Development of Novel Biosensing Techniques for Analysis of Aflatoxins in Food Products

THESIS

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LIZY KANUNGO

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Prof. Sunil Bhand

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
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CERTIFICATE

This is to certify that the thesis entitled **"Development of Novel Biosensing Techniques for Analysis of Aflatoxins in Food Products"** and submitted by **LIZY KANUNGO** ID No. **2010PHXF439G** for award of Ph. D. Degree of the Institute embodies original work done by her under my supervision.

Signature in full of the Supervisor: _____

Name in capital block letters: **Dr. SUNIL BHAND**

Designation: Professor

Date:

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ABSTRACT

Aflatoxin contamination is a worldwide problem with regard to food and feed safety. These aflatoxins are extremely potent carcinogens and can have significant economic impacts, making them important targets for detection and quantitation. Due to their toxicity, stringent limits have been imposed on aflatoxin contents in various foods and dairy products. Even though, most national and international organizations have set legal permissible limits for aflatoxins to control the quality of various food commodities, their contamination and persistence in food remain a major challenge to public health services. Apart from sensitive detection and quantification, on-line monitoring of aflatoxins in various foods is of immense importance to food processing industries. Therefore, ultrasensitive, high throughput and field portable biosensing techniques are required for the analysis of aflatoxin contamination for effective food safety monitoring programs (chapter 1).

The research work incorporated in this thesis demonstrates the development of novel biosensing techniques for aflatoxin detection in different food products such as milk, milk products and peanuts. Construction of biosensors for ultra sensitive detection of AFM1, AFM2 and AFB1 have been successfully achieved in this work.

Principle of Chemiluminescence (CL) and fluorimetric techniques were exploited for optical detection of AFM1 in 384 microwell plate (chapter 2). Simple optical setup, high sensitivity, minimum interference and capability of high sample throughput are the key reasons to exploit CL as optical detection technique for quantification of AFM1. Unlike other reported methods for AFM1 quantification, biosensor presented here is almost free from toxic organic solvents. The working range of AFM1 assay was 0.005-250 pg/mL and AFM1 as low as 0.005 pg/mL could be detected by this CL measurement.

To avoid possible matrix interference from whey proteins and fats found in milk, novel sample pre-treatment methods were investigated such as fat digestion by treatment with trichloro acetic acid (TCA) followed by centrifugation, filtration and dilution. Milk samples with different fat contents were analyzed by these pre-treatment methods.

Apart from CL technique, the analysis of AFM1 was also carried out by fluorimetric technique. Herein, specific FITC conjugated secondary antibodies were used for quantitative detection of AFM1. This technique was also ultrasensitive and could detect 1 pg/mL AFM1.

The fluorimetric analysis of AFM1 was further extended to multi analysis of different aflatoxins such as AFM2, AFB1 and AFG1 which are structurally analogous to AFM1 (chapter 3). Here cross reactivity concept was used for multianalysis of these aflatoxins. The monoclonal antibody of AFM1 was used as capture antibody that recognized AFM1 most specifically than other aflatoxins. FITC conjugated secondary antibodies detected the antigen-antibody complex and quantified aflatoxins based on their crossreactivity with AFM1-mAb. Apart from microwell plate based FL assay, quantitative image based aflatoxin detection was also carried out on a customized device by fluorescence microscope. The labeled techniques such as CL, fluorimetric and fluorescence (FL) imaging techniques have their own advantages of being ultrasensitive and capable of high throughput toxin analysis.

Recent approaches use label free detection techniques based on real time interaction such as Suface Plasmon Resonance (SPR) & Electrochemical Impedance Spectroscopy (EIS). In this context, label free detection technique based on bioimpedance was investigated for AFM1 and AFB1 analysis in milk and peanut respectively (chapter 4). The label free measurement using EIS helps reduce number of steps in analysis & also reduces the cost of reagent labels. Herein, the bioimpedance was studied by two Silver (Ag) wire electrodes. In this label free setup, the Ag wires were functionalized with primary antibodies and impedance analysis was carried out on the addition of analytes. Certified reference milk samples were artificially spiked with known [AFM1] and bioimpedance was measured. There was an increase of impedance value observed for increase in [AFM1]. Similarly AFB1 was analyzed in peanut samples. The bioimpedance was also studied for AFM2 analysis which is prevalent in milk products. Processed milk such as drinking yogurt and flavored milk samples were analyzed in a flow based set up. Two micro flow pumps were used in the flow system where analytes [AFM1] & [AFM2] were injected to the samples and impedance was measured by functionalized Ag wire electrodes. The flow system was optimized by adjusting both inlet and outlet flow to maintain the reaction volume optimum for impedance measurement. This flow based set up analyzed multianalytes such as AFM1 and AFM2 in milk and related milk products provides remarkable scope for on-line

monitoring of such hazardous toxins. Analysis of AFM1 and AFB1 on a novel IDE device was successfully carried out by EIS technique with very low sample volume. Using the developed device, AFM1 in milk was successfully quantified at stringent EU cut off of 50 pg/mL and below.

In brief, novel biosensing techniques were developed for analysis of aflatoxins in different foods such as milk, milk products and peanuts. In continuation to the development of CL sandwich ELISA, a systematic survey was conducted in the local markets of Goa, India for detection of AFM1 contamination in commercial milk samples and infant formula milk samples (chapter 6). From the analysis, it was evident that, the all the analyzed commercial milk samples were found to contain AFM1 concentrations exceeding permissible limits of EU standard and around 75% of the samples exceeded Codex, USFDA and FSSAI standards. The detected levels of AFM1 in the analyzed samples show a serious health alarm in regards to the safety limits for AFM1 levels in collected infant formula and milk samples.

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List of Abbreviations & Symbols

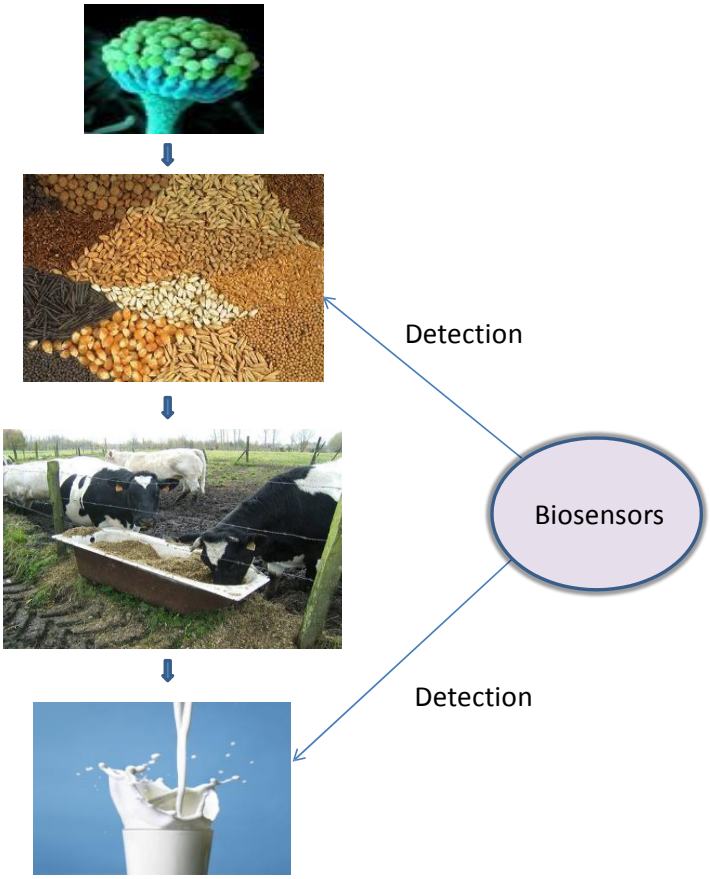
Abbreviation	Description
Ab	Antibody
AC	Alternate current
CAN	Acetonitrile
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
Ag	Silver
Al	Aluminium
AOAC	Association of analytical communities
BSA	Bovine serum albumin
CB	Carbonate buffer
CCD	Charge-coupled device
CL	Chemiluminescence
cm	Centimeter
CNT	Carbon nano tube
CR	Cross reactivity
CRM	Certified reference material
CV	Coefficient of variance
DW	Distilled water
EC	European commission
EDC	1-ethyl-3(3/-dimethylaminopropyl) carbodiimide, HCl
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EM-CCD	Electron multiplier-charge coupled device

EU	European union
FIA	Flow injection analysis
FITC	Fluorescein isothiocyanate
FL	Fluorescence
g	Gram
H	Hour
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HMs	Heavy metals
HRP	Horseradish peroxidase
I	Inhibition
IAC	Immunoaffinity column
IARC	International agency for research on cancer
IC_{50}	Half maximal inhibitory concentration
IDE	Interdigitated electrode
IgG	Immunoglobin G
IUPAC	International union of pure and applied chemistry
kDa	Kilo daltons
Kg	Kilogram
KHz	Kilo Hertz
L	Liter
LC	Liquid chromatography
LD_{50}	Lethal dose at 50 %
LOD	Limit of detection
LOQ	Limit of quantification
LRSP	Long range surface plasmon
M	Molar
mAb	Monoclonal antibody
mg	Mlligram
mM	Milli molar
min	Minute

mL	Mililiter
mV	Millivolt
MUA	Mercaptoundecanoic acid
ng	Nanogram
nL	Nanoliter
NHS	N-hydroxysuccinimide
pAb	Polyclonal antibody
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
pH	Acidity measurement unit
pg	Picogram
PMT	Photomultiplier tube
rpm	Revolutions per minute
RSD	Relative standard deviation
SAM	Self assembled monolayer
SD	Standard deviation
SPE	Solid phase extraction
SPFS	Surface plasmon-enhanced fluorescence spectroscopy
SPR	Surface plasmon resonance
TCA	Trichloro acetic acid
TLC	Thin layer chromatography
USFDA	United States food and drug administration
UV	Ultra violet
µg	Microgram
µL	Microliter
1º Ab	Primary antibody
2º Ab	Secondary antibody

Chapter 1

Introduction



Schematic representation of chapter content

1. Aflatoxins as food contaminants

Many grains and food stuff have been found to be contaminated with aflatoxins as a result of natural invasion by the molds before and during harvest, or because of improper storage. Humans may be exposed to aflatoxins in their diet either directly, by eating contaminated grains or nuts, or indirectly via animal tissues (meat) or animal products. The aflatoxins are recognized as a serious health risk to humans and animals alike. Detection and quantification of aflatoxins in various foods is of immense importance. Therefore, an ultrasensitive, high throughput field portable biosensor is required for the analysis of aflatoxin contamination for effective food and feed safety monitoring programs.

1.1 Scope of research work

1.1.1. Mycotoxins

Toxins, by definition, are compounds that are poisonous to living beings. Natural toxins found in foods can be divided into the following categories: mycotoxins, bacterial toxins, phytotoxins and zootoxins. The first three are toxic compounds produced by living organisms, and are formed directly in the food or transferred through the food chain, whereas, the latter two are inherent components (of plants or animals) that are harmful to humans and animals. (van Egmond and Dekker, 1995). Mycotoxins are toxic compounds produced by fungi. Most mycotoxins can be defined as natural products produced by fungi that evoke a toxic response in higher vertebrates and other animals when fed at low concentrations (Bhatnagar et al., 2002). Many mycotoxins, which are produced by various toxic fungi, are able to produce deleterious health effects.

Many agricultural commodities are susceptible to attack by group of fungi that produce mycotoxins. These mycotoxins are secondary metabolites of molds frequently found on and in agricultural commodities, food stuff and animal feeds. Molds are ubiquitous in nature and are universally found where environmental conditions are suitable for mold growth. Because molds are present in soil and plant debris and are spread by wind currents, insects and rain, they are frequently found in/on foods together with their associated mycotoxins (Council for Agricultural

Science and Technology, 1989). Analysis for mycotoxins is essential to minimize the consumption of contaminated foodstuff and animal feeds, for monitoring domestic and import surveillance programs, controlling the quality of products, establishing new regulatory standards and guidelines, validating decontamination procedures and preparing standard materials for use in toxicological studies.

Over 190 molds have been found to be able to produce toxins. In some cases more than one mold can produce the same toxin (Gilbert and Anklam, 2002; Moreau C. 1979). The main molds families of concern are *Fusarium, Aspergillus* (**Figure 1.1**), *Penicillium,* and *Alternaria.*

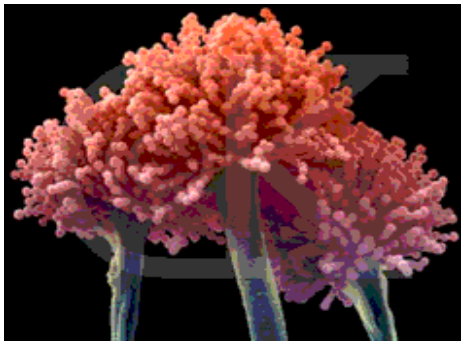


Figure 1.1 *Aspergillus flavus* (source: www.icrisat.org/aflatoxin/aflatoxin.asp).

1.1.2. Aflatoxin origin and distribution

Aflatoxins are naturally occurring mycotoxins. The survey of the outbreaks of "turkey X disease" in 1960 drew scientists' attention to study aflatoxins for the first time. The toxin producing fungus was identified as *Aspergillus flavus* (1961) and the toxin was given the name aflatoxin by virtue of its origin (i.e. the genus Aspergillus, the species flavus and the suffix toxin). The name "aflatoxin" was introduced by Nesbitt from the abbreviation of "*A. flavus* toxin" (Nesbitt et al., 1962). Aflatoxins are poisonous and carcinogenic byproducts produced by several species of *Aspergillus* family, that are found growing mainly in improperly stored food and animal feeds (**Figure 1.1**).

Among various mycotoxins, aflatoxins have striking significance due to their deleterious effects on human beings, poultry and livestock. Aflatoxins are among the most toxic of the

known mycotoxins and have been implicated in the deaths of humans and animals that have consumed moldy foods. At least 13 different types of aflatoxins are known in nature, mainly produced by 3 types of molds, namely: *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* species growing on a wide range of food and animal feedstuffs. Under favorable conditions of temperature and humidity, these fungi grow on various foods and feeds, resulting in the production of aflatoxins. Aflatoxins' affect in the food producing animals are of concern because they can be found in food produced or obtained from those animals. Human exposure to aflatoxins is predominantly to aflatoxin B1 (AFB1), or to mixtures of various aflatoxins, depends upon the geographical distribution of the stains. *A. flavus* which occurs worldwide, produces AFB1 and AFB2, while *A. parasiticus*, that occurs principally in America and Africa, produces AFB1, AFB2, AFG1 and AFG2. People, especially those who live in under developed countries, are frequently exposed to aflatoxins through food contamination.

Aflatoxins have been detected in many grains and foodstuffs including corn, peanuts, tree nuts, cottonseed, cereal crops, beans, cassava, milo, sorghum, copra, rice, dried fish and beer (Brera et al., 2011). Milk, milk products, eggs and meat products are sometimes contaminated by feeding the animal with aflatoxin-contaminated foods (Kuilman et al., 2000). While the liver is the target organ for aflatoxicosis, aflatoxins are also found in other animal tissues and products, such as meat, milk, and eggs (Van Egmond, 1989). The mature animals modify and eliminate toxins effectively; however, the main concern is long-term intake of low concentrations of these toxins, which can lead to cancer and immuno-suppression (Van Egmond, 1989; IARC, 1993; Campbell et al., 1990; Wang et al., 1996).

1.1.3 Classification and description

There are 13 different types of aflatoxins known in nature of which four are major aflatoxins; AFB1, AFB2, AFG1 and AFG2. There are two additional metabolic products, M1 and M2, which are of significance as direct contaminants of milk and related milk products (**Figure 1.2**). Their molecular formulae are established from elementary analyses and mass spectrometric determinations. AFB1 is the most frequent of these compounds present in contaminated food samples and AFB2, AFG1 and AFG2 are generally not reported in the absence of AFB1.

Aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) are hydroxylated derivatives of AFB1 and AFB2 respectively that may be found in milk and milk products (hence the designation "M"). They are a group of polyketide-derived bis-furan containing dihydro-furanofuran and tetrahydrofuran moieties (rings) fused with a substituted coumarin (Smela et al., 2001).

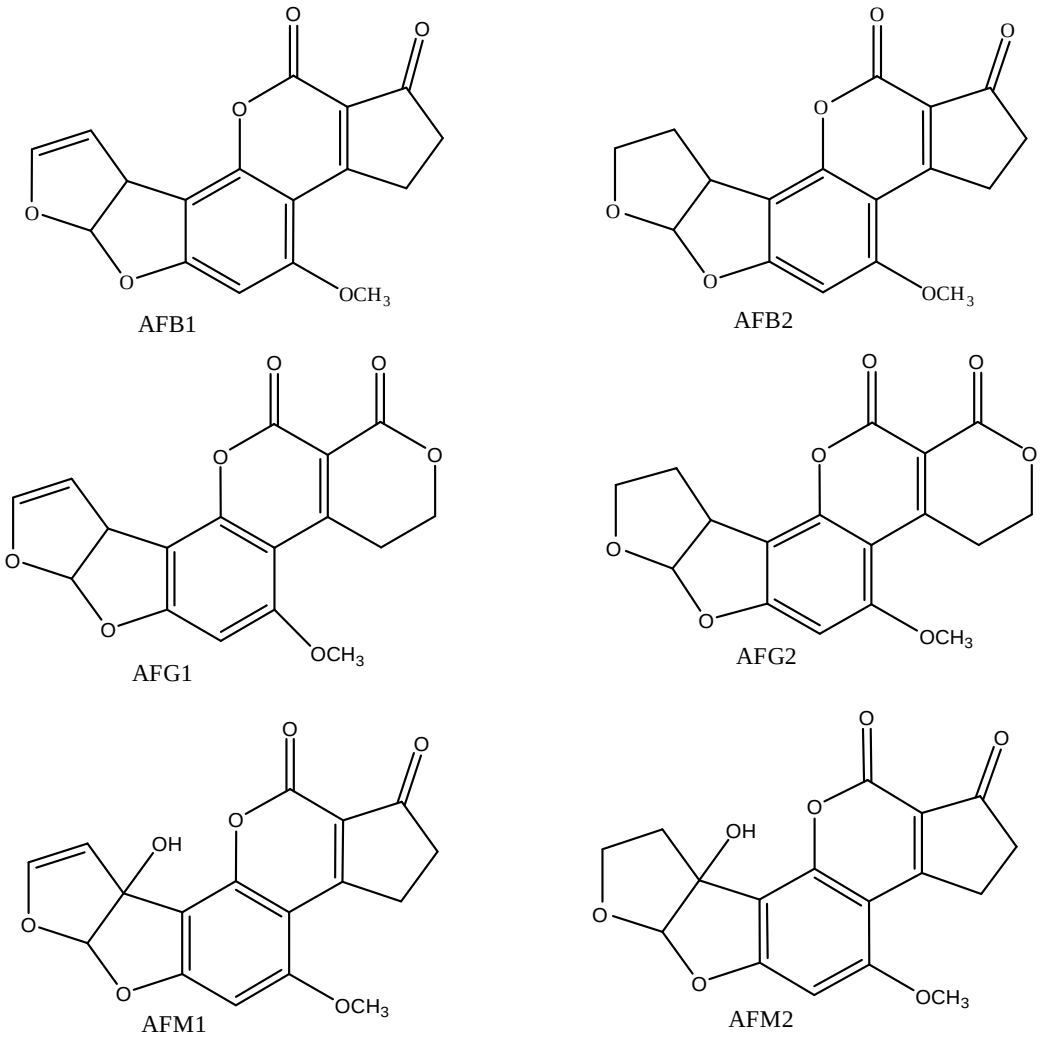


Figure 1.2 Chemical structures of aflatoxins; AFB1, AFB2, AFG1, AFG2, AFM1, AFM2.

Aflatoxins are intensely fluorescent in ultraviolet (UV) light and the four major aflatoxins are named accordingly to the colour of the emitted light; "B" for blue and "G" for green (Henry et al., 2001). The aflatoxins form a family of highly oxygenated heterocyclic compounds with closely

similar chemical structures. They are all slightly soluble in water and are heat stable. The toxicity of the major aflatoxins has been established in the following order: AFB1 > AFG1 > AFB2 > AFG2. AFM1 is 10 fold less toxic than AFB1, but its presence in milk is of concern in human health (Cullen et al., 1987; Galvano et al., 1996; Van Egmond, 1989). The chemical and physical properties of various aflatoxins are presented in **Table 1.1**.

Table 1.1 Chemical and physical properties of different aflatoxins (source: www.sigmaaldrich.com).

Aflatoxin	Molecular formula	Molecular weight	Melting point (°C)
AFB1	$C_{17}H_{12}O_6$	312	268-269
AFB2	$C_{17}H_{14}O_6$	314	286-289
AFG1	$C_{17}H_{12}O_7$	328	244-246
AFG2	$C_{17}H_{14}O_7$	330	237-240
AFM1	$C_{17}H_{12}O_7$	328	299
AFM2	$C_{17}H_{14}O_7$	330	293

1.1.4 AFM1 and AFM2

Aflatoxin M is the generic name of two compounds, AFM1 and AFM2. It has been reported that, the consumption of aflatoxin contaminated feed by the lactating animals result in the secretion of the toxic, carcinogenic and mutagenic AFM1 in milk (Henry et al., 1997). Delong et al. (1964) showed that, AFM1 is a blue-violet fluorescent compound. Holzapfel et al. (1966) reported that, aflatoxin contaminated milk contained two fluorescent compounds subsequently designated as AFM1 and AFM2. These AFM1 and AFM2 are the secondary hydroxylated metabolites of AFB1 and AFB2 respectively. The bio-transformation of AFB1 and AFB2 into AFM1 and AFM2 occur in the liver cytochrome system of lactating animals. It has been demonstrated that, up to 6% of the ingested AFB1 is secreted into the milk as AFM1 (Van Egmond and Dragacci, 2001) and because, AFM1 is relatively resistant to heat treatments

(Yousef and Marth, 1989; Galvano et al., 1996), it is almost entirely retained in pasteurized milk, powdered milk and infant formula milk powder.

AFM1 is known for its hepatotoxic and carcinogenic effects. The presence of AFM1 in milk, possess a major risk for humans especially to infants, as it has immune-suppressive, mutagenic, teratogenic and carcinogenic effects (Henry et al., 2001). AFM1 is relatively stable during milk pasteurization, storage as well as during the preparation of various dairy products (Codex Alimentarius, 2000; Badea et al., 2004). AFM2 along with AFM1 has been found in other dairy based products such as cheese and yogurt (Sharman et al., 1989; Martins and Martins, 2000; Kamkar A, 2006).

1.1.5 Chemical properties of AFM1

AFM1 is chemically stable; it is not destroyed under domestic conditions such as microwave or oven heating, however, the stability of AFM1 during pasteurization is in debate. Bakirci (2001) and Henry et al. (2001) report that, pasteurization has no effect whereas Deveci and Sezgin (2006) suggested that, pasteurization causes a 16% decrease, hypothesizing that the decrease is due to heat treatment causing casein decomposition. Chemically AFM1 is hydrophobic; studies have shown that, AFM1 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 AFM1 compared to low protein products (van Egmond, 2002). Since it is difficult to eradicate AFM1 in milk, greater monitoring of AFB1 must be performed so that AFB1 is prevented from entering into cattle feed (Bakirci, 2001).

1.1.6 AFM1 targeted organs

Liver is the primary target organ for aflatoxins' toxicity in all species studied (Wogan, 1992; Wang et al., 1996). In some cases, AFM1 has been reported and found in the muscle tissues of animals (Rodricks and Stoloff, 1976; Stubblefield et al., 1983). It was found that, when cows were fed on AFB1 contaminated feed, AFM1 was detected in the brain, gall bladder, heart, intestines, kidneys, liver, lungs, 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 respectively

(Parker et al., 2009). The precise manifestations of toxicity depend upon a number of factors, including dose and duration of exposure. However, it is the potent ability of aflatoxins to induce liver cancer when exposed to chronic low level exposure and the significant economic and public health consequences that follow, which has stimulated much of the work on these compounds over the last 50 years.

1.1.7 Toxicity of AFB1 and AFM1

The hepatotoxicity and carcinogenic effects of AFB1 have been clearly demonstrated, thus it has long been classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002). Initially, the IARC had classified AFM1 as a possible carcinogen for humans (group 2B), since toxicological data was limited (IARC, 1993). However, genotoxicity and carcinogenecity of AFM1 have been observed in vivo, although lower than those of AFB1, and its cytotoxicity has been definitively demonstrated (Caloni et al., 2006). As a result of these and other further investigations, the IARC moved AFM1 from group 2B to group 1 human carcinogen (IARC, 2002). A wide variation in lethal dose 50% (LD_{50}) values have been obtained in animal species tested with single doses of aflatoxins. For most species, the LD_{50} value ranges from 0.5 to 10 mg/kg body weight (Reddy and Waliyar, ICRISAT, 2000).

1.1.8 Permissible limits of AFM1 and AFB1

Many international agencies are trying to achieve universal standardization of regulatory limits for mycotoxins. This is a very difficult task as many factors have to be considered when deciding on regulatory standards. In addition to scientific factors, such as risk assessment and analytical accuracy, economical and political factors such as the commercial interests of each country and the constant necessity of a sufficient food supply also play a role in the decision making process. The whole process is further complicated by the fact that action levels pertain to single mycotoxin contamination, but in reality, several mycotoxins often occur in combination, which may require different and often lower action levels. Measuring the toxicological effects of a variety of different mycotoxin combinations, as they occur in nature, is an enormous and probably impossible task, especially considering that they may be mycotoxins present that have not been identified. In addition, nutritional, environmental and species all play a contributory

role in determining the effect of a combination of mycotoxins on animal health. **Table 1.2** summarizes the permissible limits of aflatoxins in food by different agencies such as; European Union (EU), US Food and Drugs Administration (USFDA), CODEX and Food Safety and Standards Authority of India (FSSAI).

Table 1.2 Permissible limits for Aflatoxins in different food (Dors et al., 2011).

Product	Maximum Permissible Level			
	EU	USFDA	CODEX	FSSAI
Groundnuts & dried fruits & their processed products for human consumption	2 µg/kg-B1 4 µg/kg-Total	20 µg/kg-Total	15 µg/kg-Total	30 µg/kg
Raw groundnuts before human consumption	8 µg/kg-B1 15 µg/kg-Total	20 µg/kg-Total	Not specified	30 µg/kg
Nuts & dried food	5 µg/kg-B1 10 µg/kg-Total	20 µg/kg-Total	Not specified	30 µg/kg
Cereals for human consumption	2 µg/kg-B1 4 µg/kg-Total	20 µg/kg-Total	Not specified	30 µg/kg
Milk & Milk based products	50 pg/mL-M1	500 pg/mL-M1	500 pg/mL-M1	0.5µg/kg
Herbs & Spices	5 µg/kg-B1 10 µg/kg-Total	20 µg/kg-Total	Not specified	30 µg/kg

1.2 Methods for analysis of Aflatoxins

Many analytical methods have been developed and are available for estimation of aflatoxins in agricultural commodities. For the detection of aflatoxins, mainly two types of methods have been used. These include; conventional and advanced.

1.2.1 Conventional methods

The conventional or routine detection methods of aflatoxins mainly comprise of High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). For conventional techniques, there are several limitations. The HPLC technique is time consuming,

frequently involving in large-scale equipment, uses large sample volumes, involves extensive extraction or derivatization steps (Tang et al., 2008), complicate clean-up, concentration and multiple centrifugation, etc. Moreover the cost per sample analysis is quite expensive. On the other hand, the TLC technique is although simple to use, it is less sensitive.

1.2.1.1 High Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique (**Figure1.3**) used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. This is a very versatile method and during the 1980ˇs popularity for HPLC increased. HPLC is an expensive technique to perform mainly due to the cost of the instrumentation and the cost of employment of technical operators. It is the generally accepted method for the analysis of aflatoxin in milk (Henry et al., 2001). It became the Association of Analytical Communities (AOAC) method in 1986. Martins and Martins (2000), Stroka et al. (2000), Farjam et al. (1992), Mortimer et al. (1987) and many others have implemented immunoaffinity columns for AFM1 determination. In all methods, the detection method was fluorescence. Recently the use of HPLC with tandem mass spectroscopy has been applied to the detection of AFM1.

Figure 1.3 HPLC setup.

1.2.1.2 Thin Layer Chromatography (TLC)

TLC, also known as flat bed chromatography or planar chromatography, is one of the most widely used separation techniques in aflatoxin analysis. Since 1990, it has been considered the

AOAC official method and the method of choice to identify and quantify aflatoxins at levels as low as 1 ng/g. 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 sensitive than HPLC (Gilbert and Anklam, 2002). TLC quantification method gives a reasonable level of selectivity and sensitivity to separate aflatoxins from other interfering compounds (**Figure 1.4**). Despite being less sensitive, TLC is still one of the method of choice for rapid screening of aflatoxins and for situations where advanced equipments are not available.

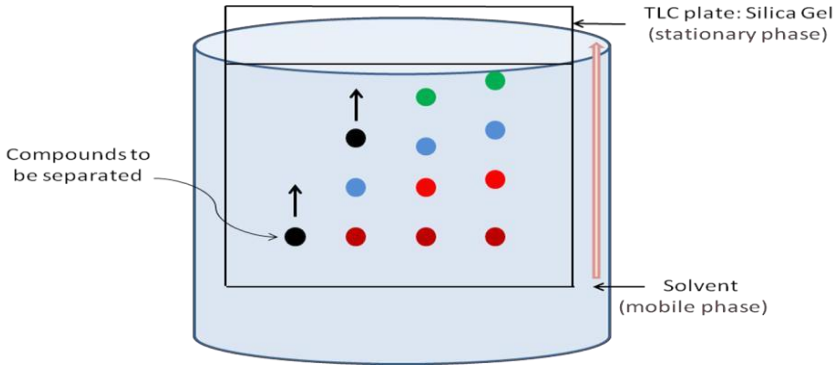


Figure 1.4 TLC technique.

1.2.2 Advanced methods

The advanced method for aflatoxin detection involves immunological methods. The sample pre-treatment for immunoassay (for instance, enzyme-linked immunosorbent assay or ELISA) is usually easier, cheap, rapid and generally without derivatization but still need clean-up and concentration.

1.2.2.1 Immunoassay

An immunoassay is a specific type of biochemical test that measures the presence or concentration of a substance (referred to as the "analyte") in solutions that frequently contain a complex mixture of substances (Gosling J. P. 2000). Immunoassays benefit from very high selectivity and affinity of antibody/antigen systems, as well as from decades of immunoassay developments in diagnostics. The antibodies bind to very specific antigens. Either the antigens or the antibodies are labeled before analysis, in order to give a measurable signal. This label can be

an enzyme, a radioactive isotope or a fluorophore. The signals obtained from an immunoassay can be radioactivity or emission of light. These signals are commonly called as responses. **Figure 1.5** shows binding of AFM1 with specific antibody.

