REPUBLIC OF TURKEY

ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

INSTITUTE OF GRADUATE SCHOOL

DEPARTMENT OF BIOENGINEERING

AFB1 RECOGNITION FROM LIVER TISSUE VIA AFB1 IMPRINTED MAGNETIC NANOPARTICLES

VELİ ZİYA ERDEM

MASTER OF SCIENCE

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ADANA 2021

I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all information that is not original to this work.

[Signature]

Veli Ziya ERDEM

ABSTRACT

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Fungi grow rapidly depending on the increase in humidity and temperature, producing harmful toxins called “mycotoxins” in cereals, feed and almost all dry foods. The most important of these toxins is Aflatoxins which are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Although there are more than 20 types of aflatoxins, types B1, B2, G1, G2 are the most important ones and Aflatoxin B1 is one of the most harmful among mycotoxins with its carcinogenic properties. As a result of consumption and metabolization of AFB1 contaminated food by animals, AFB1 and metabolites such as M1, M2, aflatoxicol can be found in meat and dairy products. People may be exposed to aflatoxicosis as a result of consumption of such contaminated animal products. Many of the methods used for the detection of aflatoxin require long extraction procedures which is time consuming, labourous and costly. In this thesis, AFB1 imprinted magnetic nanoparticles (magAFB1-MIP) were synthesized using molecular imprinting technology to be used in AFB1 recognition. Characterization studies of the synthesized polymers were carried out by swelling analysis, surface area measurements, scanning electron microscopy and particle size analysis. The effects of AFB1 concentration, temperature and interaction time on the adsorption capacity of magAFB1-MIPs from the aqueous solution were investigated. Selectivity of the magAFB1-MIP was analyzed calculating the selectivity coefficients against competitor molecules of AFB2, AFG1 and AFG2. Moreover as a final part of the thesis AFB1 recognition form liver tissue was investigated.

Keywords: Aflatoxin B1, molecular imprinting technology, magnetic nanoparticles, liver

ÖZET

AFB1 BASKILANMIŞ MANYETİK NANOPARTİKÜLLER İLE KARACİĞER DOKUSUNDAN AFB1 TANINMASI

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Eylül 2021, 72 sayfa

Tahıllarda, yemlerde ve neredeyse tüm kuru gıdalarda, rutubetin artmasına ve sıcaklığa bağlı olarak küf mantarları üreyerek "mikotoksin" adı verilen zararlı kimyasal maddeler üretirler. Bu toksinlerden en önemlisi başlıca *Aspergillus flavus* ve *Aspergillus parasiticus* küf türleri tarafından üretilen Aflatoksin'dir. 20'den fazla aflatoksin türü bulunmasına rağmen B1, B2, G1, G2 tipleri önemlidir ve Aflatoksin B1 mikotoksinler içerisinde kanserojen özelliği ile en zararlı olanlardan biridir. Hayvanlar tarafından AFB1 kontamine gıdaların tüketilmesi ve metabolize edilmesi sonucu et ve süt ürünlerinde AFB1 ve M1, M2, aflatoksikol gibi metabolitleri bulunabilir. Aflatoksinin tespitine yönelik kullanılan metotların birçoğu uzun ekstraksiyon işlemleri ile zaman almakta, işçilik gerektirmekte ve maliyetleri de yüksek olmaktadır. Bu tez çalışmasında AFB1 tanınmasında kullanılmak üzere moleküler baskılama teknolojisi kullanılarak AFB1 baskılanmış manyetik nanopartiküller (magAFB1-MIP) sentezlenmiştir. Sentezlenen polimerlerin karakterizasyon çalışmaları şişme analizleri, yüzey alanı ölçümleri, taramalı elektron mikroskobu ve zeta boyut analizleri ile gerçekleştirilmiştir. magAFB1-MIP kullanılarak sulu çözeltiden AFB1 adsorpsiyonuna, AFB1 konsantrasyonu, sıcaklık ve zamanın etkisi araştırılmıştır. Seçicilik çalışmalarında yarışmacı moleküller olarak AFB2, AFG1 ve AFG2 kullanılmıştır. Son olarak sentezlenen malzemenin karaciğer dokusundan AFB1'i tutma kapasitesi belirlenmiştir.

Anahtar Kelimeler: Aflatoksin B1, moleküler baskılama teknolojisi, manyetik nanopartiküller, karaciğer

Dedicated to my father I lost 7 years ago, who showed me to struggle, not to give up, and the power of perseverance,

and to my mother who supports me unconditionally under all circumstances.

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This thesis has given me an incredible experience and I would not have been able to complete it without the support of my supervisors, valuable teachers, friends and family.

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NOMENCLATURE

µL	: Microliter
µm	: Micrometre
mL	: Milliliter
µg	: Microgram
mg	: Milligram
cm	: Centimetre
L	: Liter
sec	: Second
min	: Minute
h	: Hour
eq.	: Equation
M	: Molarity
°C	: Celsius degree
MIP	: Molecularly Imprinted Polymer
NIP	: Non-molecularly Imprinted Polymer
PBS	: Phosphate Buffered Saline
AF	: Aflatoxin
AFB1	: Aflatoxin B1
AFB2	: Aflatoxin B2
AFG1	: Aflatoxin G1
AFG2	: Aflatoxin G2
AFM1	: Aflatoxin M1
OTA	: Ochratoxin A
ZEA	: Zearalenone
DON	: Deoxynivalenol
PAT	: Patulin
T-2 toxin	:Trichothecenes
AIBN	:Azobisizobutironitril
HEMA	: 2-Hydroxyethylmethacrylate
EGDMA	: Ethylene Glycol Dimethacrylate

VBA : 4-Vinylbenzoic Acid

UV : Ultraviolet

HPLC : High Performance Liquid Chromatography

SEM : Scanning Electron Microscopy

TLC : Thin Layer Chromatography

ELISA : Enzyme-Linked Immune-Sorbent Assay

CE : Capillary Electrophoresis

SR : Swelling Ratio

BET : Brunauer-Emmett-Teller

DR : Desorption Amount

Dc : Distribution Coefficient

IF : Imprinting Factor

K : Selectivity Coefficients

1. INTRODUCTION

Fungi reproduce rapidly depending on the increase in humidity and temperature, producing harmful toxins called “mycotoxins” in cereals, feed and almost all dry foods. Mycotoxins pose an important problem for human and animal health worldwide. The most important of these toxins is Aflatoxin (AF) by *Aspergillus flavus* and *Aspergillus parasiticus*. Although there are more than 20 types of aflatoxins, types B1, B2, G1, G2 are important and Aflatoxin B1 (AFB1) is one of the most harmful among mycotoxins. As a result of consumption and metabolization of contaminated food by animals, metabolites such as M1, M2 and aflatoxicol can be found in meat and dairy products (Agag et al., 2004; Plieva et al., 2007).

People may be exposed to aflatoxicosis as a result of consumption of such contaminated animal products. Aflatoxins generally damage the liver. Aflatoxin B1 is the most likely to cause cancer among mycotoxins. These mycotoxins can cause disease directly, as well as be a factor in the emergence of other diseases (Godfrey et al., 2013). It is not possible to remove aflatoxin from food by washing. It is therefore important to detect aflatoxin quickly, inexpensively and reliably. Many of the methods used for the detection of aflatoxin including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), mass spectroscopy, enzyme-linked immune-sorbent assay (ELISA). Most of them require long extraction procedures which are labourous and costly (Tang et al., 2007; Joint FAO/WHO food standards programme, 2013).

Molecular imprinting is a technique in which polymeric structures are synthesized that are complementary to the target molecule (target analyte, template molecule) in terms of properties such as shape, size and functional group distribution, and thus have high selectivity recognition sites against the target molecule. It can be used for purification, removal and determination in the fields of food, environment, medicine and biotechnology. Molecular imprinted polymers (MIPs) are easily prepared, low in cost, high physical strength, heat and pressure resistance (Bao-jun et al., 2006; Verheyen et al., 2011).

Nanoparticles have high surface area to volume ratio. This property increase the adsorption capacity of template molecule by molecularly imprinted nanoparticles. Moreover the high surface area ensures that the interaction surface is large and the removal process takes place

quickly. Magnetic separation techniques have some advantages. The separation procedure can be performed in a test tube. Separation is directly carried out on samples containing suspended solid particles using a magnetwithout any needs for centrifugation.

In this thesis, molecular imprinting technology was used for AFB1 recognition. Magnetic nanoparticles which is called magAFB1-MIPs have been synthesized by bulk polymerization using VBA as a monomer with the addition of iron. Initially 3 different polymers were synthesized using 3 different template molecule:monomer ratio (1:2, 1:3, 1:5; molar ratio of nAFB1:nVBA) and they are called magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$. The template removal ratios were obtained 93, 91 and 87% for the magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$ respectively. But the adsorption amount- rebinding amount- of the template obtained higher for magAFB1-$MIP_{1:3}$ then others. That is why the magAFB1-$MIP_{1:3}$ is selected as working polymer due to high AFB recognition capacity and named as magAFB1-MIP rest of this report. Swelling tests, surface area measurements, scanning electron microscopy (SEM) and particle size analysis were used for magAFB1-MIPs and magNIPs characterization. In order to determine optimum adsorption conditions of synthesized magAFB1-MIP, the effects of AFB1 concentration, ambient temperature and interaction time on the adsorption capacity of magAFB1-MIPs were investigated. The maximum AFB1 adsorption amounts were found as 33 ng/g for magAFB1-MIP for the concentration of 1 ng/mL AFB1. The reusability of magAFB1-MIP was determined with carrying out adsorption and desorption studies using same magAFB1-MIP 10 times. It was observed that the decrease of the AFB1 adsorption capacity is negligible. Also selectivity of the magAFB1-MIP was analyzed calculating the selectivity coefficients against competitor molecules of AFB2, AFG1 and AFG2. As a results, it was calculated that magAFB1-MIPs adsorb AFB1 1.74 times selectively than that of AFB2; 2.46 times selectively than that of AFG1 and 2.52 times selectively than that of AFG2 molecules. Finally AFB1 recognition of magAFB1-MIP from liver tissue was carried out. The adsorption capacity was found to be 10.4 and 54.8 ng/g for low and high AFB1 spiking levels, respectively.

2. LITERATURE REVIEW

2.1. Mycotoxins

Fungi can thrive in a wide variety of foods under favorable environmental conditions. The contamination caused by fungi in agricultural products may occur at all stages of food production, post-production storage, and this may have very critical consequences in terms of economy, food safety and human and animal health. Consumption of mycotoxin-containing feeds causes acute and chronic poisoning, loss of yield, decrease in weight gain and immunosuppression in animals. In addition, as a result of the genotoxic effects of mycotoxins, toxins such as aflatoxin, ochratoxin and fumonisin cause various types of cancer. This is very important in terms of public health, as the consumption of products obtained from animals that mycotoxin-containing feeds are consumed by humans brings serious health problems (Sonal et al., 2000; Tunail, 2000; Bryden et al., 2012).

In 1969, 100,000 turkeys were lost in England as a result of poisoning caused by peanuts used as a feed additive. The main cause of poisoning was determined as aflatoxins produced by a fungus called *Aspergillus flavus*, which completely invaded peanuts during the storage of the feed (Blout, 1961; Forgacs,1962).

Most of the known mycotoxins have very high thermal and chemical stability. This makes it possible to only partially remove mycotoxins from processed foods by conventional methods (Creppy, 2002; Devreese et al., 2012). Therefore, instead of trying to clean foodstuffs after contamination with mycotoxins, it is very important to keep the product under control during the harvest, storage and production stages, to prevent the growth of molds and to monitor the presence of mycotoxins in the product during all these stages. This can only be possible with sensitive, reliable and relatively low-cost analysis methods.

2.1.1. Important Mycotoxins and Producing Fungi

Mycotoxins are secondary metabolites produced by different types of fungi. More than a hundred mycotoxins have been identified, but only some of them have a significant toxic effect. Among the mycotoxin-producing fungi, the most well-known are; *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium* species (Huwig et al., 2001; Everley et al., 2007). Among the mycotoxins produced by these species; Aflatoxins, Ochratoxin A (OTA), Fumonisin B1 (FB1),

Trichothecenes (deoxynivalenol, T-2 toxin), Zearalenone (ZEA) and Patulin are the most emphasized mycotoxin types due to the problems they cause in terms of public health and economy.

Table 2.1. The major food-borne mycotoxins, their main producing fungal species and the most frequently contaminated commodities (Shephard, 2008).

Mycotoxin	Fungal species	Food commodity
Aflatoxins B1, B2, G1 and G2	*Aspergillus flavus* *Aspergillus parasiticus*	Maize, wheat, rice, sorghum, ground nuts, tree nuts, figs
Aflatoxin M1	Metabolite of aflatoxin B1 in mammals	Milk, milk products
Fumonisins B1, B2 and B3	*Fusarium verticillioides* *Fusarium proliferatum*	Cereal products, sorghum, asparagus
Deoxynivalenol	*Fusarium graminearum* *Fusarium culmorum*	Cereals, cereal products
T-2 toxin	*Fusarium sporotrichioides* *Fusarium poae*	Cereals, cereal products
Zearalenone	*Fusarium graminearum* *Fusarium culmorum*	Cereals, cereal products
Ochratoxin A	*Aspergillus ochraceus* *Penicillium verrucosum* *Aspergillus carbonarius*	Cereals, dried vine fruit, wine, coffee
Patulin	*Penicillium expansum*	Apples, apple juice

2.1.2. Effects of Mycotoxins on Human Health

Fruits, vegetables and cereals can cause epidemic diseases that negatively affect public health in developing countries (Hocking et al., 2003). In the case of chronic exposure, mycotoxins can trigger diseases such as cancer, for example, intense exposure of a person with hepatitis B to aflatoxin can accelerate the development of liver cancer. Especially recent studies have provided important findings on the relationship between acute aflatoxin poisoning and liver cancer. Fumonisins, Ochratoxin A (OTA) and citrinine (STR), on the other hand, can cause fatal kidney disease in the case of chronic exposure due to their nephrotoxic effects (IARC, 2012; Jaimez et al., 2000). The chemical structure of ZEA and its metabolites are similar to hormones such as estradiol, estrone and estriol, and these substances can interact with hormone receptors in the human body in competition with hormones and cause disorders in the hormonal system (Jalili et al., 2012; Jodlbauer et al., 2002). As a result, it has been determined that ingestion of mycotoxins through the nutrition and respiratory tract or absorption through the skin has acute and chronic effects on human health. These include pulmonary mycotoxicosis,

toxic mold syndrome, autoimmune diseases, allergic reactions, carcinogenic, mutagenic and teratogenic effects in chronic exposure (Josic et al., 2001; Brera et al., 2002; Kokkonen et al., 2010; IARC, 2012).

2.1.3. Effects of Mycotoxins on Animal Health

Animal food products have an important place in human nutrition and therefore in the economic system. Some short- and long-term effects may be seen in animals fed plant foods contaminated with mycotoxins. These effects may vary depending on the type of mycotoxin, the breed and age of the animal, and so on (Bayram, 2013). The effects of mycotoxins on animals are summarized in Table 2.2.

Table 2.2. Effects of mycotoxins on animal health (Gilbert et al., 2002, Bayram, 2013)

Mycotoxin	**Clinical Symptoms**
Aflatoxin	Liver damage, decrease in yield, structural defects in the eggshell and reduced resistance to diseases.
Cyclopiazonic acid	Liver, kidney and gastrointestinal system damage, weight loss, weakness, structural disorders in eggshell, anorexia, diarrhea and dehydration, depression, opistotonus, convulsion.
Zearalenon	Choking, vaginal prolapse and redness, reproductive problems.
Deoxynivalenol	Lack of appetite and refusal of food.
Trichothecenes (T-2 toxin etc.)	Anorexia, vomiting, tenderness in the skin and gastrointestinal system, neurotoxic effects, anomalies in offspring.
Fumonisin B1	Leukoencephalomalacia (ELEM), Porsin Pulmonary Edema (PPO), liver tumor in rats.
Ergot alkaloids	Nervous system disorders, involuntary spasms, tremors, miscarriages, weaning, in some cases blackening of the hooves and beak.

