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DEVELOPMENT OF AN AFFINITY SENSOR FOR THE DETECTION OF AFLATOXIN M_1 IN MILK

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ABSTRACT

Much research has been done on aflatoxins since their discovery in the 1960's where it was concluded that aflatoxins have carcinogenic, mutagenic, teratogenic and immunosuppressive properties. Aflatoxin M_1 exists in milk and since milk is a major component of the diet of infants, the maximum permissible limit set by the EU is 50 parts per trillion (ng L^{-1}).

Current methods of analysis for aflatoxin M_1 is primarily based around techniques such as HPLC and TLC which require extensively trained operators and equipped laboratories. Using antibodies as receptors in an enzyme linked immunosorbent assay (ELISA), the analysis costs can be reduced and simplified, however, an equipped laboratory is still required. Hence there is a need for a low cost, rapid, portable instrument which is easy to use at the point of source for the detection of aflatoxin M_1.

This thesis describes the development of affinity sensors to meet these requirements. Firstly the design and optimisation of an ELISA method was carried out, utilising a commercially sourced monoclonal antibody.

Once the antibodies suitability for sensing aflatoxin M_1 was determined the antibody was successfully employed as the receptor for a screen printed HRP/TMB based immunosensor. Upon the analysis of milk it was observed that milk caused extensive interference and through a series of chemical extractions the interference was attributed to whey proteins in the milk with suspicion towards α-lactalbumin. A simple pre-treatment technique of adding calcium chloride was performed and the interference from the whey proteins was removed. The resulting immunosensor achieved a sensitivity of 39 ng L^{-1} (Figure 3.26), however, poor reproducibility was observed due to the screen printed electrode production (%CV = 21% variance for screen printed electrode production).

Gold cell on a chip microelectrode arrays were used to replace the screen printed electrodes and the successful covalent attachment of the antibody to the microelectrode array through PDITC cross linking compound was monitored using atomic force microscopy and scanning electron microscopy. It was shown that the majority of the antibodies during immobilisation orientate in a 'side on' orientation and therefore a cheap capture polyclonal antibody was first immobilised before the addition of the sensing anti-aflatoxin M_1 monoclonal antibody. Using the microelectrode array an improvement of the sensitivity as well as a reduction of the milk interference was shown. Sensitivity was improved to 8 ng L^{-1} in milk (Figure 4.23).

Further work was performed to substitute the fragile antibody used in the sensing layer for a robust synthetic peptide receptor. Initially a virtual library of synthetic peptides was created using *de novo* design techniques *in silico*. Further computational techniques were performed to determine the best peptide from the library. This peptide had a sequence of PVGPRP. From literature a peptide (LLAR) was reported with affinity for aflatoxin B_1. This sequence along with the *de novo* design peptide was synthesised and tested using a host of techniques and immobilisation chemistries such as optical waveguide lightmode spectroscopy (OWLS), BIAcore and enzymatic techniques using EDC/NHS, glutaraldehyde and BS^3 cross linking methods. The affinity of both peptides to aflatoxin M_1 was demonstrated however further work is required to quantify the affinity and to incorporate the peptides into the microelectrode array.

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Special thanks to Elin Karlsson who loving encouraged me and gave me enthusiasm and determination whenever my attention waned.

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NOTATION

AAL	*Alternaria alternata* f. sp. *Lycopersici*
AFM	Atomic force microscopy
AFM1	Aflatoxin M_1
AOAC	Association of analytical communities
BLM	Bilayer lipid membrane
BSA	Bovine serum Albumin
CAS	Chemical abstracts service
DON	Dioxynivalenol
EILSA	Enzyme-linked immunosorbent assay
FDA	The US food and drug administration
FEP	Free energy perturbations
GHz	Gigahertz (1×10^9 Hertz)
HDME	Hanging drop mercury electrode
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IgG	Immunoglobin G
IUPAC	International union of pure and applied chemistry
Kcal	Kilo calories (1×10^3 calories)
kDa	Kilo Daltons
Kg	Kilogram (1000 g)
LC	Liquid chromatography
MHz	Megahertz (1×10^6 Hertz)
MIP	Molecularly imprinted polymer
MS	Mass spectroscopy
mV	Millivolt (1×10^{-3} volts)
nm	Nanometer (1×10^{-9} meters)
NMR	Nuclear magnetic resonance

OWLS	Optical waveguide light spectroscopy
PBS	Phosphate buffered saline
PDB	Protein Databank
pNPP	p-Nitrophenyl phosphate
ppb	Parts per billion (1×10^{9}), $\mu g\ L^{-1}$
ppm	Parts per million (1×10^{6}), $mg\ L^{-1}$
ppt	Parts per trillion (1×10^{12}), $ng\ L^{-1}$
PVA	Polyvinyl alcohol
PVPP	Polyvinyl pyrrolidone
SEM	Scanning electron microscopy
SPE	Solid phase extraction
SPR	Surface plasmon resonance
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
VdW	Van de Waals (energy or forces)

Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW.

1.1. History of mycotoxins.

The term mycotoxin is applied to a group of toxic secondary metabolites produced by fungi. The word mycotoxin is derived from the Greek language where *mekes* translates to mushroom and *toxikon* for toxic (Waring, 2002) or poison (Rustom, 1997). Since Roman times the knowledge that some fungal products can cause illness (mycotoxicoses) has been known.

One of the first recorded cases of mycotoxicoses occurred in 1722 when a large Russian army was gathered on the delta of the river Volga at Astrakhan under the direction of Peter the Great. During the armies formation the men were given rye flour and the horses were fed on grain and hay. Within a short period of time after consumption, the men and horses were struck by paralysis and suffered a 'fiery itch.' Thousands of men died before reaching battle with the Turkish armies. The mould *Claviceps purpurea* was the cause of the symptoms now named St. Anthony's fire or Holy Fire (Farrer, 1987).

In 1960 there was significant research into the causes of mycotoxicoses due to a mysterious new disease that swept through England which killed over one hundred thousand turkeys. This occurrence was given the name turkey 'X' disease (Bradburn *et al.*, 1994; Sargeant, 1961). The fatalities were not limited to turkeys; also ducklings and young pheasants were affected. After extensive investigation into the deaths, a link was observed that the feed had come from the same shipment of peanut meal from Brazil (Daly *et al.*, 2000) which had become mouldy during transport. Further investigations showed that the peanut meal was heavily contaminated with the organism '*Aspergillus flavus*' hence the name *Afla*toxin (Sargeant, 1961) and that the poultry died from liver cancer since the aflatoxins were highly carcinogenic.

The detection method used was thin layer chromatography (TLC). During analysis of the peanut meal four dots would appear on the paper, when illuminated with an ultra violet light, two dots would emit a blue light and two would emit a green light,

hence the structures of the aflatoxins are known as B_1, B_2 and G_1, G_2. In 1966 it was observed that cows that were fed on a diet contaminated with aflatoxin B_1 and B_2 would produce milk contaminated with new aflatoxins M_1 and M_2, denoted M for milk (Holzapfel & Steyn, 1966).

The connection with aflatoxin and poultry gave concerns into human consumption of mouldy foods. Investigations were made into the high levels of liver cancer found in Uganda and Swaziland (South Africa). When measuring the aflatoxin levels of about 500 samples of local food the levels found were about 100 μg Kg^{-1} (current European Commission limits for total aflatoxin are between 4 and 10 μg Kg^{-1} depending on the foodstuff). Later a study in Thailand showed similar levels for aflatoxins in normal foods but levels of 3,000 μg Kg^{-1} and 12,000 μg Kg^{-1} were found in corn and peanuts respectively (Waring, 2002).

In northwest India further information was discovered about the onset of *Aspergillus flavus*. During the summer of 1974 there had been chronic drought conditions affecting the maize crop, in the harvest time unseasonable rains occurred resulting in the corn being stored at high humidity. Upon consumption of the corn it was reported that nearly 400 people suffered from fevers and jaundice and 108 people died. The suspicions that the corn had caused the disease were verified by the village dogs similarly suffering with the same symptoms. Analysis of the corn showed that the levels of aflatoxin being consumed were between 2,500 μg Kg^{-1} and 15,600 μg Kg^{-1} whereas a survey of the following year's crop showed levels of less than 100 μg Kg^{-1} (Waring, 2002).

Another episode occurred in Kenya in 1981 where again chronic drought conditions and then early rains hampered the maize crop, 20 people complained of light fever and abdominal discomfort. They were admitted to hospital with jaundice and were feeling very weak. It was diagnosed that their livers were very tender.

Only 8 people made a recovery. As in India the dogs also became ill as well as the woodland birds.

1.2 The Mycotoxin family.

Over 190 moulds have been found to be able to produce toxins. In some cases more than one mould can produce the same toxin (Gilbert, 2002; Moreau, 1979). The main moulds families of concern are;
Fusarium, Aspergillus, Penicillium, and *Alternaria*. Table 1.1 shows the mycotoxins that these moulds produce.

Table 1.1: Moulds responsible for producing mycotoxins.

Mycotoxin	**Mould**
Aflatoxin	*Aspergillus*
Ochratoxin	*Penicillium*
	Aspergillus
Patulin	*Penicillium*
	Aspergillus
	Byssochylamys
Trichothecenes	*Fusarium*
Zearalenol	*Fusarium*
Fumonisins	*Fusarium*
AAL	*Alternaria*

1.2.1 Ochratoxin.

Ochratoxin can be produced by *Aspergillus ochraceus*, A*spergillus ostianius* and *Penicillium verrucosum*. Generally *Aspergillus* occurs in warmer climates where as *Penicillium* occurs in cooler climates. Ochratoxin mainly contaminates grains such as barley, corn, wheat, rye and oats and therefore products manufactured from grain, for example beer. Ochratoxin can also be found in beans, figs, olives, nuts, coffee and spices as well as grapes and grape based products such as wine. Ochratoxin A (Figure 1.1) is the main form of this toxin and has a molecular weight

of 403.8 Daltons. Ochratoxin B also exists, however it is rarer and at least one order of magnitude less toxic (Petzinger & Zeigler, 2000). Ochratoxin A has an unusually long serum half-life resulting in a high occurrence in human serum. Its half-life is 840 hours; hence from a single uptake, Ochratoxin A would still be detectable for 280 days. The European Commission maximum permissible limits for this toxin are between 1 and 10 μg Kg^{-1} depending on the foodstuff (EMAN, 2000).

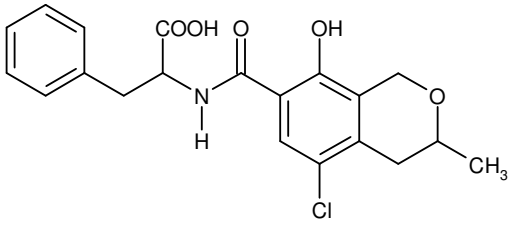


Figure 1.1: Structure of Ochratoxin A.

1.2.2 Patulin.

Patulin (Figure 1.2) is produced mainly from *Penicillium expansum* however some 60 species of mould can produce patulin (Lai *et al*., 2000). It is mainly found in apple products although pears, peaches and berries can also be affected. The toxicity of patulin affects bacteria, fungi, plants and animals. It also has mutagenicity towards *Saccharomyces cerevisiae*, a yeast used in brewing. The EU maximum limits for this toxin are between 10 and 50 μg Kg^{-1}. When raising antibodies towards this toxin, patulin has to be coupled to a protein carrier to increase its immunogenicity due to its small size (154.1 Daltons), however when using these antibodies greater preference is found for the protein carrier than patulin hence resulting in poor specificity (Sheu *et al*., 1999; McElroy and Weiss, 1993).

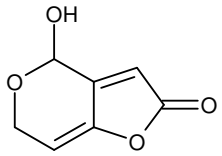


Figure 1.2: Structure of Patulin.

1.2.3 Trichothecenes.

Trichothecenes, zearalenone and fumonisins are all formed by the *Fusaria* mould. This mould has one major difference to the other mycotoxin moulds since it grows in the field whereas most moulds grow during storage. Figure 1.3 shows the basic structure of the trichothecenes. There are two groups of trichothecenes, A and B. Group A consists of T-2 and HT-2 toxin whereas group B contains fusarenon X, deoxynivalenol (DON) and nivalenol. The mycotoxins of interest in this group are deoxynivalenol and T-2. Neither deoxynivalenol nor T-2 have shown strong evidence that they are carcinogenic (Wijnands & van Leusden, 2000).

Deoxynivalenol is a concern because it is frequently found in grains however T-2 is not so common although it has a much higher toxicity. *Fusaria graminearum* is chiefly responsible for the production of deoxynivalenol. Animal feed contaminated with deoxynivalenol results in a loss of appetite and vomiting, this has given deoxynivalenol the synonym vomitoxin.

The main producer of T-2 toxin is *Fusaria sporothrichioides*, which occurs in cooler climates and grows on crops which are left over the winter in the field. The most significant effect of T-2 is its immunosuppressive activity. The European Commission limits for these compounds are; deoxynivalenol 100-1500 µg Kg^{-1}, T-2 50-500 µg Kg^{-1} (EMAN, 2000).

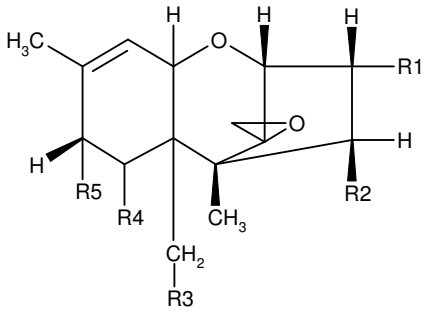


Figure 1.3: Basic structure of Trichothecenes.

1.2.4 Zearalenone.

Zearalenone (Figure 1.4) is another toxin produced by several *Fusarium* species but mainly *Fusaria graminearum*. It is a non-steroidal estrogenic mycotoxin which contaminates many cereals but mainly maize (EMAN, 2000; Wijnands & van Leusden, 2000). It is responsible for outbreaks of oestrogenic syndromes in farm animals. There is little evidence to show that zearalenone is carcinogenic for humans.

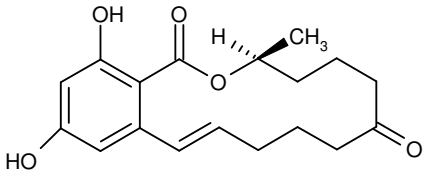


Figure 1.4: The structure of Zearalenone.

1.2.5 Fumonisins.

Fumonisins (Figure 1.5) are a relatively recent discovery having only been discovered in 1988 (EMAN, 2000). There have been six fumonisins isolated however only B_1 is of interest due to its health risks. *Alternaria alternata* produces a toxin AAL that is structurally similar to Fumonisin B_1 and has very similar toxic properties (Wijnands & van Leusden, 2000; Pinot *et al.*, 1997). The main effects of fumonisin B_1 are carcinogenic and growth problems, it accumulates in the liver and kidneys of animals so man could be exposed to the toxin upon the consumption of the animal. Fumonisins have been detected in milk (Barna-Vetró *et al.*, 2000).

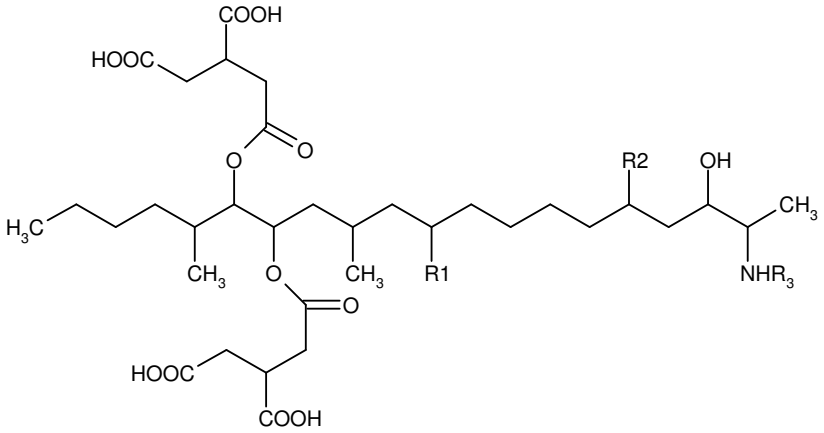


Figure 1.5: The general structure of Fumonisins.

1.2.6 Aflatoxins.

The discovery of aflatoxins has been previously discussed in Section 1.1. In this review the aflatoxin of interest is aflatoxin M_1 or AFM1. From the reported outbreaks of aflatoxins it became clear that the mould *Aspergillus* was prevalent in the warmer climates. Optimum temperature for *Aspergillus flavus* has been determined at 30^{o}C however it can grow in a range of temperatures from 10^{o}C to 45^{o}C. It was also recognised that storage of the grain after heavy rains increased the chance of spoilage. In order for *Aspergillus* to grow a relative humidity of 80% is required (Moreau, 1979) hence aflatoxin production is more of a concern in humid tropical regions, such as Brazil, Uganda, Nigeria and India, when grain is not stored within the correct parameters (Janardhana *et al*., 1999; D'Mello & mac Donald, 1997).

High aflatoxin levels are associated with stresses such as; drought, temperature, nutrients, insects and weeds, hence reducing aflatoxin contamination by plant breeding or genetic manipulations is a difficult task due to the number of sources of stress (Moreno & Kang, 1999). Aflatoxin B_1 is known to be found on grains, however it also contaminates nuts such as cashew, hazel, peanuts, walnuts, pistachios and almonds (Leszcynska *et al*., 2000; Mahoney & Rodriguez, 1996;

Steiner *et a*l., 1992) also figs, spices (chilli and cayenne pepper) (Vahl & Jorgensen, 1998) as well as rice and pulses (Begum & Samajpati, 2000).

Out of all the mycotoxins, aflatoxin causes the greatest amount of management costs and the most losses in the USA, due to its high toxicity per unit basis and long history of strict legislations. Furthermore monitoring and research of mycotoxins in the USA is estimated to cost between 0.5 and 1.5 billion dollars a year (Robens & Cardwell, 2003). Potentially 1.3 billion tonnes (Stroka & Anklam, 2002) or 25% (Moreno & Kang, 1999) of crops world wide are contaminated with aflatoxins. The toxic effects of aflatoxins have resulted in them being used in biological weapon programmes (Zilinskas, 1997; Presidential Advisory Committee on Gulf War Veterans' Illnesses, 1996).

Aflatoxin B_1 is not a primary toxin. Upon digestion of the Aflatoxin B_1 the body's response is to activate a microsomal cytochrome P450. This cytochrome is a general response to detoxification of foreign substances or 'xenobiotic compounds' (Stryer, 1988). In the removal of aflatoxins the enzymes emitted from the cytochrome are CYP1A2 and CYP3A4. Evidence shows that the CYP1A2 enzyme is responsible for the conversion to aflatoxin M_1 whereas CYP3A4 causes the production of an epoxide of the AFB_1. Figure 1.6 summarises these reaction routes. The chemically unstable epoxide then reacts with DNA, RNA and proteins (Kuilman *et al.*, 2000) in particular the epoxide seems to attack the guanine sites on the DNA.

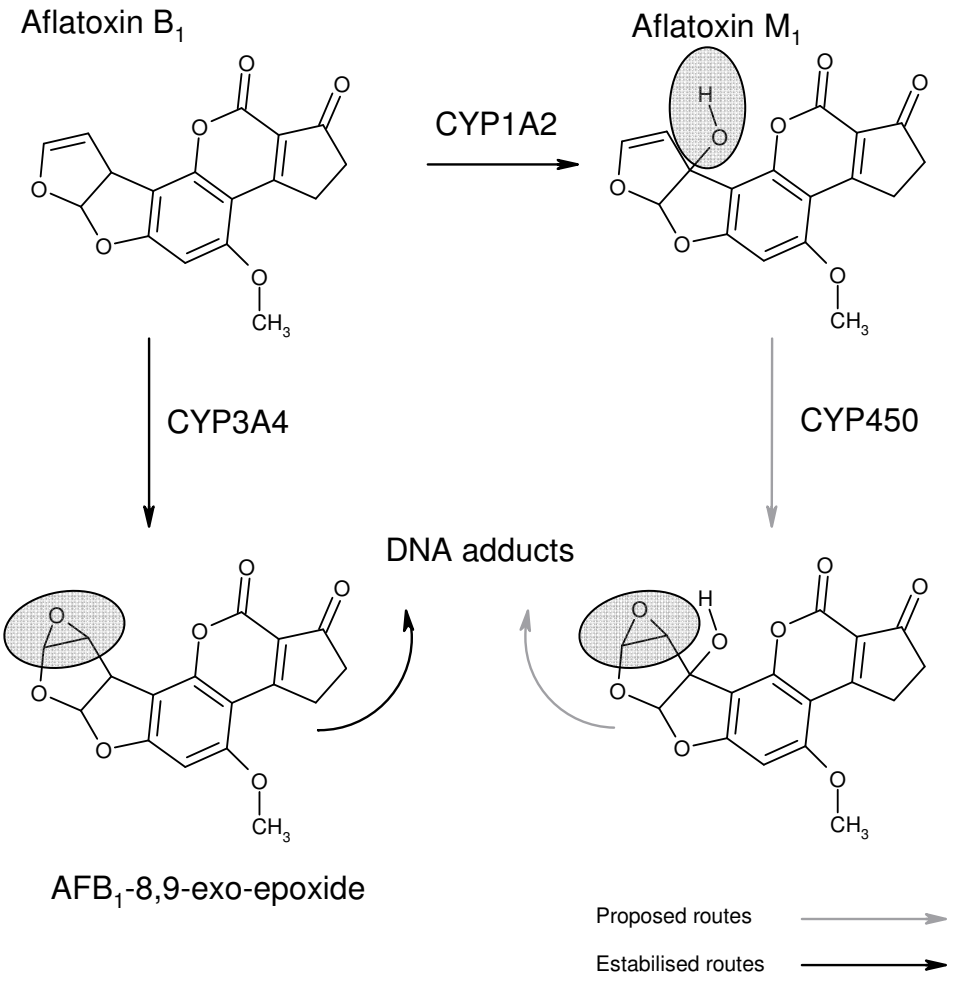


Figure 1.6: The mechanisms for DNA attack. The structural changes from aflatoxin B_1 are highlighted.

Similar to the trends found between liver cancer and aflatoxin B_1 levels in Uganda, a trend was noticed connecting levels of liver cancer and hepatitis A where aflatoxin B_1 exposure also occurred. It has been suggested that the metabolism of aflatoxin B_1 changes with people infected with hepatitis A and the occurrence of liver cancer is increased. Upon immunisation to hepatitis A the number of cases of liver cancer decreases (Henry *et al.*, 1997).

There are many conflicting reports about the conversion of aflatoxin B_1 into M_1. Reports vary between 0.003% (Rodricks and Stoloff, 1976) and the generally

accepted value of around 5% of digested aflatoxin B_1 turns into aflatoxin M_1 in milk produced by dairy cows. It should be noted however that the low ratio reported by Rodricks and Stoloff, (1976) was early work where the detection was not as precise as later reports. The conversion takes between 12 to 72 hours after consumption of feed contaminated with aflatoxin B_1 (Martins & Martins, 2004; Lopez *et al*., 2003; van Egmond, 1983). Levels of aflatoxin M_1 are seasonal due to dairy cows being fed stored feed during winter or grazing naturally on fresh grass during summer (Lopez *et al*., 2003; Bakirci 2001; Henry *et al*., 1997). It is proposed that the production of aflatoxin M_1 is a detoxification process since the carcinogenicity is one tenth of aflatoxin B_1 (Neal *et al*., 1998) and the genotoxicity is estimated between one tenth (Neal *et al*., 1998) and one third (Henry *et al*., 1997). Aflatoxins are mutagenic, teratogenic and also act as immunosuppression agents.

Aflatoxin M_1 was first discovered to exist in urine when trying to elucidate the aetiology of liver cancer from aflatoxin B_1 (Campbell *et al*., 1970). Aflatoxin P_1 and Q_1 have been recorded as homologues of aflatoxin M_1 and have also been isolated in human urine (Moreau, 1979). Figure 1.7 shows the structural differences between P_1 and Q_1 as well as other known aflatoxins. Aflatoxin Q_1 is found in greater concentrations in human urine than M_1 (Kussak, 1994) however the opposite has been reported for monkeys and dogs (Bingham, 2004; Hsieh, 1974). The conversion of ingested aflatoxin B_1 to excreted urinary aflatoxin M_1 is reported to be in the region of 1.3 to 1.5 % (Zhu, 1987).

In a study of 300 young Chinese males, Mykkänen *et al*., (2005) reported that 50% of the subjects produced detectable aflatoxin M_1 in urine (>8 ng L^{-1}) with the mean concentration of 80 µg L^{-1} furthermore the concentration of Q_1 was 60 times that of M_1 in urine and faeces. A study in Sierra Leone showed aflatoxin M_1 levels in the urine of 50 % of school children (n=334) with concentrations as high as 374 µg L^{-1} with a mean value of 7.1 µg L^{-1} (Jonsyn-Ellis, 2000a,b), similarly a study by Nyathi

et al., (1987) in Zimbabwe reported that aflatoxin M_1 levels were detected only in 4.4% of human subjects (n=1228), however the mean concentration was similar to Sierra Leone at 4.2 µg L^{-1} and maximum detected sample of 120 µg L^{-1}. The percentage detected values between Zimbabwe and Sierra Leone cannot be analytically compared since there is no detail in the reports of the level that is classified as 'detectable'.

Coulter *et al.,* (1986) reported levels in Sudanese children (n=584) with positive detection in 39% and a mean level of 490 ng L^{-1}. A study in Lagos, Nigeria reported aflatoxin M_1 detectable in 8.7% of the population (n=161) with the average concentration being much lower than other studies at 8.9 ng L^{-1} (Bean *et al*., 1989). This data shows that there is significant consumption of aflatoxin M_1 contaminated food in Africa. No literature reports were found other than Mykkänen *et al*., (2005) where aflatoxin M_1 in bodily fluids had been surveyed in a non-African country.

There are no limits set for aflatoxin P and aflatoxin Q, presumably since it does not reach the food chain and they are of lower toxicity than M_1 (Fan, 1984). Q_1 can be identified by TLC where aflatoxin Q_1 is separated out between aflatoxin M_1 and aflatoxin B_1 and emits green light under fluorescence (Hsieh, 1974).

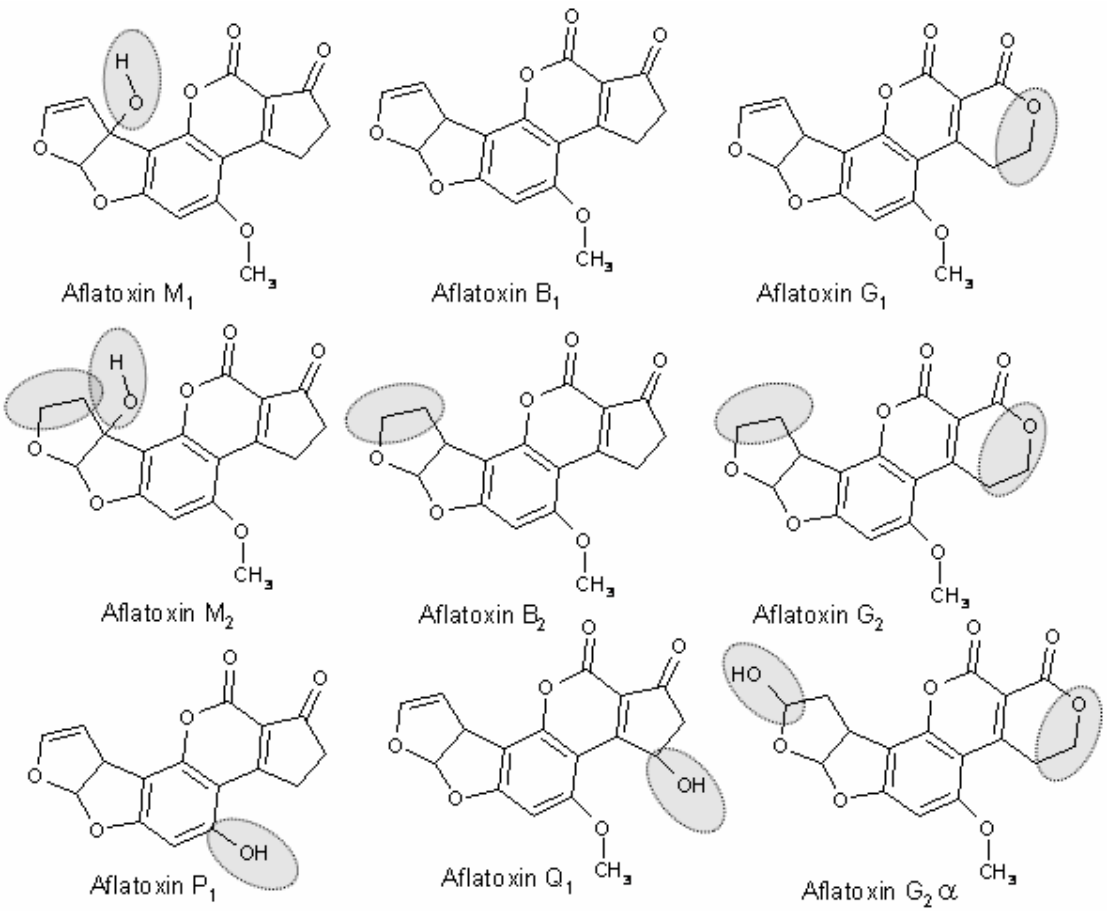


Figure 1.7: The different homologues of aflatoxin. Highlights show the differences from aflatoxin B_1.

Aflatoxin M_1 has been detected in human breast milk samples and this acts as a good biomarker for human intake for aflatoxin B_1. Table 1.2 shows reported levels of aflatoxin M_1 in human breast milk.

Table 1.2: Worldwide reported levels of aflatoxin M_1 in human breast milk (all values in ng Kg^{-1}).

Location	Percentage detection	Maximum	Minimum	Mean	Number of tests	References
Victoria, Australia	15 %	1031	28	71	73	El-Nezami *et al.*, 1995
Qalyubiyah, Egypt	35.5 %	5000	5	13.5	388	Polychronaki *et al.*, 2006
São Paulo, Brazil	2 %	20	20	20	50	Navas *et al.*, 2005
Lombardy, Italy	<1 %	194	194	194	231	Turconi *et al.*, 2004
Adu Dhabi, UAE	99.5 %	3000‡	2	187	445	Saad *et al.*, 1995
Northern Zimbabwe	11 %	14.1	49.3	~	54	Wild *et al.*, 1987
Khartoum, Sudan	37 %	3.0	19.0	64.0	99	Coulter *et al.*, 1984
Liverpool, UK	0 %	0	0	0	10	Coulter *et al.*, 1984
Accra, Ghana	34 %	1815	20	377	264	Lamplugh *et al.*, 1988
Accra, Ghana	32 %	1379	5.0	445	501	Maxwell *et al.*, 1989
Al Ain, UAE	92 %	3400	7.0	560	140	Abdulrazzaq *et al.*, 2003

‡ Samples were taken from mixed nationalities residing in Adu Dhabi.

More concerning is the fact that aflatoxin M_1 is detectable in neonatal cord blood, demonstrating that aflatoxin M_1 passes through the placenta thus reaching the foetus. Aflatoxin M_1 levels as high as 7320 ng L^{-1} has been detected in neonatal cord blood (Lamplugh *et al.*, 1988).

Due to the fact than the milk intake of infants is high, and when young they are very vulnerable to toxins, a low limit was introduced by the European Union in 1999 of maximum levels of aflatoxin M_1 in milk 0.05 µg L^{-1} and 0.025 µg Kg^{-1} for infant formulae (Henry *et al.*, 2001). This level is now enforced by Commission regulation (EC) no. 472/2002 (Gilbert and Vargas, 2003). The FDA (The US Food and Drug Administration) have set levels higher than the European Commission at 0.5 µg L^{-1}

whereas Switzerland and Austria have set limits at 0.01 μg L^{-1}. These limits have been set by the ALARA process, As Low As Reasonable Achievable rather than medically determined (Rastogi *et al.*, 2004).

Aflatoxin M_1 has also been found in other dairy based products such as cheese and yoghurt (Martins & Martins, 2004; Sharman *et al.*, 1989; van Egmond, 1983). Aflatoxin M_1 has been reported in the muscle tissues of animals (Stubblefield *et al.*, 1983; Rodricks and Stoloff, 1976). It was found that when cows were fed on aflatoxin B_1 contaminated feed, then aflatoxin M_1 was detected in the brain, gallbladder, heart, intestines, kidneys, liver, lung, mammary glands, spleen and tongue, of which the kidneys, mammary glands and liver were the highest with levels at 57.9, 25.1 and 13.2 μg L^{-1} respectively. When studying swine, although their digestive system is completely different, the highest aflatoxin levels were again found in the kidneys (Stubblefield *et al.*, 1983). In a more recent study aflatoxin M_1 has been detected as high as 1.05 μg kg^{-1} in the livers of swine (Chiavaro *et al.*, 2005). The kidneys of chickens have also been shown to contain aflatoxin M_1 if the chicken is given aflatoxin B_1 contaminated feed (Trucksess *et al.*, 1983).

Eggs of laying hens have been shown to contain aflatoxin M_1 (Kuilman *et al.*, 2000; Wolzak *et al.*, 1985). The main observation when hens were fed an aflatoxin B_1 contaminated feed was a reduction of the weight of the eggs. After being fed for four weeks the eggs had reduced in weight to 39%. The aflatoxin M_1 levels recorded were a maximum of 0.03 μg L^{-1}. Other authors have reported no aflatoxin M_1 in eggs, however there is dispute in the extraction methods (Trucksess *et al.*, 1983).

1.3 Aflatoxin M_1 and Milk.

As previously discussed the aflatoxin M_1 is a detoxification product from aflatoxin B_1. Aflatoxin M_2 is a detoxification product of aflatoxin B_2, however aflatoxin M_2 is

rarer than M_1 and not as toxic so it receives little interest. Aflatoxin M_1 has also been isolated on highly contaminated corn samples where it occurs 1000 times lower concentration than aflatoxin B_1 (Shotwell *et al*., 1976). Much less research has been done on aflatoxin M_1 compared to the parent aflatoxin B_1.

Aflatoxin M_1 has the full chemical name of; Cyclopenta (C) furo (3', 2':4,5) furo (2,3-H) (1) benzopyran-1,11-dione, 2,3, 6A, 9A tetrahydro-9°-hydroxy-4-methoxy and its CAS number is 6795-23-9. Its chemical formula is $C_{17}H_{12}O_7$ hence it molecular weight is 328.3 Daltons. As with all aflatoxins it is a highly oxygenated heterocyclic compound. Aflatoxin M_1 is chemically stable, it is not destroyed under domestic conditions such as microwave or oven heating however the stability of aflatoxin M_1 during pasteurization is in debate. Bakirci, (2001) and Henry *et al*., (1997) report that pasteurization has no effect whereas Deveci and Sezgin (2006) suggests that pasteurization causes a 16% decrease, hypothesising that the decrease is due to heat treatment causing casein decomposition.

Many methodologies have been researched to remove aflatoxin M_1 from milk by adsorption using bentonite, applying ultra-violet radiation (Henry *et al*.,1998), humic acids (van Rensburg *et al*., 2006), polyvinylpolypyrrolidone and synthetic zeolites (Keçeci, *et al.,* 1998) although little is known about the changes in biological safety or nutritional value these processes cause (Henry *et al*., 1997). Additionally it is suggested that binders may aid mycotoxin management but other methods would be required for complete control (van Rensburg *et al*., 2006). Since it is difficult to eradicate aflatoxin M_1 in milk, greater monitoring of aflatoxin B_1 must be preformed so that aflatoxin B_1 is prevented from entering cattle feed (van Egmond, 1983). Many studies have shown that aflatoxin B_1 binds reversibly with the polysaccharide surface of lactic acid bacteria (Haskard *et al*., 2001; Peltonen *et al*., 2001; Haskard *et al*., 2000).

Chemically aflatoxin M_1 is hydrophobic; studies have shown that aflatoxin M_1 in milk resides in the hydrophobic cavities of the protein casein. Therefore, casein rich foods such as cheese have a 3 to 6 fold increase in aflatoxin M_1 compared to low protein products (Bakirci, 2001; van Egmond, 1983; Brackett and Marth, 1982).

The casein content of cows milk is about 2.5% (compared to 0.4% found in human milk). Casein consists of about 200 amino acid residues. It occurs in a structure similar to denatured globular proteins due to the high number of proline residues (about 10% proline); this in turn causes a hydrophobic surface of the protein making it insoluble in water and attractive to the aflatoxin M_1 molecule (Henry *et al*., 1997). Frequently in clean-up procedures for analysis, proteins are removed by denaturing with trichloroacetic acid and then filtered or centrifuged, however in the case of aflatoxin M_1 the trichloroacetic acid derivatizes the molecule thus causing low recovery rates. Another unfavourable property of milk with respect to analysis is the fat content. Fats can cause blocking of the active sites in clean up techniques, thus ideally fats are removed in an early stage of analysis. Milk also contains about 5% sugar in the form of lactose.

Due to the high solid content of milk, direct analysis can cause blockages in narrow fluid channels found in some analytical equipment (Sibanda *et al*., 1999).

Figure 1.8 and Table 1.3 shows the levels of aflatoxin M_1 reported in literature where it is observable that the highest levels reported come from hotter climates and the lowest levels from cooler climates.

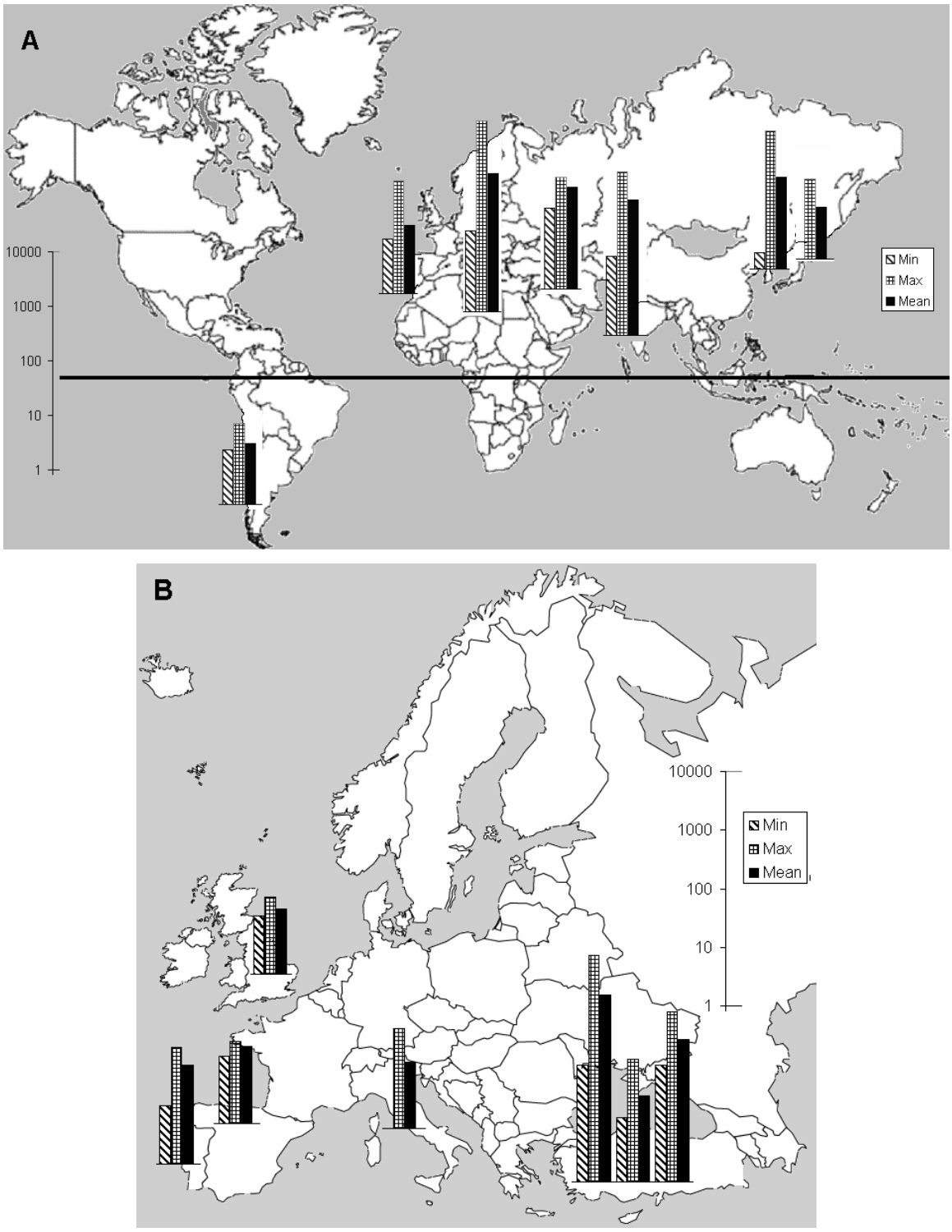


Figure 1.8: Levels of aflatoxin M_1 reported in literature for the world.
A) excluding Europe. B) Europe only. Logarithmic scale for aflatoxin M_1 (ng L^{-1}). Data obtained from Table 1.3.

Table 1.3: Reported levels of aflatoxin M_1. Table is ordered by maximum aflatoxin M_1 levels. All aflatoxin M_1 values reported as ng L^{-1}.

Country/ State	Number of samples	Method	Min	Max	Mean	Matrix	References
UK, Nationwide	~	HPLC-immuno	10	21	~	Raw and pasteurized milk	FSA, 2001
Spain, Leon	92	ELISA & HPLC	14	25	20.5	Raw milk	Rodriguez Velasco, 2003
Japan, Nationwide	208	HPLC-immuno	1	29	9	Milk	Nakajima *et al.*, 2004
Argentina, Nationwide	77	Ridascreen	<10	30	13	Milk	López *et al.*, 2003
Greece, Nationwide	297	HPLC-immuno	<5	>50	~	Milk	Roussi *et al.*, 2002
Italy, Nationwide	373	HPLC-immuno	<1	>50	13.6	Milk, yogurt	Galvano *et al.*, 2001
Portugal, Lisbon	101	HPLC-immuno	5	>50	~	Raw and UHT milk	Martins & Martins, 2000
Turkey, Ankara	27	HPLC-immuno	<10	51	22.3	UHT and pasteurized	Gürbay *et al.*, 2006
Portugal, Lisbon	96	HPLC-immuno	<10	98	48	Yogurt	Martins & Martins, 2004
Iran, Tehran	328	Ridascreen	31	113	72.2	Pasteurized milk	Oveisi *et al.,* 2007
Morocco, Rabat	54	HPLC -immuno	10	117	18	Pasteurized milk	Zinedine *et al.*, 2006
Turkey, Van province	90	TLC	12.5	123	29	Raw milk	Bakirci, 2001
Kuwait, Nationwide	54	HPLC-immuno	20	210	~	Milk, yogurt, infant formula	Srivastava *et al.*, 2001
Colombia, Bogota	~	HPLC-immuno	10.7	213	~	Pasteurized milk	Diaz *et al.*, 2004
Turkey, Ankara	223	Ridascreen	<1	>250	~	Cheese and butter	Aycicek *et al.*, 2005
Iran, Sarab city	111	TLC	15	280	24	Raw milk	Kamkar, 2005
Korea, Seoul	180	HPLC & ELISA	10 & 2	342	49	Milk, yogurt, fnfant formula	Kim *et al.*, 2000
Brazil, Campinas	204	TLC	73	370	~	Milk, cheese, yoghurt	de Sylos *et al.*, 1996
Brazil, São Paulo	139	HPLC- Silica	<15	500	~	UHT and pasteurized milk	Garrido *et al.*, 2003
Turkey, Anatolia	129	Ridascreen	0	543	108.17	UHT milk	Unusan, 2006
Turkey, Nationwide	600	ELISA	100	800	269	Cheese	Yaroglu *et al.*, 2005
Turkey, Ankara	400	Ridascreen	<50	>800	~	Cheese	Sarimehmetoglu *et al.*, 2004
India, Lucknow	87	Ridascreen	28	1012	299	Milk and infant food	Rastogi *et al.*, 2004
Brazil, São Paulo	150	TLC	100	1680	~	Milk	Sabino *et al.*, 1989
Brazil, Paraná state	42	Ridascreen-Fast	<245	1975	~	Raw milk	Sassahara *et al.*, 2005
Libya, Northwest	69	HPLC-immuno	30	3130	347	Milk, cheese	Elgerbi *et al.*, 2004

Other members of the Bovine family apart from cows also produce aflatoxin M_1 contaminated milk. Paul *et al.*, (1976) reported the occurrence of aflatoxin originating in buffalo milk from India with contamination levels similar to that of cows.

Not only Bovine milk is affected, Oliveira & Ferraz (2005) reported the natural occurrence of aflatoxin M_1 in goats milk. Goat milk has a 1% market share of the consumption in Brazil where 69% of the samples were positive with values ranging from 11 to 161 ng L^{-1} (n=36) however these values are lower than those from local bovine milk studies. In addition to goat and cow, milk from ewes is susceptible to aflatoxin M_1 contamination with the conversion ratio of ingested aflatoxin B_1 transformed into aflatoxin M_1 being nearly identical to that of cows (Allcroft *et al.*, 1968; Naybey *et al.*, 1967).

Kaniou-Grigoriadou *et al.*, (2005) observed that from 54 samples taken from Thessaloniki Greece, no samples were above EU maximum permissible limits. Furthermore from Sicily 240 samples were analysed and only 3 were above EU maximum permissible limits (Bognanno *et al.*, 2006). The results from the Ovine samples are not significantly high; however the studies were performed geographically where incidents of aflatoxin M_1 contamination in Bovine milk are not high (1% of samples above 50 ng L^{-1} reported by Roussi *et al.*, (2002) from Greece; 0% above 50 ng L^{-1} reported by Galvano *et al.*, (2001) from Italy). This data suggests that not only Bovine milk should be routinely monitored, but also Ovine and goat milk. It should be observed that when comparing geographical data, the analysis of supermarket milk gives a good indication of the levels of consumed milk. However this does not necessary give a good indication of the aflatoxin M_1 levels produced by the lactating cattle at that strict location, since at the dairies, contaminated milk is blended with non contaminated milk to comply with the maximum permissible limits (de Sylos *et al.*, 1996; Sabino *et al.*, 1989).

1.4 Analysis techniques.

The generally accepted method for the analysis of aflatoxin in milk is by high performance liquid chromatography (HPLC) (Henry *et al.*, 2001). Many other methods exist; if these methods show a positive result then HPLC is used for confirmation. For the analysis of aflatoxin B_1 the greatest source of error is due to sampling. With pistachios for example, one single heavily contaminated nut can cause a 5 Kg sample to be rejected (Mahoney & Rodriguez, 1996) hence it is very important to get a representative sample of the bulk material.

For the analysis of milk the sampling errors are reduced dramatically since the milk is assumed to be a homogeneous matrix (van Egmond, 1983) even so the FDA has guidelines stating that ten portions should be taken from a 5 Kg sample and the European Commission state that the sample size should be 10 Kg (Henry *et al.*, 1997). Analysing for aflatoxin B_1 to European Commission legislations is no longer an analytical challenge due to state of the art instrumentation and methods, however due to capacity problems in many countries only 5-25% of imported foods are screened for mycotoxins (Stroka *et al.*, 2000).

HPLC is an expensive technique to perform mainly due to the cost of the instrumentation and the cost of employment of technical operators. Unfortunately the regions of the world which are most affected by aflatoxin contamination tends to be the poorer areas. In India, a recent survey found that 87.3% of the milk based samples analysed were contaminated, of those 99% were outside European Commission limits. This is a major concern considering that India is the largest producer of milk in the world (Rastogi *et al.*, 2004). In conclusion a direct quote from United Nation states (Proctor, 1994) '*There is an urgent need for simple, robust, low-cost analysis methods, for the major mycotoxins, which can be routinely used in developing country laboratories.*'

1.4.1 HPLC (High Performance Liquid Chromatography).

This is a very versatile method and during the 1980's popularity for HPLC increased and the analysis moved away from TLC (Henry *et al.*, 1997). As previously discussed, milk is a difficult matrix to analyse. Many different clean up techniques have been reported. Some of these are; C18 SPE columns (Carisano & Torre, 1986; Bijl & van Peteghem, 1985; Takeda 1984; Winterlin, 1979), silica columns (Qian *et al.*, 1984; Chambon *et al.*, 1983; Fremy & Boursier, 1981) precipitation by zinc hydroxide (Chambon *et al.*, 1983) and precipitation by concentrated hydrochloric acid and sodium hydroxide (Gauch 1979), however the latter has shown that alkaline extracts reduce the recovery of aflatoxin M_1 (Shepherd *et al.*, 1986).

The first draft Association of Analytical Communities method (AOAC) used trifluoroacetic acid to intentionally derivatize the aflatoxin M_1 to M_{2a} which has six times greater fluorescence (Orti *et al.*, 1989; Carisano & Torre,1986; Hisada *et al.*, 1984). This reaction is shown in Figure 1.9.

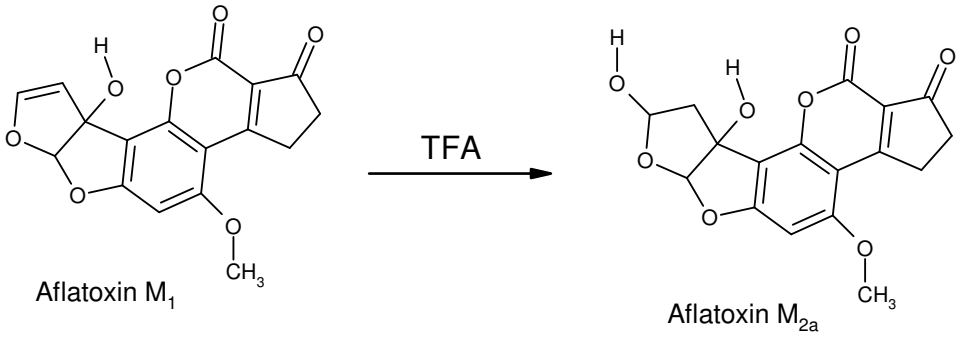


Figure 1.9: The reaction scheme for the conversion of aflatoxin M_1 to the more fluorescent M_{2a}. (Carisano & Torre,1986).

A silica SPE cartridge was used to clean up the sample and then normal phase chromatography was used for the separation. The method was revised to use reverse phase HPLC using a C18 column and acetonitrile : water as an eluent, thus reducing the amount of waste solvent and analysis time (Beebe & Takahashi,

1980). It became the AOAC method in 1986. Currently the AOAC method uses immunoaffinity columns containing monoclonal antibodies specifically for aflatoxin M_1 which are supported on Sepharose® packing material (AOAC, 1996). The following literature was among the first to implement immunoaffinity columns for aflatoxin M_1 determination; Martins and Martins, (2000); Stroka *et al.*, (2000); Farjam *et al.*, (1992); Mortimer *et al.*, (1987). In all methods the detection method is fluorescence. Recently the use of HPLC with tandem mass spectroscopy has been applied to the detection of aflatoxin M_1. Chen *et al.*, (2005) has reported impressive limits of detection to 0.59 ng L^{-1}, some 100 times lower than the current EU maximum permissible limits.

Aflatoxins have been detected using electrochemical detection. Holak *et al.* (1997) reported the use of a voltammetric detector for detecting aflatoxins. Voltammetry alone cannot distinguish between aflatoxins B_1, B_2, G_1 and G_2 since they all give a response at -1.25V vs Ag/AgCl so by coupling a hanging drop mercury electrode (HDME) to a HPLC system it was reported that it is feasible to detect aflatoxin B_1 levels down to 2.5 µg L^{-1}. Holak *et al.*, (1997) also speculated that by switching to a gold/mercury electrode then lower limits of detection could be achieved. Elizalde-Gonzalez *et al.,* (1998) continued this work and showed that sensitivity was improved using a glassy carbon electrode rather than a gold or platinum electrode.

1.4.2 TLC (Thin Layer Chromatography).

This method is older than HPLC and has many advantages. It is far cheaper than HPLC methods and it does not require extensively trained operators, however it is less accurate than HPLC (Gilbert and Anklam, 2002). Original extraction methods detailed were laborious involving a 6 hour Soxhlet extraction using acetone, chloroform and water followed by treatment with lead acetate and then petroleum ether before being spotted onto TLC plates (Goldblatt, 1969).

Subsequently the method has improved using a two dimensional development technique (Beebe & Takahashi, 1980). The milk is extracted with chloroform and spotted onto silica plates, first toluene : ethyl acetate : formic acid is used then diethyl ether : methanol : water (Henry *et al.*, 2001; Wood & Mann, 1989). In order to improve accuracy, techniques are being implemented to incorporate densitometric equipment such as a computer scanner to electronically quantify the dots rather than by visual inspection (Henry *et al.*, 2001). All these methods used long wave ultra-violet light for the detection where aflatoxin M_1 fluoresces blue.

In developing countries TLC is the preferred detection technique due to the low cost and ease of use.

1.4.3 ELISA (Enzyme Linked ImmunoSorbant Assay).

The majority of recent papers reviewed who have used ELISA as a method of detection have all used the kit made by R-Biopharm for example; Rastogi *et al.*, (2004); Sarimehmetoglu *et al.,* (2004); Lopez *et al.*, (2003); Rodriguez Velasco *et al.*, (2003). The kit is part of the RIDASCREEN® range of diagnosis. Many authors have developed themselves an ELISA protocol. Table 1.4 lists the differences in these self developed methods.

Table 1.4: The different ELISA protocols developed for aflatoxin by various authors.

Enzyme marker	**Enzyme substrate**	**Immobilised component**	**Limit of detection**	**Reference** (main author)
HRP	TMB	Aflatoxin B_1-BSA	30 ng L^{-1}	Wild, 1987
HRP	TMB	Aflatoxin B_1-BSA	5 ng L^{-1}	El-Nezami, 1995
HRP	TMB	Antibody	5 ng L^{-1}	Kim, 2000
AP	pNPP	Antibody	10 ng L^{-1}	Thirumala Devi, 2002
HRP	luminol	Aflatoxin M_1-BSA	0.25 ng L^{-1}	Magliulo, 2005

HRP, horseradish peroxidase; AP, alkaline phosphatase; TMB, 3,3′,5,5′-Tetramethylbenzidine; pNPP, 4-Nitrophenyl phosphate.

Commonly the principle of the analysis is to incorporate a monoclonal antibody which is specific to aflatoxin M_1 only. Either standards of known value or sample is added to immobilized antibodies. An additional secondary antibody with an attached enzyme or an enzyme conjugated analyte then binds to any surplus antibody sites and the excess secondary antibody with enzyme is then washed away. For the RIDASCREEN method, urea peroxide acting as an enzyme substrate is added with a chromogen and the enzyme will break down the urea peroxide and the by-product will cause a colour reaction with the chromogen from a blue colour to a yellow colour, which is then measured at 450nm (Ridascreen®, 2001). The absorbance is proportional to the amount of enzyme conjugate bound, which is therefore inversely proportional to the amount of aflatoxin M_1. This reaction process is classified as a competitive enzyme immunoassay.

Competitive assays have the disadvantage to non-competitive assays since the amount of unbound sites are measured, resulting in difficulties to distinguish low levels of analyte from a zero level i.e. blank value (Giraudi *et al.*, 1999b). A more reliable method is the non competitive sandwich technique where one antibody is bound to a fixed surface and the analyte is added, then a different antibody is added and this also binds to the analyte. Thus the detection method is directly proportional to the concentration of analyte. This method is unsuitable for small analytes such as the aflatoxins since the molecule is not large enough for two antibodies to bind to it, and therefore can not be applied to mycotoxin analysis.

The advantages of the ELISA technique is the ease of use and the cost of the equipment required. A semi-automated version of this method is available from Charm Scientific Inc. (Massachusetts, USA), however at 0.25 µg L^{-1} the detection limits are insufficient for European Commission legislations. With all ELISA techniques a positive result needs to be verified by HPLC since no ELISA method has been given AOAC approval (Henry *et al.*, 2001). Frequently ELISA methods yield results which are higher than those obtained by HPLC; it is believed the

reason for this misalignment is due to the antibodies in the ELISA procedure cross-reacting with molecules of similar structure to the analyte of interest in the sample (Kulisek & Hazebroek, 2000).

In 1999 a paper was published by Sibanda *et al.*, (1999) detailing a portable field assay for the detection of aflatoxin M_1. This used a cell which contained antibodies and reagents, so that the milk sample could be detected by a visible colour development, as with the standard ELISA test the colour development was inversely proportional to the concentration. This idea has been invested in and a company called Idexx Laboratories Inc. (Maine, USA) have produced a working commercial kit. Unlike the original paper by Sibanda which required clean up using immunoaffinity columns, this method requires no pre-treatment of the milk and a positive or negative result is known within 15 minutes. Like the Charm instrument the limits of detection are insufficient for European Commission legislations (0.50 $\mu g\ L^{-1}$). Again a positive test needs to be verified by HPLC, but this is the first signs of aflatoxin M_1 analysis can be performed away from the laboratory.

Another interesting application using antibodies is the use of a dipstick similar to a home pregnancy test. Two references of this technology have been reported for mycotoxin analysis. Delmulle *et al.*, (2005) have reported the development of a lateral flow dipstick for the detection of aflatoxin B_1 in pig feed. In the test they used monoclonal antibodies conjugated to colloidal gold particles which upon the reaction with immobilised aflatoxin B_1 – BSA yielded a visual pink band on the dipstick. Although this technology can only give a false/positive result, the reported detection limit of 5 $\mu g\ kg^{-1}$ is sufficient to meet the maximum permissible limit set by the European Union for pig feed, the accuracy of the test was reported at 90% (n=88). The other reported use of dipstick technology for mycotoxin detection was for fumonisins in corn based foods (Schneider *et al.*, 1995). The dipstick provides a positive/negative result within 60 minutes for qualitative measurement.

1.4.4 Bilayer Lipid Membranes (BLM).

Bilayer lipid membranes are classified as a non immunochemical technique and can provide rapid results (Siontorou *et al.*, 2000; Andreous & Nikolelis, 1998). Briefly, upon the interaction of aflatoxin M_1 and a lipid, channels in the lipid open up and allow the eluent to pass through. The eluent is an ionic solution so that changes in ion concentration can be monitored using an electrochemical detector (see Figure 1.10). The method has very fast analysis time (response time of 15 seconds) and the lipid membranes can be used many times. The cost of the equipment is much lower than HPLC and by altering the flow rates, protein interferences from the milk can be eliminated.

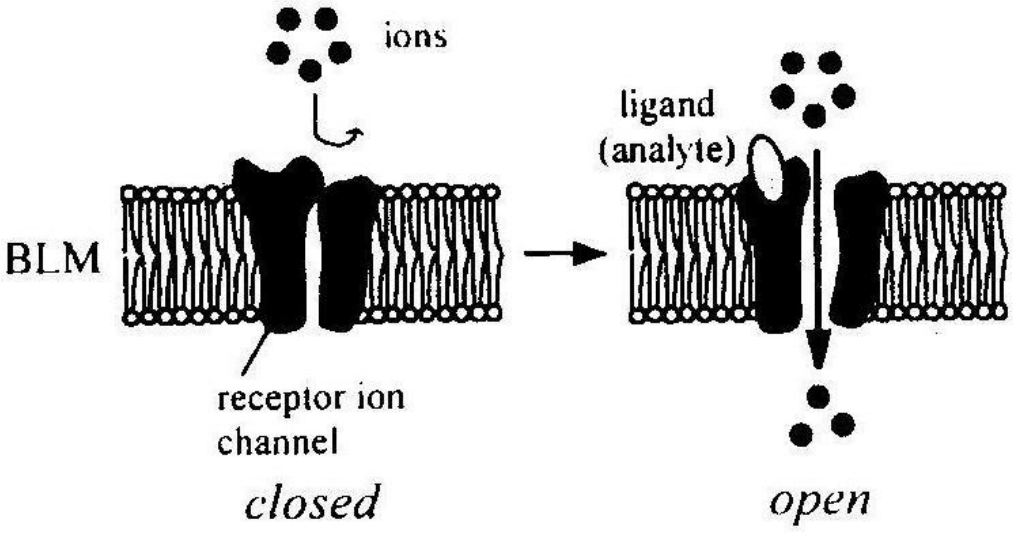


Figure 1.10: Schematic diagram of a Bilayer lipid membrane. (Reproduced from Sugawara *et al.*, 2002).

Initially detection limits were only 750 μg L^{-1} but these limits were improved by stabilising the system. Most recently single strands of DNA oligomers were incorporated into the membranes to modify the surface electrostatic properties and thus provided a system with better stability and 0.016 μg L^{-1} as a limit of detection (Gilbert and Vargas, 2002; Siontorou *et al.*, 2000).

1.4.5 Summary of conventional methods.

Figure 1.11 shows the frequency of a sample of literature published detailing the detection of aflatoxin M_1.

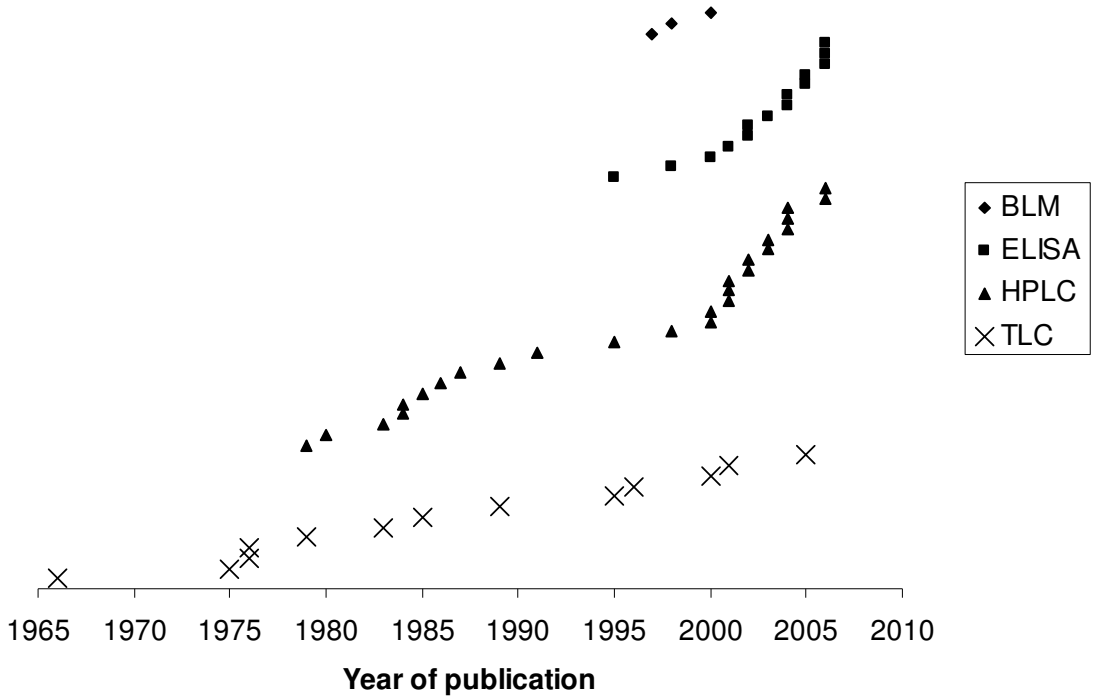


Figure 1.11: Detection of aflatoxin M_1 timeline. The x-axis states the date of publication for each method and therefore the frequency of each method and when the method was first reported.

The timeline in Figure 1.11 is compiled from; Holzapfel & Steyn,1966; Tuinstra & Bronsgeest, 1975; Paul *et al*., 1976; Shotwell *et al*., 1976; Gauch 1979, Winterlin 1979; Beebe & Takahashi,1980: Chambon *et al*., 1983; van Egmond, 1983; Qian *et al*., 1984; Takeda, 1984; Bijl & van Peteghem,1985; Carisano & Torre, 1985; Shepherd *et al*., 1986; Mortimer *et al*., 1987; Sharman *et al*., 1989; Farjam *et al*., 1991; Diaz *et al*., 1995; El-Nezami *et al*., 1995; de Sylos *et al*., 1996; Andreou & Nikolelis, 1997, Andreou & Nikolelis,1998; Sibanda *et al*., 1999; Simon *et al*., 1998; Kim *et al.,* 2000; Martins *et al*., 2000; Siontorou *et al*., 2000; Stroka *et al*., 2000; Bakirci, 2001; Galvano *et al*., 2001; Lopez *et al*., 2001; Srivastava *et al*., 2001;

Martins & Martins, 2002; Garrido *et al*., 2003; Lopez *et al*., 2003; Diaz *et al*., 2004; Elgerbi *et al*., 2004; Martins & Martins, 2004; Nakajima *et al*., 2004; Rastogi *et al*., 2004; Sarimehmetoglu *et al*., 2004; Aycicek *et al*., 2005; Sassahara *et al.,* 2005*;* Tekinşen & Tekinşen, 2005; Yaroglu *et al*., 2005; Gürbay *et al*., 2006; Oveisi *et al*., 2006; Unusan *et al*., 2006; Zinedine *et al*., 2006).

As discussed, the first identification of aflatoxin M_1 as a product of milk was in 1966 using TLC. In the late 1970's HPLC technology received much interest and more recently the use of ELISA and BLM's have been reported. TLC is still being used in developing countries.