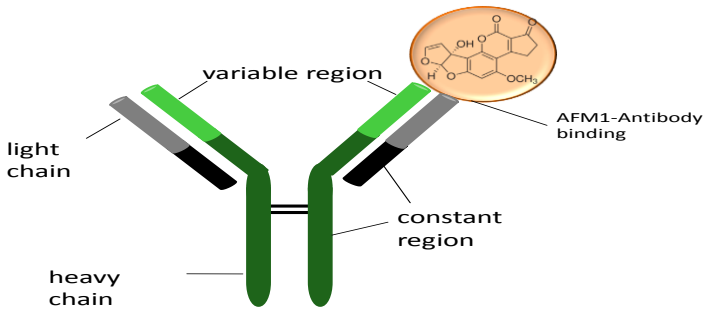


Figure 1.5 Schematic diagram of AFM1 and antibody (immunoglobulin IgG) binding.

Three types of immunochemical methods are mostly used for aflatoxin analysis: radio immunoassay, immunoaffinity column assay and ELISA.

1.2.2.2 Radioimmunoassay (RIA)

RIA is a very sensitive in-vitro assay technique used to measure concentrations of antigens by use of antibodies. Although the RIA technique is extremely sensitive and extremely specific, requiring specialized equipment, it remains the least expensive method to perform such tedious immunosorbent assays. It requires special precautions and licensing, since radioactive substances are used. RIA is not usually environment friendly and can easily harm operators, so radio markers for agricultural immunoassays have been replaced by enzymes and other signal reagents. In recent years, it has been replaced by the ELISA method.

1.2.2.3 Immunoaffinity column assay (IAC)

The IAC has been used widely for sample clean-up in the mycotoxin analysis (Goryacheva et al., 2009; Li et al., 2009). The IAC contains anti-mycotoxin antibody that is immobilized onto a solid support such as agarose gel in phosphate buffer, all of which is contained in a small plastic cartridge (**Figure 1.6**). The sample extract is applied to an IAC containing specific antibodies to

a certain mycotoxin. The mycotoxin binds to the antibody and water is passed through the column to remove any impurities. Then by passing a solvent such as methanol through the column, the captured mycotoxin is removed from the antibody and thus eluted from the column. The mycotoxin in the methanol elute is then further developed by addition of a chemical substance to either enhance the fluorescence or render the mycotoxin fluorescence before measuring in a fluorometer. Prior to adding a fluorescent enhancing chemical, the methanol solution can be used for HPLC analysis as well. With IAC clean-up, the mycotoxin can be concentrated in the column, thereby increasing the fluorometric assay sensitivity or decrease its limit of detection. However, IACs have a limited loading capacity and the sample clean-up procedures are more complicated compared to others in the rapid methods for mycotoxins. Currently the AOAC method uses immunoaffinity columns containing monoclonal antibodies specifically for AFM1 which are supported on Sepharose® packing material (AOAC, 1996).

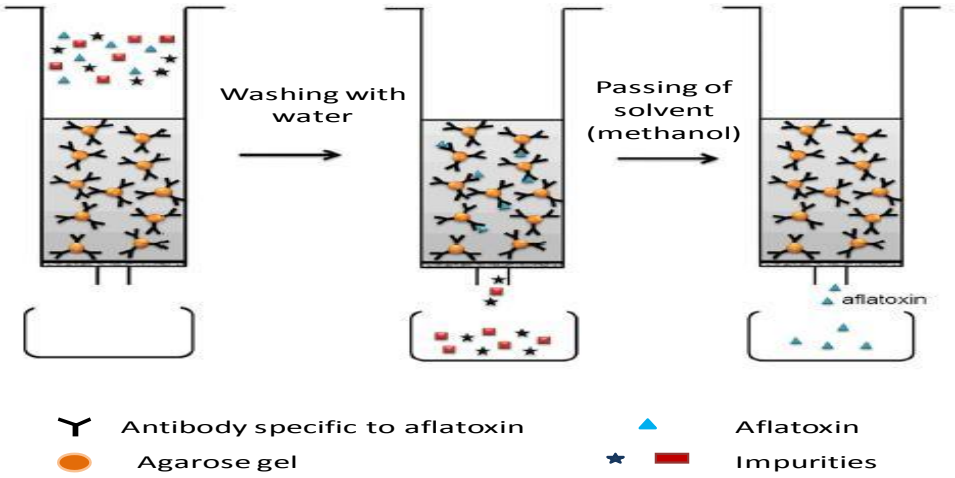


Figure 1.6 Scheme of aflatoxin immunoaffinity column for sample pretreatment (clean-up and enrichment).

1.2.2.4 ELISA

ELISA methods for mycotoxin assay have been available for more than a decade. The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin (**Figure 1.7**). A simple microtiter plate ELISA requires equilibrium of the antibody–antigen reaction that would require an incubation time of

approximately 1–2 h. Currently, most of the commercially available ELISA test kits for mycotoxins are working in the kinetics phase of antibody–antigen binding, which reduces the incubation time to minutes. Although reduction of incubation time may lead to some loss of assay sensitivity, the tests can provide accurate and reproducible results.

Figure 1.7 Types of ELISA.

ELISA techniques can be classified as homogenous and heterogeneous assays.

a) **Homogeneous ELISA**: In a homogenous immunoassay, also referred to as the competitive immunoassay, the antigen in the unknown sample competes with the labeled antigen to bind to the antibodies. The amount of labeled antigen to the antibodies is then measured by different detection techniques. The response or signal measured is inversely proportional to the concentration of the antigen in the unknown sample. This is because, greater the response, the less antigen in the unknown was able to "compete" with the labeled antigen for binding with the antibodies.

b) **Heterogeneous ELISA**: In a heterogeneous immunoassay, also referred to as the "sandwich assay," a "sandwich" complex is formed with an antigen coupled between a primary antibody and a secondary antibody. The primary antibody is immobilized onto a solid surface which may be typically a plate or the surface of a tube or a bead. The secondary antibody is labeled with an enzyme which reacts with a substrate or an introduced chemical reagent to give a relative indication of the concentration of the antigen (the extent of the reaction is a relative measure of the concentration of the antigen). The antigen of interest is captured between the two antibodies which can further be separated from the unreacted solution and analyzed. A heterogeneous immunoassay includes an extra step of removing the excess or unbound antibody or antigen from the

reaction site, using a solid phase reagent usually which is the solid wall of a tube or a plate or beads made of various materials.

Competitive assays have the disadvantage to non-competitive assays since the amount of unbound sites are measured, which result in difficulties to distinguish low levels of analyte from a zero level i.e. blank value (Giraudi et al., 1999 b). 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 sandwich method is very sensitive and specific as it involves two types of antibodies that are specific to a particular analyte (Krick and Wild, 2001). Also sandwich type of ELISA generates less toxic waste when compared to other types of ELISA.

1.3 Biosensing techniques

1.3.1 Introduction to biosensors

Biosensors are characterized by a high level of specificity generated by the bio-component, which specifically reacts with a given analyte or substrate. According to the IUPAC's (International Union of Pure and Applied Chemistry) definition, a biosensor may be defined as "A device that uses specific biochemical reactions mediated by isolated enzymes, immuno-systems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals". The combination of this specificity with a sensitive transducer gives the biosensors their unique characteristics for the detection of a variety of analytes, even when they occur in complex matrices (Zhang et al., 2005).

The advantages of biosensing techniques compared with other traditional analysis techniques are summarized below:

- ✓ Extraction and clean-up analytical steps are reduced, thereby shortening the process time, making it possible to monitor a large number of samples.
- ✓ Separation and analysis procedure could be achieved at the same time, making it suitable for online automated analysis.

- ✓ Neither high cost is involved nor skilled personnel are needed which make it very convenient to use.

Biosensors have mainly been applied for analytical purpose in environmental chemistry (Mozaz et al., 2004), clinical practice (Newman and Turner, 2005) and analysis of different foods (Neethirajan et al., 2005). The most salient advantage of biosensor is that, it enables miniaturization and *in-situ* measurement. In biosensors the following sequence of processes takes place as shown in **Figure 1.8**;

1. specific recognition of the analyte by bio-receptor
2. transduction of the physiochemical effect caused by the interaction with the receptor into an electrical signal
3. the sensor should be field deployable
4. the sensor should be cost-effective

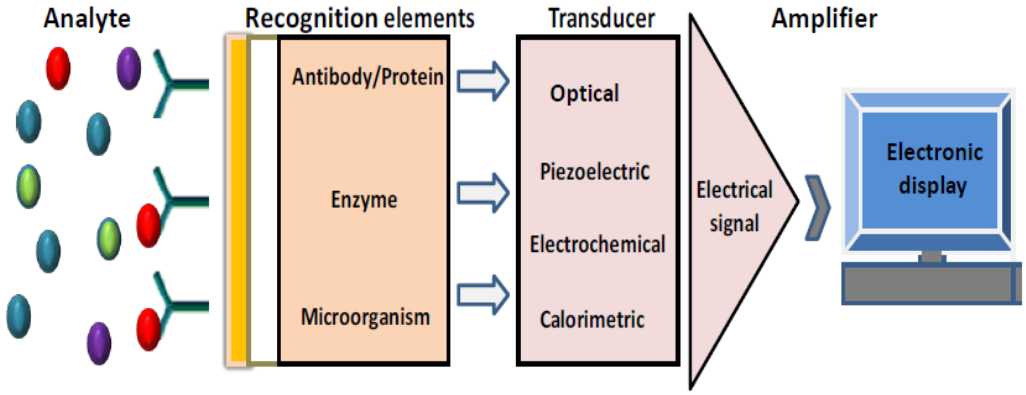


Figure 1.8 Schematic of a biosensor showing its various components; bioreceptor, transducer, signal amplifier and electronic display.

1.3.2 Classification of biosensors

Biosensors can be classified either by the type of biological signaling mechanism they utilize or by the type of signal transduction they employ. Biosensors can also be classified according to their method of signal transduction. A transducer should be capable of converting the bio-recognition event into a measurable signal. This is done by measuring the change that occurs in

the bio-receptor region. Based on the kind of transducers used, biosensors may be classified as follows:

a) **Optical transducer**: A biosensor with optical transducer converts an optical signal into an electrical signal. In this case, either an optical signal is generated (e.g. colour, luminescence or fluorescence) or a change in the optical properties of the surroundings such as absorption, emission, reflectance or a change in an inter-ferometric pattern following the antigen-antibody complex formation is measured. In principle, this happens by generating an electrical current proportional to the intensity of incident optical radiation. Signal changes are recorded by a photo-detector and then, transformed into an electrical signal. Optical transducer represents one of the largest and fastest growing areas in biosensor technology.

b) **Thermal transducer**: In this type, the biosensors measure the changes in temperature in the reaction between a bio-component and a suitable analyte. The total heat produced or consumed in a reaction is proportional to the molar enthalpy and the number of moles produced. These temperature changes are reflected in the reaction medium. Temperature changes are recorded by thermistors. Among the various thermal transducers, thermistors comprise a major share for biosensor development.

c) **Electrochemical transducer**: The third and most commonly used transducers are electrochemical. These devices measure the current produced from oxidation and reduction reactions. The current produced can be correlated to either the concentration of the electro-active species present or its rate of production or consumption (Mulchandani et al., 2001). Different types of the electrochemical transducers have been employed in the development of biosensors. The main electrochemical transducers are amperometric (measuring of current), potentiometric (measuring of electrode potential or voltage differences) and conductometric (measuring of conductivity or resistance). Amperometry is the most popular method used for electrochemical detection.

d) **Piezoelectric or Mass sensitive transducer**: These biosensors are based on mass-sensitive measurements and can detect small mass changes caused by chemical binding to small piezoelectric crystals. **Figure 1.9** represents a schematic illustration for classification of biosensors on the basis of transducers.

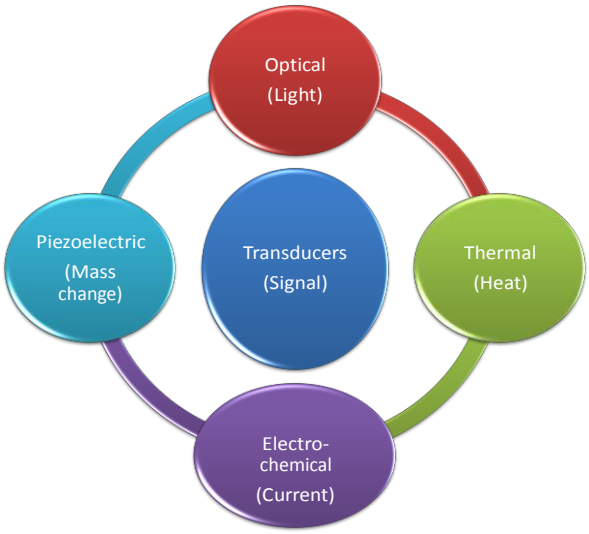


Figure 1.9 Different transduction methods of biosensor.

1.3.3 Performance criteria of biosensor

The performance of a biosensor is evaluated on the basis of various parameters. Parameters significant in evaluating biosensor performance are listed below:

a. **Specificity**: Ability of a method to detect one element in the presence of another element.

b. **Sensitivity**: The slope of the calibration curve. If the curve is in fact a 'curve', rather than a straight line, then of course sensitivity will be a function of analyte concentration or amount.

c. **Precision**: Measure of instrument reproducibility. In other words, it is the ability to obtain the same value with repeated measurements of a process variable.

d. **Stability and lifetime**: The operational stability of a biosensor may vary according to the sensor geometry, method of preparation, as well as on the applied enzyme or transducer. Storage stability is stability of biosensor if it is stored under optimum conditions. This gives idea of lifetime of the biosensor.

e. **Response time**: The time required for the detector output to go from the initial value to a percentage (e.g. 99%) of the final value.

f. **Sample throughput**: The number of results that is produced by an instrument divided by time of operation.

1.4 Present state of art and gaps in the existing research

The detection and quantification of aflatoxins in food have been achieved through several sensors. The conventional analytical methods though sensitive, suffer from several limitations and disadvantages such as;

- Lack of reusability
- Long analysis time
- Low throughput
- High solvent usage
- Generate toxic waste in high volume
- Tedious sample preparation
- High cost per sample
- Require highly trained professional to operate

In the last decade, there has been a remarkable development in the field of miniaturization and automation of chemical and biochemical sensor devices (Berthold et al., 2002). This is because, it is found that, miniaturization improves the speed and reliability of the measurements and dramatically reduces the sample volume and the system costs. Among all the analytical methods that are mostly used for aflatoxin detection, ELISA technique has become immensely popular due to its specificity and selectivity. Most immunosensors are of single use due to the high stability of the antigen-antibody complex once it is formed and the biological sensitive element is usually immobilized onto the surface of the transducer. The present scenario of aflatoxin sensors is presented in **Figure 1.10**. As compared to the period 2009-2010 (Shephard et al., 2012), ELISA remained the main analytical technique in the monitoring studies for AFM1) in milk and milk products (55%), followed by HPLC (27%), TLC (11%) and fluorometry (7%).

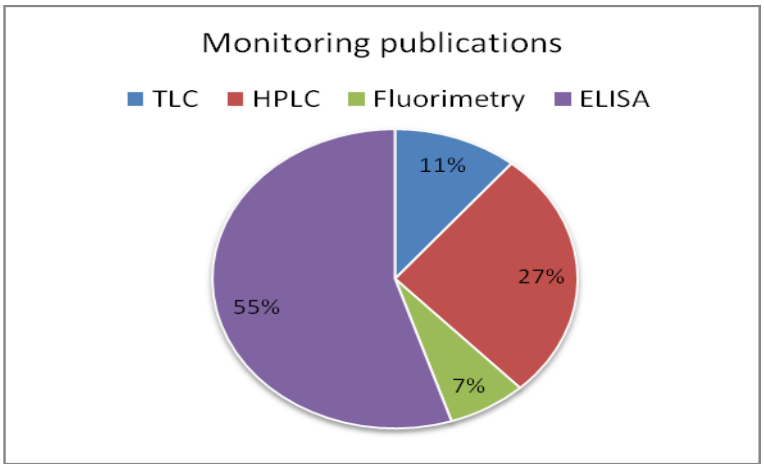


Figure 1.10 Present scenario of AFM1 analysis (Shephard et al., 2012).

ELISA tests are favored as high throughput assays with low sample volume requirements and often less sample extract clean-up procedures compared to conventional methods such as TLC and HPLC. The advantages of the ELISA technique is the ease of use and the cost of the equipment required. Moreover, the methods are quantitative. They are rapid, simple, specific and sensitive for the detection of mycotoxins in foods and feeds.

Although the antibodies have the advantage of high specificity and sensitivity because the target compounds are mycotoxins but not only the specific antigens, compounds with similar chemical groups can also interact with the antibodies. This so-called matrix effect or matrix interference and it commonly occurs in ELISA methods resulting in underestimates or overestimates in mycotoxin concentrations in commodity samples (Rubert et al., 2010). Additionally, insufficient validation of ELISA methods causes the methods to be limited to those matrices for which they were validated. Therefore, an extensive study on the accuracy and precision of an ELISA method over a wide range of food commodities is needed and a full validation for an ELISA method is essential and critical.

As discussed earlier, 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, 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 and Hazebroek, 2000).

In 1999, a paper was published by Sibanda et al. (1999), detailing a portable field assay for the detection of AFM1. 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) is producing 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 (0.5 μg/L). Again a positive test is needed to be verified by HPLC, but this was the first time when the AFM1 analysis could be performed away from the laboratory. Another interesting application using antibodies was 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 AFB1 in pig feed. In the test, they used monoclonal antibodies conjugated to colloidal gold particles which upon the reaction with immobilized AFB1–BSA yielded a visual pink band on the dipstick. Although this technology could only give a false positive result, the reported detection limit of 5 μg/kg was 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 dipstick provided a positive/negative result within 60 minutes for qualitative measurement.

Paniel et al., (2010) described the development of an electrochemical biosensor for the detection of AFM1 in milk, based on a competitive immunoassay that made use of magnetic beads, coated with anti-AFM1 antibody and screen-printed electrodes. The method was not a

new development, but rather another variant of the sensor methodology, about which, mention was made in the AFM1 reviews in the periods 2008-2009 (Shephard et al., 2010) and 2009-2010 (Shephard et al., 2012). Nivarlet et al. (2011) presented a rapid dipstick test for the determination of AFM1 in milk. This semi-quantitative test made use of gold-labeled antibodies that competed between AFM1 present in the milk sample and the toxin immobilized on the dipstick. When the sample is contaminated with AFM1, the antibodies could no longer recognize the immobilized antibody and no colour would be generated on the test line. The test did not require any pre-treatment or cleaning of the milk and gave a semi-quantitative response after 20 minutes of incubation when the strips were analyzed with an optical reader. Another strip-format test for AFM1 determination was developed by Wang et al. (2011). With this test, the whole analysis procedure of milk could be completed within 10 min, but the reported relatively high limit of detection (LOD) of 1 μg/kg drastically limited its use in practice.

In brief, the following scopes have been identified in the aflatoxins research;

1. There is a need of ultrasensitive detection system for aflatoxins in real samples.
2. The device or sensor should be used for high throughput analysis of samples.
3. The detector system should have a single platform for simultaneous analysis of different aflatoxins.
4. The sensor should be field deployable.
5. The sensor should be cost-effective.

1.5 Objectives of the work

The research work presented in this thesis is aimed at development of miniaturized immunosensing techniques for ultrasensitive, high throughput analysis of AFM1, AFM2 in milk and related products and AFB1 in groundnuts or peanuts. To accomplish these, the following objectives were set as major goals.

Objective 1. Development of novel biosensing techniques for ultrasensitive analysis of aflatoxins in various food products such as milk, milk products and groundnuts.

Objective 2. Development of miniaturized multi analyte immunosensors for simultaneous detection of AFM1 and AFM2 in milk and milk products.

Objective 3. Development of flow injection techniques for analysis of aflatoxins in milk and related products.

Objective 4. To investigate novel approaches for online monitoring of aflatoxins.

1.6 Thesis structure

The thesis comprises in six chapters and each of these chapters has been detailed below.

1.6.1 Chapter 1: Introduction

This chapter gives a description of aflatoxin contamination in various food commodities, its consequent health effects and the established quality standards set by national and international agencies. Analysis of aflatoxins (AFM1 and AFB1) in milk and food by conventional techniques, gaps in the existing research and need for biosensor is discussed here. Various aspects of biosensors, particularly immunosensors, current state of art for aflatoxins detection techniques are also reviewed. This chapter discusses about gap in the existing reported work, objective of the proposed doctoral work and finally about the thesis structure.

1.6.2 Chapter 2: Development of an ultra sensitive immunoassay for analysis of AFM1 in milk

This chapter gives an account of an ultrasensitive assay development for AFM1 in milk. This also describes the assay to be high throughput as it was performed in 384 microwell plate with very less sample volume (40µL). Milk samples with different fat percentages were analyzed. Novel sample pre-treatment procedure was investigated to avoid matrix interference due to presence of fats in milk samples. The AFM1 analysis was done by both chemiluminescence and fluorescence techniques in microwell plates and results were discussed.

1.6.3 Chapter 3: Multi analysis of aflatoxins

Simultaneous analysis of AFM1, AFM2, AFB1 and AFG1 were done in 384 microwell plate and the cross-reactivity results were analyzed. The binding specificity of monoclonal antibody of AFM1 was quantified against other aflatoxins. Cross reactivity studies of different aflatoxins were carried out. Subsequently, mixture analyses of AFM1 with AFB1 and AFM2 were investigated. The multi analysis of aflatoxins was also investigated on a customized novel device

by fluorescence imaging.

1.6.4 Chapter 4: Application of label free immunosensor for analysis of aflatoxins

This chapter describes the application of an immunosensor, based on impedance measurement of AFM1 and AFB1 in milk and peanuts respectively. Silver wires were used as two electrode set-up for impedance analysis. The immunosensor used here is label free and ultra sensitive. Flow injection analysis of AFM1 and AFM2 were done by impedance measurement in milk and related milk products such as drinking yogurt and commercially available flavored milk. Customized novel inter digitated electrode devices were tested for label free detection and quantification of AFM1 in milk sample.

1.6.5 Chapter 5: Validation of developed AFM1 assay in real samples

This chapter briefly describes about the validation of developed assay with control methods. A survey was conducted to detect the level of AFM1 contamination in commercially available milk samples and infant formula milk of local market. The developed immunoassay for AFM1 in milk by sandwich ELISA in microplate was cross validated with HPLC and an AOAC approved control kit assay.

1.6.6 Chapter 6: Conclusions and future scope of work

This chapter gives a review of all the five chapters' conclusions and proposes the future scope of work.

An outline of the different stages undertaken in this thesis to meet the aims and objectives is shown in the flowchart below (**Figure 1.11**).

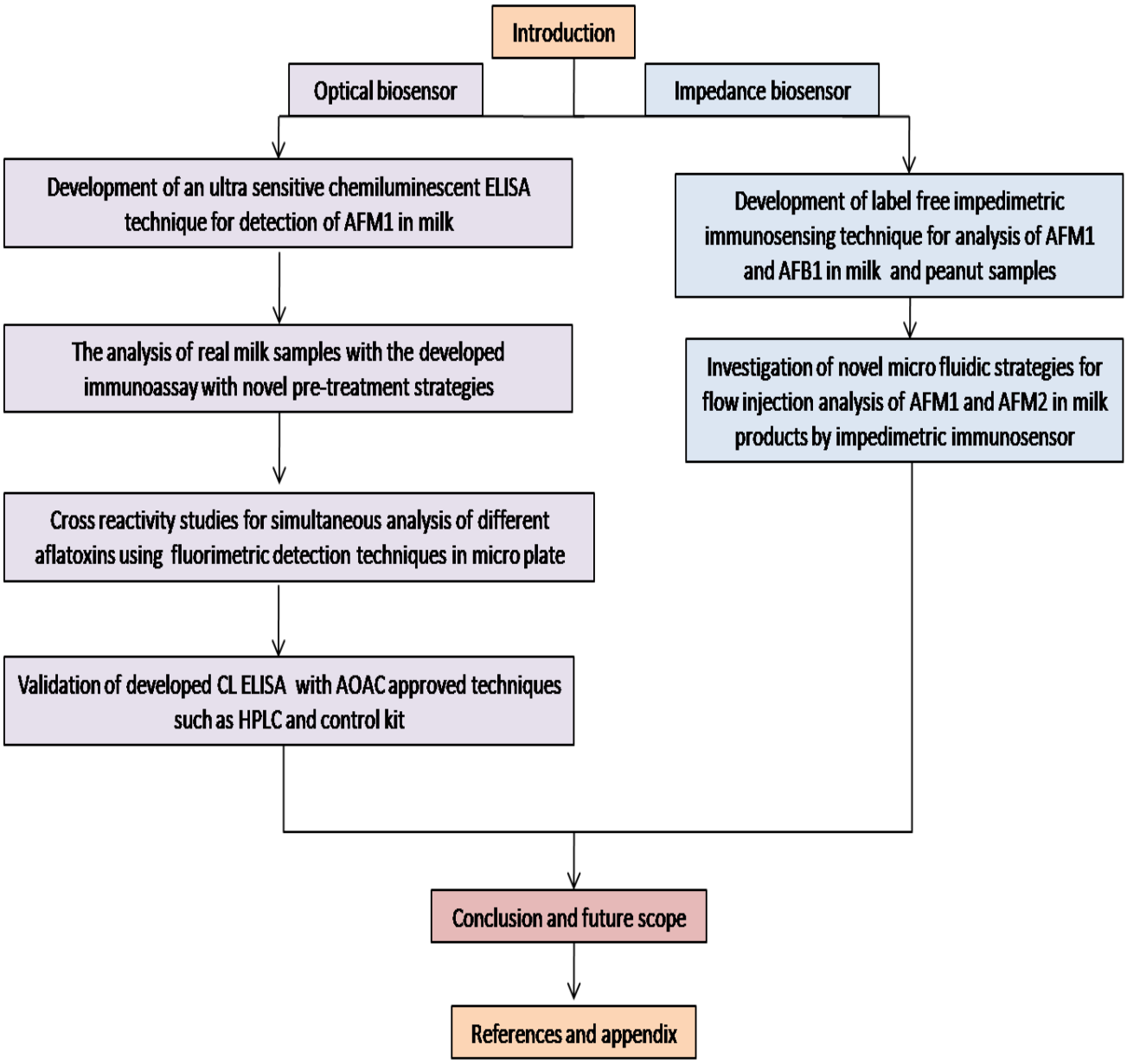
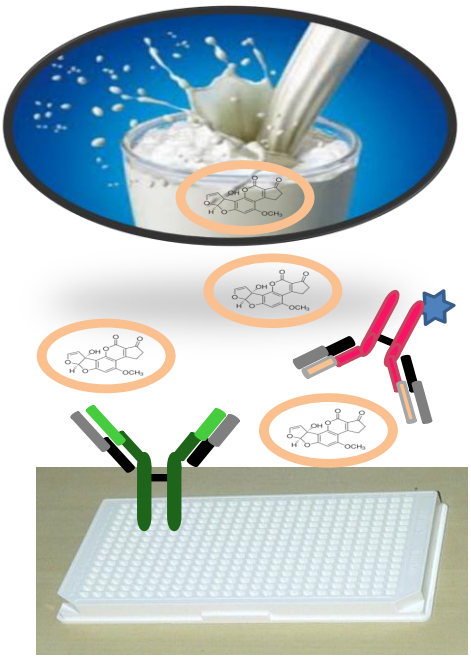


Figure 1.11 Flowchart detailing the different stages of the work in this thesis.

Chapter 2

Development of an ultra sensitive immunoassay for analysis of AFM1 in milk



Schematic representation of chapter content

2.1 Introduction

Milk is an important agricultural product and is part of the everyday nutrition of humans. As milk is the main nutrient for infants and children who are considered to be more susceptible to adverse effects of mycotoxins, the presence of AFM1 in milk is a concern. On the other hand, milk is not only consumed as liquid, but also utilized for the preparation of infant formula, yogurt, cheese, and milk-based confectioneries including chocolates, sweets and pastries. Therefore, it is important to determine AFM1 levels in milk and milk products in order to protect consumers in various age groups, from its potential hazards (Gurbay et al., 2006). It is apparent from a recent study that, the utilization of milk in infant food formulations has increased substantially in recent years (Kim et al., 2000). Therefore, the quality of milk products has increased the reflective influence on the health of people in various age groups.

Contamination of milk with AFM1 and its ill effects on humans are well documented (van Egmond, 1989; Thirumaladevi et al., 2002; Rastogi et al., 2004; Siddappa et al., 2012). European Communities have fixed the limit to a maximum of 50 ng/L (European commission, 2006). The US regulation has prescribed a limit of 500 ng/L for AFM1 in milk and dairy products. Risks (especially its carcinogenic and teratogenic effects) due to the consumption of AFM1-contaminated milk have been assessed in many countries, but there are no reports on the distribution of AFM1 in milk produced by small-scale farmers or by large dairies in India especially at the milk collection centers. India is currently the largest milk producer in the world but, testing of milk for AFM1 is not practiced by the milk industry in India. Recently, FSSAI in 2011 has set the maximum permissible limit of AFM1 in milk as 500 ng/mL (FSSAI, F.No. 2-15015/30/2010). So, the objective of this research work attributes to the lack of rapid and cost effective technologies for estimation as well as ultrasensitive detection of AFM1 in milk.

2.1.1 Milk as a complex matrix

Milk is a complex matrix consisting of a variety of compounds such as proteins, fat, minerals, vitamins, and sugars. Fat globules are the largest particles in milk that have dissimilar densitites and thereby cause matrix effects. Matrix effects are defined as induced deviation from

theoretically predicted results due to the constitution of the sample matrix (Oubiña et al., 1997). Fats can cause blocking of the active sites in clean up techniques, thus ideally fats are removed at 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 (Maqbool et al., 2009; Paniel et al., 2010).

2.1.2 AFM1 analysis in milk

From the literature, it is known that, chemically AFM1 is hydrophobic and AFM1 in milk resides in the hydrophobic cavities of the casein protein. Therefore, casein rich foods such as cheese have a 3 to 6 fold increase in AFM1 compared to low protein products (Brackett and Marth, 1974; van Egmond, 2002). The casein content of cow 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%); this in turn causes a hydrophobic surface of the protein making it insoluble in water and attractive to the AFM1 molecule (Deveci and Sezgin, 2006). According to Krska et al. (2008), AFM1 cannot be destroyed or removed readily, it can be excluded from milk only by eliminating AFB1 from the diet of animals. AFM1 has also been found in other dairy based products such as cheese and yogurt (Sharman et al., 1989; van Egmond, 1989; Martins and Martins, 2004). As formerly discussed, the AFM1 is a detoxification product from AFB1, and AFM2 is a detoxification product of AFB2, however AFM2 is rarer than M1 and not very toxic so, it receives less interest.

