DEVELOPMENT OF AN AFLATOXIN B1 SPECIFIC MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION SORBENT FOR THE SELECTIVE PRE-CONCENTRATION OF TOXIC AFLATOXIN B1 FROM CHILD WEANING FOOD, *Tsabana*

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ABSTRACT

In this thesis, the synthesis, optimization and application of a simple aflatoxin B1 (AFB1) specific extraction polymer sorbent based on molecular imprinting technology (MIT), for the selective pre-concentration of the potent AFB1 toxin from child weaning food, *Tsabana* manufactured in Serowe, Botswana is presented. As a food safety regulatory measure, the *Tsabana* must be cleared of the hazardous aflatoxins especially AFB1 before consumption as it is the most potent amongst those commonly found in cereals. Accurate analysis of AFB1 is a challenge as AFB1 exists in complex, 'dirty' matrices such as that of food in very low concentrations thus making it difficult for the existing analytical instruments to detect it despite their femto level sensitivities. The synthesized molecularly imprinted solid phase extraction sorbent in this thesis, managed to deal with the challenge by selectively pre-concentrating the AFB1 from real samples of *Tsabana* extracts by a pre-concentration factor of 5, thus the signal was greatly improved for easy detection. This was further supported by the short optimal time of 25.0 minutes needed for the maximum AFB1 extraction by the sorbent as well as the reasonable optimal dose of the MIP, 20.0 mg needed for maximum extraction of AFB1. The prepared AFB1 powder particles also exhibited good physical characteristics of a spherical geometry and reasonably small sizes of 800 nm associated with excellent sorbent materials, as demonstrated by the scanning electron micrographs.

Keywords: Aflatoxin B1, Molecularly imprinted polymer, *Tsabana*, Pre-concentration, Serowe, Botswana, Solid phase extraction

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ABBREVIATIONS

AFB1- Aflatoxin B1

SPE-Solid Phase Extraction

HPLC-FLD- High Performance Liquid Chromatography Fluorescence Detection

MIP- Molecularly Imprinted Polymer

FTIR- Fourier Transform Infrared Spectroscopy

IAC- Immuno-affinity column

LLE- Liquid-Liquid Extraction

SFE- Supercritical Fluid Extraction

PS-DVB- Polystyrene-divinylbenzene

EU- European Union

RAM- Restricted Access materials

ADS- Alkyl-diol-silica

XDS- Ion exchange diol silica

EGDMA- Ethylene gycol dimethacrylate

TRIM- trymethylolpropane trimethacrylate

AIBN- azo (bis)-isobutyronitryl

TFA- Trifluoroacetic acid

ABDV- 2,2- azobis (2,4-dimethyl-valeronitrile)

NIP- Non-imprinted polymer

SEM- Scanning Electron microscope

CHARPTER I: INTRODUCTION

1.0 General Introduction

Each year, there are over 6 million deaths of children in developing countries from food borne diseases especially malnutrition [1]. This brings food safety and security to the forefront of national and international concern. As a dietary intervention Botswana has developed *Tsabana* as a weaning food for infants at ages 6 – 36 months to improve their diet quality [2].

Tsabana is made from a combination of red sorghum and soya beans with sorghum being the main ingredient [3].Cereal grains, sorghum and soya beans included, are prone to pre- and post-harvest fungal contamination [4]. Some of the fungal species isolated from sorghum have potential to produce mycotoxins [5]. These species, mainly the *Aspergillus flavus* and *Aspergillus parasitus* are known to produce aflatoxins (a type of mycotoxin) [6][7]. There are approximately 20 different types of aflatoxins but only four are found in cereals; aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) with AFB1 being the most toxic. Aflatoxins have been found in samples of sorghum stored in silos in Botswana [5] and since sorghum is used in making *Tsabana*, the aflatoxins found in the grain maybe passed to the *Tsabana*.

The exposure of children to aflatoxins through *Tsabana* could lead to serious health risks. Ingestion of high concentrations of aflatoxins is fatal and chronic exposure is associated with liver cancer, immunosuppression, cirrhosis and other liver diseases [8]. Recent studies in Benin and Togo among children aged 9 months to 5 years have highlighted a further health consequence of aflatoxin exposure, in which growth faltering occurs at the time of weaning [9].

Given the potential adverse health effects of this dietary toxin on children, it is important that appropriate interventional strategies are introduced.

Institutions around the world have classified and regulated aflatoxins in food. The European Union (EU) has the most rigorous regulations concerning mycotoxins in food. The limits of AFB1 and total aflatoxins in foods are 5 and 10 ng/g, respectively, in more than 75 countries around the world, whilst they are 2 and 4 ng/g in the European Union. The AFB1 limit for infant food has been established by the EU as 0.10 ng/g [10]. In order to reach the regulatory levels established, it is necessary to employ highly sensitive and reliable analytical methods[11]. Contemporary analytical methods have the sensitivity required for contamination detection and quantification. These methods however, lack direct application on food samples due to the fact that contaminants are present in highly complex, 'dirty' and morphologically structured matrices, at very low concentrations [12]. Consequently, analysis can only be achieved after some extensive sample preparation steps have been applied [13].

Conventional techniques for sample preparation such as the commonly employed liquid-liquid extraction (LLE) are time consuming and require large quantities of reagents, which are expensive, generate considerable waste and contaminate the sample. Recently the most popular sample preparation technique for environmental, food and biological samples is solid-phase extraction (SPE). It is replacing conventional techniques as it offers reduction or complete elimination of solvent consumption in analytical procedures [14]. Extraction, pre-concentration and clean-up of the sample can be achieved in a single step in SPE by an appropriate selection of the type of sorbent or their combination. Classical sorbents used include graphitized or porous carbon, silica or silica bonded and polymeric sorbents, these however lack selectivity [15]. So far

the most commonly used selective sorbents are based on immuno-affinity. In this format, antibodies attached to an inert support material are employed to specifically bind the analyte while sample impurities pass through [16]. Immuno-affinity sorbents are now used less widely because a selective, monoclonal antibody must be immobilized for each analyte and this is a long process that can take as long as 12 months [17].

In recent years, interest in a new technique based on the development of molecularly imprinted polymers (MIPs) also known as man-made antibodies has grown. This is because MIPs not only allow pre-concentration and clean-up of the sample but also selective extraction of the analyte, which is important, particularly when the sample is complex, 'dirty' and there are higher chances of impurities interfering with quantification as is the case in the accurate analysis of very low concentrations of aflatoxins in food matrices such as in *Tsabana* [18]. Thus the objectives of this thesis were to synthesis, optimize and apply a simple aflatoxin B1 (AFB1) specific pre-concentration polymer sorbent based on molecular imprinting technology (MIT), to selectively pre-concentrate potent AFB1 toxin from child weaning food, *Tsabana* manufactured in Serowe, Botswana. The synthesized polymer powder was characterized by the scanning electron microscope (SEM) and Fourier transform infrared spectrometer (FTIR). The AFB1 MIP powder was optimized for MIP quantity and time needed for maximum extraction of Aflatoxin B1. Through SPE batch adsorption experiments the MIP powder's aflatoxin-binding properties were studied in comparison with the non-imprinted polymer and the selectivity of the polymer was evaluated against a structurally related compound, aflatoxin G2 (AFG2). From these, dissociation constant, selectivity factor and relative selectivity factor were calculated for the MIP. Finally, the resultant MIP was employed to selectively pre-concentrate.AFB1 from *Tsabana* prior to instrumental analysis.

1.1 Sample Handling techniques in Food Contaminant Analysis

Sample handling refers to the way in which a sample is treated prior to analysis [19]. It incorporates steps from sampling to sample preparation (pre-treatment, pre-concentration, clean-up and derivatization). Comprehensive sample handling is important to maintain the integrity of samples in terms of analyte identity and concentration. It is essential to prevent deterioration and cross contamination, to maintain sample tracking, chain of custody and ensure a clean sample ready to be fed to the instrument.

1.1.1 Sampling

Virtually no food material can be analyzed in its entirety. It is necessary to obtain a representative portion, of the food under study, this is known as sampling [20]. The portion is then divided into laboratory-sized primary samples, in addition to subsequent subsamples, or secondary samples [21]. It is important to develop a sample plan, this is a strategy employed to represent the distribution of one or multiple analytes in the object of study. The sampling plan stipulates how the sample will be selected from the bulk lot and the size of the sample [22]. When sampling, two approaches can be followed. In the first approach many small increments of a solid material can be collected and blended to represent the entire population. Whereas in the second approach a quantity of material that is large enough to be compositionally representative of the whole population can be collected and then reduced to a fine mixture before being subsampled. The first one is more practical, accurate, and reproducible. It takes into consideration the variation in the quantity of toxin in the contaminated kernels and the percentage of contaminated kernels in the lot [21]. The size of the sample is also an important

factor to consider. The required sample size is defined by the nature of the target compound, (that is, to what extent the analyte is retained in the matrix) and the relationship that exists between the mass required to adequately represent a sample and the characteristics of that sample [23]. A sufficiently large quantity of sample must be collected and analyzed in order to be able to determine trace quantities of the analyte. On the other hand, relatively small samples may be collected for the macro analysis of gross food components.

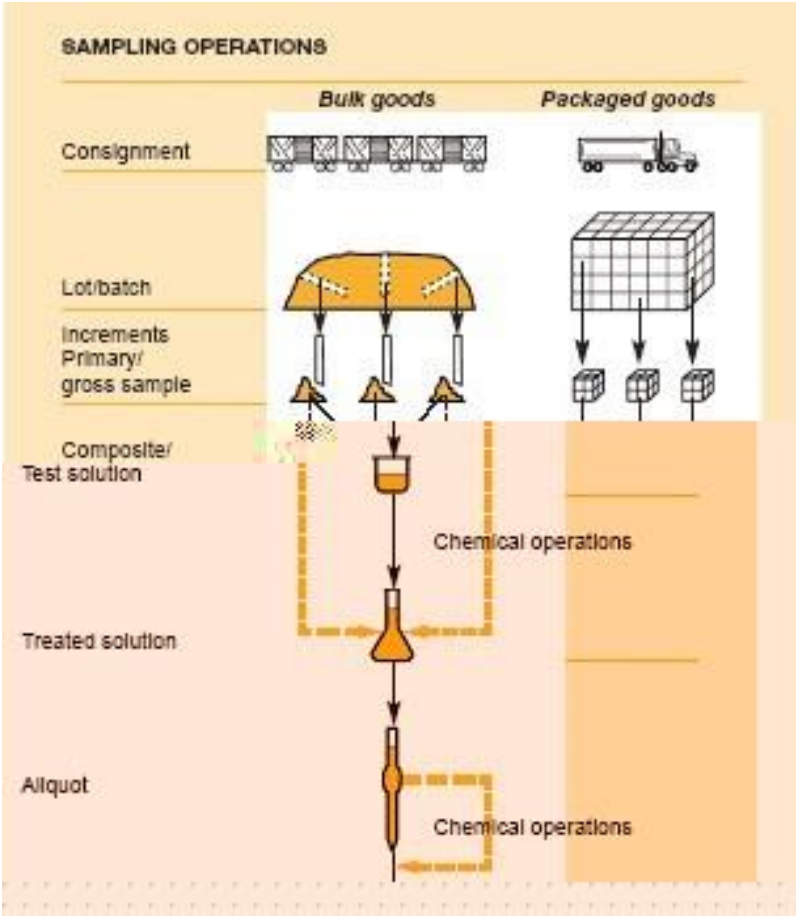


Figure 1: Operations involved in solid sampling [21]

1.1.2 Sample Preparation

In general, food and environmental samples are not ready for direct instrumental analysis without some preliminary sample preparation, because analytes of interest often exist in very low concentrations in highly complex matrices [24]. A series of steps are therefore required after sampling and before instrumental analysis. The steps include pretreatment [25], pre-concentration [26], clean up [27], extraction [16] and derivatization [28]. They are very straightforward but if not carried out properly the original integrity of the sample may be compromised.

1.1.2.1 Pretreatment

In food sample preparation, the purpose of pretreatment is to attain a perfect digestion of the organic matrix, decrease viscosity, increase shelf life of the sample, increase homogeneity so as to keep the integrity of the sample [25].

A common sample pre-treatment technique in solids is the reduction of the sample. Finely divided materials dissolve faster and are easier to extract because of their greater surface area. Sample sizes are usually reduced by grinding. In their study, *Turcotte A. et.al.* used a direct drive food processor, coarse grinding blade and knife to grind cocoa products for aflatoxin analysis [29]. In another study, *Yazdanpanaha H. et.al.* used a mill to grind rice, bread, peanuts, puffed corn snack and wheat flour for aflatoxin analysis [30].

Liquid samples can be mixed using magnetic stirrers or sonic oscillators to achieve homogeneity. Some liquid samples such as beer [31] and wine [32] need to be degassed before analysis, this is

usually done by sonication. In mycotoxin analysis, samples may be diluted as a sample pretreatment technique. This is meant to reduce matrix effect during analysis. Other sample pretreatment techniques reported include filtration, centrifugation and pH adjustment[27][29].

1.1.2.2 Pre-concentration and Clean-up

Clean-up is necessary for removing many of the co-extracted impurities and obtaining cleaner extracts for determinative, or quantitation step. Often clean-up is achieved simultaneously with pre-concentration. Pre-concentration involves isolating the analyte of interest from a large volume matrix containing many other substances [26]. The resultant is that the analyte of interest will now be contained in a smaller volume with less or no inteferents thus will be concentrated and more detectable [16].

Currently, there is a strong trend towards the use of immuno-affinity columns (IACs) in mycotoxins analysis as clean-up and pre-concentration technique for sample extracts or liquid samples [27]. A comparison has been made of a tandem liquid chromatography mass spectrometry (LC/MS/MS) method employing direct analysis of acetonitrile extracts of feed and cereal samples and the other one involving acetonitrile extracts and subsequent immuno-affinity column (IAC) cleanup. Naturally contaminated samples containing one or more of deoxynivalenol, zearalenone, T-2, and HT-2 toxins were analyzed with some test materials containing known toxin levels. It was concluded that the LC/MS/MS analysis of the samples with no cleanup was adequate for screening purposes and not for accurate quantification for food regulatory control purposes. The experiment proved that IAC cleanup is very essential [33].

1.1.2.3 Derivatization

Derivatization reactions are meant to modify an analyte's chemical structure so that it can be analyzed by the desired technique. It achieves reduction of interactions that interfere with analysis and thus improving detectability either by increasing the bulk of the compound or by introducing onto the analyte atoms or functional groups that interact strongly with the detector, hence improving sensitivity and selectivity during analysis. A modified analyte in this case will be the product, which is known as the derivative. The derivative may have similar or closely related structure, but not the same as the original non-modified chemical compound[28].

Chemical derivatization of aflatoxin B1 may be needed to enhance sensitivity during high performance liquid chromatography fluorescence detection (HPLC-FLD) analysis since the natural fluorescence of AFB1 may not be high enough to reach the required detection limits. The fluorescence intensity of AFB1 can be enhanced by pre/post-column derivatization with trifluoroacetic acid (TFA), iodine or bromine [34]. The reactions are shown in figure 2. In this thesis TFA was used to derivatize AFB1 to its highly fluorescing derivative AFB2a.

