Development of an assay for Aflatoxin B$_1$ in a range of foodstuffs using the Biacore Q Surface Plasmon Resonance biosensor

A dissertation for the degree of

Master of Science

by

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August, 2011

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DECLARATION

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ACKNOWLEDGEMENTS

PUBLICATIONS AND PRESENTATIONS

Publications

Applications of Sensors in Food and Environmental Analysis (2010)

O'Kennedy, R., Finlay, J.J., Leonard, P., Hearty, S., Brennan, J., Stapleton, S. Townsend S., Darmaninsheehan, A., Baxter, A. and Jones, C.

pp 195-232.Book chapter in:

Sensors for Chemical and Biological Applications, Edited by Manoj Kumar Ram and Venat R. Bhethanabotla

CRC Press, Taylor and Francis Group, Bocan Raton, Fl., USA.

Oral presentations

"The Detection of Aflatoxin B_1 by Optical Biosensor Analysis"

SafeFood Biotoxin Research Network, April 2007, Belfast, UK.

Poster presentations

"The Detection of Aflatoxins by Optical Biosensor Analysis"

International Association for Cereal Science and Technology, September 2007, Glasgow, UK.

"The Detection of Aflatoxins by Optical Biosensor Analysis"

XII International IUPAC Symposium on Mycotoxins and Phycotoxins, May 2007, Istanbul, Turkey.

"The Detection of Aflatoxins by Optical Biosensor Analysis"

Rapid Methods Europe 2007, January 2007, Noorwijk, The Netherlands.

"The Detection of Mycotoxins by Optical Biosensor"

3th World Mycotoxin Forum, November 2005, Noorwijk, The Netherlands.

ABBREVIATIONS

ABBREVIATION	MEANING
A_w	Arbitrary unit for water activity
Ab	Antibody
AFB_1	Aflatoxin B_1
AFB_1-FAPY	AFB_1-formamidopyrimidine adduct
AFB_1-N7-Gua	8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1
Afla	Aflatoxin
ALARA	As Low As Reasonably Achievable
AOAC	Association of Official Analytical Chemists
ASEAN	Association of Southeast Asian Nations
BF method	Best Food method
CEN	European Committee for Standardisation
ddH_2O	Double distilled water
DCC	N,N'-dicyclohexyl-carbodiimide
DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation (of the United Nations)
FAPAS	Food Analysis Performance Assessment Scheme
GC	Gas spectrometry
HBV	Hepatitis B virus
HBx	HBV X protein
HCC	Hepatocellular carcinoma
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
IFC	Integrated micro-fluidic cartridge
IRMM	Institute for Reference Materials and Methods
ISO	International Standardisation Organisation

IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LAB	Lactic acid bacteria
LD_{50}	Lethal Dose that kills 50% of a group of test animals
MERCOSUR	Mercado Común del Sur
MS	Mass spectrometry
NHS	N-hydroxysuccinimide
NOAEL	No Observable Adverse Effect Level
OCHA	Ochratoxin A
PVDF	Polyvinylidene Fluoride
ppb	parts per billion
PTW/DI	Provisional Tolerable Weekly/Daily Intake
PVP	Polyvinylpyrrolidone
RASFF	Rapid Alert System for Food and Feed
RU	Resonance units
scFv	Single chain anitbody fragment
SPE	Solid phase extraction
SPR	Surface plasmon resonance
TIR	Total internal reflection
TLC	Thin layer chromatography
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation
ZEA	Zearalenone

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ABSTRACT

Aflatoxin B_1 (AFB1) is one of the most toxic substances known to man and chronic exposure to low doses has been implicated in hepatic cancer.   Strict regulations have been imposed world-wide to significantly reduce food and animal feed contaminated with AFB1 from entering the food stream.

The purpose of this study was to develop an antibody-based diagnostic kit for aflatoxin B_1 using surface plasmon resonance (SPR). SPR is an innovative optical technique that measures biomolecular interactions on the surface of a sensor chip. Advantages of SPR are that it is label-free, high-throughput, it does not use large volumes of solvents and is both accurate and sensitive.

A wide range of crops are susceptible to AFB_1 contamination. Several of the most commonly affected were chosen for investigation. . The first studied was compound feed which was purchased from IRMM as certified reference material for aflatoxin analysis. This was chosen to investigate first as the compound feed was produced from naturally incurred aflatoxin contamination and was not a spiked sample. It therefore closely resembles a real sample. Preliminary studies showed recoveries of 98%.

The second matrix chosen was infant formula. When this study had started there were currently no high throughput assays commercially available that were sensitive enough to detect aflatoxin to the legislative levels. The purpose of this study was to eventually create and market an aflatoxin B_1 testing kit. If we could develop a kit that would detect aflatoxin B_1 to these levels it would be a unique selling point of our kit. Unfortunately, due to matrix interference problems we were not able to achieve an assay sensitive enough to detect to the legislative limits.

The third and fourth sample matrices chosen were maize and peanuts. The preliminary studies showed that recoveries of 111% and 86 – 103%, respectively, were possible.

Chapter 1: Introduction

1.1   Mycotoxins

Mycotoxins are toxic secondary metabolites produced from a range of toxigenic filamentous fungi (moulds), predominantly from the genera *Aspergillis, Penicillium* and *Fusarium* (Sweeney *et al.,* 1998).   Some originate from a particular species, for example, only *A. flavus* or *A. parasiticus* produce aflatoxins, whilst patulin is produced by a variety of different moulds from the species *Penicillium, Aspergillus* and *Byssochlamys* (Alves *et al.,* 2000).   Some other moulds can produce several mycotoxins.   *Fusarium* species can produce tricothecenes, fumonisins, zearolenone (ZEA), deoxynivalenol (DON) and monoliform amongst others (Creppy, 2002).   However, the maximum production of different mycotoxins may not occur under the same conditions.   A single strain of *Fusarium graminearum* can produce both ZEA and DON.   However, while ZEA production reaches a maximum at 25ºC, DON production increases with increased temperature, the maximal amount being produced at 30 ºC (Ramirez *et al.,* 2006)

Certain environmental conditions favour mycotoxin production. These conditions are more restricted than those needed for normal fungal growth (Kokkonen *et al.,* 2005). Production of particular mycotoxins is also dependant on the climate. Some mycotoxins, such as ochratoxin A produced by *P. verrucosum*, favour temperate climates and are, therefore, found predominantly in Europe.   Others, for example aflatoxins, occur more commonly in tropical climes. They are produced when the crop is under stressed conditions and this is accelerated by crop damage e.g. by insect infestation (Pier, 1992).

Since mycotoxins can occur all over the world, all crops are susceptible to contamination, and this can happen at any stage of the farming process, including cultivation, harvest, drying, storage, or transportation. Major commodities affected are cereals (maize, wheat, barley, oats and rice), nuts, dried fruit, coffee, cocoa, spices, beer and wine. Mycotoxins can also enter the human food chain through the meat of livestock that have eaten contaminated feed, or from their milk, cheese or eggs (Bintvihok *et al*., 2002; Battacone *et al.,* 2003; Van Eikeren *et al.,* 2006).   Many mycotoxins are stable to heat and other effects of food processing.   Therefore, processed foods, especially those intended for children, have to be tested for contamination.

The functions of mycotoxins are not fully understood. Mycotoxins are secondary metabolites, and as such they are not involved in fungal growth, respiration or reproduction. A range of theories have been put forward as to their precise physiological roles. Suggestions include that they may be waste products, that they have antibiotic properties, that they were developed as a chemical defence system or they may facilitate communication between moulds (Etzel, 2006; Ciegler, 2007).

Approximately 400 mycotoxins exist. Only six or seven, however, are considered significant, as they are causative agents for a large range of food-borne illnesses in both humans and livestock. These mycotoxins are the aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A, fumonisin, deoxynivalenol, zearalenone, tricothecenes and patulin.

The detrimental effect of fungal toxins was first uncovered in the 1960's when the consumption of contaminated peanut meal from Brazil lead to the death of 100,000 turkeys in England. Subsequently, it was discovered that the meal had been infected with the mycotoxin aflatoxin B_1 (Diaz, 2005).

Since then, mycotoxin contamination was linked to many and varied human disorders depending on the type and dose of the toxin. Acute cases of mycotoxicosis, where large amounts have been consumed, are generally confined to the developing countries. An example of this is the acute aflatoxicosis outbreak in Kenya in 2004, which resulted in 125 deaths due to the consumption of contaminated maize (Niyikal *et al*., 2004; Aziz-Baumgartner *et al.,* 2005; Lewis *et al*., 2005). Chronic cases of mycotoxin contamination, where small amounts of the mycotoxin have been ingested over long periods of time, have been associated with a wide variety of diseases targeting several organs in humans and animals. These include Balkan Nephropathy (ochratoxin A) (Petkova-Bocharova *et al.,* 1991)*,* Reye's Syndrome (aflatoxins) (Trauner *al*., 1984), alimentary toxic aleukia (tricothecenes) (Lutsky *et al.,* 1983) and oesophageal cancer (fumonisins) (Chu *et al.,* 1994).

Most of the epidemiological research has focused on the individual effects of mycotoxins. However, several mycotoxins are able to contaminate the same commodity concurrently (Fernandez *al*., 2001; Domijan *et al*., 2005). For example, corn can be infected with fumonisin, aflatoxin, zearalenone and/or ochratoxin, and there is a positive association between the presence aflatoxin and patulin in Turkish dried figs (Karaca *et al.,* 2006). Therefore, recent studies have investigated the simultaneous effects of mycotoxins, or whether they act additively, synergistically or antagonistically.

An additive effect is when the cumulative effects of two mycotoxins are equal to the sum of the separate effects. However, a synergistic effect is when the interaction between the mycotoxins causes a greater effect than just the sum of the individual mycotoxin's effect, whereas an antagonistic effect lessens the combined effect of the mycotoxins through the interference of one mycotoxin with the effect of the other.

Aflatoxin has an additive affect on both DON and fumonisin B. It acts synergistically with T2 toxin and ochratoxin A, but works antagnostically with monoliform. A study (Huff *et al.*, 1986) showed the synergism between AFB_1 and T2 by feeding broiler chickens a diet containing either:
- 2.5µg/g aflatoxin B_1
- 4.0 µg/g T-2
- 2.5µg/g aflatoxin B_1 + 4.0 µg/g T-2,

and comparing it to uncontaminated feed.

By the end of three weeks, the results show that with the combined diet there was a weight drop of 28% when compared to the control, whereas with AFB_1 and T-2 alone there was a 12% and 8% drop, respectively. A synergistic effect was also noted with respect to the increase in relative weights of the kidney, liver and spleen, and also with the reduction of serum protein, glucose, albumin and potassium levels. In a similar experiment, Huff *et al.* (1984) also showed the synergistic effect between aflatoxin and ochratoxin A. Boiler chickens given feed containing either AFB_1 (2.5µg/g) or ochratoxin A (2.0µg/g) or both combined, showed that the diet containing both toxins caused a drop in body weight of 39%

compared to aflatoxin (12%) or ochratoxin A (14%) alone. The reason for this may be that when these toxins are combined they are more effective at disrupting protein synthesis.

The FAO estimates that 25% of the world's crops are contaminated with mycotoxins. It is, however, difficult to calculate the total economic losses as, in addition to losses of crops, mycotoxin exposure to livestock can cause loss due to reduced fertility, stunted growth and impaired immunity.

1.2   Aflatoxins

Aflatoxins are produced from particular strains of the species *Aspergillus flavus* and *Aspergillus parasciticus* and only under certain environmental conditions. Aspergillus grows favourably under hot and humid tropical or sub-tropical conditions. Temperature range for growth is between 28 and 33ºC and at the water activity of about 0.83-$0.97a_w$, and are predominantly found in acidic soils and decaying vegetation (Ehrlich *et al.,* 2005). The climate in Europe does not lend itself to aflatoxin production, and so the risk for contamination is low. Nevertheless, occurrences have been reported for stored maize in Northern Italy. Aflatoxins can contaminate a wide variety of commodities, for example, cereals (maize, wheat, rice, sorghum, millet), nuts (pistachios, brazil nuts, peanuts, walnuts, coconuts), spices (chilli, tumeric, paprika, black pepper, ginger), dried fruit, seeds and milk.

There are 17 related aflatoxin metabolites (McClean and Dutton, 1995). Only four of these are the main contaminants found in food. These are aflatoxins B_1, B_2, G_1 and G_2, named after their natural fluorescence under UV light - B_1 and B_2 have a blue fluorescence, whilst G_1 and G_2 are green. B_1 is the most predominant aflatoxin, usually present in greater concentrations than the sum of the other three, and it also has the greatest toxicity. Two toxic hydroxy metabolites, M_1 and M_2, are formed and excreted via the milk and urine of the mammal that has consumed contaminated food. This is especially significant for dairy cows, as their milk will contain these toxins if they have eaten feed containing aflatoxins. $0.3 - 6.2$ % (w/v) of the AFB_1 in animal feed is transformed into M_1. M_1 can also be passed in human breast milk to nursing babies.
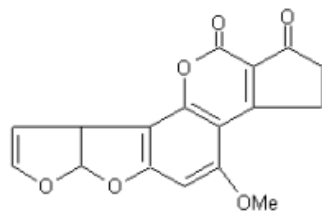
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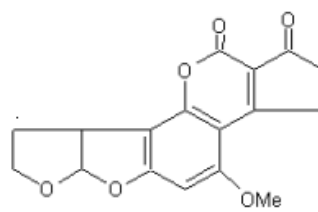
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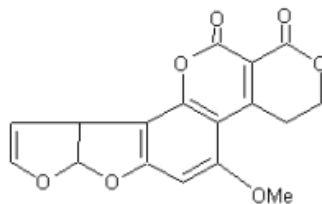
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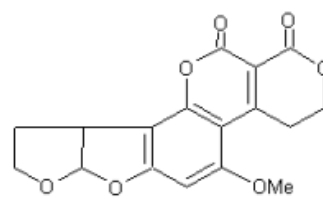
Aflatoxin G$_2$

Figure 1.1 Chemical structure of aflatoxin B$_1$, B$_2$, G$_1$ and G$_2$.

1.3 Physical and chemical properties

Aflatoxins belong in a family of chemical compounds called coumarins. They are crystalline solids that are soluble in polar solvents such as methanol, acetonitrile, acetone, chloroform, dichloroethane, ethyl acetate and dimethyl sulfoxide. They dissolve in water at a concentration of 10–20mg/L. The molecular weights of aflatoxins differ slightly. B$_1$ is 312, B$_2$ 314, G$_1$ 328 and G$_2$ is 330, and their melting points are 268°C, 286°C, 289°C and 237°C, respectively. They are very stable in the absence of light, even at temperatures above 100ºC when in a dry state. In the presence of moisture, however, long periods of elevated temperatures can cause the lactone ring to open, making them susceptible to decarboxylation.

Aflatoxins can be deactivated by alkali due to the hydrolysis of the lactone ring. Therefore, addition of ammonia or hypochlorite is often used in labs to decontaminate aflatoxins.

However, if the basic conditions are mild, acidification can reverse the reaction forming the original aflatoxin.

1.4   Epidemiology

Aflatoxins, especially B_1, are the most potent naturally occurring toxins, primarily targeting the liver. Exposure to large doses of aflatoxin B_1 can cause acute toxicity in both animals and humans, whereas chronic toxicity caused by prolonged exposure to lower doses is carcinogenic. It was described by the International Agency on Research on Cancer (IARC) in 1993 as a group I carcinogen. This means that there is sufficient evidence available to implicate aflatoxin as causative of human hepatocellular carcinoma (HCC). They are also teratogenic, mutagenic and immunosuppressive (Kihara *et al*., 2000).

Acute toxicity has been studied in a wide range of animals from trout to primates (Pier *et al*., 1992). The susceptibility of a species to aflatoxin exposure varies (Table 1.1). In most animal studies, however, exposure resulted in hepatocellular and/or cholangiocellular liver tumours, including carcinomas. The LD_{50} scores, the dose required to kill half the population, are generally between 0.5 and 10mg/kg body weight. Susceptibility to aflatoxicosis has also been shown to be gender-specific in both rat and mice studies, with males being more sensitive than females.

Table 1.1 Acute toxicity of aflatoxin B1 expressed as a single oral dose LD50 (Cardona et al., 2000)

Species	LD50 mg/kg bodyweight
Rabbit	0.30
Duckling (11 day old)	0.43
Cat	0 55
Pig	0.60
Rainbow trout	0.80
Dog	0.50 - 1.00
Sheep	1.00 - 2.00
Guinea pig	1.40 - 2.00
Baboon	2.00
Chicken	6.30
Rat (male)	5.50 - 7.20
Rat (female)	17.90
Macaque (female)	7.80
Mouse	9.00
Hamster	10.20

Adult mice are resistant to aflatoxin-induced liver cancer. However, infant mice are extremely susceptible – aflatoxin causes cancer in 4-day old mice, but mice that are a week old are resistant. Studies in changes of gene expression in mice by Essignmann (1982) may help to explain why some species, or genders, are more susceptible than others.

Exposure to fatal doses of aflatoxins (>6000mg) are rare, with outbreaks confined to the developing countries, and are caused by contamination of a staple food, such as rice, maize or wheat. The most recent outbreak of aflatoxicosis was in Kenya from January to June 2004, where maize stocks had been contaminated to concentrations as high as 4,400 ppb, over 220 times greater than the regulatory limits set for Kenya (Niyikal *et al*., 2004; Aziz-Baumgartner *et al.,* 2005; Lewis *et al*., 2005). This episode resulted in 317 cases and 125 deaths Since then several isolated instances of aflatoxin fatalities have occurred. For example, 5 deaths were reported in April 2006 in the Makueni district of Kenya. In total in Kenya alone over the last 2 years 100 known fatalities caused by aflatoxins were reported (FAO/WHO).

Aside from cases of acute aflatoxicosis, chronic doses of aflatoxin over extended periods of time can also be attributed to many deaths in the developing world.

Aflatoxin has a positive association with hepatocellular carcinoma, which is the fifth most common and the third most fatal cancer worldwide, causing an estimated 500,000 deaths annually. Other deaths that have been linked to aflatoxin contamination are caused by either the loss of immunity or malnutrition, especially to the old and very young. Aflatoxin in weaning foods or breast milk from a mother who has consumed contaminated food can cause stunted growth and underweight children. Also, reduction in immunity caused by aflatoxin consumption can lead to increased susceptibility to a range of diseases, for example AIDS, or infections from drinking unsafe water.

Fatalities from acute aflatoxicosis are not restricted to humans, a recent example was in January 2006, where over 100 dogs died due to the contamination of dog food in America (Leung *et al*., 2006; Stenske *et al*., 2006). Symptoms included loss of appetite, jaundice, severe vomiting combined with bloody diarrhoea and a fever. All dogs died of liver failure.

1.5 Mechanism of AFB$_1$-induced DNA damage

After ingestion, aflatoxin is transported to the liver, where it is oxidised by the enzyme cytochrome P450 (McClean and Dutton, 1995; Turner *et al.,* 1998). The function of cytochrome P450 is to convert toxins into a more soluble form to facilitate their removal from the body via the kidney. However, in the case of aflatoxins, the enzyme forms an intermediate through the addition of an oxygen onto the aflatoxin molecule, converting it to a highly reactive and mutagenic compound, AFB$_1$-8,9-epoxide (Figure 1.2).

The epoxide can be deactivated by addition of a glutathione molecule, facilitated through the enzyme glutathione S-transferase, making it more water soluble and easier to eliminate from the body. However, due to the reactive nature of the epoxide, there is not enough time for this reaction to be completed, and the intermediate can attack DNA. AFB$_1$-8,9-epoxide can exist as two stereoisomers in an *exo* and an *endo* conformation. AFB$_1$- *exo*-epoxide is 1000 fold more reactive than the endo form, and can react with DNA forming AFB$_1$ adducts with a yield of 98%, despite having a half-life of only one second in aqueous buffer (Bedard *et al.,* 2006). Mice are able to express constitutively an α-class glutathione-S-transferase which has higher specificity to AFB1-*exo*-epoxide, resulting in faster detoxification by glutathione conjugation. This has been linked to the relative resistance mice have to the toxicity of aflatoxins (Wang *et al.,* 2000).

The primary DNA adduct that the epoxide forms is 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B$_1$ (AFB$_1$-N7-Gua) when the C8 position of the epoxide reacts with the N7 position of guanine in DNA (Bedard *et al.,* 2006). The formation of AFB$_1$-N7-Gua is directly proportional to the amount of AFB$_1$ ingested and, as it is excreted via urine, several human studies have exploited this correlation to investigate the relationship between dietary exposure to AFB$_1$ and hepatocellular carcinoma (HCC) (Groopman *et al., 1992,* Groopman *et al.,* 1993; Groopman *et al.,* 1996*).

AFB$_1$-N7-Gua is unstable due to the positive charge on the imidazole ring, with a half-life of 7.5 hours in rat liver (Wang and Groopman, 1999). It is then broken down to form two

secondary compounds. The first is due to depurination leading to the formation of an apurinic (AP) site. The second, is produced when the mildly alkali conditions hydrolyse the imidazole ring, opening it up, resulting in the creation of the stable AFB_1-formamidopyrimidine adduct (AFB_1-FAPY) (Bailey *et al.,* 1996; Keller- Seitz *et al.,* 2004).

It is the AFB_1-FAPY adduct that has been implicated as the causative species for HCC. Both AFB_1-N7-Gua and AFB_1-FAPY adducts alter the structure of DNA. However, AFB_1-FAPY is less distortive and thus is more resistant to repair through the nucleotide excision repair pathway. This adduct is, therefore, resistant and can interfere with DNA replication. It is probably responsible for the G to T transformation in the third position of codon 249 of the p53 tumour suppressor gene that is associated with over 50% of AFB_1-related hepatocellular carcinoma (Aguilar *et al.,* 1993; Bailey *et al.,* 1996; Smela *et al.,* 2002).
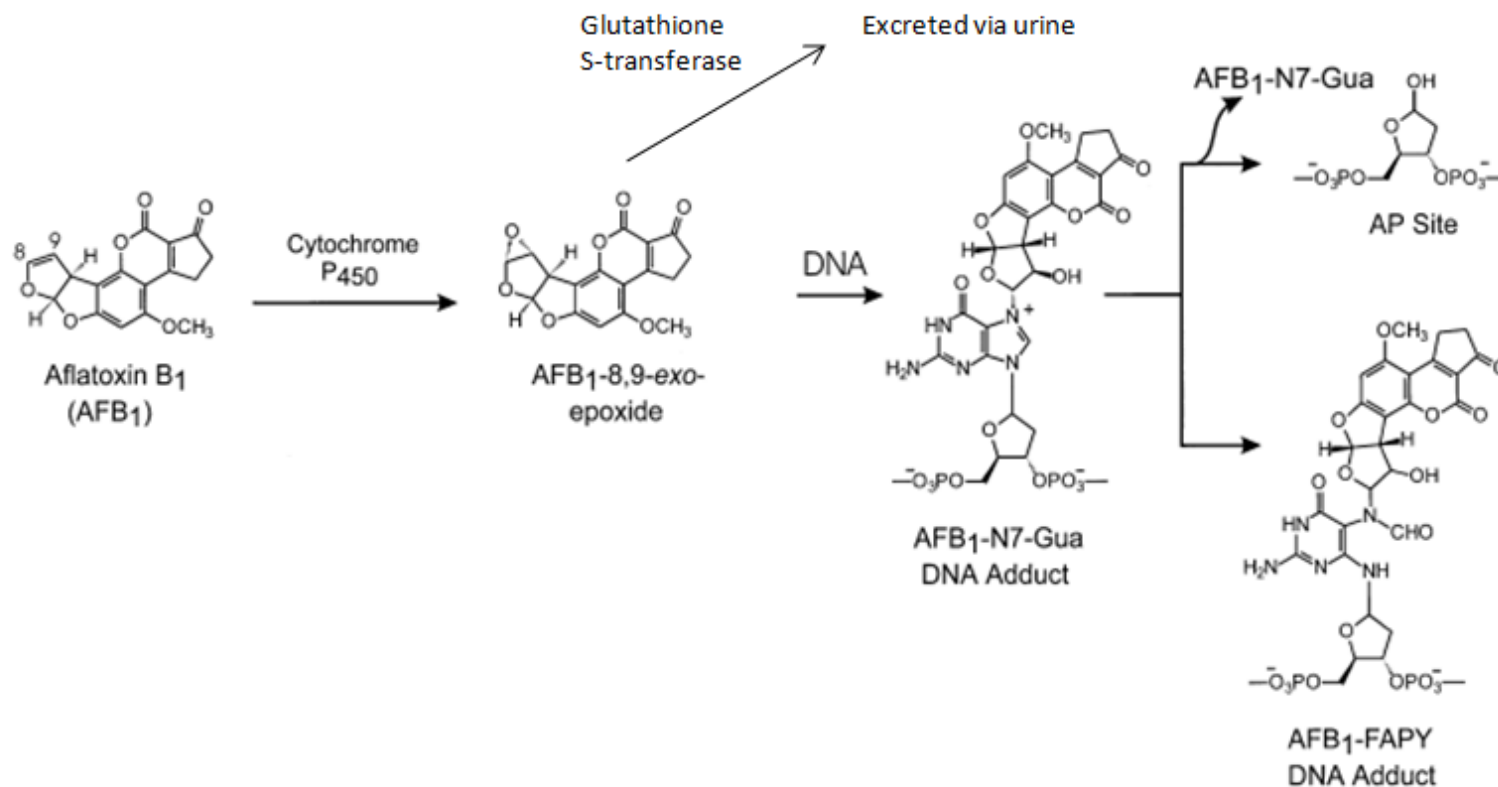
Figure 1.2 Diagram showing the Aflatoxin B₁ metabolic pathway
Aflatoxin is enzymatically altered by the liver by cytochrome P450 system producing the compound AFB1-8,9-epoxide. This epoxide can then be deactivated by the addition of glutathione mediated by the enzyme glutathione S-transferase, and is then excreted via the urine. The epoxide, however, is very reactive and can quickly form an adduct with DNA, 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B₁ (AFB1-N7-Gua). AFB1-N7-Gua can then be excreted via urine, but is extremely unstable. It can quickly break down into two secondary compounds, an apurinic site and a stable AFB1-formamidopyrimidine adduct (AFB1-FAPY). AFB1-FAPY has been implicated as the causative species for hepatocellular carcinoma (Smela et al., 2001)

1.6   p53

p53 is a transcription factor that plays a role in many anti-cancer mechanisms. It regulates the cell cycle, promotes DNA repair and initiates apoptosis (or programmed cell death). P53 consists of 3 domains, a C-terminus from amino acids 280 – 390, which is the domain that allows the molecule to self-oligomerise to form tetramers, the middle region, consisting of amino acids 80 – 290 that is the DNA binding region, and the remainder, the N-terminal region, which is the part that confers transcription activation capabilities (Wang *et al*., 1994).

