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Development of molecularly imprinted polymer based solid phase extraction sorbents for the selective clean-up of food and pharmaceutical residue samples

A thesis submitted to Rhodes University in fulfillment of the requirements for the degree of

Doctor of Philosophy (Science)

by

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Supervised by

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Dedication

There ain`t nobody deserving this dedication but me! So this is to me `WITH LOVE. `

This has long been overdue!

Acknowledgements

There are numerous people over the years who have brought me to this point, but due to space limitation I shall only highlight a few.

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Abstract

This thesis presents the development of chlorophyll, cholic acid, aflatoxin B1 molecularly imprinted polymer (MIP) particles and cholic acid MIP nanofibers for application as selective solid phase extraction (SPE) sorbents. The particles were prepared by bulk polymerization and the nanofibers by a novel approach combining molecular imprinting and electrospinning technology. The AFB1 MIP particles were compared with an aflatoxin specific immunoextraction sorbent in cleaning-up and pre-concentrating aflatoxins from nut extracts. They both recorded high extraction efficiencies (EEs) of > 97% in selectively extracting the aflatoxins (AFB1, AFB2, AFG1 and AFG2). High reproducibility marked by the low %RSDs of < 1% and low LODs of ≤ 0.02 ng/g were calculated in all cases. The LODs were within the monitoring requirements of the European Commission. The results were validated with a peanut butter certified reference material. The chlorophyll MIP on the other hand selectively removed chlorophyll that would otherwise interfere during pesticide residue analysis (PRA) from > 0.6 to <0.09 Au in green plants extracts. The extracted chlorophyll was removed to far below the level of ≥ 0.399 Au that is usually associated with interference during PRA. Furthermore, the MIP demonstrated better selectivity by removing only chlorophyll (> 99%) in the presence of planar pesticides than the currently employed graphitized carbon black (GCB) that removed both the chlorophyll (> 88%) and planar pesticides (> 89%). For the interfering cholic acid during drug residue analysis, cholic acid MIP electrospun nanofibers demonstrated to be more sensitive and possessing higher loading capacity than the MIP particles. 100% cholic acid was removed by the nanofibers from standard solutions relative to 80% by the particles. This showed that the

nanofibers have better performance than the micro particles and as such have potential to replace the particle based SPE sorbents that are currently in use. All the templates were optimally removed from the prepared MIPs by employing a novel pressurized hot water extraction template removal method that was used for the first time in this thesis. The method employed only water, an environmentally friendly solvent to remove templates to ≥ 99.6% with template residual bleeding of ≤ 0.02%.

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List of Abbreviations

µg	Microgram
1-2-MDA	1-2-chloromandelic acid
^{14}C	Carbon-14
2,4-D	2,4-dichlorophenoxyacetic acid
2-VP	2-vinylpyridine
3-D	3 dimensional
AA	Acrylic acid
Absorbance units	Au
ADS	Alkyl-diol-silica
AFB1 or B1	Aflatoxin B1
AFB2 or B2	Aflatoxin B2
AFG1 or G1	Aflatoxin G1
AFG2 or G2	Aflatoxin G2
AIBN	Azobisisobutyronitrile
AMPSA	Acryl-amido-2-methyl-1-propanesulfonic acid
APTMS	aminopropyltrimethoxysilane

ASE	Accelerated solvent extraction
BET	Brunauer, Emmett and Teller analysis
BPO	Benzoylperoxide
CEC	Capillary elecrochromatography
Chenodeoxycholic acid	CCA
Cholic acid	CA
C_i and C_f	Initial and final concentration respectively
C.f.	Compare with
cm	Centimeter
CO_2	Carbon dioxide
CRM	Certified reference material
DAD	Diode array detector
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenylethylene
DDT	Dichlorodiphenyltrichloroethane
DEAEM	Diethylamino ethyl methacrylate
Deoxycholic acid	DCA
DIP	Diisopropanyldenzene
DMF	Dimethylformamide

DNA	Deoxyribonucleic acid
DRA	Drug residue analysis
DVB	Divinylbenzene
EC	European Commission
EGDMA or EDMA	Ethylene glycol methacrylate
ELISA	Enzyme linked immuno sorbent assay
EPA	Environmental Protection Agency
eV	Electron volt
FLD	Fluorescence luminescence detector
FTIR	Fourier transformer infrared spectroscopy
g	Gram
GC	Gas chromatography
GCB	Graphitized carbon black
GC-ECD	Gas chromatography - electron capture detection
GMA	Glycidyl methacrylate
h	Hour
HCB	Hexachlorobenzene
HF-LPME	Hollow fiber protected liquid phase micro-extraction
HF-MMLLE	Hollow fiber microporous membrane liquid-liquid extraction
HPHTSE	High pressure high temperature solvent extraction

HPLC	High performance liquid chromatography
HPSE	High pressure solvent extraction
HTWE	High temperature water extraction
HWE	Hot water extraction
IARC	International Agency for Research on Cancer
ISs	Immuno-sorbents
K or K_d	Equilibrium distribution/partition coefficient
k	Selectivity coefficient
k`	Relative selectivity coefficient
kV	Kilo volts
L	Liter
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase micro-extraction
MAA	Methacrylic acid
MAAM	Methacrylamide
MAE	Microwave assisted extraction
MeCN	Acetonitrile
MeOH	Methanol

mg	Milligram
$MgSO_4$	Magnesium sulphate
Min	Minutes
MIPs	Molecularly imprinted polymers
MISPE	Molecularly imprinted solid phase extraction
mL	Milliliter
MMA	Methyl methacrylate
MMLLE	Microporous membrane liquid-liquid extraction
MS	Mass spectrometry
ng	Nanogram
NIP	Non imprinted polymers
nm	Nanometer
NMR ^{13}C-CPMAS	Carbon-13 nuclear magnetic resonance cross polarization-magnetic angle spinning spectroscopy
NMR	Nuclear magnetic resonance
Nq or nq	Not quantifiable
PAHs	Polyaromatic hydrocarbons
PCB	Polychlorinated biphenyls
PETRA	Pentaerythritol trimethylacrylate
PFE	Pressurized solvent extraction

PHSE	Pressurized hot solvent extractio
PHWE	Pressurized hot water extraction
PLE	Pressurized liquid extraction
PET	Polyethylene terephthalate
PPy	Polypyrrole
PRA	Pesticide residue analysis
Py	Pyrrole
r^2	Correlation coefficient
RAMs	Restricted access materials
RCC	Radical cross-linking copolymerization
rpm	Revolutions per minute
RSD	Relative standard deviation
SEM	Scanning electron microscopy
SFE	Supercritical fluid extraction
SHWE	Superheated solvent extraction
SPE	Solid phase extraction
SSE	Subcritical solvent extraction
ST	Styrene
SWE	Subcritical water extraction
TAIC	Triallyl isocyanurate
TEM	Transmission electron microscopy

TEOS	Tetraethyl orthosilicate
TFA	Trifluoroacetic acid
TFMAA	Trifluoromethyl acrylic acid
TGA	Thermo-gravimetric ananlysis
THF	Tetrahydrofuran
TRIM	Trimethylolpropane trimethacrylate
UAE	Ultra assisted extraction
UAEE	Urocanic acid ethyl ester
UV	Ultraviolet
v/v	Volume by volume
VP	Vinyl pyridine
w/v	Weight by volume
WHO	World Health Organization
α_2^H	Hydrogen bond acidity
β_2^H	Hydrogen bond basicity
ε	Dielectric constant

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Chapter 1 Background

1.1 Introduction

Good analytical protocols based on efficient materials and sample handling strategies are increasingly being required by legislation and health authorities for the accurate determination of residue concentrations of administered pharmaceuticals/drugs to humans or applied agrochemicals such as pesticides to crops. This is due to severe risks to human health that could result from unreliable, inefficient or inaccurate monitoring assays. Pharmaceutical/drug residue analysis (DRA) gives vital information about the efficacy of the administered drug(s) for further action in saving human life [1].

The effects of indirect exposure to food contaminated with pesticide residues on the other hand have raised the need for pesticide residue analysis (PRA) before consumption [2, 3]. However, the current residue analysis methods face a challenge of lack of direct and accurate analysis despite employing sensitive, hyphenated analytical instruments with quantification and detection limits down to femto-gram level [3-5]. The lack of direct and accurate analysis of the residues is due to the fact that they are sampled from very complex matrices of biological or food origin with analytes of interest present at very low concentrations.

Furthermore, biological and food samples are well known to be characterized by `dirty` and complex matrices which introduce severe disturbances in the analytical separation and detection steps [4]. Of consequence, quantitative analysis of the residues can be achieved only after extensive clean-up steps [6]. For optimal clean-up, sample handling strategies relying on selective, sensitive, robust, cheap and intelligent functional materials are needed prior to separation and detection. An example of such materials has recently been identified as molecularly imprinted polymers (MIPs). MIPs are synthetic, nano - porous polymers possessing specific cavities designed for a target analyte [7]. By mechanism of molecular recognition imprinted polymers are used as selective tools for the development of various analytical techniques such as liquid chromatography [8], artificial receptors, [9] binding assays, biosensors and solid phase extraction (SPE) [10-12].

The objectives of this thesis were to develop improved MIPs and optimize their application to areas where sample handling, clean-up strategies of higher selectivity and sensitivity based on SPE were highly sought. MIP materials for the clean-up & pre-concentration of aflatoxins from nuts, removal of interfering chlorophyll from green plant extracts during pesticide residue analysis and interfering bile acids during drug residue analysis were developed employing both the traditional methods and the new strategies proposed in this thesis. They were characterized and their performances evaluated and compared employing batch re-binding experiments. In some instances the custom-made MIP sorbents were compared to commercially available sorbents. New strategies particularly for optimal template removal which resulted in more recognition sites being freed and made available

for rebinding were also proposed. To further improve sensitivity and selectivity, nano scale MIP materials in the form of nano-fibers were prepared employing electro-spinning technique. The performance of the MIP-nanofibers was then evaluated relative to the traditional MIPs.

1.2 Sample handling

Sample handling refers to any action applied to the sample before the analytical procedure [13]. Sample handling incorporates a number of processes that include: sampling and sample preparation (e.g. sample pretreatment, extraction, clean up and sample enrichment). Thorough sample handling is very important as it ensures the integrity of samples as well as prevents deterioration and cross contamination. Furthermore, it helps in maintaining sample tracking and in safety measures [14]. Sampling and sample preparation generally accounts for about 80% of the whole analysis time [15-17].

1.2.1 Sampling

Sampling is a process of collecting small portions (samples) that are representative of the whole population. By sampling only a fraction of the population, quality estimates can be obtained accurately, quickly, with less expense and time than if the whole population were measured. Since virtually no food material can be analyzed in its entirety, careful sampling techniques are required to obtain representative, laboratory-sized primary samples, in addition to subsequent subsamples, or secondary samples [18]. The size of the sample selected for analysis largely depends on the expected variations in the properties of the

sample once sampled from the population, the cost of analysis and the type of analytical technique used. Based on this information it is often possible to employ statistics to design a sampling plan that specifies the minimum number of sub-samples that need to be analyzed to obtain an accurate representation of the population [19]. Furthermore, a checklist is usually devised as a guideline for carrying out an effective sampling strategy [20].

1.2.2 Sample preparation

Sample preparation is one step out of a series making up the overall sample handling and the analytical process (see Fig. 1.2.2) [21]. It consists of several procedures which are all geared towards bringing the concentration of the chemical residues of interest to detectable levels. Additionally, the residues of interest are isolated, any matrix interference that may affect the separation and detection system is removed and subsequent purification of the extract is achieved in the process.

Over the last decade much progress has been made in the field of detection technology [22]. Nowadays there are commercially available hyphenated analytical instruments with quantification and detection limits that suit the monitoring of food and drug residues. Despite these advances sample preparation is still a vital part of the analytical process. An effective sample preparation protocol is essential to achieve reliable results and maintain instrument performance. Analysis of `clean samples` also reduces the time to maintain instruments and in turn the cost of assay [23]. Sample preparation impacts nearly all the

assayed steps and is critical for unequivocal identification, confirmation and quantification of analytes. Generally, a clean sample results in improved separation and detection, while a poorly treated sample may invalidate the whole assay [24].

Sample preparation entails a series of procedures, each capable of a specific task. These procedures are the fundamental building blocks for any analytical method development that can be matched to an analytical challenge at hand [25]. Figure 1.2.2 summarizes typical sample preparation procedures that are usually employed in DRA and PRA.

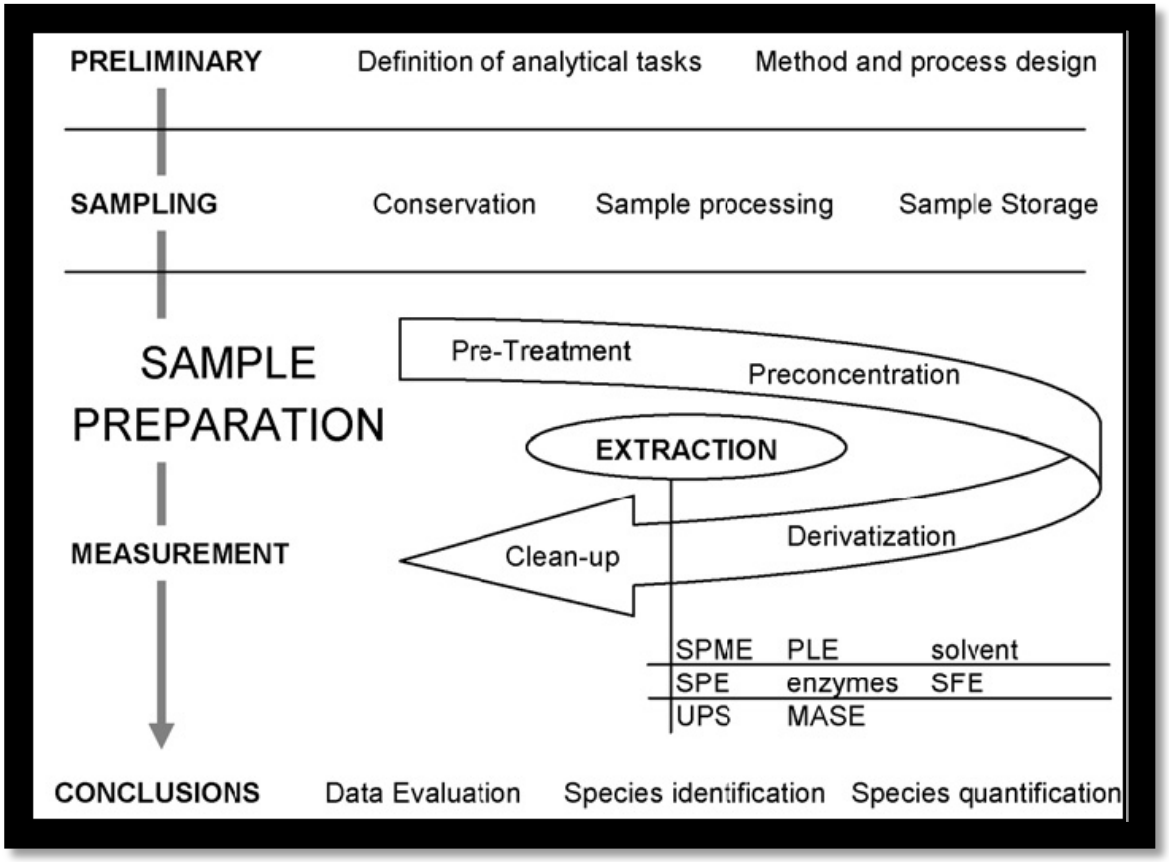


Figure 1.2.2: Sample preparation procedures within the general steps of an analytical process [21].

1.2.2.1 Sample pre-treatment

Sample pre-treatment consists of a whole series of manipulations geared toward releasing or making available the analytes of interest (residues) to the assay. Biological matrices are usually hydrolyzed to release the drug residues [26, 27]. Hydrolysis can be performed either by enzymes, acids or bases. Acidic or basic hydrolysis usually presents harsher conditions i.e. extremes of both pH and temperature can be encountered but they take less time and give cleaner extracts relative to enzymatic hydrolysis [28, 29]. Food matrices are at times subjected to hydrolysis by enzymes [30]. The obtained solution may be fit for extraction techniques to be applied to isolate the residues or, in limited cases, for direct analysis. In order to bring low residue concentrations to detectable levels, sometimes a pre-concentration step is employed.

1.2.2.2 Pre-concentration and clean-up

Drugs and food residues are usually present at trace levels in complex matrices [1, 4]. The sensitivity of the available detection system may not be adequate for their analysis. As a result, the detection limits of the method will have to be improved by selectively extracting/isolating the residues of interest from the bulk solution matrix with sample preparation techniques employing sorbents designed for that purpose. To recover the isolated residues from the sorbent, they are eluted in a relatively small quantity of the solvent, concentrating them hence the term ***pre-concentration***. Sometimes instead of targeting residues of interest, sample preparation methods aimed at removing unwanted

matrix constituents from the complex samples are developed. These are referred to as ***clean-up*** sample preparation methods.

For the increasingly sensitive chromatographic analyses good sample preparation is essential, because it protects the chromatographic columns and it allows a greater sensitivity by removal of interfering matrix components or concentrating the target residues. Thus, selective and specific sample preparation strategies are a prerequisite for reasonable, economical and sensitive analyses [31]. A number of approaches for the removal of matrix interference have been reported. For instance, solid-phase extraction (SPE) is the predominant clean-up technique [32]. However; the high matrix load of complex bio-fluids affects the efficiency of this extraction technique and gives rise to co-elution of interfering substances. This is particularly true for proteins, because many commercially available SPE sorbents are not biocompatible and cause non-specific adsorption and/or precipitation of proteins especially with on-line SPE [22, 33]. This, in turn, causes clogging of the SPE column and shortens its lifetime dramatically. Furthermore, separation and detection are eventually interfered with. As a result, most sample clean-up procedures include a protein precipitation step in order to prevent these effects [33]. Most recently, with the advent of new selective SPE sorbents like MIPs and restricted access materials (RAMs) [34], it is possible to effectively remove the unwanted matrix constituent which is what this thesis exploited.

1.2.2.3 Derivatization

Derivatization refers to all sample manipulations that transform the chemical structure of target analytes into species that can be detected with enhanced sensitivity and selectivity. In addition, derivatization may be necessary to protect species integrity through the whole analytical process as well as to produce extractable species. In LC analyses, UV chromophores and fluorophores are often introduced into sample molecules to increase their sensitivity to UV absorption and fluorescence detection respectively. Benzoyl chloride, m-toluol chloride and p-nitrobenzoyl chloride are reagents that can add a benzene ring to a solute molecule and render it UV absorbing. To introduce UV chromophores into a solute containing a carbonyl group, 3, 5-dinitrophenylhydrazine and p-nitrobenzylhydroxylamine are probably the two most common and effective reagents. To prepare fluorescent derivatives of phenols, and primary and secondary amines, dansyl chloride (5-dimethyl aminonaphthalene-1-sulphonyl chloride) is strongly recommended [35]. Another fluorescent derivatizing agent is trifluoroacetic acid (TFA). In this thesis TFA was employed to derivatize the natural food residue contaminants, aflatoxins, from the weakly fluorescing hydroxyl groups to the highly fluorescing acetal chlorides [36].

1.3 Extraction techniques for pharmaceutical and food residue analysis

The determination of trace residues and contaminants in complex matrices, such as food, often requires extensive sample extraction and preparation prior to instrumental analysis

[37]. Extraction techniques are employed to selectively separate the target species from their matrix (e.g. water, soils, sediments, biological tissue or fluids) based on differences in their chemical and physical properties. These typically include molecular weight, charge, solubility (hydrophobicity), polarity, or differences in volatility. Some extraction methods, such as immunoaffinity and imprinted polymers, utilize selectivity for specific structural groupings or mimic a biological selectivity [37]. Furthermore, extraction of analytes is influenced by the penetration of solvent into the sample (mass transfer) and matrix effects. The theory of the extraction process is covered in more detail, including theoretical equations in the book, 'Sample Preparation Techniques in Analytical Chemistry' [38]. Solid samples are usually prepared by grinding them, followed by solvent or liquid extraction. Organic or aqueous solvents are usually used to extract the analyte of interest, mostly followed by concentration or additional clean-up. These extract solutions can then be treated as liquid samples. Liquid samples can easily be handled directly by solvent–solvent extraction methods or sorption methods [39].

Several reviews have dealt with "classical" techniques of extraction [40–44]. Introduced in the mid 19th century, soxhlet extraction has been one of the classical extraction methods that have been extensively applied until the development of modern extraction techniques [40]. Classical extraction techniques, in particular soxhlet is slow (up to 24–48 h of extraction) with a very high consumption of organic solvents that have to be evaporated, although achieving high recoveries and multiple extraction possibilities [40, 41]. To address the challenges associated with the techniques, efficient and fast extraction methods for

residue analysis; like ultrasonic assisted extraction (UAE), pressurized liquid extraction (PLE) and solid phase extraction (SPE) with selective sorbents were developed. Both UAE and PLE have reduced the volume of extraction solvent required and shortened the sample preparation time (less than 1 h) when compared to conventional soxhlet extraction. The extraction recoveries attained are comparable to those of the conventional soxhlet [45].

1.3.1 Solvent extraction/Liquid-liquid extraction (LLE)

Liquid–liquid extraction (LLE) is based on the relative solubility of an analyte (residue) in two immiscible phases and the mechanism is governed by the equilibrium distribution/partition coefficient (K). Extraction of an analyte is achieved by the differences in solubilizing power (polarity) of the two immiscible liquid phases. LLE is traditionally one of the most common methods of extraction, particularly for organic compounds from aqueous matrices [46]. As a stand-alone technique it is rarely used because the pre-concentration factors achieved are typically low (1:50–1:250) and often time consuming. However, it is relatively robust and can be directly applied to a non-filtered sample, even with complex matrices and allows the transfer of analytes into a nonpolar organic solvent. The organic phase is then ready for subsequent instrumental analyses, e.g. by gas chromatography (GC).

Liquid–liquid extractions are usually accomplished with a separating funnel. The two liquids are placed in the separating funnel and shaken to increase the surface area between

the phases. When the extraction is complete, the liquids are allowed to separate; with the denser phase settling to the bottom of the separating funnel (see Fig. 1.3.1).

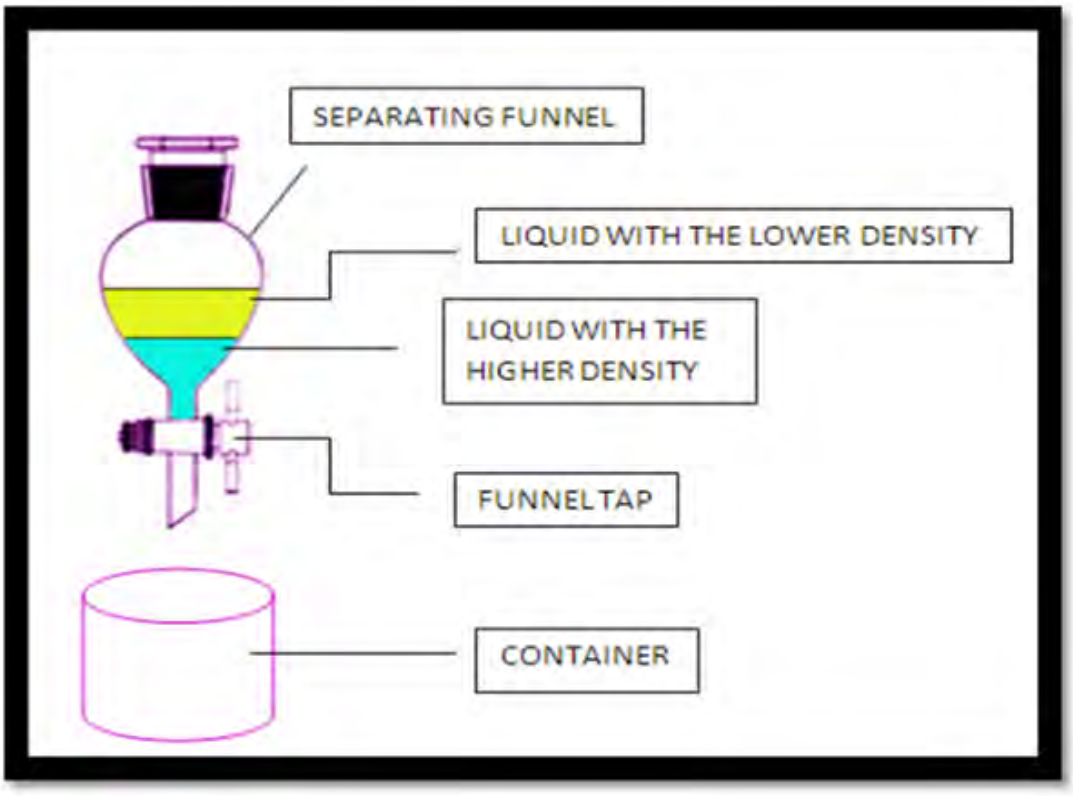


Figure 1.3.1: Liquid-liquid extraction apparatus [47]

The main advantage of LLE is the wide availability of pure solvents and the use of low cost apparatus. However, a major disadvantage of bulk LLE is the need for large volumes of organic solvents. Also, due to limited selectivity particularly for trace level analysis, there is need for clean up or analyte pre-concentration prior to instrumental analysis. The need to reduce solvent consumption led to micro extraction forms of LLE [48-51] like liquid phase micro-extraction (LPME), which has shown detection limits comparable to traditional LLE [52] but still with inadequate selectivity when coupled to MS.

1.3.2 Pressurized liquid extraction (PLE)

Several other names have been used for this technique [53], including accelerated solvent extraction (ASE®), pressurized fluid extraction (PFE), high-pressure solvent extraction (HPSE), high-pressure, high temperature solvent extraction (HPHTSE), pressurized hot solvent extraction (PHSE) and subcritical solvent extraction (SSE). When water is used as a solvent other names have also been used: superheated water extraction (SHWE), subcritical water extraction (SWE), hot water extraction (HWE), pressurized hot water extraction (PHWE) or high temperature water extraction (HTWE). The use of water at higher temperatures has specific advantages such as being cleaner, cheaper and more environmentally friendly than the organic solvents that are usually employed in PFE [37]. But PHWE is essentially a variant of PLE.

PLE is an analyte and matrix independent technique which provides cleaner extracts relative to the time-consuming classical procedures used for the extraction of compounds from complex matrices. The process is based on applying increased temperatures, elevated pressures, and keeping the solvent below its boiling point, thus accelerating the extraction kinetics and enabling safe and rapid extractions. Since the first instruments became commercially available in the mid 90's, this technique gained widespread acceptance for extraction of organic micro-pollutants such as pesticides, polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's) and dioxins from a great variety of environmental and clinical matrices [54, 55].

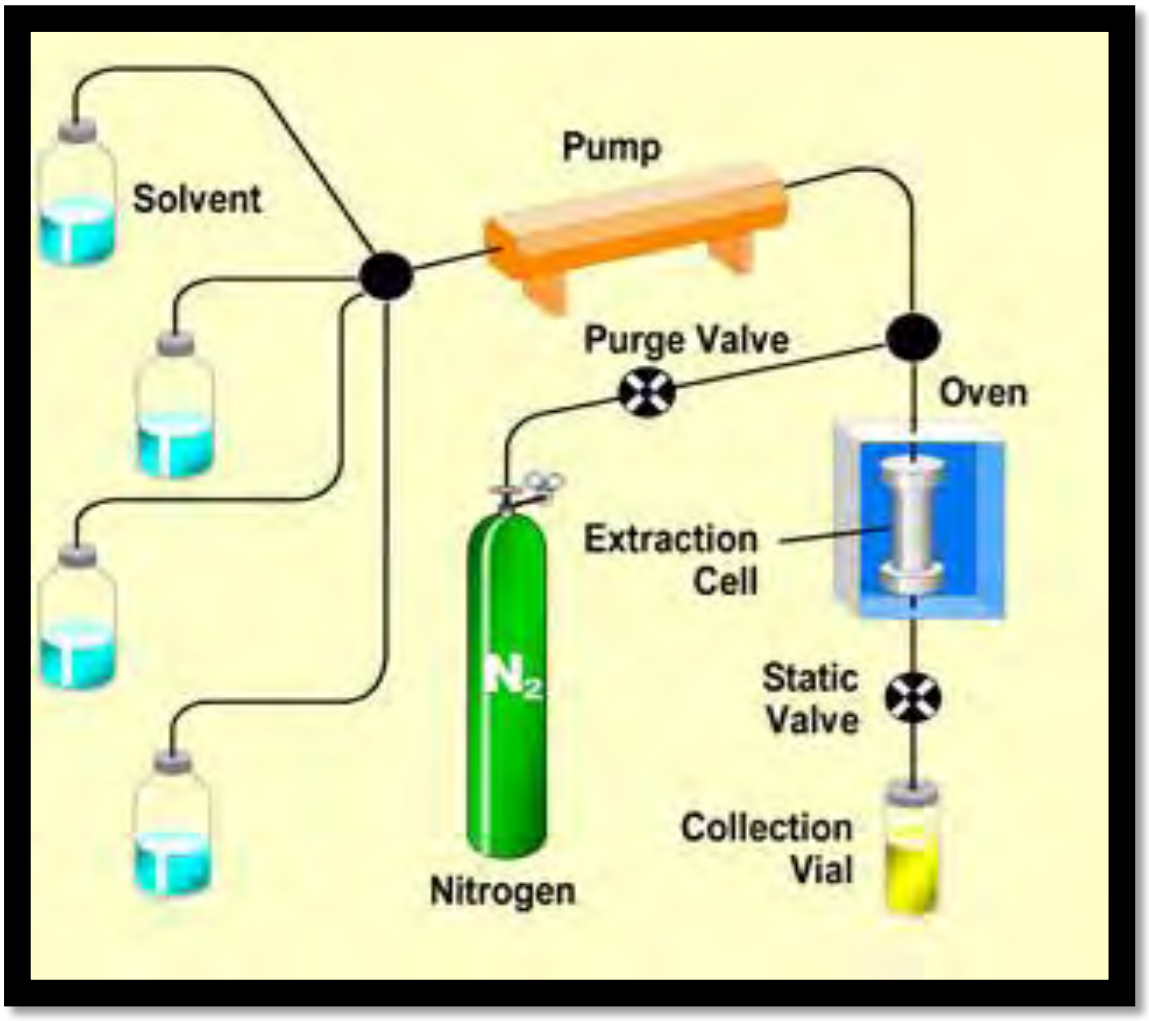


Figure1.3.2: A schematic ASE® diagram [56]

PLE can be performed in both static and dynamic (flow-through) modes, or a combination of both. In static mode, the samples are enclosed in a stainless steel vessel filled with an extraction solvent, and following extraction the remaining solvent is purged with N_2 into a collection vial (see Fig. 1.3.2). Flow-through systems continuously pump solvent through the sample, but this has the disadvantage of using larger volumes of solvent and of diluting the extract. A desiccant, such as sodium sulphate, diatomaceous earth or cellulose can be added directly to the extraction cell or sorbent materials such as MIPs can be used to

provide in situ clean-up. The latter has been employed in some of the work that will be discussed in this thesis under the experimental chapter. The extraction conditions must be optimized and this can be done using statistical 'experimental design' procedures to minimize the number of experiments [57, 58]. Modifiers can be added to the extraction solvent, for example water modified with a surfactant (sodium dodecyl sulphate) was used to extract PAHs from fish tissues [59]. For lipid containing samples, further clean-up is usually required and Gomez *et al.* [60] investigated the use of several sorbents and concluded that florisil produced the cleanest extracts for their samples. An alternative approach is to perform a preliminary PLE with a non-polar solvent to eliminate the hydrophobic compounds prior to extraction of the analytes of interest (known as selective PLE).

Carabias-Martinez *et al.* [61] reviewed the use of PLE for food and biological samples. In the review,details of several procedures for the analysis of matrix components in food and biological samples were discussed. PLE has been used as a sample preparation technique for the determination of organochlorine pesticides in fish [62] and animal feed [63]. Chuang *et al.* [64] investigated the use of PLE for the analysis of pesticides in baby food and observed matrix interferences due to the level of fat present in the samples. Although widely used as an initial extraction for solid samples, for trace analysis post-extraction procedures for analyte enrichment/concentration are often required. SPE can be coupled to the extractor outlet to enable clean-up and concentration. Coupling of PLE to other clean-up steps was detailed by Luque-García and Luque de Castro for environmental

samples [65], but has also been used for the determination of pesticides in foods [66]. Other food applications include the determination of ochratoxin A in bread [67], bisphenol A diglycidyl ether residues from canned food [68], organochlorine pesticides in vegetables [69] and persistent organic pollutants, such as PCBs and dioxins [70].

1.3.3 Supercritical fluid extraction (SFE)

A promising approach to reduce the consumption of and exposure to organic solvents is the use of fluids in the supercritical state. Among the properties attractive for extraction are the low viscosity and the low diffusion coefficients at supercritical state, contributing to a rapid mass transfer of solutions and enhanced interactions at molecular level, thus favoring solubilization processes. Unfortunately, fluids possessing critical points which are easily realizable are limited. So far CO_2 is the most prominent supercritical fluid used for extraction. It is non-toxic, non flammable, relatively cheap and possesses a low critical temperature, enabling extractions under mild conditions, thus protecting thermally labile species [71]. However, for trace analysis, a high-purity SFE grade of CO_2 can be required, unless in-house purification schemes are adopted [72, 73]. A major advantage is that the 'solvent' (supercritical fluid) is easily removed from the sample matrix after extraction by reducing the pressure and temperature.

