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GEBZE TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EFFECT OF DIFFERENT ANTI-AFLATOXIN ANTIBODY ISOTYPES ON BIOSENSOR AND IMMUNOAFFINITY COLUMN PERFORMANCE

ÖZLEM ERTEKİN

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR

PROF. DR. ZAFER ZİYA ÖZTÜRK

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2016

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AFLATOKSİNE KARŞI GELİŞTİRİLEN FARKLI İZOTİPLİ ANTİKORLARIN BİYOSENSÖR VE İMMUNOAFFİNİTE KOLON PERFORMANSLARI ÜZERİNE ETKİLERİ

ÖZLEM ERTEKİN
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. ZAFER ZİYA ÖZTÜRK

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SUMMARY

Antibody based techniques are widely used for the detection of aflatoxins which are potent toxins with a high rate of occurrence in many food and feedstuff. The success of antibody based methods inevitably depends on the choice of the antibody. In this thesis, we evaluated the structural character of the antibody determined by its isotype as an antibody selection parameter for the development of immunoanalytical systems.

We developed murine monoclonal antibodies of immunoglobulin A (IgA) and immunoglobulin G (IgG) isotypes with similar affinities to aflatoxin B1 (AFB1) in order to conduct comparative studies in immunoaffinity column (IAC) and Quartz Crystal Microbalance (QCM) immunosensor development. Both antibodies were effectively utilized in either of the systems with distinctive performances. We showed that antibody affinity, rather than isotype was determinative for the performance of IACs. In QCM biosensor development employing competitive immunoassay, the detection range for AFB1 was higher with IgA antibody than IgG due to its higher molecular weight.

The monoclonal IgA antibody developed in this research was hitherto the first presentation of quadruple antigen binding IgA monoclonal antibodies in mycotoxin analysis and also the first study of the utilization of IgA antibodies in IAC and immunosensor development. IgA antibodies are proven to be valuable alternatives for immunoassay development.

Key Words: Antibody isotype, mycotoxin, monoclonal antibody; immunoglobulin A; immunoaffinity column, QCM Immunosensor.

ÖZET

Antikora dayalı sistemler, gıda ve yemlerde sıklıkla rastlanan aflatoksinlerin tespitinde yaygın olarak kullanılmaktadır. Bu sistemlerin başarısı tamamen antikor seçimine bağlıdır. Bu çalışmada, immunoanalitik yöntemleri geliştirirken izotipe dayalı yapısal karakterin antikor seçimindeki önemi değerlendirilmiştir.

İmmunoafinite kolon (IAK) ve kuartz kristal mikrotartım (QCM) biyosensörleri geliştirmek amacıyla karşılaştırmalı çalışmalar yürütmek için aflatoksin B1 (AFB1)'e karşı benzer afinitelerde immunoglobulin A (IgA) ve immunoglobulin G (IgG) izotipli fare monoklonal antikorları geliştirildi. Her bir antikor iki sistemde de farklı ama yüksek etkinlikte kullanıldı. IAK'larda antikorların izotipinden ziyade afinitesinin performans üzerinde etkili olduğu görüldü. QCM biyosensörleri geliştirilirken yapılan antikor temelli yarışımlı çalışmalarla IgA izotipli antikorun moleküler ağırlığı daha yüksek olduğu için, IgG'ye göre AFB1 tespit ölçüm aralığının daha geniş olduğu görüldü.

Bu çalışma ile dört antijen bağlayabilen IgA antikorlarının mikotoksin analizlerinde kullanımı şimdiye dek ilk defa gösterilmiştir. Aynı zamanda IAK ve immunosensör gelişiminde IgA kullanılabilirliği de ilk defa sunulmaktadır. IgA antikorlarının antikor temelli çalışmaların geliştirilmesinde değerli bir alternatif olduğu da bu şekilde kanıtlanmaktadır.

Anahtar Kelimeler: Antikor izotipleri, mikotoksin, monoklonal antikor, immunoglobulin A, immunoafinite kolonu, QCM immunosensör.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
ε	:	Molar Extinction Coefficient
A	:	Absorbance
AF	:	Aflatoxin
AFB1- BSA	:	Conjugated Aflatoxin B1 and Bovine Serum Albumin
AFB1 oxime	:	AFB1 - o carboxymethyloxime
AFB1- OVA	:	Conjugated Aflatoxin B1 and Ovalbumin
AFB1- TF	:	Conjugated Aflatoxin B1 and Apo-Human Serum Transferrin
AP	:	Alkaline Phosphatase
BCR	:	B Cell Receptor
BSA	:	Bovine Serum Albumin
cBSA		Cationized Bovine Serum Albumin
cOVA		Cationized Ovalbumin
cTF	:	Cationized Apo-Human Serum Transferrin
IC-ELISA	:	Indirect Competitive ELISA
CMO	:	Carboxymethyl hydroxylamine HCl
DEAE-C	:	Diethylaminoethyl Cellulose
DMF	:	Dimethyl formamide
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethylsulphoxide
EDA	:	Ethylene diamine
EDC	:	1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride
ELISA	:	Enzyme Linked Immunosorbent Assay
EOA	:	Ethanolamine
Fab	:	Antigen Binding Fragment of Antibody
Fc	:	Constant Fragment of Antibody
FCS	:	Fetal Calf Serum
FLD	:	Fluorescence Detector
HPLC	:	High Performance Liquid Chromatography

Hz	:	Hertz
IAC	:	Immunoaffinity Column
IC50	:	50% Inhibitory Concentration
Ig	:	Immunoglobulin
IL	:	Interleukin
LC-MS/MS	:	Liquid Chromatography-Tandem Mass Spectrometry
MAb	:	Monoclonal Antibody
MES	:	2-(N-morpholino) - ethane sulfonic acid
MUA	:	11-mercaptoundecanoic acid
MW	:	Molecular Weight
NHS	:	N-hydroxysuccinimide
OTA	:	Ochratoxin A
OVA	:	Ovalbumin
PEG	:	Polyethylene glycol
PBS	:	Phosphate Buffered Saline
PNPP	:	Para-Nitro phenyl phosphate
QCM	:	Quartz Crystal Microbalance
SAM	:	Self-Assembled Monolayer
SDS PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TF	:	Apo-Human Serum Transferrin
TLC	:	Thin Layer Chromatography
ZEA	:	Zearelanone

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1. INTRODUCTION

Aflatoxins (AFs) are hepatotoxic mycotoxins that are produced by *Aspergillus* spp. Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are the four common naturally occurring analogues of AF. High level exposure to AFs results in acute toxicity which may lead to death, and chronic exposure often leads to liver diseases including liver cancer in humans [1–3].

AF levels in food and feedstuff is regulated in many countries due to their toxic effects [4, 5] and several methods are devised to fulfil the requirements of the regulations. Liquid chromatography based methods including high pressure liquid chromatography (HPLC) or liquid chromatography-tandem mass spectrometry (LC-MS/MS); and enzyme based immunological test methods including enzyme linked immunosorbent assay (ELISA) are most commonly used and internationally accepted methods for AF quantification. Instrumental AF analysis requires an extract cleanup step with immunoaffinity chromatography in order to concentrate and remove the AFs from complex extract matrix. Both immunoaffinity columns (IACs) and ELISA systems utilize the ability of an anti-AF antibody to specifically bind AFs. In addition to the mentioned laboratory based standard methods, there is a requirement for on-site analysis of aflatoxins to provide rapid risk assessment. Biosensors, which have gained popularity during the past decade, are the most potent solutions towards this goal. Various studies have been conducted in order to develop AF biosensors. Among these studies, immunosensors are widely preferred since they utilize the specificity of the antibodies as sensing element.

Antibody based methods are widely used for AF analysis and their performance ultimately relies on the quality of the antibody. Hence, choosing a suitable antibody for the method is the most critical factor for the success of the developed system. Although ground rules for antibody selection such as specificity, selectivity and affinity are well established, structural character of the antibody, which is determined by its isotype, is an overseen important criterion. IgG antibodies are divalent with a MW of 150.000 kDa. They are the most abundant isotype in mammals and accordingly, they are the most easily developed MAbs. 960 kDa, decavalent IgM antibodies are also abundant but their low affinity makes them questionable to be used in immunoanalysis. IgA antibodies are also high affinity

antibodies with 300 kDa MW and tetravalent structure. However, their relatively low abundance makes it harder, while possible, to develop as monoclonals. Each isotype, with their differences in terms of MW and valency, may pose some advantages and/or disadvantages depending on the requirements of the developed system which may rely on antibody immobilization or antigen immobilization.

In antibody immobilized systems such as IACs or direct immunoassays, proper orientation of the antibody so that antigen binding sites will be freely available for binding is of crucial importance. When IgG antibodies are randomly immobilized to a solid support, about 50% of the antibody activity is impaired [6]. This results in the need for purified antibody solutions for concentrated binding to the immunosorbent surface, a higher amount of the immunosorbent material and/or a means of orienting the antibody, so that the antigen binding sites are free. The use of multivalent antibodies may be a solution to this problem as there will be free antigen binding sites even when some are blocked during immobilization.

In antigen immobilized systems such as biosensors developed with competitive immunoassays, the molecular size of the antibodies comes into prominence. Molecular size is especially important in Quartz Crystal Microbalance (QCM) transducers which are sensitive to mass change on their surface. Detection of small molecule analytes as AF usually requires various labels like enzymes, nanoparticles, or fluorescent molecules in order to increase sensitivity. QCM based AF sensors also utilized signal enhancers such as nanoparticles [7] or magnetic beads [8]. With their higher MW compared to IgG, IgA antibodies are expected to generate a higher frequency shift in QCM transducer system which relies on the mass change on the sensor surface, and hence an increase in sensitivity can be expected.

The objective of this thesis is to evaluate the use of different antibody isotypes for the development of IAC and QCM immunosensor for the detection of AF. Therefore, the role of valency and molecular size of the antibodies on the performance of immunoanalytical systems will be elucidated. Previously known anti-AF antibodies are mostly IgG isotype [9,10] where few IgM isotype antibodies were reported [11]. Accordingly, AF IAC and immunosensor development literature is based on either polyclonal [12,13] or IgG [14,15] antibodies. In this thesis, AF specific antibodies of IgG, IgM and IgA isotypes were developed and their performances in IAC and QCM immunosensor were comparatively evaluated.

2. BACKGROUND

2.1. Aflatoxins

Mycotoxins are fungal metabolites which are among the most important food contaminants threatening human health. Food and Agricultural Organization reported 25% of agricultural products are contaminated with mycotoxins worldwide. Mycotoxins, which are mostly heat stable small molecules, can not be destroyed by usual cooking procedures and hence cause severe health implications including hepatotoxicity, genotoxicity, oncogenicity, nephrotoxicity, immunomodulation, fertility and reproduction perturbation, central nervous system damage or skin toxicity. Although nearly 300 types are known, AFs are the most prominent mycotoxins in accordance with their abundance and toxicity [16].

AFs are secondary metabolites of fungi from *Aspergillus* spp. which can cause acute and chronic toxicity in both humans and animals when ingested [17], [18]. High level exposure to AFs results in acute toxicity which may lead to death, and chronic exposure often leads to liver diseases including liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis in humans [1–3].

Among 20 different types of identified AFs, major members are AF B1, B2, G1, G2 and M1. AFB1 is grouped as class 1 human carcinogen by IARC [19]. AF B1, B2, G1 and G2 are the four common naturally occurring analogs of AF and AFM1 is a water-soluble AF analog found in milk produced by animal metabolism [20]. They are metabolized by cytP450 enzymes in liver. Their metabolites cause immune suppression, change in cell morphology and form DNA and protein adducts [1]. The primary metabolite which causes hepatotoxic effects is Aflatoxin-8,9-epoxide is produced by the metabolization of AFB1 by CYP1A2 and CYP3A4 [21]. This metabolite induces selective mutation in p53 oncogene, which leads to hepatocellular carcinoma [22].

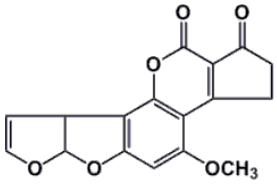
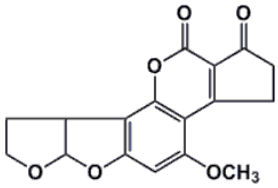
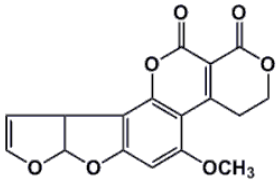
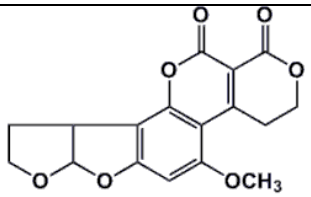
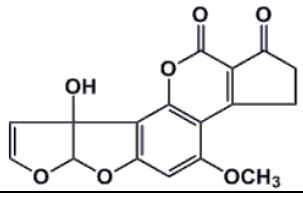
AF levels are controlled in food and feed products. Different maximum allowable AF levels for different feed products established by FDA are as follows; 20 ng/g for feed of immature animals, 100 ng/g for feed of breeding cattle, swine or poultry, 300 ng/g for the feed of finishing cattle, swine and poultry [23]. For food products, 20 ng/g limit is accepted [24], however, AFM1 level in milk and milk

products is limited by 0.5 ng/g [25]. These levels show differences in the regulations of different countries and organizations.

AFs are freely soluble in moderately polar organic solvents such as methanol, ethanol and dimethyl formamide and have limited solubility (10-20 mg/L) in water. They show spectral activity at UV range, where their maximal absorbance is between 360-363 nm and show a secondary absorption peak at 265 nm [18].

Their stability and reactivity was extensively studied in order to elucidate detoxification strategies. They are extremely stable to heat up to 100°C but unstable at extreme pH ranges (<3, >10). They are degraded by reacting with hypochloride and ammonia. Structure of common AFs are presented at Table 2. 1.

Table 2. 1: Structure of common aflatoxins.

Aflatoxin	Molecular formula	Molecular weight	Structure
B1	$C_{17}H_{12}O_6$	312	
B2	$C_{17}H_{14}O_6$	314	
G1	$C_{17}H_{12}O_7$	328	
G2	$C_{17}H_{14}O_7$	330	
M1	$C_{17}H_{12}O_7$	328	

2.1.1. Detection and Quantification

AFs are amongst the most abundant food and feed contaminants, and due to their high toxicity and carcinogenicity, they are of major concern for food producers, the food processing industry, traders and consumers [20,26]. National and international directives are employed in order to prevent the associated health risks [4, 5]. Corresponding methods have been developed for accurate and reliable analysis in various food products [27].

Internationally accepted precise AF quantification is conducted with laboratory based methods such as thin layer chromatography (TLC), HPLC, LC-MS/MS or immunological test methods including ELISA [17,18]. Most commonly used method is instrumental analysis with HPLC which requires a series of steps including sampling, homogenization, extraction, extract cleanup with IAC and detection.

Conventional AF quantification methods require expensive, sophisticated equipment and trained staff [2, 3]. The availability of rapid and on-site systems for the analysis of AF will both provide better control of AF contamination in food and feedstuff and also decrease the analytical costs. Biosensors are powerful tools which can meet this requirement.

AF specific antibodies are indispensible components of these methods. They are utilized in IACs for sample cleanup prior to instrumental methods and they are the core components of ELISA and biosensor systems.

2.2. Antibodies

Immunoglobulins (Ig) are globular glycoproteins composed of four polypeptide chains linked together with disulfide bonds. They are produced by B lymphocytes as important components of adaptive immune response in order to recognize specific antigens. Igs are found either on the surface of B lymphocytes or secreted to the bloodstream, tissues or mucosa by plasma cells. The secreted form of Igs, produced upon antigen encounter is denoted as antibodies and they exert specialized immune effector functions for host defense [28,29].

Igs are composed of 2 heavy chains (450-600 amino acids) and two light chains (~220 amino acids). N-terminal peptides of heavy and light chains are

different in each Ig and they form the variable (V) region. The remaining part of the Ig is called constant (C) region. Both heavy and light chain V regions have 3 hyper variable segments called complementarity determining regions (CDR). C region of the antibody determines the immune effector function [28,30].

2.2.1. Antigen Specific Immune Response

In mammalian systems, the availability of antibodies capable of recognizing a variety of different antigens is the result of high variability at V region. 10^{11} different Igs can be produced in the lifespan of a human. Initial source of this variability is at genetic level. The DNA coding this part of the Igs is composed of gene segments which can be used in different combinations through somatic recombination (Figure 2. 1) [31].

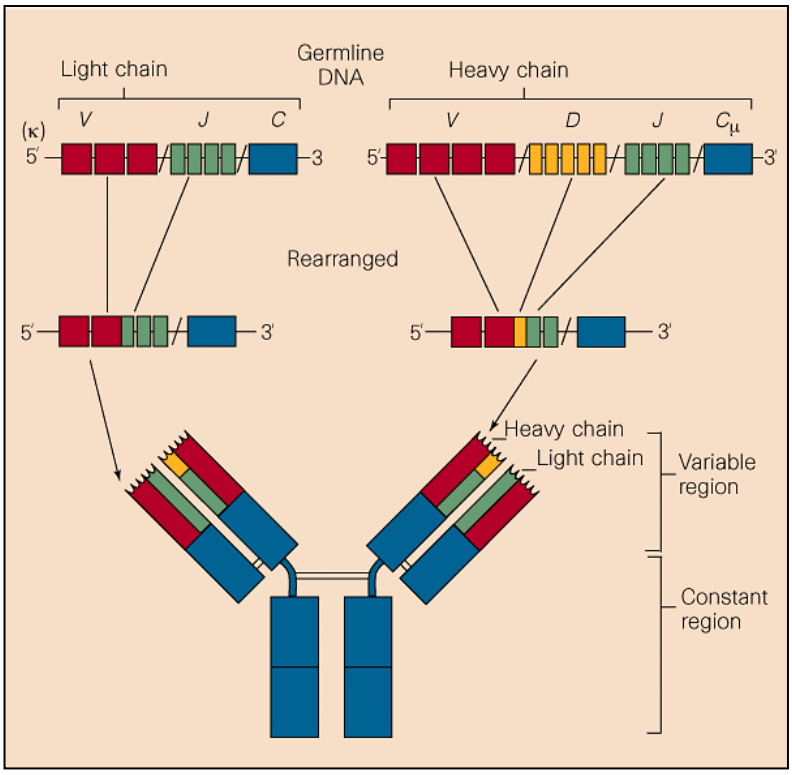


Figure 2. 1: Rearrangement of gene segments in the variable regions of an Ig gene.

In light chain, there are approximately 70 variable (V) segments and 4-5 joining (J) segments. In heavy chain, besides 40 V and 6 J segments, there are 23 diversity (D) segments. During the maturation of B cells, considering the number of

these different segments and their random recombination, there are 1.9 x 10^6 possible combinations. Besides segmental variability, "junctional diversity" which is a result of random addition and subtraction of nucleotides to the segment junctions during the recombination process adds 3 x 10^7 fold variability to the antibody repertoire. As a result of segmental and junctional diversity, mammalian B cells are capable of producing approximately $5x10^{13}$ different Igs. Considering nonproductive combinations, the variability at this level is approximately 10^{11}. B cells which successfully completed the recombination at bone marrow are immature B cells which have membrane bound IgMs, i.e. B cell receptors (BCR), on their surface. Immature B cells are exported from bone marrow to join blood circulation to become mature B cells in spleen [28,31,32]. After maturation in spleen, naive, mature B cells are ready for specific antigen encounter. The encounter may occur in spleen, circulation or tissues. Soluble antigens, as well as antigens presented by specialized antigen presenting cells (APC) may trigger activation of B cells.

The activation and antibody production process of naive B cells are explained by the clonal selection theory of Burnet [33]. According to this theory, naive B cells found in spleen or circulation dies after approximately 3 days unless they encounter their specific antigen. Naive B cells which encounter their respective antigens go through maturation and become antibody secreting plasma cells or memory B cells (Figure 2.2) [34].

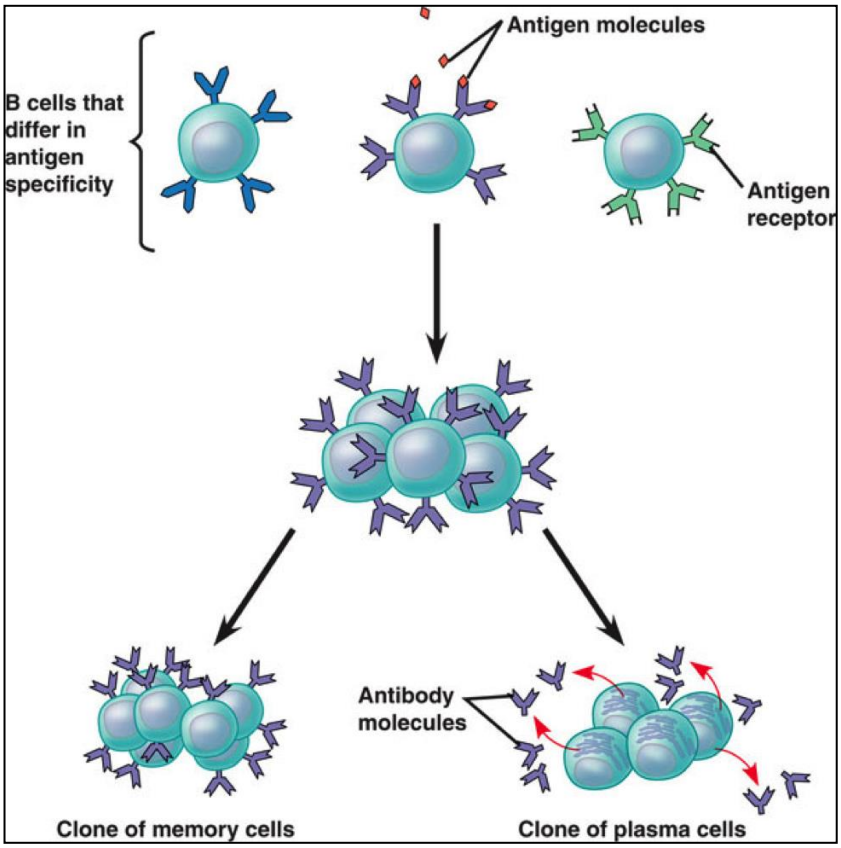


Figure 2. 2: Clonal selection of B cells.
Naive B cells which encounter with their specific antigens are activated and clonally expanded for the generation of plasma cells and memory B cells.

B cell activation process may occur with or without the involvement of $CD4^+$ helper T (TH) cells. Protein antigens usually result in T cell dependent activation and T cell independent activation is out of the scope of this thesis. Upon antigen encounter, BCR binds its specific antigen and the antigen is taken up with receptor mediated endocytosis. Endocytosed antigen is processed and presented to T_H cells via MHCII molecules (Figure 2.3) [35]. T_H cells which recognize the MHCII-peptide complex presented by B cells with T cell receptor (TCR) and express costimulatory factor CD40L and cytokines IL-4 and IL-21 as major factors that stimulate proliferation, immunoglobulin class switching and somatic hypermutation. Activated B cells initially produce short lived plasmablasts which produce IgM or IgD BCRs on their surface and secrete low affinity IgM antibodies for early response to the infection.

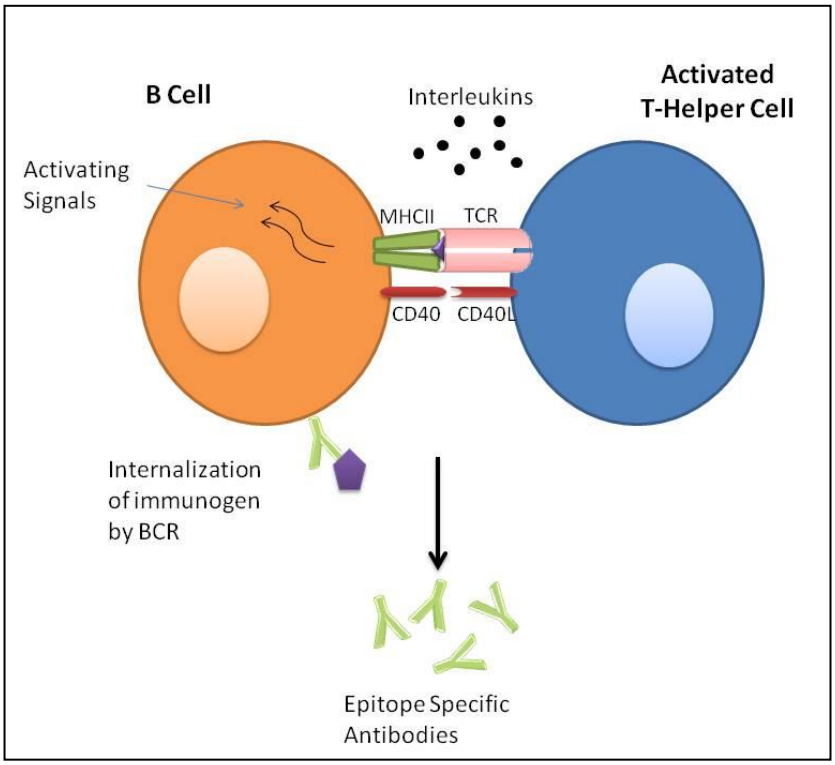


Figure 2. 3: Interaction between B cells and T_H cells for B cell activation in germinal center. Activating signals from T_H cells lead to B cell activation and antibody production.

The second step of differentiation takes place in a specialized microenvironment in a lymphoid follicle called germinal center. In germinal center, after the interaction of TH with B cell, activated B cells go through affinity maturation to the antigen directed by somatic hypermutation and class switching. Somatic hypermutation occurs in the previously combined V(D)J region DNA sequence. These mutations may lead to enhanced or diminished affinity to the respective antigen. Clones which have improved affinity are positively selected during this process.

Class switching is a recombination process taking place in the C region of the Ig gene, which results in generation antibodies with different effector functions. This is the primary immune response of the organism where initially low affinity IgM antibodies are produced by short-lived plasma cells and later, class switched, high affinity antibody producing, long-lived plasma cells and memory B cells are generated [28,36,37].

Upon subsequent antigen encounter, secondary immune response takes place where readily available long lived plasma cells and memory B cells are activated and

clonally expanded. This leads to faster response, high number of high affinity antibody producing cells and higher antibody titers in the organism [38]. Primary and secondary immune response is presented in Figure 2.4 [39].

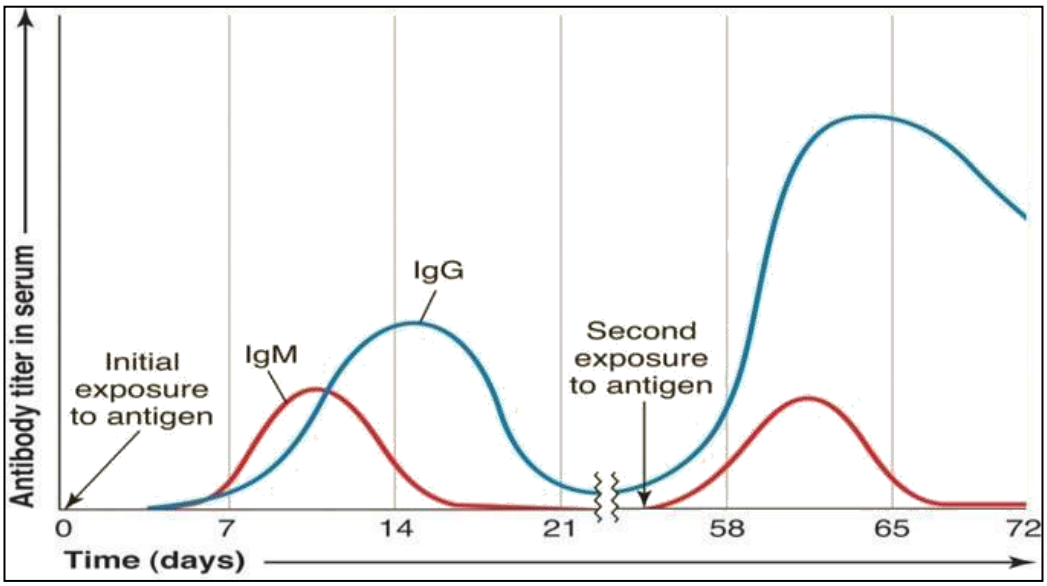


Figure 2. 4: Primary and secondary immune response.
Initial challenge with an antigen leads to high IgM antibody titers and takes about 10-14 days. Secondary exposure to the same antigen leads to high IgG titers in a shorter period.