Aflatoxin causes a mutation in the third position of codon 249, where an arginine is converted to a serine. This amino acid convertion is a 'loss-of-function' mutation, and destroys the ability of the p53 molecule to bind with DNA. Molecules that have these mutations bind to wild type p53 molecules and then prevent them from activating transcription.   If there is a mutation in the gene encoding p53, tumour suppression is drastically compromised. In fact, a mutation in this gene is implicated in 50% of all human cancers (Smela *et al*., 2001).

1.7   Hepatitis B

Studies with transgenic animals have shown that Hepatitis B virus (HBV) infection and AFB_1 exposure act synergistically in the development of hepatocellular carcinoma (HCC) (Kew, 2003). Another study has shown that a person is three times more likely to develop HCC when they test positive for AFB_1-N7-Gua and are seven times more likely when they are infected with HBV. However, when a person tests positive for both AFB_1-N7-Gua and HBV, they are sixty times more likely to develop the disease (Smela *et al*., 2001). This explains why in areas that both HBV and aflatoxin consumption are prevalent, predominantly in the developing world, instances of HCC are high. For example, in Mozambique and in some provinces in China 65-75% of males and 30-55% of females of all cancer deaths are HCC, compared to 2% in the United States (Sell, 2003).

The mechanism for synergism effect between HBV and AFB_1 is still not fully understood. Several hypotheses have been suggested (Kew, 2003). These include, the involvement of the 17kDa HBV X protein (HBx), which is required for the establishment of the virus. It is thought that this protein inhibits nucleotide excision repair, allowing the persistence of DNA-AFB_1 adducts and thus allowing an increased frequency of mutations (Smela *et al*., 2001). Another suggestion is that the HBV may induce cytochrome P450 production, which would increase the amount of AFB_1 conversion into the more reactive AFB_1-8,9-epoxide (Kew, 2003).

1.8   Other diseases associated with AFB_1

AFB_1 is characteristically associated with hepatic carcinoma, but it has also been implicated in tumourogenesis of the lung following both ingestion and inhalation of the toxin (Donnelly *al*., 1996; Desai *al*., 2003). Whereas cytochrome P450 plays the major role in the bioactivation of AFB_1 in the liver, in the lung the main mediators in the conversion of AFB_1 to the epoxide are lipoxygenase and prostaglandin H synthase.

AFB_1 has also been implicated in other diseases, for example Reye's syndrome (Trauner *et al*., 1984) and Kwashiorkor (Hendriekse *et al.,* 1982). Reyes's syndrome is a children's disease that attacks all organs of the body, but in particular the liver and brain. If left untreated this disease may progress into a deep coma, and finally death. Fatality of this disease is common, up to as many as 40% of all cases. Kwashiorkor, again, is a childhood disease, most common in developing countries, and is caused by inadequate intake of proteins. Symptoms include an extended abdomen and a reddish discolouration to the hair. Although this disease can be treated simply by adding protein to the diet, mortality can be as high as 60% or can result in arrested mental development.

Aflatoxin has also been strongly linked to immunosupression, and has been shown in many livestock animals. Poultry (chickens and turkeys), pigs and lambs in particular are at risk from immunosuppression caused by aflatoxins (Devegowda and Murphy, 2005; Smith *et al.,* 2005). Although the mechanisms of how aflatoxins do this is unknown, studies have shown that animals fed with aflatoxins show a decrease in specific antibody production and

the phagocytic activity of macrophages also falls. A reduction in both humoral and cell mediated immunity decreases the resistance to infectious diseases. This is most relevant for people in the developing world where depressed immunity caused by dietary aflatoxin intake increases the risk of contracting infectious diseases such as HIV and tuberculosis.

Reduced immunity to animals can lead to reduced weight and growth and reduced egg and milk production These have implications for the farmer e.g. losses in productivity. Aflatoxin has also been shown to be teratogenic in rats, mice and hamsters, promoting miscarriage and, thus, reducing normal birth rates.

1.9   Aflatoxin prevention

Aflatoxin contamination, under favourable conditions, is unavoidable. However, several methods can help reduce the amount of aflatoxin generated. The simplest way of reducing exposure to aflatoxins is improved storage, as most contamination occurs post-harvest. An intervention study in West Africa showed that by employing simple post-harvest methods, a drastic reduction in the blood marker aflatoxin-albumin was observed (Turner *et al.,* 2005). These methods included basic procedures to reduce fungal growth, such as hand sorting, sun drying on mats, storage in natural-fibre bags and using wooden pallets to raise the bags from the ground.

Aflatoxin can be reduced in animal feeds by either physical or chemical means. Physical methods include heat, microwaves, gamma rays, X-rays, UV light and adsorption. Adsorption methods involve the addition of inert chemicals to feed that are able to physically bind to aflatoxin (Philips *et al.,* 1999). The binding prevents the aflatoxin from becoming absorbed across the intestinal tract, and the aflatoxins, therefore, are passed out of the body via the faeces. Adsorbents include some aluminosilicates (Scheideler, 1993), polymeric glucomannan (a compound extracted from yeast cell walls) (Karaman *et al,* 2005*)*, and chlorphyllin, a chemical closely related to chlorophyll (Simonich *et al,* 2007*)*.

AFB_1 also binds to a number of different strains of lactic acid bacteria (LAB) (Haskard *et al*., 2001; Gratz *et al.,* 2004). LAB are probiotic bacteria found in healthy intestinal

microflora and have a positive effect on the removal of mutagens in the gut. AFB_1 binds non-covalently and extra-cellularly to the cell wall polysaccharide and peptidoglycan. This also has potential for future applications to remove AFB_1 from animal feed. The most successful way, however, of reducing aflatoxins in feed is ammoniation which reduces 95 – 98% of AFB1. This method, however, is used only for animal feed, and is not used for food to be consumed by humans. Methods used in developing countries to reduce aflatoxin contamination include par boiling rice and roasting pistachios in lemon juice. Diet can also be important – both vitamin A (retinol), zinc, iron and selenium seem to alleviate the effects of aflatoxin B_1. A low protein diet also has been shown to reduce the carcinogenicity of AFB_1. In a study using Fischer rats, Youngman *et al.* (1992) compared aflatoxin-induced liver tumour development with rats fed a 5% (w/v) casein diet compared with those fed a 20% (w/v) casein diet. The experiment showed that animals fed with the high protein diet had an approximately six times greater risk of developing a tumour on the liver. This research seems to contradict findings that a low protein diet in humans actually accelerated aflatoxin carcinogenesis in the case of people suffering from Kwashikor. It has been shown, however, that a low protein diet enhances the acutely toxic aflatoxin lesion but depresses the carcinogenic lesion (Appleton *et al.,* 1983).

Chemoprotection is also being examined as a means to reduce AFB_1-induced HCC in areas with high instances of hepatitis B, as well as high AFB_1 contamination risk. An example of this is the anti-schistosomal drug Oltipraz (4-methyl-5-[*N*-2-pyrazynil]-1,2-dithiole-3-thione) (Bammler *et al*., 1999). Rats treated with Oltipraz have shown resistance to the development of liver tumours (Buetler *et al.,* 1996). Although the mechanism is largely unknown, experiments have been undertaken with marmosets, as they have similar oxidative profiles to humans (Bammler *et al.,* 2000). Results have shown that Oltipraz reduces AFB_1 activation by inhibiting cytochrome P450 and decreasing AFB_1-DNA adduct formation, and it also slightly induces glutathione S-transferase. These three mechanisms in combination can all protect against AFB_1-induced mutagenicity.

1.10 Legislation and regulatory limits for aflatoxins

In an attempt to limit exposure to aflatoxins many countries (approximately 100) have imposed regulatory limits for aflatoxins levels in both food and animal feed. Limits are selected to ensure adequate protection against the toxin, but are also designed to minimise the large negative impact on trade. Regulations are based on the known toxicology of the toxin. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recently evaluated the toxicity of aflatoxin. The typical end result of this is a regulation based on the Provisional Tolerable Weekly/Daily Intake (PTW/DI), which has been evaluated using the No Observable Adverse Effect Level (NOAEL). The NOAEL is based on animal toxicology studies, incorporating an uncertainty factor of 100 – an extrapolation of 10 from animals to humans, and then a factor of 10 for the variation of an individual. However, in the case of aflatoxins, when their carcinogenicity has been proven, the level of the contaminant has to be As Low As Reasonably Achievable (ALARA). The ALARA principle is applied when the contaminant is ubiquitous and cannot fully be removed from the food. The aim is to exclude as much of the toxin as possible from the food supply (FAO Corporate Document Repository, 2004).

International limits for aflatoxins have yet to be harmonised, but the Codex Alimentarius Commission has suggested guidelines. The Food and Agriculture Organization of the United Nations (FAO), and the World Health Organization (WHO) established the Codex Alimentarius jointly in 1963 to protect consumers' health and fair trade, and to help establish coordinated regulations. In addition to the Codex regulations, many countries have set their own limits. However, there can be quite a large discrepancy between the regulatory limits in different countries (Figures 1.3 and 1.4). For example, limits for food for direct human consumption can range between 1 and 20 parts per billion (ppb) for aflatoxin B_1 and between 0 and 35 ppb for total aflatoxins.

Worldwide limits for aflatoxin B1 in food
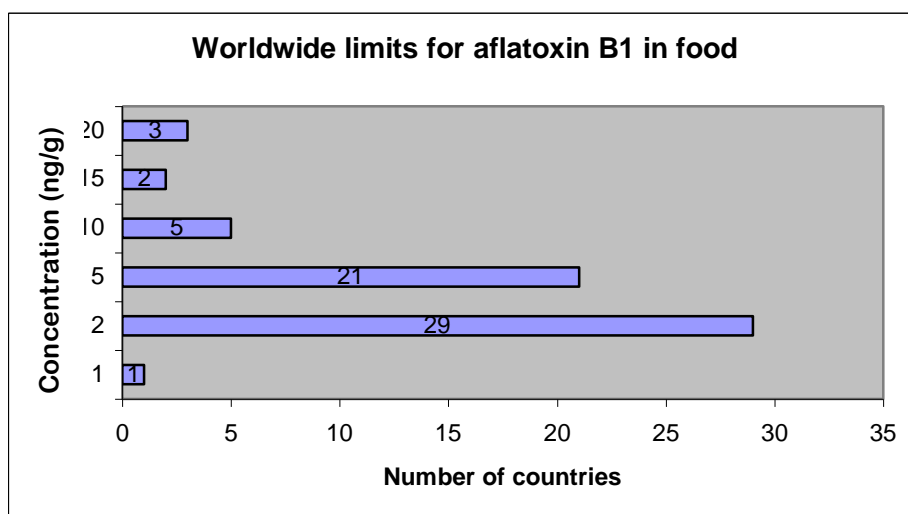
Figure 1.3 Graph showing the worldwide limits for aflatoxin B$_1$ in food
The limits for aflatoxin B$_1$ worldwide range from 1ng/g to 20ng/g. The most general
limit for aflatoxin B$_1$ in food worldwide is 2ng/g (FAO Corporate Document Repository,
2004)

The regulations of some countries are more comprehensive than others (Table 1.2). For example Australia and New Zealand have only one specific regulatory limit set, for peanuts and tree nuts at 15μg/kg total aflatoxins. All other foods have been set at 5μg/kg. The most complete set of regulations including coverage of the largest range of foodstuffs and feed was set by the EU. This was most recently updated in 2006 when new regulations were set By the European Commission for aflatoxin B$_1$ in infant formula. It was set at 0.05 μg/kg (Commission Regulation (EC) No 1881/2006).

Worldwide limits for total aflatoxin in food
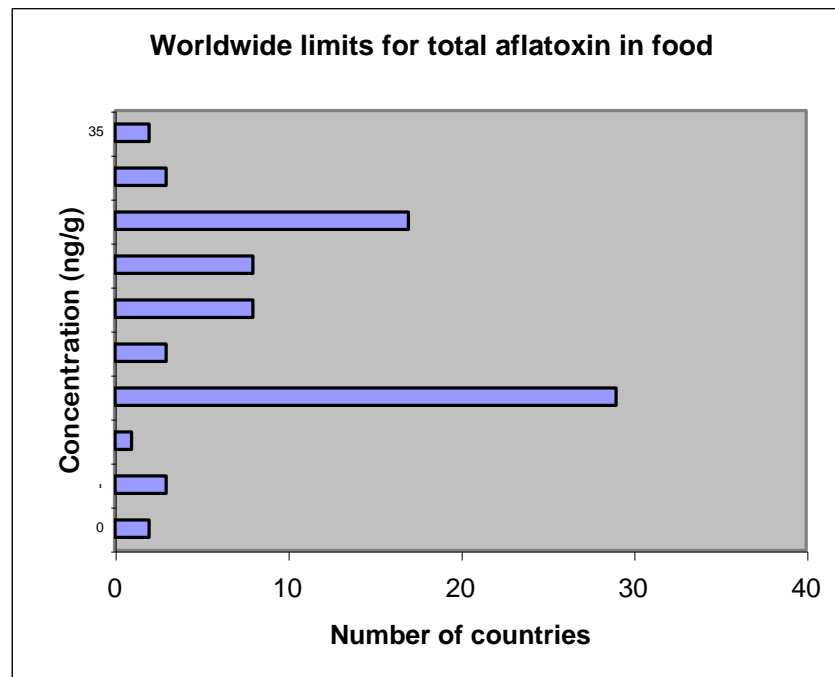
Figure 1.4 Graph showing the worldwide limits for total aflatoxins in feed
The limits for total aflatoxins worldwide range from 0ng/g to 35ng/g. The most general
limit for aflatoxin B$_1$ in food worldwide is 4ng/g (FAO Corporate Document Repository,
2004)..

Some countries (such as the US and Canada, following Codex guidelines) have set limits
for total aflatoxins only, whereas other countries (for example members of the EU) have
regulations for both total aflatoxins and aflatoxin B$_1$.

Apart from Europe other countries from the same geographical area have similar limits. In
Latin America, for example, the aflatoxin regulations have been harmonized through a
trading block of countries, MERCOSUR (Mercado Comun del Sur), which comprises
Argentina, Brazil, Paraguay and Uruguay, although other countries also follow these
regulations. Brazil and Uruguay have applied additional regulations to certain matrices.
Africa has 15 countries that have regulatory limits set for mycotoxins, most of which
include limits for aflatoxins.

ASEAN (Association of Southeast Asian Nations), consisting of Brunei Darussalam, Cambodia, Indonesia, Lao People's Democratic Republic, Malaysia, Myanmar, the Philippines, Singapore, Thailand and Vietnam, do not have harmonised regulations. These countries all have specific regulations, the most strict of which was set by Singapore.

Milk and milk products, including infant formulae, are a staple food for babies and young children, who are highly susceptible to the adverse effects of AFB_1. Some countries, therefore, have enforced stringent regulatory limits to protect those most at risk. The lowest limits that have been set for AFB_1 have been for infant formula by the EU. The limits have currently been set at 0.1ppb (0.1μg/kg).

Table 1.2 Comparison between worldwide aflatoxin regulations
Regulatory limits for aflatoxin can vary greatly between different foods and at different places around the world. This table highlights how greatly the regulations can vary (FAO Corporate Document Repository, 2004)..

Worldwide Aflatoxin Regulatory Limits (ng/g)

			EU	CODEX (US and Canada)	MERCOSUR (Latin America)	AUSTRALIA/ NEW ZEALAND	CHINA	IRAN	MOROCCO	INDIA
FOOD	Consumer-ready ground nuts, nuts and fruit	AFB₁	2					5	1	
		Total Aflatoxins	4	15	20	15		15		
	Groundnuts for further processing	AFB₁	8							
		Total Aflatoxins	15							
	Nuts and dried fruit for further processing	AFB₁	5							
		Total Aflatoxins	10							
	Cereals (except maize) and processed product	AFB₁	2				20		5	
		Total Aflatoxins	4							
	Maize for further processing	AFB₁	5					5		
		Total Aflatoxins	10		20			30		
	Spices	AFB₁	5							
		Total Aflatoxins	10							
	Rice	AFB₁					10	5		
		Total Aflatoxins						30		
	Infant food (non-dairy)	AFB₁					non-detectable	1	1	
		Total Aflatoxins	0.5							
	All other food stuffs	AFB₁				5	5		10	
		Total Aflatoxins		20						30
MILK	Raw milk	AFM₁	0.05	0.5	0.5		0.5	0.05	0.05	
	Powdered milk	AFM₁	0.4		5		0.5	0.5	0.5	
	Baby food (with milk)	AFM₁	0.025				non-detectable	0.01	0.03	
		AFB₁	0.1					0.5		
	Cheese	AFM₁	0.02					0.2		
	Butter	AFM₁	0.02					0.02		
ANIMAL FEED	All feed materials	AFB₁	20	200				50	50	120
		Total Aflatoxins			50					
	Complete feed stuffs for dairy animals	AFB₁	10	20				5	5	
								20		
	Complete feed stuffs for juvenile livestock	AFB1	5	20				5	10	
								20		
	Poultry	Total Aflatoxins						10		

1.11 Methods of Analysis

The establishment of regulatory limits for aflatoxin has necessitated reliable and sensitive analytical methods of detection and analysis. Several official or regulatory laboratories have already validated numerous methods. These organisations include the Association of Official Analytical Chemists (AOAC), the International Union of Pure and Applied Chemistry (IUPAC), International Organisation for Standardisation (ISO), and its European counterpart the European Committee for Standardisation (CEN).

The first methods that were validated to test for mycotoxins used thin layer chromatography (TLC). Aflatoxin has a natural fluorescence, and so can be detected using UV light. These methods are simple and cheap, but they are insensitive and lack precision (repeatability and reproducibility). They have been overtaken by other chromatographic methods, such as High Performance Liquid Chromatography (HPLC) (improved by derivatisation with bromine) (Dunne *et al.,* 1993; Zhang *et al.,* 2004; Seuva *et a.l,* 2005), HPLC/MS (HPLC/mass spectrometry) (Biancardi *et al.,* 2005) LC-MS/MS (Liquid Chromatography/Mass Spectrometry/Mass Spectrometry) and GC/MS (gas spectrometry/MS) (Sforza *et al.,* 2005; Cavaliere *et al.,* 2006). These methods are more sensitive than TLC, but are time consuming, labour intensive and require skilled training. They also require high consumption of hazardous solvents, and, so, are often expensive. Therefore, they are unsuitable for the analysis of large numbers of samples.

More recently, however, the advent of immunoassays has had a significant impact on aflatoxin detection and quantification. Immunassays are simple, quick and cheap to apply for routine monitoring of aflatoxin contamination. These can then be checked by an analytical method, such as HPLC. Immunological assays can come in many different formats – e.g. ELISA (enzyme-linked immunosorbant assay) (Reddy *et al*., 2000; Gathumbi *et al*., 2003) and lateral flow strips (Sibanda *et al*., 1999; Blesa *et al.,* 2003). These methods are ideal for screening large numbers of samples, but they can suffer from matrix interference depending on the food type being tested and they lack the sensitivity of

detecting aflatoxin B1 as low as the regulatory limits, especially for limits as low as 0.1ppb for infant formula.

Another immunological method that have become increasingly popular is the use of immunoaffinity columns (IAC's) (Stroka *et al.,* 2000; Senyuva *et al.*, 2005; Castegnaro *et al.*, 2006; Ip *et al.*, 2006). Immunoaffinity columns have been used for aflatoxin isolation from complex matrices prior to analysis. They consist of antibodies immobilised onto a stationary phase (e.g. sepharose). When a sample is passed through the column all the aflatoxin binds to the antibodies and remains in the column. The other components from the sample, however, are washed through the column and discarded. The aflatoxins are then eluted from the column with a solvent, resulting in a pure solution of aflatoxin, which can then be quantified using HPLC or GC methodology. IAC's are simple to use and drastically reduce sample preparation time. One drawback, however, is that they can be relatively expensive and generally cannot be re-used efficiently.

There are also several automated systems in development that can detect and quantify aflatoxins that are not yet commercially available, each utilising a different innovative technology. These include optical waveguide lightmode spectropscopy (Adanyi *et al.*, 2007), sol particle immunoassay (Brenn-Struckhofova *et al.*, 2007), fluorescence polarisation (Nasir *et al.*, 2002), affinity electrochemistry (Mascini *et al.*, 2001), fluid based-bioaerosols and surface plasmon resonance (SPR) (Daly *et al.*, 2000; Maragos *et al.*, 2002; Dunne *et al.*, 2005). Surface plasmon resonance was chosen as the sensor detection system for the study of aflatoxin determination in this research.

1.12   Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an optical technique that can measure biomolecular interactions, allowing detection and quantification of specific analytes in a solution. Although several commercial systems are available, the most universal is Biacore^{TM}, who have established a clear leadership in SPR technology, holding approximately 90% of the market (Figure 1.5).
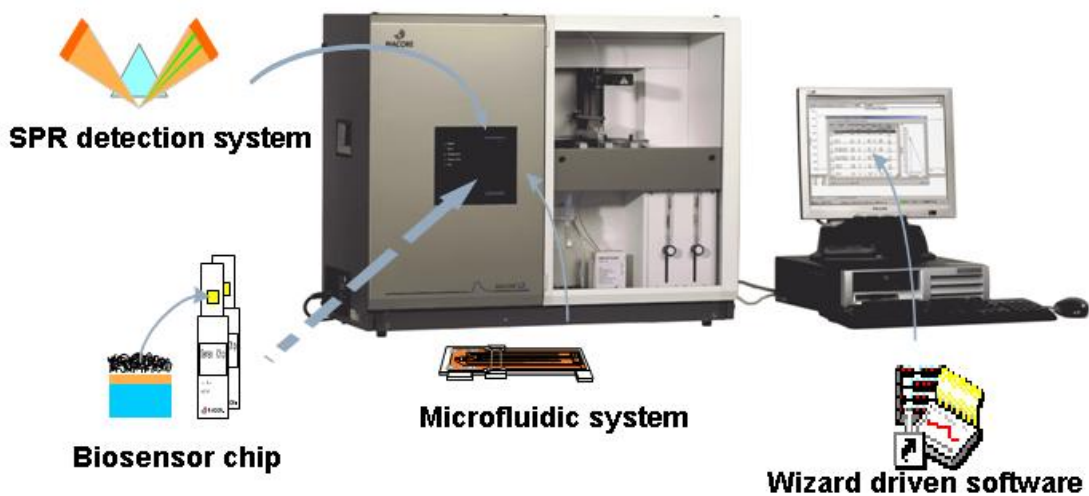
Figure 1.5 The Biacore^{TM} Surface Plasmon Resonance System
The Biacore^{TM} system combines SPR technology with a unique microfluidic system and innovative biosensor chip technology using comprehensive wizard driven software. (Image courtesy of Biacore^{TM})

SPR occurs at the interface between two media of different refractive index. In the case of Biacore^{TM}, these media are the glass of the sensor chip and the sample solution. When the beam of light passes from a dense to a less dense medium the light bends towards the plane of the interface. However, when the angle of incidence is at a critical angle, none of light is refracted across the interface, but is reflected internally. This is called the total internal reflection (TIR). At this point, an electromagnetic field penetrates a short distance, about half the wavelength of the incident light i.e. tens of nanometres, into the lower density media, the sample solution. This creates an evanescent wave, with an amplitude that decreases exponentially with increased distance from the interface surface (Liedberg *et al.,* 1993).
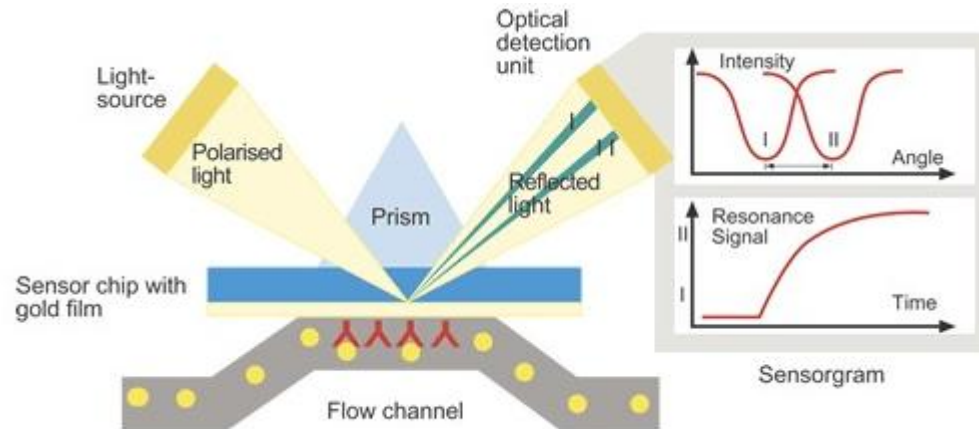
Figure 1.6 Surface Plasmon Resonance
When a sample is passed through the flow channel specific antigens (if present in the sample) will bind to the antibody coated surface. This causes a shift in the resonance angle of the refracted polarised light shone onto the chip. This change in angle is directly proportional to the amount of bound antigen. (Image courtesy of Biacore^{TM})

If the interface is covered with a thin layer (50nm) of a metal (usually gold), and the light is p-polarized and monochromatic, under conditions of TIR, photons react with the free electron cloud in the metal and are converted into surface plasmons, the particle name of electron density waves.