2.1.3 Gaps in the existing research

Although various analytical methods have been successfully applied for AFM1 estimation in milk, immunological methods for the estimation of various aflatoxins have been shown to be cost-effective (Pestka et al., 1981) and therefore preferred for surveillance. There are reports on sensitive detection of AFM1 in milk but these methods lack miniaturization and high throughput system (**Table 2.1**). Previously developed enzyme immunoassays for AFM1 analysis using conventional colorimetric detection with chromogenic substrates (Sibanda et al., 1999; Badea et al., 2004) were allowed to reach detection limits not lower than 5-10 ng/L in milk. In addition,

they usually require a 60-120 min incubation time, as well as several analytical steps, which limits their extensive use for rapid analysis of AFM1.

Table 2.1 Summary of reported ELISA for AFM1 detection.

Assay type	Range	LOD achieved	Reference
Direct competitive ELISA (MNPs)	4-250 ng/L	8 ng/L	Radoi et al., 2008
Indirect competitive ELISA	0-100 ng/L	5 ng/L	Anfossi et al., 2008
Indirect competitive ELISA	10-0.01 ng/mL	0.24 ng/mL	Thirumaladevi et al., 2002
Flow-injection immunoassay	20-500 pg/mL	11 pg/mL	Badea et al., 2004
Competitive ELISA	28-164 ng/kg	28 ng/kg	Rastogi et al., 2004
Indirect competitive ELISA	0.1-3.2 ng/mL	0.04 ng/mL	Pei et al., 2009
Competitive ELISA	0-1000 ng/L	39 ng/L	Parker et.al., 2009
IAC purification, liquid chromatography (LC) separation and fluorescence detection	0-437 ng/kg	3 ng/kg	Iha et al., 2013
Competitve ELISA using electrochemical sensor	5-500 pg/mL	10 pg/mL	Paniel et al., 2010
ELISA using surface plasmon-enhanced fluorescence spectroscopy (SPFS) through the excitation of long range surface plasmons (LRSPs)	0.01-10,000 pg/mL	0.6 pg/mL	Wang et al., 2009

2.2 Design of a sensitive immunoassay

The immunoassay design was based on the development of ultrasensitive ELISA for high throughput analysis of AFM1 in milk. The antibody's suitability was validated by designing and optimizing an ELISA protocol using the antibody by CL method. Once the antibodies were validated, they were then implemented in the immunassay. A sandwich immunoassay format was chosen for the assay configuration. As discussed, the sandwich-type immunoassay is an effective bioassay due to the high specificity and sensitivity and it avoids production of more toxic waste

as compared to other ELISA techniques which use two sets of antigens. The matrix interference was also studied which arises due to presence of fat in milk samples. In this regard, a novel sample pre-treatment of milk before analysis was investigated.

2.2.1 Principle of chemiluminescence

As discussed earlier, an optical biosensor converts an optical signal into an electrical signal. This optical property can either be absorption, emission, fluorescence, luminescence, reflectance or a change in an inter-ferometric pattern. Optical detection method is regarded as one of the sensitive techniques for mycotoxin analysis. These sensors belong to a class of a contactless method of measurement eliminating backward influence of measuring device on the object of measurement. The additional advantage that optical transducers have over other methods is the use of visible radiation that allows versatile detection in many different areas and also accommodates miniaturization. Furthermore, the optical signal is not influenced by electrical, magnetic or ionic fields.

Among the most extensively reported transducers for optical biosensors, Photo Multiplier Tubes (PMTs) and Charge-Coupled Devices (CCDs) have major contribution (Chouhan et al., 2006). PMTs are extremely sensitive detectors of light in the ultraviolet, visible and near-infrared ranges of the electromagnetic spectrum. These detectors amplify or multiply the current produced by incident light by as much as 100 million times in multiple dynode stages, enabling individual photons to be detected when the incident flux of light is very low. A CCD is a light-sensitive integrated circuit that stores and displays the data for an image in such a way that each pixel (picture element) in the image is converted into an electrical charge, the intensity of which is related to a colour in the colour spectrum (Martine et al., 1994). Mainly it comprises of an array of photosensitive elements (photoelectric convertors) configured either in a line or in a matrix. An array of photoelectric convertors where charge is accumulated, corresponds to the reflected light from the elementary area of observed object. Imaging time is shorter and multiple images can be obtained and stored easily. Although costly, their use reduces the recurring cost of purchasing film and film processing, besides the cost of the processing unit.

For an effective screening and monitoring of AFM1 in foodstuffs at ultra low (pg/mL) levels, analytical methods combining simplicity with high detectability and analytical throughput are required. This can be achieved by means of immunological methods in combination with a highly sensitive detection of the label. Enzyme labels have experienced widespread popularity since their first use in 1971 in an ELISA (Van Weeman and Schuurs, 1971). The enzyme labels are not consumed, and their reactions can be initiated and stopped. Furthermore, enzymes amplify the signal because an enzyme can produce many detectable molecules, up to 10^7 molecules of substrate per minute per enzyme molecule, by its catalysis of a substrate product reaction. They can be used in both homogeneous and heterogeneous immunoassays. Enzymes are the most commonly used labels as they can produce coloured, fluorescent, luminescent, and electroactive compounds enabling detection by a variety of techniques (Gracia et al., 2005).

Enzyme labels detected by CL substrates, such as the luminol (5-aminophthalyhydrazide)/peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase represent the most sensitive detection system in immunoassay development. CL compounds produce light in response to chemical reactions and as labels in immunoassay, they can be more sensitive than radio labeled and fluorescent forms (Krick and Wild, 2001). In addition, the CL signal detection can be performed immediately after substrate addition, thus shortening the overall analytical procedure when compared with conventional colorimetric assays (Magliulo et al., 2005). Gracia et al. (2005) have reported that luminol could be used as an enzyme substrate for HRP that yield high sensitivity. A CL sandwich ELISA format was adopted for AFM1 assay development as shown in **Figure 2.1**.

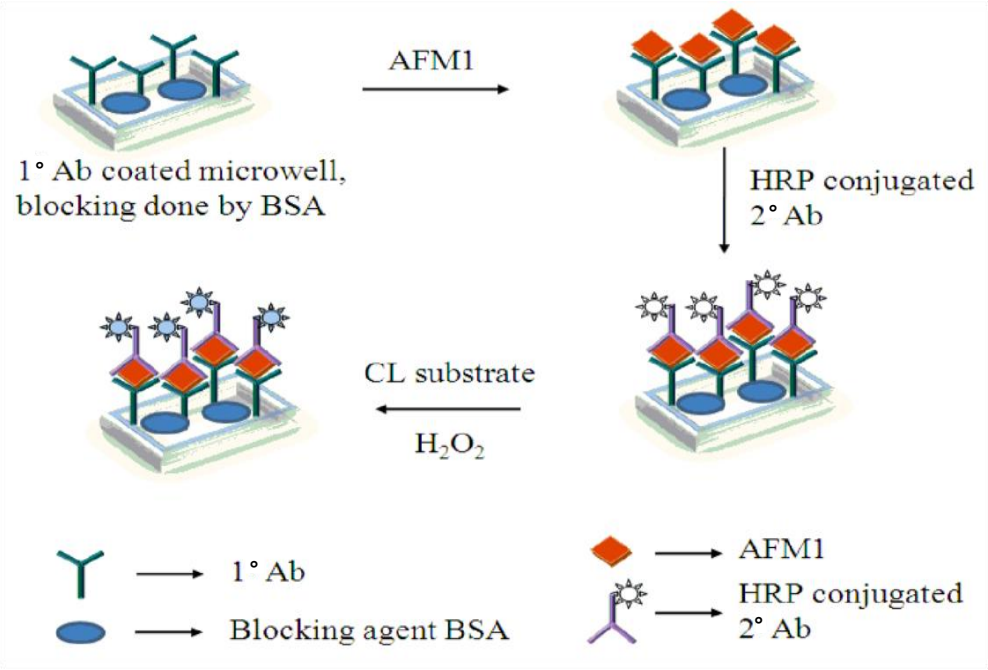


Figure 2.1 Scheme of CL sandwich ELISA for detection of AFM1 in milk.

Initially the system was optimized for the antibodies being used, with varying the antibody concentrations and time of incubation. Milk was studied in the system to determine whether there would be any matrix effect. Then, several sample pre-treatment techniques were investigated to overcome interference attributed by whey proteins and fat in complex matrix like milk.

2.2.2 Materials and methodology

AFM1, Bovine serum albumin (BSA), Tween 20, luminol and Certified Reference Material (CRM) ERM-BD282 (AFM1 in whole milk powder, < 0.02 µg/kg) were purchased from Sigma–Aldrich (USA). Hydrogen peroxide (H_2O_2) 30% (w/v), acetonitrile (ACN) HPLC grade, trichloro acetic acid (TCA), sodium chloride (NaCl), methanol (99% pure) were purchased from Merck (Germany). Sodium hypochlorite (4%) solution was purchased from Fisher Scientific (India).

All the AFM1 solutions were prepared inside a Glove box in a maintained inert (N_2) atmosphere. AFM1 stock solution was prepared by dissolving the AFM1 powder in 5% ACN

(v/v) in phosphate buffered saline (PBS) at a concentration of 5 µg/2mL and stored at –20 ºC. A wide dynamic range of working standard solutions in the range of 0.005-250 pg/mL was prepared by diluting the stock with 5% ACN.

Anti AFM1 fractionated antiserum primary antibody (1º Ab) raised from rat, Horse Radish Peroxidase (HRP) conjugated secondary antibody (2º Ab) raised from rabbit and Fluorescien isothiocyanate (FITC) conjugated 2º Ab were purchased from Abcam (UK). Upon delivery of the antibody solution (1 mg/mL), the contents were split into 5 aliquots and stored at -18 ºC to avoid repeat thaw – freeze cycles which reduce the antibody activity. The stock solution of rat monoclonal [1C6] 1ºAb, 100 µg (1 mg/mL) was diluted with 500 µL of pyrogen free de-ionized water. It was divided into 2 fractions. The first fraction containing 400 µL was stored at −20 ºC. From the second fraction, working 1º Ab solution was prepared prior to the experiment by serial dilution in coating buffer as 1:1000, 1:2000 etc. The stock solution of 1 mg (2 mg/mL) rabbit polyclonal to rat IgG-H & L (HRP) 2º Ab was diluted with 500 µL of de-ionized water. It was also divided into 2 fractions. The first fraction containing 400 µL was stored in −20 ºC. From the second fraction, working 2º Ab solution was prepared prior to the experiment by serial diluted in PBS. FITC labeled 2º Ab solutions were also prepared in the similar manner.

Centrifugation, shaking and filtration of the samples was done by Spinwin mini centrifuge, Spinix shaker and syringe filter repectively purchased from Tarsons (India). 0.22 micron filter papers (25mm diameter) were obtained from Millipore (USA). White 384 well polystyrene microtiter plates were purchased from Nunc (Denmark). For CL measurement, VictorX^4 2030 optiplate reader from Perkin Elmer (USA) was used. Glove box, Cole Parmer (USA) was used for the handling of AFM1 standard solution. Water produced in a Milli-Q system (Millipore, Beford, MA, USA) was used for preparing all the solutions. Certified ultra high pure N_2 (99.9%), pH meter (Seven Multi Mettler Toledo, 8603, Switzerland) were used in various experimental steps.

All other reagents used for CL reaction were of analytical reagent grade and prepared as per available literatures. CL measurements were done and recorded. For processing of data, Origin 6.1 (Microcal, USA) was used. Experimental data obtained was also treated statistically for

regression coefficient, equation for straight line, standard deviation and % CV using the said software.

2.2.3 General protocol for CL ELISA

Initially the dilutions of antibodies and incubation times were optimized. The antibody concentrations were optimized by taking various dilutions and checking the stable signal intensity by CL method. The developed ELISA method was performed as follows. The sandwich ELISA system was optimized by producing calibration charts using a series of standards of AFM1 ranging from 0.005 ng/L to 10,000 ng/L in 5 % ACN. The dilution of primary antibody was varied from 1:1000 to 1:64000, the dilution of HRP labeled secondary antibody was varied from 1:1000 to 1:64000 and the time of the incubation for the two antibodies were varied from 30 min to 2 h. The coating of the micro well plate was done at 4 ºC overnight. All other incubations were performed at room temperature. The plate was measured by VictorX^4 2030 (**Figure 2.2**) after addition of luminol and H_2O_2.

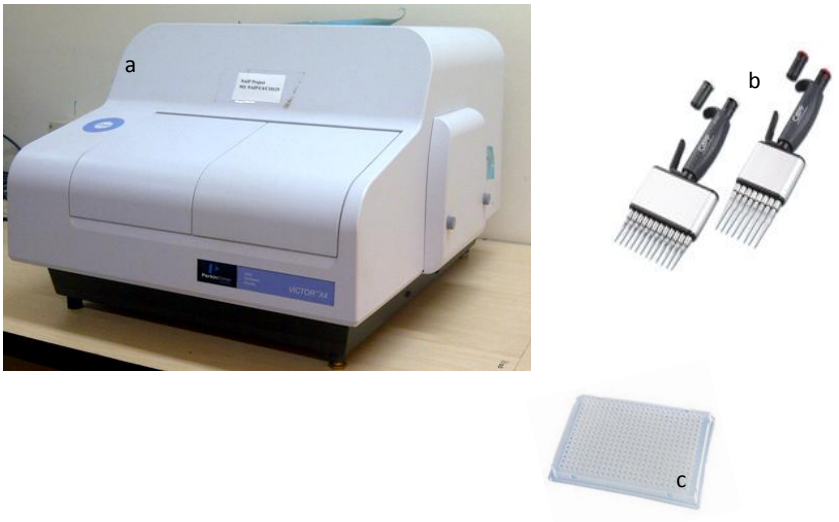


Figure 2.2 Instruments used for high throughput analysis; a:VictorX^4 2030 multi plate reader; b: multi channel pipette; c: 384 micro well plate.

2.2.4 Experimental and result analysis

2.2.4.1 Optimization of antibody dilutions

The 1º Ab solutions were prepared by serial dilutions as 1:1000 to 1:64,000 and in similar way 2º Ab solutions were prepared. Then each concentration of the 1º Ab was incubated with each of 2º Ab concentrations. From the experiments, it was observed that 1º Ab at a dilution of 1:16000 showed best signal intensity with HRP labeled 2º Ab at a dilution of 1:32000 (**Figure 2.3**).

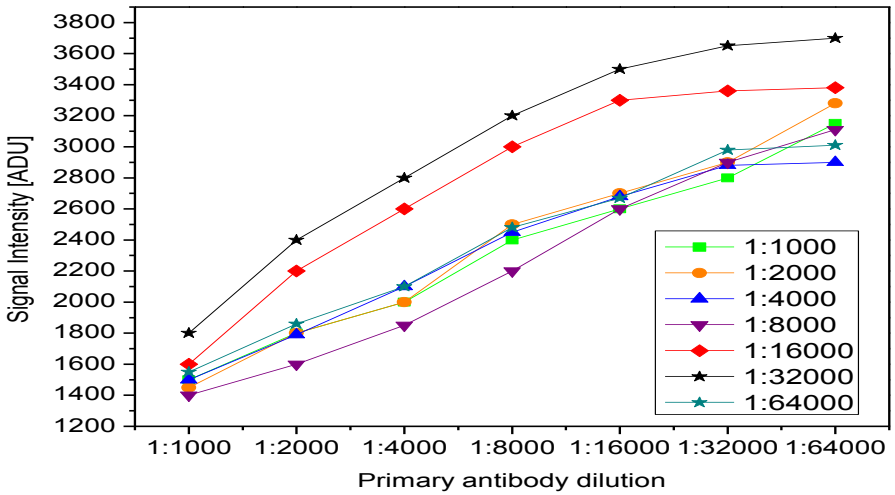


Figure 2.3 Titration curve for primary antibody and HRP labeled secondary antibody in CL assay.

2.2.4.2 Optimization of incubation time

The incubation time plays a very crucial role in an immunoassay. In sandwich ELISA, there is scope of two incubations with two different antibodies. **Figure 2.4** shows the experimental results for 1st incubation with monoclonal antibody with AFM1 antigen (50 pg/mL) keeping the second incubation time constant at 1h. From, the graph it was found that after 1.5 h to 2 h, the signal intensity did not vary much and remain almost contant. So, the incubation time was chosen as about 2 h for 1º Ab and AFM1 binding. In sandwich assay, the 2º labeled antibody recognizes the 1º antibody-antigen complex. Therefore the optimization for second phase incubation time was done. **Figure 2.5** shows the experimental results for second phase incubation time optimization. It was observed that, from the signal intensity became very stable at 45-60 min and after 70 min, it decreased. So, the second incubation time was chosen at 60 min.

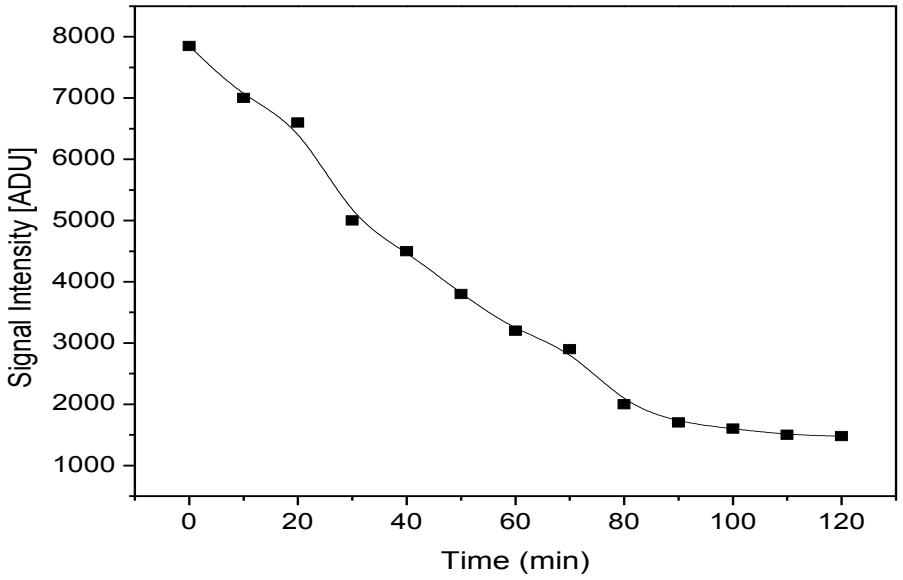


Figure 2.4 Optimization of incubation time for primary antibody and [AFM1] 50 pg/mL in CL assay.

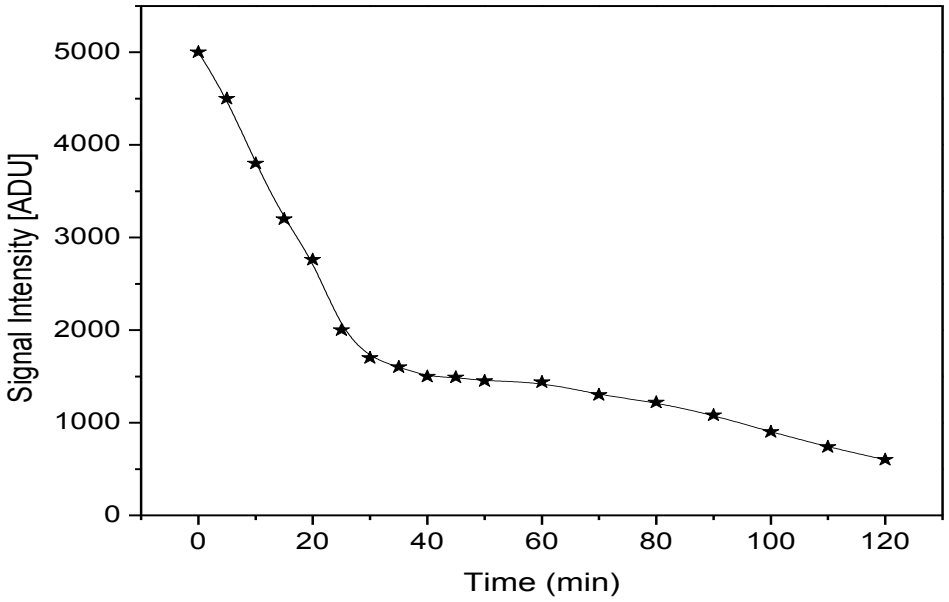


Figure 2.5 Optimization of incubation time for [AFM1] 50 pg/mL and HRP conjugated antibody in CL assay.

2.2.4.3 Standard calibration curve of developed CL assay

The assay for AFM1 analysis was done in PBS for optimization of parameters such as dilutions of antibodies, incubation time for each antibody with AFM1. ELISA was performed in 384 microwell plate as it facilitates high throughput sample analysis when compared to 96 well plate. Moreover the sample volume used in 384 well plate is less and it generates less toxic waste. Optimized 1°Ab was diluted to 1:16000 in CB and coated as 40 µL/well in triplicate. The plate was covered and kept at 4 °C for overnight. Then plate was washed 3 times by rinsing the wells with 60 µL PBS. The remaining protein binding sites in the coated wells were blocked by adding 40 µL of blocking solution and kept for about 1 h at room temperature. The plate was washed once with 40 µL PBS. Following this step, AFM1 standard solution in the range 0.005-250 pg/mL was added as 40 µL/well. The plate was incubated for about 2 h at room temperature. Then optimized 2°Ab (diluted to 1:32,000 in PBS) was added as 40 µL/well. The plate was kept for 2 h at room temperature. The excess label was removed by washing with PBS. The CL substrate 10 µL/well was added which was added in the following manner; 1.2 µL of 0.5 M H2O2 + 8.8 µL luminol. The signal intensity was kinetically measured at steady state. **Figure 2.6** shows the standard calibration curve of various concentrations of AFM1 in the PBS buffer.

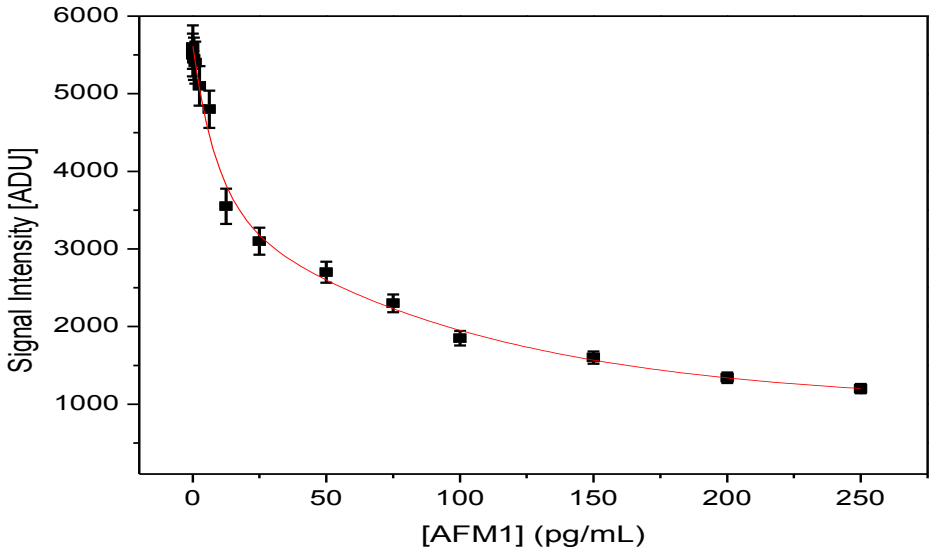


Figure 2.6 Standard calibration curve of AFM1 in PBS by CL assay.

The CL ELISA was then performed in the milk based buffer (milk: PBS at 1:1000) and the inhibition curve was obtained (**Figure 2.7**). Percentage inhibition (I %) was calculated as described by Arduini et al. (2009) in presence and absence of analyte

$$I\,\% = \left(\frac{I_0 - I_A}{I_0}\right)100$$

I_0 = signal intensity of blank and I_A = signal intensity of spiked sample.

The IC_{50} is defined as the half maximal inhibitory concentration. It is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular toxin, drug or other substance (inhibitor) is needed to inhibit a given biological process by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance. From **Figure 2.7**, the IC_{50} value was obtained as 6.25 pg/mL. The relative standard deviation (R.S.D.) was calculated as 1.1 and regression coefficient (R^2) was 0.979. The lower limit of detection (LOD) was calculated to be 0.005 pg/mL. The analytical figures of merits of the assay are presented in **Table 2.2**.

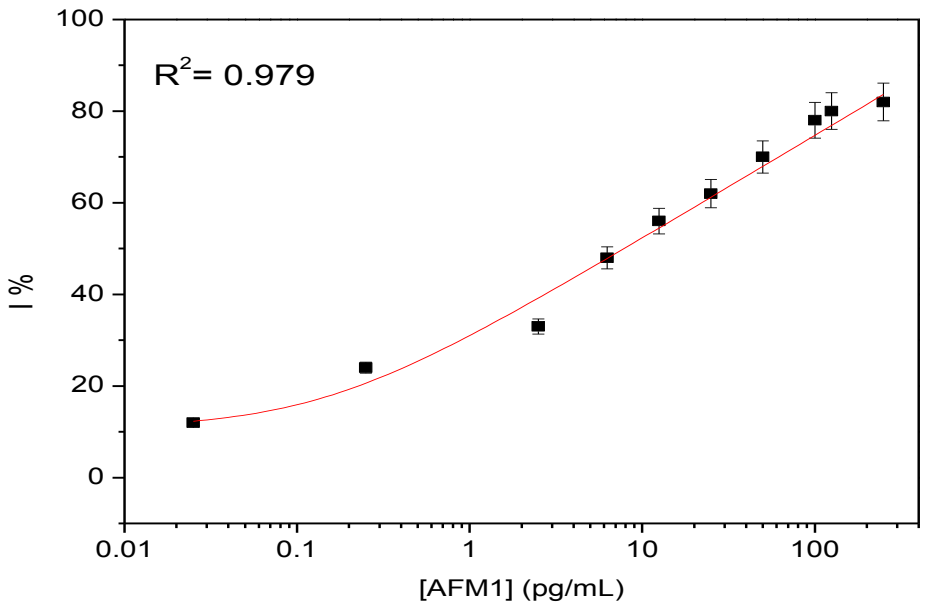


Figure 2.7 Inhibition curve of AFM1 in milk based buffer in CL assay

Table 2.2 Analytical figures of merit of the developed sandwich ELISA for AFM1 analysis in milk based buffer.

Analytical Parameters	Experimental findings
Dynamic range	0.005-250 pg/mL
Linear range	6.25-100 pg/mL
LOD	0.005 pg/mL
IC_{50}	6.25 pg/mL
R.S.D	1.1 %
R^2	0.979
Assay sample volume	40 µL
Response time	5 h
Sample throughput	384 samples in 5 h

2.2.4.4 Detection of AFM1 in real milk samples with different fat contents by CL assay

2.2.4.4.1 Milk sample collection and pretreatment

Commercial milk samples of different fat content were purchased from the local supermarket of Goa, India.

2.2.4.4.2 Matrix interference

One of the common challenges of immunoassay for food analysis is matrix interference, causing false positives by lowering the colour development or signal intensity. This occurs when either the enzyme activity is inhibited by the presence of interferences in the sample extracts or the interaction between the antigen/analyte and the antibody is hindered (Foster et al., 1983), or both of these phenomena has occurred concurrently in an immunoassay (Campbell et al., 1990). Matrix interference is a common problem for all aflatoxin-specific immunoassays, which could cause false positives.

The reported effects were either inhibiting enzyme activity only (Wilkinson et al., 1988; Ramakrishna et al., 1990; Li et al., 1994) or inhibiting both enzyme activity and antibody binding (Dell et al., 1990; Figueira et al., 1991). These matrix interferences can be reduced by a number of ways, such as dilution of sample extract or removal of interferences by sample cleanup procedures using solid-phase extraction or addition of heavy metal salts for precipitation of certain interferences. Dilution is a commonly used procedure to reduce the interferences (Chu et al., 1987; Figuera et al., 1990; Ramakrishna and Mehan, 1993), but this procedure would also reduce the quantifiable sensitivity. However, a common error occurring in an immunoassay is dilution error if the dilution factor is too great. When the second approach is used, sample cleanup procedure is generally kept as simple as possible to sustain the advantage of immunoassay as ease of use. Interferences in form of particles can be removed by centrifugation or filtration, and many sample preparation protocols have incorporated one of these procedures to remove the interferences.

2.2.4.4.3 Experimental steps adapted for analysis of milk samples containing different fat content to study matrix interference

To study the matrix effect and to circumvent the interference caused by whey proteins and fats present in milk, novel sample pre-treatment technique was carried out. For this purpose, milk samples having different fat % were collected. Three types of low fat containing milk samples were chosen such as 0.5%, 1.8% and 3% fat. In the experimental analysis, 3% fat containing milk sample was regarded as milk with high fat content where as, 1.8% fat containing milk sample was regarded as milk with medium fat content and 0.5% fat containing milk sample was regarded as milk with low fat content. The packaged milk samples containing different fat were divided into 4 fractions each. The first fraction was prepared by diluting 1 µL raw milk in 999 µL PBS making the final concentration to 1:1000. In the second fraction, 20% of 20 mM TCA was added to 1mL of milk and kept for 20 min at 4 ºC. This fraction was diluted in PBS as 1:1000. Similar to second fraction, third fraction was treated with TCA followed by centrifugation at 10,000 rpm for about 15 min. After centrifugation, the upper fat layer was completely removed. The fourth fraction was centrifuged at the 10,000 rpm for 15 min and filtered through a syringe filter using 0.22 micron filter paper and diluted to 1:1000 in PBS.

2.2.4.4.4 Results and analysis of matrix interference studies

The pre-treatment procedure carried out to avoid matrix interference was studied. It was observed that among the three tested pre-treatment procedures, milk treated with TCA, centrifuged and filtered (fourth fraction) in all cases showed best signal intensity. Milk samples having different fat content spiked with AFM1 were compared with assay in buffer. **Figure 2.8** shows the calibration curve obtained for different fat % in milk by CL assay.

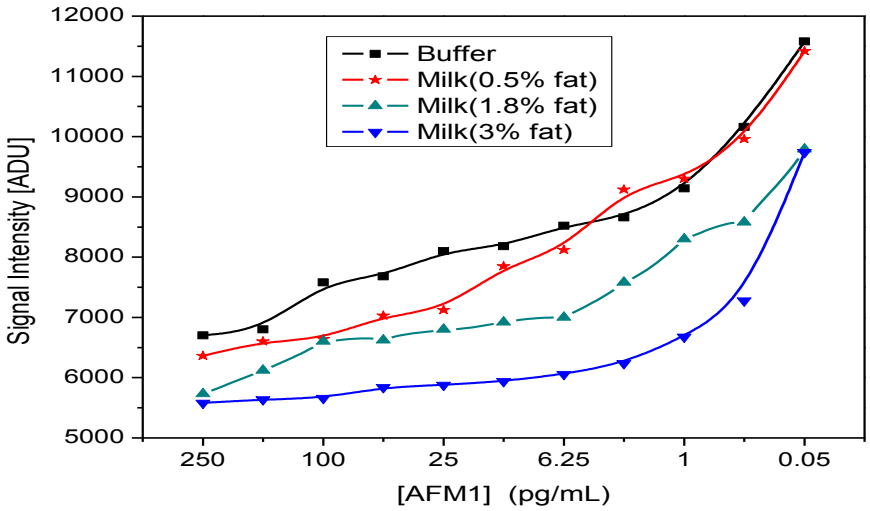


Figure 2.8 Calibration plot of [AFM1] in milk samples with different fat content by CL assay.