2.2.2. Structure of Antibodies

The basic antibody structure is composed of two antigen binding fragments (Fab) which are unique for each specified antigen and a constant fragment (Fc) where different effector molecules of immune system bind. These fragments can be separated by enzymatic digestion with pepsin, yielding two interconnected Fab fragments (F(ab)2) and the Fc fragment; or papain, yielding two separate Fab fragments and an Fc fragment [40].

Class switching recombination yields antibodies of different structure and effector functions; IgM, IgG, IgA, IgD and IgE; depending on the structure of their C regions. These isotypes differ in the length of amino acid chain, associated carbohydrate moieties and disulfide bonds. Some of them can form multimeric complexes (Figure 2.5) [28,30].

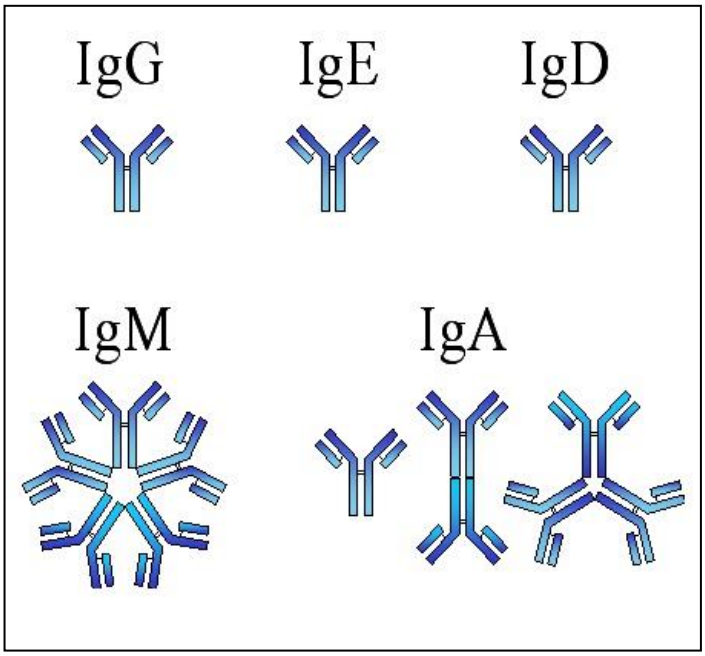


Figure 2. 5: Antibody isotypes.

IgM is the biggest immunoglobulin with approximately 960 kDa molecular weight. It is formed by disulfide linkage of 5 basic Ig molecules of two heavy and two light chains. Structure is stabilized by a small peptide called J chain. Pentameric IgM has a total of 10 antigen binding sites, which makes it a high avidity antibody. However, since IgMs are usually not affinity maturated, they are low affinity antibodies. IgM antibodies are players of early humoral immune response and due to their effectiveness in complement activation; they are important parts of defense against bacterial infection. Their high molecular weight prevents tissue penetration and they are mainly found in serum. Crystal structure of IgM molecule is presented in Figure 2.6 [41].

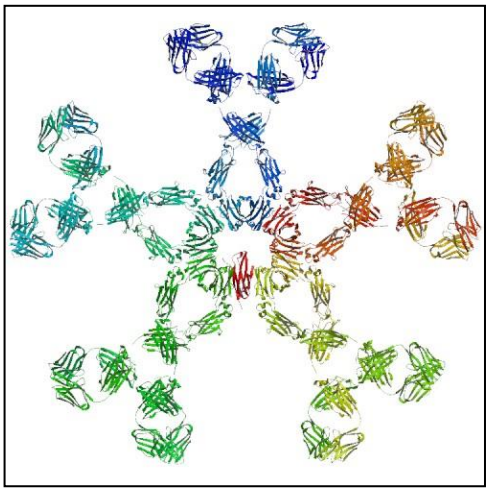


Figure 2. 6: Crystal structure of IgM molecule.

IgG is the primary antibody in the serum and comprises approximately 75% of the serum Igs. It consists of 2 heavy and 2 light chains and has two antigen binding domains. Different IgG sub-isotypes are found in different species where there are 4 in mouse; IgG1, IgG2a, IgG2b and IgG3. These antibodies are usually produced 10-14 days after initial antigen encounter. IgG also has strong complement activation efficiency and is an important part of defense against bacterial infection. IgG is the only Ig isotype that can penetrate through placenta. Crystal structure of IgG is presented in Figure 2.7 [42].

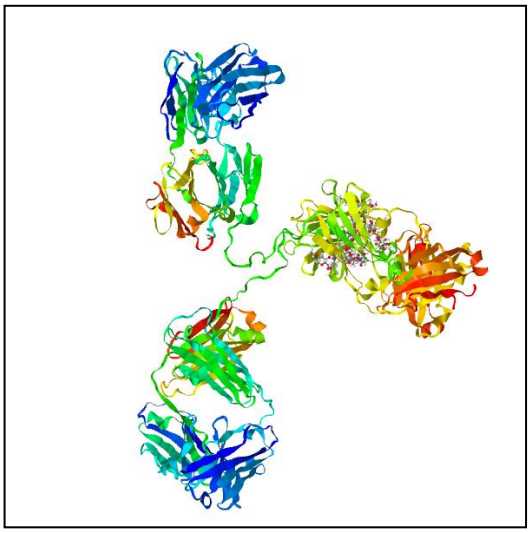


Figure 2. 7: Crystal structure of IgG molecule.

IgA is the primary antibody found in bodily secretions. It has a dimeric structure of two intact Ig molecules. Dimeric structure is stabilized with disulfide bonds and a short polypeptide chain called J chain. Additionally, there is a polypeptide chain called secretory piece in its structure, which facilitates its transport to mucosa through the inner epiderm. Dimeric IgA is found in secretions where complement system is inactive and there are no phagocytic cells. Its main function is neutralization of the antigens and cannot involve in complement activation efficiently. Its crystal structure is presented in Figure 2.8 [43].

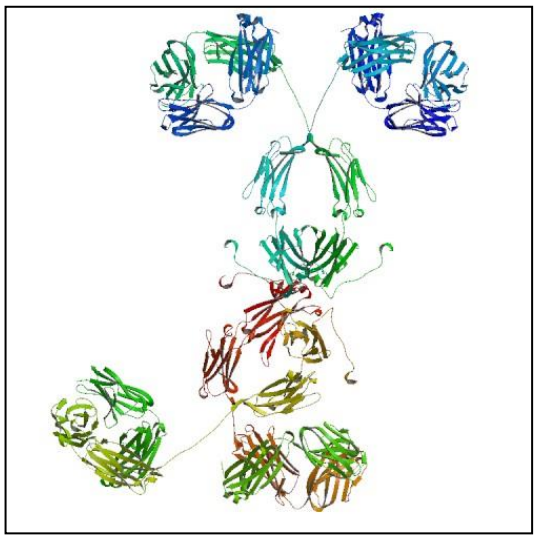


Figure 2. 8: Crystal structure of IgA molecule.

2.2.3. Antigenicity and Immunogenicity

Antigenicity is the ability of a molecule to combine specifically with the final products of immune response. Therefore, an antigen is any substance that has necessary chemical moieties to specifically bind to antibodies and/or cell surface receptors. On the other hand, immunogenicity is the ability to induce humoral and/or cell-mediated immune response. An immunogen should be able to trigger B cells to produce antibodies and/or can be presented to T cells via MHC molecules [44–46].

All immunogenic molecules are at the same time antigenic, i.e. can bind to antibodies/receptors, but the reverse is not true. There are some antigens that cannot induce humoral immune response in host organism. For instance, there are some bacterial toxins which developed certain systems in order to escape from the immune

response of the host such as superantigens or, certain groups of molecules which are too small to be immunogenic (haptens) such as mycotoxins [28].

Superantigens are protein antigens which cause non-specific activation of T-cell by binding MHC molecules. This activation results in non-specific proliferation of T cells and a massive cytokine release which in turn leads to shock and multiple organ failure [44,47]. This response also provides the pathogen to disrupt B cell activation and hence antibody production, which makes them non-immunogenic antigens.

Haptens are the low molecular weight molecules (<5000) which cannot raise an immune response in mammalian systems [48,49]. However, many environmental and food contaminants such as antibiotics, pesticides and mycotoxins are in hapten structure. In order to develop an antibody response towards these molecules, they should be chemically modified and conjugated to a bigger and immunogenic molecule such as proteins, polymers or some nanoparticles so that they will evoke an immune response [50,51]. A variety of conjugation methods specific for the hapten of interest may be chosen in order to confer the immunogenicity of these molecules (Figure 2.9) [26].

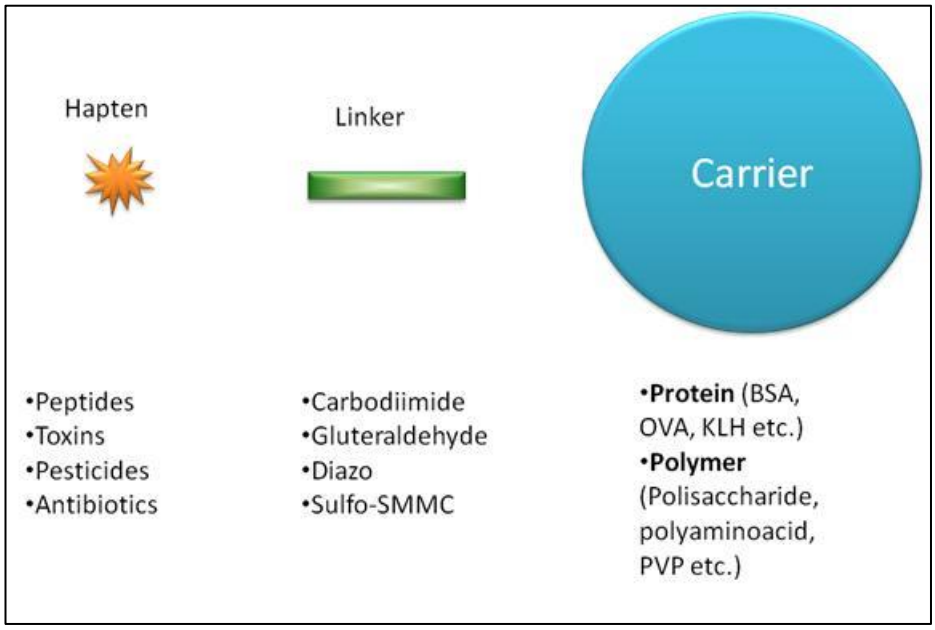


Figure 2. 9: Hapten-Carrier conjugation strategies.

When a hapten-carrier conjugate is used for immunization, antibodies against the hapten, carrier or the bond between hapten and carrier are produced; however, co-injection of the hapten and unconjugated carrier does not confer an immune response against the hapten (Table 2. 2).

Table 2. 2: Antibody response resulting from the immunization of hapten, carrier and hapten-carrier conjugate.

Injection	Antibody Response	Hapten Specific Antibody Response
Carrier	+	-
Hapten	-	-
Hapten + carrier	+	-
Hapten - Carrier conjugate	+	+

2.2.4. *in vitro* Antibody Development

Starting from the first discovery of antibodies by Emil von Behring as “serum therapy” to treat diphtheria and tetanus in 1890 [52], their importance have immediately been recognized by scientists and they have become one of the most prominent tools of biochemical applications. With their specificity and selectivity, antibodies are potent tools for selection, identification, purification of biological molecules and widely used as therapeutics. Targeted applications of antibodies require in vitro production of antibodies raised against specific antigens.

The main source of antibodies is mammalian organisms. The earliest studies directly used the antibodies present in the immunized animal sera. These antibodies are polyclonal antibodies which are produced by different sets of B cells of the animal immune system and consist of target antigen specific antibodies as well as other antibodies in the animal. Antigen specific antibodies in the polyclonal antibody pool recognize different epitopes of the same antigen. The variability of the antibodies in polyclonal sera results in low specificity and low batch to batch consistency. In 1975, Köhler and Millstein developed hybridoma technology to develop Monoclonal Antibodies (MAbs) which recognize a single and specific epitope of the antigen [53]. In this technology, B cells of the immunized animal are

fused with myeloma cells of the same species in order to immortalize B cells, which otherwise live up to 1 week under *in vitro* conditions. The immortalized B cells are hybridoma cells capable of antibody production that can be used as constant source of MAbs. Thus, MAbs overcame the challenges of polyclonal antibodies and became the initial choices for immunoassay development with their predetermined specificity and affinity as well as robust production in specialized cell lines. The ultimate success of hybridoma technology and MAbs led to new MAb and antibody fragment development strategies based on recombinant DNA techniques [54–56] or novel cell immortalization methods such as immortalization with eppstein barr virus [57] of electrofusion methods [58] . These alternative technologies obviously constitute an important part of antibody development especially for therapeutic purposes; however, they are beyond the scope of this thesis.

As there are numerous techniques for antibody production, immunization and antibody repertoire of mammals remained the primary source of MAb production. Therefore, the initial requirement for MAb production is a good, target specific immune response from the experimental animal.

2.2.4.1. Anti-Aflatoxin Antibody Production and Characterization

AFs are haptens which are not recognized by mammalian immune system. Immune response against these hapten structures can only be raised by conjugating them to bigger and immunogenic molecules such as proteins [59–61]. Reactive groups such as carboxyl, amine or thiol on haptens are commonly used for conjugation reaction. However, AFB1 does not bear such highly reactive groups (Table 2. 1). Most methods initially introduce a carboxyl group on AFB1 (Figure 2.10) [62] and use this group for conjugation to the amine groups of proteins forming a stable amide linkage [59,63–66]. Mannich type reaction can be used without prior AFB1 derivation using the α-hydrogen adjacent carbonyl in the presence of formaldehyde [44].

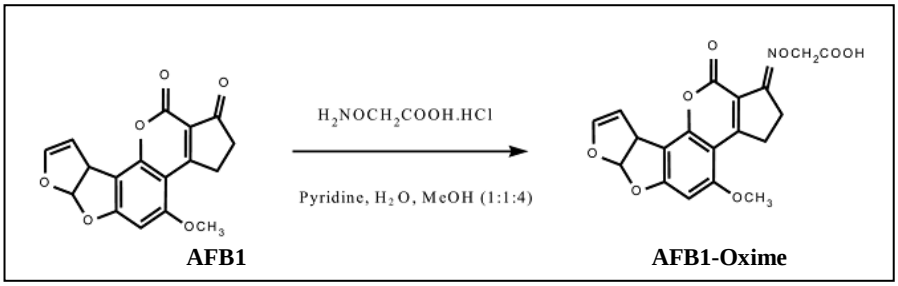
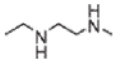
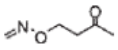
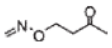
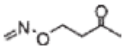


Figure 2. 10: Synthesis of AFB1-O-carboxymethyl oxime.

Several methods previously used for AFB1-protein conjugation is summarized in Table 2. 3 [60].

Table 2. 3: Comparison of the methods of preparing AFB1–carrier protein conjugate.

	Mannich-type method [a]	MA method [b]	AE method [b]	AE method [c]	WSC method [d]
AFB1 derivation	No derivation	Yes [e]	Yes [e]	Yes	Yes [e]
Derivation time		24 h	24 h	20 min	24 h
Purification		Yes	Yes	Yes	Yes
Conjugating time	24 h	10 h	2h	22h	48h
By-products	Little	Medium	Little	-	Little
Spacer arms					
Antisera titer	High	Low	Medium	-	Medium
Antisera specificity	Good	Poor	Good	-	Good
AE, activated ester; MA, mixed anhydride; WSC, water soluble carbodiimide					
a Mannich type method [60], b MA and AE methods [63], c The method induced glycolic acid to AFB1 [64], d WSC method [59], e AFB1-oxime method [67]					

Once AF is successfully conjugated to a suitable carrier, the conjugate can be used for immunizations to develop AF specific antibody response in the test animals which will subsequently be used for development of MAbs using hybridoma technology.

After the establishment of the stable hybridoma cell line producing AF specific MAbs, the developed MAbs should be thoroughly characterized for their efficient

utilization in test systems [68]. Specificity and affinity of the antibodies are common factors affecting the test performance [68–70]. However, a additional selection criteria should be employed for the characterization of AF specific MAbs. MAbs against AFs are conventionally required to measure 4 naturally occurring AF derivatives and one water soluble metabolite in food and feedstuff [10]. So, the specificity of the antibody should be assessed such that, it has affinity towards AF derivatives B1, B2, G1, G2 and M1, yet does not display cross reactivity to other mycotoxins. Furthermore, in case of AF specific antibodies, solvent tolerance and minimal matrix interference with food extracts are additional factors to be considered. AF analysis is conducted with a liquid extract of the sample to be analyzed. Preparation of the liquid extracts of solid samples is achieved by the use of organic solvents. During the extraction process, several metabolites of the sample are co-extracted with the AF. For an antibody to be effectively utilized in aflatoxin analysis it needs to be tolerant to solvents used in extraction process and should not cross-react with the sample derived metabolites present in the extract.

2.3. Antibody Based Analytical Systems

Immunoanalytical methods are antibody based methods allowing the specific detection, quantification and characterization of antigens by means of antibody binding. Specificity and selectivity of antibodies are widely utilized for the diagnosis of human, plant and animal pathogens as well as the detection of contaminants in food, feed, water, soil and air. There are a variety of immunoanalytical platforms where quantization (ELISA, RIA, biosensors etc.), characterization (western blotting, cellular localization etc.) or separation (IAC, co-immunoprecipitation etc.) of antigens can be achieved using antibodies raised against a specific antigen.

This thesis covers antibody development and selection work related with both the basic requirement of the conventional AF quantification systems, IAC, and the advancing AF analysis method, biosensors. Hence, these two methods will be discussed in detail.

2.3.1. Immunoaffinity Columns

Immunoaffinity chromatography is a liquid chromatographic method for specific separation of biological molecules by the aid of antibodies. This method utilizes the specificity and reversibility of antibody-antigen interactions. In IAC preparation, antibodies are immobilized to a solid support in order to create a stationary phase for chromatographic separation and the prepared resin is filled to appropriate columns. Antigens are specifically adsorbed to the antibody bound stationary phase in conditions that favor antigen-antibody binding. Bound antigens are eluted from the resin by changing the conditions where desorption of ligand is favorable [71,72].

There are a variety of different solid supports for IAC development such as carbohydrate based media (agarose, cellulose) and synthetic organic molecules (polymers such as polyethersulfone). Low cost and availability of carbohydrate based media made them popular choices for IAC development. For special applications specialized supports are available such as magnetic beads for magnetic separation of cells and some molecules, or pressure resistant supports such as silica, glass or azalactone beads for high pressure IAC (HPIAC) [72].

Sepharose is a cross-linked form of agarose widely used for IAC development. For effective immobilization of the antibodies, sepharose is activated by different methods such as cyanogen bromide (CNBr) or epoxy activation to generate reactive moieties for covalent attachment. When sepharose is activated with CNBr, the OH groups on the sepharose are converted to the cyanide groups, which readily react with amine groups at high pH to form isourea linkage (Figure 2.11).

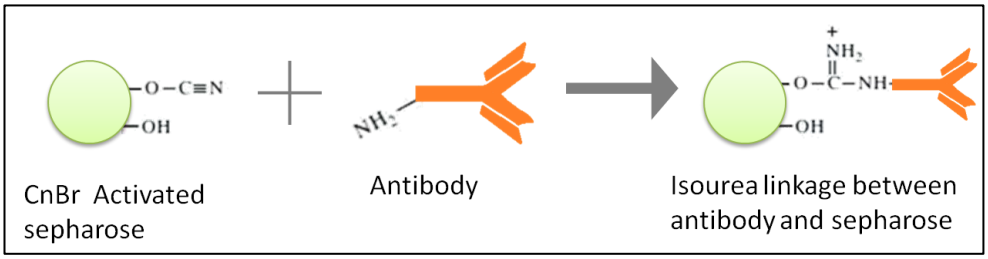


Figure 2. 11: Antibody immobilization to CNBr activated sepharose.

Antibodies should be immobilized to this support material irreversibly, so that they will not be separated from the resin during the elution step. Random chemical attachment and oriented immobilization of antibodies are the two considerable choices for immobilization. For random chemical attachment, covalent binding through the reactive moieties of the antibodies such as amine groups is widely preferred. However, antibody orientation is critical while immobilizing divalent antibodies to a support since random immobilization causes activity loss due to hindrance of variable regions which should be exposed in order to be functional [73]. When IgG antibodies are randomly immobilized to a solid support, about 50% of the antibody activity is impaired [6]. This results in the need for purified antibody solutions for concentrated binding to the immunosorbent surface, a higher amount of the immunosorbent material and/or a means of orienting the antibody, so that the antigen binding sites are free. The use of multivalent antibodies may be a solution to this problem as there will be free antigen binding sites even when some are blocked during immobilization.

IACs are routinely used in standard AF analysis methods for sample cleanup. The sample cleanup step comprises affinity based purification techniques, particularly IACs in order to concentrate AFs and purify them from complex extract matrix (Figure 2.12) [10].

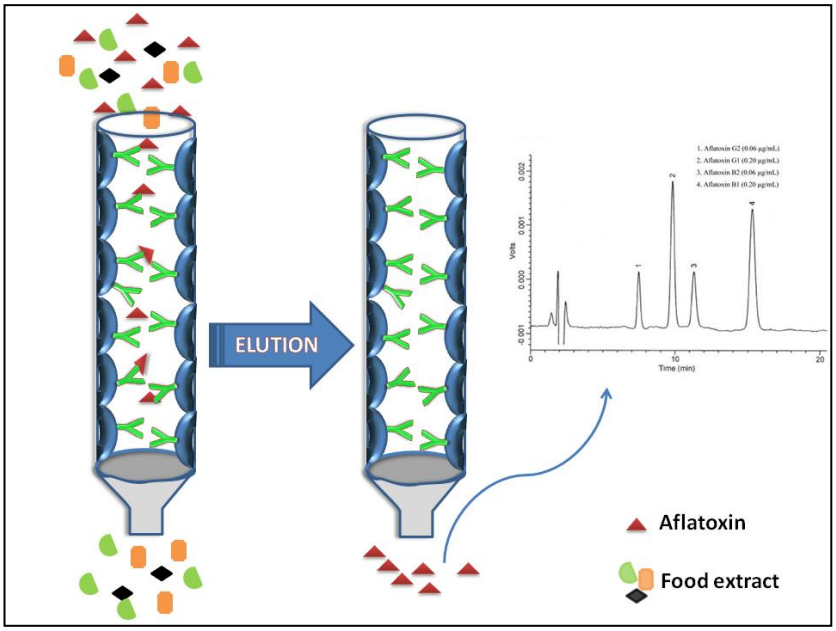


Figure 2. 12: Principle of aflatoxin immunoaffinity columns.

The quality standards for AF IACs are set by the AOAC guidelines. According to the guidelines, an acceptable AF IAC should retain at least 100 ng AFB1, and should give recovery of at least 80% of AF B1, B2 and G1 and 60% of AFG2 when aqueous solution of 10% methanol containing 5 ng of each toxin is applied to the columns [15].

2.3.2. Immunosensors

A biosensor is defined as a bioanalytical device incorporating a molecular recognition element associated or integrated with a physicochemical transducer [74]. A biosensor has two critical components: a receptor which is the biological recognition element that provides specificity and selectivity and a detector which is the transducer that translates the physical or chemical change resulting from analyte-receptor interaction to electrical signal (Figure 2.13). Several studies have been conducted in order to develop AF biosensors [71–75]. Sensors showing competitive performance with conventional analytical systems are emerging [68].

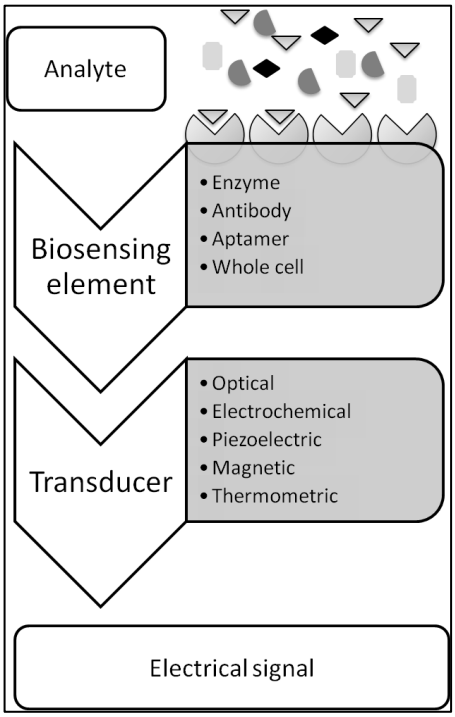


Figure 2. 13: Schematic representation of a biosensor.

Enzymes, antibodies, nucleic acids or whole cells may be used as receptors in biosensing. The receptor should be selective and exert a specific, measurable change upon interaction with the target analyte. Among these receptors, antibodies are widely preferred due to their specificity and selectivity. Biosensors that utilize antibodies as sensing element are referred to as *immunosensors*.

As a recognition element, the antibody is in close contact with a transducing element that converts the antigen–antibody binding into quantitative electrical or optical signal. The transducer is not selective and its function is to convert the result of biological recognition into a quantifiable signal. Common transducer systems employ piezoelectric, optical, electrochemical, magnetic or thermometric principles for detection [75].

In this thesis, QCM will be used as transducer. QCM is a commonly used piezoelectric sensor system for immunosensing. It is an extremely sensitive weighing device which is based on measurement of the change in mechanic resonance of the quartz crystal with changing mass. Quartz crystal has piezoelectric properties that which under mechanical stress, it produces electrical voltage. And vice versa; when an electric voltage is applied to the crystal, it produces a resonance at a certain frequency which is affected by the mass on the crystal. According to Sauerbrey's equation [76] ($\Delta F = -2F02\Delta m/A(pq\mu q)^{1/2}$ where ΔF is the counted frequency change (Hz); F0 is the fundamental resonance frequency of the quartz oscillator; Δm is the mass change; A is the area of the electrode; ρq is quartz density; and μq is the shear stress of quartz), the change in resonant frequency of a QCM is principally based on the mass of adsorbed material on the QCM surface. The shift in resonance frequency (ΔF) is proportional to the surface mass (m) of the deposit [77,78]. This mechanism allows label free detection of analytes.

After the choice of the receptor and transducer, an efficient biosensor requires a carefully designed assay format which will clearly reflect the amount of the analyte and a compatible sensor surface preparation strategy. In the next two sections, different immunoassay formats and surface preparation strategies will be discussed.

2.3.2.1. Immunoassay Development

Immunoassays use the principle of detecting the interaction of antibodies with their corresponding antigen [79]. In most immunoassays, the antigen-antibody interaction is detected using a reporter such as enzymes or fluorescent tags. However, QCM transducer systems do not require a label for the detection of antibody-antigen interaction and can directly report the signal for antigen-antibody binding as frequency change [80]. Two major immunoassay development strategies can be used for QCM immunosensor development; direct recognition and competitive assay format. In direct recognition, sample is delivered to the antibody immobilized sensor surface, and the change in mass upon antigen binding is measured (Figure 2.14). However, small molecular weight analytes such as AF are not suitable for this kind of assay format with QCM transducers since they cannot exert a high mass change upon binding.

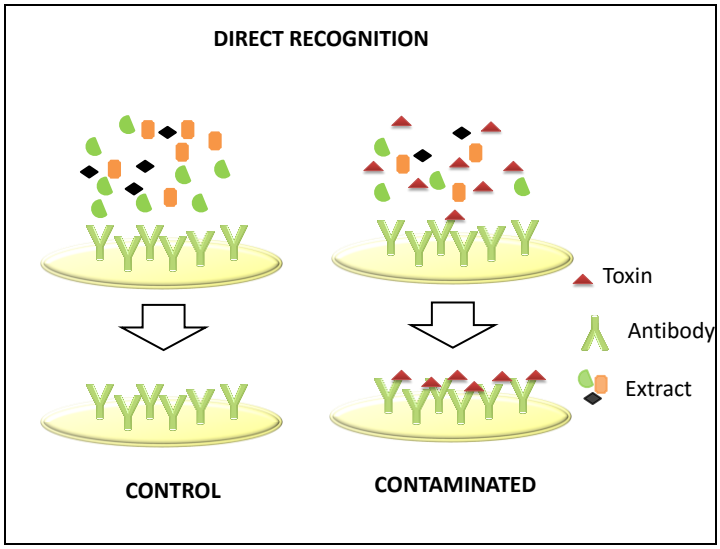


Figure 2. 14: Biosensor based on direct recognition of analytes.