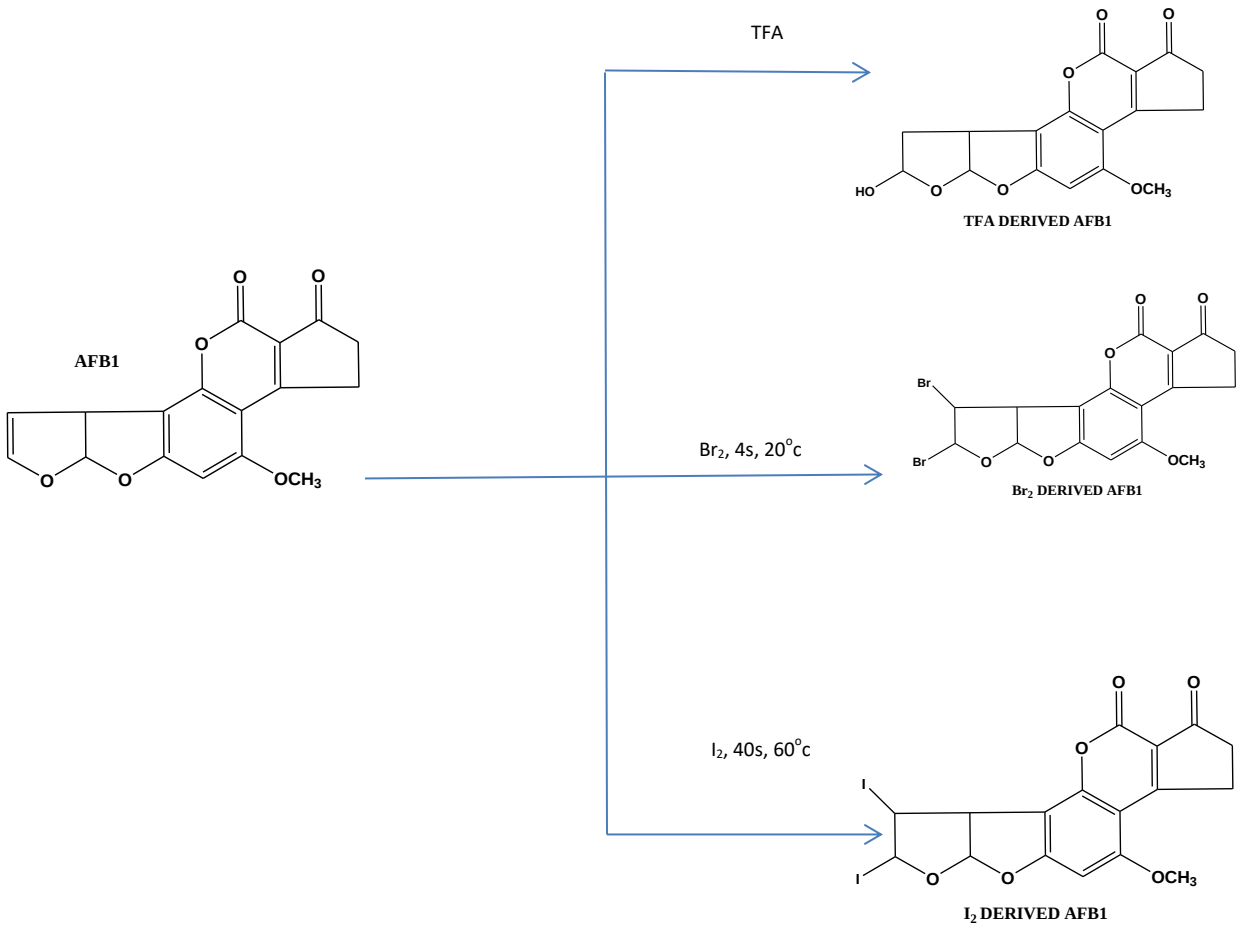


Figure 2: Derivatization of AFB1 with TFA, Bromine and Iodine

1.1.2.4 Extraction

The purpose of extraction is to release as much of the analyte of interest from the food matrix as possible from various compounds and phases into a solvent suitable for subsequent clean-up and determination as well as removal of components that may have adverse impacts on the analytical signal [16]. Extraction of dissolved chemical components from liquid phase is accomplished by bringing the liquid solution containing the chemical component into contact with a second phase, given that the two phases are immiscible. The second phase may be a solid, liquid, gas, or

supercritical fluid. A distribution of the component(s) between the immiscible phases occurs. After the analyte is distributed between the two phases, the extracted analyte is released and/or recovered from the second phase [19]. Extraction of an analyte is influenced by solubility, penetration of the sample by the solvent (mass transfer) and matrix effects [35].

1.2 Common Sample Extraction Techniques in Food Analysis

1.2.1 Liquid-Liquid Extraction

Liquid–liquid extraction is conventionally one of the most common methods of extraction, particularly for organic compounds from aqueous matrices [36]. It is based on the relative solubility of an analyte in two immiscible phases and is governed by the equilibrium distribution/partition coefficient (k_D). The differences in polarity of the two immiscible liquid phases are used to achieve extraction of an analyte [35]. When choosing an extraction solvent pair, several factors are considered. These are miscibility, density and solubility. When dealing with solid samples like cereals, polar solvents can dissolve mycotoxins and extract them from the cereals. Such solvents are methanol, acetone, acetonitrile ethyl acetate, diethyl ether, toluene, and chloroform. As water is a polar solvent, it can also be used for extraction of some mycotoxins. Small volumes of it will wet the sample and offer higher extraction efficiencies, by increasing accessibility of the solvent into the hydrophilic sample [37]. *Bertuzzi T. et al* evaluated the extraction efficiencies of the extraction methods for the analysis of Aflatoxins B1, B2, G1 and G2 from naturally contaminated maize and reported that an 8.5:1.5 or 8:2 (v/v) acetone-water showed better extraction efficiency for AFB1 determination compared to a methanol-water mixture [38]. An acidic aqueous phase also showed improved extraction as it broke down interactions between the toxins [37].

The LLE process can be accomplished by shaking the aqueous and organic phases together in a separating funnel, see figure 3.

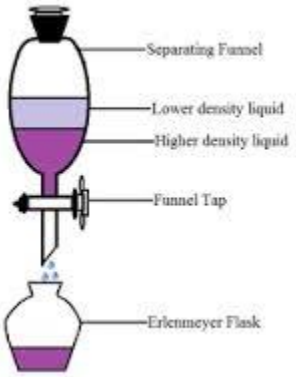


Figure 3: Liquid-Liquid Extraction apparatus [39]

The separating funnel is gently shaken for a few seconds and frequently inverted and the cock is opened to relieve excess pressure. Following which, the layers are allowed to separate. When the layers are completely separated, the lower layer is drawn off through the stopcock, and the upper layer is removed through the top of the separating funnel. The relative position of each layer depends on the relative densities of the two immiscible phases. During an extraction process, all layers should be saved until the desired analyte is isolated. A given solvent layer can easily be determined to be aqueous or organic by testing the solubility with a few drops of water [35]. To ensure improved recoveries and complete extraction of an analyte into the required phase, repeat extractions may be necessary[37], see figure 4

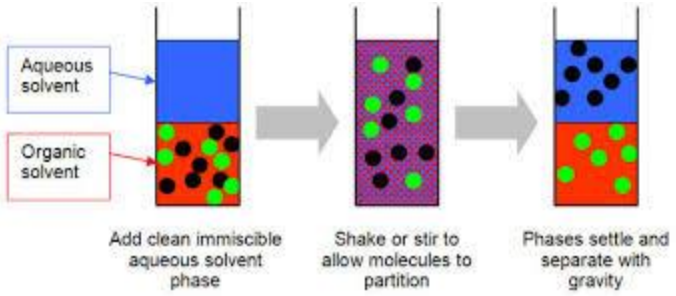


Figure 4: Schematic diagram of the LLE process [36]

The major disadvantage of liquid–liquid extraction is that it requires high volumes of organic solvents, labor intensive and it is relatively time consuming [40]. Also due to the absence of specific extraction solvents for a particular analyte there is limited selectivity, particularly for trace level analysis [41]. Consequently there is a need for selective pre-concentration/ enrichment and clean-up prior to food analysis [35].

1.2.2 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is a process of separating one component (the extract) from another (the matrix) employing supercritical fluids as the extracting solvents [42]. The technique is a powerful alternative to conventional organic solvent extraction because of its combination of gas-like mass transfer and liquid-like solvating properties [43]. Also rates of extraction and phase separation can be significantly faster because there is no surface tension and viscosities are much lower than in liquids. As a result, the solvent can penetrate into small pores within the matrix which would otherwise be inaccessible to liquids [44]. SFE offers low consumption of organic solvents thus reducing the challenges of their storage and disposal [45].

In the supercritical fluid extraction process, the mobile phase is exposed to pressures and temperatures near or above the critical point for the purpose of improving the mobile phase solvating power. The process begins with the solvent in vapor form. It is then compressed into a liquid before becoming supercritical. While the fluid is supercritical, the extraction takes place [46]. Instrument components include a fluid source, commonly a tank of carbon dioxide followed by a syringe pump having a pressure rating of at least 400 atm, then a valve to control the flow of the critical fluid into a heated extraction cell (where it is heated to supercritical conditions). The pressure vessel contains the sample. It is here that the super critical fluid would rapidly diffuse into the solid matrix and dissolve the material to be extracted. The dissolved material would then be swept from the extraction cell into a separator at lower pressure, and the extracted material settled out. Lastly, there is an exit valve leading to a flow restrictor where the solvent would then be cooled, re-compressed, recycled and transferred into a collection device or discharged to the atmosphere [42][46].

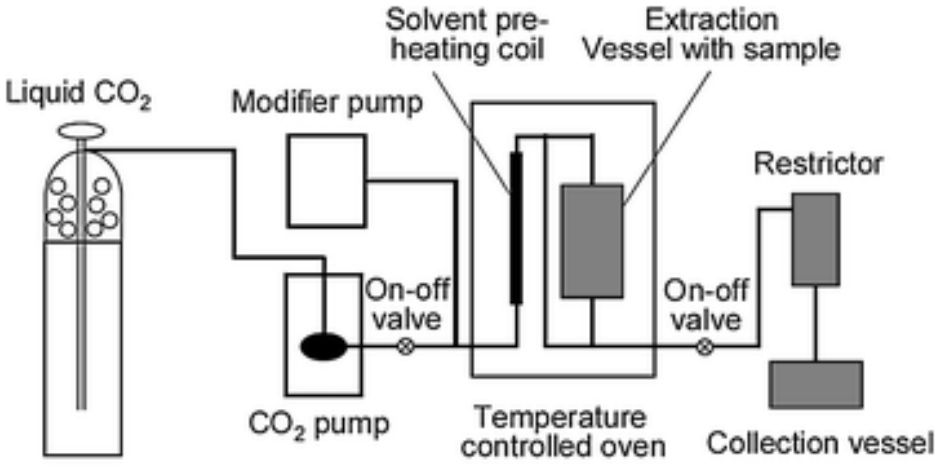


Figure 5: Schematic diagram of the SFE system [42]

Extraction is mostly dependent on the critical temperature and pressure of a supercritical fluid [46]. Super critical fluids have features which ensure high fluid phase capacity associated with favorable transport properties, making supercritical fluids attractive as the solvents for extraction in food analysis. Their densities are much greater than those of typical gases and slightly less than those of organic liquids. Several gases or liquids have been in use as solvents in supercritical fluid extraction such as ethylene, propane, carbon dioxide (CO_2), ethane, benzene with CO_2 as the most adapted in the food industry [47]. This is because it is non-toxic, non-flammable, inert, transmits in the ultraviolet, readily available in high purity, cheap, has low surface tension and viscosity, and high diffusivity [44]. In addition CO_2 is devoid of oxygen, therefore protecting samples against oxidative degradation [46]. One of the CO_2 disadvantages is its non-polar property that does not favor the extraction of polar substances [47]. The addition of a polar organic modifier, such as methanol, ethanol, dichloromethane or even water, is necessary to increase the solute solubility [48]. *Arnáiz, et al* in their study of supercritical fluid extraction of free amino acids from broccoli leaves, where methanol was used as a modifier, found that the modifier percentage had a strong effect on the extraction yield [49]. To further corroborate *Arnáiz et al* work *Taylor et al* reported that the addition of an organic modifier to the supercritical CO_2 was essential in achieving high aflatoxin B1 recoveries [43]. Furthermore, *Taylor et al* reported that an increase in modifier beyond the optimized percentage, caused an excessive pressure drop on the system that could damage it [49].

1.2.3 Solid Phase Extraction

In principle, solid phase extraction (SPE) involves extraction of solutes (analytes of interest) between two phases, the liquid phase containing the analyte of interest (solutes) and the solid (sorbent) material [50]. SPE procedures are employed not only to extract traces of organic compounds from samples but also to remove interfering components of the complex matrices in order to obtain a cleaner extract containing the analyte(s) of interest only. The most common retention mechanism in SPE is based on van der waals forces (non-polar interactions), hydrogen bonding, dipole-dipole forces (polar interactions) and cation-anion interactions (ionic interactions) [51]. Three general theories of interactions between sorbent, analyte and solvent exist; these include normal phase[14], reverse phase [52] and ion exchange [14].

The first approach involves a polar analyte, a mid- to non-polar matrix (e.g. acetone, chlorinated solvents and hexane) and a polar stationary phase functionally bonded silicas with short carbon chains [14]. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface [52]. Then, elution of the analyte is obtained by increasing the strength of the mobile phase. When the analytes are non-polar, reverse phase separation is advised. It involves a polar or moderately polar sample matrix (mobile phase) and a non-polar stationary phase. Retention of analytes from polar solutions onto the sorbent is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the sorbent surface, commonly called van der Waals forces or dispersion forces [50]. A non-polar solvent, which can disrupt the forces between the sorbent and compound, is used to elute an adsorbed compound

from the stationary phase. Materials used as sorbents include graphitized and non-porous carbon, styrene/divinyl benzene and silica bonded material [52].

Ion exchange SPE extraction is suitable when the analytes are charged. Extraction is based on electrostatic interactions between the analyte of interest and the charged groups on the stationary phase [14]. Anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to a silica surface. Cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface [52].

A typical, practical SPE procedure always consists of four successive steps, as illustrated in the figure below.

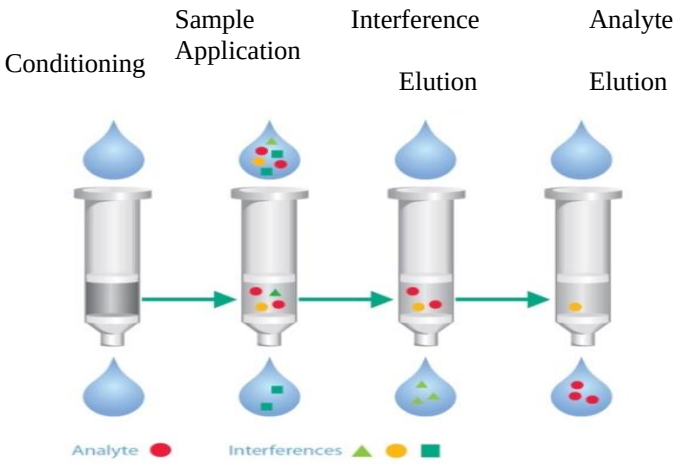


Figure 6: A typical four step SPE method [52]

Initially, the sorbent is conditioned in a solvent that allows it to swell properly, followed by the same solvent as the sample solvent. This step is crucial, as it enables the wetting of the packing material and the solvation of the functional groups. Also, it removes possible impurities initially contained in the sorbent or the packaging [53]. After sorbent conditioning follows sample

application. During this step, the analytes are concentrated on the sorbent. Even though matrix components may also be retained by the solid sorbent, some of them pass through, thus enabling some purification of the sample [52]. The next step involves interference elution; here the solid sorbent is washed with an appropriate solvent, with low elution strength, to eliminate matrix components that have been retained by the solid sorbent, without displacing the analytes [50]. In the final step the analyte is eluted with a solvent or solvent mixture that breaks the bonds between sorbent and analyte, without removing the matrix components that may have been retained [54].

SPE Sorbents

Different types of sorbents are employed in SPE, such as: silica based sorbents, carbon based sorbents, polymer based sorbents, graphitized or porous carbon, immunosorbents, restricted access materials and sorbents based on synthetic molecular recognition[55][56]. The selection of an appropriate SPE extraction sorbent depends on the mechanisms of interaction between the sorbent and analyte of interest. That in turn depends on knowledge of the hydrophobic and polar properties of both the solute and the sorbent[52]. The main requirements for substances to be used as solid-phase extraction sorbents, are as follows: the ability to extract compounds over a wide pH range, fast quantitative sorption and elution, high capacity, regenerability and accessibility[57].

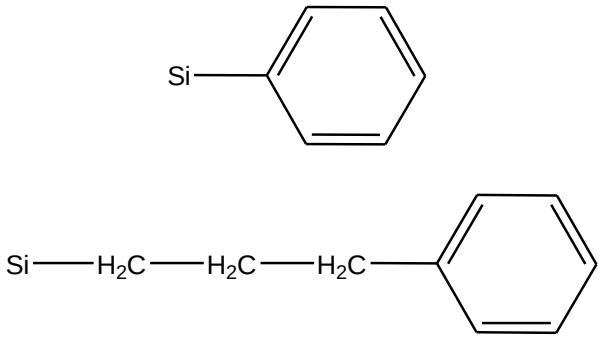
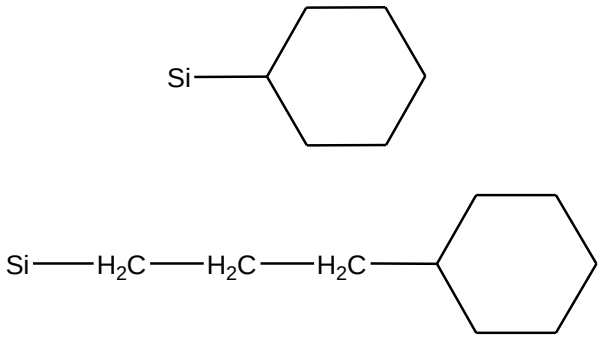
a) Conventional Sorbents

These are low specificity sorbents, which include chemically bonded silicas, porous polymers and carbon, are commonly used for the isolation of contaminants from aqueous solution. They represent the most common applications of SPE to date.

i) Silica and Bonded Silica Sorbents

The first SPE materials were silica-based. These were synthesized by the reaction of silica surface silanol groups with chloroakyl- or alkoxyakylsilanes [58] and have been modified with C18, C8, phenyl, CH, CN, or NH2 groups shown in table 1 below [15]. Their interaction mechanisms are mainly based on hydrophobic interaction (van der waals forces) [59]. However, silica-based materials present several disadvantages, such as instability at extreme pH, low recovery in the extraction of polar analytes, and the presence of residual silanol groups [15][59].