An interesting observation when reviewing the literature for this thesis was that Mass Spectroscopy (MS) as a detection technique is used rarely, presumably due to the highly fluorescent properties of the aflatoxins (sufficient limits of detection are reached with fluorescence). Mass spectroscopy was used by van Egmond (1983) for verification of TLC and more recently it has been employed by Chen *et al*., (2005) with HPLC for aflatoxin M_1 determination in milk and milk powders and by Kokkonen *et al*., (2005) again with HPLC but for aflatoxin M_1 determination in Cheese. Chen *et al.* achieved a limit of detection to 0.59 ng kg^{-1} solely looking at aflatoxin M_1 whereas Kokkonen *et al*., utilized the multianalysis advantages of MS/MS by observing 9 mycotoxins including aflatoxin B_1, B_1, G_1, G_2, M_1, ochratoxin A aswell as roquefortine C, a common mycotoxin found in roquefortine cheeses. Considering the potential sensitivity of MS/MS the detection limit for aflatoxin M_1 was a high level at 0.3 µg kg^{-1}, insufficient for the maximum permissible limits imposed by the European Union and almost 3 orders of magnitude worse than the work of Chen *et al*., (2005).

1.5 Biosensors.

The term biosensor is used for a device where a biological component attracts an analyte of interest and the reaction between the biological component and analyte

produces an electrical signal. The official IUPAC definition states *"A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an transduction element."* (Thévenot *et al.*, 1999.)

If the biological component is an antibody or antibody fragment then the term immunosensor is employed. Biosensors have the advantages over traditional techniques of; rapid analysis, continuous monitoring and reusable sensor elements. There are five classes of biosensor detection; electrochemical, optical, calorimetric, magnetic and mass (Tothill and Turner, 2003).

Many biosensors have been reported for aflatoxin B_1 however aflatoxin M_1 has received less interest. Table 1.5 summarises this area of research.

Table 1.5: Reported limits of detection from aflatoxin biosensors.

Aflatoxin	Matrix	Sensing method	Limits of detection	Reference
B_1	Maize	Optical	Not Reported	Boiarski *et al.*, 1996
B_1	Nut	Optical	4 μg Kg^{-1}	Strachan *et al.*, 1997
B_1	Maize	Optical	2 μg Kg^{-1}	Maragos & Thompson, 1999
B_1	None	Optical	1 μg L^{-1}	Carlson, 2000
B_1	Maize, Sorgum, Nut	Optical	5 μg Kg^{-1}	Nasir & Jolley, 2002
B_1	Nut, Oat	Optical	0.2 μg Kg^{-1}	Gaag *et al.*, 2003
B_1	Barley	Electrochemical	0.03 μg Kg^{-1}	Ammida *et al.*, 2004*
M_1	Milk	Electrochemical	0.02 μg L^{-1}	Badea *et al.*, 2004*
M_1	Liver	Optical	1 μg Kg^{-1}	Chiavaro *et al.*, 2005
M_1	Milk	Electrochemical	0.05 μg L^{-1}	Micheli *et al.*, 2005*

* Authors from the same research group at Universitá di Roma *Tor Vergata*.

1.5.1 Optical Biosensors.

Surface plasmon resonance (SPR) was first suggested as a detection method for biological interactions in 1990, however, the phenomenon was first described by Kretschmann in 1971 (Kurihara & Suzuki, 2002; Sarkar & Somasundaran, 2002; Kretschmann, 1971).

SPR is an increasingly popular technique for the detection of biological reactions since detection requires either substances with a high refractive index or greater than 10 kDa (Gaag *et al.*, 2003; Tudos *et al.*, 2003). SPR measures the change in mass concentration on a thin gold surface (Sarkar & Somasundaran, 2002; Mullett *et al.*, 1998). At a specific angle of incidence and wavelength, the evanescent wave component of the light will be adsorbed by the free electrons in the gold (total internal reflection) (Tudos *et al.*, 2003) therefore the amount of light being reflected will decrease. The angle at which this occurs (the critical angle) is dependant on the refractive index of the material under investigation. Figure 1.12 displays a simplified diagram of the SPR detection process.

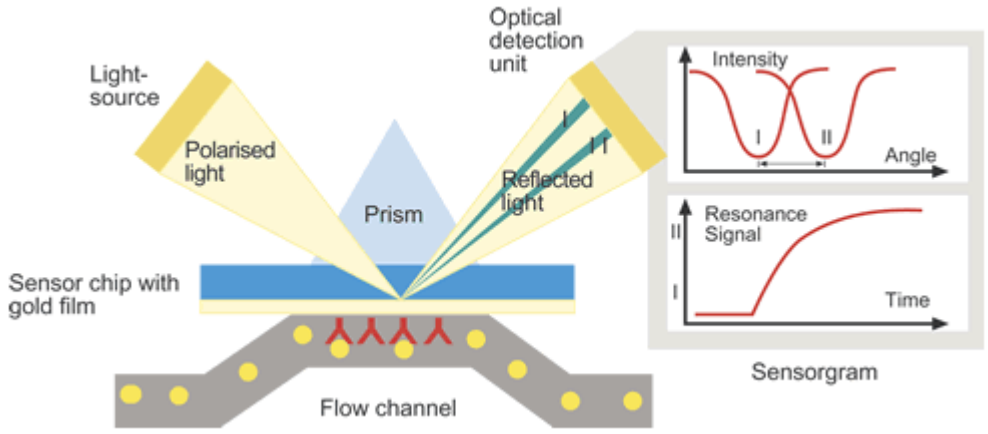


Figure 1.12: Schematic diagram of the detecting unit of the Biacore instrument. Diagram from Biacore AB (Uppsala, Sweden).

In practice a mycotoxin could be immobilized onto the sensor chip surface, the test sample would be mixed with a known excess amount of antibody and the sample would pass over the sensor surface. The non-complexed antibody would be

attached to the mycotoxin immobilized on the sensor surface and thus the mass concentration on the surface would change, hence the refractive index would change. By monitoring the change in the angle of incidence required to achieve total internal reflection then the concentration of non-complexed antibody is known, thus the amount of mycotoxin in our original sample can be back calculated. This technique is known as an inhibition assay and has been reported by Gaag *et al.*, (2003). In order to immobilise aflatoxin directly onto the sensor surface, the aflatoxin has to be carboxylated. The interactions with the antibody and mycotoxin are non-covalent therefore the sensor surface can be regenerated many times.

Mullett *et al.*, (1998) has reported the use of a homemade SPR biosensor for the detection of fumonisin B1 with a detection limit of 50 μg kg^{-1}. In opposite to Gaag *et al.*, (2003) a polyclonal antibody was immobilized onto the surface rather than the analyte, and the detection relied upon the direct detection of fumonisin B_1 (712 daltons).

The Biacore instrumentation is automated so that the analysis and regeneration is computer controlled. The sensor chip contains four channels; hence four different interactions can be studied simultaneously or one of the four channels can be used to measure a sample of known value to act as an internal control. Commonly light in the visible region is used which results in a field of detection 200 to 300 nm away from the sensor surface, hence turbid solution can be analysed.

Mobile SPR instruments have been developed such as the SPREETA range by Texas Instruments, although not as sensitive as the larger instruments its small size of 1.5 x 0.7 x 3 cm and weighing only 7 grams means that SPR can be applied in portable instrumentation (Chinowsky *et al.*, 2003; Weimar, 2000).

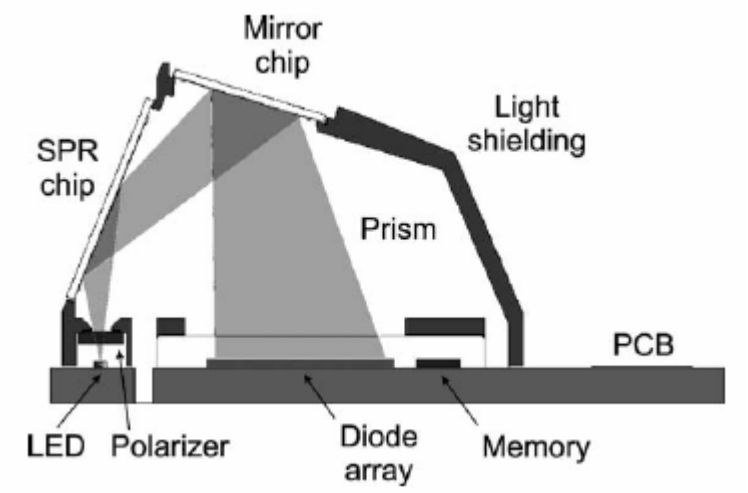


Figure 1.13: Schematic diagram of the Spreeta 2000, showing the internal components. Reproduced from Chinowsky *et al.*, (2003).

In order to regenerate the sensor an antibody only of moderate affinity should be chosen since antibodies can have very strong affinity and when used with SPR the regeneration of the temporarily complexed analyte may not be released or require a drastic regeneration solution which would destroy the antibody (Daly *et al.*, 2000; Diamond, 1998). Optical biosensors have the disadvantage that they contain expensive optical components (Tothill and Turner, 2003).

The theory of evanescent waves has been used in handheld fibre-optic devices for the detection of aflatoxin B_1 (Maragos & Thompson, 1999). Using fibre-optics has great potential for hand held devices since they can be easily miniaturised. An additional optical biosensor has been reported by Nasir & Jolley (2002) which uses a different optical method of fluorescence polarization. The fluorescence polarization method will not be discussed here, but its potential should be highlighted since the instrumentation is portable and can be powered from a laptop battery.

The biosensor reported by Boiarski *et al.*, (1996) is similar to the method reported by Gaag (2003). An aflatoxin antibody is attached to a sensor surface and the

sample is added, the aflatoxin in the sample binds to the antibody, then an excess of aflatoxin labelled with horseradish peroxidase (HRP) is added. The change in mass concentration due to the HRP is recorded and the amount of aflatoxin in the sample can be back calculated. Instead of SPR, Boiarski *et al.*, (1996) used an optical waveguide based interferometric device which can be classed as optical waveguide lightmode spectroscopy or OWLS. This device is very similar to SPR however a fixed grating diffuses the light into a waveguide rather than a prism and the detectors are perpendicular to the sample, this latter difference means that the OWLS instrumentation cannot be miniaturised (Vörös *et al.*, 2002). A comprehensive review of OWLS and SPR is given by Ramsden (1997).

Optical biosensors which don't require SPR or OWLS have been reported. Carlson *et al.*, (2000) reported the use of immunoaffinity columns to separate the aflatoxins from the impurities, and then the aflatoxin is eluted from the column and detected by fluorescence without any further separation. The detection range for the sensor was 0.1 to 50 μg Kg^{-1}. The same protocol has been adopted by Chiavaro *et al.*, (2005) for the detection of aflatoxins B_1 and M_1 in pigs liver where a detection limit of 1 μg Kg^{-1} was reported.

Cucci *et al.*, (2006) reported a fluorometer for the detection of aflatoxin M_1. The detection relied upon the monitoring of fluorescence using a small, portable fluorometer with a very sensitive PMT detector. For pure solutions of aflatoxin M_1 the fluorescence could be monitored to 50 ng L^{-1}. With no pre-treatment the detection method cannot be classed as a biosensor since no biological element is incorporated. Although a detection limit of 50 ng L^{-1} was achieved, this was only semi-quantitative and furthermore only applicable with pure solutions. Due to the fact that milk causes light scattering this sensor is not currently applicable to real samples.

1.5.2 Amperometric devices.

A large number of biosensors use amperometry as their detection technique. The first sensors were based on the oxygen electrode developed by Clark in 1973. Typically glucose and oxygen would react with glucose oxidase to produce hydrogen peroxide and gluconic acid. The electrode detected oxygen by applying a high positive potential to reduce oxygen from O_2 to O_2^-, thus the amount of glucose could be calculated. This principle has evolved into the commercially very successful glucose monitor for diabetics. With no optical system the amperometric biosensor is robust and makes it ideal for using in the field at the point of source. This is a very useful concept with regards to aflatoxin M_1 detection since much of the highly contaminated milk which is consumed originates from village dairies with 1 or 2 cows rather than large scale producers (Suzangar *et al*., 1976).

Only a few electrochemical biosensors have been reported for mycotoxin detection. A report by Moressi *et al*., (1999) demonstrated the detection of mycotoxins produced by *Alternaria*. Moressi *et al*., (1999) incorporated a polyphenol oxidase enzyme found in mushrooms (also found in pears, peaches and potatoes) to a carbon paste electrode. Mushroom tyrosinase, a member of the polyphenol oxidase family, reduces the high potential required for the detection of the alternaria mycotoxins and therefore gains specificity. The author concluded that further research was required.

Much work has been carried out at the *Dipartimento di Scienze e Tecnologie Chimiche, Universita di Roma Tor Vergata* on the electrochemical detection of aflatoxins. Ammidia *et al*., (2004) reported the development of an electrochemical screen printed device for the detection of aflatoxin B_1 in barley. This device used alkaline phosphatase as the enzyme label with a detection limit of 30 ng kg^{-1} in barley. Badea *et al*., (2004) also reported a flow injection system with electrochemical detection. In an uncommon format, free aflatoxin M_1 and aflatoxin M_1 – HRP competes for an anti-aflatoxin M_1 antibody; however the complexed anti-

aflatoxin M_1 and aflatoxin M_1–HRP is retained on a protein G column and the eluted aflatoxin M_1–HRP is detected electrochemically. The protocol reached a limit of detection of 11 ng L^{-1} in pure buffer conditions although the presence of milk required the deactivation of indigenous lactoperoxidase through heat treatment and dilution. The resulting sensor had a detection limit of 20 ng L^{-1}, however, the sensor was confined to the laboratory and not portable.

As previously stated in Section 1.3, milk is a difficult matrix to analyse. There have been some reports published on biosensors designed for analytes in milk such as Pellegrini, (2003) and Pemberton *et al.*, (1999). Pemberton *et al.* used an alkaline phosphatase based ELISA to detect progesterone in milk. By using alkaline phosphatase the products (4-aminophenol or 1-naphthol) can be detected by amperometric methods operating below +400mV. This voltage is low enough so that electro-active interfering species, such as fructose and lactose, from the milk are not activated (Mayer *et al.*, 1996). It was concluded by Pemberton *et al.* when using 1-naphthol phosphate as the enzyme substrate rather than 4-aminophenyl phosphate, better correlation with existing methods were observed.

1.6 Sensing receptors.

There are a range of different receptors that can be used as sensing layers in sensor systems. This section describes the most relevant types for this work.

1.6.1 Antibodies.

Antibodies are glycoproteins produced in response to foreign molecules or organisms in the body. Mostly they are produced by plasma cells and transported around the body by the blood system where they bind to the antigen. The affinity of the antibody towards the antigen is very high. To produce specific antibodies, a specific antigen is injected into a laboratory animal and serum samples containing the specific antibodies are collected and purified. Usually the animal will produce

many different antibodies each with different binding capabilities towards the antigen. Hence the term polyclonal is used.

Monoclonal antibodies were first synthesised in 1975 (Milstein 2000; Harlow & Lane, 1999; Kohler & Milstein, 1975). These are produced by isolating precursors of plasma cells (myeloma) and fusing them with B-cells (lymphocytes) to produce hybridoma cells (B lymphocytes). Normal B-cells have a finite lifetime; however by using them to produce hybridoma cells they become immortalised (Diamond, 1998).

In order to produce monoclonal antibodies towards a specific antigen, lymphocytes which are expected to yield specific antibodies are screened and fused with myeloma cells and then continuously produced in vitro. Monoclonal antibodies have much higher specificity towards the antigen than polyclonal antibodies (Chandrashekar & Bandyopadhyay, 2000).

Commonly when considering antibody binding, the immunoglobulin G (IgG) molecules are used since the IgG molecules have the simplest structure, a Y shape (Figure 1.14). The arms of the Y interact with the antigen and are called the Fab domain (antigen binding) whereas the base or tail interact with macrophages for transportation and is called the Fc domain (fragment that crystallises.)

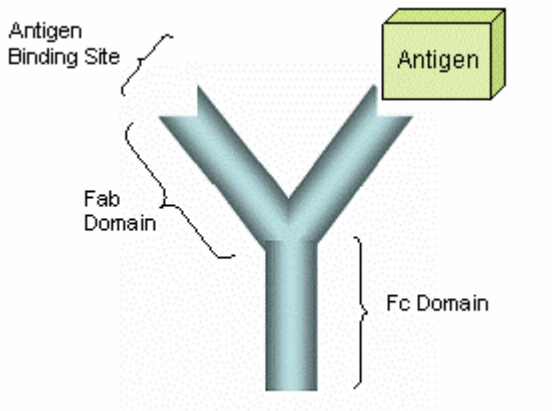


Figure 1.14: Diagram of IgG molecule.

The IgG molecule contains four polypeptides, two are called the heavy chains which consist of about 440 amino acids hence 55,000 Daltons and two are called the light chain consisting of 220 amino acids thus 25,000 Daltons. The chains are held together by disulphide bridges.

The binding of the antibody to the antigen consists of non-covalent interactions such as; van de Waals forces, coulombic interactions, hydrophobic interactions and hydrogen bonding (Harlow & Lane, 1999). This collection of interactions can make the antigen-antibody binding very specific, for instance, if two very similar antigens are present A and B, where A has an additional hydrogen bond which B does not. The strength of the interaction of A to the antigen compared to B can be 1,000 greater. Due to this great specificity shown by antibodies several techniques now employ this binding such as ELISA and immunoaffinity clean-up columns for HPLC.

1.6.2 MIPS (Molecularly Imprinted Polymers).

Molecularly imprinted polymers (MIP) have recently been researched to replace antibodies in analysis. Basically they are produced by forming a highly cross-linked organic polymer around the molecule of interest (target) then removing the target to leave a void. Figure 1.15 summaries this process. Unlike size exclusion techniques MIPs interact similarly to antibodies with the target through non-covalent bonding (Batra & Shea, 2003). MIPs have great potential since they are very robust as they are made in essence of plastic. However MIPs can suffer from either leaching of the target due to insufficient cleaning during synthesis or that they are poorly specific for one molecule from a series of homologues (Ye & Mosbach, 2001).

MIP's require a very pure analyte as a template, any impurities will obviously produce impure affinity media (Tozzi *et al.*, 2003a; Tozzi *et al.*, 2003b) and MIPs are currently only operational in non aqueous environments.

Attempts have been made to use the MIP procedure but using amino acids as the monomers (Giraudi *et al.*, 2003). As with MIP protocols the target is surrounded by amino acid monomers and then cross linked to produce peptides with memory for the target. The binding affinity for the peptide was deduced by adding the peptides to an immunoassay where the antibody was immobilised. With the addition of the peptide the concentration of antibody / antigen (target) complexes decreased. The results show that there is an advantage from MIPS that these amino acid polymers can be used in an aqueous media, however there are two distinct disadvantages, there is doubt over their stability and they cannot be fixed to a solid surface.

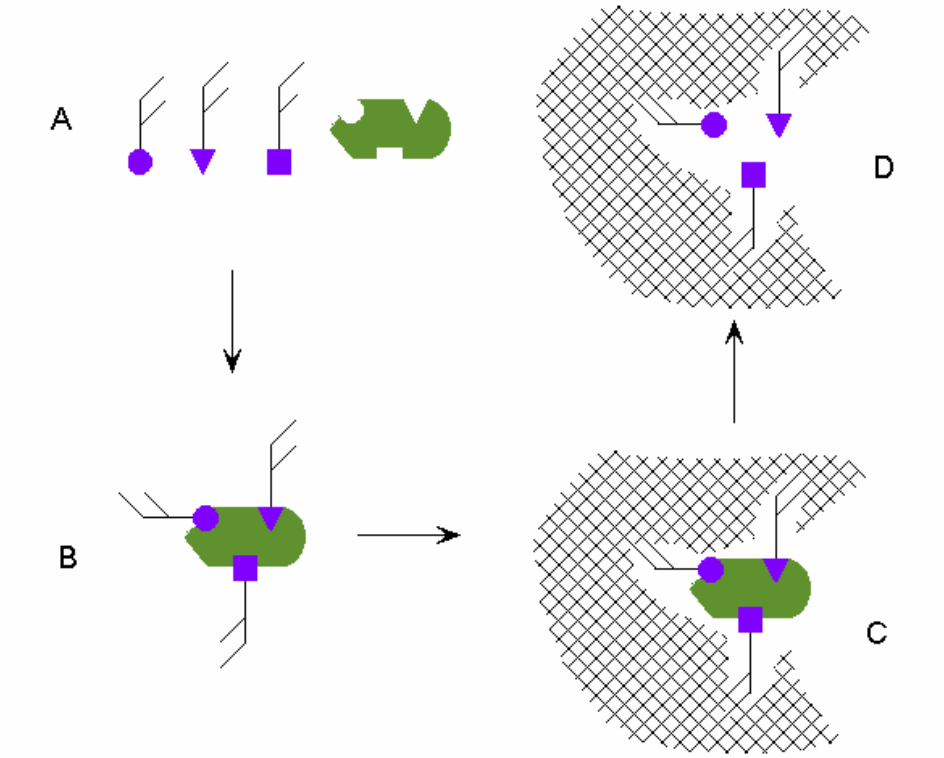


Figure 1.15: A schematic diagram showing formation of MIP.
A) Template and monomers are added together, B) assembly of monomers with the template, C) polymerisation and D removal of template.

MIPS for mycotoxin removal has been reported for zearalenone and ochratoxin A. Urraca *et al*., (2006) reported the use of a zearalenone mimic for MIP production since the aromatic ring in zearalenone becomes incorporated into the polymer during polymerization, thus blocking the binding sites and rendering the polymer with poor recognition (Urraca *et al*., 2006; Weiss, 2003). This raises concerns about the applicability of MIP technology for the development of receptors towards aflatoxins due to their highly electron rich ring structure. For ochratoxin A, Yu and Lai (2005) has reported on a MIP layer integrated onto a SPR gold surface for the detection of ochratoxin A. Unfortunately the report does not include affinity data but the sensor was capable for detecting a concentration range of 0.05 to 0.5 mg L^{-1}. Mailer *et al*., (2004) has reported on the use of a MIP as a solid phase packing material for the separation and clean-up of ochratoxin A in red wine determination. Although the MIP recognised ochratoxin A, the recovery rate was <66% due to polar acidic compounds in red wine. Upon the pre-treatment of red wine with a conventional C18 column the recovery increased to >90% showing that MIP technology can aid extraction techniques but cannot be solely employed. Maier *et al*. (2004) used a template mimic perhaps causing the low selectivity. MIPS have been developed and designed by computer molecular modelling methods by Cranfield University. Turner (2004) reported the development of MIPS for Ochratoxin A analysis, however with detection levels at 50 – 100 µg L^{-1}, further work needed to be carried out on the monomers employed to be able to detect the EU maximium permissible limits of 1 µg L^{-1}.

1.6.3 Peptides.

Peptides are compounds made from arrangements of 10 or less amino acids. There are over 500 amino acids in nature however only 20 amino acids are observed in all species, hence there are a vast number of peptides (Berg *et al*., 2006). As previously stated the biologically active site of the antibody is a polypeptide. Thus it is possible to recreate the affinity of the antibody by using a

peptide sequence (Welling *et al.*, 1990). There are many documented reports were peptides have been specifically synthesised to react with molecules of interest such as; nucleic acids (Gutte *et al.*, 1979), DDT (Moser *et al.*, 1983), estradiol (Giraudi *et al.*, 2003; Giraudi *et al.*, 1999a), estrogen (Tozzi *et al.*, 2002) and aflatoxins (Tozzi *et al.*, 2003b).

None of these studies have used anything other than the 20 common amino acids hence by additionally incorporating other amino acids, the peptide's affinity and stability may be improved. There is a great interest into the use of unnatural amino acids in the pharmaceutical industry. These unnatural amino acids are divided into two groups α and β. α amino acids have been used more than the β unnatural amino acids in order to improve availability and dynamics, and also used to reduce conformational flexibility (Ma, 2003). Recently there is increasing interest into the use of the β unnatural amino acids.

Potentially there are many advantages for using peptides rather than antibodies. For nature to remove the antibody once the antibody is attached to the antigen many other interactions have to occur (Fc interactions). It is possible that these other functions on the antibody causes a decrease in the specificity of the antibody to the antigen (Welling *et al.*, 1990).

With the current method of producing antibodies, variations in the quality and concentration of the antibodies occur between each batch (Tozzi *et al.*, 2003a) whereas since the peptide is synthesised completely by instrumentation the reproducibility will be increased as well as being prepared faster than antibodies. Peptides are also more stable than antibodies, and immunity against low molecular weight analytes such as patulin are difficult to produce (Nakamura *et al.*, 2001).

Peptides should have an advantage over MIPs as molecular receptors since they are more flexible than MIPs.

In the work by Tozzi *et al.*, (2003b) they used only eight different amino acids. They created a combinatorial library by placing the eight different amino acids in the wells on a micro titre plate in rows and then they added a second amino acid in columns in order to produce 64 different combinations. Each dipeptide was screened for the best binding properties towards the aflatoxin. To evolve the peptide further the best binding dipeptide (Leu Leu) was added to each well on the micro titre plate and then the plate was filled again with each of the eight amino acids in row and columns to form another 64 different combinations this time consisting of tetrapeptides. It was reported that the best tetrapeptide was Leu Leu Ala Arg, which had selectivity equal to the commercial antibody.

Katayama (2000) has reported using peptides as ion channel mimics. Although no application towards the aflatoxin has yet been reported, it is an area worth reviewing. A gold electrode is coated with a peptide and placed into an anionic solution. Upon the addition of the analyte of interest (cyclic AMP) the analyte binds to the peptide which in turns blocks the surface of the electrode to the anionic solution. This blocking action causes a change in the response on the electrode. Figure 1.16 shows this principle.

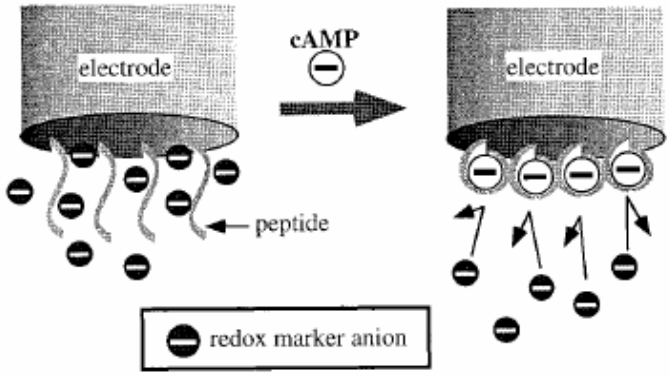


Figure 1.16: Schematic diagram of a ligand gated ion channel or 'Ion Channel Mimic' (Reproduced from Katayama, 2000).

1.7 Receptor design using computational methods.

Molecular modelling or computational chemistry is a technique which is becoming more popular. The cost of the technique is decreasing as computers are becoming

cheaper and more powerful and in turn the programs have evolved with simpler user interfaces allowing chemists access to such tools rather than computer specialists.

Since the 1980's many *de novo* design computer programs have been employed within the pharmaceutical industry to aid drug design. Drugs for HIV-1 protease inhibitors, for example, have been successfully designed and now marketed (Wlodawer & Vondrasek, 1998).

1.7.1 Peptide design using computational methods.

In this project, a peptide is to be designed and computationally tested. The peptide will be created from a minimum of twenty different amino acids and will be at least six residues in length. The number of different combinations thus is 20^6. Using the data from Abagyan and Totov (2001) this would suggest that in order to dock all the possible combinations of peptide then it would take about 120 years to screen. Subsequently Zsoldos *et al.*, (2003) has suggested a new docking method which can dock 10000 ligands in 16459 seconds. Using this rate the library would take 3.3 years, however there is still concern over the validity scoring function, furthermore this example suggests use of only twenty different amino acids, to improve interaction and stability, unnatural amino acids may be incorporated into the design.

A function called genetic search algorithm can screen through the library in an 'intelligent' approach. Leapfrog (Tripos) utilises this principle.

Leapfrog is a '*de novo*' ligand design program which creates ligands in the active site of the receptor (Bertelli *et al.*, 2001). First a program called GRID (Goodford, 1985; Moon and Howe, 1991) searches the receptor for interaction sites by using a small probe of; methyl, hydroxyl or carbonyl-oxygen. This data is then used to

describe the desirable orientations of the methyl, hydroxyl or carbonyl groups of the ligand (Goodman, 1998).

Then the ligand is built by placing fragments and linking together. The genetic algorithm calculates a binding score of the total of the contributions from steric, electrostatic and hydrogen bonding interactions, and records the score. A slight variation of the ligand is produced and the ligand is scored again, if the new ligand has a more favourable interaction than the parent then the parent is dismissed and the sibling is stored (Payne and Glen, 1993).

This principle of preservation or the survival of the fittest is likened to Darwin's natural selection (1872). In summary Darwin stated, '*it leads to the improvement of each creature in relation to its organic and inorganic conditions of life; and consequently, in most cases, to what must be regarded as advancement in organisation.*' Genetic algorithms are frequently employed in docking programs, such as Flexidock (Tripos) (Bertilli, *et al.*, 2001; Payne and Glen, 1993).

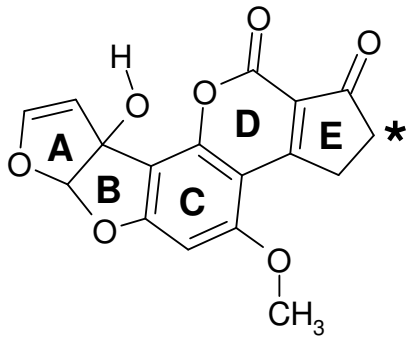
There are disadvantages of genetic algorithms. Again the scoring function frequently stores a significant number of false positive structures and some of the structures produced by *de novo* programs are synthetically difficult to produce (Schneider and Böhm, 2002; Bohacek *et al.*, 1997).

From an extensive literature search only one author has been discovered whom has used computational modelling for sensor development. Mascini *et al*. in 2004 published a report on a 5 residue peptide optimised using the *de novo* program leapfrog to act as a receptor for the detection of dioxins. It should be noted however that 4 residues of the peptide had already been discovered to have affinity by Kobayashi *et al.*, (1999) by analysing dioxins affinity in biological systems, therefore the sequence was not truly prepared from first principles by the leapfrog

program. Additionally the peptide developed was covalently attached at both ends, therefore not projecting into solution but activating as a reactive surface.

1.7.2 Previous Molecular Modelling Work.

Previous molecular mechanics work has been done on aflatoxin M_1 to produce a better understanding of the ideal attachment of the aflatoxin to the protein to form a specific antibody towards aflatoxin M_1 (Holtzapple *et al.*, 1996). It was concluded that the aflatoxins can be split into three conformational/chemical groups. Group one consists of aflatoxin M_1, B_1 and G_1, although chemically different, the rings B, C, D and E are all in the same plane with ring A at 102^{O} from the plane. The presence of the hydroxide group does not change the bond angle. Figure 1.17 shows the structure of aflatoxin M_1 and the labelling of the rings.



* point of attachment

Figure 1.17: Labelling the rings of aflatoxin M_1 and the idealistic point of attachment for covalent bonding to a protein for production of an immunogen.

Group two contains aflatoxin M_2, B_2 and G_2, these again have rings B, C, D and E in the same plane and ring A at the same angle however with the loss of the double bond in ring A, a bend or kink is formed in the ring which is not present in group one.

The third group consists of the derivatives $B_2\alpha$ and $G_2\alpha$, where rings B, C, D and E are still in the same plane, ring A is significantly altered due to the loss of the double bond and the insertion of a hydroxide group. From these observations it is

clear to see that the conformation of ring A changes the most throughout the groups, thus this ring should be furthest from the point of attachment when producing an antibody. In addition, when comparing the chemical structure of aflatoxins B and M the only difference is the extra hydroxide group on ring A.

1.8 Aims and Objectives.

The work carried out in this project was part of a European Union collaborative project, GOODFOOD (FP6-IST-1-508744-IP) which is an integrated project with aims at developing a new generation of analytical methods based on micro and nanotechnology solutions for the safety and quality assurance along the food chain in the agrofood industry.

The GOODFOOD project was split into 8 workgroups in which this project was part of the WP3 workgroup. Workgroup 3 was founded with the aim to develop microsystems technology solutions for the rapid detection of toxigenic fungi and mycotoxins by natural bioreceptors, artificial receptors technology and nano-electrode devices.

The attention of this thesis has been focused on the development of artificial and bioreceptors for the rapid detection of the mycotoxin aflatoxin M_1. The objectives of this project were:

- Sourcing a commercially available antibody and verifying its affinity to aflatoxin M_1 through development of an indirect ELISA protocol.
- Production in-house of screen printed electrode and characterisation of the sensors towards use as an immunosensor.
- Development of the screen printed immunosensor for aflatoxin M_1 utilising the developed ELISA protocol as a foundation.
- Optimisation of the screen printed immunosensor for detection of aflatoxin M_1 to meet the maximum permissible limits imposed by the European Union foods standards committee.
- The analysis of real milk samples with the developed immunosensor and the determination of pre-treatment strategies.
- Transfer of the screen printed electrode immunosensor protocol to microelectrodes to achieve high detection senstivity.
- Development of synthetic peptide receptors through computational chemistry by building a virtual receptor library. Screening the library for peptides with high affinity towards aflatoxin M_1 using computational docking programs.
- Validating the affinity of the synthetic peptides using laboratory instrumentation.
- Substitution of the bioreceptor used in the sensor for the synthetic receptor.

An outline of the different stages undertaken in this thesis to meet the aims and objectives is shown in the flowchart below (Figure 1.18).

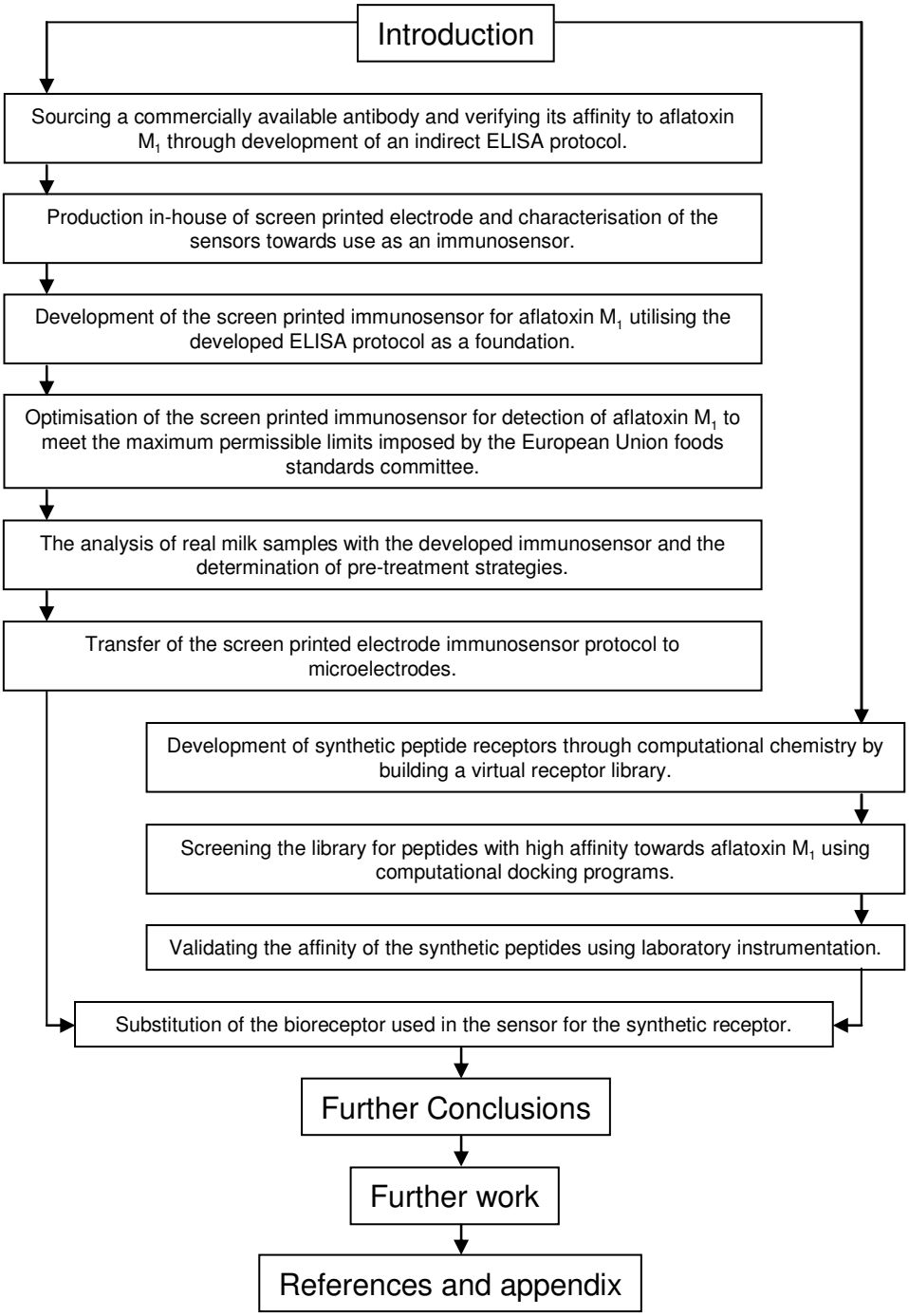


Figure 1.18: Flowchart detailing the different stages of the work in this thesis.

CHAPTER 2

DEVELOPMENT OF THE ELISA PROTOCOL AND VALIDATION OF THE ANTIBODY.

2.1 Introduction.

The initial stage of the project was to source and test an antibody for use as a sensing receptor in the electrochemical affinity sensor. The antibody's suitability was validated by designing and optimising an ELISA protocol using the antibody with spectrophotometric methods. Once the antibody was validated it was then implemented in the electrochemical sensor.

A heterogeneous indirect immunoassay format was chosen for the assay configuration since this is a common format for the analysis of small analytes, when the more common sandwich format is not suitable due to the small size of the analyte.

Initially the work published by Ammida *et al.* (2004) was used as a foundation, however the system was optimised for the antibodies being used, by changing the concentration and time of incubation. Furthermore the blocking agent was optimised and compared to other agents available. Lastly milk was studied in the system to determine whether there would be any matrix effects and how to overcome these problems.

2.2 Materials and Methods.

2.2.1 Materials.

A search was carried out to find an antibody with affinity towards aflatoxin M_1. Abcam Ltd. (Cambridge, UK) produces a rat monoclonal antibody raised against aflatoxin M_1 – BSA as the immunogen. Upon delivery of the antibody solution (1 mg mL^{-1}) the contents were split into 5 µL aliquots and stored at -18^{o}C to avoid repeat thaw – freeze cycles which reduces the antibody activity. A secondary antibody with alkaline phosphatase (polyclonal goat) enzyme was also obtained from Abcam Ltd (1 mg mL^{-1}).

Aflatoxin M_1 was obtained from Alexis Biochemicals (Nottingham, UK), made up in methanol and split into aliquots and stored under nitrogen at -18^{o}C. Working standards were made from the stock using 1% methanol in 10 mM PBS. Aflatoxin M_1- BSA from Sigma-Aldrich Co. Ltd. (Gillingham, UK) was diluted in carbonate buffer (pH 9.6) and stored as aliquots at -18^{o}C. Aflatoxin M_1 RIDASCREEN assay kit was purchased from R-Biopharm (Glasgow, UK).
Blocking agents, polyvinyl alcohol 50,000 Da (PVA) and polyvinyl pyrrolidone 360,000 Da (PVPP), p-nitrophenyl phosphate (pNPP) enzyme substrate for the alkaline phosphatase enzyme, 10% sodium hypochlorite for aflatoxin M_1 decontamination and all other general chemicals were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, UK).

ELISA experiments were measured using a BMG Fluorstar galaxy ELISA plate reader (Aylesbury, UK). The micro well plates were Nunc Immuno plates supplied by Fisher Scientific (Loughborough, UK).

2.2.2 General protocol for ELISA.

Optimisation experiments used the checkerboard design as detailed by Crowther (2001). Initially the concentrations of antibodies and buffers, incubation times and blocking agents described by Ammida *et al*. (2004) were used and then each component of the system was optimised further. Table 2.1 shows the parameters which were altered. Additionally, the time of the blocking was also investigated using the checkerboard design. This was altered from 2 hours to 15 minutes.

Table 2.1: The different reagent concentrations used to optimise the ELISA system.

Component	**Concentration range**
Anti-aflatoxin M_1 antibody	4 to 0.05 µg mL^{-1}
Alkaline phosphatase labelled antibody	4 to 0.2 µg mL^{-1}
Aflatoxin M_1 – BSA	200 to 2.5 ng per well
Blocking buffers (PVA and PVPP)	2 % to 0.25%

The ELISA system was also optimised by producing calibration charts using a series of standards of aflatoxin M_1 ranging from 10,000 ng L^{-1} to 20 ng L^{-1} in 1% methanol, 99% PBS. Three experiments were carried out; the concentration of anti-aflatoxin M_1 was varied from 4 to 0.05 µg mL^{-1}, the concentration of alkaline phosphatase labelled antibody was varied from 2 µg mL^{-1} to 1 µg mL^{-1} and the time of the incubation for the anti-aflatoxin M_1 was varied from 2 hours to 30 minutes.

Throughout the investigation the antibodies were diluted in 10 mM PBS (phosphate buffered saline) pH 7.4 buffer and the aflatoxin M_1 - BSA was diluted in a 0.1 M carbonate buffer pH 9.6.

With the exception of washing before the addition of pNPP substrate, the washing solution used was 0.05% Tween in 10 mM PBS buffer (pH 7.4). For the washing before the addition of pNPP, a 0.05 M Tris buffer was used at pH 7.5 since phosphate based buffers inhibit alkaline phosphatase.

Polyvinyl alcohol and polyvinyl pyrrolidone blocking solutions were made using cold reverse osmosis water.

The coating of the micro well plate was done at 4^{o}C overnight. All other incubations were performed at 25^{o}C. During the incubation of the antibodies, the plate was shaken using a Labsystems iMES plate incubator at 400 rpm, 25^{o}C. The plate was measured at 405 nm, 45 minutes after the addition of pNPP.

Calculations of analytical sensitivity were determined as described by Ammidia *et al*., (2004) and Draisci *et al*., (2001) as the amount of aflatoxin M_1 required to reduce the signal change by 25%.

All glassware and consumables were decontaminated from aflatoxin M_1 by soaking in 10% sodium hypochlorite for 48 hours. Then an equal amount of 5% aqueous acetone was added and left for three hours. The mixture was disposed of as general hazardous waste. This procedure is recommended by AOAC (Association Of Analytical Communities) official methods of analysis (AOAC, 1996).

The developed ELISA method was performed as follows. A 50 µl aliquot of 0.2 mg L^{-1} aflatoxin M_1 – BSA solution in 0.1 M, pH 9.6 carbonate buffer was added to the bottom of a microwell and stored at 4^{o}C overnight. After washing twice with 0.05 % Tween 20 in 10 mM PBS (pH 7.4) and once with reverse osmosis water, 50 µl of 1% PVPP in water was added and incubated at room temperature for 90 minutes. After washing again with Tween 20 and water as previously described, 25 µl of sample or standard was added to the well and incubated for 5 minutes at room temperature before the addition of 25 µl of the anti-aflatoxin M_1 antibody diluted in PBS to 1.0 µg ml^{-1}. The microwell plate was shaken during incubation, and after 90 minutes was washed again as previously described with Tween 20 and water. After washing, 50 µL of 2.0 µg ml^{-1} alkaline phosphatase labelled antibody diluted in 10 mM PBS, pH 7.4 was added to the plate and shaken at room temperature for 60 minutes. After washing with Tween 20 twice and once with 0.05 M Tris buffer, pH 7.5, pNPP substrate system was added to the microwell plate and was allowed to develop for 45 minutes at room temperature before measurement at 405 nm with a BMG Fluorstar galaxy ELISA plate reader.

2.3 Results and Discussions from ELISA development.

2.3.1 Investigating the sources of anti-aflatoxin M_1 antibody.

A search was performed to find suppliers of an anti-aflatoxin M_1 antibody. Also a literature search was executed to locate where previous authors had sourced their antibodies.

Of the reported ELISA methods for aflatoxin M_1, the authors had either used the commercial kit from R-biopharm or had produced their own supplies. No other report could be found where an ELISA system had been developed using a commercial antibody. Abcam Ltd (Cambridge, UK) was found to supply a monoclonal antibody for aflatoxin M_1 and therefore this antibody was used throughout this investigation.

2.3.2 Development of ELISA method without using free aflatoxin M_1.

The first studies were undertaken to optimise the system so that the maximum signal would be obtained with the absence of free aflatoxin M_1. Using a checkerboard experiment design, as described by Crowther (2001), different concentrations of the BSA-aflatoxin M_1 conjugate (from 250 ng per well to 2.5 ng per well) were added to the ELISA plate with each column containing a different concentration. Figure 2.1 shows a schematic of the micro well plate and concentrations used in this work.

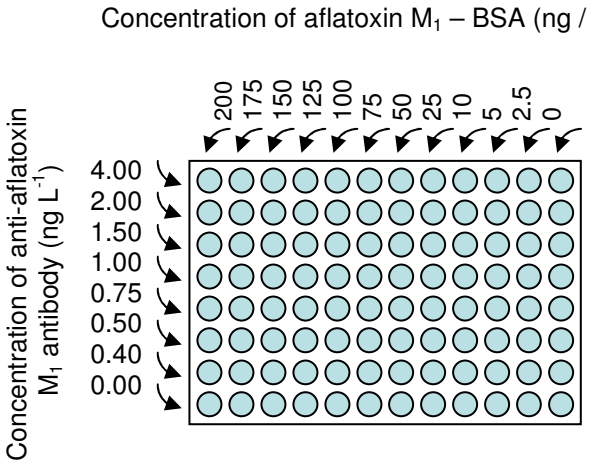


Figure 2.1: Schematic diagram showing the principle of the checkerboard design used to develop and optimise the ELISA test.

After incubation and blocking, different concentrations of the anti-aflatoxin M_1 antibody were used (from 4 µg mL^{-1} to 0.4 µg mL^{-1}) and added in rows. Thus each well had a different permutation of the amount of anti-aflatoxin M_1 and BSA-aflatoxin conjugate. The results obtained are shown in Figures 2.2, 2.3 and 2.4.

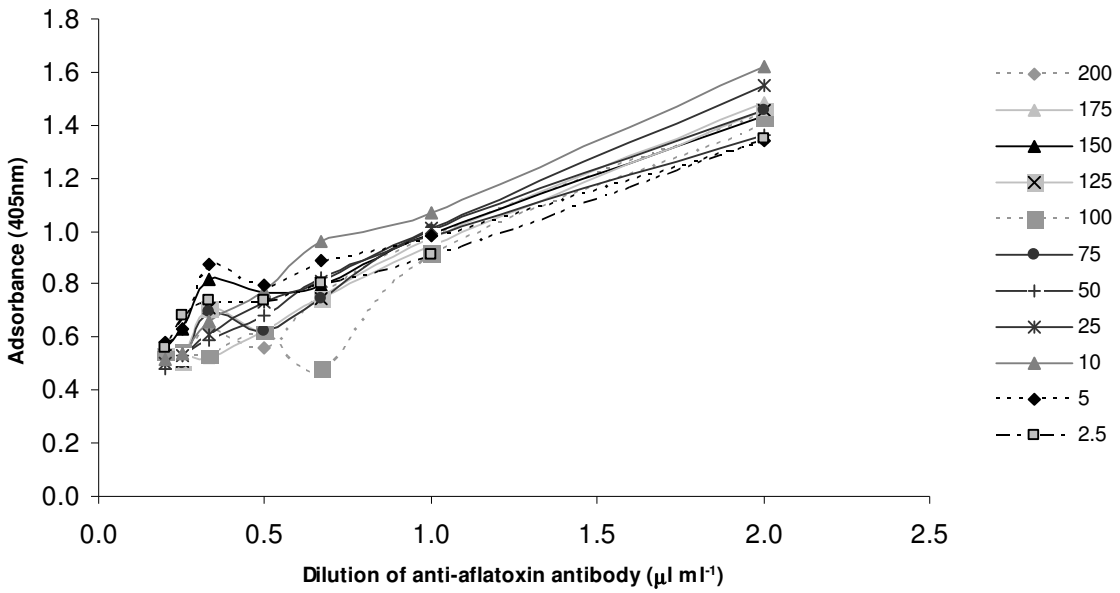


Figure 2.2: Optimisation of the signal by varying the anti-aflatoxin antibody concentration (all data shown).

The graph in Figure 2.2 was obtained by varying the aflatoxin M_1 - BSA concentration from 0 to 200 ng L^{-1} and varying the anti-aflatoxin M_1 antibody from 0.2 to 2 µg mL^{-1}. Alkaline phosphatase labelled antibody concentration was 1 µg mL^{-1}. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. PVA blocking (1% PVA) was carried out at room temperature for 1 hour as were both antibody incubations. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

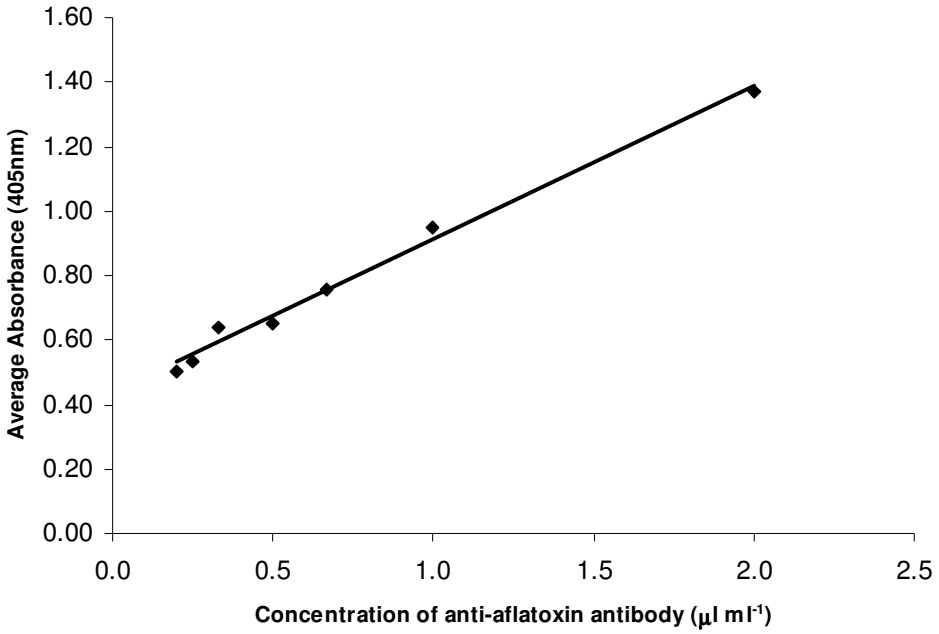


Figure 2.3: Optimisation of the signal by varying the anti-aflatoxin antibody concentration (mean only shown).

For the data shown in Figure 2.2 each plot contains the results for a different concentration of aflatoxin M_1 - BSA, however the change in dilution of anti-aflatoxin M_1 antibody is common to all the plots. Therefore, the mean of the value for the dilution of anti-aflatoxin M_1 antibody is shown in Figure 2.3 but standard deviations cannot be produced since the experiment is not repeated exactly, and hence error bars are not shown. This approach of development was recommended by Crowther (2001). The data from Figure 2.3 shows a significant difference between 2 µg mL^{-1} and 1 µg mL^{-1} dilution is observed; hence subsequent experiments used a concentration of 1 µg mL^{-1}.

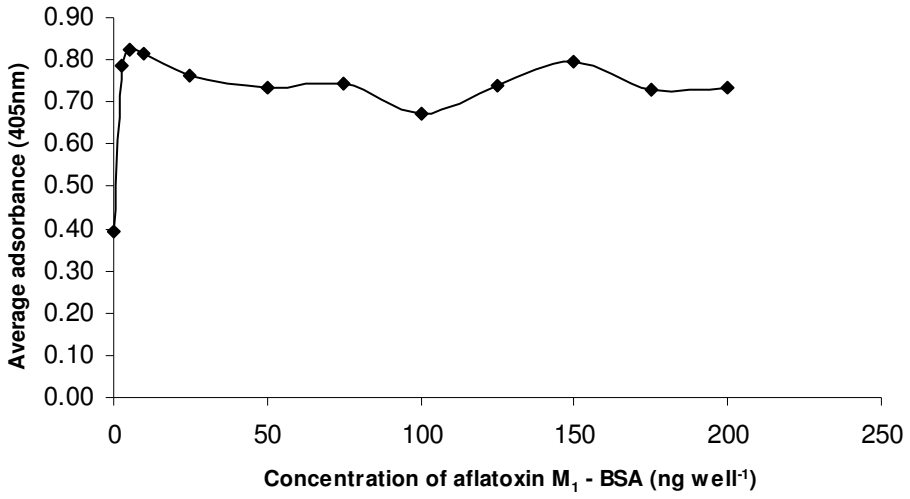


Figure 2.4: Optimisation of the signal by varying the amount of aflatoxin M_1 - BSA coated per well.

Figure 2.4 was obtained from the same run as Figures 2.2 and 2.3. Figure 2.4 shows that there is a plateau in the signal from 25 ng per well and above. Therefore the binding capacity of the surface for aflatoxin M_1 - BSA is 25 ng per well or 39.7 ng cm^{-2} (surface area of well for 50 µL is 0.63cm^2). For further experiments a slightly higher concentration of 50 ng was used to allow for some errors in reproducibility and degradation of the protein. A 50 ng per well concentration was also used by De Boevere and Peteghem (1993). The use of carbonate buffer as an immobilisation buffer was recommended by Crowther (2001) as a standard method of immobilisation and was also followed by Magliulo *et al.* (2005).

This method of immobilisation using absorption is common to all authors who have developed ELISA systems for the aflatoxins with the exception of Pestka *et al.*, (1980) where they immobilised the BSA conjugate using covalent attachment through glutaraldehyde, dried using compressed air and storing the plates in a desiccator for two weeks. The immobilisation performed by Pestka *et al.* (1980) allows the plates to be stored at room temperature whereas other authors (El-

Nezami *et al*., 1995; Wild *et al*., 1987) require refrigerated storage of plates. In this project storage of plates was not considered since the ELISA development was only to characterise the antibody.

In the second experiment the amount of alkaline phosphatase labelled antibody was optimised whilst changing the anti-aflatoxin antibody by again using the checkerboard procedure. Figures 2.5 and 2.6 show the results achieved.

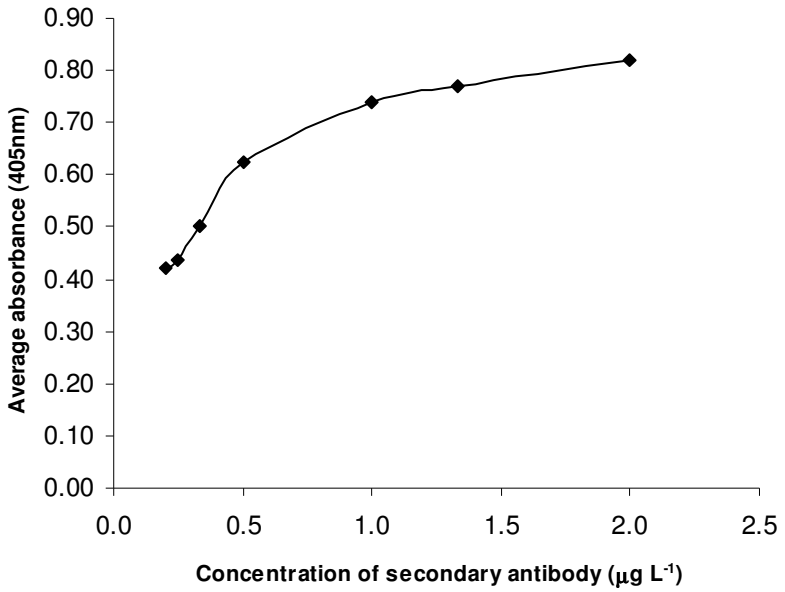


Figure 2.5: The optimisation of the alkaline phosphatase labelled antibody applied in the test.

Figure 2.5 was obtained by varying the alkaline phosphatase labelled antibody concentration from 0.2 to 2.0 µg mL^{-1}. Aflatoxin M_1 - BSA concentration was 50 ng per well and anti-aflatoxin M_1 antibody concentration was 1.0 µg mL^{-1}. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4°C. PVA blocking (1% PVA) was carried out at room temperature for 1 hour as were both antibody incubations. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

The trend in Figure 2.5 is expected since the more labelled enzyme present, the greater the signal produced. In order to keep costs down but still give a respectable signal a dilution of 1 µg mL^{-1} was chosen during optimisation of incubation times. However, as with the anti-aflatoxin M_1 antibody, the optimum concentration was re-evaluated using a competition assay.

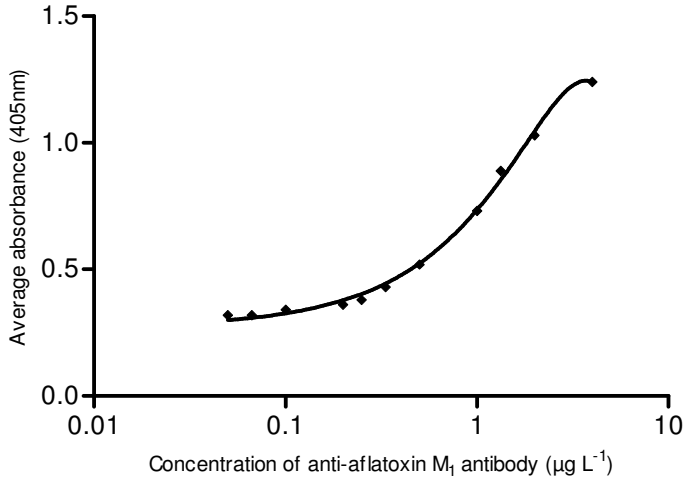


Figure 2.6: Optimisation of the ELISA by varying the amount of anti-aflatoxin M_1 antibody.

Figure 2.6 was obtained by varying the anti-aflatoxin M_1 antibody concentration from 0.05 to 4.00 µg mL^{-1}. Aflatoxin M_1 - BSA concentration was 50 ng per well and Alkaline phosphatase labelled antibody concentration was 1.00 µg mL^{-1}. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4°C. PVA blocking (1% PVA) was carried out at room temperature for 1 hour as were both antibody incubations. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

Figure 2.6 is a repeat and extension of Figure 2.3 to determine the point of inflection of the higher plateau. It is shown that the more anti-aflatoxin M_1 antibody used the higher the signal. However due to the cost of the antibody the plateau could not be determined. A concentration of 2 µg mL^{-1} was used for further

experiments since this level is about 80% of the highest signal. However if this level did not meet the required limits of detection, then the anti-aflatoxin M_1 antibody levels could be re-evaluated.

Following the work of Ammida *et al*. (2004) a PVA blocking agent was used. The immunogen used for the anti-aflatoxin M_1 antibody was aflatoxin M_1-BSA. BSA is a common blocking agent. It is a component of the immunogen for the Abcam anti-aflatoxin M_1 antibody, therefore non-specific binding could be observed resulting in high background noise. BSA can also contain Bovine IgG, causing interferences (Crowther, 2001), therefore the use of BSA was avoided. Another frequently used blocking agent is casein. Casein is a milk protein and studies have shown that aflatoxin M_1 resides in the hydrophobic cavities of this protein, therefore the use of casein has also been avoided (Bakirci, 2001). Fish gelatine has been used in the past as a blocking agent (Lee *et al.*, 2004; Crowther, 2001). However, since the end product is required to be robust, polymers were investigated as the blocking agent due to their stability rather than proteinaceous blocking agents.

The use of polymers as ELISA blocking agents is an increasing area of research (Micheli *et al.* 2005; Ammida *et al*, 2004). Work carried out by Studentsov *et al.*, (2002) compared using PVA against using PVPP. The results showed that PVPP provided better sensitivity limits. Thus the two polymers were compared against each other in the next experiment. Different concentrations of PVPP and PVA were used as well as varying the time allowed for the blocking. Figures 2.7 and 2.8 show the results.

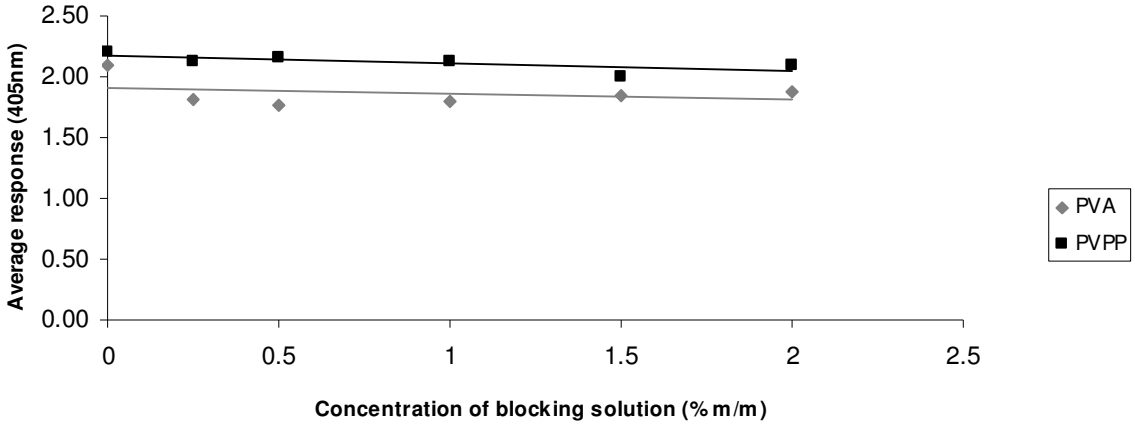


Figure 2.7: The determination of the best blocking agent and concentration for use as a blocking buffer.

Figure 2.7 was obtained by varying the PVA and PVPP concentrations from 0.25 to 2.0 % (weight by weight). Blocking was carried out at room temperature for 5 minutes to 2 hours. Aflatoxin M_1 - BSA concentration was 50 ng per well and anti-aflatoxin M_1 antibody concentration was 2.0 µg mL^{-1}. Alkaline phosphatase labelled antibody concentration was 1.0 µg mL-1. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Both antibody incubations were performed at room temperature for 1 hour. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

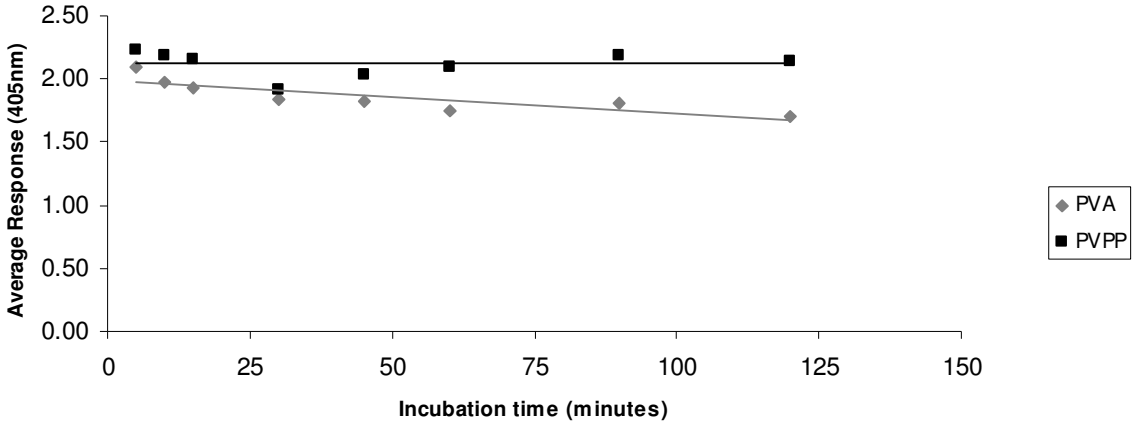


Figure 2.8: Optimising the incubation time required to achieve blocking.

Figure 2.8 was obtained using the same data as Figure 2.7.

From both Figures 2.7 and 2.8, it can be seen that a better signal response is observed using PVPP rather than PVA. From Figure 2.7, little difference is observed when using different concentrations of PVPP, so a value of 1% was used to give tolerance and allow for any decrease in concentration which may occur due to polymerisation of the blocking solution during storage, which was observed during use.

2.3.3 Optimisation of the ELISA system using the free aflatoxin M_1.

After optimising the ELISA reagents and assay procedure, the method was used to test the analyte (aflatoxin M_1) in buffer solutions. The first experiment with free aflatoxin M_1 was to perform a calibration curve experiment from 10,000 ng L^{-1} (parts per trillion) to 20 ng L^{-1}. The free aflatoxin M_1 and the anti-aflatoxin M_1 antibody were premixed in an Eppendorf tube, in triplicate, and then added to the ELISA plate. The transfer procedure involving 36 Eppendorf tubes took a substantial amount of time to perform and with the incubation time for the anti-aflatoxin M_1 antibody being just 60 minutes, a considerable error occurred hence a high standard deviation can be seen when plotting the graph with error bars. This is shown in Figure 2.9.