Competitive immunosensors employing inhibition assay are commonly used for the analysis of low molecular weight molecules such as AF. There are two forms of inhibition assay; analyte immobilized or antibody immobilized.

In antibody immobilized system, low MW toxin in the sample is delivered to the surface together with a toxin-high MW compound conjugate. The conjugated form of the toxin will provide a measurable signal. The test interprets the

competition of the toxin and its high MW, conjugated form. As the concentration of toxin in the sample increases, less conjugate will bind to the sensor surface, which will lead to a decrease in the signal (Figure 2.15).

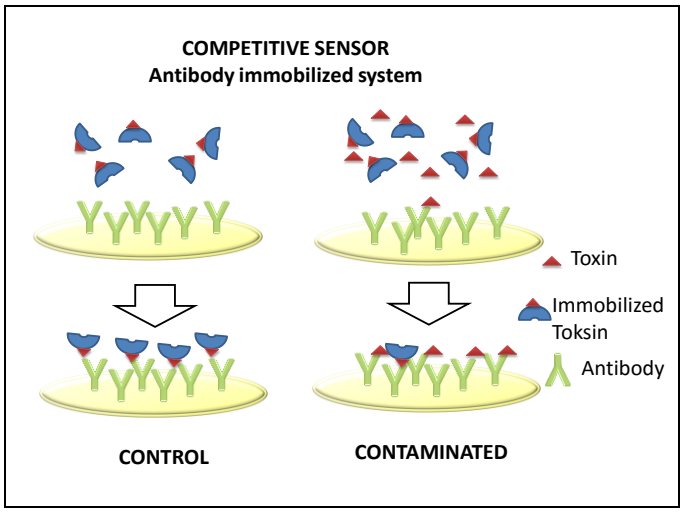


Figure 2. 15: Biosensor based on competitive assay - antibody immobilized system.

When the analyte, i.e. toxin, is immobilized on the sensor surface, the test sample is mixed with the antibody solution and delivered to the sensor surface. The test interprets the competition between the immobilized toxin on the surface and free toxin in the sample for binding to the toxin specific antibody. The more toxins in the solution, the fewer antibodies will bind to the surface. Quantification is achieved by evaluating the interaction of a fixed amount of antibody with changing analyte concentrations (Figure 2.16).

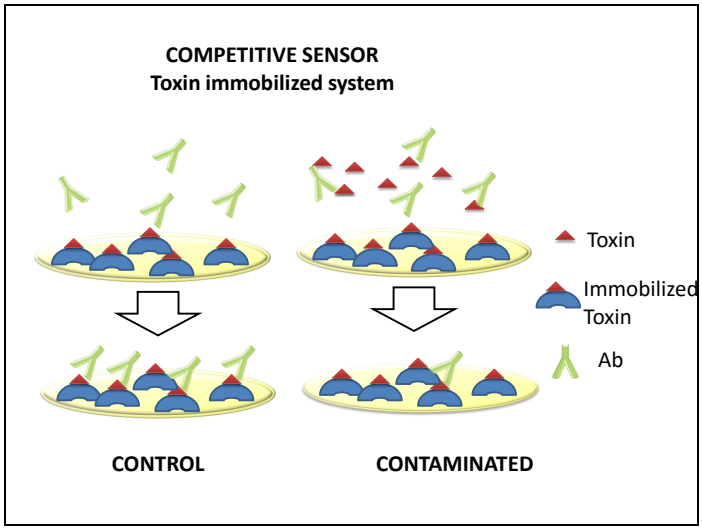


Figure 2. 16: Biosensor based on competitive assay - analyte immobilized system.

The assay methodology should be carefully planned so that a detectable signal will be achieved with changing analyte concentrations. In this thesis, a competitive immunoassay will be employed for AF quantification for the development of immunosensor.

2.3.2.2. Surface Preparation for Biosensing

An efficient immunosensor can only be developed using a stable and reproducible surface and a high quality antibody with high specificity and affinity for the analyte. Although high affinity antibodies have a major role to improve the limit of detection, they require harsh regeneration conditions; such as pH extremes, application of detergents or both. Therefore, a sensor surface should preferentially be tolerant to different regeneration conditions.

Surface development for competitive small molecule immunosensors is challenging. Direct adsorption of the analyte may be impossible if the target molecule does not bear the essential reactive groups. Even when adsorption is possible, it is very likely that binding of the molecule to the surface masks the moiety interacting with the antibody [81]. Therefore, a properly functionalized surface allowing proper antibody-antigen interaction is crucial.

Previously developed AF biosensors generally immobilized AFs to the surface through its protein conjugates, either by direct adsorption [15,82,83] or using self-

assembled monolayers (SAM) [84,85]. However, the utilization of proteins for sensor surface functionalization leads to low batch-to-batch reproducibility due to protein instability and/or aggregation. Additionally, the chips require special storage conditions in order to increase the shelf life such as cold storage and the use of preservatives in order to prevent protein degradation and contamination [86]. Furthermore, protein bearing surfaces are labile to harsh regeneration conditions which may lead to protein denaturation or delocalization preventing successive regeneration cycles.

Chemical immobilization of AFB1 to the sensor surface is a means of overcoming challenges related with reproducibility, stability and regeneration. Direct immobilization of AFB1 to Biacore CM5 sensor chip was previously described where carboxylated AFB1 derivative AFB1 - o carboxymethyloxime (AFB1-oxime) was coupled to amine groups generated on the CM5 chip [87,88]. Nevertheless, studied Surface Plasmon Resonance (SPR) systems depend on prefabricated sensor chips and do not cover AF immobilization to unmodified, gold coated surfaces which are generally the only available surfaces in other transducers such as piezoelectric transducers which are frequently used for this kind of applications.

Surface preparation not only involves antigen immobilization, but also proper blocking of the surface in order to prevent non-specific adsorption of molecules. Sensor surfaces prepared by the immobilization of analytes via SAM are usually blocked with a nonspecific protein such as Bovine Serum Albumin (BSA) in order to minimize off-target readings [85,89,90]. However, protein blocking is sensitive to harsh regeneration conditions and chemical blocking is more stable. In the mentioned SPR sensors, blocking of reactive carboxyl groups was achieved by ethanolamine [88] but blocking of unbound reactive amine groups was not evaluated. QCM is a label-free sensor system which detects not only specific binding, but also nonspecific binding. Therefore, selective binding and efficient blocking of the prepared sensor surface are crucial to avoid interferences with the original signal. This factor is particularly important for analytes with ng/g level maximum allowable limits, such as AF. Optimization of the coupling reaction, as well as proper blocking of the sensor surface to prevent nonspecific protein binding is also essential for a good performance.

3. MATERIALS AND METHODS

3.1. Equipment and Chemicals

We used most of the chemical reagents such as buffers and salts from Sigma Aldrich, Germany. Other reagents were as follow; 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Thermo Scientific, USA), AFs B1, B2, G1, G2, M1, ochratoxin A (OTA), zearelanone (ZEA) and Fumonisin B1 (FB1) (Fermentek, Israel), SDS PAGE Laemmli Sample buffer (Bio-Rad, USA), Mouse MAb isotyping kit (BD, USA), chromatography resins (GE, USA). 5 MHz AT cut quartz crystals with gold-plated electrodes on both sides were purchased from KVG Quartz Crystal Technology GmbH, Germany. Agilent Bio IEX SCX NP5 column was used for conjugate characterization and Nucleosil 100-5 C18 column (Macherey-Nagel 720014.46) column was used for AF analysis by HPLC.

Absorbance of ELISA plate wells were measured with Biotek Synergy HT microtiter plate reader, which was controlled by a personal computer containing the GEN5 standard software package from BioTek Instruments, (Vermont, USA). Biochrom Libra S2 spectrophotometer was used in spectrophotometric measurements. Agilent 1260 Infinity HPLC was used for HPLC analysis. QCM200 Quartz Crystal Microbalance of Stanford Research Systems, USA was used for QCM measurements.

3.2. Preparation of AF-Protein Conjugates

Three different protein conjugates of AFB1 were prepared. AFB1 was conjugated to Ovalbumin (OVA) and Apo-Human Serum Transferrin (TF) for use in immunizations and BSA conjugate was used in ELISA tests. Conjugation of AFB1 to proteins was achieved in two steps. In the first step, amine groups of the proteins were enriched by converting carboxyl groups to amine groups using ethylene diamine (EDA) with an EDC linker with the method described by Domen, 1992 [91] and cationized forms of the proteins were obtained. In the second step, AFB1 was conjugated to the proteins by Mannich type reaction in the presence of formaldehyde

with the modification of the method of Zhou et al [60]. The amine groups of cationized proteins were condensed with formaldehyde and the α-hydrogen adjacent carbonyl in AFB1 (Figure 3.1).

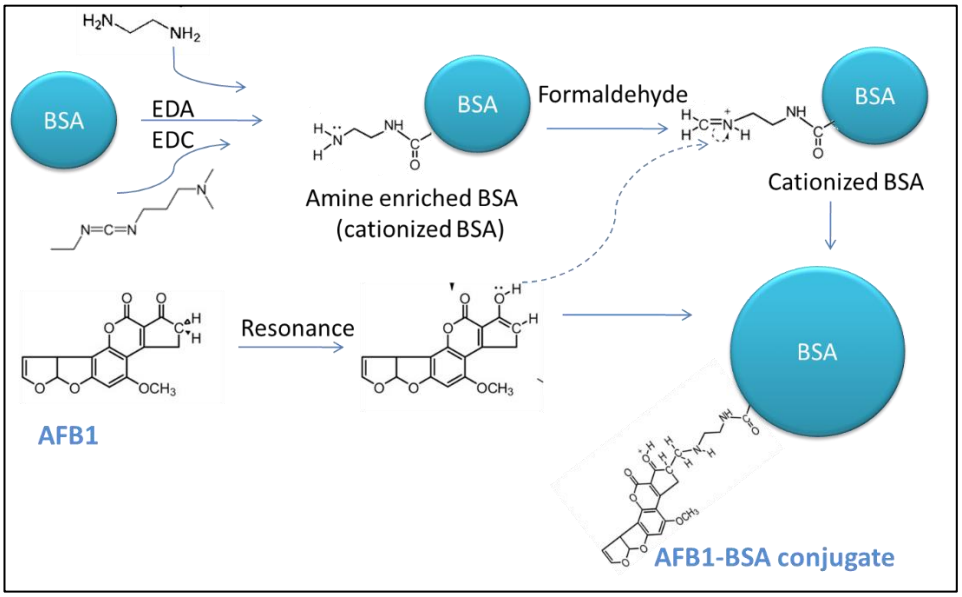


Figure 3. 1: AFB1-BSA conjugation using Mannich type method.

Cationized BSA, OVA or TF was conjugated to 240-fold molar excess of AFB1 dissolved in DMF at 2 mg/mL concentration. The reaction took place in 0.1 M MES, pH: 4.8 in the presence of formaldehyde. Conjugate proteins were exhaustively dialyzed at against 0,1 M MES, pH: 4.8 in order to remove unbound AF and chemicals.

Characterization of cationized proteins was done with Diethylaminoethyl Cellulose (DEAE-C) ion exchange chromatography and HPLC analysis with strong cation exchange column. Conjugate characterization and calculation of conjugation ratios were done with spectrophotometry using Beer-Lambert law with the following formula (Formula 2.1).

$$C(AF)/C(Protein) = \frac{A360 \text{ x } \varepsilon \text{ Protein } 280}{(A280 \text{ x } \varepsilon \text{ AF360} - \varepsilon \text{ AF280 x } A360)} \quad (3.1)$$

Where, A: absorbance, ε: molar extinction coefficient, C: concentration.

This formula was derived from A = ε x C x L (L: pathlength (1cm)) with the assumption that the absorbance characteristics of proteins and AF do not change upon conjugation. Molar extinction coefficients of cationized proteins and AFs were calculated by measuring absorbance of solutions with different concentrations at 360 nm and 280 nm. The slope of the linear graph obtained from concentration vs. absorbance plot was used as molar extinction coefficients.

3.3. Indirect ELISA

Indirect ELISA [92,93] was used for monitoring mice immune response, screening of hybridoma supernatants, antibody purification studies and antibody characterization studies including determination of solvent tolerance of the antibodies. 96 well polystyrene plates were coated with 500 ng AFB1-protein conjugate (AFB1-BSA, AFB1-TF or AFB1-OVA) in 100µl phosphate buffered saline (PBS :10 mM K_2HPO_4, 10mM KH_2PO_4, 0.15 M / L NaCl, pH 7.2), overnight at 4°C. The plates were washed three times with washing buffer (0.005 % tween-20 in PBS). Then, 0.1 % skimmed milk solution in 200µl PBS was added to the wells and the plates were incubated for 1 h at 37 °C. After washing, the culture supernatants of hybridoma, purified MAb or 2000 fold PBS diluted mice sera were added and incubated for 1 h at 37° C. Anti-AF antibody binding reaction was detected by using an alkaline phosphatase (AP) conjugated goat anti-mouse polyvalent (IgA, IgM, IgG) antibodies as secondary antibody. Primary-secondary antibody binding reaction was visualized by p-nitrophenyl phosphate (PNPP) hydrolysis reaction. Optical density at 405nm was recorded using a microplate reader.

3.4. Indirect Competitive ELISA

Interaction of antibodies with soluble mycotoxins was tested with Indirect Competitive ELISA (IC-ELISA) [94]. IC-ELISA was used to test AF specific response in mice sera, antibody specificity tests, matrix interference effect assessment and affinity determination. Mice sera were additionally tested for

interaction with the proteins used in immunization. In this assay, 500 ng AF-protein conjugate coated (AFB1-BSA, AFB1-TF or AFB1-OVA) plates were blocked with 1% skimmed milk solution in PBS. Unconjugated mycotoxins or proteins were incubated on ELISA plates together with mice sera or hybridoma culture supernatants or partially or fully purified antibodies with proper dilution. The competition reaction was allowed to proceed for 30 min. at 37°C. Visualization of the plates was achieved similarly with indirect ELISA.

3.5. SDS-PAGE and Western Blotting

Conjugate characterization and antibody purity assessment were evaluated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting [95,96]. For SDS-PAGE analysis, protein samples were mixed with 4x Laemmli Sample Buffer. DTT was included in the sample buffer for reducing gels, omitted in non-reducing analysis. Thermo pre-stained broad range protein MW marker was used in reducing gels, Sigma Aldrich Kit for MW 14.000 – 500.000 (SIGMA MWND500) was used as molecular weight marker in non-reducing gels. Proteins were loaded on discontinuous gel system with 5% (w/v) polyacrylamide loading gel and 12 % (w/v) separating gel. Electrophoresis was conducted with Bio-Rad mini protean tetra cell gel apparatus under 120 V continuous voltage. Gels were stained with silver staining [97]. For Western blot analysis, the proteins on the gels were transferred on a PVDF membrane in methanol containing transfer buffer (0.02 M Trizma base, 0.15 M Glycin, 0,0003 % SDS and 20% Methanol) using a semi-dry Western blot apparatus (Bio-Rad, Trans-Blot Turbo Transfer System) for 30 min. at 200 mA. Membrane was then blocked with 1 % skim milk powder. 1:5000 diluted AP labeled Rabbit anti-mouse polyvalent antibody was incubated with the membrane for 1 h. The blots were visualized on the membrane with AP substrate (Sigma B5655).

3.6. Antibody Development

3.6.1. Immunization

All animal experiments were approved by the ethical committee of Turkish Scientific and Research Council (TÜBİTAK), Marmara Research Center (MAM), Genetic Engineering and Biotechnology Institute. 6-8 weeks old, female BALB/C mice were intraperitoneally immunized with AFB1-TF or AFB1-OVA conjugate. Antigens were prepared in PBS and mixed with equal volumes of adjuvant. Initial immunization was done using complete Freund's adjuvant. Remaining immunizations were done with incomplete Freund's adjuvant. 20 µg of conjugate per mouse was injected in the first immunization. The second immunization was done with 50 µg of the antigens one week after the initial immunization. Three subsequent injections with 50 µg of the conjugates were given at 2-week intervals. A total of 5 immunizations were done until desired immune response was achieved. 5 mice per immunogen were used. Mice were bled to track the antibody response 10 days after each immunization. Antibody response was monitored with direct and indirect ELISA.

Mice with high antibody titers were selected for fusion and received an intravenous booster immunization with an alternate conjugate sans adjuvant, four days before fusion. AFB1-TF immunized mice received 50 µg AFB1-OVA booster and vice versa in order to enrich only AF specific B cells.

3.6.2. Development of Monoclonal Antibodies

MAbs were produced by modification of the method of Kohler and Milstein [53,98–100]. The spleen and lymph nodes of selected mice were used as lymphocyte source in the fusion studies. The lymphocytes of the immunized BALB/c mouse and mouse myeloma cells (F0 ; ATTC CRL 1646) were fused in the presence of 50% polyethylene glycol (PEG) 4000 [100–102]. Fusion product, resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal calf serum and antibiotic were distributed into the 96 wells culture plates and incubated overnight at 37°C, 5% CO_2, 95% humidity. 10-15 days after fusion, supernatants from hybridoma

colonies were screened using indirect ELISA with AFB1-BSA coated plates. Positive clones were selected for binding to free AFs with IC-ELISA with 1 μg AFB1. Selected hybridomas were subjected to three rounds of cloning using limited dilution method [103]. At each stage of growth, aliquots of hybrid cultures (3–5.10^6 cells) were frozen in liquid nitrogen in 80% DMEM, 20% Fetal Calf Serum (FCS) and 10% dimethylsulphoxide (DMSO).

3.6.3. Antibody Purification

MAbs were partially purified and concentrated from the hybridoma supernatant by ammonium sulfate (AS, $(NH_4)_2SO_4$) precipitation at 40% saturation with either crystalline AS (341 g/L) or saturated AS solution by equally mixing saturated AS solution with cell culture supernatants (1:1, v:v). Precipitated proteins were dissolved so that cell culture supernatants were 25 fold concentrated. Dissolved proteins were exhaustively dialyzed in PBS. AS precipitates of antibodies were used for further purification studies using solid phase bound protein A [104], size exclusion or ion exchange chromatography.

3.6.3.1. Purification of IgG Antibodies

IgG isotype antibodies were purified using protein A affinity chromatography. AS precipitates of the antibodies were ran through the columns in indicated loading buffers at 1 mL/min flow rate until all unbound proteins were discarded in flow through. Bound antibodies were collected with indicated elution buffers at the same flow rate. IgG1 isotype antibodies was purified, using loading buffer 1 (1 M Tris, pH: 9, 3 M NaCl) and eluted with 0.1 M glycine pH: 2.7. Other IgG sub-isotypes were purified using loading buffer 2 (20 mM K_2HPO_4, pH: 7) and eluted with 0.1 M glycine pH: 2.7. 1 mL fractions were collected and absorbance at 280 nm was recorded. Fractions were tested for antibody activity with indirect ELISA. Antibody containing fractions were pooled and exhaustively dialyzed against dH_2O and freeze dried.

3.6.3.2. Purification of IgA Antibodies

IgA isotype antibody was purified with anion exchange chromatography with DEAE-C column by modified standard protocols [105]. AS precipitated antibody was loaded to 20 mL DEAE-C column in 10 mM K_2HPO_4, pH: 7, 150 mM NaCl and column was washed at 1 mL/min flow rate until all unbound proteins were discarded in flow through. The bound antibodies were eluted with 10 mM K_2HPO_4, pH: 7, 350 mM NaCl at 1 mL/min flow rate. Column was regenerated using 10 mM K_2HPO_4, pH: 7, 500 mM NaCl. 1 mL fractions were collected and absorbance at 280 nm were recorded. Fractions were tested for antibody activity with indirect ELISA. Antibody containing fractions were pooled and exhaustively dialyzed against dH_2O and freeze dried.

3.6.3.3. Purification of IgM Antibodies

IgM isotype antibodies were purified using a two-step chromatography protocol. In the first step, antibodies were separated using size exclusion chromatography with S300 resin (GE, USA). 50 cm column with 1 cm diameter was packed with 25 mL S300 resin. 1 mL AS precipitated cell culture supernatant was loaded to the column and separated with 0.5 mL/min flow rate in PBS. 1 mL fractions were collected and absorbance at 280 nm was recorded. Fractions were tested for antibody activity with indirect ELISA. Fractions with antibody activity were collected and used in the second step of the purification with anion exchange chromatography using 20 mL DEAE-C column. 40 mg of protein in 15 mL pool from antibody containing fractions of S300 column was loaded to the DEAE-C column at 1 mL/min flow rate in 10 mM K_2HPO_4, pH: 7, 150 mM NaCl and column was washed until all unbound proteins are discarded in flow through. Bound antibodies were eluted with 10 mM K_2HPO_4, pH: 7, 250 mM NaCl at the same flow rate. Column was regenerated with sequential application of at least 5 column volume 10 mM K_2HPO_4, pH: 7, 500 mM NaCl and 10 mM K_2HPO_4, pH: 7. 1 mL fractions were collected and absorbance at 280 nm were recorded. Selected fractions were tested for antibody activity with indirect ELISA. Purified antibody containing fractions were exhaustively dialyzed against dH_2O and freeze dried.

3.6.3.4. Antibody Purity Assessment

Purity of antibodies in AS precipitates, antibody containing fractions of gel filtration chromatography, ion exchange chromatography and protein A affinity chromatography were evaluated by SDS-PAGE and western blotting as described previously. For SDS-PAGE analysis, 2 µg of purified antibodies or antibody AS precipitates were loaded on Polyacrylamide gels.

3.6.4. Antibody Characterization

MAbs selected positive in hybridoma development were characterized in terms of their specificity, isotype, solvent tolerance, interaction with different food extracts, spectral absorptivity and affinity.

3.6.4.1. Specificity Determination

Antibodies were tested for their reactivity with the proteins used in immunization and plate coating by indirect ELISA with 100 ng BSA, TF or OVA coated ELISA plates using 100 µL cell culture supernatants.

Interaction of antibodies with soluble AF isotypes and other mycotoxins were tested by IC-ELISA with AF B1, B2, G1, G2, M1, OTA and ZEA. 50 µL cell culture supernatant was mixed with 50 µL of 0.02 mg/mL solution of each specified mycotoxin and incubated for 30 min at 37°C. Cell culture supernatants mixed with PBS was used as negative control.

3.6.4.1.1. Antibody Isotype Determination

The heavy and light chain isotypes of antibodies were determined using a mouse immunoglobulin isotyping kit used according to manufacturer’s protocol (BD, 550487).

3.6.4.1.2. Solvent Tolerance

Solvent tolerance of the antibodies was determined using indirect ELISA with AFB1-BSA coated plates. 0 - 70% aqueous solutions of methanol, acetonitrile, acetone and ethanol was prepared. AS precipitates of the antibodies were 500 fold diluted with these solutions and incubated at room temperature for 5 minutes. 100 µL of the antibody solutions containing 100-200 ng antibody in differing solvent concentrations were loaded to the ELISA plates and incubated for 20 min at room temperature. Antibody binding was visualized as stated previously. Work was conducted with 3 independent replications.

3.6.4.1.3. Matrix Interference Effect Assessment

Reactivity of antibodies with corn, red pepper and hazelnut extracts prepared according to directives [106] and AF binding capability of the antibodies in these extracts were determined by indirect competitive ELISA using AFB1-BSA coated plates. 1000 fold diluted AS precipitated antibodies were prepared in PBS. 1 mL of diluted antibody was pre-incubated with 0, 1, 5, 10, 50 and 100 µL of clean or 91 ng/mL AF B1 spiked corn, red pepper and hazelnut extracts for 30 min at 37°C. Similarly spiked 70% methanol-water solution was used as positive control. Pre-incubated antibodies were added to the wells and incubated for 1 hour at room temperature. Antibody binding was visualized as stated previously.

3.6.4.1.4. Calculation of Spectral Absorptivity

Purified antibodies were exhaustively dialyzed against distilled water and lyophilized under 0.1 mbar pressure for 24 hours. Lyophilized protein samples were weighed and 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, 0.05 mg/mL and 0.01 mg/mL antibody solutions were prepared. Absorbance at 280 nm was recorded and linear graphs were plotted. 0.1% extinction coefficients ($\varepsilon^{0.1\%}$) corresponding to protein absorbance value for 0.1% (= 1 mg/mL) solutions, was calculated as the slope of the linear curve. Work was conducted with three independent replicates.

3.6.4.1.5. Antibody Affinity Determination

0.2 µg/mL purified antibody solutions in PBS were competitively interacted with AFB1, B2, G1 and G2 at concentrations ranging from 0.001 ng/mL to 100 ng/mL on AFB1-BSA coated plates. Competition mixtures were loaded to AFB1-BSA coated ELISA plates and let for interaction for 1 hour. Visualization was done with AP conjugated polyvalent antibody using 1 mg/mL PNPP as substrate. 50% inhibitory concentrations (IC50) was calculated using ED50plus v.1 (2000) software prepared by Mario H. Vargas.

3.7. Immunoaffinity Column Development

3.7.1. Antibody Immobilization to Chromatographic Resin

CNBr activated sepharose 4B was purchased in lyophilized form with sugar stabilizers. The stabilizers were washed off in acidic pH (1mM HCl, pH<3). Antibody solutions in binding buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) were used for conjugation of the antibodies to sepharose. 120 µg purified antibody per column was used for IAC development. Sepharose binding efficiency was evaluated by measuring absorbance at 280 nm wavelength prior to and after immobilization. The unbound moieties on the sepharose were blocked with 1 M Ethanolamine, pH: 8.

3.7.2. HPLC Analysis for Aflatoxin Quantification

AFs were quantified with HPLC analysis after IAC applications. Kobra cell method was employed for the derivatization of the aflatoxins [107]. 20 µL sample was loaded to 250/4,6Nucleosil 100-5 C18 column (Macherey-Nagel 720014.46) at 1 mL/min flow rate at room temperature. 55% KBr-HNO_2 buffer + 27% methanol + 18% acetonitrile was used as mobile phase. Toxins were detected with Fluorescence detector at 360 nm excitation and 430 nm emission wavelengths. Device was calibrated with certified AF standards (Supelco 46304-U) for accurate quantification.

3.7.3. Overload Test

AF binding capacity of the columns were evaluated with overflow test, where 500 ng AF B1, B2, G1 or G2 was loaded to separate columns in 23.3 % Methanol-water solution with 3 replicas. AF solution was loaded to the columns at 3 mL/min flow rate and the columns were washed with 20 mL distilled water at the same flow rate. Bound AFs were eluted with 1 mL methanol. Eluates were diluted with equal volume of water prior to HPLC analysis.

3.7.4. Limit Binding Test

Column performance was evaluated with 5 ng AF limit binding test. 70% methanol was spiked with 5 ng of AFB1, AFB2, AFG1 and AFG2. Columns were tested for recovery of 5 ng AFB1, AFB2, AFG1 and AFG2. 20 mL contaminated methanol solution was loaded to the columns at 3 mL/min flow rate and the columns were washed with 20 mL distilled water at the same flow rate. The bound aflatoxin was eluted with 1 mL methanol. Eluate was diluted with equal volume of water prior to HPLC analysis.

3.8. Biosensor Development

3.8.1. Gold Surface Preparation

3.8.1.1. Preparation of AFB1 O-carboxymethyloxime

1 mg AFB1 in 400 µL methanol, 2 mg carboxymethyl hydroxylamine HCl (CMO) in 100 µL dH_2O and 100 µL pyridine were mixed. The reaction proceeded at 100 °C for 3 hours followed by 24 h incubation at room temperature. Solvents were evaporated just to dryness under gentle N_2 flow [62]. Resulting AFB1-oxime was dissolved in PBS to get 10 mM concentration.