Table 1: Alkyl bonded functionalities of common silica bonded phases

Phase	Bonded Phase
C_{18} Octadecyl	$Si\text{-}(CH_2)_{12}CH_3$
C_8 Octyl	$Si\text{-}(CH_2)_7CH_3$
Phenyl	
CH cyclohexyl	
CN Cynopropyl	$Si\text{-}(CH_2)_3\text{-}CN$
NH_2	$Si\text{-}(CH_3)_2\text{-}NH_2$

ii) Polymer Sorbents

Traditional polymer sorbents were generally co-polymers of styrene and divinylobenzene. Polystyrene-divinylbenzene (PS-DVB) is a hydrophobic resin which has greater polar compounds retention. It interacts with analyte mainly through van der waals' forces and the π-π interactions of the aromatic rings that make up the sorbent structure [52]. However these conventional sorbents have poor capacity and selectivity. To improve their capacity hydrophilic hyper-crosslinked sorbents were introduced through a reaction with hydrophilic precursor monomer or by chemically modifying the PS-DVB polymer skeleton with acetyl, hydroxymethyl, benzoyl functional groups. The effect is increased surface polarity, due to ultra-high specific surface area hypercrosslinked sorbents thus enabling aqueous samples to make better contact with the resin surface [15].

iii) Graphitized or Porous Carbon

Carbon-based media consist of graphitic, non-porous carbon with a high attraction for organic polar and non-polar compounds from both polar and non-polar matrices. Retention of analytes is based primarily on the analyte's structure, rather than on interactions of functional groups on the analyte with the sorbent surface [54]. Recovery of adsorbed analytes from carbon is usually achieved through the use of stronger (less polar) binary solvent mixtures [60]. The challenge with the graphitized carbon sorbents is that they have excessive retention but this can be overcome by performing the elution in the backflush mode [52].

iv) Ion exchange sorbents

Ionic or ionizable analytes such as acidic and basic compounds from aqueous or non-polar organic compounds can be extracted by ion exchange sorbents [61]. Ion exchange phases are comprised of positively (aliphatic quaternary anime, aminopropyl) or negatively (aliphatic sulfonic acid, aliphatic carboxylic acid) charged groups that are bonded to silica gel [14] or polymers (usually a styrene-divylbenzene copolymers) [50]. Silica based ion exchange sorbents have a disadvantage over the other polymers of being limited to the pH range 3 to 9 and have lower capacity [61].

Extraction is based on electrostatic interaction between the analyte of interest and the charged groups on the stationary phase. Retention occurs for a sample pH that allows the analyte to be in its ionic form whereas desorption in its neutral form. A solution having a pH that neutralizes either the compound's functional group or the functional group on the sorbent surface is used to elute the compound of interest. When one of the two functional groups is neutralized, the electrostatic force that binds the two together is disrupted and the compound is eluted. Alternatively, a solution that has a high ionic strength, or that contains ionic species that displaces the adsorbed compound, is used to elute the compound [14]. If the analytes are ionic over the whole pH range, then desorption occurs by using a solution of appropriate ionic strength [61]. There should be few, if any, other species of the same charge as the compound in the matrix that may interfere with the adsorption of the compound of interest.

There are certain disadvantages associated with ion exchange, especially in the water treatment in both industrial and municipal water treatment systems. These include calcium sulfate fouling,

iron fouling, adsorption of organic matter, organic contamination from the resin, bacterial contamination and chlorine contamination [15].

b) Selective Sorbents

Drawbacks of the conventional sorbents include low breakthrough volumes for very polar compounds, and low sampling rate, and they require extensive cleaning before use[52]. Silanol groups have strong energies of absorption which leads to strong, occasionally irreversible bonding of compounds onto the silica surface. The hydroxyl groups on silica allow a large variety of different functional groups and hence lack selectivity[62]. It is clear that for optimal clean-up and pre-concentration in SPE, selectivity is vital. Restricted access materials have been introduced as well as the emerging sorbents such as immunosorbents and molecularly imprinted polymers [61][63].

i) Restricted Access Material (RAM)

RAMs are materials such as alkyl-diol-silica (ADS) and ion exchange diol silica (XDS) [64]. The specific feature of the diol silica particles is the topochemically bifunctional surface of the particles: the outer particle surface is modified with hydrophilic diol groups, whereas the inner pore surface is covered with hydrophobic alkyl chains and/or ion exchange groups. The hydrophilic barrier allows the small molecules to permeate through the hydrophobic part of the stationary phase, and, at the same time, it excludes the macromolecules [65].

ii) Immuno-sorbents

In this format, antibodies covalently bonded to an appropriate support are employed to specifically bind to the analyte of interest while sample impurities pass through [66]. The analyte is then eluted with a solvent that denatures the antibody [67]. The process is shown on figure 7 below. Immunosorbents are very specific in nature of the interaction between the mycotoxin and the antibody [16].

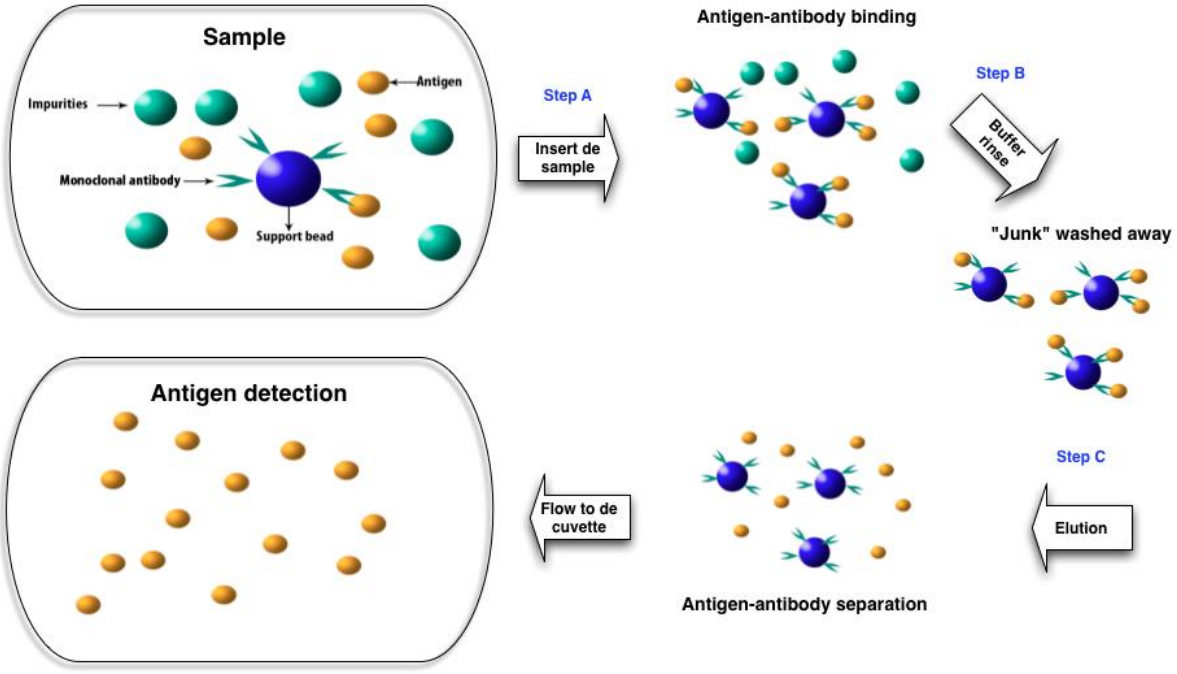


Figure 7: Immuno-affinity extraction process [67]

iii) \Molecularly Imprinted polymers

Conventional sorbents are inexpensive but show a lack of selectivity, while immunosorbents are very selective but expensive and not suitable for harsh environments and RAMs have insufficient selectivity [68]. Thus, inexpensive, rapid and selective sorbents, relying on 'intelligent' materials

are needed. Recently there has been a significant increase of molecularly imprinted polymers as solid phase extraction sorbents in food contaminant analysis[69]. Molecularly imprinted polymers (MIPs) have attracted much attention due to their outstanding advantages, such as predetermined recognition ability, stability, relative ease and low cost of preparation, and potential application to a wide range of target molecules. MIPs are synthetic polymers with highly specific recognition ability for target molecules. In the most common preparation process, monomers form a complex with a template through covalent or non-covalent interactions and are then joined by using a cross-linking agent. After the removal of the template by chemical reaction or extraction, binding sites are exposed which are complementary to the template in size, shape, and position of the functional groups, and consequently allow its selective extraction [70]. In the next chapter molecularly imprinted polymers are discussed in detail.

CHAPTER II: MOLECULARLY IMPRINTED POLYMERS

Chapter 2 discussed in detail, the background of molecularly imprinted polymers. Information on the history of molecular imprinting, the concept of molecular imprinting, the molecular imprinting process, synthesis methods, characterization and applications of MIPs were presented.

2.0 The history of molecular imprinting

The first work on molecular imprinting dates to the 1930's by a Soviet chemist Polyakov [71]. He performed investigations on silica for use in chromatography and did further investigations in 1937 on their selective molecular recognition. The work went largely unrecognized by the scientific community [72]. In 1949 another scientist Dickey published a paper describing the polymerization of sodium silicate in the presence of a dye after being inspired by Pauling's theory of production of antibodies in-vitro, where he had used dyes as antigens to study the selectivity of antibodies earlier in that decade [73]. In the 1950's, other groups continued in the same line of work [74].

In the 1970's, research transferred from silica based systems to synthetic organic polymers. Wulff and Sarhan set out the principle underpinning the molecular imprinting concept for the first time [75]. They produced molecularly imprinted polymers by synthesizing specific sugar or amino acid derivatives which contained polymerizable functional groups employing covalent imprinting methods [76]. Their work had significant impact on the research direction that was later on taken by Shea, a physical organic chemist. Shea's work brought understanding to the fundamental mechanisms of the imprinting process and subsequent recognition of the template by the imprinted polymers [77]. After attending a conference where Shea was speaking, a

German scientist Klaus Mosbach developed interest on the subject. In 1981 he published work on imprinting using only non-covalent interactions which at the time was referred to as 'guest-host polymerization', instead of the now popular term molecular imprinting which was only adopted in 1983 in an article on novel affinity technology [78]. The abbreviation of MIP was not adopted until 1993, before then, Mosbach referred to them as MIA for molecular imprinted adsorbents. In 1993, *Mosbach et. al.,* published a paper titled; 'Drug assay using antibody mimics prepared by molecular imprinting' [79]. The scientific content of this paper elevated the technique from a position of academic curiosity researched by only a few groups into the scientific mainstream for the first time.

2.1 The concept of molecular imprinting

Molecular imprinting is a synthetic strategy that is employed to assemble a molecular receptor via template guided synthesis [80]. The concept is based on the principle of molecular recognition found in biological processes, including antibody-antigen complex recognition in the immune system and enzyme-substrate complexes in enzymatic catalysis [81]. The principle asserts that there is formation of a complex as a result of intermolecular interactions between complimentary functional groups on a receptor and the desired substrate which Emily Fisher first described as the 'lock and key model' over a century ago [82]. In the birth stages of molecular imprinting, Pauling used antibodies to explain the source of complementary shape exhibited by molecularly imprinted polymers. He postulated how an otherwise nonspecific antibody molecule could be reorganized into a specific binding molecule. He reasoned that shape specificity was obtained by employing a target antigen to arrange the complementary shape of an antibody. Thus, a nonspecific molecule can be shaped to the contours of a specific target, and when the

target is removed, the shape is maintained to give the antibody a tendency to rebind the antigen [83].

2.2 The Molecular Imprinting Process

Typically, the imprinting process involves complexation of a template molecule/ion with a functional monomer(s), followed by polymerization of these monomers around the template with the aid of a cross-linker in the presence of a radical initiator, in a proper solvent (see figure 8), most often an aprotic and non-polar solvent referred to as a porogen [84]. During polymerization, the complex formed between the template molecule and the functional monomer(s) is stabilized within the resulting rigid, highly cross-linked polymer [85]. Subsequent removal of the template leaves the resultant three-dimensional cavities complimentary in size, shape and chemical functionality to the template [86]. The high level of cross-linking enables the cavities to maintain their shape after template removal, and consequently, the functional groups are held in an optimal configuration for selective rebinding of the template over structurally related analytes [85].

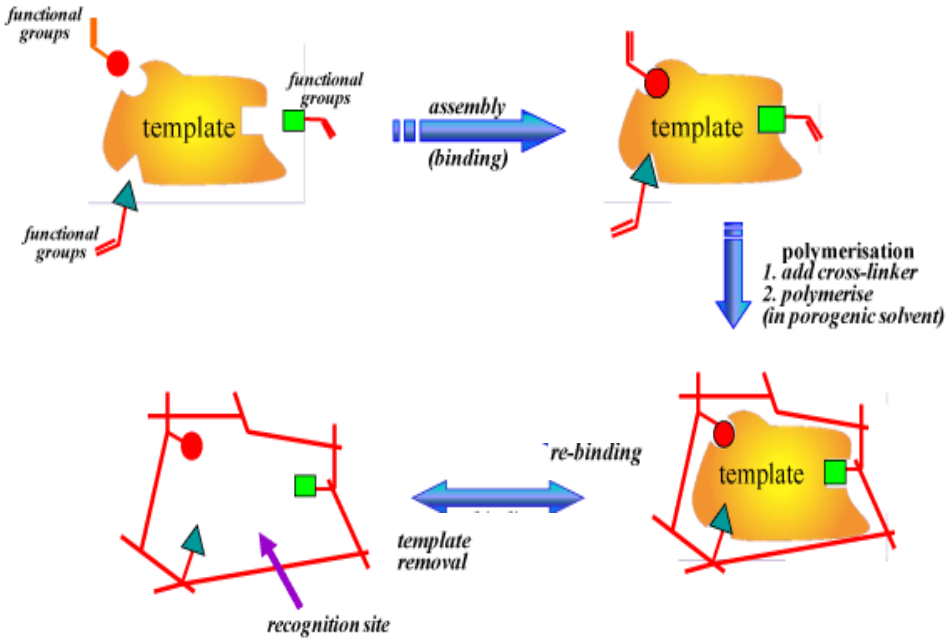


Figure 8: Schematic diagram of molecular imprinting process [86]

2.3 Types of imprinting strategies

The template–monomer interactions observed during the imprinting process can be through three types of imprinting strategies; the non-covalent interactions proposed by Mosbach , the reversible covalent bonds introduced by Wulff and the one that is a combination of the two strategies, referred to as the semi-covalent or sacrificial imprinting strategy attributed to Whitcombe [87]. Non-covalent imprinting relies on self-assembly of the monomer around the template by a series of non-covalent interactions such as hydrogen bonding, ionic interactions and hydrophobic effects, which during polymerization, form a basis of the recognition sites [88]. After polymerization and removal of the template by solvent extraction, the functionalized polymeric matrix, then rebinds to the template via the same non-covalent interactions [89]. Given that non-covalent molecular interactions are prevalent in the biological world, exploitation

of these binding forces has proven the most efficient and preferred method for generating robust, biomimetic binding materials [80]. The covalent imprinting strategy on the other hand depends on the formation of labile covalent bonds between the functional groups of the template and the complementary monomers, which become fixed at their spatial arrangement during polymerization [88]. The template is then removed by cleavage of the covalent bonds, which upon rebinding of the target molecule are re-formed. This approach is restrictive since the cleavages of covalent bonds always require harsh conditions to remove the template [90].

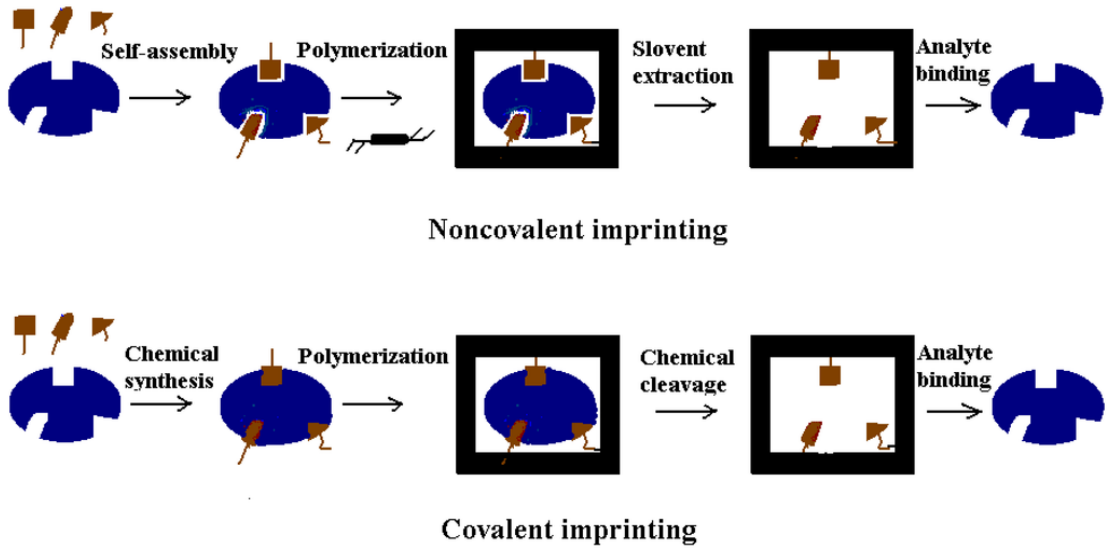


Figure 9: Types of imprinting strategies [89]

An intermediate alternative is offered by the semi covalent imprinting strategy, which relies on the template covalently binding to functional monomers as in the covalent approach and the template rebinding non-covalently. It is characterized by both the high affinity of covalent binding and mild operation conditions of non-covalent rebinding [89].