SFE works best for finely powdered solids with good permeability, such as soils and dried plant materials. Lipid-type compounds are frequently extracted along with the analytes of

interest, and one of the main applications for SFE in foods is the extraction of lipids and the determination of fat content of raw and processed foods [74]. One of the main challenges with SFE is the robustness of the method compared to other techniques; conditions must be consistent for reproducible extractions. The automated systems that are available are aimed mainly at the environmental area, rather than trace analysis in foods. The presence of water and fat in food samples may require extensive sample preparation and as such the development of more on-line clean-up procedures for SFE could enable further applications for food analysis to be developed. For example, sorbents, such as alumina, florisil, silica or even MIPs, can be placed in the extraction cell, or used as a clean-up following the extraction to increase selectivity. Sorbents in the extraction cell can also be used for 'inverse' SFE extraction, in which interfering compounds are removed by a weak supercritical extraction fluid, leaving the analyte trapped on the sorbent for subsequent extraction under stronger conditions [75].

Several food applications are detailed in the review by Zougagh *et al.* [76], and examples include the extraction of pesticides from plants [77] and honey [78], and the determination of PAHs in vegetable oil [79]. Chuang *et al.* [64] investigated the use of SFE for the analysis of pesticides in baby food, but were unable to obtain quantitative recoveries using this technique. The use of SFE for food analysis was also reviewed by Anklam *et al.* [80]

1.3.4 Microwave assisted extraction (MAE)

The efficiency of a microwave field for analyte extraction from different matrices is well known in sample preparation [81–83] as considerably efficient and faster than conventional soxhlet extraction procedures. A low power focused microwave field, typically 20–90W which is about 10% of the maximum power provided by commercial systems, is usually employed to speed the extraction [82]. At such powers the integrity of the analyte is not affected while working at atmospheric pressure [83].

MAE agitates and heats the sample during extraction, and this technique is particularly good for achieving efficient extraction from solid samples. It is only applicable to thermally stable compounds due to the increase in temperature during extraction. As non-polar solvents do not absorb microwave energy, at least some polar solvent, such as water, must be used. The main applications of MAE are as an alternative to Soxhlet extraction because good extraction efficiencies can be achieved using less solvent and shorter extraction times. It has been shown to significantly reduce both the time and volume of solvent used, compared to dialysis [84].

Most publications on MAE have been for environmental applications, although Hermo *et al* [85] have investigated the use of microwave extraction techniques for the determination of quinolone residues in pig muscle. As with Soxhlet extraction, further extraction or clean-up steps such as SPE are generally required, particularly for the determination of trace contaminants.

1.3.5 Ultrasonic assisted extraction (UAE)

In a similar way to MAE, ultrasonication can be used to enhance extraction. The solvent type or mixture can be selected to obtain maximum extraction efficiency and required selectivity. Several extractions can be performed simultaneously and as no specialized laboratory equipment is required the technique is relatively inexpensive compared to most modern extraction methods. One disadvantage of UAE is that it is not easily automated and as with MAE it is not suitable for volatile analytes. The presence of water in UAE generally decreases extraction efficiency and some sample preparation is usually required.

Ruiz-Jiménez *et al.* [86] used a dynamic UAE technique for the analysis of *trans*-fatty acids in bakery products. Other food applications include the extraction of phenolic compounds from strawberries [87], using simultaneous extraction and hydrolysis, followed by LC. In this example no further clean-up (other than filtering) was required. However, as both selectivity and sample enrichment capabilities are limited, further clean-up and/or concentration steps are usually required for the determination of trace analytes in foods biological and pharmaceutical samples.

1.3.6 Membrane extraction

Membrane extraction involves selectively extracting analytes in aqueous liquid samples by transferring them into a second, usually organic solvent by use of a porous or non-porous membrane. With the use of only small quantities of the organic collecting solvent,

membrane extraction can provide very high concentration factors. The most versatile membrane extraction technique is a three-phase system, where analytes are extracted from one aqueous phase into another through an organic phase supported by a porous hydrophobic membrane. Recent efforts in membrane extraction gear towards the acceleration of mass transfer via the application of electric fields, rendering the so called membrane extraction [88]. As this involves two different equilibria, the selectivity of extraction is increased and the applications of this technique for on-line sample preparation have also been reviewed by Van de Merbel *et al.* [89], Cordero *et al.* [90], and Jönsson and Mathiasson [91]. According to Chimuka *et al.* and Jönsson *et al.* [92, 93] this method is most suited to analytes with high or moderate polarity and is particularly useful when size or charge can be used to achieve selection. It has been applied to the determination of triazines in vegetable oils [94], vitamin E in butter [95], vanillin in sugar and chocolates [88] and pesticide residues in egg [96].

Instead of a planar membrane, a porous hollow fiber membrane can be used to support the organic solvent during extraction from the aqueous sample and this approach has been reviewed by Rasmussen and Pedersen-Bjergaard [97]. The fiber allows the use of vigorous stirring or agitation without loss of the micro-extract (as can occur in droplet LPME) and as a fresh hollow fiber can be used for each extraction, no carry-over is observed. The hollow fiber, due to the pores in its walls, also shows some selectivity, preventing the extraction of higher molecular weight materials. This technique has been referred to as hollow fiber protected liquid phase micro-extraction (HF-LPME).

1.3.7 Solid phase extraction (SPE)

Sample preparation using SPE was firstly introduced in the mid-1970s, replacing LLE due to its simplicity, selectivity and the better LODs that it provides. Since then, SPE has gained a wide popularity due to the ease of automation, high analyte recovery, extraction reproducibility, ability to increase selectively analyte concentration and commercial availability of many SPE devices and sorbents, including the use of molecular imprinted polymers (MIPs) [98, 99].

Solid phase extraction (SPE) involves the partitioning of selected analytes between a gas, fluid or liquid phase (sample matrix) into a solid (sorbent) phase [100]. The principal goals of SPE are trace enrichment (pre-concentration), matrix removal (separation) and medium exchange (transfer from the sample matrix to a different solvent or gas phase) [101, 102]. The distribution of analyte between an aqueous solution and the sorbent in SPE is based upon interaction mechanisms such as adsorption, H-bonding, polar and non-polar interactions, cation, anion exchange or size exclusion and other chemical reactions on or in the sorbents.

The basic approach involves passing the liquid sample through a column, a cartridge, a tube or a disk containing an adsorbent that retains the analytes. After the entire sample has been passed through the sorbent, retained analytes are subsequently recovered upon elution

with an appropriate solvent [103]. A typical SPE procedure consists of four successive steps which include conditioning of the solid sorbent with an appropriate solvent, percolation of the sample through the solid sorbent, washing of the sorbent with a solvent of low elution strength to eliminate matrix components that have been retained on the solid sorbent (without displacing the analytes) and finally eluting the analytes of interest by employing an appropriate solvent with a higher elution strength (Fig. 1.3.7).

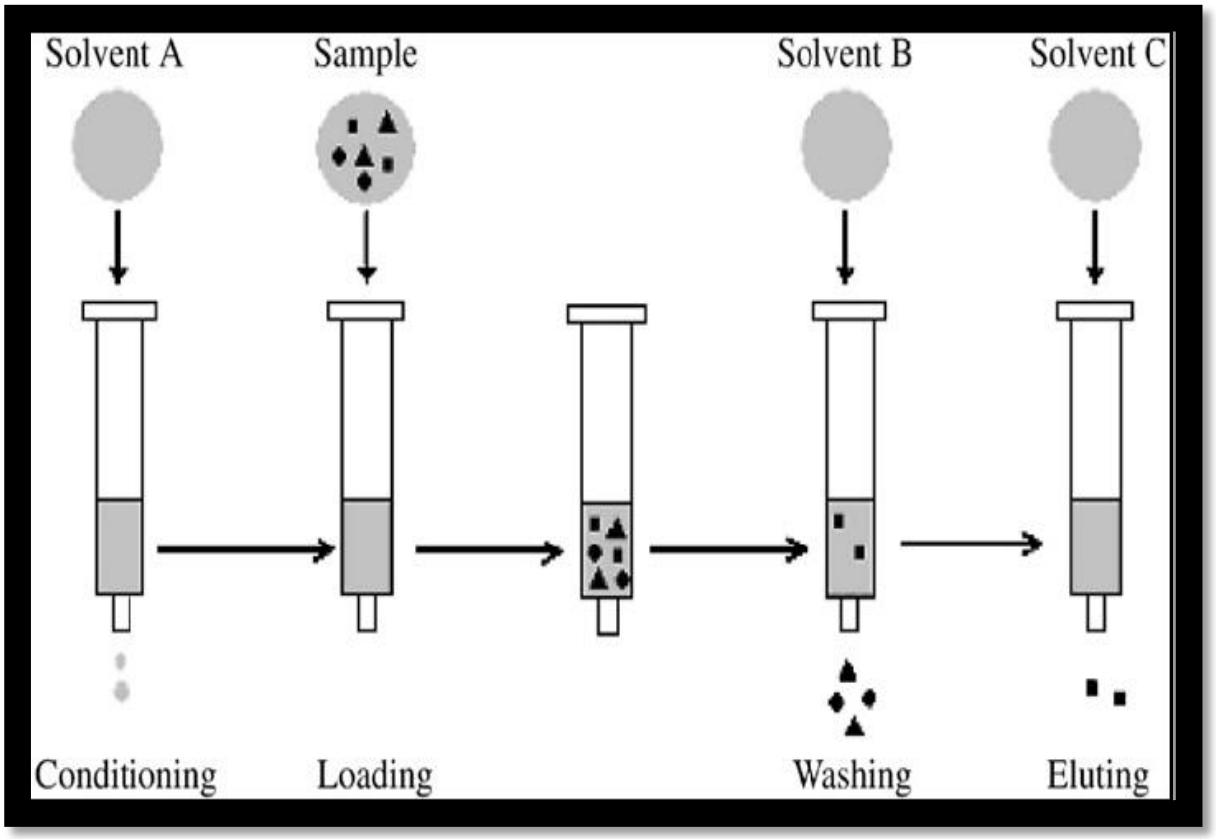


Figure1.3.7: Schematic diagram of SPE procedure [102]. The column is packed with sorbent. Solvent A, B and C are the solvents for conditioning, washing and eluting steps, respectively. The symbols of triangle and circle represent the impurities, and the symbols of square represent the target molecules.

Of the two modes of SPE, online flow injection SPE has several virtues over offline methods, viz. high sample throughput, improved precision, low sample and reagent consumption, reusability and green concept [102]. It is this mode that has further popularized SPE as a method of choice for sample preparation. For instance, when compared to the commonly used LLE method, SPE can reduce the time required (especially the automated form), can handle small sample volumes (50-100 μL), and consumes small volumes of solvent [104]. Consequently, SPE is the most popular sample preparation method to date for food, environmental and biological samples [105].

1.3.7.1 Conventional SPE sorbents

A wide range of sorbents have been used including C8 and C18 bonded phases on silica, polymeric resins (polystyrene/divinyl benzene copolymer), florisil (activated magnesium silicate), polar sorbents such as alumina, charcoal, silica and cyano & amino-bonded sorbents. Ionic functional groups such as carboxylic acid or amino groups can also be bonded to silica or polymeric sorbents to create ion-exchange sorbents. Mixed-mode sorbents are also available that use both the primary and secondary mechanisms for selective retention of analytes. Furthermore some very specific selective sorbents have been designed recently (see section 1.3.7.2).

The use of the optimum SPE cartridge can have significant effects on recoveries as shown by Posyniak *et al* [106] for the determination of fluoroquinolone residues in animal tissues, and Toribio *et al*. [106, 107] for heterocyclic amines from a lyophilized meat extract. The extensive clean-up was employed in the analysis of dioxins and PCBs [108] enabling their

determination down to part per trillion levels as required by EU legislation [109]. Similarly methods for the determination of polybrominated diphenylethers [110] utilized clean-up with silica and alumina columns following Soxhlet extraction. One of the drawbacks of SPE is that the packing must be uniform to avoid poor efficiency and although the pre-packed commercial cartridges are now considered reliable, automated systems can have difficulties with reproducibility for some sample types. The sample matrix can also affect the ability of the sorbent to 'extract' the analyte due to competition for retention. Many traditional sorbents are limited in terms of selectivity and insufficient retention of very polar compounds can also be a challenge. The use of hydrophilic materials for the improved extraction of the most polar compounds by SPE was reported by Fontanals *et al.* [111] and a number of selective sorbents have also been developed (Section 1.3.7.2).

1.3.7.2 New selective SPE sorbents

All the extraction methods including the conventional SPE sorbents discussed in the previous sections, despite their attractive features retain targeted analytes by non- selective hydrophobic interactions & polar interactions that lead to a partial co-extraction of interfering species. To enhance extraction selectivity, new selective materials based on molecular recognition were recently developed. Generally, they include restricted access media (RAMs), immuno-sorbents (ISs) and molecular imprinted polymers.

Restricted access media (RAM)

One group of selective sorbents for SPE is restricted access media [112]. These sorbents were developed particularly for analysis of biological samples, such as plasma and serum as they are designed to exclude macromolecules, such as proteins. They combine size exclusion of protein and other high molecular mass matrix components with the simultaneous enrichment of low molecular mass analytes at the inner pore surface. Macromolecules are excluded either by a physical barrier (pore diameter) or by a chemical diffusion barrier created by a protein network at the outer surface of the particle. The interaction sites within the pores are accessible to small molecules only and analytes are retained by conventional retention mechanisms such as hydrophobic or electrostatic interactions. Various RAM sorbents are available with different surface chemistries, one of the most common being alkyl-diol-silica (ADS). Several applications are given in a review by Souverain *et al.* [113], including the direct analysis of pharmaceuticals in milk [114] and tissue [115]. Both these applications used column-switching with LC for on-line extraction/clean-up.

Immuno-sorbents (ISs)

Immuno-sorbents (ISs) affinity and selectivity stem from antigen–antibody interactions thus allowing selective extraction of the targeted analyte. Several reviews have highlighted the interest in immuno-extraction as a selective sample pretreatment method [116-118]. However, the development of ISs is time consuming, and relatively expensive. The sorbents also exhibit low pH stability. These drawbacks have contributed to the recent development

of molecularly imprinted polymers (MIPs) which will be discussed in detail in the following chapter.

Chapter 2 Molecularly imprinted polymers as selective sorbents for SPE

2.1 Overview

This chapter presents background information covering the theory, imprinting process and preparation methods of molecularly imprinted polymers (MIPs). The chapter will conclude with a detailed discussion of the advanced application of MIPs as selective SPE sorbents (MISPE) and its challenges in the clean-up of food and pharmaceutical/biological samples prior to instrumental analysis.

2.2 Molecularly imprinted solid phase extraction (MISPE)

MIPs are synthetic polymeric materials with specific binding sites at molecular level designed to selectively recognize a target molecule during rebinding. MIPs like immuno-sorbents (ISs) employ a retention mechanism based on molecular recognition. As a result, they are often referred to as synthetic antibodies in comparison to ISs. Indeed both have comparable selectivities but MIPs offer better handling, stability, are cheaper and easier to prepare [119] which make them attractive for numerous applications. Over 1450 references related to the use of MIPs in a large range of application areas have been recently reported [120]. Therefore, MIPs have already been successfully used as an

alternative tool over the biological entities in several analytical fields such as separation of enantiomers in LC or CEC [121, 122], binding assays [123, 124] and sensors [125-127].

Solid phase extraction (SPE) is the most advanced application area of the MIPs [128]. The principle of selective extraction by MIPs is the same as that of immuno-sorbents and follows the SPE procedure in Fig. 1.2.7. After a conditioning step, the sample is percolated through the MIP and then a washing step removes interfering compounds that were partially retained. Desorption of analytes is achieved by percolating a solvent that is able to disrupt the selective interactions involved between the MIP and the target analyte.

In recent years the development of MISPE has been extensively reported for applications in the areas of environmental [129], food [130-132] and pharmaceutical analysis [133, 134]. In fact considering the number of articles published worldwide in peer-reviewed journals, MISPE has demonstrated to be one of the fastest growing sample preparation applications in the recent past, with more than 260 publications between 1994 and 2006 [135].

2.3 Theory of molecular imprinting

The theory of molecular imprinting emanates from understanding the fundamentals of molecular recognition as found in living organisms. It involves both selection and binding of a substrate by a given receptor resulting in a structurally well defined pattern of intermolecular interactions [136]. All the important, biological, living processes like DNA

replication, transcription and translation rely on these types of specific recognition interactions at molecular level. The processes involve the template guided coupling of various building blocks through specific binding and recognition of substrate molecules to target macromolecular receptors [137, 138].

Non-covalent intermolecular interactions e.g. ionic interactions, hydrogen bonding, Van der Waals forces and hydrophobic effects provide the required binding energy for the substrate-receptor interaction. Even though the non-covalent interactions are weak if they act independent of each other, they promote stable and specific complexation between substrate and receptor as they usually act in a polyvalent manner [139]. The binding interactions are identical to the ones employed in molecular imprinting [140].

2.4 Molecular imprinting process

The imprinting process involves complexation in solution of the template molecules with functional monomer(s), through either non-covalent or covalent interactions, followed by a polymerization reaction with an excess of cross linking monomer.

Three imprinting approaches exist; non covalent imprinting, covalent imprinting or a mixed combination of the two known as semi covalent/sacrificial imprinting. Non covalent imprinting was first proposed by Mosbach [141]. It involves employing non covalent interactions for both the imprinting and rebinding of the template to the MIP during

analysis [142]. In the covalent approach, the formation and cleavage of reversible bonds as proposed by Wulff *et al.* are responsible for the imprinting and the rebinding [143]. An intermediate approach relying on covalent interactions for imprinting but non covalent for rebinding has been proposed by Whitcombe *et al.* [144]. All the three imprinting approaches make use of a high percentage of cross-linking monomer during synthesis resulting in polymers that are very rigid and insoluble. The work in this thesis relied more on non covalent imprinting.

A general outline of the protocol used for the preparation of molecularly imprinted polymers (MIPs) is highlighted in Figure 2.4. Functional monomers arrange themselves around the template via non-covalent interactions and are "frozen" into position by polymerization with a high degree of cross-linking monomer to form 3-D, highly cross-linked polymer materials. Subsequent removal of the template from the polymer materials by solvent extraction with a suitable solvent(s) or by cleavage with a suitable chemical reaction leaves specific recognition sites within the MIP at molecular level. The induced sites have a memory of the template and are complementary in shape, size and functionality in the polymer network (Fig 2.4). Hence imprinted polymers will recognize the template during rebinding even in the presence of closely related analogues [145-147].

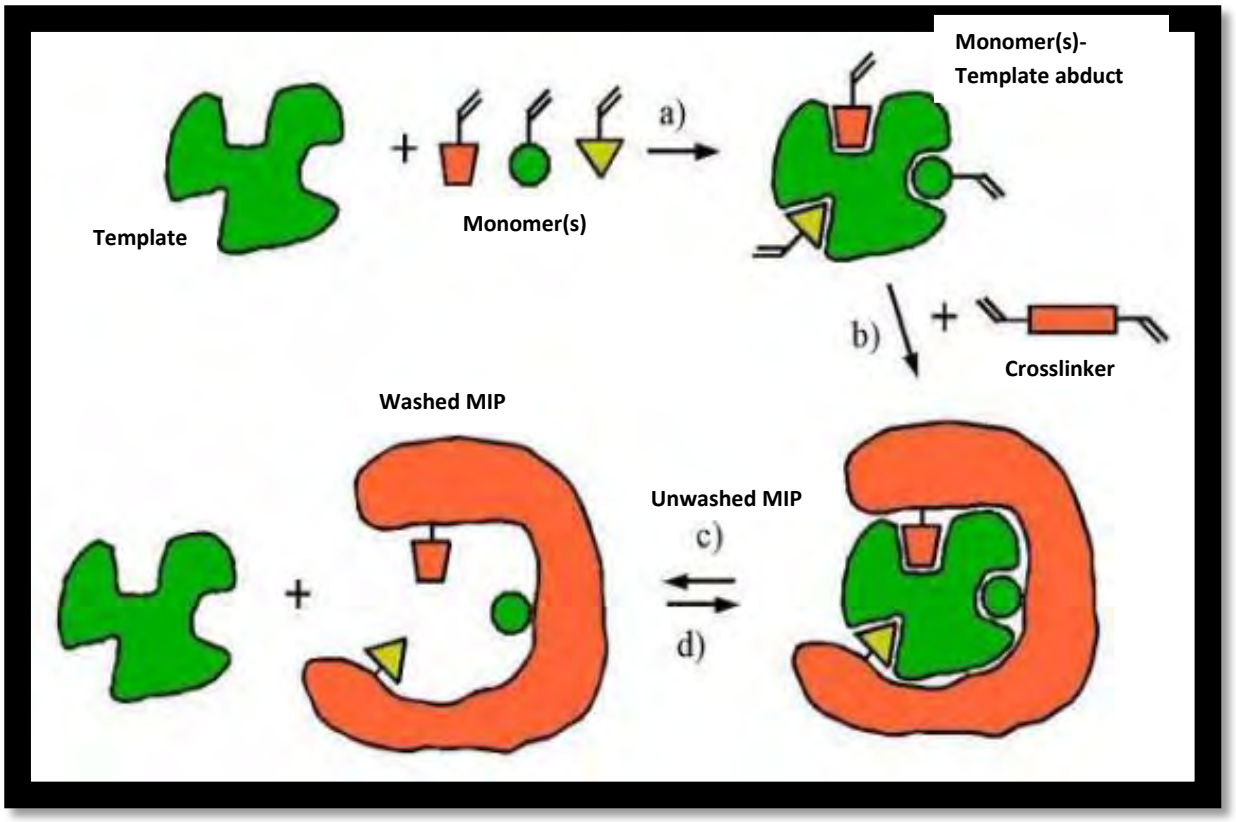


Figure 2.4: Schematic representation of the imprinting process [148], (a) Pre-assembly of functional monomer(s) guided by a template to form a firm or loose monomer-template abducts. (b) Polymerization in the presence of crosslinker, which subsequently 'glues' together the monomer-template abducts resulting in a highly networked construction (Unwashed MIP materials). (c) Removing the template (Desorption/Extraction) then leaves the construction with a binding site selective to the original template (Washed MIP). (d) Recognition of identical molecules to the template during rebinding/adsorption experiments.

2.4.1 Template (Print molecule)

In all imprinting processes the template is of central importance in that it directs the organization of the functional groups pendent to the functional monomers. For practical applications, the analyte of interest is generally used as the print molecule for preparing the imprinted polymer. The template should possess suitable functional groups for interaction with the ones on the functional monomer(s), to ensure stable complexation.

The chemical structure of the template is used as the starting point for selecting functional monomer candidates especially if the non-covalent approach is followed [149]. For example, if basic groups are present in the print molecule, an acidic functional monomer would be a choice as it may provide strong ionic interactions between the two [150].

Although the choice of the template is almost, always the target analyte, where the molecule is very expensive to buy, not readily available, very toxic or not soluble, a structural analogue termed a dummy template can be employed to deal with these challenges [151, 152]. The dummy must resemble the print molecule in terms of shape, size and functionalities and should give rise to imprints that have the ability to bind the target analyte. The dummy approach is also used to avoid the risk of residual template leaking from the MIPs and causing false positives, particularly in MISPE applied to residual analysis (see section 2.6 for a detailed discussion of template leaking).

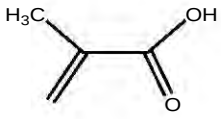
2.4.2 Functional monomers

The functional monomer ultimately becomes responsible for the binding interactions in the imprinted binding sites. Ideally it should form a stable complex with the print molecule during the pre assembly stage of the polymerization process. Functional monomers are usually used in excess relative to the number of moles of template to favor the formation of template-functional monomer assemblies. Template-functional monomer ratios of ≥1:4 are common for non-covalent imprinting [149]. It is very important to match the functionality

of the template with that of the functional monomer(s) in a complementary fashion, (e.g. H-bond donor with H-bond acceptor) in order to maximize complex formation which in turn enhances the imprinting effect.

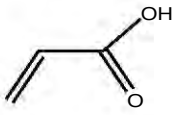
When two or more functional monomers are used simultaneously in "cocktail" polymerization [153], it is critical to bear in mind the reactivity ratios of the monomers to ensure that copolymerization is feasible. Furthermore, it is important that the different multiple interactions exhibited by the functional monomers taking part in the polymerization are exploited simultaneously. In taking advantage, it should be ensured that the different monomers do not interact with one another stronger than with the template. Some of the functional monomers widely used for non-covalent imprinting are shown in Fig. 2.4.2. methacrylic acid (MAA) is by far the most frequently used monomer due to its ability to participate in ion-ion, ion-dipole, dipole-dipole and hydrogen bonding interactions [154]

Acidic (a)

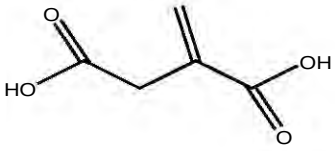


aI

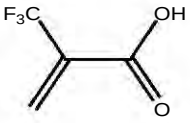
aII



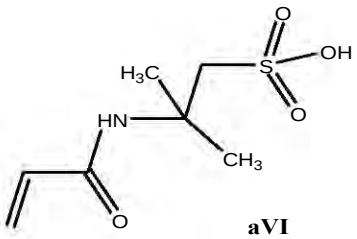
aIII



aIV

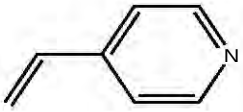


aV

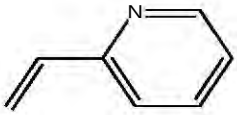


aVI

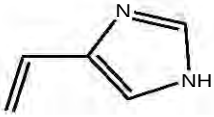
Basic (b)



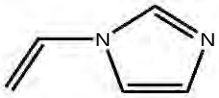
bI



bII

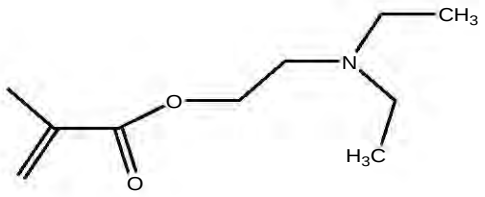


bIII



bIV

bV



bVI

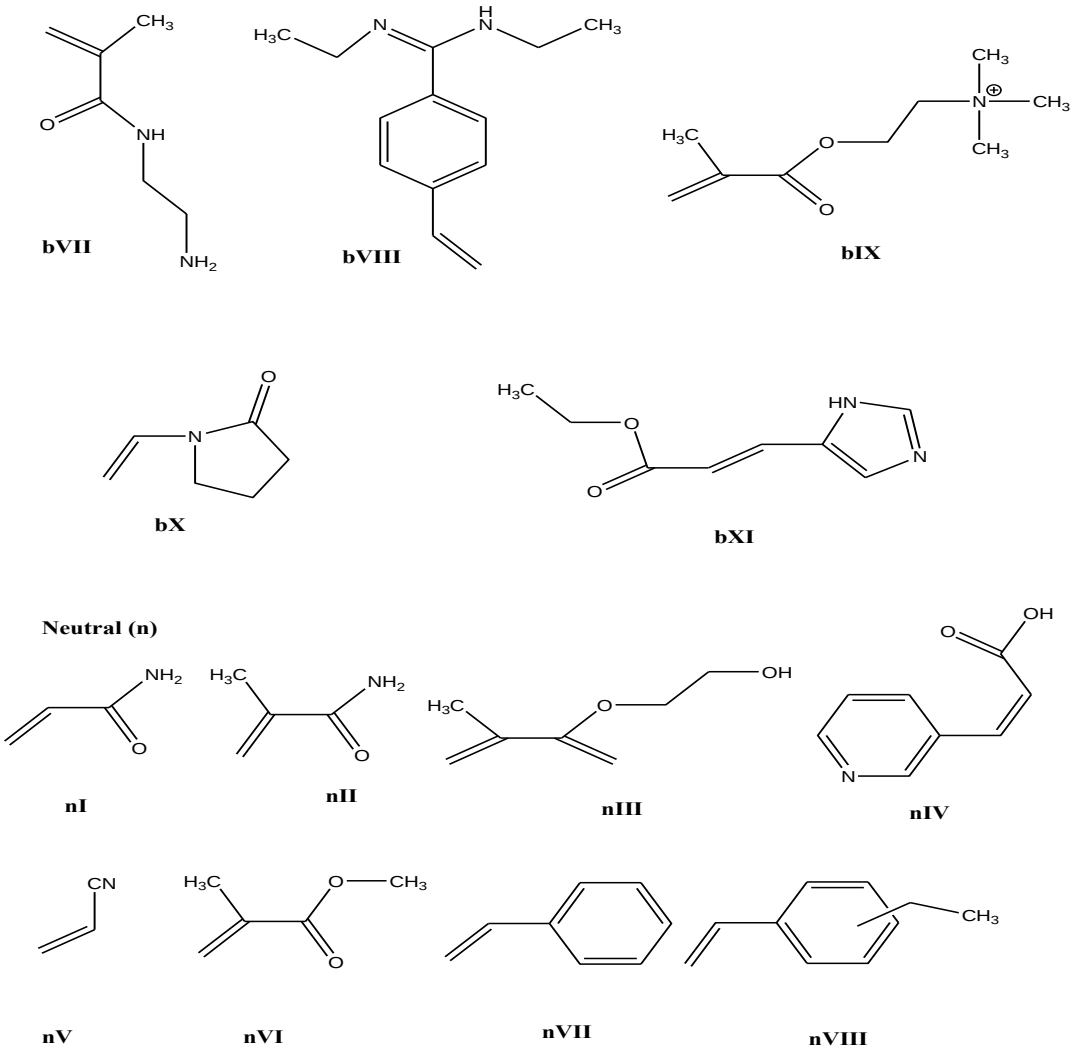


Figure 2.4.2: Selection of monomers used in the non-covalent approach. Acidic; aI: methacrylic acid (MAA); aII: p-vinylbenzoic acid; aIII: acrylic acid (AA); aIV: itaconic acid; aV: 2-(trifluoromethyl)-acrylic acid; aVI: acrylamido-(2-methyl)-propane sulfonic acid (AMPSA). Basic; bI: 4-vinylpyridine ; bII: 2-vinylpyridine (2-VP); bIII: 4-(5)-vinylimidazole; bIV: 1-vinylimidazole; bV: allylamine; bVI: N,N-diethyl aminoethyl methacrylamide, bVII: N-(2-aminethyl)-methacrylamide; bVIII: N,N-diethyl-4-styrylamidine; bIX: N,N,N,-trimethyl aminoethylmethacrylate; bX: N vinylpyrrolidone; bXI: urocanic ethyl ester. Neutral; nI: acrylamide; nII: methacrylamide; nIII: 2-hydroxyethyl methacrylate nIV: trans-3-(3-pyridyl)-acrylic acid; nV: acrylonitrile (AN); nVI: methyl methacrylate (MMA); nVII: styrene; nVIII: ethylstyrene.

The choice of functional monomer(s) that match the functionalities on the other MIP reagents particularly the template is a daunting challenge as there are usually a number of

potential monomer candidates that suit. Traditionally, the choice is based on information available from literature about the behavior of similar systems, individual experience of the researcher or extensive experimental trials [155]. Although many MIPs [156-159] that have been prepared via this approach perform effectively, there is still need for faster protocols for the choice of reagents especially when the polymer has to be designed and quickly applied to address a specific practical challenge.

Recently, computational design (modeling), sometimes called MIP dialling is used for the selection of reagents particularly functional monomers that would interact strongly with the template from a virtual library of potential monomers. This approach has been employed to select the best monomer(s) to be used for the synthesis of MIPs for, dimethoate [160], tetracycline [161], and aniline [162]. A library of 31 commonly used functional monomers was recently screened for their ability to form a complex with nonylphenol, allowing the number of monomers to be employed for the synthesis of the MIPs to five [163]. Nuclear magnetic resonance (NMR) data were also combined with a molecular modeling approach for predicting the template-monomer ratio and also for selecting the porogen [164]. The approach can be used to limit the number of polymers to synthesize and evaluate, which can be time consuming and expensive.

2.4.3 Cross-linking monomer (Crosslinker)

In many cases high binding specificity is augmented by the rigid three-dimensional (3-D) structure of the polymer, which in turn is ensured by the high cross-link density. For this

purpose, a relatively large volume of the cross-linking monomer is usually copolymerized with the functional monomer. Cross linking to functional monomer ratios of ≥1:4 are common [165]. In an imprinted polymer the cross-linker fulfils three major functions;

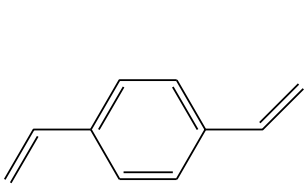
(1) The cross-linker is important in controlling the morphology of the polymer matrix, whether it is gel-type, macroporous or a microgel powder.

(2) It serves to stabilize the imprinted binding site.

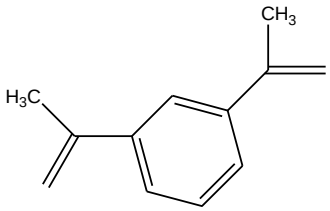
(3) It imparts mechanical stability to the polymer matrix.

The crosslinker can also play an important role in the porosity and the hydrophobicity of the MIP. Its solubility together with that of the monomer-template abduct in the pre-polymerization solution reduces the number of possibilities. Nevertheless, several different crosslinkers have been evaluated with different degrees of success. Most commonly ethylene glycol dimethacrylate (EGDMA) has been employed in several systems [154] because of its availability and ability to cross link with a wide range of functional monomers with ease.

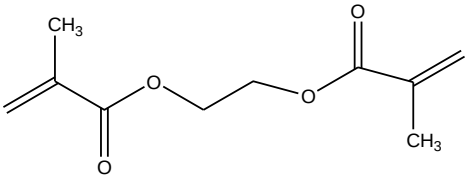
Figure 2.4.3 shows some of the commonly used cross-linking monomers. Derivatives of divinylbenzene and acrylate cross-linkers are generally hydrophobic, while acrylamide ones are relatively hydrophilic.