3.8.1.2. Amine Functionalization of the Crystals

The coating of quartz was carried out under a sterile hood to avoid contamination. Crystals were soaked in the ethanol solution of 2mM 11-mercaptoundecanoic acid (MUA) overnight at room temperature. 400 mM EDC was 1:1 mixed with 100 mM N-hydroxysuccinimide (NHS) to obtain EDC/NHS solution. MUA coated surface was activated with EDC/NHS for either 10 or 15 min. Carboxylic acid moieties of MUA were transformed to amine by incubation of activated surface with 1 M ethylenediamine (EDA), pH: 8.5 for 7 min followed by blocking with 1 M ethanolamine (EOA) pH:8.5 for 2 min.

3.8.1.3. QCM Measurements

QCM200 Quartz Crystal Microbalance connected to QCM25 5 MHz Crystal Oscillator (Stanford Research Systems, USA) was used for the measurement of frequency changes. The sensor was used in the flow mode where a constant flow of used solutions was run over the crystal using a siphon system. Flow rate was controlled with a peristaltic pump connected between the siphon and the crystal holder. In order to maintain a low noise to the frequency signal due to pressure transients, 12-roller peristaltic pump of Harvard Apparatus, USA with two channels was used.

5 MHz AT-cut chrome/gold coated polished quartz crystals with 2.4 cm diameter were used for the measurements. Gold coated quartz crystals were argon plasma cleaned with Diener Femto plasma cleaner for 3 min at 40 mV prior to surface modification. Crystals are sensitive to large changes in frequency (~8 Hz/ºC) [108]. The experiments were performed at controlled room temperature (22°C ± 1 °C) and at least 30 min. stabilization period was required before measurements in order to minimize the temperature effects. Frequencies were recorded using in-house developed software, prepared by Dr. Mika Harbeck.

A standard measurement protocol involved the application of PBS to the sensor surface in constant flow rate in order to achieve a stable baseline, delivery of test solution and application of PBS to achieve the post-measurement baseline. Signal was recorded throughout the experiment. Frequency changes were calculated using

the frequencies of pre- and post-measurement baselines. All solutions were filtered through a 0.22 μm filter and degassed to prevent bubble formation.

3.8.1.4. Chemical Blocking of Activated Surfaces

Unbound amine groups on the crystal surface were blocked with the carboxylic acid in the acetate buffer. Acetate buffers were prepared with the dilution of the Acetate buffer stock 1 M CH_3COONa, pH 4.8. pH was adjusted with acetic acid when necessary. Blocking efficiencies of 60 mM acetate buffer, pH: 4 and 1 M acetate buffer, pH: 4.8 were evaluated. Additionally, 60 mM acetate buffer, pH: 4 was activated with EDC/NHS solution for 10 min with 1:5 acetate:EDC ratio and used for blocking to test the effect of activation for blocking efficiency. The crystals prepared for comparison of 10 or 15 min EDC/NHS activation of MUA prior to amine conversion were blocked with 1 M acetate buffer, pH: 4.8. All experiments were carried out with at least three independent replicas.

Binding of nonspecific proteins to the prepared the crystal surface was evaluated by sensor measurements with 0.1 mg/mL BSA in PBS. The analysis was conducted at room temperature with a flow rate of 50 μL/min. Frequency changes resulting from protein binding were recorded with in-house developed software. Experiments were conducted with at least 3 replicas.

3.8.1.5. AFB1 Immobilization to the Quartz Surface

10 mM AFB1-Oxime was activated by EDC/NHS solution for 10 min with 1:5 AFB1-oxime : EDC ratio. Activated AFB1-oxime was incubated on the amine functionalized surface for 15 min. The surface was blocked with 1 M acetate buffer, pH: 4.8 for 10 min.

The AF immobilization strategy presented in the current study is summarized in Figure 3.2.

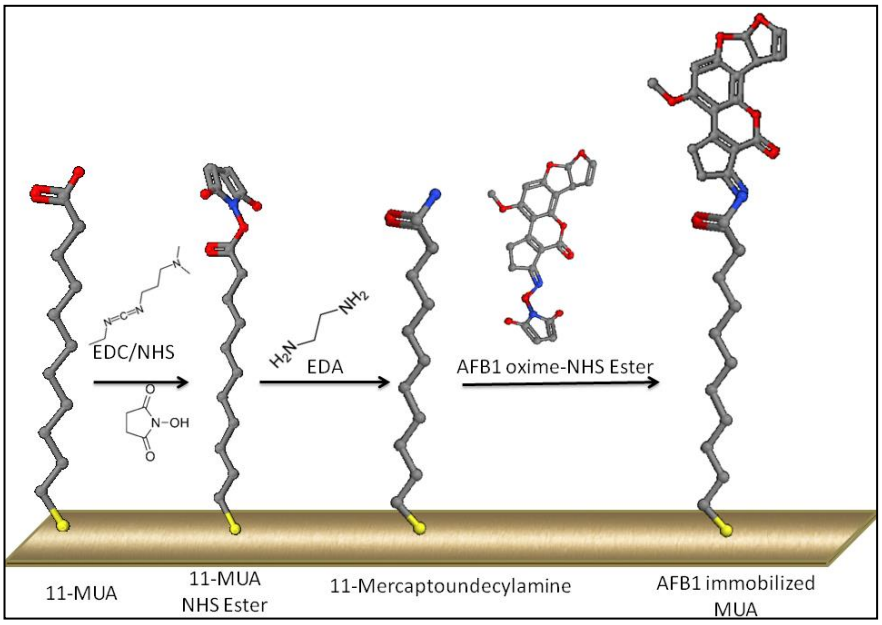


Figure 3. 2: Schematic illustration of AF immobilization strategy to gold coated crystal surface.

3.8.2. Development of Competitive Immunosensor

3.8.2.1. AFB1 Measurement Procedure

AF specific IgA and IgG antibodies were used in inhibitory immunoassay with the prepared crystals. 0.1 mg/mL of purified antibodies were competitively interacted with changing concentrations of AFB1 in PBS for comparative evaluation. Antibody-AFB1 mixtures were delivered to the prepared sensor surface at room temperature with 50 µL/min flow rate. Differential frequency shifts resulting from competition of free AFB1 at different concentrations and surface immobilized AF to bind the antibody were evaluated. X-axes of the sensograms were normalized for alignment using BIAevaluation Software version 4.1. The experiments were conducted with at least 4 replicas.

3.8.2.2. Surface Regeneration

Different solutions were used for the regeneration of antibodies from sensor surface. HPLC grade water, 0.1 M HCl, 50 % Methanol, 50 mM NaOH + 30% ACN,

100 mM Glycine HCl, pH:2, 50mM NaOH + 0.5% SDS, 50mM NaOH + 1% SDS and 1.2 mM NaOH + 30mM EDTA were injected to the sensor surface after antibody binding with 50 µL/min flow rate. Regeneration efficiencies were evaluated by frequency changes in PBS.

4. RESULTS

4.1. Antibody Development

The antibody development section consists of antibody preparation steps including immunogen preparation, immunizations, hybridoma development, selection and characterization of the MAbs and antibody purification.

4.1.1. Preparation of Immunogens

Conjugation of AFB1 to BSA, OVA and TF were achieved by modification of the method of Zhou et al. in two steps [60]. In the first step, amine groups of the proteins were enriched by converting carboxyl groups to amine groups with an EDC linker to yield cationized BSA (cBSA), cationized OVA (cOVA) and cationized TF (cTF). In the second step, AFB1 was conjugated to the proteins by Mannich type reaction in the presence of formaldehyde and the conjugates AFB1-BSA, AFB1-OVA and AFB1-TF were obtained. Characterization of cationized proteins and conjugates are presented.

4.1.1.1. Preparation and Characterization of Cationized Proteins

Initially, the yield of the chemical reaction for cationized protein preparation was determined. Spectral readings at 280 nm were used to determine the concentration of soluble proteins prior to and after the reaction. Total protein yield was calculated as percent recovery of proteins. Reaction yields for BSA, OVA and TF cationization are determined as 82%, 85% and 66%, respectively (Table 4. 1) which is sufficient for pursuing the next step of conjugation.

Table 4. 1: Net protein yield of cationization reaction.

	Starting protein (mg)	Cationized protein (mg)	Yield (%)
OVA	25.0	20.5	82.0
BSA	25.0	21.4	85.6
TF	25.0	16.6	66.4

Cationization procedure increases the number of amine groups on the surface of the protein. Hence, an increase in the isoelectric point (pI) of the protein is expected. Therefore, the protein characterization strategies were based on the identification of this pI change. pI value of BSA is 4.7 [109], OVA is 4.6 [110] and TF is 6.5 [111] and the pI values of cationized proteins are expected to be higher than 8 [112]. This opens a working pH range for the separation of cationized proteins from native proteins by ion exchange chromatography. Proteins are amphoteric molecules whose net charge depends on the pH of the containing medium. When proteins are in buffers with higher pH than their pI value, their net charge is negative and vice versa. So, at neutral pH, native forms of the proteins are expected to be negatively charged, and cationized proteins should be positively charged.

4.1.1.1.1. Anion Exchange Chromatography

With this principle, cationized proteins were characterized by DEAE-C anion exchange chromatography, which retains negatively charged molecules and does not bind to positively charged ones. At neutral pH (10 mM K2HPO4, pH: 7), negatively charged native proteins were retained in the column and cationized proteins were discarded in flow through. Bound native proteins were eluted from the column with a high ionic strength buffer (10 mM K2HPO4, pH: 7, 500 mM NaCl). The chromatograms showing characterization of cationized BSA, OVA and TF are presented in Figure 4.1, Figure 4.2 and Figure 4.3, respectively. The chromatograms showed that more than 90% of the proteins were successfully cationized as a result of the reaction.

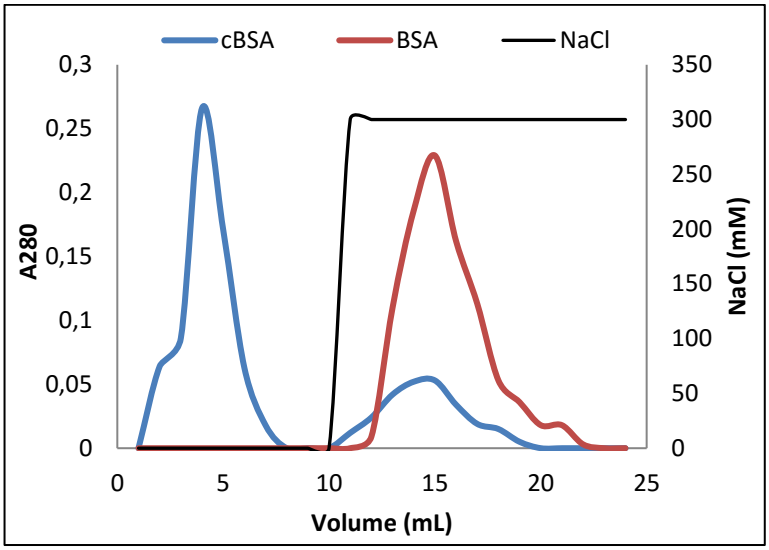


Figure 4. 1: DEAE-C anion exchange chromatogram of BSA and cBSA.

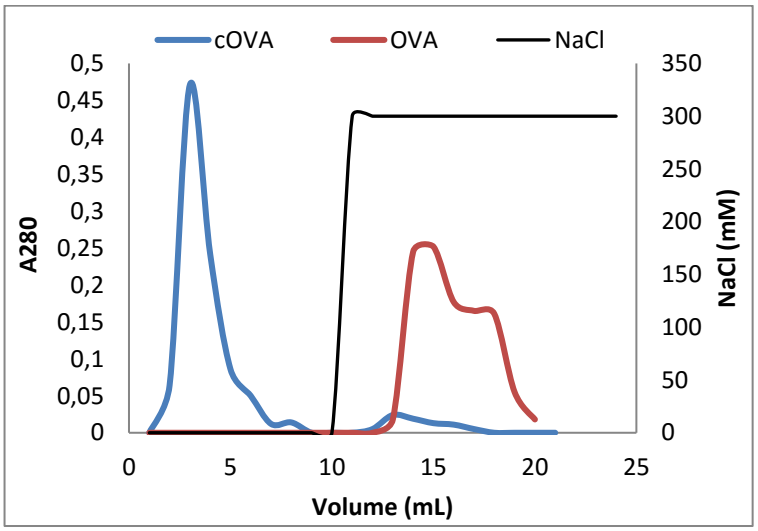


Figure 4. 2: DEAE-C anion exchange chromatogram of OVA and cOVA.

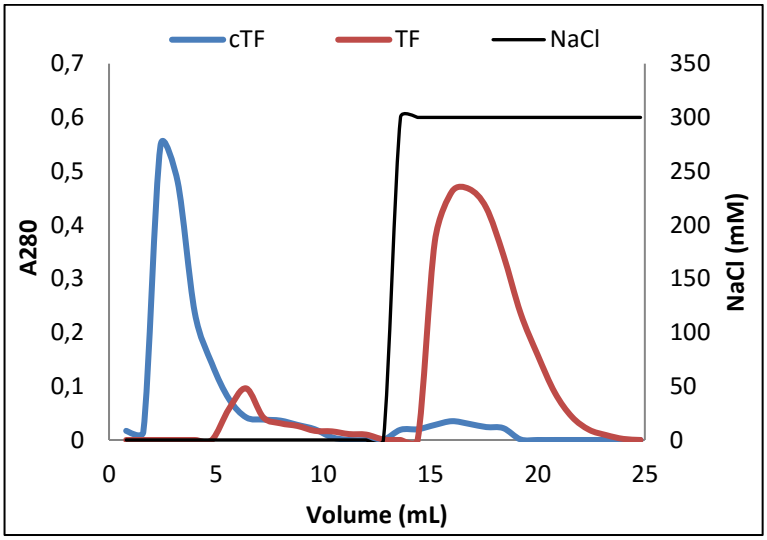


Figure 4. 3: DEAE-C anion exchange chromatogram of TF and cTF.

4.1.1.1.2. HPLC Analysis with Cation Exchange Column

Cationized BSA and TF were additionally tested with HPLC using strong cation exchange column. In this case, the column should retain positively charged cationized proteins and native proteins should be discarded in the flow through. In the analysis, 20 µg protein was loaded to the column at neutral pH (10 mM K2HPO4, pH: 7). 5 minutes after loading, the bound molecules were eluted at high ionic strength (10 mM K2HPO4, pH: 7, 1 M NaCl). Fluorescent detector with 280 nm excitation and 360 nm emission wavelength was used. In the analysis of cBSA, the peak of native BSA was observed at 1.6 min and cBSA at 12 min (Figure 4.4). In the chromatograms obtained from TF and cTF, TF peak was observed at 1.7 min and cTF peak was observed at 7 min. The efficiency of both cationization reactions were shown to be more than 90%, in conformity with DEAE-C ion exchange chromatography.

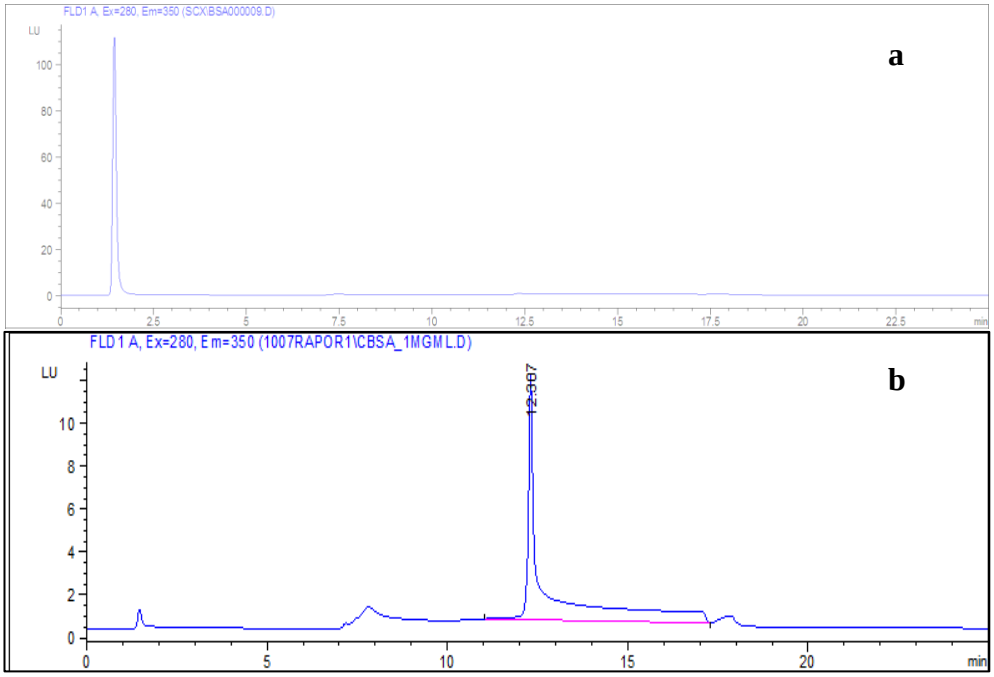


Figure 4. 4: HPLC strong cation exchange chromatogram of a) BSA b) cBSA with FLD detector (extinction: 280 nm, emmission: 350 nm).

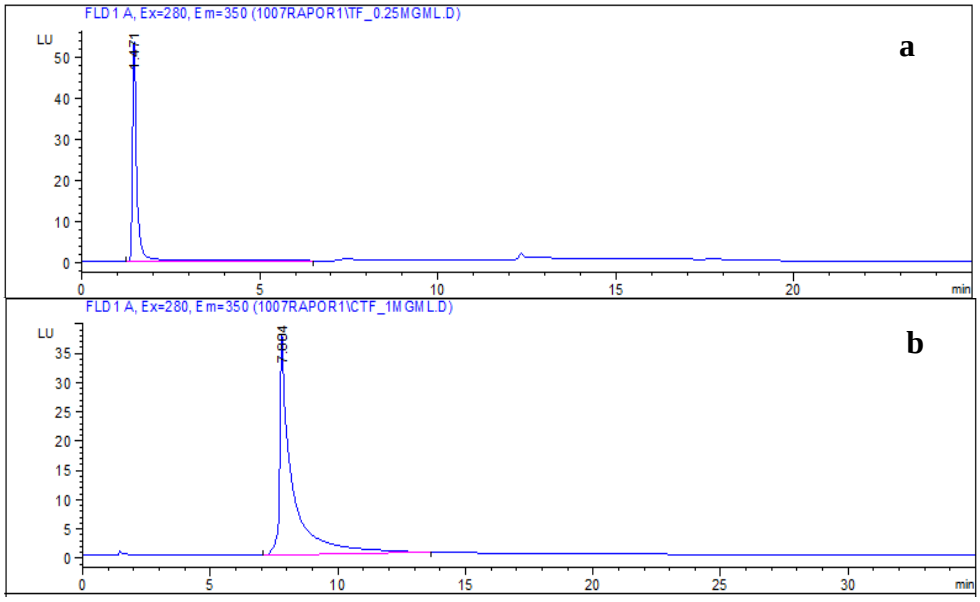


Figure 4. 5: HPLC strong cation exchange chromatogram of a) TF b) cTF with FLD detector (extinction: 280 nm, emmission: 350 nm).

4.1.1.2. Preparation and Characterization of AF-Protein Conjugates

AFB1-protein conjugation efficiencies were evaluated by spectrophotometry according to Beer-Lambert Law. Wave scans of unconjugated proteins, AFB1 and AFB1-protein conjugates are presented in Figure 4. 6 for BSA, OVA and TF conjugation reactions. Maximal absorptivity of proteins is known to be at 278 nm. AFB1 has maximal absorption wavelength of 366 nm and has a minor peak at 266 nm. Wavescan of AFB1-BSA conjugate showed a major absorption peak at 270 nm and AFB1-OVA and AFB1-TF conjugates showed the major peak at 276 nm. A minor peak at 370 nm was observed in all three conjugates. The presence of two peaks and the blue shift observed in the major protein peak indicated the success of the conjugation.

AFB1-TF and AFB1-OVA were used as immunogens and AFB1-BSA was used to test AF specific immune response in indirect enzyme linked immunosorbent assay (ELISA) test.

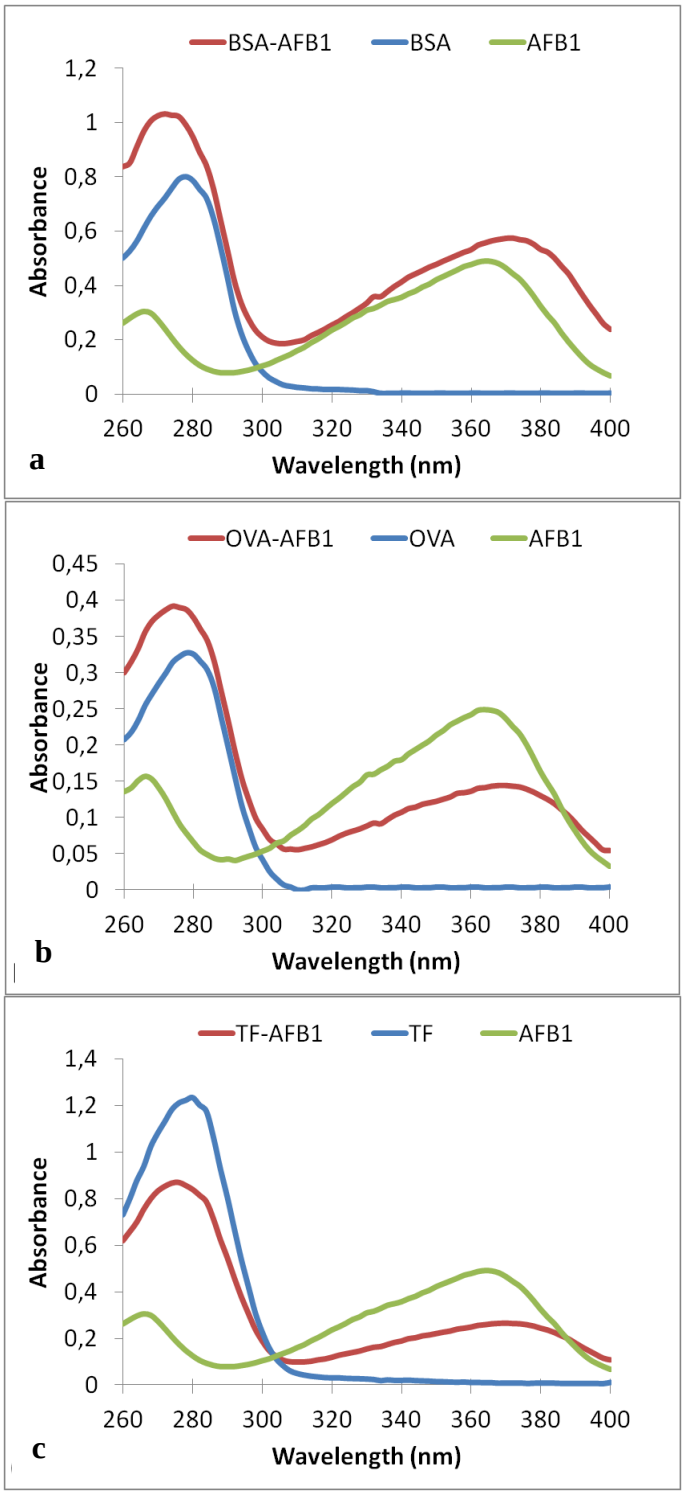


Figure 4. 6**:** UV-Vis absorption spectra of AFB1-Protein conjugates between 260 nm – 400 nm wavelengths**.** a) BSA-AFB1, BSA and AFB1; b) OVA- AFB1, OVA and AFB1; c) TF- AFB1, TF, AFB1.

4.1.2. Immune Response

Mice immunized with AFB1-protein conjugates not only respond to AF itself, but also produces antibodies against the carrier protein. The small molecular weight of AFs makes them unsuitable to be used in ELISA tests as coating antigens so; they should be coated to ELISA plates as protein conjugates. BSA conjugate of AFB1 was used as coating antigen for testing the sera of both AFB1-OVA and AFB1-TF immunized mice in order to detect only AF specific antibodies. The initial monitoring of immune response of the mice was done by indirect ELISA with plates coated with AFB1-BSA. The results of immunization showed that, TF conjugate of AFB1 produces an earlier AF specific immune response, however, after 5 immunizations, humoral response of mice immunized with both antigens reached similar levels (Figure 4.7).

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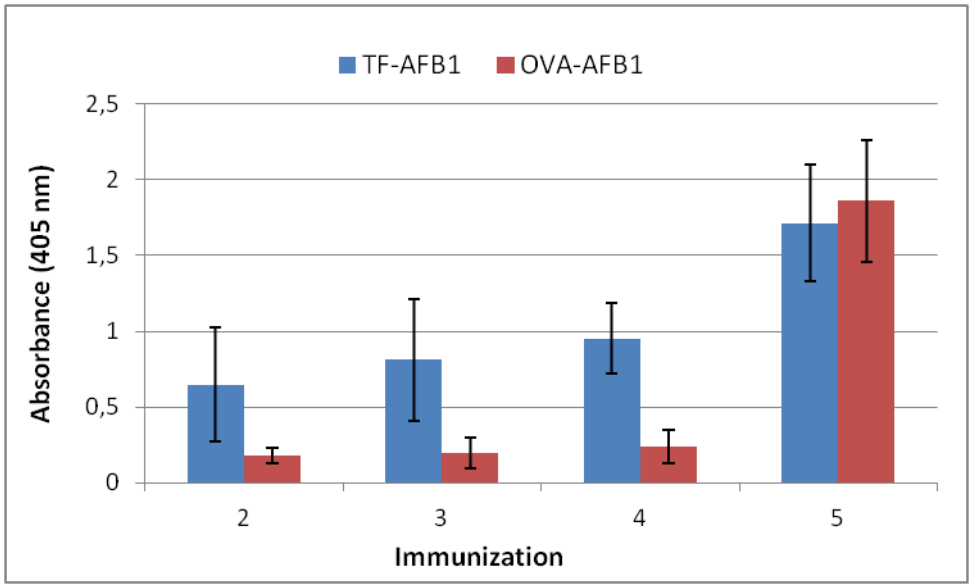

Figure 4. 7: Comparison of AF specific immune response of mice immunized with AFB1-OVA or AFB1-TF. Mice sera were 2000 times diluted with PBS. The results are presented as mean of 5 replicates. Error bars represent standard deviations.

Antibodies inevitably interact with protein conjugates of AF differently when compared to free, soluble mycotoxins. The response obtained by indirect ELISA might be against AFB1, chemical bond between AFB1 and protein or to the protein itself. So, in order to discriminate between these responses, IC-ELISA method was employed.

In the IC-ELISA tests conducted for the characterization of immune sera, we coated the ELISA plates with four different antigens;

i) AF-protein conjugate used in immunizations: The ELISA results with this coating showed the interaction of sera with either AF or the carrier protein used in immunizations.
ii) Unconjugated carrier protein used in immunizations: This will show the antibodies rose against the carrier protein by the immunized animal.
iii) AF-alternative protein conjugate: This coating will show the antibodies which recognize AF or AF-protein bond in the sera.
iv) Unconjugated alternative protein: This will serve as a negative control.

Sera was incubated with either free AFB1 or the carrier protein used in immunizations prior to loading to ELISA wells. Sera incubated for the same duration without antigen was used as negative control. The competition sowed the reactivity of the sera with unconjugated free AFB1 and free carrier protein. The antibodies in the sera which interact with AFB1 or carrier protein cannot bind to the antigens coated to the ELISA plates. So, for IC-ELISA, lower signal is the evidence of the interaction of antibody with competing antigen.

The test was used for the selection of the mice which produced highest antibody titer against free AFB1. IC-ELISA results of an AFB1-TF immunized mouse and an AFB1-OVA immunized mouse are presented in Figure 4.8 a and b, respectively.

