Volume Required (x 1)**
Tris base	2.9 g
Glycine	14.4 g
SDS	1.0 g
Deionised H_20	1 litre

The gel was run at 200v for 60 -90 min (once the tracking dye had reached the bottom of the resolving gel, electrophoresis was stopped). The gel was then ready to be removed from the cassette. Detection of the protein was possible with the aid of a Polaroid gel documentation system (used to photograph the gel).

X. Coupling of 1G7-1E2 Antibody to Activated Immunoaffinity Gels

(i) Cyanogen bromide-activated Sepharose 4B (Sigma).

The purified Ab was coupled to CNBr Sepharose 4B using 0.1M $NaHCO_3$ buffer containing 0.5M NaCl, pH 8.4 (adjusted using 1M NaOH). The CNBr activated resin was washed and left to swell in cold 1mM HCl for 30 minutes. Then, a total of 200mL/g of dry gel was added to several aliquots. A Büchner funnel was used to wash the gel by removing the supernatant using a vacuum, only applying gentle suction. The resin was then washed with 10mL nanopure water, and then by 5mL $NaHCO_3$/NaCl coupling buffer. This was immediately transferred to a solution of the Ab in coupling buffer (of which there were two concentrations; 180µg/mL and 360µg/mL Ab in 3.5mL and 7.0 mL of $NaHCO_3$/NaCl buffer). The Ab and gel were then mixed at 4°C overnight on a rocking stirrer. Unreacted Ab ligands were washed away using the filtration method mentioned above and $NaHCO_3$/NaCl coupling buffer. Un-reacted groups were blocked with 1M ethanolamine in water, pH 8 for 2 hours at room temperature. Coupling buffer was used to wash the resin extensively before washing with 0.1M acetate buffer, pH 4, containing 0.5M NaCl (pH adjusted by gradual addition of droplets of 0.2M NaOH). This wash cycle was repeated 5 times. If the resin was being used immediately, it was equilibrated in buffer, however, if it was to be stored until a later date, 1M NaCl containing 0.2% (w/v) NaN_3 was used as a storage buffer, which was then kept at 4ºC.

(ii) Affi-Gel 10 (BIO-RAD)

Before transferring the gel (21mL) to a Büchner funnel, the vial containing gel was shaken to ensure a uniform suspension. Then, the supernatant was washed from the gel using 10mL nanopure water. A vacuum was attached to the Büchner funnel to increase the flow rate of nanopure water whilst washing the gel. Once the gel was transferred to a flask, the Ab solution (180µg/mL and 360µg/mL) was added (0.5mL

per mL of gel) and agitated to form a uniform suspension. The gel was then left on a rocking stirrer at room temperature for 1 hour. After stirring, 0.1mL (of 1M ethanolamine HCL, pH 8.0) per mL of gel was added. The gel was then put into ten (Alltech) columns and washed with nanopure water until no further reactants could be detected by using a spectrophotometer at A_{280}. Further washing was carried out by putting 10mL of methanol through each of the columns (as recommended by the manufacturer, to elute substances stuck to the columns). When the columns were not in use, they were stored in 10mM PBS, pH 7.4, containing 0.2% (w/v) NaN_3 and kept at 4ºC.

XI. Production of Immunoaffinity Columns

Individual parts (reservoirs, frits, inlet and outlet caps) of the IACs were purchased from Alltech Associates Limited. Once assembled, 0.5mL of each gel (Affigel 10 and Sepharose 4B) was added to each of the 2 x 10 columns ((2x) 5 columns x 180µg/mL Ab and (2x) 5 columns x 360µg/mL Ab). The columns were stored in a cold room at 4ºC until required. Tinfoil was used to cover the columns and protect the Ab from UV light damage.

XII. Aflatoxin Standards

Four sets of standards were prepared before measuring using a Fluorescence Spectrophotometer and HPLC to produce a standard curve with which samples could be compared. Stock solutions of each of the four AFs were made using neat methanol (AFB_1 = 996.4 ng/mL, AFB_2 = 350.6 ng/mL, AFG_1 and AFG_2 = 1000 ng/mL). From these stock solutions, a series of dilutions were made to produce 10mL solution containing 30 ng/mL, 25 ng/mL, 20 ng/mL, 15 ng/mL, 10 ng/mL, 8 ng/mL, 4 ng/mL, 2 ng/mL, 1 ng/mL and 0.5 ng/mL AF in methanol.

XIII. Fluorescence Spectrophotometry

Standards for each AF were prepared as already mentioned. Since AFB_1 and AFG_1 require fluorescence boosting (as they do not naturally fluoresce well), ß-CD was added to each of them at a concentration of $6x10^{-3}$M (Chiavaro *et al*, 2001). This is a natural sugar with the ability to induce fluorescence.

A Perkin-Elmer 650-40 Fluorescence Spectrophotometer was used to measure the fluorescence of the standards. Since the standards were in methanol, neat methanol was used as a blank and any readings taken were noted. An emission spectra was generated by setting the extinction wavelength to 365nm and emission wavelength to 425nm. The slits on the Perkin-Elmer were set at 5 as this was found to give the best results. The standard curves for this were prepared by plotting the absorbances from each AF against the corresponding standard AF concentrations.

XIV. High Performance Liquid Chromatography

Chromatographic analyses of the AF standards were performed with a Gynkotek - High Precision Pump, Model 480, equipped with a Shimadzu - RF-530 Fluorescence HPLC Monitor ($\lambda ex = 365nm$ and $\lambda em = 425nm$ for AFB_1 and $\lambda ex = 360nm$ and $\lambda em = 435nm$ for AFB_2, AFG_1 and AFG_2) and a Waters Spherisorb ODS-1 (4.6 X 250mm) column. All work was carried out at room temperature and a HPLC Grade methanol:distilled water (40:60 v/v) mix was used as the mobile phase. The standard curves produced with the data obtained from the HPLC was used as a tool to determine the concentration of AF on peanuts (and indeed if the columns prepared using IG7-1E2 are of any use as a diagnostic tool).

AF standards were prepared as previously stated in the standards section. Since ß-CD can potentially block the column when mixed with the solvents in the mobile phase, it was decided to use trifluoroacetic acid (TFA) as a fluorescence booster instead. TFA (10mL) was mixed with distilled water (35mL) and glacial acetic acid (5mL) before being introduced to the standards/samples. Once this solution was ready, 700 µl of it was added to every 200 µl of standard/sample required. The standards/samples were then put in a water bath at 65˚C for 9 mins. The samples were quantified using the standard curves produced by the spectrophotometer data (by measuring the samples in the spectrophotometer and comparing to the standard curves).

XV. Preliminary Test Assay for Zearalenone-Poly L Lysine Conjugate

A zearalenone conjugate was produced in April 2005, and tested 2 weeks later, and again after 46 weeks to check for degradation. The conjugate was checked for validity using ELISA test strips. All conjugates mentioned in this thesis were produced in a similar manner.

1-ethyl-3-13-dimethylaminoproyl (EDAC) was used as a conjugation reagent to couple the carboxyl groups of the Zearalenone's oxime to amino groups on the protein, forming amide bonds. The oxime was dissolved in a 25:75 ethanol:water mix (3mg/10mL). 5mg of protein (e.g. BSA) was added followed by 188mg EDAC. This mixture was stirred continuously in darkness and with heat applied, using a Bunsen burner, until the ethanol had evaporated.

After this, the conjugate was dialysed in dialysis tubing for 2 days at 4˚C in 10mM PBS, pH 7.4 (the PBS was changed daily).

The methods for the test strips were as follows.

(i) Coating

The conjugate was diluted 1:25 in acetate-citrate buffer, pH 5.5. The microtitre plate wells were coated with 350µl/pw of Zearalenone-PLL conjugate. This was incubated overnight in a cold room at 4˚C. Afterwards, the test strips were washed x 4 with 0.1M acetate citrate buffer plus 0.05% (v/v) Tween 20® (wash buffer), then dried.

(ii) Blocking

Each well was blocked with 300µl 3% (w/v) Marvel® in acetate-citrate buffer (as defined overleaf), pH5.5. This was incubated for 1hr at 37˚C, then washed x 4 with wash buffer and dried in a 37˚C incubator before being stored overnight at 4˚C.

XVI. Enzyme Linked Immunoabsorbant Assay

The following chemical solutions were prepared to use in each ELISA produced for this project.

(i) 0.1M acetate-citrate buffer, pH 5.5

Solid citric acid was added, with stirring, to 0.1M sodium acetate to give a pH of 5.5.

(ii) ELISA coating buffer, pH 9.0

HCl was added to 0.02M Tris to give a pH of 9.0.

(iii) TMB substrate

Six mg/mL of TMB was dissolved in dimethyl sulfoxide (DMSO and stored in 250µl aliquots at -20˚C until required. All 250 µl of TMB substrate was thawed out and added to 25mL of 0.1 M acetate citrate buffer and 4 µl of H_20_2 prior to use in the ELISA.

(iv) Wash buffer, pH 7.4

Ten mM PBS, pH 7.4 was made by dissolving 8.00g NaCl, 0.20g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 800mL Nanopure water. The pH of this solution was adjusted to 7.4 by using concentrated HCl, then Nanopure water was added to make the solution up to 1L. 0.05% (v/v) Tween 20 was then added.

(v) Blocking agent

Three g of Marvel® was added to 100mL of 0.1M acetate citrate buffer and stirred on a magnetic stirrer until all the milk powder had dissolved. This was kept at 4˚C until required.

XVII. Zearalenone ELISA

(i) Coating: Zearalenone -PLL

Each well was coated with 250µl Zearalenone Poly-L-Lysin (ZEAR-PLL) conjugate, diluted 1:25 in 0.1M acetate-citrate buffer, pH 5.5. The concentration of zearalenone in conjugate was 0.342mg/mL, 1.640mg/mL poly-L-lysin (PLL). The microtitre plates were coated with a 1:25 dilution, therefore contained 11nM/well zearalenone or 0.073nM PLL/well. The plate was then stored overnight at 4˚C or in a 37˚C incubator for 2 hrs.