It was observed from **Figure 2.8** that, the milk sample containing 0.5% fat showed signal intensity close to that of buffer. From the inhibition graph in **Figure 2.9**, it was observed that 3% fat milk showed highest inhibition than 1.8% than 0.5% fat milk. The IC_{50} value for 3%, 1.8% and 0.5% fat milk were 16.95 pg/mL, 17.7 pg/mL and 39.88 pg/mL respectively. Two linear ranges in each calibration were observed for instance, in 0.5% fat milk one linear range was from 250 to 6.25 pg/mL with higher sensitivity and another was from 6.25 to 0.005 pg/mL with a lower sensitivity. From calibration curves (**Figure 2.9**), R.S.D. for 3%, 1.8% and 0.5% fat milk were obtained as 1.29, 1.95 and 1.69 and their R^2 values were 0.986, 0.965 and 0.982 respectively. Sensitivity of about 6–10% inhibition per decade change of AFM1 for 3% fat milk was observed. The LOD was found to be 0.005 pg/mL for 0.5% fat and 3% fat samples whereas,

a decrease in LOD for 1.8% fat sample was obtained as 0.05 pg/mL. In all the cases, signal suppression was found to be < 10%. The analytical figures of merit of the assay are summeraized in **Table 2.3**.

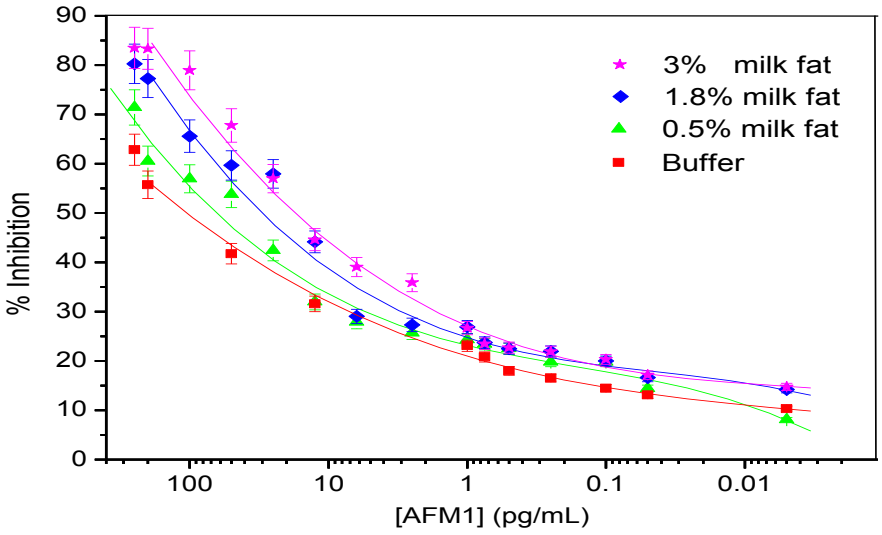


Figure 2.9 Inhibition graph of milk samples with different fat % spiked with known [AFM1] by CL assay.

Table 2.3 Summary of analytical parameters obtained in the AFM1 ELISA in milk samples with different fat content by CL assay.

Analytical parameters	**High fat (3%) content milk**	**Medium fat (1.8%) content milk**	**Low fat (0.5%) content milk**
LOD	0.05 pg/mL	0.005 pg/mL	0.005 pg/mL
IC_{50}	16.95 pg/mL	17.7 pg/mL	39.88 pg/mL
R.S.D	1.29 %	1.95 %	1.69 %
R^2	0.986	0.965	0.982

2.5 Fluorimetric analysis of AFM1

The detection and quantification of AFM1 was also investigated by fluorimetric analysis. For this purpose, fluorophore labeled specific secondary antibodies (Fluorescien isothiocyanate) were chosen. Fluorescein isothiocyanate (FITC) is a derivative of fluorescein used in wide-ranging applications including flow cytometry. FITC is the original fluorescein molecule functionalized with an isothiocyanate reactive group (**-N=C=S**), replacing a hydrogen atom on the bottom ring of the structure (**Figure 2.10**). FITC has excitation and emission spectrum peak wavelengths at 495 nm and 521 nm respectively. Like most fluorochromes, it is prone to photobleaching.

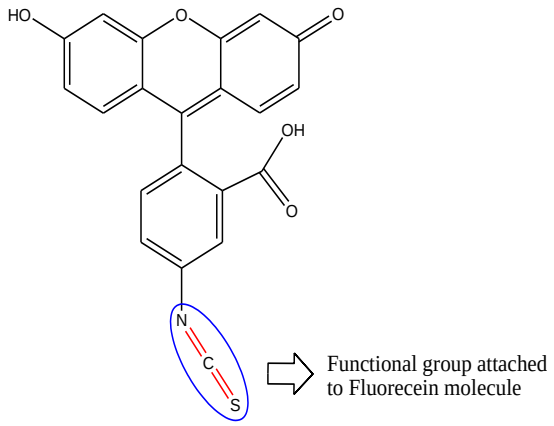


Figure 2.10 Structure of FITC molecule.

2.5.1 Design of a fluorimtric assay for analysis of AFM1

A simple ELISA was developed using fluorimetric technique. The fluorimetric analysis of AFM1 was performed using FITC conjugated 2º Ab in 384 microwell plate as shown in **Figure 2.11**.

2.5.1.1 Fluorimetric immunoassay procedure

Sandwich ELISA was performed in a 384 microwell plate. 1º Ab was diluted to 1:16000 in CB and coated as 40 µL/well. The plate was covered with parafilm and aluminium foil and kept at 4 ºC for overnight; washed 3 times by rinsing the wells with 40 µL PBS. The remaining

protein binding sites in the coated wells were blocked by adding 40 µL of blocking solution for about 1 h at room temperature. The wells were washed once with 40 µL PBST. Following this step, AFM1 standard solution in the range 1-250 pg/mL was mixed up separately with optimized 2º Ab (diluted to 1:64,000 in PBS) solution. This antigen-antibody mixture solution was then added as 40 µL/well. The plate was incubated for about 2 h at room temperature. The excess label was removed by washing with PBS. The micro plate was then analyzed by VictorX^4 2030 opti plate reader.

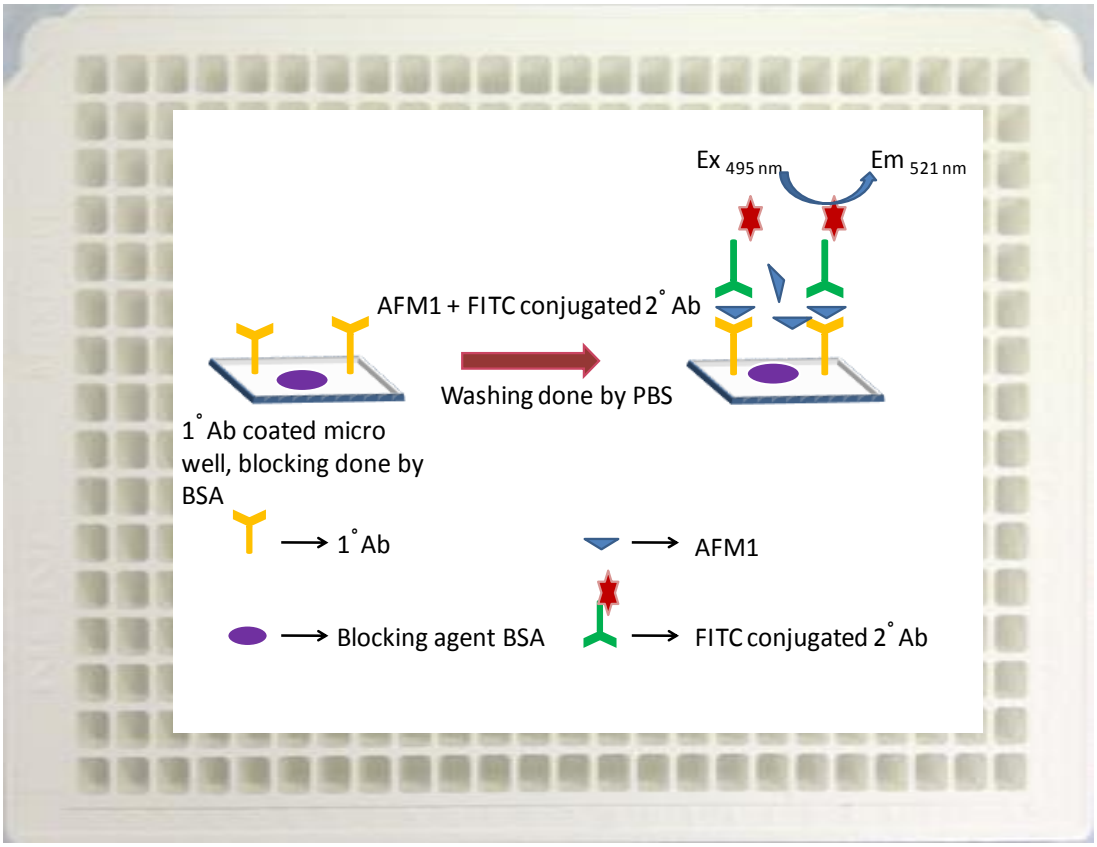


Figure 2.11 Principle of fluorimetric ELISA designed for analysis of AFM1.

2.5.1.2 Optimization of FITC conjugated secondary antibody

The FITC labeled 2°Ab solutions were made by serial dilutions as 1:1000, 1:2000 etc upto 1:64000. The ELISA designed for fluorimetric analysis AFM1 was carried out in 384 microwell plate. The incubation time of primary antibody with the analyte was not altered. All the washing

steps and blocking steps were done similar to CL sandwich method. **Figure 2.12** shows the optimization curve for FITC labeled antibody with 1ºAb in fluorimetric assay. The incubation time was again optimized for the two sets of antibodies. From **Figure 2.12** it was observed that FITC labeled antibody at 1:64000 dilution showed best signal when assayed with 1º Ab. This optimized 2º Ab dilution (1:64000) was used for subsequent experiments.

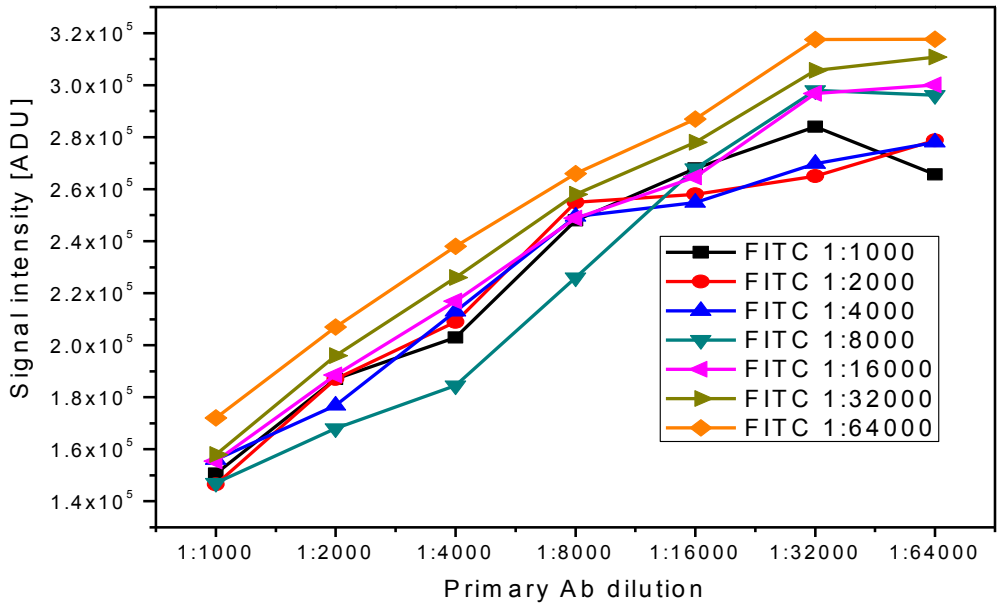


Figure 2.12 Optimization curve of FITC conjugated 2º Ab against 1º Ab .

2.5.1.3 Optimization of incubation time

Figure 2.13 shows the optimization result of incubation time of AFM1 and FITC labeled 2º Ab mixture with mAb by opti plate reader. The incubation time was varied from 10 min to 180 min. It was apparent from the graph that around 100-120 min, the fluorescence signal was stable and after 120 min, it was unstable. So, the incubation time was chosen at 120 min for further experimental analysis.

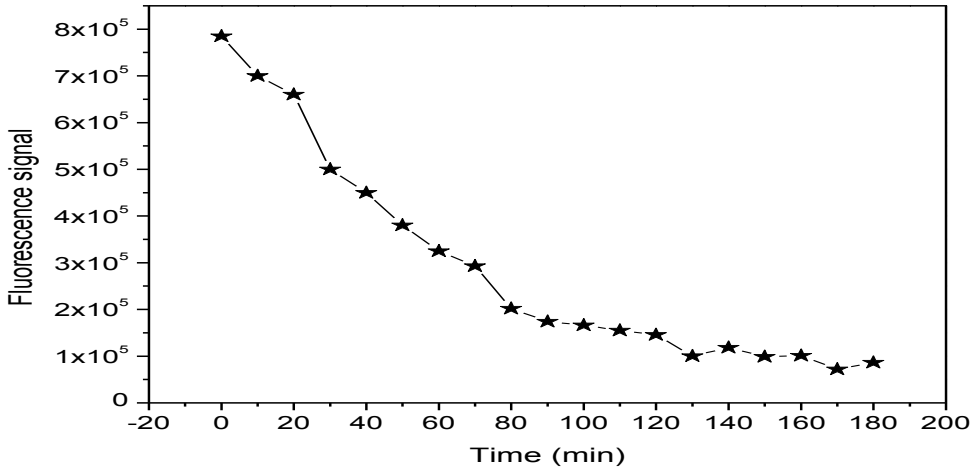


Figure 2.13 Optimization of incubation time for FITC conjugated 2º Ab.

2.5.1.4 Calibration of AFM1 by fluorimteric technique in microwell plate

Different concentrations of AFM1 were tested and calibration was done in the range 1-250 pg/mL by fluorimetric analysis. **Figure 2.14** shows calibration curve of AFM1 (1-250 pg/mL) using FITC labeled 2°Ab by multi plate reader.

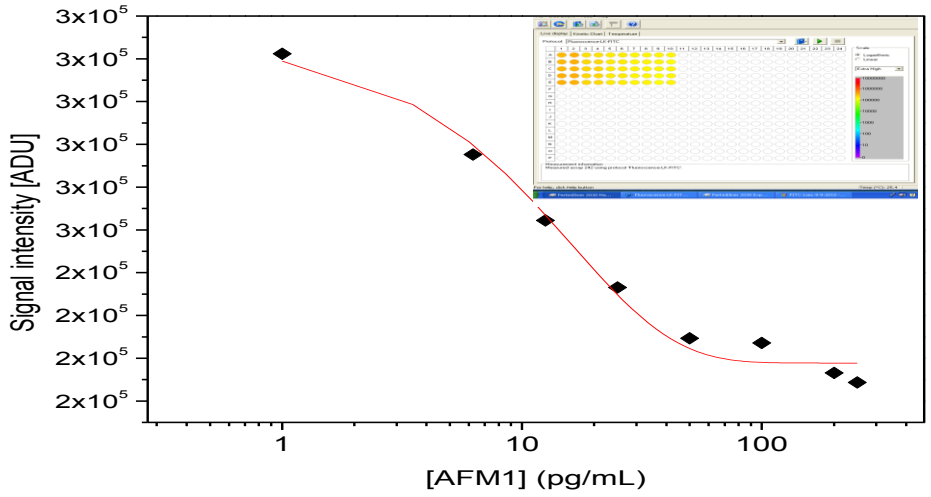


Figure 2.14 Calibration curve of AFM1 in fluorimetric assay using FITC conjugated 2º Ab, inset: snapshot of fluorimetric measurement of AFM1 by FITC conjugated 2º Ab by the opti plate reader.

A decrease in the signal intensity was observed with the increase of [AFM1]. The fluorimetric assay could quantify the [AFM1] in an ultra sensitive manner with a lower limit of detection (LOD) at 1 pg/mL. The analytical figures of merit of this AFM1 assay by fluorimetric technique is given in **Table 2.4**.

Table 2.4 Summary of analytical figures of merit of fluorimetric analysis of AFM1 in microwell plate.

Analytical Parameters	**Experimental findings**
Dynamic range	1-250 pg/mL
Linear range	6.25-50 pg/mL
LOD	1 pg/mL
IC_{50}	100 pg/mL
R.S.D	1.1 %
R^2	0.978
Assay sample volume	40 µL
Analysis time	4 h
Sample throughput	100 samples (in triplicate) in 4 h

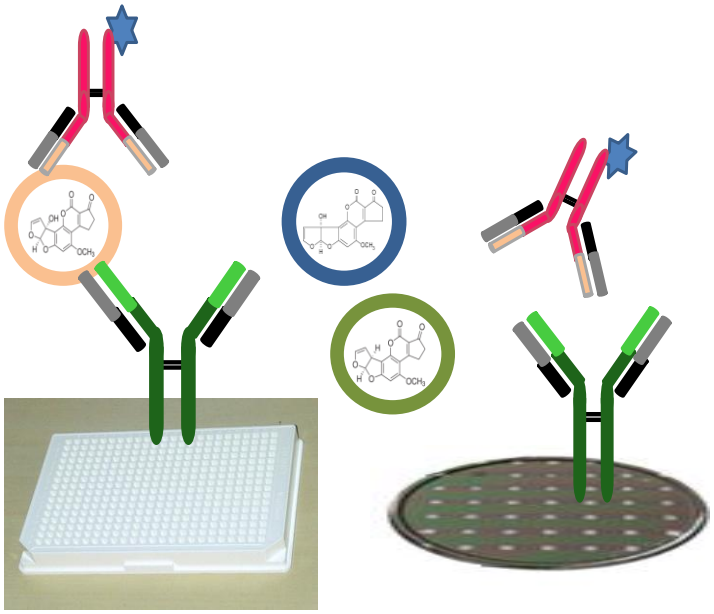
2.6 Conclusions

This work illustrates development of a high throughput microwell plate based ultra sensitive assay for AFM1 in milk. Ultra high sensitivity, lower detection limit up to 0.005 pg/mL and assay economy using very less antibodies for simultaneously screening up to 100 samples are the salient features of the developed assay. The presented assay is completely free from organic solvents. The present study investigates possibility of extending the immunoassay for real milk samples for quality analysis. Assay miniaturization in 384 micro wellplate format result in drastic reduction of toxic waste without compromising assay sensitivity. The AFM1 assay was performed using both CL and fluorimetric where signal suppression was evident with increase in

analyte concentration. The detection of AFM1 concentration was very much quantifiable when the sandwich ELISA was performed by CL technique. By fluorimetric technique, detection limit for AFM1 as low as 1 pg/mL was achieved. This fluorimetric assay provides scope for multi analysis of different aflatoxins in the microwell plate.

Chapter 3

Multi analysis of aflatoxins



Schematic representation of chapter content

3.1 Introduction

Aflatoxins contamination greatly affects the economic value of crops, reduce the efficiency of animal production, and increase the cost of surveillance. The four major naturally occurring aflatoxins viz. AFB1, AFB2, AFG1, and AFG2 belong to a class of structurally related toxic fungal metabolites (Sweeney and Dobson, 1998). As discussed in previously, there should a single platform for simultaneous analysis of these toxins. This multi analysis of aflatoxins can be achieved through the use of a miniaturized device. Miniaturized devices are promising as they facilitate in size reduction of laboratory analyzers, first to bench top and then to portable, hand-held devices and micrometer-sized microchip devices. The dramatic downscaling and integration of bioassay make these analytical devices particularly attractive for field screening tools. Major benefits particular with miniaturization are reduction in manufacturing costs, ease of transport and minimal space requirements in a laboratory. In addition, a miniaturized device reduces requirements for power and consumable reagents and offers the possibility integration of individual steps in a multistep analytical process. Significant interest toward miniaturization of analytical system is due to handling of low volume samples, reduction in waste generation and reagent consumption with increased sample throughput (Kricka, 1998). As an effect of miniaturization, analysis with reducing amount of toxic chemicals will not only have positive impact on environment but also on the assay economy.

3.2 Aflatoxin analysis by fluorimetric detection technique

The aim of this research was optimization of a broad-specificity mAb, enabling development of a sensitive and reliable immunoassay capable of detecting a broader range of aflatoxins. The aflatoxin analysis was done by fluorescence detection technique based on the principle described in Chapter 2. The fluorimetric analysis of aflatoxins was carried out in 384 micro well plate by multiplate reader and on a customized miniaturized sample holder. It is evident from the available literatures that, aflatoxins have similar structures. Thus the capture antibody that specifically recognizes it's analyte, may also partly recognize it's structural analogues. Bearing this concept in mind, crossreactivity (CR) studies were carried out for AFM1, AFB1, AFG1 and AFM2. The available mAb of AFM1 was used as capture antibody and FITC conjugated antibody was used as 2º Ab for recognition of antigen-antibody complex.

3.3 Materials and methods

3.3.1 Chemicals and instrumentation

AFB1, AFG1 were purchased from Acros Organics, USA. AFM2 was purchased from Fermentek, Israel. For the immunoassay development, all reagents and chemicals were obtained and diluted as optimized by the sandwich ELISA protocol described in Chapter 2. VictorX^4 2030 multiplate reader with FL, CL and photometric mode was used for fluorimetric analysis of aflatoxins in the 384 micro wellplate (Nunc, USA). For image based quantification and analysis, an inverted fluorescence microscope (Olympus IX71 series, Japan) was used. The CCD camera (Hamamatsu, Japan) integrated with the FL microscope was used for image capture. A software called Micromanager was used to analyze the intensity of captured images.

All the aflatoxins standard solutions were prepared in a glove box in a maintained inert (N_2) atmosphere. AFB1 stock solution 1000 µg/mL was prepared by dissolving the AFB1 crystalline in 5% ACN (v/v) in PBS and stored at 4 ºC. Working AFB1 standard solutions were prepared in the following concentrations; 1, 6.25, 12.5, 25, 50 & 100 pg/mL by diluting the stock with 5% ACN. AFM2 and AFG1 standard solutions were also made in the similar manner.

3.4 FL immunoassay for multianalysis of aflatoxins in microwell plate

3.4.1 Experimental procedure

FL ELISA was performed in a 384 micro well plate. 1º Ab was diluted to 1:16000 in CB and coated as 40 µL/well. The plate was covered with parafilm and aluminium foil and kept at 4 ºC for overnight; washed 3 times by rinsing the wells with 40 µL PBS. The remaining protein binding sites in the coated wells were blocked by adding 40 µL of blocking solution for about 1 h at room temperature. The wells were washed once with 40 µL PBST. Following this step, AFM1 standard solution in the range 1-250 pg/mL was mixed up separately with optimized 2º Ab (diluted to 1:64,000 in PBS) solution. This antigen-antibody mixture solution was then added as 40 µL/well. The plate was incubated for about 2 h at room temperature. The excess label was removed by washing with PBS. The micro plate was then analyzed by opti plate reader. Similarly the FL immunoassay for multi analysis of aflatoxins such as AFM2, AFB1 and AFG1 were carried out at different concentrations that were mixed with FITC conjugated 2º antibodies.

This mixture was subsequently added to wells and incubated for 2 h. The excess label was removed by washing with PBS. The micro plate was then analyzed by opti plate reader.

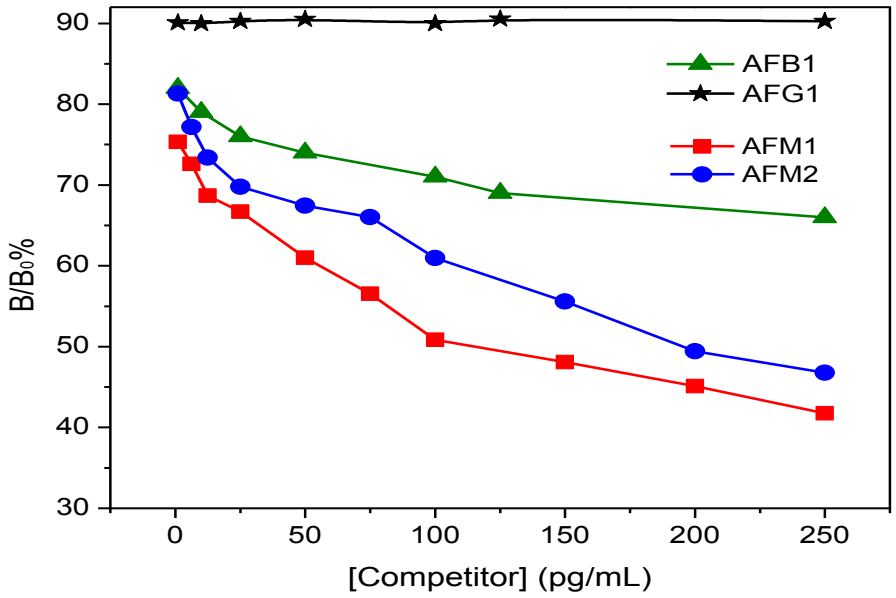


Figure 3.1 Inhibition graph for AFM1 against mAb of AFM1 and measurement of cross-reactivities with AFM2, AFB1, AFG1 in competition with FITC conjugated antibodies in micro well plate. The concentration of cross-reactant was identical in all the measurements.

3.4.2 Result and analysis of fluorimetric assay for aflatoxins in microwell plate

Sensitivity and specificity are important parameters for the mAb and any ELISA method. To evaluate the sensitivity of the ELISA, the IC_{50} was obtained from the standard curves using 384 micro well plate. The specificity of the ELISA was evaluated by determining the cross-reactivity with structurally related aflatoxins. The mAb of AFM1 was highly specific to AFM1 and showed partial recognition towards its structural analogues AFM2 and AFB1. In **Figure 3.1**, it was observed that different aflatoxins were recognized by the mAb antibody of AFM1. The concentration of competitor were plotted against B/B_0 (%), where B_0 is the maximum signal obtained in absence of analyte, and B is the signal obtained in presence of analyte concentration.The B/B_0% were summarized in **Table 3.1**.

Table 3.1 Summary of B/B_0 % of various aflatoxins using standard curve of AFM1.

[AFM1] from standard curve (pg/mL)	**B/B_0 %**			
	AFM1	**AFM2**	**AFB1**	**AFG1**
50	61	68	74	94
100	52	62	71	92
150	48	57	N.R.	N.R.
200	45	50	N.R.	N.R.

N.R. : not recorded

3.4.3 Crossreactivity analysis of different aflatoxins against anti-AFM1 mAb

The antibodies have specificity to a set of analytes and they may partly recognize other analytes known as CR. Therefore, a single antibody will respond to different analytes with varying specificity. The CR of mAb pairs was determined in competition with tracer. For standard analyte (for which CR is 100%), concentrations that result in 50% inhibition (IC_{50}) of the signal obtained form calibrations and were used to compute the CR using the following formula (Bhand et al., 2005):

$$\text{CR}\ (\%) = \frac{\text{IC}_{50}\text{value of standard analyte}}{\text{IC}_{50}\ \text{value of cross reacting analyte}} \times 100$$

The IC_{50} values of AFM1, AFM2, AFB1 and AFG1 were obtained. As indicated in **Table 3.1**, the mAb of AFM1 showed 23.2% cross-reactivitiy with AFB1, approximately 50% with AFM2 and showed negligible (<1%) cross-reactivity with AFG1. The standard curve solution of AFM1 was used as reference in the ELISA as indicated in **Figure 3.2 a** & **b**.

3.4.4 Mixture analysis of aflatoxins in microwell plate

The CR studies were further carried out using mixture analysis. The mixture analysis was done by adding 50 and 25 pg/mL of AFM2 in [AFM1] as shown in **Figure 3.2 (a)**.

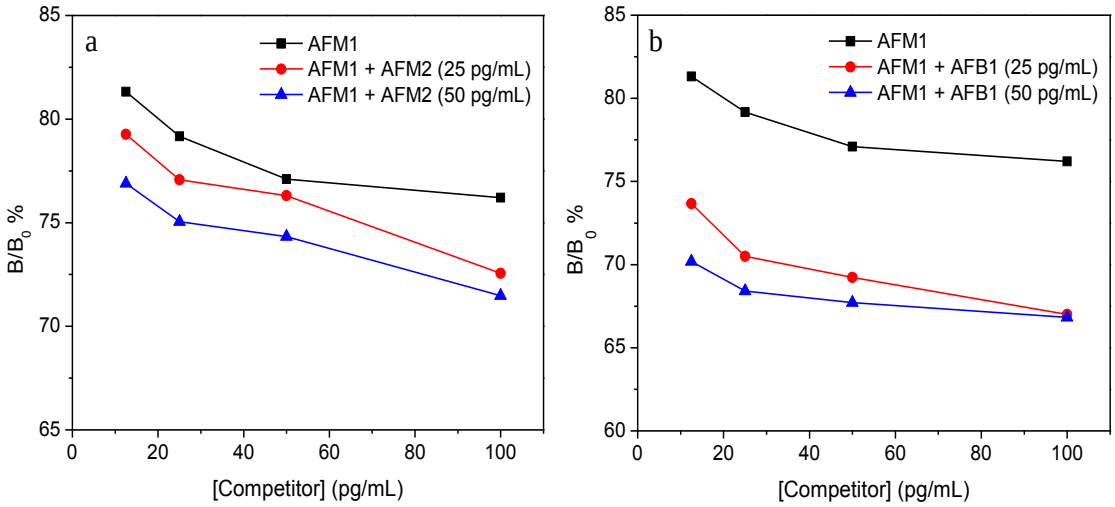


Figure 3.2 CR result showing mAb recognition towards (a) AFM1 and AFM2 mixture; (b) AFM1 and AFB1 mixture.

It was observed that, at 12.5 pg/mL of AFM1, the AFM1+AFM2 (25 pg/mL) mixture showed 4% and the AFM1+AFM2 (50 pg/mL) mixture showed 10% lesser signal intensity than that of only AFM1. Similarly at 25 pg/mL of [AFM1], the mixture of both AFM1+AFM2 (25 and 50 pg/mL) showed 2% and 4% decrease in signal intensity respectively as compared to only [AFM1]. At the EU cutoff limit or 50 pg/mL, there was no significant variation observed. Only 1.5% of decrease in signal intensity was obtained for AFM1+AFM2 (25 pg/mL) mixture. But when analyzed for AFM1+AFM2 (50 pg/mL) mixture, it showed 4% lesser signal when compared to only AFM1. At higher concentration of AFM1 (100 pg/mL), the AFM1+AFM2 (25 pg/mL) showed 4% and the AFM1+AFM2 (50 pg/mL) showed 5% lesser signal intensity when compared to that of only AFM1. This mixture analysis postulates an account of some correlation between CR and signal suppression. As AFM2 showed almost 50% CR with AFM1, its presence as co-contaminant in the mixture resulted in small variation of 1.5 to 4% decrease at lower concentration (25 pg/ml), whereas at 50 pg/ml, the signal suppression was observed in the range of 4 to 10% variation.