The evanescent wave field penetrates the sample solution. It is, therefore, sensitive to solute concentration at that point and the binding of molecules from the sample onto the gold surface can cause changes in the refractive index. This is measured as a change in resonance angle and is directly proportional to the amount of biomolecules bound. The shift in resonance angle is directly proportional to the mass increase on the chip surface.

The change of angle, or the response, is measured in arbituary units, Resonance Units (RU), where 1 RU is equivalent to a shift in angle of 0.0001° (Huang *et al*., 2007). For most proteins this is a change in concentration of 1pg/mm^2 on the sensor surface.

1.13 Sensor Surface

In the Biacore^{TM} system, the sensor surface is a removable, re-usable sensor chip, consisting of a glass surface coated with a thin layer of gold (50nm) at the interface between the glass and the buffer (Figure 1.7). A variety of other metals can be used for SPR. To be suitable for SPR a metal's conduction electrons must be able to resonate with light at a particular wavelength, generally in the visible and near infrared parts of the spectrum, as there are a number of detectors readily available for these regions. Appropriate metals include aluminium, silver, copper, sodium, indium and gold. Gold has been chosen as the ideal candidate as it is inert and resistant to oxidation, while still allowing the attachment of antibodies. It also produces a strong SPR signal in the near-infrared region.
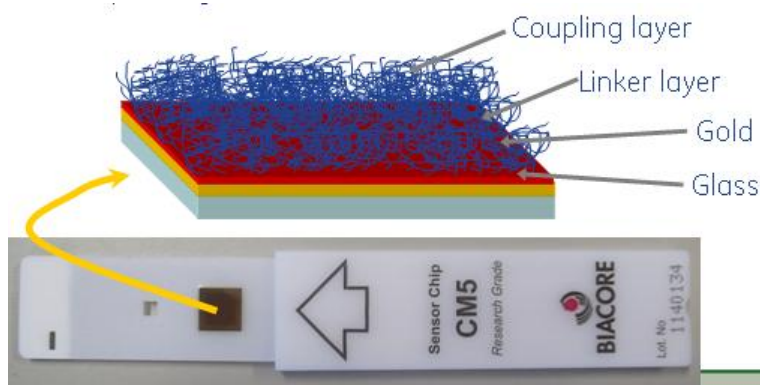
Figure 1.7 Biacore^{TM} CM5 Sensor Chip
The sensor chip consists of gold-coated glass surface. A linker layer is coated onto the gold. In the case of the CM5 chip it is carboxymethylated dextran. The dextran layer then facilitates the attachment of the coupling surface. In this assay the coupling surface consists of the aflatoxin B$_1$ derivative aflatoxin B$_1$-oxime-ethylenediamine.

To measure binding of molecules at the sensor surface one of the binding partners must be immobilised onto the gold surface, which is facilitated through covalent attachment. The most commonly used and versatile chip, the Sensor Chip CM5, is coated with a 100–200nm carboxymethylated dextran layer. This dextran hydrogel layer is hydrophilic and allows

attachment of molecules via amine, thiol, aldehyde, hydroxyl or carboxyl groups, without causing them to denature (Shankaran *et al.,* 2007). The sensor surface is generally very stable, which allows it to be reused hundreds of times whilst still maintaining high precision and accuracy in many cases.

1.14   Microfluidic System

Another feature of the Biacore^{TM} system is the unique microfluidic system, which allows a continuous and controlled flow of liquid over the sensor surface. When the sensor chip is docked in the instrument, it is pressed against the integrated microfluidic cartridge (IFC) and four flow cells are formed, which range in size from 20 to 60nl, depending on the model of the instrument. The analyte is then able to flow continuously at a constant concentration over any one of the flow cells. The sample volumes can be in the range of 5 to 450μl and with a flow rate of between 1 and 100 μl/min. This system has numerous advantages. These include the exclusion of air from the chip surface thus preventing protein denaturation or sample evaporation, low sample consumption and the removal of the necessity for washing steps.

The models Biacore-Q and Biacore-1000 only allow the monitoring of one flow cell at a time. Biacore 2000 and Biacore 3000, however, allow a sample to pass over the four flow cells sequentially, following the response from all four flow cells in parallel. This means that four different assays can potentially be performed on one sample in the same run.

1.15   Surface Plasmon Resonance Sensorgrams

The association and disassociation of analytes in the sample binding to the sensor chip surface is followed, in "real-time", on a graph, called a sensorgram. The sensorgram measures changes in response units (RU) over time, where one thousand RU is equivalent to approximately 1ng of analyte bound to the chip surface.

When a sample is injected over the chip surface, there is an interaction between the molecules in the sample and the flow cell surface. If the binding molecules are present,

they will associate, resulting in a rise in the sensorgram. Once the reaction has reached an equilibrium the sensorgram will remain constant. After the sample is injected, buffer is passed over the chip surface and the interacting partners dissociate, causing the sensorgram to fall. Not all of the analyte may dissociate from the chip surface, necessitating a regeneration step to return the sensorgram to the baseline before another sample is injected (Figure 1.8).
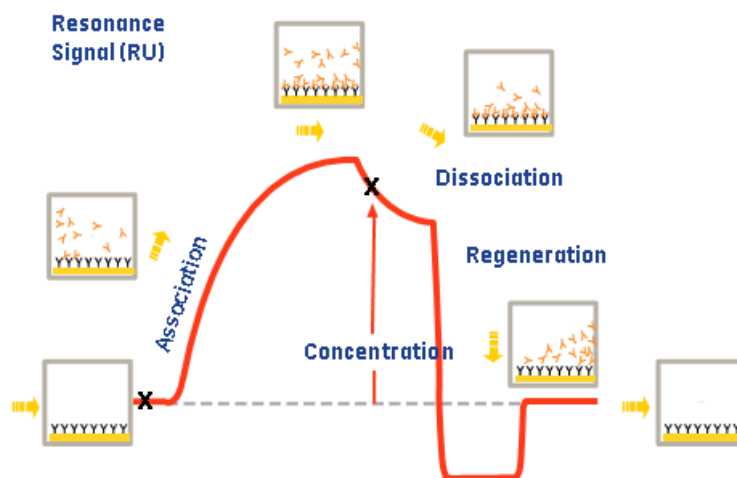
Figure 1.8 SPR Sensorgram
*If there are binding molecules in a sample there will be an association on the chip surface which result in a rise in the sensorgram. When the reaction has reached equilibrium the sensorgram will remain constant. Once the sample injection has finished buffer will be injected over the surface. At this point some of the interacting proteins will dissociate, as shown by a drop in the sensorgram. Ten seconds after the sample injection has finished the concentration of the sample bound to the surface is recorded. This is point is shown as an "X" on the diagram. To remove all the bound material a regeneration step is introduced. (**Image courtesy of Biacore^{TM}**)*

The sensorgram shows binding between the immobilised target and its corresponding ligand and can be used for kinetics, e.g. calculating association/disassociation rates. It can also show how strong the affinities of the interacting partners are.

1.16 Inhibition Assays

The smallest molecule that can be detected using SPR in the configuration described is 180 Da. Therefore, this is not sufficiently sensitive to detect small molecules such as toxins and drug residues in a direct assay format. For low molecular weight analytes the assays are formatted as inhibition assays. In this format the analyte (or its derivative) is immobilised onto the chip surface. Prior to injection a fixed amount of aflatoxin binding protein is mixed with the sample. Any analyte in the sample will bind to the binding protein and inhibit it from binding to the surface of the sensor chip when the sample is passed over it. The higher the concentration of the analyte in the sample, the higher the level of inhibition and hence the lower the response of the biosensor. A calibration curve is generated using the responses produced from known standards. The amount of analyte in a sample can then be quantified in reference to this curve as shown in Figure 1.9.
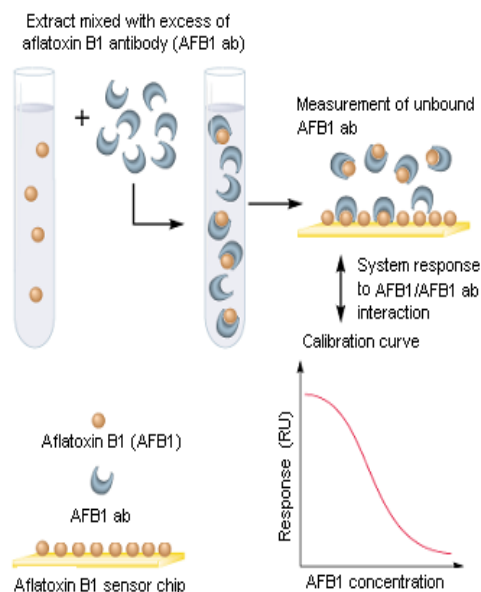
Figure 1.9 Inhibition Assay for Aflatoxin B$_1$ Determination
A mixture of sample and antibody is passed over the sensor chip surface. Any analyte in the sample will sequester antibody and prevent it from binding to the surface. The higher the amount of analyte in the sample, the higher the level of inhibition and, therefore, the lower the response on the sensorgram (Image courtesy of Xenosense Ltd.).

1.17   Binding Protein used in SPR assays

Binding proteins are proteins that are known to bind to a specific target protein. The sensitivity and the specificity of an assay is largely dependant on the specificity and strength of the binding protein to the target.   Other requisites for the binding protein are that it must be stable and robust, and that it can be immobilised onto the chip surface.

The binding proteins most often used in bioassays are antibodies. Antibodies are useful tools as they can be easily raised against a particular target and are capable of very specific recognition and high affinities. The basic structure of an antibody is depicted in fig 1.10. An antibody is made up of two identical heavy (H) and two identical light (L) chains which are joined together by disulphide bonds. A heavy chain has a molecular weight of 50kDa and has one variable region V_H and three constant regions C_{H1}, C_{H2} and C_{H3}. The light chain has a molecular weight of 25kDa and consists of one variable and one constant region, V_L and V_H. It is the variable regions of the heavy and light chains that together form the antigen binding site (Conroy *et al.,* 2009).
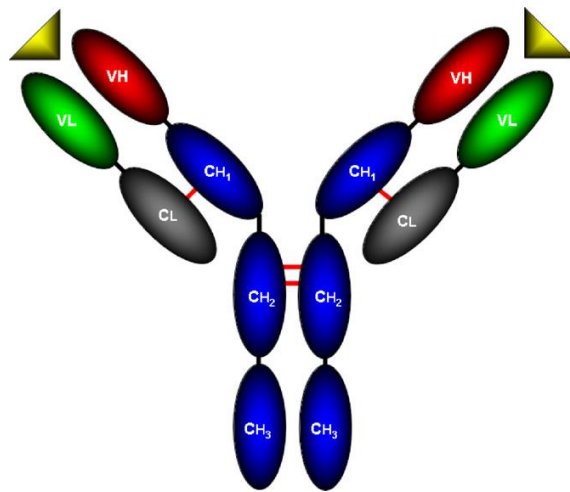
Figure 1.10 Structure of an antibody made up of two identical heavy and two identical light chains. A heavy chain is made up of one variable region (VH) and three constant regions (CH₁, CH₂ and CH₃). A light chain comprises of one variable region (VL) and one constant region (CL). The antigen binding sites are indicated by the triangles and the disulphide bridges are indicated by red lines (Conroy et al., 2009)

Antibodies used in assays can either be polyclonal, monoclonal or recombinant. Polyclonal antibodies are produced when an antigen is injected into a suitable host, e.g. mouse, rabbit, goat or rat. This induces the B-cells of the immune system to produce antibodies specific for that antigen. Each B-cell produces a slightly different antibody, either with different specificities or targeting different epitopes on the antigen. Therefore, when serum from the animal is removed and purified it will contain a mixture of antibodies, with varying affinities for the original injected antigen (Conroy *et al.,* 2009).

Monoclonal antibodies, however, are derived from a single B-cell and, so, are identical. In the generation of monoclonal antibodies B-cells from the spleen or the lymph nodes of an animal that was immunised with the antigen several times, are removed. The B-cells are then fused, either by electroporation or using polyethylene glycol, to myeloma tumour cells. Myeloma tumour cells are cancerous B-cells that are able to grow indefinitely, but have lost the ability to produce antibodies. When the two cells are fused, a hybridoma cell is produced, which is capable of reproducing quickly and indefinitely, generating large amounts of identical antibody to the target antigen (Maragos *et al.,* 2000).

The use of recombinant antibodies, however, has become increasingly more popular due to advantages over both monoclonal and polyclonal antibodies. The production of recombinant antibodies, for example, does not necessarily require the use of animals to produce an antibody library. Naïve libraries can be produced using pooled blood samples from humans. However, for certain applications immunisation of animals is necessary where antibodies to the required target are not naturally found in the blood. This is the case for toxins such as aflatoxins and would generate an immune library that would contain more antibodies specific to the target. Other advantages of recombinant antibodies are that they are theoretically far quicker than either monoclonal or polyclonal to produce, and can also be used for antigens unsuitable for conventional antibody production, for example if the antigens are non-immunogenic or are extremely toxic to the animal. However, the primary advantage of utilising recombinant antibody technology is that the engineered antibody fragments can be manipulated to improve their sensitivity, cross-reactivity and robustness.

Recombinant antibodies are artificially produced through the manipulation of genetic material. RNA, extracted from B-lymphocytes, is reverse transcribed and amplified by PCR to produce a synthetic library of recombinant antibodies. Several types of recombinant antibody can be made, including single chain antibody fragments (scFv) and Fab fragments (Morea *et al.*, 1997).

To generate an antibody to a specific antigen an antibody library must go through several rounds of screening. An example of a robust high-throughput screening procedure is phage display. Phage display was first described by Smith and co-workers in 1985 (Smith, 1985). They had discovered that when a protein is cloned into the PIII gene of the filamentous phage M13 it will be displayed on the phage coat surface as a fusion protein. A library of antibody fragments of up to 10^{10} can be cloned into the phage genome (Hoogenboom, 2005).

| Antibody molecule | Fab fragment | scFv |
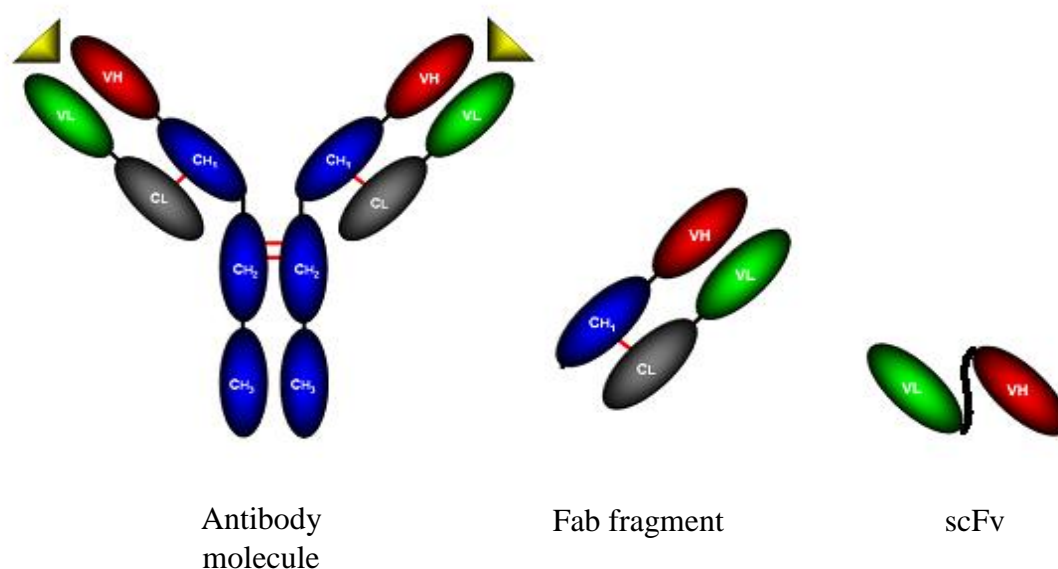
Figure 1.11 Diagram showing structures of a Fab fragment and an scFv compared to a typical IgG molecule. A Fab fragment consists of a heavy and a light variable region, V_H and V_L, which comprise the antigen binding site. Their structure also includes the constant regions of the heavy and light chains, C_H and C_L. An scFv, however, consists of only the V_H and V_L domains joined together by a (Gly$_4$Ser)$_3$ linker (Conroy et al., 2009).

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A scFv comprises of a V_H and a V_L domains of an antibody joined by a $(Gly_4Ser)_3$ linker. The glycine/serine rich sequence does not form secondary structures and is also beneficial as it is found naturally in the M13 PIII gene and is therefore tolerated in phage display (Conroy *et al*., 2009).

Fab fragments, in addition to the V_H and the V_L sections, contain constant regions of the heavy and light chains, C_H and C_L. Although Fab are more stable and less likely to dimerise, scFv libraries tend to be more popular because the expression of the smaller scFv on the phage surface has a less toxic effect on the cell, thus resulting in a better yield and, therefore, library diversity (Arndt *et al.,* 2001).

To isolate an antibody that binds specifically to the target protein, or antigen, usually requires between two and five rounds biopanning (Figure1.11). The system works on the principle that the phage displaying the antibody that shows affinity towards the target also contains the genetic material that encodes it, thus linking the antibody's phenotype with genotype.

The mixture of phage, each presenting an antibody from the library is added to a microtitre plate coated with target protein. The plates are then washed and any phage-antibodies that bind to the target remain associated to the surface. Binders are then eluted, and then used to produce more phage. This phage mixture will be "enriched" containing a higher percentage of binders. The enriched mixture is then used for further rounds of biopanning.
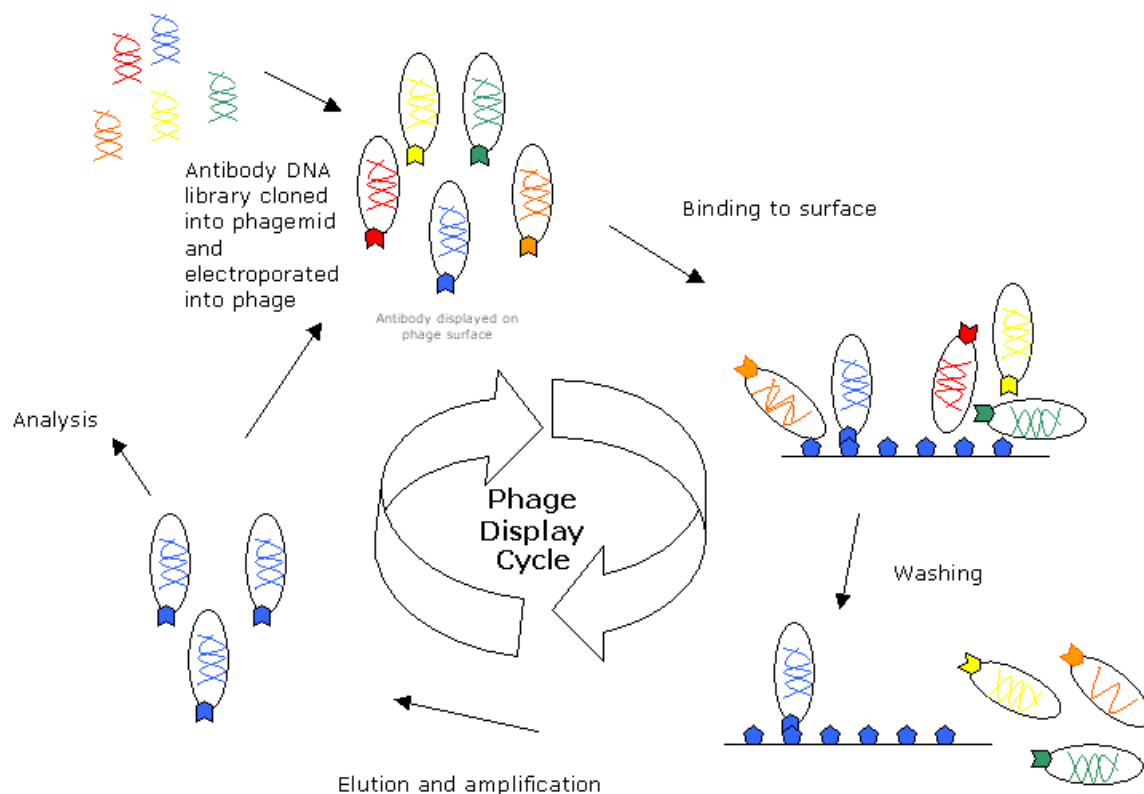
Figure 1.12 Schematic drawing of phage display and bio-panning. The DNA antibody library is ligated into a specific part of the phagemid genome. The antibody fragment is then expressed as a protein on the phage coat surface. The antigen is immobilised onto the surface of a microtitre plate. The library of phage displaying antibody on the coat surface are added to the plate and incubated to allow the phage to bind. Non-binders are washed away. Attached phage are then eluted and then re-infected into E.coli cells. The phage can then go through another round of biopanning (Wittrup et al., 1999).

1.18 Aim of Project

The aim of this project was to design a SPR sensor-based diagnostic method to measure Aflatoxin B_1 in a wide range of foodstuffs and animal feed. The kit will be designed specifically for use on a Biacore analyser, which utilises the latest SPR technology.

This project was partially funded through the FUSION Programme, which is co-ordinated by Inter*Trade*Ireland. The purpose of FUSION is to establish collaborative 3 way cross border partnerships between technology-based private companies, knowledge centres (such

as universities and colleges) and recent graduates. The strategy is to facilitate technology and knowledge transfer from universites to businesses, in order to introduce innovation, increase their capabilites, and, thus, gain competitive advantage. This project was a joint venture between Xenosense Ltd and Dublin City University.

XenoSense Ltd. is a Belfast based biotech company which develops and manufactures *in vitro* diagnostic kits for use in food safety and quality assurance applications. The kits have been designed specifically for use on optical biosensors produced by the Swedish company, Biacore^{TM}, which harness innovative SPR and sensor chip technology. Xenosense currently has a repertoire of 13 kits that fall into two categories – vitamins and drug residues.

Xenosense, as a new and expanding company, is eager to explore and break into different market sectors. The global market share for mycotoxins is estimated at approximately £75 −100 million, with rapid diagnostic methods accounting for 10 − 20% of this (CAST report, 2003). This is a fairly new market, the average annual growth is high at 10%. Aflatoxin B_1 was chosen to be the pilot assay for development as it is the most well known mycotoxin and has the most complete regulatory limits compared to other mycotoxins. There are already a number of competitors in the market. However, due to changing legislation demanding increased testing and the ability to detect lower levels, this sector is growing fast. There is niche in this market for high-throughput, automated systems that are sensitive enough to meet the demands of the recently lowered regulatory limits. This project, to develop an assay for aflatoxin B_1, will be a springboard into this market, and will give Xenosense the opportunity to expand their portfolio further still, by producing other kits to detect different mycotoxins.

1.19 Objectives

The overall objectives for the experimental research of the project could be summarized as follows.

1. Development of a sensor chip surface

   A previous study had shown the most effective way to immobilise aflatoxin B1 onto the sensor chip surface. The first objective was to recreate this and prove the efficacy by injecting over the surface an excess of antibody specific to aflatoxin B_1.

2. Prove the feasibility of an aflatoxin B_1 assay in buffer

   The next objective was that a standard curve for aflatoxin B_1 could be generated in a buffer system.

3. Evaluation of different antibodies that are specific to aflatoxin B_1

   Antibodies were supplied to us by Professor Richard O'Kennedy, School of Biotechnology, Dublin City University as part of the FUSION programme. These antibodies had to be tested individually to determine which one would be most suitable for the assay. A series of curves had to be set up using all four antibodies added in various percentage fractions, and different injection times, to determine the most sensitive antibody and assay conditions.

4. Choose which food matrices to investigate

   Aflatoxins contaminate a large and diverse range of foodstuffs. Ultimately this assay is to be marketable and therefore matrices chosen depend on a number of factors. For example, what other tests are currently available on the market, what the unique selling point of the assay will be and what matrices would be beneficial to existing customers of Xenosense Ltd.

5. Produce a sensitive and reliable assay for each matrix

   A quick and simple extraction method would then need to be developed for each matrix. To do this the matrix is spiked with a known amount of aflatoxin B_1 and then extracted. The amount recovered could be calculated by running the extract against the aflatoxin B_1 standard curve.

6. Validation

The final objective for this product was to validate the assay(s). This would be required if the test was to go on the market.