After the microtitre plates had been coated, they were washed with PBS and Tween 20 x 3, then dried by shaking off excess wash buffer and allowing to air dry.

(ii) Marvel® in 0.1M acetate-citrate buffer was used as the blocking agent (300 µl/well). The plates were then incubated for 1 hr at 37˚C. After this, the plates were washed x3 and dried as before.

(iii) A set of standard zearalenone/methanol concentrations were made, ranging from 0 to 100ppb (ng/mL). The following plate plan was derived: -

WELL	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0	0	0	0	0	0	0	0	0	0	0	0
B	20	20	20	20	20	20	20	20	20	20	20	20
C	40	40	40	40	40	40	40	40	40	40	40	40
D	50	50	50	50	50	50	50	50	50	50	50	50
E	75	75	75	75	75	75	75	75	75	75	75	75
F	100	100	100	100	100	100	100	100	100	100	100	100
G	B	B	B	B	B	B	B	B	B	B	B	B
H	B	B	B	B	B	B	B	B	B	B	S	S

Key

S = Anti-mouse IgG serum only (200 µl)
B = Blank (200 µl PBS)
0 – 100 = zearalenone in ppb

(iv) After the addition of 200 µl 1G7-1E2 Ab supernatant to each well (except blanks), the plates were incubated for an hour at 37˚C, then washed. Anti mouse IgG serum was added afterwards (1:100 in PBS, 100µl/well). This was incubated at 37˚C for 45 mins, then washed, after which a colour change reaction was induced with the addition of H_2O_2 and TMB. To prevent further reaction, when maximum colour was reached (a change of colour noticeable to the naked eye within a minute of the reaction onset), 0.1mL 10% (v/v) H_2SO_4 was added. There was a visible colour change from yellow to blue. The A_{450} was measured in an ELISA plate reader (Multiskan MCC/340 P version 2.20). All assays were performed in duplicate and the results recorded.

XVIII. Lactone Cross Reactivity in a 1G7-1E2 ELISA

(i) Coating

A third of a microtitre ELISA plate was coated with 250µl per well of 1µg/mL AFB_1-BSA conjugate in 10mM PBS, pH 7.40, another third with 250µl per well of a 1:25 dilution of a Patulin Poly-L-Lysin (PAT-PLL) conjugate in 0.1M acetate-citrate buffer, pH 5.5. The remainder of the plate was coated with 250µl per well of 1:25 dilution of ZEAR-PLL conjugate in 0.1M acetate citrate buffer, pH 5.5. This was then kept overnight at 4˚C, allowing plenty of time for the coating to take to the plate.

After coating, the plate was washed x3 with 10mM PBS, pH 7.4, containing 0.05% Tween 20®, then dried prior to blocking.

(ii) Blocking

The patulin and zearalenone conjugates were blocked with 300µl per well of 3% Marvel® in 0.1M acetate citrate buffer, pH 5.5. The AFB_1 conjugate was blocked with 3% Marvel® in 10mM PBS, pH 7.4. The plate was incubated at 37˚C for 1 hour. This was then washed x 3 with wash buffer (10mM PBS pH 7.4) and 0.05% Tween 20®, before drying.

(iii) Primary Antibody

200 µl of 1G7-1E2 was added to 12 wells, along with 100µl 10mM PBS, pH 7.4 or 100 µl 0.1M acetate-citrate buffer.

As well this, 12 wells contained the blanks (100 µl 10mM PBS, pH 7.4 in 4 wells, and 100 µl 0.1M acetate citrate buffer, pH 5.46 in 8 wells).

To the AFB_1, zearalenone and patulin conjugate sections, 100 µl 10mM PBS, pH 7.4 and 200 µl 1G7-1E2 neat supernatant was added. This was incubated for 1 hour at 37˚C before washing x 3 with wash buffer and drying.

(iv) Anti-mouse IgG

Anti-mouse IgG (100 µl) was added to each well, except the blanks. The concentration was 1:5000, in 10mM PBS, pH 7.4 for the AFB_1 section and in 0.1M acetate citrate buffer, pH 5.5 for the patulin and zearalenone sections.

Ten mM PBS (100µl) of pH 7.4 was added to the blanks. The plate was incubated for 1 hour at 37˚C before washing x 4 (10mM PBS pH 7.4 and 0.05% Tween 20®) and drying.

(v) Substrate

TMB substrate (100 µl of a solution composed of 4 µl H_2O_2, 250 µl TMB stock and 25 µl 0.1M acetate-citrate buffer, pH 5.5) was added to each well. Once development was complete, by visual change of colour from clear to blue, the process was stopped with the addition of 100 µl 10% (v/v) H_2SO_4. The A_{450} was read on a Multiskan MCC/340 P version 2.20 plate reader.

CHAPTER III

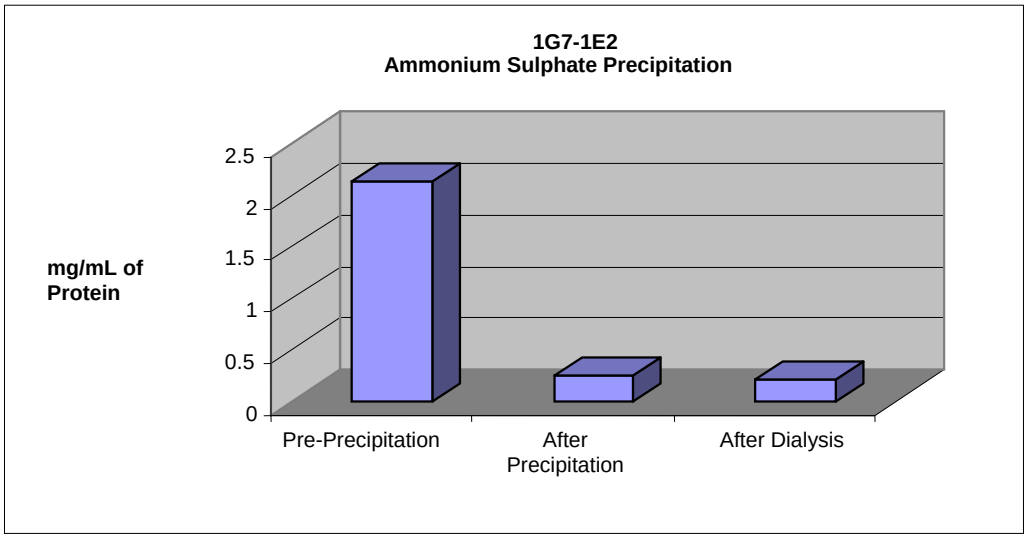
RESULTS and SYNOPSIS

Results

I. Antibody purification

Figure 3.0 shows the 3 stages of ammonium sulphate precipitation and shows how effective it was on taking away the contaminant proteins found amongst 1G7-1E2. Originally, 2.142mg/mL of protein was recovered from the frozen stock supply of protein. After precipitation with 4M $(NH_4)_2SO_4$ and centrifugation of the suspension, 0.255mg/mL of Ab was left. Post precipitation (i.e. after dialysis and filtration), 0.206mg/mL of Ab remained. Levels of protein were detected by measuring the light absorbance at A^{280} in a spectrophotometer. Purity was confirmed by SDS PAGE.

Figure 3.0 Ammonium Sulphate Precipitation Purification of Ab 1G7-1E2; After the precipitation process, it is apparent that some contaminant proteins have been removed from the MAb suspension due to the decreasing levels of protein (detected via spectrophotometry). Further still, more unwanted proteins are removed post dialysis.



II. SDS PAGE

The following pictures show the components in the MAb 1G7-1E2 preparation after it has been purified using either a T gel column, Ammonium sulphate precipitation, or both techniques.

Figure 3.1 SDS PAGE 1; 1G7-1E2 (post purification by means of T-gel and ammonium sulphate) is shown next to a protein marker. The protein marker is an indicator of the MAbs molecular weight and was provided by Dr Carter, University of Strathclyde.

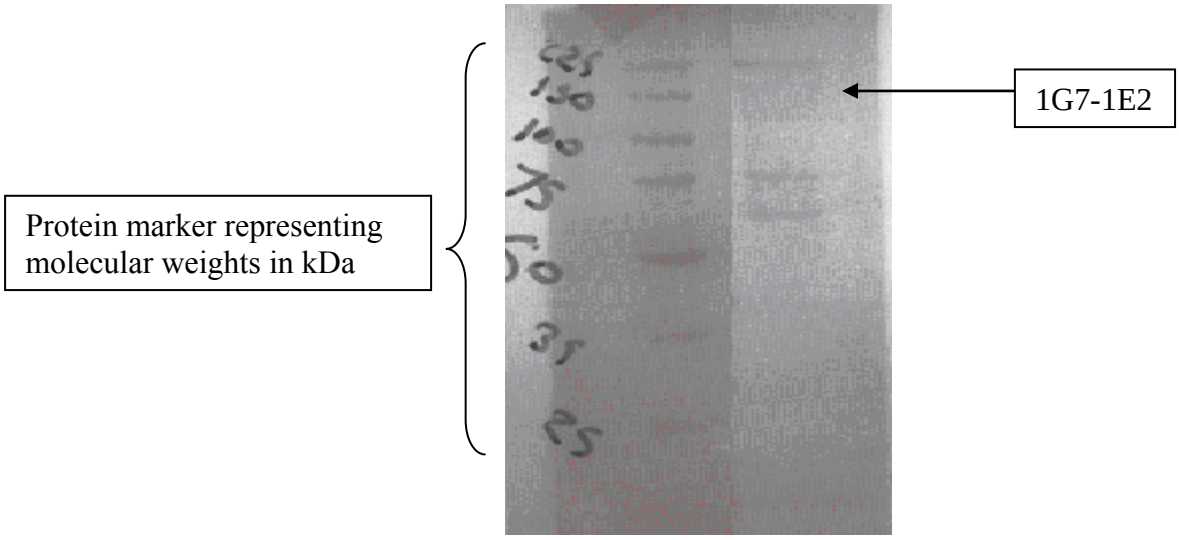


Figure 3.2 SDS PAGE 2; 1G7-1E2 (purified by means of T-gel).