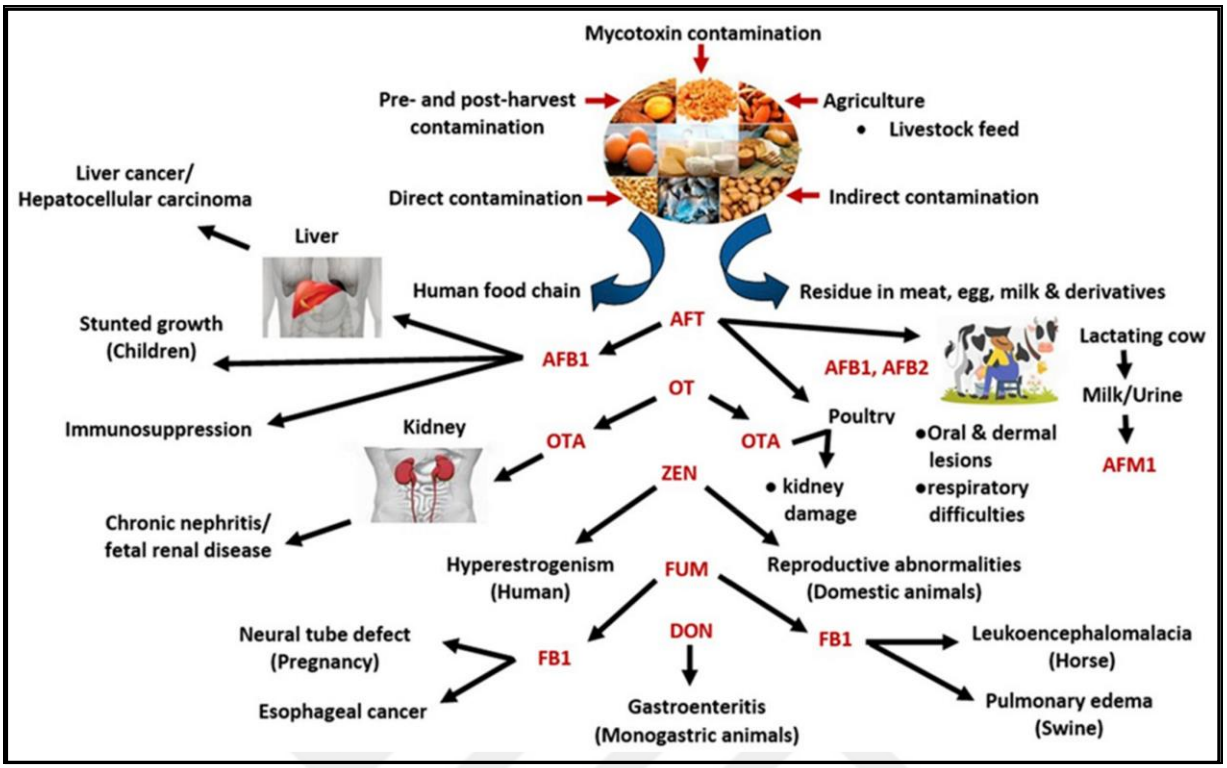


Figure 2.1. Factors affecting mycotoxin occurrence and effects on animal and human health. Factors affecting mycotoxin occurrence and effects on animal and human health (Haque et al., 2020; Fokunang et al., 2006; Vanhoutte et al., 2016)

2.1.4. Factors Affecting Formation of Mycotoxins

The most important factors affecting the growth of fungus and ultimately mycotoxin production in agricultural products can be listed as follows:

Effect of humidity: There is a linear relationship between the relative humidity of the environment and mold growth. As the humidity increases, mold growth also increases and becomes easier. Molds need moist environments to grow. While producing toxins, humidity levels can be lower than the humidity required for grow.

Effect of temperature: Temperature, which plays a very important role in the survival of many microorganisms and the realization of chemical reactions and activities in nature, is also effective on the growth and development of molds. The optimum temperature required for the formation of toxins is 20–40 °C, and the temperature at which they occur best is between 25–26 °C. Aflatoxins, on the other hand, can be produced between 5 and 40 °C, although the temperature at which they occur best is 24–35 °C.

Effect of oxygen and carbon dioxide: Molds are aerobic, and they need oxygen to grow, reproduce and synthesize toxins. By reducing the amount of oxygen in the environment below 1%, mold growth is reduced as well as toxin production. There can be significant differences in the oxygen needs of fungal species in order to grow and synthesize mycotoxins. By taking advantage of these effects of oxygen and carbon dioxide on molds, this feature can be utilized in the storage conditions of foods and feeds.

Effect of food type and structure: Molds need organic carbon and other energy sources to grow and reproduce. Molds also need to use organic substances such as peptones, polypeptides and amino acids as nitrogen sources and elements such as calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), zinc (Zn), phosphorus (P). In addition, considering that mold contamination in feed materials starts from the field, products that have been physically damaged during harvest provide an environment for fungi to reproduce and synthesize toxins.

Effect of pH: It is known that molds prefer more acid environments to grow, however they can grow between pH 1.5 - 8.5. Aflatoxin producers synthesize toxins between pH 2.5 - 6.0, but they produce high amounts at higher pH's starting from pH 5.0.

Effect of time: The longer the storage period, the mold growth and mycotoxin synthesis increase. In products containing mold spores, molds can grow and reproduce within 2 to 4 days, if suitable conditions are provided, especially temperature and humidity, and they can synthesize mycotoxins in amounts that threaten human health. A period of 16-24 days is sufficient to quadruple the amount of AF.

Other factors: Factors such as harvest time of food and feed, climatic conditions and ventilation are among the factors affecting mold growth. Broken, damaged plant product shells allow mold micelles to migrate to the inner grain and form mycotoxins. Chemical factors such as pesticides and fungicides used on the crop during the production phase are also effective in the growth of molds, such as stress caused by improper irrigation and fertilization (Whitlow, 2001; Tunail, 2000; Bryden, 2012; İpek, 2019).

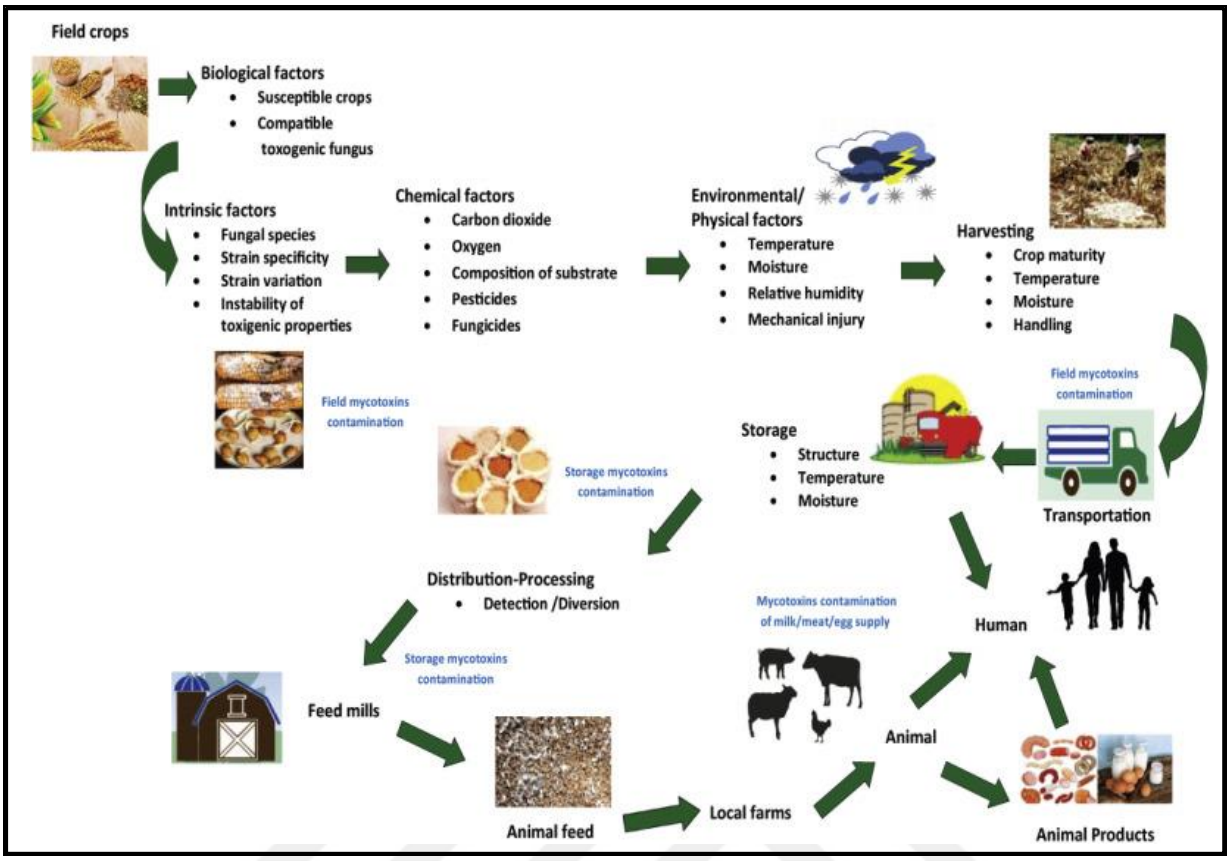
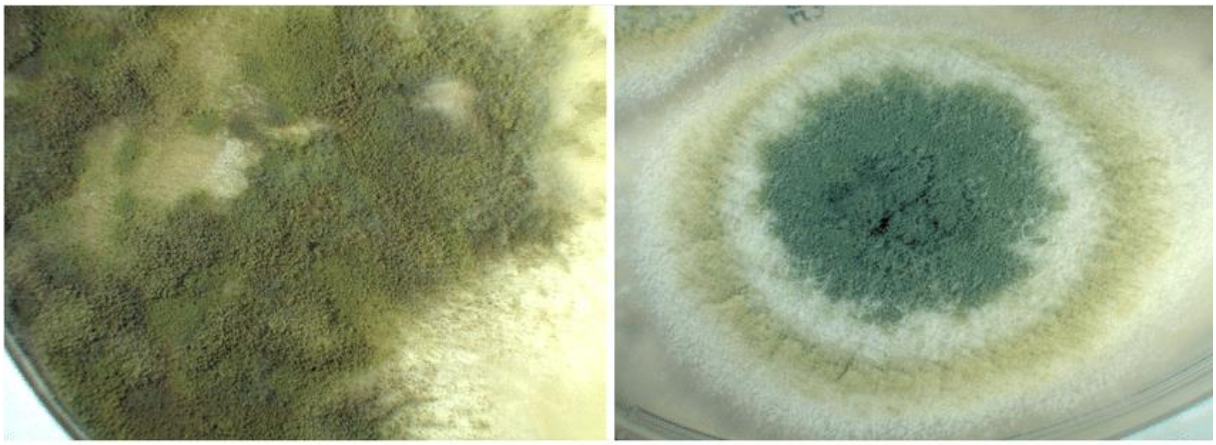


Figure 2.2. Factors affecting formation of mycotoxin and its roles in the human and animal food supply chain (Haque et al., 2020; Fernandes et al., 2017; Simion et al., 2011).

2.2. Aflatoxins

Aflatoxins, one of the most important mycotoxins, cause contamination in almost all kinds of food and feed materials. Consumption of these foods by humans or feeds animals causes serious health problems and economic losses. In addition, the consumption of products obtained from animals fed with aflatoxin-contaminated feeds by humans threatens public health. For these reasons, aflatoxins have been extensively researched since 1960 after their discovery. *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* are the most important mold fungi that synthesize aflatoxins. Aflatoxin B1, B2, G1, G2, M1 and M2 produced by these molds are the most important toxins of this group. In addition, the number of these toxins, together with those formed as metabolites in the culture medium and in the body, is over 20. The nomenclature of these toxins is based on the color of the fluorescent light emitted by aflatoxins under UV light (Agag et al., 2004; Plieva et al., 2007; Mahato et al., 2019).



A: *Aspergillus flavus* B: *Aspergillus parasiticus*

Figure 2.3. Cultures of *Aspergillus flavus* and *Aspergillus parasiticus*, respectively, both producers of aflatoxins, on Sabouraud Agar after incubation at 26 °C for 7 days (Hof, 2016)

2.2.1. Chemical Structure of Aflatoxins and Their Effects on Human Health

Although more than twenty species of aflatoxins have been identified, only four of them are of considerable importance. These are aflatoxin G2 (AFG2), aflatoxin G1 (AFG1), aflatoxin B2 (AFB2), aflatoxin B1 (AFB1), letters G (green) and B (blue) refer to the colors produced by these substances when exposed to UV light. Aflatoxins are difurancoumarin derivative compounds and are well soluble in polar organic solvents, chlorinated compounds and aromatic hydrocarbons due to their chemical structure. The metabolites of AFB1 and AFB2 in milk are AFM1 and AFM2. “M” is given as the initial letter of the word milk, which means milk in English (Piletska et al., 2010; Plieva et al., 2006).

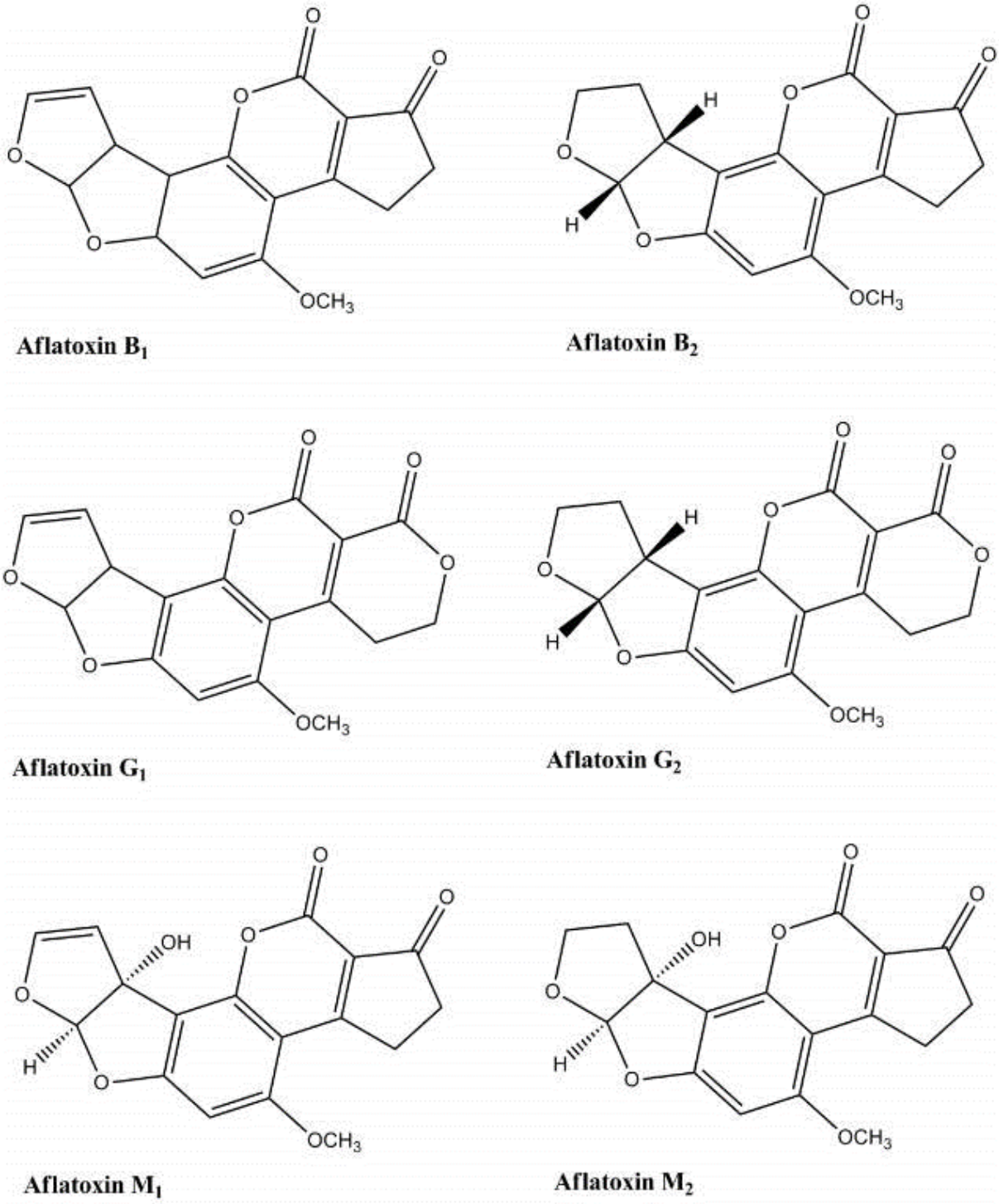


Figure 2.4. Chemical structures of Aflatoxin (Santini and Ritieni, 2013).

After aflatoxins are taken into the body, some of them are metabolized and converted into metabolite derivatives in humans and sensitive animal species. Experiments on animals have revealed that aflatoxin B1 is absorbed by the small intestine at a rate of 50% after oral ingestion, from where it is converted into metabolites by passing to the liver. Various CYP450 enzymes

isoforms occur in the liver, and they metabolize aflatoxins, particularly AFB1, to aflatoxin-8,9-epoxide a reactive oxygen form. This reactive form forms adducts by binding to DNA and albumin in blood serum, thus causes DNA damage inducing liver cancer and acute toxicity. CYP 1A2 perform hydroxylation of AFB1 to form AFM1, which is less efficient than AFB1 and weak substrate for epoxidation. This is generally the main pathway used to metabolize aflatoxins. The CYP3A5 metabolizes AFB1 mainly to the exo-epoxide and some AFQ1, a less toxic detoxification metabolite. In addition, aflatoxin P1 (AFP1) is formed by demethylation. The oxidation products (AFQ1 and AFM1) as well as AFP1 are potential detoxification products, since they represent weaker substrates for epoxidation than AFB1 (Wild et al., 2009; Wu et al., 2010; Godfrey et al., 2013; Peles et al., 2019). Major metabolites of AFB1 occur in liver can be seen in Figure 2.5.