2.4 Typical reagents in the imprinting process

Molecular imprinting technology involves polymerization of monomers around the template with the aid of a cross-linker in the presence of a radical initiator in a suitable solvent, referred to as a porogen [84].

2.4.1 Template (Print molecule)

In molecular imprinting, template molecules are the target species. The template directs the organization of the functional groups pendent on the functional monomers. The structure and functionalities of this molecule define the subsequent properties of the binding sites [84]. A variety of templates such as drugs [91][92], carbohydrates, proteins [93], amino acids [23], hormones [95], pesticides [18] and toxins [96] have all been employed.

An ideal template molecule should satisfy the following three requirements; first, it should not contain groups involved in or preventing polymerization, (it should not bear any polymerizable groups). Secondly, it should exhibit excellent chemical stability during the polymerization reaction, which is at moderately elevated temperatures or upon exposure to UV irradiation. Finally, it should contain functional groups well adapted to assemble with functional monomers [85][89].

Before the imprinted material can be used in any application, the template molecules have to be optimally removed from the polymer [97]. However, most of the time there is a challenge of incomplete removal of the template from the polymeric matrix [98]. Such an occurrence leads to contamination of the sample during the analysis by the residual template released. Removing all

the template molecules from the synthesized MIP matrix is extremely difficult and tedious but key as it is the step that is responsible for the creation of the cavities (recognition sites) needed for rebinding [99]. The most successful alternative has been to use a mimic of the target analyte as a template molecule, also referred to as a 'dummy' [87]. The use of this structural analogue should be in such a way that one can obtain imprinted binding sites with good selectivity towards the analyte and should be different from the target analyte such that the analytical separation or detection system employed after sample preparation can distinguish clearly between the analyte and the residual template [69].

2.4.2 Functional monomer

The role of the functional monomer is to provide functional groups which can form a complex with the template. It is responsible for the binding interactions in the imprinted binding sites. For non-covalent imprinting, the hydrogen bond is most often applied as a molecular recognition interaction of molecularly imprinted polymers [100]. From this, a vast majority of molecularly imprinted carriers are based on organic acrylates or acrylic polymers, since the carboxyl group found on the acrylate, functions as a hydrogen donor and a hydrogen acceptor at the same time [72]. Some researchers have employed heteroaromatic bases such as vinylpyridine as monomers [101], other examples are shown in figure 10. An optimal template /monomer ratio is achieved empirically by evaluating several polymers made with different formulations with increasing template. Normally monomers are employed in excess relative to the number of moles of template to favor the formation of template-functional monomer assemblies. A typical template/ monomer ratio is 1:4 or greater [102]. For covalent molecular imprinting, the effects of changing the template to functional monomer ratio is not necessary because the template dictates the

number of functional monomers that can be covalently attached; thus the ratio is stoichiometric [85].

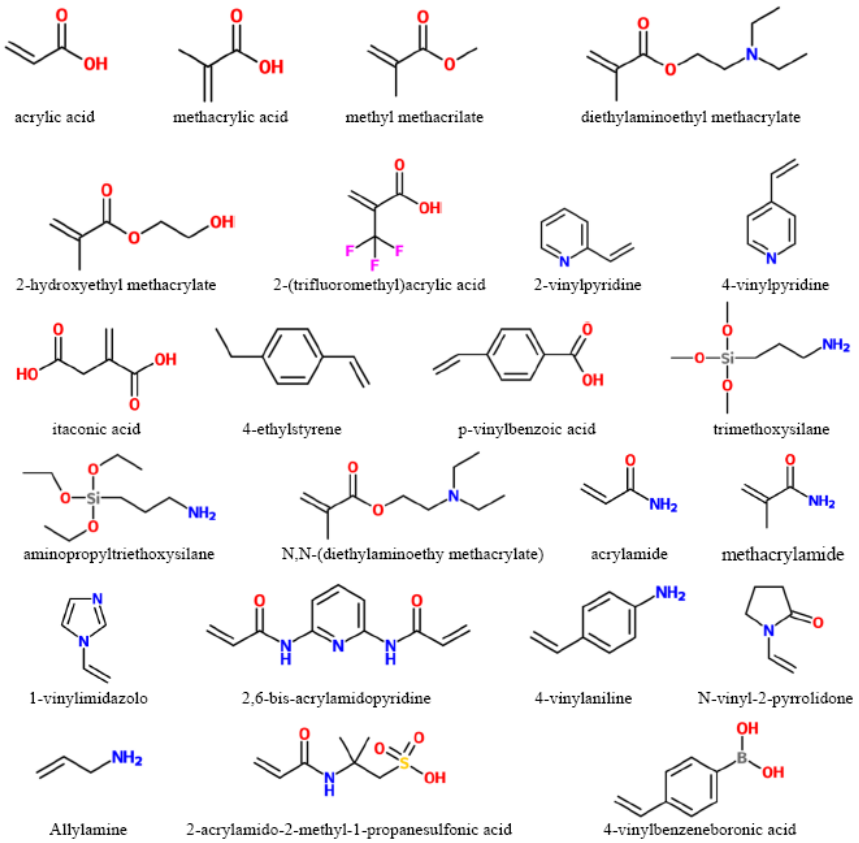


Figure 10: A selection of common functional monomers employed in molecular imprinting

2.4.3 Cross linking monomers

Cross-linking monomers (cross linkers) play an important role in the formation of molecular fingerprinting. Cross-linkers fix functional groups of the functional monomers around the template molecules, thereby forming a highly cross-linked rigid polymer [89]. It is important in controlling the morphology of the polymer matrix, whether it is gel-type, macroporous or a microgel [85]. Cross-linkers provide closure of the pre-polymerization complex in the rigid structure of the polymer, thus, forming a highly cross-linked, three-dimensional structure with recognition cavities for future molecular recognition during rebinding. In addition, they

determine the physical and chemical polymer stability. The quantity of cross-linker should be high enough to maintain the stability of the recognition sites [72]. Ethylene gycol dimethacrylate (EGDMA) and trymethylolpropane trimethacrylate (TRIM) are commonly employed. The reason being that they have multiple polymerizable acrylate groups resulting in polymers with more rigidity, structure order and effective binding sites [103] [100].

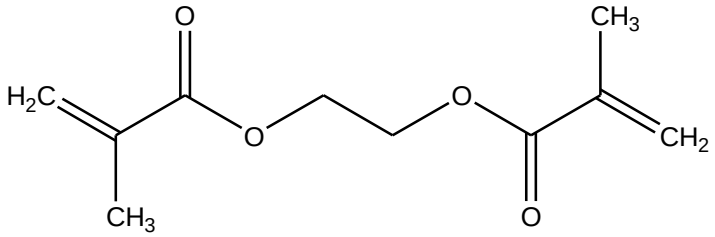


Figure 11: Structure of EDGMA

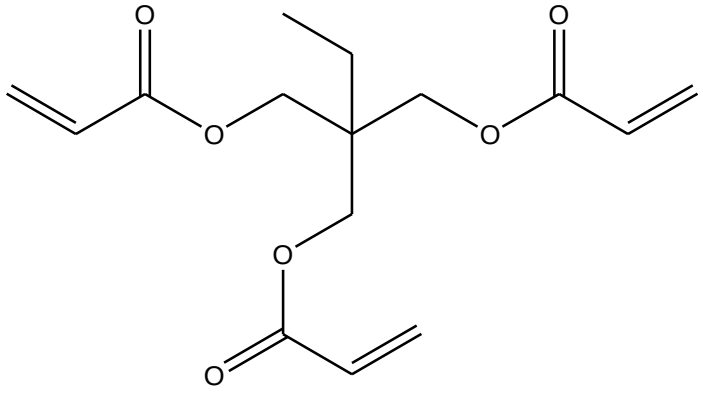


Figure 12: Structure of TRIM

2.4.4 Porogen

Solubilizing solvents are referred to as ‘porogens’. They play a dual role. In addition to mediating the interactions between the monomer functional groups and the template molecule,

porogens determine the timing of the phase separation during polymerization, which is an important determinant of polymer morphology, porosity and ultimately accessibility of the binding site [104]. Porogenic solvents with low solubility phase separate early and tend to form larger pores and materials with lower surface areas. Conversely, porogenic solvents with higher solubility phase separate later in the polymerization resulting in materials with smaller pore size distributions and greater surface area [85]. The nature of the porogen also determines the strength of non-covalent bonds in the creation of polymer's rigid structure [72]. Porogenic solvents in MIP synthesis are usually of relatively low polarity, in order to reduce the interferences during complex formation between the print molecule and the monomer. Examples of these porogens include toluene, dichloromethane, chloroform and acetonitrile. However, if hydrophobic forces are involved in template-monomer complexation, water or other protic solvents are preferred [18].

2.4.5 Initiator

The most common method of initiation in the synthesis of MIPs is by the formation of free radicals. The initiator provides a source of free radicals. These can be generated by thermal or photolytic decomposition of azobis(nitriles) or peroxides, such as azo (bis)-isobutyronitryl (AIBN), 2,2- azobis (2,4-dimethyl-valeronitrile) (ABDV) and benzoyl peroxide (BPO) [105]. The rate and mode of decomposition of an initiator to radicals can be affected by several factors, including heat, light and by chemical/electrochemical means, depending upon its chemical nature. Also the atmosphere of synthesis influences, for instance, oxygen gas slows down free

radical polymerizations. Removal of dissolved oxygen can be achieved by ultra-sonication or by purging of the monomer solution by an inert gas, e.g. nitrogen or argon [106].

2.5 Methods of MIP Synthesis/Fabrication

MIPs can be prepared in a variety of physical forms, employing various synthesis or fabrication methods, depending on their final application [100]. Conventional MIPs are prepared employing free radical bulk polymerization synthesis. The synthesis method is popular because it is simple and requires no prior skill, however it yields particles of irregular size and shape [18]. Recently efforts have been made to prepare highly uniform spherical imprinted particles, of the nanoscale magnitude. Different methods of synthesis/fabrication have been employed to prepare MIP nanoparticles, namely suspension, precipitation [107], multi-step swelling [90] , in-situ polymerization [107] and electrospinning.

2.5.1 Free radical bulk polymerization

Free radical bulk polymerization is the most popular method for preparing MIPs due to its simplicity and versatility [18]. In this method all the components are dissolved in a small volume of a suitable solvent, which serves as a porogen. The polymerization is photo-chemically or thermally initiated [107]. During polymerization, the complexes formed between the template molecule and the functional monomers are stabilized within the resulting highly cross-linked polymer matrix to produce a monolith polymer structure [85]. The resulting insoluble polymer monolith is then ground into a powder and sieved to collect homogenous powder particles. The process is laborious, time-consuming and yields only moderate quantities of useful product with low capacity and poor site accessibility by the template during rebinding experiments [108] as

each powder particle is relatively bulky. Furthermore, particles possess irregular shape and heterogeneous binding site distribution [109].

Since decreasing the size of the particles leads to larger surface areas, it seems the ideal MIPs particle sizes for many applications must be one dimensional and nano sized to attain this attribute and improve accessibility and efficiency of rebinding of the target analyte. Consequently, considerations have been geared towards achieving highly uniform spherical imprinted particles, of the nanoscale magnitude. Different methods of synthesis/fabrication, namely suspension, precipitation, multi-step swelling, in situ polymerization as well as electrospinning, have been employed to prepare uniformly shaped MIPs of very small particle sizes (nano scale) with large surface areas and easy accessibility of the recognition sites.

2.5.2 Suspension polymerization

In this synthesis method, a hydrophobic organic medium containing the monomer and an oil soluble radical initiator is dispersed as liquid droplets by vigorous stirring with the action of an impeller. Each droplet becomes a miniature "chemical reactor", within which the free radical initiator starts polymerization [107]. A major objective of suspension polymerization reaction is to control the final particle size to between 10-100μm. This is achieved by the addition of a stabilizing or suspending agent [110], either water soluble polymers, such as poly (N-vinylpyrrolidone) and poly (vinyl alcohol-co-vinyl alcohol) or insoluble organic salts such as talc, calcium and magnesium carbonates, silicates and phosphates [111]. The stabilizer improves dispersion by increasing viscosity of the aqueous phase. It acts by forming a film at the droplet or

particle surface thus controlling coalescence of the monomer droplet during the early stages of the polymerization and agglomeration of the partially polymerized particles.

This method of polymerization is advantageous due to its easy temperature control, low dispersion viscosity, and low separation costs [112]. Its disadvantages include the disruption of the monomer-template complex, due to the presence of surfactants, and the remnant quantities of stabilizers in the polymer particles after extensive washing [107].

2.5.3 Precipitation Polymerization

The precipitation polymerization approach for obtaining MIP nanoparticles was first described in 1999, where monodisperse particles were imprinted for 17β-estradiol and theophylline [113]. It is based on the precipitation of the polymeric chains out of the solvent in the form of particles as they grow insoluble in an organic continuous medium [100]. The polymer grows in the form of independent spherical nanoparticles, with a high yield and homogeneous distribution of the binding sites that are easily accessible.

This method is analogous to bulk polymerization except for the addition of higher quantities of the porogen (2 to 10 times higher) and the resulting smaller, uniform polymer particles. *Mohajeri et. al.* prepared clozapine (CLZ) imprinted polymers by bulk and precipitation methods and compared them [109]. The results demonstrated that precipitation polymerization was a more convenient synthesis method as there were no post processing steps like grinding, sieving and sedimentation.

2.5.4 Multi step Swelling

Two-step or multi-step swelling polymerization was developed by *Hosoya et.al* and further optimized by Haginaka and Sagai [90]. It requires several swelling steps before the polymerization process. The method consists of swelling seed particles (polystyrene latex) employing micro-emulsion of a low molecular weight activating solvent such as dibutyl phthalate in water containing a stabilizer in the presence of an initiator. Once the emulsion droplets have been adsorbed onto the seed particles, the dispersion is added to a second dispersion containing a porogen, functional monomers, cross-linker and the template dispersed in water, in the presence of a polymeric stabilizer such as polyvinylalcohol. The mixture is then stirred for a few hours until the droplets are absorbed onto the seed particles. Finally, the dispersion is purged with an inert gas and polymerization started [15].

The resultant particles are comparatively monodisperse in size and shape, making them suitable for chromatographic applications. The method however presents some challenges; it is time consuming and fairly complicated procedures and reactions are involved. Also the aqueous suspensions used in this method may interfere with the imprinting and thus lead to decrease in selectivity [85]. The method's applicability is limited to templates that can interact with monomers through strong electrostatic and hydrophobic interactions [15]. This was also reported by Haginaka's group; they had proposed the use of multi-step swelling combined with restricted access media to exclude macro molecules and a MIP to selectively retain target analytes from biological fluids. They found it difficult to develop a hydrophobic surface that did not modify the selectivity of the MIP [114][115].

2.5.5 In-Situ Polymerization

In-situ polymerization is carried out directly in a chromatographic column. It is a one step, free radical polymerization ‘molding’ process [107]. The method yields polymer materials with good porosity and permeability making it a favorable fabrication method in preparing stationary phases for chromatography and SPE [90]. A typical procedure starts by activating the inner surface of the silica capillary using 3-(trimethoxysilyl) propyl methacrylate for a better attachment of the monolith. The capillary is then filled with the polymerization mixture and polymerization is carried out by placing the capillary under a UV source in a heating bath. When the polymerization is achieved, the capillary is flushed with a range of solvents to remove the remaining reagents and the template as well as to equilibrate it for later use [115].

2.5.6 Electrospinning

Electrospinning is rapidly emerging as a simple and reliable technique for the preparation of smooth nanofibers with controllable morphology from a variety of polymers for application in MIPs [116]. The advantages include its relative ease, low cost, high speed, vast materials selection, and versatility. Additionally, the technique allows control over fiber diameter, microstructure, and arrangement [117]. It consists of three major components: a high voltage power generator, a syringe pump and a collector see figure 13 [118].