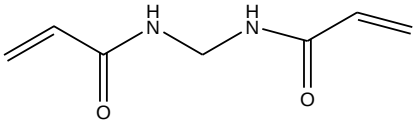
p-divinylbenzene (DVB)



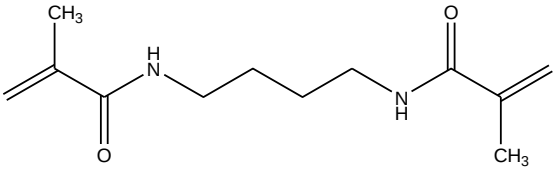
1,3 -diisoppropanyldenzene (DIP)



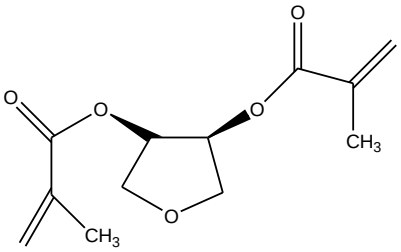
ethylene glycol dimethyacrylate (EGDMA)



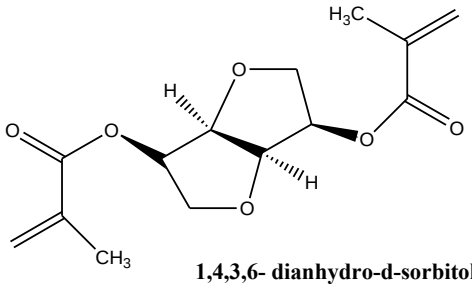
N,N-methylene bisacrylamide



N,N-tetramethylenebismethacrylamide



unhydroerythritol dimethacrylate



1,4,3,6- dianhydro-d-sorbitol-2,5-dimethacrylate

Figure 2.4.3: Selection of cross-linkers used for molecular imprinting.

2.4.4 Porogens

Besides their dual role as solvents and pore forming agents, they also help to homogenize the reaction components prior to polymerization. They must be judiciously chosen such that they maximize the likelihood of template-monomer abduct formation. Table 2.3.4 lists the imprinting solvents commonly used in the non-covalent approach, together with their dielectric constants and hydrogen bond scales [166-168]. If solubility of template is satisfied, then solvents with lower dielectric constant, (i.e. apolar, non-protic solvents) are preferred for non covalent imprinting employing ionic interactions. When hydrogen bond interactions are utilized, solvents with lower hydrogen bond acidity or basicity generally result in a better imprinting effect. If hydrophobic interactions are being used to drive the complexation then water could well be used as the solvent of choice. Porogenic solvents play a role in determining the physical characteristics of the resulting polymer i.e. morphology, surface area and porosity [169-171].

Table 2.4.4: Imprinting solvents commonly used in the non-covalent approach, together with their dielectric constants and hydrogen bond scales.

Solvent	Dielectric constant ε (20°C)	Hydrogen-bond acidity α_2^H	Hydrogen-bond basicity β_2^H
Benzene	2.3	0.00	0.14
Tolouene	2.4	0.00	0.14
Chloroform	4.8	0.200	0.02
Dichloromethane	9.1	0.13	0.05
Acetonitrile	37.5	0.09	0.44
Acetone	20.7	0.44	0.50
Tetrahydrofuran	7.6	0.00	0.51
Dimethylformamide	36.7	0.00	0.66
1-propanol	20.1	0.33	0.45
Methanol	32.6	0.37	0.41
Water	78.5	0.35	0.38

2.4.5 Initiators

Free radical polymerization is the most important synthetic method available to date for the conversion of monomers into polymers. Initiators (see Fig. 2.4.5) are normally the sources of free radicals to start the propagation of the polymerization reaction. Their concentrations influence the formation or lack of formation as well as the morphology of the prepared polymer. The rate and mode of decomposition of an initiator in the first step of polymerization to produce radicals can be triggered and controlled in a number of ways including heat (thermolysis), light (photolysis), and chemical/electrochemical (chemo/electrochemolysis).

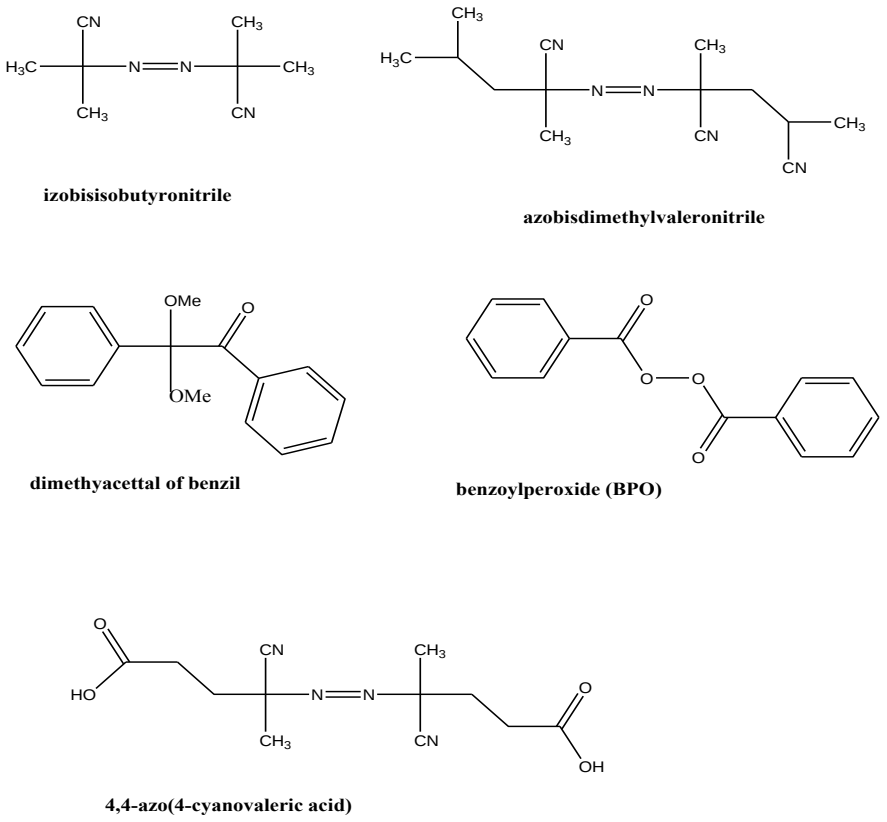


Figure 2.4.5: Chemical structures of selected chemical initiators.

2.5 Synthesis of molecularly imprinted polymers

Most methods for the preparation of molecularly imprinted polymers (MIPs) employ a synthetic, chemical process known as free radical cross linking polymerization [172, 173]. Vinyllic monomers are usually employed for this kind of polymerization. The monomers are commercially available at low prices and can efficiently be polymerized. The method can be performed under mild conditions of ambient temperature and pressure in bulk or solution. There are three steps that characterize free radical polymerization; (1) initiation, (2) propagation and (3) termination.

In a typical free radical polymerization, the rate of propagation is usually much faster than the rate of initiation such that as soon as a new polymer chain starts to grow it propagates to high molecular weight in a relatively short period of time (seconds), before it terminates [174]. It is often highly desirable, not only in imprinting to simultaneously polymerize (copolymerize) two or more functional monomers within the same reaction vessel to give linear chain copolymers as opposed to polymerizing one single polymer (homopolymerization). This allows the resulting copolymers to have chemical properties that are distinct to polymers that would have been formed if each of the monomers that were used in the copolymerization was allowed to homopolymerize.

When multi-functional monomers (cross-linkers) are polymerized either on their own or in combination with a comonomer(s), the outcome is highly cross-linked, non-linear polymer networks. These materials may be soluble or insoluble, and can be classified as branched macromolecules, microgels or macroscopic networks [175], (Fig. 2.5). This is well

described by the theory of radical cross-linking copolymerization (RCC) as defined by Funke *et al* [176]. For the copolymerization of mono- and poly-unsaturated monomers, there is a phase separation that occurs when the quantity of the cross-linker exceeds a critical value. The total monomer concentration also gets reduced to such an extent that a highly cross-linked network can no longer absorb all the porogen present in the reaction mixture. Funke proposed that if the volume of the porogen was to be further increased, the growing polymer chains would be unable to occupy the entire volume available. A dispersion of macrogel particles in the porogen would then result (see Fig. 2.5). More dilute systems (with high volume of porogen) would lead to a decrease in the size of the gel particles. At infinite dilution, macromolecules consisting of intra-molecularly cross-linked primary chains are formed, which are known as primary particles or microgels (see Fig. 2.5). This forms the basis of MIP formats (configurations).

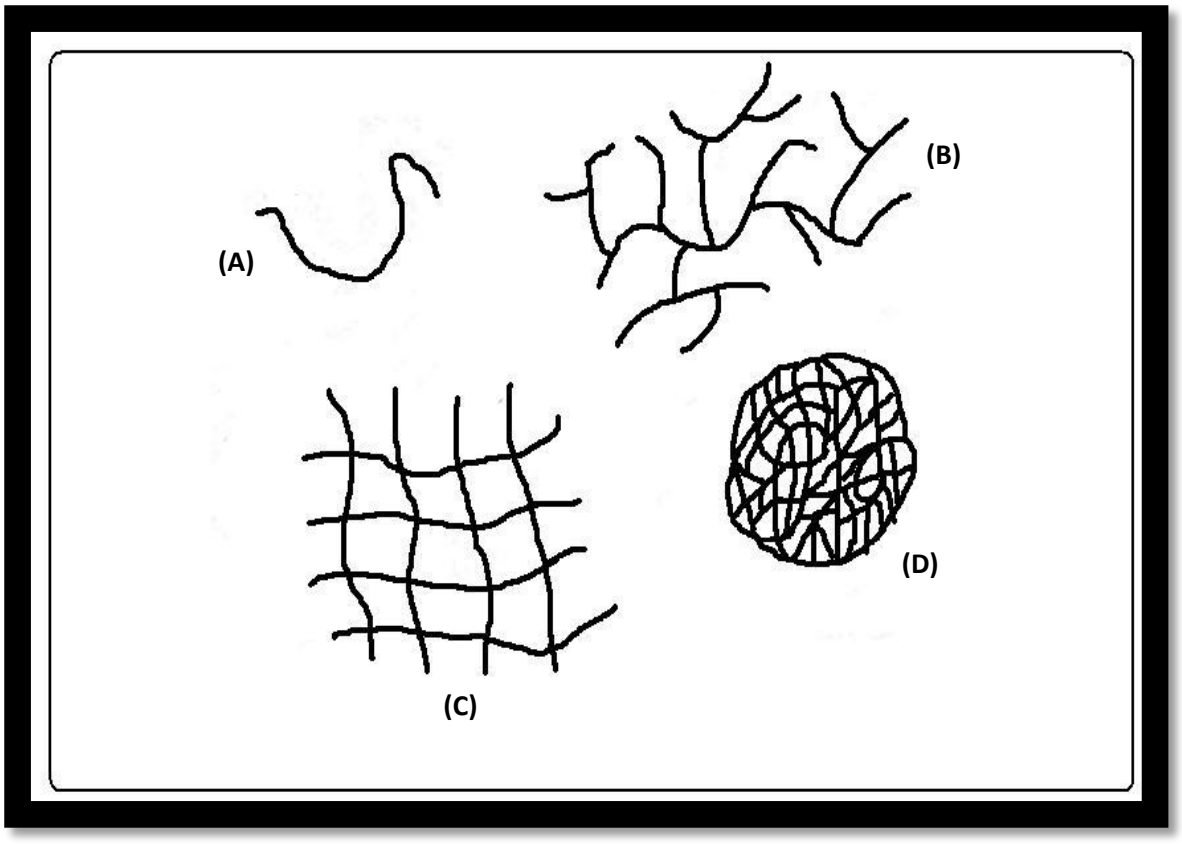


Figure 2.5: Schematic representation showing polymers with different topologies: (A) linear, (B) branched (C) macroscopic network and (D) microgel [176]

Depending on the polymerization preparation method employed, MIPs can have various physical configurations (spheres, rods, fibers etc), sizes (nano, micro etc) and morphology (rough, smooth, spongy, porous etc) [177,178]. To satisfy the ever increasing use of MIPs as selective adsorbents in a wide range of analytical applications, MIP materials of different structures and formats of varying sizes are highly desirable. MIP formats have an influence on the overall performance of the MIP. For instance, nano-MIPs are suitable for advanced applications like developing binding assays/sensors for food and pharmaceutical

industries whereas MIP micro beads are suitable for preparative liquid chromatography studies [179].

2.5.1 Bulk polymerization

Most reported imprinted polymers have been prepared by the bulk polymerization method. It is the most widely used owing to its simplicity. Most investigations still rely on it for the purpose of demonstrating the imprinting effect and potential applications [180]. The method involves mixing together in one reaction vessel the correct quantities of each of the MIP reagents. The reaction is then initiated with heat or ultraviolet light. The product is usually a monolith which would then be ground and sieved to obtain polymer particles mainly in the size range of 25-100 μm (Fig. 2.5.3A). Sedimentation then follows to remove the fine particles. Despite the ease of preparation by this polymerization technique, it is time consuming, labor intensive and wasteful as only 30-40% of the ground polymer is recovered as useable material [181]. The irregularly shaped polymer materials that result from this polymerization method together with their broad size distribution can be avoided by employing other methods. The alternative methods result in more regular, spherical mono-dispersed or even nanofibrous particles which are good geometrical features, characteristic of a potential sorbent. Other methods include suspension polymerization, dispersion/precipitation polymerization and seed polymerization [182] as well as combining molecular imprinting and electro-spinning technologies to produce composite MIP nano-fibers [183] which will be further discussed in the following sections.

2.5.2 Suspension polymerization

Suspension polymerization involves the dispersion of the polymerization mixture as droplets in a continuous phase (porogen) like water, mineral oil or perfluorocarbon. Each droplet acts like a mini bulk reactor producing spherical beads (Fig. 2.5.3C) of a broad size range, 10 μm to 5 mm size. Poor yields have been reported where this method of polymerization was employed in aqueous media [184]. However, Matsui *et al* have used the method successfully in an aqueous continuous media against atrazine, a lipophilic herbicide. They applied the synthetic receptor in the SPE of the triazine and reported group selectivity of the imprinted polymer for the triazine family [185]. In contrast, suspension polymerization in organic media like perfluorocarbon liquid continuous phases, yields good quality beads, with controlled particle sizes [186, 187]. The beads have improved adsorption capacities as well as high selectivity coefficients [188].

2.5.3 Precipitation polymerization

When large quantities of the porogen are used than the ones used in bulk polymerization (2–10 times higher) [189, 190], the growing polymer chains become insoluble in the liquid phase and precipitate out as the polymerization proceeds. If there is an accurate control of the polymerization conditions like the temperature and the cross-linker ratios [191] microspheres and nanospheres (Fig. 2.5.3B) are formed [192-197]. This kind of polymerization is referred to as precipitation polymerization method.

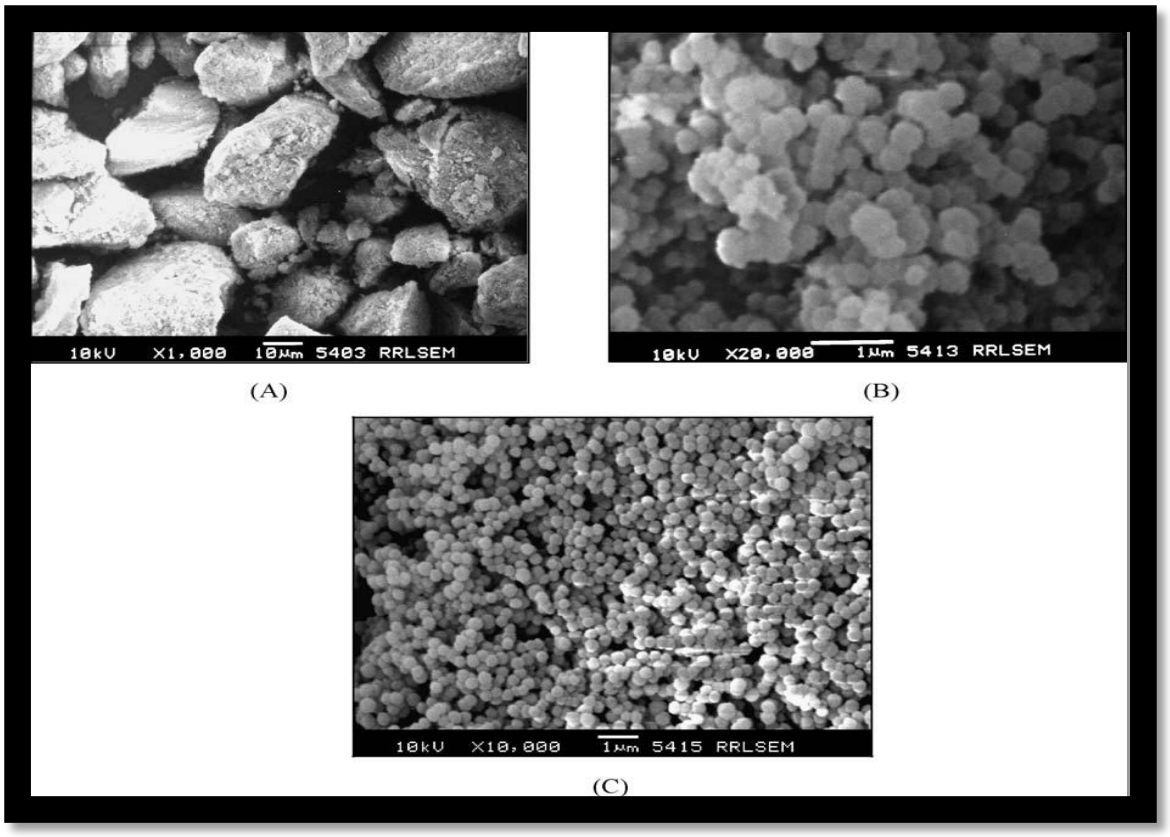


Figure 2.5.3: SEM micrographs of MIPs synthesized via bulk (A), precipitation (B) and suspension (C) polymerization [187]

2.5.4 In-situ polymerization

Imprinted polymers can also be prepared directly by filling the polymerization mixture into casts or moulds made from for example, stainless steel, silica, or glass column. This results in different configurations such as polymer rods that can be easily adapted to chromatographic applications [198-201]. There is no grinding or sieving required. The imprinted column can be used immediately following polymerization and washing. This technique has been largely reported by Schweitz *et al*. who developed MIP monoliths for capillary electrochromatography (CEC). They combined the intrinsic high efficiency and

enhanced flow dynamics of the CEC with the high selectivity of the MIP for the separation of enantiomers [202, 203]. It has been reported that strong interactions between the functional monomer and the template are essential for the polymerization to be successful since the employed specific porogens interfered with the desired hydrogen bond formation. This is said to limit the general applicability of the in-situ technique [203-206].

2.5.5 Multistep swelling polymerization

In order to better control the size distribution and the shape of the particles as well as decrease the quantity of polymer material wasted, imprinted polymer layers have been grafted onto pre-formed supporting materials (<10 μm) acting as casts or molds, for example silica particles [207, 208] or poly(TRIM) beads [209]. Polystyrene microspheres have also been used in a multistep swelling polymerization to produce uniformly sized beads against various substrates [210, 211]. The method is time consuming and sophisticated but it results in mono-dispersed spherical polymer particles of size ranges <10 μm. A one step swelling approach was recently proposed by Liu *et al.* [212] to obtain MIP particles of 7.5 μm for the selective extraction of metsulfuron-methyl from drinking water.

A more advanced and recent approach of the method is where nanomaterials like carbon nanotubes or nanofibers are employed to provide large surface area and high porosity platforms for molecular imprinting, especially surface imprinting. The thickness of the

polymer grafted around each nanomaterial can be fine-tuned to imprint different sizes of the template, yet allowing it to be thin enough to expose every imprint site to the target molecules in solution without sacrificing the capacity of the binding sites. The performance of the nanomaterial-MIP architecture that results has been assessed for caffeine with a caffeine-imprinted polypyrrole (PPy) coating on two types of carbon nanotube arrays [213] and more recently for alachlor herbicide with alachlor imprinted alumina nanofibers [214].

2.5.6 Molecularly imprinted electrospun nanofibers

To further make the MIP binding sites more accessible to the target analyte in solution and improve MIP performance, new, facile strategies for effective and versatile fabrication of high surface area to volume ratio nanomaterials are needed. Recently, the electrospinning technique has been identified as a technique to produce such materials.

Electrospinning is a technique that relies on repulsive electrostatic forces to draw a viscoelastic solution into nanofibers [215]. As displayed in Fig. 2.5.6.1, the basic requirements of an electrospinning apparatus include: (a) a mode to deliver a polymer solution (capillary tube with a needle or pipette), (b) a high power voltage supply, and (c) a collector or target [216]. Electrical wires connect the high power supply to the capillary tube, which contains a polymeric solution, as well as to the target. The capillary tube and target are held at a relatively short distance from each other. Copper plates [217, 218], aluminum foil or plates [219-222], rotating drums [223-225] and human hands [226] have been utilized as targets to collect fibers during the electrospinning process. The polymer

solution is forced through the syringe pump to the needle, either by gravity or by an advancement pump. Initially, as a result of surface tension, pendant droplets of the solution are held in place. A conical protrusion [227], known as a Taylor cone [228] (Fig 2.5.6.2) is formed when a critical voltage is applied to the system.

For a few centimeters, an approximately straight jet emerges from the cone. However, this straight segment cannot hold for long. The jet therefore emerges into a diaphanous and conical shape, within which exists the complicated path taken by the jet [229]. Bending instabilities are experienced by the conically moving jet and its field is directed towards the collector, which has the opposite electrical charge. Within the time it takes the jet to reach the collector, the solvent evaporates and dry polymer fibers are deposited [230].

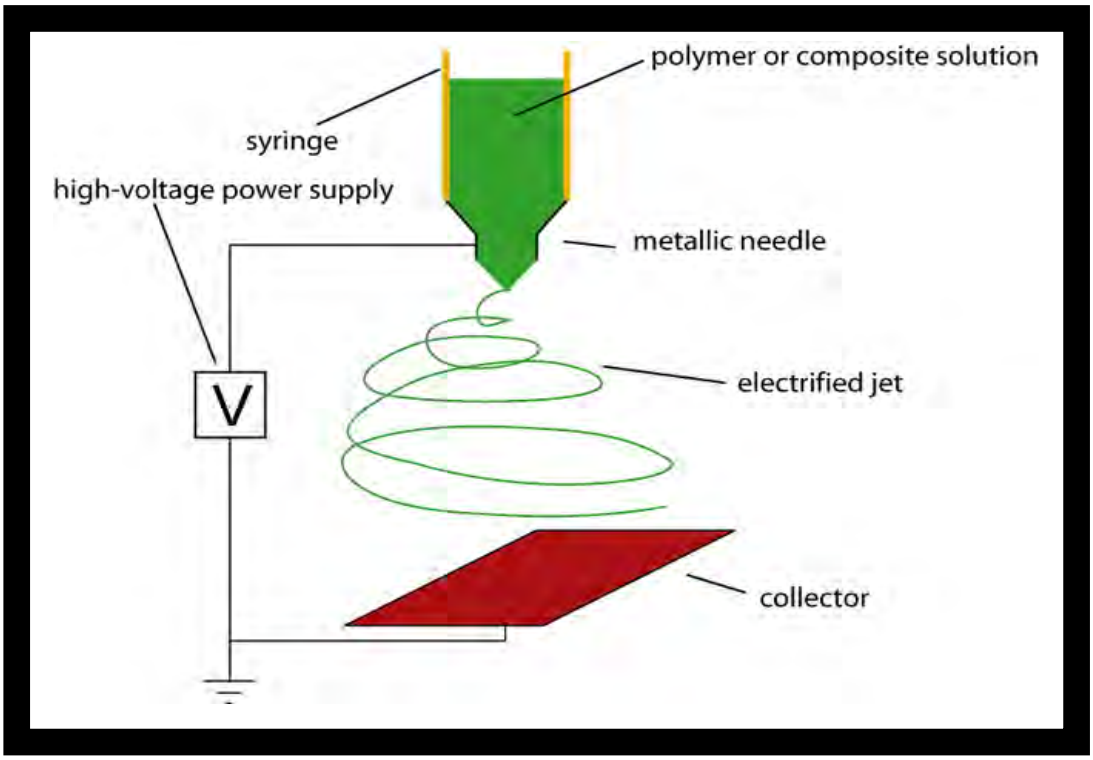


Figure 2.5.6.1: A typical electrospinning setup [215]

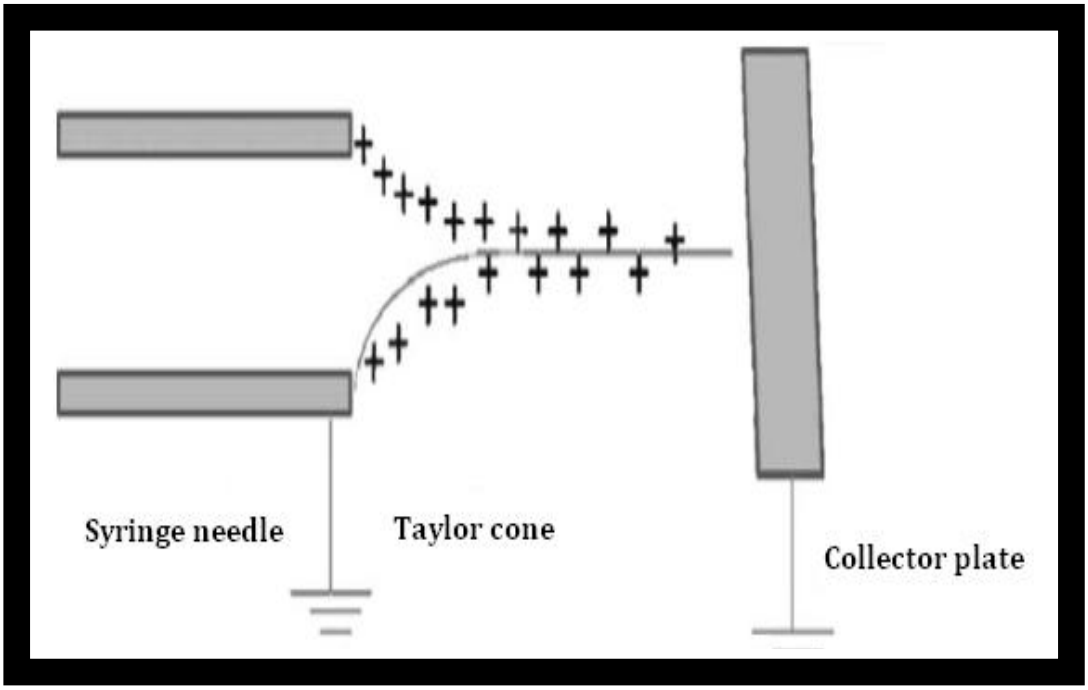


Figure 2.5.6.2: Schematic representation of the Taylor cone formation [228]

Electrospinning technique can be used with a variety of polymers to produce the nanoscale fibrous membranes. The electrospun nanofiber membranes can have approximately 1 to 2 orders of magnitude more surface area than that found in continuous thin films [231]. It is expected that this large amount of available surface area has the potential to provide unusually high sensitivity and fast response time. Combined with the imprinting technology, highly sensitive and selective imprinted nanofibers referred to as MIP-electrospun nanofibers are realized.

Currently two approaches for introducing MIPs on the nanofibers have been reported [231-233]. In the first approach, pre formed MIP particles of nano sizes are homogenously mixed with a spinnable polymer solution and electrospun. The product is nanofibers with the MIP particles incorporated within the fiber. The composite nanofibers interconnect and form non-woven mats that can be used as affinity membranes to greatly simplify advanced applications like solid phase extraction of drug residues in analytical samples. Yoshimatsu *et al.* have demonstrated this by encapsulating propranolol-imprinted nanoparticles into poly (ethylene terephthalate) nanofibers. Employing the new composite nanofiber mats as solid phase extraction materials, trace quantities of propranolol (1 ng mL^{-1}) in tap water were easily detected [233]. The main challenge with this approach is that the synthesis of nano sized MIPs needs special skills and apparatus, thus limiting a wider application of this approach.

The second approach involves mixing all the MIP reagents with a spinnable polymer to form a homogenous solution which is then optimized for electrospinning. Chronakis *et al.*

employed this strategy to fabricate MIP nanofibres for the residue analysis of 2, 4-dichlorophenoxyacetic acid (2, 4-D) [183]. A new strategy based on a slight modification of this approach, for the selective extraction of cholic acid from human bile during DRA is proposed by this thesis in the experimental section.

2.6 Characterization of molecularly imprinted polymers

This section will discuss techniques that are commonly employed to elucidate the structural features of the MIPs at both pre and post-polymerization. Methodologies for the evaluation of the performance of the prepared MIP particles will also be discussed with a bias towards the ones employed in this thesis.

2.6.1 Monomer-Template (Pre-polymerization) characterization of MIPs

The first step towards the preparation of a MIP is the formation of a self-assembled complex between the monomer and the chosen template molecule. The binding affinity between them will form the basis of the MIP recognition sites. Therefore, the ability to determine the appropriate monomer-template interactions is critical in ensuring a successful MIP preparation. Various types of spectrometric techniques such as nuclear magnetic resonance (NMR) [234] and ultraviolet (UV) [235, 236] have been employed to confirm the possible non-covalent monomer-template interactions during MIP synthesis.

2.6.1.1 Nuclear Magnetic Resonance (NMR)

Spectrometric techniques such as NMR are usually the ones used to give evidence about any existence or the extent of pre-polymerization interactions between the monomer and template. Pioneering the work was Sellergren *et al.* employing NMR studies to show the minor presence of template self-association and higher order complexes [237]. In their studies, the extent of interaction of the monomer-template was predicted by determining the chemical shifts and relating them to the dissociation constants. The NMR characterizations of functional monomer-template interactions have also been applied to the study of the interactions between many functional monomers and templates [238-247]. In most of these studies, it was possible to determine the exact composition of the complex [243], dissociation constants [244] as well as predicting binding capacities of the overall polymer materials especially where NMR chemical shifts were determined [245, 248]. For example, Nicholls investigated in detail the characterization of complexes between nicotine and methacrylic acid [245]. Based on chemical shifts of the nicotine-methacrylic acid pre-assembly abduct, binding capacities of the final MIP particles were predicted and were found to be statistically comparable to the experimental results.

2.6.1.2 Fourier transform infrared (FTIR)

Fourier transformer infrared spectroscopy (FTIR) has been employed for samples in solution and in the solid state to identify the formation or lack of new bonds by the MIP reagents. The spectra of the functional monomers and template (starting materials) before and after they have assembled during an imprinting process are compared. The imprinting

process starts with the complexation of the functional monomer and the template, in most cases via hydrogen bonding in the non-covalent approach. The formation of the bond can be readily identified by FTIR since the stretching frequency of hydroxyl or amino groups (hydrogen bond donors) and carbonyl groups (hydrogen bond acceptors) are displaced and an observable shift can be identified. The technique has been rarely applied to the pre-polymerization solution analysis [249] due to solvent interference on the determination of specific characteristics of complexation but it is a technique of choice for characterization when the imprinted polymer materials have already been formed.

2.6.1.3 Ultraviolet (UV)

Complexation or monomer-template self assembly has also been studied using UV spectroscopic titrations in order to calculate the dissociation constants for the solution adducts and the relative concentration of fully complexed templates in the polymerization mixture [250]. The main advantage of this technique is its simplicity and the possibility to control monomer-template complex formation in aqueous media [251, 252]. Ping *et al*. [253] synthesized a MIP from the photoinduced polymerization of acrylamide and butylenes diacrylate in the presence of l-2-chloromandelic acid (l-2-MDA) as a template. The MIP was used as a highly selective separation material for l-2-MDA. The UV spectra showed that the l-2-MDA and the functional monomer acrylamide formed complexes before polymerization. The structures of the complexes were simulated using HyperChem and it was found that the binding capacity of the MIP to l-2-MDA was higher than that of the competing species. Scatchard analysis suggested that the MIP recognized l-2-MDA with two

classes of binding sites, which was in agreement with the complexes that were simulated employing HyperChem.

2.6.2 Post-polymerization characterization of MIP particles

Imprinted polymers belong to a class of materials known as macro-porous polymers [254, 255]. Imprinted polymers are solids, and therefore cannot be characterized by more commonly employed polymer characterization methods that would require polymer solutions; e.g. gel permeation chromatography, solution NMR techniques, and UV measurements directly on the polymers. Furthermore, because they are amorphous, crystallographic or microscopy methods cannot be used to determine the structure of the imprinted polymer binding sites, although microscopic techniques like scanning electron microscopy (SEM), atomic force microscopy (AFM) and transmission electron microscopy (TEM) have aided the macroscopic understanding of their morphology [256]. Therefore, there are only a limited number of direct physical characterization methods for imprinted polymers. These include surface area and porosity measurements, FTIR spectroscopy, solid state carbon-13 nuclear magnetic resonance cross polarization-magnetic angle spinning (NMR ^{13}C-CPMAS) spectroscopy, and swelling experiments. The surface area, porosity, and swelling measurements characterize macroscopic features of MIPs; however, information provided by these on the binding site structure of MIPs is very limited but useful in some applications such as in drug delivery applications.

2.6.2.1 Surface area and porosity

The morphology of MIPs, as shown in Fig. 2.6.2.1, arises from the nuclei forming around the initiator and growing to sizes of 10-50 nm in diameter. The nuclei then aggregate to form microspheres which further group themselves into larger clusters to form a body of beads. The porosity and resulting surface area in MIPs is formed from the irregular voids located between clusters of the microspheres (macropores, 50 nm in diameter), or from the interstitial spaces of a given cluster of microspheres (mesopores, 2-50 nm in diameter), or even within the microspheres themselves (micropores, of 0.6-2 nm in diameter).