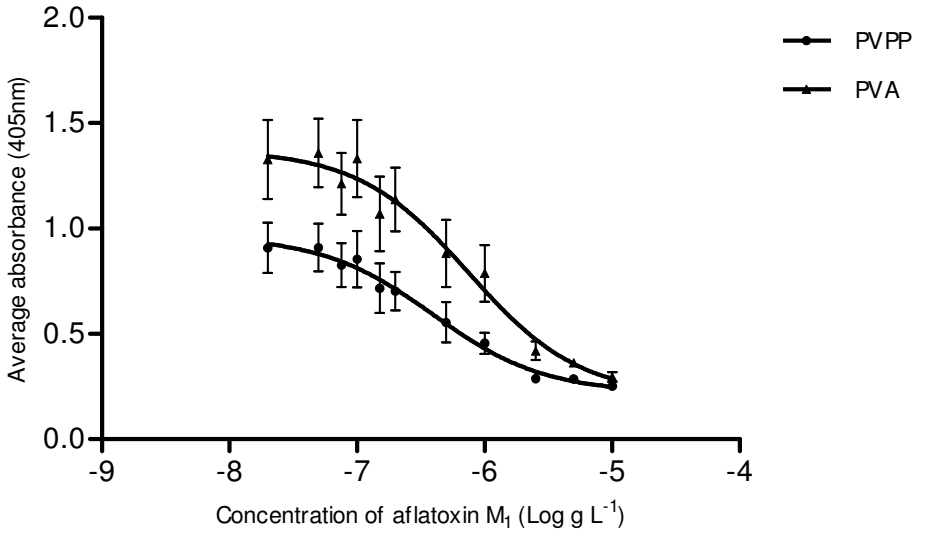


Figure 2.9: The first attempt at a calibration graph using the developed ELISA method.

Figure 2.9 was obtained with an aflatoxin M_1 - BSA concentration at 50 ng per well and anti-aflatoxin M_1 antibody at 2.0 µg mL-1. Alkaline phosphatase labelled antibody concentration was 1.0 µg mL-1. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Both antibody incubations were performed at room temperature for 1 hour. Blocking with either PVA or PVPP (1%) was performed at room temperature for 90 minutes. pNPP substrate system was added to the system and allowed to develop for 45 minutes. (Error bars show standard deviations, n=3).

Again it can be seen that PVPP gives a better response than PVA, so PVPP was used for all subsequent experiments.

To improve the reproducibility the experiment was repeated, however, the free aflatoxin M_1 was first placed into the micro wells on the ELISA plate then the anti-aflatoxin M_1 antibody was added. Previously, the free aflatoxin M_1 and anti-aflatoxin M_1 was pre-mixed to gain sensitivity. This reduced the amount of transfer time and improved the repeatability as shown in Figure 2.10.

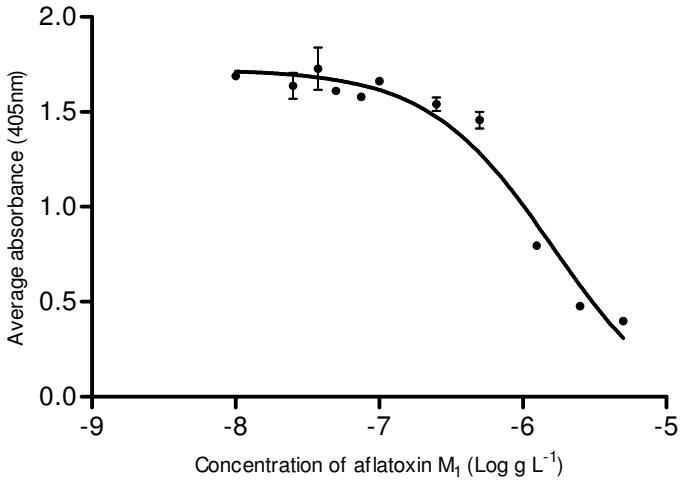


Figure 2.10: Repeat of the calibration performed by ELISA.

Figure 2.10 was obtained using an aflatoxin M_1 - BSA concentration of 50 ng per well and anti-aflatoxin M_1 antibody of 2.0 µg mL-1. Alkaline phosphatase labelled antibody concentration was 1.0 µg mL-1. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Both antibody incubations were performed at room temperature for 1 hour. Blocking with PVPP (1%) was performed at room temperature for 90 minutes. pNPP substrate system was added to the system and allowed to develop for 45 minutes. (Error bars show standard deviations, n=3).

It can be seen from Figure 2.10 that at the lower aflatoxin M_1 concentrations there is little difference between each concentration so this procedure reduced the assay sensitivity.

The European Commission maximum permissible limit for aflatoxin M_1 in milk is 50 ng L^{-1}. Additional work was done to try and 'fine tune' the assay so that the dynamic range included this level.

The concentration of the anti-aflatoxin antibody was reviewed, on the basis of the understanding that a lower amount of antibody may cause a greater competition

between the free aflatoxin and the BSA-aflatoxin conjugate as observed by El-Nezami (1995). Figure 2.11 shows the results.

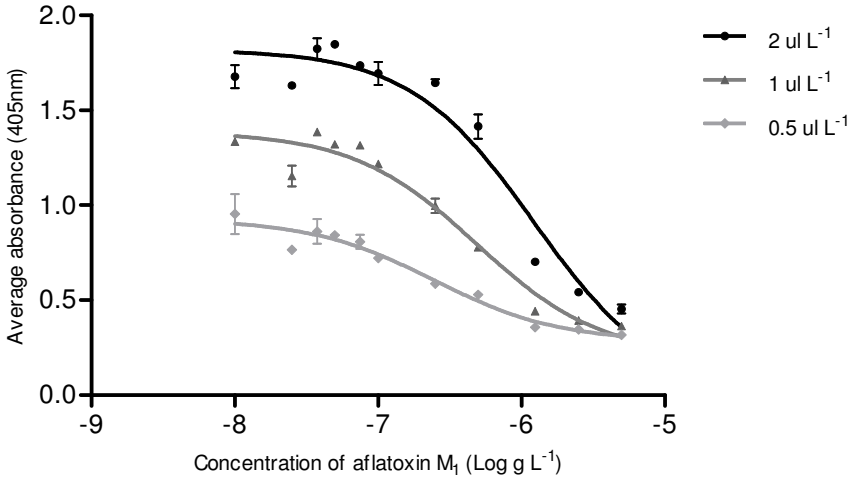


Figure 2.11: Optimising the concentration of the anti-aflatoxin M_1 antibody in the presence of the free aflatoxin M_1.

Figure 2.11 was obtained using various anti-aflatoxin M_1 antibody concentration from 0.5 to 2.0 µg mL-1. Aflatoxin M_1 - BSA concentration was 50 ng per well. Alkaline phosphatase labelled antibody concentration was 1.0 µg mL-1. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Both antibody incubations were performed at room temperature for 1 hour. Blocking with PVPP (1%) was performed at room temperature for 90 minutes. pNPP substrate system was added to the system and allowed to develop for 45 minutes. (Error bars show standard deviations, n=3).

To be able to detect 50 ng L^1, the standard curve must be able to distinguish between 50 ng L^{-1} and lower concentrations. The 0.5 µL L^{-1} plot does this, however, the signal is low. The 2 µL L^{-1} plot has the levels at 100 and 75 ng L^{-1} significantly higher than the lower concentrations; hence the use of a 1 µL L^{-1} dilution was investigated further.

The incubation time of the anti-aflatoxin M_1 antibody had not been studied. Figure 2.12 shows the effects of increasing the incubation time on the signal and specificity.

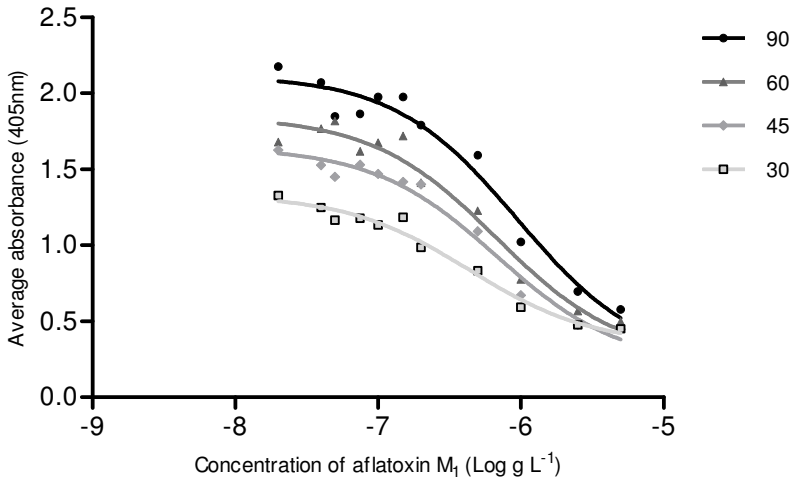


Figure 2.12: The effect of different incubation times of the anti-aflatoxin M_1 antibody and the sample on the ELISA signal.

Figure 2.12 was obtained using an anti-aflatoxin M_1 antibody concentration of 1.0 µL g^{-1} and the time of incubation was varied from 30 to 90 minutes at room temperature. Aflatoxin M_1 - BSA concentration was 50 ng per well. Alkaline phosphatase labelled antibody concentration was 1.0 µL g^{-1} and incubated at room temperature for 1 hour. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Blocking PVPP (1%) was performed at room temperature for 90 minutes. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

Figure 2.12, shows the maximum signal is obtained with the longest incubation time; however the noise has increased with increasing the incubation time. The data for 30, 45 and 60 minutes shows that there is a detectable difference between 50 ng L^{-1} and lower concentrations, however for these values the signal is low, hence the difference is slight. As an experiment 90 minutes was chosen.

The concentration of the alkaline phosphatase labelled antibody was checked. If there is not enough of this antibody in the assay, then this might be a cause of why the lower end reached a plateau. Figure 2.13 shows the results from this investigation.

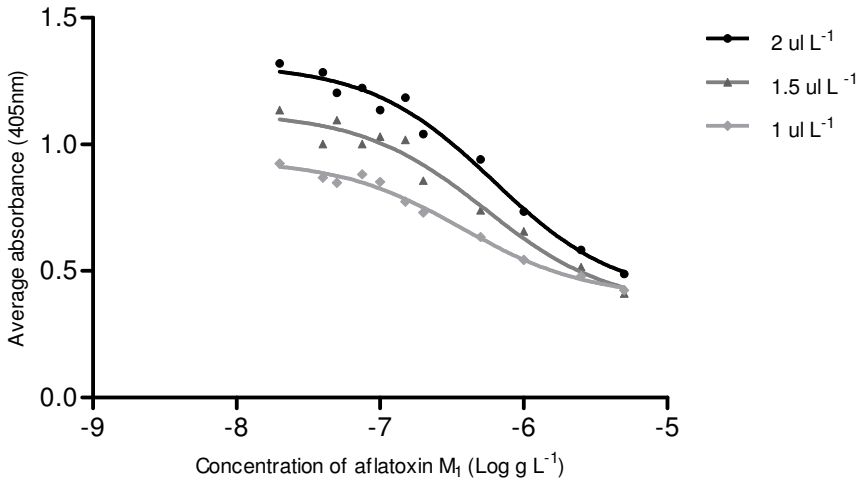


Figure 2.13: The effect of changing the concentration of the alkaline phosphate labelled antibody.

Figure 2.13 was obtained by varying the alkaline phosphatase labelled antibody concentration from 1.0 to 2.0 µg mL-1 and performed at room temperature for 1 hour. Anti-aflatoxin M_1 antibody concentration was 1.0 µg mL-1 and the time of incubation was 90 minutes at room temperature. Aflatoxin M_1- BSA concentration was 50 ng per well. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Blocking with PVPP (1%) was performed at room temperature for 90 minutes. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

Figure 2.13, shows that there is an improvement with increasing the concentration of the alkaline phosphatase labelled antibody. Furthermore all three lines show there is a detectable difference between 50 ng L^{-1} and lower concentrations. Using a concentration of 2 µL L^{-1} for the antibody would seem preferential. Figure 2.14 shows a repeat of this concentration with an r^2 value of 0.96 and an analytical

sensitivity of 50 ng L^{-1} as determined by Ammidia *et al*., (2004) and Draisci *et al*., (2001) as the amount of aflatoxin M_1 required to reduce the signal by 25%.

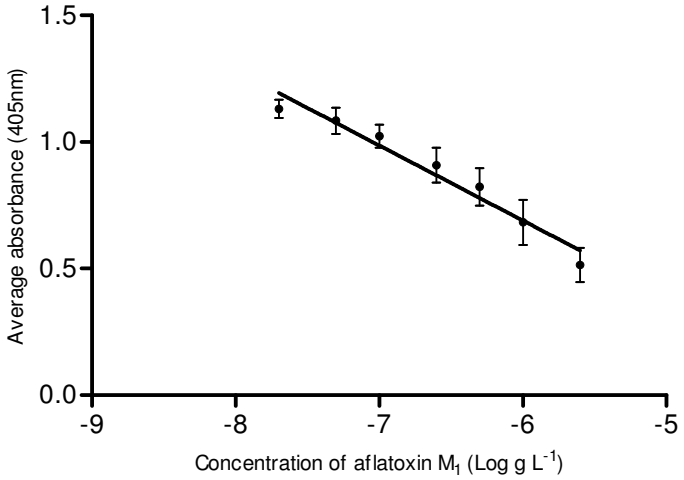


Figure 2.14: Calibration graph for aflatoxin M_1 after complete optimisation of the ELISA system.

Figure 2.14 was obtained using an aflatoxin M_1 - BSA concentration was 50 ng per well. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4^{o}C. Blocking with PVPP (1%) was performed at room temperature for 90 minutes. Anti-aflatoxin M_1 antibody concentration was 1.0 µg mL-1 and the time of incubation was 90 minutes at room temperature. Alkaline phosphatase labelled antibody concentration was 2.0 µg mL-1 and incubated at room temperature for 1 hour. pNPP substrate system was added to the system and allowed to develop for 45 minutes. (Error bars show standard deviations, n=3).

2.3.4 Determination of the interference from the milk matrix.

An initial investigation was carried out on the matrix effects which may occur from milk. Milk samples complying with British standards were purchased daily from Tesco's supermarket (Flitwick, UK). Three samples were examined; a homogenised full fat milk, a semi skimmed milk and a skimmed milk sample. Table 2.2 lists the nutritional information given for each product.

Table 2.2: The nutritional information for the three milk samples examined in this investigation.

	Full Fat (g)	**Semi Skimmed (g)**	**Skimmed (g)**
Protein	3.2	3.3	3.3
Carbohydrate	4.7	5.0	5.0
Of which are sugars	4.7	5.0	5.0
Fat	3.6	1.6	0.1
Of which is saturated	2.2	1.1	0.1

Each milk sample (25 µL) was taken with 25µL standard (1% methanol in 10 mM PBS, pH7.4) and placed into the micro well of the ELISA plate and shaken for 5 minutes using a Labsystems iMES plate incubator at 400 rpm, 25°C. The anti-aflatoxin M_1 antibody was then added and the plate was shaken for 90 minutes. Figure 2.15 shows the more fat there is then the greater the signal.

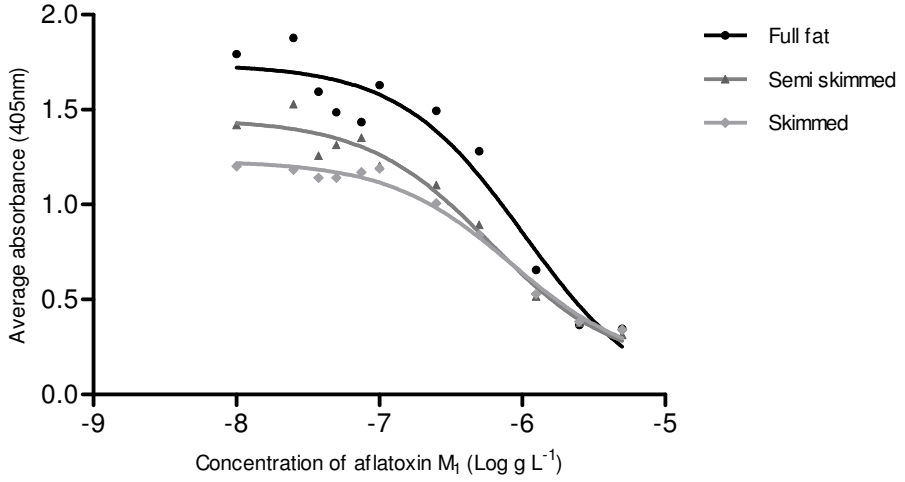


Figure 2.15: The effect of the milk matrix on the ELISA response.

Figure 2.15 was obtained using an aflatoxin M_1 - BSA concentration at 50 ng per well. Aflatoxin M_1 – BSA was immobilised at pH 9.6 overnight at 4°C. Blocking with PVPP (1%) was performed at room temperature for 90 minutes. Anti-aflatoxin M_1 antibody concentration was 1.0 µg mL-1 and the time of incubation was 90 minutes at room temperature. Alkaline phosphatase labelled antibody concentration was

2.0 µg mL-1 and performed at room temperature for 1 hour. pNPP substrate system was added to the system and allowed to develop for 45 minutes.

The presence of milk caused the loss of resolution at low aflatoxin M_1 concentrations and poorer detection limits.

Further work was carried out to clarify the milk such as the Carrez technique, to remove high molecular weight compounds such as proteins and fats (Gökmen and Şenyuva, 2006; Rufián-Henares and Morales, 2006), deproteinate using lead acetate (Goldblatt, 1969) or to remove the fats in the milk using solvents (Thomas *et al.*, 1998; Delgado Zamarreño *et al.*, 1992) to reduce the differences between each type of milk and to determine whether fat or protein causes the interferences observed.

The results of milk sample pre-treatment was not successful. Defatting the milk using hexane caused no detectable signal. Thomas *et al.*, (1998) used hexane to remove the fat from milk samples, for fat content and polychlorinated biphenyls analysis. It was noted by Thomas *et al.* (1998) that milk with hexane caused a suspension that became difficult to separate. The defatted sample in our tests had a hexane aroma which indicates residual hexane may be present in the sample and caused the loss of binding. This would have not caused a problem for Thomas *et al.* (1998) since the hexane extracts were used for HPLC analysis where the presence of solvents is not a concern.

Deproteination failed to work since the lead acetate used to denatured the protein in the milk sample, may also had the same effect on the antibody and BSA conjugate in the test. This denaturing of the test components may have been the cause of the zero signal when utilising Carrez clarification.

The pre-treatment techniques stated for the RIDASCREEN kit did not remove the matrix effects. The pre-treatment was to chill the sample to 4^{o}C and centrifuge at 10,000 rpm for 10 minutes and aliquots were taken from below the fat layer.

Upon reviewing the literature reports for self-developed ELISA methods there is little consensus whether milk causes interferences. Magliulo *et al*. (2005) reports that milk causes 'significant' interference, and commercial dried non fat milk diluted in buffer could only be used. El-Nezami *et al*., (1995) reported that extraction is 'essential' and recommends freeze drying, solvent extraction, solid phase extraction and reconstitution in buffer. Micheli *et al*., (2005) reports that milk that has only been mildly centrifuged does not cause any interference and Thirumala-Devi *et al*., (2002) reports that milk only required mild centrifugation followed by filtration through standard filter paper. It can only be postulated that milk does form some interference in the aspect of antibody binding. However, either different authors have obtained antibodies with different affinities or geographically the milk is different in its composition. Unfortunately the authors do not give data on the milk composition. From reviewing the geographical location of the authors (Table 2.3) it cannot be concluded that location is the prime cause of the sporadic interferences, However the provenance of the interference also is influencing the results in this project.

Table 2.3: Geographical location of authors for aflatoxin M_1 ELISA development.

Main author	Interference	Geographical location
El-Nezami (1995)	Yes	Australia and Thailand
Magliulo (2005)	Yes	Central Italy
Micheli (2005)	No	Central/Western Italy
Thirumla-Devi (2002)	No	Rural India

Before further investigation could be carried out supplies of aflatoxin M_1 – BSA could not be obtained from Sigma Aldrich so work was transferred to the electrochemical sensor where the assay format was then modified.

2.4 Conclusions to the ELISA development.

The purpose of the ELISA development was to investigate whether a commercially available antibody was suitable for the application in sensor development. The detection of aflatoxin M_1 in the region of the required detection range demonstrates that the antibody from Abcam Ltd is suitable for use in the immunosensor.

A schematic flow diagram of the final method developed in this chapter is shown in Figure 2.20.

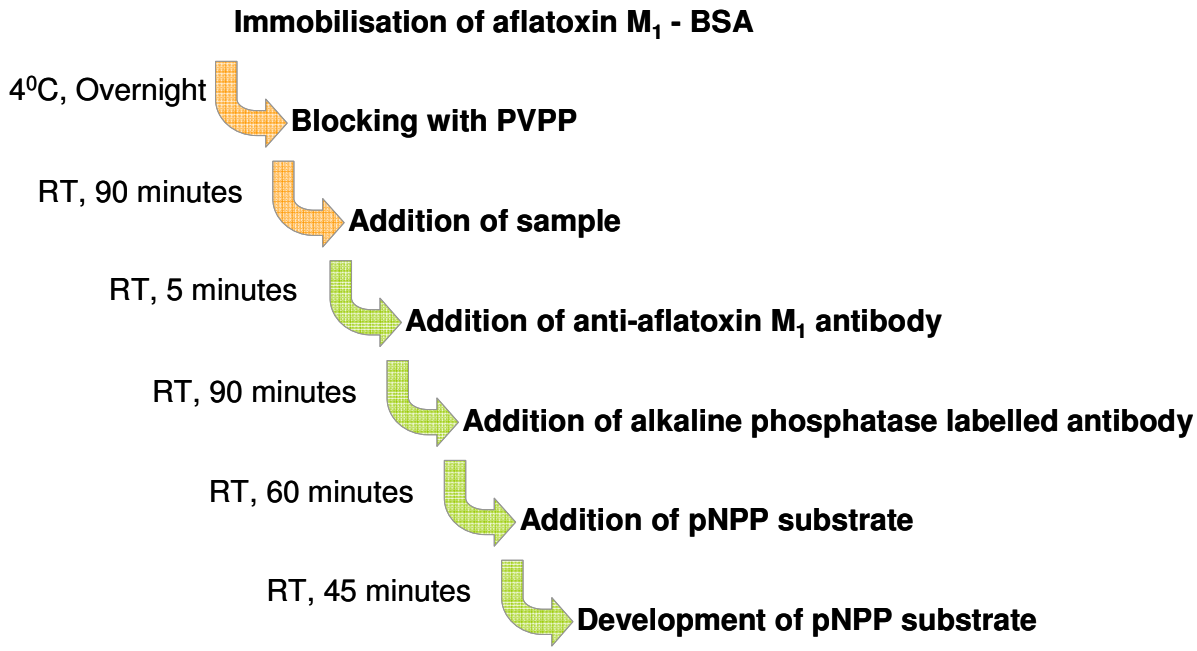


Figure 2.16: The developed ELISA scheme.
The scheme is split into two stages. First (marked with orange arrows) is the preparation of the microwell plate; immobilisation of the aflatoxin – BSA conjugate onto the microwell plate and blocking. Second is the analysis (shown in green) i.e. competition reaction and detection.

The analysis stages require about 3.5 hours to perform which is comparable to the commercial ELISA kit which requires 3 hours.

Traditional clean-up protocols stemming from TLC and HPLC methods have been demonstrated to be inadequate and not appropriate since the components employed for deproteination also have some effect on the antibody and immobilised BSA conjugate, as both are proteinaceous in nature. Techniques such as solid phase extraction (SPE) and immunoaffinity columns (IAC) are also not suitable since they require the use of solvents and are laborious techniques. Further work using the ELISA had to be suspended due to a shortage of aflatoxin M_1 - BSA supplies. To produce the aflatoxin – BSA conjugate a reactive group for the coupling reaction is first produced by preparing an aflatoxin M_1-1-(*O*-carboxymethyl) oxime before coupling can be performed (Chu *et al*., 1977). This is a complicated procedure outside the scope of this project for in-house production.

CHAPTER 3

DEVELOPMENT OF THE IMMUNOSENSOR FOR AFLATOXIN M_1 USING SCREEN PRINTED ELECTRODES

3.1 Introduction.

The aim of this chapter is to describe the coupling of the antibody receptor, optimised by ELISA, to an electrochemical interface. Thick film technology was utilised for the fabrication of screen printed carbon electrodes, which were then characterised by cyclic voltammetry and used as the transducer for the electrochemical immunosensor. A new immunochemical format was chosen for the development of the immunosensor due to the difficulty in acquiring aflatoxin M_1 – BSA. However the new format incurred many advantages such as the reduction of assay time, fewer steps and the number of components involved in the test for the final user. Initially the work described by Micheli *et al*., (2005) was used as a foundation for the immunosensor construction. However, upon reviewing the literature the initial protocol was modified and improved.

The effect of milk was reviewed with the immunosensor and through cross examining reported literature the responsible compounds for the interferences in the test were elucidated and the matrix interferences were reduced. Lastly the causes of poor reproducibility were investigated and the immunosensor was compared to other existing technologies.

3.2 Materials and methods.

3.2.1 Fabrication of electrodes.

The screen printed electrodes were manufactured in house at Cranfield university by a multistage deposition process using a DEK 248-screen printer and stencils (DEK, Weymouth, UK). The electrodes were printed using 250 μm thick polyester Melinex sheets (CMS acoustics, Colchester, UK). The print parameters were set so that the squeegee pressure was 4 psi, a carriage speed of 50 mm sec^{-1} and a print gap of 2.5 mm. For the fabrication, the basal tracks were printed first using Electrodag 423-SS graphite ink (Acheson industries, Plymouth, UK). The auxiliary electrode layer was printed using the same ink but with a different screen. The

reference electrode was printed using Electrodag 6038-SS silver / silver chloride ink and printed over the graphite basal track (left track). The blue epoxy insulating layer was printed last using 242-SB protective polymer (ESL electroscience products, Reading, UK).

Between each layer the sheets were allowed to dry for 2 hours at 60^{o}C. After the insulating layer the sheets were cured at 120^{o}C for two hours. Figure 3.1 shows a schematic diagram of the fabrication procedure.

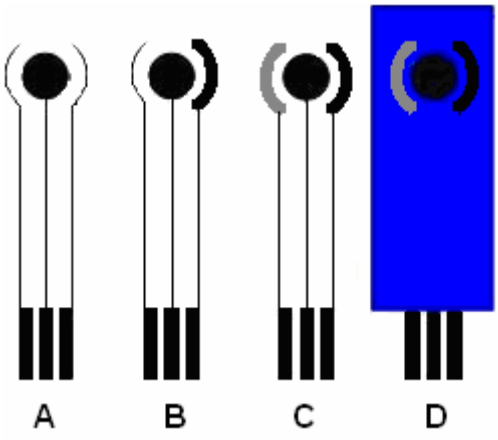


Figure 3.1: The fabrication of the sensor. A) the initial basal tracks are laid, B) the auxiliary electrode is added, C) the silver/silver chloride reference electrode is then printed and finally D) the epoxy insulating layer is placed onto the sensor.

3.2.2 Methods and parameters for the electrochemical procedures.

For the electrochemical procedures a computer controlled four channel Eco Chemie Autolab electrochemical analyser multipotentiostat (Eco Chemie, The Netherlands) was used throughout, which allows the simultaneous detection of four electrodes. Data capture was through the supplied GPES version 4.9 software to a PC.

The screen printed electrodes were connected to the Autolab using an in-house fabricated connector from a PCB edged IDC socket, ribbon cable and 4mm cable sockets. Figure 3.2 shows a photo of the instrumental components.

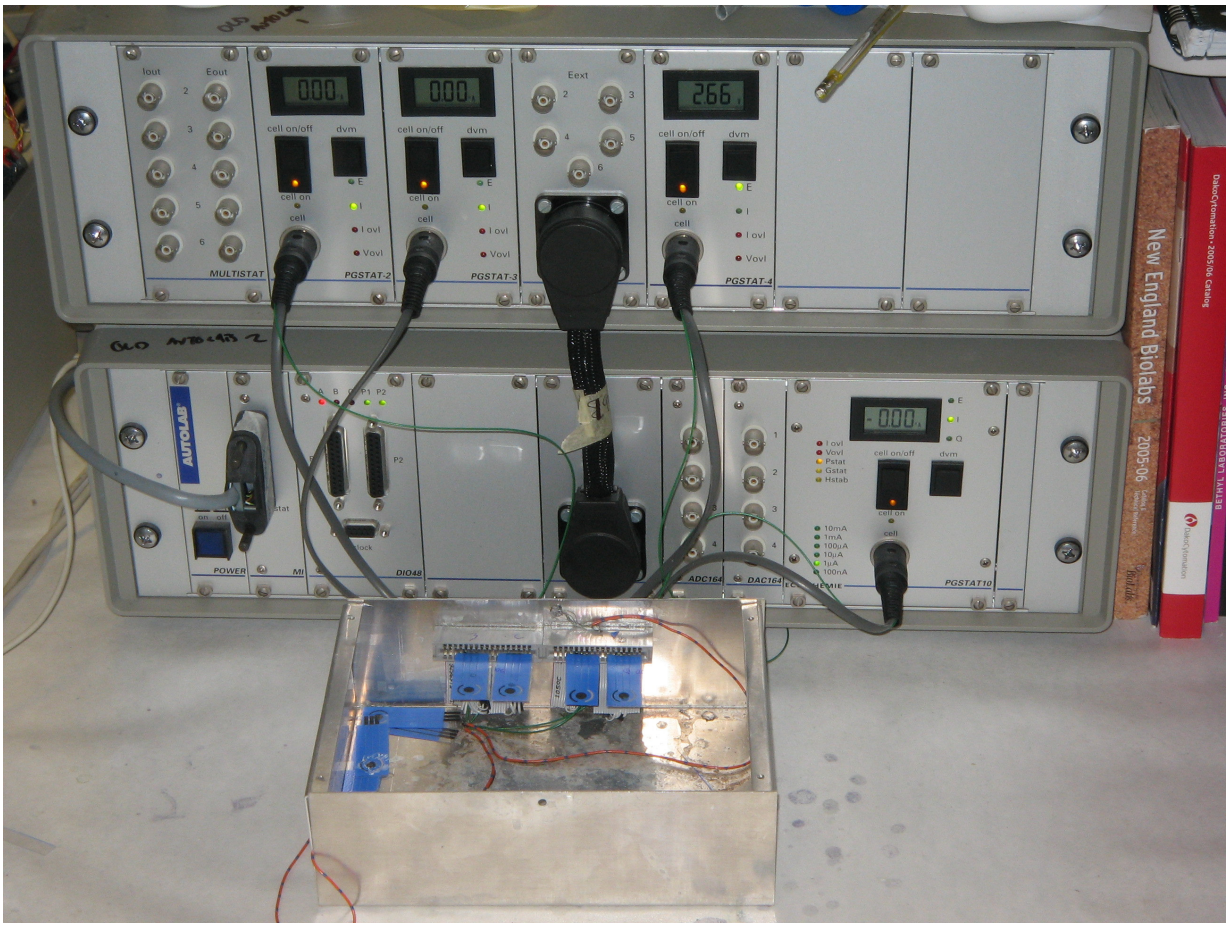


Figure 3.2: The Autolab instrumentation from Eco chemie used for the electrochemical measurement.

The parameters for the cyclic voltammetry (C.V.) scans are shown in Table 3.1

Table 3.1: The settings used for cyclic voltammetry using the Autolab instrument.

Number of cycles	5
Start potential	-1.0 V
First vertex potential	+1.0 V
Second vertex potential	-1.0 V
Step rate	0.00274 V
Scan rate	0.1 V/s (unless stated)

For the cyclic voltammetry experiments 100 µl of sample was used and the electrode was replaced after each scan. Characterisation of the electrodes was

carried out by recording cyclic voltammetry scans of potassium hexacyanoferrate (III) whilst varying the scan rate from 10 to 100 mV s^{-1}.

For the immunosensor construction all reagents and chemicals were obtained and diluted as optimised by the ELISA protocol described in Chapter 2 with some additional steps included. In general 8 µl of 96 µg mL^{-1} anti-primary antibody (capture antibody; Pierce, Cramlington, UK) with carbonate buffer pH 9.6, 0.1 M was added to the working electrode, placed into a humid environment to avoid drying and stored overnight at 4°C. The sensor was then washed twice with 0.05% Tween 20 in 10 mM PBS buffer and once with 10 mM PBS (pH 7.4), ensuring that the spray from the wash bottle did not impact directly on the working electrode but the flow washed over the working electrode. The electrode was shaken dry to remove most of the surplus buffer.

The anti-aflatoxin M_1 antibody (8 µl, 40 µg mL^{-1}) was added to the working electrode and incubated for 2 hours at 37°C for the reaction of the immobilized antibody to the anti-aflatoxin M_1 antibody. To block the surface the sensors were dipped into 1% PVA in PBS for 2 hours at 37°C then washed and dried using the same protocol as before. To the sensor 6 µl of a mixture of aflatoxin M_1 standard (in either 1% methanol PBS buffer or 1:1 dilution of milk with 1% methanol PBS) and a equal volume of 1:10 dilution of the aflatoxin M_1 - HRP conjugate from the Ridascreen kit from R-Biopharm, Glasgow, UK (diluted using 1% PVA in PBS) was added to the working electrode and incubated at 37°C for 2 hours. The sensor was again washed, dried and 100 µl of 0.5 mM 3,3′,5,5′-Tetramethylbenzidine (TMB) in 0.1 M citrate buffer (pH 5.2) with 1 mM hydrogen peroxide was added to the sensor, ensuring all three electrodes were covered. The Autolab running in chronoamperometry mode was started and the data collected for 10 minutes. During measurement the sensors were stored within an aluminium chassis case connected to the Autolab's earth point to reduce the electrical interferences from neighbouring apparatus such as computers, gas chromatographs and air

conditioning units and in turn stored the sensors away from light during measurement due to the TMB being light reactive.

Calculations of analytical sensitivity was determined as described by Ammidia *et al.*, (2004) and Draisci *et al.*, (2001) as the amount of aflatoxin M_1 required to reduce the signal change by 25%.

The settings for the chronoamperometry are listed in Table 3.2

Table 3.2: Settings for the Autolab chronoamperometry measurments.

Measurement interval Time	1s
Standby voltage	0.0 V
Preconditioning voltage for 20s	0.2 V
Equilibration voltage for 5s	0.0 V
Measuring potential for 600s	0.1 V

Step amperometry experiments were performed in triplicate where a solution of 0.1 M KCl in citrate buffer, 0.1 M pH 5.2 citrate buffer with 0.5 mM TMB and 1 mM H_2O_2 was added to the sensors with and without 20 U of peroxidase from Sigma Aldrich Ltd (Gillingham, UK). The peroxidase was added and an incubation time of 30 minutes was allowed for the reaction with peroxide. Starting from 0 V and going towards -1 or 1 V incremental steps of 0.1 V were taken, scanning for 100 seconds each. The ratio of peroxidase *versus* blank (no added peroxidase) was calculated.

For all the experiments the aflatoxin M_1 was made up in methanol to a stock concentration of 1 mg L^{-1}, aliquots were taken and stored at -18^{o}C under nitrogen. Working aflatoxin M_1 standards between 10 μg L^{-1} and 10 ng L^{-1} were made using 1% methanol in 10 mM PBS (pH 7.4).

Milk samples complying with British standards were purchased from Tesco supermarket (Flitwick, UK) and obtained on the day of analysis. 50 mL of milk sample was placed into a 100 mL volumetric flask, 10 mL of 100 mM PBS and 0.26 g of $CaCl_2$ was added and the sample was made up to 100 mL. The milk samples

were centrifuged using a Hettich D-78532 centrifuge, (Kirchlengern, Germany) at 5000 rpm for 5 minutes. Initially only centrifugation was applied to milk samples.
For all electrochemical experiments a supporting electrolyte of 0.1 M KCl was added to the scanning solution.

All glassware and consumables were decontaminated from aflatoxin M_1 by soaking in 10% sodium hypochlorite for 48 hours and then an equal amount of 5% aqueous acetone was added. The mixture was left for three hours and then disposed as general hazardous waste. This procedure is recommended by AOAC (Association Of Analytical Communities) official methods of analysis (AOAC, 1996).

For testing the non-specific binding of the aflatoxin M_1 to different blocking agents the assay for the screen printed electrode was transferred to an ELISA multiwell plate and the same concentrations of reagents were used. 50 µL of 96 µg mL^{-1} anti-primary antibody (capture antibody; Pierce, Cramlington, UK) with carbonate buffer pH 9.6, 0.1 M was added to the plate in triplicate and incubated overnight at 4^{o}C. The plate was washed twice with 10 mM 0.05% PBS-T and once with 10 mM PBS. Following drying, 50 µL of 40 µg mL^{-1} anti-aflatoxin M_1 antibody was added to the well and incubated for 2 hours at 37^{o}C using a Lab systems multiwell plate incubator. The plate was washed again and 50 µL of either 1% PVPP, 1% PVA, 0.5% BSA or 1% gelatine in 10 mM PBS buffer was added and incubated for 2 hours at 37^{o}C. Again washing of the plate was performed as before and 50 µL of aflatoxin M_1 – HRP (1:10 dilution from Ridascreen Stock) was added and incubated for a final 2 hours at 37^{o}C. The plate was then washed and 50 µL of Ultra TMB solution from Pierce (Cramlington, UK) was added to the wells and the plate was scanned using a BMG Fluorstar galaxy ELISA plate reader (Aylesbury, UK) at 450 nm after 30 minutes.

3.2.3 Calculation of indigenous lactoperoxidase.

The levels of lactoperoxidase were estimated by the addition of 0.8 mL of untreated milk and 0.2 mL of water to 0.4 mL of TMB ultra peroxidase substrate solution from Pierce (Cramlington, UK). After 10 minutes incubation the sample was quenched with 10 µL of H_2SO_4 and centrifuged at 9000 rpm to yield a transparent solution. The resulting solution was spectrophotometrically recorded using the plate reader at 450 nm. Blank readings for the milk were calculated by pre-treating the milk with the addition of 0.2 mL, 1 M trichloroacetic acid to 0.8 mL of milk, stirring and then adding to the TMB solution. A corresponding calibration curve was produced by dissolving pure peroxidase enzyme into PBS buffer and treating the same as the milk sample.

3.2.4 Optimisation of the electrochemical detection of TMB using the screen printed electrode.

During the optimisation of the detection of TMB, three experiments were performed in a similar manner. For all the experiments the immobilisation of the capture antibody, anti-aflatoxin M_1 antibody, blocking agent, aflatoxin M_1 – HRP and TMB substrate addition were performed as previously described in Section 3.2.2. Firstly the identification of the optimum potential for TMB measurement was determined by preparing sensors in triplicate and scanning the TMB at +100 mV and -100 mV using chronoamperometry. Blank values were obtained by excluding aflatoxin M_1 – HRP from the test. Secondly the effect of pre-conditioning the electrode with TMB before scanning was investigated by again producing sensors in triplicate and immediately prior to scanning either applying or not applying a potential at 200 mV for 5 seconds. A blank value was obtained by not including aflatoxin M_1 – HRP in the test. Finally the effect of electrochemical pre-cleaning of the sensor was investigated by cleaning screen printed electrodes in triplicate with water, ethanol, water and then applying a potential of 2.0 V for 30 minutes to the electrodes. Once cleaning had been completed then sensors were produced using the cleaned

electrodes. A blank value was obtained by not including aflatoxin M_1 – HRP in the test.

3.2.5 Determination of the causes of electrochemical fouling from milk.

For the elucidation of the causes of interference from milk on the electrochemical sensors several solutions of full fat commercial milk was spiked or treated. Initially milk was added to the test and a calibration was performed. This was done by mixing 1:1 full fat untreated milk with aflatoxin M_1 standards (1% methanol in PBS) and no other pre-treatment.

Further investigations were performed to determine the interfering factors. Firstly full fat milk was added 1:1 with 10 mM potassium hexacyanoferrate (III) and 0.2 M KCl to yield a test solution of 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl. This solution was then analysed by cyclic voltammetry using the setting described in Table 3.1. Secondly for the investigation of lipids, a commercial milk sample was adjusted to pH 8.6 with NaOH whilst constantly stirring, to activate indigenous lipases, and then placed into an incubator set at 37^{o}C for 24 hours. This sample and a sample of non-fat milk from Sigma Aldrich (Gillingham, UK) were added to potassium hexacyanoferrate (III) as described above and tested by cyclic voltammetry using the same settings. The non-fat milk was prepared as described by the manufactures instructions.

The investigation into the effects of lactose was performed by producing solutions of 4.6% lactose in 0.1 M KCl with and without the presence of potassium hexacyanoferrate (III) and analysed by cyclic voltammetry as described by Table 3.1.

For the investigations into the removal of proteins three solutions were prepared for potassium hexacyanoferrate additions. 1) To a 100 mL commercial sample of milk the pH was adjusted to 4.6 and the sample was centrifuged at 4000 rpm, 4^{o}C for 10 minutes and the supernatent was poured out and an aliquot was mixed with potassium hexacyanoferrate (III) and tested by cyclic voltammetry. The remaining supernatent was further treated with 50 µL of 5.5 M trichloroacetic acid and stirred

for 10 minutes to allow coagulation. The sample was centrifuged again at 4000 rpm, 4°C for 10 minutes to remove the whey proteins and tested again with potassium hexacyanoferrate. Finally a sample was produced by mixing a 50 mL commercial milk sample with 25 g finely ground (using a pestle and mortar) ammonium sulphate. The solution was incubated at 4°C for 48 hours before centrifuging at 4000 rpm and testing with potassium hexacyanoferrate (III) using cyclic voltammetry.

3.2.6 The determination of urine on the alfatoxin M_1 immunosensor.

For the determination of aflatoxin M_1 in urine, the general method for the determination of aflatoxin M_1 in milk was followed with the exception of the addition of calcium chloride. Breifly, 8 μl of 96 μg mL^{-1} anti-primary antibody (capture antibody; Pierce, Cramlington, UK) with carbonate buffer pH 9.6, 0.1 M was added to the working electrode, placed into a humid environment to avoid drying and stored overnight at 4°C. The sensor was then washed twice with 0.05% Tween 20 in 10 mM PBS buffer and once with 10 mM PBS (pH 7.4), ensuring that the spray from the wash bottle did not impact directly on the working electrode, but the flow washed over the working electrode. The electrode was shaken dry to remove most of the surplus buffer.

The anti-aflatoxin M_1 antibody (8 μl, 40 μg mL^{-1}) was added to the working electrode and incubated for 2 hours at 37°C, for the reaction of the immobilized antibody to the anti-aflatoxin M_1 antibody. To block the surface the sensors were dipped into 1% PVA in PBS for 2 hours at 37°C, then washed and dried using the same protocol as before. To the sensor 3 μl of urine (diluted 1:1 with PBS buffer, spiked with aflatoxin M_1 in methanol) and 3 μl of 1:10 dilution of the aflatoxin M_1 - HRP conjugate from the Ridascreen kit from R-Biopharm, Glasgow, UK (diluted using 1% PVA in PBS) was added to the working electrode and incubated at 37°C for 2 hours. The sensor was again washed, dried and 100 μl of 0.5 mM 3,3′,5,5′-

Tetramethylbenzidine (TMB) in 0.1 M citrate buffer (pH 5.2) with 1 mM hydrogen peroxide was added to the sensor, ensuring all three electrodes were covered. The Autolab running in chronoamperometry mode was started and the data collected for 10 minutes.

3.2.7 The development and validation of the HPLC method.

The HPLC determination was performed using a Waters 600E System Controller, a Waters 712 WISP Autosampler and a Waters 470 Scanning Fluorescence Detector set at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The Waters modules were computer controlled using Kromasystem 2000 software. A Phenomenex Luna 5u C18 analytical column was used throughout with a Security Guard ^{TM} guard column. Aflatoxin M_1 standards were made up 1% methanol, 49% PBS buffer and 50% milk and isolated using immunoaffinity columns as denoted by the manufactures R-Biopharm (Glasgow, UK). Briefly 50 ml of spiked milk was centrifuged at 3,000 RPM to isolate the fat and then passed through the immunoaffinity column at a rate of 1-2 drops per second. Once the sample had been passed then the column was washed with 2 aliquots of 10 ml H_2O and eluted into a eppendorf tube with 1.25 ml of 2:3 methanol:acetonitrile followed by 1.25 ml of H_2O. After mixing by vortex the sample was divided into three and placed into HPLC vials for triplicate analysis.

3.3 Results and discussions for the electrochemical sensor.

3.3.1 Production and characterisation of electrodes.

The screen printed electrodes were produced as described in Section 3.2.1. Thick film technology was chosen initially as the fabrication method for the sensors due to the flexibility of design, choice of materials, easy of integration with electronic circuits, low cost and fast speed of manufacturing.

Thick film technology or screen printing is a procedure where a paste of dielectric or conductive materials is applied to a substrate material using a mask. The deposition is achieved by pressing the paste through a silk screen mask using a squeegee and then curing at raised temperatures. The deposited films have a typical thickness of 10 to 50 μm. There is a variety of materials available for printing the working electrode including gold and carbon inks, as well as silver based inks for the reference electrode. Carbon inks are attractive for sensing applications since they are inexpensive, have a wide potential range with a low current background. Additionally the adsorption capacity of carbon results in easy immobilisation through adsorption of antibodies to the carbon surface. Carbon inks contain graphite particles and a polymeric binder as well as other additives which are removed upon curing. This leaves a relatively rough surface and thus a high surface area.

Sensors were printed in batches of 6000 (100 sheets) and the sensor to sensor reproducibility was quantified using cyclic voltammetry (C.V.) by varying the scan rate whilst observing the anodic to cathodic peak separation of potassium hexacyanoferrate (III) as shown in Figure 3.3.

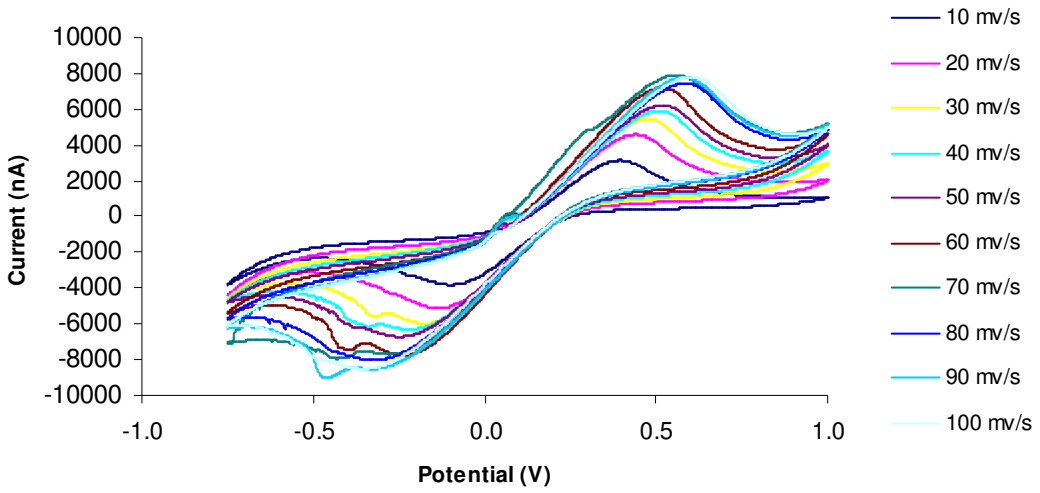


Figure 3.3: Cyclic voltammetry scans of 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl whilst varying scan rate using the in-house fabricated screen printed sensors. Plots were obtained from the average of triplicate results. Fresh sensors were used after each scan.

The redox reaction for potassium hexacyanoferrate is a classic reaction and therefore easily comparable to other electrode systems. As the potential is increased from -0.75 to 1 V in the forward scan the electrode becomes a strong oxidant and $Fe^{II}(CN)_6^{3-}$ is formed at the electrode. The current increases and forms an oxidation peak on the voltammogram until the vicinity of the electrode is depleted from $Fe^{III}(CN)_6^{4-}$ and migration of the reactant to the surface through diffusion cannot be sustained, i.e. mass transport for the unstirred reaction is slower than the redox reaction, at which point the current decreases and the potential scan is reversed. When the potential scan is in the opposite direction and back towards the origin, the reduction reaction for the cathodic peak becomes $Fe^{II}(CN)_6^{4-} \rightarrow Fe^{III}(CN)_6^{3-} + e^-$.

It can be seen in Figure 3.3, that at a low scan rate of 20 mv s^{-1} the peak separation is 59 mV and thus the reaction is reversible as the electrical equilibrium is maintained at the electrode surface (Nicholson, 1965). Upon increasing the scan

rate then the oxidation peak potential (E_{pa}) is shifted to a more positive potential and therefore demonstrates that the redox reaction is quasi-reversible because the rate of electron transfer is too slow to keep the $Fe^{II}(CN)_6^{4-}$ / $Fe^{III}(CN)_6^{3-}$ couple in equilibrium as the potential is changed.

3.3.2 Characterisation of milk samples.

Milk is a very complex solution consisting of over 100,000 compounds (Hui, 1992; Walstra, 1984). Within its constitution are electro-active species such as ascorbic acid, fructose and lactose (Mayer *et al*., 1996). Hence it is recommended to use low voltages for the electrochemical detection to avoid interference. Previous reports have stated that the detection of alkaline phosphatase or horseradish peroxidase at -100mV does not cause interferences (Pellegrinei *et al*., 2004; Pemberton *et al*., 1999).

Additionally milk contains indigenous enzymes such as lacto-peroxidase and alkaline phosphatase which may cause false positive readings.

3.3.2.1 Electrochemical interferences from electro-active compounds.

To validate these concerns, a C.V. scan of full fat milk, with no laboratory pre-treatment other than spiking with KCl was performed as shown in Figure 3.4. The C.V. scan doesn't show any peaks at the voltage of -100mV, and therefore re-enforces the observations of Pellegrinei *et al*., (2004) and Pemberton *et al*., (1999).

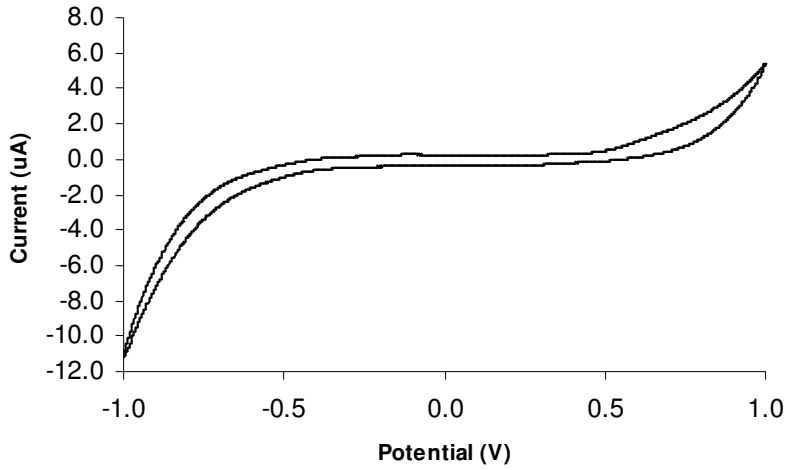


Figure 3.4: A C.V. scan of full fat milk with 1 M KCl (no other pre-treatment). The results shown are taken from the average of 4 sensors.

3.3.2.2 The determination of indigenous lactoperoxidase.

Marks *et al*., (2001) detailed a test using hydrogen peroxide strips for detecting the effectiveness of heat treatment during the packaging process by determining the lactoperoxidase activity. Furthermore reports from Badea *et al*., (2004) described an electrochemical system for the detection of aflatoxin M_1 using flow injection where the indigenous lactoperoxidase interfered with the HRP marker in the test. To determine the lactoperoxidase content a test was done to quantify the levels using the Ultra TMB determination solution from Pierce. Briefly a sample of milk was added to the Ultra TMB solution and incubated for 10 minutes before quenching the reaction with trichloroacetic acid. The solution was centrifuged at high speed to yield a transparent solution and the absorbance was measured at 450 nm. A blank reading was taken where the trichloroacetic acid was added to the milk before the addition of Ultra TMB.

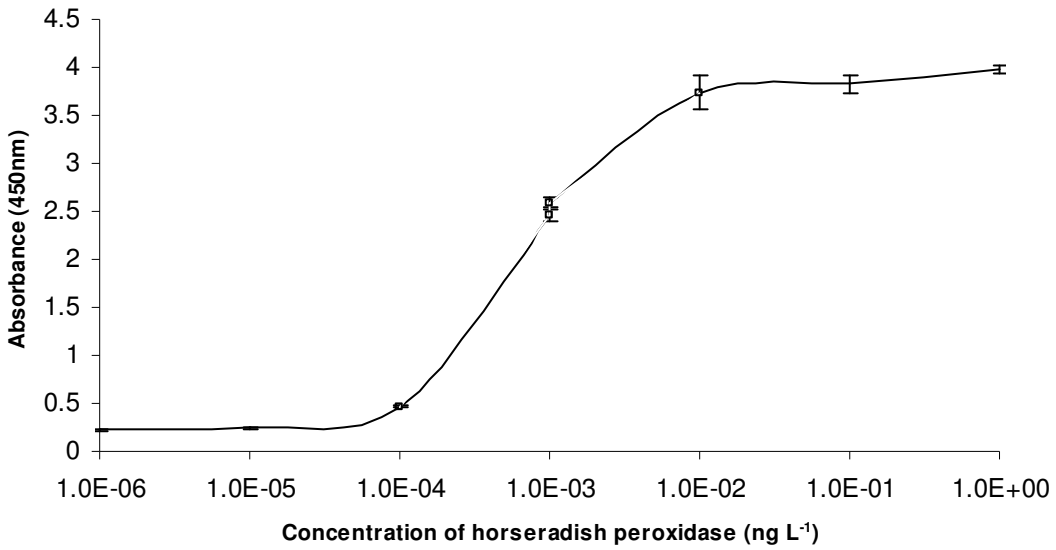


Figure 3.5: A calibration graph for the determination of peroxidase activity in milk using spectrophotometry at 450 nm. Error bars showing standard deviation (n=3).

Using the calibration graph different milk samples were tested and the responses and respective concentrations are reported in Table 3.3.

Table 3.3: The responses and concentrations of peroxidase found in different milks.

Sample	Dilution	Response	Concentration (ng L^{-1})
UHT	1	0.428 ± 0.050	$<1x10^{-4}$
Dried	1	0.222 ± 0.012	$<1x10^{-4}$
Pasteurised	10	0.382 ± 0.011	$<1x10^{-3}$
Raw	10	0.709 ± 0.062	$<1x10^{-3}$

Table 3.3 is an estimate of the lactoperoxidase activity in the milk samples. It can clearly be seen that the enzyme concentrations are very low and 4 magnitudes lower than the maximum permissible levels of aflatoxin M_1 in the milk.

3.3.3 Development of the screen printed sensor.

The aim of this project focuses on developing a sensor for aflatoxin M_1. The ELISA method developed utilised an alkaline phosphatase enzyme marker as the method

of detection, however, for the electrochemical sensor it was chosen to substitute the alkaline phosphatase enzyme for horseradish peroxidase.

Horseradish peroxidase has been extensively evaluated in the literature as an electrochemical marker. The enzymatic system monitors the consumption of hydrogen peroxide by the horseradish peroxidase enzyme, an enzyme with a high turnover rate. The electrochemical detection of hydrogen peroxide directly requires a high voltage and therefore subject to interference. In order to overcome this problem, mediators are employed. Upon undergoing a literature review for the electrochemical detection of HRP several mediators have been evaluated. The most common of these are; OPD (*o*-Phenylenediamine dihydrochloride), ABTS (2-2'-azino-di-[3-ethylbenzthiazoline sulfonic acid]) and TMB (3,3',5,5'-Tetramethylbenzidine). Volpe *et al*., (1998) concludes that alkaline phosphatase generally has superior electrochemical activity over horseradish peroxidase, but, when using TMB this difference is negligible. ABTS and OPD as mediators have shown to exhibit mutagenic or carcinogenic effects (Voogd *et al*., 1980), therefore the use of these mediators has been avoided. Alkaline phosphatase does have the drawback that there are concerns over its stability, particularly in alkaline environments. An additional disadvantage of using alkaline phosphatase is that the electrochemical product of the reaction of 4-aminophenolphosphate (*p*-NPP) is 4-aminophenol, which is prone to fouling the electrode surface upon measurement. Hence the electrochemical scanning technique can only involve one reading rather than continuous measurement. It was chosen in this project to use chronoamperometry to allow real time observation of the enzyme kinetics to aid development. Thus the employment of alkaline phosphatase was not feasible.
In a deviation to the ELISA system, the antibody has been immobilised onto the surface of the sensor, and an aflatoxin M_1 – HRP conjugate is used in the test. These changes occurred due to the shortage of the commercially available aflatoxin M_1- BSA conjugate, but also resulted in the sensor becoming much simpler for the end user since it will require fewer operator steps.

Upon immobilising the antibody onto the surface, much of the antibody is wasted due to the haphazard random orientation of the antibody during adsorption. This causes up to a 90% decrease in sensitivity (Malmsten *et al.*, 1998). The anti-aflatoxin M_1 antibody is a monoclonal antibody and hence expensive. To reduce costs and to improve the affinity of the anti-aflatoxin M_1 antibody, a cheap anti-rat polyclonal antibody from Abcam Ltd (Cambridge, UK) was immobilized onto the surface which has affinity for the Fc fragment (tail) of the anti-aflatoxin M_1 antibody. This results in the anti-aflatoxin M_1 antibody orientated in the correct position and therefore improves efficiency. Figure 3.6 shows a schematic diagram of the immobilisation steps.

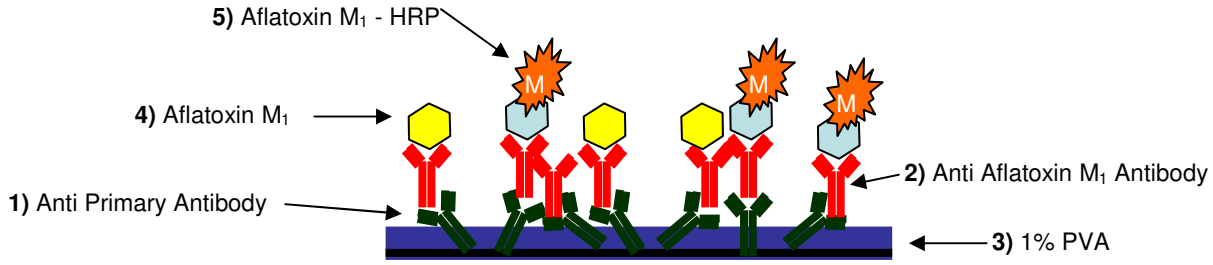


Figure 3.6: A schematic diagram of the electrochemical sensor.

Initially a polyclonal antibody with affinity for the Fc fragment (tail) of the anti-aflatoxin M_1 antibody is immobilised onto the carbon surface of the electrode (Figure 3.6 [1]). Secondly the anti-aflatoxin M_1 antibody is added to the sensor [2] and using the polyclonal antibody the anti-aflatoxin M_1 antibody is orientated into the solution and thereby improving sensitivity. The surface is then blocked with PVA [3] and for detection a competitive reaction occurs between free aflatoxin M_1 in the sample and HRP labelled aflatoxin M_1 [4,5].

Before using the new assay format on the screen printed electrode, the assay was performed using the ELISA method. This was employed to determine if this substitution improved the detection in the ELISA test. The results showed that horseradish peroxidase binds non-specifically to the PVPP blocking agent and

causes significant interference when PVPP is used as the blocking agent, as shown in Figure 3.7.

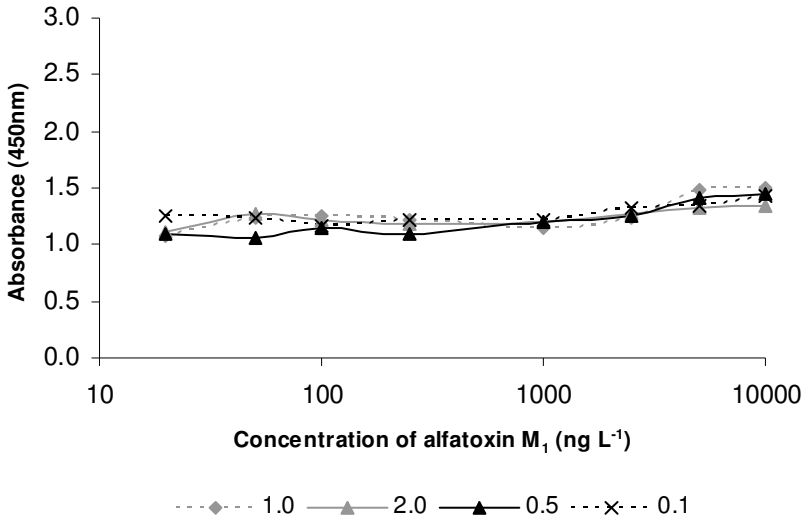


Figure 3.7: Testing the new method design using ELISA. Different concentrations of anti-aflatoxin M_1 antibody (2.0, 1.0, 0.5, 0.1 μg L^{-1}) was applied in the test with PVPP used as the blocking agent.

Little observational difference can be seen due to non-specific binding of the HRP enzyme for the PVPP blocking layer. A study was then done to show, whether, horseradish peroxidase has affinity for PVPP alone or other blocking agents. PVPP, PVA, BSA and gelatine were used as blocking agents, and background readings were obtained for each agent. Table 3.4 shows these results.

Table 3.4: Investigation of the non-specific binding from horseradish peroxidase secondary antibody with different blocking agents.

Blocking Agent	Average Absorbance (450nm)
1% PVA	0.33
1% PVPP	2.32
0.5% BSA	0.58
1% Gelatine	1.20

From the results PVPP gave high non-specific binding and thus high interference, followed by gelatine, BSA and PVA. From this evidence PVA was used as the

blocking agent with horseradish peroxidase. The use of PVA should stabilise the system for long time periods since traditional protineacous blocking agents such as gelatine and BSA could denature upon storage.

3.3.4 Electrochemical detection of TMB.

Before implementing the immuno-components onto the sensor the detection of the TMB was verified. Pure unconjugated horseradish peroxidase enzyme (Sigma Aldrich, Gillingham, UK) was immobilised onto the sensor surface (1 µL L^{-1} to 1,000,000 µL L^{-1}) through adsorption and measured using chronoamperometry (measuring potential -100 mV) with TMB (0.5 mM) and hydrogen peroxide (1 mM) in 0.1 M citrate phosphate buffer, pH 5.2, as described by Micheli *et al*., (2005). In addition to the parameters reported by Micheli *et al*., (2005) 0.1 M KCl was added additionally as a supporting electrolyte to the citrate buffer to repress the migration of electro-active species from the electrode surface (Evans *et al*., 1983). The results are shown in Figure 3.8.

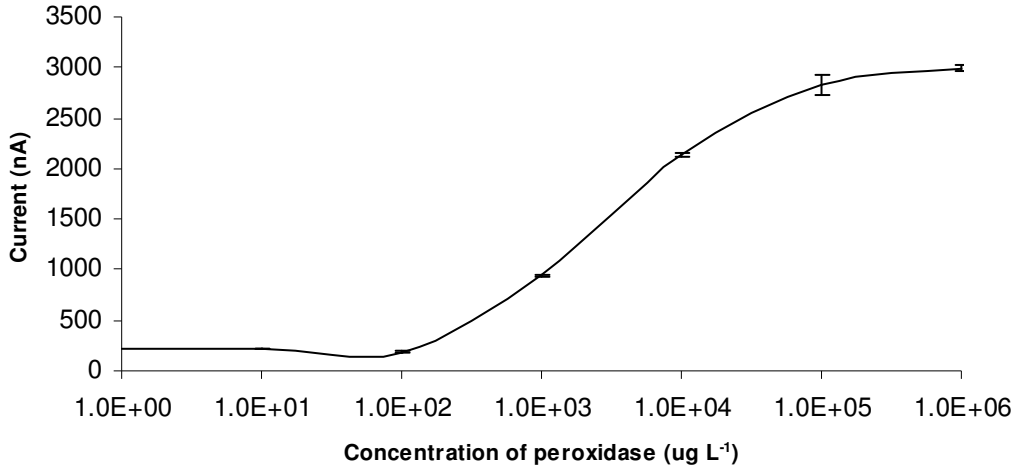


Figure 3.8: Electrochemical detection of immobilising peroxidase onto the electrode surface using TMB and H_2O_2 solution.

The data in Figure 3.8 was obtained using chronoamperometry (-100 mV) with TMB (0.5 mM) and hydrogen peroxide (1 mM) in 0.1 M citrate phosphate buffer, pH 5.2. Error bars denote standard deviations (n=3).

It can clearly be seen that the parameters were able to detect horseradish peroxidase with good reproducibility.