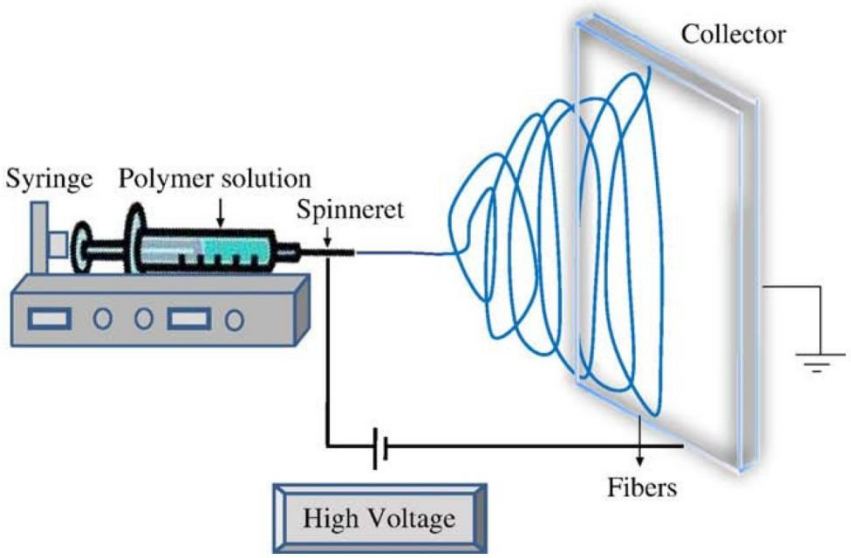


Figure 13: Typical electrospinning setup [116]

To create an electrically charged jet of polymer solution/melt out of the needle, a high voltage is applied between two electrodes connected to the spinning solution/melt and to the collector (normally grounded) [117]. Direct current power supplies are always used as electrospinning power source, though, the alternating current can also be applied [118]. The electric field at the tip of the needle electrifies the surface of the droplet of the polymer solution situated on it. Repulsion forces between charges present at the surface, as well as their attraction to the opposite electrode, induce a force that overcomes the surface tension. As a result, a charged jet is ejected from the tip of the droplet. Due to the mutually repulsive forces of the electric charges in the jets, the polymer solution jet undergoes a bending instability, thereby an elongation and thinning process. Meanwhile, evaporation of the solvent leads to the formation of a charged polymer nanofiber, collected as an interconnected web on the collector. The resulting web is composed of randomly aligned nanofibers resembling a non-woven material and a membrane [117].

The morphology and the properties of the electrospun fibers depend on the solution parameters such as the polymer and ion concentrations, the viscosity, and some operative parameters, such as the spinning distance, the needle diameter, the applied voltage and the flow rate. An important ambient parameter is the humidity which plays a fundamental role in the formation of porous structures on the surface of the nanofibers [119].

2.6 Characterization of molecularly imprinted polymers

The highly cross-linked network MIP materials formed during the molecular imprinting process are part of a class of materials known as macro-porous polymers [120]. They are challenging to characterize on account of their intractable, insoluble nature [121]. Three forms of characterization: (I) chemical characterization, (II) morphological characterization, and (III) characterization through binding studies are usually employed to physically and chemically characterize MIP materials [120].

2.6.1 Chemical Characterization

Fourier-transform infra-red spectroscopy (FTIR) is commonly employed to characterize the nature of binding interactions and the extent of complex formation between functional monomers and the template molecule in solution. FTIR characterization is performed to determine the functional groups in the MIP before and after the washing stage. The FTIR spectra give the fundamental analytical base for rationalizing the mechanisms of recognition during the imprinting process that governs interaction for selective binding site formation at molecular level. The characterization method is important when there are chemical fluctuations in the sample (e.g. functional monomer and cross-linker in an imprinted polymer) that give rise to well resolved, diagnostic signals. The interaction between the monomer and template molecule during pre-polymerization complex formation and the template incorporation into the imprinted polymer during rebinding can be studied by FT-IR [121].

2.6.2 Morphological Characterization

The morphology of MIPs arises from nuclei that form around the initiator, growing to 10-50 nm in diameter. The nuclei then aggregate to form microspheres, which in turn grow into larger clusters that form a body of beads [120]. Depending upon the method of analysis, useful information may be gathered on the particle size, shape, texture, specific pore volumes, pore sizes, pore size distributions and surface areas of the materials [106]. The scanning electron microscope (SEM) is commonly employed to examine the size, texture, structure and surface morphology of the MIP particles. Its excellent resolution makes it one of the best tools for this purpose [90]. Furthermore, some other microscopy techniques such as atomic force microscopy (AFM) and transmission electron microscopy (TEM) provide information on surface topography, crystalline structure, chemical composition and electrical behavior of the top 1μm of the specimen respectively [122].

2.6.3 Characterization through binding experiments

The defining characteristic of the binding sites of any particular molecularly imprinted material is heterogeneity: that is, they are not all identical. Therefore, it is useful to study their fundamental binding properties. In particular, it has been instructive to compare the binding properties of imprinted and non-imprinted polymers [123]. In this section the use of SPE batch analysis to evaluate the presence of binding sites in the imprinted polymers and their selectivity towards the template is discussed.

a) Equilibrium SPE Batch Rebinding Studies

Equilibrium batch rebinding is one of the most common methods for evaluating the presence of cavities. It is generally based on the use of a molecularly imprinted polymer in parallel with a non-imprinted polymer. The non-imprinted polymer is obtained by applying the same procedure of polymerization but in the absence of the template. As a result, the non-imprinted polymer (NIP) possesses the same chemical properties as those of the MIP but without the cavities. In a typical procedure a known quantity of the template is introduced to a vial with a given quantity of MIP or NIP and shaken/stirred for a known period of time to equilibrate. Once the system has come to equilibrium, the solution is separated from the polymer by filtration and the quantity of the free template is determined by a detection system that can result in quantification of the template unbound and by subtraction, the template bound is calculated.

b) Selectivity Studies

The selectivity of a MIP results from the presence of specific cavities designed for a template. It is studied by comparing the extraction performance of the MIP/NIP sorbent material in absorbing the template relative to absorbing a competing structural analogue. The choice of the analogue is according to its occurrence in the sample and its probable interaction with the MIP and NIP.

During selectivity studies an optimal quantity of MIP is added to a solution containing the target analyte (template) and/or the analogue. The solution mixture is then equilibrated for an optimal period of time. Once the system has equilibrated, the quantity of template (analogue) remaining in solution after absorption by the MIP/NIP sorbent is determined by suitable equipment. The solution is separated from the polymer by filtration. The quantity of template/target analyte or

analogue bound to the MIP/NIP sorbent is then calculated by subtraction of the concentration of the free template/target analyte or analogue from the total concentration of the template/target analyte or analogue before the MIP/NIP sorbent was added.

The distribution of the template/target analyte or analogue between the MIP sorbent and the solution mixture containing the template/target analyte or analogue is defined by a distribution coefficient, k_D, which indicates the fraction of the analyte remaining in the analyte solution mixture and the fraction absorbed by the MIP sorbent. The distribution coefficient (k_D) is calculated following equation 1:

$$k_D = \frac{conc\ of\ analyte\ in\ MIP}{conc\ of\ analyte\ in\ solution} \quad [1]$$

The selectivity of the template versus the analogue is quantified by the ratio of the two distribution coefficients $k_{Dtemplate}$ and $k_{Danalogue}$ (for template and analogue) respectively, which is referred to as the selectivity coefficient (k), see equation 2:

$$k = \frac{k_{D\,template(target\ analyte)}}{k_{D_{analogue}}} \quad [2]$$

When applied to MIPs, the selectivity coefficient indicates how many times better the template binds the target analyte (template) than it binds the analogue.

2.7 Application of MIPs

In recent years, molecular imprinting has been applied to many areas of chemistry, biochemistry, biotechnology and medicine. MIPs were initially developed as analytical materials intended to provide molecule specific separation in chromatography or solid phase extraction. The antibody like nature of these materials has provided researchers with numerous other applications including drug delivery [92], library screening [124], chiral separations [125], purification[102], biomimetic materials and sensing elements [84].

In this thesis, the synthesis, optimization and application of an aflatoxin B1 (AFB1) molecularly imprinted polymer for the accurate analysis of suspected AFB1 traces in child weaning food, *Tsabana,* produced in Serowe, Botswana were investigated. The next chapter will cover extensively the experimental procedures that were conducted in this study.

Chapter III: EXPERIMENTAL PROCEDURES

3.0 Synthesis, characterization, optimization and application of an aflatoxin B1 molecularly imprinted polymer

This chapter focuses on the experimental procedures that were performed in the study. An aflatoxin B1 selective molecularly imprinted polymer powder was prepared. The MIP powder was characterized for morphology and molecular structure by SEM and FTIR respectively. The aflatoxin B1 molecularly imprinted polymer (AFB1 MIP) powder was optimized for MIP quantity and time needed for maximum extraction of Aflatoxin B1. Aflatoxin-binding properties of the MIP powder were studied in comparison with a non-imprinted polymer (NIP) powder, a reference (control) polymer powder, synthesized in the same manner as the AFB1 MIP but in the absence of the template. The selectivity of the polymer was evaluated against a structurally related compound, aflatoxin G2 (AFG2). Finally, the AFB1 MIP powder was employed to extract aflatoxin B1 from a real sample of *Tsabana* extract.

3.1 Chemicals, Reagents and Standards

Analytical grade; Aflatoxin B1 analytical standards (3 µg/mL), aflatoxin G2 (3 µg/mL), methacrylic acid MAA (99%), ethylene glycol methacrylate (EGDMA) (98%), 1,1-Azobis (cyclohexanecarbonitrile) (AIBN), acetonitrile, toluene, methanol (99.9%), sodium chloride, HPLC grade; methanol and acetonitrile employed as HPLC mobile phases were all supplied by Sigma-Aldrich (Johannesburg, South Africa). Trifluoroacetic acid and hexane were products of Merck Schuchardt OHG (Hohenbrunn, Germany). All water used was purified using an Elgar water distiller. The *Tsabana* sample was obtained from Food Botswana (Serowe, Botswana).

3.2 Equipment Employed

Micrographs of the polymer powder particles were obtained employing a JSM-7100F Field Emission Scanning Electron Microscope (JEOL, Tokyo, Japan) to study morphology and determine size of the particles whilst the FT-IR spectra of the prepared polymer powders were obtained using a Thermoscientific Nicolet iS10 Fourier Transform Infrared Spectrometer (Johannesburg, South Africa) to identify the functional groups on the surface of the MIP particles before and after template removal as well as that of the NIP. Benchmark hot plate was purchased for general heating from Benchmark Scientific (Sayreville, NJ, USA). A scientific drying oven (TTM-J4) employed for drying the prepared polymer powders at 60℃ and a pH meter were supplied by Crison Laboratory (Liverpool, England). Centrifuge *VWR* (24/16) for separating the MIP powder from the supernatant, was purchased from VWR Catalyst (Philadelphia, PA, USA). Boeco GP Series micropipettes were from BOECO (Berlin, Germany). All glassware employed was purchased from Pyrex companies (Frankfurt, Germany). The concentrations of the aflatoxins in all solutions were determined employing an Agilent 1200 series High Performance Liquid Chromatography fitted with a fluorescence detector from Agilent Technologies (Santa Clara, CA, USA). The HPLC conditions outlined on table 2 below were employed.

Table 2: HPLC conditions

Column	Agilent Zorbax Eclipse Plus C18 4.6mm*100mm 3.8 micron
Flow rate	0.800 mL/min
Injection volume	10 µL
Column Temperature	40 ºC
Mobile phase	Water:acetonitrile:methanol (50:10:40)
Run time	5mins
Post run time	3mins
Detector	FLD at 365 λ-excitation and 455 λ-emission

3.3 Synthesis of AFB1 MIP

For the preparation of the AFB1 MIP powder, 3 µg/mL AFB1 standard (100 µL), MAA (8.1 µL), EGDMA (65.2 µL) and 1, 1-Azobis (cyclohexanecarbonitrile) (2.2 mg) were dissolved in plentiful (250 mL of toluene:acetonitrile (98:2 v/v). The mixture was refluxed at 70 ºC for 6 h after which a fine white precipitate was observed at the bottom of the round bottom flask. The precipitate was obtained as a very fine powder by simple filtration. To remove the template from the obtained fine powder, a solution mixture of the powder and solvent mix of toluene:acetonitrile:acetic acid (60:20:20 v/v) were refluxed. The powder was washed in 3hr cycles, seven times. At the end of each cycle, the concentration of AFB1 in the supernatant was

determined with the HPLC-FLD until a point where no further change in AFB1 concentration was observed with subsequent washings. A plot of the signal against concentration of AFB1 was constructed. This was performed to confirm template removal. The particles were left to dry in open air overnight and further dried in the oven at 60℃. A reference polymer referred to as a non-imprinted polymer (NIP), without the imprinting template AFB1, was prepared with a similar procedure to provide for the control in the binding experiments.

3.4 Characterization of the MIP powder

To evaluate and reveal details on the physical and chemical properties of the prepared powders, their SEM micrographs and FTIR spectra were obtained.

3.4.1 Scanning Electron Microscope

The surface morphology of the NIP, washed and unwashed MIP powders was observed with a JSM-7100F Field Emission Scanning Electron Microscope operated at an accelerating voltage of 5 kV. The powder had to be prepared before the characterization. For each powder sample, a small quantity of the powder was lightly sprinkled on a stub (sample holder) and pressed lightly to seat. To eliminate the charge effect, the powder was carbon coated employing thermal evaporation.

3.4.2 FTIR analysis

Fourier transform infrared spectroscopy (FTIR) spectra were obtained to demonstrate the existence or the inexistence of functional groups in the prepared MIP powders before and after the washing stage in order to confirm the removal of the template. The FTIR spectra were recorded in the wavelength range 500-4000 cm^{-1} on a Thermo-scientific Nicolet iS10 Fourier Transform Infrared Spectrometer. The data was collected at 2.0 cm^{-1} resolution and each spectrum was a result of 35 scans.

3.5 Pre-column derivatization procedure

All samples and standard solutions containing Aflatoxins B1 were evaporated to dryness with nitrogen in screw cap vials after which 200 µL hexane followed by 50 µL trifluoroacetic acid (TFA) was added. The mixture was vortexed for 1 min then allowed to stand for 5 min before adding 1 mL de-ionized water: methanol (9:1 v/v) solution. The resulting mixture was vortexed for 1 min and allowed to stand for the organic and the aqueous layers to separate. The aqueous layer was collected and centrifuged. The supernatant was then injected onto the HPLC-FLD.

3.6 Optimization

Molecular imprinting extraction is an equilibrium-driven process; the efficiency is dependent on the partitioning of the analyte between the liquid phase and the solid MIP powder sorbent phase. The quantity of the MIP powder sorbent and the time needed for maximum extraction were

studied and optimized. The experiments were carried out in triplicates with 15 ng/mL of AFB1standard solution at pH 7 in phosphate buffer.

3.6.1 Optimization of the MIP powder sorbent quantity needed for the maximum pre-concentration of AFB1

The doses of MIP powder sorbent were varied in the range of 5.0-30.0 mg and equilibrated in 5.0 mL solutions of 15.0 ng/mL aflatoxin B1 at pH 7 in phosphate buffer employing a water bath shaker for 6 hrs. The solution mixtures were then centrifuged for 4 mins and the supernatant collected in 10.0 mL volumetric flasks to determine the concentration of AFB1 at equilibrium employing an HPLC-FLD. The concentration of AFB1 bound to the MIP was calculated by subtracting the concentration of AFB1 at equilibrium from the initial concentration of AFB1 of the prepared standard solution. A plot of percentage of AFB1 bound against the increasing quantities of the AFB1-MIP powder was constructed.

3.6.2 Optimization of time needed for the maximum pre-concentration of AFB1

The extraction time was optimized by equilibrating the optimized dose of the MIP, with 5 mL of the 15 ng/mL of aflatoxin standard solutions at pH 7 in phosphate buffer for fixed periods from 0 mins-6 hrs. Following the same procedure as in the previous section, the concentrations of AFB1 in the supernatants at the specified times were determined. The concentrations of AFB1 bound were calculated by subtracting the concentration of AFB1 at equilibrium from the initial concentration of AFB1 of the standard solutions. A plot of percentage AFB1 bound against increasing specified time was constructed.

3.7 MIP rebinding studies

Steady state SPE batch binding studies of the template/target analyte or the analogue by the synthesized AFB1 MIP powder sorbent or the NIP were assessed. The aflatoxin B1 standard solutions were prepared with concentrations varying from 2.5-15.0 ng/mL. The optimised mass quantities of both polymers (MIP and NIP) were separately weighed into vials and mixed with 5.0 mL of the aflatoxin standard solutions of varying concentrations at optimal time. Following the procedures in the previous sections, the concentrations of the bound AFB1 were calculated. Plots of template bound (ng/mL) against initial concentration of AFB1 for the MIP and NIP were constructed and compared.