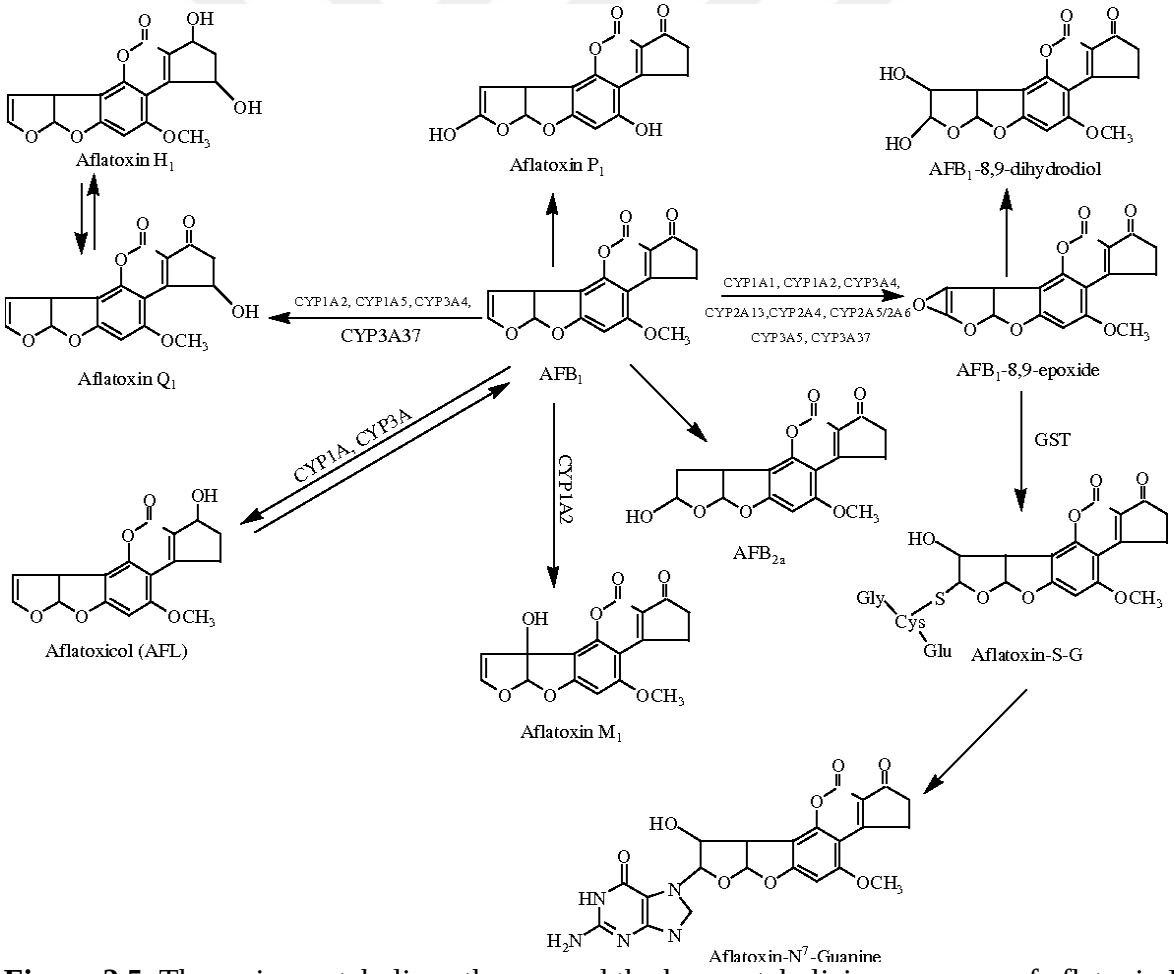


Figure 2.5. The major metabolic pathways and the key metabolizing enzymes of aflatoxin B1 (AFB1) in animals and humans (Dohnal et al., 2014)

2.2.3. Effects of Aflatoxins on Human and Animal Health

Aflatoxins have various harmful effects on human and animal health. Aflatoxins are compounds with high lipid solubility and easily pass into the bloodstream through the gastrointestinal tract and respiratory tract (Agag et al., 2004; Larsson et al., 2000).

Humans and animals are exposed to aflatoxins in two different ways. One is the digestive tract and the other is the respiratory tract. Digestive exposure occurs as a result of direct consumption of foods containing aflatoxin or consumption of dairy products such as milk and cheese and milk powder and other tissues of animals fed aflatoxin feed. On the other hand, respiratory exposure, occurs through inhalation of dust particles of aflatoxins, especially AFB1 in contaminated food in industries and factories (Coulombe et al., 1994).

After aflatoxins enter the body, they are absorbed through cell membranes and reach the circulatory system. They are distributed through the blood to different tissues and to the liver, the main organ where metabolism takes place. Aflatoxins are metabolized by the liver to a less harmful reactive epoxide intermediate or hydroxylated to become AFM1 (Coulombe et al., 1994; Wu et al., 2010). The condition caused by aflatoxins in both humans and animals is called aflatoxicosis. It has two general forms acute and chronice forms. The acute form occurs when moderate or high levels of aflatoxin are consumed. Specific acute illness symptoms may include acute liver injury, bleeding, edema, altered digestion, impaired absorption and/or metabolism of nutrients, and possibly death (Godfrey et al., 2013).

Chronic aflatoxicosis occurs when low levels of aflatoxin are consumed continuously. Decreased phagocytic activity or disruption of normal body immune function by decreasing T cell number have been reported as effects of chronic aflatoxicosis. It has been reported to suppress immunity in an animal model study. In addition, it has been reported that there is a relationship between growth rate as a result of exposure to aflatoxin in infants and children (Godfrey et al., 2013). Aflatoxins also disrupt the structure of nutrients such as vitamins A or D in animal models, rendering them unusable and thus leading to nutritional deficiencies (USAID, 2012; Peraica et al., 1999).

Aflatoxins disrupt the protein synthesis process. They bind to enzymes and substrates used in initiation, translation and transcription processes involved in protein synthesis. They interfere

with protein synthesis by interacting with purines and purine nucleosides and forming links with DNA, RNA, and proteins (Clifford et al., 1967). Aflatoxin also damages DNA-dependent RNA polymerase activity and inhibits RNA synthesis, thereby leading to degranulation of the endoplasmic reticulum. In addition, the decrease in protein in body tissues such as skeletal muscle, heart, liver and kidney may be due to necrosis in the liver and kidney (Sharma et al., 2011).

In the study conducted by Tuzcu et al., 60 one-day old goslings were divided into 3 groups of 20 each. The first and second groups were given feed containing 2.5 ppm and 5 ppm total aflatoxin, respectively. The goslings in the third control group were fed with aflatoxin-free feed. On the 30th day of the study, the remaining goslings in all groups were euthanized and systemic necropsies were performed. Macroscopic and microscopic examination of liver, kidney, intestine, spleen, bursa fabricius, lung, heart, brain and cerebellum samples taken after necropsy were performed. It was observed that a significant hyperemia developed in the livers of all the goslings given aflatoxin and the cytoplasm of the hepatocytes became cloudy (Figure 2.6.). It was also detected in multifocal necrosis which was distributed to the liver parenchyma (Figure 2.7.). It was noted that there were large fat vacuoles in the cytoplasm of hepatocytes, and a few vacuoles combined to form small fat areas. In Sudan Black staining, these vacuoles were determined to be oil vacuoles (Figure 2.7.). In bursa fabricius, emptying of lymph follicles (Figure 2.7.), decrease in lymphocyte count and shedding of interfollicular epithelium were determined (Tuzcu et al., 2009).

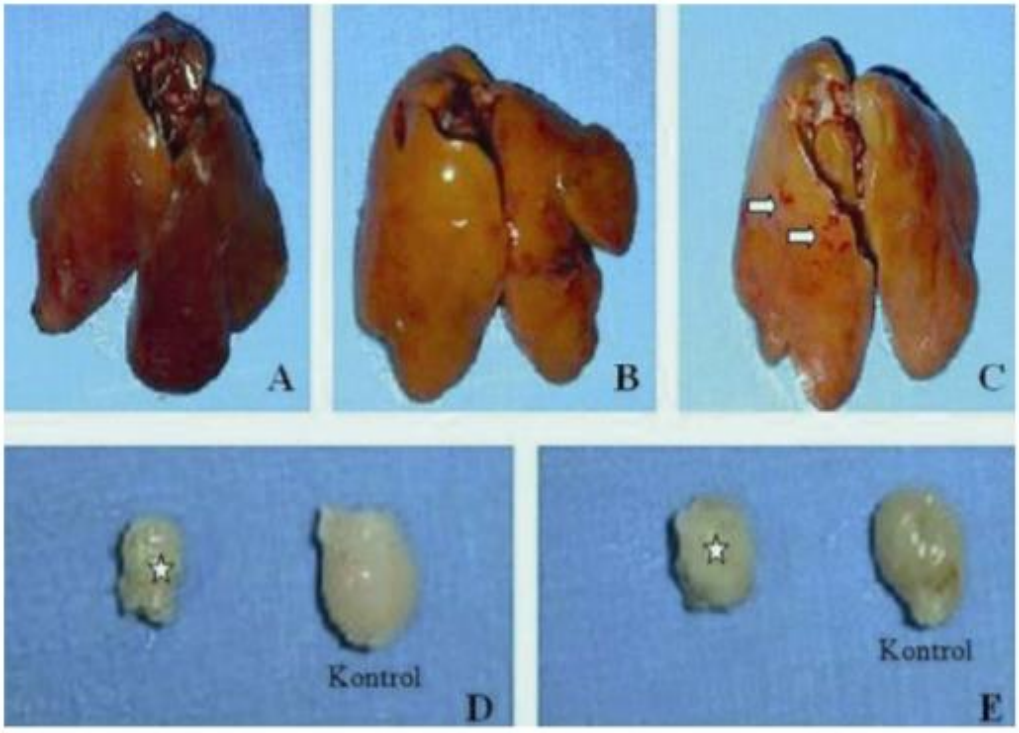


Figure 2.6. Macroscopic examination of liver and bursa fabricius of gooses fed with AF contaminated feed. Liver of a gosling from the control group (A) pale color in the liver, group given 2.5 ppm aflatoxin (B), pale color and ecchymotic hemorrhages in the liver (arrows) group given 5 ppm aflatoxin (C), group given 5 ppm aflatoxin, atrophy in bursa fabricius (star) (D) Group given 2.5 ppm aflatoxin, atrophy of bursa fabricius (star) (E).

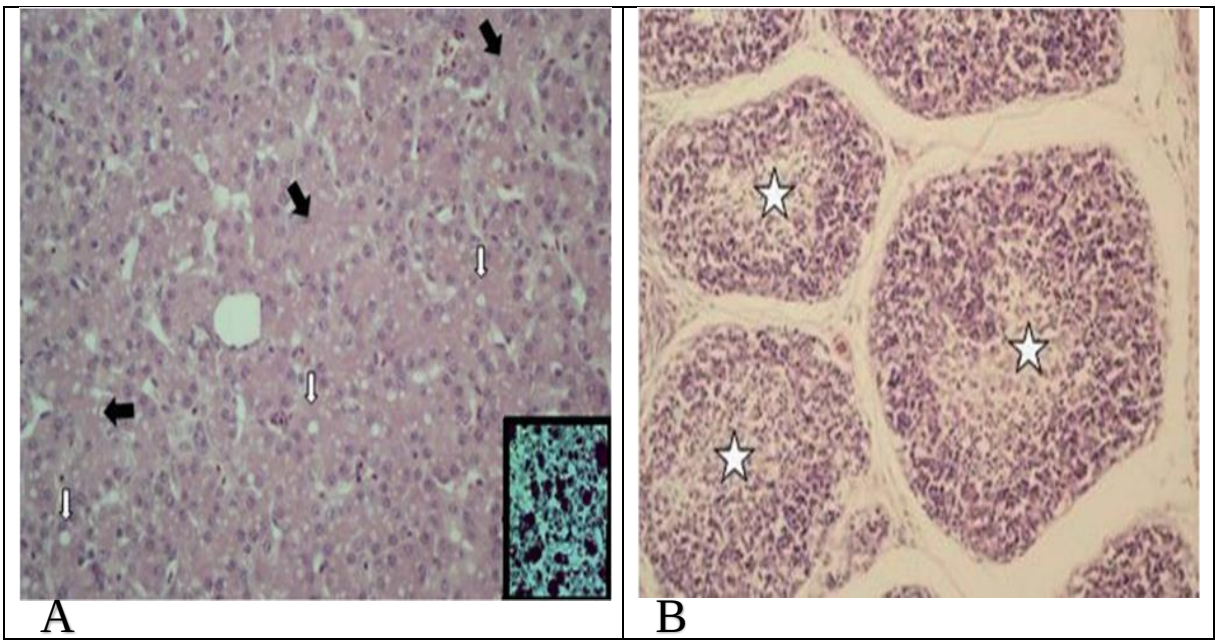


Figure 2.7. Pathological examination of liver and bursa fabricius of gooses fed with AF contaminated feed. (A) Multifocal necrosis areas (black arrows) in the livers of goose bulls given 5 ppm aflatoxin, fat vacuoles in hepatocytes (White arrows) Hematoxylin & Eosin x 300, Fat vacuoles in hepatocytes (small image), Sudan Black x 420. (B) Discharge in follicles (star) in group bursa fabricius given 5 ppm aflatoxin Hematoxylin&Eosin x 300.

Acute toxicity is less likely than chronic toxicity. Comparative LD50 or lethal values for Aflatoxin B1 for some animals are given in Table 2.3.

Table 2.3. Comparative LD50 or lethal values for AFB1 (Edds, 1973; WHO, 1979).

Species	Oral LD50/Lethal dose (mg/Kg)
Chick embryo	0.025
Chicken, New Hampshire	2.0
Chicken, Rhode Island	6.3
Turkey poultry	0.5
Duckling	0.3
Sheep	5.0
Rat(male)	7.2
Rat(female)	17.9
Rabbit	0.3
Cat	0.6
Pig	0.6
Guinea pig	1.4
Hamster	10.2
Mouse	9.0
Baboon	2.0

The causes and consequences of aflatoxicosis are summarized in Figure 2.8.

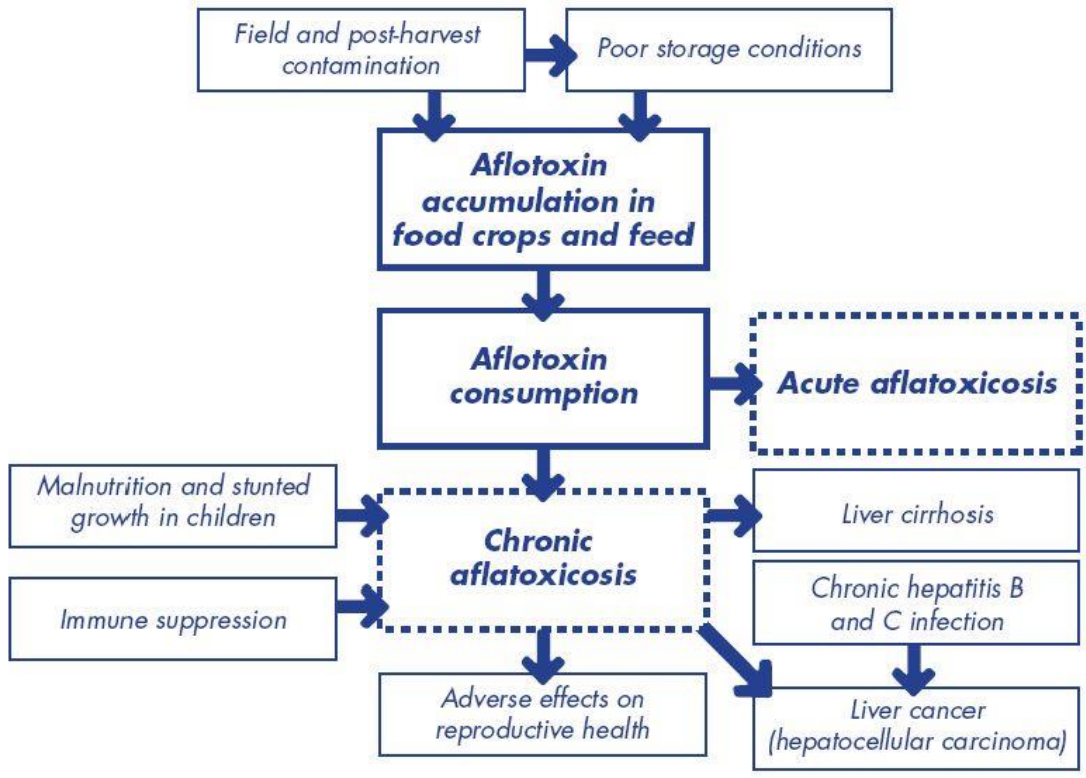


Figure 2.8. Effects of aflatocosis in humans (Wu, 2010; USAID, 2012; WHO, 2011; Wu and Tritscher, 2011)

2.2.4. Legal Requirements and Current Limits for the Analysis of Aflatoxins

As mentioned under the previous headings, aflatoxins have serious negative effects on human and animal health. In addition to health problems the inability to prevent contamination during harvest and post-harvest storage can lead the decrease in the quality of agricultural products, decreasing in productivity or death of livestock animals raised for food production. For this reason, risky foodstuffs should be analyzed in order to determine the aflatoxin content in all food production stages and just before the food reaches the end consumer.In our country and in many countries, governments have created laws to control aflatoxin B1 in food and feed and aflatoxin M1 levels in milk to protect consumers, especially children, from contaminated products.Today, almost all countries in the world set limit values for aflatoxin levels that can be found in food and feed in order to avoid this danger and reduce the return of the products they export.The Turkish Food Codex Contaminants Regulation determined by the Ministry of Agriculture and Forestry and published in the Official Gazette dated December 29, 2011 (28157) determines the upper limit values of aflatoxin that can be found in foods for Turkey. In the Table 2.5. the values determined in the relevant regulation are given.

Table 2.4. Aflatoxin maximum limit values allowed in food in Turkey (TGK, 2009)

FOODS	Maximum Limit (μg / kg)		
	Aflatoxin		
	B1	**B1+B2+G1+G2**	**M1**
Peanuts and Other oilseeds[1]	8	15	-
Almond, Pistachio and Apricot Kernel[1]	12	15	-
Hazelnut and Brazil Nut[1]	8	15	-
Nuts[1]	8	15	-
Peanuts and other oilseeds[2]	5	10	-
Almond, Pistachio and Apricot Kernel[2]	8	10	-
Hazelnut and Brazil Nut[2]	5	10	-
Nuts[2]	5	10	-
Dried fruits	8	10	-
Cereals, products derived and processed from them	2	4	-
Corn and Rice[1]	5	10	-
Raw milk (heat treated or to be used in dairy production)	-	-	0.05
Spices (Red pepper, black pepper, nutmeg / nutmeg, ginger, turmeric and their mix)	5	10	-
Supplementary foods for infants and young children	0.1	-	-
Infant and continuation formulas	-	-	0.025
Special purpose diet foods for babies	0.1	-	0.025

[1]Which will be subjected to sorting and other physical processes before being offered for direct human consumption or used as a food ingredient. [2]Intended for direct human consumption or used as a food ingredient.