The mixture analysis was also carried out for AFB1 and AFM1 as shown in **Figure 3.2 (b)**. The standard AFM1 curve was plotted against mixture of AFM1 and varying concentrations of AFM1 & AFB1 (25, 50 pg/mL). In this case, further decline in signal intensities was observed. At 12.5 pg/mL of [AFM1], [AFB1] for 50 and 25 pg/mL showed 7.5% and 11.5% decrease in signal respectively. For 25 and 50 pg/mL of [AFM1], the AFM1+AFB1 (25 pg/mL) mixture showed the same decrease in signal of about 8% but for AFM1+AFB1 (50 pg/mL) mixture it was 11% and 9.5% respectively. At higher concentration of AFM1 (100 pg/mL), the AFM1+AFB1(25 pg/mL) and AFM1+AFB1 (50 pg/mL) mixtures showed 9 and 9.5% lesser intensities respectively. The curve representing the mixture of [AFM1] with 100 pg/mL [AFB1] showed 12 and 10% less signal intensity of 25 and 50 pg/ml respectively when compared to only [AFM1]. In all the mixtures of AFB1, the signal suppression was observed in a higher order of 7.5 to 11.5% variation when compared to the mixture analysis of AFM2. This difference might have occurred for the reason of difference in CR of AFB1 with AFM1 (23%) which differs from 50% CR of AFM2 with AFM1.

The fluorescence analysis of different aflatoxins done in 384 micro well plate was quantitatively measurable with sample volume of 40 µL. This provides scope for further advancement in lowering the sample volume and analysis in a miniaturized device.

3.5 A novel device for imaging and quantification of aflatoxins

Note: The work incorporated in this section of the thesis constitutes collaborative experimental work with CARE IIT Delhi and had resulted in Intellectual Property. Thus due to non disclosure commitment, some details on device are not disclosed.

Micro fabricated device facilitates assay miniaturization and thus reduces sample volume. High-density arrays of micro reaction wells and micro fabricated devices are emerging devices in the current miniaturization trend. The micro fabricated devices can facilitate detection and analysis of many aflatoxins because it can be readily integrated into an automated process and provide multiple simultaneous testing on a simple reusable device. Micro fabricated devices represent the ability to miniaturize current "bench-top" experiments with the advantages of speed, automation, volumetric reduction of sample and waste. This ability has allowed the advancement of high-throughput analysis on micro sized chips. There are already numerous examples of assays and analytical processes that have been successfully adapted to a microchip format and the goal of a "lab-on-a-chip" is realistic. The low sample volume required for analysis on a chip also reduces analysis cost and makes the technique applicable to the analysis of extremely small volumes of highly toxic compounds such as aflatoxins. Development of chip based system can also facilitate field deployable techniques capable of analyzing trace metals in contaminants on site (Lee and Mirkin, 2008). It is worth mentioning that substrates having microarrays of bio-receptors are often referred to as biochips although most of these systems do not have integrated micro-sensor detection systems. Several biochips have been reported for environmental monitoring (Wang et al., 2004; Dounin et al., 2010; Tuantranont et al., 2006). Biochips are fabricated by various materials such as gold, titanium, silicon, glass, quartz, and plastics (Hadd et al., 1997; Meusel et al., 1998; Doong and Shih, 2010) etc.

Micro fabricated devices and "Lab-on-a- Chip" technologies (Harrison et al., 1992; Harrison, Glavina and Manz, 1993), which possess the ability to control the manipulation, distribution, and detection of minute amounts of sample (Murakami et al., 1993; McEnery et al., 1999; Xu et al., 2000; Bousse et al., 2001), by instrument miniaturization, and analytical process integration play an important role in the development of high-throughput instrumentation. The major features associated with miniaturization reside in high-speed, reduced sample volume and

reagent consumption, integration of operational elements, and high-throughput capabilities via parallelization.

3.5.1 Process fabrication of device

The novel device was designed and fabricated at Centre for Applied Research in Electronics CARE, IIT Delhi together with graduate student Ms. Ruchi Tiwari and Prof. Sudhir Chandra. The chip was fabricated using standard lithographic technique (**Figure 3.3**). The device as received was used for developing the multianalyte analysis.

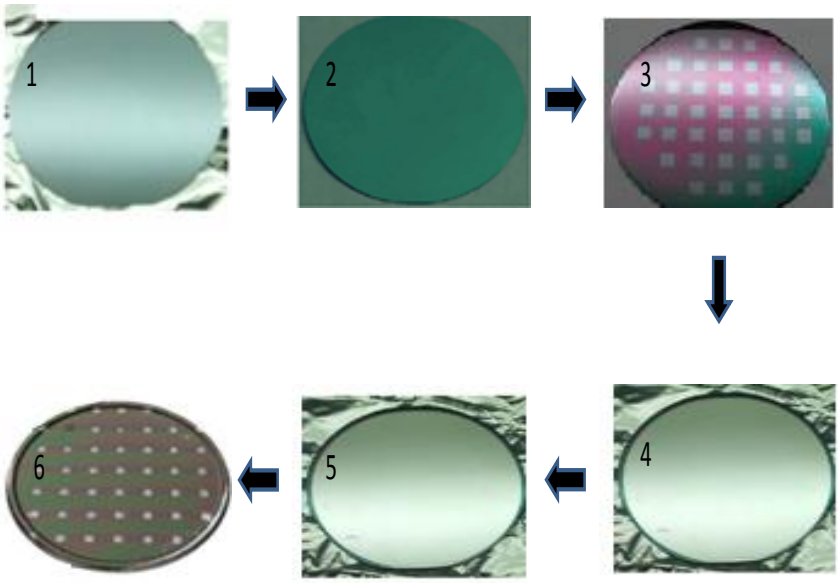


Figure 3.3 Schematic of device fabrication process.

3.6 FL immunoassay of AFM1 on novel device

3.6.1 Expeimental procedure

In the immunoassay, done on the novel device, the following steps were performed which significantly lowered sample volume without affecting its efficacy. The device were coated with 1º mAb. This was done by adding only 4 µL of mAb at 1:16000 dilution in CB and kept overnight at 4 ºC. Then washing was done by washing buffer. Blocking was done by adding 4 µL BSA. After 2 h of blocking, it was washed twice by PBS. Following this step, AFM1 standard solution in the range 1-500 pg/mL was mixed up (at equal volume) separately with

optimized 2° Ab (diluted to 1:64,000 in PBS) solution. This antigen-antibody mixture solution (8 µL) was then added. The device was incubated for about 2 h at room temperature. The excess label was removed by washing with PBS. The device was seen under fluorescence microscope.

3.6.2 Results and discussion of FL assay of AFM1 on novel device

The device was seen under fluorescence microscope. **Figure 3.4** shows the FL image of AFM1-antibody complex recognized by FITC conjugated 2º Ab.

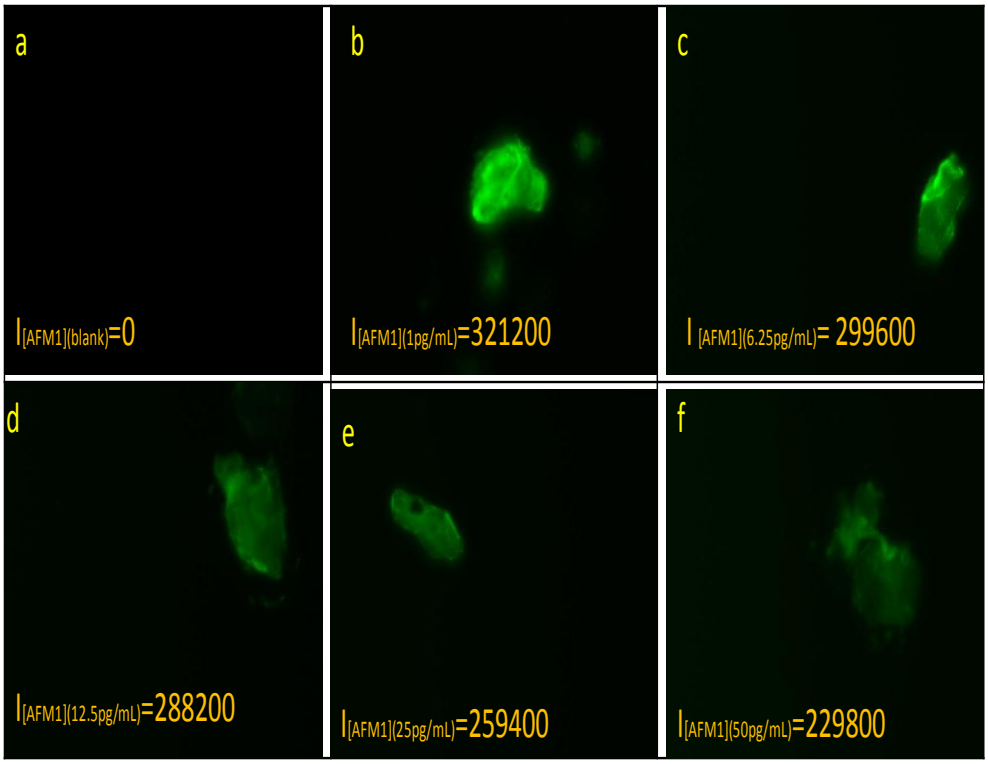


Figure 3.4 FL images showing immunoassay using FITC conjugated 2° Ab of AFM1 at different concentrations; a: blank (without toxin & FITC antibody), b: [AFM1] at 1pg/mL, c: [AFM1] at 6.25 pg/mL, d: [AFM1] at 12.5 pg/mL, e: [AFM1] at 25 pg/mL, f: [AFM1] at 50 pg/mL.

The FL images were captured (**Figure 3.4**) and analyzed and it can be seen that at low AFM1 concentration, the FL signal appeared very bright and as the concentration of analyte increased, there was a decrease in signal brightness. **Figure 3.4** (**a**), shows the image of the blank where no antigen was added. The average FL signal intensity was plotted on a graph shown in **Figure 3.5**.

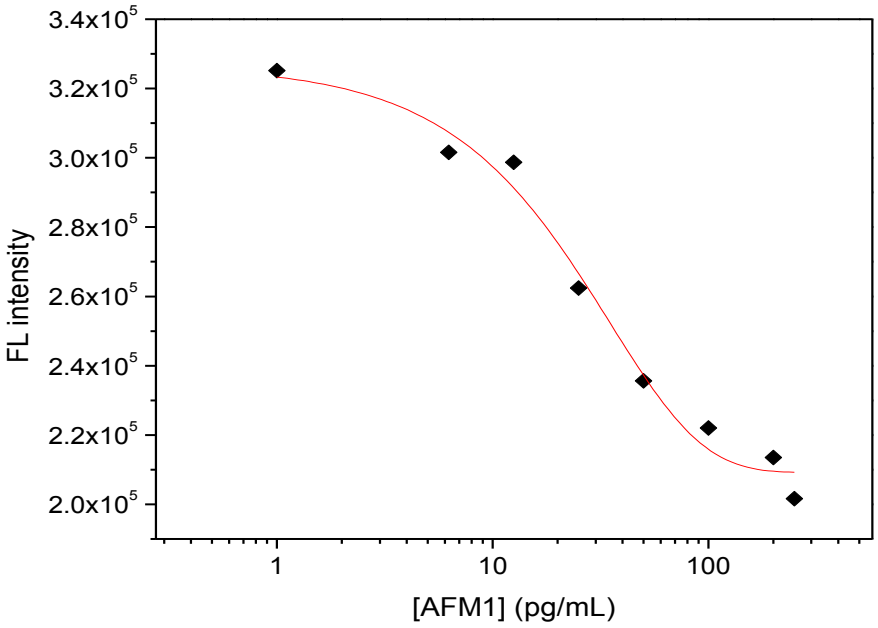


Figure 3.5 Calibration curve of AFM1 by FL imaging.

The **Figure 3.5** shows the calibration curve of different [AFM1] using the FITC labeled 2° Ab. There was a decrease in the FL signal intensity with the increase of [AFM1]. The limit of detection was 0.5 pg/mL. The FL assay could quantify the [AFM1] in an ultra sensitive manner. This arises further scope for multi analysis of aflatoxins by CR studies.

3.7 Multi analysis of aflatoxins on device

The device comprising of arrayed spots was used for multi analyte detection by FL technique. In this work, miniaturization of developed immunoassay was attempted for multi analysis of aflatoxins. The assay was developed using FL technique on a single device for simultaneous detection of various aflatoxins. This has immense potential for food quality assessment. Herein, an attempt to miniaturize immuno assay on device for highly sensitive measurement is demonstrated.

3.7.1 Result and discussion of multi analysis of aflatoxins on novel device

The miniaturized assay of aflatoxins on the novel device was studied using immobilized antibodies of AFM1 that partially recognizes other aflatoxins. Reasonably good results were observed. This study also demonstrates one of the few attempts for the development of high throughput FL based detection for on-chip analysis of several aflatoxins. The **Figure 3.6** shows the images of FL assay.

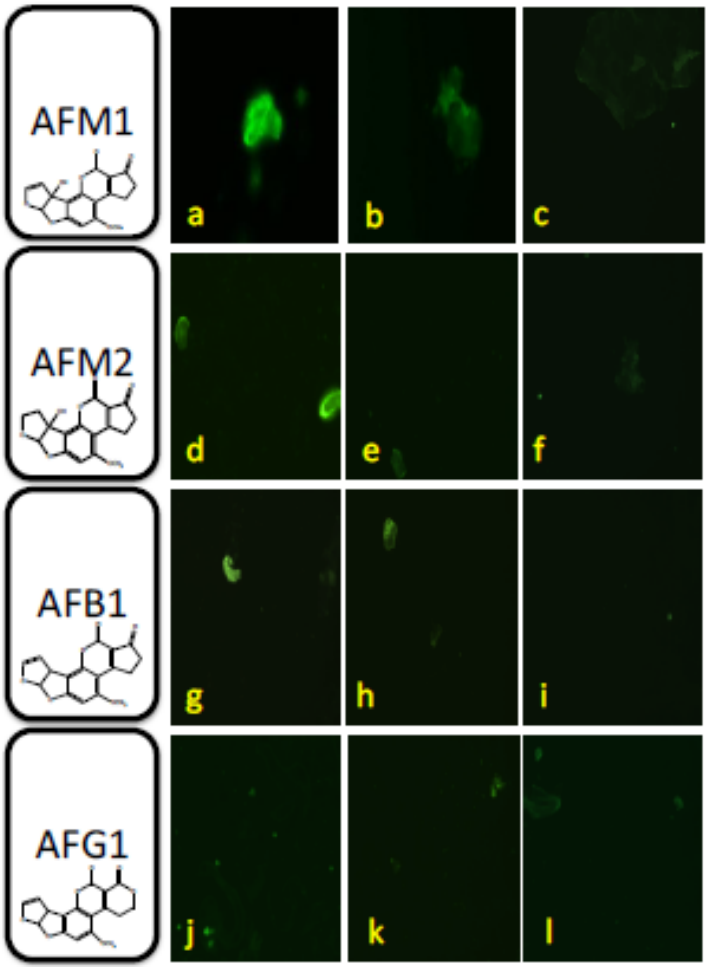


Figure 3.6 FL image of different aflatoxins as seen under microscope; a-c: [AFM1] (1, 50, 100 pg/mL); d-f: [AFM2] (1, 50, 100 pg/mL); g-i: [AFB1] (1, 50, 100 pg/mL); j-i: [AFG1] (1, 50, 100 pg/mL).

It was observed that FITC labeled anti AFM1 antibody showed remarkable and strong FL when recognized AFM1-antibody complex. With the increase in [AFM1], the signal intensity

was decreased due to inhibition. The FITC labeled anti AFM1 antibody also recognized AFM2 (**Figure 3.6**; **d-f**), AFB1 (**Figure 3.6**; **g-i**) and AFG1 (**Figure 3.6**; **j-i**). The FL images of each spot was taken by the camera one after another. The multianalysis of aflatoxins in FL assay was quantified from the average FL signal intensities plotted in **Figure 3.7**.

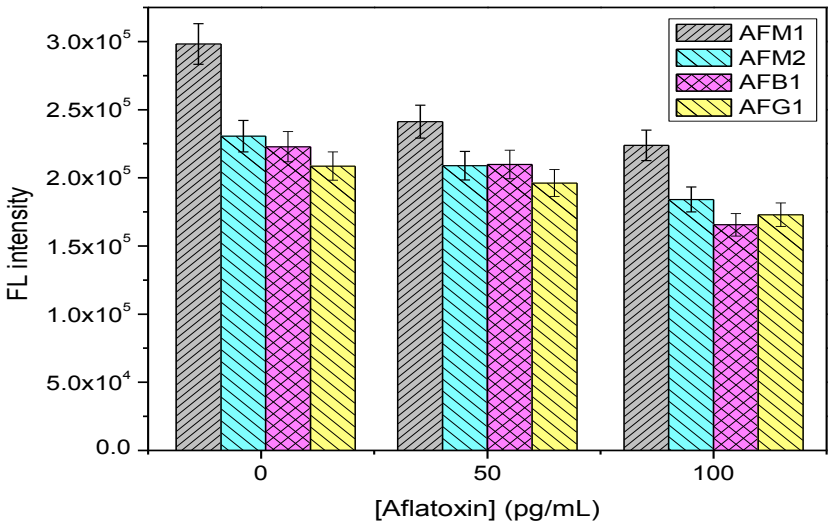


Figure 3.7 FL intensities of different aflatoxins with different concentration as seen with FL imaging in the novel device.

The cross reactivity study of FL assay shows that FITC labeled anti AFM1 antibody showed strong FL when recognized AFM1-antibody complex. The average point intensities of different aflatoxins are presented in **Figure 3.7**. With the increase in [AFM1], the signal intensity was decreased due to inhibition. The FL intensity for AFB1 was always found less than that of AFM1. It was further observed that the FL signal for AFM2 and AFG1 were significantly weaker than AFM1 and AFB1. This pattern was observed for all the notable aflatoxin concentrations.

3.8 Conclusions

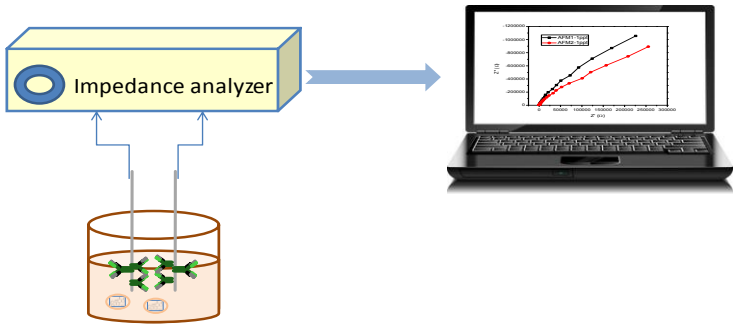
In this chapter, the principle of crossreactivity was demonstrated to analyze structurally analogous different aflatoxins using fluorimetric detection. This work illustrates a simple and

sensitive fluorimetric immunoassay for multi analysis of aflatoxins. Ultra sensitive analysis of AFM1 was successfully carried out by the developed technique with LOD as low as 1 pg/mL.The presented protocol has been improved by eliminating one step in the immunoassay. ELISA for multi analysis of different aflatoxins was investigated by fluorimetric technique in the micro well plate in presence of various types of competing analytes. From the assay, it was observed that AFM2 showed highest CR as compared with AFB1. AFG1 was least recognized by the anti AFM1 antibodies. The multi analysis of different aflatoxins was also verified by the mixture analysis of AFM1 with AFB1 and AFM2. Assay on micro well plate allowed testing of highly toxic aflatoxins done with low sample volume and with easy handling. Besides the sensitivity and minimal reagent consumption, such multi analysis would lead to simultaneous screening of different aflatoxins.

Miniaturization of ELISA for simultaneous analysis of different aflatoxins were investigated by FL technique in 384 micro well plate and on a novel device. Reduction of toxic waste without compromising assay sensitivity is the key feature of assay miniaturization. Major achievements resulting from miniaturization of assay in well plate format to device include lowering of sample volume from 40 µL to about 8 µL and ease of handling. The FL imaging by the inverted FL microscope provided vast scope for multi analysis of aflatoxins. The novel device facilitated simultaneous analysis of various aflatoxins based on their cross reacting properties. A good repeatability is observed for device without significant changes in the response signal. Assay on novel device allows testing of highly toxic aflatoxins to be performed with very less sample volume and with easy handling. Besides the drastic reduction in the size of the analytical system, such miniaturization should lead to increase speed, minimal reagent consumption and disposal.

Chapter 4

Application of label free immunosensor for analysis of aflatoxins



Schematic representation of chapter content

4.1 Introduction

Immunoassays are presently available for a more diverse range of analytes than immunosensors. Immunoassay development is paramount for integrating analytical capabilities into a portable, disposable and robust device, useful in many scenarios such as hospitals, general practitioners, airport, roadside police control and environmental measurerments (Ruano-Lopez et al., 2009). These immunoassays, for example ELISA, can be highly labour intensive, time consuming and expensive. There is an increasing demand for ultrasensitive and field deployable immunosensors for analysis of aflatoxins.

4.2 Label free detection techniques

The aflatoxin detection techniques are often classified as being label-based and label free. Label-based detection is widely used due to the common availability of reagents and simple instrument requirements. Most of the labelled detection such as FL, CL and radioactive labeling strategies have synthetic challenges, multiple label issues and may exhibit interference with the binding site. However, these labelling strategies often alter surface characteristics and natural activities of the query molecule. Moreover, the labelling procedure is laborious, lengthy and limits the number and types of query molecules that can be studied. Therefore, the development of sensitive, reliable, high-throughput, label free detection techniques are now attracting significant attention. In general, label free detection techniques monitor biomolecular interactions and simplify the bioassays by eliminating the need for secondary reactants. Moreover, they provide quantitative information for the binding kinetics. However, label free techniques measure an inherent property of the query itself (eg. mass and dielectric property) thereby avoiding modifying interactors.

A sensitive, label free detection method would represent a great advance toward portable arrayed sensors, consisting of small detection spots with high stability and label free continuous monitoring. Many label free techniques such as Surface Plasmon Resonance (SPR), Carbon Nano-Tubes (CNTs) and nanowires, nanohole arrays, interferometry, etc. have been successfully integrated with bioanalysis and are emerging rapidly as a potential complement to labelling methods (Yu et al., 2006). The label free detection techniques are progressing rapidly. Standard

label free techniques such as imaging with SPR or quartz crystal microbalance are nonspecific as they merely report the accumulation of material on the sensor surface and lack the required spatial resolution.

4.2.1 SPR

The SPR is a surface sensitive, spectroscopic method which measures change in the thickness or refractive index of biomaterials at the interface between metal surfaces. In SPR the test proteins are immobilized on a gold-surface, unlabelled query protein is added, and change in angle of reflection of light caused by binding of the probe to the immobilized protein is measured to characterize biomolecular interactions in real-time. SPR has been widely used for many biomedical, food and environmental applications. The SPR method has several potential advantages such as: (1) small sample volume (in μL unit); (2) reusability of metal chip; (3) ability to detect kinetics of antibody–antigen reaction; (4) ability to detect a range of analytes; and finally (5) user-friendly. However, sensitivity may be an obstacle for some SPR systems. The monetary investment for SPR equipment is quite high. Additionally SPR technique has following limitations such as mass transport can affect kinetic analysis, false signal due to artifactual refractive index change, one of the interacting molecules should be immobilized on the surface, the sensor surface deteriorates over time and with re-use etc (Zheng et al., 2006).

4.2.2 Electrochemical impedance spectroscopy (EIS)

Among the reported biosensors, EIS has emerged as sensitive label free technique for analysis of biomolecules and aflatoxins (Vig et al., 2009; Paniel et al., 2010). Electrochemical sensors are good choice due to their fast, simple, and low-cost detection capabilities for biological binding events (Tan et al., 2009). Only a few electrochemical biosensors have been reported for mycotoxin detection. A large number of biosensors use amperometry as their detection technique. The amperometric biosensor is robust and makes it ideal for using in the field at the point of source. EIS is suitable to analyze the electrical properties of the modified electrode, i.e. when an antibody coupled to the electrode reacts with the antigen of interest (Felice et al., 1999). In such a case, the adsorption/desorption process is the rate-determining step. This remarkable step is controlled through the appropriate choice of electrical potential. This confirms that the

antigen–antibody interaction is largely influenced by the applied potential. EIS is also a suitable technique to understand the adsorption and charge transfer process for modified electrode (Ma et al., 2006). In an EIS technique, the electrode setup can be modeled as an equivalent electrical circuit. This is further used to curve fit the experimental data and extract the necessary information about the electrical parameters responsible for the impedance change (Yang et al., 2004). This is a very useful concept with regards to AFM1 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). For rapid analysis of aflatoxins, impedimetric biosensors are promising as they are label free and are highly sensitive. Moreover they can be field portable.

Impedance changes between electrode surfaces and a surrounding solution upon a binding event can be transduced into an electrical signal using a frequency response analyzer. Several theories demonstrate that, this binding event affects the change in real and imaginary components of the system, although it is difficult to identify the origin of these changes. One theory hypothesizes that binding of larger antigen forms a resistive barrier, causing the impedance to increase whilst binding of smaller antigens can facilitate a charge transfer and lower impedance (Tully et al., 2008). Another theory is that when the antigen-antibody complex is formed, the binding events between the hyper-variable loop regions mean that conformational changes occur and potential changes are introduced into the system. Future work must establish the origin of this impedance change, whether from increase in surface density or conformational changes that modify charge transfer across the sensor interface. EIS-based immunosensors have recently received particular attention, since they possess a number of attractive characteristics associated with the use of electrochemical transducers, being considered as promising candidates for on-site applications. EIS technique and other label free detection techniques have been studied in aflatoxin research and a summary is presented in **Table 4.1.**

Table 4.1 Summary of label free detection techniques for analysis of different aflatoxins.

Label free detection technique	Analyte	Limit of detection	Reference
Long range SPR	AFM1	0.6 pg/mL	Wang et al., 2009
SPR	AFB1	3 ng/mL	Daly et al., 2000
SPR	AFB1	0.2 ng/mL	Van der Gaag et al., 2003
Spectroscopic ellipsometry in total internal reflection mode	AFB1	0.04 ng/mL	Nabok et al., 2011
Impedance	AFM1	15 ng/L	Vig et al., 2009
Impedance and cyclic voltammetry	AFM1	1 ng/mL	Dinckaya et al., 2011
Bioelectronic recognition assay	AFM1, AFB1	5 pg/mL	Larou et al., 2011
Linear sweep voltammetry	AFM1	0.054 ng/mL	Tan et al., 2009
EIS	AFB1	0.028 pg/mL	Ningning et al., 2011

4.3 Analysis of peanut

Peanut is a known product which has widespread usages in making foods and production of oil. Peanut is full of fat and protein; besides its super potential as a food, in suitable conditions of humidity, light, temperature and air flow for fungi growth, it can be contaminated by toxin (mycotoxin) producing fungi. It has been discovered that each aflatoxin producing *Aspergillus* strains is able to produce different and characteristic patterns of aflatoxins. *A. flavus* produces exclusively AFB1 and AFB2, while *A. parasiticus* produces all four major aflatoxins (AFB1, AFB2, AFG1 and AFG2) (Moss, 1989), with AFB1 and AFG1 as the major metabolites (Goldblatt, 1969).

Figure 4.1 (a) Peanuts, (b) Peanuts contaminated with AFB1/G1.

Peanut or groundnut is usually found to be contaminated with AFB1 and AFG1 and thus its occurrence is a serious threat to its consumers. Acceptable limit of Aflatoxin by European standard in food products is 2 μg/kg. **Table 4.2** summarizes different detection techniques developed for analysis of AFB1 and related aflatoxins in peanuts and other food matrices.

Table 4.2 Summary of different detection techniques used for analysis of AFB1, AFB2, AFG1 and AFG2 in peanuts and related food products.

Detection technique	Analytes	Matrix	Detection limit	Reference
EIS and cyclic voltammetry	AFB1	PBS buffer	0.1 mg/L	Owino et al., 2007
EIS	AFB1	cereals	1 ng/mL	Dinckaya et al., 2011
HPLC-FL	AFB1	cereals	0.003 ng/g	Fumani et al., 2011
UHPLC	AFB1, AFB2, AFG1, AFG2	peanut, corn	0.32 μg/kg (AFB1), 0.19 μg/kg (AFB2), 0.32 μg/kg (AFG1), 0.19 μg/kg (AFG2)	Fu et al., 2008
Adsorptive stripping voltammetry	AFB1, AFB2	groundnut	0.1-0.115 ng/mL	Hajian and Ensafi, 2009
HPLC-ESIMS/ MS	AFB1, AFB2 AFG1, AFG2	peanuts and their derivative products	0.012-0.273 μg/kg	Huang et al., 2010
Intermittent pulse amperometry	AFB1	corn	30 pg/mL	Piermarini et al., 2007
TLC-CCD	AFB1	peanuts	0.4 ng/spot	Hoeltz et al., 2010
HPLC-UV-FLD	AFB1, AFB2 AFG1, AFG2	peanuts	0.1-3.5 ng/ mL	Gonçalez et al., 2008

4.4 Application of label free impedance immunosensor for aflatoxin analysis

Development of a novel impedimetric immunosensor was envisaged under collaborative mode wherein the sensing mechanism for antigen-antibody interaction using Ag wire electrode was

developed jointly with Mr. Gautam Bacher from Dept. of EEE & I, BITS, Pilani- K. K. Birla Goa Campus and CARE, IIT-Delhi. While the bioelectronic aspect including the electrical characterization and equivalent circuit has been reported elsewhere (Bacher et al., 2012), the application of the developed immunosensor for analysis of aflatoxins in various matrices is presented in this chapter. The aim was to develop a practical and highly sensitive technique for label free analysis of aflatoxins such as AFM1, AFM2, AFB1 in various food with short analysis time. The first set up was wire based immunosensor where functionalized Ag wires were made as electrodes. In the second set up flow based impedance analysis was done for AFM1 and AFM2 in milk products.

4.4.1 Impedimetric immunosensor

The sensor constitutes two electrodes made of Ag wires functionalized with primary antibody against aflatoxin and placed at fixed distance in a liquid cell. Primary mAb specific to AFM1 was attached on the Ag wire through self assembled monolayers (SAMs) of 11-marcaptoundecanoic acid (11- MUA) as shown in **Figure 4.2**. The Ag wire set-up provides additional features such as storage, ease of handling and portability. The impedance change during antigen–antibody interaction was measured using EIS. Similarly for AFB1 immunosesnor, primary pAb against to AFB1 was attached on the Ag wire through SAMs of 11-MUA.