Serum of AFB1-TF immunized mouse which was not pre-incubated with AFB1 or TF interacted with AFB1-TF, TF and AFB1-OVA but did not bind to OVA coated wells. Pre-incubation with TF decreased the binding of sera to AFB1-TF and TF coated wells where the signal in TF coated wells decreased drastically. Pre-incubation with AFB1 completely diminished the signal from AFB1-BSA indicating that all antibodies binding to BSA-AFB1 was also binding free AFB1. AFB1 competition also decreased the signal in AFB1-TF coated wells (Figure 4.8 a).

A similar pattern was observed with AFB1-OVA immunized mouse serum. However, the response to free AFB1 was lower in this case, since sera from 4th immunization were used (Figure 4.8 b). This result also revealed the progress of the

immune response in case of hapten-protein conjugate immunogens, indicating antibody response to the carrier protein arises earlier and stronger than the response to the hapten.

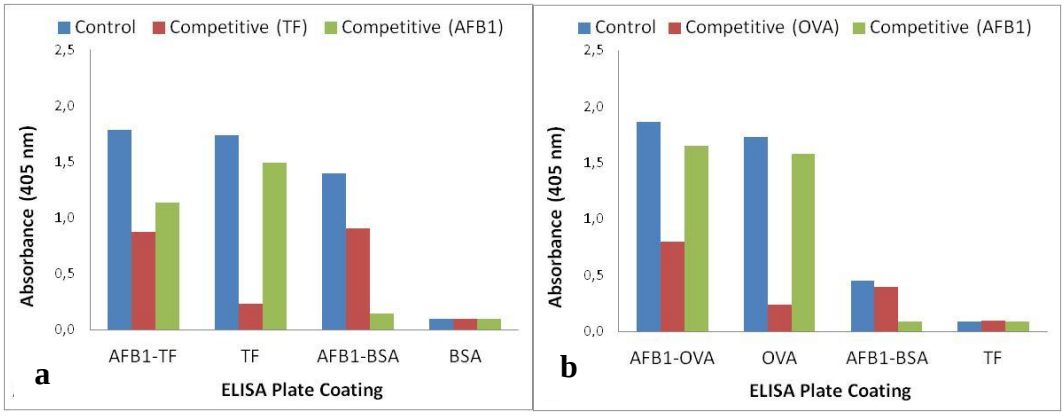

Figure 4. 8: IC-ELISA showing interaction of mice sera with AFB1 and proteins. a) AFB1-TF immunized mice serum with free AFB1 and TF. b) OVA immunized mice serum with free AFB1 and OVA. Serum dilution is 1/5000.

4.1.3. Development of MAbs

Fusions for the development of AF specific MAbs were done using the mice with highest serum antibody titers for free AFB1. Four fusions were performed with two AFB1-TF and two AFB1-OVA immunized mice. Lymphocytes derived from both spleen and lymph nodes were used for the fusions. The results of the fusions were summarized in Table 4.2. Fusions yielded 3852 hybrid clones with 685-1540 hybrid clones per fusion. Every clone was tested for antibody production with indirect ELISA using AFB1-BSA coated plates. 134 clones were selected whose cell culture supernatants interacted with AFB1-BSA.

Table 4. 2: Fusion conditions and results.

Fusion	Immunogen	Myeloma	Lymphocytes	Number of Hybrid Cells	Number of Antibody Producing Hybridomas
1	AFB1-TF	0.45×10^8	4×10^8 (S*)	748	19
		1×10^7	55×10^6 (L**)		
2	AFB1-TF	2×10^8	592×10^6 (S)	878	31
		34×10^6	76×10^6 (L)		
3	AFB1-OVA	240×10^6	1960×10^6 (S+L)	1541	13
4	AFB1-OVA	245×10^6	828×10^6 S)	685	71
		16×10^6	55×10^6 (L)		
TOTAL				3852	134
*S: Spleen Cells **L: Lymph nodes					

4.1.4. Antibody Characterization

The antibodies obtained from the above stated fusions were characterized in terms of their specificity, isotypes, solvent tolerance and affinities. In addition to the antibodies obtained from the indicated fusions, AF specific MAM-8G8 antibody, which was provided as a courtesy of Dr. Esin Akçael and Şerife Şeyda Pirinçci was also used in this thesis and subjected to similar characterization.

4.1.4.1. Specificity

Specific MAbs were selected with a series of consecutive ELISA tests. Initially, there were 134 clones identified in indirect ELISA tests, conducted with AFB1-BSA conjugate coated plates. The antibodies identified in indirect ELISA may be produced against AF itself, the bond between AF and the protein, the protein or it may be nonspecific which is reactive with even blocking agent used. So, carrier

protein cross-reactivity was determined by indirect ELISA test, and reactivity with free AF was evaluated with IC-ELISA.

4.1.4.1.1. Carrier Protein Cross-Reactivity

134 antibody producing clones were tested for their cross reactivity with proteins. Test proteins were selected from the proteins either used for immunization (TF and OVA) or in ELISA plate coating (BSA). AFB1 conjugates of the stated proteins were used as positive control. AFB2 conjugates were used for the initial demonstration of the reactivities of the antibodies with different AF types. Out of 134 antibody producing clones, 117 were cross reactive with either one or more of the selected proteins. 17 antibodies which did not react with carrier proteins are presented in Table 4.3. Hybrid cells and their corresponding antibodies were named according to their location in the screening plates. MAM- prefix represents the research institution where antibodies were developed; TÜBİTAK Marmara Research Center. High cross-reactivity of the antibodies with nonspecific proteins is the result of protein conjugates used for immunization.

Table 4. 3: Reactivities of the antibodies with carrier proteins.

Hybrid Cells	AFB1-BSA	AFB2-BSA	BSA	AFB1-TF	AFB2-TF	TF	PBS (no antigen)
MAM-D3E4	+	+	-	+	+	-	-
MAM-D4C2	+	+	-	+	+	-	-
MAM-D1H2	+	+	-	+	+	-	-
MAM-D3C6	+	+	-	+	+	-	-
MAM-D3F5	+	+	-	+	+	-	-
MAM-D4D6	+	+	-	+	+	-	-
MAM-D12E2	+	+	-	+	+	-	-
MAM-1D1	+	+	-	+	+	-	-
MAM-2B11	+	+	-	+	+	-	-
MAM-6D6	+	+	-	+	+	-	-
MAM-6E10	+	+	-	+	+	-	-
MAM-8G12	+	+	-	+	+	-	-
MAM-10E5	+	+	-	+	+	-	-
MAM-8G8	+	+	-	+	+	-	-
MAM-9F8	+	+	-	+	+	-	-
MAM-2A7	+	+	-	+	+	-	-
MAM-7B2	+	+	-	+	+	-	-

4.1.4.1.2. Mycotoxin Cross-Reactivity

Ultimate aim of the work was to produce the MAb which can react specifically with unconjugated AF derivatives. The antibodies which did not react with proteins were tested for their interaction with free mycotoxins. The interaction of selected antibodies with free AFs and cross-reactivity of the antibodies with the other mycotoxins were tested with IC-ELISA. The IC-ELISA results showed that 12 of the 17 antibodies reacted specifically with aflatoxins (Table 4.4).

Table 4. 4: Comparison of the reactivity of the MAbs with different mycotoxins with IC-ELISA.

Antibody	AFB1	AFB2	AFG1	AFG2
MAM-D3E4	+	+	+	+
MAM-D4C2	-	-	-	-
MAM-D1H2	-	-	-	-
MAM-D3C6	+	+	+	+
MAM-D3F5	-	-	-	-
MAM-D4D6	-	-	-	-
MAM-D12E2	+	+	+	+
MAM-1D1	-	-	-	-
MAM-2B11	+	+	+	+
MAM-6D6	+	+	-	-
MAM-6E10	+	+	+	+
MAM-8G12	+	+	+	+
MAM-10E5	+	+	+	+
MAM-8G8	+	+	+	+
MAM-9F8	+	+	+	+
MAM-2A7	+	+	+	+
MAM-7B2	+	+	+	+

A total of 12 AF specific antibody producing hybrid clones were obtained from 4 fusions. The clones were selected for cell viability and the affinity of their respective antibodies. 3 clones either lost antibody production capacity or cell viability because of chromosomal losses or genetic instability [113].

Mycotoxin cross-reactivities were tested for remaining antibodies. Results of the selected clones MAM-1D1 MAb, MAM-6D6 MAb, MAM-6E10 MAb, MAM-8G12 MAb, MAM-D4D6 MAb, MAM-D3E4 MAb, MAM-2B11 MAb, MAM-D12E2 MAb and MAM-8G8 MAb are presented in Figure 4. 9.

The results of mycotoxin inhibition assay are presented as inhibition rate (%) with respect to control antibody which was not subjected to competition (Figure 4.

9). MAM-1D1 MAb was inhibited by AFB1 and AFM1 less than 30%, and not inhibited by other toxins. MAM-D4D6 MAb was inhibited by AFs less than 60 %. So, these antibodies failed to interact with soluble toxins. MAM-6D6 MAb interacted with AF B1, B2 and M1, however, did not bind to AF G1 and G2 and hence did not qualify the requirement to bind to all AF isotypes. This antibody may be used for specific determination of these isotypes when required, however cannot be used for simultaneous determination all AF isotypes. Similarly, MAM-6E10 MAb preferred AF B1, B2 and M1, and inhibited by AF G1 and G2 less than 60%. However, this antibody was also inhibited by OTA and ZEA. So, this antibody both failed to recognize all AFs with good performance and was not specific. MAM-8G12 MAb showed superior performance in the recognition of AF isotypes. However, it showed cross-reactivity with other mycotoxins as well. Antibodies MAM-D3E4, MAM-2B11, MAM-8G8 and MAM-D12E2 recognized all AF isotypes and showed no cross-reactivity with OTA and ZEA (Figure 4. 9). So, MAM-D3E4, MAM-2B11, MAM-8G8 and MAM-D12E2 MAbs were used for further characterization.

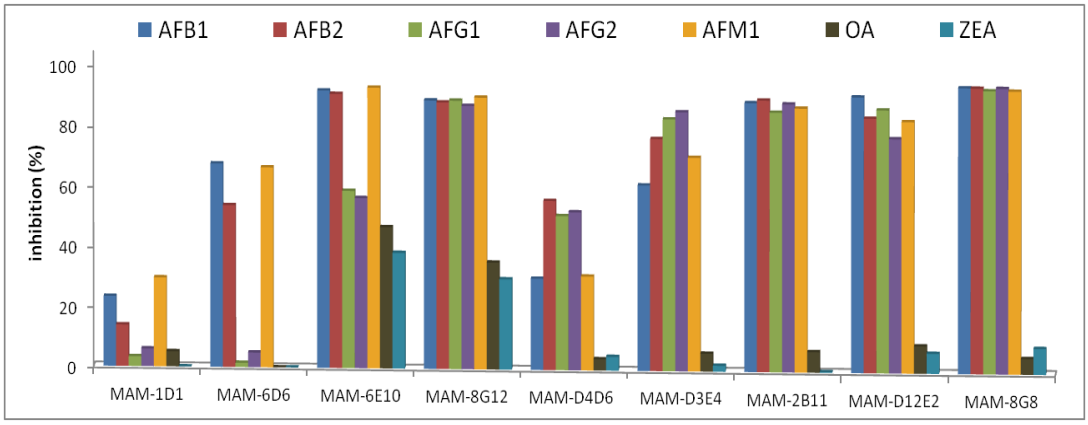

Figure 4. 9: IC-ELISA showing the interaction of antibodies with 1 µg free AFB1, AFB2, AFG1, AFG2, AFM1, OTA and ZEA. Inhibition rates were calculated with respect to no mycotoxin bearing positive control. Results are the mean of two independent replicas.

4.1.4.2. Antibody Isotype Determination

Immunoglobulin isotyping of developed MAbs were done by using hybridoma subisotyping Kit (BD Biosciences). MAM-D12E2 antibody was IgA isotype with lambda light chain, MAM-8G8 was IgG1 isotype with lambda light chain and MAM-

2B11 was determined as IgG2b isotype with lambda light chain. MAM-D3E4 was IgM MAb with lambda light chain (Figure 4. 10).

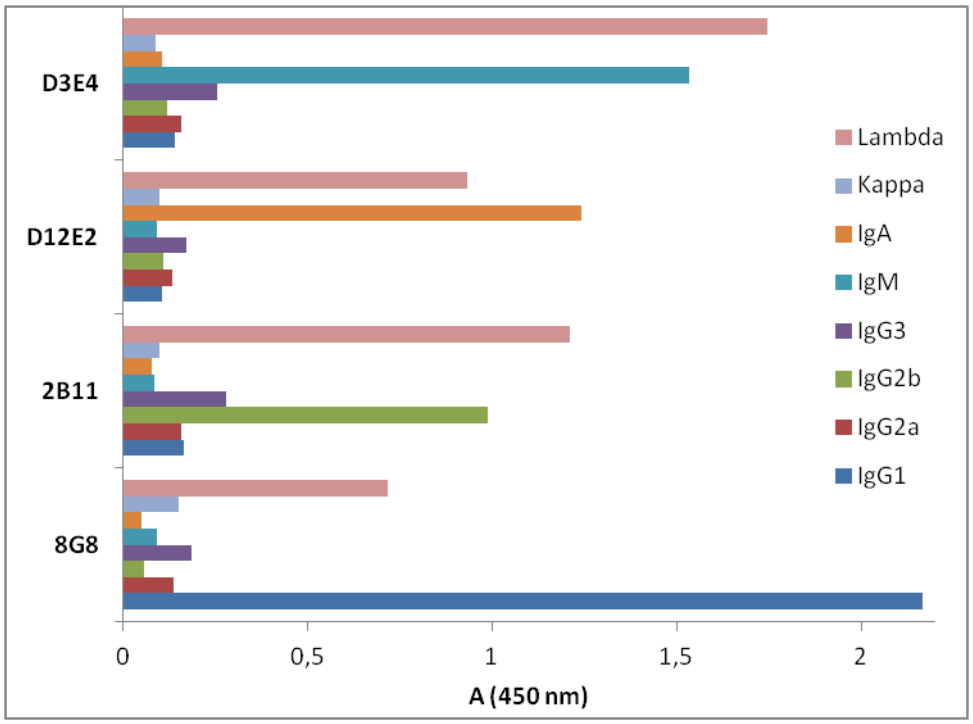

Figure 4. 10: Isotype determination of antibodies.

4.1.4.3. Determination of Antibody Extinction Coefficients

Antibody quantification is critical for immunoassay development for making accurate test design and characterization of the system. Spectral measurement is the simplest and most widely used method for quantification. Commonly known antibody absorptivities are not applicable for different antibodies and spectral absorptivities of every developed antibody have to be determined prior to use in immunoassays.

For this aim, purified MAM-D12E2, MAM-2B11, MAM-D3E4 and MAM-8G8 antibodies were exhaustively dialyzed against double distilled water in order to clear the solutions from any salt or buffer ions and lyophilized. These lyophilized pure antibodies were used for the determination of 0.1% extinction coefficients ($\varepsilon^{0.1\%}$) of the antibodies. The slopes of linear graphs showing spectral measurements of different antibody concentrations represent the $\varepsilon^{0.1\%}$ of each antibody, i.e. absorbance of 1 mg/mL solution of the antibodies. The concentration vs. absorbance

graphs are presented in Figure 4. 11. Calculations revealed $\varepsilon^{0.1\%}$ of MAM-D3E4 is 0.83, MAM-8G8 is 0.4, MAM-D12E2 is 0.65 and MAM-2B11 is 1.23 (Table 4. 5).

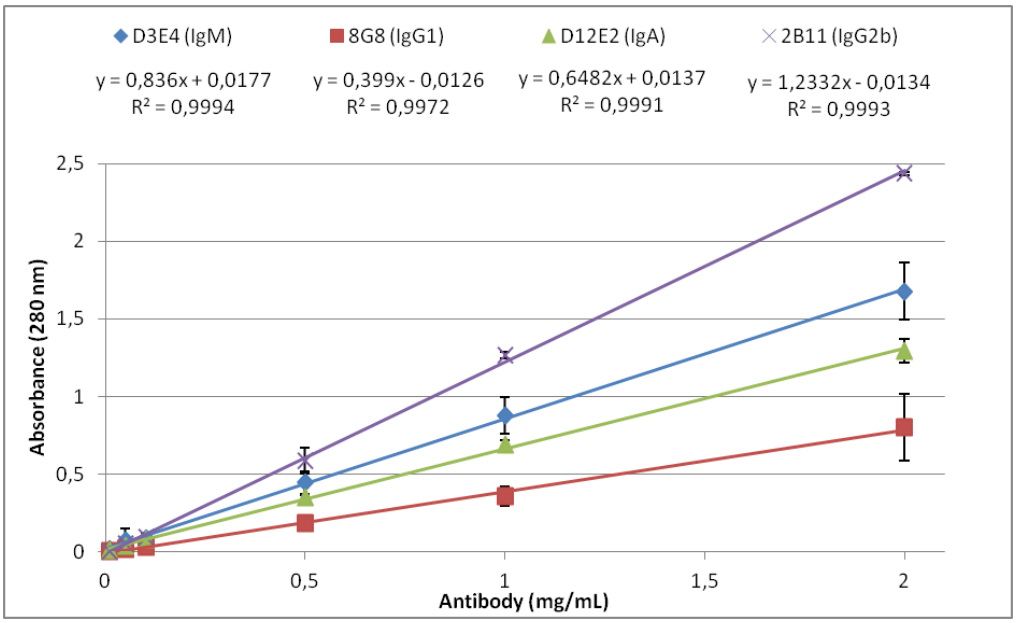

Figure 4. 11: Calculation of spectral absorptivities of MAM-D12E2, MAM-2B11, MAM-D3E4 and MAM-8G8 antibodies. Error bars represent standard deviations. Slopes of the linear curves represent 0.1% extinction coefficients of the antibodies.

Table 4. 5: Extinction coefficients ($\varepsilon^{\%0.1}$) of developed antibodies.

Antibody	$\varepsilon^{\%0,1}$
MAM-D12E2	0.65
MAM-2B11	1.23
MAM-8G8	0.4
MAM-D3E4	0.84

4.1.4.4. Solvent Tolerance

Anti-AF MAbs will be used in detection systems in order to detect and/or quantify AF contamination in food and feedstuff. Toxin extraction procedures involve the use of organic solvents, and the resulting extracts contain all solvent extractable metabolites of the sample. So, high solvent tolerance as well as low cross-reactivity with sample extracts is expected from the antibodies.

The solvent tolerance of the antibody was assessed by indirect ELISA test. Methanol, acetonitrile, acetone and ethanol stabilities of the antibodies were examined. Antibodies were diluted in 0-70% aqueous solutions of methanol, acetonitrile, acetone and ethanol. Indirect ELISA was conducted with antibodies diluted in solvents on AFB1-BSA coated wells. The results showing the binding capability of the MAbs to AFB1-BSA conjugate in solutions with increasing concentrations of solvents are presented in Figure 4. 12. MAM-D3E4 MAb was shown to be intolerant to organic solvents (Figure 4. 12 c). MAM-D12E2 and MAM-2B11 and MAM-8G8 MAbs retained their activities in 40% methanol and 30% ethanol solutions. MAM-D12E2 MAb did not lose its activity at 20% acetone solution and retained 80% activity at 30% aqueous solution of acetone. However, the activities of MAM-2B11 and MAM-8G8 gradually decreased even in 10% acetone and lost their activities after 30% acetone concentration. MAM-D12E2 and MAM 8G8 showed high tolerance to acetonitrile where no activity loss was observed in 20% acetonitrile solution. The activity of MAM-2B11 MAb decreased to 70% at 20% acetonitrile concentration. Hence, MAM-D12E2 MAb was shown to be tolerant to 40% methanol, 20% acetonitrile, 30% acetone and 40% ethanol (Figure 4. 12 a). MAM-2B11 MAb was tolerant to 40% methanol, 20% acetonitrile, 20% acetone and 30% ethanol (Figure 4. 12 b). MAM-8G8 was tolerant to 40% methanol, 20% acetonitrile, 30% acetone and 30% ethanol (Figure 4. 12 d). Solvent tolerances of MAM-2B11, MAM-D12E2 and MAM 8G8 were shown to be sufficient to be utilized in detection systems.

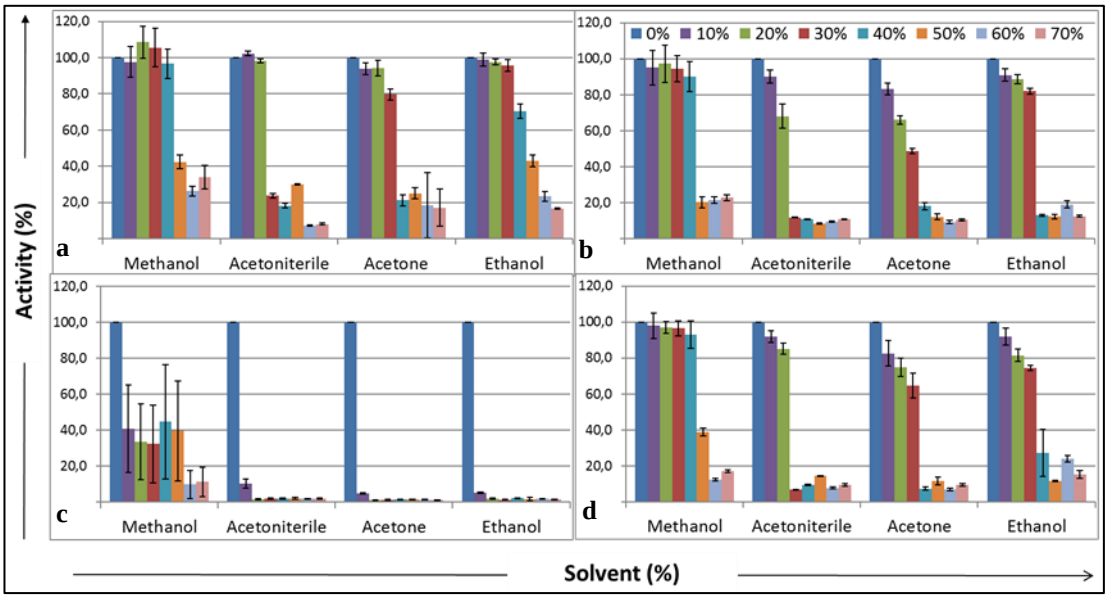

Figure 4. 12: Tolerance of antibodies to different concentrations of methanol, ethanol and acetonitrile demonstrated with ELISA. a) MAM-D12E2 MAb b) MAM-2B11 MAb c) MAM-D3E4 MAb d) MAM-8G8 MAb. The results are presented as the mean of three replicates. Error bars represent standard deviations.

4.1.4.5. Antibody Affinity

The affinities of MAM-D12E2, MAM-8G8 and MAM-2B11 were determined. Purified MAbs were competitively interacted with AFB1, B2, G1 and G2 at concentrations between 0.001ng/mL to 100ng/mL in PBS in order to determine the affinities of the antibodies.

Binding inhibition curves for AFB1 are presented in Figure 4. 13. MAM-D12E2, MAM-8G8 and MAM-2B11, which were selected as potential candidates for IAC development had 0.86 ng/mL, 086 ng/mL and 16.75 ng/mL IC50 values for AFB1, respectively.

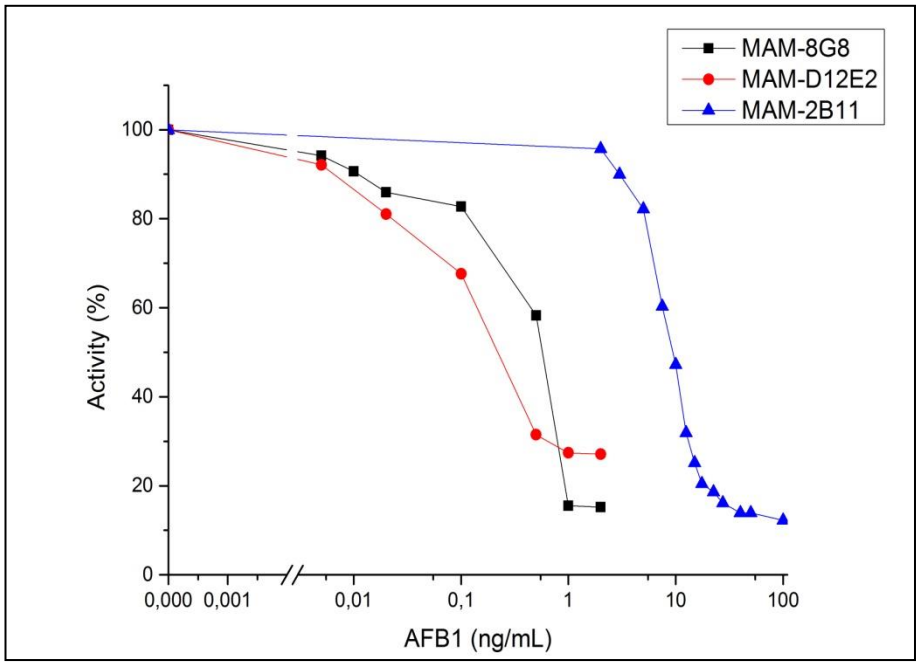

Figure 4. 13: AFB1 binding inhibition curves of MAM-D12E2, MAM-8G8 and MAM-2B11 MAbs.

AFB2 binding inhibition curves are presented in Figure 4. 14. MAM-D12E2, MAM-8G8 and MAM-2B11, had 3.33 ng/mL, 3.70 ng/mL and 5.63 ng/mL IC50 values for AFB2, respectively.

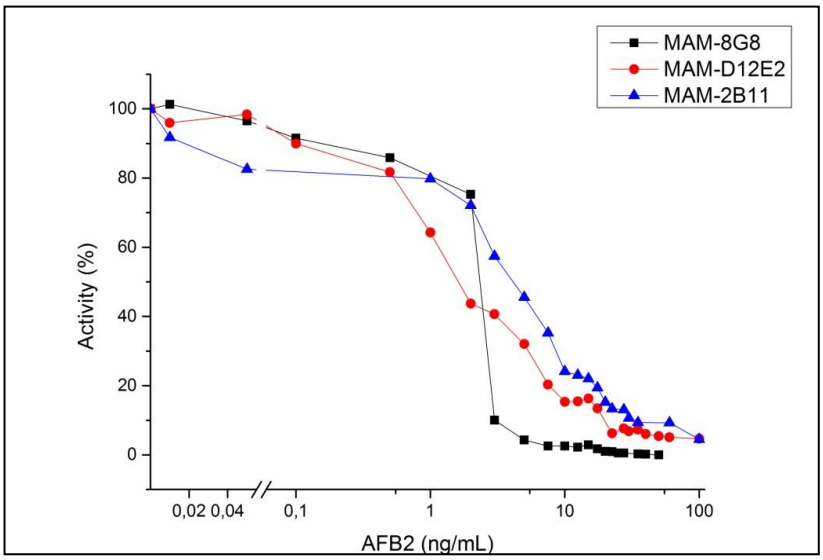

Figure 4. 14: AFB2 binding inhibition curves of MAM-D12E2, MAM-8G8 and MAM-2B11 MAbs.

AFG1 binding inhibition curves are presented in Figure 4. 15. MAM-D12E2, MAM-8G8 and MAM-2B11, had 2.95 ng/mL, 4.36 ng/mL and 36.63 ng/mL IC50 values for AFG1, respectively.