2.2.5. Aflatoxin Analysis Methods

Aflatoxins are one of the most important mycotoxins that threaten human and animal health. Since they cannot be cleaned from contaminated food and feed materials by washing and similar processes, it is important to determine their presence before the use of food and feed materials. Generally, various chromatographic and immunochemical methods are used for aflatoxin determinations. In almost all aflatoxin detection methods, it is possible to determine the toxin by extracting it from the complex mixture.

After the extraction stage, the samples are analyzed. For this purpose; enzyme-linked immunoassay (ELISA), thin layer chromatography (TLC), capillary electrophoresis (CE), high performance liquid chromatography (HPLC), Liquid chromatography single quadrupole mass spectrometry (LC-MS), liquid chromatography triple quadrupole mass (LC-MS / MS) techniques are used (Brera et al., 2002; Plieva et al., 2006; Zöllner et al., 2006; Corcueraa et al., 2011; Shimadzu et al., 2004).

After separation by chromatographic methods, analysis is performed using UV adsorption, mass spectrophotometer, fluorescence measurement or amperometric determination methods. With the fluorescence determination method, 30-40 times more sensitive measurements are obtained compared to the UV method. Many of the analysis methods mentioned above are techniques that require expertise and experience in terms of application, or some of them lose their reliability as a result of decreased accuracy and repeatability and high device and consumable costs still constitute a serious handicap for the use of these devices in routine analysis.

Aflatoxins are natural fluorescent substances. In particular, the high fluorescence intensity of AFG2 and AFB2 enables analysis even at low concentrations. The fluorescence intensity of AFG1 and AFB1 is not as high as AFG2 and AFB2. For this reason, they must be transformed into species with high fluorescence intensity through some chemical processes. This process is called derivatization and can be performed before the sample is applied to the HPLC column.

2.3. Affinity Chromatography

Chromatography is an analytical method used for the separation, identification and determination of chemical compounds in mixtures. It is based on the principle of separating the

components in the mixture by drifting over the stationary phase with the help of the mobile phase. Parameters related to some intermolecular relations such as adsorption, partition, affinity and molecular sizes are effective in the process of separation of molecules from each other. Depending on these factors, some of the components in the mixture hold on to the stationary phase longer and leave the system later while some other components adhere less on the stationary phase and therefore leave the system faster. There are various types of chromatography; liquid, gas, ion-exchange and affinity chromatography (Laurence et al., 1989).

Affinity chromatography is defined as a type of the adsorption chromatographic method. Moreover, it is defined as liquid chromatography that uses a biological interaction for the separation and analysis of specific analytes in a sample. Because it is very difficult to purify many biological materials with other methods, affinity chromatography usually preferred as a separation method in the fields of pharmaceutical and biotechnological industries (Hage, 1999). In this method, a stationary phase-dependent affinity ligand interacts with the desired analyte and separation takes place in the chromatographic column depending on the degree of this interaction. The immobilized ligand is the key factor determining the success of any affinity chromatographic method. Most of the ligands used for affinity chromatography are of biological origin. In this method, the molecule is selectively and reversibly bound to the covalently immobilized ligand. In this method in order to achieve an efficient separation first passes through a ligand that is covalently bound to the matrix. Also, one of the most important requirements for this process is that the immobilized ligand has sample specific binding affinity and there is a method that can be used for selective desorption of the bound sample after removal of impurities (Wilchek and Chaiken, 2000; Firer, 2001).

The strength of affinity chromatography depends on the specific interactions, and therefore the matrix used must show very high specific adsorption. This matrix should be able to withstand different conditions. In this method, the selected ligand must show reversible and specific binding affinity for the substance to be purified, while at the same time it must contain chemically modifiable groups that do not impair the binding affinity of the ligand and allow it to attach to the matrix. These properties are two of the essential and important properties of the ligand. At the same time, if the ligand to be used in this method has different functional groups, the region where it is attached to the matrix is also important for this ligand. Therefore, this binding should be between the ligand group and the matrix that is least likely to give a specific

interaction with the molecule to be purified. Although it can be used effectively in many fields (enzyme, protein, hormone, vitamin, receptor, antigen, antibody, carbohydrate, bacteria, virus and cell), the instability of the molecules used as ligands under working conditions prevents the industrial use of affinity chromatography. The industrial use of affinity chromatography has become increasingly widespread, with more stable structures obtained by recently produced artificial ligands (Hage, 1999; Hedhammar et al., 2006).

2.4. Molecular Imprinting Technology

Molecularly imprinted polymers are synthetic systems formed by cross-linking monomers in the environment of the target molecule. A molecule and a cavity complement each other chemically and geometrically, similar to key-lock compatibility, enzyme-substrate compatibility, or antigen-antibody compatibility. They have high selectivity for the template molecule and are resistant to mechanical stress, heat, acid, base, water and organic solvents besides they can be used again and again. In addition, their high stability, low cost, and full compatibility with micromanufacturing have enabled them to be used as synthetic recognition elements in many applications. It can be used for purification, removal and determination in the fields of food, environment, medicine and biotechnology. Since target analytes (residual chemicals, biochemical substances, elements, etc.) are mostly in a complex matrix (plant and animal tissues and liquids, soil, etc.), the sample preparation stages and the methods used in these stages are as important as the analysis method. The fact that the sample preparation method is fast, easy to apply, efficient and low cost are the prominent parameters for the evaluation of the method (Bao-jun et al., 2006; Yılmaz et al., 2009; Verheyen et al., 2011; Alexander et al., 2006).

2.4.1. Preparation of Molecular Imprinted Polymers (MIP)

Molecular imprinting method basically consists of three stages (Figure 2.9).

The molecularly imprinted polymer preparation process consists of three steps.

1- Pre-Complexation: Complexes are formed between the target molecule and functional monomers through covalent bonds or non-covalent interactions.

2- Polymerization: The monomer-target molecule complex is polymerized onto the functional monomer using an appropriate crosslinker and initiator. The functional groups of the monomers in the polymer are fixed according to a certain sequence and orientation in the

presence of the target molecule. Thus, a three-dimensional porous polymer structure with recognition ability is obtained.

3- Template Removal: The target molecules are removed from the polymer with the help of suitable solvents and memories specific to the size and functional groups of the target molecule with its three-dimensional structure are formed.

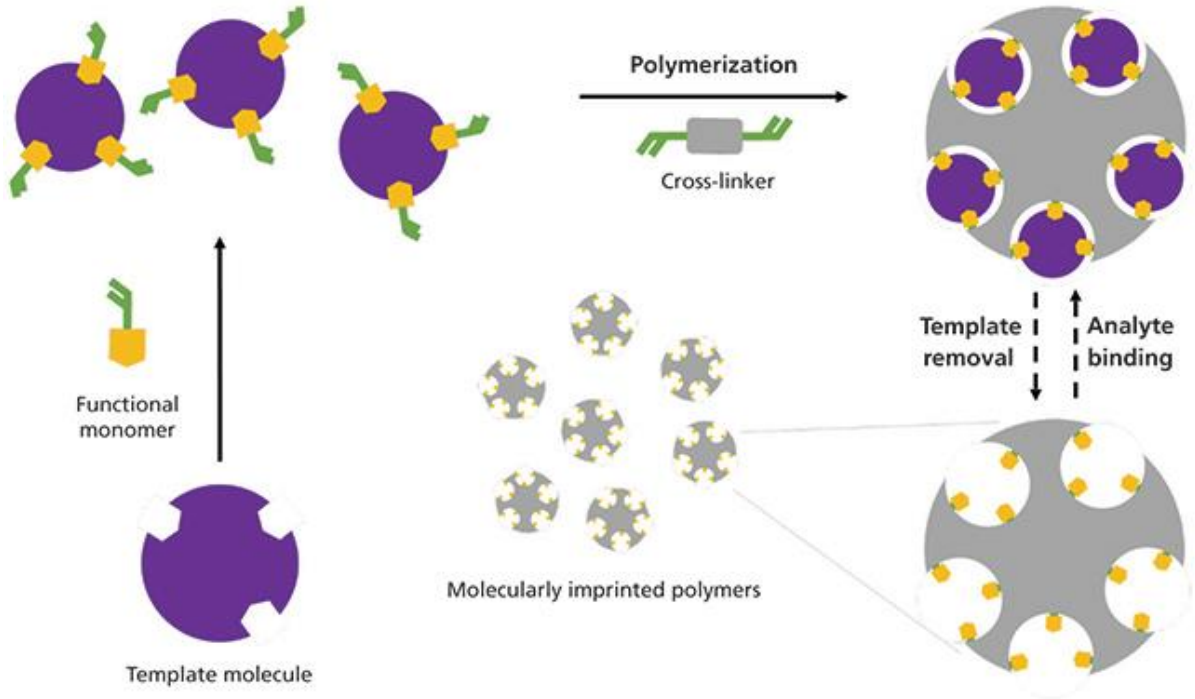


Figure 2.9. Schematic representation of the molecular printing method (LCGC North America, 2018)

2.4.1.1. Pre-complexation (Pre-organization)

The monomer with the appropriate functional group and polymerizable forms a complex (prepolymerization complex) by covalent interactions with the template molecule and non-covalent interactions such as hydrogen bonds, hydrophobic interactions, electrostatic interactions, van der waals interactions or metal ion coordination interactions. In this interaction, the three-dimensional structure and chemical properties of the template molecule are important. For ease of application, acrylic and methacrylic acids, their esters and amides have been the most widely used functional monomers.

Whether the imprinting molecule contains a group that can polymerize, whether it has a group that will prevent or slow down free radical polymerization (such as thiol group, hydroquinone group) and thermal stability are important parameters for imprinting. The solvents keep the

polymer mixture in same phase and and provides being the formation of pores in polymers.For this reason, the solvent is often referred to as "pore former".

The solvent is an important element to control the physical properties (pore structure, pore size distribution, swelling properties, strength and morphology) of the obtained polymer. Also, the solvent has an important role in pre-complex formation during printing, especially in the non-covalent approach. Generally, nonpolar and aprotic solvents such as toluene, chloroform are preferred, water or protic solvents can be used if hydrophilic forces are involved during complex formation. Crosslinkers primarily control the morphology of the imprinted polymer matrix, ensure determining the binding portions of the imprinted molecule or ion, and ensure high mechanical stability of the polymer matrix.

Covalent Printing: In this technique, before the polymerization process, strong and reversible covalent bonds are formed between the template molecule and the functional monomer. After the polymerization process, the covalent bonds are broken to remove the template molecule from the polymer matrix and the template molecule is removed from the polymer. The covalent bond is re-formed when the target molecule interacts with the polymer. Covalent bonds have high stability and this feature ensures homogeneous distribution of the binding sites. Although the presence of functional groups only in the binding site specific to the template molecule creates an advantage in terms of selectivity, the number of molecules to be suppressed by this method is limited. In addition, slow bonding and dissociation due to strong covalent bonds makes it difficult to reach thermodynamic equilibrium. (Wulff and Sarhan, 1972; Hansen, 2007; Alexander et al., 2006)

Non-Covalent Printing: The synthesis process takes place by linking the functional monomer with the template molecule through non-covalent interactions such as hydrogen bonding, electrostatic interactions and coordination bond formation in a suitable porogen (solvent). After polymerization, the template molecule is simply removed from the polymer by washing with a solution that can easily eliminate non-covalent interactions and the template molecule can be readily dissolved without any change in the polymer structure. Subsequently, rebinding of the template molecule with MIP occurs via non-covalent interactions. The template molecules can be removed from the polymeric structure by treatment with non-covalent interactions, aqueous solutions of acids and bases, or alcohol. Easy applicability, widespread and easy availability of

functional monomers, fast and reversible binding of template molecule, application of the technique to a wide variety of templates have made this method widely used in the preparation of MIPs. However, variable strengths of non-covalent interactions may result in heterogeneous binding sites that reduce rebinding activity, resulting in nonspecific binding sites. (Arshady ve Mosbach, 1981; Yan ve Row, 2006; Alexander et al., 2006).

The advantages and disadvantages of polymers prepared with covalent and non-covalent printing are summarized in Table 2.4.

Table 2.5. The advantages and disadvantages of polymers prepared with covalent and non-covalent printing

	Covalent Imprinting	**Non-Covalent Imprinting**
Synthesis of theMonomer-template molecule complex	Necessary, difficult	Unnecessary
Polymerization conditions	Flexible	Limited
Removal of template molecule after polymerization	Diffucult	Easy
Binding and separation of the molecule to be imprinted	Slow	Fast

2.4.1.2. Polymerization

Polymerization is when a large number of the same or different monomers combine with each other through a chemical process to form long chains. The monomer-template molecule complex is polymerized over the functional monomer using a suitable crosslinker and initiator (Figures 2.10 - 2.11). When preparing MIPs, free radical polymerization is generally used and azo-initiators are the most used materials for this purpose. The most commonly used azo-initiator is azobisisobutyronitrile (AIBN).

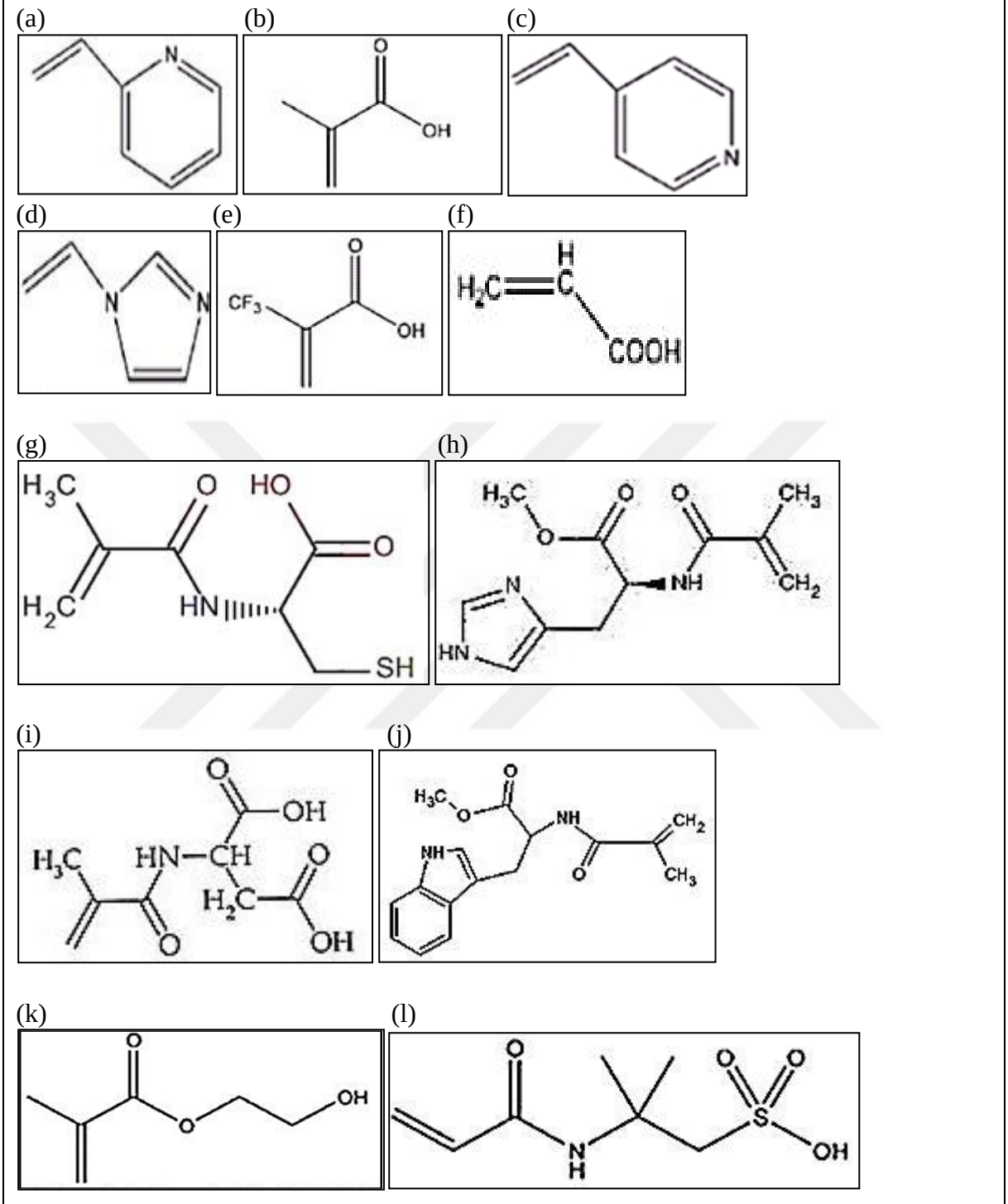


Figure 2.10. Some common monomers *.

* (a) 2-vinylpyridine (2-VPY), (b) methacrylic acid (MAA), (c) 4-vinipyridine (4-VPY), (d) 1-vinylpyridine, (e) trifluoromethyl acrylic acid (TFMAA) (f) acrylic acid, (g) methacroylamido cysteine (MAC), (h) methacroylamidohistidine (MAH), (i) methacrylamidoglutamic acid (MAGA), (j) Metacroylamidotriptophan methyl ester, (MATrp), (k) 2-hydroxyethyl methacrylate (HEMA), (1) 2-Acrylamido2-methylpropane sulfonic acid.