To investigate the selectivity of the MIP and NIP, the same experiments were performed with a structurally related mycotoxin (AFG2). The choice of the analogue mycotoxin was per its occurrence in the sample and its probable interaction with the MIP and NIP

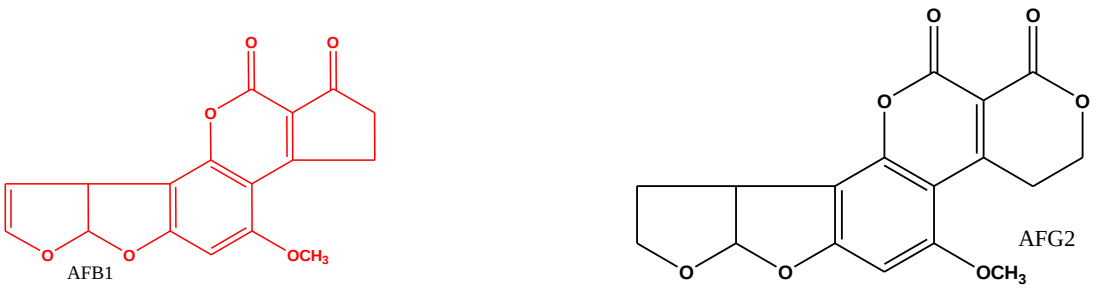


Figure 14: Structures of AFB1 and AFG2

3.8 Sample preparation

The *Tsabana* sample from the manufacturing plant in Serowe, Botswana, sample was prepared in the manner outlined below for the accurate trace analysis of AFB1.

3.8.1 Preparation of Tsabana extract

25 g of the homogenized test sample was weighed into a blending jar. Thereafter 5g of sodium chloride and 125 mL of the extraction solvent were added and the mixture was homogenized with a mixer for 2 mins at high speed. The mixture was then filtered through a fluted filter paper. 15 mL of the filtrate was pipetted into a 30 mL volumetric flask and water was added to the mark .The solution was then homogenized to produce the final *Tsabana* extract from which the suspected traces of AFB1 were analyzed.

3.8.2 Pre-concentration of AFB1 from the Tsabana extracts employing the synthesized AFB1 MIP powder sorbent

The molecularly imprinted polymer was applied to the prepared *Tsabana* extract employing optimal conditions for the selective extraction of the targeted AFB1 from the sample and its concentration determined with HPLC-FLD following the standard addition method. The obtained data was used to calculate the concentration of AFB1 in the *Tsabana* extract using the following equations:

$$\frac{[X]_i}{[S]_f+[X]_f} = \frac{I_X}{I_{S+X}} \qquad [3]$$

Where $[X]_i$ is the unknown initial concentration of the analyte with signal I_x, spiked analyte signal I_{S+X}, the diluted concentration of the original analyte is $[X]_f$. The concentration of the standard in the final spiked solution is $[S]_f$.

For an initial volume V_0 of unknown and added volume V_s of the standard with concentration $[S]_i$, the total volume is $V= V_0+V_S$ and the concentrations in equation 3 are:

$$[X]_f = [X]_i(\frac{V_0}{V}) \quad [4]$$

$$\text{and } [S]_f = [S]_i(\frac{V_S}{V}) \quad [5]$$

By expressing the diluted concentration of the analyte, $[X]_f$, in terms of the initial concentration of the analyte $[X]_i$, the concentration in ng/mL of AFB1 in the sample was determined.

The mass fraction of each aflatoxin in ng/g of the sample was calculated using equation 6 below.

$$w_i = \frac{v_5 \times m_i}{v_6 \times m_t} \quad [6]$$

Where v_5 is the volume of AFB1 in microliters and v_6 is the volume of extract injected, m_i is the mass of the aflatoxin present in the injection volume, corresponding to the measured peak in ng, m_t is the mass of the test sample in grams, present in the second filtrate taken after MIP extraction.

3.9 Method Validation

Method validation is confirmation via the provision of objective evidence that the requirements for specifically intended use or application of a method have been met. The components of method validation in the present work were linearity, limit of detection (LOD), recovery and precision (repeatability).

3.9.1 Linearity

Linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample within a defined range. Linearity of the method was evaluated by analyzing spiked *Tsabana* extracts at different concentrations ranging from 0-20 ng/g. From the data obtained a calibration curve was plotted and the square of the correlation coefficient, R^2 which is a measure of linearity and the equation of curve were obtained.

3.9.2 Limit of Detection (LOD)

The limit of detection (LOD) of an analytical procedure is the lowest concentration of an analyte in a sample that can be detected by a method but not necessarily quantified as an exact value. In the work of this study, the LODs were statistically calculated by using the intercept (y_B) and the standard error of the regression line (*SB*), at 3 times standard error and the values were calculated according to the equations:

$$y_{LOD} = y_B + 3SB \quad [7]$$

$$LOD = \frac{(y_{LOD} - y_B)}{m} \quad [8]$$

Where m is the gradient.

3.9.3 Precision and recovery

Precision is the degree of conformity between independent measurement results obtained under prescribed conditions. It is a measure of random errors and may be expressed as repeatability. Precision is expressed as a percentage of the relative standard deviation (%RSD). Repeatability and recovery studies were carried out by analyzing six replicates of *Tsabana* extracts spiked at three different concentration levels of aflatoxin B1 within a day and then introduced to the MIP extraction procedure. Recovery was calculated by comparison of the peak areas before and after MIP extraction.

The %RSD is given by:

$$\%RSD = \frac{standard\ deviation}{mean} \times 100\% \quad [9]$$

And recovery is given by:

$$\%recovery/bound = \frac{(concentration\ before\ extraction - concentration\ after\ extraction) * 100\%}{concentration\ before\ extraction} \quad [10]$$

CHAPTER IV: RESULTS AND DISCUSSION

4.0 Synthesis of the MIP and the NIP

After polymerization, fine white particles were observed suspended at the bottom of the round bottom reaction flask in a small quantity of the porogen solution (see figure 15a). The particles were optimally washed off the template, then harvested by filtration as a white fine powder (see figure 15b) and dried at 60℃.

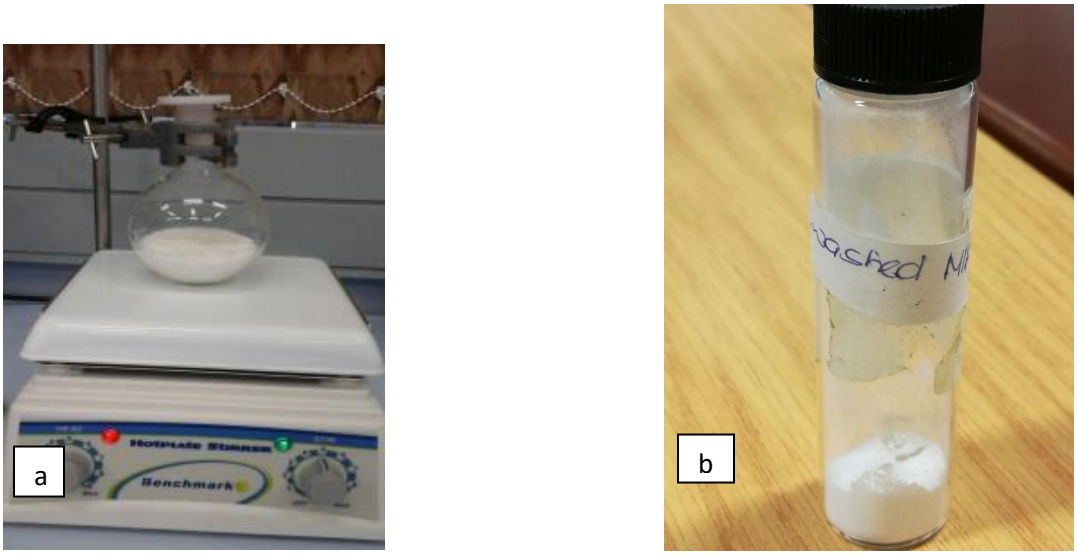


Figure 15: a) AFB1 precipitate before template removal and b) dry fine MIP powder after template removal

Optimal template removal was marked by a horizontal line plot from the 7th cycle on the plot of intensity (luminescence units) versus the number of washing cycles, see figure 16, despite continued refluxing with a fresh solvent every time. Hence, washing had to be discontinued at this point since there was no further change in the concentration of AFB1 in the washings. Extensive template removal is a key step in MIP synthesis as it is directly responsible for freeing

recognition sites for future rebinding. The choice of a particular template removal strategy is a limiting step as a poor strategy will result in few recognition sites being freed, hence poor binding recoveries and vice versa for an excellent removal strategy. Thus an excellent removal strategy is always needed.

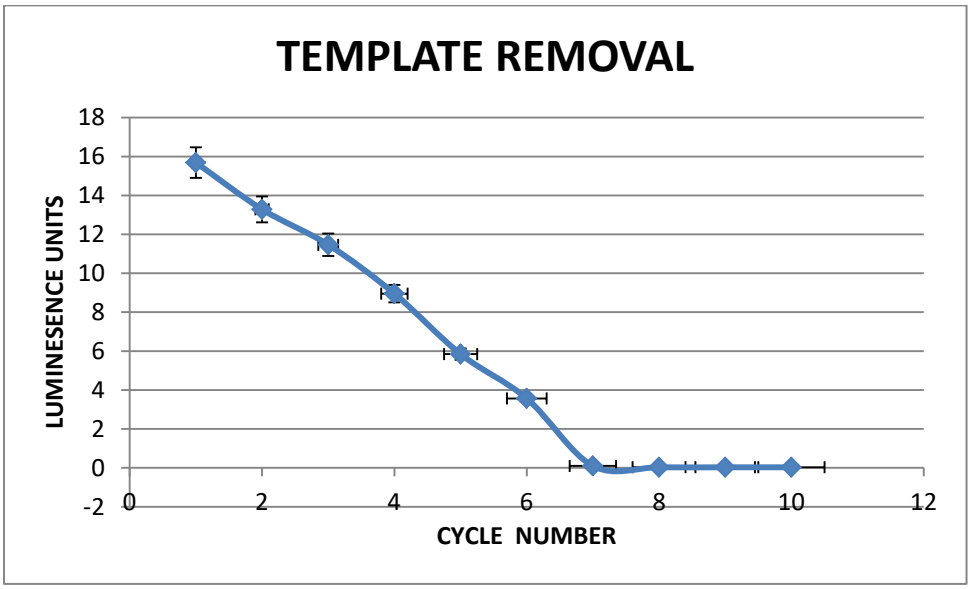


Figure 16: Template removal

4.1 Physical Characterization

4.1.1 Scanning Electron Microscope Images for the washed AFB1-MIP and unwashed AFB1-MIP

The surface morphology and the particle size of the MIP powders before and after template removal were analyzed employing a scanning electron microscopy (SEM). The SEM images for the washed and unwashed aflatoxin B1 MIP powder showed no significant difference in the morphologies of the powder when they were observed hence, only the SEM image for the washed AFB1-MIP powder is reported. From the SEM micrograph on figure 17, precipitation

polymerization had yielded MIP particles of regular spherical shapes and sizes (diameter~ 0.8 μm), which were not subjected to the destructive and time-consuming process of grinding, as they were already very small (diameter~0.8 μm), regular (figure 17) and in powdery form (figure 15b). The small MIP particles are ideal because smaller particle sizes are generally responsible for increased surface area and hence increased sorbent capacity needed for enhanced pre-concentration. The spherical geometry of the MIP particles is also ideal as it is associated with excellent sorbent materials.

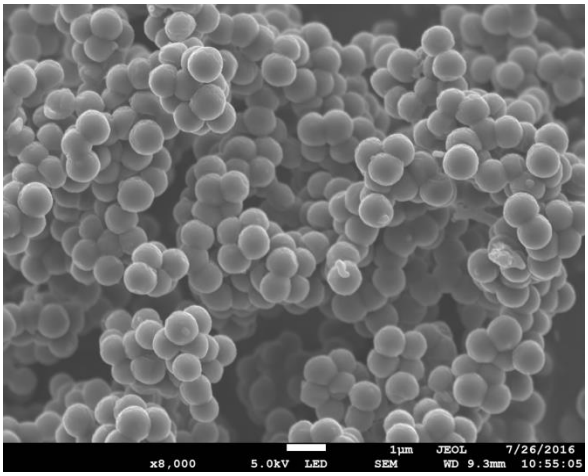


Figure 17: SEM image of washed AFB1-MIP powder

4.1.2Confirmation of the formation of AFB1-MIP powder and removal of AFB1 template from AFB1-MIP by Fourier Transform Infrared Microscopy (FTIR)

The FTIR spectra of the starting materials, MAA and EGDMA, (see figure 18 and 19 respectively) were distinctively different from that of the washed product (AFB1 MIP) (see figure 21) confirming that a product (MIP) different from the starting materials was formed. For

example, a peak at 1691 cm $^{-1}$ in the MAA spectrum (see figure 18) were found to be absent as shown in the spectrum of the washed MIP (AFB1 MIP) (see figure 21). The differences confirmed that polymerization occurred.

The IR spectra of the unwashed and washed AFB1 MIPs were recorded in the range 4000-400 nm as shown on figures 20 and 21 respectively. A band at 3661 cm^{-1} due to –OH stretching vibration of the functional monomer MAA was observed before removal of the template. The band was not observed for the washed MIP. Also the peak at 2987.94 cm^{-1} had reduced after the washing step. This confirmed removal of template and creation of recognition sites. The IR spectra showed some similarities in their backbone structure due to the incorporation of cross-linker EGDMA. The bands that were found in both spectra were the C=O stretching (≈1725 cm^{-1}), C-O stretch (≈1150 and 1250 cm^{-1}) and symmetric and asymmetric stretching of C-H due to the methyl and methylene groups existing in the polymer network (≈2980 cm^{-1}, ≈1450 cm^{-1} and ≈1390 cm^{-1}).