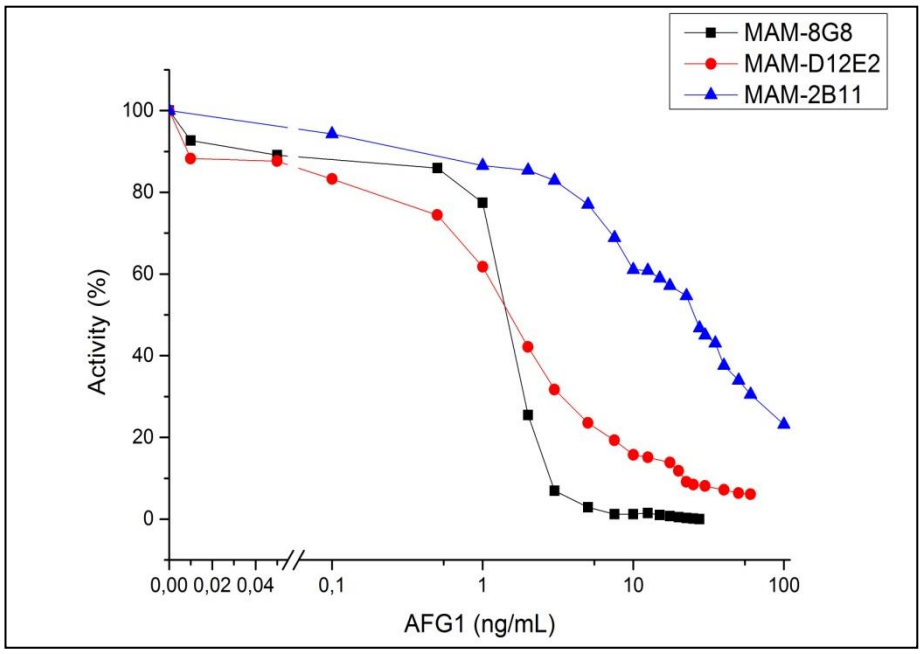

Figure 4. 15: AFG1 binding inhibition curves of MAM-D12E2, MAM-8G8 and MAM-2B11 MAbs.

AFG2 binding inhibition curves are presented in Figure 4. 16. MAM-D12E2, MAM-8G8 and MAM-2B11, had 4.30 ng/mL, 40.34 ng/mL and 27.44 ng/mL IC50 values for AFG2, respectively.

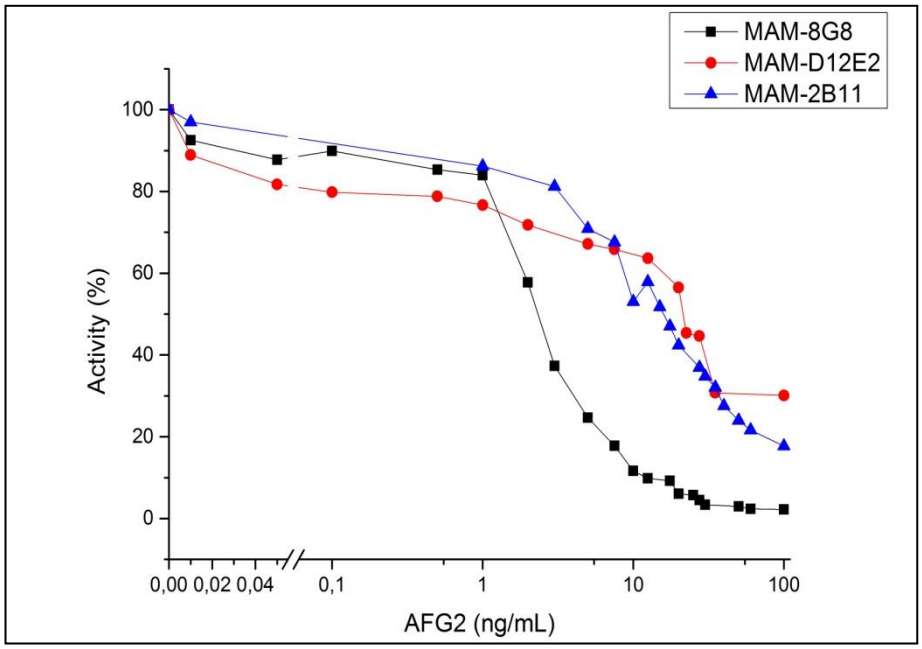

Figure 4. 16: AFG2 binding inhibition curves of MAM-D12E2, MAM-8G8 and MAM-2B11 MAbs.

The affinities of the antibodies to different AF types are presented in Table 4.6. For an accurate comparison of the effect of different antibody isotypes on immunoanalytical systems, antibodies with similar affinities should be utilized. MAM-D12E2 and MAM-8G8 will be the MAb candidates that fulfill this requirement as representatives of IgA and IgG isotype antibodies, respectively.

Table 4. 6**:** Affinities of MAM-D12E2, MAM-8G8 and MAM-2B11 MAbs.

AF	Affinity (ng/mL)		
	MAM-8G8	MAM-D12E2	MAM-2B11
AFB1	0.86	0.86	16.75
AFB2	3.33	3.70	5.63
AFG1	2.95	4.36	36.63
AFG2	4.30	40.31	27.44

4.1.4.6. Matrix Interference Effect Assessment

In order to assess the matrix interference effect, MAM-D12E2 MAb, MAM-2B11 MAb, MAM-8G8 MAb and MAM-D3E4 MAbs were evaluated with IC-ELISA with AF-free corn, red pepper and hazelnut extracts. Antibodies were pre-incubated with differing concentrations of extracts from 0.1% to 10%. MAM-D12E2 showed slight reaction with hazelnut and red pepper extracts (Figure 4. 17 a). MAM-2B11 and MAM-8G8 MAbs did not bind to any of the extracts tested for cross-reactivity (Figure 4. 17 b and d). MAM-D3E4 MAb showed strong cross reactivity with corn and red pepper extracts (Figure 4. 17 c).

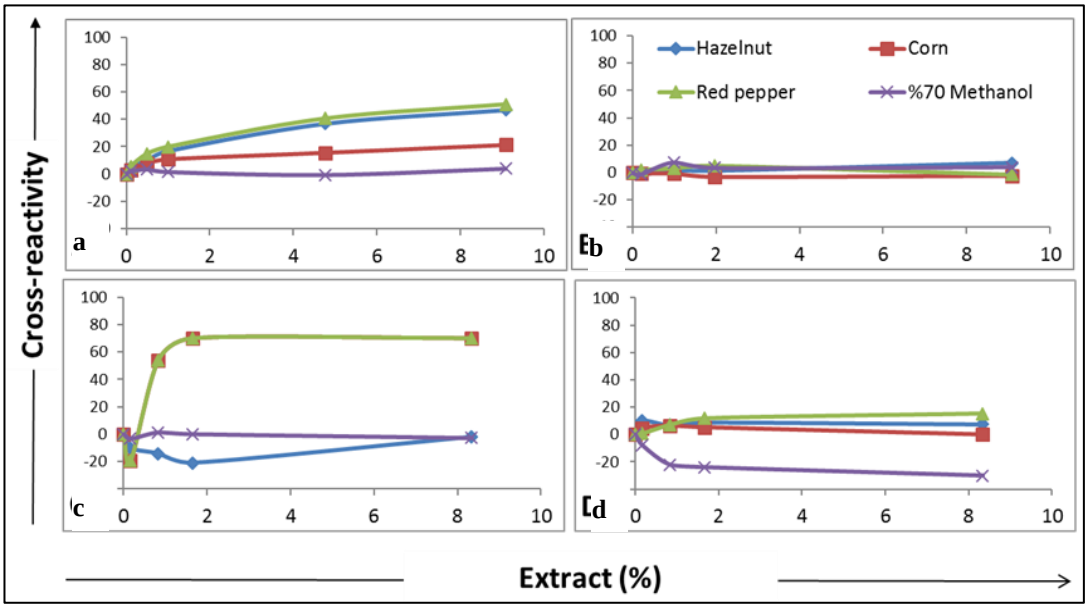

Figure 4. 17: IC-ELISA showing the matrix cross-reactivity of MAbs. Cross-reactivities of a) MAM-D12E2 b) MAM-2B11 c) MAM-D3E4 d) MAM-8G8 with AF free Corn, Pepper and Hazelnut extracts with respect to noncompetitive ELISA results with the same antibodies in PBS.

Toxin binding performance of antibodies in real sample extracts is of crucial importance. In the next step characterization, we evaluated the interaction of the antibodies with low concentrations of AFB1 in food extracts. MAbs were tested for their reactivity with 0.1 to 20 ng/mL AFB1. AF free corn, red pepper and hazelnut extracts or 70% Methanol were spiked with 9.1 ng/mL AFB1. The spiked extracts were pre-incubated with the antibodies and matrix effects are evaluated by indirect competitive ELISA. The antibodies incubated with PBS were used as positive

control and the results are presented as antibody binding ratio (%) with respect to PBS incubated antibodies. The resulting binding inhibition curves are presented in Figure 4. 18. The results showed that MAM-D12E2 and MAM-8G8 MAbs effectively bound to the AFB1 in different matrices at less than 2 ng/mL concentration (Figure 4. 18 a and d). MAM-2B11 also interacted with free AFB1 in methanol and food extracts, however, binding inhibition curves showed less effective interaction of MAM-2B11 with free AFB1 (Figure 4. 18 b). This is proposed as the consequence of lower affinity of 2B11 to AFB1. The study indicated that MAM-D12E2 and MAM-8G8 MAbs can bind to AFB1 more effectively when compared to MAM-2B11 in real samples. MAM-D3E4 failed to interact with low concentrations of AFB1 used in this study, and cross-reactivity of the antibody with corn and red pepper extracts was confirmed by the inhibition of the antibody in these extracts while it is not inhibited by AFB1 in methanol or hazelnut (Figure 4. 18 c).

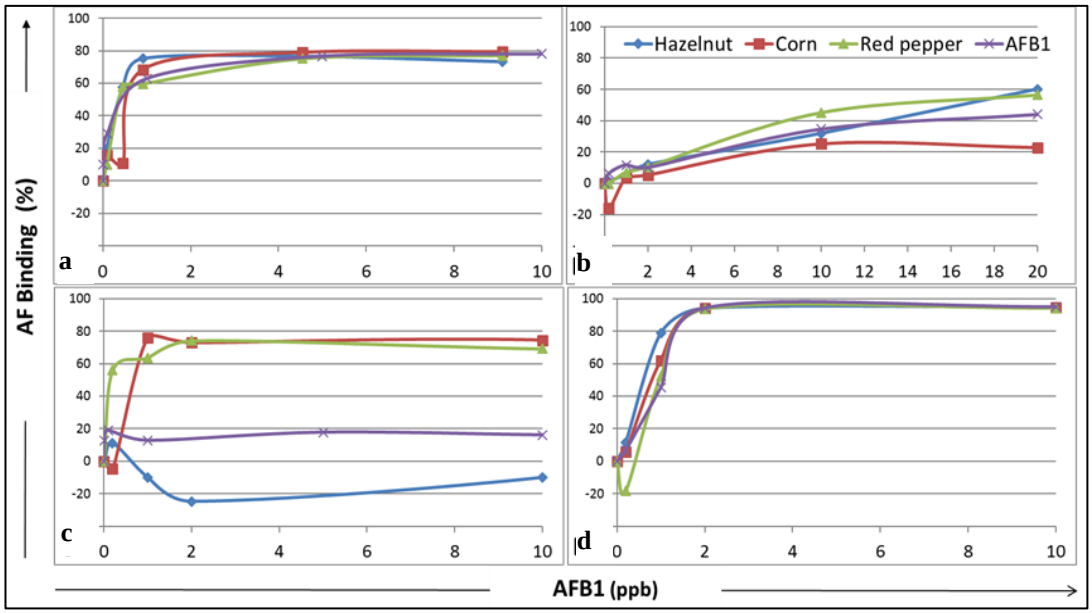

Figure 4. 18: The results of the IC-ELISA showing the interaction of antibodies with AFB1 in 70% methanol or food extracts spiked with different concentrations of AFB1. Antibody binding as presented with respect to noncompetitive binding of antibodies in PBS. Binding inhibition curves of a) MAM-D12E2 MAb, b) MAM-2B11 MAb, c) MAM-D3E4 MAb and d) MAM-8G8 MAb.

4.1.5. Antibody Purification

Antibody isotype is critical for the selection of the purification method. In this thesis, we used one IgG1 (MAM-8G8), one IgG2b (MAM-2B11), one IgA (MAM-D12E2) and one IgM (MAM-D3E4) antibody. Each antibody required a different strategy for efficient purification where IgG MAbs were purified using Protein A affinity chromatography and IgA and IgM antibodies were purified with chromatographic methods based on molecular weight and/or net charges of the antibodies.

4.1.5.1. Affinity Purification of IgG Antibodies

IgG isotype antibodies can be purified in one step using protein A/G purification columns which specifically bind to the Fc portions of immunoglobulins. Although protocols for antibody purification with this method is established, customization of the protocol for antibodies is necessary and sub-isotype differences determine the purification protocol to be used for each isotype [114].

Optimal loading and elution conditions for protein A purification were established for 8G8 and 2B11 antibodies. The interaction of protein A with immunoglobulins is stronger at high pH values, ideally at pH 8-9 and at high ionic strength (>1 M NaCl). However, milder conditions for loading will prevent exposure of antibodies to extreme conditions.

8G8 antibody could be purified at high pH and high ionic strength loading conditions, using loading buffer 1 (1 M Tris, pH: 9, 3 M NaCl) and eluted with 0.1 M glycine pH: 2.7. Purification chromatogram for 8G8 antibody is presented in Figure 4. 19.

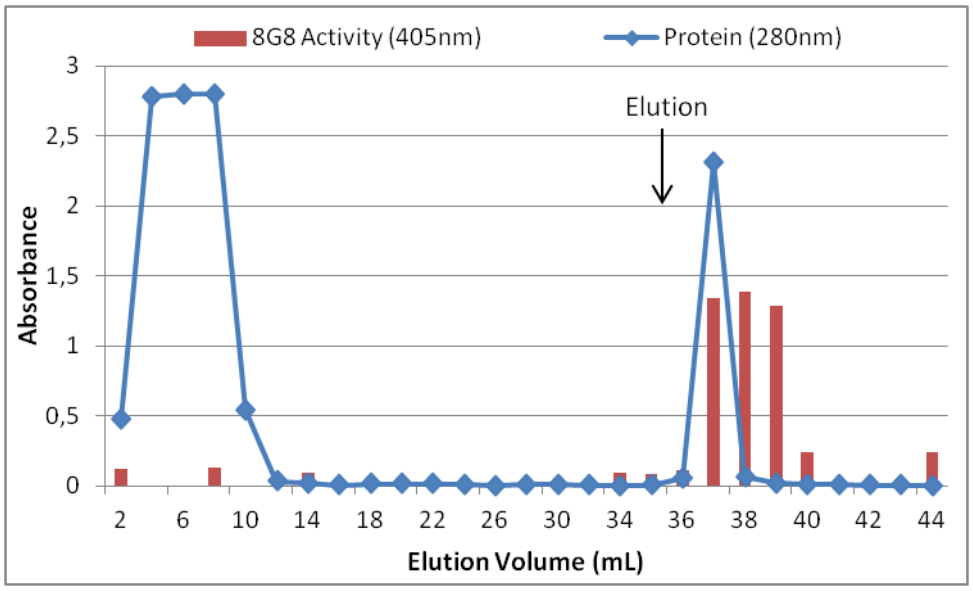

Figure 4. 19: Protein A chromatography for the purification of 8G8 antibody.

2B11 could successfully be purified with milder purification conditions. The antibody was loaded to the protein A column with loading buffer 2 (20 mM K2HPO4, pH: 7) and eluted with 0.1 M glycine pH: 2.7. The purification chromatogram for 2B11 is presented in Figure 4. 20.

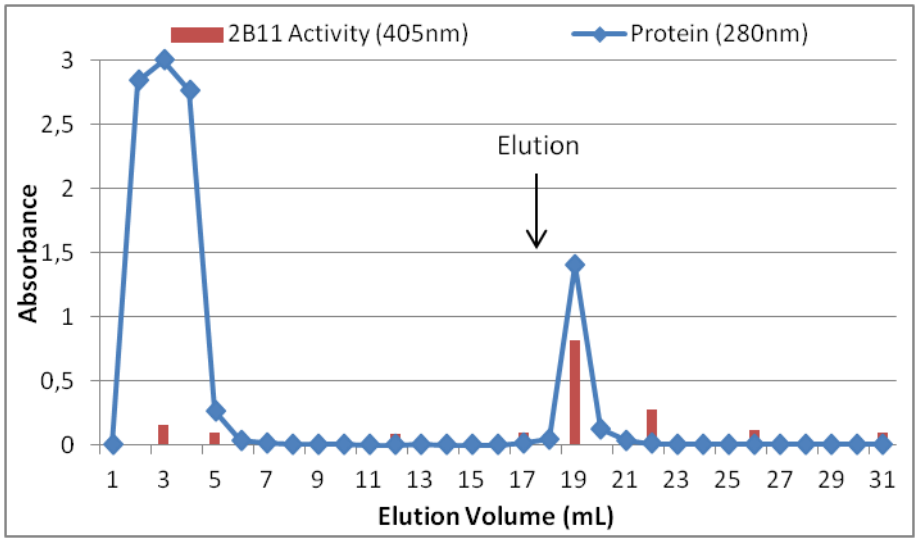

Figure 4. 20: Protein A chromatography for the purification of 2B11 antibody.

4.1.5.2. Purification of IgA Antibody

IgG antibodies were purified with protein A affinity chromatography however; IgA antibodies cannot be purified with the same method, since they do not have affinity to protein A/G. So, a robust protocol should be optimized for its purification of D12E2 based on classical chromatography methods. In the present work, we optimized a one-step purification scheme for D12E2 antibody using ion exchange chromatography. DEAE-C Cellulose column was used for the purification of the antibody. AS precipitated antibody was loaded to the column in 10 mM phosphate buffer, pH: 7 with 150 mM NaCl where most of the serum proteins were eluted and antibody was bound to the column. The bound antibody was eluted at the same pH, with 350 mM NaCl concentration. Figure 4. 21 shows the chromatogram of the purification procedure.

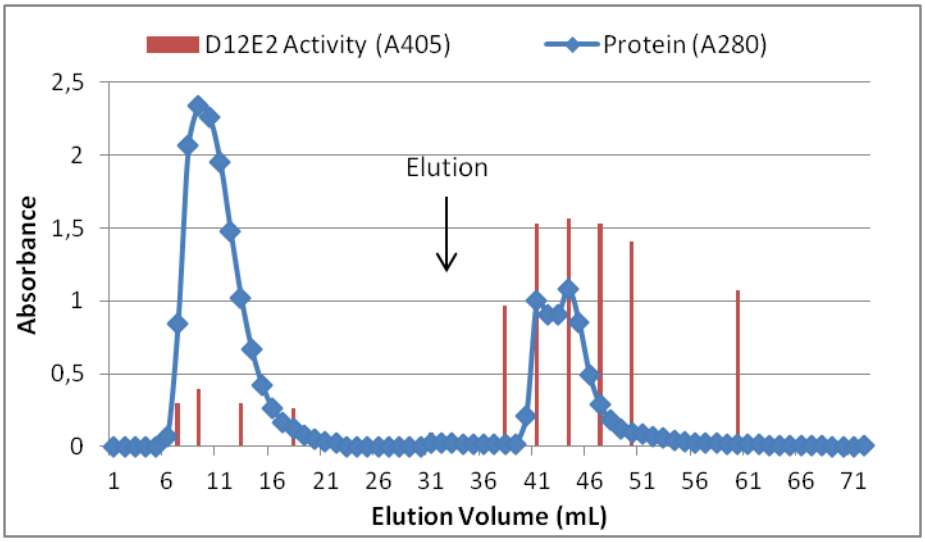

Figure 4. 21: DEAE-C ion exchange chromatogram for the purification of D12E2 antibody.

4.1.5.3. Purification of IgM Antibody

Sequential application of size exclusion chromatography and ion exchange chromatography was required to achieve sufficient purification of IgM isotype MAM-D3E4 antibody. AS precipitated antibody was used in the purification procedure.

Size exclusion chromatography is an analytical method used to separate molecules of different molecular sizes. In this method, low molecular weight proteins are retained in the pores of the chromatographic resin where high molecular weight proteins move faster by omitting the pores of the resin. So, the early chromatographic fractions contain high MW proteins and small molecules are collected in the later fractions. This method was preferred to separate 960 kDa IgM antibody from the other serum components. In order to separate IgM isotype MAM-D3E4 from the serum components, Sephacryl 300 (S300) was used as chromatographic resin.

When 1 mL of MAM-D3E4 AS was loaded to sephacryl S300 column with 0.5 mL/min flow rate in PBS, two peaks were obtained. The antibody was eluted in the first peak which consists of higher MW proteins. However, high diffusion constants of IgM antibodies resulted in low resolution of the peaks and the antibody could not be fully separated from other proteins [115]. Additionally, the antibody was obtained in a large volume of chromatographic eluate (Figure 4. 22). So, the antibody containing fractions of S300 chromatography was pooled and subjected to a second step of purification with DEAE-C ion exchange chromatography in order to concentrate the antibody and achieve a higher purity.

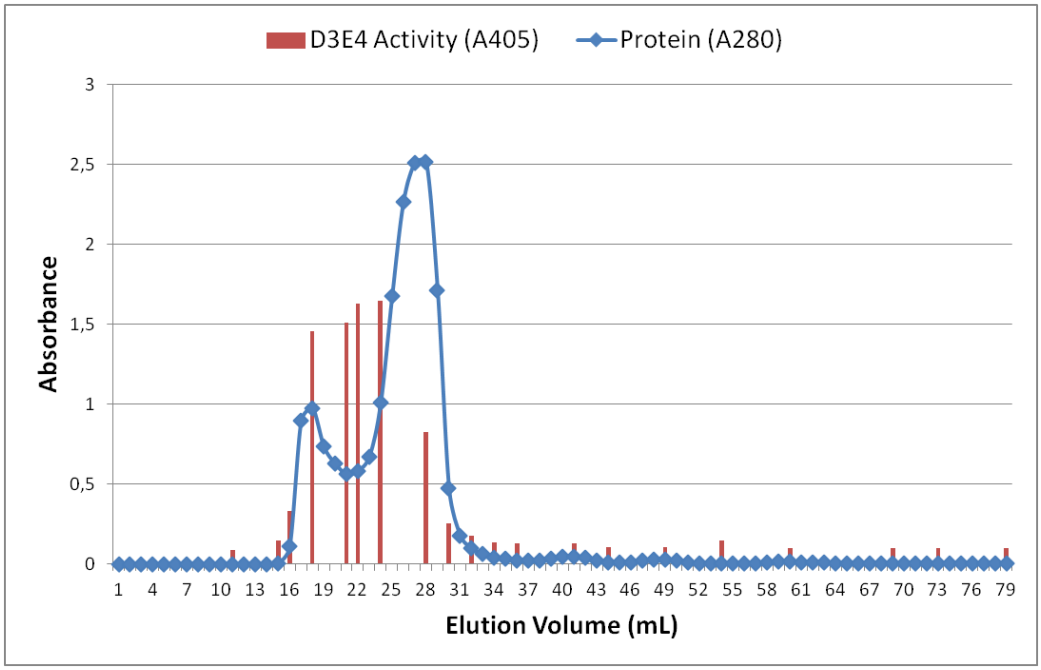

Figure 4. 22: Sephacryl S300 size exclusion chromatogram for the purification of MAM-D3E4 antibody.

The collected fractions containing MAM-D3E4 MAb which was in PBS was loaded to DEAE-C anion exchange column. Bound antibodies were eluted with 10 mM K2HPO4, pH: 7, 250 mM NaCl, and remaining protein in the column was eluted with 10 mM K2HPO4, pH: 7, 500 mM NaCl. The resulting chromatogram is presented in Figure 4. 23. Three chromatography peaks were obtained from the procedure where only the second peak, eluted with 250 mM NaCl, contained the antibodies (Figure 4. 23) which both concentrated the antibodies to a smaller volume and provided a better separation from serum proteins.

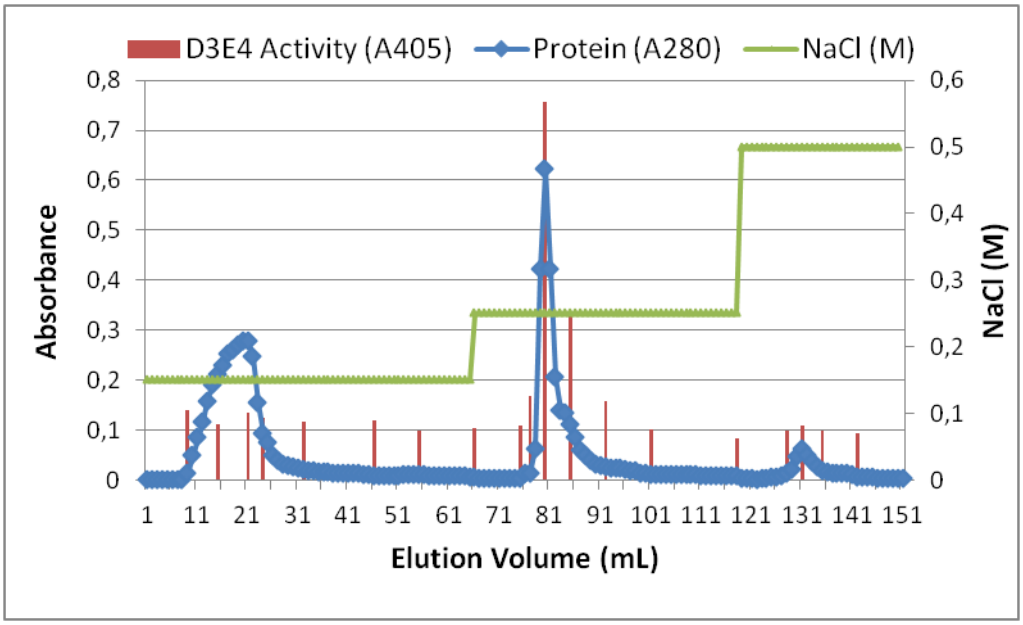

Figure 4. 23: DEAE-C anion exchange chromatogram of MAM-D3E4 antibody Sephacryl S300 fractions.

4.1.5.4. Antibody Purity Assessment

The resulting purified antibodies were analyzed by SDS PAGE and western blot which is followed by an immunoblot aiming to visualize the antibodies transferred to the membrane. In the SDS PAGE and Western blot analysis, 2 µg protein was loaded from either protein AS precipitates or purified antibodies. Analysis was done in non-reducing conditions so that antibody subunits will not be separated and antibodies are observed as one high MW band. SDS PAGE analysis shown in Figure 4. 24 shows the exact same amount of protein that was transferred to the PVDF membrane for immunoblot analysis shown in Figure 4. 25.

SDS PAGE analysis showed the BSA contamination at 66 kDa and antibody bands are observed at high MW as faint bands in unpurified antibody AS precipitates at lanes 1 (MAM-D3E4 AS), 4 (MAM-2B11 AS), 6 (MAM-D12E2 AS) and 8 (MAM-8G8 AS). Semi-purified MAM-D3E4 MAb after size exclusion chromatography can be seen in lane 3 in Figure 4. 24. Purified antibody solutions can be seen at lanes 2 (MAM-D3E4), 5 (MAM-2B11), 7 (MAM-D12E2) and 9 (MAM-8G8) where single strong antibody bands are observed. Western blot analysis presented in Figure 4. 25 showed that the bands observed at high MW were antibody bands.

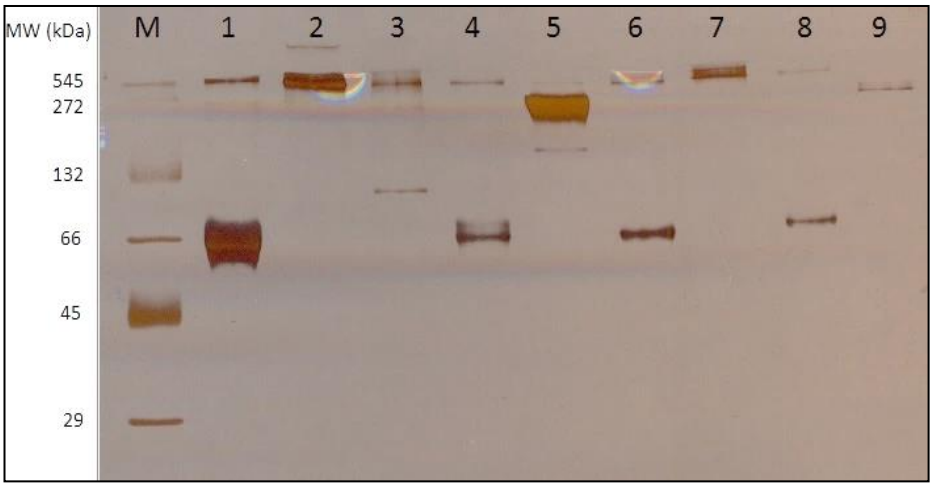

Figure 4. 24: SDS PAGE analysis of purification fractions.
M: SDS PAGE marker (Sigma MWND500), Lane 1: MAM-D3E4 AS, Lane 2: DEAE-C Purified MAM-D3E4, Lane 3: S300 purified MAM-D3E4 Lane 4: 2B11 AS, Lane 5: Purified 2B11, Lane 6: D12E2 AS Lane 7: Purified D12E2, Lane 8: 8G8 AS, Lane 9: Purified 8G8.