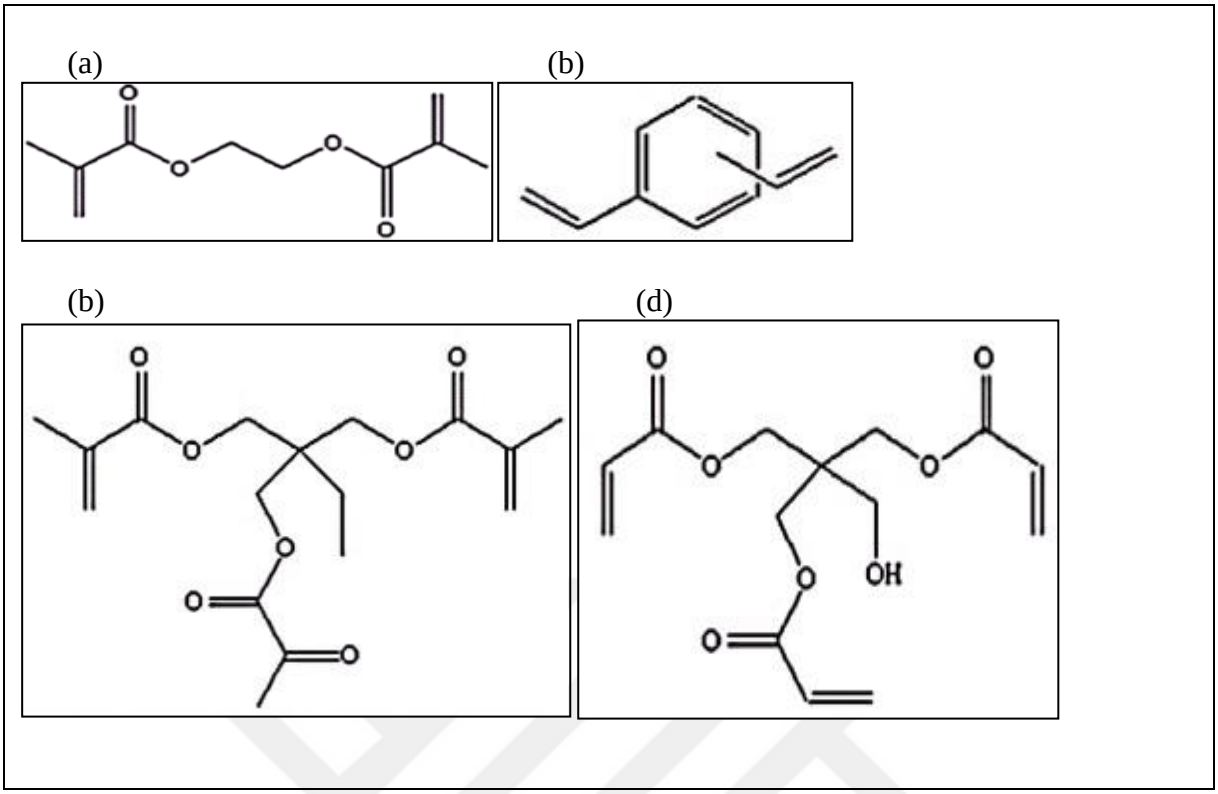


Figure 2.11. Some common crosslinkers *.
* (a) Ethyleneglycolmethacrylate (EGDMA), (b) divinylbenzene (DVB), (c) trimethylpropane trimethacrylate (TRIM), (d) pentaerythriol triacrylate (PETRA)

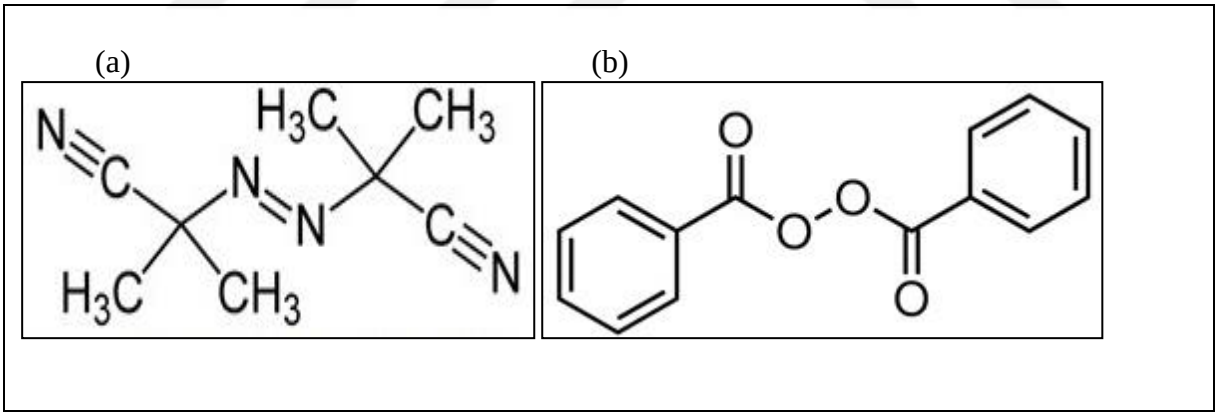


Figure 2.12. Some common initiators *.
*** (a)** Azobisizobutironitril (AIBN), **(b)** Benzoil peroksit (BPO)

It is recommended to use functional monomer at high concentrations in order to increase the imprinting efficiency. This leads to increase intramolecular and intermolecular interactions (especially if molecules are prone to such interactions) and thus the formation of the monomer-template complex and the imprinting efficiency are adversely affected.

2.4.1.3. Removal of the Template Molecule (Disassembly)

The template molecule is removed from the polymeric structure using suitable solvent or solvent mixtures. After the imprinted molecule is removed from the polymer, cavities are formed in the imprinted molecule that are compatible with the physical and chemical properties of the removed molecule. The imprinted molecule can be coated with polymer and used for selectivity and other purposes. (Tekin et al., 2012). While preparing MIPs, it should be taken into account that the functional monomer used for polymerization, the target molecule (template / analyte molecule / ion), the crosslinker, the solvent and the initiator are very important parameters in terms of properties such as the morphology, performance, imprinting efficiency of the obtained polymer. Therefore, relevant parameters should be optimized while preparing MIPs (Osman et al., 2013; Verheyen et al., 2011; Önnby et al., 2012; Tekin et al., 2009; Baur et al., 2007).

2.5. Determination of AFB1 by Molecularly Imprinted Polymer

The fact that aflatoxins are extremely toxic and carcinogenic, as well as contaminating many food and feed products around the world, has led to the need for rapid and reliable detection of these toxins. Although there are many methods for aflatoxin detection, in recent years, aflatoxin determination using molecular imprinting technology has made significant progress. Because MIP technology provides convenience in pre-analysis sample preparation, analysis time and specialized workmanship when compared to the currently used methods. In addition, it provides an important advantage in terms of being economical compared to methods. In addition, while MIPs recognize the analyte, the selectivity of the target molecule is quite high since both its 3-dimensional structure and its chemical bonds must be compatible. Various methods are applied while preparing MIPs, but the methods used for AFB1 determination can be listed as lumb-bulk polymerization, spherical MIP synthesis, surface-imprinted polymerization and electro polymerization.

In Lumb-bulk polymerization, template, functional monomers, crosslinkers are mixed in an initiator and a suitable solvent. An initiator such as light or thermal irradiation is required for polymerization to begin. After the polymerization process, the molded polymer must be crushed and ground in order to remove the template molecule more easily. Although it has advantages such as being cheap and simple, it also has disadvantages such as long and laborious processes after polymerization. In order to eliminate some of the disadvantages of this method, the in-situ

polymerization method has also been developed. Sergeyeva et al. developed an in-situ synthesis a nanostructured molecular imprinted membrane to be able to recognize AFB1. Ethyl 2-oxochlorcyclopentanoate was used as template molecule. The surface binding sites of the imprinted membrane selectively and sensitively recognized AFB1. The detection limit was 14 ng mL^{-1}, and the detection range was 14–500 ng mL^{-1}.

Although lumb-bulk polymerization gives satisfactory values in terms of selectivity and sensitivity, spherical MIP Synthesis was used to eliminate deficiencies such as low-rate mass transfer and poor site accessibility. While preparing spherical MIPs, methods such as suspension polymerization, emulsion polymerization and precipitation polymerization have been developed to make AF determination more efficient. Suspension polymerization is a heterogeneous radical polymerization process that uses mechanical agitation to mix a monomer or monomer mixture in a liquid phase such as water, during which the monomers polymerize forming polymer spheres. The monomer is polymerized by an initiator dissolved in the monomer. Stabilizers such as polyvinyl alcohols are often added to the continuous phase as a suspending agent to enhance its stability. Song et al., prepared MIP by suspension polymerization, has good detection of AFB1 in soy sauce with a recovery rate of 96%. The method has a linear coefficient of 0.9994 and a detection limit of 0.05ng mL^{-1} with a linear range of 10-1000ng mL^{-1} (Song et al., 2019).

Emulsion polymerization is a type of radical polymerization that usually starts with an emulsion containing water, monomer and surfactant. It has been developed to obtain smaller MIP particles. Wei et al. developed AF-MIP microspheres using emulsion polymerization. AFB1 was used as template, methacrylic acid as functional monomer, ethylene glycol dimethyl methacrylate as crosslinking agent and AIBN initiator. The MIPs exhibited good recognition and selectivity for AFs B1 and M1. The detection limit and quantification limit for AF M1 and B1 are 0.05 and 0.16mg kg^{-1}, respectively (Wei et al., 2015).

Precipitation polymerization is a heterogeneous polymerization process that begins initially as a homogeneous system in the continuous phase, where the monomer and initiator are completely soluble, but upon initiation the formed polymer is insoluble and thus precipitates. After precipitation, the polymerization proceeds by absorption of monomer and initiator into the polymer particles. Rui et al. prepared a surface MIPs which have good selective and

sensitive recognition using precipitation polymerization based on molecular surface imprinting techniques (Rui et al., 2019).

In Surface-imprinted polymerization technique, materials such as silica gel, magnetic spheres, carbon materials, or quantum dots (QDs) are used as surface areas. These surface areas are coated in a thin layer with the molecularly imprinted polymer. This leads to positive changes such as the formation of more binding sites, an increase in the absorption capacity, and an increase in the mass-transfer ratio. It also allows for faster elution and reduced solvent usage, which is advantageous for reuse (He et al., 2020).

Electropolymerization is the standard oxidation method to obtain electrically conductive polymers. Thanks to this method, a polymeric film is obtained on the working electrode surface. Gold nanoparticles (AuNPs) have unique electrochemical properties, high chemical stability, favorable biocompatibility, and can be easily functionalized, so they are widely used for the fabrication of sensors (Xue et al. 2019).

Biosensors

Due to the high selectivity of MIPs for analytes, their use in the sensor field has greatly improved the detection capability. A physical or chemical signal is generated when MIPs capture the target molecule. This signal is converted into a quantitative value by means of a converter and using this value, the relevant molecule is determined. Biosensors show promise for AFB1 detection because they give fast results and have high sensitivity. Many studies have been conducted for AFB1 Detection using molecular imprinting and biosensor technology (Liu et al., 2020; He et al., 2020). Guo et al. combined molecular imprinting and quantum dots (QD) technology to detect AFB1 with a fluorescent detector. The method has also been successfully applied in real samples. (Guo et al., 2019). Wang et al. developed an electrochemical sensor for the detection of AFB1 by modifying a glassy carbon electrode with functional multi-walled carbon nanotubes (MCNTs), Au/Pt bimetallic nanoparticles (Au/PtNPs), and printed films. Sensor results were analyzed by CV, differential pulse voltammetry and electrochemical impedance spectroscopy. Jiang et al. developed another sensitive electrochemical molecular imprinted sensor by electropolymerization of PATP-functionalized gold nanoparticles using AF B1 as the template molecule. Template cavities using π–π interactions were able to selectively recognize and capture AF B1 molecules (Jiang et al., 2015).

3. MATERIALS AND METHODS

3.1. Materials

Aflatoxin B1 was supplied from Acros Organics (Fisher Scientific, US). 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), Azobisizobutironitril (AIBN), 4-vinylbenzoic acid (VBA), iron powder, methanol (LC grade), acetonitrile (LC grade), dichloromethane, anhydrous sodium sulphate, potassium bromide, nitric acid, AFB2, AFG1 and AFG2 were purchased from Sigma (St. Louis, MO). All water used in the experiments was purified using a Millipore Direct-Q 3 UV Ultrapure (Type 1) water purification system (MerckAG, Darmstadt, Germany).

3.2. Methods

In this study, AFB1 imprinted polymers were synthesized for selective aflatoxin adsorption from aqueous media and AFB1 selection from liver mixture.

3.2.1. Synthesizing AFB1 Imprinted Polymers

200 nmol of AFB1 dissolved in 40% methanol in DI water (in water bath 40 °C for 15 minutes), then VBA monomer was dissolved in it to have AFB1-VBA pre-complex in 1:2; 1:3; 1:5 molar ratio (nAFB1: nVBA) and interacted 2 hours. HEMA (2000 μL), as monomer, and EGDMA (500 μL), as crosslinker, were poured into the glass test tube, then pre-complex (200 μL) was poured in it and 300 mg of iron powder (< 20 nm sized) was used in order to obtained magnetic particles. 20 mg of AIBN was added in to the final mixture and vortexed. Then polymerization reaction was initiated at 70 °C into water bath and the reaction was completed in 2 hours. Polymerization procedure were achieved for three precomplex (1:2; 1:3; 1:5) one by one, to obtain three different polymeric particles, which are called magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$. The obtained monolithic polymer was taken of the glass test tube and it was grounded (Ika-Werke, M20, Germany) until the particles which have <500 nm diameter size obtained.

After washing and drying, particles <500 nm in size and smaller were collected using woven wire mesh sieves (Retch, Mesh size 125 mm-20 μm, Germany). The resulting particles were used to evaluate the adsorption properties of AFB1-MIP. NIPs were imprinted to analyze the

non-specific adsorption predisposition of polymers. NIPs were imprinted with the same AFB1-MIP preparation method without the use of AFB1 molecules. It is very important to keep in mind that all AFB1 included solutions were protected from light and stored in dark until use.

3.2.2. Target AFB1 Removal Molecule AFB1-MIP

First of all, the obtained AFB1-MIP was washed with 200 mL DI for 2 h for removing unreacted monomers and impurities. This step was repeated 3 times and the DI was changed in each cycle. 1 g of dry AFB1-MIPs were interacted with methanol in ultrasonic bath at 40 °C for approximately for 2 hours and this step repeated until no AFB1 (template molecule) detected in elution solution, as well. The initial value of the desorption solution was measured using Flourescence spectrometer (Agilent, Cary Eclipse, US) at 365 nm excitation and 430 nm emission wavelenghts. The amount of removed AFB1 was calculated by measuring the intensity value of the elution solutions in each step by using following equations;

$$Q_{AFB1} = CAFB1 \times V/mdry \quad \text{Eq. (1)}$$

$$ARR\ (\%) = (QAFB1/Qi)x100 \quad \text{Eq. (2)}$$

Q_{AFB1} is removed AFB1 capacity in ng/g and AF% is AFB1 removal ratio.

The CAFB1 is the AFB1 concentration in elution solution in, ng/mL; V is volume of elution solution in mL and m is weight of dried AFB1-MIPs in g. QAFB1 is AFB1 removal amount in ng/g and Qi is AFB1 amount in polymerization receipt in ng/g. Eq. (2); Qi represents amount of AFB1 (ng/g) used in polymerization process.

3.2.3. Characterization Studies

SEM, BET, swelling tests and particle size analysis were used for magAFB1-MIPs and magNIPs characterization. In swelling test the swelling behavior of magnetic particles were determined via calculating swelling ratio (SR); firstly the dry particles were weighed and recorded (Wo), then they were put in water (DI) in a vessel and left at room temperature until reach equilibrium and the swollen particles were weighed and recorded, (Ws). Swelling ratio was calculated according the Eq 3;

SR (%) = [(Ws – Wo)/Wo] x 100 Eq. (3)

Polymerization efficiency was determined by calculating the percentage of polymerization yields of magAFB1-MIPs and magNIPs. In order to determine the polymerization efficiencies of the particles, the total amounts of reagents used in polymerization (m_t) and the weight of dry particles (mdry) were used.

Polymerization yield (%) = (m_{dry}/m_t) x 100 Eq. (4)

The surface area of magAFB1 particles were measured with multi-point N2 adsorption method of Brunauer-Emmett-Teller (BET) analysis (Flowsorb II 2300, Micromeritics Instrument Corp., Norcross, USA).

The surface structure of synthesized magAFB1-MIP and magNIP monoliths (before grinding) were examined using scanning electron microscopy (FEI, Quanta 650 Field Emission SEM, USA). Initially the a pieces of monoliths were dried in a freeze dryer (Biobase, BK-FD 10P, China) and SEM images were taken from their cross sectional part at different magnifications after coating the samples with gold palladium (40:60) under vacuum.

Size analysis of magAFB1-MIP and NIPs nanoparticles obtained by grinding monolithic materials was performed with Nano Zetasizer (NanoS90, Malvern Instruments, London, UK). Zeta size analysis can determine the particle size in the range of 0.3 nm-5μm by measuring with the light scattering technique. For dimensional analysis, 3 mL of magnetic nanoparticle solution was prepared in measurement cuvette and determined at 25°C.

3.2.4. AFB1 Adsorption Studies

Adsorption of magAFB1-MIP-/magNIPs were carried out using 40% methanol in DI water solutions of AFB1 in batch system. Effects of equilibrium AFB1 concentration, temperature, interaction time, were studied on adsorption of AFB1 and compared with magNIP particles for control.