Chapter 2: Materials and Methods

2.1 Suppliers

Table 2.1 List of suppliers for raw materials, consumables and equipment

Supplier	Address
Albion Chemicals	Albion House, Rawdon Park, Green Lane, Yeadon LS19 7XX, UK.
BD Plastipak	Edmund Halley Road, Oxford OX4 4DQ, UK.
BDH	603a Dalamal Chambers, New Marine Lines, Mumbai, 400 020, India.
Biacore AB	Rapgatan 7, SE754 50, Uppsala, Sweden.
BOC Group	Prince Regent Road, Belfast BT5 6RW, Northern Ireland
Chromacol	3 Mundells Industrial Centre, Welwyn Garden City, Herts AL7 1EW, UK.
Denver Instruments	Robert-Bosch-Breite 10, 37079 Gottingen, Germany.
Eppendorf	Barkhausenweg 1, 22339 Hamburg, Germany.
Fermentek	Yatziv25, POB47120, Jerusalem 97800, Israel.
Gilson	3000 Parmenter Street, P.O. Box 620027, Middleton WI53562-0027, USA.
Greiner	7 Rue Leo Lagrange, F-27950 Saint Marcel, France.
Kimberley Clark	P.O. BOX 619100, Dallas, Texas, USA.
KNF Neuberg	D-79112 Freiberg, Germany.
Nalgene	Ridderstraad 26, B3040 Neerijse, Belgium.
Prolabo	54, rue Roger Salengro, 94126 Fontenay-sous-bois Cedex, France.
R-Biopharm Rhone	West of Scotland Science Park, Unit 3.06, Kelvin Campus, Glasgow G20 0SP, Scotland.
Scientific Industries Inc	Bohemia, New York, 11716, USA.
Sigma Aldridge	Fancy Road, Poole, Dorset BH12 4QH, UK.
Stuart Scientific	Beacon Road, Stone, Staffordshire ST15 0SA, UK.
Techne	3 Terri Lane, Suite 10, Burlington, NJ 08016, USA.

Vicam	313 Pleasant Street, Watertown, MA 02472, USA.
Whatman	Springfield Mill, James Whatman Way, Maidestone, Kent ME14 2LE, UK.
Wishart Group	Milewater House, Mill Road, Ballyclare, County Antrim, BT39 9DY.

2.2 Materials

2.2.1 *Chemicals*

Table 2.2 List of chemicals used with the supplier and the catalogue number

Chemical	Supplier	Catalogue number
Acetone HPLC grade	BDH	15296 6P
Acetonitrile HPLC grade	BDH	15285 6K
Aflatoxin B_1	Fermentek	AF
Bovine Serum Albumin (BSA)	Sigma Aldridge	054KD607
Chloroform HPLC grade	BDH	152835F
Cyclohexane HPLC grade	BDH	1528965
Di-sodium hydrogen orthophosphate anhydrous, GPR	BDH	301584L
Ethylenediamine	Biacore AB	22-0526-55
Ethanol (min 99.7%, v/v), 25L	Albion Chemicals	Se 3033
Guanidine hydrochloride (99%, w/v)	Sigma Aldridge	G4505
HBS	Biacore AB	22-0527-06
HBS EP+	Biacore AB	BR-1006-69
Hydrochloric acid (2.0M)	Merck	HC753278
Methanol (HPLC grade)	BDH	15250
Monoclonal anti-Aflatoxin B_1 antibody (mouse-derived)	Sigma	A9555

N,N'-dicyclohexyl-carbodiimide	Biacore AB	22-0526-54
N-hydroxysuccinimide	Biacore AB	22-0526-53
Nitrogen ("oxygen-free")	BOC	Size W
O-(carboxymethyl)-hydroxylamine hemi-hydrochloride	Sigma Aldridge	A4508
Pyridine	Prolabo	27 197.238
Sodium azide, AnalaR	BDH	K35580906 605
Sodium chloride, AnalaR	BDH	102415K
Sodium hydrogen carbonate	Sigma Aldridge	236527
Sodium hydroxide, 0.1M, (ConvoL), 6pk	BDH	18043 5A
Sodium hypochlorite solution GPR	BDH	3016965
Sodium phosphate monobasic	Sigma Aldridge	S0751-100G

2.2.2   *Preparation of Buffers and Reagents*

0.1M 10X Phosphate Buffered Saline (PBS), pH 7.4,

Sodium phosphate monobasic	2.3g
Di-Sodium hydrogen	11.5g
Sodium chloride	87.5g

900ml of ddH$_2$O was added and stirred until all salts had dissolved. The pH was adjusted to 7.4 by addition of 1M HCl and then more ddH$_2$O added to a final volume of 1l.

0.1M NaOH:20% (v/v) acetonitrile regeneration solution

1M Sodium hydroxide	100μl
Acetonitrile	200 μl
ddH$_2$O	700 μl

<u>6M Guanidine hydrochloride, pH 4</u>

Guanidine hydrochloride	114.64g
ddH$_2$O	180ml

Adjust the pH to 4.0 with 1M HCl, then add ddH$_2$O to a final volume of 200ml.

<u>Antibody storage solution</u>

BSA	0.5g
Sodium azide	0.195g
HBS buffer	200ml

<u>HBS-EP (produced by Biacore^{TM})</u>

0.01M HEPES, pH 7.4

0.15M NaCl

2mM EDTA

0.005% (v/v) Surfactant P20

2.2.3   *<u>Consumables</u>*

Table 2.3 List of consumables and details of where they were purchased

Consumable	Supplier	Catalogue number
'Easi-extract' aflatoxin immunoaffinity columns	R-Biopharm Rhone	RP70N
Aflatest immunoaffinity columns	Vicam	G1024
Anotop 25 inorganic membrane filter (0.2µm)	Whatman	6809-2024
96 well plate	Greiner	M2186
Foil plate sealers	Biacore AB	N/A
7mm vials	Gilson	2 9508 32

4ml glass vial (6mm)	Chromacol	45V
2ml plastic microtube	Sarstedt	72.694
4ml plastic Vial (6mm)	Nalgene	215-0246
2ml syringes	BD Plastipak	300185
CM5 sensor chips (Certified)	Biacore AB	22-0310-03
Latex gloves	Kimberley Clark	112-0150
Pipettes tips 100-5000µl	Eppendorf	022492080
Pipettes tips 50-1000 µl	Eppendorf	022491555
Pipettes tips 2-200 µl	Eppendorf	022491539

2.2.4   *Equipment*

Table 2.4 List of equipment and details of where they were purchased

Equipment	Supplier
Analytical balance (3 figure) IR-403	Denver Instruments
Analytical balance (4 figure) Mettler Toledo	Wishart Group
Biacore Q instrument	Biacore
Concentrator (DRI Block DB 3D)	Techne
Magnetic stirrer	Stuart Scientific
pH meter (Ultra basic benchtop)	Denver Instruments
Pipettes (2-20µl, 20-200µl, 200-1000µl and 500-5000µl)	Eppendorf
Roller Mixer SRT2	Stuart Scientific
Vacuum manifold	Vac Master
Vacuum pump	ICNF Neuberg
Vortex	Scientific Industries Inc
Refrigerated Centrifuge (5810R)	Eppendorf

2.3 Methods

2.3.1 *Assay Development Methodology*

2.3.1a *Preparation of aflatoxin B₁-oxime-ethylenediamine*

1. Fifty mg aflatoxin B_1 was dissolved in 10mls of pyridine and 80mg carboxymethyloxime hydrochloride added.
2. The pyridine was evaporated under a stream of nitrogen and gentle heating to produce a brown oil. 9ml chloroform was added to the oil, followed by 1ml pyridine.
3. The flask was then placed on ice and 32mg N,N'-dicyclohexyl-carbodiimide (DCC) and 20mg N-hydroxysuccinimide (NHS) added. The reaction mixture was then stirred overnight at 4°C.
4. The next day it was decanted into an eppendorf tube. It was then centrifuged on a benchtop microfuge at 18000g for 10 minutes, the pellet discarded and the solvent evaporated under a stream of nitrogen and gentle heating, and kept to one side.
5. The remaining brown oil in the flask was dissolved in 2ml pyridine with 24mg DCC and 12mg NHS.
6. The reaction was stirred for the rest of the day and overnight at room temperature.
7. The mixture was again centrifuged at 18000g on a benchtop microfuge for 10 minutes and the pellet was discarded.
8. The solvent was added to the residue from step 4, and the solvent again was evaporated under nitrogen with gentle heating. This resulted in the formation of a brown residue.
9. Five ml of ethanol was added to the residue, and then a solution of $NaHCO_3$ and 50µl ethylendiamine in 1ml water was added drop wise. The reaction mixture was stirred all day and overnight at room temperature.
10. The ethanol was then evaporated under a stream of nitrogen and gentle heating (70°C).

11. Two ml of methanol was added to the end product and it was purified by preparative TLC. It was then dissolved in chloroform: methanol (7:3), and finally, the solvent was removed using a rotary evaporator. The final product was a brown residue.

2.3.1b Immobilisation of aflatoxin B₁derivative, aflatoxin B₁-oxime-ethylenediamine, onto sensor chip surface through amine coupling

1. EDC (1-ethyl-3-(-3-dimethylamino propyl) carbodiimide hydrochloride) and NHS were mixed together 50:50. 50µl was added onto a research grade sensor chip. It was left on at room temperature for 30 minutes, and then removed with tissue without touching the surface.

2. Fifty µl of derivative was made up to a concentration of 2mg/ml with 0.1M borate buffer, pH 8.5, and incubated in darkness overnight. The solution was then removed with tissue paper.

3. Fifty µl ethanolamine was added to the chip. After a 30 minute incubation at room temperature, the solution was again removed with tissue paper. Finally, the chip was washed with double deionised water and dried over a stream of nitrogen.

2.3.1c Optimisation of the immobilisation of aflatoxin B₁ derivative, aflatoxin B₁-oxime-ethylenediamine onto sensor chip surface through amine coupling

1. EDC (1-ethyl-3-(-3-dimethylamino propyl) carbodiimide hydrochloride) and NHS were mixed together 50:50. 50µl was added onto a research grade sensor chip. It was left on at room temperature for 30 minutes, and then removed with tissue paper without touching the surface.

2. Fifty µl of derivative was made up to a concentration of 2mg/ml with a 50:50 mix of borate buffer/CTAB (cetyl trimethyl ammonium bromide solution) and incubated in darkness overnight. The solution was then removed with tissue paper.

3. Fifty µl ethanolamine was added to the chip. After a 30 minute incubation at room temperature, the solution was again removed with tissue paper. Finally the chip was washed with water and dried over a stream of nitrogen.

2.3.2   *Compound Feed (IRMM Reference Material) Extraction Methodology*

2.3.2a Simple methanol extraction of aflatoxin B_1 from compound feed

1.   One g aliquots of sample (9.3ng/g) were added to 10ml of 100% (v/v) methanol.

2.   The samples were vortexed and centrifuged for 10 minutes at 2000g.

3.   Eight ml of the samples were removed and the solvent evaporated in a concentrator (Techne) at 70°C over a steam of nitrogen. An oily residue was produced and this was then reconstituted in 1ml of HBS.

4.   The samples were then diluted 1 in 2, 1in 3 and 1in 4 with HBS. A calibration curve was produced using known amounts of aflatoxin B_1 dissolved in HBS buffer. The calibrants were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1 and they were prepared in duplicate.

5.   The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions consisted of an antibody fraction of 30% (antibody and sample are mixed together by the biosensor at a ratio of 30:70) and an injection time of 480 seconds as these were found to be the optimal conditions. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.2b Extraction of aflatoxin B_1 from compound feed using "Mycosep" SPE (solid phase extraction) columns from Romer Laboratories

1.   One gram of compound feed (9.3ng/g) was added to an acetonitrile/water solution (8.4ml acetonitrile to 1.6ml water), vortexed and put on a roller mixer for 30 minutes.

2.   The sample was centrifuged at 2000g for 10 minutes and 8ml was removed.

3.   The sample was then passed through the Mycosep column.

4.   Four ml of the eluate was them removed and evaporated over heat (70°C) and under a stream of nitrogen.

5. The sample was then reconstituted in 1ml HBS to make up a final concentration of 3.72ng/g. The sample was analysed against a calibration curve made up in either HBS buffer or blank extract. The calibrants were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1 and they were prepared in duplicate.

6. The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions constitute an antibody fraction of 30% (i.e. the sample volume to antibody volume is 70 to 30) and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.2c Extraction of aflatoxin B_1 from compound feed using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone

The extraction procedure was followed as described in the protocol supplied with the columns. An acetonitrile extraction is recommended for compound feed.

1. Three 1g samples (2 blank and one low-level aflatoxin" sample (9.3ng/g)) were added to 10ml 60/40 acetonitrile/water solution. The samples were vortexed and put on a shaker for 30 minutes.

2. They were then centrifuged at 2000g for 10 minutes.

3. Two ml was removed and added to 48ml PBS, pH 7.4.

4. The columns were conditioned by passing 20ml of PBS through.

5. The diluted samples were then passed through the columns and then washed with 20ml distilled water.

6. The aflatoxin B_1 was then eluted by passing 1.5ml 100% (v/v) methanol through the column.

7. The samples were dried in a concentrator at 70°C over a stream of nitrogen.

8. The samples were then resuspended in 1ml HBS. The sample was analysed against a calibration curve made up in blank sample extract. The calibrants were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1.

9. The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions constitute an antibody fraction of 30% (antibody and sample are mixed

together by the biosensor at a ratio of 30:70) and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.2d Extraction of aflatoxin B₁ from compound feed using "Aflatest" immunoaffinity columns from Vicam

1.   One g of compound feed sample (two blank samples to make up the calibration curve, one low-level IRMM sample (9.3ng/g aflatoxin B_1) and one blank sample that had been spiked at 10ng/g) was added to 5ml 80% (v/v) methanol.

2.   The samples were filtered using Millex PVDF filters. 3ml was then removed and 12ml of distilled water added.

3.   10ml was then passed through the columns.

4.   The columns were washed with 10ml distilled water, and then the aflatoxin eluted using 1.5ml 100% (v/v) methanol and collected in a test-tube.

5.   The sample was then evaporated over nitrogen in a concentrator at 70°C and reconstituted in 1ml HBS.

6.   The calibration curve produced by reconstituting known amounts of aflatoxin B_1 in blank sample instead of HBS-EP. The calibrant concentrations were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1.

7.   The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions constitute an antibody fraction of 30% (antibody and sample are mixed together by the biosensor at a ratio of 30:70) and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.3   Infant Formula Extraction Methodology

2.3.3a Methanol extraction of aflatoxin B_1 from infant formula

1. Five 1g aliquots of infant formula were weighed out. Two were spiked with aflatoxin B_1. One sample was spiked with 25μl of a 400ng/ml standard, which is equivalent to 10ng/g. The other was spiked with 25μl of a 40ng/ml standard, which is equivalent to 1ng/g. The remaining samples were used for the calibration curve.
2. Ten ml of 100% methanol was added to each sample, which were then vortexed and put on the roller for 30 minutes.
3. The samples were then centrifuged for 10 minutes at 2000g.
4. The supernatant was removed and filtered through a Millex PVDF filter.
5. A volume of supernatant (7.5ml) was removed and then blown down on the concentrator at 70ºC over nitrogen.
6. The samples were then reconstituted in 1ml HBS.
7. Calibrants were made up in blank sample extract as before. The calibrant concentrations were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1.
8. The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions constitute an antibody fraction of 30% (antibody and sample are mixed together by the biosensor at a ratio of 30:70) and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.3b Methanol extraction of aflatoxin B$_1$ from infant formula with a cyclohexane step

1.   The above experiment was repeated from points 1 to 6.

2.   One ml of cyclohexane was then added, vortexed and then incubated at 37 ºC for 15 minutes. After centrifugation at 18000g on an Eppendorf refridgerated centrifuge for 10 minutes, two separate phases had formed.

3.   The bottom HBS layer was carefully removed so as not to disturb the top layer, and the samples filtered again with the Millex filter.

4.   The blank samples were combined and used to make the calibration curve. The calibrants and samples were then run on the Biacore Q biosensor, conditions as described in the previous assay (experiment 2.3.3a).

2.3.3c Extraction of aflatoxin B$_1$ from compound feed using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone

1.   Four 1g infant formula samples were weighed out.

2.   One sample was spiked with 25µl of a 400ng/ml standard, which is equivalent to 10ng/g. The other was spiked with 25µl of a 100ng/ml standard, which is equivalent to 2.5ng/g. The other two blank samples were be used for the calibration curve.

3.   A volume (12.5ml) of 80% (v/v) methanol were added to the samples, vortexed for 2 minutes, put on a roller mixer for 30 minutes and then centrifuged at 2000g for 10 minutes.

4.   Two ml was removed and added to 16ml of PBS, pH 7.4.

5.   The columns were conditioned by passing 20ml of PBS through them.

6.   The diluted samples were then passed through the columns and then washed with 20ml distilled water.

7.   The samples were eluted from the columns as before with 1.5ml 100% (v/v) MeOH, which was then blown down over nitrogen to dryness.

8.   The samples were reconstituted in 1ml HBS. The two blank samples were combined and used construct the calibration curve. The calibrant concentrations were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1.

9.   The calibrants and samples were then run on the Biacore Q biosensor, with conditions as described in the previous assay (experiment 2.3.3a).

2.3.3d Acetonitrile extraction of aflatoxin B_1 from infant formula

1.   Five 1g samples of infant formula samples were weighed out, and one was spiked at 10ng/g aflatoxin B_1.

2.   Ten ml of 100% (v/v) acetonitrile was added to each sample, vortexed vigorously, put on a roller mixer for 30 minutes and then centrifuged at 2000g for 10 minutes.

3.   The solvent was then filtered using Millex PVDF filters. 5ml was removed and blown down over nitrogen at 60°C.

4.   The samples were then resuspended in 1ml HBS and vortexed vigorously.

5.   1ml of cyclohexane was then added to each sample, vortexed and then incubated at 37°C for 15 minutes. The samples were then centrifuged at 18,000g and the bottom layer removed with a syringe.

6.   The cyclohexane step was repeated for a second time. The samples were filtered again using Millex PVDF filters.

7.   The blank samples were combined and used to make a calibration curve.

8.   The calibrants and samples were then run on the Biacore Q biosensor, with conditions as described in the previous assay (experiment 2.3.3a).

2.3.3e Acetic acid precipitation of the proteins in infant formula

1.   Six 1g samples of infant formula were weighed out and two spiked with 1ng and 10ng of aflatoxin B_1. The four remaining blank samples are to be used for the calibration curve.

2. Seven ml of deionised water and 1ml of 3% (v/v) acetic acid were added to the samples. The samples were then made up to 10ml with more deionised water, vortexed and then put on a roller mixer for 30 minutes.

3. The samples were centrifuged at 2500g for 15 minutes and 5ml was then removed.

4. Five ml was ethyl acetate was then added, put on a roller mixer for 30 minutes and centrifuged again for 15 minutes at 2500g.

5. The ethyl acetate layer was then removed and then evaporated in the concentrator at 70°C over a stream of nitrogen.

6. The samples were then reconstituted in 1ml HBS and washed twice with cyclohexane, as described in 2.3.3d. They were then filtered using the Millex PVDF filters.

7. The calibrants and samples were then run on the Biacore Q biosensor, with conditions as described in the previous assay (experiment 2.3.3a).

2.3.3f Hydrochloric acid precipitation of the proteins in infant formula

1. One g aliquots of infant formula were added to 8ml 100% (v/v) methanol and 2ml 0.1M hydrochloric acid, vortexed and then put on a roller mixer for 30 minutes.

2. The samples were then centrifuged for 10 minutes at 2500g. Eight ml of the supernatant was removed and 8ml of deionised water was added and mixed together.

3. Four ml of hexane was added and the samples were incubated at 37°C for 10 minutes.

4. The samples were centrifuged for 10 minutes at 2500g and 14ml of the bottom layer removed.

5. Eight ml of chloroform was added, and the samples were vortexed and then centrifuged at 2500g for 10 minutes

6. Six ml of the chloroform layer was then removed and blown down at 60°C over a stream of nitrogen.

7. The samples were then reconstituted in 1ml HBS-EP.

8. The calibrants and samples were then run on the Biacore Q biosensor. Conditions were as described in the previous assay (experiment 2.3.3a).

2.3.3g Extraction of aflatoxin B₁ from compound feed using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone using acetonitrile instead of methanol in the extraction step.

1. Five 1g aliquots of infant formula were weighed. Two were spiked with aflatoxin B_1. One sample was spiked with 25µl of a 400ng/ml standard, which is equivalent to 10ng/g. The other was spiked with 25µl of a 40ng/ml standard, which is equivalent to 1ng/g. The remaining samples were used for the calibration curve.

2. Ten ml of 100% (v/v) acetonitrile was added to each sample, which were then vortexed and put on the roller for 30 minutes.

3. The samples were then centrifuged for 10 minutes at 2000g.

4. The supernatant was removed and filtered through a Millex PVDF filter.

5. A volume of supernatant (7.5ml) was removed and then blown down on the concentrator at 70ºC over nitrogen.

6. The samples were then reconstituted in 1ml HBS.

7. Calibrants were made up in blank sample extract as before. The calibrant concentrations were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1.

8. The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions consisted of an antibody fraction of 30% (antibody and sample are mixed together by the biosensor at a ratio of 30:70) and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.4   Maize extraction methodology

2.3.4a   Extraction of aflatoxin B1 from maize using the AOAC (Association of Official Analytical Chemists) recommended "best food"(BF) method for corn.

1.   One g aliquots of maize were weighed out and one sample was spiked with aflatoxin B1 at a concentration of 10ng/g.

2.   Ten ml of 55% (v/v) methanol/water added. The sample was then vortexed and put on the roller mixer for 30 minutes.

3.   Five ml was removed and added to 5ml of hexane. It was then placed on a roller mixer for 15 minutes and centrifuged at 2000g for 10 minutes.

4.   2.5ml of the methanol phase was removed and 10ml of chloroform added. The volume of sample was again put on the roller mixer for 30 minutes and then centrifuged at 2000g for 10 minutes.

5.   Finally, 5ml was removed and concentrated on the concentrator over a stream of nitrogen. The sample was then reconstituted in 1ml of HBS.

6.   Calibrants were made up in blank sample extract as before. The calibrant concentrations were 0, 0.5, 1, 2.5, 5, 10, 2.5 and 50ng/g of aflatoxin B_1.

7.   The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions consisted of an antibody fraction of 30% and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.4b Aflatoxin B1 extraction from maize using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone

1.   Three maize samples of 1g were weighed out. One was spiked with aflatoxin to a concentration of 10ng/g and two were blanks to make the calibration curve (two blanks were required to make up the volume required for the calibration curve).

2.   Five ml of 80% (v/v) methanol was added, vortexed and then put on the roller mixer for 30 minutes.

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3. The sample was centrifuged at 2000g for 10 minutes, 2ml was removed and sixteen ml of PBS was added.

4. The R-Biopharm Rhone IAC were pretreated by passing 20ml of PBS through them.

5. The samples were then passed though the columns at a speed of 1-2 drops per second. The columns were then washed with 20ml deionised water to remove any impurities.

6. The aflatoxins were then eluted using 2 applications of 1.5ml 100% (v/v) methanol.

7. The samples were then evaporated to dryness on a concentrator over a stream of nitrogen. Finally the samples were reconstituted in 1ml HBS.

2.3.4c Amylase treatment of maize samples before they are t through"Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone

1. Three 1g samples were weighed and to each 1ml deionised water and 0.125g of amylase were added. The amylase was added to break down any starch that is present in the sample. One of the samples was spiked at 20ng/g of aflatoxin B_1, the other two were left blank as they were to be used for the calibration curve.

2. The samples were then incubated at 37°C for 30 minutes.

3. Four ml of methanol (100% (v/v)) was then added to make a final concentration of 80% (v/v). The sample was then vortexed, put on the roller mixer for 30 minutes, centrifuged at 2000g for 10 mins and 2mls solvent removed.

4. The 2mls solvent was then passed through the IAC as described in *2.3.4b*

2.3.5a Extraction of aflatoxin from peanuts using methanol and immunoaffinity column clean-up

1.   One g of blank sample of ground peanuts, or sample that had been spiked at 5ng/g was vortexed with 10ml 80% (v/v) methanol/water.

2.   The samples were then placed on a roller mixer for 30mins with the spiked samples protected from light.   The samples were then centrifuged at 1600g on a bench centrifuge for 10mins.

3.   Two ml of the sample was removed and 16ml of PBS, pH 7.4, was added.

4.   The columns were first conditioned using 20ml PBS, pH 7.4.

5.   The samples were then passed through the column, and then the columns were washed with 20ml ddH$_2$O.

6.   The samples were eluted by passing and then collecting 2 volumes of 1.5ml 100% (v/v) methanol.

7.   The eluted fraction was blown down in a concentrator at 70°C over a stream of nitrogen.

8.   The samples were then reconstituted by adding 1ml HBS and vortexing for 2mins. The blank samples that were used to make the calibration curve were all filtered using Millex 0.2µm PVDF filters.

9.   One blank sample and one spiked sample were both filtered using PDVF filters (as in 8). The other blank and spiked sample were left unfiltered.