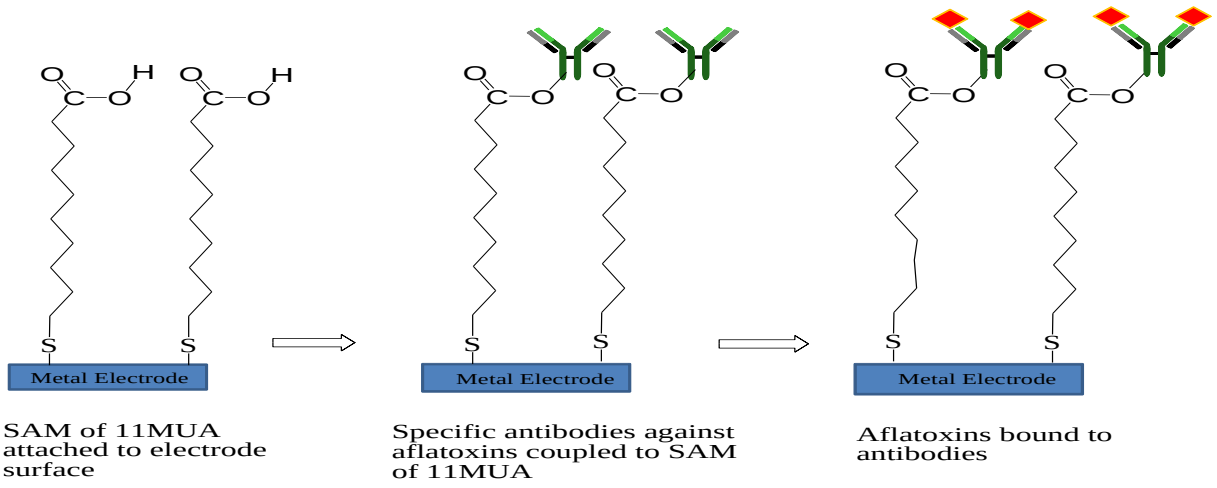


Figure 4.2 Schematic showing functionalization of electrode surface of wire based immunosensor by SAM and binding of antibody and aflatoxin.

The simple, immunosensor for aflatoxins was mounted in the single well of 384 well plate with the help of two electrode system (**Figure 4.3**).

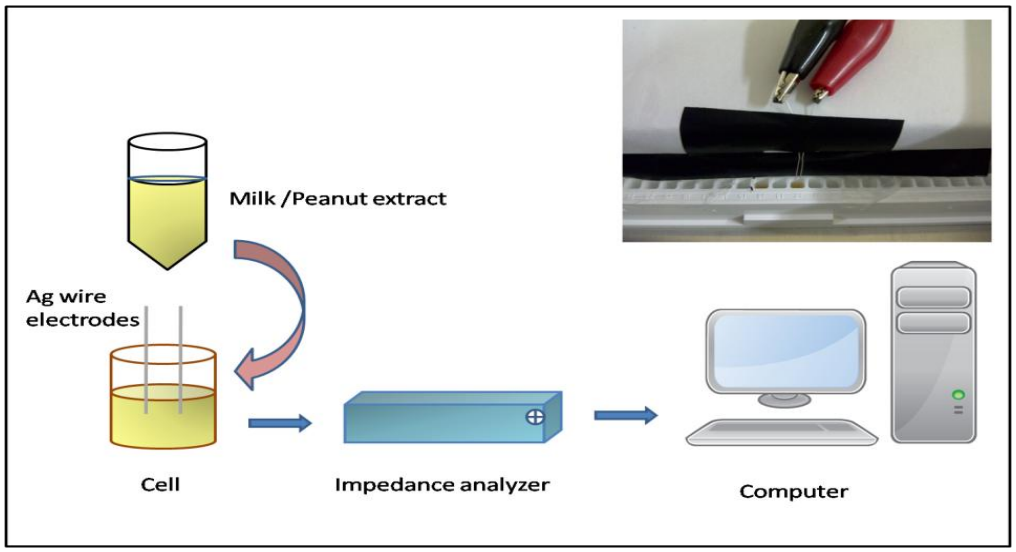


Figure 4.3 Schematic of the sensing mechanism; inset: real picture of sensor used with 384 micro well plate.

4.4.2 Materials and methodologies

Ag wire (diameter = 0.25 mm) was procured from ACROS Organics, USA. The diameter of wire was reduced to 0.18 mm by manual stretching (verified by vernier caliper). AFB1 primary pAb raised from rat, Tween 20, 11-mercaptoundecanoic acid (11-MUA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS) were purchased from Sigma–Aldrich, USA. Ethyl alcohol 200 proof was purchased from TEDIA, USA. Methanol was purchased from MERCK (Germany). Peanut samples were finely ground by a household mixer grinder. Shaking was done by Rotospin (Tarsons, India). Filtration was done by Whatman® filter paper No. 41. For flow injection analysis, a syringe pump and a multi channel peristaltic pump was used. The syringe pump and the peristaltic pump were purchased from Chemyx, US and Gilson, France respectively. All other necessary chemicals and glasswares used were described earlier in **section 2.3**. Impedance measurements were carried out using IVIUM CompactStat impedance analyzer, Netherland.

4.4.2.1 Preparation of AFM1, AFB1 and AFG1 standard solutions and antibody dilutions

All the aflatoxins standard solutions were prepared in a glove box in a maintained inert (N_2) atmosphere. AFM1, AFB1 and AFG1 standard solution and the antibody solutions were prepared as described in **Section 2.3** and **Section 3.4** respectively.

4.4.2.2 Peanut sample extraction procedure

Non contaminated peanuts were purchased from the local supermarket, Goa, India. The sample was extracted by the following method. Peanuts were finely ground by a household mixer grinder to a fine consistency. 10 g of this ground commodity was extracted with 50 mL of methanol/water mixture (80:20) by a rotospin shaker at 50 rpm for 15 min. The slurry was filtered through Whatman filter paper No. 41 and used for analysis.

4.4.3 Experimental procedure for aflatoxin analysis by label free immunosensor

The wires were washed with distilled water followed by drying under ultra pure N_2 stream. This cleaning procedure was repeated before every electrode preparation step. Initially the surfaces of the bare Ag wire electrodes was washed ultrasonically in deionized water for 5 min to remove inorganic particles. Following this, the electrodes were immersed into piranha solution (H_2O_2/H_2SO_4, 30/70 v/v) for 30s. The electrodes were dried with N_2 stream before use. The clean Ag wire electrodes were immersed overnight in 0.004 M ethanolic solution of 11-MUA under ambient condition. The electrodes covered by SAMs were gently washed with absolute ethanol to remove unbounded 11-MUA residues. Then the electrodes were dried with N_2 stream. The 1° Ab was covalently coupled on Ag wire electrode through SAMs. For coupling the 1° Ab, the carboxyl group of SAMs on modified electrode was activated by 1:1 EDC/NHS (0.1 M each) mixture for 2 h. Subsequently, the electrodes were washed with distilled water to remove excess EDC/NHS. The 1°Ab was attached to the electrode by carefully spreading Ab solution (optimized) over the activated surface followed by overnight incubation at 4 ºC. The unused antibody coupled electrodes were washed and stored at 4 ºC for future use.

The AFB1 analysis in peanut was carried out in the following manner. 102 µl of the peanut extract solution was taken in 384 microwell plate. Then 3 µl AFB1 standard solution (0.01, 0.1,

1, 10, 25, 50, 100 pg/mL) was spiked into it. The functionalized and Ab coated Ag wires were dipped into the wells (1 cm dipped) and 5 mV voltage was applied. The applied frequency was in the range 100 KHz to 1 Hz.

The AFM1 analysis in milk was done with the simple pre-treatment procedure. The CRM BD-zero level milk sample was reconstituted and centrifuged at 6000 rpm for 10 min, then the middle fat free portion was taken and filtered with 0.2 micron filter paper. Then it was diluted with PBS to 1:1 prior to analysis. The known concentrations of AFM1 (1-100 pg/mL) were spiked into the CRM milk sample. The Ag wire electrodes were dipped into well of 384 micro plate where 105 µL of milk sample was analyzed.

4.4.4 Results and discussion

4.4.4.1 Optimization of antibody dilutions for label free immunosensor

The antibody dilutions were optimized for EIS analysis. It was observed that in the wire based impedimetric immunosensor, AFM1 antibody at a dilution of 1:16000 showed good impedance value when compared to other dilutions such as 1:32000 and 1:64000 (**Figure 4.4)**.

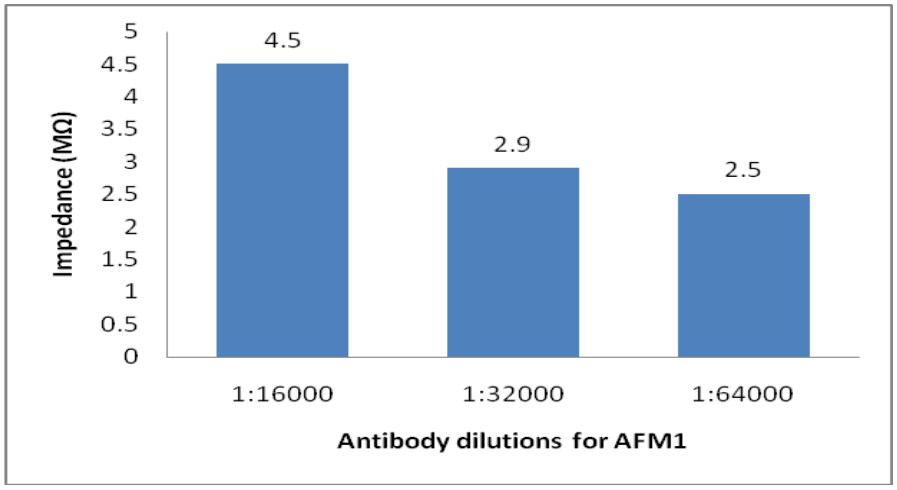


Figure 4.4 Optimization of AFM1 antibody dilution in an wire based immunosensor; mAb of AFM1 tested at different dilutions such as 1:16000, 1:32000 and 1:64000; analysis time 20 min.

The optimization of AFB1 antibody dilutions was also carried out for EIS technology. AFB1 antibody at a dilution of 1:64000 showed highest impedance value (2.5 MΩ) when compared to 1:32000 and 1:16000 (**Figure 4.5**). With increasing incubation time, the impedance response increased and reached to a constant value after 20 min.

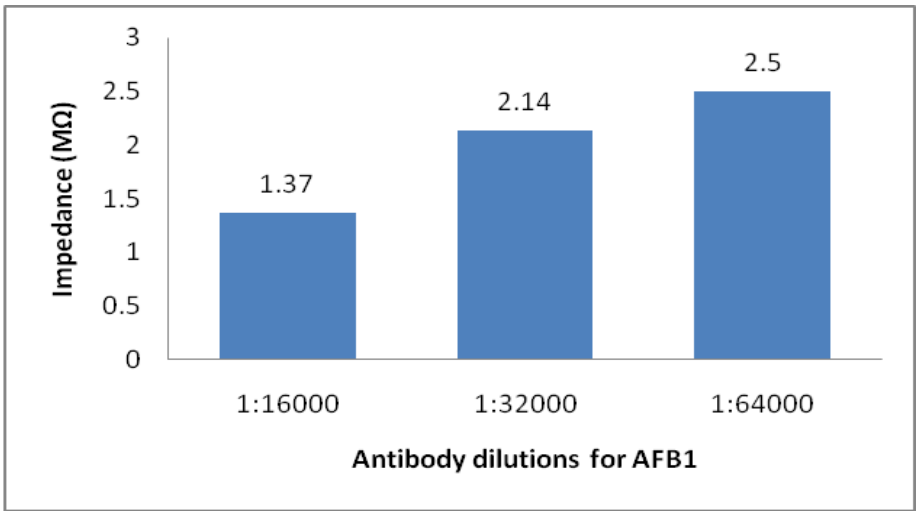


Figure 4.5 Optimization of AFB1 antibody dilution in an wire based immunosensor, pAb of AFB1 tested at different dilutions such as 1:16000, 1:32000 and 1:64000; analysis time 20 min.

4.4.4.2 Crossreactivity analysis of AFB1 and AFG1 by EIS

The immunoassay was further extended to the analysis of AFG1 (also found in peanuts). It was found from the material data sheet that antibody of AFB1 can also recognize AFG1 to some extent. So, the analysis of both AFB1 and AFG1 were done in peanuts. The crossreactivity between AFB1 and AFG1 with same AFB1 antibody dilution (1:64000) was also observed and shown in **Figure 4.6**.

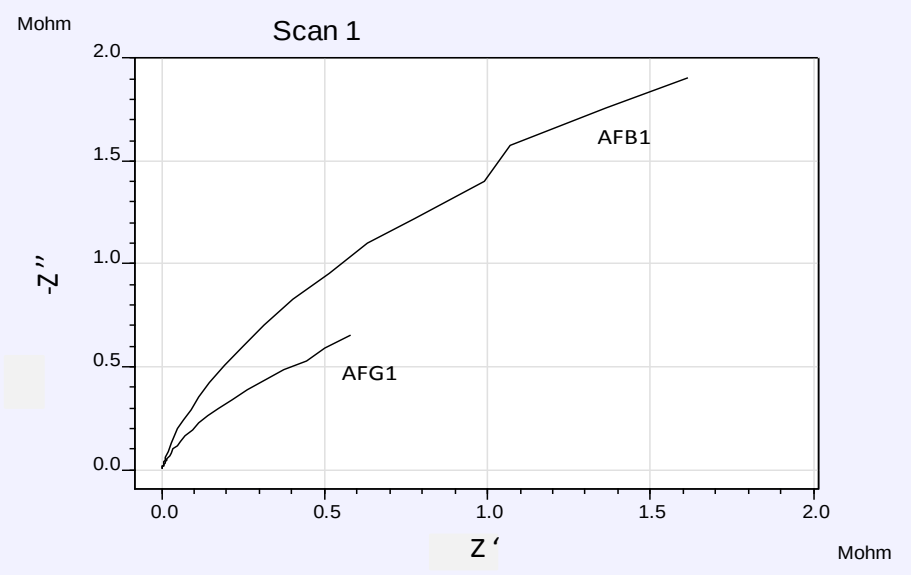


Figure 4.6 Impedance analysis of AFB1 & AFG1 [100 pg/mL] at 1:64000 AFB1 (pAb) with analysis time 20 min in peanut.

4.4.4.3 Optimization of applied voltage and frequency

For the quantification of aflatoxins using EIS, various optimized parameters were used such as 5 mV applied potential. The applied frequency was in the range from 1 Hz to 100 KHz. For the quantification of impedance response due to the binding of analyte, frequency at 1 Hz was used.

4.4.4.4 Impedance analysis of AFB1 in peanut

After optimizing the antibody dilution and applied voltage and frequency, different concentrations of AFB1 were spiked (0.01-100 pg/mL) in the non-contaminated peanut extract. **Figure 4.7** showed the impedance plot of peanut extract along with spiked 0.01, 1 and 10 pg/mL [AFB1].

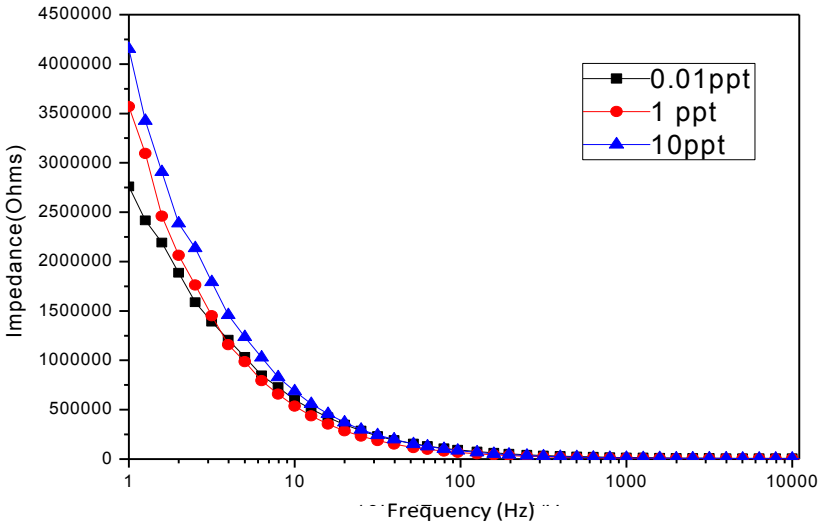


Figure 4.7 Impedance spectra (Bode plot) of different concentrations of AFB1 spiked in peanut matrix.

The complete calibration of AFB1 was carried out in peanut extract. The dynamic range was taken from 0.01-100 pg/mL. **Figure 4.8** represents Nyquist plot obtained for the AC impedance analysis of anti-AFB1 pAb following exposure to various AFB1 concentrations. It was evident that both Z´ and Z´´ component of impedance increased with decreasing frequency. It was seen from the **Figure 4.8** that, the blank peanut (matrix) showed lowest Z´´ value of -1000000 and was distinct from the spiked samples. There was an increasing trend observed with the increase in AFB1 concentration.

The interaction of antibody-antigen (aflatoxin and its specific antibody) on functionalized electrode surface was analyzed. These interactions created a new charged layer as a capacitance that was in series with the double layer capacitance. A decreased double layer capacitance and increased impedance was observed at the lower applied frequency of 1 Hz for lower concentration (0.01-10 pg/mL) and decreased impedance was observed at higher concentration (25-100 pg/mL). The change in impedance confirmed binding of the analyte (AFM1/AFB1). The decrease in impedance attributed to the limiting value of available binding sites on the electrode.

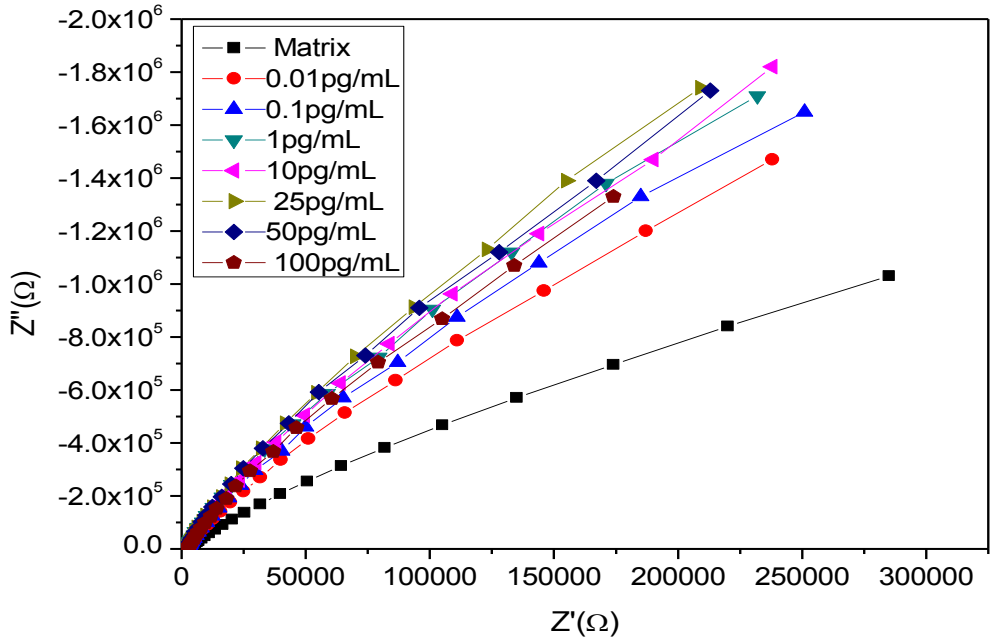


Figure 4.8 Nyquist plot of different [AFB1] spiked in peanut extract.

As regards applied frequency, it was observed that impedance remains constant in the higher frequency region (100 Hz-100 KHz). A significant change was measurable in the low frequency region (1-100 Hz). The intra day analysis of the immunosensor (n=4) showed a good repeatability with 5% variance. The analytical figures of merit of the AFB1 analysis in peanut by EIS technique is summaraized by **Table 4.3**.

Table 4.3 Summary of analytical figures of merit of EIS technique used for AFB1 analysis in peanut.

Analytical parameters	**Experimental findings**
Dynamic range	0.01-100 pg/mL
LOD	1 pg/mL
R.S.D.	0.16%
R^2	0.95
Analysis time	20 min
Minimum detection limit	0.01 pg/mL
Toal assay volume	105 µL

4.5 Flow based impedance analysis of AFM1 and AFM2 in milk and related products

The consumption of milk and various milk products is quite high in world market. The AFM1 and AFM2 possess serious health hazard as contaminants for such food commodities. AFM1 contamination in milk is a worldwide threat and it is unfortunate that, AFM1 remains in milk and milk products even after pasteurization. So, the early detection of AFM1 and AFM2 in milk and milk products is the most important requirement for any dairy industry.

There are few reports available on flow based AFM1 detection and analysis. A bi-layer lipid membrane based biosensor and related thin-film technology were investigated for AFM1 monitoring of milk using a flow-injection system by Andreou and Nikolelis, (1998). This very fast method (four samples per min) permits a continuous monitoring of milk. But the detection limit was around 200 pg/mL. Sibanda et al. (1999) have developed a membrane-based flow-through enzyme immunoassay. A detection limit of 50 pg/mL was achieved using a preconcentration step based on an immunoaffinity column. But in this case, the total assay time was 30 min. Badea et al., (2004) developed a flow-injection immunoassay method with amperometric detection for AFM1 determination in milk. The detection limit of 20 pg/mL obtained for milk samples at the rate of six samples in triplicate per hour. But the immunoassay involved a complex procedure comprising of many steps. Thus, there is a need for a simple, sensitive and rapid method for flow based AFM1 detection and analysis in milk with minimal pretreatment methods.

4.5.1 Sample preparation of milk products for analysis by EIS technique

The milk product analysis was carried out in commercially available drinking yogurt and flavored milk. The flavored milk sample was centrifuged at 6000 rpm for 10 min. The upper fatty layer was removed by spatula and middle clear portion was diluted with PBST (1:1) and used. The drinking yogurt sample was diluted with PBST (1:5) and used for analysis.

4.5.2 Experimental procedure for flow based impedance analysis

It is known from the literature that AFM2 is more prevalent in milk products than AFM1. The occurrence of AFM1 and AFM2 were analyzed by impedance in milk products such as drinking

yogurt and flavored milk in a flow based system. The flow system was designed in house as shown in **Figure 4.9**. The flow rate was optimized by adjusting both the inlet and outlet flow rates. The inlet to the cell was governed by Chemyx micro syringe pump where two fluids (PBST and milk product sample) were injected, mixed and channelized to the working cell. The flow rate of the inlet system was optimized to about 0.5 mL/min for a total syringe sample volume of 2 mL.

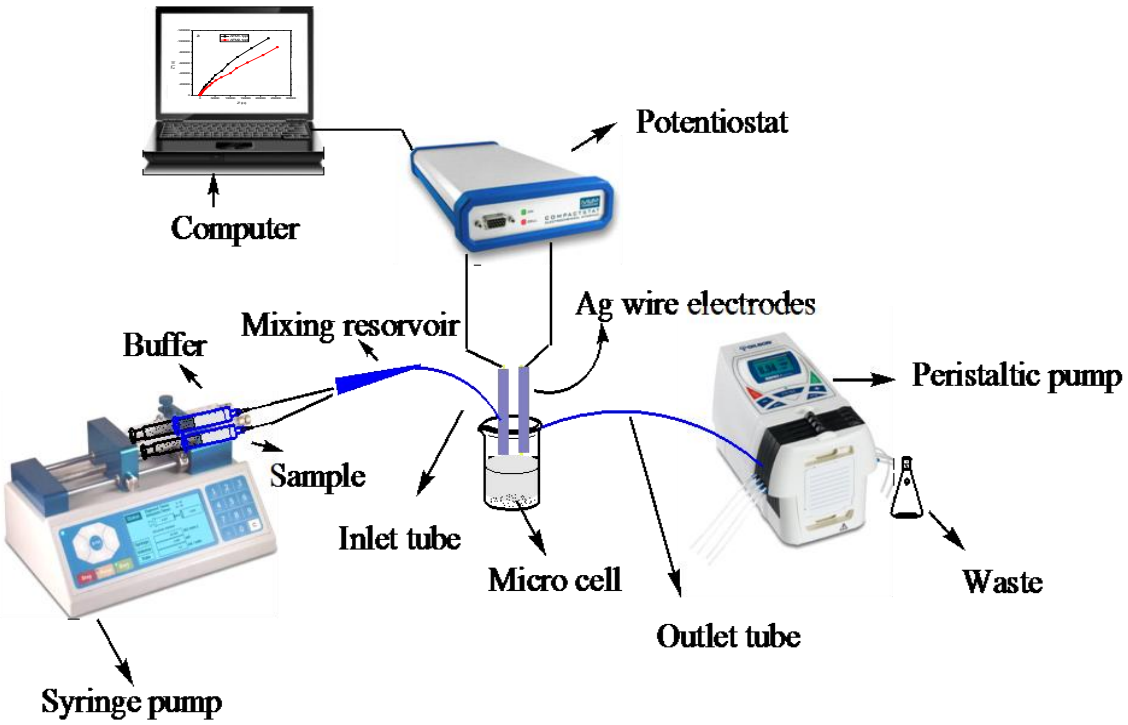


Figure 4.9 Schematic representation of flow based impedance analysis of milk products.

The sample was injected through the inlet pipe along with buffer where two solutions were mixed and directed to the cell. The milk products were spiked with known concentrations of AFM1 or AFM2 by micro pipettes. The outlet system was governed by Gilson micro flow pump where the flow rate was optimized to 2.4 rpm. These two flow rates resulted in maintaining a working volume of 0.5 mL in the cell.

4.5.3 Results and discussion of flow based impedance analysis of CRM milk samples

The flow injection analysis of milk products was done in real and spiked samples. CRM BD-zero level milk was reconstituated and spiked with known concentrations of AFM1 and AFM2. **Figure 4.10** presents the impedance values of AFM2 spiked CRM-zero level milk samples. It can be clearly observed from the **Figure 4.10** that, zero level CRM-BD sample had lowest (2.56E+05 Ω) impedance value. With the increase of AFM2 concentrations (1, 25, 50 pg/mL), the impedance values were increased respectively.

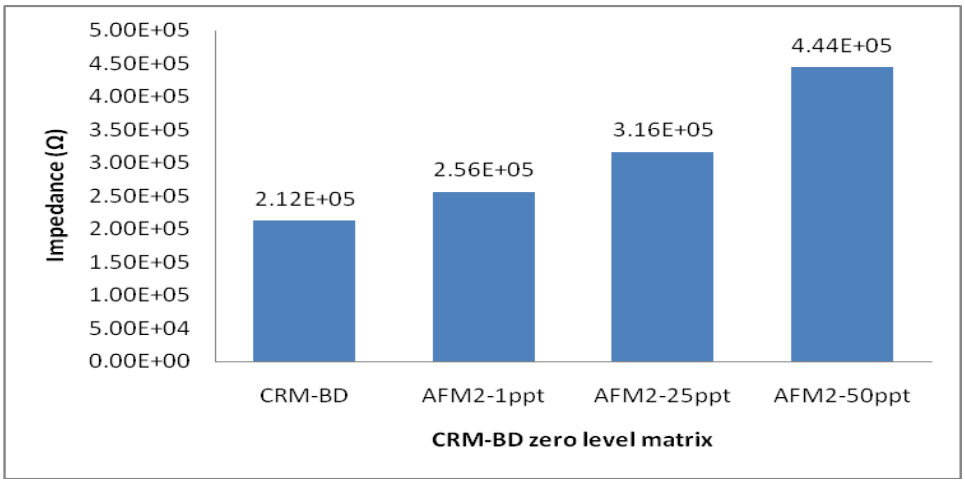


Figure 4.10 Impedance plot of different concentrations of AFM2 spiked in zero level CRM milk sample.

4.5.4 Flow injection based impedance analysis of AFM1 and AFM2 in drinking yogurt

Drinking yogurt was diluted with PBST (1:5) and used for analysis. The analysis time was 10 min. It was observed from **Figure 4.11** that, the impedance value of yogurt matrix was 4.40E+05. AFM1 at concentration of 1, 25, 50 and 100 pg/mL was spiked into yogurt sample and analyzed.

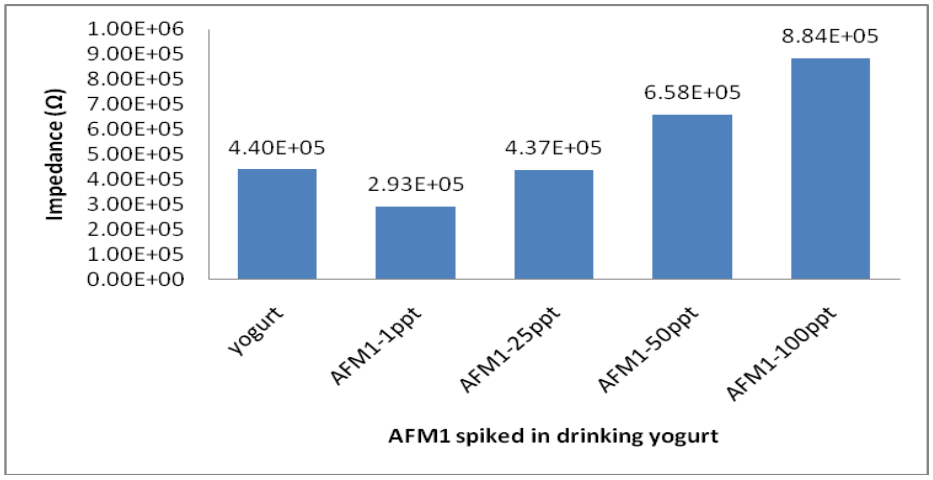


Figure 4.11 Impedance plot of AFM1 spiked in drinking yogurt by flow injection technique.

It was observed that, for 1 and 25 pg/mL, AFM1 spiked samples, the impedance values obtained were less than that of blank yogurt. The impedance values increased subsequently for 50 and 100 pg/mL AFM1 spiked samples. This was probably due to the less signal to noise ratio obtained at the lower concentrations (1 and 25 pg/mL). But at 50 pg/mL and above, the sensor was more reliable. The Nyquist plot in **Figure 4.12** shows the curves represent ing [AFM1] and [AFM2] in drinking yogurt. Both the analytes were spiked at 1 pg/mL in yogurt and EIS was studied.

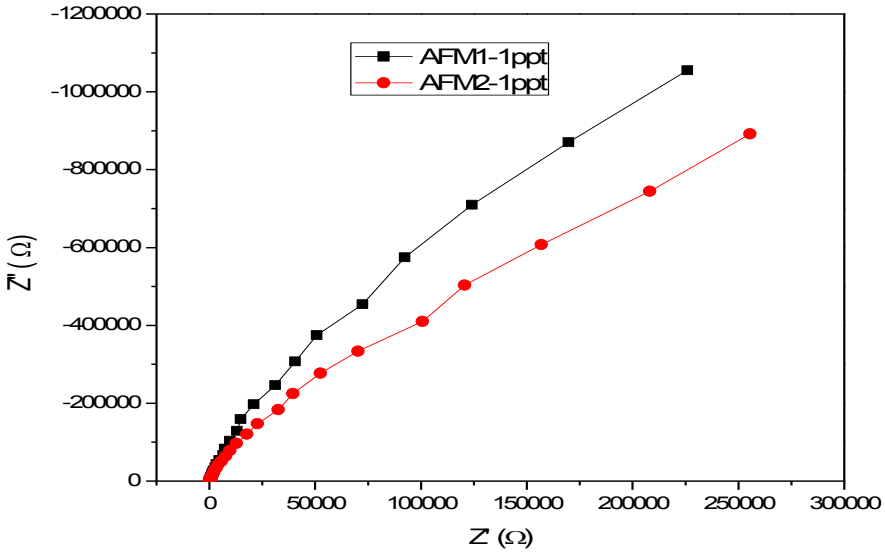


Figure 4.12: Nyquist plot showing AFM1 (1 pg/mL) and AFM2 (1 pg/mL) spiked in drinking yogurt.