Firstly 100 mg of magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$ particles interacted with 1 ng/mL AFB1 solution 40% metanol:water. And the adsorption behavior of the

synthesized particles were compared for discussing the effect of the mole ratios of the template to functional monomer.

The equilibrium AFB1 concentration was varied between 0.05 ng/mL to 10 ng/mL. 6 mL of each solution was prepared and 1 mL of that solution was taken to determine the initial AFB1 concentration. The rest of the 5 mL of AFB1 solution was interacted with 200 mg of dry magAFB1-MIPs by rotating for 2 h. After the interaction completed the particles were separated from the solution magnetically and the 1 mL of the sample was then taken to measure final AFB1 concentration. All measurements were done using fluorospectrometer (Agilent, Cary Eclipse, US), and AFB1 adsorption capacities of the particles was measured using, Eq (5),

$$Q = [(Ci-Cf)] \times V/mdry \qquad \text{Eq. (5)}$$

Ci is the initial AFB1 concentration in ng/mL. Cf is the final AFB1 concentration in ng/mL. V is volume of adsorption solution and mdry is the dry weight of magAFB1-MIP/magNIPs.
The temperature effect on AFB1 adsorption was studied. The temperature was increased from 4 °C to 40 °C at a certain 0.2 ng/mL AFB1 concentration for 2 hours. The adsorption time was also studied to determine the effect of adsorption time on AFB1 adsorption amount. In here the 5 mL 0.2 ng/mL of AFB1 was prepared and the 200 mg of dry magAFB1-MIP particles were added into this solution. Then 500 μL of the prepared solutions were taken at 0^{th}, 5^{th}, 10^{th}, 15^{th},30^{th},45^{th},60^{th}, 90^{th}, 120^{th} minutes and measured using flouro spectrometer. AFB1 concentration in each step was measured as describe in above according to Eq 5. It is important to note that all experiments were repeated at least 3 times and the avarege of the results obtained were used.

3.2.5. Desorption and Repeated Use

Methanol was used as desorption solution. The 200 mg of dry magAFB1-MIPs interacted with 0.2 ng/mL of AFB1 solutions, and the adsorption amount was recorded as decribed in section 3.2.4. Then magnetic particles were separated from this solution and incubated with 5 mL of methanol for 2 hours, then the particles were separated magnetically and the desorbed AFB1 from the magAFB1-MIPs was measured fluorospectrometer (Agilent, Cary Eclipse, US). The adsorbed and desorbed AFB1 values were used to calculate the desorption amount (DR). Related equation Eq. (6) is indicated below. After each desorption process, the particles are

washed with of 40% methanol in water before reuse. This cycle was repated 10 times to describe the reusability of the synthesized magAFB1-MIPs and NIPs.

$$\text{DR}\ (\%) = \frac{\text{Amount of the desorbed AFB1x 100}}{\text{Amount of the adsorbed AFB1}} \qquad \text{Eq. 6.}$$

3.2.5. Selectivity Studies

AFB2, AFG1 and AFG2 were chosen as competitor molecules for the purpose of testing of the selectivity of the magAFB1-MIPs by comparing mag-NIPs. AFB1, AFB2, AFG1 and AFG2 interacted with magAFB1-MIP and NIP. The obtained values were checked against each other and the selectivity of AFB1-MIP was evaluated. While performing selectivity studies, 40% methanole in DI water was prepared for 0.2 ng/mL AFB1 and competitive molecule solutions. The synthesized magAFB1-MIP and NIP particles were interacted with AFB1, AFB2, AFG1 and AFG2 solution for 2 hours at room temperature. Amount of molecules absorbed by magAFB1-MIP and NIPs were used in order to calculate selectivity constants. These values were evaluated to understand the detection of selectivity of AFB1. The adsorption amounts were evaluated using HPLC by applying following procedure; AFB1, AFB2, AFG1 and AFG2 were quantified using the Shimadzu Class10AVP Series HPLC system equipped with a fluorescence detector (Shimadzu, Kyoto, Japan) set for wavelengths 360 nm and 420 nm for excitation and emission, respectively. Separation was obtained on an ACE C18 reversed-phase column (25 cm × 4.6 i.d.; 5 µm particle size; ACE, Aberdeen, UK) at 35 °C. The mobile phase consisted of distilled water: acetonitrile:methanol (6:2:3, v/v/v) with 120 mg/L of KBr and 350 µl/L of HNO_3. The flow rate was 1 ml/min and injection volume was 100 µl. The aflatoxins were derivatised by a post-column derivatisation process (Kobra Cell, R-Biopharm AG) and data were processed by Class-Vp 5 software (Shimadzu).

3.2.6. AFB1 Detection in Liver Tissue

In order to study magAFB1-MIP adsorption efficiency from real sample, 200 g of liver tissue was provided from the supermarket. Initially tissue was grounded by using tissue grinder and 2 g ± 0.02 g weighed into centrifuge tubes and spiked with AFB1 with two concentrations, 2 ng/g and 10 ng/g. Spiked samples were left in the dark for two hours. After that AFB1 extraction from liver tissue were carried out according to method described in Cui et al. (2017). Briefly, AFB1 spiked samples were ultrasonicated for 10 min after the addition of twenty milliliters of dichloromethane followed by shaking 1 h on a shaker. Anhydrous sodium sulfate (2 g) was

added and centrifuged at a speed of 10,000 rpm for 5 min. Finally, 10 ml of supernatant was collected and dried in a water bath at 50 °C. Following to the extraction procedure magAFB1-MIP adsroption studies was conducted according to the method described in section 3.2.4. AFB1 adsorption studies.

4. RESULTS AND DISCUSSIONS

4.1. Characterization Studies

Percentage yield and swelling tests of both mag-AFB1-MIP and magNIPs were studied. The ratio of the total dry weight of the synthesized polymer to the total amount of monomer involved in the polymerization reaction is expressed as the efficiency calculation of polymeric systems. For AFB1-MIPs, the percentage yield was determined as 98% and the swelling rate was determined 81%. Yield percentage for NIP was determined 97% and swelling rate was determined 80%.

Surface area of both magAFB1-MIP and magNIP was determined using BET method and found 117 m^2/g and 105 m^2/g for magAFB-MIP and magNIP respectively. These results showed that the molecularly imprinted polymers have higher surface area.

The surface morphology of the synthesized particles was investigated by SEM studies. Figure 4.1A shows the surface morpholohy of magAFB1-MIP while Figure 4.1B shows magNIP.

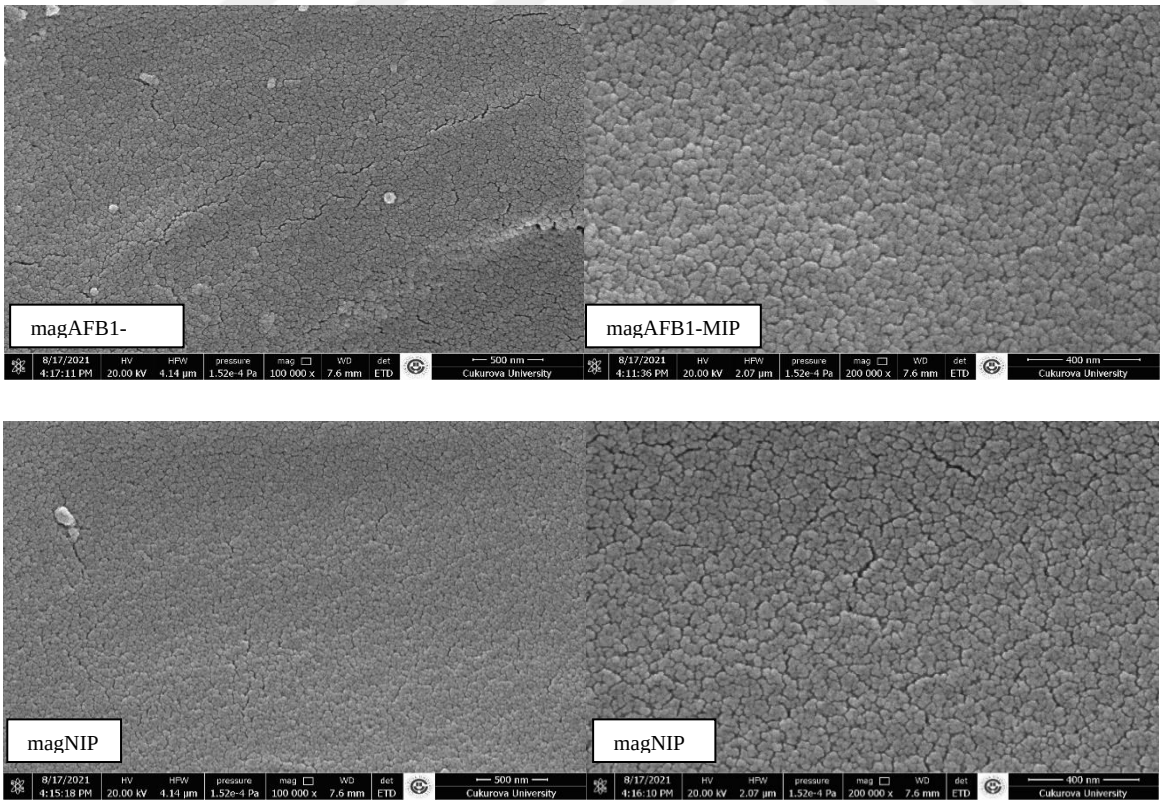
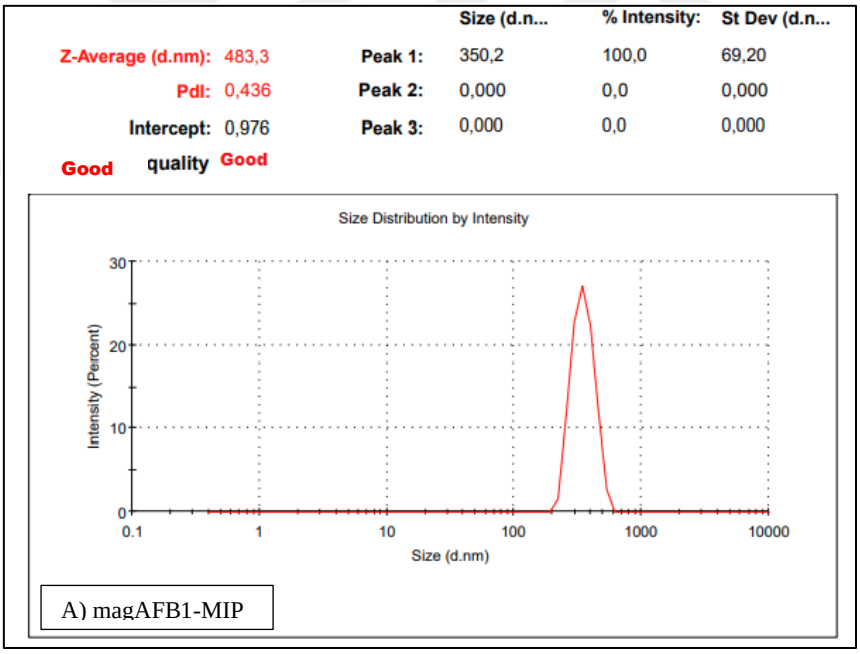


Figure 4.1. SEM photos A) SEM photos of magAFB1-MIP and B) magNIP

As it can be seen from the SEM images both magAFB1-MIP and magNIP monoliths have a rough and porous structure. As can be seen from these figures the porous structure contributed to the high surface area of the syntesized magnetic nanoparticles.

The zeta size analysis results of the magnetic nanoparticles are shown in Figure 4.2. In this analysis the density of deionized water was used as 0.88 mPa.s and the refractive index was 1.33 in the size measurement performed by zeta size analysis. The light scattering signal was calculated as the particle number (particle number/s). It is clearly seen from the graphs that the particles are obtained in nano size, but it is observed that the size distributions are not very sharp as a result of sieve separation. As a result of the measurements obtained, it was observed that the size range of magAFB-MIPs was found within the range of 200-800 nm and the average size was found as 483 nm. On the other hand the size range of mag-NIPs was found as 200-1000 nm and the average size is measured as 496 nm.



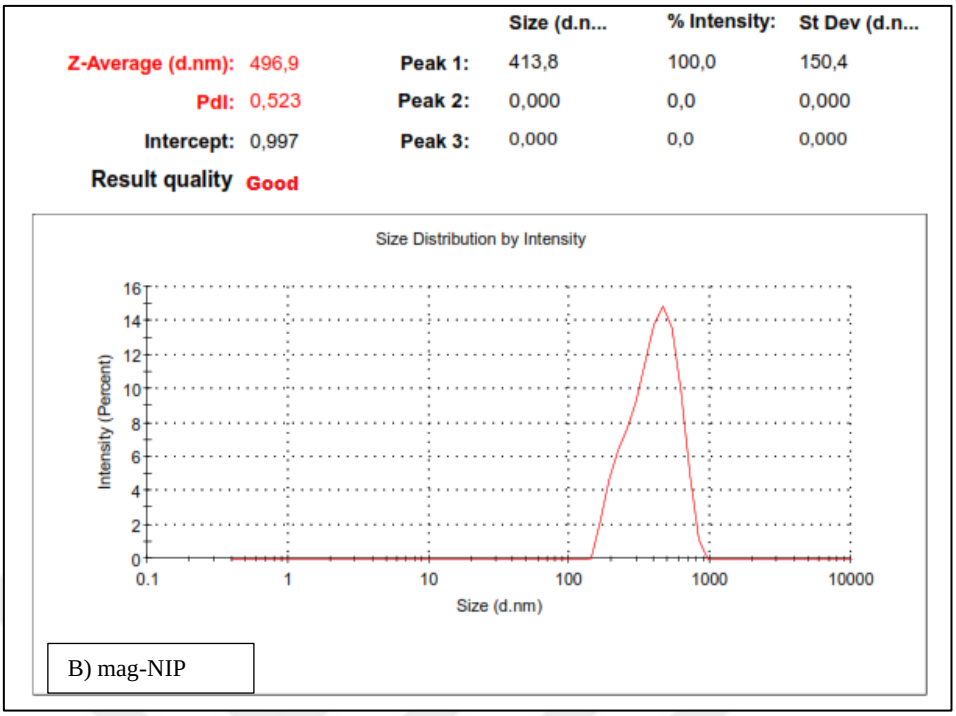


Figure 4.2. Particle size analysis results of A) magAFB1-MIPs B) magNIPs

Moreover optical photographs of magAFB1-MIPs and NIPs were taken and given in the Figure 4.3 and 4.4. Magnetic properties of the particles also can be seen in these photos. These optic photos showed the obtained monolithic polymer structures before and after grinding. As can be seen there is no big differnce between the MIP and NIPs. After grinding, the magAFB1-MIPs and NIP powders were also shown in this figure and there is no observable difference between the obtained nanoparticles. When magnetic field applied magAFB1-MIPs and NIP powders were attracted by the magnet. Because of the magnetic peroperty the particles move to the magnet and during adsorption studies the supernatant can be taken easily without centrifugation.

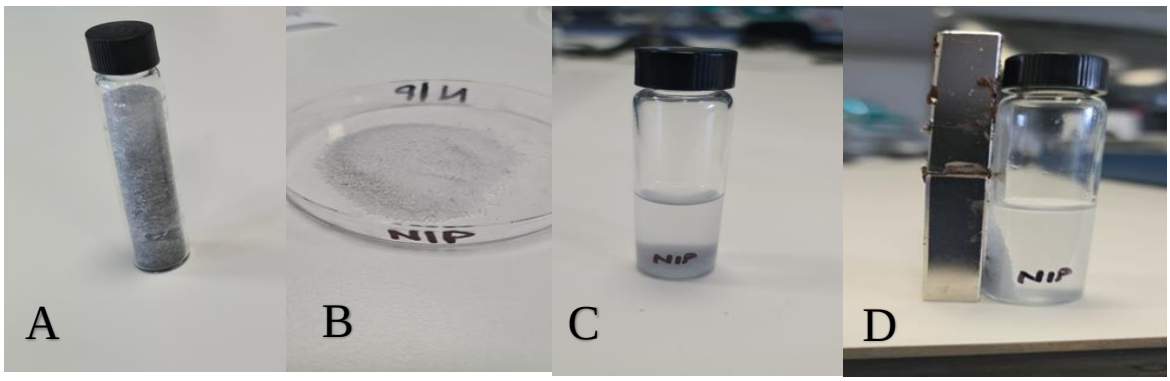


Figure 4.3. Optical photographs of NIPs. A) Monolithic polymer B) Dried and ground mag NIPs C) In methanol-water solution D) magNIP in magnetic field

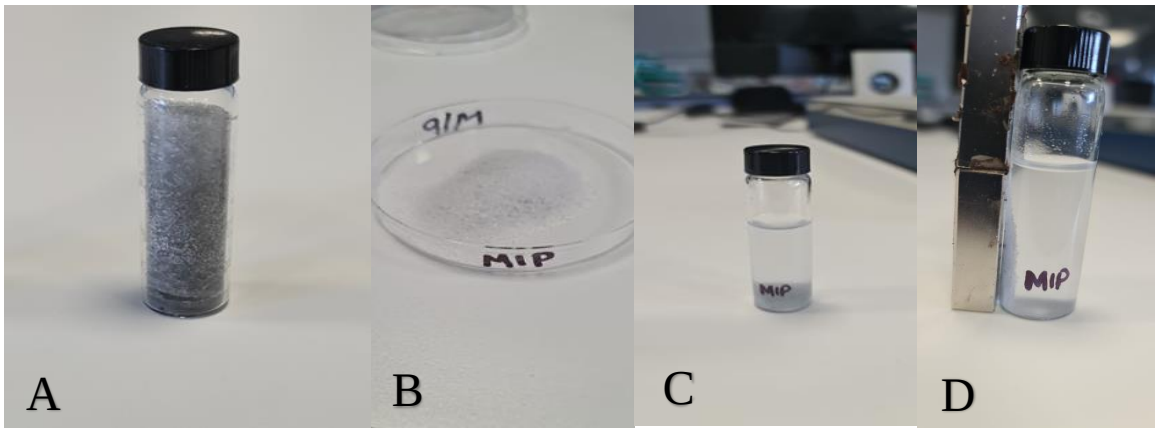


Figure 4.4. Optical photographs of magAFB1-MIPs A) Monolithic polymer B) Dried and ground magAFB1-MIPs C) In methanol-water solution D) magAFB1-MIPs in magnetic field.