10. The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions consisted of an antibody fraction of 30% and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.5b Acetonitrile extraction of aflatoxin B₁ from peanuts followed by an immunoaffinity column cleanup

1. One g of blank sample or sample that had been spiked at 5ng/g was vortexed with 10ml 60% (v/v) methanol/water.
2. The samples were then mixed on a roller for 30mins with the spiked samples protected from light. The samples were then centrifuged at 1600g for 10mins.
3. Two ml of the sample was removed and 48ml of PBS, pH 7.4, was added.
4. The columns were first conditioned using 20ml PBS, pH 7.4.
5. The samples were then passed through the column, and then the columns were washed with 20ml ddH$_2$O.
6. The samples are eluted by passing and then collecting 2 aliquots of 1.5ml 100% (v/v) methanol.
7. The methanol was blown down in a concentrator at 70°C over a stream of nitrogen.
8. The samples were then reconstituted by adding 1ml HBS and vortexing for 2mins. The blank samples that were used to make the calibration curve were all filtered using Millex 0.2µm PVDF filters.
9. One blank sample and one spiked sample were filtered using the PDVF filters. The other blank and spiked sample were left unfiltered.
10. The calibrants and samples were then run on the Biacore Q biosensor. The assay conditions consisted of an antibody fraction of 30% and an injection time of 480 seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.5c Treatment of peanuts with Polyvinylpyrrolidone (PVP) to remove tannins present in the skins of peanuts

Peanut samples caused a large amount of non-specific binding. It was thought that the non-specific binding may have been caused by the tannins present in the peanut skins. Polyvinylpyrrolidone (PVP) can be used to remove tannins.

1. The samples were treated as follows:-

Sample 1.   1g Julian Graves peanuts and 4ml PVP were vortexed for 30 secs, placed on a roller mixer for 3mins and 6ml of water added.

Sample 2. 1g Julian Graves peanuts and 4ml PVP was vortexed for 30 secs, placed on a roller mixer for 3mins and then 6ml acetonitrile added (to bring to 60% (v/v) acetonitrile/water)

Sample 3. 1g Julian Graves peanuts and 4ml 60% acetonitrile were vortexed for 30 secs, put on a roller mixer for 3mins and then 6ml 60% (v/v) acetonitrile/water added.

Sample 4. 1g FAPAS peanuts and 4ml 60% (v/v) acetonitrile/water was vortexed for 30 secs, put on a roller mixer for 3mins and then 6ml 60% acetonitrile added.

2. All the samples were put on a roller mixer for 30mins and then centrifuged.
3. Two ml from each sample was removed and added to 48ml PBS.
4. The samples were passed through the IAC, as described in the previous experiment (2.3.5b).

2.3.5d   Optimised assay for the extraction of aflatoxin from peanuts

1. Fifty g quantities of peanuts were weighed out and 500ml of 80% (v/v) methanol/water added.
2. Samples were shaken vigorously and put on a roller mixer for 30 minutes.
3. The samples were then centrifuged at 2000g on a bench centrifuge for 10 minutes, 2ml removed and mixed with 14ml PBS, pH 7.4.
4. The "Easi-extract aflatoxin" columns were first conditioned using 20ml PBS, pH 7.4.
5. The samples were then passed through the column, and then the columns were washed with 20ml ddH$_2$O.
6. The samples are eluted by passing and then collecting 2 aliquots of 1.5ml 100% (v/v) methanol.
7. The methanol was blown down in a concentrator at 70°C over a stream of nitrogen.
8. The samples were then reconstituted by adding 1ml HBS and vortexing for 2mins.
9. The calibrants for the calibration curve were made out of blank sample as follows:-

Amount and concentration of aflatoxin B$_1$ added to blank sample	Calibrant concentration (ng/g blank sample)
40µl 125ng/ml aflatoxin B$_1$ + 960µl blank sample	25
40µl 50ng/ml aflatoxin B$_1$ + 960µl blank sample	10
40µl 25ng/ml aflatoxin B$_1$ + 960µl blank sample	5
40µl 12.5ng/ml aflatoxin B$_1$ + 960µl blank sample	2.5
40µl 5ng/ml aflatoxin B$_1$ + 960µl blank sample	1
40µl 2.5ng/ml aflatoxin B$_1$ + 960µl blank sample	0.5
40µl HBS + 960µl blank sample	0

10. The calibrants and samples were then filtered using Whatman anatop filters.

11. The calibrants and samples were then run on the Biacore Q biosensor. The optimised assay conditions consisted of an antibody fraction of 20% and an injection time of 600seconds. The regeneration solution was 10mM NaOH and 20% (v/v) acetonitrile, with a 20 second injection time.

2.3.6   General Methodology

2.3.6a Decontamination of consumables and glassware that have come into contact with aflatoxin

Aflatoxin B1 is a toxic substance. Therefore, several precautions had to be carried out when handling it. Protective clothing and gloves were worn at all times. Standards were made up in a fume cupboard whilst wearing a mask. Aflatoxin B1 is sensitive to light and therefore was stored in vials that were protected form sunlight.

1. All materials and reagents that have been contaminated with aflatoxin were soaked in a solution of 5% (v/v) sodium hypochlorite for at least 30 minutes.
2. The 5% (v/v) sodium hypochlorite is then removed, and the materials are then soaked in 5% (v/v) acetone for another 30 minutes.
3. Glassware was then washed as per normal practice.
4. The consumable materials (i.e. pipette tips, eppendorfs, centrifuge tubes) were disposed of with normal lab wast

Chapter 3: Assay Development And Feasibility

3.1   Format of aflatoxin B_1 assay using a Biacore Q biosensor

Aflatoxin B_1 is a small molecule, with a molecular weight of 312 Da, which when binding to the chip surface would not create a large enough change in resonance to facilitate easy detection in complex matrices. The aflatoxin B_1 assay, therefore, was formatted as an inhibition assay, where the amount of antibody binding to the surface is measured.

In an inhibition assay the chip surface is immobilised with the analyte or a derivative of the analyte. In this assay a derivative of aflatoxin B_1 is immobilised to the chip surface. A fixed amount of the recombinant antibody is mixed with the sample prior to injection. Aflatoxin B_1 present in the sample will bind to the antibody and therefore inhibit it from binding to the surface of the sensor chip. The higher the concentration of the analyte in the sample, the more antibodies will bind to it, causing a higher level of inhibition. This produces a lower response of the biosensor. A calibration curve is generated using the responses produced from known standards. The amount of aflatoxin B1 in a sample can then be quantified in reference to this curve (Van der Gaag *et al.,* 2003).
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3.2   Development of a sensor chip for the aflatoxin B_1 assay

Zhanna Samaonova was a previous employee of Xenosense Ltd, and had previously carried out a study to determine the most efficient way of immobilising aflatoxin B_1 onto the chip surface. Direct immobilisation of aflatoxin B_1 was compared to the immobilisation of several aflatoxin B_1 derivatives and immobilisation methods. These included aflatoxin B_1-hydrazone (2 hour and overnight immobilisations), aflatoxin B_1-oxime (immobilised with hydrazine, ethylendiamine, jeffamine or 1,6-hexanediamine), an aflatoxin B_1-BSA or an aflatoxin B_1 amine derivative (both amine coupling immobilisation) and aflatoxin B_1-oxime-ethylenediamine (2 hour or overnight amine coupling immobilisation). The derivative that showed optimal immobilisation was aflatoxin B_1-oxime-ethylenediamine.

Aflatoxin B$_1$-oxime-ethylenediamine was made producing a yield of 22.8mg (Figure 3.2). This yield is poor. Another disadvantage of the method is that the purity of the amine is unknown.

carboxymethoxylamine

Aflatoxin B$_1$

Intermediate I

II DCC

NHS

Intermediate II

Aflatoxin B$_1$-oxime-
ethylenediamine
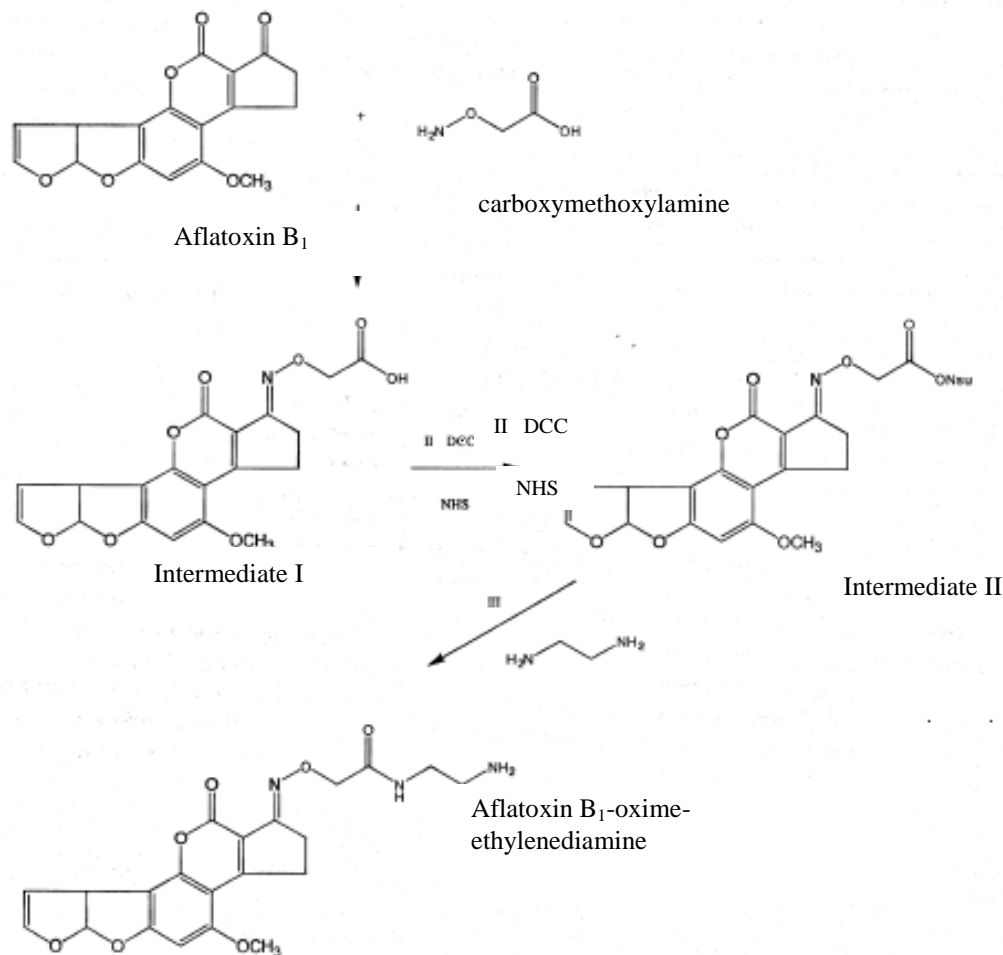
Figure 3.1 Diagram showing the reactions and chemical structures of the products and intermediates in the formation of aflatoxin B$_1$-oxime-ethylenediamine. Aflatoxin B$_1$ is dissolved in pyridine and carboxymethyloxime hydrochloride added. The pyridine is evaporated under a stream of nitrogen and gentle heating producing intermediate I. Next, 32mg N,N'-dicyclohexyl-carbodiimide (DCC) and 20mg N-hydroxysuccinimide (NHS) is added to produce intermediate II. To produce aflatoxin B1-oxime-ethylenediamine ethylenediamine is added to intermediate II.

3.3 Testing the efficacy of the surface of the aflatoxin B$_1$ assay sensor chip

Aflatoxin B_1-oxime-ethylenediamine was immobilised onto the chip surface. To determine the efficiency of immobilisation, the maximum binding capacity, R_{max}, was calculated. This was achieved by first injecting a regeneration solution over the surface, which is strong enough to remove all unbound molecules, but will not destroy the binding of the derivative with the surface of the chip. In the case of the aflatoxin assay, the most efficient regeneration solution was found to be 10mM NaOH/20% (v/v) acetonitrile. An excess of antibody was then passed over the chip surface over a long injection time of 20 minutes with a flow rate of 5μl/min.

If the immobilisation was successful, the resulting sensorgram should show binding to the surface equivalent to several thousand RU. This is shown in figure 2.2. As a control an unrelated antibody was also passed across the surface to confirm that the binding was specific (sensorgram not shown).
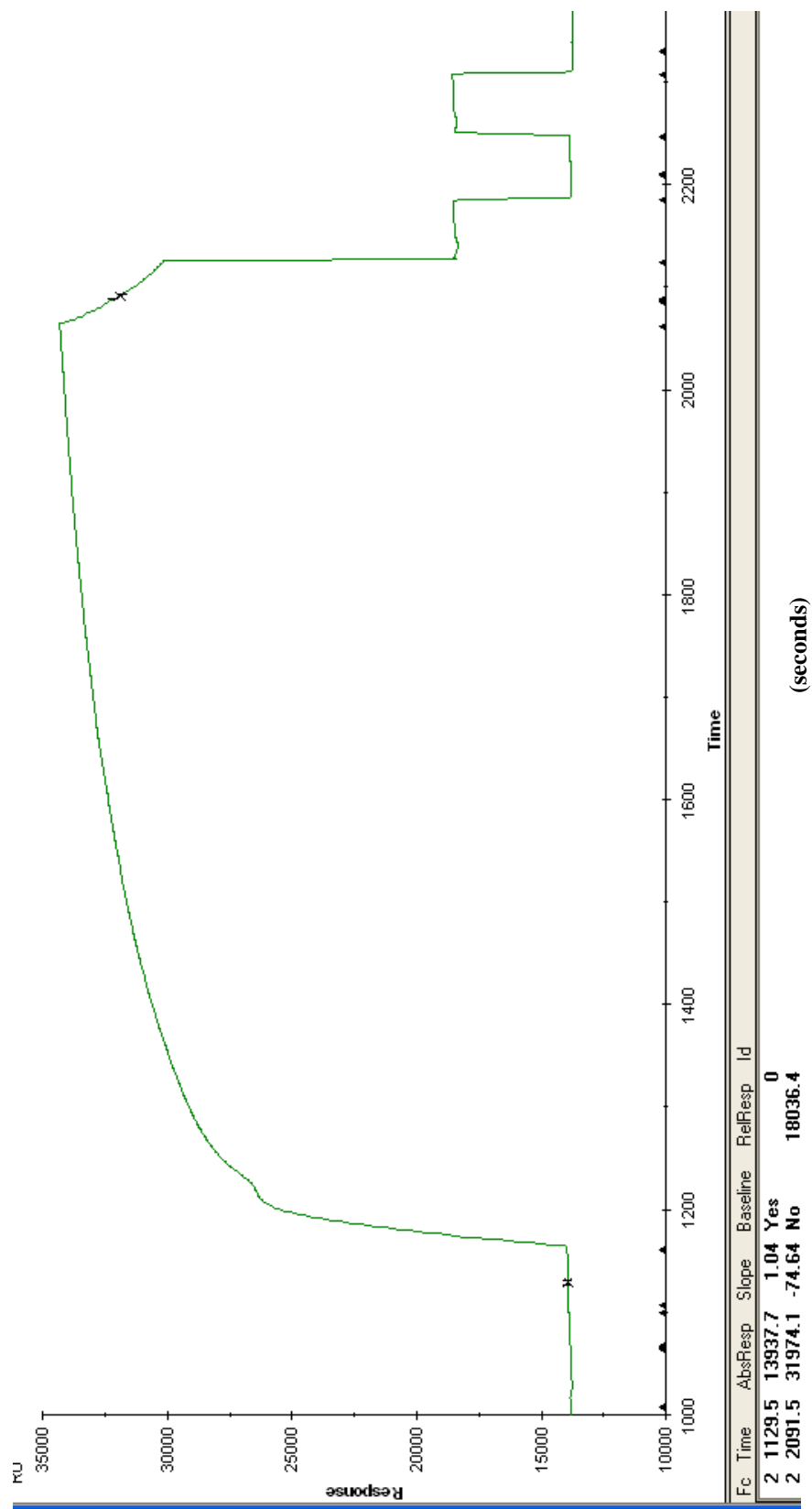
Figure 2.2 Sensorgram showing an R_{max}, the maximum amount of antibody binding to the chip surface To test the efficacy of the sensor surface anti-aflatoxin B_1 antibody was passed over the surface in large excess. If the surface is sound, the maximum amount of antibody binds to the surface and a response of several thousand RU (response units) is generated. This is called the R_{max}. In this R_{max} the response was 18,036 RU.

3.4 Evaluation of the feasibility of the aflatoxin B$_1$ assay

Initially the feasibility of the assay had to be proved by generating a standard curve in buffer. The buffer used with Biacore was HBS-EP. The standards for the calibration curve were made up from a stock solution of 1mg aflatoxin B$_1$ (Fermentek, Israel) in 1ml 100% (v/v) methanol, and the antibody was a mouse monoclonal anti-aflatoxin B$_1$ antibody produced in mouse from Sigma.

A calibration curve was produced for aflatoxin B$_1$ in a buffer system. This would indicate that an assay using food matrices may be feasible.

Cycle	Conc. (ng/ml)	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	CV (%)
10	0.001	475.2	OK	NA	
18	0.001	478.1	OK	NA	
Avg.		476.7		NA	NA
9	0.5	467.7	OK	0.498	
17	0.5	467.3	OK	0.530	
Avg.		467.5		0.514	4.3
8	1	460.2	OK	0.980	
16	1	459.7	OK	1.01	
Avg.		460.0		0.997	2.4
7	2.5	433.2	OK	2.45	
15	2.5	415.5	OK	3.35	
Avg.		424.4		2.90	22.0
6	5	390.2	OK	4.65	
14	5	382.8	OK	5.04	
Avg.		386.5		4.85	5.7
5	10	306.5	OK	9.53	
13	10	301.5	OK	9.87	
Avg.		304.0		9.70	2.5
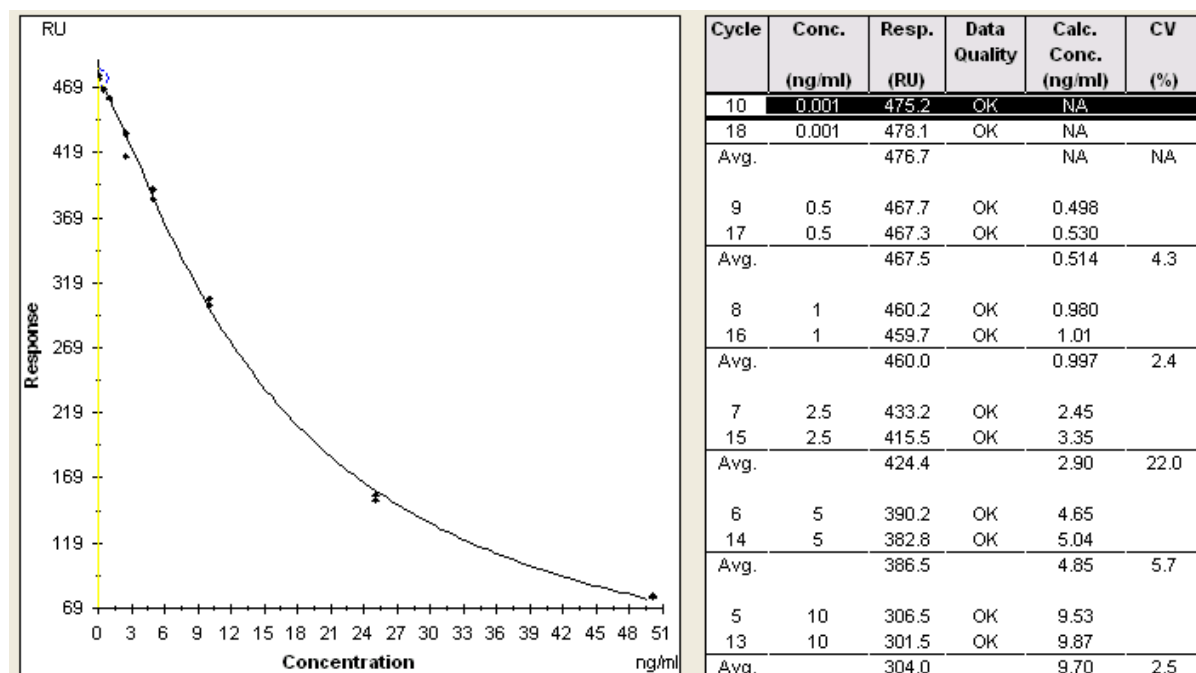
Figure 3.3 Aflatoxin B$_1$ calibration curve in HBS buffer
A calibration curve for Aflatoxin B$_1$ was produced in buffer using the calibration points 0ng/ml (as shown by 0.001ng/ml in the table as it is not possible to give a value of 0ng/ml), 0.5ng/ml, 1.0ng/ml, 2.5ng/ml, 5ng/ml and 10ng/ml of Aflatoxin B$_1$. A calibration curve was produced, demonstrating that the assay works in a buffer system. The table above shows the cycle number, the concentration of aflatoxin in ng/ml that was passed over the surface in that cycle and the respose in arbitrary response units (RU). It also shows whether the data quality was good and shows the calculated concentration of the sample when the response is read off the calibration curve. The coefficient of variation is also calculated between two samples of the same concentration. However, more than two samples would be needed for a more accurate measure of the CV.

3.5 Evaluation of the anti-aflatoxin B_1 antibodies for use in an assay using a Biacore Q biosensor

Antibodies were supplied to us by Professor Richard O'Kennedy, School of Biotechnology, Dublin City University as part of the FUSION programme. These antibodies were produced using phage display technologies. They were a monomeric (400), a dimeric (500) and 2 Fab fragments (D11 and G6). The four recombinant antibodies were compared to a commercial monoclonal anti-aflatoxin B_1 antibody.

A series of curves were set up using all four antibodies added in various percentage fractions, and comparing different injection times, to determine the most sensitive antibody and assay conditions. The antibody to sample ratio for each antibody was either 30/70 (v/v) or 50/50(v/v) and the injection times were 120 secs, 240 secs and 480 secs. The standards used in the curve were 0, 0.5, 1, 2.5, 5, 10, 25 and 50ng/g of aflatoxin B_1.

To determine which antibody is the most sensitive, and what concentration and contact time were optimal, the midpoint of the curve was calculated. This was done by halving the difference between the highest and the lowest responses on the curve and reading off the curve to give a concentration of aflatoxin. The lower the midpoint, the more sensitive the curve is. Another important factor is that the range of the curve is sufficient. It should have a range of 400-600RU.

The results are shown in Table 3.1. The Fab G6 was found to be the most sensitive for use with the biosensor, using an antibody fraction of 30% (antibody and sample are mixed together by the biosensor at a ratio of 30:70) and an injection time of 480 secs. It also had a good range at these parameters from 32.9 to 451.3RU.

Table 3.1 Table of results showing the midpoint values for antibodies G6, D11 and scFv500 each with a range of antibody fractions and contact times. To evaluate which antibody is the most sensitive and at what conditions a range of calibration curves were carried out in buffer for the anti-aflatoxin antibodies G6, D11 and scFv500. Two different antibody fractions (30 and 50% antibody) and contact times of 120, 240 and 480 secs were compared. The concentrations of aflatoxin B1 at the midpoints of each curve were compared. The lower the concentration, the more sensitive the antibody is. The range of the curve is also a factor when choosing an antibody. It should have a range of 400-600RU between 50ng/g and 0ng/g. The results show that the Fab antibody G6, with and antibody fraction of 30% and an injection time of 480secs was sufficiently sensitive and had a sufficiently wide analytical range, as this produced the lowest midpoint but with a range of over 400RU.

ANTIBODY	Ab fraction (%)	Contact time (secs)	Response at an aflatoxin B_1 concentration of 50ng/g	Response at an aflatoxin B_1 concentration of 0ng/g	Concentration of aflatoxin B_1 at the midpoint of the curve (ng/g)
G6	50	120	47.3	494.2	4.8
	30	120	21.1	307.6	2.0
	50	240	65.7	491.2	5.1
	30	240	29.7	379.8	2.5
	50	480	87.4	513.7	5.9
	30	480	32.9	451.3	3.1
D11	50	120	16.4	245.6	2.0
	30	120	4.5	120.1	1.4
	50	240	16.7	193.4	3.0
	30	240	7.1	120.4	1.7
	50	480	22.4	186.2	3.6
	30	480	7.7	149.6	1.9
scFv500	50	240	65.6	369.6	5.9
	30	240	28.2	221.8	3.3
	50	480	58.8	488.2	5.8
	30	480	70.9	393.8	3.2

Chapter 4: Matrices and Sample Extraction

4.1 Introduction

The range of commodities that are affected by aflatoxins is very broad as contamination can occur at any time from pre-harvest, from storage and during transportation (Kabak *et al.,* 2006). Cereals such as maize, rice and wheat, and nuts (e.g. peanuts, brazil nuts and pistachios) can be affected at any stage of the farming cycle, whilst spices and dried fruit are most likely to become contaminated when stored. Aflatoxins can withstand high temperatures and, therefore, can also be present in processed foods. Recently, in September 2006, snacks containing rice flour contaminated with aflatoxins were withdrawn from several large retailers in the UK (RASFF, Rapid Alert System for Food and Feed). Another product that was associated with aflatoxin contamination is peanut butter when made from contaminated peanuts. Milk and milk products may also have aflatoxin contamination due to metabolism of ingested aflatoxins by dairy cows.

Aflatoxins can be extracted from food matrices using a number of different solvents, including methanol, acetonitrile, chloroform, acetone and DMSO. However, pure solvents are not as efficient as solvents that have been diluted with water. The ratios of solvent to water are critical, as are the solvent to matrix ratio (Whitaker *et al.,* 1986). Also, some solvents work best for different matrices. For example, R-Biopharm Rhone recommend an 80% (v/v) methanol extraction for nuts, figs, maize and cereals, whereas for spices, compound feed and herbs a 60% (v/v) acetonitrile solution is advised.