The oxidation of TMB is a two step reaction. Firstly the addition of hydrogen peroxide to the heme group containing HRP enzyme reduces the HRP to form an intermediate (compound 1) involving a 2 electron process changing the heme (Fe^{3+}) group into a ferryl oxo iron (Fe^{4+}=O) and a porphyrin (P) cation radical. Upon the addition of TMB, 2 molecules of TMB are oxidised by compound 1 to form a blue coloured product. Upon the release of H_2O the peroxidase returns to the native state via a further intermediate, leaving the TMB in an oxidized state. Commonly sulphuric acid is added to the oxidised TMB to develop a stable yellow diiamine product that is measured at 450nm and can be measured electrochemically (Tanaka *et al.*, 2003; Frey *et al.*, 2000; Ruzgas *et al.*, 1996; Josephy *et al.*, 1982). Figure 3.9 shows a summary of the TMB reduction reaction.

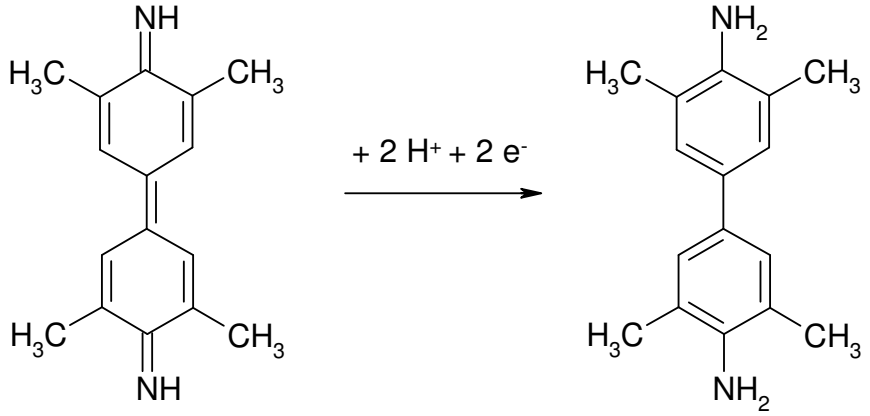


Figure 3.9: The electrochemical reaction of TMB.

3.3.5 Optimisation of anti-aflatoxin M_1 antibody concentration.

To develop the system a variety of concentrations for the anti-aflatoxin M_1 antibody was tested without the addition of the free aflatoxin M_1 on the screen printed electrode sensor surface as shown in Figure 3.10.

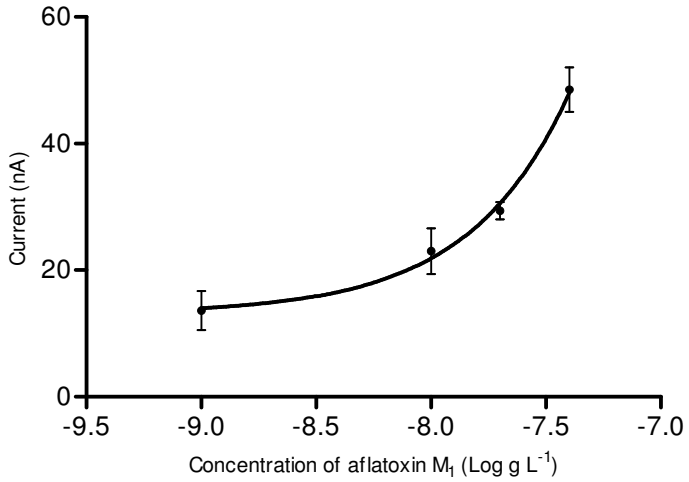


Figure 3.10: The effect of different primary antibody (anti-aflatoxin M_1) concentrations using chronoamperometry (-100 mV) for 10 minutes.

Figure 3.10 was obtained with TMB (0.5 mM) and hydrogen peroxide (1 mM) in 0.1M citrate phosphate buffer pH 5.2. Error bars denote standard deviations (n=3).

From Figure 3.10 it can be seen that the baseline response is proportional to the amount of anti-aflatoxin M_1 antibody, this is to be expected. The graph shows that a concentration of 100 μg mL^{-1} (1:10 dilution of the stock solution) yields the greatest response, however, this would incur a high cost for the sensor, hence a lower dilution of 40 μg mL^{-1} was trialled and if this had not given suitable results then a higher dilution would have been used. It was demonstrated during the ELISA development that the concentration yielding the highest signal was not required and a lower concentration could be used in order to reduce antibody consumption and hence costs.

From Micheli *et al.*, (2005) an initial concentration of 96 μg mL^{-1} (1:25 dilution of the stock) of capture (anti-anti-aflatoxin M_1 antibody) antibody was used, if this level was not sufficient then we would expect a plateau of the graph in Figure 3.10 at the highest concentrations. Therefore a concentration of 96 μg mL^{-1} is sufficient. In deviation from the reports of Micheli *et al.*, (2005) all incubations were done in a

humid environment at 37°C. This change is due to the observations during the development of single and multi-analyte affinity sensors for the rapid detection of androgen residues in live and *post mortem* animals (European commission contract QLK1-2001-01670). A humid environment was required to avoid the sensor from drying during incubations.

Using these adjustments, a calibration graph was obtained on the screen printed electrode to determine the limits of detection. Figure 3.11, shows the results obtained from the sensor.

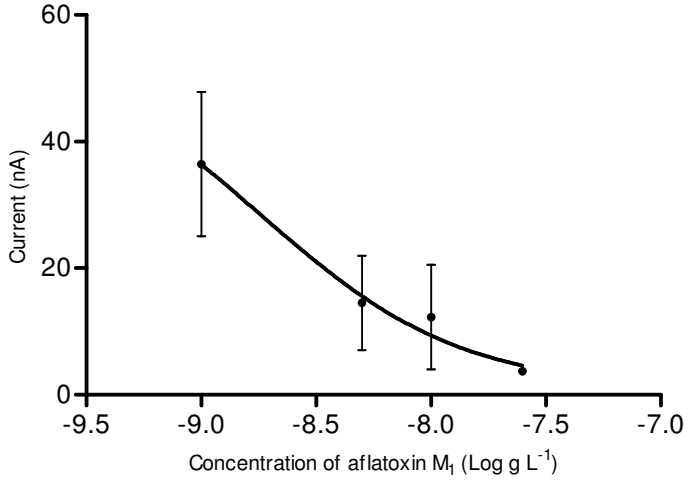


Figure 3.11: Initial results from the electrochemical sensor using chronoamperometry (-0.100 mV) for 10 minutes.

Figure 3.11 was obtained using TMB (0.5 mM) and hydrogen peroxide (1 mM) in 0.1 M citrate phosphate buffer, pH 5.2. Error bars denote standard deviations (n=3).

The calibration procedure was reproduced a different day and the same trend was obtained, although the limits of detection seem surprisingly low. Concerns were raised and the test was repeated however with the practical order of the standards reversed. Figure 3.12 shows the results for this test.

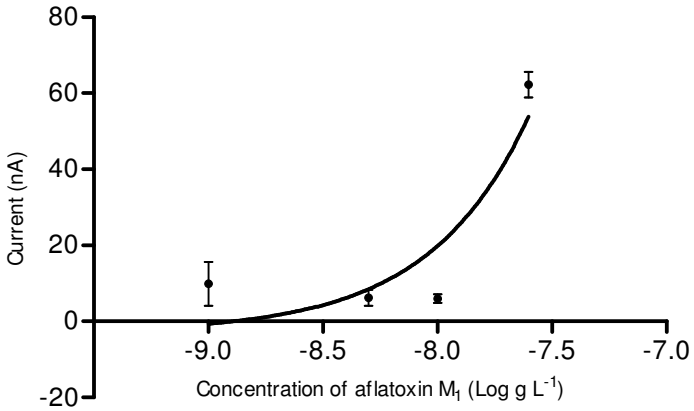


Figure 3.12: Conclusive results showing errors in the system using chronoamperometry (-100 mV) for 10 minutes.

Figure 3.12 was obtained using TMB (1 mM) and hydrogen peroxide (5 mM) in 0.1 M citrate phosphate buffer pH 5.2. Error bars denote standard deviations (n=3).

When reversing the order of analysis for the standards, the opposite trend was observed. This result was invalid. Each standard was analysed in triplicate with the electrochemical measurement taking 30 minutes to analyse. From the beginning of the run of all the standards to completion the electrochemical measurement took 3 hours. During this time the TMB mediator was found to be precipitating out of solution. This is assumed to be the reason for the decrease of electrochemical signal with time, which gave a plot similar to that expected from a sigmoidal curve seen from antibody based systems. Additionally hydrogen peroxide does cause some spontaneous oxidation with TMB, this has been quantified by Volpe *et al.*, (1998) as causing a 4 nA current change after 15 minutes although it should be noted that the TMB/H_2O_2 system is more stable than other systems with regards to oxidation such as hydroquinone.

With concerns about the solubility stability of the TMB mediator in the citrate buffer, different buffer solutions were tested. The TMB appeared stable in ultrapure water so the TMB was tested in a 1:10 dilution (10 mM) of the original citrate buffer

solution (100 mM) to investigate whether if the high ionic strength of the citrate buffer was the cause of the TMB instability.

The previous work was repeated but with the TMB solution prepared just before each measurement and using a diluted citrate buffer solution. A 1:10 dilution of the original citrate buffer was used (10 mM). This produced an expected sigmodial curve as shown in Figure 3.13.

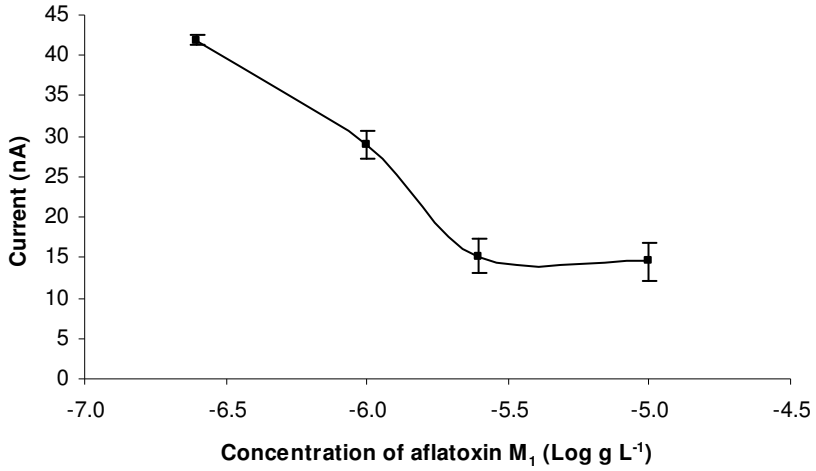


Figure 3.13: A standard curve of the optimised procedure for the electrochemical sensor using chronoamperometry (-100 mV) for 20 minutes.

Figure 3.13 was obtained using TMB (0.5 mM) and hydrogen peroxide (1 mM) in 10 mM citrate phosphate buffer pH 5.2. Error bars denote standard deviations (n=3).

The EU maximum permissible limit for aflatoxin M_1 is 50 ng L^{-1}. The calibration curve in Figure 3.13 fails to meets the requirements for the EU maximum permissible limits. Therefore further experimentation was carried out to increase the sensitivity of the sensor.

The initial immunosensor protocol was adopted from Micheli *et al.*, (2005) and was either validated or evolved to maximise the signal response and improve sensitivity. Firstly upon performing a literature review there are discrepancies into the optimum potential for electrochemical detection of TMB.

Micheli *et al.*, (2005) reported the detection of TMB at -100 mV versus Ag/AgCl whereas Butler *et al.*, (2006), Fanjul-Bolado *et al.*, (2005) Badea *et al.*, (2004), and Volpe *et al.*, (1998) suggest a voltage at +100 mV versus Ag/AgCl. To elucidate the correct potential initially step amperommetry was performed as shown in Figure 3.14.

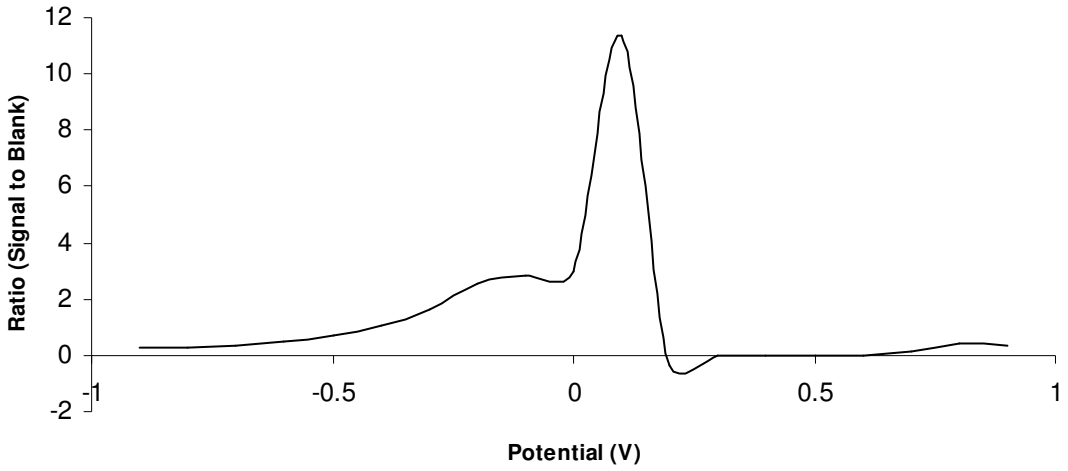


Figure 3.14: The determination of the optimum potential for horseradish peroxidase detection using TMB as the mediator.

In Figure 3.14 the ratio of the signal current to background current is plotted using step amperometry of 0.5 mM TMB / 1 mM H_2O_2 with the addition of peroxidase in pH 5.2 10 mM citrate buffer with 0.1 M KCl. The data is a result from an average of 4 electrodes.

No previous literature reports could be found where the preferential voltage for TMB detection had been discussed. All reports were either using the reduction peak or the oxidation peak at +100 mV (Butler *et al.*, 2006, Fanjul-Bolado *et al.*,

2005 Badea *et al*., 2004, Volpe *et al*., 1998) and -100 mV (Micheli *et al*., 2005), respectively, therefore a range of voltages from -900 mV to +900 mV was investigated. It is shown in Figure 3.14 that the best potential for monitoring the reduction was -100 mV and for the oxidation +100 mV. The step amperometry in Figure 3.14 suggested that +100 mV would yield a stronger signal than -100 mV therefore an additional more accurate experiment was preformed to validate this observation.

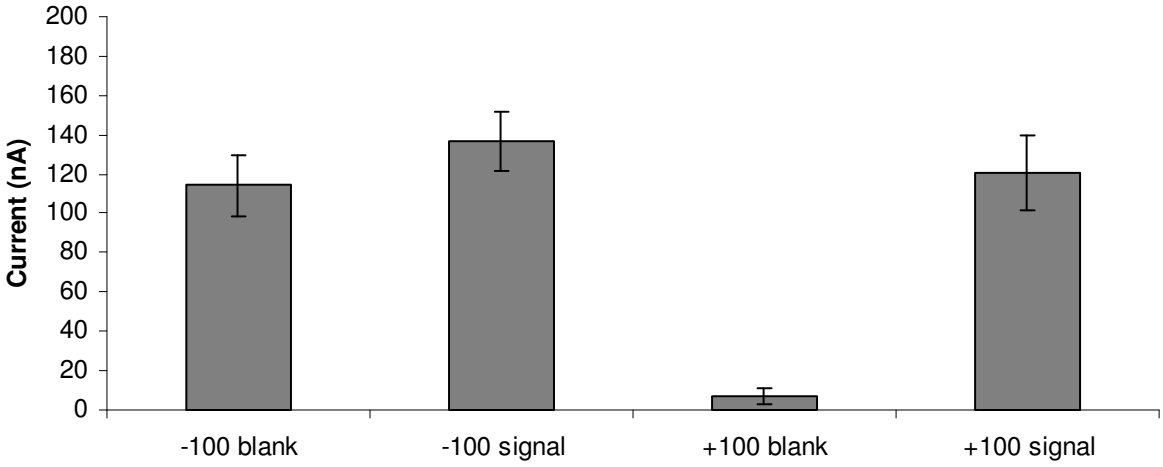


Figure 3.15: The comparison of different sensing potentials for horseradish peroxidase detection using TMB as the mediator.

In Figure 3.15 the blank comprised of the complete sensor system without the addition of aflatoxin M_1 – HRP conjugate. The buffer contained 0.5 mM TMB / 1 mM H_2O_2 in pH 5.2, 10 mM citrate buffer with 0.1 M KCl. Error bars denote standard deviation (n=3).

From Figure 3.15 although the reduction signal gave a greater signal than the oxidation signal, the reduction signal also incurred a high blank signal, hence for the development of the sensor the oxidation signal was monitored. The +100 mV did yield some background signal; this is to be expected since TMB with hydrogen

peroxide undergoes limited spontaneous reaction producing a small signal (Volpe *et al*., 1998).

Work completed in a previous EU project on the development of single and multi-analyte affinity sensors for the rapid detection of androgen residues in live and *post mortem* animals (European commission contract (QLK1-2001-01670) used electrochemical preconditioning of the electrode for TMB as the mediator for horseradish peroxidase (Conneely *et al*., 2007; Lu *et al*., 2006). No other literature reports could be discovered where a preconditioning potential was applied before detection of TMB. For the chronoamperometric detection a preconditioning potential of +200 mV for 20 s was applied before the scanning potential of +100 mV was set. Figure 3.16 shows the gain in detection signal when applying a preconditioning potential.

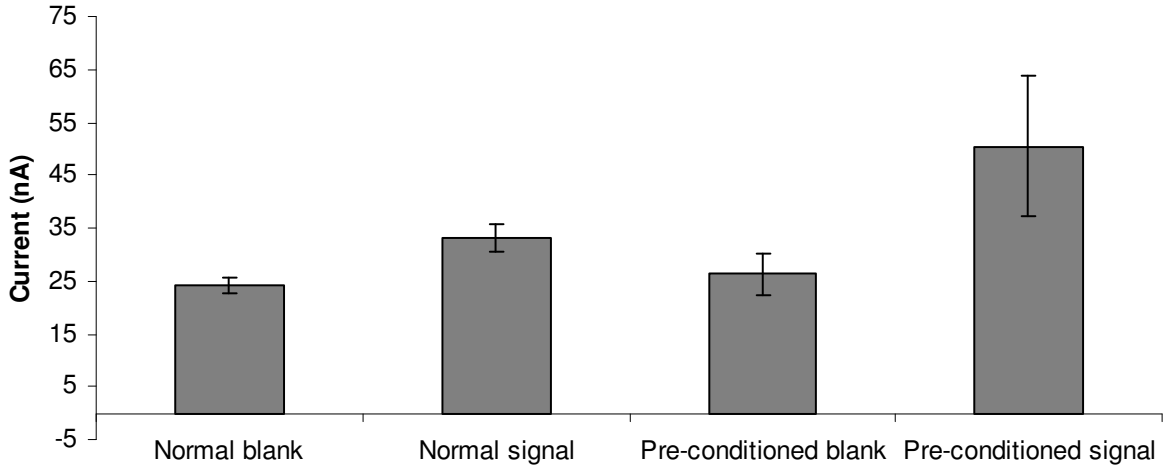


Figure 3.16: A study into the use electrode preconditioning for maximizing the signal for aflatoxin M_1 detection.

In Figure 3.16 the blank comprised of the complete sensor system without any addition of HRP- aflatoxin M_1. For preconditioning a potential of +200 mV was applied for 20 seconds followed by a 5 second equilibration stage before the data collection at an applied of potential of +100 mV. The blank comprised of the

complete sensor system without the addition of aflatoxin M_1 – HRP conjugate. The buffer contained 0.5 mM TMB / 1 mM H_2O_2 in pH 5.2, 10 mM citrate buffer with 0.1 M KCl. Error bars denote standard deviation (n=4).

Figure 3.16 shows although there is little advantage with respect to the background levels between applying a preconditioning potential or not. However there is a significant gain in signal by preconditioning the sensor before data collection.

Further electrode treatment was investigated to depolarise the electrode surface before antibody immobilization. Grennan *et al.*, (2000); Espinosa *et al.*, (1999) and Wang *et al.*, (1996) advocate the use of electrode pre-treatment. Summarising the reports, the use of a potential of 2.0 V from 30 seconds to 10 minutes was applied to increase protein immobilization capacity and electron transfer rates of the working electrode, in turn increasing the signal and reproducibility. The same treatment was performed for the in house produced electrodes to determine if this treatment would increase the signal or improve reproducibility.

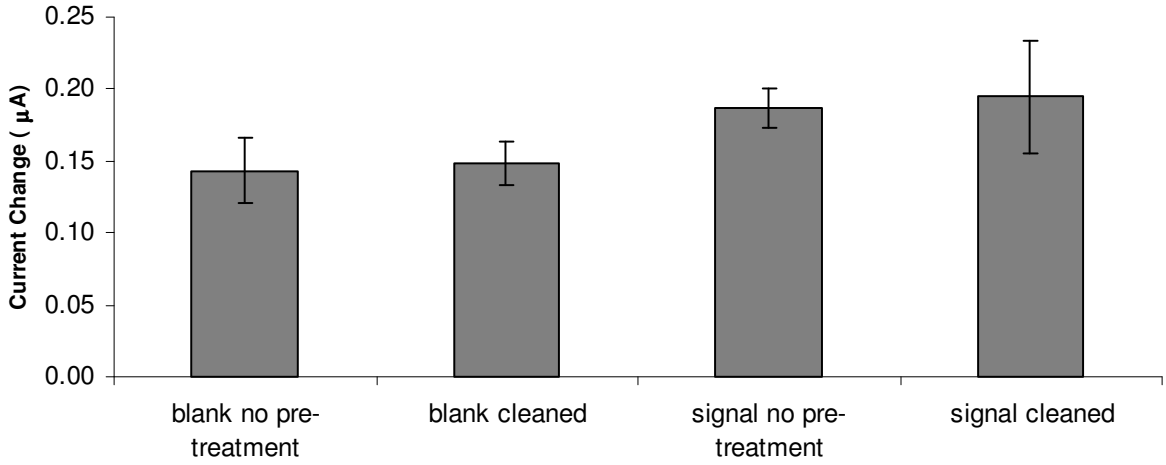


Figure 3.17: The effect of pre-cleaning of the electrodes before immobilisation of the antibodies.

In Figure 3.17 the electrodes were pre-cleaned by cleaning with water, ethanol and then applying a potential of 2.0 V for 30 minutes with the electrode covered with PBS before the application of the anti-primary antibody. The blank comprised of the complete sensor system without the addition of aflatoxin M_1 – HRP conjugate. The buffer contained 0.5 mM TMB / 1 mM H_2O_2 in pH 5.2, 10 mM citrate buffer with 0.1 M KCl. Error bars denote standard deviation (n=4).

As shown in Figure 3.17 although the depolarisation did produce a greater signal, the difference is not significant. Additionally the cleaning resulted in a high standard deviation. Considering the additional time incurred from depolarisation the electrodes it was deemed that this step was not fundamental to increasing the sensors performance.

The work of Micheli *et al.*, (2005) described the use of PVA as a blocking buffer. The use of different blocking buffers with a range of different chemistries was investigated to ensure that for electrochemical detection PVA is the most suitable blocking agent (Figure 3.18).

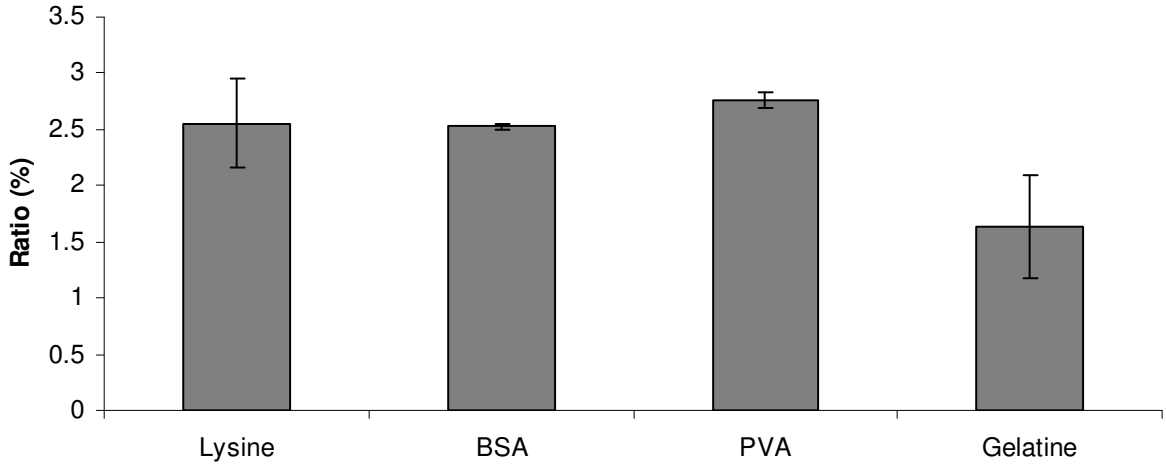


Figure 3.18: The effect of different blocking buffers on the signal.

In Figure 3.18 he different blocking buffers were made up in PBS buffer at a concentration of 1% and allowed to adsorb for 30 minutes at room temperature. The figure shows the ratio of the signal current and blank current where the blank signal was obtained using the complete sensor without the addition of aflatoxin M_1 – HRP. For all graphs error bars indicate the standard deviation (n=4). The buffer contained 0.5 mM TMB / 1 mM H_2O_2 in pH 5.2, 10 mM citrate buffer with 0.1 M KCl.

Traditional proteinaceous blocking agents such as gelatine and BSA did not perform as well as the PVA. Interestingly the signal for BSA was lower than that for gelatine; the reverse could have been postulated considering that aflatoxin M_1 - BSA was used as the immunogen for the antibody, thus demonstrating the high specificity of the monoclonal antibody.

With the signal ameliorated a calibration was performed in pure buffer undertaking the factors from the optimisation. The dynamic range from 1 to 10,000 ng L^{-1} possessed a linear r^2 value of 0.95 as shown in Figure 3.19.

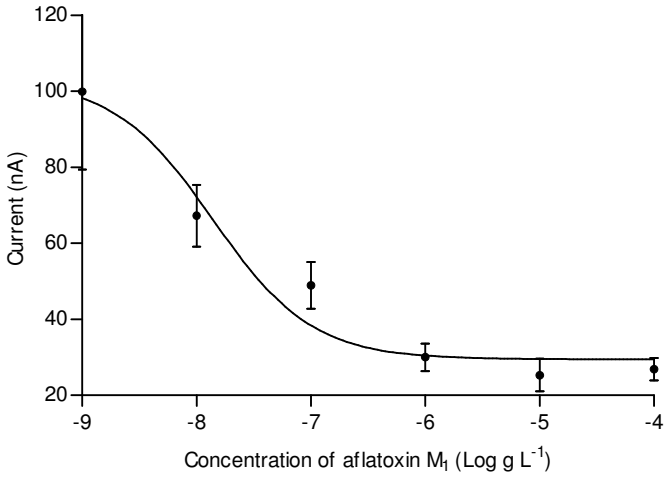


Figure 3.19: Standard curve for the detection of aflatoxin M_1 using the electrochemical sensor after optimisation.

Figure 3.19 was obtained using electrochemical preconditioning and data collection at a potential of +100 mV for 10 minutes. Error bars indicate the standard deviation (n=4) The buffer contained 0.5 mM TMB / 1 mM H_2O_2 in pH 5.2, 10 mM citrate buffer with 0.1M KCl.

With the system working well a calibration was performed in a milk sample with no pre-treatment other than centrifugation. The correlation between concentration of aflatoxin M_1 and current was not clear.
Milk was added to the system to determine what effects that may have. Figure 3.20 shows the calibration obtained with the presence of milk.

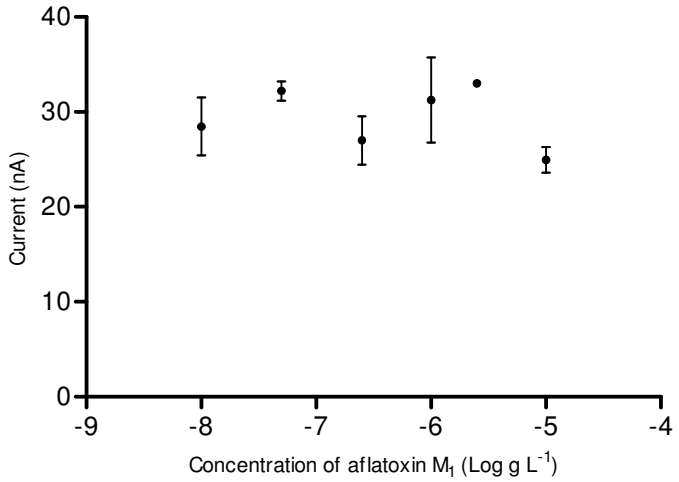


Figure 3.20: Calibration graph for aflatoxin M_1 in milk using chronoamperometry.

Figure 3.20 was obtained using TMB (0.5 mM) and hydrogen peroxide (1 mM) in 10 mM citrate phosphate buffer, pH 5.2. Error bars from standard deviations (n=4).

The milk sample selected was skimmed milk containing approximately 0% fat and was further centrifuged at 2000 g for 30 minutes. Figure 3.20 shows that there is significant interference caused by the presence of milk. Some sample treatment would be required, although the sample pre-treatment should be kept to a minimum so that the test can be carried out in the field at the point of source.

3.3.6 Electrochemical characterisation milk.

Previous reports from Pemberton *et al.*, (1999) states that electro-active species can interfere with the detection of progesterone in milk. Mayer *et al.*, (1996) have reported that milk can cause electrode fouling without pre-treatment, upon dialysis with a 12000-19000 molecular size cut off membranes the matrix effects are removed. To establish the cause of the interference several chemical clean up strategies were employed and tested by monitoring the electrochemical quenching effects of the cleanup sample to 5 mM potassium hexacyanoferrate in 0.1 M KCl.

Firstly potassium hexacyanoferrate was added to untreated milk (Figure 3.21).

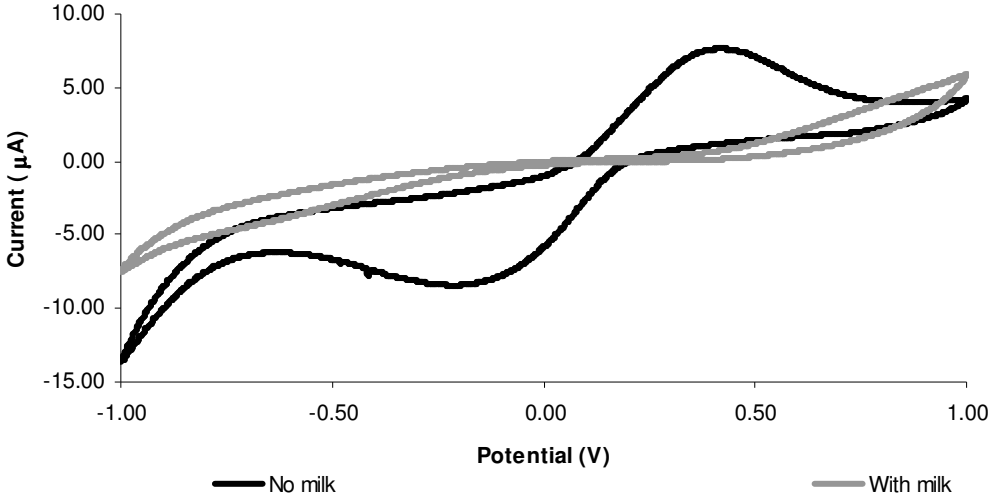


Figure 3.21: Demonstration of the quenching effect of milk on the detection of 5 mM potassium hexacyanoferrate in 0.1 M KCl. Plotted scans are from the average of 4 sensors.

To ascertain the effects of fats to the system two samples were spiked with potassium hexacyanoferrate to a concentration of 5 mM and 0.1 M KCl. Firstly a sample of milk was adjusted to pH 8.6 and incubated at 37^{o}C for 24h to activate the natural lipases in the milk and thus breaking down the fats into fatty acids (Hui, 1993). In a second sample, non-fat milk from Sigma - Aldrich (M7409) was also analysed. The resulting voltammograms are shown in Figure 3.22.

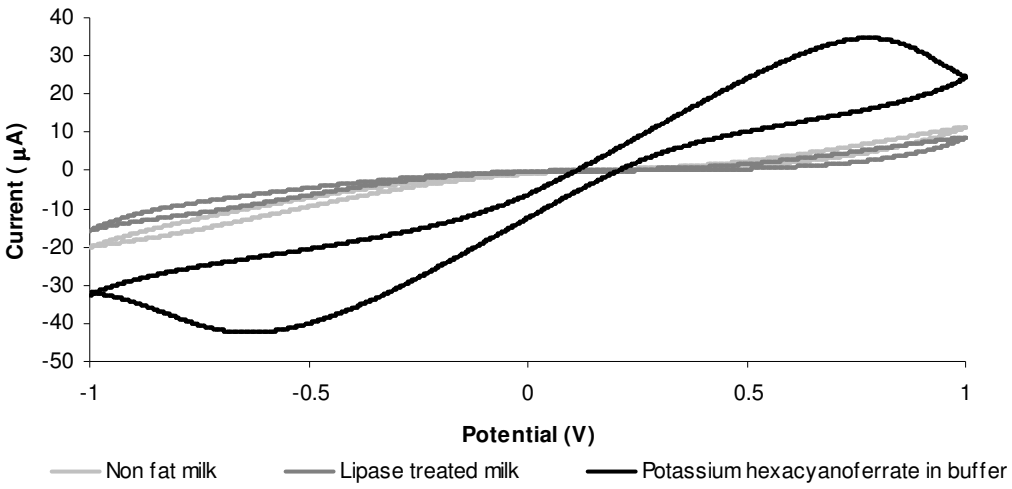


Figure 3.22: The cyclic voltammogram of 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl with and without the presence of non-fat milk or milk subjected to natural activated lipases. Plotted scans are from the average of 4 sensors.

Both of these samples quenched the electrochemical signal from potassium hexacyanoferrate suggesting that fats are not the cause of the electrochemical interference and another component of the milk is still affecting the signal.

Mayer *et al.*, (1996) reported that lactose was an interfering compound for their milk based biosensor. Furthermore the electro-active nature of lactose is taken advantage of as a method of detection for ion chromatography (Hanko and Rohrer, 2000). To determine the electrochemical effects of lactose, potassium hexacyanoferrate was spiked with 4.6% lactose to replicate the natural concentration in milk (Schrimshaw, 1988).

From Figure 3.23 it can clearly be seen that lactose is not responsible for the quenching effects seen from milk. This is to be expected since lactose is below the molecular weight which Mayer *et al.*, (1996) reported as being responsible for electrode fouling.

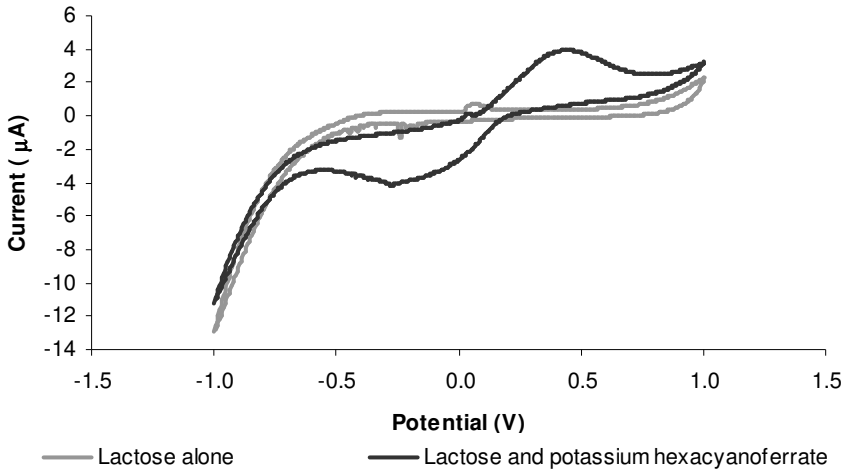


Figure 3.23: The cyclic voltammogram of 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl with and without the presence of 4.6% lactose. Plotted scans are from the average of 4 sensors.

A third major component of milk is proteins. Milk was fractionated into a casein free sample by the addition of HCl until a pH of 4.6 was obtained (Hui, 1993; Walstra, 1984) and a fraction free from both casein and whey using HCl and trichloroacetic acid using the methods described by Vernozy-Rozand *et al.*, (2004). Potassium hexacyanoferrate was added to all fractions and voltammograms were taken as shown in Figure 3.24.

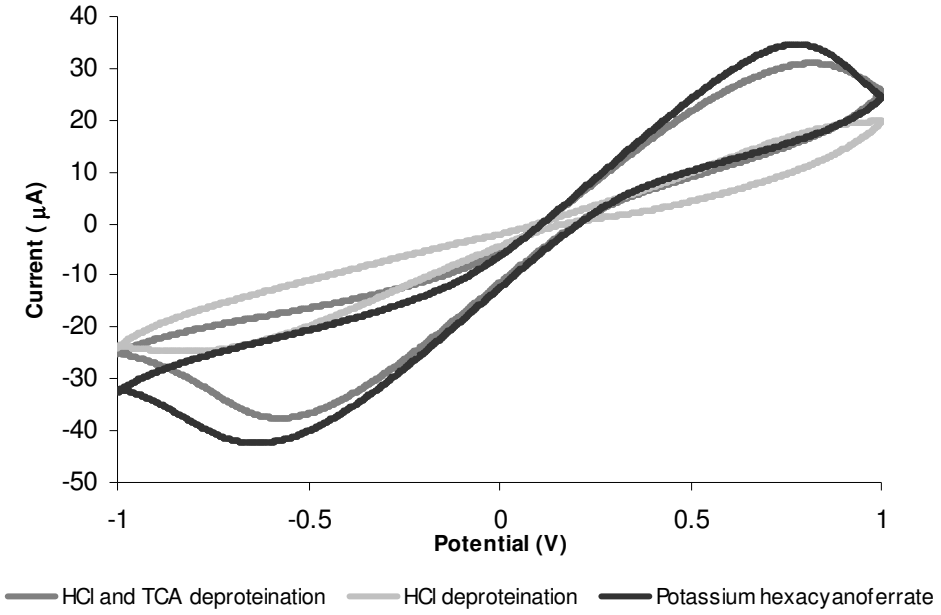


Figure 3.24: The cyclic voltammogram of potassium hexacyanoferrate (III) in 0.1 M KCl with and without the presence of milk liquor subjected to deproteination with HCl for casein removal and HCl and TCA for casein and whey protein removal. Plotted scans are from the average of 4 sensors.

The fraction from the casein only free sample still shows quenching of the signal from potassium hexacyanoferrate. Therefore the casein proteins alone are not the cause of the matrix interference.

It can be seen that although the peak height is not identical for the casein and whey free fraction, the peak separation however is similar. It should be considered that the voltammograms would not be truly identical due to the samples consisting of different ionic strengths. This does show that whey proteins are the cause of the matrix interference.

In a test to substantiate the findings from protein removal the proteins were removed in a milk sample through precipitation by saturating a milk sample with finely ground ammonium sulphate and allowing the milk proteins to completely precipitate for 48 hours at 4^{o}C. The corresponding cyclic voltammogram is shown in Figure 3.25.

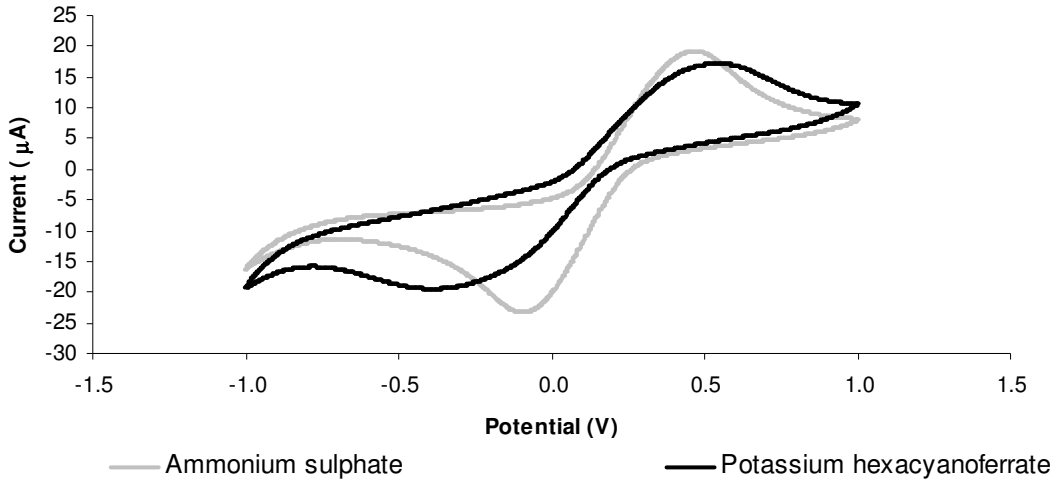


Figure 3.25: The cyclic voltammogram of potassium hexacyanoferrate (III) in 0.1 M KCl with and without the presence of deproteinated milk saturated with ammonium sulphate. Plotted scans are from the average of 4 sensors.

The pre-treatment with ammonium sulphate certainly removed all traces of the interference (the induced pH shift from ammonium sulphate is the cause of the sharper peaks) confirming that the electrochemical interference from milk is due to a proteinaceous compound.

Whey proteins otherwise known as 'milk serum' proteins are a group containing; β-lactoglobulin (18,363 Daltons), α-lactalbumin (14,176 Daltons) and bovine serum albumin (66,267 Daltons) additionally the groups also contains immunoglobins and small molecular weight peptides (Walstra, 1984). The molecular weight of β-lactoglobulin, bovine serum albumin and α-lactalbumin correlates with the reports of Mayer *et al.*, (1996) that the electrode fouling was eradicated by the use of dialysis membranes at 12,000 – 19,000 daltons. Cosman *et al.*, (2005) reinforces this observation. In a method utilising TLC as the detection method, Diaz *et al.*, (1993) suggested the use of dialysis membranes for the clean-up of milk with membranes at 8,000 to 15,000 Daltons.

Cosman *et al.*, (2005) reported on the adsorption of the milk whey proteins holo-α-lactalbumin and β-casein onto stainless steel surfaces. It was detailed that holo-α-lactalbumin spontaneously adsorbs onto the surface and thus causes fouling to the surfaces of pasteurisation elements, a phenomena known as '*milk stone*'. During the adsorption the protein goes through dramatic conformational change as it is denatured resulting in the loss of centrally bound calcium ions. Considering that the adsorption capacity for carbon is far greater than stainless steel it is possible that a similar fouling of the surface is arising from this protein.

In an experiment the calibration procedure was repeated however calcium chloride was added to the milk sample and washing buffers at a concentration of 18 mM based on the theory that through denaturation calcium is lost, then an increase in calcium ions may make it less energetically favourable for the protein to denature. The results are shown in Figure 3.26.

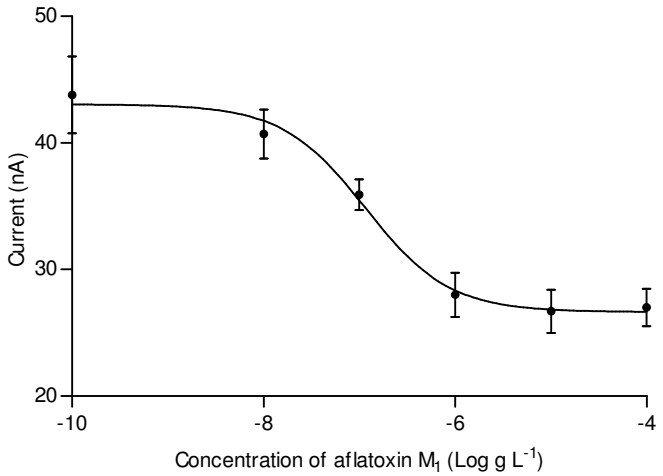


Figure 3.26: Calibration graph for aflatoxin M_1 in real milk samples using calcium chloride as pre-treatment. Error bars taken from standard deviations (n=3).

By spiking the milk sample with calcium chloride produced a working calibration graph with analytical sensitivity at 39 ng L^{-1}. Analytical sensitivity is determined as the amount of aflatoxin M_1 required to reduce the signal change by 25% (Ammidia

et al., 2004; Draisci *et al*., 2001). The concentration of 18 mM $CaCl_2$ was chosen to mimic the concentration of calcium chloride in PBS suggested by Dulbecco *et al*., (1954) upon the work with the isolation of viruses. The recipe later became known as Dulbecco's PBS and is a standard buffer for maintaining the structure of mammalian cells. However it is shown to work with antibodies. Therefore fears that an addition of calcium chloride could increase the ionic strength and affect the antibodies activity are unfounded.

Although the detection at 50 ng L^{-1} is possible, the error bars are significant and therefore accuracy with the measurement is low. To improve the accuracy, causes of the poor reproducibility was investigated.

3.3.7: Investigations into the errors associated with the screen printed sensor.

The sensors in use had been manufactured 18 months before Figure 3.26 was obtained. A new batch of sensors was produced and the calibration was repeated as shown in Figure 3.27.

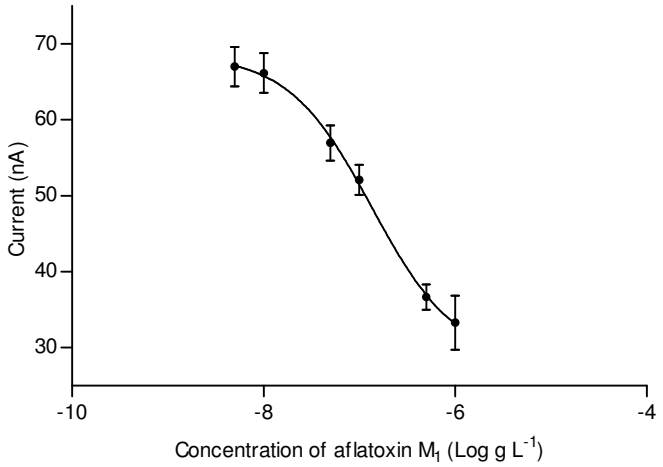


Figure 3.27: A calibration using calcium chloride for milk pre-treatment and fresh sensors. Error bars taken from standard deviations (n=3).

The relative standard deviation for the 10 ng L^{-1} point for the old electrodes is 9.5 % whereas for the new electrodes it was 7.8 % therefore showing that fresh electrodes improved the reproducibility however the error bars are still significant. Additionally the signal improved from Figure 3.26 with the working range greater than 30 nA compared to less than 20 nA, suggesting that the new electrodes had lower resistance than the old electrodes again possibly due to surface contamination. With these improvements it should be noted, however, that the analytical sensitivity became worse from 39 ng L^{-1} in Figure 3.26 to 42 ng L^{-1} for Figure 3.27, although this change is not significant.

To try to elucidate the variability between electrodes a fresh sheet of new electrodes was taken and the resistance from the carbon connector to the working electrode was taken for every sensor on the sheet (60 electrodes). The results are shown in Figure 3.28.

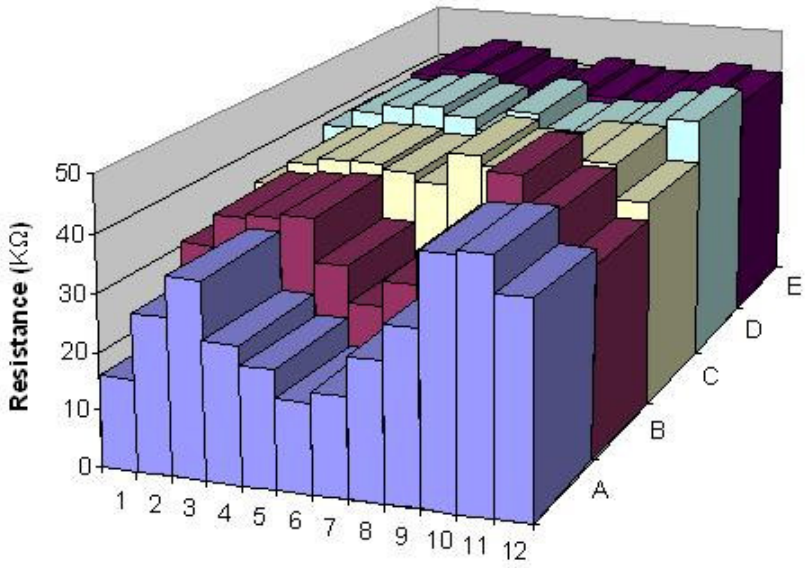


Figure 3.28: A 3D graph showing the resistance changes across a sheet of screen printed electrodes (%CV = 21%).

Figure 3.28 shows that about 60% of the sheet had unilateral resistance. However towards the top of the sheet there is a reduction in the resistance. Therefore this

would incur poor repeatability, if these sensors were used in the test, since upon measuring current at a fixed potential then the resistance must also be fixed within a test. The cause of the variation in the resistance is most probably due to two issues. Firstly if the tension varies around the printing screen during electrode production then different thicknesses of carbon ink will be deposited on the screen and thus different resistances will arise. Secondly, placement in the oven during drying can affect the thicknesses of the carbon ink, and thus the resistances, if hot air from the oven fans are directed over one part of the screen during drying (Gilleo, 1996). These issues are inherent with screen printing technology, particularly if the fabrication machine is old. In order to overcome this variability a new screen printer is required to replace the existing printer since the printing registration can affect the quality of the sensors. Also the number of sensors printed on each sheet need to be reduced from 60 per sheet to 5 or 10 sensors.
A further cause of reproducibility errors could derive from sampling and sample pre-treatment. Van Egmond (1983) stated that although the greatest source of error in analytical measurement is through sampling, for milk this error is negligible since milk is assumed to be a homogeneous matrix.

The developed pre-treatment technique with calcium chloride from the screen printed method was compared to existing technology.

Spiked milk samples prepared using the method developed for the immunosensor were analysed using the Ridascreen ELISA kit along with the standards in the kit. As shown in Figure 3.29 the calcium chloride pre-treatment did not fully isolate the aflatoxin M_1. Therefore although the calcium chloride pre-treatment is a very clean pre-treatment there is some underestimation.

Mendonça and Venâncio (2005) and Dosako *et al.* (1980) suggest that aflatoxin M_1 has affinity for casein proteins and whey proteins. The binding of aflatoxin M_1 with casein is due to the hydrophobic pockets formed by the high number of proline

residues in casein (Bakirci, 2001; Henry, *et al.*, 1997). It could be postulated that by increasing the ionic strength of the milk by adding calcium chloride then the aflatoxin M_1 has increased affinity for the casein through hydrophobic interaction and thus remains partially bound during analysis. The underestimation requires that all samples and calibration standards need to be treated the same for this bias to be uniform and thus accountable.

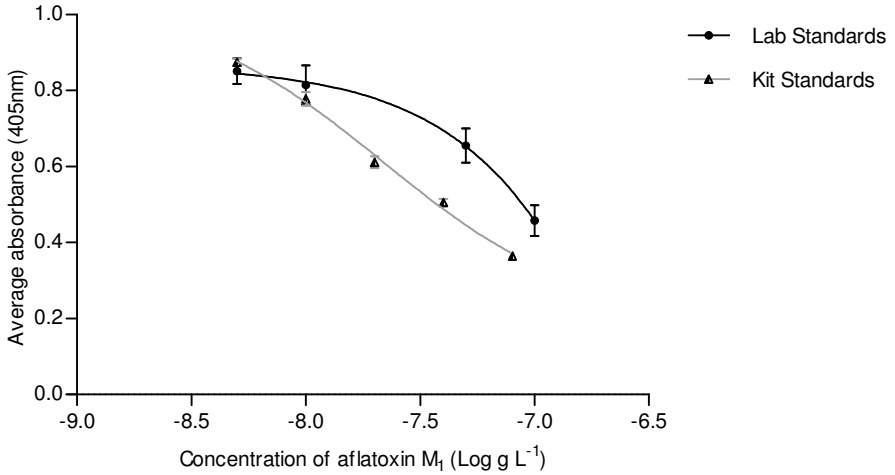


Figure 3.29: Comparison of the calcium chloride pre-treatment method with existing ELISA standards obtained with the Ridascreen kit. Error bars denote standard deviations (n=3).

More critical is the fact that the standard deviations for the laboratory prepared samples using calcium chloride are higher than those obtained using the kits and therefore more work is required to improve the repeatability of the extraction.

3.3.8 Further application of the aflatoxin M_1 immunosensor.

As detailed in Chapter 1, aflatoxin M_1 can be found in the urine, where it acts as an important biomarker for aflatoxin consumption. The reviews of Jonsyn-Ellis (2000a,b) Nyathi *et al*., (1987) and Coulter *et al.,* (1986) reported levels of aflatoxin M_1 as high as 374 µg L^{-1} and mean levels between 490 ng L^{-1} to 7.1 µg L^{-1}. With the immunosensor successfully designed and optimised for aflatoxin M_1 detection in milk to 50 ng L^{-1}, initial work was done to investigate the matrix effects of urine

on the sensor. Using the same procedure as the immunosensor for milk, however without the addition of calcium chloride to the sample, a calibration graph was obtained. The data is shown in Figure 3.30.

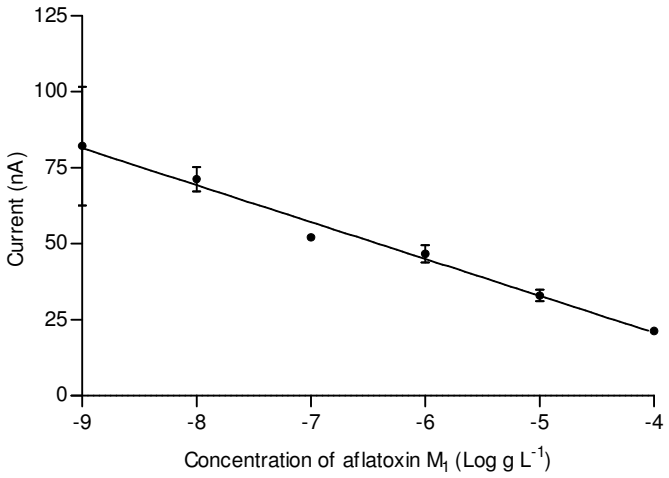


Figure 3.30: The effect of urine on the immunosensor.

Figure 3.30 was obtained using TMB (1mM) and hydrogen peroxide (5mM) in citrate phosphate buffer pH 5.2. Error bars denote the standard deviation (n=3).

Figure 3.30 clearly shows that the immunosensor is applicable for the detection of aflatoxin M_1 in urine with an analytical sensitivity of 25 ng L^{-1}. The levels of aflatoxin M_1 reported with means in the region of 490 ng L^{-1} to 7.1 µg L^{-1} lie midway in the dynamic region of the plot. Further work should be carried out to ensure that the results are reproducible. However early results are encouraging, especially since there is no reported evidence of a sensor for aflatoxin M_1 detection in urine. Furthermore this analysis provides key information on aflatoxin consumption in rural areas of Africa. In Zimbabwe 20,000 deaths per year are attributed to aflatoxin consumption (Nyathi *et al.*,1987) and the worldwide figure is estimated at over 200,000 deaths per year (Groopman *et al.*, 1993).

3.4 Comparison of the developed immunosensor to other technologies.

With the immunosensor fully functioning, comparisons were made against other technologies with regards to performance and cost. The immunosensor was compared to the Ridascreen ELISA kit and a developed HPLC method.

3.4.1 The development and validation of the HPLC method.

Originally the determination of aflatoxin M_1 was performed using the standard method for the Waters HPLC equipment which was for the determination of Ochratoxin A. The resulting chromatogram showed the elution of the aflatoxin M_1 at the start of the chromatogram and thus prone to interference from non-retained components from the sample and also interference from the solvent peak. A literature search was performed to deem the most suitable ratio of acetonitrile to water which would yield good peak separation. Table 3.5 shows the composition of the mobile phases.

Table 3.5: Literature reports for the determination of aflatoxin M_1 using a C18 analytical column.

Main author	**% Acetonitrile**	**% Methanol**	**% Water**
Winterlin *et al.*, (1979)	28	0	72
Chambon *et al.*, (1983)	20	5	75
Takeda (1984)	15	15	70
Farjam *et al.*, (1992)	20	5	75
Saad *et al.*, (1995)	25	0	75
Martins & Martins (2000)	25	0	75
Kim *et al.*, (2000)	30	50	20
Galvano *et al.*, (2001)	28	0	72
Roussi *et al.*, (2004)	25	0	75
Elgerbi *et al.*, (2004)	20	20	60
Martins & Martins (2004)	25	0	75
Gürbay *et al.*, (2006)	16	22	62
Bognanno *et al.*, (2006)	25	25	50

The original mobile phase was 57% acetonitrile, 41 % H_2O and 2% acetic acid. With the aflatoxin M_1 peak eluting early on the C18 column, the mobile phase

therefore required to be more polar by consisting of a greater percentage of water. Upon reviewing table 3.5 this is clearly seen that the majority of authors use a higher water ratio thus confirming that the polarity of the mobile phase was too low. The most common composition of mobile phase is of 25% acetonitrile and 75% water. Upon running this mobile phase the elution of aflatoxin M_1 occurred at 11 minutes rather than 2 minutes and thus isolating aflatoxin M_1 from the non-retained compounds. Figure 3.31 shows an example chromatogram.

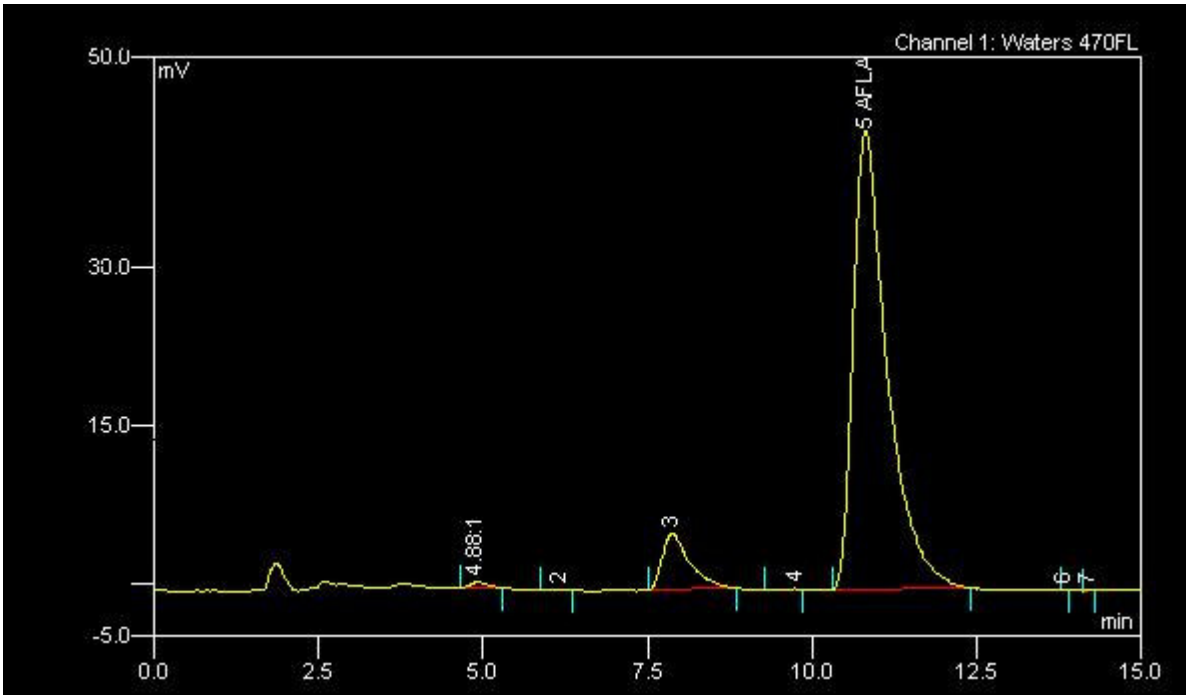


Figure 3.31: The chromatogram from the determination of aflatoxin M_1 using the Waters HPLC system.

In Figure 3.31 the aflatoxin M_1 peak has a retention time of 10.5 minutes and is clearly resolved from other interfering compounds.

Using these parameters a calibration graph was performed as shown in Figure 3.32.

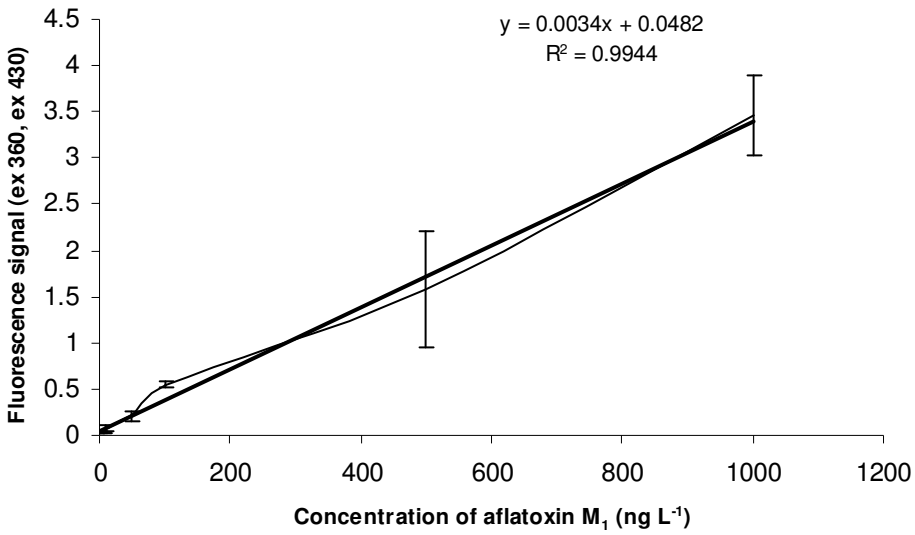


Figure 3.32: A calibration graph for aflatoxin M_1 standards extracted from milk using immunoaffinity columns. Error bars plotted from the standard deviations from triplicate injections.

The calibration from the immunoaffinity columns produced an r^2 value of 0.994 showing that the method is valid. Using the accepted definition of the limit of detection is equilivent to 3 times the standard deviation of the zero value, the limit of detection for aflatoxin M_1 by immunoaffinity SPE- HPLC is less than 10 ng L^{-1}.

3.4.2 The comparison of HPLC, ELISA and the immunosensor with respect to performance and cost.

Milk samples were prepared using calcium chloride pre-treatment and the same sample were analysed by all three methods. Figure 3.33 shows the calibration graphs for all three methods.

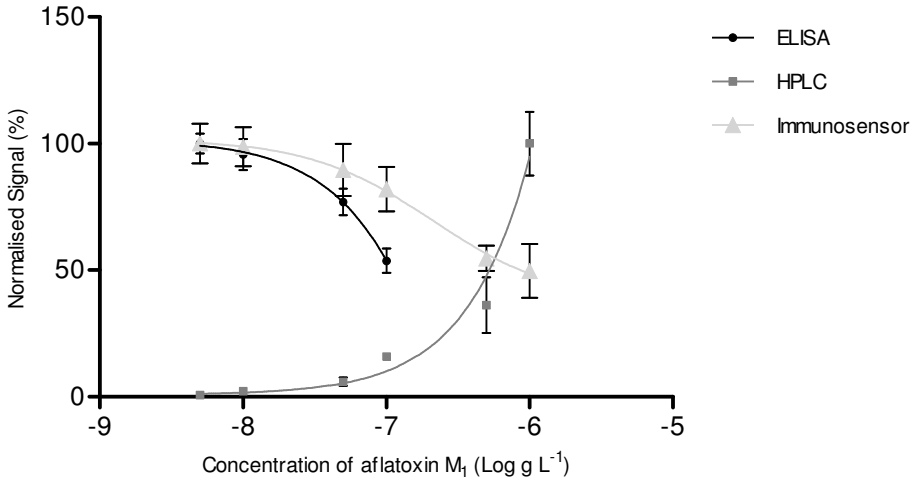


Figure 3.33: Comparison between the developed screen printed immunosensor with the developed HPLC method using immunoaffinity pre-treatment and the commercial Ridascreen ELISA kit.

In Figure 3.33 the same samples were used for all three methods and performed on the same day of analysis. For comparison the scale has been normalised to the highest signal for each method. Error bars denote standard deviations (n=3).

The plots in Figure 3.33 show the success of the immunosensor development. Compared to the ELISA method, the immunosensor has similar limits of detection and comparable repeatability although the working range of the immunosensor is far greater than the ELISA method.

Comparing the HPLC data to the immunosensor data, the HPLC and immunosensor has arguably similar sensitivity. However, at low concentrations the reproducibility of the HPLC is superior to the immunosensor. The HPLC and the immunosensor have similar dynamic ranges from 0 to 1000 ng L^{-1}, although at high concentrations the immunosensor has marginally superior repeatability. With similar performance between the HPLC and immunosensor the cost of analysis was reviewed. Figure 3.34 shows predicted costs of using the three technologies with respect to cost of purchasing the instrumentation and running the analysis (derivation of the costs are detailed in Section 8.2).