4.2. Template Removal Studies

The AFB1 removal studies were achieved for synthesized three polymer magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$, separately. The template removal ratio (%) of three polymers were calculated and given in the Table 4.1.

Table 4.1. Polymer nomination; precomplex mole ratio and template removal ratios

Polymer	Precomplex mole ratio AFB1:VBA	Template removal ratio %
magAFB1-$MIP_{1:2}$	1:2	93%
magAFB1-$MIP_{1:3}$	1:3	91%
magAFB1-$MIP_{1:5}$	1:5	87%

The obtained results can be explained as the interaction between the the AFB1 and VBA. As expected, when the target molecule is surrounded by more functional monomers, it becomes more difficult to break the interaction between them. According to results obtain the template removal ratios are nearly the same, for 1:2 and 1:3 mole ratios but for 1:5 it was decreased slightly. It can be concluded that when the AFB1 surrounded with the VBA in the mole ratio of 1:5 the secondary interactions between the AFB1 and VBA ie, hydrophobic interactions etc, achived strongly and the desorption agent can not remove AFB1 from its cavities efficiently.

It is clear that the desorption agent can desorb AFB1 from the cavities more efficiently, when the AFB1 to functional monomer ratios are 1:2 and 1:3 due to the weak intreractions.

4.3. Adsorption Studies

In order to determine optimum adsorption conditions of synthesized magAFB1-MIP, the results of AFB1 adsorption from the aqueous solution were given in this section. The effects of AFB1 concentration, ambient temperature and interaction time on the adsorption capacity of magAFB1-MIPs were investigated. Moreover to determine the reusability of magAFB1-MIP, adsorption and desorption studies carried out using same magAFB1-MIP 10 times, repeatedly. AFB1 Finally selectivity of the magAFB1-MIP was analyzed calculating the selectivity coefficients against competitor molecules of AFB2, AFG1 and AFG2.

4.3.1. Effect of Changes in AFB1 Concentration on AFB1 Adsorption

We examined the adsorption efficiencies of magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$ particles. For this purpose 200 mg of dry magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$ were interacted with 1.0 ng/mL AFB1 solution separately and the AFB1 adsorption amouns were compared. The AFB1 adsorption amounts were found as 24, 33, 20 ng/g for magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$, respectively. It is observed that the results are in agreement with section 4.2. The template removal ratios were obtained 93, 91 and 87 for the magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$ respectively. But the adsorption amount- rebinding amount- of the template obtained higher for magAFB1-$MIP_{1:3}$ then others. So, at this stage of the study the magAFB1-$MIP_{1:3}$ is selected as working polymer due to high AFB recognition capacity and named as magAFB1-MIP rest of this report.

In order to investigate the effect of equilibrium AFB1 concentration on AFB1 adsorption, 7 different concentrations of AFB1 solutions were prepared. AFB1 was added to DI water containing 40% methanol at 0.05, 0.1, 0.5, 1, 2, 5 and 10 ng/mL concentrations, respectively. The obtained solutions of different concentrations were treated with both magAFB1-MIP and magNIP particles in the same environment under the same conditions. The particles adsorbed almost all of the AFB1 at all concentrations, as the concentration increased, the percentage of adsorption increased due to driving force even at 10 ng/mL, particles did not reach the saturation. There is no need to work at higher concentrations here, because the amount of AFB1 in the samples to be analyzed is far below this value. All concentrations were also examined

using magNIPs the adsorbed AFB1 was calculated as 44.6 ng/g at 10 ng/mL, the same adsorption behavior observed for magNIPs. The particles adsorbed AFB1 by increasing equilibrium AFB1 concentration, but there is not saturation point was observed for magNIP as well. The effect of AFB1 concentration on AFB1 adsorption amount for magAFB1-MIPs and NIPs was given in the Figure 4.5.

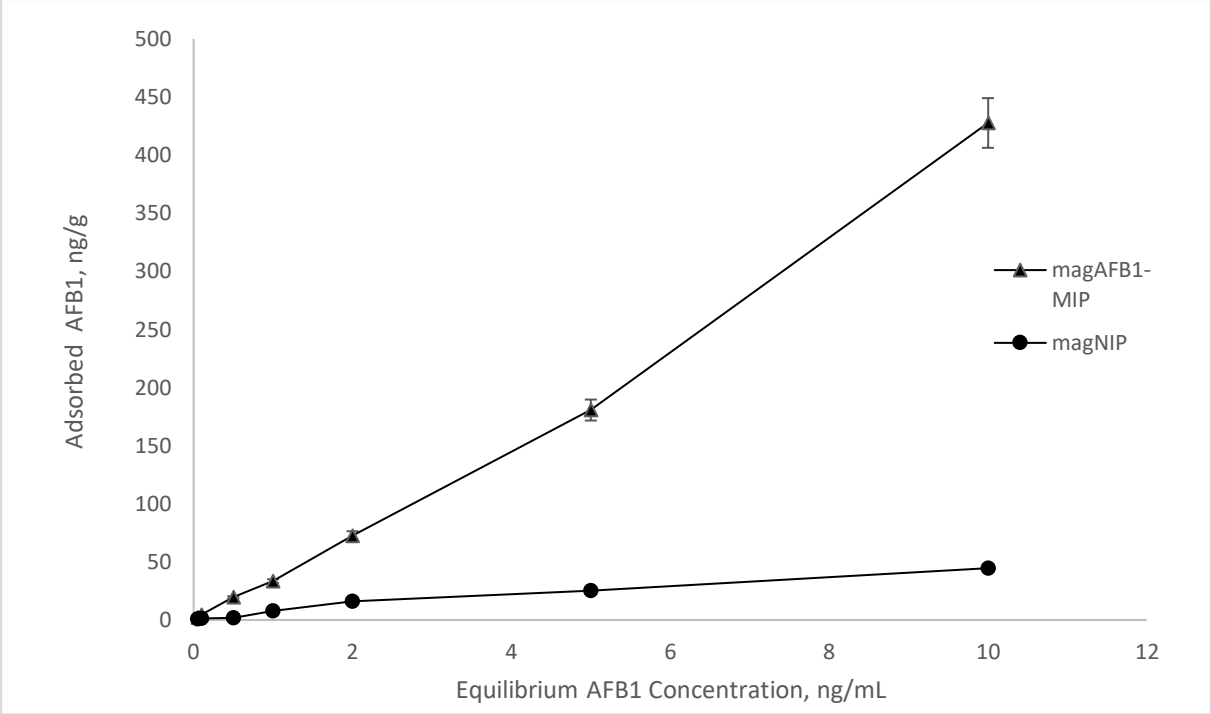


Figure 4.5. The effect of equilibrium AFB1 concentration on AFB1 adsorption amount. V: 5 mL, T: 25 ℃, time, 120 min, mdry of magAFB1-MIP and mag NIP: 200 mg.

4.3.2. Effect of Changes in Time on AFB1 Adsorption

In order to determine the effect of interaction time on AFB1 adsorption, AFB1 was treated with magAFB1-MIP and the sasmples were collected at certain time periods. Figure 4.6. shows the adsorption behavior of AFB1 particles with respect to time. The maximum AFB1 adsorption amount was reached at the 60th minute and the amount of adsorption remained constant after this point and according to this results all adsorption experiments were set as 60 minutes.

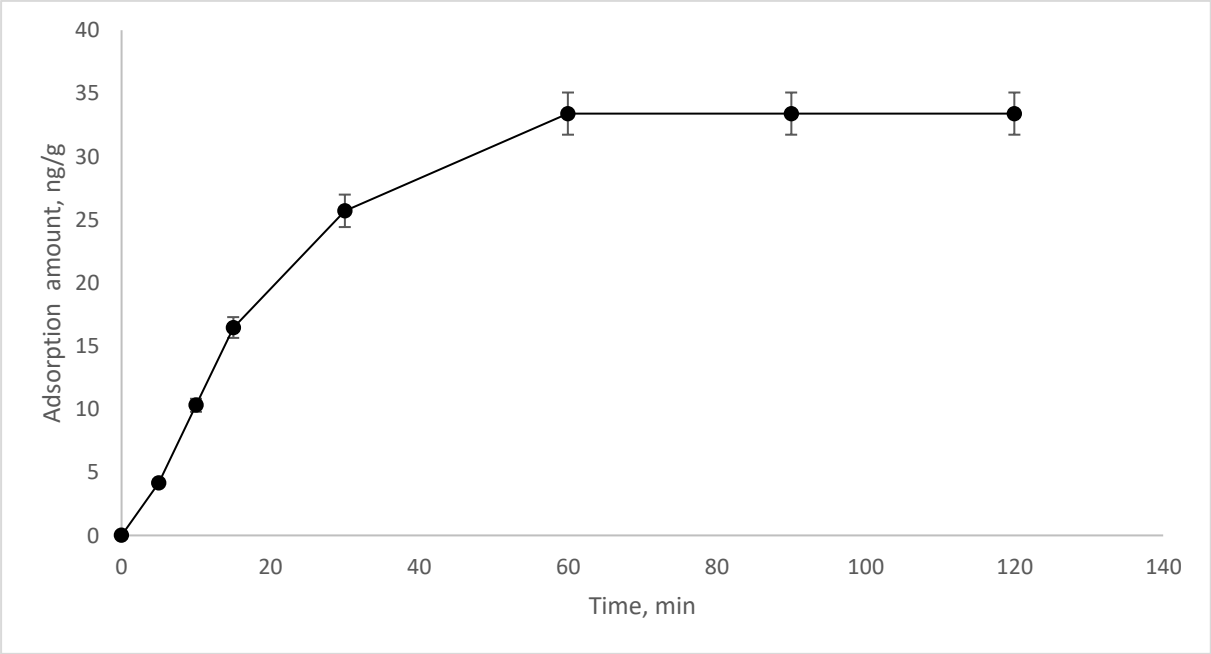


Figure 4.6. The effect of interaction time on AFB1 adsorption amount. Equilibrium AFB1 concentration: 1 ng/mL, V: 5 mL, T: 25 ℃, mdry of magAFB1-MIP and magNIP: 200 mg.

4.3.3. Effect of Temperature on AFB1 Adsorption

The effect of temperature on AFB1 adsorption amount was studying by changing temperature while keeping other conditons constant. As can be seen from Figure 4.7., increasing temperature from +4 to 40 °C has positive effect on AFB1 adsorption amount due to the hydrophobic interactions between AFB1 and VBA. The increase in adsorption capacity directly depends on the type of interactions between the monomer and AFB1 molecules. In this study the functional monomer VBA has a hydrophobic character, and it was choosen the hydrophobic properties of the AFB1. Hydrophobic interactions are interactions that proceed with an increase in entropy; temperature increase also affects these interactions to increase. Mainly in the hydrophobic effect is that nonpolar molecules interact via intermolecular hydrophobic groups or intramolecular interactions in aqueous environments. The increase in temperature strengthens the interactions between the benzene ring in the VBA structure and the AFB1s cyclopentane and cyclohexane groups via hydrophobic interactions and causes an increase in entrophy of water molecules, allowed reach higher adsorption capacities. In this study due to to easy processing the rest of studies was carried out at room temperature.

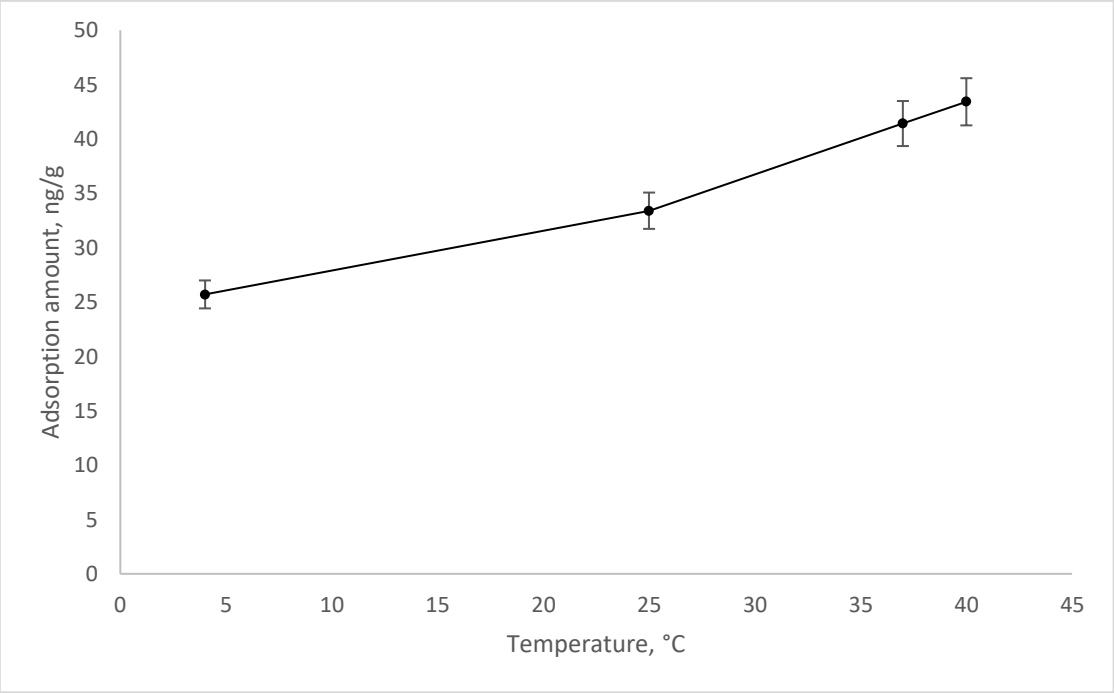


Figure 4.7. The effect of temperature on AFB1 adsorption amount. Equilibrium AFB1 concentration: 1 ng/mL, V: 5 mL, time: 120 min, mdry of magAFB1-MIP and mag NIP: 200 mg.

4.4. Selectivity Studies

The selectivity tests were studied to show selective AFB1 adsorption behavior of magAFB1-MIP in presence of competitor molecules. In this study the AFB2, AFG1 and AFG2 were choosen as competitor molecules (Figure 4.8.). Although the structure of the competitors have very similar structure with the target AFB1 molecule, all molecules were interacted with the particles at the same time. The equal amounts of molecules were dissolved in 40% methanol in water and interacted with 100 mg of dried magAFB1-MIP and magNIP separately.

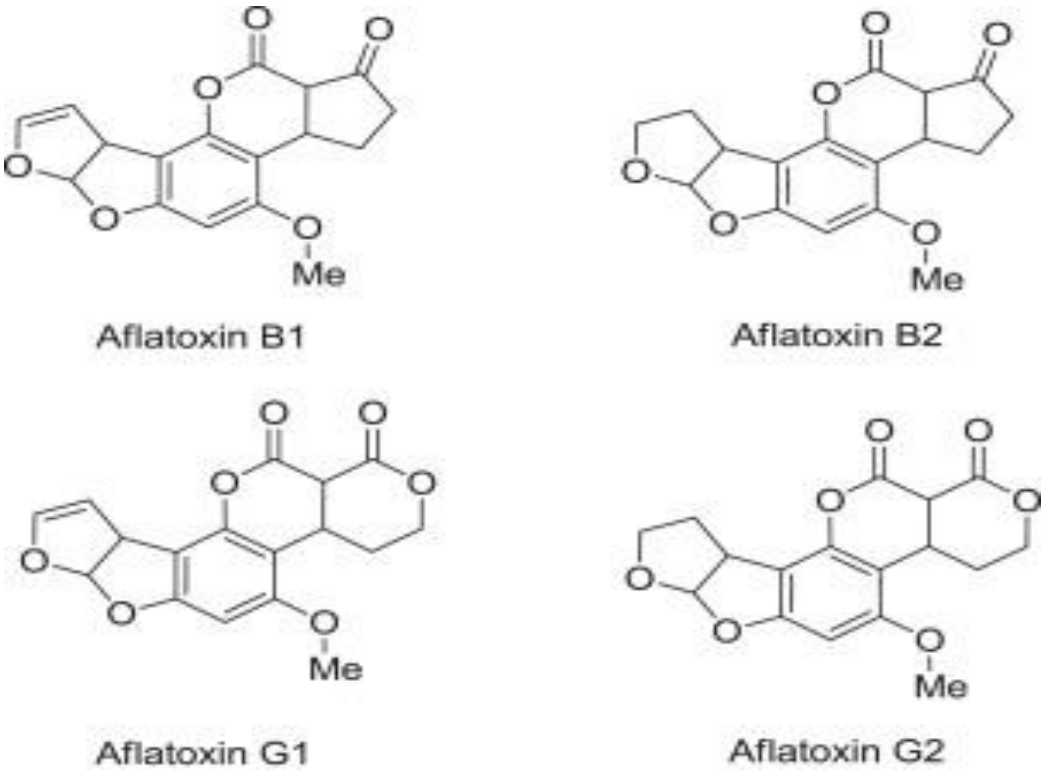


Figure 4.8. Chemical structures of competitor molecules

The adsorption amounts were evaluated according to HPLC studies and obtained chromatograms shown in Figure 4.9. When the chromatograms of the molecules given as a quaternary mixture are examined, it is clearly observed that the concentrations of the molecules are reduced due to separation and adsorption at the retention times. Figure 4.9A. is the chromatogram before interacting with magAFB1-MIP with competing molecules, and Figure 4.9B shows the post-interaction chromatogram. In Figure 4.9C, two chromatograms are overlayed for better observation of the concentration difference. With the area calculation made from the chromatograms, the concentration of each molecule was calculated separately and the results were shown in Figure 4.10. and it is given in Table 4.2.