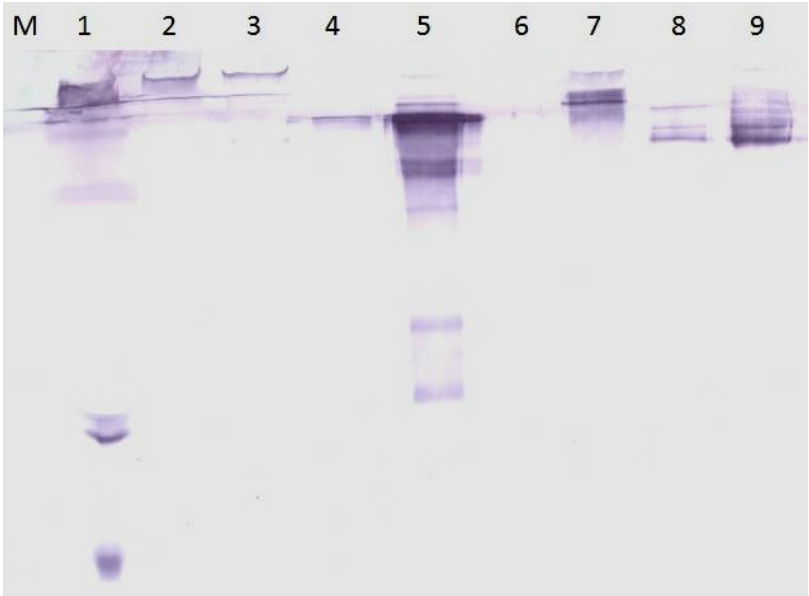

Figure 4. 25: Immunoblot analysis of purification fractions.
The antibodies were visualized with AP labeled anti-mouse polyvalent antibody. M: SDS PAGE marker (Sigma MWND500), Lane 1: MAM-D3E4 AS, Lane 2: DEAE-C Purified MAM-D3E4, Lane 3: S300 purified MAM-D3E4 Lane 4: 2B11 AS, Lane 5: Purified 2B11, Lane 6: D12E2 AS Lane 7: Purified D12E2, Lane 8: 8G8 AS, Lane 9: Purified 8G8.

4.2. Immunoaffinity Column Development

In the development of immunoaffinity chromatography systems, the antibody should irreversibly bind to the resin, retain the antigen effectively upon loading and release it under proper elution conditions. This working scheme will only be successful with the choice of the right antibody and its effective immobilization.

Antibody development and selection of antibodies to be used for the comparison of different antibody isotypes in IAC system was presented in chapter 4.1. Antibody Development. Antibody affinities directly impact the performance of the test system. So, in this chapter, antibodies with similar affinity to AFB1, MAM-8G8 and MAM-D12E2, were selected for the development of IACs in order to elucidate the effect of antibody isotype in the performance of IACs. For this purpose, protein binding to the selected resin, CNBr activated sepharose, was optimized. Then, antibodies were immobilized to the resin and IAC performances were compared in terms of quality standards set by AOAC [15] where total AF binding capacity and the ability to bind limited amount of AFs were evaluated.

4.2.1. Optimization of Resin Immobilization

The optimization of protein binding to CNBr activated sepharose was achieved using BSA as model protein. Critical parameters were tested to determine the optimal immobilization conditions. Critical parameters that were used in binding optimizations are as follows:

i) The resin is provided in a powder form where sugar is used as stabilizing agent. The sugar is washed away from the resin with 0.1 M HCl. Washing step is done at pH<3 since the azide coupling group is not active at low pH.
ii) After clearance of the resin from the sugar molecules pH is increased and resin is therefore activated. There are two common approaches for this. In one approach, initially HCl is washed away from the resin with water and then the resin is washed with coupling buffer. The second approach omits water washing step. Since the activated resin is prone to immediate nonspecific

reactions, this step is tested as a part of optimization to determine whether water washing step is necessary for efficient removal of HCl.

iii) Each binding reaction was performed using 0.05 g CNBr activated sepharose and 0.5 mg BSA. The same amount of BSA is used in 1 mg/mL or 2 mg/mL concentration to test the effect of protein concentration on binding efficiency.

iv) Duration and temperature of protein-resin binding is optimized.

The result of the optimization study revealed that there are two critical factors affecting the efficiency of protein binding; incubation temperature and protein concentration. Accordingly;

i) Excessive washing of the resin with HCl was shown to increase the binding efficiency; however, it may lead to the loss of resin. So, excessive washing can be applied in case necessary precautions are taken to prevent resin loss (Table 4. 7, columns A and B).

ii) Water washing after HCl treatment decreased the binding efficiency (Table 4. 7, columns A and C).

iii) Protein concentration was determined to be the most important criteria in binding efficiency where high protein concentration leads to higher immobilization efficiency (Table 4. 7, columns A and F).

iv) Binding at room temperature is more effective than incubation of the samples overnight at +4 °C. The duration of binding at room temperature, 2 h or 4 h, does not affect binding efficiency (Table 4. 7, columns A, D and E).

Table 4. 7: Optimization of CNBr activated sepharose-BSA binding.

Critical steps	A	B	C	D	E	F
Washing (1 mL 0.1 M HCl)	10 X	20x	10 X	10 X	10 X	10 X
Washing (1 mL dH_2O)	+	+	-	+	+	+
BSA in coupling buffer	0.5 mL (1mg/mL)	0.5 mL (1mg/mL)	0.5 mL (1mg/mL)	0.5 mL (1mg/mL)	0.5 mL (1mg/mL)	0.25 mL (2mg/mL)
Incubation with shaking	2 hours	2 hours	2 hours	4 hours	o/n +4 °C	2 hours
Column volume	100	90	100	100	100	100
A280 (before binding)	0.665	0.665	0.665	0.665	0.665	1.306
A280 (after binding)	0.493	0.488	0.479	0.492	0.533	0.702
Binding efficiency (mg/mL)	1.29	1.47	1.39	1.30	0.99	2.26

4.2.2. Immunoaffinity Column Preparation

The binding procedure was optimized with BSA; however, the protocol should be proven to be useful for the antibodies as well. Selected MAbs should bind to the IAC resin effectively, and be able to bind AFs after immobilization. We used Purified MAM-D12E2 and MAM-8G8 to prepare IACs for comparative studies. Equal amount of MAbs were bound to CNBr activated sepharose for IAC development. MAb quantification was done spectrophotometrically using the determined extinction coefficients for each antibody.

2.6 mg of each antibody was bound to 1.25 mL of resin. Efficiencies of the antibody coupling reactions are presented in Table 4. 8. Each antibody was successfully bound to the resin with more than 90% efficiency. Resulting resins contained 1.95 mg/mL antibody for MAM-D12E2 and 2.01 mg MAM-8G8 MAb / mL resin.

The antibody bound resins were used to prepare IACs that contain 150 µg antibody per column.

Table 4. 8 Sepharose binding efficiencies of MAM-D12E2 and MAM-8G8 MAbs.

	Antibody	
	MAM-D12E2	MAM-8G8
$\varepsilon^{\%0,1}$	0.65	0.4
A280 (before coupling)	1.29	0.8
A280 (after coupling)	0.188	0.071
Starting MAb in solution (mg)	2.58	2.60
Final MAb in solution (mg)	0.14	0.09
Binding efficiency (%)	94.4	96.6
Bound MAb (mg/mL resin)	1.95	2.01

4.2.3. Overload Test

Aflatoxin binding capacities of the prepared columns was evaluated by overflow test. 500 ng of each AF were loaded to the columns in separate occasions and total binding capacities of the columns were determined by HPLC analysis for each AF isotype. The overflow test results showed that the IACs prepared with MAM-D12E2 and MAM-8G8 antibodies were both able to bind all four AF types when immobilized to the columns.

The columns prepared with IgA antibody could bind 23.8, 24.4, 25.09 and 24.89 ng AFB1, AFB2, AFG1 and AFG2, respectively. The columns prepared with IgG antibody could bind 23.38, 26.8, 22.91 and 44.4 ng AFB1, AFB2, AFG1 and AFG2, respectively. Two IACs showed similar performance for AFB1, AFB2 and AFG1. However, IACs prepared with IgG isotype MAM-8G8 antibody withheld twice as much AFG2 when compared to IACs prepared with IgA isotype D12E2 antibody (Figure 4. 26).

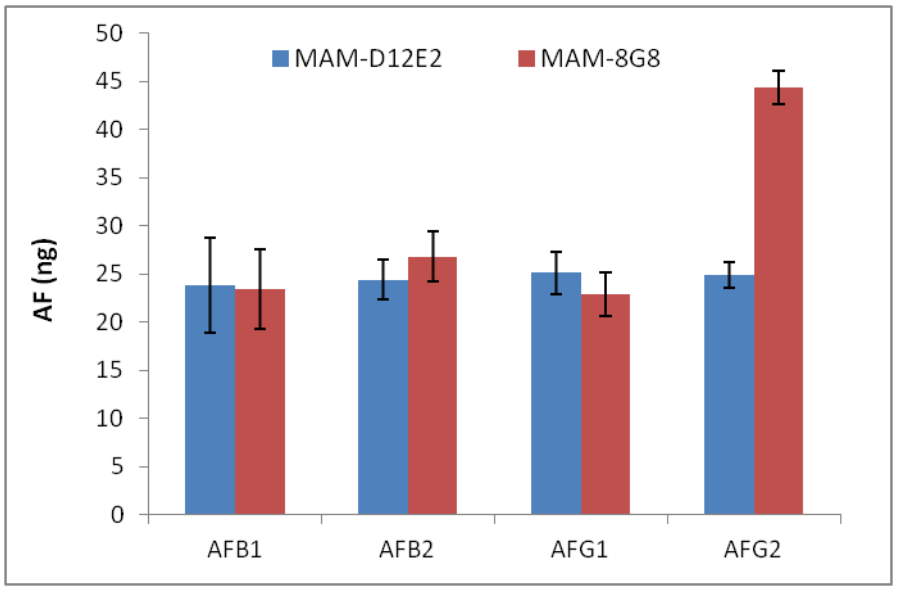

Figure 4. 26: Total AF binding to MAM-D12E2 and MAM-8G8 IACs.
Total binding capacities of the IACs were evaluated for AF B1, B2, G1 and G2.
Error bars represent standard errors of 3 independent replicas.

4.2.4. Limit Binding Test

Limit binding test was conducted using 16% methanol-water solution contaminated with a mixture of AFB1, AFB2, AFG1 and AFG2 so that 5 ng of each toxin was loaded to the columns. The percentage of the recovered AFs was used for evaluation. The limit binding test results showed that the binding performance of the immunoaffinity columns prepared with IgG and IgA MAbs showed the same performance, where full recovery of toxins were observed with AFB1 and AFG1 and more than 75% recovery for AFB2 and AFG2.

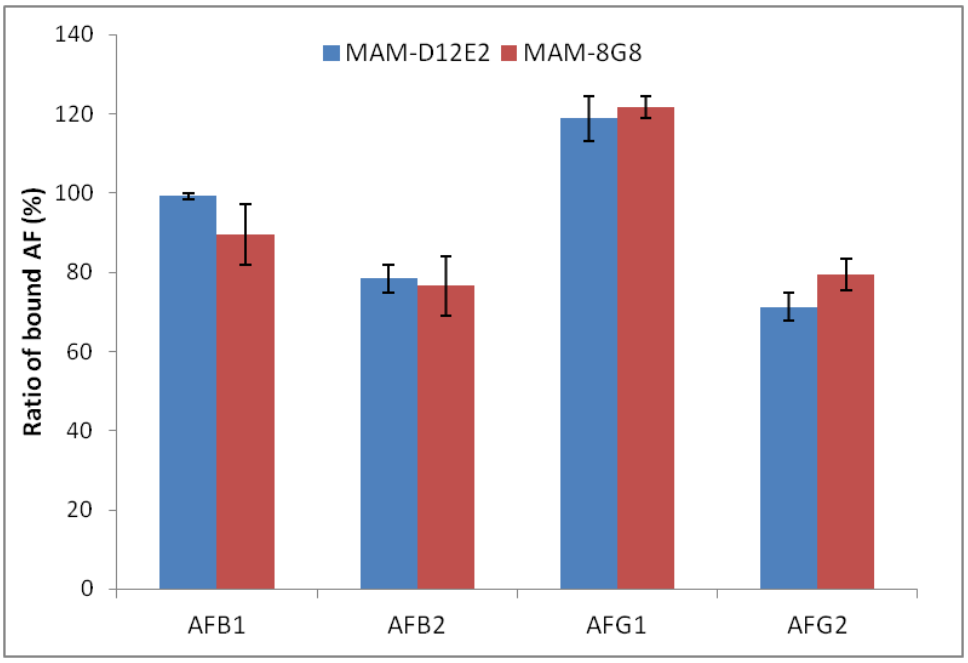

Figure 4. 27: Limit binding test performed with loading 5 ng of each AFB1, AFB2, AFG1 and AFG2 to MAM-D12E2 and MAM-8G8 IACs. Recovery rates of AFs from the IACs were evaluated. Error bars represent standard errors of 3 independent replicas.

4.3. Biosensor Development

IgA isotype MAM-D12E2 and IgG isotype MAM-8G8 MAbs were comparatively evaluated for the development of a highly sensitive QCM immunobiosensor for the detection of aflatoxin (AF) in inhibitory immunoassay format. For this aim, sensor surface was prepared by covalent immobilization of AFB1 on gold coated quartz crystal. AFB1 immobilized surface was chemically blocked without any need for protein blocking. Regeneration and assay conditions were optimized to evaluate the sensitivities of the two antibodies.

4.3.1. Surface Preparation

Reactive groups were introduced to the gold surface by the use of 11-MUA SAM. Carboxylic acid groups of 11-MUA were converted to amine in order to provide binding sites for carboxyl bearing AFB1-Oxime. The reaction involved activation of the carboxyl groups with EDC and NHS prior to the binding of EDA. The surface was activated with EDC/NHS solution for either 10 or 15 minutes to evaluate the effect of duration on nonspecific binding of proteins to the prepared sensor surface. The frequency changes upon application of 0.1 mg/mL BSA were 4 Hz on 10 minutes activated surface and 9.75 Hz on 15 minutes activated surface (Table 4. 9). It was shown that when the duration of EDC/NHS activation was extended from 10 min to 15 min, nonspecific binding increased by two fold.

Table 4. 9: Effect of different durations of EDC/NHS activation on non-specific protein binding to the MUA coated QCM crystals (uncertainty values are presented as standard deviations).

EDC/NHS application time (min)	Frequency change upon 0.1 mg/mL BSA application (Hz)
10	4.0 ± 1.4
15	9.75 ± 1.8

Free amine groups on the prepared surface were blocked with carboxylic acid in acetate buffer. Frequency shift resulting from 0.1 mg/mL BSA solution was used

to demonstrate the blocking efficiency of the surface. Amine activated surface was treated with 60 mM acetate buffer, pH: 4 or 1 M acetate buffer, pH: 4.8 to evaluate their blocking efficiencies. Additionally, carboxylic acid groups of 60 mM acetate buffer were activated with EDC/NHS and used as blocking agent.

Figure 4. 28 presents nonspecific protein binding after different blocking conditions. In control crystal, the surface bears many unblocked highly reactive amine groups. Application of acetate buffer, either 60 mM, pH: 4 or 1 M pH: 4.8 significantly reduced nonspecific binding to the crystals. Nevertheless, when carboxyl group of acetate was activated with EDC/NHS solution in order to improve the blocking efficiency, the blocking property of the solution completely vanished. This result was strongly correlated with the previous finding that the duration of EDC/NHS activation of MUA increased the nonspecific reactivity of the surface. We consider the reason for the extreme reactivity is the retreatment of the surface with EDC/NHS solution.

Application of activated acetate can be considered as a simulation of AF-oxime binding, so we cannot avoid the exposure of the surface to EDC/NHS solution. We considered controlling nonspecific reactivity of the surface by lowering the EDC/NHS reaction pH from 7 which falls within optimal working pH range of EDC/NHS activation to the suboptimal pH: 4, but no significant difference was observed in terms of nonspecific binding. Considering the better reproducibility and stability of the surface blocked with 1 M Acetate buffer, pH: 4.8, this condition was chosen for blocking of AFB1-oxime bound crystals.

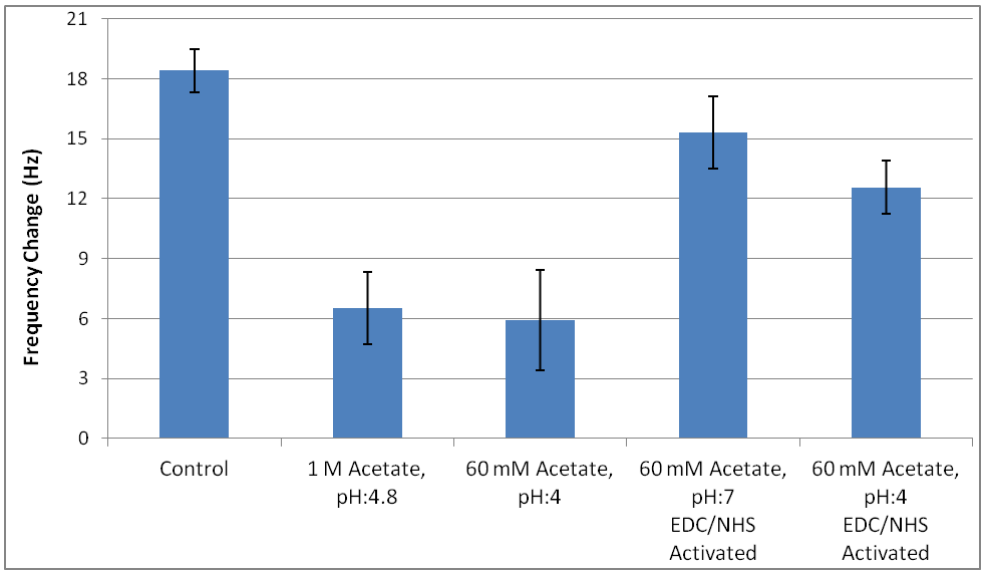

Figure 4. 28: Blocking optimization on amine activated surface.
Non-specific protein binding to sensor surface after blocking with 1 M acetate buffer, pH: 4.8, 60 mM acetate buffer, pH: 4 or EDC/NHS activated 60 mM acetate buffer. Error bars represent standard errors.

4.3.2. Inhibitory Immunoassay Using IgA or IgG Antibodies

Changing concentrations of AFB1 (5 ng/mL – 40 ng/mL) was mixed with equal volume of 0.1 mg/mL IgA isotype MAM-D12E2 antibody or IgG isotype MAM-8G8 antibody with similar affinity to AFB1. The mixture was delivered to the AF immobilized sensor surface and frequency shifts were recorded (Figure 4. 29). Maximal frequency shifts observed in control samples with no AFB1 competition were different in two antibodies as an indication of antibody structure. IgG isotype antibody (150 kDa) produced 32 Hz and IgA isotype MAM-D12E2 antibody (340 kDa) produced 55 Hz control sensor response. When we examined the frequency changes in AFB1 competition samples with respect to control samples, we showed that higher control sensor response was correlated with greater sensor signal (Table 4.10). MAM-D12E2 showed 3 times as much frequency difference with respect to control at 5-20 ng/mL AFB1 competition and twice as much frequency difference at 40 ng/mL AFB1 competition when compared to MAM-8G8. The sensor was developed using the IgA antibody due to its broader AFB1 competition range.

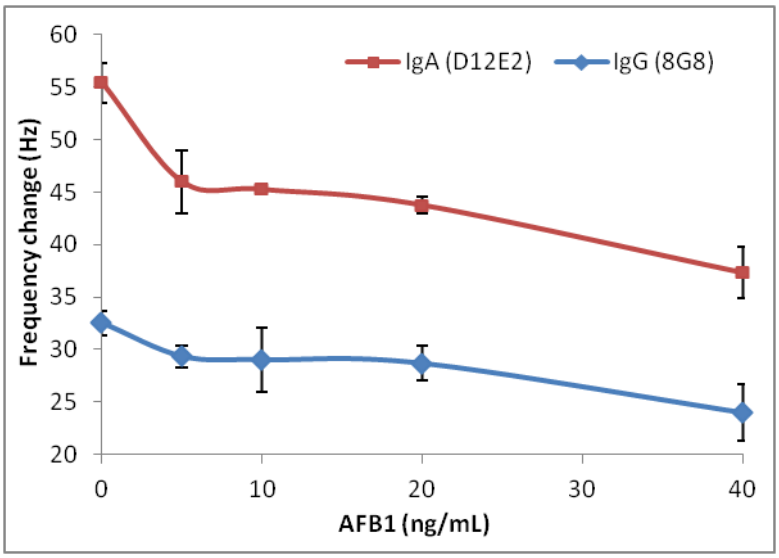

Figure 4. 29: Inhibitory AFB1 analysis with MAM-8G8 and MAM-D12E2 MAbs using AFB1 immobilized sensor chip (0-40 ng/mL AFB1). Error bars represent the standard errors.

Table 4. 10: The frequency difference between control and competition samples for MAM-8G8 and MAM-D12E2.

AFB1 (ng/mL)	MAM-8G8 (0.1 mg/mL)	MAM-D12E2 (0.1 mg/mL)	Fold difference (MAM-D12E2/MAM-8G8)
5	3.2 Hz	9.4 Hz	3.0
10	3.5 Hz	10.2 Hz	2.9
20	3.8 Hz	11.7 Hz	3.0
40	8.5 Hz	18.1 Hz	2.1

4.3.3. Sensor Surface Regeneration

Recovery of MAM-D12E2 antibody from sensor surface using different regeneration solutions is presented in Figure 4. 30. MAM-D12E2 antibody binding can be fully recovered with 50mM NaOH and 1% SDS. The antibody could not be regenerated using solutions that disrupt ionic bonds, but efficiently regenerated with a strong ionic detergent. This result is the indication of a hydrophobic interaction of the antibody with AF.

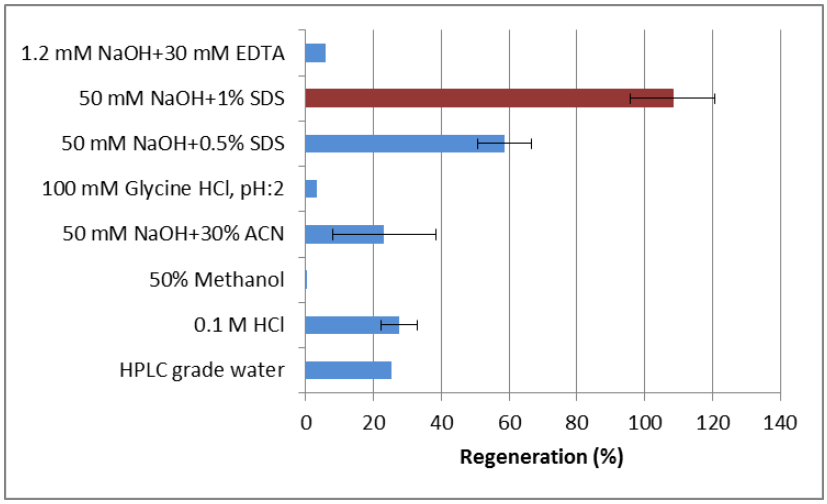

Figure 4. 30: Regeneration of AFB1 immobilized sensor surface after MAM-D12E2 application using different solutions. Results are presented as the percentage of the antibody recovered from the surface, calculated with binding and regeneration frequency shifts.

Reproducibility of regeneration was tested with sequential injections of 0.1 mg/mL MAM-D12E2 antibody to the regenerated surface. The surface could be regenerated at least 9 times without significant loss of performance (Figure 4. 31). We observed that number of regeneration cycles could be improved when we wash the surface with water after the application of regeneration solution, before PBS injection.

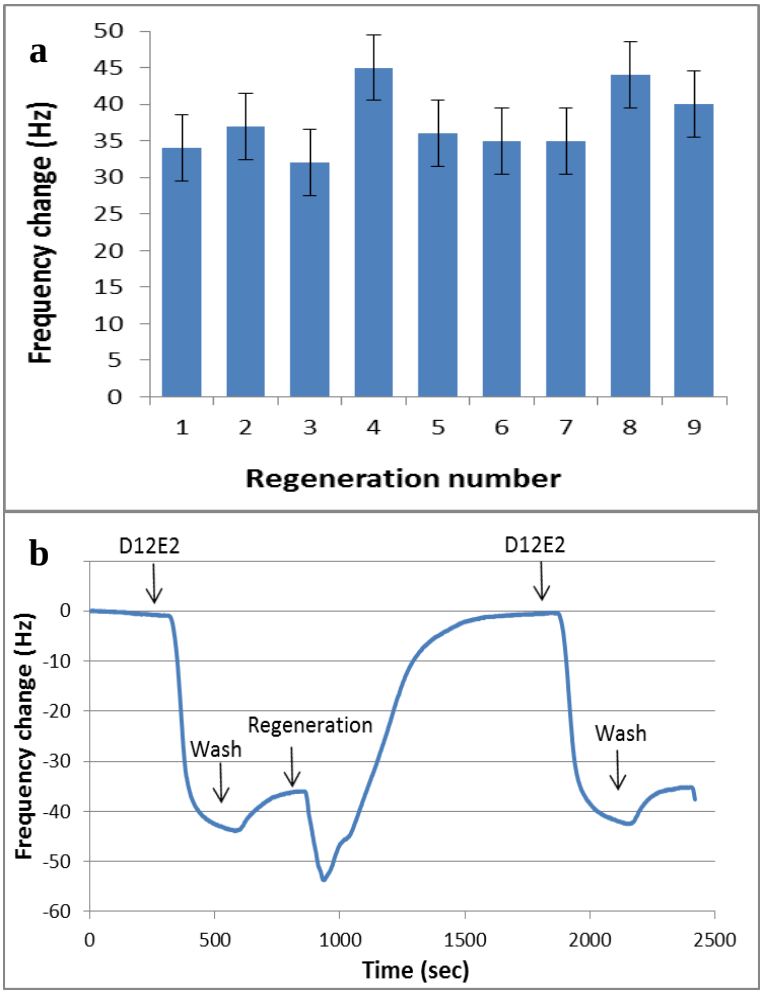

Figure 4. 31: Regeneration of AF immobilized sensor surface.
a) Sequential regeneration of the sensor surface with 50 mM NaOH and 1% SDS. Graph shows sensor response as frequency change upon delivery of 0.1 mg/mL MAM-D12E2 antibody after each regeneration. b) Sensorgram showing a typical regeneration cycle with MAM-D12E2 antibody.

4.3.4. Sensor Development with Monoclonal IgA Antibody

After the determination of the antibody to be used as sensing element and optimization of the assay conditions including regeneration and competition, we generated a standard curve for AFB1 detection using MAM-D12E2 antibody. In the work conducted for the choice of sensing element, high concentrations of AFB1 were chosen in order to be able to evaluate the working competition range for each antibody. The sensograms showing the frequency shifts upon application of different AFB1 concentrations in inhibitory immunoassays is presented in Figure 4. 32 a. Dose response curve showing the sensor response to different AFB1 concentrations

is shown in Figure 4. 32 b. The results show that, the surface is responsive to the inhibition assay conducted with IgA isotype MAM-D12E2 antibody and AFB1. Limit of detection was shown to be 1.25 ng/mL with 1.25 ng/mL - 10 ng/mL detection range.