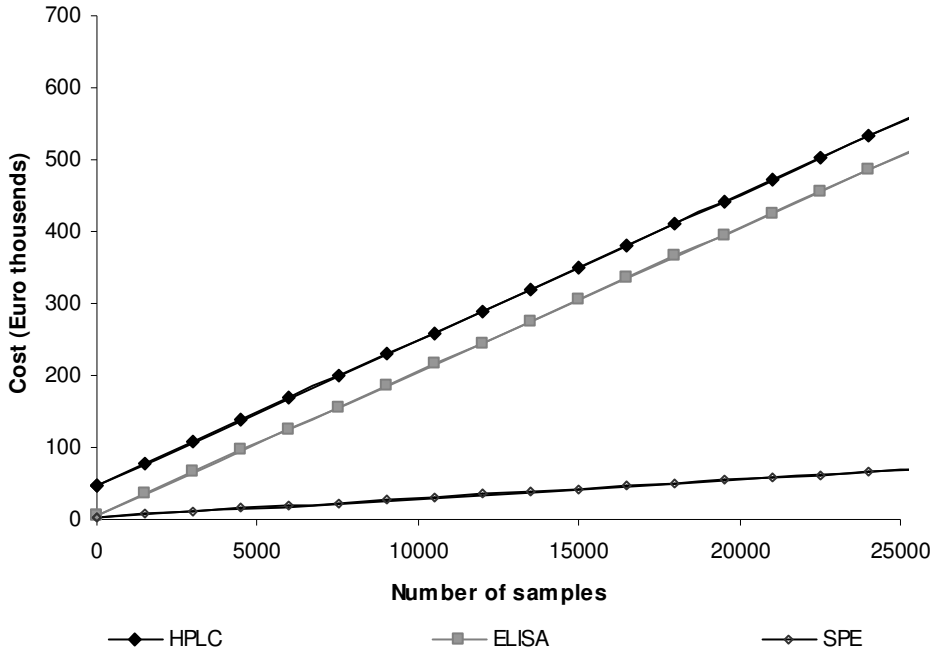


Figure 3.34: The comparison of the start-up costs and analysis costs for three different methods in terms of number of samples analysed.

All three methods rely on antibodies in the analysis. HPLC utilises antibodies in the form of immunoaffinity column pre-treatment, ELISA requires antibodies to be immobilised onto the surface of the microwell plate and the immunosensor requires antibodies on the surface of the electrode. It is this component of the analysis which is the main cost inherent to all methods. The ELISA and immunosensor methods require the same amount of financial investment. However, the cost per analysis is much cheaper for the immunosensor compared to the ELISA, which makes the former cheaper overall. The analysis cost of the HPLC and the ELISA are virtually the same as can be seen by the gradients in Figure 3.34. The main drawback of the HPLC with regards to cost is the higher start-up costs involved in purchasing the instrumentation.

The main advantage with the immunosensor is the portability of the equipment, which is difficult to quantify, but using the Palmsens^{TM} (a handheld, battery

operated electrochemical potentiostat from the Netherlands) the immunoassay can be performed away from the laboratory. Both the HPLC and ELISA methods require optical measurement which is fragile technology and cannot be miniturised. Therefore in terms of robustness the immunosensors boasts superior robustness compared to the HPLC and ELISA methods and increased portability.

The results obtained from this project shows that screen printed technology answers the criteria of a simple, robust, low-cost analysis methods for aflatoxin M_1 analysis in milk.

3.5 Conclusions to electrochemical immunosensor development.

Starting with the components from the ELISA development the system was transferred to screen printed electrodes. Initially the system was not capable for detection of aflatoxin M_1 at 50 ng L^{-1} but upon optimising the system with regards to measurement potential as well as other factors limits of detection improved. The final method scheme is detailed in Figure 3.35.

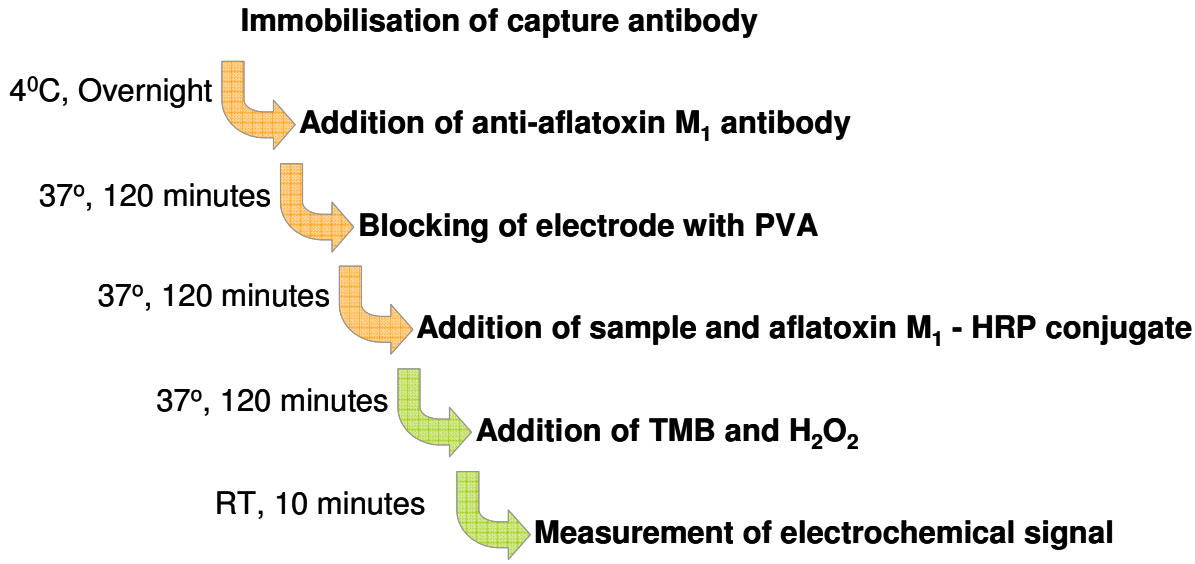


Figure 3.35: The developed immunosensor scheme.

The scheme in Figure 3.35 is split into to stages. First (marked with orange arrows) is the preparation of the sensor; immobilisation of the antibodies and blocking the working electrode surface. Second is the analysis (shown in green) i.e. competition reaction and detection.

The immunosensor scheme for the end user was faster and with fewer steps than the ELISA method. This improvement was due to the immobilisation of the antibody rather than aflatoxin M_1 – BSA. The scheme requires three steps to be performed for 2 hours. These incubation times were not optimised and therefore the method could be preformed faster upon further optimisation.

After synchronising the immuno-components to the electrode surface the effects of milk on the sensor was assessed. It was discovered that the milk matrix causes significant effects, chiefly from whey proteins. When an excess of calcium chloride was added to the milk matrix then the effects from the whey proteins was suppressed and a working calibration graph down to 39 ng L^{-1} was obtained.

The immunosensor is not solely suitable for milk, but initial investigations have shown that the immunosensor could be employed for aflatoxin M_1 determination in urine, and therefore monitor human aflatoxin M_1 consumption. The quenching effect seen from milk was not observed from urine, thus the addition of calcium chloride to the sample or any other sample pre-treatment was not required.

Large error bars were a cause for concern with the calibration graphs obtained with the immunosensor therefore the causes was investigated. It was discovered that aging of the electrodes had caused some reduction in the electron transfer and thus increased resistance. Additionally it was observed that the screen printing process was not fully reproducible and therefore partially responsible for the poor reproducibility.

To improve the performance of the immunosensor the use of substituting the screen printed electrodes with arrays of microelectrodes was investigated and shall be discussed in Chapter 4.

CHAPTER 4

DEVELOPMENT OF MICROELECTRODE ARRAY AS THE IMMUNOSENSOR

4.1 Introduction.

The European Union maximum permissible limits for aflatoxin M_1 in milk was achieved using screen printed carbon electrodes (Chapter 3). However the detection limit is close to the required limit of detection and reproducibility is poor. In this chapter microelectrode arrays were reviewed with the possibility whether it was possible to improve the signal and hence the detection limit.

The term microelectrode is described for an electrode where one of its dimensions is in the µm range (Ŝtulik *et al*., 2000). Advances have been fuelled by medical applications where microelectrodes can be implanted to monitor electrophysiological pulses such in cardiac tissues (Hoffman, 2002) or oxygen levels in living tissues (Bond, 1994). For immunosensors the advantages of microelectrodes are greater mass transport, which results in a greater sensitivity, and an increased electrode signal to surface area ratio compared to larger electrodes. This causes low noise levels for microelectrodes and therefore are very sensitive. The main disadvantage of microelectrodes is the low current generated from the devices requiring very sensitive potentiometers to record the signal. To overcome this obstacle, arrays of many microelectrodes are placed together, wired in parallel and the current is increased (Davis and Compton, 2005; Feeney *et al*., 1997; Wittkampf *et al*., 1997).

Utilising the optimisation for the immunosensor with the screen printed electrodes, the method was transferred to electrochemical microarrays developed and provided by Tyndall National Institute in Ireland (Berduque *et al*., 2007; Berduque *et al*., 2005; Arrigan, 2004) with minor modification. Competitive calibrations were performed, with aflatoxin M_1 in the milk matrix, on the microarray to determine its feasibility for the application and sensitivity improvements.

4.2 Materials and methods.

4.2.1 Materials used for the microelectrode array.

The materials used for the microelectrode array immunosensor are as described in Section 3.2.2. Briefly the capture antibody (anti-anti-aflatoxin M_1) was obtained from Peirce (Cramlington, UK) and the anti-aflatoxin M_1 antibody was obtained from Abcam Ltd (Cambridge, UK). The aflatoxin M_1 – HRP conjugate was supplied as part of a diagnostic kit from R-Biopharm (Glasgow, UK). TMB, H_2O_2 and all other general chemicals were purchased from Sigma-Aldrich LTD (Gillingham, UK). All buffers and antibody solutions used were the same concentrations and ionic strengths as reported in Section 3.2.2.

4.2.2 Antibody immobilisation onto the microelectrode array.

The gold cell-on-a-chip microelectrodes (including on-chip reference and counter electrodes) were fabricated by standard deposition, etching and lithographic techniques and were produced by Tyndall national institute (Berduque *et al*., 2007; Berduque *et al*., 2005; Arrigan, 2004) and had a modified surface allowing covalent immobilisation of the bio-recognition molecules (Figure 4.1). The working, counter and reference electrodes were made from gold. The working electrode consists of 35 elements, 20 µm x 20 µm wide, wired in parallel.

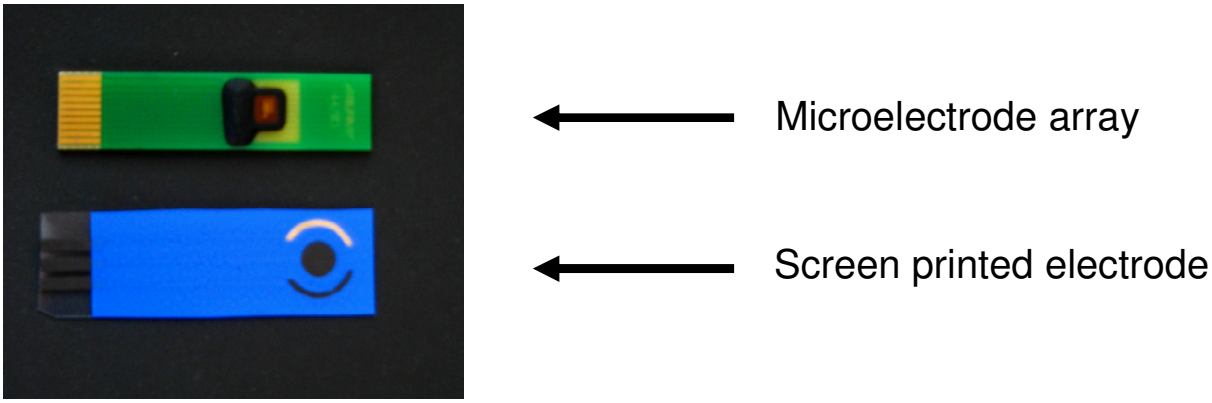


Figure 4.1: The microelectrode array from Tyndall National Institute and the in-house fabricated screen printed electrode.

The surfaces had been silanised followed by attachment of a PDITC (1,4-phenyldiisothiocyanate) cross linker compound for antibody attachment was carried out by Tyndall national institute as described by Lillis *et al*., (2006). The capture antibody (anti-anti-aflatoxin M_1) from Pierce (Cramlington, UK) was diluted (96 μg mL^{-1}) with carbonate buffer, (0.1M, pH 9.6) of which 1 μl of the antibody solution was placed onto the working microelectrode. The electrodes were then placed on a damp tissue in a Petri dish and stored overnight at 4°C to allow covalent attachment. The microelectrodes were washed with 10 mM PBS-T pH 7.4 buffer twice and once with water using a dispensing bottle, ensuring that the buffer was not directly sprayed onto the working microelectrode, and then shaken dry.

After drying, 3 μl of 0.1% NH_4OH in water was added for 60 mins at room temperature to deactivate any un-reacted PDITC cross linker and then washed and dried. A 1 μL of 40 μg mL^{-1} anti-aflatoxin M_1 antibody (Abcam, Cambridge, UK) was placed onto the microelectrode and incubated at 37°C for 2 hours in a Petri dish with damp tissue. The electrode arrays were then washed and dried as reported above and stored at 4°C until used.

4.2.3 Assay development for the microelectrode array.

To the antibody immobilised microelectrode 1 μl of sample or standard, mixed 1:1 with aflatoxin M_1 HRP (diluted 1:10 with 10 mM PBS, pH 7.4) was placed and incubated at 37°C for 120 minutes.

For the detection, 1 mg of TMB was dissolved with 150 μl de-ionised water, 20 μl of this stock solution was taken with 2 μl of 30% hydrogen peroxide and made up to 1 ml using 10 mM citrate buffer, pH 5.2, in 0.1 M KCl at 37°C. A 4 μl aliquot of the TMB solution was placed onto the micro array immediately prior to analysis. The stock solution of TMB was prepared daily and stored in the dark prior to use.

The electrochemical measurement was performed by connecting the microarray to the Autolab using an interface provided by the Tyndall National Institute and the scanning potential was set to +168 mV and a pre-conditioning potential was applied before measurement for 5 seconds at a potential of +268 mV. Figure 4.2 shows a diagram of the surface chemistry of the microelectrode array with the provided PDITC cross linker for covalent amine linkage.

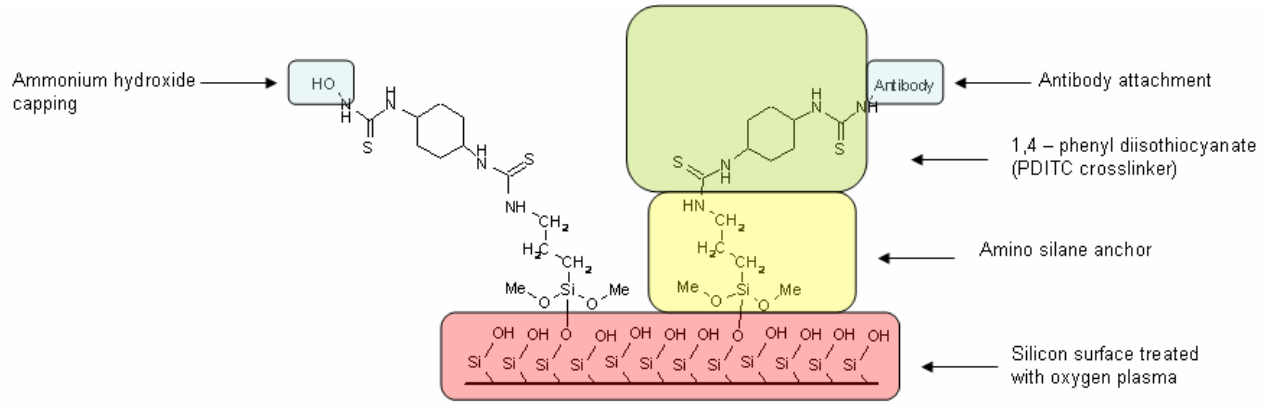


Figure 4.2: A schematic diagram for the covalent immobilisation of the capture antibody onto the microelectrode surface. The surface chemistry was performed by Tyndall National Institute and the capture antibody attachment and capping of the excess linker was performed at Cranfield.

4.2.4 Surface analysis of the microelectrode array by AFM and SEM.

The surface of two microelectrode arrays were analysed in detail to monitor and ensure correct immobilisation of the antibody. To one of these sensors the surface was prepared by immobilising the capture antibody before the surface analysis. This was done using the same concentration of reagents as the SPE immunosensor.

A 1 µL aliquot of 96 µg mL^{-1} of capture antibody solution (Pierce, Cramlington, UK) was placed onto the microelectrode surface at pH 9.6 and incubated at 4°C overnight for immobilisation. The surface was washed with 10 mM PBS-T and H_2O then the excess linker compound applied by Tyndall national institute was deactivated using 4 µL 0.1% NH_4OH for 1 hour at 37°C.

The atomic force microscopy (AFM) images were obtained using a Dimension 3000, manufactured by Digital Instruments (now Veeco Instruments, Cambridge UK). The tips used were silicon probes used in tapping mode. The probes were 225 x 38 x 7 microns with a typical resonant frequency of 160 kHz. The scan speed applied was between 0.5 to 1 Hz.

The SEM (scanning electron microscope) images and elemental scans were taken using a Philips XL30 SFEG (Guildford, UK) (scanning field emission gun).

4.3 Results and discussions for the microelectrode array immunosensor.

4.3.1 Surface characterization of the microsensors.

SEM images were taken using sFEG rather than a conventional SEM since the sFEG has a thin needle of a tungsten crystal as its filament rather than a normal tungsten filament and therefore the resolution is much better than using a standard SEM.

Using a high resolution scanning electron microscope (sFEG) at a low magnification images (x80 magnification) of the working microelectrode for the microsensors was taken. Figure 4.3 clearly shows the layout of the working microelectrode with the 35 arrays cut into the surface.

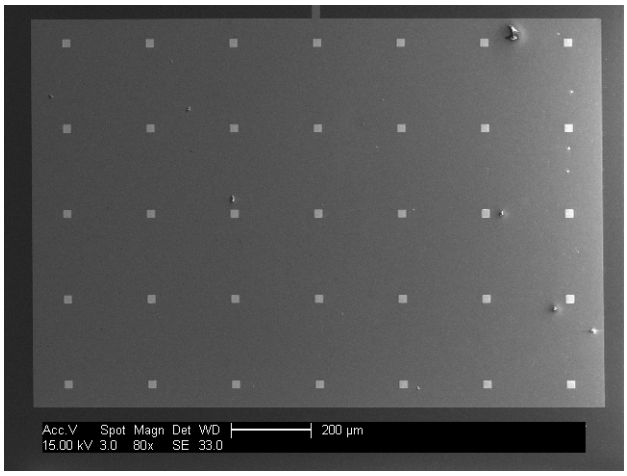


Figure 4.3: The whole working microelectrode of the untreated Tyndall microelectrode at 80x magnification using sFEG.

The magnification was increased to 3500x magnification to study the differences in the surfaces of a single element between the untreated surface and the antibody treated surface. This is shown in Figure 4.4.

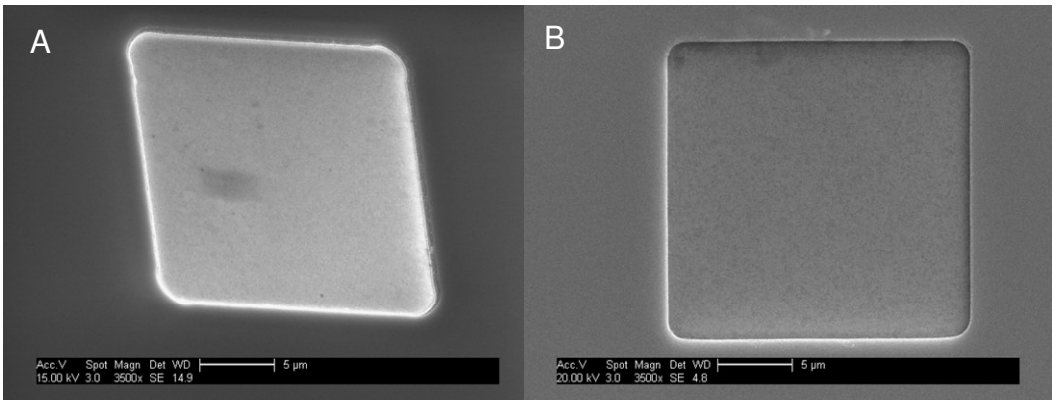


Figure 4.4: 3500X magnification of the single element in the electrode array.

Figure 4.4A shows 3500x magnification of a single element for the untreated working microelectrode. The surface is tilted 30^{o} to show the depth of the array. Figure 4.4B shows 3500x magnification of a single element for the treated working

microelectrode with the antibody present. The surface is at 0^{o}. For the treated microelectrode 1 µL of 96 µg mL^{-1} of capture antibody was immobilised at $4^{o}C$ overnight then excess linker compound was deactivated using 4µL 0.1% NH_4OH for 1 hour at $37^{o}C$ and sFEG images were taken immediately.

Although the tilt angles of the two images are different there is little to observe at this magnification. Figure 4.4A clearly shows the change in profile of the cut out of the array.
To observe differences in the surfaces the magnification was increased further to 35000 times magnification. Figure 4.5 show these images.

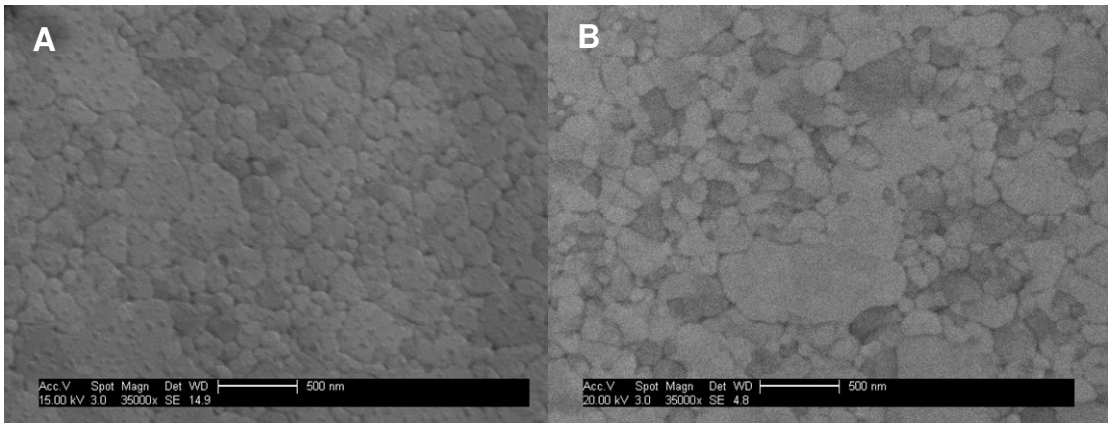


Figure 4.5: 35000X magnification of a single microelectrode array.

Figure 4.5A shows 35000X magnification of the surface of a single element for the untreated working microelectrode. Figure 4.5B shows 35000x magnification of the surface of a single element for the treated working microelectrode. For the treated microelectrode 1 µL of 96 µg mL^{-1} capture antibody was immobilised at $4^{o}C$ overnight, then excess linker compound was deactivated using 4µL 0.1% NH_4OH for 1 hour at $37^{o}C$ and finally sFEG images were taken immediately.

There are no major differences between the two images in Figure 4.5. On both there is some graining effect of the gold surface and possibly Figure 4.5B shows a

more uniform layer due to the protein surface. It should be noted though that the electron beam of the sFEG usually penetrates the surface to a depth of 10 μm therefore probably bypassing the proteinaceous surface. Furthermore the antibodies on the surface are not electron rich and hence not clearly visible by SEM techniques.

Elemental scans of the two surfaces were taken to deem if one had greater carbon content due to the proteins. Figures 4.6, 4.7 and Table 4.1 show the results.

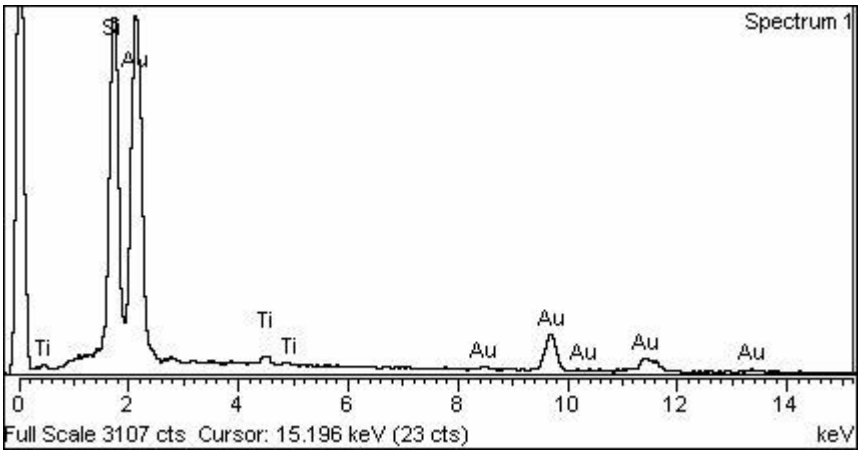


Figure 4.6: The elemental scan from the sFEG for the untreated working microelectrode surface showing the elemental composition of the surface.

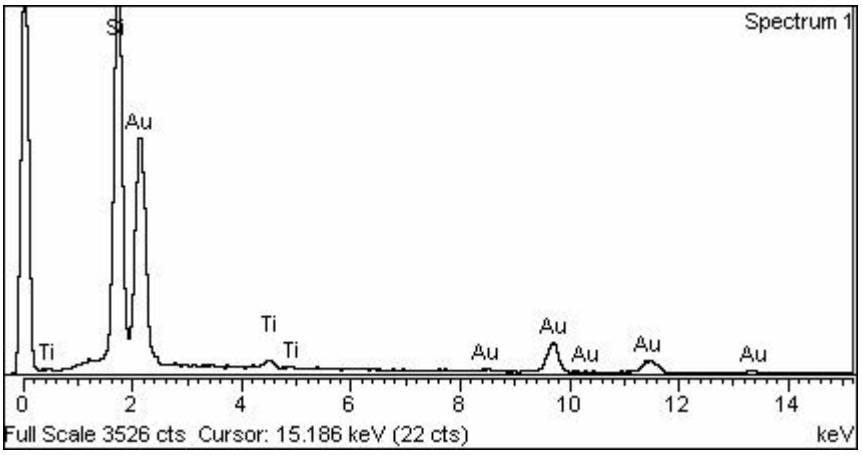


Figure 4.7: The elemental scan from the sFEG for the treated microelectrode working surface showing the elemental composition of the surface.

For the treated microelectrode 1 µL of 96 µg mL^{-1} dilution of capture antibody was immobilised at 4°C overnight, then excess linker compound was deactivated using 4 µL 0.1% NH_4OH for 1 hour at 37°C and finally sFEG images were taken immediately.

Table 4.1: The results of the elemental scans between the two surfaces.

Element	% by weight in untreated	% by weight in treated
Si	21.53	29.69
Ti	1.45	1.63
Au	77.02	68.69

The data from Figures 4.6, 4.7 and Table 4.1 shows that there is some variability between the two sensors in relation to silicon and gold, however no carbon was detected. This again was most probably due to the penetration depth of the electron beam and the low density of the carbon layer.

With scanning electron microscopy unable to provide robust evidence of the presence of proteins, atomic force microscopy was employed. Atomic force microscopy has been increasingly employed to study biological samples since

analysis can be performed at room temperature and pressure and in liquid environment whereas SEM required analysis at low pressures and in a dry or near dry state. Furthermore as stated in Section 4.3.1, the surface is required to be electron rich. Therefore to study the surface morphology of proteins a 20nm gold layer has to be coated over the proteins (de Souza Pereira, 2001).

Initially one array was isolated and scanned to quantify the dimensions of the arrays. Figure 4.8 shows a 3D image of a single element.

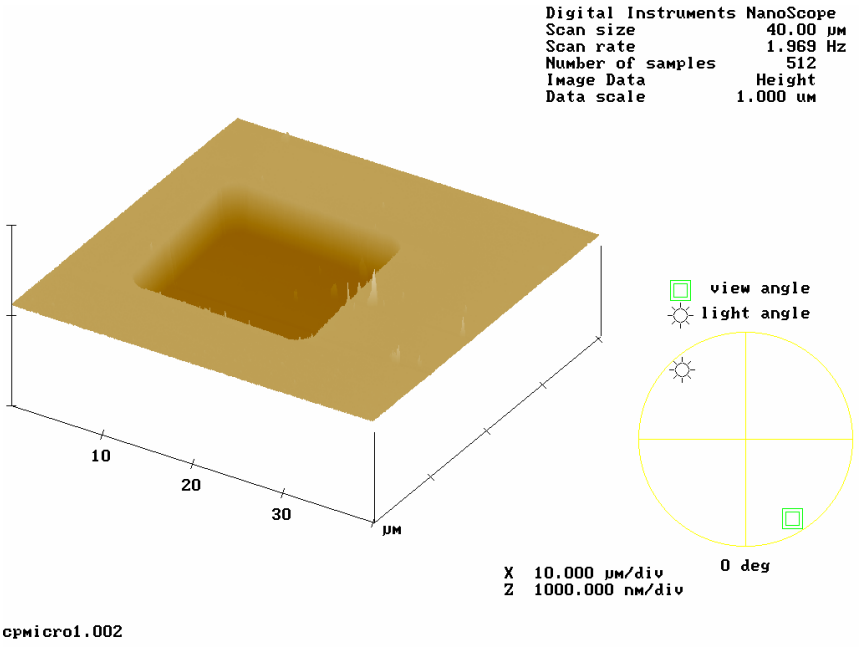


Figure 4.8: Atomic force microscopy image of a single element for the untreated working microelectrode (image 40 µm x 40 µm).

Topographic analysis of the data was performed to show that the element is 20.5 µm wide and 0.48 µm deep. The data is shown in Figure 4.9.

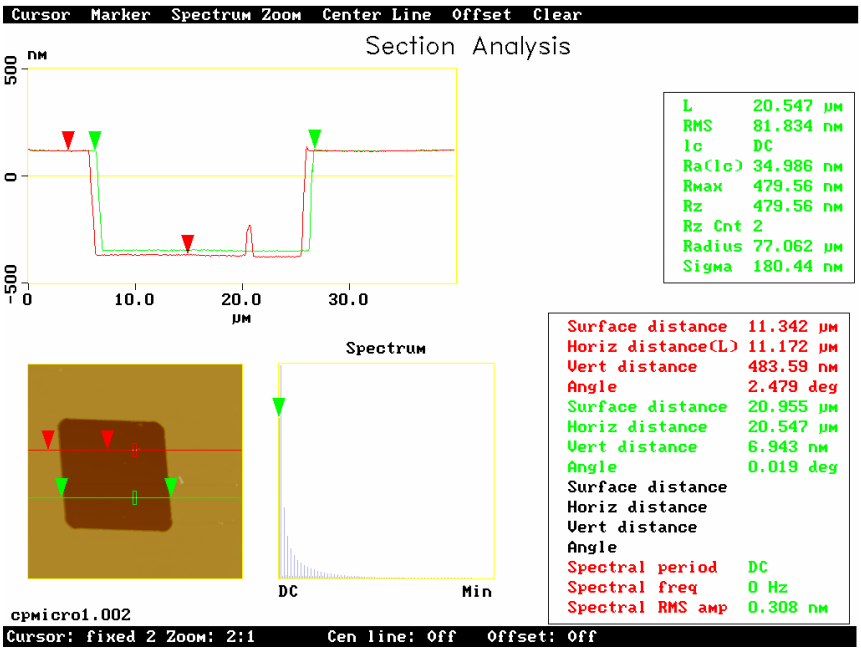


Figure 4.9: Determination of the width and depth of a single element for the working microelectrode using atomic force microscopy.

To investigate the surfaces of the two different samples firstly a detailed analysis was performed of the surface inside the arrays. Figures 4.10 and 4.11 show 3D images of the surfaces.

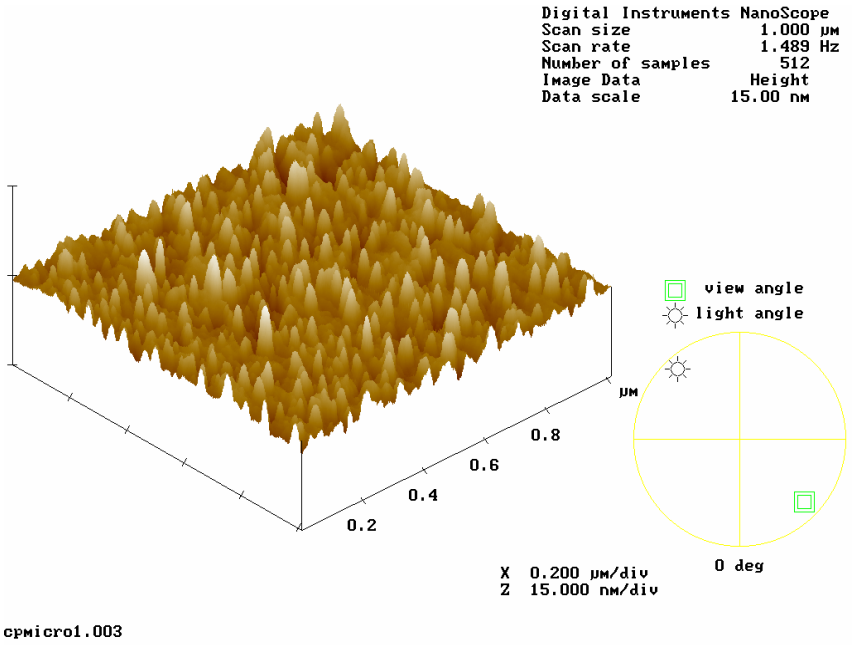


Figure 4.10: The surface of the interior of a single element for the untreated working microelectrode (1 µm x 1 µm) analysed by atomic force microscopy.

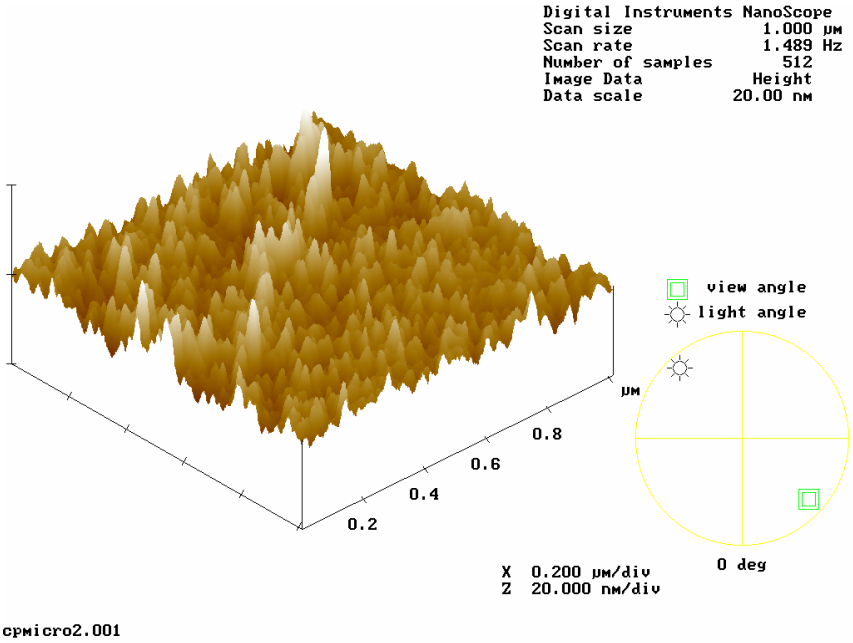


Figure 4.11: The surface of the interior of a single element for the treated working microelectrode (1 µm x 1 µm).

Visually there is not an appreciable difference between the two images. For the untreated sample there is a more uniform layer, whereas with the addition of protein the uniformity is lost to a more sporadic layer.

The roughness of the surface of the untreated working microelectrode was determined. Initially the bulk material was analysed, then the interior of the arrays was determined for both the treated and untreated. Figures 4.12, 4.13 and 4.14 show the results.

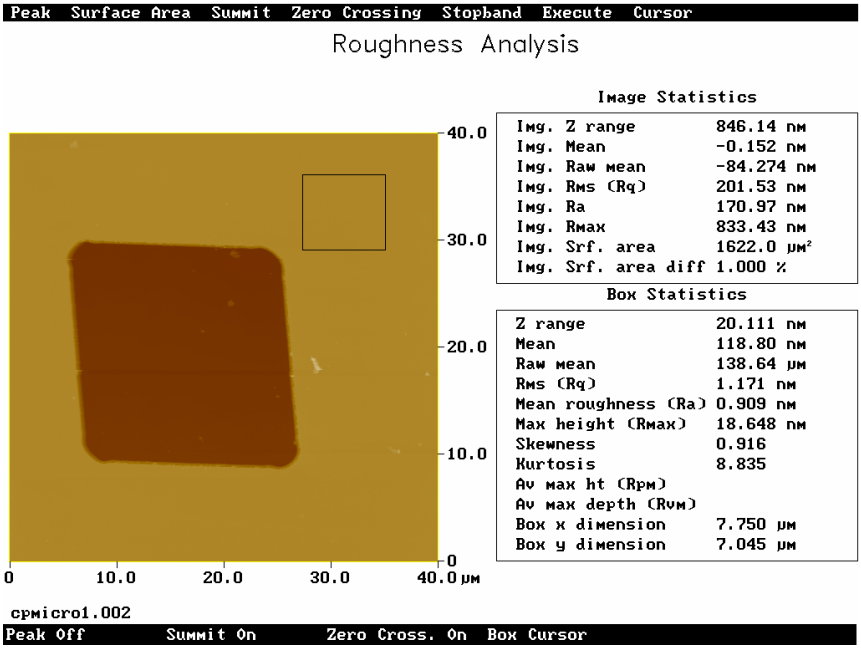


Figure 4.12: Determination of the roughness of the bulk surface for the working microelectrode analysed by atomic force microscopy.

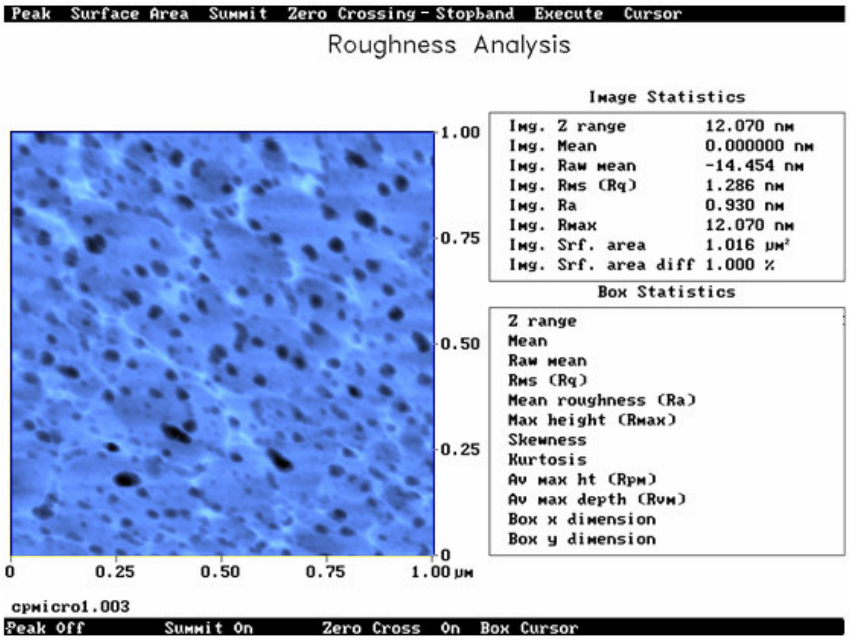


Figure 4.13: The surface roughness of the untreated working microelectrode inside the element analysed by atomic force microscopy.

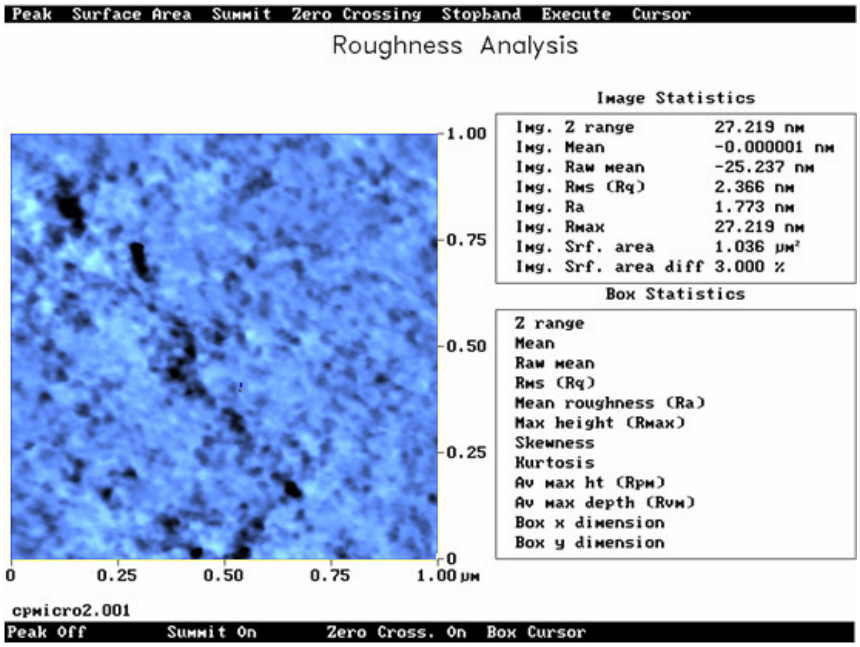


Figure 4.14: The surface roughness of the treated working microelectrode inside the element.

The mean roughness for the bulk material is 0.90 nm, for the untreated inside the element 0.93 nm and 1.77 nm inside the treated element. This does show that there is a quantifiable difference between the two samples and concurs with the visual observations that the proteins cause a sporadic effect.

Little data in the literature has been reported on the characterising effects of antibody adsorption to electrodes for immunosensor surfaces using atomic force microscopy. In the development of an ethanol enzyme based biosensor using carbon nanotubes, Tsai *et al*. (2007) reported that upon immobilising alcohol dehydrogenase onto a PVA coated nanotube, the surface roughness increased from a root mean square value of 112 nm to 127 nm.

Vianello *et al*., (2007) reported the effect of adding horseradish peroxidase to a silanised glass surface. The untreated surface had a RMS roughness of 4.2 nm, which upon the addition of a monolayer of HRP increased by 1.4 nm to 5.6 nm.

In an investigation by Parra *et al*., (2007) cholesterol oxidase was immobilised to gold electrodes and the change in RMS was 1.5 nm.

In this investigation the immobilisation of the antibodies cause a change from 1.27 nm to 2.37 nm agreeing with the observation of Tsai *et al*. (2007), Vianello *et al*., (2007) and Parra *et al*., (2007) that upon the addition of protein to a sensor surface the roughness RMS increases. Although for enzymes the increases of 1.4 nm by Vianello *et* al., (2007) and 1.5 nm by Parra *et al*., (2007) is comparable to the observations in this investigation, for an antibody, of 0.84 nm.

Protein adsorption on the carbon screen printed electrodes produced in house could not be analysed by atomic force microscopy, since the surface was too rough for the instrument so show any variations between the treated and untreated surfaces.

By using the same data as shown in Figures 4.13 and 4.14, topographic analysis was performed between the two samples. The results are shown in Figures 4.15 and 4.16.

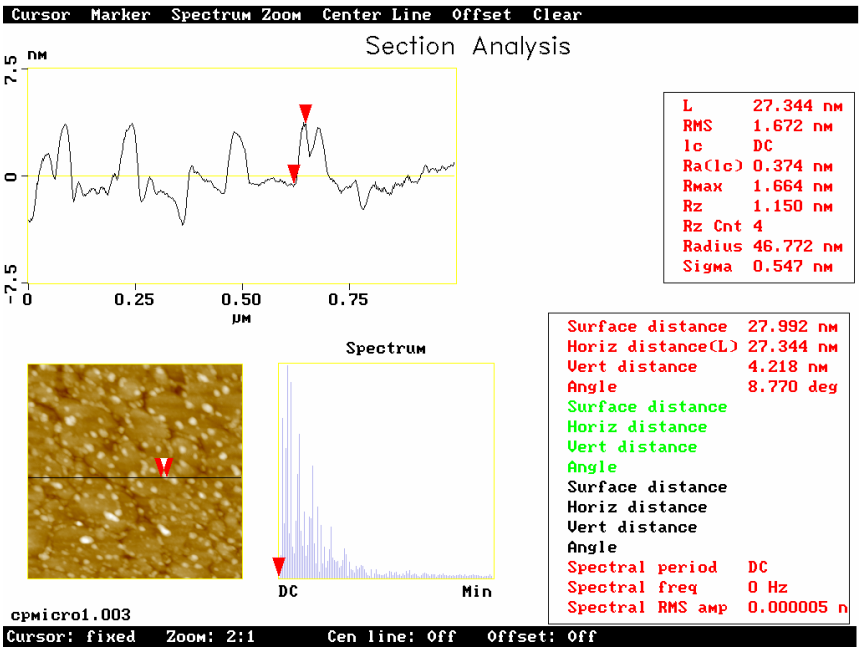


Figure 4.15: The surface topography of the untreated microelectrode analysed by atomic force microscopy.

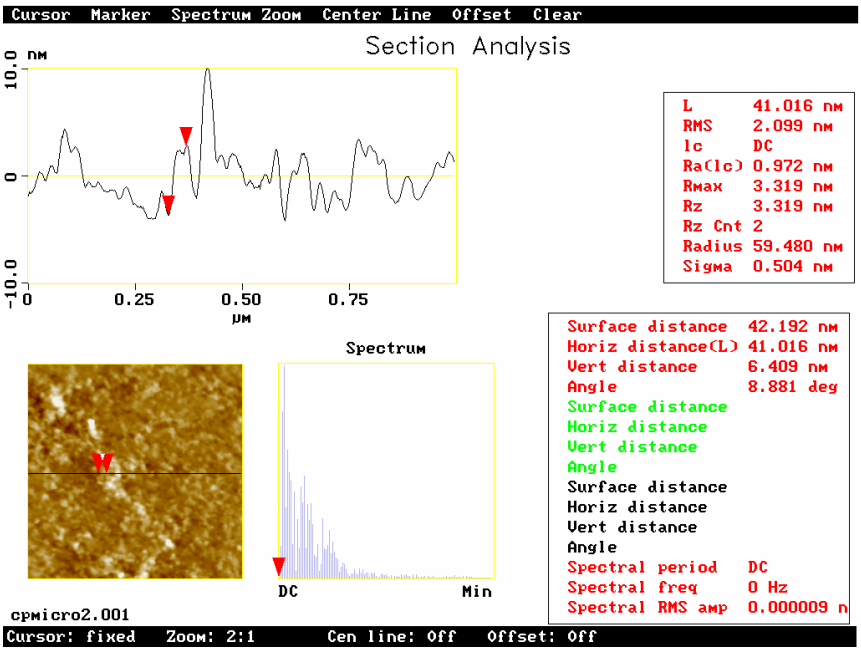


Figure 4.16: The surface topography of the treated working microelectrode.

In a report on the effect of enzyme immobilisation to glass surfaces, Zhang and Tan (2001) reported that the peak to valley distance changed with protein

concentration on the surface. Using a glass surface the untreated surface corresponded to a peak to valley distance of 6 nm. With immobilising the enzyme (glutamate dehydrogenase) onto a glutaraldehyde activated surface the peak to valley distance grew from 20 nm after 15 minutes incubation to over 200 nm after 24 hours. With depth of protein came an increase of activity as more and more protein became immobilised until aggregation occurred resulting in physical blocking of the enzyme and a decrease in activity.

In a report from Ouerghi *et al*., (2002) on the immobilisation of antibodies onto a mica surface the mean peak to valley height increased by 2.5 nm with the immobilisation of the antibodies. In an additional report by Bergkvist *et al*., (1998) a change in the height of 1.98 nm was recorded. In this investigation with the presence of antibodies the peak to valley heights on the treated surface is 6.4 nm, whereas for the untreated microelectrode the peak to valley heights are 4.2 nm, a difference of 2.2 nm and very similar to the observations of Ouerghi *et al*., (2002) and Bergkvist *et al*., (1998). The theoretical height of an IgG antibody is 4 nm (Ouerghi *et al*., 2002) suggesting that many of the antibodies are bound to the surface in a 'side-on' orientation rather than bound perfectly by the based of the Fc fragment. It is for this reason why a cheaper polyclonal antibody has been immobilised onto the surface of the immunosensor rather than the expensive monoclonal anti-aflatoxin M_1 antibody.

The final atomic force microscopy investigation studied the surface under phase control. In Figure 4.17 the graining effect seen using the sFEG was again observed but upon the addition of the proteins (Figure 4.18) this effect is completely lost and a much more irregular pattern was observed.

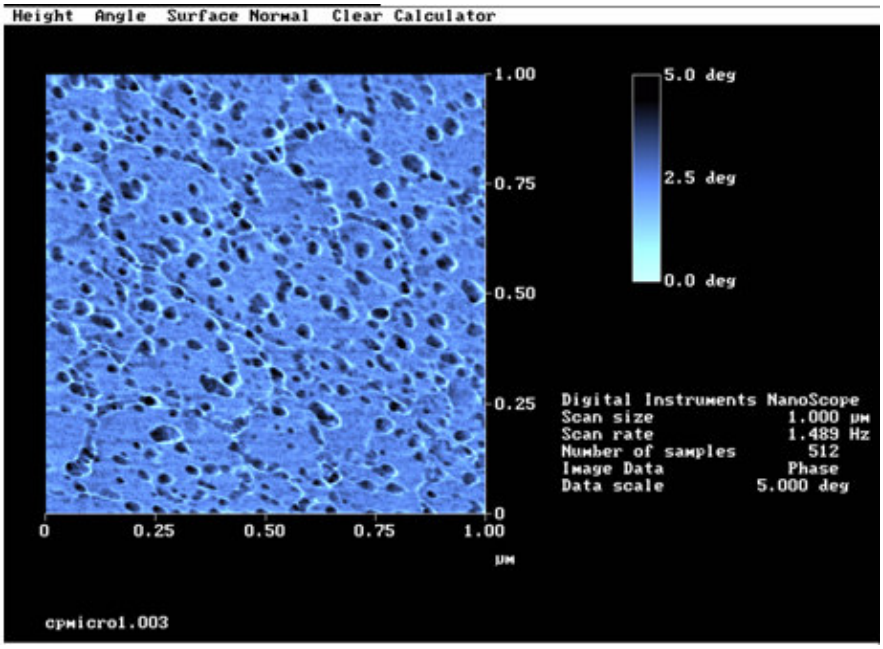


Figure 4.17: The surface image taken using phase control for the untreated working microelectrode inside the element analysed by atomic force microscopy.

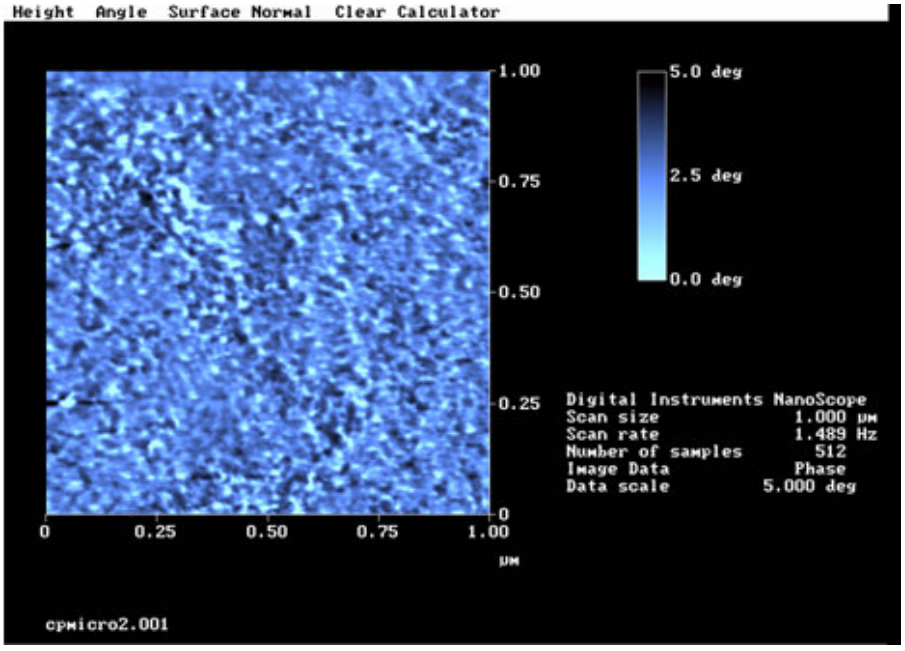


Figure 4.18: The surface image taken using phase control for the treated working microelectrode inside the element.

In conclusion the data from the sFEG was unable to quantitatively determine, if there was a difference in the surfaces between the untreated sample and the sample with the antibody attached. Using atomic force microscopy, however, showed consistent evidence that the surface does change with the addition of the proteinacieous component. Both the surface roughness and topography indicated quantifiable differences and visual evidence was seen using 3D imaging and phase control. This evidence shows that the covalent immobilisation of the antibodies to the working microelectrode is successful and agrees with literature reports.

4.3.2 Development of the microelectrode array immunosensor.

Improved mass transport is the main benefit of microelectrodes compared to planar electrodes. This results in faster diffusion of the electrochemical species under investigation. For screen printed electrodes planar diffusion perpendicular to the microelectrode surface occurs. For a microelectrode the diffusion is hemispherical and large compared to the surface area of the microelectrode. On a surface area to surface area ratio, the microelectrode has a much larger catchment of electro-active particles (Wittkampf *et al.*, 1997).

When microelectrodes are placed into arrays, the advantages of a single microelectrode are kept. The current is increased significantly, so that the same diffusional space as a screen printed electrode can be attained at a fraction of the surface area, since the active area is the sum of the individual electrodes and the intervening insulator (Wightman & Wipf, 1989).

The positioning of the microelectrode arrays is very important, so that the diffusion zones of the electrodes do not overlap as shown in Figure 4.19.

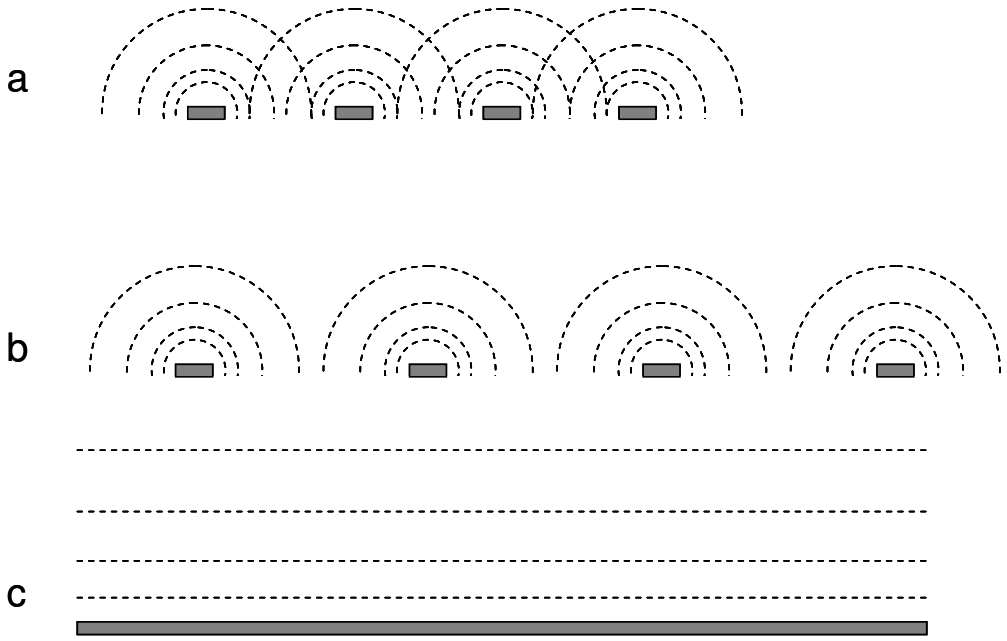


Figure 4.19: A schematic diagram of the hemispherical diffusion layers originating from microelectrode array. a) The electrodes in the array are too close together, overlapping and thus shielding causing a lower signal. b) Idealistic spacing of microelectrode where the hemispherical diffusion doesn't not impinge on its neighbours. c) Diffusion effect for planar, screen printed electrodes (Davis *et al*., 2005; Freire *et al*., 1999).

In the case of this project the microarrays was designed and optimised by Tyndall national institute and detailed by Berduque *et al.* (2007). Hence the geometric format is outside the scope of this thesis.

Using the successful immobilisation protocol several electrodes were prepared for aflatoxin M_1 measurement. Before a calibration graph could be determined the test parameters had to be optimised for the electrochemical microarrays. The reference electrode on the microelectrode is made from gold, whereas for the screen printed electrodes it is printed using Ag/AgCl ink. Differential pulsed voltammetry was employed to find the maximum detection potential of TMB. Figure 4.20 shows the voltammogram.

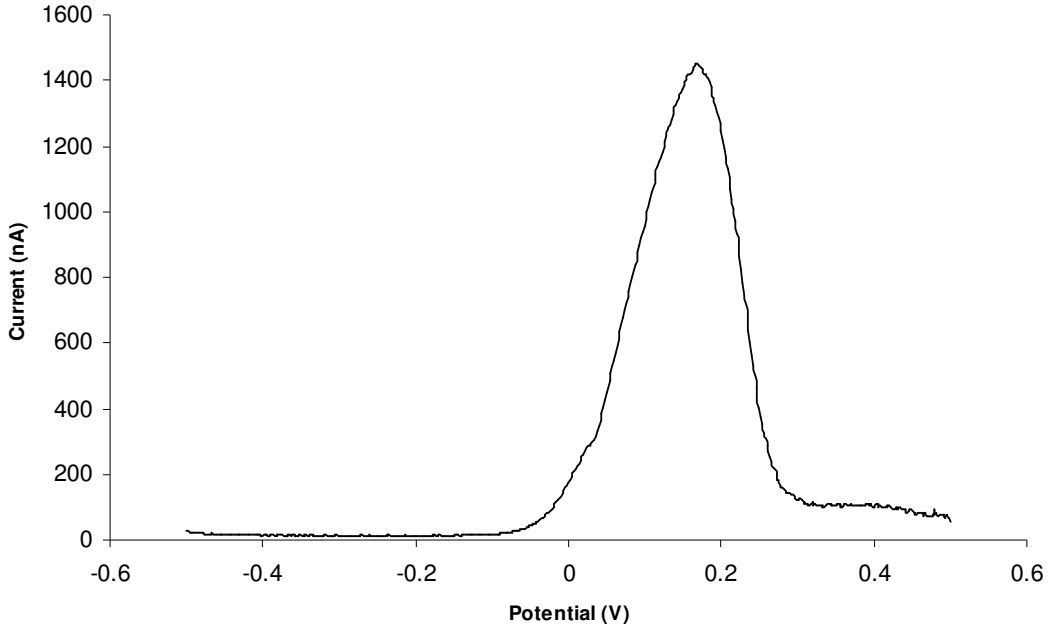


Figure 4.20: Differential pulsed voltammetry for 0.5 mM TMB on the microelectrode with 10 mM citrate buffer in 0.1 M KCl. The working microelectrode was first capped using 1% NH_4OH at room temperature for 1 hour before TMB addition.

The maximum peak signal occurred at a potential of +168 mV therefore the detection of TMB using chronoamperometry was set at +168 mV and a pre-conditioning potential of +268 mV was applied. Using these parameters a standard calibration plot was performed in pure buffer using the existing screen printed electrode method without the addition of PVA to block the surface.

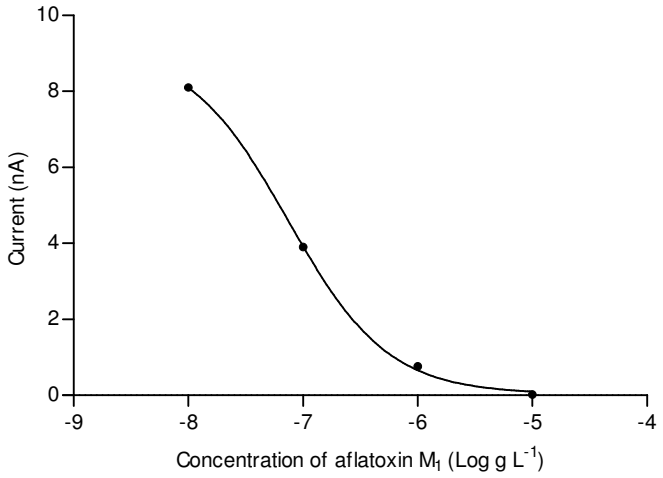


Figure 4.21: Early development using the microelectrodes in pure buffer.

The data in Figure 4.12 was obtained using chronoamperometry (+0.168 mV) for 10 minutes. A 96 µg mL^{-1} of the capture antibody solution was covalently immobilised onto the working microelectrode (pH 9.6, 4°C). A 3 µl of 0.1% NH_4OH in water was added for 60 mins at room temperature to deactivate any unreacted PDITC cross linker. Subsequently 1 µl of 40 µg mL^{-1} anti-aflatoxin M_1 antibody was placed onto the microelectrode and incubated at 37°C for 2 hours in a Petri dish with damp tissue and again washed and dried. To the microelectrode 1 µl of the sample or standard + aflatoxin M_1 HRP (diluted 1:10 with PBS) was added and incubated at 37°C for 120 mins before detection with TMB (0.5 mM) and hydrogen peroxide (1 mM) in citrate phosphate buffer pH 5.2 with 0.1 M KCl.

Initial work using same protocol as the carbon based screen printed electrodes yielded a good calibration graph with the EU maximum permissible requirements of 50 ng L^{-1} clearly detectable. The supplied information with the microelectrodes was incorrect with regards to the wiring configuration, therefore when connecting the microelectrode interface with the Autolab a low, but reliable signal was recorded. Upon configuring the interface correctly significantly higher signals were observed (substituting the working electrode connection with the auxiliary electrode connection). Due to the limited supply of electrodes available the points are not

reproduced. The calibration plot shows that levels below 10 ng L^{-1} can be detected so a further calibration plot using the same method was produced at lower concentrations. Figure 4.22 shows the results.

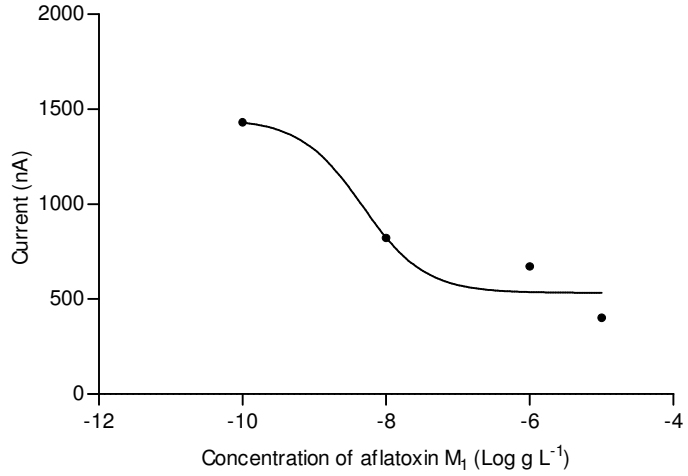


Figure 4.22: Investigation into the sensitivity of the microelectrodes for aflatoxin M_1 in pure buffer.

Figure 4.22, does show that the microelectrodes are very sensitive and with further optimisation could be used at levels sufficiently lower than the current requirements of 50 ng L^{-1}. Analytical sensitivity can be calculated by the amount of aflatoxin M_1 required to reduce the signal by 25%. In this case sensitivity is less than 1 ng L^{-1}.

With the microelectrodes performing well in pure buffer solutions, further examination was carried out to assess the performance in a milk matrix.
Using the same pre-treatment for the milk samples as for the carbon based screen printed electrodes (Section 3.2.2) a calibration was performed using spiked milk samples, (Figure 4.23).

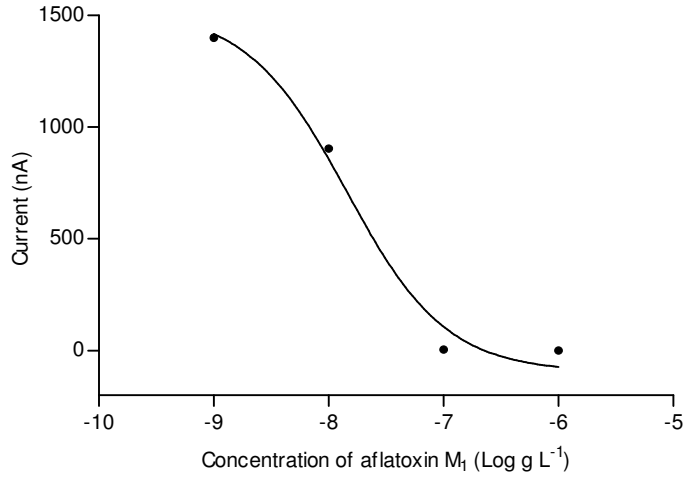


Figure 4.23: The performance of the microelectrode in milk.

Figure 4.23 was obtained using by covalently immobilising 96 µg mL^{-1} of the capture antibody solution onto the working microelectrode (pH 9.6, 4°C). A 3 µl of 0.1% NH_4OH in water was added for 60 mins at room temperature to deactivate any unreacted PDITC cross linker. Subsequently 1 µl of 40 µg mL^{-1} anti-aflatoxin M_1 antibody was placed onto the microelectrode and incubated at 37°C for 2 hours. To the microelectrode 1 µl of the sample or standard + aflatoxin M_1 HRP (diluted 1:10 with PBS) was placed and incubated at 37°C for 120 mins before detection with TMB (0.5 mM) and hydrogen peroxide (1 mM) in citrate phosphate buffer, pH 5.2, with 0.1 M KCl at +168 mV.