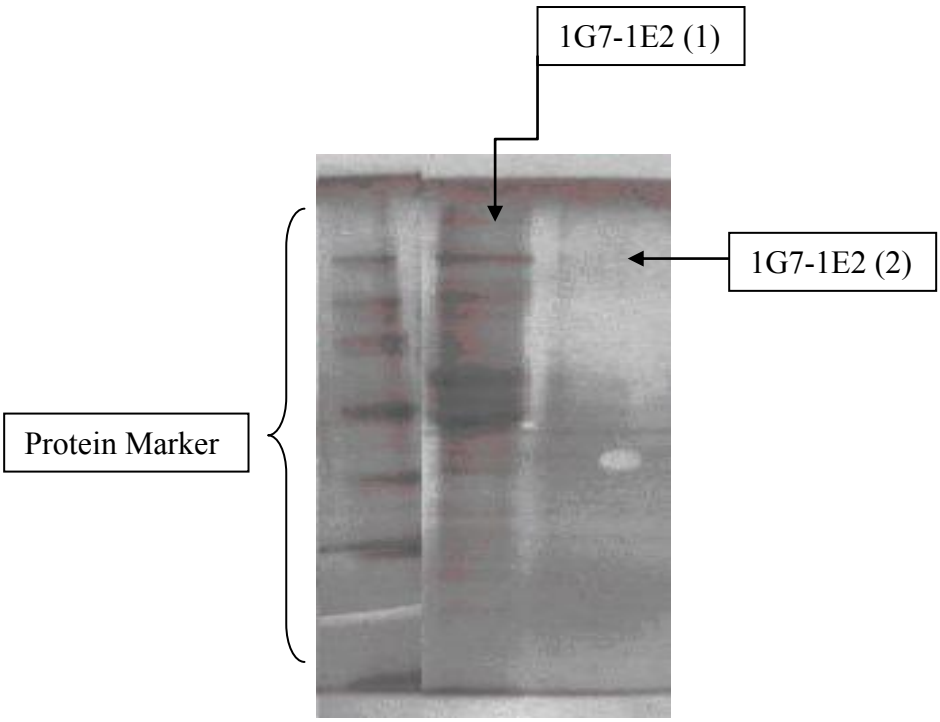
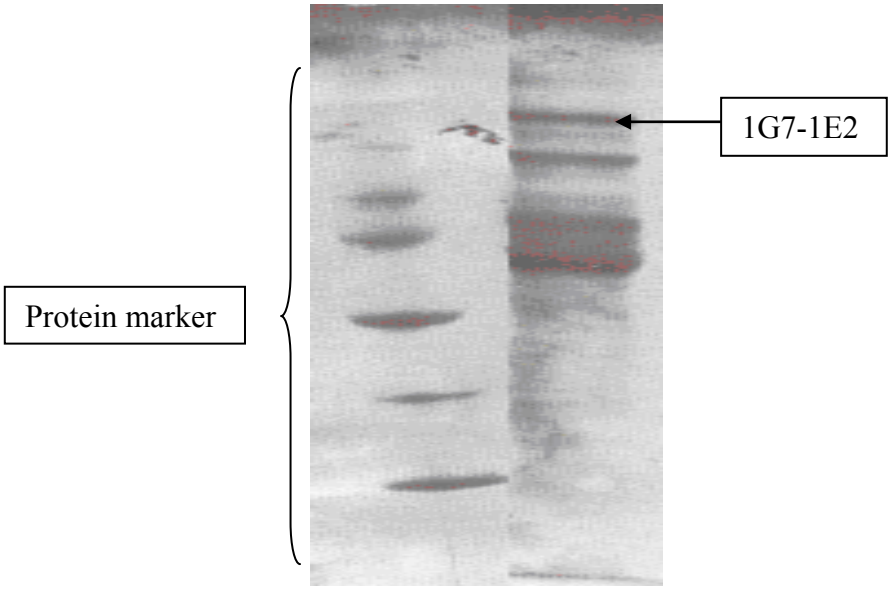


Figure 3.3 SDS PAGE 3; 1G7-1E2 (purified by means of ammonium sulphate).



In the pictures on the previous pages, it is possible to see the Ab after T-gel purification (Figure 3.2), and post ammonium sulphate purification (Figure 3.3). The various bands show that other proteins are present within the samples, however, the 1G7-1E2 bands are thick, showing that a descent amount of Ab has been obtained.

The number of bands shown in Figures 4.1, 4.2 and 4.3 is the same, which indicates that a 2-step procedure for MAb purification, as shown in Figure 3.1, is not required (i.e. the same level of purification is achieved if T-gel is used alone, or with the ammonium sulphate as an additional step). Since T-gel columns are quick and efficient to use, they are the preferred choice material for MAb purification over the ammonium sulphate, which can take days to utilise.

III. Fluorescence Detection of Aflatoxin Standards Using Spectrophotometry

The standard curves shown in this thesis were produced from the relative fluorescence (area) of each toxin, spanning from a concentration of 0.5 ng/mL to 30 ng/mL. The R^2 values are represented in a table below for easy viewing:-

Aflatoxin	**R^2 Value**
AFB1	0.9416
AFB2	0.9908
AFG1	0.9859
AFG2	0.9953

The following graphs show the standard curves used to obtain these results.

Figure 3.4 Standard Curve for AFB_1; A series of AFB_1 standards (0.5 ng/mL to 30 ng/mL) were made using methanol and were measured in a spectrophotometer to produce the relative fluorescence values with which this standard curve was created.

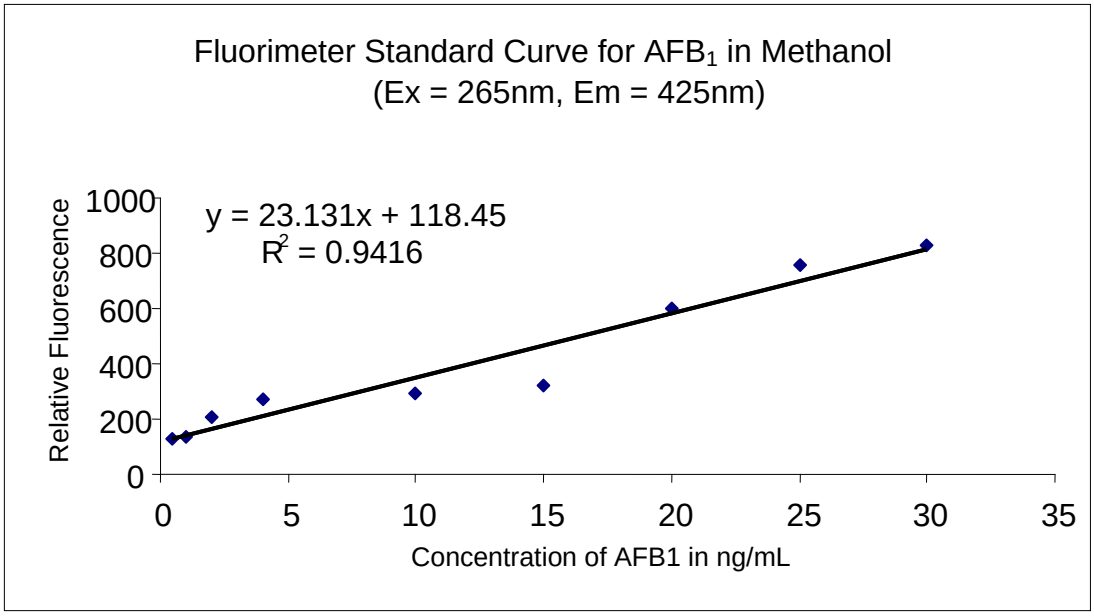


Figure 3.5 Standard Curve for AFB_2; A series of AFB_2 standards (0.5 ng/mL to 30 ng/mL) were made using methanol and were measured in a spectrophotometer to produce the relative fluorescence values with which this standard curve was created.

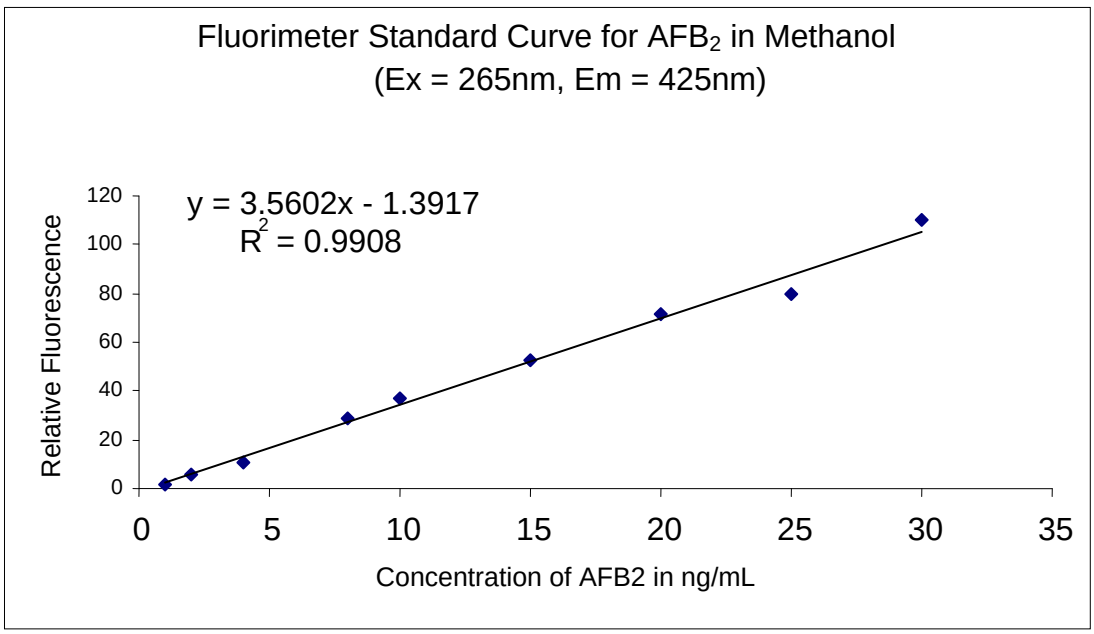


Figure 3.6 Standard Curve for AFG_1; A series of AFG_1 standards (0.5 ng/mL to 30 ng/mL) were made using methanol and were measured in a spectrophotometer to produce the relative fluorescence values with which this standard curve was created.