Typical values for the surface area of the imprinted polymers are in the range of 100-400 m^2g^{-1}. For pore size distribution there are both macropores and mesopores in the range 2 to 100 nm, and micropores of 0.6 to 2 nm in diameter [256]. The most effective variables that control surface area and pore distributions are the percentage of crosslinking monomer, the type and quantity of porogen, and the reaction temperature. Although binding and selectivity by MIPs in chromatographic or batch rebinding mode are not dependent on macroporosity, applications in drug analysis especially drug delivery may rely on mass-transfer kinetics related to porosity [257].

Surface area measurements in MIPs are primarily carried out by a nitrogen adsorption porosimeter employing a BET (Brunauer, Emmett and Teller) analysis routine that is standard to all instruments. For pore size distributions in MIPs, the same nitrogen

adsorption data can be analyzed using BJH (Barret, Joyner and Halenda) methods also available on porosimetry instruments [256].

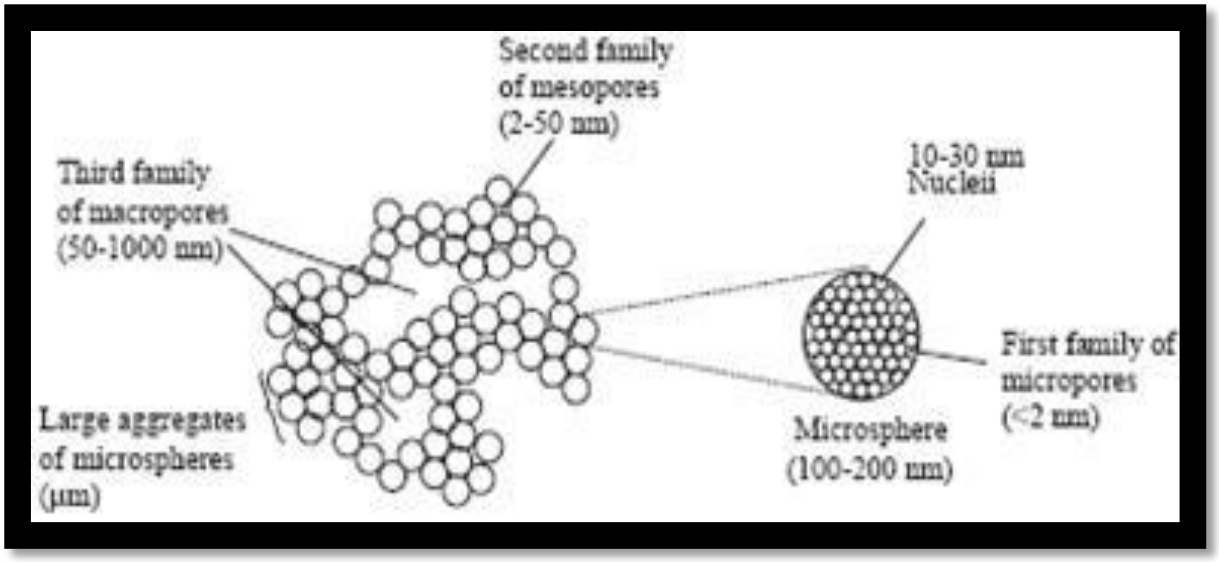


Figure 2.6.2.1: Model of morphology formation that provides the porous network in imprinted polymers [256].

2.6.2.2 Characterization of post polymerization MIP materials by spectroscopic and spectrometric analysis techniques

FT-IR and solid state NMR techniques are useful for the measurement of functional group incorporation on MIP particles. They are also employed in the quantification of the degree of polymerization and reactivity for each type of the polymerizable groups on the monomers. For example, quantitative FT-IR can be used to determine the extent of unreacted double bonds using the C-H out of plane bend at 900 to 950 cm^{-1} and the –C-C– stretch at 1639 cm^{-1}. This would give an indication of the possible recognition sites in the formed polymer.

A measure for the degree of polymerization is assessed from the number of un-reacted double bonds, which are quantified by integration of the area under the peak corresponding to the wave numbers [258]. Using computer aided programs like Origin, the integrated value is converted to the number of double bonds using a calibration curve separately developed that correlates double bonds and integration areas. A more quantitative measure of overall un-reacted double bonds in the different MIP materials can be obtained directly by NMR ^{13}C-CPMAS without the need for calibration curves. All other functional groups of interest that are carbon-based can also be quantified using this technique [256].

2.6.2.3 Characterization of imprinted polymer swelling

Swelling in MIPs has most often been evaluated using volumetric methods as has been reported by Sellergren and Shea [256]. There are some difficulties, however, due to buoyancy (i.e. the polymers float) especially for the polymers in chlorinated solvents; and inaccuracy of the volumetric methods. A more accurate technique that can be used, determines changes in volume for a single bead. Its size is then observed under a microscope in the absence and presence of solvent. The particles are then photographed in swollen and un-swollen states, and the ratios in surface area calculated to give the percent swelling [259]. In many cases the particles have irregular shape giving wide ranges of different sizes between the particles. Consequently, it is best to follow the same particle from the swollen state to the dry state.

2.6.2.4 Binding studies

Binding studies are experiments in which the presence of recognition cavities in the imprinted polymers and their selectivity in recognizing the targeted species are assessed by methods such as batch analysis [260, 261] and frontal analysis [262, 263]. In batch analysis experiments, a known concentration of the template solution (initial concentration) is introduced in a vial with a specific quantity of the prepared MIP particles and shaken/stirred for a known period of time to equilibrate. Once the system has equilibrated the concentration of the template (final concentration) in the supernatant solution is determined. By subtraction the concentration of the target analyte bound/extracted/removed by the prepared MIP can then be calculated and expressed as a percentage employing equation 2.6.2.4. The binding studies are generally carried out in parallel on a non-imprinted polymer (NIP), also referred to as the control or blank polymer. The NIP is obtained by applying the same procedure of polymerization but in the absence of the template. Hence NIPs possess the same chemical properties as the MIPs but without any specific cavities. The difference between the values obtained for the MIP and NIP give the actual occurrence of selective cavities in the MIP.

$$\%\textbf{Extracted/Removed} = \frac{\textbf{Initial concentration} - \textbf{Final concentration}}{\textbf{Initial concentration}} \times \mathbf{100} \qquad \textbf{2.6.2.4}$$

A similar procedure, including the calculations is followed for frontal analysis. The imprinted polymer is packed into a column and a solution containing the target analyte is

continuously percolated through the packed polymer material, which would now be acting as a sorbent [262]. Frontal analysis represents SPE studies better when compared to batch. Desorption and reusability experiments for the polymer are also easier to handle with frontal analysis. A reproducible way of packing the polymer materials is usually a challenge especially if the particles were produced by the common bulk polymerization method. Both batch and frontal analysis methods allow for the determination of the number of binding sites and the equilibrium adsorption constants [263].

2.6.2.5 Non selectivity/competition studies

These are rebinding experiments performed in the presence of closely related, competing species that co-exist and hence might interfere with the selective extraction of the targeted analyte. An estimation of the effect of imprinting on selectivity in the presence of competing species is given by the relative selectivity coefficient (k`), which in turn is calculated from the distribution coefficient (K_d) values of the template (print molecule) as per the following equations;

$$\mathbf{K_d} = \frac{\mathbf{C_i - C_f}}{\mathbf{C_f}} \times \frac{\mathbf{V}}{\mathbf{m}} \qquad \mathbf{2.5.2.5.1}$$

Where, K_d is the distribution coefficient, C_i and C_f, the initial and final concentrations respectively, V, the volume of the solution used for the extraction and m, the mass of the

MIP particles employed during the extraction experiments. The selectivity coefficient (k) and relative selectivity coefficient (k`) for the binding of a particular molecule in the presence of competing species can respectively be obtained by;

$$\mathbf{k} = \frac{\mathbf{k_{print\ molecule}}}{\mathbf{k_{competing\ molecule}}} \qquad \textbf{2.5.2.5.2}$$

$$\mathbf{k'} = \frac{\mathbf{k_{imprinted}}}{\mathbf{k_{non-imprinted}}} \qquad \textbf{2.5.2.5.3}$$

2.7 MISPE application and challenges to food and pharmaceutical samples

The field of contaminant analysis which includes food and drug residue analysis is highly dependent on sample preparation techniques. The complexity of food, pharmaceutical and/or biological matrices demands a highly effective clean-up approach and the high selectivity of the MISPE presents an efficient way for the removal of interfering molecule prior to instrumental analysis.

Numerous studies reported since 2000, dealing with the employment of MIPs for the extraction of organic compounds (drugs, toxins, pesticides) from real samples are described

in Table 2.7 [264-303]. Many of the successful applications have demonstrated that the use of MISPE is a powerful method for the clean-up and the direct selective extraction of compounds at trace levels from various complex matrices. This explains why MIPs are close to commercialization in some fields, such as solid-phase extraction. As a proof of concept, a few start-up companies are already producing MIPs and propose to develop custom made phases [304].

Table 2.7: MISPE of food and pharmaceutical samples from various complex matrices coupled off-line or on-line with sample pre-treatment methods.

Target analytes	Template	Matrices	Monomer/CL/solvent	Sample pretreatment	Ref.
Alkyl-phosphonates	Pynacolyl-methylphosphonate	Soil	MAA/EDMA/MeCN	Extraction with pressurized hot water	[264]
		Water	MAA/EDMA/MeCN	SPE on Oasis HLB polymer	[265]
Alkyl-phosphonates and –phosphate	Diisopropylmethylphosphonate, tributylphosphate	Diesel fuel, gasoline, air extract	4-VP/TRIM/chloroform or MAA/TRIM/chloroform	Dilution in pentane	[266]
Alfusozine	Alfusozine	Soil	MAA/EDMA/CH_2Cl_2	Soil: solvent extraction	[267]
Benzo(a)pyrene	Benzo(a)pyrene	Waters	2-VP/DVB/CH_2Cl_2,	Dilution with MeCN	[268]
Bisphenol A	Terbutylphenol	Surface water	4-VP/EDMA/**	No	[269]
Bisphenol A	Bisphenol A	Surface water	4-VP/EDMA/toluene + dodecanol	No	[270]
Bisphenol A	Bisphenol A	Tap water	APTMS/TEOS/MeOH	No	[271]
Bisphenol A	Bisphenol A-d16	Surface water	4-VP/EDMA/	Acidification	[273]
Carbamazepine	Carbamazepine	Waste water	MAA/DVB/CH_2Cl_2	pH adjustment	[274]
Carbaryl	Carbaryl	River water	AA/EDMA/MeCN	Filtration	[275]
Catechol	Cathecol	Aqueous effluent	4-VP/EDMA/MeCN	No	[276]
	Cathecol	Tap water	4-VP/EDMA/MeCN	SPE on methacrylic polymer + C18	[277]
Chloro-, nitro-phenols	Chlorophenol	River water	4-VP/EDMA/MeCN	Acidification	[278]
Chlorophenoxy acetic acids	Trichlorophenoxy acetic acid	River water	4-VP/EDMA/MeOH-H_2O	Acidification	[279]
17-βestradiol	6-Ketoestradiol	River water	4-VP/EDMA/toluene**	No	[280]
17-βestradiol	17-βestradiol	Waste water	4-VP/EDMA/acetonitrile	No	[281]
Fluoroquinolones	Ciproflaxin	Soil	MAA/EDMA/MeOH	Soil extract diluted in methanol	[282]
Methylxanthines	Caffeine	Water	APTMS/TEOS/aqueous solution	No	[283]
Metsulfuron-methyl	Metsulfuron-methyl	Drinking water	4-VP/EDMA/MeCN: Chloroform*	Addition of EDTA, filtration	[284]
Microcystin-LR	Microcystin-LR	Drinking water	AMPSA + UAEE/EDMA/DMSO	Addition of buffer (pH 4)	[285]
Monosulfuron	Monosulfuron	Soil	MAA/EDMA/DMF	Solvent extraction of soil	[286]
Naphtalene sulfonates	1-Naphtalene sulfonate	River water	4-VP/EDMA/MeOH, water	No	[287]
Nitrophenol	Nitrophenol	River water	4-VP or MAA/EDMA/ MeCN	Acidification	[288]
Non-steoridal anti-inflammatory drugs	Ibuprofen	River water	4-VP/EDMA/Toluene	No	[289]
Organotin compounds	Bu2SnO-*m*-vinylbenzoin	Certified mussel tissue	Sodium MA/EDMA/CAN	Solvent extraction of mussel tissue	[290]
Phenylureas	Isoproturon	Surface water	MAA/EDMA/toluene	SPE (PS-DVB)	[291]
	Isoproturon	Surface water	MAA/EDMA/toluene	SPE on Oasis HLB polymer	[292]
Pirimicarb	Pirimicarb	Water surface	MAA/EDMA/$CHCl_3$	No	[293]
Polyphenols	Caffeic acid p-Hydroxybenzoic	Olive mill waste water	4-VP/PETRA/THF 4-VP/EDMA/THF	pH control	[294]
Sulfonylureas	Metsulforon-methyl	Water and soil	TFMAA/DVB/CH_2Cl_2	Addition of EDTA	[295]
	Monosulfuron	Soil	MAA/EDMA/DMF	Extraction by water-MeOH	[296]
Triazines	Irgarol	River water	TFMAA/EDMA**	Filtration	[297]
	Terbutylazine	Water surface and sediment	MAA/EDMA/CH_2Cl_2	Water: no. Soil: soxhlet extraction (MeOH)	[298]
	Terbutylazine	Humic acid	MAA/EDMA/toluene	SPE (RAM)	[299]
	Terbutylazine	Surface water	MAA/EDMA/toluene	SPE (C_{18})	[300]
Triazines and metabolites	Terbutylazine	Surface water	MAA/EDMA/CH_2Cl_2	No	[301]
	Terbutylazine	Soil	MAA/EDMA/CH_2Cl_2	No	[302]
	Propazine	Water, soil	MAA/EDMA/toluene	Water	[303]

where; AA: acrylamide. AMPSA: 2-acryl-amido-2-methyl-1-propanesulfonic acid. APTMS: 3-aminopropyltrimethoxysilane. DEAEM: diethylamino ethyl methacrylate. DMF: dimethylformamide, DVB: divinylbenzene, EDMA: ethylene glycol dimethacrylate, GMA: glycidyl methacrylate, MAAM: methacrylamide. MAA: methacrylic acid. MeCN: acetonitrile; MeOH: methanol; PETRA: pentaerythritol trimethylacrylate, Py : Pyrrole, ST: styrene, TAIC: triallyl isocyanurate, TEOS : tetraethyl orthosilicate, THF: tetrahydrofuran; TFMAA: trifluoromethyl acrylic acid. TRIM: trimethylolpropane trimethacrylate, UAEE: urocanic acid ethyl ester, VP: vinyl pyridine. *: Addition of reactants required for one or multi-step swelling. **: Several solvents required for particular conditions of polymerization [304].

In academic research, many groups (see Table 2.7) including ours synthesize their own MIPs for the development of new selective extraction methods of various molecules of interest such as drugs for the drug residue analysis or pesticides for the pesticide residue analysis. Their high chemical robustness, providing the opportunity to clean and reactivate them for multiple uses in SPE application has been recently proven by our group: RSD values of <2% (n = 3) were obtained for the removal of interfering chlorophyll from green spinach extracts with % removal of >97% [305]. Nevertheless, it has to be pointed out that MIPs often present limits in terms of selectivity due to the nature of selective interactions taking place during the extraction. They are not intrinsically selective. This field of research still needs improvements particularly in the synthesis of MIPs selective to polar molecules that are not soluble in conventional solvents [304].

The main challenge of MIPs is associated with application as a MISPE protocol in the current food and pharmaceutical sample preparation methods. The challenge emanates from incomplete template removal by the current washing methods during the imprinting process and consequently the template slowly leaking during the various stages of the analysis. Such template loss is referred to as template bleeding and is usually detected at trace levels. It represents a significant source of interferences and systematic errors in trace analysis, as has been demonstrated by Martin *et al.* by using a ^{14}C-labelled template for the MISPE of propranolol [306]. Moreover, the concern for the possible contamination of the analytical samples by the residual template bleeding during analysis is one of the main obstacles to a wider practical application of the MISPE as a routine sample preparation method.

Several methods have been proposed to overcome this drawback by efficiently removing the residual template, including thermal annealing of the imprinted polymer [307] and severe washing conditions in harsh acidic or basic media [308]. Despite these efforts the removal of all template molecules from MIP materials is extremely difficult to achieve using the current template removal methods. In an effort to circumvent this challenge our group has recently employed PHWE to demonstrate its capability to optimally remove or desorb a wide range of templates [309, 310] by simply varying the temperature and pressure of the environmentally friendly water as opposed to the harmful solvents that have been traditionally employed for this purpose. In our work, templates from model MIPs of chlorophyll, quercetin and phthalocynine were removed by PHWE [309].

The most successful strategy has been revealed to be the use of a mimic of the analyte, known as the dummy template as the template molecule. The dummy approach was introduced for the first time by Andersson [311]. With this approach, a structural analogue of the molecule of analytical interest is employed as the template. The choice of the analogue requires a certain degree of creativity from the chemist as it should be made in such a way as to obtain imprinted binding sites with good selectivity for the target analyte molecules. Moreover, the structural analogue should be different from the target analyte in such a way that the analytical separation performed after the extraction step discriminates clearly between the analyte and the residual template molecules (which would in this case be the dummy template) released by the imprinted material. Differences in molecular structure between the analyte and the dummy template should be minimal and localized far from relevant structural motifs and substituent directly involved in non-covalent

interactions with the binding sites. Thus, any modification of the target involving structures critical for molecular recognition should be discarded. Although sophisticated and complicated to many researchers several authors have successfully employed this approach to circumvent the challenge of template bleeding [311-324].

Most of the drawbacks that have been described so far can be avoided through careful design of the imprinting process. Thus, through strategic imprinting process designs, our group has synthesized and successfully applied molecularly imprinted polymers for the clean-up of food and pharmaceutical samples as shall be demonstrated in the next chapter albeit the MISPE challenges.

Chapter 3 Experimental

3.1 Overview

This chapter introduces the specific analytes of interest in this thesis together with the rationale and motivation that led to their study. The analytes include chlorophyll (a green pigment that interferes with pesticide residue analysis (PRA) of green plant extracts), cholic acid (a bile salt that interferes with drug residue analysis (DRA) in human bile) and aflatoxin (a natural food toxicant commonly found in agricultural commodities produced/stored in hot and humid conditions). Detailed experimental procedures that were performed in the study are also covered in this chapter.

3.2 Specific analytes of interest

3.2.1 Chlorophyll

Chlorophyll is a green pigment found in all photosynthetic plants as well as some bacteria. It often interferes with the quantitative analysis of bio-actives in natural product research or in pesticide residue analysis (PRA) at a concentration of ≥ 2% (*w/v*) [325]. For example, when QuEChERS technique is employed in the sample clean-up of extracts of green vegetables like spinach which are highly pigmented with chlorophyll, the analytical portion of the method is challenged [326]. Such a type of matrix introduces severe disturbances in the analytical separation step and detection. Moreover, very "dirty" samples exhibit a noxious property of strongly influencing the background ion current in MS detectors, thus

reducing their sensitivity. Of consequence, quantitative analysis can be achieved only after extensive clean-up step(s) [327]. Currently graphitized carbon black (GCB) is employed as a sorbent in the dispersive SPE kits of QuEChERS to remove the high levels of the green pigment from the vegetable matrix. However GCB does not only remove chlorophyll, it also retains pesticide residues with planar structures (see Fig. 3.2.1) resulting in poor recovery and precision [328]. Moreover, it is very expensive.

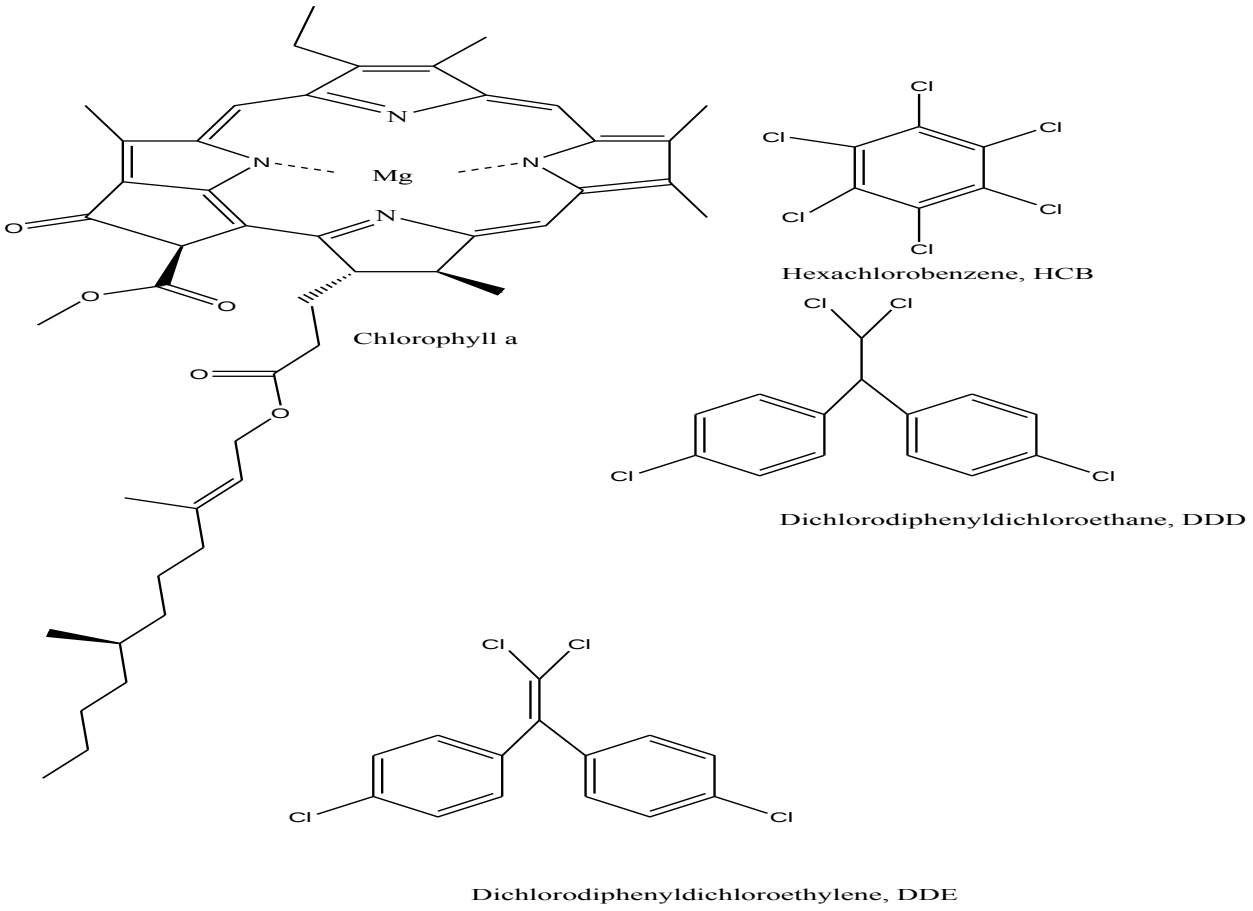


Figure 3.2.1: Structures of interfering chlorophyll and the planar pesticide residues (HCB, DDD & DDE) that were investigated.

In order to address the challenges of GCB on planar pesticide residues or bioactives recoveries, a sample preparation step employing cheap, selective materials should be developed so that only the interfering chlorophyll is removed prior to pesticide residue or bioactive analysis. An example of such materials is the molecular imprinted polymers (MIPs). MIPs have seen increased use in contaminant or trace analysis as suitable materials for applications where analyte selectivity is essential [329]. They have been shown to be suitable as sorbent materials for solid phase extraction hence providing a selective clean-up and pre-concentration step for samples normally associated with complex matrices.

This thesis presents experimental procedures for the preparation and evaluation of the effectiveness of a simply and cheaply prepared MIP for the removal of chlorophyll from green plants extracts relative to the performance offered by commercial GCB which has been in use.

3.2.2 Cholic acid

Cholic acid is a major bile acid constituting about 80-90% of the total bile acids in the human body [330]. It is a steroidal compound synthesized from cholesterol in the liver cells, stored in the gall bladder and emptied into the small intestine for the digestion of fats and lipids [331]. Cholic acid often exists in its conjugated forms as salts known as cholates [332]. Bile acids (see Fig.3.2.2 for structure) are present in the human body at micro-molar concentrations in the peripheral circulation [333].

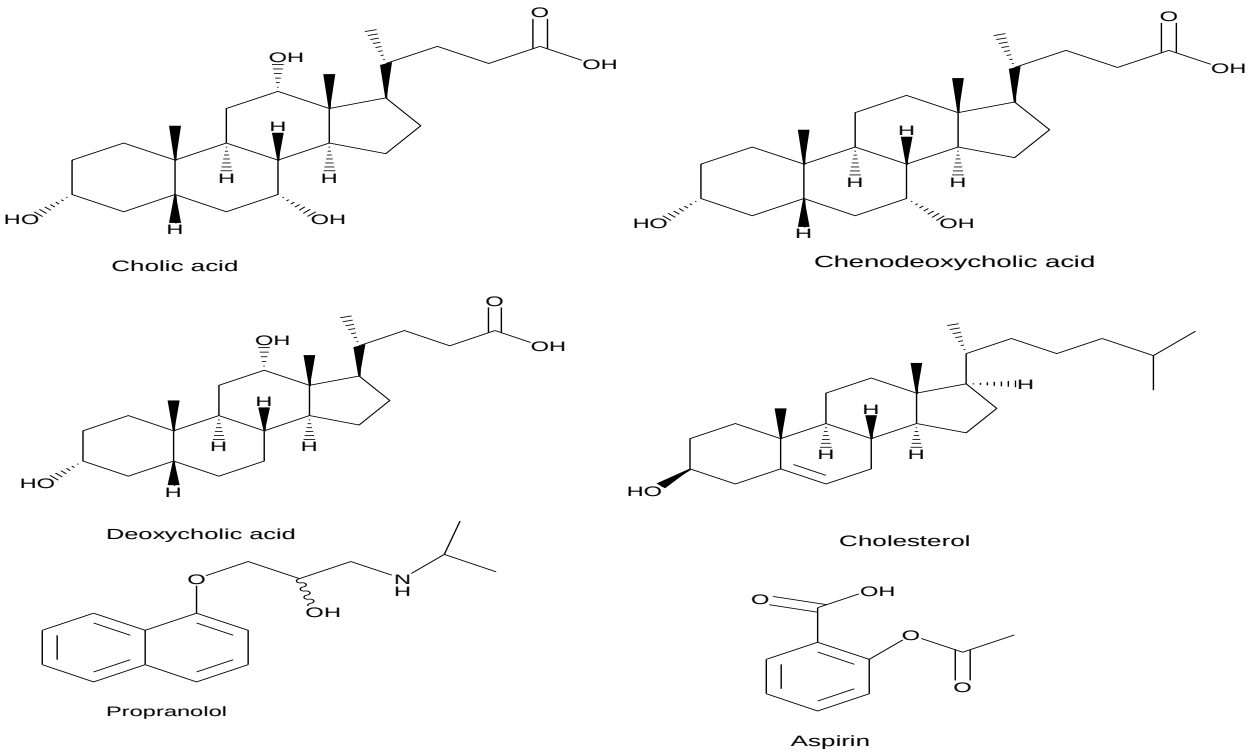


Figure 3.2.2: Structures of common bile acids and drug residues that they interfere with.

There is a novel interest by our group to selectively remove bile acids especially cholic acid from human tissue extracts like bile during drug residue analysis (DRA) or drug metabolism studies. The studies involve monitoring the efficacy of the administered drugs (pharmaceuticals) by assaying for their residues or metabolites in biological fluid extracts. The high concentrations of bile acids relative to the drug residues interfere with the isolation and accurate determination of trace quantities of the drug residues or their metabolites by the sensitive instruments like liquid chromatography-mass spectrometry (LC-MS). Cholic acid tends to cause ion suppression in the LC-MS if its concentration is more than 0.1% (w/v) [334].

In order to address the challenge presented by bile/cholic acid a sample preparation step employing cheap, selective materials was developed in the form of MIP-SPE spherical sorbent materials. Only the interfering cholic acid was removed prior to drug residue analysis. To further enhance the sensitivity of the sorbent, an electrospun molecularly imprinted nano-fibrous SPE sorbent specific to cholic acid was fabricated. The performances of the two MIP formats in removing interfering cholic acid were compared.

3.2.3 Aflatoxins

Food contamination due to natural toxicants represents a significant source of food-borne illnesses and it poses severe risks to human health [5]. Several natural contaminants represented by low mass molecules of a non-proteic nature are extremely potent acute toxins (*e.g.* T2 toxin) or are very strong carcinogens (*e.g.* aflatoxins) which are officially recognized by the World Health Organization (WHO) as bio-contaminants representing a significant source of food borne illnesses [335].

Aflatoxins belong to a class of toxins known as mycotoxins which are toxic secondary metabolites produced by several species of fungi found on agricultural commodities directly in the field or during storage. The most predominant mycotoxins are the aflatoxins produced by Aspergillus species. Among the 16 aflatoxins compounds known, only Aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (see Fig. 3.2.3) are routinely monitored [336]. The International Agency for Research on Cancer (IARC) has classified all four aflatoxins as group 1 carcinogens [337]. Among this group of toxins, AFB1 was found

to be one of the most potent environmental carcinogens. The intake of AFB1 over a long period of time, even at very low concentration, may be highly dangerous. This compound can enter into the food chain mainly by ingestion through the dietary channel of humans and animals [338]. Furthermore, aflatoxin contamination affects the economic value of the crops as well as reduces the efficiency of animal production, resulting in higher costs incurred by all sectors from production to consumption.

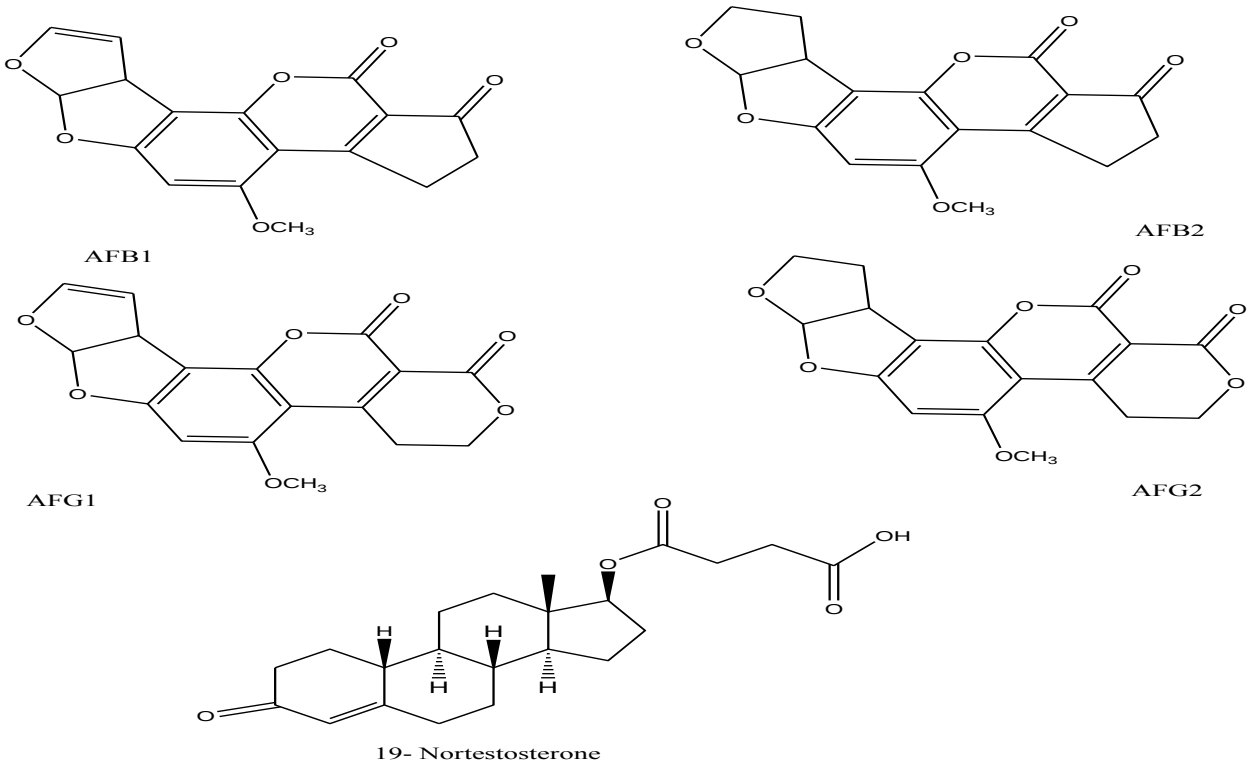


Figure 3.2.3: Structures of the four common aflatoxins found in agricultural products and an analogous molecule, 19-nortestosterone.

Due to the risk associated with aflatoxins, the European commission (EC) has established the maximum acceptable level of aflatoxins in corn, groundnut, dried fruit and cereals for direct human consumption as: 4 ng/g for total aflatoxins (AFB1, AFG1, AFB2, AFG2) and 2

ng/g for AFB1 alone [339]. In order to assess the risk associated with aflatoxin contamination, rapid, reliable and robust analytical methods that rely on intelligent materials with high selectivities and affinities for their analysis at such low concentrations and often complex matrices are highly sought.

Currently, commercially available rapid assays based on the use of IAE techniques or bio-sensing devices are widely employed, as these analytical techniques assure the feasibility of fast sample mass screenings in a more affordable fashion compared to the older thin layer chromatographic methods [340]. However, a sample which is positive to toxicant contamination should be validated by employing more quantitative analytical methods. The methods are usually based on instrumental separation techniques coupled with fluorescence luminescence or mass spectrometry detectors of varying complexity. They have the sensitivity required for contamination detection and quantification, but direct application of these techniques on food can be rarely performed [5] due to the complex matrices associated with food samples. Just like in the case of chlorophyll, quantitative analysis can only be performed after extensive clean-up and pre-concentration steps [6].