Solvent extraction, however, can be problematic because other impurities from the matrix are often extracted along with the aflatoxin. These impurities may also bind non-specifically to the surface of the sensor chip. Non-specific binding (NSB) can, therefore, lead to false results. A popular way of reducing NSB is the use of clean-up columns. SPE (solid phase extraction) columns or immunoaffinty columns (IAC) are frequently used to isolate an analyte from a matrix and also to concentrate the samples (Stroka *et al.,* 2000; Senyuva *et al.,* 2005; Ip *et al.,* 2006; Turner *et al.,* 2009).

SPE columns contain a matrix, which is known as the solid phase. The extraction process works on the premise that the analyte of interest has a far greater affinity for the matrix than the impurities in a sample. Once the sample has been extracted, using a suitable solvent and passed through the SPE column, the analyte has a stronger affinity to the solid phase than the rest of the matrix. Therefore, the impurities are washed away, and the purified analyte can be eluted from the column. A variety of different stationary phases can be used, depending on the charge of the analyte of interest. Most are based on a bonded silica material derivatised with a functional group to confer a positive or negative charge (Turner *et al.,* 2009). The main disadvantage of SPE is that the matrix may contain impurities that have the same charge as the analyte. These too will be adsorbed on to the solid phase, and so the analyte may not be pure.

In contrast, IACs are also based on a column filled with a matrix. This matrix, however, contains antibodies that have affinity specifically for the analyte of interest. Therefore when a sample is passed through the column only the analyte binds to the surface, washing away all other impurities. Therefore, the advantage of this system is that the eluate contains a pure solution of the analyte. A disadvantage is that these columns can be very expensive (Castegnaro *et al.,* 2006).

The purpose of this project was to develop a diagnostic kit to test for aflatoxins in a range of foodstuffs that will be launched as a product alongside the other food testing kits developed and manufactured by Xenosense Ltd. The matrices chosen are dependant on a number of factors. For example, what is the unique selling factor of this test? What tests are there already available on the market? At the beginning our tests will be targeted at existing customers, which include Nestle, Kraft and Analyscen. Therefore, it is very important that the commodities that are within the capabilities of testing by the kit coincide with the interests of potential customers.

4.2 Compound Feed (IRMM Reference Material)

4.2.1.Introduction

The first matrix to be investigated was a reference material. The reference material was compound feed, which was commercially available from Sigma Aldridge. Spiked samples can sometimes behave differently than real samples, so it was decided to try to optimise the assay using real samples first. The reference material was purchased from the Institute for Reference Materials and Measurements (IRMM). Two samples were bought that had been certified to contain 0 ng/g (blank) and 9.3 ng/g aflatoxin B_1.

4.2.2 Results

The most common extraction solvent for aflatoxin B_1 is methanol. Therefore a simple methanol extraction was carried out using compound feed reference material that contained 9.3 ng/g aflatoxin B_1. The sample was run against a standard curve made from aflatoxin B_1 in HBS-EP buffer. The sample, however, gave reading that were "off-scale", showing major interference effects from the matrix.

To remove the matrix effects from the sample the use of solid phase extraction (SPE) columns and immunoaffinity columns (IAC) was investigated.

"Mycosep" SPE columns, purchased from Romer Laboratories, were evaluated initially as they were considerably less expensive than the immunoaffinity columns. The first extraction was read off a standard curve that had been created using HBS-EP buffer. The results showed apparent recoveries of about 1000%. This would suggest that, again, there was interference from the sample matrix. The experiment was then repeated. This time, however, the standard curve was constructed using blank sample extract instead of buffer. The reasoning for this is that the sample and the calibration curve are directly comparable. However, the calibration points obtained were erratic, and a calibration curve was not

generated, so that it was impossible to determine the concentration of aflatoxin B_1 in the sample from the constructed standard curve. This was, again, due to matrix interference.

In an attempt to remove matrix interference immunoaffinity columns were tested. There are many IAC specific to aflatoxins available. "Easi-extract aflatoxin" IAC are mentioned frequently in papers and other published documents and were, therefore, first to be evaluated

The calibration curve was made using a blank sample extract. On this occasion the curve showed little matrix interference. The concentration of the sample when it was read off the curve was 9.3 ng/g. The compound feed was certified as containing aflatoxin B_1 at a concentration of 9.3 ng/g. This gives a recovery of 100.5%. The experiment was then repeated to prove that the results could be replicated. The recovery from the certified compound feed sample was 9.08 ng/g, which is 98%. These results are shown in Figure 4.1. This shows that the "easi-extract aflatoxin" IAC are very effective in sample clean-up.
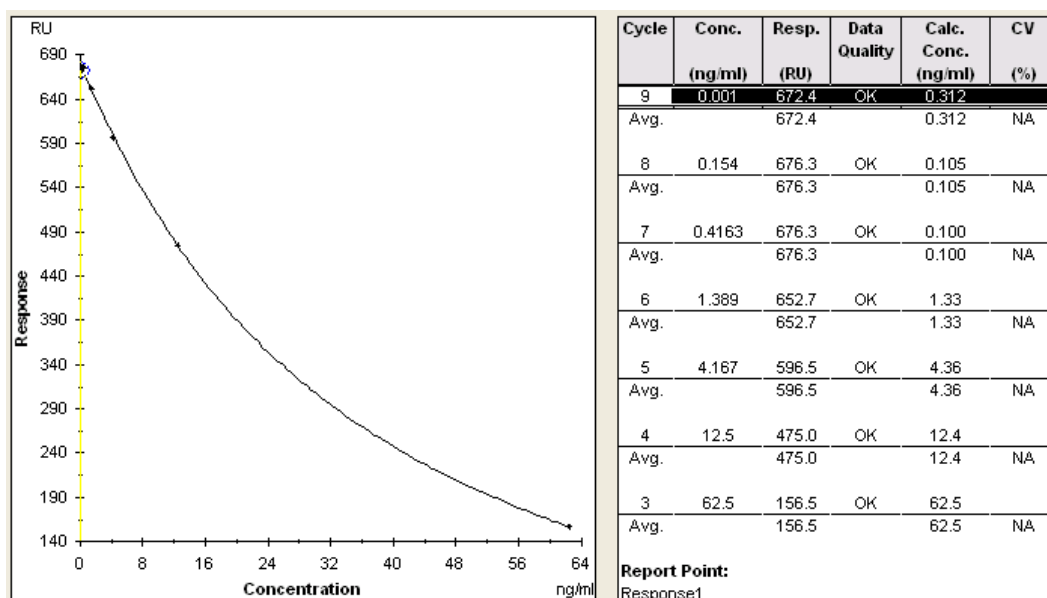
Cycle	Conc. (ng/ml)	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	CV (%)
9	0.001	672.4	OK	0.312	
Avg.		672.4		0.312	NA
8	0.154	676.3	OK	0.105	
Avg.		676.3		0.105	NA
7	0.4163	676.3	OK	0.100	
Avg.		676.3		0.100	NA
6	1.389	652.7	OK	1.33	
Avg.		652.7		1.33	NA
5	4.167	596.5	OK	4.36	
Avg.		596.5		4.36	NA
4	12.5	475.0	OK	12.4	
Avg.		475.0		12.4	NA
3	62.5	156.5	OK	62.5	
Avg.		156.5		62.5	NA

Report Point: Response1

Cycle	Sample Id	Amount (g)	Volume (ml)	Dil. Fact.	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	Calc. Amount (ng/ml)	CV (%)
10	a			1	601.9	OK	9.01	9.01	
11	a			1	604.0	OK	8.86	8.86	
12	a			1	592.2	OK	9.72	9.72	
13	a			1	595.5	OK	9.48	9.48	
14	a			1	599.5	OK	9.19	9.19	
15	a			1	608.9	OK	8.51	8.51	
16	a			1	606.4	OK	8.69	8.69	
17	a			1	599.4	OK	9.20	9.20	
Avg.								9.08	4.4

Figure 4.1 Aflatoxin B_1 in compound feed reference material assay using "Easi-extract aflatoxin" immunoaffinity columns commercially available from R-Biopharm Rhone
The table above shows the cycle number, the concentration of aflatoxin in ng/ml that was passed over the surface in that cycle and the respose in arbitrary response units (RU). It also indicates whether the data quality was good and shows the calculated concentration of the sample when the response is read off the calibration curve. The coefficient of variation is also calculated between two samples of the same concentration.
Aflatoxin B_1 was extracted from compound feed using "Easi-extract aflatoxin" immunoaffinity columns. The feed had been certified to contain aflatoxin B_1 at a concentration of 9.3 ng/g. Using a calibration curve that had been created using blank extracted sample, the calculated recovery of aflatoxin B_1 from the sample was given as 9.08 ng/g, which is 98%. The coefficient of variation (% CV) was 4.4.

One disadvantage of the columns is that they are very expensive, especially since they are recommended for single use only. To determine how many times a column could actually be used the experiment was repeated, but the column used for the sample was re-used nine times. The sample used was the compound feed certified as containing aflatoxin B_1 at a concentration of 9.3 ng/g.

The results (Table 4.1) show that the recovery dropped to 88% after one use and after 6 times the recovery was 25%. This proves that the columns are not re-usable.

Table 4.1 Table showing the re-usablity of the "Easi-extract aflatoxin" immunoaffinity columns. "Easi-extract aflatoxin" IAC were re-used several times. The eluate was collected each time and the recoveries were calculated using a calibration curve that had been constructed using a blank extracted sample. The recoveries dropped significantly each time the columns were re-used, thus proving the columns cannot be used more than once.

Sample name	Number of times the column used	% Recovery
A	1	88
B	2	73
C	3	78
D	4	67
E	5	sample lost
F	6	25
G	7	32
H	8	12
I	9	26

There are many other immunoaffinity columns for aflatoxin B_1 clean-up on the market. Another column that has been used in a variety of papers is the "Aflatest" IAC from Vicam. These columns were found to be considerably cheaper than the IAC from R-Biopharm Rhone. Therefore, these columns were used to compare their effectiveness in the compound feed assay.

The experiment was repeated using "Aflatest" IAC. IRMM compound feed (certified to contain 9.3 ng/g aflatoxin B_1) and a spiked blank sample (containing 10ng/g aflatoxin B_1) were extracted and the recoveries calculated using a calibration curve made using blank extracted sample. The columns seemed to remove most of the matrix effect because the calibration curve produced showed little interference. The recoveries of the samples, however, were lower than expected. The real sample had a recovery of 4.68 ng/g (50.3%) and the spiked sample had a recovery of 3.57 ng/g (35.7%). This would suggest that some of the aflatoxin B_1 is being lost through the "Aflatest" IAC, and that they are not as effective as "Easi-extract aflatoxin" IAC. This is illustrated in Figure 4.2.

To further test the efficacy of the columns the experiment was repeated in a buffer system. This experiment removes any interference that might have been caused by the matrix. The calibration curve was constructed using HBS-EP and the "sample" was HBS-EP buffer spiked with 10ng/ml of aflatoxin B_1. The recovery of the aflatoxin B_1 in buffer was approximately 67%, which is considerably lower than the recoveries using the "Easi-extract aflatoxin" IAC.

Top-right table:

Cycle	Conc. (ng/ml)	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	CV (%)
7	0.01	476.3	OK	0.0115	
Avg.		476.3		0.0115	NA
6	3.21	328.9	OK	3.20	
Avg.		328.9		3.20	NA
5	6.25	245.5	OK	6.30	
Avg.		245.5		6.30	NA
4	12.5	166.1	OK	12.4	
Avg.		166.1		12.4	NA
3	25	106.6	OK	25.1	
Avg.		106.6		25.1	NA

Report Point:
Response1

Fitting Function:
4 Parameter Equation

Parameters:
Rhi = 41.65

Bottom table:

Cycle	Sample Id	Amount (g)	Volume (ml)	Dil. Fact.	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	Calc. Amount [ng/ml]	CV (%)
8	SPIKE			1	306.6	OK	3.87	3.87	
9	SPIKE			1	317.6	OK	3.53	3.53	
10	SPIKE			1	324.6	OK	3.32	3.32	
Avg.								3.57	7.7
11	SAMPLE			1	285.8	OK	4.58	4.58	
12	SAMPLE			1	284.2	OK	4.64	4.64	
13	SAMPLE			1	279.5	OK	4.82	4.82	
Avg.								4.68	2.6

Figure 4.2 Aflatoxin B₁ in compound feed reference material assay using "Aflatest" immunoaffinity columns commercially available from Vicam.
Aflatoxin B₁ was extracted from compound feed using"Easi-extract aflatoxin" immunoaffinity columns. Samples used were IRMM compound feed (certified to contain 9.3 ng/g aflatoxin B₁) and a spiked blank sample (containing 10ng/g aflatoxin B₁). The real sample had a recovery of 4.68 ng/g (50.3%) and the spiked sample had a recovery of 3.57 ng/g (35.7%).

4.3 Infant Formula

4.3.1 Introduction

The feasibility of the aflatoxin assay had been proven with matrix using the compound feed reference material. The first matrix chosen to investigate was infant formula. Infant formula has regulatory limits set by the EU at 0.05ng/g. These limits are the lowest set by the EU for aflatoxin B_1 in any food. The reason why the limits are so low is that the food is designed for consumption by babies who would be extremely vulnerable towards aflatoxin toxicity. In addition, there are currently no rapid tests available in the market able to detect such low concentrations. This would be a unique selling point for the kit and the reason why infant formula was chosen as the first matrix to examine in detail.

4.3.2   Results

To minimise cost for budgetary and commercial feasibility it was decided to first investigate sample preparations that did not use expensive IACs. The simplest extraction procedure involved using methanol. A simple methanol extraction was carried out using infant formula that had been spiked with aflatoxin B_1. The calibration curve was constructed using calibrant that had been made from blank infant formula that had gone through the same methanol extraction as the spiked sample.

Unfortunately, when the calibrants were analysed there was too much interference from the matrix and a calibration curve could not be generated. Infant formula contains a high perventage of fat, which could bind to the chip surface. The experiment was, therefore, repeated except that an additional cyclohexane step was introduced. Cyclohexane is used to remove fat from the sample. Results are shown in Figure 4.3.
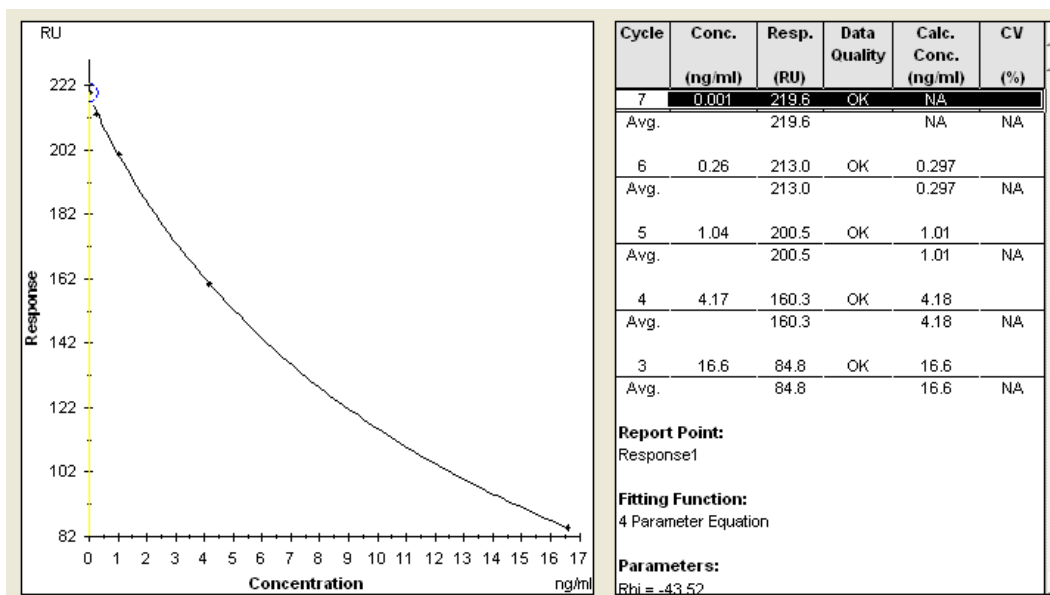
Table (top-left graph and data):

Cycle	Conc. (ng/ml)	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	CV (%)
7	0.001	219.6	OK	NA	
Avg.		219.6		NA	NA
6	0.26	213.0	OK	0.297	
Avg.		213.0		0.297	NA
5	1.04	200.5	OK	1.01	
Avg.		200.5		1.01	NA
4	4.17	160.3	OK	4.18	
Avg.		160.3		4.18	NA
3	16.6	84.8	OK	16.6	
Avg.		84.8		16.6	NA

Report Point:
Response1

Fitting Function:
4 Parameter Equation

Parameters:
Rhi = -43.52

Cycle	Sample Id	Amount (g)	Volume (ml)	Dil. Fact.	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	Calc. Amount [ng/ml]	CV (%)
9	1ng/ml			1	283.0	OK	<1.00e-3	<1.00e-3	
Avg.								<1.00e-3	NA
10	10ng/ml			1	200.6	OK	1.00	1.00	
Avg								1.00	NA

Figure 4.3 Aflatoxin B$_1$ extraction from infant formula using a simple methanol extraction and including a cyclohexane step.
Infant formula was spiked with aflatoxin B$_1$ to a concentration of 10 ng/g. This spiked and blank samples (that were later used to construct the calibration curve) underwent a simple 100% methanol extraction followed by treatment with cyclohexane to remove matrix effect caused by fat. A calibration curve was generated, but the recovery was only 10% of the spiked sample.

The matix effect seemed to be removed from the infant formula by the cyclohexane step and a calibration curve was generated. However, the recovery for the spiked sample was very low. The 10 ng/g spiked sample only had a recovery of 10%. There are several possible reasons as to why aflatoxin B$_1$ was lost in the extraction. For example, the temperature for the concentration step was too high and was somehow damaging the aflatoxin B$_1$, or that the reconstitution step in HBS-EP was not thorough enough. The experiment was repeated, but the vortexing steps for extraction and reconstitution were more vigorous, lasting 2 minutes. Also, the temperature of the concentrator was reduced

from 70 to 60˚C. The recoveries were only slightly improved, increasing from 10% to 16%. Therefore, other extraction methods had to be investigated.

Acetonitrile is another solvent that is recommended for aflatoxin B_1 extraction. The methanol extraction method, which included the cyclohexane washes, was repeated using acetonitrile instead of methanol. There was a slight improvement in the recoveries (the 10 ng/g spike had a recovery of 26%), but they were still very low. The experiment was repeated without the cyclohexane washes to determine whether the aflatoxin B_1 was lost along with the fat in the sample. The calibrants, however, made from "blank-extracted" sample had too much matrix interference and a satisfactory calibration curve could not be generated.

Infant formula also contains a high percentage of protein. It was possible that the aflatoxin was binding to the proteins in the matrix and was then being removed. Therefore, several methods were performed to precipitate out the proteins. The first method used acetic acid. This experiment was repeated using either ethyl acetate or chloroform as the extraction solvents. Again, the recoveries were low. Both solvents had recoveries of 13% for samples spiked at 10ng/g. Hydrochloric acid was then used to precipitate the proteins instead of acetic acid. A sample spiked with 4 ng/g aflatoxin B_1 was added to a mixture of 100% methanol and 0.1M hydrochloric acid. After the protein precipitation, a hexane wash was completed to remove fat from the sample. The aflatoxin B_1 was then extracted using chloroform. However, the recovery was only 15%.

Excellent results had previously been achieved for aflatoxin B_1 extraction from compound feed using "Easi-extract aflatoxin" IAC from R-Biopharm Rhone. The use of these columns had been avoided because they were so expensive, but, because of the failure of simple solvent extraction to yield good recoveries, the columns were assessed for use in the infant formula assay.

Two blank infant formula samples were spiked with aflatoxin B_1, one at 2.5 ng/g and the other at 10ng/g. An 80% methanol extraction was first carried out and then the samples

were put through the "Easi-extract aflatoxin" IAC. A calibration curve was constructed using blank samples that had also been put through the IAC. The results showed, however, that again, the recoveries were low being 24% for the 2.5ng/g spike and only 13% for the 10 ng/g spiked sample.

R-Biopharm Rhone recommends both methanol and acetonitrile as solvents for aflatoxin B_1 extraction. The experiment was repeated using 100% acetonitrile for the extraction solvent instead of 80% methanol. The IAC were conditioned, samples added and washed as before. This time, however, the 10 ng/g spike had an apparent recovery of 137%. It is impossible to have recoveries over 100%, so the results are too high. However, of major concern, was the sensitivity of the curve. The regulation limits for aflatoxin B_1 in infant formula are 0.05 ng/g. Therefore, this assay must be sensitive enough to detect to these levels. In order to increase the sensitivity of the assay the experiment was repeated using a smaller extraction volume. R-Biopharm Rhone recommend an extraction volume of 10ml. In order to increase the sensitivity of the assay, however, 5ml of 100% acetonitrile were used. Two spiked samples were used in this assay (1 ng/g and 5 ng/g). Although the recoveries for the 5ng/g spike were promising (the recoveries were an average of 78%), those for the 1ng/g were erratic. One of the recoveries was 100.4% but the other was 384%. The reason for this is that the sensitivity of the curve at this concentration is very poor. This is not acceptable for an infant formula assay as it has to be able to detect levels down to 0.05 ng/g. It seemed very unlikely, therefore, that even with the use of IAC, that a calibration curve of this sensitivity can be achieved. The outcomes of the various approaches are summarized in Table 4.2. Hence, it was decided to concentrate on other potential matrices of commercial relevance that might be more feasible for assay development.

Table 4.2 Results for experiments extracting aflatoxin B$_1$ from infant formula
Aflatoxin B$_1$ was extracted from infant formula using a variety of extraction solvents and immunoaffinity columns were evaluated. Fat and protein removal techniques were also assessed.

Title of Experiment	Calibration curve produced?	Level at which aflatoxin B$_1$ was spiked (ng/g)	% Recovery	Problem with assay
Methanol extraction of aflatoxin B$_1$ from infant formula	No	10	n/a	No calibration curve produced
Methanol extraction of aflatoxin B$_1$ from infant formula with a cyclohexane step	Yes	10	10	Recovery very poor
Methanol extraction of aflatoxin B$_1$ from infant formula with a cyclohexane step (more stringent washing, lower evaporation temperature)	Yes	10	16	Recovery very poor
Acetonitrile extraction of aflatoxin B$_1$ from infant formula	Yes	10	26	Recovery very poor
Acetonitrile extraction of aflatoxin B$_1$ from infant formula without a cyclohexane step	No	10	n/a	No calibration curve produced
Acetic acid precipitation of the proteins in infant formula using ethyl acetate as extraction solvent	Yes	10	13	Recovery very poor
Acetic acid precipitation of the proteins in infant formula using chloroform as extraction solvent	Yes	10	13	Recovery very poor
Hydrochloric acid precipitation of the proteins in infant formula	Yes	4	15	Recovery very poor
Extraction of aflatoxin B$_1$ from compound feed using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone	Yes	2.5	24	Recoveries very poor
		10	13	
Extraction of aflatoxin B$_1$ from compound feed using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone using acetonitrile instead of methanol in the extraction step	Yes	10	137%	Recoveries very high and calibration curve not sufficiently sensitive
Extraction of aflatoxin B$_1$ from compound feed using "Easi-extract aflatoxin" immunoaffinity columns from R-Biopharm Rhone using acetonitrile instead of methanol in the extraction step (more sensitive assay)	Yes	1	Too erratic	Calibration curve not sufficiently sensitive
		5	78	

4.4   Maize

4.4.1 Introduction

The FAO has estimated that 25% of grains and cereals worldwide have been contaminated by mycotoxins. This includes aflatoxins. Cereals and grains are both major constituents of the human food chain and the main food-stuff fed to livestock. Therefore, cereals contaminated with aflatoxins can have a huge adverse impact on animal husbandry, as they have been shown to reduce immunity, decrease fertility, decrease weight gain, cause a reduction in milk production and, when contamination is high enough, mortality. It is of great significance for dairy cows, where aflatoxin is metabolised and then excreted via the milk in the form of aflatoxin M_1. Thus, control of aflatoxin contamination of cereals is essential for production economics, animal health, food safety and product quality.

A cereal that is at high risk of aflatoxin contamination is maize. Maize is the major cereal for both human and livestock consumption in many African and South-East Asian diets, and in North America it is the staple food for livestock. Therefore, control of aflatoxin levels in corn is crucial and it was for this reason that it was chosen as the next sample matrix. In addition, it was thought that sample preparation would be easier than that for infant formula as it is not as complex a matrix. The lowest regulatory limits for the detection of aflatoxin B_1 in corn was set by the EU and is 2ppb (Commission Regulation (EC) No 2174/2003 of 12 December 2003).

4.4.2   Results

The AOAC recommends the "Best Food" (BF) method for the extraction of aflatoxins from corn. The method involves a 55% (v/v) methanol/water extraction, followed by a hexane step and then a final chloroform extraction. The calibration curve was generated using extracted blank sample. A calibration curve was formed. However, the background was very high caused by interference from the matrix and the sample reading was of the scale.

"Easi-extract aflatoxin" columns had previously proved successful with sample preparation of compound feed. Therefore, the columns were used in the maize assay to remove matrix effects. R-Biopharm Rhone recommended that the extraction buffer for maize should be 80% methanol. However, it was found that the background was quite high. Nonetheless, a calibration curve was generated. The recovery from a sample spiked at 10ng/g was only 60%. The reason for this may have been the high background recorded due to matrix effects.

The IAC are supposed to remove all matrix interference from the maize samples. However, the high background of the calibration curve shows that there is still some matrix effect. One attempt to remove matrix interference was to carry out a "Best Food" (BF) method extraction followed by the use of the IAC. Unfortunately, this did not fully remove the background and the recoveries of the sample spiked at 10ng/g dropped to 32%.