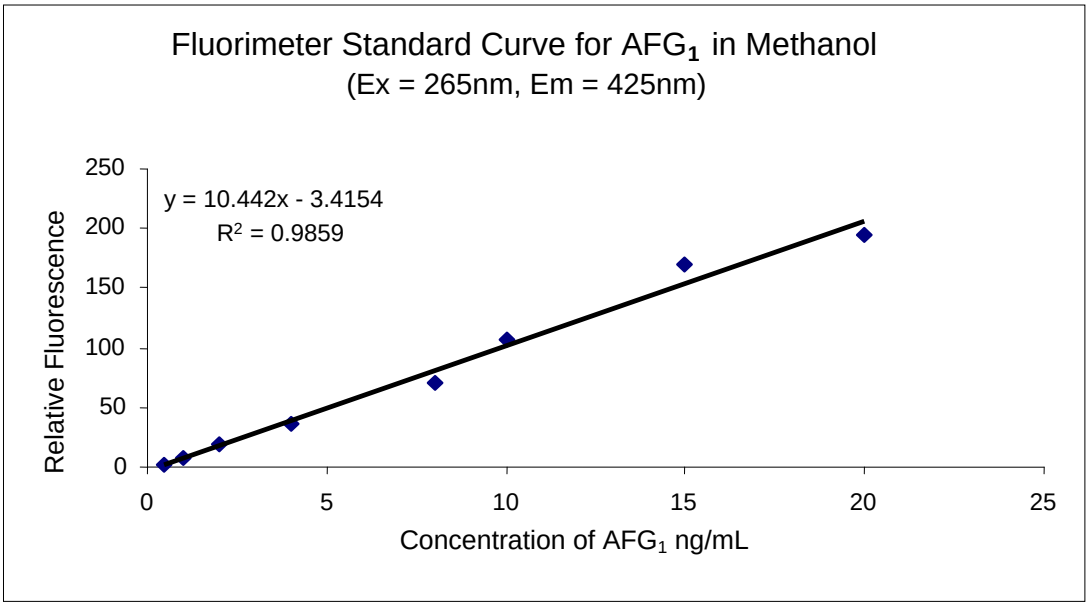
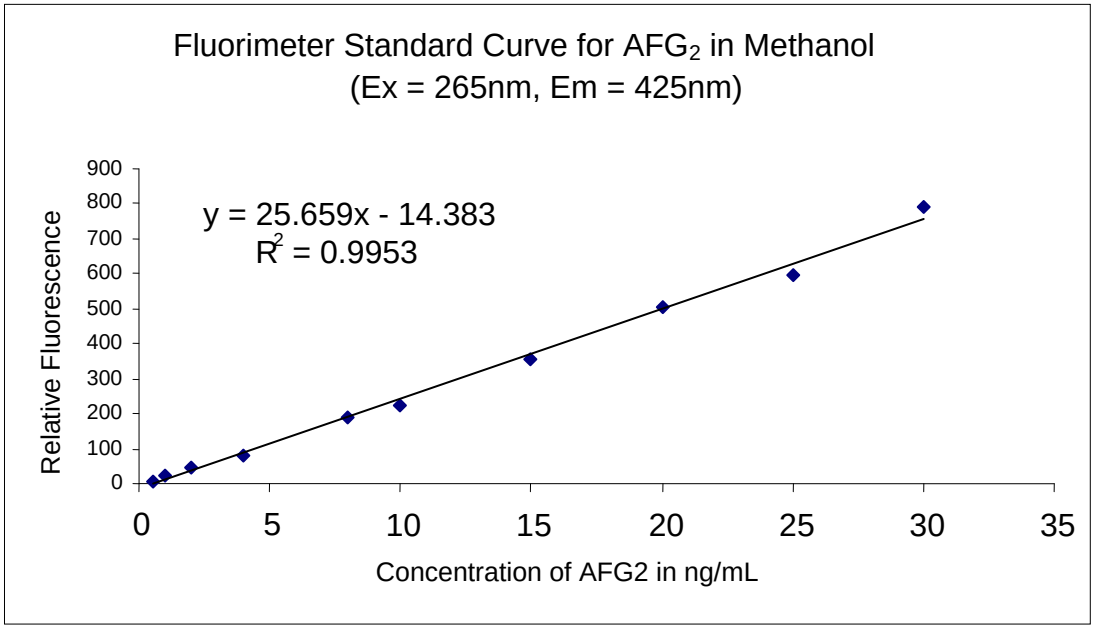


Figure 3.7 Standard Curve for AFG_2; A series of AFG_2 standards (0.5 ng/mL to 30 ng/mL) were made using methanol and were measured in a spectrophotometer to produce the relative fluorescence values with which this standard curve was created.



It was noted that secondary metabolites, AFB_2 and AFG_2 produced a better standard curve than the other toxins, even though the other toxins had their fluorescence boosted by the addition of ß-CD.

IV. Comparison of Post Column Recovery of Aflatoxins Detected by Fluorescence Spectrophotometry

Table 3.0 overleaf and the corresponding graphs (figure 3.8, 4.9, 4.10 and 4.11) show the percentage recovery for all four AFs (with the concentrations 180 µg/ml Ab, 360 µg/ml Ab or 88 µg/ml Ab) after they have been eluted and collected from IACs (produced in the laboratory or bought from Rhône Diagnostics) and measured on the fluorimeter. In the table, X represents the concentration derived from the curve and Y represents the fluorescence of the sample.

Table 3.0 Comparison Table for Percentage Recovery of Aflatoxins from Columns Containing 1G7-1E2 Ab Bound Gel. By using the equations produced by the AF standard curves for each of the AFs below and the values given as X and Y in this table, it was possible to obtain the percentage recovery of each AF run through the test columns listed here. The recovery values are shown in graph form on the following pages.

		X (ng/mL)	**Y (ng/mL)**	
Column Type	**Concentration of Antibody**	**AFB_1**	**AFB_1**	**%Recovery**
Affigel-10	180µg/mL Ab	19.65	5.97	70.8
	360µg/mL Ab	25.75	6.23	92.8
Sepharose 4B	180µg/mL Ab	24.30	6.17	87.6
	360µg/mL Ab	25.37	6.22	91.5
Rhône	88µg/mL Ab	24.40	6.18	87.0

Column Type	**Concentration of Antibody**	**AFB_2**	**AFB_2**	**%Recovery**
Affigel-10	180µg/mL Ab	27.21	8.03	98.1
	360µg/mL Ab	25.01	7.42	90.2
Sepharose 4B	180µg/mL Ab	22.85	6.81	82.4
	360µg/mL Ab	25.33	7.51	91.3
Rhône	88µg/mL Ab	30.06	8.83	108.4

Column Type	**Concentration of Antibody**	**AFG_1**	**AFG_1**	**%Recovery**
Affigel-10	180µg/mL Ab	20.66	2.31	74.5
	360µg/mL Ab	22.61	2.49	81.5
Sepharose 4B	180µg/mL Ab	24.10	2.63	86.9
	360µg/mL Ab	27.00	2.91	97.3
Rhône	88µg/mL Ab	26.95	2.91	97.2

Column Type	**Concentration of Antibody**	**AFG_2**	**AFG_2**	**%Recovery**
Affigel-10	180µg/mL Ab	3.62	0.70	13.1
	360µg/mL Ab	-1.49	0.50	-5.4
Sepharose 4B	180µg/mL Ab	4.52	0.74	16.3
	360µg/mL Ab	14.55	1.13	52.4
Rhône	88µg/mL Ab	9.28	0.92	33.5

Figure 3.8 Comparison of Percentage Recovery of AFB_1 from Columns Containing 1G7-1E2 Ab Bound Gel