It was observed from **Figure 4.12** that, the AFM1 and AFM2 at 1 pg/mL were spectacularly distinguishable from each other showing the scope for simultaneous detection of these compounds by the sensor in drinking yourt.

4.5.5 Flow injection analysis of AFM2 in flavored milk

The flow injection analysis of flavored milk was done by impedance measurement. Blank sample was first tested and its impedance value was found out to be 2.65E+05 (**Figure 4.13**). Then known concentrations of AFM2 was spiked into the sample and impedance measurements were carried out in flow state.

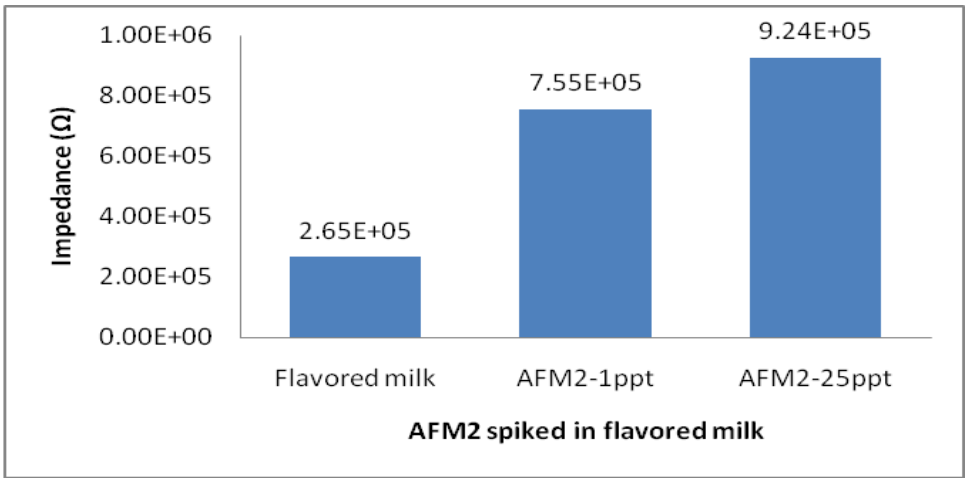


Figure 4.13 Impedance plot obtained for flow injection analysis of AFM2 in flavored milk.

The graph (**Figure 4.13)** shows different impedance values obtained for different [AFM2] (1 and 25 pg/mL) when spiked in flavored milk. At lower concentration (1 pg/mL) the impedance value of AFM2 was found to be 7.55E+05 whereas with the increase of concentration (25 pg/mL), a higher impedance value (9.24E+05) was obtained. This verifies the excellent detection ability of the sensor at ultra low concentration of AFM2 in flavored milk which is consumed world wide.

4.6 Development of an impedimetric biosensor using interdigitated electrode (IDE)

Note: The work incorporated in this section of the thesis constitutes collaborative experimental work among BITS, Pilani & CARE IIT Delhi and had resulted in Intellectual Property, which is provisionally filed as an Indian Patent with application No. 1203/MUM/2013. Thus, due to non disclosure commitment to the funding agency, some of the details are not disclosed.

Impedimetric biosensor has the advantage of being highly sensitive, rapid and low cost (Gomez et al, 2001; Owino et al., 2007). To miniaturize the sensor and improve the sensitivity, microelectrodes have been considered as a potential candidate to combine with traditional detection systems. Microelectrode arrays offer many advantages over standard planar electrodes. Microelectrodes provide stir independence sensor responses and although each separate microelectrode environment is far smaller than that of a planar electrode as a whole, collectively in an array, they frequently permit lower limits of detection for an analytes. Among microelectrodes, interdigitated electrodes or IDEs have greater advantages in terms of low ohmic drop, fast steady state response, rapid reaction kinetics and increased signal to noise ratio (Arya et al., 2010).

For selective detection, the electrodes are functionalized with specific antibody so that a specific antigen of interest can be targeted. An AC voltage is then applied to a circuit containing the electrodes, and the resulting impedance is measured. The applied voltage is small (mV) in order to minimize altering the properties of the analyte immobilized on the electrodes. Impedance depends on the frequency of the applied AC potential. Change in impedance is due to the presence of the analyte in the solution, so it is possible to detect the presence of analyte by impedance measurement. The magnitude of the impedance signal can be calibrated to to detect the concentration of the analyte.

4.6.1 Experimental procedure for AFM1 analysis in milk on novel device

The IDE devices were designed by Mr. Gautam Bacher and fabricated at Center for Applied Research in Electronics (CARE), IIT Delhi in collaboration with BITS, Pilani-K. K. Birla Goa Campus. The IDE was made by standard lithographic procedure. **Figure 4.14** represents the schematic diagram of novel device with patterned electrodes and measuring pads.The electrodes of the device were functionalized in the similar manner as Ag wires as described in **Section 4.4.3.** As the surface area of the device was much lesser than the wires, the chemicals requirement for IDE experiments was very less than that of wire set up (< 10 µL).

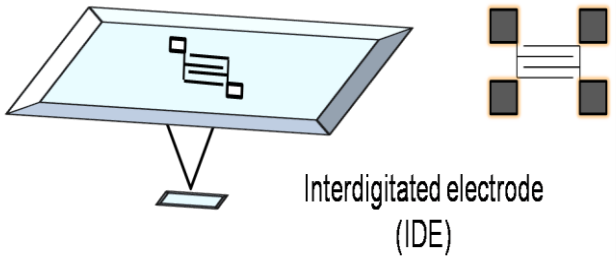


Figure 4.14 Scheme representing inter digitated electrode used for aflatoxin analysis.

4.6.2 Results and discussion for AFM1 analysis in milk on novel device

Various AC voltages were applied to device for optimization and 5 mV was found to be most suitable for the impedance measurement. Measurements were carried out with applied potential of 5 mV and frequency range of 10-10000 Hz. Calibration curve was obtained for the device (**Figure 4.15**).

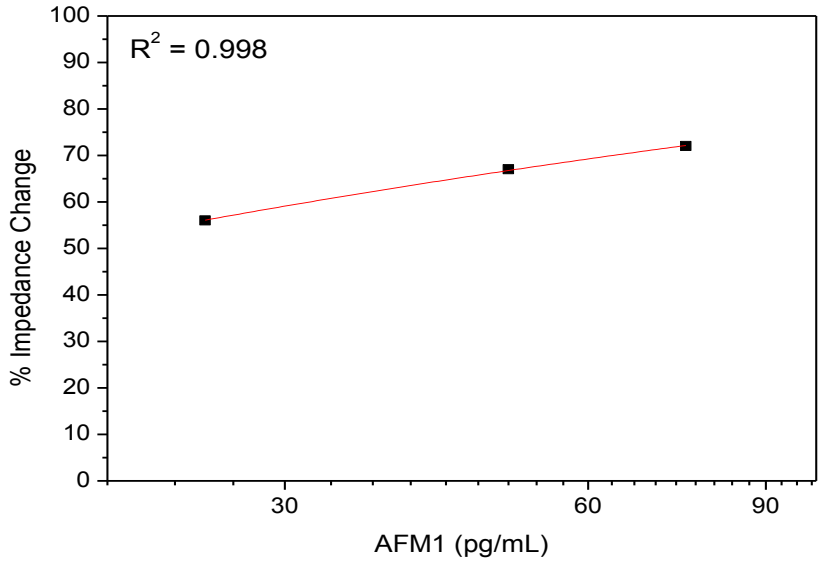


Figure 4.15 Calibration curve obtained for for AFM1 on novel device (best fit).

The linear range for AFM1 assay was from 25-75 pg/mL. The R^2 was found to be 0.998. The LOD of the assay was 25 pg/mL. This meets the stringent EU standard cutoff. The Nyquist plot was obtained for 25, 50 and 75 pg/mL of AFM1 concentration spiked in CRM-BD 282 and shown in **Figure 4.16**.

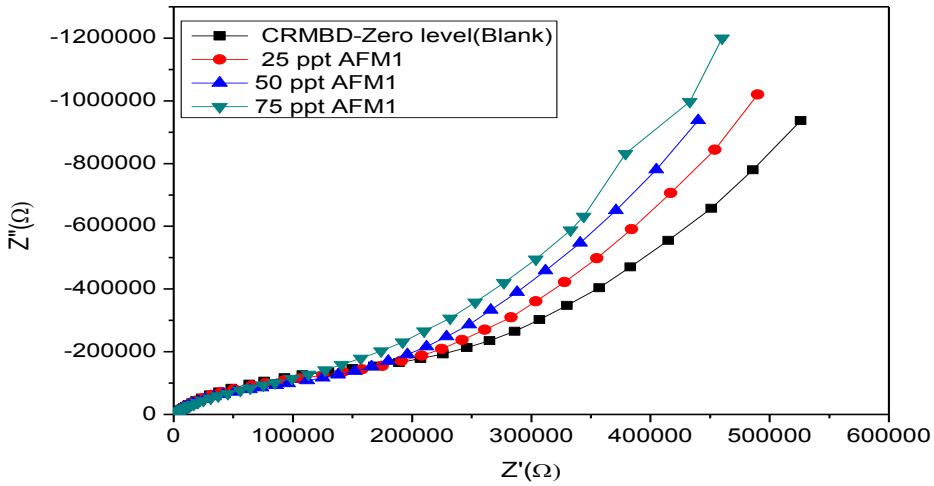


Figure 4.16 Nyquist plot (analytical signal) recordings of different [AFM1] in CRM-BD- zero level at electrode surface. EIS: 1 Hz to 1000 KHz, 10 mV AC potential.

The device which features miniaturization with a very less sample volume, provides the scope for label free and sensitive analysis of aflatoxins with field portability. The summary of analytical parameters of aflatoxin analysis on device are presented in **Table 4.4**.

Table 4.4 Summary of analytical paramaters of AFM1 analysis by EIS on novel device.

Analytical parameters	**Experimental findings**
Linear range	25-75 pg/mL
LOD	25 pg/mL
R^2	0.998
Applied potential	5 mV
Applied frequency	1Hz-100 KHz

The impedimetric micro biosensor using the novel device could detect AFM1 as low as 25 pg/mL with a very less sample volume <10 µL. The device facilitates low cost measurement per sample as against the time consuming, low throughput and expensive analytical techniques such as HPLC.

4.7 Conclusions

The label free detection and analysis of AFM1 in milk and AFB1 In peanut matrix were successfully carried out on the developed immunosensor with impedance set up. After simple pre-treatment of food samples, the immunosensor was optimized with regard interferences from different matrices. This demonstrated a simple, cost effective, label free impedimetric immunosensor for detection of AFM1, AFM2 and AFB1 in different food products with the help of two electrode system. Novel microfluidic strategies were developed for flow injection analysis of AFM1 and AFM2 in milk products. Analysis of AFM1 and AFM2 were successfully carried out in the flow system with very short analysis time of 5-10 min. The immunosensor showed an astoundingly low limit of detection (1 pg/mL) for AFB1 with a short analysis time of 20 min. The method with pre-functionalized electrodes can be usable under field conditions. This imparts

high specificity to the biosensor. This flow analysis can be attributed for on-line monitoring of AFM1 and AFM2 at milk collection centers.

Analysis of AFM1 and AFB1 on the novel IDE device was successfully carried out by EIS technique with very low sample volume (<10 µL) and short analysis time of 5-10 min. The novel IDE device facilitates miniaturization of the assay and provides greater sensitivity with minimal sample volume. The label free immunosensor was able to meet stringent regulatory standards of EU cut off of 50 pg/mL and below. Besides being low cost, the novel immunosensor was easy to handle due to the fact that sensor setup was quite simple to operate. This provides the scope for on-line monitoring and sensitive analysis of aflatoxins with field portability.

Chapter 5

Validation of developed AFM1 assay in real samples

Schematic representation of chapter content

5.1 Introduction

Milk and milk products in several countries have been widely surveyed for the natural occurrence of AFM1. Monitoring is important for not only consumer protection but also producers of the raw products prior to transport or processing. Recent literature describes the methods in which milk may be analyzed directly or after simple and limited pre-treatment (Anfossi et al., 2008; Lin et al., 2004). In India, a survey found that 87.3% of the milk-based samples analyzed were contaminated; of these 99% were much above European permissible limits (Rastogi et al, 2004). This is a major concern considering that India is the largest producer of milk in the world (Thirumala-Devi et al., 2002; Rastogi et al., 2004; Parker et al., 2009) and there are very scarce reports of AFM1 analysis. One recent report describes about the occurrence of AFM1 in raw, pasteurized and ultra high temperature treatment of milk of the major brands prevalent in the Karnataka and Tamilnadu region of India. It has been surveyed that varieties of packaged milk samples are available in the Indian market for consumption without any food safety certification (Siddappa et al., 2012).

5.1.1 Design of experiment

The developed CL sandwich ELISA for the analysis of milk and milk products was validated with AOAC approved techniques such as HPLC and commercial kit. For the validation, a survey type sampling analysis was carried out to check the occurrence of AFM1 in commercial milk samples and infant formula milk samples of Goa, India. The real milk sample comprising of both liquid milk as well as infant formula milk were analysed using all the methods. Herein, 15 milk brands and 3 infant formula milk brands (total 72 samples) were analyzed to quantify the AFM1 level as recommended by the FSSAI, Codex, USFDA and EU guidelines. One of the milk samples was artificially contaminated with known concentrations of AFM1 and this was detected and validated by HPLC for confirmation of the presence of the toxin. This AFM1 analysis using HPLC was carried out in an accredited Lab. The commercial milk and infant formula samples were tested by two commercial kits (Art. No.: R1121 & R5802) bought from Ridascreen® (approved by AOAC) and a CL sandwich ELISA (**Figure 5.1**).

5.2 AOAC approved techniques

5.2.1 HPLC analysis of real milk sample for detection of AFM1

As described earlier in **Section 1.2.1.1**, HPLC is one of the most trusted and used conventional technique for routine analysis of aflatoxins.The principle of HPLC relies upon chromatographic separation of analytes when applied pressure is very high.

The analysis of real milk sample was done by HPLC technique. This was carried out in an accredited Lab in India. The samples were artificially contaminated or spiked by known amount of AFM1 and sent for analysis. The test portion was extracted and cleaned up by passing through an immunoaffinity column containing specific antibodies bound onto a solid support. Antibodies selectively bound with any AFM1 contained in the extract, to give an antibody-antigen complex. Other components of matrix were washed off the column with water. AFM1 from the column was eluted with acetonitrile. After the elute was concentrated, the amount of AFM1 was determined by LC with fluorometric detection.

5.2.2 Commercial kit

There are several commercial kits available for AFM1 analysis such as Vicam test kit, Helica ELISA kits, Ridascreen® ELISA kits, Beacon plate and tube kits, SNAP ELISA kits, MaxSignal® ELISA kits, AuroFlow™ strip test kits etc. 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) and Rodriguez et al. (2003). The kit is part of the RIDASCREEN® range of diagnosis.

The principle of the commercial kit analysis is done by ELISA. This reaction process is classified as a competitive enzyme immunoassay. In that case, a monoclonal antibody is incorporated which is specific to AFM1 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 byproduct will cause a colour reaction with the chromogen from a blue color to a yellow color, which is then measured at 450 nm. The absorbance is proportional to the amount of enzyme conjugate bound, which is therefore inversely proportional to the amount of AFM1.

5.3 Real sample analysis

5.3.1 Samples

A total of 72 samples were purchased from retail shops in the city of Goa, India comprising of liquid milk (plain) of 15 different brands and 3 infant formula milk powders before their expiry date that is sold in the local market. 70% of these brands are also sold all over India. These samples were stored in the laboratory at a temperature of less than 4 $^{\circ}$C.

5.3.2 Chemicals

The AOAC approved commercial kits were purchased from R-Biopharm, Darmstadt, Germany. There were two kits used for validation work. Kit-1 has a dynamic range from 0-80 ng/L and Kit-2 has a wider dynamic range of 0-2000 ng/L. All other necessary chemicals and glasswares used were described earlier in **section 2.3**.

5.3.3 Sample preparation

Both milk and infant formula milk powders were randomly collected from the markets of Goa. Powder based samples (formula milk food) were suspended in warm de-ionized water as per the instructions written on the packets. The packaged milk samples as well as the formula milk samples were centrifuged at 6000 rpm for about 10 min. After centrifugation, the upper fat layer was completely removed, and the aqueous layer was filtered through a syringe filter using 0.22 micron filter paper.

Prior to the validation work, one of the milk samples was artificially contaminated with known concentrations of AFM1 and this was detected and validated by HPLC for confirmation of the

presence of the toxin. This AFM1 analysis using HPLC was carried out in an accredited Lab. Afterwards the assay results were compared against two commercial kits bought from Ridascreen® (which is also approved by AOAC), that showed good correlation among the two assays.

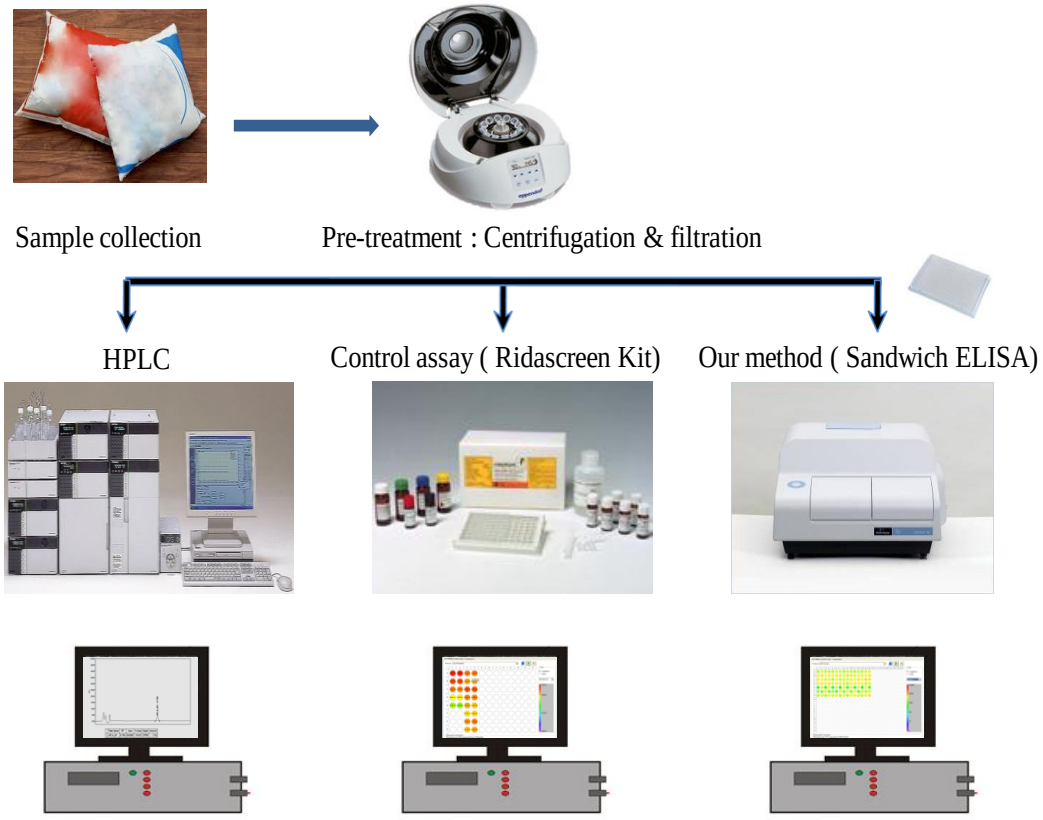


Figure 5.1 Schematic diagram of validation of AFM1 in milk by different AOAC methods and by developed CL ELISA.

5.3.4 Immunoassay procedure

To investigate the presence of AFM1, the milk samples were analyzed by ELISA. First, the samples were analyzed by sandwich ELISA. Subsequently, they were also tested by AOAC approved commercial kits from Ridascreen® where competitive ELISA was performed as per the protocol provided in the literature.

5.3.4.1 CL sandwich ELISA

Sandwich ELISA was performed in 384 microwell plate. The same protocol was followed as described in **section 2.2.1.3** with reduced incubation time of 1 h.

5.3.4.2 Competitive ELISA (using commercial Kit-1, dynamic range 0-80 ng/L)

The quantitative analysis of AFM1 in the commercial samples was first performed by competitive ELISA using Ridascreen AFM1 30/15 test kit (Kit-1) as per the instructions. The absorbance was measured within 10 min at 450 nm by the plate reader.

5.3.4.3 Competitive ELISA (using commercial Kit-2, dynamic range 0-2000 ng/L)

The analysis of AFM1 in milk samples was also done by RIDASCREEN® Fast AFM1 test, which has two types of antibodies and a wider dynamic range of antigens (0-2000 ng/L) as per the instructions. The plate was measured the absorbance at 450 nm by the plate reader. The intensity of absorbance was inversely proportional to the concentration of AFM1 in samples. The AFM1 concentration results from the ELISA assay were then analyzed.

5.4 Results and analysis

5.4.1 HPLC analysis

In HPLC analysis, the peaks generated were studied for the confirmatory test of AFM1 (**Figure 5.2**). In **Figure 5.2**, the chromatogram peaks are shown for the concentrations such as 0.625, 2.5, 5 and 10 ng/mL respectively that were spiked in certified reference milk samples and were analyzed through an accredited laboratory in India.

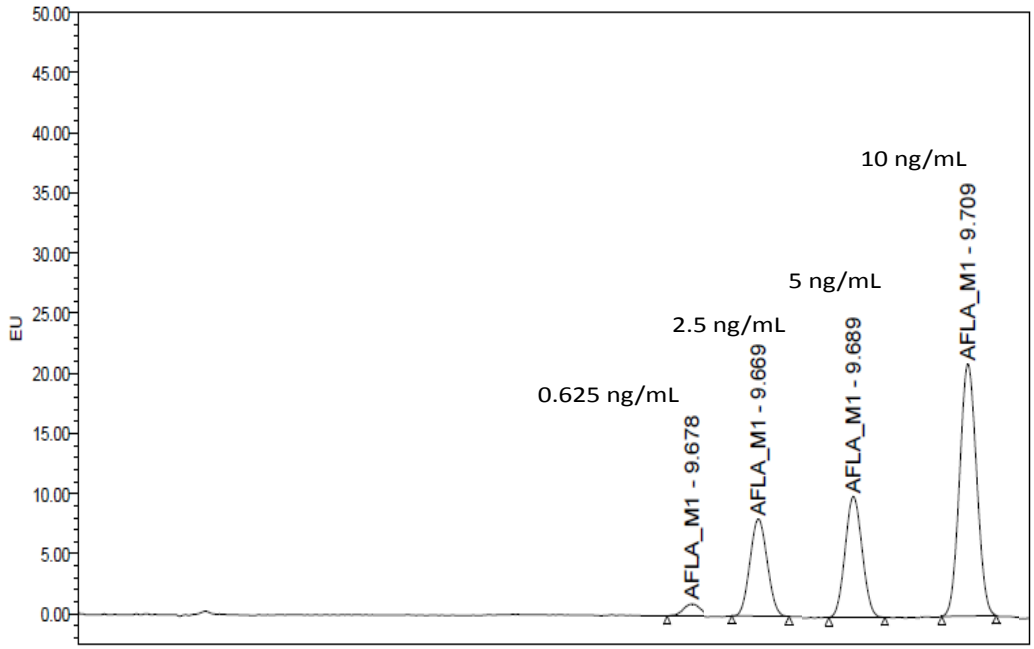


Figure 5.2 HPLC chromatogram peaks confirming AFM1 presence in the analyzed cerified reference milk samples.

5.4.2 AFM1 standard calibration curve

The calculations for CL sandwich ELISA was made by simple observations of signal intensities or photon counts plotted against various AFM1 concentrations. The signal intensity or photon counts were generated from the luminol/peroxidase reaction. The standard calibration curve for AFM1 detection by CL sandwich ELISA is shown in **Figure 5.3**. A concentration dependent decrease in signal intensity was observed for AFM1. Certified reference material ERM-BD282 was reconstituted to a liquid form which was later spiked with known amounts of AFM1 solutions in different concentrations. The assay was performed for 3 times in triplicate and the error values were plotted as a calibration curve. This curve showed the photon count in terms of signal intensity.

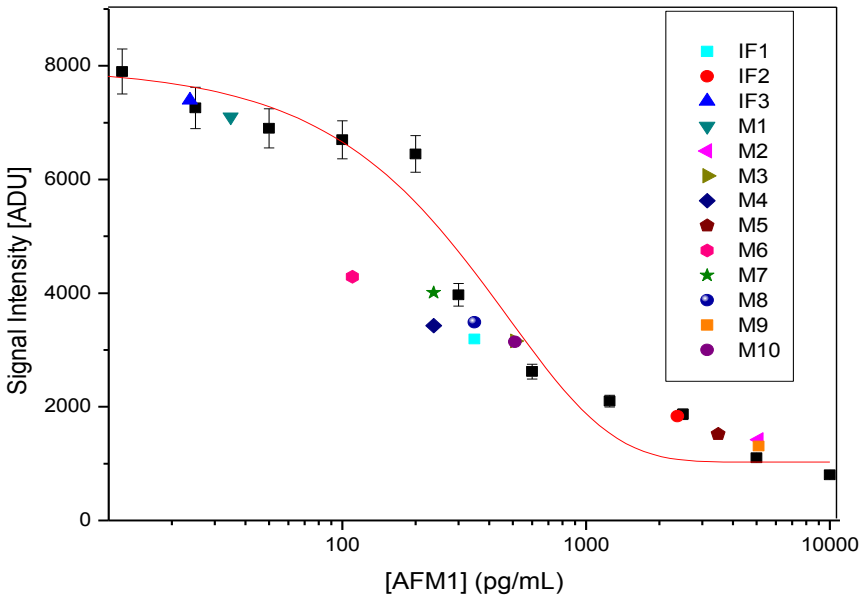


Figure 5.3 Calibration curve of CL ELISA for detection of AFM1 in real milk sample analysis; IF: infant formula milk; M: commercial milk.

The calibration curve the [AFM1] in tested infant formula milk powders as well as milk samples were obtained. The error bar indicates the standard deviation (n=3), where "n" is an independent assay by the proposed method. The S.D. and R^2 were calculated to be 1.69 and 0.89 respectively.

The calculations used for quantitative analysis by Ridascreen test kits were followed from the kit protocol. For the same, the % absorbance was plotted against the standard concentrations of AFM1 provided in the kit. The **Figure 5.4** shows the calibration curve obtained using commercial kit.

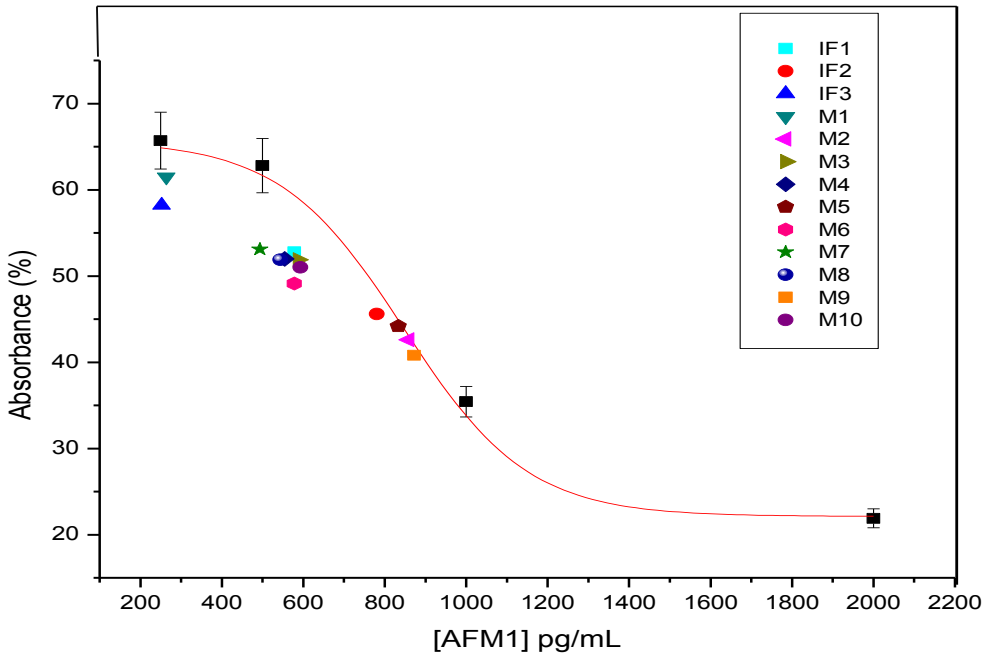


Figure 5.4 Calibration curve of AFM1 by commercial Kit-2.

The absorption intensity was found to be inversely proportional to AFM1 concentration in the sample. The calibration curve was then used to analyze several milk samples to detect AFM1 contamination levels as shown in **Figure 5.4**.

5.4.3 Recovery analysis of AFM1 spiked in milk samples

Recovery of AFM1 from spiked and CRM milk samples The developed microplate ELISA was further validated with CRM (ERM-BD282, zero level of AFM1) for milk powder. The milk powder was reconstituted as indicated in the certification report supplied by the IRMM, Belgium. To test the accuracy of developed assay, AFM1 concentrations ranging from 1 to 100 pg/mL were added to the milk samples and analyzed by ELISA. CRM milk samples containing zero AFM1 were compared with samples deliberately contaminated with known amounts of AFM1.

Recovery was assessed by spiking AFM1 with the CRM-BD282 reconstituted material and presented as **Table 5.1**. The fortified (1, 2.5, 6.25, 12.5, 25, 50 and 100 pg/mL of AFM1) milk samples (0.5% fat) were interpolated from the calibration curve performed using reconstituted

CRM. The precision and reliability of the developed assay is notable from the data presented in **Table 5.1**. The resultant data showed an excellent percentage of recovery, close to 100% for CRM. The precision was determined by calculating the relative standard deviation (R.S.D.%) for the replicate measurements and the accuracy (R.E.%) was calculated by assessing the agreement between measured and nominal concentration of the fortified samples.

$$\text{R.E. (relative error) \%} = \left[\frac{(\text{measured value- true value})}{\text{true value}}\right] \times 100$$

$$\text{R.S.D (relative standard deviation) \%} = \left[\frac{\text{standard deviation}}{\text{mean}}\right] \times 100;\ n= 3$$

Table 5.1 Summary of recovery studies of AFM1 using CL sandwich method and commercial kit.