Current sample pre-treatment methods, mostly based on the solid phase extraction technique, are very fast and economical but not selective, while methods based on immunoaffinity extraction are very selective but expensive and usually not suitable for harsh environments [341]. Thus, economical, rapid and selective clean-up and pre-concentration methods based on “intelligent” materials are needed. Molecular imprinted polymers (MIPs) have been identified as examples of such materials. They have been shown

to be suitable candidates for sorbent material hence providing a selective clean-up and pre-concentration step for samples normally associated with complex matrices.

In this thesis, the performance of a custom synthesized aflatoxin specific MIP was evaluated and compared to a commercially available aflatoxin specific immuno-sorbent as an alternative in selectively extracting aflatoxins from complex matrices of various nuts and peanut butter prior to analysis by high performance liquid chromatography coupled with a fluorescence luminescence detector (HPLC-FLD). The need to move from immuno-based sorbents to MIP based sorbents is necessitated by the MIPs ability to withstand harsh conditions, their relative inexpensiveness, ease of preparation, high mechanical and thermal stability while still possessing high selectivities and affinities comparable to their immuno-sorbents counterparts.

Other than wanting to contribute to the knowledge of science in this area of research, our group which is based at Rhodes University, Eastern Cape, South Africa was motivated by a 2001 case in the Eastern Cape where it was found out that school children were exposed to aflatoxin contamination after the peanut butter which formed part of their daily school meal tested positive when an aflatoxin mass screen analysis test was performed on the peanut butter [342]. Confirmatory tests based on efficient methods were then needed to establish the concentration levels of the aflatoxins and compare them to the legislative maximum acceptable levels for further action. The confirmatory tests proved to be expensive and the government then made a call for the search for efficient analytical methods in this area after the incident. This is our response to the call as well as the

continued global research for better, relatively inexpensive confirmatory methods of analysis for aflatoxins which are usually present in complex matrices at trace levels.

3.3 Materials and reagents

Chlorophyll *a* (99.99%), Hexachlorobenzene (HCB) (99.99%), dichlorodiphenyldichloroethylene (DDE) (99.99%), dichlorodiphenyldichloroethane (DDD) (99.99%), graphitized carbon black (GCB), Methacrylic acid (MAA) (99.99%), ethylene glycol methacrylate (EGDMA) (99.99%), dichloromethane (DCM) (99%), cholic acid (CA) (99.99%), chenodeoxycholic acid (CCA) (99.99%), deoxycholic acid (DCA) (99.99%), cholesterol (99.99%), propranolol, aspirin, Polyethylene terephthalate (PET), trifluoroacetic acid (TFA) (99.99%), Aflatoxin B1, B2, G1, G2 standards (3 µg/mL) (99.99%), Toluene (99%), Sodium phosphate (99.99%), Sodium chloride (98%), 19-Nor testosterone (99.99%), European commission (EC) community bureau reference traceable certified reference material (CRM) of peanut butter (BCR 401R, sample identification number 0085), HPLC grade methanol, ethanol, acetic acid and azobisisobutyronitrile (AIBN) (recrystalized before use) were supplied by Sigma-Aldrich (Saint Louis, MO, USA).

Working solutions for aflatoxins and 19-Nor testosterone were prepared from their standard/stock solutions by serial dilutions with a loading buffer/methanol. A 5.488 µg/mL 19-Nor testosterone stock solution was prepared. Loading buffer solution was prepared fresh from a 1:1 v/v 10 mM sodium phosphate and 150 mM sodium chloride solutions every time it was needed. The pH of the buffer was maintained at 6.8

Fresh spinach, dried-nuts and peanut butter were purchased from a local store (Grahamstown, South Africa). A contaminated peanut butter was sampled from one of the local schools (Grahamstown, South Africa). Tswii (*Nymphia lotus*) and Makgonatsotlhe (*Acalypha vilicaulis*) traditional medicine leaves were collected from the Okavango Delta area in Botswana (Okavango Delta, Botswana) and dried in ambient air for 3 weeks. All solvents used were of analytical grade or better and were used as received. Ultra pure water (18.2 MΩcm) purified using a Direct Q 3UV Millipore system (Billerica, MA, USA) was used in the analysis.

3.4 Instrumentation

After grinding the MIP monoliths Standard Test sieves by Retsch GmbH & Co., (Haan, Germany) combined with sedimentation were employed to collect polymer particles of an average size of ≤ 45 μm by screen analysis.

Custom-made pressurized hot water extraction equipment which comprised of a gas chromatographic oven with a maximum temperature of 350 °C was used for the removal of the templates; chlorophyll, AFB1 and cholic acid from their respective MIP materials. Inside the chamber, a preheated stainless steel coil was present to maintain the programmed temperature followed by a stainless steel extraction cell. All the tubings were made from stainless steel. Ultrapure water was pumped using Bio LC pump Dionex Model GS50 Gradient Pump, Dionex Corporation (Sunnyvale, CA, USA). The working range of pressure was kept at 50 bars. Soxhlet and ultrasonic extractor from Integral systems (Randburg,

South Africa) were also used for the extraction of templates so as to compare with the PHWE.

A custom made electrospinning technique set-up was employed to fabricate molecularly imprinted elecrospun nanofibers. It consisted of a power supply, a 10 mL glass syringe with stailess needle from Poulten GmbH (Berlin, Germany) mounted on a new Era, NE-1000 programmable syringe pump (New York, USA).

FTIR spectra (4000–400 cm^{-1}) of the MIP/NIP particles and nanofibers were recorded by a Bruker Tensor 27 FTIR spectrophotometer (Ettlingen, Germany) to confirm the formation/disappearance of bonds. For morphology and characterization, scanning electron microscope (SEM) micrographs of the materials were obtained at 20 kV on a JSM 840 field emission scanning electron microscope JEOL, (Tokyo, Japan).

A lamda 25 Perkin-Elmer spectrophotometer, (Santa Clara, CA, USA) was employed to detect the concentrations of chlorophyll (680 nm), cholic acid (207 nm), chenodeoxycholic acid (205 nm), deoxycholic (210 nm), cholesterol (212), propranolol (254 nm), aspirin (277 nm), 19-nortestosterone (240 nm) in every washing during template removal or in the supernatant during the MIPs rebinding experiments. A sample cell of path length 1 cm was used in the assays.

Agilent GC-6820 (Santa Clara, CA, USA) equipped with ^{63}Ni electron capture detector and DB-5 MS fused-silica capillary column (30 m length, 0.25 mm internal diameter, 0.25 μm, film thickness) was employed in the seperation and determination of the concentrations of planar pesticides that were coextracted with the interfering chlorophyll. GC analytical conditions used were as follows: Oven temperature programming: 50 °C (hold time: 1 min), raised to 145 °C at 30 °C min^{-1} (3 min), to 260 °C at 20 °C min^{-1} (3 min) and finally to 300 °C at 10 °C min^{-1} (3 min). The injector and detector temperatures were held at 280 °C and 300 °C, respectively. Helium flowing at 2 mL min^{-1} was used as the carrier gas while nitrogen was the make-up gas. 1 μL sample was injected in the splitless mode.

An Agilent 1200 series HPLC coupled with a fluorescence luminescence detector (FLD), with an Agilent ZORBAX Eclipse Plus C_{18} column (4.6 x 150 mm x 5 μm) analytical column by Agilent Technologies, (Santa Clara, CA, USA) was employed to separate and detect the concentrations of the unextracted aflatoxins in the supernatant. A 5 μL aliquot of aflatoxin B1, B2, G1, and G2 mixed standard (3 μg/mL of each) was initially injected into the HPLC column to optimize their separation. The separation of all the four aflatoxins was monitored at 333 nm excitation and 460 nm emission wavelengths. The HPLC conditions were as outlined in Table 3.4 below.

Table 3.4: HPLC conditions for the separation of four aflatoxins; B1, B2, G1 and G2.

Column	Agilent ZORBAX Eclipse Plus C_{18}, Column, 4.6 x 75 mm x 3.5 µm
Flow rate	1 ml/min
Injection volume	5 µl
Column Temperature	30 ºC
Mobile Phase	A: Methanol
	B: Water
Run time	5 min
Post time	3 min
Isocratic	40 % A 60 % B
Detection (FLD)	Excitation wavelength 333 nm
	Emission wavelength 460 nm

Commercial cartridges (Easi-Extract® Aflatoxin) for aflatoxin clean-up procedure were purchased from R-Biopharm Rhône Ltd (Glasgow, Scotland). An MSE Mistral 1000 by Sanyo Gallenkamp, (Loughborough, England), was employed for centrifugation. Jenway 3510 pH meter by Bibby Scientific Ltd., (Dunmow, United Kingdom) was employed for the pH determination of the solutions.

3.5 Preparation of MIP particles and molecularly imprinted nanofibers

3.5.1 Preparation of chlorophyll, cholic acid and AFB1 MIP particles

For the synthesis of the MIPs, a thermal bulk polymerization method (see section 2.8) was employed with MAA and EGDMA as the functional and cross linking monomers in the ratio, 1:5 respectively. The mixtures were refluxed in either DCM or ethanol at 65 or 75 ºC for 6 h for the chlorophyll, 4.5 h for cholic acid and 7 h for AFB1 MIP. The resultant polymer monoliths were ground to powders with particle sizes of ≤ 45 µm in diameter, and then introduced to the PHWE set up, soxhlet or ultrasonic extraction for template removal. Thereafter the particles were left to dry in open air overnight ready to be used for the batch rebinding experiments. Control polymers referred to as non imprinted polymers (NIPs), without the imprinting templates (chlorophyll, cholic acid and AFB1) were prepared following a similar procedure.

3.5.2 Preparation of cholic acid molecularly imprinted electrospun nanofiber

Two formats of cholic acid MIP materials; MIP nanofibers and MIP particles were synthesized and compared for the removal of interfering cholic acid in drug metabolite studies. For the preparation of the cholic acid MIP nanofiber, 6.4491 g Polyethylene terephthalate (PET) and all the cholic acid MIP reagents mentioned in section 3.5.1 were mixed with TFA (16 mL) and DCM (4 mL) to form an optimized homogenous spinnable

solution. The mixture was stirred overnight until complete dissolution. The solution contained 30% PET (w/v).

The molecularly imprinted nanofibers were fabricated by electrospinning the homogenous mixture according to the following optimized procedure: The solution was pumped at a rate of 0.005 mL h^{-1} through a steel needle of 0.84 mm internal diameter. The distance between the needle tip and the collector was kept at 15 cm, while the needle tip and the collector were held at optimized voltages of +20 and -5 kV, respectively. Continuous PET fibres were collected on a hot plate at 80 ^{0}C covered with a grounded aluminium foil in the form of a fibrous mat. The fiber diameters were measured using the Scandium 4.0 software [343]. To the best of our knowledge this is the first time electrospun fibers were collected on a hot plate.

3.6 Template removal and template bleeding

Three extraction methods (PHWE, soxhlet and ultrasonic assisted extraction (USE)) were evaluated for their ability to optimally remove templates from the prepared MIP materials. Template bleeding due to each method was also investigated.

3.6.1 PHWE

To wash off the templates, 800 mg of the MIP materials (particles/nanofiber) were extracted in a 34 mL PHWE extraction cell with water as the solvent. All extraction procedures were carried out at a flow rate of 2 mL min^{-1}; temperature, 220 °C and pressure, 50 atm for chlorophyll MIP. For cholic acid and AFB1 MIP materials the optimized temperatures were slightly lower, 195 & 180 °C respectively. Aliquots of the washings from the PHWE set-up were then collected at 10 min intervals until the detected absorbance of the templates in subsequent washings was constant.

3.6.2 Soxhlet and UAE

800 mg of the MIP materials were extracted using up to 9 times fresh 80 mL methanol aliquots at 70 °C for up to 16 h. Washings were collected every 2 h to determine the absorbance of the templates.

3.6.3 Determination of the absorbance of the templates in the washings using UV spectrophotometer

The absorbance of the different templates in the washings was determined with a UV spectrophotometer. This was carried out in triplicates for each washing method. Statistical methods were then used to determine the mean values and the %RSD. From the values, plots of absorbance against time of collection for each washing was constructed for each of the MIPs and extraction methods (see Figs. 3.6.3.1, 3.6.3.2 and 3.6.3.3).

3.6.4 Template bleeding evaluation

To assess if there were any remnants of the templates (template bleeding) in the washed MIPs, 800 mg of the dry, washed MIP powders were extracted by employing the three different extraction methods with water or methanol modified with acetic acid (9:1 *v/v*). Acetic acid was chosen as the modifier since it has been used to enhance the elution strength of solvents during desorption studies [344, 345]. Absorbance of templates from the washings was determined with the UV spectrophotometer so as to ascertain that there was no further change in the template bleeding concentrations detected by each method. The experiments were performed in triplicates.

3.7 Batch rebinding experiments of the MIP sorbent materials

To judiciously evaluate the performance of the prepared MIP materials in binding and selectively extracting the respective analytes of interest, both the quantity of the MIP materials and the time should be optimized for maximum extraction. The maximum % of analyte of interest extracted/removed by the MIP materials is calculated from these experiments employing equation 2.6.2.4. The optimal working pH and concentration range for the different analytes of interest were obtained from literature [344, 345].

3.7.1 Optimization of the quantity and the time needed for maximum removal/extraction of the analytes of interest by the prepared MIP materials

Increasing quantities of MIP materials of chlorophyll or cholic acid in the form of particles/nanofibers were added to 5 mL aliquots of concentrated 10% (w/v) chlorophyll or cholic acid standards to determine the optimal quantity needed to remove chlorophyll or cholic acid respectively. They were then equilibrated for 24 h at pH 7 in sodium phosphate buffer and centrifuged for 5 min. The absorbance of chlorophyll/cholic acid in the supernatant was determined with a UV spectrophotometer until there was a constant reading with further addition of the MIP materials. The experiments were carried out in triplicates. Statistical methods were used to determine the mean values and the percentage standard deviations (%RSD). From the values the %bound/removed/recovered were calculated employing equation 2.6.2.4. Plots of absorbance against quantity of MIP materials added as adsorbents were constructed for the cholic acid MIP nanofibers, cholic acid MIP particles and chlorophyll MIP particles.

Following the procedure for optimization of quantity, absorbance of chlorophyll/cholic acid in the supernatant after addition of the optimized quantities of cholic acid MIP nanofiber, cholic acid MIP particles and chlorophyll MIP particles were monitored at 5 min intervals until a constant value of absorbance was reached. This marked the optimum time needed by each MIP material to maximally remove the interfering chlorophyll or cholic acid.

Similar optimization experiments were conducted for the extraction and clean-up of aflatoxins by the prepared aflatoxin MIP before its application to real samples.

3.8 Sample preparation

3.8.1. Extraction

3.8.1.1 Chlorophyll

To obtain chlorophyll extracts from spinach and traditional medicine plant leaves an optimized protocol involving sampling 10 g of each and blending with 80 mL methanol for 5 min. The homogenate was then centrifuged for a further 5 min at 9000 rpm. The green supernatants obtained were used as test samples in the SPE clean-up procedures. The initial absorbance for each of the extracts was determined to provide the initial concentrations of the sample before the SPE clean-up procedures.

3.8.1.2 Aflatoxins

An optimized extraction protocol involving blending 5 g of nut samples with 30 mL of 80% methanol in water and ultrasonically extracting for 15 min was employed. The obtained extract was centrifuged for 5 min at 9000 rpm. 1 mL of the supernatant was diluted ten times with loading buffer and subjected to a derivatization procedure.

All aflatoxin samples and standard solutions were derivatized by evaporating them to dryness with nitrogen in screw cap vials. 5 mL hexane followed by 1 mL trifluoroacetic acid (TFA) was added to them. The mixture was vortexed for 1 min then allowed to stand for 5 min before adding 3 mL de-ionized water: methanol (9:1 v/v) solution. The mixture was vortexed for a further 1 min and allowed to stand for the organic and the aqueous layers to separate. The aqueous layer was collected and centrifuged to provide the test sample for the SPE clean-up procedures. The initial absorbance of the test samples were determined to provide the initial concentrations of the sample before the SPE clean-up procedures.

3.8.1.3 Cholic acid

For cholic acid, there was no extraction since there were no real samples. Only standard solutions were subjected to the SPE procedures.

3.8.2 MISPE clean-up procedures by the custom synthesized MIP materials: chlorophyll, cholic acid, AFB1 MIP particles & cholic acid MIP nanofiber.

The effectiveness of removing/extracting chlorophyll, cholic acid and aflatoxins from the extracts by the MIP materials was investigated by applying an optimized batch MISPE procedure. It involved adding an optimized quantity of the MIP material in each case to 5 mL of the extracts or spiked samples of the analytes of interest. The samples were equilibrated for an optimized duration by manually shaking continually. The mixtures of

the MIP materials and the different samples were centrifuged and the absorbance (concentration) of the supernatants determined.

To demonstrate the non-selectivity of the MISPE procedures, standard solutions of compounds known to compete with the analytes of interest were also subjected to the procedures. Their concentrations were determined thereafter. For control experiments employing the NIP materials as SPE sorbents instead of MIP materials were performed in parallel.

For comparison, all the test samples were subjected to SPE clean-up procedures of commercial materials/sorbents which have conventionally been used for the removal/extraction of the analytes of the interest. The manufacturer's SPE protocols were followed for the commercial products.

After the SPE clean-up procedures were performed the concentrations of the analytes of interest or competing compounds in the resultant sample solutions were determined employing either UV spectrophotometer, HPLC-DAAD or GC-ECD. Analytical parameters that include linearity, recovery, reproducibility, precision and accuracy were also evaluated. As a standard of validating the results, accuracy was evaluated by determining the concentrations of the analytes of interest in CRM samples after subjecting them to the SPE procedures.

Chapter 4 Results and discussion

4.1 Removal of templates from the MIP materials

Template removal

Following the procedures described in the experimental section, the templates were thoroughly washed off their MIPs so as to free recognition sites for selective binding during the rebinding experiments. The concentration (absorbance) of the templates as determined by the UV spectrophotometer decreased with time in all cases until it remained constant with continued washing. This marked the point at which the templates were completely removed by a particular method of extraction for template removal (see Figs. 4.1.1, 4.1.2 and 4.1.3). It should be noted that the results for the removal of the template from the cholic acid MIP nanofiber were not included as the results were identical to that of removing the template from the cholic acid MIP particles.

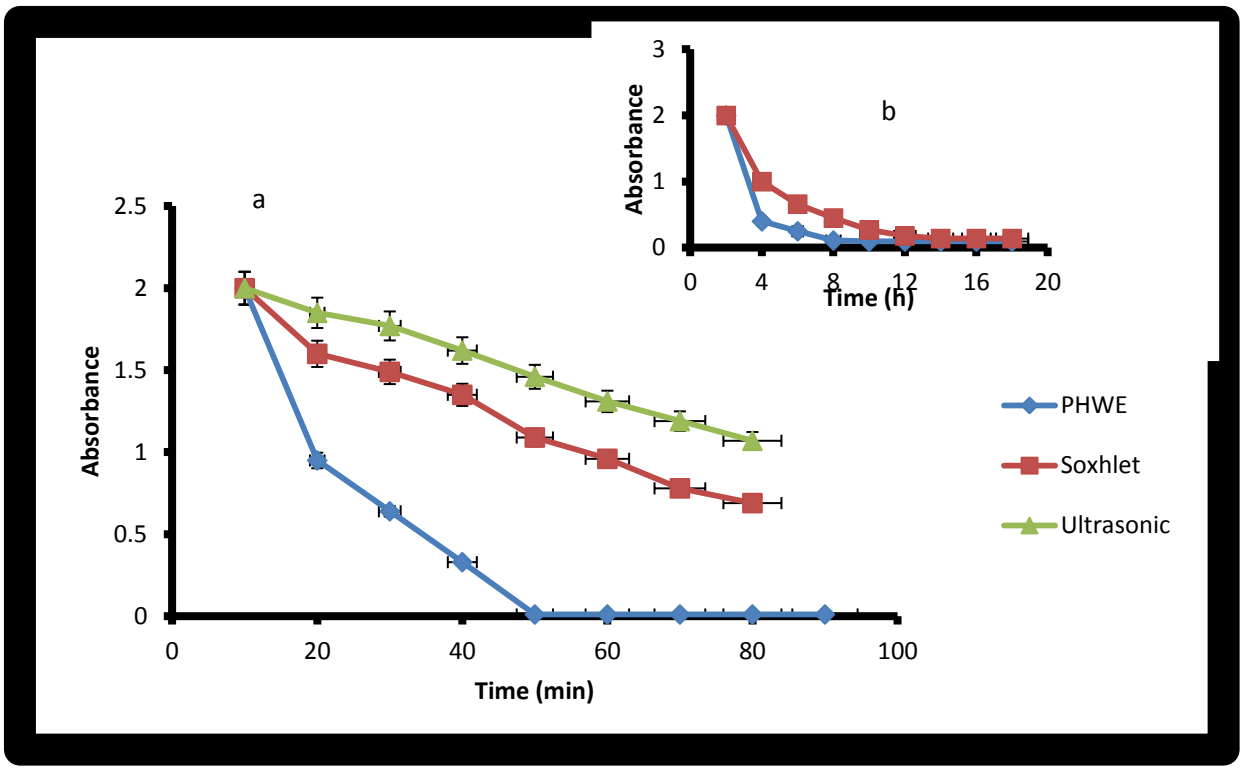


Figure 4.1.1: Absorbance of chlorophyll in each washing at (a) 10 min intervals for the three extraction methods and (b) at 2 h intervals for the two that took longer to complete the removal.

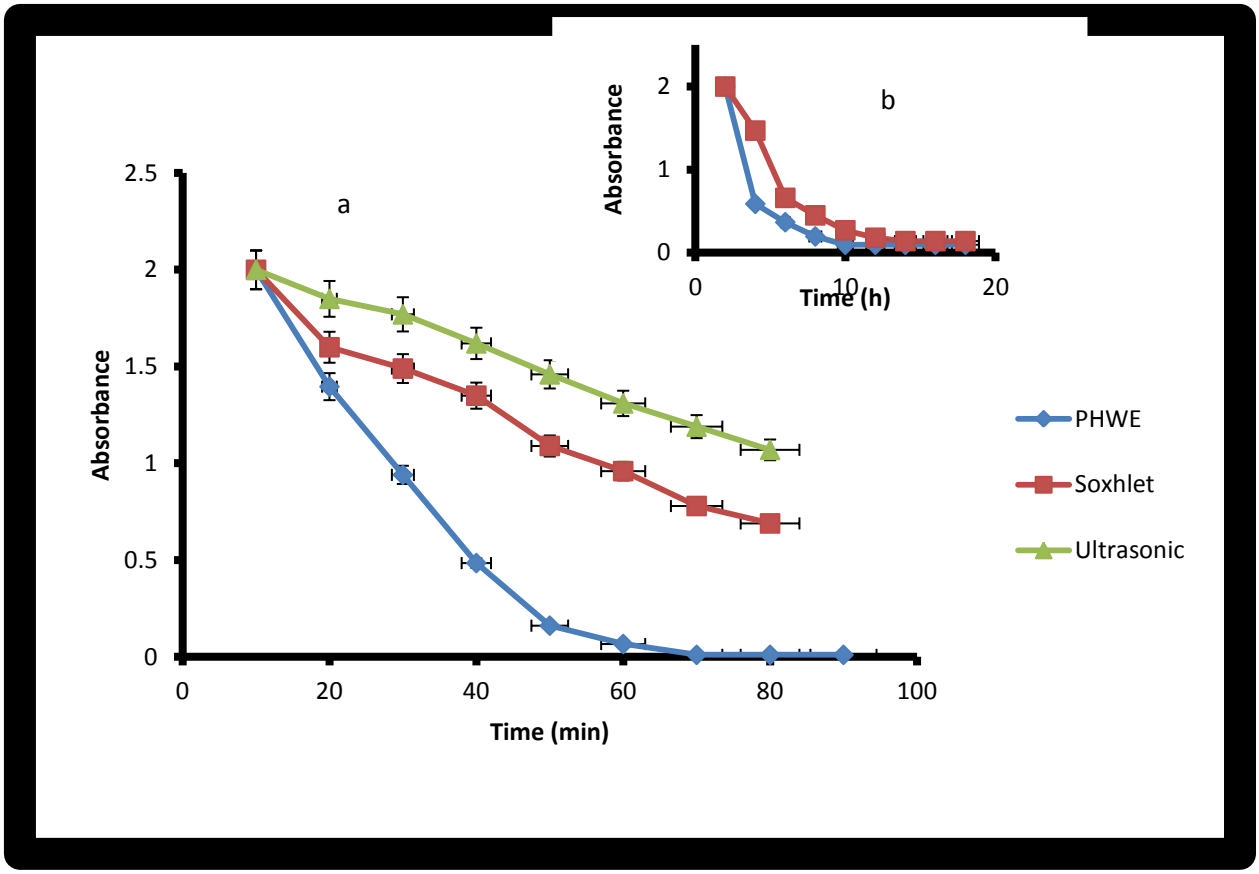


Figure 4.1.2: Absorbance of cholic acid in each washing at (a) 10 min intervals for the three extraction methods and (b) at 2 h intervals for the two that took longer to complete the removal.

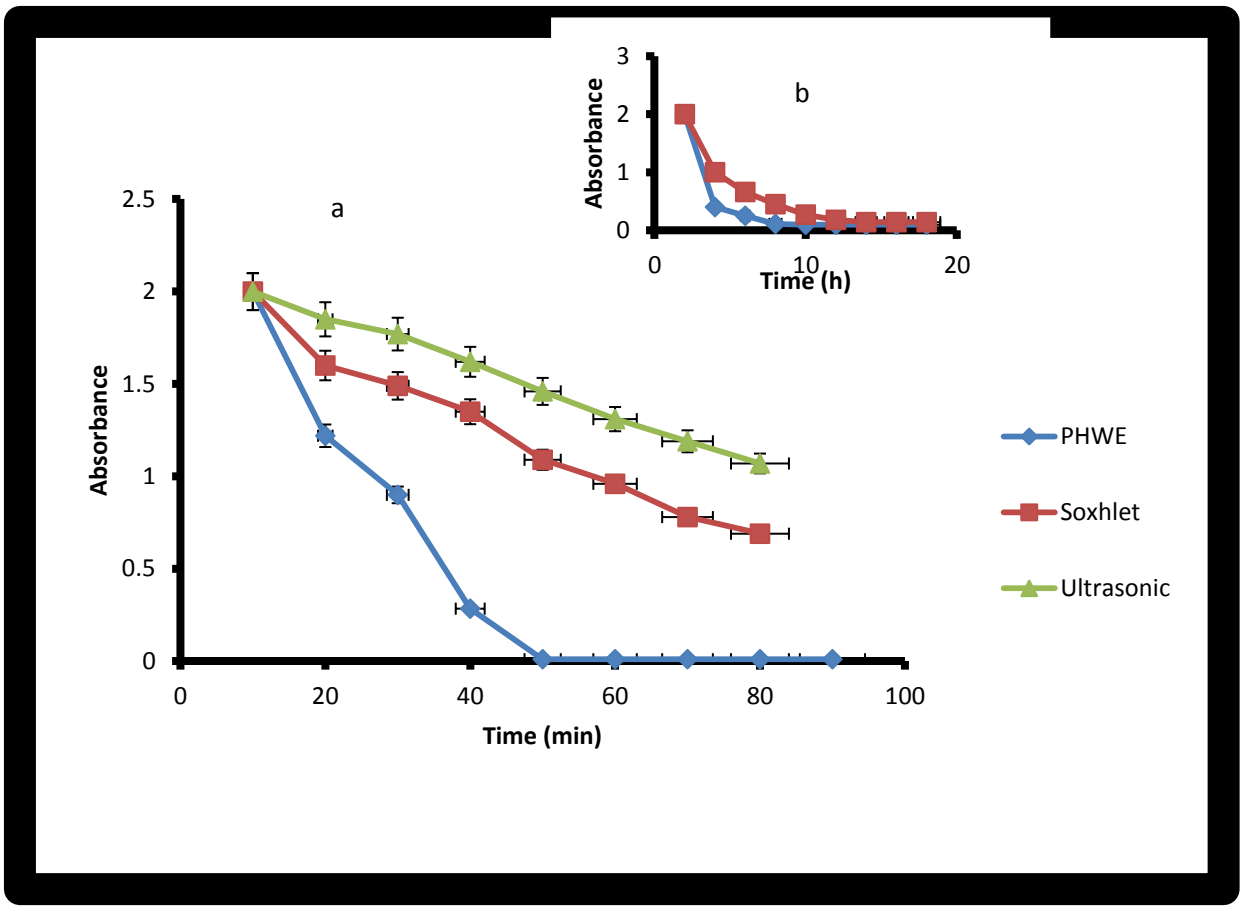


Figure 4.1.3: Absorbance of aflatoxin B1 in each washing at (a) 10 min intervals for the three extraction methods and (b) at 2 h intervals for the two that took longer to complete the removal.

According to the plots, the complete process of washing-off the templates took under 70 min for all the MIP materials when using PHWE (see Fig. 4.1.1a, 4.1.2a, 4.1.3a). This was advantageous as the extraction time was relatively very short compared to that of the soxhlet and ultrasonic extraction methods which took several hours (see Fig. 4.1.2b, 4.1.2b, 4.1.3b). Furthermore PHWE used an environmentally friendly solvent (water) to achieve the same or better results. Conventional methods of removing templates like soxhlet and ultrasonic extraction employed organic solvents to achieve optimal extraction. Organic solvents have detrimental effects to the environment.

Template bleeding

Template removal by PHWE was better than the other two methods employed as marked by the much lower template bleeding concentrations (0.02%) or non-detectable in some cases when using it (see Fig. 4.1.4). The relatively higher bleeding concentrations (over 0.1%) by soxhlet and ultrasonic methods are a clear indication that the methods are not exhaustive in washing-off templates from MIP materials.

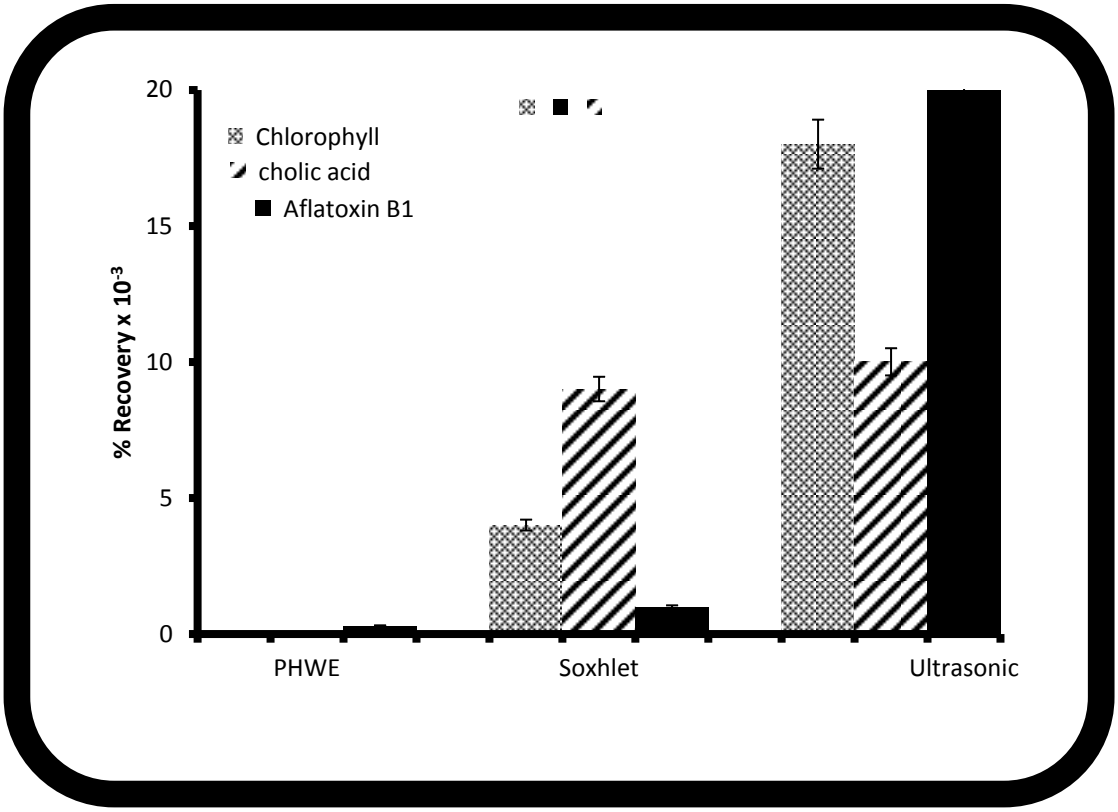


Figure 4.1.4: Template removal by the different extraction methods.

Integrity of binding sites post template removal

Rebinding experiments were used to evaluate the binding affinity of the MIPs after washing-off the templates. On average the MIPs adsorbed over 99.6-100% of templates after PHWE template removal and less than 94.5% after the other two methods were used. Statistically the %recoveries were not significantly different at 95% confidence level using the t-test hence the perfomance of the three extraction methods were comparable. The high percentage recoveries were a demonstration that the recognition sites of the MIPs were not destroyed and still had excellent selectivities even after employing the methods of extraction. Sometimes the removal methods distort the recognition sites leading to very low %recoveries on rebinding the analytes of interest.

Of the three methods of template removal that were optimized, PHWE proved to be the method of choice for optimal template removal. It recorded lowest bleeding levels and higher extraction efficiency than soxhlet or UAE in removing templates from the four different MIP materials that were synthesized. Consequently, PHWE was the method that was employed to remove templates from the MIP materials for the work in this thesis. To the best of our knowledge, this is the first time water under subcritical conditions is employed as the sole extraction solvent (without modifiers) in the removal of templates from MIP materials.