Again the maximum permissible requirements are met with the dynamic range occurring between 10 and 100 ng L^{-1} and the analytical sensitivity at 8 ng L^{-1} clearly showing that the microelectrodes could be used as a replacement for screen printed technology. It should be noted that the microelectrodes were not blocked by PVA as with the carbon based screen printed electrodes and neither are they fouled by whey proteins as with the carbon based screen printed electrodes.

Upon observing SEM images of both the carbon SPE and the gold microelectrode at similar magnification it can be seen that the carbon surface is vastly rougher and therefore has a much larger surface area. This combined with the strong absorption of carbon would explain why proteins foul the carbon surface and not the gold (Figure 4.24).

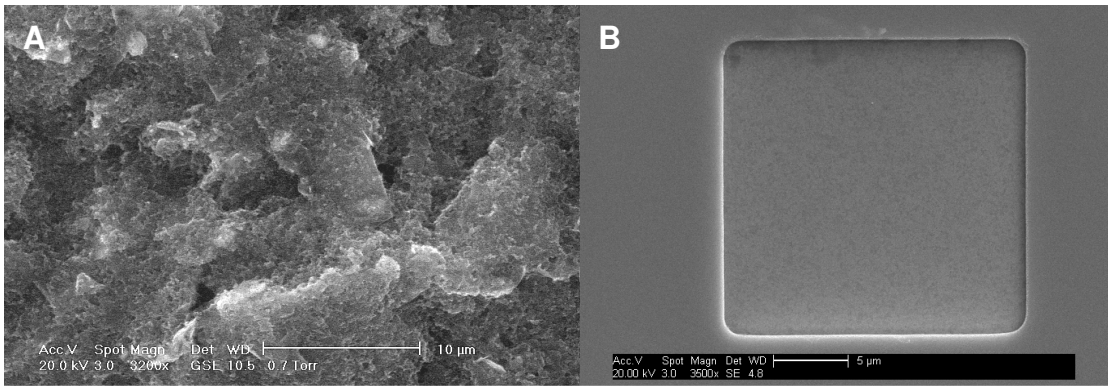


Figure 4.24: Comparison between the screen printed electrode surface and the microelectrode array using SEM.

Figure 4.24A is the surface of the carbon SPE taken using sFEG (x3200) whereas Figure 4.24B is the surface of the gold microelectrode taken by sFEG (x3500) as described for Figure 4.4B.

Since the gold surface does not require blocking with PVA makes manufacturing easier. It removed a 2 hours step and increases the electron transfer between the solution and the microelectrode making the sensor more sensitive.
Compared to the carbon screen printed sensor the signal for the microelectrode was over ten times more sensitive. The improvement is partially due to the attributes of the microsensor size and dynamics, but also due to the use of gold over carbon. The carbon paste used for printing contains organic solvents and binding polymers to aid spreading and reproducibility of the printing. This however limits conductivity and changes the electrochemical behaviour by covering the individual carbon graphite particles. Additionally the bulk resistance for graphite is

7.84×10^{-6} ohms M^{-1} whereas for gold it is 2.06×10^{-8} ohms M^{-1} (ASM, 1990). These observations help explain why the total resistance for the carbon screen printed electrode (from terminal to exposed electrode) is 36.9 KΩ (+/- 7.57 KΩ where n=60) and for the gold microelectrode 25.5 Ω (+/- 1.81 Ω where n=4) some 1000 times lower resistance. With lower resistance for the microelectrode at a fixed potential then the current will be greater and thus the sensitivity is improved.

Few microelectrode array immunosensors have been published. In a report by Katz and Willner, (1996) for the detection of a dinitrophenyl antibody, detection limit was 0.2 mg L^{-1}, although detection limit was calculated by the reduction in the current upon the antibody binding with the surface and blocking the surface, i.e. acting as an insulator.

In a more traditional approach and one matching this project, Dill *et al*., (2004) produced an immunosensor for the detection of α1 acid glycoprotein by immobilising an anti-α1 acid glycoprotein antibody to a microelectrode surface and exposing the antibody against an HRP tagged α1 acid glycoprotein. The mediator used was OPD rather than TMB. The reported detection limit for this immunosensor is 5 ng L^{-1} and comparable to the limits observed in this project.

4.4 Conclusions of the microelectrode array sensor.

Using the successful development of the carbon based screen printed immunosensor the chemistry was transferred to the microelectrode array. Using atomic force microscopy for confirmation, the immobilisation of the antibody onto the gold surface was successful and similar trends as in literature reports were observed. However, this investigation was more thorough than any reported literature discovered. The antibodies immobilised in a 'side on' orientation, which reduces the efficiency of the antibody, Therefore the use of a cheaper polyclonal capture antibody for surface attachment before attachment of a monoclonal antibody was correct. Upon utilising the microelectrode arrays for aflatoxin M_1

detection significant improvement was shown over the carbon based screen printed electrodes. This improvement was primarily attributed to the microelectrode dynamics over planar screen printed electrodes, but also the use of gold rather than graphite for the construction.

The necessity for blocking the electrode surface to avoid fouling from the whey proteins in the milk matrix for the carbon based screen printed electrodes was not the required for the gold surface. This was attributed to the micro porous structure of the carbon surface and the higher absorption capacity of carbon.
Few comparable publications for microelectode immunosensors are available. The most directly comparable report in terms of method and application is the report from Dill *et al*., (2004), who have produced an immunosensor for α1 acid glycoprotein with a detection limit of 5 ng L^{-1}. In the present project the detection limit is 8 ng L^{-1}. Improvements have been seen in this project of employing gold microelectrodes rather than carbon based screen printed electrodes. For high sensitivity applications, such as low detection limits, a boundary has been surpassed allowing for development of many new applications which have previously only been detected using elaborate instrumentation.

CHAPTER 5

DEVELOPMENT OF A SYNTHETIC PEPTIDE RECEPTOR FOR AFLATOXIN M_1

5.1 Introduction.

Chapters 3 and 4 have described the successful design and optimisation of an immunosensor for aflatoxin M_1. The aim of the project is to develop a robust sensor for the determination of aflatoxin M_1 in hot humid environments. The developed sensor at its sensing layer utilised antibodies as the receptor, which are fragile glycoproteins and prone to losing their binding affinities with incorrect storage. Additional disadvantages of antibodies are reported in Section 5.1.1. Therefore to develop new sensor strategies the use of synthetic receptors was reviewed with focus on synthetic peptides. Chapter 5 details the design, optimisation and testing of synthetic peptides which can be implemented in the sensor for aflatoxin M_1 determination.

5.1.1 Advantages of peptides over antibodies.

Currently produced antibodies are prone to many problems. Variations can occur in the quality and concentration of the antibodies in each batch. Antibodies are raised in an animal host and require a long production time for the animal immune system to raise antibodies to the antigen (for polyclonal antibodies a production time of 6 weeks is required, for monoclonal antibodies up to 6 months can be required). Additionally antibodies have a 3 dimensional proteinaceous structure and therefore susceptible to degradation upon storage (Nakamura *et* al., 2005; Tothill, *et al.*, 2003; Tothill *et al.*, 2001). The view of this project was to produce a robust and stable sensor, therefore the use of peptides as the sensing element was investigated. Peptides are synthesised completely by instrumentation and thus the reproducibility will be increased as well as being prepared faster than antibodies. Peptides are also more stable than antibodies, and also peptides are more beneficial than antibodies since immunity against low molecular weight analytes such as the mycotoxin patulin are difficult to produce (Nakamura *et al.*, 2005; Morrill *et al.*, 2002; Nakamura *et al.*, 2001).

The ability of peptides to act as receptors has been known for some time. Emil Fisher at the start of the 20th century noted that peptides had special medicinal powers but it was not until 1953 when peptides were synthesised chemically by du Vigneaud, for which he received the Noble prize, did they receive further attention (Bruckdorfer *et al*., 2004).

Today peptides are produced in the multi ton scale; for example the pharmaceutical product *Fuzeon* is a 36-mer peptide which is able to block the Human Immunodeficiency Virus (HIV) from entering the cells of human blood (Cooper and Lange, 2004).

Ligand / receptor binding interactions are a central process to a number of key biological processes, for example signal transduction, gene transcription, physiological transcription and enzymatic processes (Lybrand, 1995). These processes either involve macromolecular to macromolecular binding of proteins to proteins or proteins to DNA, or macromolecules to small molecules. Since many key biological functions are controlled by the interaction of small molecules with macromolecules, a detailed understanding of these interactions can provide essential information for the development of therapeutic products and side reactions that can occur.

With the challenge to produce anti-HIV drugs much work has been done to develop ligand/receptor computational modelling programs which have resulted in many successful peptide products for battling HIV. *Fuzeon* (or *Enfurirtide*) for example is a drug developed by computational method which inhibits the development of the HIV virus. *Fuzeon* is a peptide sequence taken from a glycoprotein, gp41, found on the surface of the virus and was extensively developed using computational chemistry.

5.1.2 Practical methods of peptide design.

Original methods of synthetic peptide design used geometry and binding prediction tables to design a synthetic peptide towards a target. These procedures resulted in limited success (Moser *et* al., 1983; Fukushima *et al*., 1979; Gutte *et al*., 1979).

A more successful approach was discovered in 1986 with the introduction of combinatorial chemistry (Tozzi *et al*., 2003a; Geysen *et al*., 1986). Combinatorial chemistry uses a large peptide library to test each peptide receptor against the target of choice. Each peptide sequence is constructed on a bead and the target is immersed in the cocktail of peptides.

The target is tagged with either a fluorescent marker or an enzyme marker, the beads with the target are collected and the sequence is determined using a peptide sequencer at a rate of 3 sequences a day (Schmuck and Hell, 2003a; Lam *et al*., 1991).

An alternative approach reported by Houghten, (1985) used 'tea bags' with each bag containing a separate sequence (Nestler, 2000). The 'tea bags' method used small polypropylene mesh bags filled with Boc amino acid resin. The bags were numbered and sealed then peptide synthesis was performed manually creating 247 different sequences with each bag containing one sequence.

Nakamura *et al*., (2005) reported that a peptide sequence of more than four residues is required for binding with small molecules therefore a pentapeptide or hexapeptide should be developed. A full library of pentapeptides using natural amino acids consists of 20^5 peptides or 9×10^{13} different sequences. In reality not all 20 natural amino acids are used to build a combinatorial library. Cysteine is usually not included to eliminate disulphide crosslinking and the formation of dimers (Lam *et al*., 1991) additionally tryptophan can be omitted (Houghten *et al*.,

1991) as well as methionine and lysine (Tozzi *et al.*, 2002) to reduce synthesis problems. Therefore combinatorial chemistry can be restricted.

5.1.3 History of computational ligand / receptor affinity calculation.

The first computation chemical simulations were performed in 1964 (Rahman, 1964) with the simulation of the motion of atoms in liquid argon. A year later computational quantium models were produced by Moody and Thomas (1965) to aid in sensor design with construction of ion selective electrodes. Decades on from the advent of computational chemical simulations, simulations were used in more complex applications such as ligand/receptor affinity calculations.

Early computational ligand/receptor docking methods such as DOCK (Kuntz, 1992) were designed to dock ligands into receptors, however they only considered orientational and translational degrees of freedom which do not take into account the changes in the ligand conformation during docking (Baxter *et al.*, 2000). More recently ligand flexibility has been considered however this increases the number of degrees of freedom and hence requires greater computing power, although the receptor is still assumed to be ridged (Alberts *et* al., 2005; Bertelli *et al.*, 2001).

To reduce computational time, further approximations can be made. CHARMm for instance (Chemistry at Harvard Macromolecular Mechanics) (Brooks, 1983) recognises amino acid groups as a single entity rather than separate atoms and therefore speeds up the computational time due to fewer degrees of freedom. The drawback of this is the difficulty in predicting hydrogen bonding since the position of the hydrogen has a large effect on the hydrogen bond strength (Goodford, 1985; Brooks, 1983). Docking methods have been developed for drug screening, so that many possible compounds can be screened in a short amount of time, but Abagyan and Totov (2001) reported that docking programs only have a 30 – 50% success rate, and each molecule takes between one to three minutes. Jackson (1995) and Illapakurthy *et al.* (2003) also reports that scoring functions are poor

and variations exist between different programs. Realising that binding prediction is the most difficult part of drug design, Wang and Wang (2001) have suggest a method of consensus scoring to reduce bias of some binding programs. By using a variety of scoring methods and converting the score into a rank, errors within the docking program can be removed. A similar approach is now taken by a program CScore by Tripos (Michigan, USA).

With these concerns in mind for the development of peptides for aflatoxin M_1 many docking programs each with different algorithms shall be employed to remove the bias that may occur if only one algorithm is utilised.

5.2 Materials and methods used to design a synthetic peptide receptor for aflatoxin M_1.

5.2.1 Computer Modelling.

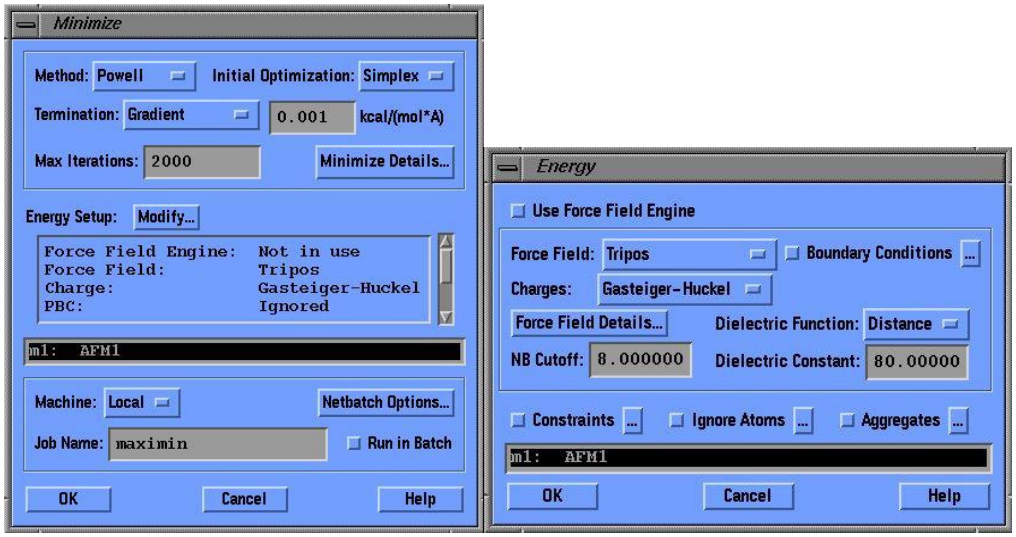
The workstation used for the modelling studies was a Silicon Graphics Octane running IRIX 6.6 operating system. The workstation was configured with two 195 MHz reduced instruction set processors, 712 MB RAM and a 12 GB fixed drive. This system was used for the software package SYBYL 6.9.1 Tripos Inc. (St. Louis, Michigan, USA).

5.2.2 Obtaining the structure of aflatoxin M_1.

The first part of the work was to determine the structural conformation of the aflatoxin M_1 molecule. A search was executed in the Protein Data Bank (PDB) for structures which contain the aflatoxin M_1 molecule. Several structures were found which contained the AFB_1 -8, 9-exo-epoxide bound to a DNA chain; however none contained the aflatoxin M_1 molecule.

Since no structure of the aflatoxin M_1 molecule was available the structure was taken from text (Moreau, 1979) and sketched into the SYBYL package. Aflatoxin M_1 does not contain any chiral centres. The molecule was then charged by the Gasteiger – Huckel method and molecular mechanics was applied to minimise the

structure using the Powell method. Figures 5.1A and 5.1B show these settings. The minimisation was run for 2000 iterations or until the convergence gradient reached 0.001 kcal mol^{-1} x A. The dielectric constant was changed from 1 to 80, thus simulating an aqueous environment.



A **B**

Figure 5.1: The settings used for minimisation of aflatoxin M_1. The main window is shown in A, and B shows the modified energy setting used.

In order to insure the structure obtained was that of the global minimum, simulated annealing was performed. The settings used were the default settings. Figure 5.2 show these settings. After simulated annealing then the minimisation was repeated. The resulting structure was saved and used in subsequent experiments.

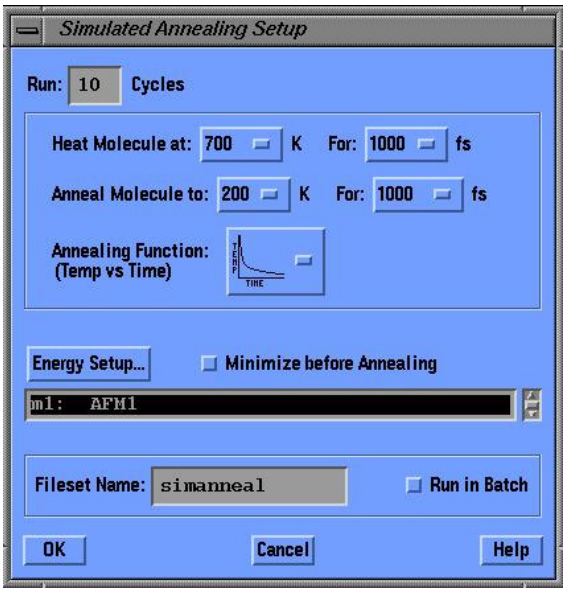
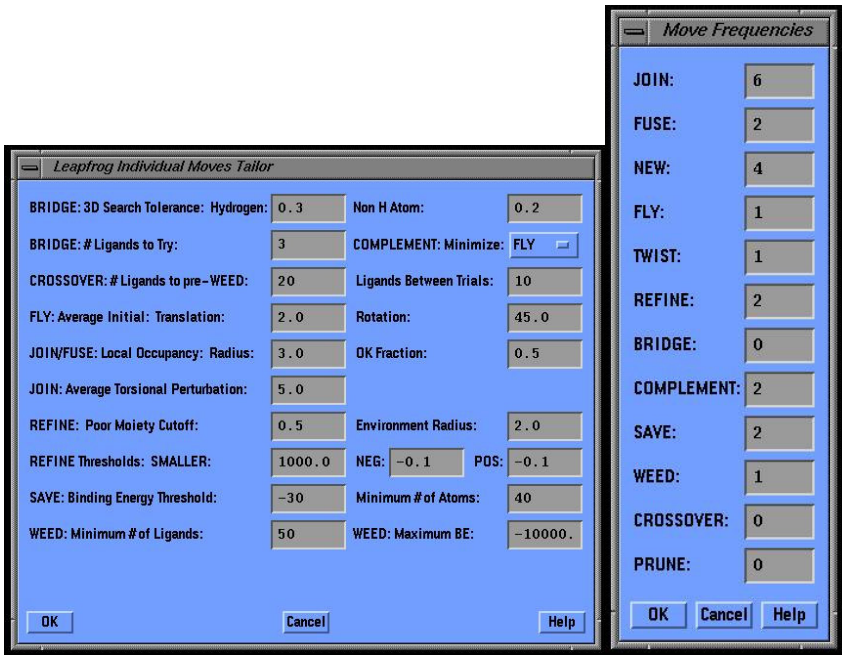


Figure 5.2: The settings used for simulated annealing. The energy setup used the same settings as shown in picture 5.1B

5.2.3 Computer aided receptor design.

Many experiments were performed to optimise the conditions required for a successful Leapfrog run. Figures 5.3A and 5.3B show the settings used. The data set for the monomers was set to the TRIPOS peptide library and the Leapfrog program was always used in the 'Dream mode'. Only the 20 natural amino acids were used as the library of building blocks.



A **B**

Figure 5.3: The tailored parameters used for Leapfrog. (A) shows the individual settings for each move type and (B) shows the frequency of these moves.

To tailor Leapfrog for our requirements many parameters were changed. From 'Energy Startup' it was decided to include hydrogen bonding in the calculations and to 'always link' through active hydrogens. The other parameters changed were within the individual move types and relative move frequencies. For every Leapfrog run, 'Bridge' within the relative move frequencies was kept at zero.

5.2.4 Investigation of 1GVE.

To analyse natural receptors with the aflatoxins a PDB file (1GVE) of aflatoxin aldehyde reductase (AKR7A1) was found on the protein data bank. This was a structure determined by X-ray diffraction (Kozma *et al.*, 2002).

The 1GVE PDB file was imported into the SYBYL package, the surface water molecules were deleted and the structure was charged with Pullman charges.

A SYBYL module called SiteID was used to find the possible binding pockets of the enzyme to determine the relevant amino acids binding with the aflatoxin. The SiteID program was used with the default settings as shown in Figure 5.4.

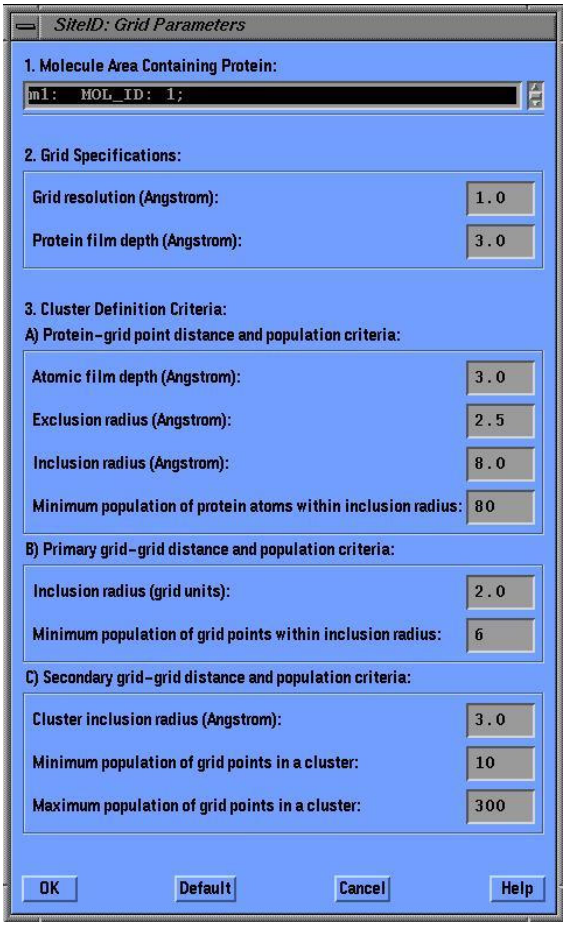


Figure 5.4: The default settings used for SiteID to elucidate the residues in the AKR7A1 enzyme with binding to aflatoxin M_1.

5.2.5 Validation of binding the scores from Leapfrog.

The results from Leapfrog and SiteID were validated using a docking program called Flexidock. Flexidock is unique since the parameters can be changed to allow the protein backbone of the receptor to 'flex' whereas most docking programs assume that the receptor is ridged. Thus by creating a copy of the system version of the flexidock.par file to a local location, the file was changed to allow flexing and to use a dielectric constant of 80. These changes are highlighted in Figure 5.5.

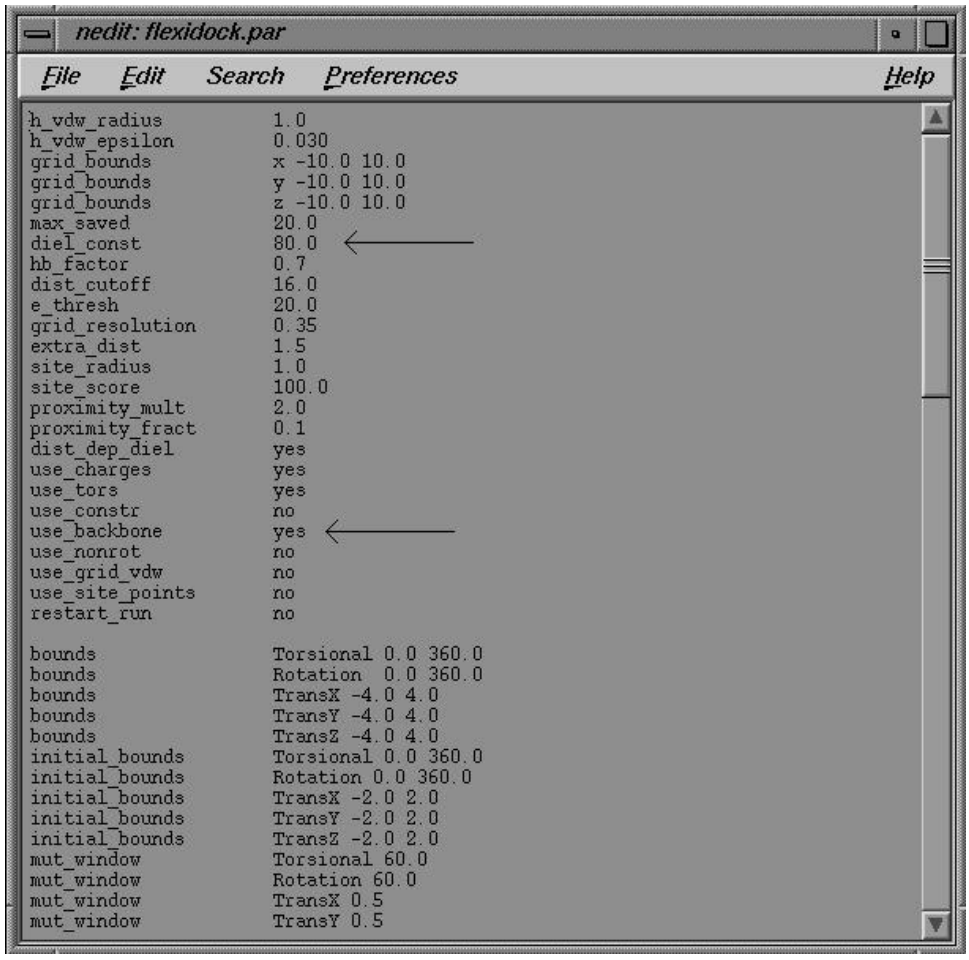
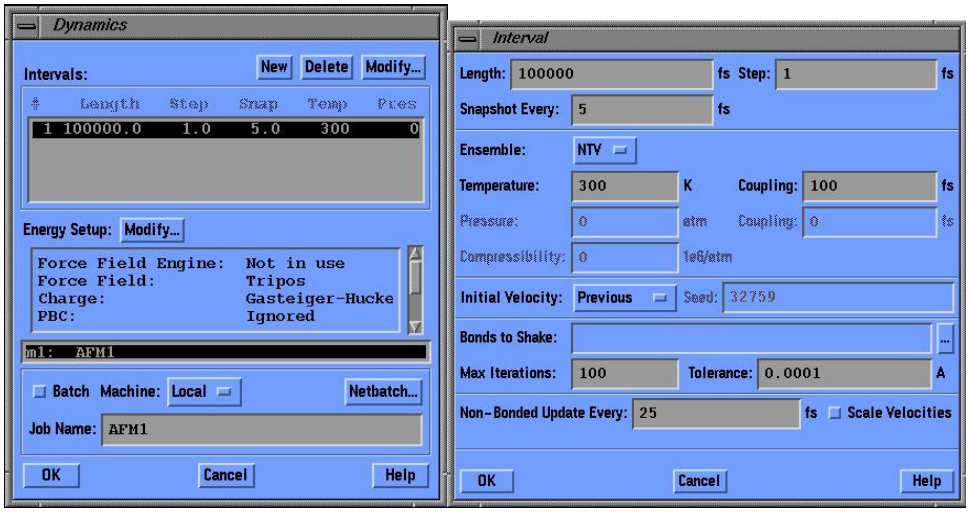


Figure 5.5: The change in the settings in the flexidock.par file to allow a dielectric constant of 80 and flexing of the backbone.

For all the experiments, all rotatable bonds and charges on the molecule were selected and the radius around the pocket was defined to 3 Å.

Dynamic runs were performed using the function ‘setup dynamics’. Depending on the experiment the lengths of the runs were altered. Initially a box was placed around the molecules of interest and the box was partially filled with water molecule so that the density was about 0.2 g ml^{-1}. Later, the dynamics run was performed without any water molecules however the dielectric constant was set to 80. This was done to reduce the amount of computer time required. In every

experiment the pressure was not set and the temperature was set to 300 K. Figure 5.6 shows these settings.



A **B**

Figure 5.6: The settings used for the dynamics simulations. (A) the initial screen to setup the dynamics, modification of the temperature and length of the run is shown in (B).

The designed peptides were synthesised, purified by HPLC and checked by mass spectroscopy by the Medical Research Council (MRC) clinical science centre (London, UK). Quality reports for the synthesised peptides can be found in the appendix (Section 8.3).

An experiment was performed to use a LINUX package called GROMACS (Groningen Machine for Chemical Simulations) in order to carry out FEP (free energy perturbation) calculations. GROMACS is a GPL (General Public License) molecular dynamics software which is known for its fast calculation speed (www.gromacs.org). The program was installed onto a COMPAC Presario Personal Computer with a 1.99GHz Intel Celeron CPU, 192 MB of random access memory and 30GB fixed disk. The operating system was SuSe Linux 9.0.

GROMACS has no graphical user interface; hence it is entirely controlled from command lines.

The approach used was to create a PDB file from the SYBYL package and to convert it into a GROMACS file using the GROMACS command

pdb2gmx –f peptide.pdb –p peptide.top –o peptide.gro

Then the peptide would be placed into a box and filled with water using the following commands;

Editconf –f peptide –o –d 0.5

Genbox –cp out –cs –p peptide –o b4em

The peptide and box of solvent would then be minimised to reduce the strain and bad VdW forces caused by the addition of the solvent this was done using the following two commands;

grompp –v –f –em –c b4em –o em –p peptide

mdrun –v –s em –o –c after_em

Finally the dynamics could be run by the following commands;

grompp –v –f full –o full –c after_em –p peptide

mdrun –v –s full –e full –o full –c after_full –g flog >& full.job &

The parameter files were all kept to the default setting with the exception of the full.mdp where the number of steps and hence the length of the run was extended.

5.2.6 Materials used in the affinity studies.

For the affinity investigations aflatoxin M_1 – BSA, lysine, glutaraldehyde, Tween 20, Tween 80, Triton X-100, and CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate) were all obtained from Sigma-Aldrich LTD (Gillingham, UK) as well as all general chemicals. Aminopropyltrioxysilane was purchased from VWR (Lutterworth, UK). The anti-aflatoxin M_1 antibody was obtained from Abcam (Cambridge, UK) as well as the secondary alkaline phosphatase labelled antibody. BIAcore buffers, reagents and consumables were purchased through BIAcore (Uppsala, Sweden). Microwell plates from Nunc were ordered through VWR (Lutterworth, UK). BS^3 linker compound was purchased from

Pierce (UK) as well as TMB ultra substrate / chromatogen solution. Aflatoxin M_1 – HRP was obtained as part of the aflatoxin M_1 Ridascreen Kit. Sodium hypochlorite was obtained from Sigma Aldrich LTD (Gillingham, UK).

5.2.7 Monitoring of the affinity of the peptide for aflatoxin M_1 using optical waveguide lightmode spectroscopy (OWLS).

For the affinity studies, an IOS-1 optical waveguide lightmode spectrometry was used powered by a PSU1-3 (Artificial sensing instruments, Zurich, Switzerland). A Gilson minipuls 3 peristaltic pump was used for the fluid handling (Middleton, USA) at a flow rate of 10 µl min^{-1}. Tygon tubing with an internal diameter of 0.51 mm (Ismatec, Switzerland) was used for the peristaltic pump.

For the data manipulation the room temperature was recorded at the start of each run to calculate the binding constants. Standard 2400 waveguides were used throughout and obtained from Microvacuum (Budapest, Hungary). The waveguides were silianized by first washing in a beaker of ultrapure water at 90^oC for 10 minutes, and then the waveguides were placed into 10% aminopropyltrioxysilane at 60^oC where the pH had been adjusted to 3.5 with HCl. The waveguides were kept at 60^oC for one hour and then washed with distilled water. After washing the waveguides were placed onto aluminium foil and baked in an oven at 100^oC overnight. Once baked, the waveguides were stored in distilled water until protein immobilisation and then stored in PBS at 4^oC. For immobilising the aflatoxin M_1 - BSA, 2.5 % glutaraldehyde was pumped through the OWLS cell for 1.5 hours. After washing with water and then equilibrated with 10 mM PBS, 100 mg L^{-1} of aflatoxin M_1 - BSA was added to the system for a further 1.5 hours. The OWLS cell was then washed with 10 mM PBS (pH 7.4) and any unbound aflatoxin M_1 - BSA was removed using 0.01 M hydrochloric acid.

To block any un-reacted sites, 10 mM lysine was added to the system in a separate operation for 1.5 hours. To confirm the affinity of the anti-aflatoxin M_1

antibody and to validate the instrument, the anti-aflatoxin M_1 antibody (8 µg L^{-1}) was allowed to react with the immobilised aflatoxin M_1 - BSA until a plateau was reached. To regenerate the waveguides initially 0.1 to 0.01 M HCl was used and then additionally a mixture of detergents was used containing 0.3% Tween 20, Tween 80, Triton X – 100 and CHAPS. For experiments with the peptides, two new waveguides were prepared as previously described, with one of the waveguides using BSA and not aflatoxin M_1 - BSA for immobilisation. The peptide concentrations used in the experiments was kept to 100 µl ml^{-1} throughout.

All glassware and consumables were decontaminated from aflatoxin M_1 by soaking in 10% sodium hypochlorite for 48 hours and then an equal amount of 5% aqueous acetone was added. The mixture was left for three hours and then disposed down the drain.

5.2.8 Monitoring of the affinity of the peptide for aflatoxin M_1 using BIAcore.

Throughout the investigation using the BIAcore (Upsala, Sweden) all buffers, reagents and consumables were supplied pre-filtered from BIAcore, and all chemicals for analysis were degassed prior to use by placing the solutions in a reduced atmosphere. For immobilisations the antibodies and peptides were diluted in 10 mM acetate buffer (pH 4.5) to a concentration of 10 µg ml^{-1} as recommended in the BIAcore user manual. High versatility CM5 chips were used throughout which have an activated carboxyl group attached to the gold surface via a dextran substrate. Covalent immobilisation to the carboxyl group occurred through EDC/NHS coupling was computer controlled.

For all experiments the running buffer was HBS-EP (0.01 M HEPES pH 7.4 buffer containing 0.15 M NaCl, 3 mM EDTA and 0.005% P20 surfactant) and 10 mM glycine in pH 2 HCl was used as a regeneration buffer. A BIAcore 3000^{TM} was used throughout in this investigation.

5.2.9 Immobilisation of the peptides onto solid supports (ELISA plates) for chemical determination of the peptide / aflatoxin M_1 complex.

To immobilise the peptides onto the microwell plates, amine activated microwell plates from Nunc were used throughout. The attachment of the peptides to the microwell surface was carried out using BS^3 linker compound at a concentration of 10 mM in de-ionised water, and various peptide concentrations from 1 to 0.1 mg ml^{-1} in immobilisation buffer. The immobilisation buffer was pH 8, 20 mM sodium phosphate with 0.15 M sodium chloride. Incubation times for the immobilisation were 30 minutes, followed by capping the excess linker compound with 1 M TRIS for 15 minutes, to stop aflatoxin M_1 – HRP attachment to the un-reacted BS^3 linker compound. For joint immobilisation, the peptide and BS^3 were added together to react in the same well for 30 minutes. For stepwise immobilisation, the BS^3 was first reacted with the amine surface of the microwell for 30 minutes, washed with de-ionised water, tapped onto tissue paper to dry, then the peptide was added and incubated for a further 30 minutes before a second washing and capping of the excess linker compound. All stages were performed in the dark at 21^{o}C.

For the testing of the peptides with aflatoxin M_1 – HRP; pH 7.4 PBS-tween 20 buffer was used as a washing buffer and the conjugate was diluted 1:5 in 10 mM PBS, pH 7.4. Once the peptide was immobilised to the microwell surface the aflatoxin M_1 – HRP conjugate was added and incubated for 2 hours at 37^{o}C. After the incubation the wells were washed with PBS-T and PBS then a ready made TMB Ultra substrate/chromogen solution (Pierce, Cramlington, UK) was placed into the wells and the colour intensity was recorded using a BMG Fluorstar galaxy ELISA plate reader (Aylesbury, UK).

5.3 Results and Discussions.

5.3.1 Computational modelling results.

Throughout this thesis the colour scheme used for the screenshots of the computer generated diagrams is as shown in Table 5.1.

Table 5.1: The general colour scheme for the figures containing computer screenshots.

Colour	**Atom/Bond**
Light blue (cyan) or white	Hydrogen
Grey	Carbon
Dark blue	Nitrogen
Red	Oxygen
Yellow	Sulphur

Initially the conformation of aflatoxin M_1 was determined by sketching the molecule into the SYBYL package. The molecule was then charged using the Gasteiger-Hückel method as described by Chianella *et al*., (2002) and with a combination of minimisation using the Powell method and with simulated annealing, the conformation of the global minimum was found, and hence the lowest energy state. The structure of this is shown in Figure 5.7

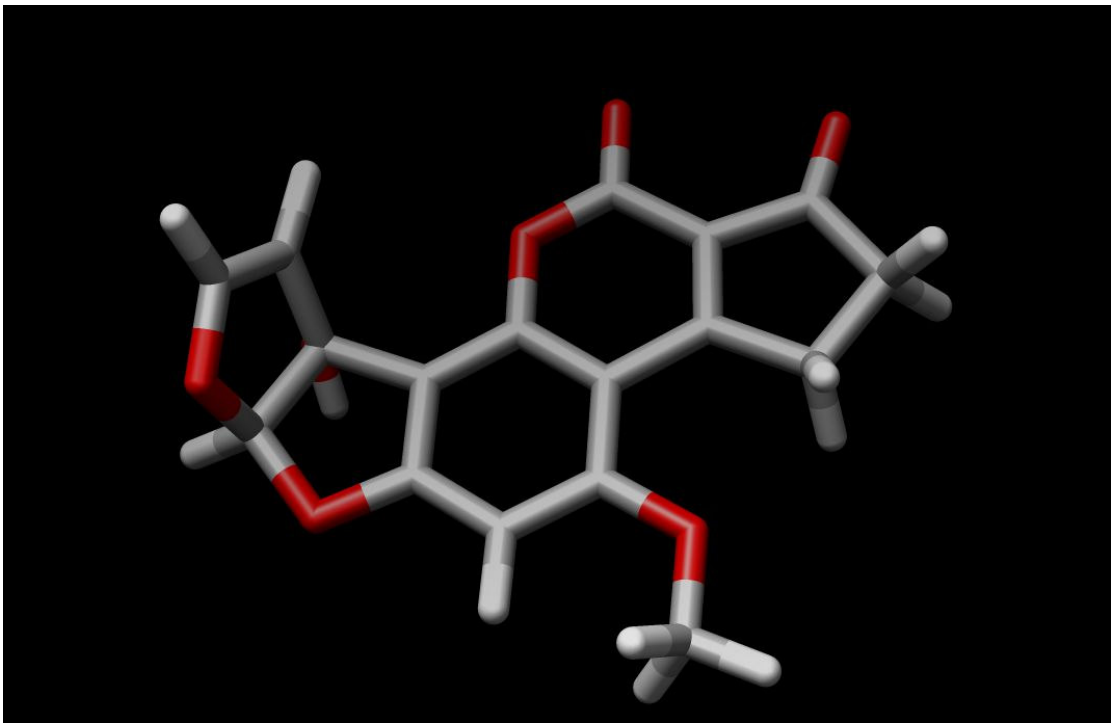


Figure 5.7: The low energy conformation of aflatoxin M_1.

The insertion of the aflatoxin M_1 structure into the SYBYL is a key and fundamental step. If there is an error in the conformation of the starting structure then all subsequent work may also be incorrect.

Typically the structure used for molecular modelling would have been experimentally found by NMR or crystal structure studies, however not all structures have been experimentally determined. In this case the aflatoxin M_1 molecule has to be minimised to its lowest steric energy and hence a good representation of nature since no molecular modelling structure has previously been recorded. For example Figure 5.8 shows a simple energy diagram for ethane.

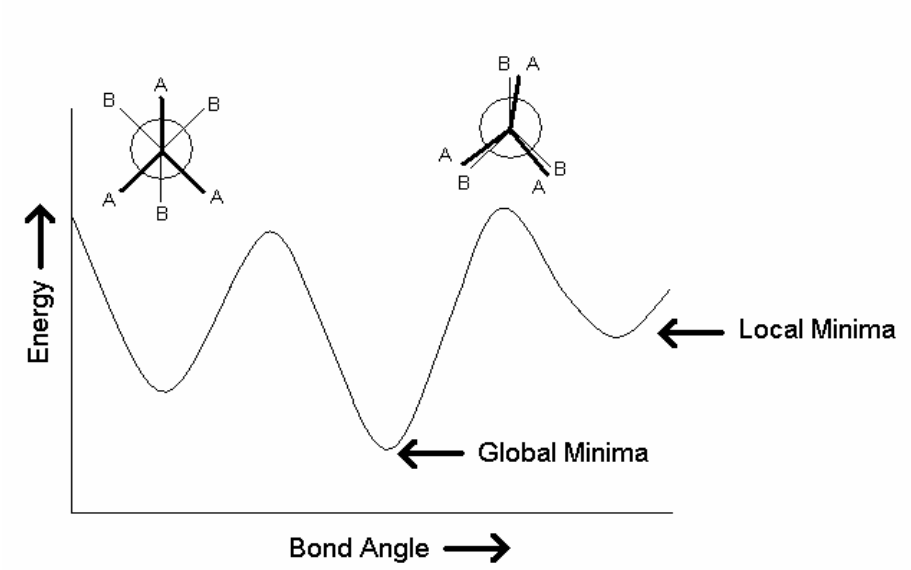


Figure 5.8: A schematic energy diagram for ethane.

As the C-C bond is rotated then the energy of the molecule increases and decreases due to steric hindrance of the hydrogens. In the favourable positions the graph shows valleys or wells, these valleys are where the molecule is most likely to reside. Upon minimising a molecule, the algorithm searches for the valleys by moving a bond and recording the new energy value, if it is a decrease in value then it keeps moving the bond in that direction. When it gets to a scenario where all movements cause an increase in energy, then the algorithm assumes that it has found the global minima, i.e. the lowest point on the graph.

On complicated systems there can be many local minima and the algorithm could incorrectly presume that the minima found is the global where actually it is only a local minima (Goodman, 1998). Simulated annealing is a process which detects the global minima and not an incorrectly assumed local minima (Donnelly, 1987). Simulated annealing was first reported by Kirkpatrick *et al.* in 1983 (Kirkpatrick *et al.*, 1983). As with the physical meaning of annealing, the molecule is given energy through temperature, allowing the molecule to break through any energy valleys. Then the amount of energy is slowly removed from the molecule to simulate

cooling. Providing the energy is removed at the correct rate then the molecule will find the global minima.

Upon comparing the result of the minimisation and simulated annealing, the structure of aflatoxin M_1 obtained is consistent with the reports from Holtzapple *et al*. (1996) as described in Section 1.7.2. The backbone (rings B, C, D and E) are in the same plane and with ring A 102^o from the plane.

5.3.2 The determination of the binding interaction of the amino acids.

To determine the best amino acids for binding, a program within the SYBYL suite called Leapfrog was employed. Leapfrog is a second generation *de novo* design application for the generation of receptor libraries. First generations programs were GROW (Moon and Howe, 1991), LUDI (Böhm, 1994). Leapfrog has previously been credited with providing structures with correct length, hydrogen bonding ability and hydrophobicity (Honma, 2003) whereas LUDI has been criticised for placing too much emphasis on van der Waals interactions and too little on hydrogen bonding (Fischer *et al*., 2001).

A run was performed where the leapfrog move frequencies were adjusted so that the amino acids did not connect to form peptides (Join = 0, Fuse = 0, Bridge = 0, Crossover = 0). Leapfrog detected that the aflatoxin M_1 has 36 active sites (see Figure 5.9), hence using the library which contains 20 amino acids, there are 720 different combinations, however the amino acid can have many different orientations thus the Leapfrog run was set for 500,000 moves. This correlates to two days computer time. The results from this Leapfrog run are shown in Table 5.2.

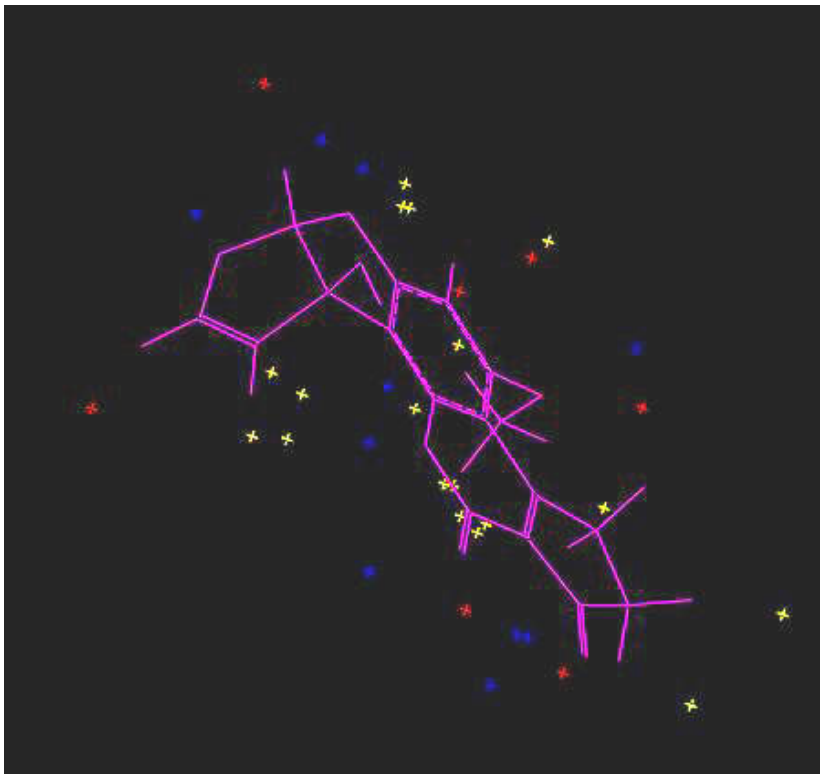


Figure 5.9: The active sites of aflatoxin M_1 determined by Leapfrog.

Table 5.2: Binding interactions of the amino acids with aflatoxin M_1 and their binding scores.

Amino Acid		**Binding Score (Kcal mol^{-1})**	**Amino Acid**		**Binding Score (Kcal mol^{-1})**
Isoleucine	Ile	-31.87	Tyrosine	Tyr	-17.12
Cysteine	Cys	-29.36	Lysine	Lys	-16.37
Phenylalanine	Phe	-29.18	Threonine	Thr	-13.44
Valine	Val	-25.88	Aspartic acid	Asp	-12.40
Leucine	Leu	-21.01	Serine	Ser	-10.28
Methionine	Met	-20.73	Glutamine	Gln	-9.77
Alanine	Ala	-20.71	Asparagine	Asn	-9.15
Tryptophan	Trp	-20.28	Glutamic acid	Glu	-8.62
Histidine	His	-17.41	Arginine	Arg	-2.26

Only 18 amino acids are shown, glycine and proline did not produce a negative binding score, hence this reaction would be exothermic. Kyte and Doolittle (1982)

produced values to predict the hydrophobicity of the amino acids. These values are shown in Table 5.3. The higher the hydrophobicity value, the greater the hydrophobic nature of the amino acid.

Table 5.3: The Hydrophobicity values of the amino acids as calculated by Kyte and Doolittle (1982).

Amino Acid	Hydrophobicity Value	Amino Acid	Hydrophobicity Value
Arginine	-4.5	Serine	-0.8
Lysine	-3.9	Threonine	-0.7
Aspartic Acid	-3.5	Glycine	-0.4
Glutamic Acid	-3.5	Alanine	1.8
Asparagine	-3.5	Methionine	1.9
Glutamine	-3.5	Cysteine	2.5
Histidine	-3.2	Phenylalanine	2.8
Proline	-1.6	Leucine	3.8
Tyrosine	-1.3	Valine	4.2
Tryptophan	-0.9	Isoleucine	4.5

When plotting the hydrophobicity value against the Leapfrog scores (Figure 5.10) it can be seen that the hydrophobic amino acids have greater binding than the hydrophilic acids towards aflatoxin M_1. This is due to aflatoxin M_1 being mildly hydrophobic.

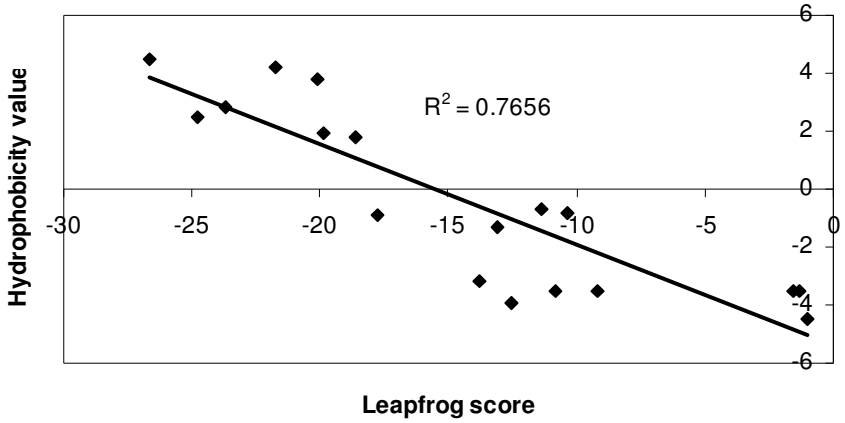


Figure 5.10: Comparison of the predicted binding affinity of the amino acid with aflatoxin M_1 against the hydrophobicity value of the amino acids.

5.3.3 Optimisation of Leapfrog.

The Leapfrog parameters were changed to; join = 6, fuse = 2, bridge = 0, complement = 2. Bridge was initially turned on, however the function forms arrogates which require a name, and until the name is given the Leapfrog run is halted. Since Leapfrog runs were set on average for 999,999 moves (if set for 1,000,000 then the program never stopped) which takes four days of computer time, with the bridge function on, the Leapfrog run took considerable longer. A value of 999,999 moves was deemed suitable since the improvement in binding score between 10,000 moves and 999,999 moves was considerable in terms of both peptide size and binding score as shown in Figures 5.11 and 5.12.

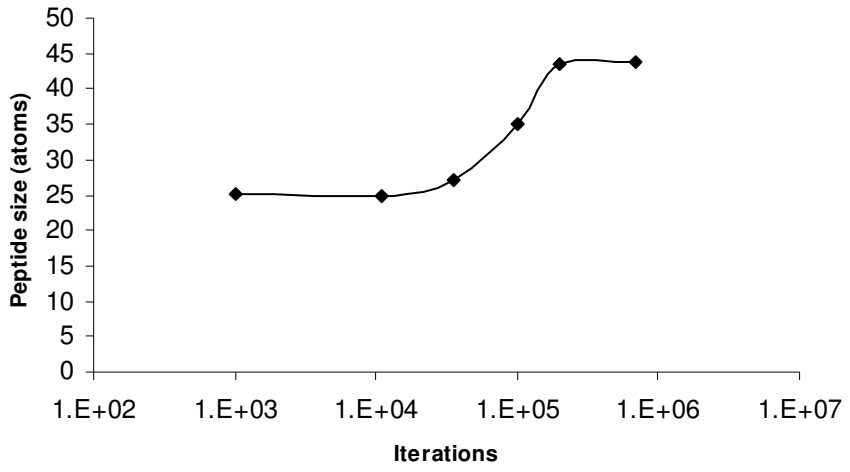


Figure 5.11: The evolution of the peptides with regards to size using the Leapfrog program.

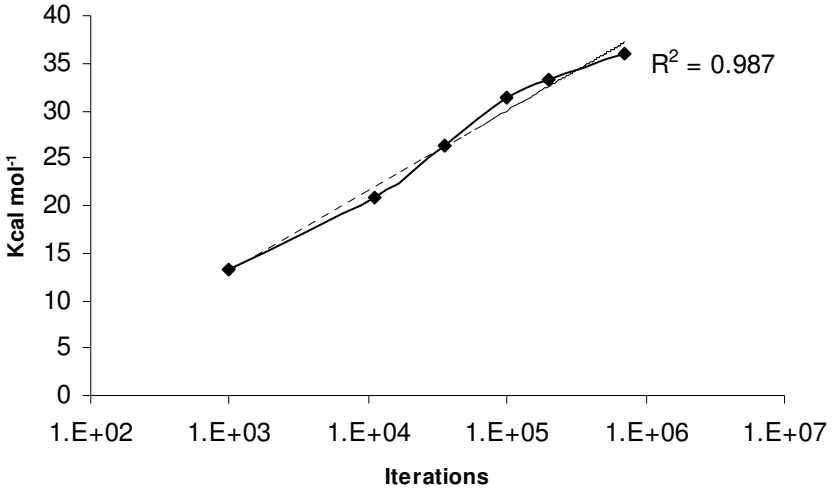


Figure 5.12: The evolution of peptides with respect to binding score. The linear trendline clearly shows that the binding score improves with the number of iterations.

Figure 5.11 shows that as the number of iterations is increased there is little increase in the size of the peptide until 5,000 moves has been performed, then between 50,000 moves and 500,000 moves the peptide size is evolves dramatically and then the speed of evolution slows down. With respect to the binding score with aflatoxin M_1, Figure 5.12 shows that the number of moves is directly proportional to the binding score and therefore the longest run would yield peptides with the best affinity for aflatoxin M_1.

Dong *et al*., (2006) has reported using Leapfrog with the building parameters activated to produce *de novo* designed ligands for the development of PPAR (peroxisome proliferator activated receptors) agonists. The development was successful with computational predictions from Leapfrog validated with laboratory screening. As with the development of receptors against aflatoxin M_1, Dong *et al*., (2006) did not use the Bridge command. Leapfrog has also been tested against receptor systems such as DHFR/methotrexate, Thermolysin and HIV protease (Cramer, 1993) and used extensively for the design of molecular imprinted

polymers (MIPS, Section 1.6.2) by Chianella *et al*., (2002), Piletsky *et al*., (2001) and Subrahmanyam *et al*., (2001). The 'bridge' command is one of Leapfrog's 12 commands, which links together two fragments using a monomer. Using the amino acid monomer set as the building blocks, the 'bridged' monomer will be identical for all amino acids and therefore is a nonessential move for building peptides.

From reviewing the literature for MIP design, the number of Leapfrog moves applied varies from 30,000 to 100,000 which Figures 5.11 and 5.12 suggest may not be suitable and is not substantiated in the MIPS reports.

A Leapfrog run of 999,999 moves was performed to test the settings. The ten best sequences achieved in this investigation against aflatoxin M_1 are shown in Table 5.4.

Table 5.4: The results from a test of the Leapfrog settings.

Sequence	**Binding score** (Kcal mol^{-1})
Phe Cys	-36.6
Pro Ser Leu Gly Leu	-36.2
Pro Ser Leu Gly	-34.5
Ser Leu Gly	-34.2
Leu Gly	-34.1
Leu Gly	-34.0
Pro Ser Leu Gly Gly Leu	-33.7
Asp Ala Val	-33.5
Arg Phe	-32.6
Ser Leu	-32.6

A further Leapfrog run was performed by taking the ten best peptides from Table 5.4 and using them as starting ligands, then continuing the development for another 500,000 moves. The results are shown in Table 5.5.

Table 5.5: The results from continuing the first Leapfrog test run.

Sequence	**Binding Score** (Kcal/mol^{-1})
Phe Cys Gly	-60.8
Pro Phe Cys Gly	-60.3
Pro Phe Cys Gly Leu	-57.0
Pro Phe Cys Gly	-56.0
Phe Phe Cys Gly	-53.5
Phe Cys Phe	-53.3
Gly Phe Cys Gly	-52.2
Phe Cys Gly Gly	-52.2
Gly Phe Cys Gly	-51.9
Gly Phe Cys Gly	-51.9

The structure of the best sequence, Phe Cys Gly, is shown in Figure 5.13. Schneider and Böhm (2002) and Bohacek and McMartin (1997) both commented that *do novo* design programs can produce results which are difficult to synthesise. It can be seen in Figure 5.13 that the peptide backbone is incorrectly formed, as a result both ends of the peptide have an amino terminal.

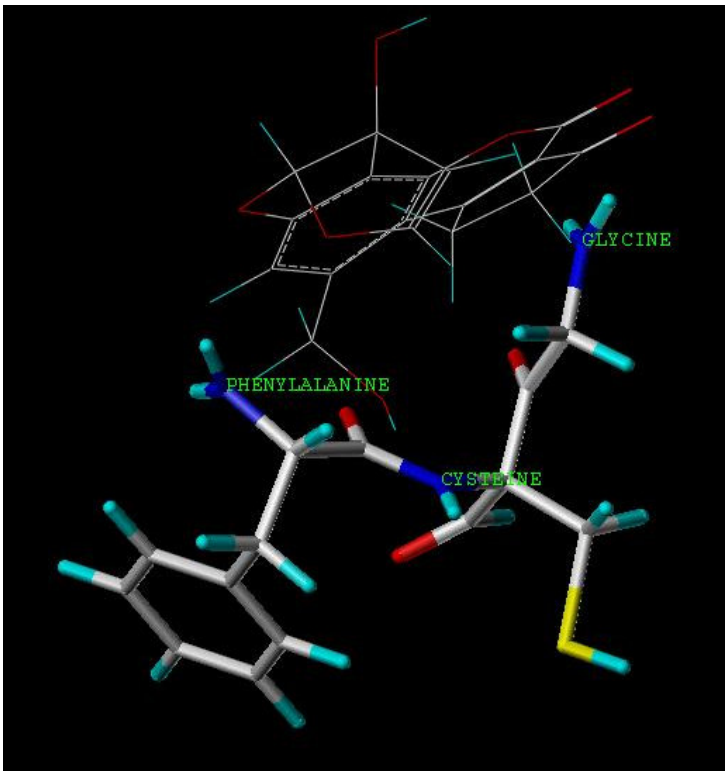


Figure 5.13: The structure of the best peptide sequence from Table 5.5 interacting with aflatoxin M_1. The Phe Cys Gly peptide is shown in 'stick' view with aflatoxin M_1 in 'line' view.

To stop Leapfrog from producing ligands with an incorrect backbone, a further restriction was activated. Leapfrog was setup to link through 'active hydrogens' only. By changing this function then the correct peptides were formed, however when taking the peptides from one run and continuing their development then the imported peptides would again cause incorrect backbones. To rectify this problem, the imported peptides had to be individually checked and the correct sites for amino acid addition selected.

Upon fault finding for incorrect peptides, it was discovered that larger peptides could be formed if the individual move type setting were changed. By changing the Weed value from 10 to 50, then a minimum of 50 ligands would be in process at any one time, thus existing peptides would be extended rather than new peptides

started from one amino acid monomer. This increased the computational time and the number of ligands being saved to the results database. To reduce computational time, the Save values were changed since accessing the results database during the run was taking considerable time. By changing the minimum number of atoms required to save to 40 and a minimum binding score to -25 Kcal mol^{-1} then small undesirable peptides were not saved.

5.3.4 Generation of peptides using Leapfrog.

With all the parameters optimised and faults rectified then a new run was started (LFPOUT20). Again this was done for 999,999 moves, the ten best results were taken and processed for a further 999,999 moves. The results of this run are shown in Figure 5.14.

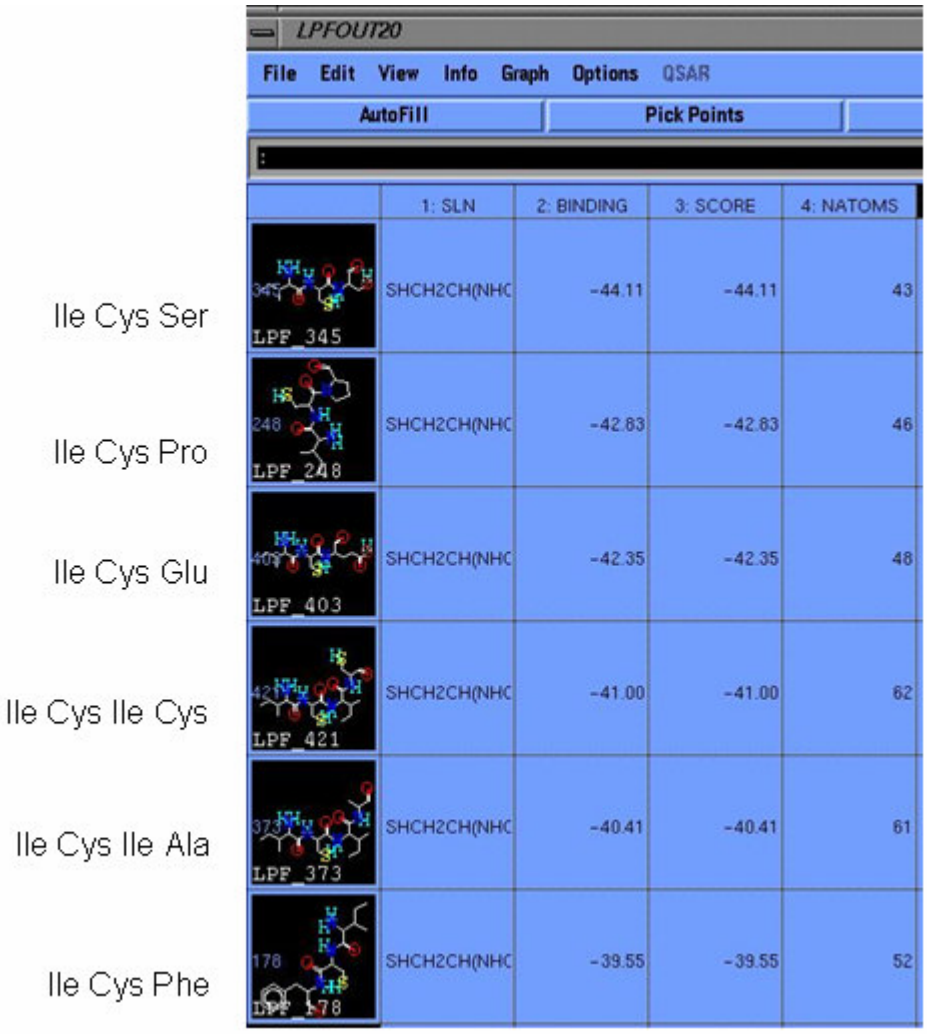


Figure 5.14: The Leapfrog output for run LPFOUT 20 with the sequences listed using SLN (sybyl line notation, Ash *et al*., 1997) and alongside in three letter code.

Further runs were done for a total of 700,000 moves (LFPOUT35) and 1,300,000 (2 x 700,000) moves (LFPOUT36) the data for these runs are shown in Tables 5.6 and 5.7.

Table 5.6: The results from LFPOUT35.

Sequence	**Binding Score** (Kcal mol^{-1})
Pro Val Gly Pro Asn	-36.0
Pro Val Gly Pro Asp	-35.3
Pro Val Gly Pro	-35.3
Pro Val Cys	-35.0
Leu Met Cys Pro	-33.2
Pro Val Ile	-32.9
Pro Val Pro	-31.8
Pro Val Gly Gly	-31.3
Pro Val Gly Ala	-31.3
Pro Val Gly Phe	-30.6

Table 5.7: The results from LFPOUT36.

Sequence	**Binding Score** (Kcal mol^{-1})
Pro Val Cys	-38.5
Pro Val Gly Pro Asp	-38.4
Pro Met Cys Pro	-37.5
Pro Val Gly Pro Asn Gly	-36.8
Pro Val Cys Pro	-36.3
Pro Val Gly Pro Asn Ala	-36.2
Pro Val Gly Pro	-35.3
Gly Met Cys Pro	-35.1
Pro Val Ile	-35.0
Pro Val Gly Pro Asn Pro	-34.0

LFPOUT36 was continued for a further 500,000 moves but no improvement in the results were seen.

To check the scoring by Leapfrog, Flexidock was used. From the three Leapfrog runs (LFPOUT 20, 35, 36) the best binding score was seen by the peptide “Ile Cys Ser”. A calculation of the scoring by Leapfrog takes seconds, for Flexidock the calculation takes about 20 minutes, hence it can be assumed that there is greater accuracy in the Flexidock scores due to the increase in the number of calculations performed.

5.3.5 Validation of peptides using Flexidock.

Flexidock, part of the SYBYL program suite, is one of two docking programs from Tripos. Its unique ‘flexing’ of the receptors backbone allows for accurate

predictions in peptide binding. Original docking programs, such as DOCK (Kuntz, 1992) calculated the binding interaction by placing many copies of the ligand into the receptor pocket simultaneously with different orientations and positions and then using energy minimisation algorithms, binding affinities were predicted. The backbone of the ligand and proteins were not flexed to reduce the number of degrees of freedom and therefore reduced computational time. The method was still computationally laborious and prone to providing false positives if the starting structures are not the same as the ideal structure (Lybrand, 1995).