Milk Samples	**AFM1 added (ng/L)**	**AFM1 found (ng/L)**		**R.S.D %**		**R.E.%**		**Recovery %**	
		CL ELISA	**kit assay**	**CL ELISA**	**kit assay**	**CL ELISA**	**kit assay**	**CL ELISA**	**kit assay**
IF*1	50.00	47	47.5	2.12	1.05	-6	-5	94	95
	500.00	486	480	1.02	0.41	-2.8	-4	97.2	96
M**1	50.00	57.5	47	4.31	2.1	15	-6	115	94
	500.00	508	534	1.18	0.56	1.6	6.8	101.1	106.8

*Infant formula milk, **Milk sample

5.4.4 AFM1 contamination level in commercial milk samples

The comparison of AFM1 contamination levels in real samples with EU, Codex, FSSAI and FDA standard is summarized in Table 5.2.

Table 5.2 AFM1 contamination in infant milk products and milk samples exceeding EC, Codex, FSSAI and USFDA regulations.

Assay type	Sample category	Samples analyzed	Positive samples	Exceeding EC regulations (Infant feed >25 ng/kg) (Liquid milk >50 ng/kg)		Exceeding Codex, FSSAI & USFDA regulations (Liquid milk >500 ng/kg)	
				Number#	Range (ng/kg)	Number#	Range (ng/kg)
Sandwich ELISA (our assay)	IF*	18	18	18 (100)	160-713	12 (66.6)	501-713
	M**	54	54	54 (100)	172-809	42 (77.7)	511-809
Competitive ELISA (AOAC control kit)	IF*	18	18	18 (100)	150-500	6 (33.3)	500-730
	M**	54	54	54 (100)	178-820	42 (77.7)	160-820
	Total	72	72	72	150-820	48 (66.6)	160-820

*Infant Formula milk, **Milk sample

#Values in parentheses indicate % samples exceeding the prescribed limits

It was observed that 100% of all the samples exceeded EC standard and around 75% of the samples exceeded Codex, FSSAI and USFDA standards. By CL sandwich ELISA method as well as by commercial kit, it was found that, out of 18 samples of infant formula milk analyzed, 100% would not pass the EC regulations, while this number is reduced to 66.6% and 33.3%

respectively if the USFDA or FSSAI regulations are applied. On the other hand, out of 54 liquid milk samples 100% exceeded EC regulations and 77.7% surpassed USFDA and FSSAI regulations.

The figures of merit of the CL sandwich ELISA were compared with Ridascreen kit and summarized in **Table 5.3**. The parameters such as detection limit, analysis time, sensitivity, sample throughput and cost per sample were compared. The dynamic range and the upper limit of detection for the milk samples using the kit were found inferior when compared against the sandwich assay. Although the analysis time of the kit was less than our assay, the detection limit and sensitivity of the CL sandwich assay was found to be more promising than the kit. The higher analysis time of CL sandwich assay can be compensated against the number of real samples per assay (n=128) in triplicate as against the commercial kit wherein 48 samples (Kit-1) and 24 samples (Kit-2) per assay can be analyzed. Moreover, the use of 384 microwell plate facilitates reduction in reagent consumption and volume of toxic waste. The comparison result suggests that the sandwich assay is better suited for ultra sensitive analysis of AFM1 contamination in milk samples and can be easily adapted for routine analysis.

Table 5.3 Summary of analytical figures of merit of the commercial kits and developed assay.

Figures of merit	**Ridascreen 30/15 kit (Kit-1)**	**Ridascreen Fast kit (Kit-2)**	**CL sandwich ELISA**
Dynamic range	0-80 ng/L	0-2000 ng/L	0-2000 ng/L
Limit of detection	5 ng/L	< 367 ng/L	0.005 ng/L
Limit of quantification	25 ng/L	500 ng/L	5 ng/L
Recovery rate with coefficient of variation (CV)	95% (CV=14%)	78-115% (CV=20%)	94-115% (CV=20%)
Time requirement	1.5 h/48 sample	0.5 h/24 sample	5 h/128 sample
High throughput	96	48	384
Sample volume (µL)	100	50	40
Cost per sample analysis (Indian Rupees)	320	164	62

5.5 Conclusions

The developed sandwich ELISA for AFM1 detection in real samples was successfully validated by AOAC approved methods. Based on the random sampling and analysis of commercial milk samples and infant formula milk samples, it is evident that the all the analyzed samples were found to contain AFM1 concentrations exceeding permissible limits of EU standard. These observations strongly suggest that it is necessary to pay attention to this subject. In this chapter both CL sandwich and competitive ELISA have been shown to be simple and useful. This developed CL sandwich technique can be used to monitor milk quality for low level AFM1 contamination. Moreover the sandwich assay could detect AFM1 contaminaiton as low as 60 pg/mL whereas commercial ELISA could detect 110 pg/mL in the commercial milk samples. From the analysis, it was found that around 75% of the samples were contaminated with AFM1 (Codex, USFDA and FSSAI). The detected levels of AFM1 in the analyzed samples show a serious health alarm in regards to the safety limits for AFM1 levels in collected infant formula and milk samples.

Chapter 6

Conclusions

This thesis has been focused on development of novel biosensing techniques for ultrasensitive, high throughput analysis of AFM1, AFM2 in milk and related products and AFB1 in groundnuts. The imaging assay as well as impedimetric immunosensor were miniaturized for aflatoxin analysis using novel devices. During the course of study, significant observations were noted. The overall conclusions are summarized below.

1. Introduction (**Chapter 1**)**:** This chapter gave a description of aflatoxin contamination in milk, milk products and in peanuts, its effect and consequent health effects and established quality standards set by national and international agencies. Analysis of aflatoxins (AFM1 and AFB1) in milk and food by conventional techniques, gaps in the existing research and need for biosensor was discussed here. Various aspects of biosensor particularly immunosensors based on optical and impedance transducers, current state of art for aflatoxins detection techniques were also reviewed. This chapter discussed about gap in the existing reported work, objective of the proposed doctoral work and finally about the thesis structure.

2. Development of an ultra sensitive immunoassay for analysis of AFM1 in milk (**Chapter 2**)**:** An ultrasensitive and high throughput micro well plate based CL sandwich ELISA technique was developed for analysis of AFM1 in milk. Extensive experiments were carried out for optimizing biochemical reaction parameters required for optical sensing followed by matrix interference studies and analysis of real samples. Ultra high sensitivity, lower detection limit up to 0.005 pg/mL and assay economy using very less antibodies for simultaneously screening up to 100 samples are the salient features of the developed assay. The presented assay was almost free from organic solvents**.** Assay miniaturization in 384 micro wellplate format resulted in drastic reduction of toxic waste without compromising the assay sensitivity. The developed ELISA was extensively used for real milk sample analysis for quality analysis. The AFM1 assay was performed using both CL and FL methods where signal suppression was evident with increase in analyte concentration. The detection of AFM1 concentration was very much quantifiable when the sandwich ELISA was performed by CL technique. By FL technique, although signal suppression was observed, the quantification of analyte concentration was also achieved.

3. Multi analysis of aflatoxins (**Chapter 3):** By FL detection technique, structurally analogous different aflatoxins were detected and quantified successfully and their cross reactivity were studied in 384 micro well plate. Simultaneous analyses of different aflatoxins were successfully investigated by FL technique. Mixture analysis was also carried out to detect the presence of co-contaminants by this fluorimetric assay. Quantitative image based aflatoxin detection was also carried out on a customized novel device by fluorescence microscope.

4. Application of label free immunosensor for analysis of aflatoxins (**Chapter 4):** Labeled techniques such as CL and FL detection were studied alongside the label free technique such as electro chemical impedance spectroscopy. The label free detection and analysis of AFM1 in milk and AFB1 in peanut matrix were successfully carried out and one model immunosensor was applied with impedance set up. This demonstrated a simple, cost effective, label-free impedimetric immunosensor for detection of AFM1, AFM2 and AFB1 in different food products. The immunosensor showed a low limit of detection (1 pg/mL) for AFB1 in peanut with a short analysis time of 20 min.

Using micro flow techniques, novel strategies were investigated for analysis of AFM1 and AFM2 in milk products. Processed milk products such as drinking yogurt and flavored milk samples along with certified reference milk samples were analyzed in the micro cell with impedimetric technique. Optimization of the inlet and outlet flow rates was done in the flow setup. Known amount of [AFM1] and [AFM2] were injected or spiked into the samples in the flow state. Analysis of AFM1 and AFM2 were successfully carried out in the flow system with very short analysis time of 5-10 min. This flow injection analysis by impedance provided scope for on-line monitoring of AFM1 and AFM2 in milk and related products.

Analysis of AFM1 and AFB1 on a novel IDE device was successfully carried out by EIS technique with very low sample volume (<10 μL) and short analysis time of 5-10 min. The IDE device featuring miniaturization with less sample volume did not compromise with assay sensitivity. The label free immunosensor was able to meet stringent regulatory standards of EU cut off at 50 pg/mL and below.

5. Validation of developed AFM1 assay in real samples (**Chapter 5):** The developed sandwich ELISA for AFM1 detection in real samples was successfully validated against AOAC approved methods. Based on the random sampling and analysis of commercial milk samples and

infant formula milk samples, it was evident that the all the analyzed samples were found to contain AFM1 concentrations exceeding permissible limits of EU standard. From the observations, it was concluded that both CL sandwich and competitive ELISA have been shown to be simple and useful. This developed CL sandwich technique can be used to monitor milk quality for low level AFM1 contamination. Moreover the sandwich assay could detect AFM1 contamination as low as 60 pg/mL whereas; commercial ELISA could detect 110 pg/mL in the commercial milk samples. From the analysis, it was found that, around 75% of the samples were contaminated with AFM1 (Codex, USFDA and FSSAI). The detected levels of AFM1 in the analyzed samples show a serious health alarm in regards to the safety limits for AFM1 levels in collected infant formula and milk samples.

Future scope of work

- The developed miniaturized bioassay for ultrasensitive analysis of AFM1 in milk can be deployed for screening of milk samples at milk collection centres or centralized laboratories.
- The developed biosensor can be extended for multi mycotoxin research.
- The developed immunosensing techniques using FL can be exploited for analysis of different analytes by tagging multiple fluorophores.
- The developed immunosensing techniques can be used for analysis of cheese for determination of AFM1 and AFM2.
- Development of novel strategies for remediation of AFB1 in cattle feed stock and other affected food commodities.
- Development of novel techniques for removal of AFM1 in contaminated milk samples to improve the milk quality.

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CERTIFICATE OF ANALYSIS

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WHOLE MILK POWDER (zero level)		
Compound	Mass fraction	
	Certified value [1] [µg/kg]	Uncertainty [µg/kg]
Aflatoxin M_1	< 0.02	-

1) Mass fraction below the limits of detection of less than 0.02 µg/kg with a probability of 95 %, independently obtained by 9 laboratories and traceable to analytical methods based on immunoaffinity clean-up, separation by high-performance chromatography and fluorometric detection of the target analyte.

This certificate is valid for one year after purchase.

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NOTE

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Accepted as an ERM®, Geel, November 2004
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Signed:

Prof. Dr. Hendrik Emons
Unit for Reference Materials
EC-DG JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

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DESCRIPTION OF THE SAMPLE

The material is a whole milk powder. It is supplied in units of at least 30 g in amber glass bottles filled and sealed under nitrogen.

ANALYTICAL METHOD USED FOR CERTIFICATION

The methods used for certification involved instrumental determination by high performance liquid chromatography using a variety of separation techniques and fluorescence detection. The methods mainly varied in their initial extraction and clean-up procedures. Details of the methods used are given in the certification report.

PARTICIPANTS

Central Science Laboratory (CSL), York (UK)
Centrum voor Landbouwkundige Onderzoek (CLO), Melle (BE)
Direct Laboratories, Wolverhampton (UK)
Eläinlääkintä - ja elintarviketutkimuslaitos (EELA), Helsinki (FI)
Eusko Jaurlaritza, Dept. de Sanidad, Bilbao (ES)
Institute for Reference Materials and Measurements (IRMM), Geel (BE)
Instituto Nacional de Engenharia e Tecnologia Industrial (INETI), Lisbon (PT)
Istituto Superiore di Sanità (ISS), Rome (IT)
Laboratory of the Government Chemist (LGC), Teddington (UK)
Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek (TNO), Zeist (NL)
Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven (NL)
Rijks-Kwaliteitsinstituut voor Land- en Tuinbouwproducten (RIKILT), Wageningen (NL)

SAFETY INFORMATION

Not applicable.

INSTRUCTIONS FOR USE

The material is intended to serve as analytical blank for the determination of aflatoxin M_1 in milk or milk powder:

a) for recovery experiments;

b) to investigate laboratory contamination during storage and analysis of samples;

c) to investigate limits of detection of analytical procedures.

The laboratory must judge whether the given limit of the aflatoxin M_1 mass fraction is sufficient for its purposes.

The bottles should be allowed to warm to ambient temperature before opening to avoid water condensation. Before sub-samples are taken the content should be thoroughly mixed. The materials may be used as received or after reconstitution with water to simulate liquid milk. Recommendations for the reconstitution procedure are given in the certification report (c.f. Instructions for use).

STORAGE

The bottles should be stored unopened at - 20 °C or less.

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NOTE

A detailed technical report is available on www.erm-crm.org. A paper copy can be obtained from IRMM on request.

European Commission - Joint Research Centre
Institute for Reference Materials and Measurements (IRMM)
Retieseweg 111, B - 2440 Geel (Belgium)
Telephone: +32-14-571.722 - Telefax: +32-14-590 406

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Enzymimmunoassay zur quantitativen Bestimmung von Aflatoxin M_1

Enzyme immunoassay for the quantitative analysis of aflatoxin M_1

Art. No.: R1121

In vitro Test

Lagerung bei 2 - 8 °C

Storage at 2 - 8 °C

R-Biopharm AG, Darmstadt, Germany
Tel.: +49 (0) 61 51 81 02-0 / Telefax: +49 (0) 61 51 81 02-20

RIDASCREEN® FAST Aflatoxin M_1

Enzymimmunoassay zur quantitativen Bestimmung von Aflatoxin M_1

Enzyme immunoassay for the quantitative analysis of aflatoxin M_1

Inmunoensayo enzimático para el análisis cuantitativo de aflatoxina M_1

Art. No.: R5812

In vitro Test
Lagerung bei 2-8 °C
Storage at 2-8 °C
Almacenar entre 2-8 °C

R-Biopharm AG, Darmstadt, Germany
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49.3.07

AOAC Official Method 2000.08
Aflatoxin M_1 in Liquid Milk
Immunoaffinity Column by Liquid Chromatography
First Action 2000

(Applicable to determination of aflatoxin M_1 in raw liquid milk at > 0.02 ng/mL).

Caution: This method requires the use of solutions of aflatoxin M_1. Aflatoxins are carcinogenic to humans. Aflatoxins are subject to light degradation. Protect analytical work from the daylight and keep aflatoxin standard solutions protected from light by using amber vials or aluminum foil. The use of non acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Special attention should be taken with new glassware. Thus, before use, soak glassware in dilute acid (e.g., sulfuric acid, 110 mL/L) for several hours; then, rinse extensively with distilled water to remove all traces of acid (check with pH paper).

See Table **2000.08** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

The test portion is extracted and cleaned up by passing through an immunoaffinity column containing specific antibodies bound onto a solid support. Antibodies selectively bind with any aflatoxin M_1 (antigen) contained in the extract, to give an antibody–antigen complex. Other components of matrix are washed off the column with water. Aflatoxin M_1 from the column is eluted with acetonitrile. After the eluate is concentrated, the amount of aflatoxin M_1 is determined by LC with fluorometric detection.

B. Performance Standards for Immunoaffinity Columns

The immunoaffinity column shall contain antibodies against aflatoxin M_1 with a capacity of not less than 100 ng aflatoxin M_1 (which corresponds to 2 ng/mL when 50 mL test portion is applied). Recovery of not less than 80% must be obtained for aflatoxin M_1 when a calibrant solution containing 4 ng toxin is applied (which corresponds to 80 ng/L for a loaded volume of 50 mL).

Any immunoaffinity column meeting the above specifications can be used. Check the performance of the columns regularly, at least once for every batch of columns.

C. Apparatus

(**a**) *Disposable syringe barrels.*—To be used as reservoirs (10 and 50 mL capacity).

(**b**) *Vacuum system.*—For use with immunoassay columns.

(**c**) *Centrifuge.*—To produce a radial acceleration of at least 2000 × *g*.

(**d**) *Volumetric pipets.*

(**e**) *Microsyringes.*—100, 250, and 500 µL (Hamilton, or equivalent).

(**f**) *Glass beakers.*

(**g**) *Volumetric flasks.*—50 mL.

(**h**) *Water bath.*—37 ± 2°C.

(**i**) *Filter paper.*—Whatman No. 4, or equivalent.

(**j**) *Conical glass tubes.*—5 and 10 mL, stoppered.

(**k**) *Spectrophotometer.*—Wavelength 200–400 nm, with quartz face cells of optical length 1 cm.

(**l**) *Liquid chromatography equipment.*—With pump delivering a steady flow rate of 0.8 mL/min; loop injection system of 50–200 µL capacity; fluorescent detection with 365 nm excitation and 435 nm emission; and recorder, integrator, or computer-based processing system.

(**m**) *Reversed-phase LC analytical column.*—The following columns have been used satisfactorily: Octadecylsilane (ODS, ODS-1, ODS-2, ODS Hypersil, Nucleosil C18 [Machery-Nagel], Chromospher C18, Nova-Pak C18 [Waters Corp.], LiChrosorb RP18 [Merck KGaA, Darmstadt, Germany], Microsphere C18); dimensions (mm): 100 × 2.3, 4.6, 5; 125 × 4; 200 × 2.1, 3, 4; 250 × 4.6; with and without guard columns.

(**n**) *Mobile phases.*—Water–acetonitrile (75 + 25) or (67 + 33); water–acetonitrile–methanol (65 + 25 + 10); or water–isopropanol–acetonitrile (80 + 12 + 8). Degas before use.

D. Reagents

(**a**) *Chloroform.*—Stabilized with 0.5–1.0% ethanol.

(**b**) *Nitrogen.*

(**c**) *Aflatoxin M_1 standard solutions.*—(*1*) *Stock standard solution.*—1 µg/mL. Suspend a lyophilized film of reference standard aflatoxin M_1 in chloroform to obtain the required concentration. Determine the concentration of aflatoxin M_1 by measuring its absorbance at the maximum (ca 365 nm) in a calibrated spectrophotometer against chloroform as a blank between 200–400 nm. Check purity by noting an undistorted shape of the recorded peak. Calculate the mass concentration (C, µg/mL) from the equation:

Table 2000.08. Interlaboratory study results for aflatoxin M_1 in liquid milk immunoaffinity column LC method

Sample ID	No. of labs, a(b)[a]	$\bar{x}$ Average (ng/mL)	r	s_r	RSD_r, %	R	s_R	RSD_R, %	HORRAT value	Rec., %
Spiked	10 (2)	0.037[b]	0.019	0.007	18	0.032	0.011	31	0.42	74
a	12 (0)	<0.005	—	—	—	—	—	—	—	—
b	12 (0)	0.023	0.011	0.004	17	0.017	0.006	27	0.33	93
c	12 (0)	0.046	0.016	0.006	12	0.029	0.010	23	0.31	94
d	12 (0)	0.103	0.022	0.008	8	0.062	0.022	21	0.33	107

[a] a = Number of labs retained after eliminating outliers; (b) = number of labs removed as outliers.

[b] Spike level = 0.05 ng/mL.

Note: Statistical analysis was not carried out on the blank milk (a).

$$C = \frac{1000AM}{\varepsilon}$$

where A is the measured absorbance at the maximum wavelength, M is the molecular mass of aflatoxin M_1 (328 g/mol), and ε is the absorption coefficient of aflatoxin M_1 in chloroform (1995 m^2/mol).

Store this stock solution in a tightly stoppered amber vial below 4°C. Solution is stable ca 1 year.

(2) *Working standard solution.*—1 µg/mL. Transfer by means of a syringe 50 µL of the standard stock solution, (c)(*1*), into an amber vial and evaporate to dryness under a steady stream of N. Dissolve the residue vigorously in 500 µL acetonitrile using a Vortex mixer. Store this solution in a tightly stoppered amber vial below 4°C. Solution is stable ca 1 month.

(3) *Calibrant standard solutions.*—Prepare on day of use. Bring working standard solution, (c)(2), to ambient temperature. Prepare a series of standard solutions in the mobile phase, **C(n)**, of concentrations that depend upon the volume of the injection loop in order to inject, e.g., 0.05–1.0 ng aflatoxin M_1.

E. Preparation of Test Solution

Warm milk before analysis to ca 37°C in a water bath, and then gently stir with magnetic stirrer to disperse the fat layer. Centrifuge liquid milk at 2000 × *g* to separate the fat and discard thin upper fat layer. Filter through one or more paper filters, collecting at least 50 mL. Let immunoaffinity columns reach room temperature. Attach syringe barrel to top of immunoaffinity cartridge. Transfer 50 mL (V_s) of prepared test portion with volumetric flask or volumetric pipet into syringe barrel and let it pass through immunoaffinity column at slow steady flow rate of ca 2–3 mL/min. Gravity or vacuum system can be used to control flow rate.

Remove syringe barrel and replace with a clean one. Wash column with 20 mL water at steady flow rate. After washing completely, blow column to dryness with N stream. Put another dry clean barrel on the cartridge. Slowly elute aflatoxin M_1 from column with 4 mL pure acetonitrile. Allow acetonitrile to be in contact with column at least 60 s. Keep steady slow flow rate. Collect eluate in conical tube. Evaporate eluate to dryness using gentle stream of N. Dilute to volume V_f of mobile phase, i.e., 200 µL (for 50 µL injections) to 1000 µL (for 250 µL injections).

F. LC Determination with Fluorescence Detection

Pump mobile phase at steady flow rate through LC column. Depending on the kind of column, the acetonitrile–water ratio and flow rate of the mobile phase may be adjusted to ensure optimal separation of aflatoxin M_1 from other extract components. As a guideline for conventional columns (with a length of 250 mm and id of 4.6 mm), a flow rate of ca 0.8 mL/min gives optimal results. Check optimal conditions with aflatoxin M_1 calibrant solution and spiked milk before analyzing test materials.

Check linearity of injection calibrant solutions and stability of chromatographic system. Repeatedly inject a fixed amount of aflatoxin M_1 calibrant solution until stable peak areas or heights are obtained. Peak areas or heights corresponding to consecutive injections must be within ± 5%. Retention times of aflatoxin M_1 can vary as a function of temperature and must be monitored by injecting a fixed amount of aflatoxin M_1 calibrant solution at regular intervals.

(*1*) *Calibration curve of aflatoxin M_1.*—Inject in sequence suitable volumes V_i, depending on the injection loop, aflatoxin M_1 standard solutions containing from 0.05 to 1 ng. Prepare a calibration graph by plotting the peak area or peak height against the mass of injected aflatoxin M_1.

(2) *Analysis of purified extracts and injection scheme.*—Inject suitable volume V_i (equivalent to at least 12.5 mL milk) of eluate into LC apparatus through injection loop. Using the same conditions as for calibrant solutions, inject calibrants and test extracts according to stipulated injection scheme. Inject an aflatoxin M_1 calibrant with every 10 injections. Determine aflatoxin M_1 peak area or height corresponding to the analyte, and calculate aflatoxin M_1 amount W_a in test material from the calibration graph, in ng. If aflatoxin M_1 peak area or height corresponding to test material is greater than the highest calibrant solution, dilute the eluate quantitatively with mobile phase and re-inject the diluted extract into the LC apparatus.

G. Calculation

Calculate aflatoxin M_1 mass concentration of the test sample, using the following equation:

$$W_m = W_a \times (V_f / V_i) \times (1/ V_s)$$

where W_m = the numerical value of aflatoxin M_1 in the test sample in ng/mL (or µg/L); W_a = the numerical value of the amount of aflatoxin M_1 corresponding to area or height of the aflatoxin M_1 peak of the test extract (ng); V_f = the numerical value of the final volume of redissolved eluate (µL); V_i = the numerical value of the volume of injected eluate (µL); V_s = the numerical value of volume of prepared test portion passing through the column (mL).

Express the results to 3 significant figures.

Reference: *J. AOAC Int.* **84**, 437(2001).

List of Publications

Patent applications

1. Inventor in Indian Patent Application "A device for analysis of aflatoxins", Application No. 1203/MUM/2013, Ownership-ICAR, Govt. of India. Inventors: Sunil Bhand, Sudhir Chandra, Hardik Pandya, Gautam Bacher, Lizy Kanungo (Output from chapter 4).

2. Inventor in Indian Patent Application "A novel sample handling device for imaging" **to be filed** through BITS, Pilani. Inventors: Sudhir Chandra, Sunil Bhand, Lizy Kanungo, Ruchi Tiwari (Output from chapter 3).

List of publications counted in Thesis

1. L. Kanungo*, S. Pal*, S. Bhand. Miniaturized hybrid immunoassay for high sensitivity analysis of aflatoxin M1 in milk. Biosensors & Bioelectronics. 2011, 26 (5), 2601-2606 (Output from chapter 2).

2. G. Bacher*, L. Kanungo*, S. Bhand. Miniaturized label-free impedimetric immunosensor for analysis of Aflatoxin B1 in peanut. IEEE explorer, DOI: 10.1109/ICSensT.2012.6461690 (Output from chapter 4).
   **Authors with equal contribution*

3. L. Kanungo, S. Bhand. A survey of Aflatoxin M1 in some commercial milk samples and infant formula milk samples in Goa, India. Submitted revised manuscript to Food and Agricultural Immunology, Manuscript ID CFAI-2012-0086.R1 (Output from chapter 5).

4. L. Kanungo, S. Bhand. Fluorimetric immunoassay for multianalysis of aflatoxins. Submitted to Special Issue of International Journal of Analytical Chemistry, Manuscript ID 584964.v1 (Output from chapter 3).

Other publication

1. G. Bacher, S. Pal., L. Kanungo, S. Bhand, A label-free silver wire based impedimetric immunosensor for detection of aflatoxin M1 in milk. Sensors & Actuators B. 2012,168, 223- 230.

Conferences and workshops attended

- Poster presented in National Conference on Emerging Trends in Life Sciences Research organized by Biological Sciences Group and Pharmacy Group BITS, Pilani-K. K. Birla Goa Campus, March 6-9, 2009.

Accepted abstracts in conferences

1. L. Kanungo, S. Pal, S. Bhand. Miniaturized Immunoassay for high sensitivity analysis of Aflatoxin M1 in milk. Biosensors 2010.20th Anniversary World Congress on Biosensors. Glasgow, UK.

2. L. Kanungo, G. Bacher, S. Pal, S. Bhand. Ultrasensitive Micro-Biosensors for Analysis of Aflatoxins. Emerging Technologies from micro to nano (ETMN-2013). BITS, Pilani-K.K. Goa Campus.

3. G. Bacher, L. Kanungo, S. Bhand. Miniaturized label-free impedimetric immunosensor for analysis of Aflatoxin B1 in peanut. ICST-2012, Kolkata, India.

Brief Biography of the Candidate

Name	Lizy Kanungo
Date of Birth	30-06-1982
Education	M. Sc. (Bioinformatics)
	Orissa University of Agriculture and Technology, Orissa (2004)
	B. Sc (Chemistry, Botany, Zoology as Honours)
	Ramadevi Autonomous College, Bhubaneswar, Orissa (2002)
Email ID	lizy_kanungo@yahoo.co.in, lizy.kanungo1@gmail.com

Research Experience (5 years)

1. Presently working as Research Associate in National Agricultural Innovation project (NAIP), ICAR (July 2012 till date).

2. Worked as Senior Research Fellow in National Agricultural Innovation project (NAIP), ICAR (March 2008 to June 2012).

Research Publications

01 Indian Patent filed & 1 in process of filing,

03 publications in international journals & 2 manuscripts submitted

Conferences/workshops participated/ attended

1 national, 1 international

Work Experience

1. 2 years of experience as a Lecturer in Bioinformatics in Royal School of Management and Technology, IDCO Tower-2000, Mancheswar, Bhubaneswar, Orissa (2004-2006).

2. 1.5 yrs of experience as Asst. Professor in the Dept. Of Bioinformatics, Centre for Post Graduate Studies,Orissa University of Agriculture & Technology, Bhubaneswar, Orissa (2006-2008).

Honors and Awards

1. Best All rounder award in school career
2. National scholarship holder in 10^{th}
3. National scholarship holder in B.Sc.
4. Topper in B.Sc

Brief Biography of the Supervisor

Name	Sunil Bhand
Date of Birth	17.03.1969
Present Position	Professor & Head, Department of Chemistry
	BITS, Pilani-K. K. Birla Goa Campus
Address	C-201, BITS, Pilani-K. K. Birla Goa Campus
	NH17B Bypass, Zuari Nagar, Goa -403726, India
Email	sunilbhand@goa.bits-pilani.ac.in, sunil17_bhand@yahoo.com
Education	Ph.D. 1996
	M.Sc. 1990 (First in University Merit)

Post Doctoral Experience

Department of Pure and Applied Biochemistry

Lund University, Sweden 2001-2002

Short term visits 2003, 2004, 2005, 2007, 2008, 2011

No. of Sponsored Research Projects

(a) Completed projects

i. Joint Indo-Swedish Project on "Biosensors for Environmental analysis". 2003-2005, funded by Swedish Research Council. Prof. B. Danielsson and Prof. Sunil Bhand as joint PIs. (35 lakhs).

ii. CSIR Project 2006-2009 on "Biosensors for analysis of pesiticides in sea water" (14.6 lakhs).

(b) Ongoing projects

i. Consortium PI for NAIP, ICAR New Delhi funded project on "Development of biosensors and micro techniques for analysis of pesticide residues, aflatoxin, heavy metals and bacterial contamination in milk" in collaboration with IIT Delhi, NDRI-Karnal and PU Patiala (729 lakhs).

ii. Consortium Co-PI, NAIP project on "Detection and mitigation of dairy pathogens and detection of adulterants using chemical biology" (45 lakhs).

Honors and awards

i. Invited as Opponent to a Ph.D. Thesis for Linkoeping University Sept. 2011.
ii. Best Poster award "Biosensors for arsenic analysis" 7th Intl. Conference on Biogeochemistry of trace elements 2003, Uppsala, Sweden.
iii. UV Rao memorial awards for young scientists by Indian Chemical Society 1998.

Publications

i. 4 Patents (1 PCT, 3 Indian) and 28 publications in international journals.
ii. Membership of societies: Affiliate member IUPAC since 2000, IAEAC Switzerland, AAAS, USA, 2012.

Reviewer for International journals

Biosensors and Bioelectronics, Analytical Letters, International Journal of Environmental & Analytical Chemistry, Applied Biochemistry and Biotechnology, Journal of Agricultural Food Chemistry.

No. of Ph.D. Students

Completed 02, Registered 04.

No. of Conferences organized: 03