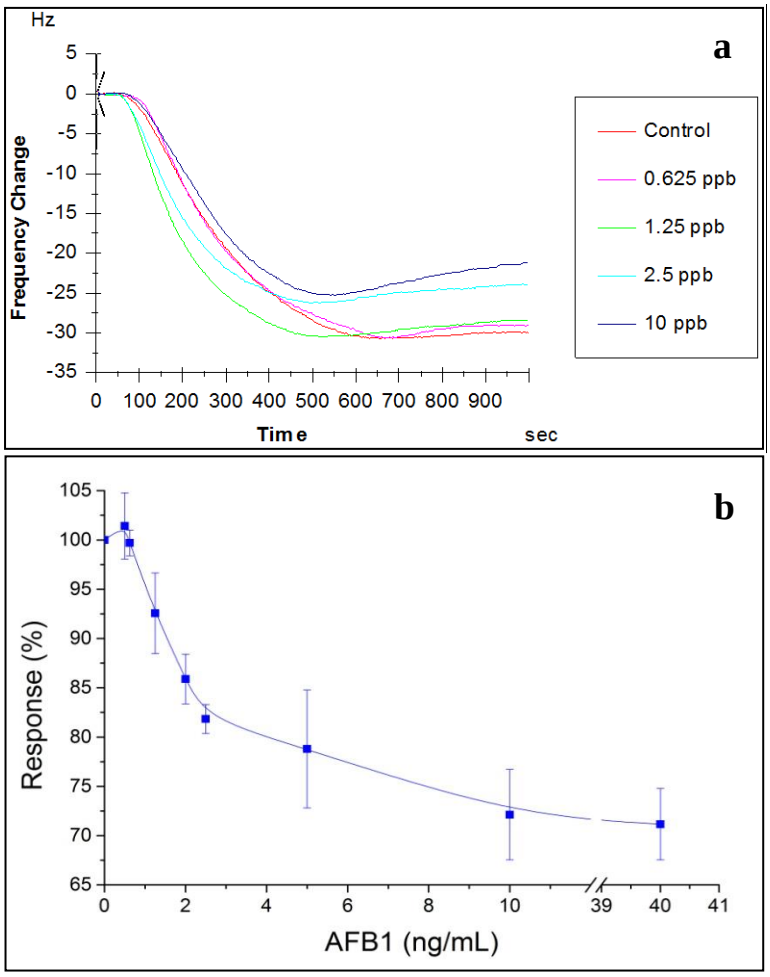

Figure 4. 32: AFB1 sensor response with IgA antibody.
a) Sensograms showing the sensor response at different concentrations of AFB1.
b) Sensor response curve for AFB1 detection. Error bars represent standard errors.

5. DISCUSSION

In this thesis, we aimed to elucidate the effect of antibody isotype used in the development of immunoanalytical systems on their performance. The work towards this aim was conducted in three stages;

i) Antibody development
ii) Comparative studies in IAC development
iii) Comparative studies in Biosensor development

We gained several perspectives from each stage, not only related with the isotype effects on the system performance, but also related with antibody development including generation of hapten specific immune response, antibody characterization and purification and the surface and regeneration properties of sensors. The discussions of all insights gained from the thesis are presented with three main sections; antibody development, IAC development and biosensor development.

5.1. Antibody Development

Antibodies against AFs are important part of AF detection systems where they are utilized for the development of various AF immunoassays. Aflatoxin is a hapten molecule which should be conjugated to a bigger immunogenic molecule in order to raise antibody response in mammals. In this study, we used two different carrier proteins, OVA and TF, for the immunization of mice which was used for monoclonal antibody development. Both immunogen preparations raised strong AF specific immune response, however, when we used TF as immunogenic carrier, we observed earlier AF specific antibody response. TF is known as a carrier for intracellular drug delivery since every mammalian cell contains TF receptors [116–118]. However, when conjugated to drugs with hapten structure, such as peptides, it has been demonstrated that, the efficiency of the drug delivery decreases and a strong anti-hapten immune response is generated [119]. Although there are numerous reports indicating the use of TF in drug delivery and drug specific immunogenicity, proteins

such as IgG, OVA or BSA were commonly used as carriers for hapten immunization [120–122] and there are few studies published presenting the use of TF for hapten immunization [123,124].

In this work, we demonstrated the efficiency of TF as carrier protein in AF immunization to obtain earlier immune response from the experimental animal. Monoclonal antibodies were developed successfully using mice immunized with either of the carrier molecules.

We developed four AF specific monoclonal antibodies; MAM-D3E4, MAM-2B11, MAM-8G8 and MAM-D12E2; and characterized them in order to determine their adequacy for AF immunoassay development.

The initial criteria for selection of the antibody producing hybridoma clones was specificity, where the produced antibodies should be able to interact with free AF isotypes but not interact with proteins used in immunization or other mycotoxins. This step was demonstrated with IC-ELISA, with 1µg of each toxin, which is excess with respect to the highest possible antibody amount in cell culture supernatants used in the study. Hybridoma clones producing specific antibodies were monitored for cell growth and antibody production stability, where chromosomal instability of the clones could result in loss of antibody production [113]. Stable hybridomas were cloned into monoclonal cell lines.

When monoclonal cell lines were established, produced antibodies were evaluated for solvent tolerance and extract cross-reactivity. For an antibody to be effectively utilized in aflatoxin analysis it needs to be tolerant to solvents used in extraction process and should not cross-react with the plant metabolites present in the extract.

The results of solvent tolerance experiments showed us the secure limits of solvent concentrations that can be used in the assay development, including loading of toxins to the IACs or use in biosensor systems. In addition to this information, the data indicates the safe elution conditions of the developed IACs as well. ELISA based optimization of elution conditions for different antigens were previously presented for protein antigens [125,126] where mainly aqueous buffers were evaluated. AFs retained in IACs are commonly eluted with 100% methanol in commercial IACs. Our results indicated that acetonitrile can be considered a good HPLC compatible choice for complete elution of the bound AFs. MAM-2B11,

MAM-8G8 and MAM-D12E2 MAbs showed high solvent tolerance where MAM-D3E4 was sensitive to solvents.

The developed antibodies are intended to be used in food extracts prepared according to directives which basically includes homogenization of the food samples and mixing them with organic solvents [106]. So, interaction of the developed antibodies with the extracts will lead to blocking of AF binding sites with the food metabolites and reduced performance in AF binding. MAM-2B11, MAM-8G8 and MAM-D12E2 MAbs showed low cross-reactivity with the extracts, and interacted with AFB1 they encounter in the food extracts. However, MAM-D3E4 strongly cross-reacted with corn and red pepper extracts. MAM-D3E4 also failed to interact with low concentrations of AFB1 in food extracts. Low solvent tolerance and high extract cross-reactivity of MAM-D3E4 MAb made the antibody unsuitable to be used in AF analysis.

Most of the antibodies require to be purified prior to use. Purification step both increases the production costs and inversely affects the activity of the antibody [127], so the availability of a feasible purification scheme is another important criteria. Antibodies were successfully purified and their activities were demonstrated with ELISA tests. The purity of the antibodies was shown with SDS PAGE and western blot analysis. IgG isotype antibodies MAM-8G8 and MAM-2B11 were purified using protein A affinity chromatography using specific protocols for IgG1 and IgG2b isotypes. IgA isotype D12E2 antibody was purified using one step DEAE-C anion exchange chromatography. To our knowledge, this was the first demonstration of the purification of a monoclonal IgA antibody using a simple, one-step purification scheme. Monoclonal IgM antibody was purified through a two-step purification scheme where Sephacryl S300 size exclusion chromatography and DEAE-C ion exchange chromatography were used consecutively.

MAM-D3E4, MAM-2B11, MAM-8G8 and MAM-D12E2 antibodies had 0.83, 1.23, 0.65 and 0.4 $\varepsilon 0.1^{\%}$ values, respectively. These values are different from commonly used 1.4 $\varepsilon^{0.1\%}$ for IgG isotype antibodies [128,129]. With this study, we highlighted the importance of the determination of molar absorptivities of each developed monoclonal antibody which can vary remarkably between different monoclonals.

We developed and elaborately characterized high affinity monoclonal antibodies which can be utilized for other antibody based detection systems such as biosensors, strip assays or immunoaffinity cleanup columns [30,130].

This thesis presents a comparative study for the evaluation of different antibody isotypes in IAC and Biosensor development. So, a successful comparison should involve antibodies of similar affinities. MAM-D3E4 was shown to be a low affinity antibody as it is cross-reactive with food extracts and is sensitive to even low concentrations of organic solvents. The affinities of MAM-2B11, MAM-8G8 and MAM-D12E2 were determined (Table 4. 6). MAM-D12E2 was a monoclonal IgA antibody and had 0.86 ng/mL IC50 for AFB1 and MAM-8G8 was IgG1 with the exact same affinity for AFB1. MAM-2B11 was IgG2b, with 16.75 ng/mL IC50 value for AFB1. Affinities of the MAbs to other AF analogs showed a greater variation where in AFB2 and ABG1, MAM-D12E2 and MAM-8G8 showed similar affinities when compared to MAM-2B11. In case of AFG2, MAM-D12E2 had significantly lower affinity than other AF analogs.

AFB1, which is the most toxic and common analog was considered as the primary measure of similarity and the comparative work was based on the data obtained with IgA isotype MAM-D12E2 and IgG1 isotype MAM-8G8 MAbs.

5.2. Immunoaffinity Column Development

In the development of IACs, purified MAM-D12E2 and MAM-8G8 were used. IACs which contain 150 μg of each antibody were prepared and tested for their performances. Two critical parameters were chosen for the comparative performance analysis [131]; total capacity of AF binding and their ability to capture AFs in small concentrations where 5 ng of each AF was loaded.

The binding capacities of the two columns were shown to be similar for AFB1, AFB2 and AFG1. IAC prepared with MAM-8G8 was shown to be superior in terms of AFG2 binding capacity Figure 4. 26. The choice of antibodies was done according to their affinities to AFB1, which is the most commonly found and most toxic AF. However, antibodies have different affinities to different AF types and when the antibody is developed with the conjugate of AFB1, it usually has the highest affinity to AFB1 and lowest affinity to AFG1 [9,132]. The affinities of

MAM-D12E2 and MAM-8G8 antibodies to four naturally occurring AF analogs are presented in Table 4.6. The affinities of the two MAbs are the same for AFB1 and very close for AFB2 and AFG1. However, there is 10-fold difference between their affinities to AFG2, where MAM-8G8 is superior. The toxin binding capacities of the columns prepared with IgG and IgA isotype antibodies were shown to be strongly correlated with their affinities to toxins and were not affected by antibody isotype.

In case of limit binding test, a mixture of toxins containing 5ng of each AF analog was loaded to the columns and recovery rates (%) were calculated. The IACs are intended to be used in food and feed analysis, and the samples usually contain small amount of toxins, where AF limits for foodstuff is usually below 5 ng/mL [133]. Therefore, it is important for an IAC to be able to bind small amount of toxins in the sample and release them upon elution. Limit binding test is considered to be the uttermost test to determine column performances.

Our work revealed that, the limit performance of the IACs prepared with IgG and IgA isotype antibodies did not show any difference in the recovery of 5 ng AF analogs, even in AFG2, where there is a significant affinity difference between the MAbs. The affinities of the antibodies were within the requirements in order to achieve sufficient recovery rates for AF analogs.

5.3. Biosensor Development

For the direct determination of AF in sample, immobilization of AF on to gold coated QCM crystal functionalized via Self Assembled Monolayers (SAM) as sensing layer was performed. Our results indicated that nonspecific reactivity of the surface increases with increasing duration of EDC/NHS application to the surface. EDC/NHS activation is commonly used in sensor development studies [134–136], and the duration of activation should carefully be optimized to obtain minimal exposure of the surface. On the other hand, nonspecific binding to the surface can be minimized by careful choice of blocking reagents. In previous studies, blocking was usually achieved with nonspecific proteins [83,137,138]. We used carboxyl group bearing acetate buffer to block unreacted amine groups on the surface. Our results showed that, 1 M acetate buffer, pH: 4.8 provided a good blocking efficiency. Prepared surface was regenerated for at least 9 sequential analyses with strong

detergent containing regeneration solution which would disrupt conventionally used protein containing surfaces. Similar surfaces were shown to be regenerated up to 75 times with antibodies that can be regenerated at milder conditions such as 10 mM NaOH application [87]. However, MAM-D12E2 is a very high affinity antibody that can only be regenerated using strong ionic detergents in addition to high pH.

We developed a QCM immunosensor for AFB1 detection using monoclonal IgA antibody and showed its advantage over a conventional AF specific IgG antibody with similar affinity to AFB1. In this hitherto first study of sensor development with IgA antibodies, we compared frequency shifts observed upon AFB1 competition for both antibodies. Three times wider working frequency range was observed with MAM-D12E2, a 340 kDa monoclonal IgA antibody when compared to MAM-8G8, 150 kDa IgG antibody, in the inhibitory immunoassay. In the immunoassay development, we did not use any labels like magnetic beads or dendrimeric material for signal enrichment. In only one step, AF was detected with monoclonal IgA antibody based inhibitor immunoassays within the limits stated in the official regulations for AF. We showed that higher molecular weight of IgA antibodies contributed to a higher sensitivity and provided a natural signal enrichment for QCM immunosensor development (Figure 5. 1).

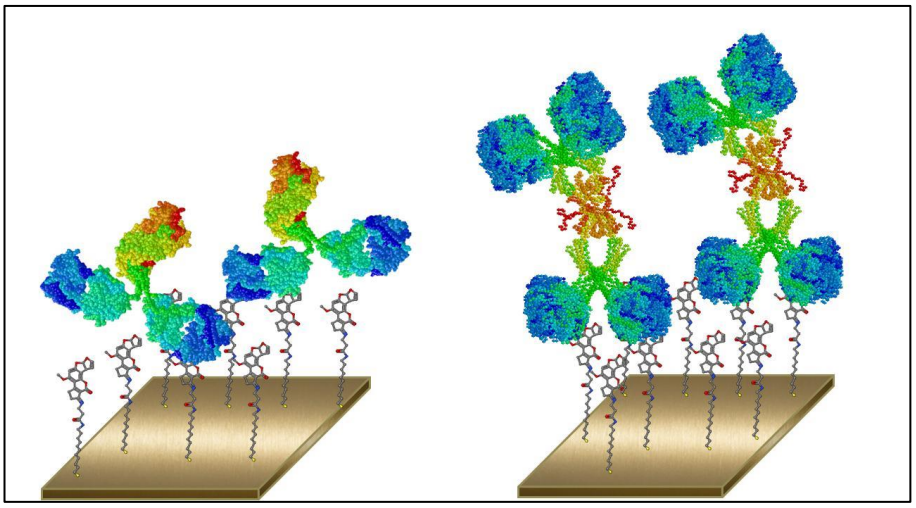

Figure 5. 1: Use of different antibody isotypes in sensor development.

The competitive QCM immunosensor prepared in this thesis utilized AFB1 immobilized surface in competition with free AFB1 in solution in order to bind to monoclonal IgA antibody and had a detection range of 1.25 – 10 ng/mL. There are

only two studies which used AF immobilized surface in QCM immunosensor development for AF detection up to date. These studies both immobilized AFB1-BSA conjugate to the gold surface and required the use of secondary antibodies for detection. The first study used an enzyme labeled secondary antibody for electrochemical QCM measurement and had a detection range of 0.01 – 10 ng/mL detection range [85]. The second study used gold nanoparticle labeled secondary antibodies and achieved a detection range of 0.1 – 100 ng/mL [134]. Other work employing a similar immunoassay strategy used optical or electrochemical transducers. Among the optical sensors developed with the same strategy, there are two SPR studies using a similar surface preparation approach. One study used monoclonal IgG antibodies and could achieve a detection range of 0.2 - 10 ng/g with prior concentration of AFs using IACs [88] and the other used multimeric recombinant antibody fragments and had 0.2 - 24 ng/mL detection range [87]. Other optical AF sensors utilized either fluorescent [15,139] or chemiluminescent [82] labels for limits of detection ranging from 0.1 to 1.5 ng/mL. The best detection range achieved with this strategy was through an electrochemical immunosensor prepared by Chen *et al.* with 0.08-100 ng/mL [83]. In this study an extensive surface enrichment was made through immobilization of gold nanoparticles to the gold coated quartz crystal surface prior to AFB1-BSA immobilization. The increased surface are obtained with this method significantly improved the detection range when compared to both optical and piezoelectric transducers as well as other electrochemical transducers [86,140–142]. This study emphasizes the importance of surface preparation for sensor development.

In this thesis, which was intended to show the effect of antibody isotype on sensor performance, we did not use any kind of weight tags such as the use of secondary antibodies or nanoparticles to enhance the signal. Furthermore, the surface used in this study was prepared with SAM and no additional strategy was utilized for increasing the surface area. Yet, a detection range compatible with international standards was achieved. This study proved IgA antibodies as promising candidates for the development of competitive immunosensors for small molecule analytes.

6. CONCLUSIONS

The work presented in this thesis was intended to show the effect of different antibody isotypes on the performance of immunoanalytical systems, specifically in IACs and biosensors. For this aim, two AF specific monoclonal antibodies of different isotypes were developed and thoroughly characterized. Two antibodies with similar affinities to AFB1 were selected; MAM-D12E2, as the representative of IgA isotype antibodies and MAM-8G8 as the representative of IgG antibodies. Both antibodies were used for the development of IACs and QCM biosensors and their performances were evaluated.

Our results showed that, antibody isotype is not a critical parameter for the performance of IACs. However, the affinities of the antibodies directly affect the performance especially in the total binding capacity of the prepared columns.

In case of QCM biosensor development studies, the detection sensitivity range with IgA antibodies was higher than IgG antibodies in QCM immunosensor developed for AFB1. The results showed that the IgA antibodies provide sufficient affinity and signal enrichment in QCM immunosensor development and this concept can be implemented for different target analytes for the development of mass sensitive sensors.

The key to develop reliable immunoanalytical systems is to choose the right antibody for the task. The results of this thesis once again highlighted the importance of assay specific requirements for antibody selection. The test design and the rationale behind the assay strongly affect the antibody selection parameters. This study introduced the use of tetravalent IgA isotype monoclonal antibodies in immunoassays and broadened the repertoire of scientists developing immunoanalytical systems, by including monoclonal IgA antibodies as an advantageous choice in addition to IgG antibodies used up to date.

7. FUTURE PERSPECTIVES

The research presented in this thesis was the first step towards the use of IgA antibodies for immunoassay development and the results were encouraging for moving this work forward.

IgA antibodies are not as abundant as IgG antibodies in mammalian systems, and as a result, IgA monoclonals are difficult to develop. In future studies, a vector with an IgA backbone can be designed for the preparation of recombinant IgA antibodies for any desired analyte which has a previously developed monoclonal antibody with known variable region sequence.

The results also emphasized assay specific variation in the effectiveness of IgA antibodies. So, the utilization of IgA antibodies for immunoanalytical systems other than IAC and immunosensors, such as ELISA or lateral flow systems will help to better understand their feasibility and practicality.

The results of the sensor development studies with the developed IgA antibody opened the door for a whole new set of studies. The sensitivity of the sensor can be enhanced with the use of alternative surface preparation methods to facilitate higher surface area. Moreover, this work was one of the few studies using piezoelectric transducers in AF detection. Transducers such as surface acoustic wave or high frequency QCM has not been previously used for AF immunosensing studies. Utilization of these systems can improve the sensor performance. Additionally, we discussed the molecular weight of the antibody as the advantageous property in microbalance transducers which are directly affected by the weight on the sensor. The very same reason may be useful in SPR studies as well, since the greater molecular size of the molecule will result in a more prominent change in the reflection angle of the light.

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BIOGRAPHY

Özlem ERTEKİN was born in 1982, Istanbul. She earned her B.Sc. degree from Middle East Technical University (METU) Department of Molecular Biology and Genetics in 2004 and M.Sc. degree at METU Department of Biology in 2007. She started her PhD studies in 2011 at Gebze Technical University, Graduate School of Natural and Applied Sciences, Department of Molecular Biology and Genetics where she worked on the development of immunodiagnostic systems for toxin detection. She is working as a senior research scientist at The Scientific and Technological Research Council of Turkey (TUBITAK), Marmara Research Center, Genetic Engineering and Biotechnology Institute since 2004. She has worked at several projects related with environmental and food safety as research scientist or work group leader.

APPENDICES

Appendix B: Publications Originating from the Thesis

Ertekin, Ö., Öztürk S., Öztürk Z.Z. (2016), "Label Free QCM Immunobiosensor for AFB1 Detection Using Monoclonal IgA Antibody as Recognition Element", Sensors, 16(8), 1274

Ertekin Ö., Pirincci Ş.Ş, Öztürk S., (2016), "Monoclonal IgA Antibodies for Aflatoxin Immunoassays", Toxins, 8(5), 148.

Ertekin Ö., Pirincci Ş.Ş, Öztürk S., (2016), "Monoclonal Antibodies MAM-D3E4, MAM-D3C6, MAM-D12E2, and MAM-D4D6 Against Aflatoxins", Monoclonal Antibodies in Immunodiagnosis and Immunotherapy, 35(2), 117-118.

Ertekin Ö., Akçael E., Yücel F., (2016), "Monoclonal Antibodies MAM-2B11, MAM-6E10, and MAM-8G12 Against Aflatoxins, Monoclonal Antibodies in Immunodiagnosis and Immunotherapy", 35(2), 117-119-120.

Öztürk S., Ertekin Ö., Pirinçci Ş. Ş., Kocaağa H., (2016), "Monoclonal IgA antibody for mycotoxins, a hybridoma cell line Producing said antibody, and specific use thereof in immunoassay", WIPO International Patent application, PCT/IB2015/052150.

Ertekin Ö., Öztürk S., Öztürk Z. Z., (2015), "Chemical Immobilization of Aflatoxins to Immunosensor Surface for Improved Regeneration and Reproducibility", 4th International Symposium on Sensor Science, 131, Basel, Switzerland, 13-15 July.

Ertekin O., Guloglu F. B., Pirincci S., Tuglu S., Akcael E., Ercan D., Hatipoğlu I, Göksel M., Ahsen V., Ozturk S., (2013), "Antibody based systems for the determination of mycotoxins in food and feed", Current Opinion in Biotechnology, 24, S23.

Ertekin Ö., Güloğlu B., Pirincci Ş.Ş., Tuğlu S., Kaymak T., Öztürk S., (2013), "Modification of small molecule chemical contaminants for immunochemical detection", IUPAC 44th World Chemistry Congress, 342, Istanbul, Turkey, 11-16 August.

Öztürk, S., Ertekin Ö., (2013), "Detection of Small Molecule Food Contaminants Using Antibody Based Systems", 4th Food Safety Congress, 97, İstanbul, Turkey, 14-15 May.

Ertekin Ö., Guloğlu F.B., Öztürk S., (2012), "Increasing the immunogenicity of non-immunogenic antigens", EMBO Young Scientists Forum, Istanbul, Turkey, 14-16 June.

Appendix A: HPLC calibration for Aflatoxin Quantification

Device was calibrated with certified AF standards (Supelco 46304-U) for accurate quantification. 6 different concentration of AF standards from 0.2 ng/mL to 100 ng/mL were injected 3 times for the generation of the calibration curves.

Calibration curves are presented in Figure A1. 1 – 4. The correlation of the curves are 0.99765 for AFG1 (Figure A1. 1); 0.99847 for AFG2 (Figure A1. 2); 0.99944 for AFB2 (Figure A1. 3) and 0.99931 for AFB1 (Figure A1. 4).

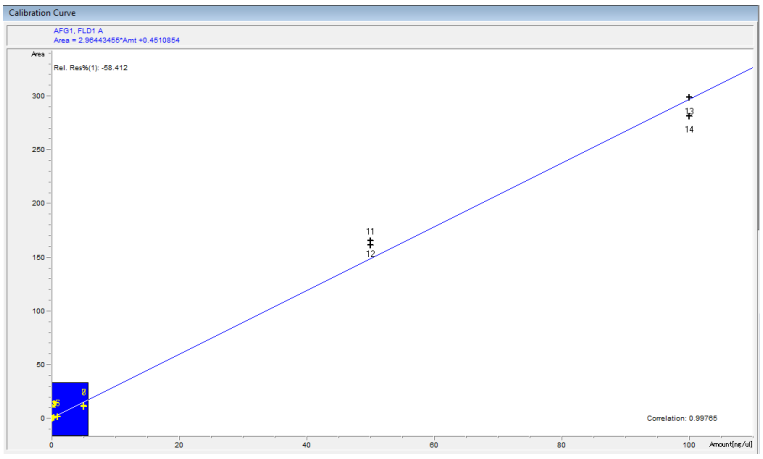

Figure A1. 1: Calibration curve for AFG1.

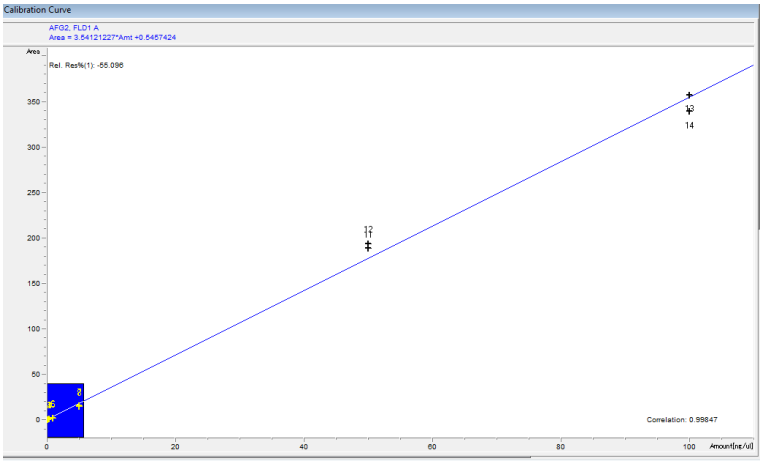

Figure A1. 2: Calibration curve for AFG2.

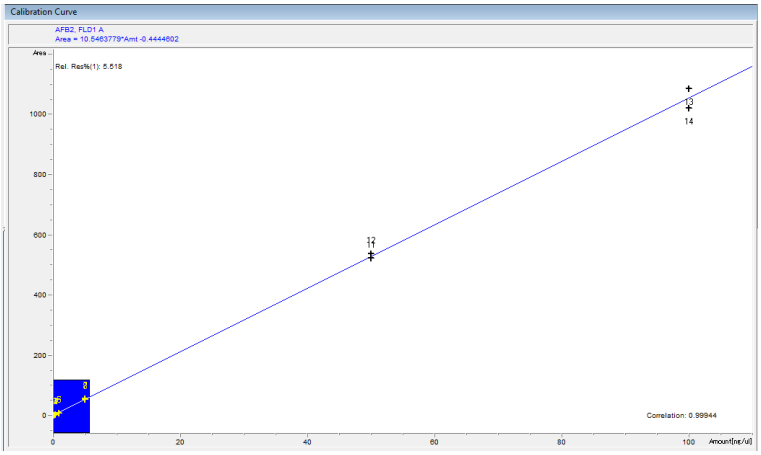

Figure A1.3: Calibration curve for AFB2.

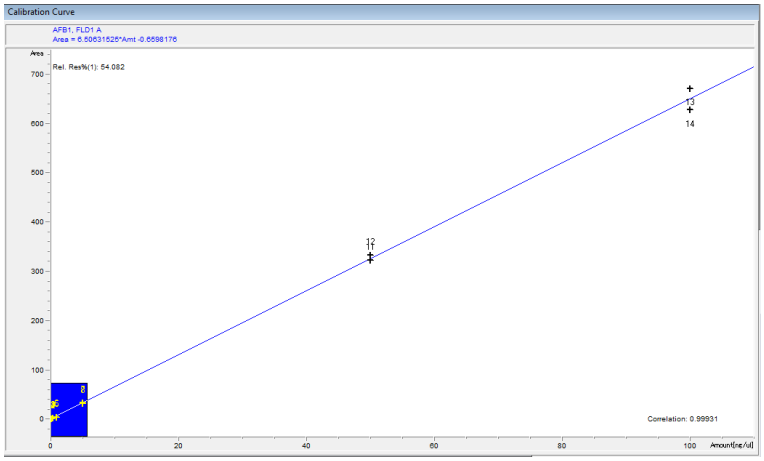

Figure A1. 4: Calibration curve for AFB1.