Flexidock is a combination of two algorithms, a genetic algorithm for changing the structure of the ligand and the binding site as well as an energy evaluation function. As with Leapfrog, the computational time is reduced since an intelligent optimisation process is applied. A starting structure is placed into the pocket and a change in the ligand is made. If the daughter structure is more preferential than the parent then the daughter structure is kept and a second evolution step is performed. If the parent structure is more preferential then the daughter is destroyed and a new daughter structure is made from the parent. As part of the genetic approach the backbone is allowed to flex and therefore simulates the binding of peptides to targets more accurately than traditional docking programs. Previously, Flexidock has been used successfully by Illapakurthy *et al*., (2003) and Bertelli *et al*., (2001) for docking ligands into cyclodextrins and 6-phosphogluconate dehydrogenase respectively.

Flexidock's binding predictions were first evaluated using the results from Tozzi *et al.* (2003b). Tozzi *et al.* reported that "Leu Leu Ala Arg" has good binding towards aflatoxin B_1 whereas "Leu Leu Ala Ser" had poor affinity. Flexidock was used to dock these two peptides to the aflatoxin B_1 molecule and it was determined that "Leu Leu Ala Arg" gave a result of -52.41 Kcal mol^{-1} whereas "Leu Leu Ala Ser" gave a result of only -9.15 Kcal mol^{-1} which confirmed the results achieved by Tozzi *et al*, (2003b) and increased the confidence in the Flexidock program.

The top ten sequences from LFPOUT20, LFPOUT35 and LFPOUT36 were recreated with a straight backbone and docked in triplicate with the aflatoxin M_1 molecule using the Flexidock program. Table 5.8 shows these results.

Table 5.8: Results from Flexidock validation of Leapfrog scores.

Sequence	**First score** (Kcal mol^{-1}) and Rank	**Second score** (Kcal mol^{-1}) and Rank	**Third score** (Kcal mol^{-1}) and Rank
Ile Cys Ser	-22.75 (29)	-22.73 (30)	-22.80 (30)
Ile Cys Pro	-26.41 (20)	-26.58 (19)	-26.21 (21)
Ile Cys	-26.26 (21)	-26.33 (20)	-25.92 (24)
Ile Cys Ile Cys	-29.43 (7)	-30.05 (8)	-29.70 (8)
Ile Cys Ile Ala	-28.02 (14)	-28.03 (16)	-28.03 (14)
Ile Cys Phe	-26.15 (22)	-26.16 (22)	-26.14 (22)
Ile Cys Ile	-28.81 (12)	-27.24 (18)	-28.79 (11)
Ile Cys Ala	-22.46 (30)	-23.93 (29)	-22.83 (29)
Ile Cys Cys	-24.44 (26)	-25.41 (24)	-25.07 (27)
Ile Cys Val	-23.30 (28)	-25.43 (23)	-27.28 (16)
Pro Val Gly Pro Asn	-31.00 (2)	-30.91 (5)	-31.18 (5)
Pro Val Gly Pro Asp	-29.88 (6)	-30.55 (6)	-28.81 (10)
Pro Val Gly Pro	-29.24 (8)	-28.55 (13)	-29.02 (9)
Pro Val Cys	-23.74 (26)	-24.83 (27)	-23.97 (28)
Leu Met Cys Pro	-30.74 (4)	-34.02 (2)	-31.86 (3)
Pro Val Ile	-27.12 (18)	-28.09 (15)	-25.16 (26)
Pro Val Pro	-27.61 (15)	-25.38 (25)	-26.57 (19)
Pro Val Gly Gly	-25.84 (23)	-23.94 (28)	-25.94 (23)
Pro Val Gly Ala	-26.44 (19)	-26.24 (21)	-27.08 (18)
Pro Val Gly Phe	-27.56 (16)	-28.22 (14)	-31.32 (4)
Pro Val Cys	-24.72 (25)	-25.15 (26)	-25.57 (25)
Pro Val Gly Pro Asp	-30.93 (3)	-30.92 (4)	-30.30 (6)
Pro Met Cys Pro	-30.71 (5)	-30.48 (7)	-30.01 (7)
Pro Val Gly Asn Gly	-28.66 (13)	-28.73 (12)	-28.30 (13)
Pro Val Cys Pro	-25.19 (24)	-28.85 (11)	-27.79 (15)
Pro Val Gly Pro Asn Ala	-29.04 (10)	-32.93 (3)	-34.24 (1)
Pro Val Gly Pro	-29.08 (9)	-29.48 (9)	-28.56 (12)
Pro Val Ile	-29.00 (11)	-27.55 (17)	-26.44 (20)
Gly Met Cys Pro	-27.22 (17)	-29.11 (10)	-27.23 (17)
Pro Val Gly Pro Asn Pro	-39.24 (1)	-34.70 (1)	-33.86 (2)

By counting the rankings (as shown in brackets), the top three sequences are Pro Val Gly Pro Asn Pro, Leu Met Cys Pro and Pro Val Gly Pro Asn Ala.

5.3.6 Optimisation of peptides for solubility and immobilisation.

With the best three peptides determined the practical aspects of solubility and immobilisation were considered.

The original strategy for analysing the binding of the peptide to the aflatoxin was to use a BIAcore instrument. The peptides would be immobilised to the gold coated BIAcore detector chip through a gold-cysteine interaction. This interaction would also be used when the peptide was transferred to a gold electrode surface as described by Katayama *et al*., (2000). Furthermore to move the active peptide region into the solution and reduce steric hindrance and improved binding capabilities (Tozzi *et al*., 2003b), two glycienes acting as a spacer arm were attached to the peptide along with the cysteine to make a distance of 8 atoms. Tozzi *et* al., (2002) suggests that an arm length of at least 4 atoms is required to remove steric hindrance.

Using the hydrophobicity data from Kyte and Doolitle (1982) the hydrophobicity prediction for these peptides are;

(1) Pro Val Gly Pro Asn Pro = -4.5 (hydrophilic)

(2) Leu Met Cys Pro = 6.6 (hydrophobic)

(3) Pro Val Gly Pro Asn Ala = -1.1 (mildly hydrophilic)

With consideration of synthesis and stability, peptide (2) is hydrophobic which will cause problems when using the peptide and methionine could become oxidised therefore further investigation was inappropriate.

To immobilise the peptides using two glycines and a cysteine, if the peptide has two cysteines then cross-linked disulphide aggregates occur. Grant, (2002) recommends replacing cysteine with serine to remove this problem; additionally it

is recommended that methionine is replaced by norleucine to increase stability, however, the tetrapeptide has now been changed significantly.

Peptide number (3) is hydrophilic however by adding two glycines and a cysteine, it becomes hydrophobic. Peptide (1) is hydrophilic and upon adding two glycines and a cysteine then it is still hydrophilic.

In order to compare the results of the simulation with practical values obtained it was decided to synthesise the peptide sequence given by the paper published by Tozzi *et al.*, (2003b) of Leu Leu Ala Arg.

To add the spacer arm to the peptide sequence the optimal attachment was determined using detailed molecular dynamics.

Molecular dynamics is based on the calculation of Newton's equations of motion for molecular systems in which the trajectory (as a function of time) of all atoms in the system is determined and therefore the total energy of a molecular system (van Gunsteren and Berendsen, 1990). Molecular dynamics is suitable for molecules less than 100 atoms (Gunsteren and Berendsen, 1990). Both peptides are less than 100 atoms.

Molecular mechanics was employed in a fashion similar to the thermodynamic cycle. First a dynamics run was performed with Cys Gly Gly Arg Ala Leu Leu and the aflatoxin M_1. Then the run was performed again without the aflatoxin M_1, and finally with the aflatoxin M_1 and not the peptide. This will give an approximation of the binding energy but will not consider the change in entropy, however it does allow for full flexibility of the ligand and receptor. The values of total energy (the addition of kinetic and potential energy) were calculated by Equation 5.1.

Change in Energy = (Energy Reactant A + Energy Reactant B) – Energy of Product AB Equation 5.1.

This calculation is similar to that described by Moon and Howe (1991). In the previous section it was discussed that Flexidock is superior to the docking calculations done by Leapfrog. The molecular dynamics performed each took about 2 hours. This results in a greater accuracy in the calculation and a more reliable result. Using the different techniques of Leapfrog, Flexidock and molecular dynamics should remove bias which maybe observed if only one technique would be performed. Although molecular dynamics is the most detailed method performed, it is impractical to use it throughout since the number of docking calculations performed, to eliminate the early contenders, would have taken considerable time.

For the dynamic runs the pressure was kept constant, allowing the temperature to rise and fall. For the lone peptide or aflatoxin M_1, 10000 moves were performed but for the aflatoxin M_1 with the peptide 100000 moves were set. After each run the data files containing total energy and time were analysed to check that the system had equilibrated and hence the run was significantly long enough.

Table 5.9 shows the results for the peptide Leu Leu Ala Arg with different modes of addition for the two glycines and cysteine.

Table 5.9: The results of the dynamic runs for the optimisation of the attachment of the spacer arm to LLAR.

Sample	**Potential Energy** (Kcal mol^{-1})	**Kinetic Energy** (Kcal mol^{-1})	**Total Energy** (Kcal mol^{-1})
AFM1 (aflatoxin M_1)	57.50	29.76	87.25
LLARGGC + AFM1	145.54	118.41	263.95
CGGRALL + AFM1	147.03	118.57	265.60
CGGLLAR + AFM1	141.94	118.54	260.47
LLARGGC - AFM1	102.31	88.01	190.32
CGGRALL - AFM1	103.23	88.57	191.80
CGGLLAR - AFM1	98.76	86.07	184.82

By incorporating Equation 5.1 yields Table 5.10.

Table 5.10: The change in energy for the optimisation of the attachment of the spacer arm for LLAR.

Peptide	**Potential Energy** (Kcal mol^{-1})	**Kinetic Energy** (Kcal/mol^{-1})	**Total Energy** (Kcal/mol^{-1})
CGGRALL	-13.699	0.24	-13.45
CGGLLAR	-14.318	2.714	-11.604
LLARGGC	-14.27	0.64	-13.62

Table 5.10 shows there is little variation in the energy between the three sequences. The sequence RALLGGC was not calculated since the synthesis of peptides with arginine on the amino terminal is difficult (Grant, 2002).

From the data it was concluded that LLARGGC would be the best peptide due to the lowest total energy. Furthermore having the arginine in the middle of the sequence should increase the solubility (Grant, 2000). This energy calculation through molecular dynamics process was repeated for the peptide PVGPNP. Through solubility concerns, a Flexidock run was performed with the asparagine replaced by arginine. The Flexidock result for PVGPNP was -29.91 Kcal mol^{-1} and for PVGPRP it was -32.61 kcal mol^{-1}. This change results in a change on the hydrophobicity of -1 or an improvement of 20%.

The change in energy calculated by dynamics for this change in the sequence is shown in Table 5.11.

Table 5.11: Comparison between the changes in energy resulting in the substitution of one amino acid.

Sample	**Potential Energy** (Kcal mol^{-1})	**Kinetic Energy** (Kcal mol^{-1})	**Total Energy** (Kcal mol^{-1})
PVGPRP + AFM1	140.03	99.22	240.05
PVGPNP + AFM1	142.53	102.74	245.28

The data from Table 5.11 is in correlation with the Flexidock scores showing PVGPRP is slightly preferential over PVGPNP.

Using the same procedure as the investigation on the placement of the spacer arm as used with LLAR the optimisation of the spacer attachment was performed again for PVGPRP. The results are shown in Table 5.12 and Table 5.13.

Table 5.12: The results of the dynamic runs for the optimisation of the attachment of the spacer arm to PVGPRP.

Sample	**Potential Energy** (Kcal mol^{-1})	**Kinetic Energy** (Kcal mol^{-1})	**Total Energy** (Kcal mol^{-1})
AFM1	57.50	29.76	87.25
CGGPVGPRP +AFM1	175.56	133.45	309.01
PVGPRPCCG +AFM1	173.98	133.30	307.27
CGGPVGPRP – AFM1	137.20	104.23	241.43
PVGPRPCCG – AFM1	137.02	101.19	238.20

Table 5.13: The change in energy for the optimisation of the attachment of the spacer arm for PVGPRP.

Sample	**Potential Energy** (Kcal mol^{-1})	**Kinetic Energy** (Kcal mol^{-1})	**Total Energy** (Kcal mol^{-1})
CGGPVGPRP	-19.14	-0.54	-19.67
PVGPRPGGC	-20.54	2.35	-18.18

So from the data in Table 5.13, CGGPVGPRP was synthesised since the total energy is more negative and therefore more preferential than PVGPRPGGC. Having a proline on the carbon terminal can result in diketopiperazine formation during peptide chain elongation, hence the final product may contain a truncated sequence minus the proline. This can delay the synthesis (Patel, 2004). An interesting observation is that the total energy change for CGGPVGPRP (-19.67 Kcal mol^{-1}) is more endothermic than the total energy change for LLARGGC (-13.62 Kcal mol^{-1}) suggesting that the computationally derived receptor should have superior binding to the reported peptide. Figure 5.15 shows the final peptide sequence binding with aflatoxin M_1.

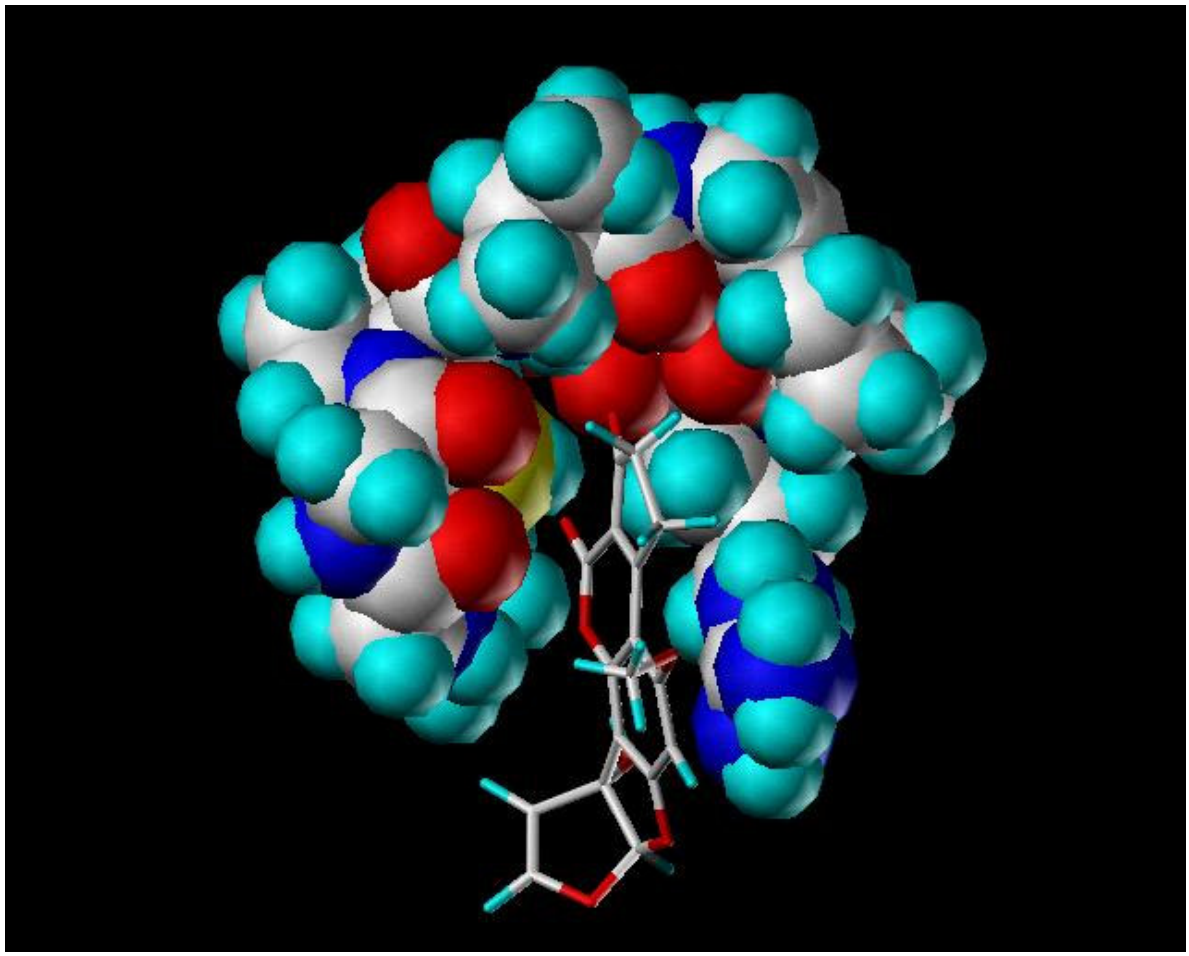


Figure 5.15: The *de novo* designed synthetic peptide receptor binding with aflatoxin M_1. The peptide receptor (CGGPVGPRP) is shown in spacefill view and the aflatoxin M_1 in stick view.

5.3.7 Studying natural receptors for aflatoxins.

To aid the design of the peptides, it was chosen to study natural receptors. Within the Protein Databank a structure of an enzyme was found for aflatoxin B_1 aldehyde reductase (AKR7A1). Figure 5.16 shows the structure of the enzyme and Figure 5.17 shows the reaction of the enzyme. From the reported literature the enzyme is found to have affinity for aflatoxin B_1 aldehyde and aflatoxin B_1. Since the structure of aflatoxin B_1 and M_1 is similar the enzyme was analysed using the SYBYL software for the amino acids which interact with the aflatoxin M_1.

The protein databank file was imported into the SYBYL suite and the water molecules were deleted. Then the program SiteID was employed to find possible binding pockets within the enzyme.

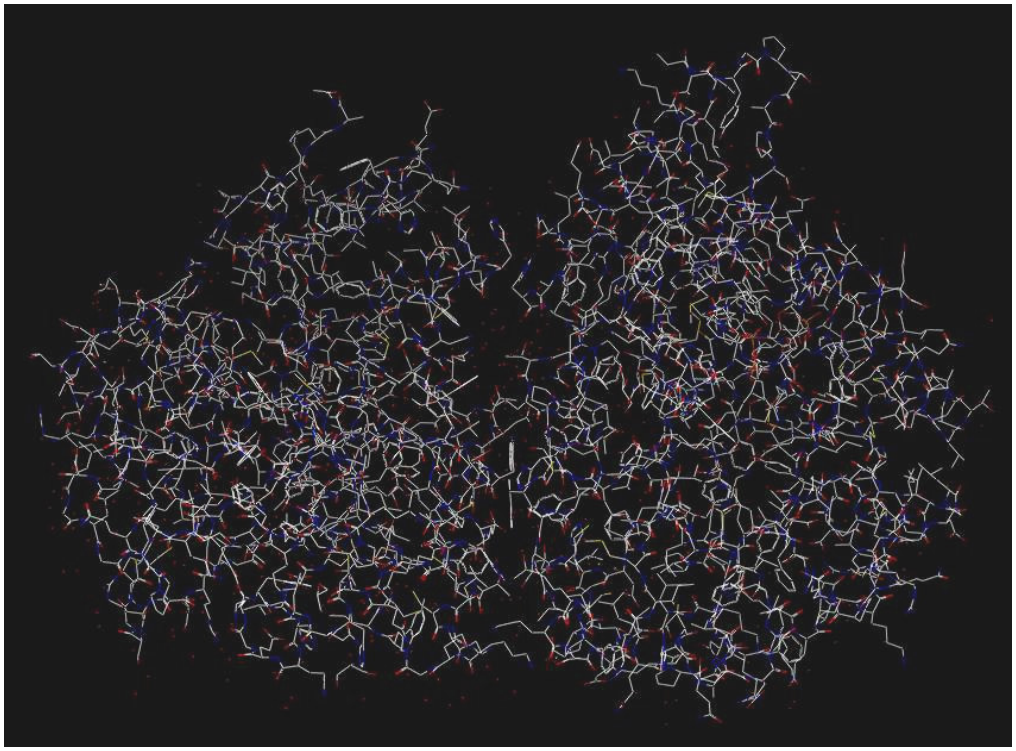


Figure 5.16: Structure of AKR7A1 (1GVE) as taken from the protein databank.

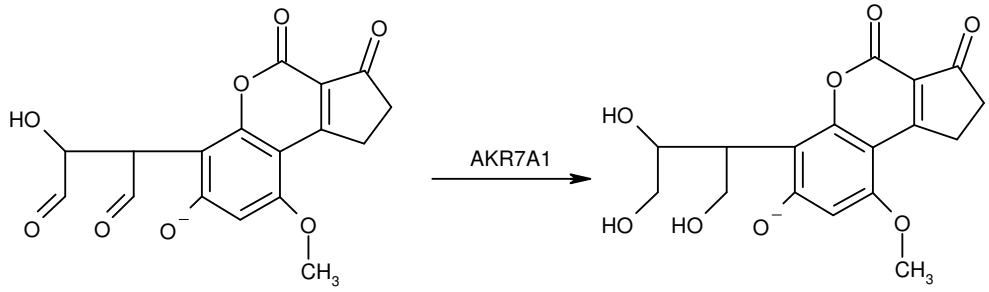


Figure 5.17: The substrate aflatoxin B_1 dialdehyde and the enzyme product dihydroxy aflatoxin B_1.

The results of the search is shown in Figure 5.18, the peptide chain is shown as a ribbon and the sphere indicate the voids in the enzyme and hence the potential binding sites.

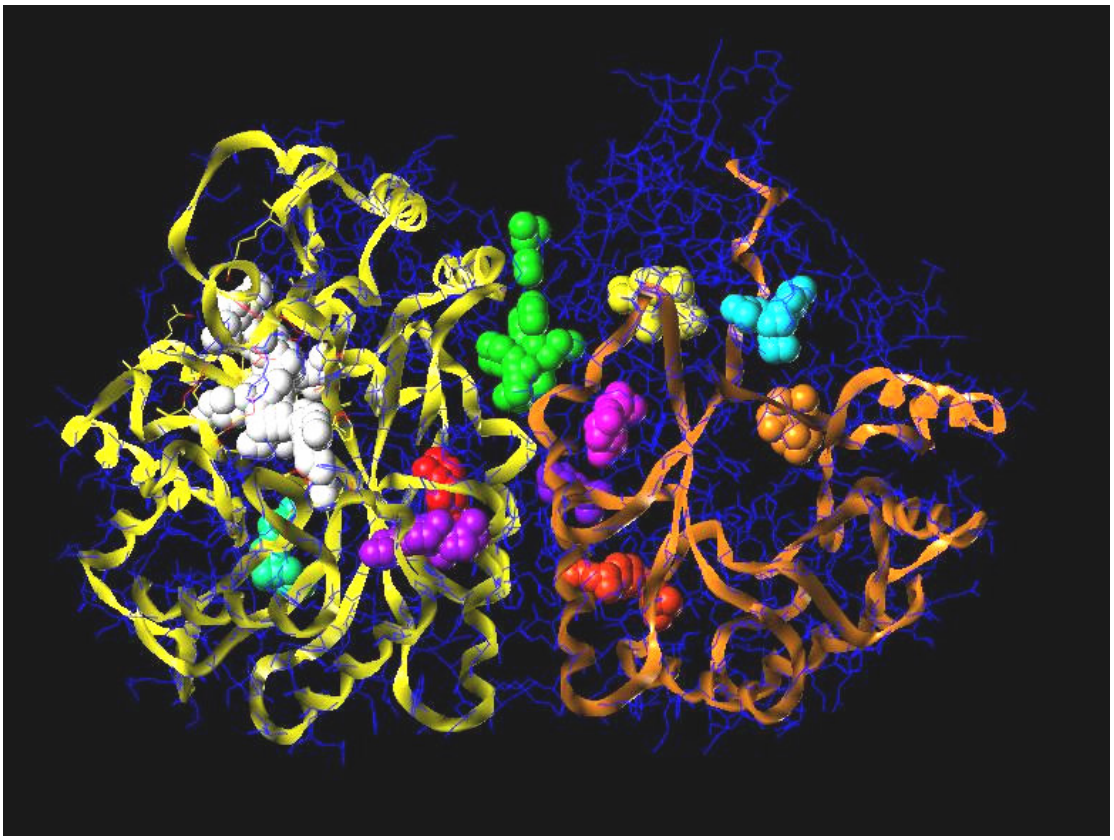


Figure 5.18: The results from SiteID showing the voids in the enzyme and thus possible binding sites.

The site corresponding to the white spheres correlates with the location identified with the published reports (Kozma *et al.*, 2002). Figure 5.19 shows in detail this area. The amino acids labelled are predicted to be involved in the binding.

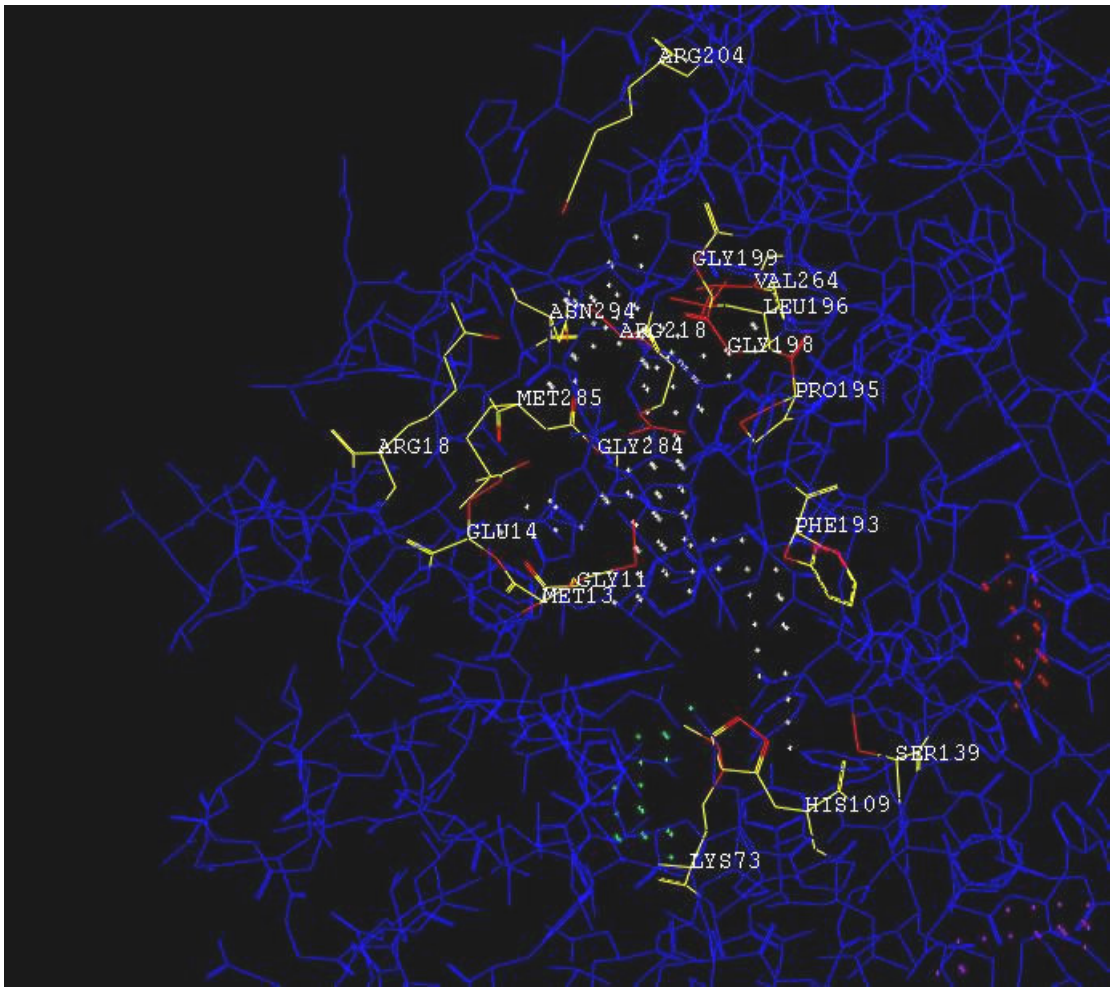


Figure 5.19: The binding site of aflatoxin B_1 aldehyde reductase.

The literature from Kozma *et al.* (2002) stated that the active amino acids were; Asp40, Lys 73, Tyr 45, His 109, Arg 17, Arg 231, Arg 327, met 13, Leu 227, Tyr 228 and Phe 224. The SiteID program predicted the relevant amino acids were; Arg 18, Glu 14, Met 13, Gly 11, Lys 73, His 109, Ser 139, Phe 193, Gly 284, Met 285, Asn 294, Pro 195, Val 264, Leu 196, Gly 198, Arg 218, Gly 199, Arg 204.
The only Met 13, Lys 73 and His 109 are in both sets.

The amino acids identified by either the literature or the SiteID program were extracted and Flexidock was used to validate the amino acids and also to remove

any amino acids not contributing to the binding. The results from Flexidock showed that the following fragments were relevant;

Arg 17, Arg 18.
Phe 193, Asn194, Pro 195, Leu 196, Ala 197
Gly 199
Arg 204
Val 9, Leu 10
Thr 202

These fragments were then taken and placed into a molecular dynamics box with the aflatoxin M_1 and the box was filled with 0.223 g ml^{-1} of water. The dynamics run was for set 100,000 fs. Upon replaying the trajectory only Val 9, Leu 10 was the only fragments which interacted with the aflatoxin M_1 molecule, presumably since both have hydrophobic properties. Figure 5.20 shows the positions of the fragments at the end of the run.

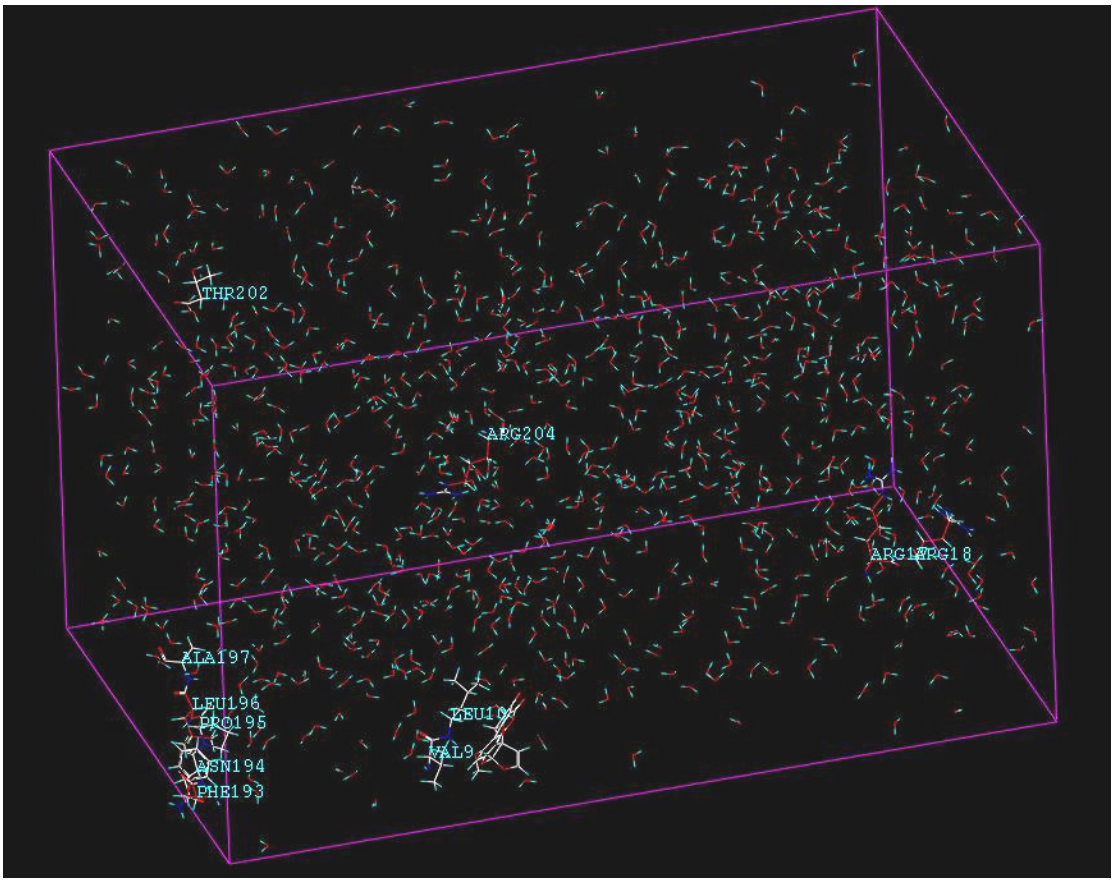


Figure 5.20: The final snapshot of the dynamics run for the interaction of fragments from 1GVE with the aflatoxin M_1, in the presence of water molecules.

This experiment gave no extra information to aid peptide design and therefore this approach was not followed further in this study.

5.3.8 Evaluation of the GROMACS software.

Democritus and Lucretius in 55 BC first suggested the idea of small hard atoms however it was van der Waals who using the Joule-Thompson effect showed that there are attractive forces between atoms (Goodford, 1985). The underlying term calculated in molecular modelling is energy. The total energy of the molecules is the sum of the potential and kinetic energy. An analogy used to describe molecules is the 'ball and spring' model. If the ball is pulled from a stationary position, the system is high in potential energy and low in kinetic energy, then when the ball

released then the ball will have high momentum and hence high kinetic energy and low potential energy, however in both scenarios the total energy is the same. This total energy is termed the 'internal energy' (ΔU) and is related to enthalpy (ΔH) by Equation 5.2 where P equals pressure and V for volume.

$$\Delta H = \Delta U + P\,\Delta V \qquad \text{Equation 5.2}$$

Molecular mechanics cannot calculate exactly the internal energy of a molecule, but it makes an approximation by adding up all the internal forces within a molecule such as van de Waals and columbic interactions. This term is called E_{MM} and often referred to as the 'Steric energy' and is calculated by Equation 5.3 where E_{bonds} is the energy due to bond stretching, E_{angles} is the energy due to bond bending, E_{vdw} is the energy due to van der Waals interactions between atoms, $E_{torsion}$ is the energy due to bond rotation, E_{charge} is the energy due to the interaction of atoms with varying charge and $E_{miscellaneous}$ is the energy due to additive effects such as if a bond is stretched then it maybe easier to bend the bond. A comprehensive account of these terms is given by Goodman, (1998).

$$E_{MM} = E_{bonds} + E_{angles} + E_{vdw} + E_{torsion} + E_{charge} + E_{miscellaneous} \qquad \text{Equation 5.3}$$

Within a mechanics model there is no external pressure (P = 0) thus from equation 5.1 $\Delta H \approx \Delta U \approx \Delta E_{MM}$ (Goodman, 1998).

If an external source of energy is applied to the molecule in the form of heat then we would expect the conformation of the molecule to change, however the total energy of the molecule does not change. The change is due to entropy.
Entropy is a measure of disorder, the higher the disorder of a molecule then the higher the entropy. It is a very difficult term to calculate through molecular dynamics however it allows us to calculate a very important value, ΔG.

ΔG is the Gibbs free energy and it allows us to tell if a reaction is favourable, it is calculated by equation 5.4.

$\Delta G = \Delta H - T\Delta S$ or $\Delta G = \Delta E_{MM} - T\Delta S$ Equation 5.4

Where ΔH is the change in enthalpy, ΔS is the change in entropy and T is the temperature measured in Kelvin. If the change in entropy is small then it can be assumed that $\Delta G \approx \Delta E_{MM}$.

ΔG is it advantageous to know since it is related to the dissociation constant as shown in Equation 5.5.

$-\Delta G = RT \ln K_d$ Equation 5.5

Where R is the gas constant (8.314 $JK^{-1}mol^{-1}$), T is the temperature in Kelvin and K_d = [Concentration of A]/[Concentration of B].

Considering the reaction, A $\rightleftharpoons$ B, if ΔG is positive (surplus energy) then there would be more of B than A hence the reaction is favourable. Using ΔG, it would be possible to predict the affinity of a receptor for a ligand (Honma, 2003). As previously stated entropy is not an easy value to calculate, hence other methods for obtaining the free energy have been reported. The most common method is called free energy perturbations (FEP) (Reddy and Erion, 2001). If there are two ligands L_1 and L_2 and one protein (P), the binding ratios for L_1 and L_2 for the protein can be calculated. By performing a run where L_1 is bound to the protein and during the dynamics run it is mutated from L_1 into L_2, this gives a value of ΔG_{PL}. This is an example of alchemy and not physically possible, however it can be performed in computational space. By setting up a second dynamics run with L_1 in solution at the start and then mutate the ligand into L_2, this gives a value of ΔG_L. Figure 5.21 shows a thermodynamic cycle for this reaction.

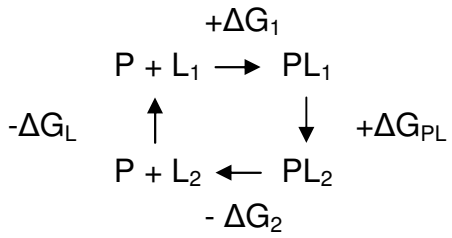


Figure 5.21: A thermodynamic cycle for a free energy perturbation calculation.

Since it is a cycle, the sum of all the ΔG values will equal zero (Equation 5.6) therefore

$\Delta G_1 - \Delta G_2 = \Delta G_L - \Delta G_{PL}$ Equation 5.6

From the dynamics run values for ΔG_{PL} and ΔG_L are known, thus a value for $\Delta G_1 - \Delta G_2$ can be obtained as shown in Equation 5.7.

$\Delta G_1 - \Delta G_2 = RT \ln (K_1 / K_2)$ Equation 5.7

Therefore the dissociation constant is determined and a ratio how favourable the protein is for ligand one compared to ligand two.
This has a drawback since it is only a relative value for the binding of one ligand compared to another.

A different technique can be performed where L_1 is mutated into dummy atoms. These are atoms which have no charge or VdW forces, in other words the ligand disappears. This would give a value of the binding constant; however this has had limited success at accurate prediction and depends on the speed and length of the dynamics run (Donnini and Juffer, 2003; Honma, 2003; Goodman, 1998; Pearlman and Rao, 1998).

FEP is slow and computer intensive, for faster predictions, docking methods can be used but at the expense of accuracy (Honma 2003, Josephy-McCarthy, 1999).

From the literature there is a strong argument stating that the prediction of binding scores is unreliable and by using molecular dynamics to determine ΔG provides a more robust prediction. The software package GROMACS was evaluated since it boasts being the fastest dynamics package available, thus able to calculate the interaction of water molecule with the aflatoxin M_1 and peptide but at a density of at 1 g ml^{-1}, allowing a good approximation of ΔG.

The package was installed onto a Laptop personal computer with an Intel processor since the software had been tailored for such an environment. The procedure described in the literature utilising the thermodynamic cycle requires atoms to change charge or weight. This function is not available in the SYBYL package. The best peptides from the Leapfrog runs were saved using the SYBYL package as a PDB file and then converted using the gromacs software into a GMX file. Unfortunately the encoding of the Leapfrog results into PDB files by SYBYL produced incompatible PDB files. An example of the errors caused is the amino acid isoleucine was encoded as ISO and not ILE. This could be adjusted manually and the error has been reported to the software manufactures. Additionally for GROMACS to run as fast as possible, only the essential hydrogen atoms of the amino acids were present, non essential hydrogen atoms were deleted. This might be appropriate if a large protein was being studied however the removal of several hydrogen atoms on a small peptide may have a significant effect on the binding prediction.

A further problem, and the greatest, was importing the aflatoxin M_1 molecule into the gromacs program. PDB files are generally used for describing proteins and some common cofactors such as NADH. To import aflatoxin M_1 a PDB file had to be created and modified. Within the GROMACS program is a database of angles, charges and weights for typical atoms and bonds found in proteins and common cofactors, these are used to create a GMX file from a PDB file. The structure of aflatoxin M_1 contains several contradictions according to the database. Ring A for

example as shown in Figure 5.22, contains a five membered ring, however it is a heterocyclic ring with an oxygen present (furan) and it contains one double bond. Hence this ring could classify as three different structure types. Thus when the aflatoxin M_1 molecule was finally accepted by the GROMACS program the structure of the aflatoxin M_1 was different to that determined from the minimisation results from SYBYL as shown in Figure 5.23. Rings D and E are not in the same plane as rings B and C, furthermore ring A is twisted, as would be seen in aflatoxin M_2.

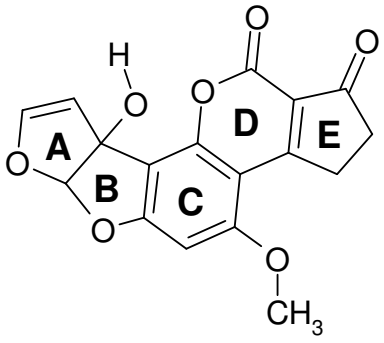


Figure 5.22: The complexity of ring A.

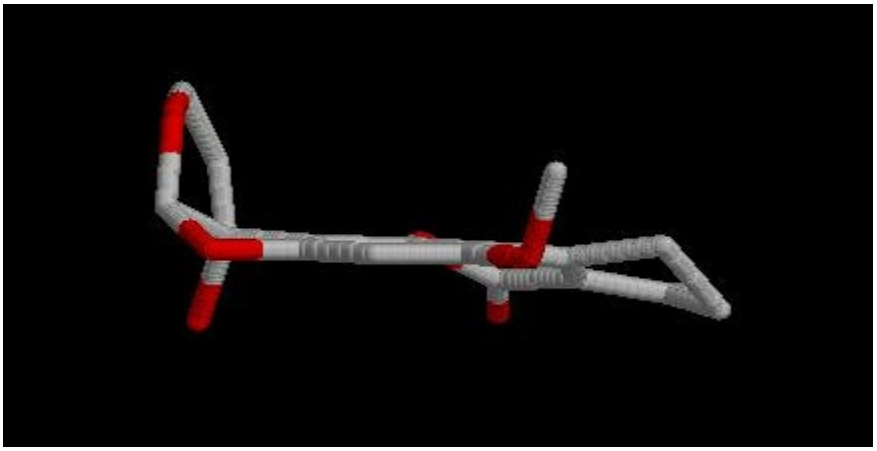


Figure 5.23: The GROMACS version of aflatoxin M_1 with a twisted backbone.

In conclusion the investigation suggests that the GROMACS software was not suitable for the calculation of ΔG since the software is developed for biological applications such as drug discovery and is unsuitable for chemical structures such as aflatoxin M_1. More time would be needed to investigate fully the conversion of

structures other than proteins or common co-factors from PDF file format into GMX file format.

In conclusions to the molecular modelling, the peptides LLARGGC and CGGPVGPRP have been deemed as suitable receptors by the computer modelling and the peptide sequences were sent to the Medical Research Council (London, UK) for synthesis.

5.3.9 Affinity studies using the OWLS instrumentation.

OWLS or optical waveguide lightmode spectroscopy is a label free method for monitoring ligand receptor reactions. The method uses a He-Ne laser to produce an evanescent field by utilising a planar waveguide. The waveguide has a diffraction grating cut into the surface to project the evanescent field into the solution. At a particular angle of incident the laser would be coupled into the waveguide and detected at the end. The angle of coupling is dependant on the refractive index of the solution and surface mass on the waveguide. By recording the intensity of the light at the end of the waveguide against the angle of incident (α) then minute changes in the mass on the surface can be detected (~1 ng cm^2). The OWLS system can be coupled to a liquid flow cell for detecting antibody and peptide ligand reactions. Figure 5.24 shows a schematic diagram of the OWLS system.

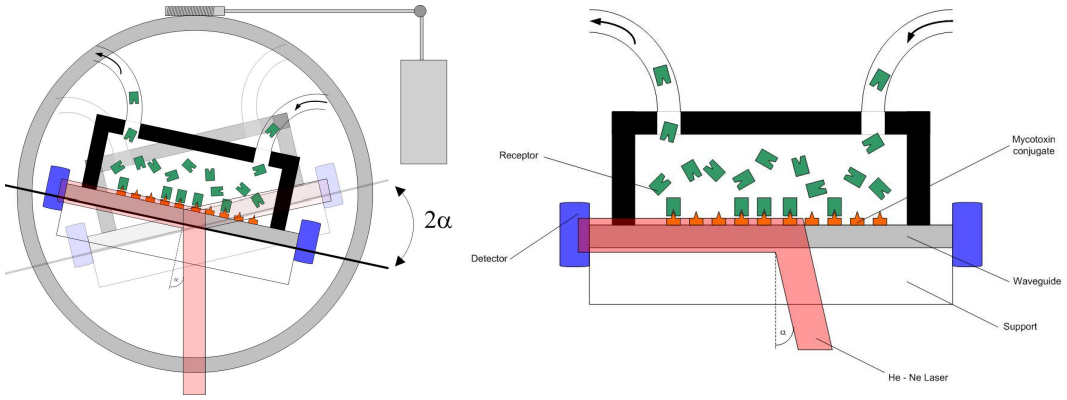


Figure 5.24: A schematic diagram of the OWLS system. Left) The whole waveguide and liquid cell is rotated back and forth by 2α, at a particular angle on incident (α) the He-Ne laser will be coupled into the waveguide and detected at the detectors. The angle α is dependant on the refractive index of the solution and the surface mass. Right) The aflatoxin M_1 – BSA is covalently immobilised onto the surface and either antibody or peptide is flowed through the system during operation.

To monitor the interaction of the peptides with aflatoxin M_1, the aflatoxin M_1 conjugate used in the ELISA (BSA - aflatoxin M_1) was immobilised onto the glass surface of the waveguide. The waveguide was silanised using 3-aminopropyltrioxysilane as described by Trummer *et al.*, (2001) in Section 5.2.7. Following silanisation, whilst monitoring using the OWLS instrument, the aflatoxin M_1 – BSA was attached. Figure 5.25 shows the sensorgram of this immobilization.

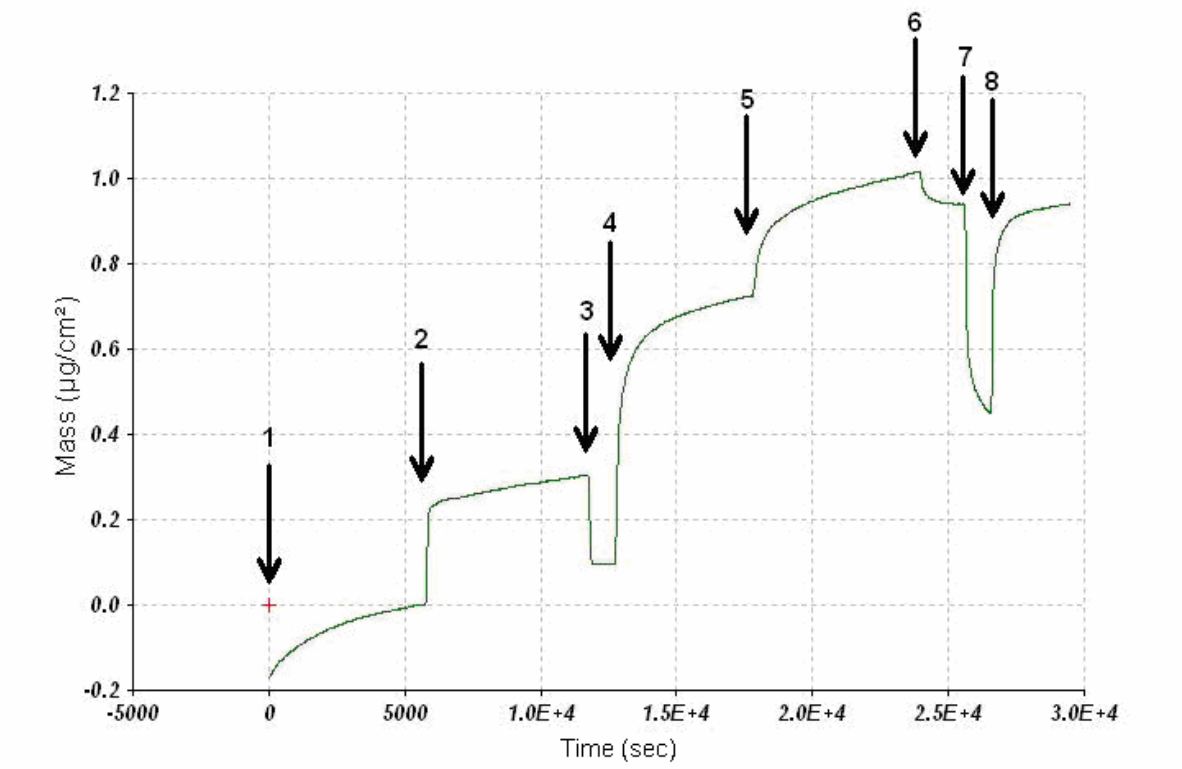


Figure 5.25: The printout of the immobilization of aflatoxin M_1 – BSA onto the silanised surface using optical waveguide lightmode spectroscopy.

In Figure 5.24 initially ultrapure water was pumped through the system (1), and then (2) 2.5 % glutaraldehyde was allowed to react to the surface amino groups of the aminopropyltrioxysilane, the system was purged with water (3) and equilibrated with PBS buffer (4). Once the signal has stabilized (5), 100 mg L^{-1} BSA - aflatoxin M_1 was added over the waveguide and allowed to react with the immobilised glutaraldehyde for 1.5 hours. 10 mM PBS (pH 7.4) was then used to remove any unbound BSA – aflatoxin M_1 (6) (a small decrease in the signal was observed as the reversibly bound aflatoxin M_1 – BSA is removed). Then the waveguide was washed with 0.01 M hydrochloric acid (7) and then stored in PBS (8).

In a subsequent operation, surplus unreacted glutaraldehyde sites on the waveguide were blocked using 10 mM lysine to stop any non-specific binding of the antibody or peptides.

To verify the dynamics of the system, the anti-aflatoxin M_1 antibody / BSA aflatoxin M_1 interaction was studied since the affinity of the antibody complex is proven. Figure 5.26 shows that the anti-aflatoxin M_1 antibody has affinity for the immobilised aflatoxin M_1, as would be expected.

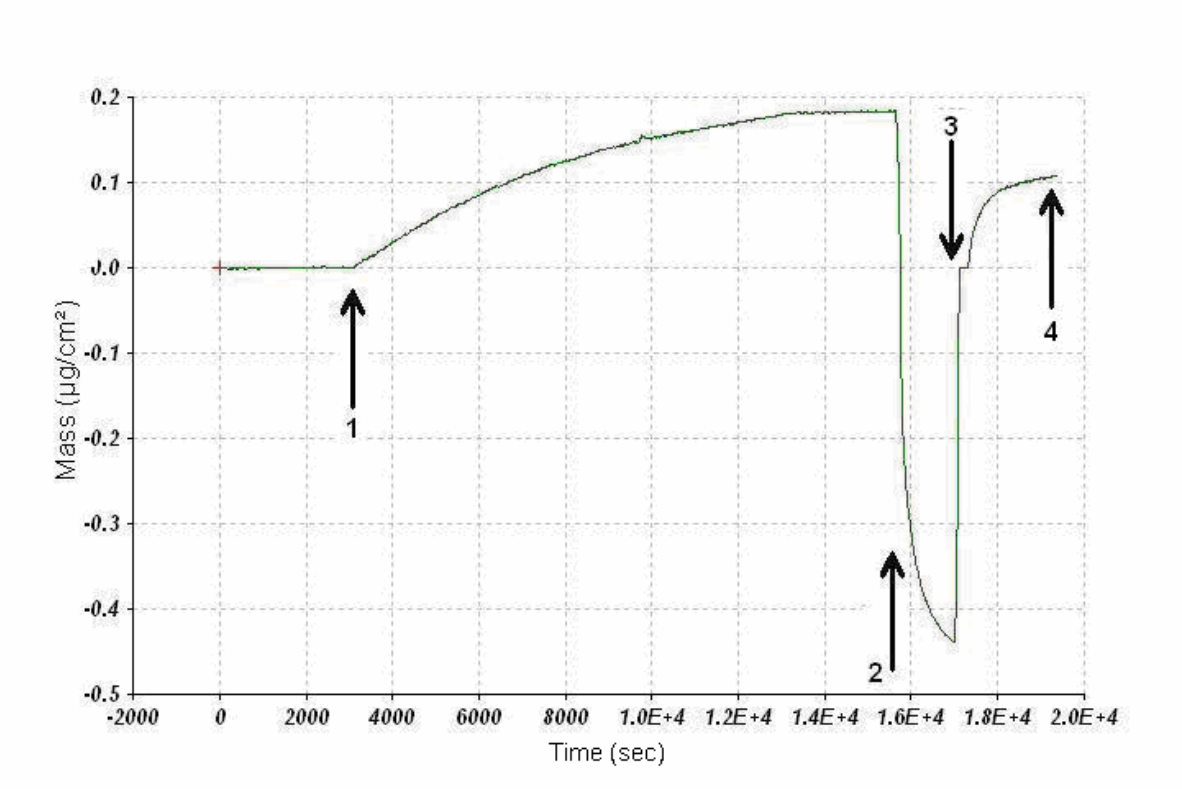


Figure 5.26: The printout of the saturation of the aflatoxin M_1 - BSA coated waveguide with anti-aflatoxin M_1 antibody using optical waveguide lightmode spectroscopy.

In Figure 5.26 the waveguide was first equilibrated with PBS and anti-aflatoxin M_1 antibody was allowed to react with the waveguide (1). The absorbance reaches a plateau (2) and the buffer was switched to 0.01 M HCl. The eluent is changed back to PBS (3) and allowed to stabilise. With PBS still in the system, the baseline at (4) is higher than at (1) suggesting that the regeneration was not completely successful.

The antibody was injected until the adsorption curve reached a maximum, at this point the mass of the antibody on the surface was 0.185 μg cm^{-2}. Assuming a relative molecular mass of 150,000 Daltons for the antibody, then 1.67 x 10^{23} molecules cm^{-2} were immobilised. From the immobilization of aflatoxin M_1 – BSA, 8.55 x 10^{21} molecules cm^{-2} were immobilised. Hence 5.12% of the aflatoxin M_1 - BSA was available for binding.

Previous reports have suggested 0.01 M HCl is suitable for regeneration however this was for a polyclonal antibody (Székács *et al.*, 2003). From the sensogram in Figure 5.26 it can be seen that 0.01 M HCl does not totally regenerate the surface presumably since the antibody binding strength is greater being a monoclonal antibody (Figure 5.26, 4). Upon restoring the system to PBS the baseline does not return to its original state before the injection of antibody. The mass of the protein on the surface after regeneration is 0.107 μg cm^{-2} hence the regeneration with 0.01 M HCl removed 42%. This shows that either longer regeneration regimes are required or different regeneration agents.

Most commonly HCl is used for the regeneration, however in a separate experiment a mixture of detergents was used for regeneration. The anti-aflatoxin M_1 antibody which survived the HCl wash was removed. The detergent mixture was; Tween 20, Tween 80, Triton X-100 and CHAPS although after exposure of the waveguide to the detergents, the anti-aflatoxin M_1 antibody / aflatoxin M_1 – BSA affinity was significantly reduced possibly due to denaturing the protein.

In order to produce affinity constants for this interaction, many different concentrations of the anti-aflatoxin M_1 antibody are required to be injected. The interaction of the anti-aflatoxin M_1 antibody and the BSA aflatoxin M_1 is so strong that it is difficult to regenerate the waveguide, so that the anti-aflatoxin M_1 antibody is removed fully from the immobilised BSA aflatoxin M_1 without denaturing the aflatoxin M_1 – BSA conjugate.

To assess the affinity of the peptides for the aflatoxin M_1 - BSA, a new waveguide with aflatoxin M_1 - BSA immobilised was prepared. Solutions of 100 μg ml^{-1} of each of the peptides were injected into the system and allowed to react for 1.5 hours. This procedure was repeated for a separate waveguide with just BSA immobilised to check for any non-specific binding of the peptide for BSA rather than aflatoxin M_1. The sensorgrams in Figures 5.26 and 5.27 below shows these interactions.

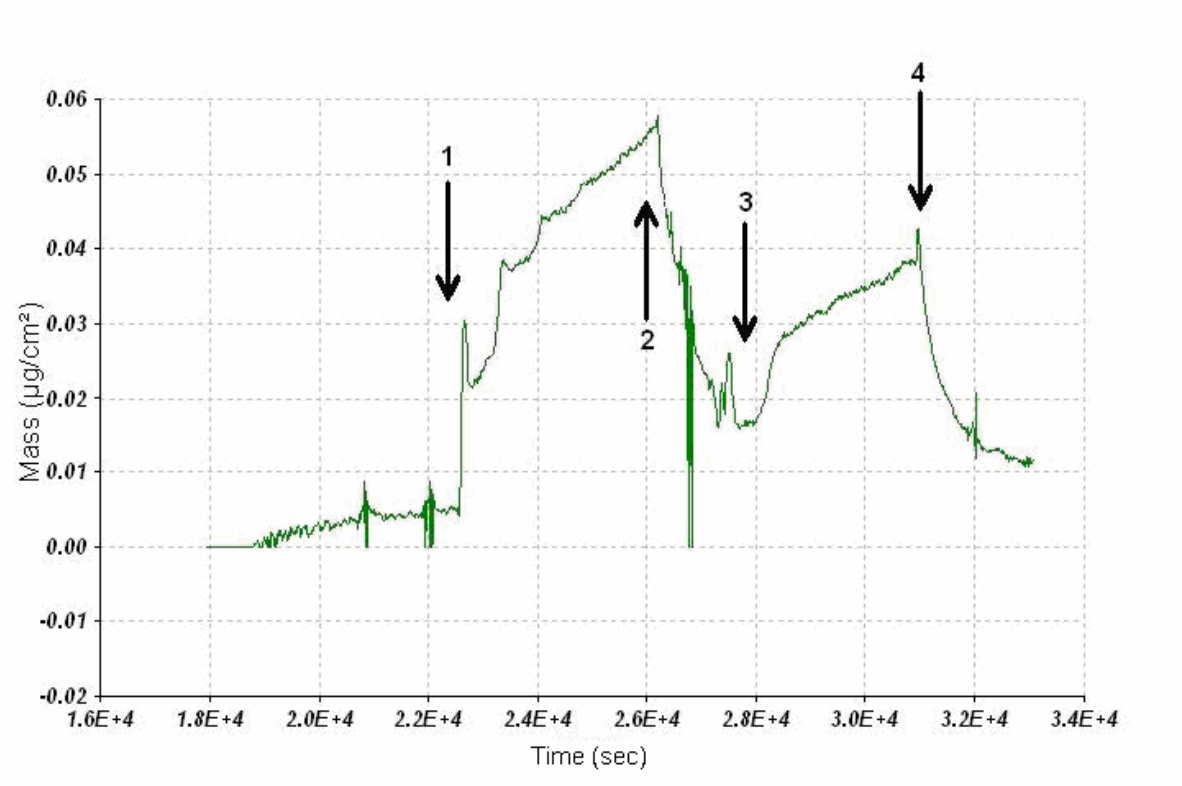


Figure 5.27: The printout showing the peptide's affinity for BSA alone to test for unspecific binding using optical waveguide lightmode spectroscopy.

In Figure 5.27 initially the system was equilibrated with PBS then (1) 100 μg ml^{-1} LLARGGC was injected onto the waveguide for 1. 5 hours. The eluent was changed to 10 mM PBS, pH 7.4 (2) then the signal decreases immediately. The signal is allowed to stabilize and then the second peptide is injected (3) 100 μg ml^{-1} CGGPVGPRP for 1.5 hours and again the eluent was changed to 10 mM PBS, pH 7.4 (4).

It can be seen from Figure 5.27 that upon the injection of LLARGGC the signal increases with time suggesting some electrostatic interaction. When changing the eluent to PBS then the signal decreases immediately thus the binding is reversible rather than irreversible. With the injection of CGGPVGPRP the same trend as the LLARGGC peptide is seen, again demonstrating little irreversible binding. Hence the two peptides have little affinity for the BSA.

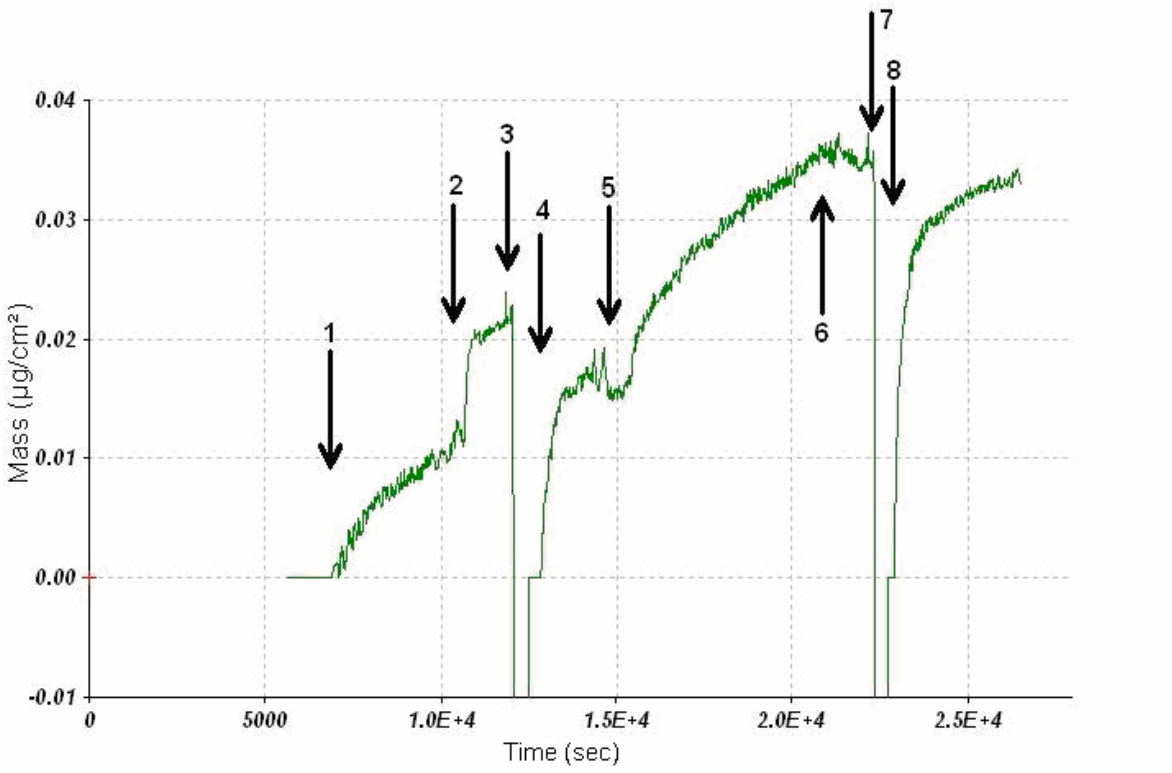


Figure 5.28: The printout of the peptide affinity for aflatoxin M_1 using optical waveguide lightmode spectroscopy.

In Figure 5.28 the system was first equilibrated with 10mM PBS, pH 7.4 and then 100 µg ml^{-1} CCGPVGPRP was injected for 1.5 hours (1). After the peptide injection the eluent was switched back to PBS to remove any unbound peptide (2). Following the PBS a small injection of 0.1 M HCl was injected to remove the bound peptide (3). After the HCl, 10mM PBS (pH 7.4) was injected to stabilize the system

(4). In the same procedure as CGGPVGPRP the LLARGGC peptide at a concentration of 100 μg ml^{-1} was injected (5) and then PBS was again applied (6).

The sensorgram in Figure 5.28 shows the reaction of the peptides to aflatoxin M_1-BSA. The mass on the surface for the PBS before the CGGPVGPRP peptide injection is considerably lower than the PBS buffer after the CGGPVGPRP injection thus suggesting that the peptide has irreversibly bound to the immobilised aflatoxin M_1. For the LLARGGC peptide, the signal steadily increases after the injection during the incubation. Upon changing the solution to PBS alone the signal stays at the same level. When comparing this to the BSA waveguide, the BSA waveguide shows an instant decrease in the signal thus demonstrating that both peptides have affinity for the aflatoxin M_1 and not BSA.

When the experiment was repeated, on a different day, the signal decreased with the peptide injection and continued to decrease during the run. This was assumed to be due to contamination of the waveguide. The waveguide had been regenerated using 0.1 M hydrochloric acid and with a mixture of detergents (CHAPS, Tween 20, Tween 80, Triton X – 100 at 0.3%) however the affinity of the peptide and antibody has decreased. This is possibly due to using too harsh regeneration conditions which has denatured the BSA – aflatoxin M_1 immobilised on the surface.

An additional problem was encountered during the OWLS work; the formation of bubbles in the measurement cell caused the termination of many runs. All solutions were degassed prior to use by degassing in a reduced atmosphere. This gave initial success however due to the experiments taking place during the summer in a room lacking temperature control, the fluctuations in temperature caused the solutions to become aerated. This effect can be reduced by storing all the solutions in a temperature controlled water bath. Unfortunately the experiments with the

OWLS instrument had to be aborted due to the unavailability of aflatoxin M_1 - BSA, as occurred with the ELISA development.

5.3.10 Affinity studies using the BIAcore™.

Using a BIAcore instrument for monitoring the peptide – aflatoxin M_1 interaction was a natural progression from the OWLS instrument. The BIAcore uses surface plasmon resonance (SPR) for monitoring receptor – ligand interaction. Reproducibility was the biggest problem with using the OWLS instrument and the BIAcore offers better sample handling through the use of micro fluidics and a temperature controlled environment. The BIAcore does have the disadvantage that it is not as sensitive as the OWLS instrument as the manufacture advises using compounds with molecular weights above 1000 Daltons. The peptide molecular weights are 689 (LLARGGC) and 849 (CGGPVGPRP) Daltons, which was the reason for utilising the OWLS first for affinity studies.