4.2 Physical characterization & performance evaluation of the prepared MIP materials

4.2.1 Chlorophyll MIP particles

4.2.1.1 SEM

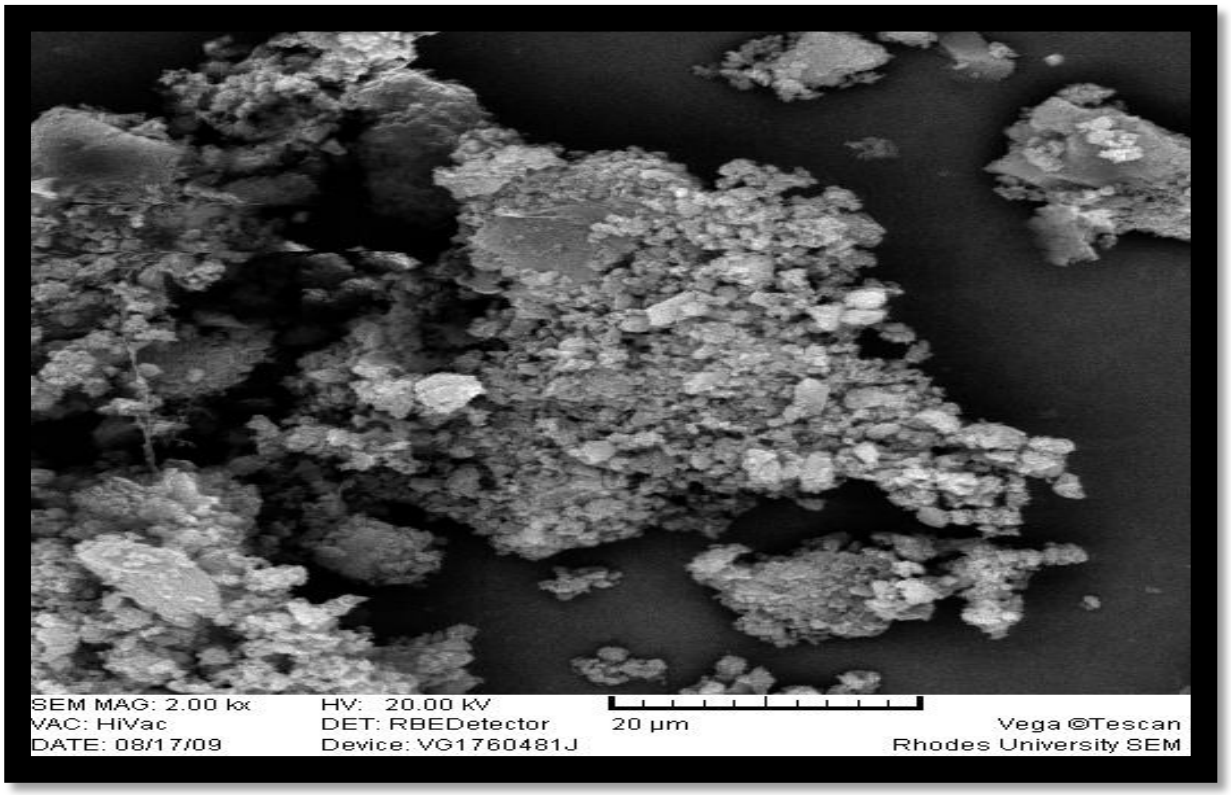


Figure 4.2.1.1: Typical SEM image of the synthesized chlorophyll, cholic or AFB1 MIP or NIP particles. NB: There were no discernible differences amongst the images.

Figure 4.2.1.1 is a typical SEM image of any of the MIP or NIP particles that were prepared (chlorophyll, cholic or AFB1 particles). The different particles (MIP or NIP) for the different

analytes; chlorophyll, cholic or AFB1 could not be differentiated based on the SEM images. The SEM only determines the shape, size and morphology, not the identity of the materials.

The particles exhibited very similar SEM images characterized by a powder like appearance (see Fig. 4.2.1.1) which confirmed the treatment (grinding) that they were subjected to just before the images were taken. The individual particles were chunky with smooth morphology which is a suitable geometrical and textural property for a material that is to be used as a SPE sorbent.

4.2.1.2 FTIR

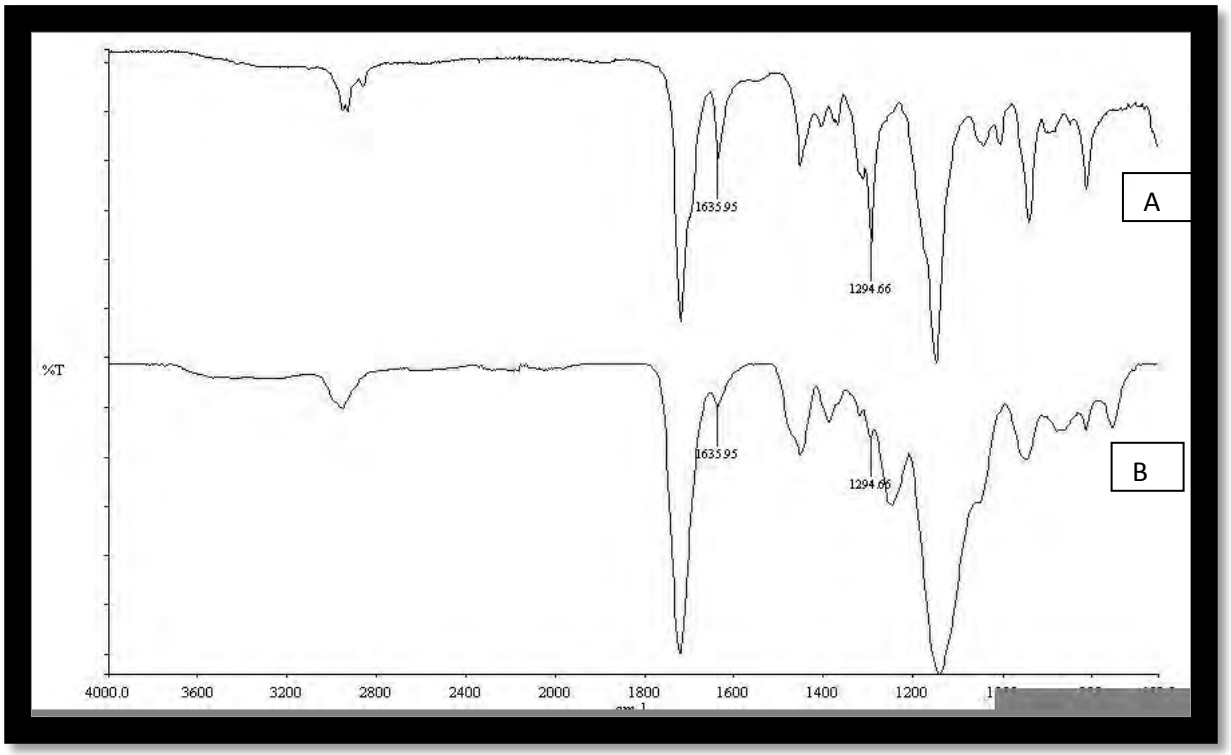


Figure 4.2.1.2: FTIR spectra of unwashed (A) and washed (B) chlorophyll MIP particles.

Charaterization of the chlorophyll MIP particles (washed MIP and the unwashed MIP) by FTIR showed similar locations and appearances of major bands (see Fig. 4.2.1.2). The observed peak around 1125 and 950 cm^{-1} were attributed to the C-O-C and C-O-H stretching vibration respectively while the peak at 790 cm^{-1} was assigned to C-O stretching. Of particular interest was the presence of a characteristic peak at 1635.94 cm^{-1} due to the existence of COO- stretching.

The peak was very strong in the unwashed MIP spectrum which confirmed the formation of the pre-assembly template-monomer complex between chlorophyll and the MAA via hydrogen bonding. The peak was very weak in the washed MIP spectrum due to the removal of most of the chlorophyll template hence minimal existence of the template-monomer complex. The MIP particles were now ready for optimization experiments once the template was removed.

4.2.1.3 Optimization of the quantity and the time needed for maximum removal of chlorophyll

The absorbance (concentration) of chlorophyll decreased with an increase in the quantity of MIP added until an optimal value of 750 mg in the 5 mL of the 10% (w/v) chlorophyll standard solutions (see Fig. 4 2.1.3.1) was reached. According to Mastovska *et al* and our own findings, 300 mg of GCB (350 mg less than the quantity of the chlorophyll MIP) was needed to remove an equivalent concentration of chlorophyll [346]. The prepared polymer removed chlorophyll up to a very low absorbance (0.081 Au) when compared to what is

said to interfere with the analysis 0.399 Au [1], which is the absorbance of a 2% chlorophyll standard. Even though a lot of the MIP was needed relative to the commercial sorbent (GCB), it was still estimated to be a lot cheaper based on the cost of the starting materials (< half the price of GCB which is $ 2500/50g).

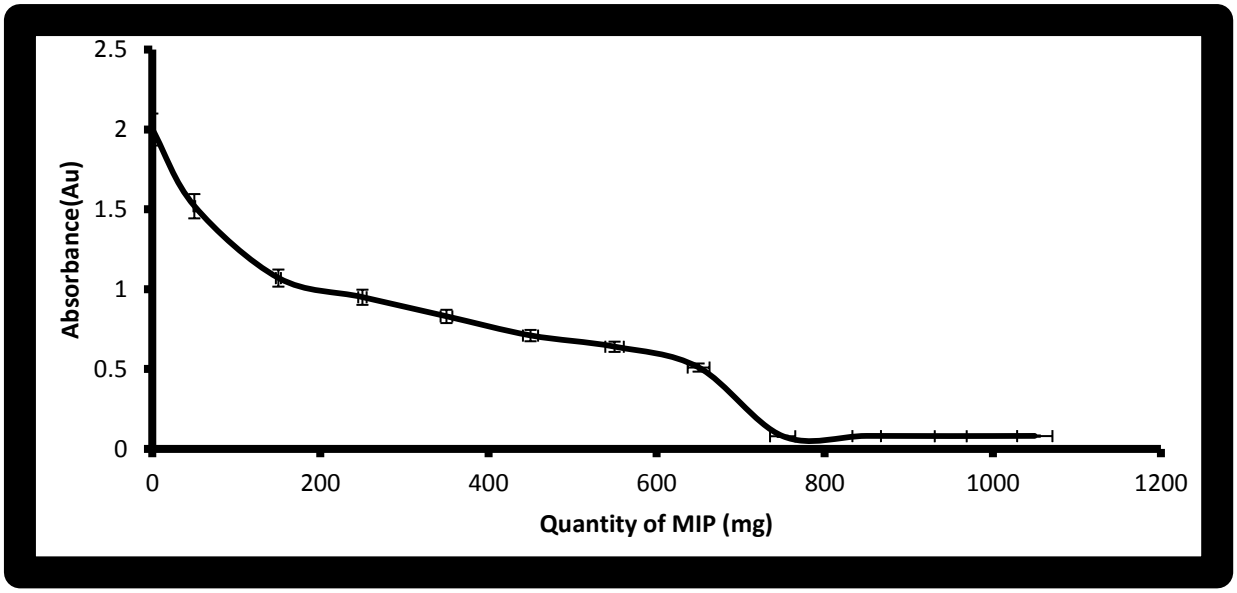


Figure 4.2.1.3.1: Optimization of the quantity of MIP needed to remove maximum chlorophyll over 24 h, for n=3.

Figure 4.2.1.3.2 below shows the time dependence of the MIP in removing chlorophyll. Optimum chlorophyll removal as indicated by the leveling off of the plot was achieved at 6 min which is comparable to the fast kinetics exhibited by GCB (4.5 min) in removing chlorophyll [347].

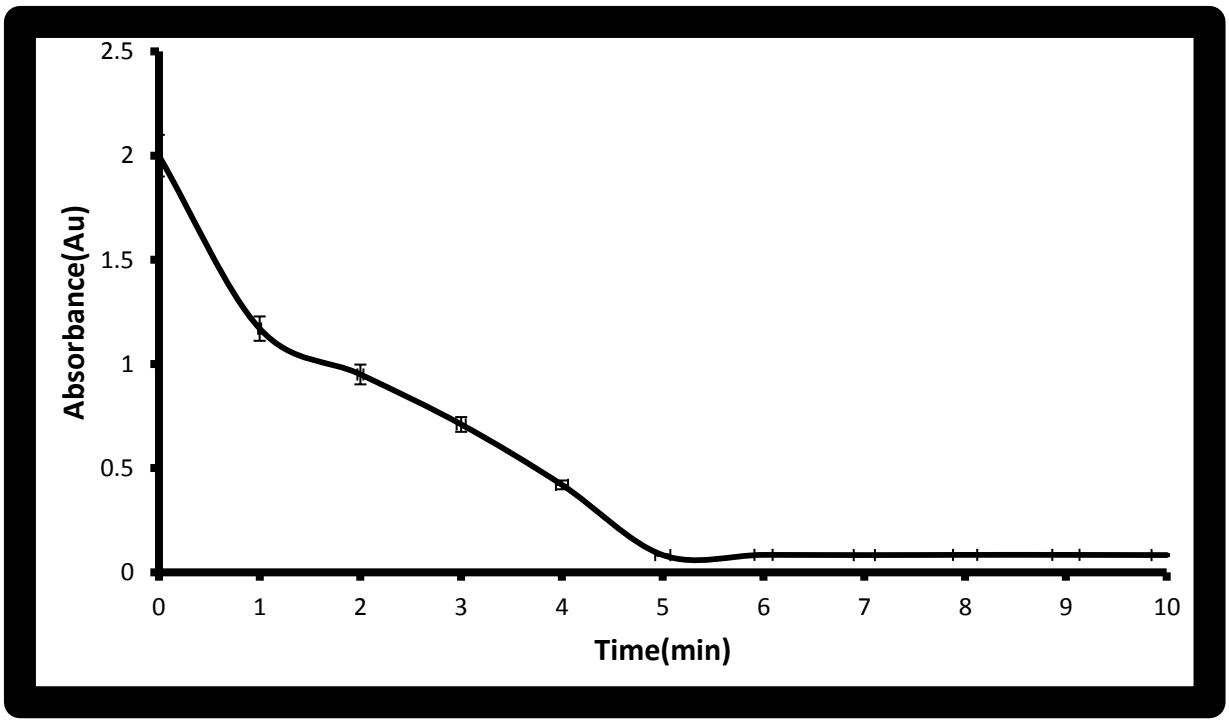


Figure 4.2.1.3.2: Optimization of the time needed for maximum removal of chlorophyll for 750 mg of MIP (optimal quantity) for n=3.

4.2.1.4 Evaluation of the non-selectivity behavior

To investigate the non-selectivity behavior of the sorbents, both the planar pesticides and the interfering chlorophyll were exposed to the MIP as well as GCB as single entities and in a mixture form at optimum conditions. In all cases the MIP did not bind any of the planar pesticides while the percentage of chlorophyll bound was over 99.75% (see Table 4.2.1.4). GCB on the other hand showed that it was not selective to any of the analytes by binding over 88% of each of the planar pesticides as well as the chlorophyll from their prepared standard solutions.

Table 4.2.1 4: Percentage of analyte bound to adsorbent at optimized conditions.

Analyte / Interferent	% bound to MIP n=3	% bound to GCB n=3	% bound to NIP n=3
Chlorophyll (Spinach extract)	99.86 (0.72)	99.92 (1.01)	2.96 (0.19)
Chlorophyll (*Nymphia lotus* extract)	99.90 (0.64)	99.95 (0.83)	3.66 (0.09)
Chlorophyl (*Acalypha vilicaulis* extract)	99.86 (0.97)	99.88 (1.06)	2.42 (0.04)
HCB	**nq**	90.41 (1.67)	3.61 (0.28)
DDE	**nq**	89.97 (0.93)	3.44 (0.01)
DDD	**nq**	91.28 (1.38)	3.29 (0.14)
Mixture			
Chlorophyll	99.79 (1.03)	88.94 (0.73)	3.22 (0.36)
HCB	**nq**	94.52 (1.84)	3.49 (0.92)
DDE	**nq**	90.26 (1.51)	2.79 (0.02)
DDD	**nq**	93.08 (1.96)	3.58 (0.16)

NB: Values in parenthesis are %RSD and **nq** is for not quantifiable.

It should be noted that the same experiments were performed with the NIP which exhibited non selectivity as all of the analytes were bound just like with GCB. However the percentages bound by the NIP were very low, less than 3.7% in all cases (see Table 4.2.1.4) and as such were considered negligible.

GCB was able to bind higher quantities of the analytes than the NIP because it has a layered, planar structure which easily flushed with those of the planar pesticides. The NIPs on the other hand have a 3-D highly cross-linked structure similar to that of the MIPs except for the recognition sites which allow them to selectively bind the target analytes.

4.2.1.6 Effectiveness of the MIP materials on removing chlorophyll from the green plant extracts

The initial absorbance of the methanolic, spinach extract was reduced from an absorbance of 0.683 to 0.092 Au after the addition of the MIP. For the traditional medicine extracts there was removal from a chlorophyll absorbance of 0.795 to 0.0897 Au in *Nymph lotus* and 0.649 to 0.0899 in *Acalypha vilicaulis*. In all cases, the MIP had significantly removed the interfering chlorophyll to a value far below what is regarded as the interfering chlorophyll concentration which is the absorbance of a 2% (w/v) chlorophyll standard which we determined to be 0.401 Au (literature value is 0.399 Au) [325]. This was also marked by the loss of the green characteristic color of the plant extracts to almost colorless solutions after exposure to the MIP powder.

Based on these results it can be concluded that a polymeric sorbent which is cheaper and with high selectivity would soon replace the expensive, challenged, non selective GCB in the dispersive SPE kits of QuEChERS. This would result in cheaper, improved and reliable analytical results of both pesticide residues and bio-actives in green vegetable extracts and natural product research respectively.

4.2.2 Cholic acid MIP particles and MIP nanofiber

4.2.2.1 SEM

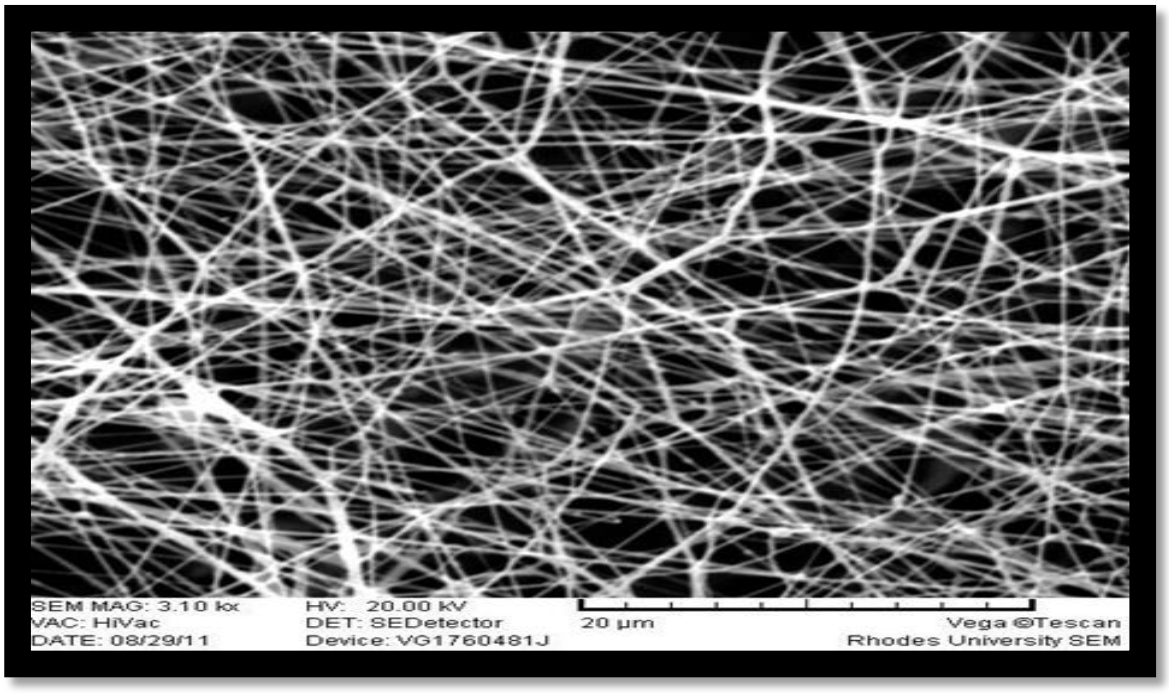


Figure 4.2.2.1: A typical SEM image of the cholic acid MIP or NIP electrospun nanofibers.

Figure 4.2.2.1 shows a typical SEM image of the prepared cholic acid MIP or NIP electrospun nanofibers. The MIP and NIP nanofiber images were similar displaying smooth, thin and

long, thread like structures (see Fig.4.2.2.1; c.f. Fig.4.2.1.1). The nanofibers had an average diameter of 660 nm as determined by the Scandium 4.0 software. All these are excellent physical characteristics for a material that is to be used as a sorbent material for SPE. The nano dimensions contribute to the high surface area to volume ratio that nano-fibrous materials are acclaimed for, which lead to improved sensitivity of the materials.

4.2.2.2 FTIR

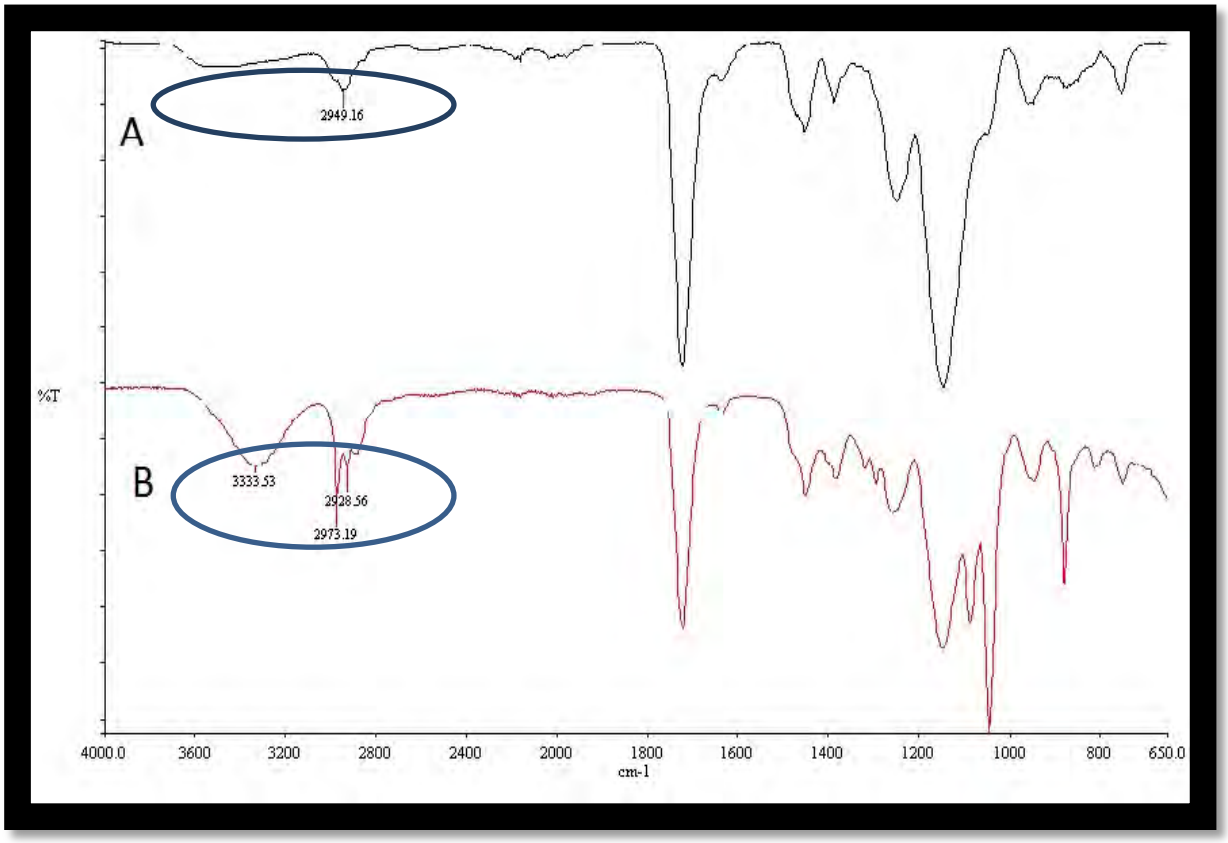


Figure 4.2.2.2: A typical FTIR spectra of the washed (A) and unwashed (B) cholic acid MIP particles/nanofiber.

The removal of cholic acid from the imprinted polymer matrix was confirmed by FT-IR spectroscopy. The MIP monolith particles and the electrospun nanofiber exhibited identical FTIR spectra with three characteristic O-H stretch bands at 3333.53, 2973.19 and 2928.56 cm^{-1} observed on the spectrum of the unwashed MIP particles/nanofibers (see Fig. 4.2.2.2). The bands disappeared on washing the MIP particles/nanofibers with the optimized PHWE procedure with the peak at 2949.16 cm^{-1} reduced and shifted (see Fig. 4.2.2.2). This

confirmed optimal template removal and creation of recognition sites. The circled parts of the spectra indicate the regions of interest.

4.2.2.3 Optimization and comparison of the performances of the cholic acid MIP particles and the MIP nanofibers

After successfully creating binding cavities, rebinding studies were performed to compare the MIP nanofiber with the MIP particles. The studies showed that the MIP nanofiber performance in selectively extracting cholic acid was better than the ordinary MIP particles. For example, Fig. 4.2.2.3.1 shows that at 100 mg of the both MIP formats reached their optimal extraction, with the nanofiber extracting 100% of cholic acid in solution while only 80% was bound to the MIP particles. This trend continued for all the quantities of the MIP materials that were investigated.

Time was also optimised to determine the time required by the MIP monolith particles and MIP nanofiber to extract the cholic acid maximally (see Fig. 4.2.2.3.2). It was observed that 4min was the optimal time needed by either the ordinary MIP particles or the MIP nanofiber for maximum extraction. 100% of cholic acid was bound by the nanofiber while only less than 70% was bound by the MIP particles for the same duration (see Fig. 4.2.2.3.2).

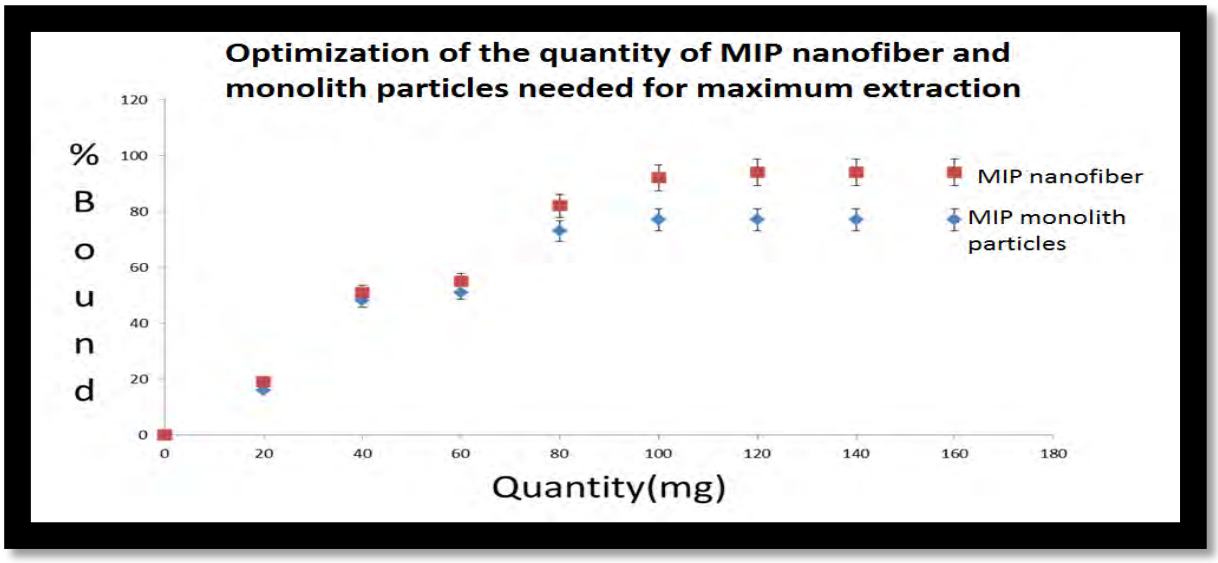


Figure 4.2.2.3.1: Optimization of the quantity of MIP nanofiber or MIP particles needed for maximum extraction of cholic acid from 5mL aliquots of a 10% (w/v) concentrated standard solution over 24 h, for n=3.

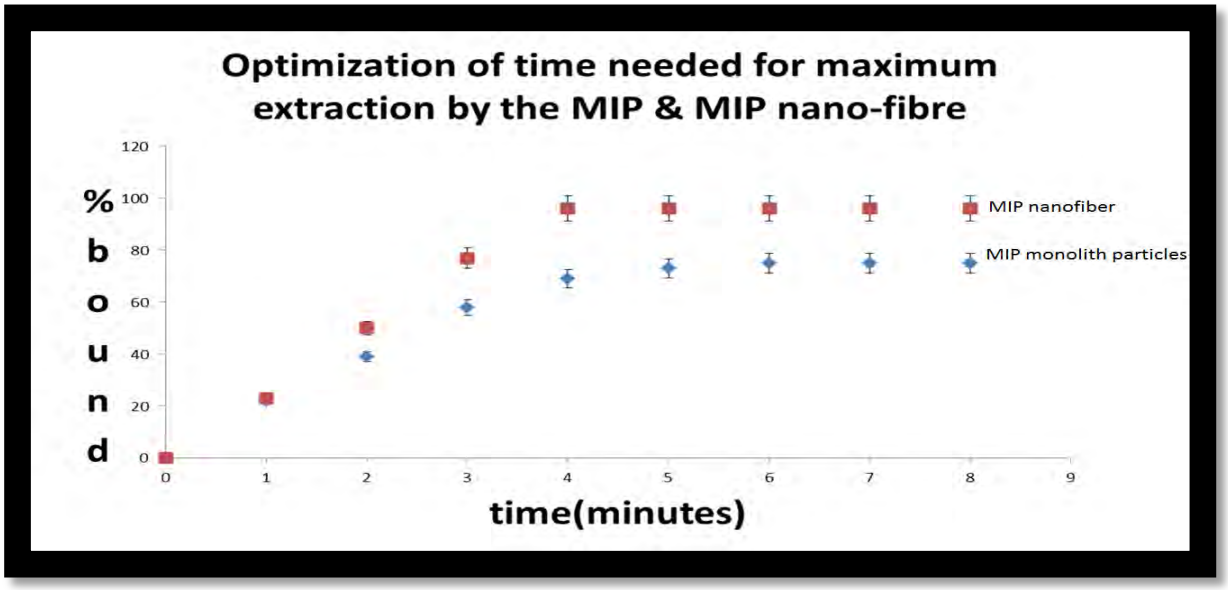


Figure 4.2.2.3.2: Optimization of the time needed by 100 mg (optimal quantity) of MIP nanofiber or MIP particles for maximum extraction of cholic acid from 5mL aliquots of a 10% (w/v) concentrated standard solution for n=3.

4.2.2.4 Evaluation of the non-selectivity behavior

Both the cholic acid MIP fibers and the particles showed greater affinity for the target analyte (cholic acid) in the non-selectivity studies (see Table 4.2.2.4). The nanofiber out competed the particles in binding the cholic acid confirming the excellent performance that nanomaterials possess as has been discussed in previous sections of the thesis. The NIP formats adsorbed negligible quantities of the analytes (results not shown).

Table 4.2.2 4: Percentage of analyte bound to adsorbent at optimized conditions

Compound	% Bound by MIP monolith particles	% Bound by MIP nanofiber
Cholic acid	79.7 (0.1)	100.1 (0.7)
Cholesterol	**Nq**	**Nq**
Deoxycholic acid	**Nq**	**Nq**
Chenodeoxycholic	**Nq**	**Nq**
Propranolol	**Nq**	**Nq**
Aspirin	**Nq**	**Nq**

4.2.2.5 Effectiveness of the MIP materials on removing cholic acid

The prepared cholic acid MIP materials were only applied to standard solutions since the procurement of human bile as a representative sample proved to be futile. The results showed potential of the materials to remove interfering cholic acid during drug residue analysis or drug metabolite studies in human biological fluids especially bile. The MIP nanofiber in particular had completely removed cholic acid from the concentrated standard solution of cholic acid, 10% (w/v). These preliminary results showed that MIP nanofibers prepared through simple methodologies could be the answer to the highly needed selective and sensitive SPE sorbent materials in DRA/PRA.

Based on these results it can be concluded that a novel polymeric sorbent with high selectivity for the removal of the interfering cholic acid has been synthesized. The sorbent would be applied for the first time in the sample preparation of drug residue analysis which is very challenging as it has been discussed in previous sections once the real samples are procured.