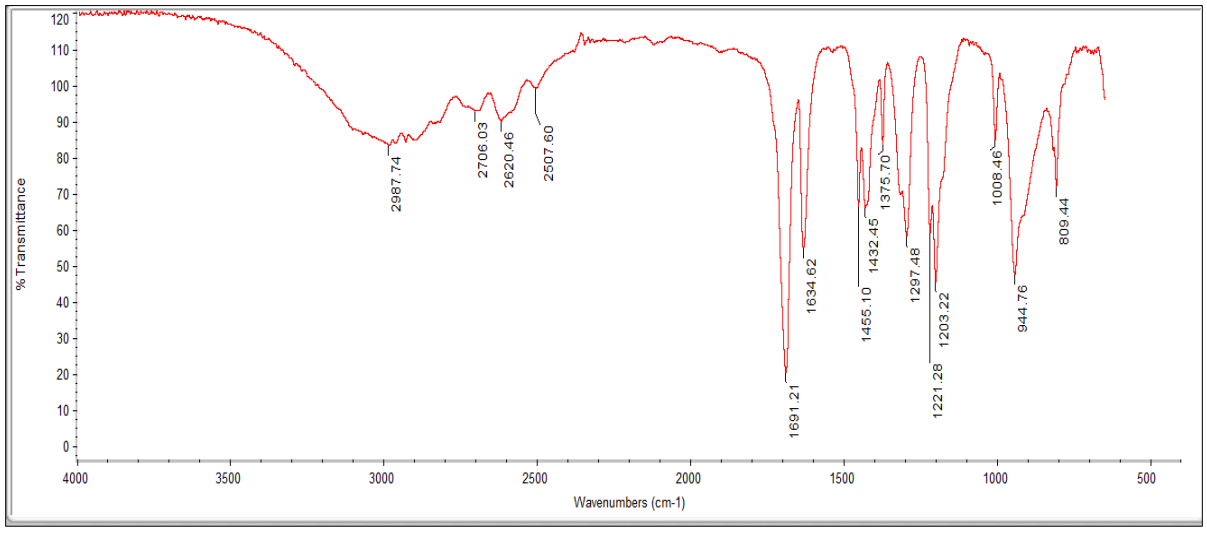


Figure 18: MAA FTIR spectrum

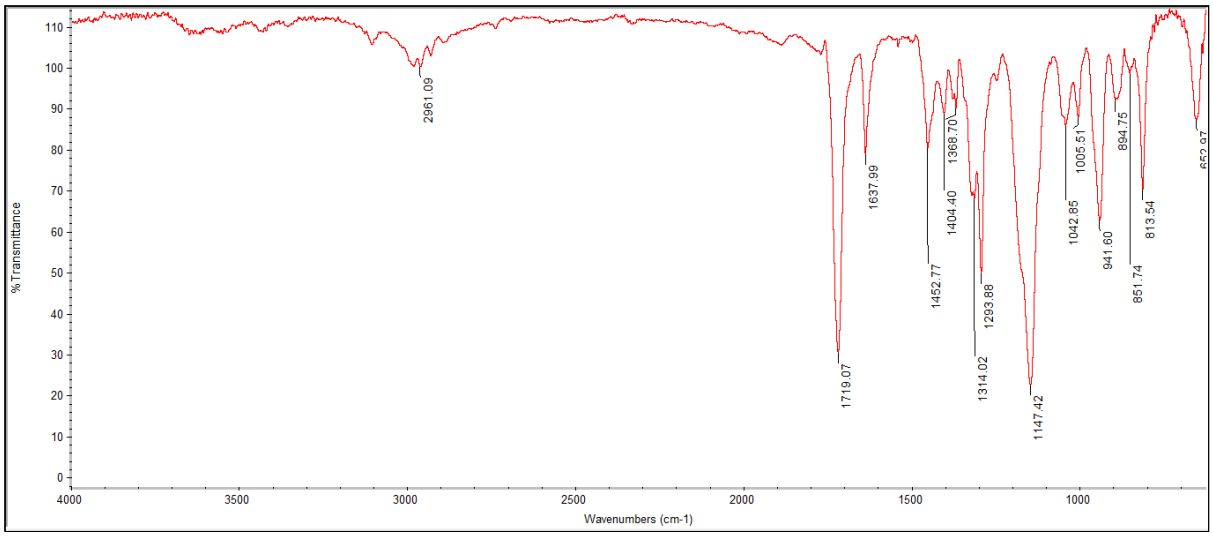


Figure 19: EGDMA FTIR spectrum

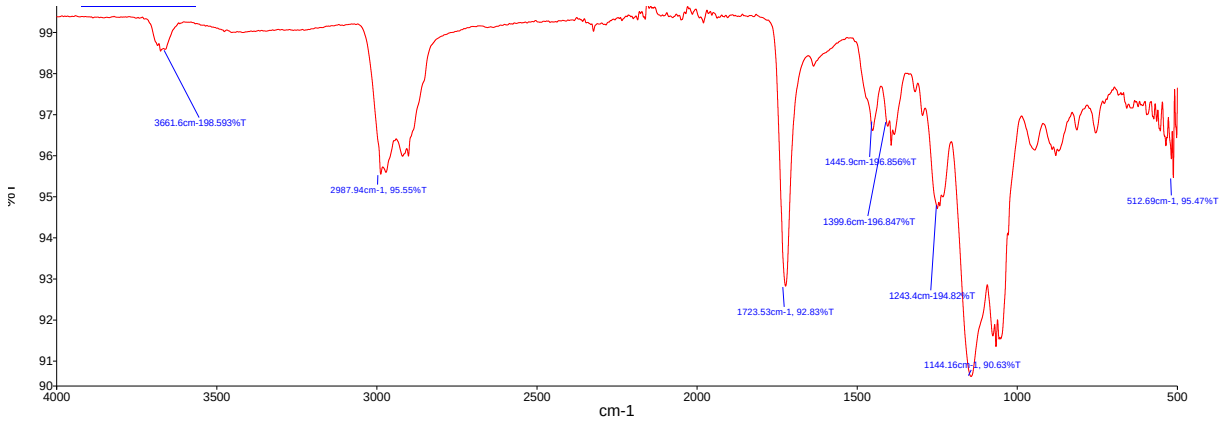


Figure 20: FTIR spectrum of unwashed MIP

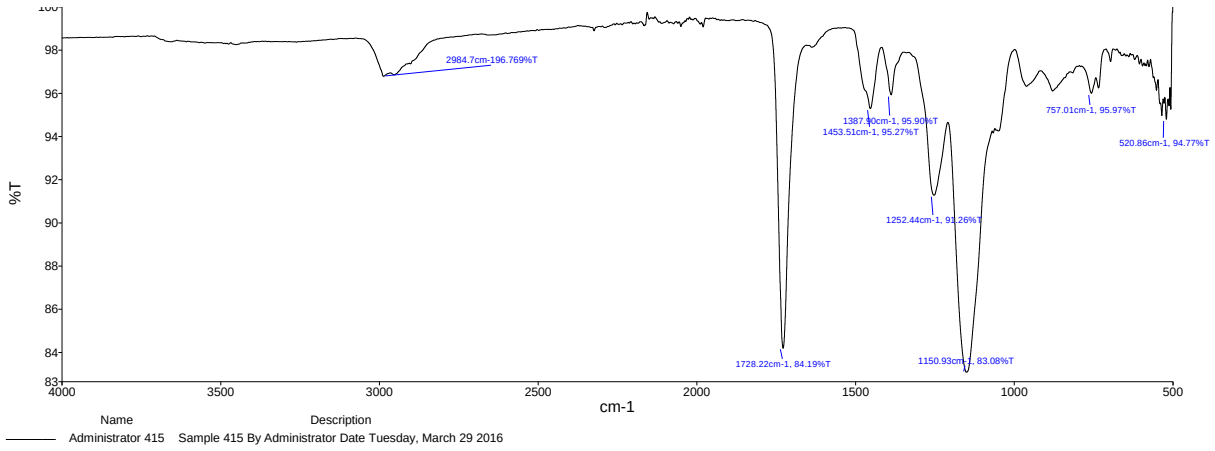


Figure 21: FTIR spectrum of washed MIP

4.2 Optimization of quantity and time needed for maximum pre-concentration of AFB1

The percentage of AFB1 template bound increased with increase in the quantity of MIP added until an optimal dose of 20.0 mg, which was marked by a point at which the plot on figure 22 started to plateau at maximum percentage of AFB1 bound, 90%. Further increase in the MIP dose did not yield any significant change in percentage of AFB1 bound, thus 20.0 mg was the optimal quantity needed for maximum pre-concentration of AFB1. The increase in percentage of AFB1 bound with increase in quantity of the MIP powder was due the availability of more binding sites from the increased MIP dose. The plateau of the plot marked the saturation point of the binding sites.

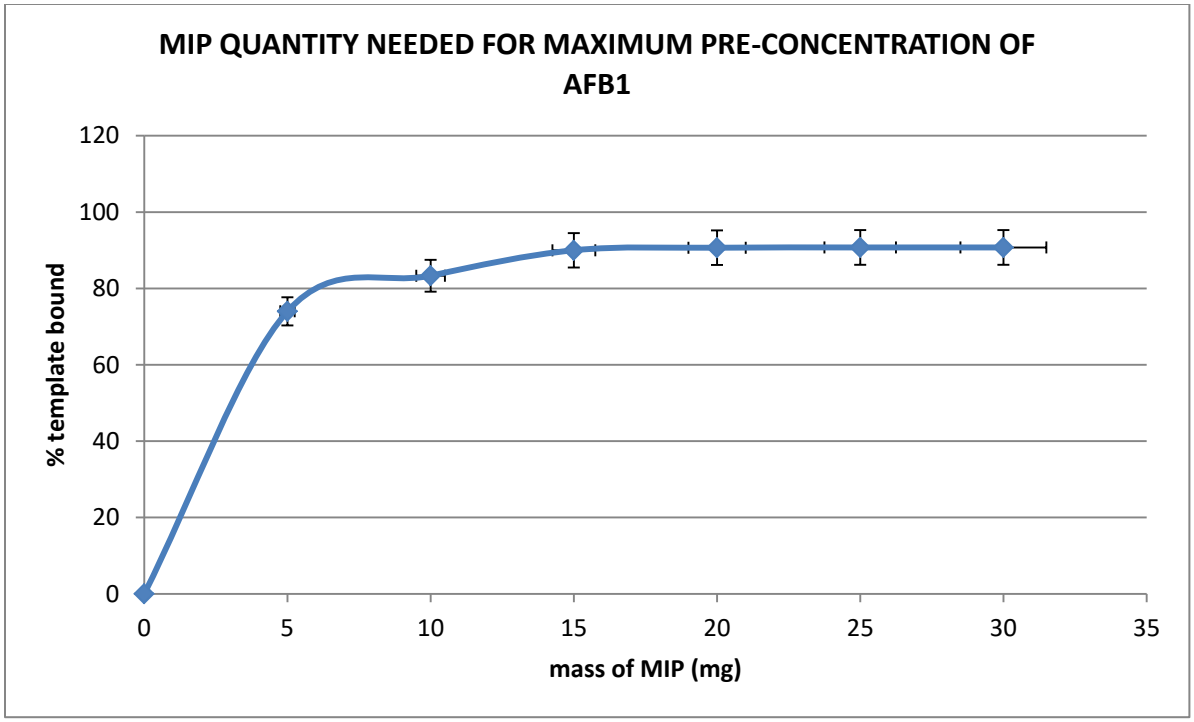


Figure 22: Optimization of the MIP quantity needed for maximum pre-concentration of AFB1

From the plot of percentage template bound against time needed for adsorption in figure 23 there was an increase in percentage of AFB1 bound to the MIP with time until an optimal time of 25 mins. Beyond this point, continued exposure of the MIP sorbent to the standard solutions of AFB1 did not translate into any further increase in percentage of AFB1 bound. This was marked by the flattening of the plot from 25 to 150 mins. The optimal time needed for maximum pre-concentration of AFB1 was as such considered to be 25 mins.

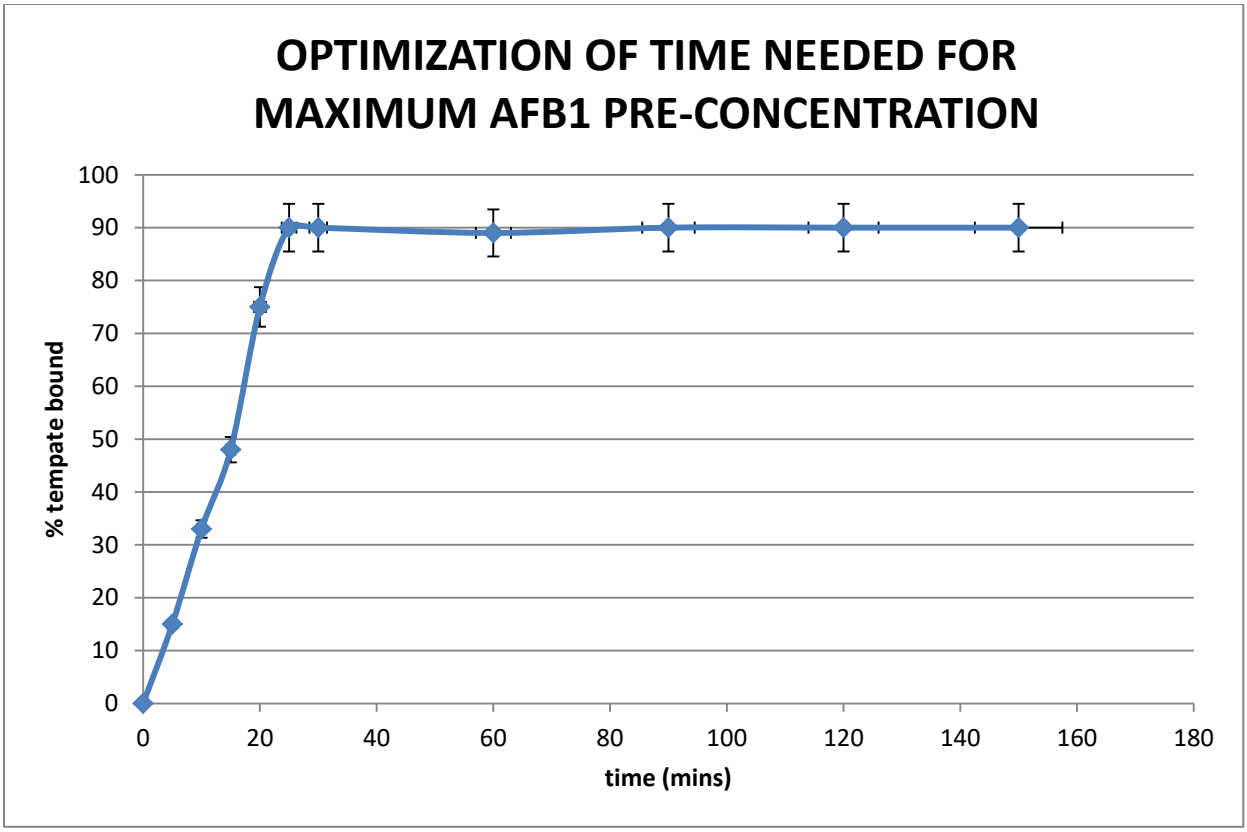


Figure 23: Optimization of time needed for maximum AFB1 pre-concentration

4.3 Rebinding of the MIP

Figure 24, compares the re-binding of AFB1 to the MIP and NIP. With each studied concentration level, more of the AFB1 was bound to the MIP than the NIP. This is because after the removal of the template from the MIP, binding sites that are complementary to the template in size, shape, and position of the functional groups were exposed, and consequently allowed rebinding, whereas the NIP with no cavities to rebind AFB1 template could not bind the AFB1 more than the MIP. Furthermore, the prepared AFB1-MIP achieved very high pre-concentration efficiencies of around 90% (compared to 8% of the NIP) at each concentration point of the AFB1 because of the increased surface area due to the small sizes of the particles yielded. Also precipitation polymerization which was the synthetic method employed, resulted in the formation of fine powdery particles with excellent binding site accessibility due to their non-bulkiness.

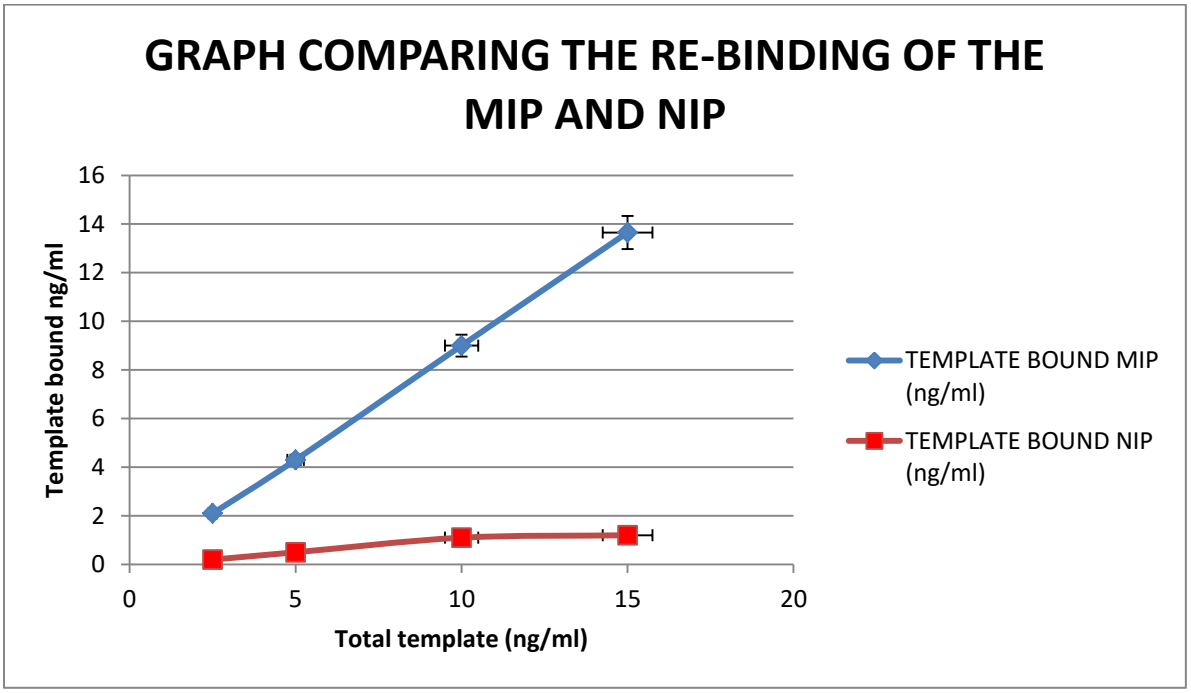


Figure 24: Re-binding of the MIP and NIP

4.4 Selectivity of the MIP

The selectivity of the MIP was studied by comparing the percentage recoveries of aflatoxin B1 and a structurally related compound, aflatoxin G2 (see figure 25). The MIP showed 90% recovery for AFB1 and 85% for AFG2. The results show that prepared MIP powder that was prepared for AFB1 equally had great affinity for AFG2 thus the prepared MIP sorbent may be employed to pre-concentrate AFG2 independently or even simultaneously with AFB1. On the other hand, the NIP showed 15% recovery for AFB1 and 25% for AFG2, further confirming that the NIP has no affinity for aflatoxins due to lack of recognition cavities.