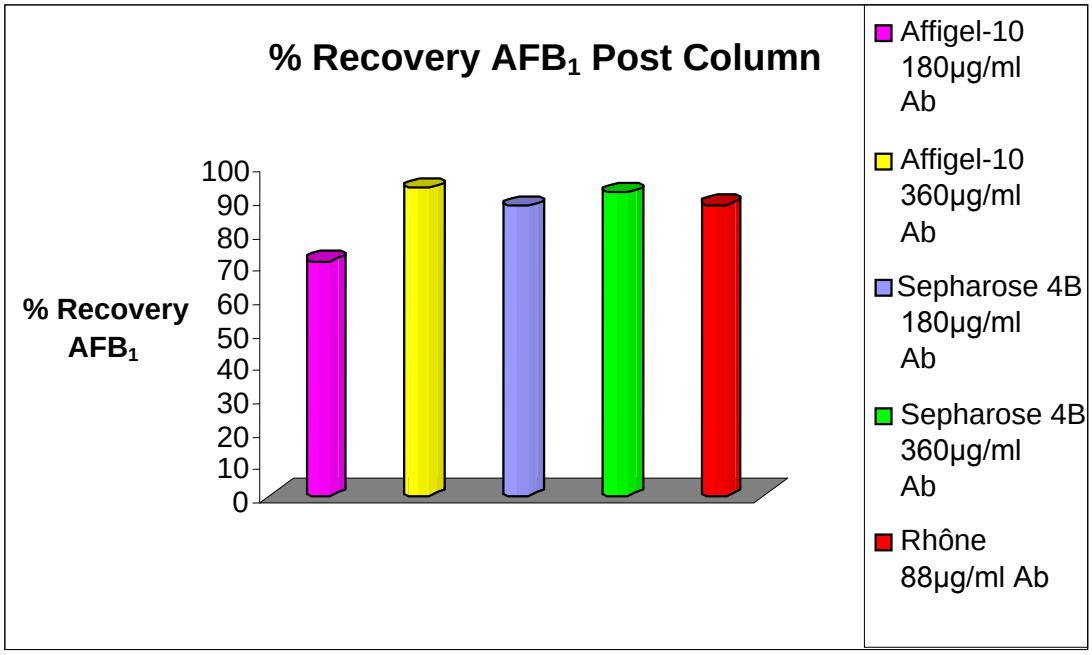


Figure 3.9 Comparison of Percentage Recovery of AFB_2 from Columns Containing 1G7-1E2 Ab Bound Gel

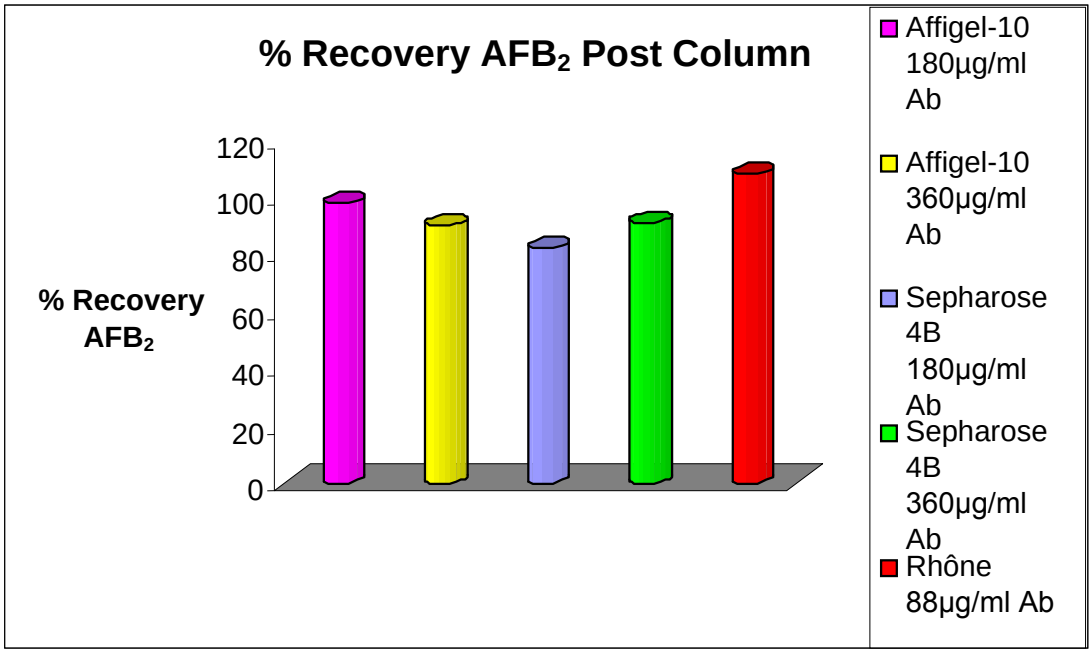


Figure 3.10 Comparison of Percentage Recovery of AFG_1 from Columns Containing 1G7-1E2 Ab Bound Gel

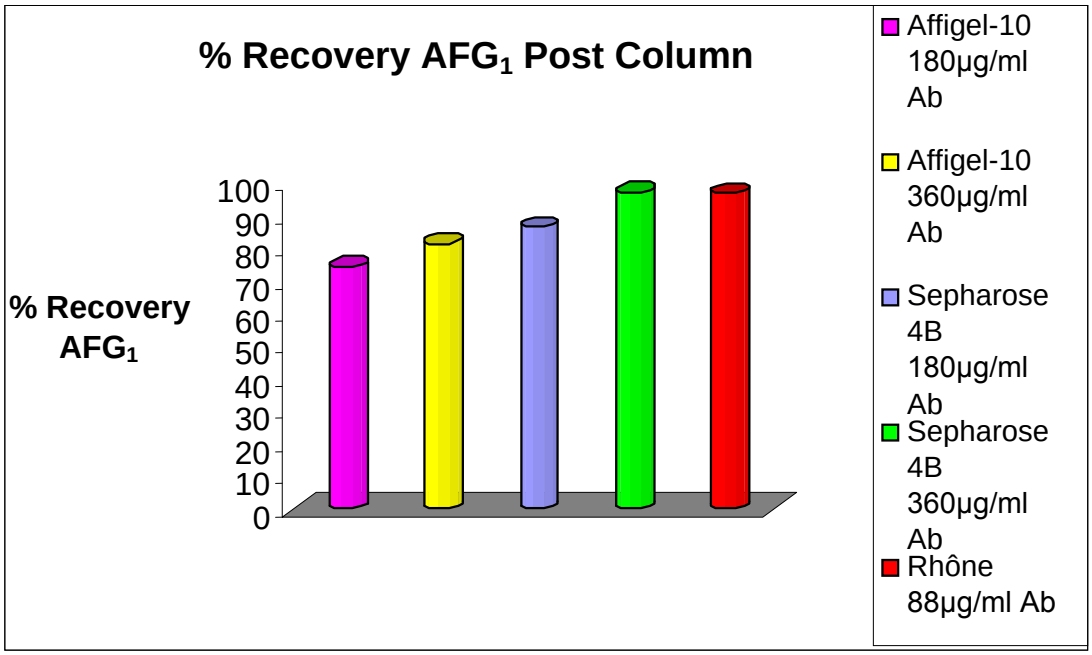
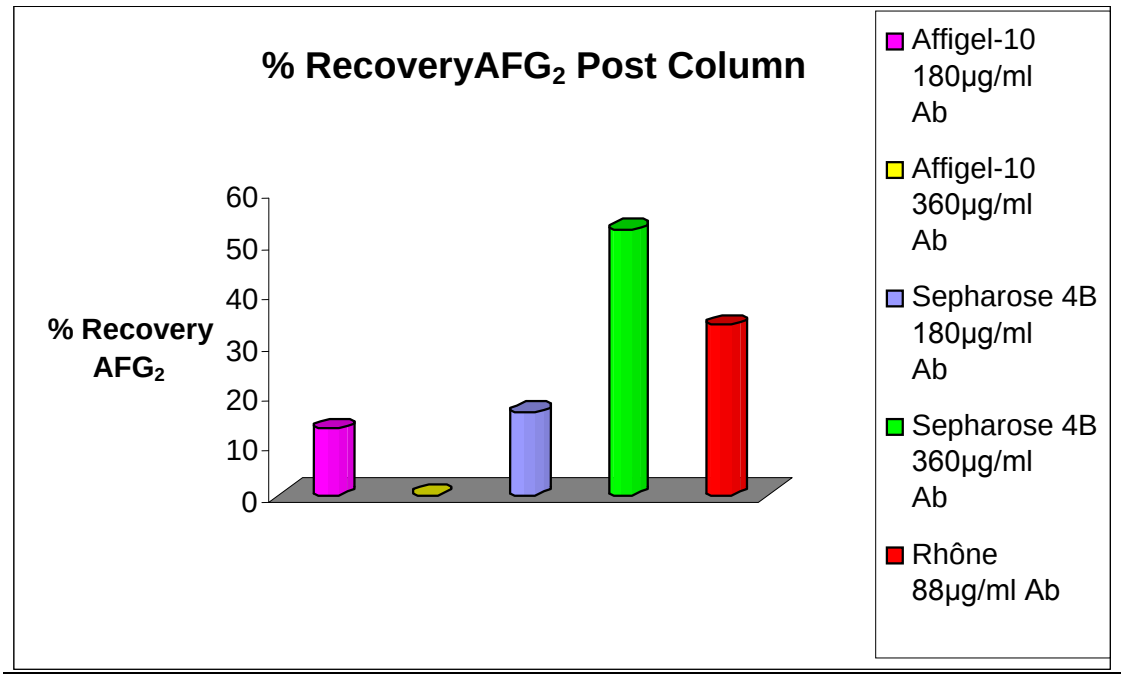


Figure 3.11 Comparison of Percentage Recovery of AFG_2 from Columns Containing 1G7-1E2 Ab Bound Gel



After analyzing these results, it was decided that CNBr Sepharose 4B would be the better gel to use in any future IACs as it produced the better overall recovery rate with the least amount of Ab bound to gel and the cost of material is low.

V. Fluorescence Detection of Aflatoxins Via HPLC

The R^2 values shown below were taken from the standard curves produced from the HPLC data obtained after analysing a series of samples for each standard toxin. The concentration range went from 0.5 ng/mL to 30 ng/mL toxin in solvent. It was not possible to obtain results for AFB_1 due to variances in equipment (there were technical problems with the original HPLC, therefore another HPLC was used for analysis, limiting time and prompting a change in the methods already used to gain results).

Table 3.1 R^2 Values for AFs obtained via HPLC, as given on the standard curves shown on the following pages.