Maize is mostly composed of starch. It was thought that the starch could be binding non-specifically to the chip surface causing the high background. The composition of maize is shown in Table 4.3.

Table 4.3 Composition of Maize **(USDA National Nutrient Database for Standard Reference)**

Component of maize	% composition
Starch	62
Corn Oil	4
Protein	8
Fibre	11
Water	15

Starch content was reduced by treatment with the enzyme amylase, which seemed to considerably improve the assay as shown by the results in Figure 4.4 and the accompaning tables. The background was reduced from 1760RU to 1280RU at the 0ng/g calibrant point

of the curve. The range of the calibration curve had also improved with a drop of 300RU between 0 and 50ng/g. The larger the drop in the calibration curve, i.e. the greater the difference in RU, the more sensitive the assay will be. Finally, the recoveries had also been greatly improved. The apparent recovery for a 20ng/g spike was 111%.

In conclusion, it would seem that it was the starch present in the maize that was binding non-specifically to the surface of the chip. The starch was successfully broken down by the amylase treatment, thus reducing the background and resulting in acceptable recoveries.

Cycle	Conc. (ng/ml)	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	CV (%)
8	0.001	1275.3	OK	NA	
Avg.		1275.3		NA	NA
7	3.125	1195.2	OK	3.96	
Avg.		1195.2		3.96	NA
6	6.25	1174.3	OK	5.53	
Avg.		1174.3		5.53	NA
5	12.5	1119.4	OK	11.0	
Avg.		1119.4		11.0	NA
4	25	1022.6	OK	29.2	
Avg.		1022.6		29.2	NA
3	50	975.2	OK	47.2	
Avg.		975.2		47.2	NA

Report Point:
Response1

Fitting Function:
4 Parameter Equation
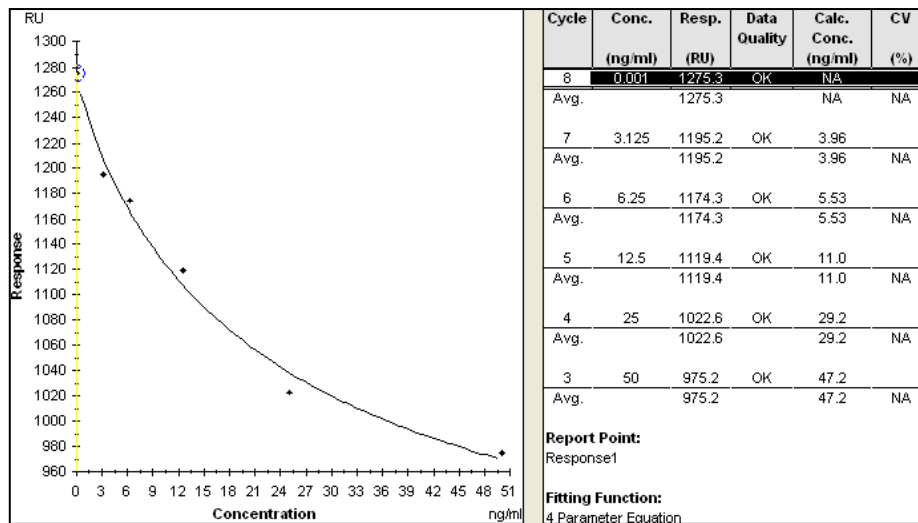
Figure 4.4 Aflatoxin B₁ extraction from maize with 55% (v/v) methanol/water, followed by a hexane step and then a final chloroform extraction after the maize had been treated with amylase.
Maize was spiked with aflatoxin B₁ to a concentration of 10 ng/g. After an amylase treatment to remove starch, the sample was then extracted with 55% (v/v) methanol/water, followed by a hexane step and then a final chloroform extraction. The recovery was found to be 111% and the coefficient of variation was 5.0%

81

4.5   Peanuts

Peanuts are very susceptible to mould growth and, therefore, it is a commodity that is notorious for aflatoxin contamination. The lowest regulatory limits that have been set for aflatoxin B_1 in peanuts are by the EU and are 2ppb (2ng/g).

The first extraction procedure attempted for the peanut assay using the "Easi-extract aflatoxin" immunoaffinity columns (IAC) was one recommended for peanuts in the protocol of the kit. This involved an extraction step using 80% (v/v) methanol/water. The peanuts used were reference material purchased from FAPAS (Food Analysis Performance Assessment Scheme). They had been tested and certified as blank peanut samples. Controls to determine whether the use of filters was needed in the assay were also included. Half the blank sample and half the spiked sample were filtered using the PVDF (Polyvinylidene Fluoride) filters and the other half remained unfiltered. This was to determine whether non-specific binding to the chip surface could be removed by the use of filters.

A calibration curve was generated, but there is still some background present. In buffer, the zero calibration gave a response of 648RU, whilst in extract the response was 1356RU. The filters did remove some non-specific binding as shown when comparing the samples that have been filtered to those that had not. However, the recoveries were poor. An average of 2.0ng/g was recovered, which was a 40% recovery. A possible reason for this was that the high background was "masking" some of the recovery i.e. the background was so high that the specific binding cannot be differentiated from the non-specific. Peanuts contain high amounts of fats, which are known to associate with the surface of the Biacore chip. Fat binding to the surface produces a characteristic bend in the sensorgram, which was seen in this experiment. Removal of fat from a sample can be achieved by incorporating a cyclohexane wash into the protocol, but when this was incorporated into the previous experiment the background actually increased.

Another extraction process described in the R-Biopharm Rhone protocol involved using 60% (v/v) acetonitrile/water instead of 80% (v/v) methanol/water. This extraction was carried out in the hope that less matrix effect would be extracted along with the aflatoxin. The background was significantly reduced using acetonitrile instead of methanol for the extraction solvent, the recoveries, however, were very poor. The samples had been spiked with the equivalent of 5ng/g and the recovery was 1.21ng/g, which is 24.2%. Interestingly, there did not seem to be too much difference between the filtered and unfiltered samples. This suggests that the filters were unnecessary and the experiment was repeated without filters. Both spiked and real samples were used. The real sample from FAPAS, which contained 2.06ng/g of aflatoxin B_1 was also included in this experiment.

The sample spiked with 5ng/g aflatoxin also showed good recovery (88%). However, with the 2.5ng/g spike had very poor recovery. The most worrying outcome of the experiment, however, was the responses from the real samples. The real samples had responses of over 2000RU. When the samples were extracted they had also looked different, with the real samples producing a reddish liquid. FAPAS were contacted to discover what was responsible for the differences between the blank and the positive samples were. The blank samples had in fact been treated differently. The blank sample had been blanched, the skin removed and milled with flour. The positive samples had only been milled, without the addition of flour. Since the blank sample was not 100% peanut it should not be used for the calibration curve. Although there is no guarantee that commercially bought peanuts are aflatoxin-free, it was decided that a sample of these should be used for the calibration curve as they would be 100% peanut. The peanuts chosen were bought from a health food shop called "Julian Graves". The reason for the high matrix effect was thought to be because the peanut skins contain tannins, which are known to bind to the sensor chip surface. Other assays have used PVP to remove tannins to great effect

The Julian Graves sample showed considerably more matrix effect than the FAPAS sample (1500-1600RU compared to approximately 800RU). This shows that the matrix effect is indeed caused by the peanut skins. The treatment of the peanuts with the PVP seemed to add to the matrix effect with both samples treated with PVP showing responses double that

than the untreated sample. It was then decided to filter the samples and test then with manual injections. Millex GS filters were selected as they had been used to remove PVP in previous assays. This time all matrix effect were removed, including the sample that had not been treated with PVP. It was concluded that the use of PVP was unnecessary, and that a simple filtration step would suffice. However, Millex GS filters were known to sequester aflatoxin. Therefore, a range of filters had to be evaluated.

Six different filters were evaluated, to determine their effectiveness at removing matrix effects. The possibility that filters might sequester aflatoxin was also investigated. To test if the matrix was removed, the peanut extract was filtered and the filtrate compared to HBS when run against a calibration curve. Blank HBS and HBS that had been spiked at a concentration of 5ng/g were also treated using the different filters. These too were compared to a calibration curve in HBS, and this enabled the calculation of recoveries.

The results are shown in Table 4.4. They show that filters do remove the matrix interferences from the samples. The response for the peanut extract without filtering was 3346RU. This shows a high level of non-specific binding to the surface when compared to the response of buffer alone (630RU). The Millex GV filters remove approximately half the non-specific binding (NSB), but the aflatoxin recovery is only 14%. The Millex GV and the Millex PVDF remove all the NSB, but the aflatoxin is completely removed from the sample by the filters. The Ministart columns remove about half the NSB and the recovery was only 61%. The Target filters removed all the NSB, but the recovery was only 59%. The best performing filters were both from Whatman. The Whatman CA w/GMF filters eliminated all NSB and the recovery was 84%. The best results, however, were using the Whatman Anotope filters which removed all NSB and had apparent recoveries of 112%. This test was performed only once. It was thought that further tests were not necessary as the experiment was designed only to give a quick comparison between the filters. The Whatman Anotope filters were found to be the most suitable for use in the aflatoxin/peanut assay.

Table 4.4 Table showing the efficacy of a range of different filters in removing matrix effect from peanuts whilst not sequestering aflatoxin B_1.

Filter	Sample Type	Response (RU)	Recovery
No filter	Peanut extract	3346	N/A
	HBS	630	N/A
	HBS spiked with 10ng/g aflatoxin	123	160%
Millex GV	Peanut extract	1757	N/A
	HBS	578	N/A
	HBS spiked with 10ng/g aflatoxin	501	14%
Millex GS	Peanut extract	663	N/A
	HBS	683.7	N/A
	HBS spiked with 10ng/g aflatoxin	686	0%
Millex PVDF	Peanut extract	681	N/A
	HBS	686	N/A
	HBS spiked with 10ng/g aflatoxin	684	0%
Ministart	Peanut extract	1761	N/A
	HBS	663	N/A
	HBS spiked with 10ng/g aflatoxin	556.8	61%
Target	Peanut extract	739	N/A
	HBS	687	N/A
	HBS spiked with 10ng/g aflatoxin	277	59%
Whatman Anatop	Peanut extract	657	N/A
	HBS	676	N/A
	HBS spiked with 10ng/g aflatoxin	169	112%
Whatman CA w/GMF	Peanut extract	1082	N/A
	HBS	643	N/A
	HBS spiked with 10ng/g aflatoxin	215	84%

The assay with the acetonitrile extraction was then repeated using Whatman Anatop filters, as they had been proven to remove the matrix effect, whilst not removing aflatoxin B_1. In this experiment, the Whatman filters removed all NSB, producing a good curve. However, the recoveries for the 20ng/g spike are only 69% and the the aflatoxin was completely lost for the 10ng/g spike. The protocol for peanuts described in the protocol for the Easi-extact columns had recommended a methanol extraction. Therefore, the experiment was repeated replacing the 60% (v/v) acetonitrile/water with 80% (v/v) methanol/water.

The recoveries, however, in this experiment were reading too high. The 20ng/g spike was off the scale, the 10ng/g spike had an apparent recovery of 190%, while the 2ng/g spike had an apparent recovery of 400%. The reasons for this are not known. In order for the assay to be as comparable as possible, all samples and calibrants should be treated in exactly the same manner. One difference between the samples and the calibrants is that all the sample is put through the filters, but the calibrants are only spiked after filtering. The next experiment, therefore, was to spike the blank sample before filtering. Two experiments were carried out. The first had peanut samples spiked at 10ng/g, and second experiment had samples spiked at 10, 5 and 2ng/g.

The first experiment, with a spiked sample of 10ng/g, had a recovery of 74%, which is an improvement on previous experiments. The recoveries for the 10ng/g spike in the second experiment were again approximately 70% and the 5ng/g spike had a recovery of 106%. However the 2ng/g spike had the disappointing recovery of 31%. This would suggest that the sensitivity of the curve is at 5ng/g. This is not sensitive enough as this assay has to have a limit of detection less than 2ng/g because the regulatory limits are at this level. The sensitivity of the assay had to be improved. To improve the sensitivity of an assay the conditions of the experiment can be altered by changing the percentage antibody fraction and the injection time. Previously the sensitivity of the assay had been optimized in an HBS buffer system, and the optimal conditions were an antibody fraction of 30% and an injection time of 8 minutes. However, a calibration curve in buffer will often be different than that produced using sample extract. A sample after extraction will frequently have

some components present that will contribute to some level of matrix effect. Therefore, it is better to create a calibration curve using standards of the sample matrix, which would then account for any matrix effect there is in the sample. In this case the matrix is peanut, and the standard samples were produced by following the same extraction procedure for that of the samples. This is described in *2.3.5d.*

This experiment was therefore repeated using peanut extract. The previous assay conditions had been an antibody fraction of 30% and an injection time of 8 minutes. To improve the sensitivity other conditions were carried out (30% antibody fraction, 10 minute injection time; 30% antibody fraction, 8 minute injection time; 20% antibody fraction, 10 minute injection time; 10% antibody fraction, 8 minute injection time; 10% antibody fraction, 10 minute injection time). The results are shown in Figure 4.5.

The optimization experiment showed that the most sensitive curve, still maintaining a good range, was an antibody fraction of 20% and an injection of 10 minutes. The more sensitive a curve is, the lower the midpoint will be. This is shown in figure 4.5., where there was the largest drop between 0.0 and 0.1ng/g for these conditions compared to the others showing the most sensitivity at this point in the calibration curve.
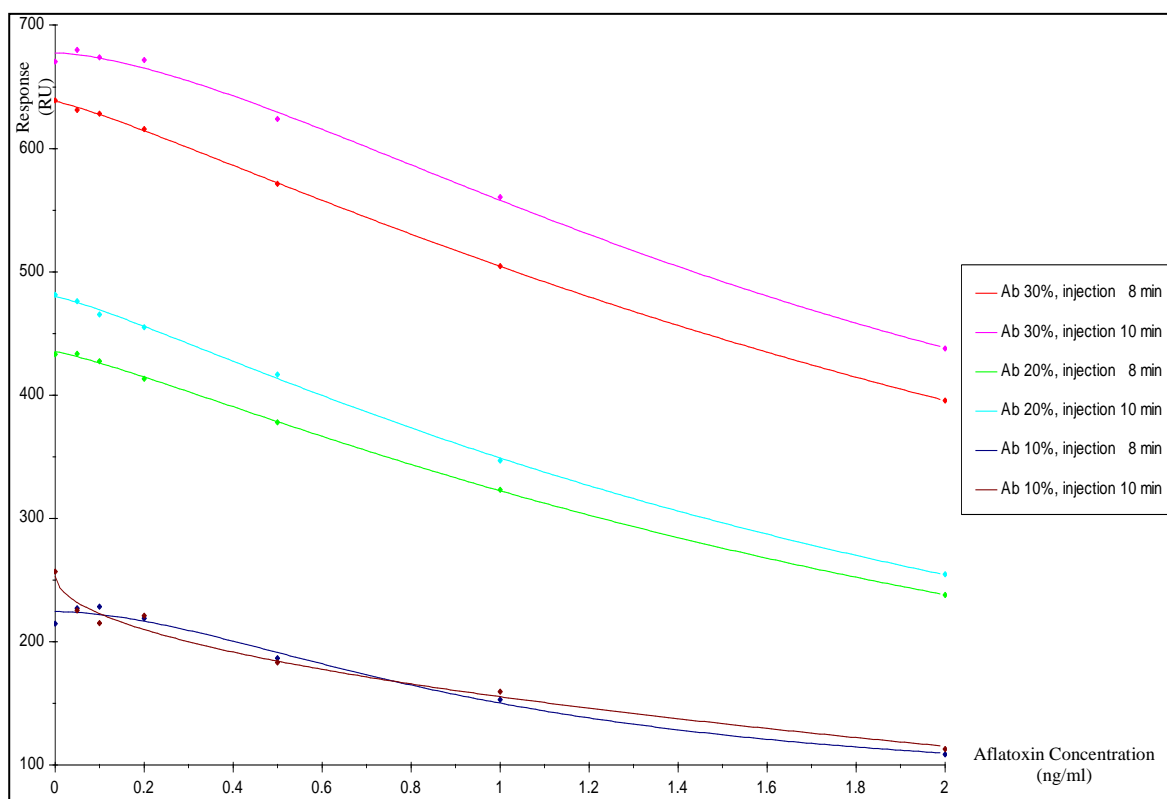
Study:			Sensitivity optimisation					
Curve:			HBS curves					
Ab conc:			1/2500	1/2500	1/2500	1/2500	1/2500	1/2500
Ab fraction:			30 %	30 %	20 %	20 %	10 %	10 %
Injection time:			8 min	10 min	8 min	10 min	8 min	10 min
Injection FR:			20 ul/min	20 ul/min	20 ul/min	20 ul/min	20 ul/min	20 ul/min
AFconc/RelResp:	ng/ml	ng/g	RU	RU	RU	RU	RU	RU
2.00	2.00	10.00	395.8	438.1	238.2	254.9	109.0	113.3
1.00	1.00	5.00	504.7	560.5	323.3	347.2	153.2	159.6
0.50	0.50	2.50	571.6	623.8	377.9	417.0	187.0	183.4
0.20	0.20	1.00	615.7	671.6	413.5	455.3	219.0	221.1
0.10	0.10	0.50	628.1	673.8	427.6	465.4	228.7	215.3
0.05	0.05	0.25	631.5	680.1	433.8	476.4	227.3	225.7
0.00	0.00	0.00	639.2	670.7	433.0	481.5	215.0	256.9
Curve range (RU):			243.4	232.6	194.8	226.6	106.0	143.6
Midpoint (RU):			517.5	554.4	335.6	368.2	162.0	185.1
Midpoint (ng/ml), BIAevaluation:			0.899	1.026	0.876	0.843	0.839	0.490

Figure 4.5 Optimisation of the aflatoxin B$_1$ assay in peanuts.
To improve the sensitivity of the assay, different antibody injection times and percentage fractions were compared using a calibration curve constructed using blank peanut sample.

An assay was set up using these new assay conditions with an antibody fraction of 20% and an injection of 10 minutes to determine if this did in fact improve the sensitivity. The assay used 80% methanol as the extraction solvent, IAC for the clean-up and spiking the calibrants before filtering using the Whatman anotop filters. Results are shown in figure 4.6.
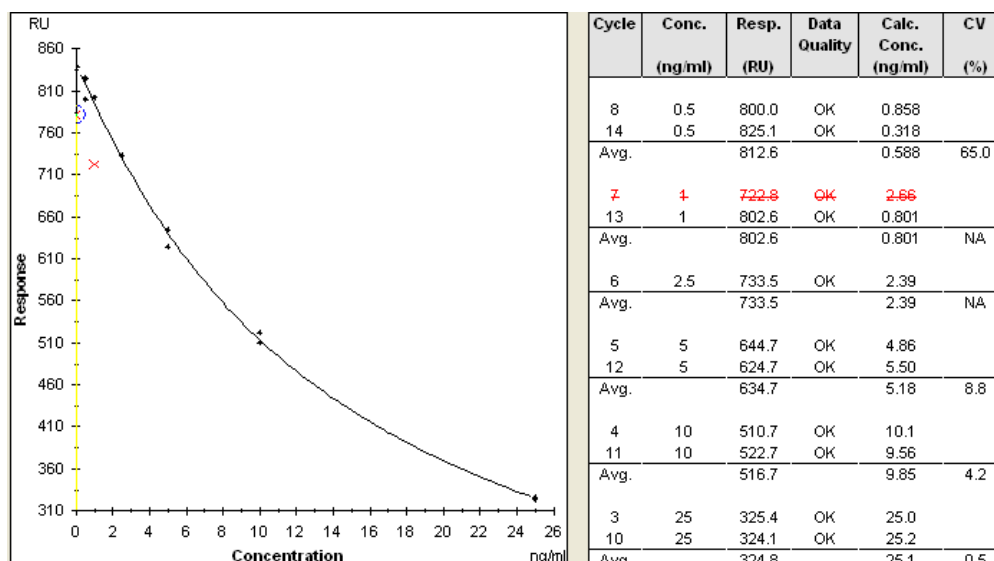
Cycle	Conc.	Resp.	Data Quality	Calc. Conc.	CV
	(ng/ml)	(RU)		(ng/ml)	(%)
8	0.5	800.0	OK	0.858	
14	0.5	825.1	OK	0.318	
Avg.		812.6		0.588	65.0
7	1	722.8	OK	2.66	
13	1	802.6	OK	0.801	
Avg.		802.6		0.801	NA
6	2.5	733.5	OK	2.39	
Avg.		733.5		2.39	NA
5	5	644.7	OK	4.86	
12	5	624.7	OK	5.50	
Avg.		634.7		5.18	8.8
4	10	510.7	OK	10.1	
11	10	522.7	OK	9.56	
Avg.		516.7		9.85	4.2
3	25	325.4	OK	25.0	
10	25	324.1	OK	25.2	
Avg.		324.8		25.1	0.5

Cycle	Sample Id	Amount	Volume	Dil. Fact.	Resp.	Data Quality	Calc. Conc.	Calc. Amount	CV
		(g)	(ml)		(RU)		(ng/ml)	[ng/ml] ▼	(%)
16	2ng/g a			1	803.5	OK	0.783	0.783	
17	2ng/g a			1	796.9	OK	0.925	0.925	
Avg.								0.854	11.8
18	2ng/g b			1	769.3	OK	1.54	1.54	
19	2ng/g b			1	735.2	OK	2.35	2.35	
Avg.								1.95	29.4
20	2ng/g c			1	735.7	OK	2.34	2.34	
21	2ng/g c			1	759.0	OK	1.78	1.78	
Avg.								2.06	19.2
22	5ng/g			1	648.5	OK	4.74	4.74	
23	5ng/g			1	661.7	OK	4.34	4.34	
Avg.								4.54	6.3
24	4ng/g			1	677.6	OK	3.87	3.87	
25	4ng/g			1	713.1	OK	2.91	2.91	
Avg.								3.39	20.1

Figure 4.6 Aflatoxin B$_1$ extraction from peanuts using the new assay conditions of an antibody fraction of 20% and an injection of 10 minutes. 80% methanol was used as the extraction solvent, IAC for the clean-up and the calibrants were spiked before filtering using the Whatman Anotop filters. Apart from the first sample where the recovery was only 42.7%, all other recoveries ranged from 85% to an apparent recovery of 103%.
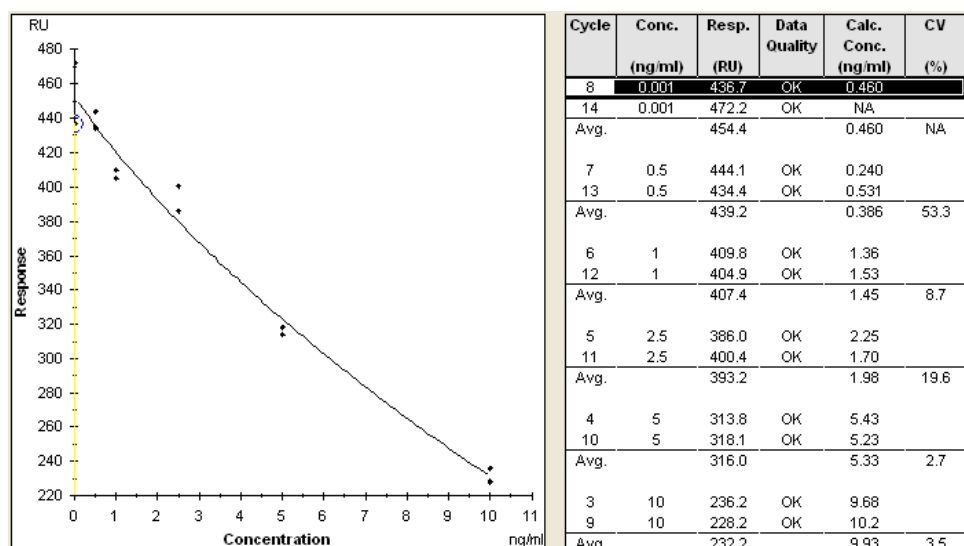
These results indicated that the sensitivity of the assay may have improved and that the assay was potentially more accurate at the lower end of the calibration curve. This is reflected in the recoveries. Although the recovery for the first sample was low, all the other recoveries were over 85%. The reason why the recovery for the first sample is so low is not known.

Table 4.5 Table showing sample recoveries from the Aflatoxin B₁ extraction from peanuts using the new assay conditions of an antibody fraction of 20% and an injection of 10 minutes. 80% methanol was used as the extraction solvent, IAC for the clean-up and the calibrants were spiked before filtering using the Whatman anotop filters. Apart from the first sample where the recovery was only 42.7%, all other recoveries ranged from over 85%.

Sample	Concentration of aflatoxin B_1 in sample (ng/g)	Apparent recovery of sample (ng/g)	Percentage recovery of sample
1	2	0.854	43%
2	2	1.95	98%
3	2	2.06	103%
4	5	4.54	91%
5	4*	3.39	85%

*Sample 5 (4ng/g) was two 2ng/g samples passed through the same column.

European sampling laws for aflatoxin B_1 states that the minimum sample size is 50g. Therefore, if this assay was to be developed into a diagnostic kit, the sample size must be increased from 1g to 50g. To test whether the assay would still be effective using the larger sample size it as scaled up by 50 times, where 50g of peanuts were spiked at 10ng/g and extracted using 500ml of 80% methanol. After incubation for 30 minutes and centrifugation at 2000g, 2ml of the solvent was removed and the assay was repeated (as described in 2.3.5d).