Initially the BIAcore was tested by monitoring the interaction of the polyclonal capture antibody used in the electrochemical sensor against the monoclonal anti-aflatoxin M_1 antibody to validate the instrument. Using the immobilisation wizard as part of the BIAcore program the optimal surface coverage of about 5000 RU as dictated by the software was easily reached (5049).

Using the kinetics wizard as part of the BIAcore control program the association constant (k_a) was calculated as $6x10^6$ M^{-1} and dissociation (k_d) at $1.7x10^{-7}$ M^{-1}. For an antibody a k_a of 10^9 would be expected however the capture antibody would be expected to lose some of its affinity during immobilisation. This experiment showed that the BIAcore is able to monitor receptor-ligand interactions. The sensorgram of the kinetics wizard is shown in Figure 5.29.

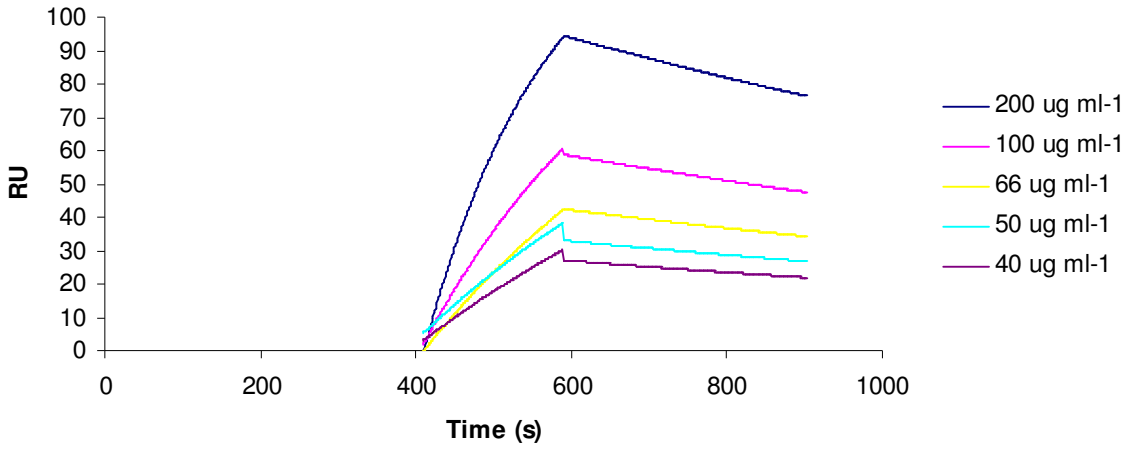


Figure 5.29: The sensorgram for the binding of immobilised capture antibody with the anti-aflatoxin M_1 antibody performed using the BIAcore kinetics wizard.

In Figure 5.29 immobilisation was achieved using the BIAcore immobilisation wizard with BIAcore 10mM acetate buffer, pH 4.5. The running buffer of BIAcore HBS-EP (0.01 M HEPES buffer (pH 7.4), 0.15 M NaCl, with 0.005% Surfactant P20) was used to dilute the anti-aflatoxin M_1 antibody. Regeneration was performed automatically using a 10 mM glycine-HCl pH 2.0 buffer. Concentrations of anti-aflatoxin M_1 antibody stated as µg ml^{-1}. Plots taken from the average of triplicate measurements.

When plotting the maximum signal for the association curve against the concentration then a linear trendline is observed as shown in Figure 5.30 demonstrating the reproducibility of the affinity.

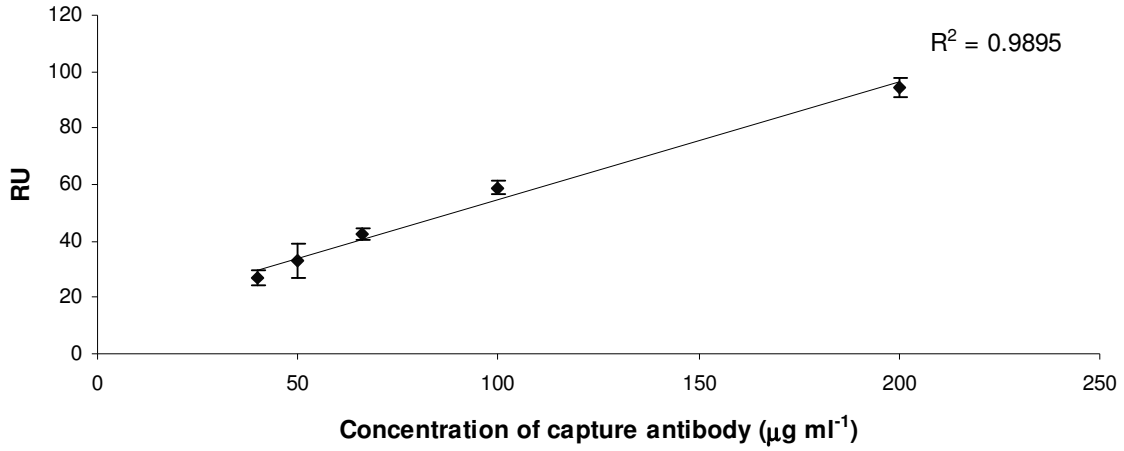


Figure 5.30: The reproducibility of the BIAcore kinetics wizard program and the capture antibody to anti-aflatoxin M_1 antibody binding. Data taken from the sensorgram at 589 seconds. Error bars denote the standard deviation of triplicate measurements.

Upon immobilising the anti-aflatoxin M_1 antibody onto the surface, again the optimal level was again reached (4813). Using the BIAcore kinetics program the interaction of aflatoxin M_1 – HRP with the anti-aflatoxin M_1 antibody was reviewed before implementing the peptide.

A problem arose with the filtering of the aflatoxin M_1 – HRP conjugate. All solutions for the BIAcore require filtration due to the narrow bore of the micro fluidic tubing; however the conjugate did not pass through the filter. Initially nylon and PVDF filters were used which are hydrophilic, meaning that they are suitable for the filtration of aqueous samples. When using the hydrophilic filters the filter quickly became blocked even though the pure conjugate solution was being used. When using PTFE filters, which are hydrophobic, again the filters became blocked.

The cause of the poor filtration is due to the aflatoxin M_1 – HRP being supplied in a 75% solution of ammonium sulphate. The high ammonium sulphate solution is required for stabilising the conjugate and results in a viscous solution that cannot be filtered (Jordan, 2007). Any solution that would pass through the filter would have a lower enzyme activity for aflatoxin M_1-HRP since filtration destabilises the

conjugate, therefore filtration is strongly not advised by the manufactures (Donnelly, 2007).

Without a source of aflatoxin M_1 – BSA, use of the BIAcore is difficult since the aflatoxin M_1 molecule alone is too small for detection. An additional problem occurred when trying to immobilize the peptides to the CM5 dextran chip. Using the same protocol as proven for the antibodies, the immobilization wizard failed to complete since the peptide did not behave as the program expected. After exhaustive attempts at trying to achieve the optimal level the program aborted.

The wizard's procedure was re-enacted using manual commands and it can be seen from Figure 5.31 that saturation was observed after the 6th injection and the change in signal was only 80 RU. This is considerably less than the expected 3000 RU stipulated by the wizard and demonstrates the difficulties in using low molecular weight compounds with the BIAcore.

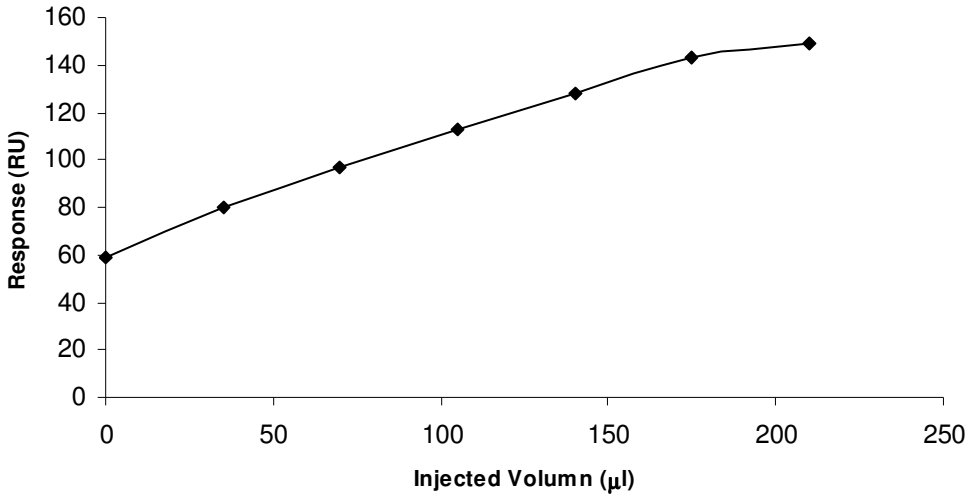


Figure 5.31: Manual immobilisation of CGGPVGPRP using the BIAcore. Injection volume was constant at 25 µl of peptide per injection.

5.3.11 Chemical methods for investigating the affinity of the peptides.

With the OWLS showing initial evidence that the peptides have affinity and the BIAcore not being applicable, chemical techniques were employed. Other authors investigating peptide / ligand interaction had used tritium labelled ligands and recorded peptide / ligand binding using a scintillation counter; Tozzi *et al*., (2002) and Giraudi *et al*., (1999a). Since this equipment was unfeasible the peptides were immobilised onto the surface of microwell plates and the interaction between the peptide and the aflatoxin M_1 – HRP conjugate was monitored through colorimetric reaction of TMB with HRP.

For the experiments involving the microwells a linker compound BS^3 was employed rather than glutaraldehyde, since it was believed, that a short spacer arm could cause steric hindrance between the surface of the plate and the HRP conjugate enzyme.
The immobilisation using BS^3 was performed as detailed with the manufactures instructions.

Initially two experiments were performed, one where the linker compound was first attached to the activated surface, washed and then the peptide attached (stepwise immobilisation); and a second experiment where the peptide and the linker compound was added to the activated surface together (joint immobilisation). Figure 5.32 shows a schematic diagram of the attachment.

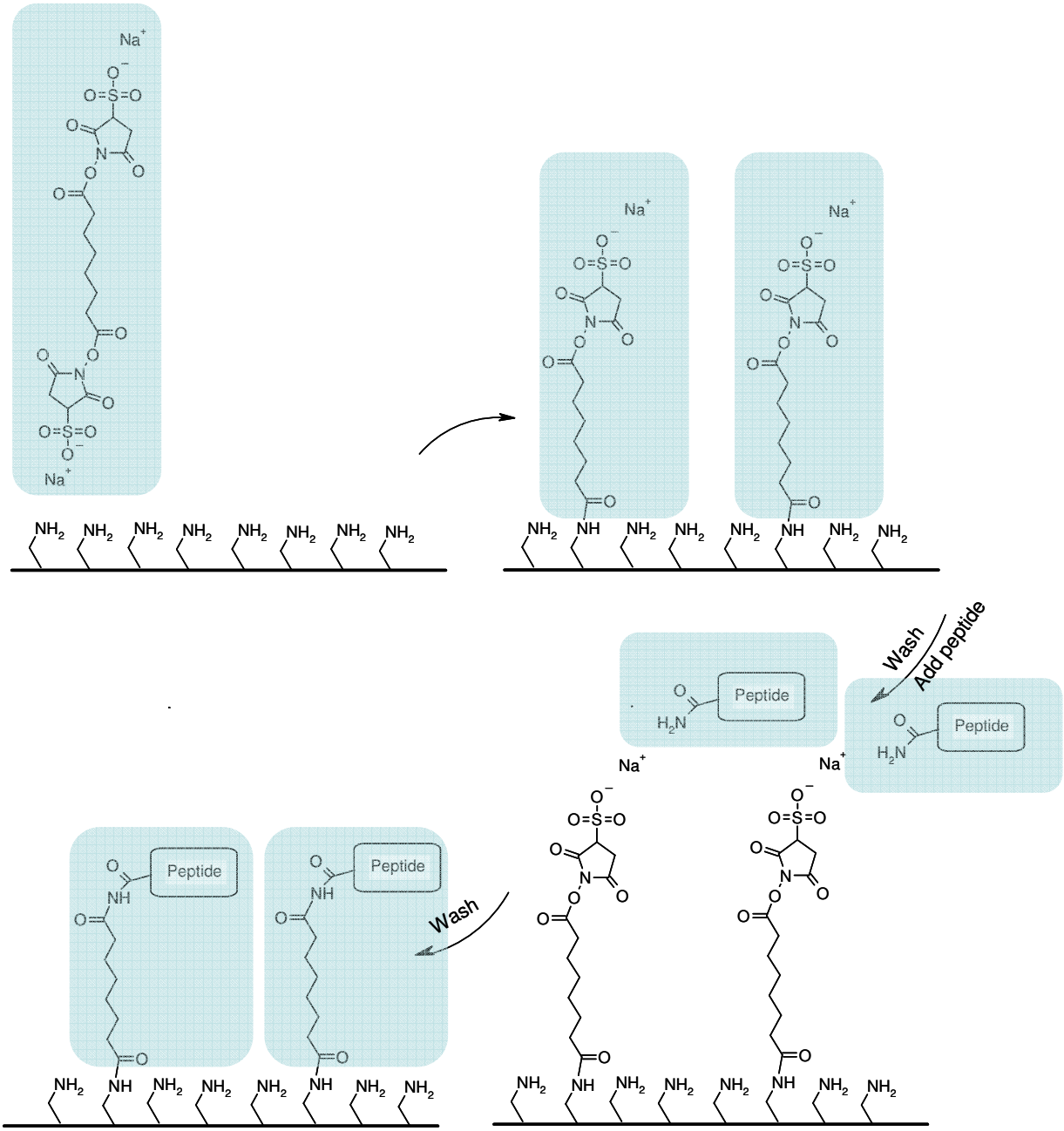


Figure 5.32: A schematic diagram of the peptide immobilisation on to the surface of a microwell plate. Clockwise: the BS^3 linker compound and the activated plate are added together, the BS^3 bind to the surface by breaking the O – N bond for a stronger C – O bond. After washing the peptide is added and the same chemical process occurs leaving the peptide covalently attached.

The immobilisation efficiency is clearly shown in Figure 5.33 and 5.34. For both the peptides greater range and the highest signal were observed when the peptide and BS^3 were added together into the activated microwell plate. This is surprising since it would be expected that by using a one step (joint) method, then an array of compounds and agglomerations would be formed as observed by Sinz, (2003) and Hermanson, (1996).

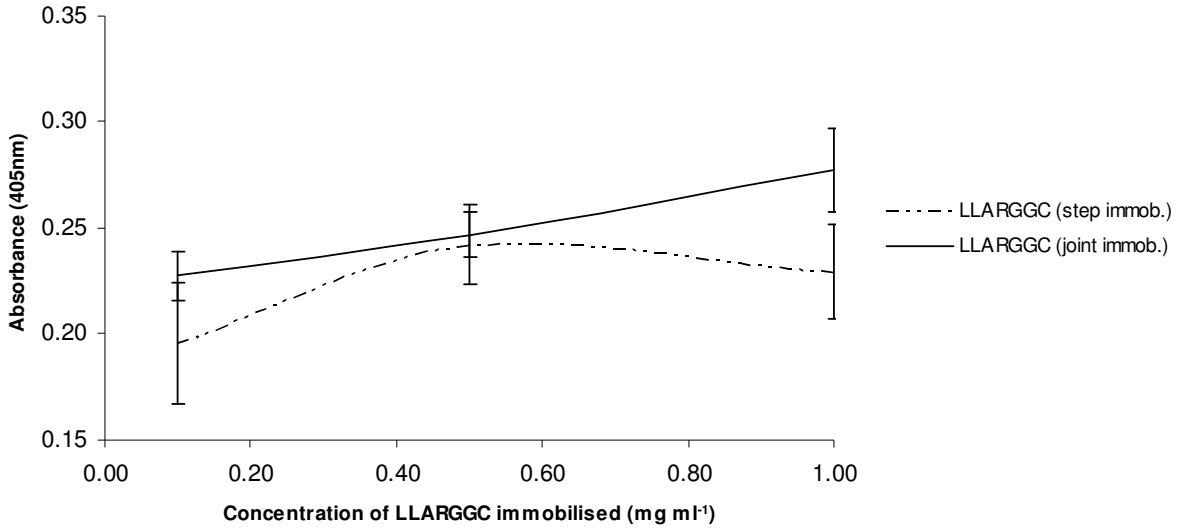


Figure 5.33: The immobilisation of LLARGGC onto the surface of a microwell plate using different protocols. Error bars denote standard deviation where n=3, blank reading = 0.20 ± 0.017).

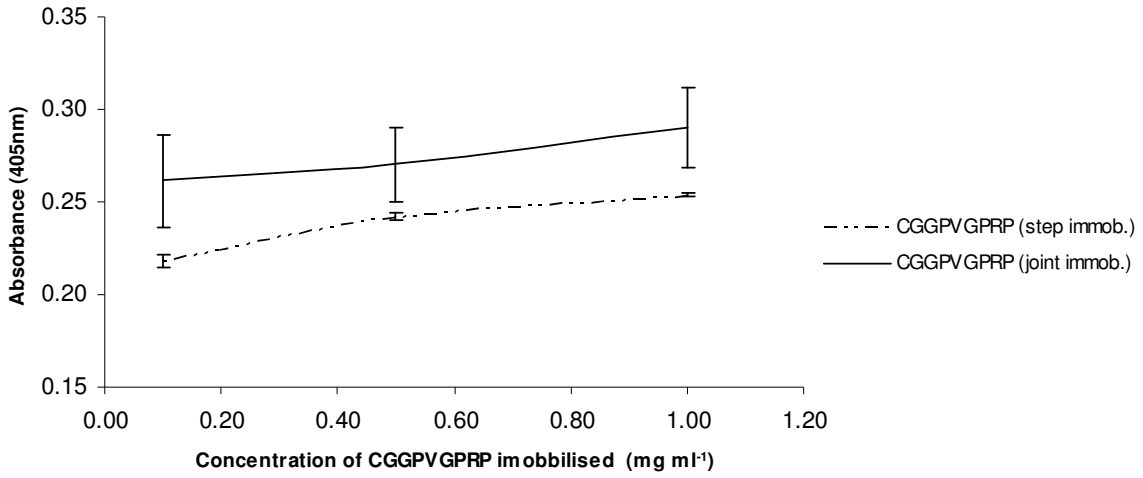


Figure 5.34: The immobilisation of CGGPVGPRP onto the surface of a microwell plate using different protocols. (Error bars denote standard deviation where n=3, blank reading = 0.20 ± 0.017).

Although the graphs only contain 3 data points, the r^2 value for LLARGGC and CGGPVGPRP are 0.9961 and 0.9513 respectively. The gradient for the LLARGGC and CGGPVGPRP plots were 0.0554 and 0.0332 respectively. These results suggest that LLARGGC has better specificity (due to a higher r^2 value) and affinity for aflatoxin M_1 - HRP since the gradient is larger for LLARGGC than CGGPVGPRP. Therefore the peptide reported in literature which had been developed using a combinatorial library out performed the *de novo* designed peptide. It should be noted however that both peptides positively recognised the aflatoxin M_1 – HRP conjugate, confirming the evidence from the OWLS machine that both peptides have affinity for aflatoxin M_1. Additionally the data shows that BS^3 is suitable as a linker compound for immobilising peptides to the surface of microwells without the loss of activity.

5.4 Conclusion.

It was shown in Chapter 4 that the microelectrode arrays improved the detection of the immunosensor from the screen printed electrodes. The aim of the project was to design and produce a robust sensor, therefore the use of chemically synthesised peptides was investigated to replace the antibody in the sensing layer and improve storage stability.

Computational chemistry has previously been carried out to design new peptide receptors for HIV drugs; therefore a similar approach was taken to produce a receptor against aflatoxin M_1. The structure of aflatoxin M_1 was successfully re-created within the SYBYL program suite and Leapfrog was employed to develop receptors against aflatoxin M_1. Previous authors have reported using *de novo* design of peptides with a pre-known starting sequence (Mascini *et* al., 2004; Schmuck and Hell, 2003b) whereas this project started *ab initio*.

A receptor library of 30 candidates was built, all with some predicted affinity. The candidates were reduced to three using a more detailed docking program called Flexidock. From the group of three one was reviewed in detail with respect to solubility and immobilisation using molecular dynamics. A frequent criticism of computational docking programs is false positive results due to bias in the calculation. It was hoped that by using three completely different algorithms then bias should be removed. From the computational work, one peptide CGGPVGPRP was chosen and laboratory tests were performed to determine the binding affinity. For comparison an additional peptide, LLARGGC, was taken from literature for having affinity with aflatoxin B_1 and investigated simultaneously. In the computational tests it was predicted that CGGPVGPRP would have better binding than LLARGGC. Additional investigations using computational methods were carried out by analysing enzyme structures and utilising different computational programs, however neither approach gave fruitful information.

Several laboratory methods were carried out. Initially optical waveguide lightmode spectroscopy was investigated, and proven effective at monitoring the interaction of alfatoxin M_1 – BSA with anti-aflatoxin M_1 antibody used in the ELISA test. Upon reviewing the peptides it was shown that both peptides have affinity towards aflatoxin M_1 however, problems with waveguide regeneration, run reproducibility, fluid handling and environmental factors caused difficulties when attempting to replicate results which were required to calculate binding affinities.

BIAcore was also investigated for determining the binding affinities. The detection of compounds using the BIAcore with a mass less than 1000 Daltons is not recommended. This investigation has validated the recommendation and has shown that the OWLS was indeed more appropriate for the analysis of small molecules than the BIAcore. The microfluidic system on the BIAcore makes operation much easier and reproducible but comes at a price since the instrument is not as robust as the OWLS instrument. The OWLS instrument does not require the filtration of the consumables since the internal diameter of the fluid handling tubing was 0.5 mm. Furthermore the OWLS can withstand the use of solvents, which could aid the dissolution of the aflatoxin M_1 – HRP conjugate which is not possible with the BIAcore. Further work would needs to be carried out on dissolution and filtration techniques for the aflatoxin M_1 – HRP conjugate or use of an aflatoxin M_1 – BSA conjugate which would allow the monitoring of a large detectable molecule by the BIAcore.

By covalently immobilising the peptides onto the surface of microwell plates and detection using a labelled HRP conjugate, the affinity was again demonstrated. It was shown that the amount of labelled HRP conjugate bound to the plates was proportional to the concentration of the immobilised peptide where the linear regression (r^2) was ≥ 0.95. These tests show conclusively that both peptides have affinity for aflatoxin M_1, however also demonstrating the difficulties in measuring

ligand / receptor affinities using small molecules which as also been observed by Giraudi *et* al., (1999b) and Falter *et al*., (1994).

The peptide sequence reported in literature (Tozzi *et al*., 2003b) was shown using practical tests to have greater affinity to aflatoxin M_1 than the computationally designed peptide. This observation is the inverse of the computer molecular dynamics predictions. Many authors have documented the inaccuracy in docking algorithms (Alberts *et al*., 2005; Abagyan and Totrov; 2001; Wang and Wang, 2001; Baxter, 2000; Jackson, 1995). Commonly docking programs assume that the protein receptor is rigid and the amino acid side chains are fixed in position, upon comparison to the receptors in this work the receptor is a highly flexible peptide where the side chains placement will have significant impact on the binding ability. This assumption error or the intelligent approach of *de novo* design, rather than a systematic search, was the fault for the literature method by Tozzi *et al*., (2003b) outperforming in the laboratory the *de novo* designed peptide.

Further work should be performed to investigate the affinities of the peptides sequences with an aim to accurately quantify the affinity of each peptide to the aflatoxin M_1.

CHAPTER 6

FINAL DISCUSSIONS AND CONCLUSIONS

6.1 Immunosensor and ELISA development.

The ELISA method has been developed and optimised using an alkaline phosphatase labelled antibody and results show that the procedure is suitable for detecting aflatoxin M_1. Initially the work by Ammidia *et al*, (2004) and recommendations by Crowther (2001) were used as a foundation for the development.

During the optimisation of the ELISA system several blocking agents were tested with particular focus on polymers due to their increased stability over protienaceous compounds. Both Micheli *et al.* (2005) and Ammida *et al*, (2004) used polyvinyl alcohol as the blocking agent however work carried out by Studentsov *et al.*, (2002) reported that using polyvinyl pyrrolidone rather than polyvinyl alcohol was found to be preferential. In this project it was confirmed that polyvinyl pyrrolidone outperformed polyvinyl alcohol as a blocking agent.

Incubation times were optimised for the test and overall the time of analysis was 3.5 hours which is comparable to the commercial tests available at 3 hours. After optimisation and validation of the ELISA test, milk was applied and the resolution of the test was reduced. Traditional clean-up methodologies were investigated to remove the matrix interference of the milk. These methods were; Carrez clarification to remove high molecular weight compounds such as proteins and fats (Gökmen and Şenyuva, 2006; Rufián-Henares and Morales, 2006), deproteinate using lead acetate (Goldblatt, 1969), the removal the fats in the milk using solvents (Thomas *et al.*, 1998; Delgado Zamarreño *et al.*, 1992) as well as cold centrifugation as recommended by the commercial kits. None of the sample pre-treatment methods removed the matrix effects observed and did not improve sensitivity of the test.

From literature reports of Magliulo *et al.* (2005) and El-Nezami *et al.*, (1995) it was suggested that extensive pre-treatment is required for the detection of aflatoxin M_1

in milk using ELISA, whereas Micheli *et al.*, (2005) reports that milk that has only been mildly centrifuged does not cause any interference and Thirumala-Devi *et al.*, (2002) reports that milk only requires mild centrifugation followed by filtration through standard filter paper is required. Hence there is a 50:50 divide to whether milk caused interferences to ELISA tests or no interferences. In this project extensive interference was observed.

Before further work could be performed, supplies of the immobilised antigen (aflatoxin M_1 – BSA) were depleted and insufficient equipment and skills were available for production of the immobilised antigen in house due to a complex procedure of synthesising an aflatoxin M_1 intermediate.
Critically, the development of the ELISA demonstrated that the commercially sourced antibody was suitable for using as a sensing receptor for the electrochemical immunosensor.

6.2 Screen printed electrode immunosensor development.

Firstly screen printed sensors were produced in-house and characterised with cyclic voltammetry by scanning potassium hexacyanoferrate (III) at different scan rates.

In a change from the ELISA protocol the antibody was immobilised onto the solid substrate via a capture antibody and a competition reaction between aflatoxin M_1 and aflatoxin M_1 – HRP was performed. An initial calibration graph was produced however the graph didn't meet the requirements for the EU maximum permissible limits of 50 ng L^{-1}. A literature review was performed on screen printed immunosensors with specific attention to HRP based systems. The main discrepancy between the work from Micheli *et al.*, (2005) with Butler *et al.*, (2006), Fanjul-Bolado *et al.*, (2005) Badea *et al.*, (2004) and Volpe *et al.*, (1998) was the measuring potential. Micheli *et al.*, (2005) reported using -100 mV for TMB

detection and other authors reported +100 mV. Upon comparing the two potentials, +100 mV was discovered to give the greatest signal.

Additional optimisation investigations were performed such as electrochemical pre-cleaning of the electrodes before antibody immobilisation, as described by Grennan *et al.*, (2000); Espinosa *et al.*, (1999) and Wang *et al.*, (1996), or using proteinaceous blocking agents. These methods did not improve the sensitivity of the test, however, pre-conditioning the electrode before detection of TMB as described by Conneely *et al.*, (2007) and Lu *et al.*, (2006) did significantly improve the performance of the sensor. When milk was applied to the test, all sensitivity was lost, therefore the causes of the matrix interference was investigated.

Milk is a highly multi-component matrix and many authors such as Pemberton *et al.*, (1999) and Mayer *et al.*, (1996) have reported that it causes electrochemical interference, however none have identified the cause. For the investigation into the electrochemical interference one component at a time was investigated. Firstly the effect of lipids was investigated. Milk was defatted enzymatically by raising the pH of the milk and allowing the natural lipases to digest the lipids to volatile fatty acids (Hui, 1993) additionally a second sample was tested, non fat milk. The samples were mixed with potassium hexacyanoferrate (III) in 0.1 M KCl and using cyclic voltammetry, voltammograms were taken. In neither case did the quenching effect from the milk subside thus demonstrating that lipids are not the cause of the interference.

Milk is high in lactose (4.6%) which is electrochemically active (Hanko and Rohrer, 2000, Mayer *et al.*, 1996) so the presence of lactose was investigated by spiking potassium hexacyanoferrate (III) in 0.1 M KCl, with 4.6% lactose, and again taking cyclic voltammograms. No quenching effect was observed with the lactose, demonstrating that lactose is not the interfering substance.

Finally milk was fractionated into a casein free sample by the addition of HCl until a pH of 4.6 was obtained (Hui, 1993; Walstra, 1984), and a fraction free from both casein and whey using HCl and trichloroacetic acid using the methods described by Vernozy-Rozand *et al.*, (2004), then both fractions were spiked again with potassium hexacyanoferrate (III). Although the casein free fraction showed quenching effects, the whey and casein free fraction showed no signs of quenching, suggesting that whey proteins were the cause of interference. In a conformational test, ammonium sulphate was added to a milk sample to precipitate the proteins and the solution was tested with potassium hexacyanoferrate (III) and again no quenching was observed.

When reviewing literature reports on the absorption of whey proteins onto the surfaces of stainless steel pasteurisation elements, α-lactalbumin has high affinity towards metal surfaces and causes 'milk stone' during milk processing (Cosman *et al.*, 2005). During adsorption the protein goes through dramatic conformational change resulting in the loss of centrally bound calcium ions. An experiment was performed in spiking the milk sample with an excess of calcium chloride and repeating the calibration where the resulting calibration did not incur interferences from the milk matrix and detection of aflatoxin M_1 was achieved. With reviewing observations of other authors with the interfering effects of milk, they correlate that α-lactalbumin is the cause of interference. The molecular weight of α-lactalbumin at 14,176 Daltons correlates with the reports of Mayer *et al.*, (1996) that the electrode fouling was eradicated by the use of dialysis membranes at 12,000 – 19,000 daltons. Cosman *et al.*, (2005) reinforces this observation. In a method utilising TLC as the detection method, Diaz *et al.*, (1993) suggested the use of dialysis membranes for the clean-up of milk with membranes at 8,000 to 15,000 Daltons. Upon the addition of $CaCl_2$ to milk as a pre-treatment step the quenching effects of milk were lost, and a successful calibration was recorded with an analytical sensitivity of 39 ng L^{-1}.

The calibration graphs using the screen printed sensors had larger error bars than other literature reports (Micheli *et al.*, 2005). Investigations were carried out to elucidate the causes of the poor reproducibility. Using new electrodes in the assay had increased the reproducibility from a relative standard deviation of 9.5% to 7.8%; however this is still significant. In a second investigation the reproducibility of the screen printer was accessed. It was observed that from a sheet of electrodes, only 60% had unilateral resistance. The causes for the poor reproducibility were attributed to the age of the screen printer and the drying process.

The efficiency of the calcium chloride pre-treatment developed in the project was evaluated by using the Ridascreen ELISA kit. ELISA kit standards and standards prepared using the $CaCl_2$ pre-treatment was tested side by side and it was shown that the $CaCl_2$ pre-treatment results showed some underestimation. Mendonça and Venâncio (2005) and Dosako *et al.* (1980) suggest that aflatoxin M_1 has affinity for casein proteins and whey proteins. The binding of aflatoxin M_1 with casein is due to the hydrophobic pockets formed by the high number of proline residues in casein (Bakirci, 2001; Henry, *et al.*, 1997). It could be postulated that by increasing the ionic strength of the milk by adding calcium chloride then the aflatoxin M_1 has increased affinity for the casein through hydrophobic interaction and thus remains partially bound during analysis.
The underestimation requires that all samples and calibration standards need to be treated the same for this bias to be uniform and thus accountable. More critical is the fact that the standard deviations for the laboratory prepared samples using calcium chloride were higher than those obtained using the kits and therefore more work is required to improve the repeatability of the extraction.

Using the calcium chloride pre-treated samples the developed immunosensor was compared to a developed HPLC method and the commercial ELISA method. The HPLC had superior limits of detection compared to the ELISA and the immunosensor which had similar limits of detection. The working range for the

ELISA was worst with the immunosensor and HPLC having similar working ranges. Although the HPLC showed better reproducibility at low levels of aflatoxin M_1 at higher levels the immunosensor had marginally better reproducibility. With consideration of cost of analysis the immunosensor is considerable cheaper than ELISA and HPLC and furthermore the immunosensor is portable and can be operated in the field. Therefore out of the technologies tested the immunosensor is best suited for fulfilling criteria of a simple, robust, low-cost analysis methods for aflatoxin M_1 analysis in milk.

In a final investigation with the screen printed electrode it was demonstrated that the developed immunosensor could be employed for the detection of aflatoxin M_1 in urine, which is very encouraging since no previously reports about biosensors for aflatoxin M_1 in urine has been published.

6.3 Microelectrode array development.

The current maximum permissible limits of detection set by the EU for aflatoxin M_1 is 50 ng L^{-1} however this level was set by the ALARA process, (As Low As Reasonable Achievable) and therefore with the increasing use of HPLC MS/MS it is reasonable to suspect that the current levels maybe decreased in future time causing further analytical challenges for other methodologies. The results obtained from this project shows that should this occur, then screen printed technology may suffer. However, the requirements of a simple, robust, low-cost analysis method for aflatoxin M_1 analysis in milk can be answered using microelectrodes.

Covalently immobilising the antibody onto the gold surface of the microelectrode array with PDITC, as described by Lillis *et al.*, (2006), was a new method of immobilisation compared to the developed screen printed electrode immunosensor which relied on passive absorption onto carbon. To ensure correct covalent attachment occurred, atomic force microscopy and scanning electron microscopy

was employed. Little reported evidence of microscopy being employed as a tool for immunosensor construction could be discovered.

Scanning electron microscopy uses a beam of electrons as the detection source, which penetrates the surface by 10 µm, bypassing the proteinaceous surface of the immunosensor and therefore not suitable for monitoring the covalent attachment of the antibodies. Atomic force microscopy does not have this limitation and gave detailed images into the immunosensor chemical construction. Quantifiable differences between the immunosensor and a blank microelectrode array were roughness and topographic measurements.

It was observed that with the immobilisation of the antibody onto the surface, the surface roughness increased from 1.27 nm to 2.37 nm. In three separate investigations into the addition of enzymes onto surfaces Tsai *et al*. (2007), Vianello *et al*., (2007) and Parra *et al*., (2007) all reported that upon the addition of protein to a sensor surface the roughness increased following the same trend as this investigation. Although for enzymes, the increases of 1.4 nm by Vianello *et* al., (2007) and 1.5 nm by Parra *et al*., (2007) which is comparable to the observations in this investigation, for an antibody, of 0.84 nm

The topographic measurements showed that with the addition of the capture antibody caused an increase of 2.2 nm which is very similar to the observations of Ouerghi *et al*., (2002) and Bergkvist *et al*., (1998) who reported changes of 2.5 nm and 1.98 nm respectively. With the theoretical height of an antibody at 4 nm, this data showed that many of the antibodies are immobilised in a ‘side on’ orientation and therefore inefficient. This proves the logic of immobilising a cheap polyclonal antibody rather than immobilising an expensive monoclonal antibody.

Initial optimisation was carried out to determine the optimum potential for TMB detection on the microelectrode array since the microelectrode array consists of a

gold working, auxiliary and reference electrode whereas for the screen printed electrode the working and auxiliary is carbon based and the reference electrode is Ag/AgCl.

Transferring the receptor chemistry from the screen printed electrode to the microelectrode array was a success with detection significantly superior to the screen printed electrode. In addition the necessary PVA blocking for the screen printed electrodes was not required for the microelectrode array and this was attributed to the comparably low active surface area of the gold electrode to the carbon. The sensitivity improved from 39 ng L^{-1} for the screen printed electrodes to 8 ng L^{-1} for the microelectrodes, both in the milk matrix. This improvement is greater than first sight considering that the sample volume for the microelectrode is only 16% of that for the screen printed electrode.

The superior detection is due to the microelectrode dynamics over planar screen printed electrodes but also the use of gold rather than graphite for the construction. Due to a limited supply of microelectrode arrays from Tyndall University (Ireland) full optimisation could not be achieved. It could be postulated that since diffusion alone is the source of mobility of the analyte in the screen printed electrodes, ELISA and microelectrode arrays, a reduction of the test volume will yield shorter incubation times and faster assay times. The incubation times for the assay at 2 hours was taken from the optimisation of the ELISA assay where the test volume was 50 times greater.

The screen printed electrode is the cheapest technology reviewed in this conclusion due to the low cost of electrode production. Compared to the cost of manufacturing for screen printed electrodes, microelectrode arrays are significantly more expensive with the microelectrode array estimated at 5 euros each by Tyndall institute.

In conclusion, employing gold microelectrodes rather than carbon based screen printed electrodes, allows new applications of high sensitivity and low detection limits to be analysed without requiring elaborate instrumentation. If future detection limits for aflatoxin M_1 are reduced then microelectrode arrays can be successfully utilised outside of the laboratory.

6.4 The development of synthetic receptors.

With the aim of producing a robust sensor, the use of a synthetic receptor rather than a natural antibody was investigated for the sensing layer. Antibodies have the disadvantages of being proteinaceous and therefore fragile due to denaturation, long production times, variations between each batch and high cost of production (Nakamura *et* al., 2005; Tothill, *et al.*, 2003; Tothill *et al.*, 2001).

Previous authors have proven that synthetic peptides have affinity for small molecules such as estradiol (Giraudi *et al.*, 2003; Giraudi *et al.*, 1999a), estrogen (Tozzi *et al.*, 2002) and aflatoxins (Tozzi *et al.*, 2003b). Using this premises synthetic receptors were selected for the replacement of the antibodies in the sensing layer.

The development of synthetic peptides as receptors in medicine has gained significant momentum due to computational drug design. Using the same approach, synthetic peptides sequences with affinity for aflatoxin M_1 were produced *in silico* by *de novo* design programs.

The structure of aflatoxin M_1 was determined using computational methods described by Chianella *et al.*, (2002) and the results concurred with the published results from Holtzapple *et al.* (1996). A list of amino acids with affinity to aflatoxin M_1 was calculated and these followed a trend with hydrophobicity. Isoleucine was found to have the greatest affinity.

Leapfrog was employed to develop peptides which would have favourable binding with aflatoxin M_1. After optimisation of the parameters, a virtual combinatorial library of 30 peptide sequences was produced by Leapfrog. The results from Leapfrog were scrutinised using Flexidock and the best peptide had a sequence of Pro Val Gly Pro Arg Pro.

A spacer/linker arm of two glycines and a cysteine was attached to the peptide. The addition of the cysteine was chosen to aid immobilisation of the peptide to the gold microelectrode surface or BIAcore surface as described by Katayama *et al*., (2000). Furthermore the addition of glycine was required to move the active peptide region into the solution and reduce steric hindrance and improved binding capabilities (Tozzi *et al*., 2003b). Detailed molecular dynamics was used to determine the most idealistic attachment of a spacer/linker arm without any loss of affinity of the peptide. As a reference, a sequence from literature with good affinity to aflatoxin B_1 was also processed by molecular dynamics for the idealistic of the placement of a linker arm with view to compare the *de novo* designed peptide to one determined combinatorially. The data from the molecular dynamics suggested that the binding of the *de novo* designed peptide sequence would be more energetically favourable than the combinatorially design sequence.

Two other computational investigations were performed to source information. A search was executed for known receptors for aflatoxins. The search resulted in only one structure, an enzyme, aflatoxin aldehyde reductase (Kozma *et al*., 2002). Upon study of this structure using computational methods, the only fragment to show binding with aflatoxin M_1 was Leu Val, hence no new information was determined. In another investigation a software package called GROMACS was evaluated for determining ΔG using the thermodynamic cycle for the binding interactions between the peptides and aflatoxin M_1, however due to the software being developed for proteins it was deemed unsuitable for experiments with aflatoxin M.

With the computational methods delivering a peptide sequence and modifying a literature sequence, the sequences were tested by a host of laboratory methods.

Firstly optical waveguide lightmode spectroscopy (OWLS) was utilised. With sensitivity expected to be greater than the BIAcore, the affinity of the peptide sequences were investigated. Initially the instrument was validated with the testing of the anti-aflatoxin M_1 antibody against aflatoxin M_1 – BSA. The OWLS instrument was able to monitor the binding interaction of the antibody, with 5% of the antibody molecules binding to the immobilised aflatoxin M_1. Upon regeneration of the sensor to remove the bound anti-aflatoxin M_1 antibody using the conditions stated by Székács *et al*., (2003), only 42% of the antibody was removed. This suggested that harsher regeneration conditions was required and that the polyclonal antibody used by Székács *et al*., (2003) had poorer binding strength than the monoclonal antibody provided by Abcam (Cambridge, UK). When harsher regeneration conditions were performed, then a decrease of the affinity of the complex was observed, due to denaturation of the BSA protein and possible formation of aflatoxin M_{2a} from aflatoxin M_1.

Without a reproducible sensor surface, kinetic data could not be determined. On new waveguides aflatoxin M_1 – BSA and BSA alone was immobilised. The peptide sequences were allowed to bind to both surfaces and the resulting sensorgrams were recorded. Both peptides showed reversible binding for the BSA alone sensor surface, but irreversible binding for the aflatoxin M_1 – BSA sensor demonstrating that the peptides has affinity for aflatoxin M_1. Due to environmental conditions and a limited supply of aflatoxin M_1 – BSA further investigations could not be performed.

To improve reproducibility between runs, BIAcore technology was investigated following the OWLS investigation. The BIAcore machine does not recommend use of receptors below 1000 Daltons since it is below the level of sensitivity and when

using the peptide sequences, both with a weight below 1000 Daltons, the instrument failed to automatically recognise the binding of the peptide to the surface of the BIAcore sensor. Furthermore BIAcore has the advantage of high reproducibility with handling low quantities of liquids. This is performed using micro-fluidics. A requirement of the micro-fluidics is for all solutions to be filtered prior to analysis, and this was not possible for the aflatoxin M_1 – HRP conjugate, which became trapped when filtered. Therefore BIAcore was not suitable for this investigation.

Initial investigations using chemical techniques, akin to ELISA, showed that when immobilising the peptide sequences to the surface of a microwell plate via BS^3 coupling, then with the addition of aflatoxin M_1 – HRP, the bound activity of HRP is proportional to the peptide concentration on the surface. The repeatability using linear regression was 0.99 and 0.95 for LLARGGC and CGGPVGPRP respectively.

Both the OWLS investigations and the chemical investigations showed that the peptides have affinity for aflatoxin M_1. Further work must be performed to quantify the binding association. Previously reported investigations in the binding affinity of small molecules have been performed using scintillation which was outside the scope of this project. There are many other possible techniques for monitoring the binding of small molecules which could be carried out as described in Section 7.2.

6.5 Future developments.

In summary, the project was to answer the criteria of Proctor, (1994) '*There is an urgent need for simple, robust, low-cost analysis methods, for the major mycotoxins, which can be routinely used in developing country laboratories.*'

Through screen printed electrodes, this criterion is answered for aflatoxin M_1 to European Union maximium permissible limits of 50 ng L^{-1}, and using

microelectrode arrays the detection limit can be lowered for future requirements. The microelectrode array, being made up of many microelectrodes, has potential for future development where each electrode is a sensor for a different mycotoxin and thus producing a multi-analyte affinity sensor. At present the microelectrode is produced so that the electrodes are wired in parallel, not individually, and therefore are not suitable for this application. Tyndall national institute are continuing the development in microelectrodes and nanoelectodes to meet this requirement.

Work to replace the antibody with a synthetic peptide receptor demonstrated that it is possible to design a *de novo* sequence which as binding comparable to literature reports. Further work is required to quantify this binding and incorporate the peptide into the immunosensor.

CHAPTER 7

FURTHER WORK

7.1 Further work for the sensor.

7.1.1 Stabilisation of reagents.

The sensor has been successfully designed to be cheap, rapid and easy to use however geographically aflatoxin M_1 contamination tends to occur in poorer countries with hot climates. To make the sensor robust, synthetic receptors have been investigated however other reagents required for the immunosensor also need to be robust.

Currently the detection for the immunosensor requires the use of hydrogen peroxide and TMB, both of which require storage at 4^{o}C. Work reported by Frey *et al*. (2000) has shown that TMB can be stabilized with the substitution of water as the solvent for N,N-dimethylacetamide and with the addition of tetrabutylammonium borohydride. Initial work carried out as part of this project has shown that this system can be incorporated into the protocol for aflatoxin M_1 determination and stable at elevated temperatures in the presence of light. Further work is required to optimise the N,N-dimethylacetamide / tetrabutylammonium borohydride for this assay.

7.1.2 Optimisation and validation of the aflatoxin M_1 immunosensor for urine.

As shown in Section 3.3.8, the analysis of urine rather than milk was briefly investigated. It was clearly shown that the immunosensor is applicable for the detection of aflatoxin M_1 in urine with an analytical sensitivity of 25 ng L^{-1}. The levels of aflatoxin M_1 reported with means in the region of 0.49 µg L^{-1} to 7.1 µg L^{-1} lie midway in the dynamic region of the plot and therefore the early investigations are very encouraging. Further work should be carried out to ensure that the results are reproducible and validated against HPLC.

7.2 Further work for synthetic peptide receptor.

Giraudi *et* al., (2000) and Falter *et al*., (1994) have reported that working with small molecules to determine binding constants is difficult and this has been demonstrated in the project. To determine the binding constant many new methods could be performed as described below.

7.2.1 Study the binding forces of the peptide and antibody using chemical force microscopy.

Chemical force microscopy is a technique very similar to atomic force microscopy. To the sensing tip an antibody is immobilised and the tip is scanned over a surface with the immobilised aflatoxin M_1 and the force required to break the antibody / aflatoxin M_1 complex is measured in piconewtons. Once the data has been obtained for the antibody, then the antibody is substituted for the peptide and the force is again measured. This technique will give a quantitative comparison for the strength of the complex for the antibody and each peptide.

7.2.2 Incorporate a marker onto the peptide to trace the peptide.

The marker could either be electrochemical, fluorescent or an enzyme, and by immobilising the aflatoxin M_1 then chemically the presence of the peptide could be monitored. If the marker is fluorescent then it might be fruitful to select a marker which has an excitation wavelength which will overlap the emission wavelength of the aflatoxin M_1. This technique is referred to as FRET (Fluorescence Resonance Energy Transfer). The transfer of energy from one component to the other only occurs if the two components are in close proximity to each other as shown in Figure 7.1.

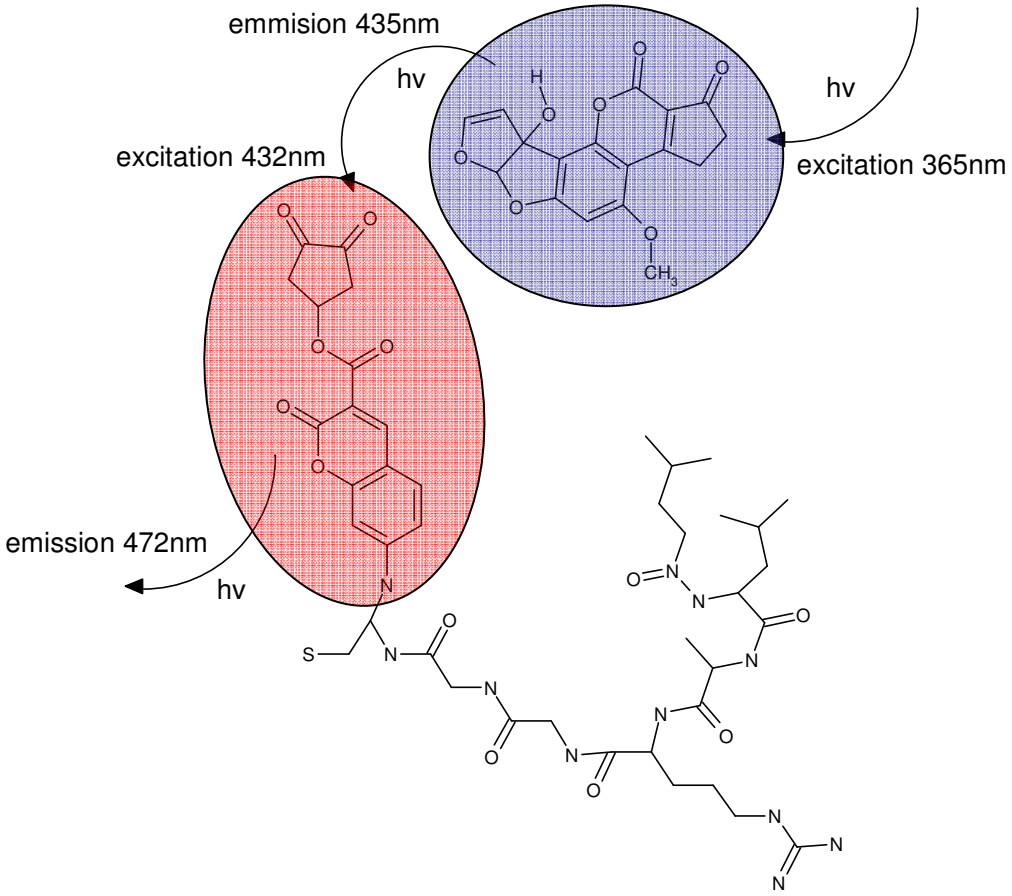


Figure 7.1: A schematic diagram of the energy transfer from aflatoxin M_1 to a fluorescent tag on the peptide. The tag shown is 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester which is attached to the peptide through amino / carboxylic acid dehydration reaction.

This is a useful technique for monitoring the binding of the aflatoxin M_1 to the peptide.

If an electrochemical or enzymatic marker is introduced then the peptide can be monitored using the current detection protocol for the sensor. One concern is that if the marker was an enzyme then the enzyme will be significantly larger than the peptide and therefore may affect the binding.

7.2.3 Monitoring the binding by Nuclear Magnetic Resonance (NMR).

Using NMR it would be able to monitor the interaction of the peptide with the aflatoxin M_1 molecule by recording the change in chemical shift of the peptide against the change in aflatoxin M_1 concentration. By plotting the data then the binding constant can be determined.

The method would also give a value closer to the theoretical value given from the computational models since both the peptide and aflatoxin M_1 molecules would be unmodified and in free solution rather than anchored, which causes a change in affinity.

Initial investigations were performed using ^{13}C NMR and ^{1}H NMR to obtain the spectra of aflatoxin M_1 and the peptides however insufficient experience at Cranfield University in NMR meant that investigations had to be aborted.

CHAPTER 8

APPENDIX

8.1 Buffers.

10 mM Phosphate buffered saline (PBS), pH 7.4

0.24 g KH_2PO4

1.44 g Na_2HPO4

8.00 g NaCl

0.20 g KCl

800 ml H_20

Adjust pH to 7.4 and bring volume to 1 litre with reverse osmosis water

0.5M Acetate Buffer, pH 5.2

68 g Sodium Acetate Trihydrate

Adjust to pH 5.2 with Acetic acid

Make up to 1 litre with reverse osmosis water

0.1 M Carbonate buffer, pH 9.6

0.85 g Na_2CO_3

1.43 g $NaHCO_3$

250 ml reverse osmosis water

0.05M Tris Buffer, pH 7.5

1.51 g Tris base

2.19 g NaCl

230 ml H_2O

Adjusted to pH 7.5 with HCl and made up to 250 ml with reverse osmosis water

0.1M Citrate buffer, pH 5.2

4.6 g citric acid

7.1 g Na_2HPO_4

500 ml reverse osmosis water

8.2 Derivation of method costs.

Table 8.1: Derivation of the costs for each method.

	Commercial ELISA
Cost of Instrument *	4000
Service *	500
Kit per sample (£360/24)	13.84
	HPLC
Cost of instrument *	30,000
Service *	3,000
Cost of immuno-affinity column per sample	11.00
Cost of reagents (mobile phase, vials, etc.) per sample	3.00
Total consumables per sample	14.00
	Immunosensor
Cost of instrument	2500
Cost of service	N/A
Cost of reagents (AB's, conjugate, sensor)	1.81

* Cost of instrumentation for ELISA and HPLC provided by Norman (2007)

Prices correct as of September 2007.

8.3 Peptide synthesis reports.

MRC Medical Research Council
Clinical Sciences Centre CSC

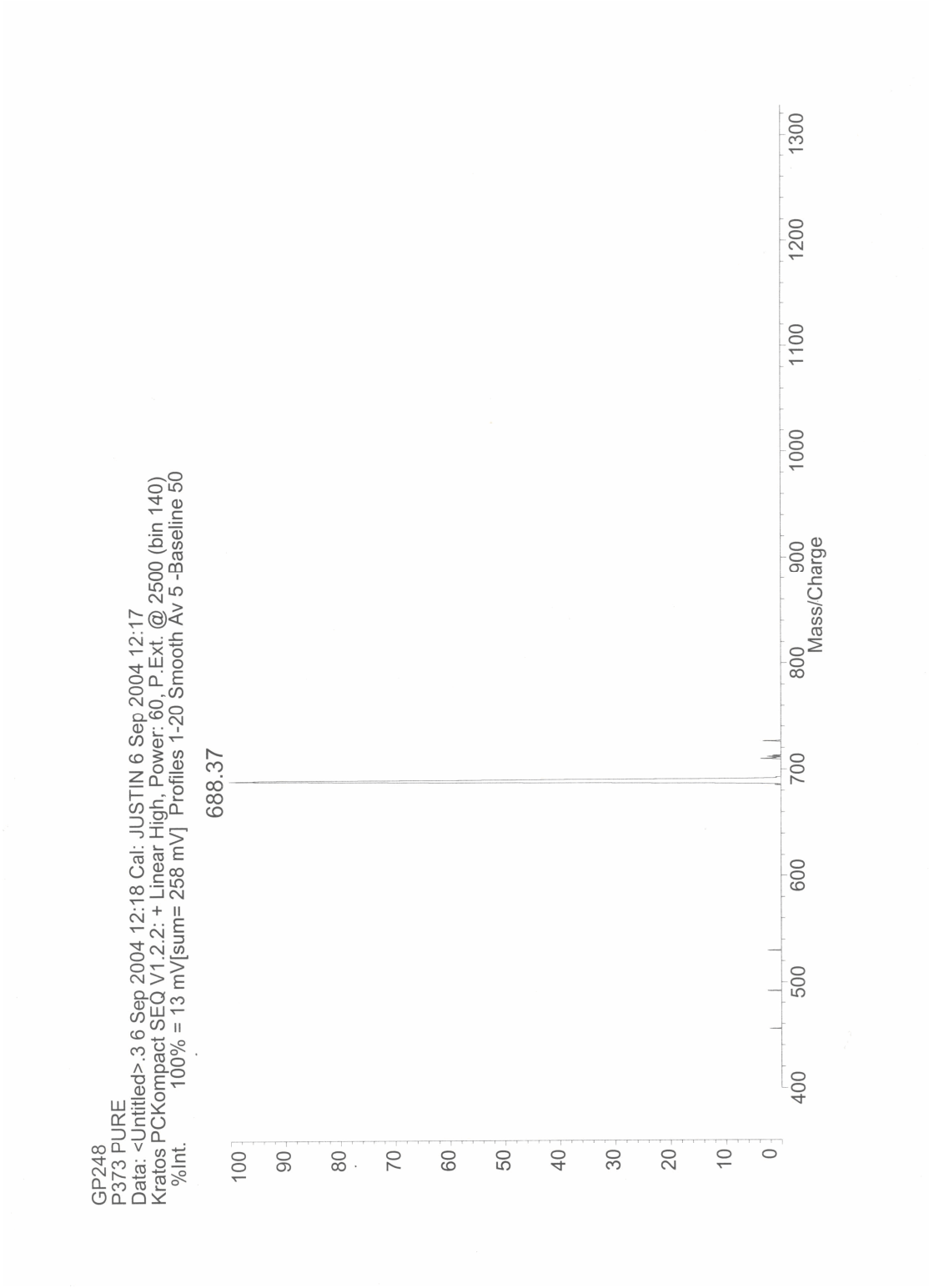
Peptide Unit, Proteomics
MRC Clinical Sciences Centre
Faculty of Medicine, Imperial College
Hammersmith Campus
Du Cane Road
London W12 0NN

Tel. *020 8383 8272 / 8257*
Fax. *020 8383 8404*
Email. *peptide@csc.mrc.ac.uk*

Peptide Report

Customer Name:	C Parker
Peptide Name:	LLARGGC
Sample Number:	P 373
Sequence:	LLARGGC
Expected Mass:	688.85
Mass Obtained:	688.37
Weight Obtained:	17.9 mg
Date on Request Form:	23/08/2004
Date delivered:	06/09/2004
Storage Recommendations:	Store lyophilised peptides at -20°C. Minimise prolonged exposure of Tryptophan (W) containing peptides to light.

Mass Spectrum attached


GP248
P373 PURE
Data: <Untitled>.3 6 Sep 2004 12:18 Cal: JUSTIN 6 Sep 2004 12:17
Kratos PCKompact SEQ V1.2.2: + Linear High, Power: 60, P.Ext. @ 2500 (bin 140)
%Int. 100% = 13 mV[sum= 258 mV] Profiles 1-20 Smooth Av 5 -Baseline 50
688.37
100
90
80
70
60
50
40
30
20
10
0
400
500
600
700
800
900
1000
1100
1200
1300
Mass/Charge

MRC Medical Research Council

Clinical Sciences Centre CSC

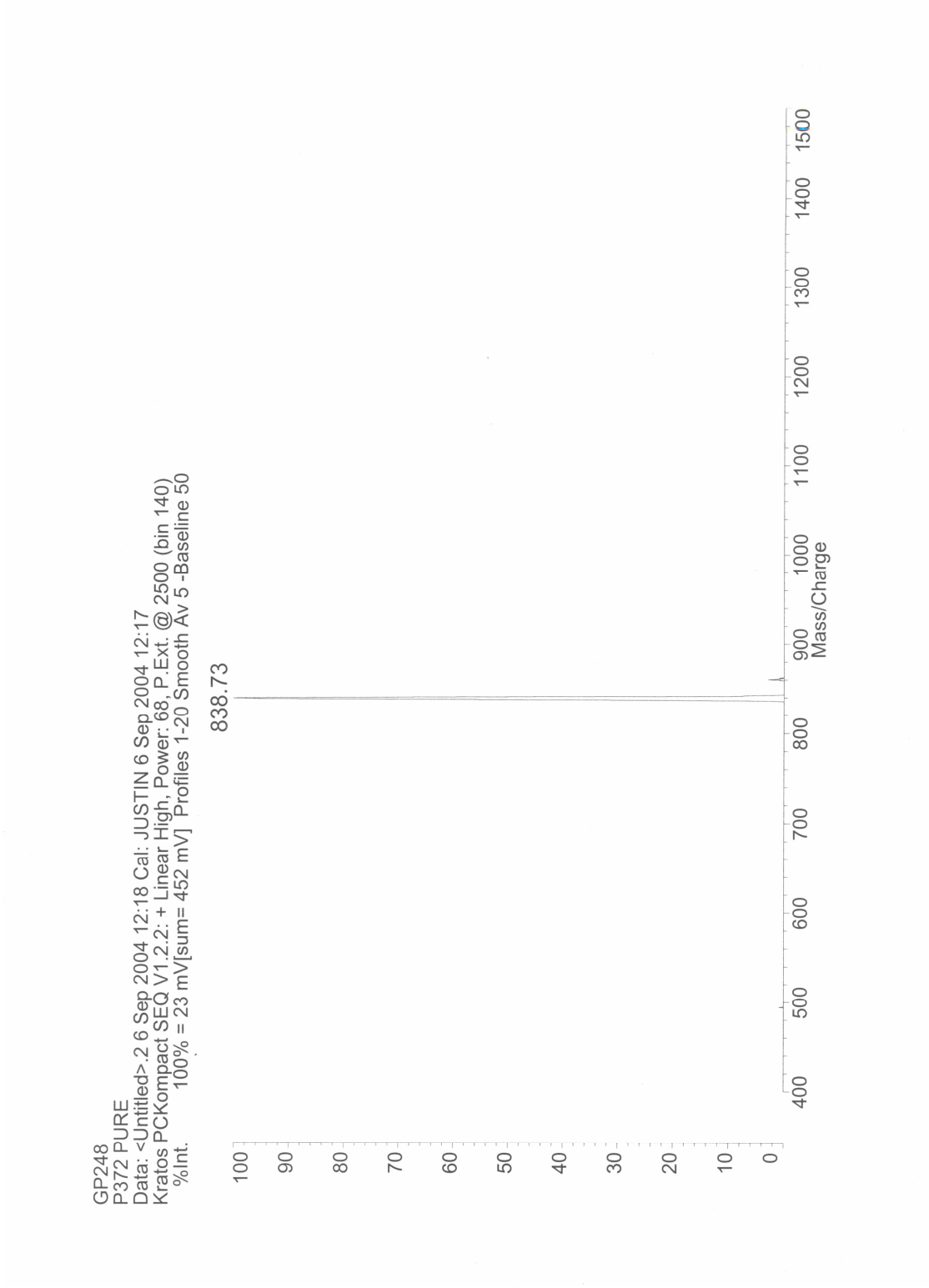
Peptide Unit, Proteomics
MRC Clinical Sciences Centre
Faculty of Medicine, Imperial College
Hammersmith Campus
Du Cane Road
London W12 0NN

Tel. *020 8383 8272 / 8257*
Fax. *020 8383 8404*
Email. *peptide@csc.mrc.ac.uk*

Peptide Report

Customer Name:	C Parker
Peptide Name:	CGGPVGPRP
Sample Number:	P 372
Sequence:	CGGPVGPRP
Expected Mass:	838.99
Mass Obtained:	838.73
Weight Obtained:	11.6 mg
Date on Request Form:	23/08/2004
Date delivered:	06/09/2004
Storage Recommendations:	Store lyophilised peptides at -20°C. Minimise prolonged exposure of Tryptophan (W) containing peptides to light.

Mass Spectrum attached


GP248
P372 PURE
Data: <Untitled>.2 6 Sep 2004 12:18 Cal: JUSTIN 6 Sep 2004 12:17
Kratos PCKompact SEQ V1.2.2: + Linear High, Power: 68, P.Ext. @ 2500 (bin 140)
%Int. 100% = 23 mV[sum= 452 mV] Profiles 1-20 Smooth Av 5 -Baseline 50
838.73
100
90
80
70
60
50
40
30
20
10
0
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
Mass/Charge

8.4 Publications.

Parker, C.O., Tothill, I.E. Heurich, M. (2004) Food safety and quality monitoring with Microsystems, *GOODFOOD 12th month project meeting, Athens, Greece, 29th November – 1st December, 2004*. (Oral Presentation)

Lanyon, Y., Watson, Y., Arrigan, D., DeMarzi, G., Quinn, A., Heurich, M., Parker, C., Tothill, I. (2004) The development of micro-nano-systems for mycotoxins detection, *GOODFOOD 12th month project meeting, Athens, Greece, 29th November – 1st December, 2004*. (Poster Presentation)

Tothill, I.E., Parker, C., Heurich, M., (2005) The development of sensing receptors for mycotoxins detection, GOODFOOD Second Review, *Montreux, Switzerland, 4th – 6th April 2005*. (Poster Presentation)

Parker, C., Tothill, I.E., (2005) Development of sensing receptor for aflatoxin M_1 detection, *Second world congress on synthetic receptors, Salzburg, Austria, 7th-9th September 2005*. (Poster Presentation)

Tothill, I.E., Parker, C., Heurich, M., (2006), Development of sensing receptors for mycotoxin detection, *GOODFOOD 24th month project meeting, Cranfield, UK, 14th – 17th March 2006*. (Poster Presentation).

Tothill, I.E., Parker, C., Heurich, M., (2006), Development of sensing receptors for mycotoxin detection, *Novobiochem – The Chemistry and Biology of Peptides. University of Nottingham, Nottingham. 30th March 2006.*

Tothill, I.E., Heurich, M., Parker, C., Lanyon, Y.H, Arrigan, D.W. (2006). Microsensors for mycotoxins detection in foods. *Myco-Globe Conference, Bari, Italy, 26 – 29th September 2006*. (Poster Presentation)

Parker, C., Heurich, M., Tothill, I.E., (2007) Development of affinity sensors for mycotoxin detection. *GOODFOOD final project meeting, Lucerne, Switzerland, 13th – 15th March 2007* (Oral demonstration and poster presentation).

Ibtisam E. Tothill, Charlie Parker, Meike Heurich, Yvonne h. Lanyon and Damien w. M. Arrigan (2007). Development of Micro/Nanosensor Arrays for Mycotoxins Analysis in Foods. *IUPAC conference, Istanbul, Turkey, May 21-25th 2007*. (Oral presentation).

Ibtisam E. Tothill, Charlie Parker , Meike Heurich, Yvonne H. Lanyon, Mary Manning And Damien W. M. Arrigan (2008). Microsensor Arrays for Mycotoxin Detection. *Biosensor 2008, Shanghai, China, 14-16th May 2008*.

CHAPTER 9

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