Aflatoxin	**R^2 Value**
AFB_2	0.9818
AFG_1	0.9842
AFG_2	0.9826

Figure 3.12 Standard Curve for AFB_2 by means of HPLC

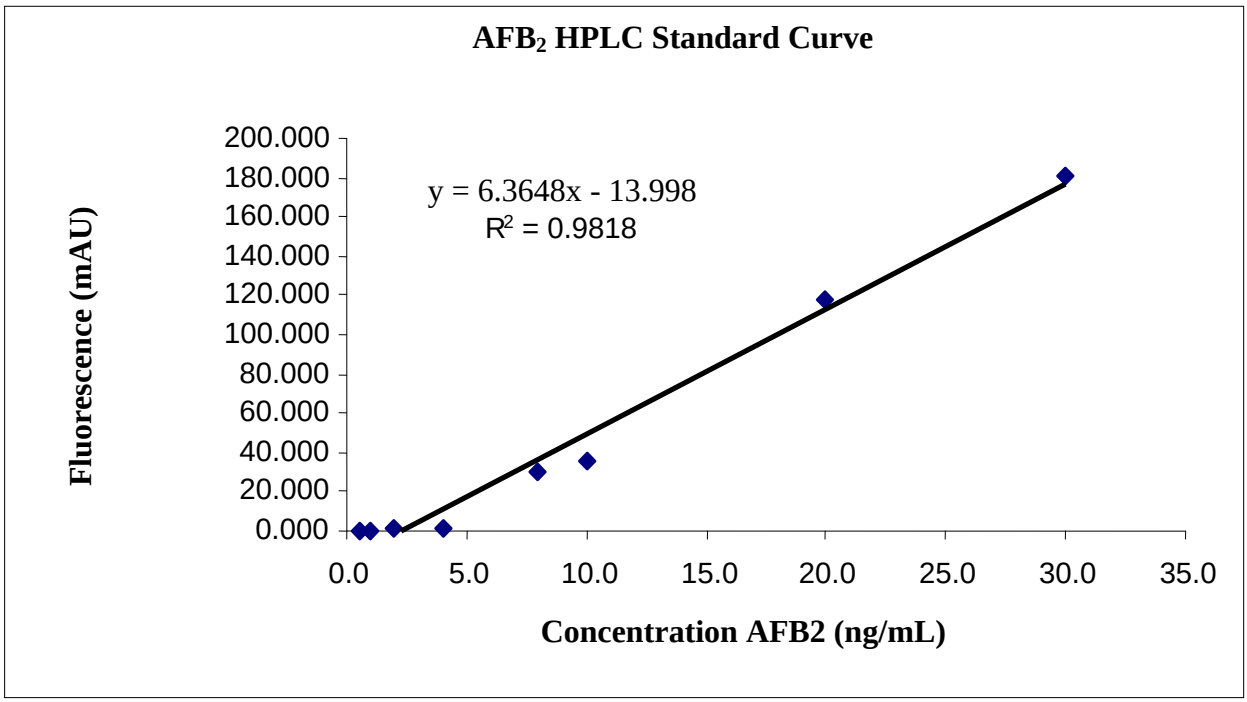


Figure 3.13 Standard Curve for AFG_1 by means of HPLC

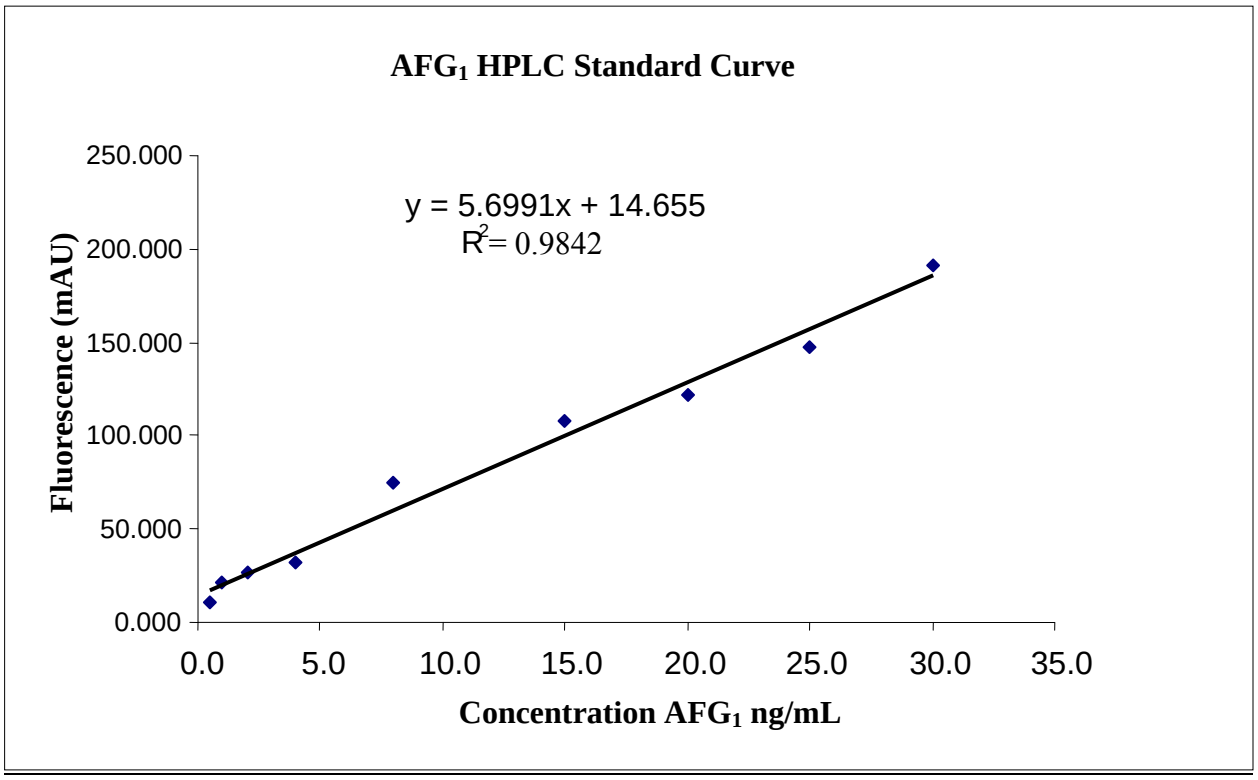
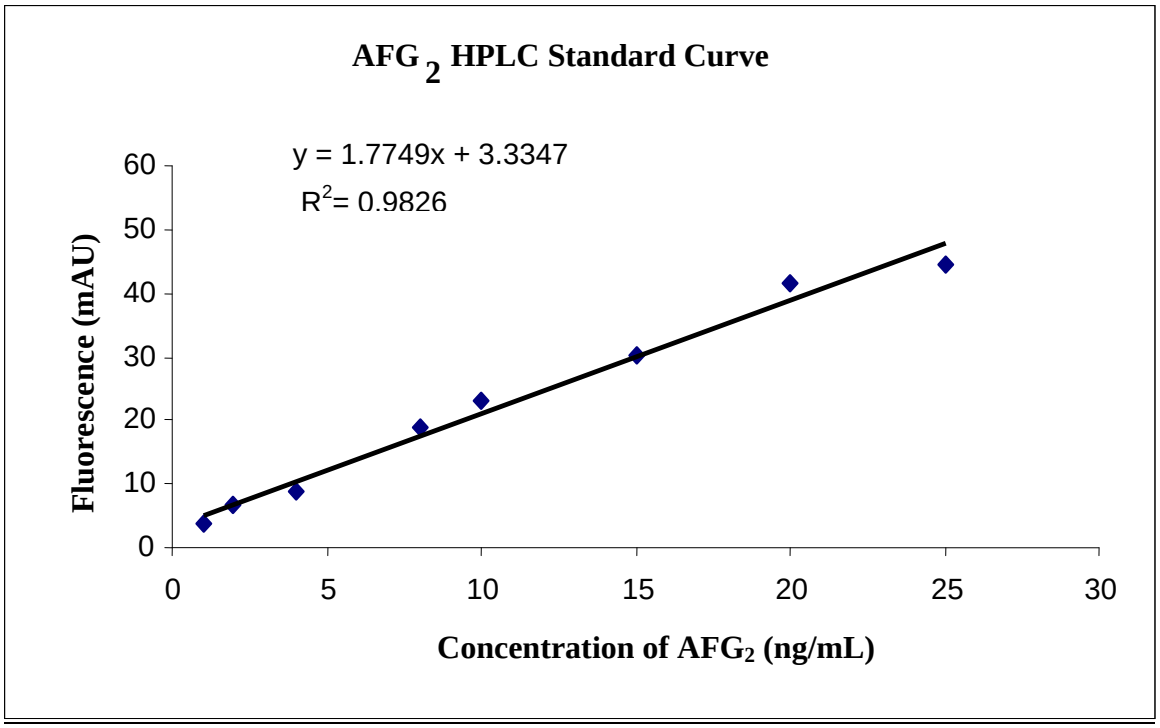