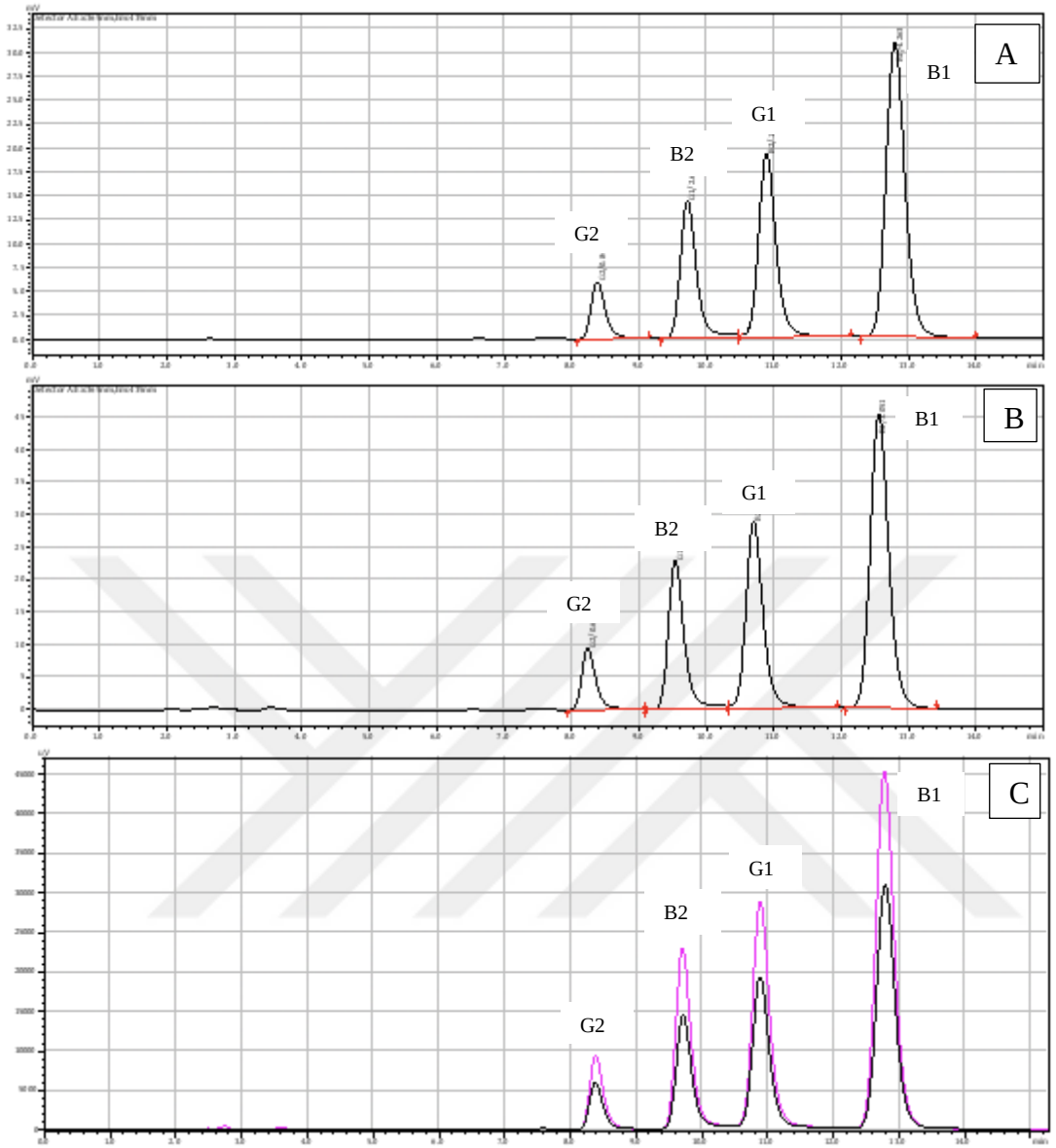


Figure 4.9. HPLC chromatagrams A) Initial solution B) Final solution C) Overlayed peaks of chromatograms.

Selectivity constants shows the selectivity properties of m molecularly imprinted materials. According to the adsorption amounts of all molecules under the same condititions the selectivity constants; distribution coefficient (Dc), imprinting factor (IF), selectivity coefficients (k) can be calculated. The distribution coefficient (Dc) expressed the distribution of adsorbed molecules in the adsorption soluion given in mL/ng and calculated as follows;

$$Dc= ((C_i-C_f)/C_f)*V/m_{dry} \quad \text{Eq. (7)}$$

In here C_i and C_f are concentrations of adsorbed molecules before and after interacted with magAFB1-MIP and magNIP respectively. V is volume in mL and the mdry is the dry mass of the magAFB1-MIP and magNIP particles in g.

Imprinting factor gives valuable information by comparing adsorption behavior of moleculary imprinted polymers with non imprinted polymers against adsorbed molecules. (IF) can be expressed as;

$$IF = \frac{\text{Dc magAFB1}-\text{MIP}}{Dc\ magNIP} \qquad \text{Eq. (8)}$$

The selectivity coefficient k' expresses the selectivity of MIP with respect to other competior molecules. k' is calculated by comparing the distribution coefficient of magAFB1-MIP particles against target AFB1 molecule with other competitors respectively.

$k = Dc_{magAFB1\text{-}MIPs}$ template/ $Dc_{magAFB1\text{-}MIPs}$ competitor Eq. (9)

The competitive molecules were choosen according to their molecular structures, competitors have similar chemical structures with the target molecule. According to the results of the selectivity tests, it was observed that AFB1 had a higher adsorption amount than the competitive molecules AFB2, AFG1 and AFG2. Table 4.2 summarizes the adsorption capacities and the selectivity constants Qd, IF and k of both magAFB1-MIP and magNIPs. According to the table, the IFs are calculated as 70.17; 45.67; 31.90; 17.90 for AFB1, AFB2, AFG1, AFG2, respectively. These results showed that AFB1-specific cavities on magAFB1-MIP structure form more effective binding sites than non-imprinted polymers. By calculating k values, the selectivity of magAFB1-MIPs was determined, by comparing the adsorbing behavior of AFB1 and competitor molecules one by one. As can be seen from the table, magAFB1-MIPs adsorb AFB1 1.74 times selective than that of AFB2; 2.46 times selective than that of AFG1 and 2.52 times selective than that of AFG2 molecules. Although AFB1, AFB2, AFG1, AFG2 structures are very similar to each other, it has been clearly shown that the imprinted structure in the magAFB1-MIP selective towards AFB1 molecule. When these results are evaluated together with their adsorption capacities (Figure 4.10), it is observed that the synthesized particles also adsorb the competitor molecules. Since the amount of AFB1 to be analyzed in food, feed tissue,

etc. is very low, it is possible to say that the magAFB1-MIPs has the potential to be used as a selective separation column specific to AFB1 as well as a pre-concentration materials for other aflatoxins.

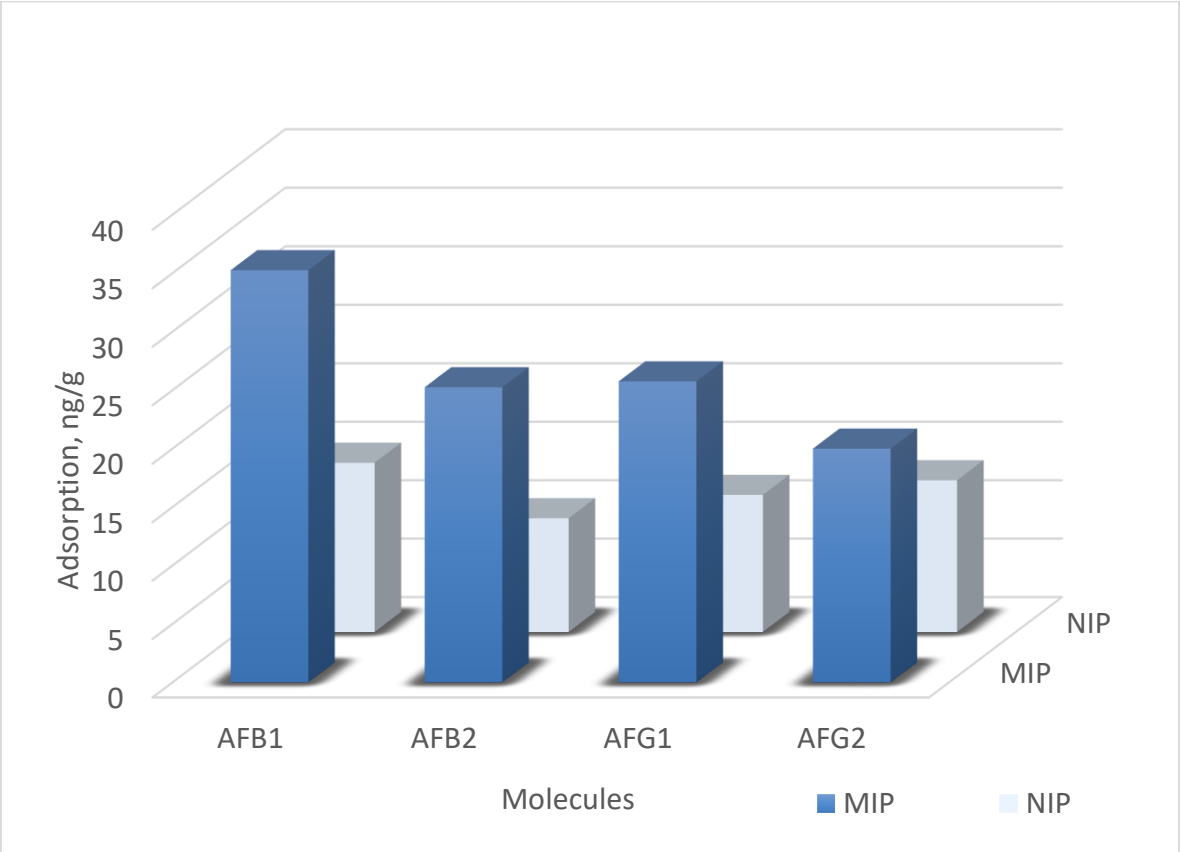


Figure 4.10. The selectivity studies. Equilibrium AFB1, AFB2, AFG1 and AFG2 concentrations: 2 ng/mL, V: 5 mL, time: 120 min, mdry of magAFB1-MIP and magNIP: 100 mg.

Table 4. 2. The adsorption capacities and the selectivity constants Qd, IF and k.

	magAFB1-MIP		magNIP			
	Q ng/g	Qd mL/g	Q ng/g	Qd mL/g	IF	k
AFB1	35.25	80.11	14.5	1.14	70.17	
AFB2	25.25	45.90	9.75	1.00	45.67	1.74
AFG1	25.75	32.59	1.,75	1.02	31.90	2.46
AFG2	20.00	18.18	13	1.01	17.90	2.52

4.5. Desorption and Reusability Studies

Reusability of synthesized magAFB1-MIP was tested by interacted particles with the same concentration of AFB1 solution in each cycle. Each one of the10 cycles adsorbed and desorbed AFB1 amount was recorded and the obtained resuts showed in Figure 4.11. As can be seen the decrease of the AFB1 adsorption capacity is very low. The calculated decrease was 3.21% in adsorption capacity is negligible, so it concluded that magAFB1-MIPs can be reuse.

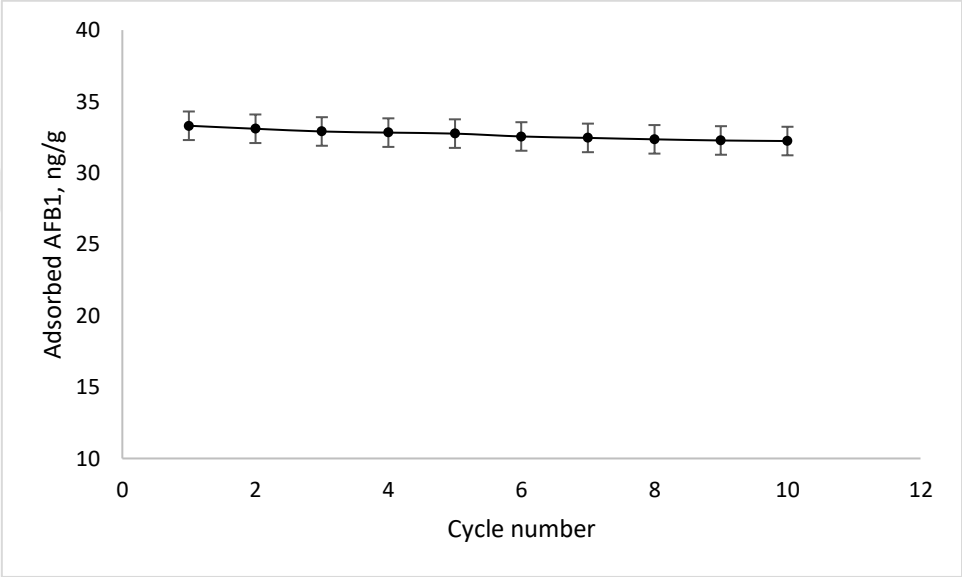


Figure 4.11. Reusability: 2 ng/mL, V: 5 mL, time: 120 min, mdry of magAFB1-MIP and magNIP: 100 mg.

4.6. AFB1 Detection in Liver Tissue

As a result of AFB1 detection from liver tissue which is spiked at 2 ng/g and 10 ng/g using magAFB1-MIP, adsorption capacity was found to be 10.4 and 54.8 ng/g with the recovery of 78% and 83%, respectively. The data suggests that synthesized magAFB1-MIP material was found to have high adsorption capacity and comparable with the studies conducted with adsorption capacity in the AFB1 aqueous solution.

5. CONCLUSIONS

- In this thesis AFB1 imprinted monolithic nanoparticles were synthesized by bulk polymerization technique using vinyl benzoic acid (VBA) as a functional monomer for the selective recognition of AFB1 from aqueous solution and liver tissue. 300 mg of iron powder (< 20 nm sized) was used in order to obtaine magnetic particles. Synthesized material was named as magAFB1-MIPs and magNIPs.

- Polymerization procedure were achieved for three precomplexes with the addition of different amounts of functional monomer to 200 nmol AFB1 (1:2; 1:3; 1:5 in molar ratio). The template removal ratios were obtained for 93, 91 and 87 for the magAFB1-$MIP_{1:2}$, magAFB1-$MIP_{1:3}$, magAFB1-$MIP_{1:5}$ respectively. But the adsorption amount- rebinding amount of the template obtained higher for magAFB1-$MIP_{1:3}$ then others. The magAFB1-$MIP_{1:3}$ is selected as working polymer due to high AFB recognition capacity.

- Percentage yield and swelling tests of both mag-AFB1-MIP and magNIPs were studied. For AFB1-MIPs, the percentage yield was determined as 98% and the swelling rate was determined 81%. Yield percentage for NIP was determined 97% and swelling rate was determined 80%.

- Surface area of both magAFB1-MIP and magNIP was determined using BET method and found as 117 m^2/g and 105 m^2/g, respectively.

- The surface morphology of the synthesized monolithic materials was investigated by SEM studies. Both magAFB1-MIP and magNIP monolithic polymers have a rough and porous structure. The porous structure contributed to the high surface area of the synthesized magnetic nanoparticles.

- Particle size analysis were conducted using Nano Zetasizer. Average diameters of magAFB1-MIPs and NIPs were determined as 483 and 496 nm, respectively. This results indicates that synthesized molithic materials were grounded and sieved succesfully in order to obtain particles with the size of <500 nm.

- AFB1 adsorption by magAFB1-MIP was investigated with 0.05, 0.1, 0.5, 1, 2, 5 and 10 ng/mL AFB1 concentrations, respectively. The obtained solutions of different concentrations were treated with both magAFB1-MIP and magNIP particles in the same environment under the same conditions. The particles adsorbed almost all of the AFB1 at all concentrations, as the concentration increased, the percentage of adsorption increased due to driving force even at 10 ng/mL, particles did not reach the saturation. The AFB1 adsorption amount was determined as 33 ng/g for of magAFB1-MIP at 1 ng/mL AFB1 concentration.

- The effect of temperature on AFB1 adsorption was investigated at 4 °C, 25 °C, 37 °C and 40 °C. It was observed that AFB1 adsorption increased as the temperature increased.

- The effect of time on AFB1 adsorption by magAFB1-MIP was investigated at 0^{th}, 5^{th}, 10^{th}, 15^{th},30^{th},45^{th},60^{th}, 90^{th}, 120^{th} minutes. The maximum AFB1 adsorption amount was reached at the 60^{th} minute and the amount of adsorption remained constant after this point.

- According to the results of the selectivity tests, it was observed that AFB1 had a higher adsorption level than the competitive molecules AFB2, AFG1 and AFG2. magAFB1-MIPs adsorbed AFB1 1.74 times selectively than that of AFB2; 2.46 times selectively than that of AFG1 and 2.52 times selectively than that of AFG2 molecules.

- Reusability of synthesized magAFB1-MIP was tested and the calculated decrease was 3.21% in adsorption capacity is negligible.

- In addition, as a result of AFB1 recognition from liver tissue which is spiked at 2 ng/g and 10 ng/g using magAFB1-MIP, adsorption capacity was found to be 10.4 and 54.8 ng/g, respectively.

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