4.2.3 AFB1 MIP particles

4.2.3.1 FTIR

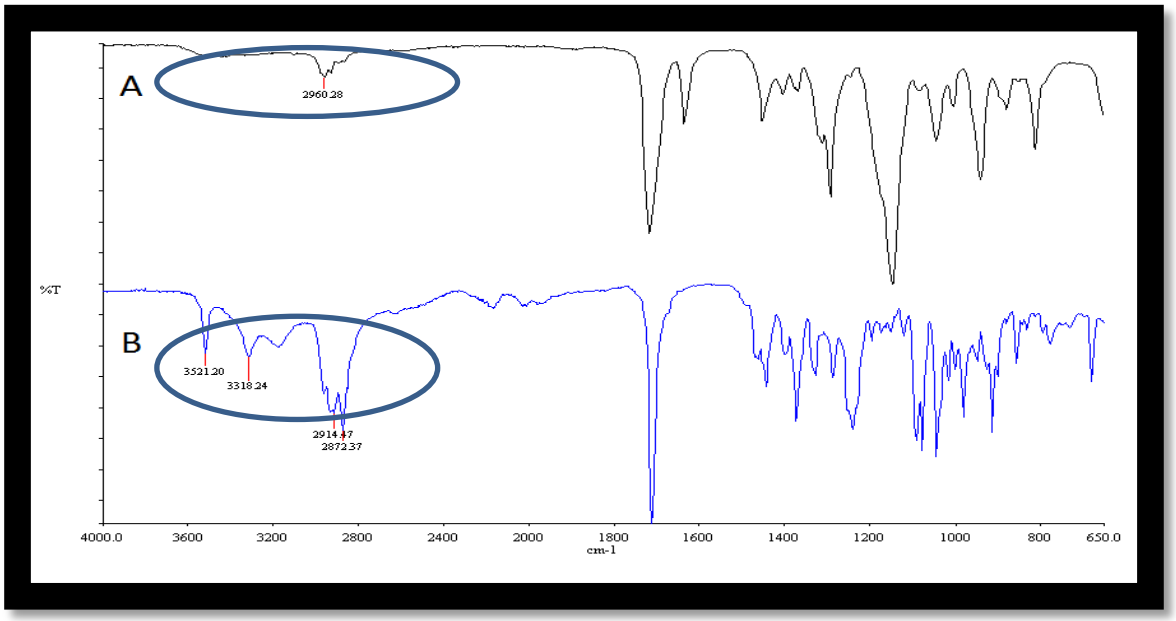


Figure 4.2.3.1: A typical FTIR spectra of the washed (A) and unwashed (B) AFB1 MIP particles

The removal of AFB1 from the imprinted polymer matrix was confirmed by FT-IR spectroscopy. The AFB1 MIP particles exhibited three characteristic O-H stretch bands at 3521.20, 3318.24 and 2914.47 cm^{-1} observed on the spectrum of the unwashed MIP (B) (see Fig. 4.2.3.1). The bands disappeared on washing the MIP particles employing the optimized PHWE procedure. This confirmed optimal template removal and creation of recognition sites. The circled parts of the spectra indicate the regions of interest.

4.2.3.2 HPLC-FLD chromatograms of aflatoxins in nuts/peanut butter samples before and after clean up by the MIP or commercial IAE sorbent

The chromatogram of a derivatized, equi-molar composite standard of Aflatoxins B1, B2, G1, G2 shows good separation of the aflatoxins (see Fig. 4.2.3.2.1), using the optimized HPLC conditions outlined in Table 3.4. The peaks were all symmetrical and the separation was obtained in less than 7 min with good resolution.

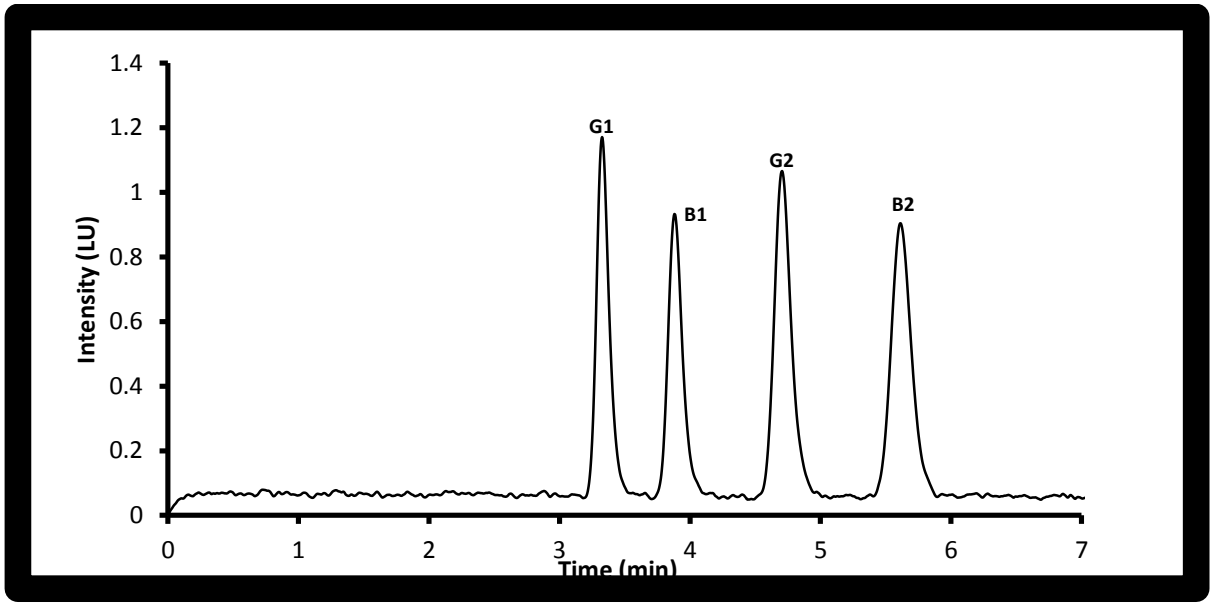


Figure 4.2.3.2 1: Chromatogram of a 1.50 ng/mL equi-molar composite standard solution of aflatoxins B1, B2, G1 and G2.

Supernatants of the derivatized nuts and peanut butter extracts were injected into the HPLC, before and after sample clean-up by the IAE or the MIP. The sample extracts did not show any discernible peaks at the positions of the aflatoxins before or after clean-up except for the contaminated peanut butter sample from Grahamstown school. Both the IAE

cartridge and the synthesized MIP resulted in the simultaneous extraction, clean up and pre-concentration of the aflatoxins from the contaminated peanut butter sample. Figure 4.2.3.2.2 and 4.2.3.2.3 show typical chromatograms, before and after clean-up by the MIP. After clean up it was evident that most of the interfering peaks were removed. The peak intensity of the selectively extracted aflatoxins B1, B2, G1, and G2 were greatly enhanced. The derivatization of the samples was carried out to enhance the signals of aflatoxins B1 and G1, which are known to be quenched by HPLC solvents especially methanol [347].

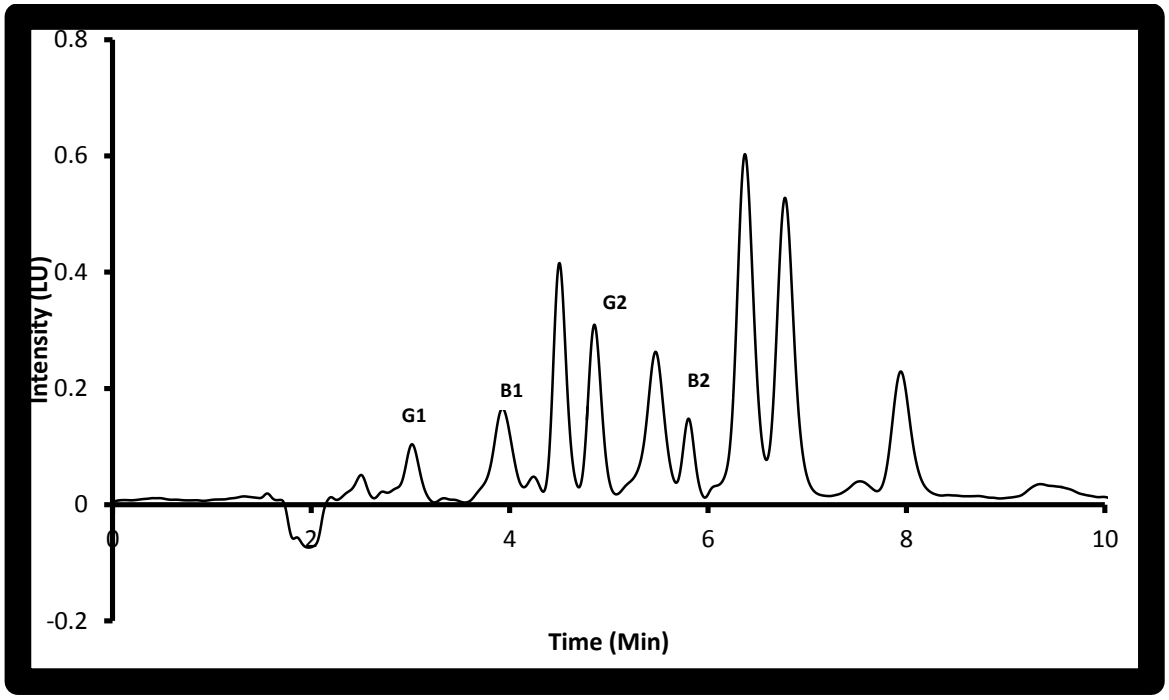


Figure 4.2.3.2.2: Typical chromatogram of a contaminated peanut butter extract prior to MIP clean-up.

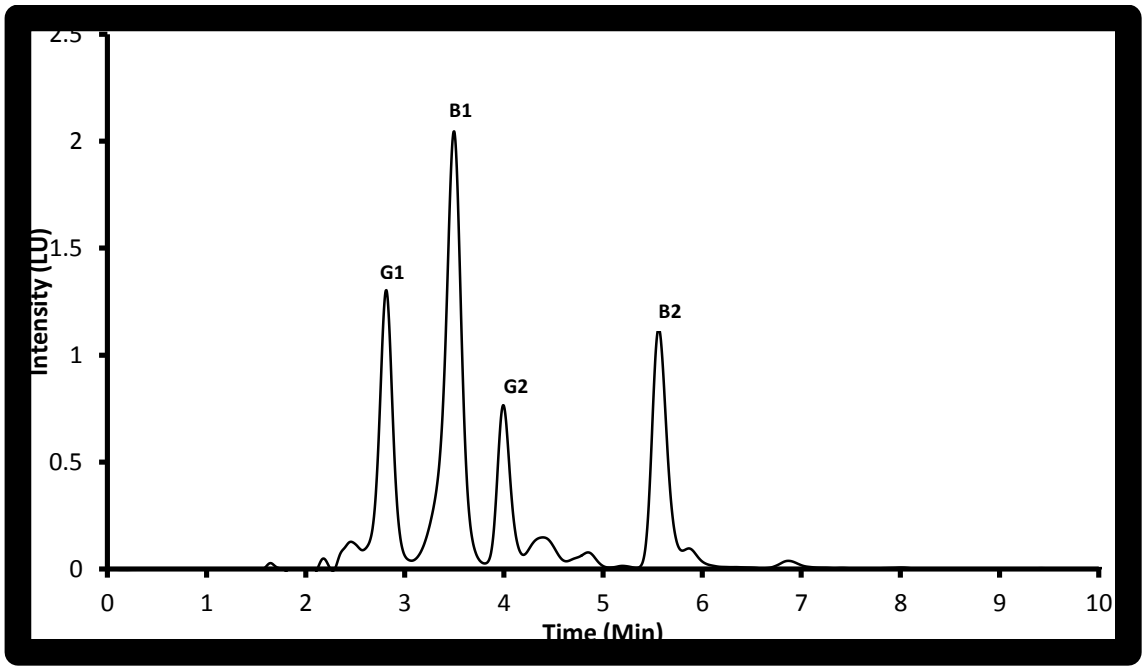


Figure 4.2.3.2.3: Typical chromatogram of a contaminated peanut butter extract after MIP clean-up. Similar results were obtained with the commercial IAE clean-up.

4.2.3.3 Recovery and reproducibility of the clean-up/pre-concentration procedure

Recovery and reproducibility studies of the clean-up procedure were evaluated by analyzing seven replicates of the nut and peanut butter samples spiked at concentrations 2 ng/mL (1 ng/mL for AFB1). A blank was run between the runs of the different samples to ensure that there were no fluctuations on the baseline. Recovery was calculated by comparison of the peak areas before and after the IAE, MIP or NIP clean up. High, comparable recoveries (≥ 97% for all aflatoxins) with percentage relative standard deviations (%RSD) of less than 1% were calculated for samples that were subjected to the MIP and IAE clean up (see Table 4.2.3.3). This indicated that the two sorbents have high affinity for all the four aflatoxins hence the prepared MIP could be classified as a class

selective MIP for the four aflatoxins even though the synthesized MIP was imprinted with AFB1 only. Samples that were subjected to the NIP clean up recorded very low percentage recoveries (≤ 11% for all aflatoxins). The low % recoveries were due to the lack of selective sites for the analytes in the NIP structure as the synthesis of NIP does not include imprinting with a template hence no recognition sites are formed in NIP structures.

Table 4.2.3.3: Average recovery (%) and % RSD (in parenthesis) data for aflatoxins B1, B2, G1 and G2 after the MIP, IAE and NIP clean up.

Sorbent	AFB1	AFB2	AFG1	AFG2
MIP	99.4 (0.6)	97.1 (0.3)	98.6 (0.4)	99.8 (0.3)
IAE	98.0 (0.2)	99.2 (0.7)	101.5 (0.2)	97.4 (0.9)
NIP	10.9 (0.4)	11.0 (0.5)	9.7 (0.2)	10.2 (0.8)

NB: Values in parenthesis are %RSD for n=7.

4.2.3.4 Evaluation of the non-selectivity behavior of the MIP and commercial IAE sorbent cartridges

The following results have been obtained for the non selectivity of testoterone standard solution (750 ng/mL) on the MIP and commercial IAE sorbent cartridges.

Table 4.2.3.4: %Recoveries for the non-selectivity experiments of testosterone on the commercial Easi-Extract® IAE cartridge.

IAE cartridges	Nonselectivity(%)
Breakthrough (4ml = 1ml std + 3ml loading buffer)	97.00 (0.71)
Washings	**nq**
Elution (2ml MeOH)	4.03 (0.48)

NB: The values in parenthesis are %RSD for n=3. **nq** is for not quantifiable.

Although testosterone is not completely recovered in the breakthrough volume (see Table 4.2.3.4), the %recoveries of testosterone in the breakthrough volume (97%) could be regarded as statistically adequate to conclude that the IAE sorbent was not selective to testosterone. The passing through of testosterone with the breakthrough volume without being extracted by the sorbent material was an indication that the sorbent materials did not have affinity for it or similar molecules. This was supported by the low %recovery (4%) of testetosterone in the elution step of the SPE (see Table 4.2.3.4 for results). Since the prepared MIP was not packed in cartridges, batch analysis was employed for the non-

selectivity experiment. The unextracted testosterone in the supernatant was determined to be 94% after the testosterone standard was exposed to the MIP under optimal conditions indicating that the MIP had very low affinity to adsorb it, hence its existence in the supernatant.

4.2.3.5 Calibration parameters

Calibration was performed using aflatoxin standards at ten different concentrations in the range of 0.0-3.0 µg/mL for each standard. The method was found to be linear for all the aflatoxins, with correlationcoefficients (r^2) values of ≥ 0.9986 (see Table 4.2.3.5). To investigate if the matrix of the samples interfered with quantification, parallel calibration plots of standards and spiked neat nut samples before and after clean-up were obtained (Data not shown). From the plots, covariance analysis employing F test at $P < 0.05$ for each aflatoxin was calculated. The calculated F Snedecor values for all the aflatoxins were lower than the tabulated ones indicating that matrix effect was negligible after clean up.

The limits of detection (LOD) values were calculated using the intercept (*yB)* and the standard error of the regression line (*SB),* at 3 times standard error and the values were calculated employing equations 4.2.3.5.1 and 4.2.3.5.2.

$$y(LOD) = yB + 3SB \qquad \textbf{(4.2.3.5.1)}$$

$$LOD = (yLOD - yB)/m \qquad \textbf{(4.2.3.5.2)}$$

where *m* is the gradient of the line.

The limits of quantification (LOQ) values were calculated employing the same method as in equations 4.2.35.1 and 4.2.3.5.2, at 10 times the standard error of the regression line, (see equations 4.2.3.5.3 and 4.2.3.5.4).

$$yLOQ = yB + 10SB \qquad \textbf{(4.2.3.5.3)}$$

$$LOQ = (yLOQ - yB)/m \qquad \textbf{(4.2.3.5.4)}$$

Table 4.2.3.5: Calibration parameters of the MIP extraction method for aflatoxins B1, B2, G1 and G2.

Parameter	B1	B2	G1	G2
LOD (ng/g)	0.004	0.019	0.020	0.011
LOQ (ng/g)	0.013	0.064	0.065	0.038
Regression line equations	y = 0.016x	y = 0.0065x	y = 0.0092x	y = 0.006x
r^2	0.9986	0.9990	0.9990	0.9990

NB: y represents peak areas (determined aflatoxin concentrations by the instrument) and x is the concentration of the prepared aflatoxins.

4.2.3.6 Validation and application to nuts and peanut butter samples

The accuracy of the MIP extraction method was validated by determining the aflatoxin concentrations in the peanut butter CRM. Table 4.2.3.6, shows the results obtained after subjecting the CRM, various nuts and peanut butter extracts to all the MIP extraction conditions and determining the concentrations of the aflatoxins afterwards. Employing the student t-test, the accuracy and precision of the method were found to be acceptable at 95% confidence limit (n=7) for the extraction and analysis of aflatoxins B1, B2, G1, and G2 in various nuts and peanut butter (see Table 4.2.3.5). Similar results were obtained by the commercial IAE method (results not shown).

Table 4.2.3.6 Incidence of aflatoxins (in ng/g) in various nut, peanut butter and CRM extracts after clean up with the MIP and subsequent analysis with HPLC-FLD.

Sample	B1(ng/g)	B2(ng/g)	G1(ng/g)	G2(ng/g)
Peanut	0.89(0.03)	**Nq**	0.64(0.01)	**Nq**
Brazil nut	Nd	**Nq**	**Nq**	**Nq**
Hazel nut	0.42 (0.03)	**Nq**	**Nq**	
Black cat peanut butter	**Nq**	**Nq**	**Nq**	**Nq**
Yum Yum peanut butter	**Nq**	**Nq**	**Nq**	**Nq**
Contaminated peanut butter	1.56(0.03)	2.50(0.07)	0.94(0.01)	1.50(0.01)
CRM (peanut butter) as determined by the MIP method	0.18(0.01)	0.15(0.03)	0.14(0.08)	0.17(0.02)
Certified values for CRM (peanut butter) as per certificate of analysis	<0.2	<0.2	<0.2	<0.2

NB: The values in parenthesis are %RSD for n=7. **nq** is for not quantifiable.

The performance of the custom synthesized MIP statistically matched that of commercial IAE sorbent in selectively extracting, cleaning up and pre-concentrating the four aflatoxins simultaneously from nuts or their derivatives within the European commission monitoring concentration levels. Excellent recoveries (> 97% for all aflatoxins), high reproducibility (%RSD < 1%) and satisfactory accuracy and precision (see Table 4.2.3.6) based on application to the peanut butter CRM, were also achieved and comparable for the two sorbents. The excellent performance by the MIP in selectively extracting aflatoxins demonstrated that analysis involving expensive IAE assays for aflatoxins could be replaced by the relatively inexpensive and robust MIP assays for aflatoxin sample preparation prior to LC separation and detection. The results also indicate that for a positive sample in the analysis of aflatoxins, ELISA (enzyme linked immuno-sorbent assay) can be employed as the initial test for screening purposes then backed by the molecularly imprinted solid phase extraction-liquid chromatography-fluorescence luminescence detection, MISPE-LC-FLD method for confirmatory as low LODs, of ≤ 0.020 ng/g which are far below the maximum acceptable levels for aflatoxins as set by the European commission were achieved with this method. A combination of ELISA and MISPE-LC-FLD method can be beneficial in the analysis of these compounds where there are large number of samples to be analyzed thus making it a cost effective exercise.

Chapter 5 Concluding remarks

This thesis presented an evaluation on the applicability of the different custom synthesized MISPE sorbents for the clean-up of food and drug residues. It was demonstrated that the prepared MIP materials posses the potential to selectively remove interference from complex food or biological matrices for accurate PRA or DRA respectively. Both synthetic and instrumental conditions were optimized in order to obtain maximum selectivity and sensitivity. The optimized procedures resulted in excellent removal and pre-concentration recoveries (>80% in all cases with 100% cholic acid removal by the prepared cholic acid MIP nanofiber), reproducibility (%RSD of <2% in all cases) good linearity and low LODs and LOQs that adhere to the monitoring requirements of governing authorities were calculated.

This thesis has shown that MIP technology can be successfully employed to fabricate `smart` materials that can be employed to selectively remove interfering molecules from complex matrices prior to instrumental analysis. Consequently, cleaner samples in which the analytes of interest were determined with ease and accurately were obtained. Based on the work reported in this thesis, MIP materials especially in the form of MIP electrospun nanofibers were shown to be potential competitors to the traditional particle based SPE sorbents for selectivity. The MIPs also challenged the IAE sorbents for their stability, ease of preparation and cost effectiveness. In particular the chlorophyll specific MIP demonstrated that with further optimization it will soon replace the expensive, non selective GCB in the QuEChERS PRA extraction method. The performance the aflatoxin specific MIP also

demonstrated that it may replace the IAE sorbents in the near future as the two displayed comparably excellent results in selectively cleaning and pre-concentrating aflatoxins from nut samples prior to separation and detection.

It has also been shown that the main challenge of MIPs; template bleeding can be circumvented with new simpler and environmentally friendlier template removal strategies such as the one that was applied successfully in this thesis resulting in negligible quantities of residual template. To further make the technology of molecular imprinting a mature technique in residue analysis, new approaches to the template removal/bleeding must be fully researched on.

Furthermore, it should be noted that despite the excellent results on application of MIPs to the analysis of natural toxins like the aflatoxins, there are inherent practical challenges that continue to prevent the roll out to a wider application of MIPs to PRA, CA and DRA. An example is the high toxicity associated with such analytes as aflatoxins. It is hazardous to directly manipulate the quantities of aflatoxin necessary to synthesize a sufficient MIP to set up an extraction protocol. In fact due care must be taken when working with these toxins. Another challenge is related to the procurement of these compounds. It is very difficult to acquire pure standards and if available the price is usually exorbitantly high. Despite all the challenges, MIPs have been successfully synthesized for these compounds as reported in this thesis and will hopefully continue to be synthesized with improved strategies to deal

with the challenges of residue analysis in complex matrices of food and pharmaceutical samples.

In future, our endevours will be geared towards further investigation and optimization of our successful preliminary attempt to fabricate imprinted electrospun nanofibers through incorporation of templates during the spinning process.

REFERENCES

[1] L.I. Andersson, J. Chromatogr. B 739 (2000) 163.

[2] A.G. Frenich, F.J.A. Liebanas, M. Mateu-Sanchez, J.L.M. Vidal, Talanta 60 (2003) 765.

[3] A. Wilkowska, M. Biziuk, Food Chemistry 125 (2011) 803.

[4] A. Polettini, J. Chromatogr. Biomed. Sci. Applications 733 (1999) 47.

[5] C. Baggiani, L Anfossi, C Giovannoli, Analyst 133 (2008) 719.

[6] M. Careri, L. Elviria, A. Mangia, I. Zagnoni, Rapid Commun. Mass Spectrom. 16 (2002) 1821.

[7] C. He, Y. Long, K, Li, F. Liu, J. Biochem. Biophy. Methods 70 (2007) 133.

[8] H.X. Chen, Q.P. Deng, L.W. Zhang, X.X. Zhang, Talanta 78 (2009) 464.

[9] A. Beltran, F. Borrull, P.A.G. Cormack, R.M. Marce, Trends Anal. Chem. 29 (2010) 1363.

[10] T. Pap, G. Horvai, J. Chromatogr. B 804 (2004) 167.

[11] F. Barahona, E. Turiel, P.A.G. Cormack, A. Martín-Esteban, J. Polym. Sci. Part A Polym. Chem. 48 (2010) 1058.

[12] A. Piletsky, S. Alcock, A.P.F. Turner, Trends Biotech. 19 (2001) 9.

[13] Http://goldbook.iupac.org/S05457.html (2011) November 18.

[14] http://www.aoac.org/dietsupp6/Dietary-Supplement-web-site/Sampling.pdf (2011) November18.

[15] X. Fu, Y. Liao, H. Liu, Anal. Bioanal. Chem. 381 (2005) 75.

[16] R.M. Smith, J. Chromatogr. A 1000 (2003) 3.

[17] J.Pawliszyn, Anal. Chem. 75 (2003) 2543.

[18] S.S. Nielsen (Ed.) (2010) Food Analysis. Springer, New York.

[19] http://www-unix.oit.umass.edu (2011) November 18.

[20] J.M. Rosenfeld,(2002) In: J. Pawliszyn (Ed.), Sampling and sample preparation for field and laboratory, Elsevier, Amsterdam, 2002, pg 609.

[21] C. Dietz, J. Sanz, E. Sanz, R. Mu˜noz-Olivas, C. C´amara, J. of Chromatogr. A 1153 (2007) 114.

[22] E. Turiel, A. Martin-Esteban, Anal. Chim. Acta 668 (2010) 87..

[23] B. Kinsella, J. O'Mahony, E. Malone, M. Moloney, H. Cantwell, A. Furey, M. Danaher, J. Chromtogr. A. 1216 (2009) 7977.

[24] Chen, Z. Guo, X. Wang, C. Qiu, J. Chromatogr. A 1184 (2008) 191.

[25] R.D. McDowell, J. Chromatogr. B, 492 (1989) 2.

[26] K.S. Boos, A. Rudolphi, LC–GC 15 (1997) 602.

[27] D.J. Anderson, Anal. Chem. 65 (1993) 434.

[28] P.H. Degen, J. Chromatogr. B 222 (1981) 437.

[29] H. Freiser, Q. Jiang, Anal Biochem. 388 (2009) 260.

[30] J. Combie, J.W. Blake, T. E. Nugent, T.Tobin, Clin. Chem. 28 (1983) 83.

[31] Y.J. Xue, J. Pursley, M.E. Arnold, J Pharm Biomed Anal 34 (2004) 369.

[32] V. Pichon, J. Chromatogr. A 1152 (2007) 41.

[33] V. Pichon, K. Haupt, J. Liq. Chromatogr. Related Technol. 29 (2006) 989.

[34] S. Kjellstrom, O.N. Jensen, Anal Chem 75 (2003) 2362.

[35] http://www.chromatography-online.org/topics/derivatization.html (2011) November 18.

[36] H. Joshua, J. Chromatogr. A 654 (1993) 247.

[37] K. Ridgway, S.P.D. Lalljie, R.M. Smith, J. of Chromatogr. A 1153 (2007) 36.

[38] S. Mitra, (Ed.) (2003) Sample Preparation Techniques in Analytical Chemistry. Wiley-Interscience, New Jersey.

[39] F. Soxhlet, Dinglers' Polyt. J. 232 (1879) 461.

[40] V. Lopez-Avila, Crit. Rev. Anal. Chem. 29 (1999) 195.

[41] J. Pawliszyn, Trends Anal. Chem. 14 (1995) 113.

[42] C. B'Hymer, J.A. Caruso, J. Chromatogr. A 1045 (2004) 1.

[43] M. Burguera, J.L. Burguera, Talanta 44 (1997) 1581.

[44] P.L. Buldini, S. Cavalli, A. Trifiro, J. Chromatogr. A 789 (1997) 529.

[45] S. Chiron, S. Roy, R. Cottier, R. Jeannot, J. Chromatogr. A 879 (2000) 137.

[46] T.A. Gehring, L.G. Rushing, M.I. Churchwell, J. Agric. Food Chem. 44 (1996) 3164.

[47] http://www.tutorbene.com/cms_images/LIQUID-LIQUID%20EXTRACTION.bmp (2011) November 18.

[48] H.H. Liu, P.K. Dasgupta, Anal. Chem. 68 (1996) 1817.

[49] D.C. Wood, J.M. Miller, I. Christ, LC-GC Euro. 17 (11) (2004) 573.

[50] M. Ma, F.F. Cantwell, Anal. Chem. 70 (1998) 3912.

[51] M. Ma, F.F. Cantwell, Anal. Chem. 71 (1999) 388.

[52] G. Shen, H.K. Lee, Anal. Chem. 74 (2002) 648.

[53] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P. Revilla-Ruiz, J. Hernandez-Mendez, J. Chromatogr. A 1089 (2005) 1.

[54] H. Giergielewicz-Mozajska, L. Dabrowski, J. Namiesnik, Crit. Rev. Anal. Chem. 31 (2001) 149.

[55] E. Björklund, T. Nilsson, S. Bøwadt, Trends Anal. Chem. 19 (2000) 434.

[56] http://www.dionex.com/en-us markets/food-beverage (2011) November 18.

[57] L. Pallaroni, C. Von Holst, J. Chromatogr. A 993 (2003) 39.

[58] C. Von Holst, F. Serano, S. Sporring, E. Björklund, A. Muller, Chromatographia 61 (2005) 391.

[59] S. Morales-Munoz, J.L. Luque-Garcia, M.D. Luque de Castro J. Chromatogr. A 978 (2002) 49.

[60] J.L. Gomez-Ariza, M. Bujalance, I. Giraldez, A. Velasco E. Morales, J. Chromatogr. A 946 (2002) 209.

[61] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P. Revilla-Ruiz, J. Hernandez-Mendez J. Chromatogr. A 1089 (2005) 1.

[62] P. Suchan, J. Pulkrabová, J. Hajšlová, V. Kocourek, Anal. Chim. Acta 520 (2004) 193.

[63] M. Gfrerer, S. Chen, E. Lankmayr, X. Quan, F. Yang, Anal. Bioanal. Chem. 378 (2004) 1861.

[64] J.C. Chuang, K. Hart, J.S. Chang, L.E. Boman, J.M. Van Emon, A.W. Reed, Anal. Chim. Acta, 444 (2001) 87.

[65] J.L. Luque- García, M.D. Luque de Castro, Trends Anal. Chem. 23 (2004) 102.

[66] M.C. Herrera, R.C. Prados-Rosales, J.L. Luque-Garcia M.D. Luque de Castro, Anal. Chim. Acta 463 (2002) 189.

[67] L. González Osnaya, J.M. Soriano del Castillo, J.C. Moltó Cortés J.M. Vinuesa, J. Chromatogr. A 1113 (2006) 32.

[68] O. Pardo, V. Yusa, N. Leon, A. Pastor, J. Chromatogr. A 1107 (2006) 70.

[69] M. Barriada-Pereira, M.J. Gonzalez-Castro, S. Muniategui-Lorenzo, P. Lopez-Mahia, D. Prada-Rodriguez, E. Fernandez-Fernandez, Talanta 71 (2007) 1345.

[70] E. Björklund, S. Sporring, K. Wiberg, P. Haglund C. Van.Holst, Trends Anal. Chem. 25 (2006) 318.

[71] G.K. Zoorob, J.W. McKiernan, J.A. Caruso, Microchim. Acta 128 (1998) 145.

[72] M.L. Hopper, J.W. King, J.H. Johnson, A.A. Serino, R.J. Butler, J. AOAC Int. 78 (1995) 1072.

[73] M.E. Zorn, R.J. Noll, M.A. Anderson, W.C. Sonzogni, Anal. Chem. 72 (2000) 631.

[74] F.J. Eller, J.W. King, J. Agric. Food Chem. 46 (1998) 3657.

[75] J.W. King, Am. Lab. 30 (1998) 46.

[76] M. Zougagh, M. Valcarcel, A. Rios, Trends Anal. Chem. 23 (2004) 399.

[77] V.G. Zuin, J.H. Yariwake, C. Bicchi, J. Chromatogr. A 985 (2003) 159.

[78] S.R. Rissato, M.S. Galhiane, F.R.N. Knoll, B.M. Apon, J. Chromatogr. A 1048 (2004) 153.

[79] M.A. Lage Yusty, J.L. Cortizo Davina, Food Control 16 (2005) 59.

[80] E. Anklam, H. Berg, L. Mathiasson, M. Sharman, F. Ulberth, Food Addit. Contam. 15 (1998) 729.

[81] H.M. 'Skip' Kingston, S.J. Haswell (Eds.), Microwave-Enhanced Chemistry. Fundamentals, Sample Preparation and Applications, American Chemical Society, Washington, 1997.

[82] V. Camel, Trends Anal. Chem. 19 (2000) 229.

[83] Q. Jin, F. Liang, H. Zhang, L. Zhao, Y. Huan, D. Song, Trends Anal. Chem. 18 (1999) 479.

[84] V. Yusa, A. Pastor, M. de la Guardia, Anal. Chim. Acta 540 (2005) 355.

[85] M.P. Hermo, D. Barron, J. Barbosa, Anal. Chim. Acta 539 (2005) 77.

[86] J. Ruiz-Jimenez, F. Priego-Capote, M.D. Luque de Castro, J. Chromatogr. A 1045 (2004) 203.

[87] M.C. Herrera, M.D. Luque de Castro, J. Chromatogr. A 1100 (2005) 1.

[88] S. Pedersen-Bjergaard, K.E. Rasmussen, Trends Anal. Chem. 27 (2008) 934.

[89] N.C. Van de Merbel, J. Chromatogr. A 856 (1999) 55.

[90] B.M. Cordero, J.L. Perez Pavon, C. Garcia Pinto, M.E. Fernandez Laespada, R. Carabias Martinez, E. Rodriguez Gonzalo, J. Chromatogr. A 902 (2000) 195.

[91] J.Å. Jönsson, L. Mathiasson, J. Chromatogr. A 902 (2000) 205.

[92] L. Chimuka, L. Mathiasson, J.Å. Jönsson, Anal. Chim. Acta 416 (2000) 77.

[93] J.Å. Jönsson, L. Mathiasson, LC–GC Eur. 16 (10) (2003) 683.