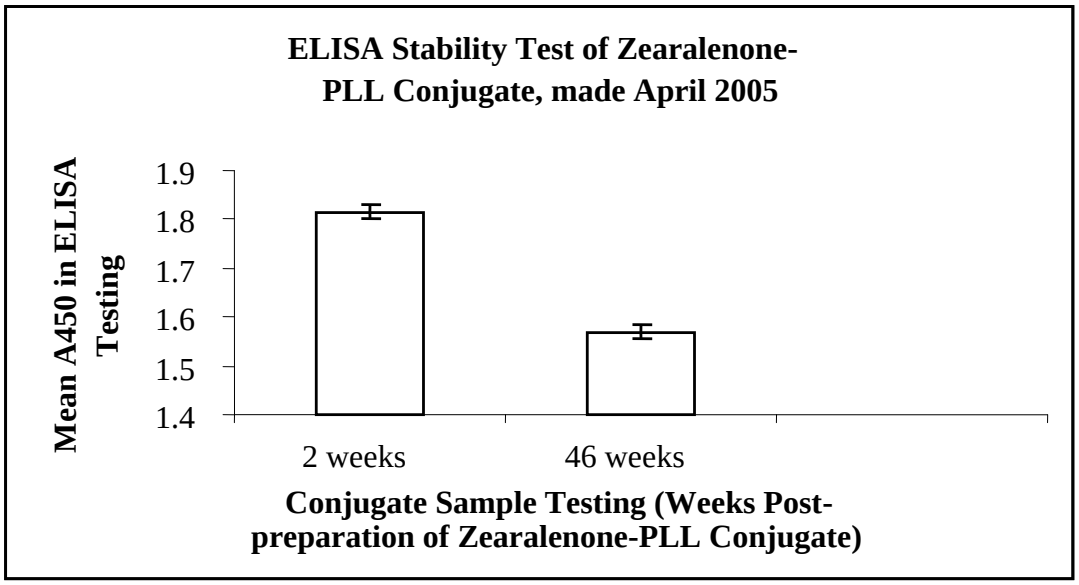
Figure 3.14 Standard Curve for AFG_2 by means of HPLC



VI. Test assay for Zear-PLL conjugate

The ELISA results shown overleaf (figure 3.15) indicate the conjugate has degraded over the interviewing 44 weeks. This was also confirmed by lower spectrophotometer readings and molar ratios. The A_{450} values were slightly low for routine use in ELISA. Therefore, a new conjugate had to be produced for further tests.

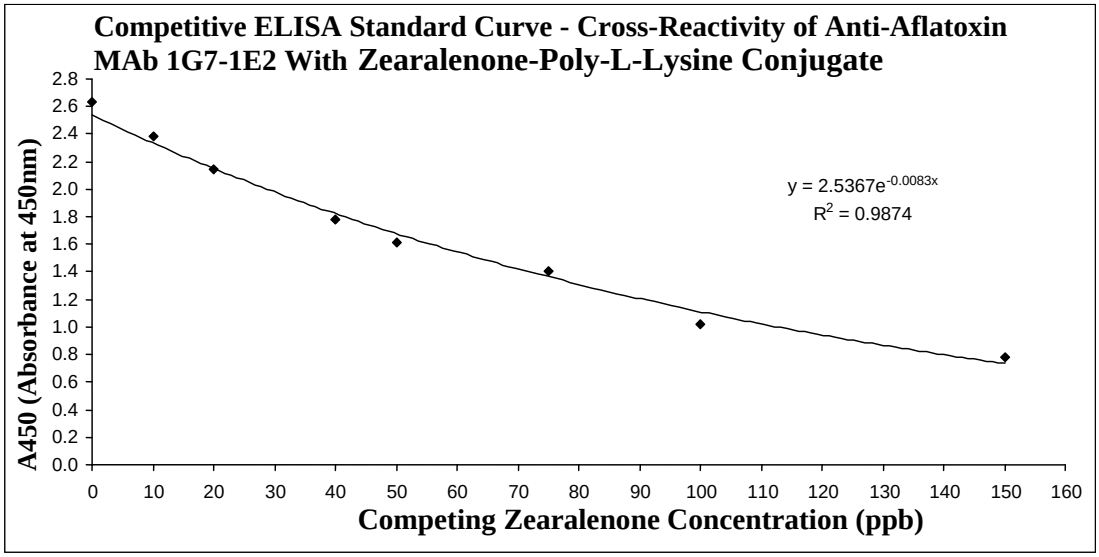
Figure 3.15 Degradation Levels of Zearalenone and PLL Conjugate 46 Weeks Post Production.



VII. Zearalenone ELISA

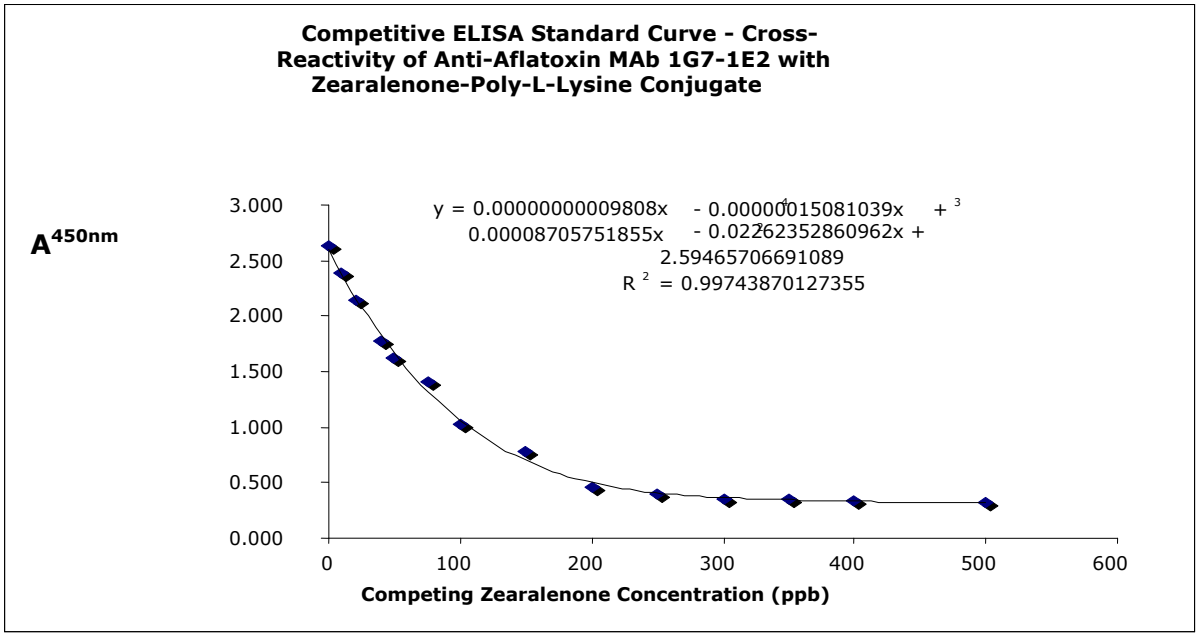
The ELISA was repeated to check for reproducibility. All results were collected and the standard curve below was produced.

Figure 3.16 Standard Curve for Zearalenone



This standard curve (fig 4.17)is additional to the one on the previous page (fig 4.16). The same methods were applied to produce this, however, the concentration of Zearalenone exceeded the last set of ELISA's.

Figure 3.17 Additional Standard Curve for Zearalenone



Another ELISA which tested a BSA/BGG + 1 – Naphthol conjugate for cross-reactivity with 1G7-1E2 was produced. It proved to be unsuccessful, specifically when compared to the Zear-PLL conjugate.

VIII. Cross Reactivity ELISA for 1G7-1E2

Figure 3.18 overleaf shows the absorbance (450nm) results for this ELISA which was produced to show the cross reactivity capabilities 1G7-1E2 has with various (lactone ring structure) mycotoxin conjugates. The best cross-reactivity is with AFB_1 in BSA, as expected (average absorbance = 2.76). This is followed by Zearalenone in PLL (average absorbance = 1.66) and then patulin in PLL (average absorbance = 1.641). The average absorbance for the blank was deducted from the average absorbance of each toxin-conjugate.

Figure 3.18 ELISA Results showing the Cross-Reactivity of Mycotoxins (that contain Lactone Rings) with 1G7-1E2.

Well	1	2	3	4	5	6	7	8	9	10	11	12
A	2.786	2.792	2.776	2.754	1.806	1.752	1.603	1.834	1.860	1.751	1.692	2.033
B	2.819	2.723	2.706	2.852	1.855	1.627	1.763	1.667	1.503	1.696	1.834	1.744
C	2.907	2.957	2.792	2.734	1.668	2.060	1.563	1.440	1.582	1.594	1.420	1.636
D	2.813	2.851	2.914	2.840	1.735	1.656	1.618	1.891	1.754	1.677	1.694	1.725
E	2.757	3.039	3.074	3.008	2.124	1.560	1.717	1.905	1.618	1.939	1.925	1.868
F	2.885	2.775	2.752	2.953	1.699	1.396	1.493	1.872	1.768	2.328	1.484	1.646
G	2.084	2.070	2.074	2.140	1.931	2.497	1.893	2.104	1.923	1.115	2.214	2.140
H	0.065	0.084	0.081	0.084	0.079	0.084	0.079	0.099	0.072	0.076	0.071	0.090

Key

AFB_1-BSA PAT-PLL ZEAR-PLL

NEAT IG7-1E2 SUPERNATANT + Buffer BLANK (PBS/Acetate citrate buffer)

The above shows that 1G7-1E2 cross-reacts with AFB_1, Patulin and Zearalenone, all of which have a common component – a lactone ring. However, does this mean all mycotoxins with a lactone ring will react to 1G7-1E2? To answer this question, further experiments will be required.