Cycle	Conc. (ng/ml)	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	CV (%)
8	0.001	436.7	OK	0.460	
14	0.001	472.2	OK	NA	
Avg.		454.4		0.460	NA
7	0.5	444.1	OK	0.240	
13	0.5	434.4	OK	0.531	
Avg.		439.2		0.386	53.3
6	1	409.8	OK	1.36	
12	1	404.9	OK	1.53	
Avg.		407.4		1.45	8.7
5	2.5	386.0	OK	2.25	
11	2.5	400.4	OK	1.70	
Avg.		393.2		1.98	19.6
4	5	313.8	OK	5.43	
10	5	318.1	OK	5.23	
Avg.		316.0		5.33	2.7
3	10	236.2	OK	9.68	
9	10	228.2	OK	10.2	
Avg.		232.2		9.93	3.5

Cycle	Sample Id	Amount (g)	Volume (ml)	Dil. Fact.	Resp. (RU)	Data Quality	Calc. Conc. (ng/ml)	Calc. Amount [ng/ml]	CV (%)
17	2ng/g b			1	406.3	OK	1.49	1.49	
18	2ng/g b			1	391.5	OK	2.04	2.04	
Avg.								1.76	22.2
19	2ng/g c			1	393.1	OK	1.98	1.98	
20	2ng/g c			1	396.5	OK	1.85	1.85	
Avg.								1.91	4.8

Figure 4.7 Aflatoxin B$_1$ extraction from peanuts using an increased sample size of 50g. 80% methanol was used as the extraction solvent, IAC for the clean-up and the calibrants were spiked before filtering using the Whatman anotop filters.

The results of the experiment using the increased sample size of 50g were promising. The apparent recoveries of the samples spiked with 2ng/g of aflatoxin B$_1$ were 1.76 and 1.91ng, which are 88% and 96%, respectively (Figure 4.7). However, the percentage coefficient t of variation between some of the points in the calibration curve were high (53.3 for the 0.5ng spike). Ideally these should be below 10.   The reason for this was because the trendplot of the baseline had risen over the course of the experiment (figure 4.8).   The baseline trendplot records the response after the regeneration solution has passed over the surface.
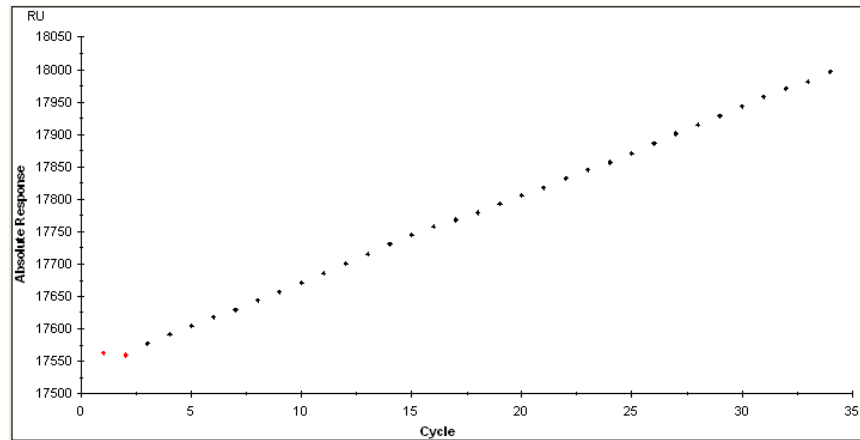
Figure 4.8 Baseline Trendplot.
This shows that the baseline has steadily increased by apporoximately 400RU over the
course of the experiment. This is due to a build up of material on the sensor ship surface
which will interfere with the efficacy of the assay.

An explanation as to why the baseline is increasing is that there is a steady build up of
material on the surface of the chip, which is not being removed by the regeneration
solution. This, in turn, was interfering with the assay. One way to resolve this would be to
optimise the regeneration solution. Therefore, although the results seem promising, further
optimisation of the assay was still required.

Chapter 5: Discussion and Conclusions

5.1 Aflatoxin B_1 Detection and Quantification

Aflatoxin B_1 is a mycotoxin, a toxic secondary metabolite produced from the toxigenic filamentous fungi *Aspergillus flavus* and *Aspergillus parasciticus*. Aflatoxin B_1 is produced in hot and humid tropical conditions and is known to contaminate a wide range of crops, including nuts (peanuts, almonds, pistachio, walnuts and brazil nuts), cereals (maize, wheat, rice, oats, sorgam) and spices (chilli, black pepper, turmeric, ginger).

Aflatoxin B_1 is highly toxic and primarily targets the liver and has a positive association with human hepatocellular carcinoma. Aflatoxin B_1 contamination can occur at anytime throughout the farming process, including storage and transportation, which makes aflatoxin contamination a worldwide problem. Therefore, approximately 100 countries have imposed regulatory limits for aflatoxin B_1 in order to limit exposure. The limits that have been set for aflatoxin B_1 are low, primarily due its possible carcinogenic effects.   The lowest regulations that have been set are to protect the most susceptible to aflatoxin B_1 toxicity, and these are for infant formula, which was set by the EU in 2006 at 0.05ng/g.

The imposture of strict regulatory limits for aflatoxin B_1 has necessitated the development of reliable, sensitive analytical methods to detect and quantify aflatoxin B_1. Several official regulatory organisations, for example Association of Official Analytical Chemists (AOAC), the International Union of Pure and Applied Chemistry (IUPAC), International Organisation for Standardisation (ISO), and its European counterpart, the European Committee for Standardisation (CEN), have validated numerous methods. Many of these methods use a chromatographic technique, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), liquid chromatography with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS).   Although these methods are sensitive enough to detect aflatoxin B_1 to the regulatory limits, they are not without disadvantages. All use large quantities of hazardous solvents and require skilled training to execute. They are also time consuming and labour intensive, and are therefore not suitable for testing large numbers of samples. They are also very expensive.

These disadvantages have necessitated the development of new diagnostic techniques for aflatoxin B_1. The assays must be reliable, simple to use and sensitive enough to detect to the lowest regulatory limits. They have to be sufficiently robust to use on the many different types of food affected by aflatoxin B_1, have a high-throughput capacity, be able to test many samples quickly and accurately, and they must also be cost effective.

The purpose of this study was to develop a diagnostic kit for aflatoxin B_1 using Surface Plasmon Resonance (SPR). SPR is an innovative optical technique that measures biomolecular interactions on the surface of a sensor chip. The biomolecular interactions are between the analyte of interest (in this case aflatoxin B_1) and its binding partner. In this assay the binding partner is an antibody. The association and disassociation of aflatoxin B_1 with its antibody can be followed in "real-time" on a graph called a sensorgram. Other advantages of SPR are that it is label-free, it is high-throughput, its associated software is windows-led and straightforward to use, it does not use large volumes of solvents and is both accurate and sensitive.

5.2 Assay development and feasibility

To determine the feasibility of an assay several aspects have to be considered. The first was the format of the assay. When the molecule to be tested is small (less than 312 Da), as is the case for aflatoxin B_1, the assay has to be an inhibition assay as the change in resonance when the molecule binds to the surface would otherwise be negligible.

The second parameter was the efficacy of the chip surface. In an inhibition assay, the analyte or a derivative of the analyte is immobilised onto the chip surface. The analyte or derivative may not be orientated correctly (i.e. with its epitope easily accessible) on the

surface, and therefore the surface should be tested by performing an Rmax (section 3.3). This is when antibody is passed across the surface in large excess. If the immobilisation is successful, the response should be several thousand RU. In the case of the aflatoxin B_1 assay, the most effective surface was with the aflatoxin B_1-oxime-ethylenediamine derivative immobilised.

Thirdly, the assay should work in a buffer system. Although the Rmax may indicate that there is binding of the antibody to the surface, a standard curve has to be generated to enable analyte quantification. A curve was produced for the aflatoxin B_1 assay in an HBS-EP buffer system using aflatoxin B_1 standards (0, 0.5, 1.0, 2.5, 5.0 and 10ng/g of aflatoxin B_1).

Finally, the sensitivity of the antibody is extremely important. Regulatory limits for aflatoxin B_1 have been set as low as 0.05 ng/g. These limits were set by the EU for infant formula. For the assay to be feasible the antibody must be sensitive enough to detect aflatoxin B_1 to these levels. Several antibodies were evaluated for this assay. The most sensitive was found to be the Fab antibody, G6.

5.3 Aflatoxin B_1 assay with compound feed (IRMM Reference Material) as the sample matrix

The first matrix that was investigated was compound feed as it could be bought as a certified reference material from Sigma Aldridge. Two samples were purchased, one that had been certified to contain no aflatoxin B_1 (blank) and another containing 9.3ng/g aflatoxin B_1.

A simple methanol extraction was first attempted. There was, however, too much interference from the sample matrix and a calibration curve using blank extracted sample could not be generated. To remove the interference, a solid phase extraction column, "Mycosep" from Romer Laboratories was used in the sample clean-up. Unfortunately,

there was still non-specific binding of the matrix onto the chip surface, and a calibration curve could not be produced.

The immunoaffinity columns "Easi-extract Aflatoxin" from R-Biopharm Rhone were then evaluated. Their use reduced the interference and a calibration curve was generated. Also, the recoveries for the certified compound feed sample were excellent. The compound feed had been certified to contain 9.3ng/g aflatoxin B_1. When read off the calibration curve the compound feed appeared to contain 9.35ng/g. This was a recovery of 100.5%. The experiment was repeated, and again the recoveries were excellent at 98%.

The major disadvantage of using immunoaffinty columns, however, is that they were very expensive, especially since it was proven that they could not be re-used. Cheaper "Aflatest" IAC columns, were therefore sourced from Vicam. However, their performance was disappointing. Although the matrix effect was again removed, the recoveries were poor at only 50%.

Overall, the preliminary assays using compound feed were promising. The feasibility of the assay, extracting Aflatoxin B_1 from a real sample, had been demonstrated. However, there were problems with interferences from the matrix resulting in non-specific binding to the Biacore chip surface. The most effective way of resolving this problem was to use immunoaffinity columns. The use of these columns, however, would result in assay costs that were potentially prohibitive.

5.4 Aflatoxin B_1 assay with infant formula as the sample matrix

The next matrix chosen for investigation was infant formula. The lowest regulatory limits for aflatoxin B_1 in any food have been set for infant formula by the EU at 0.05ng/g. There are no rapid methods on the market at present that are sufficiently sensitive to detect aflatoxin B_1 to these levels. This would be a unique selling point for the kit, and for this reason infant formula was chosen as the matrix to investigate.

Simple extraction procedures were first examined. Unfortunately, as previously found with compound feed, there were problems with matrix interference. Infant formula has a high fat content, and it was perceived that it may be the fat that is binding to the surface of the chip. A cyclohexane step was introduced to remove the fat from the sample. The non-specific binding to the chip surface was removed and a calibration curve generated, but the recoveries, however, were poor at approximately 10%. In addition, the aflatoxin B_1 seemed to be removed from the sample along with the fat.

Further attempts to reduce matrix interference were carried out by precipitating out proteins. These too resulted in low recoveries.

Promising results had been achieved for compound feed using "Easi-extract aflatoxin" immunoafinity columns. Therefore, these columns were utilised in the clean-up of the assay for infant formula. Recoveries were satisfactory for samples spiked with aflatoxin B_1 at 5ng/g (the average recovery was 78%). The recoveries of samples spiked at 1ng/g, however, were erratic. This was because the sensitivity at this point of the curve was poor. Unfortunately, with regulatory limits of 0.05ng/g for infant formula, the sensitivity of the curve at this point must be better in order to detect and quantify accurately. However, despite very significant efforts, it was not possible to achieve a calibration curve sensitive enough to test for aflatoxin B_1 in infant formula to the regulatory limits. The assay, therefore, was obsolete, and it was decided to focus on a different matrix (or matrices) that had regulatory limits for aflatoxin B_1 within the performance characteristics of the assay.

5.5 Aflatoxin B_1 assay with maize as the sample matrix

The infant formula and compound feed assays both had problems with matrix interference binding to the surface of the chip. Unlike infant formula, maize does not have a high fat content which was the possible cause of non-specific binding to the chip surface. Compound feed is composed of up to 30 different ingredients, including vitamins, minerals, fermentation products e.g. ash and antibiotics, which all could cause matrix effects. Maize was chosen to investigate primarily because it was thought that it, out of all other matrices,

it might have the least problems with matrix effects. The reduction in matrix effects would therefore negate the need for immunaffinity columns.

With maize the AOAC recommends a 55% (v/v) methanol/water extraction followed by a hexane step and then a chloroform extraction. Again, however, matrix interferences gave rise to a background that was too high. This matrix effect was still evident even after an additional clean-up step using "Easi-extract aflatoxin" immunoaffinity columns. Maize is composed of 62% starch, which could be the root cause of the matrix effect. Therefore, an amylase treatment step was introduced to the method in an attempt to remove non-specific binding. After amylase treatment, there was an 80% (v/v) methanol/water extraction followed by clean-up using the "Easi-extract aflatoxin" immunoaffinity columns. This additional step did reduce the background from 1760RU to 1280RU, but this is still approximately double the background with buffer alone. In spite of this, a calibration curve was still generated and the recovery for a 20ng/g aflatoxin B_1 spiked sample was 111%.

The purpose of the immunoaffinity column was to remove all matrix from a sample producing a pure and concentrated solution of aflatoxin B_1. Unfortunately we found that in the case of maize that some interference was still remaining in the sample that bound non-specifically to the sensor chip surface.   The assay was therefore problematic. There was pressure to produce a marketable assay as quickly as possible and so a new matrix, peanuts, was chosen to investigate.

5.6 Aflatoxin B_1 assay with peanuts as the sample matrix

The final matrix examined in this study was peanuts. "Easi-extract aflatoxin" immunoaffinity columns were used for sample clean-up to remove matrix interferences. Two extraction methods were recommended by the immunoaffinity column kit, 80% (v/v) methanol/water and 60% (v/v) acetonitrile/water. The methanol extraction still resulted in a high degree of matrix non-specific binding. Although the background with the acetonitrile extraction was reduced, the recoveries were poor at approximately 24%. There was also a difference between the backgrounds of samples that had been filtered compared

to the backgrounds of unfiltered samples. An evaluation of six different filters was carried out, first on their effectiveness on removing matrix effect and secondly on whether or not any aflatoxin B_1 was removed from the sample and sequestered by the filter. There were huge differences between the filters. Some removed all matrix interferences , whilst others only removed half (see table 4.5.1). Some of the filters also removed all of the aflatoxin B_1. The best performing filters were the Whatman Anotop filters, which removed all of the non-specific binding but none of the aflatoxin B_1.

The peanut assay was repeated using these filters. A methanol extraction was performed before clean-up using immunoaffinity columns. Also, the calibrants were spiked before extraction. Three different spiked samples were assessed. They were spiked at 10, 5 and 2ng/g. The recoveries were 70%, 106% and 31%, respectively. This would suggest that the sensitivity of the calibration curve is at 5ng/g as the readings were most accurate around this point. The regulatory limits set for aflatoxin B_1 in peanuts is 2ng/g. Therefore the assay was not sensitive enough.

The assay conditions were optimised to improve the sensitivity of the calibration curve. The assay conditions were altered slightly, which greatly improved the recoveries of the samples spiked at 2ng/g. Although the first sample had a recovery of only 43%, the other two samples spiked at 2ng/g had recoveries of 98% and 103%. For all three samples, this had an average recovery of 81%.

Sampling laws for aflatoxin B1 state that the minimum sample size is 50g. Keeping all conditions the same, the assays were scaled up from 1g to 50g of peanuts. In this assay two 50g samples were spiked at 2ng/g. The recoveries were excellent at 88% and 96%.

5.7 Comparison of assay with current market leaders

The global market share for mycotoxin testing is estimated at approximately £75 –100 million, with rapid diagnostic methods accounting for 10 – 20% of this (Council for Agricultural Science and Technology (CAST) report, 2003). This is a fairly new market

and, therefore, the average annual growth is high at 10%. Aflatoxins, and aflatoxin B_1 in particular, are the best known and studied mycotoxins and are proven to be the most carcinogenic. Therefore, most mycotoxin diagnostic kits on the market are for aflatoxin B_1 or total aflatoxins. They also have the most complete regulatory limits compared to other mycotoxins.

There are, however, already a number of competitors on the market, but due to changing legislation demanding increased testing and the ability to detect lower levels, this sector is growing fast. There is, therefore, a niche in the market for high-throughput, automated systems that is sensitive enough to meet the demands of the lowering regulatory limits.

Aflatoxin B_1 testing generally falls into two groups. The first is analytical chromatographic methods. These methods, such HPLC, TLC, HPLC/MS and GC/MS are reliable, sensitive and quantitative. These conventional analytic methods, however, are time consuming, labour intensive and require skilled training. They also require high consumption of hazardous solvents, and, so, are often expensive. These tests would be too expensive and time consuming to analysis large numbers of samples, but can be used as confirmatory tests.

Table 5.1 Table showing analytical laboratory techniques for aflatoxin analysis and their relative advantages and disadvantages

Method	Advantages	Disadvantages
TLC	Simple Cheap Rapid	Separation may be unsatisfactory Poor precision Needs confirmation
HPLC	Sensitive Selective Easy to automate	Compounds may require derivatisation Method requires skilled end-user Expensive
HPLC/MS	Provides high level of confirmation Multi-analyte detection Very sensitive	Expensive Specialist expertise required.
GC/MS	Very sensitive	Expensive Specialised expertise required Compounds must be volatile.

The second type of aflatoxin B_1 tests are antibody-based. These diagnostic tests, such as ELISAs and lateral flow tests ("dipstick" tests), are now taking over the mycotoxin testing market. They are commercially available in a kit form and are a simpler, quicker and cheaper means to routinely monitor aflatoxin contamination. The main disadvantage of immunological kits is that they are not quantitative and therefore the results may still require confirmation by an analytical method, such as HPLC. Other disadvantages are false positives or false negatives and they may have matrix interference problems.

There are currently a number of these kits on the market. The main market leaders are r-Biopharm, Romer, Diffchamp, Neogen and Charm, who between them share 60 –70% of the market share.

Romer (www.romerlabs.com)

Romer Labs is a world leader in the development of mycotoxin test kits. It has a wide and comprehensive portfolio of different tests for aflatoxins, each designed for specific analyses, depending on the number of samples to be analysed, the matrix or whether or not it is to be quantitative of qualitative. All assays are both AOAC and USDA/GIPSA approved. They currently have four kits on the market that test for aflatoxins.

i)   AgraQuant ELISA kits for total aflatoxin

This kit tests for total aflatoxins in grain, nuts, cottonseeds, cereals and other commodities such as animal feeds. It has two quantitation ranges, 1-20ppb and 4-40ppb (with limits of detection 1 and 3ppb, respectively).

ii)   FluoroQuant Total Aflatoxin test kit

FluoroQuant is a rapid, quantitative fluorometric test kit for total aflatoxin specially formulated for the needs of the grain industry. It has a limit of detection of 1ppb and can be used to test corn, wheat, soybeans, raw peanuts, rice and cottonseed.

iii)   AflaCup Kit for total aflatoxins

AflaCup is a test designed for single sample testing. It has detection level of 10ppb, but is a qualitative test, giving only yes/no answers.

iv)   AgriStrip lateral flow kit

This a one step lateral flow immunochromatographic assay to determine the presence of aflatoxins B1, B2, G1 and G2. The strips are designed for *in-situ* testing at grain elevators and peanut buying points.   The strips have cut off levels at 4, 10 and 20 ppb. Again, this is a qualitative test, giving only yes/no answers. The test involves comparison by eye, so results would be somewhat open to interpretation.

Charm (www.charm.com)

Charm Sciences have recently achieved approval from the USDA/GIPSA for their aflatoxin test.   No other test has ever received approval for such a comprehensive list of commodities. These are corn, corn flour, corn germ meal, corn gluten meal, corn meal, corn screenings, corn soy blend, cracked corn, distillers dried grains, flaking corn grits, milled rice, popcorn, rough rice, sorghum, soybeans and wheat. The procedure includes a sample extraction and a ten minute incubation with a test strip. The strip is then inserted into a reader, which then displays and records the reading. The limit of detection is 2ppb, and the range is 0-100ppb.

R-Biopharm Rhone (www.r-biopharm.com)

In addition to marketing several IAC for aflatoxin sample clean-up, R-Biopharm Rhone also produce kits for aflatoxin detection and/or quantification. They currently have two ELISAs on the market to test for aflatoxins.

i)   AflaPlate

This is a quantitative ELISA for the analysis of aflatoxin B_1. It has a limits of detection of 1ppb and the test matrices include maize, nuts and animal feed.

ii)   Aflacard B_1 and Aflacard total

These are qualititative screening cards for the detection of either aflatoxin B_1 or total aflatoxins, working in the same was as a lateral flow immunoassay. They have a detection limit of 2ppb and gives a yes/no answer by way of a colour change, and, so, the results are open to interpretation.

Diffchamp

Diffchamp also has an ELISA on the market that tests for and quatifies aflatoxin B_1. It tests matrices such as cerals, peanuts, maize, wheat, nuts, figs, spices, tea, cocoa and animal feeds. It has the lowest detection limit on the market of 0.5ppb and the test can also be automated using the transia Elisamatic II.

The number of competitors is high, but there is still a niche in the market that necessitates the development of this assay using the Biacore SPR system. The main selling point Xenosense has for this kit is product innovation, and it will be the only kit on the market that is fully automated. This will allow analysis of large numbers of samples and in a short time. The hope is that the final assay will be more reliable and robust than other quantitative techniques, with little inter or intra laboratory variation in results due to the reduction in operator handling of samples.

Aflatoxin assays have been developed on other analysers, harnessing different technologies, for example optical waveguide lightmode spectropscopy (Adanyi *et al*., 2007), sol particle immunoassay (Brenn-Struckhofova *et al*., 2007), fluorescence polarisation (Nasir *et al*.,

2002), affinity electrochemistry (Mascini *et al*., 2001), fluid based-bioaerosols and surface plasmon resonance (SPR) (Daly *et al*., 2000; Maragos *et al*., 2002; Dunne *et al*., 2005). However, only Biacore has the capability of producing and manufacturing commercial kits for the assay. Thirteen kits are presently on the market that test for either vitamins or drug residues using the Biacore system. Xenosense, with its current repertoire of kits, therefore, has a strong customer base, and some of these customers have also expressed an interest in the Aflatoxin B_1 kit. Customers include industrial end users in the food industry, such as Nestle, Fonterra and Wyeth Nutrition; and regulatory end users, e.g. Livmedelsverket (Sweden), Department of Livestock Development (Thailand), Central Sciences Laboratories (UK) and Teagasc (Ireland). These customers will have already bought the biosensors to use with either vitamin or drug residue kits.

5.8 Conclusions

The purpose of this study was to develop such an assay that combines the use of innovative technologies of surface plasmon resonance and recombinant antibody manipulation. The hope was to develop a user friendly assay that could be marketed as a kit for use on the Biacore$_{TM}$ Q biosensor.

Four different types of food matrix where investigated – compound feed (IRMM certified material), infant formula, maize and peanuts. The first material investigated, compound feed, proved that the assay was feasible in this matrix. The certified reference material used in the assay had recoveries of over 98%.

The infant formula assay was disappointing. The regulatory limits for infant formula are the lowest limits that have been set for aflatoxin B_1 and were set by the EU at 0.05ng/g. At present there are no rapid tests on the market that can detect aflatoxin B_1 to these levels. This would be the unique selling point of this kit. Unfortunately, the assay was not sensitive enough to detect and quantify aflatoxin B_1 to 0.05ng/g.

The third matrix examined was maize. This assay proved problematic due to matrix interference. There was non-specific binding of the maize matrix to the chip surface despite the use of immunoaffinity columns.

The most promising matrix investigated was peanuts. Recoveries of over 80% were achieved even when the whole process was scaled up to test aflatoxin B_1 in sample sizes of 50g. However, further optimisation of the aflatoxin B_1 assay in peanuts was still required. The trendplot for the baseline showed there was a steady increase as the assay progressed. This is indicative of build-up on the chip surface showing that the regeneration stage is not stringent enough. Addition research into different regeneration solutions and regeneration injection times is required before going to the validation stage.

The assay has two major disadvantages. The disadvantages are the labourious extraction process and the reliance on immunoaffinity columns. Simple extraction procedures were attempted, but all four foodstuffs in this study had problems with matrix effect causing non-specific binding to the chip surface. This resulted in backgrounds so high that either the required calibration curves could not be generated or the true recoveries were masked. Immunoaffinity columns are extremely expensive and cannot be re-used. Although the use of the columns could be recommended along with the kit they would cause the price per analysis to become too high and I found that despite the use of the columns the extraction procedure was still very time consuming. This would defeat two of the fundamental goals of the kit, i.e. that it should be more cost effective and it should be high-throughput. The only solution to this problem may be to improve the chip surface. This, however, would require the investigation to start again at the feasibility stage. Unfortunately, this was beyond the scope of this project. The reason for this was that, unfortunately, due to the economic downturn, Xenosense Ltd. ceased operating. Xenosense had been bought over by Biacore, which, in turn had been purchased by GE Healthcare. Global loses by GE Healthcare necessitated closures of many departments, including Xenosense.

Overall, however, this study has shown that an aflatoxin B_1 assay is possible using a Biacore Q biosensor. Two matrices have shown great promise – peanuts and compound

feed. Although some areas of the assay need further optimisation, namely the improvement of the chip surface and the development of a simpler extraction process, the investigation so far has proved feasibility. Following optimisation, the next stages of the research would be validation of the assay.

Chapter 6:
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