[94] R. Carabias-Martınez, E. Rodrıguez Gonzalo, E. Hernandez Fernandez,

J. Hernandez Mendez, Anal. Chim. Acta 304 (1995) 323.

[95] M.M. Delgado Zamarreno, A. Sanchez Perez, M. Bustamante Rangel, J. Hernandez Mendez, Anal. Chim. Acta 386 (1999) 99.

[96] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P.H. Paniagua-Marcos, J. Hernandez-Mendez, J. Chromatogr. A 869 (2000) 427.

[97] K.E. Rasmussen, S. Pedersen-Bjergaard, Trends Anal. Chem. 23 (2004) 1.

[98] G. Theodoridis, G.J. de Jong, Adv. Chromatogr. 43 (2005) 231.

[99] E.E. Stashenko, J.R. Martínez, Trends Anal. Chem. 23 (2004) 553.

[100] C.F. Poole, Trends Anal. Chem. 22 (2003) 362.

[101] I. Liska, J. Chromatogr. A 885 (2000) 3.

[102] V. Camel, Spectrochim. Acta Part B 58 (2003) 1177.

[103] C.F. Poole, Solid-phase extraction. In: Encyclopedia of Separation Science 3, Academic Press (2000) 1405.

[104] C.W. Huck, G.W. Bonn, J Chromatogr. A 885 (2000) 51.

[105] M.C. Hennion, J Chromatogr. A 856 (1999) 3.

[106] A. Posyniak, J. Zmudzki, S. Semeniuk. J. Chromatogr. A 914 (2001) 89.

[107] F. Toribio, L. Puignou, M.T. Galceran. J. Chromatogr. A 836 (1999) 223.

[108] F. Krokos, C.S. Creaser, C. Wright, J.R. Startin. Fresenius J. Anal. Chem. 357 (1997) 732.

[109] Commission Regulation (EC) No 466/2001 setting maximum levels for certain contaminants in foodstuffs, Official J. (2001) L77 (16.3.2001).

[110] J.K. Huwe, M. Lorentzsen, K. Thuresson, A. Bergman, Chemosphere 46 (2002) 635.

[111] N. Fontanals, R.M. Marce, F. Borrull, Trends Anal. Chem. 24 (2005) 394.

[112] C.P. Desilets, M.A. Rounds, F.E. Regnier. J. Chromatogr. B 544 (1991) 25.

[113] S. Souverain, S. Rudaz, J.L. Veuthey, J. Chromatogr. B 801 (2004) 141.

[114] E. Blahova, L. Bovanova, E. Brandsteterova, J. Liq. Chromatogr.24 (2001) 3027.

[115] K. Heinig, F. Bucheli, J. Chromatogr. B 769 (2002) 9.

[116] M.C. Henninon, V. Pichon, J. Chromatogr. A. 1000 (2003) 29.

[117] D.C. Hage, J. Chromatogr. A. 715 (1998) 2.

[118] V. Pichon, M.C. Henninon, Sampling and sample preparation for field and laboratory, Comprehensive analytical chemistry, Elsevier, Amsterdam, 2002.

[119] J. Svenson, I.A. Nicholls, Anal. Chim. Acta 435 (2001) 19.

[120] C. Alexander, H.S. Andersson, L.I. Andersson, R.J. Ansell, N. Kirsch, I.A. Nicholls, J. O'Mahony, M.J. Whitcombe, J. Mol. Recogn. 19 (2006) 106.

[121] Z. Liu, C. Zheng, C. Yan, R. Gao, Electrophoresis 28 (2007) 127.

[122] R.J. Ansell, Adv. Drug Deliv. Rev. 57 (2005) 1809.

[123] N. Lavignac, C.J. Allender, K.R. Brain, Anal. Chim. Acta 510 (2004) 139.

[124] R.J. Ansell, J. Chromatogr. B 804 (2004) 151.

[125] E.L. Holthoff, F.V. Bright, Anal. Chim. Acta 594 (2007) 147.

[126] M. Avila, M. Zougagh, A. Rios, A. Escarpa, Trends Anal. Chem. 27 (2008) 54.

[127] M.C. Blanco-Lopez, M.J. Lobo-Castanon, A.J. Miranda-Ordieres, P. Tunon-Blanco, Trends Anal. Chem. 23 (2004) 36.

[128] E. Turiel, A. Martin-Esteban, P. Fernandez, C. Perez-Conde, C. Camara, Anal. Chem. 73 (2001) 5133.

[129] C. He, Y. Long, J. Pan, K. Li, F. Liu, J. Biochem Biophys. Methods 70 (2007) 133.

[130] V. Pichon, J. Chromatogr. A 1152 (2007) 41.

[131] F.G. Tamayo, E. Turiel, A. Martin-Esteban, J. Chromatogr. A 1152 (2007) 32.

[132] V. Pichon, K. Haupt, J. Liq. Chromatogr. Related Technol. 29 (2006) 989.

[133] E. Caro, R.M. Marce, F. Borrull, P.A.G. Cormack, D.C. Sherrington, Trends Anal. Chem. 25 (2006) 143.

[134] Q. Fengxia, S. Hanwen, Y. Hongyuan, R. Kyung Ho, Chromatographia 64 (2006) 625.

[135] C. Alexander, H.S. Andersson, L.I. Andersson, R. J. Ansell, N. Kirsch, I.A. Nicholls, J. O'Mahony, M.J.Whitcombe, J. Mol. Recognit. 19 (2006) 106.

[136] B. Sellergren K.J. O'Shea, J. Chromatogra. A 635 31.

[137] J.M. Lehn, Angew. Chem. Int. Ed. Engl. 27 (1988) 89.

[138] J.W.R. Schwabe, D. Rhodes, Trends Biochem. Sci. 16 (1991) 291.

[139] L. Ye, Artificial Receptors: New opportuniyties for the exploitation of molecularly imprinted polymers, Doctoral Thesis, University of Lund, Sweden, (1999) pg.1-2 Chapter 1.

[140] F.J. Zeelen, Pharmacochemisty library 15 (1990) 1.

[141] R. Arshady, K. Mosbach, Makromol Chem 182 (1981) 687.

[142] K. Mosbach, O. Ramström, Bio/Technology 14 ((1996) 163.

[143] G. Wulff, A. Sarhan, Angew. Chem. 84 (1972) 364.

[144] M.J. Whitcombe, M.E. Rodriguez, P. Villar, E.N. Vulfson, J. Am. Chem. Soc. 117 (1995) 7105.

[145] Z. Wang, G. Wu, M. Wang, C. He, J. Mater. Sci. 44 (2009) 2694.

[146] P. Martin, I.D. Wilson, G.R. Jones J Chromatogr A 889 (2000) 143.

[147] R. Suedee, T. Srichana, J. Saelim, T. Thavornpibulbut, Analyst 124 (1999) 1003.

[148]http://www.mpimagderburg.mpg.mpg.deresearch/projects/1088/enansepa/chiralmem/ (2012) March 23.

[149] K. Sreenivasan, J Mater Sci 42 (2007) 7575.

[150] I.A. Nicholls, Chem. Lett. 24 (1995) 1035

[151] C. Cacho, E. Turiel, A. Martin-Esteban, C. Perez-Conde, C. Camara, Anal. Bioanal. Chem.376 (2003) 491.

[152] G. Theodoridis,A.Kantifesa, P.Manesiotis, N. Raikos, H. Tsoukali-Papadopoulou, J. Chromatogr. A 987 (2003) 103.

[153] L. Ye, Artificial Receptors: New opportuniyties for the exploitation of molecularly imprinted polymers. Doctoral Thesis, University of Lund, (1999), Sweden pg.17 Chapter 4.

[154] W.M. Mullett, Investigation of selective binding interactions for analytical separations and determination of pharmaceuticals and toxins. Doctoral Thesis, Dept. of Chemistry, Ottawa, Ontario, Canada (2000).

[155] S. Piletsky, E. Piletska, K. Karim, G. Foster, C. Legge, A. Turner, Anal. Chim. Acta 504 (2003) 123.

[156] S. Hjerten, J.L. Liao, K. Nakazato, Y. Wang, G. Zamaratskaia and H.X. Zhang, Chromatographia, 44 (1996) 227.

[157] A.L. Jenkins, O.M. Uy, G.M. Murray, Anal. Chem. 71 (1999) 373.

[158] S.A. Piletsky, T.L. Panasyuk, E.V. Piletskaya, I.A. Nicholls, M. Ulbricht, J. Membr. Sci. 157 (1999) 263.

[159] G. Vlatakis, L.I. Andersson, R. Müller, K. Mosbach, Nature, 361 (1993) 645.

[160] Y. Lv, Z. Lin, W. Feng, X. Zhou, T. Tan, Biochem. Eng. J. 36 (2007) 221.

[161] E.R. Mojica, J. Autschbach, F.V. Bright, D.S. Aga, Anal. Chim. Acta 684 (2011) 72.[162] J. Yao, X. Li, W. Qin, Anal. Chim. Acta 610 (2008) 282.

[163] A. Guerreiro, A. Soares, E. Piletska, B. Mattiasson, S. Piletsky, Anal. Chim. Acta 612 (2008) 99.

[164] K. Farrington, E. Magner, F. Regan, Anal. Chim. Acta 566 (2006) 60.

[165] O. Ramström, L.I. Andersson, K. Mosbach, J. Org. Chem. 58 (1993) 7562

[166] L Ye, Artificial Receptors: New opportuniyties for the exploitation of molecularly imprinted polymers. Doctoral Thesis, University of Lund, Sweden, (1999), pg.19, Chapter 4.

[167] M.H. Abraham, P.L. Grellier, D.V. Prior, P.P. Duce, J.J. Morris, P.J. Taylor, J. Chem. Soc.Perkin Trans. 2 (1989) 699-711

[168] M.H. Abraham, P.L. Grellier, D.V. Prior, P.P. Duce, J.J. Morris, P.J. Taylor, J. Chem. Soc.Perkin Trans. 2 (1990) 521-529

[169] M. H. Abraham, Chem.Soc.Rev., 22 (1993) 73

[170] M. Kemp, Chiral recognition Studies on chiral discrimination in enzymatic peptide synthesis and non-covalent molecular imprinting. Doctoral Thesis, University of Lund, Sweden, 1994.

[171] O. Ramström, Molecular imprinting technology. Design and recognition properties of antibody/ receptor mimics. Doctoral Thesis, University of Lund, Sweden, 1996.

[172] S. Boonpangrak, M.J. Witcombe, Prachayasittikul, K. Mosbach, L. Yei, Biosensors and Bioelectrics 22 (2006) 349.

[173] D. Lakshmi, M.J. Witcombe, Imprinted Polymers. In: S. Cornier, A. Karyakin (Eds), Electropolymerization, Wiley (2010), pg 153-150.

[174] J.M.G. Cowie, V. Arrigh, Polymers: Chemistry and Physics of Modern Materials, RC Press (2008), Scotland.

[175] R.Z. Greenley, J. Macromol. Sci.Chem. A14 (1980) 445.

[176] W. Funke, O. Okay, B. Joos-Müller, Adv. Polym. Sci. 136 (1998) 139.

[177] J. Haginaka, J. Chromatogr. B 866 (2008) 3.

[178] N. Perez-Moral, A.G. Mayes, Bioseparation 10 (2001) 287.

[179] D. Forchheimer, G. Luo, L. Montelius, L. Ye, Analyst 135 (2010) 1219.

[180] J. Haginaka, J. Chromatogr. B 866 (2008) 3.

[181] N. Perez-Moral, A.G. Mayes, Anal. Chim. Acta 504 (2004) 15.

[182] O. Bruggemann, K. Haupt, L. Ye, E. Yilmaz, K. Mosbach, J. Chromatogr. A 889 (2000) 15.

[183] I.S. Chronakis, B. Milosevic, A. Frenot, L. Ye, Macromolecules 39 (2006) 357.

[184] P.J. Dowding, B. Vincent, Colloids Surf. A: Physicochem. Eng.

Aspects 161 (2000) 259.

[185] J. Matsui, M. Okada, M. Tsuruoka, T. Takeuchi, Anal. Commun. 34 (1997) 85

[186] A.G. Mayes, K. Mosbach, Anal. Chem. 68 (1996) 3769.

[187] D.W. Zhu, Macromolecules 29 (1996) 2813.

[188] L. Ye, O. Ramström, K. Mosbach, Anal Chem. 70 (1998) 2789.

[189] R. Carabias-Martinez, E. Rodriguez-Gonzalo, E. Herrero-Hernandez, M.E. Diaz-Garcia, J. Sep. Sci. 28 (2005) 453.

[190] E. Turiel, J.L. Tadeo, P.A.G. Cormack, A. Martin-Esteban, Analyst 130 (2005) 1601.

[191] S. Wei, B. Mizaikoff, Biosensors and Bioelectronics 23 (2007) 201.

[192] Y. Lin, Y. Shi, M. Jiang, Y. Jin, Y. Peng, B. Lu, K. Dai, Environ. Pollut. 153 (2008) 483.

[193] E. Turiel, A. Martin-Esteban, Anal. Bioanal. Chem. 378 (2004) 1876.

[194] C. Cacho, E. Turiel, A. Martin-Esteban, C. Perez-Conde, C. Camara, J. Chromatogr. B 802 (2004) 347.

[195] F.G. Tamayo, J.L. Casillas, A. Martin-Esteban, J. Chromatogr. A 1069 (2005) 173.

[196] F.G. Tamayo, J.L. Casillas, A. Martin-Esteban, Anal. Chim. Acta 482 (2003) 165.

[197] O. Okay, M. Kurz, K. Lutz, W. Funke, Macromomolecules (1995) 2728.

[198] J. Matsui, T. Kato, T. Takeuchi, M. Suzuki, K. Yokoyama, E. Tamia, I. Karube, Anal. Chem. 65 (1993) 2223.

[199] B. Sellergren, J. Chrobmatogr. A 673 (1994) 133.

[200] J.C.C. Yu, S. Krushkova, E.P.C. Lai, E. Dabek-Zlotorzynska, Anal. Bioanal. Chem. 382 (2005) 1534.

[201] Z. Liu, C. Zheng, C. Yan, R. Gao, Electrophoresis 28 (2007) 127.

[202] L. Schweitz, M. Petersson, T. Johansson, S. Nilsson, J. Chromatogr. A 892 (2000) 203.

[203] L. Schweitz, L.I. Anderson, S. Nilsson, J. Chromatogr. A 817 (1998) 5.

[204] L. Schweitz, L.I. Anderson, S. Nilsson, Anal. Chem. 67 (1997a) 1179.

[205] L. Schweitz, L.I. Anderson, S. Nilsson, J. Chromatogr. A 792 (1997b) 401.

[206] O. Brüggemann, R. Freitag, M.J. Whitcombe, E.N. Vulfson, J. Chromatogr. A 781 (1997) 43.

[207] M. Glad, O. Narrlow, B. Sellergren, N. Siegbahn, K Mosbach, J. Chromatogra 347 (1985) 11.

[208] S.D. Plunkett, F.H. Arnold, J. Chromatogr. A 708 (1995) 19.

[209] M. Glad, P. Reinholdsson, K Mosbach, React. Polym 25 (1995) 47.

[210] J. Haginaka, H. Sanbe, Chem. Lett. (1998) 1089.

[211] J. Haginaka, H. Takehira, K. Hosoya, N. Tanaka, J. Chromatogr. A 816 (1998) 113.

[212] X. Liu, Z. Chen, R. Zhao, D. Shangguan, G. Liu, Y. Chen, Talanta 71 (2007) 1205.

[213] C. Choong, J.S. Bendall, W. Milner, Biosensors and Bioelectronics 25 (2009) 652.

[214] B. Paul, W.N. Martens, R.L. Frost, J. Colloid Interface sci. 360 (2011) 132.

[215] W.E. Teo, S. Ramakrishna, Nanotechnology 17 (2006) R89

[216] Z.M. Huang, Y.Z. Zhang, M. Kotaki, S. Ramakrishna, Compos. Sci. Technol. 63 (2003) 2223.

[217] J.D. Schiffman, C.L. Schauer, Biomacromolecules 8 (2007) 594.

[218] J.D. Schiffman, C.L. Schauer, Biomacromolecules 8 (2007) 2665.

[219] J.D. Schiffman, Masters of Engineering; Cornell University, 2004.

[220] L. Li, L.M. Bellan, H.G. Craighead, M.W. Frey, Polymer 47 (2006) 6208.

[221] Y. Ji, B. Li, S. Ge, J.C. Sokolov, M.H Rafailovich, Langmuir 22 (2006) 1321.

[222] R. Gopal, S Kaur, C.Y Feng, C. Chan, S. Ramakrishna, S. Tabe, T. Matsuura, J. Membr. Sci. 289 (2007) 210.

[223] L. Wannatong, A. Sirivat, P. Supaphol, Polym. Int. 53 (2004) 1851.

[224] K.W. Kim, K.H. Lee, M.S. Khil, Y.S. Ho, H.Y Kim, Fibers and Polymers 5 (2004) 122.

[225] S.Y. Chew, J. Wen, E.K.F. Yim, K.W. Leong, Biomacromolecules 6 (2005) 2017.

[226] E.R Kenawy, J.M. Layman, J.R Watkins, G.L. Bowlin, J.A. Matthews, D.G. Simpson, G.E. Wnek, Biomaterials 24 (2003) 907.

[227] Y.M. Shin, M.M Hohman, M.P. Brenner, G.C. Rutledge, Polymer 42 (2001) 09955.

[228] G. Taylor, Disintegration of water drops in an electric field", Proc. R. Soc. London, Ser. A 1964, 280, 383–397.

[229] D.H Reneker, A.L. Yarin, H. Fong, S. Koombhongse, J. Appl. Phys. 87 (2000) 4531.

[230] T. Subbiah, G.S. Bhat, R.W Tock, S. Parameswaran, S.S Ramkumar, J. Appl. Polym. Sci. 96 (2005) 557.

[231] I.S. Chronakis, A. Jakob, B. Hagström, L. Ye, Langmuir 22 (2006b) 8960.

[232] P.G.Conrad, P.T. Nishimura, D. Aherne, B.J. Schwartz, D. Wu, N. Fang, X. Zhang, M.J. Roberts, K.J. Shea, Adv.Mater. 15 (2003) 1541.

[233] K. Yoshimatsu, J. Lindberk, L. Ye, I.S. Chronakis, Biosensors and Bioelectronics 23 (2008) 1208.

[234] K.J. Shea, D.Y. Saski, Am. Chem. Soc. 113 (1991) 4109.

[235] J. Svenson, H.S. Andersson, S.A. Piletsky, I.A. Nicolls, J. Mol. Recognit. 11 (1998) 83.

[236] H.S. Andersson, I.A. Nicholls, Bioorgan.Chem.25 (1997), 203.

[237] B. Sellergren, M. Lepistö, K. Mosbach, J. Am. Chem. Soc. 110 (1988) 5853.

[238] J. Zhou, X.W. He, Anal. Chim. Acta 381 (1999) 85.

[239] C. Luebke, M. Luebke, M.J. Whitcombe, E.N. Vulfson, Macromolecules 33 (2000) 5098–5105.

[240] A. Katz, M.E. Davis, Macromolecules 32 (1999) 4113.

[241] X. Dong, H. Sun, X. Lue, H. Wang, S. Liu, N. Wang, Analyst 127 (2002) 1427.

[242] A.J. Hall, P. Manesiotis, J.T. Mossing, B. Sellergren, Mater. Res. Soc. Symp. Proc. 723 (2002) 11.

[243] F. Lanza, M. Ruther, A.J. Hall, C. Dauwe, B. Sellergren, Mater. Res. Soc. Symp. Proc. 723 (2002) 93.

[244] J.G. Karlsson, B. Karlsson, L.I. Andersson, I.A. Nicholls, Analyst 129 (2004) 456.

[245] J. Svenson, J.G. Karlsson, I.A. Nicholls, J. Chromatogr., A 1024 (2004) 39.

[246] T. Sagawa, K. Togo, C. Miyahara, H. Ihara, K. Ohkuboa, Anal. Chim. Acta 504 (2004) 37.

[247] J. O'Mahony, A. Molinelli, K. Nolan, M.R. Smyth, B. Mizaikoff, Biosens. Bioelectron 20 (2005) 1884.

[248] M.J. Whitcombe, L. Martin, E.N. Vulfson, Chromatographia 47 (1998) 457.

[249] A. Katz, M.E. Davis, Macromolecules 32 (1999) 4113.

[250] H.S. Andersson, I.A. Nicholls, Bioorg. Chem. 25 (1997) 203.

[251] J. Svenson, H.S. Andersson, S.A. Piletsky, I.A. Nicholls, J. Mol. Recognit. 11 (1998) 83.

[252] S. Striegler, E. Tewes , Eur. J. Inorg. Chem. (2002) 487.

[253] H. Guo, X. He, J. Anal. Chem. 368 (2000) 461.

[254] L. Ping, R. Fei, X.L. Zhu, J.Z. Hu, C.W. Yuan, Acta Polym. Sin. 5 (2003)724.

[255] Guyot, in: D.C. Sherrington, P. Hodge (Eds.), Synthesis and Separations Using Functional Polymers, John Wiley & Sons, New York, 1989, pg. 1-36.

[256] L. Lloyd, J. Chromatogr. 544 (1991) 201.

[257] L. Ye, Artificial Receptors: New opportuniyties for the exploitation of molecularly imprinted polymers. Doctoral Thesis, Lund University, Sweden, 1999, pg. 7 Chapter 4.

[258] B. Sellergren, K.J. Shea, J. Chromatogr. 635 (1993) 31.

[259] K.J. Shea, D.Y. Sasaki, J. Am. Chem. Soc. 113 (1991) 4109.

[260] V.K. Sarin, S.B.H. Kent, R.B. Merrifield, J. Am. Chem. Soc. 102 (1980) 5463.

[261] C. Michailof, P. Manesiotis, C. Panayiotou, J. Chromatogr. A. 1182 (2008) 25.

[262] Y. Lv, Z. Lin, W. Feng, X. Zhou, T. Tan, Biochem. Eng. J. 36 (2007) 221.

[263] C. Legido-Quigley, J. Oxelbark, E. De Lorenzi, A. Zurutuza-Elorza, P.A.G. Cormack, Anal. Chim. Acta 591 (2007) 22.

[264] S. Le Moullec, A. Begos, V. Pichon, B. Bellier, J. Chromatogr. A 1108 (2006) 7.

[265] S. Le Moullec, L. Truong, C. Montauban, A. Begos, V. Pichon, B. Bellier, J. Chromatogr. A 1139 (2007) 171.

[266] S.D. Harvey, J. Sep. Sci. 28 (2005) 1221.

[267] F. Chapuis, J.U. Mullot, V. Pichon, G. Tuffal, M.-C. Hennion, J. Chromatogr. A 1135 (2006) 127.

[268] J.P. Lai, R. Niessner, D. Knopp, Anal. Chim. Acta 522 (2004) 137.

[269] T. Kubo, K. Hosoya, Y. Watabe, T. Ikegami, N. Tanaka, T. Sano, K. Kaya, J. Chromatogr. A 987 (2003) 389.

[270] J. Ou, L. Hu, L. Hu, X. Li, H. Zou, Talanta 69 (2006) 1001.

[271] X. Jiang, W. Tian, C. Zhao, H. Zhang, M. Liu, Talanta 72 (2007) 119.

[273] M. Kawaguchi, Y. Hayatsu, H. Nakata, Y. Ishii, R. Ito, K. Saito, H. Nakazawa, Anal. Chim. Acta 539 (2005) 83.

[274] A. Beltran, E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington, F. Borrull, Anal. Chim. Acta 597 (2007) 6.

[275] I. Sanchez-Barragan, K. Karim, J.M. Costa-Fernandez, S.A. Piletsky, A. Sanz-Medel, Sens. Actuators B: Chem. 123 (2007) 798.

[276] C.R.T. Tarley, L.T. Kubota, Anal. Chim. Acta 548 (2005) 11.

[277] E.C. Figueiredo, C.R.T. Tarley, L.T. Kubota, S. Rath, M.A.Z. Arruda, Microchem. J. 85 (2007) 290.

[278] E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington, F. Borrull, J. Chromatogr. A 995 (2003) 233.

[279] C. Baggiani, C. Giovannoli, L. Anfossi, C. Tozzi, J. Chromatogr. A 938 (2001) 35.

[280] Y. Watabe, T. Kubo, T. Nishikawa, T. Fujita, K. Kaya, K. Hosoya, J. Chromatogr. A 1120 (2006) 252.

[281] M. Le Noir, F. Plieva, T. Hey, B. Guieysse, B. Mattiasson, J. Chromatogr. A 1154 (2007) 158.

[282] E. Turiel, A. Martin-Esteban, J.L. Tadeo, J. Chromatogr. A 1172 (2007) 97.

[283] R.G. da Costa Silva, F. Augusto, J. Chromatogr. A 1114 (2006) 216.

[284] X. Liu, Z. Chen, R. Zhao, D. Shangguan, G. Liu, Y. Chen, Talanta 71 (2007) 1205.

[285] I. Chianella, S.A. Piletsky, I.E. Tothill, B. Chen, A.P.F. Turner, Biosens. Bioelectron. 18 (2003) 119.

[286] X. Dong, N. Wang, S. Wang, X. Zhang, Z. Fan, J. Chromatogr. A 1057 (2004) 13.

[287] E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington, F. Borrull, J. Chromatogr. A 1047 (2004) 175.

[288] N. Masque, R.M. Marce, F. Borrull, P.A.G. Cormack, D.C. Sherrington, Anal. Chem. 72 (2000) 4122.

[289] E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington, F. Borrull, J. Sep. Sci. 28 (2005) 2080.

[290] M. Gallego-Gallegos, R. Munoz-Olivas, C. Camara, M.J. Mancheno, M.A. Sierra, Analyst 131 (2006) 98.

[291] A. Martin-Esteban, E. Turiel, D. Stevenson, Chromatographia 53 (2001) S434.

[292] R. Carabias-Martinez, E. Rodriguez-Gonzalo, E. Herrero-Hernandez, M.E. Diaz-Garcia, J. Sep. Sci. 28 (2005) 453.

[293] M.L. Mena, P. Martinez-Ruiz, A.J. Reviejo, J.M. Pingarron, Anal. Chim. Acta 451 (2002) 297.

[294] C. Michailof, P. Manesioti F. Chapuis s, C. Panayiotou, J. Chromatogr. A 1182 (2008) 25.

[295] Q.Z. Zhu, P. Degelmann, R. Niessner, D. Knopp, Environ. Sci. Technol. 36 (2002) 5411.

[296] H. Sambe, K. Hoshina, J. Haginaka, J. Chromatogr. A 1152 (2007) 130.

[297] I. Ferrer, F. Lanza, A. Tolokan, V. Horvath, B. Sellergren, G. Horvai, D. Barcelo, Anal. Chem. 72 (2000) 3934.

[298] R. Koeber, C. Fleischer, F. Lanza, K.-S. Boos, B. Sellergren, D. Barcelo, Anal. Chem. 73 (2001) 2437.

[299] T. Pap, V. Horvath, A. Tolokan, G. Horvai, B. Sellergren, J. Chromatogr. A 973 (2002) 1.

[300] F. Chapuis, V. Pichon, F. Lanza, B. Sellergren, M.C. Hennion, J. Chromatogr. A 999 (2003) 23.

[301] F. Chapuis, V. Pichon, F. Lanza, B. Sellergren, M.C. Hennion, J. Chromatogr. B 804 (2004) 93.

[302] V. Pichon, F. Chapuis-Hugon, Anal. Chim. Acta 622 (2008) 48.

[303] E.V. Piletska, A.R. Guerreiro, M. Romero-Guerra, I. Chianella, A.P.F. Turner, S.A. Piletsky, Anal. Chim. Acta 607 (2008) 54

[304] E. Turiel, A. Martin-Esteban, P. Fernandez, C. Perez-Conde, C. Camara, Anal. Chem. 73 (2001) 5133.

[305] B.S Batlokwa, J. Mokgadi, R. Majors, C. Turner, N. Torto, A molecularly imprinted polymer for the removal of interfering chlorophyll from heavily pigmented green plant extracts prior to pesticide residue analysis. (2011) (Manuscript submitted).

[306] P. Martin, I.D. Wilson, G.R. Jones, K. Jones, in: Drug Development Assay Approaches Including molecular imprinting and biomarkers, ed. H. M. Hill and I. D.Wilson, Royal Society of Chemistry, Cambridge, 1998, pg. 21–27

[307] A. Zander, P. Findlay, T. Renner, B. Sellergren A. Swietlow, Anal. Chem. 70 (1998) 3304.

[308] A. Ellwanger, C. Berggren, S. Bayoudh, C. Crescenzi, L. Karlsson, P.K. Owens, K. Ensing, P.A.G. Cormack, D.C. Sherrington, B. Sellergren, Analyst 126 (2001) 784.

[309] B.S. Batlokwa J. Mokgadi T. Nyokong N. Torto, Chromatographia 73 (2011) 589.

[310] D. Adeyemi, J. Mokgadi, J. Darkwa, C. Anyakora, G. Ukpo, C. Turner, N. Torto, Chromatographia 73 (2011) 1015.

[311] L.I. Andersson, A. Paprica T. Arvidsson, Chromatographia 46 (1997), 57.

[312] L.I. Andersson, Analyst 125 (2000) 1515.

[313] L.I. Andersson, M. Abdel-Rehim, L. Nicklasson, L. Schweitz, S. Nilsson, Chromatographia 55 (2002) S65.

[314] C. Cacho, E. Turiel, A. Martin-Esteban, C. Perez-Conde, C. Camara, Anal. Bioanal. Chem. 376 (2003) 491.

[315] G. Theodoridis, A.Kantifesa, P.Manesiotis, N. Raikos, H. Tsoukali-Papadopoulou, J. Chromatogr. A 987 (2003) 103.

[316] C. Crescenzi, S. Bayoudh, P.A.G. Cormack, T. Klein and K. Ensing, Anal. Chem. 73 (2001) 2171.

[317] C. Baggiani, G. Giraudi , A. Vanni, Bioseparation 10 (2002) 389.

[318] J. Jodlbauer, M.M. Maier, W. Lindner, J. Chromatogr. A 945 (2002) 45.

[319] N.W. Turner, E.V. Piletska, K. Karim, M. Whitcombe, M. Malecha, N. Magan, C. Baggiani, S.A. Piletsky, Biosens. Bioelectron. 20 (2004) 1060.

[320] J.L. Urraca, M.D. Marazuela, E.R. Merino, G. Orellana, M.C. Moreno-Bondi, J. Chromatogr. A 1116 (2006) 127.

[321] T. Kubo, K. Hosoya, Y.Watabe, T. Ikegami, N. Tanaka, T. Sano, K. Kaya, J. Chromatogr. A 987 (2003) 389.

[322] A. Rachkov and N. Minoura, J. Chromatogr. A 889 (2000) 111.

[323] M. Quaglia, K. Chenon, A.J. Hall, E. DeLorenzi, B. Sellergren, J. Am. Chem. Soc. 123 (2001) 2146.

[324] T. Kubo, K. Hosoya, Y.Watabe, N. Tanaka, T. Sano K. Kaya, J. Sep. Sci. 27 (2004) 316.

[325] http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/General_Information (2012) March 23.

[326] S.J. Lehotay, J. AOAC Int. 88 (2005) 615.

[327] J. Hajslova, J. Zrostlikova, J. Chromatogr. A 1000 (2003) 181.

[328] http://www.chem.agilent.com/Library/applications/5990-4248EN.

[329] C. He, Y. Long, J. Pan, K. Li, F. Liu, J. Biochem. Biophys. Methods 70 (2007) 133.

[330] E. Virtanen, E. Kolehmainen, Eur. J. Org. Chem. 22 (2004) 3385.

[331] W. Yongjilan, Z. Jie, X.X. Zhu, A. Yu, Polymer 48 (2007) 5565.

[332] P. Lefebvre, B. Cariou, F. Lien, F. Kuipers, B. Staels, Physiol Rev. 89 (2009) 147.

[333] Z. Wu, X. Hu, C. Tao, Y. Li, J. Liu, C. Yang, D. Shen, G. Li, J. Mater. Chem. 18 (2008) 5452.

[334] http://www.Biotage.com/Library/applications.

[335] J.A. Lewis, G.R. Fenwick, in: C.S. Creaser, R. Purchase, (Eds), Food contaminants: Sources and Surveillance, The Royal Society of Chemistry, Cambridge 1991, pg. 1-20.

[336] E. Papp, K.H. Otta, G. Zaray, E. Mincsovics, Microchem. J. 73 (2002) 39.

[337] International Agency for Research on Cancer., IARC monographs on the evaluations of carcinogenic risks to human, IARC, Lyon 1993, 56, pg. 489-521.

[338] M. Miraglia, C. Brera, M. Colatosti, Microchem. J. 54 (1996) 472.

[339] European Commission, Commission Regulation (98/53/EC), Off. J. Eur. Communities: Legis. 1998, L201/93.

[340] W. Horwitz, Pure Appl. Chem. 67 (1995), 331.

[341] Q. Fengxia, S. Hanwen, Y. Hongyuan, R. Kyung Ho, Chromatographia 64 (2006) 625.

[342] http://www.mrc.ac.za/promec/afloxin.htm (2012) March 23.

[343] H. Zhang, T. Song, F. Zong, T. Chen, C Pan, Int. J. Mol. Sci. 9 (2008) 98.

[344] A. Ellwanger, C. Berggren, S. Bayoudh, C. Crecenzi, L. Karlsson, P.K. Owens, K. Ensing, P. Cormack, D. Sherrington, B. Sellergren, Analyst 126 (2001) 784.

[345] W. Peng-Ju, Y. Jun, S. Qing-De, G Yun, Z. Xiao-Lan, C. Ji-Bao Chin. J. Anal. Chem. 35 (2007) 484.

[346] K. Mastovska, S.J. Lehotay, Pittcon Conference (2009).

[347] H. Joshua, J. Chromatogr. A 654 (1993) 247.