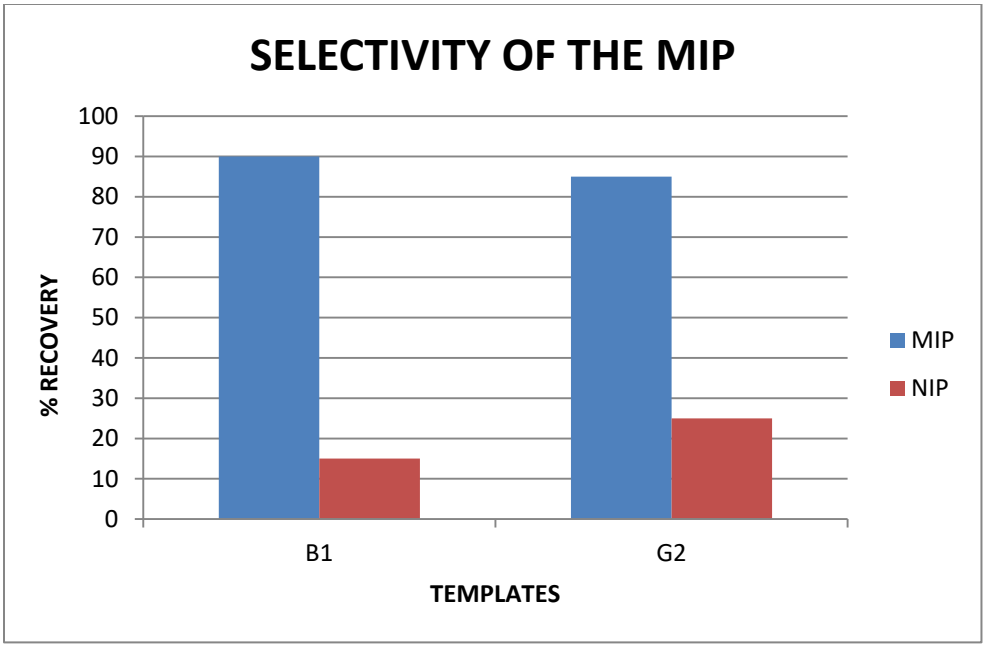


Figure 25: Selectivity of the MIP

To corroborate these results, the distribution coefficients for aflatoxins B1 and G2 in the MIP and NIP were calculated employing equation 1, (see results on table 3). The distribution coefficient for the adsorption of AFB1 to the MIP is 9.000 meaning that the concentration of AFB1 bound to the MIP is greater than that in the solution at equilibrium by a factor of 9. This shows greater affinity of AFB1 towards the MIP phase compared to the solution phase.

Table 3: Distribution coefficients for the MIP and NIP

	MIP	NIP
Aflatoxin B1	9.000	0.176
Aflatoxin G2	5.667	0.330

From the distribution coefficients calculated, the selectivity was calculated employing equation 2. The selectivity factor, k, means that although the MIP showed significant selectivity towards the analogue AFG2, the MIP was still more selective towards aflatoxin B1 than aflatoxin G2 by a factor of 1.579.

4.5 Method Validation

4.5.1 Linearity

A calibration curve was constructed using the areas of the chromatographic peaks measured at six increasing concentration levels of aflatoxin B1 spiked *Tsabana* extracts (figure 26). The square of the correlation coefficient, R^2= 0.9932 which is a measure of linearity was obtained from the calibration curve. A value of R^2 above 0.995 is deemed a good fit for most purpose but for this study the R^2 obtained is good. The value of R^2 obtained means that for the studied concentration range (0-20 ng/mL) there is a strong linear correlation between the concentration of the AFB1 spiked *Tsabana* and the intensity.

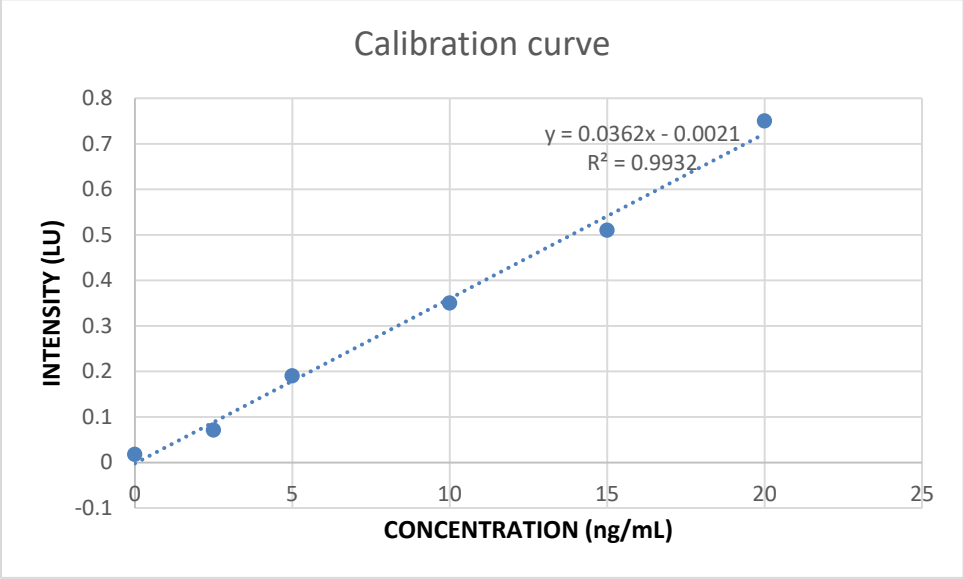


Figure 26: Standard calibration curve

4.5.2 Limit of Detection (LOD)

From the calibration curve on figure 26 and equations 7 and 8, the two LODs were calculated (see table 4). The LODs are much lower than the set regulatory levels of AFB1 in infant food

(0.1 ng/g) this means that this method is good for the determination of very low concentrations of AFB1 in *Tsabana*.

Table 4: Limits of detection

Equation of curve	y=0.0362x-0.0021
Y_{LOD} (ng/g)	0.019225±0.0002
LOD (ng/g)	0.027523±0.0005

4.5.3 Precision and recovery

Precision is expressed as a percentage of the relative standard deviation (%RSD) (see equation 9) for several replicate measurements (e.g n=6) and recovery was calculated (see equation 10) by comparison of the concentration before and after MIP extraction. The results are shown in table 5 below with low precision of %RSD of less 7% for n=6 and high %recoveries of more than 83%.

Table 5: MIP %recoveries and %RSD at three concentration levels

SPIKING CONCENTRATION (ng/ml)	% RECOVERY (n=3)	% RSD
2.50	83.51	6.41
5.00	86.10	1.75
15.00	90.03	0.81

4.6 Application to real samples

When the validated method was applied to the neat, derivatized 2 ng/mL standard AFB1 solution and real *Tsabana* extracts, employing optimized HPLC conditions as outlined in table 2, the chromatograms in figure 27 and 28 & 29 were obtained respectively. The peak for the standard

solution in figure 27 showed a symmetrical profile with a short retention time of 2.456 mins which was also emulated on the before and after application of the MIP chromatograms in figure 28 and 29 respectively.

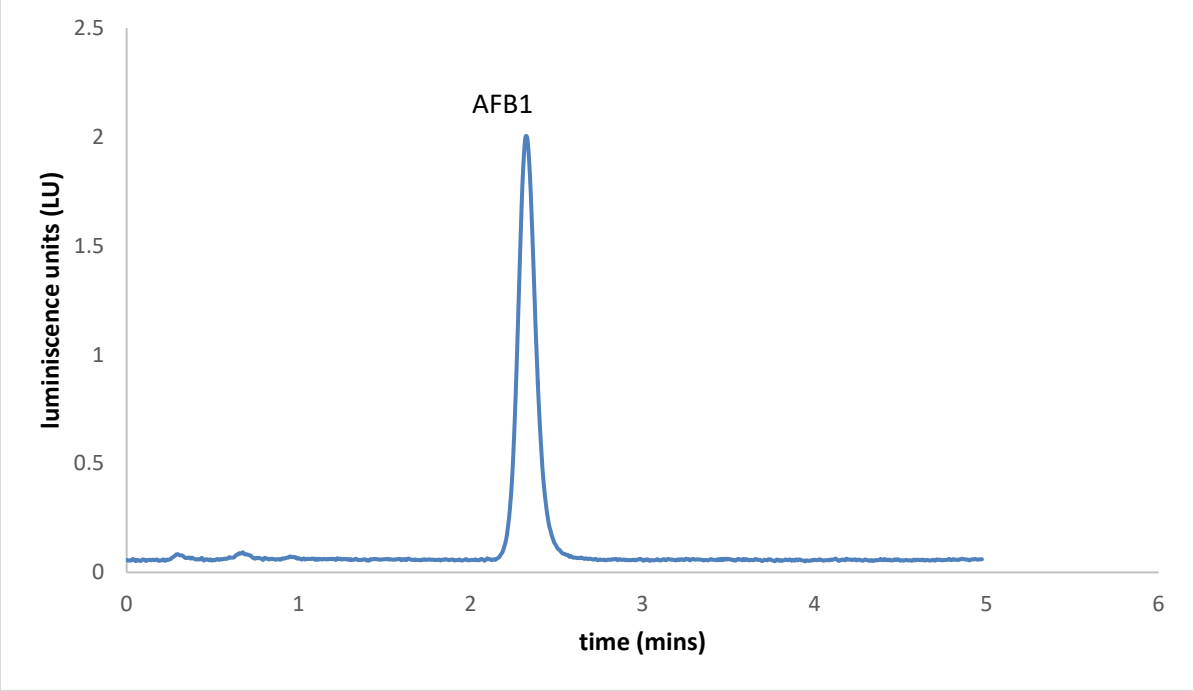


Figure 27: Chromatogram of 2ng/mL standard solution of AFB1

After pre-concentration in figure 29, it was apparent that most of the unidentified peaks were removed demonstrating that the synthesized MIP had achieved simultaneous extraction, clean up and pre-concentration of the AFB1 from the *Tsabana* sample extracts. Comparing the peak intensities of AFB1, before and after MIP application, the AFB1 intensity for before application of the MIP was 0.85 LU and it increased to 4.00 LU after pre-concentration by the MIP. The peak intensity of the selectively extracted aflatoxins B1 was significantly greater. This demonstrated synthesized molecularly imprinted solid phase extraction sorbent, managed to deal with the challenge of non-detectable low concentrations that are characterized by 'dirty' matrices by selectively pre-concentrating the AFB1 from real samples of *Tsabana* extracts by a pre-concentration factor of 5.

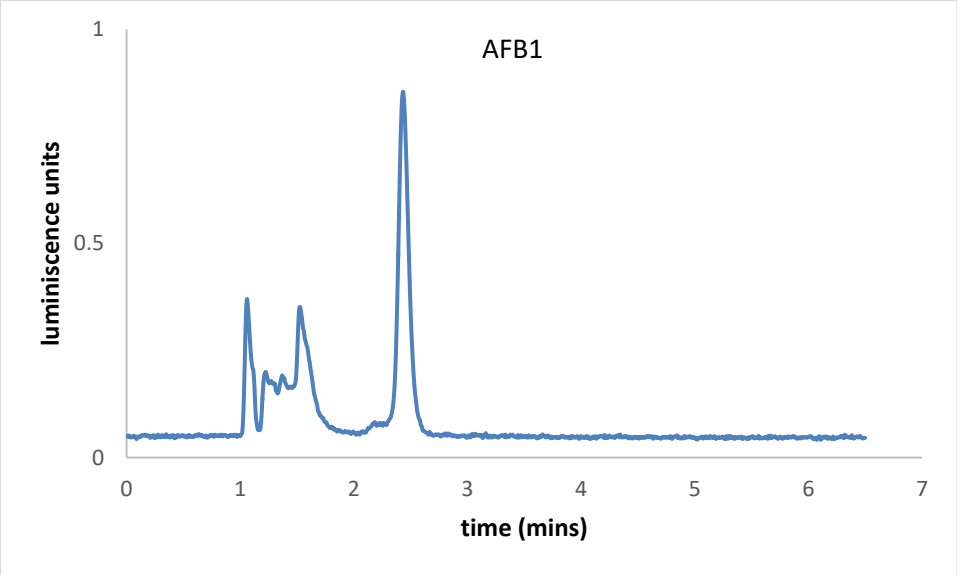


Figure 28: Chromatogram of Tsabana extract before MIP application

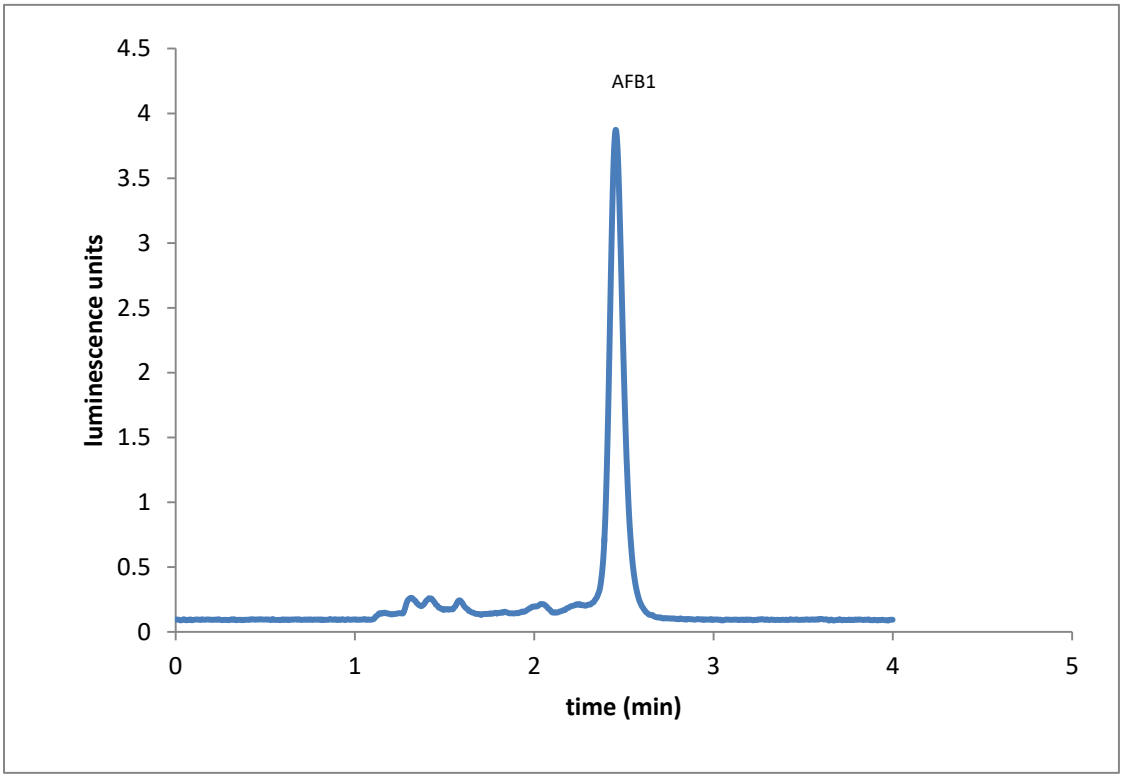


Figure 29: Chromatogram of tsabana extract after MIP application

In conclusion the mass fraction of the aflatoxin B1 in the *Tsabana* sample from Food Botswana Serowe was calculated as 0.05147 ± 0.001345 ng/g (see equations 3, 4, 5 and 6). The quantity of aflatoxin B1 present in the sample was found to be below the European Union regulated level of 0.1 ng/g. The method in this thesis allowed for the otherwise challenging AFB1 determination of concentration in complex matrices such as food to be improved and be brought to enhanced signals by selective pre-concentration employing smart functional materials such as MIPs, thus achieving easy and accurate detection.

CHAPTER V: CONCLUSION

This thesis presented the synthesis, optimization and application of a simple aflatoxin B1 (AFB1) specific extraction polymer sorbent based on molecular imprinting technology (MIT), for the selective pre-concentration of a potent AFB1 toxin from child weaning food, *Tsabana.* The prepared AFB1-MIP powder not only allowed pre-concentration of the sample but also selective extraction of the analyte, which was important, particularly because the *Tsabana* sample is characterized by complex and 'dirty' matrices which pose a challenge when the sample is directly introduced into the sensitive analytical instruments without proper sample preparation techniques. The MIP powder has demonstrated a potential for an efficient pre-concentration sorbent material that can replace commonly employed sorbent materials that are expensive and non-selective in pre-concentrating aflatoxin B1 in food. The results showed that among the variables studied, time and quantity of MIP had significant impact on aflatoxin B1 extraction efficiency. Equilibrium rebinding studies demonstrated that the MIP possessed specific binding sites for the template compared to the NIP. Validation studies verified the excellent performance of the MIP in terms of recovery, repeatability and robustness at AFB1 trace concentrations of 0-20 ng/g which includes the European Union regulatory levels of 0.1 ng/g.

Although the MIP showed high affinity for AFB1, it also had affinity for the structural analogue AFG2 and this suggests it can also be employed to selectively pre-concentrate other aflatoxins, since their structures are very much closely related. Therefore, for future work it will be necessary to develop a multi aflatoxin templated MIP which can simultaneously pre-concentrate all the main aflatoxins in complex matrices, at very low concentrations and not necessarily individual aflatoxins as is the case here. The potent nature of the template aflatoxin B1 was a challenge during MIP synthesis, thus for future work a non-toxic mimic template known as the

‘dummy’ is suggested[87]. Other than the safety advantage, the ‘dummy’ would also be beneficial because the structure would be different from the target analyte such that the analytical separation or detection system employed after sample preparation would distinguish clearly between the analyte and the residual template, thus there will be no cases of template bleeding.

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