Results Synopsis

The following is a summary of the experimental work carried out to assist in achieving the aim of this project:

- Preparation and spectrophotometric quantification of AF standards for B_1, B_2, G_1 and G_2 from 0.5 ng/mL to 30 ng/mL in solvent.
- Fluorescence enhancement for AFB_1 and G_1 using β-CD.
- Fluorescent spectrophotometer determination of signals for AFB_1, B_2, G_1 and G_2.
- Preparation of standard curves for all four toxins from fluorescence spectrophotometric readings.
- Preparation of ammonium sulphate precipitated anti-AF Ab 1G7-1E2.
- Coupling of semi-purified antibody to both Affi-Gel 10 and Sepharose 4B at concentrations of 180 μg/mL of gel and 360 μg/mL of gel.
- Preparation of IACs using the above gels and Ab.
- Comparison of commercial IACs with laboratory-produced columns.
- Passing 30 ng/mL standards through three columns being compared (commercial column, 88 μg/mL of Ab + Sepharose 4B and Affi-Gel 10 at 360 μg/mL of Ab).
- Purification of 1G7-1E2 by T-Gel column chromatography.
- SDS-PAGE to determine the purity of purified Ab.
- Preparation and spectrophotometric quantification of AFB_1, B_2, G_1 and G_2 standards from 0.5 to 30 ng/mL in solvent for HPLC.
- Fluorescence enhancement of AFB_1 and G_1 with β-CD.
- Fluorescence enhancement of AFB_1 and G_1 with TFA.
- HPLC assays of AFB_2 and G_2.
- HPLC assays of fluorescence-enhanced AFB_1 and G_1.
- Preparation of standard curves for AFB_2, G_2 and G_1 from area data of HPLC chromatograms.
- Spike and then extract AFs from peanut samples using the MAb 1G7-1E2.
- Sample extracts (of AFs) were ran through test columns.
- Sample extracts (of AFs) were used in fluorimetry and HPLC assays.

- AF concentrations determined using fluorescence spectrophotometric and HPLC assay data.
- Calculation of recovery of AFs.
- Analysis and determination of Ab/column performance for capturing AFs.
- Competitive ELISAs to determine cross-reactivity of 1G7-1E2 with zearalenone and patulin.

CHAPTER IV

DISCUSSION

Discussion

I. Cell Culturing

All laboratories that culture cells have to deal with the constant threat of microbial contamination. Microorganisms are ubiquitous and bacteria can be isolated from virtually any surface, whether on an inanimate object, or from human skin. Fungal spores are often found in the atmosphere in the laboratory due to opening of doors, and air conditioning ducts. Any windows that are within a cell culture room need to be properly sealed, to avoid getting spores and any coming into the environment (although, ideally, a cell culture room would have no windows for this purpose). If media, or even serum, is not sterilized properly, then mycoplasma infection is possible. As well as these possible routes of contamination, ALL equipment which is used in cell culturing can cause contamination if not properly disinfected. It is, therefore, important to use aseptic techniques during the process…preventive measures are far better than having to deal with a contaminated batch of cells (which often means discarding all the cells and starting the process again!). For this project, all work with regards to cell culturing was carried out using good aseptic technique as a preventative measure against contamination. An adequate amount of 1G7-1E2 for study was produced this way and was stored in a freezer until required.

II. Antibody Clean-up Processes

Any one of a whole range of techniques could have been used to purify the Abs, however, after extensive research into various protocols, it was decided to test and compare only two methods. Ammonium sulphate precipitation proved useful in "cleaning up" the Abs, although after comparing the results with that of the T-Gel purified Abs, it became clear that T-Gel is slightly more successful of the two at removing unwanted proteins. The comparison was made through SDS-PAGE. It is possible to see from the number of protein bands on the SDS PAGE photos which of the two methods is best (the least bands means less unwanted proteins). The ammonium sulphate precipitated Abs were examined by SDS-PAGE prior to and after they had undergone thiophilic adsorption chromatography with T-Gel.

Since the results show that using both techniques in succession to purify the Ab does not make a difference when compared to the straight forward purification of supernatant by T-gel, it was decided that after this point onwards, all of the 1G7-1E2 supernatant would be purified using a T-Gel column.

III. SDS PAGE

SDS-PAGE was used to ascertain the purity of 1G7-1E2. The MAb appears to have been reduced to two bands, as shown in figures 4.2, 4.2 and 4.3 on pages 53-54 of this report. The results show that there are other proteins amongst IG7-1E2.
The breakdown of the disulphide bonds within the MAb is the result of 2-mercaptoethanol, a reducing agent that denatures the protein before loading on the gel to ensure a smooth and uniform run. This is part of the SDS PAGE buffer.
If only one band of pure IG7-1E2 was produced, then it could be seen at around the 150kD mark (example of scale given on figure 3.1, page 53). When two bands are produced, they will sit at around 25kD and 80kD. This indicates that albumin was also present in the sample run. Normally, albumin can be removed (as it is will not serve any purpose in this instance) by using a protein A column, however, this will not work for IgG1's, such as 1G7-1E2 (due to weak binding affinity).

As well as 2 - mercaptoethanol being a probable cause of excess bands or fragments of protein, the heat activation stage may also have contributed by denaturing this particular Ab and therefore splitting the heavy and light chains. This is something that should be noted for future studies.

IV. Immunoaffinity Columns

It was decided to produce four different types of columns – two sets of IACs containing different gels and two subsets containing gel with different amounts of Ab attached. The columns were compared against columns purchased from an established biotech company.

A comparison was made between Sepharose 4B and Affigel 10. Overall, Sepharose 4B proved to be the better gel for use in the IACs. These two gels were then

compared to the already commercial column produced by Rhône Diagnostics. A greater percent recovery of $AFB_{1,}$ G_1 and G_2 was achieved using Sepharose 4B and 1G7-1E2 than any other gel and Ab combination. This result makes Sepharose 4B and 1G7-1E2 a possible pair for the purpose of producing and commercialising a new IAC. The comparison between columns was run only once for each set of columns, as time would not permit further experimentation. There is a need however, for this to be repeated to be able to collate the results and give a fuller comparison.

The only down side to Sepharose 4B gel is that the beads tend to crumble fairly easily, therefore, great care must be taken with packaging and use of the material. However, the benefits outweigh this potential problem. The gel is reasonable in price, easy to prepare and allows proteins to attach efficiently.

V. Spectrophotometry and HPLC

Technical difficulties meant that the HPLC work could not be completed, however, the spectrophotometric work proved very useful in determining the best materials and methods to use in constructing the IACs as well as being used to observe their analytical capabilities with the work that as carried out to create the standard curves.

Sepharose 4B is the best gel as it allows the Ab to bind well, is cheap and effective in its purpose of helping to create a column that can be used to obtain AF from samples. The amount of Ab attached to gel was 180µg and 360µg, a higher amount than competitors IACs, however, captures a good amount of toxin (as can be seen from the results page 59). The amount of Ab required is not considered problematic as it is easy to produce in abundance. Ab purification by means of T-gel was also adopted as the preferred method since Ab clean-up is possible by putting supernatant through the gel just once.

VI. Test Assay for ZEAR-PLL conjugate

As suggested by Prasertsilpa (1999), 1G7-1E2 can be used to detect total AF, given that former studies have proved there is cross-reactivity of the MAb with all four major AFs. The percentage of cross-reactivity of each toxin to Ab is shown in table 3.0 below; along with a comparative set of results from an ELISA that tested another Ab already on the biotechnology market, sold as Biokits ® Ab.

Table 3.0 Evaluation of indirect competitive ELISA's (results extracted from the thesis by Prasertsilpa, 1999)

AF standard	**% Cross-reactivity of 1G7-1E2 MAb**	**% Cross reactivity of Biokits ® Ab**
AFB_2	100%	100%
AFB_1	159.2%	60%
AFG_1	162.2%	100%
AFG_2	52.3%	60%

There is a clear difference in cross-reactivity, which shows that 1G7-1E2 is the superior Ab. With this in mind, it was decided to create additional experiments for this project, involving the production of various ELISA's. It was suggested that 1G7-1E2 is capable of reacting with other mycotoxins (personal communication with Dr D.C. Morris), as long as they have a lactone ring. To put this theory to the test, a patulin and a zearalenone ELISA were produced. Both of these toxins had some level of cross-reactivity with 1G7-1E2, as can be seen on page 67 in the results section.

Since the mycotoxins used in the ELISA's are not immunogenic (as most mycotoxins are), all of them had to be conjugated to a carrier protein to elicit the immune response (Shim *et al*, 2004).

Thus, carrier proteins such as BSA, PT and PLL were used and also compared for suitability to each toxin/ELISA. PLL was determined to be the most successful conjugate and was used in the test assay described earlier. The conjugate degrades quickly (see results page 65), therefore it is necessary to use a fresh batch when carrying out a test.

VII. Zearalenone assay

The graphs of the ELISA results show that there is a good level of cross-reactivity between the Ab and toxin (p65 and 66).

It is not clear why these results were obtained, however, from looking at the structure of zearalenone (EMAN), the lactone ring that it has is a common component which is seen in the structure of the other mycotoxins tested.

VIII. Lactone Cross Reactivity ELISA

It was thought because of previous studies that perhaps Ab 1G7-1E2 would react with any mycotoxin which contains a lactone ring, and not just the AFs as originally intended. Therefore, an ELISA was produced to test this theory.

The ELISA was split into three sections – one for each of AFB_1, Patulin and Zearalenone. All toxins reacted with the Ab, showing that 1G7-1E2 is not that specific. It is a useful Ab for obtaining any one of these mycotoxins (which would still need determined via HPLC to specifically identify the toxin bound to Ab). However, it does have a better affinity towards the AFs (as shown in fig 4.18).

CHAPTER V

CONCLUSIONS
and
REFERENCES

Conclusions

The following conclusions can be derived from this project:

1. The MAbs can be produced readily and in abundance with little nutrient supply.

2. There are several protein clean-up methods that can be used to expel unwanted proteins from the Ab supernatant, however, T-Gel proved the more favourable for its efficiency.

3. Sepharose 4B readily bound 1G7-1E2 and allowed for a higher AF recovery rate than Affi-gel 10.

4. ß-CD is useful in boosting immunofluorescence for small spectrophotometry samples, but TFA is better for HPLC samples.

5. High recovery rates for AFB_1, G_1 and G_2 was achieved 1G7-1E2, bound to Sepharose 4B using 1G7-1E2.

6. 1G7-1E2 cross-reacted with patulin and zearlenone, as proved by means of an ELISA.

7. The protein carrier PLL was the most successful for producing conjugates for the mycotoxins.

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